SHORT COMMUNICATION

Effect of oral dosing of live and autoclaved culture of *Ruminococcus flavefaciens* FD-1 on rumen bacterial and fungal populations in Murrah buffaloes

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Received: 31 May 2021 / Accepted: 19 August 2021 / Published online: 31 October 2021
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Abstract: Dietary interventions aiming at increasing the number and activities of beneficial gut microbes could enhance digestive functions as well as the long-term welfare of dairy animals. In the present experiment, the effect of supplementation of a novel bacterial culture *Ruminococcus flavefaciens* FD-1 isolated from the rumen liquor of Murrah buffaloes was studied for its effect on rumen microbial populations enumerated using the most probable number technique. Three permanently fistulated buffaloes maintained on a high fibre diet were used as the source of rumen fluid for bacterial isolation that was subsequently tested in vivo. Twelve healthy mid-to-late lactating buffaloes in their second to third parity were divided into two similar groups of six each with a mean body weight of 601.5 kg. One group (LBC) was dosed with 300 mL of live bacterial culture, whereas an equal volume of autoclaved bacterial culture was dosed in another group (ABC) for a period of one month. The treatment-wise effect of supplementation of *R. flavefaciens* FD-1 culture was evident in an increase ($P<0.05$) in total rumen fungal population by 2.7 times in LBC than ABC in the post-dosing period. However, the period-wise comparison revealed that the population of bacteria augmented ($P<0.05$) by 2.16 and 3.17 times in both the groups ABC and LBC, respectively; whereas the magnitude of increase in fungal population was 5.04 and 10.3 times, respectively for both the groups. Post-dosing effects were evident for only group LBC in both bacteria and fungi, respectively increasing ($P<0.05$) the population by 2.16 and 8.28 times than pre-dosing period. These preliminary results may foster scope for developing

species-specific (autochthonous) bacterial probiotic/direct-fed microbial based on *R. flavefaciens* FD-1 for large ruminants maintained on fibrous diets under tropical production systems.

Keywords: Direct-fed microbial, Fibrolytic bacterial isolates, Fungi, Murrah buffaloes, Rumen bacteria, *Ruminococcus flavefaciens*

Harnessing the potential of ruminants through natural feed additives targeting an improved gut microbial balance is growingly exigent today with a heightened research interest worldwide (O'Hara et al. 2020). Additive-led improvement in production performance would be of special interest for ruminants raised under agricultural residue-based low input system that is prevalent in India. Since rumen microbiota is an integral part of feed digestion and nutrient metabolism, it is imperative to maintain an optimum population of multifarious rumen microbes (Liu et al. 2021). When bacterial culture in the form of probiotic or direct-fed microbial (DFM) are supplemented, betterment in performance outcome and health status has been obtained through a better gut microbial balance (Mazon et al. 2020; Mahesh et al. 2021; Singh et al. 2021).

Ruminococcus are the predominant cellulose-digesting bacteria in rumen (Wanapat and Cherdthong, 2009; Israeli-Ruimy et al. 2017; Patra, 2020; Wang et al. 2020). A shift in microbial population was noted previously when *Ruminococcus* spp. are supplemented (Krause et al. 2001). However, it is also established

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that successful results are more likely to be obtained when bacterial culture originating from the host species (autochthonous) are supplemented (Patra, 2020; Singh et al. 2021). While the multitude of research explorations on various bacterial cultures is available, there seems limited published information on isolating fibrolytic bacteria from buffalo rumen and its subsequent evaluation on microbial population dynamics *in vivo*. Therefore, it was hypothesised in the present research that when the in-lab grown live culture of *R. flavefaciens* is orally administered, it survives in the rumen, multiplies as well as stimulates other beneficial microbes that co-exist in the ruminal consortium. Therefore, the main objective was to enumerate bacterial and fungal populations of Murrah buffaloes receiving either live or autoclaved cultures of *R. flavefaciens* before, during, and after the administration.

This research was approved by the Institutional Animal Ethics Committee of ICAR – National Dairy Research Institute, Karnal, India.

Fibre-degrading bacteria were isolated from the rumen liquor of three permanently fistulated adult male buffaloes maintained on a diet with a concentrate-to-wheat straw ratio of 40:60. Total fourty two numbers of bacterial isolates were isolated using Hungate's anaerobic roll tube technique (Hungate, 1969) using carboxymethylcellulose as the substrate in the culture medium. The culture medium for isolation of anaerobic fibrolytic bacteria was prepared as described by Bryant and Burkey (1953). Pure cultures were obtained by repeated roll tube preparation on agar medium, picking off the single bacterial colony, and subsequent sub-culturing into the broth medium. The fibre-degrading characteristics of the bacterial isolates were tested by their *in*

vitro fibre-degradation potential on pure neutral detergent fibre using *in vitro* Hohenheim gas production technique and based on *in vitro* fibre degradability (Kumar and Sirohi, 2013^a). The three most potent isolates (NB-1, NB-2 and NB-3) were selected and identified by molecular technique. The molecular characterisation of the isolates was done 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 (Kumar and Sirohi, 2013^b). All the isolates were found to be of genus *Ruminococcus*. The amplified product of three most potent fibrolytic isolates was sequenced and nearly the complete sequence data were obtained for all three isolates. Similarity values of 16S rRNA gene sequences retrieved from the select isolates have been shown in Table 1. All three isolates have shown similarity with the *Ruminococcus flavefaciens* strains. The isolate NB-1 showed 97% similarity with *Ruminococcus flavefaciens* strain FD-1 and was the most potent fibre degrader that was used later to supplement experimental Murrah buffaloes.

Twelve lactating Murrah buffaloes of mid-to-late lactation were selected from the buffalo herd of the institute and randomly distributed into two groups of six each according to their milk yield (mean: 5.9 kg/d) and live body weight (mean: 601.5 kg). The animals were housed in a well-ventilated open shed having arrangements for individual feeding and watering. Animals of both the groups were fed an experimental diet comprising of conventional concentrate:wheat straw:green maize in the proportion of 40:30:30. The overall diet had a nutrient profile of 12.1% crude protein, 2.6% ether extract, 55.6% neutral detergent fibre, 31.5% acid detergent fibre, and 9.2 MJ/kg of predicted metabolisable energy/kg dry matter (Owens et al. 2010).

Table 1 Similarity values of 16S rRNA gene sequences retrieved from three most potent ruminal fibre-degrading bacterial isolates

Isolate	Strain I.D.	Nearest relative taxon	Similarity (%)	Query coverage (%)	Gene bank accession no.
NB-1	NB-01	<i>Ruminococcus flavefaciens</i> strain FD-1	97	96	JN222390.1
NB-2	NB-02	<i>Ruminococcus flavefaciens</i> strain LP-C14	96	99	JN222391.1
NB-3	NB-03	<i>Ruminococcus flavefaciens</i> strain 007	96	97	JN222392.1

NB: NDRI buffaloes

Table 2 Ruminal populations of bacteria and fungi upon oral administration of *R. flavefaciens* FD-1 in Murrah buffaloes at different time intervals

	Bacterial population ¹		Fungal population ²	
	ABC	LBC	ABC	LBC
Pre-dosing	11.1 ^{aA} ± 4.29	7.58 ^{aA} ± 3.59	2.40 ^{aA} ± 1.72	1.22 ^{aA} ± 0.68
During dosing	24.0 ^{aB} ± 0	24.0 ^{aB} ± 0	12.1 ^{aB} ± 2.60	12.6 ^{aB} ± 3.89
Post-dosing	15.5 ^{aA} ± 4.80	16.4 ^{aC} ± 3.52	3.75 ^{aA} ± 1.55	10.1 ^{bB} ± 4.47

¹Expressed as cells × 10¹¹/mL of rumen liquor; ²Expressed as cells × 10⁵/mL of rumen liquor

^{a,b}Values with different superscripts within each row, separately for bacterial and fungal population, differ significantly (P<0.05)

^{A,B}Values with different superscripts within each column, separately for bacterial and fungal population, differ significantly (P<0.05)

After 21 days of adaptation, animals of treatment (LBC) and control (ABC) groups were orally dosed with live and autoclaved culture of *Ruminococcus flavefaciens* strain FD-1, respectively at the rate of 300 mL every alternate day. The bacterial culture contained 3×10^{12} cells per mL, and hence the total number of bacterial cells dosed per animal was 9×10^{14} cells on every alternate day continuously for a period of one month. Autoclaving of the culture was performed at 121°C for 15 minutes, thus arresting the activity of *R. flavefaciens* FD-1 and hence acted as control, and any effect detected in treatment group could be ascribed to the live cells of *R. flavefaciens* FD-1.

Rumen liquor sample was drawn individually from all the animals of both groups with the help of a stomach tube before feeding. i.e., pre-dosing (prior to the start of the dosing), during dosing (immediately after dosing), and post-dosing (after one month of completion of dosing) period for the quantification of total ruminal bacteria and fungi.

10 mL rumen liquor was taken in a sterilised beaker and transferred to a pre-gassed (CO_2) mixer-grinder containing 90 mL of anaerobic dilution medium. The mixture was thoroughly churned for 3-minutes in presence of CO_2 to dislodge microbes from feed particles. The dilution of the contents thus obtained was marked as 10^{-1} . Then, 1 mL of diluted sample was taken into dilution tube (containing 9 mL of sterilised anaerobic dilution fluid) to make the dilution to 10^{-2} . Likewise, serial dilutions were made up to 10^{-12} . The dilution ranged from 10^{-8} to 10^{-12} and 10^{-2} to 10^{-6} , and was used for the total bacterial and fungal count, respectively. Each dilution was in triplicate, and un-inoculated control tubes were also kept in triplicate. All tubes were incubated at 39°C for two weeks. After 2 weeks, pH was recorded, and a decrease of >0.3 unit pH when compared with control was considered positive for microbial growth. Three consecutive dilutions were selected in such a way that the maximum dilution contained negative tubes as well. The number of cells was enumerated from the most probable number (MPN) and the MPN media for total bacterial and fungal estimation were prepared in accordance with Grubb and Dehority (1976).

Data analysis was carried out separately for each bacterial and fungal population using one-way analysis of variance with the software package of Sigma Plot 11.0. The data were presented as mean $\pm$ SE, and the difference in mean values between ABC and LBC was tested for significance at $P < 0.05$.

The treatment-wise effect of supplementation of *R. flavefaciens* FD-1 culture was evident in an increase ($P < 0.05$) in total rumen fungal population by 2.7 times in LBC than ABC in the post-dosing period. In the both pre-dosing and dosing periods, the differences between groups ABC and LBC were non-significant for both bacterial and fungal populations. However, the period-wise comparison revealed that the population of bacteria augmented ($P < 0.05$) by 2.16 and 3.17 times in both the

groups ABC and LBC, respectively; whereas the magnitude of increase in fungal population was 5.04 and 10.3 times, respectively for both the groups. Post-dosing effects were evident for only group LBC in both bacteria and fungi, respectively increasing ($P < 0.05$) the population by 2.16 and 8.28 times than pre-dosing period.

Our objective was to test whether or not the bacterial culture of *R. flavefaciens* could able to colonise the rumen and in turn stimulate other gut microbes so that its potential to use as a DFM/probiotic for ruminants could be envisaged further.

Although *R. flavefaciens* dosing did increase microbial abundance, the effect of live and autoclaved cultures did not differentially influence microbial numbers during the dosing phase. It could be reasonable that certain residual nutrients contained in the growth media along with autoclaved cell fragments of test bacteria might have acted similar to the live culture in stimulating rumen bacteria and fungi. Such effects have already been known in ruminants, for instance, with yeast products (Shurson, 2018; Mahesh et al. 2021). In addition, our result on augmenting bacterial numbers corroborates with Ebtehag et al. (2016), who observed a significant increase in various bacterial populations in lambs after supplementation of bacterial isolate. Indeed, real-time quantification further deduced an increase in the bacterial population (Kumar and Sirohi 2013b). It could be seen that *R. flavefaciens* live culture exhibited carry-over effect by increasing fungal number many-fold, even after one month of withdrawal of supplementation. This indicates that fibrolytic bacterial culture stimulates the multiplication of fungal zoospores within rumen, albeit the mechanism underpinning such response remains unclear. Consistent with our findings, Krause et al. (2001) also documented changes in rumen microbial populations measured with 16S-rRNA-based probes upon administration of *Ruminococcus* spp. Furthermore, given the highly recalcitrant lignin-cellulose structure found in the crop residue-based diets in India (Mahesh and Mohini, 2013), the present results appear to hold promise in practically enhancing the digestion of complex forage fibre mediated by rumen fungi (O'Hara et al. 2020; Hartinger and Zebeli, 2021), which could result in better ruminant performance. Supportively, when dairy cattle were drenched with rumen bacterial culture *Megasphaera elsdenii*, the milk yield of cows was increased along with better rumen health attributes (Mazon et al. 2020). Nevertheless, a slight variation in the absolute number of microbial population than the previous studies could be due to differences in rumen microbial composition between cattle and buffaloes (Wanapat and Cherdthong, 2009; Wang et al. 2020).

It is also important to select the origin of fibrolytic bacteria in obtaining a desired response. Varel et al. (1995) could not observe multiplication of proven fibrolytic bacteria *Clostridium* spp. isolated from bison and pigs when administered to fibre-rich hay diets in bovines. Hence, it was suggested that autochthonous

(native) microbes seem advantageous over allochthonous (outside the native habitats) species for quick adaptation and establishment (Patra, 2020), as also demonstrated recently in tropical buffaloes (Singh et al. 2021).

Conclusion

The quantification of rumen microbial populations using the MPN technique showed that there was a significant increase in total fungal and bacterial population after oral administration of *R. flavefaciens* FD-1. Although the carry-over effect was persistent with live culture for the fungal population over a period of one month, it is necessary to investigate the long-term sustainable effects. Nonetheless, these preliminary findings clue that there is a potential to increase fibre digestion in ruminants with this isolate, which may open-up an opportunity to develop *R. flavefaciens*-based DFM for ruminants maintained on fibrous diets. However, in order to practically merit its use, economic feasibility needs further assessment when applying the results from lab to farm.

Acknowledgements

Authors acknowledge the research grant provided by the National Agricultural Innovation Project (NAIP-Component-4/C2128&65) of Indian Council of Agricultural Research to carry out this research work. This paper is dedicated to the memory of late Dr. S.K. Sirohi, Principal Scientist, ICAR – NDRI, Karnal, India.

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