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PROBIOTIC CHARACTERIZATION OF *Levilactobacillus brevis* CM04 ISOLATED FROM HANOI-PICKLED DONG DU (*Brassica campestris* L.)

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ABSTRACT

Hyperuricemia, characterized by increasing concentrations of serum uric acid, is associated with metabolic disorders such as gout, diabetes, and heart diseases. Hyperuricemia is a prevalent condition with a rapidly increasing global incidence. Probiotics represent the promising option for preventing and treating hyperuricemia without side effects. Microorganisms can be considered probiotics; they must pass safety evaluations. This study assessed the probiotic characteristics of *Levilactobacillus brevis* CM04. *L. brevis* CM04 was isolated from Hanoi-pickled Dong Du (*Brassica campestris* L.) and exhibited high purine degradation ability. This strain showed a strong survival capability in the oral-gastrointestinal assay and adhered to intestinal cells more effectively than *Lactobacillus rhamnosus* GG (LGG). CM04 grew well in both media with and without sodium taurocholate, however, it did not exhibit bile salt deconjugation activity. Additionally, CM04 exhibited strong antimicrobial activity against pathogenic bacteria and sensitivity to all tested antibiotics. These findings indicate that *L. brevis* CM04 possesses probiotic characteristics.

Keywords: CM04, hyperuricemia, probiotic characteristics, safety test.

INTRODUCTION

Hyperuricemia is a major risk factor for gout, cardiovascular diseases, and metabolic disorders linked to increased serum uric acid levels. In the human body, uric acid is the end product of purine catabolism (Li *et al.*, 2020). Under normal conditions, it is filtered by the kidneys and primarily excreted through urine. Blood uric acid concentrations exceeding 350 - 420 µmol/L are considered abnormal, indicating excessive uric acid production or impaired kidney function. Prolonged elevation of uric acid levels can lead to the deposition of urate crystals in soft tissues, which is the primary cause of gout. Currently, pharmacological agents such as allopurinol, febuxostat, benzbromarone, and lesinurad are used to reduce serum uric acid levels by inhibiting its synthesis, enhancing its excretion, or

promoting its catabolism. However, these drugs require long-term use and are associated with various side effects, including abdominal pain, diarrhea, nausea, gastrointestinal irritation, skin rashes, severe hypersensitivity reactions, renal failure, and elevated liver enzyme levels (Pillinger & Mandell, 2020). Given these concerns, finding a safer alternative for managing hyperuricemia is crucial.

Probiotics are living microorganisms that offer health benefits to the host when administered in appropriate doses (Reid, 2005). They help to balance the gut microflora, enhance the immune system, reduce gastrointestinal infections, and protect intestinal health from pathogenic bacteria. Probiotics have been known for their anti-colitis (Santos Rocha *et al.*, 2014), antimicrobial, anti-inflammatory, antioxidant (Kuda *et al*., 2014), and anti-diabetic (Honda et al., 2012) effects, as well as their ability to alleviate hyperuricemia (Zhao *et al.*, 2022). The promising anti-hyperuricemic potential of *Lactobacillus* was demonstrated in some previous studies. For example, *Lactobacillus brevis* MJM60390, isolated by Lee et al. (2022), exhibited the highest inosine and guanosine assimilation among 24 lactic acid bacteria (LAB) strains, reducing serum uric acid levels to normal in an *in vivo* hyperuricemia model after two weeks of oral administration (Lee *et al.*, 2022). Other LAB strains, such as *Limosilactobacillus fermentum* NCUH003018, *Limosilactobacillus reuteri* NCUH064056, and *Lactobacillus gasseri* NCUH066006, screened by Wu *et al.* (2022), also show anti-hyperuricemic potential (Lin *et al.*, 2022). However, for being probiotics, candidate strains must meet specific criteria, including the absence of hemolytic activity, the ability to adhere to intestinal cells, sensitivity to antibiotics, resistance to bile acids, and the ability to survive oral-gastrointestinal stress to exert their probiotic potential (Lee *et al.*, 2015). In this study, the commercial probiotic *Lactobacillus rhamnosus* GG (LGG) was used as a reference strain.

Isolation and selection of probiotic strains from traditional Vietnamese fermented products is an important research direction to exploit beneficial microorganisms for health. From pickled ginger and fig samples, the *L. plantarum* SM1.3 strain with high probiotic activity was selected for use in the fermentation process of a ginger-based probiotic beverage (Huong *et al.*, 2022). Another study isolated 296 lactic acid bacterial colonies from four nem chua samples, among which the *Pediococcus pentosaceus* NC4 and *Lactobacillus plantarum* NC3.1.3 strains exhibited good biological activity for application in producing probiotic products (Nhung *et al.*, 2024). Pickled Dong Du (*Brassica campestris* L.) is a traditional fermented vegetable dish in Vietnam, particularly popular in Hanoi. It is made from *B. campestris* L., salt, sugar, shallots, green onions, and chili peppers. During fermentation, the bacterial population increases (Sharma *et al.*, 2020). Pickled Dong Du is known to provide various health benefits, including rich nutritional content, improved digestion, enhanced immunity, weight reduction, stress relief, anti-cancer properties, and support for heart and bone health. In our previous study, among 15 lactic acid bacteria strains isolated from Hanoi-pickled Dong Du, the *L. brevis* CM04 strain was found to reduce 100% of purine after 2 h of treatment (Huong *et al.*, 2025).

This study focused on assessing the probiotic characteristics of the CM04 strain,

derived from Hanoi-pickled Dong Du (*B. campestris* L.). The aim was to characterize the CM04 strain to determine its suitability as a probiotic for managing hyperuricemia by evaluating its gastrointestinal survival, intestinal adhesion, bile salt tolerance, antimicrobial activity, and antibiotic sensitivity, with the goal of exploring its potential for domestic production as a safe and effective probiotic to address the rising incidence of hyperuricemia.

MATERIALS AND METHODS

Oro-gastrointestinal transit assay

An oro-gastrointestinal (OGI) transit assay was used to examine the survival of CM04 after passing several inclement environments such as acid pH, bile, and digestive juice (Palaniyandi *et al.*, 2020). In brief, 10^9 CFU/mL of *L. brevis* CM04 and *L. rhamnosus* GG (positive control) were initially treated with oral stress solution (sodium chloride, 6.2 g/L; potassium chloride, 2.2 g/L; calcium chloride, 0.22 g/L; sodium bicarbonate, 1.2 g/L; lysozyme, 0.15 g/L) for 10 min at 37°C. After incubation, cells were centrifuged at 4000 rpm for 10 min, then subjected to the first gastric stress solution (pH 3.0) for 30 min, then treated with the second gastric stress solution (pH 2.0) for 30 min. Finally, the cells were resuspended in intestinal solution (sodium chloride, 5 g/L; potassium chloride, 0.6 g/L; calcium chloride 0.25 g/L; pancreatin, 1 g/L; bile bovine, 3 g/L; pH 7.0) for 2 h. CM04 and LGG which were treated with only phosphate-buffered saline (PBS) instead of the stress solution during this process were used as the control group. At each step, cells were diluted and plated on de Man, Rogosa, and Sharpe (MRS) agar to detect live cells by counting and calculating the number of colonies after 48 h incubation.

Bile salt deconjugation

MRS agar added with or without 5 g/L sodium taurodeoxycholate monohydrate (Sigma-Aldrich, MO, USA) was used to establish the bile salt deconjugation ability of *L. brevis* CM04 according to the methodology described by Ahn *et al.* 2003). After 48 h incubation at 37°C, bile salt hydrolase (BSH) activity was observed via the opaque white precipitate and halo zone surrounding the colonies on the bile acid-containing plate.

Adhesion to HT-29 cells

The adhesion assay was performed following a previously described method using HT-29 cells with some modifications (Yu *et al.* 2019). Briefly, HT-29 cells were seeded at a density of 1×10^5 cells/well in 12-well plates and cultured in RPMI-1640 medium with 10% fetal bovine serum (FBS), and 1% penicillin-streptomycin (Avenue, Waltham, MA, USA) in an incubator at 37°C with 5% CO_2. After 24 h incubation, cells were washed 2 times with PBS and starved with the FBS-free RPMI 1640 medium for 1 h. Following the incubation, 2×10^8 CFU/mL of bacteria was inoculated into each well for 2 h. HT-29 monolayers were washed with PBS thrice to remove non-adhered LAB and incubated with 0.5 mL trypsin (Sigma-Aldrich, USA) for 3 min at 37°C. Harvest incubated cells, diluted and plated on MRS agar culture at 37°C for 48 h to determine the adherent bacterial cells. The ratio of remaining bacteria grown on MRS agar in the initial inoculation bacteria was calculated and was adhesion activity. All experiments were performed in triplicate.

Susceptibility of CM04 strain to antibiotics

Nine (9) popular antibiotic types following the European Food Safety Authority (EFSA, 2018): ampicillin, tetracycline, streptomycin, gentamycin, vancomycin, kanamycin, erythromycin, clindamycin, chloramphenicol, and two-fold broth microdilution method were used to determine the minimum inhibitory concentration (MIC) of the CM04 strain (Rychen *et al.*, 2018; Wiegand *et al.*, 2008). The MIC cutoff values for various antibiotics recommended by the EFSA (Rychen *et al.*, 2018) for obligately heterofermentative *Lactobacillus* were used to determine the antibiotic susceptibility of *L. brevis* CM04 after 48 h of treatment.

Antimicrobial assay

The antimicrobial activity of *L. brevis* CM04 was assessed using the agar well diffusion test. In short, CM04 was inoculated at 37°C in MRS broth for 24 h. The supernatant was collected by centrifugation, then filtered and dropped into wells (6 mm diameter) of LB agar plates containing one type of pathogenic strains: *Staphylococcus aureus* CCARM 3640, *S. aureus* CCARM 3090 (CCARM means Culture Collection of Antimicrobial Resistant Microbes, provided by the Korea National Research Resource Center), *Salmonella enterica* ATCC 14028, *Kocuria rhizophila* NBRC 12708 (provided by NITE Biological Resource Center (NBRC)), *Bacillus subtilis* VTCC 6633, *Enterococcus faecalis* 19433, *Klebsiella pneumoniae* ATCC 10031, *Escherichia coli* ATCC 25922 and *Vibrio* sp. MH1 (provided by the Institute of Biological and Food Technology, Hanoi Open University). After 48 h incubation at 37°C for 24 h, measuring diameters of inhibition zones to detect antibiotic susceptibility. All the tests were conducted in triplicate.

Hemolytic activity

Following the American Society for Microbiology guidelines (Gerhardt *et al.*, 1981), the hemolytic activity of *L. brevis* CM04 was confirmed. Briefly, *L. brevis* CM04 and LGG (positive control) were streaked in trypticase soy agar containing sheep blood (5 g/L), and cultured at 37°C for 24 h. Finally, the hemolytic activity was observed with backlighting. The clean zone, similar to the base medium color area surrounding the colony, was measured to determine hemolytic activity.

Statistical analysis

All data were analyzed using a T-test method on the Microsoft Excel program. The *p*-value less than 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

OGI transit assay

If probiotics are alive and in adequate amounts after entering the host, they can benefit the host's health. However, oral probiotics face multiple challenges as they pass through the mouth, stomach, intestine, and colon. Their health benefits are largely reduced due to a significant decline in viable probiotic bacteria when exposed to the harsh conditions of the oral gastrointestinal tract (Han *et al*., 2021). In this study, the tolerable ability of the CM04 strain against inclement environments was tested by subjecting it to OGI transit stresses. The survival of the CM04 is shown in Figure 1. Without stress, the cell viability of LGG and CM04 was not

changed. With oral and gastric stress, both LGG and CM04 showed a slight reduction but not significantly. However, the log unit CFU after passing through intestinal stress was significantly reduced by 1.28 log unit CFU ($p < 0.01$) and 0.86 log unit CFU ($p < 0.05$) compared to the initial counts for LGG and CM04, respectively. However, the log unit CFU of CM04 after the OGI assay was 8.18, still higher than LGG (Figure 1). This indicates that CM04 can withstand harsh conditions in the digestive tract and fulfills the criteria of a probiotic.

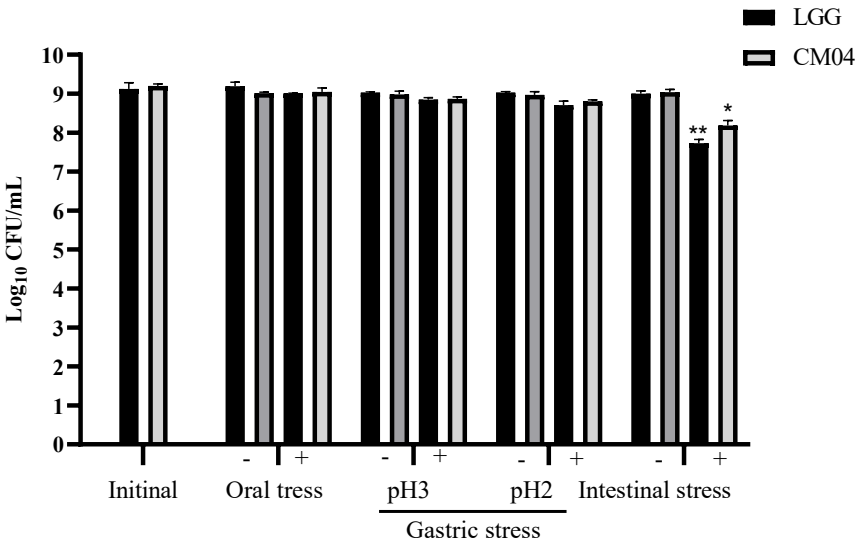

Figure 1. Oro-gastrointestinal transit assay of *L. brevis* CM04 and *L. rhamnosus* GG (LGG)**.** – indicated nontreatment, + indicated treatment. Results are presented as the mean ± standard deviation of triplicate independent experiments. *$p < 0.05$, **$p < 0.01$, compared to the LGG.

Bile salt deconjugation

Another characteristic of probiotics is bile salt deconjugation, which enhances the survival of LAB in gastrointestinal conditions. However, excessive bile salt deconjugation is harmful to the human intestine (Begley *et al.*, 2006). Therefore, it remains unclear that the BSH activity is truly a beneficial trait in a probiotic bacterium. The BSH activities of *L. brevis* CM04 and LGG were determined by using MRS agar plates supplemented with 0.05% sodium taurodeoxycholate monohydrate. In this study, CM04 grew well in both media with and without sodium taurocholate; however, there were no opaque white precipitates or halo zones surrounding the colonies on the bile acid-supplemented plate for both LGG and *L. brevis* CM04 (Figure 2). Similar to LGG, CM04 did not exhibit bile salt deconjugation.

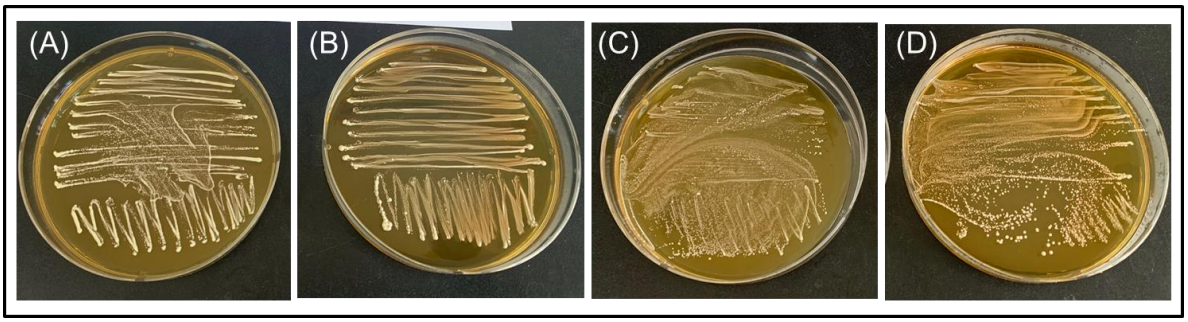

Figure 2. Deconjugation of sodium taurocholate by *Lactobacillus*. (A) LGG in an agar plate without sodium taurocholate. (B) LGG in an agar plate with sodium taurocholate. (C) CM04 in an agar plate without sodium taurocholate. (D) CM04 in an agar plate with sodium taurocholate.

Adherence of CM04 to HT-29 cells

Probiotics contribute to the host's health by acting and interacting with the host. For this, probiotics generate metabolites by producing enzymes, short-chain fatty acids, vitamins, and/or other secondary compounds. Therefore, probiotics have to bind to intestinal epithelial cells. As shown in Figure 2, CM04 and LGG strains exhibited strong adhesion to HT-29 cells. The adherence rate of *L. brevis* CM04 (5.31%) was significantly higher than *L. rhamnosus* GG (3.79%, $p < 0.001$) (Figure 3).

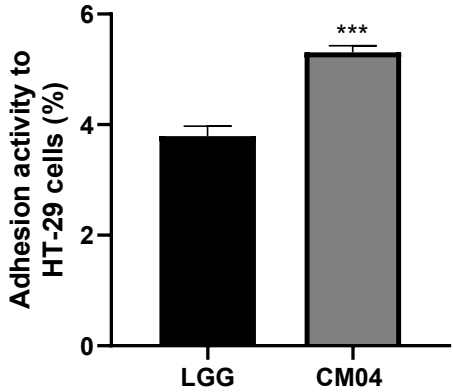

Figure 3. Adherence ability of CM04 strain to HT-29 cells. Results are presented as the mean ± standard deviation of triplicate independent experiments. *** $p < 0.001$, compared to the LGG.

Antibiotic susceptibility

The evaluation of antibiotic sensitivity is a crucial aspect of safety assessment. The overuse of antibiotics has become a significant societal issue, leading to the emergence of numerous antibiotic-resistant strains. If resistance-related factors are transferred to other microorganisms, particularly pathogens, through probiotics, they could pose serious health risks. The presence of a small number of lactobacilli with atypical and undesirable resistance to certain antibiotics indicates that not every

strain is appropriate for use as a probiotic or bacterial therapeutic agent (Anisimova *et al.*, 2022). Moreover, if probiotics are sensitive to antibiotics, they can be easily destroyed in necessary cases. Results in Table 1 demonstrated that CM04 is susceptible to 9/9 types of antibiotics tested.

Table 1. Antibiotic susceptibility assessment of LAB. *L. brevis* CM04's minimum inhibitory concentrations (MIC) against a variety of antibiotics.

Safety test	***L. brevis* CM04**	**LGG**
Antibiotics*	MIC (µg/mL)	MIC (µg/mL)
Ampicillin	1	1
Tetracycline	4	1
Streptomycin	16	32 (R)
Gentamycin	16	32 (R)
Vancomycin	256 (NR)	256 (NR)
Kanamycin	16	16
Erythromycin	1	1
Clindamycin	1	1
Chloramphenicol	4	4

*NR not required, R resistant, *MIC value for the antibiotics recommended by European Food Safety Authority (EFSA), 2012.*

Antimicrobial assay

Bacteriocins are antibacterial proteins produced by various microorganisms such as lactic acid bacteria. Unlike other antibiotics, which are secondary metabolites that may cause side effects in humans, bacteriocins are hydrolyzed in the digestive tract without leaving any residue. This makes them suitable for replacing antibiotics, preservatives, or bioregulators (Yang *et al.*, 2014). Therefore, the antibacterial activity of CM04 culture broth was performed. The results in Table 2 reveal that CM04 strains exhibited antibacterial activity against all 9 pathogenic bacteria. The antimicrobial activities of CM04 were strongest in *E. faecalis* 19433, *E. coli* ATCC 25922, and *S. aureus* CCARM 3640 with the inhibition zone diameter reaching 15 mm after 72 h of culture, followed by *S. enterica* ATCC 14028, *S. aureus* CCARM 3090, *K. pneumoniae* ATCC 1003 and *Vibrio* sp. MH1 with the inhibition zone diameter of 14 mm. The antimicrobial activity of CM04 against *B. subtilis* VTCC 6633 was the lowest (the inhibition zone diameter of 13 mm). That effect slightly decreased after 96 h of growth. This suggests that 72 h may be the optimal cultivation time for harvesting antimicrobial compounds from CM04, and extending the cultivation period could reduce activity, possibly due to antimicrobial compound degradation or changes in the culture environment.

Table 2. Antibacterial activity of *L. brevis* CM04 against intestinal pathogens.

No.	Strains	Diameter of zone inhibition of CM04 (mm)		
		48 h	72 h	96 h
1	*Bacillus subtilis* VTCC 6633	12 ± 1.0	13 ± 0.6	12 ± 0.6
2	*Enterococcus faecalis* 19433	13 ± 1.2	15 ± 0.6	14 ± 1.0
3	*Escherichia coli* ATCC 25922	15 ± 0.6	15 ± 1.5	14 ± 0.6
4	*Salmonella enterica* ATCC 14028	13 ± 1.2	14 ± 0.6	14 ± 1.2
5	*Kocuria rhizophila* NBRC 12708	13 ± 0.6	14 ± 1.0	12 ± 1.2
6	*Staphylococcus aureus* CCARM 3640	14 ± 0.6	15 ± 0.6	13 ± 1.0
7	*Staphylococcus aureus* CCARM 3090	9 ± 1.0	14 ± 0.6	13 ± 1.0
8	*Klebsiella pneumoniae* ATCC 10031	14 ± 1.5	14 ± 1.0	14 ± 0.6
9	*Vibrio* sp. MH1	13 ± 1.0	14 ± 1.2	12 ± 0.6

Hemolytic activity

Hemolysis is a major virulence factor of bacterial pathogens. A hemolysin is a substance that induces hemolysis, the breakdown of red blood cells. Hemolytic activity is used to assess the presence of safety-related virulence factors, including hemolysin production. To be considered a probiotic, LAB must lack the ability to cause hemolysis. The hemolytic activities of CM04, *Streptococcus mutans* (positive control), and LGG were evaluated on blood agar. As expected, strong hemolytic activity was observed in *S. mutans*, the positive control group, while no hemolysis was detected in the CM04 and LGG strains (Figure 4). These results are consistent with findings from other studies evaluating the safety of LAB strains for probiotic applications. For instance, Casarotti investigated the hemolytic activity of various *Lactobacillus* strains, reporting no hemolysis in probiotic candidates, confirming non-hemolytic behavior as a hallmark of safe LAB strains (Casarotti *et al.*, 2017). Similarly, Jang assessed the safety of the *L. brevis* KU15153 strain (Jang *et al.*, 2019), finding that non-hemolytic strains were suitable for probiotic use, while hemolytic strains were excluded due to potential pathogenicity. These comparisons highlight that the non-hemolytic profile of CM04 is consistent with the safety profiles of other LAB strains deemed safe for probiotic applications. These results demonstrate the safety of CM04 and its potential as a probiotic.

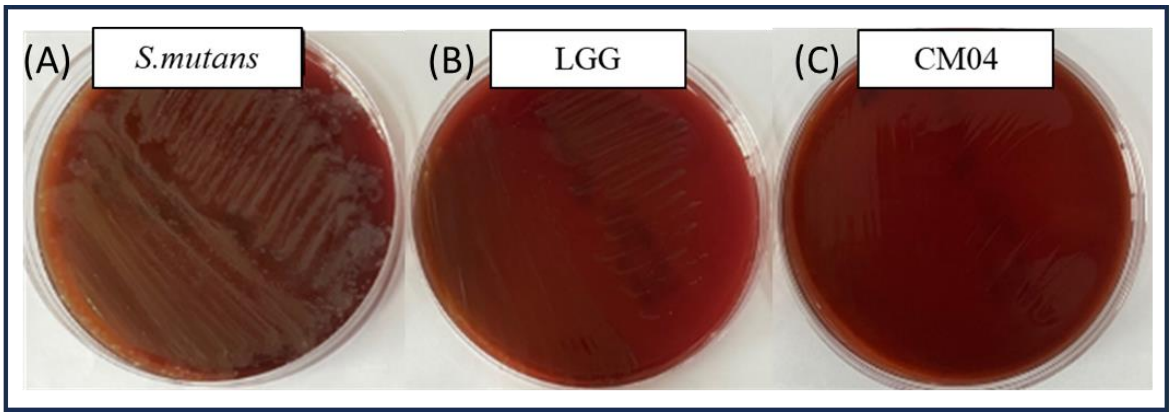

Figure 4. Hemolytic activity on the blood agar plate of (A) *S. mutans*, (B) LGG, and (C) CM04 after culturing at 37°C for 24 h.

CONCLUSION

In conclusion, this study thoroughly investigated the probiotic properties of *L. brevis* CM04, isolated from Hanoi-pickled Dong Du (*B. campestris* L.). The strain displayed robust tolerance to environmental stress conditions, acceptable antibiotic susceptibility, and pronounced adhesion to HT-29 epithelial cells. Safety evaluations, including hemolytic activity, bile salt deconjugation, and antimicrobial activity, confirmed its non-pathogenic profile. These results demonstrated *L. brevis* CM04 is a promising candidate for further research and potential application in probiotic-functional food development.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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