Indian J. Anim. Nutr. 2020. 37 (2): 132-137
doi: 10.5958/2231-6744.2020.00022.5

Indian Journal of Animal Nutrition

Effect of Supplementing Conjugated Linoleic Acid Producing *Bifidobacteria*l Strains on *In vitro* Rumen Fermentation Attributes

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ABSTRACT

The present study was undertaken to evaluate the effects of supplementation of four conjugated linoleic acid (CLA) producing strains of *Bifidobacteria* isolated from rumen fluid samples of Murrah buffalo (*Bubalus bubalis*) on ruminal fermentation attributes based on a basal diet having roughage: concentrate in a ratio of 70:30. Results of the study showed that dosing *Bifidobacterium* strains (10^9 cells/ml) separately into *in vitro* incubation tubes neither affect the total gas nor methane (CH_4) production after 24 h of incubation. Digestibility parameters showed no significant ($P>0.05$) changes with respect to *in vitro* dry matter digestibility (IVDMD), *in vitro* organic matter digestibility (IVOMD), or total ammonia-N content of fermentation fluid. However, volatile fatty acid (VFA) concentration was observed to be significantly increased ($P<0.05$) in the treatment samples with bacterial strains NB-191 and NB-184. While analyzing CLA content of incubation medium, a significant increase in the value was observed as compared to control tubes. Therefore, it can be concluded that the addition of potential CLA producing *Bifidobacteria*l isolates to mixed rumen culture increased the CLA and VFA content without affecting digestibility and fermentation parameters.

Key words: *Bifidobacteria*; Conjugated linoleic acid; IVDMD; Murrah buffaloes; Rumen fermentation

INTRODUCTION

In present time, manipulation of rumen microbial ecosystem by supplementation of probiotics and prebiotics to enhance feedstuff utilization and improve production efficiency is a key area of research pursued by both ruminant nutritionists and microbiologists (Sharma *et al.,* 2011; Chaturvedi *et al.,* 2014). Most researches using direct-fed microbials (DFM) have generally indicated positive responses (Newbold *et al.,* 1990). The bacterial strains most commonly used as DFM are *Lactobacillus*, *Lactococcus*, *Enterococcus* and *Bifidobacterium*, which are categorized as lactic acid bacteria (LAB) and are normal gut inhabitants in most of the animals. LAB is reported to improve the dry matter (DM) digestibility and decreased ruminal CH_4 production (Cao *et al.,* 2011). Among LAB, *Bifidobacteria* have been confirmed to have specific health benefits that can be mediated through enhancement of vitamin synthesis and mineral bioavailability (Jena *et al.,* 2017; Teraguchi *et al.,* 1984), improvement of immune function (Schiffrin *et al.,* 1997), reduction of gastrointestinal disturbances (Hotta *et al.,* 1987; Ballongue *et al.,* 1993). Further, Bifidobacterium may accelerate production of CLA having functional properties like anti-carcinogenic, anti-obesity, antioxidant and anti-inflammatory effects (Kim *et al.,* 2016). Ruminant products are reported to have highest concentration of CLAs (Alfaia *et al.,* 2017). Despite having such beneficial effects, *Bifidobacteria* have not been investigated as a feed additive in Murrah buffalos. To the best of our knowledge, there are no published reports documenting a response to *in vivo* and/or *in vitro* supplementation of anaerobic *Bifidobacteria*l cultures on nutrient digestion by Murrah buffaloes. Therefore, the study was undertaken to examine the effects of *Bifidobacteria* supplementation on *in vitro* rumen fermentation attributes in Murrah buffalo model.

MATERIALS AND METHODS

In vitro trials were carried out as per the procedure described by Menke and Steingass (1988) in triplicates. Rumen fluid was obtained 2 h after morning feeding from two fistulated buffalo bulls maintained on a standard diet (70 parts roughage: 30 parts concentrate) in a pre-warmed container. Ruminal feed

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particles were allowed to settle to the bottom (5 min), and finally the fluid content was strained through two layers of nylon cloth (50 mm pore size). Particle-free ruminal fluid was mixed with the buffer solution (Menke and Steingass, 1988) in a proportion 1: 2 (v/v) at 39°C anaerobically under a constant stream of O_2 free CO_2.

To evaluate the effect of supplementing different strains of *Bifidobacteria*, a substrtae was prepared (Table 1) by mixing roughage and concentrate in the ratio of 70: 30 to simulate a commonly fed total mixed ration (TMR). The roughage portion consisted of fresh berseem and the concentrate mixture consisted of maize-33%, groundnut cake-18%, mustard cake (expeller)-20%, cotton seed cake-5%, wheat bran-20%, de-oiled rice bran-6%, bajra-5%, mineral mixture-2% and common salt-1%. Proximate principles (AOAC, 2005) and fibre fractions (Van Soest *et al.,* 1991) were determined for individual ingredients and the substrate.

Four bacterial isolates namely NB-19, NB-81, NB-179 and NB-184, isolated from Murrah buffalos were used in the study. One millilitre of each bacterial isolate (10^9cells/ml) was added in each glass syringe except control (100 ml) (Fortuna Optima, Germany) containing 200±5 mg of substrate with 30 ml of buffered rumen liquor. Three blank syringes containing only 30 ml of buffered rumen fluid were included to correct gas production values for the gas released from endogenous substrates. Syringes were closed using clamps and petroleum jelly was applied on the plungers of syringes to avoid friction and any leakage. Syringes were incubated for 24 h at 39±0.5°C. The corrected gas values (net gas) were used for calculation of partitioning factor (PF) and microbial biomass production (MBP). After 24 h incubation, fermentation was arrested by chilling the syringes to 4°C, and the quantities of fermentation products were determined in each syringe. The *in vitro* gas production technique was employed to estimate the parameters such as total gas production, CH_4 production, ammonia nitrogen (NH_3-N) concentration and individual VFA's (Menke *et al.,* 1979; Menke and Steingass, 1988). Three trials were run for each treatment and each treatment was performed in triplicates. CH_4 content in fermentation gas was determined by gas chromatography (GC) using Nucon-5765 gas chromatograph equipped with a flame ionization detector (FID) and a stainless steel column packed with Porapak-Q (length 6';o.d.1/8" i.d. 2 mm; mesh range 80-100). After collection of gas sample the contents of each syringe were centrifuged at 3000 rpm for 15 min to get a clear supernatant and pellets. To estimate ammonia nitrogen (NH_3-N) an aliquot of supernatant was acidified with equal volume of 0.5 M HCl and the acidified supernatant (5 ml) was mixed with 10 ml of NaOH (1 N) and immediately steam distilled using KEL PLUS - N analyser (Pelican, India). Boric acid (20% w/v) solution was used for ammonia collection and titrated against N/100 sulphuric acid. Another aliquot of supernatant (4 ml) was added to 25% m-phosphoric acid (2 ml), kept overnight at 4°C and centrifuged at 2000 rpm for 15 min and supernatant was stored at -20°C VFA analysis (Erwin *et al.,* 1961). The pellets obtained after centrifugation were refluxed with neutral detergent solution for an hour (Van Soest *et al.,* 1991) where loss in weight was considered as true dry

Table 1. Chemical composition of ingredients and substrates used in this study

Nutrients	Berseem	Concentrate	Mixed substrate
Dry matter	14.34±0.70	88.12±0.22	51.23±0.60
Organic matter	90.22±0.41	90.24±0.48	90.21±0.21
Crude protein	16.78±0.66	18.21±0.95	17.49±0.34
Ether extract	1.01±0.36	3.47±0.22	2.07±0.12
Total	9.04±0.74	7.21±0.64	7.95±0.35
Neutral detergent fibre	54.69±1.21	23.96±0.87	45.25±0.52
Acid detergent fibre	28.78±1.35	10.64±0.45	23.22±0.10

matter digestibility. Residue was further subjected to ashing at 550°C for estimation of *in vitro* organic matter digestibility (IVOMD). The PF and MBP were calculated based on truly degraded organic matter (TDOM) as described by Blummel *et al.* (1999) and Blummel *et al.* (2005), respectively.

PF= mg TDOM/total gas production

TDOM was calculated by multiplying TOMD (%) by mg OM content of substrate.

Microbial biomass production (MBP) was calculated from TDOM using equation:

MBP (mg) = TDOM (mg) – (Net gas volume×2.20)

Where 2.20 is the stoichiometric factor

CLA content of the rumen fluid was estimated by UV-spectrophotometric method. In brief, after incubation, fermentation fluid (1 ml) was centrifuged at 18,000 rpm for 1 min, the pellet was discarded and the supernatant was mixed with 2 ml of isopropanol by vortexing and allowed to stand for 3 min. The fatty acids were extracted following the addition of 1.5 ml of hexane. The presence of CLA in the culture supernatant was assayed spectrophotometrically by dispensing 230 µl of the fat-soluble hexane layer into a UV-transparent 96-well plate for determining the absorbance at 233 nm using a 96 well plate spectrophotometer.

The data were subjected to one-way analysis of variance procedure of SPSS (2010), using the linear model. The post-hoc comparison of means was done for the significant difference by Tukey's test. Significant differences of treatments were considered at $P<0.05$.

RESULTS AND DISCUSSION

Results indicated that supplementation of bacterial isolates had no effect on gas production as compared to control (Table 2). The *in vitro* gas production technique has been used widely to study feed degradation (Rymer *et al.,* 2005); it can provide valuable information on the kinetics of feed digestion in rumen and reflect the utilisation efficiency of fermentation substrates (Metzler-Zebeli *et al.,* 2012). Previous studies also reported that LAB survive in rumen, affects rumen microflora, and changed the *in vitro* ruminal fermentation (Weinberg *et al.,* 2004; Gollop *et al.,* 2005). Our results indicated that supplementation of *Bifidobacteria* did not affect the total gas production. This agrees with Jena *et al.* (2017) who reported that *Bifidobacteria* did not produce gas on

Table 2. Effect of the *Bifidobacteria*l isolates on rumen fermentation and CLA production

Parameter	Bifidobacterium Strain				
	Control	NB-191	NB-81	NB-179	NB-184
Net gas (ml)	32.23^{a}±0.179	33.02^{ab}±0.423	33.08^{ab}±0.318	33.97^{b}±0.241	33.03^{ab}±0.453
CH_4 (%)	33.01±0.353	33.71±0.147	33.58±0.543	34.39±0.659	34.43±0.723
CH_4 (ml)	8.07±0.11	8.12±0.13	8.10±0.16	9.10±0.31	9.14±0.41
NH_3-N (mg/100 ml)	13.08±0.68	13.67±0.87	14.08±0.38	13.63±0.29	14.99±0.26
IVDMD (%)	60.48±0.63	62.26±1.04	62.03±0.25	61.25±0.46	61.59±0.19
IVOMD (%)	61.86±0.92	63.34±0.87	63.45±0.41	61.70±0.45	62.80±0.16
Acetate (mmol/L)	41.11^{a}±0.46	49.45^{b}±0.83	45.28^{ab}±1.82	43.24^{a}±0.89	49.94^{b}±1.19
Propionate (mmol/L)	14.47±1.53	18.20±0.23	17.20±0.71	17.07±1.39	15.60±0.96
Butyrate (mmol/L)	8.4±0.95	11.37±0.12	9.70±1.01	9.40±0.62	10.93±0.18
PF	3.59±.06	3.59±.09	3.59±.04	3.39±.02	3.55±.03
MBP (mg/200mg DM)	43.35±1.97	44.15±2.76	44.42±1.01	38.94±.79	43.12±.46
CLA (µg/mL)	15.23±0.11^{a}	18.09±0.62^{b}	17.74±0.34^{b}	18.84±1.00^{b}	18.52±0.41^{b}

IVDMD, *in vitro* dry matter digestibility; IVOMD, *in vitro* organic matter digestibility; P,- partitioning; MBP, microbial biomass production; CLA, conjugated linoleic acid; ^{a,b} means bearing superscript differs significantly in a row ($P<0.05$).

substrate fermentation

Approximately 6-15% of feed energy is lost in the formation of methane (Johnson and Johnson, 1995). Santoso *et al.* (2003) reported that CH_4 production could be affected by the nature of the carbohydrates being fermented; cellulose and hemicellulose are important fibre fractions that influence CH_4 production. However, we found that CH_4 production was not affected with the addition of *Bifidobacteria*l strains which may be because of differences between fermentation substrates (single fermented cell wall substrates compared to total mixed substrates).

The data revealed that incorporation of CLA producing bacteria had no significant effect on IVDMD and IVOMD (Table 2). IVDMD reflects the degree of degradation of substrates by microorganisms in fermentation systems. LAB do not possess the enzymatic ability to hydrolyse cell-wall constituents (Rooke and Hatfield, 2003), and the activity of cellulolytic bacteria may not have been affected with the addition of *Bifidobacteria.* Although Reich and Kung (2010) reported that a combination of *Lactobacillus buchneri* 40788 with *Lactobacillus plantarum* or *Pediococcus acidilactici* tended to increase *in vitro* neutral detergent fibre degradation (IVNDFD) in treated compared to untreated silage. However, no such results with supplementation of *Bifidobacteria* have been reported till now. So, further study is needed to investigate the effect of *Bifidobacteria* supplementation on *in vitro* rumen fermentation.

NH_3-N production was similar in all the isolates and varied in a range of 13.67±0.87 to 14.99±0.26 mg/ 100 ml indicating no adverse effect of bacterial isolates on this fermentation parameter (Table 2). Optimum ammonia levels vary from 50 mg/l (Satter and Slyter, 1974) to 190 mg/L. Deficiency of NH_3-N restricts microbial protein synthesis, while high concentrations of NH_3-N also inhibit microbial utilization of this compound (Hristov *et al.,* 2002). The acquisition and assimilation strategies followed by members of *Bifidobacteria* to retrieve nitrogen from the gut lumen are still largely unknown (Ferrario *et al.,* 2015). When similar experiment was conducted with *L. acidophilus* a significant increase in ammonia-N was reported (Chen *et al.,* 2017) which might be due difference in substrate availability or /and different nitrogen assimilation pathways between microbes. So, further research is required to investigate the effect of *Bifidobacteria* on protein metabolism in rumen.

PF and efficiency of MBP synthesis varied from 3.39 to 3.59, and 38.94 to 44.15 respectively; having no significant difference from control diet (Table 2). Normal physiological range for PF varies from 2.75 to 4.41 (Blummel *et al.,* 1997) and PF values of the present study lies within this normal range. Results of this experiment were in agreement with the findings of Sarkar *et al.* (2017) who reported 3.63 and 40.23 for PF and MBP, respectively on similar type of substrate.

Data on individual VFA is presented in table 2. Acetate concentration was higher in NB-191 and NB-184, and lower in control and NB-179, however, its level was similar between NB-81, NB-171 and control group. Proportions of propionate and butyrate did not differ significantly between the control and treatment groups. Kumar *et al.* (2013) reported that supplementation of *Saccharomyces cerevisiae* or lactobacilli alone or in combination of both cultures had no effect on total VFA concentration. The values of VFAs in the present study were lower than those reported by Kaur *et al.* (2017) from similar type of substrate. In another *in vitro* study performed by Zicarelli *et al.* (2011) having same roughage to concentrate ratio a higher level of VFA production was reported. The difference could be due to different green fodder used and also due to the variation in the ingredient composition of the of the substrate (Table 1).

A significant increase was observed on CLA production by supplementation of *Bifidobacteria* isolates (Table 2). Hussain *et al.* (2016), reported strain specific CLA producing ability of *Butyrivibrio spp.* Similarly Jaglan *et al.* (2019) investigated the conjugated linoleic acid production potential of *bifidobacterial* isolates from ruminal fluid samples of Murrah buffaloes and found that *B. thermophilum* and *B. pseudolongum* produced cis9, trans11 CLA isomer. Results of this study further testifies the CLA producing capability of

Bifidobacterial isolates, which is reflected in the results obtained. In covenant with the present findings various workers have also reported increase in CLA production with supplementation of different bacterial isolates (Puniya *et al.,* 2008; Shivani *et al.,* 2016). The conversion rate of linoleic acid to CLA is very high at reasonably high concentration of linoleic acid, so relatively high CLA concentration could be maintained if this strain is stable at ruminal condition.

CONCLUSIONS

The results conclusively revealed that addition of potential CLA producing *Bifidobacteria*l isolates to mixed rumen culture increased the VFA and CLA content without affecting digestibility and fermentation attributes. Such a development is particularly significant because this study demonstrates that the introduction of a microorganism with CLA producing potential into the rumen may improve functional worth of ruminant products. However, *in vivo* trials are necessary before advocating these findings to feed industry.

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Received on 10-08-2020 and accepted on 23-08-2020