J Appl Biotechnol Rep. 2026 March;13(1):1963-1971

Journal of
Applied Biotechnology Reports

doi 10.30491/jabr.2025.497733.1825

Original Article

# *Bifidobacterium bifidum* Supernatant Affects the Expression of Apoptosis Gene in Caco-2 Cells Infected with Pathogenic Group B *Streptococcus*

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**Received** January 3, 2025; **Accepted** May 21, 2025; **Online Published** March 30, 2026

**Abstract**

**Introduction:** Group B *Streptococcus* (GBS) is a significant pathogen associated with a range of infections, including those of the reproductive tract. *Bifidobacterium*, a beneficial gut microorganism, has shown promise in various health applications. This study aimed to investigate the prevalence and genetic characteristics of GBS among Iranian women with vaginitis. Subsequently, the study explored the anticancer potential of *Bifidobacterium bifidum* supernatant (BS) against GBS-infected colorectal adenocarcinoma cells (Caco-2) by examining its impact on apoptosis-related gene expression.

**Materials and Methods:** Firstly, the prevalence and genetic characteristics (virulence factor and serotype distribution) of GBS were thoroughly examined among women with vaginitis in Iran. Subsequently, Caco-2 cells were initially infected with pathogenic strains of GBS, followed by an investigation into the anticancer properties of BS on the expression of two apoptosis related genes (*Bcl-2* and *Casp-3*) in these cells at the $IC_{50}$ concentration.

**Results:** High prevalence rates of GBS (60 out of 235 (25.53%)) were detected. The dominant serotype was Ib (13.33 %) followed by serotype II (8.33%). The most common virulence genes were *lmb* (100%), *pavA* (100%), *fbsB* (100%), *fbsA* (78.33%), *pI-1* (91.66%), and *pI-2a* (95%). Notably, the expression of the anti-apoptotic gene *Bcl-2* decreased, while the pro-apoptotic gene *Casp-3* increased significantly following BS treatment.

**Conclusions:** These findings strongly suggest the anticancer properties of BS, potentially influencing key cellular pathways that regulate cancer cell survival and apoptosis. However, to gain a more comprehensive understanding and validate the findings, additional research, particularly clinical studies, is essential.

**Keywords:** *Bifidobacterium bifidum*, Apoptosis Genes, Group B *Streptococcus*, Caco-2 Cells

**Citation:** Ramezani U, Amini K, Jafari P, Tafvizi F. *Bifidobacterium bifidum* Supernatant Affect the Expression of Apoptosis Gene in Caco-2 Cells Infected with Pathogenic Group B *Streptococcus*. J Appl Biotechnol Rep. 2025;13(1):1963-1971. doi:10.30491/jabr.2025.497733.1825

## Introduction

Group B *Streptococcus* (GBS or *Streptococcus agalactiae*) an opportunistic Gram-positive pathogen, is a common cause of infections in newborn infants, including pneumonia, septicemia, and meningitis. It is also associated with stillbirth. In addition, GBS can cause infections in pregnant women, including urinary tract infections (UTI), chorioamnionitis (infection of the fetal membranes), and postpartum infections, especially in elderly and immunocompromised patients.[1] In developed countries, the implementation of screening programs for pregnant women has contributed to a notable decrease in infections caused by GBS in both newborns and expectant mothers.[2] Nevertheless, in the developing world, early-onset GBS infection remains a substantial issue, emphasizing the need for more surveillance and investigation.[3] GBS possesses a range of virulence factors that enhance its ability to cause disease. Some of these factors have been identified and characterized, including capsular polysaccharides, regulatory proteins, surface-localized proteins, and toxins. Additionally, several surface proteins serve as adhesins and may contribute to immune evasion.[4]

Over the years, diverse methodologies have been developed to facilitate GBS typing. These approaches are aimed at understanding the genetic and molecular characteristics of GBS, which can provide valuable insights into its epidemiology, transmission patterns, potential virulence factors, and treatment processes.[5] The evolution of typing methods has encompassed both traditional techniques, such as the Lancefield precipitation test (LP test), which involves the identification of bacterial cell wall

antigens, and modern methods like PCR-based approaches, which leverage the amplification of specific genetic markers for differentiation.[6]

One of the most common typing methods for GBS is based on capsule polysaccharides, through which streptococci are divided into 10 different serotypes including Ia, Ib, and II through IX.[7,8] However, only limited subtypes have been recognized in infants with invasive infections, suggesting that diversity in the expression of surface molecules is connected to the strain's pathogenicity. According to previous studies, it has been reported that serotype III is the most predominant, comprising 60-70% of the occurrences, and this serotype is identified as a marker of late-onset neonatal disease.[7,9]

Beyond its well-established role in perinatal infections, emerging evidence suggests that GBS may also play a role in the development or progression of certain cancers, particularly colorectal cancer (CRC). GBS has been detected in the tumor microenvironment of CRC tissues, suggesting that it may colonize or invade the gastrointestinal tract under certain conditions.[10] Recent *in vitro* and *in vivo* studies have demonstrated that specific GBS strains can adhere to and invade intestinal epithelial cells, disrupt tight junctions, and trigger pro-inflammatory responses-processes that can contribute to carcinogenesis. Moreover, some virulence factors of GBS, such as the β-hemolysin/cytolysin (β-H/C) toxin, have been shown to cause DNA damage and modulate host cell signaling pathways associated with apoptosis and cell proliferation.[11]

In recent years, *Bifidobacterium* species have attracted considerable attention for their potential anticancer properties, particularly in the context of CRC. These probiotic bacteria exert their anticancer effects through multiple mechanisms. One key mechanism is the modulation of the gut microbiota, leading to a more balanced microbial environment that inhibits the growth of pathogenic or carcinogenic bacteria.[12] *Bifidobacteria* also produce short-chain fatty acids (SCFAs), such as acetate and butyrate, which have been shown to induce apoptosis and inhibit proliferation in colon cancer cells. Additionally, these probiotics can enhance host immune responses by activating dendritic cells, natural killer (NK) cells, and T cells, thereby promoting tumor surveillance and destruction. They also play a role in reducing systemic and intestinal inflammation by downregulating pro-inflammatory cytokines such as IL-6 and TNF-α, which are often elevated in the tumor microenvironment. Furthermore, some *Bifidobacterium* strains are capable of binding to and inactivating mutagens or carcinogens in the gut, reducing DNA damage and the initiation of tumorigenesis. Together, these mechanisms highlight the multifaceted role of *Bifidobacterium* species in cancer prevention and provide a strong biological rationale for their use in studies aimed at combating CRC.[13]

The present study aims to investigate the effect of BS on the expression of apoptosis-related genes, including *Bcl-2* (anti-apoptotic activity) and *Casp-3* (involved in the execution of apoptosis), in the Caco-2 cell line exposed to pathogenic GBS. In the initial phase of the study, pathogenic GBS isolates containing virulence factors will be selected for use in the subsequent part of the study.

## Materials and Methods

### *Sample Collection and Bacterial Identification*

Initially, 235 vaginal samples were collected from individuals diagnosed with vaginitis at hospitals within Tehran Province. Inclusion criteria were women aged 18-50 years with clinical symptoms of vaginitis (e.g., abnormal discharge, itching, burning), and no antibiotic or antifungal treatment within the previous two weeks. Exclusion criteria included women with chronic systemic diseases (such as diabetes or immunosuppressive conditions), recent use of vaginal products or douches, and those unwilling to provide informed consent. Among the collected samples, 84 (35.74%) were obtained from pregnant women and 151 (64.26%) from non-pregnant women. The samples were promptly transferred to the laboratory under sterile conditions and maintained on ice. Subsequently, 10 µl from each sample were cultured on blood agar medium supplemented with 5% sheep blood, 10 mg/L of colistin, and 15 mg/L of nalidixic acid. The cultures were then incubated in a controlled environment for 24 hours at 37 °C. Bacterial identification was performed using standard microbiological techniques, including Gram stain, catalase, CAMP, and hippurate hydrolysis tests.[14] To confirm the identity of the isolate as GBS, the *dltS* gene was targeted by PCR.[15] The isolated bacteria were stored in Tryptic Soy Broth (TSB; Merck, Germany) containing 30% glycerol at -70 °C until further analysis.

### *DNA Extraction and PCR Assay*

DNA extraction was carried out from 10 ml overnight cultures of each isolate in tryptic soy broth (TSB) using the Cinnagen extraction kit (Cinnagen, Iran) following the manufacturer's instructions. The concentration of the extracted DNA was measured using the BioSpectrometer® (Eppendorf, Germany) and stored at -20 °C for further analysis.

### *Virulence Factors Gene Detection and Capsular Typing*

In this study, we focused on specific virulence genes (*lmb*, *pavA*, *fbsA*, *fbsB*, *pI-1*, *pI-2a*, *pI-2b*) and capsular serotypes (Ia, Ib, II, III) due to their established roles in the pathogenicity of GBS. The selected virulence genes are associated with key mechanisms of bacterial adhesion, invasion, and immune evasion. Regarding serotyping, we focused on serotypes Ia, Ib, II, and III, as they are the most

commonly reported in clinical isolates associated with invasive GBS disease, especially in pregnant women and neonates. Including these genes and serotypes allows for a comprehensive evaluation of the pathogenic potential of clinical GBS isolates. Primer function and sequences, product sizes and annealing temperatures are summarized in Table 1. Virulence genes (*lmb*, *pavA*, *fbsA*, and *fbsB*) and biofilm-related genes (*pI-1*, *pI-2a*, and *pI-2b*) were amplified using multiplex PCR. In addition, the genes for the determined capsular types, including Ia, Ib, II, and III were amplified. Table 2 presents the oligonucleotide primers used for capsular typing.

**Table 1.** Primer Sets Used for the Detection of Virulence Genes and Capsular Typing in GBS

| Function | Target gene | Primer sequence (5′–3′) | Amplicon size (bp) | Annealing temperature (°C) | Reference |
|---|---|---|---|---|---|
| laminin-binding protein | *lmb* | GACGCAACACACGGCAT<br>TGATAGAGCACTTCCAAATTTG | 397 | 55 | (17) |
| Adherence and virulence protein A | *pavA* | TTCCCATGATTTCAACAACAAG<br>AACCTTTTGACCATGAATTGGTA | 288 | 47 | (18) |
| Fibrinogen-binding protein A | *fbsA* | TGTAGCTAATGGACCGATGTT<br>TTTTCATTGCGTCTCAAACC | 156 | 58 | (19) |
| Fibrinogen-binding protein B | *fbsB* | ACAACTGCGGAAATGACCTC<br>ACGAGCGACGTTGAATTCTT | 186 | 58 | (17) |
| Pilus production | *pI-1* | AACAATAGTGGCGGGGTCAACTG<br>TTTCGCTGGGCGTTCTTGTGAC | 102 | 58 | (19) |
| Pilus production | *pI-2a* | CACGTGTCGCATCTTTTTGGTTGC<br>AACACTTGCTCCAGCAGGATTTGC | 249 | 58 | (20) |
| Pilus production | *pI-2b* | AGGAGATGGAGCCACTGATACGAC<br>ACGACGACGAGCAACAAGCAC | 175 | 58 | (20) |
| Capsular type Ia | *cps Ia* | GGTCAGACTGGATTAATGGTATGC<br>GTAGAAATAGCCTATATACGTTGAATGC | 521 | 58 | (21) |
| Capsular type Ia | *cps Ib* | TAAACGAGAATGGAATATCACAAACC<br>GAATTAACTTCAATCCCTAAACAATATCG | 770 | 58 | (21) |
| Capsular type Ia | *cps II* | GCTTCAGTAAGTATTGTAAGACGATAG<br>TTCTCTAGGAAATCAAATAATTCTATAGGG | 397 | 58 | (21) |
| Capsular type Ia | *cps III* | TCCGTACTACAACAGACTCATCC<br>AGTAACCGTCCATACATTCTATAAGC | 281 | 58 | (21) |
| Internal positive control | *dlts* | AGGAATACCAGGCGATGAACCGAT<br>TGCTCTAATTCTCCCCTTATGGC | 199 | 58 | (21) |

**Table 2.** Primer sets Used for the Quantitative Real-time RT-PCR

| Description | Target gene | Primer sequence (5′–3′) | Amplicon size (bp) | Annealing temperature (°C) | Reference |
|---|---|---|---|---|---|
| Apoptosis regulator | *Bcl-2* | ATCGCCCTGTGGATGACTGAGT<br>GCCAGGAGAAATCAAACAGAGGC | 127 | 57 | (22) |
| Apoptosis regulator | *Casp-3* | GGAAGCGAATCAATGGACTCTGG<br>GCATCGACATCTGTACCAGACC | 146 | 57 | (23) |
| Housekeeping gene | *GAPDH* | AACTTTGGCATTGTGGAAGG<br>ACACATTGGGGGTAGGAACA | 223 | 55 | (24) |

### *Selecting Invasive Strains Based on the Presence of Virulence Genes and Serotyping*

In the second part of the study, Caco-2 cells were infected with GBS, and the expression of apoptosis-related genes in these infected cells was examined under the influence of the BS. To infect the cells, more pathogenic GBS strains (those with the highest number of virulence genes) were used. Accordingly, 5 GBS isolates were selected for the second part of the study. An effort was made to select strains that had the highest number of virulence genes. Additionally, one isolate from each serotype was selected for the subsequent study.

### *Isolation of Bifidobacterium bifidum Supernatant Procedure*

*B. bifidum* strain ATCC 29521 was obtained from the Persian Type Culture Collection (Tehran, Iran). A single colony of *B. bifidum* on MRS agar was carefully selected and transferred into MRS broth (pH 5.5, Millipore-110661) with 0.05% L-cysteine (Sigma, 24850236) at 37 °C for 48 hours under microaerophilic conditions. To isolate the supernatant, the bacterial suspension was centrifuged three times at 10,000 ×g at 4 °C. The supernatant was separated and filtered using a 0.2 µm microfilter. The B. bifidum supernatant was then frozen to preserve the stability and bioactivity of the secreted molecules until they were subjected to further analysis.

### *Cell Culture and Cytotoxicity Assay*

To investigate the effect of BS on the expression of cancer-related genes in rectal cells, the toxicity level of the supernatant was first determined to ensure it would be used at a concentration lower than the toxic threshold in the main experiment. The cytotoxic effects of BS on Caco-2 cells were evaluated using the MTT (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyl-tetrazolium bromide) assay. Caco-2 cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and penicillin-streptomycin at 37 ºC in a humidified atmosphere with 5% $CO_2$. All

materials used were obtained from Gibco (UK). The Caco-2 cells were seeded into 96-well plates at a density ranging from $2\times10^4$ to $2\times10^5$ cells per well and then incubated under the same conditions for 24 hours. Subsequently, 10 µl of BS (concentrations ranging from 0.062 to 4 mg/ml) were added to the wells (each concentration in three wells) and incubated for 24 hours. For control cells, 10 µl/ml of MRS broth (pH value 5.5, Millipore-110661) was applied. After incubation, 200 µl of MTT solution (5 mg/ml in PBS) was added to each well and further incubated for 3 hours. The formazan product was dissolved in 150 µl of DMSO, and the absorbance was read in a microplate reader (ELx808, BioTek, USA) at 570 nm. Cytotoxicity was expressed as the concentration of BS inhibiting cell viability by 50% ($IC_{50}$). All measurements were performed in triplicate, and the means and standard errors were calculated.

### *Gene Expression Analysis in Treated Samples with BS*

### *RNA Extraction and cDNA Synthesis*

To extract RNA, cultured cell lines were initially plated in 6-well plates and incubated at 37 °C in a humidified atmosphere with 5% $CO_2$ for 12 hours. Subsequently, GBS bacteria were added to them at a concentration of $10^5$ cfu/ml. Two groups were then established: the first group was subjected to treatment with BS, while the second group served as a control without treatment. After 6, 12, and 24 hours of incubation, the cells were harvested by centrifugation at 3000 rpm for 3 minutes at 4 °C and immediately processed for RNA extraction using a commercial RNA extraction and purification kit (SinaClon, Iran) according to the manufacturer's instructions. Then, the purified RNAs were reverse transcribed to cDNA using a commercial cDNA synthesis kit according to the manufacturer's instructions (Takara, Japan), and the cDNA molecules were stored at -70 °C to use in the real-time PCR reactions.

### *Quantitative real-time RT-PCR*

In order to investigate the effect of BS on the expression of genes related to apoptosis in GBS-infected cells, the real-time RT-PCR technique was employed. The real-time PCR assay was performed using a commercial SYBR Green master mix (Ampliqon, Denmark) and previously described pairs of primers (Table 2). The reactions were conducted in a Corbett Life Science Rotor-Gene 6000 Cycler (Qiagen, Germany). The *GAPDH* housekeeping gene was considered as an internal control to normalize the expression levels of target genes (*Bcl-2* and *Casp-3*). The *GAPDH* gene was selected as the housekeeping gene due to its consistent and stable expression across different experimental conditions, including bacterial infections and probiotic treatments, as reported in previous studies. The amplification proceeded as follows: denaturation at 95 °C for 8 min and then 38 cycles including denaturation at 95 °C for 30 sec, annealing at 57 °C (*Bcl-2* and *Casp-3*) and 55 °C (*GAPDH*) for 30 sec, and 72 °C for 30 sec. A negative control was included in each run. All the samples were analyzed in triplicate and finally, relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method.[16]

### *Statistical Analysis*

The experiments were carried out three times, each in triplicate, and the data are represented as the mean ± SD. The statistical analysis was performed using GraphPad Prism software (version 6). The Student's t-test and one-way ANOVA were applied to examine variations among different treatments. A $p$-value of $<0.05$ was considered to be statistically significant.

## Results

### *Bacterial Isolates*

Out of the 235 vaginal samples collected from women with vaginitis, 60 (25.53%) GBS isolates were identified. Among the 84 samples from pregnant women, 33 isolates (39.28%) were obtained, while 27 isolates (17.88%) were identified from the 151 samples of non-pregnant women. All 60 isolates were Gram-positive, catalase-negative, exhibited a CAMP reaction, and demonstrated hippurate hydrolysis. The confirmation of all 60 GBS isolates was achieved using the amplification of a 199 bp fragment obtained from the *dlts* gene.

### *The Serotype Distribution of GBS Isolates*

The molecular serotype of 60 GBS isolates was determined using multiplex PCR with specific primers targeting different serotypes (Figure 1). Accordingly, serotypes Ia, Ib, II and III were identified**.** One (1.66%) isolate was classified as serotype Ia, while 8 (13.33%) isolates were identified as serotype Ib. Additionally, 5 (8.33%) isolates were categorized as serotype II, and 2 (3.33%) isolates were characterized as serotype III. The remaining isolates (44, 73.33%) were not categorized as tested serotypes in this study.

All 60 isolates possessed the *lmb*, *pavA*, and *fbsB* genes (Figure 1). The *fbsA* gene was present in 47 (78.33%) of the isolates. Regarding the *pili* genes, the *pI-1* gene was detected in 55 (91.66%) isolates, *pI-2a* in 57 (95%), and *pI-2b* in 2 (3.33%) isolates (Figure 2). Furthermore, the results indicated that a significant correlation between serotype and virulence gene prevalence was not observed ($p > 0.05$).

### *MTT Results*

The effect of BS on Caco-2 cell viability was determined by the MTT assay. The results demonstrated that 2 mg/ml of BS completely inhibited cell viability. It was found that BS inhibited Caco-2 cell viability in a dose-dependent manner, with an $IC_{50}$ value of 0.3649 mg/ml (Figure 3).

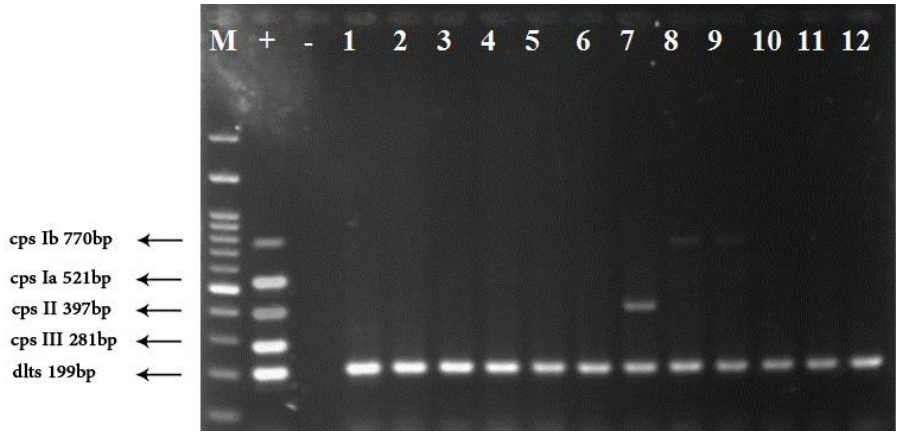


**Figure 1.** Gel Electrophoresis of the Multiplex PCR Amplification Products for the Detection of GBS Serotypes. Lane M: Molecular size standard (100-bp ladder); Lane +: Positive control (containing all amplified sequences); Lane -: Negative control (without samples); Lanes 1-12: Different capsular serotypes of GBS isolates, corresponding to specific numbered bands.

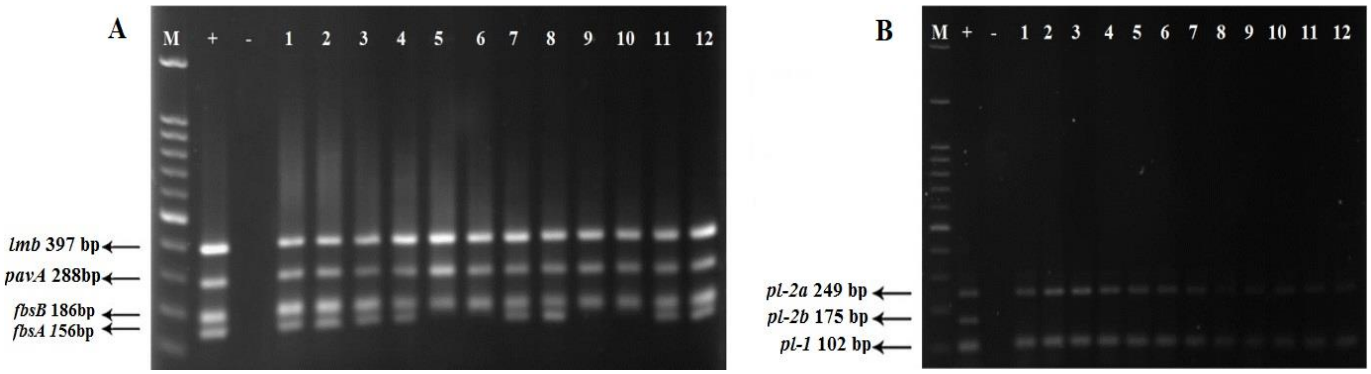


**Figure 2.** Gel Electrophoresis of Multiplex PCR Amplification Products for the Detection of GBS Virulence Factors. A shows the amplification of *fbsA*, *fbsB*, *pavA*, and *lmb* genes, while B shows the amplification of *pl-1*, *pl-2a*, and *pl-2b* genes. Lane M: Molecular size standard (100-bp ladder); Lane +: Positive control (containing DNA for all target genes); Lane –: negative control (no DNA template); Lanes 1–12: different GBS isolates.

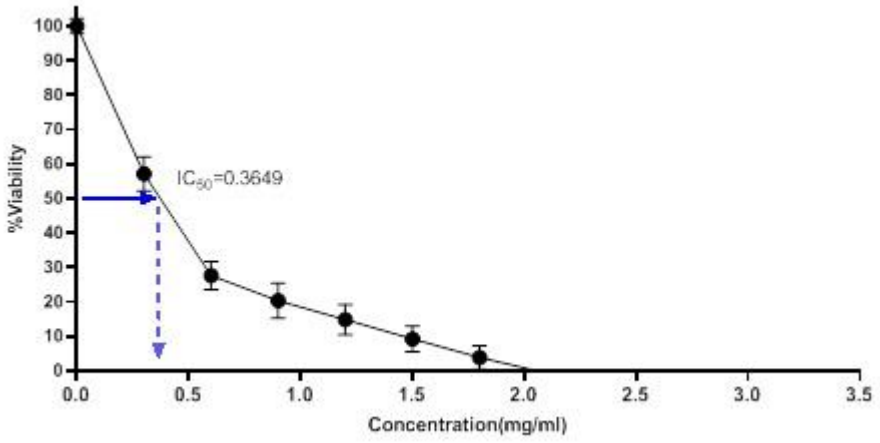


**Figure 3.** Dose-response Curve and $IC_{50}$ Value of BS in the Caco-2 Cell Line ($IC_{50}$ = 0.3649). Data are presented as mean ± SD from three independent experiments performed in triplicate.

### *Apoptosis gene Expression in Treated Samples with BS*

According to the data obtained from real-time PCR, the expression of the *Bcl-2* gene significantly decreased in response to BS treatment at the $IC_{50}$ concentration across all three sampling time points (6, 12, and 24 hours of incubation). *Bcl-2* is an anti-apoptotic gene, and its decreased expression suggests a reduction in the anti-apoptotic signals within the cells, which is a key event in promoting apoptosis. In parallel,

the expression of *Casp-3*, a pro-apoptotic gene, significantly increased following BS treatment at the same $IC_{50}$ concentration. Caspase-3 plays a central role in the execution phase of apoptosis, and its increased expression indicates that the cells are undergoing programmed cell death. These results, presented in Figure 4, suggest that BS treatment may promote cancer cell death by modulating key apoptotic pathways, favoring the activation of apoptosis while inhibiting survival signals.

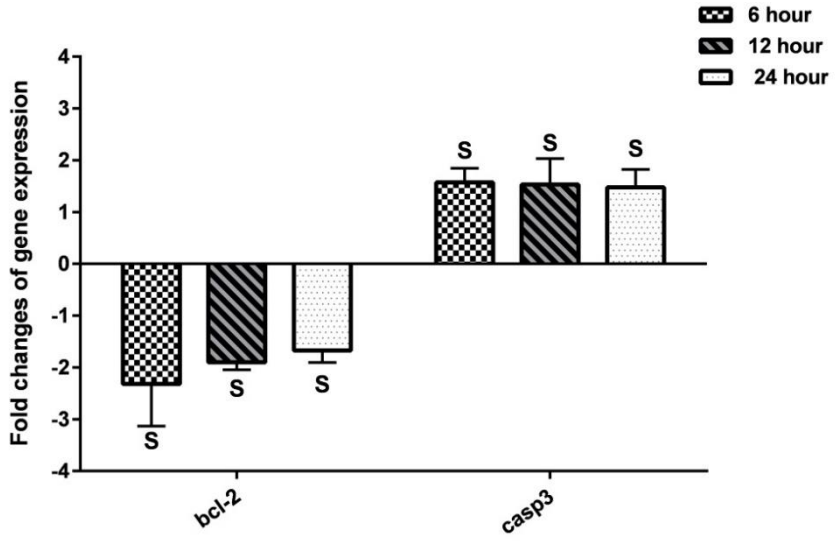


**Figure 4.** Effect of BS on the gene expression of *Bcl-2* and *Casp-3* in Caco-2 treated cells, quantified by real-time PCR. All data are presented as mean ± SD of the gene expression relative to an untreated control group, which were examined in triplicate independent experiments. (S): $p < 0.05$ indicates statistical significance versus control.

## Discussion

It is estimated that GBS colonizes the genital tract in 4-18% of healthy women. Maternal colonization by GBS varies widely across geographic regions due to factors like ethnicity, geography, and laboratory detection methods. In addition, the presence of GBS varies between healthy individuals and vaginitis cases.[25] In the present study, 60 out of 235 (25.53%) samples collected from women with vaginitis were found to be positive for GBS bacteria. This prevalence is higher compared to the most of the other previous studies. For instance, the average reported GBS prevalence in South America is 15.90%;[26] in Brazil, the range varies from 4.20% to 28.40%;[27] in Chile, approximately 19.80%;[28] in Uruguay, around 17.30%,[29], and in Mexico, it is 9.50%.[30] Regarding GBS reports in Iran, there are varying accounts of prevalence. In a meta-analysis study, the overall prevalence of this bacterium in pregnant women was reported to be 15.5%. In addition, with regards to the sampling area, the prevalence of GBS colonization was 11.1% in the vagina and 18.1% in the vagina-rectum.[31] Nevertheless, it appears that in women with vaginitis, the prevalence of GBS is higher than in healthy women, and this elevated prevalence necessitates monitoring and control measures by healthcare organizations.

We also determined the GBS serotype based on capsular serotyping. Our results demonstrated that 1.66%, 13.33%, 8.33%, and 3.33% of isolates were identified as serotypes Ia, Ib, II, and III, respectively. It is worth mentioning that due to limitations in the current study, the identification of other serotypes was not possible. We found that serotype Ib strains were the most prevalent among the strains from Iran. Considering that most previous studies conducted in Iran have identified serotype III as predominant.[32,33] it can be expressed that the findings of the current study do not align with the results of these previous studies. This, however, may be due to the limited number of cases we investigated, or it is possible that the specimens were obtained from a specific group of women in a particular geographic area.[3]

Considering the vital role of virulence genes, especially adhesion genes, in the effective colonization and pathogenesis of GBS, the next section of the study examined the prevalence of several of these genes. The results indicated that *lmb*, *pavA*, and *fbsB* were present in all 60 of the examined isolates. In addition, the prevalence rate of *fbsA*, *pI-1*, *pI-2a* and *pI-2b* was 78.33%, 91.66%, 95%, and 3.33%, respectively. However, there were no associations between virulence genes and serotype distribution.

The high prevalence of *lmb*, *pavA*, and *fbsB* genes among all isolates is particularly noteworthy, as these genes are known to encode surface proteins that facilitate bacterial adherence to host extracellular matrix components such as laminin and fibrinogen.[34] Their consistent presence in all isolates may indicate that these genes are core virulence determinants in GBS strains circulating in this population. Importantly, these adhesion-related genes have been linked to enhanced colonization efficiency, persistence in host

tissues, and increased capacity to initiate infection.[34,35] Previous studies have also associated these genes with more invasive disease outcomes, particularly in neonates and immunocompromised individuals.[35] Therefore, their universal presence in our isolates may reflect a heightened pathogenic potential of the GBS strains in this study, warranting further attention for clinical surveillance and targeted prevention strategies.

In the second part of the study, Caco-2 (a type of human CRC cell line) cells were infected with 5 selected pathogenic strains of GBS and subsequently treated with BS. The expression of two apoptotic genes, *Bcl-2* and *Casp-3*, was examined in the treated cells. The aim of this part of the study was to assess the anticancer potential of BS on GBS-infected cells. The results showed that at all three time points (6, 12, and 24 hours of incubation), at $IC_{50}$ concentration of BS the expression of the *Bcl-2* gene significantly decreased, while the expression of the *Casp-3* gene significantly increased. Both of these findings indicate the anticancer properties of BS.

The selection of Caco-2 cells for this study is significant as they serve as a valuable model for understanding cancer cell behavior and responses to various treatments.[36] By infecting them with GBS, which is known for its virulence factors, and subsequently applying *Bifidobacterium* treatment, researchers aimed to investigate whether the probiotic could mitigate the potential cancer-promoting effects of GBS. The examination of apoptotic genes, specifically *Bcl-2* and *Casp-3*, provided insights into the cellular processes at play.[37] The observed significant decrease in *Bcl-2* gene expression suggests a reduction in anti-apoptotic signals, which may contribute to a more favorable environment for apoptosis in cancer cells.[38] Conversely, the substantial increase in *Casp-3* gene expression indicates an activation of caspase-3, a key enzyme involved in apoptosis execution.[39] These dual findings strongly suggest that BS treatment holds promise as a potential anticancer agent by influencing critical cellular pathways that regulate cancer cell survival and apoptosis.[37] This shift towards favoring apoptosis and reducing the anti-apoptotic signals may hold the key to inhibiting cancer cell proliferation and promoting their elimination within the studied colon cells.

The research into the anticancer potential of *Bifidobacterium* is gaining significant attention due to its multifaceted effects on both cancer cells and the host immune system.[40] *Bifidobacterium* has shown promise in immunomodulation, reducing inflammation, and competing with pathogenic microorganisms.[41] It can also metabolize dietary compounds, produce antimicrobial substances, and enhance drug delivery to tumor sites.[42] This probiotic may play a role in cancer prevention and as an adjuvant therapy alongside traditional cancer treatments.[12]

Finally, the present study has several limitations that should be considered when interpreting the findings. The relatively small sample size and geographic limitation to Tehran province may affect the generalizability of the results. Additionally, the study only identified a limited number of GBS serotypes, and the *in vitro* Caco-2 cell model may not fully represent the complexities of *in vivo* tumor environments. Moreover, the study's short-term time points for gene expression analysis and lack of longitudinal data limit the understanding of the long-term effects of *Bifidobacterium* treatment. Finally, while the study observed significant changes in apoptotic gene expression, further research is needed to establish a causal relationship between GBS infection, *Bifidobacterium* treatment, and cancer cell apoptosis.

## Conclusion

This study represents the first exploration of BS's influence on cells affected by GBS infection. It unveils BS's ability to induce apoptosis, even when cancer cells are dealing with the presence of GBS bacteria. This result suggests that *Bifidobacterium* might be a potent ally in fighting cancer, even in challenging, infection-prone conditions. BS exhibits anticancer properties, likely by modulating the cellular pathways that control cancer cell survival and apoptosis. It highlights BS's potential as a therapeutic agent for GBS-related infections and its possible role in cancer therapy. However, further research, particularly clinical studies, is needed to fully understand and validate these findings.

## Authors' Contributions

KA planned and designed the research. UR performed experiments. PJ and FT analyzed the data and wrote the manuscript. All authors read and approved the final manuscript.

## Ethical Approval

This research was approved by the Ethical Committee of Islamic Azad University, Arak Branch, Iran (ethical code: IR.IAU. ET.REC.1400.035) which was in accordance with the ethical standards of the Helsinki Declaration in 1975 and its later amendments. In addition, informed consent form was obtained from all of the patients.

## Conflict of Interest Disclosures

The authors declare that they have no conflicts of interest.

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