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Probiotic Potential of *Lactobacillus plantarum* with the Antioxidant Properties

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Abstract
The use of microbes as functional food maintains health and prevent many disease and disorder. The isolation and research of new strains of probiotic strains especially lactobacilli proven to be useful to satisfy the increasing demand of the population. In the current study, the probiotic potential of *Lactobacillus* strains isolated from sheep milk was investigated. The whole genomic sequence proved the strain as *Lactobacillus plantarum* and same was deposited with the accession number as PYRN00000000 in world genomic bank. The Lactobacillus strains were identified and evaluated for tolerance against gastric acidity and bile toxicity, along with the antioxidant properties and antimicrobial activities. Survival of the strains was evaluated by animal model through the host intestine examination by the 12-week Wister rat feeding and fecal analysis trial. The *in-vivo* trials not only proved the antioxidant activity but also the survival of the *Lactobacillus plantarum* inside the intestinal lumen of the host. Thus, the isolated strain can act as functional food by further clinical investigation.

Keywords
Lactobacillus, antioxidant properties, antimicrobial activity, functional food.

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INTRODUCTION:
Probiotics are belonged to the category of the organism which provides the health benefit to the end user. [1] This organism have different properties which consist of muco-adhesion [2], bile tolerance and antimicrobial activity [3] and should not affect the host immunology with non-pathogenic abilities. [4, 5] Microorganisms which fall under probiotics category are lactobacillus, bacilli, bifidobacteria and other lactic acid bacteria's. [6] *Lactobacillus* are found in various dairy and non-dairy products such as cheese, butter etc. These microbes not only provide nutrition but also provides health benefits to the host. [7-13] Nowadays lactobacillus is considered as a part of a functional food with the ability to treat many disorders of the body. [1, 23, 24] This antioxidant activity of lactobacillus is found to cure dreadful diseases like cancer and degenerative disorders. [1, 34, 35] Several studies proved that *Lactobacillus* species act as a potent antioxidant and anti-bacterial agent. The current study deals with the discovery of a new strain of lactobacillus and evaluation of the various probiotics properties.

MATERIAL AND METHODS:

Isolation and identification of *Lactobacillus plantarum JDARSH:*

The bacterial isolates, *Lactobacillus plantarum JDARSH* (LAB) was isolated from sheep milk and it was characterized for its morphological and biochemical identification as mentioned by Fakruddin et al. [16] Carbohydrate utility of the isolate i.e. rhamnose, trehalose, galactose, lactose, maltose, ribose, mannitol, glucose, xylose, and sucrose, fructose) was determined according to Forouhandeh et al. [17] Identification of the phylogenetic relationship was determined by analysis of the variable region of the lactobacillus 5.8S rDNA gene as described by Fakruddin et al. [16]

Stress tolerance test of *L. plantarum JDARSH* isolate:

The pH tolerance was studied according to Fakruddin et al. [21] Bile salt tolerance of the isolates was examined as described by Kim et al. [22] Sodium chloride tolerance test was performed as described by Fakruddin et al. [18] The simulated gastric environment in vitro survival potential of the bacterial isolates (aqueous solution containing 3 g/l pepsin, and 5 g/l NaCl, pH 1.8) was determined according to Fietto et al. [20] Thermotolerance of the bacterial strains was studied according to Fakruddin et al. [14] Organic acid production was performed as described by Chowdhury et al. [4]

Probiotic nature determination:

Cholesterol utility and assimilation test were carried out as per Liong and Shah. [25] Auto-aggregation ability was determined as per Syal and Vohra. [26] Different activities of enzymes such as amylase, protease, total glutathione etc. were carried out according to Kim et al. [22]

Preparation of Lactobacillus extracts and its autolysates:

Bacterial extracts from the isolated strains were prepared as per Ali et al. [31] and further was autolysated by the method described by Hassan [29].

Antibacterial activity:

Anti-bacterial activity of the whole cell and its supernatant was performed by agar overlay method and well diffusion method respectively. [32, 33]

Antioxidant and toxicity properties:

The reducing power of isolate extracts with its autolysates was determined by the method of Mathew and Abraham. [36] The scavenging activity of the stable DPPH free radical was determined by the method described by Fakruddin et al. [37] The antioxidant activity as by nitric oxide method was carried out as described by Kumaran and Karunakaran. [35] The antioxidant activity as by hydroxyl radical scavenging activity was determined by the method described by Nagai et al. [38]

Acute toxicity study using an animal model along with haematological studies:

Ten Wister rat aged 8–9 weeks were used for the acute toxicity study, as per the Institutional animal ethical committee guidelines TKCP/13/2017. This animal was categorized into two treatment groups designated as A and B (5 rats in each group). In order to study the toxicity profile of Lactobacillus plantarum JDARSH a single dose of 250 µl (~109 cfu) was administered by per oral route to each of the test group of the rat using feeding needles. [39, 40] Rat 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. [41, 42] Simultaneously the faeces of the rat were collected from time to time on day 0, 3, 6, 9 12 and 15 to enumerate the total Lactobacillus count using MRS media. Similarly, MacConkey was used to analyze the cfu count of enterobacteria from faeces of the rat. Haematological studies with other vital organ analysis were carried out to determine the aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP) along with the liver marker estimation and different body index parameters such as the weight of liver, spleen etc., according to Kantachote et al. [43]

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 GraphPad Prism 5.01 software, USA. Standard deviations and significant differences at *P-value < 0.05, ***P-value < 0.001 were presented.

RESULTS

Isolation, identification, and preparation of Lactobacillus plantarum:

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

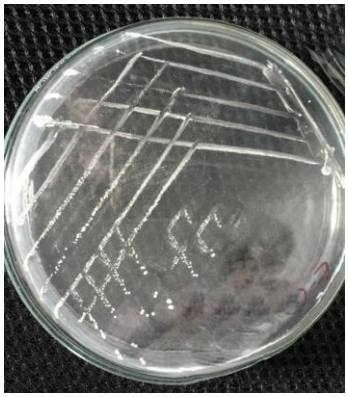

Figure 1: A white creamy colony of *Lactobacillus* on MRS agar

The whole genomic sequence proved the strain as Lactobacillus plantarum and same was deposited with the accession number as PYRN00000000 in world genomic bank. The isolate observed was rod-shaped which was gram-positive and showing positive utilization of glucose, fructose, sucrose, maltose and trehalose with negative growth on lactose and xylose, rhamnose, raffinose, and arabinose. [46] The isolate was identified as Lactobacillus plantarum by 16 S DNA analysis. This strain was later maintained at -40 OC in MRS broth with 20% glycerol. The working culture was retrieved successfully in MRS broth for 37 OC for 24 hours incubation. The phylogenetic relationship was found a close resemblance with the other L. plantarum species along with the E. coli and S. aureus (Figure 2).

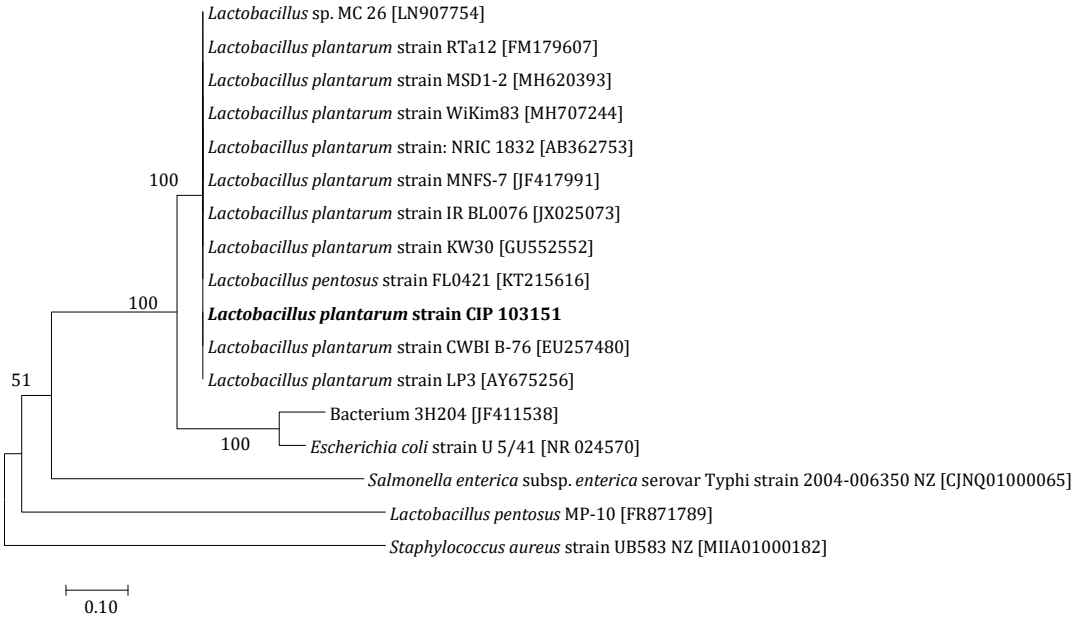

Figure 2: Phylogenetic relationship analysis

Induced stress tolerance test:

The isolate was tolerance to various stress condition of pH, temperature as shown in figure 3. It has shown the optimum growth conditions at 37 °C and pH 5.6. It has shown the high rate of bile salt tolerance, NaCl, and gastric juice tolerance. It has produced many organic acids (3.0% after 48 hours incubation).

Probiotic characteristics:

The given isolate of Lactobacillus plantarum shown various positive probiotics characteristics as in Table. 1. The isolate produced different enzymes such as protease (1800 unit/g cell), and lipase (60 unit/g cell). The isolate also assimilated of about 21% of cholesterol and produced 4.2 mg/100 ml total glutathione with 51.11% auto-aggregation abilities (Table 1).

Table 1. Probiotics properties of the Lactobacillus species

Property		Lactobacillus isolate
Cholesterol assimilation		21 %
Enzyme activity assay	lipase	60 unit/g cell
	protease	1800 unit/g cell
Total glutathione		4.2 mg/100 ml
Auto-aggregation ability		51.11%

Antibacterial activities:

Antibacterial activity of isolate containing whole cells with its supernatant and cell lysate is shown in Table 2. Comparing with amoxicillin (30 µg/disc), the isolate Lactobacillus showed moderate antibacterial activity. In general, cell lysate showed more anti-bacterial activity as compared to whole cells. Anti-bacterial activities were performed in case of both gram-positive and negative pathogens in which it was observed that gram-negative pathogens were more inhibited as compared to gram-positive by the isolated Lactobacillus.

Table 2. Zone of inhibition studies of the whole cell, supernatant, and lysate of Lactobacillus species

Test organism nature		ATCC code	Zone diameter (mm)			
			Lactobacillus			Amoxicillin
			Whole cell	Supernatant	Lysate	
Gram positive	B. subtilis	11774	7.4 ± 0.5*	6.8 ± 0.8	7.8 ± 0.7	21 ± 0.3*
	B. cereus	10876	5.4 ± 0.4*	5.2 ± 0.5	6.0 ± 0.9*	17 ± 0.4*
	E. faecalis	29212	8.2 ± 0.6	7.8 ± 0.5	9.2 ± 0.5*	14 ± 0.6*
Gram negative	P. vulgaris	13315	8.4 ± 0.8*	8.2 ± 0.9*	9.4 ± 0.6*	21 ± 0.5*
	E. coli	25922	8.6 ± 0.6*	7.2 ± 0.5	8.8 ± 0.4*	16 ± 0.4*
	P. aeruginosa	27853	11.4 ± 0.5	10.4 ± 0.2	12.4 ± 0.5*	22 ± 0.9*
	S. typhi	65154	10.8 ± 0.8	9.8 ± 0.5	11.4 ± 0.5*	24 ± 0.7*

Values are mean ± SD; (n=3), *P< 0.05

Antioxidant activity of Lactobacillus species:

The isolate showed different antioxidant activity as in Figure 4. The Lactobacillus isolate showed significant nitric oxide scavenging and hydroxyl radical scavenging activity compared with ascorbic acid. Similar antioxidant results were seen in case of the different assay such as DPPH scavenging activities and reducing power activity. The isolate is also proved as a strong antioxidant agent, especially observed by DPPH assay. It has shown good reducing power, hydroxyl radical scavenging, and nitric oxide activities. 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 was observed at the concentration of 600 µg/ ml.

Animal acute toxicity study of Lactobacillus species:

The health status was observed in the case of rat fed with Lactobacillus, which not shows any significant difference with the control group in terms of growth rate. Lactobacillus fecal matter content in log CFU count was observed significantly ($P < 0.001$) higher in the treated group as compared to the control group (Figure 5). These results were evaluated for the period of 15 days studies at a regular interval of 3 days. Liver markers such as AST level observed in case of LAB treated group was lower significantly ($P < 0.001$) as compared to control group (Figure 6). While ALT and ALP level observed in case of LAB treated group was statistically non significantly ($P < 0.05$) as compared to control group (Figure 6).

The index parameters such as the liver weight ratio and spleen weight index observed in the case of both groups show no significant differences (Figure 7). No, any diarrhoeal mortality was observed in the case of both isolates treated and control groups. This proves that the LAB is inhibiting in all part of large intestine including the colon.

There was no significant difference observed in the 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.

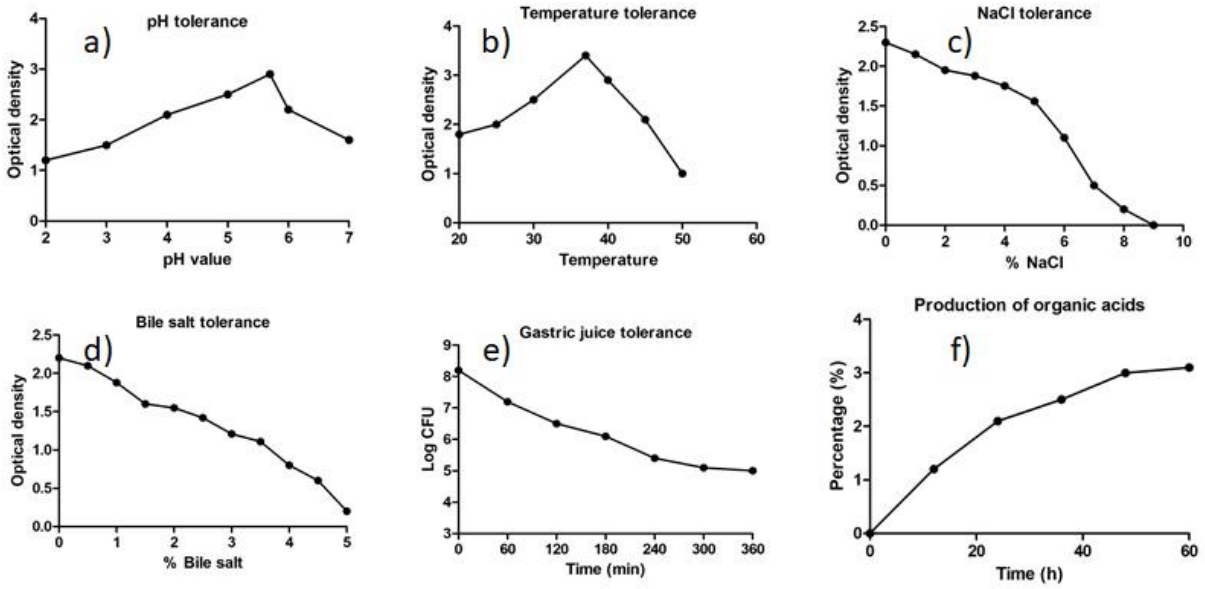

Figure 3: Stress tolerance test of a) pH, b) temperature, c) NaCl, d) Bile salt, e) Gastric juice and f) organic acid production respectively.

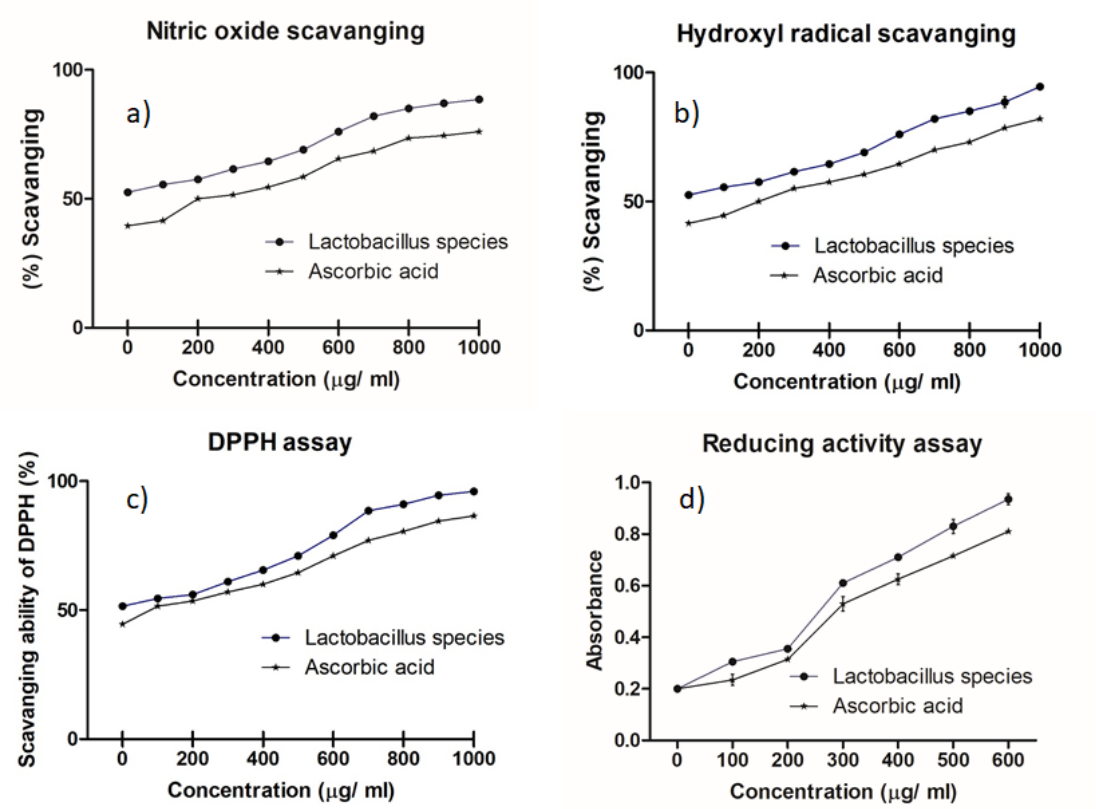

Figure 4: Antioxidant activity of Lactobacilli species with a) Nitric oxide scavenging, b) Hydroxyl radical scavenging, c) DPPH and d) Reducing activity assays respectively.

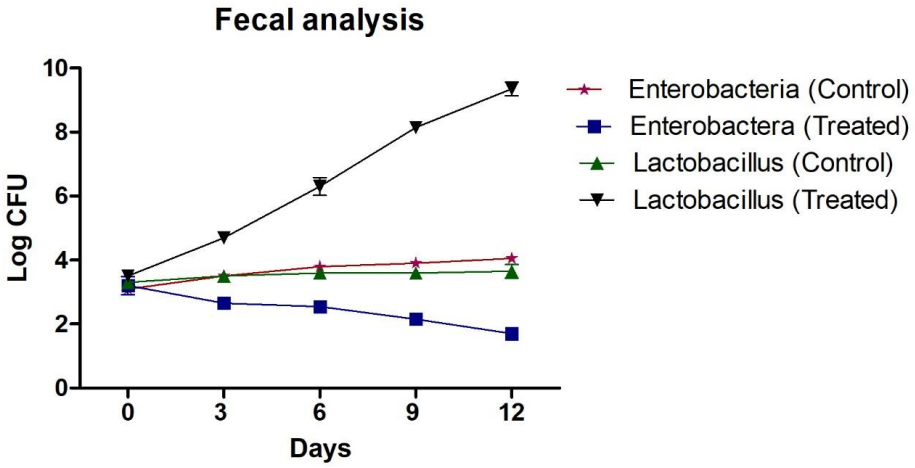

Figure 5: Analysis of fecal content of lactobacilli in the animal model

No, any significant difference was observed in both groups in the case of ALP values.

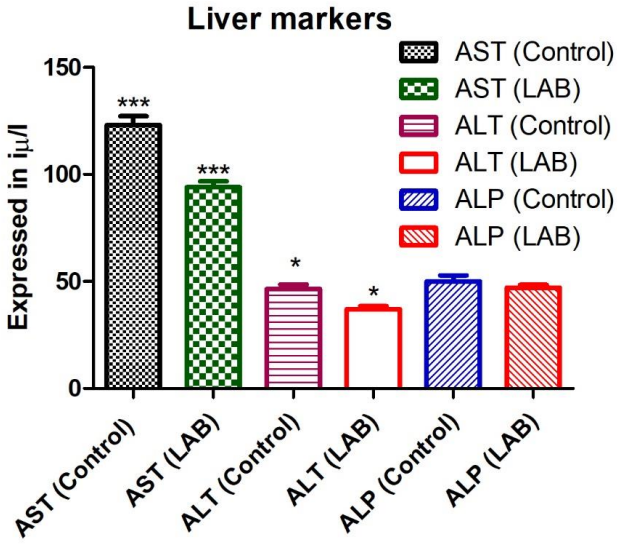

Figure 6: Analysis of the effect of *Lactobacillus* on liver markers compared with the control group

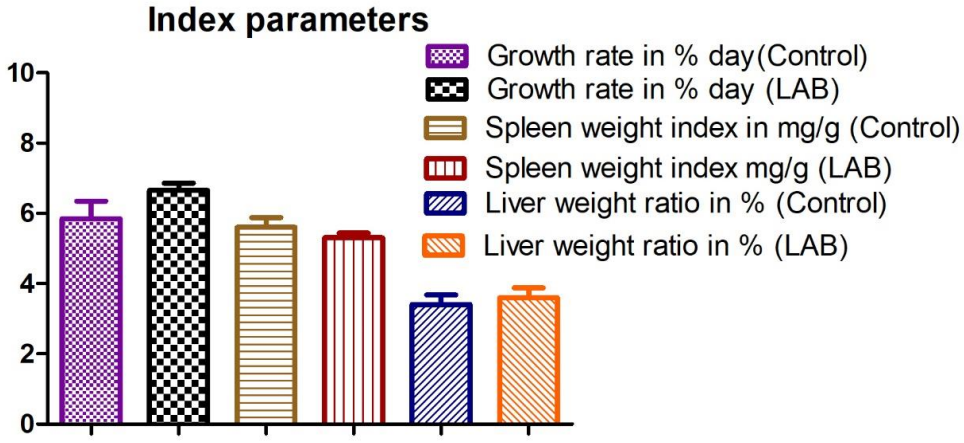

Figure 7: Analysis of the effect of *Lactobacillus* on different index parameters compared with the control group

DISCUSSION:

The isolated strains from sheep milk were identified as lactobacillus by the morphological colony forming studies and identified as Lactobacillus plantarum by 16 S DNA analysis. The phylogenetic analysis proved L. plantarum has close resemblance with the other lactobacillus species. The L. plantarum proved to be resistance against the bile salt NaCl, and gastric juice with the production of the organic acids differentiating this strain with other existing lactobacillus species. The isolated strain showed various probiotics properties such as cholesterol assimilation (21%), lipase (60 unit/g cell) and protease (1800 unit/g cell) activity with total glutathione activity around 4.2 mg/100 ml and auto-aggregation ability around 51.11%. Further, the antimicrobial activity was screened against amoxicillin as the standard in which the whole cell of L. plantarum proved more effective against B. subtilis, B. cereus, E. faecalis as Gram positive organisms and P. vulgaris, E. coli, P. aeruginosa as Gram-negative organisms as compared to the supernatant. While L. plantarum as lysate proved more effective against B. subtilis, B. cereus, E. faecalis as Gram-positive organisms and P. vulgaris, E. coli, P. aeruginosa as Gram-negative organisms as compared to the supernatant. L. plantarum also proved as strong antioxidant agent evaluated by nitric oxide scavenging, hydroxyl radical scavenging, DPPH scavenging and reducing scavenging activity as compared to ascorbic acid as the reference standard.

The acute toxicity studies proved L. plantarum as non-toxic without any diarrhoeal mortality. Further, this probiotic strain also replaced the enterobacteria from the gastrointestinal tract of Wister rat demonstrated successfully by the fecal matter analysis. The liver marker studies such as AST and ALP did not show any significant difference with the control group proved that lactobacillus not interfere the normal functionality of the host (rat) liver system and not precipitated any hepatic cellular toxicity. While AST level was significantly lower than the control group which proved that lactobacillus relax the any cellular stress level if or elevated. No, any changes were found in percentage growth rate, liver weight ratios, and spleen weight index between control and lactobacillus treated group proving lactobacillus as nutraceutical agent.

CONCLUSION:

The isolated strain Lactobacillus plantarum JDARSH, proven as ideal probiotic candidature with strong antioxidant and antimicrobial activities. Thus, the given isolate is non-toxic with non-invasive nature inside the host. It also possesses great gastrointestinal stress tolerance abilities with long resident potential evaluated by Wister rat antioxidant model studies.

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