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RESEARCH ARTICLE

Isolation, Identification, Characterization and Antimicrobial Study of Probiotic from Sauerkraut

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ABSTRACT

Background and Objectives: Long term use of Antibiotics, sedentary lifestyle, and change in the diet pattern is responsible for dysbiosis of microbiota. Dysbiosis of microbiota is the etiological factors of chronic diseases. Probiotics are live microorganisms, which show beneficial health effects on hosts once consumed in sufficient amounts may be the prophylaxis approach for chronic disease. LAB group can be isolated and characterized from traditional dairy sources and fermented product. This study aimed at isolating, identifying, and in vitro characterizing (morphology, catalase, oxidase test 16 S rRNA Sequencing and Phylogenetic Analysis) LAB strains from traditional Sauerkraut.

Materials and Methods: Isolated strains were identified by Gram staining, catalase assay, and molecular identification methods; 16S rDNA gene sequencing, phylogeny study, antipathogenic study and antibiotic susceptibility Assay.

Results: The experiments revealed that Pediococcus pentosaceus DSM 20336 (T) strains, which were isolated sauerkraut shows a desirable tolerance to low pH and high bile salts, favourable anti-pathogen activity, and acceptable antibiotic susceptibility; hence, they could be considered as novel probiotic candidates for preventing and treating the chronic disease.

KEYWORDS:
Dysbiosis, Microbiota, Sauerkraut, Pediococcus pentosanes, probiotic

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1. INTRODUCTION

The gut microbiome plays an important role in human health and influences the development of chronic diseases ranging from metabolic disease, gastrointestinal disorders to CNS diseases and colorectal cancer. Of increasing prevalence in Western societies, these conditions carry a high burden of care [1-4]

Scientific research has demonstrated that nutrition plays a crucial role in the prevention of chronic disease. Changed lifestyles caused by fast food, Western food and the increased use of antibiotic are significant factors which are responsible of dysbiosis of microbiota. [5]

The community of ~200 prevalent bacteria, virus, and fungi inhabiting the human gastrointestinal (GI) tract provide unique metabolic functions to the host and are fundamentally important in health and disease [6,7].

Probiotics are defined as “living microorganisms which, when administrated in adequate numbers, confer a health benefit to the host” [8]. In general, commercially available probiotic bacteria are from the Lactobacillus, Bifdobacterium, Streptococcus and Enterococcus genera. The health benefits of probiotics in treating disorders, including inflammatory bowel disease, irritable bowel syndrome, constipation, antibiotic-associated and acute diarrhea, allergy-related conditions, hypertension, and diabetes, have been well-documented by numerous esteemed scientific reports and systematic reviews [9].

The most common fermented foods that naturally contain probiotics, or have probiotics added to them; include yogurt, kefir, kombucha, sauerkraut, pickles, miso, tempeh, kimchi, sourdough bread and some cheeses. Most probiotics fall into the group of organisms' known as lactic acid-producing bacteria and are normally consumed in the form of yogurt, fermented milks or other fermented foods. Some of the beneficial effect of lactic acid bacteria consumption include: (i) improving intestinal tract health; (ii) enhancing the

immune system, synthesizing and enhancing the bioavailability of nutrients; (iii) reducing symptoms of lactose intolerance, decreasing the prevalence of allergy in susceptible individuals; and (iv) reducing risk of certain cancers and chronic diseases. [10] Its main properties are: protection of the microbiota against pathogenic microorganisms, stimulation of the immune system, normalization of the microbiota and reduction of intestinal permeability. [11] On the other hand, prebiotics are food elements used in the growth and maintenance of the intestinal microbiota, are metabolized in the large intestine, but are not digested in the small intestine. Prebiotics have the potential to transform the composition of the intestinal microbiota, making the beneficial bacteria become the dominant microbial profile [12]. In front of increase in chronic diseases, consumers are changing their eating habits, looking for foods with functional properties as fermented dairy products, infant formulas and dietary supplements. Basic research on probiotics has suggested several modes of action beneficial for the human body and clinical research has proven its preventive and curative features in different intestinal and extraintestinal diseases. In this sense, this article focused on the evidence of clinical benefits of prebiotics and probiotics toward prevention and treatment of chronic diseases. [13]

Sauerkraut is a type of fermented cabbage with major health benefits. It's fermentation creates conditions that promote the growth of beneficial probiotics, which are also found in products like yogurt and kefir.[14] Probiotics are bacteria that provide powerful health benefits. Sauerkraut is a source of probiotics, which provide many potential health benefits. It also contains enzymes that help body absorb nutrients more easily.[15]

Historically, it served as a source of nutrients during the winter months when fresh food was scarce, as proper fermentation preserves the nutritive value of cabbage while creating desirable sensory properties [16,17]. It is most commonly associated with Central and Eastern European cultures, though it can be found in Western European cuisine as well. Sauerkraut is thought to have been part of the American diet since the country's founding, usually as a cooking ingredient, side dish, or condiment. Its popularity declined beginning in the 1930s as a result of shifting consumer preferences and a lack of product uniformity [16,18]; however, advances in food fermentation science and modern consumer interests have brought sauerkraut renewed popularity in recent years.

Sauerkraut production and characteristics are largely dependent on the resident microbial community and the fermentation conditions [19]. Though the microbial composition of sauerkraut can vary during the initial stages of fermentation, appropriate fermentation conditions such as temperature and relative ingredient concentration ensure that lactic acid bacteria (LAB) are the dominant microorganisms in the final fermented product. These LAB are of critical importance for successful fermentation; they produce the organic acids, bacteriocins, vitamins, and flavor compounds responsible for many of the characteristic sensory qualities of fermented foods, including extended shelf life, flavor, and nutritional content [19–20]. Additionally, certain LAB have been purported to act as probiotics that contribute to human health and microbiome stability [21-22]. Though these claims have not yet been fully substantiated by scientists, this perspective has contributed to recent increased consumer popularity and consumption in the United States [23].

Historically, the important species in sauerkraut fermentation were considered to be L. mesenteroides, L. plantarum, and L. brevis, which is supported by recent studies [24, 25]. In the event of abnormally high heat or salinity, Enterococcus faecalis and Pediococcus cerevisiae are thought to play a role in the fermentation process [24]. However, these observations were drawn from studies that used culture-based techniques to isolate bacteria, which are inherently biased due to their inability to capture the range of non-culturable bacteria.

Recent advances in high-throughput sequencing technology have created the potential for highly accurate, culture-independent characterization of the sauerkraut microbiome. The advent of 16S rRNA amplicon sequencing technology has made it possible to systematically analyze the sauerkraut microbiome before, during, and after fermentation. Sauerkraut fermented at warmer temperatures has historically been considered to be of lower quality than sauerkraut fermented at low temperatures; however, current methods of industrial production are turning towards warm-temperature fermentation because it dramatically shortens production time. Here, we analyze the taxonomic composition of sauerkraut fermented at room temperature over a 14-day fermentation period. Overall, the taxonomic composition of this sauerkraut is in line with the taxonomic composition observed in sauerkraut fermented in the traditional cold temperature range, suggesting that warm-temperature fermentation may be a viable option for producing sauerkraut with a bacterial community structure that is in line with sauerkraut produced by more traditional cold-temperature fermentation. This may be of particular interest to industrial and commercial producers, who would be able to speed their production process without sacrificing the taxonomic composition that is at the heart of consumer interest in probiotics and fermented foods.

The word "Probiotic" was first introduced in 1974 by Parker who defined it as organism and substances that have a have a beneficial effect on the host animal by contributing to its intestinal microbial balance and since then, the definition of probiotic has been improved several times.[26] Probiotics not only improve the gut health but also have been documented to other health promoting effects including for prophylaxis or treatment of chronic diseases such as cancer, diabetes, obesity, allergy, Hyperlipidemia and HIV.[27]

Therefore, this study aimed at screening traditional sauerkraut to determine new strains with high probiotic

capability by employing important morphological and biochemical assays with 16 S rRNA Sequencing and Phylogenetic Analysis

2. MATERIALS AND METHODS

2.1 Collection of Cabbage:

Cabbage (Brassica oleracea L. var. capitata) is one of the most important vegetables grown worldwide. It belongs to the family Brassicaceae, which includes broccoli, cauliflower, and kale. The different cultivated types of cabbage show great variation in respect of size, shape and color of leaves as well as the texture of the head (Singh et al., 2006). [28]. The cabbage (Brassica Oleracea L. Var Capitata) was purchased from local market and authenticated in the Department of Botany, Dayanand Science college Latur, Maharashtra, India.

2.2 Preparation of Sauerkraut

Cabbage was washed with water and damaged upper green leaves were removed. The cabbage heads were trimmed and sliced into 1-2mm thick shreds. The shredded cabbage was mixed with 3% (w/w) food grade salt (NaCl) and kept 1L sterilized glass bottles; air-tight bottle was subjected to fermentation at 25 °C for 8 days.

2.3 Bacterial isolation and purification.

On eighth day of fermentation 1g paste of fermented Sauerkraut was mixed homogeneously with 10 ml of sterile distilled water in test tubes and the serial dilutions was made up to 10-6. From the above dilution 100 µl was taken and spread on 1.7 %(W/V) MRS agar plates [29]. Then the Petri dish was anaerobic cultured at 37°C for 36-48h. Bacterial colonies that exhibited clear zone on the plates were individually picked out. The bacteria colonies were streaked on plates and anaerobic cultured at 37°C for 24-36h. The bacteria colonies was streaked for 2 or 3 times to get the pure isolations

2.4 Morphological and biochemical characterization.

1. Gram Staining

Is the common, important, and most used differential staining techniques in microbiology, which was introduced by Danish Bacteriologist Hans Christian Gram in 1884. This test differentiates the bacteria into Gram Positive and Gram-Negative Bacteria, which helps in the classification and differentiations of microorganisms. Gram staining was performed to determine the cell morphology and Gram stain reaction of the isolates. [30]

2. Catalase and Oxidase test

Catalase is an enzyme found in most bacteria and is known to catalyze the breakdown of hydrogen peroxide (H_2O_2) to liberate oxygen as (O_2) in this process. The catalase test was carried out using the method of Kiss [31] (Snell et al.1999). To loopful of the culture was added a few drops of 3% Hydrogen peroxide on a slide and any reaction was observed. Evolution of gas or white froth indicates a catalase positive reaction while absence of froth indicates negative reaction. For the oxidase test, a few drops of 1% aqueous tetra methyl phenylenediamine hydrochloride was added to a piece of Whatman No.1 filter paper, with the aid of glass rod, to moisten it. The filter paper was then smeared with isolated bacterial culture at different positions. A purple color was regarded as positive if developed within a few seconds usually 5-10 seconds and a delayed reaction or no coloration was regarded as negative or weak. Catalase and oxidase tests were also performed [31].

Only those isolations which were catalase and oxidase negative and Gram-stain positive were maintained. Select the isolations which were catalase negative and Gram-stain positive to observe under 100 times oil immersion lens. Observed and recorded the characteristics of the bacterial colonies.

3. Strains Preservation

The selected strains were preserved by two methods, slant preservation and freeze-drying preservation. The first method can preserve the strains for 1 to 2 weeks, and the other method can preserve the strains for a long time.

2.3. 16 S rRNA Sequencing and Phylogenetic Analysis

The pure culture in the form of slant and Petridis submitted to National center for microbial Resources (NCMR) Pune, Maharashtra, India for 16S rRNA Gene Sequencing & Phylogenetic tree based on the 16S rDNA sequence

2.4. Evaluation of Antibacterial Activity of Identified Strains

Agar disk-diffusion testing developed in 1940 [8] is the official method used in many clinical microbiology laboratories for routine antimicrobial susceptibility testing. In this experiment Antibacterial activity of probiotic is evaluated by using disk diffusion methods. [32]

Pediococcus pentosaceus DSM 20336 (T) was grown in 10 ml MRS medium [32] at 37°C for 18–24 h. Pediococcus pentosaceus DSM 20336 (T) cells were removed by centrifugation at 8,000g and 4 ° C for 10 min and the cell-free supernatant was sterilized by filtering through 0.22 µm membrane. The petri dishes containing nutrient agar, previously inoculated with 1 % each of test microorganisms (Enterobacter, Salmonella, Bacillus megaterium, Staphylococcus aureus, Rhizopus, Aspergilllus Niger, Candida Albicans, Candida Albicans, Penicillium Chrysogenum)., were prepared. The paper disc assay is a modification where instead of making wells, discs measuring 6 mm are absorbed with aliquots of cell free supernatant and placed on the agar inoculated with indicator strains. After incubation, Antibacterial activity was evaluated by measuring the diameter of the inhibition zone (IZ) around the discs [33].

2.5 Susceptibility of Pediococcus pentosaceus DSM20336(T) to Antibiotics

Bacterial strain: Bacterial Strain was isolated from sauerkraut and further sub cultured and obtained pure culture for the antibacterial susceptibility test.

Disc diffusion method was used to obtain antibiograms of isolated Pediococcus strains following the modified standard Kirby–Bauer procedure (Rojo-Bezares et al. 2006). Disc diffusion method was used to obtain antibiograms of isolated Pediococcus strains following the modified standard Kirby-Bauer procedure (Rojo-Bezares et al. 2006). Antibiotic susceptibility pattern of isolated pediococci was assessed using commercially available antibiotic discs (Hexa G plus 2 manufactured by HiMedia, Mumbai, India) having 06 antibiotics including Penicillin G-P10 units , Clindamycin CO-2mcg, Co-trimoxazole COT 25mcg , Erythromycin –E-15 mcg, Vancomycin-VA 30 mcg, Ampicillin/ Salbactum A/S-10/10mcg . MRS agar plates were prepared and overlaid with 50 mL of MRS soft agar tempered at 45 °C and seeded with 100 µL of active Pediococcus cultures (cell density 107 cfu/mL). Plates were allowed to stand at room temperature for 15–20 min and then the antibiotic discs were dispensed onto the seeded agar plates under aseptic conditions. Inhibition zone diameters (mm) were measured using antibiotic zone scale after incubation at 37 °C for 24 h. Results were expressed in terms of resistant (R), intermediate resistant (IR) or susceptible (S) with zone diameter of ≤14, 15–19 and ≥20 mm, respectively, as per recommended standards given by Clinical and Laboratory Standards Institute (CLSI 2012) described by Charteris et al. (2000).

3. RESULTS

3.1 Morphological and biochemical characterization

1. Gram staining

Gram staining is done according to (Bartholomew JW, Mittwer T) [30] the observance of purple colour interpret the bacteria is gram positive cocci.

2. Catalase and oxidase test

For the oxidase test, a few drops of 1% aqueous tetra methyl phenylenediamine hydrochloride was added to a piece of Whatman No.1 filter paper, with the aid of glass rod, to moisten it. The filter paper was then smeared with isolated bacterial culture at different positions. A purple color was regarded as positive if developed within a few seconds usually 5-10 seconds and a delayed reaction or no coloration was regarded as negative or weak [31]. Both catalase and oxidase test show negative test conform the lactobacillus species.

Table 1: The features of the bacterial colonies observed under microscope

Sample	Diameter	Shape	Humidity	Color	Edge	Gram	Surface
Bacterial culture	0.8-1mm	Round	Slight/ Moderate	Milky white	Irregular	positive	Rough

3.2 16 S rRNA Sequencing and Phylogenetic Analysis :[34]

The bacterial sample(s) identification based on 16S rRNA, the identification report was generated using EzBioCloud Database at NCMR Pune.

Table 2: Summary of the closest neighbour(s) for sample

PRN	Strain No.	Taxonomic Designation	Accession No.	%Similarity
AUG_21_275	W/P/003	Pediococcus pentosaceus DSM20336(T)	JQBF01000022	99.93

Sequence Text (in FASTA format font: courier new 10)

TGCTCAGGATGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGA
ACGAACTTCCGTTAATTGATTATGACGTACTTGTACTGATTGAGAT
TTTAACACGAAGTGAGTGGCGAACGGGTGAGTAACACGTGGGTAA
CCTGCCCAGAAGTAGGGGATAACACCTGGAAACAGATGCTAATAC
CGTATAACAGAGAAAACCGCATGGTTTTCTTTTAAAAGATGGCTCT
GCTATCACTTCTGGATGGACCCGCGGCGTATTAGCTAGTTGGTGA
GGTAAAGGCTCACCAAGGCAGTGATACGTAGCCGACCTGAGAGGG
TAATCGGCCACATTGGGACTGAGACACGGCCCAGACTC
CTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGCAAGTC
TGATGGAGCAACGCCGCGTGAGTGAAGAAGGGTTTCGGCTCGTAAA
GCTCTGTTGTTAAAGAAGAACGTGGGTAAGAGTAACTGTTTACCCAG
TGACGGTATTTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCC
GCGGTAATACGTAGGTGGCAAGCGTTATCCGGATTTATTGGGCGTA
AAGCGAGCGCAGGCGGTCTTTTAAGTCTAATGTGAAAGCCTTCGGC
TCAACCGAAGAAGTGCATTGGAAACTGGGAGACTTGAGTGCAGAAGA
GGACAGTGGAACTCCATGTGTAGCGGTGAAATGCGTAGATATATGG
AAGAACACCAGTGGCGAAGGCGGCTGTCTGGTCTGCAACTGACGCT
GAGGCTCGAAAGCATGGGTAGCGAACAGGATTAGATACCCTGGTAG
TCCATGCCGTAAACGATGATTACTAAGTGTTGGAG

GGTTTCCGCCCTTCAGTGCTGCAGCTAACGCATTAAGTAATCCGCC
TGGGGAGTACGACCGCAAGGTTGAAACTCAAAAGAATTGACGGGG
GCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCTACGCG
AAGAACCTTACCAGGTCTTGACATCTTCTGACAGTCTAAGAGATTA
GAGGTTCCCTTCGGGGACAGAATGACAGGTGGTGCATGGTTGTC
GTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCG
CAACCCTTATTACTAGTTGCCAGCATTAAGTTGGGCACTCTAGTGA
GACTGCCGGTGACAAACCGGAGGAAGGTGGGGACGACGTCAAAT
CATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGATG
GTACAACGAGTCGCGAGACCGCGAGGTTAAGCTAATCTCTTAAAAC
CATTCTCAGTTCGGACTGTAGGCTGCAACTCGCCTACACGAAGT
CGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGT

3.3. Phylogenetic Analysis Report

The analysis is based on single gene sequence for a total of 1411 bp length of the 16S rRNA gene of sample with its closest type strains in the database. The closest phylogenetic neighbors found for sequence is Pediococcus pentosaceus. Phylogenetic tree, analysis details and the general observations are given below.

Phylogenetic Tree

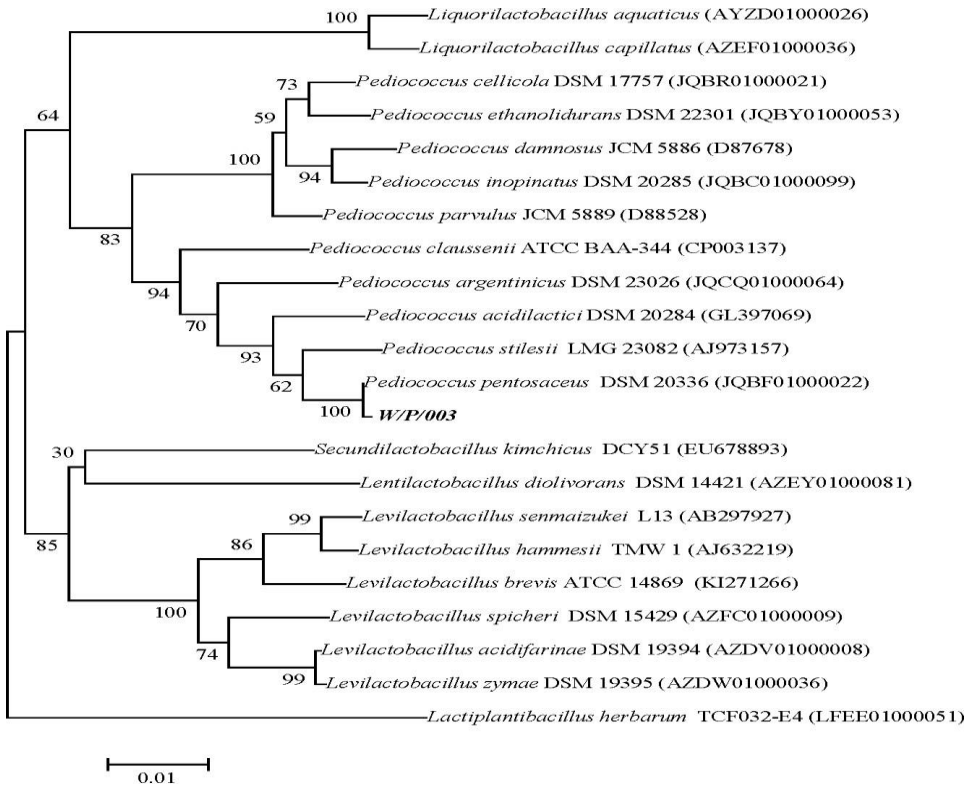

Fig.1: Evolutionary relationships of taxa

Analysis details

Table 3: 16S rRNA gene based phylogenetic analysis

Total number of sites for the analysis	1368
Conserved sites	1130
Variable site	236
Parsimony informative sites	180
Analysis	Phylogeny Reconstruction
Statistical Method	Neighbor-joining
Test of Phylogeny	Bootstrap method
No. of Bootstrap Replications	1000
Substitution Model	Kimura 2 parameter method
Substitutions to Include	Transitions + Transversions
Rates among Sites	Gamma Distributed (G)
Pattern among Lineages	Same (Homogeneous)
Gaps/Missing Data Treatment	Pairwise deletion

3.4 Evaluation of Antibacterial Activity of Identified Strains:

Pediococcus pentosaceus DSM 20336 (T) displayed antibacterial activity against all four test microorganisms. The zone of inhibition observed around Enterobacter, Salmonella, Bacillus megaterium, and Staphylococcus aureus was 15 ± 0.2 mm, 17 mm ± 0.2 mm and 13 ± 0.2 mm, and 27 ± 0.2respectively. No zone of inhibition was observed around the wells containing fungus Rhizopus, Aspergillus niger, Candida albicans , penicillium chrysogenum.

Table 4: Evaluation Antibacterial and Antifungal Effect of Probiotics (Pediococcus pentosaceus DSM 20336 (T))

S. No	Pathogen	Type	Zone of Inhibition (mm) after 24 hrs
01	Enterobacter	Bacteria	15
02	Salmonella		17
03	Bacillus megaterium		13
04	Staphylococcus aureus		27
05	Rhizopus	Fungi	6
06	Aspergilllus Niger		6
07	Candida Albicans		6
08	Penicillium Chrysogenum		6

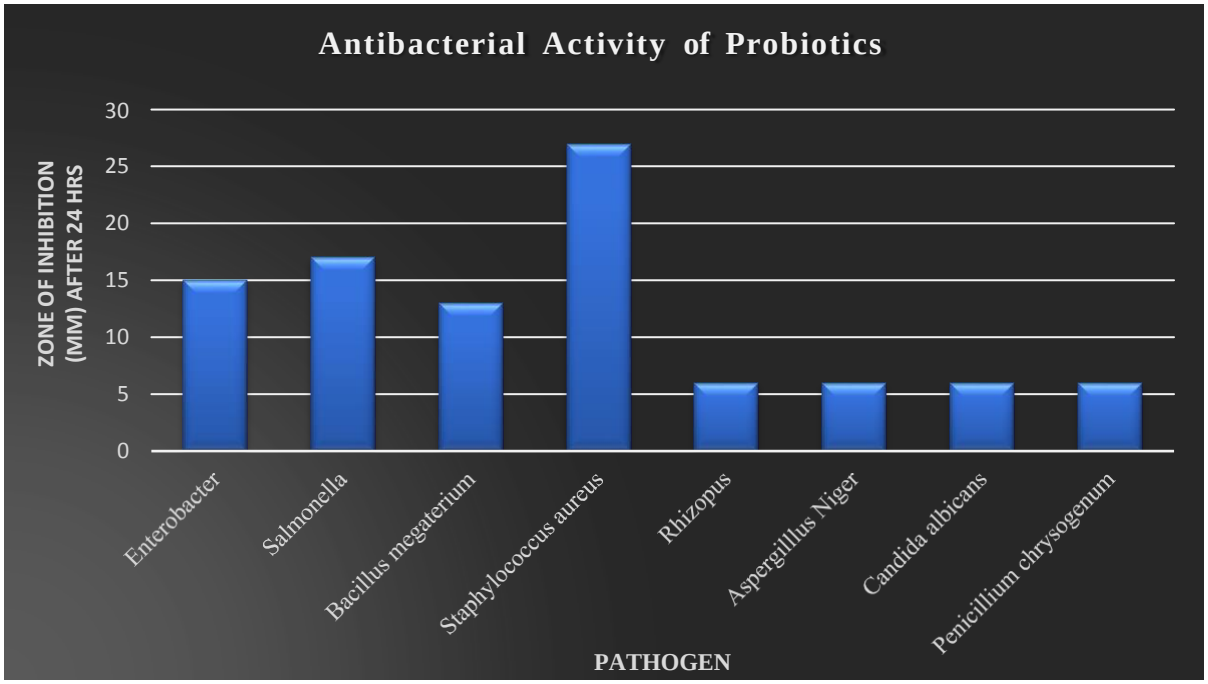

Fig.2: Evaluation Antibacterial and Antifungal Effect of Probiotics (Pediococcus pentosaceus DSM 20336 (T))

Table 5: Susceptibility of Pediococcus pentosaceus DSM20336 (T) to Antibiotics

Antibiotic	Zone of Inhibition (mm)			% of Zone of Inhibition	
	0 hrs	24 hrs	48 hrs	24 hrs	48 hrs
Penicillin G-P10 units	6	6.2	6.4	3.23	6.67
Clindamycin CO-2mcg	6	6.3	6.3	1.61	5.00
Co-trimoxazole COT 25mcg	6	6.3	6.5	4.84	8.33
Erythromycin -E-15 mcg	6	8	8	29.03	33.33
Vancomycin-VA mcg	6	22.1	22.3	259.68	271.67
Ampicillin/ Salbactum A/S-10/10mcg	6	15.3	16.5	166.13	175.00

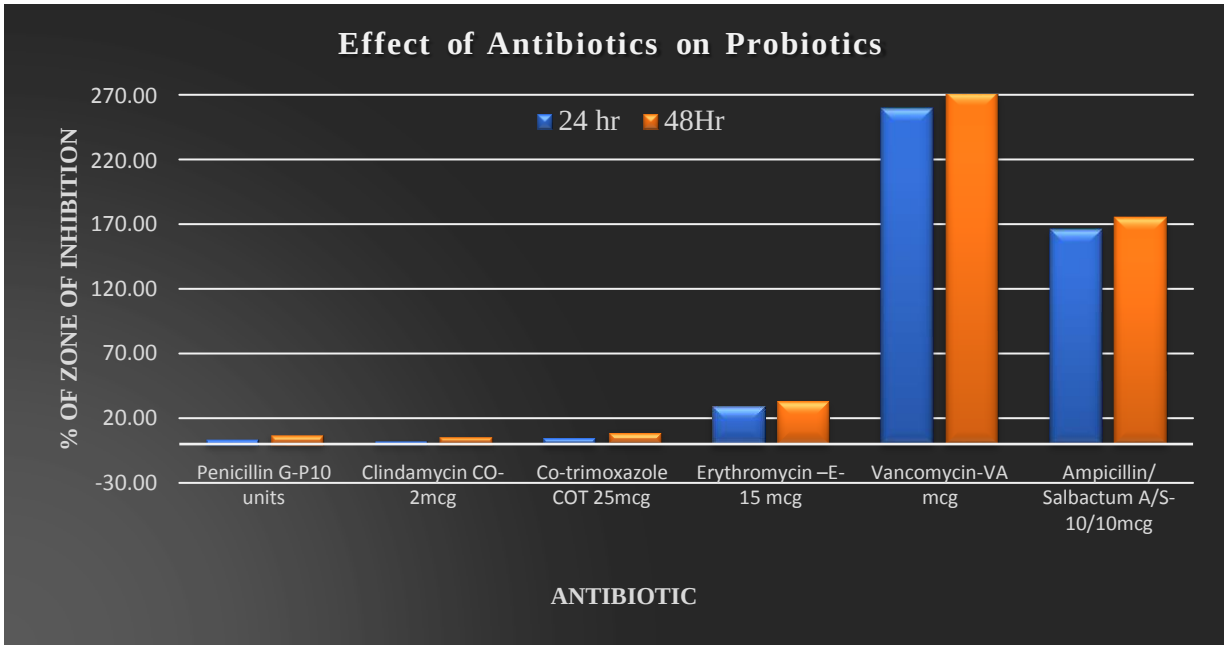

Fig.3: Susceptibility of Pediococcus pentosaceus DSM20336 (T) to Antibiotics

The antibiotic susceptibility results of isolated Pediococcus pentosaceus DSM20336 (T) against clinically important antibiotics are presented in Table 5. Based on our findings, all Pediococcus pentosaceus DSM20336 (T) were sensitive or semi-sensitive to Vancomycin and Ampicilln salbacturm and resistant to penicillin, clindamycn, cotrimaxazole and Erythromycin.

DISCUSSION

Dysbiosis of gut microbiota is causative factors for the various chronic diseases if we maintain the healthy gut flora by administration probiotic can be a prophylaxis approach for the chronic diseases. As we know that the naturally probiotic are present in various fermented product like Yogurt, Kefir, Tempeh, Kimchi, Miso, Kombucha, Pickles, Traditional Buttermilk, Natto and Sauerkraut.

The bacterium was isolated from sauerkraut and evaluated for morphological and biochemical test. As it gives oxdase, catalase negative and gram positive test indicate the bacterial strain is lactic acid bacteria. Pediococcus pentosaceus are Gram-positive, facultatively anaerobic, non motile and non spore forming lactic acid bacteria.

The evolutionary history was inferred using the Neighbor-Joining method [35]. The optimal tree with the sum of branch length = 0.32680031 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches [36]. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method [37] and are in the units of the number of base substitutions per site. The rate variation among sites was modeled with a gamma distribution (shape parameter = 1). The analysis involved 22 nucleotide sequences. All positions with less than 95% site coverage were eliminated. That is, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position. There were a total of 1338 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 [38].

A comparative 16S rRNA gene based phylogenetic analysis placed strain in a clade with the species Pediococcus pentosaceus and revealed pairwise similarities ranging from 100 to 99%. Based on the results from the phylogenetic tree using 1368 bp sequence of 16S rRNA gene and pairwise similarity results using GenBank database it can be inferred that this might be another genus of the Pediococcus. However, according to the available literature, a taxonomic group includes species/subspecies that are not distinguishable by 16S rRNA gene sequence alone.

Antibacterial effects of Pediococcus pentosaceus DSM 20336 (T) against Enterobacter, salmonella, Bacillus megaterium, and Staphylococus aureus strain. Our study showed that zone inhibition of all these species indicates Pediococcus pentosaceus DSM 20336 (T) shows antibacterial properties.

CONCLUSION

The experiments revealed that Pediococcus pentosaceus DSM 20336 (T) strains, which were isolated sauerkraut, shows a desirable tolerance to low pH and high bile salts, favourable anti-pathogen activity, and acceptable antibiotic susceptibility; hence, they could be considered as novel probiotic candidates for preventing and treating the chronic disease.

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