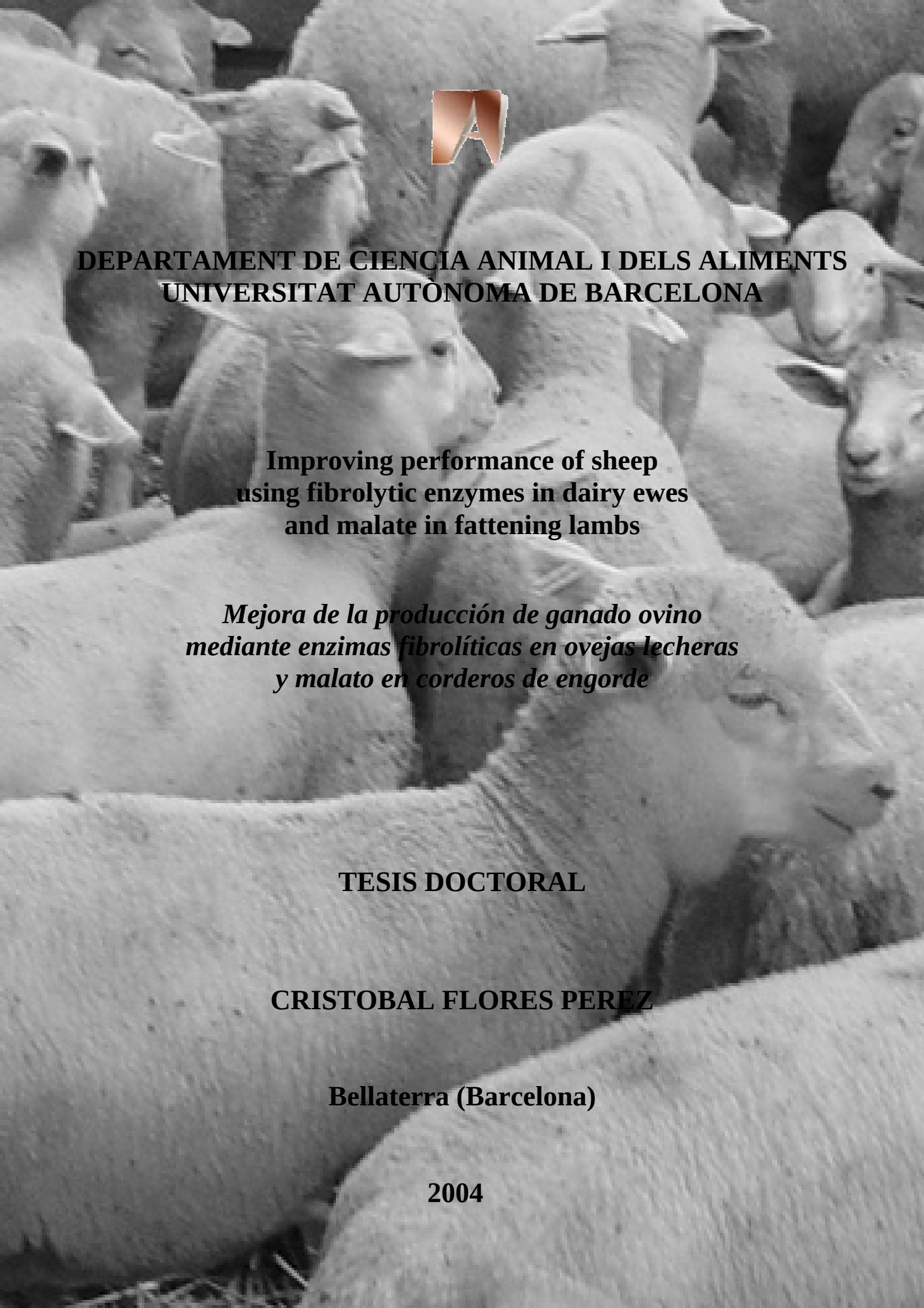

DEPARTAMENT DE CIENCIA ANIMAL I DELS ALIMENTS
UNIVERSITAT AUTÒNOMA DE BARCELONA

Improving performance of sheep using fibrolytic enzymes in dairy ewes and malate in fattening lambs

Mejora de la producción de ganado ovino mediante enzimas fibrolíticas en ovejas lecheras y malato en corderos de engorde

TESIS DOCTORAL

CRISTOBAL FLORES PEREZ

Bellaterra (Barcelona)

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Tesis Doctoral presentada por Cristobal Flores Pérez, dirigida por el Dr. Gerardo Caja López del Departament de Ciència Animal i dels Aliments de la Universitat Autònoma de Barcelona, para optar al grado de Doctor.

Bellaterra, 15 de abril de 2004

Vº Bº

Dr. Gerardo Caja López

DEDICATORIA

A la memoria de mi abuelo Valeriano, de mi tío Gilberto y de mi querida sobrina. Gracias por darme las herramientas y transmitirme su experiencia. Espero haberlas usado como ustedes me enseñaron.

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A los niños de la calle, con especial cariño para los de México.

Yo soy ese niño desagradable,
sin duda inoportuno...
de cara redonda y sucia,
que ante los grandes faroles
o bajo las grandes lámparas
bien iluminadas,
o el de las armiñas
que parecen levitar,
proyectan insulto
de su cara redonda y sucia.

Yo soy ese niño repulsivo,
que improvisa una cama...
de una vieja caja de cartón
y espera
con certeza de que tu
me harás compañía.

Si tuviera que comenzar todo de nuevo trataría, por supuesto, de evitar tal o cual error, pero en lo fundamental mi vida sería la misma... La vida es hermosa. Que las futuras generaciones la liberen de todo mal, opresión y violencia y la disfruten plenamente.

L. T. México, D. F. Febrero 27 de 1940.

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Abbreviations
Abreviaturas

(Based on the Journal of Animal Science norms)

ACL	ATP-citrate lyase
ADF	acid detergent fiber
ADG	average daily gain
AFLP	amplified restriction fragment length polymorphism
Assoc.	Association
ATP	adenosine triphosphate
BCS	body condition score
BHBA	β-hydroxybutyrate
BSE	bovine spongiform encephalopathy
BW	body weight
$BW^{0.75}$	metabolic body weight
ºC	degree Celsius
cal	calorie
CBD	cellulose-binding domain
CBP	cellulose-binding protein
CF	crude fiber
cfu	colony-forming unit
CJD	Creutzfeldt-Jacob disease
CN	casein
CoA	coenzyme A
Conf.	Conference
CP	crude protein
CSF	cerebrospinal fluid
d	day
D	dextro-
Da	dalton
DE	digestible energy
DEI	digestible energy intake
DFM	direct-fed microbial
dL	deciliter
DM	dry matter
DMI	dry matter intake
DNA	deoxyribonucleic acid
EC	European Commission
ECM	energy corrected milk
ed.	edition, editor(s)
EFSA	European Food Safety Authority
e.g.,	for example
et al.	et alia
EU	European Union
FA	fatty acids
F:C	forage concentrate ratio
FCM	fat-corrected milk
FE	fungal enzyme
g	gram

GE	gross energy
GLM	general lineal model
h	hour
i.e.,	that is
IgG	immunoglobulin G
i.m.	intramuscular(ly)
i.p.	intraperitoneal(ly)
IU	international unit
i.v.	intravenous(ly)
kDa	kilo Dalton
kg	kilogram
L	liter
LDH	lactatedehydrogenase
m*M*	millimol
M	molar
ME	metabolizable energy
mg	milligram
mL	milliliter
mo	month
n	sample size
NAD	nicotinamide adenine dinucleotide
NEFA	nonesterified fatty acid
NDF	neutral detergent fiber
NSP	non-starch polysaccharides
OM	organic matter
P	probability
pKa	the pH point of maximum buffering
ppm	parts per million
SAS	statistical analysis system
spp	several species
Suppl.	supplement
SARA	subacute ruminal acidosis
Symp.	symposium
t	metric tone (1,000 kg)
TMR	total mixed ration
TS	total solids
VFA	volatile fatty acids
µ	micron
UV	ultraviolet
vol/vol	volume/volume
vs.	versus
wk	week
×	multiplied by or crossed with

ABSTRACT
RESUMEN

The aim of this thesis was to study the effects of fibrolytic enzymes in dairy ewes and malate in fattening lambs.

A total of ninety-six Manchega and Lacaune multiparous ewes were used in three experiments to evaluate the effects of fibrolytic enzymes complex on the performance and feed intake. Ewes were fed *ad libitum* with a diet based on 70% forage and 30% concentrate to which the enzyme was added. Treatments were: control (without enzyme) and enzyme (Promote, included at 0.47 mL/kg of concentrate). In Exp. 1, a total of seventy-two ewes (Manchega, n = 36; and, Lacaune, n = 36) were used in a 2 × 2 factorial design to evaluate the effects of fibrolytic enzyme on lactational performance during suckling period (wk 1 to 4). Milk yield, milk composition, dry matter intake (DMI), lamb growth, as well as body weight (BW) change and body condition score (BCS) change, were not affected by enzyme supplementation. In Exp. 2, the ewes from the Exp. 1 were used to evaluate the effects of fibrolytic enzyme on lactation performance during milking period (wk 6 to 12). Milk yield, milk composition and DMI were not affected by enzyme supplementation, although BW change and final BCS increased ($P < 0.05$). Breed effect was significant on milk yield in both suckling and milking periods, the Manchega ewes yielding less milk ($P < 0.01$) with greater milk composition ($P < 0.01$) than the Lacaune ewes. Manchega ewes intake less DM during milking period ($P < 0.001$). In Exp. 3, twenty-four dry and open ewes (Manchega, n = 12; and, Lacaune, n = 12) were used to measure the fill value of the diet. Enzyme supplementation reduced DMI ($P < 0.001$) resulting in a greater sheep fill value ($P < 0.001$) of the diet.

On the other hand, a total of eighty-four weaned lambs (5 wk of age) were used in three experiments to evaluate the effects of malate in intensively fattened lambs. Lambs were fed *ad libitum* with barley straw and a pelleted concentrate varying on malate addition (Rumalato, 0 or 0.2%) and type of cereal (barley or corn). Treatments were: barley without malate, B0; barley with malate, CM; corn without malate, C0; and corn with malate, CM. In Exp. 1, sixty-four lambs were used in a 2 × 2 factorial design to evaluate the effects of malate on growth performances and ruminal traits at slaughter. Malate reduced ($P < 0.01$) concentrate intake, feed conversion, and ruminal parakeratosis ($P < 0.001$), and increased ($P < 0.05$) growth rate and ruminal pH at slaughter. In Exp. 2, twelve lambs were used to evaluate the effects of malate on daily variations of ruminal pH and serum metabolites at 6-h and 4-h interval, respectively. Malate increased ($P < 0.001$) ruminal pH, and decreased ($P < 0.05$) lactate, glucose, nonesterified fatty acids (NEFA,), and urea ($P < 0.01$) in serum. In Exp. 3, eight male Manchega lambs were used in a double 4 × 4 Latin square design to evaluate the effects of malate on the digestibility and energy and nitrogen (N) balances. Malate decreased total DMI ($P < 0.05$) and feed conversion ($P < 0.01$), and improved ($P < 0.001$) the digestibility of DM, OM, CP and NDF, ADF ($P < 0.05$), and GE ($P < 0.01$). As a result retained energy, and DE and ME estimation increased ($P < 0.01$). Interaction malate × cereal was significant in most of cases, and the effects of malate were more marked in the barley-based concentrates than in the corn ones. In conclusion, no lactational effects were detected when the fibrolytic enzyme complex was added to the concentrate in dairy ewes, but BW and BCS improved as an effect of the enzyme complex in late lactation. Use of malate (0.2%) is recommended as a feed additive for intensively fattening lambs and can be used to replace antibiotics as growth promoters in practice.

RESUMEN
ABSTRACT

El objetivo de esta tesis fue estudiar los efectos del uso de enzimas fibrolíticas en ovejas lecheras y de malato en corderos de engorde.

Se usaron un total de noventa y seis ovejas raza Manchega y Lacaune en tres experimentos para evaluar los efectos de un complejo de enzimas fibroliticas en la producción de leche y en la ingestion. Las ovejas fueron alimetadas *ad libitum* con una ración basada en 70% forraje y 30% concentrado al cual se adicionó la enzima. Los tratamientos fueron control (sin enzima) y enzima (Promote, incluido al 0.47 mL/ de concentrado). En el Exp. 1, se usaron setenta y dos ovejas (Manchega, n = 36; and, Lacaune, n = 36) en un diseño factorial 2 × 2 para evaluar los efectos de las enzimas fibrolíticas en la producción de leche durante el período de cría (sem. 1 a 4). La producción y composición de leche, ingestión de materia seca (MSI), crecimiento de los corderos, así como el cambio de peso vivo (PV) y de condición corporal (CC), no fueron afectados por la suplementación con enzima. En el Exp. 2, se usaron las ovejas del Exp. 1 para evaluar los efectos de las enzimas en la producción de leche durante el periodo de ordeño (sem. 6 a 12). La producción y composición de la leche, así como la MSI de la ración, no fueron afectados por la suplementación con enzima, aunque el cambio de PV y la CC final aumentaron ($P < 0.05$). El efecto de la raza fue significativo en la producción de leche en ambos períodos de cría y ordeño, las ovejas Manchega produjeron menos leche ($P < 0.01$) pero con una mejor composición ($P < 0.01$) que las ovejas Lacaune. Las MSI de las ovejas Manchegas el periodo de ordeño ($P < 0.001$) fue menor que la de las Lacaunes. En el Exp. 3, se usaron veinticuatro ovejas secas y vacias (Manchega, n = 12; y Lacaune, n = 12) para medir el el valor lastre de la ración. La suplementacion con enzima redujo la MSI ($P < 0.001$), resultando en un mayor valor lastre de la ración en ovino.

Por otro lado, se usaron ochenta y cuatro corderos destetados (5 sem. de edad) en tres experimentos para evaluar los efectos del malato en corderos en cebo intensivo. Los corderos fueron alimentados *ad libitum* con paja de cebada y concentrado granulado el cual varió según la adición de malato (Rumalato, 0 o 0.2 %) y el tipo de cereal (cebada o maíz). Los tratamientos fueron: cebada sin malato, B0; cebada con malato BM; maíz sin malato, C0; y maíz con malato, CM. En el Exp. 1, se usaron sesenta y cuatro corderos en diseño factorial 2 × 2 para evaluar los efectos de malato en el crecimiento y características ruminales al sacrificio. El malato redujo ($P < 0.01$) la ingestion de concentrado, conversión alimenticia y la paraqueratosis ruminal ($P < 0.001$), y aumentó el índice de crecimiento y pH ruminal al sacrificio ($P < 0.05$). En el Exp. 2, se usaron doce corderos para evaluar los efectos del malato sobre las variaciones diarias de pH ruminal y metabolitos en suero a 6 y 4 h de intervalo, respectivamente. El malato aumentó ($P < 0.001$) el pH ruminal, y disminuyó ($P < 0.05$) el lactato, glucosa, NEFA, y urea ($P < 0.01$) en suero. En el Exp. 3, se utilizaron ocho corderos machos de raza Manchega en un diseño en cuadrado Latino 4 × 4 repetido para evaluar los efectos de malato sobre la digestibilidad y balances de energía y nitrógeno. El malato disminuyó la MSI ($P < 0.05$) y conversión alimenticia ($P < 0.01$), y mejoró ($P < 0.001$) la digestibilidad de MS, MO, PC, y la de FND, FAD ($P < 0.05$) y EB ($P < 0.01$). En consecuencia aumentó la energía retenida ($P < 0.01$), la energía ED y la EM estimadas ($P < 0.01$). La interacción malato × cereal fue significativa en la mayoría de los casos, y los efectos de malato fueron más marcados en los concentrados basados en cebada que en los de maíz.

En conclusión, no se detectaron efectos en la producción de leche por la adición del complejo de enzimas fibrolíticas al concentrado en ovejas lecheras, pero el PV y la CC mejoraron como efecto del complejo de enzima al final de la lactancia. Se recomiendan el empleo de malato (0.2 %) como aditivo en el alimento para corderos en cebo intensivo y como alternativa para sustituir los antibióticos promotores de crecimiento en la práctica.

Chapter 1: Introduction

Capítulo 1: Introducción

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According to FAO, there are approximately one thousand million (1,024,007,820 heads) sheep in the world producing approximately 7,734,207 t of meat and 7,886,217 t of milk (FAOSTAT, 2003). This represents 3.1% and 1.3% of the total meat and milk produced in the world. Sheep in the European Union (EU-15) produced 67,400,072 t of meat and 1,265 t of milk in 2003. Mediterranean countries produce a total of two-thirds of the world's sheep milk (Ronchi and Nardone, 2003), which is of particular importance in the economy of some EU countries such as Italy, Greece, Spain, France, and Portugal (Haenlein, 2001), which produce 39, 29, 15, 11, and 4.6% of the total EU sheep milk, respectively.

In Spain, from a total of 24,399,645 sheep, approximately 3,604,860 are dairy breeds producing 306,000 t of milk (MAPA, 2002), mainly for the industrial (93%) and artisanal (6%) manufacture of cheese (25,000 t) and other dairy products (MAPA, 2002). Lambs are produced as milk fed lambs ('lechal') or young fattened lambs ('Pascual') according to breed and local production system.

Since the ban on using protein sources from animal origin (blood, meat, bone and entrails) on December 4 of 2000 (Rodríguez Ferri, 2001), forages (legumes and grass), cereals and oilseeds have been the most important sources of energy and protein for ruminant production.

Although, forages are generally the cheapest form of feeding ruminants, they can limit sheep performances when used as the only feed source. Small ruminants consuming forages may not have enough energy intake for optimum milk yield during lactation or lamb growth.

Phenol acids and lignin deposited in the maturing cell wall, can limit digestibility, intake and energy available in forages (Jung and Allen, 1995), because lignin protects structural carbohydrates from being utilized by rumen bacteria and fungi (McSweeney *et al*., 1999). Moreover, forages *per se* can limit the voluntary dry matter intake (DMI) as a result of the restricted flow of digesta through the gastrointestinal tract (Allen, 1996).

In order to increase the energetic value of ruminant diets and to improve productivity, the use of cereals is a common practice in feedlots. Nevertheless, a high percentage of cereals in the diet (> 60%) has some inconveniences including: increment in the cost of animal diets, competition between animals and humans for cereals (especially in under-developed countries), higher incidence of digestive problems (off-feed condition, acidosis, bloat, and a reduction of fiber digestion), changes in milk composition (low milk fat percentage), and

changes in carcass quality (soft fat). Of these, the main problems are bloat and rumen acidosis, which are responsible for approximately 28 and 33% of lamb deaths in USA feedlots (NAHMS, 2001).

Animal nutrition science in concordance with biotechnology and the feed industry has made a great effort to optimize the digestion of forages and cereals in the digestive tract. Manipulation of rumen fermentation by using feed additives has been a key point in ruminant nutrition and a frequent topic of study for many years. Antibiotic additives have proved to be effective in the production of ruminants, but their use is increasingly worrying, due to the resistance that they can confer to certain bacteria, which affect the respiratory and digestive tract of the animal and human beings.

Antibiotic additives, such as virginiamicyn, avoparcin, and ionophores (especially monensin, lasalosid, and salinomycin), have been extensive studied in ruminants due to their properties for improving feed efficiency and weight gain in high-concentrate based diets. However, avoparcin and virginiamicyn were prohibited as growth promoters in Sweden and Denmark in 1997, for awarding resistance in bacteria to antibiotics used in human therapies (Anadón and Martínez-Larrañaga, 1999). In 2003 the European Food Safety Authority (EFSA) regulated the use of antibiotics (Regulation EC 1831/2003) as additives and established a restriction period for avilamicin, flavophospholipol, sodium monensin, and sodium salinomicyn until 2006.

Alternatives to the use of antibiotic feed additives in ruminants are, among many others, the use of fibrolytic enzymes complexes and salts of organic acids. The fibrolytic enzyme complexes and their use in mono-gastric nutrition (poultry and pigs) to remove anti-nutritional factors (phytic acid) and toxins, increasing nutrient digestibility and performances have been extensively studied. Moreover, they also reduce the nutrient content (i.e., nitrogen and phosphorus) in animal excreta (Campbell and Bedford, 1992; Brufau *et al*., 1994; Zhang *et al*., 2003). Some commercial presentations of enzymes are available for use in ruminant diets, but their effects on milk and meat production are under study.

Some studies have reported that fibrolytic enzymes improve DMI and milk yield in dairy cows fed forage-based diets (Schingoethe *et al*., 1999), or in dairy cows fed concentrate-based diets (Sanchez *et al*., 1996). However, other authors reported no effects (Fredeen and McQueen, 1993; Sutton *et al*., 2003) or even negative effects (Dhiman *et al*., 2002; Knowlton *et al*., 2002). Currently, the effects of the method of application and diet inclusion portion, and dose of enzyme complex for dairy cows are known, but, to our knowledge, only one study on low yielding dairy sheep has been published (Titi and Lubbadeh, 2004).

Whit regard to organic acids, malic acid appears on the list of additives allowed by the European Commission (EC, guideline 70/524/CEE, E296). Most studies with malic acid and malate have been done *in vitro* and *in vivo* in cattle (dairy cows, and beef steers), but few studies have been carried out in goat (dairy goats) and in sheep (fattening lambs).

If fibrolytic enzyme complexes are capable of improving digestibility in the total gastrointestinal tract (Beauchemin *et al*., 1999b) and malate has similar effects to ionophores antibiotics (i.e., increase pH, propionate, and decrease lactate and methane concentrations in ruminal fluid) *in vitro* (Martin and Streeter, 1995; Callaway and Martin, 1996), these additives may be a useful alternative for dairy ewes fed high forage-based diets, and malate for lambs in intensively fattened conditions, respectively.

When enzymes or organic acids are administered to animals they are metabolized in the rumen or in the posterior digestive tract, and so would not leave residues in the animal products destined for human consumption. Until now, no negative effects attributed to the use of these additives have been reported in human or animal health. More studies are needed to allow a safe and effective recommendation of these types of feed additives in sheep production.

Chapter 2: Bibliographic review

Capítulo 2: Revisión bibliográfica

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2.1. Role of forages and cereals in ruminant nutrition

2.1.1. Forages

Carbohydrates are the main component of ruminant diets, and the primary source of energy in forage based diets (Wang and McAllister, 2002). Forages are the largest component in dairy rations and contribute between 40 and 70% of the net energy used for milk production (Harris, 1993).

In many areas of the world, forages are used as a unique source of feed for ruminants due to their abundance and low cost. However, their availability and quality are not constant throughout the year. Ruminants in the tropics and sub-tropics suffer marked seasonal fluctuations in pasture supply and quality and, as a result, their weight can only be maintained or reduced. Moreover, the digestion of forages in the rumen is relatively slow and incomplete, limiting animal performance and increasing the cost of livestock production. Tropical forages, in particular, have limited energy value and cell wall rich in lignin, silica and cutin, which limit fermentation of structural carbohydrates, and therefore the production of volatile fatty acids (VFA) and microbial mass in the rumen (Dominguez Bello and Escobar, 1997).

2.1.2. Cereal grains

Approximately 2,067 Mt of cereals are produced in the World (FAOSTAT, 2003). Cereals are grown over 70% of the total world harvested areas and contribute over 60% of world food production (Charalampopoulos *et al*., 2002). Although grains make a significant contribution of protein to ruminant diets, the major nutrient furnished is energy, which is primarily derived from starch with smaller amounts coming from lipids, non-essential amino acids, free sugars and non-starch polysaccharides (NSP) (Newman, 1994).

Due to the constant demand of food for a growing population, ruminants are now fed a diet that has a higher percentage of cereals than of forage in order to reduce the days on feed and to accelerate their production. The value of cereals as a feed for cattle is usually explained by their ability to provide more energy for microbial growth and animal metabolism. However, large amounts of cereal grain without a correct animal adaptation can produce metabolic disorders in ruminal fermentation resulting in negative effects on animal performances.

Cereals such as barley, wheat, and corn, are the main sources of energy in feedlot cattle diets. In Europe (Bacha, 2002), North America (Cheng *et al*., 1998) and Canada (Ghorbani *et al*., 2002), finishing cattle diets typically consist of 85 to 90% grain and 10 to 15% forage DM.

2.2. Components of forages and cereals

The cell wall of forages and cereals has received attention from a nutritional point view, not only because it contains nutrients (polysaccharides, vitamins, and minerals), but also, substances that make difficult the digestion of nutrients in the rumen. There are complex semi-rigid structures that surround the cytoplasmic membrane of the cell wall, which may be divided into primary and secondary (Theander, 1989; Reiter, 2002). Plant cell walls are mainly composed of sugars (rhamnose, fucose, arabinose, xylose, mannose, galactose, glucose, and galacturonic and glucuronic acids) arranged as complex polysaccharides with hydroxycinnamic acid, lignin, proteins, ions and water, varying in composition and structure. These polysaccharides can be grouped analytically into cellulose, hemicellulose and pectin fractions (Wang and McAllister, 2002). In cereal grains, the fiber content is usually determined as crude fiber (CF), and reflects the proportion of hull, pericarp and testa and the polysaccharide complex contained within the outer layers of the grains. Average values in maize, sorghum, barley, and oats are 2, 2, 5, and 10 g/100 g DM, respectively (Lawrence, 1988).

2.2.1. Cellulose

Cellulose is the most abundant organic molecule in Nature and consists of linear chains of several thousand glucose units, linked by β(1-4) glycosidic bonds (Theander, 1989; Uhlig, 1998). Cellulose is the basic element of the cell walls. It is present in the hulls of legumes and oilseeds (40 to 50% of DM), forages and sugar beet pulps (10 to 30% of DM), oilseeds and legume seeds (3 to 15% of DM), and grain cereals (1 to 5% of DM) (Giger-Reverdin, 1995).

2.2.2. Hemicellulose

Hemicellulose is formed by two analytical fractions, alkali soluble (α-cellulose) and acid soluble (β-cellulose) (Uhlig, 1998). Hemicellulose constitutes 10 to 25% of DM in forages and agro-industrial by-products (bran, oilseeds and legume seeds, hulls and pulps), and from 2 to 12% of DM in grains and roots (Giger-Reverdin, 1995). The most representative

hemicellulose groups founded within the cell walls are: xylans, arabinoxylans, mannans, galactomannans, glucomannans, xyloglucans, glucuromannans, arabinogalactans, and β-1,3-glucans (Heredia *et al*., 1995).

Xylans constitute the more abundant non-cellulosic structural polysaccharide (20 to 30% of DM) in angiosperms (Rodríguez-Palenzuela *et al*., 2000), wood and agricultural wastes (20 to 35% by weight) (Chen *et al*., 1997). They also constitute the major component of the cell wall in gramineous (Heredia *et al*., 1995; Matsui *et al*., 1998). Xylans are linked by acetic and phenolic acids such as ferulic and *p*-cumaric (Bhat and Hazlewood, 2001). Xyloglucans are the most important constituent of hemicellulose in the primary wall of dicotyledonous (Matsui *et al*., 1998). The β-1,3-glucans are mainly present in barley (3 to 11% of DM) and oats (3 to 7% of DM), but are lower in wheat (usually < 1% of DM) as indicated by Charalampopoulos *et al*. (2002).

2.2.3. Pectin

Pectin is a complex mixture of colloidal polysaccharides (Theander, 1989; Heredia *et al*., 1995) found in the middle lamella of the plant cell wall (Hall, 1998). Pectins constitute approximately 35% of cell wall of dicotyledonous and are rapidly and extensively degraded by rumen microbes (Rodríguez-Palenzuela *et al*., 2000). The most representative group of pectins in the cell wall are: rhamnogalacturonans I, rhamnogalacturonans II, arabinans, galactans, arabinogalactans I, arabinogalactans II and D-Galacturonans (Heredia *et al*., 1995).

2.2.4. Starch

Starch is the second most abundant polysaccharide in Nature, representing about 0.5% of the total biomass (Uhlig, 1998) and the main storage polysaccharide in forages and seeds (Hall, 1998). Cereal grains are 70 to 90% composed of starch (Newman, 1994), most of which is content in the endosperm (Lawrence, 1988). Starch averages 77% in wheat, 72% in corn and sorghum, and 57 to 72% in barley and oats, respectively (Huntington, 1997). The starch granule of cereal grains varying in size from <1 to >100 μm (Martin and Smith, 1995) and can be chemically fractioned in two groups: amylose, representing 22 to 28 g/100 g of starch (Lawrence, 1988), and amylopectin that approximately makes up 70% of starch (Martin and Smith, 1995).

2.2.5. Proteins

The proteins of the cell wall are classified into two large groups (Theander, 1989; Heredia *et al*., 1995): structural proteins (hidroxyproline and extensine, representing more than 20% of the amino acids content, and makes up 2 to 10% of the primary cell wall), and enzymes, which are represented in forages by peroxydase and hydrolases (cellulases, pectinases and glycosidases).

Cereal grains contain enzymes that can influence their solubilization and viscous properties, and include: amylolytic (α-amylase, β-amylase, glucoamylase, and pullulanase); cellulases and hemicellulases (cellulase, laminarinase, lichenase, endo-1,4-β-D-xylanase, α-L-arabinosidase, and ferulic acid esterase); proteases; lipases and esterases (lipase, lysophospholipase); 6- or 3-phytase; and oxydases (lipoxygenase, glucose oxydase), as indicated by Poutanen (1997).

2.2.6. Lignin

Lignin is the third most common constituent of cell wall. It is present in young (< 5% of DM) and in mature (> 12% of DM) forages (Giger-Reverdin, 1995). Lignin is a three-dimensional network built up by phenylpropane units, whose precursors are coniferyl, sinapyl and *p*-coumaryl alcohols, which are transformed into lignin by a dehydrogenative polymerization process (Theander, 1989).

2.2.7. Cellular content

Cellular content is an important fraction of forages and more digestible than cellulose and hemicellulose (Hodgson, 1990). It is composed for organic acids, soluble carbohydrates, crude protein, fat and soluble ash (Minson, 1990). Organic acids are not carbohydrates *per se*; they are derivatives or precursors of carbohydrates, which generally come from two main sources: growing plants and fermentation acids. The organic acids are intermediates of the citric acid and include: citrate, malate, quinate, succinate, fumarate, oxalate, shikimate, *trans*-acoinate, and manolate (Hall, 1998).

2.2.8. Lipid and mineral fraction

Cereal starches also contain lipids (lysophospholipids), making up to 1.5% of total weight (Tester and Karkalas, 2001). They are associated with carotenoids and tocopherol (Lawrence,

1988). Regarding mineral elements, the most important in cell walls are calcium, phosphorus and silica. Silica has been founded in grasses (0.6 to 2.8%), straws (0.5 to 3.4%) and legumes (0.08 to 0.4%), and in cereal plants it is found mainly in the leaves and seed germ (Theander, 1989). Approximately 60 to 65% of the phosphorus present in cereal grains exists as phytic acid (myoinositol hexaphosphate), and also is the major storage for the phosphate in plants. Phytic acid is considered antinutritional because it forms complexes with minerals such as iron and zinc preventing their assimilation by monogastric animals (Lyons, 1994).

2.3. Degradation of forages and cereals in rumen

The ruminant performance depends on the activities, ability, and capacity of their microorganisms to degrade and utilize the dietary feeds. Although substrate competition is high in the rumen, the synergism and symbiosis among microorganisms make more efficient the utilization of substrates.

The rumen is a highly complex ecosystem, which contains different microbial species with a great potential for microbial associations. Many relationships are known to exist among microorganisms in the rumen (Lee *et al*., 2000b). The microbial ecosystem comprises at least 30 bacterial species (10^{10} to 10^{11}/mL of rumen fluid) (Stewart *et al*., 1997), 40 species of protozoa (10^5 to 10^7/mL of rumen fluid) (Williams and Coleman, 1997), and 5 species of fungi ($< 10^5$/mL of rumen fluid) (Orpin and Joblin, 1997). Bacteria, fungi, and protozoa are the microorganisms involved in most plant cell wall digestion in the rumen and are responsible for 50 to 82% cell wall degradation *in vitro* (Lee *et al*., 2000b). Bacterial species are considered more important than protozoa and fungi in determining the extent and rate of feed degradation and utilization for the production of microbial protein and VFA (Stewart *et al*., 1997).

Rumen microorganisms ferment the polysaccharides (cellulose, hemicellulose and starch) to short-chain VFA, which are then used by the host, providing energy and carbon for the growth and maintenance of the microbial community (Wolin and Miller, 1997). The main VFA present in the rumen are acetic, propionic and butyric acids, at molar proportions of 70:20:10 with a hay based diet, and of 50:35:15 with a concentrate based diet, respectively (Rémond *et al*., 1996). Subsequently, the host absorbs VFA (mostly through the rumen wall) and digests proteins, lipids, and carbohydrate constituents of microbes and feed residues entering the small intestine to supply maintenance requirements and for the production of meat and milk (Miron *et al*., 2001).

During the fermentation of the cellulose and hemicelluloses, several species of cellulolytic bacteria and all species of anaerobic fungi are able to produce hydrogen. This gas does not build up, and traces of hydrogen (< 0.2% of the gas phase) are found in the rumen. The hydrogen is mainly utilized by *Archaea methanogens*, which represent the main hydrogenotrophic bacteria of the ruminal ecosystem (Morvan *et al*., 1996).

In the rumen, hydrogen, carbon dioxide and other substrates including formiate, acetate, methylamine and methanol (produced from the demethylation of plant polymers) are mainly used to produce methane (Stewart and Bryant, 1997). The bacteria assumed to play the most significant role in ruminal methanogenesis are *Methanobrevibacter ruminantium*, *Methanosarcina barkeri*, *Metanobacterium formicicum*, and *Metanomicrobium mobile*, which are present at a level of 10^6/ml in the rumen digesta of grazing animals (Stewart and Bryant, 1997; Jarvis *et al*., 2000).

Protozoa plays an important role in methane production, particularly when cattle are fed with high-concentrate based diets (Johnson and Johnson, 1995). There is also a close relationship between ruminal methanogens and ciliate protozoa such as *Polyplastron multivesiculatum*, *Isotricha prostoma*, *Oprhyoscolex caudatus*, and in a low quantity *Entodinium* spp. (Tokura *et al*., 1999).

Methane production has been estimated to represent a loss of 2 to 12% of the gross energy (GE) of feed in cattle (Johnson and Johnson, 1995; Jouany, 1994; Dominguez Bello and Escobar, 1997) but is most serious in animals fed forage (Johnson and Johnson, 1995). Pelchen and Peters (1998) estimated that methane emissions from growing and adult sheep are 23.2 (7.2% GE) and 20.5 g/d (7.2 % GE), respectively. Those authors observed that methane emission rises with increasing CF content (> 18% DM) in the ration. As a consequence, methane in forages is higher than in concentrates.

The differences between cereal and forage methane production, can be explained in two ways: 1) methanogenic bacteria need the hydrogen derived from the synthesis of acetate, which is related to the cellulose in the ration (Pelchen and Peters, 1998); and 2) differences in ruminal pH, in which the methane production decreases from 48 to 7 nmol/mg of protein at pH values from 6.5 to 5.7 (Lana *et al*., 1998).

Reduction of methanogenesis in ruminants is particularly important because it represents a loss of feed energy and increases the greenhouse effect in the world.

2.4. Factors that affect feed digestibility

According to Minson (1990) factors that may affect forage digestibility include: specie differences, cultivar differences, plant parts, stage of growth, soil fertility, nitrogen and mineral content (such as S, P, Ca, K, and Mg), climate, processing, and detrimental factors (silica, tannins, and essential oils). Also the growing conditions (weather, soil, cultural practices, developmental stage, and variety) may influence the quality of forages (Albanell *et al*., 1995). Plant digestibility is generally more dependent on the vegetation stage, decreasing with the age of the plant. As the plant aged the amount of cell content decreases and the amount of cell wall increases (Baumont *et al*., 2000). The environment can also affect the amylose and amylopectin structure, composition, physicochemical properties, hydrolysis, and starch digestibility in cereals (Tester and Karkalas, 2001).

Forages and cereals contain natural compounds that have negative effects on feed digestibility and/or gastrointestinal microflora that may limit the animal performance or produce fatal toxicosis. Among those substances are: alkaloids, glycosides, steroids and terpens, simple acids and their salts, proteins and amino acids, polyphenols, and micotoxins (Mackie and White, 1990), arabinogalactans (Weimer, 1998), minerals such as Cu (Salam Abdullah and Rajion, 1997), silica (Giger-Reverdin, 1995), and lignin (Theander, 1989). Forage degradability has been correlated particularly with silica content (Nakashima and Ørskov, 1990). Arabinogalactans prevent cellulolytic bacteria from adhering to cellulose and alkaloids inhibit certain ruminal microbial processes (Weimer, 1998). Coniferaldehyde alcohol is an inhibitor of cell wall degradation for the fungal enzymes (Grabber *et al*., 1998). Lignin has no digestible energy value (Jung *et al*. 1999), resists the ruminal fermentation, and is the main factor responsible for the difficulty in degrading plant fiber (Theander, 1989). Cooper, mimosine, tannins and steroidal saponins, contained in forages (grasses, legumes or trees) and in agroindustrial by-products, may affect the voluntary intake and the entero-hepatic function of the animals (Salam Abdullah and Rajion, 1997).

In cereal grains, internal barriers to digestion are also encountered. The epidermal plant cells are composed of highly structured cellulose and hemicellulose, which constitute a physical barrier to bacterial penetration. On the other hand, individual starch grains within endosperm cells are surrounded by a protein matrix of aleurone (e.g., gluten in wheat grains), which surrounds the grains completely in some cases (e.g., hard wheat) or incompletely in others (e.g., soft wheat) (Cheng *et al*., 1991). In sorghum and maize, the aleurone layer surrounding the starch granules severely limits bacterial access to the starch affecting its

digestibility. The protein matrix in the horny endosperm is extremely resistant to the attachment, or promotes the detachment of ruminal bacteria (McAllister *et al*., 1994).

Moreover, the rate and extent of starch digestion in the rumen are determined by several factors, such as: source of dietary starch, diet composition, and feed consumed per unit of time, mechanical alterations (e.g., grain processing, chewing), chemical alterations (e.g., degree of hydration, gelatinization), and degree of adaptation of ruminal microbiota to the diet (Huntington, 1997). Physical processing (e.g., grinding, rolling, etc…) increases the rate and extent of ruminal digestion of starch, which reduces the amount of starch available for digestion in the small intestine. The rumen microorganisms can digest from 60 to 90% of the starch, depending on the type of processing and the species of cereal grain. However, only a small proportion of the starch in these diets is actually digested by the enzymes produced by ruminants (McAllister and Cheng, 1996).

Additional to the forage factors, those related to ruminal microflora may also alter the efficiency of utilization of plant fiber by ruminants. They are kinetics of ruminal digestion, nature and population densities of the predominant species of microorganisms, and enzymatic action of fibrolytic microorganisms on the cell wall carbohydrates (Mackie and White, 1990).

2.5. Role of ruminal microbiota on the degradation of forages and cereals

2.5.1. Bacteria

The first step in feed degradation is the adhesion of bacteria to the substrate. The adhesion to cellulose ranges from 63 to 87% for *Ruminococcus albus*, 57 to 82% for *Ruminococcus flavefaciens*, and 25 to 67% for *Fibrobacter succinogenes*. The adherence of *R. flavefaciens* and *R. albus* is greater than for *F. succinogenes*, but little or no competition for adhesion is observed between *R. flavefaciens* and *F. succinogenes* (Mosoni *et al*., 1997), indicating that these two species may have different adhesion sites.

The adhesion process of the predominant cellulolytic bacteria can be divided in four phases (Miron *et al*., 2001): 1) transfer of the bacteria to the substrate; 2) nonspecific adhesion of bacteria to unprotected sites of the substrate with bacterial glycocalyx; 3) specific adhesion via with the substrate by bacterial organelles including cellulosome complexes, fimbriae connections, glycosylated epitopes of cellulose-binding protein (CBP) or glycocalyx, and cellulose-binding domain (CBD) enzymes; and 4) proliferation of the attached bacteria on the potentially digestible tissues of the substrate.

However, several factors may affect the adhesion process: 1) factors related to bacteria (age, glycocalyx condition, and microbial competition); 2) factors related to substrate (cuticle protection, surface area, hydration, ionic charge, and cation exchange capacity); and 3) environmental factors within the rumen: pH, temperature, O_2, Na^+, Ca^{+2}, Mg^{+2}, and soluble carbohydrates (Miron *et al*., 2001). The predominant ruminal bacteria are shown in Table 2.1. Lee *et al*. (2000b) demonstrated *in vitro* that bacterial fraction alone could digest 46% of orchard grass cell wall after 96 h of incubation. The most important fibrolytic bacteria were *R. albus*, *R. flavefaciens*, and *F. succinogenes*.

Table 2.1. Characteristics of predominant ruminal bacteria
Características de las principales bacterias ruminales

Specie	Substrate	Fermentation product
Fibrobacter succinogenes	Cellulose	Succinate, formiate, acetate
Ruminococcus albus	Cellulose, hemicellulose	Acetate, formiate, ethanol, H_2
Ruminococcus flavefaciens	Cellulose, hemicellulose	Succinate, formiate, acetate, H_2
Eubacterium ruminantium	Hemicellulose, dextrin, sugar	Acetate, formiate, butyrate, lactate
Ruminobacter amylophilus	Starch	Succinate, formiate, acetate, ethanol
Streptococcus bovis	Starch and sugar	Lactate, acetate, formiate, ethanol
Succinomonas amylolytica	Starch	Succinate, acetate, propionate
Prevotella ruminicola, *albensis*, *brevis,* and *bryantii*	Starch, pectin, xylan, sugar	Succinate, acetate, formiate, propionate
Butyrivibrio fibrosolvens	Starch, cellulose, pectin hemicellulose, sugar	Butyrate, formiate, acetate, H_2
Selenemonas ruminantium	Starch, dextrin, sugar, lactate, succinate	Lactate, acetate, propionate, butyrate, formiate, H_2
Megasphaera elsdenii	Lactate, sugar	Propionate, acetate butyrate, branched-chain VFA, H_2
Lachnospira multiparus	Pectin, sugar	Lactate, acetate, formiate, H_2
Succinivibrio dextrinosolvens	Pectin, dextrin, sugar	Succinate, acetate, formiate, lactate
Anaerovibrio lipolytica	Glycerol, sugar	Acetate, succinate, propionate
Peptostreptococcus anaerobius	Amino acids	Branched-chain VFA, acetate
Clostridium aminophilum	Amino acids	Acetate, butyrate
Clostridium sticklandii	Amino acids	Acetate, branched-chain VFA, butyrate, propionate
Wolinella succinogenes	Organic acids, H_2,	Succinate
Methanobrevibacter ruminantium	H_2, CO_2, formiate	Methane

Source: Russell and Rychlik (2001).

Fondevila and Dehority (1996) observed *in vitro* that *F. succinogenes* plays a predominant role on digestion of the cellulose (52.8 to 60.6%) compared to *R. flavefaciens* (21.1 to 47.0%), or *P. ruminicola* (0.9 to 7.7%). However, Matsui *et al*. (1998) reported that the DM digestion of Timothy hay at 96 h incubation was similar for *R. albus* (28.8%) and *F. succinogenes* (27.7%), and in a lesser extent by *P. ruminicola* (16.4%) and *B. fibrosolvens* (7.6%).

The main enzymes involved in cellulose hydrolysis are: endocellulase (endoglucanase, endo-β-1,4-glucanase, carboxymethylcellulase, and β-1,4-glucan glucanohydrolase), exocellulase (exoglucanase, exo-β-1,4-glucanase, and cellulase β-1,4-cellobiolase), and β-glycosidase (cellobiase or glucohydrolase) (Beauchemin *et al*., 2002).

R. albus metabolizes cellobiose mainly via phosphorolytic rather than hydrolytic enzyme, indicating that phosphorylases are key enzymes in the initial metabolism of the soluble products of cellulose degradation (Lou *et al*., 1997). Increasing ruminal ammonia (from 4.8 to 13.7 mg NH_3-N/dl) by urea infusion may enhance fibrolytic enzyme of *F. succinogenes* after feeding (Pan *et al*., 2003).

Xylans contained in the feed are mainly degraded by xylanases produced by *P. bryantii* and *P. albensis* (Matsui *et al*., 2000b). The xylanolytic enzymes include: endo-β-1,4-xylanase (1,4-β-D-xylan xylanohydrolase), β-D-xylosidase (1,4-β-xylan xylanohydrolase), and debranching enzymes (esterase). Both endolxylanase and β-xylosidase are the main enzymes involved in the hydrolysis of xylans (Chen *et al*., 1997).

Interactions and antagonism has been observed between the main ruminal fibrolytic bacteria in the *in vitro* fermentation media by Fondevila and Dehority (1996). Cellulose digestion was higher combining *R. flavefaciens* with *P. ruminicola*, than combining *F. succinogenes*, *R. flavefaciens* and *P. ruminicola*. Moreover, *R. flavefaciens* repressed the cellulolytic activity of *F. succinogenes* or its growth. These authors concluded that the synergism is the result of the ability of one organism to degrade or solubilize polysaccharides, which are then utilized by the second organism; the inhibition of *F. succinogenes* seems to be the result of the production of an inhibitory substance produced by *R. flavefaciens*. Some ruminal bacteria (*R. albus*, *B. fibrosolvens*, *S. bovis*, and *Lactocacillus fermentens*) are armed with small peptides (bacteriocins), which can act against bacteria that compete for the same substrates (Kalmokoff *et al*., 1996; Wells *et al*., 1997; Russell and Rychlik, 2001). Rumen bacteria can also inhibit the growth of fungi by using those substances (Dehority and Tirabasso, 2000).

Microbes associated with the liquid phase (20 to 30% of the total microbes) including free-living bacteria and bacteria detached from solid substrate have little direct involvement in the

digestion of insoluble feed particles. Similarly, bacteria population associated with rumen epithelium and those attached to the surface of protozoa and fungi (< 1% of total rumen population) have a minor role in the process of feed digestion (Miron *et al*., 2001).

2.5.2. Fungi

Ruminal fungi represent about 8% of the microbial biomass, but only one portion of them produces cellulases, hemicellulases and xylanases with high activity (Trinci *et al*., 1994). The most relevant fungi degrading fiber and starch are: *Neocallimastix frontalis*, *N. patriciarum*, *Piromyces communis*, *Sphaeromonas communis*, and *Orpinomyces jeyonii* (Akin, 1986; Akin and Borneman, 1990; McAllister *et al*., 1993). Their activity is responsible for most of the cell wall degradation. They alone can digest more than 50% DM of orchard grass hay after 96 h of incubation (Lee *et al*., 2000b).

The life cycle of anaerobic ruminal fungi consists of a motile stage (zoospore), which alternates with a vegetative stage (sporangium) attached with a rhizoid to plant particles. Rhizoids invade plant cell walls to obtain fermentable carbohydrate and develop sporangia, which on maturity, release zoospores to establish another cycle. All plant polysaccharides, with the exception of pectin and polygalacturonic acid can be utilized for fungi growth (Fonty and Joblin, 1991). Many of the polysaccharide hydrolyzing enzymes are produced in the vegetative stage and the zoospores of the fungi (Orpin and Joblin, 1997). These organisms are more able to colonize and degrade the lignin-contained tissues than are bacteria. Phenolic compounds are solubilized but not metabolized from the plant wall by fungi (Akin and Borneman, 1990).

To degrade plant cell walls, rumen fungi produce extracellular enzymes such as: polysaccharidases (endo-*β*-1,4 glucanase, exoglucanase, xylanase, cellodextrinase) and glycosidases (*β*-glucosidase, *β*-fructosidase, *β*-xylosidase, α-L-arabinofuranosidase), cellulases, xylanases, proteases, and α-amylase to degrade starch (Fonty and Joblin, 1991).

The enzymes released by rumen fungi for solubilizing cellulose present in plant fiber are: endo-1,4-*β*-glucanase (*N. frontalis*), exoglucanase (*P. communis*) (Orpin and Joblin, 1997). Matsui *et al*. (1998) reported that DM degradation is highest with *N. frontalis* (59.4%), followed of *P. communis* (23%) and *Piromyces* spp. (12.7%).

Whit regard to grain digestion by fungi, McAllister *et al*. (1993) observed three species of ruminal fungi capable of digesting cereal grains and those include *O. jeyonii*, *N. patriciarum*, and *P. communis*.

The anaerobic ruminal fungi also have chitin-containing cell walls and chitinase as an autolytic enzyme for maintenance of their morphology. The chitinase activity of *P. communis* is greatest at pH 6, and 50% of this remains between pH 5 and 8 (Sakurada *et al*., 1998).

Fonty *et al*. (1987) found that anaerobic fungi appear in the rumen of flock-reared lambs 8 to 10 days after birth, before the ingestion of solid feed. However their subsequent development and survival depend on the composition of the diet, as fungi disappeared from 80% of the lambs when they were fed a rich concentrate diet, compared to lambs fed a dehydrated alfalfa diet (Fonty *et al*., 1987; Fonty and Gouet, 1989). In contrast, McAllister and Cheng (1996) reported that, the quantity of fungi is similar among cattle fed 80% concentrate and those fed 100% forage diets.

2.5.3. Protozoa

Protozoa are directly involved in the digestion of plant cell walls and starch in the rumen. Two types of enzymes (polysaccharide depolymerases and glycoside hydrolases) involved in cellulose digestion have also been found in *Entodiniomorphs* (Jouany and Martin, 1997). *Polyplastron* and *Eudiplodinium* are the most active protozoa degrading and fermenting crystalline cellulose and xylans. Small *Entodinia* and *Holotrichs* have no cellulolytic activity.

Lee *et al*. (2000b) observed a range of 25 to 30% of the total fiber digestion *in vitro* of orchard grass hay. Protozoa can digest more than 40% cellulose after 48 h of *in vitro* incubation, with a greater association of *Epidinium caudatum* and *Polyplastron multivesiculatum* towards cellulose breakdown (Chaudhary and Ogra, 1995).

In regard to the digestion of cereal grains, rumen ciliate protozoa play diverse and important roles in the ruminal metabolism of starch. Starch is widely used by all protozoa as an energy source, in where *Isotricha* and small *Entodinia* have the highest fermenting activities (Jouany and Martin, 1997). Moreover, protozoa have an important role in the ruminal metabolism of cereals. They have the capacity of assimilating starch and soluble sugars and of storing these energy-rich compounds inside the cells as amylopectin (Williams and Coleman, 1997). The quick assimilation of starch and soluble sugars by protozoa can help to reduce the rate of carbohydrate fermentation, and protect against the burst of acid formation with which acidosis is associated (Asanuma and Hino, 2002).

However, protozoa are considered noxious for other ruminal microorganisms, such as ruminal bacteria. Ovine protozoa are also able to digest bacterial and fungi cells, and other protozoa (Coleman, 1992).

2.6. Cereals for fattening lambs

Many studies have demonstrated that the replacement of forages whit a high percentage of cereals improves lamb performance and carcass traits (Mahgoub *et al*., 2000; Díaz *et al*., 2002). This is a result of the positive effects of cereals on nutrient digestibility in the total tract of lambs (Matejovsky and Sanson, 1995; Fimbres *et al*., 2002). However, the use of this type of diet in ruminants has many inconveniences. The most important inconvenience is ruminal acidosis and its secondary effects.

In sheep, lactic acidosis is an acute metabolic disease, characterized by: inapetence, depression, lameness, and coma (Kimberling, 1988). It is caused by sudden engorgement or not adapted ingestion of grain and other easily fermentable sources of carbohydrates. Depending on the degree of acidosis, it may classify in two classes: 1) Acute acidosis, characterized by a fast decrease of ruminal pH (< 5.0) and a rapid accumulation of lactic acid (> 90 m*M*) in the rumen (Hibbard *et al*., 1995b), and 2) Subacute ruminal acidosis (SARA) or subclinical acidosis, a temporarily altered state of the rumen that causes some aberrations in fermentation patterns (Nocek, 1997).

Sheep may develop signs of anorexia, ruminal stasis, diarrhea and nasal discharge, within 12 h of the grain feed. Later, the signs became more severe: restlessness, staggering, and hyperpnoea, abdominal respiration, teeth grinding, recumbence, flanks watching, and coma (Patra *et al*., 1993). SARA in sheep is characterized by ruminal pH ranging from 5.5 to 5.0, with increasing VFA concentrations, but with normal (lower than 5 m*M*) lactate accumulation (Patra *et al*., 1993).

Because SARA is a subclinical disease, the effects on animal production may be more serious than acute acidosis. In cattle it has been reported inconsistent DMI, and decreasing efficiency, milk production, and milk fat, poor body condition score (BCS), diarrhea, and laminitis (Kelly and Leaver, 1990; Nocek, 1997; Green *et al*., 2002).

2.6.1. Development of ruminal acidosis

After consumption of high amounts of easily fermented carbohydrates, large increases in the ruminal concentration of free glucose (160 mg/L) can occur and favor lactic acid production (Horn *et al*., 1979). The first change occurred at the beginning of the intake of diets rich in cereals is the reduction in chewing and rumination, and saliva output, as a response to the amount of physical fiber present in the diet (Oetzel, 2001).

Cereal grains contain starch as the major polysaccharide, which is rapidly fermented by amylolytic bacteria in the rumen, thus in the subsequent hours excessive quantities of VFA are produced, resulting in a reduction of ruminal pH (Van Kruiningen, 1995). When pH drops below 6.0, *S. bovis* and *Lactobacillus* spp. multiply and replace the Gram-negative bacilli with a concurrent increase in ruminal lactate concentrations of lactic acid (Kimberling, 1988; Zorrilla and Rowe, 1993; Goad *et al*., 1998). *S. bovis* is not only the predominant bacteria, but also produces lactate as a major fermentation product and reduces the availability of substrates for other saccharolytic microorganisms (Asanuma and Hino, 2002). At a ruminal pH near 5.5, cattle suffer SARA (Nocek, 1997; Oetzel, 2001).

Lactate accumulation depresses pH more drastically than similar amounts of other ruminal acids because its pKa value (the pH point of maximum buffering) is considerably lower (3.9) than the values of the VFA produced in the rumen (4.7 to 4.9) (Owens *et al*., 1998; Asanuma and Hino, 2002). The VFA pKa values are 4.75, 4.87 and 4.81 for acetic, propionic and butyric acid, respectively (Rémond *et al*., 1996).

If ruminal pH drops to 5.0 or below, the animal is in acute ruminal acidosis. At this point, bacteria pass into the liver and may colonize lungs, heart valves, kidneys, or joints, resulting in pneumonia, endocarditis, pyelonephritis, and chronic arthritis (Oetzel, 2001).

During the development of ruminal acidosis two types of bacteria can be detected: lactate producers (*S. bovis* and *L. vitulinus*) and lactate users (*M. elsdenii*, *S. ruminantium*, *A. lipolytica*, *Veillonella parvula* and *Propionibacterium shermanii*) as indicated by Nocek (1997) and Oetzel (2001). *S. bovis* is of particular interest because of its role in the development of lactic acidosis in ruminants. It is a Gram-positive bacterium, non-motile, 0.8-1.5 μm in shape (Stewart and Bryant, 1997), relatively acid resistant and can proliferate at low pH (Asanuma and Hino, 2002).

Regarding lactate users, *S. ruminantium* is resistant to monensin, lasalocid and avoparcin (Stewart and Bryant, 1997). It has nuclease (deoxyribonucleosides, ribose or 2-deoxyribose) activity, fermenting ribose to acetate, propionate and lactate (Al-Khaldi *et al*., 2000), and high phytase activity at low ruminal pH (4.0 to 5.5) (Yanke et al., 1999). It may explain why ruminant animals have no problems with phytic acid metabolism. *M. elsdenii* is a large coccus (mostly diplococci), which is able to use lactic acid as a sole source of carbon and energy (Ouwerkerk *et al*., 2002). It is Gram-negative non-motile (2.4 μm in diameter), which occurs in pairs and in chains of more than 20 cells. Lactate is fermented by this bacterium mainly to butyrate, propionate, isobutyrate, valerate, carbon dioxide, and hydrogen, and occasionally traces of propionate (Stewart and Bryant, 1997). Maltose is hydrolyzed by this bacterium with

the help of maltose phosphorylase and maltase. Their phosphorylation activity remains fairly high between pH 6.5 and 8.0, but decreases (69%) at pH 5.0 (Martin and Wani, 2000). *M. elsdenii* can use from 60 to 80% of the fermented lactate (Counotte and Prins, 1981; Nocek, 1997), and resist additives antibiotics such as avoparcin, monensin and lasalocid (Stewart and Bryant, 1997).

Other microorganisms implicated in ruminal acidosis are protozoa. When protozoa break due to changes in acid or osmolarity associated with acidosis, they release large amounts of amylase that in turn accelerate glucose production from starch, increasing acidosis (Owens *et al*., 1998).

The total ruminal protozoa numbers in grain adapted steers, mainly consists of starch-utilizing *Entodinomorphs*, of which *Entodinium* spp. constitute 97.2% of the total; *Epidinium* is the second most numerous genus. Protozoal populations decrease extensively with increased ruminal acidity, but complete defaunation did not occur (Goad *et al*., 1998; Hristov *et al*., 2001). They can persist at levels exceeded 3×10^5/mL in steers fed 70 to 90% of barley grain, and approximately 328×10^3/mL in cattle fed 95% barley grain. *Dasytricha* spp., *Eudiplodinium*, *Diplodinium*, *Metadinium*, *Ophryoscolex*, and *Ostracodinium* spp. are present in low numbers or are not detected in ruminal fluid when steers are fed 95% concentrate (Hristov *et al*., 2001).

2.6.2. Secondary affects associated to ruminal acidosis

As a consequence of excessive lactic acid accumulation in the rumen, the animal may experience other disorders that affect directly its health and production. In cattle, negative affects associated to the ruminal acidosis, either in experimental or normal fattening conditions have been reported. They are: atony (Oetzel, 2001), changes in ruminal osmolarity (Garza *et al*., 1989; Owens *et al*., 1998), ruminal fluid viscosity and bloat (Cheng *et al*., 1998), parakeratosis and hyperkeratosis (Kimberling, 1988; Rémond *et al*., 1996), disturbances in VFA and energy absorption (Krehbiel *et al*., 1995a), disturbances in pH and electrolytes of body fluids (Patra *et al*., 1993), liver abscesses (Nagaraja and Chengappa, 1998; Ramsey *et al*., 2002), lameness (Manson and Leaver, 1988; Rajala-Schultz *et al*., 1999; Warnick *et al*., 2001), polioencephalomalacia (Patra *et al*., 1993), and sudden death (Glock and DeGroot, 1998).

The rumen epithelium is responsible for physiologically important functions, such as absorption, transport, VFA metabolism (primary acetate, propionate and butyrate) and

protection (Baldwin, 1998; Gálfi *et al*., 1991), and glucose absorption (Aschenbach *et al*., 2002). Because the ruminal epithelial cells are not protected by mucus, they are vulnerable to acids (Oetzel, 2001). In sheep and other ruminants, the integrity of ruminal epithelium during acidosis is predominantly affected by the low luminal pH, due to the accumulation of organic acids (Aschenbach and Gäbel, 2000), resulting in rumenitis (Kimberling, 1988) or hyperkeratosis (Rémond *et al*., 1996). Rumenitis is a normal consequence of SARA (Oetzel, 2001), leading to disturbances in VFA and energy absorption. The fractional rate and VFA absorption are reduced from 36 to 43%, respectively in lambs suffering acidosis. This reduction in VFA absorption would reduce total ME supply from 23 to 32% (Krehbiel *et al*., 1995a), which may explain the reduced efficiency observed in ruminants after severe ruminal acidosis.

Thus, numerous feed strategies have been developed to improve animal production reducing the risk of acidosis and its secondary effects. In steers, secondary effects included: replacement of grain cereals for fat, such as tallow or yellow grease (Krehbiel *et al*., 1995b) or other sources of starch, such as potatoes (Monteils *et al*., 2002), restriction of feed intake during the adaptation period (Choat *et al*., 2002) and feed bunk management strategies (Erickson *et al*., 2003), grain source combinations (Owens *et al*., 1997) and, grain processing (Beauchemin *et al*., 2001; Cooper *et al*., 2002; Zinn *et al*., 2002).

However, in lambs, a few strategies have been studied: feeds at free-choice (Phy and Provenza, 1998a, 1998b; Askar, 2004), forage at increasing levels (Fimbres *et al*., 2002), grain processing (Hadjipanayiotou, 1990; Hejazi *et al*., 1999), and whole grain feeding (Askar, 2004).

2.7. Biotechnology in ruminant nutrition

Agricultural biotechnology is the collection of technologies that involve the application of biologic processes and living organisms, or parts thereof (tissues, cells or enzymes) in the livestock, pharmaceutical and chemical industries, as well as for environment control (Arias, 1992; Falk *et al*., 2002).

Ruminal biotechnology applies the available knowledge on forestomach fermentation and the use and management of both natural and recombinant microorganisms to improve the efficiency of digestion of fibrous feedstuffs by ruminants (McSweeney *et al*., 1999). Methods for manipulating ruminal fermentation by biotechnology involve: microbial and antimicrobial feed additives (e.g., ionophores and antibiotics, and microbial feed additives), introduction of

genetically modified microorganisms in the rumen (cloning of genes from ruminal microorganisms, expression of foreign DNA in ruminal bacteria and establishment of new organisms in the rumen), implementing molecular techniques, rumen defaunation, and the introduction of foreign microbial species (Wallace, 1994; Dominguez Bello and Escobar, 1997).

The main applications of biotechnology in ruminant nutrition have been for enhancing the nutritive value and quality of feeds, and to optimize the nutrients in the intestinal tract by using of enzyme silage inoculants, minerals, amino acids, hormones, and direct-feed microbial (DFM).

2.8. Feed additives

For many years, animal nutrition has been interested in improving animal production by manipulating ruminal fermentation by using additives. The efforts have particularly focused on substances that increase fiber digestibility, ruminal propionic acid and saliva output, and that reduce ruminal acidosis, bloat incidence, methanogenesis, ruminal proteolysis, and deamination of dietary protein.

Additives are substances or preparations which when incorporated in feedstuffs, influence or affect food characteristics and improve animal production (Hollo *et al*., 1992; Lyons, 1994). They are administrated in a regular form in small quantities in the diet over long periods. In 2003, the EU created the European Food Safety Authority (EFSA) to be in charge of the regulation of the use of additives in animal nutrition and the prohibition of the use of antibiotics as feed additives (Regulation EC 1831/2003). For antibiotics the new offer establishes a ban, with a period of restriction (up to 1/1/2006) for those having an active principle not used in human beings (avilamicin, flavophosfolipol, monensin and salinomicyn sodium). In the regulation, the EFSA regrouped the feed additives in five new categories according to function:

- Technological (e.g., preservatives, antioxidants, emulsifiers, stabilizing agents, acidity regulators, silage additives).
- Sensory (e.g., colorants, flavors)
- Nutritional (e.g., vitamins, minerals, amino acids, trace elements)
- Zootechnical (e.g., digestibility enhancers, gut flora stabilizers)
- Coccidiostats and histomonostats.

Moreover, the cases of bovine spongiform encephalopathy (BSE) disease and its relation with the variation of Creutzfeldt-Jacob Disease (CJD) in humans caused the ban of the use of protein sources from animal origin. Thus, the use of forages and cereals for ruminants are currently more important. However, diets rich in forages or grain may affect negatively the ruminant performances creating more dependence for feed additives.

2.8.1. Substances used in ruminants to prevent acidosis

Regarding the use of high concentrate based diets the studies have focused on how control the ruminal acidosis. More studies have been carried out in beef cattle and very few in sheep.

- Buffers: Sodium bicarbonate ($NaHCO_3$) is usually included in fattening cattle diets because it is generally believed that $NaHCO_3$ increase the buffering capacity of ruminal fluid, in part, by increasing the amount of dissolved carbon dioxide (Callaway and Martin, 1996). Mandebvu and Galbraith (1999) reported that the inclusion of $NaHCO_3$ (15 g/kg feed) to the lambs diet did not affect ruminal pH, weight gain, DMI, or feed efficiency. On the contrary, Santra *et al*. (2003) observed that $NaHCO_3$ improved ruminal pH, ADG, ciliate protozoal population, and cellulose digestibility in lambs. Dry matter intake, OM, CP and GE digestibility were unaffected. Tripathi *et al*. (2004) reported in lambs that crescent levels of $NaHCO_3$ (7.5, 15, and 22.5 g/kg of DM) increased linearly ruminal pH and NDF and ADF digestibility, without improvements in DMI, DM, OM or GE. Only the intermediate dose increased lamb growth by 35%. Recently, Azcar (2004) observed a reduction in ruminal digestibility and an increase in acetate:propionate in the rumen when $NaHCO_3$ was offered at free access or incorporated in the protein supplement at 15 g/kg DM in intensively fattened lambs.

- Antibiotics: Ionophores are the most anticoccidial agent used in domestic animals. Monensin (obtained from *Streptomyces cinnamonensis*) may reduce the methane emission *in vitro* from 40 (García-López *et al*., 1996) to 78% (Dong *et al*., 1999), but may also depress digestibility of OM (15%), cellulose (27%) and hemicellulose (17%) of grass hay (Dong *et al*., 1999). In cattle, monensin has shown selective inhibition of ruminal bacteria, lactic and methane producers (Matabudul *et al*., 2001), and stimulation of propionate production by 25% (Jouany, 1994). In lambs, monensin improves weight gain and feed conversion (Martini *et al*., 1996). Lasalocid is other important ionophore studied in fattening lambs; it depressed the DMI, but did not

attenuate lactic acidosis (Phy and Provenza, 1998a). In contrast, Swanson *et al*. (2000) reported that lasalocid did not affect DMI neither body gain in lambs. Virginiamycin, which is obtained from *Streptomyces virginiae*, has been used for many years as a performance enhancer for poultry and swine, and recently has been approved in USA for cattle. Virginiamycin increased propionate and reduced deamination of amino acids i*n vitro*, but did not effect pH, total VFA, lactate concentrations, or protozoa populations (Ives *et al*., 2002). This antimicrobial feed additive improved growth performances, and prevented steers against liver abscess (Rogers *et al*., 1995). However, in sheep, virginiamycin declined drastically total ciliate protozoa (especially *Entodinium*), ruminal pH, and increased lactate and propionate concentrations (Nagaraja *et al*., 1995). Anthraquinone has increased propionic acid concentrations (26%) and also methane production *in vitro* (Garcia-Lopez *et al*., 1996). In sheep, anthraquinone increased propionic acid and reduced methane, and had no toxic effects in the lambs. Nevertheless, did not affect weight gain, and nutrient digestibility (Kung *et al*., 2003).

- Probiotics and direct-fed microbial (DFM): These types of additives, described as microbial-based feed additives (Kung, 2001) have been used to improve sheep performance, although more studies have focused on *in vitro* (*M. elsdenii*; Kung and Hession, 1995) or in dairy cattle (*Saccharomyces cerevisiae* and *Enterobacterium faecium*; Nocek *et al*., 2003) and beef cattle (*Propionebacterium spp*. and *E. faecium*; Ghorbani *et al*., 2002). *S. cerevisiae* is one of the most DFM studied in ruminants with variable effects. Results indicated an increase in ruminal pH and reduction of risk of acidosis in sheep (Brossard *et al*., 2003). However, when sheep was fed with a high 90% grain diet use of *S. cerevisiae* depressed ruminal pH and did not affect VFA concentrations or total tract digestibility of DM, CP, NDF and ADF (Angeles *et al*., 1998; Corona *et al*., 1999). Lema *et al*. (2001) studied the use of fed in the diet *Lactobacillus acidophillus*, alone or in combination with *Streptococcus faecium* or *S. faecium*, *L. casei, L. fermentum* and *L. plantarum* in lambs. Body weight and feed conversion improved (34 and 39.4%, respectively), and the fecal shedding of *Escherichia coli* was reduced in 1.3 to 2.4 times. Feed-direct microbial are capable to compete for gut colonization or nutrients with pathogenic bacteria producing inhibitory metabolites (acids, bacteriocins, antibiotics), or stimulating the activity

enzymes and nutrients (e.g., amino acids, vitamins) or other growth factors that improve the immune response in the host animal (Zhao *et al*., 1995; Kung, 2001).

Like to feed additives, the use of vaccines has increased since the last century. Shu *et al*. (1999) isolated lactic bacteria from rumen contents of cattle (*S. bovis* strain Sb-5 and *Lactobacillus* LB-27) to immunize steers. The vaccine increased the concentrations of anti-*S. bovis* and anti-*Lactobacillus* immunoglobulin G (IgG) in serum and saliva and DMI, and decreased lactate concentrations and numbers of *S. bovis* and *Lactobacillus* in the rumen.

In sheep, Shu *et al*. (2000) also reported a higher DMI and ruminal pH, and lower diarrhea incidence, when animals were immunized against *S. bovis*. Immunization by intramuscular was more effective than intraperitoneal way. The correct immunization against lactic acidosis in sheep depends on the adjuvant used to prepare the vaccine, and the time of immunization, but also the presence of adverse effects in immunized animals because it determine antibody concentrations in saliva, serum, and feces (Shu *et al*., 2001).

2.9. Results and concerns on the use of antibiotics as feed additives

Veterinary drugs are usual in the livestock industry and play an important role in increasing the productive capacity of animals. Feedlot cattle in USA are still fed with antibiotics (tylosin, bacitracin, chlortetracycline, oxytetracycline, and virginiamycin) to reduce the incidence of liver abscesses in feedlot (Nagaraja and Chengappa, 1998) and to increase the growth performance.

In Europe the Regulation EC 1831/2003 restricts the use of antibiotics as feed additives due to the possible antibiotic resistance. Virginiamicyn may confer resistance to *E. faecium* for streptogramins (pristinamicyn and synercid) potentially useful for the treatment of human enterococcal infections (Anadón and Martínez-Larrañaga, 1999).

Regarding ionophores, no negative effects have been detected on the animal health, but some studies indicate that monensin, nigericin and lonomysin produce negative cardiovascular effects in rabbits and dogs (Fahim and Pressman, 1981; Satoh and Tsuchida, 1999). Ionophore residues (monensin, lasalosid, narasin, and salinomycin) have been detected in the liver of sheep, calf and broiler, and in eggs (Martini *et al*., 1996; Blanchflower and Kennedy, 1996; Matabudul *et al*., 2001), being the ionophore residues directly proportional to the doses employed.

Other disadvantage of the ionophores is its toxic effects when fed at inadequate dose. In ruminants, signs such as anorexia, ruminal atony, weakness, ataxia, hyperpnoea, diarrhea,

dehydration, loss weight and death may be observed (Hollo *et al*., 1992). In sheep, the monensin concentration causing death is 150 to 160 ppm (Martini *et al*., 1996). In cattle lethal doses are 50 to 100 mg/kg BW for lasalosid, and 25 mg/kg BW for monensin. Concerns regarding antibiotics residues, selection of resistant populations, antibiotic toxicity, and public and regulatory acceptance have delayed the study and finally banned the use of ionophore antibiotics for dairy cattle (Kalmokoff *et al*., 1996).

Although, the use of ionophores in animal feed is not likely to have a significant impact on the transfer of antibiotic resistance from animals to man (Russell and Houlihan, 2003), and none study about the negative effects of ionophores in human have reported, their use must be controlled because this compounds have different activities, and may leave toxic residues or metabolites in milk and meat (Jouany, 1994).

Taking in account the risk of secondary effects for human and animal health, several studies focus on safe alternatives to growth promoters. Among these alternatives, the use of fibrolytic enzyme complex and organic acids are discussed below.

2.10. Fibrolytic enzymes complex

Enzymes are biological catalysts of metabolic processes, and are involved in all anabolic and catabolic pathways of digestion and metabolism (Wenk, 1992). In general, they are large and three-dimensional proteins with a molecular weight from 20 to 70 kDa. They need an optimal supply of substrate in constant and unimpaired contact, and a specific pH range for maximum enzymatic activity. The conversion rate of the enzymes is reduced when insufficient substrate is available to saturate the enzyme. The enzyme can be inhibited or completely inactivated at temperatures of 70 to 80 ºC or in presence of chemicals (heavy metals) and natural inhibitors (Uhlig, 1998).

Today the enzyme complexes have a large use in the monogastric food industry to: remove anti-nutritional factors and toxins (Brenes *et al*., 1993), increase digestibility and digestion of nutrients in broilers (Bedford *et al*., 1998), chicken (Boros *et al*., 1998) and pigs (Baidoo *et al*., 1998), and to reduce nutrient content in wastes (Matsui *et al*., 2000a; Spencer *et al*., 2000). According to Sheppy (2001), there are four main reasons for using enzymes in animal feed: 1) to break down anti-nutritional factors; 2) to increase the availability of starches, proteins and minerals enclosed within fiber-rich cell walls; 3) to break down specific chemical bounds in raw materials which are not usually broken down by the animals' own enzymes, thus releasing more nutrients; and, 4) to supplement the enzymes produced by young animals.

The particular application of this technology to poultry production has increased considerably. In the United Kingdom, as much as 60 to 70% of wheat and barley-based poultry feeds are supplemented with enzymes (Wyatt and Queenborough, 1995). Enzymes are used to improve the nutritive value of feeds for non-ruminants, particularly broilers, and as silage additives, but they are not routinely used in the ruminant diets (Yang *et al*., 1999).

The aerobic fungus *Trichoderma* is the main source of enzymes used in the feed industry (Morgavi *et al*., 2000) producing large amounts of extracellular xylanases in both shake culture and solid state (Chen *et al*., 1997). Fungi are commonly found in the environment biodegrading cellulolytic compounds (Madinaveitia *et al*., 1985) as previously discussed. They are cosmopolitan fungi, remarkable for their rapid growth, high capability of utilizing diverse substrates, and resistance to noxious chemicals (Kubicek *et al*., 2003).

Enzyme feed additives for ruminants are concentrated extracts obtained from fungal fermentations (mostly *Trichoderma longibrachiatum*, *Aspergillus niger* or *A. oryzae*) and bacterial (*Bacillus* spp.). They do not contain microbial cells, because are removed from the fermentation and finally concentrated and purified (Beauchemin, 2002; Beauchemin *et al*., 2002).

2.10.1. Physicochemical properties of the fibrolytic enzymes

The *T. longibrachiatum* xylanase and cellulase preparations contain several proteins with a molecular size of 22 kDa (Morgavi *et al*., 2000). Xylanase of *T. longibrachiatum* can rapidly hydrolyze xylans (> 8 xylose residues in length) at 5.0 of pH and 50 ºC temperature (Li *et al*., 2000). Endoxylanases hydrolyzing xylans have little activity on carboxymethyl cellulose and dextrin, and had no activity on pectins and starch. Endoxylanase has an optimum temperature of 45 ºC and pH 5, but it is less stable at pH 6.0 (Chen *et al*., 1997).

The molecular structure of enzymes can be easily altered by external factors such as heat during the pelleting process (70 to 90 ºC) or even long storage periods may modify or reduce the enzyme activity, delivering insufficient activity levels when fed to the animal. With some exceptions, bacterial enzymes are more resistant to heat than those derived from fungi (Mascarell and Ryan, 1997; Bowman *et al*., 2002).

2.10.2. Fibrolytic enzyme complex on ruminant production

Studies on the use of fibrolytic enzymes complex in ruminant diets increased in the last years. Some commercial enzyme complexes are in Table 2.2.

Table 2.2. Commercial fibrolytic enzyme complex for ruminants
Complejos comerciales de enzimas fibrolíticas para rumiantes

Product	Manufacturer	Activity IU (μmol·min·mL)
Alfa	Farmline International, Schaumburg, IL, USA	Cellulase; amylase; glucose oxidase
Grass	Farmline International, Schaumburg, IL, USA	Cellulase; glucose oxidase
Alphazyme	Finnfeeds International, Marlborough, Wiltshire, UK	Cellulase 11,500; xylanase 3,000; cellobiase 40; α-amylase 5,500; pectinase 1,250
Grasszyme	Finnfeeds International, Marlborough, Wiltshire, UK	Cellulase 23,300; xylanase 5,800; cellobiase 55; glucose oxidase 83
Xylanase B	Biovance Technologies Inc., Omaha, NE, USA	Cellulase, 32 FPU[1] g; xylanase, 4200 IU
Spezyme CP	Genencor, Rochester, NY, USA	Cellulase, 90 FPU g
Promote	Biovance Technologies Inc., Omaha, NE, USA	Cellulase 31.0; xylanase 43.4
Promote	Biovance Technologies, Inc., Omaha, NE, USA	Xylanase 26,483; endoglucanase 2,645
Promote N.E.T.	Agribrands International, St. Louis, MO	Cellulase 130/g; xylanase 120,000/g
Natugrain 33-L	BASF Corporation, Ludwigshafen, Germany	Endo-1,3(4)-β-glucanase 6000/g; endo-1,4-β-xylanase 2750/g
Bovizyme	Finnfeeds International, Marlborough, Wiltshire, UK	Cellulase 2736/g; xylanase 7995/g
Liquicell 2500	Specialty Enzymes and Biochemicals, Fresno, CA, USA	Xylanase 14,860; endoglucanase 1699; exoglucanase 2.6; *β*-D-glucosidase 45.5; *α*-L-arabinofuranosidase 1.4
Fibrozyme	Alltech Inc., Nicholasville, KY, USA	No available
Maxicel 200	George A. Jeffreys, Salem, VA, USA	Cellulase 200 IU/mg

[1]FPU = Filter paper cellulase activity

The majority of these studies have been carried out in dairy cows, in which several forms of addition (solid or liquid), and doses of enzyme complex have been assayed (Beauchemin, 2002). Also, the enzyme inclusion to different portion of the diet (forage, concentrate,

supplement or premix) has been studied in cows, fed either rich forage diets or high concentrate diets.

2.10.3. Enzymes in dairy cows fed forage-based diets

The studies summarized in Table 2.3 shown a large variability on cows performances when enzymes were used as a feed supplement. On average, cows fed enzymes ingested similar quantities than control cows (21.7 kg/d of DM). However, cows ingested 7% (+1.5 kg/d) more DM when the enzyme was added to the concentrate portion than to the forage portion (average 22.9 vs. 21.4 kg/d, respectively). Regarding to lactation stage, the use of enzymes improved DMI by 3.8% (+0.8 kg/d) in cows in middle lactation compared to cows in early lactation (average 21.8 vs. 21.0 kg/d, respectively).

A similar variability is shown in Table 2.3 with regard to milk yield. On average, cows fed enzyme slightly increased milk yield (+0.46 kg/d) compared to the control cows (average 29.2 vs. 29.7 kg/d, respectively). Nevertheless, the enzyme supplementation was more effective in early lactation cows, which produce 11.8% more milk than cows in middle lactation (31.3 vs. 28 kg/d, respectively). Those results agree with Rode *et al*. (1999), Schingoethe *et al*. (1999), and Zheng *et al*. (2000), who affirm that the better effects of fibrolytic enzymes are in cows at the beginning of lactation, when the animal is in negative nutrient balance. On the other hand, milk production was 5.7% (+1.6 kg/d) higher when the enzyme was added to the concentrate portion compared with the enzyme addition to the forage portion.

In relation to the effects of fibrolytic enzyme on milk composition, the results are also variable. Bowman *et al*. (2002) observed improvements in fat (+7%) and milk protein (+ 3%), but a diminution in milk lactose (-1.3%) was observed in cows fed a premix supplemented with fibrolytic enzymes. In contrast, Yang *et al*. (1999) did not observe changes in milk fat or milk protein attributed to the enzyme addition to the alfalfa cubes or concentrate. However, milk lactose was higher (+1.3%) in cows fed concentrate supplemented with enzymes. Greater reductions in milk fat were observed by Sutton *et al*. (2003) in cows fed enzyme added in TMR (-12%), concentrate (-10%), or in cows in which the enzyme was infused ruminally (-2.8%). Similar results were obtained Kung *et al*. (2000) in milk fat (-11%), and in milk protein (-6.2%) by adding enzyme into the forage portion at high dose (5 L/t of DM). But, when the enzyme was added at low dose (2 L/t of DM) milk fat and protein increased (+4% and +1.3%, respectively).

Table 2.3. Effects of fibrolytic enzymes on dairy cows feeding forage-based diets (≥ 50%)
Efectos de las de enzimas fibrolíticas en vacas lecheras alimentadas con dietas basadas en forraje (≥ 50%)

Author (year)	Enzyme product	Lactation stage (cows)	Ration F:C[1]	Enzyme inclusion portion and (dose DM basis)	DMI (kg/d)	Milk yield (kg/d)	Milk composition (%)		
							Fat	Protein	Lactose
Fredeen and McQueen (1993)	Grass Alfa	Middle (9)	72:38	Control	18.8	22.8	4.40	3.70	5.00
				Silage 1st cut (1.50 L/t)	+0.5	-0.8	0.00	0.00	0.00
				Silage 1st cut (1.50 L/t)	+0.4	+0.1	0.00	0.00	-0.10
	Grass Alfa	Middle (9)	72:38	Control	21.4	24.0	4.16^{a}	3.66	4.72
				Silage 2nd cut (1.50 L/t)	-0.1	+0.1	-0.06^{ab}	+0.02	+0.02
				Silage 2nd cut (1.50 L/t)	+1.1	+0.2	-0.18^{b}	-0.03	+0.01
Schingoethe *et al*. (1999)	Enzyme complex	Middle (50)	55:45	Control	20.6	25.1	3.70	3.30	4.90
				Forage (0.70 L/t)	+0.8	+1.1	+0.10	+0.10	0.00
				Forage (1.00 L/t)	-0.3	+0.9	+0.20	+0.10	0.00
				Forage (1.50 L/t)	+1.7	+2.7	+0.10	+0.10	0.00
Yang *et* al. (1999)	Pro-mote	Early (4)	55:45	Control	20.4	23.7^{b}	3.79	3.36	4.56^{b}
				Alfalfa cubes (1.00 L/t)	+0.3	+0.9^{ab}	-0.09	+0.05	+0.05^{ab}
				Alfalfa cubes (2.00 L/t)	+0.3	+1.9^{a}	-0.01	+0.12	+0.04^{ab}
				Concentrate (1.00 L/t)	+0.4	+1.6^{ab}	-0.03	+0.13	+0.06^{a}
Kung *et al*. (2000)	Enzyme complex	Middle (27)	50:50	Control	22.0	37.0^{c}	2.80^{cd}	3.14^{cd}	—
				Forage (2.00 L/t)	+0.5	+2.5^{d}	+0.11^{d}	+0.05^{d}	—
				Forage (5.00 L/t)	-0.2	-0.8^{c}	-0.28^{c}	-0.18^{d}	—
Bowman *et al*. (2002)	Promote	Middle (8)	55:45	Control	23.6	29.4^{cd}	3.91^{c}	3.59^{c}	4.51^{d}
				Concentrate (1.00 g/d)	+0.1	+0.6^{d}	+0.10^{cd}	+0.02^{cd}	-0.02^{cd}
				Supplement (1.00 g/d)	+0.4	-0.6^{cd}	+0.15^{cd}	+0.03^{cd}	-0.03^{cd}
				Premix (1.00 g/d)	+0.1	-1.5^{c}	+0.27^{d}	+0.09^{d}	-0.06^{c}
Dhiman *et al*. (2002)	Bovizyme	Early (50)	51:49	Control	27.1	39.5	3.29	3.16	4.18
				Forage (1.30 L/t)	+0.4	-0.8	-0.08	-0.07	-0.10
Knowlton *et al*. (2002)	Enzyme complex	Late (6)	61:59	Control	24.3	32.6	3.74	3.29	4.58
				Concentrate (204 g/d)	0.0	-1.2	+0.09	-0.01	+0.02
Sutton *et al*. (2003)	Enzyme complex	Early (4)	57:43	Control	20.7	34.0	4.04	3.36^{b}	4.67
				TMR (2.00 g/kg)	+0.4	+1.5	-0.43	-0.01^{b}	-0.01
				Concentrate (2.00 g/kg)	+0.2	+0.5	-0.32	+0.09^{a}	+0.03
				Rumen infusion (2.00 g/kg)	-0.3	+1.0	-0.11	-0.01^{b}	+0.01
Vicini *et al*. (2003)	Enzyme complex	Early (86)	57:43	Control	18.5	28.2	3.95	3.34	—
				Forage (1.25 L/t)	-0.2	-0.3	-0.12	+0.01	—
				TMR (2.00 L/t)	-0.2	+0.6	-0.26	+0.02	—
Mean		25	59:46	—	+0.29	+0.46	-0.04	+0.03	0.00

[a, b]Means with different letter in the same column differ ($P < 0.05$); [c, d]means with different letter in the same column differ ($P < 0.10$). [1]Forage to concentrate ratio (%)

Considering milk composition data showed in Table 2.3, enzyme supplementation slightly reduced milk fat content (-0.04 units), but increased milk protein (+0.03 units), and did not change milk lactose content. Contrary to milk yield, the cows in middle lactation fed enzymes were higher in fat (+4%), protein (+4%), and milk lactose (+1.7%) than cows in early lactation. The enzyme inclusion in the concentrate portion of the diet resulted in more fat (+5.6%), and milk protein (+3%), but less lactose (-3%).

Regarding the effects of enzymes on weight gain and body condition score (data not shown), no authors reported relevant changes in these variables, with the exception of Schingoethe *et al*. (1999) who detected an increase in body condition (+1.4%) in cows fed forage treated with enzymes at low (0.7 L/t of DM) and middle (1.0 L/t of DM) doses, but not at high doses (1.5 L/t of DM).

2.10.4. Enzymes in dairy cows fed concentrate-based diets

Although the effects of fibrolytic enzyme supplementation were more clear in dairy cows fed diets based on high concentrate (Table 2.4) than in cows fed forage-based diets, the results are vary as well.

Contrary to the results in dairy cows fed high forage diets (Table 2.3), the global data analysis in Table 2.4 shows that cows fed enzyme complex ingested 4% more DM (+0.88 kg/d) than the control cows (average 23.7 vs. 22.8 kg/d, respectively). Contrary to cows fed forage-based diets, cows in early lactation supplemented with enzymes eat 8.5% (+1.9 kg/d) more DM than cows in middle lactation (average 24.2 vs. 22.3 kg/d, respectively). Regarding enzyme inclusion portion, DMI was improved by 16% (+3.6 kg/d) for cows fed enzymes in the forage portion compared to cows fed enzymes in the concentrate (average 26 vs. 22.4 kg/d, respectively).

Taking into account all the data shown in Table 2.4, dairy cows fed fibrolytic enzymes in high concentrate rations produced 3% (+1 kg/d) more milk than controls (average 35.6 vs. 34.6 kg/d, respectively). Fibrolytic enzymes were more effective for cows in early lactation yielding 19.4% more (6.1 kg/d) than cows in middle lactation (37.5 vs. 31.4 kg/d, respectively). With regard to the enzyme inclusion portion, milk yield improved 15.9% (5.4 kg/d) in cows fed enzymes applied to the forage portion compared to those cows fed enzymes in the concentrate portion (average 39.4 vs. 34.0 kg/d, respectively).

Table 2.4. Effects of fibrolytic enzymes on dairy cows feeding concentrate-based diets (> 50%)
Efectos de las enzimas fibrolíticas en vacas lecheras consumiendo dietas basadas en concentrado (> 50%)

Author (year)	Enzyme product	Lactation stage (cows)	Diet F:C[1]	Enzyme inclusion portion (dose DM basis)	DMI (kg/d)	Milk yield (kg/d)	Milk composition (%)		
							Fat	Protein	Lactose
Sanchez *et al*. (1996)	Promote	Early (–)	–	Control	24.4[a]	39.6[b]	3.99[a]	2.95[A]	4.89[ab]
				Forage (1.25 L/t)	+1.8[b]	+1.2[b]	-0.16[ab]	-0.08[b]	+0.02[ab]
				Forage (2.50 L/t)	+1.8[b]	+6.3[a]	+0.01[a]	-0.07[b]	+0.03[a]
				Forage (5.00 L/t)	+2.2[b]	+1.6[b]	-0.24[ab]	-0.10[b]	-0.08[b]
Beauchemin *et al*. (1999b)	Promote	Middle (4)	45:55	Control	22.3	29.9	3.70	3.18[a]	4.65
				Barley (2.50 g/kg)	-0.3	+0.3	+0.20	+0.10[b]	-0.01
				Control	21.8	31.0	3.72	3.27[b]	4.69
				Hulless-barley (2.50 g/kg)	+0.7	+1.5	+0.03	+0.01[b]	+0.03
Rode *et al*. (1999)	Promote	Early (20)	39:61	Control	18.7	35.9	3.87[b]	3.24	4.73[d]
				Concentrate (1.30 g/kg)	+0.3	+3.6	-0.50[a]	-0.21	-0.11[c]
Beauchemin *et al*. (2000)	Natugrain 33-L	Early (6)	45:55	Control	20.5[b]	31.3	3.61	3.28[b]	4.49
				Concentrate (1.22 L/t)	+1.5[a]	-0.5	+0.01	+0.06[a]	-0.02
				Concentrate (3.67 L/t	+1.1[a]	-0.5	+0.01	+0.07[a]	+0.08
Kung *et al*. (2002)	Complex	Early (30)	45:55	Control	26.9	37.2	3.33	3.10	–
				Forage (10.00 L/t)[2]	+0.9	+1.8	+0.11	+0.04	–
				Forage (10.00 L/t)[3]	+0.3	-0.4	-0.04	-0.01	–
Knowlton *et al*. (2002)	Complex	Early (16)	45:55	Control	25.5	41.1	3.11	3.00	4.85
				Grain (204.00 g/t)	+1.2	+1.8	+0.03	+0.07	+0.03
	Complex	Late (18)	45:55	Control	24.3	32.6	3.74	3.29	4.58
				Grain (204.00 g/t)	0.0	-1.2	+0.09	-0.01	+0.02
Vicini *et al*. (2003)	Complex	Early (172)	48:52	Control	21.2	32.9	3.73	3.15	–
				Forage (1.25 L/t)	+0.6	-0.4	-0.03	+0.02	–
				TMR (2.00 L/t)	+0.2	-0.5	-0.02	+0.02	–
Mean		34	45:55	–	+0.88	+1.04	-0.04	-0.01	0.00

[a, b]Means with different superscript in the same column differ ($P < 0.05$); [c, d]means with different superscript in the same column differ ($P < 0.10$). [1]Forage to concentrate ratio (%); [2]carboxymetyl cellulase + xylanase; [3]cellulase + xylanase.

The higher milk yield observed in cows in early lactation feeding concentrate-based diets supplemented with enzymes agrees with the yield in cows feeding forage-based diets. As can be observed in Table 2.4, results in milk composition are also variable. Beauchemin *et al*. (1999b) observed improvements in milk fat (+5%) and milk protein (+3%) when the enzyme was added to whole barley grain, but no changes were detected when the enzyme was added to hulless-barley.

Nevertheless, the global data analysis of Table 2.4 showed that enzyme supplementation slightly reduced milk fat (-0.04 units) and milk protein (-0.01 units) and did not change milk lactose content. Cows in early lactation supplemented with enzymes produced less fat (-4%), and protein (-4.5%), but slightly more lactose (+1.3%) compared with supplemented cows in middle lactation. Cows fed enzymes in the forage portion improved milk fat (+6.7%) and milk lactose (+5.2%), but reduced milk protein by 10% compared whit cows fed enzymes in the concentrate.

In general, enzyme supplementation affected body weight and body condition score by 1.4 and 7.8 percent points, respectively (data not shown). The weight gain was slightly higher (+3%) when the enzyme was included in the forage portion. The improvements on condition score in dairy cows fed enzyme in the concentrate were also negligible (+ 2%).

2.10.5. Enzymes in beef cattle

There are few studies on the effects of fibrolytic enzyme complex in beef cattle (Table 2.5). Most of them were done by the research group of Beauchemin *et al*. in steers fed forages by using Promote, who observed a high variability in the animal response. For alfalfa cubed with an intermediate dose of enzyme (3,900 UI/kg of DM) these authors observed increases in DMI (14%), ADG (30%), and feed conversion (16%). In steers fed timothy with the enzymes, the higher dose (15,800 UI/kg) improved the DMI (5%), ADG (33%), and feed conversion (23%). The lower effects of enzyme were detected in DMI (8%), ADG (9%), and feed conversion (3%) when was added to barley silage (1,000 UI/kg).

In contrast, the enzyme inclusion (2000 UI/kg) in timothy depressed by 17% the DMI and 9.1% the ADG. Also, the same doses of enzyme applied to silage reduced the DMI (10%) and the ADG (10%). Similarly, McAllister *et al*. (1999) detected improvements on DMI (6%) in steers fed silage with enzyme at high dose (5 L/t of DM), but also a diminution in DMI of 2.6 and 6.3% was observed by the enzyme addition to barley silage (1.25 L/t of DM) or TMR (10 L/t of DM), respectively.

Table 2.5. Effects of fibrolytic enzymes on beef cattle performance
Efectos de las enzimas fibrolíticas en la producción de vacuno de carne

Author (year)	Enzyme product	Animal (n)	Diet F:C[1]	Enzyme inclusion portion (dose DM basis)	DMI (kg/d)	ADG (kg/d)	Feed/Gain (kg/kg)
Beauchemin *et al*. (1995)	Complex	Steers (24)	97:3	Control	10.2^{d}	1.0^{d}	9.9
				Alfalfa cubed (1000 UI/kg)	+0.6^{d}	+0.3^{ef}	-0.9
				Alfalfa cubed (2000 UI/kg)	+0.3^{d}	+0.3^{ef}	-1.2
				Alfalfa cubed (3900 UI/kg)	+1.5^{e}	+0.3^{ef}	-1.4
				Alfalfa cubed (7900 UI/kg)	+0.7^{d}	+0.2^{ef}	-0.3
				Alfalfa cubed (15800 UI/kg)	+0.1^{d}	+0.1^{de}	-0.4
	Complex	Steers (24)	91:9	Control	8.8^{ef}	1.2^{d}	7.3^{e}
				Timothy cubed (1000 UI/kg)	-0.5^{de}	+0.1^{d}	-0.8^{de}
				Timothy cubed (2000 UI/kg)	-1.3^{d}	-0.1^{d}	+0.2^{e}
				Timothy cubed (3900 UI/kg)	+0.4^{ef}	0.0^{d}	-1.0^{e}
				Timothy cubed (7900 UI/kg)	-0.2^{ef}	+0.1^{d}	-0.5^{de}
				Timothy cubed (15800 UI/kg)	+0.5^{f}	+0.4^{e}	-1.4^{d}
	Complex	Steers (24)	91:9	Control	7.5^{de}	1.1	7.1
				Silage (1000 UI/kg)	+0.6^{e}	+0.1	-0.1
				Silage (2000 UI/kg)	-0.7^{d}	-0.1	+0.1
				Silage (3900 UI/kg)	+0.3^{e}	-0.1	+0.5
				Silage (7900 UI/kg)	-0.2^{de}	0.0	-0.2
				Silage (15800 UI/kg)	-0.2^{de}	0.0	-0.1
Beauchemin *et al*. (1997)	Complex	Steers (28)	5:95	Control	10.0	1.4	7.1^{b}
				Barley (4.0 L/t) H[2]	-0.5	+0.1	-0.8^{a}
				Barley (4.0 L/t) L[3]	-0.1	0.0	0.0^{b}
	Complex	Steers (28)	5:95	Control	9.6	1.3	7.3^{ab}
				Corn (4.0 L/t) H	-0.3	-0.1	+0.5^{b}
				Corn (4.0 L/t) L	-0.5	0.0	-0.3^{a}
Beauchemin *et al*. (1999a)	Promote	Heifers (1200)	8:92	Control	10.7	1.4^{b}	7.7
				Concentrate (1.40 L/t)	-0.1	+0.1^{a}	-0.7
McAllister *et al*. (1999)	Complex	Steers (98)	83:17	Control	7.7^{ab}	1.3	5.9
				Silage (1.25 L/t)	-0.2^{a}	0.0	+0.2
				Silage (3.5 L/t)	0.0^{ab}	+0.1	-0.1
				Silage (5.0 L/t)	+0.5^{b}	+0.1	+0.1
	Complex	Steers (66)	70:30	Control	10.1	1.1	8.9
				TMR (10 L/t)	-0.6	+0.2	-0.3
ZoBell *et al*. (2000)	Complex	Steers (32)	65:35	Control	9.0	1.2	7.8
				TMR (2 L/t)	-0.4	0.0	-0.3
	Complex	Steers (20)	20:80	Control	9.2	1.2	8.2
				TMR (2 L/t)	-0.3	0.0	-0.5
Gómez-Vázquez *et al*. (2003)	Fibrozyme	Steers (20)	99:1	Control	15.1	0.6^{a}	26.3
				Sugar cane (15 g/d)	-1.1	+0.1^{b}	-4.1
				Sugar cane (30 g/d)	+0.4	+0.2^{c}	-6.3
Mean		142	58:42	—	-0.05	+0.09	-0.69

[a,b,c] Means with different superscript in the same column differ ($P < 0.05$).
[d,e,f] Means with different superscript in the same column differ ($P < 0.10$).
[1]Forage to concentrate ratio (%).
[2]H = high xylanase and low cellulose activity.
[3]L = low xylanase and high cellulose activity.

The ADG increased more than 7 and 18% by enzyme addition in both barley silage and TMR, respectively. Previously, Feng *et al*. (1996) reported improvements in DMI of 2 to 12% by adding enzymes to dry-grass forage immediately before feeding steers.

With respect to enzyme addition to the concentrate portion, Beauchemin *et al*. (1997) observed improvements in feed conversion (12%) in steers fed barley diets treated with an enzyme with high xylanase activity, but not with enzymes with high cellulose activity. No effects were detected in animals fed corn diets supplemented by enzymes, except for feed conversion that was improved by between 4 and 7%.

Beauchemin *et al*. (1999a) by adding enzymes into the concentrate (1.40 L/t) increased ADG (9%) and feed conversion (10%) in heifers, but DMI was not affected. Similarly, ZoBell *et al*. (2000) reported no effects on DMI or ADG by the enzyme addition to TMR in steers fed diets based on forage or concentrate. Recently, Gómez-Vázquez *et al*. (2003) reported no effects on DMI, but ADG and feed conversion were improved (15 to 42% and 25 to 40%, respectively) in steers grazing stargrass and sugar cane supplemented with concentrate to which the enzyme was included.

2.10.6. Enzymes in sheep

Most studies about the effects of enzymes in sheep have focused on digestibility and ruminal parameters instead of sheep performance. Fredeen and McQueen (1993) reported that enzymes increased DM intake and digestibility in sheep. Similar results were obtained by Pinos-Rodríguez *et al*. (2002) in lambs fed hay (alfalfa or ryegrass) with enzymes applied ruminally (5 g/d). For both hays, enzymes increased total VFA concentration and DMI, without effects on ruminal pH. The enzyme increased CP and fiber digestibility for alfalfa hay, but not for ryegrass.

In contrast, McAllister *et al*. (1999) reported that enzymes applied either in silage or directly into the rumen did not affect DMI, DM, NDF digestibility, ruminal pH, or number of cellulolytic bacteria. Furthermore, DM and NDF digestibility were lower in ruminally-dosed enzyme sheep than those consuming enzyme-treated silage. In agreement, McAllister *et al*. (2000) observed no effects on DMI, ADG, ruminal pH, or on total tract digestibility of DM, OM, and starch, NDF or ADF in lambs fed concentrate or forage-based diets. Additionally, Lee *et al*. (2000a) with sheep dosed ruminally with a fungal enzyme obtained from *Orpinomyces* (strain KNGF-2), did not observe improvement *in situ* DM disappearance, or

ruminal pH, but cellulolytic bacteria decreased slightly. These authors concluded that the enzymes may be rapidly degraded by ruminal microbes or could reduce its growth or activity.

Titi and Tabbaa (2003) reported improvements in the digestibility of DM (4%), OM (> 4%) CF (> 5%), NDF (6%), and ADF (8%) in Awassi lambs fed a concentrate based diet supplemented with enzymes. Moreover, Titi and Lubbadeh (2004) have reported increases in lamb and kid weaning weights (7 and 2%, respectively) when their mothers (Awassi ewes and Shami goats) were fed a diet supplemented with a cellulase enzyme during the last 2 mo of pregnancy and the first 2 mo of lactation. Milk fat, milk protein and total solids in milk during suckling also improved in the enzyme fed groups, but milk samples may have been unrepresentative due to the collection method. No effects were observed in lamb and kid weights at birth.

2.10.7. Enzymes on nutrient digestibility

Positive effects of fibrolytic enzymes on nutrient digestibility have been reported in different studies *in vitro* (Feng *et al*., 1996; Dong *et al*., 1999) and *in situ* (Lewis *et al*., 1996) in which the enzyme improved DM and NDF disappearance of dry forages. Dong *et al*. (1999) demonstrated that the effects of enzymes might start when the enzyme is in contact with the substrate.

Nsereko *et al*. (2000b) suggested that the enhancements of *in vitro* fiber degradation of forages for fibrolytic enzymes is due to the alteration on the structure of the feed, in which xylan is the primary site of action for *Trichoderma*, and endo-1,4-β-xylanases and acetyl-xylan esterases work to stimulate fiber digestion by rumen microorganisms.

Regarding the *in vivo* digestibility effects of fibrolytic enzymes, Rode *et al*. (1999) observed improvements in NDF (20%) and ADF (32%) digestibility in dairy cows in early lactation fed concentrate-based diets supplemented with enzymes. Whereas, Feng *et al*. (1996) reported improvements in steers in the total tract digestibility of DM, NDF, and ADF (8.5, 8.9, and 13%, respectively) by enzyme addition to dry-grass forage; and Krause *et al*. (1998) reported improvements in ADF digestibility in steers fed diets containing silage or straw (55 and 14%, respectively).

Nsereko *et al*. (2002) indicated that enzymes from *T. longibrachiatum* stimulate the growth of specific ruminal populations of dairy cows (e.g. cellobiose-utilizing, xylanolytic, and amylolytic bacteria), increasing the capacity of the rumen for digesting feed. Additionally, *T. longibrachiatum* enzymes act synergistically with enzymes from rumen microorganisms to

degrade soluble cellulose, xylan and other plant components. This cooperative effect between endogenous and exogenous enzymes may partially increase the ruminal digestion of fiber (Morgavi *et al*., 2000). Enzymes from *T. reesei*, increase the release of reducing sugars from xylan and xylanase, endoglucanase and β-D-glucosidase activities in the liquid fraction, and xylanase and endoglucanase activities in the solid fraction, indicating an increase in fibrolytic activity due to ruminal microbes in ruminal fluid of steers (Colombatto *et al*., 2003).

Knowlton *et al*. (2002) reported that DM digestibility was greater in dairy cows fed enzymes, but NDF digestibility was unaffected. In contrast, Fredeen and McQueen (1993) reported no effects attributed to enzyme addition in the digestibility of dairy cows fed barley silages.

Nsereko *et al*. (2000a) detected dietary factors such as monosaccharides and proteins in barley silage that inhibit endo-1,4-β-xylanase (23 to 51%), α-amylase (30 to 50%), and cellulase (12 to 25%) activities of the enzymes from *T. longibrachiatum*. In the opinion of these authors, it may explain why feed enzymes are generally less effective when applied to silages than to dry feeds.

Lewis *et al*. (1996) and Feng *et al*. (1996) concluded that the *in vivo* improvements in digestibility by enzymes resulted from the enhanced colonization and digestion of degradable fiber fraction by ruminal microorganisms and, consequently, as a consequence of the further increase in degradation and particle size reduction.

2.11. Action mode and discussion on the effects of enzymes in ruminants

The improvements in milk yield have been attributed to a direct effect of enzymes on nutrient digestibility in the total tract, in which different mechanisms can be involved. They are: 1) direct hydrolysis of the feed, 2) enhancement of microbial attachment, 3) changes in gut viscosity, 4) complementary action with ruminal enzymes, and 5) improvement of nutrient digestion (Beauchemin *et al*. 1999b).

The elucidation of the mechanisms by which feed enzymes increase the digestion and the utilization of feedstuffs in ruminant diets is complicated by three main factors (Colombatto *et al*., 2003): 1) feeds are structurally very complex, containing a variety of polysaccharides, proteins, lipids, lignin, and phenolic acids, often in intimate association; 2) the enzyme products are mixtures of enzymes containing many different activities, each of which differs in their optimal conditions and specificities; and 3) ruminal fluid is by nature an extremely

complex microbial ecosystem, containing many hundreds of microbial species and their secreted enzymes.

With respect to intestinal effects, *in vivo* experiments have shown the potential of enzymes for changing not only ruminal (Yang *et al*., 1999; Sutton *et al*., 2003) but also intestinal viscosity and absorption of nutrients in the small intestine (Poutanen, 1997). Beauchemin *et al*. (1999b) observed that enzyme supplementation improved more total tract digestibility of starch (5%), NDF (8%), and ADF (11%) than digestibility in the rumen, indicating that the effects of enzyme supplementation were mostly intestinal.

It is possible that enzymes binding to substrates before introduction into the rumen may protect themselves from degradation by ruminal proteases (Kung *et al*., 2000) and then pass into small intestine. The enzyme feed additives originated from the fungus *Trichoderma* are relatively stable in the ruminal fluid and resistant to microbial degradation for a long enough time to act in the rumen. They are likely to be more affected by gastrointestinal proteases and abomasum pH than by ruminal proteases. Moreover, exogenous fibrolytic enzymes can survive in the intestine and exert their action on available substrates (Morgavi *et al*., 2001). However, Yang *et al*. (1999) observed no effects of enzyme supplementation either on viscosity contents or on nutrient duodenal digestibility, concluding that the improved digestion may not be attributed to a post-ruminal effect.

As a conclusion, Beauchemin *et al*. (1999b) proposed three ways for the arrival and action of the enzymes into the intestine:

- Enzymes applied to dry feed may enhance the binding of the enzyme to the substrate, which may increase the resistance of the enzymes to proteolysis and prolong their residence time within the rumen.
- Enzymes applied to silage or TMR immediately prior to feeding, may be released into the ruminal fluid and may pass through the rumen quickly before they can be effective, which would provide larger intestinal effects.
- Exogenous enzymes may alter digestion and nutrient absorption in the small intestine.

2.12. Causes attributed to the inconsistent results of the enzymes

In practice, the effects of exogenous enzymes on ruminant performances are variable, and many factors may affect the enzyme activity and reduce its expression on the animal. Among those factors are: 1) application method and portion of the diet to which fibrolytic enzyme complex is applied (forage, concentrate, supplement or premix); 2) diet composition and

dryness of the feed; 3) type, dose, activity and stability of the enzyme; 4) time and duration of enzyme application before feeding; 5) complementation of exogenous and endogenous enzyme activities; and, 6) experimental conditions, as well as animal differences and physiological status (Feng *et al*., 1996; McAllister *et al*., 2000; Beauchemin *et al*., 2002; Bowman *et al*., 2002).

The effectiveness of enzymes may change due to the ruminal pH during the fermentation process. Each enzyme from *Trichoderma* has an optimal pH, thus the stability and enzymatic activity may vary between commercial products. Also there are differences between the maximum xylanase activity from ruminal enzymes pH (6.0 to 6.5) and from fungal enzymes (pH 5.0) (Morgavi *et al*., 2000). The cellulolytic (Yang *et al*., 2002; Sutton *et al*., 2003; Titi and Tabbaa, 2003) and xylanolytic (Kung *et al*., 2002) activity of the fibrolytic enzyme is compromised at a ruminal pH lower than 6.0. However, Vicini *et al*. (2003) observed major cellulase and hemicellulase activities at a range of pH between 4.0 and 5.0. Moreover, the maximum release of monosaccharides and evident synergism between endogenous enzymes from *T. longibrachiatum* were detected at a pH of 5.0 (Morgavi *et al*., 2000).

The application of moderate levels of enzyme preparation (1 to 2 L/t DM) to ruminant feeds causes some beneficial disruption to the surface structure of the feed either before or after ingestion. Higher concentrations of enzyme (5 to 10 L/t DM) may compete with bacteria for binding sites on feed particles, thereby reducing overall endogenous bacterial activity and hence limit digestion of the feed (Nsereko *et al*., 2002).

Development of enzyme preparations targeted for specific feeds may be necessary. However, the structural complexity of feeds and our incomplete knowledge of the factors limiting the rate and extent of feed digestion will continue to confound the development of crude enzyme preparations for overcoming the digestive constraints of a specific feed (McAllister *et al*., 1999).

Some cows may have a more favorable rumen environment for the expression of exogenous enzyme activity, and therefore they could benefit more from the addition of enzyme additives in the diet than other cows fed a similar diet (Morgavi *et al*., 2001).

Other factors such as enzyme applied prior to consumption (uniform application, ambient temperature and the length of time between treatment and consumption); rumen environment (proteolytic stability, complementation of microbial enzymes and degree of retention in the rumen); small intestine environmental (resistance to HCl-pepsin digestion and intestinal proteases, as well as activity against substrates arriving at the small intestine) may affect the effectiveness of exogenous enzymes (McAllister *et al*., 1999).

The high variability of the effects of enzyme complex on ruminant performance between enzyme types, doses used, and portion of enzyme inclusion, even in the same study, shows the high sensibility of the enzyme at different experimental conditions.

On the other hand, enzymes are proteins that are eventually degraded in the digestive tract; consequently, they pose no risk of residues in tissues or milk. Thus enzyme technology offers to ruminant producers a competitive advantage of increased production efficiency without jeopardizing product quality (Beauchemin *et al*., 1995). There is no information available on negative effects of the exogenous enzyme complex on human or animal health. However, more investigation is needed in order to recommend their utilization in ruminants.

2.13. Organic acids

Use of carboxylic acids has been proposed as an alternative to antibiotic promoters in ruminants (Caja *et al*., 2000, 2003; Flores *et al*., 2003; Castillo *et al*., 2004). The carboxylic acids and their salts are found in nature as common constituents of plants and animal tissues. According to Mateos *et al*. (1999) they can be divided into two groups:

- Organic acids (with chemical structure based on a carboxyl group), including: formic, tartaric, acetic, fumaric, citric, propionic, butyric, lactic, sorbic and malic acid.
- Salts of organic acids, including: calcium formiate, sodium formiate, calcium lactate, calcium propionate, sodium propionate, calcium malate and sodium malate.

Malic acid ($C_4H_6O_5$) is an intermediate in the Krebs or tricarboxylyc acids cycle, with solid stage, water-soluble, pKa from 3.4 to 5.1, molecular mass of 134 g, and GE of 2.39 Mcal/kg. It is normally used for improving silage fermentation and for reducing digestive problems in piglets, as an acidogenic agent. It controls the growth of microorganisms (reducing the pH in food and digesta, creating negative conditions for the bacterial growth, or passing into the microbial cytoplasm to disrupt their live cycle) (Mateos *et al*., 1999).

Malate in feeds is formed during the glycolisis process within the cytosol of the plant cell. It is imported into the mitochondria and oxidized in two ways: 1) via 'malic enzyme' producing piruvate and CO_2; and 2) via 'malate dehydrogenase enzyme', producing oxaloacetate. Piruvate is then oxidized in the tricarboxylyc acid and malate regenerated (Lambers *et al*., 1998; Castillo *et al*., 2004).

Malate is a natural constituent of forages that are commonly used in ruminant diets. Values ranged in alfalfa from 2.4% (Jung *et al*., 1999) to 7.5% DM (Callaway *et al*., 1997); Bermuda grass (1.9 to 4.5% of DM) (Callawall *et al*., 1997); Kura clover (3.8% of DM); annual medic

(7.8% of DM) and switch grass (1.0% of DM). It has not been detected in maize silage or grasses such as: orchard grass, smooth brome grass, and oat straw (Jung *et al*., 1999). Supplementation of the diet with malate may be necessary to ensure adequate ruminal concentrations in ruminant diets (Martin *et al*., 2000a; Caja *et al*., 2000, 2003; Salama *et al*., 2002).

Malate can stimulate microbial activities in the rhizosphere impacting on the availability of nutrients (Langlade *et al*., 2002). Recently, these authors using the cDNA-amplified restriction fragment length polymorphism (AFLP) technique identified a large number of genes in the roots of lupine and maize. Among these genes they identified an ATP citrate lyase (ACL) that catalyses the formation of acetyl-CoA with a concomitant hydrolysis of ATP. The highest ACL activity was found in growing tissues such as young leaves and elongation zones of roots and was implicated in lipid biosynthesis and malate excretion.

Malate concentrations in forages also depend on variety, maturity stage (young or mature), harvested type (early or late), forage process (ground), and moisture stage (hay or freshly) (Callaway *et al*., 1997; Martin *et al*., 2000a; Salama *et al*., 2002). The release of this organic acid from the forage to the rumen may be dependent on the digestion process of the plant cell wall (Callaway *et al*., 1997). However, it is necessary to take into account that malate contained in alfalfa and Bermuda grass hays disappears very quickly (within 30 min) in ground forage once it is made available in the rumen (Martin *et al*., 2000a).

Inclusion of malate as a feed additive in the diets of ruminants is currently feasible for industrial synthesis (Caja *et al*., 2000, 2003; Salama *et al*., 2002) but at a high cost (Castillo *et al*., 2004). The incorporation of forage varieties that are high in malate makes the inclusion of malate in the ruminant diet economically viable (Callaway *et al*., 1997; Salama *et al*., 2002; Caja *et al*., 2003).

2.13.1. Effects of malate on *in vitro* digestibility

Most studies with malate have been done *in vitro*, in which malate has shown effects similar to ionophores antibiotics (i.e., changes on pH of ruminal liquid, increase of propionate, and reduction of acetate:propionate ratio, lactate concentrations and methane).

Martin and Streeter (1995) incubated DL-malate with mixed ruminal microorganisms using soluble starch or cracked corn as substrate. In soluble starch incubations, all concentrations of DL-malate (0, 4, 8, and 12 m*M*) increased final pH and propionate concentrations, and decreased the acetate:propionate ratio. Methane was decreased with 8 and

12 m*M* DL-malate, and L-lactate decreased particularly at 12 m*M*. In cracked corn fermentation, 8 and 12 m*M* DL-malate increased final pH, propionate, and total VFA, but decreased L-lactate concentrations. Similarly, 8 and 12 m*M* DL-malate increased final pH, propionate and total VFA and decreased lactate concentrations (Callaway and Martin, 1996).

Carro *et al*. (1999) reported that DL-malate (5.6 m*M* DL-malate) decreased lactate and increased CO_2 production, DM, OM, NDF, and hemicellulose disappearance in semi-continuous culture Rusitec, using 50:50 forage:concentrate diet. However, there were no differences in the disappearance of ADF and cellulose and pH values. They concluded that the improvements in DM, OM and NDF were due to an increase in the number of ruminal bacteria and their activity on fiber breakdown.

In addition, Jalč and Čerešňáková (2002) observed increments in propionate (5.8%) and reductions in methane (20%) concentrations when malate was added at a rate of 8 m*M* to *in vitro* incubated oil seeds (rapeseed, sunflower, and linseed). No effects on DM disappearance were observed.

Carro and Ranilla (2003) studied the effects of malate (0, 4, 7 and 10 m*M* DL-malate) on rumen fermentation *in vitro* using different cereal grains as substrate (maize, barley, wheat and sorghum). Propionate, total VFA, CO_2 production and final pH, increased linearly and acetate:propionate ratio and L-lactate concentrations decreased in all substrates incubations, as malate concentration increased. Methane production was decreased (7 to 8%) for barley, wheat and sorghum, but no for maize. No effects were observed for OM apparent disappearance when barley, wheat and sorghum were incubated. The effect was significant when maize was incubated with 4 and 7 m*M*-malate. García Martínez *et al*. (2003) reported improvements in final pH and OM digestibility and lower methane productions by malate addition (8 m*M*/L) to high forage and concentrate diets.

Carbon dioxide is an end product of lactate fermentation to propionate via the succinate-propionate pathway (Callaway and Martin, 1996). Malate might act as an electron sink for H^+, before dehydration of malate to fumarate. As H^+ is used to reduce fumarate, there is a decrease in the availability of H^+ for methanogenesis in the rumen, which could explain the observed decrease in methane production when malate is present (Nisbet and Martin, 1991; Martin, 1998). On the other hand, the improvements in propionate, total VFA concentrations and pH, would indicate a stimulatory effect of malate on fermentation due to changes in bacterial populations and/or in their activity (Martin and Streeter, 1995; Carro and Ranilla, 2003). The stimulation of mixed ruminal microorganism by DL-malate has been demonstrated *in vitro* (Martin and Streeter, 1995; Martin *et al*., 2000b).

The effect of organic acids on ruminal traits has been mainly attributed to the *S. ruminantium* stimulation (Nisbet and Martin, 1991; Carro et al., 1999; Caja *et al*., 2000, 2003; Castillo et al., 2004). This microorganism is considered to be the predominant ruminal bacterium, representing from 21 to 51% of the total viable bacteria in the rumen (Caldwell and Bryant, 1966). Nisbet and Martin (1991) reported that addition of malate (0.03 and 10 m*M*) increased the uptake of lactate by *S. ruminantium*, causing a reduction of lactate levels. However, Jalč and Čerešňáková (2002) also reported that malate addition reduced lactate production slightly when oil seeds were used as substrate.

Malate is a key intermediate in the succinate-propionate pathway in the rumen (Carro et al., 1999; Caja *et al*., 2000, 2003; Castillo et al., 2004), which is used by *S. ruminantium* to synthesize succinate and propionate (Martin, 1998). In this pathway, malate is dehydrated to fumarate; fumarate is reduced to succinate, which is finally decarboxylated to propionate.

S. ruminantium has been implicated as the microorganism primarily responsible for succinate decarboxylation in the rumen, and therefore for most propionate production (Wolin and Miller, 1997). *S. ruminantium* is able to produce more propionate and decrease the acetate to propionate ratio when organic acids are present (Callaway and Martin, 1996).

In addition, organic acids may act by buffering the ruminal contents by a dual mechanism: 1) increasing lactate utilization, and 2) by CO_2 production by *S. ruminantium*. Therefore, it seems that stimulation of *S. ruminantium* in mixed culture by organic acid addition helps to increase ruminal pH by increasing lactate utilization as well as the concentration of CO_2 (Callaway and Martin, 1996).

Incorporation of a feed supplement that contains sugars and malate into batch culture fermentations stimulated lactate utilization by *S. ruminantium*. This stimulation of lactate utilization occurred even though final concentrations of malate in the medium were less than 1.0 m*M*/L (Martin *et al*., 2000b).

The effects of organic acids in presence of monensin have also been studied *in vitro*. Organic acids (L-aspartate, fumarate, and DL-malate), added at a rate of 0, 4, 8, or 12 m*M*/L plus monensin, stimulated the mixed ruminal microorganism fermentation producing more propionate, less lactate, and increasing the final pH (Callaway and Martin, 1996). The reduced lactate concentrations by organic acid-plus monensin-treated incubations represent an additive effect of increased lactate production by *S. bovis* and increased lactate utilization by *S. ruminantium* (Callaway and Martin, 1996). However, in severe acidotic conditions, neither organic acids nor monensin were effective to increase ruminal pH.

2.13.2. Effects of malate on metabolism and nutrient digestibility

The effects of organic acids on nutrient total tract digestibility and metabolism have been less studied than *in vitro*.

Kung *et al*. (1982) reported no effects on DMI, ruminal pH, ADF and CP digestibility, or nitrogen retention, but total VFA concentrations and propionic acid increased in steers fed malate (100 and 200 mg/kg BW). Whereas, Montaño *et al*. (1999) reported that malate (80 g/d) increased ruminal pH in rumen in steers induced to acidosis, but without affecting total tract digestion (OM, ADF, CP and starch), VFA concentrations, lactate, or microbial efficiency. Martin *et al*. (1999) with intraruminal infusions of DL-malate (0, 27, 54, or 80 g/d) in steers fed a high-grain diet (80%), also observed that malate increased ruminal pH, but total VFA and propionate concentrations were lower with the highest DL-malate dose.

In regard to the effects of malate on nutrient digestibility in lambs, the information is scarce. Cuesta *et al*. (2003) reported that malate added at a rate of 0.4% in a diet based on high grain cereal (80%) did not affect DM, CP or NDF digestibility of fattening lambs, although the dose may not have been optimal.

2.13.3. Effects of malate on ruminant performances

The evaluation of the effects of organic acids on ruminant performance is very limited and their results are variable.

In dairy cattle production, Kung *et al*. (1982) reported no effects on DMI by adding increasing doses of malic acid (70, 105, and 140 g/d) to the diet of dairy cows. Although, cows fed 140 g/d of malic acid ingested less food, and the persistency of the lactation curve and fat and total solids contents in milk was higher.

Salama *et al*. (2002) reported that supplementation with a mixture of malate and yeast culture did not affect DMI, milk yield, milk composition or the persistency of the milk production in dairy goats fed a diet based on dehydrated forage. Theses authors explain that the lack of effect was due to the high basal level of malate as a result of the use of alfalfa in the TMR fed to the goats.

In beef cattle, Martin *et al*. (1999) conducted three finishing trials in feedlot. In the first study, steers received increasing concentrations of malate (40 or 80 g/d) in a diet based on 95% concentrate plus lasalocid (360 mg/d). The high dose (80 g/d) of DL-malate had little effect on DMI. However, steers fed 40 g/d of malate ingested less DM (-0.2 and -0.9 kg/d). Average daily gain and gain efficiency increased linearly with DL-malate dose. In the second

feedlot study, steers were supplemented with higher concentrations of DL-malate (60 or 120 g/d). DL-malate supplementation had little effect on DMI, but ADG and feed efficiency linearly increased with the dose of malate. Finally, in the third feedlot trial, steers and heifers where supplemented with 100 g/d of DL-malate. Neither ADG nor gain efficiency were affected by malate addition. DL-malate supplementation resulted in a slight reduction of ADG on d 26.

Regarding effects of malate on sheep performance, the first results were reported by Garín *et al*. (2001) who observed improvements on concentrate intake and feed conversion in lambs intensively fattened lambs fed with a mixture of malate and yeast culture at different concentrations (0, 0.2, and 0.3%). The intermediate doses (0.2%) improved feed conversion by 7%. However, ADG, ruminal pH and keratinization index of ruminal mucosa at slaughter were unaffected by malate addition.

In contrast, Cuesta *et al*. (2003) reported slight improvements on concentrate intake and ADG in intensively fattened lambs fed malate, and as a consequence, feed conversion increased. In this experiment, malate was added daily in top feeding and $NaCO_3$ was incorporated as buffer in the concentrate, which may have reduced the malate effects.

If malate can reduce the effects of lactic acidosis and can improve the energy efficiency of concentrates, as suggested by Garín *et al*. (2001), it may well be a useful additive for intensively fattened lambs in which high amounts of cereals are incorporated to concentrate diets.

There is no information about possible negative effects of malate in ruminants or in humans and malate can be proposed as a substitute of ionophores antibiotics in the concentrate of fattening lambs. More research is needed to evaluate the effects of malate on ruminant performance in practice.

Chapter 3: Objectives

Capítulo 3: Objetivos

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3.1 . General Objective

The main objective of this thesis was to evaluate the effects of two feed additives included in the concentrate of sheep, consisting of: 1) a fibrolytic enzyme complex evaluated in dairy ewes, and 2) an organic acid evaluated in intensively fattening lambs.

3.2. Specific objectives

3.2.1 Evaluation of the effects of a xylanase and cellulase fibrolytic enzyme complex (Promote) on the performance of Manchega and Lacaune dairy ewes, with specific focusing on:

- Milk yield and milk composition of dairy ewes during the suckling and milking periods.
- Growth of the lambs during the suckling period.
- Dry matter intake, body weight and body condition score of dairy ewes during the suckling and milking periods.
- Effects of the fibrolytic enzyme on the fill value of the total mixed ration, body weight and body condition score of dry and open dairy ewes.

3.2.2. Evaluation of the effects of malate (Rumalato) on the performance, total tract digestibility, and metabolism of intensively fattened lambs fed different types of concentrates based on cereals (barley and corn), with specific focusing on:

- Growth and ruminal traits of intensively fattened lambs.
- Ruminal pH and blood serum metabolites of intensively fattened lambs.
- Nutrient digestibility and nutrient balance (energy and nitrogen) of intensively fattened lambs.

Chapter 4: Improving performance of Manchega and Lacaune dairy ewes by fibrolytic enzymes complex addition in the concentrate

Capítulo 4: Mejora de la producción de ovejas lecheras Manchega y Lacaune mediante la adición de un complejo de enzimas fibrolíticas en el concentrado

Chapter 4. Improving performance of Manchega and Lacaune dairy ewes by fibrolytic enzymes complex addition in the concentrate

Capítulo 4. Mejora de producción de ovejas lecheras Manchega y Lacaune mediante la adición de un complejo de enzimas fibrolíticas en el concentrado

ABSTRACT: A total of ninety-six Manchega and Lacaune multiparous ewes were used in three experiments to evaluate the effects of fibrolytic enzymes complex on lactation performance and feed intake. Ewes were fed *ad libitum* with a diet based on 70% forage and 30% concentrate to which the enzyme was added after pelleting. Experimental concentrates were control (without enzyme) and enzyme (Promote, included at 0.47 mL/kg of concentrate). In Exp. 1, seventy-two ewes (Manchega, n = 36; and, Lacaune, n = 36) were used in a 2 × 2 factorial design to evaluate the effects of diet supplementation with an exogenous fibrolytic enzyme complex on lactational performance and feed intake during suckling period (wk 1 to 4). A suckling-milking mixed period was used during wk 5. Milk yield, milk composition, dry matter intake (DMI), lamb growth, as well as body weight (BW) change and body condition score (BCS) change, were not affected by enzyme supplementation. In Exp. 2, the same ewes from the Exp. 1 were used to evaluate the effects of fibrolytic enzyme on lactation performance during milking period (wk 6 to 12). Milk yield, milk composition and DMI were not affected by enzyme supplementation, although BW change ($P < 0.01$) and final BCS ($P < 0.05$) increased. Breed effect was significant on milk yield in both suckling and milking periods, the Manchega ewes yielding less milk ($P < 0.01$) with greater ($P < 0.01$) milk composition than the Lacaune ewes. The Manchega ewes intake less DM during milking period ($P < 0.001$). The interaction treatment × breed was not significant. In Exp. 3, twenty-four dry and open ewes (Manchega, n = 12; and, Lacaune, n = 12) were used to measure the fill value of the whole diets. Enzyme supplementation reduced DMI by 9% ($P < 0.001$) resulting in a greater sheep fill value ($P < 0.001$) of the diet. In conclusion, no lactational effects were detected when the fibrolytic enzyme complex was added to the concentrate in dairy ewes, but BW and condition score improved as an effect of the enzyme complex in late lactation.

Key words: Fibrolytic Enzymes, Dairy Sheep, Milk, Intake

4.1. Introduction

More than half of plant DM consists of carbohydrates, from which starch and cellulose are considered to be the most abundant polysaccharides. Natural substances contained in forages and grains may reduce the digestibility of polysaccharides by decreasing the activity of ruminal microbiota or by protecting the cell wall (Minson, 1990). Condensed tannins and methylcellulose prevent microbial attachment or promote their detachment, inhibiting partially or completely the cellulose digestion; also, the protein matrix of cereal grains is extremely resistant to microbial attachment and penetration (McAllister *et al*., 1994).

Applied biotechnology and feed industries currently offer exogenous enzymes as feed additives for enhancing the nutritive value of animal diets. There are many studies about adding enzyme preparations to diets for non-ruminant species, but research in ruminants is more limited. Beauchemin *et al*. (2002) reviewed the use of enzymes in ruminants indicating an increase in DM and fiber digestion *in vitro*, *in situ*, or *in vivo*, and an increase in milk yield and weight gain in cattle. Nevertheless, the results of adding exogenous enzymes to the diet of ruminants have been variable in practice. Much of the variability can be attributed to factors such as: type, dose and activity of the enzyme; application method and portion of the diet (forage or concentrate) to which the enzyme is applied; and, differences in the physiological status of the test animals (Beauchemin *et al*., 2002; Bowman *et al*., 2002). Addition of enzymes in the concentrate portion of the diet is especially interesting in practice when enzyme activity is not compromised (Bowman *et al*., 2002, 2003).

The use of enzymes in sheep diets has been limited to trials of digestibility in wethers (Lee *et al*., 2000a; Pinos-Rodríguez *et al*., 2002) and to trials of growth rate in fattening lambs (McAllister *et al*., 2000). As far as I know however, one research has been carried out on milk yield in small ruminants (Titi and Lubbaden, 2004). The aims of this study were: 1) to evaluate the effects of diet supplementation with a commercial xylanase and cellulase fibrolytic enzyme complex included in the concentrate on the lactation performances of dairy ewes during suckling and milking periods; and 2) to evaluate the effects of diet supplementation with a commercial fibrolytic enzyme complex on the fill value of the TMR of dry and open ewes.

4.2. Materials and Methods

Three experiments were conducted on the Experimental Farm of the S1GCE (Servei de Granges i Camps Experimentals) of the Universitat Autònoma of Barcelona in Bellaterra. The experimental and animal care procedures were approved by the Ethical Committee on Human and Animal Experimentation of the University Autònoma of Barcelona (Reference CEEAH 03/429).

4.2.1. Experiment 1. Suckling Period

4.2.1.1. Animals and Management

Seventy-two multiparous ewes from two dairy breeds (Manchega and Lacaune) differing in milk yield performance were used according to a 2 × 2 factorial design in a lactation trial from wk 1 to 4 after lambing. Ewes (Manchega, n = 36, 74.6 ± 1.2 kg BW; and, Lacaune, n = 36, 73.1 ± 1.4 kg BW) were allocated into eight balanced groups (9 ewes per group) according to breed, number of lactation, and wk, BW and BCS at lambing. Dietary treatments started after lambing. Ewes were confined to straw bedded pens and the balanced groups of each breed randomly assigned to the experimental treatments.

Lambs were allowed to suckle from their mothers 24-h a day until wk 4 after birth. A suckling-milking mixed period was used during wk 5 to reduce weaning stress (Treacher and Caja, 2002).

4.2.2. Experiment 2. Milking Period

4.2.2.1. Animals and Management

The same ewes from Exp. 1 were used in a lactation trial from wk 6 to 12. After weaning, ewes (Manchega, n = 36, 70.0 ± 1.2 kg BW; and, Lacaune, n = 36, 66.4 ± 1.3 kg BW) were machine milked in a double-12 stall parallel milking parlor (Westfalia Landtechnik, Granollers, Spain) with recording jars. A small amount of concentrate (50 g per ewe) was also offered at each milking in the milking parlor to encourage the ewe to enter. Milking was conducted at a vacuum pressure of 42 kPa, a pulsation rate of 120 pulses/min, and a pulsation ratio of 50%, as indicated by Such *et al*. (1999) for Manchega and Lacaune ewes. Milk samples were collected weekly as a composite of the morning and afternoon milkings.

4.2.3. Experiment 3. Evaluation of TMR Fill Value

4.2.3.1. Animals and Management

Twenty-four dry and open ewes from the two dairy breeds (Manchega, n = 12, 71.4 ± 1.9 kg BW; and, Lacaune, n = 12, 70.3 ± 1.4 kg BW) were used to estimate the TMR fill value as defined in the INRA system (Jarrige, 1989) over a 2-wk adaptation period and 5-wk measurement period. The dry and open ewes were distributed into four balanced groups (6 ewes per group) according to breed, BW and BCS. Ewes were allocated in straw bedded pens next to the dairy ewes.

4.2.4. Feeds, Feeding and Treatments

Ewes received an *ad libitum* TMR based on 70% forage (dehydrated mixture of 50% alfalfa and 50% maize-whole plant) and 30% concentrate pellets to which the fibrolytic enzyme complex was or was not added. Enzyme preparation was added to the entire concentrate to maximize the proportion of the diet to which the enzyme was applied according to the conclusions of Bowman *et al*. (2002). Whit this aim, the liquid enzyme preparation was sprayed (0.47 mL/kg of concentrate) onto the previously manufactured and cooled concentrate pellets in a horizontal mixer.

Diet ingredients are given in Table 4.1. The forage mixture was offered twice daily (0900 and 1500) at a rate of 115% of the voluntary intake from the previous day. The concentrate was included in the TMR at a rate of 0.8 kg per kg of forage offered. Fresh water was permanently available in the pens.

Treatments were: control (without enzyme) and enzyme supplemented (Promote fibrolytic enzyme complex, Agribrands International, St Louis, MI). The fibrolytic enzyme complex is a commercial product containing high cellulase and xylanase activities, which were described previously by Bowman *et al*. (2002). Cellulase and xylanase activities of the commercial enzyme preparation used were 130 units/g and 120,000 units/g, respectively (P. Frumholtz, Agribrands International, St. Louis, MO, personal communication), as also measured by González *et al*. (2004).

4.2.5 Measurements, Sampling and Analysis

Individual milk yield during suckling was estimated fortnightly by using the oxytocin method (Doney *et al*., 1979) with machine milking according to Casals *et al*. (1999). Ewes

were milked twice at a 4-h interval after i.v. injections of 2 IU oxytocin (Veterin lobulor; Laboratorios Andreu, Barcelona, Spain). Between these two milkings, ewes were prevented from suckling their lambs. Milk secretion during these 4 h was assumed to be the normal rate of milk secretion and was extrapolated at 24 h to estimate daily milk yield. An individual milk sample was taken from the second milking for chemical analysis.

During the milking period, milk yield was recorded weekly for each ewe in two consecutive milkings (Tuesday 0800 and Wednesday 1700). Individual milk samples were collected fortnightly as a composite of the morning and afternoon milkings. Milk samples were preserved with potassium dichromate (0.5 ml of a 70 mg/L solution in 100 mL milk) and analyzed for TS, fat, total protein (N × 6.38), true protein, and CN by using a near-infrared spectroscopy analyzer (Technicon InfraAlyzer-450, Bran+Luebbe SL, Nordersted, Germany) according to Albanell *et al*. (1999). Calibration was checked using the AOAC (1990) reference methods. Energy corrected milk (ECM) was calculated according to Bocquier *et al*. (1993).

Table 4.1. Ingredients of the experimental diets
Ingredientes de las raciones experimentales

Ingredients, % as feed	Forage mixture	Concentrate
Maize-whole plant dehydrated	50.0	—
Alfalfa hay dehydrated	50.0	—
Alfalfa meal pellets	—	37.9
Barley meal	—	11.9
Spanish ground corn	—	11.7
Soybean-44 meal	—	22.5
Whole sunflower-seed meal	—	14.7
$CaC0_3$	—	1.0
MVM[1]	—	0.3
Promote, mL/kg	—	0.47

[1]Mineral-vitamin mix was supplied by Agribrands Europe, Barcelona, Spain. The preparation contained 10.5% Ca, 20.0 g/kg Mn, 17.5 g/kg Fe, 15.0 g/kg Zn, 250 mg/kg I, 100 mg/kg Se, 50 mg/kg Co, 3600 IU/kg of vitamin A, 700 IU/kg of vitamin D_3, 22000 UI/kg (α-tocopherol) of vitamin E.

The DMI was calculated for each ewe group as the difference between the total amount of DM offered and the amount refused daily (10 g accuracy). Daily samples of the TMR and orts were collected and composited by period for each group and treatment throughout the experiment for analysis of composition. Samples were ground through a 1-mm stainless steel screen and were analyzed for DM and OM (AOAC, 1995). The CP was determined by using a Kjeltec Auto 1030 Analyzer (Tecator, Hogänäs, Sweden). The method of Van Soest *et al*. (1991) was used to analyze NDF and ADF using the ANKOM^{200} Fiber Analyzer incubator (Ankom Technology, Fainport, NY).

Individual lamb BW, and BW (0.1 kg accuracy) and BCS of the ewes were recorded weekly throughout the experiment. The BCS was measured on a scale of 0 to 5 (Russell *et al*., 1969) to the nearest 0.25.

4.2.6. Statistical Analysis

Individual data for BW of lambs and for milk yield and composition, BW and BCS of the ewes, and group data for DMI of the ewes at each period (suckling or milking) were analyzed using the PROC MIXED procedure with repeated measures of SAS (SAS v. 8.1; SAS Inst., Inc., Cary, NC). The statistical model contained the fixed effects of treatment, parity, prolificacy, breed, and wk of lactation as the repeated factor; the random effects of the animal inside the group; the first order interactions of these factors; and, the residual error.

The PROC MIXED procedure was also used in the TMR fill value experiment to analyze the effects of treatments on DMI, and BW and BCS change. The covariance structure that yielded the smallest Schwartz Bayesian criterion was considered to be the most suitable analysis (Littell *et al*., 1998). Differences were tested using the PDIFF option of SAS and were considered significant at $P < 0.05$; trends were discussed at $P < 0.10$.

4.3. Results and Discussion

4.3.1. Nutritive Value of Feeds

Chemical composition and nutritive value of forage mixture, concentrate and TMR are listed in Table 4.2. The concentrate fed to the enzyme group contained slightly more CP (4.1 g/kg) than the control group due to differences in the manufacturing process. This difference in CP supply supposed a slightly lower CP intake (< 1%), which was not relevant for the treatment comparison. No relevant differences between experimental diets were observed for any other nutrient either.

Table 4.2. Chemical composition and nutritive value of feeds used in the experimental diets
Composición química y valor nutritivo de los alimentos usados en las raciones experimentales

Item	Forage mixture	Concentrates		TMR	
		Control	Enzyme[1]	Control	Enzyme
DM content, %	93.61	91.82	91.98	93.07	93.12
Composition, % DM					
OM	92.2	90.1	90.6	91.6	91.7
CP	11.72	24.00	24.41	15.39	15.53
Fat	—	2.69	2.58	—	—
Crude Fiber	28.8	17.6	18.7	25.4	25.7
NDF	45.8	27.5	28.3	40.3	40.5
ADF	27.3	16.7	18.0	24.1	24.5
NEL, Mcal/kg DM[2]	1.36	1.56	1.51	1.41	1.41
Ca, g/kg DM[2]	13.8	11.5	11.3	12.9	12.9
P, g/kg DM[2]	3.0	4.3	5.0	3.7	3.7

[1]Fibrolytic enzyme mixture (Promote, Promote Technologies Group, Agribrands, Minnetonka, MN) applied by spraying onto the concentrate at 0.47%.
[2]Estimated from INRA tables (Jarrige, 1989) by using the PreValim 2.7 software.

4.3.2. Experiment 1. Suckling Period

Intake of TMR during the suckling period was high (Table 4.3 and Figure 4.1) for both breeds and both treatments, averaging 3.0 kg DM/d. Despite the differences in milk yield between the two breeds (0.52 L/d; $P < 0.001$), as estimated by the oxytocin method, no DMI differences were detected between them.

Values of DMI were greater than those observed by Molina *et al*. (2001) in Manchega and Lacaune dairy ewes with similar diets, stage of lactation and level of production, reaching 4.3% of BW in my results. The effects of enzyme supplementation treatments on DMI were also not significant. Interaction between treatment and breed was significant ($P < 0.001$); as a result of the different trend of DMI change according to time for each breed (Figure 4.1). An initial decrease in DMI in all ewe groups was observed after wk 1, most probably due to the adaptation of the ewes to the lactation diet.

Supplementation with fibrolytic enzyme complex did not affect actual (average 2.41 L/d) and ECM (average 2.10 L/d) milk yield or milk efficiency (average 0.70 L/kg) during the suckling period (Table 4.3 and Figure 4.2), but Lacaune ewes yielded more actual (22%; $P < 0.05$) and ECM (28%; $P < 0.001$) milk than Manchega ewes. Moreover, Lacaune ewes were 30% ($P < 0.01$) more efficient than Manchega ewes transforming DMI into milk during the suckling period (Table 4.3) as a consequence of their greater milk yield for a similar DMI as was observed by Marie *et al*. (2002) in dairy sheep during milking.

Table 4.3. Lactational effects of fibrolytic enzyme supplementation of Manchega and Lacaune dairy ewes during the suckling period (values are LSM)
Efectos productivos de la suplementación con enzimas fibrolíticas de ovejas lecheras Manchega y Lacaune durante el periodo de cría (medias por mínimos cuadráticos)

Item	Treatment		Ewes breed		Effect ($P <$)		
	Control	Enzyme	Manchega	Lacaune	Enzyme	Breed	E × B1
DMI							
kg/d	2.99	2.99	2.98	2.99	0.851	0.843	0.001
% of BW	4.26	4.16	4.14	4.30	0.436	0.773	0.353
Milk, L/d							
Actual	2.42	2.40	2.17	2.65	0.930	0.004	0.529
ECM[2]	2.09	2.10	1.84	2.36	0.922	0.001	0.579
ECM/DMI	0.70	0.70	0.61	0.79	0.864	0.008	0.141
BW, kg							
Initial	73.5	74.4	74.6	73.1	0.864	0.008	0.141
Final	68.7	69.8	70.5	67.7	0.663	0.610	0.230
Change	-4.8	-4.6	-4.0	-5.4	0.785	0.008	0.580
BCS[3]							
Initial	2.93	2.94	2.96	2.90	0.885	0.444	0.831
Final	2.33	2.35	2.40	2.26	0.486	0.437	0.060
Change	-0.60	-0.59	-0.56	-0.64	0.802	0.240	0.276

[1]Interaction enzyme × breed.
[2]Estimated according to equation of Bocquier *et al*. (1993): ECM = Milk yield, L/d [0.071 + 0.15 × Fat (%) + 0.043 × Protein (%) + 0.2224].
[3]Body condition score (scale of 0 to 5 to the nearest 0.25). Measured according to Russell *et al*. (1969).

Contrary to my results, Titi and Lubbaden (2004) reported increases in milk yield (10%) in Awassi ewes fed a diet supplemented with a cellulase enzyme during the last 2 mo of pregnancy and the first 2 mo of lactation. No effects were observed in DMI. Moreover, Rode *et al*. (1999) observed an increase in milk yield (10%) in dairy cows by adding the same enzyme complex as in this experiment to the concentrate, and concluded that this enzyme complex has a great potential application in ruminants in negative energy balance. This result was not observed in my case.

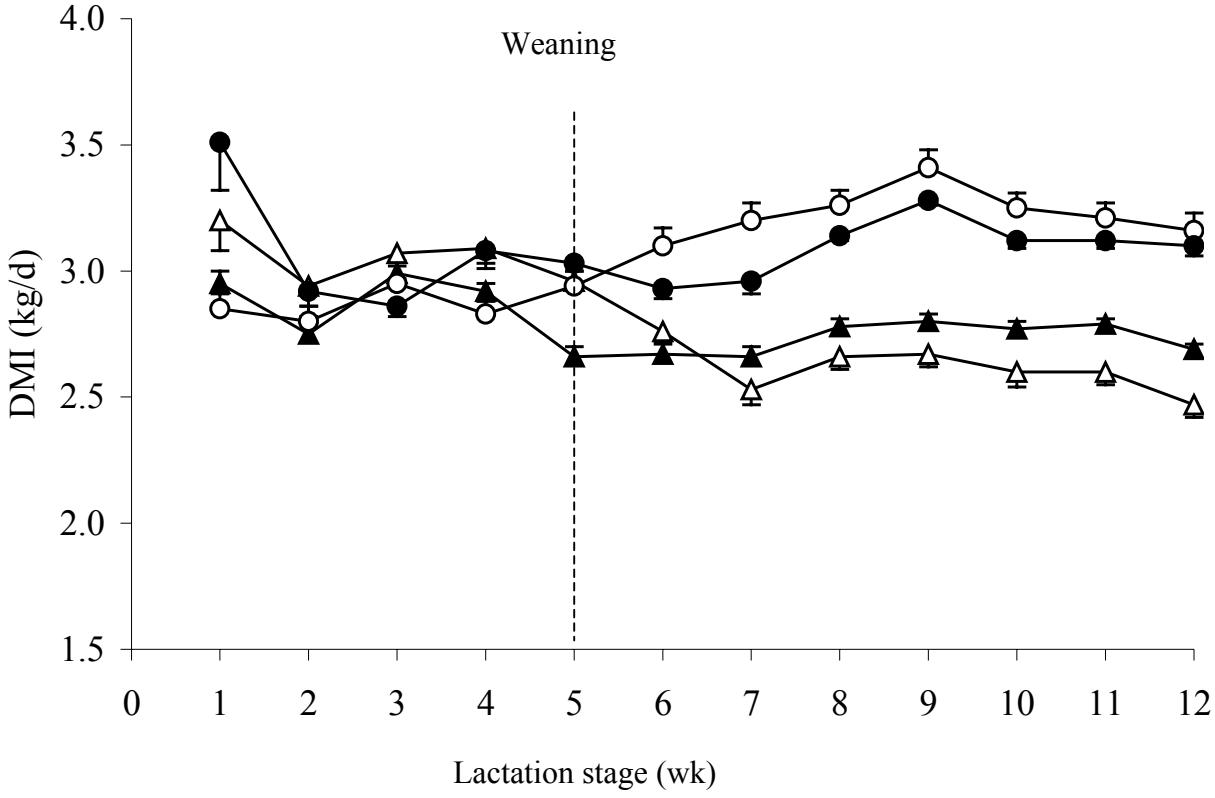

Figure 4.1. Effects of adding fibrolytic enzymes to the concentrate of lactating dairy ewes on dry matter intake. (Each point represents the mean of 18 observations; Manchega ewes: control, Δ; and, enzyme, ▲; Lacaune ewes: control, ○; and, enzyme, ●).

Efectos de la adición de enzimas fibrolíticas en el concentrado de ovejas lecheras en el consumo de materia seca de la ración. (Cada punto representa la media de 18 observaciones; ovejas raza Manchega: control, Δ; y enzima, ▲; ovejas raza Lacaune: control, ○; y enzima, ●).

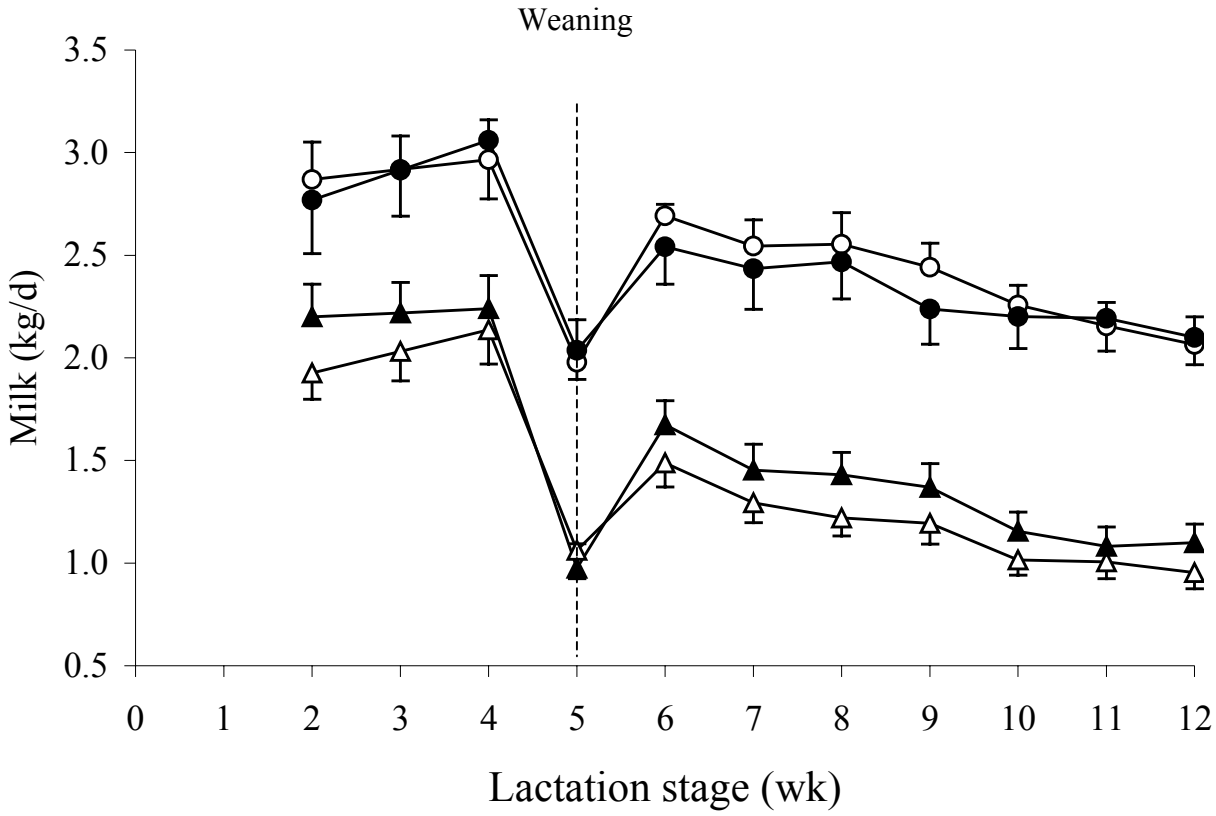

Figure 4.2. Effects of adding fibrolytic enzymes to the concentrate of lactating dairy ewes on milk yield. (Each point represents the mean of 18 observations; Manchega ewes: control, Δ; and, enzyme, ▲; Lacaune ewes: control, ○; and, enzyme, ●).

Efectos de la adición de enzimas fibrolíticas en el concentrado de ovejas lecheras en la producción de leche. (Cada punto representa la media de 18 observaciones; ovejas raza Manchega: control, Δ; y enzima, ▲; ovejas raza Lacaune: control, ○; y enzima, ●).

The enzyme supplementation affected neither final BW nor change in BW, nor BCS during suckling (Figure 4.3). Ewes of both breeds and both treatments lost BW (-4.7 kg) and BCS (-0.60) during suckling period (Table 4.3), indicating a similar negative energy balance for both dietary treatments. Loss of BW was greater in Lacaune than in Manchega ewes (35%; $P < 0.01$) as a consequence of their greater milk yield with similar DMI and maintenance requirements.

Milk composition during suckling period was not affected by enzyme supplementation. Bred effect was not significant (Table 4.4). In contrast, Titi and Lubbaden (2004) reported that milk fat, milk protein and total solids in milk improved (0.84, 0.67 and 1.42 percentage points, respectively) during suckling in the enzyme fed groups. However, milk samples may have been unrepresentative due to the collection method.

Table 4.4. Effects of fibrolytic enzymes supplementation on milk composition of Manchega and Lacaune dairy ewes during the suckling period (values are LSM)
Efectos de la suplementación con enzimas fibrolíticas en la composición de leche de ovejas lecheras Manchega y Lacaune durante el periodo de cría (medias por mínimos cuadráticos)

Item	Treatment		Ewes breed		Effect ($P <$)		
	Control	Enzyme	Manchega	Lacaune	Enzyme	Breed	E × B[1]
Composition, %							
Total solids	17.07	17.41	17.02	17.46	0.280	0.162	0.659
Fat	5.92	6.09	5.89	6.12	0.351	0.219	0.706
Crude protein	5.21	5.30	5.23	5.28	0.170	0.458	0.840
True protein	4.84	4.94	4.86	4.91	0.146	0.415	0.871
Casein	3.96	4.03	3.98	4.01	0.116	0.431	0.718
Component, g/d							
Total solids	407	403	360	450	0.839	0.001	0.363
Fat	149	152	129	172	0.818	0.003	0.659
Crude protein	124	125	111	138	0.907	0.002	0.570
True protein	115	116	102	128	0.859	0.002	0.582
Casein	94	95	84	105	0.855	0.002	0.537

[1]Interaction enzyme × breed.

Lamb growth was not affected by the dietary treatments and averaged 276 g/d (Table 4.5). Breed of ewe affected significantly lamb BW at birth ($P < 0.001$) but not at weaning. Lacaune lambs were lighter than Manchega lambs at birth (-0.6 kg; $P < 0.001$) but the difference was not significant at weaning ($P = 0.117$). Lamb growth rate was similar to the values previously reported by Casals *et al*. (1999) in Manchega sheep but milk conversion was low in all groups (average 0.113 kg/L). Titi and Lubbadeh (2004) reported increases in lamb weaning weights (7%) when their mothers were fed with a diet supplemented with a cellulase enzyme during the last 2 mo of pregnancy and the first 2 mo of lactation. No effects were observed in lamb and kid weights at birth.

Manchega lambs were apparently more efficient ($P < 0.001$) than Lacaune lambs due to the lower milk yield of their mothers as estimated by the oxytocin method. Interaction enzyme × breed was not significant. The results on lamb growth were congruent with the lack of effect of the enzyme supplementation on milk yield and composition during suckling.

Table 4.5. Effects of fibrolytic enzymes supplementation to the concentrate on growth of Manchega and Lacaune lambs during the suckling period (values are LSM)
Efectos de la suplementación con enzimas fibrolíticas en el crecimiento de corderos raza Manchega y Lacaune durante el periodo de cría (medias por mínimos cuadráticos)

Item	Treatment		Lamb breed		Effect ($P<$)		
	Control	Enzyme	Manchega	Lacaune	Enzyme	Breed	E × B[1]
Animals	49	51	52	48			
Birth BW, kg	4.3	4.2	4.5	3.9	0.633	0.001	0.301
Weaning BW, kg	11.5	12.2	12.2	11.5	0.113	0.117	0.393
BW gain, g/d	267	284	276	275	0.168	0.838	0.217
Milk conversion[2]	0.109	0.117	0.134	0.093	0.188	0.001	0.345

[1]Interaction enzyme × breed; [2]measured as kg lamb gain/kg milk.

4.3.3. Experiment 2. Milking Period

Intake of DM was not affected by fibrolytic enzyme supplementation during the milking period (Table 4.6). This result agrees with other studies reporting no differences in DMI when the enzyme complex was added to the diet of dairy cows (Beauchemin *et al*., 1999b; Kung *et al*., 2000) or lambs (McAllister *et al*., 2000). In contrast, a positive effect of enzyme supplementation on DMI has been reported in dairy cows (Beauchemin *et al*., 2000) and in steers (Krause *et al*., 1998).

Although DMI was relatively steady after weaning in both breeds (Figure 4.1), DMI varied by ewe's breed according to milk yield, being greater in Lacaune than in Manchega ewes. On average, Lacaune ewes ingested 18.8% more than Manchega ewes during the milking period ($P < 0.001$). The greater value of DMI for Lacaune ewes was consistent throughout the entire milking period (Figure 4.1). Differences between breeds were significant for daily DMI and for percentage of BW. Marie *et al*. (2002) also reported a greater DMI value throughout lactation for Lacaune, compared to Manchega ewes. This is a consequence of differences in energy demand for mammary development and milk production (Molina *et al*., 2001). As milk production increases, glucose uptake by the mammary gland for synthesis of lactose, fatty acids, and protein increases (Freetly and Ferrell, 1999). Interaction enzyme × breed was significant ($P < 0.001$).

Supplementation with fibrolytic enzyme complex to the concentrate did not affect milk production (actual and ECM milk) or milk efficiency during the milking period (Table 4.6). The lack of effect of fibrolytic enzyme on milk yield and milk efficiency agree with the results of previous studies in dairy cows for which, a similar enzyme was added to the TMR (Beauchemin *et al*., 1999a), or a different enzyme was added to the concentrate (Beauchemin *et al*., 2000) or to ensiled forages (Fredeen and McQueen, 1993).

Table 4.6. Lactational effects of fibrolytic enzymes supplementation of Manchega and Lacaune dairy ewes during the milking period (values are LSM)
Efectos productivos de la suplementación con enzimas fibrolíticas de ovejas lecheras Manchega y Lacaune durante el periodo de ordeño (medias por mínimos cuadráticos)

Item	Treatment		Ewes breed		Effect ($P<$)		
	Control	Enzyme	Manchega	Lacaune	Enzyme	Breed	E × B[1]
DMI							
kg/d	2.90	2.91	2.66	3.16	0.839	0.001	0.001
% of BW	4.28	4.24	4.15	4.40	0.737	0.022	0.550
Milk, L/d							
Actual	1.79	1.82	1.26	2.35	0.797	0.001	0.188
ECM[2]	1.67	1.68	1.22	2.13	0.970	0.001	0.118
ECM/DMI	0.56	0.57	0.46	0.67	0.765	0.001	0.385
BW, kg							
Initial	68.2	68.6	70.0	66.4	0.864	0.060	0.604
Final	69.0	70.6	71.9	67.0	0.424	0.009	0.627
Change	0.8	2.0	1.9	0.7	0.005	0.002	0.812
BCS[3]							
Initial	2.41	2.42	2.53	2.28	0.597	0.015	0.069
Final	2.63	2.76	2.90	2.43	0.038	0.001	0.239
Change	0.27	0.36	0.41	0.19	0.272	0.001	0.189

[1]Interaction enzyme × breed.
[2]Estimated from equation of Bocquier *et al*. (1993): ECM (L/d) = Milk yield (L/d) × [0.071 × Fat (%) +0.043 × Protein (%) + 0.2224].
[3]Body condition score (scale of 0 to 5) measured according to Russell *et al*. (1969).

In contrast, other authors have reported increments in milk yield from 7 (Yang *et al*., 1999) to 10% (Rode *et al*., 1999) in dairy cows when a similar enzyme complex was added to alfalfa hay cubes or to the concentrate, respectively.

Milk composition during the milking period (Table 4.7) was not affected by enzyme supplementation in the concentrate, except for milk fat, which tended to decrease by 5% (P = 0.060) during the milking period. On the contrary, Beauchemin *et al*. (1999b) and Bowman *et al*. (2002) reported slight increases in milk fat and milk protein in cows supplemented with a similar fibrolytic enzyme mixture. Reduction in milk fat content by effect of enzyme supplementation agrees with previous results in dairy cows indicating a reduction of 4 to 13% in cows fed enzyme-treated forages (Fredeen and McQueen, 1993; Rode *et al*., 1999). Nevertheless, the effects of enzyme supplementation on milk fat are not consistent. In a two-year experiment in dairy cows, Kung *et al*. (2000) reported changes in milk fat content ranging from an increase of 4% to a decrease of 4 to 11%.

Table 4.7. Effects of fibrolytic enzymes supplementation on milk composition of Manchega and Lacaune dairy ewes during the milking period (values are LSM)
Efectos de la suplementación con enzimas fibrolíticas en la composición de leche de ovejas lecheras Manchega y Lacaune durante el periodo de ordeño (medias por mínimos cuadráticos)

Item	Treatment		Ewes breed		Effect (P<)		
	Control	Enzyme	Manchega	Lacaune	Enzyme	Breed	E × B[1]
Composition, %							
Total solids	17.19	17.01	17.60	16.60	0.423	0.001	0.350
Fat	6.83	6.53	7.07	6.28	0.060	0.001	0.620
Crude protein	5.85	5.76	6.00	5.60	0.223	0.001	0.660
True protein	5.50	5.42	5.67	5.24	0.230	0.001	0.551
Casein	4.48	4.41	4.60	4.29	0.294	0.001	0.545
Component, g/d							
Total solids	304	306	220	391	0.870	0.001	0.235
Fat	120	117	88	149	0.683	0.001	0.142
Crude protein	102	101	77	126	0.834	0.001	0.055
True protein	96	95	71	119	0.808	0.001	0.099
Casein	78	77	58	98	0.826	0.001	0.098

[1]Interaction enzyme × breed.

Whereas, Zheng *et al*. (2000) studied the effects of enzyme supplementation and lactation stage, reporting a moderate increase in milk fat content (2 to 2.5%) without effects of lactation stage. The reason for the depression in milk fat content in my results and in the above discussed experiments is unclear but it may be attributed to an increase in the ruminal propionate as well as to an increase in the blood insulin.

Rode *et al*. (1999) suggested that the lower yield in milk fat for cows fed the enzyme-treated is as a result of the increase in rumen propionate and blood glucose from increased post-ruminal digestion, leading to an increase in insulin release and to a net effect of depressing milk fat synthesis by increasing adipose tissue lipogenesis. Moreover, the authors also suggest that the increase in fiber digestion by effect of the fibrolytic enzyme may reduce the effective NDF content of the diet, resulting in a greater requirement of fiber to maintain milk fat content.

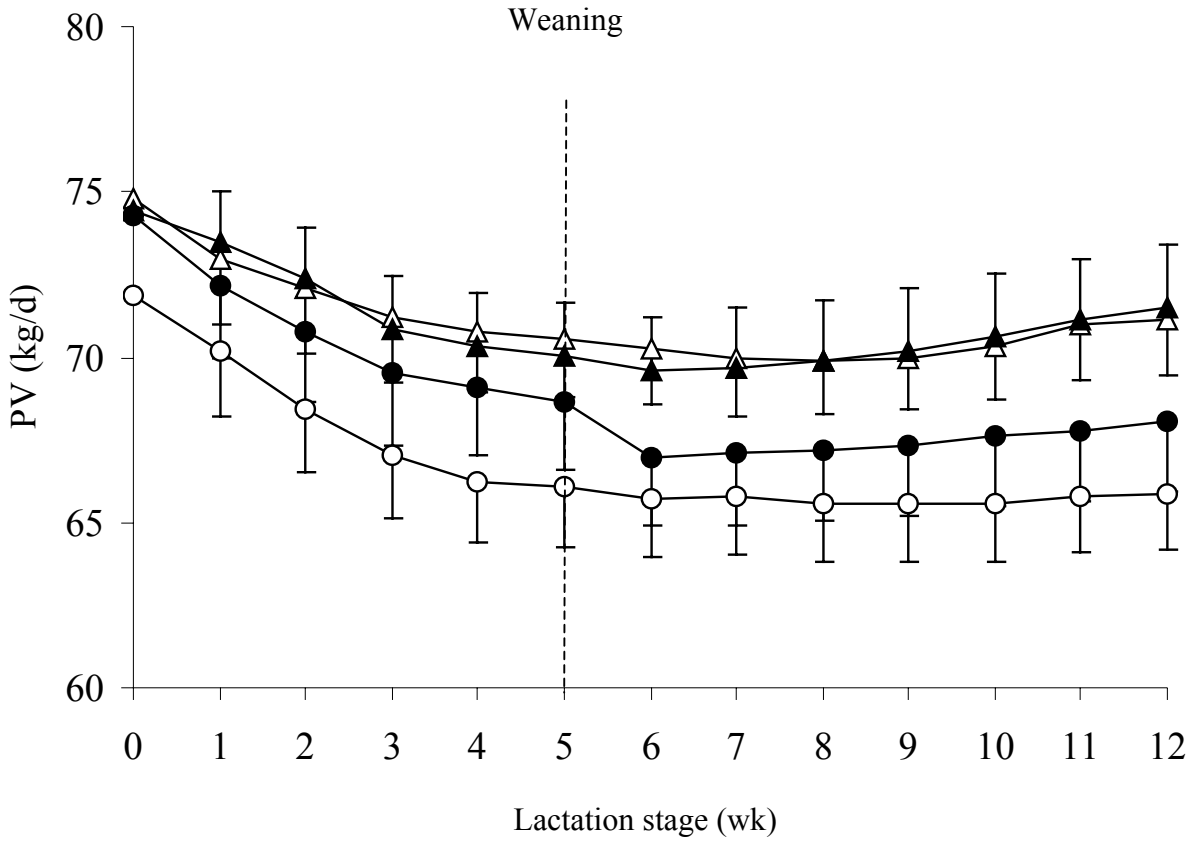

Figure 4.3. Effects of adding fibrolytic enzymes to the concentrate of lactating dairy ewes on change of body weight. (Each point represents the mean of 18 observations; Manchega ewes: control, Δ; and, enzyme, ▲; Lacaune ewes: control, ○; and, enzyme, ●).

Efectos de la adición de enzimas fibrolíticas en el concentrado de ovejas lecheras en la variación de peso vivo. (Cada punto representa la media de 18 observaciones; ovejas raza Manchega: control, Δ; y enzima, ▲; ovejas raza Lacaune: control, ○; y enzima, ●).

Nevertheless, this explanation is not supported by the values observed in milk protein content in my results that decreased numerically ($P = 0.223$) due to enzyme supplementation. Sanchez *et al*. (1996) also reported decreases in milk fat and milk protein when a fibrolytic mixture was sprayed onto the TMR of dairy cows.

With regard to body reserves, enzyme supplementation increased BW change ($P < 0.01$) and final BCS ($P < 0.05$) during the milking period (Table 4.6, Figure 4.3 and Figure 4.4). These results agree with those of Sanchez *et al*. (1996) in which BW and BCS of dairy cows improved in the enzyme-supplemented group. However, in other cases, BW and BCS were unaffected by addition of a similar enzyme complex to the concentrate (Rode *et al*., 1999), or when a different enzyme complex was added to alfalfa hay cubes (Zheng *et al*., 2000). Ewe breed significantly affected ($P < 0.05$) the initial BW and BW change, and values were greater in Manchega than Lacaune ewes according to milk yield. Moreover, final BCS was greater ($P < 0.001$) in Manchega than in Lacaune ewes. Interaction enzyme × breed was not significant.

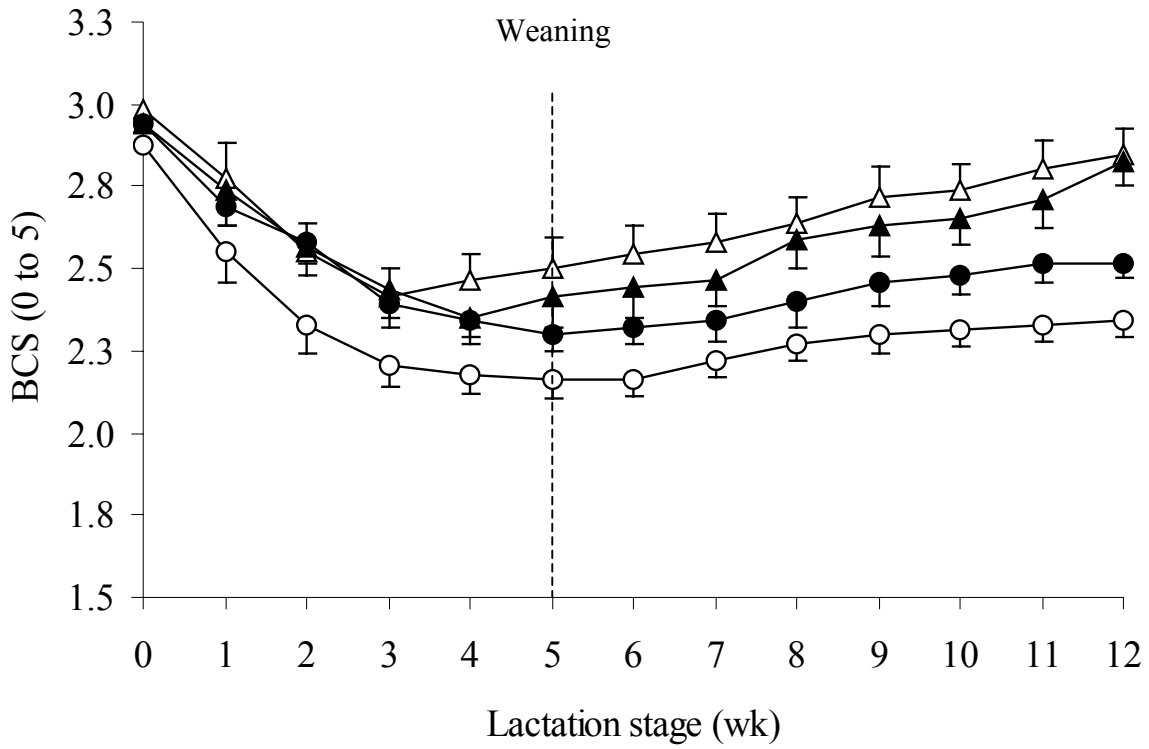

Figure 4.4. Effects of adding fibrolytic enzymes to the concentrate of lactating dairy ewes on change of body condition score. (Each point represents the mean of 18 observations; Manchega ewes: control, Δ; and, enzyme, ▲; Lacaune ewes: control, ○; and, enzyme, ●).

Efectos de la adición de enzimas fibrolíticas en el concentrado de ovejas lecheras en la variación de condición corporal. (Cada punto representa la media de 18 observaciones; ovejas raza Manchega: control, Δ; y enzima, ▲; ovejas raza Lacaune: control, ○; y enzima, ●).

4.3.4. Experiment 3. Evaluation of TMR Fill Value

The dry and open ewes used to evaluate the effect of enzyme supplementation on the fill value of the TMR increased BW and BCS during the ingestion experiment (Table 4.8) as expected because of the high nutritive value of the TMR (Table 4.2). No significant differences were found according to breed and treatment, although Manchega ewes tended ($P < 0.10$) to be heavier than Lacaune at the end of the experiment.

The DMI of TMR was high for ewes at maintenance requirements and significant effects ($P < 0.001$) were observed according to breed and enzyme treatment (Table 4.8). Breed differences disappeared when intake was expressed as percentage of BW, being 2.6% BW on average. Nevertheless, Lacaune ewes showed a numerically greater ingestibility than Manchega ewes when DMI was expressed per metabolic BW ($P = 0.112$).

Enzyme supplementation reduced DMI by 9.1% (2.03 *vs*. 1.86 kg/d; $P < 0.001$) and ingestibility by 8.8% (80 *vs*. 73 g/kg $BW^{0.75}$; $P < 0.001$). Differences in intake results between lactation and ingestion experiments may be a consequence of the differences in the rate of passage of the diet through the gastrointestinal tract according to physiological stage, as reported by Molina *et al*. (2001) in dairy ewes.

Beauchemin *et al*. (1995) also reported differences in ration DMI according to the enzyme dose. Thus, in steers fed enzyme treated forages, DMI was reduced for medium doses whereas it increased for high and low doses. Moreover, Beauchemin *et al*. (2000) indicated greater improvements in DMI when the enzyme was added to the concentrate portion of the TMR at a low dose rather than at a high dose in dairy cows.

As a consequence of the reduced voluntary intake, sheep fill values estimated according to the INRA system (Jarrige, 1989) methodology increased due to effect of enzyme supplementation, being 0.94 and 1.03 ($P < 0.001$) for the control and enzyme supplemented TMR, respectively. The difference in fill value can justify approximately 90 g/kg of DMI. These sheep fill values were used to calculate the intake capacity of the ewes during the lactation experiment using the equation proposed by Caja *et al*. (2002) for dairy ewes. Predicted DMI values were 3.20 and 2.95 kg/d for control and enzyme treatments, respectively, giving an overestimation of 10.3 and 1.5%, respectively, for the intake of each diet.

Table 4.8. Effects of fibrolytic enzymes supplementation on intake and body changes of Manchega and Lacaune dry ewes during the ingestion trial (values are LSM)

Efectos de la suplementación con enzimas fibrolíticas en la ingestión y variación de ovejas secas de raza Manchega y Lacaune durante el experimento de ingestión (medias por mínimos cuadráticos)

Item	Treatment		Ewes breed		Effect ($P <$)		
	Control	Enzyme	Manchega	Lacaune	Enzyme	Breed	E × B[1]
BCS[2]							
Initial	3.02	3.04	3.06	3.00	0.748	0.340	0.340
Final	3.35	3.44	3.41	3.38	0.269	0.576	0.269
Change	0.33	0.40	0.35	0.38	0.257	0.701	0.701
BW, kg							
Initial	70.6	71.1	71.4	70.3	0.670	0.840	0.574
Final	74.2	74.6	74.7	74.1	0.548	0.095	0.735
Change	3.6	3.5	3.3	3.8	0.955	0.552	0.645
Average $BW^{0.75}$	25.4	25.7	25.1	26.0	0.974	0.131	0.755
DMI							
kg/d	2.03	1.86	1.78	2.02	0.001	0.001	0.158
% of BW	2.74	2.47	2.56	2.64	0.004	0.411	0.636
Ingestibility[3]	80	73	75	78	0.001	0.112	0.520
Fill value[4]	0.94	1.03	1.01	0.96	0.001	0.111	0.520

[1]Interaction enzyme × breed.
[2]Body condition score (ranging from 0 to 5) measured according to Russell *et a*l. (1969).
[3]$kgDM/kgBW^{0.75}$
[4]Estimated from INRA tables (Jarrige, 1989).

4.4. Conclusions

No lactational effects were detected when the fibrolytic enzyme complex was added to the concentrate in dairy ewes, but BW and BCS improved as an effect of the enzyme in late lactation. Enzyme dose (medium) and application method (sprayed onto the concentrate) may have affected the response to enzyme supplementation in dairy ewes, which showed a high level of intake and presumably have a short time of residence of the diet in the rumen.

Chapter 5: Improving performance and digestion of fattening lambs by malate addition in the concentrate

Capítulo 5: Mejora de la producción y digestibilidad de corderos en engorde mediante la adición de malato en el concentrado

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Capítulo 5. Mejora de la producción y digestibilidad de corderos en engorde mediante la adición de malato en el concentrado

ABSTRACT: A total of eighty-eight Manchega and Lacaune weaned lambs (5 wk of age) were used in three experiments to evaluate the effects of adding malate to the concentrate of intensively fattened lambs. Lambs were fed ad libitum with barley straw and a pelleted concentrate varying in malate addition (0 or 0.2%) and type of cereal (barley or corn). Treatments were: barley without malate, barley with malate, corn without malate, and corn with malate. In Exp. 1, sixty-four lambs were used in a 2 × 2 factorial design to evaluate growth performances and rumen traits at slaughter. Malate reduced concentrate intake and feed conversion ($P < 0.01$), increased growth rate and ruminal pH at slaughter ($P < 0.05$), and reduced parakeratosis of ruminal mucosa ($P < 0.001$). In Exp. 2, twelve lambs were used to evaluate the effects of malate on daily variations of ruminal pH and serum metabolites at 6-h and 4-h interval, respectively. Malate increased ruminal pH ($P < 0.001$), and decreased lactate, glucose, NEFA ($P < 0.05$), and urea ($P < 0.01$) in serum. In Exp. 3, eight male Manchega lambs were used in a double 4 × 4 Latin square design to evaluate the effects of malate on the digestibility and energy and N balances. Malate decreased total DMI and feed conversion, and improved the digestibility of DM, OM, CP and NDF ($P < 0.001$), ADF ($P < 0.05$), and GE ($P < 0.01$). As a result retained energy and DE and ME estimation increased between 12 and 18% ($P < 0.01$). Interaction malate × cereal was significant in most cases, and the effects of malate were grater in the barley-based concentrates than in the corn ones. Malate (0.2%) is recommended as a feed additive for intensively fattening lambs and can be used to replace antibiotics as growth promoters in practice.

Key Words: Feed Additive, Fattening Lambs, Digestibility, Malate.

5.1. Introduction

A high percentage of cereal (70 to 90%) in the diet is a common practice in intensively fattened ruminants. Difficulty in adapting to a diet rich in cereals is one of the main factors producing acute or subacute ruminal acidosis. This disorder is a direct consequence of the rapid fermentation of non-structural carbohydrates in the rumen, leading to accumulation of lactate (Cheng *et al*., 1998; Owens *et al*., 1998). Lactic acid causes not only local ruminal damage (i.e., atony, bloat, and parakeratosis), but also, secondary effects such as laminitis (Nocek, 1997; Oetzel, 2001), liver abscesses (Nagaraja and Chengappa, 1998) and changes in body fluids (Patra *et al*., 1996).

The papillae and the entire inner rumen wall are covered with a keratinized multilayer epithelium (Steven and Marshall, 1970; Rémond *et al*., 1996). As the ruminal epithelial cells are not protected by mucus they are vulnerable to acid (Oetzel, 2001). Integrity of ruminal epithelium during acidosis is mainly affected by the luminal pH, due to the accumulation of organic acids (Aschenbach and Gäbel, 2000), resulting in rumenitis (Kimberling, 1988) or hyperkeratosis (Rémond *et al*., 1996). These negative effects may be avoided by using feed additives (e.g., buffers and ionophore antibiotics).

An interesting approach to reducing the lactate accumulation in the rumen is the use of salts of dicarboxylic acids (e.g., malate and fumarate), as indicated by Martin (1998) and Caja *et al*. (2000). Malate is a common constituent of forages, which represents 1 to 8% of DM; legumes are richer than grasses (Callaway *et al*., 1997). It has similar *in vitro* effects such as ionophores, diminishing lactate and methane, and increasing pH and propionate concentrations in ruminal fluid (Martin and Streeter, 1995; Carro *et al*., 1999).

Organic acids have been studied *in vitro* (Martin and Streeter, 1995; Callaway and Martin, 1997; Carro *et al*., 1999), in lactating dairy cows (Kung *et al*., 1982; Vicini *et al*, 2003) and in feedlot steers (Martin *et al*., 1999), but the effects on performance and nutrient digestibility in fattening lambs are limited (Garín *et al*., 2001). The aim of this study was to evaluate the effects of adding malate to the concentrate of lambs on growth performances and digestive traits in intensive fattening conditions.

5.2. Materials and Methods

5.2.1. Experimental Design and Animal Care

A total of eighty-eight lambs from two dairy breeds (Manchega and Lacaune) were used in three experiments to evaluate the effects of adding malate to the fattening concentrates.

Lambs were reared suckling from their mothers and supplemented with creep feeding by using a commercial starter concentrate (DM, 87.9%; CP, 19.3%; ether extract, 3.3%; NDF, 17.0%, DM basis, Fimsa, La Bisbal del Penedés, Tarragona, Spain).

Weaning was performed at wk 5 of age (12 to 14 kg BW). Lambs were fed the same concentrate for a transition period of three weeks (wk 5 to 8 of age) to adapt to the intensive fattening diets. Lambs had permanent free access to barley straw, water and concentrate. The experiments were carried out at the Experimental Farm of the S1GCE (Servei de Granges i Camps Experimentals) of the Universitat Autònoma de Barcelona in Bellaterra, Spain. Experimental and animal care procedures were approved by the Ethical Committee on Human and Animal Experimentation of the Universitat Autònoma de Barcelona (Reference CEEAH 02/360).

5.2.2. Experiment 1. Growth Performance and Rumen Traits at Slaughter

5.2.2.1. Animals and Management

Sixty-four weaned lambs were used according to a 2 × 2 factorial design (cereal × treatment) fattening trial. Lambs (Manchega, n = 40, 16.3 ± 1.9 kg BW; and, Lacaune, n = 24, 16.5 ± 1.7 kg BW) were allocated into eight balanced groups of eight lambs each according to breed, sex and BW. Lambs were kept in straw bedded pens equipped with a hopper for concentrate and a rack for straw. An automatic drinker was also available. The lamb groups were randomly assigned to experimental treatments, with two repetitions per treatment. Concentrate orts by lamb pen were removed and weighed weekly during the experiment. Barley straw and fresh water were permanently available in the pens, but their intake was not recorded. The hygiene of the feeders and drinkers, and the presence of concentrate and straw were checked daily. Lambs were individually weighed weekly and slaughtered when they reached the typical 'Pascual' weight (25 kg BW approximately) after fasting overnight (8 h on average). The rumen of each lamb was collected and processed immediately to measure pH and sampled for histology.

5.2.3. Experiment 2. Ruminal pH and Serum Metabolites

5.2.3.1. Animals and Management

Twelve fattening lambs, previously adapted to the intensive fattening and experimental concentrates for two wk, were used to evaluate the effects of concentrates on daily variations

of ruminal pH and blood serum metabolites. Lambs (Manchega, n = 8, 19.5 ± 1.6 kg BW; and, Lacaune, n = 4, 20.0 ± 0.9 kg BW) were allocated into four balanced groups (3 lambs per group) according to breed, sex and BW, and kept in the same conditions as lambs in Exp. 1. Lamb groups were randomly assigned to the experimental diet treatments. Management and BW at slaughtering were similar to those used in Exp. 1. Blood and ruminal fluid samples were collected from each lamb at different times of day on d 15 of the experiment. Lambs were fattened thereafter until they reached the slaughtering BW (25 kg). Ruminal pH was also measured in these lambs at slaughtering.

5.2.4. Experiment. 3. Digestibility and Nutrient Balance

5.2.4.1. Animals and Management

Twelve weaned Manchega male lambs (eight experimental and four in reserve) were used in this trial. The eight experimental lambs (14.9 ± 0.6 kg BW) were arranged according to a double 4 × 4 Latin square design to evaluate the digestibility of experimental concentrates. Lambs were randomly assigned to one of the four dietary treatments. Lambs were kept in individual metabolic crates equipped with an automatic drinker, two feeders (one for concentrate and the other for straw, and containers for total feces and urine collection). Concentrate and barley straw (chopped coarsely to approximately 5 cm in length) were offered once daily (0800) at a rate of 115% of the voluntary intake from the previous day. Fresh water was permanently available in the crates, but its consumption was not measured. The experiment was divided into four periods of 21 d (14-d for diet adaptation and 7-d for intake measurement, and orts, feces and urine total collection). Concentrate and straw refusals were weighed daily. Urine was collected in a container and 20 ml of 10% H_2SO_4 (vol/vol) was added each morning to prevent ammonia loss.

The four lambs from the reserve group were allocated to a straw bedded pen, and fed *ad libitum* with a mixture of the four experimental diets. Barley straw and water were also permanently available in the pen. No reserve lambs were needed for replacement of the experimental lambs, and they were slaughtered at the end of the experiment.

5.2.5. Feeds and Treatments

Lambs were fed *ad libitum* a diet of pelletized concentrate varying in composition according to the inclusion of a commercial malate salt (Rumalato, Norel & Nature Nutrición S.A., Lliçà de Vall, Barcelona, Spain) and type of cereal (barley or corn). Concentrate ingredients are

shown in Table 5.1. Rumalato is a stabilized and micronized commercial product composed of salts of malic acid (16% disodium malate and 84% calcium malate), with a pH greater than 8. Concentrates were: B0 (barley without malate), BM (barley with malate), C0 (corn without malate), and, CM (corn with malate).

Table 5.1. Ingredients of the concentrates
Ingredientes de los concentrados

Ingredients, % as feed	Concentrate[1]			
	B0	BM	C0	CM
Barley meal	66.3	66.3	—	—
Corn meal	6.0	6.0	60.0	60.0
Manioc	—	—	6.5	6.5
Gluten feed	8.0	8.0	7.0	7.0
Soybean meal	13.4	13.4	16.5	16.5
Soybean hulls	2.0	2.0	4.0	4.0
Cane molasses	—	—	2.1	2.1
Animal fat	0.9	0.9	—	—
Urea	0.6	0.6	0.8	0.8
Premix[2]	2.8	2.8	3.0	3.0
Rumalato[3]	—	0.2	—	0.2

[1]B0 = barley without malate; BM = barley with malate; C0 = corn without malate; CM = corn with malate.

[2]Contained per kg 3.5 mg $CuSO_4(OH)_5$ as sulfate 5-hidrated, 8000 IU of vitamin A, 2500 IU of vitamin D_3, 5 mg of vitamin E (α-tocopherol).

[3]Norel & Nature Nutrición S.A., Lliçà de Vall, Barcelona, Spain.

5.2.6. Measurement, Calculations and Analysis

Feed intake was calculated as the difference between the total amounts of DM offered and refused (10 g accuracy). Individual lamb BW was recorded weekly (0.1 kg accuracy) before offering feed throughout the experiments. Daily samples of the concentrate (composited by treatment) and barley straw were collected for chemical analysis for periods of 15 d throughout the experiment. Refusals of concentrate and straw were discarded in order to offer fresh feed. During the collection periods, the total weight of orts, feces, and urine volume were recorded daily, and then, a 10% sample was collected and composited by period and treatment. Samples

of feces and urine were frozen (-20ºC) and orts were stored in a cold chamber (4ºC) until analysis. Feed and feces were dried in an air-forced oven at 60ºC for 96 h and occasional contamination particles were hand removed (lamb hair and wool, concentrate, and straw).

Samples of feed, orts, and feces were ground through a 1-mm stainless steel screen and analyzed for DM, OM and fat (except for feces and straw) according to AOAC (1995). Crude protein was determined by using a Kjeltec Auto 1030 Analyzer (Tecator, Hogänäs, Sweden). The method of Van Soest *et al*. (1991) was used to analyze NDF and ADF by an Ankom^{200} Fiber Analyzer incubator (Ankom Technology, Fainport, NY). Starch content was derived from FEDNA tables (de Blas *et al*., 1999). Urine samples were also analyzed for DM and CP. Gross energy of feed, feces, and orts was determined using an adiabatic oxygen bomb calorimeter (Calorimeter C4000, Janke & Kunkel, IKA Analysentechnik, Heitersheim, Germany) and DE was calculated. Estimation of ME was derived from calculated urinary and methane energy losses, according to the equation of Blaxter *et al*. (1969).

Ruminal fluid was collected at 6-h intervals (0800, 1400 and 2000) on the same day via a stomach tube, as described by Geishauser and Gitzel (1996). Ruminal fluid samples were squeezed through two layers of cheesecloth and the pH was measured immediately. Rumen pH was measured in 50 mL of ruminal fluid by using a pH meter (Nb 547, series 7489, Crison Instruments, S. A. Barcelona, Spain) equipped with an immersible probe (Cat. 52-00, Crison Instruments, S. A. Barcelona, Spain).

Samples of ruminal wall tissue were dissected from the dorsal sac**,** and fixed in 10% buffered neutral formalin. Two sections per sample were trimmed, placed in tissue cassettes, dehydrated and embedded in paraffin. Sections were cut (approximately 4 μm thick) in duplicate, mounted on glass slides and stained with hematoxylin-eosin (Kiernan, 1990).

Ruminal preparations were used for morphometric measurements of ruminal papillae by light microscopy (Olympus series 218223 BHS, Barcelona, Spain). With this aim, 10 intact, well oriented, and transversely cut papillae were measured per lamb. Papillae length was defined as the distance from the tip to the base of the papillae. Keratinized epithelium and length of ruminal papillae were measured using a graduate ocular with an objective of 20× and 10×, respectively. Keratin stains red in hematoxylin and eosin, thus allowing for the differentiation of papillae keratinized epithelium (i.e., tip, middle and base). Additionally, a 1-cm^2 portion of ruminal tissue was cut in duplicate in order to manually count the ruminal papillae using a stereoscopic light microscopy (Bausch & Lomb, model A5Z45E, Rochester, NY).

Blood samples were collected at 4-h intervals (0800, 1200, 1600, and 2000) via jugular puncture by using 10 ml vacuum tubes (Venoject, Leuven, Belgium), and centrifuged immediately at 1,000 × g for 15 min at room temperature. Blood serum was frozen (-20ºC) until analysis. Blood serum samples were analyzed with a colorimetric autoanalyzer (Roche Cobas Mira 89, series 27-6151, Walpole, MA) using different analysis kits.

Glucose (UV enzymatic test, hexokinase/glucose-6-phosphate dehydrogenase method), lactate (lactate dehydrogenase method), total serum protein (biuret reaction method), serum albumin (green of bromocresol method), creatinine (method of Jaffé), lactate dehydrogenase (method of the Chemical Clinic German Society), and urea (UV enzymatic test, urease/GLDH method) were analyzed by using kits from ABX diagnostics (Montpellier, France). Beta-hydroxybutyrate was analyzed by the enzymatic method based on D-3-hydroxybutyrate oxidation with the enzyme 3-hydroxybutyrate dehydrogenase using NAD+ as cofactor (Boehringer Mannheim GmbH, Mannheim, Germany). The NEFA were analyzed by enzymatic method based on the acyl reaction catalyzed by the enzyme acylCoA synthetase (NEFA-C, Wako Chemicals USA, Dallas, TX).

For malate determination, 0.5 g of ground concentrate samples were mixed with 12.5 mL of deionized water in a glass tube and were boiled in a 100ºC water bath for 30 min. The samples were then mixed using a glass-stirring rod to release organic acids from the cellular membranes. The slurry was filtered and the tube and the residue were rinsed with 12.5 mL of deionized water to remove malate residues. The filtrate was collected for the analysis of malate by High-Performance Liquid Chromatography (Waters 600E, Waters 486 UV absorption detector at 214 nm, D-2500 Merck-Hitachi integrator, 100-µL loop; Waters HPLC system, Waters Corporation, Milford, MA) at 35ºC using a Bio-Rad-HPX-87H Aminex organic column (Bio-Rad Laboratories, Hercules, CA). The samples were eluted from the column with 4 m*M* H_2SO_4 at a flow rate of 0.5 mL/min and malate was expressed as the anion of malic acid.

5.2.7. Statistical Analysis

In Exp. 1, weekly data for group DMI and individual lamb BW were analyzed using the PROC MIXED procedure with repeated measures of SAS (SAS v. 8.1; SAS Inst. Inc., Cary, NC). The statistical model contained the fixed effects of malate treatment, lamb, breed, sex, and type of cereal; week as the repeated factor; the random effects of the lamb inside the group; the first order interactions of these factors; and, the residual error. The PROC GLM

procedure of SAS was used for analyzing lamb group feed conversion, individual ADG, days at slaughter (actual and corrected for 25 kg BW), ruminal pH at slaughter, and keratinization degree of ruminal papillae. The statistical model used in this case contained the fixed effects of malate treatment, lamb, breed, sex, and cereal type; the first order interactions, and the residual error.

In Exp. 2, weekly data for group DMI and individual lamb BW, and hourly individual data for ruminal pH and blood serum metabolites, were analyzed using the PROC MIXED procedure with repeated measures of SAS. The statistical model contained the fixed effects of malate treatment, breed, sex, and type of cereal; week as the repeated factor; the random effects of the lamb inside the group; the first order interactions; and, the residual error.

In Exp. 3, in which the lambs were located in individual crates, the PROC GLM procedure was also used to analyze data of lamb BW, DMI, ADG, feed conversion, digestibility (DM, OM, CP, NDF and ADF), and nutrient balance (energy and nitrogen). The statistical model contained the fixed effects of malate treatment and type of cereal; the first order interactions; and, the residual error.

For the PROC MIXED procedure, the covariance structure that yielded the smallest Schwartz Bayesian criterion was considered to be the most suitable analysis (Littell *et al*., 1998). Differences were tested using the PDIFF option of SAS. For PROC GLM procedure, differences were tested using the SNK option of SAS. For all experiments, when the probability of an interaction term was not significant ($P > 0.20$), it was discarded from the model. The differences were considered significant at $P < 0.05$, and trends were discussed at $P < 0.10$.

5.3. Results and Discussion

5.3.1. Nutritive Value of Feeds

Chemical composition and nutritive value of concentrates and barley straw are listed in Table 2, which shows the high CP (18.2% on average, DM basis) and starch (40.7% on average, DM basis) contents commonly used in the diets of intensively fattened ‘Pascual’ type lambs. The concentrates based on barley contained slightly more CP and fiber than the corn-based concentrates, but were similar in other nutrients.

Table 5.2. Chemical composition and nutritive value of experimental concentrates and barley straw

Composición química y valor nutritivo de los concentrados experimentales y paja de cebada

Item	Barley straw	Concentrate[1]			
		B0	BM	C0	CM
DM, %	90.18	89.28	89.48	87.76	87.44
Composition, % DM					
OM	93.37	93.31	93.24	93.10	93.04
CP	5.60	18.53	18.18	18.10	18.00
Fat	—	3.14	3.29	3.03	3.00
Ash	7.03	6.69	6.76	6.90	6.96
Crude Fiber	39.41	5.23	5.23	5.18	5.18
NDF	77.40	15.60	15.78	12.14	11.83
ADF	44.20	6.14	6.49	5.47	5.28
Starch	—	40.32	40.32	40.99	40.99
Malate	—	0.20	0.29	0.11	0.18

[1]B0 = barley without malate; BM = barley with malate; C0 = corn without malate; CM = corn with malate.

Although malate was not added to the control concentrates, analysis showed concentrations of 0.20 and 0.11% malate in B0 and C0 concentrates, respectively. Addition of malate (0.2% concentrate, as feed) substantially augmented the malate content of control diets, increasing malate content in BM and CM concentrates by 45 and 64%, respectively.

5.3.2. Experiment 1. Growth Performance and Rumen Traits at Slaughter

Two lambs (one from B0 and another from CM) died during the first week of the experiment due to accidental causes not related to the dietary treatments. Addition of malate to the concentrate ($P < 0.01$) and cereal type ($P < 0.05$) affected concentrate intake during fattening (Table 5.3), but the interaction was not significant. Lambs fed CM ingested less concentrate (8%; $P < 0.05$) compared with C0 lambs, and 10.8% less than B0 and BM lambs (average 0.936 kg/d). Moreover, malate increased ADG by 14% on average ($P < 0.05$) and, as a consequence, reduced feed conversion by 18% ($P < 0.01$) in the supplemented lambs.

Table 5.3. Effects of malate addition to the concentrate on the performance and ruminal pH of fattening lambs (values are LSM)

Efectos de la adición de malato en el concentrado en el crecimiento y pH ruminal de corderos de engorde(medias por mínimos cuadráticos)

Item	Treatment[1]				Effect ($P <$)		
	B0	BM	C0	CM	Malate	Cereal	M × C[2]
Lambs, n	16	16	16	16	—	—	—
Intake							
kg DM/d	0.948[a]	0.923[a]	0.913[a]	0.844[b]	0.007	0.046	0.122
BW, kg							
Initial	16.4	16.3	16.4	16.4	0.999	0.943	—
Final	25.1	25.9	25.1	26.1	0.139	0.747	0.064
ADG, g/d	259[b]	330[a]	299[ab]	307[ab]	0.013	0.601	0.005
Feed conversion	3.81[a]	2.88[b]	3.25[b]	2.90[b]	0.002	0.155	0.001
Days at slaughter							
Actual	34	30	31	31	0.697	0.693	0.310
For 25 kg BW	34	28	31	29	0.124	0.693	0.061
Ruminal pH at slaughter	6.87[b]	7.05[ab]	6.94[ab]	7.13[a]	0.017	0.338	0.025

[1]B0 = barley without malate; BM = barley with malate; C0 = corn without malate; CM = corn with malate.

[2]Interaction malate × cereal.

[a,b,]Row means with different superscript are different $P < 0.05$.

Significant interactions were observed between malate and cereal type ($P < 0.01$), with the effects of malate being greater in the barley-based concentrates.

Despite the effects on ADG, no significant effects on days at slaughter for constant weight were detected in my results (32 d on average), although numerically lower values were observed in lambs fed malate ($P = 0.124$). Similar results obtained Garín *et al*. (2001) in intensively fattened lambs fed barley based concentrates supplemented with a mixture of malate and yeast, significant reductions were observed in intake (11%) and feed conversion (7%) but not in ADG. However, Cuesta *et al*. (2003) did not observe effects on the performance of Merino lambs fed the same commercial product used in this trial, which was included into the concentrate at a rate of 0.4% of fresh mater.

The effects of malate have also been studied in beef cattle with quadratic responses according to dose. Streeter *et al*. (1994) studied three malate doses (60, 80 and 120 g/d) in steers fed rolled corn based diets, and reported the greatest improvements in feed conversion (8%) and ADG (9%) with the intermediate dose. Moreover, Martin *et al*. (1999) reported that intake decreased for low (40 g/d; from 3.8 to 8.9%) and high (120 g/d; from 0.9 to 5.8%) doses, but increased for medium doses (60 and 80 g/d; from 1.7 to 7.3%). Similar results with a greater variability depending on the length of fattening period were observed in ADG. Despite the lambs fasting before slaughtering, addition of malate to concentrates increased ruminal pH at slaughter ($P = 0.017$; Table 5.3), although differences between B0 and BM, and between C0 and CM were not significant ($P > 0.05$). Garín *et al*. (2001) reported no differences in rumen pH at slaughter in lambs fed malate added concentrates after 12 h fasting. Range of values of rumen pH obtained in this work agreed with Garín *et al*. (2001) but were higher than those obtained in lambs fed cereal based concentrates a few hours after feeding (pH 5.9, Fimbres *et al*., 2002) and than those reported as a daily average (pH 5.8; Brossard *et al*., 2003).

Type of cereal affected concentrate intake (Table 5.3), which was 6% greater ($P < 0.05$) in lambs fed barley compared to corn. Significant malate × cereal interaction ($P < 0.05$) was observed for ADG, feed conversion, and ruminal pH at slaughter. Tendencies were also observed for final BW and days at slaughter for constant weight.

There was no evidence of inflammation, microabcesses, parakeratosis or bacteria adhering to the ruminal papillae of any lamb. Dietary treatments did not affect number of papillae per cm^2 or papillae length in the rumen, but papillae length tended ($P = 0.109$) to be longer in lambs fed malate added concentrates when compared to control lambs (Table 5.4). Shorter and cauliflower shaped ruminal papillae are normally associated to rumen acidosis (McGavin and Morrill, 1976). Moreover, ruminal papillae showed a lower keratinization degree in lambs fed malate added concentrates (Table 5.4). Lambs fed BM or CM concentrates have less keratinized ruminal papillae, from the tip to the base, than those fed B0 and C0 ($P < 0.001$). Colors of ruminal epithelium varied noticeably between treatments, the lambs fed malate and barley based concentrates showing the clearest colors (Table 5.4), as a consequence of lesser effects of acidosis. Rumenitis is a normal consequence of subacute ruminal acidosis, leading to disturbances in volatile fatty acids and energy absorption, which may explain the reduced efficiency of ruminants after severe acidosis (Krehbiel *et al*., 1995a; Oetzel, 2001).

Table 5.4. Effects of malate addition to the concentrate on morphometric characteristics of ruminal papillae of fattening lambs (values are LSM)

Efectos de la adición de malato en el concentrado en las características morfométricas de las papilas ruminales de corderos de engorde (medias por mínimos cuadráticos)

Item	Treatment[1]				Effect ($P <$)		
	B0	BM	C0	CM	Malate	Cereal	M × C[2]
Samples, n	16	16	16	16			
Rumen epithelium color[3]	Grey	White	Grey	White	—	—	—
Papillae/cm^2	180	188	170	231	0. 913	0.791	0.643
Keratinization, µ 20×							
Tip	7.9^a	5.6^b	7.1^a	4.6^b	0.001	0.354	0.001
Middle	7.6^a	5.5^b	6.8^a	4.6^b	0.001	0.237	0.001
Base	6.6^a	5.3^b	6.4^a	4.7^b	0.001	0.914	0.001
Length, µ 10×	263	294	283	327	0.109	0.112	0.092

[1]B0 = barley without malate; BM = barley with malate; C0 = corn without malate; CM = corn with malate.
[2]Interaction malate × cereal.
[3]Subjective assessment.
[a,b]Row means with different superscript are different $P < 0.05$.

Although no evidence of rumenitis was detected in our results, the thinner keratinized epithelium and the higher ruminal pH observed in lambs fed malate may explain the improvements in their fattening performances, when compared to the control lambs. Changes in the ruminal epithelium depend on the amount of easily digestible carbohydrates (Gálfi *et al*., 1991)**,** which induce important differences in fermentation. However, cereal type did not significantly affect the keratinization degree of ruminal papillae in our study (Table 5.4). The malate × cereal interaction affected ($P < 0.001$) the keratinization of ruminal papillae, and tended to affect papillae length ($P = 0.092$), with corn being more improved than barley.

5.3.3. Experiment 2. Ruminal pH and Serum Metabolites

As in Exp. 1, lambs fed BM consumed less concentrate ($P < 0.05$) than those fed B0 or CM (Table 5.5). Differences in intake between C0 and CM lambs were not significant. Malate improved numerically ADG in lambs fed BM or CM when compared to those fed B0 or C0

concentrate treatments. As a consequence, feed conversion numerically improved by 44 and 18% for BM and CM, respectively, when compared to their control groups.

Sampling of rumen fluid at 6-h intervals indicates significant effects of concentrates on lamb ruminal pH ($P < 0.001$; Table 5.5). Lambs fed BM showed greater pH than those fed B0 ($P < 0.05$), although ruminal pH in lambs fed CM or C0 did not differ. Nevertheless, no differences in rumen pH were observed when measured at slaughter, probably as a result of lamb fasting and of the buffering properties of saliva during rumination. Increase in ruminal pH by malate agrees with Streeter *et al*. (1994), who reported a linear increase in ruminal pH when steers received a malate ruminal infusion at different doses (27 to 80 g/d). In contrast, Kung *et al*. (1982) reported a slight decrease in the ruminal pH of dairy cows fed corn and corn silage with increasing doses of malic acid (0 to 140 g/d), although differences in the form of the acidic ion must be stressed. In steers fed malic acid (80 g/d) Montaño *et al*. (1999) observed an increase in ruminal pH for the first 3 h after a ruminal infusion of glucose, although pH decreased after 4 h.

Table 5.5. Effects of malate addition to the concentrate on lamb performance and ruminal pH of fattening lambs (values are LSM)

Efectos de la adición de malato en el concentrado en el crecimiento y pH ruminal de corderos de engorde (medias por mínimos cuadráticos)

Item	Treatment[1]				Effect ($P <$)		
	B0	BM[1]	C0	CM	Malate	Cereal	M × C[2]
Intake kg DM/d							
Concentrate	0.954[a]	0.843[b]	0.916[ab]	0.944[a]	0.040	0.202	0.028
BW, kg							
Initial	19.9	19.3	19.7	19.6	0.996	0.944	0.900
Final	25.1	25.9	25.1	26.1	0.738	0.927	0.706
ADG, g/d	275	349	286	342	—	—	—
Feed conversion	3.50	2.43	3.30	2.80	—	—	—
Ruminal pH at							
6-h intervals	5.88[b]	6.44[a]	5.90[b]	6.02[b]	0.001	0.671	0.371
Slaughter	6.96	7.10	6.96	6.86	0.531	0.230	0.452

[1]B0 = barley without malate; BM = barley with malate; C0 = corn without malate; CM = corn with malate.

[2]Interaction malate × cereal.

[a,b]Row means with different superscript are different $P < 0.05$.

The increase of ruminal pH in lambs fed malate may be attributed to the decrease in ruminal lactate caused by the stimulation of lactate utilization by specialized bacteria. In the rumen of animals fed concentrate diets, *Selenomonas ruminantium* accounts for approximately 50% of the ruminal microbiota (Caldwell and Bryant, 1966), and is cable of up taking L-lactate in the presence of L-malate (Nisbet and Martin, 1991, 1994). Moreover, *Megasphera elsdenii* has the capacity to use up to 80% of the lactate produced in the rumen (Counotte and Prins, 1981; Nocek, 1997; Ouwerkerk *et al*., 2002) as the sole source of carbon and energy. Utilization of lactate would lead to a decrease in rumen acidosis and lactate concentration in blood.

Average serum concentrations of lactate varied by effect of malate addition to the concentrate ($P < 0.05$; Table 5.6). Effect of cereal type was not significant, but a malate × cereal interaction was also detected indicating more noticeable effects in the corn-based diet.

Table 5.6. Effects of malate addition to the concentrate on serum metabolites of fattening lambs (values are LSM)

Efectos de la adición de malato en el concentrado en los metabolitos en suero de corderos de engorde (medias por mínimos cuadráticos)

Item	Treatment[1]				Effect ($P <$)		
	B0	BM[1]	C0	CM	Malate	Cereal	M × C[2]
Lambs, n	3	3	3	3	—	—	—
Lactate, m*M*/L	1.87[b]	1.47[b]	2.58[a]	1.47[b]	0.026	0.257	0.002
LDH[3], U/L	1369	1226	1228	982	0. 837	0.566	0.675
Glucose, mg/dL	86.45[b]	74.70[c]	102.47[a]	95.61[ab]	0.011	0.044	0.240
BHBA[4], m*M*/L	0.52	0.59	0.52	0.53	0.956	0.648	0.851
NEFA[5], m*M*/L	0.14[b]	0.23[a]	0.12[b]	0.11[b]	0.001	0.326	0.012
Urea, mg/dL	38.17[a]	37.33[a]	37.55[a]	25.32[b]	0.002	0.020	0.002
Creatinine, mg/dL	0.77[a]	0.83[a]	0.73[a]	0.61[b]	0.001	0.001	0.001
Total protein, g/dL	5.41	5.34	5.87	5.42	0.612	0.529	0.940
Albumin, g/dL	2.95	2.96	3.39	3.05	0.363	0.004	0.011

[1]B0 = barley without malate; BM = barley with malate; C0 = corn without malate; CM = corn with malate.

[2]Interaction malate × cereal.

[3]LDH = lactate dehydrogenase; [4]BHBA = *β*-hydroxybutyrate; [5]NEFA = nonesterified fatty acids

[a,b]Row means with different superscript are different $P < 0.05$.

Serum lactate in lambs fed the corn control concentrate was greater ($P < 0.05$) than in lambs fed other concentrates. Although no differences ($P > 0.05$) in serum lactate were detected between B0 and BM lambs, values agreed with the corresponding ruminal pH values in these lambs (Table 5.5). Concentrations of lactate in serum of C0 lambs were close to those reported by Krehbiel *et al*. (1995a) and Patra *et al*. (1996) in lambs induced to acidosis (2.25 to 2.95 m*M*/L) agreeing with our data on ruminal epithelium in Exp. 1.

Lactate contents in the serum of B0, BM and CM were under the above referred values (1.7 m*M*/L on average) indicating less subacute acidosis. Nevertheless, Martin *et al*. (1999) did not observe changes in lactate plasma concentrations in beef cattle supplemented with 100 g/d of malate. Serum glucose concentration was also affected by malate treatment ($P < 0.05$) and cereal type ($P < 0.05$) showing greater values in the lambs fed control and corn based concentrates (Table 5.6). Nevertheless, differences were only significant ($P < 0.05$) in lambs fed BM compared to B0 lambs. As expected, greater glucose and lactate values in serum occurred in the lambs from the same dietary treatments, according to the contribution of lactate on glucose turnover in growing lambs (19.5%) as estimated by Prior and Christenson (1977).

Values of NEFA in serum were affected by the malate treatment ($P < 0.001$) although the difference was only significant for the lambs fed B0 and BM concentrates (Table 5.6). Increased NEFA values in malate supplemented lambs agreed with the decrease in glucose and lactate discussed above. The high concentration of NEFA in lambs fed BM may be related to an increase in triglycerides synthesis in the liver (Brown *et al*., 2000) and an increased mobilization of lipids from adipose tissue (Smith *et al*., 1997). Serum concentrations of LDH and BHBA, also indicators of the glucose-lactate and energy metabolism in ruminants, were unaffected by the dietary treatments.

Protein metabolism indicators, such as total protein and albumin in serum, were unaffected by the malate treatment. On the contrary, malate treatment caused urea and creatinine in serum to decrease ($P < 0.01$). Cereal type also affected values of urea and creatinine in serum, being significant the malate × cereal interaction. Lambs fed CM showed the lowest urea and creatinine values in serum ($P < 0.05$) when compared to other concentrates (averages: urea, 37.9 mg/dL; and, creatinine, 0.80 mg/dL). The rate of blood creatinine production may be considered as an index of endogenous protein catabolism (Hatfield *et al*., 1998).

Kung *et al*. (1982) reported no effects on urea or glucose concentrations in plasma of dairy cows fed different doses of malic acid in three consecutive experiments. Additionally, Martin

et al. (1999) did not observe changes in plasma components (i.e., urea, glucose, cholesterol, triglycerides, amylase, and lactate) attributable to malate.

5.3.4. Experiment. 3. Digestibility and Nutrient Balance

During the first period of this experiment, one lamb from B0 treatment (wk 1), and another lamb from the C0 treatment (wk 2) showed symptoms of severe acidosis (bloat, diarrhea, increased respiratory frequency, ruminal atony, abdominal pain, and decreased DMI). These lambs were substituted by two lambs from the reserve group thereafter. The incident delayed the experiment by two weeks but no lambs were replaced. Concentrate and barley straw intakes were not affected by treatments (Table 5.7).

Despite the differences observed in ruminal pH (Exp. 2, Table 5.5) and rumen epithelium keratinization (Exp. 1, Table 5.4), observed values of straw intake were very low (5 to 6% total DMI) and were not affected by the dietary treatments. This result indicates that adjusting straw intake cannot regulate the pH of the young lambs. Measured values of straw intake were lower than those reported in intensively fattened lambs by Bocquier (1989) and Azkar (2004) who reported average values of 10% and 6.7%, respectively.

Type of cereal tended to affect ($P = 0.066$) concentrate intake, and significantly affected ($P < 0.05$) total DMI (Table 5.7). Mean BW and ADG of lambs were not affected by any treatments although ADG values improved numerically ($P = 0.151$) in the malate fed lambs (BM and CM). The lower DMI and higher BW gain in lambs fed malate added concentrates affected feed conversion, which significantly decreased ($P < 0.01$) by 20% in lambs fed BM and CM (Table 5.7). A significant malate × cereal interaction ($P < 0.001$) was detected for feed conversion. These results confirm the positive effects of malate obtained in Exp. 1 and 2.

Malate addition to barley and corn concentrates also affected the total tract apparent digestibility of nutrients (Table 5.7). Malate increased the digestibility of all nutrients studied in both cereal-based concentrates ($P < 0.05$ to $P < 0.001$), but the response was greater in fiber (NDF and ADF) than in other components (DM, OM, CP, and GE). The increase in GE digestibility improved the energetic value of the malate added concentrates by 3.6% on average ($P < 0.01$), justifying the more efficient feed conversion of the BM and CM fed lambs. In contrast, Cuesta *et al*. (2004) reported slight decreases on the digestibility of OM (-0.6%), CP (-0.5%) and NDF (-3.5%) in Merino lambs fed the same organic acid used in this trial.

Table 5.7. Effects of malate addition to the concentrate on performance and nutrient digestibility of fattening lambs (values are LSM)
Efectos de la adición de malato en el concentrado en el crecimiento y digestibilidad de corderos de engorde (medias por mínimos cuadráticos)

Item	Treatment[1]				Effect ($P <$)		
	B0	BM	C0	CM	Malate	Cereal	M × C[2]
Intake, kg DM/d							
Concentrate	1.09	1.07	1.03	0.97	0.132	0.066	0.543
Straw	0.049	0.052	0.059	0.055	0.775	0.370	0.879
Total	1.14[a]	1.12[ab]	1.09[ab]	1.03[b]	0.054	0.035	0.434
BW, kg	29.5	29.0	28.4	27.7	0.556	0.254	0.602
ADG, g/d	277	324	290	308	0.151	0.952	0.054
Feed conversion	4.20[a]	3.43[b]	3.97[a]	3.31[b]	0.003	0.291	0.001
Digestibility, %							
DM	78.11[b]	82.14[a]	79.28[b]	83.05[a]	0.001	0.083	0.001
OM	80.98[b]	84.48[a]	82.22[b]	85.58[a]	0.001	0.041	0.001
CP	78.33[b]	82.16[a]	76.05[b]	81.17[a]	0.001	0.038	0.003
NDF	39.82[b]	49.73[a]	42.06[b]	48.64[a]	0.001	0.790	0.004
ADF	47.44[b]	55.85[a]	54.92[b]	59.18[a]	0.017	0.023	0.026
GE	80.61[b]	83.44[a]	81.37[b]	84.49[a]	0.003	0.169	0.003

[1]B0 = barley without malate; BM = barley with malate; C0 = corn without malate; CM = corn with malate.
[2]Interaction malate × cereal.
[a,b]Row means with different superscript are different $P < 0.05$.

Cereal type affected OM, CP and ADF digestibility ($P < 0.05$) but digestibility of NDF and GE did not vary. Digestibility of DM tended to vary ($P < 0.10$). Values of digestibility were generally greater in the corn-based concentrate. Significant interactions were observed in malate × cereal ($P < 0.05$ to $P < 0.001$), indicating a greater increment of digestibility in the barley than in the corn-based concentrates.

Unexpectedly, ADF digestibility was superior (6 to 13 percentage points) to NDF digestibility as shown in all cases in Table 5.7, but reasons are uncertain. Similar results were also reported in fattening lambs by Ahmad *et al*. (1995) and McAllister *et al*. (2000), and in

fattening kids (Bueno *et al*., 2002) indicating this effect can be a consequence of the acidic conditions found in the rumen of this type of intensively fattened animals.

The effects of malate on nutrient digestibility may be explained by the improvement in ruminal fermentation conditions (Martin and Streeter, 1995; Martin *et al*., 2000b) and by the increase of pH as a consequence of the reduction of lactic acid in the rumen (Callaway and Martin, 1997), which positively affected lamb performance (i.e., ADG and feed conversion; Table 5.7) in this experiment. No effects on digestibility were reported in steers supplemented with malic acid at increasing levels (0, 100, 200 mg/kg BW daily) by Kung *et al*. (1982) and at 80 g/d by Montaño *et al*. (1999). This lack of effect when malic acid was used instead of malate is consistent with the differences in the form of the acidic ion previously discussed (Exp. 2).

Regarding energy balance (Table 5.8), lambs fed malate added concentrates ingested less energy ($P < 0.05$), and excreted less by feces ($P < 0.01$) than those fed control concentrates.

Estimated methane losses were also reduced ($P < 0.05$) by the addition of malate. As a consequence, estimations of DE and ME increased 2 to 4% by effect of malate ($P < 0.05$), supporting the greater ADG of lambs fed with the malate added concentrates (Exp. 1). No effects in urinary energy were detected between treatments, however total energy excreted tended ($P = 0.066$) to be lower in lambs fed malate concentrates.

Lambs fed malate ingested more N ($P < 0.05$) and tended to excrete less N by feces ($P < 0.10$). No differences in urinary N were attributed to the treatments (Table 5.8). As a consequence, N retention increased between 10 and 16% by effect of malate ($P < 0.01$) in agreement with the above mentioned energy results.

Type of cereal affected both energy and N intake, output and retentions, with the exception of methane ($P = 0.109$) and urinary N ($P = 0.360$). Malate × cereal interaction affected fecal and urinary energy ($P = 0.048$) and tended to affect N output ($P = 0.099$), but did not affect the energy and N intakes or the total outputs. Factors such as fermentability of dietary carbohydrate and N intake relative to nutrient requirements are responsible for differences in N utilization as indicated by Huntington and Archibeque (1999).

Table 5.8. Effects of malate addition to the concentrate on energy and nitrogen balances of fattening lambs (values are LSM)

Efectos de la adición de malato en el concentrado en los balances de energía y nitrógeno de corderos de engorde (medias por mínimos cuadráticos)

	Treatment[1]				Effect ($P <$)		
Item	B0	BM[1]	C0	CM	Malate	Cereal	M × C[2]
Energy, Mcal/d							
Intake	4.43[a]	4.36[a]	4.04[ab]	3.87[b]	0.012	0.004	0.615
Fecal	1.11[a]	0.97[a]	1.00[a]	0.81[b]	0.005	0.038	0.046
Urinary	0.75	0.85	0.85	0.83	0.410	0.555	0.605
Total excreted	1.86	1.82	1.85	1.63	0.066	0.225	0.146
Methane, %GE	6.17[a]	5.83[ab]	6.11[a]	5.65[b]	0.029	0.373	0.021
Methane	0.28[a]	0.26[ab]	0.26[ab]	0.22[b]	0.034	0.058	0.153
DE	3.32[ab]	3.39[a]	3.04[b]	3.06[b]	0.017	0.005	0.818
ME	2.28[a]	2.28[a]	1.93[b]	2.01[ab]	0.015	0.002	0.759
Nitrogen, g/d							
Intake	34.7[ab]	36.6[a]	32.4[b]	32.7[b]	0.042	0.013	0.455
Fecal	7.3	6.4	7.5	5.9	0.072	0.822	0.099
Urinary	10.9	10.9	9.9	10.3	0.682	0.360	0.960
Total excreted	18.1	17.3	17.4	16.2	0.394	0.398	0.438
Retained	16.6[ab]	19.3[a]	15.0[b]	16.5[ab]	0.004	0.058	0.042

[1]B0 = barley without malate; BM = barley with malate; C0 = corn without malate; CM = corn with malate.

[2]Interaction malate × cereal.

[a,b]Row means with different superscript are different $P < 0.05$.

5.4. Conclusions

The results obtained in our experiments showed consistent beneficial effects of the use of malate (0.2% in concentrate) as a natural feed additive in the concentrate of fattening lambs. As well as the growth and digestibility improvements, which lead to a reduction in feed conversion, use of malate reduced acidosis and parakeratosis in the rumen of lambs, which should improve their health condition and welfare. As a consequence, use of malate is recommended in practice as a tool for improving rumen function and as an alternative to the use of antibiotics as a feed additive in intensively fattened lambs.

Chapter 6: Conclusions

Capítulo 6: *Conclusiones*

Chapter 6. Conclusions

Capítulo 6. Conclusiones

Based on the experimental results obtained in the evaluation of the effects of including the fibrolytic enzymes complex (Promote) and the organic acid (Rumalato) in the concentrate of sheep, the following specific and general conclusions are extracted:

6.1. Specific Conclusions

6.1.1. Effects of the fibrolytic enzymes complex on the performance of Manchega and Lacaune dairy ewes:

- The fibrolytic enzymes complex did not affect the dry matter intake, milk yield, or milk composition of Manchega and Lacaune dairy ewes during suckling or milking periods, with the exception of milk fat which tended to decrease in the enzyme fed ewes during the milking period.
- No significant interaction between breed and enzyme was observed, and the Manchega ewes yielded less milk with a greater milk composition than the Lacaune dairy ewes.
- The fibrolytic enzymes complex did not improve growth parameters of the lambs during suckling period.
- The fibrolytic enzymes complex did not affect body weight or body condition score of Manchega and Lacaune dairy ewes during suckling period, but both traits improved in late lactation in the enzyme fed ewes.
- The fibrolytic enzymes complex reduced the dry matter intake of dry and open ewes, resulting in a greater sheep fill value of the diet.

6.1.2. Effects of malate on the performance, total tract digestibility and metabolism of intensively fattened lambs:

- Malate reduced the feed intake and increased growth rate, ruminal pH and improved lamb feed conversion. Moreover, an improved health of ruminal mucosa was observed at slaughter.

- According to the fattening performance and slaughter results, malate increased ruminal pH, and decreased lactate, glucose, nonesterified fatty acids, and urea in the serum of fattening lambs.
- Malate improved the total tract digestion of main nutrients in the concentrate and energy and nitrogen balances of intensively fattened lambs.

6.2. General Conclusions

- The addition of a fibrolytic enzymes complex to the concentrate of a 70:30 forage:concentrate mixed ration, failed to improve the digestive and lactational performances of high and medium yielding dairy ewes. Enzyme dose, application method and portion of the ration to which the enzyme was applied may have compromised this lack of response in the dairy ewes.
- The use of malate showed positive effects on the growth performances and metabolic indicators of intensively fattened lambs. This was due to an enhancement of rumen function and to improvements in nutrient utilization in the digestive tract of the lambs.
- The consistent beneficial effects of malate addition to the concentrate (0.2%) observed in all experiments done, favor the use of malate as a natural feed additive for fattening lambs and as a replacement for the use of antibiotics as growth promoters in practice.

Chapter 7: References

Capítulo 7: Referencias

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