Postbiotic and Anti-aflatoxigenic Capabilities of *Lactobacillus kunkeei* as the Potential Probiotic LAB Isolated from the Natural Honey

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Abstract

The use of probiotic, postbiotic, and anti-aflatoxigenic capabilities of the lactic acid bacteria (LAB) isolated from stressful niches is a major strategy to prepare functional cultures and bio-preservatives for food industries. In the present study, abundant LAB strains isolated from natural honey were screened based on their tolerance to continuous pH and bile salt treatments. Then, the pro-functional properties of the selected LAB were investigated. In accordance with the screening data, a bacilli isolate was selected for further characterization. Sequencing results led to the identification of *Lactobacillus kunkeei* as the selected LAB isolate. In vitro antibacterial and antifungal activities of the LAB and in situ antifungal activity of the isolate cell-free supernatant (CFS) were verified against food-borne indicators. Accordingly, in vitro antibacterial and antifungal effects of *Lact. kunkeei* ENH01 on respective *Escherichia coli* and *Aspergillus niger* were significantly ($P < 0.05$) higher than the other indicators. Furthermore, in situ inhibitory effect of *Lact. kunkeei* CFS on *Candida albicans* (as the highest in situ effect) was equal to 76.36%. The presence of three antibacterial peptides was also verified in the *Lact. kunkeei* CFS according to the results of liquid chromatography mass spectrometry (LC/MS) assay. Antibiotic susceptibility profile and auto-aggregation ability of the isolate were noticeable. Anti-mycotoxigenic capabilities of *Lact. kunkeei* ENH01 as viable and heat-killed cells were also revealed against total aflatoxins according to the HPLC-based analysis. In vivo safety of the isolate was also attested through the evaluation of blood biochemistry and hematological parameters in the *Lact. kunkeei* ENH01 fed-mice compared with the control. Based on the findings, probiotic properties of *Lact. kunkeei* ENH01 and postbiotic capabilities of the isolate CFS and its heat-killed cells were approved.

Keywords Honey LAB isolate · In situ antifungal · Anti-mycotoxigenic · In vivo safety · Postbiotic

Introduction

Previous researches have shown that health benefit potentials of honey are attributed to the plant-derived phytochemicals, its prebiotic effects, and endogenous probiotics [1]. Probiotics as live microorganisms confer health promoting nutritional and physiological effects on the host when consumed in adequate amounts. Lactic acid bacteria (LAB) as Gram-positive, catalase-negative, and non-spore forming bacteria are the major group of probiotic technologically suitable microorganisms [2].

Usually, pro-functional properties of probiotics are correlated to their viability; however, these microorganisms have postbiotics or paraprobiotics potentials which refer to the released soluble metabolites or products of cell lysis of the probiotics with specific physiological functions. These compounds may affect through the mechanisms not entirely elucidated. In other words, dead cells and cell-free supernatants (CFS) of the probiotics harbor some functional features like bio-preservative and anti-mycotoxigenic effects. Accordingly, it is not necessary to consume viable probiotics for health benefits [3].

Survival in simulated gastrointestinal (GI) conditions is the unique capability of probiotics. Meanwhile, as mentioned, some of the pro-functional properties of these microorganisms are independent of their viability. LAB and their probio-active specific cellular components exert many pivotal effects on the

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ecosystem of the human GI tract including maintenance of the gut microbiota and control of the enteric mucosal pathogens, as well as modulation of the cell-mediated immune responses [4].

From an ecological perspective, natural honey as a potential probiotic vehicle is a proper niche for probiotic LAB due to several oligosaccharides, reducing sugars, organic acids, and phenolic compounds. Aforementioned ingredients promote viability of these bacteria through different mechanisms like prebiotic function, oxidative-stress protection, and reduction of the redox potential [1].

In various reports, several probiotic LAB were isolated from honey or honeybee, and then, they were characterized. Esawy et al. [5] approved different probiotic functionalities of the LAB isolated from honey including acid and bile salt tolerance, antibacterial activity against enteric pathogens and antibiotic resistance patterns. Nutraceutical potential of honey and probiotic properties of its innate microflora was also investigated in Begum et al. [6] survey. Bulgasem et al. [7] verified inhibitory effect of the LAB strains isolated from natural honey on pathogenic *Candida* species. Protective activity of the endogenous LAB against mycotic contamination of honeybee beebread was also revealed in Janashia et al. [8] study. Isolation of osmotolerant probiotic LAB from honey reflects the ubiquitous nature of these bacteria and their abilities to survive in the stressful conditions [9]. The aim of the present study was to characterize pro-functional and postbiotic capabilities of the potential probiotic LAB isolated from natural honey.

Materials and Methods

Raw Materials

Unpasteurized natural honey was harvested directly from a honeycomb that was prepared from a local jungle (Caspian Hyrcanian mixed forests, North of Iran). The characteristics of this honey were analyzed in accordance with the FDA standard methods. All the chemical reagents and microbial media used in this research were also purchased from Merck (Darmstadt, Germany) and Sigma-Aldrich (Saint Louis, MO, USA) companies with analytical grade. Food-borne indicator bacteria (*Bacillus cereus* ATCC 14579, *Escherichia coli* ATCC 11229, *Salmonella enterica* ATCC 35664, *Staphylococcus aureus* ATCC 23235) and fungi (*Aspergillus flavus* ATCC 26771, *Aspergillus niger* ATCC 1015, *Candida albicans* ATCC 10231) used in the present project were also purchased from American type culture collection, and then, they were activated in specific media.

Isolation of the Abundant Honey LAB

Approximately 10 g of the natural honey sample was suspended in 90 mL modified de Man, Rogosa, and Sharpe (MRS) broth (Merck; containing 5 g/L yeast extract as an enrichment medium) incubated at 37 °C for 48 h in microaerophilic condition. Then, 10-fold serial dilutions of the sample were spread plated on MRS and modified MRS agar. Subsequently, the plates were incubated under identical conditions. After that, Gram-positive and catalase-negative colonies were streaked on MRS agar to obtain pure colonies. The single pure colonies (stationary phase) were maintained in the MRS broth with 25% glycerol and kept at − 80 °C.

Screening Based on pH and Bile Tolerance

Continuous acidic (pH 2.0 for 1.5 h) and bile salt (Merck; 0.3% *w/v* at pH 6.0 for 1.5 h) treatments of the isolates in the MRS broth incubated at 37 °C were used to screen the LAB according to Rolim et al. [10] method with some modifications. To prepare these conditions, the population of each LAB isolate was adjusted to 10^8 colony forming units (CFU)/mL, and then, the pH of the medium was altered to 2 using 1 N HCl. Subsequently, bile salt was added and the pH was readjusted to 6 using 1 N NaOH. Finally, the most resistant LAB in comparison with its corresponding control (without pH and bile treatments) was selected for further study. *Lactobacillus rhamnosus* (ATCC 7469) was also employed as a reference probiotic LAB in the screening bioassay.

Molecular Identification of the LAB Isolates

The genomic DNA was extracted from the pure single colony of the isolates in accordance with the instructions of the kit manufacturer (Bioneer; Daejeon, South Korea). Then, the 16S rRNA gene was PCR amplified using the universal F44 (5′-RGTTYGATYMTGGCTCAG-3′) and R1543 (5′-GGNTACCTTKTTACGACTT-3′) primers. The amounts of PCR reagents (Ampliqon; Odense, Denmark) and conditions of the thermal cycling (Corbett thermal-cycler; Sydney, Australia) were also optimized in accordance with Abnous et al. [11] survey. Subsequently, the PCR products were checked through agarose gel electrophoresis and sequencing (Bioneer) assays. Next, the basic local alignment search tool (BLAST) algorithm and phylogenetic evolutionary tree (drawn with 16S rRNA genes obtained from the gene bank via the MEGA 6 software) were used to identify the isolates [12].

Antibacterial Activity of the Selected LAB and Its CFS

Antibacterial activity of the selected LAB isolate was evaluated against food-borne indicator bacteria using the dual

agar overlay method. LAB isolate was spotted on MRS agar plates and incubated at 37 °C for 48 h. Then, the plates were overlaid with 10 mL of brain heart infusion (BHI) agar (Merck) containing the target bacteria with 10^5 CFU/mL. Following that, the diameter of inhibitory zone was measured 24 h after incubation. LAB CFS was also prepared through centrifugation (Hanil Combi; Gimpo, South Korea; 6500 g for 15 min at 4 °C) and microfiltration of the overnight culture of the LAB isolate. After that, the crude CFS was naturalized (pH 6.5), and then, it was catalase (200 U/mL, 2 h, 37 °C) and protease (10 μg/mL, 1.5 h, 37 °C) treated, respectively. Inhibitory activity of the CFS was also determined through the broth microdilution assay [13]. In order to purify the antibacterial peptides, a technique was adopted based on the pH-dependent adsorption-desorption principle [14]. Finally, the extract was dialyzed, freeze-dried (Operon; Gimpo, South Korea), and kept at − 20 °C before analysis. Detection of proteinaceous metabolites was also carried out through liquid chromatography mass spectrometry (LC/MS) analysis, using Alliance, Waters 2695 system (Milford, MA, USA), which was equipped with electrospray ionization (ESI) according to the procedure proposed by Zendo et al. [15].

Inhibition of Fungal Growth

In vitro anti-mold effect of the LAB isolate on *A. niger* and *A. flavus* was determined using overlay method. Briefly, an overnight activated culture of the isolate was streaked on MRS agar. After incubation at 37 °C for 48 h under microaerophilic conditions, the plate was overlaid with soft yeast extract glucose chloramphenicol (YGC) agar (Merck) containing 10^5 spores/mL of the indicator fungi, and then, it was incubated at 25 °C for 7 continuous days. Anti-yeast activity of the isolate against *C. albicans* was also studied through the dual agar plate. Microtiter bioassay was utilized to investigate in vitro and in situ antifungal activity of the LAB CFS following the methodology proposed by Espinel-Ingroff et al. [16]. To prepare sugar-free lemonade containing honey for in situ study, the brix of sterile water was reached to 11.5 ± 0.5 with honey, and then, its pH was adjusted to 3.0 ± 0.2 with commercial sterile lemon juice. Subsequently, 10^8 CFU/mL of the LAB isolate was inoculated to the beverage and incubated at 37 °C for 24 h. Finally, the suspension was centrifuged, and the produced supernatant was used to assess its inhibitory effect against indicator fungi. It should be noted that the percentage of growth inhibition (I) in antimicrobial assays was calculated as $I = 100(C - T)/C$, where T and C are the population of indicator bacteria or yeast (or the area of mold growth) in both treated and control plates, respectively.

Aggregation and Cell Surface Hydrophobicity

Auto-aggregation experiment was performed based on the modified method described by Del Re et al. [17]. The selected LAB isolate was cultured for 24 h on MRS broth at 37 °C. The cells were then centrifuged (5000 ×g for 15 min at 4 °C) and washed three times with phosphate buffered saline (PBS). Subsequently, they were re-suspended in sterile PBS to obtain 10^8 CFU/mL. After 24 h incubation at 37 °C, the auto-aggregation was measured (spectrophotometer, PG Instruments; Leicestershire, England) and expressed as auto-aggregation (%) = $[(OD_0 - OD_{24})/OD_0] \times 100$, where OD_0 and OD_{24} represent the optical density (600 nm) at 0 and 24 h incubation, respectively. Cell surface hydrophobicity was also determined through bacterial adhesion to xylene (Merck). In brief, a suspension of activated isolate in sterile PBS (10^8 CFU/mL) was mixed with xylene (3/1 *v/v*). The absorbance of the isolate suspension (A_0) and the aqueous phase of the mixture (A_F) were then measured at 600 nm. Finally, the percentage of hydrophobicity was calculated using the Eq. $[1- (A_F/A_0)] \times 100$.

Antibiotic Susceptibility Test

The antibiotic susceptibility of the isolate was evaluated through the disk-diffusion method, as performed by Temmerman et al. [18]. Briefly, pure culture of the selected LAB was swabbed on MRS agar plates. Then, the commonly used antibiotic disks (Padtan Teb, Tehran, Iran, Table 3) were placed on the surface of the plates. Afterwards, the plates were incubated at 37 °C for 24 h, and the diameter of inhibition zone around the disks was measured.

Anti-aflatoxigenic Effect

Effects of the selected LAB as viable and heat-killed (autoclaved at 121 °C for 15 min) cells on the reduction of total aflatoxins (B_1, B_2, G_1, and G_2; Sigma-Aldrich) were investigated according to the high performance liquid chromatography-based (HPLC; Waters 2475, Multi λ Florescence Detector) validated method reported by Sadeghi et al. [19] with acceptable ranges of linearity ($r^2 = 0.997$–0999), limit of detection (0.04–0.13 μg/L), and limit of quantification (0.07–0.15 μg/L). Anti-aflatoxigenic capabilities were determined compared with the control, which contained a mixture of the toxin and PBS. The studied samples were also comprised of the LAB isolate (as viable or heat-killed), PBS, and each toxin. Finally, the percentage of removed aflatoxins (AF) was calculated by the following formula: $100 \times [1 - (AF$ peak area of sample/AF peak area of control)].
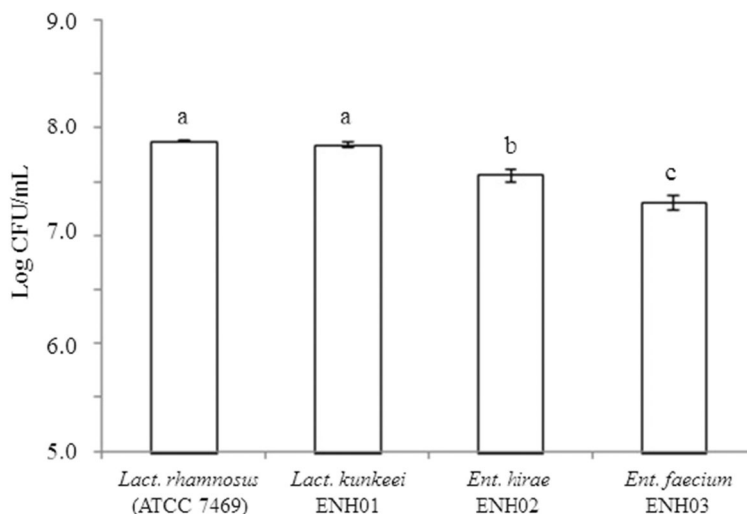
Hemolytic Activity and In Vivo Safety of the Isolate

The selected LAB isolate was cultured on blood agar supplemented with 5% sheep blood at 37 °C for 48 h. The plates were analyzed for the presence of green-hued (α-hemolysis), white or transparent-hued zones (β-hemolysis) or absence of hemolysis (γ-hemolysis) around the colonies. The in vivo safety was also evaluated following the guidelines of OECD using male mice without any sign of infections in the same age and weight group. Prior to the experiment, the mice were kept 2 weeks under regular 12-h light-dark periods at 25 °C. Then, the mice were orally administered with the LAB isolate (10^8 CFU/kg of the mouse weight) in 14 continuous days, and blood biochemistry (platelet, white and red blood cell counts, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, and mean corpuscular volume), and hematological parameters (aspartate aminotransferase, alanine aminotransferase, and alkaline phosphatase) of the mice were checked compared with the control (PBS-fed mice). All the biochemical tests were performed using commercial kits via semiautomatic biochemical analyzer [20].

Statistical Analysis

All the experiments were performed in duplicates. The means were compared through one-way analysis of variance and the least significant difference post-hock at $P < 0.05$ by the SPSS 20 software. The data were shown as mean ± standard deviation. It should be noted that the significance of differences between two different samples (inhibitory activities of the LAB isolate and its CFS), or between a sample and its corresponding control (in vivo safety assay) was also assessed using the independent-samples t test.
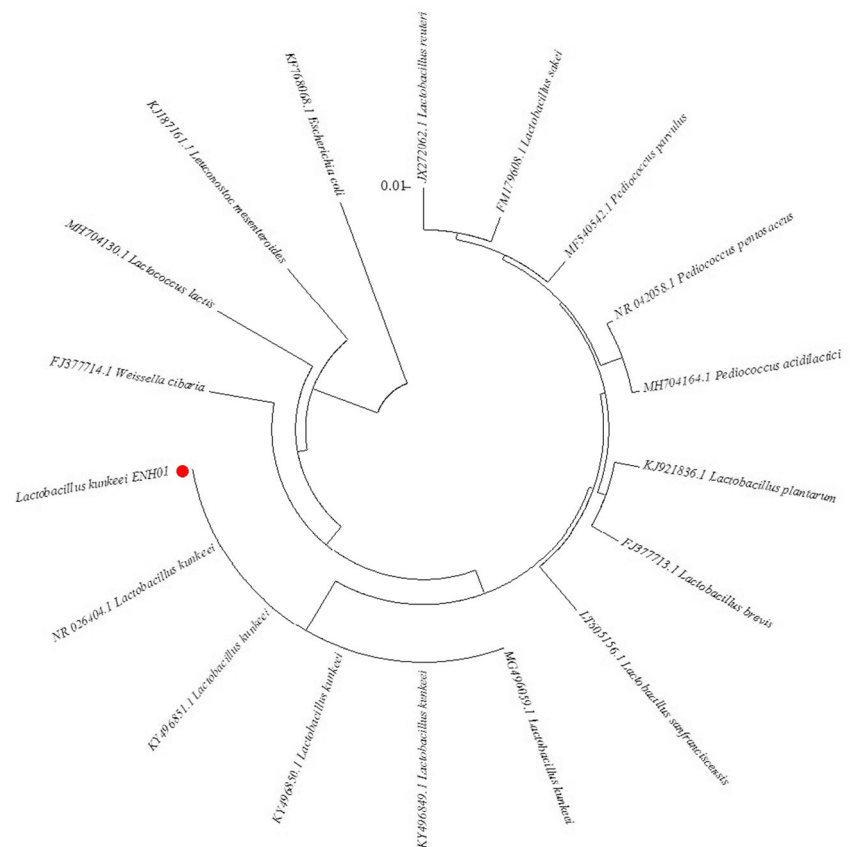
Results

LAB Survival Under pH and Bile Stress

Chemical composition of the natural honey used in the present study included 12.7% moisture, 2.6% sucrose, pH 3.6, and 78.2% total reducing sugar, and it was free from hydroxyl methyl furfural. Furthermore, in accordance with the preliminary results of micro morphological study, three different LAB including bacilli and cocci were isolated from the natural honey. The survival (Log CFU/mL) of a bacilli isolate under pH and bile stress was significantly ($P < 0.05$) higher than the other LAB isolates (Fig. 1); therefore, it was subjected to further characterization. It should be noted that there was no significant difference between selected LAB isolate (ENH01) and reference probiotic LAB (*Lact. rhamnosus*) in terms of viability in low pH and bile stress. The selected isolate showed the maximum survival (74.28%) with a Log CFU value of 7.84, but experienced inhibition of 0.16 Log cycle.

Molecular Identification of the Selected and Non-selected LAB Isolates

The sequencing results of the PCR products led to the identification of *Lactobacillus kunkeei* as the selected LAB isolate. The evolutionary position of the isolate was also verified through a phylogenetic tree which was drawn with the correlated 16S rRNA sequences retrieved from the GenBank database (Fig. 2). Based on the sequencing results, non-selected LAB isolates were also identified as *Enterococcus hirae* and *Enterococcus faecium*. The selected (ENH01) and non-selected (ENH02 and ENH03) LAB isolates are stored in the reference Laboratory of Drug & Food Control (Golestan University of Medical Sciences, Gorgan, Iran) with *Lact. kunkeei* ENH01, *Ent. hirae* ENH02, and *Ent. faecium* ENH03 designations.

Fig. 1 Log CFU/mL of the abundant LAB isolated from natural honey (*Lactobacillus kunkeei* ENH01, *Enterococcus hirae* ENH02, and *Enterococcus faecium* ENH03) in continuous pH and bile salt treatments in comparison with *Lactobacillus rhamnosus* (ATCC 7469) as a reference probiotic LAB. Different letters indicate significant differences at $P < 0.05$ among the viability of the LAB

Fig. 2 Phylogenetic associations constructed using the neighbor-joining method with 1000 bootstrapping replicates giving the position of *Lactobacillus kunkeei* ENH01. The bar shows 1% sequence divergence

Antibacterial Activity

Table 1 shows the in vitro antibacterial effects of *Lact. kunkeei* ENH01 on different food-borne indicators. According to the results, inhibitory activity of the isolate against *E. coli* was significantly ($P < 0.05$) higher than the others, whereas there was no significant difference among the antibacterial effect of the *Lact. kunkeei* ENH01 on the other indicators. Interestingly, the inhibitory activity of the CFS was significantly (*$P < 0.05$, t test) higher than the LAB effect on the same indicator bacterium (in the same row of Table 1) probably due to its low pH (about

Table 1 Antibacterial effects of *Lactobacillus kunkeei* ENH01 and its cell-free supernatant (CFS) on the food-borne indicator bacteria (percentage of growth inhibition)

Indicator bacteria	LAB	CFS
Bacillus cereus ATCC 14579	34.55 ± 1.24^b	89.75 ± 1.34^b
Escherichia coli ATCC 11229	44.75 ± 3.05^a	93.39 ± 1.49^a
Salmonella enterica ATCC 35664	33.82 ± 4.68^b	83.15 ± 1.39^c
Staphylococcus aureus ATCC 23235	37.01 ± 3.45^b	87.40 ± 2.64^b

Data are expressed as means ± standard deviation of two independent experiments. Values in the same column with different letters are significantly different ($P < 0.05$)

3.5). The highest inhibitory effect of the crude CFS obtained from *Lact. kunkeei* ENH01 was also observed on *E. coli*, which was significantly higher than its inhibitory activity against the other microorganisms. In addition, the CFS effect on *B. cereus* was remarkably higher than its effect on *Salm. enterica* and *Staph. aureus*. After protease treatment, the inhibitory effect of the CFS was completely removed. Accordingly, protein-based nature of the antibacterial compounds was verified. CFS of the isolate was subsequently analyzed to characterize the antibacterial peptides. The CFS was applied to ESI-LC/MS and total ion chromatograms were obtained. Three peaks at 22.1, 28.4, and 31.6 retention times (min) were identified as peptide-derived peaks. The mass spectrum at the mentioned times showed that these peptides were detected as mass-to-charge ratio (m/z) 473–873, 701–1053, and 743–1096 (Fig. 3). The molecular mass of the antibacterial peptides was also determined to be 9183.82, 16,643.77, and 18,339.65 Da, respectively.

Antifungal Activity

The potent in vitro antifungal effect of *Lact. kunkeei* ENH01 on the studied fungi was clarified (Fig. 4). Based on the results of the overlay bioassay, the

Fig. 3 Liquid chromatography (LC) chromatogram obtained from cell-free supernatant (CFS) of *Lactobacillus kunkeei* ENH01 (**a**), electrospray ionization (ESI) mass spectrum of the fractions eluting at 22.1 (**b**), 28.4 (**c**), and 31.6 (**d**) min, which corresponded to I, II, and III peaks of the LC chromatogram, respectively. According to these *m/z* values, the presence of three different anti-bacterial peptides with 9183.82, 16,643.77, and 18,339.65 Da molecular mass was verified in the LAB CFS
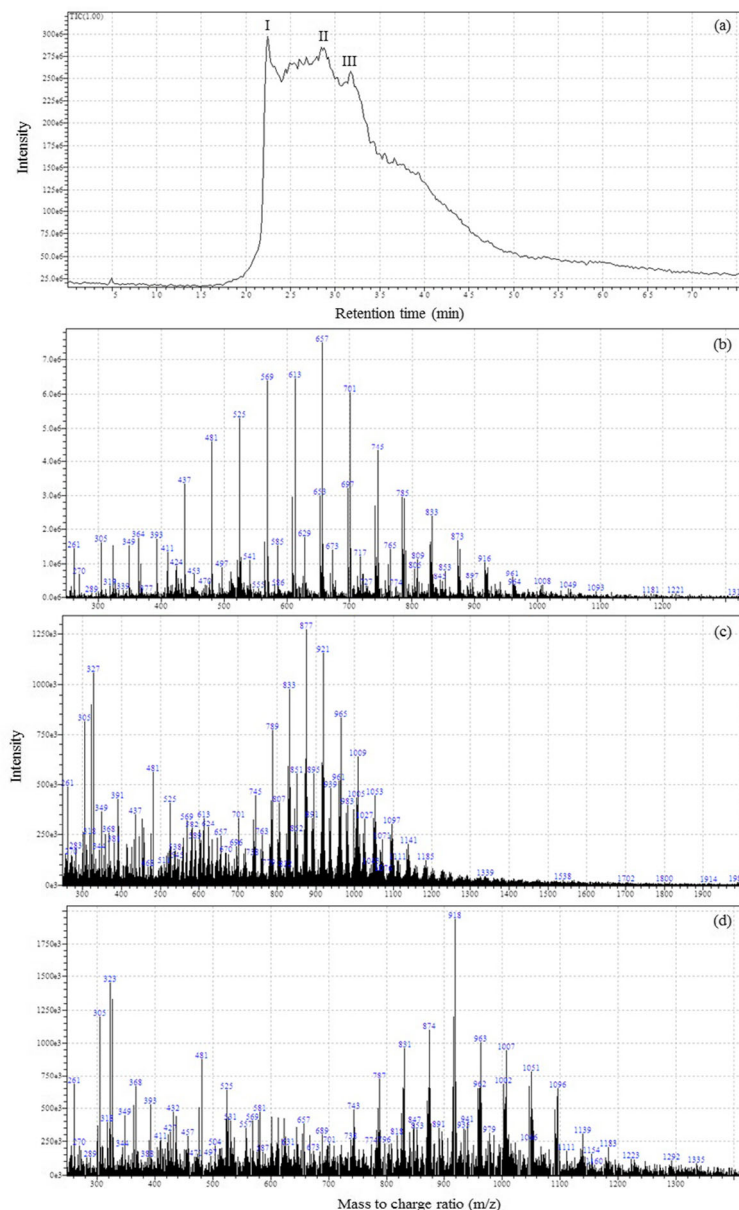
percentage of inhibition against *A. flavus* and *A. niger* was 55.94 ± 2.38 and 71.18 ± 4.5, respectively. In vitro and in situ anti-mold activities of the LAB CFS against *A. flavus* and *A. niger* were also equal to 50.32, 36.36 and 27.83, 10.70 inhibition percentage, respectively. Furthermore, anti-yeast activity of the *Lact. kunkeei* CFS against *C. albicans* was significantly ($P < 0.05$) higher than its anti-mold effects (Table 2).

Auto-aggregation and Cell Surface Hydrophobicity

Auto-aggregation capability of *Lact. kunkeei* ENH01 was $83.65\% \pm 4.07$. Cell surface hydrophobicity of the isolate was calculated at $33.18\% \pm 1.79$.

Antibiotic resistance

The antibiogram profile of *Lact. kunkeei* ENH01 revealed high resistance to several antibiotics tested (Table 3). The diameter of inhibition zone varied between 23.33 and 0 mm. The LAB isolate was also susceptible towards penicillin, and it proved semi-resistant to cefazolin, ampicillin, and cephalothin.

Anti-aflatoxigenic Effect

Lact. kunkeei ENH01 as viable and heat-killed (autoclaved) cells reduced 45.55 and 54.56; 38.44 and 39.57; 39.40 and 42.48; and 19.27 and 20.52% of B_1, B_2, G_1, and G_2 aflatoxins compared with the control sample, respectively (Fig. 5).

Fig. 4 Inhibitory activity of *Lactobacillus kunkeei* ENH01 against indicator fungi (**b** co-culture of the LAB and *Aspergillus niger* ATCC 1015; **d** co-culture of the LAB and *Aspergillus flavus* ATCC 26771), compared with the controls (without LAB) of *A. niger* (**a**) and *A. flavus* (**c**) in overlay bioassay
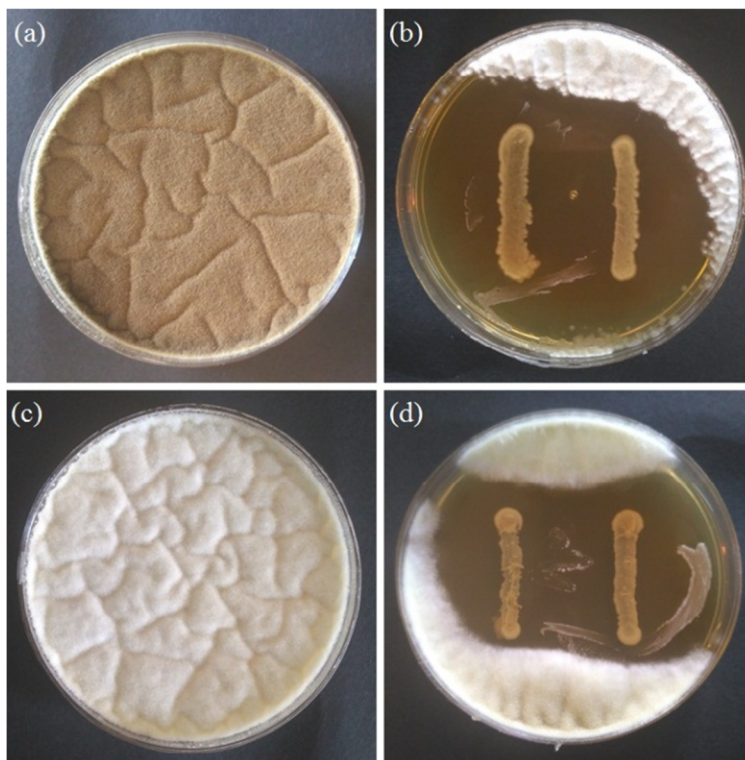
LAB Safety

In vitro safety of the *Lact. kunkeei* ENH01 was ensured by negative blood haemolytic activity. No detrimental effects were observed in the behavior, general well-being, weight gain, and water consumption of the mice receiving the isolate. Furthermore, there was no significant difference between the treated and control mice in terms of liver enzymes and blood biochemical parameters (Table 4). Accordingly, *Lact. kunkeei* ENH01 had no diverse effect on blood biochemistry and liver enzyme function, compared with the control mice.

Table 2 In vitro antifungal activity of *Lactobacillus kunkeei* ENH01 and its cell-free supernatant (CFS) and in situ inhibitory activity of the isolate CFS against *Aspergillus flavus* ATCC 26771, *Aspergillus niger* ATCC 1015, and *Candida albicans* ATCC 10231 (percentage of growth inhibition)

	LAB	CFS	In situ
A. flavus	$55.94 \pm 3.36^{B,a}$	$50.32 \pm 3.18^{B,b}$	$10.73 \pm 0.01^{C, c}$
A. niger	$71.18 \pm 4.52^{A,a}$	$27.83 \pm 2.70^{C,c}$	$36.36 \pm 6.42^{B, b}$
C. albicans	$46.83 \pm 2.00^{B,c}$	$86.80 \pm 0.77^{A,a}$	$76.36 \pm 2.11^{A,b}$

Values labeled by different uppercase letters in the same column are significantly different ($P < 0.05$). Furthermore, different lowercase letters indicate significant differences among the inhibitory effects of the LAB, its CFS, and in situ antifungal effect on the same indicator fungus (in the same row)

Discussion

High tolerance towards extreme acidic conditions and bile salts is the unique characteristic of microorganisms to be selected as probiotic. Therefore, in the present study, tolerance

Table 3 Antibiotic susceptibility profile of *Lactobacillus kunkeei* ENH01

Antibiotic (μg per disk)	Inhibitory diameter (mm)	Relative resistance
Penicillin (10)	23.33 ± 0.32^a	Susceptible
Cefazolin (30)	19.31 ± 1.28^b	Semi resistance
Ampicillin (10)	18.86 ± 3.15^b	Semi resistance
Cephalothin (30)	18.57 ± 0.05^b	Semi resistance
Imipeneme (10)	12.52 ± 0.31^c	Resistant
Ceftriaxone (30)	12.51 ± 0.20^c	Resistant
Novobiocin (5)	11.50 ± 1.20^c	Resistant
Clindamycin (2)	9.24 ± 0.27^d	Resistant
Ciproflaoxacin (5)	0.00 ± 0.00^e	Resistant
Sterptomycin (10)	0.00 ± 0.00^e	Resistant
Nalidixic acid (30)	0.00 ± 0.00^e	Resistant
Gentamycin (10)	0.00 ± 0.00^e	Resistant
Vancomycin (30)	0.00 ± 0.00^e	Resistant
Kanamycin (30)	0.00 ± 0.00^e	Resistant

Different letters indicate significant difference at $P < 0.05$ among the inhibitory diameter of the studied antibiotics

Fig. 5 Anti-aflatoxigenic effects of *Lactobacillus kunkeei* ENH01 as viable and heat-killed cells in comparison with the control sample in HPLC-based analysis. The percentage of removed aflatoxins (AF) was calculated by the following formula: $100 \times [1 - (\text{AF peak area of sample/AF peak area of control})]$. Accordingly, anti-aflatoxigenic effect of the LAB was revealed. Furthermore, LAB dead cells had noticeable anti-aflatoxigenic capability, and therefore, a postbiotic functionality of the *Lact. kunkeei* ENH01 was also verified
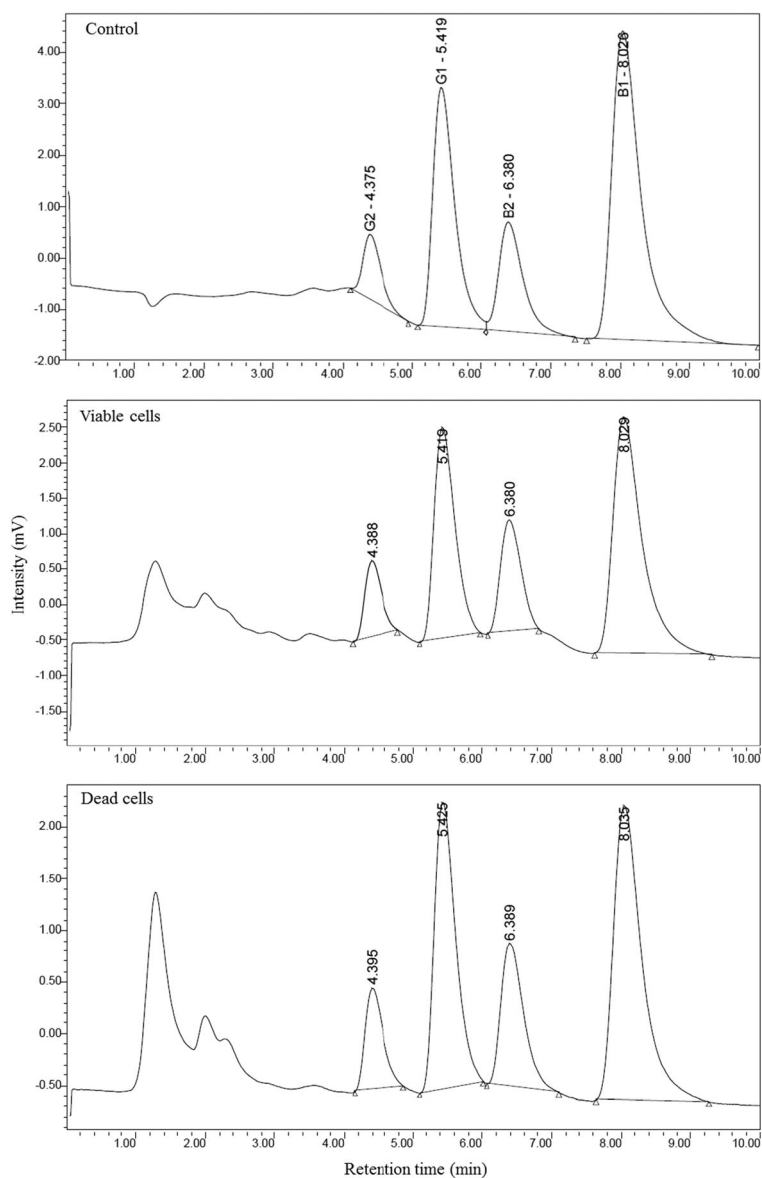
Table 4 Blood biochemistry and hematological parameters in the *Lactobacillus kunkeei* ENH01 fed-mice compared with the control

Blood biochemistry	Treated group	Control group	*t* test	Normal range
PLT (×10³/μL)	855.00 ± 13.22	831.00 ± 26.87	ns	500–1300
WBC (×10³/μL)	7.96 ± 0.45	7.41 ± 0.34	ns	6–18
RBC (×10³/μL)	6.44 ± 0.15	6.35 ± 0.12	ns	6.76–9.75
MCH (pg)	21.3 ± 0.60	21.03 ± 0.97	ns	–
MCHC (%)	31.56 ± 0.44	32.53 ± 0.74	ns	40
MCV (fL)	62.54 ± 0.41	62.60 ± 1.85	ns	48–70
Hematological parameters				
ALT (IU/L)	58.22 ± 1.76	56.08 ± 0.84	ns	17–77
AST (IU/L)	100.41 ± 1.17	101.89 ± 1.88	ns	54–298
ALP (IU/L)	63.87 ± 1.37	63.07 ± 2.06	ns	35–96

PLT platelet, *WBC* white blood cell, *RBC* red blood cell, *MCH* mean corpuscular hemoglobin, *MCHC* mean corpuscular hemoglobin concentration, *MCV* mean corpuscular volume, *ALT* alanine aminotransferase, *AST* aspartate aminotransferase, and *ALP* alkaline phosphatase. Data are expressed as means ± standard deviation of two independent experiments. Treated and control mice in terms of the same analyzed parameter in the same row were compared by *t* test (ns: not significant)

of the isolates to continuous treatment of pH 2.0 and 0.3% bile salt was investigated. Considering the count results, *Lact. kunkeei* ENH01 as the selected LAB isolate exhibited good survival under this treatment. Accordingly, the isolate is regarded as LAB strain that can be probiotic candidate. In accordance with Begum et al. [6] findings, *Gluconobacter oxydans* isolated from honey showed 54.83% survival after 3 h exposure to pH 2 and 2% bile salt. The significant growth promoting effect of sesame honey on probiotic *Lactobacillus acidophilus* (MTCC 447) and *Bifidobacterium bifidum* (ATCC 700541) was also reported [21]. Kenfack et al. [22] studied the acid and bile resistance of the LAB isolated from honeybee gut and found that 12 isolates were able to tolerant these treatments. The gene expression data of the bile salt hydrolase (BSH) enzyme were also analyzed in the isolates in order to identify potentially probiotic bacteria. In the published literature, it has been stated that the resistant of LAB to low pH and bile salt as important criteria for their colonization and metabolic activity are strain-dependent potentials. Common processes responsible for acid resistance in LAB are alteration of metabolic pathways, formation of a protective cloud of ammonia, repair or protection of macromolecules, and biofilm formation. Specific strategies involved in probiotic bile resistance and adaptation also include bile salt deconjugation, bile efflux, and exopolysaccharide production [23, 24].

Lact. kunkeei (which was molecular identified as the selected isolate in the present study) is a lactic acid bacterium that inherently inhabits fructose-rich sources. This fructophilic, osmotolerant, and acid-resistant bacterium was also predominantly found in specific honey of Nepal and Africa. Its origin is pollen and nectar, and it was also predominant in the stomach of honeybees [25]. It is reported that the bacterium could originate from honeybee hives and intestines, and therefore, it can be hypothesized that the pollen or honeybees are the source of *Lact. kunkeei* in honey. Different biochemical and metabolic activities of particular LAB species isolated from specific niches have been demonstrated by comparative genomics. The innate microflora of honey is limited due to its high sugar and low-water contents, as well as the presence of phenolic acids and lysozyme. However, microbial ecology of this stressful ecosystem consists of probiotic and pro-functional microorganisms [6]. A direct correlation was reported between honey moisture and population of *Gluconobacter* and *Lactobacillus* during ripening of honey. Furthermore, LAB microbiota of honey is associated with consumed pollen grains and flower nectar, as well as microbial ecology of the honeybees. Microaerophilic environment, proper temperature, and nutrients of the honeybee stomach are ideal conditions for improve symbiosis between LAB and this insect. Prebiotic substrates such as nondigestible oligosaccharides are also important in the modulation of the honey probiotic balance [26].

The inhibitory activity of *Lact. kunkeei* ENH01 against foodborne bacteria was also investigated in order to determine the isolate potential as proper probiotic/protective starter culture. *Lact. kunkeei* ENH01 identified in the present study was found to be effective against tested indicators. Antagonistic effects of honeybees LAB on endogenous pathogens such as *Paenibacillus larvae* BMR43-81 have been reported. Daisley et al. [27] realized that consortium of *Lactobacillus plantarum* Lp39, *Lact. rhamnosus* GR-1, and *Lact. kunkeei* BR-1 probiotics was able to inhibit *P. larvae* cells through the modulation of host-innate immunity response and improvement of honeybee survival in the presence of the pathogenic agents. Aween et al. [28] investigated antibacterial effects of the *Lact. acidophilus* strains isolated from natural honey and its CFS against *Enterobacter aerogenes*, *Salmonella Typhimurium* (ATCC 13311), and *E. coli* (ATCC 25922). There was a wide range of antimicrobial effects at 7.5–30 mm diameter of growth inhibition zone in overlay, and 0.72–24% of inhibitory effect in microtiter bioassay. Antibacterial activities of the LAB can guide us through selecting protective cultures in food fermentations, or screening starter cultures to obtain bio-preservative metabolites. Therefore, it is a key feature of probiotic microorganisms. There are three main phenomena affecting antibacterial activity of the LAB. The first is the competition with unwanted microorganisms for nutrients. The second is the production of antibacterial metabolites like organic acids, bacteriocins, bacteriocin-like substrates (BLS), deconjugated bile acids, alcohols, and H_2O_2. The effect of these compounds usually takes place through a variety of different mechanisms including acidification, inhibition of DNA gyrase or RNA polymerase activities, formation of pores in cytoplasmic membrane, and oxidation and alteration the activity of lipid II. The third is the interaction between these inhibitory metabolites. The pH-dependent activities of bacteriocins or microbial hurdles are the well-known examples of this phenomenon [29, 30].

Furthermore, the ability to produce metabolites with growth inhibition potential against important food-borne pathogens by probiotic LAB has been considered promising and applicable function. Some LAB strains are reported to produce multiple antibacterial peptides. The metabolites discussed are important elements in competition among bacteria and complementation of antimicrobial effects. In an ESI-LC/MS system, peptides can be clearly distinguished by accurate retention time and molecular mass. The *m/z* spectrum shows a Gaussian-type distribution of multiply charged ions. Each peak represents the intact protein molecule carrying a different number of charges. The *m/z* values can be expressed as follows:

$$m/z = (MW + nH+)/n.$$

Where "m/z" is the mass-to-charge ratio of the spectrum, "MW" is the molecular weight of the peptide, "n" is the number of charges on the ions, and "H" is the mass of a proton (1.008 Da). If the number of charges on an ion is known, then it is simply a matter of reading the m/z value from the spectrum to determine the molecular weight of the peptide. Usually, the number of charges is not known, but can be calculated if the assumption is made that any two adjacent members in the series of multiply charged ions differ by one charge. Therefore, to calculate the molecular weight of the protein, one can use two simultaneous equations derived from two adjacent ions [15].

Accordingly, production of four antimicrobial peptides with m/z 948–1581 (corresponding to 2843–6316 Da) was revealed in *Ent. faecium* NKR-5-3 through ESI-LC/MS analysis [31]. It is also reported that the inhibitory activity of *Lact. kunkeei* FF30-6 was tentatively attributed to the production of an antibacterial peptide or protein in the Endo and Salminen [32] study. Researchers revealed the key mechanisms through which antibacterial peptides could contribute to probiotic functionality. In brief, these metabolites act as killing, colonizing, and/or signaling peptides, and therefore, they inhibit the invasion of pathogens, modulate the host immune system, and modify the composition of the GI microbiota [33].

In vitro and in situ antifungal activities of *Lact. kunkeei* ENH01 and its CFS were also verified in the present study. Four different LAB strains isolated from honey samples in Bulgasem et al. [7] survey had proper antifungal activities against *Candida* spp. Rao et al. [34] reported that the multiple antifungal components of *Lact. plantarum* MYS44 CFS exhibited inhibitory effects on aflatoxigenic *Aspergillus parasiticus* (MTCC 411). Overall morphological changes, destroying hyphal wall and inhibition of conidial germination, were revealed as the main effects of the antagonistic compounds produced by the LAB in the research. Janashia et al. [8] found that endogenous LAB isolated from beebread had no inhibitory activity in overlay assay against pathogenic fungal strains which were isolated from the same niche, whereas the antagonistic activity was observed in simulated beebread substrate. In accordance with the findings of these researchers, the antifungal activity of LAB isolates depends on their ecological conditions because of adaptation to the environment and competition ability. In this respect, the results were similar to those reported in our study. LAB antifungal activities include limitation of fungal surface expansion, control of sporulation, reduction of hyphal biomass, and repression of metabolic biosynthetase activity. Antifungal activity of LAB as a well-known bio-preservation phenomenon is also associated with their competitiveness for nutrients (as simpler organisms with faster growth rate compared with fungi), acidification capabilities, and release of inhibitory compounds including several organic acids, phenyllactic derivatives, hydroxyl fatty acids, peptide mixtures, cyclic dipeptides, and antioxidant compounds. The most important modes of action involved in inhibitory effect of these metabolites are intercellular acidification, fungal membrane destruction, disruption of essential metabolic reactions, accumulation of toxic anions, and quorum sensing [35, 36]. In situ antifungal activity of the *Lact. kunkeei* CFS in honey-containing lemonade (especially against *Candida*) revealed that honey as a proper niche for probiotics possesses antimicrobial effects. Consequently, it is hypothesized that in situ inhibitory activity of the LAB may be influenced by the precursors of antimicrobial metabolites and prebiotic activity of the substrate.

Cell surface hydrophobicity and auto-aggregation are essential characteristics for the adhesion of probiotic LAB to the intestinal epithelial cells and their colony formation. Furthermore, these abilities offer a competitive advantage during adhesion and prevent probiotics elimination from human gut during peristalsis. Cell surface hydrophobicity of the honey isolate in our study was placed in low hydrophobicity group, according to standard classifications [37]. In contrast to our results, Sakandar et al. [38] reported that *Lact. kunkeei* (JNGBKS6-8) isolated from Chinese fruits and flowers had high hydrophobicity. Since this capability is a strain-specific feature and it depends on the glyco-proteinaceous compounds of the microbial cell surface, a substantial difference may be observed in hydrophobicity even within the same species. Audisio et al. [39] studied the auto-aggregation ability of three different *Lactobacillus johnsonii* strains (including CRL1647) isolated from bee gut, and reported that these isolates showed 77–93% auto-aggregation. Furthermore, analyzing the proteinaceous compounds involved in the bacterial surface adhesion revealed the key role of lipopetide and glicopeptide in the auto-aggregation of different isolates in aforementioned study. Mallappa et al. [40] investigated the cell surface hydrophobicity of the probiotic *Lactobacillus* isolates and revealed different hydrophobic properties. Several authors reported that surface adhesive properties of the LAB such as their hydrophobicity and aggregation are important capabilities in probiotic screening and characterization. These properties are correlated with adhesion to abiotic and biotic surfaces, and they influence microbial interaction with their environment. It is believed that extracellular matrix proteins in Gram-positive bacteria act as colonization bridges with endothelium cells, and they play a crucial role in adhesive contact with the host cells in GI tract. Bacterial adhesion to the GI tract is a complex process involving several non-specific and specific extracellular and cell surface ligand-receptors. Thus, variation in the composition of surface components associated with the hydrophobic (proteinaceous materials) and hydrophilic (polysaccharides) interactions, as well as their physicochemical characteristics, is responsible for differences in cell surface hydrophobicity values among probiotic isolates. Furthermore,

it is reported that hydrophobicity may affect auto-aggregation potential [17].

Probiotic LAB should have intrinsic antibiotic resistance, without resistance transfer ability. Maintenance of intestinal microflora by these LAB strains is important in antibiotic treatments. Concerning the antibiotic resistance, some *Lactobacillus* reportedly had less resistance to β-lactam antibiotics such as penicillin, whereas most of them have a high natural resistance towards glycopeptides like vancomycin. Minimum values of antibiotic susceptibility were observed for the *Lact. kunkeei* strains in Sakandar et al. [38] survey. A similar profile was also determined in the present project. Wahab et al. [41] investigated probiotic potentials of three levan producing Bacillus strains isolated from a different honey. They reported that the vegetative cells of the isolates were sensitive to the tested antibiotics. Begum et al. [6] revealed that *G. oxydans* isolated from honey as a potential probiotic bacterium with siderophorogenic potential was resistant to penicillin and rifampicin, whereas it was susceptible to gentamycin, tetracycline, kanamycin, and erythromycin. Enzymatic alteration, modification and inactivation of the antibiotics, hydrolyzing and destruction of the antibiotic molecules, reduced antibiotic permeability, and efflux pump are the well-known ways of antibiotic resistance in LAB. Some chromosomal or plasmid genes and episomal proteins involved in the mechanisms were revealed. Antibiotic mode of actions also includes interference with the cell-wall synthesis and changes in membrane permeability, inhibition of metabolic pathways, and interference with protein and nucleic acid biosynthesis [42].

Lact. kunkeei ENH01 demonstrated potent anti-aflatoxigenic activity based on the results. Chlebicz and Śliżewska [43] investigated the effect of different strains of *Lactobacillus* on common feed mycotoxins like aflatoxin B_1, deoxynivalenol, fumonisins, and zearalenone. They realized that all the strains had proper detoxification capability. The highest reduction (62–77%) was observed in fumonisins. Guimarães et al. [44] reported that the anti-aflatoxigenic effect of *Lact. plantarum* UM55 CFS was independent of the fungal growth. They demonstrated that the low pH of CFS due to the presence of organic acids like lactic, phenyllactic, hydroxyl phenyllactic, and indole lactic acids was the main reason to inhibit aflatoxin production. Compared with the physico-chemical approaches, bioremediation of mycotoxins is a safer and more specific reaction. There are several mechanisms for biological detoxification including microbial degradation of the toxins to less harmful metabolites, repression of toxin expression, inhibition of mycotoxin production, changing their structure or conjugation with these compounds by LAB metabolites such as enzymes (deepoxidases, epoxidases, and oxidase) and phenyllactic acid, and surface binding to the microbial cell wall. Hydrophobic interactions between mycotoxins and the cell wall components (peptidoglycans, polysaccharides, and teichoic acid) are significant in the latest mechanism. Furthermore, the influence of microbial catabolic pathways and their specific enzymes on altering coumarin structure and cleavage of the difuran ring has been clarified during microbial degradation of aflatoxins [36, 45]. Considering the stronger effect of the heat-killed cells compared with viable cells in the present study, it can be assumed that physical absorption has a key role in anti-aflatoxigenic capability of the isolate.

According to our results, in vivo safety of *Lact. kunkeei* ENH01 was verified. Similar findings were reported by Sahoo et al. [46] and Sadeghi et al. [19]. These researchers revealed no significant difference with respect to the level of liver enzymes and blood biochemical parameters among the LAB fed-mice and control. Oral administration of probiotic LAB is well tolerated and proven to be safe because of their long use as microbial cultures in fermented foods, and the time has come to carefully explore the prophylactic and therapeutic applications of these bacteria. Meanwhile, blood biochemical profiles and hematological parameters as important up-to-date diagnostic tools reflect the general metabolism status and physiological conditions of the fed-mice. Additionally, liver enzymes are common biomarkers used to investigate liver function and general health status. Alteration in the level of these enzymes in blood serum due to liver damage or injury has been revealed in several types of inflammatory and infections. Overall, the in vivo safety parameters studied in the present study were within the normal range reported in literature [47].

Conclusion

Evaluation of probiotic and postbiotic properties of the LAB isolated from non-dairy substrates is a promising approach to prepare functional microbial cultures or bio-preservatives. In the present study, *Lact. kunkeei* ENH01 was selected as probiotic LAB isolated from natural honey. In vitro antibacterial and antifungal effects of the isolate were also verified on foodborne indicators. LC/MS analysis led to the detection of three antibacterial peptides in *Lact. kunkeei* CFS. Furthermore, potent in situ inhibitory activity of the isolate CFS against *C. albicans* was approved in sugar-free lemonade containing honey. No adverse effect was found considering the blood biochemistry and hematological parameters in the LAB fed-mice. Anti-aflatoxigenic capabilities of *Lact. kunkeei* ENH01 as viable and heat-killed cells were also noticeable. Accordingly, probiotic potentials of *Lact. kunkeei* ENH01 and postbiotic functionalities of the isolate CFS and its heat-killed cells were revealed.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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