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Optimization of using ruminal microbiota as pro and prebiotic to optimize rumen development and the efficient use of forage or concentrate diets by goats

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ABSTRACT

Optimization of using ruminal microbiota as pro and prebiotic to optimize rumen development and the efficient use of forage or concentrate diets by goats

The aim of this work was to elucidate the best conditions for preparing rumen microbiome as pro and prebiotic by the analysis of fermentation parameters resulting from *in vitro* incubations (trials 1 and 2), for subsequent use in *in vivo* trial (trial 3) consisting of an inoculation of newborn goat kids with the ruminal microbiota developed in the *in vitro* experiment. This study used a 2×3×2×2 factorial design to evaluate the effects of two diets (forage and concentrate), three buffers (6.80, 6.25 y 5.75), two sample times (0 vs 3h) and two ruminal fractions (liquid vs whole content) of the ruminal content. Eight Murciano-Granadina goats were used as a donor of the ruminal content; each four animals were assigned to one group: FOR received 100% forage (based on 50% alfalfa and 50% oat hay) and CON fed with concentrate and forage (80:20) with the same forage composition. In the *in vivo* experiment, seventy four goat kids were randomly distributed into 4 identical groups with 16 and 4 animals (4x4) per group and experimental group respectively: CTL (control group without any inoculation), PRE (group inoculated with ruminal liquor autoclaved), LRC (probiotic group inoculated with ruminal liquor of animals fed concentrate diet) and LRF (probiotic group inoculated with ruminal liquor of animals fed forage diet). Experimental groups had similar average birth weight and the same number of males and females. Newborn goats were daily inoculated with fresh rumen fluid obtained from the same donors used in *in vitro* experiment during the first 12 weeks of life. The feed intake (milk, concentrate and forage), body weight and rumen fermentation were studied during the same inoculation period. In the first trial, both diet (FOR vs CON) and buffer were studied; the result showed that independently of the diet, the buffer which generates higher pH promotes higher fermentation activity in terms of VFAs (P=0.001), ammonia (P=0.005), fermentable organic matter (P= 0.006) and gas production (P <0.001). In the second trial, it was observed that the type of diet exerts a very significant effect on ruminal fermentation *in vitro*. Compared to the FOR diet, CON promoted greater ruminal fermentation with ruminal contents collected at 3h post-feeding than at 0h. The type of ruminal fraction considered did not show significant differences in relation to the fermentation pattern, gas production and concentration of ruminal microorganisms. Only a lower concentration of anaerobic fungi (P=0.002) was detected in the liquid fraction as a result of the tropism of these microorganisms towards the solid fraction. In the third trial, the inoculation no had an effect on milk or concentrate intakes, however, it had significant effect (P<0.05) on forage intake in the weeks 5, 6, 8, 10, 11 and 12 of life with higher values for LRC group (the accumulated intake was 1.2±0.31 kg Dry Matter). In addition, the inoculation no affected the body weight of animals, but had modulated some aspects of rumen fermentation like butyric production.

Keywords: Probiotic, prebiotic, goat kids, ruminal microbiota, inoculation, fermentation, diet, buffer, sampling time, *in vitro, in vivo.*

RESUMEN

Optimización del uso de la microbiota ruminal como pro y prebiótico para optimizar el desarrollo ruminal e el uso eficiente de forraje o dietas concentradas en cabritos

El objetivo de este trabajo fue elucidar las mejores condiciones para usar el contenido ruminal como pro y prebiótico analizándose los parámetros de fermentación resueltos de las incubaciones *in vitro* (ensayos 1 y 2) para su posterior uso en el ensayo *in vivo* (trial 3) que consiste en una inoculación de cabritos recién nacidos con la microbiota ruminal desarrollado en el ensayo *in vitro*. Este estudio usó un diseño factorial de 2×3×2×2 para evaluar los efectos de dos dietas (forraje y concentrado), tres tampones, dos momentos de muestreo (0 vs 3h) y dos fracciones (líquido vs contenido total) del contenido ruminal. Ocho cabras Murciana-Granadinas fueron usadas como donantes de contenido ruminal; cada cuatro animales fueron asignados a un grupo distinto: FOR recibió 100% de forraje (basado en 50% de heno de alfalfa y 50% de heno de avena) y CON alimentado con concentrado y forraje (80:20) con la misma composición de forraje. En la experimentación *in vivo*, sesenta y cuatro cabritos fueron asignados aleatoriamente en 4 grupos idénticos con 16 y 4 animales (4x4) por grupo y grupo experimental respectivamente: CTL (grupo control sin inoculación alguna), PRE (grupo prebiótico inoculado con líquido ruminal autoclavado), LRF (grupo probiótico inoculado con líquido ruminal de animales alimentados con dieta forrajera) y LRC (grupo probiótico inoculado con líquido ruminal de animales alimentados con dieta concentrada). El grupo experimental tuvo una media similar de la fecha de nacimiento y un mismo número de machos y hembras. Los cabritos fueron inoculados diariamente con el líquido ruminal fresco obtenido de las mismas donantes usadas en la experimentación *in vitro* durante las 12 semanas de vida. La ingestión (leche, concentrado y forraje), el peso vivo y la fermentación ruminal se estudiaron durante el mismo periodo de inoculación. En el primer ensayo, ambos dieta (FOR vs CON) y tampón se estudiaron, el resultado mostro que independientemente de la dieta, el tampón que genero un pH alto promovió una mayor actividad fermentativa en términos de AGVs (P=0,001), amonio (P=0,005), materia orgánica fermentable (P=0,006) y producción de gas (P <0,001). En el segundo ensayo, se observó que el tipo de la dieta ejerció un efecto muy significativo sobre la fermentación ruminal *in vitro.* En comparación con la dieta FOR, la dieta CON promovió una mayor fermentación ruminal con contenido ruminal recogido a las 3h post-ingestión que a 0h. En cuanto al tipo de fracción ruminal considerada, no se encontraron diferencias significativas entre la utilización del líquido ruminal vs contenido ruminal. Únicamente se detectó una menor concentración de hongos anaerobios (P=0,002) en la fracción líquida como consecuencia al tropismo de estos microorganismos hacia la fracción sólida. En el tercer ensayo, la inoculación no tuvo efecto sobre las ingestiones de leche y de concentrado, sin embargo, tuvo efecto significativo (P<0,05) en la ingestión de forraje en las semanas 5, 6, 8, 10, 11 y 12 de vida con valores mayores en el grupo LRC (la ingestión acumulada fue de 1,2±0.31 kg Materia Seca). En adición, la inoculación no afecto el peso vivo de los animales, pero moduló algunos aspectos de la fermentación ruminal como la producción del butirato.

Palabras claves: Probiótico, prebiótico, cabritos, microbiota ruminal, inoculación, fermentación, dieta, tampón, momento de muestreo, *in vitro*, *in vivo*.

RÉSUMÉ

Optimisation de l'utilisation du microbiote ruminal comme pro et prébiotique pour optimiser le développement ruminal et l'usage efficient de fourrage ou de rations concentrées par les chevreaux

L'objectif de ce travail est d'élucider les meilleures conditions pour utiliser le contenu ruminal comme pro et prébiotique en analysant les paramètres de fermentation résultant des incubations *in vitro* (essais 1 et 2) pour un usage ultérieur dans l'essai *in vivo* (essai 3) qui consiste en une inoculation de chevreaux récemment nés avec du microbiote ruminal développé dans l'essai *in vitro*. Cette étude a utilisé un dispositif factoriel de 2×3×2×2 afin d'évaluer les effets de deux rations (fourragère et concentrée), trois tampons, deux moments d'échantillonnage (0 vs 3h) et deux fractions (liquide vs tout le contenu) du contenu ruminal. Huit chèvres de race Murciana-Granadina ont été utilisées comme donneuses du contenu ruminal; chaque quatre animaux ont été affectés à un groupe distinct : FOR recevant 100% fourrage (50% foin de luzerne y 50% foin d'avoine) et CON alimenté en concentré et fourrage (80:20) avec la même composition de fourrage. Dans l'expérimentation *in vivo*, soixante-quatre chevreaux ont été affectés aléatoirement à 4 groupes identiques avec respectivement 16 et 4 animaux (4x4) par groupe et groupe expérimental : CTL (groupe control sans aucune inoculation), PRE (groupe prébiotique inoculé avec le liquide ruminal autoclavé), LRC (groupe probiotique inoculé par le liquide ruminal issu d'animaux recevant le fourrage). Le groupe expérimental avait une date moyenne de naissance et un nombre similaire de mâles et femelles. Les chevreaux ont été inoculés quotidiennement avec le liquide ruminal frais obtenu des mêmes donneuses utilisées dans l'expérimentation *in vitro* durant 12 semaines de vie. L'ingestion (lait, concentré et fourrage), le poids vif et la fermentation ruminal ont été étudiés durant la même période d'inoculation. Dans le premier essai, l'ensemble ration (fourragère et concentrée) et tampon a été étudié, le résultat a montré qu'indépendamment de la ration, le tampon qui génère un pH élevé a promu une activité fermentative supérieure en termes de AGVs (P=0,001), ammonium (P=0,005), matière organique fermentescible (P=0,006) et production de gaz (P <0,001). Dans le second essai, il a été observé que la ration a exercé un effet très significatif sur la fermentation ruminal *in vitro*. En comparaison avec la ration FOR, la ration CON a promu une meilleure activité fermentative avec un contenu ruminal collecté à 3h post-ingestion qu'avec celui de 0h. Quant au type de la fraction ruminale considérée, il n'y avait pas de différences significatives entre l'utilisation du liquide ruminal vs contenu ruminal. Uniquement, une basse concentration de champignons anaérobiques (P=0,002) a été détectée dans la fraction liquide comme conséquence au tropisme de ces microorganismes à l'égard de la fraction solide. Dans le troisième essai, l'inoculation n'a pas eu d'effet sur les ingestions de lait et concentré, cependant elle avait un effet significatif (P<0,05) sur l'ingestion de fourrage dans les semaines 5, 6, 8, 10, 11 et 12 de vie avec des valeurs élevées pour le groupe LRC (l'ingestion accumulée était de 1,2±0.31 kg Matière Sèche). En outre, l'inoculation n'a pas affecté le poids vif des animaux, or elle a pu moduler quelques aspects de la fermentation ruminale comme la production du butyrate.

Mots clés: Probiotique, prébiotique, chevreaux, microbite ruminal, inoculation, fermentation, ration, tampon, moment d'échantillonnage, *in vitro*, *in vitro*.

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LIST OF ABREVIATIONS

ADF: Acid Detergent Fiber

ADL: Acid Detergent Lignin

BE: Brute Energy

BW: Body Weight

CON: Concentrate

CTL: Control

DFM: Direct Fed Microbial

DM: Dry Matter

DNA: Deoxyribonucleic Acid

EE: Ethereal extract

FM: Fresh Matter

FOM: Fermentable Organic Matter

FOR: Forage

LRC: Ruminal Liquor Concentrate

LRF: Ruminal Liquor Forage

NDF: Neutral Detergent Fiber

OM: Organic Matter

PRE: Prebiotic

RNA: Ribonucleic Acid

VFA: Volatile Fatty Acid

INTRODUCTION

The growing development of antimicrobial resistance genes in livestock and its subsequent transfer to humans (Castanon, 2007) has led the European Union, since 2006, to prohibit the systematic use of antibiotics as growth promoters. This prohibition, coupled with the greater production requirement in intensive systems, has generated a demand in the sector for products with similar effects as antibiotics. Probiotics may represent an alternative, they are living microorganisms that remain active in the host gastrointestinal tract and have been shown that affect advantageously the host animal by improving its gastrointestinal microbial balance (Fuller, 1989) and promoting positive effects on health, stress and improvement of milk production, food efficiency and weight gain (Quadis *et al*., 2014). Most probiotics marketed for animal use are specific strains or mixture of pure cultures of microorganisms such as yeasts (*Saccharomyces cerevisiae*), fungi (*Aspergillus oryzae*), lactic acid bacteria (*Lactobacillus spp., Enterococcus*), pH-controlling bacteria (*Bifidoacterium Bacillus spp. Propionibacterium*) and ruminant bacteria (*Megasphaera elsdenii, Prevotella bryantii and Selenomonas ruminantium*). These probiotics are administered continuously during the productive animal phase, so they are called “Direct Fed Microbials” (DFM), (Krehbiel *et al*., 2003). Although the use of probiotics in monogastrics has shown promising results, in ruminants there is a greater discrepancy due to the considerable complexity of the ruminal microbial ecosystem and the lack ecology knowledge (McAllister *et al*., 2011). In this sense, ruminants at birth lack a ruminal microbiota, which progressively colonizes the rumen as a result of contact with adult animals. Unfortunately, such colonization can be compromised on dairy farms when newborn animals are separated from their mothers after birth and fed with artificial milk without contact with adult animals. In this sense, recent studies have shown that the use of probiotics in ruminants at early ages exerts a beneficial effect on ruminal microbial colonization (Yáñez-Ruiz *et al*., 2015). Therefore, it could be thought that the inoculation of animals at an early age with adult ruminal microbiota, as a probiotic, could accelerate the microbiological development of the rumen, and even program it for an earlier and more efficient use of a given diet depending on the production system (intensive based on concentrate vs. extensive on the basis of forage). However, to date there is no evidence of which type of ruminal microbiota would be more likely to succeed as a probiotic. Therefore the objective of the present work was to identify the factors that maximize the activity of the ruminal microbiota for its later use as a probiotic. For this purpose, two *in vitro* trials were

carried out, one to select the phosphate buffer that best promote the fermentation of different types of diet, and a second trial to determine the effect of diet, sampling time and ruminal fraction on the activity of the ruminal microbiota, and a third *in vivo* trial that aims to: i) evaluate the effect of daily inoculation of ruminal microbiota on the physiological and microbiological development of the rumen, as well as the productive parameters, ii) determine if such intervention at early ages lasts in time and allows a more efficient use of diet.

LITERATURE REVIEW

I- Importance of livestock sector and alternative sources for greater production

1- Importance of livestock ruminant sector

The world population is expected to reach more than 9 billion by 2050, imposing food security challenges particularly for developing countries. Moreover, economic growth has increased the demand for livestock products putting pressure on the livestock sector to produce more with limited resources (FAO, 2016). This increased demand brings challenges in terms of global resource usage and food security. In this current context, possibly two of the most important challenges facing animal agriculture are: i) direct competition with humans for available nutrients as, much of what is currently fed to farm animals could be consumed directly by the human population and ii) reduce the use of antimicrobials in animal feeding. Sub-therapeutic use of antibiotics as growth promoters in animal feed has evoked widespread concern, with their use banned in many countries, including the European Union (EU), due to the potential to develop antibiotic resistance in microbial populations associated with human and animal diseases (Animal Task Force, 2013).

2- Alternative sources for greater production

Ruminants, in contrast to monogastrics, have the ability to utilize human inedible ligno-cellulose material and convert non-protein nitrogen into microbial protein, which in turn is utilized by the animal for growth and productivity. Future ruminant production systems will need to capitalize on these important advantages so that ruminant agriculture continues to play a key role in maintaining and enhancing provision of protein and essential micronutrients to man. The key to understanding and indeed manipulating ruminant production is the rumen. Microbial fermentation in the rumen is conducted by a complex and diverse microbial population (bacteria, protozoa, fungi and archaea) and plays a central role in the ability of ruminants to utilize fibrous substrates. The manipulation of rumen fermentation to maximize the efficiency of feed utilization to increase ruminant productivity, i.e., increase milk, meat, and wool production, is of great interest commercially. In simplistic terms, the aim of rumen manipulation is to enhance fermentation processes that are beneficial to the host and minimize, alter, or delete inefficient or deleterious processes. Current targets for rumen manipulation include (Hart et al., 2008):

- Increase microbial degradation of fiber plant components;

- Decrease protein degradation, ammonia production in the rumen and increase net microbial protein synthesis;
- Decrease the environmental impact of ruminant agriculture;
- Decrease production and increased utilization of lactate;
- Minimize feed-related health risks;
- Prevent and control disease by management of the rumen microbiome.

One of the strategies to modulate rumen fermentation to achieve the above described objectives is the use of probiotics. Probiotics (or direct fed microbials) are becoming increasingly popular as one of the alternatives to Antibiotic Growth Promoters (AGP). The most important objectives for using probiotics in animal feed are to maintain and improve the performance (productivity and growth) of the animal and prevent and control enteric pathogens. In the context of the growing concern with the sub-therapeutic use of AGP in animal feed and greater appreciation of the role of the microbial ecology of the gastro-intestinal tract (GIT) in determining animal productivity, increasing numbers of probiotic products are being developed and used in animal nutrition.

II- Probiotics and prebiotics in animal nutrition

1- Definitions

1-1- Probiotic

Since the 1960s, the definition of the term probiotic has evolved. Firstly, it has been considered by Lilly and Stillwell (1965) as "*unknown growth promoting substances produced by a ciliate protozoan that stimulated the growth of another ciliate"*. Then Parker (1974) defined it as "*organisms and substances which contribute to intestinal microbial balance*". However, Fuller (1989) criticized the use of the term "substances" and consequently redefined probiotic as "*a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance"*. Nowadays, the FAO and WHO (2001) defined probiotic as "*live micro-organisms which when administered in adequate amounts confer a health benefit on the host*".

1-2- Prebiotic

Gibson *et al*. (2004) defined the prebiotic as "*a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastro-intestinal microflora*

that confers benefits upon host wellbeing and health". Recently, another definition was proposed by Huff *et al.* (2015) and Lee *et al.* (2016): "*the prebiotic is a non-digestible food ingredient that can stimulate the growth of one or more beneficial bacteria in the gastrointestinal tract*".

2- Use of probiotics

The probiotics are used in different livestock production systems. In poultry nutrition, probiotics can improve broiler chicken growth rates (Afsharmanesh and Sadaghi, 2014; Mookiah *et al*., 2014; Zhang and Kim, 2014; Lei *et al*., 2015) and control or prevent enteric diseases, including; salmonellosis (Haghighi *et al*., 2008; Tellez *et al*., 2012; Biloni *et al*., 2013), necrotic enteritis (Jayaraman *et al*., 2013) and coccidiosis (Dalloul *et al*., 2003). In the swine industry, sub-therapeutic use of antibiotics in feed to prevent diarrhea and improve performance is still common. Therefore, substitution of AGPs (Antibiotic Growth Promoter) with probiotics to address the issue of antibiotic resistance is critical in pig production. In ruminant nutrition, probiotics are used to modify the microbial population of the rumen and breakdown nutrients. Probiotics can improve the milk yield in dairy animals. They also can increase the weight gain of ruminants (FAO, 2016).

3- Use of prebiotics

Prebiotics have the ability to modulate the balance and activities of the gastrointestinal (GI) microbiota, and are, thus, considered beneficial to the host animal and have been used as functional foods. Previous studies reported the potential of prebiotics in animal nutrition; however, their efficacies often vary and are inconsistent, possibly, in part, because the dynamics of the GI community have not been taken into consideration. Under stressed conditions, direct-fed microbials may be used to reduce the risk or severity of scours caused by disruption of the normal intestinal environment. The observable benefits of prebiotics may also be minimal in generally healthy calves, in which the microbial community is relatively stable (Yutaka *et al*., 2015).

III- Development of the rumen and diet effect

1- Anatomic, physiological and microbial development

From birth, ruminants are physiologically distinguished from monogastric animals in their gastrointestinal system; however, the low development of reticulum-rumen and omasum, the

presence of the oesophageal groove and the incipient development of the abomasum and intestinal enzymes, make the animal behaves like a monogastric (Longenbach and Heinrichs, 1998) that needs in this period dairy feed, which is digested more efficiently (Davis and Drackley, 1998). The transition to a diet based on forage and concentrate should be performed when the animal has developed reticulo-rumen efficiently. In fact, there are three phases which clearly distinguish the development of the rumen (Jiao *et al*., 2015):

- Non-rumination phase: 0-3 weeks;
- Transitional phase: 3-8 weeks;
- Rumination phase: from 8 weeks.

Within these three phases, there are three rumen maturation elements that occur simultaneously:

- Microbial colonization;
- Functional maturation of anaerobic fermentation; and
- Anatomical development of the rumen.

1-1- Non-rumination phase

At birth, the rumen of a newborn is sterile, but it is rapidly colonized in the first 24h of life, reaching microbial concentrations of circa 10^9 cells/mL rumen (Abecia *et al*., 2014). The colonization of the rumen by microorganisms starts from the moment that the animal crosses the birth canal, but at this moment the fermenting capacity of the rumen do not exist because it is undeveloped. It also presents an omasum and abomasum covering 70% of its digestive system compared to the adult ruminant, when the rumen and reticulum form 70% of the digestive system (Figure 1). The colonization of microorganisms continues after birth by direct contact of the young animal with other adult animals, feed, feeders, drinking troughs, etc. contaminated with saliva and feces of adults (Dehority, 2003).

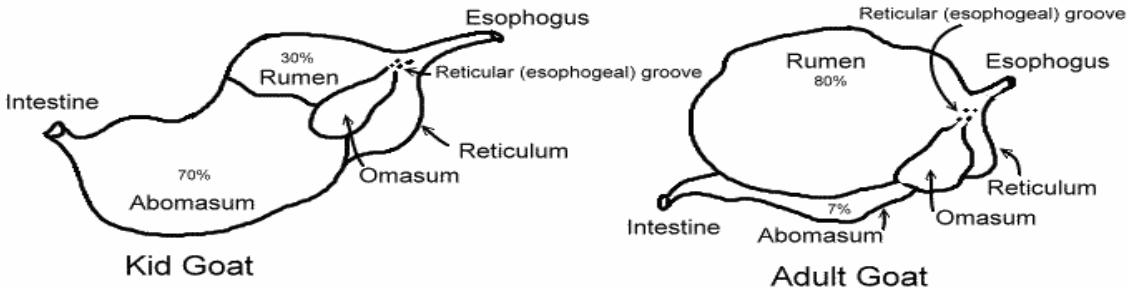

Figure 1. Difference in full functioning adult stomach vs mono-stomach function of kid

During this phase the animal gets all the energy through milk digestion, it is digested in the abomasum, which is the most developed compartment in this phase and the only glandular, where the enzymes and hydrochloric acid required are secreted to coagulate and degrade casein. The direct milk passage to the abomasum is due to a reflex that connects the esophagus directly with the abomasum, without passing through the rumen, since the milk would be fermented and would cause digestive discomfort to the animal, this reflex is denominated esophageal groove and comes stimulated by the action of sucking the milk.

The critical point of this phase is the birth, because the newborn moves from placental feeding to digestive one and the survival of the animal in first days will be linked to the composition of colostrum that will provide nutrients, passive immunity and contribute to the maintenance of the body temperature (Ørskov and Ryle, 1998).

However, the chemical composition of milk, either breast milk or milk replacer, and esophageal groove limit rumen development capacity (Warner *et al*., 1956), so that a progressive introduction of solid feeds will achieve development of this cavity.

1-2- Transitional phase

During this phase the animal begins to introduce solid foods to its feed, until now, liquid. This results in a series of changes towards rumen maturation, such as the anatomical development of this compartment, the establishment of a stable microbial population, and a papillary and metabolic differentiation in order to be able to absorb and use fermentation products (Warner, 1991). All these changes are interrelated to ensure the success of weaning without the growth or health of the animal (Klein *et al*., 1987).

1-3- Rumination phase

Although the rumen has the innate capacity to develop, it will be the consumption of solid food that is the true trigger of ruminal development, and the milk consumed during the lactation phase will stimulate or delay this development. Insufficient consumption of nutrients from milk stimulates the consumption of solid foods and the development of pre-stomachs. If animals have access to decreasing amounts of milk, approximately 7 days later they will begin to consume solid foods in larger quantities (Roy, 1980).

2- Diet effect on the rumen development

The composition and physical state of breast milk or milk replacer do not stimulate rumen development (Warner *et al*., 1956). However, the solid feed is preferably directed towards the reticulo-rumen for its digestion (Church, 1988). This stimulates the microbial proliferation and the production of its final metabolic compounds, such as VFAs with rumen stimulatory capacity (Greenwood *et al*., 1997). Independently of the milk type received during lactation, separate or keep newborn ruminants with the mother until weaning (as in dairy or meat production systems, respectively) influences the later development of this organ, despite having subsequently access to the same type of forage and concentrate offered *ad-libitum* (Abecia *et al*., 2014).

As for the diet influence of ruminants during ruminal development, two elements can be distinguished:

- ✓ Rapidly fermenting concentrate with high propionic and butyric production promotes accelerated development of the epithelial mucosa and its absorption capacity (Bach, 2014). In addition, the type of cereal and its processing must be taken into account (Gimeno *et al*., 2014).
- ✓ Forage supplied in the diet appears to be the primary stimulator of rumen muscle development and increase in volume (Zitnan *et al*., 1998). The factors that influence the effect of forage are the size, effective fiber content and volume of ingestion (Vazquez-Anon *et al*., 1993).

In intensive farming, supplemental feeding is a preferred method of providing nutrients with emphasis on offering young ruminants concentrate solid starter at a relative early age (Jiao *et al*., 2015). During the past few decades, research on rumen development has been mainly focused on this type of feeding system. Concomitantly, factors affecting rumen development processes in ruminants receiving supplemental feeding have been extensively illustrated (Owens *et al*., 1993), with the primary attention on diet composition. In contrast to concentrate feeds role in epithelial development, forages appear to be the primary stimulators of rumen muscularization development and increased rumen volume (Zitnan *et al*., 1998). As stated above, large particle size, high effective fiber content, and increased bulk of forages or high fiber sources physically increase rumen wall stimulation, subsequently increasing rumen motility, muscularization, and volume (Zitnan *et al*., 1998). Several recent studies have shown

that another effective method to foster solid feed intake in calves, contrary to what it has been traditionally recommended, is to provide ad libitum access to poor quality (nutritionally) chopped straw or chopped grass hay (Jiao *et al*., 2015). Castells *et al*. (2013) conducted a meta-analysis and concluded that there were no differences in gut fill between calves consuming no forage and calves consuming forage up to 5% of total solid feed consumption. Thus, it can concluded that when forage consumption is less than 5% of the total solid feed intake, gut fill is negligible and thus advantages reported in performance and efficiency when feeding chopped forages to calves are not an artefact due to gut fill.

IV- Nutritional interventions at an early age to program the rumen microbial ecosystem

1- Microbial modulating compounds

When addressing the development of the rumen, the question arises: does the development of the organ determine which microbes colonize the rumen or do the microbes themselves shape the rumen development through their activity and specific signaling? In the adult animal, the diet is the main driver of the microbial community structure (McCann *et al*., 2014), but in the pre-ruminant both microbial colonization and rumen development may interact in a way that one influences the other. Also, it is still unknown to what extent the animal is genetically pre-determined to develop a certain type of rumen (i.e. epithelium, muscularization, contractions).

2- Direct microbial inoculation

2-1- Yeast

Supplementing ruminant diets with yeast live culture (YLC) as feed additives is widely utilized for ruminant animals in many parts of the world to modulate ruminal fermentation in animals fed highly fermentable diets. Using YC has been proved to stimulate lactate- utilizing bacteria thus stabilizing ruminal pH and favoring fiber degradation. With this effect, using YC during rumen development could potentially represent a beneficial approach to favor the colonizing of desirable microorganisms. Regulation of ruminal pH and reduced lactic acid production are of interest in rumen development re- search because of the influence of these parameters on intake and parakeratosis (Bull *et al*., 1965; Hinders and Owen, 1965). Although supplementing the young animal with YC has been tested before (Leismester *et al*., 2004; Hucko *et al*., 2009), these studies did not address the effect on microbial colonization and animal performance after weaning.

2-2- Direct fed microbials

Bacteria used as DFM have been defined as single or mixed cultures of live organisms, which, when fed to animals, beneficially affect the host. The original concept of feeding DFM to man and livestock was based primarily on the potential for beneficial intestinal effects, including the establishment of a desirable gut microflora and/or prevention of the establishment of pathogenic organisms. More recently, however, there has been some indication that certain bacterial DFM might have beneficial effects in the rumen, such as decreasing the potential for ruminal acidosis (Krehbiel *et al*., 2003)

3- Physiological response of adult animals according to nutritional interventions received in early live

Although it is increasing the number of studies looking at the manipulation of the diet of ruminants in early life, very few have followed up the physiological response later in life (i.e. post weaning). This is particularly relevant in dairy animals as the time window between weaning and becoming a productive animal (first lactation) is rather long (15 months in small ruminants and 24 months in cattle). As stated earlier, providing ad libitum specific 'starter' concentrate to accelerate rumen development is becoming the norm in most production systems. However, depriving calves from forage during the pre-weaning phase may offer yet another physiological and dietary adaptation challenge to young calves during the transition when presented with forage for the first time. Phillips (2004) reported that calves fed fresh grass during the milk-feeding period spent more time eating on a pasture compared with those that received no forage before weaning. Recent data also shows that 22% of the variation in milk yield in first lactation is associated to the average daily gain during the first weeks of life (Soberon *et al*., 2012). However, the long-term effects of early life nutritional management in relation to rumen development are still largely unknown and there are factors that still need to be carefully considered such as composition of the starter, type of forage and timing of its introduction

OBJECTIVES

The aim of this work was to develop a rumen probiotic to enhance rumen microbial colonization and development and to promote the best conditions for animals to make the best use possible of forage or concentrate based diets after weaning.

The specific objectives were to:

- Identify using *in vitro* systems the factors that determine the most active rumen digesta to be used as probiotic;
- Assess *in vivo* the effect inoculating new born kid goats with rumen content form adult animals on the growth, intakes and rumen fermentation pre and post weaning;
- Evaluate whether using rumen fluid from adult animals fed either forage or concentrate based diets determine a different response in young animals.

MATERIALS & METHODS

This work is divided into two experiments:

- ***In vitro* experiment**: consists of an optimization of probiotic preparation based on ruminal content adopting two trials which aim for an evaluation of:
  - **a-** The best buffer to use (**Trial 1**);
  - **b-** The best diet, sampling time and inoculum process (**Trial 2).**
- ***In vivo* experiment**: (**Trial 3**) consists of an inoculation of newborn goat kids with the ruminal microbiota developed in the *in vitro* experiment.

The experiments were conducted at the Estación Experimental del Zaidín (CSIC, Granada) All management and experimental procedures involving animals were carried out by trained personnel in strict accordance with the Spanish guidelines (RD 1201/2005 of 10th October 2005) for experimental animal protection at the Estación Experimental del Zaidín. Experimental protocols were approved (09/03/2017) by the Ethics Committee for Animal Research at the Animal Nutrition Unit.All experiments and protocols were conducted in accordance to the approved by the Animal Welfare regulation.

I- *In vitro* experiments

1- Animals and treatments

Eight Murciano-Granadina goats (47±5.6 kg) were used as donors of rumen microbiota; they were randomly allocated into two groups of 4 animals and were assigned to one experimental diet: FOR received 100% forage (based on 50% alfalfa and 50% oat hay in DM basis) and CON fed with concentrate and forage (80:20) with the same forage composition than described for FOR diet. All animals had free access to fresh water and mineral block throughout all experiment. This experiment lasted 5 months, starting in 13/11/2016 and ending in 19/04/2017. During the first week feed intake was measurement to make sure that all of animals have similar intakes and the two following week were considered as an adaptation phase to the diets. The chemical composition of diets used for the both groups is presented in the Table 1.

Table 1. Chemical composition of forage and concentrate used for donor goats

Parameter	Alfalfa hay	Oat hay	Concentrate
DM (%)	89.4	91.5	90.1
OM (%)	87.6	93.5	95.1
BE (MJ/kg DM)	18.5	18.4	19.2
EE (%)	1.42	1.82	4.52
NDF (%)	50.8	68.1	25.4
ADF (%)	31.3	41.9	7.27
ADL (%)	10.3	8.66	2.11
Cellulose (%)	21.0	33.3	5.17
Hemicellulose (%)	19.5	26.2	18.1

2- Rumen inoculum preparation

During the first week of this trial, animals were sampled during 3 days at 0, 2, 4 and 6 h after feeding to monitor rumen pH (Figure 2). This information was needed to determine the buffer composition to mimic *in vitro* the rumen pH under different diets. On week two, rumen fluid was taken at 0 h and 3 h after feeding from all animals and incubated with three incubation buffers containing different concentration of bicarbonate to generate three pH (high 6.80, medium 6.25 and low 5.75) (Trial 1). Rumen contents were collected separately from all of cannulated goats fed experimental diets (FOR vs CON). Rumen contents were transferred to the laboratory in thermos bottles preheated to 39º C and filtered through cheesecloth under CO_2.

A second experiment (**Trial 2**) was designed to explore: i) two sources of ruminal inoculum (IF vs IC which concern respectively animals fed forage and animals fed concentrate); ii) two sampling times (0 and 3h after feeding) and; iii) two inoculum types: liquid rumen microbiota (LIQ) vs Liquid + Solid rumen microbiota (LS). This latter microbial inoculum was composited by mixing 75% of rumen liquid and 25% of rumen solids based on the previous measurement of the rumen content composition.

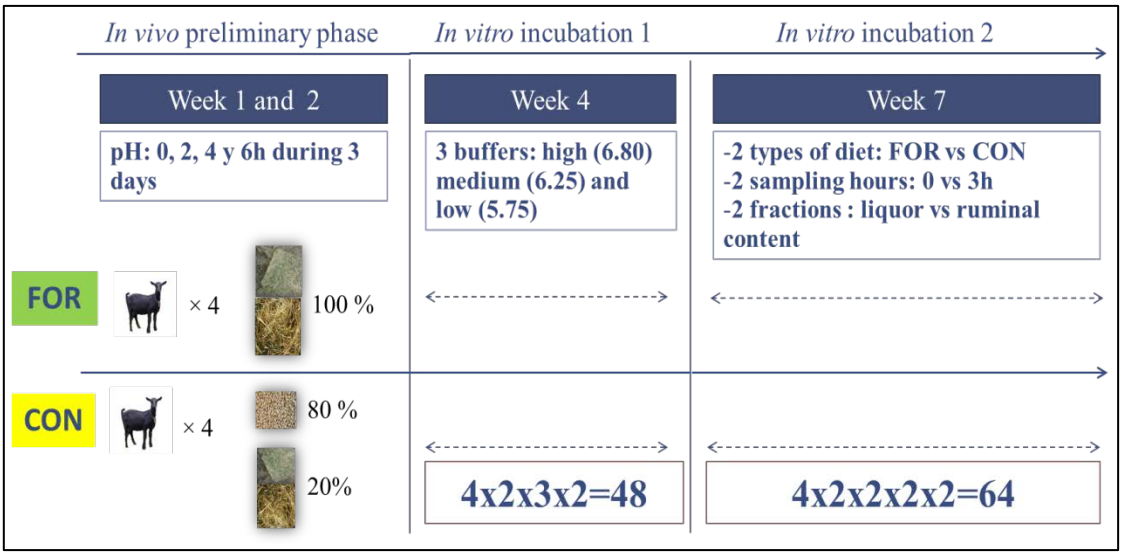

Figure 2. *In vitro* experiment schedule

3- Incubation procedures

<u>Trial 1: Identification of the best buffer</u>

All *in vitro* incubations were performed following the procedure described by Theodorou *et al*. (1994). The substrates weight and buffer concentrations are summarized in the Table 2. Briefly, the incubation mixture (mL/L) contains: 474 mL distilled water, 237 mL buffer solution made up with 35, 10.3 and 3.17 g/L sodium bicarbonate ($NaHCO_3$) and 4, 1.07 and 0.25 g/L ammonium bicarbonate ((NH_4) HCO_3), calculated to establish respectively pH of 6.80 (Trial 1 and 2), 6.25 and 5.75, 237 mL macro-mineral solution made up with 5.7 g disodium hydrogen phosphate (Na_2HPO_4), 6.2 g potassium di-hydrogen orthophosphate (KH_2PO_4) and 0.6 g magnesium sulphate ($MgSO_4.7H_2O$) and 50 mL reducing solution made up with 47.5 mL distilled water, 2 mL sodium hydroxide (NaOH) 1N and 0,285g $Na_2S.9H_2O$. Micro-minerals solution made up with 13 g ($CaCl_2.2H_2O$), 10 g ($MnCl_2.4H_2O$), 1 g ($CoCl_2.6H_2O$) and 0,8g ($FeCl_2.6H_2O$). 49.5 mL reducing solution made up with 100 mg resazurin (equivalent of 0.1%). Ingredients were mixed and reduced under a CO_2 atmosphere during 1 hour. Bottles were filled with the incubation solution (33.3 mL) under a CO_2 stream, sealed with rubber caps and aluminum rings, and pre-heated at 39º C. Finally, rumen inoculum (16.7 mL) was mixed with incubation buffer (33.3 mL) and incubated in Wheaton bottles containing substrate (0.5 g MS of experimental diets). In the first trial, two runs of incubation (0 and 3 h after feeding) with duplicated eight bottles per treatment (3 buffers) and were carried out for 72 h with each type of diet (FOR and CON) which made a total of 96 Wheaton bottles. Pressure was measured at 2, 4, 7, 10, 24 hours of incubation using a

manometer: Pressure readings were converted into volume using the ideal gas law. Each bottle was sampled (3 ml) at 2, 4, 7, 10 and 24 h to describe the fermentation parameters in terms of pH and VFA.

Trial 2: Effect of diet received by the donor animal, sampling time and type of rumen inoculum

In the second trial, specific bicarbonate buffer was used based on the results from Trial 1. In this trial were explored:

- Two sources of rumen inoculum:
  - ✓ Animals fed forage (FI) as alfalfa and oat hay
  - ✓ Animals fed concentrate (CI) with a F:C=20:80
- Two inoculum processes:
  - ✓ Liquid (LIQ)
  - ✓ Liquid and solid (LS) by mixing 75% liquid and 25% solids
- Two sampling times:
  - ✓ Before feeding (0 h)
  - ✓ After feeding (3 h)

Before starting the experiment, rumen solids from all 8 animals were collected, freeze dried (a DM determination was also conducted) and autoclaved. This material was added to the LIQ diets in order to compensate the fermentation material provided by the solid fraction but preventing the supply of solid associated microbes. Incubation method was the same than described for Trial 1. Each inoculum was incubated with its own substrate (i.e. FI with FOR and IC with CON). The two sampling times (0 and 3h after feeding) were chosen based on the differences in the diurnal changes in rumen pH during the rumen monitoring phase. Total number of bottles was 128 divided in two identical batches of 64 samples:

Samples: 2 inoculums x 2 processes x 2 time point x 4 animals x 2 replicates = 64

Blanks: (inoculum without substrate): 2 inoculums x 2 processes x 2 sampling times x 4 animals x 2 replicates = 64

Table 2. Incubation mixture composition in glass bottles in *in vitro* experiment

Trials	Inoculum source	Containing substrate (mg FM)			Incubation solution (mL)	Rumen inoculum (mL)	Incubation mixture (mL)
		Alfalfa hay	Oat hay	Concentrate			
1	FOR	250	250	0	50	16.67	33.33
	CON	60	60	380	50	16.67	33.33
2	FOR	250	250	0	50	16.67	33.33
	CON	60	60	380	50	16.67	33.33

4- Chemical analysis

All incubations were conducted in 120 mL Wheaton bottles under CO_2 and sealed with an airtight seal, and incubated at 39º C. Gas production was measured at 0, 2, 4, 7, 10, 24, 36, 48 and 72 h of incubation. At 24 h incubation the fermentation pattern was described. A head space gas sample was taken for CH4 and H_2 measurement, moreover bottles content (5 mL) was sample dosing a 13G needle. Then pH was measured and 4 subsamples taken to describe the fermentation pattern:

For NH_3; 0.8 ml bottle fluid in 0.2 ml of TCA at 25% (stored at 4° C)

For VFA; 1.6 ml bottle fluid in 0.4 ml of 20% orthophosphoric congaing crotonic acid as internal standard (stored at -20° C)

For lactate; 0.8 ml bottle fluid will be freeze a -20° C

5- Protozoal counting and microbial characterization

A microbial characterization of the rumen fluid/solid used as probiotics was performed. Inoculum from each animal after been pooled with the incubation buffer was sampled:

Trial 1: 8 animals x 2 processes x 2 times = 32 samples

Trial 2: 8 animals x 3 preservations = 24 samples

For DNA analysis (qPCR) two 10 mL aliquots were taken and snapped freeze in liquid N.

For protozoal counting 0.8 mL of incubation fluid was mixed with 0.8 mL of formaldehyde at 4% (containing NaCl 0.9%). Protozoal cells were stained with Brilliant Green colorant (10µL into 2mL of sample) and incubated at room temperature for 24h.

Then 0.15 mL of ruminal sample was poured on a dry clean slide which was then carefully covered by a dry clean cover slide. The stained samples were observed under the optical microscope (400x magnifications) to count protozoa cells and then calculated the protozoa

number based on the dilution factors used. The main porotoal group (subfamily Entodiniinae, Subfamily Diplodiniinae, Ophryoxcolex spp. Isotricha spp and Dasytricha spp.) based on the taxonomic classification described by Dehoriy *et al*., (2003).

6- Statistical analysis

Results were analyzed by factorial ANOVA using the Genstat 18 software package. The main effects were the diet, buffer and its interaction in Trial 1, while in Trial 2 the main factors were diet, inoculum process, sampling time and their interactions. The animal effect was considered as random in both trials. The differences were considered significant when $P<0.05$ and differences between means were assessed based on the least significant difference test.

II- *In vivo* experiment

1- Animals, treatments and experimental design

Seventy four Murciano-Granadina goat kids were randomly distributed into 4 identical groups (16 animals per group):

- **CTL**: control group without any inoculation;
- **PRE**: prebiotic group inoculated with ruminal liquor autoclaved (LRF and LRC mixture);
- **LRC**: probiotic group inoculated with ruminal liquor of animals fed concentrate diet;
- **LRF**: probiotic group inoculated with ruminal liquor of animals fed forage diet.

The animals were distributed in the four experimental groups containing four animals each. Experimental groups had similar average birth weight and the same number of males and females. Although the mother effect was not considered a priori, two siblings were never assigned to the same experimental group. During the first day after birth, animals were stayed with the mother for a minimum of 24 h and a maximum of 36 h to guarantee that they took enough colostrum. During this period, the goat kids were monitored at least twice. Animals that not suckle were stomach tubed to provide colostrum and / or milk during the first days of life. All kids were trained to suck from a teat connected to a unit for feeding milk replacer. All kids were treated intramuscularly with Vitamin E and selenium at 24h after birth and assigned to an experimental group. The animals of each group were divided in four identical pens based on the date of birth. All animal shared the same building but separate by treatments to prevent potential cross contaminations between treatments. At 15 days of age, vitamin

E+Selenium and a preventive oral coccidiostatic (VECOXAN) were injected again. At 21 days of age were vaccinated against enterotoxemia.

Table 3. Goat kids distribution by treatment

Group 1 (oldest)	CTL (n=4)	PRE (n=4)	LRF (n=4)	LRC (n=4)
Group 2	CTL (n=4)	PRE (n=4)	LRF (n=4)	LRC (n=4)
Group 3	CTL (n=4)	PRE (n=4)	LRF (n=4)	LRC (n=4)
Group 4 (youngest)	CTL (n=4)	PRE (n=4)	LRF (n=4)	LRC (n=4)

2- Feed intake

2-1- Milk feeding

During 7 weeks, the animals were fed milk replacer *ad libitum*, starting with concentrations equal to 135, 150, 160 and 170 g/L at week 1 and 2, week 3 and 4, week 5, 6 and 7 respectively. During the first 2 weeks artificial milk was prepared three times a day (9:00, 14:00 and 19:00 h) and twice a day thereafter (9:00 and 17:00 h). All cubes, tubes and teats were cleaned daily with soap, hot water and disinfectant before the morning feeding. The last 5 weeks of lactation were done with cold milk to avoid excessive consumption and promote the intake of solid feed. The goat kids had free access to fresh water and compound feed (Starter powder during the first 3 weeks and commercial concentrate thereafter). The forage (alfalfa hay) was introduced from the third week of age. The animals litter was wood chip-based to facilitate discrimination of forage refusals.

2-2- Weaning and post-weaning

At 7 weeks of age, goat kids were gradually weaned. The concentration of milk replacer was gradually reduced during 4 days at a rate of 20% per day (from 100% to 0%). From week 2 all animals had free access to fresh water, concentrate and alfalfa hay.

3- Sampling and measurements

At the birth, mother tag number, day and time of birth, weight, sex and animal tag number were recorded during the lactation period diet offered (milk, compound feed and forage) and feed refusals were collected and weighted daily per pen, the BW of each goat kid were recorded weekly.

4- Inoculation

The same goats of *in vitro* experiment were used as donors of fresh rumen fluid for the newborn goat kids. The diet was distributed in two times (at 8:30 h and 15:00 h) in order to extract the fresh rumen fluid at 11:30 h (3 h post feeding). Every day two goats were sampled alternately from each diet group (approximately 60 mL per animal). The content of each group was transferred to the laboratory in thermos bottles preheated to 39º C, filtered through cheesecloth under CO_2, iso-volumetrically pooled and a subsample of the mixed rumen fluid was put in sealed containers until its use to orally inoculate LRC and LRF groups. On Mondays, fresh rumen fluid was collected from all animals and used for three purposes:

- Inoculate the animals with fresh ruminal liquid on that day;
- Describe the inoculum in terms of pH, VFA, ammonium, lactate, protozoa and microbiology;
- Divide it in 7 bottles (80 mL/bottle) that were autoclaved and kept at 4º C to be used as a prebiotic (PRE) during the following week.

The goat kids were daily inoculated at 12:00 h using a syringe at a rate of 0.5 mL/kg BW corrected weekly based on the average weight of each pen. The inoculation was done after the milk intake in the morning, for a total of 11 weeks (4 weeks after weaning)

5- Chemical analysis

5-1- Inoculum characterization

To characterize the inoculum used for goat kids, ruminal fluid was collected from individual animals fed forage vs. concentrate (n = 4), it was added (16.67 mL) in bottles with 0.5 g of substrate (50% forage; 50% concentrate) and 33.3 mL of anaerobic buffer at 4º C. Then it was immediately autoclaved at 120° C for 15 minutes and stored for the next day in which samples of ruminal fluid were taken from the same animals and the incubation *in vitro* was done (mixing 33% inoculum and 67% autoclaved buffer joined to the diet). The incubation followed the same protocol as in *in vitro* experiment and all of bottles (except blanks bottles) received the same substrate (50% forage and 50% concentrate). From the ruminal liquid of the second day, one part was used to determine VFA, ammonium, lactate, DNA and RNA of each fistulated goat and another part was autoclaved to measure the same parameters.

Total number of bottles = 64

2 inoculum x 4 animals x 2 diets x 2 replicas = 32

Blanks (without substrates) 2 inoculums x 8 animals x 2 diets x 2 replicas = 16

At 24 h of incubation, samples of VFA, ammonium, protozoa and DNA were done and the gas pressure was recorded at 2, 4, 7, 10, 24, 36, 48 and 72 h.

5-2- Feed composition

Composite feed was chemically analyzed according to AOAC (2005) for dry matter (DM), organic matter (OM), crude protein (CP). Neutral Detergent Fiber (NDF), Acid Detergent Fiber (ADF) and Acid Detergent Lignin (ADL) were determined according to Goering and Van Soest (1970). Chemical compositions of tested ingredients are presented in the table 4.

Table 4. Chemical composition of alfalfa and concentrates (1 and 2) used for goat kids

Parameter (%)	Alfalfa	Concentrate 1	Concentrate 2
DM	92.6	92.4	91.9
OM	86.2	94.1	92.6
NDF	96.3	19.0	30.76
ADF	74.5	5.05	13.47
ADL	58.6	0.74	4.14
Cellulose	15.9	4.32	9.34
Hemicellulose	21.8	13.9	17.3

5-3- Rumen liquor sampling

The ruminal content was sampled from all of goat kids in three times: at 5, 7 and 9 weeks of age which is the equivalent respectively of early weaning, weaning and post- weaning, by means of a stomach tube used to the oral cavity. Each sample was strained through a layer of cheesecloth and used to measure the following parameters: pH, VFA, ammonium and lactate using the same protocol as in the *in vitro* experiment.

6- Statistical analysis

Results were analyzed by factorial ANOVA using the Genstat 18 software package. The main effects were the treatment and experimental group. The animal effect was considered as random. The differences were considered significant when $P<0.05$ and differences between means were assessed based on the least significant difference test.

RESULTS & DISCUSSION

I- *In vitro* experiment

1- pH evolution

The monitoring of ruminal pH *in vivo* (Figure 3) showed a more pronounced decrease for the CON diet (7.02, 5.79, 5.96 and 5.98) than for the FOR diet (7.39, 6.97, 6.78 and 6.92 at 0, 2, 4 and 6 h post-feeding respectively) between 2 and 4h post-feeding due to the rapidly fermentation of non-fibrous carbohydrates (starch, soluble sugars and pectin) in the rumen (Van Soest *et al*., 1991) and because they not stimulate rumination neither saliva production, promote acid production, decreasing the normal-optimal pH of the rumen which oscillates around of 6.2-7 (Calsamiglia and Ferret, 2002) and increasing the risk of ruminal acidosis, being able to inhibit the fiber fermentation.

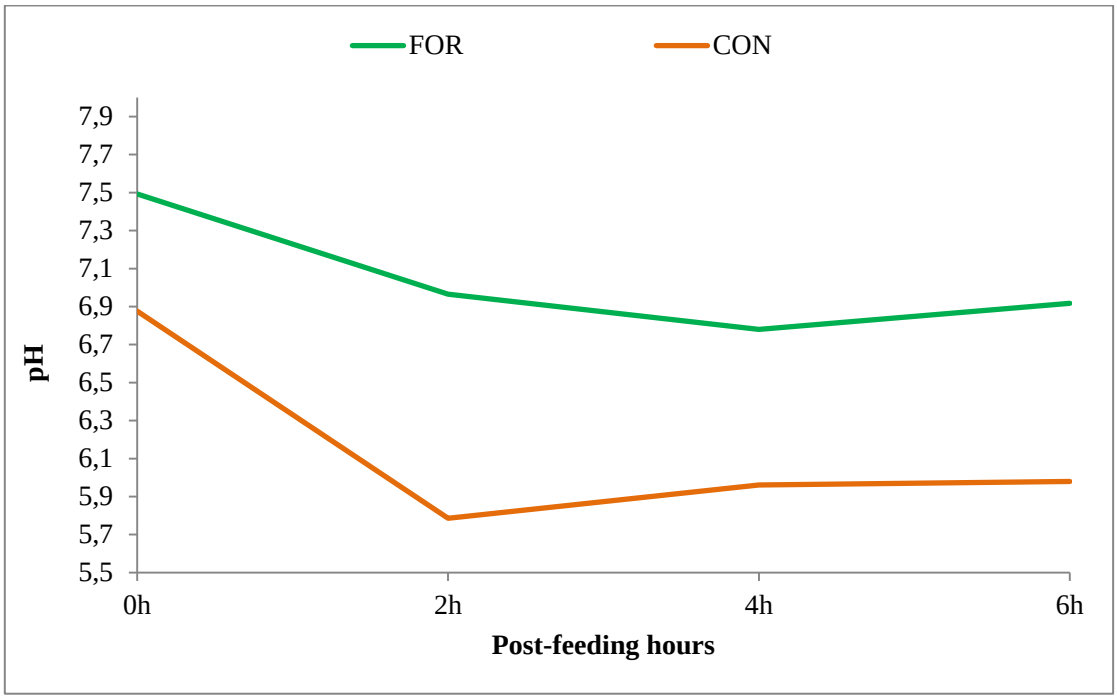

Figure 3. pH evolution *in vivo* at four hours of sampling

2- Buffer used

Based on the pH evolution results, three buffer mixtures (high: 6.80, medium: 6.25 and low: 5.75) were selected to achieve a better *in vitro* simulation of *in vivo* fermentation conditions. This assay showed that the buffer which generates a higher pH promotes a higher fermentation activity (Table 5) in terms of VFAs (P=0.001), ammonium (P=0.005), FOM (P=0.006) and gas production (P <0.001). In addition, it was observed that this effect is independent of the type of diet (absence of significant interactions), so that it was used in successive trials. The rumen pH depends on VFAs accumulation

level and the proportion of them depends on the diet nature (Tamminga and Van Vuuren, 1988). We observed that a forage diet allows a constant pH due to the buffer capacity of the hay and saliva produced in large quantities during chewing (Michalet-Doreau and Sauvant, 1989). However, Mackie *et al*. (1979) report that rations rich in grain lead to a significant amount of rapidly fermentable starch, causing an increase of the concentration VFAs and an important drop in pH.

Table 5. Diet and buffer effect on *in vitro* ruminal fermentation

Parameter	Diet		Buffer pH				*P-value*	
	FOR	CON	High	Medium	Low	SED	Diet	Buffer
pH	6.13	5.67	6.48[a]	5.79[b]	5.44[c]	0.173	0.003	<0.001
NH_3-N, mg/100 mL	23.3	48.6	48.2[a]	26.4[b]	33.2[b]	15.05	0.043	0.005
VFAs, mM	70.8	91.6	89.2[a]	80.9[b]	73.5[b]	11.27	0.056	0.001
Acetic (%)	70.0	54.0	61.9	62.0	62.1	2.51	<0.001	0.926
Propionic (%)	16.3	23.0	20.0	19.5	19.5	2.28	0.010	0.805
Isobutyric (%)	1.05	1.47	1.39	1.23	1.15	0.335	0.065	0.290
Butyric(%)	10.1	15.9	12.5	13.2	13.4	1.65	<0.001	0.544
Isovaleric (%)	1.36	2.37	2.12	1.82	1.67	0.567	0.020	0.219
Valeric (%)	1.11	3.30	2.19	2.27	2.17	0.657	<0.001	0.949
FOM (mg)	346	475	448[a]	411[ab]	374[b]	59.5	0.029	0.006
Gas production								
B[1] (mL)	63,6	106,1	103,9	84,2	66,5	14,98	0,007	<,001
C[2] (mL/h)	0.11	0.14	0.117	0.127	0.140	0.021	0.142	0.012

[a-c] Means within a row with unlike superscripts differ (P<0.05); SED-Standard Error of Difference. [1]Potential gas production. [2] Gas production rate

3- Diet, sampling time and ruminal fraction effects on ruminal fermentation

3-1- **Diet effect**

The diet had a significant effect on the parameters studied (**table 6**); on pH (P=0.005), VFAs (P=0.008), FOM (P=0.003), potential gas production (P=0.03), gas production rate (P=0.001), methane production (P=0.03), fungi (P=0.007) and protozoa concentrations (P=0.01). In contrast to the forage diet, the CON diet allowed increased production of VFAs (+21%) with greatest fraction of butyric (+48%), isobutyric (+25%), valeric (+72%) and isovaleric (+52%), gas production (+30%), FMO (+13%) and protozoa concentration (+5%). However, the FOR diet allowed a larger fraction of acetic (+14%) and anaerobic fungi concentration (+9%).

3-2- **Sampling time effect**

The sampling time showed significant effect (Table 6) on pH (P=0.001), VFAs (P=0.03), FOM (P=0.02), potential gas production (P=0.001), methane production (P=0.003) and fungi concentration (P=0.001). In a comparison of two time of sampling (0 vs 3h post-feeding), it observed that at 3h, we had more VFAs production (+12%) with biggest acetic fraction (+9%), more FOM (+13%), higher potential production gas (+24%), gas production rate (+11%) and methane production (+12%). Concerning 0h, the results showed fractions three times superior for isobutyric and isovaleric and two times superior for valeric and also in terms of fungi concentration (+8%).

3-3- **Inoculum process of ruminal content effect**

No significant effect of the inoculum process of ruminal content was observed (Table 6) except for isobutyric fraction (P=0.001) and fungi concentration (P=0.002) and they were superior in liquor and ruminal content respectively. There were not significant interactions between all of factors studied. That is why we represented all of them separately.

Table 6. Effects of diet (D), sampling time (T), inoculum process of ruminal content (P) on fermentation and ruminal microbiota *in vitro*.

Parameter	Diet		Time		Process			*P-value*		
	FOR	CON	0h	3h	L	LS	SED[3]	D	P	T
pH	6,63	6.01	6.57	6.07	6.38	6.25	0.204	0.005	0.14	0.001
VFA, mM	85.8	103.6	89.5	99.9	95.9	93.5	9.040	0.008	0.59	0.03
Acetic, %	69.2	60.7	62.1	67.7	64.2	65.6	2.010	0.001	0.11	0.001
Propionic, %	17.6	19.7	19.2	18.1	19.1	18.1	1.603	0.16	0.08	0.06
Butyric, %	9.93	14.7	12.6	12.0	12.3	12.3	1.191	0.003	0.95	0.17
Isobutyric, %	0.95	1.19	1.66	0.48	1.15	0.99	0.156	0.143	0.001	<0.001
Valeric, %	0.96	1.65	1.78	0.83	1.31	1.30	0.208	0.002	0.868	<0.001
Isovaleric, %	1.41	2.15	2.68	0.88	1.87	1.69	0.341	0.04	0.103	<0.001
FOM, mg	419	527	444	502	479	467	45.2	0.003	0.62	0.02
Gas production										
B[4], mL	176	229	181	224	198	207	22.5	0.03	0.21	0.001
C[5], mL/h	51.4	69.9	57.5	63.8	58.2	63.2	6.78	0.001	0.17	0.10
CH_4, mM	5.45	6.36	5.56	6.25	5.77	6.04	1.17	0.08	0.20	0.003
CH_4, mM/d	0.67	1.15	0.72	1.10	0.86	0.95	0.198	0.03	0.16	0.001
Microbiota, log /mL										
Bacteria	11.8	12.0	11.8	12.0	11.9	11.9	0.304	0.36	0.59	0.09
Archaea	8.66	8.71	8.57	8.80	8.78	8.59	0.241	0.78	0.11	0.05
Fungi	7.24	6.64	7.21	6.67	6.79	7.08	0.204	0.007	0.002	0.001
Protozoa	9.45	9.93	9.83	9.56	9.70	9.69	0.280	0.01	0.96	0.08

[3]SED-Standard Error of Difference. [4]Potential gas production. [5]Gas production rate

II- *In vivo* experiment

1- Goat kids intake

1-1- Milk

Weekly consumed milk decreased by animals inoculated with fresh rumen liquor (Figure 4), but the difference was not significant. According to Ørskov *et al*. (1970), the lack of ruminal development in milk fed newborn animals may be due to the effective shunting of milk directly to abomasum by the reflexive closure of the reticular groove, thus preventing substrate for the establishment of ruminal fermentation from entering the rumen.

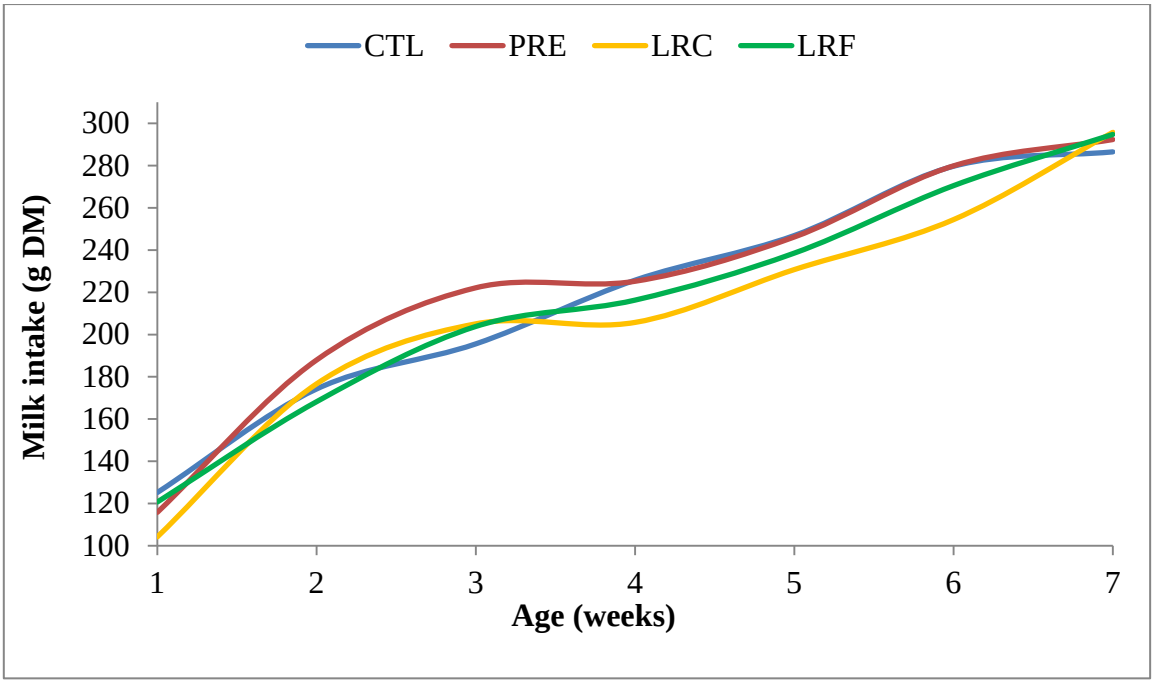

Figure 4. Milk intake of goat kids during the first 7 weeks of life

1-2- Concentrate

The inoculation had no effect on the DM concentrate intake. Indeed, evolution intake was similar for the different groups studied (Figure 5). The accumulated concentrate intake was 1.85±0.80, 1.64±0.14, 1.79±0.49 and 1.73±0.18 kg DM for CTL, PRE, LRC and LRF groups respectively. The same result was found by Zhong et al. (2003) when they inoculated weaned lambs.

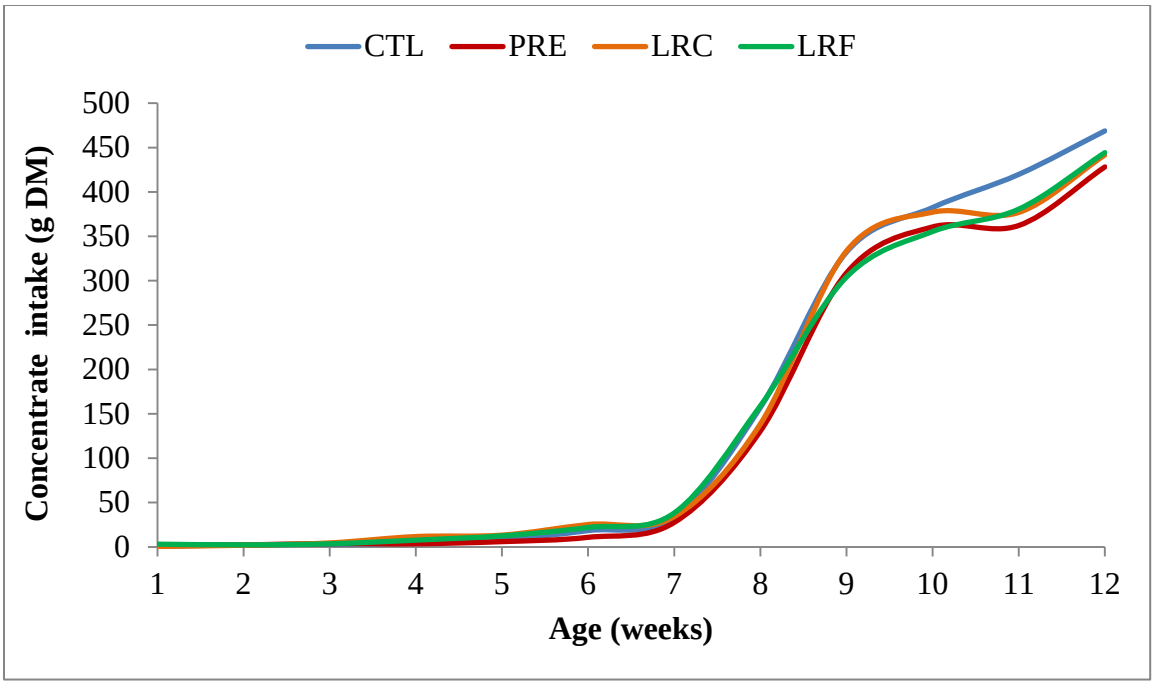

Figure 5. Concentrate intake of goat kids during the first 12 weeks of life

1-3- Forage

The inoculation had significant effect (P<0.05) on the DM forage intake from 5 weeks of life of goat kids. The evolution intake (Figure 6) showed that the LRC group had the highest ingestion value followed by the LRF group. The accumulated forage intake was 1.20±0.31, 0.98±0.11, 0.85±0.35 and 0.83±0.07 kg DM for LRC, LRF, CTL and PRE groups respectively.

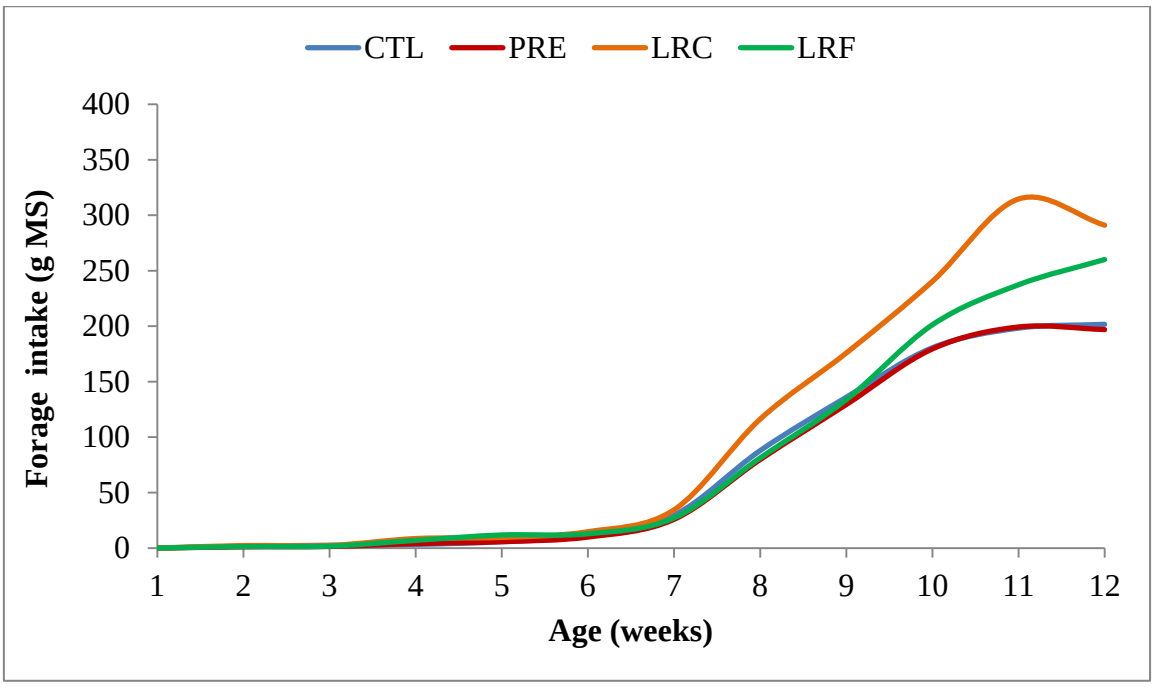

Figure 6. Forage intake in goat kids during the first 12 weeks of life

1-4- Solid ingestion in three different ages of life of goat kids

According to the results obtained (Table 7), there were no significant differences between the three periods of life of goat kids in terms of ingesting solid feeds, except in the week 5 which corresponds to an early weaning and where the difference was highly significant (P=0.002).

Table 7. Accumulated solid intake in three different ages (5, 7 and 9 weeks) of goat kids

Parameter	CTL	PRE	LRC	LRF	SEM[6]	*P-value*
Intake (g DM)						
Week 5[7]						
Milk	245.4	246.2	234	238.6	16.93	0.895
Concentrate	7.7	5.7	12.9	12.5	2.28	0.099
Forage	5.7[a]	5.8[a]	9.5[b]	11.8[b]	1.006	0.002
Week 7[8]						
Milk	285.5	292.4	294.3	294.8	10.60	0.926
Concentrate	37.6	24.9	33.9	37.8	9.12	0.777
Forage	26.8	25.8	33	26.8	4.03	0.562
Week 9[9]						
Milk	-	-	-	-	-	-
Concentrate	330.6	308.7	330.2	304.1	30.1	0.853
Forage	134.8	129.4	175.2	133.8	13.73	0.112

[6]SEM-Standard Error of Mean. [7]Early weaning. [8]Weaning. [9]Post weaning.

2- Growth performance

The inoculation no had significant effect on body weight of animals (Table 8). Contrary to this result, Abo-Donia *et al.* (2011) showed that inoculation increase the body weight.

Table 8. Effect of inoculation on body weight of goat kids

BW (kg)	CTL	PRE	LRC	LRF	SEM	*P-value*
Week 5	7.4	7.8	7.5	7.3	0.202	0.475
Week 7	10	10.3	9.9	9.9	0.254	0.608
Week 9	11.7	11.7	11.7	11.5	0.346	0.941

3- Ruminal fermentation

3-1- Week 5 of life

Table 9. Ruminal fermentation parameters in the week 5 of life of goat kids

Parameter	CTL	PRE	LRC	LRF	SEM	*P-value*
pH	6.63	6.69	6.72	6.74	0.059	0.555
VFA, mM	30.4^{b}	19.1^{a}	31.5^{b}	32.6^{b}	3.22	0.018
Acetic, %	73.5	74.3	75.3	73.8	1.053	0.609
Propionic, %	13.8	15	14.4	13.9	0.645	0.569
Butyric, %	5.5^{b}	2.9^{a}	4.5^{ab}	5.8^{b}	0.776	0.040
Isobutyric, %	2.3^{a}	2.9^{b}	2^{a}	2.2^{a}	0.1373	<.001
Valeric, %	1.6	1.2	1.2	1.4	0.1417	0.138
Isovaleric, %	3.2^{ab}	3.7^{b}	2.7^{a}	3^{a}	0.1989	0.006
Ac/Prop	5.5	5.1	5.5	5.5	0.253	0.608

3-2- Week 7 of life

Table 10. Ruminal fermentation parameters in the week 7 of life of goat kids

Parameter	CTL	PRE	LRC	LRF	SEM	*P-value*
pH	6,35	6,49	6,49	6,50	0,087	0.621
VFA, mM	34.4	30.5	32.5	32.3	3.78	0.914
Acetic, %	69.7	66.6	71	72.4	1.597	0.079
Propionic, %	15.6	16.4	14.2	14.7	0.791	0.237
Butyric, %	9.4	10	9.6	7.7	1.24	0.609
Isobutyric, %	1.5	1.9	1.6	1.5	0.143	0.166
Valeric, %	1.6^{ab}	1.9^{b}	1.4^{a}	1.3^{a}	0.113	0.003
Isovaleric, %	2.2^{a}	3.2^{b}	2.2^{a}	2.3^{a}	0.244	0.010
Ac/Prop	4.7	4.4	5.1	5.3	0.330	0.167

3-3- Week 9 of life

Table 11. Ruminal fermentation parameters in the week 9 of life of goat kids

Parameter	CTL	PRE	LRC	LRF	SEM	*P-value*
pH	6.68	6.68	6.75	6.61	0.116	0.909
VFA, mM	50.3^{ab}	58.3^{b}	46.7^{a}	42.1^{a}	3.59	0.017
Acetic, %	58.3	54.4	59.3	57	1.742	0.222
Propionic, %	25.5^{b}	30.1^{c}	20.3^{a}	23.3^{ab}	1.362	<0.001
Butyric, %	11.2^{a}	11.2^{a}	15.7^{b}	14.8^{b}	1.012	0.003
Isobutyric, %	0.9	0.9	1.1	1.1	0.115	0.239
Valeric, %	2.9^{b}	2.1^{a}	1.8^{a}	2.1^{a}	0.1812	<0.001
Isovaleric, %	1.2	1.2	1.7	1.7	0.206	0.161
Ac/Prop	2.5^{ab}	2^{a}	3.1^{c}	2.6^{bc}	0.1954	0.002

CONCLUSIONS

- The *in vitro* experiment showed that the rumen microbiota sampled at 3h post-feeding from animals fed concentrate has the highest activity and should be tested as probiotic;
- Inoculating newborn goat kids with rumen fluid from adult animals promotes higher forage intakes after weaning and suggests it could be used as strategy to alleviate the physilogical stress associated to the transition from liquid to solid milk feeding at weaning.

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