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Assessment on *in vitro* probiotic attributes of *Lactobacillus plantarum* isolated from horse feces

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Abstract

This study was designed to assess *in vitro* probiotic attributes of potent bacterium isolated from the feces of healthy horse. Initially, a total of 8 bacteria were isolated from the feces and evaluated their antibacterial activities against indicator bacterial pathogens using agar well diffusion assay. Results showed significant (P<0.05) antibacterial property of *Lactobacillus plantarum* strain LF4 against pathogens tested with maximum growth inhibitory activity of 320.16±3.4 AU/mL against *Staphylococcus aureus*. Further, *in vitro* probiotic properties of strain LF4 were determined using standard methodologies. Strain LF4 maintained its viability towards acidic condition (pH 2.0) and simulated gastric juice (pH 2.0) with total cell counts of 1.6±0.18 and 1.7±0.18 log cfu/mL, respectively. Moreover, the strain was observed resistant to oxgall (0.5% w/v) up to 36 h. The isolate showed significant (P<0.05) hydrophobicity property (60.3±1.6%), auto-aggregation trait (41.31±1.5%), and moderate proteolytic activity. Strain LF4 revealed significant (P<0.05) rate of DPPH scavenging (15.3±1.3-69.7±1.3%) and hydroxyl radical scavenging (11.3±1.3 to 56.4±1.3%) in a concentration dependent manner. Additionally, the isolate was observed susceptible to all the conventional antibiotics tested, thereby indicating its safer utilization. In conclusion, findings suggested the colossal applications of *L. plantarum* strain LF4 as an ideal probiotic bacterium in equine industries.

Keywords: Feces; Horse; *Lactobacillus plantarum*; Probiotic properties

1. Introduction

Probiotics are live microbial community which when administered in adequate doses confer health benefits to the host [1]. The selection of potential microbes with desirable characteristics is crucial in the development of probiotics. Resistance towards acidic environment, tolerance to bile, production of antimicrobial components, exhibition of antioxidant traits, cell surface hydrophobicity and auto-aggregation characteristics, sensitivity to antibiotics, and secretion of hydrolytic enzymes are some of the common desirable attributes of probiotics [2]. In this regard, *Lactobacillus* spp., *Bifidobacterium* spp., *Pediococcus* spp., *Bacillus* spp., *Enterococcus* spp., coagulase-negative *Staphylococcus* spp., and *Saccharomyces* spp., isolated from distinct sources have been identified as potent probiotic microorganisms in the past [3-5]. However, isolation of new strain of probiotic bacteria with high efficacy and extensive applications from disparate resources still requires desperate investigation.

In the recent years, isolation of probiotics from unconventional sources for disparate therapeutic and industrial applications has increased. The intestinal tract of horses is considered an unconventional hub of unique and diversified ranges of microbiota, including bacteria, fungi, and protozoa [6]. These intestinal microbial communities, particularly probiotic bacteria exhibit colossal effects on the health and growth performances of horses [7]. In addition, these microorganisms provide substantial amount of daily energy requirements to the horses by fermenting feeds into short-chain fatty acids [8].

Horses are sensitive to the alterations in the diets, thereby causing disturbances in the fermentative microbes of the large intestine [9, 10]. Every horse consists of unique category of probiotics which generally affect the immunity and metabolic processes. The intestine of

each horse is dominated by bacteria belonging to the phylum Firmicutes, as identified in the feces of horses [11]. Although, the presence of distinct microbiota in the feces of animals has been reported earlier, but investigating the desirable functional characteristics of single species of bacteria present in animal's feces is very limited. In view of this, this study was investigated to isolate new strain of bacteria from the feces of horse and assess its *in vitro* probiotic properties for its extensive roles in equine industries.

2. Materials and methods

2.1. Collection of feces sample

Feces were collected from the stable in the early morning by spreading a clean sheet close to the standing horse. A small quantity of the collected feces was transferred into a sterile collecting tube and brought to the laboratory. Samples were stored at room temperature for further experimental purposes.

2.2. Bacterial isolation

One gram of the collected feces was added in 2 mL of phosphate buffered saline (PBS; pH 6.8) and mixed homogeneously. The mixture was centrifuged at 2500 *g* for 10 min for excluding the heavy constituents and the supernatant was collected in a sterile tube. The collected supernatant was serially diluted and 0.1 mL of the suspension was spread onto sterile De Man Rogose Sharpe (MRS) agar medium (HiMedia, India) plates aseptically. Plates were incubated at 30°C for 48 h and observed for the appearance of different colonies. Pure bacterial cultures of the selected colonies were prepared by quadrant streaking on newly prepared MRS agar medium plates. Pure culture of each isolate was stored at 4°C for further experiments.

2.3. Antibacterial activities of isolates

Each isolate was sub-cultured in freshly prepared MRS broth medium under aseptic conditions and incubated at 30°C for 48 h at 130 rpm in a rotatory shaker. After required incubation period, each culture was centrifuged at 8000 *g* for 10 min. The collected cell-free supernatant from each isolate was filtered and neutralized using 1N sodium hydroxide solution. Further, the cell-free neutralized supernatant (CFNS) of each isolate was treated with catalase at 37°C for 2 h in order to eliminate the antibacterial trait of hydrogen peroxide. Meanwhile, indicator pathogens such as *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Micrococcus luteus*, *Bacillus subtilis*, *Staphylococcus saprophyticus*, and *Proteus vulgaris* were grown in Tryptone Soya broth (g/L: pancreatic digest of casein – 17.0, papaic digest of soyabean meal – 3.0, sodium chloride – 5.0, dextrose – 2.5, dibasic potassium phosphate – 2.5, and pH – 7.2) medium and incubated at 37°C for 24 h. After required incubation period, indicator bacterial pathogens were swabbed onto sterile Mueller Hinton agar (g/L: acid hydrolysate of casein – 17.5, beef extract – 2.0, starch – 1.5, agar – 18.0, and pH – 7.2) medium plates. Antibacterial properties of the CFNS of each isolate and streptomycin (10 µg; positive control) were carried out using well diffusion method, and results were expressed in arbitrary units (AU/mL) [12].

2.4. Identification of potential isolate

The isolate revealing promising antibacterial activity was identified using various standard biochemical tests such as gram staining, indole, methyl red, voges-proskauer, citrate utilization, ONPG, nitrate reductase, arginine, and malonate. Further, the isolate was subjected to molecular characterization test by amplifying its genomic DNA using polymerase chain reaction method with universal primers. The 16S rRNA sequences of the isolate were further deposited into GenBank for assigning the accession number.

2.5. Probiotic characteristics of potent isolate–

2.5.1. Resistance to acidic pH

The ability of the selected isolate to tolerate acidic pH was determined as per the modified methodology of Ramos et al. [13]. The selected isolate was grown in MRS broth up to log phase at 30°C and then centrifuged at 6000 *g* at 4°C for 15 min. The pellet obtained was further mixed in sterile distilled water and mixed homogenously. Meanwhile, fresh MRS broth media were prepared aseptically and its pH was adjusted from 6.0 to 2.0. The broth medium of pH 6.5 represents control medium. The culture was re-suspended in MRS broth of different pH ranges and incubated at 30°C up to 3 h. Serial dilution of the suspension was performed using PBS and plated on sterile MRS agar medium plates. Plates were incubated at 30°C for 48 h and the viability (log cfu/mL) was calculated.

2.5.2. Simulated gastric juice resistivity

The resistance trait of isolate towards simulated gastric juice was assessed according to the modified protocol of Charteris et al. [14]. Simulated gastric juice of pH 2.0-4.0 was prepared using pepsin (3 mg/mL) and sodium chloride (0.5% w/v) solution. The isolate was grown up to log phase and centrifuged at 6000 *g* for 15 min. The obtained pellet or cells were washed with 10 mL of K_2HPO_4 solution (50mM), and re-suspended in 3 mL of K_2HPO_4 solution of similar molarity. The prepared simulated gastric juice was added into the cell suspension and incubated at 30°C for 3 h. The suspension was plated on sterile MRS agar medium plates. Plates were incubated at 30°C for 48 h and the viability (log cfu/mL) was calculated.

2.5.3. Bile salt resistance

Bile salt resistance potency of the isolate was estimated according to the method of Aarti and Khusro [2]. The log phase grown isolate was inoculated into sterile MRS broth medium constituting 0.5% w/v oxgall. The culture was incubated at 30°C for 72 h and

aliquots of the suspension were withdrawn at regular interval. The viability was calculated against the control culture (without oxgall) by reading absorbance at 600 nm.

2.5.4. Cell surface hydrophobicity and auto-aggregation

The adherence properties of isolate towards different hydrocarbons (chloroform, toluene, and ethyl acetate) were determined according to the method of Khusro et al. [12]. The percentage (%) cell surface hydrophobicity was estimated as:

$$\% \text{ Hydrophobicity} = [(\text{Absorbance}_{initial} - \text{Absorbance}_{final})/\text{Absorbance}_{initial}] \times 100$$

The cellular auto-aggregation trait of the isolate was assessed as per the method of Khusro et al. [12]. The auto-aggregation property was estimated as mentioned below:

$$\% \text{ Auto-aggregation} = [(\text{Absorbance at 1-3 h} - \text{Absorbance at } 0^{th} \text{ h})/\text{Absorbance at 1-3 h}] \times 100$$

2.5.5. Protease activity

The isolate was grown in MRS broth medium and incubated up to the log phase. After required incubation period, the supernatant was collected by centrifuging the culture at 8000 *g* for 15 min at 4°C. Meanwhile, skim milk agar medium (% w/v: skim milk 1.0 and agar 1.8) plate was prepared and cooled under aseptic condition. The skim milk agar medium was punched using sterile cork borer for preparing wells and the collected supernatant was added into the well. The plate was incubated at 30°C for 24 h and protease production was observed in terms of zone of hydrolysis [15].

2.5.6. Antioxidant properties-

2.5.6.1. DPPH (2,2-Diphenyl-1-picrylhydrazyl) degradation

The DPPH free radical scavenging potential of the isolate (100-1000 µL) was evaluated using ascorbic acid as standard according to the method of Khusro et al. [12]. The DPPH degradation potency was estimated as:

171 $$\text{DPPH scavenging (\%)} = [(A_{sample} - A_{blank}) / A_{control}] \times 100$$

172 ***2.5.6.2. Hydroxyl radical scavenging***

173 The hydroxyl radical scavenging trait of the isolate (100-1000 µL) was depicted using
174 ascorbic acid as standard according to the method of Khusro et al. [12]. The hydroxyl radical
175 scavenging property was determined as:

176 $$\text{Hydroxyl radical scavenging (\%)} = [(A_1 - A_0) / (A - A_0)] \times 100$$

177 where, A_1 = absorbance of sample, A_0 = absorbance of control, and A = absorbance without
178 the sample and the Fenton reaction system.

179 ***2.5.7. Antibiotics sensitivity test***

180 The sensitivity of isolate towards different antibiotics was analyzed according to the
181 method of Salem et al. [16].

182 ***2.6. Statistical analysis***

183 Experiments were performed in triplicate and values were expressed as
184 mean±standard deviations (mean±SD). Data were tested using one way ANOVA and value
185 $P \leq 0.05$ was considered significant.

186 **3. Results**

187 ***3.1. Antibacterial activities***

188 Of 8 bacteria isolated from the feces of horse, isolate LF4 exhibited maximum
189 antibacterial activity of 320.16±3.4 AU/mL against *S. aureus*, followed by *S. saprophyticus*
190 (310.33±3.3 AU/mL), *S. epidermidis* (300.33±3.8 AU/mL), *B. subtilis* (200.63±2.8 AU/mL),
191 *M. luteus* (180.36±2.1 AU/mL), and *P. vulgaris* (140.36±2.AU/mL). Isolate LF3 showed
192 comparatively lower antibacterial activities against pathogens in the order of *S. saprophyticus*
193 (308.66±4.1 AU/mL) > *S. aureus* (300.43±4.6 AU/mL) > *S. epidermidis* (280.36±3.6
194 AU/mL) > *B. subtilis* (188.64±2.3 AU/mL) > *M. luteus* (160.66±2.3 AU/mL) > *P. vulgaris*

(135.65±3.5 AU/mL). On the other hand, isolate LF1, LF2, LF5, and LF8 showed significantly (P<0.05) lower antibacterial activities with respect to the isolate LF3 and LF4. Isolate LF6 and LF7 depicted lack of antibacterial activities against all the pathogens tested. The antibacterial potency of streptomycin was estimated significantly (P<0.05) higher than that of all the isolates against respective pathogens, ranging from 167.68±2.8-420.34±2.3 AU/mL (Table 1).

3.2. Identification of potent isolate

Based on the antibacterial activities results, isolate LF4 was selected and identified using standard biochemical tests and molecular techniques. The colonies of isolate LF4 grown on MRS agar medium were small, smooth, round, and creamy white in colour. Gram staining results indicated gram positive and rod-shaped morphology of bacteria. Biochemical tests showed negative results for certain biochemical tests viz. indole, methyl red, voges-proskauer. citrate utilization, arginine, and malonate tests. In contrary, the isolate showed positive results for ONPG and nitrate reductase tests (figure not shown). The 16S rRNA sequencing and BLAST, NCBI search results revealed similarity of the isolate with *Lactobacillus plantarum*, and thus, identified as *L. plantarum* strain LF4 (Accession number – MT488481).

3.3. Resistance to acidic conditions and bile salt

The growth characteristic of strain LF4 at different acidic pHs is shown in Fig. 1a. The isolate exhibited significant (P<0.05) reduction in its viability from 8.2±0.18 log cfu/mL (pH 6.5) to 1.6±0.18 log cfu/mL (pH 2.0). However, no significant differences in the viability of strain LF4 was observed at pH 6.5 (control; 8.2±0.18 log cfu/mL) and pH 6.0 (7.7±0.16 log cfu/mL). Likewise, strain LF4 exhibited resistivity towards simulated gastric juice with significant (P<0.05) viabilities of 4.2±0.18, 3.1±0.17, and 1.7±0.18 log cfu/mL at pH 4.0, 3.0,

and 2.0, respectively (Fig. 1b). Furthermore, the strain was observed resistant to oxgall (0.5% w/v) up to 36 h. A further increase in the incubation period caused significant reduction in the absorbance values (Fig. 1c).

3.4. Adhesion, auto-aggregation traits, and proteolytic activity

Strain LF4 showed significantly (P<0.05) potential hydrophobicity trait towards toluene (60.3±1.6%), followed by chloroform (41.6±1.5%) and ethyl acetate (36.2±1.5%) (Fig. 2a). Similarly, strain LF4 exhibited significant (P<0.05) auto-aggregation characteristics of 30.25±1.6, 41.31±1.5, and 36.64±1.6% at 24, 48, and 72 h, respectively (Fig. 2b). Strain LF4 revealed proteolytic property by showing moderate level of zone of hydrolysis on agar medium containing skim milk as substrate (figure not shown).

3.5. Antioxidant properties

Strain LF4 showed significant (P<0.05) DPPH scavenging rate of 15.3±1.3-69.7±1.3% at varied concentrations (100-1000 µL). Likewise, the strain depicted significant (P<0.05) rate of hydroxyl radical scavenging, ranging from 11.3±1.3 to 56.4±1.3%. Ascorbic acid showed higher rate of antioxidant activities at all concentrations as compared to the strain LF4 (Table 2).

3.6. Antibiotic sensitivity test

Strain LF4 was observed sensitive to all the tested antibiotics with maximum and minimum zone of inhibition of 32.6±0.6 and 18.3±0.6 mm against penicillin G and streptomycin, respectively (Fig. 3).

4. Discussion

Isolation of potential probiotic microbes from unconventional resources such as non-dairy food items, non-intestinal sources, and digestive tracts of animals has surged in recent years. These probiotics are beneficial not only for humans but also for improving animals'

243 health [1, 17-19]. Microbes residing in the digestive tract have colossal impact on the host
244 health. Over the past few years, several groups of probiotic bacteria have been isolated from
245 the digestive tract and feces of animals [20]. Feces from infant animals are considered a
246 pivotal source of probiotics since they rely on mother's milk which is enriched with
247 diversified nutrients, thus, favouring the growth of bacteria [21]. In the present investigation,
248 total 8 bacteria were successfully isolated from the horse feces. Among them, the potent
249 isolate was further identified as *L. plantarum* strain LF4. Recent studies reported the isolation
250 of *Lactobacillus* spp. and *Weisella* sp. from equines feces [21, 22]. On the other hand,
251 intestines of pigs were observed a potential source of *Lactobacillus* sp., *Pediococcus* sp., and
252 *Enterococcus* sp. [23].

253 The production of antibacterial substances such as bacteriocins, bacteriocins-like
254 inhibitory substances, organic acids, and hydrogen peroxide is one of the most important
255 criteria of probiotic bacteria [24]. Probiotic bacteria with promising rate of antimicrobial
256 characteristics are often considered an auspicious alternative to the conventional antibiotics.
257 In this context, the CFNS of strain LF4 exhibited antibacterial activity against indicator
258 bacterial pathogens tested which might be due to the secretion of bacteriocin-like inhibitory
259 substances into the growth medium. According to Westgate et al. [25], most of the indicator
260 bacteria tested in this study is causative agents of wound infection in equines. In view of this,
261 strain LAF4 showed its pivotal role as promising antibacterial agent against equine
262 pathogens. Similar to our findings, Xia et al. [22] demonstrated antibacterial activity of
263 *Weisella* sp. against certain gram positive and gram negative. In contrary, Kathade et al. [21]
264 reported lack of antibacterial activity of *Lactobacillus* sp. against *S. aureus*.

265 The viability at low pH conditions is one of the most important criteria for selecting
266 potential probiotic bacteria. In general, the tolerance to acidic conditions indicates the

267 survival ability of bacteria in the gastro-intestinal tract. The pH of equine stomach ranges
268 from 1.0 to 7.0 [26]. In the present study, strain LF4 revealed its ability to resist high acidic
269 conditions (up to pH 2.0). Findings of this context were observed to be in complete
270 agreement with the report of Prittesh and Vrutika [27] who depicted resistivity of lactobacilli
271 at acidic pH ranges with noticeable reduction in viabilities from pH 5.0 to pH 3.0. The
272 resistance towards bile salts is another essential parameter of any probiotic bacterium. In this
273 study, strain LF4 showed resistant to oxgall (0.5% w/v) up to 36 h. Similar finding was
274 illustrated by Kathade et al. [21] too who observed high bile salt concentration tolerance
275 abilities of lactobacilli.

276 Strain LF4 showed significant (P<0.05) hydrophobicity and auto-aggregation
277 properties, thereby indicating its ideal probiotic nature. In general, cell hydrophobicity
278 represents the unique characteristics of bacteria to adhere due to the presence of
279 glycoproteinaceous substances on its surface [28]. Likewise, auto-aggregation indicates the
280 ability of cells to colonize the colon [29]. In this study, the potentiality of strain LF4 to
281 adhere hydrocarbons and show auto-aggregation trait indicated its potency to colonize
282 intestinal epithelia. Additionally, strain LF4 revealed proteolytic property by hydrolyzing
283 skim milk agar medium. The production of protease is an important feature of probiotic
284 bacteria, as suggested by previous reports [5, 12].

285 Natural antioxidative agents reduce the oxidative damages caused by free radicals [30]. In
286 this study, strain LF4 showed its potentiality as an ideal antioxidant agent by scavenging
287 DPPH and hydroxyl radicals at diversified concentrations. Similar findings were reported by
288 Aarti and Khusro [2] who depicted concentration dependent antioxidant activity of
289 *Lactobacillus* sp. Moreover, Mishra et al. [31] demonstrated antioxidative attribute of
290 probiotic bacteria a strain-dependent process.

Probiotic bacteria may carry antibiotic resistant genes which can be pathogenic to humans and animals [32-34]. Therefore, the sensitivity of lactic acid bacteria towards antibiotics is considered as one of the leading parameters of probiotics. Findings of our study revealed susceptibility of strain LF4 to all the tested antibiotics, thereby indicating safety aspects of bacterium. In contrary to our results, probiotic bacteria isolated from equine feces were found resistant to some of the conventional antibiotics used [18, 19]. The variations in the outcomes of our findings with prior reports might be due to the differences in the bacterial strain types.

5. Conclusions

In summary, *L. plantarum* strain LF4 isolated from the horse feces exhibited antibacterial potential against indicator bacterial pathogens with maximum activity of 320.16±3.4 AU/mL against *S. aureus*. The strain maintained its viability towards low acidic conditions, simulated gastric juice, and bile salt. The isolate not only showed significant rate of hydrophobicity towards toluene (60.3±1.6%) but also depicted noticeable auto-aggregation characteristic (41.31±1.5%). Furthermore, strain LF4 showed concentration dependent antioxidant activities by scavenging DPPH and hydroxyl radicals. Additionally, sensitivity of strain LF4 to the conventional antibiotics indicated its safer utilization. Further studies are required to determine disparate techno-functional characteristics and *in vivo* safety aspects of strain LF4 for future applications in equine industries.

Conflict of interest

None declared.

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