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IN-VITRO EVALUATION OF PROBIOTIC BACTERIA SUPPLEMENTATION TO RUMINANT RATIONS

[31]

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ABSTRACT

The aim of this study was to evaluate effect of different level of probiotic supplementation to ruminant rations, using *in-vitro* batch culture technique to determine degradation and fermentation parameters. *In vitro* experimental ration was formulated, the ration consisted of 40% alfalfa hay and 60% concentrate feed mixture. Three level of probiotic supplementation (10^6, 10^8, 10^{10} cfu/kg DM) were evaluated. DM and total gas production as well as fermentation parameters of the incubated samples were determined after 24 hrs. of fermentation. Slightly increases (P>0.05) in *in-vitro* dry matter degradability were observed for the ration supplemented with probiotics bacteria at different levels (10^6,10^8 and 10^{10} cfu/ kg DM) compared to control ration. Probiotics bacteria supplementation with different level (10^6,10^8 and 10^{10} cfu/ kg DM) led to significant (P<0.001) increases in organic matter degradability and total gas production per sample and per g DM, OM, NDF and ADF compared to the not supplemented ration (control ration), and no significant differences were observed among the different levels of probiotics supplementation. Significant increase in total volatile fatty acid concentration after 24 hours' incubation period compared to the not supplemented ration. On the other hand, the treatment supplemented with probiotic recorded lower ammonia concentration compared to the control group. It could be concluded that, adding probiotics bacteria supplementation to experimental ration resulted increase DM and OM degradability and using dose 10^6 CFU/kg DM feed is sufficient to induce improvement in degradability and fermentation parameters.

Keywords: *in-vitro*, probiotic, ruminant, fermentation.

INTRODUCTION

Enhancement of animal productivity, efficiency of feed utilization and animal health are the main goal of rumen microbial studies. These aims could be achieved by producing a desirable fermentation product as probiotics or direct fed microbial (DFM). Many of the feed additives have been used to improve animal productivity and feed utilization efficiency. The probiotics are microbial growth promoters that could be manipulating the rumen fermentation characteristics in intestinal tracts of livestock animals **(Weiss et al 2008).**

The name probiotic comes from the Greek 'pro bios' which means 'for life'. The term “probiotic” has been defined as “a live microbial feed supplement, which affects beneficially of the host animal through improving the microbial balance in the intestine” **(Fuller, 1989).** Also, they are known as direct-fed microbial (DFM). Probiotic or DFM have been used to describe viable microorganisms, culture extracts, enzymes, exopolysaccharides or

various combinations of them **(Yoon and Stern, 1995).**

The use of probiotic additives has been developed as alternatives to antibiotics to improve animal health and productivity **(Allen et al 2013),** Probiotic supplements were also shown to increase carcass output and water holding capacity, and decrease cooking loss and meat hardness **(Ceslovas et al 2005).** Lactobacillus bacillus as a probiotic has several potential benefits like growth promotion of farm animals **(Tripathi and Karim, 2009),** protection against pathogens **(Casas and Dobrogosz, 2000),** alleviation of lactose intolerance **(Mustapha and Savaiano, 1996)**, relief of constipation, antic-holesterolemic effect, reduction of gut pH by stimulating the lactic acid producing microflora, competition with pathogens for a viable nutrient **(Edens, 2003)** and immunomodulation **(Aottouri et al 2002).**

The objectives of this study were to compare the effect of different levels of probiotic supplementation to ruminant ration on in-vitro degradation and fermentation parameters.

MATERIAL AND METHODS

Probiotic bacteria

Microbial strains and growth condition

The probiotic bacteria used in this study is a mixture of 15 isolate of *lactobacillus sp*. Lactobacilli isolates were grown on MRS broth (Oxoid) and Streptococci isolates were grown on M17 broth (Difco), after that the broth media incubated for 24 h at 37 ºC. The strains were activated two or three times in order to obtain high biomasses in the stationary phase

Experimental ration and treatments

In-vitro experimental ration was formulated; the tested ration contains 60:40 concentrate: roughage ratio. **The CFM consisted of** 60.89 % corn, 27.13 % soybean, 8.23 % flaxseed, 0.79% limestone 0.99 % sodium bicarbonate, 0.59 di-calcium phosphates, 0.40 trace premix and 0.79 salt. The data of chemical composition of the feed ingredients and tested rations are presented in **Table (1).** Four level of probiotic bacteria supplementation were applied 0, 10^6, 10^8 and 10^{10} CFU /kg DM of the tested ration.

Table 1. The chemical composition of the feed ingredients and tested rations

Item	alfalfa	Concentrate feed mixture
Dry matter	889.5	890.9
Organic matter	878.7	933.7
Neutral detergent fiber	460.6	184.3
Acid detergent fiber	359.7	59.4
Acid detergent lignin	41.6	10.4
Crude protein	208.5	157.3
Ether Extract	28.4	47.4
Ash	121.3	66.3
Non-fiber carbohydrate	181.2	544.7

In-vitro gas production technique

Two days before beginning of the experiment, 400 (240 mg concentrate +160 mg alfalfa hay) ± 4 mg of sample for each treatment was weighed into 125 mL glass bottles. These bottles have a total volume of 125±2 mL. A buffer solution was prepared before addition of rumen fluid as described by **McDougall (1948)** and flushed continuously with CO2 at 39°C during sample inoculation. Rumen fluid was obtained from slaughter house and it was collected from beef steers. The collected rumen fluid was mixed into a bottle (1L) with an O_2-free headspace and immediately transported to laboratory at 39°C. Upon arrival at the laboratory, the rumen fluid was filtered through four layers of cheesecloth to eliminate large feed particles. The buffer solution was added to rumen fluid at ratio 4:1. forty mL of this inoculum was added to each bottle, then the headspace of each bottle was flushed with CO_2, and closed. The initial pH of the inoculums was from 6.8-6.9. Triplicates of each sample were used for each treatment.

Degradability

Dry matter degradability (% DMD) was calculated as the (difference between the sample DM content and that in the residual after 48 h incubation / sample DM content * 100).

Total gas production

After 24 h of samples incubation, the total gas production was estimated by the displacement of syringe piston, which was connected to the serum flasks. The gas produced due to fermentation of

substrate was calculated by subtracting gas produced in blank vessels (without substrate) from total gas produced in the vessels containing buffered rumen fluid and substrate.

Calculation

In-vitro organic matter digestibility (OMD, g/kg OM) were estimated according to **(Menke and Steingass, 1988)** as:

OMD= 14.88+ 0.889 GP+ 4.5 CP (%)+ 0.0651 ash (%)

where GP is net GP in mL from 200 mg of dry sample after 24 h of incubation

After 24 hr of incubation, the filtrated rumen liquor for each sample was subjected for further investigation. The pH of rumen fluid was measured (pH meter) and quantitative analysis of ammonia concentration was carried out by Nesler method modified by **Szumacher-Strabel et al (2002).** Total volatile fatty acids (TVFA's) **(Barnett and Reid, 1957).**

Gas production calculation

After 24 hours' gas production was calculated as followed

GPDM= total gas production (ml)/ substrate DM (g)
GPOM= total gas production(ml)/ substrate OM (g)
GPNDF= total gas production (ml)/ substrate NDF (g)
GPADF= total gas production(ml)/ substrate ADF (g)

Chemical analysis of feed ingredients

Ration ingredients were analyzed for DM and ash, Crude fiber (CF); Crude protein (CP) (Nitrogen x 6.25) and ether extract (EE) contents according to **AOAC (1997).** Neutral detergent fiber (NDF), acid detergent fiber (ADF) and (ADL) acid detergent lignin contents were analyzed sequentially **(Van Soest et al 1991)** using the Ankom^{200} Fibre Analyzer for NDF and ADF. The NDF content was analyzed with 2 additions of heat-stable α-amylase and 1:1 g sodium sulfite per g sample in the neutral detergent solution**.** NDF and ADF are expressed inclusive of residual ash. Non-fiber carbohydrate (NFC) was calculated according to the following formula:

NFC(%)= 100−(%ND+%CP + %fat + %ash) **(NRC, 2001).**

Statistical analysis

The data of *In-vitro* degradability and fermentation parameters were statistically analyzed according to statistical analysis system User's Guide, **(SAS, 1998).** Separation among means was carried out by using Duncan Multiple test, **(Duncan, 1955).** The following model was used:

$$Y_{ij} = \mu + T_i + e_{ij}$$

Where: Y ij = the observation of the model, μ = General mean common element to all observation, Ti = the effect of the treatment i, and e ij = the effect of error

RESULTS AND DISCUSSION

Dry matter and organic matter degradability

The data of **Table (2)** showed Effect of probiotics supplementation doses (0, 10^6,10^8 and 10^{10} CFU/ kg DM) on *in-vitro* dry matter and organic matter degradability. The data clearly showed that, slightly (P>0.05) increases in *in-vitro* dry matter degradability were observed for the experimental ration supplemented with probiotics bacteria at different levels (10^6,10^8 and 10^{10} CFU/ kg DM) compared to control ration (not supplemented). The heights dry matter degradability was recorded for level of 10^6 CFU/ kg DM (46.45 g/kg) followed by level 10^{10} CFU/ kg DM (45.77 g/kg) then 10^8 CFU/ kg DM (43.48 g/kg), while the lowest value was recorded for control (not supplemented) (43.21 g/kg).

Table 2. Effect of probiotics supplementation doses (0, 10^6,10^8 and 10^{10} CFU/ kg DM) on *in-vitro* dry matter and organic matter degradability (DMD and OMD).

Degradation	control	Probiotic level CFU/kg DM			SE	P value
		10^6	10^8	10^{10}		
Dry matter, %	43.21	46.45	43.48	45.77	1.13	0.245
Organic matter,%	33.97^b	36.53^a	36.04^a	36.00^a	0.37	0.001

Different superscript are significantly different (P<0.05)

On the other hand, Probiotics bacteria supplementation with different level (10^6,10^8 and 10^{10} CFU/ kg DM) led to significant (P<0.001) increases in organic matter degradability (%) compared to the not supplemented ration (control ration) (Table,

2), moreover no significant differences were observed among the different levels of probiotics supplementation (10^6,10^8 and 10^{10} CFU/ kg DM). These may be due to the probiotic supplementation which stimulate rumen bacteria growth **(Chiquette et al 2008)** and fermentation **(Stein et al 2006),** consequently improve DM degradation. The heist OM degradability was recorded for level of 10^6 CFU/ kg DM (36.53 g/kg) followed by 10^8 CFU/ kg DM (36.04 g/kg) then 10^{10} CFU/ kg DM (36.00 g/kg), while the lowest value was recorded for control (33.97 g/kg). The data point to that it could be used the probioyics at level of 10^6 CFU /kg DM.

These results are in line with the earlier report of **Sheikh et al (2017)** when add probiotic mix contains Saccharomyces and Lactobacillus acidophilus to the ration which found increase in DM and OM degradability as well as gas production compared to control. Also **Ganai et al (2015)** recorded higher *in-vitro* DM and OM digestibility values at supplementation of yeast to bajra straw based complete ration using goat rumen liquor. **Malik and Singh (2009)** also reported improvement in *in-vitro* or *in sacco* degradability pattern of nutrients due to supplementation of yeast culture.

Gas production

Gas production is a good indicator of microbial ferment ability, digestibility and rumen protein production (**Salem et al 2014**). *In-vitro* gas production per g dry matter (GP/g DM), organic matter (GP/g OM), degraded dry matter (GP/g dDM), degraded organic matter (GP/g dOM), neutral detergent fiber (GP/g NDF) and acid detergent fiber (GP/g ADF) after 24 hours' incubation period as a response to increasing probiotics bacteria supplementation level (0, 10^6,10^8 and 10^{10} CFU/ kg DM) to the experimental ration are presented in **Table (3).** Probiotics bacteria supplementation with different level (10^6,10^8 and 10^{10} CFU/ kg DM) resulted significant increases in *in-vitro* total gas production per sample and per g DM, OM, NDF and ADF after 24 hours' incubation period compared to the not supplemented experimental ration (control ration). While, no significant differences were observed among the different levels of probiotics supplementation (10^6,10^8 and 10^{10} CFU/ kg DM). This increase in total gas accumulation may be attributed to effect of probiotic that led to increase in OM degradability (table 2). These results are agree with **Sheikh et al (2017)** who found increase in total gas production when add probiotic mix contains Saccharomyces and Lactobacillus acidophilus to the ration compared to control. Also **Ganai et al (2015)** recorded higher *in-vitro* total gas production when supplemented bajra straw based diet with yeast. In this connection **Blümmel and Ørskov (1993)** reported that fermentation of organic compounds produces gas as one of the end-products providing the foundation of the strong correlation between OM digestibility and volume of gas produced.

Also significant increase was observed in *in-vitro* total gas production per g dDM was recorded for level of 10^8 CFU/ kg DM compared to the control ration, while both treatment not significantly differed with 10^6 and 10^{10} CFU/ kg DM. On the other hand, no significant differences were observed among the different experimental ration in total gas production (ml) per g dOM **(Table 3).**

Fermentation parameters

In-vitro fermentation parameters pH value, ammonia and volatile fatty acids (VFA's) concentration after 24 hours' incubation period with increasing probiotics bacteria supplementation (0, 10^6,10^8 and 10^{10} CFU/ kg DM) are presented in **Table (4).** Probiotics bacteria supplementation with different level (10^6,10^8 and 10^{10} CFU/ kg DM) resulted significant increase in total volatile fatty acid concentration after 24 hours' incubation period compared to the not supplemented ration. The highest VFA's

Concentration was recorded for level of 10^6 CFU/ kg DM (7.71 mg %) followed by 10^8 CFU/ kg DM (7.69 mg %) then 10^{10} CFU/ kg DM (6.96 mg %), while the lowest value was recorded for control (6.04 mg %). These results may be due to effect of probiotic supplementation which led to improve degradability and total gas production as indicated in **Tables (2 and 3)** consequently led to increase rumen fermentation. Volatile fatty acids are the ultimate product of microbial fermentation in the rumen and they are the main source of metabolizable energy for ruminants **(Van Soest, 1982)**.

On the other hand, the treatment supplemented with probiotic recorded lower ammonia concentration compared to the control group. This may be due to *lactobasillus sp* is the main strain in our probiotics which improve carbohydrate fermentation.

Table 3. Effect of probiotics supplementation doses (0, 10^6,10^8 and 10^{10} CFU/ kg DM) on *in-vitro* gas production as ml per g DM, OM, dDM, dOM, NDF and ADF after 24 hours' incubation period.

Total gas production	control	Probiotic level, CFU/kg DM			SE	P value
		10^6	10^8	10^{10}		
per sample	37.89 [b]	41.78 [a]	41 [a]	40.38 [a]	0.62	0.0008
GP/g DM, ml	104.68	115.30	112.78	111.54	1.7	0.012
GP/g dDM, ml	87.94 [b]	90.17 [ab]	94.32 [a]	87.30 [b]	1.75	0.037
GP/g OM, ml	114.98 [b]	126.65 [a]	123.88 [a]	110.47 [a]	5.71	0.195
GP/g dOM, ml	111.50	114.36	113.74 [a]	112.11 [a]	1.48	0.499
GP/ g NDF, ml	322.57 [b]	355.17 [a]	347.23 [a]	306.97 [a]	17.18	0.212
GP/ g ADF, ml	530.15 [b]	583.54 [a]	570.23 [a]	502.08 [a]	29.12	0.221

Different superscript are significantly different (P<0.05)

Probiotics bacteria supplementation with different level (10^6,10^8 and 10^{10} CFU/ kg DM) resulted significant reduction in pH value after 24 hours incubation period compared to the not supplemented experimental ration (control ration). These may be due to the effect of the probiotic supplementation on TVFA's and ammonia concentration **(Table 4),** which the pH is affected by TVFA's an ammonia concentration.

Table 4. Effect of probiotics supplementation doses (0, 10^6,10^8 and 10^{10} CFU/ kg DM) on in-vitro fermentation parameters after 24 hours' incubation period.

Item	control	Probiotic level, CFU/kg DM			SE	P value
		10^6	10^8	10^{10}		
pH	5.77 [a]	5.56 [c]	5.50 [c]	5.64 [b]	0.02	0.0001
Ammonia, mg/dl	14.42	13.196	12.85	12.96	0.61	0.2839
Volatile fatty acid, meq/dl	6.04 [b]	7.71 [a]	7.69 [a]	6.96 [a]	0.27	0.0028

Different superscript are significantly different (P<0.05)

CONCLUSION

It could be concluded that, adding probiotics bacteria supplementation to experimental ration resulted increase DM and OM degradability and using dose of 10^6 CFU/kg DM feed is sufficient to induce improvement in degradability and fermentation parameters.

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تقييم بكتيريا البروبيوتيك معمليا على علائق المجترات

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الموجـــــز

تهدف الدراسة لتقييم مستويات مختلفة من البروبيوتيك فى علائق المجترات، وذلك بإستخدام التخمر المعملى لتحديد مقدار التحلل ومقاييس التخمر، وتتكون العليقة من 40% دريس البرسيم الحجازى و60% مخلوط علف مركز، وكانت مستويات إضافة البروبيوتيك هى 10^6، 10^8، 10^{10} خليه لكل كجم مادة جافه من العليقه، تم تقدير المادة الجافه وإنتاج الغازالكلى وكذلك مقاييس التخمر بعد مرور 24 ساعه من التخمر، لوحظ زيادة غير معنويه ($P> 0.05$) لتحلل المادة الجافة بإستخدام بكتيريا البروبيوتيك عند مستويات مختلفة (10^6، 10^8، 10^{10} وحدة خليه لكل كجم مادة جافه) مقارنة بالعليقه الضابطه، كما أدت إضافة بكتيريا البروبيوتيك بمستويات مختلفة (10^6، 10^8، 10^{10} وحدة خليه لكل كجم مادة جافه) إلى زيادة معنويه ($P<0.001$) فى تحلل المادة العضويه ومجموع إنتاج الغاز لكل عينة ولكل جرام مادة جافة ومادة عضويه والألياف الذائبه فى الوسط المتعادل والألياف الذائبه فى الوسط الحامضى مقارنة بالعليقه الضابطه، ولم يلاحظ أى فروق معنويه بين المستويات المختلفة من البروبيوتيك. ويوجد زيادة معنويه فى إجمالى تركيز الأحماض الدهنيه الطياره بعد فترة تحضين 24 ساعه مقارنة بالمجموعة الضابطة، من ناحيه أخرى وجد إنخفاض تركيز الأمونيا فى المجموعة المعاملة بالبروبيوتيك مقارنة بالمجموعة الضابطه؛ يستنتج من ذلك أن إضافة بكتيريا البروبيوتيك للعلائق التجريبيه أدى إلى زيادة تحلل المادة الجافه والمادة العضويه وإستخدام الجرعة 10^6 وحدة خليه لكل كجم مادة جافه كافيه لتحسين التحلل ومقاييس التخمر.

الكلمات الداله: الهضم المعملى، البروبيوتيك، المجترات، التخمر

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