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# Research Article
# Isolation and Characterization of Anaerobic Bacteria from Frozen Rumen Liquid and its Potential Characterizations

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## Abstract

**Background and Objective:** Anaerobic bacteriadiversity in the rumen could be one of the keys of enhancing ruminant performance and productivity. The anaerobic population by its great diversity could be possible solution for some nutritional properties and enhance animal resistance to pathogenic microbes. Antimicrobial resistance for antibiotics used in animal production. The main effects of probiotics are the improved resistance to pathogenic bacteria colonization and enhanced host mucosa immunity; thus resulting in a reduced pathogen load, an improved health status of the animals and a reduced risk of food-borne pathogens in foods. This study aimed to isolate anaerobic probiotic from bovine rumen samples. **Materials and Methods:** Isolation were carried out using classical method by using selective medium then the isolates were identified using modern techniques by real time PCR and DNA sequencing techniques. **Results:** Results showed identified isolates were as *Ruminococcus flavefaciens*, *Blautia obeum* and *Clostridium butyricum*. These isolates showed a great cellulytic activity by producing cellulase enzyme with activity degree as 6543, 8555 and 5179 IU $g^{-1}$ for *Ruminococcus flavefaciens, Blautia obeum* and *Clostridium butyricum,* respectively. Moreover, the activity of isolates for degrading tannins were 598, 1402 and 866 U $g^{-1}$ for *Ruminococcus flavefaciens, Blautia obeum* and *Clostridium butyricum,* respectively. *Clostridium butyricum* showed superiority for inhibiting pathogenic bacteria (*Yersinia enterocolitica*, *Bacillus cereus* and *Staphylococcus aureus*) while, *Blautia obeum* recorded the highest antimicrobial activity against *Salmonella typhimurium*. *Ruminococcus flavefaciens* showed the higher activity against *Listeria monocytogenes*, *Klebsiella pneumonia* and *Clostridium perfringens* **Conclusions:** The results showed a potential properties of *Ruminococcus flavefaciens*, *Blautia obeum* and *Clostridium butyricum* to be used in as supplement to ruminant diet to enhance its performance and improve the health status through the antimicrobial activity of the isolated bacteria (*Ruminococcus flavefaciens*, *Blautia obeum* and *Clostridium butyricum*).

Key words: *Rumincocccus* spp., *Clostridium* spp., cellulase, ruminant, antimicrobial properties

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Data Availability: All relevant data are within the paper and its supporting information files.

## INTRODUCTION

Rumen microbes play an important role for livestock and its ability of utilization of plant nutrients especially cellulytic feedstuffs as a source of energy. Those microbes degrade cellulosic materials and produce short chain fatty acids which are the main source of energy for ruminant. It well known that farm animals are often subjected to environmental stresses (management methods, diet, etc.) which can cause imbalance in the intestinal ecosystem and could be a risk factor for pathogen infections. Recently, probiotics play a role in maintaining intestinal health in the pre-ruminant. At the rumen level, probiotics have been shown to improve anaerobiosis, stabilize pH and supply nutrients to microbes in their microenvironment. Neonatal-calf diarrhoea, most often caused by enterotoxigenic *E. coli*, is an important cause of morbidity and mortality in young ruminants[1,2]. Therefore, the microbes which habitat the rumen are matchless and for better understanding of rumen environment, identification and molecular characterization of microbes are highly demanded[3].

Recently, many rumen bacteria have been isolated and characterized approximately one-half of the bacteria showing carboxy-methyl cellulase in the rumen are uncharacterized and/or unclassified bacteria[3,4] and thus, many uncultured/unknown cellulolytic bacteria are presumed to be involved in cellulose degradation in the rumen. Studies including comparative sequence analysis of rumen bacterial 16S rRNA gene clone libraries have shown the dominance of two phyla in the rumen: Low GC Gram-positive bacteria and the *Bacteroides* group. Within the *Bacteroides* group, *Prevotella*-related sequences were found to be predominantly associated with the rumen[5].

*Fibrobacter succinogenes, Ruminococcus albus* and *Ruminococcus avefaciens* are the predominant cellulolytic bacteria in the rumen[6,7]. Moreover, some rumen bacteria including *Clostridium longisporum*, *Clostridium lochheadii*, *Butyrivibrio brosolvens, Prevotella ruminicola, Eubacterium ruminantium* and *Eubacterium cellulosolvens* are known to be brolytic bacterial species[4]. Cellulases enzymes produced by a wide range of microorganisms, including aerobes, anaerobes; cellulytic bacteria enzymes are extracellular cellulases and hemicellulases.

Anaerobic bacteria especially those habitat in rumen (*Clostridium thermocellum*, *C. cellulovorans*, *Ruminococcus albus*, *R. flavefaciens*, *Fibrobacter succinogenes* and *Acetivibrio cellulolyticus*) which could be able to degrade cellulytic materials produce cellulase in the form of a complete cellulase enzyme system[7-11].

Some of the microbes or its produced enzymes (especially cellulase) have also been recommended as feed additive for improving the overall growth or production of animals. A cellulolytic enzyme system is a complex system of enzymes composed of endoglucanase, exo-glucanase and β-glucosidase that acts synergistically to degrade cellulosic substrate.

Earlier reports stated that microbial activity at temperatures below -12°C were unsubstantiated[12-14]. Neonatal-calf diarrhea, most often caused by enterotoxigenic pathogens especially, *E coli* is an important cause of morbidity and mortality in young ruminants[15]. Different probiotic preparations, containing 6 *Lactobacillus* spp., of bovine and human origin were successful in reducing the overall mortality, incidence of diarrhea and fecal coliforms counts in veal calves[16].

In our study it is aimed to isolate some anaerobic bacteria from frozen bovine liquor and characterize its ability to produce cellulase and tanninase enzymes and its antimicrobial activity.

## MATERIALS AND METHODS

**Isolation of anaerobic bacteria:** Rumen liquor of Egyptian cattle were collected after slaughtered and anaerobically transferred to laboratory and then stored at -20°C for 12 month. Isolation procedures were carried out by classical techniques by sequent dilutions and repeating enriched microbial culture[3] known aliquots of the different homogenates were sown on selective medium as described by Khattab and Ebeid[3] (Table 1). The tubes were incubated at 37° C for 24-48 h under anaerobic conditions. In all cases, the incubation period was extended until visible colonies appeared. White and yellow, small, colonies were recovered and placed into MRS broth and incubated at 37°C.

**Properties of isolated bacteria:** The fermentation reactions of the *Clostridium* and *Enterococcus* spp., were tested by the methods given in Manual of Microbiological Methods[17].

**Bacterial isolates identification:** Complete DNA of the isolates were carried out as described by Khattab and Ebeid[3]. Obtained sequences were compared and aligned with sequences from the GenBank database by using the BLAST program of the National Center for Biotechnology Information (NCBI; http://www. ncbi.nlm.nih.gov) network server.

**Determination of cellulase activity:** Pure anaerobic isolates were examined for carboxy-methylcellulase activity by

Table 1: Composition of isolation culture

| Component | Quantity (L) |
|---|---|
| Yeast extract (g) | 0.5 |
| Bacto-casiton (g) | 2 |
| Mineral solution I (mL) | 38 |
| Mineral solution II (mL) | 38 |
| Hemin (mL) | 10 |
| VFA mix (mL) | 3.1 |
| Resazurin (0.1%) | 0.5 |
| Microelements (mL) | 0.5 |
| Vitamins (mL) | 2 |
| Rumen fluid (mL) | 200 |
| Water (mL) | 809 |
| Cellulose (g) | 4 |
| Cysteine hydrochloride (g) | 0.5 |
| $NaHCO_3$ (g) | 4 |
| Agar (g) | 14 |

Mineral solution (I): (6 g $K_2HPO_4$ $L^{-1}$), mineral solution (II): (12 g NaCl+12 g $(NH_4)_2SO_4$+6 g $KHPO_4$+1.2 g $CaCl_2$+2.5 g $MgSO_4.7H_2O$ $L^{-1}$), microelements: (253 mg $NiCl_2$, 333 mg $H_3PO_4$, 500 mg $Na_2MoO_4.2H_2O$, 11 g $FeSO_4.7H_2O$, 500 mg $MnSO_4.4H_2O$, 500 mg $ZnSO_4.7H_2O$, 253 mg $CuSO_4.5H_2O$, 253 mg $CoCl_2.6H_2O$, 100 g chelaton I, 100 mg $KAI(SO_4)_2.12H_2O$, 253 mL 5N NaOH, vitamins: (200 mg pyridoxine, 200 mg riboflavin, 200 mg nicotinamide, 200 mg thiamine, 200 mg panthotenate B, 1 mg p-aminobenzoic acid, 0.5 mg biotin, 0.5 mg cobalamine, 5 mg folic acid were dissolved in 100 mL distilled water), VFA mix: 10 mL methylbutyric acid, 170 mL acetic acid, 60 mL propionic acid, 40 mL butyric acid, 10 mL valeric acid, 10 mL isovaleric acid, 10 mL isobutyric acid, 100 mg phenylacetic acid, 100 mg phenylpropionic acid

determination of amount of reducing sugar release from carboxy-methylcellulose. The reaction mixture contained 1.0 mL phosphate buffer (0.1 M pH 6.8), 0.5 mL of 1% carboxy-methylcellulose solution prepared in 0.1 M phosphate buffer (pH 6.8) and 0.5 mL enzyme and incubated for 60 min at 39°C. The reaction was halted and reducing sugars were determined by the addition of 3.0 mL of dinitrosalicylic acid reagent[18]. Glucose was used as standard for determination of reducing sugars. The enzyme activity is expressed as International Unit (IU) which is micromole of glucose released per milliliter per hour.

**Determination of tannase activity:** Tannase enzyme activity was determined by the method of Mondal *et al.*[19]. One unit of the tannase enzyme was defined as the amount of enzyme, which is able to hydrolyze 1 µmol of ester linkage of tannic acid in 1 min at specific condition (pH 5.0 and 40°C).

**Determination of antibacterial activity:** The antimicrobial activity of the isolates from frozen bovine rumen liquor was determined by the agar well diffusion method[20]. Ten pathogenic indicator bacteria strains were obtained from the stock cultures of the Dairy Microbiological Lab, National Research Center: *Escherichia coli* O157: H7 ATCC 6933, *Bacillus cereus* ATCC 33018, *Staphylococcus aureus* ATCC 20231, *Salmonella typhimurium* ATCC 14028, *Pseudomonas aeruginosa* ATCC 9027, *Listeria monocytogenes* ATCC 7644, *Yersinia enterocolitica* ATCC 9610, *Enterobacter sakazakii*, *Klebsiella pneumonia* and *Clostridium perfringens*. Each strain was activated in tryptone soy broth by fermentation at 37°C for 24 h except, *Clostridium Perfringens* incubated under anaerobic condition. One militer culture of the activated indicator strain ($10^4$ cells $mL^{-1}$) was inoculated into 20 mL of Mueller-Hinton agar (Becton Dickinson, USA) and poured in petri dishes. After solidification of the agar, wells of 5 mm in diameter were cut from the agar with a sterile borer and 50 µL of isolates delivered in each well.

The antimicrobial activity was expressed as the diameter of the zone of inhibition (ZOI); whereby a diameter >1 mm around the well was considered as a positive result and the greater the diameter of the ZOI, the higher is the antimicrobial activity. The percentage inhibition was calculated according to National committee for the Clinical Laboratory (NCCLS).

The zone diameter of wells cut in nutrient agar medium was 5.0 mm and the diameter of inhibition zone (DIZ) of negative a control for each bacterium was also 5.0 mm. If the DIZ value is 5.0 mm, that means the sample has no inhibitory activity against that bacterium.

## RESULTS AND DISCUSSION

**Bacterial identification and characteristics:** Different types of colonies developed on the surface of agar plate after 24-48 h of incubation at 37°C, three microorganisms were purely isolated and showed biochemical characteristic anaerobic bacteria: Small, round, opaque, yellow and white colonies, sporulated rods and non-sporulated cocci, Gram-positive; the isolates were grouped into the *Enterococcus* and *Clostridium* genus. Three strains were selected due to their species-specific identifications were derived using a 16S rRNA sequence analysis. Comparison of the near complete 16S rRNA gene sequence confirmed as *Blautia obeum*, *Ruminococcus flavefaciens* and *Clostridium butyricum* with a 99% identity.

Table 2 shows that all isolates fermented arabinose, maltose, cellulose, lactose and galactose, while, only *Clostridium butyricum* fermented sucrose.

**Bacterial enzymes activity:** The results showed that all isolated had a noticed cellulytic activity as shown in Table 3. *Blautia obeum* recorded the highest value of cellulytic activity (8555 IU $g^{-1}$) followed by *Ruminococcus flavefaciens* (6543 IU $g^{-1}$) while *Clostridium butyricum* recorded the lowest activity value as 5179 IU $g^{-1}$.

Also, *Blautia obeum* recorded the higher activity on degrading tannins as 1402 U $g^{-1}$ followed by *Clostridium*

Table 2: Characteristics and reactions of identified bacterial isolates

| Characteristics | *Blautia obeum* | *Ruminococcus flavefaciens* | *Clostridium butyricum* |
|---|---|---|---|
| Gram's reaction | + | + | + |
| Catalase | + | - | - |
| Arabinose | + | + | + |
| Maltose | + | + | + |
| Raffinose | + | - | ± |
| Cellulose | + | + | + |
| Galactose | + | + | + |
| Lactose | + | + | + |
| Sucrose | - | - | + |

Table 3: Cellulase and tannase activity produced from bacterial isolates

| Activities | *Blautia obeum* | *Ruminococcus flavefaciens* | *Clostridium butyricum* |
|---|---|---|---|
| Carboxy-methylcellulase (IU $g^{-1}$) | 8555 | 6543 | 5179 |
| Tannase (U $g^{-1}$) | 1402 | 598 | 866 |

Table 4: Antimicrobial activity of bacterial isolates (mm)

| Bacterial isolates | *Blautia obeum* | *Ruminococcus flavefaciens* | *Clostridium butyricum* |
|---|---|---|---|
| *E. coli* O157: H7 | 19 | Nil | 22 |
| *Pseudomonas aeruginosa* | Nil | Nil | Nil |
| *Salmonella typhimurium* | 20 | 12 | 15 |
| *Yersinia enterocolitica* | 16 | 16 | 20 |
| *Bacillus cereus* | 15 | 15 | 20 |
| *Listeria monocytogenes* | 11 | 20 | 11 |
| *Staphylococcus aureus* | 10 | 17 | 20 |
| *Enterobacter sakazakii* | Nil | Nil | Nil |
| *Klebsiella pneumonia* | 13 | 20 | 19 |
| *Clostridium perfringens* | 18 | 20 | 18 |

Nil: Non detected

*butyricum* as 866 U $g^{-1}$ and the lower value were recorded for *Ruminococcus flavefaciens* (598 U $g^{-1}$).

**Antimicrobial activity of isolates:** These isolates proved to inhibit growth of one or more of the indicators bacteria. In this case, their effect was based on the action of their metabolites produced in their isolates including their proteinaceous synthesizing substances (bacteriocin) as well as a combination of antimicrobial substances such as hydrogen peroxide and organic acids.

Table 4 shows that a wide range of the reported inhibition zones ranged from 10-30 mm, for all isolates, against the various tested indicators was obtained. The biggest diameter (20 mm) was obtained using of *Clostridium butyricum* against *E. coli* O157:H7 however, the smallest diameter (11 mm) was obtained by *Blautia obeum* and *Clostridium butyricum* against *Listeria monocytogenes*.

*Clostridium butyricum* recorded the highest antimicrobial activity against *Yersinia enterocolitica*, *Bacillus cereus* and *Staphylococcus aureus* as inhibition zone 20 mm for each one. While, *Blautia obeum* recorded the highest antimicrobial activity against *Salmonella typhimurium* as 20 mm. *Ruminococcus flavefaciens* showed the higher activity against *Listeria monocytogenes*, *Klebsiella pneumonia* and *Clostridium perfringens* as 20 mm for each one.

Previous studies noticed that *Ruminococcus flavefaciens* had little antimicrobial effect on bacterial population in the rumen[21]. The effect of bacteriocins on ruminal ecology diversity had not clearly defined. Because either bacteriocin-producing or others bacteriocin-sensitive strains can be readily isolated from rumen, the potentiality of bacterium to produce an antimicrobial components does not confer an absolute growth advantage[22]. Because most ruminal bacteria are attached to feed particles, cell associated bacteriocins could be a critical factor in colonization[23].

## CONCLUSION

It could be concluded that ruminal anaerobic bacteria (*Ruminococcus flavefaciens*, *Blautia obeum* and *Clostridium butyricum*) have a potential properties as safe antimicrobial treat could enhance the resistance of ruminant against pathogenic microbes could infect them, in the other hand had nutritional advantage by its cellulytic degradation activity by cellulase and tanninase enzymes. The topic need more applicable studies to prove these findings.

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