EFFECTS OF COMMONLY USED PROBIOTICS ON THE RUMEN PROTOZOA AND THEIR ACTIVITIES IN SHEEP : BIOPHYSICAL CHARACTERISTICS AND MICROSCOPICAL EXAMINATION

Fouda, T. A.; Al-Sultan, S. I.* and Hussein, Y. A.

Department of Clinical Studies and Department of Animal Management and Public Health

College of Veterinary Medicine & Animal Resources, King Faisal University

Al-Ahsa, 31982 P.O. Box: 1757, Kingdom of Saudi Arabia

Email: foudataha@yahoo.com

ABSTRACT

Ten apparently healthy sheep of 1-2 years old and weighing between 35-45 kg were used in this study. All animals were closely observed for one week before the experiment and were allowed to have regular feeding regimen. During that period, they were subjected to detailed physical examination. After that all animals were subjected to oral administration of probiotics and yeast (lactobacillus and Saccharomyces) once/ day for four consecutive days.

The rumen liquor and blood samples were obtained from each animal in two occasions, the first before administering the drugs (base or control), and the second after four days post-treatment. Each rumen fluid sample was subjected for biochemical analysis and determination of the biophysical characteristics and microscopical examination. The blood sera samples were subjected for biochemical analysis in order to determine the concentrations of the selected parameters.

The obtained results indicated that there were significant reduction in the time required for Methylene blue reduction test (6.20 minutes) and cellulose digestion test (21.20 hrs) in ruminal fluid after oral administration of the probiotics and yeast culture when compared with their values before administration. Meanwhile the pH value (7.09), ammonia concentration (112.7 mg/l) and sedimentation and flotation test showed significant elevations (27.3 minutes) in ruminal fluid after administration of the probiotics and yeast culture if compared with their values before administration. The color, smell and consistency of ruminal fluid showed non-significant variation when compared with their normal characteristics before administration.

The obtained results revealed that all supplemented sheep had numerically higher

protozoal counts over than their values before administration. Consequently, there were significant increases in the mean values of total protozoal counts (718.0 $x10^3$), the mean values of differential count particularly holotrichs protozoa (53.20 $x10^3$) and entodinium species (626.5 $x10^3$) in ruminal fluid following administration of the probiotics and yeast culture.

The results of biochemical analysis of blood revealed significant elevations in the mean values of blood glucose (68.60 mg/dl), total proteins (7.09 gm/dl), albumen (4.16 gm/dl), and blood urea nitrogen (18.80 mg/dl) four days after administration of the probiotics and yeast culture if compared with their values before administration.

Probiotics have been used successfully as feed additives and improving the total and differential counts of rumen protozoa with subsequent improving the energy status and protein levels. In addition yeast culture reducing the lactic acid concentration and maintaining the desired pH value of ruminal fluid.

INTRODUCTION

The ruminants are dependent on the fermentation of their food constituents by the rumen microorganisms. The microbial community is accommodated in a complex forestomach, the ruminoreticulum, which provides a highly specialized anaerobic environment (**Williams, 1986**).

The rumen protozoa are highly specialized for growth in the rumen ecosystem. The majority of the protozoa are ciliates (10^5 to 10^6 protozoa per ml), although flagellates are found in both the rumen and the cecum and are more numerous in animals lacking ciliates (**Clarke, 1977 and Hungate, 1966**).

Modern animal production requires the use of safe and effective feed additives as rumen manipulators to increase animal productivity. Of late, the use of antibiotics and growth promoters in animal production has been strongly discouraged in most nations. One of the potential alternatives for antibiotics are direct-fed microbials which known as probiotics (**Mwenya et al., 2006**).

Although the results are not consistent, probiotics are known to improve the establishment of beneficial gut microflora and reduce the risk of acidosis (**Ghorbani et al., 2002**); increase milk production and weight gain (**Yoon and Stern, 1995**) as well as the stimulating cellulolytic and lactate-utilizing bacteria; increase fiber digestion; and increase flow of microbial protein from rumen (**Martin and Nisbet, 1992; Newbold et al., 1996**). In addition to that, the use of feed enzymes in ruminant diets is a technology in development. Recent research has demonstrated that

supplementing diets of dairy cows and feedlot cattle with fiber degrading enzymes has significant potential to improve feed utilization and animal performance (**Nsereko et al., 2002**).

Probiotics contain normal healthy commensal (meaning naturally occurring) bacteria and yeast, and are used to re-colonize the gastrointestinal tract when it is suspected the normal balance of microflora (bacteria) has become disturbed. Many products are available, containing a variety of species. Numerous attempts have been made to stimulate rumen development in pre-ruminants in order to wean them at an earlier age and to avoid digestive disorders due to feed transition. Supplementation of the diets with feed additives would therefore be a very useful tool to achieve these goals (**Chaucheyras et al., 1997**).

Consequently, the main objective of this study was to declare the most probable effects of feed additives particularly the probiotics (lactobacillus and yeast Saccharomyces), on the total number and activities of rumen protozoa and their activities in sheep.

MATERIAL AND METHODS

Animals :

Ten apparently healthy sheep of 1-2 years old and weighing between 35-45 kg are used in this study. All animals were closely observed for one week before the experiment and were allowed to have regular feeding regimen. During that period they were subjected to detailed physical examination. After that all animals were subjected to oral administration of probiotics and yeast (lactobacillus and saccharomyces) once/day for four consecutive days

Samples and sampling protocol :

The rumen liquor was obtained in the early morning before the first feeding of the animals. The samples of rumen liquor were obtained from each animal in two occasions, the first before administering the drugs, and the second was four days post-treatment. Each rumen fluid sample was divided into two portions. The first portion was sieved and then centrifuged; only clear supernatant fluid was used for further biochemical analysis. The second portion of the rumen fluid sample was used to carry out the biophysical characteristics and microscopical examination (**Dirksen and Smith, 1987 and Fouda, 1998 &1999**).

In addition, blood samples were obtained through jugular venipuncture in plain vacculainer tubes in order to obtain blood serum. Only clear and non-hemolysed sera were used for further biochemical analysis of the selected parameters (**Coles, 1984**).

The adopted methods

A) Physical examination of the ruminal fluid

1. Color, smell and consistency: All these physical characters were judged immediately after obtaining the samples. The color was judged as green (ranged from light green to olive green) and yellow (ranged from yellowish, yellowish green to yellowish brown). The smell of the ruminal fluid was judged as pleasant aromatic, putrefactive, souring or offensive. The consistency was expressed as slimy, viscid or aqueous (**Alonso, 1979; Dirksen, 1983; Dirksen & Smith, 1987; Roussel, 1990; Fouda & Mohamed, 1999**).

2. Sedimentation and floatation time (SAT), cellulose digestion (CDT) and pH of the ruminal fluid were evaluated according to (**Dirksen, 1983 and Fouda & Mohamed, 1999**).

3. Methylene blue reduction test (Redox potential): This test involved mixing 1.0 ml of 0.03% methyelene blue solution with 20.0 ml strained fresh ruminal fluid. The mixture was incubated at 250C in transparent glass cylinder. The time required for decolorization of the mixture was calculated (**Dirksen, 1983 and Roussel, 1990**).

B) Microscopical examination and identification of the rumen protozoa:

1. The activity and population density of rumen protozoa were evaluated by using fresh unstained gently wormed rumen liquor on glass slides and cover slips by using binuclear research microscope (**Alonso, 1979 and Fouda, 1995**). The activity and density of the rumen protozoa were judged as following:

Highly motile and abundant	(+++)
Motile and moderate density	(++)
Sluggish and low density	(+)
Non-motile, sporadic alive	(±)

2. Total and differential counts of rumen protozoa were carried out according to the methods described by (**Naga, 1967**). Meanwhile the protozoal identification was carried out according to the method illustrated by **Hungate (1966), Church (1988) and Williams & Coleman (1988).**

C) Biochemical analysis of the ruminal fluid for the selected parameters was carried out spectrophotometerically using the available test kits supplied by BioMerlux/France and Stanbio/USA (**Dumas & Biggs, 1972 and Henry et al., 1974**).

Biochemical analysis of blood sera: the concentrations of the selected blood parameters, particularly, total proteins, albumin, blood urea nitrogen, glucose, sodium, potassium and chloride

were measured colorimetrically using (Ellipse DDI) machine.

Statistical analysis: The obtained data were statistically analyzed. The mean values and SE were calculated and the significance was tested by ANOVA test using SPSS computer program.

RESULTS AND DISCUSSION

The obtained results for biophysical characteristics and mean values of biochemical analysis of ruminal fluid in sheep before and after treatment are tabulated in table (1). While the mean values of total and differential counts of rumen protozoa are summarized in table (2). The values of blood biochemical analysis are tabulated in table (3).

The obtained results indicated that there were significant reduction in the time required for Methylene blue reduction test (MBT), cellulose digestion test (CDT) and lactic acid concentration in ruminal fluid after oral administration of the probiotics and yeast culture when compared with their values before treatment. Meanwhile the pH value (7.09), ammonia concentration (112.7 mg/l) and sedimentation and floatation test showed significant elevations in ruminal fluid after administration of the probiotics and yeast culture if compared with their values before administration. The color, smell and consistency of ruminal fluid showed non-significant variation when compared with their values before administration.

The reduced CDT and MBT might be a result of the positive effects of yeast cells on growth and activity of fiber-degrading bacteria and fungi, on stabilization of rumen pH and prevention of lactate accumulation, Modes of action of yeast probiotics depend on their viability and stability in the rumen ecosystem (**Fonty and Durand, 2006**).

Increased concentration of ammonia and pH of ruminal fluid are agreement with those reported by **Williams (1986)** The concentrations of ammonia and VFA in the rumen are frequently, but not always, higher in faunated animals. Reduced lactic acid and consequently pH values in ruminal fluid could be attributed to the role of which make an important contribution to rumen metabolism The holotrichs not only contribute to short-chain VFA production, but also to some extent control the overall rate at which the acids are formed. Substrate removal by the protozoa prevents a rapid bacterial fermentation to lactic acid. It has been proposed that the protozoal ingestion of starch grains or soluble sugars is beneficial to the host animal because the alternative bacterial fermentation would lead to an accumulation of lactate in the rumen and a detrimental lowering of pH. Starch is ingested actively by Isotricha spp. and soluble sugars are ingested by both holotrichs genera. On high sugar diets the holotrichs protozoa may help to prevent the onset of lactic acid acidosis by rapidly assimilating soluble sugars into amylopectin.

The pH stabilization is generally associated with decreased levels of lactic acid in rumen. The stimulation of lactic acid-utilizing bacteria could account for Saccharomyces cerevisiae-induced decreases in lactic acid concentrations and the corresponding moderation of ruminal pH. Mannitol utilizing bacteria like S. ruminantium, one of the most important consumers of lactic acid, have been shown to be stimulated in vitro by yeast in an incubation of mixed rumen fluid (**Newbold et al., 1998**). Yeast is also able to compete with Streptococcus bovis, the main lactic acid producer in the rumen, for soluble sugars uptake (**Chaucheyras et al., 1997**). **Mathieu et al. (1996)** have found an increase of the pH with yeast only in faunated sheep and not in defaunated sheep, suggesting that protozoa are involved in the effect of Saccharomyces cerevisiae on the increase of rumen pH.

Regarding the total and differential counts of rumen protozoa, the obtained results revealed that all supplemented sheep had numerically higher protozoa counts over than their control values. Consequently, there weresignificant increases in the mean values of total protozoal counts (718.0 $x10^3$), the mean values of differential count particularly holotrichs protozoa (53.20 $x10^3$) and entodinium species (626.5 $x10^3$) in ruminal fluid after administration of the probiotics and yeast culture. These results are endorsed by the findings of **Plata et al. (1994)** who stated that protozoal number was increased in cows fed supplemented diet with yeast culture. Increased levels of rumen protozoa following Saccharomyces cerevisiae ingestion were also reported by **Miranda et al., 1996**.

The results of biochemical analysis of blood revealed significant elevations in the mean values of blood glucose (68.60 mg/dl), total proteins (7.09 gm/dl), albumen (4.16 gm/dl), and blood urea nitrogen (18.80 mg/dl) four days after administration of the probiotics and yeast culture if compared with their values before administration.

Such elevation in the mean values of glucose could be attributed toincreased concentrations of VFA in the rumen in faunated animals and the relative proportions of the VFA also differ, with faunated animals having increased butyrate or propionate levels (**Williams, 1986**) with consequent increase of blood glucose through the metabolic pathways

Increased concentrations of total proteins and albumin could be ascribed for the retention of protozoa within the rumen; a significant proportion of the microbial protein available to the host is protozoal in origin (**Coleman, 1979**). A dairy cow on a maintenance ration requires 500 g of protein per day. Approximately 33 g of holotrich protein would be available to the host daily from a bovine rumen containing a holotrich population of 3,000 Isotricha spp. and 5,000 Dasytricha sp. per ml. In addition , the holotrichs may accumulate and conserve amino acids that are deficient in plants (**Coleman, 1975**). Although the biological values of bacterial and protozoal pro-

teins are similar, the protozoa are more susceptible to digestion.

It could be concluded from this study that the rumen microbial ecosystem is greatly affected by the feed additives offered to the animals. The well being of ruminant animals depends mainly on the maintenance of an appropriate microbial population and fermentative process within the compound stomach. Probiotics have been used successfully as feed additives and improving the total and differential counts of rumen protozoa with consequence improving the energy status and protein levels. In addition , yeast culture reducing the lactic acid concentration and maintaining the desired pH value of ruminal fluid.

Table (1): The biophysical characteristics and the mean values of biochemical analysis of ruminal fluid in sheep before and after treatment

Treatment	Color	Smell	Consistency	SAT min	MBRT min	CDT hrs	Protozoal activity	pH	Cl mmol/l	NH^3 Mg/l
Before treatment	Yellow	Aromatic	Slimy	23.0[a] ± 0.83	7.70[a] ± 0.33	27.80[a] ± 0.84	+++	6.66[a] ± 0.79	16.23[a] ± 1.20	99.30[a] ± 2.39
Four Days After treatment	yellow	aromatic	viscid	27.3[b] ± 0.89	6.20[b] ± 0. .32	21.20[b] ± 0.64	++++	7.09[b] ± 0.57	17.11[a] ± 1.30	112.7[b] ± 2.83

[a, b] Means with the same superscripts in the same column are not significantly different, while means with different superscripts are significantly different at 0.05 level of probability

Table (2): The mean values of total and differential counts of rumen protozoa in sheep before and after administration of the drugs.

Treatment	Total count x 10^3	Holotrichs X 10^3/ml		Entodiniomorphs (Oligotrichs) X 10^3/ml			
		Isotricha	Dasytricha	Entodinum	Epidinium	Polyplastron	Ophryoscolex
Before treatment	665.0[a] ± 13.09	24.80[a] ± 0.62	10.0[a] ± 0.85	593.7[a] ± 12.49	10.6[a] ± 0.79	20.9[a] ± 0.58	13.0[a] ± 0.57
Four Days After treatment	718.0[b] ± 9.40	33.70[b] ± 1.22	19.5[b] ± 1.04	626.5[b] ± 11.42	10.7[a] ± 0.49	21.7[a] ± 0.84	13.20[a] ± 0.57

[a, b] Means with the same superscripts in the same column are not significantly different, while means with different superscripts are significantly different at 0.05 level of probability

Table (3): The mean values of blood biochemical parameters in sheep before and after administration of the drugs.

Treatment	Na mmol/l	K mmol/l	Cl mmol/l	Glucose mg/dl	TP gm/dl	Alb gm/dl	BUN mg/dl
Before treatment	135.90[a] ± 1.86	4.68[a] ± 0.16	100.20[a] ± 1.26	60.90[a] ± 0.99	6.35[a] ± 0.20	3.90[a] ± 0.15	16.60[a] ± 0.66
Four Days After treatment	136.70[a] ± 1.32	4.70[a] ± 0.11	101.60[a] ± 1.15	68.60[b] ± 0.56	7.09[b] ± 0.66	4.16[b] ± 0.21	18.80[b] ± 0.46

[a, b] Means with the same superscripts in the same column are not significantly different, while means with different superscripts are significantly different at 0.05 level of probability

Figure (1): Different forms of rumen protozoa stained with iodine (arows)

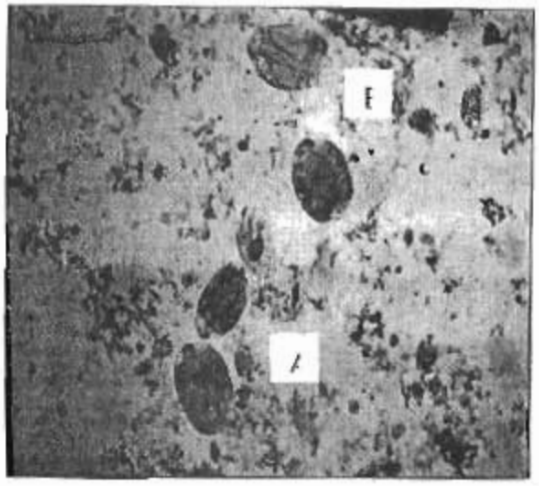

Figure (2): Rumen protozoa entodinium (a) and polyplastron (b)

Figure (3): Isotricha intestinalis in the rumen fluid of sheep stained with iodine solution

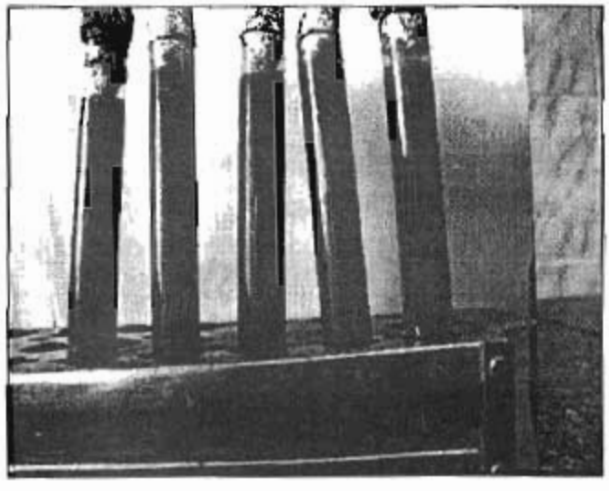

Figure (4): Sedimentation and floatation test of ruminal fluid

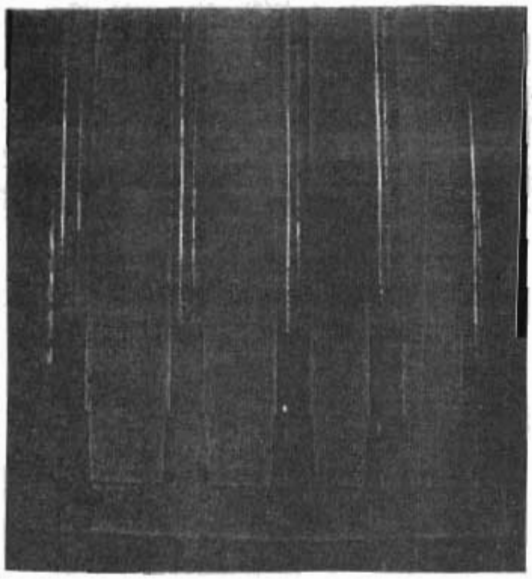

Figure (5): Methylene blue reduction test (notice the blue color of ruminal fluid before reduction)

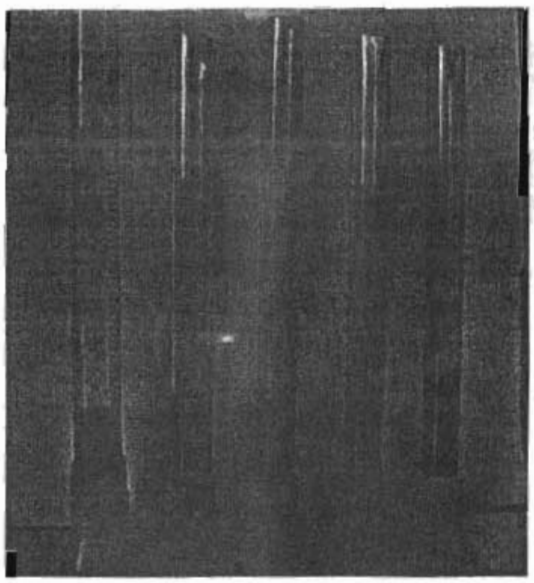

Figure (6): Methylene blue reduction test (notice the discoloration of ruminal fluid after reduction)

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الملخص العربى

تأثير الخمائر الشائع إستخدامها على أوليات الكرش ونشاطها فى الأغنام :
الخصائص البيوفيزيقية والفحص المجهرى

طه عبدالمنعم فوده - سعد السلطان - يحيى أحمد حسين

قسم الدراسات الإكلينيكية، قسم الصحة العامة - كلية الطب البيطرى والثروة الحيوانية - جامعة الملك فيصل - الإحساء، ٣١٩٨٢، ص. ب. ١٧٥٧، المملكة العربية السعودية

أجريت هذه الدراسة على عدد عشرة أغنام تراوحت أعمارهم مابين سنة إلى سنتين بمعدل وزن ٣٥ - ٤٥ كجم. تم وضع هذه الأغنام تحت الملاحظة الدقيقة لمدة إسبوع قبل بدء التجربة حيث خضعت الأغنام للفحص الإكلينيكى ومن ثم تم تجريعهم بمنشطات أو محفزات النمو والخمائر بمعدل مرة واحدة يومياً لمدة أربعة أيام متتالية.

تم الحصول على عينات من سائل الكرش كما تم الحصول على عينات دم من الوريد الوداجى للأغنام تحت الدراسة بمعدل مرتين حيث كانت المرة الأولى قبل بداية التجريع باستخدام الخمائر بينما كانت المرة الثانية بعد أربعة أيام من التجريع. هذا وقد تم إجراء الفحص المجهرى لأوليات الكرش كما تم تحديد العدد الكلى والتصنيفى لهذه الأوليات بالإضافة إلى دراسة الخصائص البيوفيزيقية لسائل الكرش فى كل العينات. كما تم تحديد تركيز الأمونيا والكلورايد فى هذه العينات، بالإضافة إلى ذلك فقد أجريت التحاليل البيوكيميائية لمصل الدم لتحديد مستويات العناصر الكيميائية مثل البروتين الكلى والزلال وسكر الدم واليوريا.

وقد أظهرت النتائج حدوث نقص معنوى فى الوقت اللازم لاختزال المثيل الأزرق وهضم السليلوز فى حين كانت هناك زيادة معنوية فى درجة الأس الهيدروجينى (pH) وتركيز الأمونيا وكذلك الوقت اللازم لعملية الترسيب والطفو فى سائل الكرش بعد إستخدام هذه الخمائر إذا ماقورنت بالنتائج قبل إستخدام هذه المركبات. كما لم تحدث أية تغيرات معنوية فى اللون أو الرائحة أو القوام لعينات سائل الكرش قبل وبعد إستخدام هذه المركبات، كما أوضحت النتائج حدوث زيادة معنوية فى العدد الكلى لأوليات الكرش بالإضافة إلى حدوث زيادة معنوية فى بعض الأنواع مثل Holotrichs and Entodinium. أما فيما يتعلق بالتحليل البيوكيميائى لمصل الدم فقد أظهرت النتائج حدوث إرتفاع معنوى فى مستويات البروتين الكلى والزلال وسكر الدم واليوريا بعد أربعة أيام من إستخدام هذه المركبات إذا ماقورنت بمستوياتها قبل إضافتها.

ويمكننا أن نستخلص من هذه الدراسة أن إستخدام أو إضافة مثل هذه الخمائر كإضافات أو محفزات للهضم قد أدت إلى زيادة فى العدد الكلى لأوليات الكرش وزيادة نشاطها وبالتالى زيادة عملية الهضم وانتاج الطاقة مماانعكس إيجابياً على مستويات سكر الدم والبروتين الكلى والزلازل، فضلاً عن ذلك فإن إستخدام هذه الخمائر أدى إلى خفض مستوى حمض اللبنيك (lactic acid) فى سائل الكرش الأمر الذى يؤدى إلى توازن فى درجة الأس الهيدروجينى لمايقى الحيوان من مخاطر حموضة الكرش ومحتوياته.