CIHEAM

FEDNA
Fundación Española para el Desarrollo de la Nutrición Animal

Thèse / *Thesis*

requise pour
l'obtention du Titre

submitted
for the Degree of

Master of Science

LACTATIONAL, DIGESTIVE AND METABOLIC EFFECTS OF SUPPLEMENTING DAIRY EWES WITH BARLEY β-GLUCANS

Neus TORRENT AMPLE

Zaragoza, Octubre 2015

Institut Agronomique Méditerranéen de
Mediterranean Agronomic Institut of
Zaragoza

Université de Zaragoza
University of Zaragoza

Fondation Espagnole pour le Développement
de la Nutrition Animale
Spanish Foundation for the Development of Animal Nutrition

INTERNATIONAL CENTRE FOR ADVANCED MEDITERRANEAN AGRONOMIC STUDIES MEDITERRANEAN AGRONOMIC INSTITUTE OF ZARAGOZA

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Agraïments

Vull donar les gràcies al Dr. Gerardo Caja per la seua motivació i entusiasme contagiós per l'aprenentatge.

Agraeixo al Dr. Ahmed Salama, a la Dr. Elena Albanell, al Dr. Xavier Such, a la Dr. Andreia Castro i a Sandra Contreras el "suport tècnic i moral" rebut.

M'agradaria agrair a Paco Batlle i al Dr. Ignacio Romagosa la seva implicació en aquest treball, així com a la Dr. Marion Boutinaud el oferir-me una meravellosa i enriquidora estada formativa.

Finalment, i no menys important, vull donar les gràcies als meus companys Wellington, Samantha, Alí, Elena i, sobretot Nabil, per la seva ajuda moral incondicional.

Abstract

Lactational, digestive and metabolic effects of supplementing dairy ewes with barley β-glucans: A set of 5 conventional and new varieties of barley with different features (waxyhull-less, waxy-hulled, non-waxyhulled) were evaluated by analyzing its nutritive composition, β-glucans content and *in situ* rumen degradability for dairy ruminants. The working hypothesis was that new varieties may have higher nutritive value and functional properties (i.e., lactogenic) for livestock. Barley waxy varieties showed higher crude protein (+2.3% units) and β-glucans (+2.6% units) contents than non-waxy varieties, whereas hull-less varieties had less crude fiber content (−2.1% units) and higher energy value (4 to 7% UFL), according to INRA (2007) and PrevAlim (v.3.23) than the hulled varieties. With regard to the rumen degradability, all the barley varieties had a similar and high DM, CP and β-glucans degradability (on average, 93.8 ± 1.6, 97.6 ± 0.3 and 100.0 ± 0.3%, respectively), being 85% of components immediately soluble. In a second experiment, a commercial barley β-glucans extract (Glucagel) was used, to evaluate the *in vivo* effects of β-glucans supplementation in the performance and metabolic profile of lactating dairy ewes at the short-term. With this aim, a new design of metabolic cages specially conceived for lactating dairy ewes was developed. Five Lacaune lactating dairy ewes (66.7 ± 2.6 kg BW; 202 ± 22 DIM and 1.58 ± 0.12 kg/d milk yield) were allocated in the metabolic boxes and submitted consecutively to 2 dietary treatments (C, control low in β-glucans; BG, β-glucans supplementation at a rate of 1.62 g/kg metabolic BW) during 10 d (C, d1 to 5; BG, d6 to 10). No changes in milk yield, milk composition and digestibility were detected for BG, but a higher feed efficiency (BG vs. C., 1.69 vs.1.77 kg DM intake/ kg ECM) with a 6% reduction in feed intake were observed. Plasma cholesterol and albumin tended to be lower in BG vs. C ewes, whereas plasmatic glucose and NEFA did not change. Metabolomic analyses (600 MHz ^{1}H NMR spectroscopy) showed higher β-glucose in plasma of BG ewes, when compared to C, which was supported by specific dynamic ELISA analysis of β-glucans (Fungitell), indicating that they left the rumen not fully degraded and that were absorbed. On the other hand, less carnitine, lecithin, creatine and 3-metylhistidine in milk were observed in the BG ewes, agreeing with a better nutritive stage. Moreover, less acetic and higher hippuric acids were found in the urine of BG vs. C ewes. In conclusion, new waxy barley varieties, high in β-glucans, showed higher nutritive value and positive metabolic effects at the short-term, recommending their use in dairy ruminants. Further research is needed a mid and long-terms.

Key words: Lactating ewes, barley, β-glucans, plasma, milk, urine, metabolomic analyses.

Resumen

Efectos de la suplementación con β-glucanos de cebada sobre la producción de leche y parámetros digestivos y metabólicos en ovejas lecheras: Cinco convencionales y nuevas variedades de cebada con diferentes características (waxy-desnudas, waxy-vestidas, no-waxy-vestidas) fueron evaluadas mediante el análisis de su composición nutritiva, contenido en β-glucanos y degradabilidad ruminal *in situ*. La hipótesis de trabajo fue que las nuevas variedades de cebada podrían tener mayor valor nutritivo y propiedades funcionales para el ganado. Las variedades waxy presentaron un mayor contenido en PB (+2.3%) y β-glucanos (+2.6%) respecto las variedades convencionales, mientras que las variedades desnudas tuvieron un contenido menor en FB (-2.1%) y mayor valor energético (de 4 a 7% UFL) respecto las variedades vestidas, según INRA (2007) y PrevAlim (v.3.23). Todas la variedades tuvieron una degradación de MS, PB y β-glucanos alta y similar (93.8 ± 1.6, 97.6 ± 0.3 and 100.0 ± 0.3%, de media, respectivamente), siendo los componentes inmediatamente solubles el 85%. En un segundo experimento, un extracto comercial de β-glucanos de cebada (Glucagel) fue usado para evaluar los efectos *in vivo* de la suplementación de β-glucanos en ovejas lecheras lactantes a corto plazo. Con este objetivo, fue desarrollado un nuevo diseño de cajas metabólicas especialmente concebido para ovejas lecheras lactantes. Cinco ovejas lecheras lactantes Laucane (66.7 ± 2.6 kg PV; 202 ± 22 DEL and 1.58 ± 0.12 kg/d leche) fueron alojadas en las cajas metabólicas y sometidas a 2 tratamientos alimentarios consecutivamente (C, control bajo en β-glucanos; BG, suplementación con β-glucanos a una dosis de 1.62 g/kg de PV metabólico) durante 10 d (C, d1 a 5; BG, d6 a 10). No se detectaron cambios por BG en producción y composición de leche y digestibilidad, pero se observó una mayor eficiencia alimentaria con una reducción de la ingestión de alimento del 6%. El colesterol plasmático y la albúmina tendieron a ser menores en las ovejas BG vs. C, mientras que la glucosa plasmática y los NEFA no cambiaron. Los análisis de metabolómica (600 MHz ^{1}H NMR espectroscopia) mostraron un mayor contenido de β-glucosa en plasma de las ovejas BG, lo cual fue apoyado por los análisis ELISA de dinámica específica de β-glucanos (Fungitell), indicando que estos abandonan el rumen no completamente degradados y que fueron absorbidos. Por otra parte, se observó menos carnitina, lecitina, creatina y 3-metylhistidina en leche en las ovejas BG, indicando un mejor estado nutritivo. Además, de menos ácido acético y más ácido hipúrico en la orina. En conclusión, las nuevas variedades waxy de cebadas, altas en β-glucanos, mostraron un valor nutritivo mayor y efectos metabólicos positivos a corto plazo, siendo recomendado su uso en rumiantes lecheros. Más investigación en condiciones a medio y largo plazo es necesaria.

Palabras clave: Ovejas lecheras, cebada, β-glucanos, plasma, leche, orina, metabolomica.

Résumé

Effets de la suplementation avec β-glucanes de l'orge sur la production du lait et les parametres digestifs y metaboliques chez les brebis laitières: Un ensemble de 5 variétés conventionnelles et nouvelles de l'orge avec des caractéristiques différentes (waxy sans coque, waxy avec coque, no-waxy avec coque) ont été évalués par l'analyse de leur composition nutritive, le contenu β-glucanes et la dégradabilité ruminale *in situ* chez les ruminants laitiers. L'hypothèse de travail était que les nouvelles variétés peuvent avoir une valeur nutritive et des propriétés fonctionnelles (ex. lactogènes) plus élevée pour le bétail. Les variétés waxy d'orge ont montré un contenu supérieur de protéines brutes (+ 2.3%) et des β-glucanes (+ 2.6%) que les variétés conventionnelles, tandis que les variétés sans coque avaient moins teneur en cellulose brute (-2.1%) et une valeur énergétique plus élevée (4 à 7% UFL), selon l'INRA (2007) et PrevAlim (v.3.23) que les variétés avec coque. En ce qui concerne la dégradabilité ruminale, toutes les variétés d'orge avaient un similaire et haute MS, PB et dégradabilité de β-glucanes (en moyenne, 93.8 ± 1.6, 97.6 ± 0.3 et 100.0 ± 0.3%, respectivement), étant 85% de composants immédiatement soluble. Dans une deuxième expérience, un extrait d'orge commerciale β-glucanes (Glucagel) a été utilisé, pour évaluer les effets *in vivo* de β-glucanes supplémentation dans la performance et le profil métabolique de brebis allaitantes journal à court terme. Dans une deuxième expérience, un extrait de β-glucanes d'orge commerciale (Glucagel) a été utilisé, pour évaluer in vivo les effets de la supplémentation en β-glucanes sur la performance et le profil métabolique de brebis laitières allaitantes à court terme. Dans ce but, une nouvelle conception de cages métaboliques spécialement conçus pour les brebis laitières en lactation a été développée. Cinq brebis laitières en lactation de la Lacaune (66.7 ± 2.6 kg PV; 202 ± 22 jour en production et 1.58 ± 0.12 kg/j rendement de lait) ont été attribuées dans les cages métaboliques et soumises consécutivement à 2 traitements alimentaires (C : contrôle, teneur faible en β-glucanes; BG : supplémentation en β-glucanes à 1.62 g/kg de poids métabolique) pendant 10 jours (C, d1 à 5 ; BG, d6 à 10). Aucun changement dans la production laitière, la composition du lait et la digestibilité n'a été détectés pour le traitement BG, mais une efficacité alimentaire supérieure a été observée avec une réduction de 6% de l'ingestion. Le cholestérol plasmatique et de l'albumine avaient tendance à être plus faible chez les ovins BG vs. C, tandis que le glucose plasmatique et NEFA n'étaient pas changées. Les analyses métabolomiques (600 MHz ^{1}H NMR spectroscopie) ont montré une teneur plus élevée en β-glucose dans le plasma des brebis BG, qui a été soutenu par les analyses ELISA de dynamique spécifiques de β-glucanes (Fungitell), indiquant que ceux-ci quittent le rumen sans être complètement dégradés et qu'ils ont été absorbés. En revanche, moins de la carnitine, la lécithine, la créatine et 3-metylhistidine dans le lait ont été observés chez les brebis BG, en accord avec un meilleur stade nutritif. D'autre part, il a été observé moins de carnitine, lécithine, créatine et de 3-metylhistidine dans le lait de brebis BG, ce qui indique un meilleur état nutritionnel. En outre, il a été observé moins d'acide acétique et d'acide hippurique dans l'urine. En conclusion, les nouvelles variétés waxy d'orge, riche en β-glucanes ont montré une plus grande valeur nutritionnelle et des effets métaboliques positifs à court terme, étant recommandé pour une utilisation chez les ruminants laitiers. Autres recherches de ces effets sur le moyen et long terme est nécessaire.

Mots-clés: brebis laitières, orge, β-glucane, lait, urine, plasma, analyses métabolomiques.

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List of abbreviations

AUC	Area under the curve
d_a	Apparent digestibility
ADF	Acid detergent fiber
BW	Body weight
CCK	Cholecystokinin
CBo	Content of carbohydrates
CE	Crude energy
CF	Crude fiber
CP	Crude protein
CPo	Content of crude protein
dE	Digestibility of the energy
DM	Dry matter
DMI	Dry matter intake
DNF	Detergent neutro fiber
DP3	Trisaccharide
DP4	Tetrasaccharide
dr	Digestibility of the amino acids from the feed in the intestine
ECM	Energy corrected milk
ED	Effective degradability
eDT	Theoric degradability of the feed in the rumen
EE	Ether extracts
FDA	Food and drug administration
FNE	Free nitrogen extracts
FOM	Fermentable organic matter
GGT	Gamma-glutamil transpeptidase
GH	Growth hormone
HR-MRS	High resolution magnetic resonance spectroscopy
I-NSP	Insoluble non-starch polysaccharides
LDL	Low density lipoprotein
LV	Latent variables
ME	Metabolic energy
MM	Mineral matter

NE_L	Net energy for milk
NEFA	Non-esterified fatty acid
NL	Alimentary level
NMR	Nuclear magnetic resonance
NSP	Non-starch polysaccharides
OM	Organic matter
OMd	Digestibility of the organic matter
PCA	Principal components analyses
PC	Principal component
PDIA	Protein digested in the small intestine supplied by rumen undegradable protein from the feed
PDIE	Protein digested in the small intestine supplied by microbial protein from rumen-fermented OM
PDIM	Digested protein in the small intestine supplied by the ruminal micro biome.
PDIME	Digested protein in the small intestine supplied by the ruminal micro biome limited by the fermentable energy
PDIMN	Digested protein in the small intestine supplied by the ruminal micro biome limited by the degradable nitrogen
PDIN	Protein digested in the small intestine supplied by microbial protein from ruminal degradable protein
pDT	Theoric degradability of the crude protein
PLS-DA	Partial least Squares-Discriminant Analysis
PRL	Prolactin
PYY	Peptide YY
RUP	Rumen undegradable protein
S-NSP	Soluble non-starch polysaccharides
TD	Total degradability
UFL	Feed units for lactation

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I. INTRODUCTION

I. INTRODUCTION

I.1. History of the barley and current situation of the crop

There is considerable historical and archaeological evidence documenting the role of barley as a sustaining food source in the evolution of humankind. Newman and Newman (2008) reviewed the barley-human joint evolution in the book "Barley for food and health: science, technology and products". It is generally accepted as a fact that the transformation of "wild barley" into "cultivated barley" crop occurred over many millennia. However, there is a wide controversy about the exact site(s) of where these events occurred.

The theory that has been widely accepted is that barley was first domesticated in the Fertile Crescent in the Near East (Israel, northern Syria, southern Turkey, eastern Iraq and western Iran in present day).

Considerable evidences indicate that agriculture and barley use spread from the Fertile Crescent, following Neolithic migrations and agricultural trade routes to North Africa, European continent and many parts of Asia (China, Japan, India). Nevertheless, there other theories propose Ethiopia, Tibet-Nepal or western Mediterranean region for the site of barley domestication. Evidences presented over the past 20 yr suggest a multicentric origin hypothesis for barley.

Wild barley was an original food utilized by humans and a key in the development of many civilizations (i.e., Egyptian, Greek, Arabian and Roman) in which barley was used for food, to make drinks and medicines, and for offerings to gods. As other food grains became more abundant, barley was relegated to the status of "poor man's bread".

Grant (2014) reported that barley allowed to the human population to shift up the Tibetan Plateau, because barley cultivation provided people sustained food supplies during winter. Before this, the humankind just couldn't hack it above 2500 m high, because the weather was just too cold and frosty for most grain crops.

The story of barley in the American continent is short and its main use was beer and animal feed production.

Currently, the total land area devoted to grow barley has been reduced significantly over the past 25 to 30 yr due to the increased demand for others cereals, although the total

production of barley grain increased. This fact may be attributed to the improved genotypes and the modern cultural practices.

According to FAO (2015), barley is at nowadays one of the 7 internationally grown cereal grains, currently ranking fourth in world production behind maize, wheat and rice. In 2013, barley world production was almost 144 Mt, which represents 5% cereal world production, approximately. The principal producers are Canada and the European continent (i.e., Russia, Germany, France and Spain in order of importance).

With regard to the barley trade, the main exporter countries in 2011 were France, Australia, and the principal importers were Saudi Arabia and China (FAO, 2015).

Newman and Newman (2008) listed the main uses of the barley at nowadays, which are: animal feed (60%), malt (30%), seeds (7%) and human food (3%). In terms of human consumption, barley gradually became associated almost exclusively with malt and brewing, but barley, especially pearled barley, continues to be a major dietary constituent for humans in parts of Asia and North Africa.

According to FEDNA (2015), barley is the main cereal used in the Spanishfeed industry, the total quantity consumed being near 3.5 Mt/yr, which suppose a third of the total cereals consumed. There are two types of barley in the Spanish market, 2 rows and 6 rows barley, according to the alignment of the grains in the ear.

I.2. Description and composition of thebarley grain

FEDNA (2015) describe in detail the barley grain, which is composed by the germ (3.5%), the pericarp (18%) and the endosperm (78.5%), as shown in **Figure 1**. The germ is rich in sugars and the pericarp is lignified and abrasive because there is silica in the epidermis. The aleurone layer, which is located between the pericarp and the endosperm, is rich in fiber, protein, triglycerides and sugars. The endosperm hasa flour consistency and a protein matrixwhere the starch granules are found.

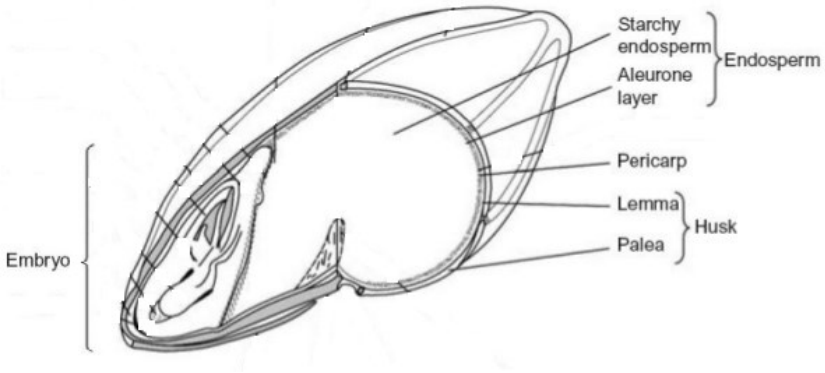

Figure 1. Diagram of the barley grain showing different organs and tissues (Modified from Harris and Fincher, 2009).

The FEDNA analyses of Spanish barley samples during the last 6 yr show a trend towards the reduction of protein content and an increase of the starch content, most probably as a consequence of using lower N fertilization. Furthermore, there are big differences according to the climatology and area of precedence.

Large biochemical variations occurred during the maturation of cereal grains as described in detail by Colonna et al. (1995). This is the case of the starch to amylose ratio, which increases throughout grain growth. So, stage of maturation is essential to determine the anatomic and biochemical composition of the grain.

In barley, the starch represents between 55-69% of the grain weight, depending on hull content. Starch is composed by:

- Amylose, a lineal homo-polymer of glucose linked by α(1→4) bonds, and
- Amylopectin, a branched homo-polymer of glucose linked by α(1→4) and α(1→6)bonds, which are disposed forming semi-crystalline granules.

In conventional barley varieties the amylose represents about 25% of the total starch, but currently there are new varieties with large variations in the amylose-amylopectin ratio.

The protein content of barley ranges around 11% but it can vary according to many variation factors. It should be stressed that the Kjeldahl factor for cereals crude protein is 5.88 as a consequence of its composition in aminoacids (Müller, 2014). Approximately, the 50% of them are glutelins, and the rest, mainly, are prolamins with specific names according to the cereal (hordein in barley) and albumins.

Newman and Newman (2008) indicate that the non-starch polysaccharides (**NSP**), like the total dietary fiber, are not digested by the mammalian digestive enzymes, thus providing little or no-digestible energy, but they are valuable diet constituents for other reasons. The main components of the NSP of the cereals are shown in **Table 1**. Of all the components of total fiber dietary in barley, the β-glucans are probably the most important in terms of human diet and health benefits.

Table 1. Composition of the NSP of the cereals grain (Colonna et al., 1995).

Item, % DM basis	Grain component	
	Endosperm	Husk
Pectin	0-0.5	0-0.5
Cellulose	3-5	30-35
Hemicellulose	80-85	45-50
Glycoproteins	10	5-10
Lignin and phenolic acids	5	15

I.3. β-glucans

Distribution of β-glucans in the grain

Amounts of NSP components vary in concentration in the different tissues of the cereal kernel. In barley, arabinoxylan and β-glucans are predominant integral components of the cell wall structure in both aleurone layer and starchy endosperm tissue, but vary inversely in their ratios in the two tissues, as shown in **Table 2**.

Scheller and Ulvskov (2010) reported that β-glucans are abundant in the *Poaceae* family (grasses) where they play a key role in cell expansion, being their amount growth-stage dependent.

Izydorczyk and Dexter (2008) indicated a heterogeneous distribution of β-glucans within barley endosperm which is related to the own content of β-glucans in the grain. In barleys with low β-glucans content, levels are relatively higher in the subaleurone layer region than in the endosperm, whereas barleys with high average content of β-glucans contained more β-glucans in the endosperm than in the subaleurone layer.

Table 2. Composition of the hemicelluloses in the cell wall of the barley grain (Modified from Colonna et al., 1995).

Item, % DM basis	Arabinoxylan	Xylan	β-glucans
Endosperm	9	10	79
Aleurone layer	23	44	29

Regarding oats and wheat, Lazaridou and Biliaderis (2007) and Newman and Newman (2008), indicated that the highest β-glucan concentration is found in the outer portion of the kernel (subaleurone layer).

Values of β-glucans in the main commercial cereals range between: barley, 2.5-11.3%; oat, 2.2-7.8%; rye, 1.2-2.0%; and, wheat, 0.4-1.4% (Izydorczyk and Dexter, 2008).

Factors affecting β-glucans content

Content of β-glucans vary widely in barley according to genetic (starch types and hull content) and environmental factors (Newman and Newman, 2008), the β-glucans being under genetic control, but their concentration is often modified by the environment. Most especially, hot and dry conditions during kernel maturation produce increased levels of β-glucans. On the other hand, under high moisture conditions, kernel β-glucans decreased.

Genetic background is the most important factor of final β-glucan content of the barley kernel. Holtekjolen et al. (2006) compared the typical and atypical barley amylose varieties and determined that the atypical amylose varieties had a significantly higher concentration of β-glucans, protein and soluble fiber, than the normal amylose ones. Newman and Newman (2008) reach the same conclusion and reported that in barley waxy varieties, which are rich in amylopectin, the change of amylose to amylopectin had a significant effect on increasing β-glucans content. In addition, similar increases of β-glucans were reported in high-amylose varieties.

The β-glucans content is influenced by the hull content of the grain. With the removal of the hull, components of the aleurone, endosperm and embryo increase in relative proportions. Holtekjolen et al.(2006) reported that the hulled varieties have higher content of total NSP than do the hull-less, probably due to the contribution of cellulose

and arabinoxylans from the hull. The hull-less varieties have lower contents of insoluble NSP (**I-NSP**) than the hulled varieties. So, this might indicate that there was more I-NSP in the hull than in the kernel. In the other hand, the highest amount of soluble NSP (**S-NSP**) was found in the hull-less varieties, and the samples with the lowest amount of I-NSP were highest in S-NSP.

Holtekjolen et al. (2006) reported that the main S-NSP component in barley was β-glucans, which was also indicated by a strong correlation between β-glucans and S-NSP and protein. On the contrary, β-glucans content was significantly and negatively related to starch, cellulose and arabinoxylans contents.

In conclusion, the hull-less and atypical amylose varieties have higher contents of β-glucans, S-NSP, protein and lower starch content.

Molecular structure

Brennan and Cleary (2005) and Izydorczyk and Dexter (2008) described the molecular structure of β-glucans as linear homopolymers of D-glucopyranosyl, mostly linked via 2 or 3 consecutive β-(1→4)bonds (70% of total linkages) that are separated by a single β-(1→3)bond (30% of total linkages), as shown in **Figure 2**.

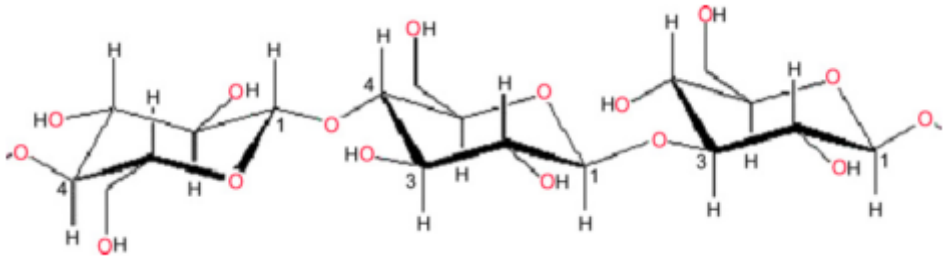

Figure 2. Structure of (1→3)β-glucans with ramifications in (1→4) according to Mantovani et al. (2008).

The molecular features of β-glucans are usually determined from the analysis of the oligomers obtained by digestion of the polymers with a specific (1→3, 1→4)-β-D-glucan hydrolase, called lichenase. The enzyme lichenase releases trisaccharide (**DP3**) and tetrasaccharide (**DP4**) units (see **Figure 3**), accounting for 90-95% of the total oligosaccharides and the longer oligosaccharides only accounting for 5-10% of the total.

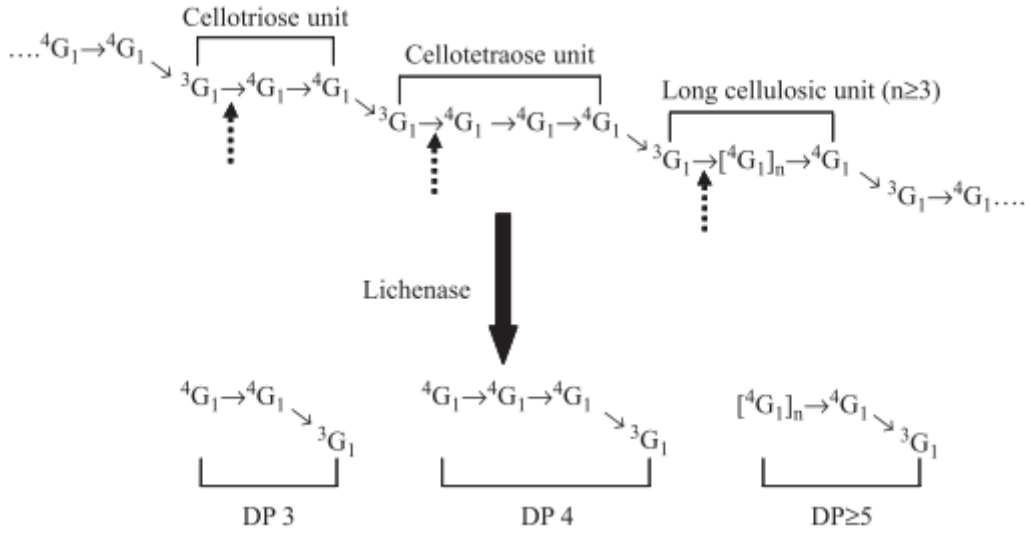

Figure 3. General structure of cereals β-glucans and its debranching process with the enzyme lichenase (Lazaridou and Biliaderis, 2007).

β-glucans from different genera of cereals share the same general molecular structure, butthe different species have differences in their structural features such as the ratio of β-(1→4)/β-(1→3) linkages, presence and amount of long cellulose-like fragments, and molecular size, which were proposed for Izydorczyk and Dexter(2008) as a tool to difference between them.

The ratio DP3:DP4 is considered to be a fingerprint of the structure of cereal β-glucans. Literature data indicate that there also are some differences in the ratio of DP3:DP4 within the same genera, which is attributed to genotypic and environmental factors (Lazaridou and Biliaderis, 2007), or to differences on the kernel tissues (Izydorczyk and Dexter, 2008).

Lazaridou and Biliaderis (2007) determined the relative amount of DP3 and DP4 in barley β-glucans, which were 52-69% and 25-33%, respectively. So, the DP3:DP4 ratio was 1.8-3.5. But, β-glucans in barley waxy varieties have a higher DP3:DP4 ratio compared to those from non-waxy cultivars. Moreover, the DP3:DP4 ratios in β-glucans from oats and barley aleurone tissues are higher than those from starchy endosperm tissues.

Physical characteristics of β-glucans

Solubility

The β-glucans are soluble components and have the capacity to make viscous solutions and this physical propriety is related to the physiological effects which are beneficial for health.

The solubility of the β-glucan is attributed to their molecular features as described by Lazaridou and Biliaderis (2007) and Izydorczyk and Dexter (2008).The β-(1→3) linkages break up the regularity of the β-(1→4) linkages sequence, which have a tendency for interchain aggregation (and hence lower solubility) via strong hydrogen bonds, making the molecule more soluble. On the other hand, it has been reported that at least 3 consecutive cellotriosyl residues form a helical segments, which may constitute a conformationally stable motif, making the molecule lower soluble. In accordance with this aggregation mechanisms,the higher amounts of cellotriosyl fragments and the lower ratio of (1→4):(1→3) linkages, might explain the solubility differences between cereal β-glucans.

Starch types are also related to the solubility of β-glucan. According to Izydorczyk and Dexter (2008), among the hull-less barley genotypes with altered starch characteristics and increased content of total β-glucans, the waxy types show slightly higher solubility of β-glucans than the high amylose genotypes.

Viscosity

β-glucans form solutions with high viscosity, as a consequence to the ability of their chain to occupylarge hydrodynamic volumes. Izydorczyk and Dexter (2008) and Lazaridou and Biliaderis (2007) reported that polymer concentration and its molecular weight affect this physical characteristic, and suggest that with increasing molecular weight and/or concentration of polysaccharide, there is an increase in viscosity.

Other sources of β-glucans

There are other natural sources of β-glucans like fungi (mainly ascomycetes and basidiomycetes), yeast, seaweed, bacteria and lichens. In these organisms, β-glucans are linear homo-polymers of D-glucose linked in the β(1→3) position, like cereal β-glucans, but with side branches of glucose of various sizes linked as β(1→6), which

occur at different intervals along the central backbone. **Figure 4** shows this molecular structure.

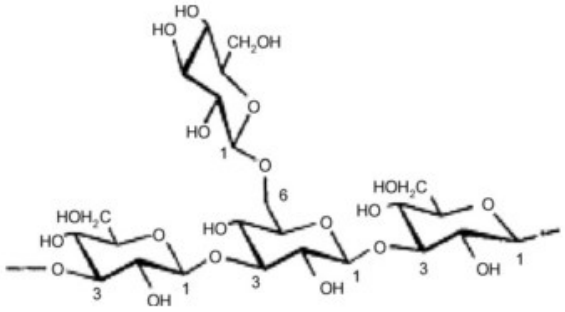

Figure 4. Structure of fungi, yeast, seaweed, bacteria and lichen (1→3)β-glucans with branches in (1→6) (Mantovani et al., 2008).

Mantovani et al. (2008) located it in the intermediate layer of the cell wall, adjacent to the plasma membrane, with the function of maintaining the rigidity and shape of the cell.

I.4. State-of-the-art of the use of β-glucans in animals

There are many plant extracts traditionally used by their pharmacological properties and some classically recognized to be lactogenic, such as barley (beer), apple, citrus, and sugar beet. Sawadogo et al. (1988) observed that, the fractions with enriched lactogenic activity contain a large quantity of pectins, polygalacturonic acid and β-glucans.

Sawadogo et al. (1988) showed that those substances markedly stimulated blood prolactin (**PRL**), growth hormone (**GH**) and cortisol secretion, when injected intravenously to ewes, and induced casein synthesis in the mammary gland when administered orally to rats. Martinet et al. (1999) obtained the same results supplementing lyophilized beer administered by the same way in ewes and rats. Moreover, *in vitro* studies have shown that extracts from lactogenic plants induce β-casein synthesis in epithelial cells of rat mammary glands.

Eslime et al. (2008) found the same effects after supplementing pituitary cells of rats with pectic acid, which stimulate the secretion of PRL, GH, luteotropin hormone and β-endorphin.

The action mechanisms of pectins on PRL production by anterior pituitary cells is not well understood, but the observations of Eslime et al. (2008) suggest that pectic acid effects are likely exerted directly at lactotropic level and do not depend on another complex interactions. Consequently, they hypothesized that pectic substances have some structural homologies with the extra cellular matrix of mammalian cells and, the active compounds of plant extracts, might affect the cellular secretion by binding to cell membrane receptors.

I.5. Health beneficial effects of β-glucans

In 2006, the Food and Drug Administration (FDA) of the USA approved a rule that allows food containing barley to claim that they reduce the risk of coronary heart disease. This mention is part of a recommended diet low in saturated fat and cholesterol, and high in vegetables, fruit and grains (FDA, 2015).Specifically, the FDA claim refers to foods containingmore than 0.75 g of β-glucans per serving, with the aim of reaching an intake for humans of 3 g/d of soluble fiber.

Plasmatic cholesterol and glucemic index reduction

The mechanism by which a soluble fiber, such as β-glucan, exerts hypocholesterolemic and hypoglycemic effects is still debated, but the most common hypothesis is based on increasing intestinal lumen viscosity. Wood (2007) suggested that the viscosity of β-glucan could account for 70-96% of the changes in plasma glucose and insulin response.

According to Wood (2007), Regand et al. (2011) and Cloetens et al. (2012), the action mechanism of β-glucans are related to the increased digesta viscosity, which causes a delayed gastric emptying after the intake of liquid or solid meals, and leads to the formation of an unstirred water layer adjacent to the gastric mucosa.That causes a slower digestion and absorption of nutrients, due to a decreased enzymatic activity, such as pancreatic amylase or bile acids, and lower mucosal absorption. Moreover, Regand et al. (2011) suggested that the viscosity of β-glucan plays a key role in reducing starch digestibility by limiting the amount of water available for starch hydration, because the greater affinity of β-glucans for hydration.

Finally, Cloetens et al. (2012) reported that the slower appearance of glucose in plasma, after the consumption of a meal supplemented with β-glucans, is the result of an extended insulin secretion and an inhibited mobilization of endogenous glucose.

Immune systemresponse improvement

Hong et al. (2004) consider that β-glucans are response modifiers that function as immunostimulants against infectious diseases and cancer.

When β-glucans are given orally, they are taken up by gastrointestinal macrophages mediated by Dectin-1 receptor. Hong et al.(2004) suggested that macrophages shuttle β-glucans to spleen, lymph nodes and bone marrow. Nevertheless, Volman et al. (2008) questioned that because it has not been currently demonstrated. However, Rice et al. (2005) and Sandvik et al. (2007) showed that β-glucans can be detected in plasma after oral administration and that hepatic uptake does not significantly contribute to the removal of β-glucans from the systemic circulation. Plasma macrophages break down the β-glucans into smaller fragments, which are released into the medium and are taken by bone marrow granulocytes mediated by CR3 receptor, actuating like small biologically active fragments.

Infectious diseases

Novak (2008) affirms that the main effect of β-glucans consists on augmentation of phagocytosis and proliferative activities of professional phagocytes-granulocytes, monocytes, macrophages and dendritic cells, not only against the activator, but also against any present antigen.

Binding β-glucans to the macrophages triggers several interconnected processes including increased chemotaxis and migration, intracellular processes (respiratory burst and content and activity of enzymes) and signaling processes leading to activation of other phagocytes and initiating inflammation reactions.

Moreover, Volman et al. (2008) suggested that when β-glucans are orally given, they modulate the immune response of intestinal mucosa cells locally by mean of the Peyer's patches as well as by mean of intestinal intraepithelial lymphocytes.

Cancer

Once recruited from the bone marrow by an inflammatory stimulus, granulocytes could kill tumor cells. Hong et al. (2004) and Vetvicka et al. (2007) reported that oral β-glucans mediate inhibition of cancer growth and tumor regression. On other hand, Mantovani et al. (2008) have shown that fungal β-glucans can also act as chemopreventive agents by inhibiting the enzymes involved in the first activation stage of carcinogens. Moreover, Vetvicka et al. (2007) and Novak (2008) stated that β-glucans are used as an immunoadjuvant therapy for cancer, because they stimulate the hematopoiesis and reverse the myelosuppression.

Voluntary food intake reduction

Cloetens et al. (2012) reported several studies in humans where subjective satiety scores increased after consumption of β-glucans. Is considered that the satiety feeling triggered after β-glucans ingestion is caused by the viscous gel matrix formed in the gastrointestinal tract. This increases the viscosity of the chime after ingestion and delays the gastric emptying and increases the satiety by modifying the secretion of gut hormones regulating voluntary food intake. Cloetens et al. (2012) observed increases of peptide YY (**PYY**) and cholecystokinin (**CCK**) and a reduction of ghrelin after β-glucans supplementation which supported the increase in satiety.

I.6. Barley β-glucans applications in the food industry

Nowadays, for meeting the consumer demands, the food industry is developing new products with improved health and functional attributes, as well as organoleptic characteristics. Barley received attention for its fiber content and atypical amylose profile. Holtekjolen et al. (2006) recommended the hull-less and atypical amylose varieties for human consumption because of their desirable nutritive composition and high soluble fiber content (i.e., high β-glucans, S-NSP and protein, low starch).

Wheat is the ideal cereal for most human foods, but the substitution of part of wheat flour by barley, or enriching the food with a β-glucan extract, can provide more soluble dietary fiber to improve the health properties of the product, keeping its sensorial features and the acceptance of consumers.

Newman and Newman (2008) summarized the main effects of including barley in human foods (i.e., bread and pasta).

In the case of bread, the level of barley flour inclusion can range between 5-40%, the main differences found with regard to the control breads (100% wheat flour breads) being less loaf volume, increased darkness and reduction of crumb quality. These sensorial characteristics vary according to the starch type and β-glucans level of the barley flour used. Due to this, the ideal bread type for including barley flour is flatbread, like *tortitas* or *chapattis*. As it discussed above, the hypocholesterolemic effect of the β-glucans is related of their viscous capacity (i.e., the size of β-glucans molecules), so, to keep it in those breads it is necessary to reduce the mixing and fermentation time during the fabrication process.

In the pasta case, inclusion of barley flour produces dark color and less firm products, but the sensorial quality is acceptable for consumers. Like in the bread, those characteristics are affected by the barley variety, especially, when the waxy varieties are used and, as the products obtained had a different texture, it would be necessary to reduce the cook time.

All those products are considered functional foods according to the FDA 2006 regulation.

II. OBJECTIVES

II. OBJECTIVES

II.1. General objective

Barley, despite being the reference for net energy source in some feeding systems (i.e., INRA), it is not the most used cereal grain in animal feeding at an international level. Reasons for that, a part of their local availability, is its high fiber and lower energy content when compared to other cereals (i.e., wheat and maize) and the soft feces problem that produces in monogastrics, especially in the avian sector.

In the recent decades, barley geneticists have developed new varieties that overcome some of the above commented problems (i.e., decreased amylose-amylopectin ratio, hull-less and high β-glucans content). Nevertheless, few is known on the metabolic and performance effects of using those news barley varieties in animal feeding, especially in the case of ruminants.

The general aim of this M.Sc. Thesis is to estimate the nutritive value of new barley varieties for feeding dairy ruminants and to study the performances and metabolic effects of supplementing the diets of lactating dairy ruminants with barley β-glucans, to mimicker the new barley varieties high in β-glucans content.

II.2. Specific objectives

Experiment 1

Five different barley varieties were selected to evaluate the following items:

- Nutrient composition (DM, Ash, EE, CP, CF, DNF, ADF and MELN)
- Nutritive value according to the INRA (2007) and Prevalim 3.23
- β-glucans content
- Ruminal degradability (DM, CP, β-glucans)

Experiment 2

β-glucans were used as a supplement in the diet of lactating dairy ewes. The first objective, which allowed working with lactating dairy ewes, was:

- Develop a new model of metabolic cages able to be used for lactating dairy sheep.

After that, the following specific objectives were boarded:

- Lactational performances:
  - Milk yield and composition
- Nutritional:
  - Dry matter intake
  - Feed efficiency
  - Digestibility
- Metabolites in blood:
  - Albumin
  - Cholesterol
  - Gamma-glutamil transpeptidase
  - Urea
  - Glucose
  - NEFAs
  - β-glucans metabolites
- Metabolomics:
  - Plasma
  - Milk
  - Urine

III. MATERIALS AND METHODS

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III.1. Experiment 1

New varieties of barley (*Hordeum vulgare* L.),recently availablein the international market, and with different compositional features, were chosen and tested for their use in the nutrition oflactating ruminants. In this experiment, 5 different barley varieties obtained from Semillas Batlle (Bell-llochd'Urgell, Lleida, Spain) were characterized by analyzing its chemical composition, nutritive value, β-glucans content and *in situ* rumen degradability.

III.1.1. Barley varieties studied

Table 3 summarizes the main features of the barley varieties used. Two of them were conventional brewing and feeding varieties, whereas the other 3 were "waxy"(looking like wax) varieties with branched starch (amylopectin) and atypically low amylose/amylopectin ratio. Two varieties were "naked" (without hull or pales), and the other 3 were hulled (**Figure 5**).The naked or nude is an old recessive mutation which is not found in wild barley.

III.1.2. Nutrient composition

Barley grain samples were milled through a 1 mm screen and analyzed for dry matter (**DM**), ash, ether extracts (**EE**) and crude fiber (**CF**) according to the Weende method, using standard procedures as described by the Association of Official Analytical Chemists International (Ref. 934.01, 923.03, 920.39, AOAC International, 2005; and Ref. 962.09,AOAC International, 2010). The nitrogen-free extract and moisture were determined by difference, according to the Weende method. Neutral detergent fiber (**NDF**) and acid detergent fiber (**ADF**) were determined sequentially according to the methods of VanSoest et al. (1991) by using the Ankom apparatus (Ankom Tech. Co., Fairport, NY, USA). For the NDF analysis, samples were treated with sodium sulphite and thermostable α-amylase, and corrected for ash. The Dumas method (968.06, AOAC International, 2003) with a Leco analyzer (Leco Corp., St. Joseph, MI, USA) was used

Table 3. Barley varieties description and characteristics of their cultivation (Batlle, 2015).

	Barley variety				
Item	Streif	Doña Pepa	BetaNud AD-1	BetaNud AD-2	BetaNud AD-3
Spike rows, n	2	6	6	2	2
Hull	Hulled	Hulled	Nude	Nude	Hulled
Edge color	Red	Light	Green	Light	-
Genotype	Conventional	Conventional	Waxy	Waxy	Waxy
B-glucans content	Low	Low	High	High	High
Land areas	All types	Unirrigated	Unirrigated	Irrigated and subdesert unirrigated land	Irrigated and subdesert unirrigated land
Soil traits	Clay	Clay	Clay	Clay	Clay
	pH 7.5-8	pH 7.5-8	pH 7.5-8	pH 7.5-8	pH 7.5-8
Sowing date	Nov-Feb	Oct-Dec	Nov-Dec	Nov-Dec	Nov-Dec
Seed dose, kg/ha	180-200	180-200	150-200	150-200	150-200
Harvest date	June	June	June	June	June
Yield, t/ha	3-5 to 8-9	2-6	2-5	2-7	2-8
Use	Brewery	Feed	-	-	-
Photo	1	2	5	3	4

for N determinations and crude protein (**CP**) was calculated as percentage of N × 6.25. All chemical analyses were performed in triplicate and expressed on a dry matter basis.

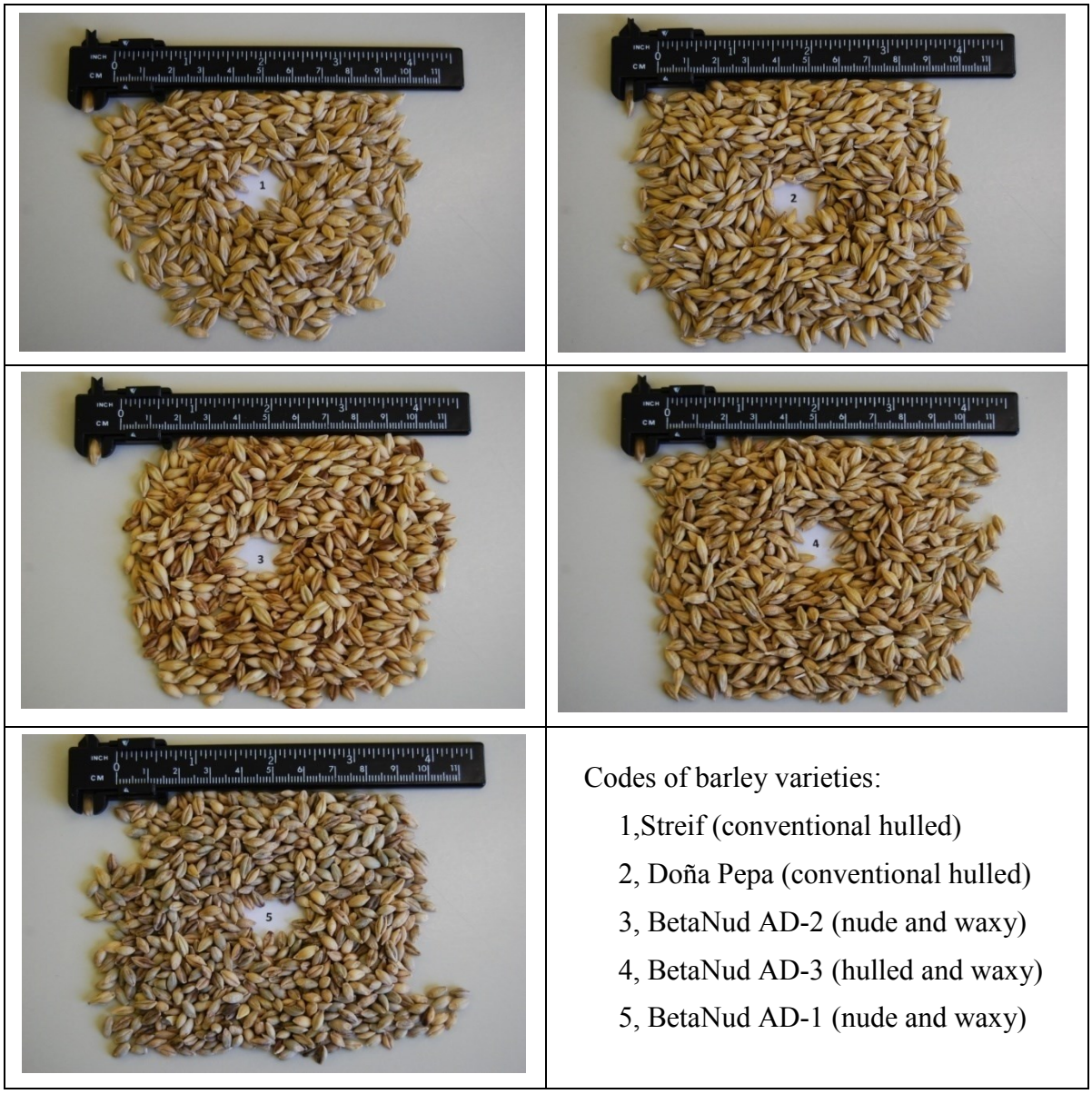

Figure 5. Macroscopic morphology of the grains of the barley varieties used.

III.1.3. Nutritive value

The software Prevalim 3.23 (INRA-UCACIG, Educagri éditions, Dijon, France) and the nutrient composition obtained previously by analysis were used to assess the nutritive value for lactation of each barley variety. Moreover, accurate nutritive values were also determined for lactating ruminants according to Demarquilly et al. (1995) and INRA (2007) methodology, being:

Energy value

Metabolizable energy (**ME**, kcal/kg): $ME = CE \times dE \times (ME/DE)$

Where,

CE is the crude energy of the feed $CE = 9.4\,EE + 5.65\,CP + 4.15\,(CF + FNE)$

dE is the digestibility of the energy with regard to the OMd

$dE = OMd - 3.94 + 0.0104\,CP + 0.0149\,EE + 0.0022N\,DF + 0.02\,MM$

q is the concentration of ME in the feed $q = ME/CE$

OMd is the digestibility of the organic matter $OMd = -1.54CF + 93.5$

ME/DE considers the energy loses because the gas formation and urine

$$\frac{ME}{DE} = (84.17 - 0.0099CBo - 0.0196CPo + 2.21\,NL)/100$$

CBo is the content of carbohydrates in g/kg OM

CPo is the content of CP in g/kg OM

NL is the feeding level, being considered as 1.2 in this case.

Net energy lactation (**NE_L**, kcal/kg): $NEL = ME \times kl$

Where,

kl is the efficiency of utilization of the metabolizable energy (**ME**) during the lactation $kl = 0.60 + 0.24\,(q - 0.57)$

And finally,

Feed units (unites fourragères) for lactation (**UFL**, units/kg): $ULF = NEL/1700$

Protein value

Protein digestible in the intestine as limited by N (g/kg): $PDIN = PDIA + PDIMN$

Protein digestible in the intestine as limited by energy (g/kg): $PDIE = PDIA + PDIME$

Where,

PDIA is the protein digested in the small intestine supplied by the rumen undegradable protein from the feed $PDIA = CP \times \big(1.11\ \times (1 - pDT)\big) \times dr$

pDT is the theoric degradability of the CP of the feed in the rumen. The values used were obtained from the *in situ* degradability test.

Dr is the digestibility of the amino acids from the feed in the intestine. In this case, the value used was 0.85 for all the varieties according to INRA (2007).

PDIM is the protein digested in the small intestine supplied by the ruminal micro biome. This can be limited by the degradable nitrogen (**PDIMN**) or by the fermentable energy (**PDIME**), being:

$PDIMN = CP \times \big(1 - 1.11(1 - pDT)\big) \times 0.9 \times 0.8 \times 0.8$

$PDIME = FOM \times 0.145 \times 0.8 \times 0.8$

FOM is the fermentable organic matter from the feed $FOM = (OMd \times OM) - EE - RUP$

RUP is the ruminal undegradable protein $RUP = CP \times (1.11 \times (1 - eDT))$

eDT is the theoretical degradability of the feed in the rumen obtained from the *in situ* degradability test.

III.1.4. B-glucans analysis

The β-glucan content was determined in the Departament de Química of the Universitat de Lleida (Lleida, Spain), using the enzymatic procedure of mixed-linkage β-glucan (Mixed-linkage β-glucan Kit, Megazyme, Wicklow, Ireland). Samples were milled at 1 mm of screen and the results were expressed on a dry matter basis.

III.1.5. Rumen degradability

Rumen incubation in situ

The rumen degradability was determined by the in situ technique with nylon bags using the method of Ørskov and McDonald (1979). Procedure was approved by the Ethical Committee of Animal and Human Experimentation of the UAB (CEEAH reference 14/2724).

One Holstein non-lactating dairy cow fitted with a rumen cannula was used. The cow was fed alfalfa hay *ad libitum* (DM, 87.3%; CP, 8.84%; CF, 25.02%; NDF, 35.92%; and ADF, 27.07%, DM basis) once-a-day (0800 h).

The nylon bag size was 11 × 5cm with a pore size of 50 μm (Ankom Technology, Ref. R510). Given the high degradability expected for the barley flour (1.0 mm sieve), a previous test was done to assess the quantity of initial sample necessary to obtain enough residues to allow the DM, CP and β-glucan determination and to asses if the sample/bag surface ratio conditioned the sample degradability. With this aim, nylon bags with 2, 4 and 6 g of sample of the Streif and BetaNud AD-2 barley varieties were incubated in the rumen for 72 h. No differences between samples sizes were reported in both barley varieties, concluding that 5 g of sample was adequate.

Bags were filled with 5 g of barley's sample and closed at approximately 1 cm of the top, being inserted in the rumen of the cow before feeding (0750 h) to be removed at 10 time points (2, 4, 6, 8, 10, 12, 16, 24, 48 and 72h) of incubation. Four bags were prepared for each barley variety and point-time. After removal from the rumen, the bags were immediately washed in cold water for 15 min (3 cycles of 5 min) using a portable washing machine (Jata, Traña-Matiena, Bizkaia, Spain). Four bags more were prepared per barley sample and only water washed (not incubated) and used for the h 0 time-point. Finally, the bags were dried at 55ºC for 24 h in an oven (Raypa, Terrassa, Barcelona, Spain), and then weighed.

The degradability study was repeated in a second serial in the following week. The bag residues of the same barley variety and time-point of both incubations serials were then mixed and analyzed as a single sample for DM, nitrogen, and β-glucans. The degradability at each time-point resulted as the value from the 8 bags (four bags per serial).

Bag residues analysis

After drying (DM, 55ºC) and weighing the incubated bag samples, the bag residues were analyzed for CP as previously indicated. Approximately 2 g of the bag residue for the 2400 h time-point of each barley variety were used to test the possible microbial contamination. Decontamination was made by using the freezing method (Zakraoui et al., 1995) and the decontaminated residues were analyzed in duplicatefor CP and compared to the intact rest of the bag residues.The CP values of the decontaminated and no decontaminated residues for the same variety were no different (P = 0.300). So, it was concluded that no significant microbial contamination ofthe bag residues was observed and no decontamination of the rest of bag residues was done.

The β-glucans content in the bag residues of composited samples from each barley variety at chosen time points (h 0, 6, 16 and 48) was analyzed using the previously described method.

Degradability modeling

The percentage of nutrients degraded (**D**, % DM basis) in the rumen was obtained from the exponential model developed by Ørskov and McDonald (1979):

$$D = a + b \cdot (1 - e^{-ct})$$

Where, e is the base of natural logarithms, the parameter 'a' represents the soluble and very rapidly degradable component and 'b' represents the insoluble but potentially degradable component, which is degradedat a constant fractional rate (c) per unit time.

Rumen effective degradability (**ED**) was then estimated by the following equation:

$$ED = a + (b \cdot c)/c + k$$

Where, 'k'refers to the fractional outflow rate of small particles from the rumen, being k = 0.06 for most lactation feeding conditions.

Rumen temperature and pH monitoring

Two wireless rumen boluses (model KB1001, Kahne, Auckland, New Zealand) were used for recording rumen pH and temperature. The boluses were calibrated before use according to the manufacturer instructions and configured for recording data every 15 min. Rumen content was sampled as close as possible to the bolus location at the same 11 time-points of incubation before indicated. Moreover, rumen pH was verified using a pH meter (pH25, Crison Instruments, Barcelona, Spain), to register the extreme pH variations.

III.2. Experiment 2

A commercial barley β-glucans extract was used to evaluate the *in vivo* effects of β-glucans supplementation in lactating diary ewes. With this aim, a new model of metabolic cages specially designed for lactating dairy ewes was developed.

III.2.1. Metabolic cages design

A new model of metabolic cages was own developed using different types of storage plastic boxes (Auer Packaging, Amerang, Germany). Boxes were modified and combined to build up a double cage with all the elements of a metabolic cage (i.e., feeders, urine-feces collector).

A collapsible bulk container made of PVC and with smooth double-walled walls (120 × 100 × 122 cm, GLT1210/125RB, Auer) was used for the main cage's body (**Figure 6**). Four wheels with breaks (10 cm diameter) and high supplements were disposed to allow the passage of the feces-urine boxes under the container. A gap of 0.5 cm between the front and the rear wheels was previewed for urine and feces drainage.

The container was divided by a middle wall following the longitudinal axis to produce 2 stalls of 44.5 × 100 cm. This wall was done with 4 independent pieces of double-walled extruded PVC (110 × 25 × 3.5 cm) allowing using different heights according to the animal size.

Six holes, framed with stainless steel u-shaped profiles, were done in the cage's body to give access to feeders (front, 20 × 30 cm), water bowls (sides, 20 × 30 cm) and milking (rear, 28 ×80 cm). The rear holes were closed with plastic guillotine doors easily removable for milking. A final hole was done in the posterior part of the cage's body floor and an ovine slat piece (40 × 80cm, Mik trapper, Ransbach-Baumbach, Germany) was inserted for allowing the passage of feces and urine, covering the rest of the floor with a plastic rug.

Feeders were made of plastic boxes with a window (30 × 40 × 32 cm, SLK 64-42, Auer) which were hanged in the cage's body using a u-shaped stainless steel profile. Water bowls (Suevia 125, Kinchheimam Neckar, Germany) were also hanged by u-shaped stainless steel profiles and connected by silicone tubes to individual plastic tanks (20 L) placed on the top of the cage's body.

Two pile-up plastic boxes (blind, 60 × 40 × 12 cm, EG64-12; and latticed, 60 × 40 × 17 cm, EO64-17/22, Auer) were used for individually collecting the urine and faeces, respectively. The feces box was arranged above the urine box, and covered with plastic mesh (0.5 ×0.5 cm) to allow the passage of urine and to retain the feces.

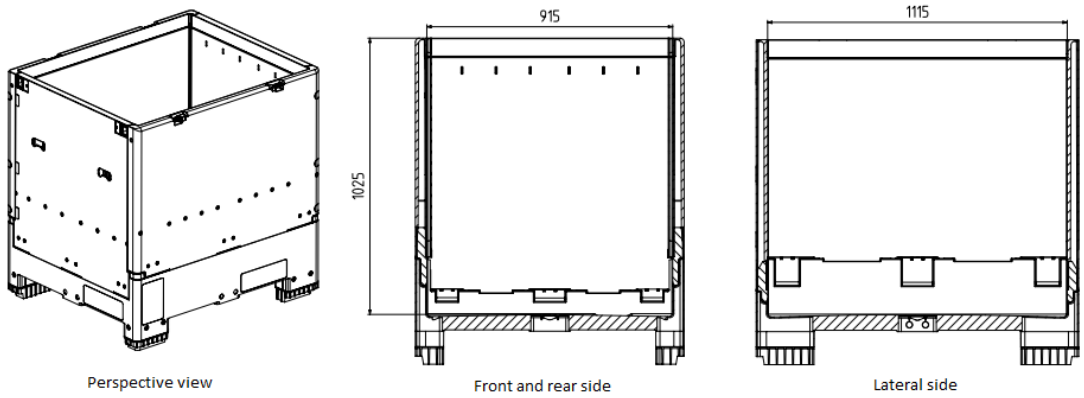

Figure 6. Collapsible bulk container used for the cage's body.

III.2.2. Animals and management

Eleven multiparous lactating Lacaune ewes (66.7 ± 2.6 kg BW; 202 ± 22 DIM) producing 1.58 ± 0.12 kg/d of milk yield, were used. The trial was carried out in the experimental farm of the SCCE (Servei de Granges i Camps Experimentals) of the UAB in Bellaterra (Barcelona, Spain), and the experimental procedures were approved by the Ethical Committee of Animal and Human Experimentation of the UAB (CEEAH reference 3195).

Ewes were initially kept in a group and adapted to the diet and milking conditions (1 mo). The diet fit the lactation requirements of the ewes according to INRA (2010) and was based on alfalfa hay *ad libitum*, offered twice a day (0930 and 1730 h) with 0.8 kg/d of concentrate [soybean hulls, 50%; barley grain, 10%; oat grain, 10%; gluten feed, 10%; rapeseed 00 cake meal, 5%; soybean oil, 5%; corn grain, 4%; sugar cane molasses, 2%;Vitafac ovino 0.3% (Vitafac, DSM Nutritional Products, Kaiseraugst, Switzerland), 1%; sodium chloride, 0.5%; as fed] and 0.4 kg/d of whole corn grain, bothoffered at milking. The β-glucan content of the basal diet was negligible (0.77% DM).

The animals were milked twice a day at 0900 and 1700 h using a portable milking machine (Westfalia-Separator Ibérica, Granollers, Spain) set at 38 kPa and 180 pulses/min with a pulsation rate of 50%, provided of a collecting jar of 20 L. Milking routine included cluster attachment, without udder preparation or teat cleaning, machine milking and machine stripping before clusters removal. Individual milk yields was

recorded at each milking. Ewes were trained for using the metabolic cages as milking stalls during the last days (5 d) of the adaptation period.

After the adaptation period, the ewes were maintained in the metabolic cages for the experimental period lasting 2 wk, in which intake and feces and urine output were recorded individually. The last 10 d of the experimental period (sampling period) were used for comparing the dietary treatments consisting of the control diet (d 1 to 5) or the supplemented diet with β-glucans (d 6 to 10).

The product used for the supplementation was Glucagel (Zeus Ibérica, Barcelona, Spain), a 77.5% barley β-glucan extract, which was added and mixed twice a day with the concentrate (daily dose, 1.62 g/kg metabolic BW). The ewe's BW at the first day of the experimental period was used for calculating the supplementation dose.

III.2.3. Measurements, sampling and analyses

Feed, water and feces

During the experimental period, daily intakes of forage, concentrate and water, as well as feces, were measured individually by weight (MobbaXie, Mobba, Barcelona, Spain) after the morning milking. Daily samples from each feed offered, orts, and feces of each ewe were taken and composited by period. Samples were stored frozen (−20ºC) until analyses.

Feces samples were conditioned to 60ºC before processing for analysis. Feed and conditioned feces were milled through a 1 mm screen and analyzed for DM, ash and NDF as described before.

Daily apparent digestibility ($\mathbf{d_a}$) was calculated individually during the sampling period according to Demarquilly et al. (1995) according to the expression:

$$(da)X = (Xingested - Xexcreted)/Xingested.$$

Milk

Milk yield was recorded and sampled individually at each milking during the experimental period. Milk samples were composited from the morning (60 ml) and afternoon (40 ml) milking, preserved with antimicrobial tablets (Bronopol, Broad Spectrum Microtabs II, D&F Control System, San Ramon, CA, USA) and kept at 4ºC

until compositional analyses. Moreover, a second composited sample (20 mL), without preservative, was taken at d 5 and 10 of the sampling period and frozen for metabolomic analyses.

Milk composition was analyzed for total fat, crude protein, true protein, casein and total solid contents, using near infrared spectroscopy (Foss NIRSystem 5000, Foss, Hillerød, Denmark).

Urine

Urine output was weighed during the sampling period and individual samples collected at d 5 and 10. Samples were stored frozen (−20ºC) until analyses.

Blood

Blood samples from the jugular vein were taken at d 5 and 10 of the sampling period using the vacutainer system with EDTA tubes (Venoject, Leuven, Belgium). Blood sampling was carried out in 8 time-points (h −1, 0, 2, 4, 6, 8, 12 and 24), starting at 0800 h, before milking and feeding. Samples were centrifuged 10 min at 2000 *g* (AlresaDidacen II, Ajalvir, Madrid, Spain) to obtain the plasma which was frozen until analyses (−20ºC).

Plasma samples were analyzed to determine the glucose and NEFA concentrations in the Servei de Bioquímica Clínica Veterinària of the UAB (Bellaterra, Spain) and the kinetics of their profiles were obtained. The NEFA were analyzed by the colorimetric enzymatic ACS-ACOD method using a commercial kit (Wako Chemicals, Neuss, Germany) and glucose was determined by the hexokinase method using the ORS (Reagent System Olympus, Beckman Coulter, Ireland) and an Olympus AU400 analyzer (Olympus Europa, Hamburg, Germany). Moreover, basal levels (h −1) of albumin (green bromocresol method), cholesterol (cholesterol esterase/peroxidise enzymatic method), GGT (gamma-glutamil-3-carboxi-4-nitroanilida, substrate concentration > 4 mmol/L) and urea (urease method and ultraviolet reading) were analyzed.

The β-glucans content from h 0, 2, 4 and 6 plasma samples, collected at d 5 and 10, were analyzed as (1→3) β-D-glucan by the kinetic chromogenic (Fungitell kit,

Associates of Cape Cod, East Falmouth, MA, USA) in Fontlab2000 (Santa Eulalia de Ronçana, Barcelona, Spain).

Metabolomics

Plasma, milk and urine from h 6 samples, collected at d 5 and 10 of the sampling period, were used for metabolomics analyses.

Before thawing samples a phosphate buffer was made containing Na_2HPO_4 (2.8%; Sigma Aldrich, 30427), NaH_2PO_4 (0.525%; Sigma Aldrich, S2554), NaN_3 (3 mmol/L, 0.02%; Sigma Aldrich, S2002) and D_2O (20%; Sigma Aldrich, 298387). Samples then were thawed at room temperature and mixed thoroughly.

Milk samples (4 mL) were transferred to filtration tubes (Amicon Ultra-4, membrane PLGC Ultracel-PL, 10 kDa; EMD Millipore, Billerica, MA, USA) and centrifuged at 5000 *g* for 20 min at 22ºC (Universal 32 R, Hettich, Tuttlingen, Germany). Then, 400 µL of the ultrafiltrated milk were pippeted out of the tube into a 1.5 mL Eppendorf containing 200 µL of the phosphate buffer solution. After mixing well, 550 µL of this mixture was pippeted into a 5 mm-NMR tube (VWR International, Eurolab, Barcelona, Spain) and put on ice ready to be run.

Urine samples were filtrated by a filter attached to a syringe and 400 µL of the filtrate were mixed with 200 µL of the phosphate buffer into a 1.5 mL Eppendorf. Then, it was centrifuged at 12000 *g* for 5 min at 4ºC (Microfuge 22R, Beckman, Coulder's, California, USA) and 550 µL of the supernatant were transferred into a 5 mm-NMR tube and put on ice ready to be run.

For the plasma samples a saline solution with 0.18 g of NaCl (121659.1211, Panreac, Castellar del Valles, Barcelona, Spain) and 2 mL of D_2O (20%; 298387, Sigma Aldrich, Saint Louis, Missouri, USA) was prepared. Thereafter, 200 µL of plasma sample and 400 µL of cold saline solution were mixed in a 1.5 mL Eppendorf, which was centrifuged at 12000 *g* for 5 min at 4ºC, and 550 µL of the supernatant were transferred into a 5 mm-NMR tube and put on ice ready to be run.

Metabolomic analyses were done using an ^1H High Resolution Nuclear Magnetic Resonance (NMR) equipment (spectrometer 600 MHz proton frequency, BrukerAvance III with a TBI 5 mm and z-gradient probe; Bruker) at the SRMN (Servei de Resonància Magnètica Nuclear) of the UAB (Bellaterra, Barcelona, Spain). The procedures

followed were as described in Beckonert et al. (2007). In the plasma samples case, a second metabolomic analyzes of small components was done with the aim to detect β-glucose, a possible metabolite from the β-glucans metabolism.

III.3. Statistical analyses

Nutritional composition, β-glucans content and nutritive value of barley varieties

Comparison between barley varieties was analyzed using the GLM procedure of SAS version 9.1.3 (SAS Institute, Cary, NC, USA). The model contained the fixed effects of the group (hulled vs.hull-less, and waxy vs.non-waxy) and the residual error. Significance was declared at $P<0.05$ and tendencies at $P<0.10$.

Rumen degradability

Rumen degradation parameters, according the equation of Ørskov and McDonald (1979), were computed using the NLIN regression procedure of SAS. Differences between parameters for each prediction equation were determined by significance test ($X \pm 1.96 \cdot SE$ for $P < 0.05$).

Dry matter intake, milk yield and composition and biochemical analyses

Data were analyzed by the MIXED procedure for repeated measurements of SAS (v. 9.0.3). The model contained the fixed effects of the dietary treatment (control and β-glucan supplementation),the random effect of the animal, the interaction treatment × time and the residual error. Lactational performances data were analyzed on a daily basis. Significance was declared at $P < 0.05$ and tendencies at $P < 0.10$.

The model was:

$$Y_{ijkl} = \mu + G_i + T_j + S \times T_{ij} + A_k + \varepsilon_{ijkl}$$

Being,

Y_{ijkl} = dependent variable,

μ = overall mean,

G_i = dietary treatment fixed effect (i = G and C),

T_j= time fixed effect (j = d 1 to 10 or h −1 to 24),

$G \times T_{ij}$ = interaction between dietary treatment and time,

A_k = individual animal's random effect (k = 1 to 5),

ε_{ijkl} = residual error effect.

Metabolomics

The metabolomics data obtained from Servei de Resonància Magnètica Nuclear (UAB, Bellaterra, Spain), were baseline corrected. The water region of the spectra was removed (O 4.69-4.90 ppm).

The sum of all intensities was set to 100 for each spectrum to normalize the signal intensity and to allow the identification of relative changes in the metabolite levels for the different spectra. In the urine sample case, the intensities were standardized based on creatinine (4 ppm). Furthermore, each HR-MRS spectrum was reduced to smaller number of variables calculated by integrating regions using the R softwarev. 2.12.2 (R Core Team, 2013). So, bucket sizes were checked and discarded to increase the separation between peaks in sample's spectra. The datasets were rearranged in such a way that the rows of each data matrix represent the samples and the columns represent chemical shifts (variable) with a matrix dimension of 3219 × 10.

The data were then subjected to multivariate statistical analysis using R software 3.0.2 version. Initially, principal component analyses (**PCA**) were performed without considering the class information, in order to get a general overview of the data and search for outliers. Visualization of the data was accomplished by inspection of the PCA score plot and no outliers were found.

Partial Least Squares-Discriminant Analysis (**PLS-DA**) multivariate analysis was also performed on the datasets (n=10) using the pls package of R software (Mevik and Wehrens, 2007). The PLS-DA is a qualitative method that has been applied for dimensionality reduction in order to visualize the high-dimensional and which allows classifying the individual samples by their respective class prior to analysis. The bucketing parameters were identical to those used for PCA, and each dataset was assigned to a class based on dietary treatment and the PLS-DA scores were plotted.

The most important changes in metabolomic profile in urine and plasma samples were achieved through PLS-DA analysis, selecting the highest absolut loadings values.

Furthermore,in the milk samples case, the integration of peak areas of milk metabolites described in Sundekilde et al. (2013) were calculated in order to compare the relative proportion of metabolites between dietary treatments (quantitative approach). Comparisons of means of each metabolite between groups were achieved using a Student's t-test for paired samples.

Both techniques (qualitative and quantitative) are complementary allowing obtaining the major information as possible in metabolic profiles.

IV. RESULTS AND DISCUSSION

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IV.1. Experiment 1

IV.1.1. Nutrient composition of barley varieties

Nutrient composition and nutritive values of the barley varieties analyzed are summarized in **Table 4**. According to the reported data, marked differences were observed when comparing hulled vs. hull-less and waxy vs. non-waxy varieties, the hulled varieties being especially rich in fiber and minerals and the waxy varieties being high in the amylopectin/amylose ratio and β-glucans. Use of INRA (2007) or PrevAlim 3.23 software conditioned the energetic values obtained for all barley varieties, being lower for PrevAlim than for INRA (**Table** 4 and **5**). Despite the PrevAlim version needing update, most likely the reason should be the use of different dE predictions in the two models.

Hulled vs. hull-less

Hulled varieties showed higher fiber contents (i.e., CF, NDF and ADF) than those hull-less (Table 5). Moreover, the ash and OM contents correlated moderately and positively with the fiber contents (r^2 = 0.54 to 0.62; $P < 0.01$) as expected because the main minerals of the barley are found in the hull (Newman and Newman, 2008).

Hull type markedly affected the nutritive value of barley varieties, as calculated by using the INRA (2007) equations and PrevAlim software (Table 5). The INRA (2007) energetic values, excluding CE, tended to be higher ($P < 0.10$) in the hull-less vs. hulled varieties as a consequence of the higher dE, which is related to OM digestibility and dependent on the CF content, according to Demarquilly et al. (1995) and INRA (2007) equations. Furthermore, for the same reason, the PDIE values were higher in the hull-less than hulled varieties (Table 5) but no differences were observed for PDIN value, which is related to the CP content.

Differences of energy values, when expressed as UFL, were detected between barley varieties according the hull type by using the PrevAlim, but no differences were observed for the PDIE and PDIN values (Table 5).

Table 4. Nutrient composition and nutritive value of the different barley varieties.

Item	Streif	Doña Pepa	BetaNud AD-1	BetaNud AD-2	BetaNud AD-3
Nutrient composition, % DM					
DM	90.19	90.57	90.45	90.81	90.88
Ash	2.29	2.10	1.71	1.77	2.24
OM	97.71	97.90	98.29	98.23	97.76
EE	2.33	2.12	2.30	2.80	2.49
CP[1]	10.96	11.19	11.07	13.32	13.45
CF	2.94	4.14	1.34	1.21	3.00
NDF	11.26	13.93	8.56	9.47	13.12
ADF	2.63	4.17	1.15	0.72	2.69
FNE	81.48	80.45	83.58	80.90	78.82
β-Glucans	5.76	4.27	5.38	7.62	7.63
Nutritive value INRA, kg DM					
CE, Mcal	4.336	4.336	4.360	4.417	4.383
ME, Mcal	3.216	3.151	3.321	3.372	3.249
NE_L, Mcal	2.159	2.115	2.229	2.264	2.181
UFL[2]	1.27	1.24	1.31	1.33	1.28
PDIA[3], g	16	16	14	15	15
PDIE[4], g	92	91	94	94	92
PDIN[5], g	68	70	68	82	82
Nutritive value PrevAlim 3.23[6], kg DM					
UFL[2]	1.17	1.16	1.24	1.25	1.19
PDIA[3], g	18	22	12	16	22
PDIE[4], g	91	94	89	92	95
PDIN[5], g	70	79	68	83	86

[1]CP= N x 6.25 (for CP = N x 5.70 is Doña Pepa, 9.23; Streif, 9.03; BetaNud AD-1, 9.12; BetaNud AD-2, 11.02; BetaNud AD-3, 11.15).[2]Feed units for lactation (1 UFL = 1.7 Mcal of NE_L).[3]Protein digested in the small intestine (PDI) from the ruminal undegradable protein. [4]PDI supplied by microbial protein from rumen-fermented OM. [5]PDI supplied by microbial protein from ruminal degradable protein.[6]Software Prevalim 3.23 (Prevalim 3.23, INRA-UCACIG, Educagri éditions, Dijon, France).

Table 5. Nutrient composition and nutritive value comparison of different barley varieties according hull and starch types.

	Hull type					Starch type				
Item	Hulled	±SE	Hull-less	±SE	*P* value	Waxy	±SE	Non-waxy	±SE	*P* value
Nutrient composition, % DM										
DM	90.55	0.24	90.63	0.25	0.830	90.38	0.27	90.71	0.16	0.188
Ash	2.21	0.07	1.74	0.04	0.006	2.20	0.13	1.91	0.21	0.188
OM	97.79	0.07	98.26	0.04	0.006	97.81	0.13	98.09	0.21	0.371
EE	2.31	0.13	2.55	0.35	0.647	2.23	0.15	2.53	0.18	0.563
CP[1]	11.87	0.97	12.19	1.60	0.996	11.08	0.16	12.61	0.95	0.397
CF	3.36	0.48	1.27	0.09	0.002	3.54	0.85	1.85	0.71	0.444
NDF	12.77	0.97	9.01	0.65	0.092	12.60	1.89	10.38	1.71	0.783
ADF	3.16	0.62	0.94	0.31	0.016	3.40	1.09	1.52	0.73	0.451
FNE	80.25	0.95	82.24	1.90	0.384	80.97	0.73	81.10	1.69	0.903
β-Glucans	5.71	0.66	6.50	1.58	0.906	5.02	1.05	6.88	0.92	0.533
Nutritive value INRA, kg DM										
CE, Mcal	4.352	0.019	4.388	0.040	0.515	4.336	0.000	4.387	0.020	0.266
ME, Mcal	3.205	0.035	3.347	0.036	0.064	3.184	0.046	3.314	0.044	0.303
NE_L, Mcal	2.152	0.024	2.247	0.024	0.066	2.137	0.031	2.225	0.029	0.306
UFL[2]	1.27	0.01	1.32	0.01	0.057	1.26	0.02	1.31	0.02	0.334
PDIA[3], g	16	1	14	1	0.293	16	1	15	1	0.184
PDIE[4], g	92	1	94	1	0.001	92	1	93	1	0.423
PDIN[5], g	73	6	75	10	1.000	69	1	77	6	0.423
Nutritive value PrevAlim 3.23[6], kg DM										
UFL[2]	1.17	0.01	1.25	0.01	0.028	1.17	0.01	1.23	0.02	0.266
PDIA[3], g	21	2	14	3	0.168	20	3	17	4	0.840
PDIE[4], g	93	1	91	2	0.423	93	2	92	2	0.800
PDIN[5], g	78	6	76	11	0.841	75	6	79	7	0.504

[1]CP= N x 6.25 (for CP = N x 5.70 is Hulled, 9.80 ±0.83; Hull-less, 10.07 ± 1.34; Waxy, 10.43± 0.80; Non-waxy, 9.13 ± 0.15). [2]Feed units for lactation (1 UFL = 1.7 Mcal of NEL). [3]Protein digested in the small intestine (PDI) from the ruminal undegradable protein. [4]PDI supplied by microbial protein from rumen-fermented OM. [5]PDI supplied by microbial protein from ruminal degradable protein. [6]Software PrevAlim 3.23 (INRA-UCACIG, Dijon, France).

When compared to corn, which is the main cereal used as energy ingredient (1.22 UFL/kg DM according to INRA, 2007) for the feed industry in the world (FAOstat, 2015), it is reasonable to consider the hull-less barley varieties as a possible substitute of corn in the future diets of lactating dairy ruminants, most likely in Europe.

Waxy vs. non-waxy

As expected, the waxy varieties had higher protein and β-glucans contents than the non-waxy varieties (Table 5), agreeing the results reported by Holtekjolen et al. (2006) and Newman and Newman (2008). Moreover, CP and β-glucans contents correlated positively ($r^2 = 0.81$; $P < 0.01$). The differences between waxy types were more evident for BetaNud AD-2 and BetaNud AD-3 varieties, than for the BetaNud AD-1 and, on average, did not differ from the non-waxy (Table 4). Nevertheless, when the BetaNud AD-1 variety was not included in the comparison, the differences between waxy and non-waxy types for protein and β-glucans content were higher and significant (CP, $P = 0.030$; β-glucans, $P = 0.003$).

On the other hand, as PDIN values were related with the CP content, the waxy varieties (when the abnormally low CP values of BetaNud AD-1 were not included in the comparison), showed higher PDIN values when compared to the non-waxy barley varieties (Table 5). A possible reason of the low values of CP and β-glucans in BetaNud AD-1 may be the climatologic conditions during cultivation of the crop, which are known to modify the CP and β-glucans contents of barley phenotypes independently of the genetic potential of the variety (Newman and Newman, 2008; FEDNA, 2015).

According to the higher amylopectin/amylose ratio of the waxy barley varieties, it may be expected possible changes on the rumen degradability and intestinal digestibility of the starch which may alter the fermentation pattern and the digestive utilization under *in vivo* conditions (i.e., rumen degradability, digestaand feces viscosity).

IV.1.2. Rumen degradability

Rumen environment conditions

According to the diet used, exclusively based on alfalfa hay, daily rumen pH recorded by the sensors used was high (6.67 ± 0.02, on average), ranging between 6.45 and 7.05. Maximum pH was observed immediately before feeding, most probably as a result of

saliva secretion, decreasing gradually thereafter (**Figure 7**), and reaching the nadir at 1700 h (9 h postfeeding). Decrease of rumen pH after feeding was produced by the microbial degradation of the feed which released volatile fatty acids that acidified the rumen fluid, as indicated by McDonald et al. (2011).

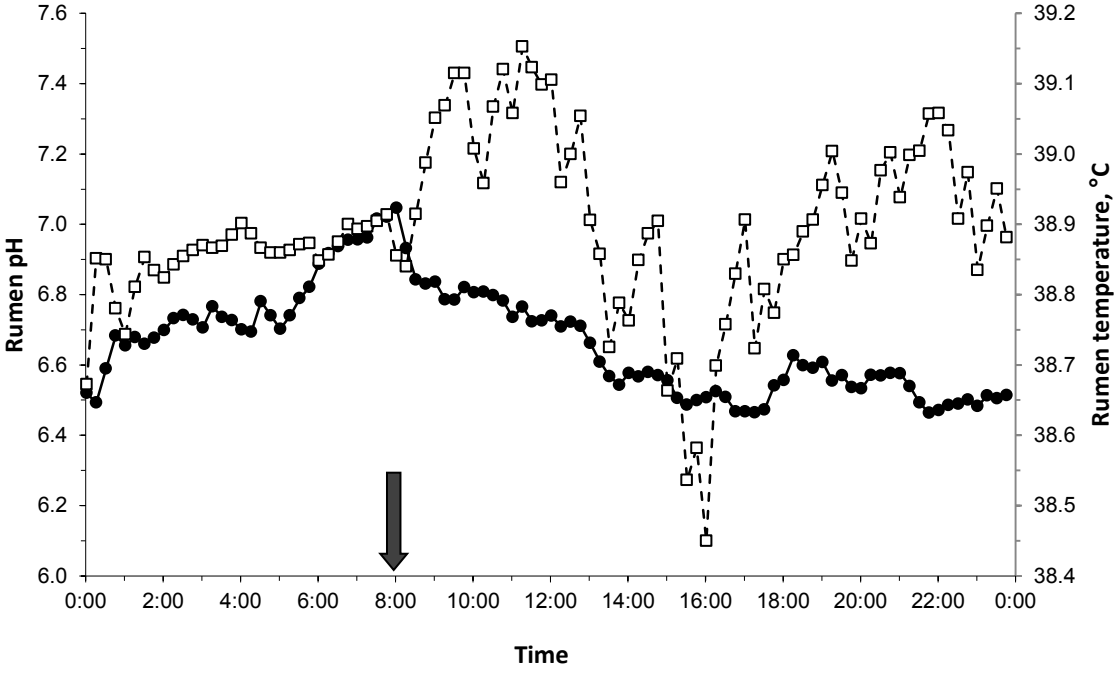

Figure 7. Rumen pH (●) and temperature (□) of the non-lactating dairy cow used for the in situ degradability study. The values represented are the mean of the 2 boluses and the 2 periods of measurement done. The solid arrow indicates the time of feeding. Mean standard errors were ±0.02 and ±0.01ºC for pH and temperature, respectively.

On the other hand, rumen temperatures averaged 38.89 ± 0.01ºC, ranging between 38.45 and 39.15ºC. Rumen temperature increased rapidly after feeding, reaching the zenith at 3 h postfeeding, agreeing the pH pattern and expected ruminal degradation of the feed, which is considered the main thermogenic activity of the ruminant (McDonald et al., 2011). A rapid drop of temperature occurred at 1600 h, which was related to a large intake of water (Figure 7). Moreover, other smaller temperature drops were also observed throughout the whole day. These pH and temperature data are consistent with the observations of Castro-Costa et al. (2014) and with a healthy rumen function, optimal for the degradation activity of the microbiota.

Ruminal degradability in situ

Values of degradability in the rumen for DM, CP and β-glucans of all the studied varieties of barley are shown in **Table 6** and **Figures 8** to **12**.

Dry matter degradability was similar between barley varieties ($P > 0.05$), being TD and $ED_{16\text{-}h}$ values 94.6 ± 1.6% and 85.4 ± 1.6%, on average for all barley varieties, respectively (Table 6). Moreover, no differences were detected between the parameters (a, b and c) of the equation defining the prediction model of Ørskov and MacDonald (1979) for different barley varieties or when the hulled vs. hull-less, or the waxy vs. non-waxy types were compared.

With regard to CP degradability, all barley varieties showed similar values of TD and $ED_{16\text{-}h}$ being their values 97.6 ± 0.3% and 86.8 ± 0.7%, on average, respectively. In this case, slight differences were detected in the "a" parameter (hidrosoluble and immediately degradable component) of the model which was lower in the BetaNud AD-1 than in the rest of barley varieties ($P < 0.05$). No differences were detected for the "b" and "c" parameters, as well for the hulled vs. hull-less and the waxy vs. non-waxy type comparisons.

The $ED_{16\text{-}h}$ values obtained in this study were higher than those reported by Yang et al. (1997), who compared hulled and hull-less barley varieties, reporting differences for DM (78 vs. 82%, respectively) and for CP (80 vs. 77%, respectively). Moreover, the feed tables of INRA (Andrieu et al., 1989) report an $ED_{16\text{-}h}$ for barley CP of 74%, also lower than the values obtained in the current study. The differences may be in part attributed to the greater particle size used by Yang et al. (1997) in the *in situ* procedure (i.e., 2 mm), reducing the access of the rumen microbiota, as indicated by Vanzant et al. (1998) who discussed the standardization of *in situ* techniques for ruminant feedstuffs and recommended 1.5 to 3 mm for concentrates. Moreover, bacterial attachment may have increased the CP contents of the bag residues, decreasing the apparent degradability of barley, especially in the case of the hulled varieties.

Finally, no differences were detected between barley varieties for the TD and $ED_{16\text{-}h}$ values of β-glucans, as well as between its parameters in the Ørskov and McDonald (1979) equation, which showed a fast and complete degradability, with values of 100.0 ± 0.3% and 95.6 ± 0.3%, respectively. This was a consequence of the high solubility of

Table 6. Degradability models for the different components of barley varieties according to the equation D = a + b • (1 – e–ct) (Ørskovand McDonald, 1979)

Item	Barley type	a[1]	b[2]	c[3]	TD, %[4]	$ED_{16\text{-}h}$, %[6]
DM						
Streif	Hulled	0.657±0.022	0.269±0.025	0.126±0.025	92.6	83.9
Doña Pepa	Hulled	0.607±0.023	0.294±0.025	0.153±0.029	90.1	81.8
BetaNud AD-1	Waxy-nude	0.655±0.019	0.314±0.021	0.190±0.027	96.9	89.4
BetaNud AD-2	Waxy-nude	0.663±0.029	0.309±0.033	0.144±0.033	97.2	88.1
BetaNud AD-3	Waxy-hulled	0.669±0.026	0.255±0.030	0.113±0.030	92.4	83.6
Mean		0.650 ± 0.012	0.288 ± 0.013	0.145 ± 0.015	93.8 ± 1.6	85.4 ± 1.5
CP						
Streif	Hulled	0.657±0.025[ab]	0.315±0.030	0.098±0.021	97.2	85.2
Doña Pepa	Hulled	0.657±0.025[ab]	0.314±0.029	0.107±0.022	97.2	85.2
BetaNud AD-1	Waxy-nude	0.611±0.024[a]	0.366±0.027	0.150±0.024	97.6	87.2
BetaNud AD-2	Waxy-nude	0.698±0.034[b]	0.284±0.040	0.104±0.033	98.1	87.8
BetaNud AD-3	Waxy-hulled	0.725±0.027[b]	0.255±0.033	0.089±0.027	98.0	87.7
Mean		0.670 ± 0.022	0.307 ± 0.021	0.110 ± 0.012	97.6 ± 0.3	86.8 ± 0.7
β-glucans						
Streif	Hulled	0.841±0.014	0.165±0.018	0.136±0.037	100[5]	95.5
Doña Pepa	Hulled	0.839±0.018	0.167±0.024	0.134±0.049	100[5]	95.4
BetaNud AD-1	Waxy-nude	0.854±0.010	0.150±0.012	0.157±0.033	100[5]	96.3
BetaNud AD-2	Waxy-nude	0.857±0.027	0.152±0.037	0.106±0.063	100[5]	95.4
BetaNud AD-3	Waxy-hulled	0.850±0.002	0.150±0.003	0.173±0.008	100[5]	96.2
Mean		0.848 ± 0.004	0.157 ± 0.004	0.141 ± 0.013	100.0 ± 0.3	95.6 ± 0.3

[1]Soluble and very rapidly degradable components.[2]Insoluble but potentially degradable components.[3]Constant fractional rate of degradation per unit of time.[4]Total degradability predicted at 72 h.[5]Total degradability predicted at 48 h.[6]Effective degradability predicted at 16 h with a 6% passage rate. [a-b]Values within a column with different superscript differ ($P < 0.05$).

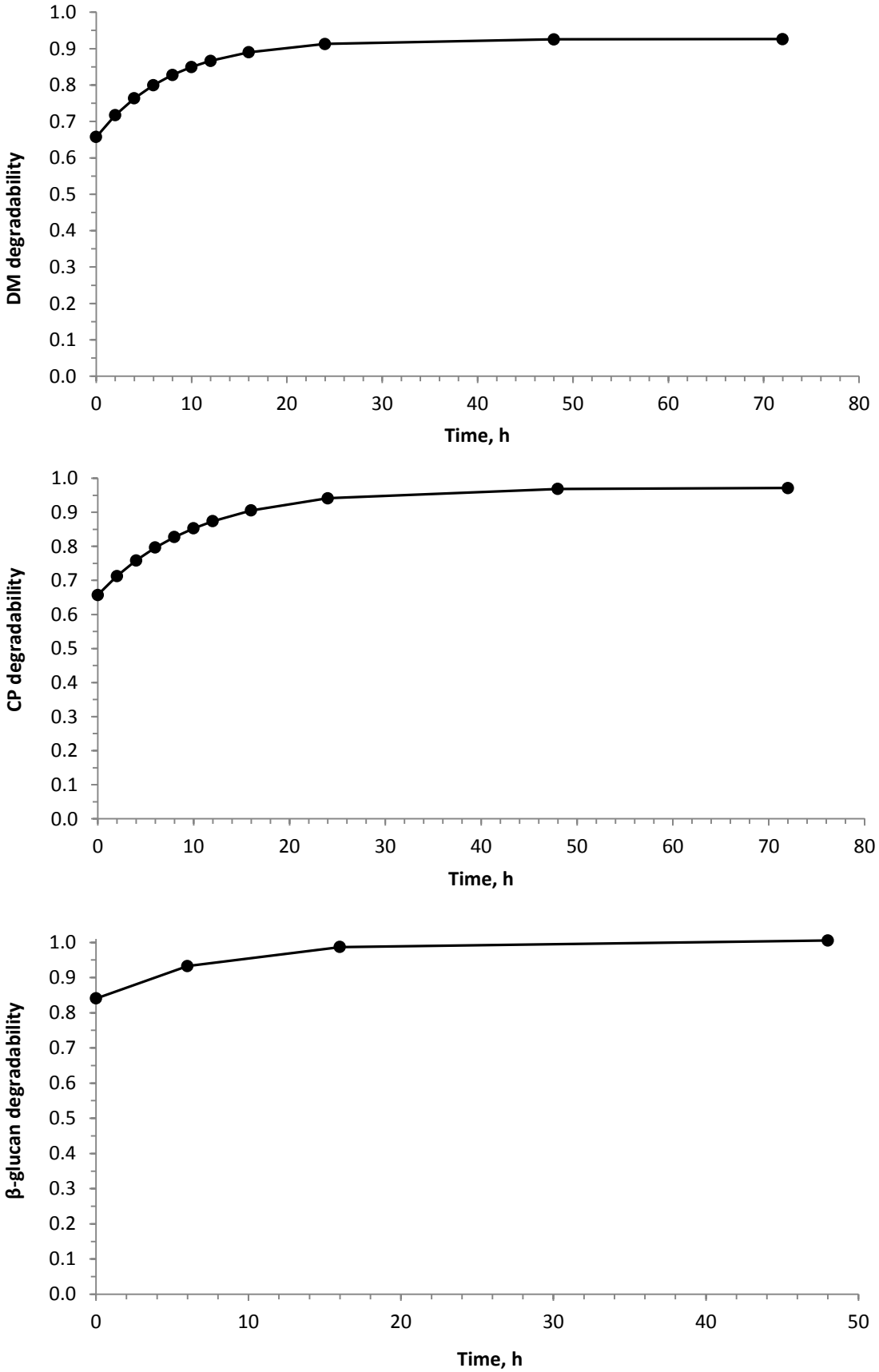

Figure 8. Ruminal degradabilities of the Streif barley variety under in situ conditions.

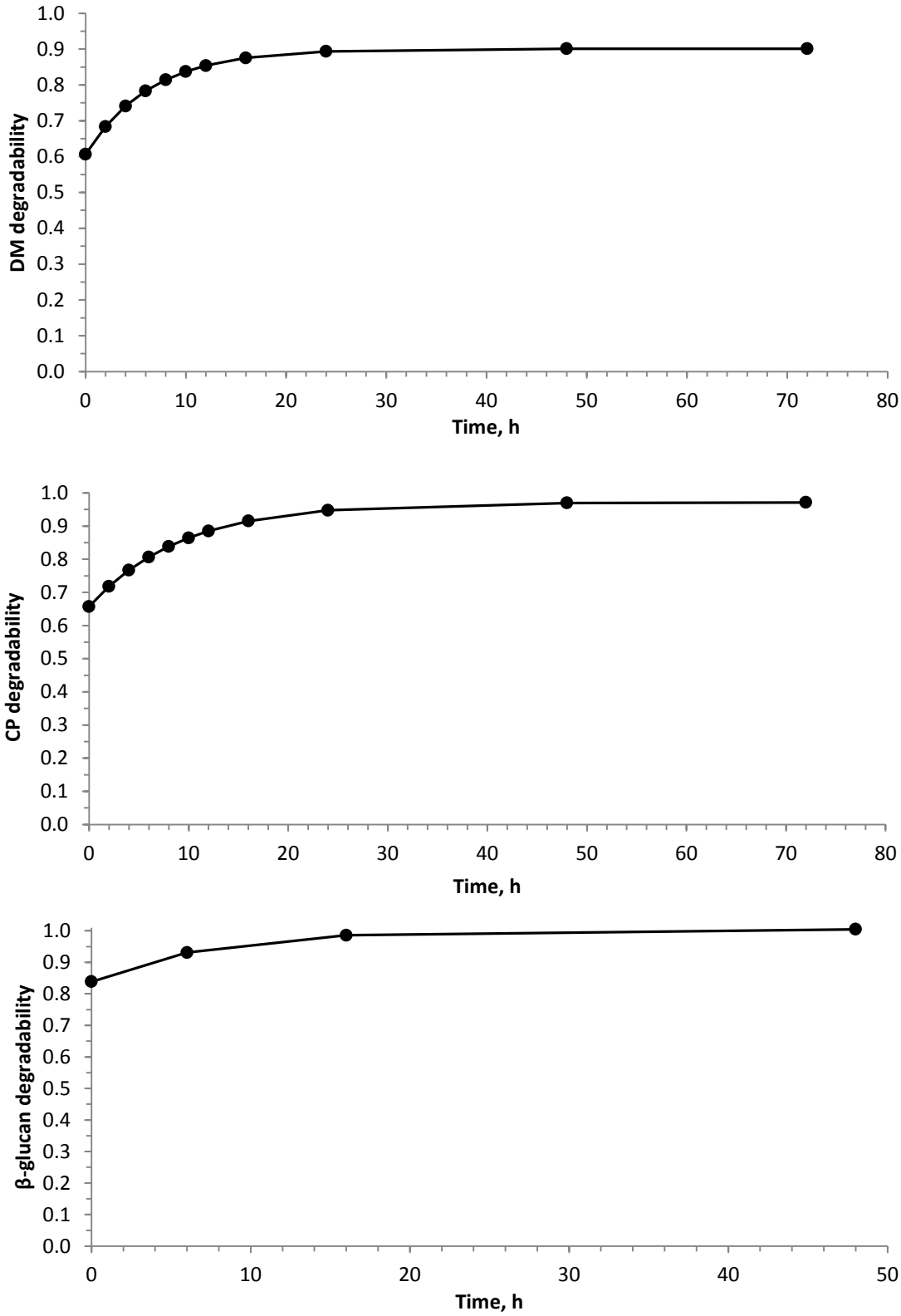

Figure 9. Ruminal degradabilities of the Doña Pepa barley variety under in situ conditions.

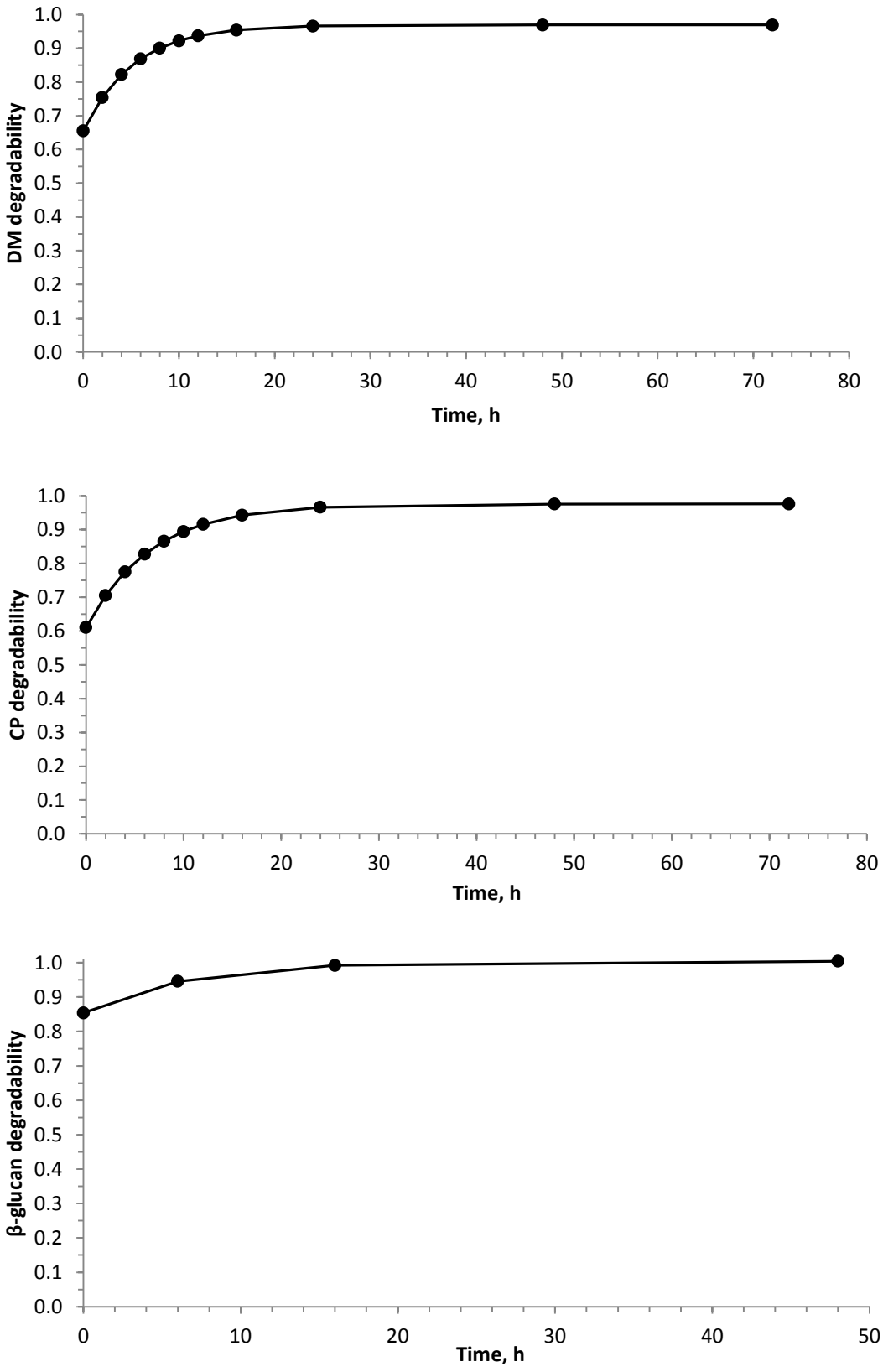

Figure 10. Ruminal degradabilities of the BetaNud AD-1 barley variety under in situ conditions.

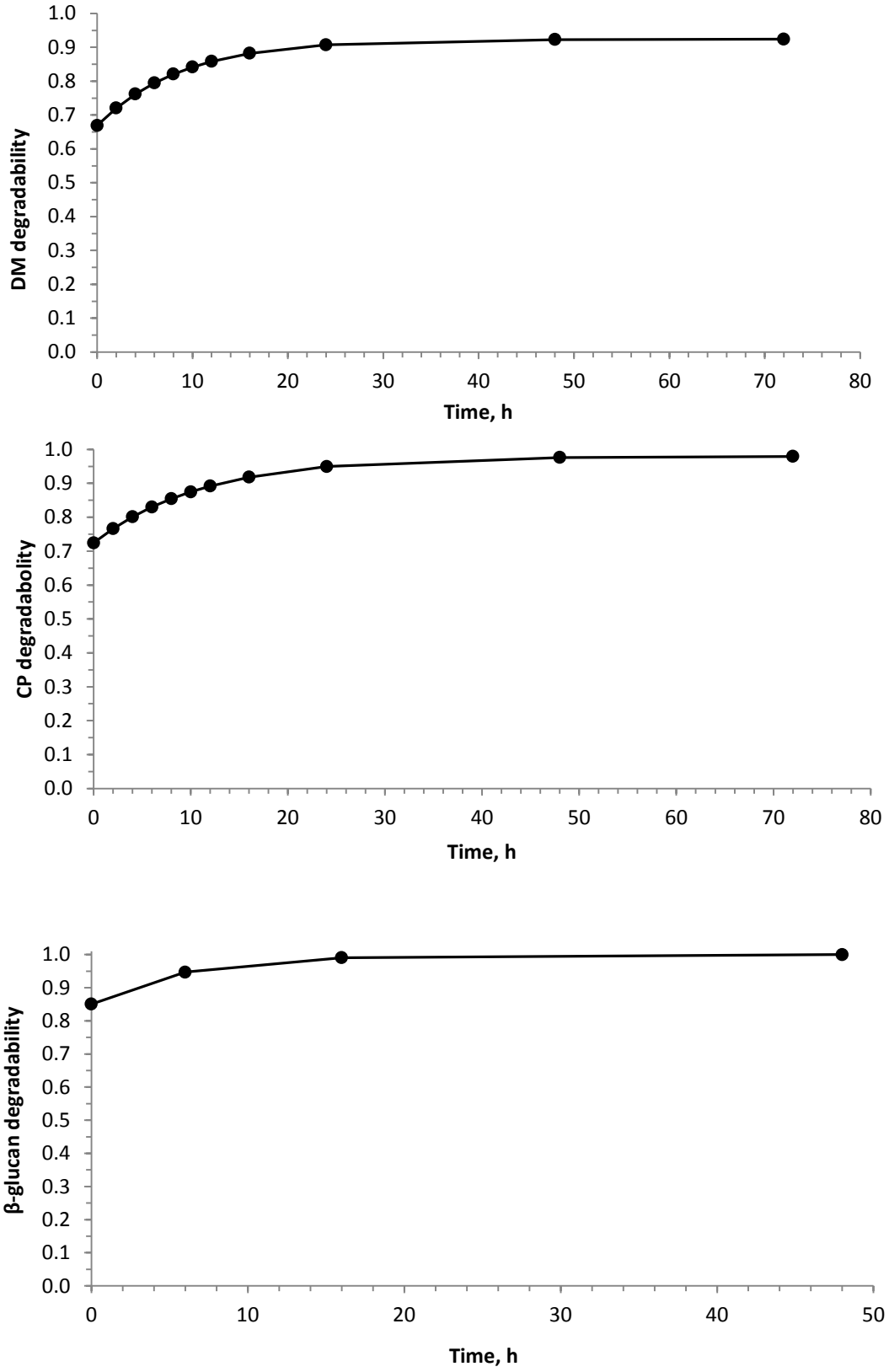

Figure 11. Ruminal degradabilities of the BetaNud AD-2 barley variety under in situ conditions.

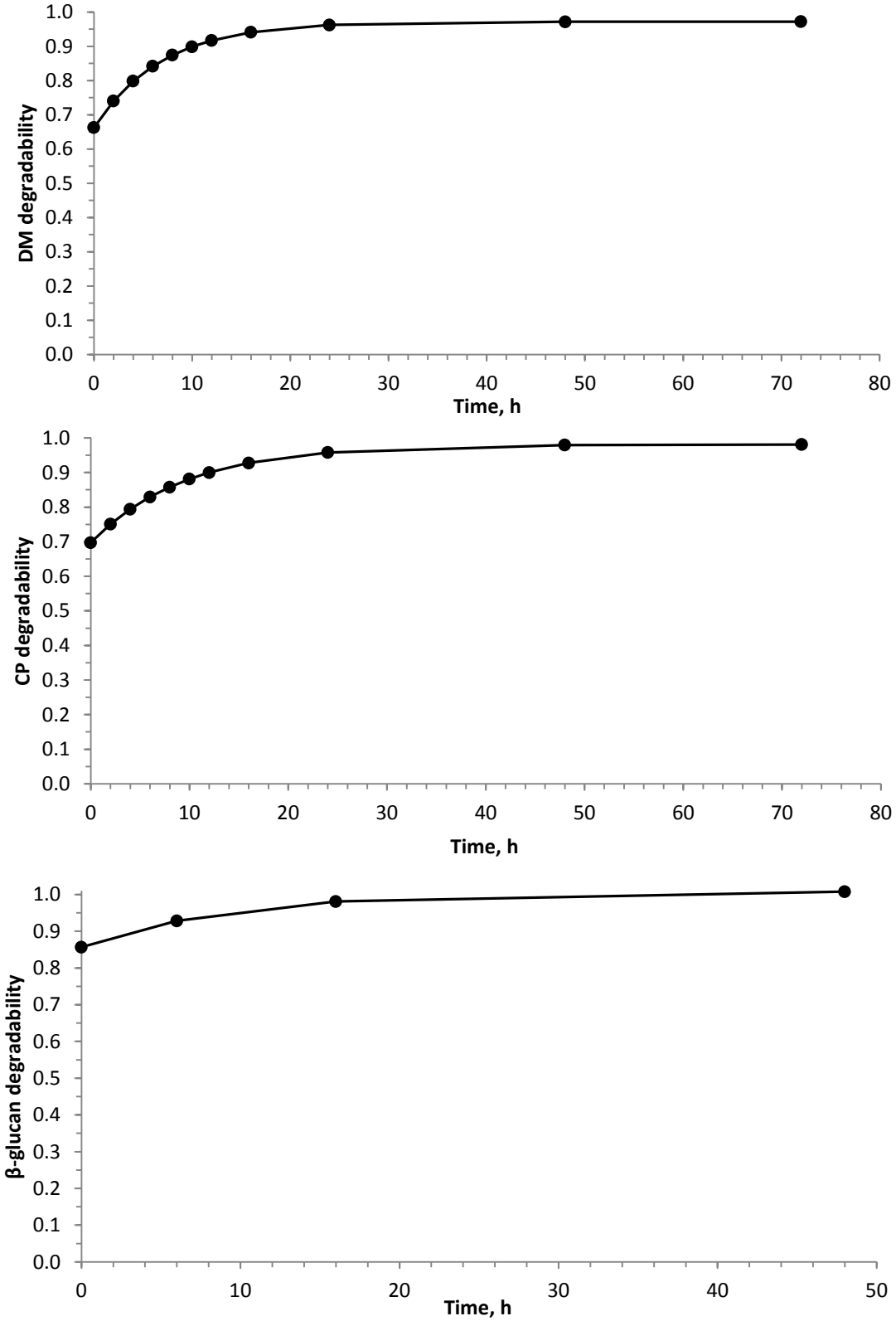

Figure 12. Ruminal degradabilities of the BetaNud AD-3 barley variety under in situ conditions.

the β-glucans under the *in situ* conditions, 84.8 ± 0.4% of which were immediately removed at 0 h as indicated by the "a" parameter (after washing in water) in Table 6.

Obtained values of DT were greater than $ED_{16\text{-}h}$ in all cases, as expected, the values of observed and predicted DT fitting tightly for DM. Moreover, significant correlations between observed and predicted values of DT and $ED_{16\text{-}h}$ were observed for DM (r^2 = 0.98 to 0.99; $P < 0.05$), but not for CP and β-glucans, as shown in **Figure 13**. In this figure it can be appreciated that hulled-less barley types tended to be more degradable than the hulled types, although the differences were not significant due to their high degradability of the barley grain and to the own precision of the *in situ* method.

Obtained results were consistent with the physical-chemical features of the barley β-glucans described by Lazaridou and Biliaderis (2007) and Izydorczyk and Dexter (2008), and demonstrated that β-glucans are rapidly solubilized and most likely completely degraded in the rumen.

Nevertheless, taking into account the dynamics of the digestion in the rumen, in which the liquid phase has a fixed capacity, as indicated by McDonald et al. (2011), the liquid or solid feeds entering the rumen will displace the same volume of rumen liquor throughout the reticulo-omasal orifice, dragging at least a part of the soluble feed components.

Consequently, it may be possible that the β-glucans could escape with the liquid phase of the rumen immediately after solubilizing, avoiding the ruminal degradation and being absorbed in the stomach or the small intestine. There are not data supporting this hypothesis. In any case, to warrant the functional effects of β-glucans in ruminants, a rumen protection method is recommended and should be developed.

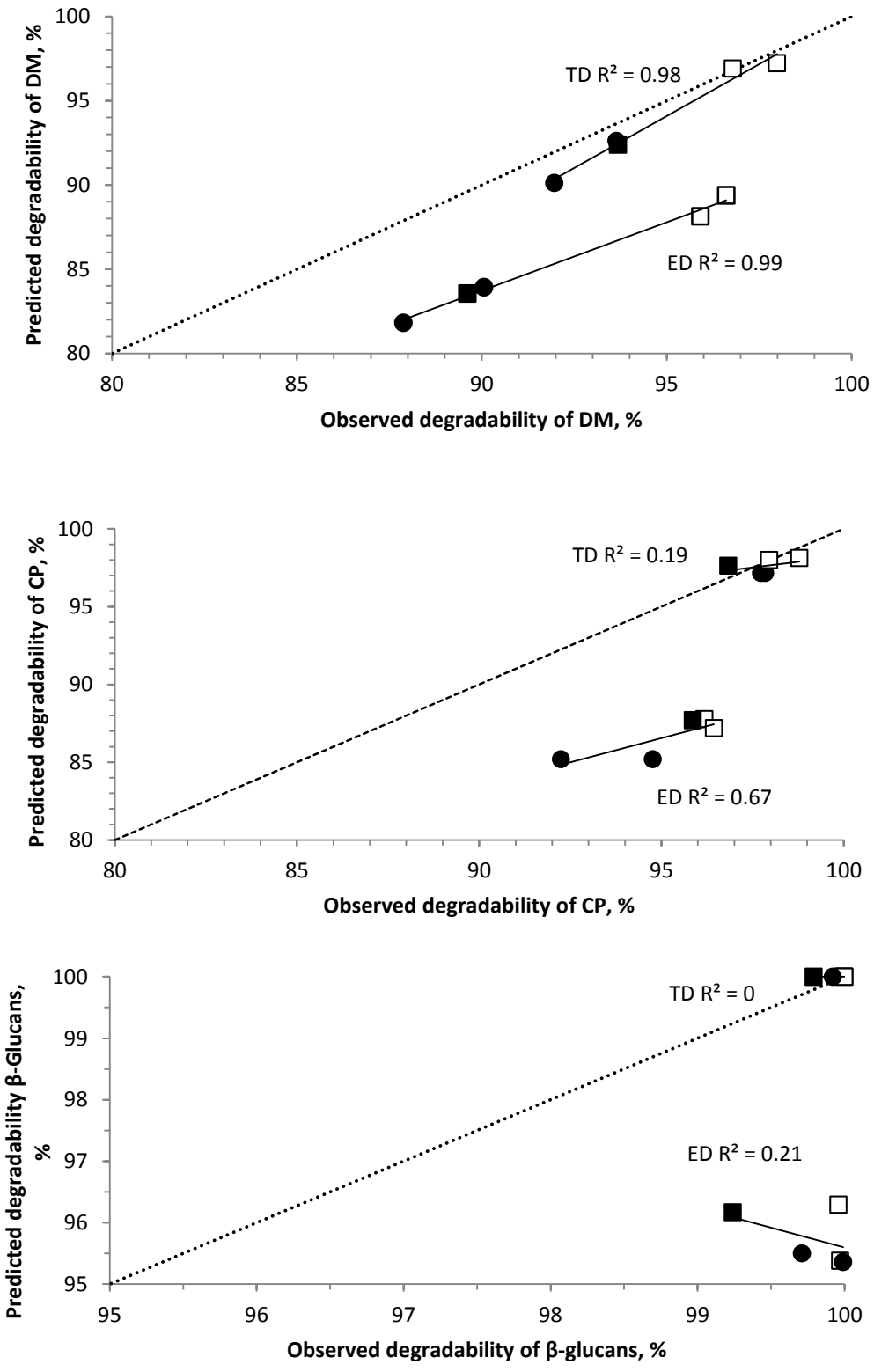

Figure 13. Observed vs. predicted values of in situ degradabilities of the barley varieties (●■, hulled; ○□, nude; □■, waxy; ○●, non-waxy; TD = total degradability, ED = effective degradability).

IV.2. Experiment 2

IV.2.1. Milk yield and composition

Milk yield

On average, milk yield and energy corrected milk during the collecting period of the experiment was 1.80 ± 0.12 kg/d and 1.59 ± 0.14 kg/d, respectively, and no differences were detected between dietary treatments (**Table 7**). However, the interaction time period × diet was significant ($P < 0.05$), indicating that milk yield and energy corrected milk slightly increased approximately 1% in the second period (i.e., control vs. β-glucans supplemented diets) as it can be observed in **Figure 14**.

Table 7. Lactational performance of Lacaune dairy ewes according to the dietary treatments (C, control diet; BG, β-glucans supplemented). Values are least squares means (LSM) and standard error of the means (SEM).

Item	Diet		SEM	Effects ($P<$)	
	C	BG		Treatment	T ×D[1]
Milk yield, kg/d	1.80	1.81	0.12	0.889	0.043
Milk composition, %					
Protein	5.60	5.44	0.11	0.159	0.129
Fat	6.25	6.05	0.22	0.249	0.599
Total solids	17.47	17.08	0.22	0.064	0.696
Lactose[2]	4.65	4.69	0.16	0.643	0.828
Casein	4.02	3.95	0.08	0.347	0.256
Energy corrected milk[3], kg/d	1.58	1.59	0.14	0.899	0.032
Feed intake, kg DM/d	2.82	2.64	0.18	0.001	0.001
Efficiency, kg DM/L ECM	1.77	1.69	0.13	0.041	0.565
Apparent digestibility					
DM	0.63	0.63	0.09	0.638	0.031
OM	0.79	0.77	0.01	0.682	0.206
NDF	0.55	0.53	0.01	0.423	0.030

[1]Time period × Diet interaction.[2]Estimated by difference considering 0.9% ashes; [3]ECM = milk yield × [0.071 × (fat, %) + 0.043 × (protein, %) + 2.224] according to INRA (2007).

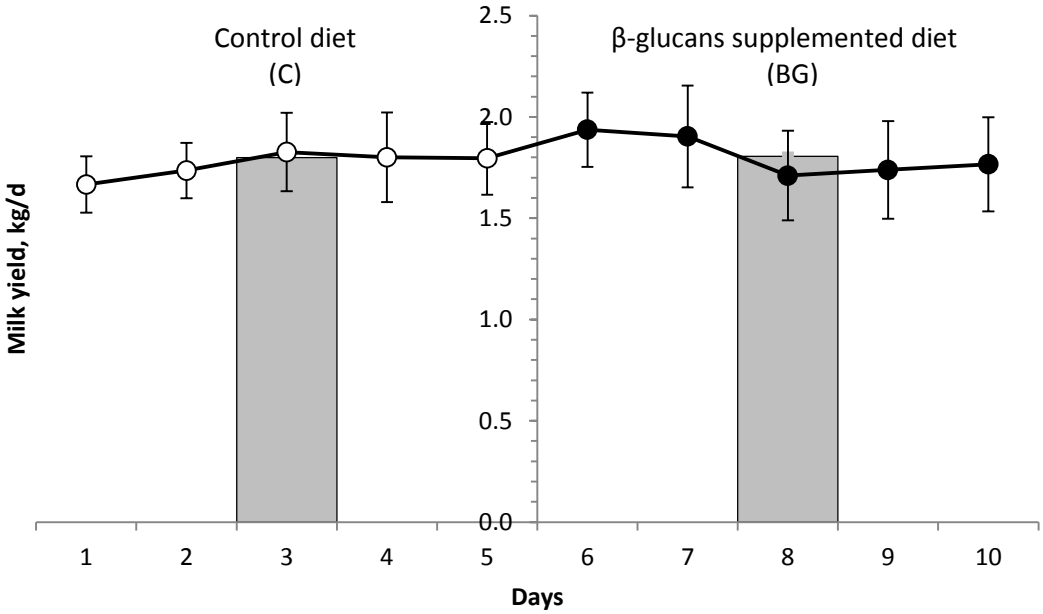

Figure 14. Milk yield during the collecting period of the experiment in which the dietary treatments were applied (control, ○; β-glucans supplemented, ●). The grey columns represent the average milk yield of the 5 din which the control or the β-glucans supplemented diets were fed to the ewes (bars are ±SEM).

Milk composition

With regard to milk composition, only a slight tendency (P = 0.064) to decrease the total milk solids was detected after β-glucans supplementation, the milk fat and protein contents being similar and averaging 6.14 ± 0.14 and 5.51 ± 0.10%, respectively, for the whole sampling period (Table 7 and **Figures 15** and **16**). Moreover, there was not difference in the casein content of the milk by dietary treatment (Table 7), averaging 3.99 ± 0.06% (72% of milk protein).

Sawadogo et al. (1988ab;1993) reported increases of β-casein synthesis in the mammary cells of rats under *in vitro* and *in vivo* conditions, when supplemented with pectic acid or extracts of lactogenic plants high in β-glucans. Given that β-casein is the main casein of ewe's milk (Richardson and Mercier, 1979; Park et al., 2007), the Sawadogo et al. (1988ab;1993) findings were not supported by the results of the current study in which content of total milk casein did not vary. In agreement, no differences were detected in the estimated lactose content by dietary treatment (4.74 ± 0.08%, on average).

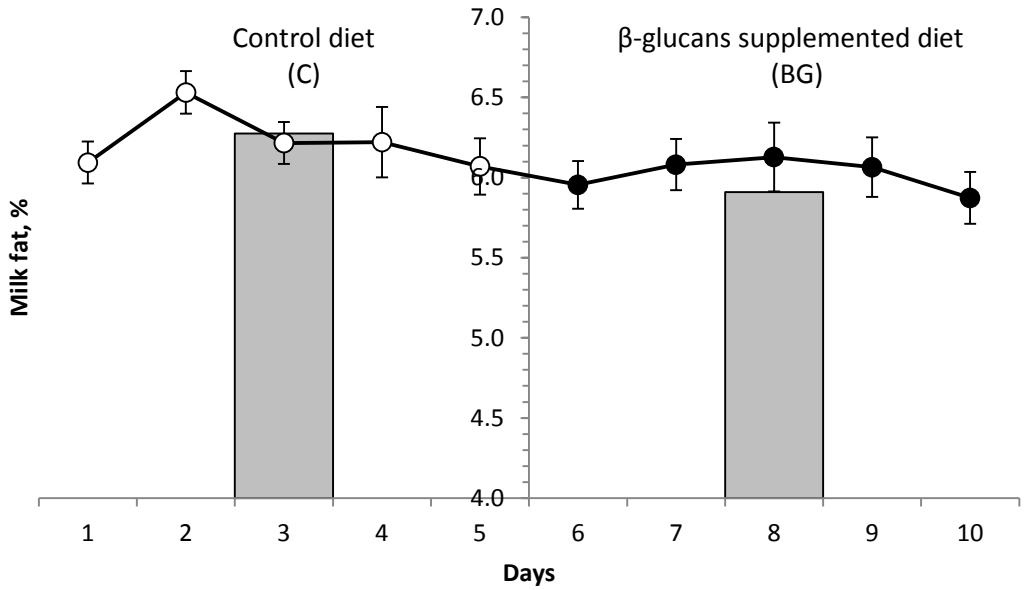

Figure 15. Milk fat content during the collecting period of the experiment in which the dietary treatments were applied (control, ○; β-glucans supplemented, ●). The grey columns represent the average fat content of the 5 din which the control or the β-glucans supplemented diets were fed to the ewes (bars are ± SEM).

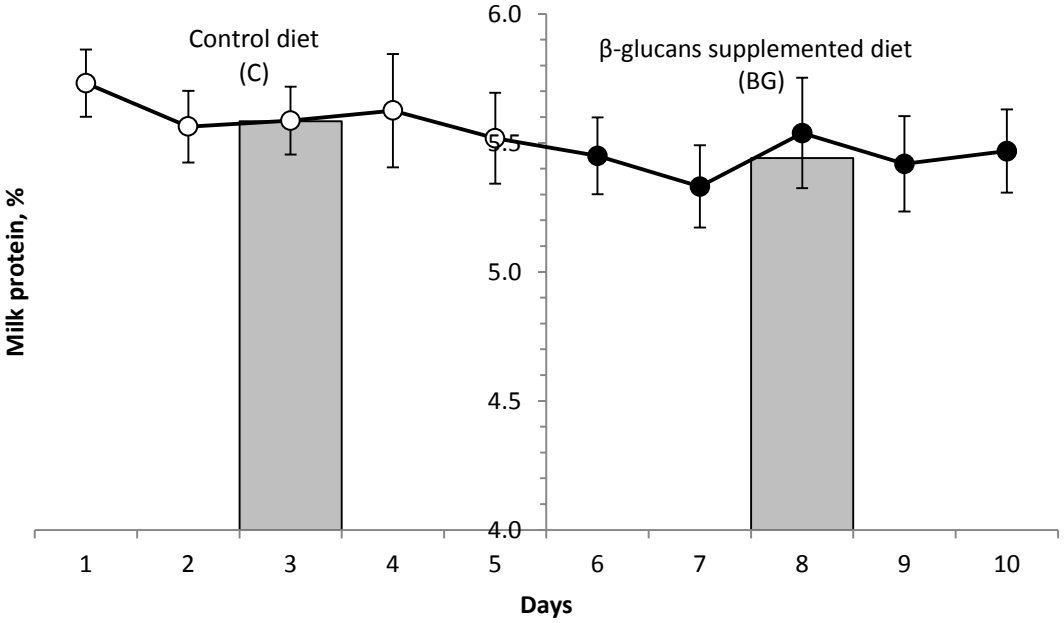

Figure 16. Milk protein content during the collecting period of the experiment in which the dietary treatments were applied (control, ○; β-glucans supplemented, ●). The grey columns represent the average protein content of the 5 din which the control or the β-glucans supplemented diets were fed to the ewes (bars are ± SEM).

IV.2.2. Feed intake and feed efficiency

Dry matter intake varied by effect of the dietary treatments, decreasing 6% ($P < 0.001$) when the β-glucans were supplemented (Table 7 and **Figure 17**). As for milk yield, there was a time period × diet interaction ($P < 0.001$), indicating that DM intake trend changed after β-glucans supplementation, the ewes reducing their intake in the first 3 d after starting the supplementation, but increasing thereafter.

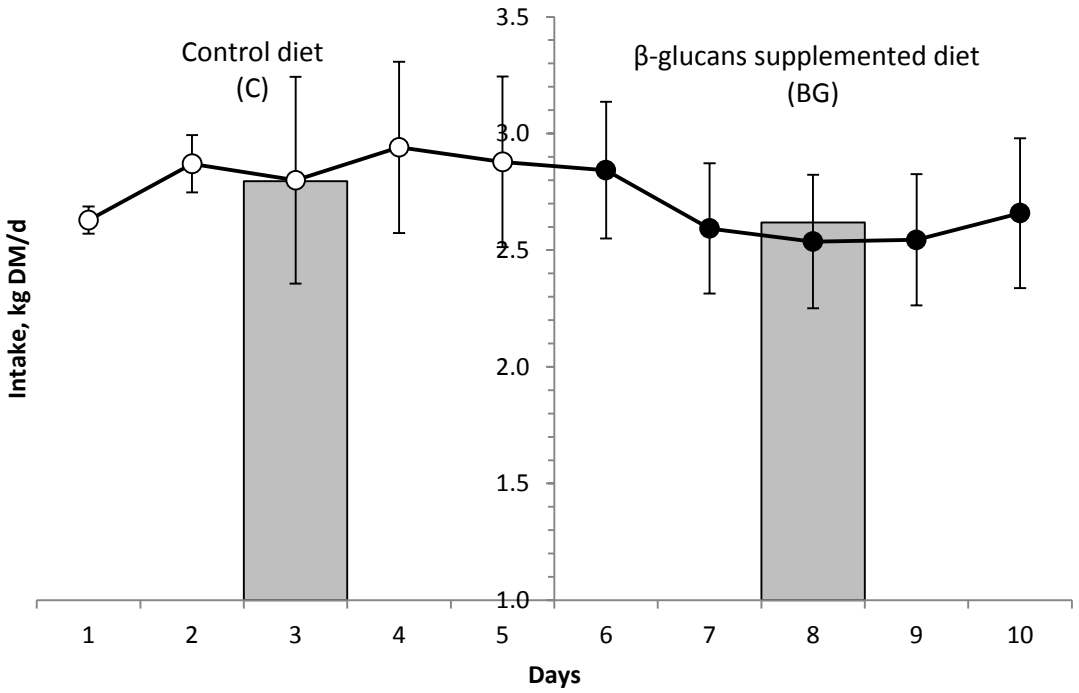

Figure 17. Dry matter intake during the collecting period of the experiment in which the dietary treatments were applied (control, ○; β-glucans supplemented, ●). The grey columns represent the DM intake of the 5 din which the control or the β-glucans supplemented diets were fed to the ewes (bars are ± SEM).

Reduction of DM intake in the diet supplemented with β-glucans was consistent with the results of Cloetens et al. (2012) in humans, who reported a satiety feeling after β-glucans consumption which was related to the increase in diet viscosity. A similar finding may have occurred in the case of the ewes fed with the β-glucans extract-concentrate mixture. Due the capacity of the β-glucans to form viscous solutions, it should be expected that, when mixed with the saliva in the mouth, they will produce a

paste of rubbery texture, making difficulties for swallowing, reducing the appetence and increasing the time for consuming the concentrate. Moreover, as a consequence, the offer of alfalfa hay was delayed during the supplementation days (approximately, 1 to 2 h).

Daily apparent digestibility was used to check the immediate effects of β-glucans supplementation on the transit and features of the digesta at whole digestive tract level (i.e., occurrence of soft feces and flatulencies…). Values of DM, OM and NDF apparent digestibility did not vary during the whole sampling period, being on average 0.63 ± 0.01, 0.78 ± 0.01 and 0.54 ± 0.01, respectively ($P > 0.05$) (Table 7 and **Figure 18**). It should be stressed that ewes supplemented with barley β-glucans did not show diarrhea or soft feces, which are negative side effects widely described in the case of monogastrics in the bibliography.

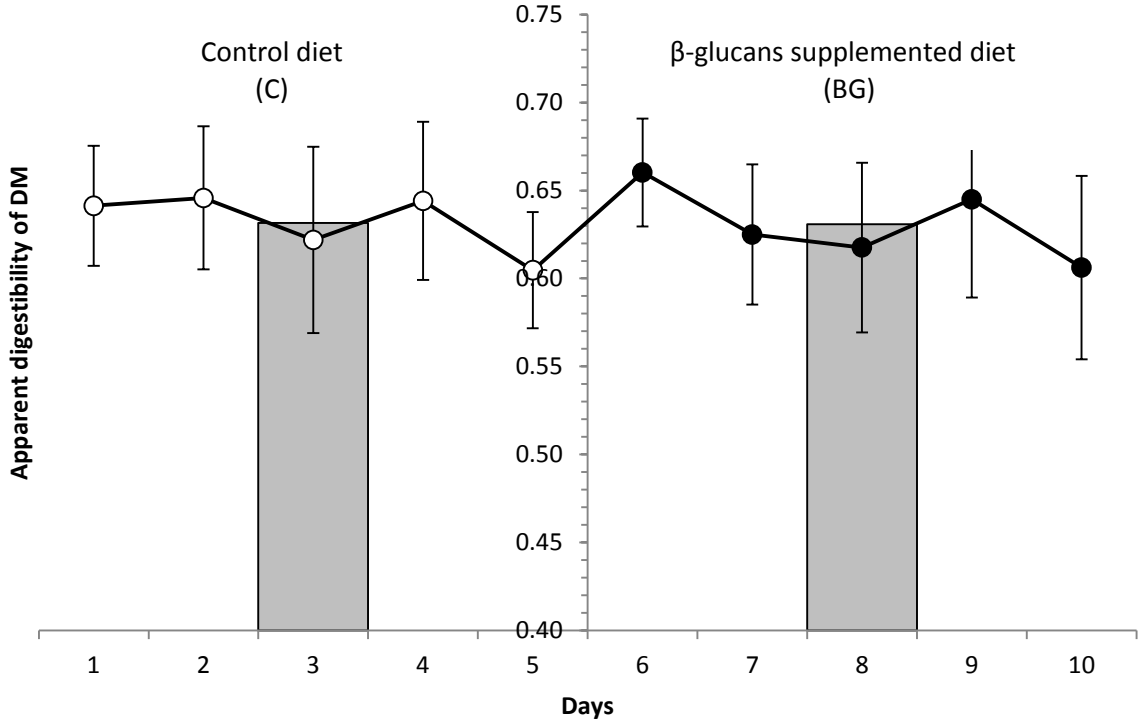

Figure 18. Apparent digestibility of DM during the collecting period of the experiment in which the dietary treatments were applied (control, ○; β-glucans supplemented, ●). The grey columns represent the average apparent digestibility of DM of the 5 d in which the control diet or the β-glucans supplemented diets were fed to the ewes (bars are ± SEM).

Because no differences in digestibility were observed, it was expected that the negative effect of β-glucans on intake was adaptive and transitory.

Consequently with the reduction of feed intake (−6%) and the slight increase in milk yield (+0.1%), feeding efficiency (dry matter ingested/energy corrected milk produced) improved 5% in the β-glucans supplemented diet ($P < 0.05$), without time period × diet interaction (Table 7 and **Figure 19**).

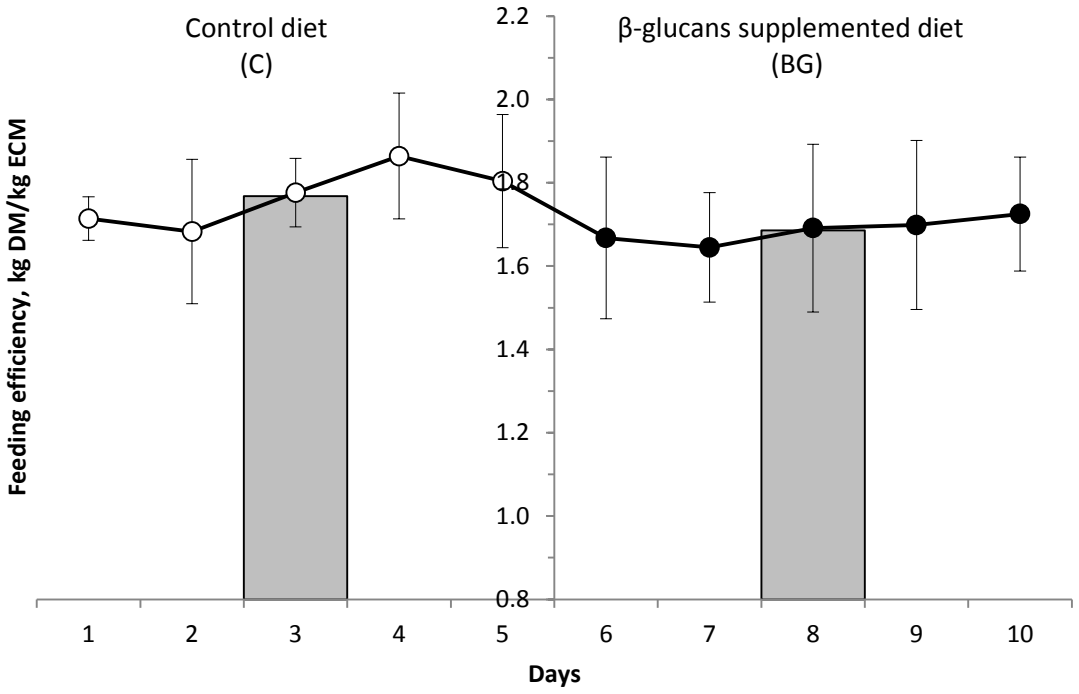

Figure 19. Feeding efficiency during the collecting period of the experiment in which the dietary treatments were applied (control, ○; β-glucans supplemented, ●). The grey columns represent the average feed efficiency of the 5 d in which the control diet or the β-glucans supplemented diet were fed to the ewes (bars are ± SEM).

This increase in lactation efficiency may have been a result, as suggested by Eslime et al. (2008), of the lactogenic effects of the pectic acid (main component of the β-glucans) and its derivatives at an extracellular level where they are supposed to bind to the receptors of lactotropic hormones (i.e., prolactin, growth hormone,…). Binding of β-glucans to cell wall receptors have been confirmed as responsible of macrophage

activation by endogenous danger signals, developing the so called trained immunity (Zhang and Mosser, 2008; Netea et al., 2011).

IV.2.3. Plasma metabolites and metabolic indicators

There were not differences between dietary treatments in plasma contents of gamma-glutamyl transpeptidase (GGT)and urea ($P > 0.05$), both indicators of liver activity, but a tendency to decrease ($P < 0.10$) was found in the case of albumin and cholesterol in the plasma of the ewes supplemented with β-glucans (**Table 8**).

Table 8. Plasmatic indicators of Laucane dairy ewes fed control or β-glucans supplemented diets (Values are LSM).

Item	Treatment		SEM	Effects ($P <$)	
	C	BG		Treatment	T ×D[2]
Albumin, g/dL[1]	3.15	3.02	0.10	0.070	-
Cholesterol, mg/dL[1]	101.2	94.6	9.5	0.053	-
GGT, U/L[1]	75.4	72.6	6.4	0.226	-
Urea, mg/dL[1]	42.3	41.4	2.2	0.489	-
Glucosa, mg/dL	62.8	60.8	1.6	0.210	0.001
NEFA, mmol/L	0.129	0.135	0.040	0.695	0.210
β-glucans, pg/mL	588	648	92	0.426	0.673

[1]Basal values.[2]Time period × Diet interaction.

These changes agreed with the hypocholesterolemic effect attributed to the β-glucans, one of its most relevant health beneficial effects, as it is widely supported in the literature (Wood, 2007; Regand et al., 2011; Cloetens et al., 2012). The advantage of reduced albumin values may be related with lower blood pressure and lower risk of vascular diseases (Guyton and Hall, 2011) and cannot be related with a significant reduction in the protein intake of the ewes.

As expected, the glycaemia values of the ewes of both treatments were high and agreed with the level of nutrition of high yielding dairy ruminants. Moreover, they showed the effects of feeding with similar evolution patterns, reaching peaks 4 h after the a.m. meal and went down thereafter (**Figure 20**). These patterns agree with the post feeding profile described by Bergman (1983) in ruminants.

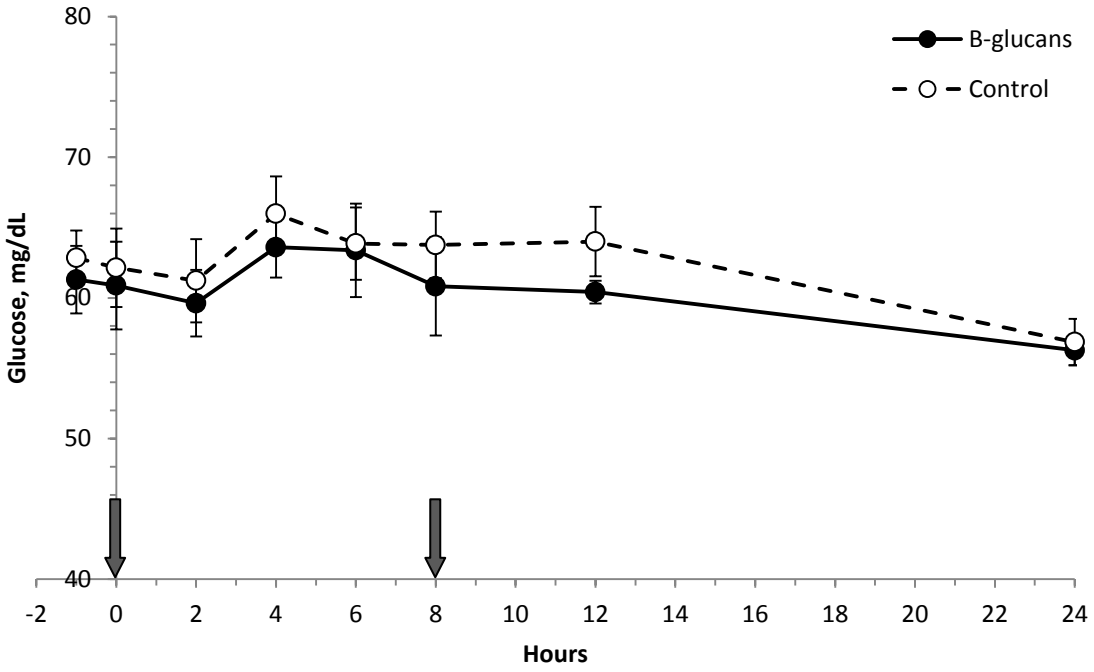

Figure 20. Daily glycaemia kinetics in the plasma of dairy ewes fed control or β-glucans supplemented diets (control, ○; β-glucans supplemented,●). The arrows show the feeding-milking times (×2 daily).

On average, no glycaemia differences were found between dietary treatments (Table 8) but the ewes supplemented with β-glucans had numerically lower basal glycaemia values than the control.

Furthermore, when the glycaemia kinetics of the second meal was considered (i.e., feeding at h 8), the ewes supplemented with β-glucans showed a tendency to differ from the control at 12 h (P= 0.083; Figure 19).

Regarding to NEFAs, obtained values were low, agreeing the high plane of nutrition of the dairy ewes, and showed peaks before each meal (**Figure 21**) according with the results of Marie et al. (2001) and Radunz et al. (2011). Peaks of NEFAs before feeding

were not related to a drop in the glycaemia (Giesecke, 1983) or with a negative energy balance and may be associated to the lipolytic effect induced by the rise of catecholamines at feeding. On the other hand, plasmatic values of NEFAs did not vary according to dietary treatments as shown in Figure 21and Table 8.

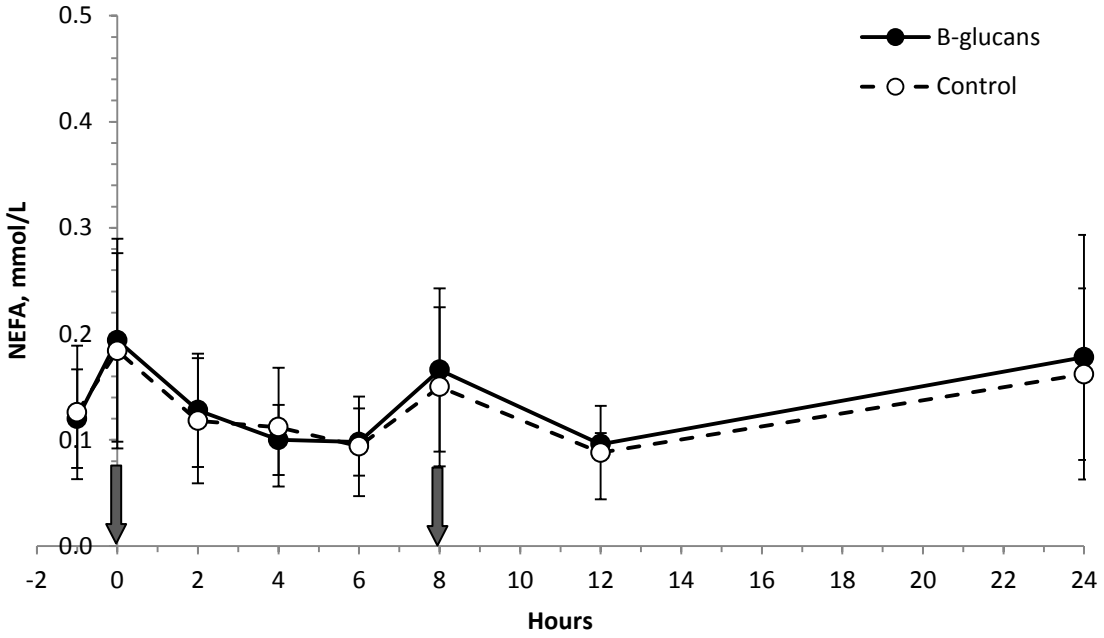

Figure 21. Daily NEFAs kineticsin the plasma of dairy ewes fed control or β-glucans supplemented diets (control, ○; β-glucans supplemented, ●). The arrows show the feeding-milking times (×2 daily).

β-glucans were detected immunologically in the plasma of dairy ewes by using the Fungitell kit, but no differences were found between dietary treatments, being on average 618 ± 43 pg/mL for all the sampling period (Table 8 and **Figure 22**). Furthermore, plasmatic β-glucans at 0 and 2 h time-point were higher in ewes fed with β-glucans supplemented diet than the ewes fed with the control diet.

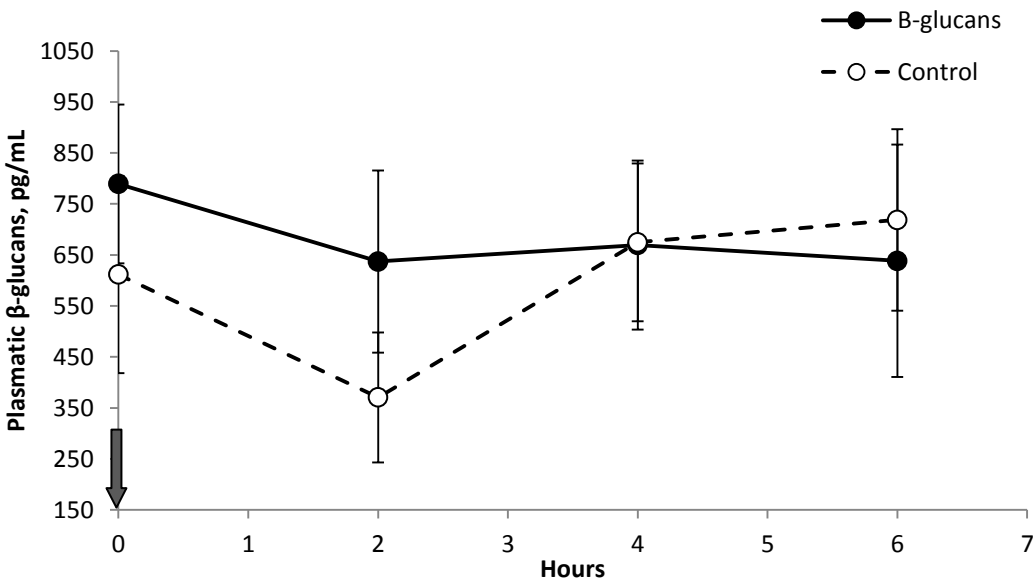

Figure 22. Daily β-glucans kinetics in the plasma of dairyewes fed control or β-glucans supplemented diets (control, ○; β-glucans supplemented, ●). The arrows show the feeding-milking times.

IV.2.4. Metabolomics

According to the NMR sample preparation (i.e., removal of fat and filtration) and principles of functioning, only compounds smaller than 1 kDa were considered in the metabolomics analyses. Moreover, water peak in each spectrum was removed. Identification of NMR peaks in each type of sample was done according to known references (Nicholson and Foxall, 1995; Bouatra et al., 2013; Sundekilde et al., 2013).

There were some metabolites found that remained unidentified as they were hidden by large molecules and it was not possible to explore them fully at this time. Using carbon spectroscopy alongside with proton spectroscopy (2D NMR correlation experiment $^{1}H/^{13}C$ HSQC experiment) would increase the accuracy of the metabolite detection and is reasonable to suggest its use in further milk metabolome studies.

Plasma

Metabolomics general spectrum of plasma samplesis shown in **Figure 23.** Identification of NMR peaks between 0.0 and 9.0 ppm in plasma samples was done according to the ^{1}H chemical shift (ppm) reported by Nicholson and Foxall (1995).

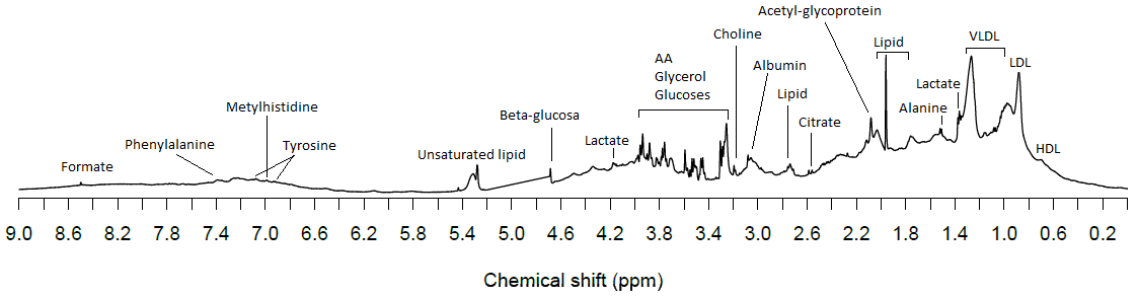

Figure 23. General ^{1}H NMR g spectrum at 600 MHz of ewe plasma samples.

Lipids were the main metabolites detected in the general spectrum of plasma samples, and were placed in 17 peaks (0.84, 0.87, 0.93, 1.22, 1.25, 1.26, 1.29, 1.30, 1.32, 1.57, 1.69, 1.97, 2.00, 2.23, 2.69, 2.71 and 2.72 ppm). Moreover, other relevant components, as amino acids, were detected. Nevertheless, these large peaks of lipids could hide other metabolite peaks of less prominent but important compounds, like β-glucose (here located at 4.64 ppm), a possible metabolite from metabolized barley β-glucans.

Amplified metabolomic spectra of small components analyses from 0 to 5 ppm (**Figure 24**) was also done to identify the small metabolites that were not visible in the general metabolomic spectrum (Figure 23).

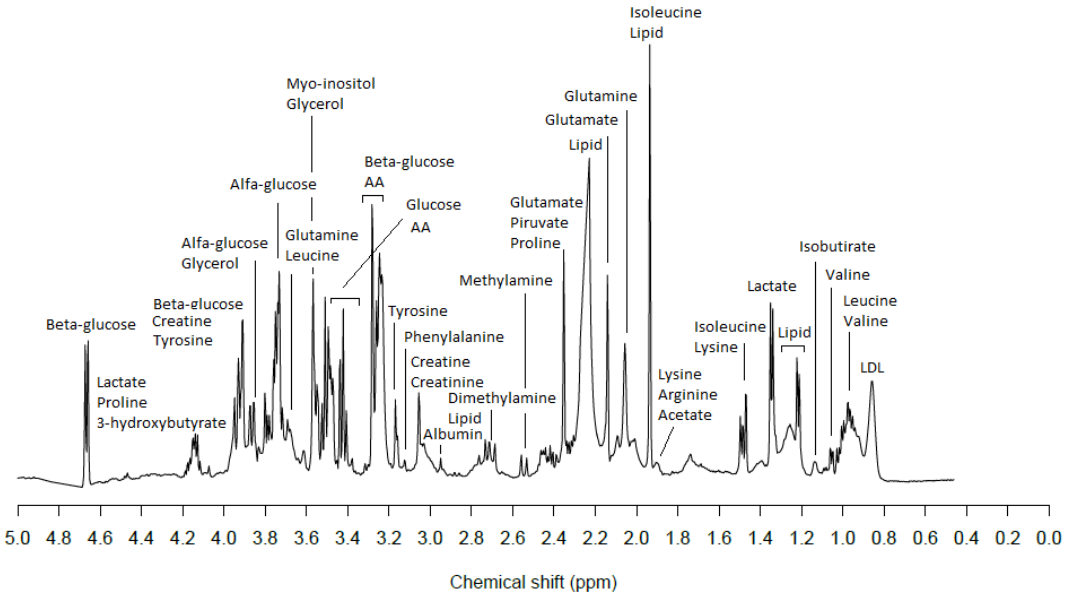

Figure 24. ^{1}H NMR g spectrum at 600 MHz of small component analyzes of plasma samples from 0 to 5.0 ppm.

Most relevant metabolites in Figure 24 were amino acids (valine, leucine, iso-leucine, lysine, arginine, proline, phenylalanine and tyrosine) and the glucose anomers (i.e., α and β), which were placed between 3 to 5 ppm. In detail, β-glucose peaks were placed in 3 peaks at 3.24, 3.90 and 4.64 ppm. Moreover, other relevant components detected were: iso-butirate (1.13 ppm), lactate (1.33 and 4.11 ppm), glutamine (2.08, 2.09 and 3.68 ppm), glutamate (2.00, 2.14 and 2.36 ppm), pyruvate (2.36 ppm), myo-inositol (3.56 ppm), glycerol (3.56, 3.64 and 3.87 ppm), creatine (3.04 and 3.93 ppm), creatinine (3.05 ppm) and 3-hydroxibutirate (4.13 ppm).

With regard to PCA, a score plot of the general spectra data (1 to 9 ppm) was done for 2 first principal components to get a general overview of plasma sample small metabolites (**Figure 25**). The PCA components accounted for the 81.5% of the cumulative proportion of variance, being the 68.0% explained by PC1 (x-axis) and 13.5% by PC2 (y-axis).

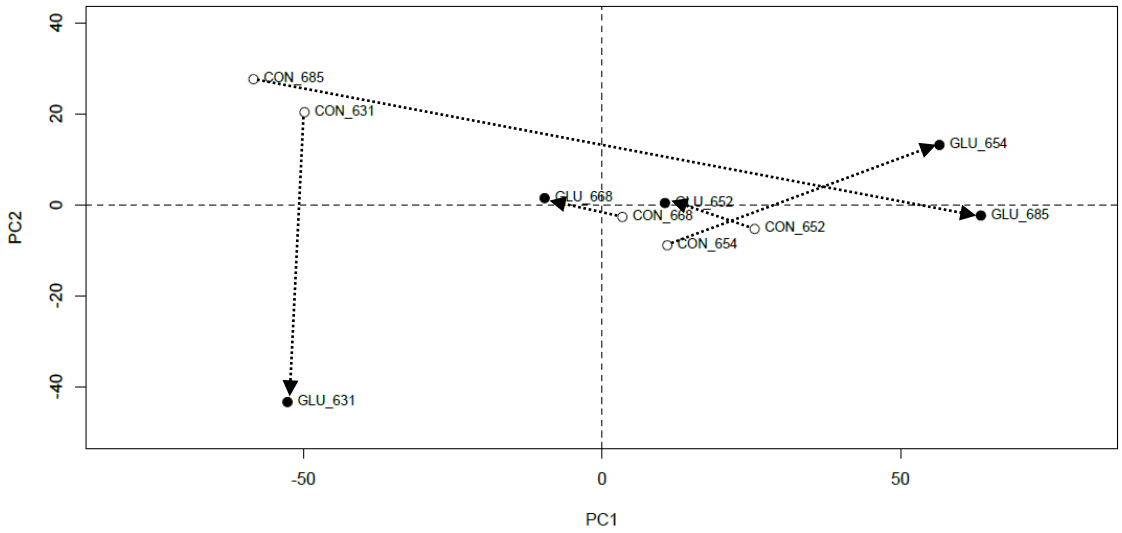

Figure 25. Principal components analysis of the 2 first principal components of plasma small components metabolome of dairy ewes (n= 5) fed control (CON) or β-glucans supplemented (GLU) diets. Arrows represent the change of individual plasma samples before or after the β-glucans supplementation.

No outlier points were observed and all paired samples (Control vs. β-glucans supplemented) showed different change pattern in each ewe. No explanation was found for this unexpected trend.

Results of the PLS-DA are shown in **Figure 26**. As it can be observed, the samples of both dietary treatments were separated in 2 clusters along the y-axis. The PLS-DA components accounted for the 75.6% of the cumulative proportion of variance, being the 58.8% explained by LV1 (x-axis) and 16.8% by LV2 (y-axis). Within component LV2 the first responsible of the variance between dietary treatments was β-glucose, which showed the highest loading (**Table 9**). So, despite the high rumen degradability measured *in sacco* conditions, barley β-glucans escaped rumen degradation and were absorbed in the intestine increasing the plasma β-glucose content in ewes supplemented with β-glucans. Moreover, ewes fed with the β-glucans supplemented diet also showed high positive loadings of histidine, acetate and choline in plasma, and high negative loads of myo-inositol and threonine when the β-glucans supplemented ewes were compared to those fed with the control diet.

The decrease in plasmatic myo-inositol agrees with a decreased muscle breakdown by effect of the β-glucans supplementation.

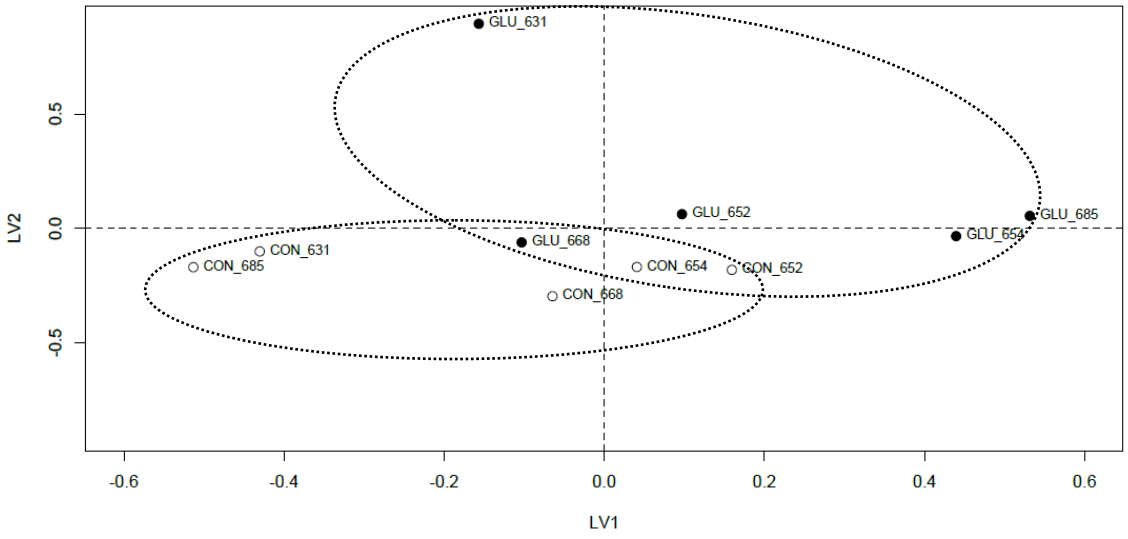

Figure 26. Partial Least Squares–Discriminant Analysis (PLS-DA) scores plot of the first 2 latent variables (LV) of 1H NMR-metabolomics general spectra of dairy ewes fed control or β-glucans supplemented diets. Each point represents an individual plasma sample.

Table 9. Plasma metabolites mainly explaining the component LV2 from the PLS-DA analysis on the effects of β-glucans supplementation on the diet of dairy ewes.

Metabolite	ppm	Loading
β-glucose	3.396	2.91
histidine	3.136	2.68
acetate	1.910	2.68
histidine	3.147	2.67
choline	3.206	2.66
myo-inositol	3.296	−2.88
threonine	3.586	−2.82
myo-inositol	3.293	−2.82
myo-inositol	3.301	−2.79
myo-inositol	3.304	−2.78

Milk

Metabolomics general pectrum of milk samples is shown in **Figure 27.** Identification of NMR peaks between 1.0 and 9.0 ppm in milk samples was done according to the ^{1}H chemical shift (ppm) reported by Sundekilde et al. (2013).

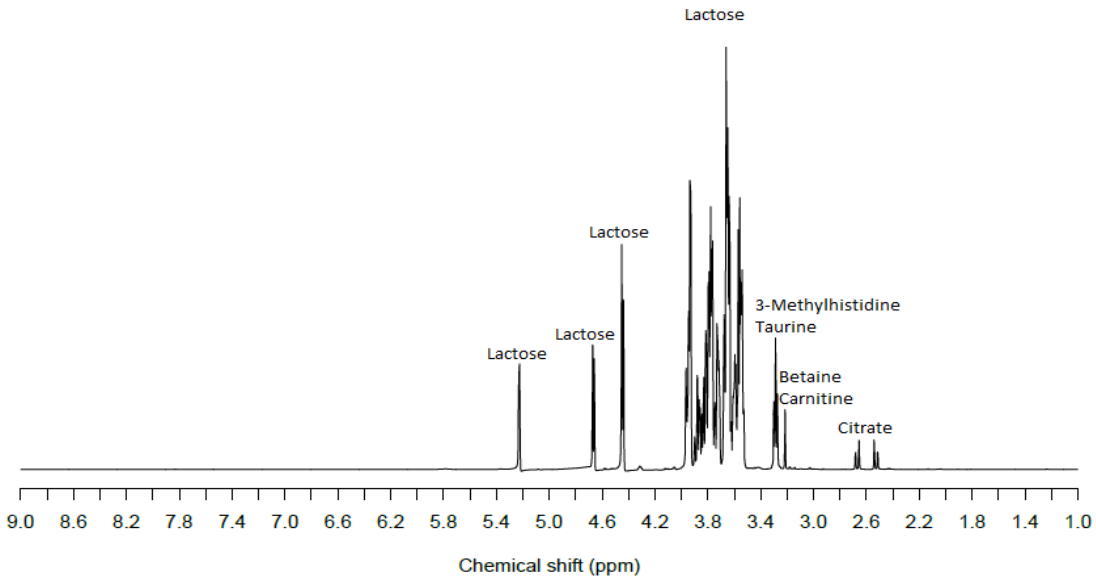

Figure 27. General ^{1}H NMR g spectrumat 600 MHz of filtrated (1 kD) ewe skimmed milk samples.

Lactose, as the most abundant component milk sugar, was placed in 17 peaks at 3.12, 3.29, 3.54, 3.59, 3.60, 3.66, 3.67, 3.73, 3.78, 3.81, 3.84, 3.88, 3.94, 3.96, 4.45, 4.67 and 5.23 ppm. Nevertheless, these large peaks of lactose could hide other metabolite peaks of less prominent compounds. Moreover, other relevant components detected were: 3-methyl histidine (3.30, 3.74 and 3.97 ppm), taurine (3.27 and 3.43 ppm), betaine (3.26 ppm), carnitine (3.21, 3.43 and 4.57 ppm) and citrate (2.52 and 2.72 ppm).

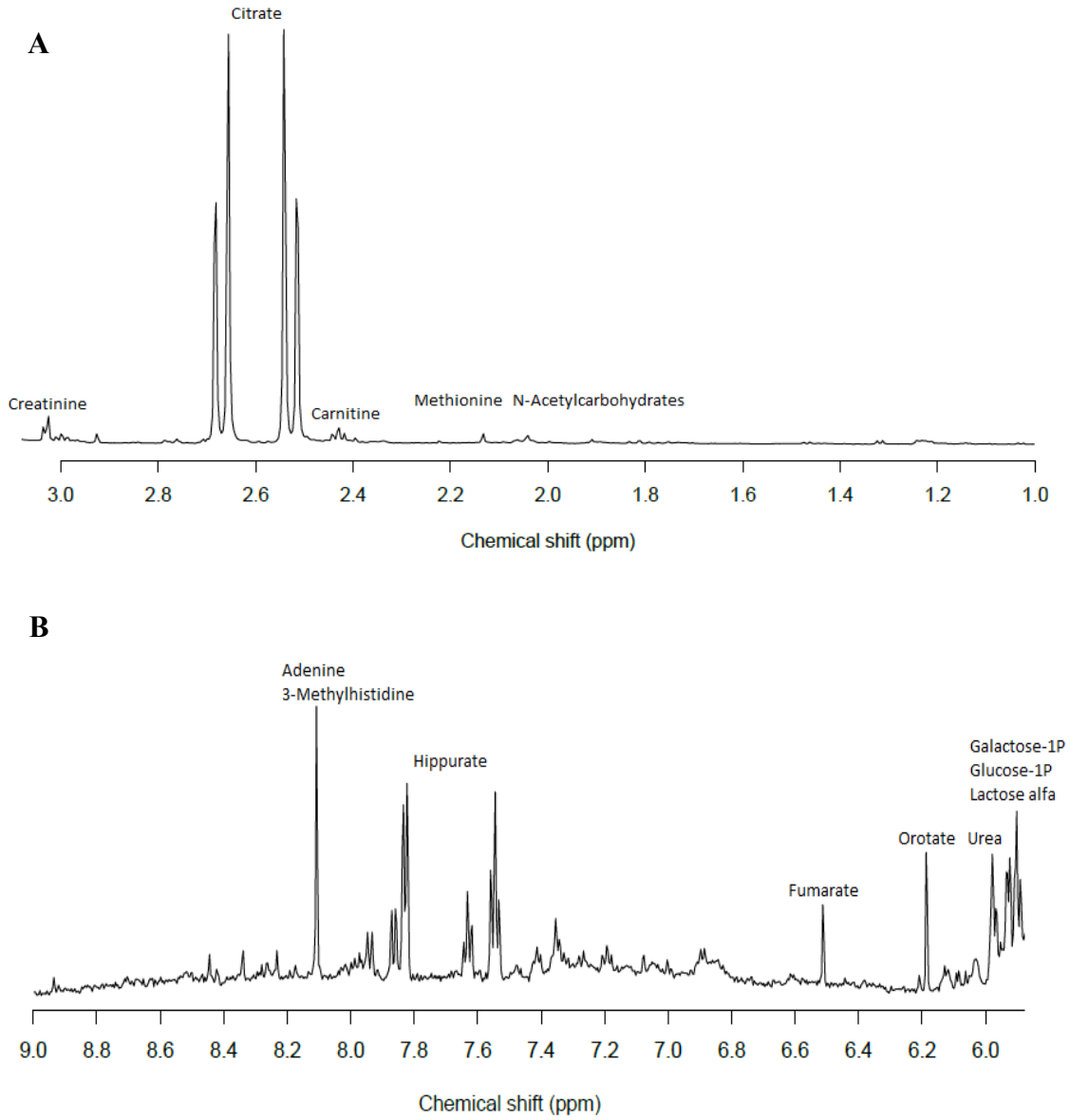

Figure 28. ^{1}H NMR g spectrum at 600 MHz of filtrated (1 kD) ewe skimmed milk samples: A) Span from 1.0 to 3.2 ppm; B) Span from 5.8 to 9 ppm.

An amplified metabolomic spectrum from 9 to 5.8 ppm (**Figure 28A**) and from 3.2 to 1 ppm ranges (**Figure 28B**) were also done in order to identify small peaks that were not visible in the Figure 27. Most relevant metabolites in Figure 28A were: creatinine (3.05 and 4.06 ppm), citrate (2.52 and 2.72 ppm), carnitine (2.44 ppm), methionine (2.15 ppm) and N-acetyl carbohydrates (2.06 ppm). For Figure 28B the relevant metabolites were: adenine (8.12 and 8.13 ppm), 3-methyl histidine (7.14 and 8.09 ppm), hippurate (7.54, 7.64 and 7.84 ppm), fumarate (6.52 ppm), orotate (6.20 ppm), urea (5.79 ppm), galactose-1P (5.38 ppm) and glucose-1P (5.51 ppm).

With regard to PCA, ascore plot of the general spectra data (1 to 9 ppm) was done for 2 first principal components to get a general overview of milk sample metabolites (**Figure 29**). The PCA components accounted for the 92.9% of the cumulative proportion of variance, being the 80.9% explained by PC1 (x-axis) and 11.9% by PC2 (y-axis).

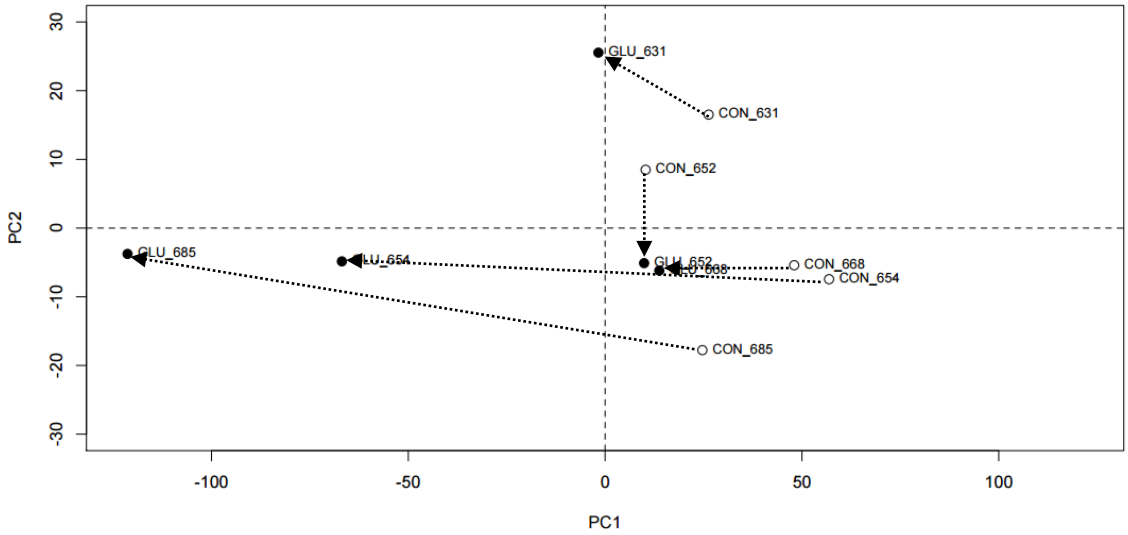

Figure 29. Principal components analysis of the 2 first principal components of milk metabolome of dairy ewes (n= 5) fed control (CON) or β-glucans supplemented (GLU) diets. Arrows represent the change of individual milk samples before or after the β-glucans supplementation.

No outlier points were observed and all paired samples (Control vs. β-glucans supplemented) showed the same change pattern in each ewe, except for ewe 652. No explanation was found for this unexpected trend.

Results of the PLS-DA and Student's t test for the paired samples are shown in **Figure 30**. As it can be observed, the samples of both dietary treatments were mainly separated in 2 clusters along the x-axis, the milk samples from ewes supplemented with β-glucans having a larger Euclidean distance from the core of the cluster than the samples from the ewes fed the control diet. For this reason, prediction of cluster core obtained by an analysis of quality (Q^2) would be dramatically affected by a cross validation procedure and, consequently, a further analysis was needed.

With this aim, the spectra were manually divided to focus on the metabolites of interest and to compute a comparison of means according to the dietary groups. Thus, 38 milk components were identified in the metabolomic analysis and compared with a paired *t*-test. Milk metabolites showing at least a tendency ($P < 0.10$) to differ between dietary treatments are shown in **Table 10**.

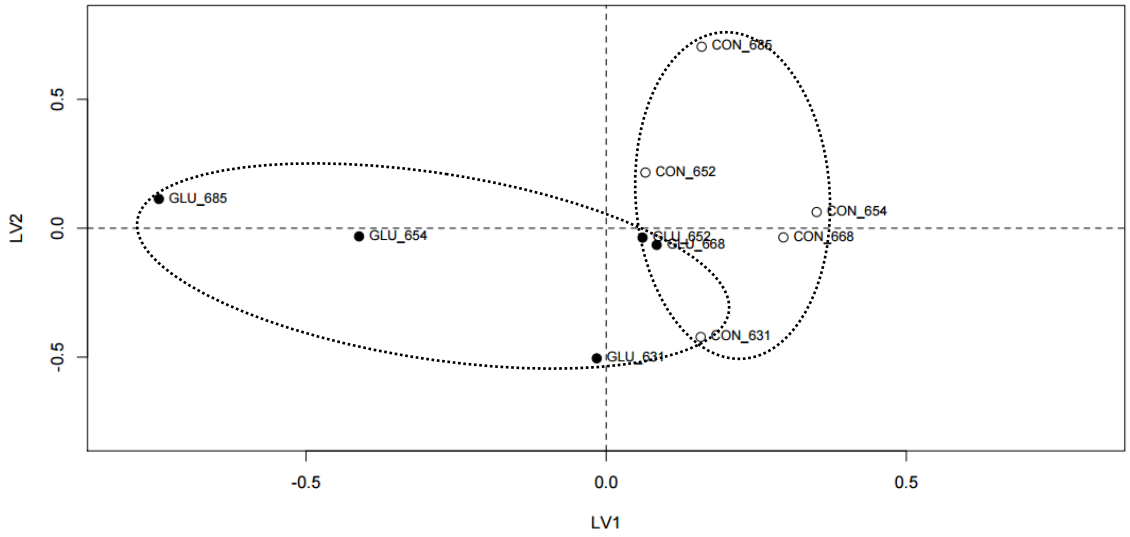

Figure 30. Partial Least Squares–Discriminant Analysis (PLS-DA) scores plot of the first 2 latent variables (LV) of 1H NMR-metabolomics general spectra of dairy ewes fed control or β-glucans supplemented diets. Each point represents an individual milk sample.

As shown in Table 10, supplementation with β-glucans decreased carnitine ($P < 0.01$), creatine ($P < 0.05$), lactose-α ($P < 0.05$), lecithin ($P < 0.05$) and 3-methylhistidine ($P < 0.05$) in milk.

Table 10. Selected metabolomic effects of β-glucans supplementation on milk composition of dairy ewes.

Milk component	Fold change[1]	Effects (*P* =)
Adenine	2.93	0.076
Alanine	0.87	0.064
Betaine	1.40	0.067
Carnitine	0.91*	0.010
Creatine	0.86*	0.022
Formate	2.97	0.074
Fumarate	2.99	0.078
Galactose-α	0.71	0.061
Glucose	2.80	0.072
Glucose-1P	2.99	0.078
Hippurate	2.56	0.079
Lactose	0.87	0.078
Lactose-α	0.87*	0.049
Lactose-β	0.87	0.053
Lecithin	0.88*	0.014
Malonic	2.82	0.092
Methionine	2,58	0.099
3-methyl histidine	0.89*	0.034
Ornithine	2.66	0.094
Orotate	3.09	0.075
Phosphocreatine	0.87	0.060
Urea	1.81	0.083
Valine	1.81	0.083

[1]Supplemented with β-glucans to control ratio.*Significant effect ($P < 0.05$).

Carnitine, creatine and 3-methyl histidine are compounds related with the energetic metabolism and degradation of muscle cells. According to Ruckebusch et al. (1991), creatine is produced in the liver and kidney from arginine and glycine (as part of the

ornithine or urea cycle), as a high-energy compound to replace ATP in the muscular cells. Carnitine is known as an enzyme involved in the oxidative processes (Madsen, 1983a;Vaz and Wanders, 2002) and specifically involved in the transport of NEFAs and acetate across the mitochondria membrane where they are oxidized. Finally, 3-methylhistidine is a biomarker of muscle breakdown (Madsen, 1983b; Rohads et al, 2013).

Specific change in lactose-α were considered no relevant given that lactose was the most important metabolite found in milk, as previously indicated, needing to relativize its change according to the total lactose changes in the spectra.

Lecithin is a molecule related to the esterification of cholesterol and also decreased in the milk of the β-glucans supplemented ewes. According to Glomset (1968), fatty acids from the n2 position of lecithin are transferred to cholesterol by lecithinase B and cholesterol esterase enzymes, which are present in high concentrations in plasma. These cholesterol esters are transported to the peripheral tissues via LDL and can enter the endothelial cell membranes. Avoiding the deposition of cholesterol on the vascular endothelium of arteries will reduce the risk of heart attacks and embolism, as indicated by Ruckebusch et al. (1991).

The decrease of lecithin is consistent with the cholesterol reduction observed in animals supplemented with β-glucans (Wood, 2007; Regand et al., 2011; Cloetens et al., 2012) and the previously mentioned hypocholesterolemic effect observed in the current study.

Urine

Metabolomics general spectrum of urine samplesis shown in **Figure 31.** Identification of NMR peaks between 1.0 and 9.0 ppm in urine samples was done according to the ^{1}H chemical shift (ppm) reported by Bouatra et al. (2013).

Urea and creatinine were the main urine components identified and were placed in 3 peaks (urea: 5.8 ppm; creatinine: 3.0 and 3.9 ppm). Moreover, other relevant components detected were: Hippuric acid (7.55, 7.67 and 7.85 ppm), sucrose (5.40 ppm), lactic acid (4.05 ppm), acetone (2.38 ppm), acetic acid (1.95 ppm) and different amino acids.

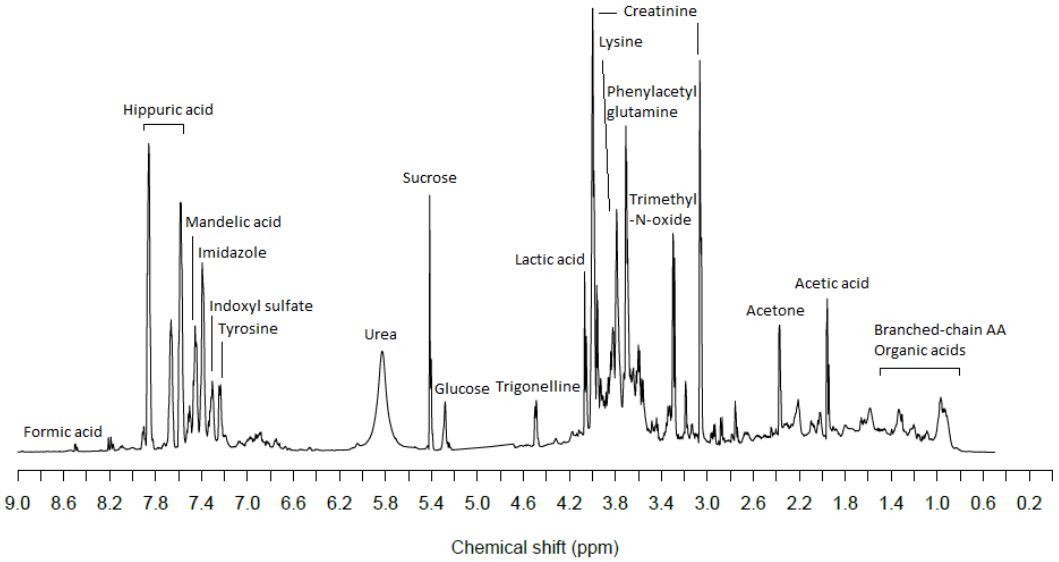

Figure 31. General ^{1}H NMR g spectrum at 600 MHz of urine samples.

With regard to PCA, a score plot of the general spectra data (1 to 9 ppm) was done for 2 first principal components to get a general overview of urine sample metabolites (**Figure 32**). The PCA components accounted for the 73.8% of the cumulative proportion of variance, being the 58.7% explained by PC1 (x-axis) and 15.1% by PC2 (y-axis).

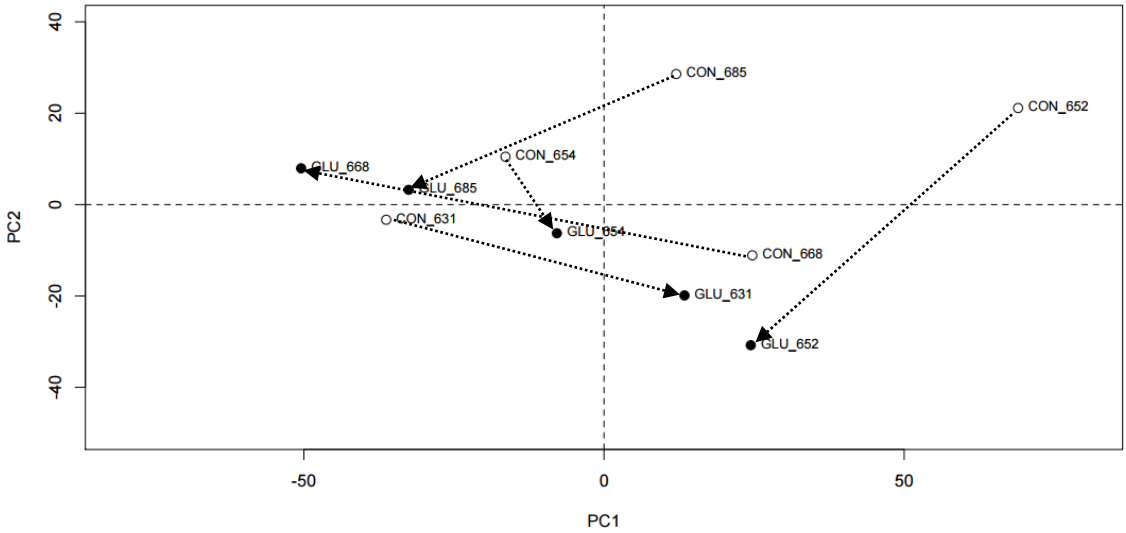

Figure 32. Principal components analysis of the 2 first principal components of urine metabolome of dairy ewes (n= 5) fed control (CON) or β-glucans supplemented (GLU) diets. Arrows represent the change of individual urine samples before or after the β-glucans supplementation.

No outlier points were observed and all paired samples (Control vs. β-glucans supplemented) showed different change pattern. No explanation was found for this unexpected trend.

Results of the PLS-DA are shown in **Figure 33**. As it can be observed, the samples of both dietary treatments were separated in 2 clusters along the x-axis and y-axis. The PLS-DA components accounted for the 71.88% of the cumulative proportion of variance, being the 50.7% explained by LV1 (x-axis) and 21.1% by LV2 (y-axis). Within component LV1 and LV2 the firsts responsible of the variance between dietary treatments were hippuric and acetic acid, respectively, which showed the highest loading. Moreover, ewes fed with the β-glucans supplemented diet also showed high positive loadings of histamine, sucrose and hippuric acid in urine, and high negative loadings of acetic acid, urea, lysine and citrate when the β-glucans supplemented ewes were compared to those fed with the control diet.

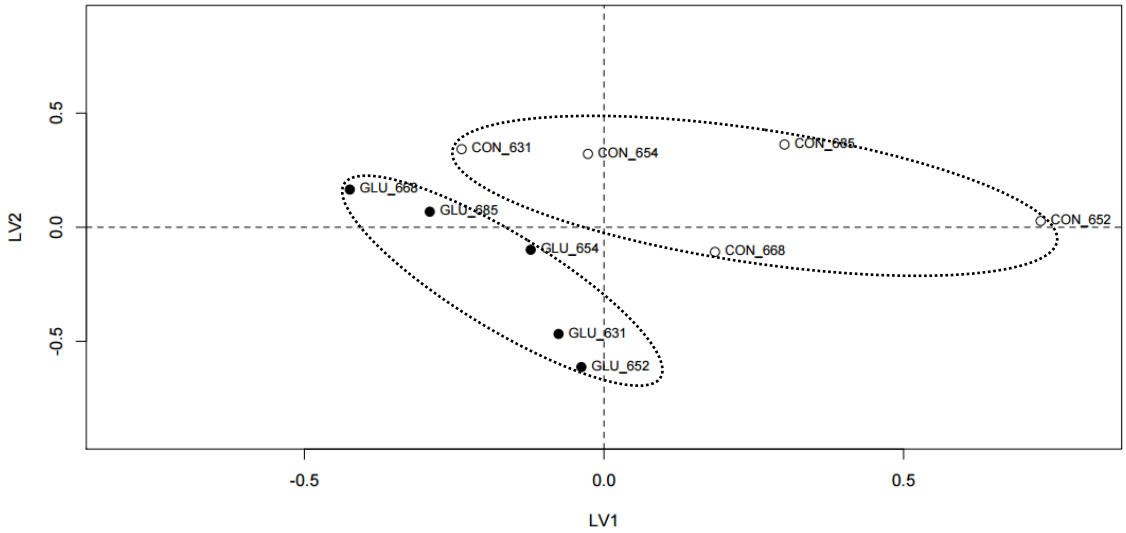

Figure 33. Partial Least Squares–Discriminant Analysis (PLS-DA) scores plot of the first 2 latent variables (LV) of 1H NMR-metabolomics general spectra of dairy ewes fed control or β-glucans supplemented diets. Each point represents an individual urine sample.

Hippuric acid is excreted daily in urine herbivores and is synthesized from dietary proteins and quinic acid, which is re-synthesis by intestinal microflora (Pero, 2010). In

other hand, acetic acid is related with some inflammatory process (Pernow and Waldenström, 1957; Bolandparvaz et al., 2004) agreeing with the better immunological status because β-glucans supplementation.

Moreover, should be mentioned that probably the sucrose detected in the general spectrum is related with the lactose excretion in urine of lactating animals (Wheelock and RooK, 1966).

V. CONCLUSIONS

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The results of the present study describe the potential (i.e. nutritive value and functional properties) of the new waxy barley varieties for lactating dairy ruminants.

First experiment: Barley varieties characterization:

- Waxy barley varieties were characterized by high crude protein and β-glucans content.
- Hull-less varieties had lower crude fiber content and higher energy value.
- All barley varieties showed high and similar DM, CP and β-glucans rumen degradability under *in sacco* conditions.
- The main fraction of barley β-glucans was immediately soluble in rumen.

Second experiment: *in vivo* effects of β-glucans supplementation in lactating diary ewes:

- No differences of barley β-glucans supplementation in lactating dairy ewes were found regarding to milk yield, milk composition and digestibility.
- Reduction in feed intake and increase of feed efficiency were observed in the ewes fed with β-glucans supplemented diet.
- With regard to the metabolic profile, plasma cholesterol and albumin were reduced in ewes supplemented with β-glucans.
- No differences were observed in plasmatic glucose and NEFA.
- Metabolites from the β-glucans metabolism (i.e. β-glucose and β(1→3)linkages) were detected in plasma of the ewes supplemented with barley β-glucans and differences between dietary treatments were found, supporting the hypothesis that they left the rumen not fully degraded and that were absorbed.
- Reduction of carnitine, lecithin, creatine and 3-metylhistidine in milk were observed in the ewes fed with β-glucans supplemented diet.
- Moreover, less acetic acid and higher hippuric acids in the urine were observed in the β-glucan supplemented ewes.
- The differences founded in milk and urine metabolic profiles were agreeing with a better nutritive and immunological status of the ewes supplemented with barley β-glucans.

- Finally, new waxy barley varieties showed higher nutritive value and positive metabolic effects at the short-term, recommending their use in dairy ruminants.

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