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Postbiotic metabolites derived from lactobacillus fermentum as potent antiproliferative bioresources on HeLa cells with promising biocompatibility

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Abstract

Chemotherapy administrations for cervical malignancy possess a variety of unfavorable influences on the human body. Scientists are interested in microbial-derived biomolecules or postbiotics as an alternative therapeutic strategy in malignant patients. This research investigated the mechanisms related to the function of two potential postbiotic *Lactobacillus* isolates, *Lactobacillus fermentum* CH and *L. fermentum* KH, isolated from indigenous Iranian dairy products. The *Lactobacillus* isolates were recognized through 16S rDNA sequence analysis followed by characterization using morphological and biochemical assays. The bioactivity of postbiotics on the cervical cancer model was also assessed through a cytotoxic study and apoptosis analysis. In addition, the anticancer activity was evaluated by qPCR, followed by a confirmation of the flow cytometry. The results of the bioactivity assay revealed that these postbiotics had suitable anticancer influences on the cervical cancer model (HeLa cells) by increasing BAX, caspase8, and caspase9, followed by a decrease in BCl-2, iKB (Inhibitor of nuclear factor kappa-B), and RelA gene expressions. Thus, the findings of this study signify that the postbiotic derivate from *Lactobacillus* strains isolated from indigenous Iranian dairy products could be regarded as a topical treatment with a promising curative index due to their effectiveness on cervical malignancy cells.

Keywords Indigenous dairy products, Apoptosis, Postbiotics, Cervical cancer

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Introduction

Cervical cancer is the third most frequent form of malignancy among women and the fourth cause of mortality among women worldwide [1–3]. This cancer arises from the cervix due to an irregular cell growth with the ability to metastasize to other human parts of the body [4]. No indications are generally observed during the initial stages; however, later presentations may include pelvic pain, pain during sexual intercourse, and abnormal vaginal bleeding [5]. The human body hosts thousands of bacterial species, which helps maintain the host's health [6, 7]. Based on the total human microbiome, 9% of microbial species are associated with the urogenital system [6]. Hence, there is a need for a balancing connection between the urogenital system microbiome and the host immune system to support healthy urogenital system homeostasis. Any distress in microbiota balance can lead to urogenital tract infections, which finally may result in cervical cancer [8, 9]. Most chemical curative treatments presently administrated for cervical cancer have been cytotoxic to the human body. Consequently, it is crucial to recognize an alternative anticancer remedy with reduced side effects [10, 11].

Lactobacillus fermentum species are the dominant constituent of the gastrointestinal tract microbiome and enhance vaginal tract health by reducing infection risks [12, 13]. Moreover, they are frequent genera called probiotics [14, 15]. Probiotics are live microorganisms that usually benefit the host's health after administration in adequate amounts [16, 17]. Probiotics' mechanisms for protection against malignant diseases include hampering the pro-carcinogens to carcinogen conversion, deactivating mitogenic substances, reducing the growth of pro-carcinogenic bacteria, diminishing the mitogen absorption, and enhancing the immune system performance against cancerous cells [14, 18].

Recently, the term "Postbiotic" has been proposed as a novel word alongside Probiotic [19]. According to the International Scientific Association of Probiotics and Prebiotics (ISAPP), postbiotics are the "preparation of inanimate microorganisms and/or their components that confers a health benefit on the host" [20]. In this context, the postbiotic point out that unanimated probiotic bacteria can offer optimistic health benefits to humans. In contrast, numerous features connected to probiotic bacteria' therapeutic and efficiency traits are not dependent on their survival. More interestingly, previous designated systematic studies demonstrated that postbiotics have anticancer activities [21–24]. The key advantage of postbiotics is that their beneficial effects do not rely on bacterial survival in the GI tract. Instead, they consist of already-produced bioactive compounds, metabolites, and cell components that can directly interact with the host. This characteristic makes postbiotics potentially more stable and consistent in their effects compared to live probiotics. However, the mechanistic concepts behind using postbiotics to exert their anticancer activities remain unknown.

Programmed cell death generally occurs as a homeostatic mechanism for keeping up the cell population in the body [25–27]. Cancer cell proliferation abnormally increases due to functional mutations in apoptosis-associated genes; thus, targeting apoptosis is one of the primary goals in non-invasive cancer treatment [28–31]. Consequently, the anticancer outcome was assessed through alterations in the expression of apoptosis-related genes (BAX/Bcl2, Casp8, Casp9, RelA, and IKB (Inhibitor of nuclear factor kappa-B)).

Unfortunately, the safe administration of live probiotic bacteria, including *Lactobacillus* species, has not yet been fully validated. In this regard, caution should be applied for particular patients, such as those with compromised immunity and elderly individuals, those with cardiac valve disease, and those with short bowel syndrome [32]. Moreover, live probiotic bacteria should be adapted to the digestive system to stay alive and be administered in adequate numbers to provoke biological reactions. On the contrary, postbiotics can offer anti-inflammatory effects similar to probiotic bacteria, higher shelf life, and sustainability. Notably, the risk of pathological side effects and the infection transmission rate have been well-reduced in immunocompromised patients [33–35].

Several investigations on alternatives, including postbiotics, have been actively performed to reduce the possible risk after probiotic administration [36, 37]. Although there is a limitation among cancer individuals with considerable immune deficiencies to intake probiotics, few studies related to their anticancer activities are available. To this end, the current research aims to inspect the effect of postbiotics from two *Lactobacilli* isolated from native Iranian dairy products on cell proliferation and apoptosis in normal (HUVEC (Human umbilical vein endothelial cells)) and cervical cancer (Hela) cell lines.

Materials and methods

Bacterial isolation and growth conditions

Sixty-eight samples of traditional dairy products, including yogurt, were collected from 10 places in Gilan province, Iran, as provided in the flowchart (Fig. 1). One gram of each dairy sample was suspended in 9 ml of 2% w/v peptone water and vigorously vortexed for 30 s. One mL of each sample was added to 9 mL of MRS (Man, Rogosa, and Sharpe) broth (Merck, Germany) and made serial dilutions. These diluted solutions (0.01 mL) were spread on MRS agar plates (Merck, Germany) supplemented

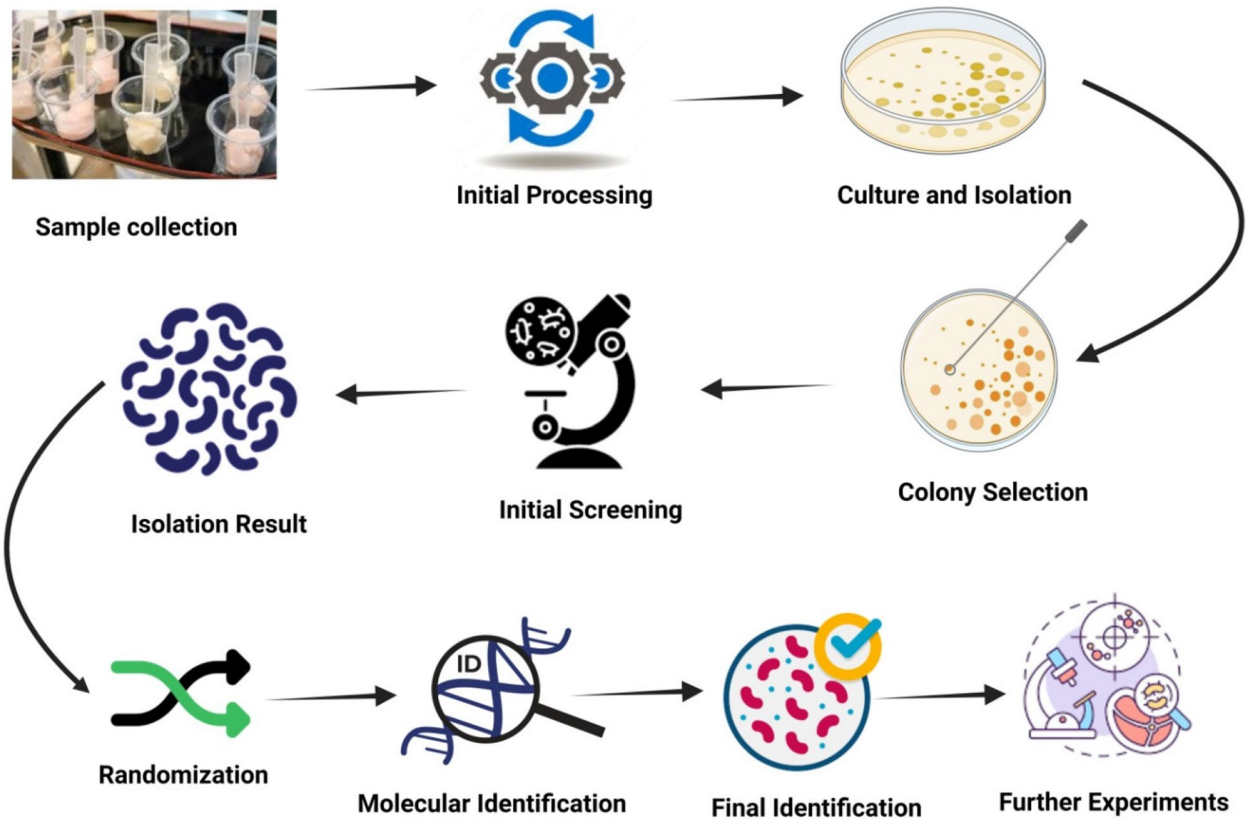

Fig. 1 Methodology flowchart for isolation and analysis of *Lactobacillus fermentum*

with 0.05% cysteine and incubated for 48 h. The single colonies on the growth agar plate were selected and transferred to a 15 mL broth culture medium for 24 h at 37 °C. Finally, two isolates were randomly chosen to evaluate the morphology, motility, oxidase, catalase, and gram's reaction. The non-motile, catalase, oxidase negative, and bacilli-shaped Gram-positive isolate was deduced to be *lactobacilli* and then stored in 30% (w/v) glycerol plus 10% (w/v) skim milk at − 70 °C for further assessments.

Identification of Lactobacillus isolates

The molecular technique was utilized to identify isolates. The genomic DNA of selected *Lactobacillus* isolates was extracted using the boiling method protocol [38]. The primers used to amplify the 16S rDNA sequences of these strains included RW01 5'-AACTGGAGGAAG GTGGGGAT-3' and DG74 5' -AGGAGGTGATCCAAC CGCA-3' [39, 40]. The fragments were amplified in a BioRAD Thermocycler (USA) using the following circumstances: 94 °C for 2 min, 30 cycles of 94 °C for 40 s, 50 °C for 30 s, and finally, 72 °C for 10 min. The obtained amplicons were run on an agarose gel and sequenced by Cinnagen Co, Tehran, Iran. All obtained sequences were searched using the Basic Local Alignment Tool (BLAST) program (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Preparation of cell-free postbiotic extract

The 10% of overnight cultivated *Lactobacillus* isolates were inoculated in a fresh MRS-Broth medium and incubated for 48 h at 37 °C in microaerobic conditions. Then, the cultures were centrifuged for 30 min at 5000 rpm, and the supernatant was removed. The bacterial cell precipitates were rinsed with buffered phosphate saline (PBS) at pH 7.2. To prepare the cell-free postbiotic extract, bacterial cell precipitates were first adjusted to different OD_{600} values (0.5, 0.75, 1.0, 1.5, and 2.0) to determine the bacterial concentration (in cfu/ml). Then, the suspension was sonicated at 12 Watts for 30 s, with 60-s intervals between cycles [41]. The filtration was used for supernatant sterilization, followed by evaluating the absence of growth in MRS broth after incubation for 48 h at 37 °C. All the specimens were maintained at − 80 °C.

Cell culture handling

The HeLa (Human Cervical Adenocarcinoma Cells, IBRC C11311) and Human Umbilical Vein Endothelial Cells (HUVEC, C554) were purchased from the

Iranian Biological Resource Center (IBRC). The HeLa and HUVEC cell lines were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% streptomycin-penicillin at 37 °C with 5% CO_2 in a humidified atmosphere. The healthiness, morphology, and cell numbers were screened using an inverted microscope. After reaching a minimum of 70% cell confluency, the cells were separated from the flask bottom by 0.05% trypsin, followed by centrifuging at 1500 rpm for 5 min. The obtained cell precipitate was suspended, and a Neubauer chamber and a Trypan blue stain measured the living cell percentage of this suspension. Cells with more than 90% viability were utilized for the trials after ensuring without contamination [42].

MTT assay

The MTT assay determined the cytotoxic impacts of two postbiotics obtained from indigenous probiotic isolates on HeLa and HUVEC cell lines. Each cell line was divided into a 100-mL aliquot containing 1×10^4 cells (in separately cultured HeLa and HUVEC cell lines) and seeded into 96-well plates following incubation for 24 h. The control groups were treated with the RPMI 1640 medium, the same solvent used for dissolving the postbiotics, without adding postbiotics. The concentrations mentioned above were selected based on the previous studies to cover a range that spans from sublethal to potentially cytotoxic concentrations, facilitating the identification of concentration-dependent trends in cell viability. Then, the various concentrations of two postbiotics (the OD_{600} value of 1.2×10^8, 1.8×10^8, 2.4×10^8, 3.6×10^8, and 4.8×10^8 cfu/ml) were separately added to each well, following the incubation of plates for additional 24 h. Afterward, 100 mL of MTT reagent (3-(4,5-dimethylthiazol- 2-yl)-2,5-diphenyltetrazolium bromide; Sigma, Germany) were added into each well at 0.5 mg/mL concentration. Then, the plates were incubated at 37 °C for 4 h in a humidified atmosphere with 5% CO_2 [43]. The formation of purple formazan crystals was dissolved by adding a dimethyl sulfoxide (DMSO) solution to each well. The optical density of each suspension was measured using an ELISA reader (Biotek, Power Wave, Winooski, VT, USA) at 540 nm. Finally, the outcomes were reported as the half-maximal inhibitory concentration (IC_{50}) values and the viability percentage.

Assessment of apoptosis by flow cytometry

The induction of apoptosis in the cells was evaluated using an Annexin V-FITC kit (Sigma-Aldrich, UK), as described by the manufacturer's instructions. The IC_{50} concentrations of postbiotic content of both isolates were used to treat the cells for 24 h. The analysis was conducted using a flow cytometry apparatus (BD FACSCalibur flow cytometer, Erembodegem, Belgium).

Gene expression analysis by Real-Time PCR

Total RNA of HeLa and HUVEC cell lines were extracted from treated cells with postbiotic for 24 h with a total RNA extraction kit (Pars Tous, Mashhad, Iran) following the manufacturer's protocol. The obtained cDNA was synthesized using the Revert Aid First Strand cDNA Synthesis Kit (Norgene Biotech, Canada) according to the manufacturer's protocols. The expression quantities of apoptosis-related genes (RelA, Casp9, Casp8, BAX/Bcl2, and IKB) in HeLa and HUVEC cell lines were determined using SYBR Green PCR Master mix with semi-quantitative RT-PCR (BioRad, USA). The primers used are listed in Table 1.

The PCR conditions were as follows: 94 °C for 2 min, followed by 35 cycles at 94 °C for 15 s, 55 °C for 30 s, annealing at 68 °C for 60 s, and a final extension at 72 °C for 5 min. The analysis of outcomes was performed using the delta–delta Cq approach. A melting curve was applied to analyze reaction specificity. In the end, the relative gene expression of the RelA, Casp9, Casp8, BAX/Bcl2, and IKB were calculated with the $\Delta\Delta C_t$ formol. So all the data were analyzed using the $2^{-\Delta\Delta Ct}$ formol, wherever $\Delta\Delta C_t = (C_{t,\ Target} - C_{t,\ Control})_{\ Time\ x} - (C_{t,\ Target} - C_{t,\ Control})_{\ Time\ 0}$ is each sample's treatment time and Time 0 represents the non-treatment control sample. The fold change in the target gene (RelA, Casp9, Casp8, BAX/Bcl2, and IKB), regulated to the reference gene (B-actin) and also relative to the expression in the non-treatment samples, was calculated for each sample using the $2^{-\Delta\Delta Ct}$ formol.

Statistical analysis

All experiments were conducted three times, and the results are presented as the mean and standard deviation.

Table 1 The primer's sequences used in qPCR

Gene	Forward (5′→3′)	Reverse (5′→3′)
BAX	ATCCAGGATCGAGCAGGGCG	GGTTCTGATCAGTTCCGGCA
Bcl-2	GTTCCCTTTCCTTTCCATCC	TAGGCCAGTCCAGAGGTGAAG
Caspase 8	CTGGGAAGGATCGACGAA TTTA	CATGTCCTGCATTTTGATGGG
Caspase 9	AGCCAGATGCTGTCCCATAC	CAGGAGACAAAACCTGGG AAA
RelA	CTGTGCGTGTCTCCCATGCA	TCGTCTGTATCTGGCAGGTA
IKB	GCTGAAGAAGGAGGCGGC TAC	TCGTACTCCTCGTCTTTTCAT
B-actin	ATGATGATATCGCCGGCC GCTC	CCCACCATCACGCCCTGG

A one-way analysis of variance (ANOVA) was used to identify significant differences. Statistical analyses were performed using SPSS software (Version 22, NY, USA). All analyses considered a *P*-value of less than 0.05 statistically significant.

Results

Isolation and identification of isolates

In the current study, 25 isolates were isolated from native dairy products. Among these, two isolates with the appearance of *Lactobacilli* on MRS agar were randomly chosen for additional experiments. In short chains, these isolates were Gram-positive, catalase, oxidase-negative, and non-motile rod-shaped. Finally, the molecular identification technique based on 16srRNA gene sequencing revealed that CH and KH isolates belonged to *Lactobacillus fermentum* with nearly 99% homology. The accession numbers of bacteri in NCBI databank are OP168796 and OP164565.

Cell proliferation assay

The cell viability of two postbiotic derivates from *L. fermentum* CH and *L. fermentum* KH were calculated against cancer and normal cells using an MTT assay. The viability of HUVEC cells treated with various postbiotic concentrations of both isolates was higher than 100%. In this regard, any increase in postbiotic concentrations showed no decrease in cell viability. However, only higher concentrations of both postbiotics (3.6×10^8 and 4.8×10^8 cfu/ml) showed significant antiproliferative effects on HeLa tumor cells in a dose-dependent manner (Fig. 2). The IC_{50} value of two postbiotics for HeLa cell lines after 24 h was 10 µg/mL.

Assessment of apoptosis by flow cytometry

The apoptosis induction outcomes in the HeLa and HUVEC cell lines upon treatment with two postbiotics at IC_{50} concentrations are presented in Fig. 3. In this context, the flow cytometry analysis was performed to determine apoptosis quantitatively in HeLa and HUVEC (as control) cells treated with two postbiotics for 24 h. The results of flow cytometry revealed 18.95% and 14.15% of total apoptotic cells for *L. fermentum* CH and *L. fermentum* KH postbiotic samples, respectively. On the other hand, treated HUVEC cells with two postbiotics of *L. fermentum* CH and *L. fermentum* KH at IC_{50} concentrations indicated 2% and 3.3% of total cell death, respectively. Moreover, the necrosis levels in treated HeLa cells

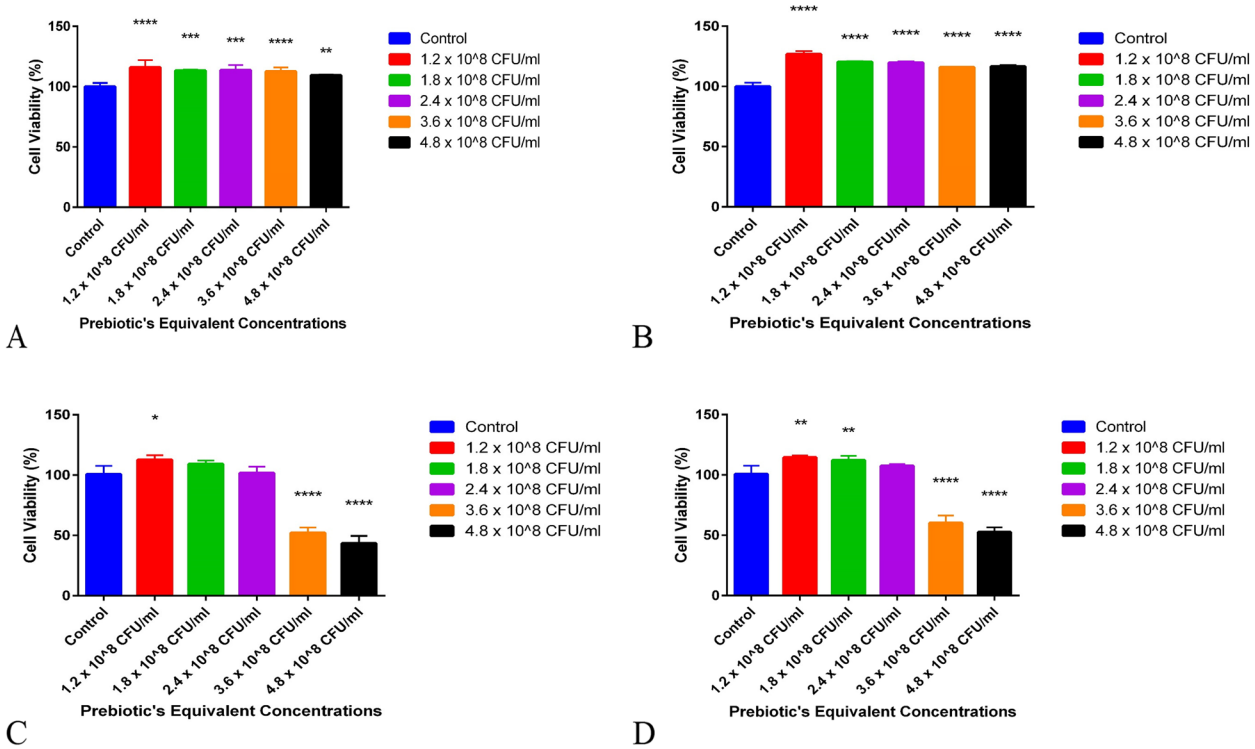

Fig. 2 The viability of examined cell line treated with postbiotics is presented: **A** Treatment of HUVEC cell line with postbiotic derivate from *L. fermentum* CH., **B** Treatment of HUVEC cell line with postbiotic derivate from *L. fermentum* KH, **C** Treatment of HeLa cell line with postbiotic derivate from *L. fermentum* CH, and **D** Treatment of HeLA cell line with postbiotic derivate from *L. fermentum* KH. The outcomes are provided as survival percentages compared to the triplicate controls ($n=5$; **** $p<0.0001$*** $p<0.001$; ** $p<0.01$; * $p<0.5$). SD: standard deviation

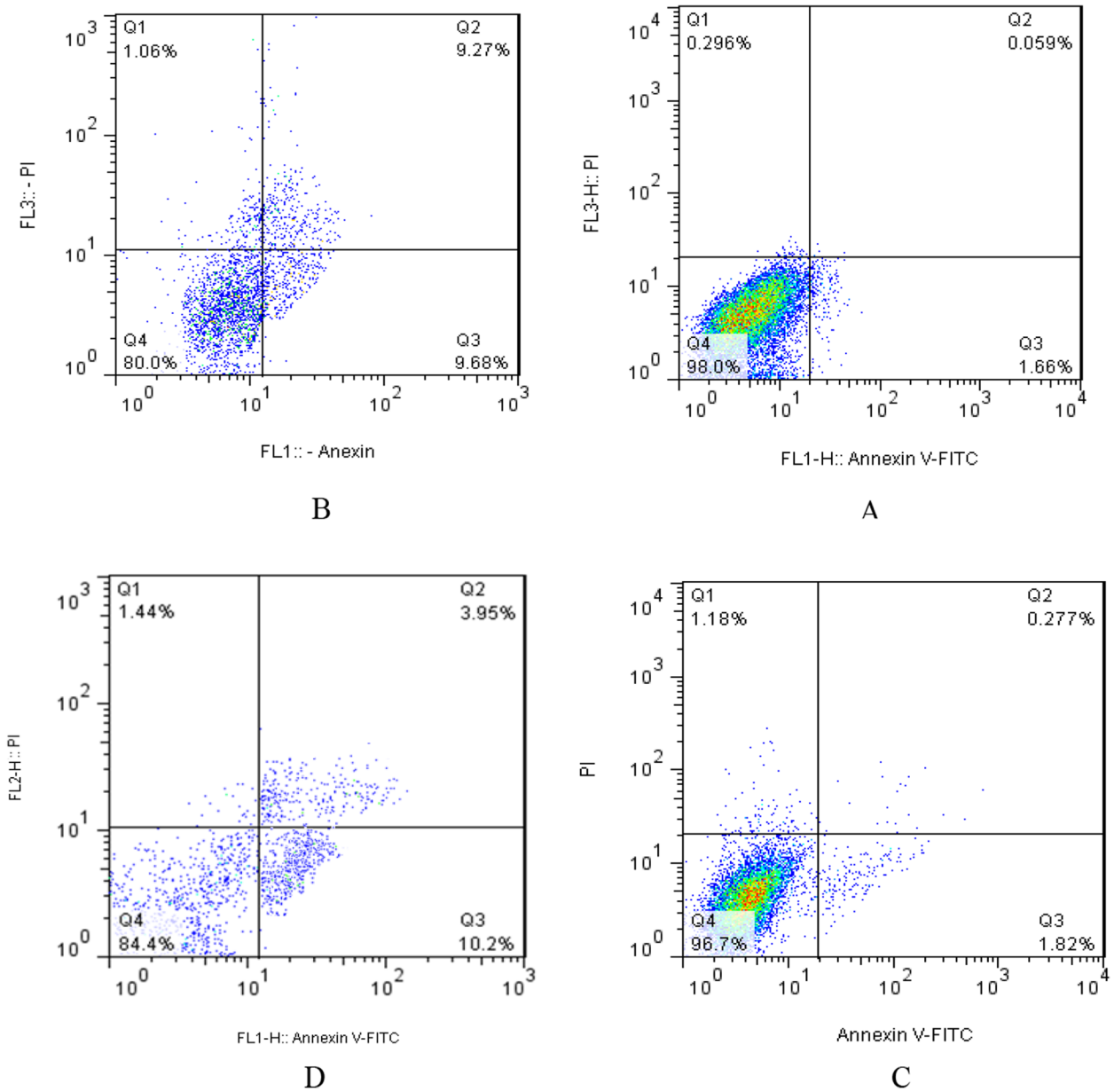

Fig. 3 Flow cytometer graphs are presented for assessing apoptosis provoked by postbiotic derivate from *Lactobacilli* sp. at 24 h in **A** control cell, **B** IC_{50} concentration of postbiotic derivate from *L. fermentum* CH., **C** control cell, and **D** IC_{50} concentration of postbiotic derivate from *L. fermentum* KH. Analysis was performed by Propidium iodide (PI)/Annexin V-stained cells. Representative figures demonstrate the population of late apoptotic (Q2) and early apoptotic (Q3) cells

with *L. fermentum* CH and *L. fermentum* KH postbiotic samples were 1.06% and 1.44% compared to control cells (0.29% and 1.18%), respectively.

Gene expression analysis by Real-Time PCR

To assess the molecular mechanism associated with the selected postbiotic samples for induction of apoptosis in cytochrome c as a cervix cancer model, the level of mRNA expression of RelA, Casp9, Casp8, BAX/Bcl2, and IKB was examined using Real-Time PCR. The β-actin gene was selected as the control due to the constant expression in all cells. The treatment of HeLa cells with IC_{50} concentration of both postbiotic samples (CH and KH) resulted in various expression levels of the genes mentioned above in HeLa cells compared to the control cell line. Significant upregulation was observed in Caspase 9, Caspase 8, and Bax genes in treated HeLa cells ($P < 0.001$) after 24 h compared to the control cells. In

contrast, remarkable downregulation was seen for Bcl2, RelA, and ikB genes in treated HeLa cells ($P < 0.001$) after 24 h compared to the control cell (Fig. 4). As an overall apoptosis induction estimation, the expression ratio of Bax/Bcl2 demonstrated a statistically remarkable rise after 24 h compared with the control cell ($p < 0.05$).

Discussion

Cervical cancer is regarded as the third most frequently recognized malignancy and the fourth foremost cause of cancer-associated mortality among women across the world [44]. The critical characteristic of malignancy cells is their resistance to apoptosis and uncontrolled cell propagation; thus, apoptosis induction in the cancer cells via simultaneous factors would be considered a novel way of cancer therapy.

Probiotics have several health benefits through modulating microbiomes and techno-functional restrictions, including lower shelf-life, that hinder their complete potential functions in the pharmaceutical sectors [21, 45]. Hence, the interest is progressively changing from live probiotic bacteria to non-viable postbiotic products. Postbiotics are emerging in the pharmaceutical and medical fields because they confer several health-supporting features. Postbiotics are usually defined as the multifaceted mixture of metabolic products, including excreted proteins, enzymes, vitamins, short-chain fatty acids, excreted biosurfactants, peptides, amino acids, and organic acids, into the culture medium or crude cell extracts [22]. Although various studies demonstrated that the effectiveness of probiotics could be harmless and impressive in individuals with no serious diseases or weak immune systems, some reports described several side effects in vulnerable patients, who need to be aware of its benefits and probable risks [46]. Therefore, we evaluated the postbiotic impact on the cervical cancer in vitro model.

Literature investigations revealed postbiotics' effectual and protective functions against malignancy cases in different in vitro, in vivo, and epidemiological studies [21, 33, 35, 41]. However, the possible impacts of postbiotics on cervical cancer have not been reported yet. In this regard, two bacterial isolates named *L. fermentum* CH and *L. fermentum* KH were used in the current study, and they were isolated from Iranian indigenous dairy products.

The comparison of various postbiotic concentrations showed that superior levels of postbiotic concentrations (3.6×10^8 and 4.8×10^8 cfu/ml) were needed to prohibit the proliferation of HeLa cell lines. In contrast, by increasing the postbiotics concentration, no inhibition was seen on HUVEC cell lines as a normal cell line. In this regard, postbiotics derived from *L. fermentum* CH showed more inhibitory activity than *L. fermentum* KH; however, the differences were not statistically significant. In total, these postbiotics obtained from the two isolated bacteria showed inhibitory activity against

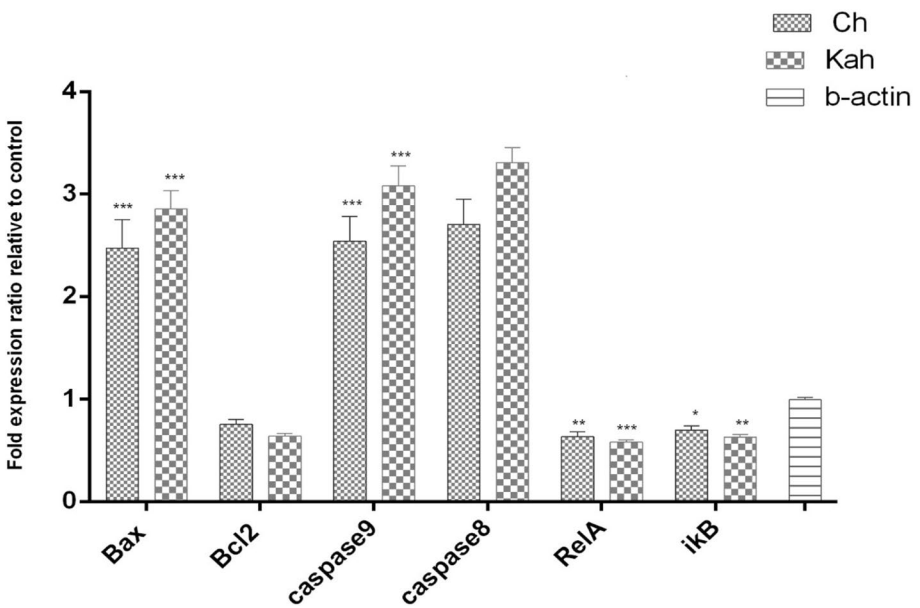

Fig. 4 The comparison of relative mRNA expression of apoptosis-associated genes in HeLa cell line is presented 24 h after treatment with postbiotic derivate from *L. fermentum* CH and *L. fermentum* KH (IC_{50} concentration). The data are provided as the mean + SD of triplicate experiments ($n = 3$, *** $p < 0.001$; ** $p < 0.01$; * $p < 0.5$). SD: standard deviation

cancerous cell line proliferation (HeLa cells) in a dose and strain-dependent manner.

Apoptosis, the programmed cell death, is initiated by extrinsic and intrinsic pathways. Signaling of these pathways actuates cysteine proteases and caspases to remove dead cells. The discrepancy among them is that each type of stress induces apoptosis inside or outside [47]. Thus, intrinsic stresses such as DNA damage or oncogenes led to intrinsic apoptosis. In this context, the intrinsic apoptosis pathway was regulated by the Bcl-2 family [48]. Several Bcl-2 family members can operate as proapoptotic proteins (e.g., Bax and Bak), while Bcl-2 can be an antiapoptotic protein [49]. By detecting an internal signal such as DNA damage, the Bax and Bak proteins are actuated to generate pores in the outer mitochondrial membrane. This phenomenon leads to the interruption of the membrane potential of mitochondria and provokes the release of cytochrome c from the internal membrane of mitochondria [11, 50, 51]. In the next step, the released cytochrome c attaches to apoptotic protease activating factor 1 (APAF1) following by induction of the caspase-9 activation, which resulted in actuates further activated caspases, like caspase-8 and caspases-3, leading to apoptosis. In the current study, a series of trials demonstrated that the antiproliferative influence of *L. fermentum* CH and *L. fermentum* KH on HeLa cells resulted from apoptosis rather than necrosis. Figure 4 depicts the proapoptotic gene upregulation (Bax, caspase-8, and caspase-9) and downregulation of the Bcl-2 gene following treatment with postbiotics. This can illustrate the process of intrinsic apoptosis due to the elevated Bax gene expression as a member of the Bcl-2 gene family, which finally leads to the activation of caspase-8 and caspase-9. In this regard, the higher expression of the Bax/Bcl-2 ratio can confirm the susceptibility of cervical cancer to apoptosis as one of the primary anticancer mechanisms.

In addition, the expression of iKB and RelA genes was also evaluated by q-PCR, showing a 0.5-fold reduction of these gene expressions compared to the control (Fig. 3). RelA or P65 is one of the central subunits of NF-κB (Nuclear factor kappa B), typically maintained in the cytoplasm and available in an inactive type related to inhibitors of κB (IκB) as the regulatory proteins [52, 53]. NF-κB is prohibited in the cytoplasm by interaction with IκBα. Bacterial products, principally LPS (lipopolysaccharide), produce a signal that causes the IκBα ubiquitination along with NF-κB translocation to the nucleus, leading to the induction of cell proliferation genes and the pro-inflammatory transcription. In this context, Sambrani et al. reported the downregulation of Bcl-2 and RelA after probiotic administration [54]. It was recently shown that iKB(α) is also located at the outer mitochondrial membrane (OMM), leading to apoptosis inhibition. In mitochondria, IκBα stabilizes the hexokinase II (HKII) and VDAC1 complex, thus hampering the recruitment of Bax to VDAC1 and release of cytochrome c in apoptosis provocation [55]. Therefore, our results revealed that the reduction of iKB by these postbiotics is attributed to inducing apoptosis in HeLa cell lines.

A flow cytometry analysis was also conducted to confirm apoptosis. Based on ANNEXIN V/PI flow cytometric analysis (Fig. 2), this research discovered an elevation in the early apoptotic cell population compared to the control and postbiotic-treated cells. Therefore, the findings of this research disclose that the antiproliferative consequence of postbiotics against HeLa cells was mainly via apoptosis induction.

Although the current research offers valuable insights into the potential of postbiotics derived from *Lactobacillus fermentum* isolates as anticancer agents, it is crucial to recognize certain limitations. Initially, the generalizability of our findings is restricted by our emphasis on two specific *L. fermentum* isolates from Iranian dairy products. Future research should examine a broader selection of probiotic strains to better comprehend the postbiotic effects on cervical cancer cells. Secondly, while informative, the in vitro nature of our experiments may not completely represent the complex in vivo environment. These findings would require additional research, including animal models and, ultimately, clinical trials, to be validated.

Furthermore, although we investigated numerous apoptosis-related genes, a more thorough examination of molecular pathways could provide a more profound understanding of the mechanisms of postbiotic action. The clinical potential of these postbiotics would be evaluated by evaluating long-term exposure and potential resistance development, as our study concentrated on short-term effects. Finally, although we employed HUVEC cells as a control for normal cells, evaluating the postbiotics on a broader array of normal and cancer cell lines would be beneficial to determine their specificity and potential off-target effects. By addressing these limitations in future research, we can better understand postbiotics as potential therapeutic agents for cervical cancer.

Considering that our study has yielded promising results, several avenues of research could ensue regarding postbiotic effects on cervical cancer cell lines. One is the possibility of expanding the investigation to a broader range of Lactobacillus strains and other species of probiotics. Second, in-vivo research using animal models is of utmost importance for validating our in vitro results and assessing the efficacy and safety of postbiotics in a more complex biological system. A full investigation into molecular mechanisms underlying the observed anticancer effects, including comprehensive proteomics and metabolomics analyses, can

potentially reveal new therapeutic targets and improve our understanding of postbiotic action. Long-term studies on the impact of extended exposure to postbiotics and the potential development of resistance in cancer cells will be helpful before concluding clinical potential. Testing on a number of cancer and normal cell lines would help establish their therapeutic index. Indeed, in-vivo studies on optimal delivery methods and the bioavailability of postbiotics will further help in translational studies to possible clinical studies. These future studies would considerably increase the development of postbiotics as a new, safe, and effective modality in treating cervical cancer. It's also worth noting that postbiotics may have advantages over live probiotics in specific applications, particularly in immunocompromised individuals or those with compromised GI tracts, where the administration of live bacteria might be contraindicated.

The current study clearly showed the two postbiotics derived from Iranian indigenous dairy products provoke apoptosis with a decrease in iKB, Bcl-22, and RelA genes and, conversely, an increase in caspase-8, caspase-9, and Bax genes. Therefore, these results confirmed the release of cytochrome c after activation of the mitochondrial pathway by the downregulation of RelA and iKB genes, along with elevating the expression of Bax/Bcl2, thus consequently enhancing apoptosis. The cell survival rate of the HUVEC and HeLa cells treated with two postbiotics shows that the indigenous *L. fermentum* KH and *L. fermentum* CH had low toxicity effects. This points out that the impact of these postbiotics differs among cancerous and normal cells, which could be considered an optimistic feature of the treatment and control of cervical malignancy. Therefore, indigenous postbiotics can be regarded as promising potential drugs owing to their apoptotic action against cervical cancer cells.

Availability of data and materials

This published article and its supplementary information files include all the critical data generated or analyzed during this study. If required, any additional data will be available from the corresponding author upon request.

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Authors' contributions
Abbas Asoudeh-Fard and Ahmad Gholami designed the study. Data was collected by Moein Beygi Yeylagh and Asghar Parsaei. Abbas Asoudeh-Fard carried out statistical data analysis. The manuscript was written by Milad Mohkam and the figures and table are designed by Mohadeseh Asoudeh-Fard reviewed by Abbas Asoudeh-Fard and Ahmad Gholami. All authors approved the final manuscript.

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Data availability
All data generated or analyzed during this study are included in this published article.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
This study was done under the ethics committee number code: IR.SUMS.REC.1399.238.

Competing interests
The authors declare no competing interests.

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