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ORIGINAL RESEARCH

Effect of isolate of ruminal fibrolytic bacterial culture supplementation on fibrolytic bacterial population and survivability of inoculated bacterial strain in lactating Murrah buffaloes

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Abstract

Aim: The present study was conducted to evaluate the effect of bacterial culture supplementation on ruminal fibrolytic bacterial population as well as on survivability of inoculated bacterial strain in lactating Murrah buffaloes kept on high fibre diet.

Materials and Methods: Fibrolytic bacterial strains were isolated from rumen liquor of fistulated Murrah buffaloes and live bacterial culture were supplemented orally in treatment group of lactating Murrah buffaloes fed on high fibre diet to see it's effect on ruminal fibrolytic bacterial population as well as to see the effect of survivability of the inoculated bacterial strain at three different time interval in comparison to control group.

Results: It has been shown by real time quantification study that supplementation of bacterial culture orally increases the population of major fibre degrading bacteria i.e. *Ruminococcus flavefaciens*, *Ruminococcus albus* as well as *Fibrobacter succinogenes* whereas there was decrease in secondary fibre degrading bacterial population i.e. *Butyrivibrio fibrisolvens* over the different time periods. However, the inoculated strain of *Ruminococcus flavefaciens* survived significantly over the period of time, which was shown in stability of increased inoculated bacterial population.

Conclusion: The isolates of fibrolytic bacterial strains are found to be useful in increasing the number of major ruminal fibre degrading bacteria in lactating buffaloes and may act as probiotic in large ruminants on fibre-based diets.

Key words: fibrolytic bacterial isolates, real time PCR, *Ruminococcus*, Murrah buffaloes

Introduction

The primary sources of energy found in forages are the structural polysaccharides, cellulose, hemi-cellulose and pectin. These three components generally account for about 400-600 g/kg forage dry matter. In tropical countries of the world, the ruminants are fed on lignocellulosic agricultural by-products like cereal straws, stovers, sugarcane bagasse, tree foliages and cakes of oil seeds like groundnut, cotton, mohua, neem and mustard. The efficiency of ruminants to utilize such a wide variety of feeds is due to highly diversified rumen microbial ecosystem consisting of bacteria (10^{10}–10^{11} cells/ml, representing more than 50 genera), ciliate protozoa (10^{4}–10^{6}/ml, from 25 genera), anaerobic fungi (10^{3}–10^{5} zoospores/ml, representing five genera) and bacteriophages (10^{8}–10^{9}/ml) [1]. These numbers might even be larger as majority of them are non-culturable. Because cellulose is the most abundant component of plant cell walls, ruminal cellulolytic microorganisms play a central role in the nutrition of ruminant animals fed diets based on forage. Digestibility of fibre in ruminants may be improved by the introduction of highly fibrolytic strains of bacteria in the rumen. This approach may be feasible only if the inoculated bacterial isolates survive in the system and express the ability to rapidly digest the fibre with other strains present in rumen. As the rumen microbial ecosystem is not fully explored and majority of the rumen microbes are unculturable and not fully identified therefore, there is a need to generate more information on rumen microbes using recent molecular tools.

Thus, the aim of present study was to identify potent fibre degrading bacteria from the rumen of buffaloes and their use as probiotic supplement for the manipulation of rumen microbial ecosystem towards improving utilization of low quality forage feeds.

Materials and Methods

Isolation of fibre degrading bacteria:

Fibre-degrading bacteria were isolated from rumen liquor of three permanently fistulated buffaloes maintained at National Dairy Research Institute herd and fed on high fibre diet (concentrate: roughage: 40:60). Freshly collected rumen liquor was used for isolation of fibre degrading bacteria. Prior permissions for taking rumen liquor from experimental male fistulated buffalo already taken from Institutional Animal Ethics Committee (IAEC).

Total forty-two numbers of bacterial isolates were isolated by Hungate's anaerobic roll tube technique using carboxymethylcellulose as substrate in the broth

Table-1. Rumen bacteria targeted and primers used

Order Name	Primers sequence	Amplicon size	Annealing temperature	References
Ruminococcus albus	Fwd- 5'-CCCTAAACAGTCTTAGTTCG-3 Rev- 5'-CCTCCTTGCGGTTAGAACA-3'	175	56	[7]
Ruminococcus flavefaciens	Fwd- 5'-CGAACGGAGATAATTTGAGTTTACTTAGG-3' Rev- 5'-CGGTCTCTGTATGTTATGAGGTATTA-3'	132	56.3	[5]
Fibrobacter succinogenes	Fwd- 5'-GTTCGGAATTACTGGGCGTAAA-3' Rev- 5'-CGCCTGCCCCTGAACTATC-3'	121	60.6	[5]
Butyrivibrio fibrisolvens	Fwd- 5'-TCTGGAAACGGATGGTA-3' Rev- 5'-CCTTTAAGACAGGAGTTTACAA-3'	284	50	[6]

medium. Pure cultures were obtained by repeated roll tube preparation on agar medium, picking of single bacterial colony and sub culturing into broth medium. The fibre degrading characteristic of the bacterial isolates were tested by their *in-vitro* fibre degrading potential on pure NDF by using *in-vitro* gas production technique and based on *in-vitro* fibre degradability three most potent isolates were selected for molecular study. Molecular characterization of the isolates was carried out using conventional PCR technique. The genomic DNA extracted from the isolate was amplified using universal as well as gene specific primers for ruminal fibre degrading bacteria. All the isolates found to be of genus *Ruminococcus*. The amplified product of three most potent fibrolytic isolates based on their *in-vitro* true dry matter digestibility potential of pure NDF isolated from wheat straw, were sequenced and nearly the complete sequence data were obtained for all the three isolates. All three isolates have shown similarity with the *Ruminococcus flavefaciens* strains. The isolate NB-1 showed 97% similarity with *R. flavefaciens strain FD-1* and was most potent fibre degrader used as supplement for experimental lactating Murrah buffaloes.

Selection, distribution and feeding of animals:

Twelve lactating Murrah Buffaloes (Mid to Late lactation) were selected from the buffalo herd of National Dairy Research Institute, Karnal. Approval of use of animals to conduct nutritional studies was taken from Institute Animal Ethics Committee .Animals were divided into two groups of six each according to their milk yield, live body weights and days in lactation. Animals were fed with experimental diets (concentrate:wheat straw:green maize in the ratio of 40:30:30) plus live and autoclaved culture of *R. flavefaciens* strain FD-1 after 21 days adaptation of animals. The animals were housed in open shed having proper arrangement of individual feeding and watering.

Dosing of experimental animals:

Treatment group of animals were fed experimental diet plus live bacterial culture of *Ruminococcus flavefaciens* strain FD-1 (300 ml) orally on alternate day for a period of one month whereas, control group was fed control diet plus autoclaved bacterial culture (300ml) on alternate day for a period of one month. The bacterial culture contained $3x10^{12}$ number of cells per ml of diluent so total number of bacterial cells dosed in an animal was $9x10^{14}$ cells on alternate day basis continuously for one month period.

Sampling from experimental animals:

The representative rumen liquor sample was collected individually from all the animals of treatment as well as control group with the help of stomach tube before feeding once in a month i.e. Pre-dosing (prior to start of the dosing), during dosing (immediately after completion of dosing) and post-dosing (after one month of completion of dosing) period for quantification of inoculated bacteria as well as ruminal cellulolytic bacteria. The samples were squeezed through two layers of muslin cloth to remove any impurities (plant extracts, undigested matter, and so on) and processed for DNA extraction.

DNA extraction:

Total genomic DNA was extracted by using CTAB method [2]. DNA concentrations were measured at 260 nm with a Nanodrop. The DNA used for these experiments possessed an *A*260/*A*280 ratio higher than 1.8. To minimize animal to animal variations, the equal amount of extracted DNA of these animals was mixed together before PCR.

Conventional PCR:

PCR amplification was conducted with MyCycler™ thermal cycler (Bio-Rad, USA) as described [3]. The PCR conditions were 95°C for 5 min, followed by 35 cycles consisting of 95°C for 30 sec, various annealing temperatures (Table 1) for 30 sec, 72°C for 1 min, and a final extension period of 72°C for 10 min.

Real-time PCR:

To establish a quantitative assay, all the targeted groups (Table-1) were amplified using specific primers and cloned by using the Stratagene Blunt End cloning kit (Stratagene, USA) and purified by QIAprep kit (Qiagen, Venlo, The Netherlands). The purified plasmids were quantified by Nano-drop (ND-1000; V3.5.2) spectrophotometer (Thermo Fischer, USA) with multiple dilutions. The target DNA was quantified by using 10-fold serial dilutions from 10^{-1} to 10^{-8} plasmid copies of the previously quantified plasmid standards. Real time PCR amplifications and detections were performed in a MJ MiniTM personal Thermal cycler system (Bio-Rad,USA) as described [4]. In brief, SYBR Green Master mix (Fermentas, USA) was used for PCR amplification. Samples were assayed in triplicate in a 25 µl reaction mixture containing 5 mM MgCl2, 12.5 µl of 10× Mastermix (including Fast start enzyme, Fast start Taq DNA

Table-2a: Real time quantification of ruminal fibrolytic bacterial strain in experimental buffaloes

	Butyrivibrio fibrisolvens			*Fibrobacter succinogenes*	
Period(Dosing)	**Control**	**Treatment**	**Period(Dosing)**	**Control**	**Treatment**
Pre X 10^7	2.50^{a}±0.52	3.29^{b} ±0.19	Pre X 10^6	1.82^{a}±0.35	1.66±0.08
During $X10^7$	3.04±0.61	3.19±0.12	During $X10^6$	1.99^{a}±0.41	1.95^{a}±0.02
Post $X10^7$	2.87±0.62	3.21±0.02	Post X 10^6	1.26^{b} ±0.28	1.51^{b}±0.02

Table-2b: Real time quantification of ruminal fibrolytic bacterial strain in experimental buffaloes

	Ruminococcus flavefaciens			*Ruminococcus albus*	
Period(Dosing)	**Control**	**Treatment**	**Period(Dosing)**	**Control**	**Treatment**
Pre X 10^7	1.86^{a}±0.37	1.46^{ac}±0.02	Pre X 10^6	1.72±0.36	1.73±0.01
During $X10^7$	2.33±0.48	2.23^{b}±0.02	During $X10^6$	1.77±0.36	1.96±0.08
Post $X10^7$	2.58^{b}±0.54	2.52^{b}±0.01	Post X 10^6	1.78±0.37	1.87±0.02

Different superscripts in columns differs significantly

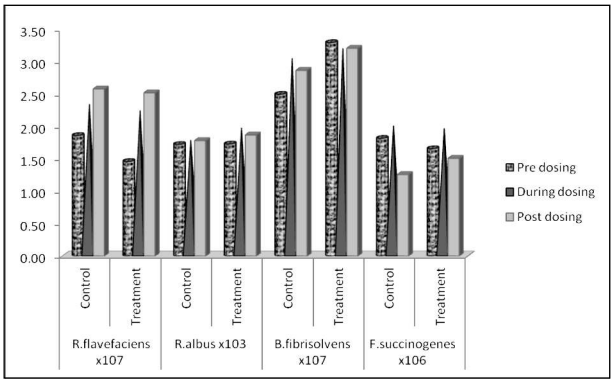

Figure-1: Real Time Quantification of Ruminal Fibrolytic Bacterial Strain in Experimental Buffaloes at Different Time Period

polymerase, reaction buffer, dNTP mixture, MgCl2, and SYBR Green dye), 50 ng of template DNA, and 0.5 µM of each primer. All PCR reactions were performed in triplicate.

Results and Discussion

For absolute quantification of different ruminal microbes, external standards were prepared from a simulated rumen matrix. For each standard, linear regressions derived from the threshold cycle [Ct] of each DNA dilution versus the threshold cycle were calculated. Logarithms of the DNA concentrations were plotted against the threshold cycles with a linear correlation coefficient (r^2) ranging from 0.993 to 0.997 for different ruminal microbes. The standard curves obtained were used to quantify DNA of different ruminal microbes.

The population sizes of targeted ruminal microbes are given in Table-2a and Table-2b respectively. Results indicated that dosing increases *R.flavefaciens* population significantly (P<0.05) from $1.46x10^7$/ml of rumen liquor to $2.23x10^7$/ml during dosing period and to $2.52x10^7$/ml after post dosing period in treatment group whereas in control group the increase was significant (P<0.05) only during post dosing period ($2.58x10^7$/ml of RL) in comparison to pre dosing ($1.86x10^7$/ml of RL). The increase in *R.albus* population was also noticed in treatment and control group and the increase was from $1.73x10^3$/ml of RL to $1.96x10^3$/ml and from $1.72x10^3$ to $1.77x10^3$/ml of RL, respectively during dosing period although the effect was not significant. The *B.fibrisolvens* population increased in control group and the increase was from $2.5x10^7$ to 3.04 x 10^7/ml of RL whereas in treatment group the population of *B.fibrisolvens* decreased from $3.29x10^7$ to 3.19 $x10^7$/ml of RL, which shows antagonistic relation in terms of population growth with *R.flavefaciens*. The population of *F.succinogenes* was increased during dosing in both group but it decreased after post dosing and the decrease was significant (P<0.05) in comparison to during dosing population. The graphical representation of real time quantification study of ruminal fibrolytic bacterial strain in experimental buffaloes at different time interval has been shown in Figure-1.

The present finding was very much identical with the finding of [5] who found that predominant cellulolytic bacteria in the rumen of swamp buffalo were 8.01×10^6, 5.76×10^5 and 2.75×10^4 copies/ml for *F. succinogenes*, *R. flavefaciens*, and *R. albus*, respectively [5]. It was found that *R. albus* was more abundant than both *F. succinogenes* and *R. flavefaciens* in batch and continuous culture [6] while [7] found *R. flavefaciens* was slightly more abundant than *F. succinogenes* with both species outnumbering *R. albus*.

The level of dosed *Ruminococcus*, and total *Ruminococcus*, *Fibrobacter succinogenes* and eukaryotes measured by 16S rRNA probes increased significantly (*P<0.05*) during the dosing period, but declined post-dosing in the sheep dosed with 500 ml medium containing strain of *Ruminococcus* continuously for two weeks [8] matches with the result of present finding as in present finding also population of *Ruminococcus flavefaciens, Fibrobacter succinogenes* and *Ruminococcus albus* has shown marked increase during dosing period whereas declined post dosing except in dosed strain.

Conclusions

The quantification studies showed that there was increase in major ruminal fibre degrading bacterial population whereas this increase was significant in inoculated bacterial population even after dosing period of one month in treatment group as compare to control group of animals during dosing and post dosing period which may lead to increased fibre digestion in rumen.

Author's contribution

BK carried out the experiment, analysis and drafted manuscript under guidance of SKS. Both the authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

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