Heliyon 10 (2024) e36554

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Research article

The inhibitory effects of the novel *Lactobacillus* cocktail on colorectal cancer development through modulating BMP signaling pathway: *In vitro* and in *vivo* study

Amin Sepehr [a], Shadi Aghamohammad [a], Roya Ghanavati [b], Ali Karimi Bavandpour [c], Malihe Talebi [d], Mahdi Rohani [a,*], Mohammad Reza Pourshafie [a,**]

[a] *Department of Bacteriology, Pasteur Institute of Iran, Tehran, Iran*
[b] *Behbahan Faculty of Medical Sciences, Behbahan, Iran*
[c] *Department of Cell and Molecular Biology Program, Michigan State University, East Lansing, MI, USA*
[d] *Department of Microbiology, Faculty of Medicine, Iran University of Medical Sciences, Tehran, Iran*

ARTICLE INFO

Keywords:
Lactobacillus cocktail
Colorectal cancer
BMP signaling pathway
Probiotics

ABSTRACT

This study investigates the impact of a five-strain *Lactobacillus* cocktail (comprising two strains of *L. plantarum*, and one strain each of *L. brevis*, *L. reuteri*, and *L. rhamnosus*) on colorectal cancer (CRC) modulation by targeting the bone morphogenetic proteins (BMP) signaling pathway. Both *in vitro* and *in vivo* (models were employed. The antiproliferative effects of the *Lactobacillus* cocktail on HT-29 cells were assessed via the MTT assay. Mice were divided into three groups: a negative control (treated with PBS), a positive control (treated with azoxymethane (AOM)/dextran sulfate sodium (DSS) + PBS), and a test group (treated with AOM/DSS + *Lactobacillus* cocktail in PBS). The role of the *Lactobacillus* cocktail in inhibiting the BMP signaling pathway was evaluated using qRT-PCR for gene expression analysis and western blotting for β-catenin protein assessment in both models. The MTT assay results demonstrated a significant, time-dependent reduction in HT-29 cell proliferation. qRT-PCR indicated downregulation of the BMP signaling pathway in treated cells, which subsequently led to decreased expression of the hes1 gene, crucial for cell differentiation and proliferation control. This inhibitory effect was corroborated in the mice model, showing significant downregulation of BMP pathway genes and *hes1* in the AOM/DSS/*Lactobacillus* cocktail-treated group. Additionally, western blotting revealed a marked decrease in β-catenin expression in both *in vitro* and *in vivo* experiments. Collectively, these findings suggest that the *Lactobacillus* cocktail may aid in CRC prevention by downregulating the BMP signaling pathway.

1. Introduction

Colorectal cancer (CRC) is responsible for almost 881,000 cancer-related deaths in 2018 [1]. Currently, CRC ranks third and fourth

* Corresponding author. Department of Bacteriology, Pasteur Institute of Iran, Tehran, Iran.
** Corresponding author. Department of Bacteriology, Pasteur Institute of Iran, Tehran, Iran.
E-mail addresses: M_rohani@pasteur.ac.ir (M. Rohani), pour62@yahoo.com (M.R. Pourshafie).

https://doi.org/10.1016/j.heliyon.2024.e36554
Received 14 May 2023; Received in revised form 16 August 2024; Accepted 19 August 2024
Available online 19 August 2024

in cancer-related deaths among Iranian men and women, respectively, and it is one of the most common cancers in Iran [2]. Unfortunately, the incidence of CRC increases with age, and it has been reported that 92 % of cases occur in the population over 50 years of age [3]. It has been proven that the style of living that may increased threat of CRC includes a absence of healthy lifestyle and being overweight and obese [4]. Therefore, a lifestyle change and searching for new strategies to prevent this malignant disease seem to be necessary.

According to studies, it has been suggested that the regulation of intestinal cell proliferation might led to the development of CRC [5,6]. It is worth noting that the colon crypt has various signaling pathways. Namely, Notch and Wnt signaling pathways are most prominent at the crypt base and stem cell compartment. On the other hand, bone morphogenetic proteins (BMP) components are found in the lower part of the colon crypt. The BMP signaling pathway is inactive in the stem cell stage due to an inhibitor called Noggin and is more active in the cell differentiation stage [7]. As intracellular signaling mediators and transcription factors, SMAD proteins are critical in the BMP signaling pathway. So far, eight SMAD proteins have been identified in mammalian genomes and classified into three functional groups, including five receptor-regulated SMADs (SMAD1/5/8 in BMP and SMAD2/3 in TGF-β signaling pathway), one Co-SMAD (*e.g.* SMAD4), and two inhibitory SMADs (SMAD6/7) [8]. In the BMP signaling pathway, phosphorylation of cellular receptors (*e.g. bmpR*) activates R-SMAD (SMAD1/5/8), which leads to the phosphorylation of SMAD4. This cascade regulates cell proliferation via the Hes1 (hairy and enhancer of split-1) transcription factor [9]. It has been previously approved that BMP components are changed in up to 87 % of gastrointestinal (GI) tumors [10]. Therefore, this pathway's genetic/epigenetic alterations in the gastrointestinal tract could be an essential factor associated with GI cancer development [11,12].

The colon contains a diverse population of microorganisms that affect the host's homeostasis and disease. Changing the symbiotic relationship within the colon microbiome is a critical point in the etiology of colon disorders. On the other hand, different studies have demonstrated that the colon microbiome is significantly changing in people with chronic diseases such as CRC [13,14]. Therefore, the dysbiotic/symbiotic state of the gut microbiota can influence some mechanisms that can lead to CRC. It has been demonstrated that taking probiotics is a prophylactic method to maintain healthy gut microbiota and reduce the risk of colon diseases. Lactic acid bacteria especially *Lactobacillus* species are the most common probiotic, which exerts health benefits. Studies demonstrated that probiotic bacteria such as *Lactobacillus* species and their metabolites could be considered a putative complementary against colon cancer and have beneficial effects on inhibiting cancer cell proliferation [15]. Although the antiproliferative effects of probiotics are not well known, they may be involved in altering the colon microbiome, promoting apoptosis, producing exopolysaccharides with diverse biological functions, and improving immune response [16].

This study used a five-strain *Lactobacillus* cocktail from different strains of *Lactobacillus* species. Indeed, the results of the previous studies confirmed that this cocktail has antipathogenic and antiproliferative effects by modulating Notch and Wnt signaling in the human colon cancer cell line and animal model [17–19]. Therefore, to understand the effects of our *Lactobacillus* cocktail on the BMP as another effective signaling pathway in the cell development process, the present study was performed *in vitro* and *in vivo* (HT-29 cancer cells and BALB/c mice respectively).

2. Materials and methods

2.1. Preparation of the Lactobacillus cocktail

This study's methodologies and techniques were crafted in compliance with the 1975 Declaration of Helsinki and its updates, receiving approval from the ethical guidelines committee of the IUMS (ethical code: IR.IUMS.FMD.REC.1397.066). This research employed a unique combination of *Lactobacillus* strains, including two strains of *L. plantarum (PRP42 and PRP128*), and single strains of *L. brevis (PRP205), L. reuteri (PRP100*), and *L. rhamnosus (PRP195)*. As documented in prior studies [17], the species in this *Lactobacillus* cocktail were initially derived from the stool samples of healthy volunteers. The *Lactobacillus* strains were grown in MRS broth (Sigma-Aldrich Co., UK) for 16 h at 37 °C. Following centrifugation at 4000×*g* for 10 min, the culture pellets of each *Lactobacillus* strain were resuspended in fresh RPMI-1640 with 10 % FBS to reach an optical density of 108 CFU/mL for *in vitro* studies, and in saline solution at 5 × 1011 CFU/mL for *in vivo* analysis. Equal volumes of each dilution were freshly combined to create the *Lactobacillus* cocktail, mixing all components into a single tube.

2.2. HT-29 - Human colon adenocarcinoma cancer cell line

The HT-29 cells with NCBI number C466 was acquired from the Pasteur Institute of Iran's Cell Bank. HT-29 cells were maintained in RPMI1460 medium, enriched with 10 % FBS (Biochrom, Germany) and 1 % penicillin/streptomycin (Sigma-Aldrich, UK), at 37 °C with 5 % CO2.

2.3. MTT assay and cell viability assessment

The impact of the *Lactobacillus* cocktail on HT-29 cancer cell proliferation was assessed using an MTT assay, following the protocol provided with the MTT kit (Bioidea, Iran). HT-29 cells were cultured in 96-well plates. To identify the most effective treatment dose, the *Lactobacillus* cocktail was applied to HT-29 cells at multiplicities of infection (MOI) of 10 and 100, with incubation periods of 24 to 120 h at 37 °C in a 5 % CO2 environment. Control wells contained untreated HT-29 cells under identical conditions. Every 6 h, fresh medium was added and cells were rinsed with PBS. MTT assays were performed at 24-h intervals up to 120 h for both treated and control groups. The 570 nm of absorbance was recorded using an ELISA reader to determine colorimetric intensity. To calculate the

anti-proliferative effect of the *Lactobacillus* cocktail, the following formula was used to compare treated wells with control wells [20]:

$$\text{The proliferation of cells} = \frac{(\text{OD sample- OD medium})}{(\text{OD control-OD medium})} \times 100$$

OD sample represents the absorbance of treated cells.
OD medium refers to the absorbance of the background.
OD control indicates the absorbance of control cells.

2.4. Animals treatments

The anticancer effects of the *Lactobacillus* cocktail were further evaluated *in vivo* following the *in vitro* experiments with HT-29 cells. Five female BALB/c mice, aged six to eight weeks, were sourced from the Institute Pasteur-Iran and housed in polycarbonate cages under standard conditions for each group. The mice were allocated into three distinct groups: I) a negative control group receiving PBS treatment, II) a positive control group treated with a combination of AOM and DSS plus PBS, and III) a test group administered AOM/DSS along with the *Lactobacillus* cocktail (L.C) in PBS.

2.5. Tumor-induction and tissue preparation

The initial two groups (negative and positive controls) were given 0.2 mL of PBS daily, while the test group received 0.2 mL of *Lactobacillus* cocktail in PBS orally. Administration of PBS or *Lactobacillus* cocktail in PBS via orogastric tube started one week prior to tumor induction with AOM/DSS and continued until the end of the experiment. For colon cancer induction, the positive and test were administered using a 10 mg/kg injection of AOM as carcinogen groups. Afters seven day, two percent DSS added to water for this group for five consecutive days, followed by a 14-day recovery period during which the DSS water was replaced with regular water. At the end the mice were euthanized at the conclusion of the third DSS/water cycle.

2.6. Tumor assessment and histopathological examinations

Ten weeks after start injection, all mice were euthanized by rapid neck dislocation. Their bodies were dissected lengthwise to extract the colon, which was then rinsed with PBS. The length of the colon and the number of tumors from the cecum to the anus were recorded and compared with those of the negative control group. Rectal specimens (~1 cm) frozen in liquid nitrogen, storing them at −80 °C for subsequent histopathological analysis, gene expression studies, and protein level assessments. Then the distal colon was fixed and prepared for histopathological examination. Tissues were analyzed under an Olympus-BX51 microscope at × 100 magnification. Cancer grading and staging were determined using a scoring system [21], which evaluated five parameters: inflammation severity, muscle thickening, crypt architecture, depletion of goblet, and crypt abscess in crypt. Changes observed during colitis were categorized as: (0) none, (1) mild, (3) moderate, and (4) severe.

2.7. Total RNA extraction and cDNA synthesis

RNA was extracted from HT-29 cancer cells and distal colon tissue of mice using the high-purity RNA isolation kit (Roche), adhering to the provided protocol. The integrity and concentration of the RNA were evaluated using a NanoDrop1000 (Thermo Scientific, USA), with a focus on the 260/280 nm absorbance ratio. Reverse-transcribed one μg of RNA for cDNA synthesis using the cDNA Synthesis Kit from Takara.

2.8. Quantitative real-time PCR of the target genes

GAPDH primers were used as internal controls to normalize the data. The expression of genes involved in the BMP signaling pathway was measured with specific primers obtained from the website of Primer-Bank[1] (refer to Table 1). Each qRT-PCR reaction contain 4 μl of cDNA, 0.5 μl of primers and 15.5 μl of SYBR Premix Ex Taq Master Mix. The thermal cycling protocol was as follows: 95 °C for 5 min (denaturation), 95 °C for 10 s (40 cycles as amplification) and 60 s as annealing step using the StepOnePlus system. Each reaction was performed in triplicate to ensure accuracy. Gene expression analysis using the $RQ = 2^{-\Delta\Delta Ct}$ method, comparing each sample to its corresponding control, which included both *in vitro* conditions and PBS-treated mice *in vivo*. Any data points that exhibited irregular distributions were reviewed and excluded from the final analysis.

2.9. Western blotting of the β-catenin protein

Protein samples from each treatment group were resolved to detect β-catenin and β-actin on 12 % gel. The concentration of total protein in each sample was analysis using the Bradford assay, with bovine serum albumin (Sigma-Aldrich) as the reference. The

[1] http://pga.mgh.harvard.edu/primerbank.

Table 1
The sequences, length, and annealing temperature of primers used in this study.

Gene Names	Sequences	Amplicon Size (bp)	Tm (°C)
bmpR1-human	F: AGATGACCAGGGAGAAACCAC	111	61
	R: CAACATTCTATTGTCCGGCGTA		60.4
bmpR1-mouse	F: TGGCACTGGTATGAAATCAGAC	76	60
	R: CAAGGTATCCTCTGGTGCTAAAG		60.1
bmpR2-human	F: CACTCAGTCCACCTCATTCATTT	131	60.1
	R: TTGTTTACGGTCTCCTGTCAAC		59.2
bmpR2-mouse	F: GTGTTATGGTCTGTGGGAGAAAT	154	60
	R: AAAGCGGTACGTTCCATTCTG		60.6
smad1-human	F: AGAGACTTCTTGGGTGGAAACA	157	61
	R: ATGGTGACACAGTTACTCGGT		60.7
smad1-mouse	F: CTCATGTCATTTATTGCCGTGTG	136	60.2
	R: CGCTTATAGTGGTAGGGGTTGA		61
smad4-human	F: CCACCAAGTAATCGTGCATCG	76	61
	R: TGGTAGCATTAGACTCAGATGGG		60.4
smad4-mouse	F: ACACCAACAAGTAACGATGCC	83	60.8
	R: GCAAAGGTTTCACTTTCCCCA		61
smad5-human	F: TCTCCAAACAGCCCTTATCCC	113	60.7
	R: GCAGGAGGAGGCGTATCAG		60.1
smad5-mouse	F: GAGCCATCACGAGCTAAAACC	120	61
	R: ACTGGAGGTAAGACTGGACTCT		61.5
smad8-human	F: TCTTTGTGCAGAGCCGGAAC	92	59
	R: GAAGACCTTGAGGCTGCAGC		60.4
smad8-mouse	F: TCCAGCAGTCTCTCTGTCCG	98	60.3
	R: GTGCTGGGGTTCCTCGTAG		59.8
gapdh-human	F: GGAGCGAGATCCCTCCAAAAT	197	60
	R: GGCTGTTGTCATACTTCTCATGG		61.8
gapdh-mouse	F: TGACCTCAACTACATGGTCTACA	85	60.2
	R: CTTCCCATTCTCGGCCTTG		60.2
hes1-human	F: TCAACACGACACCGGATAAAC	153	59
	R: GCCGCGAGCTATCTTTCTTCA		60.1
hes1-mouse	F: TCAGCGAGTGCATGAACGAG	119	62.5
	R: CATGGCGTTGATCTGGGTCA		62.2

membrane was subsequently probed overnight at 4 °C with primary antibodies: rabbit anti-human/mouse β-actin and β-catenin (catalog numbers 4970 and 8814, respectively) from Cell Signaling Technology, Inc (UK). Protein detection and quantification were performed using enhanced chemiluminescence and the results were analyzed with ImageJ software [22]. β-actin was utilized as a loading control across all samples to ensure normalization.

2.10. Statistical analysis

Data are reported as mean values with standard deviation (SD). Student's t-test was applied for comparisons between two groups, whereas one-way ANOVA was used for analyzing differences among multiple groups. All statistical computations were carried out using GraphPad Prism version 9.3.1. A P-value of less than 0.05 was deemed statistically significant for all tests.

3. Result

3.1. Cell proliferation and MTT assay

The antiproliferative effects of the *Lactobacillus* cocktail were examined *in vitro* using the HT-29 cancer cells in MOI 10 and 100. After 120 h, growth reduction in HT-29 cells was 80 % for MOI 100 and 47 % for MOI 10 (*P*-values <0.05); therefore, MOI 100 was used for further *in vitro* investigations. The MTT assay results indicated that the *Lactobacillus* cocktail notably impacts cell viability and suppresses the proliferation in both a time- and dose-dependent fashion (*P*-values <0.05) (Fig. 1). The MTT assay confirmed that an incubation time of 24 to 120 h is a proper optimum time for the treatment of HT-29 cancer cells with the *Lactobacillus* cocktail. Moreover, an optical microscope was used to monitor the inhibitory effect of the *Lactobacillus* cocktail.

3.2. Result of animals experiment

The result showed that tumor formation in the distal region of the colon is more common than in other regions (Fig. 2A). In the *Lactobacillus* cocktail-treated mice, there was a significant reduction in histopathological scores for inflammation, muscle thickening, and goblet cell depletion (*P-values* < 0.05). However, the crypt architecture and abscess showed a slight decrease in the *Lactobacillus*-induced group compared with the AOM/DSS group but this change was not significant (Fig. 2B and C).

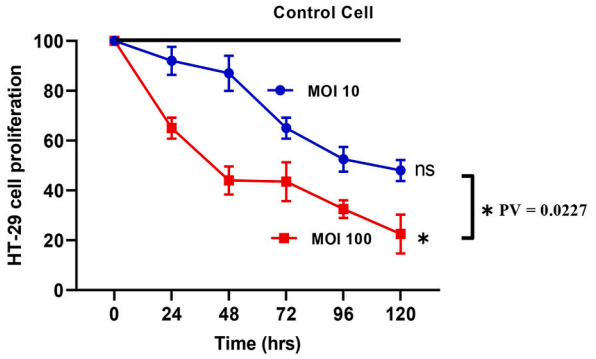

Fig. 1. The effect of the *Lactobacillus* cocktail at MOI 10 and 100 on the viability of HT-29 cells compared to untreated cells. Data were represented as mean ± SD. (* Indicaed that *P*-values <0.05).

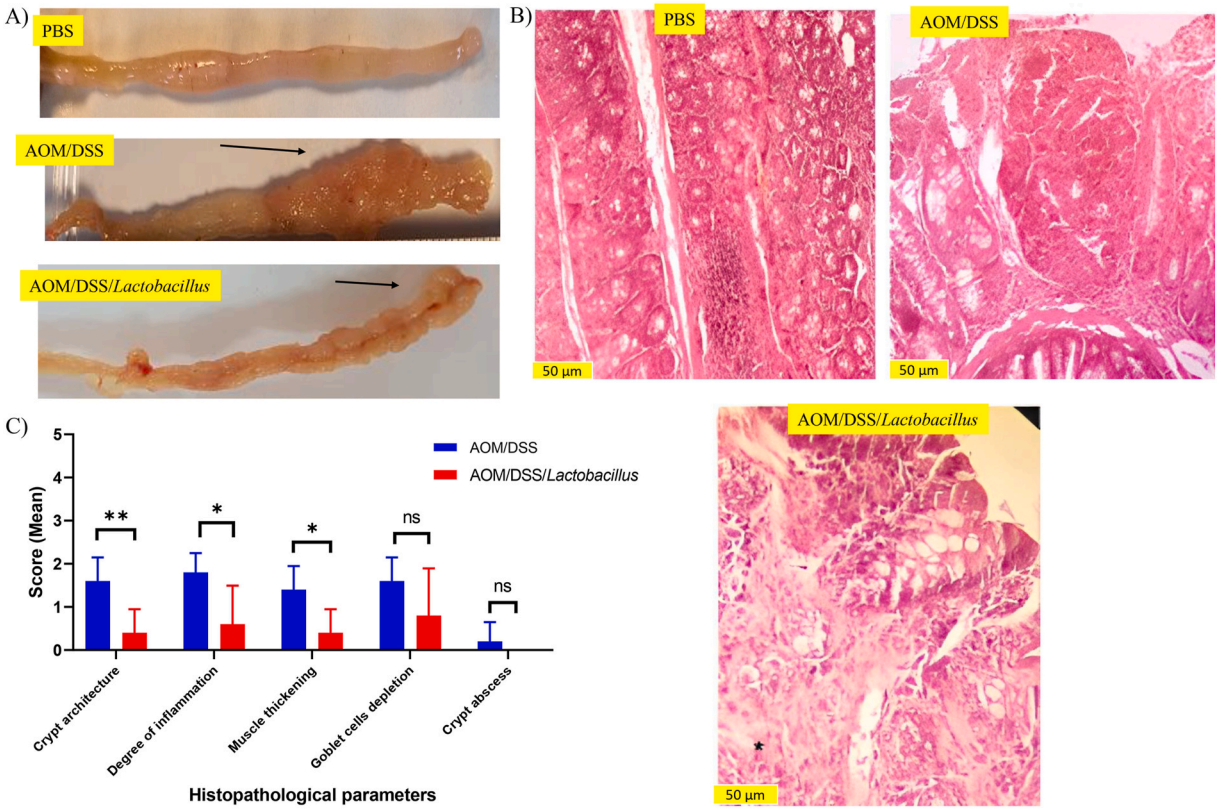

Fig. 2. Histopathological evaluation of colic tumors and inflammation in mice. A) Macroscopic appearance of colic tumors, B) Representative H&E-stained images of distal colon tissues from PBS-treated mice, AOM/DSS/L.C-treated mice, and AOM/DSS-treated mice; scale bars, 50 µm. C) Semi-quantitative assessment of histopathological parameters of mouse colon cancer tissue. PBS: PBS-treated mice as negative control; AOM/DSS/L.C: azoxymethane/dextran sodium sulfate/*Lactobacillus* cocktail-treated mouse; AOM/DSS: azoxymethane/dextran sodium sulfate-treated mouse. *Data are represented as the mean of each group ± SD (n = 5 mice) in an *in vivo* experiment. (**P*-values <0.05, ** *P*-values <0.01, *** *P*-values <0.001, and **** *P*-values <0.0001).

3.3. Quantitative real-time PCR of the genes involved in the BMP signaling pathway

Given that probiotics can mitigate inflammation-induced colon tumorigenesis by modulating various signaling pathways, the expression of BMP signaling-related genes was evaluated in HT-29 cancer cells and the distal colon of mice treated with the *Lactobacillus* cocktail.

The findings demonstrated that HT-29 cells treated with the *Lactobacillus* cocktail significantly downregulated *bmpR1/2* genes over time (~0.81–~0.29 and ~0.49 to ~0.12 respectively) during 24 to 120 h incubation time by a decreasing trend (*P*-values <0.05).

Similarly, the obtained results demonstrated that the expression of the R-*smads* genes (*e.g. smad1, 5, and 8*) (~1.02–~0.32, ~0.7 to ~0.62, and ~0.74 to ~0.68 respectively) and *smad4* (~0.96–~0.33) was decreased significantly in a time-dependent manner. The *Lactobacillus* cocktail downregulated the *hes1* gene by ~0.97 to ~0.19 fold, during the incubation times of 24 to 120 h (Fig. 3).

The qRT-PCR analysis of distal colon tissue from mice indicated that treatment with the *Lactobacillus* cocktail in AOM/DSS-induced colon cancer led to a downregulation of *bmpR1* and *bmpR2* genes, with mRNA levels reduced to approximately 0.75 and 0.66, respectively, compared to the AOM/DSS-only group. Additionally, in the AOM/DSS/L.C-treated mice, the mRNA levels of *smad1/5/8*, *Smad4*, and *hes1* were also decreased compared to test group, showing reductions to about 2.4, 0.29, 0.8, 0.41, and 0.79, respectively (Fig. 4).

3.4. Western blotting of the β-catenin protein

Western blot analysis corroborated a significant reduction in β-catenin expression following treatment with the *Lactobacillus* cocktail, observed in both *in vitro* and *in vivo* models. In the *in vitro* experiments, untreated HT-29 cells were used as controls. The Western blot results demonstrated that the *Lactobacillus* cocktail led to a substantial decrease in β-catenin protein levels in HT-29 cells

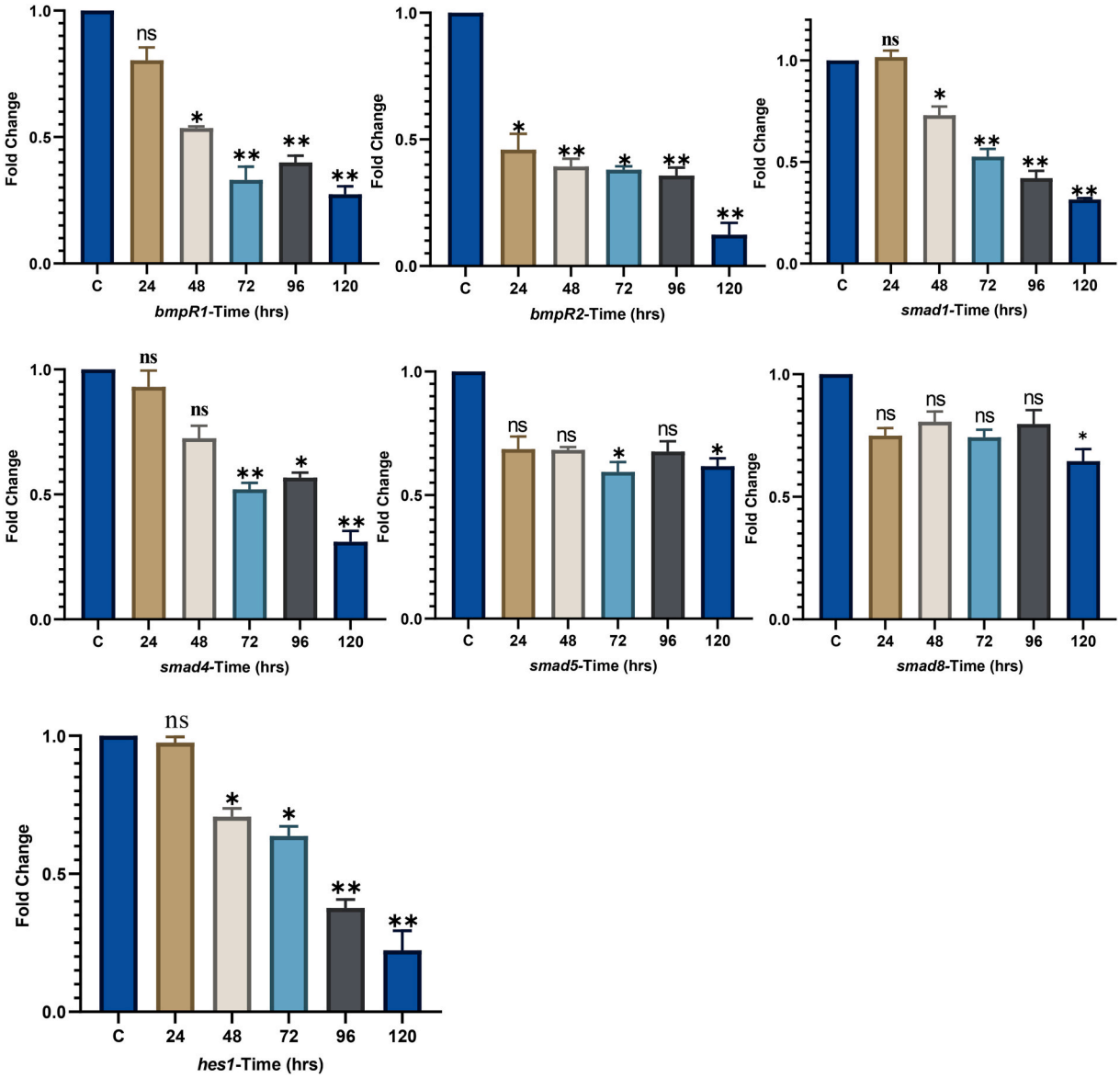

Fig. 3. Relative fold change of the *bmpR1/2, smad1/4/5/8,* and *hes1* genes in HT-29 cancer cells. Results were expressed as mean; error bars (SD);. (**P*-values <0.05, ** *P*-values <0.01, *** *P*-values <0.001, and **** *P*-values <0.0001) by comparison of each treatment with control (C). Untreated HT-29 cells in corresponding incubation time were used as the control.

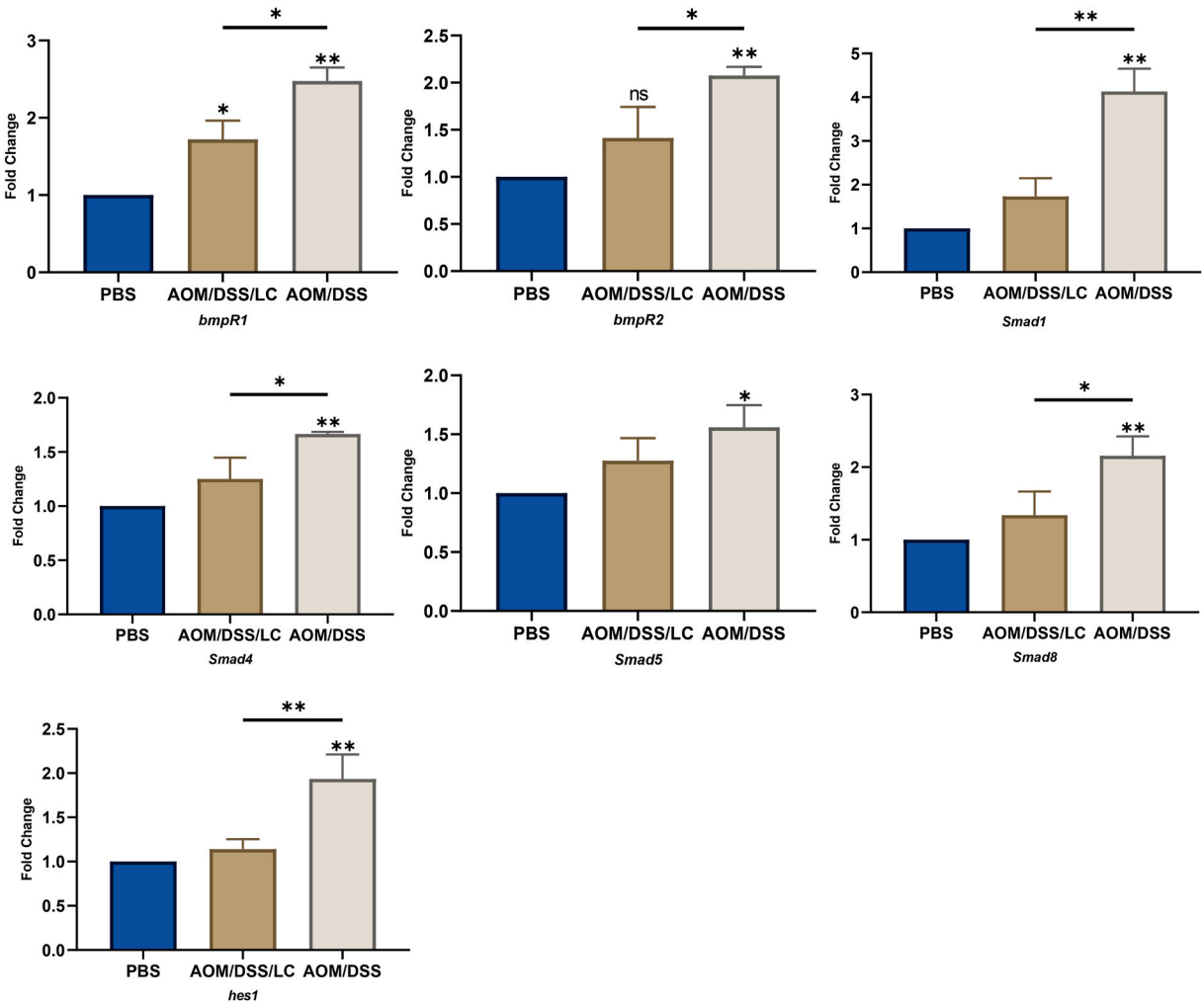

Fig. 4. Relative fold change of the *bmpR1/2, smad1/4/5/8, and hes1* genes in the mice distal tissues by comparison of each treatment with the PBS-treated mice. Results were expressed as mean; error bars (SD); (*P-values <0.05, ** P-values <0.01, *** P-values <0.001, and **** P-values <0.0001). PBS: PBS-treated mice as negative control; AOM/DSS/L.C: azoxymethane/dextran sodium sulfate/*Lactobacillus* cocktail-treated mouse; AOM/DSS: azoxymethane/dextran sodium sulfate-treated mouse.

over a range of 24 to 120 h, with statistical significance (*P-values < 0.05*). Furthermore, β-catenin levels were notably higher in AOM/DSS-induced colorectal cancer (CRC) mice compared PBS-treated mice (P-values <0.05). In contrast, β-catenin expression was significantly reduced in the mice that received the *Lactobacillus* cocktail, when compared to the positive group. (*P-values < 0.05)* (Fig. 5a, b).

4. Discussion

CRC is second-highest cancer mortality rate in world. Although the removal of the tumor and chemotherapy have achieved some success in the treatment of colon cancer, side effects and long-term complications of this type of therapy can affect the survival of CRC patients [1]. This issue has encouraged researchers to find alternative therapeutic methods such as adjuvant therapies for more appropriate treatment of malignant diseases. The use of probiotics such as *Lactobacillus* species as adjuvant therapy in the clinical treatment of colon cancer appears to be a promising strategy with many advantages, such as increased safety, better tolerability, and negligible gastrointestinal side effects [23]. Recently, studies have shown that the gut microbiota with probiotic characteristics has a beneficial effect on CRC through various mechanisms such as cross-talk with host intestinal cells, production of bile acids, or enhancement of the immune response [24,25]. In addition, studies showed that certain probiotics limit the activation of colitis-associated CRC signaling [26].

On the other hand, different studies suggested that signalling pathways including Notch, Wnt, and BMP, play an essential role in regulating normal epithelial cell proliferation; therefore, alterations in these pathways are associated with different outcomes such as tumorigenesis [7,27]. The colonic crypt reflects a gradient of signaling pathways starting at the base with the highest levels of Notch

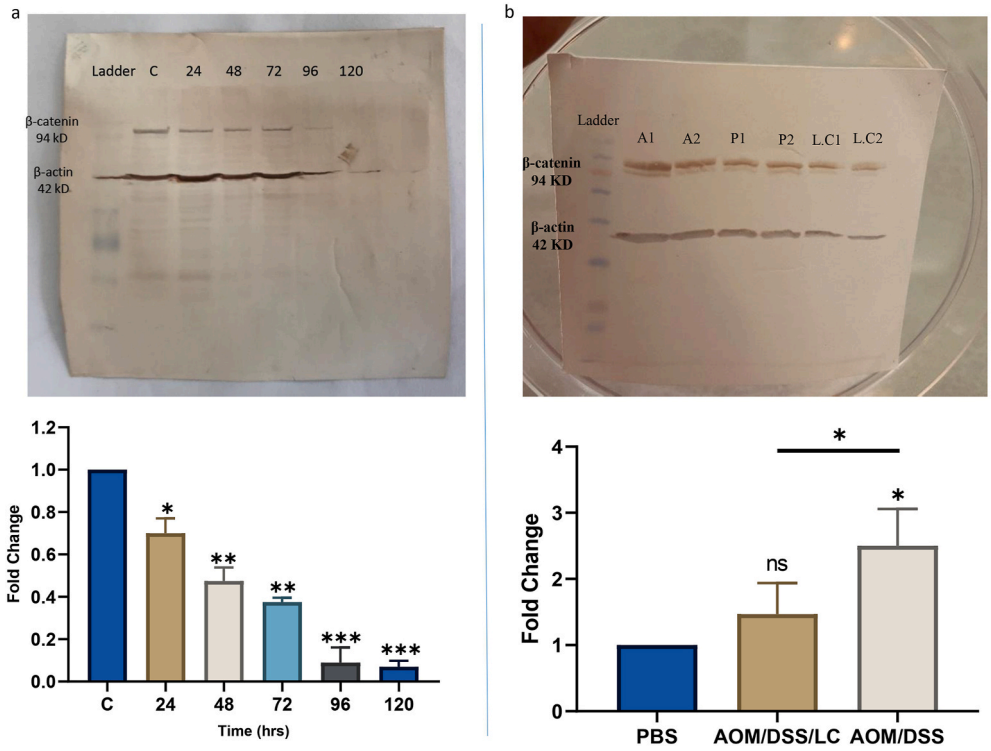

Fig. 5. Western blot analysis demonstrating the expression of β-catenin proteins in HT-29 cancer cells (a) and mice distal colon tissues (b). β-actin was used as the normalizer. The results of Image J analysis of β-catenin Western blotting for cancer cell and mice models were shown under the related image as densitometry analysis. a) 24, 48, 72, 96, and 120 are different incubation times used for HT-29 cancer cells treated with the *Lactobacillus* cocktail; b) A1 and A2: AOM/DSS-treated mouse, L.C1 and L.C2: AOM/DSS/*Lactobacillus* cocktail treated mouse, P1 and P2: PBS-treated mice as control. (*P-values <0.05, ** P-values <0.01, *** P-values <0.001, and **** P-values <0.0001). AOM: azoxymethane, DSS: dextran sodium sulfate, L.C: *Lactobacillus* cocktail.

and Wnt at the earliest stages of proliferation and then moving upward to the differentiating areas with the highest BMP signaling [7]. In our previous studies, this *Lactobacillus* cocktail has shown antiproliferative and inhibitory effects on CRC by modulating Wnt and Notch signaling pathways [18,19]. Although the role of our *Lactobacillus* cocktail in the earliest stages of the proliferation was confirmed, other studies were needed to show the effect of this cocktail in differentiation. Therefore, this study was designed to evaluate the inhibitory effects of the *Lactobacillus* cocktail on the colon singnalling.

In the present study, obtained results suggested that the *Lactobacillus* cocktail has a modulatory effect on the proliferative process in HT-29 cancer cells. The MTT assay showed that our *Lactobacillus* cocktail with MOI 100 significantly inhibited the proliferation of HT-29 cancer cells time-dependently compared to the control (P-values <0.05). The results were consistent with our previous studies on our native *Lactobacillus* cocktail conducted by Ghanavti et al. [18,19]. In their study, they observed a rise in both early and late-stage apoptotic cells in colon cancer cell lines treated for 48 and 120 h, as determined through flow cytometry analysis [18]. In this regard, a study conducted by Awaisheh and colleagues demonstrated that *Lactobacillus* species (*L. acidophilus* LA102 and *L. casei* LC232) could inhibit the proliferation of Caco-2 and HRT-18 cell lines [28]. Moreover, Chuah et al. investigated the role of bacterial metabolites of *L. plantarum* and found that these metabolites can selectively exert a cytotoxic effect on cancer cells via the anti-proliferative mechanism [29]. It can be deduced that different *Lactobacillus* species and their metabolites can modify the mechanisms involved in cell proliferation.

The expression of *bmpR1* and *2* in HT-29 cells treated with the *Lactobacillus* cocktail was decreased over time. Similarly, the study conducted by Zhiwei Sun et al. showed that in digestive tract tumors, the expression level of *bmpR2* was significantly increased [30]. Moreover, the study conducted by Liudmila et al. demonstrated a higher expression of *bmpR2* in advanced cancer cases as compared to the earlier stage of the disease [31]. The survival analysis in the Cancer Genome Atlas and Gene Expression databases has shown that patients with higher expression of *bmpR1/2*, have shorter survival time than the patients with low expression of these genes [30].

In this study, the expression of regulatory *smads* (*1/5/8)* in the HT-29 cancer cells treated with the *Lactobacillus* cocktail was lower than the control cells during the defined incubation time. In the canonical BMP signaling pathway, *smad4* is located downstream of R-*smads*. *smad4* overexperssion was significantly decreased in a time-dependent manner in this study. Consistent with our result, the study by Yi Yu et al. showed that in ALK-positive tumors, *smad4* is directly phosphorylated and its DNA binding ability is reduced, leading to blocking its tumor suppressive responses [32]. However, due to the dual role of the BMP signaling pathway in cancer development and suppression, the expression level of the *smad4* is debatable in CRC [10,33]. In the early stages of tumorigenesis, the

BMP signaling pathway has tumor-suppressive roles, mainly by arresting the cell cycle and triggering the apoptosis cascade. Conversely, the BMP signaling pathway serves as a promoter of tumor progression and metastasis in the later stages of cancer [32]. Similarly, a study by Jia et al. on 209 sporadic CRC tissues showed an increased expression of the *smad4* gene [34]. The present study showed that treatment with the *Lactobacillus* cocktail downregulated different components of the BMP from ligands to R-SMADs. Indeed, negative regulation of the BMP affects cell proliferation and differentiation, which may lead to cell cycle arrest. Regardless of the complexity of cancer development, these probiotics appear to inhibit the progression of CRC by modulating the BMP signaling pathway.

In this study, the *hes1* gene was evaluated, and the result showed that the *hes1* gene expression decreased in treated HT-29 cells compared to control cells (untreated cells). Indeed, *hes1,* as the downstream signaling pathway, is key important in regulatiog hemostasis of cell [35]. Studies reported that upregulation of *hes1* level frequently happens in cancer cells [36]. Consequently, the reduced expression of this crucial gene offers additional evidence for the *Lactobacillus* cocktail's role in inhibiting CRC. This effect is likely mediated by its impact on signaling pathways associated with cell proliferation and regulation. This result is promising due to the role of the *hes1* gene in intestinal development, stem cell self-renewal, and tumor formation.

The connection between the BMP signaling pathway and Wnt/β-catenin have been well-established for a long time and are likely the most extensively researched [37,38]. Numerous studies have explored the relationship between the BMP signaling pathway and the Wnt/β-catenin signaling pathways, highlighting their combined influence on colorectal cancer [39,40]. In the present study, we used β-catenin as a multifunctional protein involved in tumorigenesis to evaluate our results at the protein level. The western blotting results demonstrated that the expression of β-catenin in protein levels decreased in a time-dependent manner in the HT-29 cancer cells.

To further validate the HT-29 cell model findings, *in vivo* experiments were performed using a murine model of colitis-associated colorectal cancer (CRC). The *Lactobacillus* cocktail significantly reduced inflammation in the CRC model induced by AOM and DSS after 70 days in our study. Additionally, qRT-PCR analysis revealed that *Lactobacillus* cocktail treatment led to decreased expression levels of bmpR, smads, and *hes1* genes compared to the cancer control group. These *in vivo* results confirmed the *in vitro* observations, demonstrating a reduction in BMP signaling pathway-related gene expression in the colon. Western blotting also indicated reduced β-catenin levels in *Lactobacillus* cocktail-treated mice compared to the AOM/DSS group. The *Lactobacillus* cocktail may act by producing metabolites that neutralize carcinogenic compounds, converting AOM into an inactive form and thereby reducing its mutagenic effects and CRC risk [41]. Another study involving an animal model demonstrated that elevated luminal butyrate levels significantly increased the rate of apoptosis in the basal and proliferative zones of the distal colon in rats treated with AOM [42]. Furthermore, the *Lactobacillus* cocktail generates beneficial metabolites that are integral to both preventing carcinogenesis and maintaining a balanced intestinal environment. It also enhances the expression of factors involve in inflammation inhibition while reducing the production of ROS response. This dual action helps to alleviate colon damage and inhibit tumor growth, contributing to overall cancer prevention and improved intestinal health [43].

5. Conclusion

In summary, the present study showed that treatment with our defined *Lactobacillus* cocktail significantly suppressed cancer cell proliferation and tumor growth *in vivo* models through the reduction of the genes involved in the BMP signaling pathway. Moreover, the antiproliferative effects of this cocktail on Wnt and Notch signaling pathways have already been confirmed in our previous studies. As mentioned above, Notch and Wnt are two pathways that started at the earliest stages of proliferation and then moved upward to the differentiating areas with the highest BMP signaling pathway. Therefore, assessing the roles of the *Lactobacillus* cocktail on these pathways could provide a comprehensive overview of how these probiotic strains could have molecular anti-cancer effects. Taking everything into account, accurate knowledge of probiotic roles on signaling pathways can be helpful to find new solutions to control CRC.

5.1. Ethics statement

All animal studies were carried out in compliance with the Declaration of Helsinki and were authorized by the ethics committee of Iran University of Medical Sciences, under ethical code IR.IUMS.FMD.REC.1397.066.

Funding

This work was supported by the Pasteur Institute of Iran under Grant B-9531.
The author(s) declare that there are no conflicts of interest.

Data availability statement

The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

CRediT authorship contribution statement

Amin Sepehr: Writing – original draft, Software, Investigation, Formal analysis. **Shadi Aghamohammad:** Writing – original draft.

Roya Ghanavati: Investigation. **Ali Karimi Bavandpour:** Formal analysis. **Malihe Talebi:** Formal analysis, Data curation. **Mahdi Rohani:** Writing – review & editing, Supervision, Conceptualization. **Mohammad Reza Pourshafie:** Writing – review & editing, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

I would like to express my sincere gratitude to the Pasteur Institute of Iran for their generous support and funding for this project [with grant number B-9531].

References

[1] G. Ianiro, et al., Therapeutic modulation of gut microbiota: current clinical applications and future perspectives, Curr. Drug Targets 15 (8) (2014) 762–770.
[2] H.M. Vardanjani, et al., Estimation and projection of prevalence of colorectal cancer in Iran, 2015–2020, Adv. Biomed. Res. (2018) 7.
[3] M.C. Wong, et al., *Prevalence and risk factors of colorectal cancer in Asia.* Intestinal research 17 (3) (2019) 317.
[4] T. Sawicki, et al., A review of colorectal cancer in terms of epidemiology, risk factors, development, symptoms and diagnosis, Cancers 13 (9) (2021) 2025.
[5] M. De Robertis, et al., Current understanding and clinical utility of miRNAs regulation of colon cancer stem cells, in: Seminars in Cancer Biology, Elsevier, 2018.
[6] S.N. Devenport, et al., Colorectal cancer cells utilize autophagy to maintain mitochondrial metabolism for cell proliferation under nutrient stress, JCI insight 6 (14) (2021).
[7] F.E. Bertrand, et al., Developmental pathways in colon cancer: crosstalk between WNT, BMP, Hedgehog and Notch, Cell Cycle 11 (23) (2012) 4344–4351.
[8] C. Sánchez-de-Diego, et al., Interplay between BMPs and reactive oxygen species in cell signaling and pathology, Biomolecules 9 (10) (2019) 534.
[9] C.R. Hopkins, Inhibitors of the bone morphogenetic protein (BMP) signaling pathway: a patent review (2008-2015), Expert Opin. Ther. Pat. 26 (10) (2016) 1115–1128.
[10] A.J. Pellatt, et al., The TGFβ-signaling pathway and colorectal cancer: associations between dysregulated genes and miRNAs, J. Transl. Med. 16 (1) (2018) 1–22.
[11] M. Sonoshita, et al., Suppression of colon cancer metastasis by Aes through inhibition of Notch signaling, Cancer Cell 19 (1) (2011) 125–137.
[12] F. Barrow, et al., Microbiota-driven activation of intrahepatic B cells aggravates NASH through innate and adaptive signaling, Hepatology 74 (2) (2021) 704–722.
[13] E. Thursby, N. Juge, Introduction to the human gut microbiota, Biochemical journal 474 (11) (2017) 1823–1836.
[14] H. Nalluri, S. Subramanian, C. Staley, Intestinal organoids: a model to study the role of microbiota in the colonic tumor microenvironment, Future Microbiol. 15 (16) (2020) 1583–1594.
[15] R. Ghanavati, et al., Lactobacillus species inhibitory effect on colorectal cancer progression through modulating the Wnt/β-catenin signaling pathway, Mol. Cell. Biochem. 470 (1) (2020) 1–13.
[16] J. Wu, et al., The anti-cancer effects and mechanisms of lactic acid bacteria exopolysaccharides in vitro: a review, Carbohydrate polymers 253 (2021) 117308.
[17] M. Rohani, et al., Highly heterogeneous probiotic Lactobacillus species in healthy Iranians with low functional activities, PLoS One 10 (12) (2015) e0144467.
[18] R. Ghanavati, et al., Inhibitory effects of Lactobacilli cocktail on HT-29 colon carcinoma cells growth and modulation of the Notch and Wnt/β-catenin signaling pathways, Microb. Pathog. 139 (2020) 103829.
[19] R. Ghanavati, et al., Lactobacillus species inhibitory effect on colorectal cancer progression through modulating the Wnt/β-catenin signaling pathway, Mol. Cell. Biochem. 470 (1–2) (2020) 1–13.
[20] S. Wang, et al., Whole peptidoglycan extracts from the Lactobacillus paracasei subsp. paracasei M5 strain exert anticancer activity in vitro, BioMed Res. Int. 2018 (1) (2018) 2871710.
[21] J. Hu, et al., Anti-tumour immune effect of oral administration of Lactobacillus plantarum to CT26 tumour-bearing mice, Journal of biosciences 40 (2015) 269–279.
[22] L. Miller, Analyzing gels and western blots with ImageJ, Lukemiller. org Miscellaneous Topics Vaguely Related to Science (2010).
[23] R. Sambrani, et al., Recent advances in the application of probiotic yeasts, particularly Saccharomyces, as an adjuvant therapy in the management of cancer with focus on colorectal cancer, Mol. Biol. Rep. 48 (1) (2021) 951–960.
[24] W. Jia, G. Xie, W. Jia, Bile acid–microbiota crosstalk in gastrointestinal inflammation and carcinogenesis, Nat. Rev. Gastroenterol. Hepatol. 15 (2) (2018) 111–128.
[25] S.H. Wong, J. Yu, Gut microbiota in colorectal cancer: mechanisms of action and clinical applications, Nat. Rev. Gastroenterol. Hepatol. 16 (11) (2019) 690–704.
[26] L. Drago, Probiotics and colon cancer. Microorganisms 7 (3) (2019) 66.
[27] A.A. Farooqi, et al., Overview of the oncogenic signaling pathways in colorectal cancer: mechanistic insights, in: Seminars in Cancer Biology, Elsevier, 2019.
[28] S. Awaisheh, et al., In vitro cytotoxic activity of probiotic bacterial cell extracts against Caco-2 and HRT-18 colorectal cancer cells, Milk Science International-Milchwissenschaft 69 (7) (2016) 33–37.
[29] L.-O. Chuah, et al., Postbiotic metabolites produced by Lactobacillus plantarum strains exert selective cytotoxicity effects on cancer cells, BMC Compl. Alternative Med. 19 (1) (2019) 1–12.
[30] Z. Sun, et al., Deregulated bone morphogenetic proteins and their receptors are associated with disease progression of gastric cancer, Comput. Struct. Biotechnol. J. 18 (2020) 177–188.
[31] L.L. Kodach, et al., The bone morphogenetic protein pathway is active in human colon adenomas and inactivated in colorectal cancer, Cancer: Interdisciplinary International Journal of the American Cancer Society 112 (2) (2008) 300–306.
[32] Y. Yu, X.-H. Feng, TGF-β signaling in cell fate control and cancer, Curr. Opin. Cell Biol. 61 (2019) 56–63.
[33] I. Mármol, et al., Colorectal carcinoma: a general overview and future perspectives in colorectal cancer, Int. J. Mol. Sci. 18 (1) (2017) 197.
[34] X. Jia, et al., Prognostic value of loss of heterozygosity and sub-cellular localization of SMAD4 varies with tumor stage in colorectal cancer, Oncotarget 8 (12) (2017) 20198.
[35] K. Zhang, et al., Hes1, an important gene for activation of hepatic stellate cells, is regulated by Notch1 and TGF-β/BMP signaling, World J. Gastroenterol.: WJG 21 (3) (2015) 878.
[36] Z.-H. Liu, X.-M. Dai, B. Du, Hes1: a Key Role in Stemness, Metastasis and Multidrug Resistance, Cancer biology & therapy, 2015.
[37] H.C. Webber, et al., Crosstalk between TGFβ and Wnt signaling pathways in the human trabecular meshwork, Exp. Eye Res. 148 (2016) 97–102.
[38] R. Wang, et al., Notch and Wnt/β-catenin signaling pathway play important roles in activating liver cancer stem cells, Oncotarget 7 (5) (2016) 5754.
[39] F.G. Petit, C. Deng, S.P. Jamin, Partial Müllerian duct retention in Smad4 conditional mutant male mice, Int. J. Biol. Sci. 12 (6) (2016) 667.
[40] M. Pelullo, et al., Wnt, Notch, and TGF-β pathways impinge on hedgehog signaling complexity: an open window on cancer, Front. Genet. 10 (2019) 711.

[41] P. Ambalam, et al., Probiotics, prebiotics and colorectal cancer prevention, Best Pract. Res. Clin. Gastroenterol. 30 (1) (2016) 119–131.
[42] J.M. Clarke, et al., Butyrate delivered by butyrylated starch increases distal colonic epithelial apoptosis in carcinogen-treated rats, Carcinogenesis 33 (1) (2012) 197–202.
[43] S. Aghamohammad, et al., Anti-inflammatory and immunomodulatory effects of Lactobacillus spp. as a preservative and therapeutic agent for IBD control, Immunity, Inflammation and Disease 10 (6) (2022) e635.