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INFLUENCE OF DIFFERENT SPICES ON BACTERIAL PROFILES WITH OR WITHOUT RICE STRAW

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Abstract

This paper includes three studies. In the first study, different types of bacterial population (total viable, cellulolytic, proteolytic, amylolytic and lypolytic) were counted from the rumen fluid (RF) of fistulated sheep. In the second study, total viable bacteria were counted from the (RF) following its individual incubation with five spices (cinnamon, cumin, coriander, turmeric and clove) for 48 h. In the third study, Gram positive and Gram negative bacteria were considered from RF collected from sheep, after its *in vitro* incubation with rice straw in the presence and absence of spices for 120 h. Total viable counts were highest in the presence of cumin and lowest in the presence of cinnamon. The existence of Gram negative bacteria was increased but Gram positive bacteria was decreased in the presence of cumin, coriander and turmeric. Reduction of Gram positive bacteria representing methanogens in these three spices suggested that these could be used as additives to reduce methane production in ruminants.

Keywords: Spices, degradability, bacterial count, methane production, Gram positive bacteria.

Introduction

Rumen microorganisms play a vital role in ruminant digestion. The efficiency of ruminants to utilize a wide variety of feeds depends on highly diversified rumen microbial ecosystem consisting of bacteria (10^9 - 10^{11} ml^{-1}), protozoa (10^4 - 10^6 ml^{-1}), anaerobic fungi (10^3 - 10^5 ml^{-1}) and bacteriophages (10^8 - 10^9 ml^{-1}) (Rajvir *et al.,* 2006; Kamra, 2005). The rumen ecosystem is dynamic as the microbial population changes considerably with the change of diet so as to adapt it to the new feed ingredients. These microbes survive in the rumen under different constraints which may be either natural or feed associated as some of the feeds contain a significant amount of anti-nutritional factors which sometimes limit the growth of some of these natural microbial inhabitants (Kamra, 2005). Among these different types of microorganisms the anaerobic bacteria have very important functions in the degradation of plant cell walls in the rumen. In favourable environments anaerobic bacteria will increase their number and will be more active to increase forage degradability. Therefore, the determination of the bacterial population is very important to a ruminant nutritionist. Thus it will be helpful to verify the bacterial population of the rumen fluid from fistulated sheep, before monitoring different bacterial populations in the presence of different forages and spices.

Most of the methane producing rumen bacteria (*Methanobacterium ruminatium, Methanobacterium formicum*) including acetate and butyrate producing bacteria are Gram positive (Miller *et al.,* 1986; Banerjee, 1999; Busquet *et al.,* 2006). As methane is produced as a by product during acetate production, reduction in Gram positive bacteria can affect methane production. Thus it may help if the effect of these spices on Gram positive and Gram negative bacteria were monitored. This paper includes three studies. In the first study, different types of bacterial population (total viable, cellulolytic, proteolytic, amylolytic and lypolytic) were counted from the rumen fluid (RF) of fistulated sheep. In the second study, total viable bacteria were counted from the (RF) following its individual incubation with five spices (cinnamon, cumin, coriander, turmeric and clove) for 48 h. In the third study, Gram positive and Gram negative bacteria were considered from RF collected from sheep, after its *in vitro* incubation with rice straw in the presence and absence of spices for 120 h.

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Materials and Methods

Feed Materials: Five spices were selected on the basis of their availability, cost and potential for use as feed additives. Here spices represented by cumin (Cm = *Cuminum cyminum*), coriander (Cr = *Coriandum sativum)*, clove (Cl = *Syzygium aromaticum*), cinnamon (Ci = *Cinnamomum cassia*) and turmeric (Tu = *Curcuma longa*) were collected from the local market of Newcastle upon Tyne, UK. Samples of rice straw (RS = *Oryza sativa*, Asian rice) variety (IR50) were collected from Kushtia, Bangladesh. The detailed chemical compositions of spices were carried out in this lab as shown in Table 1 and 2 by using procedures as described by Chaudhry and Khan (2012) and Khan and Chaudhry (2010).

Table 1. Proximate composition (g kg^{-1} DM, unless stated otherwise) and fibre content, total sugars, starch, total phenolics, total tannins, condensed tannin and saponins of different spices (Modified from Khan and Chaudhry, 2010 and Chaudhry and Khan, 2012).

Spices	Ci	Cl	Cr	Cm	Tu
DM (g kg^{-1} fresh basis)	950	920	940	960	950
Crude Protein	74	102	106	223	86
Ash	43	85	65.6	80	69.4
Ether Extract	34	73	155	146	26
Neutral detergent fibre	450	280	540	550	380
Acid detergent fibre	540	250	380	240	300
Acid detergent Lignin	300	180	140	110	90
Total sugar	26	53	75	65	68
Starch	169	103	111	80	542
Total Phenol	51	214	11	19	22
Total tannin	36	109	2.8	4	11
Condensed tannin	30	NP	NP	NP	NP
Saponin	62	47	17	44	38

Ci = Cinnamon; Cl = Clove; Cr = Coriander; Cm = Cumin; Tu = Turmeric; TE = Tannic acid equivalent; CE = Catechin equivalent; DE = Diosgenin equivalent; NP = Not present;

Table 2. Mean mineral components (mg kg^{-1} DM) of different spices (Modified from Khan and Chaudhry, 2010).

	Ci	Cl	Cr	Cm	Tu
Ca	9630	9999	5970	8303	1539
K	3758	16403	15015	14180	24126
Mg	735	2794	2760	2700	2418
Na	360	2700	1275	2296	788
P	442	1135	3945	3969	2309
Cu	2.93	4.08	10.34	5.50	8.24
Co	2.11	1.97	1.76	1.78	0.68
Mn	196	612	27.9	50	22
Se	3.56	6.65	4.88	9.20	1.94
Zn	18.2	17.7	42.6	50.4	11.1

Ci = Cinnamon; Cl = Clove; Cr = Coriander; Cm = Cumin; Tu = Turmeric

Collection of rumen fluid from fistulated sheep: Rumen fluid (RF) was obtained from two fistulated sheep (Lleyn breed) with mean live-weight of 81 kg just before their morning feeding. These sheep were managed under the Animal and Scientific Procedures Act 1986 of the UK. These sheep were consuming fixed amounts (1200 g day^{-1}) of a diet comprising 65 % chopped hay and 35 % concentrate to fulfil their maintenance requirement (AFRC, 1993). The concentrate consisted of (% Dry Matter) soybean meal (20), maize gluten feed (15), rolled barley (27.5), sugar beet pulp (25), soy pass (2.5), molasses (7.5) and vitamin and mineral supplement (2.5). The RF was transported in insulated flasks under anaerobic conditions to the laboratory. The RF was strained through four layers of a cheese cloth into pre-warmed flasks under CO_2 before its mixing with the pre-warmed phosphate-bicarbonate (McDougall, 1948) buffer at 1:4 ratio to prepare the inoculum. The flasks were then screw capped and kept at 39°C in a water bath until used.

Counting of bacterial population

Preparation of different types of Media: Five types of media and one type of anaerobic dilution fluid were prepared to count five types of bacterial population (total viable, cellulolytic, proteolytic, amylolytic and lypolytic) according to Rajvir *et al.* (2006) with some modifications. The chemical composition of different types of media and anaerobic dilution fluids are given in Table 3. The culture media were used to count total viable bacteria. In contrast anaerobic dilution fluid was used to dilute the RF, so the number of bacteria should be in visible range (30 - 300, as bacterial colonies of more than 300 were not countable). Anaerobic dilution fluid was prepared by adding 12.5 g anaerobic agar (Collected from VWR) with 1 l buffer solution (McDougall, 1948) which was maintained in 40°C in a water bath. The cellulolytic, proteolytic, amylolytic and lypolytic media were used to count cellulolytic, proteolytic, amylolytic and lypolytic bacteria, respectively.

Table 3. Chemical composition for different types of bacterial media g l^{-1} (mentioned otherwise).

Constituents	Anaerobic dilution fluid	Media				
		Culture medium	Celluloly. Bacteria	Proteoly. Bacteria	Amyloly.B acteria	Lipoly. Bacteria
Anaerobic agar	12.5	50	20	20	20	20
Agar			30	30	30	30
Egg albumin				10		
Starch (10 g 30 ml^{-1})					200 ml	
Cellulose suspension *			200 ml			
Glucose		1				
Linseed oil emulsion						100 ml
$NaHCO_3$	9.8	9.8	9.8	9.8	9.8	9.8
$NaHPO_4.12H_2O$	9.3	9.3	9.3	9.3	9.3	9.3
NaCl	0.47	0.47	0.47	0.47	0.47	0.47
KCl	0.57	0.57	0.57	0.57	0.57	0.57
$CaCl_2$ anhyd	0.04	0.04	0.04	0.04	0.04	0.04
$MgCl_2$ anhyd	0.06	0.06	0.06	0.06	0.06	0.06

**Cellulose suspension – 24 g of filter paper into small squares (1 cm × 1 cm) soaked overnight in HCL (270 ml 300 ml^{-1}). Filter paper was washed under running tap water until pH of water became within the physiological limits (5 - 7) of rumen; filter paper was ground in a mixer with 250 ml of distilled water for 1 h.*

Counting of bacterial population from sheep RF: RF mixed with buffer solution as mentioned earlier was used to count total viable bacteria using the anaerobic culture roll tube technique of Hungate as described by Rajvir *et al.* (2006) with some modifications. Anaerobic condition was maintained strictly by purging the tubes with CO_2. The culture media and the Hungate tubes with caps were sterilised at 121°C for 30 min. Serial dilutions from 1×10^{-1} to 10^{-9} of the RF from each sheep were made by using the anaerobic dilution fluid (Table 3). About 0.5 ml inoculum from each of the 10^{-9} to 10^{-3} serial dilutions was transferred to 2 ml of anaerobic culture medium that was maintained in roll tubes at 45°C. The tubes were rolled and incubated at 39 - 40°C for 3 days. The bacterial colonies were counted and expressed as total viable count of rumen bacteria ml^{-1} by multiplying the number of colonies with the relevant dilution factor. To count specific types of bacteria (cellulolytic, proteolytic, amylolytic and lypolytic) specific types of media as listed in Table 3 were used, where the rest of the procedure was same as described above.

Total viable bacterial count from RF incubated with different spices: In this experiment, Completely Randomized Design (CRD) in duplicate were carried out to count total viable bacteria from RF incubated with five spices. The incubations of forages were conducted in 50-ml polypropylene tubes containing 0.4 g of each of the ground rice straw with 40 ml of buffered RF (1:4 ratio). After incubation for 48 h at 39 - 40°C, the supernatant was collected and diluted in serial dilutions made in anaerobic dilution fluid from 1×10^{-1} to 10^{-9}. Inoculum of 0.5 ml was transferred from 10^{-9} to 10^{-3} serial dilutions to 2 ml of anaerobic culture medium maintained at 45°C. The tubes were rolled and incubated at 39 - 40°C for 3 days. The number of colonies was counted and total viable count of rumen bacteria ml^{-1} was calculated by multiplying the number of colonies with the relevant dilution factors.

Measuring Gram positive and Gram negative bacteria in RF before and after its incubation with rice straw alone or with spices: The effects of adding three spices (coriander, cumin and turmeric) on the basis of result of previous experiment at a fixed level of 40 mg g^{-1} rice straw as the common substrate were compared in duplicate with rice straw alone (Control = Ct) for Gram positive and Gram negative bacteria. RF was collected from the same sheep and same procedure was followed as discussed above. On a glass slide (7.5 cm × 2.5 cm × 0.1 cm) one drop of RF was spread using another slide and the slide containing RF was placed on a hot plate for few seconds until it is dried to ensure that the bacteria were firmly fixed to the slide.

Staining the slides: Gram stain, containing four bottles, was purchased from Fischer Scientific, UK (PL 7000/25, PL 7003/25, PL 7006/25 PL and 7012/25). Primary stain crystal violet was added on the slide and waited for 1 minute before it was rinsed gently with water. In this step colours of all bacterial cells were observed to be violet. After that, Gram's iodine was added and washed after 30 seconds with acetone for 5 seconds. The secondary stain, safranin, was added on this slide which was then washed after 1 minute with water for a maximum of 5 seconds. The Gram-positive bacteria retained the primary stain, and so appeared as black-violet whereas the Gram-negative bacteria lost the primary stain and acquired the secondary stain and so appeared as red-pink. The stained slide was kept in a slide box for future use.

In vitro incubation trial:The RF collected from two sheep were mixed with the pre-warmed buffer at 1:1 ratio by mixing one part of RF and one part of the buffer solution. *In vitro* incubations were conducted in 50-ml polypropylene tubes containing 0.4 g of the rice straw, three spices were added according to 30 g spices kg^{-1} straw basis. After that 40 ml of buffered RF was added. The tubes were purged with CO_2, sealed with rubber stoppers fitted with pressure released valves and incubaed at 39°C in water bath for 120 h. When the incubation was completed, one drop of RF from each tube was spread on the slide as described above. A similar procedure was followed for staining the slides as described above. The stained slide was kept in a slide box for future use.

Microscopic observation: To monitor the Gram positive and Gram negative bacteria the slides were examined by Vickers microscope (Vickers Ltd, England) at 250× magnification and photographed by Moticam 1000 (Motic R, China).

Statistical Analyses: In the first study, the average of different types of bacteria from RF collected from two sheep in duplicate was calculated individually and collectively. In the second experiment CRD in duplicate were carried out to monitor the main effects of spices on total viable bacteria after incubation of 48 h. The data were compared using the Tukey's test for their significance at $P<0.05$ by using Minitab 15. In the third experiment CRD in duplicate were carried out to monitor the effect of spice supplementation on Gram positive and Gram negative bacteria of RF collected from the two sheep in the absence or presence of forage alone.

Results and Discussion

Results

Counting of bacterial population from sheep RF: Different types of bacterial counts of RF collected from two sheep are given in Table 12. The total viable counts of bacteria in sheep and 1 and 2 were 8.7 × 108 ml^{-1} and 8.1 × 108 ml^{-1} respectively with an average count of 8.4 × 108 ml^{-1}. Cellulolytic bacterial counts was very near to the total viable count (6.4 × 108 ml^{-1} and 6.2 × 108 ml^{-1} in sheep 1 and 2 respectively). After cellulolytic bacteria next group of bacteria were proteolytic bacteria. The number of proteolytic bacteria in sheep 1 and 2 were 9.9 × 107 ml^{-1} and 8.9 × 107 ml^{-1} respectively. Amylolytic bacteria were 6.4 × 107 ml^{-1} and 6.8 × 107 ml^{-1} respectively in sheep 1 and 2. The lypolytic bacterial count was lowest among all types of bacteria and the values were 2.5 × 107 ml^{-1} and 2.9 × 107 ml^{-1}, respectively in two sheep.

Total viable bacterial count from RF incubated with different spices: Significant difference was observed among the spices for viable bacterial count as shown in Table 4. Total viable count was highest in the presence of cumin followed by coriander and it was lowest in the presence of cinnamon. The total viable count in cumin was higher than the viable count of RF collected from sheep, though no statistical comparison was done.

Measuring Gram positive and Gram negative bacteria in RF: The microscopic picture of RF of two sheep after Gram staining is shown in Fig. 1 and 2. From the pictures it was clear that the RF contained both Gram positive and Gram negative bacteria with a larger amount of Gram positive bacteria. Gram staining slide of control after 120 h of incubation was shown in Fig. 3. Like RF from sheep, in control both Gram positive and Gram negative bacteria were present and a large number of bacteria were Gram positive. The microscopic slides that were prepared using RF from the tubes containing turmeric, coriander and cumin were added are shown in Fig. 4, 5 and 6, respectively. From the pictures, it can be noticed that in the presence of these three spices after 120 h of incubation the existence of Gram negative bacteria were increased and the existence of Gram positive bacteria were decreased in these tubes.

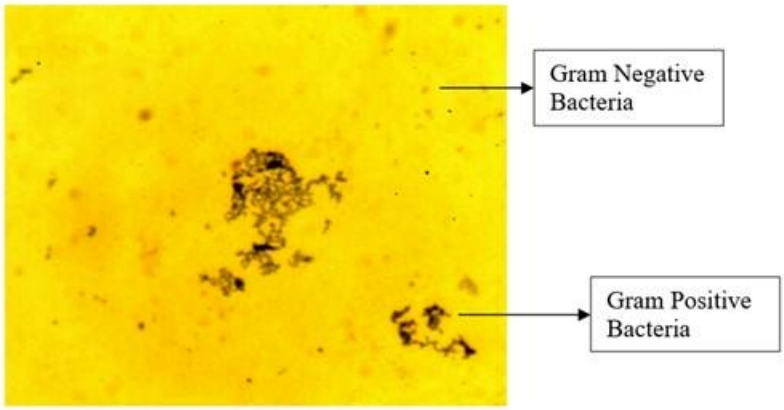

Fig. 1. Gram staining of rumen fluid collected from sheep 1

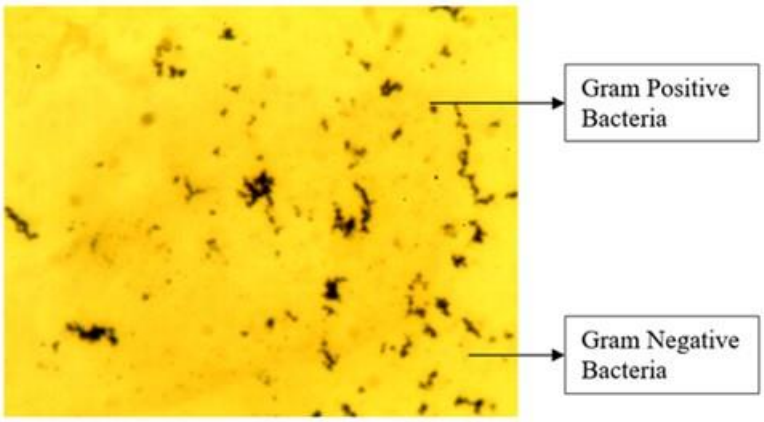

Fig 2. Gram staining of rumen fluid collected from sheep 2

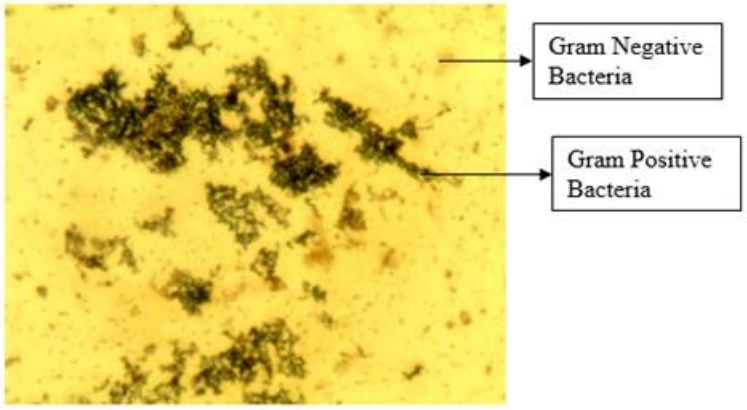

Fig. 3. Gram staining of rumen fluid collected after 120 h of incubation with rice straw.

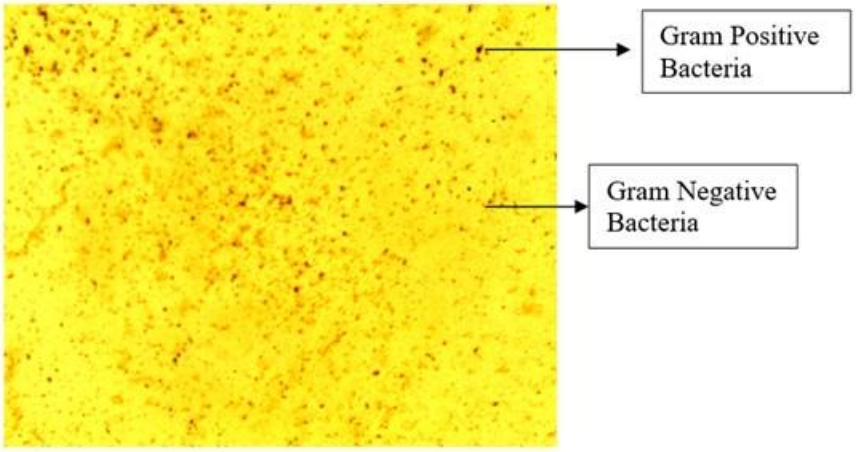

Fig. 4. Gram staining of rumen fluid collected after 120 h of incubation with rice straw added with turmeric.

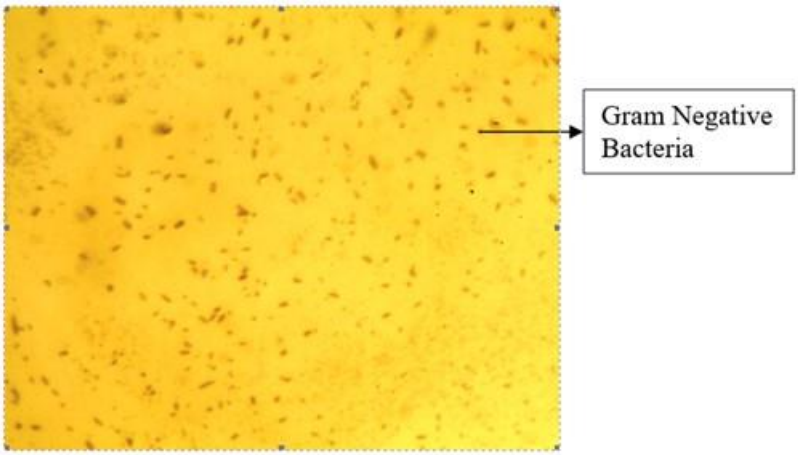

Fig. 5. Gram staining of rumen fluid collected after 120 h of incubation with rice straw added with coriander.

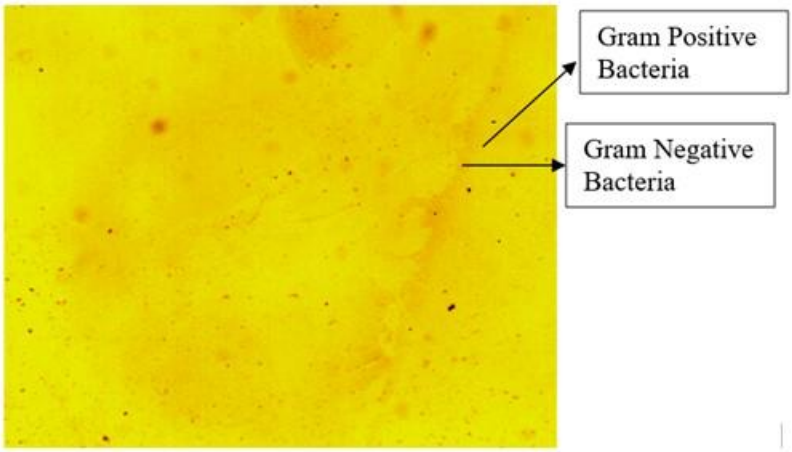

Fig. 6. Gram staining of rumen fluid collected after 120 h of incubation with rice straw added with cumin.

Table 4. Different types of bacterial count in two sheep number ml^{-1}.

Type of bacteria	Sheep 1 average	Sheep 2 average	Average of two sheep
Total viable count	$8.7 \pm 0.3 \times 10^8$	$8.1 \pm 0.4 \times 10^8$	$8.4 \pm 0.35 \times 10^8$
Cellulolytic	$6.4 \pm 0.3 \times 10^8$	$6.2 \pm 0.4 \times 10^8$	$6.3 \pm 0.3 \times 10^8$
Proteolytic	$9.9 \pm 0.8 \times 10^7$	$8.9 \pm 0.6 \times 10^7$	$9.4 \pm 0.7 \times 10^7$
Amylolytic	$6.4 \pm 1.2 \times 10^7$	$6.8 \pm 0.8 \times 10^7$	$6.6 \pm 1.0 \times 10^7$
Lypolytic	$2.5 \pm 0.8 \times 10^7$	$2.9 \pm 0.7 \times 10^7$	$2.7 \pm 0.8 \times 10^7$

Table 5. Total viable bacterial counts from rumen fluid after *in vitro* incubation with different spices.

Spice type	Total viable bacteria (number ml^{-1})
Cumin	$9.1 \pm 0.8 \times 10^8$
Coriander	$8.9 \pm 0.4 \times 10^8$
Clove	$7.6 \pm 0.6 \times 10^7$
Turmeric	$6.3 \pm 1.2 \times 10^7$
Cinnamon	$7.5 \pm 0.8 \times 10^6$
	P<0.001
SEM	14×10^7

Discussion

Total bacterial counts in rumen are normally 10^9 - 10^{11} ml^{-1}, but it depends on many factors like species, feed, time of collection, age of animal etc (Rajvir *et al.,* 2006; Kamra, 2005). Total viable count was lower than the total count (Rajvir *et al.,* 2006). Several researchers (Zawadzki and Zawadzki, 1988; Lopez *et al.,* 1999; Giraldo *et al.,* 2008) found total viable counts as 2.3×10^8 ml^{-1} to 23.5×10^8 ml^{-1} of RF if it was either directly collected from sheep or from the *in vitro* tubes containing RF from sheep. The huge variation was due to the type of feed supplied to the animal. Total viable count in sheep supplied with hay or straw based diet were 2.3×10^8 ml^{-1} to 6.43×10^8 ml^{-1} (Lopez *et al.,* 1999; Giraldo *et al.,* 2008) which were comparable to the results of this study. The sheep used in the present study consumed a hay based diet that might have caused very high population of cellulolytic bacteria in their RF compared to other types of bacteria. Pietraszek (1970) observed that cellulolytic bacteria were increased in the presence of cellulose based diet. Several researchers (Lopez *et al.,* 1999; Giraldo *et al.,* 2008; Latham *et al.,* 1971) reported cellulolytic bacterial count from 0.88×10^6 ml^{-1} to 6.43×10^8 ml^{-1} in hay or straw based diet whereas Rajvir *et al.* (2006) observed 6.45×10^8 ml^{-1} cellulolytic bacterial counts in buffalo. Yang *et al.* (2007) and Wanapat *et al.* (2008) observed 6.45×10^8 ml^{-1} and 9.0×10^8 ml^{-1} cellulolytic bacterial counts, respectively in cattle. Pietraszek (1970) mentioned that the number of proteolytic bacteria depended on the protein content of the diet, and these were decreased with the increasing amounts of soluble sugars in the diet. Wanapat *et al.* (2008) counted 1.4×10^8 ml^{-1} proteolytic bacteria in cattle fed on 5 % urea supplemented rice straw and a concentrate. In the present study, sheep were supplied with less amount of concentrate that might have caused lower proteolytic bacteria than cellulolytic

bacteria. The counts of amylolytic bacteria in the present study are comparable with the counts of Wanapat *et al.* (2008). The viable count of lipolytic bacteria in the rumen of sheep fed on hay and dried grass was 2.7×10^7 ml^{-1} (Henderson, 1975) which was very near to the value of present study. From the above discussion it can be concluded that different bacterial counts from the RF obtained from two sheep were in acceptable range. So this RF can be used for further research to observe the effect of different forages and spices on bacterial population.

Higher CP, soluble sugar and minerals present in cumin and coriander (Khan and Chaudhry, 2010) might have caused higher viable count in the presence of these two spices. Higher tannin in clove and turmeric than cumin and coriander might have caused lower bacterial count in these two spices as Sotohy *et al.* (1997) reported that the number of total bacteria in the rumen of goats decreased significantly when the animals were fed tannin-rich plant. Cinnamon was very high in condensed tannins that might have caused very low bacterial count in the presence of cinnamon. Savitri *et al.* (1986) observed that, 0.5 % cinnamon reduced count of anaerobic bacteria *Clostridium perfringens* in 1 % pigeon pea containing Brain Heart Infusion broth. Lower microbial count in the presence of cinnamon might have reduced *in vitro* degradability (IVD) of forages as reported by Khan and Chaudhry (2010) and also the IVD of cinnamon itself (Khan and Chaudhry, 2016).

In the present study, the microbial count for turmeric was lower than the microbial count of RF collected from sheep. It was observed that the IVD of turmeric increased greatly at longer incubation time (144 h). The IVD of forages was also increased greatly in the presence of turmeric at higher incubation time (Khan and Chaudhry, 2010). In the current study, the microbial counts of spices were carried out after 48 h of incubation whereas longer incubation time would have increased the total microbial count in the presence of turmeric.

Turmeric also showed reduction of Gram positive bacteria. Negi *et al*. (1999) observed that turmeric oil obtained from curcumin industry as a by product had an antibacterial activity which can reduce the Gram positive bacteria rather than Gram negative bacteria. Shankar and Murthy (1979) reported that both curcumin and turmeric oil can suppress Gram positive bacteria. Higher percentages of poly unsaturated fatty acids (PUFA) were present in coriander, cumin and turmeric (Chaudhry and Khan, 2012). PUFA have direct toxic effects on ruminant microorganisms especially the Gram positive bacteria (Broudiscou *et al.,* 1994; Dong *et al.*,1997). Demeyer and Henderickx (1967) concluded that the inhibition of Gram-positive bacterial growth was increased with the number of the double bonds of fatty acids. Large amount of dietary EE and PUFA present in coriander and cumin might have reduced Gram positive bacteria. Recently researchers (Calsamiglia *et al.,* 2007; Benchaar *et al.,* 2008) reported that essential oils have some antimicrobial activity and these microbial activities were mainly more relevant to Gram positive bacteria. All three spices used in the present study contained large amount of essential oils which also might have reduced Gram positive bacteria. By reducing Gram positive bacteria with the help of coriander, cumin and turmeric as additives, methane production may also be reduced. Chaudhry and Khan, (2012) reported lower methane production for forages and wheat as substrates in the presence of these three spices.

The bacterial population of RF collected from the two sheep were in acceptable range; hence this can be used to study the effect of spices on bacterial populations. The higher nutritive value of some spices also resulted in increased total microbial count. Antimicrobial activity of cinnamon had caused very low amount of microbial count in the presence of cinnamon. Reduction of Gram positive bacteria in the presence of turmeric, coriander and cumin suggested that these three spices could be used as additives to reduce methane production in ruminants. Effects of these spices on methane production can also be considered for the future research on animal studies.

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