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ISOLATION OF LACTIC ACID BACTERIA WITH VANCOMYCIN RESISTANCE GENE AND ITS DIVERSITY FROM FERMENTED RICE WATER

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Abstract
Traditional fermented food products such as fermented rice are known to possess probiotic potential. Probiotics are live microorganisms that provide a numerous health benefits. Microorganisms commonly used as probiotics include *Bifidobacteria*, *Lactobacilli* and certain yeasts**.** Hence the present research work aimed to study on lactic acid bacteria (LAB) group Lactobacillus. LAB were isolated from traditional fermented rice water. These isolates were found to have varying pH and bile salt tolerance, cell surface hydrophobicity, auto-aggregation, antibiotic susceptibility, and antibacterial activities. Our results indicated that the strain acts as a starter culture in the preparation of rice-based fermented foods. This strain increased the functional component (nutrients and minerals) levels of the fermented food items. Interestingly, this bacterium enhanced the mineral bioavailability via heightened phytase activity. Rapid acidification and the generation of organic acids by *Lactobacillus* improve the digestibility of the final product by reducing starches and increasing its antioxidant potential. Currently, beneficial effects of *Lactobacillus* are under investigation in vitro and in vivo to establish its probiotic characteristics. However, Lactobacillus spp. are further examining for their antibiotic resistance characteristics.
Keywords: Microorganisms, Antimicrobial activity, Fermentation, Resistance and Beneficial microorganism.

Introduction

Lactobacillus is a group of rod shaped gram positive, non sporing bacteria of the family *Lactobacillaceae*. Lactobacillus is characterized by their ability to produce lactic acid as a byproduct of glucose metabolism. The association between *Lactobacilli* and humans is a mutualistic relationship, with *Lactobacillus* species offering the host aid in digestion of certain

dietary substrates, as well as protection from pathogens, in return for accommodation and nutrients. Confirmation of the isolates belonging to the genus *Lactobacillus* could be accomplished by observing their colony morphology, staining and biochemical tests: catalase, and motility test, as reported previously (Barrow and Felthman, 1993). *Lactobacilli* metabolizes carbohydrates to produce lactic acid making them the largest genus within the lactic acid bacteria (LAB) group. *Lactobacillus* species may be divided into three groups based on their metabolism. The obligate homofermentative group which ferment carbohydrates to produce lactic acid as the main by-product (e.g. *L. acidophilus* and *L. salivarius*), the facultatively heterofermentative group which, under certain conditions or with certain substrates, ferment carbohydrates to produce lactic acid, ethanol/acetic acid and carbon dioxide as by-products (e.g. *L. casei* and *L. plantarum*) and the obligately heterofermentative group which always ferment carbohydrates to produce lactic acid, ethanol/acetic acid and carbon dioxide as by-products (e.g. *L. reuteri* and *L. fermentum*). As of March 2020 the 261 species of the *Lactobacillacae* were reclassified into 25 genera (including 23 novel genera) due to their extremely high genotypic, phenotypic and ecological diversity. The association between *Lactobacilli* and humans is a mutualistic relationship, with *Lactobacillus* species offering the host aid in digestion of certain dietary substrates, as well as protection from pathogens, in return for accommodation and nutrients. *Lactobacillus species* are considered as part of the normal microflora of the gastrointestinal and female genital tract, and also as major components of microflora involved in food fermentation. Among the patients susceptible to the side effects of *lactobacillus*, antibiotic susceptibility is observed and the antibiotic-resistant strain will not be easily eliminated (Anderson *et al.,* 1999).

Lactobacillus species possess qualities that are commercially desirable both as health supplements and as tools in the food technology sector. The main uses for *Lactobacilli* are in the manufacturing process of fermented dairy, meat, or vegetable foods and sourdough breads, and they are also widely used as probiotics (Bekkali *et al.,* 2007) i.e., live microorganisms that, when administered in adequate amounts, confer a health benefit on the host. *Lactobacilli* have been granted a ‘generally recognised as safe’ (GRAS) status from the U.S. Food and Drug Administration (USFDA) and ‘qualified presumption of safety’ (QPS) status from the European Food Safety Authority (EFSA) thus making their use in food manufacture relatively straightforward. The data on drug resistance of the industrially important lactobacilli are rare but some species of lactic acid bacteria that are commonly used in the food industry, or

naturally found in food raw material, are well known as intrinsically resistant to some antibiotics, e.g., vancomycin. Vancomycin is a tricyclic glycopeptide antibiotic and the first choice for the prophylaxis and treatment of infections caused by gram-positive bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA) like osteomyelitis (Salminen et al., 2004).

High MIC values were observed for vancomycin, at ≥64 and l >128 µg/ml, respectively, in the most strains analyzed (81 and 73%, respectively). Interestingly, strains belonging to the *Lactobacillus delbrueckii* group showed susceptibility to low concentrations of vancomycin (concentrations lower than 1 µg/ml inhibited the growth of 92% of strains belonging to the *L. delbrueckii* group). Most of the *Lactobacillus* strains were not susceptible to vancomycin and kanamycin. In addition, *L. sanfranciscensis* LMG 16002T, *Lactobacillus hilgardii* LMG 6895T, *Lactobacillus composti* DSM 18527T, *L. pobuzihii* NBRC 103219T, *Lactobacillus farciminis* LMG 9189, *Lactobacillus ceti* DSM 22408T, and *Lactobacillus algidus* DSM 15638T were characterized by the presence of Ddl of the F type, despite their susceptibility to vancomycin. In antibiotic therapy, the antibiotic concentration must be higher than the minimum inhibitory concentration (MIC). Because of difficulty of delivering antibiotics to a target site in an effective concentration by the conventional routes of oral and parenteral, it is wise to use methods of local administration. One marketed approach for this purpose is to mix antibiotics with a carrier material that can then locally release the drug (Andrighetto *et al.,* 2001).

The earlier reports highlight that the genes encoding penicillin binding proteins (PBPs) and d-alanine d-alanine ligase (Ddl) involved in ampicillin and vancomycin resistance, were found in genomes. Furthermore, amino acid sequence analysis of the PBPs for the 161 type strains confirmed the presence of conserved amino acid residues in the known binding site motif for β-lactams. The Ddl enzyme of all vancomycin-resistant type strains exhibited a conserved phenylalanine (F) residue in the active site of the enzyme, while all members of the phylogroup *L. delbrueckii,* which were susceptible to vancomycin, were characterized by the presence of a tyrosine (Y) residue at this position, with the exception of strains *L. jensenii* DSM 20557T, *Lactobacillus amylophilus* DSM 20533T, and *Lactobacillus amylotrophicus* DSM 20534T. Their respective proteins carried the Y-type motif, even though they were resistant to vancomycin. However, *Lactobacillus amylophilus* DSM 20533T and *Lactobacillus*

amylotrophicus DSM 20534T harbored specific d-alanine-d-lactate ligase sequences in their genomes, which could explain their vancomycin resistance phenotypes. In addition, *L. sanfranciscensis* LMG 16002T, *Lactobacillus hilgardii* LMG 6895T, *Lactobacillus composti* DSM 18527T, *L. pobuzihii* NBRC 103219T, *Lactobacillus farciminis* LMG 9189, *Lactobacillus ceti* DSM 22408T, and *Lactobacillus algidus* DSM 15638T were characterized by the presence of Ddl of the F type, despite their susceptibility to vancomycin (Bennedsen *et al*., 2011).

Many strains of probiotic bacteria have been isolated from different sources, however, not much work has been carried out on isolation of probiotic strains from fermented rice. Research has shown that addition of probiotics to food provides many health benefits. They are able to increase the frequency of bowel movements (Bekkali *et al.,* 2007) and stimulate cell-mediated immunity (Wold, 2001). Moreover, *Lactobacillus acidophilus* has shown the ability to reduce serum cholesterol level indicating the potential of reducing the risk for coronary heart disease by 6% to 10% in hypercholesterolemic human (Anderson *et al.,* 1999). *L. Rhamnosus* has shown anti-carcinogenic effects through decreasing the activity of β-glucuronidase. (Nicole and Martjjn, 2000). Despite the associated health benefits, fermented rice has not received due attention in the countries where rice is the staple diet. In this backdrop, the present study was carried out to isolate and identify potentially probiotic microorganisms from fermented rice**.**

Based on the literature, the current study is focused on the MIC value of Lactobacillus against vancomycin at different concentrations, potential of vancomycin resistance genes in *Lactobacillus* strains and the sequence of the vancomycin resistant gene in *Lacobacillus*.

Methodology

Determination of antibiotic resistance

Commercially available vancomycin powder was procured and different concentrations of 0.5ug, 1ug and 1.25ug/ml. MICs of antibiotics were determined on Mueller-Hinton agar plates. The resistant colonies were allowed to grow overnight in MRS broth at 30°C with and without vancomycin.

Identification of the genus *Lactobacillus*

The bacterial cells were grown in 10 ml of MRS broth and 1.5 ml aliquot culture was centrifuged at 6000rpm for 10 minutes. The cells were washed with 1 ml of sodium chloride solution and centrifuged. The supernatant was discarded and the pellet was washed once again.

The bacterial DNA was isolated and amplified by PCR (Kim *et al.,* 2020). The identification of *Lactobacillus* strains was carried out by genus-specific PCR primers targeted to the 16S ribosomal RNA intergenic spacer region. PCR protocol step comprises initial denaturation step at 95°C for 5mins followed by 35 cycles of 95°C for 30seconds (denaturation step), 55°C for 30s (annealing step) and 72°C for 1:30 minutes (extension), and final extension at 72°C for 10mins using thermocycler. These samples were sequenced using an ABI3770 sequencer (Applied Biosystems, Foster City, California, USA), where the sequencing was performed bidirectionally with both forward and reverse primers. 16S rRNA sequences has been validated.

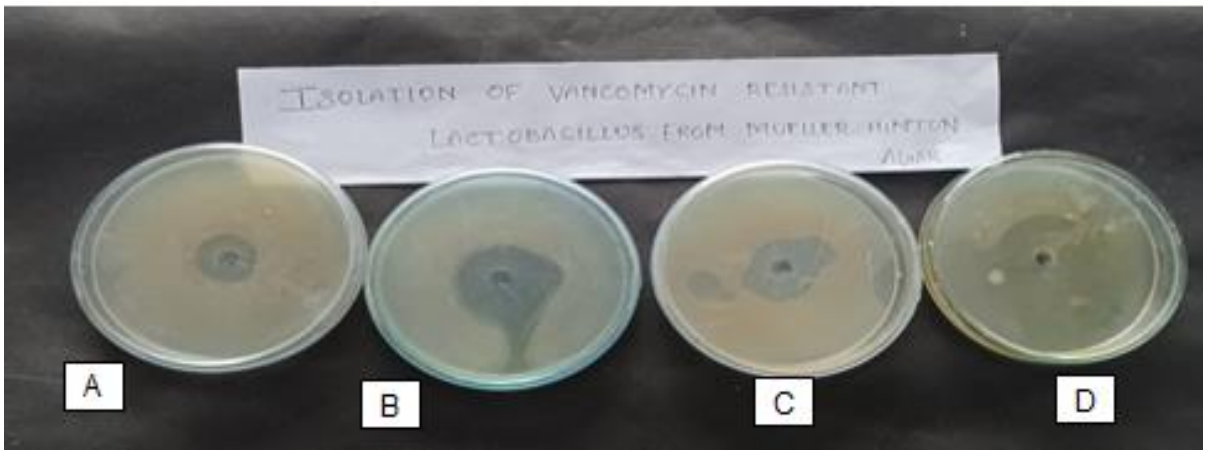
Results and Discussion

Antimicrobial Susceptibility test

Antimicrobial susceptibility test is used for to determine the vancomycin resistance at different concentrations. The bacteria were found to be resistant when the concentration of antibiotic was gradually increased during culturing. This could be due to the replacement of terminal D-alanine residue of the muramyl pentapeptide by D-lactate or D-serine, which provides resistance to the glycopeptide antibiotic vancomycin as per the report of Delcour (1999). The wide range of physiological functions may be assigned to the cell wall, which contributes to the technological and health-related attributes of Lactic acid bacteria. The resistance of the colonies on Mueller Hinton Agar when spreaded on the concentration of 1.25ug/ml was effective than other concentrations. These varying results might be due to the factors such as inoculum size, incubation time, incubation temperature, aeration and composition of growth medium. An increased inoculum size and an extended incubation time resulted in higher antibiotic MICs. The resistant colonies were picked up from the MHA plates and cultured on MRS agar (Figure:1). The screening and characterization of LAB for the presence of antibiotic resistance determinants requires the combined use of phenotypic properties and molecular methods (Figure:2).

Figure 1: Lactobacillus Colonies on MRS Agar Plate:

Figure 2: Vancomycin Resistant Colonies On Mueller Hinton Agar Plate:



a. 0.1ug/ml concentration of vancomycin b. 0.5ug/ml concentration of vancomycin
c. 1ug/ml1 concentration of vancomycin d. 1.25ug/ml concentration of vancomycin

The amplified PCR product was used to determine the gene sequence (Figure:3&4). The annotated sequences of the available genomes for the *Lactobacillus sp.* were correlated with the gene of interest or amplicon using NCBI (Figure:5). Pairwise alignments were carried out for *Lactobacilli* and the accession numbers were obtained. The results were documented in public sources with the accession number OR878453 for the isolated natural *Lactobacillus sp.* and OR865319 for vancomycin resistant gene of *Lactobacillus sp.* Comparison of Vancomycin resistant and non-resistant Lactobacilli with KP789054.1 were analysed and documented in NCBI with accession number, score value, identities, gaps and strand nature. These were tabulated in table1.

Table 1: Comparison of Vancomycin resistant and non-resistant Lactobacilli with

KP789054.1

Accession number	**Score**	**Identities**	**Gaps**	**Strand**
OR865319	789bits (427)	573/639 (90%)	28/639 (4%)	Plus/Plus
OR878453	824bits (446)	766/918 (83%)	31/918 (3%)	Plus/Plus

Figure 3: Gene sequence of Non-resistant *Lactobacilli.*

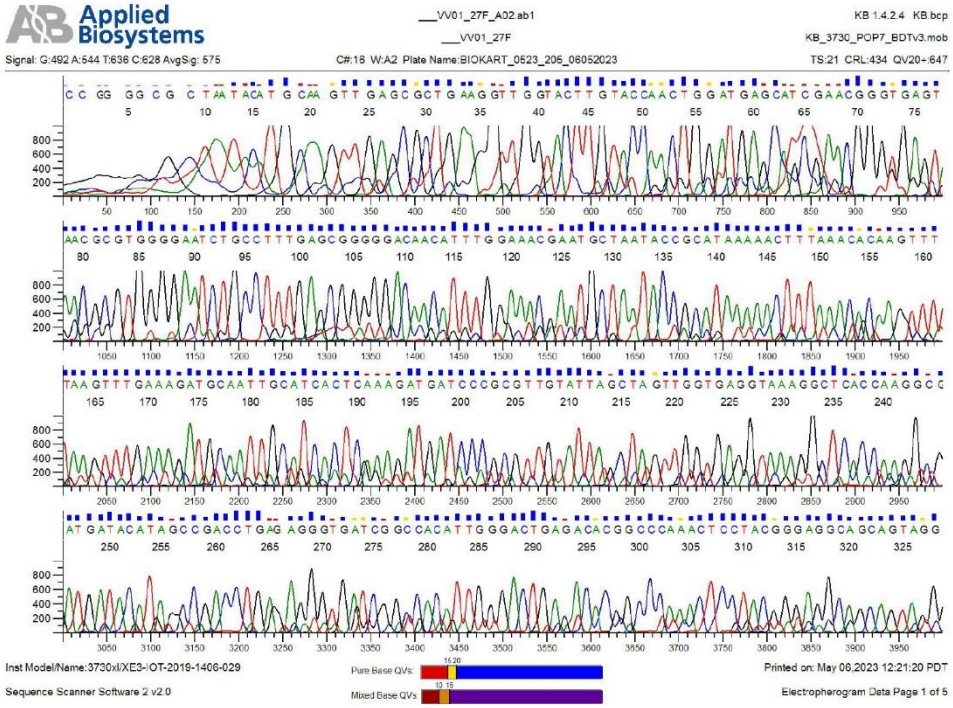


Figure 4: Gene sequence of Resistant *Lactobacilli.*

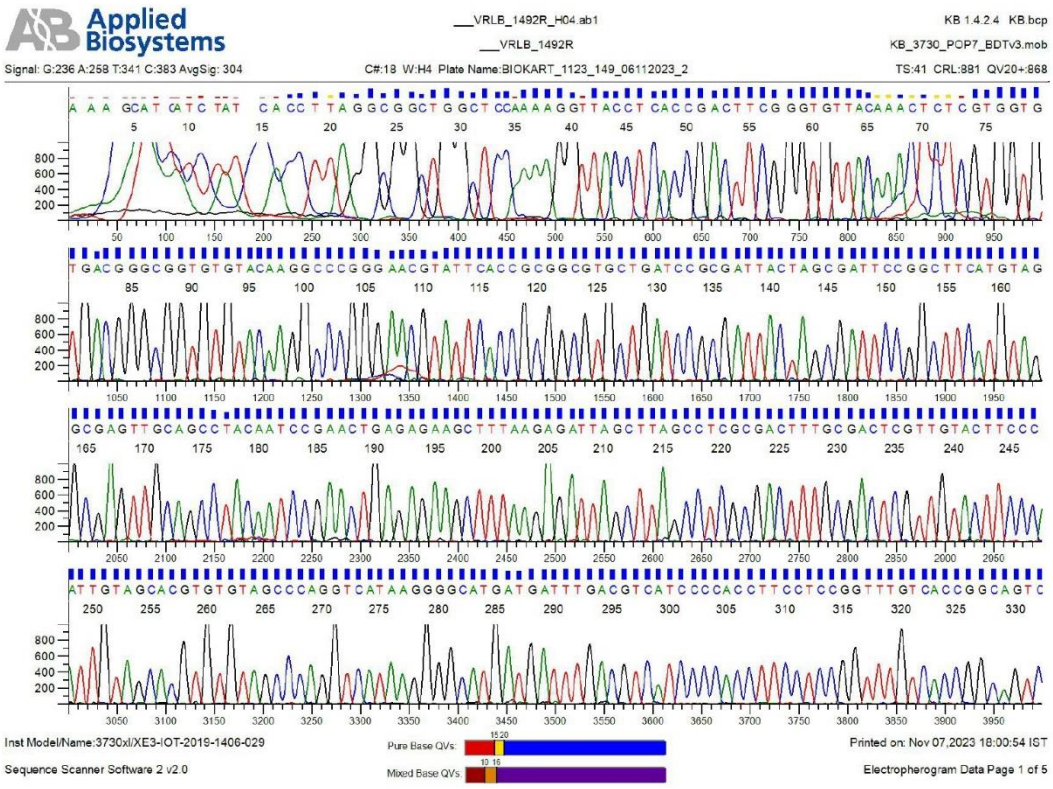
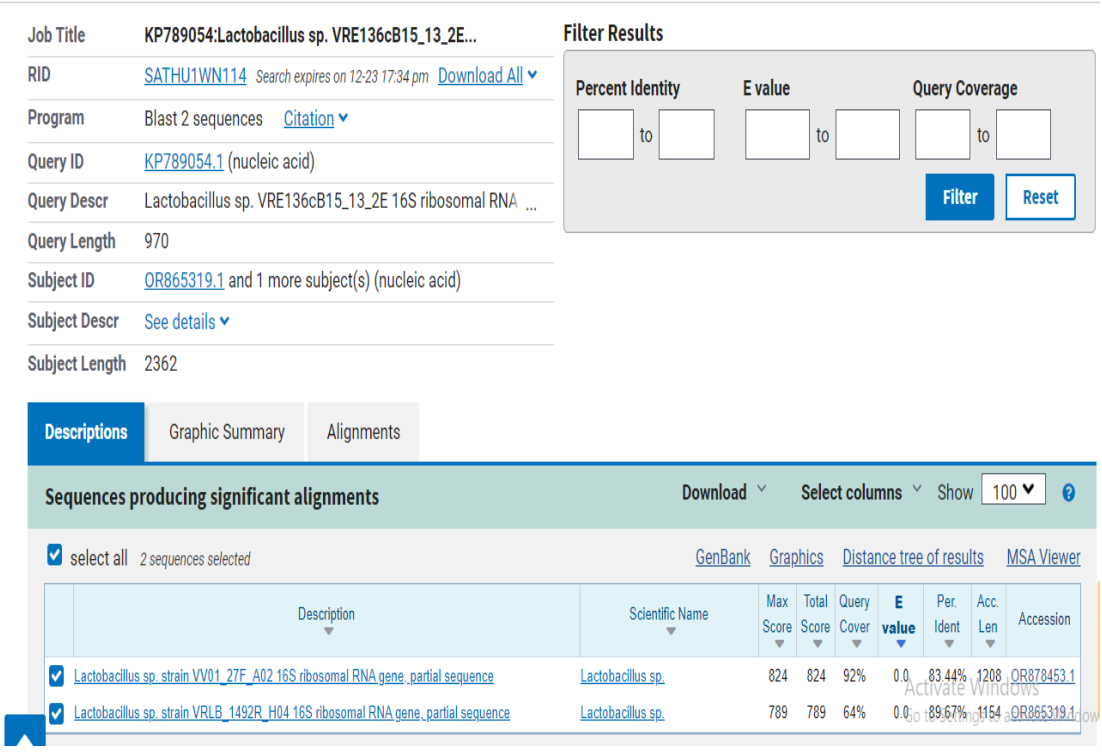


Figure 5: BLAST analysis of Lactobacilli gene sequence.



83.44% similarities only was identified in the BLAST analysis. So, the present study

found that some of the new sequence arrangements in resistant lactobacilli. According to Ilenia Campedelli *et al.,* (2019) *Lactobacillus* species are generally considered to be nonpathogenic and are used in a wide variety of foods and products for humans and animals. However, many of the species examined in this study have antibiotic resistance levels which exceed those recommended by the EFSA.

Lactobacillus is naturally resistant to a wide range of clinically important antibiotics and this could enable the development of probiotic combination therapies for treatment of diarrhea and urogenital tract infection. *Lactobacillus* has desirable probiotic properties including antimicrobial activity and antibiotic resistant characters particularly for vancomycin. Ingestion of the vancomycin resistant microbial sources could influence the presence, establishment of normal microflora in our body. LAB in dairy products contains antibiotic resistance genes and distributes the resistance through food products and further screening of antibiotic resistance in foods should be regularly monitored for food safety. In the present study, the isolates of vancomycin resistant LAB were amplified using PCR. These results may help us to use this microbe for human consumption as probiotic preparations or dietary supplements. The study also suggested that still more researches are needed in *Lactobacilli.*

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