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IN VITRO EVALUATION OF ENCAPSULATED PROBIOTIC BACTERIA SUPPLEMENTATION TO RUMINANT RATIONS

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ABSTRACT

The aim of this study was to *in-vitro* evaluate encapsulated probiotic supplementation to ruminant rationson degradation and fermentation parameters. The ration consisted of 40% alfalfa hay and 60% concentrate feed mixture. Encapsulated and not encapsulated probiotic were supplemented with level of 10^6cfu/kg of the total dry matter of ration (DM) and compared with encapsulation media (Sodium Alginate, SA) and control (not supplemented ration). DM and OM degradation and total gas production as well as fermentation parameters of the incubated samples were determined after 24 h of fermentation. Significant (P<0.01) increases in *in-vitro* DM degradability was observed for the experimental ration supplemented with encapsulated or not encapsulated probiotics at levels (10^6 CFU/ kg DM) and SA treatment compared to control ration. Also, significant (P<0.05) improvement in OM degradability was recorded for the ration supplemented with not encapsulated probiotics bacteria compared to the other treatments. Moreover no significant differences were observed between the control ration and the rations supplemented with encapsulated probiotics or SA only, as well as no significant difference was recorded between the ration supplemented with encapsulated probiotics and the ration supplemented with SA only. Probiotics bacteria supplementation in the form of not encapsulated probiotic resulted significant increases in *in vitro* total gas production per sample and per g DM, OM, dDM, NDF and ADF after 24 hours incubation period compared to the other experimental rations (control, encapsulated probiotic and SA). While significant increase in total gas production per g dOM was observed for not encapsulated probiotic compared to encapsulated probiotic only. It could be concluded that, using encapsulated probiotics bacteria had no significant effect on DM degradability and may be induce decrease in gas production and fermentation parameters.

Key words: *Probiotics, Encapsulation, in-vitro, fermentation, Degradation*.

INTRODUCTION

Improvements of animal productivity, feed utilization and animal health are the aims of rumen microbial studies. These aims could be achieved by producing desirable fermentation products 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 (direct-fed microbial, DFM) 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, enzymes, culture extracts, exopolysaccharides or any 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, anti-cholesterolemic effect, reduction of gut pH by stimulating the lactic acid producing micro-flora, competition with pathogens for a viable nutrients **(Edens,2003)** and immune-modulation **(Aottouri et al 2002)**.

The encapsulation process for probiotics may be increase the number of the probiotic escape to the intestine, consequently acts it role in the intestine and increase animal immunity. So, the objectives of this study were to evaluate effect of encapsulation probiotic supplementation to ruminant ration on in vitro degradation and fermentation parameters.

MATERIALS AND METHODS

Microbial strains and growth condition

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 37º C. The strains were activated two or three times in order to obtain high biomasses in the stationary phase then the cell pellets were harvested by centrifugation at 5000rpm, for 20 min at 4º C. The pellets were washed by sterile saline solution (0.9% (w/v) NaCl) and recovered under the same centrifugation conditions then stored at -8º C till be encapsulated.

Preparation of Encapsulated mixed strains using extrusion method

Generally, the microencapsulation process was performed using the extrusion technique **(EL-Shafei et al 2018).** One part of the cells of different isolates suspension was mixed with three parts of the freshly prepared sodium alginate (3%) with gentle stirring for 10-20 min. The mixture was then extruded into the hardening solution ($CaCl_2$, 0.2 M) through sterile syringe (25 G, 0.5 mm) with gentle stirring for 30 min to ensure complete solidification. The formed microcapsules were harvested by filtration then washed by sterile saline solution.

Enumeration of the microencapsulated cells

The viability of mixed strains was assessed as described by (**Chávarri et al 2010).** One gram of the microcapsules was dissolved in 9 ml of sterile tri-sodium citrate solution (2% w/v) and vortexes till complete dissolution then the samples were serially diluted to appreciate concentration using saline solution and pour plated in MRS agar for lactobacilli and in M17 agar for streptococii. The plates were incubated 48 h at 37º C. The viable cell number was expressed as colony forming unit per gram of microcapsule (cfu/g).

Experimental ration and treatments

The tested ration contained 60:40 concentrate: roughage ratio, the concentrate portion was composed of corn, soyabean, wheat bran, flaxseed, $CaCO_3$, salt and mineral mixture while the roughage portion was alfalfa hay. The data of chemical composition of the feed ingredients and tested rations are presented in **Table (1).** Encapsulated and not encapsulated probiotic supplementation with level of 10^6cfu/kg of the total ration DMwerecompared with encapsulation media (Sodium Alginate, SA) and control (not supplemented).

In vitro gas production technique

Two days before beginning of the experiment, 400± 4 mg (240 mg CFM +160 mg alfalfa hay) 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 CO_2 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 thisinoculums 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.

Table 1. The chemical composition of the concentrate feed mixture and alfalfa hay

Item	Alfalfa hay	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

Degradability

Dry matter degradability (% dDM) 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 hrs of incubation, the filtrated rumen liquor for each sample was subjected for further investigation. The pH of rumen fluid was measured using pH meter pen, Quantitative analysis of ammonia concentration was carried out by Nesler method modified by **Szumacher-Strabel et al (2002)** and total volatile fatty acids(TVFA's) was determined according to**(Barnett and Reid, 1957).**

Gas production calculation

After 24 hours gas production was calculated as followed

GP per g DM = total gas production (ml) / substrate DM (g)

GP per g OM = total gas production (ml) / substrate OM (g)

GP per g NDF = total gas production (ml) / substrate NDF (g)

GP per g ADF = total gas production (ml) / substrate ADF (g)

Chemical analysis of feed ingredients

Concentrate feed mixture and alfalfa hay were analyzed for DM, ash, (CF) crude fiber; 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, **(S.A.S., 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 degradation

The data of **Table (2)** showed effect of not encapsulated, encapsulated probiotics and SA supplementation to the tested ration compared to the control on in vitro dry matter and organic matter degradability.The data showed significant (P<0.05) increase in i*n-vitro* DM degradability was observed for the experimental ration supplemented with encapsulated or not encapsulated probiotics at levels (10^6cfu/ kg DM) and SA compared to control ration (not supplemented).While no significant differences were observed among the supplemented ration with probiotics bacteria encapsulated or not encapsulated at levels (10^6cfu/ kg DM) and Sodium Alginate treatments. 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. Also, may be due to effect of encapsulated medium(Sodium Alginate) for the treatments supplemented with encapsulated probiotic and SA only.

Table 2. Effect of encapsulated and not encapsulated probiotics supplementation on in vitro DM and OM degradability after 24 hours incubation period.

Degradation	Control	Probiotic	Alginate	Encapsulated probiotic	SE	P value
Dry matter,%	43.21 [b]	46.45 [a]	48.108 [a]	50.08 [a]	0.96	0.0005
Organic matter,%	33.97 [b]	36.53 [a]	34.459 [b]	34.12 [b]	0.400	0.0308

[a] and [b] Different superscript are significantly different (P<0.05)

Concerning to effect of experimental treatments on OM degradability (%), the data indicated that significant (P<0.05) improvement in OM degradability was recorded for the ration supplemented with not encapsulated probiotics bacteria compared to the other treatments. Moreover no significant differences were observed between the control ration and the rations supplemented with encapsulated probiotics or encapsulation medium only (Sodium Alginate), as well as no significant difference was recorded between the ration supplemented with encapsulated probiotics and the ration supplemented with encapsulated material only. This may be due to the stimulation effect of probiotics for rumen flora and fermentation **(Stein et al 2006)**. Which, the encapsulation process protect the probiotic bacteria and prevent its effect on rumen flora.

Gas Production

Gas production is a good indicator of microbial ferment ability, digestibility and rumen protein production (**Salem et al 2014**).Effect of encapsulated and not encapsulated probiotics supplementation on *In-vitro* total gas production per g DM, OM, dDM, dOM, NDF and ADF after 24 hours incubation period are presented in **Table (3).** Probiotic bacteria supplementation in the not encapsulated form resulted significant increases in *in vitro* total gas production per g DM, OM, dDM, NDF and ADF after 24 hours incubation period compared to the other experimental rations (control, encapsulated probiotic and SA medium). While significant increase in total gas production per g dOM was observed for not encapsulated probiotic compared to encapsulated probiotic only. This may be due to effect of DM and OM degradation improvement for the not encapsulated probiotic treatment compared to the other experimental treatments **(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 productionwhensupplementedbajra straw baseddietwith 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.

Table 3. Effect of encapsulated and not encapsulated probiotics supplementation on *in-vitro* total gas production per g DM, OM, dDM, dOM, NDF and ADF after 24 hours incubation period.

Item	Control	Probiotic	Alginate	Encapsulated probiotic	SE	P value
GP/g DM, ml	104.70 [b]	115.3 [a]	104.90 [b]	101.94 [b]	1.772	0.0001
GP/g dDM, ml	87.94 [a]	90.16 [a]	79.23 [b]	73.70 [c]	1.75	0.0001
GP/g OM, ml	115.00 [a]	126.60 [a]	115.23 [b]	111.98 [b]	1.95	0.0001
GP/g dOM, ml	111.50[ab]	114.40 [a]	110.55[ab]	108.08 [b]	1.53	0.0517
GP/ g NDF, ml	322.60 [b]	355.20 [a]	323.08 [b]	313.97 [b]	5.527	0.0001
GP/ g ADF, ml	530.10 [b]	583.50 [a]	530.71 [b]	515.75 [b]	9.18	0.0001

[a] and [b] Different superscript are significantly different (P<0.05)

Fermentation parameters

Effect of encapsulated and not encapsulated probiotics supplementation on *In-vitro* fermentation parameters pH value, ammonia and total volatile fatty acids (TVFA's) concentration after 24 hours incubation period are presented in **Table (4).** Totalvolatile 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)**.The not encapsulated and encapsulated probiotics bacteria and Sodium Alginate supplementation resulted significant increase in total volatile fatty acid concentration after 24 hours incubation period compared to the not supplemented experimental ration (control ration).These may be due to the higher DM and OM degradation rate **(Table 2)** and gas production **(Table 3)** recorded for the treatment supplemented with not encapsulated probiotic. The heist TVFA's concentration was recorded for not encapsulated probiotic (7.71 mg %) followed by sodium alginate medium (7.69 mg %) then encapsulated probiotic (7.01 mg %), while the lowest value was recorded for control (6.04 mg %).

Concerning to ammonia concentration the data of **Table (4)** showed that the treatments supplemented with encapsulated probiotics and SA medium recorded significantly lower ammonia concentration compared to the control treatment and the treatment supplemented with not encapsulated probiotics. And no significant difference between control treatment and the treatment supplemented with not encapsulated probiotics.

Encapsulated and not encapsulated probiotics bacteria supplementation resulted in significant reduction in pH value after 24 hours incubation period compared to the not supplemented experimental ration (control ration). These results may be due to effect of the increase of total volatile fatty acids recorded for the treatments supplemented with not encapsulated and encapsulated probiotics bacteria and Sodium Alginate compared to the control treatments **(Table 4).**

Table 4, Effect of encapsulated and not encapsulated probiotics supplementation on in vitro fermentation parameters after 24 hours incubation period.

Item	**Control**	**Probiotic**	**Alginate**	**Encapsulated probiotic**	**SE**	**P value**
pH	5.77 [a]	5.56 [d]	5.61 [c]	5.71 [b]	0.015	0.0001
Ammonia, mg/dl	14.42 [a]	13.19[ab]	10.47 [c]	11.55[bc]	0.565	0.0017
Total Volatile fatty acid, meq/dl	6.04 [b]	7.71 [a]	7.76 [a]	7.01 [a]	0.295	0.0045

[a], [b] and [c] Different superscript are significantly different (P<0.05)

CONCLUSION

It could be concluded that, adding probiotics bacteria at dose of 10^6 CFU/kg DM feed to experimental ration resulted increase DM and OM degradability. Also using encapsulated probiotics bacteria had no significant effect on DM degradability and may be induce decrease in gas production and some fermentation parameters

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الهدف من هذه الدراسه هو تقييم مستويات مختلفه لاضافه البروبيوتيك في علائق الحيوانات المجتره بإستخدام التخمرات المعمليه وتقدير مستويات التخمر والهضم. وتتكون العليقه من 40% دريس حجازي و60% خليط مواد مركزه. تم اضافه البروبيوتك المغلفه وغير المغلفه بتركيز 10^6cfu/kg علي أساس الماده الجافه بالعليقه والبيئات غير المغلفه (ألجينات الصوديوم) تم استخدامها للمقارنه بالمجموعه الضابطة. تم قياس الماده الجافه والغاز الكلي المنتج ومقاييس التخمر للعينات المحضنه بعد 24 ساعه من التخمرات. لوحظت زياده معنويه ($P<0.01$) لتحلل الماده الجافه في العلائق المضاف لها البروبيوتيك المغلفه وغير المغلفه بتركيز (106 CFU/ kg DM) وعلائق ألجينات الصوديوم (البيئه المغلفه) مقارنه بالمجموعه الضابطة (بدون اضافه). وأيضا لوحظ وجود فرق معنوي ($P<0.05$) كتحسن في تحلل الماده العضويه في العلائق المضاف لها البروبيوتيك غير المغلفه مقارنه بباقي المعاملات. وعلاوه علي ذلك لم تلاحظ اختلافات معنويه بين العليقه الضابطة والعلائق المضاف لها البروبيوتيك المغلفه أو البيئه المغلفه بمفردها (ألجينات الصوديوم)، وكذلك لم تلاحظ إختلافات معنويه بين العلائق المضاف لها البروبيوتيك المغلفه والعلائق المضاف لها البيئات المغلفه بمفردها. كما لوحظ ان اضافه البروبيوتيك في الصورة غير المغلفه كانت النتيجه زياده معنويه في الغاز الكلي المنتج في العلائق المختبره معمليا وأيضا خلال g DM, OM, dDM, NDF, ADF بعد 24 ساعه من فتره التحضين مقارنه بالعلائق المختبره (الضابطة والبروبيوتيك المغلفه والبيئات المغلفه فقط). بينما حدثت زياده معنويه في الغاز الكلي المنتج المقدر بـ g/dOM في البروبيوتيك المغلفه. ويمكن من خلال هذه النتائج استنتاج أن استخدام البروبيوتيك المغلف ليس لها تأثير معنوي في تحلل الماده الجافه ويحتمل أن يكون لها دور فى تقليل حجم الغاز المنتج ومقايس التخمر.

الكلمات الدالـة: بروبيوتيك 'التغليف' التقييم المعملى' مقاييس التخمر 'مقاييس الهضم"

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