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Lactic Acid Bacteria Isolated from Dairy Products Exhibit Probiotic Potential and Anticancer Activity

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ABSTRACT

Background: In this study, we aimed to isolate bacterial strains from curd and buttermilk samples to explore their probiotic and anticancer potential.

Methods: We isolated five bacterial strains from curd and buttermilk samples sourced from various origins. Identification was performed using 16S rRNA gene sequencing. Probiotic potential was assessed through tolerance tests to low pH and bile salt concentration. Antimicrobial and co-aggregation activities were evaluated for selected strains. Cell-free supernatants (CFS) were tested for anticancer activity against cervical and breast cancer cell lines.

Result: Among the isolated strains, *Limosi lactobacillus* fermentum K-2, *L. fermentum* D-1, Weissella confusa U-2, Lactobacillus delbrueckii Br and L. delbrueckii M-2 were identified by 16S rRNA gene sequencing. Strains K-2, D-1 and U-2 exhibited probiotic potential with high tolerance (>90%) to low pH (3.0) and bile salt (0.3%) concentration. Strain K-2 showed efficient antimicrobial and co-aggregation activity against clinical pathogens. Additionally, CFS of the selected LAB strains displayed anticancer activity against cervical and breast cancer cell lines. The findings suggest that the isolated dairy probiotic has potential utility in functional food and therapeutic applications.

Key words: Anticancer, Auto-aggregation, Co-aggregation, *Limosi lactobacillus*, Probiotic.

INTRODUCTION

Breast and cervical cancers are the major causes of cancer incidence and mortality among Indian women (Sathishkumar *et al.*, 2021; Kulothungan *et al.*, 2024). Among molecular subtypes of breast cancer, triple-negative [ER-/PR-/HER2-] breast cancer (TNBC) is the most prevalent (Arpita *et al.*, 2021), followed by luminal A [ER$^+$/PR$^+$/HER2-] (Jonnada *et al.,* 2021) subtypes in the Indian women. In India, infection with human papillomavirus (HPV) types 16 and 18 account for nearly 76.7% of cervical cancer cases (Kaarthigeyan, 2012; Ramamoorthy *et al.,* 2022).

Cancer patients undertaking chemotherapy or radiation therapy often experience side effects such as nausea, vomiting, diarrhoea and loss of appetite, leading to lower dietary intake and weight loss, drug resistance and recurrence. Thus, there is an urgent need to explore adjunct therapeutic approaches for effectively managing breast and cervical cancer.

Recently, various studies have shown that intake of dietary supplements, probiotics and fermented food products, could overcome chemo- and radiation therapy-associated side effects and reduce cancer development (Wei *et al.*, 2018; Legesse Bedada *et al.*, 2020). Lactic acid bacteria (LAB) from the genera Lactobacillus, Bifidobacterium, Pediococcus and Streptococcus are generally considered as safe (GRAS) probiotics due to their non-pathogenic nature and beneficial effects (FAO/WHO, 2001). Fermented dairy products have been reported to be good sources of LAB (Sugandhi, 2018) with anticancer activity against cervical and breast cancer (Ayyash *et al.,* 2018; Krishnaprabha *et al.*, 2024; Asoudeh-Fard *et al.*, 2024).

In the present study, we have isolated and identified five different LABs from commercial dairy products such as curd and buttermilk and characterized them for their *in vitro* probiotic potential. Out of all the tested microorganisms, L. fermentum K-2, L. fermentum D-1 and Weissella confusa U-2 showed significant anticancer activity against HPV 16 (SiHa) and HPV 18 (HeLa) positive cervical and triple negative (ER$^-$/PR$^-$/Her2$^-$) (MDAMB231)

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and ER^+/PR^+/Her2-(MCF-7) breast cancer cell lines. Among these, *L. fermentum* K-2 was found to be the best probiotic with promising anticancer activity.

MATERIALS AND METHODS

Samples, media and bacterial strains

Five samples of curd and five of buttermilk were collected from different areas of Pune, Maharashtra. The samples collected in sterile glass containers, either used immediately for microbial isolation or kept at 4°C until further use. All the microbial growth media were purchased from HiMedia, Mumbai, India. Clinical pathogens, Staphylococcus aureus 2043 and Staphylococcus epidermidis 2044, Pseudomonas aeruginosa 2081 and Escherichia coli 2412 were procured from National Centre for Microbial Resource (NCMR), National Centre for Cell Science (NCCS) Pune.

Cultivation, isolation and maintenance of bacteria

Curd and buttermilk samples were diluted in sterile phosphate buffered saline (PBS, pH 7). For isolating lactic acid bacteria, aliquots from dilution were spread onto the De Man, Rogosa and Sharpe **(**MRS) agar (pH 6.5) plates and incubated at 37°C for 48 h using Gas Pak system. The purified isolates with rod shape cells, Gram positive in nature and with catalase negative test was selected and preserved in 25% (v/v) glycerol at -80°C or subjected for further identification using 16S rRNA gene sequencing from commercial services of NCMR, Pune. The clinical pathogens were also maintained and propagated in nutrient media at 37°C for 24 h. The bacterial growth was analysed as optical density (OD) at 600 nm using microplate reader (Epoch, BioTek Instruments, Inc, USA) and determined by colony count method.

In vitro confirmation of pathogenicity and probiotic traits

Isolates were characterized for probiotic traits such as non-pathogenicity, acid and bile tolerance study, antibacterial and aggregation activities. Non-pathogenicity of the selected LAB using hemolysis assay was performed as described previously (Kamble *et al.*, 2022). The ability of the strains to survive under gastric and intestinal conditions was evaluated by in vitro acid and bile tolerance studies (Kamble *et al.*, 2022).

The antibacterial property of the selected isolates was evaluated by agar well diffusion method (Khalil *et al.*, 2018). Cell-free supernatant (CFS) was prepared as described previously (Kamble *et al.,* 2022). 150 µl CFS was loaded into the wells (8 mm diameter). The plates were kept in the refrigerator (4°C) for CFS diffusion into agar and incubated at 35°C for 24 h. Antibacterial activity was determined by measuring the diameter of the zone of inhibition (mm) around the well. All the grown cultures were centrifuged at 3000 rpm for 5 min for the auto-aggregation assay. The resultant cell pellets were washed and resuspended in PBS (pH 7.0) buffer to reach OD_{600nm} of 0.5±0.1. Then, 2 ml of each bacterial suspension was mixed for 10s and incubated at 35°C without agitation. An aliquot was taken from the top of the suspensions after 0 and 5 h of incubation and measured absorbance at 600 nm. The auto-aggregation percentage was calculated as described in using following equation:

$$\%\ \text{autoaggregation}: 1 - \frac{OD_t}{OD_0} \times 100$$

Where,

OD_0 = Represents the absorbance of the mixture at 0 h.

OD_t = The absorbance of the mixture at 5 h.

Co-aggregation between selected probiotic strains and pathogens was carried out by the same method by only mixing the different strains in the ratio of 1:1. Control tubes containing 2 ml of each bacterial cell suspension alone were also made. The percentage of co-aggregation was calculated using the following equation (Janković *et al.*, 2012):

$$\%\ \text{co-aggregation} = [(OD1+OD2)/2]-OD3/(OD1+OD2)/2\times100$$

Where,

OD_1 and OD_2 = Optical densities of LAB and pathogen alone at 5 h, respectively.

OD_3 = Optical density of suspension of bacterial and pathogenic culture at 5 h.

Cell culture and cell viability

The human cervical (HeLa and SiHa) cancer cell lines were obtained from the National Centre for Cell Science (NCCS), Pune, Maharashtra, India. Breast (MCF-7 and MDA-MB231) cancer cell lines were procured from American Type Culture Collection (ATCC, Manassas, USA). The cells were grown in Dulbecco's Modified Eagle Medium (DMEM) with 10% Fetal Bovine Serum (FBS) supplemented with 2 mM L-glutamine and antibiotics (100 U/ml penicillin and streptomycin). The cells were incubated in a humidified 5% CO_2 incubator at 37°C.

The effect of different concentrations (0, 4, 8, 16, 32 mg/ml) of lyophilized CFS of *L. fermentum* K-2, L. fermentum D-1 and W. confusa U-2 on the cell viability (at density of 1×10^4 cells/ml) of Hela, SiHa, MCF-7 and MDA-MB231 cells was determined by (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) assay in 96-well plates as previously described (Deshpande *et al.*, 2016).

Statistical analysis

The data were presented as mean ± standard deviation (SD) and analyzed for statistical significance by two-way analysis of variance (ANOVA) using Tukey's multiple comparisons test. All the statistical analyses were performed by using Graph Pad Prism version 9 (GraphPad, San Diego, USA).

RESULTS AND DISCUSSION

Twenty-one bacterial isolates from different curd and buttermilk samples were isolated. All the isolates were studied for their colony and cell morphology characteristics and catalase activity. A total of 5 isolates (Br, D-1, K-2, M-2 and U-2) showing *Lactobacillus*-like morphology such as rods, Gram positive and catalase negative activity, were subjected to 16S rRNA sequencing. The sequencing data (Table S1) showed that these five isolates belonged to three different genera that includes *Limosi lactobacillus* (K-2 and D-1), *Lactobacillus* (Br and M-2) and Weissella (U-2). The sequences of the strains, *L. fermentum* K-2, *L. fermentum* D-1, W. confusa U-2, *L. delbrueckii* Br and L. delbrueckii M-2 have been deposited in GenBank (https://www.ncbi.nlm.nih.gov/) with accession numbers MZ066814, MZ066815, MZ066816, MZ066817 and MT093470, respectively.

The selected strains did not show any hemolytic zone around the colonies, revealing their γ-hemolytic activity and, thus, non-pathogenic nature.

In the acid tolerance study, among all the strains, W. confusa U-2 showed significantly ($p<0.0001$) higher viable cell count (9.25±0.03 log CFU/ml; SR: 97.99%) at acidic conditions, followed by *L. fermentum* K-2 (8.89±0.05 log CFU/ml; SR: 94.47%) and *L. fermentum* D-1 (8.88±0.06 log CFU/ml; SR: 95.28%) (Table 1). In the bile tolerance study, at 0.3% bile salt concentration, among all the strains tested, *W. confusa* U-2 showed a significantly higher viable count (8.96±0.04 log CFU/ml; SR: 94.92%) and higher bile salt tolerance ($p<0.0001$) (Table 1). One study has reported 86.20 and 84.99% survival rates at pH 3.0 and 0.3% bile concentration, respectively, for *L. fermentum* DUR 18 isolated from fermented food (Khalil *et al.*, 2018). W. confusa PUFSTMO55 isolated from idli batter showed good survival under acidic and bile conditions (Sharma *et al.*, 2018). The present study showed that *L. fermentum* K-2, L. fermentum D-1 and *W. confusa* U-2, displayed excellent acidic and bile tolerance properties and thus could be good candidates for future clinical use.

The antibacterial activity of CFS of K-2, D-1, U-2, Br and M-2 was assessed based upon their zone of inhibition (ZOI) against four clinical pathogens (Table 2). Overall, The CFS of *L. fermentum* K-2 exhibited higher antibacterial activity against S. aureus 2043, S. epidermidis 2044, E. coli 2412 and P. aeruginosa 2081 (Table 2). Similar results have been reported for CFS from *L. fermentum* 89-1 (Dallal *et al.*, 2016) against P. aeruginosa. CFS from the curd isolates, *Lactobacillus* casei, *L. delbrueckii*, *L. fermentum*, *L. plantarum* and *L. pentosus*, were reported to exhibit weak activity against P. aeruginosa, without inhibiting S. aureus and E. coli (Sharma *et al.*, 2017). On the other hand, Weissella confusa DD_A7 and W. confusa K3 isolated from kimchi and dairy products, respectively, exhibited antibacterial activity against *E. coli* (El-Mekkawy *et al.*, 2023; Krishnan *et al.*, 2019). The antimicrobial activity of each probiotic is strain-specific and thus it is an essential criterion for selecting the best probiotic. Probiotic bacteria

Supplementary Table 1: Taxonomic identity and sources of probiotic strain cultures.

Isolate	Source	Closest relative	Similarity (%)	Accession no. for deposited culture*
K-2	Buttermilk K	*Limosilactobacillus fermentum* CECT562^T	99.8	MCC 4683
D-1	Curd D	*Limosilactobacillus fermentum* CECT562^T	99.9	MCC 4682
U-2	Buttermilk U	*Weissella confusa* JCM1093^T	100	MCC 4684
Br	Curd Br	*Lactobacillus delbrueckii* ZN7a-9^T	99.8	MCC 4689
M-2	Curd M	*Lactobacillus delbrueckii* JCM15610^T	100	MCC 4688

Buttermilk sample K (BK) and sample U (BU) represent buttermilk obtained from different sources; Curd sample D (CD), Br (CBr) and M (CM) represent curd obtained from different sources. *All the identified cultures have been deposited at the National Centre for Microbial Resource (NCMR), Pune, Maharashtra, India.

Table 1: pH and bile salt tolerance of the identified strains.

Strains	pH Tolerance			Bile salt tolerance(0.3%, w/v)	
	Log CFU/ ml0 h	Log CFU/ ml3 h	Survival rate (SR) (%)	Log CFU/ ml3 h	Survival rate (SR) (%)
L. fermentum K-2	9.41 ± 0.04^{A}	8.89 ± 0.05^{A}	94.47	8.79 ± 0.08^{A}	93.41
L. fermentum D-1	9.32 ± 0.02^{AaB}	8.88 ± 0.06^{AaB}	95.28	8.63 ± 0.06^{AcB}	92.60
W. confusa U-2	9.44 ± 0.04^{AaBbC}	9.25 ± 0.03^{AdBdC}	97.99	8.96 ± 0.04^{AdBdC}	94.92
L. delbrueckii Br	9.22 ± 0.02^{AdBaCcD}	7.61 ± 0.19^{AdBdCdD}	82.54	7.30 ± 0.0^{AdBdCdD}	79.18
L. delbrueckii M-2	9.13 ± 0.04^{AdBdCcDa}	7.40 ± 0.15^{AdBdCdDd}	81.05	7.30 ± 0.0^{AdBdCdDa}	79.96

The data has been presented as mean±SD of three independent experiments, each performed in triplicates. The data was analyzed for statistical significance by two-way analysis of variance (ANOVA) with multiple comparisons followed by Tukey's test. Aap>0.05, Acp<0.001 and Adp<0.0001 compared to A (*L. fermentum* K-2); Bap>0.05, Bb*p*<0.05 and Bdp<0.0001 compared to B (*L. fermentum* D-1); Ccp<0.001 and Cdp<0.0001 compared to C (*W. confusa* U-2); Dap<0.0001 and Ddp<0.0001 compared to D (*L. delbrueckii* Br).

are known to secrete different antimicrobial substances or metabolites such as short-chain fatty acids (lactic, acetic or butyric acids), hydrogen peroxide, diacetyl, bacteriocins, peptides (Sharma *et al.*, 2017), hygroline, taraxinic acid glucosyl ester, avocadyne 2-acetate, hydroxypentadecanedioic acid (Rocchetti *et al.*, 2020; Kamble *et al.*, 2022).

The auto-aggregation assay was examined for the selected three probiotic strains on the basis of their sedimentation characteristics. *L. fermentum* K-2 exhibited significantly higher auto-aggregation (70.23±1.92%; p<0.0001), followed by *L. fermentum* D-1 (47.09±1.10%) and W. confusa U-2 (36.68±1.10%) (Table S2). Studies have reported 30% auto-aggregation for *L. fermentum* (Padmavathi *et al.*, 2018) and 70.19% for *L. fermentum* TCUESC01 (Melo *et al.*, 2017). The co-aggregation studies indicated that *L. fermentum* K-2 exhibited higher co-aggregation (58.75±1.42%; p<0.0001) with P. aeruginosa compared to either *L. fermentum* D-1 (40.96±1.55%; p<0.0001) or *W. confusa* U-2 (20.37±1.46%; p<0.0001) (Table S2). L. fermentum has been previously reported to show good co-aggregation with *P. aeruginosa* (Batoni *et al.*, 2023).

In the present study, we have evaluated the anticancer activity of CFS from *L. fermentum* K-2, *L. fermentum* D-1 and *W. confusa* U-2 against HPV 18 (HeLa) and HPV 16 (SiHa) positive cervical cancer cell lines; and ER^{+}/PR^{+}/Her2 (MCF-7) and $ER^{-}/PR^{-}/Her2^{-}$ (MDA-MB-231) breast cancer cell lines. The cells were treated with lyophilized CFSs at different concentrations (0-32 mg/ml) for 24 h. At higher dose of 32 mg/ml, CFS of K-2 and D-1 significantly (p<0.0001) reduced the viability of the tested cell lines (Fig 1 A-D). CFS of U-2, at a dose of 32 mg/ml, significantly (p<0.0001) reduced the viability of HeLa, SiHa and MDA-MB231 cancer cell lines, except for MCF-7.

Substantial data is available on the anticancer properties of probiotic strains, *L. fermentum* and *W. confusa*, against colorectal cancer (Arian *et al.*, 2019; Du *et al.*, 2023; Liu *et al.*, 2023) with very few studies against breast and cervical cancer cells. For instance, exopolysaccharide (EPS), isolated from *L. fermentum* DSM 20049, reduced the viability of MCF-7 cells with IC_{50} of 22.97 mg/ml (Khalil *et al.,* 2022). The intracellular total protein of *L. fermentum* K81, *L. fermentum* K85 exhibited 69.84±0.73 and 63.47±2.90% cytotoxicity against HeLa, respectively (Purkayastha *et al.*, 2020). Camel milk-derived probiotic strains, *L. plantarum*, *L. acidophilus*, *L. reuteri* and *L. lactis*, significantly inhibited the proliferation of MCF7 and HeLa (Ayyash *et al.,* 2018). Lactobacillus species, isolated from mulberry silage (Shokryazdan *et al.*, 2017), has shown anticancer activity against MDA-MB-231. Anticancer and apoptotic activity of conditioned medium from *L. fermentum* Ab.RS22, isolated from dairy products, was reported against HeLa *via* upregulation of PTEN and downregulation of AKT genes (Asoudeh-Fard *et al.,* 2024). The anticancer activity of cytoplasmic and cell wall extracts of commercial *L. delbrueckii* capsules have been reported against SiHa via the expression of apoptotic genes BCL2, caspase-3, caspase-9 and BAX (Bi *et al.*, 2023). EPS-5, isolated from *L. delbrueckii* DSM 20081, has shown anticancer activity against MCF-7 with IC_{50} of 7.91 mg/ml (Khalil *et al.*, 2022).

Table 2: Antibacterial potential of selected probiotics against pathogens.

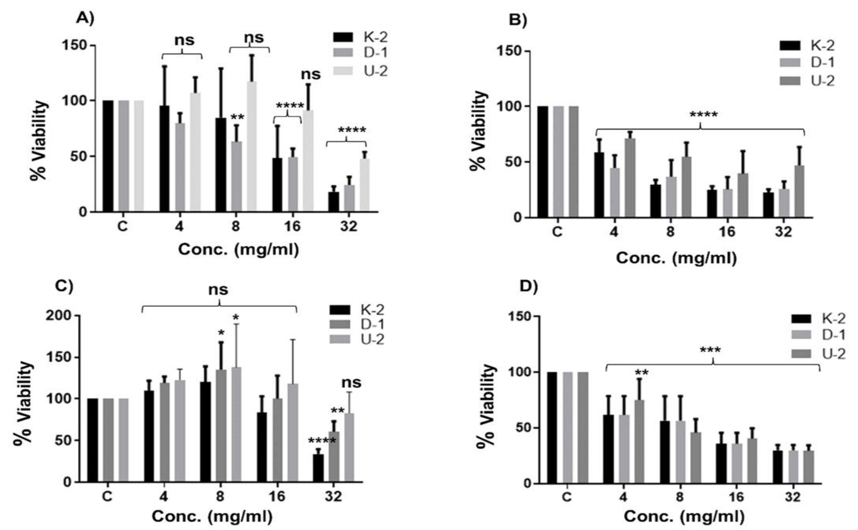
Probiotic strains	Zone of inhibition (mm)			
	S. aureus	*S. epidermidis*	*E. coli*	*P. aeruginosa*
L. fermentum K-2	16.78 ± 0.97^{A}	15.78 ± 0.67^{A}	18.00 ± 0.87^{A}	20.33 ± 0.50^{A}
L. fermentum D-1	15.78 ± 0.44^{AbB}	13.00 ± 0.87^{Ac}	14.78 ± 0.83^{AcB}	17.78 ± 0.83^{AcB}
W. confusa U-2	10.33 ± 1.12^{AcBc}	NA	13.33 ± 1.00^{AcBc}	17.00 ± 0.71^{AcBaC}
L. delbrueckii Br	NA	NA	NA	15.00 ± 0.50^{AcBcCcD}
L. delbrueckii M-2	NA	NA	NA	$10..00\pm0.71^{AcBcCcDc}$

NA indicates no activity. The data have been presented as mean±SD of three independent experiments, each performed in triplicates. The data was analyzed for statistical significance by two-way analysis of variance (ANOVA) with multiple comparisons followed by Tukey's test. Abp<0.05 and Acp<0.0001 compared to A (*L. fermentum* K-2); Bap>0.05 Bcp<0.0001 compared to B (*L. fermentum* D-1); Ccp<0.0001 compared to C (*W. confusa* U-2); Dcp<0.0001 compared to D (*L. delbrueckii* Br).

Supplementary Table 2: Auto-aggregation and co-aggregation properties of the selected probiotic strains.

Probiotic strains	Auto-aggregation	Co-aggregation (%)			
	(%)	*S. aureus*	*S. epidermidis*	*P. aeruginosa*	*E. coli*
L. fermentum K-2	70.23 ± 1.92^{A}	38.45 ± 1.07^{A}	31.93 ± 2.00^{A}	58.75 ± 1.42^{A}	47.64 ± 1.75^{A}
L. fermentum D-1	47.09 ± 1.10^{AcB}	36.96 ± 0.64^{AaB}	31.82 ± 0.75^{AaB}	40.96 ± 1.55^{AcB}	43.56 ± 1.98^{AbB}
W. confusa U-2	36.68 ± 1.10^{AcBc}	NA	NA	20.37 ± 1.46^{AcBc}	4.60 ± 1.98^{AcBc}

NA indicates no activity. The data have been presented as mean±SD of three independent experiments, each performed in triplicates. The data was analyzed for statistical significance by two-way analysis of variance (ANOVA) with multiple comparisons followed by Tukey's test. Aa*p*>0.05, Ab*p*<0.005 and Ac*p*<0.0001 compared to A (*L. fermentum* K-2); Bc*p*>0.0001 compared to B (*L. fermentum* D-1).



The effect of CFS from K-2, D-1 and U-2 was tested on the viability of A) HeLa, B) SiHa, C) MCF-7 and D) MDA-MB 231) by MTT assay. Data has been represented as Mean+SD; n=8. p>0.05 considered to be non-significant and ***(p<0.0001) indicate statistically significant difference.

Fig 1: Cell-free supernatant (CFS) of probiotic strains reduced the viability of cervical and breast cancer cell lines.

Probiotics have been shown to exhibit anticancer activity by a) secreting short-chain fatty acids (SCFAs) such as acetate, butyrate, propionate (Thirabunyanon and Hongwittayakorn, 2013) that lower the vaginal pH to 4.5 and prevent the growth of carcinogen-producing pathogens and increase mucosal viscosity of vaginal epithelium, thereby preventing the virus entry (Sharifian *et al.*, 2023b) binding of probiotics to genotoxic carcinogens (Legesse Bedada *et al.,* 2020c) producing anti-tumor compounds such as bacteriocin (Chuah *et al.,* 2019; Saeed *et al.,* 2025), peptidoglycan, exopolysaccharide (Khalil *et al.*, 2022), acetamide, thiocyanic acid (Sharma and Shukla, 2020) that inhibit cell proliferation; d) inducing apoptosis (Hadid *et al*., 2025) through upregulation of pro-apoptosis proteins (Bi *et al*., 2023e) induction of cell cycle arrest by upregulating p21, p53 (Liu *et al*., 2023f) activating immune system (Kim *et al*., 2021) and in effect exerting anti-proliferating activity. The overall data suggests that the anticancer activity of the CFS from the selected probiotic strains could be due to the secretion of various bioactive metabolites.

The present study has shown that dairy probiotics with anticancer potential could be explored as adjuncts to conventional therapies against cervical and breast cancer.

CONCLUSION

The isolated LAB from dairy samples exhibited potential anticancer activity against breast and cervical cancer cells. Such LAB strains could be proposed as functional food supplements to be used as adjuncts during cancer therapies. However, elaborate studies are warranted to understand the underlying mechanisms of these probiotics and their role in mitigation of the risk of cancer. Further studies, including therapeutic efficacy and adverse reactions of probiotics such as bacteremia, gastrointestinal side effects, antibiotic resistance and abnormal stimulation of the immune system in cancer patients, need to be carefully evaluated in the future.

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Data and materials availability

The obtained data will be available upon request.

Ethics approval

This study does not involve any animals or human participants.

Conflict of interest

The authors declare that there are no conflicts of interest.

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