International Journal of Genetic Engineering and Biotechnology.
ISSN 0974-3073 Volume 2, Number 1 (2011), pp. 1-7

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Bacteriocin Producing Probiotic *Lactobacillus sp.* from Cow's Milk

Nishant Toomula, Satya Harshada Dabbiru, Rama Rao Vachalapu, Aruna Lakshmi Komarraju and Satyanarayana Rentala*

Department of Biotechnology, GITAM Institute of Technology, GITAM University, Gandhi Nagar, Rushikonda, Visakhapatnam – 530045, Andhra Pradesh, India.
**Corresponding Author E-mail: s.rentala@yahoo.com*

Abstract

Bacteriocins are proteinaceous toxins ribosomally synthesized single polypeptides produced by bacteria to inhibit the growth of similar or closely related bacterial strains. Probiotic *Lactobacillus* species have been previously identified on the basis of its phenotypic characteristics, such as sugar fermentation patterns and cell morphology. These bacteria have high physiological similarity and this could inevitably lead to misidentification of the Lactobacillus strains. In the presence study we isolated *Lactobacillus* species, which is more resembling *Lactobacillus breve.* This species was identified by using microbiological and biochemical tests. Further studies on the species showed the expression of bacteriocin, which play an important role in the inhibition of pathological bacteria.

Introduction

Human intestinal tract is filled with an enormous number of helpful bacteria called probiotic bacteria. They are called probiotic bacteria because the term "probiotic" means "for life," as opposed to the term "antibiotic" which means "against life." Human bodies are actually designed to have symbiotic relationships with these probiotic bacteria. They help in digestion if food, killing harmful microorganisms and keep the body functioning properly in a number of ways.

Lactic acid bacteria (LAB) and *bifidobacteria* are the most common types of microbes used as probiotics; but certain yeasts and bacilli may also be helpful. Probiotics are commonly consumed as part of fermented foods with specially added active live cultures; such as in yogurt, soy yogurt, or as dietary supplements. *Lactobacillus brevis* is a species of lactic acid bacteria. It can be found in many different environments and in fermented foods such as sauerkraut and pickles. It is

also one of the most common causes of beer spoilage. Ingestion has been shown to improve human immune function, and it has been patented several times. L. brevis is one of the major *Lactobacillus* species found in tibicos grains (aka water kefir grains), and has been identified as the species responsible for the production of the polysaccharide (dextran) that forms the grains. Major metabolites of *L. brevis* include lactic acid and ethanol. Strains of *L. brevis* and *L. hilgardii* have been found to produce the biogenic amines tyramine and phenylethylamine. Bacteriocins are proteinaceous toxins ribosomally synthesized single polypeptides produced by bacteria to inhibit the growth of similar or closely related bacterial strain(s). They are typically considered to be narrow spectrum antibiotics, though this has been debated. They are phenomenally analogous to yeast and paramecium killing factors, and are structurally, functionally, and ecologically diverse. In the presence study we isolated *Lactobacillus* species, which is more resembling *Lactobacillus breve.* This species was identified by using microbiological and biochemical tests. The species was characterized by using 16s rDNA ribotyping. Further studies on the species showed the expression of bacteriocin, which play an important role in the inhibition of pathological bacteria.

Materials and Methods

Isolation of Lactic Acid Bacteria

Samples of milk and curd (24hrs) form buffalo and cow was collected. Samples were kept at refrigerated condition until analysis. For isolation of LAB, the serial dilutions of the samples (up to 10^{-5}) were inoculated into Lactobacillus MRS agar by pour plate method and incubated in anaerobic condition at 37°C for 48 h for the colonies to develop. Following incubation, 3 colonies for each sample were randomly selected from the MRS agar plates. The colonies were propagated on the same media until the pure cultures were obtained. Purification of the cultures was confirmed by Gram's staining. Pure colonies were again cultured on MRS agar slants and broth (in duplicates) and stored at 4°C until used.

Antimicrobial Activity

The antimicrobial activities of these isolates were studied by the disc diffusion procedure. A loopful of each of the LAB isolates from the MRS agar slants was inoculated into tubes containing 10 mL of sterile MRS broth. These broth cultures were incubated at 37°C for 48 h. After incubation, the cultures were centrifuged (5000 rpm for 35 min at 4°C) to obtain the Culture Free Supernatant. Sterile cotton swabs were taken and dipped into the cultures of the test microorganisms (*staphylococcus, salmonella, micrococcus, and shigella*) and inoculated by swabbing over the entire surface of the pre-set Mueller-Hinton agar plates.Sterile filter paper discs of 4mm diameter were prepared from Whatman filter paper. Each disc was saturated with the respective culture supernatant, air dried and placed on a 150 mm plate, within 5 to 15 min after swabbing the test pathogens. After 18 to 24 h of incubation at 37°C each plate was examined for the zone of inhibition. The diameters of the inhibitory zones were measured.

Antibiotic resistance

300ul and 500ul of streptomycin is mixed in 50ml MRS agar media. After sterilization media was poured in 2 petri plates.Allow it to solidify, after that *Lactobacillus* culture was swabbed on MRS agar. Then the cultures were incubated at 37°C for 48 h.

Molecular Weight Determination using SDS-PAGE

The isolates were grown in MRS broth for 48 h at 37°C. Following incubation, the cultures were centrifuged at 5000 rpm for 30 min at 4°C, after which the bacteriocins were precipitated from the supernatant with 45% saturated ammonium sulphate and kept overnight at -10°C for precipitation. After precipitation, centrifugation of the supernatants resulted in the formation of pellets, which were collected and stored in phosphate buffer (pH 7.0). The molecular weights of the bacteriocins were determined using SDS-PAGE. Molecular weight markers ranging from 10 to100 kDa was used. Following electrophoresis, the gel was stained with Coomassie Brilliant Blue. The apparent molecular weights of the samples were determined by comparison with the mobility of the standard markers.

Results

Bacterial strains were isolated from 2 types of milk and 2 types of curd Microscopic identification determined the rod shaped cells. Gram's staining and catalase test and acid fast test supported the characterization of *lactobacilli*. After taking these criteria into account, 4 strains were found to be, Gram positive, rod shaped, non-spore forming and catalase negative acid fast negative, which indicated the typical basic characteristics of lactobacilli. Among these 4 strains, 2 are isolated from curd, 2 from milk.

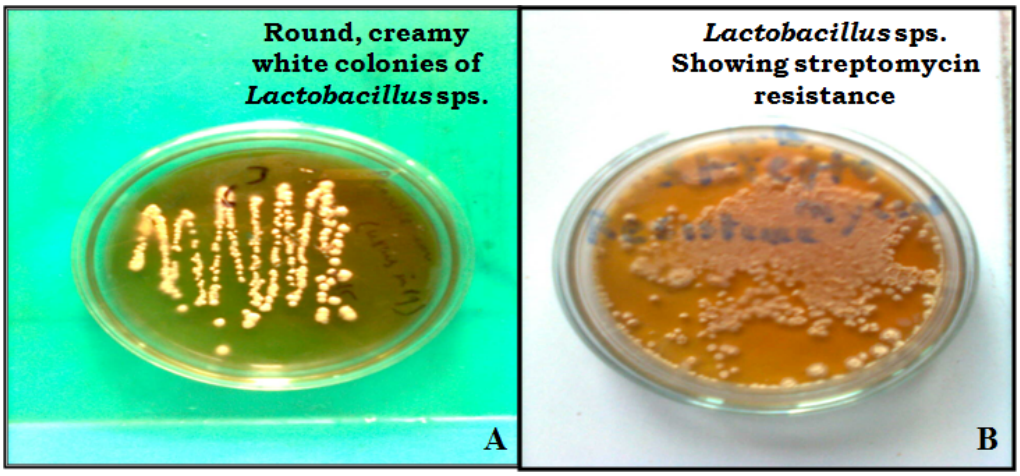


Figure 1A: Colonies of lactobacillus on MRS agar media Antibiotic resistance. Figure 1B represents Effect of streptomycin on *Lactobacillus* sps.

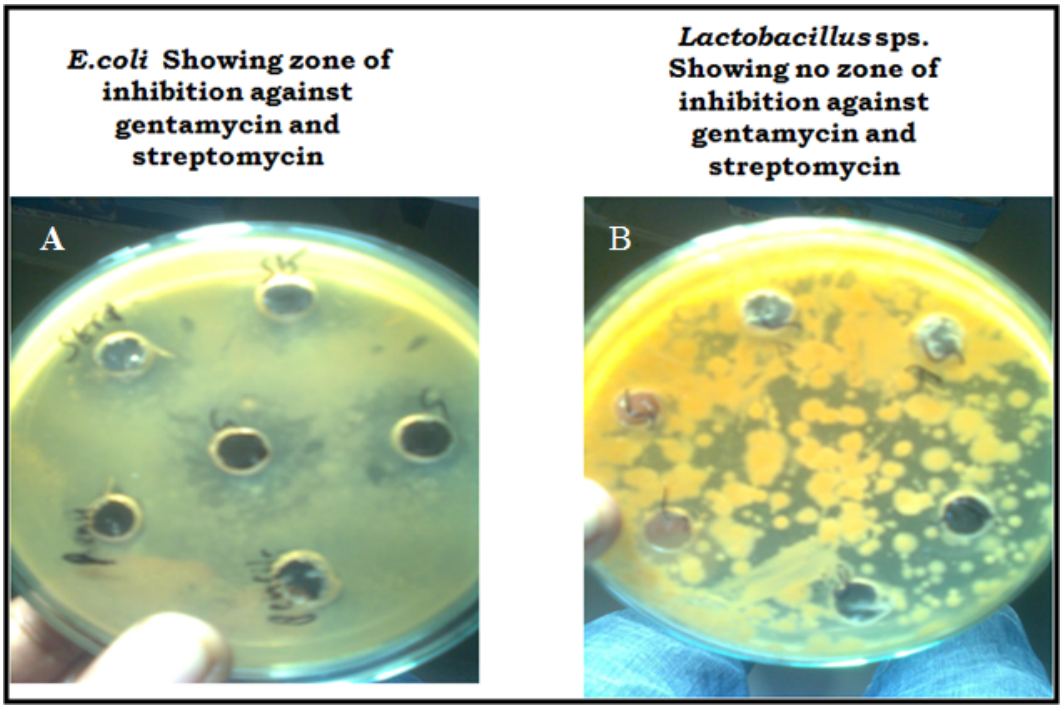
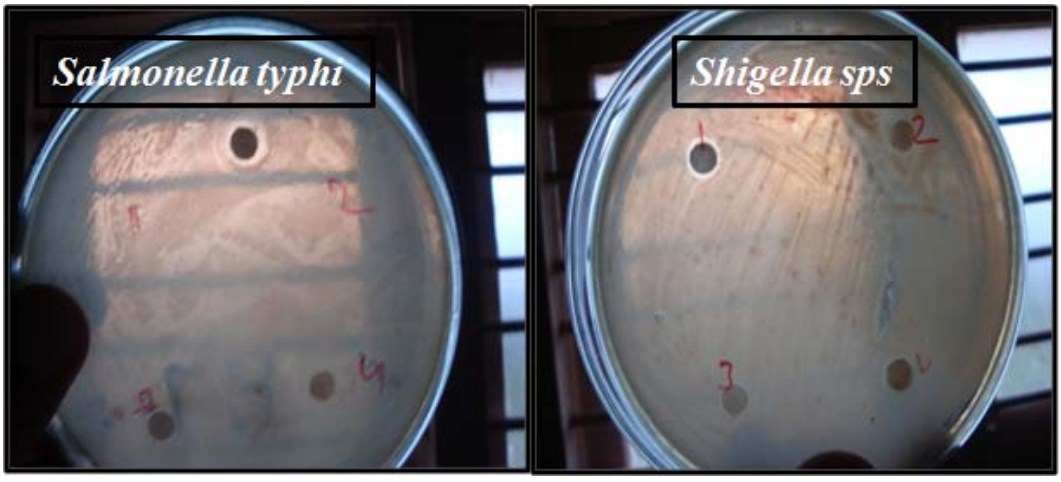


Figure 2A: represents the effect of Gentamycin, Streptomycin, on E.cloi and Figure 2B represents the effect of Gentamycin, Streptomycin, on Lactobacillus sps.

Antimicrobial activity

The antimicrobial activity of the LAB and their zone of inhibition against the various test pathogens were studied. The culture supernatant of lactobacillus showed zones of inhibition when tested against the indicator strains and the values are represented in Table. The diameters of the inhibition zones ranged from 2 to 5 mm. The highest diameter (5 mm) was recorded on *Salmonella typhi* and the smallest of 2 mm on *Shigella sps.*



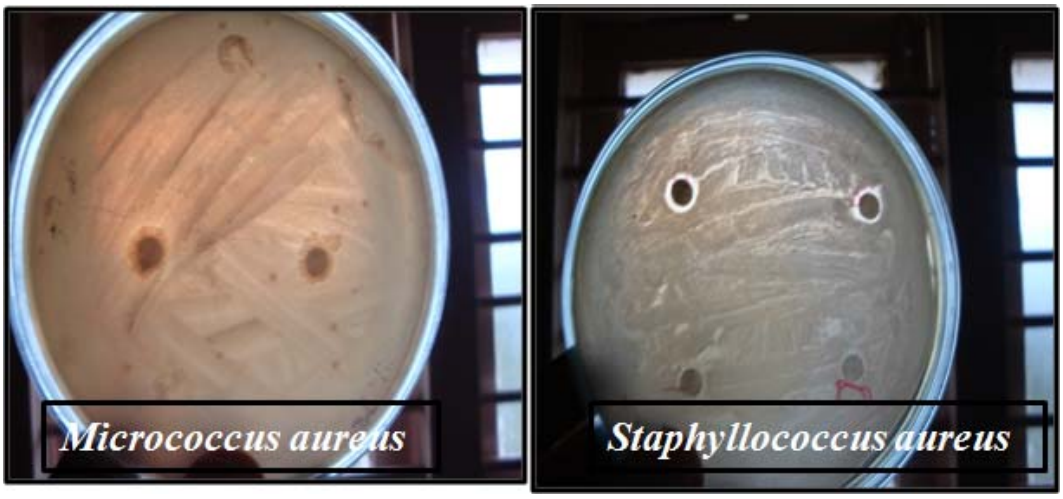


Figure 3: Antimicrobial activity of lactobacillus against various microorganisms.

Test organism	Zone of inhibition in mm
Salmonella typhi	5mm
Shigella	2mm
Micrococcus aureus	4mm
Staphyllococcus aureus	3mm

Molecular weight determination using SDS - Polyacrylamide Gel Electrophoresis (PAGE)

The above gel contains the proteins which were in the molecular weight range of 16 kDa to 32 kDa. We will further investigate the bands obtained in the gel using western blot and sequencing techniques.

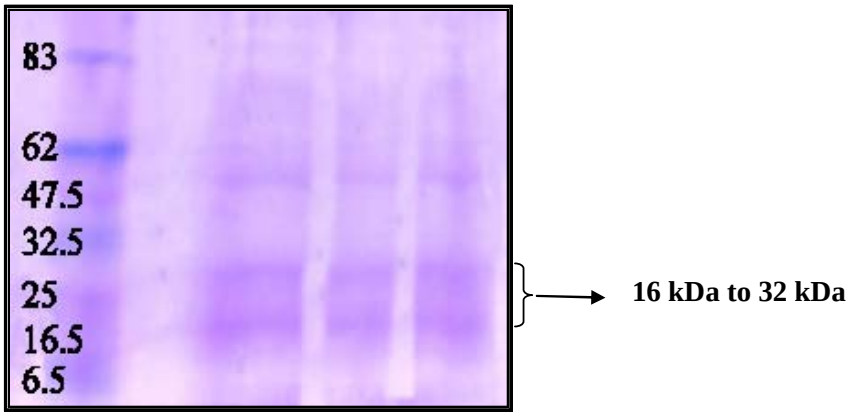


Figure 4: SDS-PAGE gel showing bands of bacteriocin.

Discussion

Previous reports demonstrated that only 30.2% of intestinal and vaginal Lactobacillus strains were correctly identified by the API 50 CHL kit at species level. In another report, only 4 out of 97 (4.12%) strains of commensal Lactobacillus showed similar identification results when the results of API 50CH strips were compared with molecular method. Several limitations have been observed with respect to the use of phenotypic test systems as identification tool for bacteria. As opposed to genotypic methods, phenotypic properties can be unstable and are dependent upon changes in cultural conditions. A further problem with commercially available phenotypic test systems is that the corresponding database is limited; usually the database is not up to date or novel, or species not yet described are not included in the database. Moreover, the identification results of the commercial kit may rely on individual and subjective interpretation. In contrast to phenotypic method, sequencing of 16S rRNA gene has been regarded as a powerful tool for the identification and phylogenetic analysis of bacteria. As compare to the commercial phenotypic test, 16S rRNA gene analysis is useful for identification of all bacteria without much constraint by using the public database. Even though the information regarding bacterial diversity is still very limited, the public database covers the whole spectrum of known phylogenetic diversity. With 16S rRNA gene analysis, newly or not yet described species can be clustered into its related bacteria group. The data obtained from the present study indicated that comparison of 16S rRNA genes is useful for the determination of phylogenetic relationship of bacteria. Similar observations have been reported. In conclusion, DNA sequencing of 16S rRNA gene could provide useful strategies for inferring inter- and intrageneric relationships of bacteria.

Conclusion

In previous investigations, the identification of *Lact. brevis* and its differentiation from closely-related species (such as Lact. hilgardii) has been assessed using carbohydrate fermentation capacities and total genomic DNA hybridization with oligonucleotide probes. However, neither method allowed clear identification owing to the fact that interstrain variability in fermentation ability, or some cross-hybridization between *Lact. hilgardii* and *Lact. buchneri* using DNA probes, were often observed. Vogel et al. (1994) developed oligonucleotide probes specific for different Lactobacillus species and based on 16S rRNA gene sequences. However, the *Lact. brevis* probe, which was used as forward PCR primer in the present study, was tested by these authors on only one strain. In this study,we isolated *Lact. brevis relating sps.* The isolated strain also produced bacteriocin, a bactericidal protein.

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