Isolation and identification of probiotic lactobacilli from ruminant animals

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Background
Lactobacillus strains are a group of bacteria that provide health benefits to their hosts when consumed in proper amounts. They are which exhibit an important for intestinal microflora that constitutes a beneficial mechanism responsible for antimicrobial activity. Ruminant animals such as cattle, buffalo, goat, and sheep have formed a symbiotic relationship with ruminal microorganisms that synthesize fiber digesting enzymes. The microbial flora obtained from ruminal ingesta is an output of the composition, nature, and quality of the animals' feed. It is found that the dominating flora are usually starch and complex polysaccharide degrading microbiota.

Objective
The aim of the present study was to isolate and identify probiotic lactobacilli present in some ruminant animals to investigate interspecies differences in probiotic Lactobacillus contents.

Materials and methods
Sixty samples were collected in triple manner under aseptic conditions from buffalo, cattle, sheep and goats including rectal, buccal, and nasal swabs. Following DNA extraction from the isolated bacteria, 16S rRNA multiplex polymerase chain reaction analysis was performed to recognize the obtained isolates.

Results and conclusion
A total of 38 isolates were identified as lactobacillus species including 7, 14, 8, and 9 isolates from buffalo, cattle, sheep, and goat, respectively. The buffalo samples displayed the lowest variability in lactobacilli with the identification of *Lactobacillus delbrueckii* only. Cattle, goat, and sheep samples showed the presence of *Lactobacillus casei*, *Lactobacillus acidophillus*, *L. delbrueckii*, *Lactobacillus gasseri*, and *Lactobacillus rhamnosus*. It is found that *L. gasseri* was the most frequently isolated species in cattle followed by *L. rhamnosus,* while *L. delbrueckii* was the most frequently isolated species in goat followed by *L. casei. L. gasseri* was the most frequently isolated strain in sheep followed by *L. acidophillus* and *L. rhamnosus.* These strains should be investigated in more detail, individually or in combination, for their potential health benefits. Understanding how these species interact with other microbiota community members in each host as well as how they interact with host cells, particularly immune cells, can provide valuable insight into their function both in health and disease.

Keywords:
Microbiota, Ruminant, Probiotics, multiplex PCR

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Introduction

The discovery of antibiotics to treat infectious diseases has phenomenally impacted human and animal health since the 1940s. Unfortunately, antibiotics and disinfectants have been misused in haphazard ways worldwide, resulting in unprecedented health problems worldwide, leading to increased mortality and morbidity among humans and animals due to the spread of multiple drug-resistant bacteria. As a result, ICU settings in developing countries are experiencing greater economic costs [1]. By developing antibiotics from natural scaffolds, we may be able to counteract antibiotic resistance in the short term. Thus, in the ongoing scenario, reconnaissance and utilization of natural resources, comprising probiotics rather than their metabolites, will gain prominence as a means of developing functional biomolecules against multidrug resistance infections. It is essential to promote alternative nonantibiotic

protocols that are safer for humans and livestock but effective *versus* infectious pathogens. Among the alternative unconventional strategies are the use of antimicrobial peptides or bacteriocins [2], fecal microbiota transplant, nanomaterials, and nanoparticles [3] as well as competitive exclusion of pathogens via genetically modified probiotics and postbiotics [4].

Probiotics have received escalating attention in the scientific, healthcare, and public arenas in recent years. Public awareness of microbiome research has also led to a more rational view of microorganisms, which incorporates an understanding of their beneficial roles in human health. This is contributing to a growing public awareness and acceptance of probiotics [5], with an estimated 7% annual growth in the probiotic industry over the next 8 years [6].

A variety of lactic acid-producing bacterias (LABs) have traditionally been used as probiotics, including lactobacilli and bifidobacteria. These bacteria are found primarily in fermented dairy products and the human gut microbiome [7]. The most common natural source of LAB probiotic strains is fermented foods, which have been linked to significant health benefits, such as a lower risk of diabetes and cardiovascular disease [8] in addition to beneficial metabolic profiles [9]. In the human gut microbiome, these foods are most likely the major sources of LAB [10], and they may prove useful in the development of future probiotics. Probiotics may be found in fermented and unfermented foods, comprising grains/cereals, fruits, vegetables, dairy, meat, and fish products, as well as honey [11]. Besides gut and immune status, emerging goals for probiotic therapy include subfertility [12], liver illness [13], mood disorders [14], oral health [15], asthma [16], metabolic diseases [17], hypercholesterolemia [18], and obesity [19].

In addition, probiotics also interact with the microbiome through molecular effectors that attach to the cell structure or output as metabolic products. Microbiota can be affected by probiotic metabolites in several ways, including crossfeeding interactions, gastrointestinal microenvironment alterations (e.g. low pH), rivalry for nutrients and binding sites, as well as prohibition of growth via the production of strain-specific antibacterial compounds, involving bacteriocins [4,20]. These impacts of probiotics on the microbiota demonstrate their ability to benefit health in status of pathogen overgrowth, such as oral and vaginal dysbioses [21]. The probiotic effector molecules can act directly on receptors on intestinal epithelium, enteroendocrine cells, immune cells, and vagal afferent fibers of host cells. In addition to local gut impacts, such as promoting intestinal barrier integrity and inflammation (e.g. via Toll-like receptors). These interactions also have systemic impacts via immune, endocrine, and nervous system mediators [4,20,21]. Furthermore, probiotics are capable of performing enzymatic metabolization of ingested xenobiotics and bile salts [4]. There are many surface-related molecules linked with probiotics, such as pili, exopolysaccharides, lipoteichoic acids, and proteins; abundent of which are strain-specific and therefore have strain-specific acts [20,21].

Rumens act as an anaerobic and methanogenic fermentation chamber, which houses microorganisms that are capable of utilizing, and increasing the productivity of, cellulolytic feed (straw, hay, silage, grass). Rumen microbiomes contain a variety of bacteria, archaea, protozoa, and fungi, and are characterized by their high density, diversity, and complexity [22]. As a result of continuous fermentation, ingested compounds are broken down into their subcomponents by these microorganisms. Rumen microorganisms are either anaerobic or optionally anaerobic, and produce end outputs that are either consumed directly by the host or consumed by other microorganisms as energy [23]. The host and microbes' interaction in the rumen is synergistic, in which the host supplies moisture, heat, and food, while the microorganisms secrete proteins and digestion byproducts [24].

We have previously investigated the interspecies differences in probiotic lactobacilli among different animals including nonruminant animals [25–27]. We have also investigated the biological activity of some isolates of lactobacilli including their anti-parasitic activity [28], and the activity of some polysaccharides produced by *Lactiplantibacillus plantarum* and *Bacillus subtilis* including antioxidant and burn healing activity [29,30].

The target of the current study was the isolation and identification of naturally existing probiotic lactobacillus species in crtain farm ruminant species using multiplex polymerase chain reaction (PCR) to investigate interspecies differences.

Materials and methods

Collection of samples

Three swabs from different body cavities of each animal including rectal, buccal, and nasal swabs were gathered under aseptic stasuses from ruminant (buffalo,

cattle, sheep, and goat). The samples were gathered from individually owned apparently healthy animals in Giza governorate, Egypt to ensure the diversity of bacterial strains. All samples were collected in the presence of the owners after oral acceptance, and they were informed with the sampling procedure and very brief note about what the samples will be used for. The samples were collected in sterile carriers containing 5 ml MRS broth medium and stored on ice till transportation to the laboratory. Once transported to the laboratory, the samples were incubated at 37°C for 2 days in anaerobic conditions.

Isolation of probiotic strains

After incubation, the sampling containers were shaken homogeneously and a total of 10 µl of the liquid culture were transferred into test tubes containing 5 ml fresh MRS broth as selective media to grow Lactobacillus as well as other lactic acid bacteria (LAB). The tubes were homogeneously shaken and incubated at 37°C for 2 days in anaerobic conditions. Inoculum from each tube was subcultured at 37°C under anaerobic conditions in the presence of 10% CO_2 to prevent the growth of other bacteria. After several subcultures, the obtained cultures were streaked onto MRS agar media. Finally, single colonies with different morphology were isolated and streaked again onto fresh MRS agar media. The obtained pure colonies were stained with Gram and examined under optical microscope then tested for catalase production. LAB was identified by being rod and coccoid shaped, Gram-positive and catalase-negative bacteria [31].

Morphological and biochemical characterization

Gram staining test

Conventional procedure of Gram staining was performed and bacterial cells were examined microscopically (magnification ×1000) [32].

Catalase test

A single bacterial colony was picked up and streaked on a glass slide then mixed with one drop of 3% hydrogen peroxide (Merck, Germany). The effervescence of oxygen indicated the positive response of the bacteria to catalase test [33].

All catalase-negative and Gram-positive bacilli were classified as potential lactobacilli strains.

DNA extraction for molecular identification of probiotic lactobacillus strains

A total of 1.5 ml of overnight culture (of each of the mixed colony cultures representing the bacterial content of the original samples) in MRS broth was centrifuged at 5000*g* for 10 min at 25°C. The obtained cell pellet was used for total genomic DNA extraction using the G-spin total DNA extraction kit (Intron, Korea).

Molecular identification of probiotic strains

The obtained Lactobacillus isolates were species identified using multiplex PCR analysis of genomic 16S DNA extracted from mixed bacterial cultures. Multiplex PCR assays were conducted using a mixture of two primers for bacterial conserved genes rather than 7 group-specific and species-specific primers for *Lactobacillus* casei-group, *Lactobacillus acidophilus*, *Lactobacillus bulgaricus*, *Lactobacillus delbrueckii*, *Lactobacillus gasseri*, *Lactobacillus plantarum*, *Lactobacillus rhamnosus*, and *Lactobacillus reuteri* (Table 1). The PCR products were exposed on 1.5% agarose gel electrophoresis, then stained with RedSafe Nucleic Acid Staining Solution (Intron Biotechnology, Korea). Lactobacillus species was distinguished based on the of the PCR product's size [34].

Results and discussion

A total of 60 samples were collected from different ruminant species including buffalo (n=5), cattle (n=5), goat (n=5), and sheep (n=5). Three swabs were collected from each animal under aseptic conditions including a swab from rectum, buccal cavity, and nasal cavity. A representative picture of Gram-stained lactobacilli isolated from different animal species.

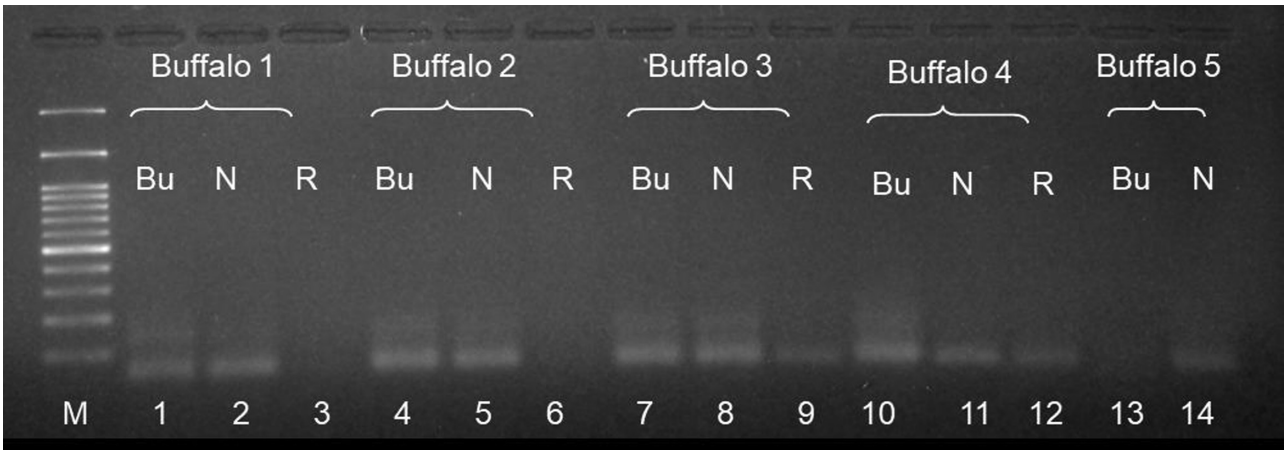
Table 1 Multiplex polymerase chain reaction primers

Target bacteria	Sequence (5'–3')	Target site	Product (bp)
Lactobacillus Acidophilus	AACTATCGCTTACGCTACCACTTTGC	2079–2104	606
Lactobacillus casei-group	TGGTCGGCAGAGTAACTGTTGTCG	472–495	727
Lactobacillus Delbrueckii	CTGTGCTACACCTAGAGATAGGTGG	1015–1039	184
Lactobacillus Gasseri	ATTTCAAGTTGAGTCTCTCTCTC	1748–1770	272
Lactobacillus plantarum	CTAGTGGTAACAGTTGATTAAAACTGC	1900–1926	428
Lactobacillus reuteri	ACCTGATTGACGATGGATCACCAGT	94–118	1105
Lactobacillus rhamnosus	GCCAACAAGCTATGTGTTCGCTTGC	1922–1946	448
All Lactobacillus species	CCACCTTCCTCCGGTTTGTCA	1178–1198	—
All Lactobacillus species	AGGGTGAAGTCGTAACAAGTAGCC	1499–1522	—

The isolated colonies' morphology was visually noticed on the surface of MRS agar medium. The color scoped from white to pale creamy, circular in shape, and 0.5–4 mm in diameter. It is known that Lactobacillus species are beneficial for their output of various antimicrobial compounds [35], which are probably important mechanism responsible for antimicrobial activity of intestinal microflora [36]. Ruminants are belonged to order Artiodactyla; a four-chambered stomach's mammals; made up of the rumen, reticulum, abomasum, and omasum [37,38]. Ruminant animals such as cow, buffalo, goat, and sheep have formed a symbiotic relationship with ruminal microorganisms that synthesize fiber digesting enzymes [37]. There were variations in the LAB species isolated from each ruminant animal in a certain sampling site, and are largely relied on health status and nutrition [39]. The microbial flora obtained from ruminal ingesta is an output of the composition, nature and quality of the animals' feed, so it is found that the dominating flora are usually starch and complex polysaccharide degrading microbiota. It has been reported that lactic acid bacteria (LAB), as well as other bacteria and fungi, are commonly encountered in the ruminants' ingesta [39,40].

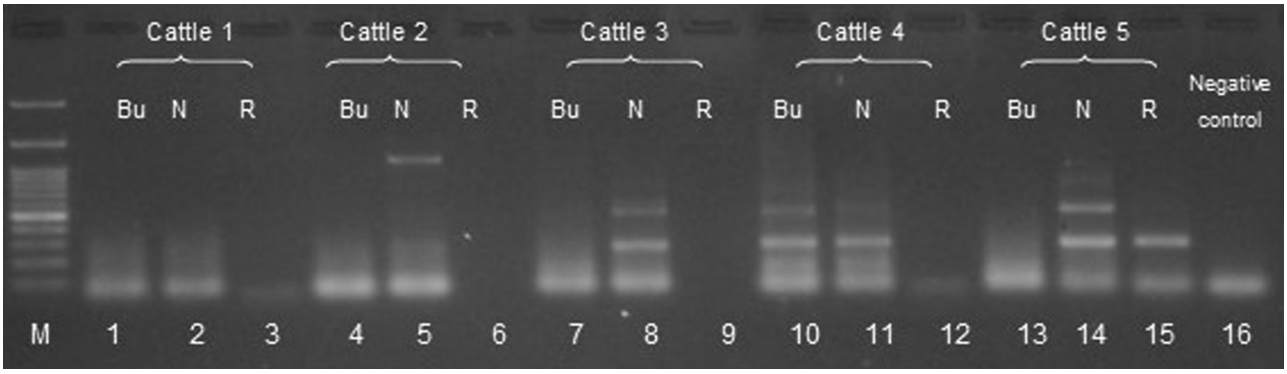
The multiplex PCR products of genomic DNA extracted from buccal, nasal, and fecal swabs taken from buffalo showed a total of seven isolates that were identified as lactobacillus strains represented as *L. delbrueckii* which was amplified from buccal and nasal swabs of first, second, and third buffalo. *L. delbrueckii* was amplified also from buccal swab of fourth buffalo (Fig. 1). The multiplex PCR products of lactobacilli isolated from cattle indicated the presence of five isolates of lactobacilli represented as

Figure 1



Multiplex polymerase chain reaction (PCR) assay of genomic DNA exposed on agarose gel electrophoreses. PCR products were obtained from suspension of mixed naturally occurring lactobacilli cell in buffalo; buccal (Bu), nasal (N), and rectal (R) swabs. Lane M: 100 bp-DNA ladder, while lanes 1, 2, 4, 5, 7, 8, and 10, representing *L. delbrueckii*.

Figure 2

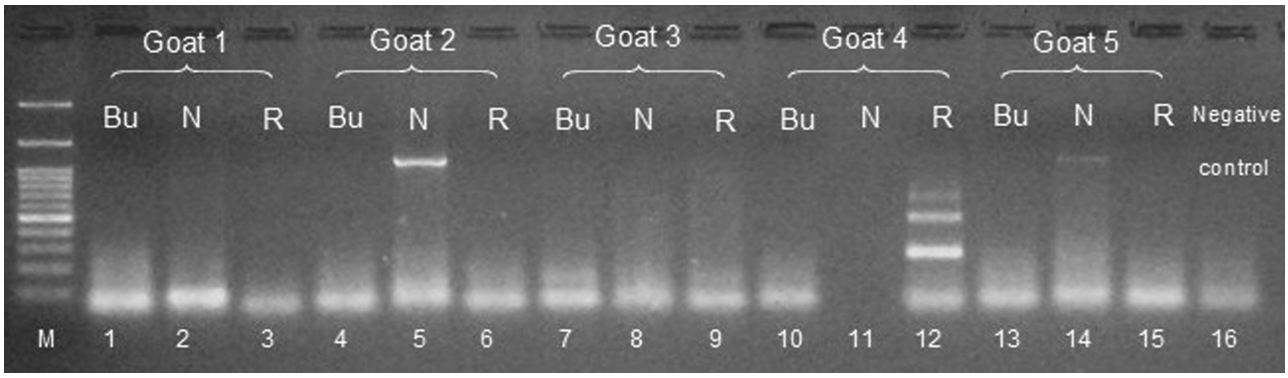


Multiplex polymerase chain reaction (PCR) assay of genomic DNA exposed on agarose gel electrophoreses. PCR products were obtained from suspension of mixed naturally occurring lactobacilli cell in cattle; buccal (Bu), nasal (N), and rectal (R) swabs. Lane M: 100 bp-DNA ladder. Lane 5, *L. casei*; lane 8, faint band of *L. acidophillus, L. rhamnosus, L. gasseri;* lanes 10, and 11; *L. gasseri, L. rhamnosus,* faint band of *L. delbrueckii*; Lane 14, faint band of *L. acidophillus, L. rhamnosus, L. gasseri*; lane 15, *L. gasseri;* lane 16, negative control.

L. acidophillus, L. rhamnosus, L. gasseri, L. delbrueckii, and L. casei. The results showed the presence of *L. acidophilus* in nasal swabs from the third and fifth cattle, while *L. rhamnosus* and *L. gasseri* were isolated from nasal swab of third, fourth, fifth cattle, and buccal swab of fourth cattle at the same time *L. gasseri* was also isolated from fecal swab of fifth cattle. *L. delbrueckii* was isolated from buccal and nasal swabs of the fourth cattle. *L. casei* was isolated from nasal swabs of second cattle (Fig. 2). Figure 3 shows the results of the multiplex PCR products of lactobacilli isolated from swabs taken from goats. A total of six isolates were identified as lactobacillus strains represented as *L. acidophillus, L. rhamnosus, L. gasserI, L. delbrueckii,* and *L. casei*. The nasal swabs from second and fifth goat showed the presence of *L. casei*. The buccal, nasal, and fecal swabs of third goat showed the amplification of *L. delbrueckii*, while *L. acidophillus, L. rhamnosus*, and *L. gasseri* were isolated from fecal swab of the fourth goat. Figure 4 shows the results of the multiplex PCR products of lactobacilli isolated from swabs taken from sheep. A total of four isolates were identified as lactobacillus strains represented as *L. acidophillus, L. rhamnosus, L. gasseri, and L. delbrueckii*. Bucacal and nasal swabs from first sheep showed amplification of *L. acidophillus, L. rhamnosus*, and *L. gasseri*. *L. gasseri* was isolated from nasal swab of the third sheep. *L. delbrueckii* was amplified from buccal swab of the fourth sheep. The results also revealed the absence of both *L. plantarum* and *L. reuteri* from the collected samples.

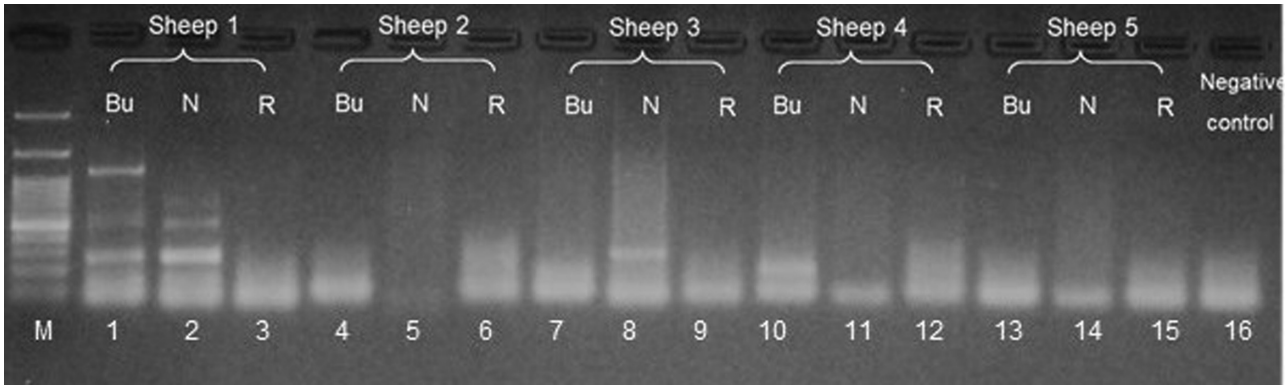
Lactobacillus strains were also isolated and identified from goats' rumen fluid and were characterized as Gram-positive and catalase-negative bacteria [41]. Many researchers have also isolated Lactobacillus strains from the calves' guts [42,43]. Also, several LAB species were also determined in other animals;

Figure 3



Multiplex PCR assay of genomic DNA exposed on agarose gel electrophoreses. PCR products were obtained from suspension of mixed naturally occurring lactobacilli cell in goat; buccal (Bu), nasal (N), and rectal (R) swabs. Lane M: 100 bp-DNA ladder. Lane 5, *L. casei*; lane 7, lane 8 and lane 9, faint bands of *L. delbrueckii*; lane 12, *L. acidophillus, L. rhamnosus, L. gasseri*; lane 14, *L. casei*; lane 16, negative control.

Figure 4



Multiplex polymerase chain reaction (PCR) assay of genomic DNA exposed on agarose gel electrophoreses. PCR products were obtained from suspension of mixed naturally occurring lactobacilli cell in sheep; buccal (Bu), nasal (N), and rectal (R) swabs. Lane M: 100 bp-DNA ladder. Lane 1, *L. casei*; faint band of *L. acidophillus, L. rhamnosus, L. gasseri*; lane 2, faint band of *L. acidophillus, L. rhamnosus, L. gasseri;* lane 8, *L. gasseri*; lane 10, *L. delbrueckii*; lane 16, negative control.

poultry [44], dogs [45], and pigs [46] and reported to have *in vitro* probiotic activity. Our results also displayed some similarities on species level; close relative's similarity between goat and sheep in their buccal and nasal samples.

Probiotic strains have the ability to improve growth performance of animals including ruminants by increasing the digestion rat and they also have the ability to improve the functions of the immune system by boosting both humoral and cellular immunity. Some probiotic strains were also able to increase milk production and inhibit milk allergic reactions [47]). Consequently, further studies are required to investigate the prospect beneficial effects of the isolated strains on both human and animal health.

The current study has assayed species specific 16S rRNA using multiplex PCR analysis to recognize and compare probiotic lactobacillus isolates recovered from various ruminants involving buffalo, cattle, goat, and sheep. Three swabs from rectum, buccal cavity, and nasal cavity were gathered from each species under aseptic conditions. Different Lactobacillus strains were isolated and identified. The results revealed the existence of lactobacilli diversity on both the individual and the species level as various lactobacilli were identified even among the same species.

Conclusion

A total of 38 lactobacillus strains were identified by the multiplex PCR into the following species of *L. acidophilus*, *L. casi*, *L. delbrucckii*, *L. gasseri*, and *L. rhamnosus*, following DNA extraction of the bacteria isolated from buffalo, Cattle, sheep and goats. The diversity in lactobacilli isolated from different ruminant animals could be attributed to dietary as well as environmental factors. The isolated strains should be further investigated in more detail, individually or in combination, for their potential health benefits.

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Conflicts of interest

There are no conflicts of interest.

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