eman ta zabal zazu

Universidad del País Vasco
Euskal Herriko Unibertsitatea

NUTRITIONAL QUALITY OF MILK FROM GRAZING MARES: EFFECT OF LACTATION STAGE

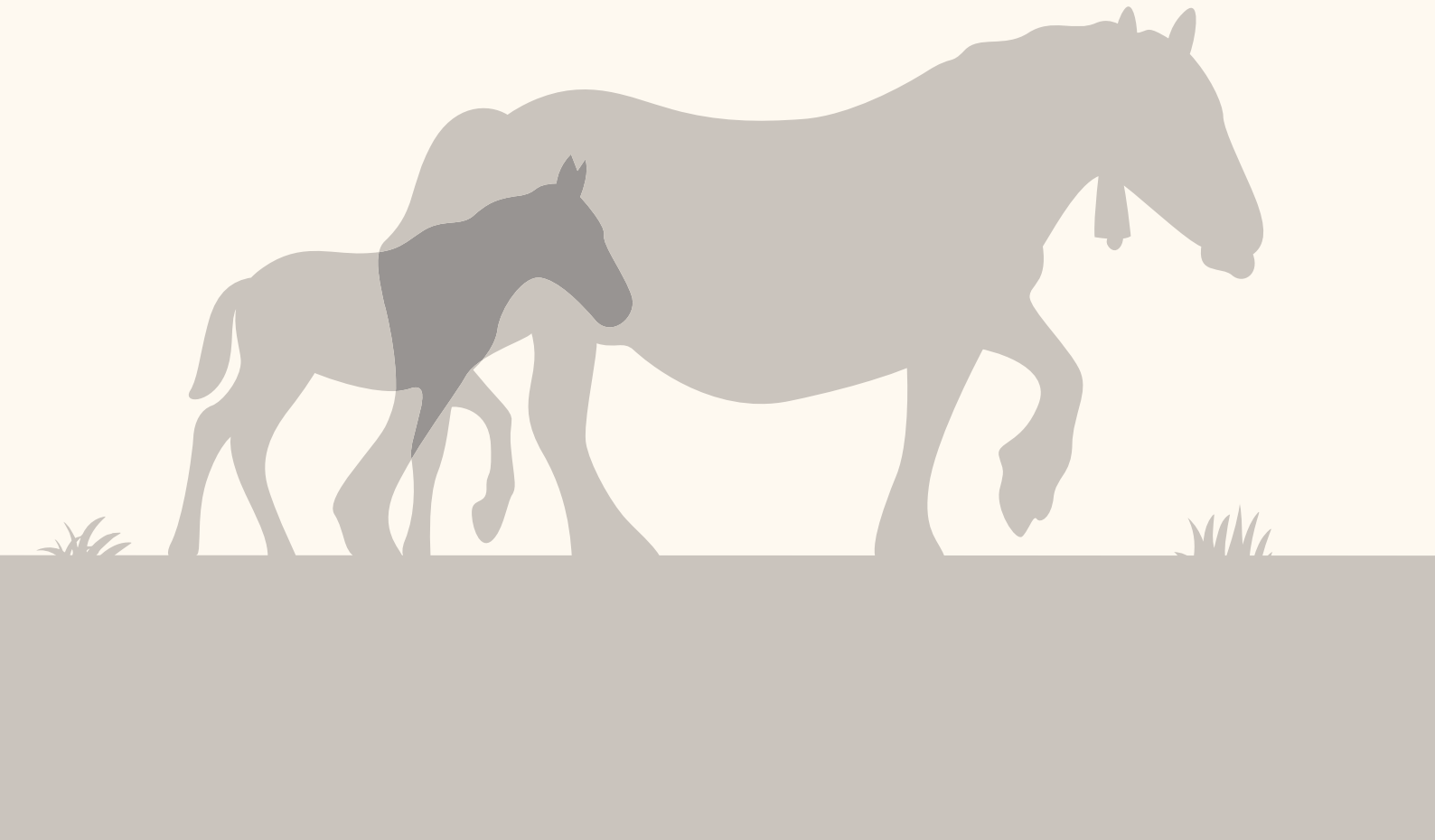
LARRATZE BEHORREN ESNEAREN NUTRIZIO KALITATEA: EDOSKITZAROAREN ERAGINA

CALIDAD NUTRICIONAL DE LA LECHE DE YEGUAS EN PASTOREO: EFECTO DEL PERIODO DE LACTACIÓN

PhD thesis | Doktorego tesia | Tesis doctoral

Ana Blanco Doval

2024



Universidad del País Vasco Euskal Herriko Unibertsitatea

FACULTY OF PHARMACY
UNIVERSITY OF THE BASQUE COUNTRY

NUTRITIONAL QUALITY OF MILK FROM GRAZING MARES: EFFECT OF LACTATION STAGE

LARRATZE BEHORREN ESNEAREN NUTRIZIO KALITATEA: EDOSKITZAROAREN ERAGINA

CALIDAD NUTRICIONAL DE LA LECHE DE YEGUAS EN PASTOREO: EFECTO DEL PERIODO DE LACTACIÓN

Ana Blanco Doval

PhD thesis | Doktorego tesia | Tesis doctoral

Supervised by

Dr. Noelia Aldai Elkoro-Iribe

Dr. Luis Javier Rodríguez Barron

Grupo de Investigación Lactiker Research Group

Lactiker Research Group

Faculty of Pharmacy

University of the Basque Country (UPV/EHU)

Vitoria-Gasteiz, 2024

But we must not forget that when radium was discovered no one knew that it would prove useful in hospitals. The work was one of pure science. And this is a proof that scientific work must not be considered from the point of view of the direct usefulness of it. It must be done for itself, for the beauty of science, and then there is always the chance that a scientific discovery may become like the radium a benefit for humanity.

Marie Skłodowska Curie, 1921

Acknowledgements for the financial support

This doctoral thesis has been funded by the Basque Government through ELKARTEK 2019 (BIOTASMA), Cooperation 2020 (BEHOR ESNE) and IT944-16 and IT1568-22 projects. Ana Blanco Doval gratefully acknowledges the doctoral fellowship provided by the Department of Education of the Basque Government.

Acknowledgements to the editorials

Authors thank the editorials for granting permission to reuse the published articles in this thesis. The published versions can be accessed at the following links:

Blanco-Doval *et al.* (2024) *Foods, 13*, 1412. (**Appendix III.I**) https://doi.org/10.3390/foods13091412

Blanco-Doval *et al.* (2023) *J Food Compos Anal*, *123*, 105629. (**Appendix III.II)** https://doi.org/10.1016/j.jfca.2023.105629

Blanco-Doval *et al.* (2024) *Food Chem*, *445*, 138766. (**Appendix III.III)** https://doi.org/10.1016/j.foodchem.2024.138766

Blanco-Doval *et al.* (2024) *J Dairy Sci*, in press. (**Appendix III.IV)**

Other works sent for publication that support the results of the present PhD Thesis are the following:

Appendix I.I – Manuscript 1. Sent to *Journal of Food Composition and Analysis*. Under review.

Appendix I.II – Manuscript 2. Sent to *Journal of the Science of Food and Agriculture*. Under review.

Con esta tesis doctoral doy fin a la que ha sido una de las etapas más duras, pero a la vez más enriquecedoras de mi vida. Han sido más de cuatro años de duro trabajo. Años que han traído consigo quebraderos de cabeza, pero también muchas alegrías y personas maravillosas con las que he tenido el placer de cruzarme por el camino. Y es que con esto cumplo el sueño de mi vida: involucrarme de manera activa en la ciencia. No deja de fascinarme la capacidad que tenemos de entender el mundo, y me siento enormemente agradecida a los gigantes que me han ofrecido sus hombros para poder ver más allá.

En primer lugar, agradezco a Luis Javier Rodríguez Barron y a Noelia Aldai Elkoro-Iribe por haberme enseñado tanto. Dejo el doctorado aún más fascinada por la ciencia de lo que estaba antes de empezar, y os debo todo lo que he aprendido a lo largo de estos años. Pero, sobre todo, gracias por ser guía y no imposición, por haberme dejado decidir qué camino tomar en esta tesis, por haber estado presentes y por haberme aconsejado siempre que lo he necesitado. Eskerrik asko bioi.

Gracias también a todas las personas que se han cruzado en mi camino durante estos años. Por supuesto, a las que estaban ahí cuando yo llegué, en especial a Lara, Lorea y Maider, por acogerme con los brazos abiertos y por hacer que el inicio fuera más fácil. Ondoren etorri zirenei ere, bereziki Nagoreri, amaiera polita egiteagatik. Thank you Lotti, Reto and Raquel for making my stay in Bern so nice and fruitful, and for giving me a great future opportunity. I am looking forward to working in your team. Eskerrik asko CIC bioGUNE-ko jendeari, Felix, Mikel, Ibon eta Jabi, ezagutu dudan zientzarik politenetako bat erakusteagatik, eta daukazuen ezagutzarekiko pasioa nirekin konpartitzeagatik.

Agradezco también a todas las personas de Lactiker, pero en especial a Marian por todo lo que me has enseñado, por las horas que invertiste en mis experimentos, y por haber aprendido conmigo a reparar cualquier tipo de error que un cromatógrafo pueda dar. Me llevo todos esos conocimientos conmigo.

Por supuesto, nada de esto habría sido posible sin el apoyo moral de las personas que comparten la vida conmigo. Gracias a Isra, Lorenzo, Iván, Alain, Erlantz, Sara y Eder por haber sido mis compañeros de viaje durante tantísimos años. A las hijas de Eustaquio y a Janire, porque a pesar de la distancia, siempre seguimos encontrando un ratito para juntarnos y reírnos. Eta eskerrik asko, bihotz bihotzez, Adurtza Dantza Taldeko *familiari*, baina bereziki Mirari, Itzal, Andrea, Titxu, Ieltxu, Alazne, Uhma eta Maitaneri. Erromerietan ikusiko dugu elkar!

Dejando lo más importante para el final, gracias a mi familia por haberme apoyado siempre, en especial a mis padres y a mi hermana, por haberme inculcado la pasión por el conocimiento y unas insaciables ganas de vivir. Eskerrik asko Igor urte hauetan zehar emandako babesagatik eta bizitza den bide luze honetan zehar nire alboan ibiltzeagatik. ¡Y por supuesto, también a Ortzito!

Por último, gracias a Dani Urraca por el diseño gráfico de la portada, y a Igor Martin, Julen Mtz. de Santos y Unai Fernández por las fotografías incluidas en la tesis.

CONTENTS

SUMMARY

Mare milk is a traditional food in many countries of Central Asia. It is valuable not only as a source of nutrients, but also for a series of attributed functional properties. Both raw and fermented mare milk (this last one named *kumis* or *koumiss*) have historically been used as medicine in patients with tuberculosis, chronic hepatitis, peptic ulcers, heartburn, etc. It has also been used as an alternative to human milk for infants due to the similarities in composition between mare and human milk. Moreover, mare milk presents a low allergenic response, being adequate also for infants and children with cow milk protein allergy, which is a common immune reaction among young age children.

However, the beneficial functions attributed to mare milk are based on experience and traditions rather than on scientific evidence, which is truly scarce compared with other well-known functional foods. In fact, even the chemical composition of mare's milk is not fully known today. A small number of studies, many of them dated back in the late 20th century, and other experimental and analytical limitations restrict the understanding of mare milk composition. Scarce information was also found as it respects to the impact of different production factors on mare milk composition, or regarding the use of grazing systems for mare milk production. However, considering its potentially beneficial attributes, a comprehensive characterization of mare milk composition would be of great interest.

In Europe, mare milk production and consumption are very low, almost negligible, but there is a minor but still relevant equine sector that focuses on horse meat production. In the case of Spain, equine breeding for meat production is concentrated in northern mountainous regions that use extensive livestock systems. Unfortunately, the current sector is structured in such a way that young horses are moved to eastern regions of Spain for fattening and slaughtering, not only losing the added value obtained from grazing, but also preventing original farmers from getting involved in the complete productive process. In this scenario, complementing horse meat production with mare milk in the equine rural farms from northern regions of Spain like the Basque Country would benefit the sector. Moreover, the use of the breed Basque Mountain Horse, an autochthonous horse breed in risk of extinction that is currently being used for meat production, would

also help preserve the gene pool and the associated cultural heritage. This breed has never been milked for commercial purposes nor studied from a scientific perspective before.

Given this, one of the objectives of the present PhD Thesis was to explore the interest of mare milk production using pasture-based management systems. For this, an extensive literature review was conducted focusing on the socio-ecological benefits of mare milk produced under grazing conditions. This included several aspects such as equine adaptation to extensive management, health and welfare of grazing mares, environmental impact of grazing equines, and impact on rural vitality and cultural heritage. This review exposed evidence that incorporation of mare milk production into local farming systems could benefit both farmers and society while providing a number of environmental ecosystem services. For instance, extensive equine breeding improves plant and animal diversity, soil quality and the aesthetic value of rural landscapes. Due to the equine physiology and grazing behaviour, it also mitigates the emission of greenhouse gases from the livestock sector and prevents wildfires. From the standpoint of rural and social development, supporting equine extensive farming could contribute to rural employment, the increase of farm income, and to population fixation and the consequent development of social services in rural areas (**Publication 1**).

After reviewing the potential benefits of mare milk production under extensive management systems and the existing knowledge on its nutritional composition, the present PhD Thesis aimed to fully characterize mare milk from the breed Basque Mountain Horse in order to establish a solid base of scientific evidence that supports its use for human consumption. In addition, this research work examined the effect of important production factors that could potentially influence milk composition, *i.e.* management system and lactation stage of the mares. To achieve this, mare milk samples from three semi-extensive commercial rural farms with different grazing intensities were collected frequently (every seven or fourteen days) during six months of lactation, from May to October. Milk samples were analysed to characterize the gross composition, mineral elements, fatty acids and water- and fat-soluble vitamins, and to measure total polyphenol content and total antioxidant capacity. A special focus was put on milk proteins, first, through a proteomic approach that allowed a complete description of mare milk proteome and its functionality; and second, through the study of the behaviour of mare milk major proteins during human gastrointestinal digestion.

The analysis of gross composition revealed that milk from this local horse breed contains a particularly low abundance of fat compared with milk from other horse breeds. Regarding fat composition, mare milk presented an interesting fatty acid profile, with 26.5 % of total fatty acids being polyunsaturated species. Polyunsaturated fatty acids are closely related to human health, and are searched nutrients in balanced diets. Despite this, it should be noted that the low fat content might limit the intake of polyunsaturated fatty acids from raw mare milk. The most abundant fatty acids in mare milk from Basque Mountain Horse breed were palmitic acid (16:0), oleic acid (9*c*-18:1), linoleic acid (18:2n-6) and linolenic acid (18:3n-3). However, a high abundance of even-chain saturated fatty acids with a length from 8 to 14 carbon atoms was also found. A number of individual branched-chain and *trans* fatty acids were also identified, expanding the knowledge on minor fatty acids in mare milk. This is of great importance considering that, first, previous studies overlooked these isomers in the analysis of mare milk, and second, that these minor fatty acids often present positive effects on human health, and might be in part responsible for the functionality of the food product. Results also showed a low abundance of species that are intermediate in the conversion from 18:3n-3 to docosahexaenoic acid (22:6n-3), supporting the idea that this metabolic pathway is limited in horses (**Manuscript 1**).

Main micronutrients in mare milk were calcium, potassium and phosphorous among macrominerals, zinc and iron among microminerals and vitamins C, A and E. Conversely, manganese and vitamin B_1 were not found in milk samples from Basque Mountain Horse breed. In the case of vitamins, some forms of specific vitamins were also absent in mare milk samples. Interestingly, vitamin E was only found in the form of α-tocopherol, and other isomers or esters of α-tocopherol were absent in mare milk samples. Similarly, vitamin A was only present as retinyl palmitate ester, and free retinol or other esters were not found. This provides some evidence to better understand the metabolism of equids, which is fairly unknown compared to more popular species for human consumption (cattle for instance). Related to these minor compounds known for their bioactivity, the antioxidant capacity of mare milk was mainly derived from the activity of caseins, which exerted about half of the antioxidant capacity of raw milk. This is consistent with results in milk from other species (**Publication 2** and **Manuscript 2**).

Besides the characterization of milk macro and micronutrients, a proteomic approach allowed to deepen in mare milk proteome. The proteomic study was done using two

complementary sample preparation methodologies: one based on an in-solution tryptic digestion of proteins, and a second one that included a protein separation step using gel electrophoresis (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) prior to tryptic digestion. The complementary use of both methods improved the coverage of unique proteins in mare milk, resulting in 469 proteins identified. The majority of these proteins were enzymes, proteins involved in nutrient transportation, structural proteins and immune-related proteins. Results demonstrated that mare milk proteome is particularly enriched by proteins that participate in lipid metabolism and inflammation, which responds to the two main roles of milk: nutrition and immunological protection of the newborn (**Publication 3**).

Finally, the present PhD Thesis studied the performance of mare milk proteins during human gastrointestinal digestion, mainly accounting for two parameters: the degree of protein degradation after intestinal digestion (protein digestibility) and the release of peptides after gastric and intestinal digestion. To achieve this, the standardized INFOGEST 2.0 protocol was used for mare milk sample digestion. This is a static *in vitro* human digestion simulation procedure constituted by three phases: oral, gastric and intestinal. The protein digestion study proved that mare milk is among the most digestible dietary protein sources with more than 90 % of the protein fraction degraded after intestinal digestion. This reinforces the interest of mare milk for infant nutrition and for individuals with digestion issues. When digestibility of individual proteins was assessed, caseins and α-lactalbumin A were highly digestible, whereas lysozyme C was resistant to human digestion, as also observed in other types of milk. Previous studies in mare milk reported that equine β-lactoglobulin was more susceptible than the bovine counterpart to proteolysis during digestion. However, this protein is present in two different isoforms in mare milk (I and II), and results obtained in the present study demonstrated differences in their behaviour during digestion. In this sense, β-lactoglobulin I was susceptible to proteolysis during digestion, whereas β-lactoglobulin II showed a resistant behaviour. Although no bioactive peptides were released after milk digestion, some precursors that contain previously defined bioactive sequences were found. These precursor peptides could release bioactive sequences in further hydrolysis steps during digestion, however, this requires further research (**Publication 4**).

Additionally to the characterization study, the effect of lactation stage and management of the mares was analysed. This is relevant not only to understand the dynamics of the

food matrix composition, but also to give some insights on how to optimize the production of a high quality milk. Based on this study, mare milk was more strongly influenced by lactation stage than by management of the mares. In fact, most individual compounds in mare milk were significantly affected by stage of lactation. In general terms, those compounds influenced by diet tended to decrease during lactation, but peaking at mid lactation. On the contrary, compounds that are synthetized in the animal and depend on metabolic aspects tended to decrease during lactation. However, the most evident changes in mare milk composition occurred at the end of the lactation period, and were principally attributed to a decrease in the content of mineral elements, short-chain fatty acids, pyridoxal and total protein.

When the effect of management system was investigated, grazing intensity was the main aspect that differentiated the three farms in the present study. Although in a lesser extent than lactation stage, management system significantly affected mare milk composition as well. This was mainly observed in the abundance of calcium, phosphorous, sulphur and sodium, and in the profile of fatty acids. Focusing on milk fat, a transfer of polyunsaturated fatty acids, particularly those from the n-3 group, from dietary plants to milk was evidently enhanced when mares were fed a pasture-rich diet. In samples from the system with a low grazing intensity, a lower polyunsaturated fatty acid content was found, and this was probably compensated with a higher saturated and monounsaturated fatty acid metabolic synthesis. The statistical analysis applied to data confirmed that milk samples from high grazing activity farms differed from those from low grazing farms, mainly due to differences in the content of unsaturated fatty acids and pyridoxine.

Overall, these findings provide a thorough characterization of mare milk composition, accounting for both major and minor compounds. They also demonstrate that a high quality milk can be obtained from the autochthonous breed Basque Mountain Horse, particularly when milk is produced from mares raised under a grazing system and during the initial and mid stages of lactation.

LABURPENA

Behor esnea edari tradizionala da Erdialdeko Asiako herrialde askotan, ez bakarrik nutriente iturri izateagatik, baizik eta hainbat ezaugarri funtzional esleitzen zaizkiolako. Bai behor esne gordina bai hartzitua (azken hau *kumis* edo *koumiss* izenekoa) sendagai gisa erabili izan dira historian zehar tuberkulosia, hepatitis kronikoa, ultzera peptikoak, bihotzerrea eta abar dituzten pazienteak tratatzeko. Era berean, giza esnearen alternatiba gisa erabili izan da bularreko umeentzat, behor eta giza esnearen arteko antzekotasunak direla eta. Gainera, behor esneak erantzun alergeniko baxua du, eta beraz, egokia da behi-esnearen proteinari alergia dioten bularreko haurrentzat. Izan ere, hori erreakzio immunologiko arrunta da jaioberrien eta adin txikiko haurren artean.

Hala ere, behor esneari egozten zaizkion propietate onuragarrien ebidentzia esperientzian eta tradizioetan oinarritzen da ikerkuntza zientifikoan baino. Izan ere, behor esnearen konposizio kimikoa ere ez da guztiz ezagutzen gaur egun, ikerketa gutxi eta horietako asko XX. mendearen amaierakoak direlako, eta baita ere beste muga esperimental eta analitiko batzuk eman direlako. Gainera, ikerlan gutxik aztertu dute ekoizpen faktore desberdinek edo behorren artzaintza estentsiboak esnearen kalitatean daukaten eragina. Hala ere, bere erabilera historikoak kontutan hartuta, interesgarria litzateke behor esnearen konposizioa era sakonean aztertu eta ulertzea.

Europan behor esnearen ekoizpena eta kontsumoa oso txikia bada ere, badago zaldi haragiaren ekoizpenera bideratutako abeltzaintza sektorea. Espainiar estatuan, zaldi haragiaren ekoizpena iparraldeko eskualdeetan biltzen da, non orografia menditsua izategatik abeltzaintza sistema estentsiboak erabiltzen diren. Hala ere, zaldiak Espainiar estatuko ekialdeko eskualdeetara eraman ohi dira gizendu eta hiltzeko, eta honek bai produktuaren kalitatean bai iparraldeko abeltzainengan kalteak eragiten ditu. Hiltze aurreko gizentze prozesuarekin batera, animaliak jandako larreetatik lortutako konposatu kimiko onuragarrien metaketa galtzen da esnean. Bestalde, animalien mugimenduak iparraldeko abeltzainek ekoizpen-prozesu osoan parte hartzea eragozten du. Hori ikusita, Espainiar estatuko iparraldeko eskualdeetan, Euskadin esaterako, ekoizten den zaldi haragiarekin batera behor esnea ekoizteak onurak ekarriko lizkioke abeltzaintza sektoreari. Halaber, Euskal Herriko Mendiko Zaldia erabiltzeak bai arrazaren eta bai horri

lotutako kultura ondarearen babespena bultzatuko luke. Izan ere, Euskal Herriko Mendiko Zaldia desagertzeko arriskuan dagoen zaldi arraza autoktonoa da, gaur egun haragi ekoizpenerako erabiltzen dena. Nolanahi ere, arraza hau ez da inoiz jetzi, ez salmentarako ezta ikuspegi zientifikotik aztertzeko ere.

Hori guztia kontuan hartuta, doktorego tesi honen helburuetako bat sistema estentsiboetan oinarritutako behor esnearen ekoizpenaren interesa aztertzea da. Horretarako, literatura berrikuspen zabala egin zen ekidoen artzaintza estentsiboaren onura sozio-ekologikoak ezagutzeko. Horrek hainbat alderdi hartzen zituen barne, hala nola zaldien egokitzea kudeaketa estentsibora, sistema estentsiboetan hazitako behorren ongizatea eta inpaktua ingurumenean, eta horrek landa bizitasunean eta kultura ondarean duen eragina. Berrikuspen bibliografiko horrek agerian utzi zuen behor esnearen ekoizpena tokiko nekazaritza sistemetan sartzeak mesede egin ziezaiekeela nekazariei zein gizarteari, eta, aldi berean, hainbat ingurumeneko ekosistema-zerbitzu eman zitzakeela. Adibidez, zaldien hazkuntza estentsiboak landareen eta animalien biodibertsitatea, lurzoruaren kalitatea eta landa paisaien balio estetikoa hobetzen ditu. Zaldien fisiologiaren eta bazkatzeko moduaren ondorioz, zaldien kudeaketa estentsiboak abeltzaintzatik datozen berotegi efektuko gasen emisioa arintzen du eta basoko suteak saihesten ditu. Landa eta gizarte garapenaren ikuspegitik, landa eremuetan enplegua sortzen laguntzen du, nekazaritza ustiategien errentak handitzen ditu eta biztanleria finkatzen laguntzen du, aldi berean landa eremuetako gizarte zerbitzuak garatuz (**1. Argitalpena**).

Behin zaldien ekoizpen estentsiboaren onura sozio-ekologikoak aztertuta, doktorego tesi honek Euskal Herriko Mendiko Zaldiaren behor esnea erabat ezaugarritzea du helburu, giza kontsumorako erabilera bermatzen duen ebidentzia zientifikoen oinarri sendoa ezartzeko. Gainera, ikerketa lan honek esnearen konposizioan eragina izan zezaketen ekoizpen faktore garrantzitsuen eragina aztertu zuen, hala nola, behorren ekoizpen sistemarena eta edoskitzaroarena. Hori lortzeko, sistema erdi-estentsibo ezberdinak erabiltzen zituzten hiru abeltegietako esne laginak hartu ziren, zazpi edo hamalau egunean behin, edoskitzaro osoan zehar (sei hilabete, maiatzetik urrira). Esne laginetan konposizio gordina, elementu mineralak, gantz azidoak eta bitamina hidro- eta liposolbagarriak ezaugarritu ziren, baita polifenol totalen edukia eta gaitasun antioxidatzaile totala ere. Arreta berezia jarri zen esnearen proteinetan, lehenik eta behin, behor esnearen proteoma eta haren funtzionalitatea erabat deskribatzea ahalbidetzen zituen ikerketa proteomikoa

eginez; eta, bigarrenik, giza digestioan zehar behor esnearen proteina nagusien portaera aztertuz.

Lortutako emaitzen arabera, Euskal Herriko Mendiko Zaldia arrazako behor esneak gantz kopuru bereziki txikia zeukan beste zaldi arraza batzuetako esnearekin alderatuta. Gantzaren osaerari dagokionez, gantz azido guztien % 26,5 poliasegabeak ziren, giza osasunarekin erlazionatuta dauden konposatuak. Hala eta guztiz ere, behor esnearen gantz kopuru urriak gantz azido poliasegabeen iturri mugatua izatea eragiten du. Euskal Herriko Mendiko Zaldia arrazako behor esnearen gantz azido ugarienak azido palmitikoa (16:0), azido oleikoa (9*c*-18:1), azido linoleikoa (18:2n-6) eta azido linolenikoa (18:3n-3) ziren. Hala ere, 8 eta 14 karbono atomo bikoiti arteko gantz azido aseen kantitate nabarmena aurkitu zen. Era berean, adarkatutako eta *trans* gantz azido ezberdinak identifikatu ziren. Hau interesekoa da kontutan izanda, lehenik eta behin, isomero horien identifikazioa oso eskasa izan dela behor esnea aztertu duten aurreko ikerketetan, eta bigarrenik, gantz azido txiki horiek giza osasunean ondorio positiboak izan ohi dituztelako (**1. Eskuidatzia**).

Behor esnearen bitamina nagusiak C, A eta E ziren, kaltzioa, potasioa eta fosforoa mikromineral nagusiak, eta zinka eta burdina mikromineral ugarienak. Bitaminen kasuan, E bitamina α-tokoferol eran baino ez zen aurkitu, eta A bitamina erretinil palmitato ester gisa bakarrik. Beste tokol batzuk, α-tokoferolaren esterrak, erretinol askea edo beste erretinil esterrik ez ziren aurkitu, ezta B_1 bitamina eta manganesoa ere. Honek ekidoen metabolismoa hobeto ulertzeko informazio baliagarria ematen du, giza kontsumorako ohikoagoak diren espezieekin alderatuta (behia, adibidez) nahiko ezezaguna baita. Bestalde, ikerketa honetako emaitzek behor esnearen ahalmen antioxidatzailearen ia erdia kaseinetatik datorrela frogatu zuten. Hori bat dator beste espezie batzuetako esnean ikusitakoarekin (**2. Argitalpena** eta **2. Eskuidatzia**).

Halaber, azterketa proteomikoaren bidez behor esnearen proteoman sakondu zen. Horretarako, bi metodologia osagarri erabili ziren: batean, esneko proteinak gel elektroforesiaren bidez (sodio dodezilsulfato-poliakrilamida gel elektroforesia) banandu ziren tripsina bidezko digestioaren aurretik, eta, bestean, digestio triptikoa laginean bertan egin zen, fase likidoan. Bi metodoak erabiliz, 469 proteina ezberdin identifikatu ziren behor esnean. Proteina horietako gehienak entzimak, mantenugaien garraioan parte hartzen duten proteinak, egitura proteinak eta immunitateari lotutako proteinak ziren. Gainera, behor esnearen proteoma metabolismo lipidikoan eta hanturan parte hartzen

duten proteinetan bereziki aberastuta zegoen. Hori esneak dauzkan bi funtzio nagusiekin bat dator: jaioberriaren nutrizioa eta babes immunologikoa (**3. Argitalpena**).

Azkenik, doktorego tesi honek behor esnearen proteinen digestio gastrointestinala aztertzen du, bi parametro kontuan hartuz: heste meheko digestioaren ondorengo proteinen degradazio maila (proteinen digerigarritasuna) eta peptidoen askapena bai urdaileko bai heste meheko digestioaren ondoren. Behor esne laginen digestio gastrointestinala INFOGEST 2.0 protokolo estandarizatua jarraituz egin zen. INFOGEST protokoloak digestioa simulatzeko *in vitro* prozedura estatiko bat deskribatzen du, hiru fasez osatua dagoena: ahokoa, urdailekoa eta heste mehekoa. Emaitzen arabera, behor esnearen proteinek digerigarritasun altua daukate, % 90 baino gehiagokoa. Hortaz, behor esnea elikagai interesgarria izan daiteke bai haurren bai digestio arazoak dituzten pertsonen elikadurarako. Proteina indibidualen digerigarritasuna ebaluatu zenean, kaseinak eta laktalbumina A oso digerigarriak zirela ikusi zen, eta C lisozima, berriz, digestioarekiko erresistentea zela. Aldiz, β-lactoglobulinak digestioarekiko zuen sentikortasuna isoformaren araberakoa zen; β-lactoglobulina I digestioan zehar degradatzen zen bitartean, β-lactoglobulina II-k erresistentzia erakusten zuen. Esnearen digestioaren ondoren peptido bioaktiborik askatu ez zen arren, askatutako peptido batzuen sekuentziaren barruan aurretik definitutako sekuentzia bioaktiboak aurkitu ziren. Sekuentzia bioaktibo horiek ondorengo digestio etapetan zehar askatu litezke, hala ere, ikerketa gehiago behar da gai honen inguruan (**4. Argitalpena**).

Behor esnearen konposizioa ezaugarritzeaz gain, doktorego tesi honen helburua edoskitzaroak eta behorren maneiuak esnearen kalitatean izan dezaketen eragina aztertzea zen. Horrekin, behor esnearen konposizioaren aldakortasuna ulertzeaz gain, hasierako irizpide batzuk emango lirateke kalitate handiko esne ekoizpena hobetzeko. Azterketa horretan oinarrituta, edoskitzaroak eragin handiagoa izan zuen behor esnearen konposizioan behorren maneiu sistemak baino. Izan ere, behor esnearen konposatu indibidual gehienen kopurua era esanguratsuan aldatzen zen edoskitzaroan zehar. Oro har, elikadurak eragiten dituen konposatuen edukia handitu egiten zen edoskitzaroaren hasieratik erdialdera, baina murriztu egiten zen gero. Aitzitik, animalien metabolismoaren mende dauden konposatuak edoskitzaroan zehar gutxitzen ziren. Hala ere, behor esnearen konposizioaren aldaketa nabarienak edoskitzaroaren amaieran gertatu ziren nagusiki, elementu mineralen, kate laburreko gantz azidoen, piridoxalaren eta proteina totalaren edukia gutxitzearen ondorioz.

Maneiu sistemaren eragina ikertu zenean, animaliak larreetan bazkatzen zuten denbora izan zen hiru ustiategiak bereizten zituen alderdi nagusia. Maneiu sistemak eragin esanguratsua izan zuen, batez ere, kaltzio, fosforo, sufre eta sodio ugaritasunean eta gantz azidoen profilean. Esnearen gantzari dagokionez, larreetan denbora luzez bazkatu zuten behorren esnea belarretik jasotako gantz azido poliasegabeetan, batez ere n-3 taldekoak, aberatsagoa zen. Bazkatze denbora laburragoa izan zuten behorren esneak gantz azido poliasegabe gutxiago baina gantz azido ase eta monoasegabe gehiago zituen, ziurrenik, konpentsazio metaboliko baten ondorioz. Bazkatze denbora ugariko eta urriko esneen arteko ezberdintasun nabarmenenak gantz azido asegabeen eta piridoxinaren edukietan aurkitu ziren.

Aurkikuntza hauek behor esnearen konposizioaren karakterizazioa zehazten dute, konposatu ugarienen zein gutxiengoen deskribapena sakonduz. Era berean, ikerketa honek Euskal Herriko Mendiko Zaldia arrazatik kalitate handiko esnea lortu daitekeela frogatzen du, batez ere esnea edoskitzaroaren hasierako eta erdiko faseetan jasotzen denean, eta behorrak maneiu sistema estentsibopetan hazten direnean.

RESUMEN

La leche de yegua es un alimento popular en varios países de Asia Central, no solo por ser una fuente de nutrientes, sino porque a este producto se le atribuyen una serie de propiedades funcionales. Así, tanto la leche de yegua cruda como su versión fermentada (llamada *kumis* o *koumiss*) han sido históricamente utilizadas como medicina contra la tuberculosis, hepatitis crónica, úlceras pépticas, ardor de estómago, etc. Por su similitud con la leche humana y porque presenta baja respuesta inmune en pacientes con alergia a la proteína de la leche de vaca, la leche de yegua también ha tenido un amplio uso como alternativa a la leche materna en infantes. Esto es de gran interés considerando la alta incidencia de este tipo de alergia en personas de edad temprana.

Sin embargo, las propiedades beneficiosas que se le atribuyen a la leche de yegua están principalmente basadas en la experiencia y en tradiciones, mientras que la evidencia científica es muy limitada, sobre todo en comparación con la que existe acerca de otros alimentos funcionales más conocidos. De hecho, ni siquiera su composición química es del todo conocida a día de hoy. Esto se debe a la existencia de pocos estudios, muchos de ellos realizados a finales del siglo XX, además de otras limitaciones experimentales. Existe también información limitada respecto a cómo diferentes factores de producción o el uso de sistemas de pastoreo afectan a la composición de la leche de yegua. Vistos los potenciales beneficios de la leche de yegua, conocer en profundidad su composición química y bioquímica sería de gran interés para entender mejor su funcionalidad.

En Europa, tanto la producción como el consumo de leche de yegua son limitados, pero existe un sector equino enfocado principalmente a la producción de carne de potro. En España, la producción equina destinada a carne de potro se concentra principalmente en zonas del norte que, por ser montañosas, hacen uso de sistemas de manejo extensivos. Por la estructura propia del sector, los caballos no suelen ser sacrificados en estas zonas de producción, sino que se trasladan al Levante peninsular para su engorde y sacrificio. Esta etapa de engorde previa al sacrificio conlleva una pérdida del valor añadido derivado de la acumulación de compuestos beneficiosos obtenidos a través del pastoreo. Por otro lado, el traslado de animales para su sacrificio impide que los ganaderos de las zonas de producción sean partícipes del ciclo completo de producción, desde la cría del caballo

hasta la venta del producto final. Ante esta situación, complementar la producción de carne de potro con leche de yegua en las zonas rurales del norte de España, como es el País Vasco, podría beneficiar al sector. Además, el uso de razas autóctonas como el Caballo de Monte del País Vasco, raza en peligro de extinción que actualmente se utiliza para la producción de carne, ayudaría a conservar tanto la raza como la tradición y cultura asociadas a ésta. Cabe decir que esta raza nunca ha sido ordeñada antes y, por tanto, su composición específica es desconocida.

Teniendo en cuenta lo mencionado, uno de los objetivos de esta tesis doctoral fue explorar el potencial interés de producir leche de yegua bajo sistemas de pastoreo. Para ello, se llevó a cabo una revisión bibliográfica exhaustiva mediante la cual se identificaron los beneficios socio-ecológicos de la producción equina bajo sistemas de manejo extensivos, incluyendo aspectos como adaptación equina al manejo extensivo, salud y bienestar de yeguas cridas bajo sistemas de pastoreo, impacto medioambiental de équidos en pastoreo, e impacto de estos sistemas en el desarrollo rural y en el legado cultural. De esta revisión bibliográfica se concluyó que incorporar la producción de leche de yegua en los sistemas ganaderos locales dedicados a carne de potro podría beneficiar tanto al sector ganadero como a la sociedad, además de brindar una serie de servicios ecosistémicos. De hecho, el manejo extensivo de équidos mejora la biodiversidad animal y vegetal, la calidad del suelo y el valor estético del paisaje rural. Debido a la fisiología y a las preferencias alimentarias de los caballos, este tipo de sistemas ayudan a prevenir incendios forestales y a mitigar la emisión de gases de efecto invernadero procedente de la ganadería. Además, fomentar los sistemas equinos de pastoreo podría ayudar al empleo rural, al estado económico de las explotaciones rurales, a la fijación de la población y al desarrollo de los recursos sociales en áreas rurales (**Publicación 1**).

Una vez identificado el potencial interés de la producción de leche de yegua desde un punto de vista socio-económico, en esta tesis doctoral se caracterizó la leche de yegua de la raza Caballo de Monte del País Vasco con el fin de aportar evidencias sólidas que respalden su uso para el consumo humano. Además, en esta investigación se examinó el efecto de algunos factores de producción que podrían influir en la composición de la leche de yegua, como son el tipo de manejo y el periodo de lactación. Para ello, se recogieron muestras de leche de yegua procedentes de tres explotaciones rurales semi extensivas con diferentes intensidades de pastoreo. Las muestras se recogieron de manera frecuente (cada siete o catorce días) a lo largo de todo el periodo de lactación, que fue de seis meses desde

mayo hasta octubre. El análisis de las muestras consistió en la caracterización de la composición grosera, elementos minerales, ácidos grasos y vitaminas hidrosolubles y liposolubles, en la cuantificación de polifenoles totales y determinación de la capacidad antioxidante total de la leche. Para el estudio detallado de la fracción proteica de la leche se realizó, por un lado, un análisis proteómico mediante el cual se caracterizó el proteoma completo de la leche de yegua asociándolo además a la función de las proteínas. Por otro lado, se estudió el comportamiento de las principales proteínas de la leche durante el proceso de digestión gastrointestinal humana.

Los resultados del análisis de composición general mostraron que la leche de yegua de la raza Caballo de Monte del País Vasco contiene menos grasa total que la descrita en la leche de otras razas de caballo. A pesar de su bajo contenido en grasa, la leche de yegua presenta una interesante composición de ácidos grasos siendo el 26.5 % ácidos grasos poliinsaturados, especies moleculares conocidas por sus propiedades saludables y necesarias en una dieta equilibrada. Aun así, cabe destacar que el bajo contenido en grasa de la leche de yegua podría limitar la ingesta de ácidos grasos poliinsaturados. Los ácidos grasos mayoritarios en las leches analizadas fueron ácido palmítico (16:0), ácido oleico (9*c*-18:1), ácido linoleico (18-2n-6) y ácido linolénico (18:3n-3), aunque también se encontraron altas proporciones de ácidos grasos saturados de longitud entre 8 y 14 carbonos pares. Se encontraron también una serie de compuestos minoritarios como ácidos grasos ramificados y algunas especies con conformación *trans*. Así, este estudio aporta una descripción más detallada sobre la composición lipídica de la leche de yegua. En estudios anteriores de caracterización del perfil de ácidos grasos en la leche de yegua generalmente se ha pasado por alto este tipo de ácidos grasos minoritarios, los cuales a menudo presentan efectos positivos para la salud humana, pudiendo ser en parte responsables de las propiedades saludables atribuidas a la leche de yegua. En la investigación que se presenta en esta tesis doctoral se observó también una baja presencia de ácidos grasos poliinsaturados n-3 de cadena larga, que son especies intermedias en la conversión de 18:3n-3 a ácido docosahexaenoico (22:6n-3). Esto aporta cierta evidencia de que esta ruta metabólica se ve probablemente limitada en el caballo (**Manuscrito 1**).

En cuanto a los micronutrientes presentes en la leche de yegua, los compuestos más abundantes fueron calcio, potasio y fósforo entre los macrominerales, zinc y hierro entre los microminerales, y vitaminas C, A y E. La vitamina E se encontró únicamente en forma de α-tocoferol, mientras que solo se identificó vitamina A en forma de retinil palmitato.

En cambio, no se encontraron manganeso, vitamina B_1, otros tocoles, ésteres de α-tocoferol, retinol libre u otros ésteres de retinol en la leche de Caballo de Monte del País Vasco. Estos resultados aportan indicios sobre el metabolismo de vitaminas y minerales en los équidos, que es considerablemente menos conocido que el de otras especies más utilizadas para el consumo humano (por ejemplo, el vacuno). En cuanto a la capacidad antioxidante de la leche, ésta provenía principalmente del efecto de las caseínas, que contribuyeron a casi la mitad de la capacidad antioxidante total, tal como se ha observado en la leche de otras especies (**Publicación 2** y **Manuscrito 2**).

Por otro lado, para el estudio proteómico se utilizaron dos metodologías complementarias: una en la que las proteínas se separaron por electroforesis en gel (electroforesis en gel de poliacrilamida con dodecilsulfato sódico) previamente a la digestión con tripsina, y otra en la que la tripsinización se realizó directamente en la leche, previa preparación de la muestra. El uso complementario de ambos métodos resultó en una caracterización profunda del proteoma de la leche de yegua, donde se identificaron un total de 469 proteínas. La mayoría de estas proteínas eran enzimas, transportadoras de nutrientes, proteínas estructurales y aquellas que participan de procesos inmunes. Además, los resultados demostraron que el proteoma de la leche de yegua está particularmente enriquecido en proteínas que participan del metabolismo de lípidos y de procesos inflamatorios, lo que se corresponde con las dos principales funciones de la leche: proveer al neonato con nutrición e inmunidad (**Publicación 3**).

En cuanto al estudio de digestión de proteínas, en la presente tesis doctoral se estudió tanto el grado de degradación de la fracción proteica tras la digestión intestinal (digestibilidad proteica) como la liberación de proteínas tras la digestión gástrica e intestinal. Para ello, las muestras de leche se digirieron siguiendo el protocolo INFOGEST 2.0, que consiste en una simulación *in vitro* de la digestión humana constituida por tres fases: fase oral, gástrica e intestinal. Este estudio demostró que la leche de yegua es fuente de proteínas altamente digeribles, con una digestibilidad proteica mayor del 90 %, lo que soporta el interés de la leche de yegua para su consumo en infantes y en personas con ciertos problemas digestivos. Cuando se consideró la digestibilidad individual de cada una de las proteínas mayoritarias en la leche de yegua se observó que tanto las caseínas como la α-lactoalbúmina A son altamente digeribles, mientras que la lisozima C presenta cierta resistencia proteolítica. En cuanto a la β-lactoglobulina, existen dos isoformas en la leche de yegua (I y II), y según los resultados de este estudio, cada

isoforma presenta un comportamiento diferente durante la digestión humana. Así, la β-lactoglobulina I fue susceptible de hidrólisis durante la digestión, mientras que la β-lactoglobulina II presentó resistencia proteolítica. Aunque en el proceso de digestión no se generó ningún péptido bioactivo, entre los péptidos liberados tras la digestión tanto gástrica como intestinal se encontraron precursores que contienen secuencias con bioactividad previamente descrita. Estos precursores podrían dar lugar a péptidos bioactivos durante procesos de hidrólisis posteriores, aunque esto requiere de una investigación más profunda (**Publicación 4**).

Además del estudio de caracterización, en esta tesis doctoral se investigó el efecto que tienen el periodo de lactación y el sistema de manejo en la composición, funcionalidad y digestión proteica de la leche de yegua. Comprender el efecto de estos factores es imprescindible no solo para comprender mejor la composición y funcionalidad de esta matriz alimentaria, sino para facilitar la optimización de la producción de leche de yegua de alta calidad. En base a esto, la leche de yegua se vio influenciada principalmente por el periodo de lactación, afectando a la mayoría de compuestos individuales. A grandes rasgos, aquellos compuestos que se ven altamente influidos por la dieta de las yeguas tendían a disminuir a lo largo de la lactación, pero presentaban un pico de abundancia a mitad de la lactación. En cambio, aquellos compuestos que dependen principalmente del metabolismo del animal tendían a disminuir continuamente. Aun así, los cambios más evidentes ocurrieron durante la etapa final de la lactación debido a la disminución del contenido en algunos elementos minerales, ácidos grasos de cadena corta, piridoxal y proteína total.

Al estudiar el efecto del sistema de manejo, se estableció la intensidad de pastoreo como el principal factor de influencia sobre la composición y funcionalidad de la leche. Así, el manejo tuvo un efecto significativo en la composición de la leche, aunque menor que el del periodo de lactación, afectando principalmente al contenido de algunos elementos minerales y al perfil de ácidos grasos. Los resultados demostraron que una mayor intensidad de pastoreo incrementó la transferencia de ácidos grasos poliinsaturados de los pastos a la leche, sobre todo de especies del grupo n-3. Por el contrario, las leches procedentes de yeguas con baja intensidad de pastoreo contenían menor abundancia de ácidos grasos poliinsaturados pero mayor contenido en ácidos grasos saturados y monoinsaturados, probablemente debido a una compensación mediante síntesis metabólica. De hecho, las diferencias entre leche de yeguas criadas con alta y baja

intensidad de pastoreo se debieron principalmente a un diferente contenido en ácidos grasos insaturados, además de en piridoxina.

Con esta investigación se ha caracterizado de manera profunda la composición de la leche de yegua, incluyendo no solo los compuestos mayoritarios sino también compuestos minoritarios que han sido escasamente descritos previamente. Los resultados obtenidos en este estudio demuestran que es posible obtener leche de yegua de alta calidad a partir de la raza Caballo de Monte del País Vasco, particularmente cuando la leche se produce a partir de yeguas criadas en pastoreo y durante las etapas iniciales e intermedias de lactación.

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
ANOVA	Analysis of variance
AOAC	Association of Official Agricultural Chemists
BCFA	Branched-chain fatty acid
BOB	Boletín Oficial de Bizkaia
BOPV	Boletín Oficial del País Vasco
BOTHA	Boletín Oficial del Territorio Histórico de Álava
c	*Cis*
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
CLA	Conjugated linoleic acid
DHCR24	24-dehydrocholesterol reductase gene
DIAAR	Digestible indispensable amino acid ratio
DIAAS	Digestible indispensable amino acid score
DTT	Dithiothreitol
ESI	Electrospray ionization
FAME	Fatty acid methyl ester
FAO	Food and Agriculture Organization of the United Nations
FAOSTAT	Food and Agriculture Organization Corporate Statistical Database
FID	Flame ionization detector
FLD	Fluorescence detector
FXR/RXR	Farnesoid X receptor/retinoid X receptor
GAE	Gallic acid equivalents

GC	Gas chromatography
GLM	General linear model
GO	Gene ontology
HPLC	High-performance liquid chromatography
Ig	Immunoglobulin
LMM	Linear mixed model
LOQ	Limit of quantification
LTQ	Linear ion trap mass spectrometry
LXR/RXR	Liver X receptor/retinoid X receptor
MALDI	Matrix-assisted laser desorption/ionization
MAPA	Ministerio de Agricultura, Pesca y Alimentación del Gobierno de España
MS/MS	Tandem mass spectrometry
MUFA	Monounsaturated fatty acid
nLC	Nano-liquid chromatography
OPA	O-phthalaldehyde
PBS	Phosphate buffer solution
PUFA	Polyunsaturated fatty acid
R^2	Coefficient of determination
RSD	Relative standard deviation
SDA	Stepwise discriminant analysis
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SFA	Saturated fatty acid
SITRAN	Sistema Integral de Trazabilidad Animal

t	*Trans*
TFA	Trifluoroacetic acid
TIMS/TOF	Trapped ion mobility and time of flight tandem mass spectrometry
TOF/TOF	Time of flight tandem mass spectrometry
Trolox	6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid
UHPLC	Ultra-high-performance liquid chromatography
UPV/EHU	Universidad del País Vasco/Euskal Herriko Unibertsitatea (University of the Basque Country)
UV	Ultraviolet
UV/VIS	Ultraviolet and visible

AMINO ACID NOMENCLATURE

Common name	3-letter code	1-letter code	Systematic name
Alanine	Ala	A	2-aminopropanoic acid
Arginine	Arg	R	2-amino-5-guanidinopentanoic acid
Asparagine	Asn	N	2-amino-3-carbamoylpropanoic acid
Aspartic acid	Asp	D	2-aminobutanedioic acid
Cysteine	Cys	C	2-amino-3-mercaptopropanoic acid
Glutamine	Gln	Q	2-amino-4-carbamoylbutanoic acid
Glutamic acid	Glu	E	2-aminopentanedioic acid
Glycine	Gly	G	Aminoethanoic acid
Histidine	His	H	2-amino-3-(1*H*-imidazol-4-yl)-propanoic acid
Isoleucine	Ile	I	2-amino-3-methylpentanoic acid
Leucine	Leu	L	2-amino-4-methylpentanoic acid
Lysine	Lys	K	2,6-diaminohexanoic acid
Methionine	Met	M	2-amino-4-(methylthio)butanoic acid
Phenylalanine	Phe	F	2-amino-3-phenylpropanoic acid
Proline	Pro	P	Pyrrolidine-2-carboxylic acid
Serine	Ser	S	2-amino-3-hydroxypropanoic acid
Threonine	Thr	T	2-amino-3-hydroxybutanoic acid
Tryptophan	Trp	W	2-amino-3-(l*H*-indol-3-yl)-propanoic acid

Amino acid nomenclature

Tyrosine	Tyr	Y	2-amino-3-(4-hydroxyphenyl)-propanoic acid
Valine	Val	V	2-amino-3-methylbutanoic acid

FATTY ACID NOMENCLATURE

	Common name
8:0	Caprylic acid
10:0	Capric acid
12:0	Lauric acid
14:0	Myristic acid
16:0	Palmitic acid
17:0	Margaric acid
18:0	Stearic acid
20:0	Arachidic acid
22:0	Behenic acid
24:0	Lignoceric acid
iso-16:0	Isopalmitic acid
*i*so-18:0	Isostearic acid
9*c*-14:1	Myristoleic acid
9*c*-16:1	Palmitoleic acid
6*c*-18:1	Petroselinic acid
9*c*-18:1	Oleic acid
11*c*-18:1	Asclepic acid
13*c*-22:1	Erucic acid
9*t*-18:1	Elaidic acid
11*t*-18:1	Vaccenic acid
9*c*,11*t*-18:2	Rumenic acid
18:2n-6	Linoleic acid
20:3n-6	Dihomo-γ-linolenic acid
20:4n-6	Arachidonic acid (ARA)
22:4n-6	Adrenic acid
18:3n-3	α-linolenic acid

20:5n-3	Eicosapentaenoic acid (EPA)
22:5n-3	Docosapentaenoic acid (DPA)
22:6n-3	Docosahexaenoic acid (DHA)

SECTION I

1 STATE OF THE ART

1.1. General framework

First evidence of mare milk consumption and processing dates back to the mid-fourth millennium Before Current Era, in the Eneolithic Botai Culture of Kazakhstan (Outram et al., 2009). Since then, mare milk production and consumption has mainly been centralised in Central Asia. Tajikistan, Uzbekistan, Tibet, Xinjiang, southern Russia and northern China traditionally produce mare milk for human consumption purposes (Doreau & Martin-Rosset, 2002), and in Mongolia, Kazakhstan, Kyrgyzstan and Belarus mare milk accounts for 8 % of total pastoral milk produced (Gall, 2013). In Europe, mare milk production is less common and it is centralised in Ukraine, Hungary, Austria, Bulgaria and Germany. It is believed that 30 million people in the world consume mare milk on a regular basis (Doreau & Martin-Rosset, 2002); however, real data is unknown.

The idea that foods exert medicinal properties is widespread in Asian traditional medicine (Díaz et al., 2020). Hence, mare milk has been used for therapeutic purposes in many Asian regions (Doreau & Martin-Rosset, 2002). Mare milk and its fermented version (*kumis* or *koumiss*) have been used to treat tuberculosis, chronic hepatitis, peptic ulcers and heartburn (Park et al., 2006; Pieszka et al., 2016). It was also recommended to expectorate, facilitate digestions, lower fever, stimulate the nervous system and boost strength (Pieszka et al., 2016). Based on this, mare milk might have potential functional properties that could be of great interest from a human health perspective. However, the various medicinal uses historically given to this dairy product have led to a general assumption that mare milk possesses such beneficial properties, although these assumptions are primarily based on experience and traditions rather than on scientific evidence, as already outlined by Park et al. (2006).

Strong evidence supports that dietary habits have a strong impact on human health, and this has raised awareness on consumers, particularly in developed countries. In this context, nutritional and hedonic value of food products are not enough to fully meet consumer needs, and more and more functional foods with additional health promoting properties are being requested (Díaz et al., 2020; Pappalardo & Lusk, 2016). Consumers often prefer functional foods over their non-functional counterparts (Pappalardo & Lusk,

2016), so promotion of the health effects of certain food products needs to be carefully addressed.

Mare milk is definitely an interesting dairy alternative, and during the last few decades, the scientific community has focused on studying mare milk from a functionality perspective. In these studies, some positive preliminary outcomes have been obtained regarding antioxidant activity (Ugwu et al., 2019; Waili et al., 2021), bacterial infection (Guri et al., 2016; Zinger-Yosovich et al., 2010), immunomodulation (Fotschki et al., 2015, 2016; Srivastava et al., 2014), hypertension (Ugwu et al., 2019), diabetes (Song et al., 2017), fatigue (Hsu et al., 2021), cancer (Guri et al., 2016; Rahmat et al., 2006; Shariatikia et al., 2017), wound healing (Zava et al., 2009), atopic dermatitis (Foekel et al., 2009), psoriasis (Algazina et al., 2020), non-alcoholic steatohepatitis (Bimbetov et al., 2021), periodontal health (Kostelac et al., 2022) and sleeping habits and other symptoms in children with attention deficit hyperactivity disorder (Jokar et al., 2021, 2023). These studies often refer to particular compounds in mare milk (unsaturated fatty acids, amino acids, vitamins…) as responsible for such health effects. However, milk is a complex matrix, and the chemical composition of mare milk still remains poorly understood if compared with milk from other mammal species such as cow, sheep or goat. Many studies on mare milk composition are from the late 20th century and used available analytical techniques with low accuracy. Low sample sizes and accounting for only a few compounds are also common limitations among these research works. Moreover, there is little knowledge on how different factors, such as animal diet and lactation stage, affect mare milk nutritional composition. These are features that need to be profoundly understood in order to get a holistic conception of the relevance of mare milk consumption for human nutrition and health. A comprehensive characterisation of mare milk composition would therefore establish a solid base to further study its bioactivities and functional properties.

Regarding the Spanish equine sector, a total of 648,277 horses and 184,185 equine farms are registered in Spain in 2024. Among these equine farms, 38 % rear horses for leisure, sports or non-commercial purposes, 9 % exclusively use horses for meat production, and 0.13 % are fattening farms. In Spain, equine farms for meat production are concentrated at both sides of the Cantabrian Mountains range (93 % in Galicia, Asturias, Cantabria, Basque Country, Navarre and Castile and Leon) (SITRAN, 2024). These are mountainous regions that provide high quality natural resources, and therefore, farmers commonly rely

on extensive grazing systems and on local and well-adapted horse breeds for equine breeding (Insausti et al., 2021). Conversely, fattening and slaughtering farms are mainly located in eastern regions of Spain (50 % in Aragon, Catalonia and Valencia) (SITRAN, 2024), which implies that foals are mainly moved from northern to eastern regions for fattening and slaughter. This involves a loss of the added value obtained through animal grazing, an incomplete use of natural resources for animal growth, and a negative impact on the rural economy of northern areas that cannot be part of the complete meat production cycle, from breeding to meat sale (Insausti et al., 2021). In this context, mare milk production could be an interesting option to enhance and preserve equine livestock systems in northern regions, majorly close to the Cantabrian Mountains range, maintaining traditional extensive management and supporting the economy of rural areas.

1.2. Current knowledge on mare milk production and chemical composition

1.2.1. Milk production traits

Horses are seasonal polyoestrous species that usually foal in spring (Nagy et al., 2000). Their gestation is 11 months long, and when human handled, milk production lasts about 6 months, but in wild conditions, mares are able to nurse their foals for up to 12 months (Doreau & Boulot, 1989; Gall, 2013). Capacity of the equine udder is low – it can accumulate less than 2 L – and mares produce an average daily yield of 2.5-3.0 L milk/100 kg live weight (Doreau et al., 1990). However, milk yield depends not only on mare's body weight, but also on body condition at foaling, number of parturitions and age of the mares (Auclair-Ronzaud et al., 2022). In terms of milk yield, the low milk production of equids might be compensated by frequent milkings on a daily basis (more than twice *per* day) (Doreau & Martin-Rosset, 2002; Gall, 2013).

During the lactation period, physiological and biochemical changes in the mammary gland affect not only milk yield (Doreau et al., 1990), but also milk composition. During the first two days after parturition, equids secrete colostrum, which is richer in fat, protein and overall, dry matter compared to mature mare milk (Csapó et al., 1995; Csapó-Kiss et al., 1995). Mare milk colostrum also shows particularly high immunoglobulin (Ig) concentrations to compensate the inability of placental Ig transfer, which makes

colostrum consumption essential for neonate foals (Perkins & Wagner, 2015). As lactation progresses, a gradual transition to mature milk occurs, which implies changes in milk composition as well. Overall, lactose contents increase during the lactation period, whereas protein and fat contents decrease (Hachana et al., 2022; Markiewicz-Kęszycka et al., 2015). **Publication 1** extends information on mare milk production.

1.2.2. Mare milk nutrients

Mare milk is known for its composition similar to that of human milk. Overall, both milks have lower fat and protein but higher lactose contents compared to ruminant milk, as well as unique fat and protein profiles. Mare milk is also considered to be rich in bioactive compounds that contribute to its potential functional properties (Sheng & Fang, 2009). However, literature on mare milk composition, including characterization of either major or minor compounds, is overall old (late 20th century or first years of the 21st century). Due to the technological limitations available at that time, studies often accounted for major compounds leaving minor compounds or isomers aside. They also used analytical methodologies with low accuracy, considered a small sample size, and did not perform an appropriate statistical analysis. This results in a limited and incomplete understanding of the chemical composition of mare milk, which is indeed essential to make the leap to more complex studies assessing its functionality. After revising available literature, this section exposes the most important features of mare milk composition. A more detailed description of the composition of mare milk can be found in **Publication 1**.

Fat content in mature mare milk usually ranges between 0.3 and 2.0 g/100 g milk (Barłowska et al., 2023a; Barreto et al., 2020; Czyżak-Runowska et al., 2021; Minjigdorj et al., 2012a; Pikul et al., 2008) and decreases during lactation; at the end of the lactation period fat contents of 0.4 g/100 g milk are usually found (Markiewicz-Kęszycka et al., 2015; Navrátilová et al., 2018; Pikul et al., 2008). Content and composition of mare milk lipids can also be influenced by breed, number of parturitions and age of the mares (Barłowska et al., 2023b; Czyżak-Runowska et al., 2021; Navrátilová et al., 2018; Pietrzak-Fiećko et al., 2013).

Lipids in milk are gathered in a fat globule surrounded by a milk fat globule membrane. This membrane is a three-layer structure, with an internal layer of proteins, an intermediate layer of phospholipids, and an external layer of glycoproteins (Malacarne et al., 2002). Fat globules in mare milk have an average diameter of 1.5-3.0 μm (García et

al., 2012; Malacarne et al., 2002), which is slightly smaller than the diameter of cow (2.8-4.6 μm), sheep (3.0-3.8 μm) and goat (2.6-3.5 μm) milk fat globules (Claeys et al., 2014). Inside the fat globule, the lipid fraction of mare milk is composed of 86.5 % triglycerides, 2.6 % diglycerides (Wu et al., 2023) and about 3.5 % cholesterol (Navrátilová et al., 2018). Phospholipids – essential components of cell and milk fat globule membranes – account for 1.1-3.7 % of mare milk fat, and the main phospholipids present in mare milk are sphingomyelin, phosphatidylcholine and phosphatidylethanolamine (García et al., 2012; Wu et al., 2023).

Equid milk contains a particular fatty acid profile due to the equine monogastric digestion system. Unlike ruminants, horses have a single stomach and lack a rumen. They are hindgut fermenters, so their fermentation chamber (caecum-colon) is located after the absorption site in the small intestine. Whereas, in ruminants, dietary fatty acids are modified by rumen microbiota (biohydrogenation) and then absorbed, horses lack this modification step prior to absorption. Consequently, horses can absorb and deposit into tissues unmodified dietary fatty acids, but in contraposition, do not accumulate modification products such as *trans* (*t*) fatty acids or branched-chain fatty acids (BCFA) (Belaunzaran et al., 2015, 2018; Clauss et al., 2009). In addition to the monogastric physiology, an abundant pancreatic lipase secretion and the continuous secretion of bile salts make horses particularly efficient at solubilizing, hydrolysing and absorbing dietary lipids (Belaunzaran et al., 2015; Warren & Vineyard, 2013). Horses also liberate a specific pancreatic lipase related to protein 2 (PLRP2) that catalyses lipid release from plant galactolipases (Sahaka et al., 2020). This is of particular relevance considering that grass lipids are primarily composed of galactolipids and that these contain up to 95 % of long chain trienoic polyunsaturated fatty acids (PUFA), mainly linolenic acid (18:3n-3) (Sahaka et al. 2020; Valdivielso et al., 2016; Warren & Vineyard, 2013). As a result, horses can accumulate high amounts of n-3 PUFAs in meat and milk, while ruminant tissues like beef and cow milk contain higher amounts of saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and modified fatty acids (BCFAs and *trans* fatty acids) (Belaunzaran et al., 2015, 2018; Clauss et al., 2009; Devle et al., 2012). Because humans are also monogastrics and easily absorb and deposit dietary fatty acids, mare and human milks resemble in terms of lipid composition considering both length and saturation degree of fatty acids (Wu et al., 2023). A high n-3 PUFA content in mare milk, as well as a low n-6/n-3 ratio (0.35-1.7; Barreto et al., 2020; Devle et al., 2012; Haddad

et al., 2011; Pikul et al., 2008), make mare milk a favourable dairy product from a human health perspective (Simopoulos, 2001).

The contents of SFA, MUFA and PUFA in mare milk are 34-53 %, 23-38 % and 17-31 %, respectively. The main SFA is palmitic acid (16:0), whereas the main MUFA is oleic acid (9*cis* (*c*)-18:1) and the main PUFAs are 18:3n-3 and linoleic acid (18:2n-6) (Barreto et al., 2020; Czyżak-Runowska et al., 2021; Markiewicz-Kęszycka et al., 2014; Navrátilová et al., 2018; Pikul et al., 2008). Unfortunately, the abundance of other fatty acids is not always determined in studies on mare milk, and some minor components like *trans* fatty acids or BCFAs have poorly been described (Devle et al., 2012; Haddad et al., 2011, 2012; Pietrzak-Fiećko et al., 2013). Reports on conjugated linoleic acids (CLA) are also scarce and incomplete, reporting only a single isomer (Barłowska et al., 2023a,b; Czyżak-Runowska et al., 2021; Devle et al., 2012) or not specifying the CLA isomers at all (Minjigdorj et al., 2012b; Naert et al., 2013; Pelizzola et al., 2006; Pikul et al., 2008). In fact, few studies have determined a fatty acid diversity over 30 isomers (Devle et al., 2012; Haddad et al., 2012). Additionally to the scarce and incomplete information available, most of the reports on mare milk fatty acids are given in percentage instead of in absolute basis (content *per* milk mass). This might lead to an overestimation of the fatty acid abundance, particularly when a low number of compounds are included in the analysis. An additional issue observed in literature is that studies often used short chromatographic columns (< 100 m) and analytical conditions that appear to be insufficient to separate potentially co-eluting fatty acids such as some long-chain PUFAs, CLA and *trans* fatty acid isomers (Delmonte et al., 2011; Kramer et al., 2008). Seeing all this, a comprehensive profiling of mare milk fatty acids that also accounts for minor species is still needed. In addition, the fatty acid profile of mare milk significantly changes throughout lactation (Barreto et al., 2020; Czyżak-Runowska et al., 2021; Haddad et al., 2011; Navrátilová et al., 2018; Pikul et al., 2008). Studies differ, though, and many of those that monitored lipid changes during lactation followed just a few fatty acids. Therefore, clear dynamics of evolution of these compounds in mare milk remain unknown. Other factors such as breed, age of the mares and parturition number can significantly affect the content of some fatty acids as well (Czyżak-Runowska et al., 2021; Navrátilová et al., 2018; Pikul et al., 2008), but once again, research is limited.

One of the most valuable characteristics of mare milk in terms of chemical composition lies in the protein fraction. Mare milk contains approximately 1-3 g protein/100 g milk

(Claeys et al., 2014). Whereas proteins in ruminant milk are 80-85 % (w/w) caseins, mare milk only contains about 45-50 % of caseins, the rest being whey proteins. This resembles the proportion in human milk, with an approximate casein/whey protein ratio of 30/70 (Miranda et al., 2004; Uniacke-Lowe et al., 2010). For this reason, cow milk is sometimes referred as "casein type milk" while human and mare milks are called "albumin type milk" (Malacarne et al., 2002).

Caseins in milk are structured in micelles. In mare milk, casein micelles have an approximate diameter of 255 nm, which is larger than in human (64 nm) and cow (182 nm) milk (Malacarne et al., 2002; Pieszka et al., 2016). The main casein in mare milk is β-casein (1.1 g/100 g milk), followed by α_{s1}-casein (0.24 g/100 g milk), whereas amounts of α_{s2}- and κ-casein (0.02 g/100 g milk) are low. A similar whey protein profile is found in human milk, although this one lacks α_{s2}-casein. On the contrary, main caseins in cow milk are α_{s1}- and β-caseins, and considerable amounts of κ-casein (0.31 g/100 g milk) are also found (Uniacke-Lowe et al., 2010). Low levels of κ-casein in mare milk might respond to a lower surface to milk volume ratio due to the larger casein micelles (Egito et al., 2001). Probably to compensate the low content, κ-caseins in mare milk are highly glycosylated, and these are in fact the only glycosylated caseins in mare milk. Only glycosylated κ-caseins are present in mare milk, while in cow milk both glycosylated and non-glycosylated forms have been detected (Jaeser et al., 2023).

Whey proteins in mare milk are mainly composed of similar amounts of β-lactoglobulin and α-lactalbumin (0.24-0.26 g each/100 g milk) (Miranda et al., 2004; Uniacke-Lowe et al., 2010). Compared to ruminant species (Claeys et al., 2014), mare milk is also rich in lactoferrin (0.03-0.07 g/100 g milk) and lysozyme (0.06-0.27 g/100 g milk) (Barłowska et al., 2023b; Naert et al., 2013). Interestingly, equine lysozyme has the ability to bind calcium (Nitta et al., 1987), and it is thought to be an intermediate molecular species in the evolution from non-calcium-binding lysozymes to α-lactalbumin (which possesses calcium-binding activity) (Tada et al., 2002). On the other hand, a high content of Igs (0.16 g/100 g milk) has also been observed in mare milk (Miranda et al., 2004; Uniacke-Lowe et al., 2010). In equids, IgG is not transferred to the foetus *via* utero, so the newborn foal relies on colostrum to acquire the needed immunity. For this reason, IgG is the most abundant Ig in equine colostrum, but in mature milk, IgG contents are diminished and IgA predominates (Bondo & Jensen, 2011; Kenzig et al., 2009; Uniacke-Lowe et al., 2010). High abundance of lactoferrin, lysozyme and Igs might contribute to mare milk

functionality due to their reported antimicrobial effect (Alichanidis et al., 2016; Uniacke-Lowe et al., 2010).

The non-protein nitrogen fraction constitutes between 10 and 15 % of total nitrogen in mare milk, and it is primarily composed of urea, peptides, free amino acids and ammonia (Uniacke-Lowe et al., 2010). The free amino acid content of mare milk accounts for 31 mg/100 g milk, which is about 16-20 % of the non-protein nitrogen fraction of milk (Csapó-Kiss et al., 1995). The most abundant free amino acids are glutamic acid and serine, followed by threonine, valine, proline and glycine. Other individual free amino acids appear in less than 1 mg/100 g milk (Csapó-Kiss et al., 1995; Rassin et al., 1978).

Even though major proteins in mare milk have been well described, other protein fractions have generally been disregarded. Recently, proteomic tools have proven to be successful in the study of specific fractions in mare milk, for instance, proteins associated with milk fat globules (Barello et al., 2008; Lv et al., 2024), glycosylation of κ-casein (Jaeser et al., 2023) or lactoferrin (Narmuratova et al., 2022). Moreover, recent advances in proteomics, bioinformatics and associated technologies (Pedrouso et al., 2022) provide new opportunities to explore milk proteins from a comprehensive perspective. Using these technologies, Ji et al. (2024) recently published a list of 965 proteins found in the whey fraction of milk from different species, revealing that equine milk proteins noticeably differ from human, cow, camel, goat, sheep, buffalo and yak milk proteins. This study has opened the way for further research on mare milk complete proteome.

Mare milk is considered a low allergenic dairy product for individuals with cow milk protein allergy. For this reason, and due to its high similarity with human milk, mare milk has been proposed by several researchers as a substitute for infant formula (Pieszka et al., 2016; Sheng & Fang, 2009). In relation to this, only five cases of mare milk allergy have been reported in the scientific literature, three related to mare milk consumption (Fanta & Ebner, 1998; Gall et al., 1996; Robles et al., 2007) and two after cutaneous application of mare milk-based cosmetics (Doyen et al., 2013; Verhulst et al., 2016). Even though few studies have addressed mare milk protein allergenicity (Businco et al., 2000; Curadi et al., 2001; Zhao et al., 2023a), these obtained promising results. However, the factors causing low allergenicity of mare milk are still uncertain. Additionally to the low allergenic response, the protein profile of mare milk is valued for being easily digestible. Low protein and, specifically, low casein content in equine milk (especially α_{s1}- and κ-caseins), big casein micelles and low susceptibility of κ-casein to proteolysis might be

responsible for creating a weak coagulation that forms a soft, fine curd (Claeys et al., 2014; Inglingstad et al., 2010; Sheng & Fang, 2009; Uniacke-Lowe et al., 2010). This is thought to facilitate the digestion of proteins (process of 2 h for equine milk *vs.* 3-5 h for cow milk; Inglingstad et al., 2010; Sheng & Fang, 2009). However, human digestion of mare milk has scarcely been studied (Inglingstad et al., 2010; Xiao et al., 2023), so the detailed behaviour of mare milk proteins during human gastrointestinal digestion is not yet well understood. After foregut digestion in humans, milk is fermented in the hindgut. Once there, mare milk shows physicochemical (pH and formed gas pressure) and microbiota modulation effects that are more similar to human milk than other milk types, and mare milk is the main promoter of Bifidobacteriaceae, Lactobacillaceae and *Akkermansia* growth (Li et al., 2020).

Since it does not coagulate into a firm curd, mare cheese cannot be easily manufactured (Doreau & Martin-Rosset, 2002). Instead, it is mainly consumed as *kumis* or *koumiss* (Bat-Oyun et al., 2015), a spontaneously fermented alcoholic beverage (Lv & Wang, 2009), very popular in western Asia, Mongolia, northern China and Russia (Park et al., 2006).

Regarding carbohydrates, lactose is the main compound present in mare milk, which accounts for 5.6-7.2 g/100 g raw milk. This places mare milk, together with human milk, among the richest in lactose, compared to ruminant milk containing less than 6.2 g/100 g milk (Claeys et al., 2014). Other minor carbohydrates present in mare milk are oligosaccharides, which can be either free or bounded to proteins (glycoproteins) or lipids (glycolipids) (Karav et al., 2018). The main beneficial effect of milk oligosaccharides is their prebiotic activity based on their ability to promote the growth of specific gut bacteria such as *Bifidobacterium* (Karav et al., 2018; Srivastava et al., 2014).

Mare milk is a rich source of free oligosaccharides, with higher diversity and total concentration than bovine milk, although lower than human milk (Albrecht et al., 2014; Difilippo et al., 2015; Karav et al., 2018). The type of free oligosaccharides found in mare milk resembles that of human milk, while bovine milk oligosaccharides are considerably different. In fact, a number of oligosaccharides reported in mare milk are also present in human milk, but appear in trace amounts in cow milk (Karav et al., 2018). In addition, some unique free oligosaccharides have been identified in mare milk (Difilippo et al., 2015; Karav et al., 2018), although diversity of free oligosaccharides depends on the equine breed (Difilippo et al., 2015). A second important group of carbohydrates in milk

are those contained in glycoproteins. As mentioned, κ-casein, located on the surface of the casein micelle, is the only glycosylated casein in mare milk (Jaeser et al., 2023). Glycans in κ-casein are bound to the C-terminal or caseinomacropeptide region of the protein and expanded to the surrounding aqueous phase in milk to aid solubilisation and stabilization *via* electrostatic repulsion (Jaeser et al., 2023; Uniacke-Lowe et al., 2010). This glycosylation is also thought to provide resistance to κ-casein against proteolytic enzymes (Egito et al., 2001; Jensen et al., 2015).

In general terms, mare milk is a poor source of vitamins and minerals compared to ruminant milk, but contents are similar to human milk (Claeys et al., 2014). However, the content of micronutrients is not well documented in mare milk. The number of studies is relatively small (compared to milk from other species), and many of those studies accounted for a low sample size and considered few factors known to affect the content of nutrients in milk, such as lactation stage. This leads to an incomplete understanding of the micronutrient profile and dynamics in mare milk, but still, the available information is often used as a reference.

As in cow milk (Claeys et al., 2014), calcium (Ca) is the main macromineral in mare milk (261-1,380 μg/g milk), followed by potassium (K; 341-701 μg/g milk) and phosphorous (P; 152-884 μg/g milk), sodium (Na; 107-220 μg/g milk), and magnesium (Mg; 29-104 μg/g milk). Regarding microminerals, zinc (Zn) is the main trace element found in mare milk (1.1-4.1 μg), followed by iron (Fe; 0.18-1.30 μg/g), copper (Cu; 0.07-0.64 μg), and manganese (Mn; 0.18-1.30 μg/g) (Alipour et al., 2023; Anderson, 1991, 1992; Csapó-Kiss et al., 1995; Grace et al., 1999; Schryver et al., 1986a,b; Summer et al., 2004; Ullrey et al., 1966, 1974). Unfortunately, few studies have addressed the content of some other minerals in mare milk, including sulphur (S) (Grace et al., 1999) and chlorine (Cl) (Summer et al., 2004), and studies reporting mare milk micromineral contents are also quite limited. Studies that monitored changes in the mineral content during the lactation period (Grace et al., 1999; Schryver et al., 1986b; Summer et al., 2004; Ullrey et al., 1966, 1974) demonstrated the effect lactation stage. Unfortunately, some of the older studies did not subject data to statistical analysis, so significance of this effect could not always be verified. In addition, different evolution trends were observed among research works, so to date a clear pattern could not be determined.

In respect to vitamins, not only scarce research has been performed, but also many studies used analytical technologies such as microbiological, colorimetric or titration methods

that only allow an indirect quantification (Collins et al., 1951; Holmes et al., 1946; Markiewicz-Kęszycka et al., 2015; Pearson, 1947; Teichert et al., 2021). Moreover, analyses are often limited to the main vitamin isomers, and secondary isomers are poorly described; this is the case of vitamins A, E and B_3. Mare milk is a poor source of fat-soluble vitamins compared to ruminant milk (Claeys et al., 2014; Medhammar et al., 2012), probably due to its particularly low fat content. Vitamins A and E are the main fat-soluble vitamins in mare milk, and vitamins D and K remain in lower amounts (Csapó et al., 1995). Vitamin E is also more abundant than vitamin A (Csapó et al., 1995; Navrátilová et al., 2019). In fact, horses are thought to have a poor efficiency in the conversion of β-carotene into retinol or vitamin A. This is probably the result of a low enzymatic activity of intestinal β-carotene 15,15′-monooxygenase, which is responsible for the conversion of β-carotene into retinal (precursor of retinol) in cattle (Álvarez et al., 2015; Mora et al., 2000).

Mare milk stands out for its high vitamin C content, whereas the content of other water-soluble vitamins has been reported to be lower than in ruminant milk (Claeys et al., 2014; Kneifel & Mayer, 1991; Medhammar et al., 2012). There are particularly few citations on vitamins B_5, B_9, B_{12} and the pyridoxine isomer of vitamin B_6 (Collins et al., 1951; Kneifel & Mayer, 1991; Navrátilová et al., 2019; Pearson, 1947; Teichert et al., 2021), so their abundance in mare milk is still relatively unknown. Elucidating the water-soluble vitamin profile of mare milk is of particular interest considering the different metabolism between ruminants and non-ruminants like horse. In ruminants, most water-soluble vitamins including thiamine, riboflavin, niacin, pyridoxine, biotin and cobalamin are not only acquired through diet, but are also produced in the rumen (Magan et al., 2020). These vitamins are then absorbed in the small intestine and deposited into other tissues and fluids such as muscle and milk. In horses, because they have the fermentative chamber located after the small intestine (Janis, 1976), bacterial synthesis and absorption of water-soluble vitamins is probably reduced. However, metabolism of water-soluble vitamins in equids remains to be fully understood. On the other hand, the influence of lactation stage on mare milk vitamins has been poorly studied, and few vitamins have been monitored during lactation in a limited number of samples (Markiewicz-Kęszycka et al., 2015; Navrátilová et al., 2019).

1.3. Equine production under pasture-based systems

Nowadays, mare milk production is low compared to other domestic dairy species (Doreau & Martin-Rosset, 2002; FAOSTAT, 2023), but considering its physicochemical properties, commercialization of high-quality mare milk is worth exploring. Extensive livestock management systems, where natural resources are used for animal feeding and husbandry, have positive impacts at different levels, from product quality to environmental, social-economic and cultural benefits. The potential impacts at different levels of milk production from grazing mares will be briefly explored in this section, while they are discussed more extensively in **Publication 1**.

1.3.1. Impact on the food quality

Plants are richer in PUFAs, particularly in 18:3n-3, than concentrates (Jahreis & Dawczynski, 2020; Sahaka et al. 2020). Due to the equine monogastric digestive system, these dietary lipids can easily get absorbed and deposited into mare milk (Belaunzaran et al., 2015, 2018; Clauss et al., 2009; Devle et al., 2012). So presumably, grazing horses would accumulate higher quantities of PUFAs in milk and meat than animals managed under more intensive systems. In this sense, studies with pasture-based fed horses reported higher mare milk PUFA percentages (Minjigdorj et al., 2012b; Pikul et al., 2008) than studies with horses fed concentrates (Markiewicz-Kęszycka et al., 2014; Navrátilová et al., 2018). Unfortunately, differences in the experimental conditions complicate comparison among studies. Recently, a lipidomic study on mare milk (Deng et al., 2022) demonstrated the positive impact of feeding mares with pasture on the abundance of lipids that participate in PUFA pathways, although authors did not confirm these results with the analysis of individual fatty acids. Moreover, the fatty acid profile of forage differs among plant species (Valdivielso et al., 2016), so plant biodiversity and seasonal changes might also affect the fatty acid profile of milk from grazing mares. Based on this, the effect of grazing on mare milk lipids remains to be demonstrated by studies that compare the fatty acid profile of mare milk produced under different grazing intensities.

Building on the impact of extensive equine production systems on milk lipids, it has been demonstrated that dietary α-tocopherol (vitamin E) supplementation increases α-tocopherol levels in mare milk (Bondo & Jensen, 2011). Similar results were observed in milk β-carotene contents when mares were supplemented with β-carotene at initial lactation stages (Kuhl et al., 2012). Even so, studies comparing the influence of different

grazing activities, or grazing *versus* non-grazing, on mare milk vitamins are still lacking. Grasses are a great source of tocopherols and carotenoids (Valdivielso et al., 2016), and when ingested, these compounds can easily be absorbed and transferred into other horse tissues (Schweigert & Gottwald, 1999). In addition, natural forms of α-tocopherol and β-carotene seem to be better absorbed than synthetic forms (Greiwe-Crandell et al., 1997; Pagan et al., 2005). So, presumably, grazing would positively impact the abundance of α-tocopherol and β-carotene in mare milk.

Interestingly, the effect of grazing on milk composition is not solely attributed to a higher intake and deposition of specific nutrients, but it can also modulate the expression of particular genes in the animal. For instance, a long-term pasture feeding depresses the expression of the gene that encodes stearoyl-CoA desaturase (responsible for the unsaturation of 18:0 to 9*c*-18:1) in cattle (Smith et al., 2009). Moreover, grazing can alter the expression of a number of genes, some of them related to lipid metabolism (Zhao et al., 2023b). Inclusion of some specific fatty acids in diet can also modulate the expression of diverse genes associated with cellular development, lipid and protein metabolism and physiology, and immune response (Mach et al., 2011). Changing the expression of proteins can possibly have an impact on various metabolic pathways and, therefore, in various different molecules. In fact, such modulations are probably responsible for the previously reported significant effect of pasture-based dietary regimes on the protein profile of mare milk (Barłowska et al., 2023b). To date, there is scarce evidence on the genetic implications of grazing systems in equids, and this is definitely a pathway worth exploring. Fortunately, recent proteomic advances allow a deep and accurate description of the proteome and protein functionality of complex matrixes (Pedrouso et al., 2022), which is also applicable to milk.

1.3.2. Related ecosystem services

Extensive equine production for human consumption is gaining relevance in line with the promotion of sustainable livestock systems by the European Union. This is due to their favourable adaptability to harsh environments, the lower contribution of equids to greenhouse gas emissions and their selective feeding preference that promotes landscape modelling and flora and fauna diversity, among others. A diagram summarizing the ecosystem services provided by equine extensive farming is shown in **Figure 1.1**.

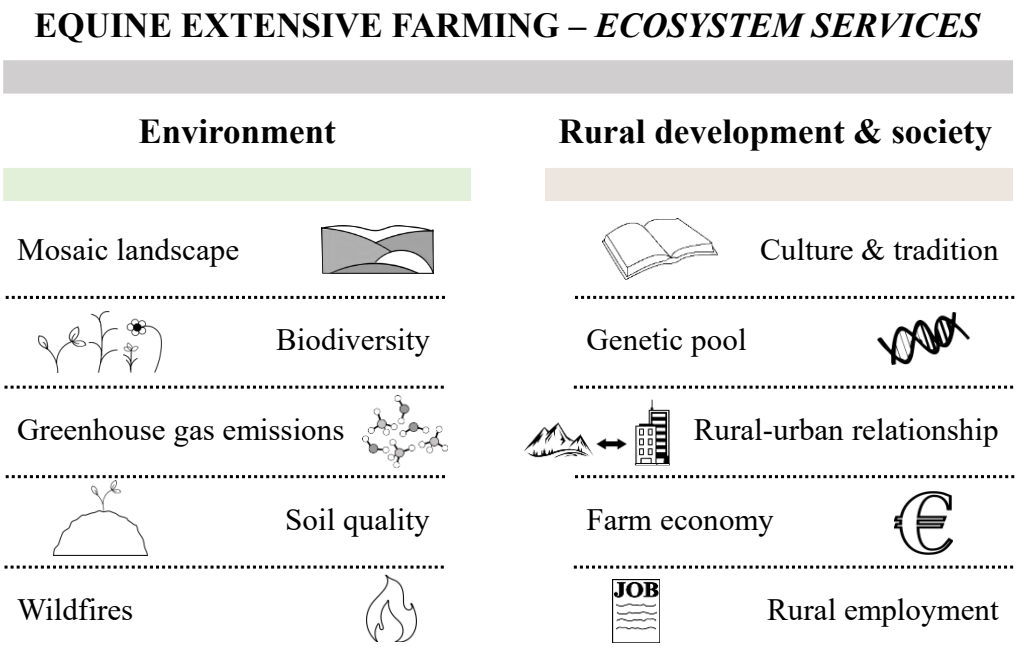


Figure 1.1. *Types of ecosystem services provided by equine extensive farming systems related to the environment (left) and rural development and society (right). Adapted from Beldarrain (2022).*

In broad terms, horse grazing displays a number of positive outcomes related to environment and rural development. On one hand, maintaining grazing systems is key to prevent land abandonment, which leads to the growth of highly flammable dead biomass and woody vegetation, therefore increasing the prevalence of wildfires (Bernués et al., 2005; Ferreira et al., 2013; Osoro et al., 2017; Rigueiro-Rodríguez et al., 2012). Horse grazing also improves soil quality and productivity through deposition and accumulation of animal wastes (dung and urine) (Bloor et al., 2012), and due to a selective grazing behaviour, it creates a mosaic landscape of short sward patches within tall vegetation, enhancing animal and plant biodiversity (Fleurance et al., 2016; Loucougaray et al., 2004). Horses, as hindgut fermenter herbivores, generate less methane during digestion than ruminants (Franz et al., 2010), so equine production could also be a strategy to somehow mitigate greenhouse gas emissions from livestock. On the other hand, enhancing extensive livestock systems could contribute to the improvement of rural employment, population growth and fixation, and subsequently higher investment in technological and social services that improve life quality in rural areas (Cobano-Delgado & Llorent-Bedmar, 2020; FAO, 2016; Reist et al., 2007). Preserving animal grazing in mountain areas also contributes to the aesthetic value, which in turn attracts tourism

(Bernués, 2017; Bernués et al., 2016, 2019). Moreover, autochthonous equine breeds are part of the culture and identity of a region (Marsoner et al., 2018), so preserving these breeds that often are in risk of extinction is important to maintain not only the genetic pool, but also cultural heritage. **Publication 1** gives an extended description of the ecosystem services and other beneficial traits that equid extensive systems provide.

1.4. Basque Mountain Horse breed

Basque Mountain Horse is an autochthonous equine breed from the Basque Country (northern Spain). These horses are medium size and stocky, and can present brown, sorrel, roan, peach or black coats (**Figure 1.2**). The breed standards were legislated for the first time in 1999 by the Basque Government (BOPV, 1999), and they are controlled by the Federation of the Basque Mountain Horse breed since 2014.

Figure 1.2. *Basque Mountain Horses grazing at a local farm in Araba, northern Spain (photo A. Blanco Doval).*

This breed is well adapted to rural areas in the Basque Country, and farmers make use of this rurality to manage Basque Mountain Horses under extensive systems. The most feral horse populations usually live in high mountains during summer, and descend to valleys and lower grasslands during winter. Conversely, more domesticated horses live in valleys

and grasslands all year long. This extensive management systems utilize natural sources to feed the animals, which reduces costs to the farmer while contributing to the preservation of rural areas.

FAO recognises the Basque Mountain Horse as a breed in risk of extinction (FAO, 2024). As a response, some public entities from the Basque Country (BOB, 2017; BOTHA, 2023) offer financial aids to buy Basque Mountain Horse studs as an attempt to preserve the local breed. Nowadays, the Basque Mountain Horse is mainly used for meat production due to its adequate muscle physiology, although it is sometimes used as a draft and pack animal as well. There are currently more than 6,000 animals of this breed, about 60 % being breeding females, in approximately 300 different farms (MAPA, 2022). Unfortunately, there are no official records of Basque Mountain Horse meat production. Equine production is still minor in the livestock sector, and most Basque Mountain Horses that are raised in the Basque Country are often moved to eastern Spain for fattening and slaughter. This not only involves the loss of added value gained through extensive management, but also a loss of proper tracking of the autochthonous breed to evaluate its relevance in the Spanish equine meat sector (Aldai et al., 2018).

This autochthonous breed has never been milked for commercial purposes before, and this PhD Thesis is the first documented evidence of the milking of Basque Mountain Horse breed on rural commercial farms, done at experimental level.

2 HYPOTHESIS AND OBJECTIVES

The Basque Mountain Horse is an autochthonous equine breed in risk of extinction that is currently being used for meat production purposes. This local horse breed is usually managed under grazing conditions, which not only makes use of natural resources, but also provides a number of ecosystem services that support the environment, society and culture. In addition, grazing animals have been shown to produce food products (meat and milk) of higher nutritional quality. In this context, mare milk production could be an interesting strategy to improve the equine livestock sector in rural areas of the Basque Country. Mare milk is a highly valued dairy product often promoted as a functional food with a wide variety of reported benefits for human health. Unfortunately, there is still limited scientific knowledge on mare milk chemical composition and functional properties, as well as on the effect that different production factors have on its nutritional quality. Milk is a complex matrix that requires the use of advanced analytical methods and technologies that allow the quantification of major but also minor compounds, which are usually key molecules in terms of food functionality. In this sense, there is still need for a comprehensive characterization of mare milk chemical composition and changes in response to important factors such as lactation stage or management system. This would set a solid scientific basis to account for the production and promotion of this dairy product.

The hypothesis of the present study was that Basque Mountain Horse breed mares managed under pasture-based systems produce milk of high quality in terms of nutrient composition, and that both the milk quality and the socio-ecological benefits derived from grazing management could be explored to improve the performance of equine farms in mountainous regions like the Basque Country. Therefore, the overall objective was to perform a comprehensive nutritional and compositional characterization of mare milk from the Basque Mountain Horse breed reared under different grazing management systems, and to investigate changes in the milk composition that occur throughout the lactation period.

The present work has been performed following four specific objectives:

Objective I. To characterize the chemical and biochemical composition and nutritional quality of mare milk, and to discuss in depth, through an exhaustive review of the scientific literature, the contribution of extensive grazing equine farms to other socio-ecological benefits generated.

Objective II. To assess *in vitro* digestibility and proteolytic patterns of mare milk proteins during human gastrointestinal digestion.

Objective III. To understand changes that occur during the entire lactation period on mare milk chemical composition and on digestibility and peptide release of mare milk proteins.

Objective IV. To compare the nutritional quality and protein performance during gastrointestinal digestion of milk from mares managed under distinct grazing intensities.

Publication 1 addressed Objective I, Publications 2 and 3 addressed Objectives I, III and IV, and Publication 4 addressed Objectives II, III and IV. In addition, Manuscripts 1 and 2 addressed Objectives I, III and IV.

3 MATERIALS AND METHODS

3.1. Experimental design

Mare milk samples were collected from eighteen Basque Mountain Horse mares that belonged to three different equine commercial farms (n = 6 *per* farm) located in Araba, Basque Country (northern Spain). The commercial farms bred horses for meat production and the mares had never been milked before. Mares averaged 9.53 ± 3.76 years old and 5.68 ± 3.56 parturitions. Parturitions were not synchronized and occurred within a range of 39 days from late March to early May, 2021. All mares were milked only at the sampling days, which happened once every 7 days from May to July, and once every 14 days from August to October. This encompassed the complete lactation period of the mares. A total of 311 individual milk samples were collected (farm I, n = 82; farm II, n = 125; farm III, n = 109) with replication based on the number of milk samples from individual mares *per* farm and lactation sampling day.

The three commercial farms differed in the animal management system, with main dissimilarities found in mares' diet (**Figure 3.1**). Briefly, mares from farm I grazed only during May, and were fed a mixture of alfalfa, silage, hay, fruits and potatoes from June until the end of the lactation period. Mares from farms II and III were kept in a pasture-based system during the whole lactation period, supplementing their diet with hay (farm II) or silage (farm III) only after July, when grass availability was insufficient to maintain animal energy balance. In addition, all mares were provided a mixture of cereals and alfalfa or pea in the milking parlour in order to get them comfortable during the milking process. Farm I moved mares to the milking parlour only during milk sampling days (once every 7 or 14 days). Conversely, farms II and III moved mares to the milking parlour (and provided the cereal and pea mixture) every day from May to October, but milking only during sampling days. With this, farmers aimed to get animals accustomed to the milking process until completion of the experiment.

Mares from farm I were milked using a portable milking machine fitted with two sheep teat-cups. Mares from farms II and III were manually milked. All mares were milked as completely as possible (visually was checked that no more milk was coming out of the udder). Before milking, foals were prevented from suckling for 2 to 3 h in order to

maximize accumulation of milk in the udder. Then, mares were moved one by one to the milking parlour and milked. No oxytocin was administered to mares, but foals were very close in an adjoining area. Individual milk samples were collected in plastic containers and transported to the laboratory in portable coolers. There, samples were individually weighed, pH was measured, and 200 mL of each sample were separated for cream separation. The rest of the milk was subsampled and kept at -80 ºC until analysis. The study of milk yield was out of the scope of this work; however, the milk quantity obtained from individual milkings was weighted (average milk yield of 880 ± 267 g was obtained *per* mare and *per* milking). All milk samples were analysed for gross composition, fatty acid, mineral, water- and fat-soluble vitamin, total polyphenol and antioxidant capacity determinations.

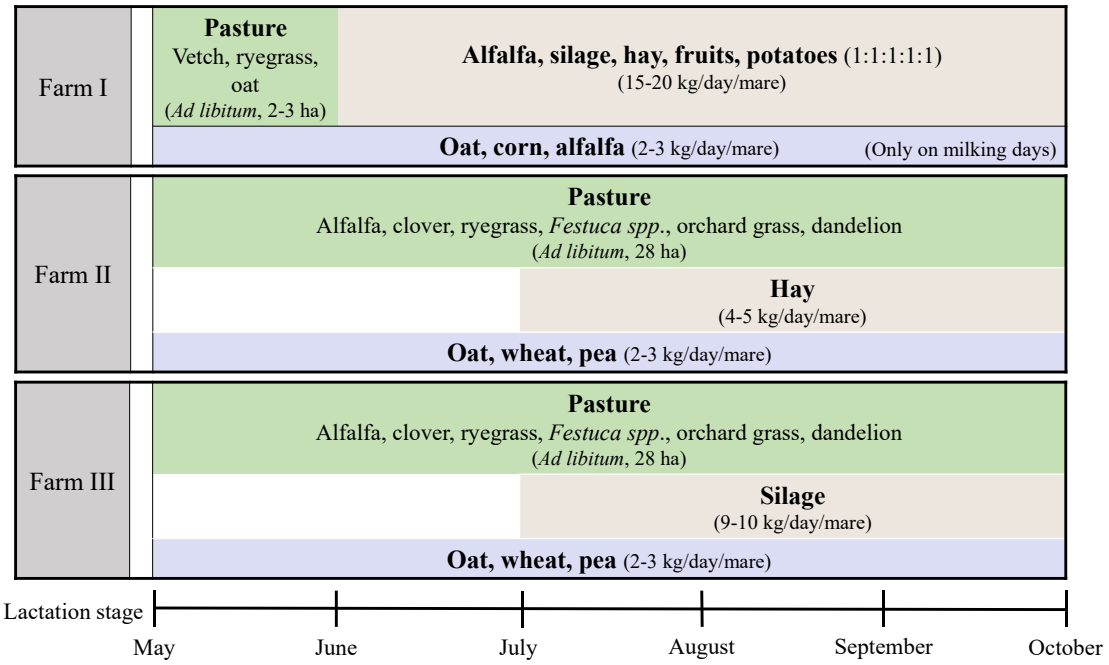


Figure 3.1. *Feeding regime of the mares from the commercial farms during the lactation period. Percentages and weights of the feeding ingredients are approximate.*

Among the 311 individual milk samples collected, a smaller set of samples was selected for proteomic analysis and protein digestion assays. This subsample was of 54 individual samples belonging to three mares *per* farm and to weeks 6, 10, 14 18, 22 and 26 of lactation.

3.2. Analysis of mare milk chemical composition

3.2.1. Determination of gross composition

Gross composition was analysed at the Lekunberri Milk Institute (Lekunberri, Spain), which is accredited (174/LE381) by the Spanish National Accreditation Body (ENAC) and performs routine milk analyses. Total lactose, fat, protein and dry matter contents (expressed as weight percentage) in mare milk were determined using near infrared spectroscopy (internal analytical method PE/ALVO/02).

3.2.2. Mineral element analysis

A semi-quantitative screening of 75 different elements was performed, in triplicate, in one mare milk sample. Those elements that were present in higher relative abundance and that were of nutritional interest were selected for further quantification in mare milk samples: Ca, K, P, S, Na, Mg, Cl, Zn, Fe, Cu and Mn were determined in the present study.

Mare milk samples were thawed in a water bath at 25 ºC for 60 min. After this, 0.2 (± 0.1) mg of milk were digested with 5 mL nitric acid (65 %, v/v) and 1 mL hydrogen peroxide (30-32 %, v/v). Digestion was carried out in a Speedwave-four microwave assisted digester (Berghof Products + Instruments, Berlin, Germany) at 190 ºC maximum temperature and 80 % power.

Samples diluted in Milli-Q water (Millipore, Merck) were analysed by inductively coupled plasma mass spectrometry (Agilent Technologies) equipped with an 8900 triple quadrupole, a MicroMist concentric nebulizer, and a Fassel-type torch (2.5 mm internal diameter). Plasma was generated with argon gas. A tandem mass spectrometry (MS/MS) scan was performed for P, S and Ca analysis, whereas a single Quad scan was used for the rest of the elements. Cell gases (ultrapure grade, 99.9999 % purity; Air Liquide, Madrid, Spain) were oxygen (30 % flow rate) for P and S, hydrogen (4.7 mL/min flow rate) for Ca, and helium (4.6 mL/min flow rate) for the remaining mineral elements. For quantification, rhodium was used as internal standard and calibration curves with five to eight levels at concentrations between 0.05 and 2,400 ng/mL were built with Na, Mg, K, Ca, P, S, Mn, Fe, Cu and Zn standard solutions (Inorganic Ventures, Christiansburg, USA; and SCP Science, Quebec, Canada). Coefficients of determination (R^2) higher than or equal to 0.994 validated the linearity of the quantitative analysis. In addition, relative

standard deviation (*RSD*) values lower than 8.29 % (except for S) and a recovery higher than 95 % were obtained for six replicates of standard solutions at the lowest and highest concentrations. Limits of quantification (LOQ) ranged between 4.4 and 2,688 µg/100 g milk and were defined as the lowest concentration at which a *RSD* value lower than 20 % was obtained by analysing ten replicates of standard solutions. Cl was analysed in semi-quantitative mode based on predefined default values (Krzciuk, 2016). Milk mineral elements were expressed as µg/100 g milk.

Mineral element analyses were assisted by the SGIker – Advanced Research Facilities of the University of the Basque Country (UPV/EHU, Vitoria-Gasteiz, Spain).

3.2.3. Fatty acid analysis

Mare milk samples (200 mL) were tempered in a water bath at 37 ºC for 20 min and centrifuged at 1,000 *g* and 4ºC for 20 min (SORVALL RC 5B Plus, Thermo Fisher Scientific, Waltham, USA) for cream separation following the procedure by Luna et al. (2005). The cream was further centrifuged at 22,000 *g* and 20 ºC for 30 min (SORVALL ST 16R, Thermo Fisher Scientific), and the upper lipid layer was collected for analysis.

Fatty acids in the lipid fraction of mare milk were derivatized following a miniaturized base-catalysed methylation (Aldai et al., 2012; Cruz-Hernandez et al., 2004) using 50 µL of an internal standard containing 13:0 (4 mg/mL in toluene) and 23:0 (4 mg/mL in toluene) added to a lipid drop of 4.90 mg average weight. This was mixed with 1.6 mL hexane, 40 µL methyl acetate and 100 µL sodium methoxide in methanol (0.5 N). After mixing, tubes were maintained at room temperature for 15 min and then at -20 ºC for 10 min. After, 60 µL oxalic acid in diethyl ether (at saturation) and 100 µL distilled water were added. The mixture was centrifuged at 800 *g* and room temperature for 5 min (SORVALL ST 16R, Thermo Fisher Scientific), and the upper phase was transferred to a gas chromatography (GC) vial containing anhydrous sodium sulphate crystals for analysis.

Mare milk fatty acid methyl esters (FAME) were analysed by GC coupled to a flame ionisation detector (FID; model 7890A) and equipped with a model 7693 autosampler (Agilent Technologies, Santa Clara, USA). A Supelco SP2560 capillary column (100 m x 0.25 mm internal diameter, 0.2 m film thickness; Merck, Darmstadt, Germany) was used for FAME separation. Analytical conditions were set as follows: 1 µL injection volume with 50/1 split ratio, hydrogen at 1 mL/min constant flow as carrier gas, a gradient

temperature program described by Kramer et al. (2008) with a plateau at 175 ºC, and injector and detector port temperature at 250 ºC.

Peak identification was done by comparison with #463 and #603 FAME reference standard mixtures, 13:0, 21:0 and 23:0 methyl ester individual reference standards (Nu-Chek Prep, Elysian, USA), linoleic acid and linolenic acid isomers, Supelco 37 Component FAME Mix (Sigma-Aldrich, Madrid, Spain), and bacterial FAME mixture containing BCFAs (Matreya, Pleasant Gap, USA). When a reference standard was not available, peaks were identified using retention times and elution orders as previously reported (Belaunzaran, 2017). For peak quantification, internal standards and theoretical response factors of individual fatty acids to GC-FID were used (Wolff et al., 1995). Internal validation of the GC-FID method is detailed in **Manuscript 1**. Limit of detection and LOQ were set as 0.0125 and 0.0250 mg/100 g milk, respectively. R^2 of calibration lines were above 0.999 and repeatability of quantitative data expressed as *RSD* was below 5 %. Milk fatty acids were expressed as mg/100 g milk.

3.2.4. Water-soluble vitamin analysis

Mare milk samples were thawed in a water bath at 25 ºC for 60 min. Then, they were prepared and analysed according to the method developed by Zafra-Gómez et al. (2006). In brief, 5 (± 0.0001) g of milk were mixed with 750 µL of a precipitation solution containing zinc acetate dihydrate (91 g/L), tungstophosphoric acid hydrate (55 g/L) and glacial acetic acid (5.8 %, v/v) in Milli-Q water (Millipore, Merck). After mixing well, clarification was allowed for 15 minutes, and the solution was centrifuged at 3,500 *g* and 20 ºC for 5 min (SORVALL ST 16R, Thermo Fisher Scientific). Supernatants were filtered through OlimPeak 0.2 µm pore size nylon filters (Teknokroma, Barcelona, Spain) and transferred into amber analytical vials. Dark conditions were maintained throughout the entire sample preparation process.

For water-soluble vitamin analysis, a high-performance liquid chromatography (HPLC) separation equipment (model 2695) coupled to fluorescence (FLD; model 2475) and diode-array (model 2998) tandem detectors (Waters, Milford, USA) was used. Compounds were separated with a C18 Spherisorb ODS-2 column (25 cm x 4.6 mm, 3 µm pore size; Waters). The mobile phase consisted of a gradient of methanol and an aqueous phosphate buffer at pH 2.95 (Zafra-Gomez et al., 2006). Injection volume was 50 µL, flow rate 1 mL/min, and column temperature was 40 ºC. FLD was programmed at

290/410 nm (excitation/emission) wavelength for pyridoxine and pyridoxal, and at 400/520 nm for riboflavin. Diode-array detector was programmed at 195 nm for pantothenic acid, 245 nm for thiamine, 261 nm for nicotinic acid and niacinamide, 282 nm for folic acid and ascorbic acid, and 370 nm for cyanocobalamin. Identification was done by comparing retention times with high purity (> 90 %; Sigma-Aldrich) reference standards. Quantification was performed using calibration curves with reference standards of individual vitamins at 4-6 concentration levels within a range of 0.001 to 90 µg/mL (depending on the vitamin). All R^2 obtained for the calibration lines were higher than 0.999 except for riboflavin (0.957), and *RSD* values were lower than 6.8 % for six replicates of standard solutions at a concentration range of 4.5-9.0 µg/mL. Recovery values of standard solutions analysed in triplicate overall ranged between 80.4 and 104.0 %. LOQs were established as the lowest concentration of individual standards that followed a linear correlation with higher concentrations and that presented a *RSD* lower than 12 %. LOQs of water-soluble vitamins ranged between 0.076 and 90 µg/100 g milk. Milk water-soluble vitamins were expressed as µg/100 g milk.

3.2.5. Fat-soluble vitamin analysis

Tocols (tocopherols, tocotrienols and their esters) and retinoids (retinol and retinyl esters) were simultaneously extracted from individual mare milk samples following the liquid-liquid extraction procedure without saponification described by Valdivielso et al. (2015). Mare milk samples were thawed in a water bath at 25 ºC for 60 min. Fat-soluble vitamins were extracted from 2 ± (0.0001) g of milk individual samples with the addition of ethanol, hexane/ethyl acetate (90/10, v/v), ultrapure water and diethyl ether in several steps. Butylated hydroxytoluene (100 µL *per* sample) was also added to protect samples against lipid oxidation. Samples were kept in an ice-bath and under dim light throughout the entire extraction process. Polar and non-polar phases were separated using centrifugation at 4 ºC for 5 to 10 min (SORVALL RC 5B Plus, Thermo Fisher Scientific), and the upper organic phase containing fat-soluble vitamins was collected. Before analysis, the organic phase was evaporated under a nitrogen flux, dissolved in 1.5 mL hexane/isopropanol (99/1), and filtered through 0.22 µm pore size Durapore polyvinylidene fluoride membrane filters (Millipore, Merck).

Extracts containing fat-soluble vitamins were analysed using a HPLC separation equipment (model 1260) coupled to FLD (model 1260 Spectra) (Agilent Technologies). A Luna Silica column (100 x 3 mm, 3 µm particle size; Phenomenex, Torrance, USA)

was used for separation, using 1,4-dioxane and n-hexane as mobile phase. Injection volume was 20 µL, flow rate was set at 1 mL/min, and column temperature was 22 ºC. FLD operated at 298 nm excitation wavelength, and 328 and 475 nm emission wavelengths for tocols and retinoids, respectively. External calibration with standard solutions (> 90 % purity; Sigma-Aldrich) was used for quantification. Calibration curves were built at four concentration levels from 0.01 to 0.5 µg/mL. Linear calibrations presented R^2 higher than 0.996, and *RSD* values lower than 6.9 % for five replicates of standard solutions at low and high concentrations. Six replicates of standard solutions at low and high concentrations were used for accuracy evaluation, and recovery values between 88.1 and 110.9 % were found. LOQs were established as the lowest concentration of standard solutions with a *RSD* value lower than 20 % for ten replicates. LOQ was established as 0.75 µg/100 g milk for all tocols and retinoids. Milk fat-soluble vitamins were expressed as µg/100 g milk.

Fat-soluble vitamin analyses were assisted by the SGIker – Advanced Research Facilities of the University of the Basque Country (UPV/EHU, Vitoria-Gasteiz, Spain).

3.2.6. Total polyphenol analysis

The content of phenolic compounds in mare milk was measured with spectrophotometry following the method described by Vázquez et al. (2015). Mare milk samples were thawed at 37 ºC for 45 min in a water bath. Then, 8 (± 0.0001) g of milk were clarified with 500 µL Carrez I solution (hexacyanoferrate (II) trihydrate in ultrapure water, 0.15 g/mL), 500 µL Carrez II solution (zinc sulfate heptahydrate in ultrapure water, 0.30 g/mL), and 5 mL acetonitrile. The solution was adjusted to 25 mL with a methanol/ultrapure water solution (1/1, v/v) and clot precipitation was allowed for 25 min at room temperature. Clarified samples were centrifuged at 4,700 *g* and room temperature for 15 min (SORVALL ST 16R, Thermo Fisher Scientific).

For analysis, 60 µL of supernatant, standard solution (for calibration curves) or distilled water (for blanks) were mixed with 65 µL of Folin-Ciocalteu reagent (2 N), 625 µL sodium carbonate (7 % in water, w/v) and 750 µL ultrapure water. Reaction was allowed for 90 minutes at room temperature, and absorbance was measured at 750 nm in a Cary 50 Bio ultraviolet and visible (UV/VIS) spectrophotometer (Varian, Agilent Technologies). The entire procedure was done under dark conditions. For quantification, calibration curves at five concentration levels from 1.25 to 250 µg/mL were built with

gallic acid as standard. Linear calibrations showed mean R^2 values of 0.9995, and *RSD* for five milk sample replicates was 2.82 %. Results were expressed as gallic acid equivalents (GAE)/100 g milk.

3.2.7. Determination of the total antioxidant capacity

For the antioxidant capacity assay, the 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) method was followed as described by Gila-Díaz et al. (2020). The antioxidant capacity was measured in whole mare milk and in the whey fraction (after casein precipitation) to evaluate the contribution of caseins to the total antioxidant activity of milk. A phosphate buffer solution (PBS; pH 7.4, 5 mM) was prepared with sodium chloride (4 g/L), potassium chloride (0.10 g/L), disodium phosphate (0.72 g/L) and potassium phosphate monobasic (0.12 g/L) in ultrapure water. The $ABTS^{\bullet+}$ stock solution consisted of 7 mM ABTS and 2.45 mM potassium persulfate in ultrapure water (final concentrations). This solution was oxidized in agitation for 12-16 h. For $ABTS^{\bullet+}$ working solution preparation, $ABTS^{\bullet+}$ stock solution was diluted in PBS until an absorbance of 0.70 ± 0.02 at 734 nm wavelength measured in a Cary 50 Bio UV/VIS spectrophotometer (Varian, Agilent Technologies).

Mare milk samples were thawed at 25 ºC for 60 min in a water bath. The whey fraction of mare milk samples was obtained by casein acidic precipitation (Ochirkhuyag et al., 2000). A total of 10 (± 0.0001) g of 20 randomly selected mare milk samples were acidified with lactic acid (10 %, v/v) until pH 4.2 (isoelectric point of mare milk according to Egito et al. (2001)). Once the clot was formed, samples were centrifuged at 3,500 *g* and 22 ºC for 30 min using a SORVALL ST 16R equipment (Thermo Fisher Scientific). The supernatant was collected and casein pellets were washed with 10 mL ultrapure water at pH 4.2 and 22 ºC. The supernatant was collected and pooled with the first supernatant.

Mare milk samples (75-150 µL) were diluted in PBS (whole milk 1/20 and whey fraction 1/10, v/v). After this, 150 µL of diluted sample, standard (calibration) or PBS (control) were mixed with 1,350 µL $ABTS^{\bullet+}$ working solution, and the reaction was let for 10 min at 25 ºC. To assess the turbidity of each milk sample, 150 µL of diluted sample were mixed with 1,350 µL PBS. Absorbance was read at 734 nm wavelength in a Cary 50 Bio UV/VIS spectrophotometer (Varian, Agilent Technologies). The whole process, from preparation of reagents to absorbance measurement, was done under darkness. For quantification purposes, calibration curves at five levels and concentrations between 0.01

and 0.25 mM were built using 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) as standard. Mean R^2 values were 0.994, and *RSD* values for five sample replicates were 0.53 % for whole milk and 2.84 % for the whey fraction.

Results were expressed as Trolox equivalents (based on calibration curves) or as radical scavenging activity (percentage of $ABTS^{\bullet+}$ radical cation inhibition) calculated as follows:

$$\text{Inhibition } \% = \left[\frac{A_{control} - A_{sample}}{A_{control}}\right] \times 100$$

where $A_{control}$ was absorbance of the control, and A_{sample} was the absorbance of the sample corrected with the absorbance of turbidity.

3.2.8. Proteomic analysis

The workflow of the proteomic analysis carried out on the selected milk sample subset (n = 54) is illustrated in **Figure 3.2**. This analysis was performed with the technical support of the Proteomics Platform from the CIC bioGUNE research centre (Derio, Spain).

3.2.8.1. Peptide and protein extraction

The first step in the proteomic analysis of mare milk consisted of a purification step of milk proteins and peptides with the intention of avoiding potential interferences with other components. First, mare milk samples were thawed overnight at 4 ºC and a pool of equal volumes of all the individual mare milk samples was prepared. Then, peptides and proteins present in individual mare milk samples and in the pool were isolated by addition of acetone at -20 ºC in a 1/6 milk/acetone ratio (v/v). Samples were centrifuged at 15,000 *g* and room temperature for 10 min using Microfuge 18 and Microfuge 22R equipment models (Beckman Coulter, Pasadena, USA). Supernatants from individual milk samples were transferred to separate microtubes (S1 fraction; **Figure 3.2**), whereas the pool supernatant was discarded. Precipitated proteins from the pool (P1 fraction) and from individual milk samples (P2 fraction) (**Figure 3.2**), and the S1 fraction were dried in a miVac centrifugal concentrator (Genevac, Ipswich, UK). All the dried fractions were kept at -20 ºC until analysis.

Two protein digestion methods were applied to milk samples in order to acquire a broader spectrum of protein identifications.

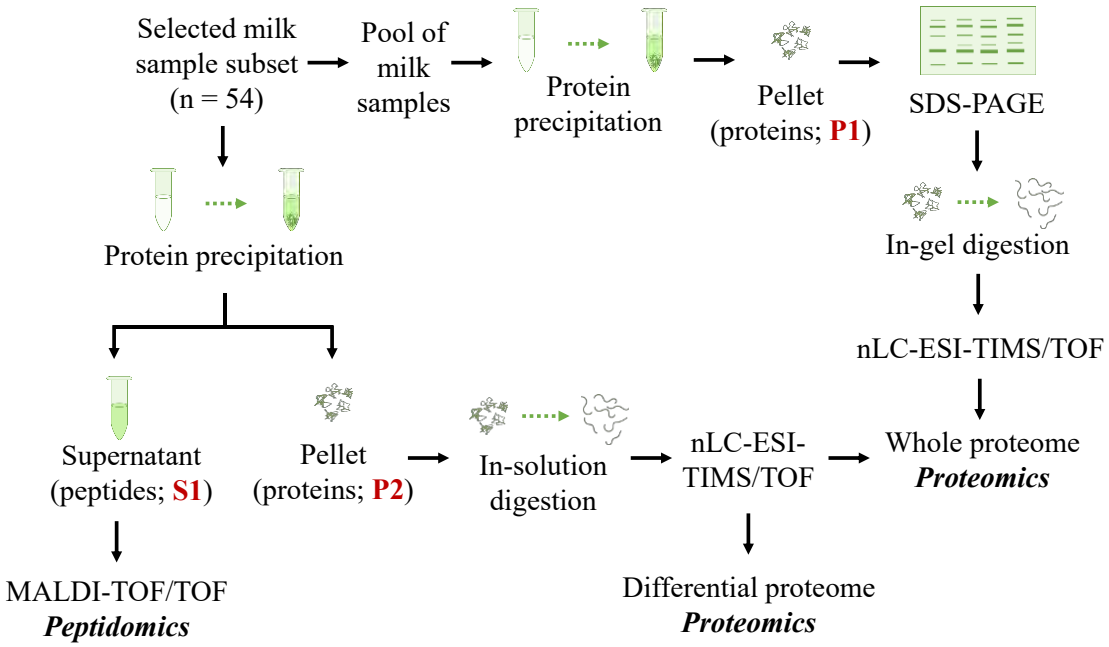


Figure 3.2. *Workflow of the proteomic analysis of mare milk samples. ESI: electrospray ionization; MALDI: matrix-assisted laser desorption/ionization; nLC: nano-liquid chromatography; SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TIMS/TOF: trapped ion mobility and time of flight mass spectrometry; TOF/TOF: time of flight tandem mass spectrometry.*

3.2.8.2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and in-gel tryptic digestion

The first one consisted of a protein separation step using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by an in-gel tryptic digestion. This method allowed the description of the whole proteome of mare milk.

Protein separation with gel electrophoresis was optimized by testing three different non-gradient gels: tris-glycine 10 % acrylamide, tris-glycine 15 % acrylamide, and tris-tricine 10 % acrylamide. Tris-glycine 10 % was a precast gel (Sigma-Aldrich), whereas tris-glycine 15 % and tris-tricine gels were prepared in the laboratory following standardized internal methods. As depicted in **Figure 3.3**, tris-tricine gels are more efficient at separating proteins with low molecular mass, whereas tris-glycine gels with 10 % acrylamide perform better at resolving larger proteins, and tris-glycine gels with 15 % acrylamide separate proteins with intermediate molecular mass.

The P1 fraction (proteins precipitated from the pool) was suspended in a buffer containing 2 M thiourea, 7 M urea, 4 % detergent (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; CHAPS) and 200 mM dithiothreitol (DTT). The protein solution was diluted with a 6x sodium dodecyl sulfate (SDS) sample buffer in ratios that ranged from 1/48 to 1/3 sample/buffer (v/v). Samples were subjected to SDS-PAGE using the BenchMark protein ladder solution (Invitrogen, Thermo Fisher Scientific) as marker. After electrophoresis, gels were washed with ultrapure water and immersed into a 40 % ethanol and 10 % acetic acid aqueous solution (v/v) for protein fixation. After 30 min, the fixation solution was discarded, and protein bands were stained overnight and under dark conditions using the SYPRO Ruby protein gel stain solution (Invitrogen, Thermo Fisher Scientific). For de-staining, gels were submerged into a 10 % ethanol and 7 % acetic acid aqueous solution (v/v) for 30 min. Gels were washed twice with ultrapure water and scanned in an ultraviolet (UV) gel imaging system (Bio-Rad ChemiDoc XRS System with Universal Hood II, Bio-Rad, Hercules, USA). Gel images are shown in **Figure 3.3**. Based on the results obtained from gel scanning, tris-glycine 15 % acrylamide gel was selected for protein digestion and proteomic analysis.

Separated protein bands (**Figure 3.3**, tris-glycine 15 % acrylamide gel, dilution 1/48) were excised into 10 separate fragments with a scalpel. The 10 fragments were cut into 1 mm^3 fragments, washed with ultrapure water and with ammonium bicarbonate (50 mM in water), and dehydrated by adding pure acetonitrile. All supernatants were discarded. Then, proteins in the gels were reduced with DTT (10 mM, in ammonium bicarbonate 100 mM in water) at 56 ºC for 30 min. After reduction, proteins were alkylated with chloroacetamide (55 mM, in ammonium bicarbonate 100 mM in water) for 30 min at room temperature and under dark conditions. Gel pieces were dehydrated with pure acetonitrile and incubated in trypsin (12.5 ng/µL, in ammonium bicarbonate 50 mM in water) overnight at 37 ºC. After tryptic digestion, the supernatants containing the released peptides were collected, and in order to ensure an optimum peptide recovery, gel pieces were dehydrated with pure acetonitrile and rehydrated with 0.1 % TFA (in water, v/v) several times. All liquid phases extracted after this washing process were pooled with the first supernatant, and the peptide solution was dried in the centrifugal concentrator and kept at 4 ºC until analysis. SDS-PAGE and in-gel trypsinization are regular procedures used in proteomics that, as in the present study, are usually based in standardized internal methods.

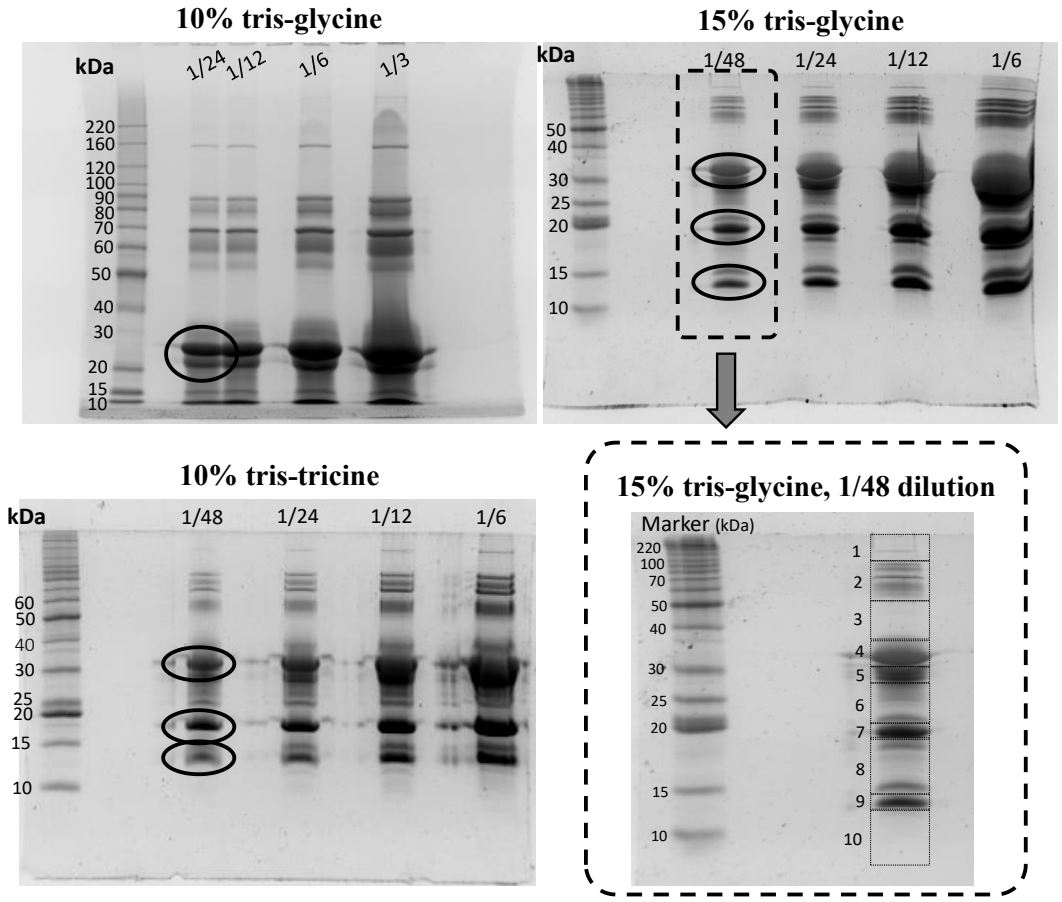


Figure 3.3. *Protein separation from a pool of mare milk samples using different sodium dodecyl sulfate-polyacrylamide gels (10 % and 15 % tris-glycine, and 10 % tris-tricine), and ten excisions made for in-gel digestion (15 % tris-glycine, 1/48 dilution). The first row in all gels corresponds to the marker (BenchMark protein ladder solution, Thermo Fisher Scientific), and other rows correspond to the mare milk sample pool diluted with 6x sodium dodecyl sulfate sample buffer at different ratios, ranging from 1/48 to 1/3. The most intense gel bands are marked with an ellipse.*

3.2.8.3. Filter-aided sample preparation and in-solution tryptic digestion

The second protein digestion method consisted of an in-solution digestion based on filter-aided sample preparation, without prior protein separation. This method allowed the description of the whole proteome of mare milk, as well as a differential proteomic analysis in which the effect of management system and lactation stage were studied.

The P2 fraction (proteins precipitated from individual milk samples) was suspended in a buffer containing 2 M thiourea, 7 M urea, 4 % CHAPS and 200 mM DTT. Sample preparation was conducted following a filter-aided sample preparation method (Berger et al., 2015; Wiśniewski et al., 2009). In 96-well plates (MultiScreen HTS-HV 96-well

plates with 0.45 μm hydrophilic Durapore polyvinylidene fluoride membrane, Merck), a sample volume adjusted to 30 μg of protein was washed twice with urea 8 M, discarding the filtrates. Then, proteins were alkylated with chloroacetamide (25 mM, in ammonium bicarbonate 50 mM in water) for 30 min at room temperature and under dark conditions. Alkylated proteins were washed three times with ammonium bicarbonate (50 mM in water) and incubated with 1 μg trypsin overnight at 37 ºC. The liquid phase containing released peptides was filtered through the plate membranes aided by centrifugation at 200 *g* for 2 min (Allegra X-14R, Beckman Coulter, L'Hospitalet de Llobregat, Spain). Filters were washed twice with aqueous solutions of acetonitrile (40 %, v/v) and formic acid (0.1 %, v/v) and centrifuged again at 200 *g* for 2 min. All filtrates obtained after trypsin digestion were pooled, dried in the centrifugal concentrator and kept at 4 ºC until analysis.

3.2.8.4. Peptide purification

Peptides formed after tryptic digestion of proteins (P1 fraction, in-gel digestion; and P2 fraction, in-solution digestion) were suspended in a formic acid aqueous solution (0.1 %, v/v). Then, peptides were purified and concentrated using C18 pipette tips (OMIX C18, Agilent Technologies) previously washed with pure acetonitrile and with trifluoroacetic acid (TFA) aqueous solution (0.1 %, v/v). Samples were loaded into the pipette tips and acidified with TFA aqueous solution (0.1 %, v/v). Peptides were then eluted with a solution of 70/30 (v/v) pure acetonitrile/TFA aqueous solution (0.1 %, v/v) and dried in the centrifugal concentrator.

3.2.8.5. Nano-liquid chromatography mass spectrometry

After purification and concentration of peptides using C18 pipette tips, an additional purification step prior to chromatographic analysis was conducted according to manufacturer instructions (Evosep, Odense, Denmark). Samples (P1 and P2 fractions) were analyzed by nano-liquid chromatography (nLC) with electrospray ionization (ESI; Evosep) coupled to trapped ion mobility and time of flight tandem mass spectrometry (TIMS/TOF) (timsTOF PRO, Bruker Daltonics, Billerica, USA). For separation purposes, a C18 nano-flow column (15 cm x 150 μm internal diameter, 1.9 μm particle size; EV1106, Evosep) was used, operated according to Evosep 30SPD standardized method (44 min gradient, 500 nL/min flow). Scans were acquired using a data-dependent approach and positive ion polarity, and were limited to 100-1,700 *m/z*. Proteins were identified and quantified with the PEAKS Xpro software (Bioinformatics solutions,

Waterloo, Canada). The score threshold providing a 1 % identification false discovery rate was automatically calculated by the software. Precursor and fragment tolerances were 20 ppm and 0.05 Da, respectively. Carbamidomethylation of cysteine and oxidation of methionine were considered fixed and variable modifications, respectively

3.2.8.6. Peptidomic analysis

In addition to the study of mare milk proteome, this work searched for differences in the profile of endogenous peptides in mare milk from different management systems or lactation stages. In this case, individual peptides were not identified, but peptide profile fingerprints were taken from each sample and milk samples from different farms and lactation stages were compared.

To accomplish this, S1 fractions (**Figure 3.2**) containing endogenous peptides were suspended in an aqueous TFA solution (0.1 %, v/v), and 2 µL were purified using ZipTip C18 micro-columns (Millipore, Merck). Pipette tips were previously washed with pure acetonitrile and an aqueous TFA solution (0.1 %, v/v). Endogenous peptides in the tip were acidified with an aqueous TFA solution (0.1 %, v/v) and eluted with α-cyano-4-hydroxycinnamic acid (10 mg/mL in 70/30 pure acetonitrile/TFA aqueous solution 0.1 %, v/v). Elutions were placed on a GroundSteel massive 384 target plate (Bruker Daltonics) and allowed to air dry at room temperature. Analysis was performed using matrix-assisted laser desorption/ionization (MALDI) coupled to time of flight tandem mass spectrometry (TOF/TOF) in reflector mode (Autoflex III, Bruker Daltonics). Analytical conditions were set as 1,000-6,000 Th window, refletron positive mode, ion source 1 at 17 kV, ion source 2 at 18.5 kV, lens at 9 kV, pulsed ion extraction of 120 ns, and high gating ion suppression up to 600 Mr. External calibration was made using pepmix standard calibration mixture (Bruker Daltonics). FlexControl software (version 3.0; Bruker Daltonics) was used for data acquisition, and FlexAnalysis (version 3.0) and ClinProtTools (version 3.1) softwares (Bruker Daltonics) were used for spectra analysis.

3.3. Protein digestion

The workflow followed for the study of mare milk protein digestion is shown in **Figure 3.4**. This study aimed to describe protein digestibility and peptide release from mare milk proteins during human gastrointestinal digestion, following a static *in vitro* simulated

digestion method applied to the selected milk sample subset (n = 54). For protein digestibility assessment, individual milk samples that corresponded to two consecutive lactation weeks of each animal were pooled, reducing sample size to 27 pool samples that belonged to three lactation stages (early lactation, weeks 6 and 10; mid lactation, weeks 14 and 18; late lactation, weeks 22 and 26). These pool samples were individually digested (intestinal digestion) in triplicate at different digestion sets and days. On the other hand, the study of peptide release comprised gastric and intestinal digestion of individual milk samples.

For both digestibility and peptide release assays, mare milk was subjected to a static *in vitro* simulated gastrointestinal digestion following the method developed by Minekus et al. (2014), which was later improved by Brodkorb et al. (2019). This last method is known as the INFOGEST 2.0 protocol which divides the gastrointestinal digestion into three consecutive steps: i) oral digestion, which includes the addition of amylase free simulated salivary fluid (pH 7); ii) gastric digestion, which includes the addition of simulated gastric fluid (pH 3) containing pepsin (2,000 U/mL of digesta); and iii) intestinal digestion, which includes the addition of the simulated intestinal fluid (pH 7) containing pancreatin (100 U trypsin activity/mL digesta) and bile (10 mM final concentration). Fluids were prepared in the laboratory as described by the INFOGEST 2.0 protocol.

Prior to gastrointestinal digestion, 800 µL of the simulated salivary fluid were dried in a centrifugal concentrator (CentriVap, Labconco, USA). A mare milk volume normalized to 40 mg of protein (volumes were normalized individually for each sample/pool) was added to the dried salivary fluid. Then, 1.6 mL of simulated gastric fluid were incorporated and the solution was incubated at 37 ºC for 120 min at constant rotation. To continue with intestinal digestion, 4 mL of simulated intestinal fluid were added to gastric digesta and this was incubated at 37 ºC for 120 min at constant rotation. In order to stop gastric digestion, pH was increased to 7 with sodium hydroxide (2 M). To stop intestinal digestion, 16 µL of a protease inhibitor (4-(2-aminoethyl)benzenesulfonyl fluoride 500 mM solution; Pefabloc, Roche, Basel, Switzerland) was added. After stopping the reaction, all digesta were immediately immersed in liquid nitrogen and kept at -20 ºC until analysis. In this work, gastric digestion will refer to the oral plus gastric digestion sequence, and intestinal digestion will refer to oral plus gastric plus intestinal digestion sequence.

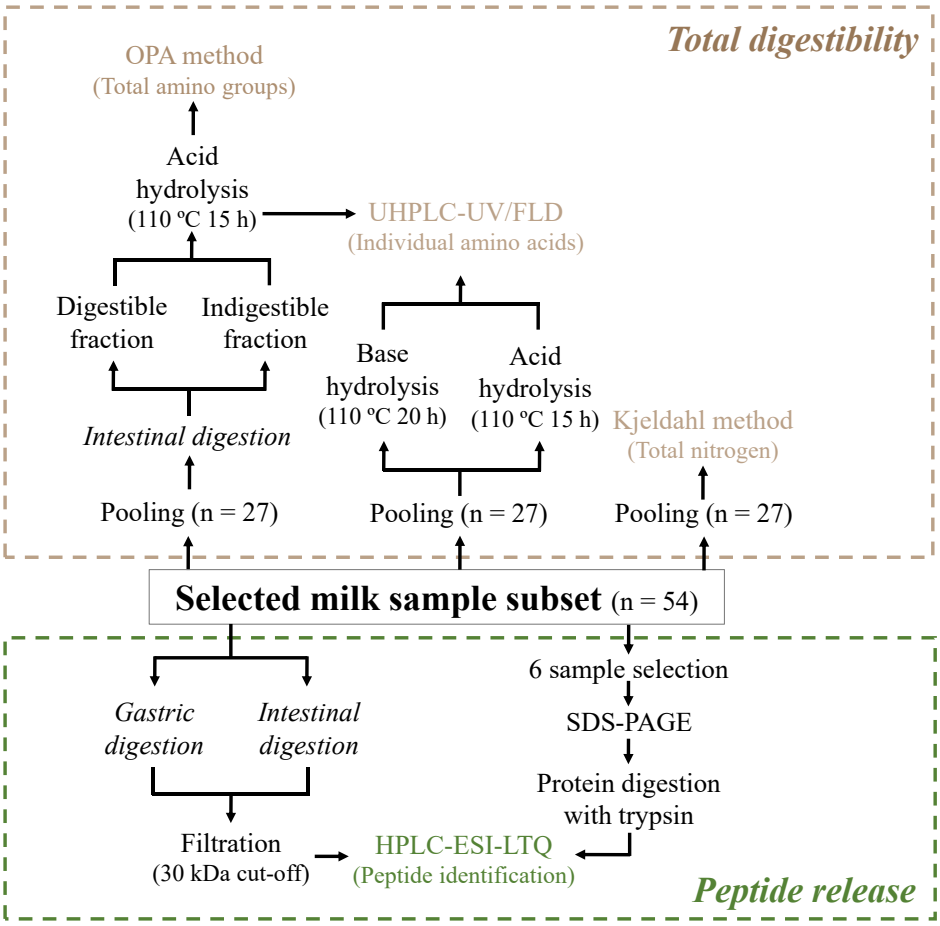


Figure 3.4. *Workflow of the protein digestion study of mare milk. ESI: electrospray ionization; FLD: fluorescence detector; HPLC: high-performance liquid chromatography; LTQ: linear ion trap mass spectrometer; OPA: o-phthalaldehyde; SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis; UHPLC: ultra-high-performance liquid chromatography; UV: ultraviolet (detector).*

For the protein digestibility assay, a protein-free cookie (Moughan et al., 2005) was digested as blank parallel with milk digestions. This allowed to estimate the enzyme background for corrections. One cookie was digested within each set of digestions, which consisted of 5-6 pool milk samples.

3.3.1. Protein digestibility assay

3.3.1.1. Sample preparation

Pool samples that underwent intestinal digestion were fractionated into digestible (absorbable) and indigestible (non-absorbable) phases (Sousa et al., 2023). Precipitation of the indigestible phase of food digesta was induced by addition of methanol (80 % final concentration, v/v) at -20 ºC for 1 h. Phases were separated aided by centrifugation at

2,000 *g* and 4 ºC for 15 min (Sorvall Legend XTR, Thermo Fisher Scientific). The digestible phase (supernatant) was collected, and a subsample of 220 µL was transferred to 2 mL glass vials, evaporated in a centrifugal concentrator (CentriVap) and kept at -20 ºC until analysis. The indigestible phase (pellet) was washed twice with methanol (100 %, v/v) at -20 ºC, discarding supernatants. Washed indigestible phases (whole pellet) were dried in the centrifugal concentrator, transferred to 10 mL glass vials and kept at -20 ºC until analysis.

Digestible and indigestible phases were separately hydrolysed for subsequent analysis (Sousa et al., 2023). Briefly, 220 µL ultrapure water, 120 µL 3,3'-dithiodipropionic acid (0.1 % in sodium hydroxide 0.2 M), 120 µL hydrochloric acid aqueous solution (0.2 M), 40 µL norvaline (10 mM in 0.1 M hydrochloric acid; internal standard), and 500 µL hydrochloric acid aqueous solution (37 %, v/v) were incorporated into digestible phase vials. For hydrolysis of the indigestible phase, 880 µL ultrapure water, 480 µL 3,3'-dithiodipropionic acid (0.1 % in sodium hydroxide 0.2 M), 480 µL hydrochloric acid aqueous solution (0.2 M), 160 µL norvaline (10 mM in 0.1 M hydrochloric acid; internal standard), and 2 mL hydrochloric acid aqueous solution (37 %, v/v) were incorporated. Samples were hydrolysed at 110 ºC for 15 h. Two cysteine standards (20 and 200 µM final concentrations) as well as undigested raw mare milk were also hydrolysed following the same procedure (Sousa et al., 2023). In the case of raw milk, a base hydrolysis was also done to quantify tryptophan. Briefly, 500 mg of milk were mixed with 170 mg starch, 200 µL 1-methionine-L-tryptophan standard (447439, Sigma-Aldrich) and 2 mL sodium hydroxide aqueous solution (8.4 M), and diluted to 4 mL with ultrapure water. The same procedure was repeated with an L-tryptophan high purity standard (Sigma-Aldrich). Base hydrolysis was performed at 110 ºC for 20 h.

3.3.1.2. Total amino group determination

Total amino groups (R-NH_2) present in hydrolysed digestible and indigestible phases were determined following the o-phthalaldehyde (OPA) method (Sousa et al., 2023). Samples were diluted with perchloric acid (0.5 M; 1/5 for digestible phase, 1/10 for indigestible phase), and derivatized with an OPA working aqueous solution containing sodium borate 50 mM, SDS 1 % (v/v), OPA 800 µg/mL, 2-mercaptoethanesulfonic acid sodium salt 4 mg/mL and Triton X-100 5 mg/mL (Sigma-Aldrich). Measurements were done in a UV/VIS spectrophotometer (Spectramax iD3, Molecular Devices, Wokingham,

UK) at 340 nm wavelength. A calibration curve with glutamic acid was built for quantification purposes, and perchloric acid (0.5 M) was used as blank.

3.3.1.3. Individual amino acid profile

Individual amino acids of the digestible and indigestible phases were quantified and compared with the undigested food (raw mare milk). The amino acid profiling was done according to Sousa et al. (2023), who based their procedure on the AOAC method 2018.06 for infant formula (Jaudzems et al., 2019). Hydrolysed digestible and indigestible phases, cysteine standards and raw mare milk (acid and base hydrolysis) were derivatized with an AccQ-Tag Ultra Derivatization Kit (Waters) as described by the manufacturer (Waters, 2007). Amino acids were separated using an ultra-high-performance liquid chromatography (UHPLC) equipment (Vanquish Flex, Thermo Fisher Scientific) and an Acquity UPLC BEH C18 column (150 x 2.1 mm, 1.7 µm film thickness; Waters). Detection was performed using an UV detector (Vanquish, Thermo Fisher Scientific) for individual amino acid determination, and a FLD (Vanquish, Thermo Fisher Scientific) for tryptophan determination. Analytical conditions were as described by the method of Waters (2007) for individual amino acids, and by ISO/DIS-13904 (2014) for tryptophan.

Total protein of raw mare milk pool samples was calculated from total nitrogen, which was analysed by the Kjeldahl method according to ISO 8968-3 (2007), using a nitrogen to protein conversion factor of 6.25.

3.3.1.4. Calculations

Results on protein digestibility were expressed as *in vitro* total digestibility (%), digestible indispensable amino acid ratio (DIAAR; %) and digestible indispensable amino acid score (DIAAS) as described by Sousa et al. (2023). The percentage of total digestibility and digestibility of individual amino acids was calculated based on the amount of total amino acids, total primary amines or individual amino acids, as follows:

$$In\ vitro\ \text{digestibility}\ (\%) = \frac{(\mathrm{Fs} - \mathrm{Cs})}{(\mathrm{Fs} - \mathrm{Cs}) + (\mathrm{Fp} - \mathrm{Cp})} \times 100$$

where Fs was the digestible phase (supernatant) of food digesta; Cs, the digestible phase (supernatant) of cookie digesta; Fp, the indigestible phase (pellet) of food digesta; and Cp, the indigestible phase (pellet) of cookie digesta. All values were expressed as mg of amino acid.

In vitro DIAAR was calculated with individual amino acid data using the following formula:

$$In\ vitro\ \text{DIAAR}\ (\%) = \frac{\text{EAA}/_{\text{P}}\ \text{sample} \times \text{IVD}}{\text{Reference requirements}}$$

where EAA was the individual essential amino acid content (mg) in food, P was the total protein content (g) in food, IVD (%) was the *in vitro* digestibility of the corresponding individual essential amino acid, and reference requirements were the reference daily requirements of individual essential amino acids (mg/g protein) according to FAO (2013) for each population group (infant, child or adult).

In vitro DIAAS is the lowest DIAAR of a food or sample, and the essential amino acid corresponding to the DIAAS represents the limiting amino acid.

3.3.2. Peptide release

3.3.2.1. Identification of major proteins in raw mare milk

In the present study, only peptides released from major proteins in mare milk were considered. This required a previous characterization of major mare milk proteins. Six undigested mare milk samples belonging to six lactation weeks from the same animal were diluted 1/25 with ultrapure water and combined with a buffer containing tris-hydrochloric acid (350 mM, pH 6.8), SDS (10 %, w/v), DTT (100 mM) and glycerol (50 %, v/v). Samples were loaded into a 15 % polyacrylamide gel and proteins were separated by SDS-PAGE (Kopf-Bolanz et al., 2012) using the BenckMark protein ladder solution (Invitrogen, Thermo Fisher Scientific) as marker. Then, gel bands were excised and digested with trypsin overnight at 37 ºC.

3.3.2.2. Peptide isolation from milk digesta

Peptides that were released from mare milk proteins after gastric and intestinal digestion were monitored to study the pattern of hydrolysis. Individual digested mare milk samples (gastric and intestinal digestion) were filtered through 30 kDa molecular weight cut-off micro-spin filters (UFC5030; Millipore, Merck) aided by centrifugation at 18,000 *g* and 4 ºC for 10 min (5427R, Eppendorf, Basel, Switzerland). Eluted samples were placed into 300 µL HPLC vials and kept at -20 ºC until analysis.

3.3.2.3. Protein and peptide identification

Major proteins in mare milk separated by SDS-PAGE and peptides released after gastrointestinal digestion were analysed with an HPLC equipment (Rheos 2200 Micro HPLC, Flux Instruments, Basel, Switzerland) coupled with an ESI source and attached to linear ion trap mass spectrometry (LTQ; LTQ XL, Thermo Fisher Scientific). Proteins were separated using an X Terra MS C18 column (100 × 2.1 mm, 3.5 µm film thickness; Waters), and the analysis was performed as previously described (Egger et al., 2019).

Peptides with at least five amino acids and an *m/z* ratio between 300 and 2,000 were considered. MS/MS spectra were analysed using the Mascot search engine (Matrix Science, London, UK) under data-dependent analysis. Proteins were checked against an in-house created database that included milk protein references from UniProtKB (2023) database. Bioactive peptides were searched in the Milk Bioactive Peptide Database (MBPDB Search; Nielsen et al., 2017), which compiles all milk-derived bioactive peptides reported in the scientific literature.

3.4. Data treatment

Mare milk characterization results were statistically analysed considering two main factors: commercial farm (farm I, II and III) and lactation time (lactation weeks or lactation stage, depending on the sample pooling performed for the analytical procedures). Time of lactation was calculated based on parturition dates of individual mares.

All data was log transformed and normality and homoscedasticity were checked for milk sample groups defined by commercial farm and lactation time using Shapiro-Wilk and Levene's tests, respectively. Logarithmic data was checked by box-plots, and values lower or higher than three times the interquartile range were identified as outliers and excluded from the datasets.

Data obtained from the analysis of all milk samples (n = 311) for gross composition, fatty acids, mineral elements, water- and fat-soluble vitamins, total polyphenols and total antioxidant capacity were subjected to analysis of variance (ANOVA) using the linear mixed model (LMM) procedure. Prior to log transformation of raw data, results from individual milk samples were pooled in ranges of 14 lactation days, establishing the

earliest lactation time at weeks 3-4, and the last time at weeks 25-26. The LMM was constructed including individual animal as subject, commercial farm as fixed factor, and lactation time as repeated measure factor. The interaction term between farm and lactation time was also included in the model. The restricted maximum likelihood method was used to estimate the parameters of the model, and the compound symmetry matrix was selected for the repeated measures covariance structure according to the Akaike information criterion. The least square means of the dependent variables were included in a Tukey's test to compare different levels of the fixed factor (I = 3). Since some of the analysed compounds appeared under the LOQ in a number of milk samples, only variables presenting values over the LOQ in milk samples from at least two mares *per* farm and *per* lactation time were considered for the ANOVA.

Data obtained from the analysis of the subset of samples (n = 54) selected for proteomic analysis and protein digestion assays were subjected to the general linear model (GLM) of the ANOVA. In the case of the proteomic analysis, only data obtained from the analysis of the P2 fraction after in-solution digestion was selected for GLM analysis. In terms of protein digestion, results obtained from all determinations were included. Only variables with values over the LOQ in milk samples from at least two animals *per* farm and *per* lactation week were considered for statistical analysis. The GLM included farm (I = 3) and lactation stage (I = 3; considering lactation weeks 6 and 10 as early lactation, weeks 14 and 18 as mid lactation, and weeks 22 and 26 as late lactation) as fixed factors, individual animal (I = 9) as random factor nested within farm, and the interaction term between farm and lactation stage. Pairwise comparisons of different levels within the fixed factors were performed using Fisher's least significance difference test for proteomic analysis, and Tukey's test for protein digestion results.

A stepwise discriminant analysis (SDA) was applied to data from mare milk gross composition, mineral elements, fatty acids, water- and fat-soluble vitamins, polyphenols and total antioxidant capacity in order to classify mare milk samples according to management system (farms I, II and III) or lactation stage (early, mid and late lactation). In this case, lactation times from weeks 3 to 10 were considered as early lactation, weeks 11 to 18 as mid lactation, and weeks 19 to 26 as late lactation. The Wilks lambda method was used for SDA, and classification results after cross validation were only considered.

All the statistical analyses were carried out by the IBM SPSS Statistic software (version 28.0, IBM, Armonk, USA) and the significance level was declared at $P \leq 0.05$.

Bioinformatic tools were used to study the biological function of the proteins identified in the proteomic analysis. Gene symbols corresponding to each protein were searched in the UniProtKB (2023) database. All non-redundant proteins identified through nLC-MS/MS were uploaded to the PANTHER (2023) classification system (version 17.0) considering *Equus caballus* genome as background. This system classified proteins according to class and function (gene ontology (GO) analysis for molecular function, biological process and cellular component). A subsequent analysis of biological function was performed using ingenuity pathway analysis (Qiagen, Madrid, Spain) within the *Equus caballus* genome, which included ingenuity canonical pathways and upstream regulators related to the identified proteins in mare milk. Thresholds for statistical significance were set as –log (*P*-value) > 1.30 for canonical pathways, and as *P*-value overlap < 0.05 for upstream regulators.

The heatmaps used for the graphical description of peptide release patterns after mare milk digestion were created using R programming (RStudio version 4.3.9, Posit PBC, Boston, USA).

4 RESULTS AND DISCUSSION

The results included in this section comprise the analysis of mare milk samples for a wide range of chemical compounds, a proteomic approach, a simulation of protein digestion, and the study on the effect of lactation stage and management system on mare milk composition.

4.1. Characterization of the chemical composition of pasture-based mare milk

This subsection includes a brief description of the profile of different chemical compounds found in mare milk from Basque Mountain Horse breed, together with a comparison with available literature on mare milk composition.

4.1.1. Gross composition

Average gross composition of mare milk, which includes total fat, protein, lactose and non-fat dry matter, is reported in **Table 4.1**. Milk from Basque Mountain Horse mares presents similar protein, lactose and dry matter contents compared to data reported in literature from other horse breeds, which are about 1.3-2.8 g protein, 6.0-7.2 g lactose and 6.1-10.1 g dry matter *per* 100 g milk (Barreto et al., 2020; Čagalj et al., 2014; Cais-Sokolińska et al., 2018; Hachana et al., 2022; Markiewicz-Kęszycka et al., 2013).

Table 4.1. *Gross composition (g/100 g milk) of mare milk samples from the commercial farms over the lactation period (from May to October).*

	Mean	Min	Max	SEM
Fat	0.270	0.110	0.720	0.007
Protein	1.78	1.42	2.94	0.15
Lactose	6.75	5.59	7.40	0.02
Dry matter (non-fat)	9.36	8.49	10.4	0.02

Max: maximum; Min: minimum; SEM: standard error of the mean.

Average mare milk fat contents reported in literature for other horse breeds (0.38-2.11 g/100 g milk; Barreto et al., 2020; Čagalj et al., 2014; Cais-Sokolińska et al., 2018; Hachana et al., 2022; Kaić et al., 2019; Markiewicz-Kęszycka et al., 2013; Minjigdorj et al., 2012a) are overall higher than those found in milk from Basque Mountain Horse breed. Therefore, the low fat content appears to be a distinctive feature of the milk from this autochthonous horse breed.

4.1.2. Mineral element composition

Among all the mineral elements analysed, Mn was the only one found below the LOQ (4.38 µg/100 g milk) in most of the samples, regardless of individual animal, farm and lactation time. As reported in **Table 4.2**, Ca was the major mineral, followed by similar contents of K and P. Among trace elements, Zn and Fe were the most abundant. Average contents of most minerals found in the present research agree with values reported in literature for mare milk (26.1-138.0 µg Ca, 34.1-70.1 µg K, 15.2-88.4 µg P, 10.7-22.0 µg Na, 2.9-10.4 µg Mg, 0.11-0.41 µg Zn and 0.007-0.064 mg Cu *per* 100 g milk). Conversely, higher Fe contents were found in the present research compared to other studies (0.018-0.130 µg/g or mL) (Alipour et al., 2023; Anderson, 1991, 1992; Grace et al., 1999; Schryver et al., 1986a,b; Summer et al., 2004; Ullrey et al., 1966, 1974). Scientific literature concerning other minerals in mare milk is particularly scarce, but compared with the present study, others have reported higher abundance of Cl (15.0-19.8 mg/100 g; Summer et al., 2004) and S (19.6-23.0 mg/100 mL; Grace et al., 1999) in mare milk. Lower Cl content in Basque Mountain Horse milk samples might be due to a balance with lactose, Na or K contents, since all these components are involved in the osmotic pressure regulation of milk (Bijl et al., 2013). The lower S content in Basque Mountain Horse milk compared to Thoroughbred mares (Grace et al., 1999) could be the result of a lower total protein content (1.78 *vs.* 2.10 g/100 g milk, respectively), since protein, and specifically the S containing amino acids methionine and cysteine, are the main source of S (Nimni et al., 2007). Detailed information on mineral element composition in mare milk samples is given in **Publication 2**.

Table 4.2. *Macromineral and trace element composition (mg/100 g milk) of mare milk samples from the commercial farms over the lactation period (from May to October).*

	Mean	Min	Max	SEM
Ca	95.8	50.3	169	1.8
K	58.1	38.5	103	0.9
P	45.4	19.3	83.7	1.2
S	15.6	8.66	28.7	0.3
Na	14.4	6.90	62.0	0.4
Mg	6.41	2.92	11.8	0.11
Cl	4.93	1.65	26.2	0.24
Zn	0.250	0.0647	0.743	0.006
Fe	0.243	0.0323	2.45	0.018
Cu	0.0343	0.00503	0.126	0.0013

Max: maximum; Min: minimum; SEM: standard error of the mean.

4.1.3. Fatty acid composition

The average content of fatty acids in mare milk samples is shown in **Table 4.3**. About half of the fatty acids in mare milk (47.5 % of total fatty acids) were SFAs, whereas the other half were MUFAs (25.3 %) and PUFAs (26.5 %). This fatty acid distribution makes mare milk particularly rich in PUFAs, especially when compared with ruminant milk, in which only 2-7 % of fatty acids are PUFAs (Claeys et al., 2014). Such a high proportion of PUFAs in mare milk is a consequence of the equine digestive tract physiology and metabolism. Since equids are quite efficient at liberating and absorbing dietary triglycerides, and due to the post-absorptive location of the fermentative caecum-colon chamber, PUFAs obtained mainly from dietary forages and pasture are taken up directly and incorporated into milk with no, or very little, modifications (Belaunzaran et al., 2015; Warren & Vineyard, 2013). The SFA fraction of mare milk lipids was mainly composed of medium-chain fatty acids, being the major one palmitic acid (16:0) followed by lauric (12:0), myristic (14:0), capric (10:0) and caprylic (8:0) acids. MUFAs mainly consisted of *cis* isomers and oleic acid (9*c*-18:1) was the main one, followed by palmitoleic acid (9*c*-16:1). Regarding PUFAs, linolenic (18:3n-3) and linoleic (18:2n-6) acids were the most abundant ones, whereas other isomers were present in a concentration lower than 1 mg/100 g milk. This profile of major fatty acids in mare milk agrees with previous reports (Czyżak-Runowska et al., 2021; Devle et al., 2012; Pikul et al., 2008). In the present study, only the most abundant fatty acids of each group, *i.e.* 16:0, 9*c*-18:1, 18:2n-6 and 18:3n-3, accounted for 61 % of total fatty acids in mare milk.

Table 4.3. *Major fatty acid composition (mg/100 g milk) of mare milk samples from commercial farms over the lactation period (from May to October).*

	Mean	Min	Max	SEM
Saturated fatty acids				
8:0	7.41	0.977	30.6	0.33
10:0	15.9	2.03	66.4	0.8
11:0	3.39	0.0261	12.3	0.13
12:0	16.9	2.52	73.0	0.7
14:0	16.6	3.09	74.1	0.6
16:0	45.6	9.26	200	1.6
18:0	2.25	0.453	8.40	0.09
Branched-chain fatty acids				
iso-14:0	0.123	0.0172	0.435	0.005
iso-15:0	0.122	0.0183	0.339	0.004
anteiso-15:0	0.147	0.0236	0.499	0.006
iso-16:0	0.408	0.110	1.30	0.014
anteiso-17:0	0.325	0.0705	0.892	0.011
iso-18:0	0.155	0.0418	0.521	0.005
Monounsaturated fatty acids				
9*c*-14:1	1.46	0.314	4.94	0.04
9*c*-16:1	12.9	2.87	49.1	0.4
9*c*-17:1	1.06	0.0350	5.34	0.04
9*c*-18:1	38.5	7.14	152	1.6
11*c*-18:1	1.98	0.400	6.85	0.08
t-15:1	0.0548	0.0101	0.209	0.0023
9*t*-16:1	0.0571	0.0136	0.309	0.0021
14*t*-16:1	0.0325	0.00830	0.486	0.0025
9*t*-18:1	0.112	0.0305	0.506	0.005
11*t*-18:1	0.0411	0.0104	0.294	0.0020
Polyunsaturated fatty acids				
16:2n-6	0.206	0.0453	0.626	0.008
18:2n-6	20.9	5.04	87.1	0.8
20:2n-6	0.466	0.0964	1.73	0.015
20:4n-6	0.150	0.0392	0.479	0.005
16:3n-3	0.434	0.0320	1.90	0.021
18:3n-3	36.6	4.65	187	1.5
20:3n-3	0.943	0.200	4.07	0.033
20:4n-3	0.121	0.0318	0.488	0.004
20:5n-3	0.0899	0.0213	0.278	0.0033
22:5n-3	0.216	0.0600	0.966	0.008
22:6n-3	0.0501	0.0152	0.159	0.0018
9*t*,12*t*,15*c*-18:3	0.251	0.0408	0.954	0.009
9*c*,11*t*-18:2	0.158	0.0313	0.946	0.009
Fatty acid groups				
SFA	111	19.4	473	4
BCFA	1.31	0.301	3.55	0.04
cis-MUFA	58.2	11.4	225	2.2
trans-MUFA	0.390	0.108	1.17	0.013
MUFA	58.6	11.5	226	2.2
n-6 PUFA	21.9	5.31	90.3	0.8
n-3 PUFA	38.5	5.10	194	1.6
PUFA	60.7	12.4	285	2.3

BCFA: branched-chain fatty acid; Max: maximum; Min: minimum; MUFA: monounsaturated fatty acid; PUFA: polyunsaturated fatty acid; SEM: standard error of the mean; SFA: saturated fatty acid.

Among minor compounds, *iso*-16:0 and *anteiso*-17:0 were the principal BCFAs, the triene 9*t*,12*t*,15*c*-18:3 was the most abundant *trans* fatty acid and rumenic acid (9*c*,11*t*-18:2) was the only CLA isomer in mare milk. Milk BCFAs and *trans* fatty acids, including CLA, are mainly ruminal fermentation products formed from amino acids and lipids, respectively (Aldai et al., 2013; Taormina et al., 2020), so their content in mare milk is very low. Most studies describing the profile of mare milk fatty acids failed to identify and quantify BCFAs and other minor unsaturated fatty acid isomers, probably due to the inability to resolve these isomers through chromatography. This is often the case when using columns shorter than 100 m, inadequate stationary phase materials or inadequate analytical conditions (Aldai et al., 2012). Even so, Devle et al. (2012) reported a decent number of BCFA isomers in mare milk, *i.e. iso*-13:0, *iso*-14:0, *iso*-15:0, *anteiso*-15:0, *iso*-16:0, *iso*-17:0, *anteiso*-17:0 and *iso*-18:0. These authors found lower percentages (in total fatty acids; w/w) of all individual BCFAs, except for *iso*-17:0 and *iso*-18:0, compared to the results of the present study. As in the present study, Devle et al. (2012) identified *iso*-16:0 as the main BCFA in mare milk. However, *anteiso*-17:0 was the second main BCFA in the present study, whereas the mentioned authors did not detect this isomer. Similar percentage of 9*t*-18:1 but 10 times lower percentage of 9*t*-16:1 were also found by Devle et al. (2012) compared to the results in the present study.

Overall, the content of intermediates in the conversion of 18:3n-3 to docosahexaenoic acid (22:6n-3) was limited and only docosapentaenoic acid (22:5n-3) contents excelled. The low content of n-3 long-chain PUFAs comes along with previous studies in mare milk (Czyżak-Runowska et al., 2021; Devle et al., 2012). This is probably because mammals show a low enzyme activity of Δ6-desaturase, which is responsible for the first step in the conversion of 18:2n-6 to arachidonic acid (20:4n-6) and 18:3n-3 to eicosapentaenoic acid (20:5n-3) and 22:6n-3 (Marangoni et al., 2020; Napier et al., 2015; Saini et al., 2021). The low efficiency in the conversion from 18:3n-3 to n-3 long-chain PUFAs is also reflected in a lower n-3 long-chain PUFA to 18:3n-3 ratio in milk from mares (0.05) compared to other species (0.35 in cow milk or 0.96 in human milk; Coppa et al., 2011; Loza et al., 2023; Yuhas et al., 2006). Detailed information on fatty acid composition of mare milk samples is shown in **Manuscript 1**.

4.1.4. Vitamin composition, total polyphenol content and total antioxidant capacity

Regarding water-soluble vitamins, ascorbic acid (vitamin C), thiamine (vitamin B_1), riboflavin (vitamin B_2), nicotinic acid and niacinamide (vitamin B_3), pantothenic acid (vitamin B_5), pyridoxal and pyridoxine (vitamin B_6), folic acid (vitamin B_9) and cyanocobalamin (vitamin B_{12}) were determined. Analysis of fat-soluble vitamins included α-tocopherol acetate, α-, β-, γ- and δ-tocopherol, α-, β-, γ- and δ-tocotrienol (vitamin E, isomers and esterified form), retinol, retinyl acetate, retinyl propionate and retinyl palmitate (vitamin A, free and esterified forms). Among all the vitamins considered in the study, thiamine, retinyl acetate, retinyl propionate, retinol, α-tocopherol acetate, β-, γ- and δ-tocopherol, and α-, β-, γ- and δ-tocotrienol were not detected in mare milk samples or were found under the LOQ. Nicotinic acid was only present above the LOQ in 27 % of the samples, with no particular discrimination among farms, individual animals or lactation times. Therefore, the vitamin profile of mare milk was limited to ascorbic acid, pantothenic acid, niacinamide, pyridoxal and pyridoxine, folic acid, cyanocobalamin and riboflavin among water-soluble vitamins, and α-tocopherol and retinyl palmitate among fat-soluble vitamins (**Table 4.4)**.

The most abundant vitamin in mare milk samples was ascorbic acid, followed by pantothenic acid. The content of other water-soluble vitamins and of fat-soluble vitamins was considerably lower, and cyanocobalamin and riboflavin were the least abundant compounds. Niacinamide was the main vitamin B_3 form in mare milk samples, probably because in addition to dietary intake, niacinamide can be produced by mucosal enzymes. Nicotinic acid can also be produced from deamination of niacinamide by gut microbiota (Bogan & Brenner, 2008), but its absorption might be limited in hindgut fermenters like horses. Niacinamide has also been reported as the main vitamin B_3 form in cow milk (Fox et al., 2015). Similarly, pyridoxal was the main form of vitamin B_6 in mare milk, in accordance to previous observations in ruminant milk (Fox et al., 2015).

Compared to ruminant milk, mare milk contains a lower abundance of water-soluble vitamins, particularly thiamine, riboflavin, niacin and pyridoxine. In ruminants, there is a relevant synthesis of water-soluble vitamins in the rumen (Magan et al., 2020), but equids might have the synthesis-assimilation of water-soluble vitamins limited. However, similar contents of pantothenic acid, folic acid and ascorbic acid, and lower contents of cyanocobalamin have been reported in cow milk (Claeys et al., 2014) compared to results

in mare milk samples in the present study. Considering that equids lack a rumen, the main source of water-soluble vitamins in these mammal species is probably diet. Compared to mare milk from the Basque Mountain Horse breed, previous studies in milk from other horse breeds found higher riboflavin and similar or higher ascorbic acid contents (Csapó et al., 1995; Holmes et al., 1946; Navrátilová et al., 2019; Pearson, 1947; Teichert et al., 2021). Differences among studies could come from differences in the analytical method (some of the methods used for vitamin quantification were indirect and less accurate), or from milk characteristics. However, the very scarce literature on mare milk water-soluble vitamins makes comparison difficult. For instance, only one or two studies have quantified pantothenic acid, pyridoxine, folic acid and cobalamin in mare milk, and enzymatic, microbiologic or spectrophotometric assays were used in most cases (Collins et al., 1951; Navrátilová et al., 2019; Pearson, 1947; Teichert et al., 2021).

Table 4.4. *Water- and fat-soluble vitamin composition (µg/100 g milk), total polyphenol content (mg GAE/100 g milk) and total antioxidant capacity (expressed as Trolox equivalents (mM) or inhibition percentage) in mare milk samples from commercial farms over the lactation period (from May to October).*

	Mean	Min	Max	SEM
Water-soluble vitamins				
Ascorbic acid	$118 \cdot 10^1$	147	$620 \cdot 10^1$	57
Pantothenic acid	366	26.7	$284 \cdot 10^1$	21
Niacinamide	19.8	2.27	105	1.0
Pyridoxal	14.2	3.22	29.8	0.3
Folic acid	7.57	5.00	13.0	0.13
Pyridoxine	2.25	0.265	18.8	0.10
Cyanocobalamin	1.51	0.932	2.30	0.01
Riboflavin	1.16	0.0760	4.96	0.05
Fat-soluble vitamins				
α-tocopherol	10.5	1.92	59.7	0.4
Retinyl palmitate	3.66	0.619	20.5	0.14
Total polyphenol content				
Total polyphenols	7.15	3.88	11.2	0.09
Total antioxidant capacity				
Trolox equivalents	3.88	2.55	4.88	0.03
Inhibition percentage	82.1	54.5	102	0.4

GAE: gallic acid equivalent; Max: maximum; Min: minimum; SEM: standard error of the mean.

Respecting fat-soluble vitamins, vitamin E was more abundant than vitamin A, as also corroborated by others (Navrátilová et al., 2019). Vitamin A was only present in mare milk samples as an ester (retinyl palmitate), as previously reported by Stowe (1982). This is because when animals consume plants, the enzyme β-carotene 15,15'-monooxygenase converts dietary β-carotene (obtained from grass and forages) into all-*trans*-retinal (precursor of retinol, the active form) in the intestinal mucosa. Then, retinol is esterified in the mammary gland (Cabezuelo et al., 2020; Fox et al., 2015) and, in consequence, retinoids are mainly transferred to milk as esters. In the case of vitamin E, this was only present as α-tocopherol in mare milk, which is the isomer with highest vitamin activity (Fox et al., 2015).

To the best of our knowledge, the total polyphenol content in mare milk was reported in the present study for the first time (**Table 4.4**). Polyphenols are plant secondary metabolites (Lang et al., 2024) that mammals can take up from diet. Consequently, milk and dairy products can also contain phenolic compounds (Sik et al., 2023), although this field has been poorly investigated. Phenolic compounds can exhibit antioxidant, anti-inflammatory, anticoagulation and immunological functions among others, as Vázquez et al. (2015) summarized. Therefore, evaluating the contribution of mare milk consumption to the human dietary polyphenol intake is of high interest. In this regard, mare milk samples presented an average total polyphenol content (7.15 mg GAE/100 g milk) fairly close to that of goat (~ 7 mg GAE/100 g milk) and human (~ 8 mg GAE/100 g milk) milks, higher than cow milk (~ 5 mg GAE/100 g milk) but lower than sheep milk (~ 17 mg GAE/100 g milk) (Vázquez et al., 2015).

On the other hand, not only phenolic compounds but also a number of other compounds that, as presented in the results of this PhD Thesis, are present in mare milk exhibit antioxidant activity. This is the case of vitamins A, E and C, proteins and, presumably, n-3 PUFAs, among others (Blaner et al., 2021; Grażyna et al., 2017; Richard et al., 2008). So as a preliminary approach to the functional properties of mare milk from Basque Mountain Horse breed, total antioxidant capacity was measured using the ABTS method (**Table 4.4**). Few studies investigated total antioxidant capacity of mare milk with this method, but similar results to the present study were reported (86-90 % inhibition; Cosentino et al., 2017). In addition to the total antioxidant capacity of whole mare milk, the role that caseins play in this antioxidant activity was assessed, and results showed that caseins contribute to 49.9 ± 8.0 % of the total antioxidant capacity of mare milk. The fact

that caseins are main contributors to the total antioxidant capacity of milk has already been demonstrated in cow milk (Zulueta et al., 2009), and now, the same effect is demonstrated in mare milk. Interestingly, some peptides that exert certain antioxidant activity have been isolated from mare milk caseins (Ugwu et al., 2019) and whey proteins (Waili et al., 2021), although knowledge on this regard is still narrow. Detailed information on vitamin composition, total polyphenol content and total antioxidant capacity of mare milk samples is given in **Manuscript 2**.

4.1.5. Proteomic approach

Characterization of the protein fraction of mare milk was performed using a proteomics approach, using in-gel (after SDS-PAGE separation) and in-solution tryptic digestions and nLC-MS/MS for analysis. In addition, MALDI-TOF/TOF was used to investigate patterns in the natural peptidome of raw mare milk that could discriminate milk samples from different management systems or lactation stages. Regarding the study of mare milk proteome, the SDS-PAGE revealed that proteins with a mass ranging between 10 and 30 kDa predominated in mare milk (**Figure 3.3**). These included milk-specific proteins previously described in mare milk (Godovac-Zimmermann et al., 1987; Miranda et al., 2004; Uniacke-Lowe et al., 2010) such as α_{s1}- (26 kDa), α-$_{s2}$ (27 kDa), β- (27 kDa) and κ-caseins (21 kDa), α-lactalbumins A (14 kDa) and B/C (15 kDa), β-lactoglobulin I and II (20 kDa), lysozyme C (15 kDa) and lactotransferrin fragments. The β-casein that was identified in gel bands relative to 10-15 kDa might reveal the presence of a phosphorylated variant with lower molecular mass (11 kDa) previously identified in equine milk (Miclo et al., 2007). Direct nLC-MS/MS analysis after in-solution digestion resulted in 247 proteins identified, whereas after in-gel digestion it revealed 331 protein identifications. Combination of both complementary approaches resulted in 469 unique proteins identified, which is quite similar to the total number of proteins identified in milk from other horse breeds (465-504; Lv et al., 2024) and in cow, goat (Chen et al., 2023) and sheep milk (Anagnostopoulos et al., 2016). The complete list of identified proteins in mare milk samples is detailed in **Publication 3**. Among all unique proteins, the majority were enzymes and proteins involved in nutrient transport (binding, transfer/carrier, and transporter proteins), as well as cytoskeletal and immune-related proteins (**Figure 4.1**). On the other hand, the peptidomic analysis using the MALDI-TOF/TOF analytical instrument demonstrated that mare milk naturally contains peptides with a size of 3,600

Da or lower, although peptides with 4,000-4,600 and 5,200-6,600 Da were also considerably abundant.

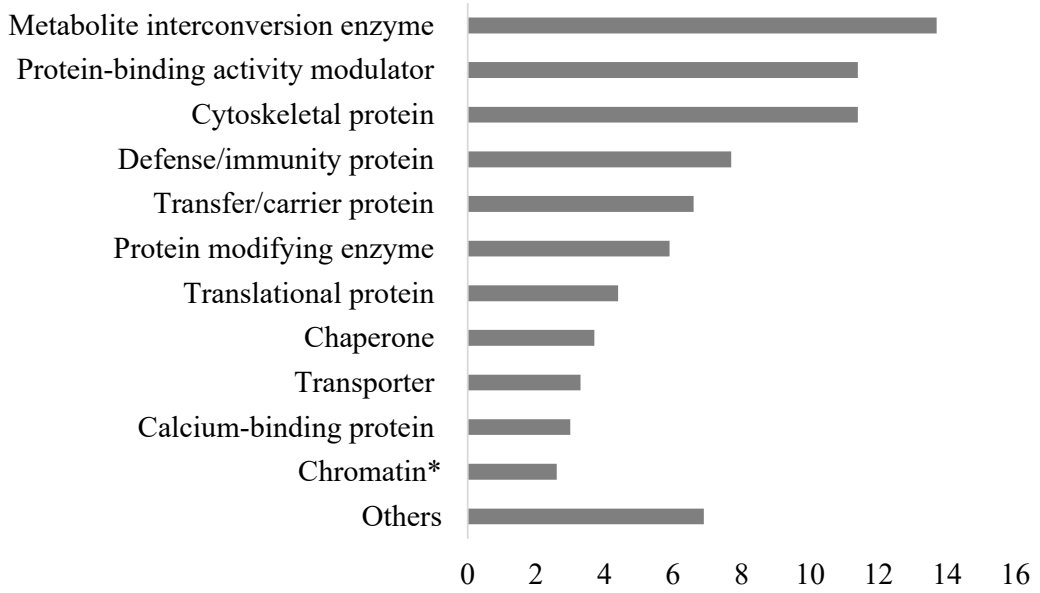


Figure 4.1. *Classification of mare milk proteins according to protein class. Numbers in the x-axis represent the percentage (%) of gene hits against total function hits. *Chromatin/chromatin-binding or -regulatory protein.*

The functional study of mare milk proteome provided a GO classification of proteins according to molecular function, biological process and cellular component. Classification according to molecular function revealed that most proteins had binding function (27.6 % of total proteins) or catalytic activity (19.8 %). Accounting for biological processes, most proteins participated in cellular (22.7 %) or metabolic processes (12.0 %). Considering cellular components, 55.7 % of the proteins were part of cell anatomy, whereas 12.1 % belonged to protein-containing complexes. Proteins that were cellular anatomical entities were mainly located in the intracellular region (16.1 % of total cellular anatomical entities), cytoplasm (12.4 %) cell membrane (12.1 %) or organelles (12.0 %). Among proteins that belonged to protein-containing complexes, 26.1 % were part of membrane protein complex, 21.7 % of ribonucleoprotein complex and 17.4 % of catalytic complex. Considering GO classifications, a large number of mare milk proteins were nutrient transporters, enzymes, structural proteins and Igs. Detailed information on GO classifications is given in **Publication 3**.

In addition, the ingenuity pathway analysis was able to identify 433 canonical pathways in which mare milk proteins participated. The canonical pathways that were most significantly enriched in mare milk were related to lipid metabolism (liver X receptor/retinoid X receptor (LXR/RXR) activation, farnesoid X receptor/retinoid X receptor (FXR/RXR) activation and 24-dehydrocholesterol reductase gene (DHCR24) signaling pathway) and inflammation (acute phase response signaling). Proteins that participated in the lipid metabolism-related pathways (apolipoproteins, fatty acid synthase, lipoprotein lipase and transthyretin) perform fatty acid acquisition from triglycerides, *de novo* fatty acid synthesis and lipid transportation, and ensure the delivery of required bioavailable lipids to the newborn (Irshad & Dubey, 2005; Liz et al., 2020; Mendelson et al., 1977; Suburu et al., 2014). Proteins from the acute phase response signaling pathway are associated with inflammation processes and with initial stages of mammary gland involution after weaning (Stein et al., 2004). Most significantly enriched canonical pathways, and function (according to GO) and class of mare milk proteins were aligned with the two main roles of milk: providing neonates with nutrients and immunity (Sun et al., 2020).

4.2. Mare milk protein digestibility and peptide release during gastrointestinal digestion

Beyond characterization of mare milk composition from a physicochemical perspective, performance of mare milk proteins during human gastrointestinal digestion was studied as an approach to its potential interest for human consumption. Briefly, digestibility of total proteins and individual amino acids was measured (in a percentage basis) after simulated *in vitro* intestinal digestion of mare milk samples. In addition, the peptides released after gastric and after intestinal digestion were identified to understand proteolytic patterns, resistance of proteins to digestion, and the release of bioactive peptides.

Total protein digestibility of mare milk was 96.0 ± 4.7 % when measured based on total amino groups (OPA method) and 93.7 ± 3.3 % when calculations were made with individual amino acid digestibility. These results demonstrate that mare milk is one of the most digestible dietary protein sources, as it is cow milk (~ 95 % digestibility; Dupont & Tomé, 2020). Digestibility of individual amino acids was higher than 90 % in all cases

except for alanine (89.2 ± 5.7 %), isoleucine (88.3 ± 3.8 %), serine (83.8 ± 5.8 %) and threonine (88.9 ± 5.2 %). Average DIAAR values in mare milk, which represent the percentage of digested indispensable amino acids in relation to daily requirements, were higher than 100 % in all amino acids for child and adult nutrition and between 74.5 and 98.7 % for infant nutrition. According to the DIAAS values, which refer to the lowest DIAAR values and are associated with the limiting amino acids in a food, the limiting amino acids were threonine for infants (DIAAS: 74.5 %) and histidine for children and adults (DIAAS: 101.5 and 126.9 %, respectively).

Additionally to protein digestibility, the release of peptides during gastrointestinal digestion was studied. In this case, only peptides released from major mare milk proteins that were identified by SDS-PAGE and HPLC-MS/MS were evaluated. Eight major proteins identified in mare milk samples were α_{s1}-, α_{s2}-, β- and κ-caseins, β-lactoglobulin I and II, α-lactalbumin A and lysozyme C. The size (mass) of peptides that came from caseins and from β-lactoglobulin I and α-lactalbumin A significantly lowered from gastric to intestinal digestion, which is a sign of further degradation during intestinal digestion of peptides released after gastric digestion. Similar results were observed in the peptide patterns after gastric and intestinal digestion, showing that caseins, β-lactoglobulin I and α-lactalbumin were hydrolysed (to a greater or lesser extent) during gastrointestinal digestion of mare milk. These results are shown in detail in **Publication 4**. Caseins being more intensely degraded than whey proteins during digestion has previously been observed in milk from various mammal species (Xiao et al., 2023), and probably occurs because caseins form a clot in the stomach induced by acidic conditions and pepsin, whereas whey proteins are soluble in the gastric fluid. In this scenario, casein clots are retained longer in the stomach and are more susceptible to proteolysis, while whey proteins pass through the digestion system faster and with lower proteolysis (Ye et al., 2016). However, structural factors also play an important role in protein susceptibility to proteolysis. Since caseins are mobile and loosely structured proteins, they are more susceptible to the action of pepsin than proteins with a globular structure, like whey proteins (Dupont & Tomé, 2020). Among whey proteins, peptides released from β-lactoglobulin II and lysozyme C did not show higher fragmentation during intestinal digestion according to peptide size. Moreover, a very low release of peptides from β-lactoglobulin II and lysozyme C was observed after both gastric and intestinal digestion, suggesting that these two mare milk whey proteins are resistant to human digestion.

Resistance of equine lysozyme was already observed by Inglingstad et al. (2010). Interestingly, resistance of β-lactoglobulin to proteolysis with pepsin has also been reported in bovine milk (Ye et al., 2016), but a study in mare milk digestion (Inglingstad et al., 2010) disclosed a higher susceptibility to hydrolysis of equine β-lactoglobulin (no isoform distinction) compared to ruminant β-lactoglobulin. In the present study, only β-lactoglobulin II was resistant to proteolysis during digestion, whereas β-lactoglobulin I was already hydrolysed after the gastric phase. This demonstrates that different protein isoforms of β-lactoglobulin present different resistance to proteolysis.

The main source of peptides was β-casein, with 247 and 226 unique peptides released after gastric and intestinal digestion, respectively. In fact, β-casein is highly digestible, but it is also the most abundant protein in mare milk (Miranda et al., 2004). On the other hand, κ-casein, α-lactalbumin A and lysozyme C were poor sources of peptides, with less than 80 unique peptides identified after gastric and intestinal digestion. Low peptide release from κ-casein is most likely due to its low content in mare milk (Miranda et al., 2004), whereas limited peptide release from lysozyme C probably comes from its resistance to digestion. The small number of α-lactalbumin A peptides released after digestion might reveal a high degradation of this protein during digestion, considering that the analytical instrument (LC-MS/MS) was unable to identify peptide sequences with less than five amino acids. In fact, the size of peptides formed from α-lactalbumin A was significantly reduced from gastric to intestinal digestion. This brings some clarity to conflicting results regarding digestibility (or resistance) of α-lactalbumin among studies (Dupont & Tomé, 2020). Digestion traits of individual milk proteins are detailed in **Publication 4**.

In addition, bioactive peptides were searched among all the unique peptides released after mare milk *in vitro* digestion. Although none of the peptides released from mare milk (≥ 5 amino acids) has been described as bioactive before, a number of precursors of known bioactive peptides were released. Some of these precursors were parent proteins of VAPFPQPVVP (fragment f(191-200) of β-casein), a bioactive peptide reported in donkey milk after *in vitro* simulated gastrointestinal digestion that presents angiotensin-converting enzyme inhibitory activity (Bidasolo et al., 2012). Although this bioactive peptide was not liberated during simulated *in vitro* gastric or intestinal digestion of mare milk following the INFOGEST protocol, it could potentially be formed in further hydrolysis of parent peptides during the next digestion steps. In fact, the digestion model

used in the present study lacks brush border enzymes from the small intestine, which further fragment peptides that reach brush border cells prior to absorption (Sousa et al., 2023).

4.3. Effect of lactation stage and management system on mare milk composition and protein digestion

In general, the statistical results showed that lactation stage had a great impact on mare milk composition as it presented a significant effect on most of the individual compounds analysed. On the contrary, the management system was only significant for some specific compounds. Lactation stage and management system also affected some parameters monitored in the study of protein digestion, mainly some DIAAR values and the release of some specific peptides after mare milk gastrointestinal digestion. The interaction effect between management system and lactation stage was significant for very few compounds, showing that changes in mare milk composition during lactation were, in general, similar regardless of the management system used (**Table 4.5**). **Publications 2-4** and **Manuscripts 1-2** detail the statistical significance of the effect of lactation stage and management system (farm), and the interaction between both factors, on the chemical composition and protein digestion of mare milk.

4.3.1. Effect of lactation stage

Lactation stage significantly affected the content of all individual compounds identified and quantified in mare milk except for ascorbic acid, pyridoxine, and 10:1, 9*c*-15:1, 9*c*-17:1, *t*-15:1 and 11*t*-18:1 MUFAs (**Table 4.5**). Significance of the effect of lactation stage, and evolution of individual chemical compounds during lactation are detailed in **Publications 2-3** and **Manuscripts 1-2**. Regarding gross composition, total fat and protein content decreased during lactation, whereas the content of lactose increased. Protein content gradually decreased from initial stages of lactation until weeks 15-16, stabilizing afterwards. The opposite trend was observed in lactose contents, which increased from the beginning of the lactation until weeks 15-16, remaining quite stable afterwards. On the other hand, the decrease in total fat content in mare milk samples was particularly evident after weeks 19-20. No clear evolution trend was observed in non-fat dry matter content, probably due to the opposite dynamics between protein and lactose

(**Figure 4.2**). A few authors also reported that mare milk fat and protein contents decreased and lactose increased during lactation (Cais-Sokolińska et al., 2018; Hachana et al., 2022). Other studies that found non-significant changes (Markiewicz-Kęszycka et al., 2015) or different evolution patterns (Barreto et al., 2020) in mare milk fat, lactose or protein contents often considered only between four and six lactation times, which hindered the complete picture of the changes that can occur throughout lactation in mare milk gross composition.

Table 4.5. *Average values of gross composition (g/100 g milk), total antioxidant capacity (mM Trolox equivalents), and content of mineral elements (mg/100 g milk), water- and fat-soluble vitamins (μg/100 g milk), total polyphenols (mg GAE/100 g milk), and fatty acids (mg/100 g milk) of mare milk samples classified according to the commercial farm, and significance of the effect of management system, lactation stage and the interaction effect of both.*

	Farm			*P*-value		
	I	II	III	Farm	Lactation	Farm*Lactation
Gross composition						
Fat	0.306	0.267	0.247	0.310	< 0.001	0.446
Protein	1.80	1.79	1.77	0.457	< 0.001	< 0.001
Lactose	6.80	6.76	6.70	0.115	< 0.001	0.693
Dry matter (non-fat)	9.45 ^{a}	9.38 ^{a,b}	9.27 ^{b}	0.026	0.002	0.260
Mineral elements						
Ca	104 ^{a}	92.8 ^{b}	93.6 ^{a,b}	0.025	< 0.001	0.857
K	58.2	56.0	60.4	0.461	< 0.001	0.104
P	50.2 ^{a}	43.1 ^{b}	44.7 ^{a,b}	0.013	< 0.001	0.471
S	16.5 ^{a}	15.3 ^{b}	15.4 ^{a,b}	0.015	< 0.001	0.493
Na	17.9 ^{a}	13.4 ^{b}	12.3 ^{b}	< 0.001	< 0.001	0.005
Zn	0.226	0.267	0.247	0.061	< 0.001	0.491
Fe	0.275	0.231	0.232	0.427	0.006	0.035
Water-soluble vitamins						
Ascorbic acid	$127 \cdot 10^{1}$	$126 \cdot 10^{1}$	$104 \cdot 10^{1}$	0.092	0.252	0.719
Pantothenic acid	375	302	431	0.056	<0.001	0.713
Niacinamide	13.2^{b}	24.5^{a}	18.8^{a}	0.012	<0.001	0.477
Pyridoxal	13.6	15.1	13.7	0.098	<0.001	0.031
Folic acid	8.04	7.66	7.04	0.061	<0.001	0.700
Pyridoxine	2.61	2.19	2.06	0.475	0.098	0.161
Cyanocobalamin	1.51	1.53	1.49	0.479	0.010	0.106
Riboflavin	0.786	1.35	1.16	0.273	0.004	0.112
Fat-soluble vitamins						
α-tocopherol	10.4	10.8	10.2	0.542	<0.001	0.076
Retinyl palmitate	3.64	3.67	3.66	0.896	<0.001	0.927
Total polyphenol content and antioxidant capacity						
Polyphenols	7.61	7.08	6.90	0.519	<0.001	0.159
Antioxidant capacity	3.95	3.84	3.87	0.889	<0.001	0.179

Table 4.5. *(continuation)*

	Farm			*P*-value		
	I	II	III	Farm	Lactation	Farm*Lactation
Saturated fatty acids						
8:0	10.2[a]	6.57[b]	6.32[b]	<0.001	<0.001	0.672
9:0	0.110[a]	0.0557[b]	0.0666[b]	0.013	<0.001	0.030
10:0	22.1[a]	13.9[b]	13.6[b]	<0.001	<0.001	0.496
11:0	4.23	3.23	2.95	0.232	<0.001	<0.001
12:0	22.1[a]	15.4[b]	15.0[b]	0.007	<0.001	0.283
14:0	20.2[a]	15.6[b]	15.2[b]	0.049	<0.001	0.295
16:0	53.8	42.7	42.8	0.122	<0.001	0.502
18:0	2.69	2.11	2.10	0.083	<0.001	0.628
Branched-chain fatty acids						
iso-14:0	0.128	0.127	0.113	0.444	<0.001	0.818
iso-15:0	0.131	0.123	0.114	0.391	<0.001	0.676
anteiso-15:0	0.126	0.157	0.150	0.632	<0.001	0.550
iso-16:0	0.353	0.438	0.411	0.317	<0.001	0.522
iso-17:0	0.0330	0.0315	0.0317	0.676	<0.001	0.968
anteiso-17:0	0.303	0.342	0.321	0.640	<0.001	0.614
iso-18:0	0.165	0.154	0.150	0.763	<0.001	0.614
Monounsaturated fatty acids						
9*c*-14:1	1.58	1.47	1.34	0.322	<0.001	0.073
7*c*-16:1	0.887[a]	0.584[b]	0.586[b]	0.011	0.003	0.443
9*c*-16:1	15.1	12.4	11.7	0.115	<0.001	0.274
9*c*-17:1	1.21	1.04	0.967	0.139	0.072	0.705
9*c*-18:1	55.4[a]	33.0[b]	32.4[b]	0.002	0.002	0.502
11*c*-18:1	2.86[a]	1.72[b]	1.62[b]	<0.001	<0.001	0.396
11*c*-19:1	0.155[a]	0.0996[b]	0.0973[b]	0.005	<0.001	0.677
11*c*-20:1	0.872[a]	0.421[b]	0.390[b]	<0.001	<0.001	0.692
13*c*-22:1	0.0980[a]	0.0573[b]	0.0524[b]	0.002	<0.001	0.868
15*c*-24:1	0.0460[a]	0.0301[b]	0.0283[b]	0.001	<0.001	0.926
9*t*-18:1	0.172[a]	0.0955[b]	0.0881[b]	<0.001	<0.001	0.371
Polyunsaturated fatty acids						
16:2n-6	0.136[b]	0.229[a]	0.230[a]	0.009	<0.001	0.923
18:2n-6	15.6[b]	22.0[a]	23.4[a]	0.017	<0.001	0.815
18:3n-6	0.0409[b]	0.0649[a]	0.0659[a]	0.024	0.003	0.038
20:3n-6	0.0947	0.0946	0.0898	0.715	<0.001	0.851
22:2n-6	0.0348[b]	0.0517[a]	0.0471[a]	0.044	<0.001	0.757
16:3n-3	0.163[b]	0.500[a]	0.558[a]	<0.001	<0.001	0.002
18:3n-3	22.0[b]	39.6[a]	43.9[a]	<0.001	<0.001	0.009
9*t*,12*t*,15*c*-18:3	0.172[b]	0.271[a]	0.287[a]	<0.001	<0.001	0.009
Fatty acid groups						
SFA	138[a]	102[b]	101[b]	0.020	<0.001	0.497
BCFA	1.24	1.37	1.29	0.729	<0.001	0.635
MUFA	79.7[a]	52.1[b]	50.5[b]	0.006	0.002	0.489
PUFA	40.1[b]	65.1[a]	70.8[a]	<0.001	<0.001	0.302
n-6/n-3	0.848[a]	0.557[b]	0.572[b]	<0.001	<0.001	0.010
PUFA/SFA	0.305[b]	0.686[a]	0.739[a]	<0.001	<0.001	0.003

[a,b] *Different letter superscripts indicate significant differences (P ≤ 0.05) among farms. GAE: gallic acid equivalents; MUFA: monounsaturated fatty acid; PUFA: polyunsaturated fatty acid; SFA: saturated fatty acid.*

When changes during lactation in the content of individual components were monitored, some common patterns were observed (**Figures 4.3-4.5**). In general, the content of Ca, P, S, PUFAs and total polyphenols peaked at mid lactation, between weeks 13-14 and 17-18, but decreased from initial to final stages of lactation (except for polyphenols that remained at similar concentration). MUFAs, BCFAs, very long n-3 PUFAs (20:5n-3, 22:5n-3 and 22:6n-3) and Zn contents peaked twice between weeks 15 and 22 of lactation, but remained more or less constant from initial to final stages of lactation. Besides, the content of K, Mg, SFAs, pyridoxal, niacinamide, pantothenic acid, folic acid, retinyl palmitate and α-tocopherol decreased throughout the lactation period, while the content of Na and Cu increased. Finally, the content of Cl, Fe, riboflavin and cyanocobalamin fluctuated with no clear patterns.

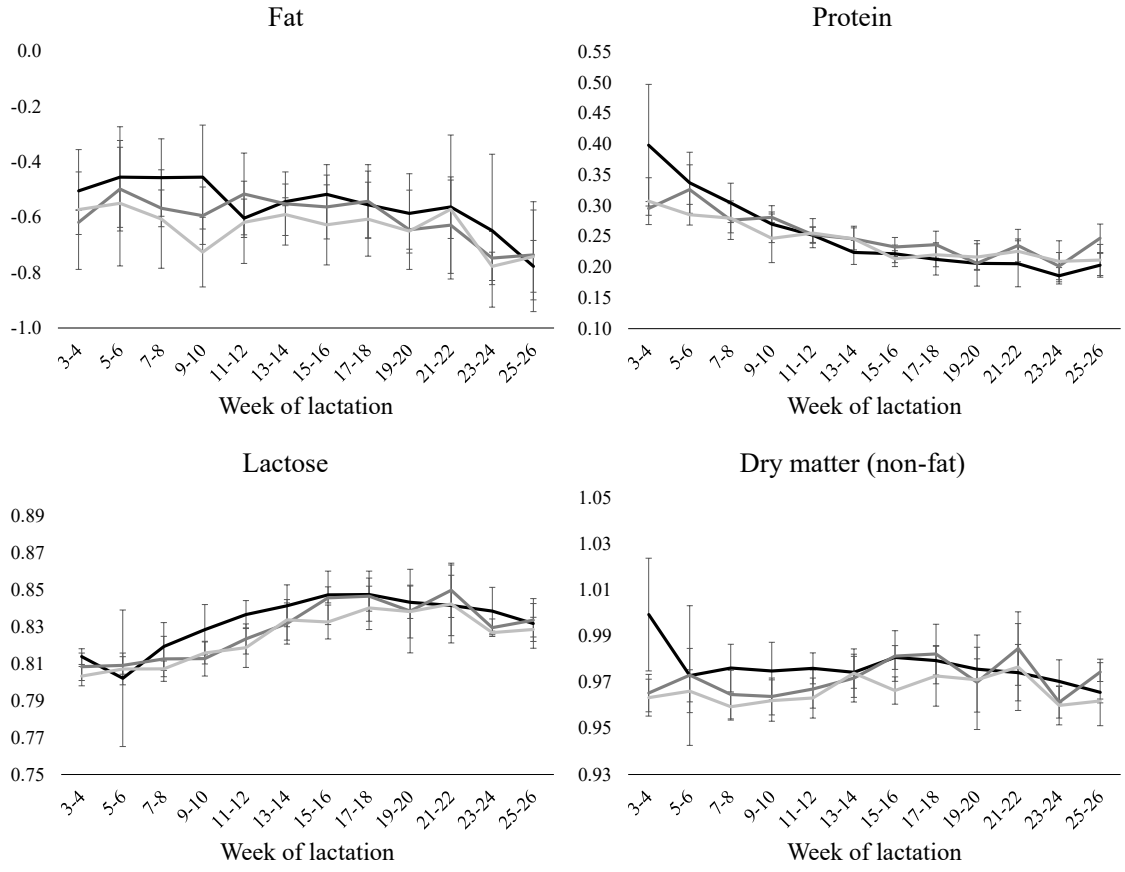


Figure 4.2. *Changes during lactation in total fat, protein, lactose and non-fat dry matter content (log (g/100 g milk)) of mare milk samples.* ▬, *Farm I;* ▬, *Farm II;* ▬, *Farm III.*

Individual compounds of the same nature (*i.e.* fatty acids with different unsaturation degree, water- or fat-soluble vitamins, etc.) tended to follow similar evolution trends, while very different evolution patterns were observed among mineral elements. Homeostasis of mineral elements is critical for the normal functioning of the body (Kronqvist, 2011), so different evolution trends during lactation among individual mineral elements could have been related with a strong regulatory effect occurring in animal tissues during lactation. Conversely, most fatty acids either peaked at mid lactation or decreased during lactation depending on the degree of unsaturation. Most individual PUFAs presented a prominent concentration increase during mid lactation, and most MUFAs peaked only at specific times around mid-late lactation. In the case of SFAs, these just decreased during lactation (**Figures 4.3-4.5**). Concentration of water- and fat-soluble vitamins predominantly decreased during lactation (**Figure 4.4**), except for riboflavin and cyanocobalamin, which showed the lowest contents in the mare milk samples, and fluctuated with no specific pattern.

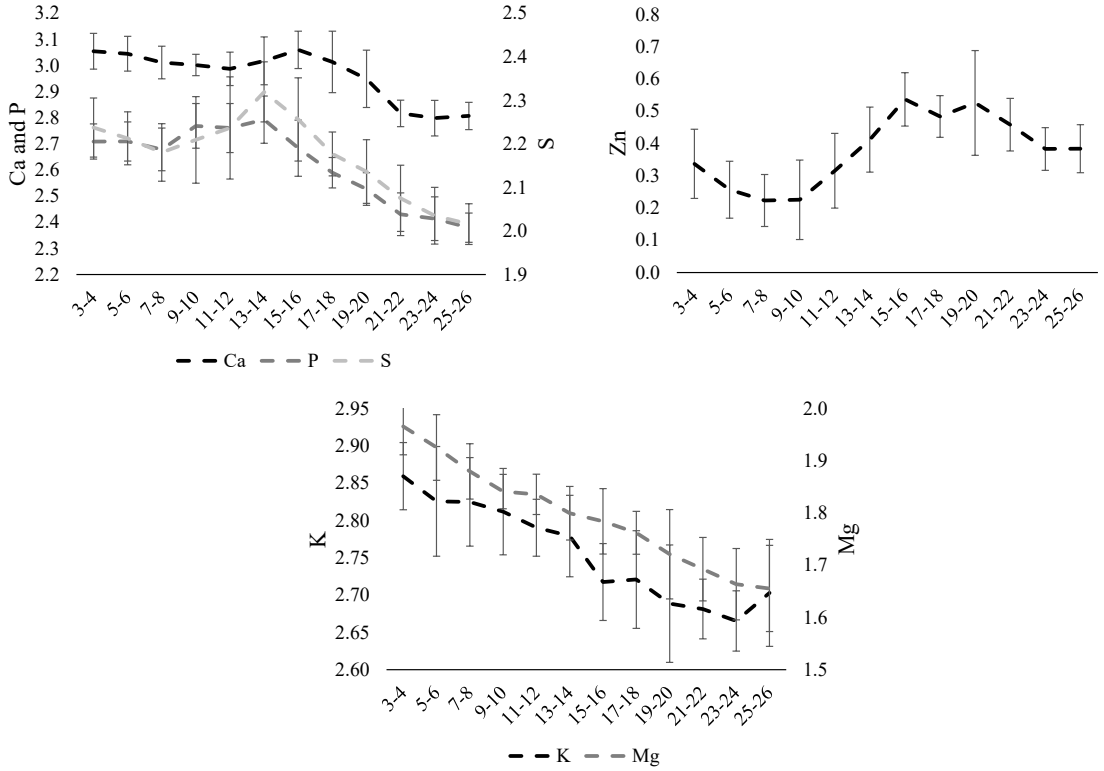


Figure 4.3. *Main evolution trends followed by individual mineral elements in mare milk samples, expressed as log-transformed units (µg/g milk).*

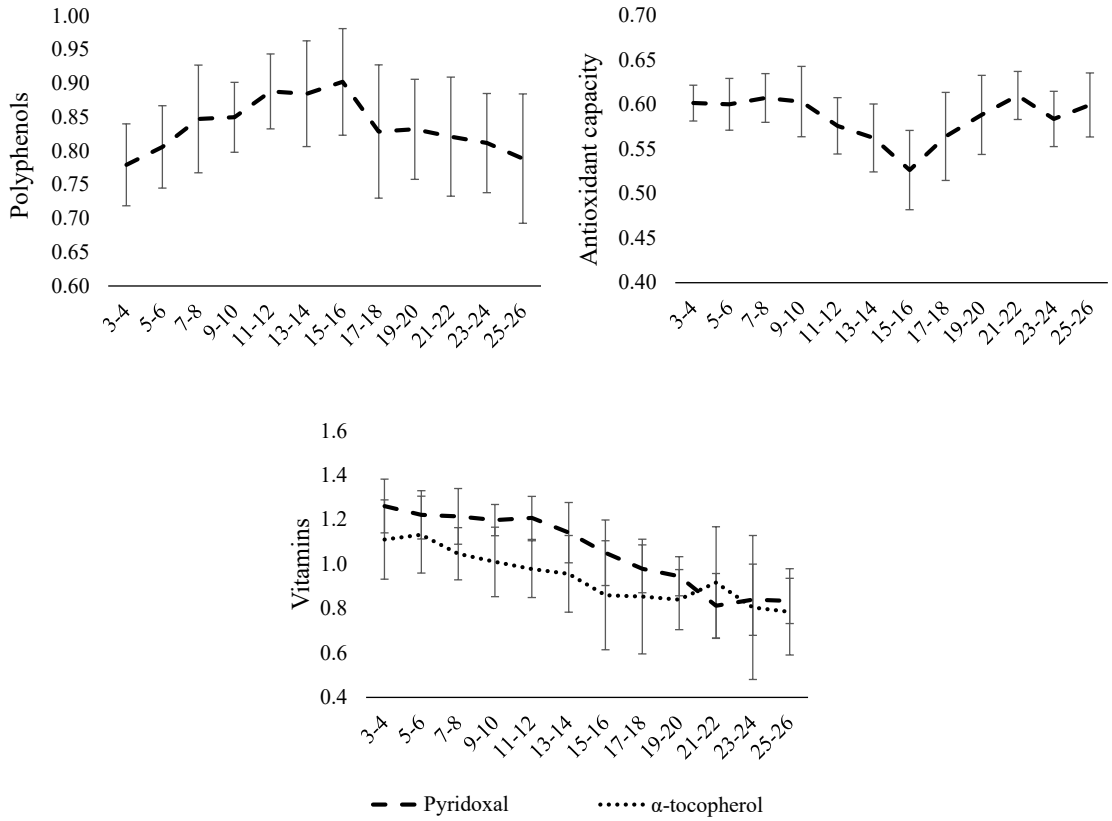


Figure 4.4. *Main evolution trends followed by bioactive compounds and total antioxidant capacity in mare milk samples, expressed as log-transformed units (total polyphenols, mg GAE/100 g milk; total antioxidant capacity, mM Trolox equivalents; individual water- and fat-soluble vitamins, µg/100 g milk). ABTS: 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); GAE: gallic acid equivalents; Trolox: 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid.*

Interestingly, Ca, P, S, polyphenols and PUFAs, which peaked during mid lactation, are known to be influenced by diet. As discussed later in this section, the contents of Ca, P and S were significantly influenced by management of the mares, which is consistent with results reported in other cow milk studies. As discussed earlier, PUFAs in mare milk also have a dietary origin due to the post absorptive location of the fermentation chamber in the equine digestive system (Belaunzaran et al., 2015; Warren & Vineyard, 2013), and polyphenols are plant secondary metabolites that must be sourced from dietary plants (Lang et al., 2024). In this regard, strong changes at mid lactation (mainly between weeks 13 and 18) might come from modifications in dietary intakes occurring in the three farms. Lactation weeks 13-18 corresponded to the months between June and August. During these months, some changes occurred in the feeding regime of mares (**Figure 3.1**). Specifically, farm I switched the feeding of mares from pasture to forage, fruits and

potatoes in June, and farms II and III started dietary supplementation with either hay or silage after July. With this, changes in the dietary supply of specific plant-derived compounds could have occurred. Then, decreasing tendencies could respond to changes occurring in the quality of forages, which is significantly influenced by several factors, including vegetation stage, plant species and preservation method of harvested forages (Glasser et al., 2013).

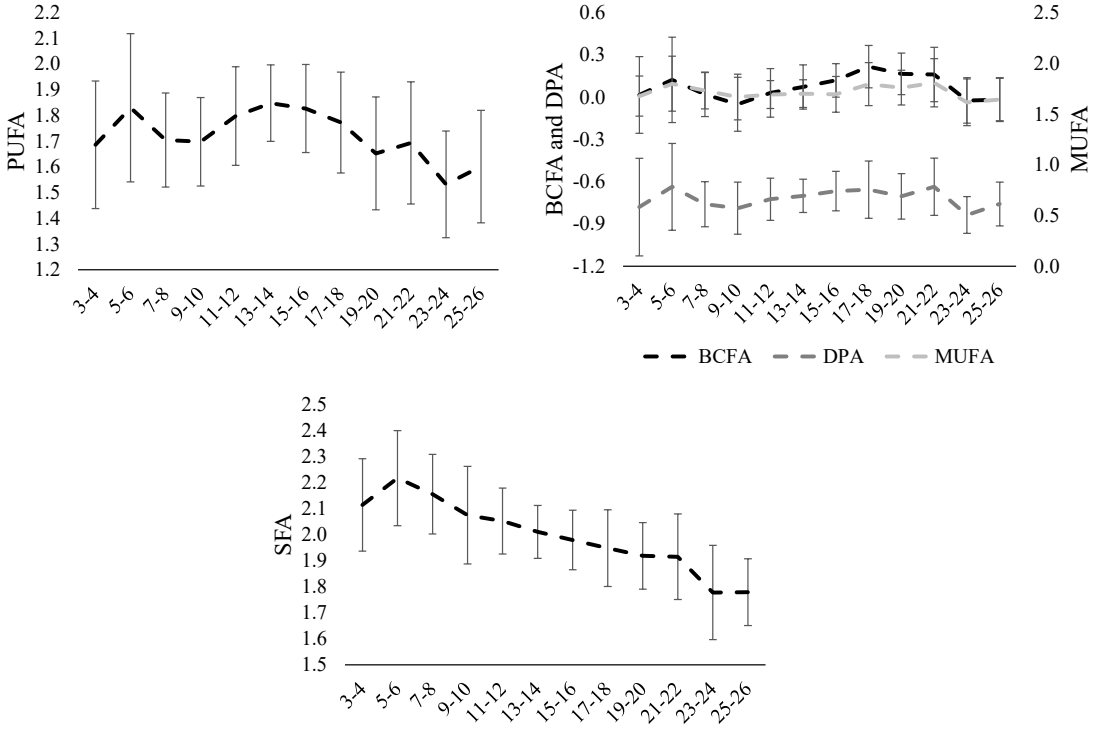


Figure 4.5. *Main evolution trends followed by individual fatty acids and fatty acid groups in mare milk samples, expressed as log-transformed units (mg/100 g milk). BCFA: branched-chain fatty acid; DPA: docosapentaenoic acid (22:5n-3); MUFA: monounsaturated fatty acid; PUFA: polyunsaturated fatty acid; SFA: saturated fatty acid.*

On the other hand, SFAs, major water-soluble vitamins and retinyl palmitate, all of which showed a decreasing trend in mare milk during lactation (**Figures 4.4-4.5**), have a closer relation with animal metabolism. Most of SFAs are synthetized *de novo* in the mammary gland (Palmquist & Harvatine, 2020), whereas the content of retinyl palmitate depends on the enzymatic conversion of its precursor β-carotene (Fox et al., 2015). Although diet is the main source of water-soluble vitamins in non-ruminants, the mammary gland seems

to have a complex regulation system to control levels of water-soluble vitamins in milk. In fact, studies in human milk have observed that dietary water-soluble vitamin levels do not always relate to vitamin levels in milk (Montalbetti et al., 2014). Moreover, a decreasing evolution trend in the content of α-tocopherol has been observed in human milk, which might indicate that the concentration of vitamin E in non-ruminant milk is unrelated to diet (Lima et al., 2014).

Other compounds such as MUFAs, BCFAs and n-3 long-chain PUFAs presented quite constant abundances except for two peaks after week 15. A common feature of these fatty acids is that they are all synthetized from other fatty acids or from amino acids. Most MUFAs are synthetized in the mammary gland by desaturation (stearoyl-CoA desaturase enzyme) of SFAs (Palmquist & Harvatine, 2020). On the other hand, n-3 long-chain PUFAs such as 20:5n-3, 22:5n-3 and 22:6n-3 are originated from elongation and desaturation of 18:3n-3 in a metabolic pathway that is initiated by the enzyme Δ6-desaturase, which has low activity in mammals (Saini et al., 2021). Considering the high 18:3n-3 content in mare milk samples found in the present study, and the low enzymatic activity of Δ6-desaturase, the contents of n-3 long-chain PUFAs were quite constant during the lactation period probably due to the limitation in this conversion step. Regarding BCFAs, these acids are mainly originated from the fermentation of dietary leucine, isoleucine and valine in the hindgut (Kaneda, 1991). Because horses lack a rumen and have their fermentation chamber located after the small intestine (Belaunzaran et al., 2015, 2018; Clauss et al., 2009), production of BCFAs is not only low but might be limited throughout the complete lactation period.

Total antioxidant capacity of mare milk was also significantly affected by stage of lactation. In this case, lowest values were found at mid lactation between weeks 15 and 18, while initial and final values were similar (**Figure 4.4**). Because caseins are main contributors to milk antioxidant activity, as the present research work has evidenced, a decrease in the antioxidant capacity from early to mid lactation stages could be related with the decrease found in total protein content (**Figure 4.2**). Unfortunately, the subsequent increase until the end of the lactation period could not be related with any of the antioxidant compounds analysed in this study. Anyway, the evolution pattern of the antioxidant activity was opposite to that of total polyphenols, demonstrating the low contribution of polyphenols to the total antioxidant capacity of mare milk.

The significant effect of lactation stage observed for the different chemical compounds was corroborated by multivariate SDA performed with all compositional data excluding the proteomic profile. This analysis was able to correctly classify more than 86 % of mare milk samples into early, mid or late lactation stage (**Figure 4.6**).

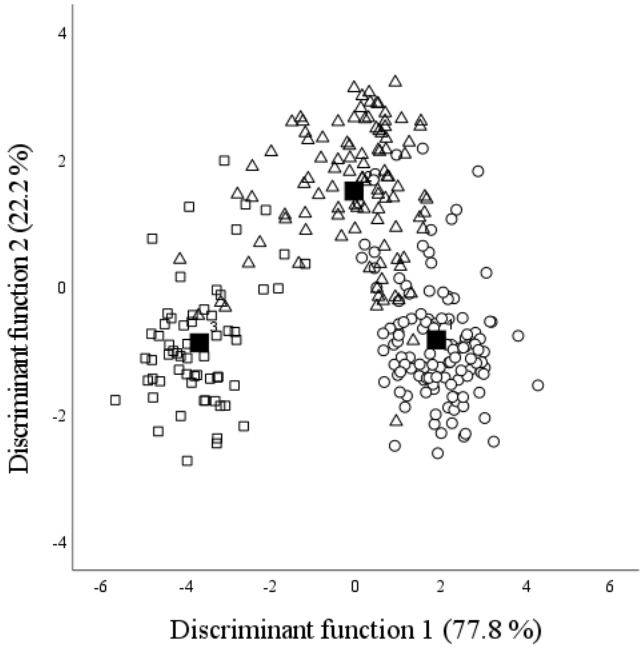


Figure 4.6. *Distribution of mare milk samples according to lactation stage (○, early; △, mid; □, late; ■, group centroid) obtained from the Stepwise Discriminant Analysis applied to compositional data (excluding the proteomic profile) of mare milk samples.*

Canonical discriminant function 1 mainly discriminated samples at late lactation stage from samples at early and mid lactation stages, while function 2 discriminated samples at mid lactation stage from the rest of the samples. Based on the highest correlations between the variables and the discriminant functions, the compounds that mainly discriminated late lactation from early and mid lactation samples (canonical discriminant function 1) were some mineral elements (Mg, P, K and Ca), short-chain SFAs (from 4:0 to 14:0), pyridoxal and total protein content. In accordance to this, the individual evolution trends depicted in **Figures 4.2-4.5** show that these compounds were in fact the ones that most intensely decreased at the end of the lactation period. On the other hand, the compounds that mainly differentiated mid lactation stage samples from early and late lactation stage samples (canonical discriminant function 2) were S and Zn, lactose, total antioxidant

capacity, 18:2n-6, 18:3n-3 and some other minor unsaturated fatty acids (6*c*-18:1, 16:2n-6, 20:2n-6, 22:2n-6, 20:3n-3, 9*t*,12*t*,15*c*-18:3). However, considering that canonical function 1 explains most of the variance, and that it mainly discriminates samples from late lactation stage, it can be concluded that the highest differences during lactation in mare milk chemical composition occurred at the end of the lactation period.

Differences in mare milk composition from different lactation stages were also significant for the protein profile (proteome), but not for the peptide profile (peptidome) (**Publication 3**). Among the 469 proteins identified in mare milk, only six were significantly affected by lactation stage. These proteins were β-lactoglobulin II, lactotransferrin, transforming acidic coiled-coil containing protein 2 and interferon α-inducible protein 27 with an increasing trend during lactation, and Ig-like domain-containing protein and polypeptide N-acetylgalactosaminyltransferase that decreased from early to mid lactation and increased afterwards. Proteins with an increasing tendency during lactation were either binding proteins and therefore nutrient transporters (β-lactoglobulin II and lactotransferrin), or proteins participating in cell differentiation and apoptosis (transforming acidic coiled-coil containing protein 2 and interferon α-inducible protein 27) (Adlerova et al., 2008; Cheng et al., 2010; Uniprot, 2023; Wodas et al., 2020). Considering their functionality, changes in these proteins during lactation could be related with early phase mammary gland involution. In addition, polypeptide N-acetylgalactosaminyltransferase is responsible for post-translational protein glycosylation (Tenno et al., 2002). Since IgA, the main Ig in mature mare milk (Perkins & Wagner, 2015), is also glycosylated (Froehlich et al., 2010), the evolution of these two proteins during lactation could be interrelated.

Lactation stage did not only affect the profile of specific mare milk proteins, but also some milk protein digestion features. Detailed information on this effect is given **Publication 4**. On one hand, lactation stage affected the DIAAR values (*i.e.* the ratio of digested essential amino acids relative to daily reference requirements established by FAO (2013)) of histidine, leucine, lysine and valine. However, these differences were due to changes in the amino acid content of raw milk during lactation rather than to changes in digestibility of individual amino acids. Changes in the amino acid profile of raw mare milk during lactation might be mainly related to dietary changes, as already demonstrated in donkey (also equid) milk (Liang et al., 2022). When the pattern of peptides released after digestion was studied, lactation stage also affected the relative abundance of six

unique peptides that were released after gastric digestion of β-casein. Modulation of protein digestion according to lactation stage probably comes from changes in mare milk composition. In fact, fat content (Egger et al., 2017) and casein phosphorylation (Liu et al., 2019) can alter milk protein digestion. Supporting this idea, the highest differences in mare milk protein digestion were observed at late lactation compared to early and mid lactation stages, similar to results depicted in the SDA using chemical composition data.

4.3.2. Effect of management system

When addressing the effect of management system, grazing intensity was considered as one of the most relevant factors, which was considerably different among farms. In general terms, farm I followed a low grazing system, while mares from farms II and III were in a pasture/forage-based system for the complete lactation period (**Figure 3.1**). Results showed that management system only affected the content of some specific individual compounds. Detailed information regarding the effect of management system on the chemical composition of mare milk is detailed in **Publications 2-3** and **Manuscripts 1-2**.

Regarding gross composition, the management system only affected total non-fat dry matter content, which was higher in milk produced under low grazing activity (farm I) (**Table 4.5**). Total protein and lactose contents were statistically similar among the three farms, so differences in total non-fat dry matter must come from the cumulative effect of differences (significant or not) among farms in the content of protein, lactose and other compounds.

Among all the mineral elements quantified in the present study, only the contents of Ca, P, S and Na were affected by the management system (**Table 4.5**). In this case, milk from the low grazing system (farm I) contained higher abundance of Ca, P and S than milk from high grazing and hay supplementation (farm II), and farm III remained in between. Na content was significantly higher in milk from low grazing (farm I) compared to high grazing (farms II and III) management systems, and changes during lactation were also significantly different between low (farm I) and high grazing farms (farms II and III). Interestingly, similar results have been observed in cow milk. In fact, a number of studies reported that cow milk produced under different grazing intensities, or under pasture-based *vs.* non-pasture-based systems, revealed significant differences in protein (the main source of S; Nimni et al., 2007), Ca, P and Na abundance (Gulati et al., 2018; Stergiadis

et al., 2021). Supporting this, a recent study developed a predictive model that was able to discriminate grass-fed *vs.* non-grass-fed cow milk according to chemical composition, and observed that milk protein, Ca and P contents, among other compounds, were good indicators of animal grazing (Soyeurt et al., 2022). As it will be discussed later, mineral elements were not the main contributors to the discrimination between high- and low-grazing farms, but they were definitely affected by management system.

The content of vitamins was overall unaffected by management of the mares as their abundance in milk was quite similar among the three farms. As an exception, a significantly higher niacinamide content was found in mare milk samples from high grazing farms (farms II and III) compared to the low grazing farm (farm I). Studies in human milk have also observed that the content of water-soluble vitamins in milk does not always respond to dietary changes, suggesting that this type of nutrients likely has independent mechanisms of metabolism and secretion to milk (Montalbetti et al., 2014). Similar results were observed in human milk when studying the effect of natural dietary changes (not supplementation) in the content of vitamin E in milk (Lima et al., 2014). On the other hand, no differences in the content of vitamin A among milk samples from the three farms could be related with previous observations suggesting that cattle (Mora et al., 2000), and probably horses as well (Álvarez et al., 2015), have a low efficiency in the conversion of β-carotene into retinal (precursor of retinol).

On the other hand, it is worth mentioning that no significant differences were observed among management systems in total polyphenol contents, probably because all feeding regimes were a good source of phenolic compounds. Phenolic compounds are plant secondary metabolites, so grasses and legumes are rich in phenolic compounds (Amrit et al., 2023), and some studies have demonstrated that grazing can improve the abundance of total phenolic contents in ruminant milk (Cabiddu et al., 2019; Delgadillo-Puga et al., 2019). However, fruits and vegetables, which were highly present in the low-grazing system in the present study (**Figure 3.1**), are also a great source of phenolic compounds (Lang et al., 2024). Management system did not significantly affect total antioxidant capacity either, probably because it did not have an impact on caseins, which were main contributors to antioxidant capacity, or other antioxidant compounds such as α-tocopherol, ascorbic acid or polyphenols (**Table 4.5**).

With regard to the lipid fraction, management system significantly affected the content of some individual fatty acids including the most abundant unsaturated species (9*c*-18:1, 18:2n-6 and 18:3n-3; **Table 4.5**), which resulted in a significant effect in total SFAs, MUFAs and n-6 and n-3 PUFAs as well. In general terms, SFAs and MUFAs were more abundant in milk samples from the low grazing system (farm I) whereas PUFAs, including n-6 and n-3 species, were more abundant in milk samples from high grazing systems (farms II and III). However, the n-6/n-3 ratio was higher in milk from the low grazing system, meaning that pasture-based feeding majorly improves the accumulation of n-3 PUFAs. Grass is rich in PUFAs, particularly 18:3n-3 (Sahaka et al., 2020), so these grass-derived PUFAs would have been accumulated in milk from mares with a feeding regime principally based on pasture (farms II and III). In the case of low-grazing mares (farm I), a lower acquisition of dietary PUFAs resulted in significantly lower PUFA but higher SFA contents in milk, probably derived from an enhanced *de novo* synthesis of short- to medium-chain SFAs. In fact, most even-chain SFAs shorter than 16:0 are synthetized *de novo* in the mammary gland (Palmquist & Harvatine, 2020), and low fat diets (such as in farm I, which was in part composed of fruits and potatoes) have been shown to enhance the *de novo* synthesis of fatty acids (Duran-Montgé et al., 2010), perhaps as a compensation strategy. Because MUFAs are mainly synthetized in the mammary gland from desaturation of SFAs, in a reaction catalysed by the enzyme stearoyl-CoA desaturase (Barber et al., 1997; Smith et al., 2009), higher MUFA contents were also found in milk samples from low-grazing mares. Probably, MUFA contents in low grazing mare milk did not only come from higher SFA contents but also from a superior SFA to MUFA conversion, since pasture feeding depresses the gene expression of stearoyl-CoA desaturase (Smith et al., 2009). In addition, a significant interaction effect between farm and lactation stage was observed for the content of 9:0, 11:0, 18:3n-6, 16:3n-3, 18:3n-3, 9*t*,12*t*,15*c*-18:3 and total n-3, and for PUFA/SFA and n-6/n-3 ratios (**Table 4.5**). In all cases, changes during lactation were different between milk samples from low grazing (farm I) and high grazing management systems (farms II and III). As highlights, milk samples from high grazing management systems (farms II and III) presented an increasing PUFA/SFA ratio during lactation, and milk samples from low grazing management (farm I) presented a decreasing trend in the content of n-3 PUFAs and an abrupt increase in the n-6/n-3 ratio after weeks 15-16 of lactation. These changes classified according to management system are depicted and further described in **Manuscript 1**.

All compositional data (excluding the proteomic profile) were submitted to a SDA in order to verify the differences among the management systems. This multivariate analysis was able to discriminate more than 79 % of mare milk samples according to farm. When discriminating samples from low grazing (farm I) and high grazing mares (farms II and III), more than 98 % of milk samples were correctly classified. Canonical discriminant function 1 successfully classified most samples from high *vs.* low grazing activity (farm I *vs*. farms II and III), whereas canonical function 2 mainly discriminated between samples from the two farms with a high grazing activity (farms II and III) (**Figure 4.7**). Considering the correlation between the variables and the discriminant functions, differences between milk samples from high *vs.* low grazing management systems were mainly due to the content of some *cis*-MUFAs and n-3 PUFAs (including 9*c*-18:1 and 18:3n-3), 9*t*,12*t*,15*c*-18:3 and pyridoxine. On the other hand, samples from the two farms with high grazing intensity (farms II and III) were mainly differentiated by the content of K, Na, pantothenic acid, some *cis*-MUFAs, some odd-chain fatty acids (11:0, 19:0, 9*c*-17:1, 11*c*-19:1) and 9*t*-16:1.

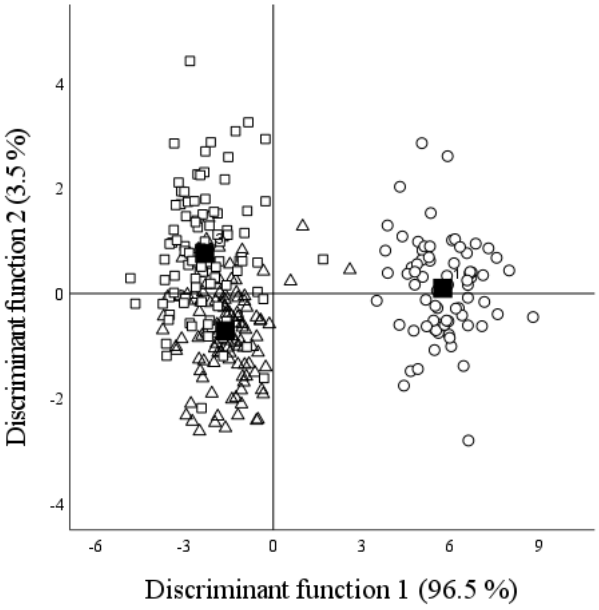


Figure 4.7. *Distribution of mare milk samples according to farm (○, Farm I; △, Farm II; □, Farm III; ■, group centroid) obtained from the Stepwise Discriminant Analysis applied to compositional data (excluding the proteomic profile) of mare milk samples.*

Regarding the protein fraction of mare milk, as previously discussed for the rest of the chemical compounds analysed, management system had a lower impact than lactation stage on mare milk proteome, whereas no effect was observed on the peptidomic profile. Detailed information on the effect of management system on mare milk proteome is given in **Publication 3**. This effect was actually only significant for the abundance of α1-antitrypsin, a serine protease inhibitor that acts against elastase from neutrophil leucocytes (Carrell & Travis, 1985). Abundance of this protein was enhanced in high grazing milk samples (farms II and III), probably as a result of a higher dietary PUFA intake. There is evidence that dietary unsaturated fatty acid supplementation can modulate the expression of SERPINA1, the gene that encodes α1-antitrypsin, in cattle mammary gland (Mach et al., 2011). SERPINA1 is involved in the acute phase response signaling pathway, so changes in its expression may affect the immune response of the mammary gland, as suggested by Mach et al. (2011). On the other hand, a significant interaction between farm and lactation stage was found for perilipin 2 and butyrophilin subfamily 1 member A1 protein, which are two of the most abundant proteins in the milk fat globule membrane (Han et al., 2020). A decrease in these two proteins at late lactation in cow milk has been related with mammary gland involution (Zhang et al., 2015), and dietary unsaturated fatty acids have also been proved to modulate the expression of perilipin 2 in cow milk. Therefore, these factors could have affected the abundance of milk fat globule membrane proteins also in mare milk.

When considering protein digestion, management of the mares somehow affected mare milk protein digestibility as well. For instance, milk from the low grazing system (farm I) presented higher DIAAR values for tryptophan and sulphur-containing amino acids, whereas milk from the high grazing system supplemented with hay (farm II) presented higher DIAAR values for aromatic amino acids and lower DIAAR values for lysine. As mentioned in the discussion about the effect of lactation stage, this was a consequence of a different amino acid composition in undigested raw mare milk, which can be modulated by diet (Liang et al., 2022). When peptides released after gastric and intestinal digestion of mare milk were monitored, the abundance of 31 peptides (28 and 3 formed after gastric and intestinal digestion, respectively) was significantly affected by management of the mares, with an overall higher peptide release in samples from low grazing system (farm I). As also discussed in the section about lactation effect, differences in protein digestion as affected by management of the mares probably come from differences in chemical

composition of mare milk samples, since other compounds in milk can affect protein gastrointestinal digestion (Egger et al., 2017; Liu et al., 2019). Overall, results in this PhD Thesis demonstrate that mare milk protein digestion can be affected by different management factors, but it remains unclear whether this is a consequence of milk composition or if other factors contribute to variations in protein digestion, so further research would definitely be needed. An extended discussion regarding the effect of management system on mare milk protein digestion can be found in **Publication 4**.

5 REFERENCES

Adlerova, L., Bartoskova, A., & Faldyna, M. (2008). Lactoferrin: A review. *Veterinarni Medicina, 53*, 457-468.

Albrecht, S., Lane, J. A., Marino, K., Al Busadah, K. A., Carrington, S. D., Hickey, R. M., & Rudd, P. M. (2014). A comparative study of free oligosaccharides in the milk of domestic animals. *British Journal of Nutrition, 111*, 1313-1328.

Aldai, N., de Renobales, M., Barron, L. J. R., & Kramer, J. K. (2013). What are the trans fatty acids issues in foods after discontinuation of industrially produced trans fats? Ruminant products, vegetable oils, and synthetic supplements. *European Journal of Lipid Science and Technology, 115*, 1378-1401.

Aldai, N., Kramer, J., Cruz-Hernandez, C., Santercole, V., Delmonte, P., Mossoba, M. M., & Dugan, M. (2012). Appropriate extraction and methylation techniques for lipids analysis. In G. Cherian & R. Poureslami (Eds.), *Fat and Fatty Acids in Poultry Nutrition and Health* (pp. 249-278). Context.

Aldai, N., Lavín, M. P., Belaunzaran, X., & Mantecón, A. R. (2018). Carne de caballo: Sistemas de explotación, evolución del sector y calidad nutricional de la carne. *Eurocarne: La Revista Internacional Del Sector Cárnico, 263*, 58-68.

Algazina, T., Gulyayev, A., Aimoldina, A., Kushugulova, A., & Yermekbayeva, B. (2020). Clinical assessment of the use of sublimated mare's milk in complex therapy of mild and moderate psoriasis. *Journal of Global Pharma Technology, 12*, 18-26.

Alichanidis, E., Moatsou, G., & Polychroniadou, A. (2016). Composition and properties of non-cow milk and products. In E. Tsakalidou & K. Papadimitriou (Eds.), *Non-bovine milk and milk products* (pp. 81-116). Elsevier.

Alipour, A., Mohajeri, A. F., Javdan, G., Pourramezani, F., Fallahzadeh, H., & Sadrabad, E. K. (2023). Concentration of mineral and heavy metals in raw mare (horse) milk consumed in Yazd, Iran: A risk assessment study. *Veterinary Medicine and Science, 9,* 1592-1598.

Álvarez, R., Meléndez-Martínez, A. J., Vicario, I. M., & Alcalde, M. J. (2015). Carotenoids and fat-soluble vitamins in horse tissues: A comparison with cattle. *Animal, 9*, 1230-1238.

Amrit, B. K., Ponnampalam, E. N., Macwan, S., Wu, H., Aziz, A., Muir, S., Dunshea, F. R., & Suleria, H. A. (2023). Comprehensive screening and characterization of polyphenol compounds from pasture grasses used for livestock production under temperate region. *Animal Feed Science and Technology, 300*, Article 115657.

Anagnostopoulos, A. K., Katsafadou, A. I., Pierros, V., Kontopodis, E., Fthenakis, G. C., Arsenos, G., Karkabounas, S. C., Tzora, A., Skoufos, I., & Tsangaris, G. T. (2016). Milk of Greek sheep and goat breeds; characterization by means of proteomics. *Journal of Proteomics, 147*, 76-84.

Anderson, R. R. (1991). Comparison of minerals in milk of four species. *Comparative Biochemistry and Physiology Part A: Comparative Physiology, 100*, 1045-1048.

Anderson, R. R. (1992). Comparison of trace elements in milk of four species. *Journal of Dairy Science, 75*, 3050-3055.

Auclair-Ronzaud, J., Jaffrézic, F., Wimel, L., Dubois, C., Laloë, D., & Chavatte-Palmer, P. (2022). Estimation of milk production in suckling mares and factors influencing their milk yield. *Animal, 16*, Article 100498.

Barber, M. C., Clegg, R. A., Travers, M. T., & Vernon, R. G. (1997). Lipid metabolism in the lactating mammary gland. *Biochimica Et Biophysica Acta (BBA) – Lipids and Lipid Metabolism, 1347*, 101-126.

Barello, C., Garoffo, L. P., Montorfano, G., Zava, S., Berra, B., Conti, A., & Giuffrida, M. G. (2008). Analysis of major proteins and fat fractions associated with mare's milk fat globules. *Molecular Nutrition & Food Research, 52*, 1448-1456.

Barłowska, J., Polak, G., Janczarek, I., & Próchniak, T. (2023a). Chemical composition, whey protein profile, and fatty acid profile of milk from Sokólski horses in relation to Polish Halfbred horses. *Annals of Animal Science, 23,* 587-596.

Barłowska, J., Polak, G., Janczarek, I., & Tkaczyk, E. (2023b). The influence of selected factors on the nutritional value of the milk of cold-blooded mares: The example of the Sokólski breed. *Animals, 13*, Article 1152.

Barreto, I. M., Urbano, S. A., Oliveira, C. A., Macêdo, C. S., Borba, L. H., Chags, B. M., & Rangel, A. H. (2020). Chemical composition and lipid profile of mare colostrum and milk of the Quarter horse breed. *PLoS One, 15*, Article e0238921.

Bat-Oyun, T., Erdenetsetseg, B., Shinoda, M., Ozaki, T., & Morinaga, Y. (2015). Who is making airag (fermented mare's milk)? A nationwide survey of traditional food in Mongolia. *Nomadic Peoples, 19*, 7-29.

Belaunzaran, X. (2017). *Lipidomics of horse-meat: Methodological aspects and factors affecting its composition.* [PhD Thesis]. University of the Basque Country (UPV/EHU).

Belaunzaran, X., Bessa, R. J., Lavín, P., Mantecón, A. R., Kramer, J. K., & Aldai, N. (2015). Horse-meat for human consumption – Current research and future opportunities. *Meat Science, 108*, 74-81.

Belaunzaran, X., Lavín, P., Mantecón, A. R., Kramer, J., & Aldai, N. (2018). Effect of slaughter age and feeding system on the neutral and polar lipid composition of horse meat. *Animal, 12*, 417-425.

Beldarrain, L. (2022). *Effect of ageing time on horse meat quality: Instrumental and sensory approach*. [PhD Thesis]. University of the Basque Country (UPV/EHU).

Berger, S. T., Ahmed, S., Muntel, J., Polo, N. C., Bachur, R., Kentsis, A., Steen, J., & Steen, H. (2015). MStern blotting-high throughput polyvinylidene fluoride (PVDF) membrane-based proteomic sample preparation for 96-well plates. *Molecular & Cellular Proteomics, 14*, 2814-2823.

Bernués, A. (2017). Animals on the land. Ecosystem services and disservices of grazing livestock systems. In J. D'Silva & J. Webster (Eds.), *The meat crisis: Developing more sustainable and ethical production and consumption* (pp. 67-92). Routledge.

Bernués, A., Alfnes, F., Clemetsen, M., Eik, L. O., Faccioni, G., Ramanzin, M., Ripoll-Bosch, R., Rodríguez-Ortega, T., & Sturaro, E. (2019). Exploring social preferences for ecosystem services of multifunctional agriculture across policy scenarios. *Ecosystem Services, 39*, Article 101002.

Bernués, A., Riedel, J. L., Asensio, M. A., Blanco, M., Sanz, A., Revilla, R., & Casasús, I. (2005). An integrated approach to studying the role of grazing livestock systems in the conservation of rangelands in a protected natural park (Sierra de Guara, Spain). *Livestock Production Science, 96*, 75-85.

Bernués, A., Tello-García, E., Rodríguez-Ortega, T., Ripoll-Bosch, R., & Casasús, I. (2016). Agricultural practices, ecosystem services and sustainability in high nature value farmland: Unraveling the perceptions of farmers and nonfarmers. *Land use Policy, 59*, 130-142.

Bidasolo, I. B., Ramos, M., & Gomez-Ruiz, J. A. (2012). In vitro simulated gastrointestinal digestion of donkeys' milk. Peptide characterization by high performance liquid chromatography-tandem mass spectrometry. *International Dairy Journal, 24*, 146-152.

Bijl, E., Van Valenberg, H., Huppertz, T., & Van Hooijdonk, A. (2013). Protein, casein, and micellar salts in milk: Current content and historical perspectives. *Journal of Dairy Science, 96*, 5455-5464.

Bimbetov, B., Shanazarov, N., Aitbaeva, S., Bakytzhanuly, A., & Utepbergenova, G. (2021). Clinical-biochemical and fibroelastometric results of the mare's milk application in non-alcoholic steathepatitis. *Revista Cubana De Medicina General Integral, 37*, Article e1953.

Blaner, W. S., Shmarakov, I. O., & Traber, M. G. (2021). Vitamin A and vitamin E: Will the real antioxidant please stand up? *Annual Review of Nutrition, 41*, 105-131.

Bloor, J., Jay-Robert, P., Le Morvan, A., & Fleurance, G. (2012). Déjections des herbivores domestiques au pâturage: Caractéristiques et rôle dans le fonctionnement des prairies. *INRAE Productions Animales, 25*, 45-56.

BOB Boletín Oficial de Bizkaia. Decreto Foral de la Diputación Foral de Bizkaia 45/2017, de 4 de abril, por el que se modifica el Decreto Foral 59/2016, de 22 de marzo, por el que se establecen ayudas de agroambiente y clima y agricultura ecológica, al sector agrario en el territorio histórico de Bizkaia, y se efectúa convocatoria de estas ayudas para 2017 (2017).

Bogan, K. L., & Brenner, C. (2008). Nicotinic acid, nicotinamide, and nicotinamide riboside: A molecular evaluation of NAD precursor vitamins in human nutrition. *Annual Review of Nutrition, 28*, 115-130.

Bondo, T., & Jensen, S. K. (2011). Administration of RRR-α-tocopherol to pregnant mares stimulates maternal IgG and IgM production in colostrum and enhances vitamin E and IgM status in foals. *Journal of Animal Physiology and Animal Nutrition, 95*, 214-222.

BOTHA Boletín Oficial del Territorio Histórico de Álava. Decreto Foral 25/2023, del consejo de gobierno foral de 30 de mayo. Aprobar las bases reguladoras para la concesión de las ayudas destinadas a la adquisición de sementales equinos de raza "Caballo de monte del País Vasco-Euskal Herriko Mendiko Zaldia" por explotaciones ganaderas del territorio histórico de Álava, así como la convocatoria para el año 2023 (2023).

BOPV Boletín Oficial del País Vasco. Agindua, 1999ko uztailaren 21ekoa, nekazaritza eta arrantza sailburuarena, Euskal Herriko Mendiko Zaldia / Caballo de Monte del País Vasco zaldi-arrazari buruzko arau bereziak onartzen dituena (1999).

Brodkorb, A., Egger, L., Alminger, M., Alvito, P., Assunção, R., Ballance, S., Bohn, T., Bourlieu-Lacanal, C., Boutrou, R., Carrière, F., Clemente, A., Corredig, M., Dupont, D., Dufour, C., Edwards, C., Golding, M., Karakaya, S., Kirkhus, B., Le Feunteun, S., … & Recio, I. (2019). INFOGEST static in vitro simulation of gastrointestinal food digestion. *Nature Protocols, 14*, 991-1014.

Businco, L., Giampietro, P. G., Lucenti, P., Lucaroni, F., Pini, C., Di Felice, G., Iacovacci, P., Curadi, C., & Orlandi, M. (2000). Allergenicity of mare's milk in children with cow's milk allergy. *Journal of Allergy and Clinical Immunology, 105*, 1031-1034.

Cabezuelo, M. T., Zaragozá, R., Barber, T., & Viña, J. R. (2020). Role of vitamin A in mammary gland development and lactation. *Nutrients, 12*, Article 80.

Cabiddu, A., Delgadillo-Puga, C., Decandia, M., & Molle, G. (2019). Extensive ruminant production systems and milk quality with emphasis on unsaturated fatty acids, volatile compounds, antioxidant protection degree and phenol content. *Animals, 9*, Article 771.

Čagalj, M., Brezovečki, A., Mikulec, N., & Antunac, N. (2014). Composition and properties of mare's milk of Croatian Coldblood horse breed. *Mljekarstvo: Časopis Za Unaprjeđenje Proizvodnje i Prerade Mlijeka, 64*, 3-11.

Cais-Sokolińska, D., Danków, R., Bierzuńska, P., Kaczyński, Ł. K., Chudy, S., Teichert, J., Dobek, A., Skotarczak, E., & Pikul, J. (2018). Freezing point and other technological properties of milk of the Polish Coldblood horse breed. *Journal of Dairy Science, 101*, 9637-9646.

Carrell, R., & Travis, J. (1985). A1-antitrypsin and the serpins: Variation and countervariation. *Trends in Biochemical Sciences, 10*, 20-24.

Chen, L., Hong, T., Li, Z., Shen, G., Gu, Y., & Han, J. (2023). A comparison of milk fat globule membranes and whey proteomes: New insight into variation nutrient differences between buffalo, cow, goat, and yak. *Food Chemistry, 429*, Article 136845.

Cheng, S., Douglas-Jones, A., Yang, X., Mansel, R. E., & Jiang, W. G. (2010). Transforming acidic coiled-coil-containing protein 2 (TACC2) in human breast cancer, expression pattern and clinical/prognostic relevance. *Cancer Genomics & Proteomics, 7*, 67-73.

Claeys, W. L., Verraes, C., Cardoen, S., De Block, J., Huyghebaert, A., Raes, K., Dewettinck, K., & Herman, L. (2014). Consumption of raw or heated milk from different species: An evaluation of the nutritional and potential health benefits. *Food Control, 42*, 188-201.

Clauss, M., Grum, C., & Hatt, J. (2009). Polyunsaturated fatty acid content in adipose tissue in foregut and hindgut fermenting mammalian herbivores: A literature survey. *Mammalian Biology, 74*, 153-158.

Cobano-Delgado, V., & Llorent-Bedmar, V. (2020). Women's well-being and rural development in depopulated Spain. *International Journal of Environmental Research and Public Health, 17*, Article 1966.

Collins, R. A., Harper, A. E., Schreiber, M., & Elvehjem, C. A. (1951). The folic acid and vitamin B12 content of the milk of various species. *The Journal of Nutrition, 43*, 313-321.

Coppa, M., Ferlay, A., Monsallier, F., Verdier-Metz, I., Pradel, P., Didienne, R., Farruggia, A., Montel, M. C., & Martin, B. (2011). Milk fatty acid composition and cheese texture and appearance from cows fed hay or different grazing systems on upland pastures. *Journal of Dairy Science, 94*, 1132-1145.

Cosentino, C., Notarnicola, G., Paolino, R., D'Adamo, C., Labella, C., Musto, M., & Freschi, P. (2017). Colostrum and milk characteristics in Murgese breed mares. *Czech Journal of Animal Science, 62*, 466-472.

Cruz-Hernandez, C., Deng, Z., Zhou, J., Hill, A. R., Yurawecz, M. P., Delmonte, P., Mossoba, M. M., Dugan, M. E. R., & Kramer, J. K. G. (2004). Methods for analysis of conjugated linoleic acids and trans-18:1 isomers in dairy fats by using a combination of gas chromatography, silver-ion thin-layer chromatography/gas chromatography, and silver-ion liquid chromatography. *Journal of AOAC International, 87*, 545-562.

Csapó, J., Stefler, J., Martin, T. G., Makray, S., & Csapó-Kiss, Z. (1995). Composition of mares' colostrum and milk. Fat content, fatty acid composition and vitamin content. *International Dairy Journal, 5*, 393-402.

Csapó-Kiss, Z., Stefler, J., Martin, T. G., Makray, S., & Csapó, J. (1995). Composition of mares' colostrum and milk. Protein content, amino acid composition and contents of macro and micro-elements. *International Dairy Journal, 5*, 403-415.

Curadi, M. C., Giampietro, P. G., Lucenti, P., & Orlandi, M. (2001). Use of mare milk in pediatric allergology. *ASPA Congress in Animal Production Science, 2*, 647-649.

Czyżak-Runowska, G., Wójtowski, J. A., Danków, R., & Stanisławski, D. (2021). Mare's milk from a small Polish specialized farm – Basic chemical composition, fatty acid profile, and healthy lipid indices. *Animals, 11*, Article 1590.

Delgadillo-Puga, C., Cuchillo-Hilario, M., León-Ortiz, L., Ramírez-Rodríguez, A., Cabiddu, A., Navarro-Ocaña, A., Morales-Romero, A. M., Medina-Campos, O. N., & Pedraza-Chaverri, J. (2019). Goats' feeding supplementation with Acacia farnesiana pods and their relationship

with milk composition: Fatty acids, polyphenols, and antioxidant activity. *Animals, 9*, Article 515.

Delmonte, P., Kia, A. F., Kramer, J. K., Mossoba, M. M., Sidisky, L., & Rader, J. I. (2011). Separation characteristics of fatty acid methyl esters using SLB-IL111, a new ionic liquid coated capillary gas chromatographic column. *Journal of Chromatography A, 1218*, 545-554.

Deng, L., Yang, Y., Li, Z., Li, J., Zhu, Y., Meng, Q., Liu, J., & Wang, X. (2022). Impact of different dietary regimens on the lipidomic profile of mare's milk. *Food Research International, 156*, Article 111305.

Devle, H., Vetti, I., Naess-Andresen, C. F., Rukke, E., Vegarud, G., & Ekeberg, D. (2012). A comparative study of fatty acid profiles in ruminant and non-ruminant milk. *European Journal of Lipid Science and Technology, 114*, 1036-1043.

Díaz, L. D., Fernández-Ruiz, V., & Cámara, M. (2020). The frontier between nutrition and pharma: The international regulatory framework of functional foods, food supplements and nutraceuticals. *Critical Reviews in Food Science and Nutrition, 60*, 1738-1746.

Difilippo, E., Willems, H., Vendrig, J. C., Fink-Gremmels, J., Gruppen, H., & Schols, H. A. (2015). Comparison of milk oligosaccharides pattern in colostrum of different horse breeds. *Journal of Agricultural and Food Chemistry, 63*, 4805-4814.

Doreau, M., & Boulot, S. (1989). Recent knowledge on mare milk production: A review. *Livestock Production Science, 22*, 213-235.

Doreau, M., & Martin-Rosset, W. (2002). Dairy animals: Horse. In H. Roginski, J. A. Fuquay & P. F. Fox (Eds.), *Encyclopedia of Dairy Sciences* (pp. 630-637). Academic Press Inc.

Doreau, M., Boulot, S., Barlet, J., & Patureau-Mirand, P. (1990). Yield and composition of milk from lactating mares: Effect of lactation stage and individual differences. *Journal of Dairy Research, 57*, 449-454.

Doyen, V., Leduc, V., Corazza, F., Mairesse, M., Ledent, C., & Michel, O. (2013). Protein contact dermatitis and food allergy to mare milk. *Annals of Allergy, Asthma & Immunology, 110*, 390-391.

Dupont, D., & Tomé, D. (2020). Milk proteins: Digestion and absorption in the gastrointestinal tract. In M. Boland & H. Singh (Eds.), *Milk proteins from expression to food* (pp. 701-714). Academic Press.

Duran-Montgé, P., Realini, C. E., Barroeta, A. C., Lizardo, R. G., & Esteve-Garcia, E. (2010). De novo fatty acid synthesis and balance of fatty acids of pigs fed different fat sources. *Livestock Science, 132*, 157-164.

Egger, L., Ménard, O., & Portmann, R. (2019). Quantitative characterization of digestion processes. In O. Gouseti, G. M. Bornhorst, S. Bakalis & A. Mackie (Eds.), *Interdisciplinary Approaches to Food Digestion* (pp. 159-184). Springer.

Egger, L., Schlegel, P., Baumann, C., Stoffers, H., Guggisberg, D., Brügger, C., Dürr, D., Stoll, P., Vergères, G., & Portmann, R. (2017). Physiological comparability of the harmonized INFOGEST in vitro digestion method to in vivo pig digestion. *Food Research International, 102*, 567-574.

Egito, A. S., Girardet, J., Miclo, L., Mollé, D., Humbert, G., & Gaillard, J. (2001). Susceptibility of equine κ- and β-caseins to hydrolysis by chymosin. *International Dairy Journal, 11*, 885-893.

Fanta, C., & Ebner, C. (1998). Allergy to mare's milk. *Allergy, 53*, 539-540.

FAO (2013). Dietary protein quality evaluation in human nutrition. Report of a FAO expert consultation. *FAO Food and Nutrition Paper, 92*, 1-66.

FAO (2016). *Migration, agriculture and rural development: Addressing the root causes of migration and harnessing its potential for development.* FAO Reports.

FAO (2024). Domestic animal diversity information system (DAD-IS). Retrieved from https://www.fao.org/dad-is/browse-by-country-and-species/en/.

FAOSTAT (2023). Food and Agriculture Organization Corporate Statistical Database. Retrieved from https://www.fao.org/faostat/en/#home.

Ferreira, L. M. M., Celaya, R., Benavides, R., Jáuregui, B. M., García, U., Santos, A. S., García, R. R., Rodrigues, M. A. M., & Osoro, K. (2013). Foraging behaviour of domestic herbivore

species grazing on heathlands associated with improved pasture areas. *Livestock Science, 155*, 373-383.

Fleurance, G., Farruggia, A., Lanore, L., & Dumont, B. (2016). How does stocking rate influence horse behaviour, performances and pasture biodiversity in mesophile grasslands? *Agriculture, Ecosystems & Environment, 231*, 255-263.

Foekel, C., Schubert, R., Kaatz, M., Schmidt, I., Bauer, A., Hipler, U., Vogelsang, H., Rabe, K., & Jahreis, G. (2009). Dietetic effects of oral intervention with mare's milk on the severity scoring of atopic dermatitis, on faecal microbiota and on immunological parameters in patients with atopic dermatitis. *International Journal of Food Sciences and Nutrition, 60*, 41-52.

Fotschki, J., Szyc, A. M., Laparra, J. M., & Wróblewska, B. (2015). Mare's and cow's milk: Promote similar metabolic effects and expression of innate markers in Caco-2 cells? *Food Research International, 72*, 184-190.

Fotschki, J., Szyc, A. M., Laparra, J. M., Markiewicz, L. H., & Wróblewska, B. (2016). Immune-modulating properties of horse milk administered to mice sensitized to cow milk. *Journal of Dairy Science, 99*, 9395-9404.

Fox, P. F., Uniacke-Lowe, T., McSweeney, P., & O'Mahony, J. A. (2015). Vitamins in milk and dairy products. In P. F. Fox, T. Uniacke-Lowe, P. L. H. McSweeney & J. A. O'Mahony (Eds.), *Dairy chemistry and biochemistry* (pp. 271-297). Springer.

Franz, R., Soliva, C. R., Kreuzer, M., Steuer, P., Hummel, J., & Clauss, M. (2010). Methane production in relation to body mass of ruminants and equids. *Evolutionary Ecology Research, 12*, 727-738.

Froehlich, J. W., Dodds, E. D., Barboza, M., McJimpsey, E. L., Seipert, R. R., Francis, J., An, H. J., Freeman, S., German, J. B., & Lebrilla, C. B. (2010). Glycoprotein expression in human milk during lactation. *Journal of Agricultural and Food Chemistry, 58*, 6440-6448.

Gall, C. F. (2013). Production systems around the world. In Y. W. Park & G. F. W. Haenlein (Eds.), *Milk and dairy products in human nutrition: Production, composition and health* (pp. 1-30). John Wiley & Sons.

Gall, H., Kalveram, C. M., Sick, H., & Sterry, W. (1996). Allergy to the heat-labile proteins α-lactalbumin and β-lactoglobulin in mare's milk. *Journal of Allergy and Clinical Immunology, 97*, 1304-1307.

Garcia, C., Lutz, N. W., Confort-Gouny, S., Cozzone, P. J., Armand, M., & Bernard, M. (2012). Phospholipid fingerprints of milk from different mammalians determined by 31P NMR: Towards specific interest in human health. *Food Chemistry, 135*, 1777-1783.

Gila-Díaz, A., Herranz Carrillo, G., Cañas, S., Saenz de Pipaón, M., Martínez-Orgado, J. A., Rodríguez-Rodríguez, P., López de Pablo, A. L., Martin-Cabrejas, M. A., Ramiro-Cortijo, D., & Arribas, S. M. (2020). Influence of maternal age and gestational age on breast milk antioxidants during the first month of lactation. *Nutrients, 12*, Article 2569.

Glasser, F., Doreau, M., Maxin, G., & Baumont, R. (2013). Fat and fatty acid content and composition of forages: A meta-analysis. *Animal Feed Science and Technology, 185*, 19-34.

Godovac-Zimmermann, J., Shaw, D., Conti, A., & McKenzie, H. (1987). Identification and the primary structure of equine α-lactalbumin B and C (Equus caballus, Perissodactyla). *Biological Chemistry, 368*, 427-434.

Grace, N. D., Pearce, S. G., Firth, E. C., & Fennessy, P. F. (1999). Concentrations of macro- and micro-elements in the milk of pasture-fed Thoroughbred mares. *Australian Veterinary Journal, 77*, 177-180.

Grażyna, C., Hanna, C., Adam, A., & Magdalena, B. M. (2017). Natural antioxidants in milk and dairy products. *International Journal of Dairy Technology, 70*, 165-178.

Greiwe-Crandell, K. M., Kronfeld, D. S., Gay, L. S., Sklan, D., Tiegs, W., & Harris, P. A. (1997). Vitamin A repletion in Thoroughbred mares with retinyl palmitate or β-carotene. *Journal of Animal Science, 75*, 2684-2690.

Gulati, A., Galvin, N., Lewis, E., Hennessy, D., O'Donovan, M., McManus, J. J., Fenelon, M. A., & Guinee, T. P. (2018). Outdoor grazing of dairy cows on pasture versus indoor feeding on total mixed ration: Effects on gross composition and mineral content of milk during lactation. *Journal of Dairy Science, 101*, 2710-2723.

Guri, A., Paligot, M., Crèvecoeur, S., Piedboeuf, B., Claes, J., Daube, G., Corredig, M., Griffiths, M. W. & Delcenserie, V. (2016). In vitro screening of mare's milk antimicrobial effect and antiproliverative activity. *FEMS Microbiology Letters, 363*, Article fnv234.

Hachana, Y., Nasraoui, C., Frija, I., & Fortina, R. (2022). Arabian mare's milk characterisation and clotting ability. *Journal of Food Science and Technology, 59*, 1840-1846.

Haddad, I., Mozzon, M., Strabbioli, R., & Frega, N. G. (2011). Fatty acid composition and regiodistribution in mare's milk triacylglycerols at different lactation stages. *Dairy Science & Technology, 91*, 397-412.

Haddad, I., Mozzon, M., Strabbioli, R., & Frega, N. G. (2012). A comparative study of the composition of triacylglycerol molecular species in equine and human milks. *Dairy Science & Technology, 92*, 37-56.

Han, L., Zhang, M., Xing, Z., Coleman, D. N., Liang, Y., Loor, J. J., & Yang, G. (2020). Knockout of butyrophilin subfamily 1 member A1 (BTN1A1) alters lipid droplet formation and phospholipid composition in bovine mammary epithelial cells. *Journal of Animal Science and Biotechnology, 11*, Article 72.

Holmes, A. D., McKey, B. V., Wertz, A. W., Lindquist, H. G., & Parkinson, L. R. (1946). The vitamin content of mare's milk. *Journal of Dairy Science, 29*, 163-171.

Hsu, Y., Jhang, W., Lee, M., Bat-Otgon, B., Narantungalag, E., & Huang, C. (2021). Lactose-riched Mongolian mare's milk improves physical fatigue and exercise performance in mice. *International Journal of Medical Sciences, 18*, 564-574.

Inglingstad, R. A., Devold, T. G., Eriksen, E. K., Holm, H., Jacobsen, M., Liland, K. H., Rukke, E. O., & Vegarud, G. E. (2010). Comparison of the digestion of caseins and whey proteins in equine, bovine, caprine and human milks by human gastrointestinal enzymes. *Dairy Science & Technology, 90*, 549-563.

Insausti, K., Beldarrain, L. R., Lavín, M. P., Aldai, N., Mantecón, A. R., Sáez, J. L., & Canals, R. M. (2021). Horse meat production in northern Spain: Ecosystem services and sustainability in high nature value farmland. *Animal Frontiers, 11*, 47-54.

Irshad, M., & Dubey, R. (2005). Apolipoproteins and their role in different clinical conditions: An overview. *Indian Journal of Biochemistry and Biophysics, 42*, 73-80.

ISO 8968-3 (2007). International Organization for Standardization (8968-3:2007/IDF 20-3:2007).

ISO/DIS 13904 (2014). International Organization for Standardization/Draft International Standard (13904:2014(E)) Animal feeding stuffs – Determination of tryptophan content.

Jaeser, M., Wunderlich, C., & Henle, T. (2023). Characterization of the glycans of equine κ-casein. *Journal of Agricultural and Food Chemistry, 71,* 9460-9468.

Jahreis, G., & Dawczynski, C. (2020). Trans and conjugated fatty acids in dairy products: Cause for concern? In I. Givens (Ed.), Milk and dairy foods (pp. 87-120). Academic Press.

Janis, C. (1976). The evolutionary strategy of equidae and the origins of rumen and cecal digestion. *Evolution, 30,* 757–774.

Jaudzems, G., Guthrie, J., Lahrichi, S., & Fuerer, C. (2019). Total amino acids by UHPLC-UV in infant formulas and adult nutritionals, first action 2018.06. *Journal of AOAC International, 102*, 1574-1588.

Jensen, H. B., Pedersen, K. S., Johansen, L. B., Poulsen, N. A., Bakman, M., Chatterton, D. E., & Larsen, L. B. (2015). Genetic variation and posttranslational modification of bovine κ-casein: Effects on caseino-macropeptide release during renneting. *Journal of Dairy Science, 98*, 747-758.

Ji, Z., Dong, R., Du, Q., Jiang, H., Fan, R., Bu, D., Wang, J., Yu, Z., Han, R., & Yang, Y. (2024). Insight into differences in whey proteome from human and eight dairy animal species for formula humanization. *Food Chemistry, 430*, Article 137076.

Jokar, S. Z., Sadeghieh, T., Sadeghmanesh, R., & Shakiba, M. (2021). Comparison of horse and cow milk on sleep disorders in children with attention deficit hyperactivity disorder: A doubleblind randomized clinical trial study. *TMR Non-Drug Theraphy, 4*, 1-9.

Jokar, S. Z., Sadeghieh, T., Shakiba, M., Emtiazy, M., Sadeghmanesh, R., Hashemi, M. S., & Mousavi, Z. (2023). Comparison of horse and cow milk on the symptoms of attention deficit

hyperactivity disorder in children: A cross-over clinical trial study. *Traditional and Integrative Medicine, 8*, 48-55.

Kaić, A., Luštrek, B., Simčič, M., & Potočnik, K. (2019). Milk quantity, composition and hygiene traits of routinely machine milked Lipizzan mares. *Slovenian Veterinary Research, 56*, 115-123.

Kaneda, T. (1991). Iso- and anteiso-fatty acids in bacteria: Biosynthesis, function, and taxonomic significance. *Microbiological Reviews, 55*, 288-302.

Karav, S., Salcedo, J., Frese, S. A., & Barile, D. (2018). Thoroughbred mare's milk exhibits a unique and diverse free oligosaccharide profile. *FEBS Open Bio, 8*, 1219-1229.

Kenzig, A. R., O'Meara, K. M., Kremer, C. J., Jogan, K. S., Jack, N. E., & Cole, K. (2009). Colostral, milk and serum immunoglobulin G concentrations in Quarter horse mares and their foals. *Journal of Equine Veterinary Science, 29*, 486-487.

Kneifel, W., & Mayer, H. K. (1991). Vitamin profiles of kefirs made from milks of different species. *International Journal of Food Science & Technology, 26*, 423-428.

Kopf-Bolanz, K. A., Schwander, F., Gijs, M., Vergères, G., Portmann, R., & Egger, L. (2012). Validation of an in vitro digestive system for studying macronutrient decomposition in humans. *The Journal of Nutrition, 142*, 245-250.

Kostelac, D., Susac, M., Sokac, K., & Frece, J. (2022). Equid milk is a source of probiotic bacteria with potential in caries reduction and preservation of periodontal health. *Journal of Microbiology, Biotechnology and Food Sciences, 12*, Article e5485.

Kramer, J. K., Hernandez, M., Cruz-Hernandez, C., Kraft, J., & Dugan, M. E. (2008). Combining results of two GC separations partly achieves determination of all cis and trans 16:1, 18:1, 18:2 and 18:3 except CLA isomers of milk fat as demonstrated using Ag-ion SPE fractionation. *Lipids, 43*, 259-273.

Kronqvist, C. (2011). *Minerals to dairy cows with focus on calcium and magnesium balance.* [PhD Thesis]. Swedish University of Agricultural Sciences.

Krzciuk, K. (2016). Intelligent analysis of samples by semiquantitative inductively coupled plasma-mass spectrometry (ICP-MS) technique: A review. *Critical Reviews in Analytical Chemistry, 46*, 284-290.

Kuhl, J., Aurich, J. E., Wulf, M., Hurtienne, A., Schweigert, F. J., & Aurich, C. (2012). Effects of oral supplementation with β-carotene on concentrations of β-carotene, vitamin A and α-tocopherol in plasma, colostrum and milk of mares and plasma of their foals and on fertility in mares. *Journal of Animal Physiology and Animal Nutrition, 96*, 376-384.

Lang, Y., Gao, N., Zang, Z., Meng, X., Lin, Y., Yang, S., Yang, Y., Jin, Z., & Li, B. (2024). Classification and antioxidant assays of polyphenols: A review. *Journal of Future Foods, 4*, 193-204.

Li, N., Li, B., Guan, J., Shi, J., Evivie, S. E., Zhao, L., Huo, G., & Wang, S. (2020). Distinct effects of milks from various animal types on infant fecal microbiota through in vitro fermentations. *Frontiers in Microbiology, 11*, Article 580931.

Liang, X. S., Yue, Y. X., Zhao, Y. L., Guo, Y. M., Guo, X. Y., Shi, B. L., & Yan, S. M. (2022). Effects of dietary concentrate to forage ratio on milk performance, milk amino acid composition and milk protein synthesis of lactating donkeys. *Animal Feed Science and Technology, 292*, Article 115444.

Lima, M. S., Dimenstein, R., & Ribeiro, K. D. (2014). Vitamin E concentration in human milk and associated factors: A literature review. *Jornal De Pediatria, 90*, 440-448.

Liu, D., Zhang, J., Wang, L., Yang, T., Liu, X., Hemar, Y., Regenstein, J. M., & Zhou, P. (2019). Membrane-based fractionation, enzymatic dephosphorylation, and gastrointestinal digestibility of β-casein enriched serum protein ingredients. *Food Hydrocolloids, 88*, 1-12.

Liz, M. A., Coelho, T., Bellotti, V., Fernandez-Arias, M. I., Mallaina, P., & Obici, L. (2020). A narrative review of the role of transthyretin in health and disease. *Neurology and Therapy, 9*, 395-402.

Loucougaray, G., Bonis, A., & Bouzillé, J. (2004). Effects of grazing by horses and/or cattle on the diversity of coastal grasslands in western France. *Biological Conservation, 116*, 59-71.

Loza, C., Davis, H., Malisch, C., Taube, F., Loges, R., Magistrali, A., & Butler, G. (2023). Milk fatty acids: The impact of grazing diverse pasture and the potential to predict rumen-derived methane. *Agriculture, 13*, Article 181.

Luna, P., Juárez, M., & de la Fuente, M. A. (2005). Validation of a rapid milk fat separation method to determine the fatty acid profile by gas chromatography. *Journal of Dairy Science, 88*, 3377-3381.

Lv, J., & Wang, L. (2009). Bioactive components in kefir and koumiss. In Y. W. Park (Ed.), *Bioactive components in milk and dairy products* (pp. 251-262). Wiley-Blackwell.

Lv, K., Yang, Y., Li, Q., Chen, R., Deng, L., Zhang, Y., & Jiang, N. (2024). Identification and comparison of milk fat globule membrane and whey proteins from Selle Français, Welsh pony, and Tieling Draft horse mares milk. *Food Chemistry, 437*, Article 137915.

Mach, N., Jacobs, A., Kruijt, L., Van Baal, J., & Smits, M. A. (2011). Alteration of gene expression in mammary gland tissue of dairy cows in response to dietary unsaturated fatty acids. *Animal, 5*, 1217-1230.

Magan, J. B., O'Callaghan, T. F., Zheng, J., Zhang, L., Mandal, R., Hennessy, D., Fenelon, M. A., Wishart, D. S., Kelly, A. L., & McCarthy, N. A. (2020). Effect of diet on the vitamin B profile of bovine milk-based protein ingredients. *Foods, 9*, Article 578.

Malacarne, M., Martuzzi, F., Summer, A., & Mariani, P. (2002). Protein and fat composition of mare's milk: Some nutritional remarks with reference to human and cow's milk. *International Dairy Journal, 12*, 869-877.

MAPA Ministerio de Agricultura, Pesca y Alimentación (2022). Raza equino caballar Caballo de Monte del País Vasco. Retrieved from https://www.mapa.gob.es/es/ganaderia/temas/zootecnia/razas-ganaderas/razas/catalogo-razas/equino-caballar/caballo-monte-pais-vasco/default.aspx.

Marangoni, F., Agostoni, C., Borghi, C., Catapano, A. L., Cena, H., Ghiselli, A., La Vecchia, C., Lercker, G., Manzato, E., Pirillo, A., Riccardi, G., Risé, P., Visioli, F., & Poli, A. (2020). Dietary linoleic acid and human health: Focus on cardiovascular and cardiometabolic effects. *Atherosclerosis, 292,* 90-98.

Markiewicz-Kęszycka, M., Czyżak-Runowska, G., Wójtowski, J., Jóźwik, A., Pankiewicz, R., Łęska, B., Krzyżewski, J., Strzałkowska, N., Marchewka, J., & Bagnicka, E. (2015). Influence of stage of lactation and year season on composition of mares' colostrum and milk and method and time of storage on vitamin C content in mares' milk. *Journal of the Science of Food and Agriculture, 95*, 2279-2286.

Markiewicz-Kęszycka, M., Wójtowski, J., Czyżak-Runowska, G., Kuczyńska, B., Puppel, K., Krzyżewski, J., Strzałkowska, N., Jóźwik, A., & Bagnicka, E. (2014). Concentration of selected fatty acids, fat-soluble vitamins and β-carotene in late lactation mares' milk. *International Dairy Journal, 38*, 31-36.

Markiewicz-Kęszycka, M., Wójtowski, J., Kuczyńska, B., Puppel, K., Czyżak-Runowska, G., Bagnicka, E., Strzałkowska, N., Jóźwik, A., & Krzyżewski, J. (2013). Chemical composition and whey protein fraction of late lactation mares' milk. *International Dairy Journal, 31*, 62-64.

Marsoner, T., Vigl, L. E., Manck, F., Jaritz, G., Tappeiner, U., & Tasser, E. (2018). Indigenous livestock breeds as indicators for cultural ecosystem services: A spatial analysis within the alpine space. *Ecological Indicators, 94*, 55-63.

Medhammar, E., Wijesinha-Bettoni, R., Stadlmayr, B., Nilsson, E., Charrondiere, U. R., & Burlingame, B. (2012). Composition of milk from minor dairy animals and buffalo breeds: A biodiversity perspective. *Journal of the Science of Food and Agriculture, 92*, 445-474.

Mendelson, C. R., Zinder, O., Blanchette-Mackie, J. E., Chernick, S. S., & Scow, R. O. (1977). Lipoprotein lipase and lipid metabolism in mammary gland. *Journal of Dairy Science, 60*, 666-676.

Miclo, L., Girardet, J., Egito, A. S., Mollé, D., Martin, P., & Gaillard, J. (2007). The primary structure of a low-Mr multiphosphorylated variant of β-casein in equine milk. *Proteomics, 7*, 1327-1335.

Minekus, M., Alminger, M., Alvito, P., Ballance, S., Bohn, T., Bourlieu, C., Carrière, F., Boutrou, R., Corredig, M., Dupont, D., Dufour, C., Egger, L., Golding, M., Karakaya, S., Kirkhus, B., Le Feunteun, S., Lesmes, U., Macierzanka, A., Mackie, A., … & Brodkorb, A. (2014). A

standardised static in vitro digestion method suitable for food – An international consensus. *Food & Function, 5*, 1113-1124.

Minjigdorj, N., Baldorj, O., & Austbø, D. (2012a). Chemical composition of Mongolian mare milk. *Acta Agriculturae Scandinavica, Section A – Animal Science, 62*, 66-72.

Minjigdorj, N., Haug, A., & Austbø, D. (2012b). Fatty acid composition of Mongolian mare milk. *Acta Agriculturae Scandinavica, Section A – Animal Science, 62*, 73-80.

Miranda, G., Mahé, M., Leroux, C., & Martin, P. (2004). Proteomic tools to characterize the protein fraction of equidae milk. *Proteomics, 4*, 2496-2509.

Montalbetti, N., Dalghi, M. G., Albrecht, C., & Hediger, M. A. (2014). Nutrient transport in the mammary gland: Calcium, trace minerals and water soluble vitamins. *Journal of Mammary Gland Biology and Neoplasia, 19*, 73-90.

Mora, O., Romano, J. L., Gonzalez, E., Ruiz, F., & Shimada, A. (2000). Low cleavage activity of 15,15'dioxygenase to convert beta-carotene to retinal in cattle compared with goats, is associated with the yellow pigmentation of adipose tissue. *International Journal for Vitamin and Nutrition Research, 70*, 199-205.

Moughan, P. J., Butts, C. A., van Wijk, H., Rowan, A. M., & Reynolds, G. W. (2005). An acute ileal amino acid digestibility assay is a valid procedure for use in human ileostomates. *The Journal of Nutrition, 135*, 404-409.

Naert, L., Verhoeven, G., Duchateau, L., De Smet, S., & Coopman, F. (2013). Assessing heterogeneity of the composition of mare's milk in Flanders. *Vlaams Diergeneeskundig Tijdschrift, 82,* 23-30.

Nagy, P., Guillaume, D., & Daels, P. (2000). Seasonality in mares. *Animal Reproduction Science, 60*, 245-262.

Napier, J. A., Usher, S., Haslam, R. P., Ruiz-Lopez, N., & Sayanova, O. (2015). Transgenic plants as a sustainable, terrestrial source of fish oils. *European Journal of Lipid Science and Technology, 117,* 1317-1324.

Narmuratova, Z., Hentati, F., Girardet, J., Narmuratova, M., & Cakir-Kiefer, C. (2022). Equine lactoferrin: Antioxidant properties related to divalent metal chelation. *LWT – Food Science and Technology, 161*, Article 113426.

Navrátilová, P., Borkovcová, I., Kaniová, L., Dluhošová, S., & Zachovalová, H. (2019). The content of selected vitamins and iodine in mare's milk. *Acta Veterinaria Brno, 88*, 473-480.

Navrátilová, P., Pospíšil, J., Borkovcová, I., Kaniová, L., Dluhošová, S., & Horáková, S. (2018). Content of nutritionally important components in mare milk fat. *Mljekarstvo: Časopis Za Unaprjeđenje Proizvodnje i Prerade Mlijeka, 68*, 282-294.

Nielsen, S. D., Beverly, R. L., Qu, Y., & Dallas, D. C. (2017). Milk bioactive peptide database: A comprehensive database of milk protein-derived bioactive peptides and novel visualization. *Food Chemistry, 232*, 673-682.

Nimni, M. E., Han, B., & Cordoba, F. (2007). Are we getting enough sulfur in our diet? *Nutrition & Metabolism, 4*, Article 24.

Nitta, K., Tsuge, H., Sugai, S., & Shimazaki, K. (1987). The calcium-binding property of equine lysozyme. *FEBS Letters, 223*, 405-408.

Ochirkhuyag, B., Chobert, J., Dalgalarrondo, M., & Haertlé, T. (2000). Characterization of mare caseins. Identification of αS1- and αS2-caseins. *Le Lait, 80*, 223-235.

Osoro, K., Ferreira, L., García, U., Martínez, A., & Celaya, R. (2017). Forage intake, digestibility and performance of cattle, horses, sheep and goats grazing together on an improved heathland. *Animal Production Science, 57*, 102-109.

Outram, A. K., Stear, N. A., Bendrey, R., Olsen, S., Kasparov, A., Zaibert, V., Thorpe, N., & Evershed, R. P. (2009). The earliest horse harnessing and milking. *Science, 323*, 1332-1335.

Pagan, J. D., Kane, E., & Nash, D. (2005). Form and source of tocopherol affects vitamin E status in Thoroughbred horses. *Pferdeheilkunde, 21*, 101-102.

Palmquist, D. L., & Harvatine, K. J. (2020). Milk fat: Origin of fatty acids and influence of nutritional factors on milk fat. In P. F. Fox & P. L. H. McSweeney (Eds.), *Advanced Dairy Chemistry* (pp. 43-92). Springer.

Pappalardo, G., & Lusk, J. L. (2016). The role of beliefs in purchasing process of functional foods. *Food Quality and Preference, 53*, 151-158.

Park, Y. W., Zhang, H., Zhang, B., & Zhang, L. (2006). Mare milk. In Y. W. Park & G. F. W. Haenlein (Eds.), *Handbook of Milk of Non-bovine Mammals* (pp. 275-296). Blackwell Publishing.

Pearson, P. B. (1947). The thiamine, riboflavin, nicotinic acid and pantothenic acid contents of mare's colostrum and milk and ascorbic acid content of the milk. *Journal of Dairy Science, 30*, 73-77.

Pedrouso, M. L., Ruiz, D. F., & Lorenzo, J. M. (2022). *Food proteomics: Technological advances, current applications and future perspectives*. Elsevier.

Pelizzola, V., Contarini, G., Povolo, M., & Giangiacomo, R. (2006). Chemical-physical characteristics and fatty acid composition of mare's milk. *Milchwissenschaft, 61*, 33-36.

Perkins, G. A., & Wagner, B. (2015). The development of equine immunity: Current knowledge on immunology in the young horse. *Equine Veterinary Journal, 47*, 267-274.

Pieszka, M., Łuszczyński, J., Zamachowska, M., Augustyn, R., Długosz, B., & Hędrzak, M. (2016). Is mare milk an appropriate food for people? – A review. *Annals of Animal Science, 16*, 33-51.

Pietrzak-Fiećko, R., Tomczyński, R., & Smoczyński, S. S. (2013). Effect of lactation period on the fatty acid composition in mares' milk from different breeds. *Archives Animal Breeding, 56*, 335-343.

Pikul, J., Wójtowski, J., Danków, R., Kuczyńska, B., & Łojek, J. (2008). Fat content and fatty acids profile of colostrum and milk of primitive Konik horses (Equus caballus gmelini ant.) during six months of lactation. *Journal of Dairy Research, 75*, 302-309.

Rahmat, A., Rosli, R., Tan, M. H., Umar-Tsafe, N., Ali, A. M., & Bakar, M. F. A. (2006). Comparative evaluation of cytotoxic effects of milk from various species on leukemia cell lines. *Malaysian Journal of Medicine and Health Sciences, 2,* 1-10.

Rassin, D. K., Sturman, J. A., & Gaull, G. E. (1978). Taurine and other free amino acids in milk of man and other mammals. *Early Human Development, 2*, 1-13.

Reist, S., Hintermann, F., & Sommer, R. (2007). The livestock revolution: An opportunity for poor farmers? *InfoResources Focus, 1/07*, 1-16.

Richard, D., Kefi, K., Barbe, U., Bausero, P., & Visioli, F. (2008). Polyunsaturated fatty acids as antioxidants. *Pharmacological Research, 57*, 451-455.

Rigueiro-Rodríguez, A., Mouhbi, R., Santiago-Freijanes, J. J., Gonzalez-Hernandez, M. D. P., & Mosquera-Losada, M. R. (2012). Horse grazing systems: Understory biomass and plant biodiversity of a Pinus radiata stand. *Scientia Agricola, 69*, 38-46.

Robles, S., Torres, M. J., Mayorga, C., Rodríguez-Bada, J. L., Fernández, T. D., Blanca, M., & Bartolomé, B. (2007). Anaphylaxis to mare's milk. *Annals of Allergy, Asthma & Immunology, 98*, 600-602.

Sahaka, M., Amara, S., Wattanakul, J., Gedi, M. A., Aldai, N., Parsiegla, G., Lecomte, J., Christeller, J. T., Gray, D., Gontero, B., Villeneuve, P., & Carrière, F. (2020). The digestion of galactolipids and its ubiquitous function in nature for the uptake of the essential α-linolenic acid. *Food & Function, 11*, 6710-6744.

Saini, R. K., Prasad, P., Sreedhar, R. V., Naidu, K. A., Shang, X., & Keum, Y. (2021). Omega-3 polyunsaturated fatty acids (PUFAs): Emerging plant and microbial sources, oxidative stability, bioavailability, and health benefits – A review. *Antioxidants, 10*, Article 1627.

Schryver, H. F., Oftedal, O. T., Williams, J., Cymbaluk, N. F., Antczak, D., & Hintz, H. F. (1986a). A comparison of the mineral composition of milk of domestic and captive wild equids (Equus przewalski, E. zebra, E. burchelli, E. caballus, E. asinus). *Comparative Biochemistry and Physiology Part A: Comparative Physiology, 85,* 233-235.

Schryver, H. F., Oftedal, O. T., Williams, J., Soderholm, L. V., & Hintz, H. F. (1986b). Lactation in the horse: The mineral composition of mare milk. *Journal of Nutrition, 116*, 2142-2147.

Schweigert, F. J., & Gottwald, C. (1999). Effect of parturition on levels of vitamins A and E and of β-carotene in plasma and milk of mares. *Equine Veterinary Journal, 31*, 319-323.

Shariatikia, M., Behbahani, M., & Mohabatkar, H. (2017). Anticancer activity of cow, sheep, goat, mare, donkey and camel milks and their caseins and whey proteins and in silico comparison of the caseins. *Molecular Biology Research Communications, 6*, 57-64.

Sheng, Q., & Fang, X. (2009). Bioactive components in mare milk. In Y. W. Park (Ed.), *Bioactive Components in Milk and Dairy Products* (pp. 195-213). Wiley-Blackwell.

Sik, B., Buzás, H., Kapcsándi, V., Lakatos, E., Daróczi, F., & Székelyhidi, R. (2023). Antioxidant and polyphenol content of different milk and dairy products. *Journal of King Saud University-Science, 35*, Article 102839.

Simopoulos, A. P. (2001). n-3 Fatty acids and human health: Defining strategies for public policy. *Lipids, 36,* 83-89.

SITRAN (2024). Informe del Sistema Integral de Trazabilidad Animal. Retrieved from https://www.mapa.gob.es/eu/ganaderia/temas/trazabilidad-animal/sitran/default.aspx.

Smith, S. B., Gill, C. A., Lunt, D. K., & Brooks, M. A. (2009). Regulation of fat and fatty acid composition in beef cattle. *Asian-Australasian Journal of Animal Sciences, 22*, 1225-1233.

Song, J. J., Wang, Q., Du, M., Ji, X. M., & Mao, X. Y. (2017). Identification of dipeptidyl peptidase-IV inhibitory peptides from mare whey protein hydrolysates. *Journal of Dairy Science, 100*, 6885-6894.

Sousa, R., Recio, I., Heimo, D., Dubois, S., Moughan, P. J., Hodgkinson, S. M., Portmann, R., & Egger, L. (2023). In vitro digestibility of dietary proteins and in vitro DIAAS analytical workflow based on the INFOGEST static protocol and its validation with in vivo data. *Food Chemistry, 404*, Article 134720.

Soyeurt, H., Gerards, C., Nickmilder, C., Bindelle, J., Franceschini, S., Dehareng, F., Veselko, D., Bertozzi, C., Gengler, N., Marvuglia, A., Bayram, A., & Tedde, A. (2022). Prediction of indirect indicators of a grass-based diet by milk Fourier transform mid-infrared spectroscopy to assess the feeding typologies of dairy farms. *Animals, 12*, Article 2663.

Srivastava, A., Tripathi, R., Soni, V. K., Misra-Bhattacharya, S., & Deepak, D. (2014). Isolation of mare's milk oligosaccharide fraction of colostrum, transitional, and mature phases promotes in vitro oxidative burst in murine macrophages. *Journal of Equine Veterinary Science, 34*, 1009-1015.

Stein, T., Morris, J. S., Davies, C. R., Weber-Hall, S. J., Duffy, M., Heath, V. J., Bell, A. K., Ferrier, R. K., Sandilands, G. P., & Gusterson, B. A. (2004). Involution of the mouse

mammary gland is associated with an immune cascade and an acute-phase response, involving LBP, CD14 and STAT3. *Breast Cancer Research, 6*, Article R75.

Stergiadis, S., Qin, N., Faludi, G., Beauclercq, S., Pitt, J., Desnica, N., Pétursdóttir, A. H., Newton, E. E., Angelidis, A. E., Givens, I., Humphries, D. J., Gunnlaugsdóttir, H., & Juniper, D. T. (2021). Mineral concentrations in bovine milk from farms with contrasting grazing management. *Foods, 10*, Article 2733.

Stowe, H. D. (1982). Vitamin A profiles of equine serum and milk. *Journal of Animal Science, 54*, 76-81.

Suburu, J., Shi, L., Wu, J., Wang, S., Samuel, M., Thomas, M. J., Kock, N. D., Yang, G., Kridel, S., & Chen, Y. Q. (2014). Fatty acid synthase is required for mammary gland development and milk production during lactation. *American Journal of Physiology − Endocrinology and Metabolism, 306*, E1132-E1143.

Summer, A., Sabbioni, A., Formaggioni, P., & Mariani, P. (2004). Trend in ash and mineral element content of milk from Haflinger nursing mares throughout six lactation months. *Livestock Production Science, 88*, 55-62.

Sun, Y., Wang, C., Sun, X., & Guo, M. (2020). Proteomic analysis of whey proteins in the colostrum and mature milk of Xinong Saanen goats. *Journal of Dairy Science, 103*, 1164-1174.

Tada, M., Kobashigawa, Y., Mizuguchi, M., Miura, K., Kouno, T., Kumaki, Y., Demura, M., Nitta, K., & Kawano, K. (2002). Stabilization of protein by replacement of a fluctuating loop: Structural analysis of a chimera of bovine α-lactalbumin and equine lysozyme. *Biochemistry, 41*, 13807-13813.

Taormina, V. M., Unger, A. L., Schiksnis, M. R., Torres-Gonzalez, M., & Kraft, J. (2020). Branched-chain fatty acids – An underexplored class of dairy-derived fatty acids. *Nutrients, 12*, Article 2875.

Teichert, J., Cais-Sokolińska, D., Bielska, P., Danków, R., Chudy, S., Kaczyński, Ł. K., & Biegalski, J. (2021). Milk fermentation affects amino acid and fatty acid profile of mare milk from Polish Coldblood mares. *International Dairy Journal, 121*, Article 105137.

Tenno, M., Toba, S., Kézdy, F. J., Elhammer, Å. P., & Kurosaka, A. (2002). Identification of two cysteine residues involved in the binding of UDP-GalNAc to UDP-GalNAc: Polypeptide n-acetylgalactosaminyltransferase 1 (GalNAc-T1). *European Journal of Biochemistry, 269*, 4308-4316.

Ugwu, C. P., Abarshi, M. M., Mada, S. B., Sanusi, B., & Nzelibe, H. C. (2019). Camel and horse milk casein hydrolysates exhibit angiotensin converting enzyme inhibitory and antioxidative effects in vitro and in silico. *International Journal of Peptide Research and Therapeutics, 25*, 1595-1604.

Ullrey, D. E., Ely, W. T., & Covert, R. L. (1974). Iron, zinc and copper in mare's milk. *Journal of Animal Science, 38*, 1276-1277.

Ullrey, D. E., Struthers, R. D., Hendricks, D. G., & Brent, B. E. (1966). Composition of mare's milk. *Journal of Animal Science, 25*, 217-222.

Uniacke-Lowe, T., Huppertz, T., & Fox, P. F. (2010). Equine milk proteins: Chemistry, structure and nutritional significance. *International Dairy Journal, 20*, 609-629.

Valdivielso, I., Bustamante, M. A., Buccioni, A., Franci, O., Ruiz de Gordoa, J. C., de Renobales, M., & Barron, L. J. R. (2015). Commercial sheep flocks – Fatty acid and fat-soluble antioxidant composition of milk and cheese related to changes in feeding management throughout lactation. *Journal of Dairy Research, 82*, 334-343.

Valdivielso, I., Bustamante, M. A., Aldezabal, A., Amores, G., Virto, M., Ruiz de Gordoa, J., de Renobales, M., & Barron, L. J. R. (2016). Case study of a commercial sheep flock under extensive mountain grazing: Pasture derived lipid compounds in milk and cheese. *Food Chemistry, 197*, 622-633.

Vázquez, C. V., Rojas, M. G. V., Ramírez, C. A., Chávez-Servín, J. L., García-Gasca, T., Martínez, R. A. F., García, O. P., Rosado, J. L., López-Sabater, C. M., Castellote, A. I., Montemayor, H. M. A., Carbot, K. T. (2015). Total phenolic compounds in milk from different species. Design of an extraction technique for quantification using the Folin-Ciocalteu method. *Food Chemistry, 176*, 480-486.

Verhulst, L., Kerre, S., & Goossens, A. (2016). The unsuspected power of mare's milk. *Contact Dermatitis, 74*, 376-377.

Waili, Y., Gahafu, Y., Aobulitalifu, A., Chang, Z., Xie, X., & Kawuli, G. (2021). Isolation, purification, and characterization of antioxidant peptides from fresh mare's milk. *Food Science & Nutrition, 9*, 4018-4027.

Warren, L. K., & Vineyard, K. R. (2013). Fat and fatty acids. In R. Geor, M. Coenen & P. Harris (Eds.), *Equine Applied and Clinical Nutrition* (pp. 136-155). Saunders.

Waters. (2007). Waters UPLC Amino Acid Analysis Solution System Guide: 71500129702/Revision B (Vol. 71500129702/Revision B): Waters Corporation.

Wiśniewski, J. R., Zougman, A., Nagaraj, N., & Mann, M. (2009). Universal sample preparation method for proteome analysis. *Nature Methods, 6*, 359-362.

Wodas, L., Mackowski, M., Borowska, A., Puppel, K., Kuczynska, B., & Cieslak, J. (2020). Genes encoding equine β-lactoglobulin (LGB1 and LGB2): Polymorphism, expression, and impact on milk composition. *PLoS One, 15*, Article e0232066.

Wolff, R. L., Bayard, C. C., & Fabien, R. J. (1995). Evaluation of sequential methods for the determination of butterfat fatty acid composition with emphasis on trans-18:1 acids. Application to the study of seasonal variations in French butters. *Journal of the American Oil Chemists' Society, 72*, 1471-1483.

Wu, Y., Sun, Y., Chen, R., Qiao, Y., Zhang, Q., Li, Q., Wang, X., Pan, Y., Li, S., & Wang, Z. (2023). Analysis for lipid nutrient differences in the milk of 13 species from a quantitative non-targeted lipidomics perspective. *Food Chemistry: X, 20*, Article 101024.

Xiao, T., Zeng, J., Zhao, C., Hou, Y., Wu, T., Deng, Z., & Zheng, L. (2023). Comparative analysis of protein digestion characteristics in human, cow, goat, sheep, mare, and camel milk under simulated infant condition. *Journal of Agricultural and Food Chemistry, 71*, 15035-15047.

Ye, A., Cui, J., Dalgleish, D., & Singh, H. (2016). Formation of a structured clot during the gastric digestion of milk: Impact on the rate of protein hydrolysis. *Food Hydrocolloids, 52*, 478-486.

Yuhas, R., Pramuk, K., & Lien, E. L. (2006). Human milk fatty acid composition from nine countries varies most in DHA. *Lipids, 41*, 851-858.

Zafra-Gómez, A., Garballo, A., Morales, J. C., & García-Ayuso, L. E. (2006). Simultaneous determination of eight water-soluble vitamins in supplemented foods by liquid chromatography. *Journal of Agricultural and Food Chemistry, 54*, 4531-4536.

Zava, S., Barello, C., Pessione, A., Garoffo, L. P., Fattori, P., Montorfano, G., Conti, A., Giunta, C., Pessione, E., Berra, B., & Giuffrida, M. G. (2009). Mare's colostrum globules stimulate fibroblast growth in vitro: A biochemical study. *Journal of Medicinal Food, 12*, 836-845.

Zhang, L., Boeren, S., Hageman, J. A., van Hooijdonk, T., Vervoort, J., & Hettinga, K. (2015). Perspective on calf and mammary gland development through changes in the bovine milk proteome over a complete lactation. *Journal of Dairy Science, 98*, 5362-5373.

Zhao, S., Pan, F., Cai, S., Yi, J., Zhou, L., & Liu, Z. (2023a). Secrets behind protein sequences: Unveiling the potential reasons for varying allergenicity caused by caseins from cows, goats, camels, and mares based on bioinformatics analyses. *International Journal of Molecular Sciences, 24*, Article 2481.

Zhao, X., Zuo, S., Guo, Y., Zhang, C., Wang, Y., Peng, S., Liu, M., Wang, B., Zhang, H., & Luo, H. (2023b). Carcass meat quality, volatile compound profile, and gene expression in tan sheep under different feeding regimes. *Food Bioscience, 56*, Article 103213.

Zinger-Yosovich, K. D., Iluz, D., Sudakevitz, D., & Gilboa-Garber, N. (2010). Blocking of Pseudomonas aeruginosa and Chromobacterium violaceum lectins by diverse mammalian milks. *Journal of Dairy Science, 93*, 473-482.

Zulueta, A., Maurizi, A., Frígola, A., Esteve, M. J., Coli, R., & Burini, G. (2009). Antioxidant capacity of cow milk, whey and deproteinized milk. *International Dairy Journal, 19*, 380-385.

Web References

UniProtKB (2023). Retrieved from www.uniprot.org/. Accessed October 2023.

PANTHER (2023). Retrieved from www.pantherdb.org. Accessed October 2023.

APPENDIX I.I – Manuscript 1

Blanco-Doval, A., Barron, L. J. R., Kramer, J. K. G., Aldai, N. (2024). **Complete characterization of the n-3 rich fatty acid profile of mare milk from commercial semi-extensive farms during lactation**. *Journal of Food Composition and Analysis,* under review.

Sent to: ***Journal of Food Composition and Analysis****,* 2024

Status: Under review

Manuscript ID: JFCA-D-24-01014

Complete characterization of the n-3 rich fatty acid profile of mare milk from commercial semi-extensive farms during lactation

Ana Blanco-Doval[a], Luis Javier R. Barron[a], John K. G. Kramer[b], and Noelia Aldai[a*]

[a]Lactiker Research Group, Department of Pharmacy and Food Sciences, University of the Basque Country (UPV/EHU), Paseo de la Universidad 7, 01006 Vitoria-Gasteiz, Spain.

[b]Research and Development Centre, Agriculture and Agri-Food Canada, Guelph, ON, Canada.

*Corresponding author: Noelia Aldai (noelia.aldai@ehu.eus)

Abstract

The present study aimed to thoroughly describe the profile of fatty acids in mare milk from farms with different management systems and changes during lactation. Eighteen mares belonging to three commercial farms (six mares *per* farm) were milked during the complete lactation period (six months). Fat content was determined by infrared spectroscopy, while FA methyl esters were analysed using GC-FID. Mare milk contained 47.5% saturated, 25.3% monounsaturated, and 26.5% polyunsaturated fatty acids. A high proportion of 18:2n-6 and 18:3n-3 but a low concentration of their long-chain metabolites was observed, and seven branched-chain FAs were identified. Stage of lactation significantly influenced the content of most individual FAs, and in general, milk from mares managed under pasture-based feeding systems showed a higher content of n-3 PUFAs. This study provides new insights into mare milk FA composition and into dynamics of mare milk nutritional quality as affected by lactation stage and grazing intensity.

Keywords: Grazing, lipid, equid, branched-chain fatty acid, CLA, *trans* fatty acid.

Abbreviations: BCFA: branched-chain fatty acid; DHA: docosahexaenoic acid; DPA: docosapentaenoic acid; EPA: eicosapentaenoic acid; FA: fatty acid; GC: gas chromatography; LA: linoleic acid; LC-PUFA: long-chain polyunsaturated fatty acid; LNA: linolenic acid; MUFA: monounsaturated fatty acid; PUFA: polyunsaturated fatty acid; SFA: saturated fatty acid.

1. Introduction

Horses are hindgut (post-gastric) fermenters, whereas in ruminants (foregut fermenters) dietary fatty acids (FA) are modified by rumen microbiota (biohydrogenation) before absorption in the small intestine. In horses, such modifications occur after absorption, in the caecum-colon region. Therefore, the equine digestion system can absorb higher amounts of non-modified FAs, such as grass-derived polyunsaturated FAs (PUFA), and deposit them into tissues including muscle (meat) and milk (Belaunzaran et al., 2015, 2018; Clauss et al., 2009). As a result, ruminant tissues (*i.e.*, beef) and fluids (*i.e.*, cow milk) contain higher amounts of monounsaturated FAs (MUFA) and saturated FAs (SFA), whereas tissues and fluids from hindgut fermenter monogastrics, such as horse meat and mare milk, are richer in PUFAs (Belaunzaran et al., 2015, 2018; Clauss et al., 2009; Devle et al., 2012).

Contribution of PUFAs to human health is well known. Linoleic acid (LA; 18:2 n-6) and linolenic acid (LNA; 18:3 n-3) are essential FAs, first, because human metabolism (and overall, animal metabolism) is unable to synthetize them; and second, because they are precursors of long-chain PUFAs (LC-PUFA; ≥ C20) that have well-described positive effects on human health. However, some of the LNA-derived LC-PUFAs such as eicosapentaenoic acid (EPA; 20:5 n-3) and docosahexaenoic acid (DHA; 22:6 n-3) are not biosynthesized in sufficient amount in humans (Burdge & Calder, 2005). Essential FAs are incorporated into cell membrane structures where they play orientation-, location- and inflammation-related roles, and are involved in the synthesis of intermediates that take part in vascular physiology, cell growth and proliferation, and immune response. Moreover, they have a protective effect in several chronic and metabolic disorders and contribute to adequate infant development (Capra et al., 2023; Saini et al., 2021). Horse meat contains a considerable amount of EPA, DHA and docosapentaenoic acid (DPA; 22:5 n-3; precursor of DHA) (Belaunzaran et al., 2017), but lower amounts have been found in mare milk (Devle et al., 2012; Haddad et al., 2011).

To date, some studies have described the content of major FAs in mare milk, but excluded minor FAs such as branched-chain FAs (BCFA) and some LC-PUFAs, which leads to an incomplete description of the lipid profile of the product. In addition, changes in the mare milk FA profile during lactation have been poorly described. Only a few FAs and few periods throughout lactation have been considered (Navrátilová et al., 2018; Pikul et al.,

2008), providing a limited description of changes over time. Data is scarce on the effect of in-farm management (feeding, etc.) on mare milk composition, and only a few studies reported the effect of different pasture-based systems in mare milk lipids (Barłowska et al., 2023; Deng et al., 2022; Minjigdorj et al., 2012).

The aim of this study was to thoroughly describe the profile of the major and minor FAs present in mare milk. The changes occurring during the six months of lactation (considering twelve lactation times) were also investigated, and milk from farms with different management systems and grazing activities were compared. The present study is part of a wider research where a complete characterization of mare milk properties was performed in rural equine farms.

2. Materials and methods

2.1. Experimental design and milk sample collection

Milk was collected from eighteen Basque Mountain Horse breed mares belonging to three commercial farms (six mares *per* farm) located in the region of Araba (northern Spain). Mares were individually milked during the six months of lactation, from May to October 2021. During the first half of the lactation, mares were milked once a week, and during the second half, they were milked every fourteen days. A total of 311 individual milk samples were collected.

Management and milking process of mares have been previously detailed in Blanco-Doval et al. (2023). **Figure 1** describes the proximate feeding ingredients used in each farm during the lactation period. Briefly, one of the farms (farm I) kept all mares on pasture during the first month of lactation, providing a mixture of alfalfa, dry grass, silage, fruits and potatoes along the rest of the lactation period. In the other two farms (farms II and III), mares grazed on pasture during all the lactation period, although after July, when the availability of grass was low, mares were supplemented either with hay (farm II) or silage (farm III). The milking process was performed using a portable milking machine in farm I and manually in farms II and III. After each collection, individual milk samples were transported to the laboratory under cooling conditions. Then, milk was subsampled for different analyses.

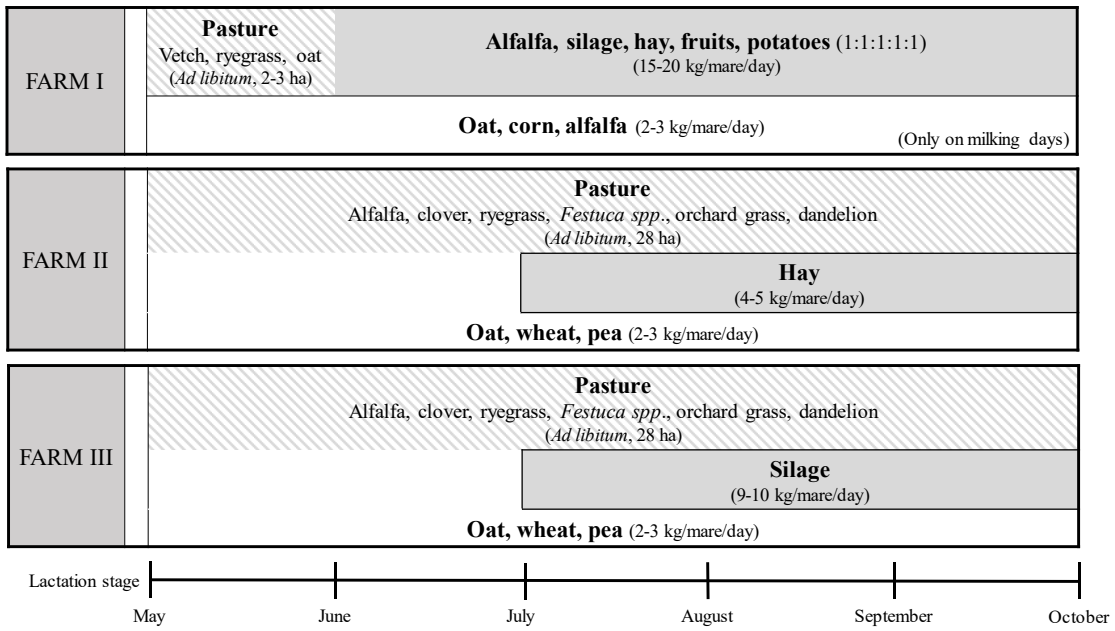


Figure 1. *Proximate feeding ingredients used in each commercial farm during the lactation period.*

2.2. Determination of fat content

Fat content of the milk samples (g/100 g of milk) was determined by infrared spectroscopy in the laboratories of the Lekunberri Dairy Institute (Lekunberri, Spain) following the method PE/ALVO/02 (2005).

2.3. Determination of total fatty acid content

Milk samples were tempered in a water bath at 37 ºC for 20 minutes as suggested by Devle et al. (2012), and total fatty acids were extracted following the double centrifugation method validated by Luna et al. (2005), with minor modifications. The first centrifugation was performed at 1,000 *g* and 4 ºC for 20 minutes (SORVALL® RC 5B Plus, Thermo Scientific^{TM}, MA, USA), and the second centrifugation was performed at 22,000 *g* and 20 ºC for 30 minutes (SORVALL® ST 16R, Thermo Scientific^{TM}, MA, USA). The upper lipid layer was collected for fatty acid methyl ester (FAME) analysis.

For derivatization, as the presence of volatile FAMEs requires the headspace to be kept to a minimum during methylation and subsequent handling, a previously described miniaturized base-catalysed methylation was used (Aldai et al., 2012; Cruz-Hernandez et al., 2004). Briefly, one drop of milk fat (4.5 to 5.0 mg) was added to a 2 mL autosampler vial. For quantitative purposes, 50 µL of an internal standard consisting of a mixture of

methyl esters of 13:0 (4 mg/mL in toluene) and 23:0 (4 mg/mL in toluene) was added to each sample. Then, 1.6 µL of hexane and 40 µL of methyl acetate were added and vials were well mixed. To this, 100 µL of 0.5 M sodium methoxide in methanol (92446 Supelco, Merck) were added, and the mixture was allowed to sit for 15 min at room temperature with occasional shaking. Vials were cooled at -20 ºC for 10 min, and 60 µL of oxalic acid (0.5 g in 15 mL of diethyl ether) were added and mixed thoroughly. After adding 100 µL of distilled water, vials were centrifuged (800 *g* during 5 min) to settle the Na-oxalate precipitate at the bottom of the vial. The upper phase was collected directly in a 2 mL autosampler vial and analysed.

The analysis of FAMEs was performed using a gas chromatograph coupled to a flame ionization detector (GC-FID; Model 7890A, Agilent Technologies, Wilmington, DE, USA), and equipped with an automatic injector (Model 7693, Agilent Technologies, Wilmington, DE, USA). FAMEs were separated in a SP2560 capillary column (100 m x 0.25 mm internal diameter, 0.2 µm film thickness; Supelco, Bellefonte, PA, USA) operated at a temperature program plateauing at 175 ºC described by Kramer et al. (2008). Samples were injected with a 50:1 split ratio, hydrogen set at a 1 mL/min constant flow rate was used as carrier gas, the injection volume was 1 µL, and both injector and detector ports were set at a 250 ºC temperature.

For peak identification purposes, #463 and #603 FAME mixtures, and individual FAME reference standards (13:0, 21:0, 23:0) were purchased from Nu-Check Prep (Elysian, MN, USA). LA (CRM47791) and LNA (CRM47792) isomers, and Supelco 37 Component FAME Mix (CRM47885) were purchased from Sigma-Aldrich (San Luis, MO, USA), and a bacterial FAME mixture (#1114) containing BCFAs was purchased from Matreya (Pleasant Gap, PA, USA). Some of the FAs not included in the reference standards were identified using the retention times and elution orders previously reported in the literature (Belaunzaran et al., 2017).

For quantification, chromatographic peak areas were considered and these peaks were then corrected according to theoretical response factors (Wolff et al., 1995). Additionally, an in-house validation of the chromatographic method included linearity, repeatability and detection and quantification limits of FAMEs using the #463 mixture. Six different concentrations of the mixture ranging from 0.005 to 0.500 mg/mL (in isooctane, Merck, Sigma-Aldrich, Madrid, Spain) were analysed. Determination coefficient values of the linear calibrations were higher than 0.998. Relative standard deviations for five replicates

of the standard mixtures at these concentrations were lower than 8.0 %. The detection and quantification limits were determined using the mean area of the noise by analysing 10 blanks (isooctane) plus 2 or 4 times the standard deviation of the noise, respectively. The detection and quantification limits established for all the FAMEs contained in the mixture were 0.0125 and 0.0250 mg/100 g of milk, respectively. Concentration of FAs in milk samples was expressed as mg/100 g of milk.

2.4. Statistical analysis

Statistical analysis was conducted using IBM-SPSS Statistics Software version 28 (IBM, Corporation, NY, USA). FA content data was log transformed, and normality and homoscedasticity were checked. In order to increase the robustness of the statistical analysis, data were grouped into two-week intervals starting at weeks 3-4 of lactation and ending at weeks 25-26. This resulted in a total of twelve lactation times. Three significant figures were used to express the FA content of the milk samples. Data were subjected to mixed linear model of analysis of variance, setting the individual animal as subject, farm (management system) as fixed factor and lactation time as repeated measure factor. The interaction term between lactation time and farm was also included in the model. The Restricted Maximum Likelihood Method was used to set the parameters of the linear model, and the compound symmetry matrix was selected for the repeated measures covariance structure following the Akaike information criteria. Least square means of dependent variables for the three levels of the fixed factor (farm) were compared using the Tukey's test. In addition, a stepwise discriminant analysis (SDA) was applied to the FA composition of the mare milk samples to separately classify milks according to farm or lactation stage, respectively. In case of SDA for lactation stage, milk samples were labelled as early lactation (from week 3 to 10), mid lactation (from week 11 to 18) and late lactation (from week 19 to 26). Significance was declared at $P \leq 0.05$.

3. Results

3.1. Fatty acid profile of mare milk

Mare milk contained, on average, 47.5 % SFAs, 25.3 % MUFAs, and 26.5 % PUFAs. **Tables 1-4** show individual FA contents in mare milk (mg/100 g of milk). The main SFA was 16:0 (palmitic acid), followed by 12:0 (lauric acid), 14:0 (myristic acid) and 10:0

(capric acid). A considerable content of 8:0 (caprylic acid) was also found. The abundance of BCFAs was low and consisted mainly of *iso*-16:0 and *anteiso*-17:0 (**Table 2**). MUFAs where primarily composed of *cis* isomers and among *cis*-MUFAs, 9*c*-18:1 (oleic acid) was the main one, followed by 9*c*-16:1 (palmitoleic acid). On the other hand, *trans* isomers comprised only 0.17 % of the total FAs in milk, with 9*t*-18:1 (elaidic acid) being more abundant than 11*t*-18:1 (vaccenic acid) (**Table 3**). Of the PUFAs, LA and LNA were the most abundant, with an average ratio of 0.64. The rest of the PUFAs were each present at less than 1 mg/100 g of milk. The major n-6 and n-3 LC-PUFAs in mare milk were 20:2n-6 and 20:3n-3, respectively. Among other LC-PUFAs of interest, DPA was relatively abundant, whereas EPA and DHA showed low contents in mare milk, even though they are intermediates in the conversion of LNA to DHA. Other intermediates in the LA and LNA pathways were also present in low abundance such as 18:3n-6 (γ-linolenic acid), 20:3n-6, and 20:4n-3. In this study, 9*c*,11*t*-18:2 (rumenic acid) was the major conjugated linoleic acid (CLA) while 9*t*,12*t*,15*c*-18:3 was also present (**Table 4**).

3.2. Effect of lactation time

The effect of lactation time was significant ($P \leq 0.05$) for all the FAs determined, except for a few MUFAs (**Tables 1-4**). Interestingly, the contents of most FAs in milk peaked at weeks 5-6 of lactation (**Figure 2**); thereafter, the FA changes could be classified into four different patterns.

In general, the contents of total SFAs as well as most individual SFAs continuously decreased to much lower levels, except 20:0 and 22:0. However, the SFAs of C14 and greater showed fluctuations, peaking at 2-3 times their concentration between weeks 11 and 21 (**Figure 2**).

At first, the content of BCFAs decreased to a minimum at week 9, but subsequently increased temporarily to their initial content between weeks 17 to 21, and then, decreased again by the end of lactation to minimum values. The MUFAs followed the same pattern as the BCFAs (**Figure 2**), however, the contents of 11*c*-16:1 and some 20 C and 22 C MUFAs (*i.e.*, 9*c*-20:1, 11*c*-20:1, 13*c*-20:1, and 13*c*-22:1) did not fall to minimum at week 9, but fluctuated at high concentrations throughout the lactation period from week 5 to 21 instead.

Table 1. *Average content of total fat (g/100 g of milk) and saturated fatty acids (mg/100 g of milk) in mare milk samples from the commercial farms over the lactation period (from May to October), and the statistical significance (P-value) of the effect of farm, lactation and interaction of both.*

						Significance (*P*-value)		
Fatty acid	Farm I	Farm II	Farm III	Total	SEM	Farm	Lactation	Farm*Lactation
Total fat	0.306	0.267	0.247	0.270	0.007	0.310	<0.001	0.446
4:0	0.459	0.414	0.463	0.442	0.023	0.885	<0.001	0.088
6:0	0.758	0.625	0.632	0.661	0.028	0.398	<0.001	0.426
7:0	0.0589	0.0408	0.045	0.0468	0.0023	0.143	<0.001	0.709
8:0	10.2[a]	6.57[b]	6.32[b]	7.41	0.33	<0.001	<0.001	0.672
9:0	0.110[a]	0.0557[b]	0.0666[b]	0.0731	0.0062	0.013	<0.001	0.030
10:0	22.1[a]	13.9[b]	13.6[b]	15.9	0.8	<0.001	<0.001	0.496
11:0	4.23	3.23	2.95	3.39	0.13	0.232	<0.001	<0.001
12:0	22.1[a]	15.4[b]	15.0[b]	16.9	0.7	0.007	<0.001	0.283
14:0	20.2[a]	15.6[b]	15.2[b]	16.6	0.6	0.049	<0.001	0.295
15:0	0.751	0.740	0.735	0.741	0.027	0.770	<0.001	0.518
16:0	53.8	42.7	42.8	45.6	1.6	0.122	<0.001	0.502
17:0	0.570	0.534	0.519	0.538	0.018	0.544	<0.001	0.816
18:0	2.69	2.11	2.10	2.25	0.09	0.083	<0.001	0.628
19:0	0.0443	0.0433	0.0405	0.0426	0.0014	0.682	<0.001	0.838
20:0	0.114	0.0982	0.0975	0.102	0.0055	0.196	0.006	0.969
22:0	0.0377	0.0314	0.0308	0.0328	0.0014	0.202	0.008	0.097
24:0	0.0442	0.0416	0.0399	0.0417	0.0019	0.412	<0.001	0.407
SFA	138[a]	102[b]	101[b]	111	4	0.020	<0.001	0.497

[a,b]: differences among farms (P ≤ 0.05). SEM: standard error of the mean; SFA: saturated fatty acids.

Table 2. *Average content of branched chain saturated fatty acids (mg/100 g of milk) in mare milk samples from the commercial farms over the lactation period (from May to October), and statistical significance (P-value) of the effect of farm, lactation and interaction of both.*

						Significance (*P*-value)		
Fatty acid	Farm I	Farm II	Farm III	Total	SEM	Farm	Lactation	Farm*Lactation
iso-14:0	0.128	0.127	0.113	0.123	0.005	0.444	<0.001	0.818
iso-15:0	0.131	0.123	0.114	0.122	0.004	0.391	<0.001	0.676
anteiso-15:0	0.126	0.157	0.150	0.147	0.006	0.632	<0.001	0.550
iso-16:0	0.353	0.438	0.411	0.408	0.014	0.317	<0.001	0.522
iso-17:0	0.033	0.0315	0.0317	0.032	0.0012	0.676	<0.001	0.968
anteiso-17:0	0.303	0.342	0.321	0.325	0.011	0.640	<0.001	0.614
iso-18:0	0.165	0.154	0.150	0.155	0.005	0.763	<0.001	0.614
BCFA	1.24	1.37	1.29	1.31	0.04	0.729	<0.001	0.635

BCFA: branched-chain fatty acids; SEM: standard error of the mean.

Table 3. *Average content of monounsaturated fatty acids (mg/100 g of milk) in mare milk samples from commercial farms over the lactation period (from May to October), and statistical significance (P-value) of the effect of farm, lactation and interaction of both.*

						Significance (*P*-value)		
Fatty acid	Farm I	Farm II	Farm III	Total	SEM	Farm	Lactation	Farm*Lactation
10:1	0.0945	0.0688	0.0277	0.0617	0.0215	0.142	0.098	0.463
9*c*-14:1	1.58	1.47	1.34	1.46	0.04	0.322	<0.001	0.073
9*c*-15:1	0.0348	0.0332	0.0353	0.0343	0.0016	0.956	0.080	0.805
7*c*-16:1	0.887[a]	0.584[b]	0.586[b]	0.662	0.027	0.011	0.003	0.443
9*c*-16:1	15.1	12.4	11.7	12.9	0.4	0.115	<0.001	0.274
11*c*-16:1	0.153	0.136	0.127	0.137	0.004	0.250	<0.001	0.343
7*c*-17:1	0.0547	0.0525	0.0762	0.0609	0.0077	0.912	0.017	0.794
9*c*-17:1	1.21	1.04	0.967	1.06	0.039	0.139	0.072	0.705
6*c*-18:1	0.141	0.178	0.161	0.163	0.011	0.851	<0.001	0.157
9*c*-18:1	55.4[a]	33.0[b]	32.4[b]	38.5	1.6	0.002	0.002	0.502
11*c*-18:1	2.86[a]	1.72[b]	1.62[b]	1.98	0.08	<0.001	<0.001	0.396
13*c*-18:1	0.106	0.0905	0.0869	0.0931	0.0028	0.224	0.002	0.940
11*c*-19:1	0.155[a]	0.0996[b]	0.0973[b]	0.113	0.0049	0.005	<0.001	0.677
7*c*-20:1	0.158	0.149	0.145	0.150	0.005	0.691	0.010	0.919
9*c*-20:1	0.171	0.123	0.112	0.131	0.005	0.067	<0.001	0.851
11*c*-20:1	0.872[a]	0.421[b]	0.390[b]	0.525	0.027	<0.001	<0.001	0.692
13*c*-20:1	0.0958	0.0892	0.0848	0.0894	0.0032	0.537	<0.001	0.965
13*c*-22:1	0.0980[a]	0.0573[b]	0.0524[b]	0.0660	0.0029	0.002	<0.001	0.868
15*c*-24:1	0.0460[a]	0.0301[b]	0.0283[b]	0.0336	0.0015	0.001	<0.001	0.926
cis-MUFA	79.2[a]	51.7[b]	50.1[b]	58.2	2.2	0.006	0.002	0.486
t-15:1	0.0477	0.0566	0.0578	0.0548	0.0023	0.517	0.524	0.489
8t-16:1	0.105	0.0888	0.0848	0.0916	0.0034	0.193	<0.001	0.590
9*t*-16:1	0.0632	0.0553	0.0548	0.0571	0.0021	0.317	0.019	0.482
14*t*-16:1	0.0360	0.0284	0.0351	0.0325	0.0025	0.173	0.017	0.912
9*t*-18:1	0.172[a]	0.0955[b]	0.0881[b]	0.112	0.0047	<0.001	<0.001	0.371
11*t*-18:1	0.0482	0.0378	0.0399	0.0411	0.0020	0.352	0.052	0.264
trans-MUFA	0.472	0.362	0.360	0.390	0.013	0.073	0.001	0.743
MUFA	79.7[a]	52.1[b]	50.5[b]	58.6	2.2	0.006	0.002	0.489

[a,b]: differences among farms (P ≤ 0.05). MUFA: monounsaturated fatty acids; SEM: standard error of the mean.

Table 4. *Content of polyunsaturated fatty acids and total fatty acids (mg/100 g of milk) in mare milk samples from the commercial farms over the lactation period (from May to October), and statistical significance (P-value) of the effect of farm, lactation and interaction of both.*

						Significance (*P*-value)		
Fatty acid	Farm I	Farm II	Farm III	Total	SEM	Farm	Lactation	Farm*Lactation
16:2n-6	0.136[b]	0.229[a]	0.230[a]	0.206	0.008	0.009	<0.001	0.923
18:2n-6	15.6[b]	22.0[a]	23.4[a]	20.9	0.8	0.017	<0.001	0.815
18:3n-6	0.0409[b]	0.0649[a]	0.0659[a]	0.0591	0.0025	0.024	0.003	0.038
20:2n-6	0.429	0.479	0.477	0.466	0.015	0.513	<0.001	0.962
20:3n-6	0.0947	0.0946	0.0898	0.0930	0.0029	0.715	<0.001	0.851
20:4n-6	0.127	0.159	0.155	0.150	0.005	0.177	<0.001	0.787
22:2n-6	0.0348[b]	0.0517[a]	0.0471[a]	0.0459	0.0020	0.044	<0.001	0.757
22:4n-6	0.0365	0.0379	0.0340	0.0362	0.0013	0.315	0.002	0.841
n-6	16.5[b]	23.1[a]	24.5[a]	21.9	0.8	0.019	<0.001	0.815
16:3n-3	0.163[b]	0.500[a]	0.558[a]	0.434	0.021	<0.001	<0.001	0.002
18:3n-3	22.0[b]	39.6[a]	43.9[a]	36.6	1.5	<0.001	<0.001	0.009
20:3n-3	0.811	0.987	0.991	0.943	0.033	0.073	<0.001	0.176
20:4n-3	0.125	0.123	0.116	0.121	0.004	0.633	<0.001	0.194
20:5n-3	0.0727	0.0971	0.0939	0.0899	0.0033	0.117	0.008	0.407
22:3n-3	0.0537	0.0651	0.0621	0.0612	0.0020	0.159	<0.001	0.586
22:5n-3	0.196	0.225	0.218	0.216	0.008	0.687	0.035	0.833
22:6 n-3	0.0520	0.0509	0.0477	0.0501	0.0018	0.728	0.007	0.881
n-3	23.5[b]	41.7[a]	46.0[a]	38.5	1.6	<0.001	<0.001	0.014
20:3n-9	0.111	0.203	0.291	0.209	0.033	0.444	<0.001	0.275
9*t*,12*t*,15*c*-18:3	0.172[b]	0.271[a]	0.287[a]	0.251	0.009	<0.001	<0.001	0.009
9*c*,11*t*-18:2	0.168	0.150	0.162	0.158	0.009	0.294	<0.001	0.324
PUFA	40.1[b]	65.1[a]	70.8[a]	60.7	2.3	<0.001	<0.001	0.302
LA/LNA	0.871[a]	0.558[b]	0.575[b]	0.643	0.022	<0.001	<0.001	0.008
n-6/n-3	0.848[a]	0.557[b]	0.572[b]	0.636	0.021	<0.001	<0.001	0.010
P/S	0.305[b]	0.686[a]	0.739[a]	0.607	0.019	<0.001	<0.001	0.003

[a,b]: differences among farms (P ≤ 0.05). LA: linoleic acid; LNA: linolenic acid; PUFA: polyunsaturated fatty acids; P/S: polyunsaturated fatty acids/saturated fatty acids; SEM: standard error of the mean; SFA: saturated fatty acids.

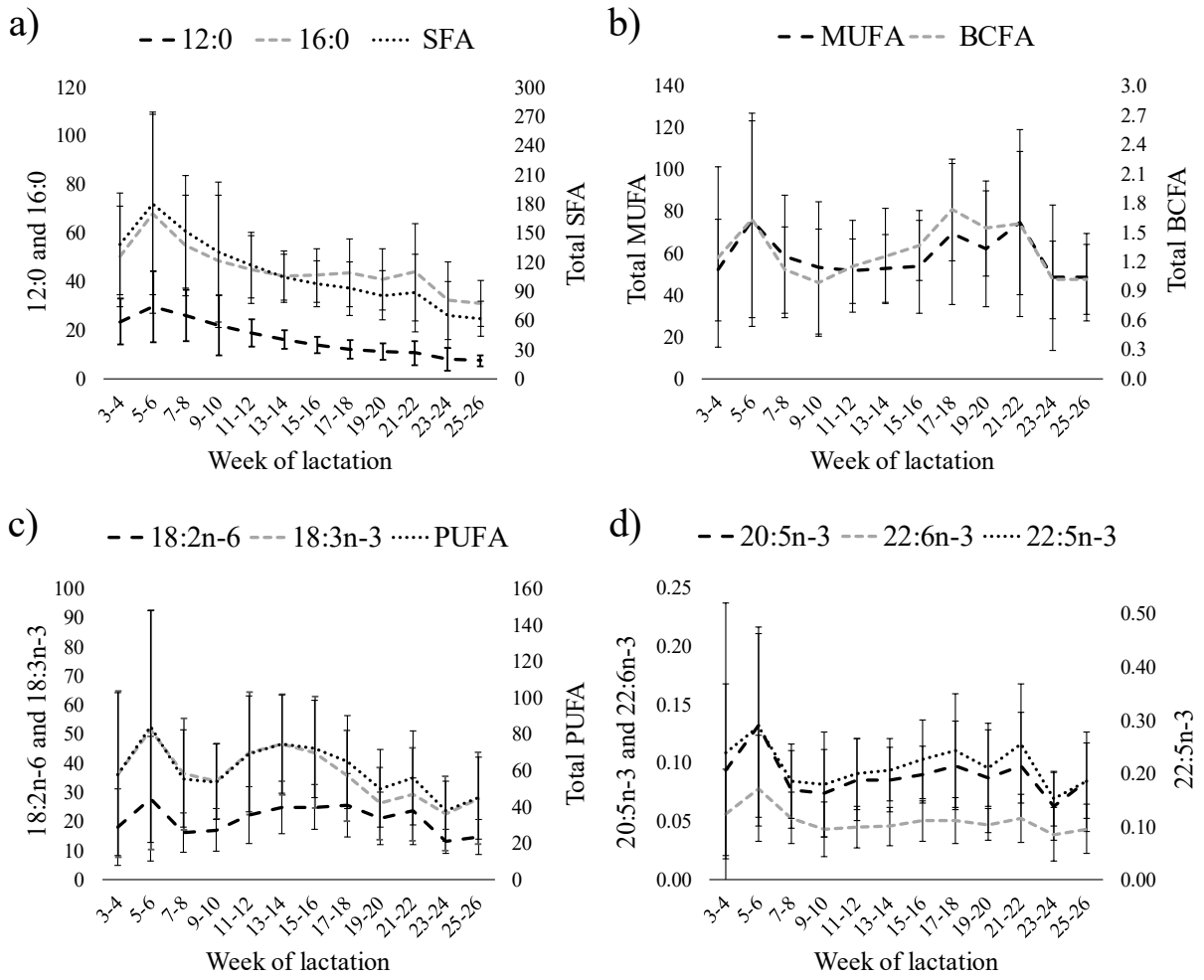


Figure 2. *Changes in the average content (mg/100 g of milk) of individual fatty acids and groups in mare milk samples from the three commercial farms along six months of lactation. BCFA: branched-chain fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; SFA: saturated fatty acids.*

The content of PUFAs showed a unique pattern. After an initial increase at week 6, they decreased at week 10 and, then, increased again to peak between weeks 13 and 17, and generally decreased towards the end of lactation (**Figure 2**). Interestingly, the longer and more unsaturated n-3 PUFAs (20:4, 22:3 and 22:4) showed the same pattern but the peak at mid-lactation was more moderate. Lastly, the EPA, DPA and DHA contents followed a pattern more similar to that of BCFAs and MUFAs, peaking twice, first at week 17 and later at week 21 (**Figure 2**).

Interaction between farm and lactation was also investigated for all the FAs identified. Very few significant ($P \leq 0.05$) interactions were observed for individual FAs (9:0, 11:0,

18:3n-6, 16:3n-3, 18:3n-3 and 9*t*,12*t*,15*c*-18:3) and groups/ratios (n-3, n-6/n-3, P/S (PUFA/SFA ratio)) of FAs (**Tables 1-4**; **Figure 3**). In the case of 9:0 and 11:0, milks from the three farms had similar contents at the beginning and the end of lactation, but those from farms II and III dropped faster and had overall lower values throughout lactation compared with farm I. The n-3 PUFA content remained higher in milks from mares with high grazing activity (farms II and III) and decreased during lactation in milks from low grazing mares (farm I). The 18:3n-6 and P/S ratios increased during lactation in milks from high grazing mares (farms II and III) while remaining lower in milks from mares with low grazing activity (farm I). The n-6/n-3 ratio in milk from farm I drastically increased after the week 16 and peaked at week 21 of lactation, whereas contents in milk from farms II and III remained lower throughout lactation (**Figure 3**).

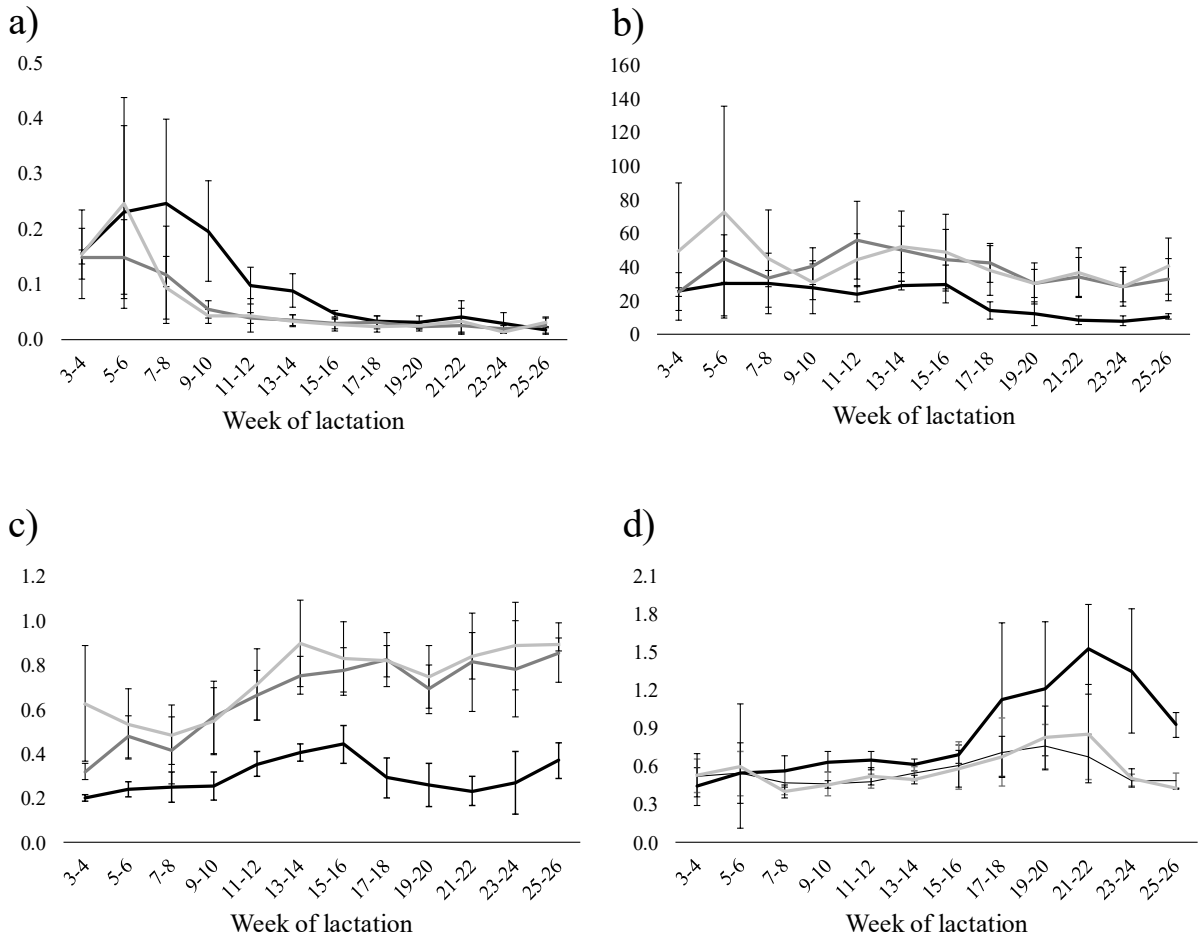


Figure 3. *Farm and lactation interaction effect for the changes in the average content (mg/100 g of milk) of various fatty acids and ratios in mare milk samples from the three commercial farms along six months of lactation. (a) 9:0 (also representative of 11:0); (b) 18:3n-3 (also representative of 16:3n-3 and 9t,12t,15c-18:3); (c) P/S (polyunsaturated/saturated fatty acids ratio; also representative of 18:3n-6); (d) n-6/n-3. Farm I (▬), farm II (▬), farm III (▬).*

3.3. Effect of management system

Management of the mares significantly ($P \leq 0.05$) affected the content of several milk FAs reported (**Tables 1-4**). Compared to mares managed under high grazing activity (farms II and III), those under low grazing activity (farm I) produced a milk with a higher content in some major SFAs (8:0, 10:0, 12:0, and 14:0), 9:0, and several individual *cis*-MUFAs (7*c*-16:1, 9*c*-18:1, 11*c*-18:1, 11*c*-19:1, 11*c*-20:1, 13*c*-22:1 and 15*c*-24:1). Among the *trans*-MUFAs, only 9*t*-18:1 was affected by the management practice. This effect was also reflected in the content of total SFAs and MUFAs, but not in individual or total contents of BCFAs. Interestingly, the content of 16:0 and 9*c*-16:1, which were major FAs in mare milk, did not significantly ($P > 0.05$) differ among milks from different farms.

It was noteworthy that milk from high grazing activity farms (farms II and III) contained significantly ($P \leq 0.05$) higher concentrations of 16:2n-6, 18:2n-6, 18:3n-6, 22:2n-6, 16:3n-3, 18:3n-3 and 9*t*,12*t*,15*c*-18:3, and consequently, higher concentrations of n-6, n-3 and total PUFAs than milk from the low grazing activity farm (farm I). However, the n-6/n-3 ratio was significantly ($P \leq 0.05$) higher in low grazing activity milk. Furthermore, the observed P/S ratio was higher in milk from high grazing farms (0.686 in farm II and 0.739 in farm III, *vs.* 0.305 in farm I).

4. Discussion

Production of horse meat and milk products has increasing acceptance in Europe because of their nutritional and potential therapeutic value (Czyżak-Runowska, et al., 2021; Naert et al, 2013; Markiewicz-Kęszycka et al., 2014; Mazhitova et al., 2015; Pietrzak-Fiećko et al., 2013; Pikul et al., 2008). For this reason, we have undertaken a thorough examination of horse meat to provide an accurate and complete FA (Belaunzaran et al., 2017) and plasmalogen (Beldarrain et al., under review) composition, and now we wish to extend this to the FA composition of mare milk and the changes that occur throughout the lactation period and under different grazing intensities. As aforementioned, this study is part of a wider research where a complete characterization of mare milk was performed in rural equine farms.

As described in section 2, a methylation procedure that prevents FAME isomerization and aldehyde formation, a long 100 m highly polar capillary GC column to resolve FA isomers, a temperature program that maximizes the separation of most FAMEs up to DHA, and appropriate FAME standards were used. Moreover, twelve lactation intervals were investigated. The total milk fat content and that of the individual SFAs, BCFAs, MUFAs, and PUFAs from the three farms over the six months lactation period are presented in **Tables 1-4**, and expressed in mg/100 g of milk fat. The results of twelve milk collection intervals of selected FAs, or sums of FAs, from all three farms over the six-month period are shown in **Figure 2**.

Since horses are hindgut fermenters and lack a rumen, the dietary unsaturated FAs are not subjected to extensive biohydrogenation, but are taken up directly and incorporated into their tissue and milk lipids instead. Lipase is the main digestive enzyme secreted by the pancreas, allowing an efficient separation of FAs from ingested acylglycerols. This, together with a continuous secretion of bile salts that have lipid solubilizing activity, makes the horse digestion system particularly efficient at liberating and absorbing dietary FAs (Belaunzaran et al., 2015; Warren & Vineyard, 2013). Moreover, the secretion of a specific pancreatic lipase related to protein 2 (PLRP2) allows the liberation of lipids esterified in galactolipids, which are the most abundant lipid species in plants (Sahaka et al., 2020). The efficient lipid digestion and the post-absorptive location of the fermentative caecum-colon chamber leads to a high absorption and deposition of non-modified PUFAs in horse tissues (Belaunzaran et al., 2017, 2018; Clauss et al., 2009; Devle et al., 2012; Gupta & Hilditch, 1951), thus becoming a good fingerprint of the dietary lipid composition (Deng et al., 2022).

The total fat content in the pasture-fed mare's milk ranged from 0.247 to 0.306 g/100 g of milk fat, and it significantly ($P \leq 0.05$) decreased during lactation (data not shown). This is consistent with the results of other free range-fed horse breeds (Barłowska et al., 2023; Barreto et al., 2020; Czyżak-Runowska et al., 2021; Navrátilová et al., 2018; Pikul et al., 2008), but not all observed a decrease in fat content during lactation (Barłowska et al., 2023; Barreto et al., 2020; Navrátilová et al., 2018). The milk fat content in human milk ranges from 2 to 4 g/100 mL of milk, whereas in ruminants it may often be about 2-9 g/100 mL (Claeys et al., 2014; Devle et al., 2012; Markiewicz-Kęszycka et al., 2014; Pelizzola et al., 2006), so mare milk is characterized for its low fat content. The average composition of mare milk fat consists mainly of SFAs (47.5 %) and about equal amounts

of MUFAs (25.3 %) and PUFAs (26.5 %). In marked contrast, the lipid composition of ruminant milk consists mainly of SFAs (about 56-73 %) with lower levels of MUFAs (about 22-30 %) and smaller amounts of PUFAs (about 2-6 %) (Claeys et al., 2014), an effect attributed to rumen biohydrogenation.

The SFAs in mare milk are principally composed of medium even chain FAs (8-16 C), whereas short-chain (< 8 C) and long-chain (> 18 C) SFAs are usually present at less than 1 % of total FAs, which is consistent with results reported by others (Barłowska et al., 2023; Czyżak-Runowska et al., 2021; Devle et al., 2012; Pikul & Wójtowski, 2008). The lower grazing activity of mares resulted in greater SFA contents in milk, although this effect was not significant ($P > 0.05$) for the major SFA (16:0). Most even-chain SFAs are synthetized *de novo* in the mammary gland from glucose (primarily up to 16 C), while dietary lipids are the source of FAs 16:0 and longer. The stage of lactation significantly decreased most SFAs in this study (**Figure 2**; **Table 1**), which was also reported by Czyżak-Runowska et al. (2021), but not consistently by others (Barłowska et al., 2023; Barreto et al., 2020; Haddad et al., 2011; Pikul & Wójtowski, 2008). Normally, changes in the total SFA content during lactation would correspond to changes in either *de novo* synthesis in the mammary gland or body fat mobilization, in response to the energy balance of the mares (Inostroza et al., 2020). Considering that horses have a low efficiency at mobilizing body reserves (Deichsel & Aurich, 2005), regulation was probably due to *de novo* synthesis of FAs. Conversely, some minor odd-chain SFAs (11:0, 15:0 and 17:0) have been proven to change differently compared to even-chain FAs (Czyżak-Runowska et al., 2021; Haddad et al., 2011; Pikul & Wójtowski et al., 2008), probably because they have a different origin (gut fermentation; Santos et al., 2013). Haddad et al. (2011) investigated the regiodistribution of mare milk triacylglycerol and found that the *sn*-2 position was primarily esterified by even SFAs from 10:0 to 16:0, which was consistent with that reported by Parodi (1982). A region-specific distribution of SFAs was also observed in cow's milk, where 8:0 to 14:0 is preferentially located at the *sn*-2 position (Parodi, 1982), and in human milk with 14:0 and 16:0 predominating at the *sn*-2 position (Jensen & Jensen, 1992). The greater proportion of 16:0 in the *sn*-2 position resulted in a lower excretion of this FA in the stool, reduced stool hardness, and a higher whole-body bone mass in infants (Kennedy et al., 1999). Based on the similarity of the FA composition and distribution of human and horse milk triglycerides (Claeys el

al., 2014; Haddad et al., 2011), some have suggested that mare milk could serve as a substitute for human milk (Barłowska et al., 2023; Markiewicz-Kęszycka et al., 2014).

BCFAs are fermentation products formed in the hindgut from dietary amino acids (leucine, isoleucine and valine) (Gozdzik et al., 2023; Santos et al., 2013). Their presence in mare milk is low, and even though no differences were found on an absolute basis (**Table 2**), significantly ($P \leq 0.05$) higher contents were found in grazing than non-grazing horses as evidenced in significantly higher total BCFA percentages in mare milk samples from farms II (0.62%) and III (0.59%) compared to low grazing animals from farm I (0.49%). Diet can alter the hindgut microbial population (Garber et al., 2020), and some authors suggested that the profile of BCFAs together with odd-chain FAs relies on the relative abundance of specific bacterial populations (Santos et al., 2011). Our results demonstrated the presence of seven BCFAs, which showed great variability in their contents (mg/100 g of milk) among farms and lactation times, with *iso*-16:0 as the main isomer and *anteiso*-17:0 as the second in abundance (**Table 2**). Devle et al. (2012) confirmed the presence of *iso*-16:0, although their overall content of BCFAs was much lower, and Pietrzak-Fiećko et al. (2009, 2013) confirmed the presence of *iso*-14:0. However, most studies do not report the second most abundant BCFA, *anteiso*-17:0, which may be due to the inability to resolve *anteiso*-17:0 and 7*c*-16:1. In the present study, an appropriate GC column and conditions, as well as a BCFA standard confirmed these two FAMEs as described by Kramer et al. (2008). Even though an in-depth understanding is lacking on how lactation stage and management of the mares affect the content of BCFAs, some authors have suggested that BCFA contents tend to increase during lactation (Pikul & Wójtowski, 2008), which we confirmed by analysing biweekly intervals throughout the lactation period (**Figure 2**).

Results in this study confirm that MUFA percentages are higher in milk from non- or low-grazing compared to high-grazing mares (Barłowska et al., 2023). The major MUFAs found in milk from farms II and III (9*c*-18:1 and 9*c*-16:1) were similar in percentages as found in other pasture-based milks (13.7-19.8 % and 3.21-6.95 %, respectively; Barłowska et al., 2023; Minjigdorj et al., 2012; Pikul et al., 2008), while percentages found on farm I were closer to values reported in non-pasture-based milks (20.8-25.0 % and 5.51-7.62 %; Barłowska et al., 2023; Czyżak-Runowska et al., 2021; Markiewicz-Kęszycka et al., 2014). In contrast to the decreasing trend of SFAs in mare milk, the percentage of MUFAs tended to increase over lactation as reported by Barłowska et al.

(2023), Czyżak-Runowska et al. (2021) and Haddad et al. (2011). However, when the FA content was expressed as mg/100 g of milk, the content of SFAs decreased over lactation whereas that of MUFAs remained relatively constant throughout the lactation period, except for a rise during mid lactation (**Figure 2**). In line with this, Navrátilová et al. (2018) observed a strong negative correlation between SFA and MUFA percentages in mare milk. In general, MUFAs are synthesized in the mammary gland by Δ9-desaturase enzyme, also called stearoyl-CoA desaturase, transforming individual SFAs into MUFAs (Barber et al., 1997; Smith et al., 2009). Differences among management systems in the total content of MUFAs (**Table 3**) presumably came from lower SFA intakes in pasture-based systems and, thus, less desaturation of SFAs to MUFAs in the udder. In addition, a long-term pasture feeding depresses the expression of the gene that encodes stearoyl-CoA desaturase in cattle, resulting in a higher SFA content in beef fat (Smith et al., 2009). Interestingly, a lower MUFA/SFA ratio was observed in long-term grazing mare milk in the present study. In essence, grazing results in lower MUFA contents in mare milk, probably not only due to a lower SFA intake and deposition, but also due to an impaired conversion of SFAs to MUFAs.

In mare milk, PUFAs mainly consist of LA and LNA, while their very long-chain PUFA metabolites were present at less than 1 % of total FAs (Barłowska et al., 2023; Czyżak-Runowska et al., 2021; Minjigdorj et al., 2012; Pikul & Wójtowski, 2008). Milk from long-term grazing mares (farms II and III) presented a lower n-6/n-3 ratio compared to short-term grazing mares' milk (farm I) due to an accumulated increase in total n-3 content (**Table 4**) (Barłowska et al., 2023; Minjigdorj et al., 2012; Pikul et al., 2008). It is known that grass is more abundant in PUFAs, particularly in n-3, than concentrates (Jahreis & Dawczynski, 2020; Sahaka et al., 2020), and therefore, pasture-based management improves the accumulation of n-3 PUFAs (mainly LNA) in mare milk. As confirmed by the present research (**Figure 3**), some studies described an increasing trend of n-6/n-3 ratio from early to mid lactation (Barreto et al., 2020; Haddad et al., 2011) and a decrease afterwards (Barreto et al., 2020; Gregic et al., 2022). In mares reared under extensive management systems, changes in milk FA profile that occur during lactation could also be derived from changes in forage lipid composition due to the growth stage of the several botanical species present during the season (Boufaïed et al., 2003).

The content of LC-PUFAs is very low in mare milk (Barłowska et al., 2023; Czyżak-Runowska et al., 2021; Devle et al., 2012; Minjigdorj et al., 2012; Orlandi et al., 2003;

Pikul et al., 2008; Pikul & Wójtowski, 2008). This is probably related to a limitation in the enzymatic activity of Δ6-desaturase, which is responsible for the first step in the conversion from LA to arachidonic acid (20:4n-6) and subsequent metabolites, and from LNA to EPA and DHA (Marangoni et al., 2020; Saini et al., 2021). The activity of this enzyme is low in mammals, which explains the very low percentages of intermediates such as 18:3n-6 (0.02-0.74 %), 20:3n-6 (0.03-0.10 %); 20:4n-6 (0.08-0.60 %), 20:5n-3 (0.01-0.12 %), 22:5n-3 (0.03-0.22 %), and 22:6n-3 (0.01-0.05 %) in mare milk (Barłowska et al., 2023; Czyżak-Runowska et al., 2021; Devle et al., 2012; Minjigdorj et al., 2012; Orlandi et al., 2003; Pikul et al., 2008; Pikul & Wójtowski, 2008). As evidenced in the present study, modulating the content of LC-PUFAs through grazing is difficult, except for the main precursors LA and LNA. However, supplementing mares' diet with ingredients rich in LC-PUFAs can significantly increase the content of n-3 LC-PUFAs in milk (Hodge et al., 2017; Kouba et al., 2019; Saini et al., 2021). When the average content of n-6 and n-3 PUFAs in mare milk was compared to milk from other species (human, cow; **Figure 4**), a higher LNA content was observed in mare milk. In other species, however, the n-3 LC-PUFA to LNA ratio was greater (**Figure 4**) indicating a more efficient LNA conversion to other n-3 LC-PUFAs.

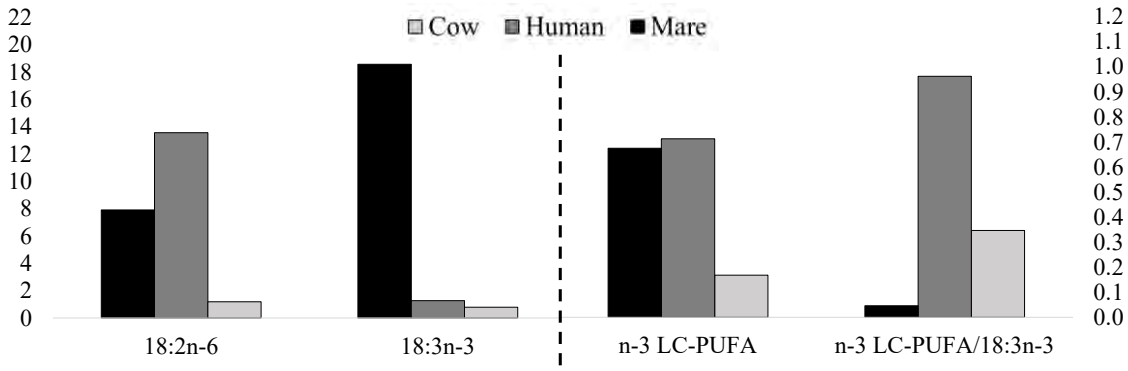


Figure 4. *Comparison of the average content (expressed as percentage of total fatty acids) of some fatty acids and ratios of nutritional interest in milk from mare, human and cow. Data was obtained from the present study and Pikul et al. (2008) for mare milk; from Capuano et al. (2014), Coppa et al. (2011) and Loza et al. (2023) for cow milk; and from Miliku et al. (2019), Yuhas et al. (2006) and own data for human milk. LC-PUFA: long chain polyunsaturated fatty acids.*

Desaturation and elongation steps from LA and LNA to LC-PUFA products were limited in mare milk. However, considering the PUFA profile, mare milk could be a source of n-6 and n-3 PUFAs, and in particular LNA. Such products have been used historically for their functional properties, such as in the treatment and prevention of gastrointestinal, respiratory (mainly tuberculosis), and skin (acne, psoriasis and atopic eczema) diseases, among others (Jastrzębska et al., 2017; Sheng and Fang, 2009).

Mare milk contains only traces of CLA isomers derived either from diet – by intestinal absorption of these isomers produced by microbial modification in the hindgut –, or from incorporation of the precursor into the mammary gland and subsequent Δ9-desaturation activity (Jahreis & Dawczynski, 2020). In non-ruminants, some microbial fermentation occurs in the stomach, by acid-tolerant bacteria, and in the small intestine (Ericsson et al., 2016). However, the post-absorptive location of the main digestive fermentation chamber (caecum-colon) leads to a low absorption and deposition of these isomers. Therefore, both horse meat and mare milk contain low quantities of CLA (around 0.1 % of total FAs or less) compared to ruminant derived foods (Belaunzaran et al., 2017; Devle et al., 2012). In the literature, only rumenic acid (9*c*,11*t*-18:2) (Barłowska et al., 2023; Czyżak-Runowska et al., 2021; Devle et al., 2012) or the total CLA contents (Minjigdorj et al., 2012; Naert et al., 2013; Pelizzola et al., 2006; Pikul et al., 2008) were reported. Some authors have reported an increase in CLA content from the first to the fifth month of lactation (Pikul et al., 2008; Pikul & Wójtowski, 2008), or from summer to autumn (Minjigdorj et al., 2012). We observed low levels of rumenic acid in mare milk that did not significantly ($P > 0.05$) vary in content between farms (**Table 4**), while Barłowska et al. (2023) found significantly more rumenic acid in milk from mares fed on pasture.

From a multivariate perspective, the results of the SDA confirmed the size of management system and lactation effects on the FA composition of mare milk. In this regard, **Figure 5** shows the distribution of mare milk samples according to the first two canonical functions obtained to discriminate either among management systems (farms I, II and III) or lactation stages (early, mid and late lactation). As depicted, milk samples from low grazing animals (farm I) were clearly discriminated from high grazing animals (farms II and III), and SDA did not differentiate between the latter. All mare milk samples from farm I were correctly classified, and 99.4 % of samples from farm II and III were considered as a single group. After cross-validation, the percentage of classification was 98.1 % in both cases. Furthermore, the milk samples from high grazing and low grazing

farms were mainly discriminated along the discriminant function 1 axis (**Figure 5**). This function showed the highest correlations with P/S ratio, total n-3 PUFA and PUFA, *cis*-MUFA and some individual FAs such as LNA, 9*c*-18:1 and 16:3n-3, which is in agreement with the results discussed above for the management effect on mare milk FA composition. In relation to the size effect of lactation stage, SDA showed that milk samples from early and late lactation were clearly differentiated (100 % percentage of classification after cross-validation) whereas, as expected, mid lactation samples were distributed at an intermediate location between early and late lactation samples along the discriminant function 1 axis (**Figure 5**). This function showed the highest correlations with most short-chain SFAs, 12:0, 14:0 and total SFA. Overall, after cross-validation, the 87.3 % of the milk samples were correctly classified in their group according to lactation stage. In short, these multivariate results were in agreement with those discussed for changes in mare milk composition during lactation.

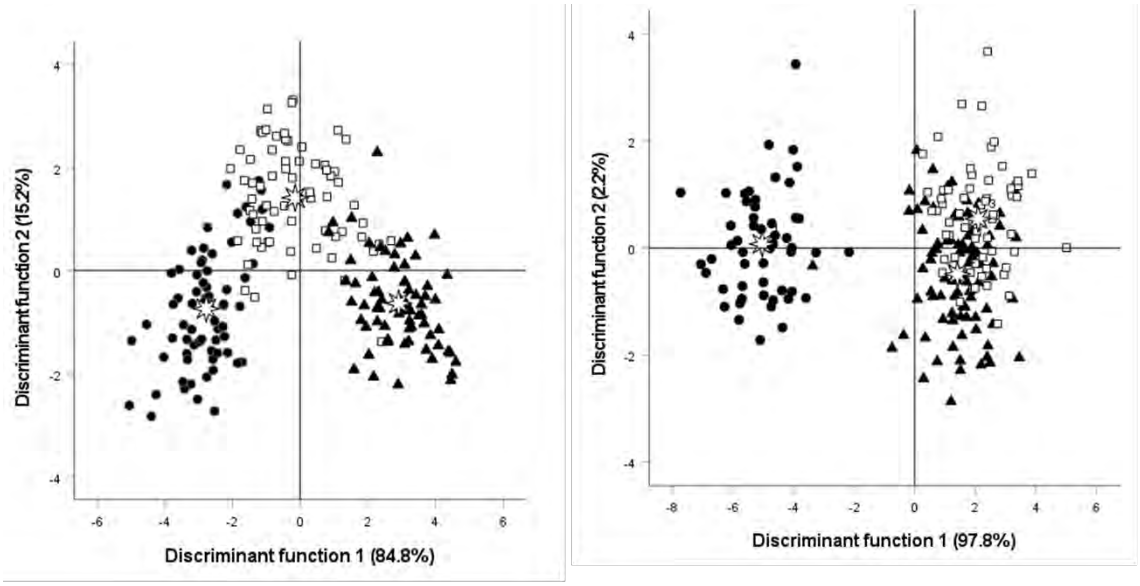


Figure 5. *Graphs for the two first canonical discriminant functions corresponding to the stepwise discriminant analysis on fatty acid composition of mare milk samples from the commercial farms (graph on the right; ●, farm I; □, farm II; ▲, farm III), and from different lactation stages (graph on the left; ●, early-; □, mid-; ▲, late-lactation). , group centroid.*

5. Conclusions

This study allowed a broad understanding of the FA profile of mare milk reared under different semi-extensive management systems, including not only major but also minor and poorly described FAs. Overall, mare milk contained a high proportion of LA and LNA but a low concentration of their long-chain metabolites, and a low proportion of BCFAs. Frequent monitoring of mare milk revealed that lactation stage significantly influenced the content of most individual FAs: some decreased (SFAs), some remained fairly constant (MUFAs and BCFAs) while the PUFAs showed a large variation throughout lactation. Milk from mares managed under pasture-based feeding systems showed a FA profile of interest from a human health perspective, with a higher content of n-3 PUFAs. The results support that mare milk production under grazing conditions could be explored as a commercial strategy to improve the performance and resilience of equine rural farms currently dedicated to meat production, or even as the establishment of new farms for mare milk production.

Author contributions

A. Blanco: Methodology, formal analysis, investigation, data curation, writing – original draft, visualization. L.J.R. Barron: Conceptualization, methodology, resources, data curation, writing – review & editing, supervision, project administration, funding acquisition. J.K.G. Kramer: Methodology, data curation, writing – review & editing. N. Aldai: Conceptualization, methodology, resources, data curation, writing – review & editing, supervision, project administration, funding acquisition, formal analysis.

Declaration of interest

None.

Acknowledgements

Authors thank the commercial farms that participated in the study.

Funding

This work was supported by the Basque Government through Biotasma (Elkartek, 2019), Behor Esne (Cooperation, 2020) and IT944-16 and IT1568-22 projects. A. Blanco-Doval received a predoctoral fellowship from The Department of Education of the Basque Government.

References

Aldai, N., Kramer, J. K. G., Cruz-Hernandez, C., Santercole, V., Delmonte, P., Mossoba, M. M., & Dugan, M. E. R. (2012). Appropriate extraction and methylation techniques for lipids analysis. In G. Cherian, & R. Poureslami (Eds.), Fat and fatty acids in poultry nutrition and health (pp. 249-278). Context.

Barber, M. C., Clegg, R. A., Travers, M. T., & Vernon, R. G. (1997). Lipid metabolism in the lactating mammary gland. Biochimica and Biophysica Acta - Lipids and Lipid Metabolism, 1347, 101-126.

Barłowska, J., Polak, G., Janczarek, I., & Tkaczyk, E. (2023). The influence of selected factors on the nutritional value of the milk of cold-blooded mares: The example of the Sokólski breed. Animals, 13, Article 1152.

Barreto, Í. M., Urbano, S. A., Oliveira, C. A., Macêdo, C. S., Borba, L. H., Chags, B. M., & Rangel, A. H. (2020). Chemical composition and lipid profile of mare colostrum and milk of the Quarter horse breed. PLoS One, 15, Article e0238921.

Belaunzaran, X., Bessa, R. J., Lavín, P., Mantecón, A. R., Kramer, J. K. G., & Aldai, N. (2015). Horse-meat for human consumption – Current research and future opportunities. Meat Science, 108, 74-81.

Belaunzaran, X., Lavín, P., Barron, L. J. R., Mantecón, A. R., Kramer, J. K. G., & Aldai, N. (2017). An assessment of the fatty acid composition of horse-meat available at the retail level in northern Spain. Meat Science, 124, 39-47.

Belaunzaran, X., Lavín, P., Mantecón, A. R., Kramer, J. K. G., & Aldai, N. (2018). Effect of slaughter age and feeding system on the neutral and polar lipid composition of horse meat. Animal, 12, 417-425.

Beldarrain, L. R., Belaunzaran, X., Sentandreu, M. A., Kramer, J. K. G., & Aldai, N. (2024). Characterization of methanolysis products from plasmalogenic lipids in horse muscle tissue. Lipids (under review).

Blanco-Doval, A., Barron, L. J. R., & Aldai, N. (2023). Changes during lactation in the mineral element content of mare milk produced in semi-extensive rural farms. Journal of Food Composition and Analysis, 123, Article 105629.

Boufaïed, H., Chouinard, P. Y., Tremblay, G. F., Petit, H. V., Michaud, R., & Bélanger, G. (2003). Fatty acids in forages. I. Factors affecting concentrations. Canadian Journal of Animal Science, 83, 501-511.

Burdge, G. C., & Calder, P. C. (2005). Conversion of α-linolenic acid to longer-chain polyunsaturated fatty acids in human adults. Reproduction Nutrition Development, 45, 581-597.

Capra, M. E., Stanyevic, B., Giudice, A., Monopoli, D., Decarolis, N. M., Esposito, S., & Biasucci, G. (2023). Long-chain polyunsaturated fatty acids effects on cardiovascular risk in childhood: A narrative review. Nutrients, 15, Article 1661.

Capuano, E., Van der Veer, G., Boerrigter-Eenling, R., Elgersma, A., Rademaker, J., Sterian, A., & Van Ruth, S. M. (2014). Verification of fresh grass feeding, pasture grazing and organic farming by cows farm milk fatty acid profile. Food Chemistry, 164, 234-241.

Claeys, W. L., Verraes, C., Cardoen, S., De Block, J., Huyghebaert, A., Raes, K., Dewettinck, K., & Herman, L. (2014). Consumption of raw or heated milk from different species. An evaluation of the nutritional and potential health benefits. Food Control, 42, 188-201.

Clauss, M., Grum, C., & Hatt, J. (2009). Polyunsaturated fatty acid content in adipose tissue in foregut and hindgut fermenting mammalian herbivores: A literature survey. Mammalian Biology, 74, 153-158.

Coppa, M., Ferlay, A., Monsallier, F., Verdier-Metz, I., Pradel, P., Didienne, R., Farruggia, A., Montel, M. C., & Martin, B. (2011). Milk fatty acid composition and cheese texture and appearance from cows fed hay or different grazing systems on upland pastures. Journal of Dairy Science, 94, 1132-1145.

Cruz-Hernandez, C., Deng, Z., Zhou, J., Hill, A.R., Yurawecz, M.P., Delmonte, P., Mossoba, M. M., Dugan, M. E. R., & Kramer, J. K. G. (2004). Methods for analysis of conjugated linoleic acids and trans-18:1 isomers in dairy fats by using a combination of gas chromatography, silver-ion thin-layer chromatography/gas chromatography, and silver-ion liquid chromatography. Journal of AOAC International, 87, 545-562.

Czyżak-Runowska, G., Wójtowski, J. A., Danków, R., & Stanisławski, D. (2021). Mare's milk from a small Polish specialized farm – Basic chemical composition, fatty acid profile, and healthy lipid indices. Animals, 11, Article 1590.

Deichsel, K., & Aurich, J. (2005). Lactation and lactational effects on metabolism and reproduction in the horse mare. Livestock Production Science, 98, 25-30.

Deng, L., Yang, Y., Li, Z., Li, J., Zhu, Y., Meng, Q., Liu, J., & Wang, X. (2022). Impact of different dietary regimens on the lipidomic profile of mare's milk. Food Research International, 156, Article 111305.

Devle, H., Vetti, I., Naess-Andresen, C. F., Rukke, E., Vegarud, G., & Ekeberg, D. (2012). A comparative study of fatty acid profiles in ruminant and non-ruminant milk. European Journal of Lipid Science and Technology, 114, 1036-1043.

Ericsson, A. C., Johnson, P. J., Lopes, M. A., Perry, S. C., & Lanter, H. R. (2016). A microbiological map of the healthy equine gastrointestinal tract. PloS One, 11, Article e0166523.

Garber, A., Hastie, P., & Murray, J. (2020). Factors influencing equine gut microbiota: Current knowledge. Journal of Equine Veterinary Science, 88, Article 102943.

Gozdzik, P., Magkos, F., Sledzinski, T., & Mika, A. (2023). Monomethyl branched-chain fatty acids: Health effects and biological mechanisms. Progress in Lipid Research, 90, Article 101226.

Gupta, S. S., & Hilditch, T. P. (1951). The component acids and glycerides of a horse mesenteric fat. Biochemical Journal, 48, 137-146.

Haddad, I., Mozzon, M., Strabbioli, R., & Frega, N. G. (2011). Fatty acid composition and regiodistribution in mare's milk triacylglycerols at different lactation stages. Dairy Science and Technology, 91, 397-412.

Hodge, L. B., Rude, B. J., Dinh, T. N., & Lemley, C. O. (2017). Effect of ω-3 fatty acid supplementation to gestating and lactating mares: On milk IgG, mare and foal blood concentrations of IgG, insulin and glucose, placental efficiency, and fatty acid composition of milk and serum from mares and foals. Journal of Equine Veteterinary Science, 51, 70-78.

Inostroza, K., Bravo, S., Larama, G., Saenz, C., & Sepúlveda, N. (2020). Variation in milk composition and fatty acid profile during the lactation of Araucana creole ewes in a pasture-based system. Animals, 10, Article 92.

Jahreis, G., & Dawczynski, C. (2020). Trans and conjugated fatty acids in dairy products: Cause for concern? In I. Givens (Ed.), Milk and dairy foods. Their functionality in human health and disease (pp. 87-120). Academic Press Elsevier.

Jastrzębska, E., Wadas, E., Daszkiewicz, T., & Pietrzak-Fiećko, R. (2017). Nutritional value and health-promoting properties of mare's milk − A review. Czech Journal of Animal Science, 62, 511-518.

Jensen, G. L., & Jensen, R. G. (1992). Specialty lipids for infant nutrition. II. Concerns, new developments, and future applications. Journal of Pediatric Gastroenterology and Nutrition, 15, 382-394.

Kennedy, K., Fewtrell, M. S., Morley, R., Abbott, R., Quinlan, P. T., Wells, J. C. K., Bindels, J. G., & Lucas, A. (1999). Double-blind, randomized trial of a synthetic triacylglycerol in formula-fed term infants: Effects on stool biochemistry, stool characteristics, and bone mineralization. The Americal Journal of Clinical Nutrition, 70, 920-927.

Kouba, J. M., Burns, T. A., & Webel, S. K. (2019). Effect of dietary supplementation with long-chain n-3 fatty acids during late gestation and early lactation on mare and foal plasma fatty acid composition, milk fatty acid composition, and mare reproductive variables. Animal Reproduction Science, 203, 33-44.

Kramer, J. K. G., Hernandez, M., Cruz-Hernandez, C., Kraft, J., & Dugan, M. E. (2008). Combining results of two GC separations partly achieves determination of all cis and

trans 16:1, 18:1, 18:2 and 18:3 except CLA isomers of milk fat as demonstrated using Ag-ion SPE fractionation. Lipids, 43, 259-273.

Loza, C., Davis, H., Malisch, C., Taube, F., Loges, R., Magistrali, A., & Butler, G. (2023). Milk fatty acids: The impact of grazing diverse pasture and the potential to predict rumen-derived methane. Agriculture, 13, Article 181.

Luna, P., Juárez, M., & de la Fuente, M. A. (2005). Validation of a rapid milk fat separation method to determine the fatty acid profile by gas chromatography. Journal of Dairy Science, 88, 3377-3381.

Marangoni, F., Agostoni, C., Borghi, C., Catapano, A. L., Cena, H., Ghiselli, A., La Vecchia, C., Lercker, G., Manzato, E., Pirillo, A., Riccardi, G., Risé, P., Visioli, F., & Poli, A. (2020). Dietary linoleic acid and human health: Focus on cardiovascular and cardiometabolic effects. Atherosclerosis, 292, 90-98.

Markiewicz-Kęszycka, M., Wójtowski, J., Czyżak-Runowska, G., Kuczyńska, B., Puppel, K., Krzyżewski, J., Strzalkowska, N., Jozwik, A., & Bagnicka, E. (2014). Concentration of selected fatty acids, fat-soluble vitamins and β-carotene in late lactation mares' milk. International Dairy Journal, 38, 31-36.

Mazhitova, A. T., Kulmyrzaev, A. A., Ozbekova, Z. E., & Bodoshev, A. (2015). Amino acid and fatty acid profile of the mare's milk produced on Suusamyr pastures of the Kyrgyz republic during lactation period. Procedia – Social and Behavioral Science, 195, 2683-2688.

Method PE/ALVO/02 of the Lekunberri Dairy Institute certified by the National Accreditation Body (ENAC, Spanish Government) according to the criteria described in the Norm UNE-EN ISO/IEC 17025:2005 from https://www.alvo-ill.org/sites/default/files/2021-12/CERT_387_LE_381_rev2.pdf

Miliku, K., Duan, Q. L., Moraes, T. J., Becker, A. B., Mandhane, P. J., Turvey, S. E., Lefebvre, D. L., Sears, M. R., Subbarao, P., Field, C. J., & Field, C. J. (2019). Human milk fatty acid composition is associated with dietary, genetic, sociodemographic, and environmental factors in the CHILD cohort study. The American Journal of Clinical Nutrition, 110, 1370-1383.

Minjigdorj, N., Haug, A., & Austbø, D. (2012). Fatty acid composition of Mongolian mare milk. Acta Agriculturae Scandinavica Section A – Animal Science, 62, 73-80.

Naert, L., Verhoeven, G., Duchateau, L., De Smet, S., & Coopman, F. (2013). Assessing heterogeneity of the composition of mare's milk in Flanders. Vlaams Diergeneeskundig Tijdschrift, 82, 23-30.

Navrátilová, P., Pospíšil, J., Borkovcová, I., Kaniová, L., Dluhošová, S., & Horáková, S. (2018). Content of nutritionally important components in mare milk fat. Mljekarstvo: Časopis Za Unaprjeđenje Proizvodnje i Prerade Mlijeka, 68, 282-294.

Orlandi, M., Goracci, J., & Curadi, M. C. (2003). Fat composition of mare's milk with reference to human nutrition. Annali Della Facolta Di Medicina Veterinaria, 56, 97-106.

Parodi, P. W. (1982). Positional distribution of fatty acids, in triglycerides from milk of several species of mammals. Lipids, 17, 437-442.

Pelizzola, V., Contarini, G., Povolo, M., & Giangiacomo, R. (2006). Chemical-physical characteristics and fatty acid composition of mare's milk. Milchwissenschaft, 61, 33-36.

Pietrzak-Fiećko, R., Tomczyński, R., & Smoczyński, S. S. (2013). Effect of lactation period on the fatty acid composition in mares' milk from different breeds. Archives Animal Breeding, 56, 335-343.

Pietrzak-Fiećko, R., Tomczyński, R., Świstowska, A., Borejszo, Z., Kokoszko, E., & Smoczyńska, K. (2009). Effect of mare's breed on the fatty acid composition of milk fat. Czech Journal of Animal Science, 54, 403-407.

Pikul, J., & Wójtowski, J. (2008). Fat and cholesterol content and fatty acid composition of mares' colostrums and milk during five lactation months. Livestock Science, 113, 285-290.

Pikul, J., Wójtowski, J., Danków, R., Kuczyńska, B., & Łojek, J. (2008). Fat content and fatty acids profile of colostrum and milk of primitive Konik horses (*Equus caballus gmelini* Ant.) during six months of lactation. Journal of Dairy Research, 75, 302-309.

Sahaka, M., Amara, S., Wattanakul, J., Gedi, M. A., Aldai, N., Parsiegla, G., Lecomte, J., Christeller, J. T., Gray, D., Gontero, B., Villeneuve, P., & Carrière, F. (2020). The digestion of galactolipids and its ubiquitous function in nature for the uptake of the essential α-linolenic acid. Food and Function, 11, 6710-6744.

Saini, R. K., Prasad, P., Sreedhar, R. V., Naidu, K. A., Shang, X., & Keum, Y. (2021). Omega-3 polyunsaturated fatty acids (PUFAs): Emerging plant and microbial sources, oxidative stability, bioavailability, and health benefits – A review. Antioxidants, 10, Article 1627.

Santos, A. S., Jerónimo, E., Ferreira, L. M., Rodrigues, M., & Bessa, R. (2013). Fatty acids and purine profile of cecum and colon bacteria as indicators of equine microbial metabolism. Journal of Animal Science, 91, 1753-1757.

Santos, A. S., Rodrigues, M., Bessa, R., Ferreira, L. M., & Martin-Rosset, W. (2011). Understanding the equine cecum-colon ecosystem: Current knowledge and future perspectives. Animal, 5, 48-56.

Sheng, Q., & Fang, X. (2009). Bioactive components in mare milk. In Y. W. Park (Ed.), Bioactive components in milk and dairy products (pp. 195-213). Wiley-Blackwell.

Smith, S. B., Gill, C. A., Lunt, D. K., & Brooks, M. A. (2009). Regulation of fat and fatty acid composition in beef cattle. Asian-Australasian Journal of Animal Sciences, 22, 1225-1233.

Warren, L. K., & Vineyard, K. R. (2013). Fat and fatty acids. In R. J. Geor, P. A. Harris, M. & Coenen (Eds.), Equine applied and clinical nutrition (pp. 136-155). Saunders Elsevier.

Wolff, R. L., Bayard, C. C., & Fabien, R. J. (1995). Evaluation of sequential methods for the determination of butterfat fatty acid composition with emphasis on trans-18:1 acids. Application to the study of seasonal variations in French butters. Journal of the American Oil Chemists' Society, 72, 1471-1483.

Yuhas, R., Pramuk, K., & Lien, E. L. (2006). Human milk fatty acid composition from nine countries varies most in DHA. Lipids, 41, 851-858.

APPENDIX I.II – Manuscript 2

Blanco-Doval, A., Barron, L. J. R., Bustamante, M. A., Aldai, N. (2024). **Characterization and monitoring of changes during lactation in total antioxidant capacity and profile of multiple bioactive compounds of milk from grazing mares**. *Journal of the Science of Food and Agriculture,* under review.

Sent to: ***Journal of the Science of Food and Agriculture,*** 2024

Status: Under review

Manuscript ID: JSFA-24-0558

Characterization and monitoring of changes during lactation in total antioxidant capacity and profile of multiple bioactive compounds of milk from grazing mares

Effect of lactation stage on bioactive compounds and antioxidant capacity of mare milk

Ana Blanco-Doval, Luis Javier R. Barron, María Ángeles Bustamante, Noelia Aldai*

Lactiker Research Group, Department of Pharmacy and Food Sciences, University of the Basque Country (UPV/EHU), Paseo de la Universidad 7, 01006 Vitoria-Gasteiz, Spain

* Corresponding author: Noelia Aldai (noelia.aldai@ehu.eus)

Abstract

Mare milk has often been considered a food product with potential functional properties. However, the bioactive compound composition of mare milk, including vitamins and other minor bioactive compounds, as well as factors affecting this composition have scarcely been studied. Therefore, the present study aimed to characterize the changes during lactation in the content of water- and fat-soluble vitamins and total polyphenols, and the total antioxidant capacity of mare milk from semi-extensive farms. A total of 311 individual milk samples from eighteen mares belonging to three commercial farms and twelve lactation times were analysed. Ascorbic acid (vitamin C), thiamine (vitamin B_1), riboflavin (vitamin B_2), nicotinic acid and niacinamide (vitamins B_3), pantothenic acid (vitamin B_5), pyridoxal and pyridoxine (vitamins B_6), folic acid (vitamin B_9), cyanocobalamin (vitamin B_{12}), tocopherols and tocotrienols (vitamin E), and retinol and retinyl esters (vitamin A) were quantified using liquid chromatography. Total polyphenols and antioxidant capacity assays were analysed by spectrophotometry. Results showed a general downward trend as lactation progressed in the content of most bioactive compounds, with the exception of polyphenols and the total antioxidant capacity that showed an oscillating variation during lactation. On the other hand, the effect of the different semi-extensive management of the farms was only significant for vitamin B_3 content. To the best of our knowledge, the present study provides the most in-depth description of the vitamin profile of mare milk as well as new insights into polyphenol content and antioxidant capacity of mare milk.

Keywords: Hydrosoluble vitamin, liposoluble vitamin, pasture, polyphenol, antioxidant activity.

1. Introduction

Mare milk is a traditional dairy product in Central Asia and Eastern Europe, but its consumption has expanded to some other European countries during the last century[1,2]. Mare milk is highly valued for its composition similar to human milk, low allergenicity, lower casein to whey protein ratio[2,3] and its abundance in polyunsaturated fatty acids[4]. Mare milk has traditionally been used for the prevention and treatment of several pathologies, reason why its potential as a functional food product has been widely assumed[1,3]. Following such historical uses and assumptions, recent studies have moved towards studying mare milk functional properties in terms of fatigue[5], cancer[6], diabetes[7] and wound healing[8], among others. However, the bioactive compound composition of mare milk has been substantially understudied compared to other more consumed milk types, such as cow, sheep or goat milk. For instance, minor bioactive components including vitamins have been barely investigated, although some studies that address mare milk as a high-value food product sometimes refer to its high vitamin content[1].

Few studies have analysed this chemical fraction of the product, particularly regarding water-soluble vitamins. Among the available studies, many of them are outdated but have been cited time after time due to the lack of recent research, as also discussed by Navrátilová et al.[9]. Most studies on mare milk water-soluble vitamins used analytical methodologies such as microbiological, colorimetric or titration methods that only allowed an indirect approximation to the content[10,11,12,13,14]. Small sample sizes and few influencing factors have often been considered[11,12,15,16], and only a few studies accounted for changes during the lactation period[9,13], using up to six sampling times and analysing few vitamins.

Regarding fat-soluble vitamins, literature is also limited and studies have usually focused on a single isomer or form of vitamins A and E[9,13,17,18,19], providing an incomplete composition of mare milk while changes in the content of these vitamins during lactation have been poorly studied[9]. To the best of our knowledge, grazing effect on the content of mare milk vitamins has not been investigated.

On the other hand, polyphenols are plant secondary metabolites that exert antioxidant activity and contribute to the functional value of foods[20]. Even though fruits and vegetables are the main dietary source of polyphenols[20,21], these are also present in milk and dairy products[22], but up to date their presence in mare milk has not been reported,

while studies assessing the total antioxidant capacity of mare milk are also scarce. Characterization of these two parameters in mare milk is necessary for a better understanding of its composition and bioactivity, which in turn is essential to establish a basis for future research that evaluates mare milk as a functional food.

In the present study, a thorough characterization of bioactive compounds in mare milk was performed. This included a broad vitamin profiling, accounting for water- and fat-soluble vitamins, and total polyphenol quantification, as well as the determination of total antioxidant capacity. Moreover, the effect of lactation (with twelve sampling times) and different feeding management systems (with different grazing intensities) was investigated in commercial farms.

2. Materials and methods

2.1. Experimental design and milk sampling

Individual milk samples were collected from eighteen mares belonging to three different commercial farms (n = 6 mares *per* farm) located in Araba (northern Spain). All mares were from the breed Basque Mountain Horse. Mares were milked during the complete lactation period (six months), once a week from early May to late July, and once every fourteen days from early August to mid October. The semi-extensive management system used for animal rearing differed among farms. Briefly, farm I used a pasture-based system only during May, and fed the animals with a variable mixture of alfalfa, silage, hay, fruits and potatoes from June onwards. On the contrary, farms II and III maintained a grazing management during the complete lactation period, supplementing with hay or silage, respectively, only after July in order to deal with low grass availability. Details in animal and sampling conditions were described in Blanco-Doval et al.[23]. A total of 311 individual milk samples were obtained. After milking, the samples were immediately refrigerated (4 ºC), transported to the laboratory, subsampled and preserved at -80 ºC until analysis.

2.2. Chemicals and reagents

2.2.1. Water-soluble vitamins

Acetic acid glacial was purchased from Panreac (Barcelona, Spain), 1-octanesulfonic acid sodium salt from ACROS Organics (Thermo Fisher Scientific, Madrid, Spain), and

trimethylamine from Thermo Fisher Scientific. Methanol for chromatographic analysis was purchased from Romil (Teknokroma, Barcelona, Spain) and potassium phosphate monobasic from Scharlab (Barcelona, Spain). Ultrapure water was obtained from a Milli-Q system (Merck Millipore, Madrid, Spain). Other chemicals were purchased from Merck (Madrid, Spain).

Precipitation solution was prepared with 9.1 g zinc acetate dihydrate, 5.46 g tungstophosphoric acid hydrate and 5.8 mL acetic acid glacial in 100 mL ultrapure water. The precipitation solution was prepared fresh every week. The aqueous mobile phase buffer was daily prepared and contained 6.8 g potassium phosphate monobasic, 1.1 g 1-octanesulfonic acid sodium salt and 5 mL triethylamine in 1 L ultrapure water. Ortophosphoric acid was used to adjust the pH to 2.95.

High purity (> 90%) commercial standards of calcium D-pantothenate, cyanocobalamin, folic acid, nicotinamide, pyridoxine hydrochloride and riboflavin were purchased from Supelco (Merck), and L-ascorbic acid, nicotinic acid, pyridoxal hydrochloride and thiamine hydrochloride from Sigma-Aldrich (Merck).

2.2.2. Fat-soluble vitamins

Isopropanol, n-hexane and 1,4-dioxane were purchased from Merck. High purity (> 90%) commercial standards of α-, β-, γ-, and δ-tocopherol, α-tocopherol acetate, α-, β-, γ-, and δ-tocotrienol, retinol, retinyl acetate, retinyl propionate and retinyl palmitate were purchased from Sigma-Aldrich (Merck).

2.2.3. Total polyphenols

Acetonitrile was purchased from Romil (Teknokroma). Carrez I and II solutions were prepared according to manufacturer instructions. Carrez I solution was prepared with potassium hexacyanoferrate (II) trihydrate in distilled water (0.15 g/mL). Carrez II solution was prepared with zinc sulfate heptahydrate in distilled water (0.30 g/mL). All the chemicals used for Carrez I and II solutions were purchased from Merck.

2.2.4. Antioxidant capacity

Potassium and sodium chloride were purchased from Panreac and potassium phosphate monobasic from Scharlab. Trolox® reagent was purchased from ACROS Organics. The phosphate buffer solution (PBS, pH 7.4, 5 mM) was prepared with 4 g sodium chloride, 100 mg potassium chloride, 720 mg disodium phosphate and 122.5 mg potassium

phosphate monobasic in 1 L ultrapure water. The pH was adjusted with a 1 M sodium hydroxide or hydrochloric acid solution. The 2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) stock solution consisted of a solution of 7 mM ABTS and 2.45 mM potassium persulfate (final concentration) in ultrapure water. The solution was oxidized in agitation, dark conditions and room temperature for at least 12-16 h. $ABTS^{\bullet+}$ working solution was daily prepared by diluting the $ABTS^{\bullet+}$ stock solution in PBS until an absorbance of 0.70 ± 0.02 at 734 nm was reached. All the chemicals used for $ABTS^{\bullet+}$ stock solution were purchased from Sigma-Aldrich (Merck).

2.3. Water-soluble vitamin analysis

Water-soluble vitamins were analysed following the method by Zafra-Gómez et al.[24] with minor modifications. Briefly, mare milk was thawed in a water bath at 25 ºC for 60 min. Around 5 (± 0.0001) g of liquid mare milk were mixed with 750 µL of the precipitation solution, and clarification was allowed for 15 min. Clarified samples were centrifuged for 5 min at 3,500 *g* and 20 ºC using a ST 16R centrifuge (Sorvall, Thermo Fisher Scientific), and supernatants were filtered through 0.2 µm pore nylon filters (OlimPeak, Teknokroma). Filtered samples were transferred to 2 mL amber vials and kept at -80 ºC until analysis. All the procedure was performed under dark conditions.

Water-soluble vitamins were separated using a high-performance liquid chromatograph (model 2695, Waters, Barcelona, Spain) coupled to fluorescence (model 2475 FLR, Waters) and diode array (model 2998 PDA, Waters) tandem detectors. For molecular separation, a C18 Spherisorb ODS-2 column was used (25 cm long, 4.6 mm i.d., 3 µm particle size; Waters). Analytical conditions were as follows: 40 ºC column temperature, 1 mL/min flow rate, 50 µL injection volume, and gradient conditions of the mobile phase (phosphate buffer/methanol) as previously described by Zafra-Gómez et al.[24]. Fluorescence detector was set at 290/410 nm (excitation/emission) wavelength for pyridoxine and pyridoxal, and 400/520 nm for riboflavin. Diode array detector was set at 245 nm for thiamine, 261 nm for nicotinic acid and niacinamide, 195 nm for pantothenic acid, 282 nm for folic and ascorbic acids and 370 nm for cyanocobalamin.

Water-soluble vitamins were identified by comparing their retention times with those of high purity commercial standards. Quantification was done by external calibration using between four and six concentration levels depending on each vitamin. Ranges were as follows: 0.9-90 µg/mL for ascorbic acid; 0.005-0.5 µg/mL for cyanocobalamin; 0.05-5

µg/mL for folic acid; 0.02-2.5 µg/mL for nicotinic acid; 0.02-10 µg/mL for niacinamide; 0.2-6 µg/mL for pantothenic acid; 0.015-1.5 µg/mL for pyridoxal; 0.001-0.3 µg/mL for pyridoxine; 0.001-0.03 µg/mL for riboflavin; and 0.02-10 µg/mL for thiamine. Determination coefficient values (R^2) were higher than 0.999 except for riboflavin (0.957). Relative standard deviation (*RSD*) for six replicates of the standard solutions at a concentration range of 4.5-9.0 µg/mL for ascorbic acid and of 0.1-0.6 µg/mL for the rest of vitamins was lower than 6.8 %. Accuracy was evaluated in terms of recovery by analysing three replicates of standard solutions, and the recovery values ranged between 80.4 and 104.0 % for all water-soluble vitamins except for pantothenic acid (73.4 %) and cyanocobalamin (123.8 %). The limit of quantification (LOQ) was determined experimentally for each vitamin. It was set as the lowest concentration at which the standard followed a linear correlation with the higher concentrations used for the calibration, and could be detected with an *RSD* lower than 12 % between triplicates. LOQs were 90 µg/100 g milk for ascorbic acid, 0.48 µg/100 g for cyanocobalamin, 5.0 µg/100 g for folic acid, 2.5 µg/100 g for nicotinic acid, 2.0 µg/100 g for niacinamide, 23 µg/100 g for pantothenic acid, 0.28 µg/100 g for pyridoxal, 0.13 µg/100 g for pyridoxine, 0.076 µg/100 g for riboflavin, and 1.9 µg/100 g for thiamine. The concentrations of water-soluble vitamins in mare milk samples were expressed as µg/100 g milk.

2.4. Fat-soluble vitamin analysis

Mare milk was thawed in a water bath at 25 ºC for 60 min. Tocols (tocopherols and tocotrienols) and retinoids (retinol and retinyl esters) were simultaneously extracted from 2 (± 0.0001) g of liquid milk using two liquid–liquid extraction steps without saponification following the procedure described by Valdivielso, et al.[25]. Tocols and retinoids were simultaneously analysed using a high-performance liquid chromatograph (model 1260, Agilent Technologies, Madrid, Spain) coupled to a fluorescence detector (1260 FLD Spectra, Agilent Technologies). Separation was done at 22 °C on a Luna Silica column (10 cm long, 3.0 mm i.d., 3 µm particle size) (Phenomenex, Madrid, Spain) protected by a guard column and using a non-linear gradient elution of 1,4-dioxane/n-hexane from 3/97 up to 25/75 (v/v) for 15 min as mobile phase. Then, the mobile phase was returned to initial conditions and the column was re-equilibrated for 3 min. Flow-rate was 1 mL/min and injection volume was 20 µL. Fluorescence detector operated at 298 nm for excitation wavelength, and 328 nm and 475 nm for emission wavelength, respectively for tocols and retinoids. The compounds were identified by comparing the

retention time of sample chromatographic peaks with that of high purity commercial standards. Quantification of tocols and retinoids in milk samples was done by external calibration using standard solutions in n-hexane/isopropanol 99/1 (v/v) analysed in triplicate in four concentration levels from 0.01 to 0.5 µg/mL. R^2 values of the linear calibrations were higher than 0.997, except for α-tocopherol acetate (0.996). *RSDs* for five replicates of the standard solutions at the low and high concentrations were lower than 6.9 %. In the same way, accuracy was evaluated in terms of recovery by analysing six replicates of standard solutions at these concentrations, and the recovery values ranged between 88.1 and 110.9 % for all tocols and retinoids studied. LOQs were determined by analysing ten replicates of the lowest concentration of the standard solution that obtained an *RSD* value lower than 20 %. This lowest concentration was established for all the tocols and retinoids in the milk samples as 0.750 µg/100 g milk. The concentrations of tocols and retinoids in mare milk samples were expressed as µg/100 g milk.

2.5. Total polyphenol analysis

Total phenolic content of mare milk was measured using the Folin-Ciocalteu method adapted for milk by Vázquez et al.[26]. In brief, milk samples were thawed in a water bath at 37 ºC for 45 min under dark conditions. Then, milk was homogenised through agitation, 8 (± 0.0001) g were weighed and 10 mL methanol/distilled water (1/1, v/v), 500 µL Carrez I solution, 500 µL Carrez II solution and 5 mL acetonitrile were added for clarification. The solution was adjusted to 25 mL using methanol/distilled water (1/1, v/v) and was let for 25 min in darkness until complete clot precipitation. Phases were separated by centrifugation for 15 min at 4,700 *g* and 20 ºC using a ST 16R centrifuge (Sorvall, Thermo Fisher Scientific). The supernatant (polyphenol extract) was collected. In dim light and at room temperature, 60 µL of extract, standard solution or ultrapure water (blank), 250 µL of ultrapure water and 65 µL of Folin-Ciocalteu reagent (2 N) were mixed in 2 mL microcentrifuge tubes. Then, 625 µL of sodium carbonate solution (7 % in water, w/v) and 500 µL of ultrapure water were added. Solutions were allowed to react for 90 min in dark conditions and at room temperature. Absorbance was read at 750 nm in a Cary 50 Bio UV-VIS spectrophotometer (Varian, Agilent Technologies).

Total polyphenol concentrations in mare milk were performed using external calibration. A calibration curve was built with gallic acid standard solutions in methanol/water (1/1, v/v/) at five concentration levels from 1.25 to 250 µg/mL. Mean R^2 values of the linear

calibrations were 0.9995. Blanks were done with ultrapure water instead of standard or extract (no differences were observed when using methanol/water (1/1, v/v) as blank; data not shown). *RDS* for five replicates of polyphenol extraction and analysis was 2.82 %. The concentrations of polyphenols in mare milk samples were expressed as mg of gallic acid equivalents (GAE)/100 g milk.

2.6. Determination of total antioxidant capacity

The total antioxidant capacity of mare milk was determined according to the procedure of Gila-Díaz et al.[27], with minor modifications. Mare milk samples were thawed in a water bath at 25 ºC for 60 min and diluted 1/20 (v/v) in PBS (75 µL milk and 1.425 mL PBS). For spectrophotometric analysis, 150 µL of diluted sample, standard or PBS (control) were mixed with 1,350 µL $ABTS^{\bullet+}$ working solution. For turbidity corrections, 150 µL of each sample were diluted in 1,350 µL of PBS. Reaction was allowed for 10 min at 25 ºC under dark conditions. Absorbance was read at 734 nm in a Cary 50 Bio UV-VIS spectrophotometer (Varian, Agilent Technologies). Absorbance of individual milk samples was corrected with the corresponding turbidity absorbance value.

To measure the contribution of caseins to whole mare milk antioxidant capacity, the whey fraction was isolated through isoelectric precipitation of caseins based on the method by Ochirkhuyag et al.[28], with some modifications. For this, 10 (± 0.0001) g of twenty randomly selected mare milk samples were weighted, and lactic acid (10 %, v/v) was added until pH 4.2 (isoelectric point of mare milk caseins according to Egito et al.[29]). Then, samples were centrifuged at 3,500 *g* and 22 ºC for 30 min in a ST 16 R centrifuge (Sorvall, Thermo Fisher Scientific). Supernatants were collected and casein pellets were washed with 10 mL ultrapure water at pH 4.2 (pH adjusted with hydrochloric acid 1 M). The supernatant was pooled with the first extracted whey fraction, and it was diluted 1/10 (v/v) in PBS. Analysis was performed as for whole milk.

Total antioxidant capacity was estimated as Trolox equivalents (mM) and as radical scavenging activity (percentage of $ABTS^{\bullet+}$ radical cation inhibition). Quantification (Trolox equivalents) was done with calibration curves constructed using five calibration levels of standard Trolox solutions at different concentrations from 0.01 to 0.25 mM in PBS. Mean R^2 values of the linear calibrations were 0.994. Percentage of inhibition was estimated using the following equation:

$$Inhibition\ (\%) = \left[\frac{A_{control} - A_{sample}}{A_{control}}\right] \times 100$$

where $A_{control}$ was the absorbance of PBS (with no sample or standard added), and A_{sample} was the absorbance of the sample corrected with the absorbance of turbidity. Total antioxidant capacity of mare milk samples was expressed as Trolox equivalents (mM) and as percentage of inhibition. *RDS* was 0.53 % for five replicates of whole milk analysis and 2.84 % for five replicates of whey fraction separation and subsequent analysis.

2.7. Statistical analysis

Statistical analysis was performed using the IBM-SPSS Statistics software version 28.0 (IBM, Endicott, NY, USA). Data was treated as described in Blanco-Doval et al.[23]. Briefly, all data were log transformed, and values higher than 3 times the interquartile range and values below LOQ were identified as outliers and excluded from the dataset. The data was pooled in ranges of 14 days maintaining animal individuality, resulting in 12 lactation times (earliest lactation time at weeks 3 and 4, and latest at weeks 25 and 26). Lactation time was estimated based on parturition dates of individual mares. Results from the analysis of ascorbic acid, pantothenic acid, niacinamide, pyridoxal, folic acid, pyridoxine, cyanocobalamin, riboflavin, α-tocopherol, retinyl palmitate, total polyphenols and antioxidant capacity were analysed using the linear mixed model (LMM) of analysis of variance as described in Blanco-Doval et al.[23]. For the analysis of the variance, same data (except for folic acid) were subjected to multivariate analysis using a stepwise discriminant analysis (SDA) in order to classify samples according to farm or lactation stage. In this case, lactation stage was defined as follows: early lactation, weeks 3 to 10; mid lactation, weeks 11 to 18; late lactation, weeks 19 to 26. Significance level was declared at $P \leq 0.05$. Results were expressed with three significant figures.

3. Results

3.1. Content of bioactive compounds and antioxidant capacity of mare milk

Thiamine, retinyl acetate, retinyl propionate, retinol, α-tocopherol acetate, β-, γ- and δ-tocopherol, and α-, β-, γ- and δ-tocotrienol were not found in mare milk samples or were found under the LOQ. From the 311 milk samples analysed, only 84 presented nicotinic acid contents above the LOQ. These 84 samples were randomly distributed during

lactation, among farms, and among individual animals. Therefore, the vitamin profile of mare milk was limited to ascorbic acid, pantothenic acid, niacinamide, pyridoxal and pyridoxine, folic acid, cyanocobalamin and riboflavin among water-soluble vitamins, and α-tocopherol and retinyl palmitate among fat-soluble vitamins. The content of bioactive compounds (water- and fat-soluble vitamins and total polyphenols) and the antioxidant capacity of commercial mare milk is summarized in **Table 1**. The content of water-soluble vitamins was considerably higher than that of fat-soluble vitamins. Among water-soluble vitamins, the major one was ascorbic acid, showing an average concentration of 1,184 ± 941 μg/100 g milk. This was followed by a considerable amount of pantothenic acid (366 ± 348 μg/100 g milk), whereas other water-soluble vitamins were found at much lower concentrations. Average contents of minor water-soluble vitamins *per* 100 g milk were 19.8 ± 15.6 μg for niacinamide, 14.2 ± 5.8 μg for pyridoxal, 7.57 ± 1.78 μg for folic acid, 2.25 ± 1.74 μg for pyridoxine, 1.51 ± 0.24 μg for cyanocobalamin and 1.16 ± 0.87 μg for riboflavin. Among the nine vitamin E forms studied (four tocopherols, four tocotrienols and a tocopherol ester), only one (α-tocopherol) was present in mare milk samples above the LOQ. This was, in fact, the main fat-soluble vitamin identified in the present study (10.5 ± 6.6 μg/100 g milk). Regarding vitamin A, retinol was only found as retinyl palmitate, showing an average concentration of 3.66 ± 2.34 μg/100 g milk. The non-esterified molecule (free retinol) was not quantifiable in most mare milk samples. The average content of total polyphenols in mare milk samples was 7.15 ± 1.42 mg GAE/100 g milk, and the total antioxidant capacity was on average 3.88 ± 0.41 mM of Trolox equivalents, resulting in an average Trolox inhibition of 82.1 ± 7.3 %. After casein precipitation at pH 4.2 (whey fraction), the capacity of milk to inhibit Trolox almost halved compared to values observed in whole milk. Thus, results showed that caseins contributed to 49.9 ± 8.0 % of the total antioxidant capacity of mare milk.

Table 1. *Average concentration of bioactive compounds in mare milk samples expressed as μg/100 g milk (vitamins), mg GAE/100 g milk (polyphenols), or mM Trolox equivalents (total antioxidant capacity), and statistical significance (P-value) of farm, lactation and interaction effects.*

	Farm				Significance (*P*-value)		
Bioactive compound	I	II	III	SEM	Farm	Lactation	Farm * Lactation
Water-soluble vitamins							
Ascorbic acid	$127 \cdot 10^{1}$	$126 \cdot 10^{1}$		$104 \cdot 10^{1}$	57	0.092	0.252	0.719
Pantothenic acid	375	302		431	21	0.056	<0.001	0.713
Niacinamide	13.2^{b}	24.5^{a}		18.8^{a}	1.0	0.012	<0.001	0.477
Pyridoxal	13.6	15.1		13.7	0.3	0.098	<0.001	0.031
Folic acid	8.04	7.66		7.04	0.13	0.061	<0.001	0.700
Pyridoxine	2.61	2.19		2.06	0.10	0.475	0.098	0.161
Cyanocobalamin	1.51	1.53		1.49	0.01	0.479	0.010	0.106
Riboflavin	0.786	1.35		1.16	0.054	0.273	0.004	0.112
Fat-soluble vitamins							
α-tocopherol	10.4	10.8		10.2	0.4	0.542	<0.001	0.076
Retinyl palmitate	3.64	3.67		3.66	0.14	0.896	<0.001	0.927
Total polyphenols	7.61	7.08		6.90	0.09	0.519	<0.001	0.159
Total antioxidant capacity	3.95	3.84		3.87	0.03	0.889	<0.001	0.179

[a,b] *Different superscripts refer to significant differences ($P \leq 0.05$) among farms. GAE, gallic acid equivalents; SEM, standard error of the mean.*

3.2. Effect of management system and lactation stage

The effect of lactation stage and management system (farm) is given in **Table 1**. Farm effect was only significant ($P \leq 0.05$) for niacinamide contents, which were higher in milk from farms with a high grazing activity (farm II and III) compared to low grazing activity (farm I). The lack of significant effect among milks from different farms in most compounds analysed is more than likely due to the large variability observed among milk samples within each farm (**Table 1**), evidencing a strong individual animal effect on mare milk bioactive compounds. On the other hand, lactation stage significantly ($P \leq 0.05$) affected the content of most water- and fat-soluble vitamins analysed, except for ascorbic acid and pyridoxine. The effect of lactation stage was also significant ($P \leq 0.05$) for total polyphenols and the total antioxidant capacity of milk (**Table 1**). **Figures 1-3** show the changes during lactation in the content of individual water- and fat-soluble vitamins and

total polyphenols, as well as in the values of the total antioxidant capacity of mare milk samples. In terms of vitamins, the content of pyridoxal, niacinamide, pantothenic acid, retinyl palmitate and α-tocopherol decreased from initial (weeks 3-4) to final (weeks 25-26) stages of lactation. For niacinamide and retinyl palmitate the decrease was more evident after weeks 13-16 of lactation, whereas pantothenic acid contents mainly decreased from initial stages to weeks 15-16 with moderate changes afterwards (**Figures 1 and 2**). On the other hand, riboflavin and cyanocobalamin contents remained quite constant but with fluctuations during lactation. Folic acid contents remained stable from initial stages of lactation until weeks 13-14. Then, these decreased to values under the LOQ in most milk samples independently of farm and lactation time (**Figure 1**). Regarding total polyphenols, these peaked at mid lactation (weeks 11-18), but similar values were found at early (weeks 3-4) and late (weeks 25-26) lactation stages (**Figure 3**). Interestingly, the opposite pattern was observed in the total antioxidant capacity of mare milk, which was lowest at mid lactation (weeks 15-18). The interaction effect between farm and lactation stage was only significant for pyridoxal contents (**Table 1**). In this respect, milk from the low grazing activity farm (farm I) followed a different pattern during lactation compared to milk from high intensity grazing mares (farms II and III) (**Figure 1**).

The multivariate analysis confirmed the effect of lactation stage on the content of mare milk bioactive compounds, whereas milk samples were not effectively discriminated according to farm. Canonical distribution of mare milk samples in the first two canonical functions obtained according to management system (farm I, II and III) or lactation stage (early, mid and late lactation) are depicted in **Figure 4**. Regarding the effect of lactation stage, 79.5 and 84.1 % of the samples from early and late lactation, respectively, were correctly classified into their corresponding groups. However, milk samples from mid lactation were mixed with the other two groups. After cross-validation, 74.0 % of classification was obtained. Function 1 axis was the function that discriminated mainly between samples from early and late lactation, and was mainly correlated with pyridoxal, retinyl palmitate and α-tocopherol contents in mare milk samples. This is in agreement with a clear decreasing trend in the content of these three vitamins during lactation (**Figures 1-2**). On the other hand, function 2 axis was responsible for discriminating mid lactation from early and late lactation samples, and was mainly correlated with the total antioxidant capacity and total polyphenol content. These results support the evolution

trends depicted in **Figure 3**, which show maximum and minimum values at mid lactation respectively for total polyphenols and total antioxidant capacity.

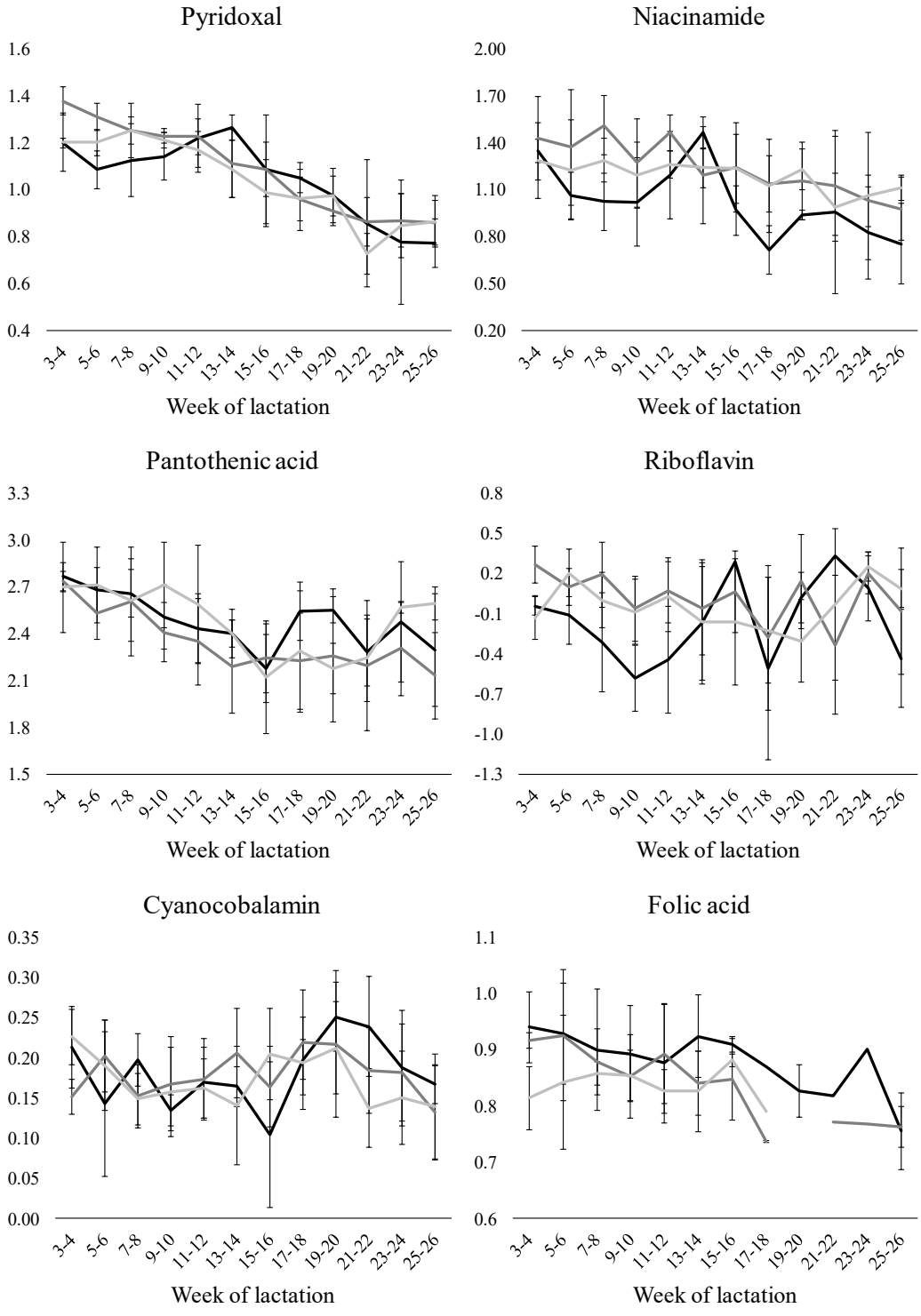


Figure 1. *Changes during lactation in the content of water-soluble vitamins (expressed as log (μg/100 g milk)) in mare milk samples belonging to three commercial farms: ▬ , farm I; ▬ , farm II; ▬ , farm III.*

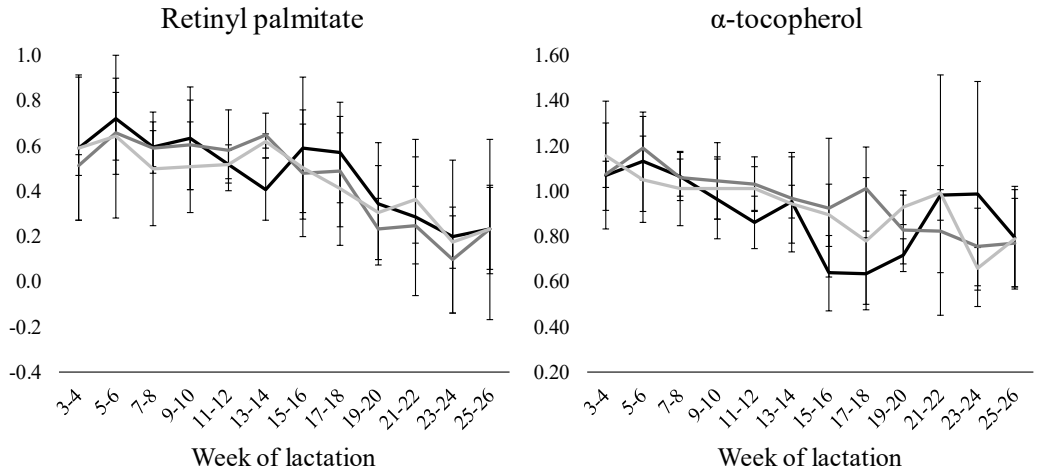


Figure 2. *Changes during lactation in the content of fat-soluble vitamins (expressed as log (μg/100 g milk)) in mare milk samples belonging to three commercial farms: ▬ , farm I; ▬ , farm II; ▬ , farm III.*

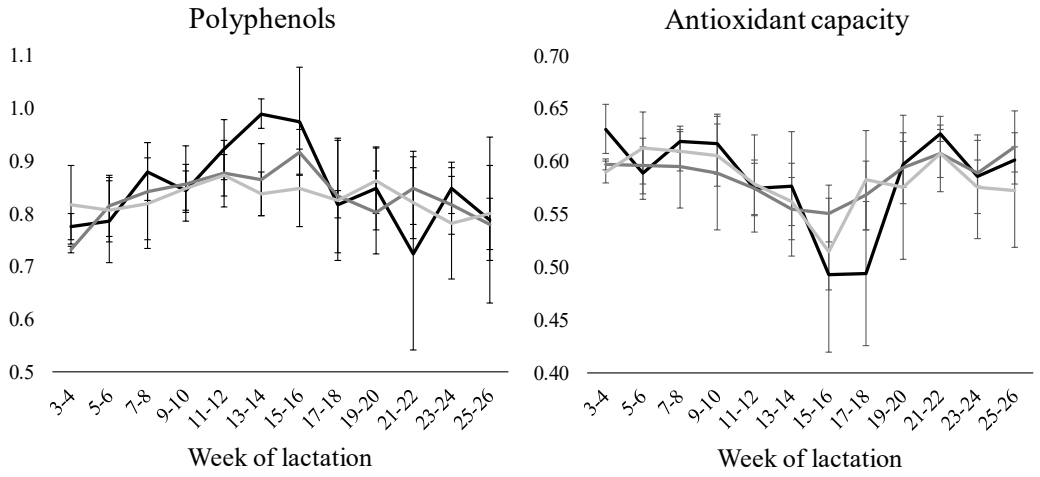


Figure 3. *Changes during lactation in polyphenol content (expressed as log (mg gallic acid equivalents/100 g milk)) and antioxidant capacity (expressed as log (mM trolox equivalents)) in mare milk samples belonging to three commercial farms: ▬ , farm I; ▬ , farm II; ▬ , farm III.*

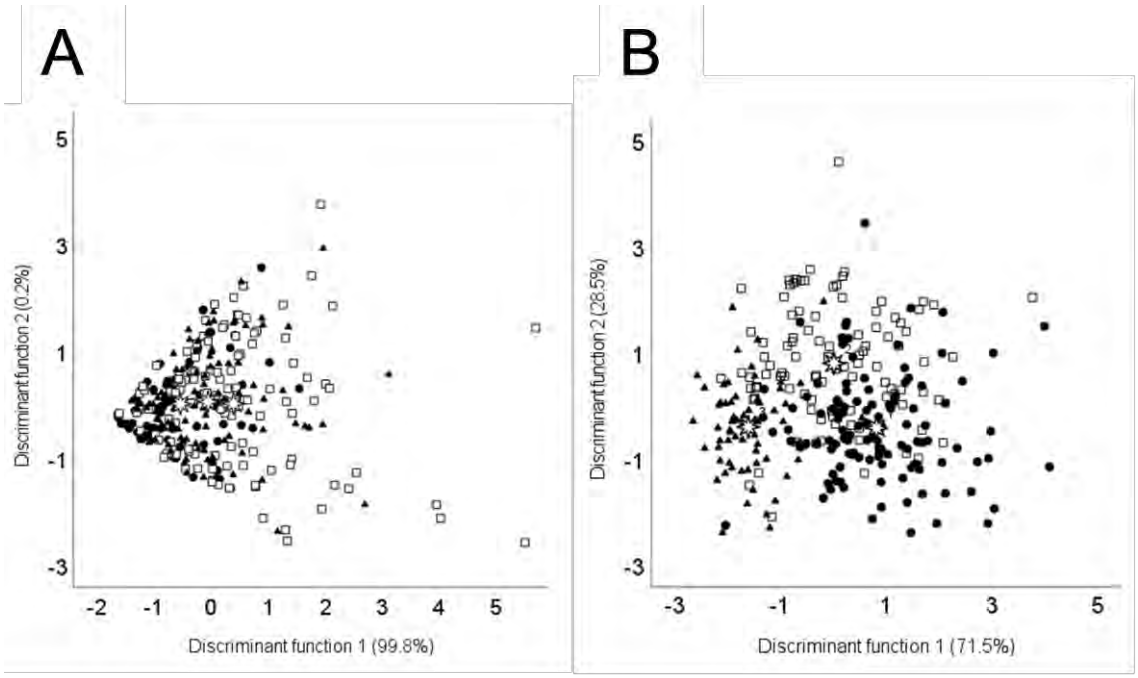


Figure 4. *Graphical representation of the two first canonical discriminant functions for stepwise discriminant analysis on mare milk bioactive compounds and antioxidant capacity based on A) management system (●, farm I; □, farm II; ▲, farm III); and B) lactation stage (●, early lactation; □, mid lactation; ▲, late lactation).* ✡*, group centroid.*

4. Discussion

4.1. Fat- and water-soluble vitamins

In the present study, the content (in absolute basis) of water-soluble vitamins in mare milk was considerably higher than that of fat-soluble vitamins. Mare milk is very poor in fat compared to milk from ruminant species[30]. Particularly, milk from the Basque Mountain Horse breed contains up to 0.306 g fat/100 g milk (data not reported), limiting the accumulation of fat-soluble vitamins.

According to literature, among fat-soluble vitamins, vitamins A and E are the major ones, and vitamins D and K contents fall below[30]. The present study corroborates that, in mare milk, vitamin E is more abundant than vitamin A as also evidenced by others[9,15,16]. Csapó et al.[15] reported higher values of fat-soluble vitamins in mature milk than those found in the present study, which could be due to a higher total fat content in milk (1.25 g/100 g milk) or milk being from early lactation stages (up to six weeks of lactation), since a decrease of the content of fat-soluble vitamins during lactation was demonstrated in the

present study. However, high variability regarding fat-soluble vitamin content in mare milk has been observed in the literature[9,16,31,32], which could be a consequence of different experimental and analytical conditions among studies.

Among the eight isomers that conform vitamin E (α-, β-, γ- and δ-tocopherol and α-, β-, γ- and δ-tocotrienol), mare milk in the present study mainly contained α-tocopherol, whereas other isoforms were not detected. This was also observed by Marconi and Panfili[16]. In the case of retinoids, only retinyl palmitate was present in mare milk, whereas free retinol or other esterified forms were almost negligible. According to Stowe[33], the main retinoid forms in mare milk are ester forms. Vitamin A or retinol is a product of the hydrolysis of its precursor β-carotene. Plants and plant food products are a good source of β-carotene[34], and horses, as well as cattle, are quite efficient at absorbing dietary carotenoids, unlike goat and sheep[35]. When animals consume plants, an enzyme present at the intestinal mucosa, named β-carotene 15,15'-monooxygenase, converts the precursor molecule into all-*trans*-retinal (precursor of the active form retinol), and then, retinol is esterified in the mammary gland[34,36]. Therefore, retinoids are mainly transferred into milk in an esterified form.

In contrast to results by Navrátilová et al.[9] that exposed a decreasing but not significant trend of fat-soluble vitamins during mare lactation, we found a significant ($P \leq 0.05$) decrease, which is in accordance with a significant reduction of the total fat content in mare milk[37]. Stowe[33] suggested that high vitamin A concentrations in mare colostrum could respond to the needs of the neonate. This might be extrapolated to the complete lactation period as a response to a decreasing reliance of the foal on milk as it consumes more and more feedstuff. On the other hand, higher grazing activities did not result in different fat-soluble vitamin contents in the present study, probably because all the three diets contained some kind of forages (fresh pasture, hay or silage) that are a good source of vitamins or vitamin precursors[34,38]. However, a high intra-farm variability was observed, reflecting how the individual animal strongly influences the abundance of fat-soluble vitamins in mare milk.

Regarding water-soluble vitamins, these are generally less abundant in mare milk than in ruminant milk[30]. In ruminants, there is a relevant synthesis of water-soluble vitamins in the rumen[39] and, therefore, equids might have the synthesis-assimilation of water-soluble vitamins limited. Moreover, each vitamin has a particular metabolism[34,39] that leads to

different transfer rates into milk, also affected by different responses to external factors (such as diet and lactation stage).

Overall, lower riboflavin contents in mare milk were found in the present study compared to previous reports[9,10,11,14]. Differences between studies could come from variability among analytical methods (some of the methods used for vitamin quantification were indirect and low-accurate), or from milk characteristics. However, the very scarce literature on mare milk water-soluble vitamins makes comparison difficult. This is the case of pantothenic acid, pyridoxine, folic acid and cobalamin, which have been reported only in one or two studies that used enzymatic, microbiologic or spectrophotometric assays (except for pyridoxine that was determined by liquid chromatography[9,11,12,14]). In the case of ascorbic acid, most reports on mare milk are similar or slightly higher than those reported in this study[10,14,15]. Thiamine was not found in mare milk samples in the present study. Thiamine in ruminant milk mainly comes from ruminal synthesis[34], but in monogastrics it is synthesized by the microbiota in the large intestine while being absorbed in the small intestine[40]. Therefore, lower absorption rates and transfer to milk are expected in monogastrics, as confirmed by the present study. Niacin or vitamin B_3 is made up by two vitamers: nicotinic acid and nicotinamide (the amide form of nicotinic acid[40]). In mare milk, only few studies determined niacin. These studies analysed nicotinic acid only, with no mention to nicotinamide, and used microbiological methods for quantification[10,11]. Both nicotinic acid and niacinamide are absorbed from diet, although niacinamide can also be produced by mucosal enzymes and nicotinic acid from deamination of niacinamide by gut microbiota[41]. In this regard, the present study demonstrated that the main vitamin B_3 form in mare milk was niacinamide, as also found in cow milk[34]. Similarly, pyridoxal contents were on average more than five times higher than pyridoxine contents, which is in agreement with pyridoxal being the main vitamin B_6 form in ruminant milk[34].

The present study demonstrated a significant impact of lactation stage on most mare milk water-soluble vitamins. The decreasing trend of mare milk vitamins follows the pattern previously described in some macrominerals of the same milk samples[23]. As confirmed by a multivariate statistical analysis, most evident changes were observed between early and late lactation stages and mainly due to variations in pyridoxal contents. There was a significant interaction between farm and lactation stage for pyridoxal, which in fact peaked at some point during mid lactation only in samples from low grazing intensity

(farm I), whereas it continuously descended in those from high grazing intensity (farms II and III). Therefore, grazing activity could modify the content of vitamin B_6 in mare milk during lactation. Similarly, Navrátilová et al.[9] observed that pyridoxine contents in milk from warmblood mares without grazing peaked at mid lactation and decreased to minimum values after six months of lactation. However, only niacinamide was significantly affected by management of the mares, being milk samples from high grazing activity richer in this vitamin. Niacin can be synthesized from L-tryptophan, and therefore, not only the niacin content but also the protein content and profile of the feeding administered to animals could affect niacin presence in milk[40].

4.2. Total polyphenols and antioxidant capacity

Some studies in ruminant milk observed an increase in the total phenolic content when animals were reared under pasture-based systems[42,43] due to the high polyphenol content and diversity of pasture grasses and legumes[44]. However, no differences in the phenolic compound content were observed between low and high grazing farms in the present study. This was probably because the addition of forages, fruits and potatoes into the low grazing diet balanced the intake of dietary polyphenols among farms. For instance, fruits are a great source of phenolic compounds[20,21], and changes in the phenolic content of milk during lactation could be attributed to seasonal variations of the phenolic profile of pastures or forage species[44].

On the other hand, the total antioxidant capacity of mare milk samples (inhibition of 82 %) was similar to that observed by Cosentino et al.[45] in milk from Murgese mares (86-90 %). Caseins are important contributors to milk antioxidant capacity[46]. Specifically, the present study showed that after casein precipitation, the antioxidant capacity of milk halved. Therefore, the decrease of the total antioxidant capacity from early to mid lactation could be linked to a decrease in total protein content in mare milk[47]. However, milk is a complex matrix, and a number of antioxidant compounds can also be present in the whey fraction of milk. Vitamins E and C are well known antioxidants[48,49], but other compounds such as vitamin A[49], vitamin D_3, coenzyme Q_{10}, some whey proteins[46] and polyphenols[20], among others, also contribute to the antioxidant activity. Dynamics of individual antioxidant compounds during lactation are probably responsible for changes in the antioxidant capacity of milk, particularly after mid lactation when it increases. In this case, polyphenols in mare milk are probably poor contributors to total antioxidant

capacity, since they changed differently to total antioxidant capacity during lactation. Among the studied parameters, total polyphenols and total antioxidant capacity were the ones that mainly changed during mid lactation.

Regarding the effect of management system, milk from the three commercial farms presented a similar total antioxidant capacity among milk samples from different farms. These results differ with studies in ruminant milk that observed an improved antioxidant capacity of milk when animals grazed[42,43]. In the present study, a low grazing activity in farm I could have been compensated, in terms of antioxidant capacity, with the inclusion of ingredients rich in direct or indirect antioxidant compounds, for example, fruits and potatoes that are rich in vitamins A, E and C, minerals and phenolic compounds[20,21,50,51].

5. Conclusion

To the best of our knowledge, the present study provides the most in-depth characterization of mare milk bioactive compounds and antioxidant capacity to date, and elucidates changes during lactation and among different management systems. The content of some of the vitamins studied was clearly different between early and late lactation, whereas total polyphenols and antioxidant capacity of mare milk were different mainly during mid lactation. Conversely, the management system had a low impact on mare milk bioactive compounds. In addition, the present study demonstrates that the antioxidant capacity of mare milk is greatly influenced by milk caseins, whereas polyphenols are poor contributors.

This study updates and deepens into understanding mare milk composition with a special focus on compounds that exhibit biological functions, and establishes a basis for future research on the value of mare milk as a functional food.

Conflict of interest

None.

Acknowledgements

The Department of Education of the Basque Government for the predoctoral fellowship granted to A. Blanco-Doval. The authors thank the technical and human support provided by SGIker UPV/EHU (Vitoria-Gasteiz, Spain) and the commercial farms that participated in the study. This work was supported by the Basque Government through Biotasma (Elkartek, 2019), Behor Esne (Cooperation, 2020) and IT-944-16 and IT1568-22 projects.

References

1. Sheng Q and Fang X, Bioactive components in mare milk, in Bioactive components in milk and dairy products, ed. by Park YW. Wiley-Blackwell, Iowa, USA, pp. 195-213 (2009).

2. Salimei E and Fantuz F, Equid milk for human consumption. Int Dairy J 24:130-142 (2012).

3. Pieszka M, Łuszczyński J, Zamachowska M, Augustyn R, Długosz B, Hędrzak M, Is mare milk an appropriate food for people? – A review. Ann Anim Sci 16:33-51 (2016).

4. Devle H, Vetti I, Naess-Andresen CF, Rukke E, Vegarud G, Ekeberg D, A comparative study of fatty acid profiles in ruminant and non-ruminant milk. Eur J Lipid Sci Technol 114:1036-1043 (2012).

5. Hsu Y, Jhang W, Lee M, Bat-Otgon B, Narantungalag E, Huang C, Lactose-riched Mongolian mare's milk improves physical fatigue and exercise performance in mice. I J Med Sci 18:564 (2021).

6. Shariatikia M, Behbahani M, Mohabatkar H, Anticancer activity of cow, sheep, goat, mare, donkey and camel milks and their caseins and whey proteins and *in silico* comparison of the caseins. Mol Biol Res Commun 6:57 (2017).

7. Song JJ, Wang Q, Du M, Ji XM, Mao XY, Identification of dipeptidyl peptidase-IV inhibitory peptides from mare whey protein hydrolysates. J Dairy S 100:6885-6894 (2017).

8. Zava S, Barello C, Pessione A, Garoffo LP, Fattori P, Montorfano G, Conti A, Giunta C, Pessione E, Berra B, Giuffrida MG, Mare's colostrum globules stimulate fibroblast growth in vitro: A biochemical study. J Med Food 12:836-845 (2009).

9. Navrátilová P, Borkovcová I, Kaniová L, Dluhošová S, Zachovalová H, The content of selected vitamins and iodine in mare's milk. Acta Vet Brno 88:473-480 (2019).

10. Holmes AD, McKey BV, Wertz AW, Lindquist HG, Parkinson LR, The vitamin content of mare's milk. J Dairy Sci 29:163-171 (1946).

11. Pearson PB, The thiamine, riboflavin, nicotinic acid and pantothenic acid contents of mare's colostrum and milk and ascorbic acid content of the milk. J Dairy Sci 30:73-77 (1947).

12. Collins RA, Harper AE, Schreiber M, Elvehjem CA, The folic acid and vitamin B12 content of the milk of various species. J Nutr 43:313-321 (1951).

13. Markiewicz-Kęszycka M, Czyżak-Runowska G, Wójtowski J, Jóźwik A, Pankiewicz R, Łęska B, Krzyżewski J, Strzałkowska N, Marchewka J, Bagnicka E, Influence of stage of lactation and year season on composition of mares' colostrum and milk and method and time of storage on vitamin C content in mares' milk. J Sci Food Agric 95:2279-2286 (2015).

14. Teichert J, Cais-Sokolińska D, Bielska P, Danków R, Chudy S, Kaczyński ŁK, Biegalski J, Milk fermentation affects amino acid and fatty acid profile of mare milk from Polish coldblood mares. Int Dairy J 121:105-137 (2021).

15. Csapó J, Stefler J, Martin TG, Makray S, Csapó-Kiss Z, Composition of mares' colostrum and milk. Fat content, fatty acid composition and vitamin content. Int Dairy J 5:393-402 (1995).

16. Marconi E and Panfili G, Chemical composition and nutritional properties of commercial products of mare milk powder. J Food Compos Anal 11:178-187 (1998).

17. Bondo T and Jensen SK, Administration of RRR-α-tocopherol to pregnant mares stimulates maternal IgG and IgM production in colostrum and enhances vitamin E and IgM status in foals. J Anim Physiol Anim Nutr 95:214-222 (2011).

18. Álvarez R, Meléndez-Martínez AJ, Vicario IM, Alcalde MJ, Carotenoids and fat-soluble vitamins in horse tissues: A comparison with cattle. Animal 9:1230-1238 (2015).

19. Danyer E and Bilal T, Effects of dietary fish oil and alpha-tocopherol supplementation on selected blood parameters and fatty acid profiles in mares and their foals. J Anim Physiol Anim Nutr 105:3-17 (2020).

20. Lang Y, Gao N, Zang Z, Meng X, Lin Y, Yang S, Yang Y, Jin Z, Li B, Classification and antioxidant assays of polyphenols: A review. J Future Foods 4:193-204 (2024).

21. Andrés-Lacueva C, Medina-Remon A, Llorach R, Urpi-Sarda M, Khan N, Chiva-Blanch G, Zamora-Ros R, Rotches-Ribalta M, Lamuela-Raventós RM, Phenolic compounds: Chemistry and occurrence in fruits and vegetables, in Fruit and vegetable phytochemicals: Chemistry, nutritional value, and stability, ed. by de la Rosa LA, Alvarez-Parrila E, González-Aguilar GA. Blackwell Publishing, Iowa, USA, pp. 53-88 (2009).

22. Sik B, Buzás H, Kapcsándi V, Lakatos E, Daróczi F, Székelyhidi R, Antioxidant and polyphenol content of different milk and dairy products. J King Saud Univ Sci 35:102839 (2023).

23. Blanco-Doval A, Barron LJR, Aldai N, Changes during lactation in the mineral element content of mare milk produced in semi-extensive rural farms. J Food Compos Anal 123:105629 (2023).

24. Zafra-Gómez A, Garballo A, Morales JC, García-Ayuso LE, Simultaneous determination of eight water-soluble vitamins in supplemented foods by liquid chromatography. J Agric Food Chem 54:4531-4536 2006.

25. Valdivielso I, Bustamante M, Buccioni, A, Franci O, de Gordoa, JCR, de Renobales M, Barron LJR, Commercial sheep flocks–fatty acid and fat-soluble antioxidant composition of milk and cheese related to changes in feeding management throughout lactation. J Dairy Res 82:334-343 (2015).

26. Vázquez CV, Rojas MGV, Ramírez CA, Chávez-Servín JL, García-Gasca T, Martínez RAF, García OP, Rosado JL, López-Sabater CM, Castellote AI, Montemayor HMA, Carbot KT, Total phenolic compounds in milk from different

species. Design of an extraction technique for quantification using the Folin-Ciocalteu method. Food Chem 176:480-486 (2015).

27. Gila-Díaz A, Herranz Carrillo G, Cañas S, Saenz de Pipaón M, Martínez-Orgado JA, Rodríguez-Rodríguez P, López de Pablo AL, Martin-Cabrejas MA, Ramiro-Cortijo D, Arribas SM, Influence of maternal age and gestational age on breast milk antioxidants during the first month of lactation. Nutrients 12:2569 (2020).

28. Ochirkhuyag B, Chobert J, Dalgalarrondo M, Haertlé T, Characterization of mare caseins. Identification of α_{S1}- and α_{S2}-caseins. Lait 80:223-235 (2000).

29. Egito AS, Girardet J, Miclo L, Mollé D, Humbert G, Gaillard J, Susceptibility of equine κ-and β-caseins to hydrolysis by chymosin. Int Dairy J 11:885-893 (2001).

30. Claeys WL, Verraes C, Cardoen S, De Block J, Huyghebaert A, Raes K, Dewettinck K, Herman L, Consumption of raw or heated milk from different species: An evaluation of the nutritional and potential health benefits. Food Control 42:188-201 (2014).

31. Schweigert FJ and Gottwald C, Effect of parturition on levels of vitamins A and E and of β-carotene in plasma and milk of mares. Equine Vet J 31:319-323 (1999).

32. Markiewicz-Kęszycka M, Wójtowski J, Czyżak-Runowska G, Kuczyńska B, Puppel K, Krzyżewski J, Strzałkowska N, Jóźwik A, Bagnicka E, Concentration of selected fatty acids, fat-soluble vitamins and β-carotene in late lactation mares' milk. Int Dairy J 38:31-36 (2014).

33. Stowe HD, Vitamin A profiles of equine serum and milk. J Anim Sci 54:76-81 (1982).

34. Fox PF, Uniacke-Lowe T, McSweeney PLH, O'Mahony JA, Vitamins in milk and dairy products, in: Dairy chemistry and biochemistry, ed. by Fox PF, Uniacke-Lowe T, McSweene, PLH and O'Mahony JA. Springer, Berlin, Germany, pp. 271-297 (2015).

35. Schweigert FJ, Metabolism of carotenoids in mammals, in Carotenoids, ed. by Britton G, Liaaen-Jensen S, Fander HP. Birkhäuser, Berlin, Germany, pp. 249-284 (1998).

36. Cabezuelo MT, Zaragozá , Barber, T, Viña JR, Role of vitamin A in mammary gland development and lactation. Nutrients 12:80 (2020).

37. Pikul J and Wójtowski J, Fat and cholesterol content and fatty acid composition of mares' colostrums and milk during five lactation months. Livest Sci 113:285-290 (2008).

38. Valdivielso I, Bustamante MA, Aldezabal A, Amores G, Virto M, de Gordoa JCR, de Renobales M, Barron LJR, Case study of a commercial sheep flock under extensive mountain grazing: Pasture derived lipid compounds in milk and cheese. Food Chem 197:622-633 (2016).

39. Magan JB, O'Callaghan TF, Zheng J, Zhang L, Mandal R, Hennessy D, Fenelon MA, Wishart DS, Kelly AL, McCarthy NA, Effect of diet on the vitamin B profile of bovine milk-based protein ingredients. Foods 9:578 (2020).

40. Ball GF (ed). Vitamins: Their role in the human body, 1st edn. John Wiley & Sons, Oxford, UK (2004).

41. Bogan KL and Brenner C, Nicotinic acid, nicotinamide, and nicotinamide riboside: A molecular evaluation of NAD precursor vitamins in human nutrition. Annu Rev Nutr 28:115-130 (2008).

42. Cabiddu A, Delgadillo-Puga C, Decandia, M, Molle G, Extensive ruminant production systems and milk quality with emphasis on unsaturated fatty acids, volatile compounds, antioxidant protection degree and phenol content. Animals 9:771 (2019).

43. Delgadillo-Puga C, Cuchillo-Hilario M, León-Ortiz L, Ramírez-Rodríguez A, Cabiddu A, Navarro-Ocaña A, Morales-Romero AM, Medina-Campos ON, Pedraza-Chaverri J, Goats' feeding supplementation with *Acacia farnesiana* pods and their relationship with milk composition: Fatty acids, polyphenols, and antioxidant activity. Animals 9:515 (2019).

44. Amrit BK, Ponnampalam EN, Macwan S, Wu H, Aziz A, Muir S, Dunshea FR, Suleria HA, Comprehensive screening and characterization of polyphenol compounds from pasture grasses used for livestock production under temperate region. Anim Feed Sci Technol 300:115657 (2023).

45. Cosentino C, Notarnicol, G, Paolino R, D'Adamo C, Labella C, Musto M, Freschi P, Colostrum and milk characteristics in murgese breed mares. Czech J Anim Sci 62:466-472 (2017).

46. Grażyna C, Hanna C, Adam A, Magdalena BM, Natural antioxidants in milk and dairy products. Int J Dairy Technol 70:165-178 (2017).

47. Hachana Y, Nasraoui C, Frija I, Fortina R, Arabian mare's milk characterisation and clotting ability. J Food Sci Technol 59:1840-1846 (2022).

48. Traber MG and Stevens JF, Vitamins C and E: Beneficial effects from a mechanistic perspective. Free Radic Biol Med 51:1000-1013 (2011).

49. Blaner WS, Shmarakov IO, Traber MG, Vitamin A and vitamin E: Will the real antioxidant please stand up? Ann Rev. Nutr 41:105-131 (2021).

50. Hellmann H, Goyer A, Navarre DA, Antioxidants in potatoes: A functional view on one of the major food crops worldwide. Molecules 26:2446 (2021).

51. Arias A, Feijoo G, Moreira MT, Exploring the potential of antioxidants from fruits and vegetables and strategies for their recovery. Innov Food Sci Emerg Technol 77:102974 (2022).

SECTION II

CONCLUSIONS

1. In terms of gross and mineral composition, fatty acid profile and bioactive compounds, mare milk reflected a high nutritional quality, mainly due to a low fat content and a beneficial fatty acid profile that could positively affect human health. In particular, a high proportion of n-3 polyunsaturated fatty acids, and the presence of specific minor fatty acids as well as both water- and fat-soluble vitamins make mare milk a high quality food matrix.

2. The proteomic analysis of mare milk using in-solution and in-gel digestion methods complementarily allowed an accurate identification of 469 unique proteins. These proteins were principally transporter of other molecules, enzymes and structural molecules. Mare milk proteome was particularly enriched by proteins that participate in lipid metabolism and inflammation.

3. The high digestibility of mare milk proteins makes it one of the most digestible dietary protein sources. Specifically, caseins and α-lactalbumin A were degraded during gastric and intestinal digestion, whereas lysozyme C was resistant to digestion. Conversely, the two isoforms of β-lactoglobulin (I and II) performed differently during digestion, and β-lactoglobulin I was highly degraded while β-lactoglobulin II showed resistance to digestion.

4. Lactation stage significantly affected most of the individual compounds studied in mare milk, but the pattern during lactation depended on the nature of the chemical compound. The most evident differences in mare milk composition occurred at late lactation due to a decrease in the content of some mineral elements, short-chain saturated fatty acids, pyridoxal and total protein content.

5. The management system also affected mare milk chemical composition, in particular when farms with high and low grazing intensities were compared. In general, grazing improved the nutritional quality of mare milk due to an enhanced accumulation of unsaturated fatty acids, especially of n-3 polyunsaturated species, and pyridoxine.

6. Overall, these findings provide an accurate description of mare milk chemical composition, emphasizing that pasture-based management systems enhance a favourable nutrient profile that could also be optimized by selecting milk from early and mid lactation stages that are quite rich in beneficial nutrients. Considering the socio-ecological benefits of pasture-based equine production systems extensively described in the scientific literature, mare milk production from Basque Mountain Horse breed could be considered within the current horse meat production sector while contributing to the sustainable livestock farming.

SECTION III

APPENDIX III.I – Publication 1

Blanco-Doval, A., Barron, L. J. R., Aldai, N. (2024). **Nutritional quality and socio-ecological benefits of mare milk produced under grazing management**. *Foods, 13,* Article 1412.

DOI: 10.3390/foods13091412

Published in: ***Foods,*** 2024

Publisher: MDPI

ISSN: 2304-8158

Impact Factor year: 2022

Impact Factor: **5.2**

Category: **Food Science & Technology**

Position in the category: 34/142 (**Q1**)

foods

MDPI

Review

Nutritional Quality and Socio-Ecological Benefits of Mare Milk Produced under Grazing Management

Ana Blanco-Doval, Luis Javier R. Barron and Noelia Aldai *

Lactiker Research Group, Department of Pharmacy and Food Sciences, Faculty of Pharmacy, University of the Basque Country (UPV/EHU), 01006 Vitoria-Gasteiz, Spain; ana.blancod@ehu.eus (A.B.-D.); luisjavier.rbarron@ehu.eus (L.J.R.B.)

* Correspondence: noelia.aldai@ehu.eus; Tel.: +34-945-014501

Abstract: This review discusses the scientific evidence that supports the nutritional value of mare milk and how its properties are essentially achieved when mares are managed under grazing conditions. Mare milk's similarity with the chemical composition of human milk makes this food and its derived products not only suitable for human consumption but also an interesting food regarding human health. The contribution of horse breeding under grazing management to other socio-ecological benefits generated by equine farms is also highlighted. Both the high added value of mare milk and the socio-ecological benefits derived from pasture-based systems could be explored to improve the performance of equine farms located in arid and semi-arid areas or in regions with moderately harsh environmental conditions as equids have a strong adaptation capacity.

Keywords: horse; milk nutritional value; grazing management; socio-ecological benefits

check for updates

Citation: Blanco-Doval, A.; Barron, L.J.R.; Aldai, N. Nutritional Quality and Socio-Ecological Benefits of Mare Milk Produced under Grazing Management. *Foods* **2024**, *13*, 1412. https://doi.org/10.3390/foods13091412

Academic Editors: Klemen Potocnik, Ana Kaić and Elisabetta Salimei

Received: 14 March 2024
Revised: 23 April 2024
Accepted: 24 April 2024
Published: 4 May 2024

1. Introduction

The most primitive evidence of horse domestication and mare milk production is attributed to the Botai culture, from the Eurasian steppe of northern Kazakhstan, dated around 3500 years before the Christian era [1]. There is also evidence of domesticated horses from the late Shang Dynasty in China (the second century before the Christian era [2]). Currently, the production and consumption of mare milk is concentrated in Mongolia, Russian Buryatia and Kalmykia, Bashkortostan, Kazakhstan, Kyrgyzstan, Tajikistan, Uzbekistan, northern China, Tibet, and Xinjiang [2,3], where about 8% of pastoral milk production comes from mares [4]. In Mongolia, pastoral nomads traditionally consume meat during the cold season and milk products during the warm season. Among these, airag (fermented mare milk) is the most popular traditional food, but it is not evenly produced throughout Mongolia, presumably due to cultural and ethnicity factors [5].

During the last few decades, mare milk consumption has extended to Europe, mainly to Belarus, Ukraine, France, Belgium, Germany, the Netherlands, Norway, Austria, Hungary and Bulgaria [2,6]. While cow milk accounts for 83% of world milk production, only less than 0.1% corresponds to mare milk [7]. However, there are no official data about mare milk production and consumption worldwide, and it is not easy to estimate as it is assumed that most of it is self-consumed in households. Uniacke-Lowe and Fox [8] estimated a production of 1–1.3 million L of equine milk per year in Europe, whereas, according to Minjigdorj and Austbø [9], 8 million L of mare milk is annually produced in Mongolia. In the past decade, it was estimated that 30 million people consumed mare milk regularly worldwide [10]. However, real data are uncertain.

Mare milk productivity is an important matter of concern due to two main factors. First, horse breeds have generally not been selected for improved milk production (except for some Asian breeds), although any horse breed can become a dairy herd as long as mares accept being milked [2]. Consideration of the integration of mare milk production into future selection schemes for some breeds could be an option. Second, due to mare

mammary biology, milk production is low. These two factors can result in low milk yields, which could be limiting depending on the characteristics of the farm and the objectives associated with milk production.

Horses are a seasonal polyoestrous species that impregnate between May and October. With gestation periods of approximately 11 months [11], lactation occurs between spring and autumn, lasting 5 to 6 months. Milking usually starts 20 days after parturition to allow the foal to suckle the colostrum and the initial milk, as it is necessary for its adequate development and growth [10]. The aforementioned small udder capacity of mares (approximately 2.0–2.5 L in heavy horse breeds [12,13]) can be, according to Turabayev et al. [14], compensated by more efficient milk production. Approximately 75–85% of the milk produced corresponds to alveolar milk, with a very low cisternal capacity [10], and the udder can be refilled in 1.5–2 h, allowing foals to suckle every 0.75–2 h [12], but also permitting several milkings per day. Overall, mare milk production is estimated at an average of 2.5 kg per 100 kg of live weight per day in heavy breeds [10], peaking somewhere between the first and the third month of the lactation period [13]. According to Mongolian data under extensive systems, 580–640 mL of milk can be obtained in each milking [15] and, after multiple interventions, up to 1.7–5.0 L of milk per day can be obtained from light breeds, which is two to three times less compared to heavy horse breeds [14–16]. It has been described that Mongolian extensive farming systems can allow 5–8 milkings per day during summer [5,15,16]. More milkings result in lower yields at each milking but a higher total yield per day [14].

Milk ejection is a consequence of oxytocin release, which, in mares, is often insufficient to completely empty the alveoli. As a result, it is common to have some residual milk, which is rich in fat, from one milking/suckling in the next one [13,17]. Mare–foal physical contact during milking [10,17,18] and oxytocin injections can effectively increase the fat content of milk [17]. Another strategy to maintain milk productivity is to supplement a mare's diet with compound feeds when grass availability decreases since they contain protein, starch and micronutrients that can increase milk production. Daily energy and nitrogen requirements for milk production in early lactation (until 3 months) are 2.0–2.3 and 3.0–3.5 times the maintenance requirements, respectively. In late lactation (4 months and over), energy and nitrogen requirements decrease to between 1.6 and 2.4 times the maintenance requirements [10].

Another factor influencing milk yield is the milking method. According to Caroprese et al. [18], in Murgese mares, machine-milking is more effective than hand-milking in collecting residual milk, resulting in higher milk yields (7.7 kg and 4.9 kg of milk, respectively) and higher fat content, with the added benefit of saving effort and time for the farmer. Conditions are commonly set at a vacuum level of 45–50 kPa and 120–160 pulsations per minute [13,14,18]; slower pulsations can result in incomplete udder emptying, whereas faster pulsations can create anxiety for mares and inflammatory edema in their nipples [14]. Maximum milk production yields are reached at the age of 7–15 years for mares [19], and multiparous mares produce higher milk yields than primiparous ones [20].

As mentioned before, mare milk is a traditional dairy product in some regions of Asia and Russia, and it is mainly consumed as koumiss (also called airag or chigee in Mongolia and Inner Mongolia), a fermented alcoholic beverage very popular in western Asia, Mongolia, northern China and Russia [5,21]. In Asia, mare milk (either raw or fermented) has extensively been used as a medicine to treat tuberculosis, chronic hepatitis, peptic ulcers and heartburn [3,21]. In Europe, mare milk is consumed raw or frozen, as well as freeze-dried in the form of capsules, pills or powder packages, and it can also be found as an ingredient in cosmetics [3]. The European Union establishes that raw milk can be destined for human consumption as long as it comes from healthy animals, following the corresponding health and quality verifications [22]. Considering that mares are unlikely to suffer from mastitis [21] and that the biological quality of their milk is usually acceptable [10], raw mare milk should be easily marketable in Europe, avoiding the alteration and loss of thermosensitive components. However, it is a perishable product

and, as indicated below, the high polyunsaturated fatty acid (PUFA) content might suffer from oxidation processes, which is why its transformation might improve conservation.

Mare milk is an interesting dairy product due to its high nutritional value, as will be discussed later in this work. Specifically, it has lower casein and higher whey protein proportions than ruminant milk and higher lactose contents (Table 1). In addition, the monogastric digestive system of horses and direct absorption of dietary lipids makes mare milk rich in unsaturated fatty acids, particularly forage-derived n-3 PUFA, compared to ruminant milk (Table 2). This makes mare milk an interesting food in terms of human health.

Table 1. Comparison of the content of lipids, proteins and other compounds among cow, mare and ewe milk.

	Cow	Mare	Ewe
Lipid fraction			
Fat content (%)	3.4–5.0 [G]	1.3–2.1 [G]	6.3–10 [G]
Triacylglycerides (% fat)	97–98	80–85	97–98
Phospholipids (% fat)	0.5–1.0	0.8–10	0.2–1.0
Free fatty acids (% fat)	0.1–0.2	9.4–9.6	0.1–0.2
Cholesterol (mg/L)	13–26 [G]	5.0–9.8 [G]	15–33 [G]
Fat globule size (μm)	2.8–4.6	2.0–3.0	3.0–3.8
Protein fraction			
Protein content (%)	3.3–3.7 [G]	2.1–3.9 [G]	4.5–8.6 [G]
Casein (% protein)	71–84	49–53 [G]	75–78 [G]
α-casein (% casein)	46–50	19–23	30–50
β-casein (% casein)	33–40	79–93	42–62
κ-casein (% casein)	10–12	1.8–2.1	7.5–8.9
Whey protein (% protein)	16–20	36–44 [G]	14–21 [G]
α-lactalbumin (% whey protein)	15–24 [G]	36–38 [G]	13–24 [G]
β-lactoglobulin (% whey protein)	64–88 [G]	29–30 [G]	64–87 [G]
Immunoglobulins (% whey protein)	8.0–16 [G]	15–17 [G]	4.5–6.6
Lactoferrin (% whey protein)	2.3–2.7 [G]	7.0–9.2 [G]	6.4–8.2
Lysozime (% whey protein)	nd	4.4–5.0 [G]	nd
Micelles size (nm)	150–182	255–312	180–210
Lactose (%)	4.7–5.4 [G]	6.4–6.7 [G]	4.1–5.1 [G]
Ash (%)	0.6–0.8 [G]	0.3–0.5 [G]	0.2–0.5 [G]
Calcium (mg/100 mL or g milk)	119–134 [G]	85–99 [G]	55–218 [G]
Potassium (mg/100 mL or g milk)	135–151 [G]	54–73 [G]	104–132 [G]
Phosphorous (mg/100 mL or g milk)	86–109 [G]	52–66 [G]	103–133 [G]
Ca/P	1.2–1.3	1.5–1.7	1.2–1.3
Vitamins			
Fat soluble vitamins			
Vitamin A (μg/100 mL or g milk)	62–285 [G]	35–104 [G]	72–393 [G]
Vitamin E (μg/100 mL or g milk)	81–166 [G]	nd-117 [G]	167–318 [G]
Water soluble vitamins			
Vitamin C (mg/100 mL or g milk)	0.3–2.3	0.7–8.1	0.4–6.0
Vitamin B1 (μg/100 mL or g milk)	28–90	20–52	28–80
Vitamin B2 (μg/100 mL or g milk)	116–202	5.0–48	160–429
Vitamin B3 (μg/100 mL or g milk)	50–130	70–140	300–500
Vitamin B5 (μg/100 mL or g milk)	260–490	277–300	350–430
Vitamin B6 (μg/100 mL or g milk)	30–70	8.0–61	27–80
Vitamin B9 (μg/100 mL or g milk)	1.0–18	0.13	0.2–6.0
Vitamin B12 (μg/100 mL or g milk)	0.3–0.7	0.3–2.0	0.3–0.7

nd, not detected. [G] Data from animals bred under extensive grazing management. Sources: cow [6,23–35], mare [6,11,13,15,27–29,35–42], ewe [6,27,35,43–57].

Table 2. Comparison of fatty acid profiles (g/100 g total fatty acids) among cow, mare and ewe milk from animals under extensive grazing management.

Fatty Acid	Cow	Mare	Ewe
4:0	0.020–3.3	0.090–0.16	1.9–3.6
6:0	1.1–1.8	0.19–0.39	1.3–2.8
8:0	0.95–2.2	0.58–5.2	1.0–2.7
10:0	1.9–3.7	2.6–11	3.3–7.9
11:0	0.038–0.060	0.030–0.050	0.030–0.33
12:0	2.1–4.1	4.2–9.9	2.4–4.1
13:0	0.070–0.15	0.040–0.19	0.070–0.096
14:0	7.4–12	6.0–9.7	8.5–10
15:0	1.0–2.6	0.22–0.56	0.97–1.2
16:0	19–34	18–27	19–25
17:0	0.53–2.4	0.35–0.53	0.44–0.83
18:0	9.0–17	0.83–4.9	9.3–13
20:0	0.13–0.21	0.080–0.10	0.20–0.36
21:0	0.050–0.49	0.56–0.77	0.010–0.10
22:0	0.060–0.57	0.030–0.30	0.11–0.18
23:0	0.020–0.063	nd	0.064–0.092
24:0	0.040–0.18	nd	0.040–0.080
SFA	52–69	43–58	55–74
10:1	0.24–0.27	1.1–1.7	0.15–0.25
9*c*-12:1	nd–0.080	0.15–0.26	0.030–0.11
9*c*-14:1	0.71–3.4	0.18–0.89	0.14–0.32
9*c*-15:1	0.23–0.27	nd–0.32	0.090–0.14
9*c*-16:1	1.0–3.1	3.2–7.0	0.79–1.3
9*c*-17:1	0.24–1.2	0.27–0.87	0.26–0.38
9*c*-18:1	17–22	14–22	18–21
11*c*-18:1	0.44–0.56	0.71–1.4	0.21–0.29
12*c*-18:1	0.23–0.25	0.69–0.74	0.37–0.46
t-18:1	1.3–6.5	nd	2.7–7.0
7*c*-20:1	0.040–1.5	0.25–0.44	0.22–0.32
11c-22:1	0.040–0.54	nd	nd
15*c*-24:1	0.010–0.061	nd	nd
MUFA	24–39	18–32	23–31
NC-dienes	0.58–1.5	0.030–0.14	0.88–1.8
CLA	0.49–2.4	0.0010–0.14	1.2–2.8
18:2n-6 (LA)	1.3–3.9	6.2–18	1.7–2.8
18:3n-6	0.040–0.63	0.15–1.3	0.054–0.080
20:2n-6	0.027–0.59	0.12–0.47	0.057–0.070
20:3n-6	0.010–0.11	0.090–0.10	0.030–0.16
20:4n-6	0.040–0.14	0.080–0.60	0.15–0.21
22:2n-6	<0.010–0.14	nd	0.090–0.12
18:3n-3 (LNA)	0.43–1.6	3.7–23	0.82–1.7
20:5n-3 (EPA)	0.060–0.15	nd	0.046–0.17
22:5n-3 (DPA)	0.050–0.10	0.080–0.12	0.11–0.23
22:6n-3 (DHA)	<0.010–0.010	nd	0.020–0.11
PUFA	2.8–7.2	18–31	2.6–7.9

nd, not detected; SFA, saturated fatty acids; c, cis; t, trans; MUFA, monounsaturated fatty acids; NC, non-conjugated; CLA, conjugated linoleic acids; LA, linoleic acid; LNA, linolenic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexadecanoic acid; PUFA, polyunsaturated fatty acids. Sources: cow [23–26], mare [36–40], ewe [43–46,57].

Currently, mare milk production is not high compared to other dairy domestic species, but its commercialization as a quality product obtained under extensive breeding systems could provide an added-value product. Considering this, the present review explores the potential benefits of implementing pasture-based mare milk production in current equine farms that produce horses for human consumption (mainly producing horse meat). As discussed below, due to its beneficial health properties, the production of mare milk in Europe could progressively increase by reaching a niche market within the functional foods

market and particularly addressing those consumers with special dietary requirements, such as children and the elderly, and/or by including mare milk into other products such as cosmetics.

2. Nutritional Value of Mare Milk

Mare milk production under extensive farming could contribute to the provisioning of high-quality food to society. In this section, the characteristics of mare milk are described and compared to those of cattle (large frame) and sheep (small frame) species. The composition of cow, mare and ewe milk—including lipids, proteins and other compounds—is detailed in Table 1.

2.1. Source of Lipids

Bovine and ovine milk lipids consist mainly of triglycerides (98%), whereas mare milk has been reported to have a higher content of phospholipids (5%), sterols (4.5%), glycolipids (1%), and free fatty acids [11]. Mare milk contains a broad diversity of lipids [58] and a lower cholesterol content compared to cow and ewe milk [6].

The total fatty acid (FA) composition of extensively reared mare milk differs considerably from grazing ruminant milk (Table 2), which is primarily related to digestive physiology, and, as in other milk-producing species, it can be affected by the feeding and lactation stage [36]. Mare milk is considerably richer in PUFA than ruminant milk [3–6], which is of interest for human nutrition. This is because, unlike ruminants, horses' digestive fermentation takes place in the caecum–colon compartment, allowing for the earlier absorption of dietary FAs (before microbial biohydrogenation). Moreover, the continuous secretion of biliary salts and high concentration of pancreatic lipases optimize the digestion of dietary lipids in the small intestine. In addition, it seems that high galactolipase activity in the horse digestion tract benefits the absorption of pasture lipids—rich in 18:3n-3 (α-linolenic acid, LNA)—which are present as galactolipids in a high proportion [59]. Galactolipase activity has been partially attributed to pancreatic lipase-related protein 2, which is present in horses [60,61]. This enzyme could be responsible for the efficient liberation of PUFAs from galactolipids and their high deposition in horse tissues and fluids, especially when managed under grazing [59,62].

Overall, grass-based mare milk is characterized by 18–31% PUFA and 43–58% saturated FA (SFA) (Table 2). The major FAs are palmitic (16:0), oleic (9*c*-18:1), linoleic (LA; 18:2n-6) and LNA (18:3n-3) acids; LA and LNA proportions can vary depending on the lipid composition of grasslands [37,45]. Compared to ewe and cow milk, the stearic (18:0) acid content is particularly low while the capric (10:0), lauric (12:0) and palmitoleic (9*c*-16:1) acid contents are higher in mare milk. On a percentage basis, mare milk is considerably rich in n-3 PUFAs [63], even though long-chain PUFA contents, such as eicosapentaenoic (20:5n-3), docosapentaenoic (22:5n-3) and docosahexaenoic (22:6n-3) acids, are low (Table 2). Taking into account equid digestive physiology, the content of both *trans*-18:1 and conjugated LA isomers is low, and their minor presence is an indicator of their limited microbial metabolism in the intestine [59,64].

As with other mammalian species [63], the FA composition of mare milk varies with breed [65,66], the age of the mare, foaling number [39] and, mainly, lactation [37,39,65,66] and nutrition [66–68]. At the beginning of lactation, when a high metabolic load is required, FAs derived from adipose tissue mobilization predominate in ewe and cow milk, while dietary FAs are relevant later [69,70]. Horses, however, are less able to mobilize body reserves and compensate for their high voluntary intake [71], which explains why dietary FAs are so abundant in mare milk.

Concerning the influence of diet, ruminants fed on pasture contain more unsaturated FAs than those fed with concentrates, particularly LNA, rumenic acid (9*c*,11*t*-18:2) and some biohydrogenation products derived from PUFA. This is due to a higher availability of LNA from forages [45,72]. Overall, very few studies have analyzed the effect of diet on mare milk FA composition. A recent lipidomic study comparing milk from mares fed pasture, silage

or stover found that grazing mares' milk contained significantly more lipids related to PUFA and lipid digestion and absorption metabolic pathways [68]. Otherwise, knowledge regarding the composition of pasture-based mare milk lipids relies on studies performed under extensive mountain conditions in the Mongolian steppe (>1000 m of altitude [40]) and Kyrgyz Republic (2200 m approx. [73]), along with others where the lactation period occurred during the grazing period (i.e., from spring to autumn [36–39]). But, unfortunately, differences in the experimental conditions related to the scientific literature have resulted in high variability of reported milk FA composition. Indeed, some of the reported variations in fat content and FA composition may be related to differences in extraction, separation and identification methods where, in some cases, the chromatographic columns and conditions used might not have been sufficient to separate potentially co-eluting FAs [74,75]. As a whole, it can be concluded that grazing management systems provide milk with a higher PUFA (n-3 and/or n-6 FAs derived from botanical species) in most mammalian species, but particularly in equids characterized by monogastric physiology and low efficiency of mobilizing body reserves.

In relation to the distribution of lipids in the milk matrix, fat forms dispersed globules of a smaller size (2–3 μm diameter; Table 1) and different layer structure compared to ewe and cow milk [47,76]. Mare milk fat globules are covered with three layers; the external layer is comprised of high-molecular-weight glycoproteins coated with a branched oligosaccharide structure. This layer might enhance the binding of lipases to the fat globules, and both the size and the cover composition have been reported to facilitate the digestion of milk lipids by humans [76].

2.2. Source of Proteins

The total protein content of mare milk is lower than that of ruminant milk, especially compared to ewe milk. One of the most remarkable differences is that the whey protein content is about 20% and casein is about 80% in cow and ewe milk, whereas it is 45% and 55%, respectively, in mare milk (Table 1). In fact, cow milk is commonly named a "casein type milk", while mare milk is considered an "albumin type milk" [3].

Mare milk contains less β-lactoglobulin but more α-lactalbumin, immunoglobulins (Ig) and lysozymes compared to bovine and ovine milk (Table 1). Mare milk contains similar quantities of α-lactalbumin and β-lactoglobulin, whereas β-lactoglobulin is the predominant whey protein in most ruminants [77]. Three genetic variants of α-lactalbumin (A, B and C) and two β-lactoglobulin isomers (I and II) are present in mare milk [77]. β-lactoglobulin has a monomeric form in horse milk, whereas it is a dimer in ruminant milk [13], and the equine isomer II has been described to be structurally similar to the human retinol-binding protein [2]. The biological function of equine β-lactoglobulin is not clear, but, unlike ruminant β-lactoglobulin, it is known to lack the ability to bind FAs. In fact, serum albumins are the only proteins with the ability to bind FAs in mare and human milk, whereas, as mentioned, ruminant β-lactoglobulin can perform that task too [2,77].

Lactoferrin, lysozyme and Igs, together with the lactoperoxidase system, have been reported to contribute to the antimicrobial effect of milk [27,77]. Mare milk is particularly rich in lactoferrin and lysozyme, except for comparable contents of lactoferrin in ewe milk (Table 1). Lactoferrin is an iron-binding enzyme that has shown antimicrobial, antiviral, anti-inflammatory, anti-oxidative and immunomodulatory properties, and it is an enhancer of the growth of specific probiotic strains [77,78]. The iron-binding capacity of equine lactoferrin, related to an antibacterial mechanism, is similar to human and greater than bovine lactoferrin [77].

On the other hand, equine lysozyme is particularly interesting due to its calcium-binding capacity, which results from the integration of active sites from α-lactalbumins (calcium-binding proteins) while maintaining the enzymatic activity of non-calcium-binding lysozymes [77]. Since equine lysozyme is resistant to acid and proteolysis, it might reach the human intestine practically intact [6]. In addition to its antimicrobial effect, antiviral, anti-inflammatory, immunomodulatory, antifungal and anti-carcinogenic properties have

been attributed to lysozyme [3,78]. So, considering the bioactive properties of these two enzymes—lysozyme and lactoferrin—the high abundance in mare milk is an attractive feature from product functionality and human nutrition perspectives.

The abundance of Igs in mare milk is considerably high (Table 1), but, interestingly, its profile differs from that of other species. IgG predominates in bovine colostrum and milk, whereas, in human colostrum and milk, IgA is the main Ig. Conversely, IgG predominates in mare colostrum, but IgA predominates in mare milk. This happens because, contrary to humans, the transfer of IgG to the fetus via utero is inefficient in ruminants and equids, so it needs to occur via colostrum [77]. Igs in milk have antibacterial, anti-inflammatory and immunomodulatory properties [78], and their main function is to protect the newborn [3].

In terms of caseins, these are conformed in micellar structures in which calcium is transported, and mare casein micelles have a wider diameter than cow or ewe micelles (Table 1). In this sense, the bigger mare milk micelles imply a low micelle surface-to-milk volume ratio. The casein profile of mare milk is very similar to that of human milk. While ruminant milk contains high and similar concentrations of αs1- and β-caseins, mare and human milk are principally composed of β-casein, and αs1-concentrations are significantly lower. Both human and equine milk contain notably low αs2- and κ-casein levels (Table 1 [6,41]). The only and always glycosylated casein in equine milk is κ-casein, which is located on the micellar surface. Its main function is to stabilize the casein micelle, and the high glycosylation makes it more resistant to hydrolysis by chymosin than bovine κ-casein. This fact, together with the low protein content and casein/whey protein ratio, as indicated before, makes mare milk unsuitable for cheesemaking, with low cheese yields due to difficulties in renneting and curd forming. From a human nutrition perspective, low contents of casein, αs-casein and β-lactoglobulin and high contents of lysozymes in mare milk resemble the protein composition of human milk [77].

Mare milk is often promoted as low-allergenic milk that could be an adequate substitute for infant formula for infants with a cow milk protein allergy. This low allergenicity has been speculated to derive from a high susceptibility of equine β-lactoglobulin to gastrointestinal digestion and the low content of some allergenic proteins (such as αs2-casein) in milk [3]. However, few studies have addressed mare milk protein allergenicity using either in silico, in vitro or animal models or clinical studies [79–81]. Moreover, up-to-date limited studies on the digestibility of equine proteins (including β-lactoglobulin) have been performed [82,83], so firm conclusions cannot be drawn. However, these studies suggest that mare milk does indeed present low allergenicity in individuals with a cow milk protein allergy, so this is a topic worth studying.

2.3. The Source of Other Components

Considering that extensive breeding systems have an impact on the lipid composition of mare milk, it is expected that other high-nutritional-value compounds, such as fat-soluble vitamins, are also affected. Vitamins A and E are the major fat-soluble vitamins present in mare milk [37], even though their content is lower than in milk from other mammal species (Table 1), probably due to a lower total fat content. Horses are known as "yellow fat animals" for their ability to absorb dietary β-carotene (precursor of retinol or vitamin A) and transfer it to tissues including milk [84]. Pasture is a great dietary source of carotenes for horses and, as a result, the seasonality and composition of the botanical species can influence the retinol status of mare serum, which might, at the same time, impact mare milk retinol content. In fact, unsupplemented mares on pasture can achieve similar serum retinol levels than mares kept indoors and supplemented with retinyl palmitate [85]. Other authors also found increased β-carotene levels in mares' plasma once the grazing period started. Supplementation with β-carotene can increase its presence in mare milk, although it might not be reflected in the vitamin A content [86], probably due to metabolic regulation processes in the horse [28], resulting in an overall low vitamin A content in mare milk (Table 1). Overall, the content of α-tocopherol in mare milk is low (Table 1). However, two independent studies have demonstrated that α-tocopherol supplementation during

pregnancy significantly affects mare milk and colostrum α-tocopherol contents [87,88], demonstrating the positive effect of dietary α-tocopherol intake in mare milk vitamin E levels. Despite all this, and due to limitations in the current knowledge regarding the contribution of pasture-based diets to fat-soluble vitamin contents in mare milk, further research is essential.

Other minor compounds of high nutritional value present in mare milk are water-soluble vitamins and minerals (Table 1). In this regard, vitamins of the B group have a different metabolism in ruminants and monogastrics. In ruminants, rumen microorganisms can synthetize most of them, while monogastrics depend on their diet to fulfill their vitamin requirements. Thus, the influence of feeding reported in ruminant studies is not applicable to horses [89]. Overall, milk water-soluble vitamins are more affected by diet than fat-soluble vitamins, although factors affecting water-soluble vitamin concentration in mare milk as well as their composition under extensive systems have not been studied in depth. The scientific literature shows that mare milk is particularly poor in vitamins B2 and B9, but, together with ewe milk, it contains higher amounts of water-soluble vitamins compared to cow milk, mainly due to a higher ascorbic acid (vitamin C) content [6] (Table 1).

Equid milk, together with ewe milk, is among the mammal milk with the lowest total mineral content. Despite the low calcium and phosphorus concentration in mare milk (Table 1), its higher calcium/phosphorus ratio compared to cow and ewe milk makes it more favorable for human nutrition [63]. Other trace elements have been found in lower or similar amounts than in cow [42,90] and ewe milk produced under extensive systems [48–50], except for copper, which is 1.5–2.5 times higher in mare compared to cow [42] and ewe milk [49,50].

Regarding carbohydrates, equine milk is richer in lactose (6.5 %) than most other mammalian milk (Table 1) [63]. Oligosaccharides, mainly linked to the surface of the external glycoprotein layer in the fat globule membrane [2], are of lower concentration and diversity in bovine and ovine milk [91], whereas mare colostrum and milk provide a great oligosaccharide quantity and structural diversity [92,93]. Some of the oligosaccharides seem to be specific to mares [93]; however, this may depend on the breed or genetics [92].

3. Socio-Ecological Benefits of Horse Production under Grazing Management

Extensive equine production is slowly gaining relevance in line with environmental alternatives that attempt to move away from industrial agriculture. Equines need to be introduced in international political debates, especially those dealing with the agro-ecological transition of animal production systems and their contribution to sustainable development goals. At the European Union level, we need to better understand and use equine green assets so that the equine sector can contribute to an agro-ecological transition and regional development [94]. In this regard, the new legislation of the Common Agricultural Policy for the period 2023–2027 includes the professionals of the equine sector as eligible for some subsidies under particular conditions [95].

European Union policies promote innovations in sustainable production systems within the current societal systems [94]. Livestock sustainability assessments mainly consider their economic and environmental contributions and often leave out other services such as product quality, rural vitality and cultural heritage. In fact, only food provisioning has a clear market price (monetary metrics), while other goods/services from livestock activity do not have such a tangible economic value, but should be quantified in some way and, of course, considered in order to assess the positive impact of pastoral equine farming systems [96]. In line with Ryschawy et al. [97] and Dumont et al. [98], we consider that there is a need to assess and value all the services that livestock provide to society and their interrelations. In this section, after discussing how horse characteristics allow them to adapt to graze vegetation from pastures and the animal welfare of mares managed under extensive grazing, we analyze the environmental benefits of grazing horses and the consequences for rural vitality and cultural heritage.

3.1. Equine Adaptation to Extensive Management in Less Favoured Areas

According to the Food and Agriculture Organization (FAO) of the United Nations [99], 41% of the global land surface can only be utilized for food production by autochthonous domestic herbivores that are able to transform vegetation from grasslands and shrublands into food. In the context of climate change, local breeds that are well adapted to harsh environmental conditions will possibly be the key to maintaining food production in less favored regions, particularly in Mediterranean areas [100]. With adverse hydrological conditions and strong human impacts, these Mediterranean areas are particularly susceptible to desertification [101], and locally adapted equids, which are part of the traditional grazing system, could play an important role in nature conservation and the prevention of land degradation [100].

Equines are non-ruminant herbivores. They have a mono-chambered stomach where non-fermentative digestion takes place (foregut), while the fermentation process takes place in the large intestine or caecum–colon compartment (hindgut fermentation) [102,103]. Horses strongly select bites dominated by grasses [104–106] and generally use dicotyledons to a lower extent compared to cattle because they are less able to detoxify their secondary metabolites [104,107]. Horses graze longer than ruminants (15 h per day on average vs. 8 h per day in ruminants) and their food intake is less constrained by the particle size of digesta. They are thus able to ingest larger amounts of forages, especially roughages, than ruminants [104,108,109].

In spring, when sward biomass is abundant and of high quality, horses preferentially graze in habitats consisting of improved pastures or grasslands, where they benefit from high-quality grasses [109–116]. Doing this, they select a diet containing more protein and/or energy and less fiber than available vegetation [109,116]. In these grasslands, horses mainly use tall vegetative sward areas that are more accessible until these become mature [117]. Then, they switch to high-quality short swards (below 8 cm) and avoid areas of tall mature grass where they concentrate their droppings [117,118]. This behavior was explained as an anti-parasitic strategy [119], although some more recent works indicate that the nutritional characteristics of grass also play an important role in the choice of feeding sites [117,120,121]. The continuous exploitation of previously grazed patches by horses is a key mechanism through which they shape the structure of the whole plant community by creating stable patches of short swards within a matrix of tall vegetation contaminated with their feces [122]. In general, cattle are excluded from the shortest swards below 4 cm where their bite depth is limited [123] and, therefore, leave these areas [112], while horses can stay, taking advantage of their two sets of incisors [104]. In cases where sward availability in grasslands becomes limiting also for horses, they generally switch to areas of poorer nutritional quality, such as heathlands [109,112,113,116]. Horses can travel long distances to new feeding sites in order to satisfy their feeding requirements [102]. In heathlands, horses were observed to select highly nutritive legumes like gorse and avoid other woody species (heather and other shrubs) [110,113–116]. Overall, horses and lactating mares have shown good adaptation capacity in shrublands and heathlands due to their ability to select highly nutritive leaves, green stems, flowers and pods among woody plant material [109,110,116]. In contrast, horses are limited when mobilizing body reserves [71], and, therefore, milk production by mares could be compromised when the availability of green biomass becomes low [110] as their nutrient requirements are 75% higher than those of dry mares [11].

The long daily grazing time of horses allows them to compensate for their lower fiber digestion efficiency compared to ruminants so that they maintain their daily intake even in poor-quality environments [104,116]. Several works have proved that grazing horses achieve adequate growth performance and can maintain their body weight all along the grazing season [106,113,114,124]. Lactating mares of light breeds under unlimited herbage conditions were shown to maintain their daily intake, meet their dietary requirements and produce foals with satisfactory growth and conformation while relying only on herbage [125]. Mares were, however, underfed and fell short of their requirements when

[125]. Mares were, however, underfed and fell short of their requirements when the daily herbage allowance was less than 3.7 kg of dry matter (DM) per mare per day [125] or when fed on heather-dominated heathlands (due to a lower preference for this type of vegetation) [114].

Horses can adapt to extensive management during winter (Figure 1) [126,127] better than cattle [128]. As an example, Brinkmann et al. [126] showed that horses (Shetland ponies (*Equus ferus caballus*) and Dülmen wild horses (*Equus ferus przewalskii* f. *caballus*)) were able to adapt their body temperature, which is similar to the pyrome and heart rate strategy of their wild ancestor, the Przewalski horse (*Equus ferus przewalskii*, originally from the Mongolian steppe) [129]. Horses are able to limit their walking activity during winter as an energy saving strategy, limiting most of their movement time to feeding and avoiding lying down [126]. Coat hair density also varies according to temperature, especially in free-ranging animals [130]. Among those facing extreme environmental conditions, horses of the Yakutian breed can be found in the northeastern part of the Russian Federation, where temperatures range from −60 °C in winter to 40 °C in summer [131]. People who emigrated there in the 13–15th centuries brought along already domesticated horse breeds that had developed additional physiological and morphological adaptations (i.e., improved hair density, fat accumulation and body size compaction) to maintain body temperature and survive under severe climatic conditions. Yakutian horses graze all year long, consuming vegetation even when it is buried under a thick layer of snow [132]. Horses are also able to adapt to semi-desert conditions [133] or short periods of water deprivation [134].

Figure 1. Basque mountain horses feeding in a mountain area in the Basque Country (Spain) in winter conditions.

It is recognized that native breeds are better adapted to physiological, nutritional and disease stress and, hence, to living in natural wild conditions [96,130,135]. In the context of climate change, autochthonous horse breeds offer opportunities for sustainable agricultural production [135] and extensive areas [136] as they can survive extreme environmental conditions like prolonged droughts or severe winters that cause the death of many livestock [137]. Moreover, horses are easy to manage and have been reported to have a low predation compared to other large herbivores [138].

3.2. Health and Welfare of Grazing Mares

Animal welfare is an indicator of sustainable livestock production systems [139]. According to the Animal Welfare Indicators (AWIN) Welfare Assessment Protocol for Horses, it has to do with adequate feeding and housing conditions and good health, but also with the expression of social and other behaviors, good human–animal relationships and positive emotional states of the animals [140]. Horses are defined as social animals since they naturally live in hierarchized groups and need social interactions with conspecifics [141]. In this sense, extensive livestock production systems where animals spend time outdoors, live through natural experiences and perform species-specific behavior are in line with the adequate practices reported, as long as the extreme environmental conditions (e.g., harsh climatic conditions in winter or food and water scarcity) are not harmful to the animals. It

has been described that horses with access to paddocks show less stress and aggressive behavior, lower risk of injury, lower prevalence of stereotypies (repetitive and invariant behaviors) and, in general, better welfare and positive social behavior [142,143].

European legislation only compels animal welfare labeling for egg production, and welfare standards and labeling for other animal products are scarce. Simultaneously, consumers are not adequately informed about the management practices used for the foods they are consuming, such as dairy products. In response to this situation, some organisms and institutions at national and international levels have implemented their own labeling systems to encourage animal welfare on farms. Some examples are Freedom Food in the United Kingdom, Label Rouge in France, some foods under Protected Designations of Origin and Protected Geographical Indications [144] and the Welfair™ certificate in Spain [145], which is based on the Welfare Quality® and AWIN® projects. None of these are related to equid milk or meat. Promisingly, the European Commission announced that harmonized labeling for sustainable food choices will be proposed within the Farm to Fork Strategy [146]. For now, the European Commission has established a sub-group within the European Union Platform on Animal Welfare dedicated to labeling [147]. There is also a European regulation that contemplates animal grazing with regard to organic production and labeling [148]. This regulation limits animal density on pastures and establishes minimum pasture-derived feed rates. It includes equids, but not the ones destined for milk production.

An important pillar of animal welfare is animal health. Good health valorization includes the absence of injuries, disease and pain. The use of antibiotics is an issue of concern in human health due to the development of resistant pathogens as well as the spread of antibiotic resistance genes through the food chain. Dairy cattle are usually administered antibiotics for the prevention and treatment of intra-mammary infections, mainly mastitis [149]. However, mastitis is uncommon in mares [21], so antibiotics as a prevention tool seem less needed. In organic dairy systems (in which extensive management systems are often included), the use of antibiotics as prophylactics or growth promoters is forbidden, and they are only accepted in extreme cases in which phytotherapeutic or other treatments are not effective. If animals are administered antibiotics or antiparasitic medication more than three times within a year (or more than one time in yearlings), they must stay under strict organic conditions for six months before their milk can be commercialized as organic again [148].

3.3. Environmental Impact of Grazing Equines

The maintenance of grassland-based systems is key for a number of ecosystem services (ES) related to the high natural value of landscapes, biodiversity conservation, and the preservation of soil and water quality [97,150]. Permanent grasslands have a key role in terms of carbon sequestration and, thus, in climate regulation and the maintenance of soil fertility [151]. The moderate intensification of nutrient-poor permanent grasslands, use of light grazing instead of intensive grazing and conversion of grass leys to grass–legume mixtures or permanent grasslands, also related to equine livestock, can increase carbon stocks [152].

It is known that grassland biodiversity can be compromised by both overgrazing and abandonment of pastoral lands [127,153–156]. Under moderate (approximately 660–675 kg of live weight/ha) to high (up to 1080 kg of live weight/ha) stocking rates, horse grazing preserves the structural heterogeneity of grasslands [106,157]. As indicated, horses create a grassland habitat made of short sward patches within a mosaic of tall vegetation. The inter-annual stability of these grazing patterns [122] benefits the co-existence of different animal and plant species with different environmental preferences, enhancing pasture biodiversity [106,157]. The grazing of equines influences pasture structure and composition differently compared to that of cattle, sheep or goats. Grazing impacts also depend on whether horses graze alone or mixed with ruminant species [109]. Under a moderate stocking rate, co-grazing horses and cattle would maximize botanical diversity compared

to only horse grazing as cattle increase the diversity in tall vegetation areas that horses avoid [157]. Horses are also less selective than cattle or sheep on forbs, so their direct grazing impact on flowering plants and flower-visiting insects is smaller [158,159]. Consequently, horses have a strong potential to enhance pasture biodiversity [124,158–160], except when the stocking rate increases considerably and horse trampling decreases the abundance of flowering plants [161].

The high voluntary intake of roughage by horses controls competitive grasses, which maintains "open" pastures and enables the coexistence of many plant and animal species [127,159]. While horses seem less able than cattle to control woody plants under extensive conditions [114,127], they can reduce some species, especially gorse, through grazing and trampling [110,115,160,162] and enhance grassland species diversity through selective grazing [110,115,162]. The reduction in gorse accumulation also opens the canopy and facilitates the access of other grazers to expanding grassland areas [163]. Mixed grazing with cattle generally improves the control of shrubs, especially when sward availability decreases in grasslands [116,127]. These strategies to improve pasture quality in shrub-dominated areas permit the recovery of abandoned lands by increasing biodiversity and nutritive plants and soils, without compromising animal performance and health.

Herbivores also benefit from the dispersal of plant seeds, attached to their coat or through their digestive tract [164], especially if grazing occurs after flowering [165]. Moreover, moderate equid grazing also contributes to the accumulation of organic matter (animal wastes) and, hence, the improvement of soil quality and productivity. Horse dung and urine deposited directly on the soil act as a natural fertilizer via the accumulation of nutrients [166]. Irrespective of the animal species, feces consist of water, undigested fodder, residues, animal metabolites, microorganisms and microbial metabolites [167]. In horses, the nitrogen excreted in feces is made up of 85–95% protein (57% microbial protein and 43% endogenous protein). Horse feces only contain 5–8% ammonia nitrogen and are rich in phosphorus (organic and inorganic; 75–85 mg per kg of body weight) and other minerals such as calcium (90–100 mg per kg of body weight), magnesium (15–20 mg per kg of body weight), potassium (15–25 mg per kg of body weight) and sodium (8–30 mg per kg of body weight) [168]. Moreover, they contain small quantities of other elements like heavy metals, which are not assimilated by the animal following the ingestion of vegetable matter or soil. It is known that herbivore species play a key role in nutrient fluxes among plants–animals–soils and in soil fertility. In this sense, not only are the nutrient contents and their chemical form important but also the mass of these nutrients related to the number and quantity of droppings [167]. In saddle horses of 500 kg fed on green or dry fodder, the average daily production of feces is estimated at 8–9.5 g DM per kg of body weight. As previously indicated, among domestic herbivores, horses are known to maintain areas of short grass within plots by grazing and avoid areas of tall grass where they concentrate their droppings. This leads to phosphorus and potassium depletion in heavily grazed areas and the enrichment of latrines [168]. Repeated grazing and trampling in short grass areas have positive impacts, especially in environments that are initially saturated with an excess of organic matter. Soils benefit from the unclogging of the catabolic chain of organic matter and are improved in their nitrogen mineralization potential. Plants, however, are limited in their growth by their small leaf area index and the low potassium availability in these areas [168]. At a local scale, heavily grazed areas are composed of short vegetation with little standing biomass, and low but high-quality primary production (young leaves). Plants rebuild their reserves to a limited extent. The root system is poorly developed and often weakened by the small amount of assimilate allocated to it, most of which is used to reconstitute leaves. In these conditions, small, prostrate (stoloniferous species) or rosette species, favored by their grazing avoidance strategy, are selected. Conversely, the high biomass accumulation in areas avoided by horses is associated with a decrease in vegetation quality. The local nutrient enrichment stimulates primary production, leading to strong competition for light. In these areas, large, highly competitive or eutrophic species are selected. These plants present a ruderal strategy that enables them to develop rapidly

in areas enriched with mineral elements and maintain their dominance by taking up space (large rosettes, broad and spreading leaves) and producing a large number of seeds [168].

When livestock is managed extensively, soil and water contamination with chemicals and other residues is limited, and water pollution by fecal elements is mitigated, although not totally eliminated [169]. In extensive systems, especially in mountain grasslands, no water for human consumption is used, and livestock consumes "green water"—the water fraction stored as soil moisture. In addition, animals that graze in pastures not only do not compete with human food production but also convert non-human-edible matter (grass and forages) into human-edible foods (meat and milk) [170].

Agricultural land abandonment leads to the invasion of highly flammable dead biomass and woody vegetation (i.e., shrubs, heather and gorse), increasing the prevalence of wildfires [112,116,160,171], which will probably be more frequent with worsening climate change [172]. Great problems after a fire are soil erosion [173,174] and biodiversity loss [110], in addition to the devastation of established fauna and flora and organic matter quality and quantity. For instance, some nutrients are affected, pollutants appear, soil microbiota is lost and water infiltration and soil water holding capacity are hindered [174]. This displays an imperative need for implementing prevention mechanisms [175]. Horses showed good potential to remove more herbaceous vegetation per unit of body weight than cattle and graze closer to the ground, as reported by Pardini et al. [176] in a study performed on firebreaks using sown botanical species and grazing horses.

Greenhouse gas emissions from livestock account for 14.5–18% of global emissions [158,177], while livestock enteric fermentation accounts for 33–39% of total methane emissions derived from agriculture [178]. Hindgut fermenter herbivores, such as equids, generate methane in the caecum–colon [103] but in much lower amounts than ruminant species in the rumen [179]. This may be explained by a shorter retention time in the hindgut than in the rumen, resulting in lower fermentative degradation of plant cell walls and an alternative non-methane-producing hydrogen sinking route (acetogenesis) occurring more actively in the horse hindgut [103]. The annual release of enteric methane by equids in France has been calculated to be 20,202 tons from a total of 975,000 animals, giving an emission number of 20.7 kg per animal per year. This value increases to 29.7 kg when calculated for lactating mares, representing just 34% of the value calculated for milking cows [168,180]. Some authors have proposed a shift from ruminants to monogastrics as an alternative to mitigate greenhouse gas emissions [181]. However, when referring to monogastrics, most authors include porcine and poultry, and only a few consider equids as a high-quality protein source. It is true that equids represent only 4×10^{-3} % of livestock for meat production in Europe [182] but, due to its high nutritional value, horse meat consumption is gaining some interest in several countries [59]. In addition, recent studies showed that there is a rising potential to increase the consumption of horse meat in European countries [183,184]. However, there are still strong emotional and cultural reasons preventing consumers from eating horse-derived foods [185].

3.4. Rural Vitality

Indicators of rural vitality (rural activation and development) are mainly related to the contribution of livestock to rural employment and stability. Rural employment concurrently entails population growth and fixation but also requires investments in other social aspects such as social and institutional organizations; educational, technological and social services; infrastructures; gender perspectives; etc., which impact the overall life quality of the rural population [186–188]. As reported by Cooper et al. [189], to maintain traditional agricultural systems and associated knowledge (traditional education), an adequate population density is needed in rural and less favored areas. However, rural abandonment and consequent depopulation are occurring in Europe [190], and significantly lower economic development and employment rates are happening in rural compared to more urbanized communities [191]. This is driven by different factors such as unfavorable biophysical conditions of agricultural lands and socioeconomic factors of farms and farmers

that are poorly supported by public administration policies and national and international markets (depending on the European region) [192].

Agricultural areas comprise almost half of the European territory, and one-third of them correspond to permanent grasslands and meadows [193]. On average, the percentage of workers dedicated to agriculture in the European Union has decreased to less than half in the last 30 years [194], although the farming sector still employs 4 million people in Europe, of which 25% are related to the dairy industry [195]. In relation to the equine sector, this provides the equivalent of 400,000 full-time jobs in Europe [196] and, in France, almost 100 different professions have been related, directly or indirectly, to the equine industry [197].

One of the strategies proposed to reinforce rural development and vitality is to make economies thrive through the diversification of economic activities [190]. Other diversification activities such as leisure, sport or equestrian activities are a source of direct economic benefit in local areas [198]. Therefore, new sources of profit, such as the production of non-traditional agricultural products (i.e., mare milk), could also be decisive in increasing farm incomes [199], especially in those equine farms exclusively dedicated to horse meat production. Moreover, grazing practices allow for lower investments in feeding and can somehow compensate for the low inputs of equine breeding [200]. Keeping in mind that a considerable proportion of citizens aim to recover relationships with the traditional rural life and are more attracted to natural environments for different reasons (i.e., close contact with nature, recreation and sports, spiritual development, cultural experiences, landscape, because they look for traditional education or want to escape from pandemic-related risk and isolation) [156,201], horse breeding for milk production could be another option that could be complemented by other activities.

3.5. Cultural Heritage

In this subsection, indicators such as heritage landscape, agrotourism, heritage animal products and genetic resources are considered [97]. European landscapes have been shaped through a long period of agricultural activities such as livestock grazing. Characterized by their heterogeneity, they are considered part of the cultural identity of many regions [189]. Landscape heterogeneity is related to biodiversity [202] and aesthetic value [156,201,203], which are directly compromised by land abandonment and the associated encroachment and homogenization of shrubs and forests [204] and urbanization, but also by agricultural intensification [155,205] and other economic activities such as intense touristic and recreational exploitation [171,205]. Mosaic-rich landscapes, open meadows and permanent grasslands with traditional agricultural buildings, farming systems and the presence of moderate livestock grazing are the elements of hedonic preference that most attract tourism [156,201,203].

Focusing on equines, cultural heritage and identity are usually linked to autochthonous horse breeds [135], which are largely being substituted with more productive international breeds for agricultural purposes [135,206], resulting in a loss of genetic biodiversity. Taking into account the overall genetic pool, horses are recognized as the second mammalian species (after rabbits) with the highest global at-risk breed percentage (33%) [207]. Therefore, autochthonous horse breeds need special attention, protection and support.

Equines can be connected with cultural heritage in many different ways such as part of leisure and sports activities (equitation), gastronomy or because they have traditionally been work animals [208], being an important part of the identity of regions. For instance, a number of equine-related affairs are a good example of “Intangible Cultural Heritage”, recognized by the United Nations Educational, Scientific and Cultural Organization [209].

Farms and households based on pastoralism usually work with autochthonous and well-adapted breeds [135], being a tool for gene preservation (primarily of those associated with the adaptation to free-ranging conditions) and promoting cultural heritage and the protection of traditional education and knowledge [136,204]. In this sense, it is recognized that extensive equine farming can contribute to the conservation of traditional and/or

cultural landscapes [171,201], given that it creates a mosaic landscape due to species-specific grazing preferences.

4. Conclusions

Mongolia, Kazakhstan and other Asian countries are the main producers of mare milk in the world, to the point of considering it a traditional dairy food. Even though mare milk consumption has extended to several European countries, its production in Europe is still scarce, first of all, because equids are not great milk producers (due to the udder structure), and, second, because the production of mare milk and its derived products, as well as its benefits for human health, have not been deeply studied or adequately promoted.

From what is known today, any horse breed could be a potential milk producer under sustainable livestock management systems in order to produce milk with high nutritional value. Horses are part of the culture in many European and worldwide regions. Their incorporation into local farming could help protect local and regional breeds as well as provide other ecosystem services that would benefit both the farmer and society. This strategy could be of interest in arid and semi-arid regions (i.e., Mediterranean areas) or areas with moderately harsh environmental conditions (i.e., northern Europe, mountainous regions) as equids have good adaptation capacity.

In this respect, equine farmers need public authorities at different levels to recognize the ecosystem services provided in terms of not only food provisioning but also the maintenance of mountains and other natural areas (including biodiversity, landscape, soil and water quality, and the prevention of forest fires), the support of rural economies, and the preservation of the genetic pool of local horse breeds and cultural heritage.

Author Contributions: Conceptualization, A.B.-D., L.J.R.B. and N.A.; Writing—Original Draft Preparation, A.B.-D.; Writing—Review and Editing, L.J.R.B. and N.A. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Basque government through ELKARTEK 2019 (BIOTASMA), Cooperation 2020 (BEHOR ESNE) and the IT944-16 and IT1568-22 projects.

Data Availability Statement: No new data were created or analyzed in this study. Data sharing is not applicable to this article.

Acknowledgments: The authors are thankful to G. Fleurance (IFCE, France) and B. Dumont (INRAE, France) for their helpful comments and advice. A. Blanco-Doval thanks the Department of Education of the Basque government for the predoctoral fellowship.

Conflicts of Interest: The authors declare no conflicts of interest.

References

1. Outram, A.K.; Stear, N.A.; Bendrey, R.; Olsen, S.; Kasparov, A.; Zaibert, V.; Thorpe, N.; Evershed, R.P. The earliest horse harnessing and milking. *Science* **2009**, *323*, 1332–1335. [CrossRef]
2. Sheng, Q.; Fang, X. Bioactive components in mare milk. In *Bioactive Components in Milk and Dairy Products*; Park, Y.W., Ed.; Wiley-Blackwell: Sussex, UK, 2009; pp. 195–213.
3. Pieszka, M.; Łuszczyński, J.; Zamachowska, M.; Augustyn, R.; Długosz, B.; Hędrzak, M. Is mare milk an appropriate food for people?–a review. *Ann. Anim. Sci.* **2016**, *16*, 33–51. [CrossRef]
4. Gall, C.F. Production systems around the world. In *Milk and Dairy Products in Human Nutrition—Production, Composition and Health*; Park, Y.W., Haenlein, G.F.W., Eds.; John Wiley & Sons: Sussex, UK, 2013; pp. 1–30.
5. Bat-Oyun, T.; Erdenetsetseg, B.; Shinoda, M.; Ozaki, T.; Morinaga, Y. Who is making airag (fermented mare's milk)? A nationwide survey of traditional food in Mongolia. *Nomadic Peoples* **2015**, *19*, 7–29. [CrossRef]
6. Claeys, W.L.; Verraes, C.; Cardoen, S.; De Block, J.; Huyghebaert, A.; Raes, K.; Dewettinck, K.; Herman, L. Consumption of raw or heated milk from different species: An evaluation of the nutritional and potential health benefits. *Food Control* **2014**, *42*, 188–201. [CrossRef]
7. Faye, B.; Konuspayeva, G. The sustainability challenge to the dairy sector—The growing importance of non-cattle milk production worldwide. *Int. Dairy J.* **2012**, *24*, 50–56. [CrossRef]
8. Uniacke-Lowe, T.; Fox, P.F. Equid milk: Chemistry, biochemistry and processing. In *Food Biochemistry and Food Processing*; Simpson, B.K., Ed.; John Wiley & Sons, Inc.: New York, NY, USA, 2012; pp. 491–530.

9. Minjigdorj, N.; Austbø, D. Production of Mare's Milk in Mongolia. 2009. Available online: http://www.umb.no/statisk/husdyrforsoksmoter/2005/088.pdf (accessed on 1 July 2023).
10. Doreau, M.; Martin-Rosset, W. Animals that produce dairy foods: Horse. In *Encyclopedia of Dairy Sciences*; Fuquay, J.W., Fox, P.F., McSweeney, P.L.H., Eds.; Academic Press: San Diego, CA, USA, 2011; pp. 358–364.
11. Salimei, E.; Park, Y.W. Mare milk. In *Handbook of Milk of Non-Bovine Mammals*; Park, Y.W., Haenlein, G.F.W., Eds.; Wiley-Blackwell Publishing: Oxford, UK, 2017; pp. 369–375.
12. Doreau, M.; Boulot, S. Methods of measurement of milk yield and composition in nursing mares: A review. *Le Lait* **1989**, *69*, 159–171. [CrossRef]
13. Salimei, E.; Fantuz, F. Equid milk for human consumption. *Int. Dairy J.* **2012**, *24*, 130–142. [CrossRef]
14. Turabayev, A.; Nurmakhanbetov, D.; Rakhmanov, S.; Baktybayev, G.; Aldabergenov, M. Control model elements of production processes of mare's milk. *Eurasia J. Biosci.* **2019**, *13*, 1169–1176.
15. Minjigdorj, N.; Baldorj, O.; Austbø, D. Chemical composition of Mongolian mare milk. *Acta Agric. Scand. A Anim. Sci.* **2012**, *62*, 66–72. [CrossRef]
16. Ishii, S.; Hosino, B.; Komiyama, H.; Uehara, A.; Nurtazin, S. Study on production and properties of kumiss of herders in Mongolian dry steppe. *J. Arid Land* **2014**, *24*, 195–197.
17. Doreau, M.; Boulot, S.; Martin-Rosset, W.; Dubroeucq, H. Milking lactating mares using oxytocin: Milk volume and composition. *Reprod. Nutr. Dev.* **1986**, *26*, 1–11. [CrossRef]
18. Caroprese, M.; Albenzio, M.; Marino, R.; Muscio, A.; Zezza, T.; Sevi, A. Behavior, milk yield, and milk composition of machine-and hand-milked Murgese mares. *J. Dairy Sci.* **2007**, *90*, 2773–2777. [CrossRef] [PubMed]
19. Doreau, M.; Martuzzi, F. Milk yield of nursing and dairy mares. In *Nutrition and Feeding of the Broodmare*; Miraglia, N., Martin-Rosset, W., Eds.; Wageningen Academic Publishers: Wageningen, The Netherlands, 2006; Volume 120, pp. 57–64.
20. Auclair-Ronzaud, J.; Jaffrézic, F.; Wimel, L.; Dubois, C.; Laloë, D.; Chavatte-Palmer, P. Estimation of milk production in suckling mares and factors influencing their milk yield. *Animal* **2022**, *16*, 100498. [CrossRef] [PubMed]
21. Park, Y.W.; Zhang, H.; Zhang, B.; Zhang, L. Mare milk. In *Handbook of Milk of Non-Bovine Mammals*; Park, Y.W., Haenlein, G.F.W., Eds.; Wiley-Blackwell Publishing: Oxford, UK, 2006; pp. 275–296.
22. European Commission. *Commission Regulation No 1662/2006 of 6 November 2006 Amending Regulation No 853/2004 of the European Parliament and of the Council Laying Down Specific Hygiene Rules for Food of Animal Origin*; European Commission: Brussels, Belgium, 2006.
23. Collomb, M.; Bütikofer, U.; Sieber, R.; Jeangros, B.; Bosset, J.O. Composition of fatty acids in cow's milk fat produced in the lowlands, mountains and highlands of Switzerland using high-resolution gas chromatography. *Int. Dairy J.* **2002**, *12*, 649–659. [CrossRef]
24. Bonfoh, B.; Zinsstag, J.; Farah, Z.; Simbé, C.F.; Alfaroukh, I.O.; Aebi, R.; Badertscher, R.; Collomb, M.; Meyer, J.; Rehberger, B. Raw milk composition of Malian Zebu cows (*Bos indicus*) raised under traditional system. *J. Food Compos. Anal.* **2005**, *18*, 29–38. [CrossRef]
25. Capuano, E.; Gravink, R.; Boerrigter-Eenling, R.; van Ruth, S.M. Fatty acid and triglycerides profiling of retail organic, conventional and pasture milk: Implications for health and authenticity. *Int. Dairy J.* **2015**, *42*, 58–63. [CrossRef]
26. Sharma, R.; Ahlawat, S.; Aggarwal, R.A.K.; Dua, A.; Sharma, V.; Tantia, M.S. Comparative milk metabolite profiling for exploring superiority of indigenous Indian cow milk over exotic and crossbred counterparts. *J. Food Sci. Technol.* **2018**, *55*, 4232–4243. [CrossRef] [PubMed]
27. Alichanidis, E.; Moatsou, G.; Polychroniadou, A. Composition and properties of non-cow milk and products. In *Non-Bovine Milk and Milk Products*; Tsakalidou, E., Papadimitrioupp, K., Eds.; Academic Press Elsevier: London, UK, 2016; pp. 81–116.
28. Álvarez, R.; Meléndez-Martínez, A.J.; Vicario, I.M.; Alcalde, M.J. Carotenoids and fat-soluble vitamins in horse tissues: A comparison with cattle. *Animal* **2015**, *9*, 1230–1238. [CrossRef] [PubMed]
29. Anderson, R.R. Comparison of minerals in milk of four species. *Comp. Biochem. Physiol. A Physiol.* **1991**, *100*, 1045–1048. [CrossRef]
30. Mackle, T.R.; Bryant, A.M.; Petch, S.F.; Hooper, R.J.; Auldist, M.J. Variation in the composition of milk protein from pasture-fed dairy cows in late lactation and the effect of grain and silage supplementation. *N. Z. J. Agric. Res.* **1999**, *42*, 147–154. [CrossRef]
31. Precht, D. Cholesterol content in European bovine milk fats. *Nahrung* **2001**, *45*, 2–8. [CrossRef] [PubMed]
32. MacGibbon, A.K.H.; Taylor, M.W. Composition and structure of bovine milk lipids. In *Advanced Dairy Chemistry, Lipids*, 4th ed.; McSweeney, P.L.H., Fox, P.F., O'Mahony, J.A., Eds.; Springer Nature: Cham, Switzerland, 2006; Volume 2, pp. 1–42.
33. Adesina, K. Effect of breed on the composition of cow milk under traditional management practices in Ado-Ekiti, Nigeria. *J. Appl. Sci. Environ. Manag.* **2012**, *16*, 55–59.
34. Brodziak, A.; Barłowska, J.; Król, J.; Litwińczuk, Z. Effect of breed and feeding system on content of selected whey proteins in cow's milk in spring-summer and autumn-winter seasons. *Ann. Anim. Sci.* **2012**, *12*, 261–269. [CrossRef]
35. Fantuz, F.; Salimei, E.; Papademas, P. Macro-and micronutrients in non-cow milk and products and their impact on human health. In *Non-Bovine Milk and Milk Products*; Tsakalidou, E., Papadimitrioupp, K., Eds.; Academic Press Elsevier: London, UK, 2016; pp. 209–261.
36. Rutkowska, J.; Adamska, A.; Bialek, M. Comparison of fatty acid composition in mare and cow milk fat. *Zywn-Nauka Technol. Jakosc* **2011**, *1*, 28–38.

37. Csapó, J.; Stefler, J.; Martin, T.G.; Makray, S.; Csapó-Kiss, Z. Composition of mares' colostrum and milk. Fat content, fatty acid composition and vitamin content. *Int. Dairy J.* **1995**, *5*, 393–402. [CrossRef]
38. Pikul, J.; Wójtowski, J. Fat and cholesterol content and fatty acid composition of mares' colostrums and milk during five lactation months. *Livest. Sci.* **2008**, *113*, 285–290. [CrossRef]
39. Pikul, J.; Wójtowski, J.; Danków, R.; Kuczynska, B.; Lojek, J. Fat content and fatty acids profile of colostrum and milk of primitive Konik horses (*Equus caballus gmelini* Ant.) during six months of lactation. *J. Dairy Res.* **2008**, *75*, 302–309. [CrossRef] [PubMed]
40. Minjigdorj, N.; Haug, A.; Austbø, D. Fatty acid composition of Mongolian mare milk. *Acta Agric. Scand. A Anim. Sci.* **2012**, *62*, 73–80. [CrossRef]
41. Miranda, G.; Mahé, M.; Leroux, C.; Martin, P. Proteomic tools to characterize the protein fraction of *Equidae* milk. *Proteomics* **2004**, *4*, 2496–2509. [CrossRef]
42. Csapó-Kiss, Z.; Stefler, J.; Martin, T.G.; Makray, S.; Csapó, J. Composition of mares' colostrum and milk. Protein content, amino acid composition and contents of macro and micro-elements. *Int. Dairy J.* **1995**, *5*, 403–415. [CrossRef]
43. Ostrovský, I.; Pavlíková, E.; Blaško, J.; Górová, R.; Kubinec, R.; Margetín, M.; Soják, L. Variation in fatty acid composition of ewes' milk during continuous transition from dry winter to natural pasture diet. *Int. Dairy J.* **2009**, *19*, 545–549. [CrossRef]
44. Valdivielso, I.; Bustamante, M.A.; Buccioni, A.; Franci, O.; de Gordoa, J.C.R.; de Renobales, M.; Barron, L.J.R. Commercial sheep flocks–fatty acid and fat-soluble antioxidant composition of milk and cheese related to changes in feeding management throughout lactation. *J. Dairy Res.* **2015**, *82*, 334–343. [CrossRef]
45. Bravo-Lamas, L.; Aldai, N.; Kramer, J.K.G.; Barron, L.J.R. Case study using commercial dairy sheep flocks: Comparison of the fat nutritional quality of milk produced in mountain and valley farms. *LWT—Food Sci. Technol.* **2018**, *89*, 374–380. [CrossRef]
46. Caprioli, G.; Nzekoue, F.K.; Fiorini, D.; Scocco, P.; Trabalza-Marinucci, M.; Acuti, G.; Tardella, F.M.; Sagratini, G.; Catorci, A. The effects of feeding supplementation on the nutritional quality of milk and cheese from sheep grazing on dry pasture. *Int. J. Food Sci. Nutr.* **2020**, *71*, 50–62. [CrossRef] [PubMed]
47. Recio, I.; de la Fuente, M.A.; Juárez, M.; Ramos, M. Bioactive components in sheep milk. In *Bioactive Components in Milk and Dairy Products*; Park, Y.W., Ed.; Wiley-Blackwell: Oxford, UK, 2009; pp. 83–104.
48. Sosa, J.; Althaus, R.L.; Scaglione, L.M.; Roldan, V.; Moreyra, E. Composición química y mineral de la leche de ovejas Corridale y Hampshire down. *FAVE* **2001**, *15*, 7–21. [CrossRef]
49. Khan, Z.I.; Ashraf, M.; Hussain, A.; McDowell, L.R.; Ashraf, M.Y. Concentrations of minerals in milk of sheep and goats grazing similar pastures in a semiarid region of Pakistan. *Small Rumin. Res.* **2006**, *65*, 274–278. [CrossRef]
50. Yabrir, B.; Hakem, A.; Mostefaoui, A.; Titouche, Y.; Bouzidi, A.; Mati, A. Nutritional value of Algerian breed ewe's milk related to its mineral content. *Pak. J. Nutr.* **2014**, *13*, 176–180. [CrossRef]
51. Barron, L.J.R.; de Labastida, E.F.; Perea, S.; Chávarri, F.; de Vega, C.; Vicente, M.S.; Torres, M.I.; Nájera, A.I.; Virto, M.; Santisteban, A.; et al. Seasonal changes in the composition of bulk raw ewe's milk used for Idiazabal cheese manufacture. *Int. Dairy J.* **2001**, *11*, 771–778. [CrossRef]
52. Lipko-Przybylska, J.; Albera, E.; Kankofer, M. Comparison of antioxidant defence parameters in colostrum and milk between Berrichon du Cher ewes and Uhrusk ewes. *J. Dairy Res.* **2010**, *77*, 117–122. [CrossRef]
53. Hejtmánková, A.; Pivec, V.; Trnková, E.; Dragounová, H. Differences in the composition of total and whey proteins in goat and ewe milk and their changes throughout the lactation period. *Czech J. Anim. Sci.* **2012**, *57*, 323–331. [CrossRef]
54. Alizadeh, A.; Ehsani, M.R.; Rofehgarinejad, L. Fatty acid profile and cholesterol content of Ghezel sheep milk during lactation period. *Acta Aliment.* **2017**, *46*, 457–463. [CrossRef]
55. Valenti, B.; Luciano, G.; Morbidini, L.; Rossetti, U.; Codini, M.; Avondo, M.; Priolo, A.; Bella, M.; Nataleno, A.; Pauselli, M. Dietary pomegranate pulp: Effect on ewe milk quality during late lactation. *Animals* **2019**, *9*, 283. [CrossRef]
56. Kawęcka, A.; Pasternak, M.; Słoniewska, D.; Miksza-Cybulska, A.; Bagnicka, E. Quality of mountain sheep milk used for the production of traditional cheeses. *Ann. Anim. Sci.* **2020**, *20*, 299–314. [CrossRef]
57. Valdivielso, I.; Bustamante, M.A.; Aldezabal, A.; Amores, G.; Virto, M.; de Gordoa, J.R.; de Renobales, M.; Barron, L.J.R. Case study of a commercial sheep flock under extensive mountain grazing: Pasture derived lipid compounds in milk and cheese. *Food Chem.* **2016**, *197*, 622–633. [CrossRef] [PubMed]
58. Garcia, C.; Lutz, N.W.; Confort-Gouny, S.; Cozzone, P.J.; Armand, M.; Bernard, M. Phospholipid fingerprints of milk from different mammalians determined by 31P NMR: Towards specific interest in human health. *Food Chem.* **2012**, *135*, 1777–1783. [CrossRef] [PubMed]
59. Belaunzaran, X.; Bessa, R.J.B.; Lavín, P.; Mantecón, A.R.; Kramer, J.K.G.; Aldai, N. Horse-meat for human consumption—Current research and future opportunities. *Meat Sci.* **2015**, *108*, 74–81. [CrossRef] [PubMed]
60. De Caro, J.; Eydoux, C.; Chérif, S.; Lebrun, R.; Gargouri, Y.; Carrière, F.; De Caro, A. Occurrence of pancreatic lipase-related protein-2 in various species and its relationship with herbivore diet. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **2008**, *150*, 1–9. [CrossRef] [PubMed]
61. Amara, S.; Lafont, D.; Parsiegla, G.; Point, V.; Chabannes, A.; Rousset, A.; Carrière, F. The galactolipase activity of some microbial lipases and pancreatic enzymes. *Eur. J. Lipid Sci. Technol.* **2013**, *115*, 442–451. [CrossRef]
62. Sahaka, M.; Amara, S.; Wattanakul, J.; Gedi, M.A.; Aldai, N.; Parsiegla, G.; Lecomte, J.; Christeller, J.T.; Gray, D.; Gontero, B.; et al. The digestion of galactolipids and its ubiquitous function in nature for the uptake of the essential α-linolenic acid. *Food Funct.* **2020**, *11*, 6710–6744. [CrossRef]

63. Medhammar, E.; Wijesinha-Bettoni, R.; Stadlmayr, B.; Nilsson, E.; Charrondiere, U.R.; Burlingame, B. Composition of milk from minor dairy animals and buffalo breeds: A biodiversity perspective. *J. Sci. Food Agric.* **2012**, *92*, 445–474. [CrossRef]
64. Belaunzaran, X.; Lavín, P.; Barron, L.J.R.; Mantecón, A.R.; Kramer, J.K.G.; Aldai, N. An assessment of the fatty acid composition of horse-meat available at the retail level in northern Spain. *Meat Sci.* **2017**, *124*, 39–47. [CrossRef]
65. Pietrzak-Fiećko, R.; Tomczyński, R.; Smoczyński, S.S. Effect of lactation period on the fatty acid composition in mares' milk from different breeds. *Arch. Anim. Breed.* **2013**, *56*, 335–343. [CrossRef]
66. Navrátilová, P.; Pospíšil, J.; Borkovcová, I.; Kaniová, L.; Dluhošová, S.; Horáková, S. Content of nutritionally important components in mare milk fat. *Mljekarstvo* **2018**, *68*, 282–294. [CrossRef]
67. Kouba, J.M.; Burns, T.A.; Webel, S.K. Effect of dietary supplementation with long-chain n-3 fatty acids during late gestation and early lactation on mare and foal plasma fatty acid composition, milk fatty acid composition, and mare reproductive variables. *Anim. Reprod. Sci.* **2019**, *203*, 33–44. [CrossRef] [PubMed]
68. Deng, L.; Yang, Y.; Li, Z.; Li, J.; Zhu, Y.; Meng, Q.; Liu, J.; Wang, X. Impact of different dietary regimens on the lipidomic profile of mare's milk. *Food Res. Int.* **2022**, *156*, 111305. [CrossRef] [PubMed]
69. Wohlt, J.E.; Kleyn, D.H.; Vandernoot, G.W.; Selfridge, D.J.; Novotney, C.A. Effect of stage of lactation, age of ewe, sibling status, and sex of lamb on gross and minor constituents of Dorset ewe milk. *J. Dairy Sci.* **1981**, *64*, 2175–2184. [CrossRef]
70. Bruckmaier, R.M.; Gross, J.J. Lactational challenges in transition dairy cows. *Anim. Prod. Sci.* **2017**, *57*, 1471–1481. [CrossRef]
71. Deichsel, K.; Aurich, J. Lactation and lactational effects on metabolism and reproduction in the horse mare. *Livest. Prod. Sci.* **2005**, *98*, 25–30. [CrossRef]
72. Aldai, N.; de Renobales, M.; Barron, L.J.R.; Kramer, J.K.G. What are the trans fatty acids issues in foods after discontinuation of industrially produced trans fats? Ruminant products, vegetable oils, and synthetic supplements. *Eur. J. Lipid Sci. Technol.* **2013**, *115*, 1378–1401. [CrossRef]
73. Mazhitova, A.T.; Kulmyrzaev, A.A.; Ozbekova, Z.E.; Bodoshev, A. Amino acid and fatty acid profile of the mare's milk produced on Suusamyr pastures of the Kyrgyz Republic during lactation period. *Procedia Soc. Behav. Sci.* **2015**, *195*, 2683–2688. [CrossRef]
74. Kramer, J.K.G.; Hernandez, M.; Cruz-Hernandez, C.; Kraft, J.; Dugan, M.E.R. Combining results of two GC separations partly achieves determination of all cis and trans 16:1, 18:1, 18:2 and 18:3 except CLA isomers of milk fat as demonstrated using Ag-ion SPE fractionation. *Lipids* **2008**, *43*, 259–273. [CrossRef] [PubMed]
75. Delmonte, P.; Ali-Reza, F.K.; Kramer, J.K.G.; Mossoba, M.; Sidisky, L.; Rader, J.I. Separation characteristics of fatty acid methyl esters using SLB-IL111, a new ionic liquid coated capillary gas chromatographic column. *J. Chromatogr. A* **2011**, *1218*, 545–554. [CrossRef] [PubMed]
76. Malacarne, M.; Martuzzi, F.; Summer, A.; Mariani, P. Protein and fat composition of mare's milk: Some nutritional remarks with reference to human and cow's milk. *Int. Dairy J.* **2002**, *12*, 869–877. [CrossRef]
77. Uniacke-Lowe, T.; Huppertz, T.; Fox, P.F. Equine milk proteins: Chemistry, structure and nutritional significance. *Int. Dairy J.* **2010**, *20*, 609–629. [CrossRef]
78. Foekel, C.; Schubert, R.; Kaatz, M.; Schmidt, I.; Bauer, A.; Hipler, U.; Vogelsang, H.; Rabe, K.; Jahreis, G. Dietetic effects of oral intervention with mare's milk on the severity scoring of atopic dermatitis, on faecal microbiota and on immunological parameters in patients with atopic dermatitis. *Int. J. Food Sci. Nutr.* **2009**, *60*, 41–52. [CrossRef] [PubMed]
79. Businco, L.; Giampietro, P.G.; Lucenti, P.; Lucaroni, F.; Pini, C.; Di Felice, G.; Iacovacci, P.; Curadi, C.; Orlandi, M. Allergenicity of mare's milk in children with cow's milk allergy. *J. Allergy Clin. Immunol.* **2000**, *105*, 1031–1034. [CrossRef]
80. Curadi, M.C.; Giampietro, P.G.; Lucenti, P.; Orlandi, M. Use of mare milk in pediatric allergology. Recent progress in animal production science. In Proceedings of the Animal Science and Production Association XIII Congress, Piacenza, Italy, 21–24 June 1999.
81. Zhao, S.; Pan, F.; Cai, S.; Yi, J.; Zhou, L.; Liu, Z. Secrets behind protein sequences: Unveiling the potential reasons for varying allergenicity caused by caseins from cows, goats, camels, and mares based on bioinformatics analyses. *Int. J. Mol. Sci.* **2023**, *24*, 2481. [CrossRef]
82. Inglingstad, R.A.; Devold, T.G.; Eriksen, E.K.; Holm, H.; Jacobsen, M.; Liland, K.H.; Rukke, E.O.; Vegarud, G.E. Comparison of the digestion of caseins and whey proteins in equine, bovine, caprine and human milks by human gastrointestinal enzymes. *Dairy Sci. Technol.* **2010**, *90*, 549–563. [CrossRef]
83. Xiao, T.; Zeng, J.; Zhao, C.; Hou, Y.; Wu, T.; Deng, Z.; Zheng, L. Comparative analysis of protein digestion characteristics in human, cow, goat, sheep, mare, and camel milk under simulated infant condition. *J. Agric. Food Chem.* **2023**, *71*, 15035–15047. [CrossRef]
84. Schweigert, F.J.; Gottwald, C. Effect of parturition on levels of vitamins A and E and of β-carotene in plasma and milk of mares. *Equine Veter J.* **1999**, *31*, 319–323. [CrossRef]
85. Greiwe-Crandell, K.M.; Kronfeld, D.S.; Gay, L.S.; Sklan, D.; Tiegs, W.; Harris, P.A. Vitamin A repletion in Thoroughbred mares with retinyl palmitate or β-carotene. *J. Anim. Sci.* **1997**, *75*, 2684–2690. [CrossRef] [PubMed]
86. Kuhl, J.; Aurich, J.E.; Wulf, M.; Hurtienne, A.; Schweigert, F.J.; Aurich, C. Effects of oral supplementation with β-carotene on concentrations of β-carotene, vitamin A and α-tocopherol in plasma, colostrum and milk of mares and plasma of their foals and on fertility in mares. *J. Anim. Physiol. Anim. Nutr.* **2012**, *96*, 376–384. [CrossRef] [PubMed]
87. Bondo, T.; Jensen, S.K. Administration of RRR-α-tocopherol to pregnant mares stimulates maternal IgG and IgM production in colostrum and enhances vitamin E and IgM status in foals. *J. Anim. Physiol. Anim. Nutr.* **2011**, *95*, 214–222. [CrossRef]

88. Danyer, E.; Bilal, T. Effects of dietary fish oil and alpha-tocopherol supplementation on selected blood parameters and fatty acid profiles in mares and their foals. *J. Anim. Physiol. Anim. Nutr.* **2021**, *105*, 3–17. [CrossRef]
89. Magan, J.B.; O'Callaghan, T.F.; Zheng, J.; Zhang, L.; Mandal, R.; Hennessy, D.; Fenelon, M.A.; Wishart, D.S.; Kelly, A.L.; McCarthy, N.A. Effect of diet on the vitamin B profile of bovine milk-based protein ingredients. *Foods* **2020**, *9*, 578. [CrossRef]
90. Blanco-Doval, A.; Barron, L.J.R.; Aldai, N. Changes during lactation in the mineral element content of mare milk produced in semi-extensive rural farms. *J. Food Compos. Anal.* **2023**, *123*, 105629. [CrossRef]
91. Albrecht, S.; Lane, J.A.; Marino, K.; Al Busadah, K.A.; Carrington, S.D.; Hickey, R.M.; Rudd, P.M. A comparative study of free oligosaccharides in the milk of domestic animals. *Br. J. Nutr.* **2014**, *111*, 1313–1328. [CrossRef]
92. Difilippo, E.; Willems, H.; Vendrig, J.C.; Fink-Gremmels, J.; Gruppen, H.; Schols, H.A. Comparison of milk oligosaccharides pattern in colostrum of different horse breeds. *J. Agric. Food Chem.* **2015**, *63*, 4805–4814. [CrossRef] [PubMed]
93. Karav, S.; Salcedo, J.; Frese, S.A.; Barile, D. Thoroughbred mare's milk exhibits a unique and diverse free oligosaccharide profile. *FEBS Open Bio* **2018**, *8*, 1219–1229. [CrossRef]
94. Rzekęć, A.; Vial, C.; Bigot, G. Green assets of equines in the European context of the ecological transition of agriculture. *Animals* **2020**, *10*, 106. [CrossRef]
95. European Commission. The Common Agricultural Policy: 2023-27. 2023. Available online: https://agriculture.ec.europa.eu/common-agricultural-policy/cap-overview/cap-2023-27_en (accessed on 26 June 2023).
96. Hoffmann, I. Livestock biodiversity and sustainability. *Livest. Sci.* **2011**, *139*, 69–79. [CrossRef]
97. Ryschawy, J.; Disenhaus, C.; Bertrand, S.; Allaire, G.; Aznar, O.; Plantureux, S.; Josien, E.; Guinot, C.; Lasseur, J.; Perrot, C.; et al. Assessing multiple goods and services derived from livestock farming on a nation-wide gradient. *Animal* **2017**, *11*, 1861–1872. [CrossRef] [PubMed]
98. Dumont, B.; Ryschawy, J.; Duru, M.; Benoit, M.; Chatellier, V.; Delaby, L.; Donnars, C.; Dupraz, P.; Lemauviel-Lavenant, S.; Méda, B.; et al. Review: Associations among goods, impacts and ecosystem services provided by livestock farming. *Animal* **2019**, *13*, 1773–1784. [CrossRef] [PubMed]
99. FAO. *Livestock Keepers—Guardians of Biodiversity*; Food and Agriculture Organization: Rome, Italy, 2009.
100. Caballero, R.; Fernandez-Gonzalez, F.; Badia, R.P.; Molle, G.; Roggero, P.P.; Bagella, S.; D'Ottavio, P.; Papanastasis, V.P.; Fotiadis, G.; Siddiropoulou, A.; et al. Grazing systems and biodiversity in Mediterranean areas: Spain, Italy and Greece. *Pastos* **2009**, *39*, 9–154.
101. Salvia, R.; Egidi, G.; Vinci, S.; Salvati, L. Desertification risk and rural development in southern Europe: Permanent assessment and implications for sustainable land management and mitigation policies. *Land* **2019**, *8*, 191. [CrossRef]
102. Brown, J.H.; Pilliner, S.; Davies, Z. *Horse and Stable Management*; John Wiley & Sons: Sussex, UK, 2003.
103. Elghandour, M.M.M.; Adegbeye, M.J.; Barbabosa-Pilego, A.; Perez, N.R.; Hernández, S.R.; Zaragoza-Bastida, A.; Salem, A.Z.M. Equine contribution in methane emission and its mitigation strategies. *J. Equine Veter Sci.* **2019**, *72*, 56–63. [CrossRef]
104. Menard, C.; Duncan, P.; Fleurance, G.; Georges, J.; Lila, M. Comparative foraging and nutrition of horses and cattle in European wetlands. *J. Appl. Ecol.* **2002**, *39*, 120–133. [CrossRef]
105. Karmiris, I.; Platis, P.D.; Kazantzidis, S.; Papachristou, T.G. Diet selection by domestic and wild herbivore species in a coastal Mediterranean wetland. *Ann. Zool. Fenn.* **2011**, *48*, 233–242. [CrossRef]
106. Fleurance, G.; Farruggia, A.; Lanore, L.; Dumont, B. How does stocking rate influence horse behaviour, performances and pasture biodiversity in mesophile grasslands? *Agric. Ecosyst. Environ.* **2016**, *231*, 255–263. [CrossRef]
107. Nolte, S.; van der Weyde, C.; Esselink, P.; Smit, C.; van Wieren, S.E.; Bakker, J.P. Behaviour of horses and cattle at two stocking densities in a coastal salt marsh. *J. Coast. Conserv.* **2017**, *21*, 369–379. [CrossRef]
108. Duncan, P.; Foose, T.J.; Gordon, I.J.; Gakahu, C.G.; Lloyd, M. Comparative nutrient extraction from forages by grazing bovids and equids: A test of the nutritional model of equid/bovid competition and coexistence. *Oecologia* **1990**, *84*, 411–418. [CrossRef] [PubMed]
109. Celaya, R.; Ferreira, L.; García, U.; García, R.R.; Osoro, K. Diet selection and performance of cattle and horses grazing in heathlands. *Animal* **2011**, *5*, 1467–1473. [CrossRef] [PubMed]
110. Celaya, R.; Ferreira, L.; García, U.; García, R.R.; Osoro, K. Heavy grazing by horses on heathlands of different botanical composition. In *Forages and Grazing in Horse Nutrition*; Saastamoinen, M., Fradinho, M.J., Santos, A.S., Miraglia, N., Eds.; Wageningen Academic Publishers: Wageningen, The Netherlands, 2012; pp. 219–226.
111. Bigot, G.; Brétiére, G.; Micol, D.; Turpin, N. Management of cattle and draught horse to maintain openness of landscapes in French Central Mountains. In Proceedings of the 17th Meeting of the FAO-CIHEAM Mountain Pasture Network—Pastoralims and Ecosystem Conservation, Trivero, Italy, 5–7 June 2013.
112. Ferreira, L.M.M.; Celaya, R.; Benavides, R.; Jáuregui, B.M.; García, U.; Santos, A.S.; García, R.R.; Rodrigues, M.A.M.; Osoro, K. Foraging behaviour of domestic herbivore species grazing on heathlands associated with improved pasture areas. *Livest. Sci.* **2013**, *155*, 373–383. [CrossRef]
113. López, C.L.; Celaya, R.; Ferreira, L.M.M.; García, U.; Rodrigues, M.A.M.; Osoro, K. Comparative foraging behaviour and performance between cattle and horses grazing in heathlands with different proportions of improved pasture area. *J. Appl. Anim. Res.* **2019**, *47*, 377–385. [CrossRef]
114. López, C.L.; Ferreira, L.M.M.; García, U.; Moreno-Gonzalo, J.; Rodrigues, M.A.M.; Osoro, K.; Ferre, I.; Celaya, R. Diet selection and performance of horses grazing on different heathland types. *Animal* **2017**, *11*, 1708–1717. [CrossRef] [PubMed]

115. López, C.L.; García, R.R.; Ferreira, L.M.M.; García, U.; Osoro, K.; Celaya, R. Impacts of horse grazing on botanical composition and diversity in different types of heathland. *Rangel. J.* **2017**, *39*, 375–385. [CrossRef]
116. Osoro, K.; Ferreira, L.; García, U.; Martínez, A.; Celaya, R. Forage intake, digestibility and performance of cattle, horses, sheep and goats grazing together on an improved heathland. *Anim. Prod. Sci.* **2017**, *57*, 102–109. [CrossRef]
117. Edouard, N.; Fleurance, G.; Dumont, B.; Baumont, R.; Duncan, P. Does sward height affect feeding patch choice and voluntary intake in horses? *Appl. Anim. Behav. Sci.* **2009**, *119*, 219–228. [CrossRef]
118. Odberg, F.O.; Francis-Smith, K. A study on eliminative and grazing behavior—The use of the field by captive horses. *Equine Veter J.* **1976**, *8*, 147–149. [CrossRef]
119. Taylor, E.L. Grazing behavior and helminthic disease. *Br. J. Anim. Behav.* **1954**, *2*, 61–62. [CrossRef]
120. Fleurance, G.; Duncan, P.; Fritz, H.; Cabaret, J.; Cortet, J.; Gordon, I.J. Selection of feeding sites by horses at pastures: Testing the antiparasite theory. *Appl. Anim. Behav. Sci.* **2007**, *108*, 288–301. [CrossRef]
121. Fleurance, G.; Duncan, P.; Fritz, H.; Cabaret, J.; Gordon, I.J. Importance of nutritional and anti-parasite strategies in the foraging decisions of horses: An experimental test. *Oikos* **2005**, *110*, 602–612. [CrossRef]
122. Dumont, B.; Rossignol, N.; Loucougaray, G.; Carrère, P.; Chadoeuf, J.; Fleurance, G.; Bonis, A.; Farruggia, A.; Gaucherand, S.; Ginane, C.; et al. When does grazing generate stable vegetation patterns in temperate pastures? *Agric. Ecosyst. Environ.* **2012**, *153*, 50–56. [CrossRef]
123. Illius, A.W.; Gordon, I.J. The allometry of Food intake in grazing ruminants. *J. Anim. Ecol.* **1987**, *56*, 989–999. [CrossRef]
124. Ringmark, S.; Skarin, A.; Jansson, A. Impact of year-round grazing by horses on pasture nutrient dynamics and the correlation with pasture nutrient content and fecal nutrient composition. *Animals* **2019**, *9*, 500. [CrossRef]
125. Collas, C.; Fleurance, G.; Cabaret, J.; Martin-Rosset, W.; Wimel, L.; Cortet, J.; Dumont, B. How does the suppression of energy supplementation affect herbage intake, performance and parasitism in lactating saddle mares? *Animal* **2014**, *8*, 1290–1297. [CrossRef]
126. Brinkmann, L.; Gerken, M.; Riek, A. Adaptation strategies to seasonal changes in environmental conditions of a domesticated horse breed, the Shetland pony (*Equus ferus caballus*). *J. Exp. Biol.* **2012**, *215*, 1061–1068. [CrossRef] [PubMed]
127. Köhler, M.; Hiller, G.; Tischew, S. Year-round horse grazing supports typical vascular plant species, orchids and rare bird communities in a dry calcareous grassland. *Agric. Ecosyst. Environ.* **2016**, *234*, 48–57. [CrossRef]
128. Gilhaus, K.; Hölzel, N. Seasonal variations of fodder quality and availability as constraints for stocking rates in year-round grazing schemes. *Agric. Ecosyst. Environ.* **2016**, *234*, 5–15. [CrossRef]
129. Arnold, W.; Ruf, T.; Kuntz, R. Seasonal adjustment of energy budget in a large wild mammal, the Przewalski horse (*Equus ferus przewalskii*) II. Energy expenditure. *J. Exp. Biol.* **2006**, *209*, 4566–4573. [CrossRef]
130. Stachurska, A.; Pluta, M.; Wójcik, M.; Giżejewski, Z.; Janczarek, I.; Różańska-Boczula, M. Coat cover hair density is a symptom of primitive horse adaptation to the environment. *Pferdeheilkunde Equine Med.* **2018**, *34*, 550–556. [CrossRef]
131. Habeck, J.O. Republic of Sakha (Yakutia). 2000. Available online: https://www.spri.cam.ac.uk/resources/rfn/sakha.html#ref (accessed on 26 June 2023).
132. Librado, P.; Der Sarkissian, C.; Ermini, L.; Schubert, M.; Jónsson, H.; Albrechtsen, A.; Fumagalli, M.; Yang, M.A.; Gamba, C.; Seguin-Orlando, A.; et al. Tracking the origins of Yakutian horses and the genetic basis for their fast adaptation to subarctic environments. *Proc. Natl. Acad. Sci. USA* **2015**, *112*, E6889–E6897. [CrossRef] [PubMed]
133. Pereladova, O.B.; Sempéeré, A.J.; Soldatova, N.V.; Dutov, V.U.; Fisenko, G.; Flint, V.E. Przewalski's horse—Adaptation to semi-wild life in desert conditions. *Oryx* **1999**, *33*, 47–58. [CrossRef]
134. Sneddon, J.C.; Van Der Walt, J.G.; Mitchell, G. Water homeostasis in desert-dwelling horses. *J. Appl. Physiol.* **1991**, *71*, 112–117. [CrossRef] [PubMed]
135. Marsoner, T.; Egarter Vigl, L.; Manck, F.; Jaritz, G.; Tappeiner, U.; Tasser, E. Indigenous livestock breeds as indicators for cultural ecosystem services: A spatial analysis within the alpine space. *Ecol. Indic.* **2018**, *94*, 55–63. [CrossRef]
136. Miraglia, N. Equids contribution to sustainable development in rural areas: A new challenge for the third millennium. In *Forages and Grazing in Horse Nutrition*; Saastamoinen, M., Fradinho, M.J., Santos, A.S., Miraglia, N., Eds.; Wageningen Academic Publishers: Wageningen, The Netherlands, 2012; pp. 439–452.
137. Degen, A.A. Sheep and goat milk in pastoral societies. *Small Rumin. Res.* **2007**, *68*, 7–19. [CrossRef]
138. Boyce, P.N.; McLoughlin, P.D. Ecological interactions involving feral horses and predators: Review with implications for biodiversity conservation. *J. Wildl. Manag.* **2021**, *85*, 1091–1103. [CrossRef]
139. Richmond, S.E.; Wemelsfelder, F.; Beltrán de Heredia, I.; Ruíz, R.; Canali, E.; Dwyer, C.M. Evaluation of animal-based indicators to be used in a welfare assessment protocol for sheep. *Front. Veter Sci.* **2017**, *4*, 210. [CrossRef] [PubMed]
140. Minero, M.; Dalla Costa, E.; Dai, F. AWIN welfare assessment protocol for horses. In *AWIN Animal Welfare Indicators*; European Union's Seventh Framework Programme: Milan, Italy, 2015.
141. Feh, C. Relationships and communication in socially natural horse herds. In *The Domestic Horse: The Origins, Development and Management of Its Behavior*; Mills, D.S., McDonnell, S.M., Eds.; Cambridge University Press: Cambridge, UK, 2005; pp. 83–94.
142. Waters, A.J.; Nicol, C.J.; French, N.P. Factors influencing the development of stereotypic and redirected behaviours in young horses: Findings of a four year prospective epidemiological study. *Equine Veter J.* **2002**, *34*, 572–579. [CrossRef]
143. Popescu, S.; Lazar, E.A.; Borda, C.; Niculae, M.; Sandru, C.D.; Spinu, M. Welfare quality of breeding horses under different housing conditions. *Animals* **2019**, *9*, 81. [CrossRef]

144. European Commission. *Feasibility Study on Animal Welfare Labelling and Establishing a Community Reference Centre for Animal Protection and Welfare—Part 1: Animal Welfare Labelling*; Directorate-General for Health and Food Safety: Brussels, Belgium, 2009.
145. Animal Welfair. Available online: https://www.animalwelfair.com/es/ (accessed on 13 March 2024).
146. European Commission. For a fair, healthy and environmentally-friendly food system farm to fork strategy. In *Communication (2020) 381 from the Commission to the European Parliament, the Council, the European Economic and Social Committee and the Committee of the Regions*; European Commission: Brussels, Belgium, 2020.
147. European Commission. Available online: https://food.ec.europa.eu/animals/animal-welfare/eu-platform-animal-welfare/thematic-sub-groups/animal-welfare-labelling_en (accessed on 26 June 2023).
148. European Commission. *Commission Regulation No 889/2008 of 5 September 2008 Laying Down Detailed Rules for the Implementation of Council Regulation No 834/2007 on Organic Production and Labelling of Organic Products with Regard to Organic Production, Labelling and Control*; European Commission: Brussels, Belgium, 2008.
149. Loo, E.; Lai, K.; Mansor, R. Antimicrobial usage and resistance in dairy cattle production. In *Veterinary Medicine and Pharmaceuticals*; Bekoe, S.O., Saravanan, M., Adosraku, R.K., Ramkumar, P.K., Eds.; IntechOpen: London, UK, 2019; pp. 7–16.
150. Rodríguez-Ortega, T.; Oteros-Rozas, E.; Ripoll-Bosch, R.; Tichit, M.; Martín-López, B.; Bernués, A. Applying the ecosystem services framework to pasture-based livestock farming systems in Europe. *Animal* **2014**, *8*, 1361–1372. [CrossRef]
151. Gocht, A.; Espinosa, M.; Leip, A.; Lugato, E.; Schroeder, L.A.; Van Doorslaer, B.; Paloma, S.G. A grassland strategy for farming systems in Europe to mitigate GHG emissions—An integrated spatially differentiated modelling approach. *Land Use Policy* **2016**, *58*, 318–334. [CrossRef]
152. Soussana, J.; Tallec, T.; Blanfort, V. Mitigating the greenhouse gas balance of ruminant production systems through carbon sequestration in grasslands. *Animal* **2010**, *4*, 334–350. [CrossRef] [PubMed]
153. Milchunas, D.G.; Sala, O.E.; Lauenroth, W.K. A generalized model for the effects of grazing by large herbivores on grassland community structure. *Am. Nat.* **1988**, *132*, 87–106. [CrossRef]
154. FAO. *Livestock's Long Shadow: Environmental Issues and Options*; Food and Agriculture Organization: Rome, Italy, 2006. Available online: https://www.fao.org/3/a0701e/a0701e00.htm (accessed on 27 July 2023).
155. Dumont, B.; Tallowin, J.R. Interactions between grassland management and species diversity. In *Grassland Productivity and Ecosystem Services*; Lemaire, G., Hodgson, J., Chabbi, A., Eds.; Centre for Agricultural Bioscience International: Surrey, UK, 2011; pp. 129–137.
156. Bernués, A. Animals on the land. Ecosystem services and disservices of grazing livestock systems. In *The Meat Crisis: Developing More Sustainable and Ethical Production and Consumption*, 2nd ed.; D'Silva, J., Webster, J., Eds.; Taylor & Francis Group: London, UK, 2017; pp. 67–92.
157. Loucougaray, G.; Bonis, A.; Bouzillé, J. Effects of grazing by horses and/or cattle on the diversity of coastal grasslands in western France. *Biol. Conserv.* **2004**, *116*, 59–71. [CrossRef]
158. Stewart, G.B.; Pullin, A.S. The relative importance of grazing stock type and grazing intensity for conservation of mesotrophic 'old meadow' pasture. *J. Nat. Conserv.* **2008**, *16*, 175–185. [CrossRef]
159. Garrido, P.A.; Marell, A.; Öckinger, E.; Skarin, A.; Jansson, A.; Thulin, C.G. Experimental rewilding enhances grassland functional composition and pollinator habitat use. *J. Appl. Ecol.* **2019**, *56*, 946–955. [CrossRef]
160. Rigueiro-Rodríguez, A.; Mouhbi, R.; Santiago-Freijanes, J.J.; Gonzalez-Hernandez, M.D.P.; Mosquera-Losada, M.R. Horse grazing systems: Understory biomass and plant biodiversity of a Pinus radiata stand. *Sci. Agric.* **2012**, *69*, 38–46. [CrossRef]
161. Nolte, S.; Esselink, P.; Bakker, J.P. Flower production of *Aster tripolium* is affected by behavioral differences in livestock species and stocking densities: The role of activity and selectivity. *Ecol. Res.* **2013**, *28*, 821–831. [CrossRef]
162. Mosquera Losada, M.R.; Mouhbi, R.; González-Hernández, M.P.; Rigueiro Rodríguez, A. Efecto del Pastoreo con Caballo de Monte Sobre la Diversidad de Plantas Vasculares en dos Sistemas de Pastoreo (Rotacional y Continuo) con Sotobosque Dominado por *Ulex europaeus*. In Proceedings of the 5° Congreso Forestal Español—Montes y Sociedad: Saber qué Hacer. Sociedad Española de Ciencias Forestales-Junta de Castilla y León, Pontevedra, Spain, 21–25 September 2009.
163. Celaya, R.; Martínez, A.; Osoro, K. Vegetation dynamics in Cantabrian heathlands associated with improved pasture areas under single or mixed grazing by sheep and goats. *Small Rumin. Res.* **2007**, *72*, 165–177. [CrossRef]
164. Rook, A.J.; Tallowin, J.R. Grazing and pasture management for biodiversity benefit. *Anim. Res.* **2003**, *52*, 181–189. [CrossRef]
165. Milotic, T.; Quide, S.; Van Loo, T.; Hoffmann, M. Linking functional group richness and ecosystem functions of dung beetles: An experimental quantification. *Oecologia* **2017**, *183*, 177–190. [CrossRef] [PubMed]
166. Bloor, J.M.G.; Jay-Robert, P.; Le Morvan, A.; Fleurance, G. Déjections des herbivores domestiques au pasturage: Caractéristiques et role dans le fonctionnement des prairies. *INRAE Prod. Anim.* **2012**, *25*, 45–56. [CrossRef]
167. Haynes, R.J.; Williams, P.H. Nutrient cycling and soil fertility in the grazed pasture ecosystem. *Adv. Agron.* **1993**, *49*, 119–200.
168. INRA. *Equine Nutrition: INRA Nutrient Requirements, Recommended Allowances and Feed Tables*; Martin-Rosset, W., Ed.; Wageningen Academic Publishers: Wageningen, The Netherlands, 2015.
169. Parvage, M.M.; Ulén, B.; Kirchmann, H. Are horse paddocks threatening water quality through excess loading of nutrients? *J. Environ. Manag.* **2015**, *147*, 306–313. [CrossRef] [PubMed]
170. Gill, M.; Smith, P.; Wilkinson, J.M. Mitigating climate change: The role of domestic livestock. *Animal* **2010**, *4*, 323–333. [CrossRef] [PubMed]

171. Bernués, A.; Riedel, J.L.; Asensio, M.A.; Blanco, M.; Sanz, A.; Revilla, R.; Casasús, I. An integrated approach to studying the role of grazing livestock systems in the conservation of rangelands in a protected natural park (Sierra de Guara, Spain). *Livest. Prod. Sci.* **2005**, *96*, 75–85. [CrossRef]
172. Whitman, E.; Parisien, M.; Thompson, D.K.; Flannigan, M.D. Short-interval wildfire and drought overwhelm boreal forest resilience. *Sci. Rep.* **2019**, *9*, 18796. [CrossRef] [PubMed]
173. De Luis, M.; González-Hidalgo, J.C.; Raventós, J. Effects of fire and torrential rainfall on erosion in a Mediterranean gorse community. *Land Degrad. Dev.* **2003**, *14*, 203–213. [CrossRef]
174. Certini, G. Effects of fire on properties of forest soils: A review. *Oecologia* **2005**, *143*, 1–10. [CrossRef]
175. Insausti, K.; Beldarrain, L.R.; Lavín, M.P.; Aldai, N.; Mantecón, Á.R.; Sáez, J.L.; Canals, R.M. Horse meat production in northern Spain: Ecosystem services and sustainability in high nature value farmland. *Anim. Front.* **2021**, *11*, 47–54. [CrossRef] [PubMed]
176. Pardini, A.; Natali, F.; Tallarico, R. Horse grazing in firebreaks sown with *Trifolium brachycalycinum* (Katznl. & Morley) and *Cynodon* dactylon (L.). Pers. *Agrofor. Syst.* **2007**, *70*, 17–24.
177. Gerber, P.J.; Steinfeld, H.; Henderson, B.; Mottet, A.; Opio, C.; Dijkman, J.; Falcucci, A.; Tempio, G. *Tackling Climate Change through Livestock: A Global Assessment of Emissions and Mitigation Opportunities*; Food and Agriculture Organization: Rome, Italy, 2013.
178. Moss, A.R.; Jouany, J.; Newbold, J. Methane production by ruminants: Its contribution to global warming. *Ann. Zootech.* **2000**, *49*, 231–253. [CrossRef]
179. Franz, R.; Soliva, C.R.; Kreuzer, M.; Steuer, P.; Hummel, J.; Clauss, M. Methane production in relation to body mass of ruminants and equids. *Evol. Ecol. Res.* **2010**, *12*, 727–738.
180. Vermorel, M.; Jouany, J.P.; Eugene, M.; Sauvant, D.; Noblet, J.; Dourmad, J.Y. Evaluation quantitative des emissions de methane enterique par les animaux d'elevage en 2007 en France. *INRAE Prod. Anim.* **2008**, *21*, 403–418.
181. Steinfeld, H.; Gerber, P. Livestock production and the global environment: Consume less or produce better? *Proc. Natl. Acad. Sci. USA* **2010**, *107*, 18237–18238. [CrossRef] [PubMed]
182. FAOSTAT. Production Data of FAOSTAT. 2021. Available online: http://www.fao.org/faostat/en/#home (accessed on 26 June 2023).
183. Lamy, A.; Costa, S.; Vial, C.; Badji, I.; Carrere, M.; Rollet, P.; Amiot, M.J. Horsemeat consumption in France: Determinants and sustainable market perspectives. *Meat Sci.* **2023**, *198*, 109083. [CrossRef]
184. Sebbane, M.; Vial, C.; Lamy, A. A horse in your plate? A cluster analysis of French consumers hippophagy acceptance. *Meat Sci.* **2023**, *203*, 109220. [CrossRef]
185. Jaskari, M.; Leipämaa-Leskinen, H.; Syrjälä, H. Revealing the paradoxes of horsemeat—The challenges of marketing horsemeat in Finland. *Nord. J. Bus.* **2015**, *64*, 86–102.
186. Reist, S.; Hintermann, F.; Sommer, R. The Livestock Revolution: An Opportunity for Poor Farmers? InfoResources Focus (1/07). 2007; 1–16. Available online: www.inforesources.ch/pdf/focus07_1_e.pdf(accessed on 14 March 2023).
187. FAO. *Migration, Agriculture and Rural Development: Addressing the Root Causes of Migration and Harnessing Its Potential for Development*; Food and Agriculture Organization: Rome, Italy, 2016.
188. Cobano-Delgado, V.; Llorent-Bedmar, V. Women's well-being and rural development in depopulated Spain. *Int. J. Environ. Res. Public Health* **2020**, *17*, 1966. [CrossRef]
189. Cooper, T.; Hart, K.; Baldock, D. *Provision of Public Goods through Agriculture in the European Union*; Institute for European Environmental Policy: London, UK, 2009.
190. ESPON. Shrinking rural regions in Europe: Towards smart and innovative approaches to regional development challenges in depopulating rural regions. In *ESPON Policy Brief*; ESPON: Luxembourg, 2017.
191. European Union. Rural Areas and the Primary Sector in the EU. 2018. Available online: https://ec.europa.eu/info/food-farming-fisheries/farming/facts-and-figures/rural-areas_en (accessed on 26 June 2023).
192. Ustaoglu, E.; Collier, M.J. Farmland abandonment in Europe: An overview of drivers, consequences, and assessment of the sustainability implications. *Environ. Rev.* **2018**, *26*, 396–416. [CrossRef]
193. European Commission. Food and farming: Focus on land. In *The 2015 EU Agricultural Outlook Conference*; European Commission: Brussels, Belgium, 2015.
194. ILOSTAT. *Employment in Agriculture (% of Total Employment) (Modeled ILO Estimate)—European Union*; ILOSTAT, 2020. Available online: https://ilostat.ilo.org/data/ (accessed on 22 April 2024).
195. Dumont, B.; Dupraz, P.; Sabatier, R.; Donnars, C. A collective scientific assessment of the roles, impacts, and services associated with livestock production systems in Europe. *Forages* **2017**, *229*, 63–76.
196. European Horse Network. The European Horse Industry in the European Regions—Key Figures 2010. 2010. Available online: http://parimutuel-europe.org/Download/Key_Figures_2010_EN.pdf (accessed on 27 July 2023).
197. Cordilhac, C.; Abellan, C. Jobs in the French equine sector. In *The New Equine Economy in the 21st Century*; Vial, C., Evans, R., Eds.; Wageningen Academic Publishers: Wageningen, The Netherlands, 2015; pp. 55–60.
198. Vial, C.; Barget, E.; Clipet, F. Economic impact of equestrian events, examples from France. In *The New equine Economy in the 21st Century*; Vial, C., Evans, R., Eds.; Wageningen Academic Publishers: Wageningen, The Netherlands, 2015; pp. 21–32.
199. Meert, H.; Van Huylenbroeck, G.; Vernimmen, T.; Bourgeois, M.; van Hecke, E. Farm household survival strategies and diversification on marginal farms. *J. Rural. Stud.* **2005**, *21*, 81–97. [CrossRef]

200. Jouven, M.; Vial, C.; Fleurance, G. Horses and rangelands: Perspectives in Europe based on a French case study. *Grass Forage Sci.* **2016**, *71*, 178–194. [CrossRef]
201. Bernués, A.; Tello-García, E.; Rodríguez-Ortega, T.; Ripoll-Bosch, R.; Casasús, I. Agricultural practices, ecosystem services and sustainability in high nature value farmland: Unraveling the perceptions of farmers and nonfarmers. *Land Use Policy* **2016**, *59*, 130–142. [CrossRef]
202. Adler, P.; Raff, D.; Lauenroth, W. The effect of grazing on the spatial heterogeneity of vegetation. *Oecologia* **2001**, *128*, 465–479. [CrossRef] [PubMed]
203. Bernués, A.; Alfnes, F.; Clemetsen, M.; Eik, L.O.; Faccioni, G.; Ramanzin, M.; Ripoll-Bosch, R.; Rodríguez-Ortega, T.; Sturaro, E. Exploring social preferences for ecosystem services of multifunctional agriculture across policy scenarios. *Ecosyst. Serv.* **2019**, *39*, 101002. [CrossRef]
204. Faccioni, G.; Sturaro, E.; Ramanzin, M.; Bernués, A. Socio-economic valuation of abandonment and intensification of alpine agroecosystems and associated ecosystem services. *Land Use Policy* **2019**, *81*, 453–462. [CrossRef]
205. Van Eetvelde, V.; Antrop, M. Analyzing structural and functional changes of traditional landscapes—Two examples from southern France. *Landsc. Urban Plan.* **2004**, *67*, 79–95. [CrossRef]
206. Scherf, B.D.; Pilling, D. *The Second Report on the State of the World's Animal Genetic Resources for Food and Agriculture*; Commission on Genetic Resources for Food and Agriculture Assessments, Food and Agriculture Organization: Rome, Italy, 2015.
207. FAO. *Status and Trends of Animal Genetic Resources*; Commission on Genetic Resources for Food and Agriculture, Food and Agriculture Organization: Rome, Italy, 2022.
208. Hall, S.J. The horse in human society. In *The Domestic Horse. The Evolution, Development and Management of Its Behavior*; Mills, D., McDonnell, S., Eds.; Cambridge University Press: Cambridge, UK, 2005; pp. 23–32.
209. UNESCO. Browse the Lists of Intangible Cultural Heritage and the Register of Good Safeguarding Practices. 2023. Available online: https://ich.unesco.org/en/lists (accessed on 26 June 2023).

APPENDIX III.II – Publication 2

Blanco-Doval, A., Barron, L. J. R., Aldai, N. (2023). **Changes during lactation in the mineral element content of mare milk produced in semi-extensive rural farms**. *Journal of Food Composition and Analysis, 123*, Article 105629.

DOI: 10.1016/j.jfca.2023.105629

Published in: ***Journal of Food Composition and Analysis***, 2023

Publisher: Elsevier

ISSN: 0889-1575

Impact Factor year: 2022

Impact Factor: **4.3**

Category: **Food Science & Technology**

Position in the category: 43/142 (**Q2**)

Journal of Food Composition and Analysis 123 (2023) 105629

Contents lists available at ScienceDirect

Journal of Food Composition and Analysis

journal homepage: www.elsevier.com/locate/jfca

Changes during lactation in the mineral element content of mare milk produced in semi-extensive rural farms

A. Blanco-Doval, L.J.R. Barron, N. Aldai *

Lactiker Research Group, Department of Pharmacy and Food Sciences, University of the Basque Country (UPV/EHU), Paseo de la Universidad 7, 01006 Vitoria-Gasteiz, Spain

ARTICLE INFO

Keywords:
Macrominerals
Trace elements
Equid milk
Basque mountain horse breed
Grazing
Lactation stage

ABSTRACT

This study characterizes, for the first time, the content of individual mineral elements in mare milk from Basque Mountain Horse breed, as well as changes that occur during lactation and among semi-extensive rural farms. Individual milk samples from eighteen mares belonging to three commercial farms were collected along six months of lactation, and the contents of eleven mineral elements in milk were determined using a triple quadrupole inductively coupled plasma mass spectrometer (ICP-MS). Basque Mountain Horse milk contained calcium (Ca; 958 ± 248 μg/g milk), potassium (K; 581 ± 117 μg/g milk), phosphorus (P; 454 ± 160 μg/g milk), sodium (Na; 141 ± 38 μg/g milk), magnesium (Mg; 64.1 ± 15.7 μg/g milk), zinc (Zn; 2.50 ± 0.88 μg/g milk) and copper (Cu; 0.343 ± 0.179 μg/g milk) amounts similar to those reported in other horse breeds. Conversely, low sulphur (S; 156 ± 42 μg/g milk) and chlorine (Cl; 49.3 ± 33.4 μg/g milk), and high iron (Fe; 2.43 ± 2.44 μg/g milk) contents were found. Mn was only quantifiable in 12% of the samples. The content of all macrominerals, except Na and Cl, decreased at the end of lactation, while that of trace elements (Zn, Cu and Fe) either increased or fluctuated. In addition, the content of Ca, P, S and Na differed significantly among semi-extensive rural farms, probably due to differences in feeding management. From a multivariate perspective, the mineral element composition of the milk made it possible to differentiate samples from early, mid and late lactation stage.

1. Introduction

Minerals are inorganic compounds that play a key role in the functioning of human organism. Calcium (Ca), chlorine (Cl), cobalt (Co), copper (Cu), iodine (I), iron (Fe), magnesium (Mg), manganese (Mn), molybdenum (Mo), phosphorous (P), potassium (K), selenium (Se), sodium (Na), and zinc (Zn) are known to be essential for human physiological functions (Hunt and Nielsen, 2009); besides, sulphur (S) is detrimental for the synthesis of several metabolic intermediates (Nimni et al., 2007). Overall, mineral elements conform structural tissues and take part in numerous metabolic reactions, so deficiencies can lead to serious health disorders. Their bioavailability depends on many factors, and even with an adequate consumption of minerals, deficiencies may appear due to an inefficient absorption (European Food Safety Authority, 2017).

Mammalian milk, particularly cow milk, and dairy products, are the main food source of bioavailable Ca, a good source of P, and a moderate source of K, Na, Mg and Cl. Conversely, dairy products are poor in trace elements such as Fe, Cu, Mn and Zn. Despite the low concentration of Zn in milk and dairy products, these are one of the main sources of this trace element for children (Fantuz et al., 2016; Hunt and Nielsen, 2009). Significant differences in the concentrations of mineral elements have been reported among mammalian species (Claeys et al., 2014). Moreover, the bioavailability of mineral elements varies according to their distribution among milk fractions as well as the presence of other compounds such as ascorbate, citrate, lactose, or casein-ophosphopeptides (Bouhallab and Bouglé, 2004; Fantuz et al., 2016; Zamberlin et al., 2012).

The studies carried out to date on different breeds showed that equid milk is chemically different to that from other mammalian species, but more similar to human milk. Compared to ruminant milk, mare milk showed lower fat, protein, ash and dry matter contents (0.3–4%, 1–3%, 0.3–0.5% and 9–12%, respectively), but it was richer in lactose (6–7%) (Claeys et al., 2014; Uniacke-Lowe et al., 2010). Despite its lower macronutrient content, some authors have reported that mare milk is rich in other nutritionally significant and bioactive compounds which may be responsible for different functional properties (Doreau and Martin-Rosset, 2002; Sheng & Fang, 2009), yet knowledge on this topic

* Corresponding author.
E-mail address: noelia.aldai@ehu.eus (N. Aldai).

https://doi.org/10.1016/j.jfca.2023.105629
Received 22 June 2023; Received in revised form 31 July 2023; Accepted 20 August 2023
Available online 24 August 2023

remains scarce. In essence, considering the essential action of mineral elements in human, these (or some of these) could contribute to mare milk functionality. However, very limited data is available concerning mare milk, very especially about the content of some of them such as S, Fe, Mn and Cl. Previous studies have reported that, in general, the content of mineral elements in mare milk was not significantly affected by the dietary intake of these, with exception of Se and I (Fantuz et al., 2016; Hunt and Nielsen, 2009). On the contrary, lactation stage has shown to affect the mineral element content of mare milk, but only a few scientific works have monitored the lactation period (Grace et al., 1999; Schryver et al., 1986b; Smolders et al., 1990; Summer et al., 2004; Ullrey et al., 1966, 1974). To the best of our knowledge, only three studies were performed under grazing conditions (Grace et al., 1999; Ullrey et al., 1966, 1974). Exploring changes in the chemical composition of milk along lactation – and potential interactions with animal feeding – could lead to a better understanding of the factors that affect equine milk quality while providing tools for optimising the production.

Therefore, the aim of this study was to characterize the composition of macrominerals and trace elements, and their concentration changes over six months of lactation in mare milk from Basque Mountain Horse breed reared in semi-extensive rural farms. In this respect, it should be noted that these mares had never been milked before. In addition, it was also investigated whether differences in animal management among farms had any impact on the mineral element content of mare milk. Mare milk from this breed has never been analysed from a scientific perspective before and, considering that different horse breeds have shown variations in milk mineral element composition (Sheng and Fang, 2009), the characterisation of milk from Basque Mountain Horse is of great interest.

2. Materials and methods

2.1. Experimental design and milk sampling

Eighteen lactating mares from three different commercial equine farms (six mares *per* farm; from now on, farm I, farm II and farm III) located in the same region of Spain (Araba) were milked. All mares belonged to Basque Mountain Horse breed, and averaged (± standard deviation) 9.53 ± 3.76 years old and 5.68 ± 3.56 parturitions. Mares were milked only in the sampling days. Parturitions were not synchronized, and happened within a range of 39 days from late March to early May 2021; therefore, lactation time of each mare was established according to parturition day. Individual milk samples were collected during the lactation period, which was from May 4th until October 13th, 2021. During the first half of the lactation (until end July), samples were collected every 7 days, whereas during the second half (from early August to mid-October), collections happened every 14 days. In total, 304 milk samples were obtained (farm I, n = 75; farm II, n = 125; farm III, n = 104).

Table 1 shows the feeding management followed by the three commercial farms during the lactation period. In farm I, mares grazed on pastures only during May and they were fed with a mixture (15–20 kg *per* mare) of alfalfa, silage, hay, fruits and potatoes afterwards. Farms II and III kept mares on pasture all along the lactation period, and from the beginning of July, onwards, when grass availability was scarce, farm II supplemented mares with hay (4–5 kg *per* mare) and farm III with grass and alfalfa silage (9–10 kg *per* mare). All the three farms provided a mixture (2–3 kg *per* mare) of cereals and alfalfa or pea in the milking parlour in order to ease and get the animals accustomed to the milking process. Farm I performed this accustoming management only at the sampling days, whereas farms II and III got the mares to the milking parlour and repeated the process every day during the lactation period. All farms supplemented mare feeding with salt blocks.

Mares were milked as completely as possible using either automatic or manual milking. Farm I used a portable milking machine (model Canarias-1, Ultramilk, Socuéllanos, Spain) fitted with two sheep teatcups, whereas farms II and III used manual milking. In the three farms, milking happened in the presence of foal after suckling deprivation of 2–3 h. After milk sampling at the farm, these were transported in portable coolers to the laboratory and subsequently subsampled and frozen at − 80 °C until analysis. In order to avoid degradation of compounds, milk samples were thawed at 25 °C for 60 min in a water bath for the analysis of different compounds, including mineral elements in this study.

2.2. Chemicals

Nitric acid (HNO_3; 68–70%, v/v) was purchased from Fisher Chemical (Madrid, Spain) and redistilled in the laboratory for purification; hydrogen peroxide (H_2O_2; 30–32%, v/v) was Optima™ grade and purchased from Fisher Chemical; CMS-5 standard solution (in HNO_3; 5% v/v) containing 10 μg/mL each of the mineral elements Ca, Mg, Na, K, Fe, Cu, Zn and Mn was purchased from Inorganic Ventures (Christiansburg, USA); standard solutions (1000 μg/mL in water) of S, P and rhodium (Rh) were purchased from SCP Science (Quebec, Canada); and ultrapure (99.9999%) oxygen, hydrogen and helium were purchased by Air Liquide (Madrid, Spain). Millipore Milli-Q grade water (18.2 MΩ/cm) was used for dilutions.

2.3. Sample preparation and mineral element analysis

For digestion of milk samples, 5 mL of 65% (v/v) HNO_3 and 1 mL H_2O_2 were added to 0.20 ± 0.01 mg of liquid milk, and the mixture was submitted to a Speedwave-four microwave assisted digester (Berghof Products + Instruments, Berlin, Germany) at a maximum of 190 °C and 80% power. Digested samples were diluted in Milli-Q water, and

Table 1
Feeding management used by each commercial farm during the lactation period of mares (from May to October).

	Grazing regime				Supplements*		
Farm	Pasture type	Quantity	Time period	Meadow size	Type	Quantity (*per* mare)	Time period
I	Vetch, ryegrass, oat	*Ad libitum*	May	2–3 ha	Alfalfa (20%), silage (20%), hay (20%), fruits (20%), potato (20%)	Daily ration of 15–20 kg of mixture	June-October
					Alfalfa, oat and corn	2–3 kg of mixture	During the milking process
II	Alfalfa, clover, ryegrass, *Festuca spp.*, orchard grass, dandelion	*Ad libitum*	May-October	28 ha	Hay	Daily ration of 4–5 kg	July-October
					Oat (60%), wheat (20%) and pea (20%)	2–3 kg of mixture	During the milking process
III	Alfalfa, clover, ryegrass, *Festuca spp.*, orchard grass, dandelion	*Ad libitum*	May-October	28 ha	Grass and alfalfa silage	Daily ration of 9–10 kg	July-October
					Oat (60%), wheat (20%) and pea (20%)	2–3 kg of mixture	During the milking process

* Percentages and weights of feeding ingredients are approximate.

analysed in an 8900 triple quadrupole inductively coupled plasma mass spectrometer (ICP-MS; Agilent, Santa Clara, USA). The ICP-MS system was equipped with a MicroMist concentric nebulizer and a Fassel-type torch with an internal diameter of 2.5 mm. Plasma was generated with argon gas as follows: 1550 W radio frequency power, 1.2 V radio frequency matching, 15 L/min plasma gas flow, 1.05 L/min nebulizer gas (argon) flow, 0.9 L/min auxiliary gas (argon) flow. An MS/MS scan was used for P and S with oxygen as cell gas (flow 30%), and for Ca with hydrogen as cell gas (flow 4.7 mL/min). For the rest of the elements, a single Quad scan was performed using helium at a flow-rate of 4.6 mL/min. Rh was used as internal standard, and for mineral element quantification, Na, Mg, K, Ca, P, S, Mn, Fe, Cu and Zn standard solutions were analysed by triplicate in a range greater than 0.05 and less than 2400 ng/mL. These concentrations were prepared from commercial standard solutions using 1% (v/v) HNO_3 in Milli-Q grade water. Table 2 shows the analytical parameters evaluated for method validation. Linearity was assessed by calculating the determination coefficient (R^2) of the calibration curves adopting between five and eight calibration levels depending on each mineral element. Precision was estimated as the percent relative standard deviation (RSD) for six replicates of standard solutions at the lowest and highest concentration for each mineral element (Table 2). In the same way, accuracy was evaluated in terms of recovery by analysing six replicates of mineral element standard solutions at these concentrations. Limits of quantification (LOQ) were determined, for each element, by analysing ten replicates of the lowest concentration of the mineral element standard solution that obtained an RSD value less than 20%. LOQ values were expressed as μg/g referring to the wet weight of the milk sample, considering the dilution step occurred in the sample preparation. Data in Table 2 showed a high linearity with R^2-values higher than or equal to 0.9940 for all the mineral elements studied, as well as a good precision with RSD values less than 8.50% except for S at high concentration. Recovery values (accuracy) ranged between 95.39% and 105.13% for all mineral element and concentration, which was an acceptable value for macromineral and trace element quantification. Although LOQ values for macromineral elements were provided in mare milk, these elements were far above their LOQ values. On the other hand, those limits were of interest for those trace elements ranging between 0.650 and 0.044 μg/g milk (for Fe and Mn, respectively).

The amount of Cl in the milk samples was estimated by using the semi-quantitative mode of ICP-MS. This method is based on predefined default values, and the approximate concentration of the mineral element is calculated from the counts *per* second measured by ICP-MS (Krzciuk et al., 2016).

2.4. Statistical analysis

Software version 28.0 of IBM-SPSS Statistics (IBM, New York, USA) was used to perform statistical data treatment. Three significant figures were used to express the mineral element content of the milk samples. Mineral element content data were log transformed, and normality, homoscedasticity and residual randomness were checked. Outliers (values higher or lower than 3 times the interquartile range) checked by box-plots were excluded from the data set. In order to make more comprehensible the study of the changes in the mineral element content of mare milk samples during lactation, the data was pooled in several lactation ranges of 14 days with the earliest lactation range corresponding to weeks 3 and 4, and the last range to weeks 25 and 26. Data (excluding Mn) were analysed using the Linear Mixed Model (LMM) procedure of analysis of variance including individual animal as subject, farm as fixed factor and lactation time as repeated measure factor. The interaction term between farm and lactation time was also included in the model. The parameters of the LMM were estimated using the Restricted Maximum Likelihood Method and the compound symmetry matrix was selected for the repeated measures covariance structure following the Akaike information criterion. Least square means of dependent variables for the levels of the fixed factor (farm) were compared using the Tukey's test. In addition, a Stepwise Discriminant Analysis (SDA) was applied to the mineral element composition (excluding Mn and Cl contents) of the milk samples from the commercial farms to classify milks according to mare lactation stage. For this purpose, milk samples were labelled as early lactation (from week 3–10), mid lactation (from week 11 to week 18) or late lactation (from week 19–26). Significance level was declared at $P \leq 0.05$.

3. Results

In mare milk samples analysed from all commercial farms over lactation, Ca, K and P were the major mineral elements with an average (± standard deviation) content of 958 ± 248, 581 ± 117 and 454 ± 160 μg/g milk, respectively, and an average Ca:P ratio of 2.26 ± 0.60. Among other macrominerals, an average of 156 ± 42 μg S, 141 ± 38 μg Na, 64.1 ± 15.7 μg Mg and 49.3 ± 33.4 μg Cl *per* g of milk were found (Table 3). Except Mn, the trace elements were found above the LOQ in most milk samples collected during the lactation period. The average content of trace elements was of 2.50 ± 0.88 μg Zn, 2.43 ± 2.44 μg Fe and 0.343 ± 0.179 μg Cu *per* g of milk. Mn was only present above the LOQ in 35 out of the 304 samples analysed, with an average of 0.118 ± 0.230 μg/g milk. Milk samples containing quantifiable amounts of Mn were from different animals and were randomly distributed along lactation and among farms; therefore, no further statistical data treatment was performed for Mn.

Table 3 shows the content of all the macrominerals and trace elements by farm, as well as the effect of the factors lactation time and farm. Farm effect was significant ($P \leq 0.05$) for Ca, P, S and Na contents in mare milk samples. For these minerals, milk samples from farm I (mares grazing only during the first month of lactation) contained higher amounts than those from farm II and III (mares grazing along lactation and supplemented after July with hay or silage, respectively). On the other hand, lactation time significantly ($P \leq 0.05$) influenced the

Table 2
Validation parameters of the ICP-MS analytical method used to quantify macrominerals and trace elements in mare milk samples.

				RSD (%)		Recovery (%)	
Mineral element	Calibration range (ng/mL)	R^2	LOQ (μg/g milk)	LConc	HConc	LConc	HConc
Ca	43.00 – 2147.00	0.9999	26.875	2.12	0.11	95.76	101.86
K	12.36 – 2074.91	0.9999	7.725	2.89	3.86	99.29	98.82
P	1.54 – 2336.11	1.0000	0.963	2.86	7.79	103.66	104.84
S	1.40 – 2074.91	0.9999	0.875	11.20	8.29	103.11	97.42
Na	12.36 – 746.49	0.9940	7.725	3.83	5.70	103.37	104.51
Mg	0.37 – 372.70	0.9998	0.231	5.46	6.05	95.39	98.65
Zn	0.70 – 372.70	0.9999	0.438	7.73	4.85	103.01	99.22
Fe	1.04 – 372.70	0.9998	0.650	3.01	4.00	105.13	100.40
Cu	0.13 – 175.14	0.9999	0.081	5.88	5.81	98.57	100.93
Mn	0.07 – 57.80	1.0000	0.044	1.53	5.43	97.25	102.37

RSD, relative standard deviation; R2, determination coefficient; LOQ, limit of quantification; LConc; lowest concentration; HConc, highest concentration.

Table 3
Average concentration (μg/g milk) and range (minimum-maximum) of macrominerals and trace elements in mare milk samples from the commercial farms over the lactation period (from May to October), and statistical significance (*P*-value) of the principal factors (farm and lactation) and their interaction. The total number of milk samples collected in commercial farms were 75, 125 and 104 for farm I, II and III, respectively.

	Farm							*P*-value		
Mineralelement	I	n	II	n	III	n	SEM	Farm	Lactation	Farm * Lactation
Ca	$104{\cdot}10^{1}$ [a] ($654–169{\cdot}10^{1}$)	75	928[b] ($504–153{\cdot}10^{1}$)	125	936[a,b] ($503–146{\cdot}10^{1}$)	104	18	0.025	< 0.001	0.857
K	582 (448–856)	75	560 (385–755)	125	604 ($414–103{\cdot}10^{1}$)	104	9	0.461	< 0.001	0.104
P	502[a] (201–837)	75	431[b] (193–781)	125	447[a,b] (202–806)	104	12	0.013	< 0.001	0.471
S	165[a] (96.7–287)	75	153[b] (86.6–259)	125	154[a,b] (90.1–253)	104	3	0.015	< 0.001	0.493
Na	179[a] (111–304)	75	134[b] (89.6–180)	125	123[b] (69.0–198)	104	4	< 0.001	< 0.001	0.005
Mg	65.4 (38.2–118)	75	61.6 (29.2–114)	125	66.2 (41.5–103)	104	1.1	0.493	< 0.001	0.013
Cl	41.2 (16.5–143)	75	52.3 (18.1–202)	125	51.5 (19.6–262)	104	2.4	0.193	< 0.001	0.895
Zn	2.26 (1.15–4.06)	75	2.67 (1.18–7.43)	125	2.47 (1.21–5.13)	103	0.06	0.061	< 0.001	0.491
Fe	2.75 (0.709–24.5)	73	2.31 (0.707–8.97)	118	2.32 (0.651–18.0)	100	0.18	0.427	0.006	0.035
Cu	0.329 (0.120–1.06)	70	0.327 (0.101–1.03)	121	0.372 (0.134–0.956)	98	0.013	0.056	< 0.001	0.080
Mn	0.0665 (0.0477–0.0903)	5	0.0735 (0.0450–0.127)	20	0.200 (0.0441–1.39)	15	0.040			
Ca:P	2.24 (1.29–3.54)	–	2.30 (1.14–4.45)	—	2.24 (1.00–3.55)	—	0.04	0.794	< 0.001	0.707

[a,b] Different letter superscripts indicate significant differences ($P \leq 0.05$) among farms.
n, number of milk samples from each farm in which mineral elements were found to be above the limit of quantification (see Table 2); SEM, standard error of the mean.

concentrations of all the minerals and the Ca:P ratio. Figs. 1 and 2 depict the changes in the mineral element content of milk samples from each farm. Contents of Ca, K, P, S and Mg showed a downward trend throughout lactation (Fig. 1). Contents of Mg and K steadily decreased during lactation. Moreover, the evolution of Mg content differed among farms (significant interaction, Table 3): in farm I and farm III, average Mg content continuously decreased except for one time point in farm I (weeks 17–18), whereas the decrease in farm II was discontinuous (Fig. 1). The evolution of Ca, P and S contents in milk samples along lactation followed similar patterns in all farms, with a slight reduction during the first weeks of lactation, peaking at mid-lactation (Ca: at weeks 15–16; P and S: at weeks 13–14), and noticeably decreasing afterwards. In farm I, the peaking time of Ca and S appears one or two weeks delayed. On the other hand, Na contents slightly increased (2.77% on average from weeks 3–4 to weeks 25–26) and Cl contents fluctuated considerably in all farms during lactation (Fig. 1). However, Na content changed differently in milk from the different farms with significant ($P \leq 0.05$) interaction between farm and lactation time (Table 3). In fact, Na content increased during the second half of lactation in farms I and III, remaining more constant in farm II.

The content of the trace elements either increased (Cu and Zn) or fluctuated (Fe) along the lactation period (Fig. 2). Both Cu and Zn contents remained more or less constant until they increased from weeks 9–12 until weeks 15–20, with a later decreasing trend. The content of Fe did not show a clear pattern during lactation apart from final contents being overall lower than initial ones. An interaction between farm and lactation stage was seen in Fe concentration as well (Table 3), since the evolution along lactation appears one or two weeks delayed in farm III compared to farms I and II. On average, Ca:P ratio decreased from 2.22 to 1.68 during the first half of lactation, and then increased for the second half, achieving values as high as 2.78 (Fig. 2).

4. Discussion

According to the results obtained (Table 3), the major mineral element in Basque Mountain Horse breed milk was Ca, followed by K and P in approximately half the amount of Ca (as confirmed by the Ca:P ratio). Average macromineral content found in this study falls within the range reported in literature for other equine breeds: 261–1380 μg Ca, 341–701 μg K, 152–884 μg P, 107–220 μg Na, and 29–104 μg Mg *per* g or mL raw milk (Alipour et al., 2023; Anderson, 1991; Bilandžić et al., 2014; Csapó-Kiss et al., 1995; Grace et al., 1999; Schryver et al., 1986a, b; Smolders et al., 1990; Summer et al., 2004; Ullrey et al., 1966) for mare milk samples collected at 14 or more days of lactation. The content of S was on average lower in Basque Mountain Horse breed milk than in pasture-fed Thoroughbred breed milk reported by Grace et al. (1999), who found 196–230 μg S/mL milk. This could be a consequence of lower protein content found in Basque Mountain Horse breed milk samples in the present study (1.84 vs. 2.10 g protein/100 g milk, on average). In fact, proteins, particularly the sulphur containing amino acids methionine and cysteine, are the main source of dietary S (Nimni et al., 2007). In addition, Cl content in the milk samples of the present study was 3–4 times lower than that reported by Summer et al. (2004) in Haflinger breed milk (150–198 μg Cl/g milk). Changes in the chlorine content in bovine and human milks are known to be mainly due to pathological processes or microbial contamination, as well as to lactation stage, but not to dietary factors (Chen et al., 2018; European Food Safety Authority, 2019; Gaucheron, 2005). However, being involved – together with lactose, Na and K – in the balancing of the osmotic pressure of milk, Cl content has been also correlated with the content of lactose and/or Na in bovine milk (Bijl et al., 2013).

Regarding trace elements, Zn and Cu contents (Table 3) were in accordance to those found in the literature (1.1–4.1 μg Zn and 0.07–0.64 μg Cu *per* g or mL milk), whereas Fe contents in milk samples from Basque Mountain Horse breed were higher than those reported in other equine breeds (0.18–1.30 μg/g or mL) (Alipour et al., 2023; Anderson, 1992; Bilandžić et al., 2014; Csapó-Kiss et al., 1995; Grace et al., 1999; Schryver et al., 1986a, b; Ullrey et al., 1974). Reports on Mn concentration vary from less than 0.01 μg/g milk up to 0.05 μg/g milk (Alipour et al., 2023; Anderson, 1992; Bilandžić et al., 2014; Csapó-Kiss et al., 1995). In the present work, Mn contents lower than 0.0438 μg/g milk were below the LOQ established in the ICP-MS methodology

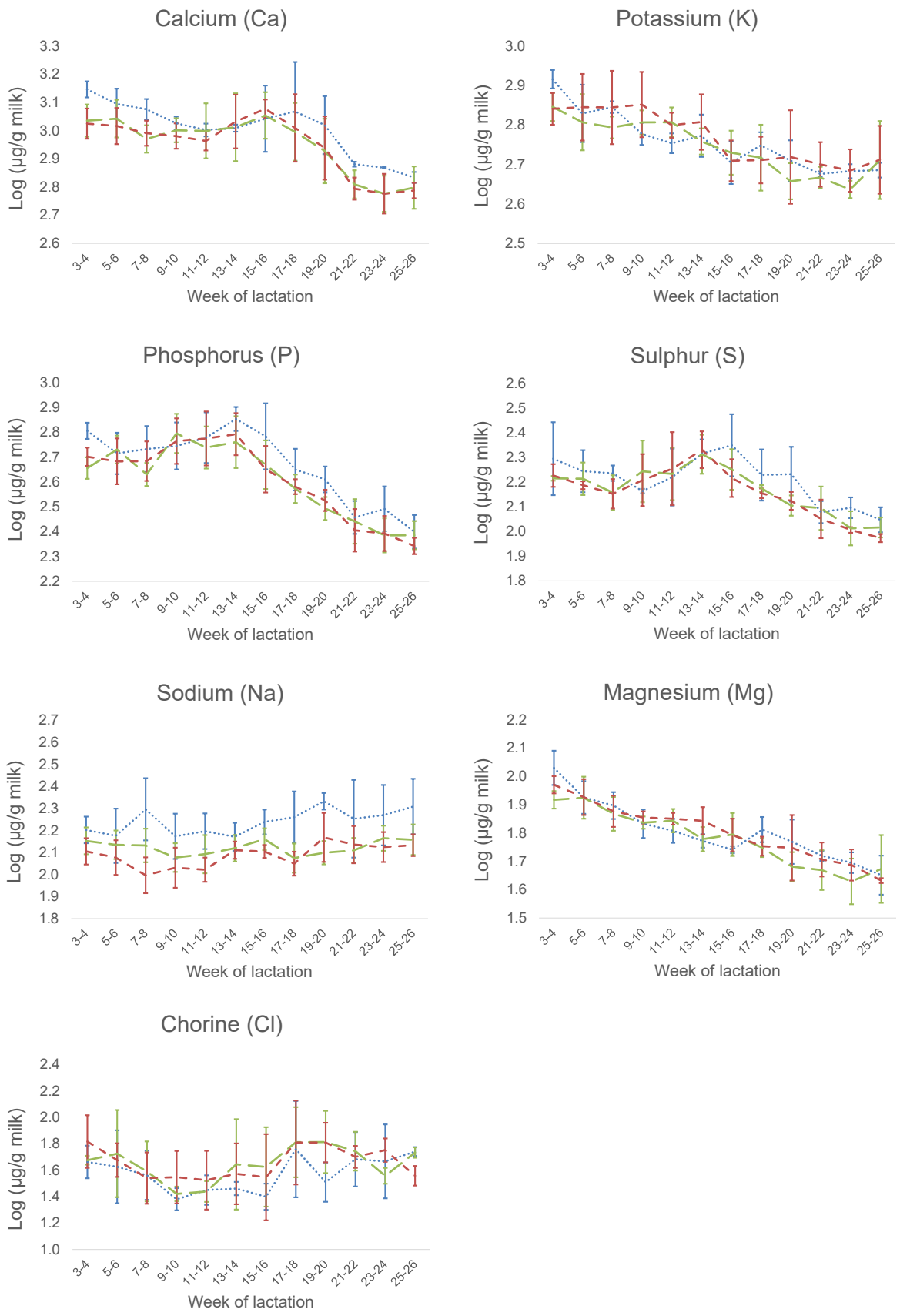


Fig. 1. Changes in macromineral content of mare milk samples from semi-extensive rural farms during lactation. – , farm I; – , farm II; – , farm III.

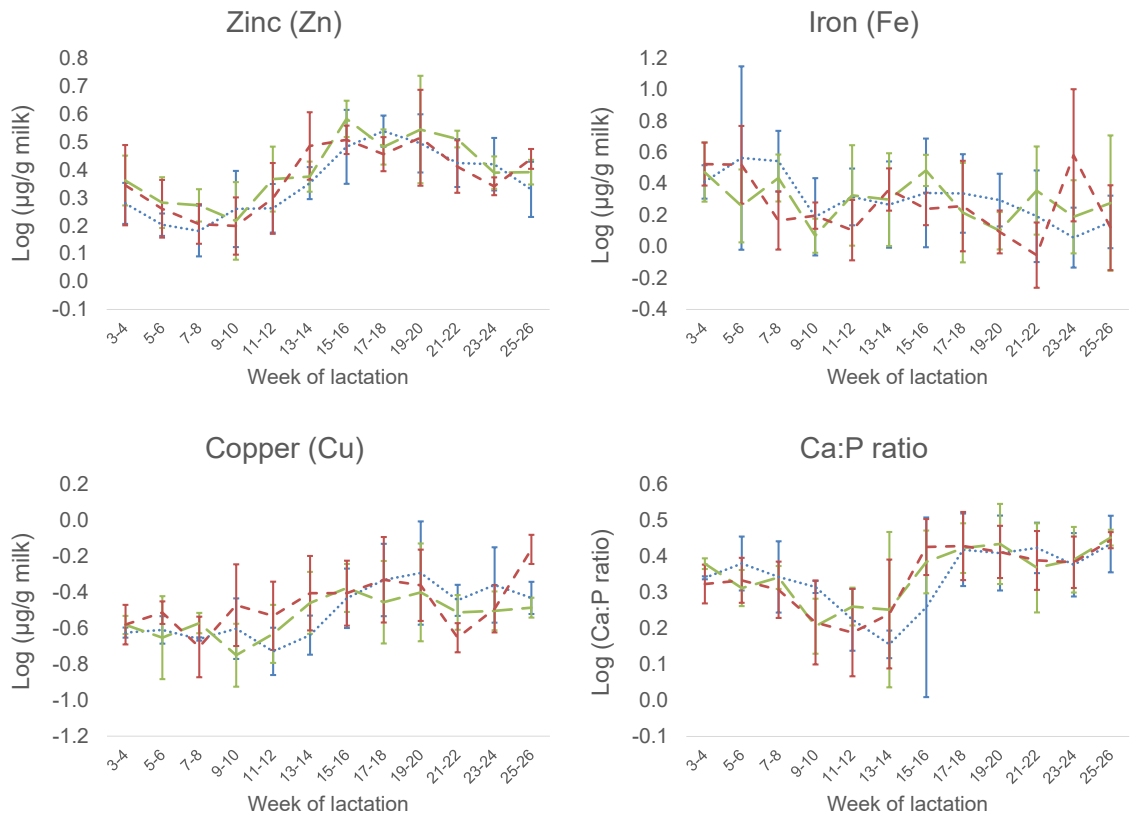


Fig. 2. Changes in trace element content and Ca:P ratio of mare milk samples from semi-extensive rural farms during lactation. – , farm I; – , farm II; – , farm III.

(Table 2). In consequence, comparison with literature data was difficult. Anyway, both human and animal milks have been considered a poor source of Mn (Fantuz et al., 2016).

Results (Table 3, Figs. 1 and 2) confirmed the significant ($P \leq 0.05$) effect of lactation time on the content of mare milk mineral elements previously reported by other authors (Grace et al., 1999; Summer et al., 2004). According to literature, Ca, P, Mg, Na, K, Cu, Fe and Zn contents decrease from week 3 to mid- (Schryver et al., 1986b; Smolders et al., 1990; Ullrey et al., 1966, 1974) or late-lactation (Martuzzi et al., 2004; Summer et al., 2004). Conversely, Grace et al. (1999) did not find significant differences in S, Cu and Fe contents after the 3rd week of lactation. Higher mineral element contents within the initial stages of lactation are inversely related to lactose content (which increases throughout lactation; Fantuz et al., 2016; Uniacke-Lowe et al., 2010). This negative correlation between mineral elements and lactose contents has also been observed in milk from other mammalian species, and has been described as responsible for osmotic regulation. In this sense, mainly Na^+, K^+ and Cl^- ions, and Ca and P associated to caseins, control milk volume in early lactation, and this role is taken up by lactose afterwards (Kobeni et al., 2020; Shennan & Peaker, 2000).

Overall, the evolution of mineral elements over the lactation period slightly differed among mare milk studies. The most variable macrominerals seemed to be Na and K since the content of both fluctuate along lactation (as the Na content in the present study; Fig. 1), but differently among studies (Grace et al., 1999; Schryver et al., 1986b; Summer et al., 2004). In the case of K content, present results resemble those reported by Ullrey et al. (1966), with a more consistent decrease and less fluctuation during lactation (Fig. 1). Similar to the present study, Summer et al. (2004) found that Cl content fluctuated without specific pattern. In the present research, mineral element changes along lactation were, in general, similar to those obtained by Grace et al. (1999) in pasture-fed mare milk, with higher content fluctuations, more prominent decreases after mid-lactation (particularly in Ca and P), and low content of Na and Zn found around weeks 7–9, also reported by Ullrey et al. (1974) for Zn. Interestingly, the peaking of some mineral elements in Basque Mountain Horse breed milk at mid-lactation has not been previously described in other mare milk studies.

From a multivariate perspective, the results of the SDA applied to the mineral element composition of mare milk samples collected in commercial farms made it possible to classify more than 86% of the samples into their lactation stage (early-, mid- and late-lactation). The milk sample distribution of Fig. 3 displayed the two canonical discriminant functions obtained. As depicted, the three lactation stages were considerably discriminated along the function 1 axis, whereas function 2 mainly contributed to discriminate between mid-lactation from the other two lactation stages (early- and late-). This multivariate approach confirmed the previously mentioned results about the individual mineral element content changes throughout lactation period. Despite of inter-farm variability, principally related to feeding differences among semi-extensive rural farms, the SDA was able to differentiate mare milk samples from early-, mid- and late-lactation.

The Ca:P ratio can affect both Ca and P bioaccessibility and bioavailability in humans. This ratio ranges between 1.9 and 2.4 in human milk, and is generally lower in cow, goat and ewe milk (Bass & Chan, 2006; Bonjour, 2011), yet there is high variability among studies. The Ca:P ratio obtained in the present study was, overall, higher than that previously reported in mare milk in other equine breeds (Alipour et al., 2023; Schryver et al., 1986b; Smolders et al., 1990; Summer et al., 2004) probably due to slightly higher Ca and slightly lower P concentrations. Other authors also found average Ca contents 1.4–1.9 times higher than P contents in mare milk (Anderson, 1991; Martuzzi et al., 2004; Schryver et al., 1986a). In accordance to the present study (Fig. 2), literature showed a decrease in the Ca:P ratio from early- to mid-lactation in mare milk (Schryver et al., 1986b; Smolders et al., 1990; Summer et al., 2004) and a posterior increase after the 16th week (Summer et al., 2004).

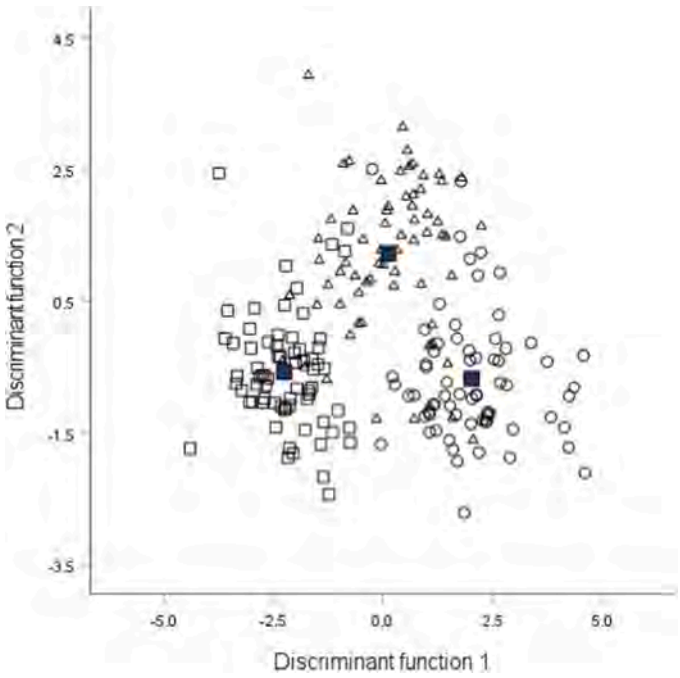


Fig. 3. Distribution of mare milk samples collected during early- (○), mid- (Δ), and late- (□) lactation stages according to Stepwise Discriminant Analysis performed using the mineral element composition (■, group centroid). Canonical functions 1 and 2 explained 85.7% and 14.3% of the total variance, respectively.

Other factors (apart from lactation) affecting mare milk mineral element composition are poorly understood and, to our knowledge, there is no previous data analysing the effect of management system. Actually, the understanding of this effect might be relevant when considering mare milk production for human consumption, specifically from a commercial perspective. Results from this study showed that mineral element composition of mare milk coming from different semi-extensive rural farms was generally similar, although Ca, P, S and Na contents significantly ($P \leq 0.05$) varied (Table 3). These variations could be related to differences associated to the management practices – including feeding – followed by each farm (Table 1). Except for Se and I, it is known that dietary mineral intake is unlikely to affect milk mineral element composition (Asai et al., 1995; Fantuz et al., 2016; Grace et al., 1999; Hunt & Nielsen, 2009; Kavazis et al., 2002). However, grazing could contribute to mineral element changes in milk. In a study by Soyeurt et al. (2022), they were able to discriminate between grass-fed and non-grass-fed cows according to protein, predicted Ca and, in a lower extent, predicted P and Mg contents in milk. Another study found that protein (and casein), Ca, P, Na, Zn, Cu, Mn and Se contents significantly varied among milks from cows fed concentrates and grazing on different pastures (Gulati et al., 2018). Grazing activity (low, moderate or high) also affected protein, Ca and P contents in cow milk (Stergiadis et al., 2021). In this regard, the results of the present study showed significantly ($P \leq 0.05$) higher contents of Ca, P and S (this last one related to proteins) in milk samples from low grazing mares (farm I) than in those samples from mares that grazed for a longer time and were supplemented with hay (farm II). Significantly higher Na contents were also found in milk samples from low grazing (farm I) than in those from long-term grazing mares (farms II and III). Furthermore, the peaks of concentration found in Ca, P and S at mid-lactation could be related to changes in grass availability after July.

Other factors such as age of the animal (Alipour et al., 2023; Asai et al., 1995), number of parturitions or health status of the mammary gland (Linn, 1988) could also have had a significant effect on the content of specific mineral elements in milk, but scientific literature on this topic remains very scarce. Since the present study was performed in commercial farms, other management practices apart from feeding could not be controlled. Consequently, correlation of the variability in milk mineral element content among farms with specific factors was not possible.

As previously indicated, milk is a great source of some essential elements described in the present work, yet not every milk type contributes equally to fulfil human daily mineral requirements. Compared to other animal species, mare milk is not the richest milk in terms of mineral element concentrations. Despite this, its mineral element composition is very similar to that of donkey (also an equid) and human milks, containing, overall, less mineral elements than other mammalian species (Claeys et al., 2014; Fantuz et al., 2016; Garhwal et al., 2023). On the other hand, mineral bioavailability of milk differs among mammalian species and can be affected by various factors. Therefore, not only mineral element content but also their bioavailability affects the nutritional value of the product, so the contribution of each type of milk to the recommended daily mineral intake should be carefully considered. Further research should address the bioavailability of mineral elements present in mare milk in order to properly evaluate the contribution of this food product to human health.

5. Conclusions

Milk from Basque Mountain Horse breed mares contains similar average mineral element concentrations compared to those described in the scientific literature in other equine breeds, except for lower S and Cl, and higher Fe contents. The macromineral (Ca, K, P, S, Na, Mg and Cl) and trace element (Zn, Fe and Cu) contents studied, as well as the Ca:P ratio, were significantly influenced by lactation time. Overall, the content of all macrominerals except Na and Cl decreased, Na, Zn and Cu increased, and Cl and Fe fluctuated throughout lactation. In addition, the management system used in farm affected Ca, P, S and Na concentrations in milk. This could be related to differences in the feeding management of mares in semi-extensive rural farms. These findings can contribute to the promotion of mare milk as a diversification option for the current equine meat production system performed in semi-extensive rural farms, and overall, to livestock grazing system sustainability.

CRediT authorship contribution statement

Ana Blanco-Doval: Methodology, Formal analysis, Investigation, Writing – original draft. **Luis Javier R. Barron:** Conceptualization, Methodology, Formal analysis, Writing – review & editing. **Noelia Aldai:** Conceptualization, Methodology, Formal analysis, Writing – review & editing.

Declaration of Competing Interest

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome. We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us. We confirm that we have given due consideration to the protection of intellectual property associated with this work and that there are no impediments to publication, including the timing of publication, with respect to intellectual property. In so doing we confirm that we have followed the regulations of our institutions concerning intellectual property.

We understand that the Corresponding Author is the sole contact for the Editorial process (including Editorial Manager and direct communications with the office). She is responsible for communicating with the other authors about progress, submissions of revisions and final approval of proofs. We confirm that we have provided a current, correct

email address which is accessible by the Corresponding Author and which has been configured to accept email from noelia.aldai@ehu.eus.

Data Availability

Data will be made available on request.

Acknowledgements

The Department of Education of the Basque Government for the predoctoral fellowship of A. Blanco-Doval. This work was supported by the Basque Government through Biotasma (Elkartek, 2019), Behor Esne (Cooperation, 2020) and IT944-16 and IT1568-22 projects. Authors thank the commercial farms that participated in the study, and also the SGIker – Advanced Research Facilities of the University of the Basque Country (UPV/EHU) for assisting in sample analysis.

References

Alipour, A., Mohajeri, F.A., Javdan, G., Pourramezani, F., Fallahzadeh, H., Sadrabad, E. K., 2023. Concentration of mineral and heavy metals in raw mare (horse) milk consumed in Yazd, Iran: a risk assessment study. Vet. Med. Sci. 9, 1592–1598.

Anderson, R.R., 1991. Comparison of minerals in milk of four species. Comp. Biochem. Physiol. Part A: Physiol. 100, 1045–1048.

Anderson, R.R., 1992. Comparison of trace elements in milk of four species. J. Dairy Sci. 75, 3050–3055.

Asai, Y., Katsuki, R., Matsui, A., Nanbo, Y., 1995. Effects of rations and age on mineral concentrations of Throughbred mare's colostrum. J. Equine Sci. 6, 21–24.

Bass, J.K., Chan, G.M., 2006. Calcium nutrition and metabolism during infancy. Nutrition 22, 1057–1066.

Bijl, E., van Valenberg, H., Huppertz, T., van Hooijdonk, A., 2013. Protein, casein, and micellar salts in milk: Current content and historical perspectives. J. Dairy Sci. 96, 5455–5464.

Bilandžić, N., Sedak, M., Đokić, M., Kolanović, B.S., Varenina, I., Božić, Đ., Končurat, A., 2014. Differences of the essential mineral element levels in the milk of Croatian Coldblood horse and Littoral-Dinaric donkey. Mljekarstvo 64, 12–18.

Bonjour, J.P., 2011. Calcium and phosphate: A duet of ions playing for bone health. J. Am. Coll. Nutr. 30, 438S–448S.

Bouhallab, S., Bouglé, D., 2004. Biopeptides of milk: Caseinophosphopeptides and mineral bioavailability. Reprod. Nutr. Dev. 44, 493–498.

Chen, X., Chu, B., Xi, H., Xu, J., Lai, L., Peng, H., Deng, D., Huang, G., 2018. Determination of chlorine ions in raw milk by pulsed amperometric detection in a flow injection system. J. Dairy Sci. 101, 9647–9658.

Claeys, W.L., Verraes, C., Cardoen, S., De Block, J., Huyghebaert, A., Raes, K., Dewettinck, K., Herman, L., 2014. Consumption of raw or heated milk from different species: An evaluation of the nutritional and potential health benefits. Food Control 42, 188–201.

Csapó-Kiss, Z., Stefler, J., Martin, T.G., Makray, S., Csapó, J., 1995. Composition of mares' colostrum and milk. Protein content, amino acid composition and contents of macro- and micro-elements. *Int. Dairy J.* 5, 403–415.

Doreau, M., & Martin-Rosset, W. (2002). Dairy animals: Horse. In H. Roginski (Ed.), Encyclopedia of dairy sciences (pp. 630–637). Massachusetts, MA, USA: Academic Press.

European Food Safety Authority, 2017. Dietary reference values for nutrients: summary report. EFSA Support. Publ. 14. Article e15121E.

European Food Safety Authority, 2019. Dietary reference values for chloride. EFSA J. 17. Article e05779.

Fantuz, F., Salimei, E., Papademas, P., 2016. Macro- and micronutrients in non-cow milk and products and their impact on human health. In: Tsakalidou, E., Papadimitriou, K. (Eds.), Non-Bovine milk and Milk Products. Academic Press, Massachusetts, MA, USA, pp. 209–261.

Gaucheron, F., 2005. The minerals of milk. Reprod. Nutr. Dev. 45, 473–483.

Garhwal, R., Bhardwaj, A., Sangwan, K., Mehra, R., Pal, Y., Nayan, V., Iquebal, M.A., Jaiswal, S., Kumar, H., 2023. Milk from Halari donkey breed: nutritional analysis, vitamins, minerals, and amino acids profiling. Foods 12, 853.

Grace, N.D., Pearce, S.G., Firth, E.C., Fennessy, P.F., 1999. Concentrations of macro-and micro-elements in the milk of pasture-fed thoroughbred mares. Aust. Vet. J. 77, 177–180.

Gulati, A., Galvin, N., Lewis, E., Hennessy, D., O'Donovan, M., McManus, J.J., Fenelon, M.A., Guinee, T.P., 2018. Outdoor grazing of dairy cows on pasture versus indoor feeding on total mixed ration: Effects on gross composition and mineral content of milk during lactation. J. Dairy Sci. 101, 2710–2723.

Hunt, C.D., & Nielsen, F.H.. (2009). Nutritional aspects of minerals in bovine and human milks. In P. McSweeney, & P. Fox (Eds.), Advanced dairy chemistry, Vol. 3: Lactose, water, salts and minor constituents (3rd ed) (pp. 391–456). New York, NY, USA: Springer.

Kavazis, A.N., Kivipelto, J., Ott, E.A., 2002. Supplementation of broodmares with copper, zinc, iron, manganese, cobalt, iodine, and selenium. J. Equine Vet. Sci. 22, 460–464.

Kobeni, S., Osthoff, G., Madende, M., Hugo, A., Marabini, L., 2020. The dynamic changes of African elephant milk composition over lactation. Animals 10, 948 (Article).

Krzciuk, K., 2016. Intelligent analysis of samples by semiquantitative inductively coupled plasma-mass spectrometry (ICP-MS) technique: A review. Crit. Rev. Anal. Chem. 46, 284–290.

Linn, J.G., 1988. Factors affecting the composition of milk from dairy cows. In National Research Council (Ed.), Designing foods (pp). Animal Product Options in the Marketplace. National Academies Press (US), Washington DC, CO, USA, pp. 224–241 (pp).

Martuzzi, F., Summer, A., Formaggioni, P., Mariani, P., 2004. Milk of Italian saddle and haflinger nursing mares: physico–chemical characteristics, nitrogen composition and mineral elements at the end of lactation. Ital. J. Anim. Sci. 3, 293–299.

Nimni, M.E., Han, B., Cordoba, F., 2007. Are we getting enough sulfur in our diet? Nutr. Metab. 4, 24 (Article).

Schryver, H.F., Oftedal, O.T., Williams, J., Cymbaluk, N.F., Antczak, D., Hintz, H.F., 1986a. A comparison of the mineral composition of milk of domestic and captive wild equids (Equus przewalski, E. zebra, E. burchelli, E. caballus, E. asinus). Comp. Biochem. Physiol. Part A: Physiol. 85, 233–235.

Schryver, H.F., Oftedal, O.T., Williams, J., Soderholm, L.V., Hintz, H.F., 1986b. Lactation in the horse: the mineral composition of mare milk. J. Nutr. 116, 2142–2147.

Sheng, Q., Fang, X., 2009. Bioactive components in mare milk. In: Park, Y.W. (Ed.), Bioactive Components in Milk and Dairy Products. Wiley-Blackwell, Iowa, IA, USA, pp. 195–213.

Shennan, D.B., Peaker, M., 2000. Transport of milk constituents by the mammary gland. Physiol. Rev. 80, 925–951.

Smolders, E.A.A., van der Veen, N.G., van Polanen, A., 1990. Composition of horse milk during the suckling period. Livest. Prod. Sci. 25, 163–171.

Soyeurt, H., Gerards, C., Nickmilder, C., Bindelle, J., Franceschini, S., Dehareng, F., Veselko, D., Bertozzi, C., Gengler, N., Marvuglia, A., Bayram, A., Tedde, A., 2022. Prediction of indirect indicators of a grass-based diet by milk Fourier transform mid-infrared spectroscopy to assess the feeding typologies of dairy farms. Animals 12, 2663 (Article).

Stergiadis, S., Qin, N., Faludi, G., Beauclercq, S., Pitt, J., Desnica, N., Pétursdóttir, A.H., Newton, E.E., Angelidis, A.E., Givens, I., Humphries, D.J., Gunnlaugsdóttir, H., Juniper, D.T., 2021. Mineral concentrations in bovine milk from farms with contrasting grazing management. Foods 10, 2733 (Article).

Summer, A., Sabbioni, A., Formaggioni, P., Mariani, P., 2004. Trend in ash and mineral element content of milk from Haflinger nursing mares throughout six lactation months. Livest. Prod. Sci. 88, 55–62.

Ullrey, D.E., Ely, W.T., Covert, R.L., 1974. Iron, zinc and copper in mare's milk. J. Anim. Sci. 38, 1276–1277.

Ullrey, D.E., Struthers, R.D., Hendricks, D.G., Brent, B.E., 1966. Composition of mare's milk. J. Anim. Sci. 25, 217–222.

Uniacke-Lowe, T., Huppertz, T., Fox, P.F., 2010. Equine milk proteins: Chemistry, structure and nutritional significance. Int. Dairy J. 20, 609–629.

Zamberlin, Š., Antunac, N., Havranek, J.J., Samaržija, D., 2012. Mineral elements in milk and dairy products. Mljekarstvo 62, 111–125.

APPENDIX III.III – Publication 3

Blanco-Doval, A., Azkargorta, M., Iloro, I., Beaskoetxea, J., Elortza, F., Barron, L. J. R., Aldai, N. (2024). **Comparative proteomic analysis of the changes in mare milk associated with different lactation stages and management systems**. *Food Chemistry, 445*, Article 138766.

DOI: 10.1016/j.foodchem.2024.138766

Published in: ***Food Chemistry***, 2024

Publisher: Elsevier

ISSN: 0308-8146

Impact Factor year: 2022

Impact Factor: **8.8**

Category: **Food Science & Technology**

Position in the category: 9/142 (**Q1**)

Food Chemistry 445 (2024) 138766

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Comparative proteomic analysis of the changes in mare milk associated with different lactation stages and management systems

Ana Blanco-Doval [a], Mikel Azkargorta [b], Ibon Iloro [b], Jabier Beaskoetxea [b], Felix Elortza [b], Luis Javier R. Barron [a], Noelia Aldai [a,*]

[a] Lactiker Research Group, Department of Pharmacy and Food Sciences, University of the Basque Country (UPV/EHU), Paseo de la Universidad 7, 01006 Vitoria-Gasteiz, Spain
[b] Proteomics Platform, CIC bioGUNE, Basque Research and Technology Alliance (BRTA), CIBERehd, Bizkaia Science and Technology Park, 48160 Derio, Spain

ARTICLE INFO

Keywords:
Horse
Grazing
Gene ontology
Ingenuity pathway analysis
Milk proteomics
Milk peptidomics

ABSTRACT

Mare milk has traditionally been attributed a number of health promoting properties. However, knowledge on its composition and functionality remains scarce, with particularly limited studies on mare milk proteomics. This study deeply characterized mare milk proteome accounting for both caseins and proteins in the whey fraction, also addressing the impact of lactation stage and different management systems. Milk samples from Basque Mountain Horse breed mares belonging to three different farms and three lactation stages were analysed after in-gel and in-solution digestion using nLC-MS/MS. Among the 469 proteins identified, the content of alpha-1 antitrypsin was significantly higher in pasture-based compared to other systems. Moreover, lactation stage significantly affected the content of beta-lactoglobulin II, immunoglobulin-like domain-containing protein, interferon alpha-inducible protein 27, lactotransferrin, polypeptide *N*-acetylgalactosaminyltransferase, and transforming acidic coiled-coil containing protein 2. This study contributes to the deep characterization of mare milk proteome and provides new insights into the effect of different production factors.

1. Introduction

Mare milk is a traditionally consumed food in several regions of the world due to its high nutritional value and potential beneficial effects on human health. In Russia and Asia, and to a lesser extent in Europe, mare milk (and its fermented version, kumis) has been used as a health promoting food for the treatment of chronic hepatitis, peptic ulcers, tuberculosis, bronchitis, asthma, anaemia, nephritis, diarrhoea or gastritis, among others (Jastrzębska et al., 2017; Park et al., 2006; Pieszka et al., 2016). It has also been valued as a human milk substitute for infant nutrition; first, because its chemical composition is very close to that of human milk (Pieszka et al., 2016); and second, because it has shown low allergic responses in infants with cow milk protein allergy (Businco et al., 2000; Zhao et al., 2023b). In view of the historical uses and healthy effects of mare milk, it has gained interest from a human nutrition perspective, and recent studies have addressed its potential functional value (Fotschki et al., 2016; Guri et al., 2016; Shariatikia et al., 2017). However, milk is a complex matrix, and compared to cow, human, sheep or goat milks, there is still limited knowledge about mare milk composition, especially considering minor components.

Compared to other mammals, protein abundance in mare milk is low but rich in whey proteins, similar to donkey and human milks. In fact, this protein fraction accounts for approximately 35 % (by weight), which significantly differs from ruminant milk, containing about 80 % caseins and only 20 % whey proteins (Miranda et al., 2004; Uniacke-Lowe et al., 2010). During late 20th century and early years of the present century, major individual proteins in mare milk (mainly alpha-, beta- and kappa-caseins, alpha-lactalbumin, beta-lactoglobulin and lysozyme C) were characterized (Curadi et al., 2000; Egito et al., 2002; Godovac-Zimmermann et al., 1987; Iametti et al., 2001; Miclo et al., 2007; Ochirkhuyag et al., 2000). In 2004, Miranda et al. (2004) performed, for the first time, a systematic and thorough analysis of the

Abbreviations: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; DHCR24, 24-dehydrocholesterol reductase gene; DTT, dithiothreitol; FXR/RXR, farnesoid X receptor/retinoid X receptor; HCCA, alpha-cyano-4-hydroxycinnamic acid, Ig: immunoglobulin; LXR/RXR, liver X receptor/retinoid X receptor; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid.

* Corresponding author.
E-mail addresses: ana.blancod@ehu.eus (A. Blanco-Doval), mazkargorta@cicbiogune.es (M. Azkargorta), iiloro@cicbiogune.es (I. Iloro), jbeaskoetxea@cicbiogune.es (J. Beaskoetxea), felortza@cicbiogune.es (F. Elortza), luisjavier.rbarron@ehu.eus (L.J.R. Barron), noelia.aldai@ehu.eus (N. Aldai).

https://doi.org/10.1016/j.foodchem.2024.138766
Received 22 November 2023; Received in revised form 24 January 2024; Accepted 13 February 2024
Available online 17 February 2024

entire protein fraction of mare milk using proteomic tools where eight major and milk-specific proteins were successfully identified in Welsh pony mare milk samples. Since then, proteomics has been further used focusing on specific fractions (proteins associated with milk fat globules, (Barello et al., 2008); glycosylation of kappa-casein, (Jaeser et al., 2023); and lactoferrin, (Narmuratova et al., 2022); among others) or technological properties (renneting, (Uniacke-Lowe et al., 2013)). All this research provided a general description of mare milk major proteins. In this regard, it contains low amounts of alpha-s1-, alpha-s2- and kappa-caseins but high amounts of beta-casein, alpha-lactalbumin and lysozyme C compared to ruminant milk. Two isoforms of beta-lactoglobulin (I and II; (Miranda et al., 2004)) and three of alpha-lactalbumin (A, B and C; (Godovac-Zimmermann et al., 1987)) have been identified. Moreover, kappa-casein is the only glycosylated casein in equine milk (Jaeser et al., 2023) while equine lysozyme C has calcium-binding activity similar to alpha-lactalbumin (Nitta et al., 1987).

Therefore, proteomics - bioinformatics technologies have provided important advances expanding the knowledge in food science (Picariello et al., 2012) and providing improvement opportunities in mare milk proteome profiling. Recently, Ji et al. (2024) have published a list of milk proteins (whey fraction only) from human and several dairy animals (including horse) and revealed that equine milk proteins remarkably differ from human, cow, camel, goat, sheep, buffalo and yak milk proteins. Additionally, Lv et al. (2024) revealed that milk fat globule membrane and whey proteins differ among horse breeds. However, more research is required to achieve a comprehensive characterization of mare milk proteins. In this respect, a global analysis of proteins would not only elucidate mare milk proteome, but also contribute to a better understanding of equine mammary gland biology and the nutritional and functional quality of milk.

In the present study, a direct and an unbiased approach to perform mare milk proteomic characterization accounting for caseins, whey proteins and milk fat globule membrane proteins has been utilized. Protein functionality aspects and changes in mare milk proteins associated with lactation stages and feeding management of Basque Mountain Horse autochthonous breed (Basque Country, northern Spain) have been addressed. This breed is at risk of extinction and its production in the equine sector is encouraged as a tool for its preservation. Actually, it is present in about 300 extensive farms dedicated to meat production (MAPA, 2022).

2. Materials and methods

2.1. Animals and sample collection

Milk from nine Basque Mountain Horse breed mares belonging to three different commercial farms (three mares/farm) was collected at weeks 6, 10, 14, 18, 22 and 26 of lactation (n = 54). All farms were located in the region of Araba (northern Spain), and differed in animal management. In brief, farm I fed the mares on pasture during the first month of the lactation period only (from early May to mid October) and fed with a mixture of alfalfa, silage, hay, fruits and potatoes during the remaining five months. On the other hand, farms II and III kept mares under grazing over the six months of lactation and supplemented with hay or silage, respectively, after July due to pasture scarcity. Further details about management of mares have been previously reported in Blanco-Doval et al. (2023). In each milking day, samples were collected in plastic bottles, transported to the laboratory under cooling conditions, subsampled and immediately frozen at −80 °C until analysis.

2.2. Chemicals

3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), alpha-cyano-4-hydroxycinnamic acid (HCCA), acetic acid, acetone, acetonitrile, ammonium bicarbonate, chloroacetamide, dithiothreitol (DTT), ethanol, formic acid, thiourea, trifluoroacetic acid (TFA) and urea were purchased from Merck (Darmstadt, Germany). SYPRO ruby protein gel stain solution and BenckMark protein ladder solution were obtained from Invitrogen (Thermo Fisher Scientific, MA, USA). Trypsin was purchased from Promega Biotech Ibérica (Madrid, Spain).

2.3. Peptidomic and proteomic analysis of mare milk samples

The workflow for peptidomic and proteomic analysis of mare milk has been depicted in Fig. 1.

2.3.1. Milk peptide and protein extraction

Individual milk samples were thawed at room temperature, and milk proteins were precipitated using acetone (ratio 1/6 milk to acetone, by volume) at −20 °C. The same procedure was repeated with a pool of equal volumes of all the 54 milk samples. After precipitation, the samples were centrifuged at 15,000 *g* for 10 min at room temperature (Microfuge 18 and Microfuge 22R, Beckman Coulter, CA, USA). Supernatants from individual samples containing endogenous peptides (S1 fraction; Fig. 1) were collected in separate microtubes and dried at room temperature using a miVac centrifugal concentrator (Genevac, Ipswich, UK); then, they were kept at −20 °C until analysis. Pellets with precipitated proteins (from the pool, *P*1 fraction; and from individual samples, *P*2 fraction; Fig. 1) were also dried using the centrifugal concentrator and suspended in a 2 M thiourea, 7 M urea, 4 % CHAPS, and 200 mM DTT buffer, aided with sonication.

2.3.2. Milk protein separation and digestion procedures

Proteins precipitated from the pooled sample (*P*1 fraction) were subjected to gel electrophoresis followed by in-gel protein digestion using the BenchMark protein ladder solution as marker. For protein separation, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used. Different non-gradient gels were tested for optimization of protein separation: tris–glycine 10 % acrylamide, tris–glycine 15 % acrylamide, and tris-tricine 10 % acrylamide (Fig. 2). Proteins were fixated with a 40 % ethanol 10 % acetic acid aqueous solution (by volume) and stained overnight with the SYPRO ruby protein gel stain solution. De-staining was performed with a 10 % ethanol 7 % acetic acid aqueous solution (by volume). All SDS-PAGE gels were scanned in an ultraviolet gel imaging system (ChemiDoc XRS System with Universal Hood II, Bio-Rad, CA, USA). Among all gels tested, tris–glycine 15 % acrylamide gel was selected for tryptic digestion.

Tris-glycine 15 % acrylamide gel bands were excised and dehydrated adding acetonitrile. Then, proteins in gel spots were reduced with DTT 10 mM, and alkylated with chloroacetamide 55 mM. For in-gel protein digestion, samples were incubated in a trypsin solution in ammonium bicarbonate 50 mM in water (12.5 ng/μL) overnight at 37 °C. Peptides released in the supernatant were recovered, and gel spots were washed several times with acetonitrile and 0.1 % TFA for higher recovery. Extracted peptides were dried in the centrifugal concentrator and kept at 4 °C until analysis.

Precipitated proteins from the individual milk samples (*P*2 fraction) were submitted to in-solution digestion following a previously described filter-aided sample preparation method (Berger et al., 2015; Wiśniewski et al., 2009). Briefly, 30 μg of protein from each sample were washed twice with urea 8 M, and alkylated with chloroacetamide 25 mM in a 96-well plate (MultiScreen® HTS-HV 96-well plates with 0.45 μm hydrophilic Durapore® polyvinylidene fluoride membrane, Merck). For protein digestion, 1 μg trypsin was added to each well and the plate was incubated overnight at 37 °C. Released peptides were filtered through the 96-well plate membrane using centrifugation (200 *g*, 2 min; Allegra X-14R, Beckman Coulter); then, filters were washed twice with 40 % acetonitrile and 0.1 % formic acid aqueous solutions (by volume). All the filtered fractions were collected, dried in the centrifugal concentrator and kept at 4 °C until analysis.

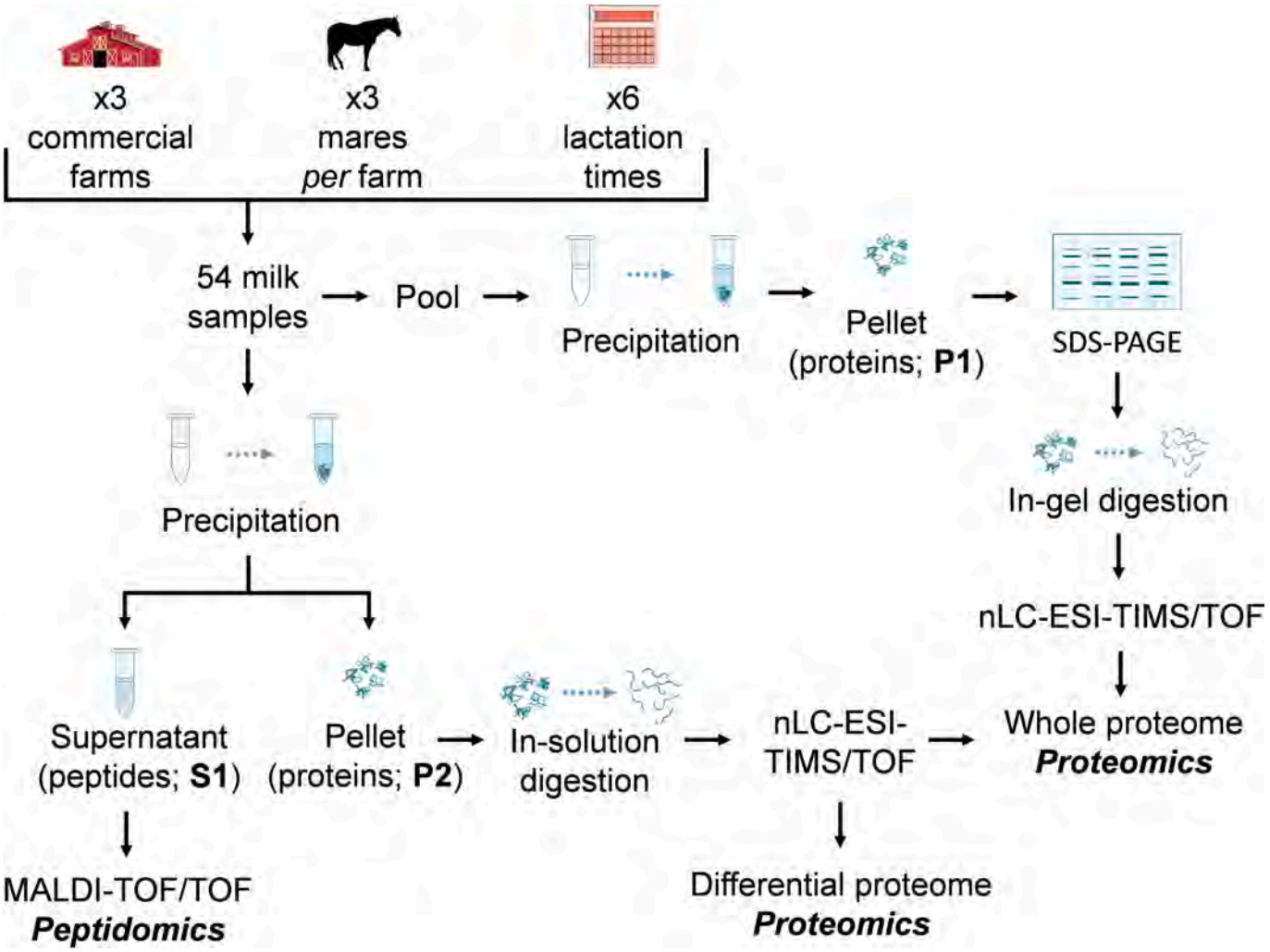


Fig. 1. Diagram of the workflow followed for mare milk peptidomic and proteomic analyses. Differential proteome refers to changes in mare milk proteins associated with different lactation stages and farm managements. SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis; nLC-ESI-TIMS/TOF: nano-liquid chromatography with electrospray ionization coupled to trapped ion mobility/time-of-flight tandem mass spectrometry; MALDI-TOF/TOF: matrix-assisted laser desorption/ionization coupled to time-of-flight tandem mass spectrometry.

2.3.3. Proteomic analysis

Extracted peptides from both the pool (*P*1 fraction; in-gel digestion) and individual milk samples (*P*2 fraction; in-solution digestion) were suspended in 0.1 % formic acid in water (by volume), followed by a purification and concentration step using C18 pipette tips (OMIX C18, Agilent Technologies, Santa Clara, CA, USA). Pipette tips were washed with pure acetonitrile followed by 0.1 % TFA in water (by volume). Then, samples were loaded into the pipette tips, acidified with a 0.1 % TFA in water solution (by volume) and eluted with 70/30 acetonitrile to 0.1 % TFA in water ratio (by volume). Eluted fractions were dried in the centrifugal concentrator.

Before proteomic analysis, an additional purification step was performed using Evotip Pure tips (Evosep, Odense, Denmark) according to manufacturer instructions. Proteomic analysis of the samples was carried out in a nano-liquid chromatograph with electrospray ionization (nLC-ESI) equipment (Evosep One, Evosep) coupled to a trapped ion mobility/time of flight (TIMS/TOF) tandem mass spectrometer (timsTOF PRO, Bruker Daltonics, Billerica, MA, USA). Peptides from digested proteins were separated in a C18 nano-flow UPLC column (15 cm x 150 μm internal diameter, 1.9 μm particle size; EV1106, Evosep) using the Evosep 30SPD standardized method (44 min gradient, 500 nl/min flow). TIMS/TOF was operated using the standard 1.1 s long gradient method (scan from 100 to 1700 *m/z*, positive ion polarity, data-dependent acquisition scan mode, $1/k_0$ start 0.60 V·s/cm^2, $1/k_0$ end 1.60 V·s/cm^2, ramp time 100 ms, collision energy ramp applied 20 eV 0.60 $1/k_0$ – 59 eV 1.60 $1/k_0$). The individual protein content was identified and quantified as area abundance (arbitrary units) using the PEAKS Xpro software (Bioinformatics solutions, Waterloo, ON, Canada). The score threshold providing a 1 % identification false discovery rate was automatically calculated by PEAKS Xpro software. Precursor and fragment tolerances used for the searches were 20 ppm and 0.05 Da, respectively. Carbamidomethylation of cysteine was considered as fixed modification, and oxidation of methionine as variable modification.

For the complete characterization of mare milk proteome, in-gel and in-solution digested samples were used, and for the differential proteome analysis related to changes in mare milk proteins associated with different lactation stages and management systems, only in-solution digested samples were used (Fig. 1).

2.3.4. Peptidomic analysis

Endogenous peptides (S1 fraction) extracted from the 54 individual samples were analysed by matrix-assisted laser desorption/ionization coupled to time-of-flight tandem mass spectrometry (MALDI-TOF/TOF) in reflector mode (Autoflex III, Bruker Daltonics). For this, dried supernatants were suspended in 0.1 % TFA in water (by volume) and desalted (2 μL sample) using ZipTip® C18 micro-columns (Merck

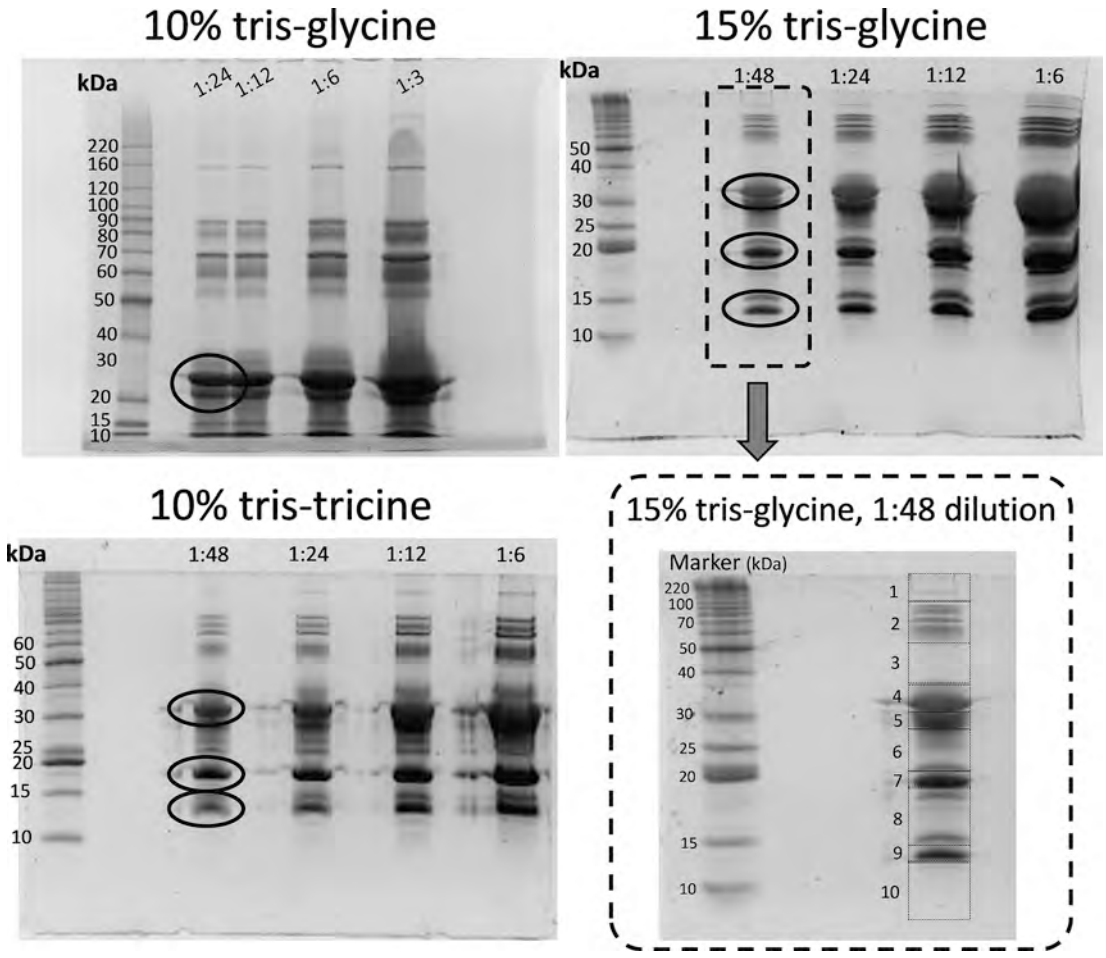


Fig. 2. Separation of proteins from mare milk samples (n = 54) pool in three different sodium dodecyl sulfate polyacrylamide (10 % and 15 % tris–glycine, and 10 % tris-tricine) gels (SDS-PAGE) using the same commercial marker, and the ten excisions made in 15 % tris–glycine gel 1:48 dilution for in-gel digestion. Different lanes within a gel correspond to different dilutions (1:48; 1:24; 1:12; 1:6; 1:3; by volume) of the precipitated proteins from the milk pool. Most abundant protein bands are marked in an ellipse.

Millipore, Burlington, MA, USA) that were prewashed with acetonitrile and TFA 0.1 % in water (by volume). Peptides were acidified with TFA 0.1 % in water (by volume), and eluted with 0.5 μL of the corresponding matrix (HCCA 10 mg/mL in 70/30 acetonitrile to 0.1 % TFA in water ratio (by volume)) on a GroundSteel massive 384 target plate (Bruker Daltonics). Eluted samples were allowed to air dry at room temperature, and MALDI-TOF/TOF analysis was performed. Mass spectrometry was operated as follows: 1000–6000 Th window, refletron positive mode, ion source 1 at 17 kV, ion source 2 at 18.5 kV, lens at 9 kV, pulsed ion extraction of 120 ns, high gating ion suppression up to 600 Mr. Mass calibration was performed externally with pepmix standard calibration mixture (Bruker Daltonics) in the same range as the samples. A total of 6000 scans were collected for each sample. Data acquisition was performed using FlexControl 3.0 software (Bruker Daltonics), and peak peaking and subsequent spectra analysis was performed using FlexAnalysis 3.0 software and ClinProtTools 3.1 (Bruker Daltonics).

2.4. Bioinformatics and statistical analysis

For whole proteomic analysis, all non-redundant proteins identified in the mare milk samples were compiled and classified using PANTHER classification system (2023) (version 17.0), considering *Equus caballus* genome as background. This bioinformatics tool classified proteins according to class and function (gene ontology analysis for molecular function, biological process and cellular component). In addition, ingenuity pathway analysis (QIAGEN, Madrid, Spain) checked against *Equus caballus* was used for subsequent bioinformatics analysis, including ingenuity canonical pathways and upstream regulators. For canonical pathway analysis, the –log (*P*-value) $>$ 1.30 was set as threshold; for upstream regulators, the threshold was defined as *P*-value overlap $<$ 0.05. Gene symbols assigned to proteins were obtained from UniProtKB (2023).

For differential proteomic analysis, data obtained from the 54 individual samples (*P*2 fraction) after in-solution digestion was analysed using the IBM-SPSS statistics software (version 28.0, IBM, Armonk, NY, USA). Among all the proteins found, only those identified in the milk from a minimum of two animals *per* lactation week and *per* farm were included in the statistical analysis. Data was log transformed, and normality and homoscedasticity were checked. The general linear model of analysis of variance was applied to data including farm (l = 3) and lactation stage (l = 3; considering weeks 6 and 10 as early lactation, weeks 14 and 18 as mid lactation, and weeks 22 and 26 as late lactation) as fixed factors, individual animal (l = 3 *per* farm) as a random factor nested within farm, and interaction effect between lactation stage and farm. Fisher's least significance difference test was used for pairwise comparisons. Significance level was declared at *P*-value $\leq$ 0.05.

Peptidomic data analysis was carried out using the ClinProTools software version 2.2 (Bruker Daltonics) including a principal component analysis (PCA) of the intensities of mass spectra signals in order to identify endogenous peptide profiles able to differentiate between mare milk samples from different farms and lactation stages (early, mid and late lactation).

3. Results

3.1. Mare milk proteome

Protein separation using SDS-PAGE revealed that 10–15, 20 and 30 kDa were the three predominant protein bands in mare milk (Fig. 2). These three bands contained alpha-s2- and beta-caseins and the major whey proteins alpha-lactalbumins A and B/C, beta-lactoglobulin I and II, and lysozyme C. The 30 kDa fraction also contained other milk-specific proteins such as alpha-s1-casein, kappa-casein, lactose synthase B protein, lactoperoxidase and a lactotransferrin precursor, as well as a high number of immunoglobulin (Ig)-related proteins and others related to lipid metabolism (apolipoproteins, lipoprotein lipase, milk fat globule epidermal growth factor 8 fragments…). Bands at 60, 70 and 80 KDa were also abundant and were mainly composed of albumin, keratins and other milk-specific proteins such as caseins, beta-lactoglobulin, alpha-lactalbumin and lysozyme C, as well as a lactotransferrin precursor. Protein resolution in SDS-PAGE gels relies on electrophoretic mobility, and therefore, not only protein mass but additional features that affect overall charge, such as post-translational modifications, may affect the way proteins get resolved in a polyacrylamide gel (Tiwari et al., 2019).

Direct nLC-MS/MS (nLC-ESI-TIMS/TOF) analysis after in-solution digestion and SDS-PAGE followed by in-gel digestion and nLC MS/MS analysis (Fig. 1) resulted in a different variety of proteins identified (Supplementary Material 1). Combining both complementary approaches improved mare milk proteome coverage resulting in 469 proteins identified. Only 109 protein accessions were shared between the two digestions, whereas 222 and 138 accessions were sorted as unique for in-gel and in-solution digestion, respectively. Based on gene ontology analysis for protein classification, in-gel digestion isolated one pigmentation-related protein that was not isolated with in-solution digestion. Based on protein class, extracellular matrix proteins were only identified after in-gel digestion, whereas structural proteins and transmembrane signal receptors were only isolated using in-solution digestion. In addition, in-gel digestion was more efficient at recovering cytoskeletal proteins, translational proteins and chaperones, while with in-solution digestion more protein modifying enzymes were identified (Supplementary Material 2).

3.2. Functional analysis of mare milk proteome

All proteome (considering in-gel and in-solution digestions combined) from mare milk was subjected to functional classification using gene ontology analysis (Table 1). Unfortunately, not all the proteins identified in mare milk could be assigned to a gene ontology annotation due to lack of data in the classification system. The occurrence of genes unclassified into total function hits was 34.2 % for molecular function, 19.2 % for biological process, and 32.2 % for cellular component. Additionally, 19.2 % of genes could not be assigned to a protein class either.

Considering the genes of all proteins identified, 55.7 % were a cellular anatomical entity, and 12.1 % belonged to protein-containing complexes. Among cellular anatomical entity proteins, the most frequent ones were those belonging to intracellular anatomical structures (16.1 % of genes classified as cellular anatomical entity), followed by proteins from cytoplasm (12.4 %), membrane (12.1 %), and organelles (12 %). Other proteins showing percentages between 8.3 and 9.1 % were classified as proteins from cell periphery, the extracellular region or the extracellular space. The rest of proteins showed percentages lower than 5 % in all cases, and often belonged to very specific regions of the cell. In the case of protein-containing complexes, most proteins belonged to the membrane protein complex (26.1 %), the ribonucleoprotein complex (21.7 %), or catalytic complex (17.4 %). The rest of the proteins belonging to other complexes was below 7 % in all cases. Regarding molecular function, most of the genes identified had binding function (27.6 %), followed by catalytic activity (19.8 %), whereas

Table 1
Gene ontology classifications of mare milk proteome obtained from PANTHER classification system, expressed as percentage (%) of gene hits against total function hits in each gene ontology domain.

Function	%
Molecular function	
Binding	27.6
Catalytic activity	19.8
Structural molecule activity	6.9
Molecular function regulator	4.3
Transporter activity	2.3
Adenosine triphosphate-dependent activity	2.0
Molecular transducer activity	0.9
Translation regulator activity	0.6
Transcription regulator activity	0.6
Molecular adaptor activity	0.6
Low-density lipoprotein particle receptor activity	0.3
Biological process	
Cellular process	22.7
Metabolic process	12.0
Biological regulation	9.4
Localization	8.1
Response to stimulus	7.6
Developmental process	5.2
Multicellular organismal process	5.2
Immune system process	2.9
Signaling	2.7
Growth	1.3
Locomotion	1.2
Biological process involved in interspecies interaction between organisms	1.0
Biological adhesion	1.0
Reproductive process	0.2
Reproduction	0.2
Pigmentation	0.2
Cellular component	
Protein-containing complex	12.1
Membrane protein complex	26.1
Ribonucleoprotein complex	21.7
Catalytic complex	17.4
Nuclear protein-containing complex	6.5
Intracellular protein-containing complex	6.5
Endoplasmic reticulum protein-containing complex	6.5
Transporter complex	4.3
Intraciliary transport particle A	2.2
Intraciliary transport particle	2.2
Sm-like protein family complex	2.2
High-density lipoprotein particle	2.2
Prp19 complex	2.2
Cellular anatomical entity	55.7
Intracellular anatomical structure	16.1
Cytoplasm	12.4
Membrane	12.1
Organelle	12.0
Cell periphery	9.1
Extracellular region	8.6
Extracellular space	8.3
Supramolecular complex	3.4
Cytosol	3.4
Endomembrane system	2.3
Cell junction	1.7
Leaflet of membrane bilayer	1.1
Side of membrane	1.1
Cell projection	1.1
Cell surface	1.0
Membrane-enclosed lumen	0.9
Extrinsic component of membrane	0.7
Intrinsic component of membrane	0.7
External encapsulating structure	0.6
Somatodendritic compartment	0.4
Perinuclear region of cytoplasm	0.4
Postsynapse	0.4
Envelope	0.3

(continued on next page)

Table 1 (*continued*)

Function	%
Organelle subcompartment	0.3
Endoplasmic reticulum exit site	0.3
Nucleoplasm	0.3
Apical part of cell	0.1
Cell body	0.1
Actin cortical patch	0.1
Actin filament bundle	0.1
Cell division site	0.1
Chromatin	0.1

proteins with other functions were less frequent (less than 7 % in each function). In the case of biological processes, the majority of proteins identified participated in cellular (22.7 %) or metabolic processes (12 %). A number of proteins taking part in biological regulation (9.4 %), localization (8.1 %) or response to stimulus (7.6 %) were also identified (Table 1). Classification according to protein class (Table 2) confirmed that the majority of proteins in mare milk were enzymes and proteins involved in nutrient transport (binding, transfer/carrier, and transporter proteins).

In addition, all proteins identified in mare milk were explored under ingenuity pathway analysis. The results showed that, in total, mare milk proteins participated in 433 canonical pathways, among which 194 were found to be significantly enriched by the genes expressed in mare milk. The top four canonical pathways were liver X receptor/retinoid X receptor (LXR/RXR) activation, acute phase response signaling, farnesoid X receptor/retinoid X receptor (FXR/RXR) activation, and 24-dehydrocholesterol reductase gene (DHCR24) signaling pathway. Milk molecules that participated in LXR/RXR activation, FXR/RXR activation and DHCR24 signaling pathway (pathways related to lipid metabolism) were almost identical, and included alpha-1-B-glycoprotein, angiotensinogen, alpha-2-HS-glycoprotein, albumin, alpha-1-microglobulin/bikunin precursor (protein AMBP), apolipoproteins A1, A2, A4, B and E, beta-2 glycoprotein I, clusterin, fibrinogen alpha chain, inter-alpha-trypsin inhibitor heavy chain 4, kininogen 1, sterol regulatory element binding transcription factor 1, serotransferrin, and transthyretin. Molecules only participating in LXR/RXR activation were CD36 molecule, lysozyme C, and tumour necrosis factor receptor superfamily member 11B. Fetuin B only participated in FXR/RXR activation, and amyloid-beta A4 protein, KRAS proto-oncogene guanosine triphosphatase and RAP1B Ras protein only in DHCR24 signaling pathway. Fatty acid synthase and lipoprotein lipase only participated in LXR/RXR activation and FXR/RXR activation. Conversely, some of the proteins contributing to acute phase response signaling were also part of lipid-related pathways (angiotensinogen, alpha-2-HS-glycoprotein, albumin, protein AMBP, apolipoproteins A1 and A2, beta-2 glycoprotein I, fibrinogen alpha chain, inter-alpha-trypsin inhibitor heavy chain 4, KRAS proto-oncogene, RAP1B, and serotransferrin), but other exclusive proteins were also found: ceruloplasmin, pentraxin family member, fibrinogen beta and gamma chains, fibronectin, plasminogen, serpin family D member 1, and serpin family G member 1. Additionally, identified enriched upstream regulators included transcription and translation regulators, peptidases and other enzymes, endogenous mammalian chemicals, kinases, transporters, receptors, cytokines, growth factors, and protease and kinase inhibitors. Among endogenous mammalian chemicals, the following types of molecules were enriched: amino acids, mono and disaccharides, hormones, fatty acids, bile acids, minerals, water-soluble vitamins, cholecalciferol, and other molecules (Supplementary Material 2).

Table 2
Classification of mare milk proteins according to the protein class, expressed as percentage (%) of gene hits against total function hits.

Function	%
Metabolite interconversion enzyme	13.7
Cytoskeletal protein	11.4
Protein-binding activity modulator	11.4
Defense/immunity protein	7.7
Transfer/carrier protein	6.6
Protein modifying enzyme	5.9
Translational protein	4.4
Chaperone	3.7
Transporter	3.3
Calcium-binding protein	3.0
Chromatin/chromatin-binding, or -regulatory protein	2.6
Scaffold/adaptor protein	1.1
Cell adhesion molecule	1.1
Intercellular signal molecule	0.7
Ribonucleic acid metabolism protein	0.7
Membrane traffic protein	0.7
Structural protein	0.7
Storage protein	0.7
Extracellular matrix protein	0.4
Deoxyribonucleic acid metabolism protein	0.4
Transmembrane signal receptor	0.4

3.3. *Effect of lactation stage and farm on mare milk proteome*

Proteins significantly affected (P-value ≤ 0.05) by the lactation stage and farm factors included in the analysis of variance are shown in Table 3, including the effect of individual animal and the interaction between farm and lactation stage. Farm and individual animal had little impact on mare milk proteome. In fact, farm only significantly changed (P-value ≤ 0.05) the abundance of alpha-1 antitrypsin (SPI2 gene), which was 2–3 times lower in farm I (low grazing activity) than in farms II and III (high grazing activity). Significant differences (P-value ≤ 0.05) among individual mares were only found in the abundance of interferon alpha-inducible protein 27 (IFI27 gene) and perilipin 2 (PLIN2 gene). Interestingly, there was a significant interaction (P-value ≤ 0.05) between farm and lactation stage in perilipin 2 abundance. In this regard, although perilipin 2 abundance slightly increased from early to mid lactation in milk from all farms, milk from farm I showed a decrease in perilipin 2 from mid to late lactation, whereas it continued increasing in milk from farms II and III. However, these differences did not translate into significantly different perilipin 2 average contents between farms. Another significant interaction was found in the abundance of butyrophilin subfamily 1 member A1 protein (BTN1A1 gene), but in this case, milk from farm I showed an opposite evolution compared to farm II, and milk from farm III changed differently (Fig. 3). Lactation stage affected the largest number of proteins, *i.e.* beta-lactoglobulin II (LGB2 gene), Ig-like domain-containing protein, interferon alpha-inducible protein 27, lactotransferrin (LTF gene), polypeptide *N*-acetylgalactosaminyltransferase (GALNT7 gene), and transforming acidic coiled-coil containing protein 2 (TACC2 gene). The abundance of Ig-like domain-containing protein and polypeptide *N*-acetylgalactosaminyltransferase decreased from early to mid lactation and increased afterwards, whereas the other four proteins increased from early to late lactation (Fig. 4). Despite this, most proteins identified in mare milk proteome were not significantly (P-value > 0.05) affected by lactation stage.

Genes of the proteins significantly affected by farm and/or lactation stage were searched within the canonical pathways and the upstream regulators enriched in mare milk. Only two proteins were involved in any of the canonical pathways identified in this study: lactotransferrin as part of the neutrophil extracellular trap signaling pathway, and perilipin 2 as part of the chaperone mediated autophagy signaling pathway. In addition, 198 enriched upstream regulators were associated with at least one of the following proteins: butyrophilin subfamily 1 member A1, polypeptide *N*-acetylgalactosaminyltransferase, lactotransferrin, perilipin 2, and transforming acidic coiled-coil containing protein 2. The protein related with the highest number of upstream regulators (113) was perilipin 2, followed by lactotransferrin (67). Other proteins were related to 14 or less upstream regulators. Upstream regulators associated with perilipin 2 were mainly chemical drugs, transcription regulators and endogenous mammalian chemicals (beta-estradiol, cholesterol,

Table 3
Mare milk proteins whose abundance was significantly (*P*-value ≤ 0.05) affected by the factors lactation stage, farm, or individual animal, and/or the combined effect between farm and lactation stage.

		Significance (*P*-value)			
Protein	Gene	Farm	Lactation stage	Individual animal	Farm*Lactation stage
alpha-1 antitrypsin	SPI2/SERPINA1	0.001	0.486	0.998	0.525
beta-lactoglobulin II	LGB2	0.815	< 0.001	0.484	0.125
butyrophilin subfamily 1 member A1	BTN1A1	0.533	0.796	0.227	0.017
immunoglobulin-like domain-containing protein	–	0.639	0.020	0.551	0.152
interferon alpha-inducible protein 27	IFI27	0.761	0.006	0.004	0.250
lactotransferrin	LTF	0.954	0.004	0.621	0.343
perilipin 2	PLIN2	0.685	0.670	0.007	0.035
polypeptide *N*-acetylgalactosaminyltransferase	GALNT7	0.466	0.037	0.889	0.640
transforming acidic coiled-coil containing protein 2	TACC2	0.464	< 0.001	0.773	0.401

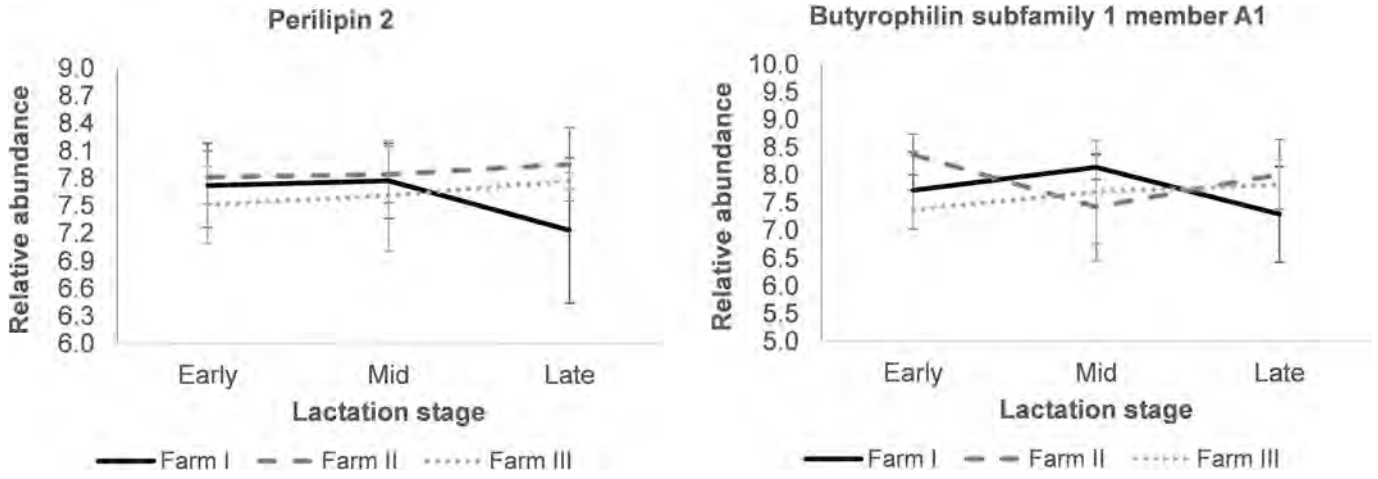


Fig. 3. Changes during lactation in the mean relative abundance (arbitrary units) of perilipin 2 and butyrophilin subfamily 1 member A1 in mare milk samples from the commercial farms (n = 54).

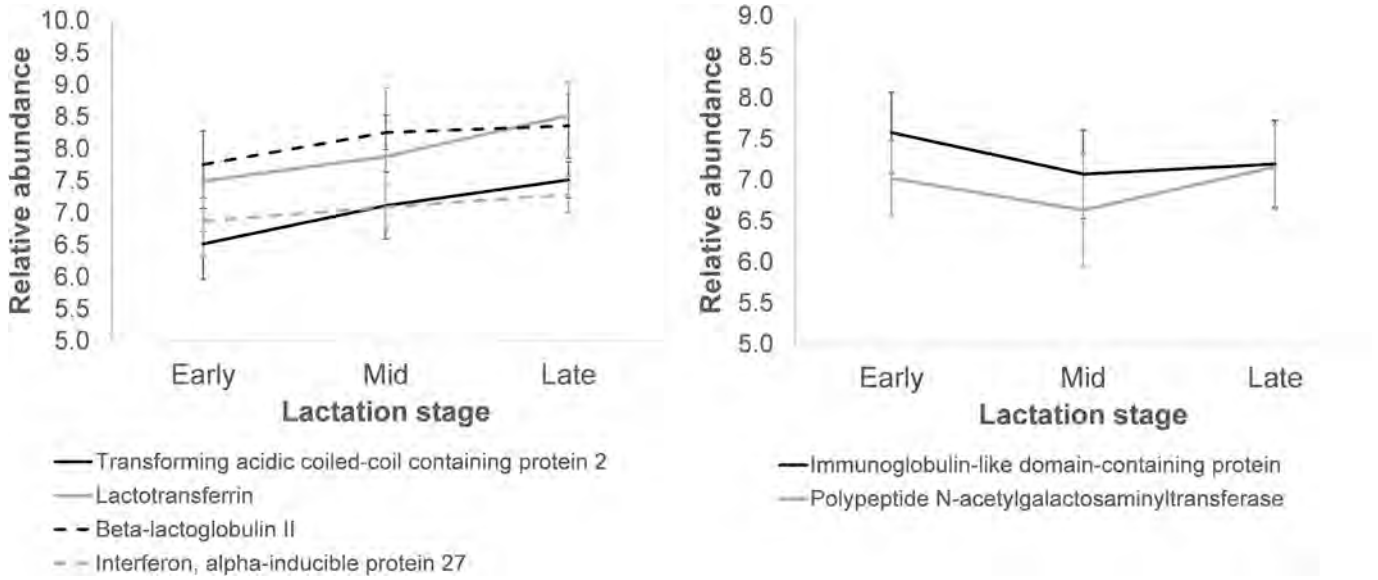


Fig. 4. Changes during lactation in the mean relative abundance (arbitrary units) of those proteins of mare milk samples from the commercial farms (n = 54) significantly affected by lactation stage.

oleic acid, palmitic acid, linoleic acid, gamma-linolenic acid, 10*t*,12*c*-octadecadienoic acid, other fatty acids, L-glutamic acid, and anandamide). Upstream regulators associated with lactotransferrin were mainly transcription regulators and chemical drugs, whereas only four endogenous mammalian chemicals, all hormone-related (beta-estradiol, progesterone, 2-methoxyestradiol, and 4-hydroxy-17-beta-estradiol), were related to lactotransferrin.

3.4. Mare milk endogenous peptidome

Most endogenous peptides found in all mare milk samples had a molecular mass lower than 3600 Da, although some relatively abundant peptides with masses ranging between 4000 and 4600 and 5200–6600 Da were also present (Fig. 5). However, no clear differentiation among the studied farms or lactation stages was observed according to the results of the PCA considering the peptide molecular sizes. In other terms, no differential patterns in mare milk peptidome were observed among farms or during lactation.

4. Discussion

Based on protein separation using SDS-PAGE, the most abundant proteins found in mare milk samples were those with a mass between 10 and 30 kDa. Within this mass range, proteins previously described in mare milk (Godovac-Zimmermann et al., 1987; Miranda et al., 2004; Uniacke-Lowe et al., 2010) were present: alpha-s1-casein (26 kDa), alpha-s2-casein (27 kDa), beta-casein (27 kDa), kappa-casein (21 kDa), alpha-lactalbumin A and B/C (14 and 15 kDa, respectively), beta-lactoglobulin I and II (20 kDa), lysozyme C (15 kDa), and lactotransferrin fragments. Beta-casein identified in spots equivalent to 10–15 kDa might reveal the presence of a phosphorylated variant with lower molecular mass (11 kDa) previously reported in equine milk (Miclo et al., 2007).

Beyond caseins and whey proteins, milk contains a large number of other proteins. In the present study, a combination of in-gel and in-solution digestion of the samples notably improved the coverage of mare milk proteins. As expected, proteome analysis after in-gel digestion resulted in a higher number of proteins identified, probably due to the extra step of fractionation at protein level using SDS-PAGE. A total of 469 non-redundant proteins were identified in the mare milk samples of Basque Mountain Horse breed, similar to the number of proteins identified in milk from Tieling Draft Horse (465), Selle Français (467) and Welsh Pony (504) breeds (whey fraction only; (Lv et al., 2024)). These results also agree with previous reports in cow (239–573 protein identifications; (Chen et al., 2023; D'Alessandro et al., 2011)), goat (418–595; (Anagnostopoulos et al., 2016; Chen et al., 2023; Sun et al., 2020; Zhao et al., 2023a)), and sheep (550–685; (Anagnostopoulos et al., 2016)) whey fractions. Conversely, Ji et al. (2024) provided a list of 687 protein identifications in mare whey proteome. However, more than 85 % of the identifications in mare milk were matched with a protein database from an organism other than horse (namely yak, cattle, buffalo, camel, goat, donkey, human, sheep, and mouflon). Probably for this reason, the study by Ji et al. (2024) shared only 33 UniProtKB primary accessions with the present study, which only considered the *Equus caballus* protein database.

With regard to protein function, similar gene ontology classifications

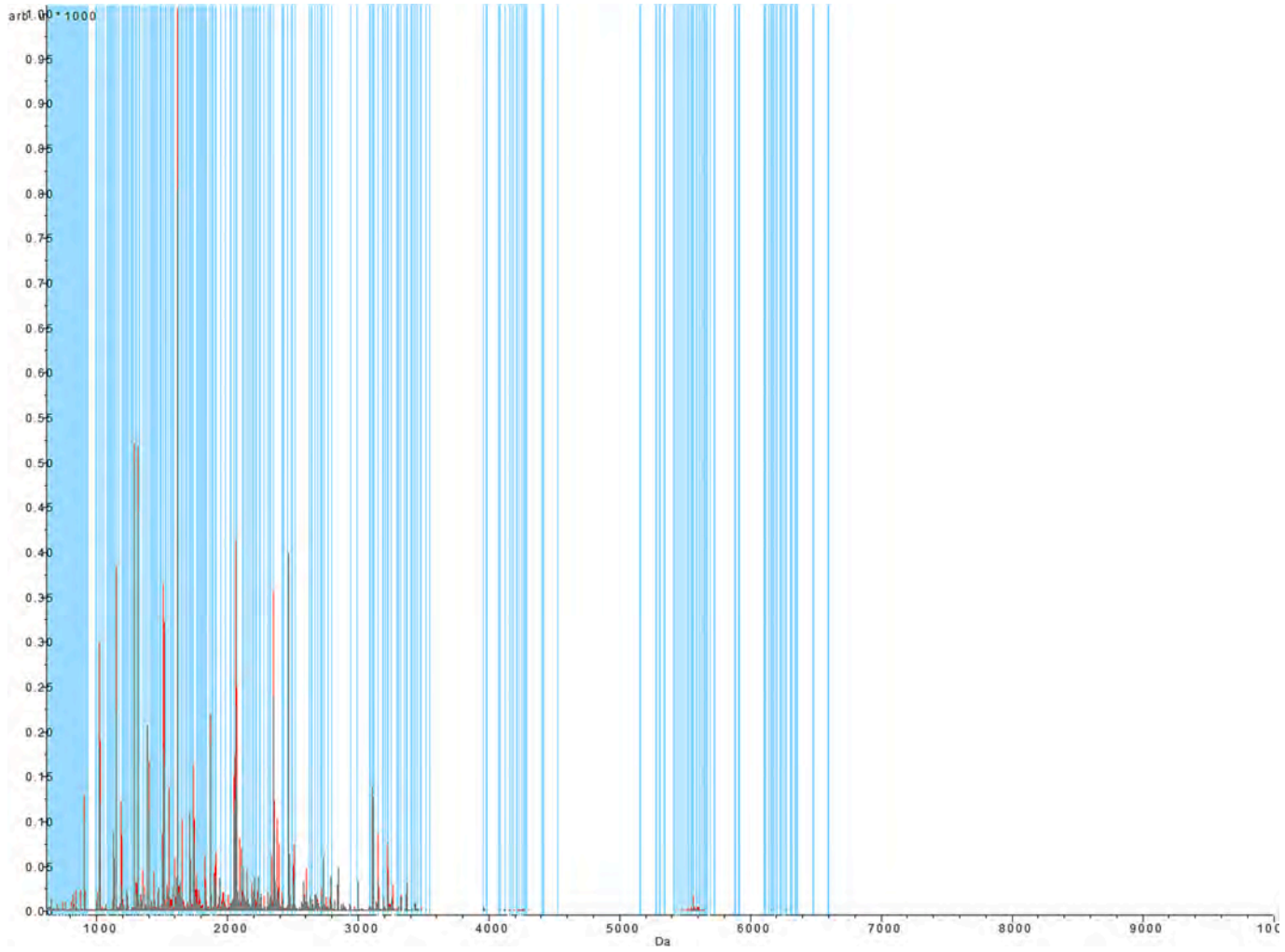


Fig. 5. Mass spectra abundances (arbitrary units) of the endogenous peptides according to their molecular size (Da) from mare milk samples (n = 54). Red spectrum represents average abundance in early lactation samples; grey spectrum represents average abundance in all samples. Blue bands correspond to the mass ranges that follow the S/N > 3 criteria (s: signal intensity; n: noise). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

were found in Basque Mountain Horse milk compared to other horse breeds (Lv et al., 2024) and other mammal species (Anagnostopoulos et al., 2016; Ji et al., 2024; Sun et al., 2020; Zhao et al., 2023a), suggesting that milk proteome from different species is comprised by proteins with similar functions that are likely elementary constituents of the matrix. Overall, main molecular functions of milk proteome are binding, catalytic activity, structural molecule activity and transporter activity. In other terms, the majority of proteins in milk are nutrient transporters, enzymes, structural proteins, and Ig-related proteins (binding function). In the present study, this was confirmed by the protein class distribution of mare milk, aligning with the main two roles of milk: providing neonates with nutrients and immunity (Sun et al., 2020). On the other hand, main biological processes reported in milk are the cellular process, metabolic process, biological regulation, response to stimulus and localization (Anagnostopoulos et al., 2016; Ji et al., 2024; Sun et al., 2020; Zhao et al., 2023a). A large number of extracellular components in milk proteome exhibits that milk contains a high number of secreted proteins, which is the case of caseins, whey proteins, and proteins associated with the milk fat globule membrane (Burgoyne & Duncan, 1998). Despite these similarities, Ji et al. (2024) confirmed that the proteome of equine (mare and jenny) milk differed from that of human, camel and ruminant milk.

Most significantly enriched pathways in mare milk are those related to lipid metabolism (LXR/RXR activation, FXR/RXR activation, and DHCR24 signaling pathway) and inflammation (acute phase response signaling). LXR and RXR are nuclear receptors that form a heterodimer with a role in lipid (particularly cholesterol) metabolism (Murthy et al., 2002). FXR is another nuclear receptor that participates in bile acid homeostasis and lipid, glucose and amino acid metabolism (Massafra et al., 2018). DHCR24 is the enzyme that catalyses the final step in the Bloch cholesterol synthetic pathway (Luu et al., 2014). In this sense, mare milk contains an enhanced accumulation of molecules that participate in lipid metabolism, including apolipoproteins (A1, A2, A4, B, E, and clusterin), fatty acid synthase, lipoprotein lipase or transthyretin. These proteins perform fatty acid acquisition from triglycerides, *de novo* fatty acid synthesis, and lipid transportation, and ensure the delivery of required bioavailable lipids to the newborn (Irshad & Dubey, 2005; Liz et al., 2020; Mendelson et al., 1977; Suburu et al., 2014). Fatty acid synthase is also detrimental for the functional development and maintenance of the lactating mammary gland (Suburu et al., 2014). On the other hand, acute phase response signaling is an inflammatory response against tissue injury or infection, which in the case of the mammary gland, also stimulates initial stages of involution after weaning (Stein et al., 2004). Proteins in mare milk that belong to this pathway are mainly associated with blood pressure regulation (Hussain & Awan, 2018), coagulation (Brighton et al., 1996; He et al., 2002; Kamath & Lip, 2003; Law et al., 2006), and inflammation (Kashyap et al., 2009; Wang et al., 2020).

Beyond the deep characterization of mare milk proteome, the present study aimed to investigate the effect of management of the mares and lactation stage on the protein profile. In this regard, a high-grazing activity of the mares increased the abundance of alpha-1 antitrypsin (encoded by SPI2 gene (UniProt), also named SERPINA1 (Ensembl Genome Browser, 2023) in milk. This protein is a serine protease inhibitor that primarily inhibits elastase from neutrophil leucocytes (Carrell & Travis, 1985). There is evidence that dietary unsaturated fatty acid supplementation (mainly oleic, linoleic or linolenic acids) modulates SERPINA1 gene expression in cattle mammary gland tissue (Mach et al., 2011). Other studies also observed that butyrate (Elce et al., 2017) and n-3 long-chain polyunsaturated fatty acids (Gladine et al., 2012) can change the expression of SERPINA1 gene in intestinal and hepatic tissues, respectively. Given that fresh grass is rich in n-3 polyunsaturated fatty acids (Valdivielso et al., 2016; Sahaka et al., 2020), a high grazing activity may have modulated the encoding of alpha-1 antitrypsin in mare mammary gland. It should also be noted that, unlike cattle, horses are non-ruminants and absorb dietary unsaturated fatty acids with only slight modification (Belaunzaran et al., 2015), which might affect SERPINA1 gene expression differently. Since SERPINA1 is involved in the acute phase response signaling pathway, it may be associated with mammary gland development and gradual involution (D'Alessandro et al., 2011), and changes in its expression may affect the immune response of the mammary gland as suggested by Mach et al. (2011).

Lactation stage showed a significant effect on mare milk proteins. Among the proteins affected, transforming acidic coiled-coil containing protein 2, beta-lactoglobulin II, lactotransferrin, and interferon alpha-inducible protein 27 steadily increased during lactation. Beta-lactoglobulin II and lactotransferrin are binding proteins and, therefore, nutrient transporters that bind to small lipophilic molecules (*e.g.* retinol, (Pérez & Calvo, 1995; Wodas et al., 2020)) and iron (Alderova et al., 2008), respectively. They are synthetized in mammary epithelial cells under hormonal, nutrient (amino acids and glucose), mammalian target of rapamycin (mTOR) signalling, and Janus kinase 2-Signal transducers and activators of transcription 5 (Jak2-Stat5) pathway regulation (Burgoyne & Duncan, 1998; Bionaz & Loor, 2011). The increasing trend of the abundance of beta-lactoglobulin II and lactotransferrin during lactation has also been reported in cow milk (Yang et al., 2020), and might respond to an upregulation in the expression of genes positively involved in milk protein synthesis (Bionaz & Loor, 2011). However, no changes in the abundance of caseins or other whey proteins were observed in the present study. In the case of lactotransferrin, changes due to hormonal regulation may have been of particular relevance, considering that four hormone upstream regulators (beta-estradiol, progesterone, 2-methoxyestradiol, and 4-hydroxy-17-beta-estradiol) were found to regulate this protein. The increase of lactotransferrin during lactation might play a role in early phase mammary gland involution by reducing viability of mammary epithelial cells. It might also protect mammary gland from infections during early involution (Riley et al., 2008; Zhang et al., 2015), since, immediately after weaning, the mammary gland is highly susceptible to infections driven by milk accumulation in the udder (Ollier et al., 2013).

Other proteins that were increased throughout lactation were transforming acidic coiled-coil containing protein 2 and interferon alpha-inducible protein 27. Transforming acidic coiled-coil containing protein 2 (TACC2 gene) participates in mitotic cell cycle, *i.e.* cell differentiation, and has repeatedly been associated with breast cancer and other tumour modulation (Cheng et al., 2010). Its implications in healthy mammary gland development along lactation remain unclear, although there is evidence that TACC2 expression in mammary gland increases during pregnancy and is highest at lactation (Bargo et al., 2010). Interferon alpha-inducible protein 27 plays a role in the apoptotic signaling pathway and programmed cell death. Therefore, the increasing expression of this protein during lactation could be related with a gradual involution of the mammary gland in response to a lower suckling frequency. In fact, foals start to graze or consume feedstuff at a very early stage of life, gradually increasing forage intake at the expense of milk (Bolzan et al., 2020).

Lactation stage also affected the content of Ig-like domain-containing protein and polypeptide *N*-acetylgalactosaminyltransferase, but in this case, the content of both proteins decreased from early to mid lactation, increasing afterwards. Polypeptide *N*-acetylgalactosaminyltransferase is a glycosyltransferase that participates in post-translational protein glycosylation (Tenno et al., 2002). There is evidence of a dynamic glycosylation of proteins during lactation, which affects protein function, stability and structure (Froehlich et al., 2010). However, glycosylation dynamic of IgA, the main Ig in mature mare milk (Perkins & Wagner, 2015), has been described as low, with similar variations of IgA expression and glycosylation profile (Froehlich et al., 2010). Therefore, evolution of glycosyltransferase and IgA abundances could be related in mare milk. In accordance to the present results, cow milk proteome also shows changes during lactation in Igs (Yang et al., 2020) and, overall, enzymes, transport proteins, and immune-related proteins (Zhang et al., 2015).

In the present study, a significant interaction effect between management and lactation stage was observed for perilipin 2 and butyrophilin subfamily 1 member A1 protein, which are two of the most abundant proteins present in the milk fat globule membrane (Cebo et al., 2012; Han et al., 2020). However, no significant differences among farms were observed. These two proteins associate with xanthine oxidoreductase both in the apical membrane of the lactating mammary epithelial cells and in the milk fat globule membrane, creating an interaction that might regulate milk lipid secretion (McManaman et al., 2002, 2007). In dairy cows, a significant effect of lactation stage on the abundance of bovine milk fat globule membrane proteins (including butyrophilin and perilipin 2) has been postulated (Mondy & Keenan, 1993; Zhang et al., 2015). In the present study, the effect of lactation stage might have been hindered by opposite evolution patterns among farms. In fact, the abundance of butyrophilin subfamily 1 member A1 protein followed an opposite evolution in milk produced under low grazing activity compared to high grazing activity supplemented with hay, whereas evolution in the farm with high grazing activity but silage supplementation was different. Changes observed in the abundance of perilipin 2 during lactation were also opposite in the farm with low grazing activity compared to farms with high grazing activity, but only after mid lactation. Zang et al. (2015) related a decrease in the abundance of cow milk perilipin 2 and butyrophilin subfamily 1 member A1 protein at late stages of lactation with mammary gland involution. Yet in the present study, such a decrease at the end of the lactation period was only evident in the low grazing mare milk. Results showed that perilipin 2 is regulated by a high number (113) of upstream regulators, including hormones and dietary substances like fatty acids (linoleic acid, gamma-linolenic acid, 10*t*,12*c*-octadecadienoic acid). As mentioned, polyunsaturated fatty acids are essentially obtained from dietary forage. In this sense, a pasture-based feeding regime of mares might have resulted in a higher assimilation of these fatty acids, and consequently, a different expression of perilipin 2 during lactation compared to low grazing mares. Supporting this, Qin et al. (2018) found that supplementation with unsaturated fatty acids (conjugated linolenic acid, or sunflower and fish oils) resulted in a higher expression of perilipin 2 in cow milk. In consequence, we hypothesize that changes in equid milk fat globule membrane proteins during final stages of lactation can be modulated through diet with a potential impact on the functions that these proteins perform (milk lipid secretion and mammary gland involution).

5. Conclusion

The comprehensive analysis of mare milk proteome performed in this study revealed that it is particularly enriched by proteins that participate in lipid metabolism and inflammation which are related to the newborn nutrient provisioning and probably also to the regulation of the mammary gland maturity during lactation. Mare milk proteome is quite stable against factors like mare management and lactation stage, although some proteins were altered. In this respect, management system, and presumably the feeding regime of mares, can modulate a protein related with the immune response (alpha-1 antitrypsin), as well as the expression of milk fat globule membrane proteins during lactation. In addition, lactation stage can affect the content of proteins related to nutrient transport, apoptosis and immunity, with overall high abundance at the end of lactation that could be related with mammary gland involution.

This study provides initial insights towards a better understanding of mare milk proteome, metabolism and functionality, which is essential for an adequate understanding of beneficial properties of mare milk. These results establish the basis for further research in equine mammary gland structure and metabolism as affected by diet and lactation.

CRediT authorship contribution statement

Ana Blanco-Doval: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Mikel Azkargorta:** Writing – review & editing, Writing – original draft, Validation, Supervision, Software, Methodology, Investigation, Formal analysis, Data curation. **Ibon Iloro:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Software, Methodology, Formal analysis, Data curation. **Jabier Beaskoetxea:** Writing – review & editing, Writing – original draft, Visualization, Software, Methodology, Formal analysis, Data curation. **Felix Elortza:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Formal analysis, Conceptualization. **Luis Javier R. Barron:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition. **Noelia Aldai:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Investigation, Funding acquisition, Conceptualization, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

The Department of Education of the Basque Government for the predoctoral fellowship of A. Blanco-Doval, and the University of the Basque Country (UPV/EHU) for the mobility funding. This work was supported by the Basque Government throughout Biotasma (Elkartek, 2019), Behor Esne (Cooperation, 2020) and IT944-16 and IT1568-22 projects. Authors thank the commercial farms that participated in the study.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2024.138766.

References

Adlerova, L., Bartoskova, A., & Faldyna, M. (2008). Lactoferrin: A review. *Veterinární Medicína, 53*, 457–468. https://doi.org/10.17221/1978-VETMED

Anagnostopoulos, A. K., Katsafadou, A. I., Pierros, V., Kontopodis, E., Fthenakis, G. C., Arsenos, G., Karkabounas, S. C., Tzora, A., Skoufos, I., & Tsangaris, G. T. (2016). Milk of Greek sheep and goat breeds; characterization by means of proteomics. *Journal of Proteomics, 147*, 76–84. https://doi.org/10.1016/j.jprot.2016.04.008

Barello, C., Garoffo, L. P., Montorfano, G., Zava, S., Berra, B., Conti, A., & Giuffrida, M. G. (2008). Analysis of major proteins and fat fractions associated with mare's milk fat globules. *Molecular Nutrition & Food Research, 52*, 1448–1456. https://doi.org/10.1002/mnfr.200700311

Bargo, S., Raafat, A., McCurdy, D., Amirjazil, I., Shu, Y., Traicoff, J., Plant, J., Vonderhaar, B. K., & Callahan, R. (2010). Transforming acidic coiled-coil protein-3 (Tacc3) acts as a negative regulator of Notch signaling through binding to CDC10/Ankyrin repeats. *Biochemical and Biophysical Research Communications, 400*, 606–612. https://doi.org/10.1016/j.bbrc.2010.08.111

Belaunzaran, X., Bessa, R. J., Lavín, P., Mantecón, A. R., Kramer, J. K., & Aldai, N. (2015). Horse-meat for human consumption – Current research and future opportunities. *Meat Science, 108*, 74–81. https://doi.org/10.1016/j.meatsci.2015.05.006

Berger, S. T., Ahmed, S., Muntel, J., Polo, N. C., Bachur, R., Kentsis, A., Steen, J., & Steen, H. (2015). MStern blotting–high throughput polyvinylidene fluoride (PVDF) membrane-based proteomic sample preparation for 96-well plates. *Molecular & Cellular Proteomics, 14*, 2814–2823. https://doi.org/10.1074/mcp.O115.049650

Bionaz, M., & Loor, J. J. (2011). Gene networks driving bovine mammary protein synthesis during the lactation cycle. *Bioinformatics and Biology Insights, 5*. https://doi.org/10.4137/BBI.S7003. Article S7003.

Blanco-Doval, A., Barron, L., & Aldai, N. (2023). Changes during lactation in the mineral element content of mare milk produced in semi-extensive rural farms. *Journal of Food Composition and Analysis, 123*, Article 105629. https://doi.org/10.1016/j.jfca.2023.105629

Bolzan, A. M. S., Bonnet, O. J. F., Wallau, M. O., Basso, C., Neves, A. P., & de Faccio Carvalho, P. C. (2020). Foraging behavior development of foals in natural grassland. *Rangeland Ecology & Management, 73*, 243–251. https://doi.org/10.1016/j.rama.2019.10.011

Brighton, T. A., Hogg, P. J., Dai, Y., Murray, B. H., Chong, B. H., & Chesterman, C. N. (1996). Beta2-glycoprotein I in thrombosis: Evidence for a role as a natural anticoagulant. *British Journal of Haematology, 93*, 185–194. https://doi.org/10.1046/j.1365-2141.1996.446981.x

Burgoyne, R. D., & Duncan, J. S. (1998). Secretion of milk proteins. *Journal of Mammary Gland Biology and Neoplasia, 3*, 275–286. https://doi.org/10.1023/A:1018763427108

Businco, L., Giampietro, P. G., Lucenti, P., Lucaroni, F., Pini, C., Di Felice, G., Iacovacci, P., Curadi, C., & Orlandi, M. (2000). Allergenicity of mare's milk in children with cow's milk allergy. *Journal of Allergy and Clinical Immunology, 105*, 1031–1034. https://doi.org/10.1067/mai.2000.106377

Carrell, R., & Travis, J. (1985). α1-Antitrypsin and the serpins: Variation and countervariation. *Trends in Biochemical Sciences, 10*, 20–24.

Cebo, C., Rebours, E., Henry, C., Makhzami, S., Cosette, P., & Martin, P. (2012). Identification of major milk fat globule membrane proteins from pony mare milk highlights the molecular diversity of lactadherin across species. *Journal of Dairy Science, 95*, 1085–1098. https://doi.org/10.3168/jds.2011-4455

Chen, L., Hong, T., Li, Z., Shen, G., Gu, Y., & Han, J. (2023). A comparison of milk fat globule membranes and whey proteomes: New insight into variation nutrient differences between buffalo, cow, goat, and yak. *Food Chemistry, 429*, Article 136845. https://doi.org/10.1016/j.foodchem.2023.136845

Cheng, S., Douglas-Jones, A., Yang, X., Mansel, R. E., & Jiang, W. G. (2010). Transforming acidic coiled-coil-containing protein 2 (TACC2) in human breast cancer, expression pattern and clinical/prognostic relevance. *Cancer Genomics & Proteomics, 7*, 67–73. https://doi.org/10.1158/0008-5472.SABCS-09-3159

Curadi, M. C., Orlandi, M., Greppi, G. F., Toppino, P. M., Barzaghi, S., & Cattaneo, T. (2000). Identification of protein fractions in mare's colostrum and milk. *Milchwissenschaft, 55*, 446–449.

D'Alessandro, A., Zolla, L., & Scaloni, A. (2011). The bovine milk proteome: Cherishing, nourishing and fostering molecular complexity. An interactomics and functional overview. *Molecular BioSystems, 7*, 579–597. https://doi.org/10.1039/C0MB00027B

Egito, A. S., Miclo, L., Lopez, C., Adam, A., Girardet, J. M., & Gaillard, J. L. (2002). Separation and characterization of mares' milk αs1-, β-, κ-caseins, γ-casein-like, and proteose peptone component 5-like peptides. *Journal of Dairy Science, 85*, 697–706. https://doi.org/10.3168/jds.S0022-0302(02)74126-X

Elce, A., Amato, F., Zarrilli, F., Calignano, A., Troncone, R., Castaldo, G., & Canani, R. B. (2017). Butyrate modulating effects on pro-inflammatory pathways in human intestinal epithelial cells. *Beneficial Microbes, 8*, 841–847. https://doi.org/10.3920/BM2016.0197

Fotschki, J., Szyc, A. M., Laparra, J. M., Markiewicz, L. H., & Wróblewska, B. (2016). Immune-modulating properties of horse milk administered to mice sensitized to cow milk. *Journal of Dairy Science, 99*, 9395–9404. https://doi.org/10.3168/jds.2016-11499

Froehlich, J. W., Dodds, E. D., Barboza, M., McJimpsey, E. L., Seipert, R. R., Francis, J., An, H. J., Freeman, S., German, J. B., & Lebrilla, C. B. (2010). Glycoprotein expression in human milk during lactation. *Journal of Agricultural and Food Chemistry, 58*, 6440–6448. https://doi.org/10.1021/jf100112x

Gladine, C., Roy, N. C., Rigaudière, J., Laillet, B., Da Silva, G., Joly, C., Pujos-Guillot, E., Morio, B., Feillet-Coudray, C., McNabb, W. C., Chardigny, J., & Comte, B. (2012). Increasing intake of long-chain n-3 PUFA enhances lipoperoxidation and modulates hepatic gene expression in a dose-dependent manner. *British Journal of Nutrition, 107*, 1254–1273. https://doi.org/10.1017/S0007114511004259

Godovac-Zimmermann, J., Shaw, D., Conti, A., & McKenzie, H. (1987). Identification and the primary structure of equine α-lactalbumin B and C (Equus caballus, Perissodactyla). *Biological Chemistry, 368*, 427–433. https://doi.org/10.1515/bchm3.1987.368.1.427

Guri, A., Paligot, M., Crèvecoeur, S., Piedboeuf, B., Claes, J., Daube, G., Corredig, M., Griffiths, M. W., & Delcenserie, V. (2016). In vitro screening of mare's milk antimicrobial effect and antiproliverative activity. *FEMS Microbiology Letters, 363*. https://doi.org/10.1093/femsle/fnv234. Article fnv234.

Han, L., Zhang, M., Xing, Z., Coleman, D. N., Liang, Y., Loor, J. J., & Yang, G. (2020). Knockout of butyrophilin subfamily 1 member A1 (BTN1A1) alters lipid droplet formation and phospholipid composition in bovine mammary epithelial cells. *Journal of Animal Science and Biotechnology, 11*. https://doi.org/10.1186/s40104-020-00479-6. Article 72.

He, L., Vicente, C. P., Westrick, R. J., Eitzman, D. T., & Tollefsen, D. M. (2002). Heparin cofactor II inhibits arterial thrombosis after endothelial injury. *The Journal of Clinical Investigation, 109*, 213–219. https://doi.org/10.1172/JCI13432

Hussain, M., & Awan, F. R. (2018). Hypertension regulating angiotensin peptides in the pathobiology of cardiovascular disease. *Clinical and Experimental Hypertension, 40*, 344–352. https://doi.org/10.1080/10641963.2017.1377218

Iametti, S., Tedeschi, G., Oungre, E., & Bonomi, F. (2001). Primary structure of κ-casein isolated from mares' milk. *Journal of Dairy Research, 68*, 53–61. https://doi.org/10.1017/S0022029900004544

Irshad, M., & Dubey, R. (2005). Apolipoproteins and their role in different clinical conditions: An overview. *Indian Journal of Biochemistry & Biophysics, 42*, 73–80.

Jaeser, M., Wunderlich, C., & Henle, T. (2023). Characterization of the glycans of equine κ-casein. *Journal of Agricultural and Food Chemistry, 71*, 9460–9468. https://doi.org/10.1021/acs.jafc.3c00452

Jastrzębska, E., Wadas, E., Daszkiewicz, T., & Pietrzak-Fiećko, R. (2017). Nutritional value and health-promoting properties of mare's milk – a review. *Czech Journal of Animal Science, 62*, 511–518. https://doi.org/10.17221/61/2016-CJAS

Ji, Z., Dong, R., Du, Q., Jiang, H., Fan, R., Bu, D., Wang, J., Yu, Z., Han, R., & Yang, Y. (2024). Insight into differences in whey proteome from human and eight dairy animal species for formula humanization. *Food Chemistry, 430*, Article 137076. https://doi.org/10.1016/j.foodchem.2023.137076

Kamath, S., & Lip, G. (2003). Fibrinogen: Biochemistry, epidemiology and determinants. *QJM: An International Journal of Medicine, 96*, 711–729. https://doi.org/10.1093/qjmed/hcg129

Kashyap, R. S., Nayak, A. R., Deshpande, P. S., Kabra, D., Purohit, H. J., Taori, G. M., & Daginawala, H. F. (2009). Inter-α-trypsin inhibitor heavy chain 4 is a novel marker of acute ischemic stroke. *Clinica Chimica Acta, 402*, 160–163. https://doi.org/10.1016/j.cca.2009.01.009

Law, R. H., Zhang, Q., McGowan, S., Buckle, A. M., Silverman, G. A., Wong, W., Rosado, C. J., Langendorf, C. G., Pike, R. N., Bird, P. I., & Whisstock, J. C. (2006). An overview of the serpin superfamily. *Genome Biology, 7*. https://doi.org/10.1186/gb-2006-7-5-216. Article 216.

Liz, M. A., Coelho, T., Bellotti, V., Fernandez-Arias, M. I., Mallaina, P., & Obici, L. (2020). A narrative review of the role of transthyretin in health and disease. *Neurology and Therapy, 9*, 395–402. https://doi.org/10.1007/s40120-020-00217-0

Luu, W., Zerenturk, E. J., Kristiana, I., Bucknall, M. P., Sharpe, L. J., & Brown, A. J. (2014). Signaling regulates activity of DHCR24, the final enzyme in cholesterol synthesis. *Journal of Lipid Research, 55*, 410–420. https://doi.org/10.1194/jlr.M043257

Lv, K., Yang, Y., Li, Q., Chen, R., Deng, L., Zhang, Y., & Jiang, N. (2024). Identification and comparison of milk fat globule membrane and whey proteins from Selle Français, Welsh pony, and Tieling Draft horse mare's milk. *Food Chemistry, 437*, Article 137915. https://doi.org/10.1016/j.foodchem.2023.137915

Mach, N., Jacobs, A., Kruijt, L., Van Baal, J., & Smits, M. A. (2011). Alteration of gene expression in mammary gland tissue of dairy cows in response to dietary unsaturated fatty acids. *Animal, 5*, 1217–1230. https://doi.org/10.1017/S1751731111000103

MAPA. (2022). *Datos censales de la raza Caballo de monte del País Vasco*. Retrieved from: https://servicio.mapa.gob.es/arca/flujos.html?_flowId=datosCensalesRazaflow&tipoOperacion=CONSULTA&formatoPagina=0&id=100466. Accessed January 11, 2024.

Massafra, V., Pellicciari, R., Gioiello, A., & van Mil, S. W. (2018). Progress and challenges of selective Farnesoid X Receptor modulation. *Pharmacology & Therapeutics, 191*, 162–177. https://doi.org/10.1016/j.pharmthera.2018.06.009

McManaman, J. L., Palmer, C. A., Wright, R. M., & Neville, M. (2002). Functional regulation of xanthine oxidoreductase expression and localization in the mouse mammary gland: Evidence of a role in lipid secretion. *The Journal of Physiology, 545*, 567–579. https://doi.org/10.1113/jphysiol.2002.027185

McManaman, J. L., Russell, T. D., Schaack, J., Orlicky, D. J., & Robenek, H. (2007). Molecular determinants of milk lipid secretion. *Journal of Mammary Gland Biology and Neoplasia, 12*, 259–268. https://doi.org/10.1007/s10911-007-9053-5

Mendelson, C. R., Zinder, O., Blanchette-Mackie, J. E., Chernick, S. S., & Scow, R. O. (1977). Lipoprotein lipase and lipid metabolism in mammary gland. *Journal of Dairy Science, 60*, 666–676. https://doi.org/10.3168/jds.S0022-0302(77)83916-7

Miclo, L., Girardet, J., Egito, A. S., Mollé, D., Martin, P., & Gaillard, J. (2007). The primary structure of a low-Mr multiphosphorylated variant of β-casein in equine milk. *Proteomics, 7*, 1327–1335. https://doi.org/10.1002/pmic.200600683

Miranda, G., Mahé, M., Leroux, C., & Martin, P. (2004). Proteomic tools to characterize the protein fraction of Equidae milk. *Proteomics, 4*, 2496–2509. https://doi.org/10.1002/pmic.200300765

Mondy, B. L., & Keenan, T. W. (1993). Butyrophilin and xanthine oxidase occur in constant molar proportions in milk lipid globule membrane but vary in amount with breed and stage of lactation. *Protoplasma, 177*, 32–36. https://doi.org/10.1007/BF01403396

Murthy, S., Born, E., Mathur, S. N., & Field, F. J. (2002). LXR/RXR activation enhances basolateral efflux of cholesterol in CaCo-2 cells. *Journal of Lipid Research, 43*, 1054–1064. https://doi.org/10.1194/jlr.M100358-JLR200

Narmuratova, Z., Hentati, F., Girardet, J., Narmuratova, M., & Cakir-Kiefer, C. (2022). Equine lactoferrin: Antioxidant properties related to divalent metal chelation. *LWT, 161*, Article 113426. https://doi.org/10.1016/j.lwt.2022.113426

Nitta, K., Tsuge, H., Sugai, S., & Shimazaki, K. (1987). The calcium-binding property of equine lysozyme. *FEBS Letters, 223*, 405–408. https://doi.org/10.1016/0014-5793(87)80328-9

Ochirkhuyag, B., Chobert, J., Dalgalarrondo, M., & Haertlé, T. (2000). Characterization of mare caseins. Identification of αs1- and αs2-caseins. *Le Lait, 80*, 223–235. https://doi.org/10.1051/lait:2000121

Ollier, S., Zhao, X., & Lacasse, P. (2013). Effect of prolactin-release inhibition on milk production and mammary gland involution at drying-off in cows. *Journal of Dairy Science, 96*, 335–343. https://doi.org/10.3168/jds.2012-5955

Park, Y. W., Zhang, H., Zhang, B., & Zhang, L. (2006). Mare milk. In Y. W. Park, & G. F. W. Haenlein (Eds.), *Handbook of milk of non-bovine mammals* (pp. 275–296). Blackwell Publishers.

Pérez, M. D., & Calvo, M. (1995). Interaction of β-lactoglobulin with retinol and fatty acids and its role as a possible biological function for this protein: A review. *Journal of Dairy Science, 78*, 978–988. https://doi.org/10.3168/jds.S0022-0302(95)76713-3

Perkins, G. A., & Wagner, B. (2015). The development of equine immunity: Current knowledge on immunology in the young horse. *Equine Veterinary Journal, 47*, 267–274. https://doi.org/10.1111/evj.12387

Picariello, G., Mamone, G., Addeo, F., & Ferranti, P. (2012). Novel mass spectrometry-based applications of the 'omic' sciences in food technology and biotechnology. *Food Technology and Biotechnology, 50*, 286–305.

Pieszka, M., Łuszczyński, J., Zamachowska, M., Augustyn, R., Długosz, B., & Hędrzak, M. (2016). Is mare milk an appropriate food for people? – a review. *Annals of Animal Science, 16*, 33–51. https://doi.org/10.1515/aoas-2015-0041

Qin, N., Bayat, A., Trevisi, E., Minuti, A., Kairenius, P., Viitala, S., Mutikainen, M., Leskinen, H., Elo, K., Kokkonen, T., & Vilkki, J. (2018). Dietary supplement of conjugated linoleic acids or polyunsaturated fatty acids suppressed the mobilization of body fat reserves in dairy cows at early lactation through different pathways. *Journal of Dairy Science, 101*, 7954–7970. https://doi.org/10.3168/jds.2017-14298

Riley, L. G., Williamson, P., Wynn, P. C., & Sheehy, P. A. (2008). Lactoferrin decreases primary bovine mammary epithelial cell viability and casein expression. *Journal of Dairy Research, 75*, 135–141. https://doi.org/10.1017/S0022029907002920

Sahaka, M., Amara, S., Wattanakul, J., Gedi, M. A., Aldai, N., Parsiegla, G., Lecomte, J., Christeller, J. T., Gray, D., Gontero, B., Villeneuve, P., & Carriere, F. (2020). The digestion of galactolipids and its ubiquitous function in nature for the uptade of the essential α-linolenic acid. *Food and Function, 11*, 6710–6744. https://doi.org/10.1039/D0FO01040E

Shariatikia, M., Behbahani, M., & Mohabatkar, H. (2017). Anticancer activity of cow, sheep, goat, mare, donkey and camel milks and their caseins and whey proteins and in silico comparison of the caseins. *Molecular Biology Research Communications, 6*, 57–64. https://doi.org/10.22099/mbrc.2017.4042

Stein, T., Morris, J. S., Davies, C. R., Weber-Hall, S. J., Duffy, M., Heath, V. J., Bell, A. K., Ferrier, R. K., Sandilands, G. P., & Gusterson, B. A. (2004). Involution of the mouse mammary gland is associated with an immune cascade and an acute-phase response, involving LBP, CD14 and STAT3. *Breast Cancer Research, 6*. https://doi.org/10.1186/bcr753. Article R75.

Suburu, J., Shi, L., Wu, J., Wang, S., Samuel, M., Thomas, M. J., Kock, N. D., Yang, G., Kridel, S., & Chen, Y. Q. (2014). Fatty acid synthase is required for mammary gland development and milk production during lactation. *American Journal of Physiology – Endocrinology and Metabolism, 306*, 1132–1143. https://doi.org/10.1152/ajpendo.00514.2013

Sun, Y., Wang, C., Sun, X., & Guo, M. (2020). Proteomic analysis of whey proteins in the colostrum and mature milk of Xinong Saanen goats. *Journal of Dairy Science, 103*, 1164–1174. https://doi.org/10.3168/jds.2019-17159

Tenno, M., Toba, S., Kézdy, F. J., Elhammer, Å. P., & Kurosaka, A. (2002). Identification of two cysteine residues involved in the binding of UDP-GalNAc to UDP-GalNAc: Polypeptide N-acetylgalactosaminyltransferase 1 (GalNAc-T1). *European Journal of Biochemistry, 269*, 4308–4316. https://doi.org/10.1046/j.1432-1033.2002.03123.x

Tiwari, P., Kaila, P., & Guptasarma, P. (2019). Understanding anomalous mobility of proteins on SDS-PAGE with special reference to the highly acidic extracellular domains of human E-and N-cadherins. *Electrophoresis, 40*, 1273–1281. https://doi.org/10.1002/elps.201800219

Uniacke-Lowe, T., Chevalier, F., Hem, S., Fox, P. F., & Mulvihill, D. M. (2013). Proteomic comparison of equine and bovine milks on renneting. *Journal of Agricultural and Food Chemistry, 61*, 2839–2850. https://doi.org/10.1021/jf3045846

Uniacke-Lowe, T., Huppertz, T., & Fox, P. F. (2010). Equine milk proteins: Chemistry, structure and nutritional significance. *International Dairy Journal, 20*, 609–629. https://doi.org/10.1016/j.idairyj.2010.02.007

Valdivielso, I., Bustamante, M. A., Aldezabal, A., Amores, G., Virto, M., Ruiz de Gordoa, J. C., de Renobales, M., & Barron, L. J. R. (2016). Case study of a commercial sheep flock under extensive mountain grazing: Pasture derived lipid compounds in milk and cheese. *Food Chemistry, 197*, 622–633. https://doi.org/10.1016/j.foodchem.2015.10.133

Wang, Z., Wang, X., Zou, H., Dai, Z., Feng, S., Zhang, M., Xiao, G., Liu, Z., & Cheng, Q. (2020). The basic characteristics of the pentraxin family and their functions in tumor progression. *Frontiers in Immunology, 11*. https://doi.org/10.3389/fimmu.2020.01757. Article 1757.

Wiśniewski, J. R., Zougman, A., Nagaraj, N., & Mann, M. (2009). Universal sample preparation method for proteome analysis. *Nature Methods, 6*, 359–362. https://doi.org/10.1038/nmeth.1322

Wodas, L., Mackowski, M., Borowska, A., Puppel, K., Kuczynska, B., & Cieslak, J. (2020). Genes encoding equine β-lactoglobulin (LGB1 and LGB2): Polymorphism, expression, and impact on milk composition. *PLoS One, 15*. https://doi.org/10.1371/journal.pone.0232066. Article e0232066.

Yang, Y., Zheng, N., Zhao, X., Yang, J., Zhang, Y., Han, R., Zhao, S., Li, S., Wen, F., & Wang, J. (2020). Changes in whey proteome with lactation stage and parity in dairy cows using a label-free proteomics approach. *Food Research International, 128*, Article 108760. https://doi.org/10.1016/j.foodres.2019.108760

Zhang, L., Boeren, S., Hageman, J. A., van Hooijdonk, T., Vervoort, J., & Hettinga, K. (2015). Perspective on calf and mammary gland development through changes in the bovine milk proteome over a complete lactation. *Journal of Dairy Science, 98*, 5362–5373. https://doi.org/10.3168/jds.2015-9342

Zhao, Q., Li, K., Jiang, K., Yuan, Z., Xiao, M., Wei, G., Zheng, W., Wang, X., & Huang, A. (2023a). Proteomic approach-based comparison of metabolomics pathways and functional activities of whey proteins derived from Guishan and Saanen goat milk. *Journal of Dairy Science, 106*, 2247–2260. https://doi.org/10.3168/jds.2022-22404

Zhao, S., Pan, F., Cai, S., Yi, J., Zhou, L., & Liu, Z. (2023b). Secrets behind protein sequences: Unveiling the potential reasons for varying allergenicity caused by caseins from cows, goats, camels, and mares based on bioinformatics analyses. *International Journal of Molecular Sciences, 24*. https://doi.org/10.3390/ijms24032481. Article 2481.

Web references

Ensembl Genome Browser. (2023). Retrieved from https://www.ensembl.org/index.html. Accessed October 2023.

Panther classification system. (2023). Retrieved from www.pantherdb.org. Accessed October 2023.

Uniprot. (2023). Retrieved from www.uniprot.org/. Accessed October 2023.

APPENDIX III.IV – Publication 4

Blanco-Doval, A., Sousa, R., Barron, L. J. R., Portmann, R., Egger, L., Aldai, N. (2024). **Assessment of in vitro digestibility and post-digestion peptide release of mare milk in relation to different management systems and lactation stages**. *Journal of Dairy Science,* in press.

Accepted: **8 May 2024**

Published in: ***Journal of Dairy Science****,* 2024

Publisher: Elsevier

ISSN: 0022-0302

Impact Factor year: 2022

Impact Factor: **3.5**

Category: **Agriculture, Dairy & Animal Science**

Position in the category: 9/62 (**Q1**)

Preview (JDS.2024-24807.R1)

From: robert.ward@usu.edu

To: noelia.aldai@ehu.eus, ana.blancod@ehu.eus, raquel.sousa.bq@gmail.com, luisjavier.rbarron@ehu.eus, reto.portmann@agroscope.admin.ch, charlotte.egger@agroscope.admin.ch

CC: ana.blancod@ehu.eus, raquel.sousa.bq@gmail.com, luisjavier.rbarron@ehu.eus, reto.portmann@agroscope.admin.ch, charlotte.egger@agroscope.admin.ch, noelia.aldai@ehu.eus

Subject: Journal of Dairy Science - Decision on Manuscript ID JDS.2024-24807.R1

Body: 08-May-2024

Dear Dr. Noelia Aldai:

Manuscript ID: JDS.2024-24807.R1
Title: Assessment of in vitro digestibility and post-digestion peptide release of mare milk in relation to different management systems and lactation stages

I am pleased to inform you that your manuscript has been accepted for publication in the Journal of Dairy Science. We expect your uncorrected proof to be published on the *Journal of Dairy Science* website within 30 days. You should anticipate receiving a PDF proof of your final manuscript within approximately 8-10 weeks.

Please ensure that all authors of this manuscript are listed below and that each has an ORCiD (www.orcid.org) associated with their account. **This information must be in our peer-review system within 24 hours of acceptance so that it can be published with the manuscript. Late ORCiDs cannot be added to manuscripts.**

Blanco-Doval, Ana - https://orcid.org/0000-0001-8754-8578, Sousa, Raquel - https://orcid.org/0000-0002-7738-9961, Barron, Luis Javier - https://orcid.org/0000-0002-2563-1082, Portmann, Reto - https://orcid.org/0000-0001-8979-7439, Egger, Charlotte - https://orcid.org/0000-0001-6501-5703, Aldai, Noelia - https://orcid.org/0000-0002-5308-9665

Note that this journal is now open access. We are collecting open access licenses from corresponding authors through ScholarOne. A link to this license was sent to the corresponding author in a separate email. Your manuscript cannot be published until the corresponding author has submitted the completed license. Only the corresponding author needs to complete this license.

If you need assistance with this process, please contact our system administrator for help (shaunam@assochq.org).

All invoicing for the *Journal of Dairy Science* will be handled by Elsevier. If you have any questions about your invoice once you receive it, please contact the American Dairy Science Association at adsa@adsa.org.

To receive the ADSA member rate for publishing (APC), at least one author must be a professional member of ADSA (https://www.adsa.org/Membership/Benefits-of-Membership).
For details on rates for APC, see our FAQ page (https://www.adsa.org/News/ArtMID/390/ArticleID/48/Journal-of-Dairy-Science174-to-Become-Open-Access-January-2022).

Now that the manuscript has been accepted, the corresponding author has earned loyalty points toward a discounted or waived future publication in the *Journal of Dairy Science* or *JDS Communications* as long as the corresponding author is a current professional member of the American Dairy Science Association and their ORCID number is associated with both their author account in ScholarOne and their account within the ADSA membership site. Please note that only the corresponding author will receive points for a paper. Find out more about the ADSA Loyalty Rewards Program here: https://www.adsa.org/Publications/Journal-Rewards.

On behalf of the editors of the Journal of Dairy Science, we look forward to your continued contributions to the Journal.

Sincerely,

Dr. Robert Ward
Section Editor, Journal of Dairy Science

Assessment of *in vitro* digestibility and post-digestion peptide release of mare milk in relation to different management systems and lactation stages

Ana Blanco-Doval[1], Raquel Sousa[2], Luis Javier R. Barron[1], Reto Portmann[2], Lotti Egger[2], Noelia Aldai[1*]

[1] Lactiker Research Group, Department of Pharmacy and Food Sciences, University of the Basque Country (UPV/EHU), Paseo de la Universidad 7, 01006 Vitoria-Gasteiz, Spain

[2] Agroscope, Schwarzenburgstrasse 161, 3003 Bern, Switzerland

Interpretive Summary

The present study describes the impact of human gastrointestinal digestion on mare milk proteins through an *in vitro* simulated digestion trial. Digestibility of total proteins and individual amino acids was measured, and the peptide profile released after gastric and intestinal digestion was characterized. These parameters were also studied as affected by management and lactation stage of the mares. Results support that mare milk contains one of the most digestible dietary proteins, and that protein digestion significantly differs depending on key production factors. This emphasises the relevance of the production stages on the protein digestion traits of mare milk.

*Corresponding author: Noelia Aldai (noelia.aldai@ehu.eus).

Abstract

Mare milk has a unique protein composition that makes it a preferred option for adult and infant nutrition. A number of functional properties have been attributed to this milk but with little evidence yet. In fact, knowledge on mare milk composition is still limited. In particular, studies addressing the performance of mare milk proteins during human gastrointestinal digestion are scarce, which limits the understanding of mare milk nutritional quality and functionality. For this reason, the present study describes the digestibility of mare milk proteins and the release of peptides as affected by management and lactation stage, factors known to affect milk composition. Mare milk samples from three different farms, and collected during six months of lactation (n = 54), were subjected to a static *in vitro* gastrointestinal model to measure peptide release and protein digestibility. In the present study, a detailed description of protein and individual amino acid behavior during the digestion process was given. For the first time, digestion of the two equine β-lactoglobulin isoforms (I and II) was described individually. In addition, it was found that lactation stage and management system can significantly affect protein digestibility and peptide release during gastrointestinal digestion of mare milk. Presumably, differences in the composition of mare milk influence the protein structure and enzyme accessibility, which might have an impact on digestion behavior. Despite no specific bioactive peptides were identified, a number of precursors of previously described bioactive peptides were found. These findings could support the idea of mare milk as a food with added value.

Keywords: Bioactive peptide, equid, grazing, INFOGEST, static *in vitro* digestion.

Abbreviations: AAA = aromatic amino acids (phenylalanine and tyrosine), DIAAR = digestible indispensable amino acid ratio, DIAAS = digestible indispensable amino acid score, OPA = o-phthalaldehyde, SAA = sulphur-containing amino acids (cysteine and methionine).

1. Introduction

Mare milk is one of the animal milks most similar to human milk (Uniacke-Lowe et al., 2010), and appears to have a low allergenic response in patients with cow milk protein allergy (Businco et al., 2000; Zhao et al., 2023) – although this still needs to be confirmed by clinical studies. These properties make mare milk a suitable option for infant nutrition. Moreover, it has an interesting protein profile. Whereas bovine milk is mainly composed of caseins (~80%), mare milk protein distribution (~55/45 caseins/whey proteins) is more similar to that of human milk (~30/70). The main protein in mare milk is β-casein, followed by similar levels of αs1-casein, β-lactoglobulin and α-lactalbumin. Conversely, low contents of αs2- and κ-casein have been reported. Despite being a minor protein, lysozyme contents are particularly high in mare milk compared to milk from other species, and lactoferrin contents are also considerable (Miranda et al., 2004; Uniacke-Lowe et al., 2010). Some particularities of mare milk

proteins are the presence of two β-lactoglobulin isoforms (I and II; Godovac-Zimmermann et al., 1985; Halliday et al., 1991; Miranda et al., 2004), three genetic variants of α-lactalbumin (A, B and C; Godovac-Zimmermann et al., 1987), and a highly glycosylated κ-casein (Jaeser et al., 2023). In addition, equine lysozyme has the ability to bind calcium similar to α-lactalbumin, a property thought to be an evolutionary linkage between lysozymes with non-calcium binding activity and α-lactalbumin (Nitta et al., 1987).

In addition to knowing the protein composition and characteristics of a product, understanding the behavior of these proteins during gastrointestinal digestion is key for a comprehensive understanding of a protein source quality. To date, very few studies have been conducted on the human digestion of mare milk (Inglingstad et al., 2010; Xiao et al., 2023), and these focused on comparing mare milk with human and other animal species' milk rather than on understanding the effect of different factors on mare milk protein digestion. Moreover, Xiao et al. (2023) used simulated infant digestion conditions, which are different from adult digestion conditions, while Inglingstad et al., 2010 used a two-step (stomach and duodenum, 30 min each) adult digestion simulation that might be limited at resembling real digestive conditions. Despite their limitations, these studies claim that mare milk proteins are highly digestible, with a higher protein degradation degree in mare milk than in ruminant milk, but lower than in human milk. In the mentioned studies, most of the proteins were overall degraded already in the gastric phase, followed by a rapid intestinal hydrolysis of the proteins remaining in the gastric digesta. However, protein digestion is a complex process, and more research is needed to fully understand the particularities of mare milk protein changes during gastrointestinal digestion. This requires the application of the latest methodologies that are constantly being improved and updated. In this sense, the INFOGEST 2.0 protocol setup (Brodkorb et al., 2019) proved useful for characterizing the digestibility and release of peptides in a wide range of dietary proteins (Portmann et al., 2023; Sousa et al., 2023), and could also be useful for mare milk studies.

Milk is a fluid with a variable composition that can be modified in response to several factors. For instance, the feeding regime of the animal as part of the management system, and lactation stage can significantly affect mare milk nutritional composition. Overall, the content of most chemical compounds (fat, proteins, mineral elements…) tends to decrease from initial to final stages of lactation, except for lactose that increases. In addition, forage feeding can increase the content of n-3 polyunsaturated fatty acids in milk, but decrease the abundance of some mineral elements such as calcium, phosphorous, sulphur and sodium (Salimei and Park, 2017; Barłowska et al., 2023; Blanco-Doval et al., 2023). For an adequate and comprehensive characterization of mare milk, consideration of these factors in research is of great importance. Unfortunately, to the best of our knowledge, previous studies about mare milk digestion and peptide release did not account for external factors that might affect its performance in the human digestive system.

The aim of the present study was to describe the digestibility of mare milk proteins during simulated *in vitro* human gastrointestinal digestion, using the extended INFOGEST protocol; to monitor the peptide release during gastrointestinal digestion and identify potential bioactive peptides; and to assess the effects of management and lactation stage on the studied parameters.

2. Materials and methods

2.1. Animals and sample collection

Fifty-four milk samples were collected from Basque Mountain Horse breed mares belonging to three commercial farms located in Araba region (northern Spain). Nine mares (3 *per* farm) were milked at weeks 6, 10, 14, 18, 22 and 26 of lactation, between May and October, 2021. Farms were primarily dedicated to horse meat production but with differences in management among them. Briefly, in farm I mares were pasture fed only during May, and after, mares were switched to a mixture of alfalfa, silage, hay, fruits and potatoes until weaning in October. In farms II and III, mares were kept on grazing during the complete lactation period, and only after July mares were supplemented with hay (farm II) or silage (farm III). More detailed information about the management of mares is available at Blanco-Doval et al. (2023). Because samples were obtained from commercial farms by standard milking procedures, institutional animal use approval was not required. Immediately after milking, samples were transported to the laboratory under refrigeration, subsampled and kept at -80 ºC until analysis.

2.2. Chemicals and reagents

Except otherwise specified, all chemicals, reagents and enzymes were purchased from Merck (Zug, Switzerland). Enzyme and bile salt characteristics and preparation of gastrointestinal fluids (simulated salivary, gastric and intestinal fluids) are described in Supplementary material 1.

2.3. Static *in vitro* simulation of mare milk gastrointestinal digestion

Milk samples were thawed at 4 ºC and individually digested following the INFOGEST protocol for static *in vitro* simulation of gastrointestinal digestion (Minekus et al., 2014) with the latest improvements (Brodkorb et al., 2019). In the oral phase, 800 µL amylase free simulated salivary fluid (pH 7, 37 ºC) were dried in a centrifugal concentrator (CentriVap, Labconco, Kansas City, MO, USA), in order not to exceed the 2 mL volume of the oral phase. Then, quantities of milk normalized to 40 mg of protein (corresponding to an approximate volume of 2 mL) were added. For the gastric phase, 1.6 mL of simulated gastric juice (pH 3, 37 ºC) containing pepsin (2000 U/mL of digesta) were added, and the solution was incubated for 2 h at 37 ºC in constant rotation. Finally, for the intestinal phase, 1.7 mL of simulated intestinal fluid (pH 7, 37 ºC) containing pancreatin (100 U trypsin activity/mL of digesta) and bile (10 mM final concentration) were added to the gastric digesta, and the solution was incubated for 2 h at 37 ºC in constant rotation. Samples used for peptide release evaluation were stopped after the gastric phase by increasing the

pH to 7 with sodium hydroxide (2 M), and after the intestinal phase by adding a protease inhibitor (4-(2-aminoethyl) benzenesulfonylfluoride 500mM, Roche, Basel, Switzerland) to the digesta. Then, digested samples were immediately immersed in liquid nitrogen and kept at -20 ºC until analysis.

2.4. Digestibility of mare milk proteins

For analysis of protein digestibility, the procedure by Sousa et al. (2023) was followed. Mare milk samples corresponding to two consecutive lactation weeks of each animal were pooled, resulting in 27 pooled samples and three lactation stages (early: weeks 6 and 10; mid: weeks 14 and 18; late: weeks 22 and 26). The pooled samples were individually digested in triplicate (section 2.3 until intestinal digestion). Simultaneously, a protein-free cookie was digested as a blank (Moughan et al., 2005) in order to determine the enzyme background. Digestions were performed in sets of 5-6 pooled samples and one cookie. Replicates were digested in different sets at different days.

2.4.1. Separation of digestible and indigestible fractions

Digested milk samples were fractionated into digestible (potentially absorbable) and indigestible (potentially non-absorbable) fractions (Sousa et al., 2023). Briefly, 32 mL of ice-cold methanol were added to samples after intestinal digestion in order to precipitate the indigestible fraction. The solution was incubated for 1 h and subsequently centrifuged for 15 min at 2,000 *g* and 4 ºC (Sorvall Legend XTR, Thermo Scientific, Reinach, Switzerland). Collected supernatants (digestible fraction) were kept at -20 ºC until analysis. Pellets (indigestible fraction) were washed twice with ice-cold pure methanol, dried in the centrifugal concentrator (CentriVap), and kept at -20 ºC until analysis. Weights of supernatant and pellet tubes (± 0.0001 g accuracy) were monitored for digestibility calculation.

2.4.2. Hydrolysis of digested samples

Previously dried pellets were transferred to 10 mL vials, and 220 µL of each supernatant were transferred to 2 mL glass vials and evaporated using the centrifugal concentrator (CentriVap). Both pellets and supernatants were hydrolyzed in hydrochloric acid and 3,3'-dithiodipropionic acid (acid hydrolysis) at 110 ºC for 15 h, with norvaline as internal standard. Two cysteine standards (20 and 200 µM final concentrations) were hydrolyzed in parallel following the same procedure (Sousa et al., 2023).

2.4.3. Determination of total amino groups using the o-phthalaldehyde method

After hydrolysis, total amino groups ($R\text{-}NH_2$) present in the digestible and indigestible fractions of the samples were analyzed using the o-phthalaldehyde (**OPA**) method (Sousa et al., 2023). Samples were diluted with perchloric acid (0.5 M; 1/5 supernatants, 1/10 pellets, v/v), derivatized with OPA and 2-mercapto-ethansulfonic acid, and analyzed by ultraviolet/visible spectrophotometry at 340 nm (Spectramax iD3, Molecular Devices, San José, CA, USA). A calibration curve was built using nine concentration levels of a glutamic acid standard solution ranging from 0.25 to 8 mM and

perchloric acid (0.5 M) as a blank. All samples and calibration levels were measured in duplicate.

2.4.4. Analysis of individual amino acids by ultra-high performance liquid chromatography

Individual amino acid contents in digested and hydrolyzed samples were measured according to the method 2018.06 of the Association of Official Analytical Chemists for infant formula (Jaudzems et al., 2019) adapted by Sousa et al. (2023). Prior to analysis, samples were derivatized with AccQ-Tag reagent (Waters, Baden, Switzerland). Individual amino acid contents were analyzed according to Waters (2007) instructions, using an ultra-high performance liquid chromatography (UHPLC) equipment (Vanquish Flex, Thermo Scientific) coupled to an ultraviolet detector (Vanquish, Thermo Scientific) and an Acquity UPLC BEH C18 column (150 mm length, 2.1 mm internal diameter, 1.7 µm particle size; Waters). Analytical conditions were set to 2 µL injection volume, 50 ºC column temperature, 260 nm detection wavelength, and 0.4 mL/min flow rate. AccQ-Tag Eluent A (diluted to 15% in ultrapure water, v/v; Waters) and formic acid 2% (v/v) in acetonitrile were used as mobile phase within a 32 min gradient.

2.4.5. Analysis of undigested mare milk

Individual amino acid content of raw (undigested) pooled mare milk samples (n = 27) was also determined after acid hydrolysis (110 ºC, 24 h) (sections 2.4.2 and 2.4.4). For tryptophan determination, alkaline hydrolysis with sodium chloride 4.2 M for 20 h at 110 ºC was used. Tryptophan was determined using an UHPLC equipment (Thermo Ultimate 3000, Thermo Scientific) coupled to a fluorescence detector (Vanquish, Thermo Scientific) and an Acquity UPLC BEH C18 column (150 mm length, 2.1 mm internal diameter, 1.7 µm particle size; Waters) operated as described in the International Organization for Standardization/Draft International Standard 13904 (2014). Analytical conditions were 2 µL injection volume, 50 ºC column temperature, 285/340 nm excitation/emission wavelengths, and 0.4 mL/min flow rate. The mobile phase consisted of an isocratic flow of 99.9% (v/v) AccQ-Tag Eluent A (diluted to 15% in ultrapure water, v/v; Waters) and 0.1% (v/v) formic acid (2%, v/v) in acetonitrile. Total nitrogen content of raw mare milk pooled samples was analyzed by Kjeldahl method (International Organization for Standardization 8968-3, 2007) to calculate the total protein content using a nitrogen-to-protein conversion factor of 6.25.

2.4.6. Calculation of in vitro digestibility and digestible indispensable amino acid ratio

The calculations for the protein digestibility of mare milk (Supplementary material 1) were performed (Sousa et al., 2023). The digestible indispensable amino acid ratio (**DIAAR**) represents the relation between digested indispensable amino acids *per* gram of food protein and the reference requirement values of individual amino acid for infants, children and adults (Food and Agriculture Organization, 2013). The digestible indispensable amino acid score (**DIAAS**) refers to the lowest DIAAR of a protein product, and represents the limiting amino acid in a food.

2.5. Peptide release after gastric and intestinal digestion

2.5.1. Sample preparation

For the study of peptide release after digestion, all individual mare milk samples were digested (section 2.3), with two digestions *per* sample: one stopped after the gastric phase (gastric digestion) and one subjected to the entire digestion process (intestinal digestion). All digested samples were filtered through 30 kDa molecular weight cut-off micro-spin filters (UFC5030, Millipore, Merck) aided by centrifugation at 18,000 *g* for 10 min at 4 ºC using a 5427R centrifuge (Eppendorf, Hamburg, Germany).

2.5.2. Identification of major proteins in mare milk

In order to relate released peptides with their parent proteins, major proteins in mare milk were identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Kopf-Bolanz et al., 2012). Six undigested milk samples from one mare and six lactation weeks were selected, diluted 1/25 (v/v) with ultrapure water, and separated with SDS-PAGE in a 15% polyacrylamide gel with a BenckMark protein ladder solution (Invitrogen, Thermo Fisher Scientific). Gels were stained with colloidal Coomassie Blue, and the bands were excised and digested with trypsin overnight at 37 ºC.

2.5.3. Identification of released peptides after gastric and intestinal digestion

Digested samples after filtration (*in vitro* gastrointestinal digestion; section 2.5.1) or trypsin digestion (section 2.5.2) were injected into a HPLC system (Rheos 2200 Micro HPLC, Flux Instruments, Basel, Switzerland) equipped with an electrospray ionization source and coupled to a linear ion trap mass spectrometer (LTQ XT, Thermo Fisher Scientific). A C18 column (X Terra MS, 100 mm length, 2.1 mm internal diameter, 3.5 µm particle size; Waters) was used for peptide separation. Analytical conditions were previously described (Egger et al., 2019). Peptides with at least five amino acids and m/z ratio between 300 and 2,000 were considered, and repetitive fragmentation was applied to the 5 most intense mass spectrometry (MS) signals. MS/MS spectra were matched against an in-house database containing only milk proteins obtained from the UniProt database (https://www.uniprot.org/), including the eight major mare milk proteins identified by gel electrophoresis: αs1-casein (CASA1_EQUAS), αs2-casein (CASA2_EQUAS), β-casein (CASB_HORSE), κ-casein (CASK_HORSE), β-lactoglobulin I (LACB1_HORSE), β-lactoglobulin II (LACB2_HORSE), α-lactalbumin A (LALB1_HORSE) and lysozyme C (LYSC1_HORSE). Peptides were identified using Mascot search engine (Matrix Science, London, UK) and considering some technically induced optional modifications (deamidation of asparagine and glutamine, oxidation of methionine), as well as phosphorylation of serine and threonine as post-translational modifications. Among the peptides identified in mare milk digested samples, bioactive peptides were searched using the Milk Bioactive Peptide Database (MBPDB), which collects milk-derived bioactive peptides identified in literature (Nielsen et al., 2017).

2.6. Statistical analysis and data visualization

Heatmaps were created with R programming (RStudio version 4.3.0, Posit PBC, Boston, MA, USA) using the individual amino acid counts within all identified peptides from the corresponding protein. Peptides liberated from the same protein were grouped by farm or lactation stage, and the average number of individual amino acids within protein sequences that belonged to the same group was used.

In order to explore the effect of management (farm) and lactation stage on mare milk protein digestibility and gastrointestinal fragmentation, the IBM-SPSS statistics software (version 28.0, IBM, New York, NY, USA) was used. In this case, the relative abundance of each individual peptide given by the MS/MS spectra was considered. All data was log transformed, and tested for normality and homoscedasticity. The general linear model of analysis of variance was applied to all data. Farm ($I = 3$) and lactation stage ($I = 3$; referring to weeks 6 and 10 as early lactation, weeks 14 and 18 as mid lactation, and weeks 22 and 26 as late lactation) were included as fixed factors, and individual animal ($I = 9$) as a random factor nested within farm. The interaction effect between lactation stage and farm was also included in the model and Tukey's test was applied for pairwise comparisons. For released peptides data, only those peptides present in at least two animals *per* farm and *per* lactation week were subjected to statistical analysis. In addition, a multivariate analysis using the IBM-SPSS statistics software was applied to peptide release results. Log transformed data were submitted to stepwise discriminant analysis to discriminate milk samples by farm or by lactation stage. Only peptides that appeared in at least two animals *per* farm and *per* lactation week were included in the multivariate analysis. Significance level was declared at *P*-value ≤ 0.05.

3. Results

3.1. Digestibility of mare milk proteins

Mare milk proteins exhibited an average digestibility (± standard deviation) of 93.7 ± 3.3% in calculations based on individual amino acids, and 96.0 ± 4.7% in calculations based on total amino groups (R-NH_2; OPA assay). No significant differences were observed in milk protein digestibility among farms. However, a significant effect of lactation stage was found only when calculations were made with OPA method. In this case, milk proteins showed a significantly ($P = 0.005$) lower digestibility at late lactation (93.3 ± 3.9%) compared to early (97.0 ± 1.9%) and mid lactation (98.1 ± 0.6%). The interaction effect between farm and lactation stage was also non-significant ($P > 0.05$).

Digestibility of individual amino acids was on average (± standard deviation) higher than 90% in all cases except for alanine (89.2 ± 5.7%), isoleucine (88.3 ± 3.8%), serine (83.8 ± 5.8%) and threonine (88.9 ± 5.2%) (Supplementary material 1). Overall, farm and lactation stage had little effect on digestibility of individual amino acids. However, significant ($P \leq 0.05$) influence of farm and lactation stage was observed for some DIAAR values (**Table 1**). In the case of farm effect, DIAAR values of sulphur-containing amino acids and tryptophan where higher in low grazing (farm

I) *versus* high grazing farms (farms II and III). Conversely, milk from high grazing activity and hay supplementation (farm II) exhibited highest DIAAR values of aromatic amino acids, and lowest DIAAR values of lysine only until mid lactation (at late lactation, the lysine ratios were equalized). These differences were mainly explained by differences in the amino acid content of undigested raw milk (**Table 1**). Overall, raw mare milk from farm I contained higher amounts of methionine (the major sulphur-containing amino acid), tryptophan, and lysine (only until mid lactation) whereas milk from farm II contained higher amounts of phenylalanine (the major aromatic amino acid). Regarding changes during lactation, DIAAR values of histidine, leucine, lysine and valine were significantly ($P \leq 0.05$) higher at late lactation compared to early and mid lactation. This was again explained by the higher amino acid contents in undigested raw mare milk at late lactation (**Table 1**). As an exception, lysine content increased at late lactation only in milk from high grazing farms whereas it decreased from early to late lactation in milk from the low grazing farm. This resulted in a significant ($P = 0.029$) interaction between farm and lactation stage in lysine content in raw mare milk. A similar interaction was found in the leucine content of raw mare milk, which changed differently during lactation in milk from high grazing farms than in low grazing milk samples. Despite significant changes observed in DIAAR values depending on farm and lactation stage, the essential to non-essential amino acid ratio remained stable in all mare milk samples (data not shown).

Mean DIAAR values calculated for all mare milk samples based on infant, child and adult nutrition are shown in **Figure 1**. Considering DIAAR values, the limiting amino acids were threonine for infants (DIAAS 74.5%) and histidine for children and adults (DIAAS 101.5 and 126.9%, respectively).

3.2. Peptide release after gastrointestinal digestion of mare milk

Since major proteins in mare milk (identified through gel electrophoresis) were αs1-, αs2-, β- and κ-caseins, β-lactoglobulin I and II, α-lactalbumin A and lysozyme C, peptides released from these eight proteins were also monitored. β-casein was the predominant source of peptides with 386 non-redundant peptides found after gastric and intestinal digestions, while less than 80 non-redundant peptides were released, respectively, from κ-casein, α-lactalbumin A and lysozyme C (**Table 2**). Considering peptide size, peptides released from caseins, β-lactoglobulin I and α-lactalbumin A had significantly ($P \leq 0.05$) lower mass after intestinal than after gastric digestion. In contrast, peptides released from β-lactoglobulin II and lysozyme C did not show higher fragmentation after intestinal digestion (**Figure 2**).

Heatmaps in **Figure 3** describe the pattern of peptides released from each major mare milk protein during gastric and intestinal digestion. Interpretation of peptide patterns was done as suggested by Portmann et al. (2023). The results confirmed that β-casein was the main source of peptides, followed by αs1-casein and β-lactoglobulin I. In accordance to the significant

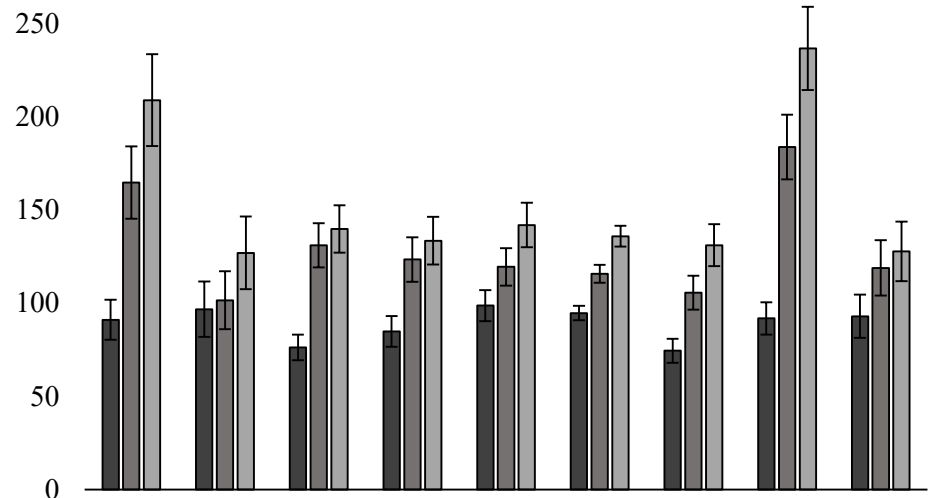
reduction of peptide size from gastric to intestinal digestion (**Figure 2**), most peptides formed during gastric digestion of αs1-, αs2-, β- and κ-casein, β-lactoglobulin I and α-lactalbumin A were further hydrolyzed during intestinal digestion. In contrast, β-lactoglobulin II and lysozyme C exhibited low proteolysis throughout the entire gastrointestinal digestion process. Peptides from the 175-186 region of αs1-casein, the 133-145 region of αs2-casein, the 18-26 region of κ-casein, the 156-167 region of β-lactoglobulin II, and the 57-63 region of lysozyme C were resistant to gastric digestion and were mainly formed during intestinal digestion (**Figure 3**).

Table 1. *Statistical significance[1] (P-values) of farm and lactation stage effects on individual amino acid (AA) content in undigested raw mare milk, AA digestibility and DIAAR values.*

Amino	Farm			Lactation			Lactation*Farm interaction		
acids	Raw	Digestibility	DIAAR	Raw	Digestibility	DIAAR	Raw	Digestibility	DIAAR
Essential AA									
Cys	0.016	0.008	-	0.166	0.188	-	0.862	0.598	-
Met	0.050	0.277	-	0.105	0.730	-	0.355	0.193	-
SAA	-	-	0.003	-	-	0.802	-	-	0.971
His	0.080	0.313	0.052	0.024	0.134	<0.001	0.963	0.784	0.771
Ile	0.199	0.369	0.971	0.003	0.187	0.271	0.051	0.545	0.918
Leu	0.214	0.286	0.712	<0.001	0.063	0.006	0.032	0.406	0.989
Lys	0.214	0.356	0.043	0.002	0.563	0.015	0.029	0.727	0.723
Phe	0.053	0.357	-	0.081	0.105	-	0.905	0.516	-
Tyr	0.124	0.109	-	0.004	0.141	-	0.118	0.829	-
AAA	-	-	0.004	-	-	0.099	-	-	0.993
Thr	0.144	0.292	0.961	0.002	0.098	0.594	0.088	0.479	0.884
Trp	0.043	0.148	0.018	<0.001	0.002	0.353	0.527	0.467	0.999
Val	0.214	0.260	0.118	<0.001	0.157	<0.001	0.050	0.543	0.579
Non-essential AA									
Ala	0.241	0.162	-	0.013	0.107	-	0.064	0.667	-
Arg	0.600	0.232	-	0.038	0.046	-	0.143	0.456	-
Asp	0.060	0.134	-	0.001	0.021	-	0.035	0.300	-
Glu	0.167	0.244	-	0.003	0.234	-	0.046	0.635	-
Gly	0.016	0.366	-	0.114	0.082	-	0.989	0.271	-
Pro	0.141	0.156	-	<0.001	0.042	-	0.030	0.368	-
Ser	0.008	0.767	-	0.542	0.127	-	0.590	0.484	-

[1]*Significance declared at $P \leq 0.05$. AAA: aromatic amino acids (phenylalanine and tyrosine); Ala: alanine; Arg: arginine; Asp: aspartic acid; Cys: cysteine; Glu: glutamic acid; Gly: glycine; His: histidine; Ile: isoleucine; Leu: leucine; Lys: lysine; Met: methionine; Phe: phenylalanine; Pro: proline; SAA: sulphur-containing amino acids (cysteine and methionine); Ser: serine; Thr: threonine; Trp: tryptophan; Tyr: tyrosine; Val: valine.*

Some individual peptides released from gastrointestinal digestion showed significantly ($P \leq 0.05$) different relative abundances in milk samples belonging to different farms or lactation stages (**Table 3**). Farm significantly affected the relative abundance of 31 different peptides (5 from αs1-casein; 19 from β-casein; 5 from β-lactoglobulin I; 1 from α-lactalbumin A; and 1 from lysozyme C) formed after gastric digestion, whereas only 3 peptides (2 from αs1-casein and 1 from β-casein) were affected by intestinal digestion. Overall, a higher peptide release occurred when milk samples belonged to low grazing activity (farm I) compared to high grazing and silage supplementation (farm III), whereas samples from high grazing and hay supplementation (farm II) were usually intermediate (**Figure 3**). In addition, lactation stage significantly ($P \leq 0.05$) affected the relative abundance of 6 different peptides (all derived from β-casein) formed during gastric digestion, but none of the peptides from intestinal digestion. In this regard, a higher peptide release was observed when milk samples were from late lactation (**Figure 3**).



	AAA	His	Ile	Leu	Lys	SAA	Thr	Trp	Val
Infant	91.1	96.7	76.2	84.8	98.7	94.7	74.5	91.9	92.9
Child	164.6	101.5	131.0	123.4	119.4	115.7	105.7	183.7	118.9
Adult	208.8	126.9	139.8	133.5	141.8	135.9	131.0	236.6	127.8

Figure 1. *In vitro digestible indispensable amino acid ratio (DIAAR) percentage values based on infant (■), child (■), and adult (■) nutrition. Numbers in the table represent the mean DIAAR value of each nutritional group. Error bars represent standard deviation. DIAAR values are based on reference requirements for infants (birth to 6 months), children (6 months to 3 years) and adults (older child, adolescent and adult) according to Food and Agriculture Organization (2013). AAA: aromatic amino acids (tyrosine and phenylalanine), HIS: histidine, ILE: isoleucine, LEU: leucine, LYS: lysine, SAA: sulphur-containing amino acids (cysteine and methionine), THR: threonine, TRP: tryptophan, VAL: valine.*

The multivariate discriminant analysis supported the differences observed in the relative abundance of individual peptides in mare milk samples. In fact, the discriminant functions were able to classify more than 60% of milk samples into their respective farm (farm I, II and III) or lactation stage (early, mid and late lactation) group based on the peptide profile after gastric or intestinal digestion, respectively. Overall, the multivariate analysis strongly discriminated between samples from early and late lactation, while samples from mid lactation were often intermingled with early or late lactation stages (**Figure 4**). The most accurate classification for lactation stage groups was achieved using the peptide profile after gastric digestion (84% of samples correctly classified; **Figure 4c**). In terms of farm differentiation, discrimination was slightly poorer. After gastric digestion, milk samples from farm II separated from the rest of the samples (**Figure 4a**), while after intestinal digestion, samples from farm I were mainly discriminated from the other two farms (**Figure 4b**).

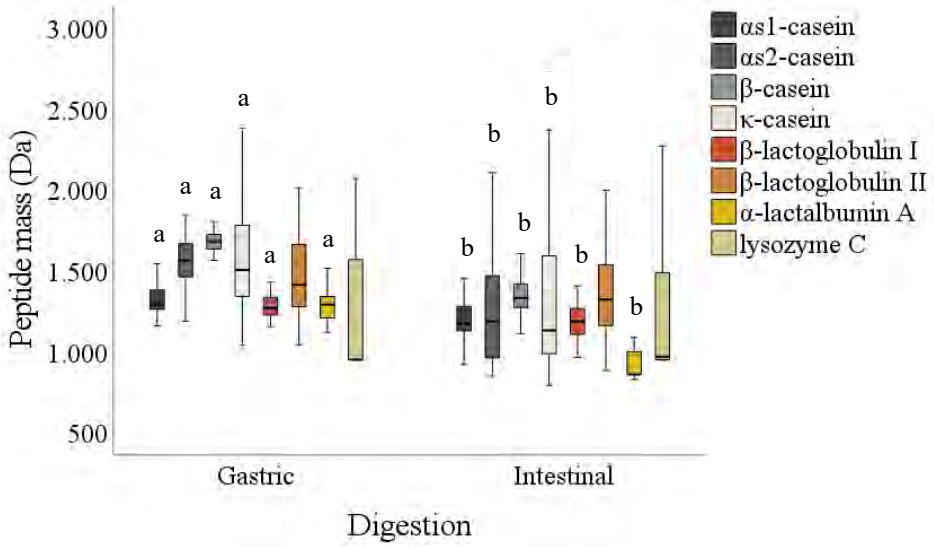


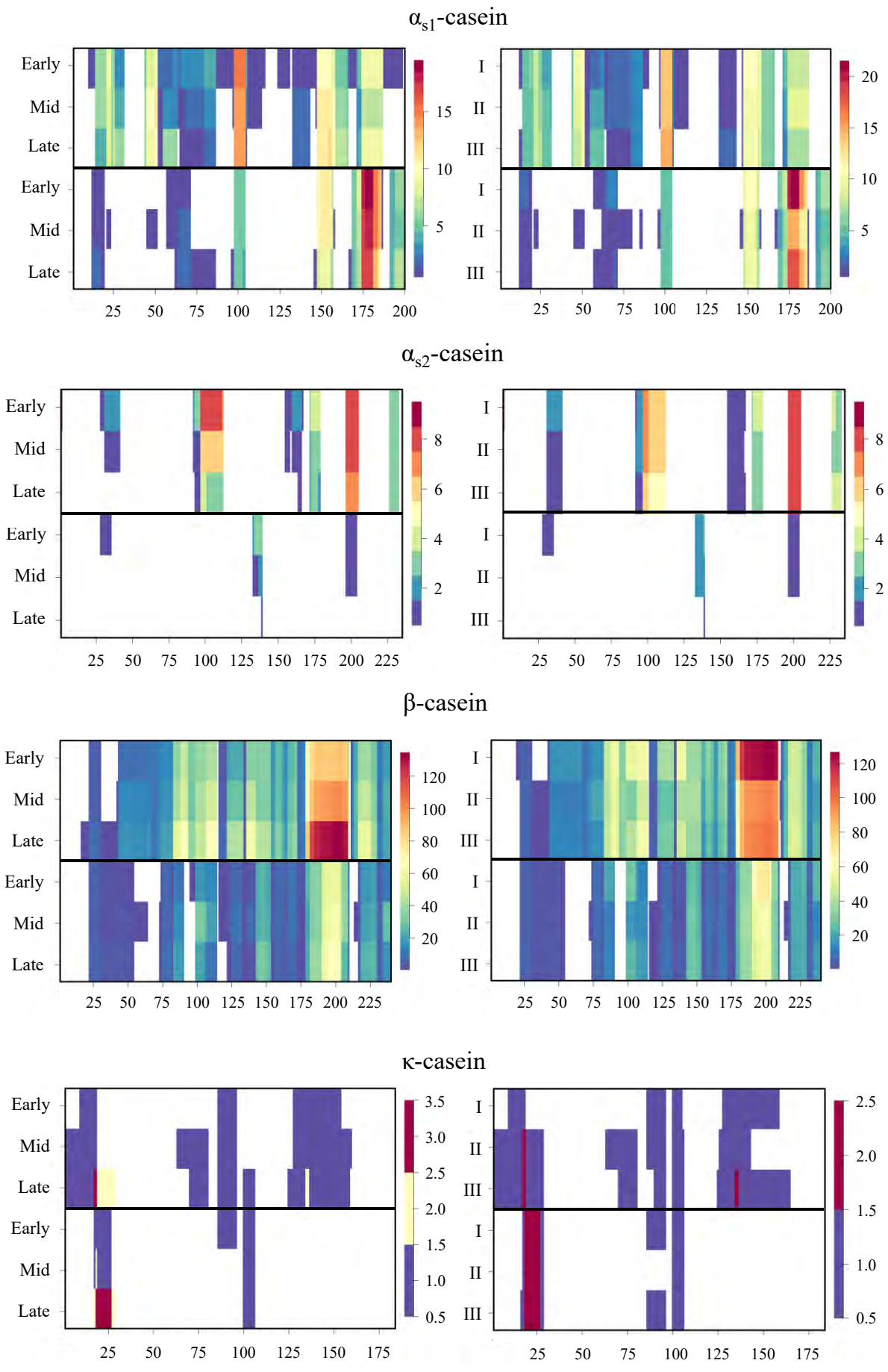
Figure 2. *Size mass of the peptides released after gastric and intestinal digestion classified according to the protein of origin. Letters (a,b) refer to statistically significant ($P \leq 0.05$) differences between gastric and intestinal digestion from the same parent protein.*

Table 2. *Number of unique peptides identified after gastric and intestinal digestion and total number of unique (non-redundant) peptides classified according to the protein of origin.*

	α_{s1}-casein	α_{s2}-casein	β-casein	κ-casein	β-lactoglobulin I	β-lactoglobulin II	α-lactalbumin A	Lysozyme C	All proteins
Gastric	81	94	247	43	101	62	23	29	676
Intestinal	66	71	226	41	94	60	27	28	603
Total	128	149	386	78	169	111	45	54	1106

Table 3. *Peptides identified in mare milk samples after gastric and intestinal digestion significantly ($P \leq 0.05$) affected by farm and/or lactation stage. Peptide sequence and protein of origin are given.*

Farm effect		Lactation effect	
Peptide sequence	Protein	Peptide sequence	Protein
Gastric digestion		Gastric digestion	
IMQHVAYSPFHDTAKL	αs1-casein	ISRFVQPQPVVY	β-casein
NRQRELL	αs1-casein	KSPIVPFSE	β-casein
YQHTLEQ	αs1-casein	PRLGPTGELD	β-casein
YQHTLEQL	αs1-casein	PYAEPVPY	β-casein
DVYPYAAW	αs1-casein	LRLPVHL	β-casein
FVQPQPVVY	β-casein	KVAPFPQPVVPYPQRDTPVQ	β-casein
PATQPIVA	β-casein		
LRLPVHLIQ	β-casein		
PIVPFSE	β-casein		
RQILNPTNGEN	β-casein		
VQPQPVVYPYAEPVPYAVV	β-casein		
PYAVVPQSIL	β-casein		
PFLQPEIM	β-casein		
HQVPQSL	β-casein		
SLLQTLMLPSQPVLSPPQS	β-casein		
ITHINKEKLQKFKHEGQQQREVE	β-casein		
PVHLIQPFM	β-casein		
PRLGPTGELDPATQ	β-casein		
ISRFVQPQPVVY	β-casein		
LYQDPRLGPTGELDPATQ	β-casein		
VPYAVVPQSIL	β-casein		
PYAEPVPYAVV	β-casein		
VQPQPVVY	β-casein		
ILNPTNGEN	β-casein		
KNAATPGQSL	β-lactoglobulin I		
TNIPQTMQD	β-lactoglobulin I		
TNIPQTMQ	β-lactoglobulin I		
VAGKWHSVA	β-lactoglobulin I		
DSESAPLRV	β-lactoglobulin I		
KSMDGYKGVTL	α-lactalbumin A		
LDENIDDD	lysozyme C		
Intestinal digestion			
YLEPFQPS	αs1-casein		
NSEKTDIIPE	αs1-casein		
VAPFPQPVVPYPQ	β-casein		


αs1-casein
Early
Mid
Late
Early
Mid
Late
25 50 75 100 125 150 175 200
15
10
5
I
II
III
I
II
III
25 50 75 100 125 150 175 200
20
15
10
5
αs2-casein
Early
Mid
Late
Early
Mid
Late
25 50 75 100 125 150 175 200 225
8
6
4
2
I
II
III
I
II
III
25 50 75 100 125 150 175 200 225
8
6
4
2
β-casein
Early
Mid
Late
Early
Mid
Late
25 50 75 100 125 150 175 200 225
120
100
80
60
40
20
I
II
III
I
II
III
25 50 75 100 125 150 175 200 225
120
100
80
60
40
20
κ-casein
Early
Mid
Late
Early
Mid
Late
25 50 75 100 125 150 175
3.5
3.0
2.5
2.0
1.5
1.0
0.5
I
II
III
I
II
III
25 50 75 100 125 150 175
2.5
2.0
1.5
1.0
0.5

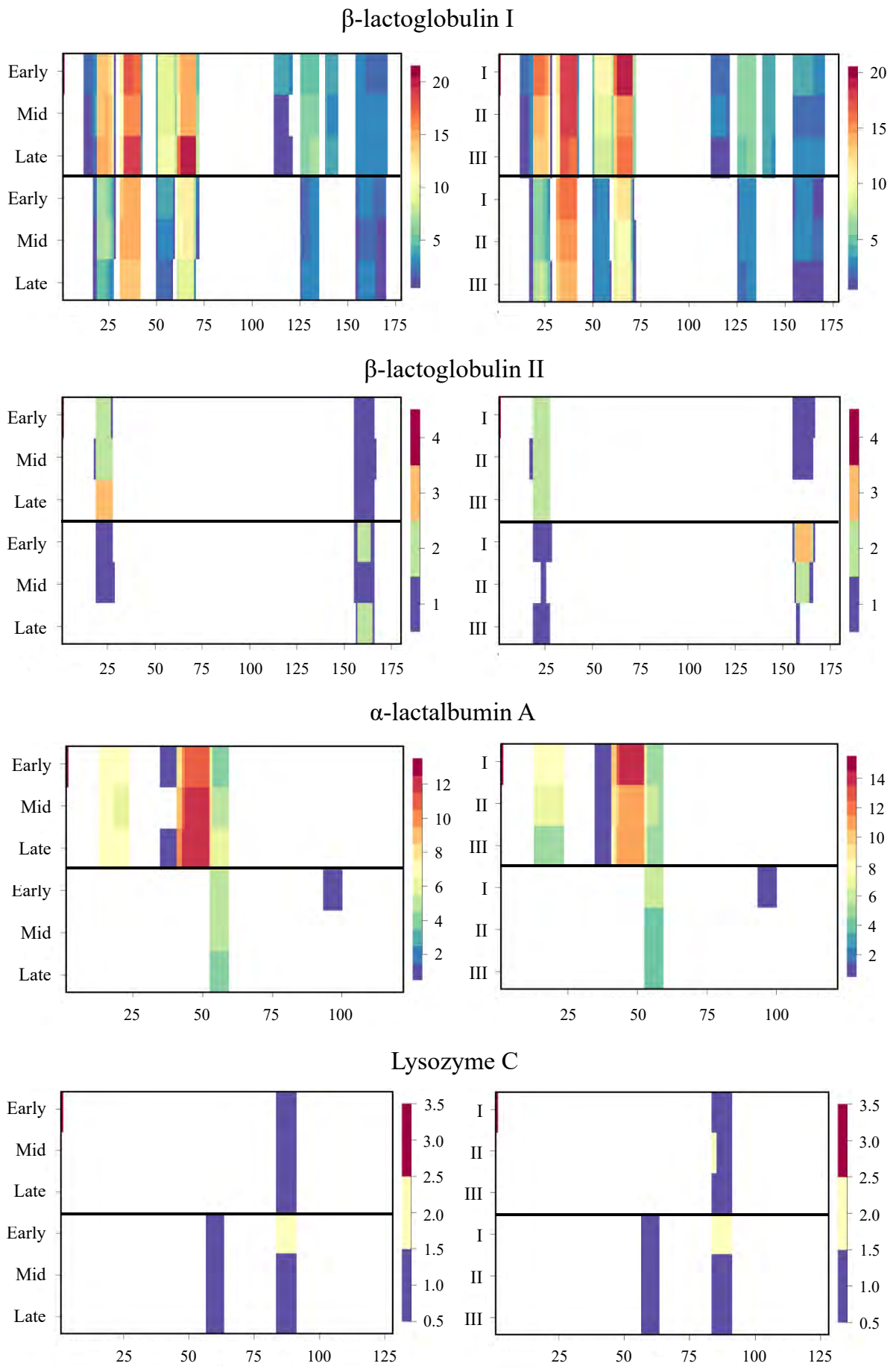

β-lactoglobulin I
Early
Mid
Late
Early
Mid
Late
25 50 75 100 125 150 175
20 15 10 5
I
II
III
I
II
III
25 50 75 100 125 150 175
20 15 10 5
β-lactoglobulin II
Early
Mid
Late
Early
Mid
Late
25 50 75 100 125 150 175
4 3 2 1
I
II
III
I
II
III
25 50 75 100 125 150 175
4 3 2 1
α-lactalbumin A
Early
Mid
Late
Early
Mid
Late
25 50 75 100
12 10 8 6 4 2
I
II
III
I
II
III
25 50 75 100
14 12 10 8 6 4 2
Lysozyme C
Early
Mid
Late
Early
Mid
Late
25 50 75 100 125
3.5 3.0 2.5 2.0 1.5 1.0 0.5
I
II
III
I
II
III
25 50 75 100 125
3.5 3.0 2.5 2.0 1.5 1.0 0.5

Figure 3. *Heatmaps showing a qualitative description of the behavior of peptide patterns at the end of gastric (upper half of the panels) and intestinal digestion (lower half of the panels), classified according to farm or lactation stage. Intensities are expressed as colors that range from purple (low intensity) to red (high intensity), and represent the mean frequency of each amino acid identified as part of the protein sequence. Numbers in x-axis indicate the position of the amino acid in the protein sequence, and the y-axis shows group classification according to lactation stage (left panels; early, mid and late lactation) or farm (right panels; farms I, II and III).*

Among all peptides released after gastric and intestinal digestion, precursors of 70 different bioactive peptides were identified using the MBPDB database (Supplementary material 2). A total of 35 peptides formed after gastric (21 peptides) and intestinal (14 peptides) digestion of mare milk β-casein were found to be precursors of VAPFPQPVVP (fragment f(191-200)), a bioactive peptide previously reported in donkey milk (Bidasolo et al., 2012). Interestingly, two of those precursors were significantly affected by farm or lactation stage. Specifically, the peptide KVAPFPQPVVPYPQRDTPVQ, released after gastric digestion, was significantly ($P \leq 0.05$) affected by lactation stage (highest relative abundance when milk was from late lactation), whereas the peptide VAPFPQPVVPYPQ, released after intestinal digestion, was significantly affected by farm (highest relative abundance when milk was from farm I).

4. Discussion

The present study addressed the performance of mare milk proteins during human digestion using the simulated *in vitro* INFOGEST gastrointestinal digestion model. Total digestibility of mare milk proteins was estimated as 93.7 or 96.0% depending on the method used (by considering individual amino acids or total amino groups, respectively), which was similar to cow milk (around 95%; Dupont and Tomé, 2020). This highlights mare milk as one of the most digestible protein sources. Digestibility of mare milk samples significantly decreased during lactation only when measuring total amino groups. In the analysis of total amino groups, a high variability among samples at late lactation, which probably came from this being a less specific method than the analysis of individual amino acids, could have led to an overestimation of the lactation effect on the digestibility of mare milk proteins. This is consistent with a non-significant effect of lactation stage on total digestibility considering individual amino acids, as well as with the results on peptide release described later in this section, which show that lactation had no significant effect on major peptide release after intestinal digestion (which is indirectly related to digestibility).

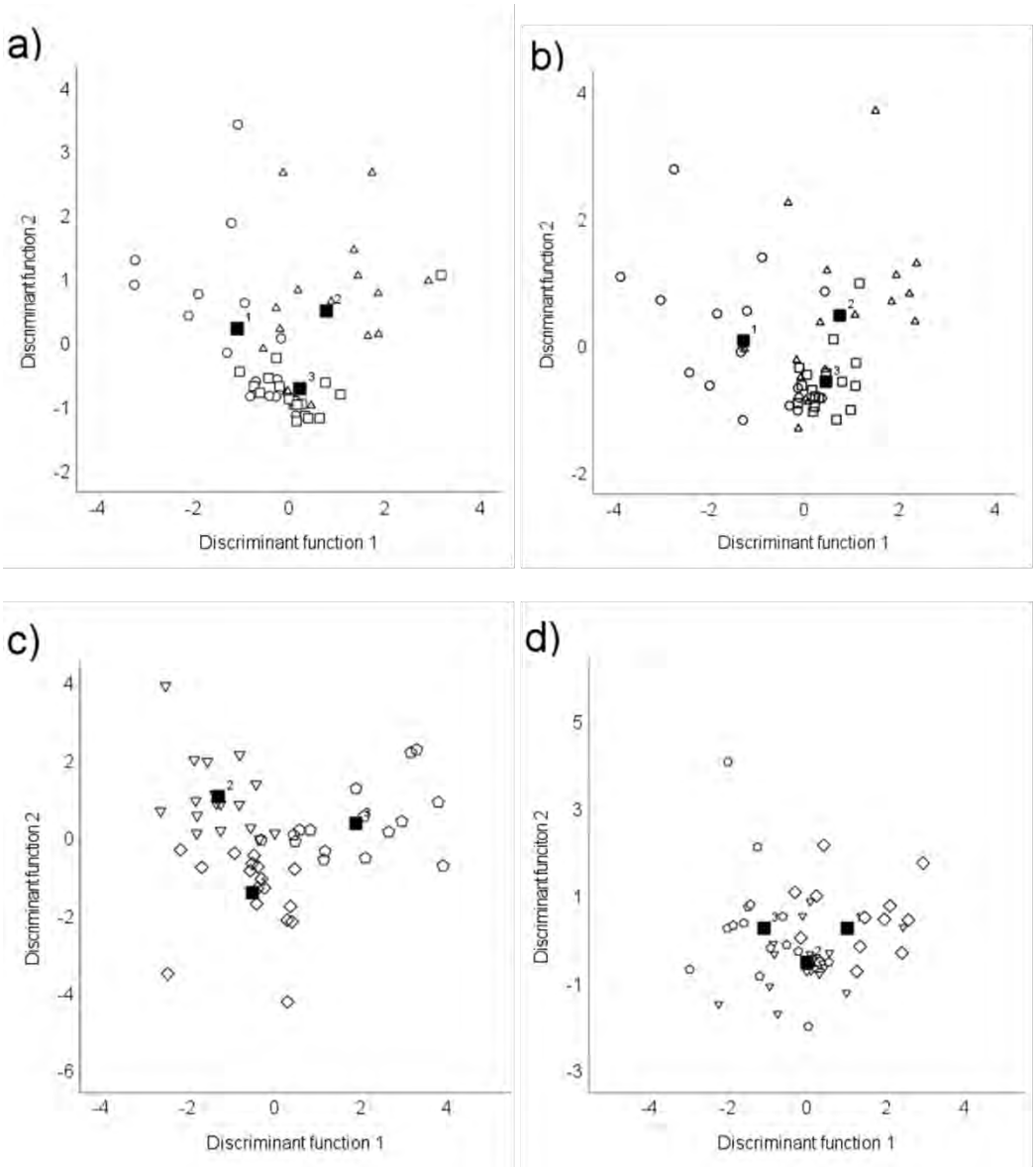


Figure 4. *Distribution of mare milk samples according to stepwise discriminant analysis using the released peptide profile after digestion. Only peptides present in at least two animals per farm and per lactation week were included in the analysis. a) Gastric digestion, classification according to farm (60% classification success); b) intestinal digestion, classification according to farm (64% classification success); c) gastric digestion, classification according to lactation stage (84% classification success); d) intestinal digestion, classification according to lactation stage (62% classification success). Farm I (○), farm II (△), farm III (□); early lactation (◇), mid lactation (▽), late lactation (⬠); group centroid (■).*

The DIAAR values, which express the ratio of digested essential amino acids relative to daily requirements, were affected by lactation stage and farm. These differences were due to changes in the amino acid content of undigested mare milk, and not to changes in digestibility of individual amino

acids. Forage feeding, and therefore grazing, can affect the amino acid composition of milk. In this sense, it has previously been reported that increasing forage intake of lactating donkeys (also equids) can change the levels in milk of threonine, phenylalanine, lysine and serine (Liang et al., 2022). In the present study, animal management affected the content of methionine, tryptophan, lysine and phenylalanine, as well as the evolution pattern of lysine and leucine during lactation. These changes could also derive from differences in the composition and/or quality of the feedstuffs used in the farms.

In mare milk, the limiting amino acids were threonine for infants, and histidine for children and adults. On the contrary, it has been reported that the limiting amino acids in cow milk are threonine and tryptophan for infants, and sulphur-containing amino acids for children and adults, while DIAAR values for histidine are quite high in cow milk (Mathai et al., 2017; Walther et al., 2022). Based on this, mare milk seems to be a better source of sulphur-containing amino acids but a poorer source of histidine than cow milk for children and adults, whereas both milk types are a good source of lysine, histidine and valine for infants (Mathai et al., 2017).

All proteins in mare milk were degraded after gastrointestinal digestion to a greater or lesser extent. However, degradation of caseins was much more intense than that of whey proteins, in agreement with previous studies (Xiao et al., 2023). In fact, the whey proteins β-lactoglobulin II and lysozyme C were resistant to gastrointestinal proteolysis. The different digestion behavior of caseins and whey proteins might be due to caseins forming a clot in the stomach induced by acidic conditions and pepsin, and therefore retaining longer in the stomach. On the other hand, whey proteins are soluble in the gastric fluid and pass through the digestion system faster and with lower proteolysis (Ye et al., 2016b). However, *in vitro* studies, in which all proteins spend the same time at each digestion phase, are comparable to *in vivo* studies in terms of protein digestion (Egger et al., 2017), suggesting that other factors, such as protein structure, also contribute to these differences. In fact, mobile and loosely structured proteins like caseins are more susceptible to the action of pepsin than proteins with a globular structure, like whey proteins (Dupont and Tomé, 2020). Caseins have been described to be almost completely degraded during the gastric phase (Inglingstad et al., 2010; Ye et al., 2016b; Egger et al., 2017; Xiao et al., 2023), whereas equine lysozyme is resistant to gastrointestinal digestion (Inglingstad et al., 2010). In this respect, equine lysozyme has shown resistance to acidic conditions (Jauregui-Adell, 1975), and being a c-type lysozyme, its calcium-binding site confers stability against protease digestion (Kuroki et al., 1989). Resistance of β-lactoglobulin to proteolysis with pepsin has previously been reported in bovine milk (Ye et al., 2016b; Egger et al., 2017; Liu et al., 2019). However, Inglingstad et al. (2010) reported higher hydrolysis of equine β-lactoglobulin (unspecified isoform) than that of bovine and caprine β-lactoglobulins after duodenal digestion. Interestingly, in the present study only β-lactoglobulin II was resistant to proteolysis, whereas β-lactoglobulin I was hydrolyzed already during the gastric phase. This might indicate a difference in the digestion

behavior of the two β-lactoglobulin isoforms in mare milk, which in the study by Inglingstad et al. (2010) could have been hindered by considering the two β-lactoglobulin isoforms together. In fact, horse β-lactoglobulin I and II isoforms present a homology of only 70%, with 48 amino acids being exchanged (Conti et al., 1984; Godovac-Zimmermann et al., 1985). This might have detrimental implications on its structure and interaction with proteases. Degradation of α-lactalbumin during gastrointestinal digestion was evident in the present study, although literature discloses conflicting results regarding digestibility (or resistance) of α-lactalbumin (Dupont and Tomé, 2020).

Comparing gastric and intestinal digestion of mare milk proteins, slightly more but also larger peptides were formed during gastric digestion, which were further degraded into smaller peptides or sequences of 5 or less amino acids (this is a limitation of MS identification using the Mascot algorithm). The main source of peptides was β-casein, which has been reported as the main (Miranda et al., 2004) and highly digestible (Xiao et al., 2023) protein in mare milk. Although, to the best of our knowledge, the native mare milk peptidome has not been characterized yet, the majority of native peptides in human milk are derived from β-casein (Dallas et al., 2013), so endogenous peptides formed in the mammary gland could have in part contributed to the high amount of β-casein fragments found after gastric digestion of mare milk. However, further research that addresses the performance of endogenous peptides during digestion of mare milk is needed. In opposition, the low peptide formation from κ-casein observed in the present work could be a consequence of its low abundance in mare milk (Miranda et al., 2004), although the protein was already highly degraded in the gastric simulation. As known, pepsin rapidly hydrolyses κ-casein into para-κ-casein destabilizing casein micelles and facilitating coagulation. Subsequently, proteolysis continues on the formed clots and on liberated para-κ-casein (Ye et al., 2016b). On the other hand, the low amino acid counts observed in lysozyme C were derived from its relatively low abundance in mare milk (Miranda et al., 2004) and its resistance to digestion.

Lactation stage and management of the mares also affected peptide release during digestion. As mentioned, a higher number of peptides were released from β-casein since it is the major mare milk protein, and therefore, more peptides derived from β-casein than from any other protein were affected by either farm or lactation stage. These differences were more evident for gastric than for intestinal peptides, probably because of the higher degree of proteolysis at intestinal level for proteins that are susceptible to gastrointestinal digestion. This is particularly relevant considering that the small intestine is the site of peptide absorption. Overall, mare milk samples from late lactation and low grazing activity showed the highest peptide release, which suggested a more efficient protein digestion of these samples. According to Egger et al. (2017), milk fat might affect protein digestion, so the differences in peptide release from mare milk samples observed among farms and during lactation could also be attributed to a different sample fat composition. However, some studies in cow milk reported that although milk fat globules

get trapped into casein clots, the structure and digestion traits of the clot are not altered (Ye et al., 2016a). On the other hand, it has also been reported that the mineral composition of milk can affect protein digestion (Liu et al., 2019). The observation was that a reduced phosphorylation improved casein digestibility and proteolysis, and consequently raised peptide release during gastrointestinal digestion (Girardet et al., 2006; Liu et al., 2019). In this regard, the phosphorus content of the milk samples from the present study halved from early to late lactation (Blanco-Doval et al., 2023) and this could be correlated with a lower casein phosphorylation of the milk samples and consequent higher digestibility and peptide release at late lactation.

Milk has been shown to be a great source of bioactive peptides (Ning et al., 2022). However, information regarding bioactive peptides released from minor dairy species is limited up to date, with little knowledge on mare milk peptides (Guha et al., 2021). In fact, to the best of our knowledge, bioactive peptides released exclusively during gastrointestinal digestion of raw mare milk have not been reported yet. In the present study, no bioactive peptides (≥ 5 amino acids) were found in mare milk after gastrointestinal digestion, while a number of precursors of bioactive peptides were identified. Most of these precursors encoded bioactive sequences of 2-3 amino acids previously identified in bovine milk. Interestingly, some precursors of the bioactive peptide VAPFPQPVVP (fragment f(191-200) of β-casein) were found. This peptide was also reported in donkey milk after *in vitro* simulated gastrointestinal digestion, and was characterized as presenting angiotensin converting enzyme inhibitory activity (Bidasolo et al., 2012). Although digested mare milk samples in the present study did not contain this bioactive peptide *per se*, it should be highlighted that the static *in vitro* model used lacks brush border enzymes from the small intestine, which further fragment peptides that reach brush border cells prior to absorption (Sousa et al., 2023). Therefore, the bioactive peptide precursors formed during gastrointestinal digestion could potentially lead to the formation of absorbable bioactive peptides. In addition, the results obtained in this work suggested that management of the mares and lactation stage could influence the release of bioactive peptides from milk, being a topic worth exploring. These results demonstrated that some regions of mare milk proteins were resistant to gastric and/or intestinal digestion. Peptides resulting from these digestion resistant sites are more likely to be absorbed in the small intestine without further degradation (Egger et al., 2017), so they could be interesting regions to search for potential bioactive peptides in future research.

The present study provided a thorough description of the human gastrointestinal digestion of the main proteins present in raw mare milk, but processed (homogenized or heated, for instance) mare milk samples were not considered, being this a limitation of the research. Raw mare milk usually has a high biological quality due to low microbiological and somatic cell counts (Dankówet al., 2006), which makes it adequate for human consumption (Regulation (EC) No 853/2004). However, milk is a perishable product, and heating treatments like pasteurization or

sterilization might be required in order to increase milk shelf life for commercialization. Heat treatments could alter milk proteins at molecular, microstructural and macrostructural level (protein denaturation, aggregation, crosslinking…), which may influence their digestive kinetics and digestibility (Li et al., 2021). Additionally, homogenization decreases the size of milk fat globules, and in this process, caseins and whey proteins are adsorbed into the surface of fat globules, altering clot formation during digestion (Ye et al., 2017). Considering differences in protein composition among cow and mare milk, further research is necessary to better understand the impact of mare milk processing on protein digestion traits.

Furthermore, recent research found that the freeze-thaw process enhanced the aggregation of (human) milk fat globules and proteins, and therefore hindered the hydrolysis of milk lipids and proteins during digestion due to physical impediment. However, milk frozen at low temperature (-60 ºC) and thawed at high temperature (45 ºC) performed similarly to fresh milk during digestion (Zhang et al., 2022). Considering this, freezing conditions in the present study (-80 ºC) probably preserved the protein digestion characteristics of raw mare milk, but a thawing process at low temperature (4 ºC) could have slightly decreased total protein digestibility. Anyway, since all mare milk samples were treated equally during sample preparation, potential changes in milk proteins occurring at the freezing-thawing step should not have interfered with the effect of lactation stage and management system observed in the present study. Nevertheless, the effect of the freeze-thaw process on mare milk remains to be further studied.

5. Conclusions

The content of highly digestible proteins in mare milk makes it one of the most digestible dietary protein sources. As far as we know, this is the first study to report different digestion behaviors of I and II isoforms of β-lactoglobulin in mare milk, deepening into the understanding of this protein source. Additionally, degradation of α-lactalbumin A was demonstrated, bringing some clarity to discrepancies among literature studies. Lactation stage and management system significantly affected some of the parameters related to protein digestion determined after gastrointestinal digestion of mare milk using the INFOGEST method, i.e., amino acid profile of raw mare milk, DIAAR values and peptide release. Therefore, behavior of mare milk proteins during gastrointestinal digestion could be modulated through diet or optimized by selecting milk from specific lactation stages, which is definitely a topic worth exploring. Further research remains essential to elucidate the role that human digestion has on the digestion and functionality of mare milk proteins.

Supplementary material

Available at http://hdl.handle.net/10810/66250.

Supplementary material 1. Supplementary data regarding enzyme characteristics and activity, chemical composition of simulated salivary, gastric and intestinal fluids, calculations for

protein digestibility, and digestibility of individual amino acids.

Supplementary material 2. List of peptides liberated after digestion of mare milk samples that were identified as bioactive peptide precursors for containing a bioactive sequence previously described in milk from mammal species other than horses, according to the Milk Bioactive Peptide Database (MBPDB).

Notes

Ana Blanco-Doval acknowledges the Department of Education of the Basque Government for the predoctoral fellowship (PRE_2022_2_0078) and mobility funding (EP_2023_1_0037). The authors thank the commercial farms that participated in the study. This work was supported by the Basque Government through Biotasma (Elkartek, 2019), Behor Esne (Cooperation, 2020) and IT944-16 and IT1568-22 projects. The authors have not stated any conflicts of interest. This is a commercial study and, therefore, institutional animal use approval was not required.

References

Barłowska, J., G. Polak, I. Janczarek, and E. Tkaczyk. 2023. The influence of selected factors on the nutritional value of the milk of cold-blooded mares: The example of the Sokólski breed. Animals 13:1152. https://doi.org/10.3390/ani13071152.

Bidasolo, I. B., M. Ramos, and J. A. Gomez-Ruiz. 2012. In vitro simulated gastrointestinal digestion of donkeys' milk. Peptide characterization by high performance liquid chromatography – tandem mass spectrometry. Int. Dairy J. 24:146-152. https://doi.org/10.1016/j.idairyj.2011.04.014.

Blanco-Doval, A., L. J. R. Barron, and N. Aldai. 2023. Changes during lactation in the mineral element content of mare milk produced in semi-extensive rural farms. J. Food Compos. Anal. 123:105629. https://doi.org/10.1016/j.jfca.2023.105629.

Brodkorb, A., L. Egger, M. Alminger, P. Alvito, R. Assunção, S. Ballance, T. Bohn, C. Bourlieu-Lacanal, R. Boutrou, F. Carrière, A. Clemente, M. Corredig, D. Dupont, C. Dufour, C. Edwards, M. Golding, S. Karakaya, B. Kirkhus, S. Le Feunteun, U. Lesmes, A. Macierzanka, A. R. Mackie, C. Martins, S. Marze, D. J. McClements, O. Ménard, M. Minekus, R. Portmann, C. N. Santos, I. Souchon, R. P. Singh, G. E. Vegarud, M. S. J. Wickham, W. Weitschies, and I. Recio. 2019. INFOGEST static in vitro simulation of gastrointestinal food digestion. Nat. Protoc. 14:991-1014. https://doi.org/10.1038/s41596-018-0119-1.

Businco, L., P. G. Giampietro, P. Lucenti, F. Lucaroni, C. Pini, G. Di Felice, P. Iacovacci, C. Curadi, and M. Orlandi. 2000. Allergenicity of mare's milk in children with cow's milk allergy. J. Allergy Clin. Immunol. 105:1031-1034. https://doi.org/10.1067/mai.2000.106377.

Conti, A., J. Godovac-Zimmermann, J. Liberatori, G. Braunitzer, and D. Minori. 1984. The primary structure of monomeric β-lactoglobulin I from horse colostrum (*Equus caballus*, Perissodactyla). Hoppe Seylers Z. 365:1393-1401. https://doi.org/10.1515/bchm2.1984.365.2.1393.

Dallas, D. C., A. Guerrero, N. Khaldi, P. A. Castillo, W. F. Martin, J. T. Smilowitz, C. L. Bevins, D. Barille, J. B. German, and C. B. Lebrilla. 2013. Extensive *in vivo* human milk peptidomics reveals specific proteolysis

yielding protective antimicrobial peptides. J. Proteome Res. 12:2295-2304. https://doi.org/10.1021/pr400212z.

Danków, R., J. Wójtowski, J. Pikul, R. Niżnikowski, and D. Cais-Sokolińska. 2006. Effect of lactation on the hygiene quality and some milk physicochemical traits of the Wielkopolska mares. Arch. Tierz. 49:201-206.

Dupont, D., and D. Tomé. 2020. Milk proteins: Digestion and absorption in the gastrointestinal tract. Pages 701-714 in Milk proteins. Elsevier.

Egger, L., O. Ménard, and R. Portmann. 2019. Quantitative characterization of digestion processes. Pages 159-184 in Interdisciplinary Approaches to Food Digestion. Springer.

Egger, L., P. Schlegel, C. Baumann, H. Stoffers, D. Guggisberg, C. Brügger, D. Dürr, P. Stoll, G. Vergères, and R. Portmann. 2017. Physiological comparability of the harmonized INFOGEST in vitro digestion method to in vivo pig digestion. Food Res. Int. 102:567-574. https://doi.org/10.1016/j.foodres.2017.09.047.

Food and Agriculture Organization. 2013. Dietary protein quality evaluation in human nutrition. Report of a FAO expert consultation. FAO Food and Nutrition Paper, 92:1-66.

Girardet, J., L. Miclo, S. Florent, D. Mollé, and J. Gaillard. 2006. Determination of the phosphorylation level and deamidation susceptibility of equine β-casein. Proteomics 6:3707-3717. https://doi.org/10.1002/pmic.200500728.

Godovac-Zimmermann, J., A. Conti, J. Liberatori, and G. Braunitzer. 1985. The amino-acid sequence of β-lactoglobulin II from horse colostrum (*Equus caballus*, Perissodactyla): β-lactoglobulins are retinol-binding proteins. Hoppe Seylers Z. 366:601-608. https://doi.org/10.1515/bchm3.1985.366.1.601.

Godovac-Zimmermann, J., D. Shaw, A. Conti, and H. McKenzie. 1987. Identification and the primary structure of equine α-lactalbumin B and C (*Equus caballus*, Perissodactyla). Biol. Chem. Hoppe-Seyler 368:427-443. https://doi.org/10.1515/bchm3.1987.368.1.427.

Guha, S., H. Sharma, G. K. Deshwal, and P. S. Rao. 2021. A comprehensive review on bioactive peptides derived from milk and milk products of minor dairy species. Food Prod. Process. Nutr. 3:1-21. https://doi.org/10.1186/s43014-020-00045-7.

Halliday, J. A., K. Bell, and D. C. Shaw. 1991. The complete amino acid sequence of feline β-lactoglobulin II and a partial revision of the equine β-lactogobulin II sequence. Biochim. Biophys. Acta. 1077:25-30. https://doi.org/10.1016/0167-4838(91)90521-Z.

Inglingstad, R. A., T. G. Devold, E. K. Eriksen, H. Holm, M. Jacobsen, K. H. Liland, E. O. Rukke, and G. E. Vegarud. 2010. Comparison of the digestion of caseins and whey proteins in equine, bovine, caprine and human milks by human gastrointestinal enzymes. Dairy Sci. Technol. 90:549-563. https://doi.org/10.1051/dst/2010018.

International Organization for Standardization 8968-3. 2007. ISO (8968-3:2007/IDF 20-3:2007).

International Organization for Standardization/Draft International Standard 13904. 2014. ISO/DIS (13904:2014(E)) Animal feeding stuffs – Determination of tryptophan content.

Jaeser, M., C. Wunderlich, and T. Henle. 2023. Characterization of the glycans of equine κ-casein. J. Agric. Food Chem. 71:9460-9468. https://doi.org/10.1021/acs.jafc.3c00452.

Jaudzems, G., J. Guthrie, S. Lahrichi, and C. Fuerer. 2019. Total amino acids by UHPLC-UV in infant

formulas and adult nutritionals, first action 2018.06. J. AOAC Int. 102:1574-1588. https://doi.org/10.5740/jaoacint.19-0036.

Jauregui-Adell, J. 1975. Heat stability and reactivation of mare milk lysozyme. J. Dairy Sci. 58:835-838. https://doi.org/10.3168/jds.S0022-0302(75)84646-7.

Kopf-Bolanz, K. A., F. Schwander, M. Gijs, G. Vergères, R. Portmann, and L. Egger. 2012. Validation of an in vitro digestive system for studying macronutrient decomposition in humans. J. Nutr. 142:245-250. https://doi.org/10.3945/jn.111.148635.

Kuroki, R., Y. Taniyama, C. Seko, H. Nakamura, M. Kikuchi, and M. Ikehara. 1989. Design and creation of a Ca^{2+} binding site in human lysozyme to enhance structural stability. Proc. Natl. Acad. Sci. 86:6903-6907. https://doi.org/10.1073/pnas.86.18.6903.

Li, S., A. Ye, and H. Singh. 2021. Impacts of heat-induced changes on milk protein digestibility: A review. Int. Dairy J. 123:105160. https://doi.org/10.1016/j.idairyj.2021.105160.

Liang, X. S., Y. X. Yue, Y. L. Zhao, Y. M. Guo, X. Y. Guo, B. L. Shi, and S. M. Yan. 2022. Effects of dietary concentrate to forage ratio on milk performance, milk amino acid composition and milk protein synthesis of lactating donkeys. Anim. Feed Sci. Technol. 292:115444. https://doi.org/10.1016/j.anifeedsci.2022.115444

Liu, D., J. Zhang, L. Wang, T. Yang, X. Liu, Y. Hemar, J. M. Regenstein, and P. Zhou. 2019. Membrane-based fractionation, enzymatic dephosphorylation, and gastrointestinal digestibility of β-casein enriched serum protein ingredients. Food Hydrocoll. 88:1-12. https://doi.org/10.1016/j.foodhyd.2018.09.032.

Mathai, J. K., Y. Liu, and H. Stein. 2017. Values for digestible indispensable amino acid scores (DIAAS) for some dairy and plant proteins may better describe protein quality than values calculated using the concept for protein digestibility-corrected amino acid scores (PDCAAS). Br. J. Nutr. 117:490-499. https://doi.org/10.1017/S0007114517000125.

Minekus, M., M. Alminger, P. Alvito, S. Ballance, T. Bohn, C. Bourlieu, F. Carrière, R. Boutrou, M. Corredig, D. Dupont, C. Dufourt, L. Egger, M. Golding, S. Karakaya, B. Kirkhus, S. Le Feunteun, U. Lesmes, A. Macierzanka, A. Mackie, S. Marze, D. J. McClements, O. Ménard, I. Recio, C. N. Santos, R. P. Singh, G. .E. Vegarud, M. S. J. Wickham, W. Weitschies, and A. Brodkorb. 2014. A standardised static in vitro digestion method suitable for food – an international consensus. Food Funct. 5:1113-1124. https://doi.org/10.1039/C3FO60702J.

Miranda, G., M. Mahé, C. Leroux, and P. Martin. 2004. Proteomic tools to characterize the protein fraction of Equidae milk. Proteomics 4:2496-2509. https://doi.org/10.1002/pmic.200300765.

Moughan, P. J., C. A. Butts, H. van Wijk, A. M. Rowan, and G. W. Reynolds. 2005. An acute ileal amino acid digestibility assay is a valid procedure for use in human ileostomates. J. Nutr. 135:404-409. https://doi.org/10.1093/jn/135.3.404.

Nielsen, S. D., R. L. Beverly, Y. Qu, and D. C. Dallas. 2017. Milk bioactive peptide database: A comprehensive database of milk protein-derived bioactive peptides and novel visualization. Food Chem. 232:673-682. https://doi.org/10.1016/j.foodchem.2017.04.056.

Ning, J., M. Li, W. Chen, H. Zhao, J. Chen, M. Yang, X. Cao, and X. Yue. 2022. Peptidomics as a tool to analyze endogenous peptides in milk and milk-related peptides. Food Biosci. 50:102199. https://doi.org/10.1016/j.fbio.2022.102199.

Nitta, K., H. Tsuge, S. Sugai, and K. Shimazaki, K. 1987. The calcium-binding property of equine lysozyme. FEBS Lett. 223:405-408. https://doi.org/10.1016/0014-5793(87)80328-9.

Portmann, R., P. Jiménez-Barrios, J. Jardin, L. Abbühl, D. Barile, M. Danielsen, Y. Huang, T. K. Dalsgaard, B. Miralles, V. Briard-Bion, S. Cattaneo, C. Chambon, B. Cudennec, I. De Noni, B. Deracinois, D. Dupont, A. Duval, C. Flahaut, R. López-Nicolás, D. N. El, V. Pica, V. Santé-Lhoutellier, M. Stuknytė, L. Theron, T. Sayd, I. Recio, and L. Egger. 2023. A multi-centre peptidomics investigation of food digesta: Current state of the art in mass spectrometry analysis and data visualisation. Food Res. Int. 169:112887. https://doi.org/10.1016/j.foodres.2023.112887.

Regulation (EC) No 853/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific hygiene rules for food of animal origin.

Salimei, E., and Y. W. Park. 2017. Mare milk. Pages 369-408 in Handbook of Milk of Non-Bovine Mammals. Wiley Blackwell.

Sousa, R., I. Recio, D. Heimo, S. Dubois, P. J. Moughan, S. M. Hodgkinson, R. Portmann, and L. Egger. 2023. In vitro digestibility of dietary proteins and in vitro DIAAS analytical workflow based on the INFOGEST static protocol and its validation with in vivo data. Food Chem. 404:134720. https://doi.org/10.1016/j.foodchem.2022.134720.

Uniacke-Lowe, T., T. Huppertz, and P. F. Fox. 2010. Equine milk proteins: Chemistry, structure and nutritional significance. Int. Dairy J. 20:609-629. https://doi.org/10.1016/j.idairyj.2010.02.007.

UniProt database. 2023. Accessed Dec. 2023. https://www.uniprot.org/.

Walther, B., D. Guggisberg, R. Badertscher, L. Egger, R. Portmann, S. Dubois, M. Haldimann, K. Kopf-Bolanz, P. Rhyn, O. Zoller, R. Veraguth, and S. Rezzi. 2022. Comparison of nutritional composition between plant-based drinks and cow's milk. Front. Nutr. 9:2645. https://doi.org/10.3389/fnut.2022.988707.

Waters. 2007. Waters UPLC Amino Acid Analysis Solution System Guide: 71500129702/Revision B (Vol. 71500129702/Revision B): Waters Corporation.

Xiao, T., J. Zeng, C. Zhao, Y. Hou, T. Wu, Z. Deng, and L. Zheng. 2023. Comparative analysis of protein digestion characteristics in human, cow, goat, sheep, mare, and camel milk under simulated infant condition. J. Agric. Food Chem. 71:15035-15047. https://doi.org/10.1021/acs.jafc.3c03123.

Ye, A., J. Cui, D. Dalgleish, and H. Singh. 2016a. The formation and breakdown of structured clots from whole milk during gastric digestion. Food Funct. 7:4259-4266. https://doi.org/10.1039/C6FO00228E.

Ye, A., J. Cui, D. Dalgleish, and H. Singh. 2016b. Formation of a structured clot during the gastric digestion of milk: Impact on the rate of protein hydrolysis. Food Hydrocoll. 52:478-486. https://doi.org/10.1016/j.foodhyd.2015.07.023.

Ye, A., J. Cui, D. Dalgleish, and H. Singh. 2017. Effect of homogenization and heat treatment on the behavior of protein and fat globules during gastric digestion of milk. J. Dairy Sci. 100:36-47. https://doi.org/10.3168/jds.2016-11764.

Zhang, L., J. Qu, T. Huppertz, J. Liu, Z. Sun, and P. Zhou. 2022. Effects of different freeze-thaw processes on the bioactivity and digestibility of human milk. LWT 156:113025. https://doi.org/10.1016/j.lwt.2021.113025.

Zhao, S., F. Pan, S. Cai, J. Yi, L. Zhou, and Z. Liu. 2023. Secrets behind protein sequences: Unveiling the

potential reasons for varying allergenicity caused by caseins from cows, goats, camels, and mares based on bioinformatics analyses. Int. J. Mol. Sci. 24:2481. https://doi.org/10.3390/ijms24032481.