Original Article

Apoptosis Induction by *Lactobacillus casei* Acidic Proteins in the Colorectal Cancer Cell Line

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Abstract

Background: Colorectal cancer (CRC) ranks third in cancer prevalence. *Lactobacillus casei*, a probiotic bacterium, can optimize the microbiota population of the gastrointestinal tract and prevent the growth of harmful bacteria that induce carcinogenesis. In this study, we investigated the effect of *L. casei* acidic proteins on apoptosis in the SW480 cell line to identify a drug protein for treating CRC.

Materials and Methods: We assayed the effect of the isolated acid-resistant proteins of the Chaperonin bacterium, a metal-dependent Hydrolase, and Lysozyme on the SW480 colorectal cancer cell line apoptosis pathway gene expression with a Real-Time RT-PCR.

Results: All three proteins induced apoptosis in the cells treated separately with each of the proteins. The results showed that the up-regulation of BAX and P53 gene expression and the apoptosis pathway were significantly induced. Also, BCL2 expression was down-regulated, and significant anti-apoptotic was observed atp<0.0001. Moreover, the cells treated with these three proteins down-regulated the expression of MAP2K1 and provoked the opposite apoptosis pathway.

Conclusion: Our results found that these proteins would be a good choice for potential CRC treatment.

Keywords: Colorectal cancer, *Lactobacillus casei*, Acid-resistant proteins, Apoptosis

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Introduction

According to Globocan 2020 data, colorectal cancer (CRC) is the third most deadly and most commonly diagnosed cancer globally. Nearly 2 million new cases and about 1 million deaths are expected in 2020[1]. Several factors, such as genetics, lifestyle, and environment, increase the incidence of this cancer[2-4]. Another critical factor in the induction of CRC is a change in the diversity of the gut microbiota population or the digestive symbiosis, which has a role in the emergence and progression of cancer in intestinal cells[1-3]. The human body has a symbiosis with microbiota, including numerous different bacteria[4,5]. Under normal circumstances, this bacterial population produces some of the essential metabolites needed by the human body[8-10]. Therefore, a change in intestinal cell metabolites or the diversity of microbial populations and their metabolites will have a two-way effect on the human body and its microbiota[8]. Evidence

suggests that a diet rich in probiotic bacteria is essential and efficacious in preventing CRC[6,7]. Probiotics are a type of microorganism that, if present in large numbers in the gastrointestinal tract, can optimize the microbiota population and prevent the growth of harmful bacteria that induce carcinogenesis[6,8]. *Lactobacillus casei* is a gram-positive probiotic bacterium [9] in the dairy industry[10-11]. The beneficial health effects of this bacterium via stimulation and regulation of the immune system have been documented[17]. However, *L.casei* and gut pathogens compete for survival in the gastrointestinal tract. This bacterium can decrease the pH of the gut by producing acid, which leads to a reduction in pathogen bacteria growth[10].

On the other hand, studies on the effect of the supernatant of *L.casei* on cancer cells have shown that this bacterium contains proteins or metabolites that prevent cell proliferation and cell escape from apoptosis[18]. Unfortunately, the type of proteins or how the proteins influence cancer cell lines in *L.casei* is unclear. Therefore, it is necessary to determine the mechanism of action for each protein produced by this bacterium[12]. Therefore, this study investigated the effect of specific acidic proteins of *L.casei* on the apoptosis pathway of the SW480 colorectal cancer cell line.

Methods

Selected Bacterial proteins: Following our previous study, we selected three secreted *L.casei* proteins from a culture media with pH 5. They were identified as Chaperonin, Metal-dependent Hydroxylase, and Lysozyme with 80, 60, and 70 kDa molecular weights, respectively. The protein spots in the two-dimensional stained gel were first excised and crushed using Amicon Ultra-0.5 Centrifugal Filter Devices; then, the PBS solution with 50 µg/µL protein was prepared for further analysis[10].

Cell culture: In the study's next steps, the human colorectal cancer SW480 cell line was first received from the ATCC. Then, the effect of three *L.casei* proteins on the apoptosis pathway of the cell line was investigated[19] by seeding $5x10^2$ cells inside a 24-well plate. The plates were then cultured on RPMI media supplemented by 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37°C under 5% CO2 conditions[13,14].

Treatment of the SW480 Colon Cancer cell line with the three target proteins: We categorized two groups of cell lines for separate treatment with each of the three proteins. The first group was a cell line treated with 50µg/ml of each protein and then incubated for 48 hours at 37°C under 5% CO2. The second group was treated with 50µg/ml of each protein and incubated for 120 hours under 5% CO2 conditions. Also, two additional cell lines were used, one as the control group without treatment and the other treated with the PBS buffer solution.

RNA extraction, cDNA preparation, and genes expression analysis by Real-time RT-PCR: First, RNA extraction of the SW480 cell line was performed

Table 1: The expression of Apoptosis genes evaluated in this study.

Gene	Primer sequence (5′-3′)	Product length (bp)
β-actin	F: GTT GGA GTG GAT CCG CCG CACAA R: ATC CTG CCC TCA GAG GGC ATGAA	224
Bax	F: CGG GGA GCA GCC CAGAG R: CCC AGT TGA AGT TGC CGT CAG	112
Map2k1	F: GCG GAG ACC AAC TTG GAG CG R: CAT CCT TCA GTT CTC CCA CC	250
Bcl-2	F: GCG ACT CCT GAT TCA TTG G R: GTC TAC TTC CTC TGT GAT GTTG	162
P53	F: AGC ACT AAG CGA GCA CTG R: CTG GGC ATC CTT GAG TTC C	156

using a GeneAll® Hybrid-RTM kit (Korea). Then, the cDNA template was synthesized from 1µg RNA with the oligo dT primer and reverse transcriptase enzyme (Fermentas, USA). Next, five critical genes were selected from the SW480 cell line for assaying (Table 1). Each of these genes plays an essential role in the apoptosis pathway[15]. The Real-time RT-PCR reaction mixture, a 12 µl total volume solution, consisted of 6 µl SYBR Premix EX Taq (2X) Master Mix and 0.25µl ROX (50X) (Takara, Japan), 1 µl (2 pmol) forward and reversed primers (Metabion- Germany), a 50 ng cDNA template, and PCR-grade H2O up to 12 µl. The reaction was run on a StepOne plus PCR system (Applied Biosystems, Foster City, CA). The PCR condition was 95°C for 10 min, followed by 40 cycles of 95°C for 10 s, 56°C for 15 s, and 72°C for 25 s. It is necessary to note that in this study, the primer specificity was verified by melting curve analysis, and the β-actin was considered as the internal reference gene. The comparative quantification Ct method (ΔΔCt) assessed mRNA expression levels[15].

Results

Selected proteins for the experiment: As mentioned above, three acid-resistant proteins of *L. casei* ATCC39392 were selected from the 2D electrophoresis gel and labeled from 1 to 3, with a molecular weight of 80, 60, and 70 kDa. These proteins were identified using MALTI TOF mass spectrometry. As a result, proteins 1, 2, and 3 were identified as Chaperonin, Metal-dependent Hydrolase, and Lysozyme, respectively[10].

Treatment of the SW480 Colon Cancer cell line with the three target proteins: During the evaluation, gene expression of *BAX* and *P53* up-regulated significantly after being treated with all three proteins for 48 hours of incubation (Fig. 1). But at 120 hours of incubation, the cell treatment by the three proteins no longer had any significant effect on the expression of these genes P>0.05 (Fig.1). It should be noted that the treatment of cell groups with the three target proteins, whether for 48 hours or 120 hours, P>0.05. In contrast, at an equal level, all three proteins down-regulated the BCL2 expression significantly under 48 hours of treatment, P<0.0001 (Fig. 2). Furthermore, a significant down-regulation of expression of this gene was observed with the Chaperonin and Metal-dependent Hydrolase proteins at 120 hours of cell treatment (Fig. 2). Moreover, the MAP2K1 expression was significantly down-regulated during 48 hours of treatment with all three proteins (Fig. 2). However, there was no significant effect on the expression after the three protein treatments at 120 hours, P>0.05 (Fig. 2).

Discussion

An imbalance of microbiota populations and their metabolites can subsequently contribute to the development and progression of CRC[1,6]. This imbalance of microbiota, or dysbiosis, is an effective agent for tumorigenesis and metastasis in this syndrome[5,23]. It is said that a diet rich in probiotic bacteria can help maintain the microbial balance of the

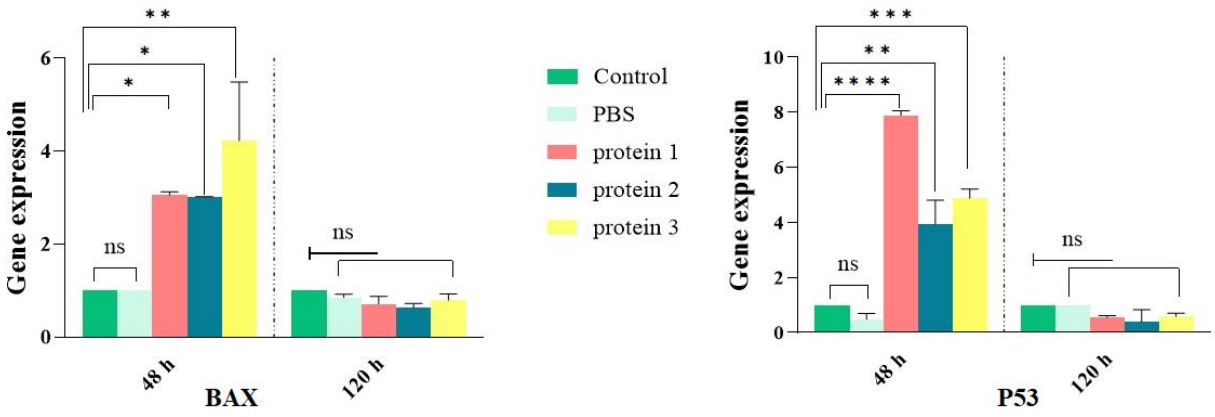

Figure 1. The diagram of Real-time RT-PCR analysis of *BAX* and *P53* expression during 48 and 120 hours of treatment with three target proteins. Protein1= Chaperonin, protein2= Metal dependent Hydrolase, and protein3= Lysozyme. For *BAX* expression, * protein1= 0,042, * protein2= 0.046, ** protein3= 0.0068. For P53 expression, ** protein1= p<0.0001, ** protein2= 0.0032, *** protein3= 0. 0009.ns.**

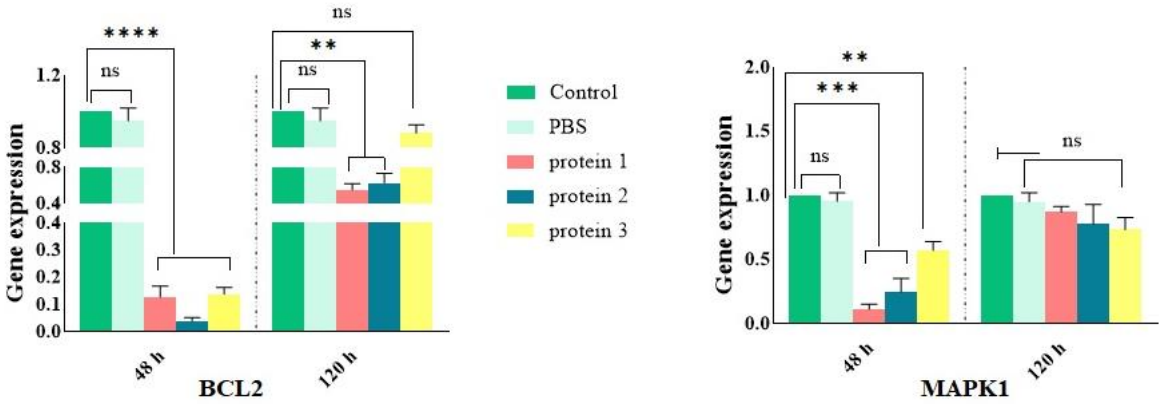

Figure 2. The diagram of Real-time RT-PCR analysis of *BCL2* and *MAP2K1* expression during 48 and 120 hours of treatment with three target proteins. Protein1= Chaperonin, protein2= Metal dependent Hydrolase, and protein3= Lysozyme. For *BCL2* expression, ** three proteins under 48h treatment= p<0.0001 and ** protein1&2 under 120h treatment = 0.0037 & 0.0085. For *MAP2K1* expression, ***protein1 &2= 0.0001 & 0.0003, ** protein3= 0.0038. ns.**

gut and prevent dysbiosis and cancer [6,16]. Indeed, probiotic bacteria are considered a candidate for the treatment of various cancers of the gastrointestinal tract, especially CRC [6,16]. The metabolites of one probiotic bacteria, *L.casei*, have been shown to have anti-cancer properties by inducing apoptosis in most cancer cells, especially in CRC[16-18].

The apoptosis pathway is disrupted in all cancerous cells, resulting in a cell cycle leading to cell proliferation [19]. The intrinsic apoptotic pathway is activated when the body faces DNA damage and oncogenes. In this situation, tumor suppressor *P53* is activated and subsequently activates *BAK* and *BAX*, two pro-apoptotic genes[19,20]. The apoptosis pathway depends on the *BAX* and *BCL-2* gene expression balance. Overexpression of the *BAX* or low expression of the *BCL-2* is one signal for cell apoptosis[15].

Opposite the apoptosis pathway is the *ERK/MAPK* (kinase/mitogen-activated protein kinase) signal pathway[29]. The mitogen-activated protein kinase1 (*MAP2K1*) is the upstream protein kinase of the extracellular signal-regulated kinase (*ERK*), which can activate the extracellular signal-regulated (*ERK/MAPK*) signal pathway. In abnormal conditions, the up-regulation of *MAP2K1* correlated with onset, progression, and metastasis[29,30]. Many studies have investigated the importance of the anti-cancer effect of *L.casei* metabolites. They have confirmed the effect of apoptosis induction of the supernatant and ferrichrome of this bacterium on the apoptosis pathway of several cell lines[8,21,22].

In this regard and confirmation of the past studies[15], we investigated the bacterial proteome to gain a drug protein for treating CRC. In this study, we assayed the effect of three acid-resistant proteins of this bacterium, Chaperonin, Metal-dependent Hydrolase, and Lysozyme, on the apoptosis pathways of the SW480 colorectal cancer cell line.

Our results showed that all three proteins could induce apoptosis in cells treated with each of the proteins. Genes expression of *BAX* and *P53* were up-regulated in the treated cells and was followed by a significant induction of the apoptosis pathway (Fig. 1). Also, a significant down-regulation of the *BCL2* gene expression (an anti-apoptotic agent) was observed, P<0.0001 (Fig. 2). Moreover, down-regulation of MAP2K1 expression was observed in cells treated with each of these three proteins, which caused the opposite pathway of apoptosis (Fig. 2).

The induction of apoptosis in colorectal cancer cells by selected proteins and the reduction of this effect after 120 hours indicates their controlled function. This therapeutic effect can potentially be beneficial for a specific and limited time. However, conversely, their long-term effect will be harmful to other healthy cells.

Conclusion

Since the Chaperonin, Metal-dependent Hydrolase, and Lysozyme had apoptosis induction potential on the SW480 colorectal cancer cell line, they appear to be a good choice of drug proteins for the treatment of CRC

with further *in vitro* and *in vivo* assay.

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