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Effects of Different Levels of Selected Probiotic Strains and Highly Concentrated Based Diet on Methane Emission, Rumen Fermentation Parameters, and Nutrient Degradability in Sheep *In vitro*

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Abstract

THE CURRENT study aimed to investigate the effects of nine probiotic bacterial strains with different levels of supplementation in a highly concentrated diet in sheep on ruminal fermentation parameters in in vitro. the examined bacterial strains were *lactobacillus cassia* (LC), *lactobacillus plantarum* (LP), *lactobacillus acidophilus* (LA), *lactobacillus bulgaricus* (LB), *bacillus subtillus* (BS), *bacillus lichnoformis* (BL), *bifidobucterium bifidum* (BB), *enterococcus faecium* (EF), and *clostridium butyricum* (CB). the probiotics were tested at 0 (control), 0.25, 0.5, 1, 2, and 4×109 cfu/g feed. The gas production value decreased by LA and LP strains, while BL, EF, BB, and CB strains increased gas production. methane production was reduced by LC, LP, and BS strains, whereas it increased by BL and EF strains. BS, CB, and LP strains improved dry matter degradability (DMD), while LC, LB, and BB strains resulted in lower values. organic matter degradability was enhanced by the addition of strains such as BL, CB, LA, and LB. certain strains, including LA, LC, LP, and LB, reduced NH3-N production, while EF supplementation increased NH3-N levels. total volatile fatty acid production was generally enhanced by the addition of bacterial strains, except for LA and BS strains, which showed lower production. pH values were influenced by bacterial strains. the LC and LP exhibited the lowest pH, while the CB, LB, and LA strains had the highest pH values. in conclusion, the best strain was LP which reduced methane and NH_3-N production and improved DMD. The best improvement occurred with the high levels of addition.

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

Introduction

Manipulating the rumen microbial environment can improve ruminant animal productivity. Supplementing with probiotics is a safe and viable alternative to antibiotics. Using probiotics is better than antibiotics which don't have side effects like toxicity in livestock products and leave no residue [1]. Probiotics are live bacteria that improve the host's health and performance. [2]. The general health benefits of probiotic supplementation include the reduction of methanogens, control of acidosis, improved digestion, encouraging the growth of the rumen and intestinal epithelium, and increased nutrient absorption [3].

Microbial fermentation in the digestive system of ruminants produces methane (CH_4) and carbon dioxide (CO_2) [4]. Ruminants have energy utilization losses (2 to 12% of gross energy intake) due to CH_4 emission. Probiotics demonstrate the potential to manipulate rumen fermentation and increase livestock performance, which can help reduce emissions of greenhouse gases. [5]. Probiotic additives have been used to control ruminal fermentation, and prevent nutritional disorders [6]. Probiotics can enhance the growth of ruminal bacteria and increase the population of bacteria [7] by providing them with some nutrition, such as metabolic intermediates and vitamins [8]. A different theory is that probiotics may promote lactic acid-utilizing bacteria, resulting in a reduction in the production of lactic acid and therefore stimulating the growth of cellulolytic bacteria, which improves fiber digestion [8]. Furthermore, probiotics inhibit some ruminal bacteria producing H_2 or methyl-containing substances; hence, CH_4 will be lowered [9].

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Many kinds of Lactic acid bacteria (LAB) strains, the genera *Lactobacillus*, *Bifidobacterium*, and *Enterococcus*, are considered beneficial to the animal host and have been used as probiotics [10]. LAB have been used as probiotics in ruminant diets to increase the beneficial microflora population and reduce pathogenic microbial development. The LAB positively affects the ecosystem of microbes by establishing native gastrointestinal bacteria in newborn calves and contributing to the balance of microbial groups in the gastrointestinal system [11, 12]. Moreover, LAB reduces oxygen from the rumen environment, prevents excess of ruminal lactate production, and modulates the microbial balance [13]. However, Previous studies showed that LAB increases the yield of microbial biomass [14], reduces methane [15], and increases dry matter digestibility by ruminants [16].

Bacillus spp. can generate and release a wide variety and quantity of enzymes that may increase feed or nutrient utilization in ruminants [17, 18]. *Clostridium butyricum* can increase rumen fermentation and nutrient degradability in ruminants [19]. Few studies were carried out on using bacterial strain additives in greenhouse gas production and ruminal fermentation. Based on the beneficial effect of tested probiotic strains, it was hypothesized that bacterial strain additives could positively affect methane emission, ruminal fermentation parameters, and feed degradability.

This study aimed to evaluate the effect of using different bacterial strains as probiotics with different levels and high concentrate diet on *in vitro* gas production, methane emission, some ruminal fermentation parameters, and nutrient degradability

Material and Methods

The present study was carried out in the Laboratory of Animal Nutrition, Animal Production Department, Faculty of Agriculture, Zagazig University, Zagazig, Egypt.

Experimental Design and Probiotic Strain

Fifty-four treatments (9 × 6 factorial arrangement) were used to investigate the effects of nine strains of probiotics with six levels on rumen fermentation characteristics using *in vitro* gas production technique. The nine bacterial strains used were *Lactobacillus cassia, Lactobacillus plantrum, Lactobacillus Acidophillus, Lactobacillus Bulgaricus, Bacillus subtillus, Bacillus Lichnoformas, Bifidobuctrium bifidum, Enterococcus faecium,* and *Colostredium butyricum.* The probiotic strains were obtained from a commercial company in 10th of Ramadan city, Egypt. The preparations were in powder form consisting of the bacteria. The bacteria strains used were at levels 0 (control), 0.25, 0.5, 1, 2 and 4×10^9 cfu /g feed.

Diet and Chemical Analysis

The basal diet used was composed of 30% berseem hay (*Trifolium alexandrinum*) and 70% concentrate mixture (70% corn grain, 15% soybean meal, 13% wheat bran, 1.2% limestone, 0.5% salt, and 0.3 premix). The concentrate and berseem hay were finely powdered (1 mm) and mixed at a percent of 70:30 respectively. This dried diet was used for chemical analysis and *in vitro* gas production studies, the chemical composition of the diet is provided in Table 1. According to the Association of Official Analytical Chemists AOAC (2006), the sample was analyzed for dry matter (DM), ash, organic matter (OM), ether extract (EE), and crude protein (CP). Neutral detergent fiber (NDF) was analyzed by using the method of [20].

In vitro Incubations

Fresh rumen fluid was collected from five male Baladi sheep (8 months of age) using a soft plastic stomach tube before morning feeding to obtain stable rumen microbial cultures. Animals were fed on *ad libitum* a ration based on 50% forage (berseem hay) and 50% concentrate. The animals were fed the diet for 1 month before the rumen liquor samples were collected. Rumen fluids were quickly transported to the laboratory in a pre-warmed (39 °C) isolation flask and stored under anaerobic conditions until used. The rumen liquid was filtered using four layers of cheesecloth, then incubated in a water bath at 39 °C and saturated with CO_2 until inoculation.

The buffered incubation media (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). The MB9 media pH was adjusted to 6.8, and to maintain anaerobic conditions the CO_2 was flushed for 30 minutes [21]. The MB9 media was mixed with the rumen fluid at a 2:1 ratio (v/v). The incubation glass tubes that contain 200 mg of the diet (30% berseem hay and 70% concentrate) and probiotic strain at various levels were injected with thirty millimeters of mixed ruminal fluid, closed rapidly with a gas-release rubber stopper connected with a tri-way valve and a measured plastic syringe for measuring gas production. The gas production volume was measured during incubation times 3, 6, 12, 24, 36, and 48 hours, and a blank tube was used to adjust the total gas volume. Each run has four blank bottles (without substrate) and six bottles for each treatment. the model of Ørskov and McDonald [22] was used to calculate The kinetics of gas production: $y = a+b (1-e^{-ct})$

Where y = gas produced in ml at time t; a = The gas produced by the immediately soluble parts (ml); b = the gas produced from the insoluble fraction (ml); c = the gas production rate constant for the insoluble fraction b (h); a+b = the potential gas production in ml; t = incubation time (h).

At the end of incubation and after recording the final gas volume the methane emission was estimated by

using NaOH (10 M) according to Fievez, Babayemi [23], and the methane intensity (CH_4 ml/ TDDM, CH_4 ml/ TDOM, CH_4 percentage from total gas) was calculated.

Estimation Of pH, Ammonia-N, Volatile Fatty Acids Concentration, Partitioning Factor, and True Nutrient Degradation

At the end of in vitro incubation, a digital pH meter was used to measure the ruminal pH immediately. After 48 hours of incubation, 30 mL of neutral detergent solution was added to the contents of three tubes from each treatment and placed at 105 °C for three hours to determine truly degraded dry matter (DMD). Then, the residual DM weight was estimated after filtering each sample through pre-weighed Gooch crucibles and drying it at 105°C for three hours [24]. After that, it was used to estimate truly organic matter degradability (TOMD) according to AOAC [25]. The contents of another three tubes of each treatment were used to determine the concentration of NH_3-N and total volatile fatty acids (TVFA). TVFA concentration was determined using the steam distillation method, according to Warner [26]. The ruminal NH3-N concentration was measured by the method proposed by Conway [27]. The partitioning factor (PF) was estimated as the ratio of OM (mg) degradability to gas production volume (in mL after 24 h) [24]

Calculations

The equation of Menke and Steingass [28] was used to calculate the metabolizable energy and net energy of lactation. The concentration of short-chain fatty acids (SCFA) was calculated according to Getachew, Makkar [29]. Microbial crude protein biomass production was estimated, according to Blümmel, Steingaβ [24].

Statistical *Analysis*

The data in the main study were analyzed as a 9 x 6 factorial arrangement, with nine probiotic strains and 6 levels using SPSS 21 (Chicago, IL) software, based on the 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 probiotic strain, βj is the effect of levels, $\alpha\beta ij$ is the interaction between treatments (probiotic strain × levels), and e_{ijl} is the standard error of term. The significant differences in mean were analyzed by Duncan's multiple comparison test at $P < 0.05$ [30].

Results

Effect of Probiotics on Gas Production and Gas Kinetics

There were significant effects ($P < 0.001$) of bacterial strains on gas production and gas kinetics as presented in Table 2. Strains *L. cassia* and *L. Plantarum* exhibited the lowest values of gas production and gas kinetics compared to the other strains. Conversely, using of *B. lichnoformas, E. faecium, B. bifidum,* and *C. butyricum* strains resulted in an increase in gas production and gas kinetics values throughout the entire incubation period up to 48 hours. Furthermore, increasing the level of probiotic addition led to a significant increase in gas production throughout the incubation period from 6 hours of incubation up to 48 hours ($P < 0.001$). A similar trend of increasing gas kinetics values was observed with a higher dose of probiotics. Additionally, the interaction between the strain and the level of probiotics had a significant effect on both gas production during different incubation periods and gas kinetics values.

Effect of Probiotics on Methane Emission

The tested strains reduced CH_4 production in the form of ml /1g DM, ml /1g TDDM, ml /1g TDOM and % of total gas ($P < 0.001$) as provided in **Table 3**. Notably, *B. lichnoformas* and *E. faecium* strains exhibited the highest methane production. Among the strains, *L. cassia*, *L. plantrum* and *B. subtillus* showed the most decrease in CH_4 production compared to the other strains. Furthermore, an increase in the level of probiotic addition led to a significant decline ($P < 0.001$) in CH_4 emission (ml /1g DM, ml /1g TDDM, ml /1g TDOM and % of total gas). The interaction between probiotics and the level of addition had a significant ($P < 0.001$) impact on methane production.

Effect of Probiotics on Degradability Parameters

The addition of probiotic strains had a significant ($P < 0.001$) impact on DMD. Specifically, the supplementation of strains *B. subtillus*, *C. butyricum*, and *L. plantrum* led to an increase in DMD. Conversely, the addition of strains *L. cassia*, *L. bulgaricus*, and *B. bifidum* had lower values of DMD compared to the other strains. Increasing the dosage of probiotic addition contributed to an increase in DMD ($P < 0.001$). A significant ($P < 0.001$) interaction impact was observed between the probiotic strain and the addition level on DMD.

The degradability of organic matter was significantly ($P < 0.001$) influenced by all bacterial strains tested. Notably, strains *L. cassia*, *L. plantrum*, and *B. bifidum* exhibited the lowest values of OMD. In contrast, the addition of strains *B. lichnoformas*, *C. butyricum*, *L. acidophillus* and *L. bulgaricus* increased the OMD. Increasing the level of probiotic strains led to a significant ($P < 0.001$) improvement in the rate of OMD. Importantly, the interaction between probiotics and the level of addition demonstrated a significant ($P < 0.001$) effect on the OMD.

Effect of Probiotics on Fermentation Parameter

Data presented in Table 4 showed the bacterial strains employed in the study exerted a significant effect on ammonia-N production ($P < 0.001$). Specifically, the addition of strains *L. acidophillus, L. cassia, L. plantrum, and L. bulgaricus* resulted in the lowest levels of NH3-N production. In contrast, the supplementation of strain *E. faecium* led to the highest NH_3-N level among all the strains tested. Moreover, the addition of probiotics at level 2×10^9 cfu demonstrated a significant ($P < 0.001$) decrease in NH_3-N concentration compared to the control group. Conversely, the addition of probiotics at level 4×10^9 cfu resulted in the highest NH_3-N production when compared to the other levels. Notably, a significant interaction was observed between probiotics and supplement levels ($P < 0.001$), indicating their combined influence on NH_3-N production.

The supplementation of bacterial strains contributed to an overall significant ($P < 0.001$) increase in the production of TVFAs, except strains L. acidophillus and B. subtillus, which exhibited the lowest production compared to the other strains. Conversely, strains C. butyricum and B. bifidum showed the highest production values of TVFAs. Furthermore, increasing the level of probiotic addition was associated with an increase in the production of TVFAs ($P < 0.001$). There was a significant ($P < 0.001$) effect observed due to the interaction between probiotics and the level of addition in the production of TVFAs.

The obtained results of NH_3-N and TVFAs reflected the values of pH. A significant effect of bacteria strains on pH value was shown ($P < 0.001$). *L. cassia* and *L. plantrum* strains showed the lowest pH value. On the other hand, *C. butyricumits, L. bulgaricus* and *L. acidophillus* strains had the highest pH value compared to the other strains. Furthermore, increasing the level of probiotic addition led to a significant ($P < 0.001$) decrease in the pH value compared to the control group. The interaction between probiotics and the level of addition had a significant effect on the pH values ($P < 0.001$).

Effect of Probiotics on Predicted Value

The addition of all strains had a significant ($P < 0.001$) impact on the production of SCFA (mmol), ME (MJ/kg DM), NE_L (MJ/kg DM), MCP (mg/g DM), and PF (mgTDOM/mL gas). Strains *B. lichnoformas* and *E. faecium* resulted in increased values of SCFA, ME and NE_L compared to the other strains. Conversely, strains *L. cassia* and *L. plantrum* showed a noticeable decrease in the values of SCFA, ME and NE_L. Furthermore, increasing the level of probiotics led to an increase in the values of SCFA, ME and NE_L compared to the unsupplemented group ($P < 0.001$). There was a significant ($P < 0.001$) interaction observed between the probiotic strain and the level of addition on the values of SCFA, ME and NE_L.

The supplementation of strains *L. cassia* and *L. bulgaricus* showed a decrease in the MCP value compared to the other strains. In contrast, the addition of strains *L. plantrum* and *B. subtillus* led to an increase in the MCP values compared to the other strains. Increasing the level of probiotic addition led to an increase in the MCP value ($P < 0.001$). There was a significant ($P < 0.001$) interaction between the probiotic strains and the level of addition on MCP value.

Strains *L. cassia* and *L. plantrum* exhibited the highest PF values, whereas strains *B. bifidum* and *E. faecium* demonstrated the lowest PF values compared to the other strains. The addition of probiotics at different levels had significant ($P < 0.001$) effects on the PF value, with the lowest value observed at level 2×10^9 cfu. Importantly, there was a significant ($P < 0.001$) interaction observed between the probiotics and the level of addition on the PF value.

Discussion

The fermentation of nutrients in the feed is directly related to *in vitro* gas production and feed degradation. In the present study, the total gas production after 48 h of incubation increased significantly by supplementation of bacterial strains in all levels compared with control. Similarly, in another experiment, 14 *L. plantarum* strains increased gas production more than the control [1]. Increasing gas production by lactic acid bacteria may result from its survival in the rumen, affect the rumen microbiota, and change in vitro the fermentation parameter in the rumen [31, 32] However, the volume of gas produced by LAB may be different due to the strain and substrate used. Because LAB produces lactic acid, acetic acid, CO_2, and ethanol, it can be homo-fermenters or hetero-fermenters [9]. According to Getachew, Blümmel [33] The percentage of soluble, insoluble but degradable, and undegradable particles in the diet impacts the gas production kinetics. In the current study, the supplementation of a probiotic strain to highly concentrated degradable feed enhanced both the gas production from the insoluble but degradable component of the feed substrate and the potential gas production. Furthermore, There were negative values for gas production from the soluble fraction, similar trend was observed by Blümmel and Becker [34] and Chanthakhoun and Wanapat [35] in their studies of in vitro gas production. They attributed these results to a delay in fermentation due to late microbial colonization or an increase in the period of lag after the soluble fraction of the substrate was consumed but before the start of cell wall fermentation [34]. The different strains of lactic acid bacteria and

addition levels could have different impacts on rumen fermentation.

Preventing the generation of H_2 in the rumen or consuming it is one strategy to keep it out of the CH_4 production cycle. In the present study, the supplementation of probiotic strains had a significant reduction in methane emission parameters (CH_4 ml /1g DM, CH_4 ml /1g TDDM**,** CH_4 ml /1g TDOM**,** percentage of CH_4). Propionate and butyrate production in the rumen produces less H_2 than acetate production. This activity will be possible through the growth and promotion of the lactic acid-utilizing bacteria (LUB) [36]. LAB's effect on reducing CH_4 production could be attributed to its beneficial influence on LUB. LAB promotes the growth of LUB by continuously producing low concentrations of lactic acid [37], leading to improving ruminal pH [38] and causing a ruminal bio-fermentation shift to produce propionate and butyrate. The production of propionate is an H_2-consuming reaction [39]. Another beneficial effect of LAB on reducing CH_4 production could be attributed to the synthesis of bacteriocin. *Streptococcus equinus* produced bacteriocin (Bovicin HC5), which lowered the quantity of CH_4 by 53% [40]. Also, *Bacillus* species produce a variety of antimicrobials, including bacteriocins [41]. The variances between strains or their metabolites will provide varied abilities to modify rumen fermentation patterns and inhibit certain rumen microbes that produce H_2 or methyl-containing substances, which are the substrates for methanogenesis [9].

Increased net gas production, volume of gas produced from insoluble parts, and potential extent of gas production suggest an increase in substrate digestibility and activity of fiber-degrading microorganisms. In the current study, the supplementation with bacterial strains led to an increase in DMD and OMD. Ridwan, Bungsu [42] it was proposed that probiotics' beneficial stimulatory effects on the process of fermentation caused an increase in nutrient digestion. Weinberg, Muck [43] suggested that the interaction of rumen microorganisms with lactic acid bacteria improves rumen fermentation and prevents harmful microbes due to the production of antimicrobial compounds such as bacteriocins by lactic acid bacteria. supplementation of Probiotic has been suggested to promote the adaptability of ruminal microorganisms to the presence of lactic acid or decrease the accumulation of lactic acid in the rumen by degrading lactic acid to acetic acid [44, 45]. Jiao, Liu [46] suggested that these conditions support the cellulolytic bacteria activities and improve the digestion of feed and fibrous feeds by ruminal microbiota. This agrees with the present study, which improved DMD and OMD levels by supplementation probiotics. Cai, Hartanto [47] reported a significant increase in DMD by adding *Clostridium butyricum*. The ability of *Clostridium butyricum* to provide animals with short-chain fatty acids, amino acids, and vitamin B may be responsible for its effect on nutrient digestibility. Furthermore, it can produce several digestive enzymes such as lipase, amylase, and protease, which could improve the digestion of nutrients [47, 48].

Bacterial probiotics have a positive impact on the rumen environment by enhancing its development and promoting the stability of ruminal fermentation. To determine the effects of dietary treatments on a host animal, ruminant nutrition experiments often include measuring multiple parameters such as rumen ammonia-N, VFA, and pH value. It is commonly known that these parameters are closely related to the rumen microbes that are affected by the feed substrates and bioactive substances.

The decrease in ammonia-N concentration obtained may be due to the inclusion of more ammonia-N in the microbial protein Synthesis [49]. *Bacillus* probiotic supplementation reduces ruminal NH_3-N, which is likely associated with the increased ruminal capacity to absorb due to the larger surfaces of the rumen papillae [50], increased the population of total ruminal bacterial with a reduced population of protozoa [51], and enhanced ruminal nitrogen absorption by ruminal bacteria to synthesize microbial protein [52]. On the other hand, supplementing with 4×10^9 cfu of a probiotic strain increased ruminal NH_3-N concentration. The results were consistent with the results of [51, 52] They found that supplementing a *B. subtilis* in dairy cattle increased ruminal NH_3-N, which was attributed to improved degradation of dietary protein by increased populations of proteolytic bacteria in the rumen. In the current study, the high value of ammonia with *B. subtillus* is accompanied by a lower value of TVFA and a higher value of DMD and MCP, this may be related to the shifting in bacterial species and their ability to use ammonia.

TVFA in rumen fluid was significantly ($P < 0.001$) increased by different bacterial strains at all levels. Our results agree with [52] who found that dietary *B. licheniformis* supplementation increased rumen TVFA in dairy cows. Increased TVFA may be connected with the *B. licheniformis* specific specialization in the hydrolysis of starch and the usage of propionate as a carbon source [53]. Soriano, Mamuad [54] found a significant increase in individual and total VFA concentrations with the addition of 1% *L. mucosae* in *in vitro* incubation for 48 hours. In contrast, O'Brien, Hashimoto [55] reported that 5% (v/v) of *L. plantarum* TUA1490L lowered individual and TVFA levels, which the authors attributed to the presence of significant concentrations of hydrogen peroxide in the supernatant. Several in vitro experiments using microbial feed additives showed no impact on TVFA [56, 57] These inconsistent results reflect variances

among research in microbial species and strains, dosage, feeding regimens, physiological conditions, animal species, and other factors. Cai, Hartanto [47] reported a significant increase in ruminal pH and ammonia-N concentrate, TVFA by adding *Clostridium butyricum*.

The pH value is considered an effective indicator of suitable rumen conditions for fermentative activity and nutrient digestibility [58, 59]. In our study, the obtained results of NH_3-N and VFA reflected the pH values. All bacterial strains and all levels led to a decrease in the pH values. Several mechanisms have been suggested to explain the effect of microbial additions on pH, such as the competition with *S. bovis* and other lactobacillus species for the use of glucose [60], stimulation LUB [61] and modification of protozoa in the rumen [62] which compete with LAB for glucose absorption. Rapid fermentation of materials can cause significant changes in rumen conditions, such as increased lactic acid levels and lowered pH, contributing to metabolic acidosis [63]. The lower tendency of the pH with supplementation of probiotic strains could be related to the production of organic acids by the bacterium.

Probiotic effects utilized to regulate rumen fermentation were effective in terms of energy efficiency when the SCFA concentration changed because the volatile fatty acid met the majority of the daily energy requirements of ruminants [64]. This is consistent with the current study, where the supplementation of probiotic bacteria led to an increase in SCFA, ME (MJ/kg DM), and NEL (MJ/kg DM).

Conclusion

Supplementing the diet with all tested strains had different effects on feed degradability and the rumen fermentation parameters. The methane emission was reduced by *L. cassia, L. plantrum,* and *B. subtillus* strains, while *B. Lichnoformas* and *E. faecium* strains resulted in higher methane production. Specific strains, such as *L. acidophillus, L. cassia, L. plantrum,* and *L. bulgaricus*, reduced ammonia-N production, while *E. faecium* supplementation increased NH4-N levels. In addition, DMD increased with *B. subtillus, C. butyricum,* and *L. plantrum* strains. Additionally, OMD was enhanced by the addition of strains such as *B. lichnoformas, C. butyricum, L. acidophillus,* and *L. bulgaricus*. 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 concentrate mixture, berseem hay, and basal diet.

Nutrient (% on DM basis)	Concentrate mixture [a]	berseem hay	basal diet [b]
Organic matter	95.82	89.94	94.06
Crude protein	14.74	16.41	15.24
Ether extract	4.50	2.26	3.83
Neutral detergent fiber	47.59	53.46	49.35
Ash	4.18	10.06	5.94
Non-structural carbohydrates [c]	28.99	13.76	24.42

[a] concentrated mixture contains 70% corn grain, 15% soybean meal, 13% wheat bran, 1.2% limestone, 0.5% salt, and 0.3 premix
[b] The basal diet was a total mixed ration containing 30% berseem hay (*Trifolium alexandrinum*) and 70% concentrate mixture.
[c] Non-structural carbohydrates = 100 - (Neutral detergent fiber + Crude protein + Ether extract + Ash)

TABLE 2. Effect of probiotic strain, level, and interaction on cumulative gas production and gas kinetics.

	Gas production (ml/g DM)						Gas kinetics		
	3h	6h	12h	24h	36h	48h	B	c	ab
Effect of probiotic strain									
L. cassia	47.47^{bc}	93.37^{d}	128.06^{c}	155.87^{f}	172.88^{e}	179.13^{d}	186.78^{d}	0.12^{ab}	183.29^{c}
L.plantrum	58.65^{a}	105.31^{c}	128.19^{c}	153.89^{f}	175.28^{e}	181.94^{d}	166.84^{e}	0.10^{b}	187.23^{c}
L.Acidophillus	44.03^{cd}	102.47^{c}	151.04^{b}	188.75^{cd}	208.58^{bc}	213.75^{bc}	244.98^{b}	0.12^{ab}	214.04^{b}
L.Bulgaricus	42.78^{cd}	103.02^{c}	148.68^{b}	181.77^{de}	204.13^{cd}	213.19^{bc}	235.73^{bc}	0.12^{ab}	211.86^{b}
B. subtillus	57.53^{a}	112.01^{bc}	145.97^{b}	174.41^{e}	196.32^{d}	205.64^{c}	216.88^{c}	0.13^{ab}	208.41^{b}
B.Lichnoformas	63.82^{a}	122.50^{a}	167.64^{a}	203.68^{ab}	229.79^{a}	242.01^{a}	236.72^{bc}	0.11^{b}	241.95^{a}
Bifidobuctrium bifidum	51.11^{b}	117.50^{ab}	166.94^{a}	194.38^{bc}	213.13^{bc}	222.05^{b}	249.41^{b}	0.14^{a}	216.39^{b}
Enteroccous faecium	40.80^{d}	108.82^{bc}	167.33^{a}	213.23^{a}	238.47^{a}	249.44^{a}	276.16^{b}	0.11^{b}	247.66^{a}
Colostredium butyricum	58.58^{a}	124.24^{a}	169.41^{a}	198.78^{bc}	216.22^{b}	222.33^{b}	241.69^{a}	0.14^{a}	220.06^{b}
Effect of level ($\times 10^9$ cfu)									
0	69.93^{a}	109.81^{abc}	148.91^{b}	175.63^{c}	194.88^{d}	197.29^{d}	184.59^{c}	0.11^{c}	205.44^{c}
0.25	46.34^{bc}	102.75^{c}	145.28^{b}	179.86^{bc}	200.69^{cd}	210.12^{c}	226.80^{b}	0.12^{bc}	210.24^{c}
0.5	44.44^{c}	104.12^{bc}	152.87^{ab}	186.11^{b}	207.29^{abc}	216.64^{abc}	235.56^{b}	0.12^{bc}	213.53^{bc}
1	49.49^{bc}	111.13^{ab}	153.31^{ab}	183.82^{bc}	205.35^{bc}	214.42^{bc}	233.78^{b}	0.13^{ab}	213.63^{bc}
2	50.74^{b}	116.78^{a}	160.30^{a}	196.04^{a}	215.05^{a}	222.41^{ab}	254.94^{a}	0.15^{a}	220.14^{ab}
4	48.89^{bc}	114.88^{a}	154.84^{ab}	188.38^{ab}	213.26^{ab}	225.45^{a}	234.44^{b}	0.11^{c}	224.29^{a}
SEM	1.06	1.37	1.88	2.20	2.31	2.45	3.54	0.003	2.35
P-value									
probiotic strain	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.009	< 0.001
Level	< 0.001	< 0.001	0.020	< 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

[a–f] Means in the same column bearing different letters differ significantly (P < 0.05); SEM indicates the standard error of the mean; 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).

TABLE 3. Effect of probiotic strain, level, and interaction on methane emission parameter after 48 hours of incubation.

	methane emission parameter			
	ml /1g DM	ml /1g TDDM	ml /1g TDOM	% of total gas
Effect of probiotic strain				
L. cassia	25.81^{d}	39.45^{e}	44.83^{e}	14.79^{c}
L.plantrum	26.42^{d}	36.75^{e}	41.15^{f}	14.68^{c}
L.Acidophillus	36.49^{b}	52.23^{bc}	51.33^{cd}	17.13^{ab}
L.Bulgaricus	36.46^{b}	53.86^{b}	53.40^{c}	17.25^{ab}
B. subtillus	27.53^{d}	37.33^{e}	41.37^{f}	13.70^{c}
B.Lichnoformas	43.18^{a}	61.07^{a}	58.87^{b}	18.17^{a}
Bifidobuctrium bifidum	31.23^{c}	45.21^{d}	48.58^{d}	14.36^{c}
Enteroccous faecium	41.20^{a}	58.23^{a}	62.86^{a}	16.61^{b}
Colostredium butyricum	37.27^{b}	50.39^{c}	51.65^{cd}	16.84^{b}
Effect of level ($\times 10^9$ cfu)				
0	35.37^{a}	57.77^{a}	56.66^{a}	18.30^{a}
0.25	32.70^{b}	45.39^{b}	50.19^{bc}	15.56^{bc}
0.5	35.23^{a}	46.99^{b}	51.61^{b}	16.26^{b}
1	33.47^{b}	46.66^{b}	47.90^{c}	15.59^{bc}
2	34.12^{ab}	45.10^{b}	48.40^{c}	15.21^{c}
4	32.85^{b}	47.77^{b}	47.92^{c}	14.77^{c}
SEM	1.41	2.14	1.88	0.511
P-value				
probiotic strain	< 0.001	< 0.001	< 0.001	< 0.001
Level	0.003	< 0.001	< 0.001	< 0.001
Interaction	< 0.001	< 0.001	< 0.001	< 0.001

[a–f] Means in the same column bearing different letters differ significantly (P < 0.05); SEM indicates the standard error of the mean.

Table 4. Effect of probiotic strain, level, and interaction on fermentation and degradability parameter.

	Degradability parameter		Fermentation parameter		
	DMD	**OMD**	**AMONIA mg/100 ml**	**TVFA Meq/L**	**pH**
Effect of probiotic strain					
L. cassia	66.14^{f}	67.40^{c}	26.91^{c}	217.28^{cd}	4.84^{d}
L.plantrum	74.03^{b}	67.58^{c}	26.01^{c}	215.89^{cd}	4.89^{d}
L.Acidophillus	70.28^{cde}	74.57^{a}	25.98^{c}	192.11^{e}	5.27^{a}
L.Bulgaricus	68.03^{ef}	74.92^{a}	27.81^{c}	215.67^{cd}	5.26^{a}
B. subtillus	77.17^{a}	70.53^{b}	33.31^{b}	197.22^{e}	5.09^{bc}
B.Lichnoformas	71.00^{cd}	76.88^{a}	29.31^{bc}	226.33^{c}	5.14^{b}
Bifidobuctrium bifidum	69.97^{de}	67.61^{c}	32.53^{b}	243.11^{b}	5.01^{c}
Enteroccous faecium	72.75^{bc}	68.86^{bc}	41.39^{a}	212.67^{d}	5.06^{bc}
Colostredium butyricum	74.22^{b}	75.88^{a}	29.95^{bc}	256.72^{a}	5.30^{a}
Effect of level (× 10^{9} cfu)					
0	61.28^{d}	65.90^{b}	30.50^{b}	175.11^{d}	5.26^{a}
0.25	73.30^{b}	72.24^{a}	27.89^{bc}	220.52^{bc}	5.08^{b}
0.5	76.52^{a}	73.28^{a}	28.49^{bc}	218.44^{c}	5.14^{b}
1	73.56^{b}	72.90^{a}	29.62^{bc}	229.67^{ab}	5.08^{b}
2	75.52^{a}	73.47^{a}	26.63^{c}	234.37^{a}	5.11^{b}
4	68.89^{c}	71.68^{a}	39.02^{a}	239.89^{a}	4.90^{c}
SEM	0.66	0.48	0.68	3.31	0.02
P-value					
probiotic strain	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
Level	< 0.001	< 0.001	< 0.001	0.001	< 0.001
Interaction	< 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; TVFA is the total volatile fatty acids; DMD, Dry matter degradability; OMD, organic matter degradability.

TABLE 5. Effect of probiotic strain, level, and interaction on predictive value

	Predictive value				
	SCFA mmol	**ME (MJ/kg DM)**	**NEL (MJ/kg DM)**	**MCP (mg/g DM)**	**PF (mgTDOM/mL gas)**
Effect of probiotic strain					
L. cassia	0.68^{f}	6.10^{f}	3.31^{g}	590.90^{de}	2.23^{ab}
L.plantrum	0.69^{f}	6.12^{f}	3.34^{g}	641.47^{a}	2.34^{a}
L.Acidophillus	0.83^{cd}	7.14^{cd}	4.07^{de}	600.29^{cde}	2.01^{cde}
L.Bulgaricus	0.80^{de}	6.98^{de}	3.96^{ef}	586.36^{e}	2.09^{bcd}
B. subtillus	0.78^{e}	6.75^{e}	3.79^{f}	627.55^{ab}	2.17^{abc}
B.Lichnoformas	0.90^{ab}	7.67^{ab}	4.47^{ab}	603.4^{cde}	1.95^{de}
Bifidobuctrium bifidum	0.85^{c}	7.31^{c}	4.21^{cd}	609.83^{bcd}	1.86^{ef}
Enteroccous faecium	0.94^{a}	7.92^{a}	4.64^{a}	601.86^{cde}	1.71^{f}
Colostredium butyricum	0.87^{bc}	7.46^{bc}	4.31^{bc}	621.41^{abc}	1.98^{cde}
Effect of level (× 10^{9} cfu)					
0	0.77^{c}	6.78^{c}	3.82^{c}	529.19^{d}	2.06^{ab}
0.25	0.79^{c}	6.89^{c}	3.89^{c}	627.16^{b}	2.16^{a}
0.5	0.84^{b}	7.19^{b}	4.11^{b}	644.87^{a}	1.98^{b}
1	0.80^{bc}	6.97^{bc}	3.95^{bc}	623.55^{b}	2.07^{ab}
2	0.88^{a}	7.50^{a}	4.34^{a}	626.85^{b}	1.93^{b}
4	0.81^{bc}	6.97^{bc}	3.95^{bc}	603.74^{c}	2.03^{ab}
SEM	0.01	0.07	0.05	4.56	0.04
P-value					
probiotic strain	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
Level	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
Interaction	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001

$^{a–f}$ 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.

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تأثير مستويات مختلفة من سلالات مختارة من البروبيوتيك والنظام الغذائي عالي المركزات على انبعاث الميثان وقياسات التخمر في الكرش وهضم العناصر الغذائية في الأغنام معملياً

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الملخص

هدفت الدراسة الحالية للتحقيق في تأثيرات تسع سلالات بكتيرية كبروبيوتيك بمستويات مختلفة كإضافات لنظام غذائي عالي التركيز على معايير تخمير الكرش في المختبر. كانت سلالات البكتيريا المدروسة هي (Lc) *Lactobacillus cassia*، *Lactobacillus plantarum* (Lp)، *Bacillus subtillus* (Bs) ، *Lactobacillus bulgaricus* (Lb) ، *Lactobacillus acidophilus* (La) ، *Bacillus lichnoformis* (Bl) ، *Enterococcus faecium* (Ef) ، *Bifidobucterium bifidum* (Bb) و *Clostridium butyricum* (Cb). تم اختبار البروبيوتيك عند 0,0 (المجموعة الضابطة)، 0.25، 0.5 ، 1 ، 2 و4 × 10^9 وحدة تشكيل مستعمرة / جرام من العلف. انخفضت قيمة إنتاج الغاز بواسطة سلالات La وLp، بينما ادت سلالات Bl، Ef، Bb وCb الى زيادة إنتاج الغاز. انخفض إنتاج الميثان بواسطة سلالات Lc، Lp وBs، بينما زاد الميثان بواسطة سلالات Bl وEf. حسنت سلالات Bs، Cb وLp هضم المادة الجافة، بينما ادت سلالات Lc، Lb وBb الى قيم أقل. تم تحسين هضم المادة العضوية عن طريق إضافة السلالات Bl، Cb، La و Lb. في حين قللت سلالات معينة، مثل La، Lc، Lp وLb من إنتاج الامونيا، بينما أدت اضافة Ef إلى زيادة مستويات الامونيا. تم تعزيز إنتاج إجمالي الأحماض الدهنية الطيارة بشكل عام عن طريق إضافة السلالات البكتيرية، باستثناء سلالات La و Bs والتي أظهرت إنتاجًا أقل. تأثرت قيم الأس الهيدروجيني بالسلالات البكتيرية حيث أظهرت سلالات Lc وLp أدنى درجة حموضة، بينما كان لدى سلالات Cb، Lb وLa أعلى قيم الأس الهيدروجيني. في الختام، كانت أفضل سلالة هي Lp التي قللت من إنتاج الميثان والامونيا وحسنت هضم المادة الجافة. لقد حدث أفضل تحسن مع المستويات العالية من الإضافة.

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