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Influence of Different Probiotic Combinations Supplementation in a Highly Concentrated Diet on *In Vitro* Gas Production, Methane Emission, and Nutrient Degradability in Sheep

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Abstract

THIS study investigated the effects of different probiotic mixes on fermentation parameters, methane emissions, and nutrient degradability of the diet *in vitro*. The examined bacterial probiotic mixes were 1- *Lactobacillus acidophillus* + *Lactobacillus bulgaricus* (AB), 2- *Lactobacillus cassia* + *Lactobacillus plantrum* (CP), 3- *Bacillus lichnoformas* + *Bacillus subtillus* (LS), 4- *Lactobacillus acidophillus* + *Lactobacillus bulgaricus* + *Bacillus lichnoformas* (ABL), 5- *Lactobacillus cassia* + *Lactobacillus plantrum*+ *Bacillus subtillus* (CPS),6- *Lactobacillus acidophillus* + *Lactobacillus bulgaricus* + *Bacillus lichnoformas* + *Bifidobuctrium bifidum* (ABLB) and 7- *Lactobacillus cassia* + *Lactobacillus plantrum*+ *Bacillus subtillus* + *Bifidobuctrium bifidum* (CPSB). The probiotic mixes were used at levels 0 (control), 2, and 4×10^9 cfu /g feed. Significant effects were observed in gas production across all incubation times, with the CPS mix exhibiting the highest production after 48 hours. Methane emissions significantly decreased with all mixes of probiotics, with the CP mix demonstrating the most substantial reduction. The degradability of dry matter and crude fiber was significantly influenced by supplementation levels, peaking at 2 and 4×10^9 cfu/g feed. Total volatile fatty acid (TVFA) production was significantly affected, with ABLB and CPSB mixes producing the highest TVFA. Furthermore, significant effects of supplementation levels were noted on ammonia-N and TVFA production. Additionally, pH value was significantly affected by the mixes of probiotics and supplementation levels. In conclusion, the probiotic combinations enhanced the rumen fermentation and degradability. besides, it reduced the methane emission. So, it is able to be used in an applicable *in vivo* study.

Keywords: probiotic combinations, supplementation, feed degradability, methane emission, *in vitro.*

Introduction

Interest in using feed additives like probiotics or direct-fed microbial (DFM) has increased due to growing concerns about the usage of antibiotics. Probiotics are living microorganisms that have been found to improve the performance and health of animals when used as a feed supplement [1, 2]. Probiotics have been receiving interest due to their beneficial effects on the performance and health of beef and dairy cattle herds [3, 4], besides it can reduce public scrutiny of animal farming by replacing antibiotics. Probiotics have many beneficial effects which maintain gastrointestinal health and improve gut function which leads to promoting productive performance and avoiding disease [5]; improve the rumen microbial environment [6]; reduce methane emission [7-9]; increase nutrient digestibility [10]; enhance nutrient absorption [11, 12], and avoid lactic acid accumulation [13].

A variety of non-pathogenic microorganisms, including Lactic acid bacteria (LAB), *Bacillus* spp., lactic acid-utilizing bacteria (LUB), and several yeast strains, are utilized as probiotics [14]. Many Lactic acid bacteria (LAB) strains from the genera *Lactobacillus (*such as *L. acidophilus, L. casei,*+ *Lactobacillus bulgaricus, Lactobacillus plantrum* and *L. rhamnosus), Bifidobacterium (such as B. bifidum, B. breve, and B. longum), Streptococcus spp.* (such as *S. thermophilus) and Enterococcus* are considered beneficial to the animal host and have

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been utilized as probiotics [15, 16]. LAB has been shown the ability to interact with the rumen microorganisms and keep the balance of the rumen environment, enhance the activity of the beneficial microbes, promote the degradability of nutrients in the rumen, and reduce methane emissions [17, 18]. These LABs constantly produce lactate in the rumen, stimulating the growth of lactic acid-utilizing bacteria (LUB) that maintain ruminal pH. As a result, these LABs have been recommended as direct-fed microbes (DFMs) [14, 19].

Bacillus spp. (such as *Bacillus licheniformis and Bacillus subtilis)* is popular as a probiotic due to its ability to produce and release a wide type and quantity of extracellular enzymes that improve the digestion of nutrients in the animal's gastrointestinal tract [20-23]. there are other mechanisms, including immunomodulation and production of antimicrobial substances, including surfactin, bacillomycin D, and fengycin, which have high antifungal, antibacterial, and antiviral properties [24].

Probiotics containing various microbial components have been proven to promote ruminal fermentation by stimulating the rumen microbiota [25, 26]. Few studies have investigated the effect of using probiotic combinations (a mix of probiotics) as a feed supplementation on methane emissions and ruminal fermentation. Based on the findings from our previous study, which investigated the beneficial effects of every single tested probiotic strain at various levels on the *in vitro* gas production technique [27], it was hypothesized that combinations of different probiotic strains may be more effective than single-strain probiotics and could positively affect feed degradability, methane emission, and ruminal fermentation parameters.

The aim of this study was to assess the impact of various probiotic combinations containing *Lactobacillus*, *Bifidobacterium*, and *Bacillus* at different concentrations on *in vitro* gas production, methane emissions, feed degradability, and certain ruminal fermentation kinetics.

Material and Methods

The current investigation was conducted in the Laboratory of Animal Nutrition, Department of Animal Production, Faculty of Agriculture, Zagazig University, Zagazig, Egypt.

Experimental design and Probiotic combinations

A factorial arrangement (7 × 3) was used to investigate the effects of 7 probiotic combinations with three levels on rumen fermentation characteristics using an *in vitro* gas production technique. The probiotic combinations used were 1- *Lactobacillus acidophillus* + *Lactobacillus bulgaricus* (AB), 2- *Lactobacillus cassia* + *Lactobacillus plantrum* (CP), 3- *Bacillus lichnoformas* + *Bacillus subtillus* (LS), 4- *Lactobacillus acidophillus* + *Lactobacillus bulgaricus* + *Bacillus lichnoformas* (ABL), 5- *Lactobacillus cassia* + *Lactobacillus plantrum*+ *Bacillus subtillus* (CPS), 6- *Lactobacillus acidophillus* + *Lactobacillus bulgaricus* + *Bacillus lichnoformas* + *Bifidobuctrium bifidum* (ABLB) and 7- *Lactobacillus cassia* + *Lactobacillus plantrum*+ *Bacillus subtillus* + *Bifidobuctrium bifidum* (CPSB). The probiotic strains were obtained from a commercial company in 10th of Ramadan city, sharkia, Egypt. The bacterial preparations were in powder form, mixed to form the combination being studied. The probiotic combinations used were at levels 0 (control), 2 and 4×10^9 cfu /g feed. The tested levels of different probiotics were selected based our earlier study [27].

Diet and chemical analysis

The basal diet was composed of 30% forage (alfalfa hay) and 70% concentrate (70% corn grain, 15% soybean meal, 13% wheat bran, 1.2% limestone, 0.5% salt, and 0.3 premixes) The chemical composition of the diet is provided in Table 1. The concentrate and alfalfa hay were finely grounded (1 mm) and mixed at a percent of 70:30, respectively. This dried diet was utilized for chemical analysis and *in vitro* gas production trials. The sample was analyzed for dry matter (DM), organic matter (OM), ash, ether extract (EE), and crude protein (CP) according to the AOAC [28] method. The method described by [29] was used to analyze neutral detergent fiber (NDF).

In vitro incubations

Fresh rumen fluid was collected from five male Baladi sheep (40.14 ± 1.67 Kg body weight, and 8 months of age) that were used as inoculum donor using a soft plastic stomach tube before morning feeding to obtain stable rumen microbial cultures. Animals were fed *ad libitum* twice a day (08:00 and 16:00) with a ration composed of 50% roughage (alfalfa hay) and 50% concentrate (70% corn grain, 15% soybean meal, 13% wheat bran, 1.2% limestone, 0.5% salt, and 0.3% premixes). Water was freely accessible by the sheep.

Animals were subjected to this ration for one month before collecting rumen liquor samples. Rumen fluid was immediately transported to the laboratory in pre-warmed isolation flasks (39 °C) and stored anaerobically. Four layers of cheesecloth were used to filter the rumen fluid, which was incubated in a water bath at 39 °C, and CO_2 was saturated until inoculation.

The content of the buffer incubation medium (MB9) has NaCl (2.8g/l), $CaCl_2$ (0.1g/l), $MgSO_4.7H_2O$ (0.1g/l), Na_2HPO_4 (6g/l) and $KH_2PO_4.H_2O$ (2g/l) as mentioned by Abd-Elkerem, Bassiony [30]. The pH of MB9 media was adjusted to 6.8, and CO_2 was saturated for 30 minutes to maintain anaerobic conditions [31]. The MB9 media was mixed with the rumen fluid at a ratio of 2:1 (v/v), and then 30 millimeters were placed into

calibrated glass tubes holding 200 mg of the diet (70% concentrate + 30 alfalfa hay) mixed with a probiotic combination in different levels, quickly closed by a gas-release rubber stopper with a tri-way valve with a calibrated plastic syringe to measure gas production. The gas production volume was measured after 3, 6, 12, 24, 36, and 48 hours of incubation. The total gas volume was adjusted using a blank tube. Each run included four blank bottles (no substrate) and six bottles for each treatment. The kinetics of gas production were calculated following the model of Ørskov and McDonald [32].

At the end of incubation and after recording the final gas volume the methane emission was estimated by using NaOH (10 M) according to [33], and the methane intensity (CH_4 ml/ TDDM, CH_4 ml/ TDOM, CH_4 percentage from total gas) was calculated.

Determination of true nutrient degradation, partitioning factor, ammonia-N, pH, and volatile fatty acids concentration

A digital pH meter (model 6010N, Jenco Instruments Inc., San Diego, CA, USA) was used to measure the ruminal pH immediately after the end of in vitro incubation. After 48 hours of incubation, 30 mL of neutral detergent solution was added to the contents of three tubes of each treatment, and the bottles were placed at 105 °C for three hours to detect the truly degraded dry matter (TDMD). The residual DM weight was determined after filtering each sample through pre-weighed Gooch crucibles and drying it at 105°C for three hours [34]. After that, it was used to estimate crude fiber degradability (CFD) according to AOAC [28]. Total volatile fatty acids (TVFA) and ruminal ammonia-N concentrations were measured using the contents of another three tubes from each treatment. The concentration of ruminal NH_3-N was estimated using the method described by Conway [35]. The steam distillation method was used to determine the TVFA concentration, according to Warner [36]. The ratio of OM (mg) degradability to gas production volume (in mL after 24 hours) was used to calculate the partitioning factor (PF) [34].

Calculations

The metabolizable energy (ME, MJ/kg DM) and net energy of lactation (NEL, MJ/kg DM) were calculated using The equation of Menke and Steingass [37] as follows:

ME (MJ/kg DM) = (0.157×GP) + (0.0084×CP) + (0.022×EE) – (0.0081×CA) +1.06

NEL (MJ/kg DM) = (0.115×GP) + (0.0054×CP) + (0.014×EE) – (0.0054×CA) - 0.36

Where: GP = net gas production (ml/0.2 g DM) at 24 h of incubation; EE= ether extract; CP= crude protein; CA= crude ash.

the concentrations of short-chain fatty acids (SCFA) were calculated using The equation of Getachew, Makkar [38] as:

SCFA (mmol/200 mg DM) = (0.0222 × GP) − 0.00425

Where GP is the 24-hour net gas production (ml/200 mg DM).

The microbial crude protein biomass production was estimated, according to Blümmel, Steingaβ [34] as follows:

MCP (mg/g DM) = mg DMD − (ml gas × 2.2 mg/ml)

Where: 2.2 mg/mL is a stoichiometric factor that expresses mg of C, H, and O required to produce SCFA gas associated with production of 1 mL of gas.

Menke, Raab [39] equation was used to calculate the *in vitro* organic matter digestibility (OMD %) as OMD (%) = 14.88+ (0.889 × GP) + (0.45 ×CP) + (0.0651× XA)

Where XA = Ash (%).

Statistical analysis

The data in the main study were analyzed as a 7 x 3 factorial arrangement, with 7 probiotic combinations and 3 levels using SPSS 21 (Chicago, IL) software, based on the following statistical model:

$y_{ijl} = \mu + \alpha_i + \beta_j + \alpha\beta_{ij} + e_{ijl}$. Where y_{ijl} is observation, μ is the general mean, α_i is the effect of first factor (probiotic combinations), β_j is the effect of second factor (levels), $\alpha\beta_{ij}$ is the interaction between treatments (probiotic combinations × levels), and e_{ijl} is the standard error of term. The significant differences in mean were analyzed by Duncan's multiple comparison test at $P < 0.05$ [40].

Results

Effect of probiotic mixtures on gas production and gas kinetics.

The different probiotic mixtures significantly affected gas production throughout all incubation times ($P < 0.001$) (Table 2). After 48 hours of incubation, the highest gas production was for the CPS mix, while the lowest was in the quaternary mixes of ABLB and CPSB. Additionally, increasing the level of probiotic addition led to a significant increase in gas production ($P < 0.001$). The highest gas production was observed at level 4×10^9 cfu/g feed. Mixes of probiotics didn't lead to significant differences in the gas production from the immediately soluble fraction (a), but there were significant differences in the gas production from the insoluble fraction (b), the gas production rate constant for the insoluble fraction(c), and potential gas production (a+b) ($P < 0.001$). Also, there was a significant effect of levels in gas production from the immediately soluble fraction, and the minimum value

was at level 4× 10^9 cfu/g feed, Conversely, it led to a significant increase in the gas production from the insoluble fraction, the gas production rate constant for the insoluble fraction, and potential gas production. the interaction between the probiotic mixtures and the level of probiotics had a significant effect on both gas production during different incubation periods and gas kinetics values ($P < 0.001$).

Effect of probiotic mixtures on methane emissions parameter.

The supplementation of all mixtures of probiotics resulted in a significant decrease in methane (CH_4) emission in the form of CH_4 ml/g DM, CH_4 ml/g TDDM, and CH_4 ml/g TDOM as appeared in Table 3. The mix of CPS probiotics achieved the highest methane production, while the mix of CP probiotics showed the most significant decrease in methane production compared to the other mixes. However, there were no significant differences between different probiotic mixes in CH_4% of total gas production. Additionally, there were significant effects of levels of addition ($P < 0.001$) on CH_4 ml/g DM, CH_4 ml/g TDDM, CH_4 ml/g TDOM, and CH_4% of total gas production, the highest decrease in methane production was observed at level 4× 10^9 cfu/g feed. Furthermore, the interaction between the probiotic mixes and the addition level had no significant effect on methane production.

Effect of probiotic mixtures on degradability of nutrient

The probiotic mixtures did not exhibit significant differences in dry matter and crude fiber degradability. However, the supplementation level of the probiotic mixes significantly influenced the degradability of dry matter and crude fiber ($P < 0.001$). Notably, the greatest improvement in dry matter and crude fiber degradability was observed at supplementation levels of 2 and 4 × 10^9 cfu/g of feed. The interaction between the probiotic mixtures and the addition level did not have a significant effect on the degradability of dry matter and crude fiber.

Effect of probiotic mixtures on fermentation parameter

The data presented in Table 4 demonstrate that the mixtures of probiotics had no significant effect on ammonia-N (NH_3-N) production. However, the supplementation levels significantly influenced NH_3-N production. The lowest production of NH_3-N was observed at a level 4 × 10^9 cfu/g of feed. In contrast, the supplementation of probiotic mixtures resulted in significant differences in the production of total volatile fatty acids (TVFAs). Specifically, the ABLB and CPSB mix had the highest production of TVFAs, while the mix of AB and CP produced the lowest. Additionally, the supplementation levels significantly affected the production of TVFAs. The highest production was observed at level 4× 10^9 cfu/g feed. The obtained results of NH_3-N and TVFAs reflected the values of pH. The pH value was significantly influenced by the bacterial mixes, with a mix of ABLB and CPSB resulting in a significant decrease in pH value in comparison to the other mixes. Additionally, the supplementation levels significantly influenced pH, with the lowest value recorded at the addition level of 2 × 10^9 cfu/g of feed. The interaction between the probiotic blends and the supplementation levels did not significantly affect NH_3-N production. Conversely, it did have a significant impact on the production of TVFAs and the pH values.

Effect of probiotic mixtures on the predicted value

The supplementation of mixtures of bacterial probiotics led to significant differences in the values of short-chain fatty acids (SCFA), metabolizable energy (ME), net energy for lactation (NEL), and organic matter digestibility (OMD). Mixtures AB, CP, and CPSB showed the lowest values for these parameters, while the mix of CPS resulted in the highest values. In contrast, there were no significant differences between the bacterial mixes in the value of microbial crude protein (MCP). However, the bacterial mixes had a significant effect on the partitioning factor (PF), with a mix of CPSB achieving the highest PF, while a mix of CPS resulting in the lowest PF value. Regarding the effect of supplementation levels, the highest addition level (4× 10^9 cfu/g feed) led to the highest values of SCFA, ME, NEL, OMD, and MCP. Conversely, increasing the addition level resulted in a decrease in the PF value, with the largest rate of decrease observed at level 4× 10^9 cfu/g feed. Furthermore, there were significant differences in the interaction between the probiotic mixtures and the supplementation levels for all the predictive values mentioned.

Discussion

The microbial feed degradation and the buffering effect of acids produced during ruminal fermentation are the causes of gas production [41]. Fermentation gas is produced during the fermentation of feed to acetate, butyrate, and propionate, which is indicative of the production of VFA [41, 42]. Measuring *in vitro* gas production offers considerable information about the digestion kinetics of soluble and insoluble feed fractions [43]. Increased net gas production, volume of gas produced from insoluble fraction, and potential extent of gas production suggest an improvement in substrate digestibility and activity of fiber-degrading microorganisms [44]. Our findings showed that supplementing postbiotic mixtures improved gas production and gas kinetics by enhancing rumen fermentation rates, dry matter, and crud fiber degradability. Rumen microorganisms, such as bacteria and protozoa, have a well-known function in digesting soluble and insoluble feed fractions. So, the enhancement of gas production and

gas kinetics due to probiotic blend supplementation may be explained by the improvement in the primary microbial population. Previous research has demonstrated that the effect of bacterial DFM in the rumen can vary depending on the kind of DFM strain, the physiological conditions of the animal [45], and the diet composition [46, 47].

Ruminants produce methane, which represents an energy loss for the animal and accounts for 3-10% of their gross energy intake [48] and contributes to global warming [49]. Therefore, decreasing methane emissions will increase the energy available to the animal, thereby enhancing productivity. In the current investigation, the supplementation of probiotic mixtures resulted in a significant decrease in methane (CH_4) emission in the form of CH_4 ml/g DM, CH_4 ml/g TDDM, and CH_4 ml/g TDOM. Preventing or consuming H_2 in the rumen prevents it from entering the CH_4 production cycle. This activity will be achievable due to the stimulation and growth of LUB [50]. LAB is predicted to promote lactic acid production, which may stimulate the growth of lactate-utilizing microbes, finally leading to the production of propionic acid through their metabolism [51]. Propionic acid production consumes H_2 in the rumen, which may correspond with reduced methane production from rumen fermentation. [52]. The supply of H_2 for methane generation decreases as propionic acid production increases. Competition for H_2 substrates will affect methanogen growth in the rumen. Probiotics are hypothesized to reduce enteric methane emission in ruminants through four different mechanisms: (1) probiotics directly impact methanogens; (2) Probiotics impact microorganisms, such as hydrogen producers, which provide the substrates needed for methanogenesis; (3) Bacteriocins and other probiotic metabolites have a direct influence on methanogens; or (4) The metabolites influence bacteria that produce the substrates needed for methanogenesis [17]. However, other results suggest that the effect of direct-fed microorganisms on enteric methane emission could depend on bacterial species, dose, strain, diet, or interaction of all factors [53, 54].

The rumen pH decreased when probiotics were added, probably because this LAB produces lactic acid. The *Lactobacillus* probiotic supplementation was proven to reduce the pH compared to the control [51]. A significant increase in total VFA production after supplementation of probiotic mixtures demonstrates that the drop in pH did not significantly affect rumen fermentation. VFAs are metabolic products of feed digestion by rumen microorganisms; thus, an increase in their synthesis after probiotic supplementation suggested an increase in rumen microbial metabolic activity [51, 53]. Higher GP, DMD, and total VFA concentrations in high-grain diets were associated with increased ruminal microbial activity and elevated ruminal pH. LABs like *Lactobacillus* and *Enterococcus* may help prevent ruminal acidosis [3], possibly by helping the ruminal microorganisms to adapt to the presence of lactate in the rumen [55].

Supplementing ruminants with probiotics has been shown to improve dry matter and fiber digestibility and fermentation [1]. Lactic acid bacteria's interaction with rumen microorganisms promotes rumen fermentation and inhibits harmful microbes by producing antimicrobial compounds like bacteriocins [56]. Probiotic supplementation has been suggested to promote the adaptability of ruminal microorganisms to the presence of lactic acid or to restrict lactic acid accumulation in the rumen through lactic acid breakdown to acetic acid [55, 57]. Jiao, Liu [18] proposed that these conditions may boost the activities of cellulolytic bacteria and increase the microbial digestion of fibrous foods. This agrees with the present findings that the supplementation of probiotic mixtures improved DMD, OMD, and CFD. Pan, Harper [58] found that a combination of *B. licheniformis* and *B. subtilis* increased the *in vitro* dry matter (DM), starch, and neutral detergent fiber (NDF) digestibility of forage-based and concentrate feedstuffs, indicating *Bacillus* spp.'s ability to produce and release a varied variety of amounts of enzymes of interest in ruminant nutrition, such as amylolytic, lipolytic, proteolytic, and fibrolytic [20-22].

Conclusion

Supplementing the diet with all tested probiotic mixtures had different effects on feed degradability and rumen fermentation parameters. The methane emission was reduced by the CP and CPSB mix. All the probiotic mixtures reduced ammonia-N production. The mixtures of ABLB and CPSB had the highest TVFAs production, and the highest production was observed at level 4× 109 cfu/g feed. In addition, all the probiotic mixtures enhanced the degradability of dry matter and crude fiber. The greatest improvement in dry matter and crude fiber degradability was observed at supplementation levels of 2 and 4×10^9 cfu/g of feed. So, we recommended using a mix of CPSB as a feed additive in a highly concentrated diet for sheep. However, more studies are needed to apply these results *in vivo*.

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Declaration of Conflict of Interest

The authors declare no competing interests.

Ethical of approval

The animal study was reviewed and approved by the Zagazig University animal ethics committee.

Table 1. Chemical composition of the experimental diet [a]

Chemical composition (g/kg DM)	
Organic matter	955.5
Crude protein	137.3
Ether extract	47.1
Neutral detergent fiber	493.5
Ash	44.5
Non-structural carbohydrates [b]	277.6

[a] Experimental diet comprised 30% forage (alfalfa hay) and 70% concentrate (70% corn grain, 15% soybean meal, 13% wheat bran, 1.2% limestone, 0.5% salt, and 0.3 premixes).

[b] Non-structural carbohydrates = 100 - (Neutral detergent fiber + Crude protein + Ether extract + Ash).

TABLE 2. Cumulative gas production and kinetics of gas as affected by different probiotic combinations and dosage levels.

	Gas production (ml/g DM)						gas kinetics			
	3h	**6h**	**12h**	**24h**	**36h**	**48h**	**a**	**B**	**C**	**a+b**
Effect of a probiotic mixture										
AB	44.17^{d}	75.69^{c}	104.17^{d}	129.58^{c}	145.07^{cd}	152.57^{cd}	15.95	136.92^{bc}	0.09^{bc}	152.86 ^{d}
CP	43.89^{d}	74.03^{bc}	106.25^{d}	130.42^{c}	146.74^{cd}	153.61^{cd}	13.66	140.26 ^{b}	0.09^{bc}	153.92 ^{cd}
LS	51.39^{bc}	82.29 ^{b}	115.63^{bc}	141.18 ^{b}	156.81 ^{b}	163.68 ^{b}	18.42	148.25^{ab}	0.09^{bc}	166.66^{ab}
ABL	46.18^{cd}	77.99^{bc}	111.53^{cd}	134.93^{bc}	153.61^{bc}	162.08^{bc}	16.11	146.56^{ab}	0.08 ^{c}	162.67^{bc}
CPS	54.72^{b}	92.43 ^{a}	127.50 ^{a}	152.78 ^{a}	169.10 ^{a}	176.60 ^{a}	17.75	156.34 ^{a}	0.10 ^{b}	174.10 ^{a}
ABLB	61.39 ^{a}	94.58 ^{a}	120.35^{ab}	137.29^{bc}	144.93^{cd}	151.18^{d}	20.61	127.12^{cd}	0.14 ^{a}	147.73 ^{d}
CPSB	61.67^{a}	93.33 ^{a}	118.06^{bc}	134.65^{bc}	142.22^{d}	148.13^{d}	22.00	123.12^{d}	0.13 ^{a}	145.12 ^{d}
Effect of level (× 10^{9} cfu/g feed)										
0	45.60 ^{c}	71.85 ^{c}	96.01 ^{c}	116.01^{c}	127.89 ^{c}	133.72 ^{c}	21.50 ^{b}	111.16 ^{c}	0.09 ^{b}	132.66 ^{c}
2	59.43 ^{a}	87.11 ^{b}	118.45 ^{b}	138.57^{b}	155.09 ^{b}	159.29 ^{b}	27.63 ^{a}	134.35 ^{b}	0.09 ^{b}	161.98 ^{b}
4	50.71 ^{b}	94.05 ^{a}	129.88 ^{a}	157.20^{a}	170.65 ^{a}	181.79 ^{a}	4.22 ^{c}	173.88 ^{a}	0.12 ^{a}	178.10 ^{a}
SEM	1.23	1.54	1.87	2.18	2.38	2.52	1.60	3.19	0.003	2.45
P-value										
probiotic mixtures	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.543	< 0.001	< 0.001	< 0.001
Level	< 0.001	< 0.001	0.020	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
Interaction	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001

[a–d] Means in the same column bearing different letters differ significantly (P < 0.05); SEM indicates the standard error of the mean; a = the gas production from the immediately soluble fraction (ml); b = the gas production from the insoluble fraction (ml); c = the gas production rate constant for the insoluble fraction b (h); a+b = potential gas production (ml) ; AB= *L.Acidophillus* + *L.Bulgaricus* ; CP= *L.casia* + *L.plantrum* ; LS= *B.Lichnoformas* + *B.subtillus*; ABL= *L.Acidophillus* + *L.Bulgaricus* + *B.Lichnoformas* ; CPS= *L.casia* + *L.plantrum* + *B.subtillus*; ABLB= *L.Acidophillus* + *L.Bulgaricus* + *B.Lichnoformas*+ *Bifidobuctrium bifidum*; CPSB= *L.casia* + *L.plantrum* + *B.subtillus*+ *Bifidobuctrium bifidum.*

TABLE 3. Methane emission parameters after 48 hours of incubation as affected by different probiotic combinations and dosage levels.

	Methane emission parameter			
	CH_4 ml/1g DM	**CH_4 ml /1g TDDM**	**CH_4 ml /1g TDOM**	**CH_4 %**
Effect of a probiotic mixture				
AB	28.12^{bc}	46.27^{cd}	66.22^{b}	19.17
CP	27.02^{c}	44.24^{d}	63.90^{b}	18.43
LS	30.43^{ab}	49.69^{abc}	74.25^{a}	19.63
ABL	30.31^{ab}	50.79^{ab}	69.81^{ab}	19.50
CPS	30.8^{a}	53.04^{a}	67.05^{b}	18.48
ABLB	27.81^{bc}	45.86^{cd}	64.49^{b}	18.85
CPSB	28.09^{bc}	48.12^{bcd}	65.66^{b}	19.35
Effect of level (× 10^9 cfu/g feed)				
0	35.29^{a}	61.86^{a}	90.06^{a}	26.43^{a}
2	26.43^{b}	42.63^{b}	60.45^{b}	16.83^{b}
4	25.00^{b}	40.20^{b}	51.18^{c}	13.85^{c}
SEM	0.53	1.06	1.70	0.54
P-value				
probiotic mixtures	0.007	< 0.001	0.018	0.764
Level	< 0.001	< 0.001	< 0.001	< 0.001
Interaction	0.409	0.075	0.532	0.476

$^{a–d}$ Means in the same column bearing different letters differ significantly (P < 0.05);
SEM indicates the standard error of the mean; **AB**= *L.Acidophillus* + *L.Bulgaricus* ; **CP**= *L.casia* + *L.plantrum* ; **LS**= *B.Lichnoformas* + *B.subtillus*; **ABL**= *L.Acidophillus* + *L.Bulgaricus* + *B.Lichnoformas* ; **CPS**= *L.casia* + *L.plantrum* + *B.subtillus*; **ABLB** = *L.Acidophillus* + *L.Bulgaricus* + *B.Lichnoformas*+ *Bifidobuctrium bifidum*; **CPSB**= *L.casia* + *L.plantrum* + *B.subtillus*+ *Bifidobuctrium bifidum.*

TABLE 4. Degradability and fermentation parameters as affected by different probiotic combinations and dosage levels.

	Degradability parameter		Fermentation parameter		
	DMD %	**CFD %**	**AMONIA mg/100 ml**	**TVFA Meq/L**	**pH**
Effect of a probiotic mixture					
AB	61.71	51.07	15.65	202.56^{d}	5.84^{a}
CP	62.22	51.57	16.41	211.89^{cd}	5.79^{a}
LS	62.11	50.25	16.46	218.67^{bc}	5.73^{a}
ABL	60.56	50.79	17.23	220.67^{bc}	5.76^{a}
CPS	58.57	51.52	16.51	226.78^{b}	5.83^{a}
ABLB	61.70	52.65	16.73	254.22^{a}	5.54^{b}
CPSB	59.11	50.23	16.78	255.00^{a}	5.59^{b}
Effect of level (× 10^9 cfu/g feed)					
0	57.11^{b}	48.21^{b}	18.82^{a}	202.38^{b}	5.76^{a}
2	62.54^{a}	53.27^{a}	17.64^{b}	206.90^{b}	5.68^{b}
4	62.91^{a}	51.98^{a}	13.15^{c}	272.05^{a}	5.74^{ab}
SEM	0.67	0.44	0.38	5.67	0.02
P-value					
probiotic mixtures	0.529	0.577	0.516	< 0.001	< 0.001
Level	< 0.001	< 0.001	< 0.001	0.001	0.054
Interaction	0.641	0.587	0.032	< 0.001	< 0.001

a–d Means in the same column bearing different letters differ significantly (P < 0.05); SEM indicates the standard error of the mean; TVFA is the total volatile fatty acids; DMD, Dry matter degradability; CFD, crude fiber degradability; AB= *L.Acidophillus* + *L.Bulgaricus* ; CP= *L.casia* + *L.plantrum* ; LS= *B.Lichnoformas* + *B.subtillus*; ABL= *L.Acidophillus* + *L.Bulgaricus* + *B.Lichnoformas* ; CPS= *L.casia* + *L.plantrum* + *B.subtillus*; ABLB= *L.Acidophillus* + *L.Bulgaricus* + *B.Lichnoformas*+ *Bifidobuctrium bifidum*; CPSB= *L.casia* + *L.plantrum* + *B.subtillus*+ *Bifidobuctrium bifidum*.

TABLE 5. Predictive value as affected by different probiotic combinations and dosage levels.

	Predictive value					
	SCFA mmol	**ME (MJ/kg DM)**	**NEL (MJ/kg DM)**	**MCP (mg/g DM)**	**OMD (%)**	**PF (mgTDOM/Ml gas)**
Effect of a probiotic mixture						
AB	0.59^{c}	5.47^{c}	2.85^{c}	554.46	45.32^{c}	1.67^{ab}
CP	0.59^{c}	5.48^{c}	2.86^{c}	559.65	45.39^{c}	1.64^{ab}
LS	0.65^{b}	5.85^{b}	3.13^{b}	557.04	47.46^{b}	1.66^{ab}
ABL	0.62^{bc}	5.64^{bc}	2.98^{bc}	543.23	46.32^{bc}	1.62^{ab}
CPS	0.70^{a}	6.19^{a}	3.38^{a}	517.95	49.43^{a}	1.61^{b}
ABLB	0.61^{bc}	5.56^{bc}	2.92^{bc}	553.89	45.83^{bc}	1.64^{ab}
CPSB	0.60^{c}	5.47^{c}	2.86^{c}	531.36	45.40^{c}	1.70^{a}
Effect of level (× 10^{9} cfu/g feed)						
0	0.51^{c}	4.89^{c}	2.43^{c}	517.90^{b}	42.06^{c}	1.75^{a}
2	0.61^{b}	5.59^{b}	2.94^{b}	562.59^{a}	46.04^{b}	1.65^{b}
4	0.74^{a}	6.52^{a}	3.62^{a}	555.61^{a}	51.26^{a}	1.55^{c}
SEM	0.01	0.08	0.06	6.55	0.46	0.02
P-value						
probiotic mixtures	< 0.001	< 0.001	< 0.001	0.473	< 0.001	0.255
Level	< 0.001	< 0.001	< 0.001	0.009	< 0.001	< 0.001
Interaction	< 0.001	< 0.001	< 0.001	0.290	< 0.001	0.001

$^{a–c}$ Means in the same column bearing different letters differ significantly (P < 0.05); SEM indicates the standard error of the mean; SCFA, short-chain fatty acids; ME, metabolizable energy; NEL, net energy lactation; MCP, microbial crude protein production; PF, partitioning factor at 72 h of incubation; OMD, organic matter degradability; AB= *L.Acidophillus + L.Bulgaricus;* CP= *L.casia + L.plantrum;* LS= *B.Lichnoformas + B.subtillus;* ABL= *L.Acidophillus + L.Bulgaricus + B.Lichnoformas;* CPS= *L.casia + L.plantrum + B.subtillus;* ABLB= *L.Acidophillus + L.Bulgaricus + B.Lichnoformas+ Bifidobuctrium bifidum;* CPSB= *L.casia + L.plantrum + B.subtillus+ Bifidobuctrium bifidum.*

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تأثير اضافة تركيبات بروبيوتيك مختلفة في نظام غذائي عالي المركزات على إنتاج الغاز في المختبر وانبعاث الميثان وقابلية تحلل العناصر الغذائية في الأغنام

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الملخص

بحثت هذه الدراسة في تأثير خلطات مختلفة من البروبيوتيك على معايير التخمير وانبعاثات الميثان وقابلية تحلل العناصر الغذائية في النظام الغذائي في المختبر. كان خلطات البروبيوتيك البكتيرية المستخدمة هى:

1. *Lactobacillus acidophillus + Lactobacillus bulgaricus (AB).*
2. *Lactobacillus cassia + Lactobacillus plantrum (CP).*
3. *Bacillus lichnoformas + Bacillus subtillus (LS).*
4. *Lactobacillus acidophillus + Lactobacillus bulgaricus + Bacillus lichnoformas (ABL).*
5. *Lactobacillus cassia + Lactobacillus plantrum + Bacillus subtillus (CPS).*
6. *Lactobacillus acidophillus + Lactobacillus bulgaricus + Bacillus lichnoformas + Bifidobuctrium bifidum (ABLB).*
7. *Lactobacillus cassia + Lactobacillus plantrum + Bacillus subtillus + Bifidobuctrium bifidum (CPSB).*

تم استخدام مخاليط البروبيوتيك بمستويات صفر (كنترول)، 2، 4 × 10^9 وحدة تشكيل مستعمرة/جرام علف. لوحظت تأثيرات كبيرة في إنتاج الغاز عبر جميع أوقات الحضانة، حيث أظهر مزيج CPS أعلى إنتاج بعد 48 ساعة وأدنى مستوى لوحظ في ABLB وCPSB. انخفضت انبعاثات الميثان بشكل ملحوظ مع جميع مخاليط البروبيوتيك، حيث أظهر مزيج CP أكبر انخفاض. تأثرت قابلية تحلل المادة الجافة والألياف الخام بشكل كبير بمستويات الاضافة، وبلغت ذروتها عند 2 و4 × 10^9 وحدة تشكيل مستعمرة/جرام علف. تأثر إنتاج إجمالي الأحماض الدهنية المتطايرة (TVFA) بشكل كبير، حيث أنتجت مخاليط ABLB وCPSB أعلى مستوى من TVFA. علاوة على ذلك، لوحظت تأثيرات كبيرة لمستويات المكملات على إنتاج الأمونيا- نيتروجين والأحماض الدهنية المتطايرة الكلية. بالإضافة إلى ذلك، تأثرت قيم الأس الهيدروجيني بشكل كبير بمزيج البروبيوتيك ومستويات الاضافة، مع تسجيل درجة حموضة أقل عند مستويات مكملات أعلى. أشارت معظم النتائج إلى أن مزيج CPSB كان أفضل بروبيوتيك.

الكلمات الدالة: تركيبات البروبيوتيك، مكملات عذائية ، هضم الأعلاف، انبعاث الميثان، في المختبر.