

# Preliminary study of the effects of *Apilactobacillus kunkeei* EIR/BG-1 and *Enterococcus hirae* EIR/CM-2 supplementation on *in vitro* rumen fermentation and microbial population

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**ABSTRACT.** The aim of the study was to assess the impact of potential probiotic strains of lactic acid bacteria (LAB), *Apilactobacillus kunkeei* EIR/BG-1 isolated from the gut of honeybees, and *Enterococcus hirae* EIR/CM-2 isolated from cow's milk, on rumen fermentation parameters and microbial population in a high-concentrate diet using a rumen simulation technique (Rusitec). The experiment consisted of 7 days of adaptation and 7 days of data collection. The dietary treatments were as follows: no additives (control), addition of 1 ml/fermenter ( $10^8$  CFU) of *Al. kunkeei* EIR/BG-1, and addition of 1 ml/fermenter ( $10^8$  CFU) of *E. hirae* EIR/CM-2. Alongside rumen fermentation characteristics, rumen microbial composition was investigated using real-time PCR. Supplementation with LAB strains did not affect ruminal pH, production of methane, and total and individual short chain fatty acids, ammonia-N concentration, dry matter digestibility and total protozoa. However, the abundance of *Ruminococcus flavefaciens* increased in the *Al. kunkeei* EIR/BG-1 treatment ( $P < 0.05$ ). Moreover, the size of *R. flavefaciens* population in *E. hirae* EIR/CM-2 was comparable to that in the *Al. kunkeei* EIR/BG-1 treatment. *Selenomonas ruminantium* was more abundant in the *Al. kunkeei* EIR/BG-1 treatment compared to the *E. hirae* EIR/CM-2 treatment ( $P < 0.05$ ). The abundance of *Streptococcus bovis* and *Megasphaera elsdenii* decreased with both *Al. kunkeei* EIR/BG-1 and *E. hirae* EIR/CM-2 supplementations ( $P < 0.05$ ). In conclusion, *Al. kunkeei* EIR/BG-1 and *E. hirae* EIR/CM-2 exhibited beneficial effects on some members of the rumen microbial population, although these effects did not manifest in significant alterations in ruminal fermentation. Further research is required to clarify the probiotic potentials of these LAB strains as feed additives for ruminant rations.

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## Introduction

Antibiotic feed additives have been used for more than forty years to enhance ruminant performance by modulating rumen microbiota and fer-

mentation processes. However, concerns regarding antibiotic residues in milk and meat, as well as the emergence of resistant strains of pathogenic bacteria led to a ban on the use of antibiotics as growth promoters in the European Union in 2006

(Vieco-Saiz et al., 2019). Since that time, extensive research efforts have been dedicated to finding safer alternatives to antibiotics as feed additives. One promising alternative is the use of probiotics in ruminant diets (Kulkarni et al., 2022).

The term ‘probiotic’ means ‘for life’ and has the opposite meaning to the word ‘antibiotic’. Probiotics have been defined as live microbial feed supplements that exert beneficial effects on the host animal by improving its microbial balance (Fuller, 1989). Probiotics are also referred to as direct feed microorganisms (DFM), which mainly include bacterial species belonging to the genera *Lactobacillus*, *Enterococcus*, *Bifidobacterium*, *Streptococcus*, *Propionibacterium* and *Bacillus*, and fungal species such as *Saccharomyces* and *Aspergillus* (Kulkarni et al., 2022). *Lactobacillus* and *Enterococcus* are lactic acid-producing bacteria and are most commonly applied as probiotics among the bacterial strains (Chen et al., 2017). Lactic acid bacteria (LAB) are generally considered safe for human and animal consumption. They have obtained Qualified Presumption of Safety (QPS) status in the European Union and Generally Recognized as Safe (GRAS) status in the United States. This is because LAB have long been used as starter cultures in the fermentation process of various dairy, meat, and plant products (Bintsis, 2018).

The potential impact of bacterial probiotics on human health as modifiers of the intestinal microbiota has already been extensively studied. In recent years, bacterial probiotics have also been recommended as alternatives to antibiotic growth promoters to improve animal production (Vieco-Saiz et al., 2019). However, the efficacy of bacterial probiotics has been primarily studied in pre-ruminants, with reported benefits including reduced coliform infections and diarrhoea incidence, lower morbidity rates, promotion of rumen development, improved feed efficiency and increased body weight gain (Krehbiel et al., 2003). For instance, administration of *Lactobacillus rhamnosus* GG in preweaning Holstein calves increased growth performance, improved rumen fermentation, diversified rumen microbial community composition and regulated rumen and gut microbial balance (Zhang et al., 2019). In adult ruminants, the use of bacterial probiotics also generally aims to improve the performance and health of animals (Doyle et al., 2019). *Lactobacillus* has been widely employed as a feed supplement in the dairy industry to promote gut health, increase milk production (Chen et al., 2017) and reduce the risk of mastitis (Beecher et al., 2009).

The addition of *Lactobacillus casei* Zhang and *Lactobacillus plantarum* P-8 to the diets of dairy cows increased milk yield by 37% and reduced the number of opportunistic pathogens in the faecal microbiota (Xu et al., 2017). A similar favourable effect was observed in beef cattle, where the addition of *Lactobacillus acidophilus* NP51 to the diet of beef feedlot cattle reduced the likelihood of faecal shedding of *Escherichia coli* O157:H7 (Peterson et al., 2007). Dietary supplementation of *Enterococcus* is also widely practiced in chickens, sows, finishing pigs, piglets, fattening cattle and calves in Europe (Becquet, 2003). Furthermore, a newly developed strain of *Enterococcus faecium* isolated from fresh dairy products was demonstrated to improve feed efficiency and production performance in lactating Holstein cows (Azzaz et al., 2022). These probiotics have also been applied as bacterial inoculants for ensiling ruminant feeds (Guo et al., 2020). However, there is limited literature available regarding the effects of LAB supplementation on rumen fermentation and microbial population.

*Apilactobacillus*, which were formerly classified as *Lactobacillus*, have recently been reclassified to underline specific adaptation to bees. Indeed, *Apilactobacillus kunkeei* (basonym *Lactobacillus kunkeei*) is an important component of the gut microbiota of honeybees and is considered as probiotic for these insects due to the bacteriocin-like substances produced in the honeybee gut, among other properties (Simsek et al., 2022). *Enterococcus hirae*, on the other hand, is a LAB species isolated from dairy products, and has been reported to exhibit probiotic potential (Melo et al., 2021). To our knowledge, there is no literature on the effects of these LAB species on ruminal fermentation and microbial population. Therefore, we aimed to evaluate the impact of two potential probiotic LAB isolates, *Apilactobacillus kunkeei* EIR/BG-1 and *Enterococcus hirae* EIR/CM-2, on rumen fermentation parameters and microbial population when added to a high-concentrate diet using a rumen simulation technique (Rusitec).

## Material and methods

### Bacterial strains and growth conditions

*Apilactobacillus kunkeei* EIR/BG-1, isolated from the gut microbiota of honeybees (Kiran et al., 2023), and *Enterococcus hirae* EIR/CM-2, isolated from the microbiota of cow milk (Sevin et al., 2021), were kindly provided by the Pharmabiotic

Technologies Research Laboratory at Ankara University (Turkey). Briefly, homogenised gut samples of honeybees in 1 ml phosphate buffer saline (PBS) and 1 ml cow milk samples were serially diluted 10-fold, and each dilution was pour-plated on De Man Rogosa and Sharpe agar (MRS; Merck, Darmstadt, Germany) plates. Following the incubation at 37 °C for 48 h, bacterial colonies were randomly selected, and pure cultures were transferred to MRS broth supplemented with 50% glycerol at -80 °C for long-term storage (Arredondo et al., 2018). Species identification from colony isolates was carried out by sequencing the 16S ribosomal-RNA (16S rRNA) subunit gene using the protocol of Kiran et al. (2023). Based on the sequencing results of the 16S rRNA gene region (approximately 1492 bp) and subsequent Basic Local Alignment Search Tool (BLAST) searches against the GenBank Bacteria and Archaea 16S rRNA sequences database, the isolates were found to closely match *Apilactobacillus kunkeei* and *Enterococcus hirae* (99% similarity), and were registered in the National Center for Biotechnology Information (NCBI).

For rumen applications, the isolates were cultured under static conditions in De Man Rogosa and Sharpe medium (MRS, Merck, Darmstadt, Germany) at 37 °C for 24 h. After 24 h of growth, broth cultures were centrifuged at 15000 g for 20 min, and viable bacteria were counted using the spread plate method to determine the number of colony-forming units (CFU). Live cells were subsequently suspended in physiological saline (0.9% NaCl solution) at a concentration of 10<sup>8</sup> CFU/ml for further analyses.

### Rusitec procedure

The assay was conducted using a Rusitec apparatus with nine 750-ml fermenters (custom-made by the Institute for Physiology and Cell Biology, University of Veterinary Medicine Hannover, Germany), as described by Czerkawski and Breckenridge (1977). Inoculum for fermenters was collected from a non-lactating rumen-fistulated Holstein cow weighing 450 kg, housed at the Ministry of Agriculture and Forestry, International Center for Livestock Research and Training, Turkey, before morning feeding. The donor cow was maintained in accordance with the animal welfare guidelines of this research centre. The local ethics committee of the International Center for Livestock Research and Training approved the previous fistulation of the donor cow under decision number 104/29.12.2014. The inoculum

was transported to the *in vitro* system in insulated flasks at a temperature of 39 °C within 30 min. To initiate the experiment, Rusitec fermenters were inoculated with 750 ml of strained rumen fluid. Two separate 80 × 120 mm nylon bags with a pore size of 150 µm were placed in each fermenter. One of the bags contained 80 g of solid ruminal digesta, while the other contained 10 g of the experimental diet consisting of 2 g of barley straw cut into 1 cm long fragments and 8 g of commercial concentrate feed. After 24 h, the nylon bags containing the rumen solid digesta were substituted with new bags containing the experimental diet, thus the feed bags with the experimental diet were incubated in the fermenter for 48 h. The donor animal was fed a diet (12 kg dry matter/day) containing barley straw and commercial concentrate feed. The commercial concentrate feed was composed of barley, maize, wheat bran, sunflower meal, maize distiller's dried grains with solubles (DDGS), molasses, marble powder, sodium chloride, and a pre-mixed blend of vitamins and minerals. The same feed sources were also used in the Rusitec experiments (Table 1). The fermenters were supplied with a constant flow of buffer solution (pH 7.4) at a rate of 750 ml/day. The chemical constituents of the buffer solution are shown in Table 2.

**Table 1.** Chemical composition of the experimental feed sources

Nutrients, %*	Barley straw	Concentrate
Dry matter (DM)	94.62	91.84
Organic matter	93.16	94.24
Ash	6.84	5.76
Crude fibre	45.67	7.27
Crude protein	5.52	13.65
Ether extract	0.70	0.59
Starch	1.84	51.96
Neutral detergent fibre	69.86	20.36
Acid detergent fibre	43.33	7.84
Acid detergent lignin	5.18	1.85
Non-fibre carbohydrates	17.09	59.64
Metabolizable energy, MJ/kg DM	5.20	11.84

\* dry matter basis

**Table 2.** Chemical composition of buffer solution

Chemicals	mmol/l
NaCl	28.00
KCl	7.69
1N HCl	0.50
CaCl <sub>2</sub> ·2H <sub>2</sub> O	0.22
MgCl <sub>2</sub> ·6H <sub>2</sub> O	0.63
Na <sub>2</sub> HPO <sub>4</sub> ·12H <sub>2</sub> O	10.00
NaH <sub>2</sub> PO <sub>4</sub> ·H <sub>2</sub> O	10.00
NaHCO <sub>3</sub>	97.90

## Experimental design and treatments

The study used a completely randomised design with three treatments, three replicates each ( $n = 3$ ). The trial consisted of a 7-day adaptation phase (day 1 to day 7) to obtain stable conditions, followed by a 7-day experimental phase (day 8 to day 14). Bacterial strains were introduced into their respective fermenters at the beginning of the experimental period. The following treatments were applied; no additives (control), addition of 1 ml/fermenter ( $10^8$  CFU) of *Al. kunkeei* EIR/BG-1, and addition of 1 ml/fermenter ( $10^8$  CFU) of *E. hirae* EIR/CM-2. The concentrations of bacteria were adjusted in 1 ml physiological saline, and an equal volume of physiological saline was added to the control group. The doses of bacterial strains used in the trial were determined based on preliminary screening of the effects of bacterial probiotics on rumen fermentation (Soriano et al., 2014; Chen et al., 2017).

## Sample collection and analyses

The pH of rumen fluids in each fermenter was measured daily at feeding time using an WD-35801-00 epoxy body pH electrode (Oakton, VA, USA) coupled to an Ion 6 pH-meter (Acorn series, Oakton, VA, USA). Samples for ammonia-N and short chain fatty acids (SCFA) analyses were taken daily during the experimental period from liquid effluents collected to overflow flasks. To preserve the fermentation products and prevent microbial activity, the overflow flasks were kept on ice. Five millilitres of samples collected for each analysis were stored at  $-20$  °C until measurements. Fluid samples for DNA extraction were collected daily from the fermenters during the feed bag exchange, thus they contained both planktonic and solid-phase-associated microorganisms (Demirtas et al., 2021). The collected samples were immediately transferred to liquid nitrogen and stored at  $-20$  °C until DNA extraction.

Ammonia-N concentration was determined with a UV-150-02 spectrophotometer at 546 nm (Shimadzu, Kyoto, Japan) using the indophenol blue method (Chaney and Marbach, 1962). Samples collected for SCFA analysis were centrifuged at 15000 rpm for 15 min at 4 °C. The resulting supernatant was acidified with 0.1 ml of 25% metaphosphoric acid, cooled in a refrigerator for 30 min, and then centrifuged at 15000 rpm for 15 min at 4 °C. The supernatant was analysed for SCFA by gas chromatography (ACME-6100, Younglin, Republic of Korea) using an HP Innovax capillary column

(30 m  $\times$  0.25 mm, 0.25  $\mu$ m film thickness; Hewlett Packard, Palo Alto, CA, USA) and flame-ionisation detector (FID). Helium was used as a carrier gas at a flow rate of 1.8 ml/min. Samples were introduced into the column via a split injector (with a split ratio of 1:40). The injection volume for each sample was 1  $\mu$ l. The injector temperature was set to 250 °C, while the detector temperature was set to 300 °C. The oven temperature was maintained at 120 °C for 1 min and then programmed to increase at a rate of 10°/min to 265 °C which was maintained for 2 min. To estimate daily SCFA production, the concentration of each SCFA was multiplied by the volume of the effluent collected daily.

The calculation of methane production was based on the stoichiometry of Wolin (1960) and the equations proposed by Abdl-Rahman (2010), as shown below:

$$\text{fermentative CO}_2 = A/2 + P/4 + 1.5 B$$

$$\text{fermentative CH}_4 = (A + 2 B) - \text{CO}_2,$$

where: A – mole of acetate, P – mole of propionate, B – mole of butyrate.

To count the protozoa, 1 ml of methyl green-formalin-saline solution (8 g NaCl, 0.6 g methyl green, 100 ml formaldehyde (37%) and distilled water to a final volume of 1000 ml) was added to 1 ml of liquid samples taken daily from the fermenters. The resulting mixture was pipetted into a counting chamber (Fuchs-Rosenthal: 0.0625 mm<sup>2</sup>, 0.2 mm deep; Marienfeld, Lauda-Königshofen, Germany). The count of total protozoa was determined under a light microscope (Leica CME, Morrisville, NC, USA).

After 48 h of fermentation, the feed bags were removed from the Rusitec vessels and washed by gently squeezing them in nylon bags containing 50 ml of buffer solution. In order to reintroduce solid-phase associated microorganisms into the system, the residual buffer in the nylon bags was returned to the vessel. Subsequently, the feed bags were dried at 65 °C for 48 h. To calculate the dry matter digestibility (DMD) after 48 h, the difference between the weight of the original dry matter (DM) sample and the weight of the DM residue was divided by the original sample weight. Then, this value was multiplied by 100 to obtain the percentage DMD (Demirtas et al., 2021).

Crude fibre, crude protein (CP), ether extract (EE), DM and ash contents of the nutrients in the experimental diet are shown in Table 1. These analyses were conducted using the procedures outlined by the Association of Official Analytical Chemists

(AOAC International, 2000). The organic matter content was calculated by subtracting the ash content from the DM content. The starch content was determined using the polarimetric method described in the ISO 6493 standard (ISO, 2000). Neutral detergent fibre (NDF), acid detergent lignin (ADL) and acid detergent fibre (ADF) were measured (Van Soest et al., 1991) using an ANKOM<sup>200</sup> Fiber Analyzer (ANKOM Technology Corp., Fairport, NY, USA). The NDF content was determined using sodium sulphite and heat stable amylase. NDF and ADF values included residual ash. Non-fibre carbohydrates (NFC) were calculated using the following equation: %NFC = (100% – CP% – NDF% – EE% – Ash%) (NRC, 1985). Metabolizable energy was calculated according to the methods of TSE (1991).

### DNA extraction and real-time PCR

The effects of LAB supplementation on the abundance of bacterial species representative of the main rumen fermentation pathways were quantified using real-time PCR. The bacterial species tested included *Ruminococcus albus* and *Ruminococcus flavefaciens* as hydrogen, formate and acetate producers, *Butyrivibrio fibrisolvens* as a butyrate producer, *Streptococcus bovis* as a lactate producer, and *Fibrobacter succinogenes*, *Megasphaera elsdenii* and *Selenomonas ruminantium* as succinate and propionate producers (Watanabe et al., 2010;

Demirtas et al., 2019). These bacteria were also selected based on their role in the degradation of fibre (*R. albus*, *R. flavefaciens*, *F. succinogenes* and *B. fibrisolvens*), starch and sugars (*M. elsdenii*, *S. ruminantium* and *S. bovis*) (Ouwerkerk et al., 2002; Wang et al., 2009; El-Nor et al., 2010). Additionally, the abundance of methane-producing (methanogenic) archaea, hyper-ammonia-producing bacteria (HAP bacteria; *Clostridium sticklandii*, *Peptostreptococcus anaerobius* and *Clostridium aminophilum*) and total bacteria was quantified by targeting specific genes. The primers used for real-time PCR are presented in Table 3.

Prior to DNA extraction, frozen fluid samples were thawed and briefly vortexed to mix the samples, and then the samples from days 8 to 14 were pooled for each fermenter. From the pooled sample, 4-ml aliquots were used for DNA extraction. After thawing, the samples were centrifuged at 10000 rpm for 10 min at room temperature, and the resulting pellets were washed twice with phosphate-buffered saline (Demirtas et al., 2021). Total DNA was extracted from the pellets using the E.Z.N.A.<sup>TM</sup> stool DNA isolation kit (Omega Bio-tek, Norcross, GA, USA). DNA purity and concentration were determined spectrophotometrically at 260 and 280 nm using an Epoch microplate reader (Bio-tek, Winooski, VT, USA). Quantification of rumen microorganisms was performed according to the real-time PCR assay (Denman and McSweeney, 2005)

**Table 3.** Primers used for real-time PCR

Target species	Forward primer (5'→3')	Reverse primer (5'→3')	Reference
Total bacteria (16S rRNA)	CGGCAACGAGCGCAACCC	CCATTGTAGCACGTGTGTAGCC	Denman and McSweeney, 2006
Methanogenic archaea ( <i>mcrA</i> )	TTCGGTGGATCDCARAGRGC	GBARGTCGWAWCCGTAGAATCC	Denman et al., 2007
HAP bacteria (16S rDNA)	GAGTTTGATCCTGGCTCAG	AAGGAGGTGATCCAGCC	Attwood et al., 1998
<i>Ruminococcus albus</i> (16S rDNA)	CAAAACCCTAAAAGCAGTCTTAGTTCG	GACGGGCGGTGTGTACAAG	Li et al., 2014
<i>Ruminococcus flavefaciens</i> (16S rRNA)	CGAACGGAGATAATTTGAGTTTACTTAGG	CGGTCTCTGTATGTTATGAGGTATTACC	Denman and McSweeney, 2006
<i>Butyrivibrio fibrisolvens</i> (16S rDNA)	ACACACCGCCCCGCACA	TCCTTACGGTTGGGTCACAGA	Klieve et al., 2003
<i>Streptococcus bovis</i> (16S rDNA)	CTAATACCGCATAACAGCAT	AGAAACTTCCTATCTCTAGG	Tajima et al., 2001
<i>Fibrobacter succinogenes</i> (16S rRNA)	GTTTCGGAATTACTGGGCGTAA	CGCCTGCCCTGAACTATC	Denman and McSweeney, 2006
<i>Megasphaera elsdenii</i> (16S rDNA)	GACCGAACTGCGATGCTAGA	CGCCTCAGCGTCAGTTGTC	Ouwerkerk et al., 2002
<i>Selenomonas ruminantium</i> (16S rDNA)	TGCTAATACCGAATGTTG	TCCTGCACTCAAGAAAGA	Tajima et al., 2001

HAP bacteria – hyper-ammonia producing bacteria

using SYBR Green and specific primers (Table 3) in a Real-Time PCR system (Roche). The total reaction mixture (20  $\mu$ l) included 10  $\mu$ l of LightCycler<sup>®</sup> 480 SYBR Green I Master mix (Roche, Mannheim, Germany), 6  $\mu$ l of nuclease free water, 0.5  $\mu$ l of each forward and reverse primer (10  $\mu$ M) and 3  $\mu$ l (approximately 30 ng) of template DNA. Duplicate reactions were carried out for each amplification reaction to ensure the accuracy of the results. The amplification program was as follows: initial denaturation at 95 °C for 10 min, followed by 55 cycles of denaturation at 95 °C for 10 s, annealing at a specific primer temperature for 15 s, and extension at 72 °C for 20 s. Melting curve analysis was carried out to confirm that each primer pair amplified a single product. To determine the counts of microorganisms, calibration curves were constructed by plotting microbial concentrations in 10-fold serial dilutions of reference microorganisms against the crossing point (Cp) values for each target (Jiao et al., 2013).

was used as the experimental unit. Fermenter was considered a random factor. Treatment and day of sampling were considered fixed factors, with day of sampling being treated as a repeated measure. Post hoc multiple comparisons between means were conducted using the Tukey test. Effects were considered significant at  $P \leq 0.05$ .

## Results

The effects of supplementing *Al. kunkeei* EIR/BG-1 and *E. hirae* EIR/CM-2 on rumen fermentation characteristics in Rusitec are summarised in Table 4. The addition of these potential probiotic LAB strains did not affect ruminal pH, methane and total and individual SCFA production, ammonia-N concentration, DMD and total protozoa. However, it is important to note that sampling days had a statistically significant impact on the fermentation parameters except for the total protozoa

**Table 4.** Effects of *Apilactobacillus kunkeei* EIR/BG-1 and *Enterococcus hirae* EIR/CM-2 supplementation on rumen fermentation characteristics in Rusitec

Parameters	Treatments			SEM	P-values		
	control	<i>Al. kunkeei</i> EIR/BG-1	<i>E. hirae</i> EIR/CM-2		treatment	day	T × D
Ruminal pH	6.74	6.74	6.74	0.014	0.999	<0.001	0.999
Total SCFA, mmol/day	23.65	22.94	23.32	0.674	0.770	<0.001	0.297
Individual SCFA, mmol/day							
acetate	12.48	12.13	12.40	0.313	0.721	<0.001	0.203
propionate	6.29	6.20	6.21	0.237	0.958	<0.001	0.785
butyrate	3.14	2.96	3.00	0.163	0.734	<0.001	0.099
isobutyrate	0.19	0.18	0.18	0.008	0.724	<0.001	0.201
isovalerate	0.55	0.52	0.52	0.019	0.414	<0.001	0.317
valerate	1.02	0.97	1.01	0.032	0.589	<0.001	0.616
DMD, %	47.38	48.11	47.04	1.007	0.754	0.010	0.493
Methane, mmol/day	6.24	5.99	6.15	0.188	0.669	<0.001	0.068
Ammonia-N, mmol/l	5.61	5.84	5.89	0.162	0.471	<0.001	0.424
Total protozoa, log <sub>10</sub> /ml	3.80	3.89	3.89	0.076	0.668	0.941	0.486

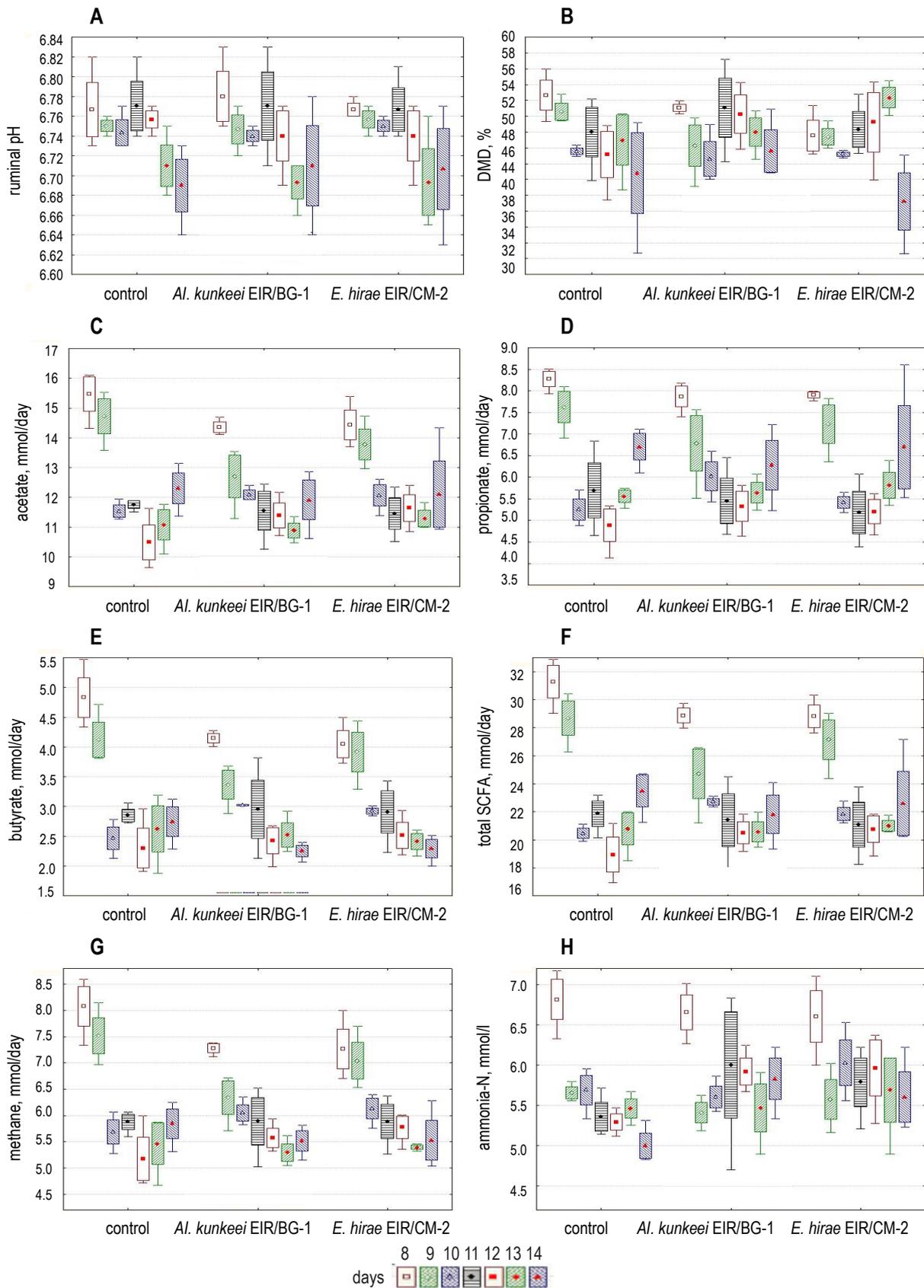
SCFA – short chain fatty acids, DMD – dry matter digestibility, SEM – standard error of the mean, T × D – treatment × day interaction

## Statistical analyses

Data on rumen fermentation characteristics were subjected to repeated analysis of variance (ANOVA) to examine the effect of treatment, day, and treatment × day interaction (SigmaStat Program, version 3.1, Systat Software, Erkrath, Germany). For representative rumen bacterial species and groups, samples pooled from days 8 to 14 for each fermenter, the general linear model (GLM) was used to analyse the effects of treatments using the same statistical software. A single fermenter

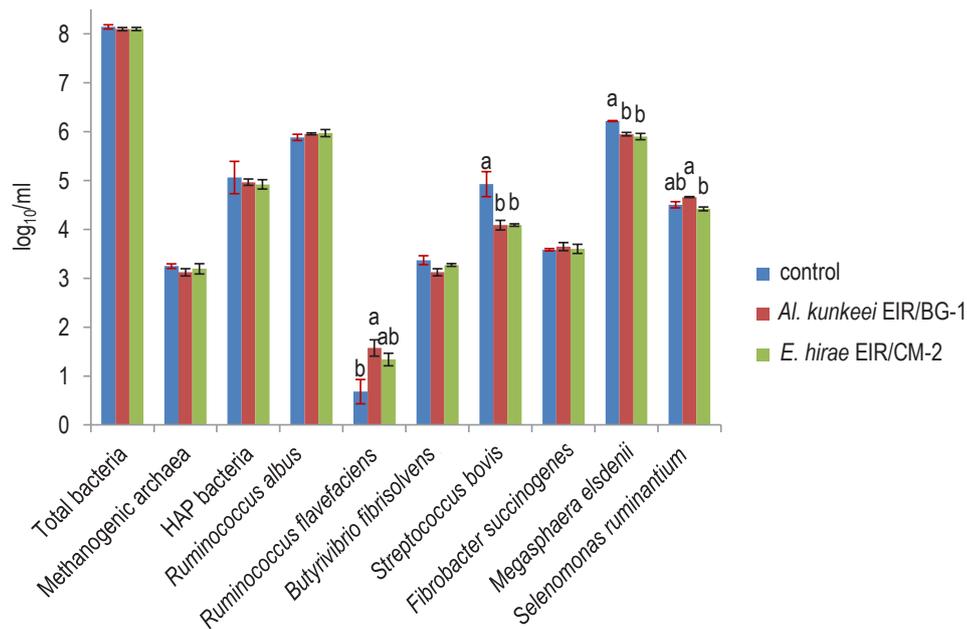
count, regardless of the treatment ( $P \leq 0.01$ ). The daily effects of LAB strains on the main rumen fermentation parameters are shown in Figure 1.

LAB supplementation affected the abundance of certain bacterial populations in Rusitec fermenters (Figure 2). The abundance of *R. flavefaciens* increased in the *Al. kunkeei* EIR/BG-1 treatment ( $P < 0.05$ ). In addition, the counts of *R. flavefaciens* cells were statistically similar between *Al. kunkeei* EIR/BG-1 and *E. hirae* EIR/CM-2 treatments. *S. ruminantium* was more abundant in the



**Figure 1.** Daily effects of *Apilactobacillus kunkeei* EIR/BG-1 and *Enterococcus hirae* EIR/CM-2 supplementation on the main rumen fermentation characteristics in Rusitec. (A) ruminal pH; (B) DMD, %; (C) acetate, mmol/day; (D) propionate, mmol/day; (E) butyrate, mmol/day; (F) total SCFA, mmol/day; (G) methane, mmol/day; (H) ammonia-N, mmol/l. Box-plots with whiskers show means and standard error of the means from minimum to maximum values (see Table 4 for *P*-values)

DMD – dry matter digestibility, SCFA – short chain fatty acids



**Figure 2.** Effects of *Apilactobacillus kunkeei* EIR/BG-1 and *Enterococcus hirae* EIR/CM-2 supplementation on the abundance of representative species and groups of rumen bacteria in Rusitec determined by real-time PCR. Graph shows means and standard error of the means; ab – bars with different superscripts for each microorganism differ at  $P \leq 0.05$

HAP bacteria – hyper-ammonia producing bacteria

*Al. kunkeei* EIR/BG-1 treatment compared to the *E. hirae* EIR/CM-2 group ( $P < 0.05$ ). On the other hand, the counts of *S. bovis* and *M. elsdenii* were lower with both *Al. kunkeei* EIR/BG-1 and *E. hirae* EIR/CM-2 supplementations ( $P < 0.05$ ). LAB supplementation did not significantly affect the abundance of total bacteria, HAP bacteria, methanogenic archaea, *R. albus*, *B. fibrisolvens* and *F. succinogenes*.

## Discussion

Probiotic supplementation of livestock feed has increased considerably in recent decades. Several LAB species are commonly used as bacterial probiotics, particularly in preweaning calves and dairy cattle (Kulkarni et al., 2022). In the present study, we investigated the *in vitro* effects of the addition of two newly isolated strains, *Al. kunkeei* EIR/BG-1 and *E. hirae* EIR/CM-2, on ruminal fermentation parameters and microbial populations in a high-concentrate diet.

The LAB strains employed in this study did not affect *in vitro* rumen fermentation characteristics. These results are consistent with *in vivo* studies, in which oral administrations of several LAB did not affect rumen fermentation parameters in preweaning calves (Zhang et al., 2017; Stefańska et al., 2021) and dairy cows (Raeth-Knight et al.,

2007). Nevertheless, the applied LAB have been reported to improve substrate efficiency of some dietary components in *in vitro* rumen studies (Soriano et al., 2014; Kim et al., 2016). For instance, *Enterococcus faecium* SROD5 and *E. faecium* SROD elevated *in vitro* total SCFA, propionate and butyrate concentrations, while reducing methane production when maize silage was used as a substrate (Kim et al., 2016). Supplementation with *Lactobacillus mucosae* during *in vitro* fermentation of dried brewer grain resulted in increased production of total SCFA and ammonia, but did not affect digestibility (Soriano et al., 2014). However, these short-term fermentation experiments used single dietary components rather than a total mixed ration (TMR) applied in the present study. The positive effects of supplementation with bacterial probiotics were also observed in a limited number of studies using TMR as a substrate. The addition of 0.1% *E. faecium* SROD, when a TMR containing 40% rice straw and 60% concentrate feed was used as a substrate, increased propionate and total SCFA concentrations and reduced methane production after 12 h of *in vitro* incubation (Mamuad et al., 2019). Moreover, Azzaz et al. (2022) reported that a novel strain of *E. faecium* isolated from fresh dairy products elevated *in vitro* total SCFA levels and decreased methane production during 48 h fermentation of a TMR with a 20:80 forage to

concentrate ratio. The differences in results between the present study and previous reports regarding ruminal SCFA and methane production could be attributed to variations in the bacterial strains used, fermentation time, forage-to-concentrate ratios, and the specific contents of the rations. Nevertheless, ruminal ammonia concentration did not change in any of those studies (Mamuad et al., 2019; Azzaz et al., 2022), which was consistent with the results of the present work. The abundance of HAP bacteria and methanogenic archaea also remained unchanged in the current study, consistent with the relevant fermentation variables.

The supplemented LAB strains exerted noticeable effects on certain members of the rumen microbial population in this study. The abundance of *R. flavefaciens* increased with *Al. kunkeei* EIR/BG-1 supplementation. In addition, the population size of this bacterium in the *E. hirae* EIR/CM-2 treatment was similar to the *Al. kunkeei* EIR/BG-1 treatment, although it did not differ from the control group. Guo et al. (2020) reported that the relative proportions of *R. flavefaciens* and cellulolytic enzyme activities during *in vitro* rumen incubation were higher for silages inoculated with *Lactobacillus plantarum* and *Enterococcus faecalis*. Additionally, a previous *in vitro* study by Mamuad et al. (2019) found that supplementation with 0.1% *Enterococcus faecium* SROD resulted in elevated levels of *R. flavefaciens* and total bacteria. *R. flavefaciens*, together with *R. albus* and *F. succinogenes*, are generally considered the dominant cellulolytic microorganisms digesting fibre in the rumen. *R. flavefaciens* was found to be the most prevalent species among the cellulolytic flora (Mosoni et al., 2007). Yeasts, such as *Saccharomyces cerevisiae*, can also promote the growth of fibre-digesting bacteria (Amin and Mao, 2021). In this regard, a probiotic mixture composed of *L. acidophilus* and *S. cerevisiae* increased the abundance of *R. flavefaciens*, *R. albus* and *F. succinogenes* in sheep fed a paddy straw-based diet (Sheikh et al., 2022). Several mechanisms have been proposed to explain the aforementioned stimulatory effect of probiotics on certain gut bacteria (Kulkarni et al., 2022). Both yeasts (Amin and Mao, 2021) and LAB (Indira et al., 2019) can produce nutrients and growth factors such as vitamins, amino acids and precursors of enzymes that stimulate the metabolism and growth of beneficial microorganisms in the gut. LAB, particularly lactobacilli, also have the ability to produce large quantities of exopolysaccharides (EPSs), which have potential prebiotic functional-

ity (Badel et al., 2011). It has been reported that *Apilactobacillus* species, mainly *Al. kunkeei*, harbour genes encoding glucosyltransferases responsible for the synthesis of prebiotic glucan-type EPS (Meng et al., 2018; Ahmad et al., 2022). Therefore, these factors may be involved in the potential mechanism of the stimulatory effects of *Al. kunkeei* EIR/BG-1 on *R. flavefaciens* in this study. Additionally, Weinberg et al. (2007) suggested that LAB inoculants could potentially compete with lactate-producing bacteria such as *S. bovis* and *Ruminobacter amylophilus*, thereby reducing lactate production and pH decline in the rumen. This, in turn, could increase the activity of cellulolytic populations and improve the digestibility of NDF and DMD. LAB supplementation did not alter DMD, although the count of lactate-producing *S. bovis* was also lower with both supplementations in the current study. The lack of effect on DMD could be related to the relatively lower forage-to-concentrate ratio (1:4) of the experimental diet compared to the feed used by Weinberg et al. (2007) (2:1 and 1:2). Considering that the total number of bacteria did not change in the current study, it can also be assumed that bacterial groups whose growth was increased may have compensated for the lower abundance of the suppressed ones, preventing noticeable alterations in fermentation and digestibility parameters.

On the other hand, both LAB supplementations led to a decrease in the counts of *S. bovis* and *M. elsdenii* cells. *S. bovis* is an amylolytic lactic acid bacterium that grows more readily when animals are fed concentrate-rich diets, and it causes rumen acidosis (Mombach et al., 2021). Although *S. bovis* enhances the degradation of simple sugars and starches, previous research has indicated that *Lactobacillus* species are the dominant microorganisms adhering to barley and maize grain particles in the rumen (Yang et al., 2018). Consequently, it is plausible that the supplemented LAB strains could compete with *S. bovis* for the substrates, leading to the observed reduction in its population, a phenomenon consistent with previous findings (Mombach et al., 2021). In addition, Morovský et al. (1998) reported that the growth of *S. bovis* was inhibited by a bacteriocin produced by *E. faecium* BC25. The latter authors also observed that various strains of *R. albus*, *B. fibrisolvans*, *M. elsdenii* and *S. ruminantium* were not sensitive to the bacteriocin isolated from *E. faecium* BC25. Therefore, *S. bovis* inhibition in the *E. hirae* EIR/CM-2 groups in the present study may be attributed to bacteriocin-mediated effects.

Reducing the abundance of *S. bovis* has also been suggested as a strategy to prevent ruminal lactic acidosis, since this bacterium is a major contributor to this condition (Gill et al., 2000). However, no significant increase in ruminal pH was observed in this study as a result of bacterial probiotic supplementation. The available literature data regarding the impact of bacterial probiotics on *in vitro* ruminal pH are contradictory. While some studies found no effects of *L. acidophilus* (Chen et al., 2017) and *E. faecium* SROD (Mamuad et al., 2019), others reported that *L. mucosae* supplementation led to a decrease in ruminal pH (Soriano et al., 2014) in the short-term batch culture experiments. In addition to differences in the bacterial species used, the lack of effect on rumen pH in the present study could be due to the high buffering capacity of the buffer solution used in the Rusitec fermenters, as reported previously by Gómez et al. (2005).

The lower abundance of *S. bovis* appears to cause reduced lactic acid availability, and thus a lower number of lactic acid-utilising *M. elsdenii* in the LAB-supplemented groups in the current study. Similarly, Zhu et al. (2017) reported reduced abundance of *M. elsdenii* along with *S. bovis* following yeast inclusion in dairy cows' diet. *M. elsdenii* normally metabolises 60 to 80% of lactate and converts it mainly to butyrate (Counotte and Prins, 1981). In the present study, the abundance of *M. elsdenii* also showed a positive correlation with butyrate production ( $r = 0.78$ ,  $P = 0.02$ ; data is not shown). However, the count of *S. ruminantium*, other lactic acid-utilising species, did not change with LAB supplementation. This was most likely due to fact that *S. ruminantium* has a more diverse substrate range, including starch, pectins and proteins when compared to *M. elsdenii* (Counotte and Prins, 1981). The size of *S. ruminantium* population also remained stable in a previous study when intensively finished beef cattle was supplemented with a combination of yeast and bacterial probiotics (Mombach et al., 2021). On the other hand, the abundance of *S. ruminantium* in the present study was higher in the *Al. kunkeei* EIR/BG-1 treatment compared to the *E. hirae* EIR/CM-2 treatment. Although not evaluated in this study, *Al. kunkeei* species is capable of synthesising bioactive molecules with prebiotic properties, such as LPS, as mentioned before (Meng et al., 2018; Ahmad et al., 2022). However, none of the nineteen *E. hirae* isolates from raw cow's milk were able to produce EPS in a previous study by Espeche et al.

(2012). This suggests that the potential growth-stimulating factors produced by *Al. kunkeei* EIR/BG-1 in this study may have contributed to its promoting effects on both *R. flavefaciens* and *S. ruminantium*, which were superior to those of *E. hirae* EIR/CM-2.

## Conclusions

Potential probiotic lactic acid bacteria (LAB) strains exhibited beneficial effects on the abundance of certain rumen bacteria, although these results were not reflected in rumen fermentation parameters. The promoting effects of *Apilactobacillus kunkeei* EIR/BG-1 on *Ruminococcus flavefaciens* and *Selenomonas ruminantium*, which outperformed stimulatory activity of *Enterococcus hirae* EIR/CM-2 may be attributed to its ability to produce growth factors or nutrients with potential prebiotic functionality. On the other hand, the inhibitory effect of both LAB on lactic acid-producing *Streptococcus bovis* was likely due to substrate competition or bacteriocin mediated mechanisms. This reduction in *S. bovis* may have contributed to the decrease in lactic acid-utilising *Megasphaera elsdenii* due to limited access to lactate. The inhibitory influence of LAB on *S. bovis* growth was promising, but additional confirmation through improvements in ruminal pH is needed. Further research is required to clarify the potential of these newly developed LAB strains as probiotic feed additives for ruminant rations.

## Conflict of interest

The Authors declare that there is no conflict of interest.

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