NEWFEED

Turn Food Industry By-products into Secondary Feedstuffs via Circular-Economy Schemes

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Grape stem-based ingredient feeding strategy: including nutritional value, in-vitro digestibility, the ruminal fermentation kinetics determination and methane production determination

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Task 2.1	Case study 1: grape stem-based ingredients for dairy sheep and cattle
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Foreword

The work described in this report was developed under the project NEWFEED: Turn Food Industry By-products into Secondary Feedstuffs via Circular-Economy Schemes (Grant Agreement number: 2013/ Call 2020 Section 1 Farming IA). If you wish any other information related to this report or the NEWFEED project please visit the project web-site (www.newfeed-prima.eu) or contact:

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Executive Summary

This deliverable provides the grape stem-based ingredient feeding strategy including the grape stems nutritional value, in-vitro digestibility, the ruminal fermentation kinetics determination and methane production determination. This process is included in the first case study developed in the project:

1) Use of **grape stems** from wineries for sheep and dairy cattle.

The analysis of the data obtained in the deliverable D2.1 allowed the detection of the most suitable process to increase the nutritional value of the ingredient and, therefore, will help to increase the percentage of inclusion described in this deliverable (D2.4) in the validation trials with ruminants in WP3. Determining the factors that affect the ingredient nutritional value is of vital importance to define the industrial process and to valorise as much ingredient as possible. The first case study will evaluate the use of grape stems from wineries as a second generation ingredient for ruminant feed (sheep and dairy cows). This case study is led by AZTI (www.azti.es) and will be validated in Spain.

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1. Introduction

The overall objective of the project is to develop alternative feed ingredients using a circular economy approach through the conversion of food by-products into high-value secondary animal feed.

The purpose of this Deliverable is to define the grape stem-based ingredient feeding strategy including the grape stems nutritional value, in-vitro digestibility, the ruminal fermentation kinetics determination and methane production determination. This process is included in the first case study developed in the project:

- ✓ The first case study will evaluate the use of grape stems from wineries as a second generation ingredient for ruminant feed (sheep and dairy cows). This case study is led by AZTI (www.azti.es) and will be validated in Spain.

The analysis of the data obtained in the Deliverable D2.1 allowed to define the best hydrolysis process for increasing the digestibility and nutritional value of the ingredients. The selected prototypes were

1. Hydrolysed ingredient (NaOH 0.5 % hydrolysis for 2.3 h and 90 ºC)
2. Washed and non-hydrolysed ingredient as control

The definition of the feeding strategy and the percentage of inclusion for the feeding trials that will be developed in WP3 have been defined in this deliverable to ensure the successful valorisation of these by-products as alternative ingredients for animal feed.

2. Methodology

2.1 Antioxidant activity

Antioxidant activity of samples was measured using the DPPH radical scavenging activity (DRSA) method based on Brand-Williams et al. (1995) with slight modifications. DPPH (2,2-Diphenyl-1-picrylhydrazyl, D9132 Sigma Aldrich, Steinheim, Germany) in methanol (40 ppm) was prepared and 280 µl of this solution were added to 20 µl of sample solution. The mixture was incubated at room temperature in the dark for 30 min. Absorbance was measured at 515 nm. The standard comprised of water–methanol (50 % v/v) and different concentrations of Trolox (218940050, Acros Organics, New Jersey, USA). The antioxidant capacity was expressed as mg Trolox equivalent antioxidant capacity (TEAC) per litter of solvent or per g of DM using the calibration curve. The same samples were used as for the determination of polyphenols.

2.2 Total phenolic determination

Total phenolic content (TPC) was measured using the Folin–Ciocalteu method (Singleton 1965) with modifications. Initially, 30 µl of Folin–Ciocalteu (J/4100/08, Fischer Scientific, Loughborough, UK) solution were added to 140 µl sample, blank or standard and 140 µl of Na_2CO_3 7 % (w/v) (Sigma Aldrich, Steinheim,

Germany). The mixture was incubated at room temperature in the dark for 1 h and the absorbance was measured at 750 nm. Gallic acid (G7384, Sigma Aldrich, Steinheim, Germany) was used as standard at a concentration range of 1.4–20 ppm and results were expressed as mg gallic acid equivalent (GAE) per litter of solvent and per g of DM sample.

2.3 Chemical analysis

The chemical analyses were carried out by the Department of Animal Nutrition of the Provincial Council of Alava (Vitoria-Gasteiz, Spain) in accordance with Royal Decree 2257/1994, of 25 November, which approves the official methods of analysis of animal feeds or foodstuffs and their raw materials (BOE, 1995).

Dry matter (DM) content was determined by oven drying at 103°C (method 6). Ash content was determined by incineration of the samples at 550°C (method 12). Nitrogen content (method 3) was determined using the macro-Kjeldahl procedure on a Kjeltec Foss (Foss, Hillerød, Denmark). Neutral detergent fibre (NDF) and acid detergent fibre (ADF) content were determined by the method of Van Soest et al. 1991. Acid detergent lignin was determined subsequent to ADF by calcination at 525 ºC. Crude fat content was determined by extraction of the sample with petroleum ether (method 4). Neutral detergent insoluble protein (NDICP) and acid detergent insoluble protein (ADICP) was determined by analyzing the NDF and ADF residues, respectively, for Kjeldahl nitrogen. The determination of Ca, P, K and Mg minerals was done by Perkin Elmer 2100DV Optical Emission Spectrophotometry (OES).

Total reducing sugars were determined by the Dinitrosalicylic acid (DNS) method adjusted to the microplate assay procedure. The DNS acid reagent was prepared by dissolving 8 g of NaOH in 100 mL of distilled water. Then, 5 g of DNS (Fischer Scientific, Loughborough, UK), 250 mL of distilled water and 150 g of potassium sodium tartrate tetrahydrate (Sigma-Aldrich, Steinheim, Germany) were added and made up to the volume (500 mL). Sample, blank or standard (25 µL), different concentrations of D-glucose (Fischer Scientific, Loughborough, UK), and 25 µL of DNS reagent were added to each well and incubated for 10 min at 100 ^{o}C. The microplate was rapidly cooled in an ice bath and 250 µL of distilled water was added to each well. Absorbance was read at 540 nm.

In vitro organic matter digestibility in the short term in vitro trial was calculated as described by Pell and Schofield (1993), where by 45 mL of a neutral detergent solution was added to each bottle and warmed at 105 °C for 1 h; then, the bottles were cooled, filtered through glass filter crucibles (Porosity 2) and washed with distilled water, ethanol and acetone. The remaining sample was dried at 100 °C overnight and then burned in a muffle furnace at 550 °C to obtain true IVOMD values.

The analysis of SCFA (acetic, propionic, butyric, isobutyric, valeric, isovaleric and caproic) of the samples of caecal contents was performed by gas chromatography using a flame ionization detector. A volume of 4 mL

of diluted cecal contents mixed with one mL of a solution of 20 g/L of 4-metil-valeric acid as an internal standard, in 0.5 N HCl, was centrifuged (15,000×g for 15 min at 4◦C) to separate the liquid phase from the feed residuals. After, 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 directly injected in the chromatograph (Agilent 6890 N, Agilent, Spain) using a capillary column (30 m× 530 um; 1-µm particle size; HP- FFAP, Agilent, Spain).

Stoichiometric methane values were estimated using equations proposed by Blümmel et al. (1999) based on the stoichiometry of Wolin (1960).

Where:

CO_2= (C2/2) + (C3/4) + (1.5 x C4)

CH_4= C2 + (2 x C4) -CO_2

Being C2: individual concentration of acetic acid; C3: individual concentration of propionic acid and C4: individual concentration of butyric acid.

2.3.1 Calculations and statistical analysis

Fermentation kinetics were described according to the exponential model described by Krisnamoorthy et al. (1991) as:

$$Y = A(1 - e^{-c(t-L)})$$

where Y is gas production (mL/g DM) at time t, A is gas production from the insoluble fraction (mL/g DM), c is the gas production rate constant for fraction A (h−1) and L is the lag time prior to gas production (h).

The parameters A, c and L for each bottle were calculated using a non-linear regression procedure, which minimizes actual distances of data points to fitted curves by Marquardt's algorithm.

Data was subjected to analysis of variance using the mixed procedure considering the series as random effect and washing (W), hydrolysis (H) and the interaction (W x H) as fixed effects. Results are reported as least-squares means. Significant differences between treatments were declared at P < 0.05 using the Tukey's multiple-comparison test.

2.4 Short-Term In Vitro Batch Fermentation Trial

Samples were tested 3 in vitro series. In each series, ruminal fluid was collected from one multiparous Latxa ewe slaughtered for production purposes. Before slaughtering, ewes were fed a basal diet (80% meadow hay and 20% compound feed) for 3 weeks and had free access to fresh water and feed. Ruminal fluid was collected before the morning feeding and filtered through four layers of cheesecloth into a volumetric flask. Then, was diluted in culture medium in a 1:4 ratio (ruminal fluid and phosphate-bicarbonate buffer, respectively) under anaerobic conditions according to Menke et al. (1979).

2.5 Long-Term in Vitro Batch Fermenation Trial

Approximately 500 mg of grape stem samples were weighed into 125 mL serum bottles. Each sample was incubated with 50 mL of culture medium in triplicate, the bottles were crimp sealed and incubated at a constant temperature (39°C) in the incubator for 24 hours. Gas production was released at 2, 4, 6 and 22 hours post-inoculation to avoid the pressure in the bottle headspace exceeding 48 kPa, as suggested by Theodorou et al. (1994). After 24 hours of incubation, bottles were put in the fridge for 20 min to stop fermentation for subsequent sampling for short chain fatty acids (SCFA) and in vitro organic matter digestibility (IVOMD) determination.Long-Term In Vitro Batch Fermentation Trial

The animals, substrates and incubation procedures were the same as those described in the previous section. Approximately 1 g of each sample and 100 mL of culture medium was incubated for 96 hours at 39°C in glass bottles with an actual volume capacity of approximately 307 mL. The kinetics of gas production were recorded using the ANKOMRF gas production system (ANKOM Technology, Macedon, NY, USA). The release of the accumulated gas was done automatically (or manually in case of technical problems) through a valve attached to the module after setting the configuration parameters in the ANKOMRF software. The recording interval was set at 10 min. A threshold of 1 psi for automatic release of accumulated gases to avoid supersaturation of CO2 in the medium and a valve opening time of 500 mseg were set. Pressure values, corrected for the quantity of substrate organic matter (OM) incubated, were used to generate gas volume estimates.

3. Ingredients nutritional value characterization

3.1 Nutritional composition

Next table shows the nutritional composition of the selected prototypes for the feeding trials in WP3.

Table 1. Nutritional composition of the selected prototypes for feeding trials.

Response factors	Hydrolysed sample	Non-hydrolysed samples
Sugars (mg/g)	192,714	121,36
Polyphenols (mg/g)	29,638	30,55
Antioxidant activity (m/g)	33,486	35,34
Protein (%)	6,141	5,66
Ash (%)	13,682	7,42
ADF (%)	47,718	50,95
Lignin (%)	29,865	22,36
NDF (%)	47,347	53,18
Total VFA (mmol/100 mL)	5,351	5,45

- Hydrolysed sample: Grinded, hydrolysed with NaOH and frezee-dried sample (Values estimated by the stategraphics second experimental design (Deliverable D2.4)).
- Non-hydrolysed sample: Grinded,washed and frezee-dried sample

3.2 In vitro digestibility value

In vitro digestibility value of the prototypes is higher in the hydrolysed sample compared to the selected control (grinded and washed sample).

Table 2. In vitro dry matter digestibility value of the hydrolysed sample and the control.

Response factors	Hydrolysed sample	Non-hydrolysed samples
Digestibility (%)	41,984	21,94

- Hydrolysed sample: Grinded, hydrolysed with NaOH and frezee-dried sample (Values estimated by the stategraphics second experimental design (Deliverable D2.4)).
- Non-hydrolysed sample: Grinded,washed and frezee-dried sample

3.3 Ruminal fermentation kinetics and methane production

Table 3. Ruminal fermentation kinetics of the hydrolysed samples and the control prototype (mmol/100mL and CH4 mmol).

Samples	Total VFA	Acetic	Butiric	Isobutiric	Isovaleric	Propionic	Valeric	c2c3	c2c4c3	BCVFA	CH4
Hydrolysed sample	5,39	71,84	7,33	0,26	0,27	19,90	0,46	3,62	3,99	0,53	0,88
Non-hydrolysed sample	5,45	71,07	8,50	0,27	0,28	18,88	0,60	3,79	4,25	0,55	0,79

- Hydrolysed sample: Grinded, hydrolysed with NaOH and frezee-dried sample. Condition 3 in the second experimental design (Deliverable D2.1)
- Non-hydrolysed sample: Grinded,washed and frezee-dried sample

4. Feeding strategy

Two concentrates will be formulated to be used in WP3. One of the concentrates will be a commercial one commonly used to feed dairy Latxa ewes at the beginning of lactation (CTR). The other concentrate will be

formulated to contain 10 % grape stems in its composition. The composition of both concentrates can be found in Table 4.

Table 4. Ingredients of the concentrates containing grape stems (EXP) and control (CTR).

Ingredients	CTR	EXP
Barley	5	20
Oat	53	28
Corn	10	10
Rapeseed meal	21	21
Rapeseed oil	5	5
Molasses	3	3
Grape stems		10
VIT-MIN	3	3

5. General conclusions

Hydrolysing the grape stems improves its digestibility without altering the fermentation pattern and total methane output. Hydrolysing the grape stems also increases ash and lignin content and reduces the NDF and sugar content.

As much as 10% grape stems can be formulated in the concentrate without imparing its nutritional value.

6. References

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