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Health endorsing potential of *Lactobacillus plantarum* MBTU-HK1 and MBTU-HT of Honey bee gut origin

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ABSTRACT

Lactobacillus plantarum, is considered as an evergreen beckoning of probiotics. The literature available on the health benefits of *L. plantarum* within the gut, *Apis cerana indica* is very limited. The present study explores the health benefits of *L. plantarum* in *Apis cerana indica* (Indian honey bee). The study aimed to determine the health endorsing role of probiotic strains MBTU-HK1 and MBTU-HT (Accession no: KX519413 and KX519414) existed in the gut, *Apis cerana indica.* In the current study, the functionality among strains was evaluated *in vitro* for bile salt hydrolase (BSH) activity, Hypercholesteraemic activity, β-galactosidase activity, antioxidative potential, angiotensin-converting enzyme (ACE) inhibitory potency, and Folate production. The test strains possess BSH activity and anti-cholesteric activity, testifying to a role in cholesterol removal. The production of β-galactosidase enzyme suggested that they are effective in the improvement of lactose intolerance. The antioxidant activity revealed their effective free radical scavenging activity. The ACE inhibitory potency indicated their involvement in blood pressure control. The ability to produce folate by the test strains was also verified. The current investigation proves probiotic role with health benefits among MBTU-HK1 and MBTU HT in reducing risks associated with cardiovascular diseases, lactose intolerance, and hypertension management.

1. INTRODUCTION

The definition for Probiotics is the "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" [1]. The extensively studied and generally employed probiotics are the lactic acid bacteria (LAB). As per the American Food and Drug Administration, LAB subsists as "generally regarded as safe" (GRAS) status because of their occurrence in the gut of healthy animals and also an evident history of safe utility in fermented foods. LAB are harmless bacteria, mainly exploited for beneficiation in human health. LAB is widely experimented with to enhance their utilization [2]. Several species of LAB, including *Lactobacillus plantarum*, obtained a Qualified Presumption of Safety status granted from the European Food Safety Authority [3]. *Lactobacillus plantarum*, one of the main LABs, is recommended [4] for a probiotic bacterium because of their apparent probiotic potencies and ability to withstand gastrointestinal conditions (acid and bile) moreover their inhibitory action on intestinal pathogens [5]. The physiological benefits of probiotics include antioxidant activity, lowering of cholesterol, immune-stimulating and allergy lowering effect, synthesis and enhancing the bioavailability of nutrients, and alleviation of lactose intolerance [6,7]. With the modern lifestyle, the global market for health products is predicted to grow rapidly for creating new health food types. In this aspect, the interest in probiotics has increased in humans and animals due to the high index of safety and therapeutic value, reducing drug dependence and medical expenses [8–10]. The upsurge in population and consumer awareness of probiotics on the immune system and gut health homeostasis promotes enhanced probiotics demand. Thus, novel LAB is perpetually explored by researchers. Potent LAB from honey bee gut serves as a useful source for novel probiotics and has been accepted with probiotics' health-conferring property [11]. Our previous communication, isolated and characterized *L. plantarum* MBTU-HK1 and MBTU-HT within the gut of *Apis cerana indica* have excellent probiotic capabilities [9]. The present communication reports the health endorsing functional/health-promoting properties of *L. plantarum* isolated within the gut of

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Indian honeybee- *Apis cerana indica*. As far as we know, our study is the first analysis highlighting the functional/health-promoting properties of *L. plantarum* of Indian honeybee gut origin.

2. MATERIALS AND METHODS

2.1. Selection of the Bacterial Isolates

Two strains of *L. plantarum* MBTU-HK1 and MBTU-HT, obtained from the gut of *Apis cerana indica* (accession no: KX519413 and KX519414) were chosen for the evaluation of the health-promoting property. The same has been hitherto testified to possess active probiotic potencies and relevant cell surface properties [9].

2.2. Bile salt Hydrolase Activity and Cholesterol Diminution

An overnight culture of *L. plantarum* MBTU-HK1 and MBTU-HT were spotted on De Man Rogosa Sharpe (MRS) agar supplemented with 0.5 g/l cysteine and 1 mM sodium taurocholate as bile salt. Incubated the plates anaerobically under 37°C for 72 hours and examined for the precipitation zone nearby colonies [10].

Test strains *L. plantarum* MBTU-HK1 and MBTU-HT were inoculated to sterile modified MRS broth containing 1% glucose and supplemented with bile salt (0.2% and 0.4%) and cholesterol (with an absolute concentration of 100 mg l^{-1}). The cholesterol content in spent broth and uninoculated control broths was evaluated using a cholesterol colorimetric enzymatic assay kit (Agappe Diagnostics). The test strain's ability to eliminate cholesterol within the media was determined by using the equation: $A = 100 - (B/C) \times 100$. Here, A is the % of cholesterol eliminated, B is the absorbance of the sample including the cells, and C is the absorbance of the sample devoid of cells (Danielson *et al*. [11] with modifications).

2.3. Alleviation of Lactose Intolerance by β Galactosidase Activity

2.3.1. Qualitative test for β galactosidase production

The test was performed using 2-Nitrophenyl β-D-galactopyranoside (ONPG) Test Disks (Sigma) [12].

2.3.2. Quantitative test

A quantitative examination of β-hydrolase activity was determined by Gheytanchi *et al.* [12]. Overnight cultures of *L. plantarum MBTU*-HK1 and MBTU-HT were centrifuged separately at 12,000 × g for 5 minutes at 5°C and washed in phosphate buffer (pH 7) twice. The cells were inoculated in MRS-lactose broth and incubated at 37°C for 24 hours. Cells were collected by centrifugation and adjusted as OD 0.1 at 560 nm with the buffer. Then permeabilized each cell suspension (1 ml) with 50 µl Toluene/Acetone (1:9 *v/v*) and vortexed for 7 minutes. The β-galactosidase activity was analyzed by using Ortho Nitro Phenyl β-D Galactopyranoside (Sigma). Each of the permeabilized cell suspension (100 µl) were kept in a microtube and mixed with phosphate buffer (900 µl) and ONPG (2 mg/ml, 200 µl). The microtubes were incubated in a water bath for 15 minutes under 37°C. To stop the reaction, added 1 M Na_2CO_3 (0.5 ml) to each tube. Centrifuge the microtubes' contents at 12,000 × g at 5°C for 5 minutes to eliminate the cells. Read the absorbance at both 420 and 560 nm. Then, the β-galactosidase activity was determined in Miller units as follows:

$$1{,}000 \times [A420 - (1.75 \times A2560)] \div [T \times V \times A1560]$$

$A1$ = absorbance immediately afore analysis, $A2$ = Cell intensity in the reaction mixture, where T = 15 minutes, V = 1 ml. The experiment was conducted in triplicate.

2.4. Free Radical Scavenging Assay

2,2-diphenylpicrylhydrazyl (DPPH) radical scavenging capacity of *L. plantarum* MBTU-HK1 and MBTU-HT were verified using the method of Brand-Williams *et al*. [13]. The freshly prepared methanolic solution of DPPH (60 µmol l^{-1}) serves as the working solution. An aliquot (25 µl) of a cellular suspension of an overnight culture of test strain was kept in a tube and mixed with DPPH (1 ml) solution. Incubated the tubes under dark for 45 minutes at 37°C. Centrifuge the mixture after incubation and read the absorbance spectrophotometrically. The scavenging activity indicates a reduction in absorbance at 517 nm. 1.15 g KCL l^{-1} serves as the blank. The test was performed with uninoculated MRS broth and ascorbic acid (1 mg/ml, positive control).

$$\% \text{ of scavenging activity} = 1 - [A517 \text{ nm sample}/A517 \text{ nm blank}] \times 100$$

2.5. Regulation of Hypertension by angiotensin converting enzyme (ACE) Inhibitory Peptides

The ACE inhibitory activity was measured by the method of Cushman [14] with some modification. The reconstituted skim milk was inoculated with the test isolates and incubated for 24–48 hours. The fermented milk thus obtained was centrifuged at 4,000 rpm for 15 minutes at 4ºC. The supernatant thus obtained was adjusted to a pH of 8.3 using 10 M NaOH. Centrifuge the suspension at 14,000 × g 4ºC for 5 minutes and collect the supernatant. The hippuryl-histidine-leucine (Hip-His-Leu) was suspended in 0.1 M sodium borate buffer (pH 8.3) containing 0.3 M NaCl. A solution of Hip-His-Leu (200 µl) was mixed with supernatant (80 µl) and incubated at 37ºC for 3 minutes. Initiation of the reaction was carried out by supplementing the inhibitory solution [ACE-rabbit lung (0.1 unit/ml)] and incubated for 60 minutes at 37ºC. Stopped the reaction by adding 250 µl of 1 N HCl and also added 1.7 ml of ethyl acetate. After the centrifugation of the mixture, transfer the organic phase (1.4 ml) to a fresh tube and kept on a water bath at 100ºC for 30 minutes for the evaporation of ethyl acetate. Then dissolve the remaining residue (containing Hippuric acid) in 1 ml of deionized water. Then read the absorbance at 228 nm by spectrophotometrically against water as a blank. The following formula determined the rate of ACE inhibition.

ACE inhibitor activity (%) = $(B-A)/(B-C) \times 100$. A is the optical density with ACE and ACE inhibitory component, B is the optical density without ACE inhibitory component, and C is the optical density without ACE.

2.6. Production of Folate

Folate production by the isolates was verified in the folate-free semi-synthetic medium (SM) SM7 with modifications (Bacto

vitamin assay Casamino Acids and glucose are used, and yeast nitrogen base is replaced with pyridoxine, nicotinic acid, thiamine, calcium pantothenate, riboflavin, Para-aminobenzoic acid, and biotin). The test strains were cultured in MRS broth having L-cysteine HCl and incubated for 24 hours at 37°C. Cells from the MRS cultures were inoculated into 10 ml of SM7, incubated for 48 hours at 37°C, and growth was determined by measuring the optical density at 600 nm [15].

The amount of folate in cell extracts and cell-free supernatants was determined by folate assay Centrifuge the test cultures (30 ml) at 13,000 × g for 10 minutes at 0°C to collect the cell pellet. The supernatant obtained was filtered through a filter (0.22-µm-pore-size). Washed the cell pellet with 0.05 M K-phosphate buffer (pH 6.5) and resuspended in the same buffer (1:1 *w/v*). Cells suspended in phosphate buffer are disrupted by sonication. The cell extract was heat-treated (100°C for 3 minutes) to release and precipitate folate from folate binding proteins, and then the contents were centrifuged (13,000 × g, 15 minutes, 4°C) and filtered (0.22-µm-pore-size filter). The microbiological bioassay has been used for the evaluation of folate concentration [15]. The bioassay was performed as per the medium manufacturer's procedure, and the assay was carried out with folic acid assay medium (Himedia) with *Enterococcus hirae* American Type Culture Collection 8043 as the test organism.

2.7. Statistical Analysis

The results obtained were statistically analyzed by performing the Students *T*-test, and One-way analysis of variance was done by Tukey's test and significance difference taken at $p \leq 0.05$. The values have been expressed as mean ± SE values. All statistical analyses were executed by the software Graph Pad Prism 5.

3. RESULTS

3.1. Bile salt Hydrolase (BSH) Activity and Cholesterol Diminution

Lactobacillus plantarum MBTU-HK1 and MBTU HT exhibited fine precipitated white granules around their colonies indicating BSH activity (Fig. 1). These deconjugating bile acids would lead to a fall in serum cholesterol amount. From the results, it was inferred that the test strains could assimilate cholesterol. From the data presented in Figure 2, it was observed that *L. plantarum* MBTU-HK1 could remove 19.03% ± 1.21% and 27.8% ± 2.9% of cholesterol from media containing 0.2% and 0.4% bile salt, respectively. At the same time, *L. plantarum* MBTU-HT removed 12.06% of cholesterol from the medium containing 0.2% bile salt and 26% of the medium containing 0.4% bile salt.

3.2. Alleviation of Lactose Intolerance by β-Galactosidase Activity

Results presented in Figure 3 for the qualitative test evidenced production of the enzyme β- galactosidase by the test strains indicated by the yellow coloration. In the quantitative method, values of β-galactosidase enzyme activities of the strains were in the range of 96 ± 1.4–93.4 ± 2.6 (Miller Units/ml). Amid the two strains tested, *L. plantarum* MBTU-HK1 possesses the maximum β-galactosidase enzyme activity of 96% ± 1.4% (Table 1).

3.3. Free Radical Scavenging Assay

The DPPH is the most widely employed free radical for the determination of scavenging activities of antioxidants. The culture supernatant of the test strains exhibited 96.3% ± 0.2% (MBTU-HK1) and 95.7% ± 0.88% (MBTU-HT) of DPPH

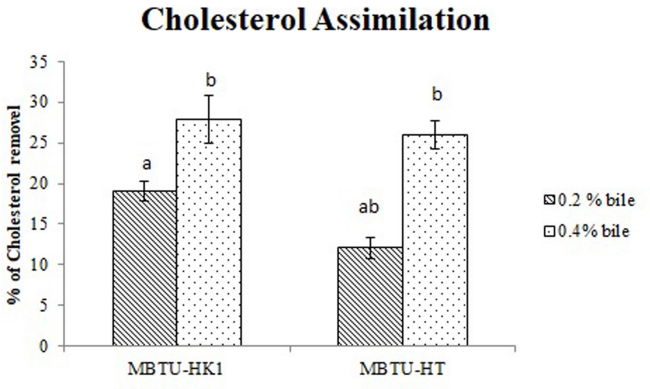

Figure 2: Cholesterol assimilation ability (%). The data are presented as mean ± SE. Data with distinct superscripts are significantly precise, where $p < 0.05$.

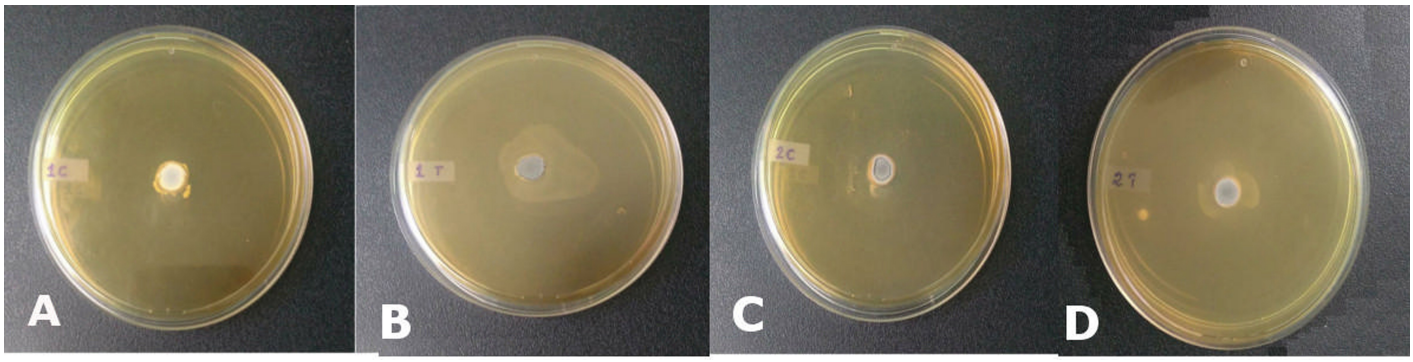

Figure 1: The BSH activity of test isolates grown on bile salt – MRS medium while exhibited by the developement of precipitation zone nearby the colony. (A) Control (without bile salt), (B) *L. plantarum* MBTU-HK1 with BSH activity, (C) Control (without bile salt) and (D) *L. plantarum* MBTU-HT with BSH activity on bile salt -MRS agar plate.

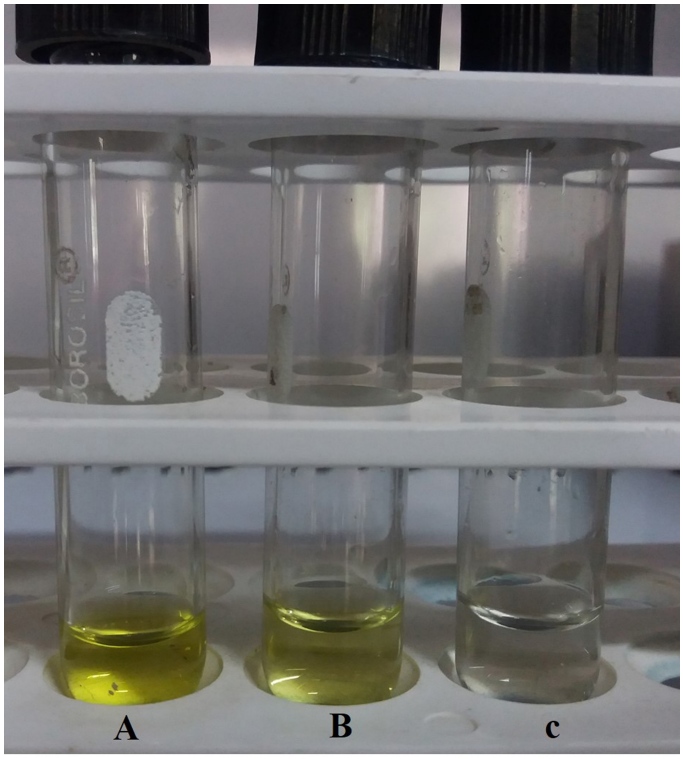

Figure 3: Qualitative analysis using ONPG test disc to examine the production of β-galactisidase enzyme by (A) *L. plantarum* MBTU-HK1, (B) MBTU-HT and (C) Control.

Table 1: Quantitative determination of the β-galactosidase activity of *L. plantarum* MBTU-HK1 and MBT-HT expressed in Miller Unit. The data are presented as mean ± SE. where $p < 0.05$.

Honeybee isolates	Values of β-galactosidase enzyme
MBTU-HK1	96 ± 1.4 Miller units/ml[a]
MBTU-HT	93.41 ± 2.6 Miller units/ml[a]

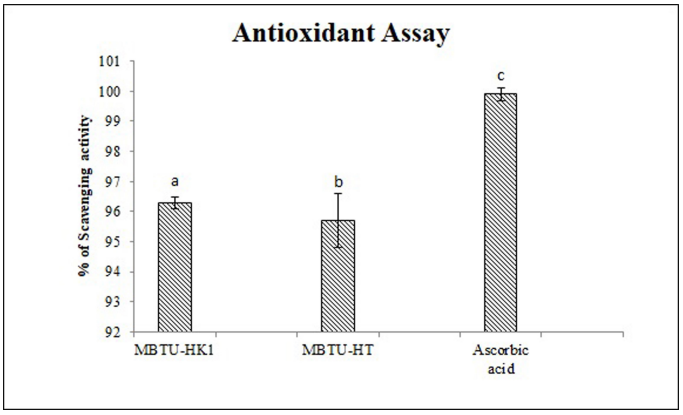

Figure 4: Antioxidant activity of *L. plantarum* MBTU-HK1 and MBTU-HT. The data are presented as mean ± SE. Data with distinct superscripts are significantly precise, where $p < 0.05$.

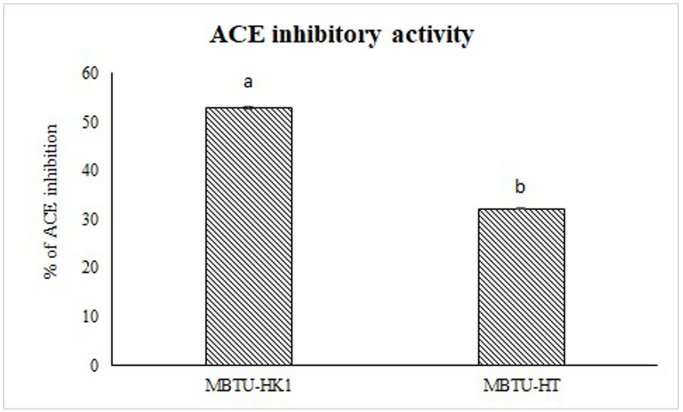

Figure 5: ACE inhibitory activity of *L. plantarum* MBTU-HK1and MBTU-HT. The data are presented as mean ± SE. Data with distinct superscripts are significantly precise, where $p < 0.05$.

radical scavenging activity (Fig. 4). Ascorbic acid at 1 mg/ml concentration, as the positive control, showed an activity of 99.94% ± 0.02%, which was almost equivalent to the activity of the test strains.

3.4. Regulation of Hypertension by ACE Inhibitory Peptides

The evaluation of ACE inhibitory activity determined antihypertensive peptides of milk proteins. Test strains possesses ACE inhibitory activity in varying levels. *Lactobacillus plantarum* MBTU-HK1 showed 52.9% ± 0.28% and *L. plantarum* MBTU-HT recorded 32.2% ± 0.06% of ACE inhibitory activity (Fig. 5).

3.5. Production of Folate

Lactobacillus plantarum MBTU-HK1 and MBTU-HT were tested for their growth in the SM7, which was deficient for folic acid but included all nutrients for the growth. Growth and folate concentration in SM7 was determined subsequently with seven subcultures. Both the strains retained growth in the SM7 medium even after seven passages and possessed folate concentration in their culture supernatants. The test strains were observed to record variation in folate concentration that ranged between 0.6 ± 0.003 and 1.4 ± 0.003 ng ml^{-1}. The test strains showed 1.46 ± 0.002 ng ml^{-1} (MBTU-HK1) and 1.202 ± 0.001 ng ml^{-1} (MBTU-HT) of extracellular folate production, and 0.66 ± 0.003 ng ml^{-1} and 0.6 ± 0.006 ng ml^{-1} of intracellular folate production correspondingly. Intracellular and extracellular folate concentrations of test strains are documented in Figure 6.

4. DISCUSSION

LAB, well known as probiotic candidates, provide several LAB strains as sources for the plenteous manufacture of probiotic-based functional foods and pharmaceutical products [9]. Based on these contexts, novel and spanking sources of potential probiotic LAB are insistently explored by the researchers. However, studies on functional properties/health-promoting effects of LAB associated to honeybee-gut are rather limited. Hence, the current research was conducted to determine the health-promoting potential of probiotic *L. plantarum* MBTU-HK1, and MBTU-HT obtained from the gut of Indian honeybee [9]. The present study's significant outcome was the health endorsing property of *L. plantarum* strains of *A. cerana indica* gut origin, not yet reported so far.

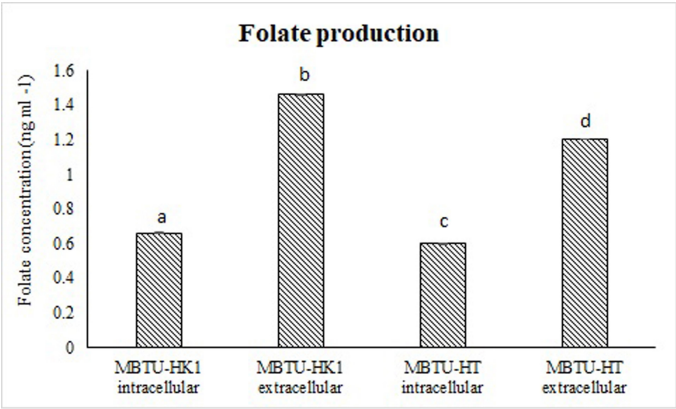

Figure 6: Folate production by *L. plantarum* MBTU- HK1 and MBTU-HT. The data are presented as mean ± SE. Data with distinct superscripts are significantly precise, where $p < 0.05$.

The BSH activity, considered as an advantageous trait, while choosing a strain for employing as a nutritional adjuvant, enabling them to survive the intestinal stress [16]. The test strains *L. plantarum* MBTU-HK1 and MBTU-HT were capable of producing BSH. Bacteria producing BSH have the efficiency to deconjugate bile salts that play a role in gut microflora equilibrium and reduce cholesterol [17,18]. In both developed and developing countries, coronary heart diseases are regarded as a treat factor mainly contributed by hypercholesterolemia. *Lactobacillus* sp. can lower cholesterol through specific processes comprising bile salt deconjugation [19]. Our findings revealed the cholesterol-lowering property of the test strains *L. plantarum* MBTU-HK1 and MBTU-HT in addition to BSH activity. In our study, increased elimination of cholesterol by the test strains has been observed by an enhancing concentration of bile salt. This may be due to the co-precipitation of cholesterol with deconjugated bile salts. Thus, it is inferred that the two LAB candidates have the potential for utilization as a good starter culture in the dairy industry and functional food products that prevent the threats associated with cardiovascular diseases. A similar finding was reported earlier by Pereira and Gibson [20]. β-galactosidase production is considered as one of the prime probiotic capability of LAB characterized so far [21]. The enzyme is extensively employed within the dairy industry and is yielded through a majority of *Lactobacilli*. The enzyme catalyzes the breakdown of lactose within the milk to glucose and galactose, engrossed through the intestinal epithelium [22]. The test strains in the present study produced the enzyme β-galactosidase with a value of 93–96 activity. Vinderola and Reinheimer [23] observed that the value of β-galactosidase activity ranged from 0 to 2,053 Miller units among various probiotic strains of *Lactobacillus delbruecki* subsp. *bulgaricus*. The symptoms of lactose intolerance can be reduced by removing lactose from the dietary regimen or by supplementing β-galactosidase enzyme [22]. LAB is one of the significant microbial reserves of β-galactosidase because of their GRAS status, and according to World Health Organization guidelines for an ideal probiotic, the ability to produce β-galactosidase is considered mandatory. The addition of bacteria capable of producing the enzyme as probiotic to infant formula could improve lactose digestion and thus helps to reduce lactose intolerance symptoms. The detection of novel strains generating a tremendous amount of the enzyme has acquired significance for promising application in probiotic cultures.

Significant scavenging activity was observed for the test strains, *L. plantarum* MBTU-HK1 and MBTU-HT indicating the production of antioxidants. Recent studies on the antioxidant properties of LAB have exposed that metabolic products decrease the build-up of reactive oxygen species by food ingestion and destroy superoxide anion and hydrogen peroxide [24]. Both strains showed scavenging activity nearly equivalent to the ascorbic acid. Our previous study [9] reported that these two strains used in this study isolated from honeybee gut are excellent producers of exopolysaccharides which reinforces their antioxidant property. Most of the studies on exopolysaccharides of LAB should be targeted on antioxidant potency for possible applicability in the food industry [25]. Nedelcheva *et al*. [27] suggested that probiotic bacteria with antioxidant activity in foods increase their biological activity, quality, as well as their shelf life.

In the present study, the test strains also proved their ability to inhibit ACE. Nakamura *et al*. [27] reported that hypertension could be mediated through inhibition of the ACE, which functions as a vital role in the control of peripheral blood pressure. Conditions of hypertension and heart failure, ACE inhibitors are well-known remedy for their treatment and also possessed an organ protective effects. According to Praveesh and Muthusamy [28], peptide production depends on the source of milk and is also strain dependent. Folic acid, vitamin B, retains the great potential to prevent disorders like cardiovascular disease, cancer, and neuropsychiatric disorders. In developed countries, folate deficiency is considered as a common vitamin deficiency [29]. In the present investigation, analysis of folate production demonstrated that the test strains were capable of growing on SM7 and generate folate in the level of 0.6–1.4 ng ml^{-1}. Pompei *et al*. [15] reported that the amount of folate in cell-free supernatants of *Bifidobacterium* ranging from 0.6 and 82 ng ml^{-1}. Thus, localized folate deficiency may overcome by the application of folate-producing probiotic strains [15]. The usage of probiotic strains with folate production can be considered as an innovative approach to enrich fermented dairy products (poor source of folate) which may prevent folate deficiency related to pre-carcinogenic variations among colonic epithelia [30].

5. CONCLUSION

Lactobacillus plantarum MBTU-HK1 and MBTU-HT of Indian honeybee gut origin are credible probiotic candidates with potent health conferring properties that have enormous applications in the food and nutraceutical industry. In the current COVID-19 pandemic situation, the role of nutraceuticals as immune boosters have much attention. Currently, there is no preventive or curative medicine available, and a lot of herbal therapeutic formulations are practiced without proper scientific study. These novel candidate strains are suitable to formulate a synbiotic-based nutraceutical with suitable prebiotic.

6. ACKNOWLEDGMENTS

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7. CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

8. AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

9. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

10. PUBLISHER'S NOTE

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