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Research Article

Lactobacillus rhamnosus ARJD as a Functional Food with Potential Antioxidant and Antibacterial Abilities

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Abstract

Lactic acid bacteria are considered as probiotics with its use as a functional food. Consumption of these article above threshold deliberates health benefits. On this basis, *Lactobacillus rhamnosus* ARJD (LAB) was isolated from sheep milk with its deposition as PYRN00000000-MCC 3594 in the gene bank. The isolate was screened for probiotics nature and its antioxidant properties. The *In-vitro* studies show that LAB is resistant to change in pH, bile concentration, gastric and intestinal content. Further assay like 2,2-diphenyl-1-picrylhydrazyl (DPPH), hydroxyl radical scavenging, reducing activity assay and nitric oxide showed antioxidant nature of LAB. An *in-vivo* study carried out on Wistar rats by its liver marker investigation showed the antioxidant nature of LAB. The fecal analysis showed that LAB not only shows various probiotics properties but also thrive and get colonized inside the gastrointestinal tract. The screening of LAB against the pathogenic bacteria showed a zone of inhibition, indicating the antibacterial properties. Finally, the isolated LAB shows antibacterial and antioxidant activity conferring the health benefits.

Keywords: Probiotics; Functional Food; Antioxidant; Antibacterial; Wistar Rats

Introduction

Currently, probiotics are considered as the up-coming alternative source of the antioxidant category nutraceuticals products against synthetic xenobiotics or drugs [1]. *Lactobacillus* genera fall under the category of the probiotics are considered as a vital functional food [2]. These microbes are obtained from many animal milk sources and their secondary product such as cheese, butter, buttermilk, etc. *Lactobacillus* is considered as a promising candidate in food and pharmaceutical industries showing the antioxidant activity [3]. These microbes also show positive health benefits against different metabolic disorders caused by the problems associated with gastrointestinal tract [4,26]. These probiotics thrive inside the gastrointestinal tract and make use of the undigested material from the bowel of the host as their own source of energy called prebiotics [5]. Different enzymes released by lactic acid bacteria such as lipase, amylase, and protease contributed greatly in antioxidant activity [6]. The free radicals generated in the host body cell not only degrade the cellular integrity of the cell but also damage their functionality. Probiotics have proven positive in neutralizing these reactive oxygen species responsible for the damage of the cell. Many antioxidant scavenging assays by using *Lactobacillus* was demonstrated by a mechanism such as a hydroxyl radical and superoxide anions [7]. *Lactobacillus* is found with strong antioxidant abilities by neutralizing the free reactive oxygen radicals by various *in-vitro* studies [8]. It was also found that daily consumption of *Lactobacillus* yogurt improves the level of the plasma folate and vitamin B-12, with the enhancement of antioxidant properties which was demonstrated successfully in women with menopause conditions [9]. Glutathione (GSH) level was enhanced in the rats, relieving acute pancreatitis by consumption of *Lactobacillus* [10].

Cell-free extract of the *Lactobacillus* shown the maximum antioxidant activity than the whole organism [11].

Many researchers used fruit juices as natural media to enhance the growth and survival of these bacteria. The most important properties of *Lactobacillus* is its survival in the gastrointestinal tract so that pharmacological activities can be delivered to the host cells [12]. Various stress boosters present in the gut region of the host interact with the microbial flora affecting viability and survival. These stress conditions include the effects of different enzyme and enzymatic system [13]. The major other factors affecting the growth and survival of the probiotics are the bile salt secretion and the effects of the cellular immunity system inside the gut of the host. The stress-resistant ability of the LAB against the enzyme not only greatly affect the survival of *Lactobacillus* against the dysbiosis condition but also helps in maintaining the microbiota of the same. Similarly, the stomach duodenum passage is the area of higher lysozyme and bile salt hydrolase activity [14]. The probiotics abilities to by-passing these stress conditions and tolerance against gastrointestinal micro-environment contribute the desired pharmacological activities to the host.

The present study deals with the isolation of LAB from the sheep milk with the investigation of its antioxidant and antibacterial activities. Further, the stress tolerance abilities of isolated *Lactobacillus* strain is evaluated by *in-vitro* simulation studies.

Material and Methods

Isolation and culturing of *Lactobacillus rhamnosus* ARJD

The bacterial isolate, *Lactobacillus rhamnosus* ARJD (LAB) was isolated from sheep milk using selective MRS (De Man, Rogosa and Sharpe agar) media. It was incubation for at 37°C, for 24 hr in an anaerobic jar and was characterized for its morphological and biochemical identification as described by Fakruddin., *et al* [15]. After incubation, the selected colonies were transferred into MRS broth media at 37°C with an adjusted pH of 6.5 for 18 h. The freshly prepared culture of LAB with a cell count of 10^8 CFU/ml (colony forming unit), was centrifuged at 3000 rpm for 5 min at 4°C. The supernatant is separated and pellet formed was added to 0.5 ml of PBS and was used for further experiments. Carbohydrate utility of the isolate i.e. rhamnose, trehalose, galactose, lactose, maltose, ribose, mannitol, glucose, xylose, and sucrose, fructose) was determined as the methods described by Patil., *et al* [1].

Stress tolerance test of *L. rhamnosus* ARJD isolate

The pH tolerance of LAB was studied at variable pH range from 2 to 7 pH value until no CFU count was observed in MRS plate [1,15]. Gastrointestinal stress resistance in the form of bile salt tolerance was examined by the *in-vitro* method as described by Kim., *et al* [16]. Sodium chloride tolerance test was performed to demonstrate osmotic stress threshold by varying the concentration from 0 to 10% as described by Fakruddin., *et al* [15]. The *in-vitro* simulated gastric environment survival potential was determined in stress condition for a period of 360 min [2]. The thermo-stress analysis till the extreme temperature of 50^0C and time-dependent production of the organic acid was determined as demonstrated by Fietto., *et al* [17].

Probiotic nature determination

Cholesterol utility and assimilation test were carried according to Liong., *et al* [18]. Auto-aggregation ability was determined according to Balakrishna., *et al* [19]. Different activities of enzymes such as amylase, protease, etc., were carried out according to Kim., *et al* [16].

Preparation of cell-free extracts and lysate of *Lactobacillus*

The culture of LAB was incubated in MRS broth for 24 h at 37°C. The cell-free extracts were prepared by centrifugation of broth at 4°C for 5000 g [20]. Final content obtained was passed through the 0.2 µm microsyringe and was used for further experimentation [21].

Antibacterial activity

Agar disc diffusion method was carried out to study the antimicrobial activity as described by Kirby-Bauer., *et al* [22] using amoxicillin (30µg/ml) as standard drug. The Gram-positive and Gram-negative strains used for antibacterial activity were *B. cereus* ATCC 11774, *B. subtilis* 10876, *E. faecalis* 29212, *P. vulgaris* 13315, *E. coli* 25922, *P. aerugionsa* 27853, and *S. typhi* 65154. These ATCC strains were obtained from D. Y. Patil Medical College, Department of Microbiology, Kolhapur; India in order to carry the anti-microbial studies. Further, these bacterial strains were maintained at -80°C in soy broth and maintained in vials supplemented with the 20% glycerol. These bacterial suspensions were inoculated overnight in nutrient broth for 24 h at 37°C. Final inoculum suspension was prepared by standards of 0.5 Mcfarlands with a cell count of 108 CFU. In this technique, the suspension of bacteria was inoculated on Mueller- Hinton agar (MHA, Hi-media, India) and simultane-

ously implanted with 6 mm disc of cell-free extract, lysate of LAB and standard for 24 hr at 37^{0}C. The zone of inhibition in the form of diameter was measured using the vernier caliper.

Antioxidant and toxicity properties

The reducing power of isolate extracts with its autolysates was determined by the method described by Ding., *et al* [23]. The scavenging activity of the stable DPPH free radical was determined according to the method described by Fakruddin., *et al* [15]. The antioxidant activity by nitric oxide method was determined according to the method described by Rahman., *et al* [24]. The antioxidant study of hydroxyl radical scavenging activity was determined by the method described by Amaretti., *et al* [7].

An antioxidant study using Wister rat animal model

The animal model for antioxidant activity was approved from the Institutional animal ethical committee (IAEC) committee TKCP/13/2015 from TKCP, Warananagar, India. 10 Wistar rats aged 8–9 weeks were used for the acute toxicity study. These animals were categorized into two treatment groups designated as A and B (5 rats in each group). In order to study the toxicity profile of *Lactobacillus rhamnosus* ARJD, a single dose of 250 µl (~10^9 CFU) was administered by per oral route to each of the test group of rats using feeding needles. The rats of the control group received the sterile saline solution containing 5% of dextrose. Animals were monitored for about 15 days regularly to see any changes in activities, behavior and general health condition along with its weight [25]. Simultaneously the feces of rats were collected from time to time on day 0, 3, 6, 9 and 12 in order to enumerate the total LAB count using MRS media and count of Enterobacteria in MacConkey agar. Haematological studies with other vital organ analysis were carried out to determine the aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP) [8, 25]. The liver weight ratio and spleen weight index along with the percentage growth rate difference among both groups were evaluated and a significant difference among them was determined.

Statistical analysis

One way analysis of variance (ANOVA) with Tukey's multiple comparison tests was used to compare the results of the probiotic and control groups using Graph-pad prism 5.01 software, USA. Standard deviations and significant differences at *P-value < 0.05, ***P-value < 0.001 were presented.

Result and Discussion

Isolation, identification and preparation of *Lactobacillus rhamnosus*

The isolated strain initially are identified as Lactobacillus by morphological characteristics, showing the white and creamy texture of colony (Figure 1.A).

The isolate observed are rod-shaped with Gram-positive nature (Figure 1.B) and shows positive utilization of glucose, fructose, sucrose, maltose and trehalose with negative growth on lactose and xylose, rhamnose, raffinose, and arabinose. The isolate is identified as *Lactobacillus rhamnosus* by 16 S DNA analysis and was deposited in (National collection of Industrial Micro-organism) NCMR, Pune, India as *Lactobacillus rhamnosus* ARJD MCC 3594 and resisted as PYRN00000000-MCC 3594 in the gene bank. This strain is later maintained at -40^{o}C in MRS broth with 20% glycerol.

Figure 1: *Lactobacillus rhamnosus* with A) white pin-point, cream color colonies on MRS plate, and B) Gram-positive and rod shape appearance

Induced stress tolerance test

The isolate is found tolerant to various stress condition of pH, temperature as shown in figure 2 (A-D).

LAB shows the optimum growth conditions at 37°C and pH 5.6. The high rate of bile salt tolerance, NaCl, gastric juice and intestinal environment tolerance is observed in case of LAB as seen in figure 2 (F, C, E and B respectively). LAB has produced many organic acids (3.0% after 48 hours incubation) as seen in figure 3.

Figure 2: Gastrointestinal *in-vitro* stress tolerance exhibited by LAB at A) variable temperature, B) time-dependent intestinal environment condition, C) variable concentration of NaCl, D) change in pH condition, E) time dependant gastrointestinal condition and F) variable concentration of bile salt.

Figure 3: Quantification of organic acid production by LAB at a different time period.

Analysis of the probiotic characteristics

The given isolate of Lactobacillus rhamnosus shows various positive probiotics characteristics as in Table. 1. The isolate produced different enzymes such as protease and lipase. The isolate also assimilated the cholesterol and produced total glutathione with auto-aggregation abilities.

Property		***Lactobacillus isolate***
Enzyme activity assay	lipase	60 unit/g cell
	protease	1800 unit/g cell
Total glutathione		4.2 mg/100 ml
Production of siderophore		+
Auto-aggregation ability		51.11%

Table 1: Probiotics properties and its quantification.

Determination of the antibacterial activities

Antibacterial activity of isolate containing whole cells with its supernatant and cell lysate is shown in Table 2.

Test organism nature		ATCC code	Zone diameter (mm)			
			Lactobacillus		Doxycycline	
			Whole cell	Supernatant	Lysate	
Gram positive	*B. subtilis*	11774	7.4	6.8	7.8	21
	B. cereus	10876	5.4	5.2	6.0	17
	E. faecalis	29212	8.2	7.8	9.2	14
Gram negative	*P. vulgaris*	13315	8.4	8.2	9.4	21
	E. coli	25922	8.6	7.2	8.8	16
	P. aeruginosa	27853	11.4	10.4	12.4	22
	S. typhi	65154	10.8	9.8	11.4	24

Table 2: Antibacterial activity of LAB against Gram-positive and Gram-negative bacteria.

Comparing with amoxicillin (30 µg/disc), the isolate LAB shows moderate antibacterial activity. In general, cell lysate shows more anti-bacterial activity as compared to whole cells. Most advantageous part observed in the case of a LAB, its antibacterial property against both Gram-positive and Gram-negative bacteria. Cell lysate acts as a best antimicrobial agent as compared to the supernatant and whole cell. This shows that cells after disintegration inside host gastrointestinal tract release endogenous enzymes that later get assimilated in the blood showing antibacterial characteristics. Thus, the isolate LAB inhibited bacterial growth of *B. cereus, B. subtilis, B. E. faecalis, P. vulgaris, E. coli, P. aerugionsa*, and *S. typhi* demonstrated by *in-vitro* studies.

Determination of the antioxidant activity

The isolate demonstrated different antioxidant activities as seen in figure 4. The LAB isolate showed significant nitric oxide scavenging and hydroxyl radical scavenging activity compared with ascorbic acid as seen in figure 4 (A and B). Similar antioxidant results were seen in case of the different assay such as DPPH scavenging activities and reducing power activity as seen in figure 4 (C and D).

The DDPH, hydroxyl radical scavenging and nitric oxide scavenging assay at concentration 950 µg/ml of LAB have shown maximum scavenging abilities as compared to the ascorbic acid. Similarly, optimal reducing abilities of LAB are observed at the concentration of 600 µg/ml.

Figure 4: *In-vitro* antioxidant assay A) Nitric oxide scavenging, B) Hydroxyl radical scavenging, C) DPPH assay and D) Reducing activity assay.

Acute animal toxicity study and *in-vivo* antioxidant activity

The health status is observed in case of rats fed with LAB, which not shown any significant difference with the control group in terms of growth rate and body weight. LAB content in log CFU count is observed significantly ($P < 0.001$) higher in the treated group in the fecal matter as compared to the control group which is observed till the period of 12 days at a regular interval of 3 days as seen in figure 5. The study demonstrated that LAB becomes the part of the host gastrointestinal tract which was evaluated by its appearance in the fecal matter without altering the count of Enterobacteria in the test group (Figure 5).

Figure 5: The fecal microbial load of *Enterobacteria* and *Lactobacillus* observed in a rat model.

AST level observed in case of LAB treated group is highly significantly ($P < 0.001$) as compared to the control group. While ALT level observed in case of LAB treated group is statistically significantly ($P < 0.05$) as compared to the control group as seen in figure 6 (A). No, any significant difference was observed in both groups in case of ALP values. The liver weight ratio and spleen weight index observed in the case of both groups shows no significant differences. No, any diarrhoeal mortality is observed in the case of both treated and control groups seen as in figure 6 (B).

After 12 days post ingestion studies no post diarrhoeal mortality or morbidity are observed in case of LAB treated group. AST and ALT level generally get increased in the host, due to any cellular injury or disease condition. While the increase in ALT level specifically defines hepatic cellular damage. Blood sample analysis shows that LAB treated group is with significant minimal AST ($P < 0.001$) and ALT ($P < 0.05$) level as compared to the control group as in figure 6 (A). While the ALP level observed in both treated and untreated group did not differ significantly ($P < 0.05$). This result shows that the LAB does not develop any oral toxicity in LAB treated group. There is a significant difference ($P < 0.001$) of LAB content in the fecal matter of LAB treated the group as compared to enterobacteria. The treated group shows a significant increase in LAB content in the fecal matter as compared to enterobacteria. This shows that the LAB is inhabiting in all part of large intestine including the colon. There was no significant difference observed in case of both treated and untreated groups in term of percentage growth rate, liver weight ratios, and spleen weight index. This indicates that the LAB is non-invasive and precipitate any systemic infection in the host.

Figure 6: Marker studies observed in a rat model with A) Liver marker study involving AST, ALP and ALT analysis, along with B) Growth rate, spleen weight index and liver weight index analysis.

Conclusion

The isolated strain *Lactobacillus rhamnosus* ARJD observed as an ideal probiotic candidature with strong antioxidant and antimicrobial activities. Thus, the given isolate is found to be non-toxic with non-invasive nature inside the host by the preliminary investigation. This probiotics strain also possesses great gastrointestinal stress tolerance abilities with long resident abilities in the host gastrointestinal tract.

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