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Evaluation of Valorisation Strategies to Improve Grape Stems' Nutritional Value as an Ingredient for Ruminants' Diets

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Abstract: Grape stems are a by-product of wine production which is managed as a waste. Animal feeding arises as a potential alternative. However, its practical use may be compromised by its high lignin content. In this sense, hydrolysis emerges as a strategy to increase fibre digestibility. In addition, due to its high and variable moisture content, it should be dried to prevent microbial deterioration and a washing pre-treatment to reduce sugar content becomes necessary to minimize drying problems due to sugar melting at high temperatures. Within this framework, this study assessed the effect of washing and three different hydrolysis on the nutritive value of grape stems. A factorial design was carried out, with washing (with or without) and hydrolysis (without, enzymatic, alkali, and alkali-enzymatic) as factors. When the washing pre-treatment was not applied, only the alkali hydrolysis process maintained in vitro digestibility, but at the expense of a lower fermentation efficiency. When the washing pre-treatment was applied, fibre contents were similar among hydrolysis processes, but the alkali hydrolysis improved in vitro digestibility with similar fermentation efficiency. In conclusion, the alkali hydrolysis maintained or improved the grape stem nutritive value depending on whether the washing pre-treatment was applied or not.

Keywords: winery by-products; washing; sugar content; hydrolysis; digestibility; animal feed; circular economy

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1. Introduction

The production of wine is widespread throughout the world. According to the International Organisation of Vine and Wine [1], world wine production has remained stable at around 260 million HL from 2019 to 2022. In the European Union, wine production volume was approximately 157 million HL in 2022, excluding juices and musts, showing an annual increase of 3.5 million HL (+2%) compared to 2021.

Destemming is one of the first steps of winemaking and aims to reduce the excessive astringency and negative strong flavours that stems can transmit to the wine if they are crushed and pressed with the grapes [2]. Stems constitute approximately 3 to 6% (w/w) of the total weight of the bunch of grapes, depending on the variety, the quality of grapes in the bunch, and their health status. Therefore, they are one of the most important organic by-products of the winemaking process [3–7]. At present, grape stems are mostly not valorised as a by-product and are sent for composting or discarded in open areas in the vineyard [8].

The livestock sector has an increasing demand for more sustainable and economic ingredients to ensure the sourcing of feed diets [9]. Some studies describe the use of

plants and other lignocellulosic wastes sources for animal feeding [10,11]. Within this background, animal feeding arises as a potential alternative since they are a good source of nutrients and high-value active substances, such as phenolic compounds, with potential health-promoting effects [8,12,13].

Moreover, the waste hierarchy stablished by the Directive 2008/98/EC of the European Parliament and of the Council [14] to prioritise waste management strategies establishes that human consumption should be the first option to reuse food waste. However, the high lignin content of grape stems [15] limits their use as an ingredient in the food industry. Although there are other potential applications for re-use this raw material, such as biomass for energy or their use as fertilizers, the second option should be to reuse them for animal feeding, so this is the preferred application for this raw material.

Grape stems are by-products with a high moisture content which should be dried to prevent microbial deterioration that could compromise its use as animal feed. At the same time, grape stems include a significant number of grapes that have not been separated during destemming. These grapes have a high sugar content [3,4] that could involve drying problems due to sugar melting at high temperatures [16]. With the aim of avoiding these problems, a previous washing step, traditionally applied in the citrus juice industry [17], is proposed as an efficient technique to reduce this sugar content, obtaining a liquor rich in sugar with food applications [18,19].

On the other hand, grape stems are also rich in lignin and, although the benefits of using lignocellulosic ingredients in ruminants have already been proven, several studies have shown that lignin limits microbial enzymatic access to cellulose and hemicellulose [20], which may affect the digestibility [21]. Thus, the dry matter digestibility significantly increases when the lignin content decreases [22]. Therefore, different treatments to break the lignocellulosic bounds in the feed fibre fraction have been considered as a valuable strategy to improve the digestibility of fibrous feedstuffs [15].

Hydrolysis emerges as a strategy with great potential to increase fibre digestibility since it is a chemical reaction to break down the bonds of a particular substance to obtain smaller and more digestible fractions [23]. There are different types of hydrolysis; while enzymatic hydrolysis uses enzymes [24], alkaline hydrolysis uses alkaline chemicals [25] to accelerate the decomposition of a molecule, leaving fragments and a neutral liquid called effluent.

In this framework, this study aimed to evaluate the effect of washing pre-treatment and enzymatic and alkali hydrolysis on the nutritive value, ruminal in vitro organic matter digestibility (IVOMD), and ruminal fermentation product production of grape stem-based ingredients.

2. Materials and Methods

2.1. Samples and Experimental Design

The grape stems samples came from Baigorri winery located in Samaniego (Basque Country—Spain). The initial sample was portioned into 500 g bags and was maintained as frozen (−20 °C) until processing.

A 2 × 4 factorial design was carried out to analyse the effect of a washing pre-treatment and different hydrolysis processes, with washing (with or without) and hydrolysis (without, enzymatic, alkali, and alkali-enzymatic) as factors.

Half of the grape stem sample was subjected to a washing pre-treatment and the other half was not washed. The washing process was carried out at a 1:1.5 solid–liquid ratio for 45 min with manual agitation every 5 min. The sample was filtered through a 250 µm mesh. The remaining solid samples were used for the subsequent hydrolysis processes, and liquid samples were discarded.

Following that, both unwashed and washed samples were divided into two subsamples. One of the subsamples was preserved without hydrolysis and served as control (washing and without washing). The other subsamples for hydrolysis were divided in three different subsamples and each of them was hydrolysed in a different way (enzymatic

hydrolysis, alkali hydrolysis, and a combination of alkali and enzymatic hydrolysis). All the processes were carried out in triplicate.

Both alkali and enzymatic hydrolysis were carried out using a Sell Symphony 7100 Bathless Dissolution Distek equipment (Distek Inc., North Brunswick, NJ, USA), controlling and monitoring temperature, time and stir speed.

Enzymes for the enzymatic hydrolysis were supplied by Novozymes (NovozymesA/S, Bagsvaerd, Denmark). Viscozyme® is an endo-beta-glucanase that hydrolyses (1,3)- or (1,4)-linkages in beta-D-glucans with high mannase activity and Ultimase® is a cellulase that hydrolyses (1,4)-beta-D-glucosidic linkages in cellulose and other beta-D-glucans. Hydrolysis conditions were pH 5, 55 °C, 20 h, 250 rpm, ratio 1:1 solid:water, and 1% of each enzyme with regards to fibre. The pH of each enzymatic hydrolysis was manually controlled and then adjusted with NaOH 1 M in a total volume of 500 mL. The enzymatic hydrolysis runs were finished by enzyme inactivation by a temperature at 90 °C for 15 min.

Alkali hydrolysis was carried out with NaOH, 1% w/v, at a sample:solvent ratio of 1:1.25 (w/w). The conditions selected were 3 h, 90 °C 250 rpm. The initial pH was not adjusted.

When the two hydrolyses were applied to the samples, the alkali hydrolysis was performed to favour delignification and then an enzymatic hydrolysis was performed. The conditions were the same as those described for each process.

Finally, the hydrolysates were centrifuged (2650 g; 15 min; ambient T[a]), and two parts were recovered: a liquid and a solid fraction. The solid samples were freeze-dried for further chemical and in vitro digestibility determinations.

2.2. In Vitro Organic Matter Digestibility

Each of the obtained solid samples were applied as a medium in a short-term in vitro batch fermentation assay. Samples were tested in three vitro runs. In each run, ruminal liquid was taken from one multiparous Latxa ewe that was sacrificed for productive purposes. Prior to slaughter, ewes were fed a forage-based diet for 3 weeks and had free access to fresh water and feed. Ruminal liquid was filtrated through four layers of cheesecloth into a volumetric glass. It was then dissolved in culture medium in a 1:4 ratio (ruminal liquid and phosphate–bicarbonate tampon, respectively) at anaerobic conditions in accordance with Menke et al. [26].

500 mg of grape stem samples were measured into 125 mL serum flasks. Each sample was incubated with 50 mL of growing medium in triplicate, and the flasks were sealed under pressure and incubated at 39 °C in the laboratory incubator during 24 h. Gas generation was discharged at 2, 4, 6, and 22 h after inoculation to prevent the pressure in the flask headspace overcoming 48 kPa, as described by Theodorou et al. [27]. Following 24 h of fermentation, flasks were placed in the refrigerator during 20 min to pause fermentation and proceed with short chain fatty acids (SCFA) sampling and IVOMD determination.

In vitro organic matter digestibility was determined, as suggested by Pell and Schofield (1993) [28]. 45 mL of a neutral detergent solution was poured into each flask and heated at 105 °C during 1 h; the flasks were cooled, filtrated by glass filter crucibles (Porosity 2), and washed using distilled water, ethanol, and acetone. The rest of the sample was dried at 100 °C overnight and was afterwards burned in a muffle at 550 °C to obtain true IVOMD values.

2.3. Chemical Analyses

The solid samples of grape stems were characterized by analysing dry matter (DM, method 934.01), ash (method 942.05), and nitrogen (method 984.13) contents following Association of Official Analytical Chemists [29]. Neutral detergent fibre (NDF) was analysed by the method of UNE EN ISO 16472 [30] with the addition of an alpha amylase, and without adding sodium sulphite, and was referred without ashes. Acid detergent fibre (ADF) and acid detergent lignin (ADL), referred without residual ash, were analysed by method 973.18 of AOAC. Total reducing sugars (TRS) in the solid parts were anal-

ysed by the Dinitrosalicylic acid (DNS) method [31] in concordance with the microplate assay technique [32].

The antioxidant capacity (AOC) of the solid fractions was determined utilizing the DPPH radical scavenging activity (DRSA) method according to Brand-Williams et al. [33] with minor variations. 280 µL of a solution of DPPH (2,2-Diphenyl-1-picrylhydrazyl, D9132 Sigma Aldrich, Steinheim, Germany) in methanol (40 ppm) were aggregated to 20 µL of sample solution. The mix was cultured at ambient temperature in the darkness during 30 min. Absorbance was determined at 515 nm (Varioskan™ LUX multimode microplate reader, Thermo Fisher Scientific, Waltham, MA, USA). The standard consisted of water–methanol (50% *v*/*v*) and various concentrations of Trolox (218940050, Acros Organics, Fair Lawn, NJ, USA). The antioxidant capacity was referred as mg Trolox equivalent antioxidant capacity (TEAC) per g of dry matter (DM) by applying the calibration graph.

Total phenolic content (TPC) in the solid fractions was determined by applying the Folin–Ciocalteu method [34] with variations. 30 µL of the Folin–Ciocalteu (J/4100/08, Fischer Scientific, Loughborough, UK) solution was mixed with 140 µL sample, blank or standard, and 140 µL of Na_2CO_3 7% (*w*/*v*) (Sigma Aldrich, Steinheim, Germany). The mix was incubated at ambient temperature in the dark during 1 h and the absorbance was determined at 750 nm (Varioskan™ LUX multimode microplate reader, Thermo Fisher Scientific, Waltham, MA, USA). Gallic acid (G7384, Sigma Aldrich, Steinheim, Germany) was applied as standard at a concentration range of 1.4–20 ppm and values were reported as mg gallic acid equivalent (GAE) per g of DM sample.

The characterization of SCFA in the liquid supernatant collected in the short-term in vitro batch fermentation assay (acetic, propionic, butyric, isobutyric, valeric, isovaleric, and caproic) was developed by gas chromatography by utilizing a flame ionization detector, in concordance with [35]. Briefly, 4 mL of culture medium and 1 mL of a solution of 20 g/L of 4 metil-valeric acid as an internal standard, in 0.5 N HCl, was mixed and centrifuged (15,000× *g* for 15 min at 4 °C) to obtain the liquid phase separated from the feed residuals. Following that, the liquid phase was microfiltered (premium syringe filter regenerated cellulose, 0.45 µm 4 mm, Agilent Technologies, Madrid, Spain), and 0.5 µL of liquid phase was introduced in the chromatograph (Agilent 6890 N, Agilent, Spain) by utilizing a capillary column (30 m× 530 um; 1 µm particle size; HP-FFAP, Agilent, Spain).

2.4. Calculation and Statistical Analysis

Normal data distribution was tested by applying the Shapiro Wilk test and the Levene test to analyse the equality of variances.

The physicochemical characterization data of the solid portions of the grape stems were assessed using an analysis of variance by the GLM technique of Statistical Analysis System (SAS) version 9.4M5 (SAS, 2017), adding the fixed effects of the washing, hydrolysis, and the interaction among them. Regarding the in vitro digestibility trial, the total number of observations was three series of processing (hydrolysis) × 2 pre-treatment × 4 hydrolysis conditions × 3 in vitro cultivation runs × 3 laboratory repetitions = 216; nonetheless, after averaging incubation series and laboratory repetitions, the remained 24 observations were tested for variance analysis by GLM procedure (SAS, 2017). The statistical model consists of the same effects as the physicochemical characterization ones. Least squares mean for treatments are described for all traits. Treatment means were separated applying Tukey adjustment, and significant effects were stated at $p < 0.050$.

3. Results

3.1. Effect of Washing and Hydrolysis of Grape Stems on Chemical Composition

The effect of washing and the hydrolysis processes of grape stems on chemical composition can be seen in Table 1. A significant interaction between washing and the hydrolysis process was found for NDF, ADF, and ADL contents.

Table 1. The effect of washing and hydrolysis of grape stems on chemical composition, reducing sugar content, total polyphenols, and antioxidant capacity.

	With Washing				Without Washing					*p*-Value		
Item	**CTR**	**AH**	**EH**	**AEH**	**CTR**	**AH**	**EH**	**AEH**	**SEM**	**H**	**W**	**H × W**
DM (%)	93.4	92.4	94.1	93.8	92.6	93.2	93.7	92.1	1.07	0.767	0.434	0.681
Ash (%DM)	6.22	12.7	6.77	8.73	4.72	10.6	5.97	8.14	0.596	<0.001	0.011	0.601
CP (%DM)	5.27	5.17	6.09	5.21	4.84	4.45	5.67	5.82	0.360	0.043	0.374	0.371
NDF (%DM)	51.1	52.9	61.9	58.6	26.4 ^{c}	41.6 ^{b}	55.7 ^{a}	64.2 ^{a}	2.28	<0.001	<0.001	<0.001
ADF (%DM)	43.7 ^{b}	47.3 ^{ab}	56.3 ^{a}	57.6 ^{a}	24.8 ^{c}	40.6 ^{b}	50.4 ^{ab}	61.1 ^{a}	2.14	<0.001	<0.001	0.002
ADL (%DM)	17.7 ^{b}	18.2 ^{b}	26.2 ^{a}	25.3 ^{a}	10.6 ^{c}	18.1 ^{b}	23.3 ^{ab}	27.2 ^{a}	1.40	<0.001	0.060	0.038
TRS (mg/g)	108	115	72.2	107	350 ^{a}	198 ^{b}	119 ^{b}	145 ^{b}	45.2	0.005	<0.001	0.018
TPC (mg GAE/g)	17.8 ^{b}	29.7 ^{a}	7.18 ^{c}	16.6 ^{b}	25.3 ^{a}	22.4 ^{a}	7.33 ^{b}	13.4 ^{b}	2.473	<0.001	0.567	0.005
AOC (mg TEAC/g)	18.0 ^{b}	33.4 ^{a}	11.0 ^{b}	24.6 ^{b}	26.7 ^{a}	25.4 ^{a}	11.5 ^{b}	19.6 ^{ab}	2.63	<0.001	0.476	0.002

CTR: control without hydrolysis; AH: Alkali hydrolysis; EH: Enzymatic hydrolysis; AEH: Alkali-enzymatic hydrolysis; SEM: standard error of the mean; H: Hydrolysis; W: Washing; DM: dry matter; CP: crude protein; NDF: neutral detergent fibre; ADF: acid detergent fibre; ADL: acid detergent lignin; TRS: total reducing sugars; TPC: total phenolic compounds; GAE: gallic acid equivalent; AOC: antioxidant capacity; TEAC: Trolox equivalent antioxidant capacity. Different superscripts within washing treatment indicate significant differences between the hydrolysis processes ($p < 0.050$).

When grape stems were previously washed, non-significant differences among hydrolysis treatments for the NDF content were found. However, both the enzymatic hydrolysis (56.3 vs. 43.7%, $p = 0.015$) and alkali-enzymatic hydrolysis (57.6 vs. 43.7%, $p = 0.007$) increased the ADF compared to control, with differences among all other treatments not being significant. Similarly, ADL contents were significantly increased with both enzymatic hydrolysis (26.2%) and alkali-enzymatic hydrolysis (25.3%) compared to control (17.7%) or alkali hydrolysis (18.2%), with differences among all other treatments not being significant.

On the contrary, when grape stems were not washed, all hydrolysis processes increased the NDF content compared to control. In addition, both alkali-enzymatic hydrolysis (64.2 vs. 41.6%, $p < 0.001$) and enzymatic hydrolysis (55.7 vs. 41.6%, $p = 0.009$) increased the NDF content compared to alkali hydrolysis. Differences between alkali-enzymatic hydrolysis and enzymatic hydrolysis were found not to be significant ($p = 0.323$). All hydrolysis processes increased ADF compared to control, but significantly to a higher extent with alkali-enzymatic hydrolysis compared to alkali hydrolysis. The same was observed for ADL contents, where all hydrolysis treatments increased compared to control, with differences between alkali-enzymatic hydrolysis and alkali hydrolysis being significant.

No interaction was observed for ash and CP contents. Regarding the washing effect, ash content increased when the grape stems were washed (8.60 vs. 7.35%, $p = 0.011$), but washing did not have an effect on CP content (5.20 vs. 5.44%, $p = 0.374$).

In terms of the hydrolysis effect, both alkali hydrolysis (11.6 vs. 5.47%, $p < 0.001$) and alkali-enzymatic hydrolysis (8.43 vs. 5.47%, $p = 0.001$) processes increased ash content compared to the control. In addition, alkali hydrolysis increased the ash content compared to alkali-enzymatic hydrolysis ($p < 0.001$) or enzymatic hydrolysis (11.6 vs. 6.37%, $p < 0.001$), with differences between the latter being significant ($p = 0.024$). In addition, enzymatic hydrolysis increased the CP content compared to alkali hydrolysis (5.88 vs. 4.81%, $p = 0.042$), with differences among all other treatments not being significant.

The effect of the washing and hydrolysis process on the AOC, TPC, and TRS content of the final solid ingredient can be seen in Table 1. A significant interaction between washing and hydrolysis was found for TRS content, TPC, and AOC. When grape stems were washed

TRS were not different among the different hydrolysis processes, but when grape stems were not washed all the hydrolysis processes showed lower TRS contents compared to control, with differences among them not being significant. Regarding TPC, when grape stems were washed alkali hydrolysis increased the content compared to all other hydrolysis processes, and enzymatic hydrolysis reduced it compared to alkali-enzymatic hydrolysis ($p = 0.026$) and control ($p = 0.010$), with differences among the latter being non-significant ($p = 0.999$), but when samples were not washed, greater TPC were found in control and alkali hydrolysed samples compared to enzymatic hydrolysed and alkali-enzymatically hydrolysed grape stems, being differences between control and alkali hydrolysis, and between enzymatic hydrolysis and alkali-enzymatic hydrolysis were non-significant.

Similar trends were observed for AOC. When samples were washed alkali hydrolysis increased it compared to all other treatments, with differences among them not being significant. When the samples were not washed greater AOC was found in control and alkali hydrolysis compared to enzymatic hydrolysis, with alkali-enzymatic hydrolysis having differences between control and alkali hydrolysis, and this was not significant between enzymatic hydrolysis and alkali-enzymatic hydrolysis.

3.2. Effect of Washing and Hydrolysis of Grape Stems on In Vitro Organic Matter Digestibility and Main Fermentation Parameters

The effect of washing and different hydrolysis processes of grape stems on IVOMD and fermentation parameters is shown in Table 2. A significant interaction between washing and hydrolysis was found for all studied variables.

Table 2. The effect of washing and hydrolysis of grape stems on in vitro digestibility and main fermentation parameters.

	With Washing				**Without Washing**					***p*-Value**		
	CTR	**AH**	**EH**	**AEH**	**CTR**	**AH**	**EH**	**AEH**	**SEM**	**H**	**W**	**H × W**
IVOMD (%)	37.6 ^{b}	48.6 ^{a}	27.5 ^{c}	35.0 ^{bc}	61.6 ^{a}	57.2 ^{a}	36.7 ^{b}	39.9 ^{b}	1.34	<0.001	<0.001	<0.001
SCFA (mmol/100 mL)	4.39 ^{a}	4.17 ^{ab}	2.81 ^{c}	3.34 ^{bc}	6.00 ^{a}	5.13 ^{a}	3.78 ^{b}	3.50 ^{b}	0.241	<0.001	<0.001	0.017
Individual SCFA proportions (mmol/100 mmol)												
C2	71.2	73.2	70.5	67.4	64.3 ^{b}	69.9 ^{ab}	69.7 ^{ab}	71.8 ^{a}	1.44	0.083	0.103	0.007
C3	18.5	17.4	19.3	21.8	24.1	20.3	20.4	18.8	1.30	0.287	0.083	0.020
C4	7.80	7.06	7.51	7.54	9.25 ^{a}	7.67 ^{b}	7.55 ^{b}	6.87 ^{b}	0.338	<0.001	0.046	0.001
C2:C3	3.85 ^{ab}	4.20 ^{a}	3.66 ^{ab}	3.54 ^{b}	2.69 ^{b}	3.44 ^{a}	3.42 ^{a}	3.81 ^{a}	0.1405	0.002	<0.001	<0.001

CTR: control without hydrolysis; AH: Alkali hydrolysis; EH: Enzymatic hydrolysis; AEH: Alkali-enzymatic hydrolysis; SEM: standard error of the mean; H: Hydrolysis; W: Washing; IVOMD: in vitro organic matter digestibility; SCFA: short chain fatty acids; C2: acetate; C3: propionate; C4: butyrate. Different superscripts within washing treatment indicate significant differences between hydrolysis processes ($p < 0.050$).

When grape stems were washed, alkali hydrolysis improved IVOMD ($p < 0.001$) compared to control, whereas enzymatic hydrolysis reduced it ($p < 0.001$).

Enzymatic hydrolysis ($p < 0.001$) and alkali-enzymatic hydrolysis ($p = 0.014$) reduced total SCFA production compared to control, with differences between the latter not being significant ($p = 0.605$). In addition, non-significant differences were found between control and alkali hydrolysis ($p = 0.995$) and between alkali hydrolysis and alkali-enzymatic hydrolysis ($p = 0.102$).

Regarding individual molar proportions of SCFA, non-significant differences were found in acetate (C2), propionate (C3), and butyrate (C4) molar proportions among the different treatments. None of the hydrolysis processes affected the acetate to propionate (C2:C3) ratio compared to control. However, the alkali-enzymatic hydrolysis reduced C2:C3 ($p = 0.031$) compared to alkali hydrolysis.

When grape stems were not washed, none of the hydrolysis processes improved IVOMD compared to control. Indeed, enzymatic hydrolysis, and alkali-enzymatic hydrolysis reduced it ($p < 0.001$), with differences between these treatments not being significant.

Similarly, enzymatic hydrolysis ($p < 0.001$) or alkali-enzymatic hydrolysis ($p < 0.001$) reduced SCFA production compared to control or alkali hydrolysis. Differences between

enzymatic hydrolysis and alkali-enzymatic hydrolysis ($p = 0.989$), or between control and alkali hydrolysis ($p = 0.068$), were not significant.

As for individual molar proportions, the alkali-enzymatic hydrolysis increased C2 molar proportions compared to control ($p = 0.037$), with differences among all other treatment being non-significant. All the hydrolysis processes reduced C4 molar proportions compared to control, with differences among them being non-significant. All the hydrolysis processes resulted in higher C2:C3 ratio compared to control, with differences among them being non-significant.

4. Discussion

Grape stems are rich in bioactive secondary metabolites, especially phenolic compounds, which make them interesting as a functional ingredient in animal feed [8,12,13]. However, their high fibre content, especially in lignin, limits their use to animals able to digest fibrous feed, such as ruminants [20,21].

Ruminant animals are capable of digesting and fermenting fibrous feed due to the symbiotic relationship with the microorganisms inhabiting their specialized digestive systems. However, although they are able to digest this fibrous feed, fibre fractions are slowly fermented in the rumen [36]. Therefore, a very high undigestible fibre content in the diet could limit intake [37] and the 'productive performance' of animals [38] and could consequently be uninteresting from an economic point of view. In this sense, despite the potential beneficial effects of grape stems for improving the health of ruminant animals, from a sustainability point of view, it is necessary to search for strategies that allow us to maximize ingredient inclusion levels in order to address the large amount of by-product generated.

It is therefore vital to find strategies to decrease the undigestible fibre content of grape stems and thus increase their digestibility. Several strategies have been proposed as a way to reduce fibre fraction in grape stems [39,40], however the main objective of those studies was to release sugars and bioactive compounds. Alkali hydrolysis is normally presented as an effective process for the pre-digestion of fibre fractions, mainly lignin, but, depending on the severity of the process, hemicellulose can also be hydrolysed due to the rupture of the ester bonds [41]. Lignin is a physical barrier to cellulose enzymatic hydrolysis, so the reduction of lignin and hemicellulose increases cellulose surface and pore size, improving enzymatic hydrolysis [42]. In addition, enzymatic action is also facilitated due to the weakening of the hydrogen bonds linking the glucans [43].

In the present study, with the aim of improving grape stems' digestibility to increase the percentage of inclusion of this by-product in the final diets, several valorisation processes were applied based on previous results in the literature. A washing pre-treatment aiming to reduce sugar content in order to avoid problems during the drying process was also tested.

The washing pre-treatment showed a significant effect on the following hydrolysis treatments.

Grape stems include a significant number of grapes with a high sugar content [3] that were not separated during destemming. This increases the sugar content of this by-product. Initial TRS in the sample without washing and hydrolysis was 350 mg/g of DM sample. Amendol et al. [44] found lower values of TRS; 143.5 mg glucose/g DM and 140.6 mg fructose/g of DM, which were likely associated with the grape juice that had impregnated stems during destemming operations. Other authors, however, found much lower initial TRS in grape stems 54.4 mg/g DM [40]. Multiple reviews have examined the nutritional composition of grape stems and other by-products from wineries. The primary conclusion is that comparing the data is complex due to variations in samples, which is attributed to factors such as grape variety, vintage, maturity, and variances in extraction processes [4].

After the washing process the TRS content in the samples was 108 mg/g DM, indicating that the washing pre-treatment, as expected, reduced TRS in the remaining samples. With this washing process as the TRS content decreased, the fibrous fractions increased (NDF, ADF, and ADL), and thus digestibility decreased about 39%.

Digestibility is closely related to the physicochemical composition of feedstuffs. Fibre fraction, mainly its degree of lignification, is one of the components which most negatively affects digestibility [45–47]. On the contrary, TRS are easily fermented and, therefore, are usually positively related to digestibility [48].

The effect of the washing pre-treatment in grape stems' physicochemical composition, with less TRS and higher fibre fraction and therefore less digestibility, of the initial ingredient affected the observed results to a great extent with the different hydrolysis processes.

It seems that washing is a crucial process. When it was not performed, the sample, without any other processing (control), retained more sugars than the hydrolysed samples. However, as all samples experienced a significant initial loss of free sugars, washing resulted in fewer sugars being released during the subsequent hydrolysis processes compared to control in these washed samples. Consequently, all fibre fractions increased with all the hydrolysis treatments compared to control when the washing pre-treatment was not applied, but when it was applied this increase was only observed in the more undigestible fibre fractions (ADF and ADL), and only with enzymatic hydrolysis and alkali-enzymatic hydrolysis treatments.

The changes observed in the physicochemical characteristics influenced the digestibility of the grape stems. In this case, consequently, the washing pre-treatment also influenced the results observed for the different hydrolysis processes.

When the grape stems were not previously washed the enzymatic and alkali-enzymatic hydrolysis, as commented, this reduced the TRS content and of grape stems increased fibre components, leading to a reduced digestibility of the final product compared to control samples (35% and 40% decrease, respectively). San Martin et al. (2023) [49] observed the same trend using similar enzymes and spent coffee grounds as substrates. This reduced digestibility was coherent with the reduced SCFA production observed during fermentation, since SCFA are end-products of microbial fermentation [26].

This effect of the enzymatic hydrolysis could be related to the hydrolysis process. The hydrolysis processes performed with enzymes implied the incorporation of water that was subsequently discarded when the hydrolysis ended. Enzymatic action caused a release of TRS to the liquid medium, and as the liquid fraction was removed, as observed, a loss of TRS in the solid fraction destined for animal feeding occurred in comparison to the non-processed samples. This release and elimination of TRS lead to a higher content of insoluble and recalcitrant compounds (NDF, ADF, and ADL) in the remaining solid part.

Although alkali hydrolysis also reduced TRS content and increased fibre components when the grape stems were not washed, no differences were observed in digestibility compared to control. Other authors pointed out that sodium hydroxide reduced the level of polymerization and crystallinity of cellulose, leading to an alteration of the lignin structure [50]. Thus, it could be hypothesized that in the present study the alkali hydrolysis succeeded in acting into the lignocellulosic bounds, leading to a more degradable fibre fraction. Therefore, although a loss of soluble sugars occurred in the hydrolysis process, this fact was offset by a more digestible fibre fraction.

This hypothesis is coherent with the observed fermentation pattern with a higher acetate to propionate ratio in the in vitro fermentation of grape stems hydrolysed with alkali compared to control samples. The physicochemical properties of an ingredient influence the ruminal fermentative digestion and the SCFA produced. Generally, more digestible fibre results in a ruminal fermentation process with acetic acid as the major end product, to the detriment of propionic production.

This effect of the alkali hydrolysis on digestibility also occurred when the grape stems were previously washed. As commented before, no differences in the physicochemical composition were observed between hydrolysed alkali and control samples when the washing pre-process was applied. However, an increase of 29% in digestibility was observed in the alkali hydrolysed samples, which should be due to the action of the alkali reagent on the lignocellulosic bonds present in the grape stems which improved the accessibility of digestible fibre fractions to the ruminal microorganisms.

On the other hand, when grape stems were washed, although less sugar release was observed with the enzymatic hydrolysis compared to control than the one observed for not washed samples, an increase in ADF and ADL was also observed, leading to a decrease of 26% in digestibility and a reduction in SCFA production. In this case the negative effect of the enzymatic hydrolysis observed in the digestibility of grape stems was less pronounced in the washed samples, probably due to the different composition of the initial ingredient, with less TRS and more NDF, ADF, and ADL contents.

Regarding bioactive compounds, similar TPC values were found in other studies [4]. Nevertheless, a high variation can be found depending on several factors [51]. Grape stems are characterized by a high presence of tannins which are linked to lignin [52,53] These tannins represent approximately 80% of the TPC in grape stems [54]. Polyphenols present in the wine and wine by-products have been widely studied for their antioxidant properties [51,55,56]. Therefore, it would be desirable that any attempt to process these by-products could maintain the concentration of these bioactive compounds, which would be potentially beneficial for improving animal health.

The levels of TPC and AOC also denoted an interaction between the washing and the hydrolysis processes. In the case of TPC, only alkali hydrolysis maintained the initial TPC in the sample compared to the unprocessed samples when no previous washing was applied. When samples were washed, alkali hydrolysis was the treatment that maintained a higher concentration of TPC in samples, even more than unprocessed samples. These results are coherent with the results observed for digestibility and could be explained similarly. Polyphenols in plants are intimately linked to the cell wall structure [57], and any effort to rupture the lignocellulosic bounds of the cell wall can result in their release. With the enzymatic hydrolysis, these polyphenols would be solubilized and removed when the liquid fraction was eliminated after the hydrolysis. In addition, as explained above, alkali hydrolysis is normally used for delignification of the matrix [41]. This delignification process could make polyphenol compounds more extractable and, consequently, increase TPC in alkali hydrolysed samples.

AOC exhibited comparable patterns, validating the hypothesis regarding the facilitation of the release of phenolic compounds with antioxidant capacity after a delignification process. However, in contrast to TPC, no differences were found between alkali-enzymatic hydrolysis and CTR and alkali hydrolysis when samples were not washed, nor between CTR, enzymatic hydrolysis, and alkali-enzymatic hydrolysis when samples were washed.

Several studies have demonstrated that the correlation between antioxidant capacity and TPC determined by the Folin-Ciocalteu method is not always linear [58]. Additionally, antioxidant compounds exhibit distinct mechanisms of action that are correlated with their structural specificity [59]. Therefore, the results may differ depending on the compounds in the sample [60].

Considering that the washing step is necessary to avoid drying problems at an industrial scale and, under that condition, that the alkali hydrolysis is the most efficient strategy to improve the digestibility of grape stems for animal feeding, further studies to optimise the parameters of the alkali hydrolysis, such as temperature, time, and solid–liquid concentration, should be necessary.

5. Conclusions

The washing pre-treatment affects the hydrolysis process. When the washing pre-treatment was not applied only the alkali hydrolysis process maintained in vitro digestibility, but at the expense of a lower fermentation efficiency. When the washing pre-treatment was applied, differences in fibre contents were not so pronounced among hydrolysis processes, but the alkali hydrolysis improved in vitro digestibility with similar fermentation efficiency, improving polyphenol concentrations and the antioxidant capacity of the ingredient at the same time. In conclusion, the alkali hydrolysis is regarded as the most suitable hydrolysis process because it either maintained or improved the grape stem nutritive value, depending on whether the washing pre-treatment was applied or not. Depending on

the initial content in soluble sugars of the grape stem, the washing process would be a necessary first step to avoid problems in the drying process. When this occurs, an alkali hydrolysis would improve the characteristics of the ingredients for animal feed.

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Glossary

ADF	Acid Detergent Fibre
ADL	Acid Detergent Lignin
AOC	Antioxidant capacity
C2	Acetate
C3	Propionate
C4	Butyrate
CP	Crude Protein
CTR	Control
DM	Dry Matter
DPPH	2,2-Diphenyl-1-picrylhydrazyl
FEFAC	European Feed Manufacturers' Federation
GAE	Gallic Acid Equivalent
IVOMD	In Vitro Organic Matter Digestibility
NDF	Neutral Detergent Fibre
OM	Organic Matter
SCFA	Short Chain Fatty Acids
SEM	Standard Error of the Mean
TEAC	Trolox Equivalent Antioxidant Capacity
TPC	Total Polyphenol Content
TRS	Total Reducing Sugars

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