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Ruminal bacteria and protozoa present in sheep supplemented with probiotics identified by counting and PCR endpoint

Bacterias y protozoarios ruminales presentes en ovinos suplementados con probióticos identificados por conteo y PCR punto final

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ABSTRACT

Some microbial cultures, mainly the use of probiotics, have been used in ruminant nutrition, generating a positive effect by solving imbalances due to dietary changes in the rumen. The objective was to identify and evaluate bacteria and ruminal protozoa present in sheep supplemented with biopreparation of microorganisms (PNC) vs commercial probiotic REVET® (PCRE) at different concentrations by Neubauer chamber count and PCR endpoint. Twenty-one Katahdin and Dorper cross sheep of 3 months of 18-25 kg were used, they were supplemented with PNC and PCRE, at different concentrations: PNC 100%, 66%, 33%, control, PCRE 100%, 66%, 33%. The ruminal liquid was obtained through a probe, the ruminal microorganisms were counted in a Neubauer chamber every six hours. Genomic DNA extraction using the Ultra Clean Microbial DNA kit, DNA quantification was performed in a spectrophotometer, and PCR reactions were performed with oligonucleotides synthesized by Invitrogen®. Statistical analysis was through the GENMODE procedure for counting bacteria and protozoa. The highest amount of protozoa was at 24 hours in PNC at 100%, followed by PNC 33% at 18 hours, for commercial probiotic PCRE at 66% at 12 hours. Regarding bacteria, they showed statistically equal values. Genomic DNA quantification was greater than 25 ng/µL. The inhibitory effect of the probiotic on *Fibrobacter succinogenes* at a concentration of 100% was demonstrated. Total bacteria were not affected by probiotic supplementation. Therefore, it is concluded that the non-commercial probiotic can be an alternative to supplement the diet of growing sheep, observing an increase in bacteria and protozoa. Likewise, probiotics are an additive that can be used successfully since they did not modify the population of total bacteria in the rumen.

Keywords: sheep, ruminal fermentation, bacteria, protozoan, PCR.

RESUMEN

Algunos cultivos microbianos principalmente el uso de probióticos se ha utilizado en la nutrición de rumiantes, generando un efecto positivo al solucionar los desbalances debido a cambios dietéticos en el rumen. El objetivo fue identificar y evaluar por conteo en cámara de Neubauer y PCR punto final bacterias y protozoarios ruminales presentes en ovinos suplementados con biopreparado de microorganismos (PNC) vs probiótico comercial REVET® (PCRE) a diferentes concentraciones. Se utilizaron 21 ovinos cruza Katahdin y Dorper de 3 meses de 18-25 Kg, se suplementaron con PNC y PCRE, a diferentes concentraciones: PNC 100%, 66%, 33%, testigo, PCRE 100%, 66%, 33%. El líquido ruminal se obtuvo a través de sonda, los microorganismos ruminales se contaron en cámara de Neubauer cada seis horas. La extracción de ADN genómico utilizando el kit Ultra Clean Microbial DNA, la cuantificación de ADN se realizó en un espectrofotómetro y las reacciones de PCR se realizaron con oligonucleótidos sintetizados por Invitrogen®. El análisis estadístico fue mediante el procedimiento GENMODE para el conteo de bacterias y protozoarios. La mayor cantidad de protozoarios fue a las 24 horas en PNC al 100%, seguido del PNC 33% a las 18 horas, para probiótico comercial PCRE al 66% a 12 horas. En cuanto a bacterias mostraron valores estadísticamente iguales. La cuantificación de ADN genómico fue mayor a 25 ng/µL. Se demostró el efecto inhibitorio del probiótico sobre *Fibrobacter succinogenes* en una concentración de 100%. Las bacterias totales no se vieron afectadas con la suplementación del probiótico. Por lo que se concluye que el probiótico no comercial puede ser una alternativa para complementar la dieta de los ovinos en crecimiento observándose un incremento de bacterias y protozoarios. Así mismo, los probióticos son un aditivo que puede ser utilizado con éxito ya que no modificó la población de bacterias totales en el rumen.
Palabras clave: ovinos, fermentación ruminal, bacterias, protozoario, PCR.

INTRODUCTION

Livestock production systems develop dietary formulations using feed additives that have the potential to modify the rumen environment by enhancing or inhibiting specific microbial populations, probiotics have been used to enhance the rumen microbiota, which is responsible for the degradation of cellulose and hemicellulose, which allows ruminants to feed on pasture and forage consumption. In addition, probiotics can be used to modulate rumen fermentation and native microbiota, as they are a source of potentially useful microorganisms (Fraga *et al*., 2014).

Microbial probiotics (*Saccharomyces cerevisiae*, *Lactobacillus plantarum* and *Bacillus subtilis*) used in ruminants improve dry matter intake and productivity. *S. cerevisiae* yeast is important and its inclusion in diets is known to improve utilization of poor quality forages (Shriver-Munsch *et al*., 2011; Khattab *et al*., 2020), increases fiber digestibility and stabilizes rumen pH (Moallem *et al*., 2009; Degirmencioglu *et al*., 2013; Meller *et al*., 2014; Khattab *et al*., 2020).

Lactobacillus plantarum improves nutrient intake, growth performance and ruminal fermentation in lambs (Izuddin *et al*., 2019; Khattab *et al*., 2020). Qiao *et al*. (2010) mentioned that *B. subtilis* in lambs reduced the incidence of diarrhea, increased dry matter intake and daily weight gain before weaning. Probiotics influence the intestinal tract with symbiosis of beneficial bacteria, on host health which may also involve growth stimulation and contribute to higher productivity (Markowiak & Ślizewska, 2018).

The concentration of microbial populations living in the rumen in anaerobiosis, specifically for bacteria, protozoa and fungi are 10^{10} cells/mL, 10^{6} cells/mL and 10^{4} cells/mL

respectively (Jouany, 1994; Cardona-Iglesias *et al*., 2017). To allow slow-growing organisms; such as fungi and ruminal protozoa to reproduce, prolonged residence of the feed inside the rumen for periods of 48 to 72 hours is needed to sustain the concentration of microbial populations (McAllister *et al.,* 1994, Gharechahi *et al*., 2021). Castillo-Lopez & Domínguez-Ordóñez (2019), Castillo-Lopez *et al*. (2014) and Petri *et al*. (2012) report that by using high-throughput DNA sequencing they have revealed the presence of 13 major bacterial phyla in the rumen, which include 40 bacterial orders, about 80 bacterial classes, and at least 180 bacterial families, about 320 bacterial genera and more than 2,000 operational bacterial taxonomic units. Bacterial density in the rumen is in the range of 107 to 1010 cells/mL of rumen fluid (Castillo-Lopez *et al*., 2014; Danielsson *et al*., 2017). The most abundant ruminal bacterial genus is *Prevotella*, which accounts for 20% of the bacterial community (Castillo-Lopez & Domínguez-Ordóñez, 2019).

Although the number of protozoan genera is smaller than that of bacteria, protozoa are physically larger than bacteria and may constitute approximately half of the total ruminal microbial biomass. Of the protozoa, more than 20 species have been identified whose concentration in the rumen is approximately 10^6 cells/mL of rumen fluid (Martin, 1994; Bodas *et al*., 2012; Castillo-Lopez & Domínguez-Ordóñez, 2019).

Fibrobacter succinogenes is a dominant species in the gastrointestinal system of herbivorous animals; it is characterized as a Gram-negative, anaerobic bacterium with the ability to degrade fiber and in greater quantity (Kobayashi, 2006; Jun *et al.*, 2007). This bacterium, which is also one of only two cultivated species, is an efficient cellulose degrader. Specifically, it has a particularly high activity against crystalline cellulose that requires close physical contact with this substrate (Suen *et al.*, 2011). Therefore, the objective was to identify and evaluate by counting and endpoint PCR ruminal bacteria and protozoa present in sheep supplemented with microorganism biopreparation (PNC) vs. commercial probiotic REVET® (PCRE) at different concentrations.

MATERIAL AND METHODS

Experimental design for animals

The experiment was conducted in El Remolino community, Juchipila municipality, Zacatecas, Mexico, located between 103˚07'26.15" N and 21˚21'48.10" W, at 1220 m a.s.l. The study was conducted in the dry season (dry season with average high temperatures of 38-40°C) starting at the end of March and ending with the first rains in June. It included 21 growing hair lambs, Katahdin X Dorper cross, healthy, aged 59 ± 5 days, with average weights of 14.3 ± 1. 7 kg, housed in homogeneous conditions in individual wire mesh pens, canoe feeders, 20 liter bucket as water trough for each pen, supplementation with the different doses of the non-commercial probiotic biopreparation and commercial probiotic REVET® was added in water, which was *ad libitum* (El-Sayed & Mousa, 2020). Growing sheep were allocated into two groups and distributed as shown

in Table 1. A diet based on ground corn stubble 70%, ground alfalfa 15%, corn grain 5%, molasses 8%, bicarbonate 1.5%, vitamin and mineral premix 0.1%, common salt 0.4% was fed. The experimental units were subjected to a 17-day adaptation period prior to the dry season, from March to June.

Table 1. Treatments used in drinking water

Treatments	% Dose	Quantity of animals
T1 PNC	100% of the recommended dosage in 6 L of water + diet	3
T2 PNC	66% of the recommended dosage in 6 L of water + diet	3
T3 PNC	33% of the recommended dosage in 6 L of water + diet	3
T4 control	0% of the recommended dosage in 6 L of water + diet	3
T5 PCRE	100 % of the recommended dosage in 6 L of water + diet	3
T6 PCRE	66 % of the recommended dosage in 6 L of water + diet	3
T7 PCRE	33% of the recommended dosage in 6 L of water + diet	3

PNC: biopreparation of microorganisms. PCRE: commercial probiotic REVET®

Preparation of the solid biopreparation of microorganisms (PNC)

For the preparation of the solid PNC, 40 kg of wheat bran, 20 kg of forest probiotic (decomposing organic matter) containing the efficient solid microorganisms (biopreparation), 0.5 kg molasses solution in five liters of water were mixed in a plastic barrel with a capacity of 100 liters. The container was opened every two to three days to release gases generated during the 30 days it took for the PNC to be ready and as reported by Kyan *et al*. (1999).

Preparation of liquid PNC

To prepare the liquid PNC, 8 kg of solid PNC were taken, were wrapped in a blanket and placed in 100 L of water with 5 kg of molasses, and left to stand for two hours (Kyan *et al*., 1999), after which time it was ready to be used. Subsequently, the content of microorganisms was characterized by using selective media sown on a plate. The administration NCP contained: mesophiles $1.94x10^7$ CFU/100mL, *Lactobacillus* sp. $1.6x10^6$ CFU/100mL, *Staphylococcus aureus* $4.2x10^4$ CFU/100mL, *Candida* sp. $5.5x10^3$ CFU/100mL, *E. coli* $1.18x10^6$ CFU/100mL, fungi $4.0x10^5$ CFU/100mL and yeasts $4.27x10^7$. Table 1 shows the PNC supply in the different treatments.

Preparation of the liquid commercial probiotic REVET® (PCRE)

The probiotic for administration contributes to the balance of intestinal microorganisms in sheep, prevents ruminal dysfunction, increases microbial synthesis, and maintains the balance and optimal conditions of the rumen flora. The doses recommended by the manufacturer are 3 g per day in sheep, which were diluted in 6 liters of water (100%), 1.32 g diluted in 6 liters of water (66%) and 0.99 g diluted in 6 liters of water (33%). According to the supplier's specifications, it contains *Lactobacillus acidophilus* $7.3x10^{18}$ CFU/100mL,

Streptococcus faecium $1.1x10^6$ CFU/100mL and *Saccharomyces cerevisiae* $3.6x10^{11}$ CFU/100mL. The probiotic was offered in the 6 liters of water every day at 08:00 hours.

Obtaining the rumen liquid

The ruminal fluid was obtained by means of a 0.5" and 1m long ruminal probe attached to a syringe. The experiment lasted 110 days; of which the sampling times were m1 (time 0), m2 (30 days), m3 (60 days) and m4 (110 days); m3 sampling was performed every 6 hours at eight time intervals (0, 6, 12, 12, 18, 24, 30, 36 and 48 hours). Approximately 30 mL of ruminal fluid was collected.

Obtaining and quantification of bacteria and protozoa

The rumen liquid obtained was mixed and filtered to obtain a homogeneous sample and filtered. With a pipette, 1 mL of ruminal liquid was extracted, to which 9 mL of saline solution with 10% formalin were added, then the samples were refrigerated for later analysis, 2 mL of this last solution were obtained and 8 mL of distilled water were added, which was centrifuged at 2000 xg for 20 minutes, from the supernatant a sample was taken for counting in the Neubauer chamber with the support of a Carl Zeiss microscope at 40X.

PCR endpoint

Total bacteria, *Fibrobacter succinogenes* and ruminal anaerobic fungi were identified by endpoint PCR; the method consisted of obtaining ruminal liquid through a 0.5" and 1m long probe attached to a syringe; this aliquot was kept at -20°C for later processing in the laboratory. DNA extraction was performed with the Ultra Clean Microbial DNA Isolation kit from MO BIO Laboratories Inc and its quantification was carried out in a spectrophotometer (NanoDrop ND-1000 LabTech), its purity was determined considering absorbance ratios of 260/280 and 260/230 nm (Green *et al*., 2012; Daza *et al*., 2014).
Dilutions of the DNA extracted from each sample were performed in order to place 50 ng/µL equimolar concentrations. The sequence of the oligonucleotides used is shown in Table 2 in addition to the molecular weight of each fragment.

Table 2. Sequence of oligonucleotides

Destination species	Oligonucleotide sequence	pb
Total bacteria	f- CGGCAACGAGCGCGAACCC	130
	r- CCATTGTAGCACGTGTGTAGCC	
Fibrobacter succinogenes	f- GTTCGGAATTACTGGGCGTAAA	121
	r- CGCCTGCCCCTGAACTATC	
Anaerobic fungi	f- GAGGAAGTAAAAGTCGTAACAAGGTTTC	120
	r- CAAATTCACAAAGGGTAGGATGATT	

(Denman & McSweeney, 2006)

PCR mix had a final volume of 25 µL, which contained: reaction buffer 1X (Tris-HCl 20 mM pH 8.4, KCL 50 mM (Invitrogen®)), MgCl2 1. 2 mM (Invitrogen®)), $MgCl_2$ 1.25mM (Invitrogen®), dNTPs 0.25 µM (Invitrogen®), each primer 0.5 µM of, Platinum Taq DNA Polymerase 1U (Invitrogen®), and ampoule water. To avoid contamination, the reaction mixture was carried out in a laminar flow hood (FH1200). The amplified products were subjected to electrophoresis in a horizontal chamber (Thermo® EC 330) in 0.8% agarose gels in TAE 1X with ethidium bromide, using Wide Range DNA Marker from 250 to 10,000 bp (Sigma-Aldrich®) as a reference marker.

Statistical analysis

An analysis of orthogonal contrasts and the chi-square test was performed in the SAS program (SAS, 2011), and the GENMOD procedure of SAS was used for counting protozoa and bacteria (Castañeda *et al*., 2021).

RESULTS AND DISCUSSION

The bacteria, protozoa and fungi that make up the rumen ecosystem differ in their nutrient requirements and metabolism (Bach *et al*., 2005; Rodríguez *et al*., 2007; Matthews *et al*., 2018). Anaerobic rumen bacteria, protozoa and fungi degrade fibrous material, which allows ruminants to utilize plant fiber for nutrition (Rodríguez *et al*., 2007). Bacteria are the most numerous microorganisms and, like the previous ones, play an important role in the biological degradation of dietary fiber, Rodríguez *et al*. (2012) state that there are many bacteria and yeasts that can be used in a beneficial way to maintain a healthy and balanced digestive flora.

Table 3 shows the results of the protozoa count carried out every six hours, where it is observed that the 100% PCRE treatment presents the lowest values at time zero ($7.5x10^3$ cells/mL), when in general the average is 10^5 cells/mL.

Table 3. Quantification of protozoa (cells/mL) treatment Probiotic Non-Commercial (PNC) and Probiotic Commercial REVET® (PCRE) at different time (hours)

HOURS	0	6	12	18	24	30	36	48
CONTROL	$1.5x10^5$	$8.7x10^4$	$2.5x10^4$	$1.2x10^5$	$1.5x10^5$	$7.2x10^4$	$5.5x10^4$	$2.5x10^4$
PNC 100%	$1.0x10^5$	$1.1x10^5$	$5.2x10^4$	$1.0x10^5$	$1.5x10^5$	$7.5x10^4$	$7.2x10^4$	$8.2x10^4$
PNC 66%	$1.0x10^4$	$6.5x10^4$	$1.3x10^5$	$1.2x10^5$	$8.7x10^4$	$3.0x10^4$	$5.7x10^4$	$4.0x10^4$
PNC 33%	$3.5x10^4$	$9.5x10^4$	$3.7x10^4$	$1.5x10^5$	$4.7x10^4$	$2.5x10^4$	$1.7x10^4$	$1.1x10^5$
CONTROL	$1.5x10^5$	$8.7x10^4$	$2.5x10^4$	$1.2x10^5$	$1.5x10^5$	$7.2x10^4$	$5.5x10^4$	$2.5x10^4$
PCRE 100%	$7.5x10^3$	$1.7x10^4$	$4.7x10^4$	$2.2x10^4$	$3.5x10^4$	$1.1x10^5$	$2.7x10^4$	$1.0x10^4$
PCRE 66%	$1.2x10^5$	$2.4x10^5$	$2.4x10^5$	$9.7x10^4$	$1.1x10^5$	$6.0x10^4$	$6.5x10^4$	$6.0x10^4$
PCRE 33%	$1.7x10^5$	$4.2x10^4$	$1.9x10^5$	$3.2x10^4$	$3.5x10^4$	$7.5x10^3$	$9.7x10^4$	$2.1x10^5$

PNC: biopreparation of microorganisms. PCRE: commercial probiotic REVET®

Table 4 and 5 present the results of the statistical analysis showing that the comparison between treatments is significant (P<0.05), but not between counting times (P>0.05).

Table 4. Comparison of protozoan counts between PNC and PCRE treatments

Treatment	Average estimate	Mean Confidence limit		Estimate β	Standard error	Chi Square	P> Chi Square
PNC100%-PCRE100%	0.9618	0.7958	0.9939	3.2258	0.9518	11.49	0.0007
PNC100%-PCRE66%	0.3547	0.1004	0.7303	-0.5985	0.8136	0.54	0.462
PNC100%-PCRE33%	0.6563	0.2025	0.9349	0.6468	1.0294	0.39	0.5298
PNC66%-PCRE100%	0.9012	0.6083	0.9817	2.2106	0.9033	5.99	0.0144
PNC66%-PCRE66%	0.1661	0.0368	0.5096	-1.6137	0.843	3.66	0.0556
PNC66%-PCRE33%	0.4089	0.0853	0.8369	-0.3684	1.0223	0.13	0.7185
PNC33%-PCRE100%	0.8515	0.4865	0.972	1.7468	0.9188	3.61	0.0573
PNC33%-PCRE66%	0.1113	0.0207	0.4259	-2.0775	0.9075	5.24	0.0221
PNC33%-PCRE33%	0.3032	0.0513	0.7779	-0.8322	1.0641	0.61	0.4342
PNC100%-CONTROL	0.5822	0.2133	0.8775	0.3319	0.8352	0.16	0.6911
PNC66%- CONTROL	0.3355	0.0871	0.7277	-0.6834	0.8501	0.65	0.4215
PNC33%- CONTROL	0.241	0.0501	0.6567	-1.1472	0.9163	1.57	0.2106
PCRE100%-CONTROL	0.0525	0.008	0.2758	-2.894	0.984	8.65	0.0033
PCRE66%- CONTROL	0.7171	0.313	0.9338	0.9303	0.8757	1.13	0.2881
PCRE33%- CONTROL	0.4219	0.0832	0.8544	-0.3149	1.0637	0.09	0.7672

PNC: biopreparation of microorganisms. PCRE: commercial probiotic REVET®

Between-treatment test contrasts show that the PNC treatment at 100% is different (P<0.05) from its counterpart PCRE at 100%, PNC at 66% is different (P<0.05) from PCRE at 100% and 66% (P=0.055) and PNC at 33% is different (P>0.057) from PCRE at 100% and 66% (P<0.05), as well as PCRE 100% was different (P>0.05) from the control.

Table 5. Analysis of variance

Source	Degrees of freedom	Chi Square	P> Chi Square
Treatment	6	18.43	0.0052
Time	7	7.17	0.4118

The chi-square test for the number of protozoa in the PNC vs. PCRE treatments at 66% of the recommended dose. It did not show a higher number of protozoa in the PNC, although a more favorable growth was observed with respect to the PCRE at 66% with a P<0.0556. In the PNC vs. PCRE treatments at 33% concentration, no statistically significant difference was observed between the counts of protozoa in the 33% PNC and PCRE treatments (P<0.05).

Bacterial counts were performed every 6 hours in eight time intervals obtaining average concentrations of 106 cells/mL in most of the times and treatments, being the 66% PNC treatment the one that presented the highest concentration of bacteria ($1.49x10^7$ cells/mL), while in the 30-hour time the lowest concentration was identified in the different treatments (Table 6).

Table 6. Quantification of bacteria in the PNC and PCRE treatments (cells/mL) at different times (hours)

HOURS	0	6	12	18	24	30	36	48
Control	$3.1x10^6$	$1.6x10^6$	$1.0x10^6$	$2.75x10^6$	$9.5x10^5$	$2.8x10^6$	$1.6x10^6$	$1.65x10^6$
PNC 100%	$2.7x10^6$	$2.5x10^6$	$1.4x10^6$	$2.5x10^6$	$2.6x10^6$	$8.0x10^5$	$1.7x10^6$	$8.5x10^5$
PNC 66%	$2.55x10^6$	$1.25x10^6$	$1.49x10^7$	$2.8x10^6$	$8.75x10^6$	$5.0x10^5$	$1.25x10^6$	$2.5x10^6$
PNC 33%	$3.5x10^6$	$8.45x10^6$	$1.25x10^6$	$6.05x10^6$	$1.6x10^6$	$1.5x10^5$	$2.9\ x10^6$	$4.45x10^6$
Control	$3.1x10^6$	$1.6x10^6$	$1.0x10^6$	$2.75x10^6$	$9.5x10^5$	$2.8x10^6$	$1.6x10^6$	$1.65x10^6$
PCRE 100%	$7.5x10^5$	$1.35x10^6$	$1.35x10^6$	$3.65x10^6$	$2.5x10^6$	$3.7x10^6$	$3.35x10^6$	$1.05x10^6$
PCRE 66%	$3.2x10^6$	$5.3x10^6$	$1.45x10^6$	$2.8x10^6$	$1.4x10^6$	$5.6x10^6$	$3.9x10^6$	$5.15x10^6$
PCRE 33%	$4.05x10^6$	$2.15x10^6$	$2.8x10^6$	$5.9x10^6$	$7.0x10^6$	$4.5x10^5$	$1.7x10^6$	$1.85x10^6$

PNC: biopreparation of microorganisms. PCRE: commercial probiotic REVET®.

In work by Uchida *et al*. (1987) reported that a single protozoan can take up to 104 bacteria per hour. These estimates indicate that protozoan predation can renew the entire bacterial biomass in the rumen at high protozoan density (10^5 to 10^6). Sheep supplemented with PNC and PCRE benefit in protozoan and bacterial populations.

Table 7. Comparison of bacterial counts between PNC and PCRE treatments

Treatment	Average estimate	Mean Confidence limit		Estimate β	Standard error	Chi Square	P> Chi Square
PNC100%-PCRE100%	0.4364	0.1282	0.803	-0.2557	0.8475	0.09	0.7629
PNC100%-PCRE66%	0.1458	0.0299	0.4859	-1.7679	0.8732	4.1	0.0429
PNC100%-PCRE33%	0.2255	0.0538	0.5987	-1.2339	0.8338	2.19	0.1389
PNC66%-PCRE100%	0.5539	0.1625	0.8882	0.2164	0.947	0.05	0.8192
PNC66%-PCRE66%	0.2149	0.0401	0.6421	-1.2958	0.9594	1.82	0.1768
PNC66%-PCRE33%	0.3183	0.0712	0.7398	-0.7618	0.9218	0.68	0.4086
PNC33%-PCRE100%	0.7429	0.3163	0.9475	1.0611	0.9348	1.29	0.2563
PNC33%-PCRE66%	0.3891	0.0952	0.794	-0.4511	0.9186	0.24	0.6234
PNC33%-PCRE33%	0.5207	0.1567	0.864	0.0829	0.901	0.01	0.9267
PNC100%-CONTROL	0.4713	0.1579	0.8091	-0.1148	0.7955	0.02	0.8852
PNC66%- CONTROL	0.5884	0.2011	0.8903	0.3573	0.886	0.16	0.6867
PNC33%- CONTROL	0.7689	0.3639	0.9509	1.202	0.8983	1.79	0.1809
PCRE100%- CONTROL	0.5352	0.1744	0.8625	0.1409	0.8652	0.03	0.8707
PCRE66%- CONTROL	0.8393	0.4782	0.9675	1.6531	0.8879	3.47	0.0626
PCRE33%- CONTROL	0.7538	0.366	0.942	1.1191	0.8513	1.73	0.1886

PNC: biopreparation of microorganisms. PCRE: commercial probiotic REVET®

Table 7 and 8 shows the chi-square test in which it is observed that there is no statistically significant difference between the bacterial counts with the different doses of PNC vs PCRE studied (P<0.05). The only comparison showed differences between PNC 100% vs PCRE 66%, being higher in PCRE 66%.

Table 8. Analysis of variance

Source	Freedom degrees	Chi Square	P> Chi Square
Treatment	6	7.01	0.399
Time	7	7.17	0.2574

Sheep supplemented with PNC and PCRE are benefited in the populations of protozoa and bacteria. According to Williams & Coleman (2012) the multiplication times for protozoa vary from 5-14 hours, coinciding with the times of concentrations in the PCRE the maximum populations were presented at 12 hours at 66% and at 18 hours with 33%, Jouany (1994) and Williams & Coleman (2012) found that several species of ruminal protozoa have alpha amylase and one of those with the highest amylolytic activity of this type is *Entodinium caudatum*, likewise, Mould & Thomas (1958) and Arcos-García *et al*., (2007), found alpha and beta amylase in holotrophic protozoa that promote the splitting of the reserve and structural sugar units of plants.

In the nutritional part, probiotics and enterobacteria compete for essential amino acids and sugars, reduce the production of toxic amines, this is remedied with acidophilic *Lactobacillus* important to the health status of the animal (Castillo-Lopez *et al*., 2013). The bacterial density in the rumen 107 and 1010 rumen fluid cells, the most abundant bacteroid phyla include 75% of the total bacterial population (Castillo-Lopez & Domínguez-Ordóñez, 2019).

Non-commercial probiotics (NCPs) can be an alternative to supplement the diet of growing sheep, it is necessary to indicate that NCPs in sheep could be used to optimize production in the integral diet of animals, without causing negative impact on the ecology which can be achieved through the direct supply of NCPs and PCRE.

Regarding the DNA concentrations recorded for the control treatment, an average concentration of 58.36 ng/µL was obtained, with a minimum value of 28 ng/µL and a value of 100 ng/µL. Table 9 shows each of the concentrations obtained, as well as the 260/280 ratio, which indicates that the DNA purity is adequate for PCR protocols.

In sheep supplemented with CNP, genomic DNA was obtained at the following average concentrations 77.6 ng/µL, 69.7 ng/µL and 70.18 ng/µL, in the 100%, 66% and 33% treatments, respectively (Table 9). The concentrations of DNA obtained from rumen fluid microorganisms of sheep supplemented with PCRE averaged 46.81 ng/mL in 100%, 46.09 ng/mL in 66% and 36.36 ng/mL in 33%.

Table 9. DNA concentrations of samples in different treatments

		Control	PNC 100%	PNC 66%	PNC 33%	PCRE 100%	PCRE 66%	PCRE 33%
Sampling				**Concentration (ng/mL)**				
M1 (0 Days)		48	----	-----	72	103	65	52
M2 (30 Days)		65	94	84	64	60	72	--------
	6hr	57	136	41	34	36	37	28
	12hr	28	45	95	127	59	30	30
	18hr	100	26	78	199	37	36	29
	24hr	88	52	109	34	28	61	42
	30hr	58	234	48	23	25	53	------
M3 (60 Days)	36hr	70	46	49	42	48	31	85
	42hr	31	38	74	37	55	48	55
	48hr	55	44	50	71	25	29	45
M4 (110 Days)		42	61	69	69	39	45	34

PNC: biopreparation of microorganisms. PCRE: commercial probiotic REVET®

The diversity of total bacteria in the rumen is important during feed degradation and fermentation, since this allows having a wide variety of enzymes, as well as precise biochemical reactions that help hydrolyze the plant material to simpler structures and can have greater availability for the microorganisms and increase the fermentation products (López *et al*., 2020).

With respect to total bacteria, the PCR amplification band corresponds to 130 bp, being able to identify this characteristic band in the control gel, which corresponds to Figure 1 (A). According to these results, it can be mentioned that the probiotic with the best adaptation effect on total bacteria was the PNC, since it allowed the bacterial microbiota to continue growing in the rumen and, therefore, did not affect feed degradation and ruminal fermentation.

Rumen bacteria are involved in cellulose degradation, this species presents more than 100 sequences coding for enzymes that degrade polysaccharides (Morrison *et al*., 2003; Jun *et al*., 2007; Firkins, 2021), most of them are responsible for the degradation of cellulosic substrates, in addition these bacteria degrade xylan, hemicellulose and the monosaccharides of plant walls (Mirón, 1991; Hobson & Stewart, 2012).

It was demonstrated that PCRE has an inhibitory effect on *Fibrobacter succinogenes* at a concentration of 100%, since the specific band for the oligonucleotide (121 bp) is not observed compared to the control as seen in Figure 1 (H), however, for the concentrations of 66 and 33% this band is present (Figure 1, J, K).

Ruminal anaerobic fungi play a strategic role in the digestion of fibrous feeds, since they present a great ability to colonize lignified cellulose walls and to weaken fibrous plant tissues, as well as the degradation of the structural components of their cell wall (Galindo *et al.,* 2017). For anaerobic fungi (120 bp), these were not affected with probiotic supplementation at any of their concentrations (Figure 1, P, Q, R, S, T, U).

The effect of probiotics seems to be related to the mechanisms and metabolic processes carried out by the microorganisms involved in ruminal fermentation and methane formation, Rodríguez *et al*. (2013) state that there are many bacteria and yeasts that can be used in a beneficial way to maintain a healthy and balanced digestive flora. The most commonly used microorganisms are *Lactobacillus* sp., *Sreptococcus faeccium*, *B*. *subtilis*, *B*. *cereus*, *B*. *licheniformis*, *B*. *stearothermophyllus* and *S*. *cerevisiae*.

Lactobacillus grow rapidly in the intestine and are perhaps the best-known bacteria that can transform lactose into lactic acid. This increase in lactic acid decreases the intestinal pH, which affects the survival of microorganisms that are not beneficial for the ruminal flora, pathogens, among others.

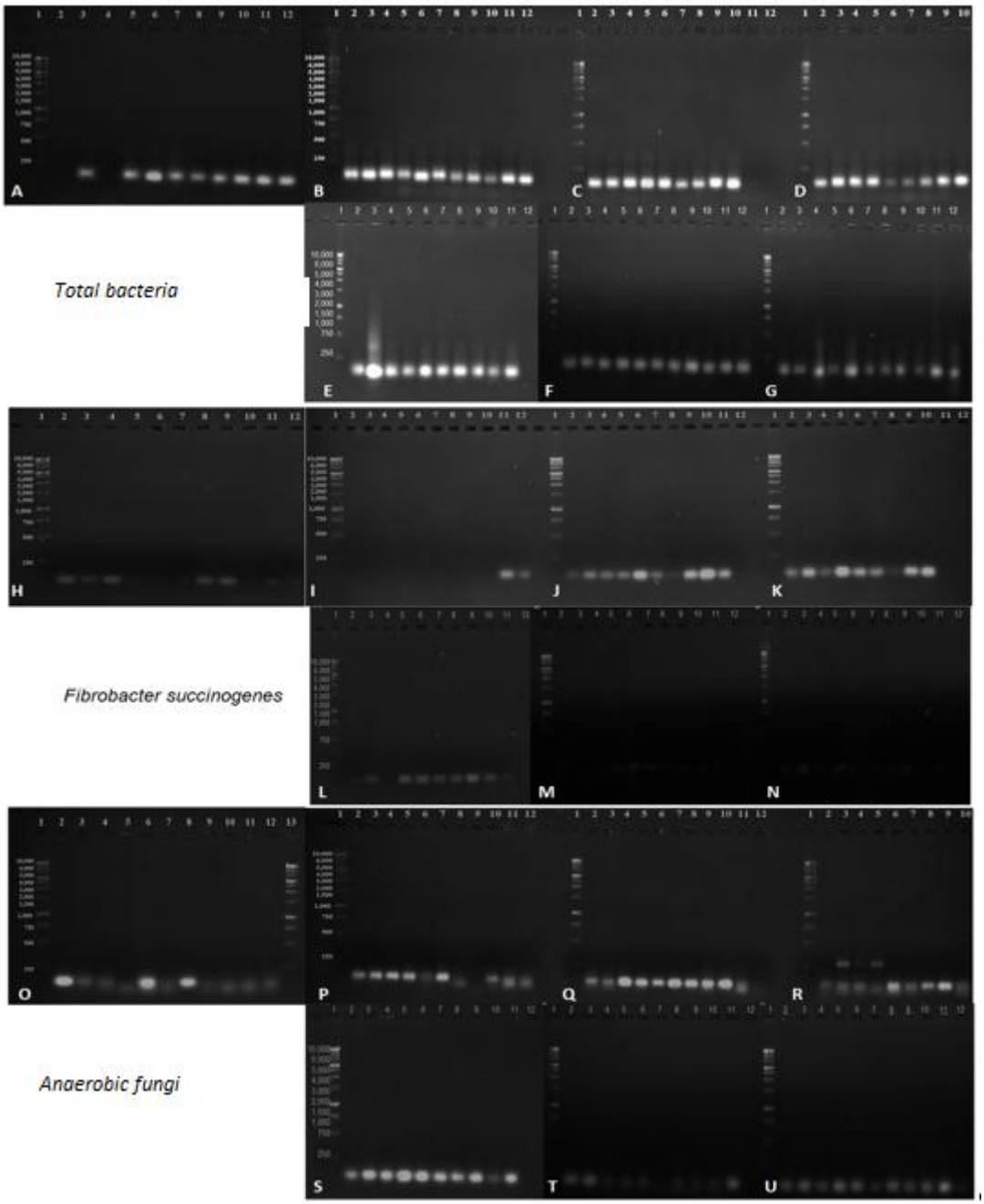

Figure 1. Band amplification by PCR. Lane 1, Molecular weight marker (250 to 10,000 bp); MP range Lane 2, M1 (0 days); Lane 3, M2 (30 days); Lane 4 to 11, M3 (60 days -6, 12, 18, 24, 30, 36, 42, 48 h-); Lane 12, M4 (110 days). Total bacteria (A, B, C, D, E, F, G), *Fibrobacter succinogenes* (H, I, J, K, L, M, N) and anaerobic fungi (O, P, Q, R, S, T, U) in each of the treatments (Control, 100%, 66% and 33%) with commercial probiotic (PCRE) and non-commercial probiotic (PNC)

CONCLUSIONS

The non-commercial probiotic can be an alternative to supplement the diet of growing sheep in the canyon region of Juchipila, Zacatecas, Mexico, by generating an increase in bacteria and protozoa. Probiotics are an additive that can be used successfully since it did not modify the population of total bacteria and anaerobic fungi in the rumen.

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Errata Erratum

https://abanicoacademico.mx/revistasabanico-version-nueva/index.php/abanico-veterinario/errata