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RESEARCH ARTICLE

PROBIOTIC AND FUNCTIONAL ATTRIBUTES OF PROBIOTIC LEUCONOSTOC CITREUM STRAIN MRKAK2

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Abstract

The development of functional foods and their safety assessment is required for well-being of the human health. In the present study, we have isolated and identified a potential probiotic bacterium from the fermented garlic pickle that exhibits an improvement in health status upon consumption. In order to assess the probiotic potential of isolated bacterium**,** *in vitro* probiotic tests were performed, including tolerance to acid, pancreatin, bile, and adhesion ability to gastrointestinal cell lines. The safety assessment of probiotic strain was evaluated using an antibiotic susceptibility test. The obtained results showed that the isolated strain was identified as *Leuconostoc citreum* strain MRKAK2 based on 16S rRNA sequencing. The isolate demonstrated tolerance to acidic pH (pH 1.0 and pH 3.0), 0.3% pepsin (pH 2.0), 0.1% pancreatin (pH 8.0), bile (0.3% and 1%) and efficient adhesion to gastrointestinal cell line HT-29. The strain also exhibited susceptibility towards antibiotic ampicillin, tetracycline, streptomycin, and sulphatriad, whereas it showed resistance against chloramphenicol and penicillin-G. The antibiotic sensitivity pattern of the strain was similar to other *Lactobacillus* strains. Thus, our results exhibited that *L. citreum* strain MRKAK2 is a potential probiotic strain for developing fermented functional foods.

Introduction:-

The worldwide demand for healthy functional foods promotes the innovations and development of new products in the food industry. Notably, probiotics are actively being used in fermented food products due to their health-promoting effects [1]. The lactic acid bacteria (LAB) are generally regarded as safe (GRAS) in the food industry as these are used as a starter culture and have a long history of health safety [2]. The common probiotic bacterial genera include *Lactobacillus***,** *Leuconostoc***,** *Lactococcus***,** *Pedicoccus*, and *Streptococcus* [3]. Probiotics are defined as live microorganisms which when consumed in an adequate amount confer a health benefit to the host [4]. The assessment of LAB for their probiotic attributes is evaluated by their gastric and bile tolerance, antagonistic activity against pathogens, and adherence to gut epithelial surfaces [2]. LAB has also been shown to produce antimicrobial peptides, metabolites, and an organic acid, which limit the growth of pathogenic bacteria [5]. In addition, they also lack antibiotic resistance properties and any harmful adverse effects on invertebrate and vertebrate models [6]. Recent evidence also claimed their role in treating several diseases, such as allergy, diabetes, hypertension,

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diarrhoea, genetic disorders, and cancers, and improving the host's immunity [6]. The health-promoting role allows the identification of novel potential probiotic candidates for developing beneficial fermented food products for consumption in humans and animals. In addition, the potential probiotic bacterium should also be assessed for their safety status.

The consumption of functional foods with probiotic bacteria modulates the environment of the gastrointestinal tract [3]. Notably, the potential effect of probiotic bacterium is mainly strain-dependent, suggesting all strains are not equally effective in their probiotic and functional attributes and treatment of diseases [7]. Reports show LAB can efficiently tolerate the lower pH of 1.0 for several hours of exposure. The LAB can also tolerate the bile salts, pepsin, and pancreatin and adhere to the mucus-secreting cell lines such as HT-29 and $CaCo_2$ [1]. In addition, LAB strains also show susceptibility to antibiotics and anti-bacterial activity against pathogenic bacteria [8].

In the context of our study, the garlic pickle is frequently consumed in rural areas of Assam, India. Several studies suggested that the lactic acid bacterium ferments fermented pickles. The traditional belief also suggests that fermented pickles improve digestion, maintain good health, and relieve constipation is scientifically unexplored.

Material and Methods:-

Sample collection and processing

The fermented garlic pickle sample was collected from the Maligaon local market, Assam, India (26.1504° N, 91.6960° E). 1 g of the samples was suspended in 9 ml of a sterile physiological saline solution [0.85% NaCl (w/v)], shaken vigorously and kept for 10 min at room temperature for sedimentation. Then, the suspension was serially diluted in sterile physiological saline solution, and each dilution from 10^{-1} to 10^{-6} was plated on de Man, Rogosa& Sharpe (MRS) agar (with 1% calcium chloride). The MRS agar plates were incubated at 30 °C for 24–48 h to collect colonies. The selected colonies were then streaked on their respective agar plates, inoculated in broth, and maintained at −80 °C with 25% glycerol as a cryoprotectant.

DNA extraction and 16S rRNA sequencing

The genomic DNA of the isolate was extracted using a genomic extraction kit (Sigma, Germany). The PCR reaction of the 16S rRNA gene was set up with a total reaction mixture of 25 µl containing 50 ng of bacterial DNA, 10 pmol of each primer pair {27-F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′)}, 1.5 U of Taq DNA polymerase (Sigma, Germany), 2.5 µL of 10X Taq buffer, 100 µM of dNTP mixture and 1.5 mM of MgCl2. The PCR was performed using the following conditions in a PCR thermal cycler (Eppendorf, Nexus gradient, Thermal cycler, Germany): 95 °C for 5 min, followed by 35 cycles of 94 °C for 30 sec, 52 °C for 30 sec, 72 °C for 1 min, and 72 °C for 5 min. The amplified product was run on 1.2% agarose gel and imaged under a UV transilluminator (Vilber, France). The 16S rRNA gene amplified PCR product sequencing was sequenced from Macrogen Inc. (Korea) and further submitted to the NCBI database.

Bile acid tolerance assay

Initially, we tested the bile salt tolerance of strain MRKAK2 using a procedure described by Vinderola et al. [9]. Briefly, 10^9 CFU/mL cells of the strain MRKAK2 were washed twice with PBS, treated with different concentrations of Ox-gall bile solution (0.3 and 1%) (Sigma, Germany), and further incubated for 0 h and 4 h. The viability of the strain was measured by spread-plating on MRS agar plates, incubated at 30 °C, and counted after 2 days of incubation.

Survival to the gastrointestinal (GIT) transit

The preparation of simulated gastric juice was performed using a procedure described by Conway et al. [10]. Firstly, 10^9 CFU/mL cells of the isolate were treated with gastrointestinal juice (0.3% pepsin, pH 2.0) for 0 h, 1 h, and 3 h and incubated at 30 °C for 2 days. Secondly, 10^9 CFU/mL cells of the isolate was treated with intestinal juice (0.1% porcine pancreatin, pH 8.0) for 4 h and incubated at 30 °C for 2 days. Thirdly, the acid tolerance of the isolate was measured at different pH ranges (1.0, 3.0, and 7.0) of PBS. Briefly, 10^9 CFU/mL cells of the strain MRKAK2 were treated with PBS of different pH range solutions for 4 h, plated at MRS plate, and incubated at 30 °C for 2 days.

Adhesion to gastrointestinal cell line HT-29

Next, the adhesion ability of the isolate was evaluated by a procedure described by Ayeni et al. [11]. The adhesion ability of the isolate was tested on the intestinal epithelial cell line HT-29 (Procured from the National Center of Cell

Sciences (NCCS), Pune, India) for incubation at 4 h compared to initial bacterial cell numbers in DMEM suspension.

Antibiotic sensitivity assay

The Hexa G-PLUS 6 antibiotic rings containing 6 antibiotics with different concentrations were used for antibiotic sensitivity assay on bacterial isolate. Briefly, the 10^9 CFU/ml of isolate spread onto the MRS agar plate, and Hexa G-plus antibiotic rings were placed aseptically onto the spreaded plate. The plates were then incubated at 30 °C, and the zone of inhibition of the spread isolate was measured after 2 days.

Results:-

Molecular characterization of the strain MRKAK2

The isolate MRKAK2 was firstly identified using 16S rRNA sequencing and BLAST results showed 99% identity to *Leuconostoc citreum*; therefore, the strain was submitted to NCBI as *Leuconostoc citreum* strain MRKAK2 (Accession no. OP218267).

***In vitro* probiotic tests**

The isolate MRKAK2 was further subjected to *in vitro* probiotic tests for its probiotic attributes, including acid tolerance, bile salt tolerance, adhesion to gastrointestinal cell line, and antibiotic resistance. All these *in vitro* probiotic tests were performed as per the guidelines of the Indian Council of Medical Research (ICMR) and the Department of Biotechnology (DBT), Govt. of India [2].

Initially, the bile salt tolerance was evaluated for *L. citreum* strain MRKAK2. The results showed no significant change in the strain's viability, suggesting tolerance against bile salt at different concentrations (Table 1). Next, *L. citreum* strain MRKAK2 was subjected to tolerance against gastrointestinal juice (0.3% pepsin, pH 2.0) after 1 h and 3 h. The results showed good survival of the isolate against gastrointestinal juice (Table 1). Furthermore, the results showed that the strain MRKAK2 tolerated intestinal juice after 4 hours of exposure (Table 1).

Lastly, the acid tolerance of the strain MRKAK2 was evaluated at pH 1.0 and pH 3.0. We observed no significant change in the viability of the strain MRKAK2 after 4 hours of exposure to PBS with pH 1.0 and pH 3.0 (Table 1).

Table 1:- Survival and adhesion of MRKAK2 under in vitro simulated gastro-intestinal conditions.

Gastrointestinal juice tolerance											
Treatment	pH 1.0		pH 3.0		pH 7.0		Pepsin (pH 2.0)			Pancreatin (pH 8.0)	
Time	0 h	1 h	0 h	1 h	0 h	1 h	0 h	1 h	3 h	0 h	4 h
Mean* ± SEM	9.13 ± 0.05	8.76 ± 0.03	9.22 ± 0.06	8.83 ± 0.05	9.05 ± 0.04	9.01± 0.07	9.10 ± 0.02	8.91 ± 0.11	8.74 ± 0.07	9.09 ± 0.04	8.95 ± 0.11
#Survival %	95.77		95.58		99.55		95.88			98.43	
P value	p< 0.05		p< 0.05		p> 0.05		p< 0.05 for 1 h and 3 h			p< 0.05	

	Bile salt tolerance				Adhesion in HT-29 cell line	
Treatment	0.3%		1%			
Time	0 h	4 h	0 h	4 h	0 h	4 h
Mean* ± SEM	9.15 ± 0.09	9.08 ± 0.06	9.03 ± 0.04	8.95 ± 0.07	9.21 ± 0.10	8.68 ± 0.15
#Survival %	99.22		99.10		93.89	
P value	p> 0.05		p>0.05		p< 0.05	

* The values represent mean log (CFU/ml) ± standard error mean (SEM).

Survival percentage (%) is expressed as the percentage of 1– [(log CFU per ml at T= initial) – (log CFU per ml at T = incubation hour)/ (log CFU per ml at T= initial)].

The results showed no significant decrease in the adhesion ability of the strain MRKAK2 to gastrointestinal cell line HT-29. All experiments were performed in triplicate after the completion of incubation time, and each treatment was plated on MRS agar plates at 30 °C for 24-48 h (Table 1).

Antibiotic sensitivity

The antibiotic sensitivity pattern of the strain MRKAK2 was described in Table 2.

Table 2:- Antibiotic sensitivity of the strain MRKAK2.

Antibiotic	Sensitivity
Ampicillin (10 µg)	S
Chloramphenicol (25 µg)	R
Streptomycin (10 µg)	MS
Penicillin-G (10µg)	R
Sulphatriad (300 µg)	MS
Tetracycline (25 µg)	S

S = Sensitive; MS = moderately sensitive; R = resistant

Discussion:-

The present study showed that gram-positive *L. citreum* strain MRKAK2 was isolated from garlic pickle. The strain survived the stress conditions in the gastrointestinal tract of humans, including the lower pH, bile salts, pancreatin, and gastric juice. The isolated strains could tolerate a lower pH of 3.0, which is considered the pH of the gastric mucosal layer. The present study showed a more than 90% survival rate of the strain MRKAK2 at pH 3.0 and pH 1.0, which indicates its efficacy against lower pH. The physiological bile concentration is approximately 0.3% in the duodenum [9]. Our results showed no significant changes in the survival of the isolate MRKAK2 at 0.3% and 1% bile concentration. The 0.1% pancreatin treatment to the strain MRKAK2 showed no significant change in the survival of the strain MRKAK2. Generally, the studies showed that bacteria isolates showed a decline in their growth after increasing the exposure time of pancreatin [10]. Therefore, survival in the presence of pancreatin enzymes has become an essential criterion for probiotic selection.

The adhesion ability of the bacteria with the gastrointestinal mucosal layer is highly strain- and species-dependent [11]. Our results showed that the strain MRKAK2 could effectively adhere to the gastrointestinal cell line HT-29. Similar results were also observed with probiotic strains *L. plantarum*, *L. johnsonii*, and *L. acidophilus* [7, 11]. The presence of glycoproteins. lipoteichoic and teichoic acid help bacteria adhere to the gastrointestinal mucosal layer[1].

The strain MRKAK2 was found to be sensitive to antibiotic ampicillin, tetracycline, streptomycin, and sulphatriad, while the strain showed resistance against chloramphenicol and penicillin-G. Our results showed that the antibiotic sensitivity of the strain MRKAK2 resembles other *Lactobacillus* strains, as reported by Costa et al., 2013 [12].

Conclusion:-

All *in vitro* probiotic tests showed that *L. citreum* strain MRKAK2 could successfully tolerate the harsh gastrointestinal conditions of the stomach and small and large intestine in humans and colonize efficiently to the intestinal mucosa. Therefore, the strain could be used as a potential probiotic bacterium for developing fermented functional foods.

Author's contributions

The authors AK and MRK have planned the experimental work of the paper. The author AK has performed the experiments and written the first draft of the manuscript. The authors MRK and MCK have further revised the manuscript. All the authors have provided approval for the publication of the final manuscript.

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Availability of data

The complete relevant data was provided in the final manuscript. The raw fastq files of 16S sequence for the probiotic bacteria *L. citreum* strain MRKAK2 have been submitted in the GenBank accession number (OP218267).

References:-

1. Ahmadi, S., et al., A human-origin probiotic cocktail ameliorates aging-related leaky gut and inflammation via modulating the microbiota/taurine/tight junction axis. JCI insight, 2020. 5(9).
2. Ganguly, N., et al., ICMR-DBT guidelines for evaluation of probiotics in food. The Indian journal of medical research, 2011. 134(1): p. 22.
3. Azad, M., et al., Probiotic species in the modulation of gut microbiota: an overview. BioMed research international, 2018. 2018.
4. Hill, C., et al., The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. Nature Reviews Gastroenterology & Hepatology, 2014. 11(8): p. 506-514.
5. Krishna Rao, R. and G. Samak, Protection and restitution of gut barrier by probiotics: nutritional and clinical implications. Current Nutrition & Food Science, 2013. 9(2): p. 99-107.
6. Sánchez, B., et al., Probiotics, gut microbiota, and their influence on host health and disease. Molecular nutrition & food research, 2017. 61(1): p. 1600240.
7. Suez, J., et al., The pros, cons, and many unknowns of probiotics. Nature medicine, 2019. 25(5): p. 716-729.
8. Chelliah, R., et al., In vitro and in vivo defensive effect of probiotic LAB against *Pseudomonas aeruginosa* using *Caenorhabditis elegans* model. Virulence, 2018. 9(1): p. 1489-1507.
9. Adak, A. and M.R. Khan, An insight into gut microbiota and its functionalities. Cellular and Molecular Life Sciences, 2019. 76(3): p. 473-493.
10. Jena, P.K., et al., Isolation and characterization of probiotic properties of lactobacilli isolated from rat fecal microbiota. Microbiology and immunology, 2013. 57(6): p. 407-416.
11. Kumar, R., S. Grover, and V.K. Batish, Molecular identification and typing of putative probiotic indigenous *Lactobacillus plantarum* strain Lp91 of human origin by specific primed-PCR assays. Probiotics and antimicrobial proteins, 2011. 3(3-4): p. 186-193.
12. Costa, H., et al., Probiotic potential of lactic acid bacteria isolated from minas artisanal cheese from Serra da Canastra, MG. ArquivoBrasileiro de MedicinaVeterinária e Zootecnia, 2013. 65: p. 1858-1866.