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Article

Combined Lactobacillus Postbiotics with Carrageenan and Soy Protein Induce Sustained Apoptosis Through Multi-Pathway Survival Disruption in Colon Cancer Cells

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Abstract

Background: Colorectal cancer remains a major cause of cancer-related mortality, highlighting the need for effective and low-toxicity therapeutic approaches. This study examined the time-dependent anticancer effects of Lactobacillus postbiotics alone and in combination with a carrageenan/soy protein blend in HCT-116 colorectal cancer cells. **Methods:** Cells were divided into three groups: untreated control (NH), postbiotics alone (LPH), and the combined formulation (LPCS). Cell viability, morphological alterations, apoptotic stages, gene expression, and temporal gene–gene correlations were analyzed at 24, 48, and 72 h. **Results:** The combined formulation induced the strongest and most sustained cytotoxic effects, with maximal late apoptosis and necrosis observed at 72 h. Postbiotics alone mainly triggered early apoptosis at 24 h, which diminished over time. Dynamic, time-dependent modulation of apoptotic regulators (*BAX* and *BCL2*), inflammatory signaling (*NF-κB*), and survival-related pathways (*Notch-1*, *Notch-2*, *JAG-1*, *HES-1*, and *CXCR4*) were observed. Combination treatment led to early stress responses followed by suppression of survival signaling and terminal disruption of apoptotic balance. **Conclusion:** These findings demonstrate that combining Lactobacillus postbiotics with carrageenan and soy protein enhances both the potency and durability of anticancer effects, supporting the development of multi-component postbiotic strategies for colorectal cancer.

Keywords: postbiotics; lactobacillus; colorectal neoplasms; apoptosis; *NF-κB* signaling pathway; notch signaling pathway; *CXCR4* chemokine receptor; cell survival

1. Introduction

The rising incidence of cancer worldwide demands new, effective treatments that may minimize side effects and prevent drug resistance [1]. Traditional therapies like chemotherapy and radiation, though effective, often lead to severe side effects and resistance. These side effects have sparked interest in alternative therapies that target cancer cells more selectively and spare normal cells [2]. Scientists are increasingly recognizing natural compounds for their ability to disrupt key cellular pathways that drive cancer progression [3]. Regarding colon cancer, apoptotic genes such as *BAX* and *BCL2* play crucial roles in immunity against tumor progression [4]. *BAX* (the pro-apoptotic factor) promotes programmed cell death [5], but *BCL2* acts to prevent apoptosis, potentially aiding in tumor survival and resistance to treatments [4]. The critical balance between these genes (*BAX* and

BCL2) determines whether apoptosis is triggered, which can lead to tumor reduction [5,6]. So, targeting these genes to enhance apoptosis offers an effective treatment strategy for colon cancer, thereby activating the body's natural ability to eliminate cancer cells and slow disease progression [7].

Inflammation-related genes (*NF- κ B*, *COX-2*, and *TNF- α*) are pivotal in colon cancer as they drive pathways that support tumor growth, angiogenesis, metastasis, and resistance to cell death [8]. Overactivation of these genes activated an inflammatory environment conducive to cancer progression [9]. Targeting these genes to mitigate inflammation presents a strategy to impede tumor growth and potentially enhance the efficacy of other cancer treatments, emphasizing the importance of managing gene expression in the development of treatments for colon cancer [10].

Notch signaling has a significant impact on colon cancer by affecting cell differentiation, proliferation, and interactions within the tumor microenvironment, as well as angiogenesis [11]. When dysregulated, this pathway leads to abnormal cell proliferation and sustains cancer stem cell populations, which are crucial for continued tumor growth and resistance to treatment [12]. Notch signaling enhances the tumor-supportive environment and promotes angiogenesis through regulatory factors, such as VEGF [13]. Additionally, Notch plays a role in helping cancer cells escape from the apoptosis process by promoting their survival signals during treatment [13,14]. Due to their vast influence, targeting the Notch signaling pathway is considered a promising strategy in cancer therapy, in particular colon cancer, aiming to interfere with these critical processes and potentially improve treatment outcomes [15].

Lactobacillus postbiotics, sourced from probiotics [16], combat colon cancer by balancing gut microbiota [17], supporting intestinal barriers [18], and enhancing immune responses [19], effectively reducing tumor inflammation and triggering cancer cell death [20]. Meanwhile, carrageenan, a natural polysaccharide from red seaweed [21], despite concerns about its inflammatory properties at high doses [22], demonstrates potent anti-cancer capabilities by inhibiting cell growth, promoting apoptosis, and acting as an antioxidant to minimize oxidative stress [23] [24]. Additionally, soy protein that is rich in isoflavones like genistein and daidzein [25] offers protective effects against colon cancer by inhibiting tumor proliferation [26], regulating hormone levels, and showcasing anti-inflammatory properties, thus collectively offering a multi-faceted approach to preventing and combating colon cancer [27].

This study aimed to assess whether combining *Lactobacillus* postbiotics with a carrageenan/soy protein blend enhances anticancer efficacy in HCT colorectal cancer cells compared with postbiotics alone. Specifically, the objectives were to evaluate time-dependent effects on cell viability and apoptosis, to examine modulation of key apoptotic, inflammatory, and survival pathways (*BAX*, *BCL2*, *NF- κ B*, Notch signaling, and *CXCR4*), and to elucidate treatment-induced changes in regulatory networks underlying sustained apoptotic commitment and resistance suppression.

2. Results

2.1. Morphological Evidence of Apoptosis in HCT Cells Following Postbiotic and Combination Treatments

Untreated HCT cells consistently preserved their typical round-to-oval morphology throughout the experimental period, exhibiting an intact centrally located nucleus and abundant surface microvilli (Figure 1A). HCT cells treated with *Lactobacillus* postbiotics alone showed clear time-dependent cytotoxic and apoptotic responses. After 24 h, treated cells displayed characteristic apoptotic features, including cell shrinkage, membrane blebbing, and nuclear condensation and fragmentation (Figure 1B). These morphological changes persisted at 48 h, accompanied by a further increase in overall cell death (Figure 1C). By 72 h, *Lactobacillus* postbiotics alone continued to induce substantial toxicity, with pronounced apoptotic morphology and increased cell detachment relative to earlier time points (Figure 1D).

In contrast, HCT cells exposed to the combined treatment of *Lactobacillus* postbiotics with the carrageenan/soy protein blend exhibited markedly enhanced anticancer activity at all examined time

points. After 24 h, the combination treatment produced stronger cytotoxic effects than postbiotics alone, as evidenced by more pronounced apoptotic morphology (Figure 1E). This enhanced response was further amplified at 48 h, resulting in extensive cell shrinkage, detachment, and a substantial rise in apoptotic cell death (Figure 1F). After 72 h, cells treated with the combination therapy demonstrated the most severe morphological alterations, including widespread apoptosis and marked cellular detachment, exceeding the effects observed with *Lactobacillus* postbiotics alone (Figure 1G).

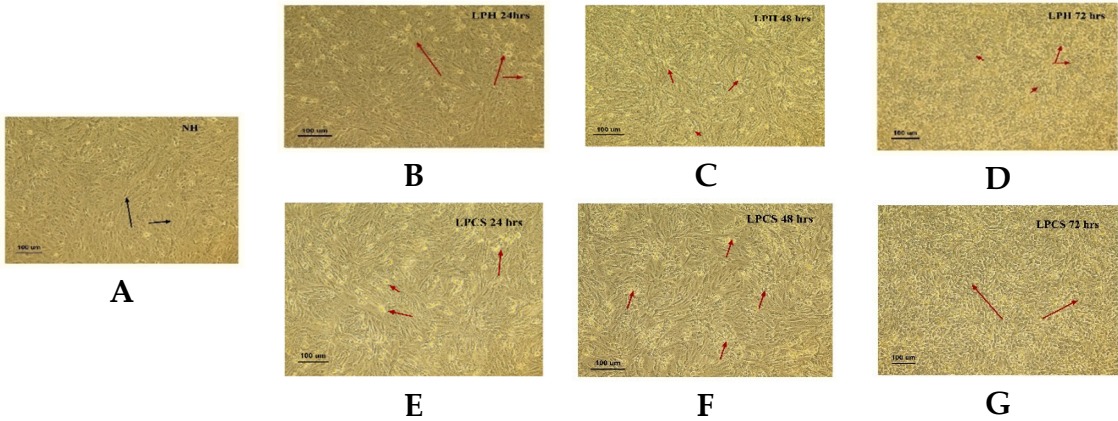


Figure 1. Morphological assessment of apoptosis features in HCT cells following treatments at different time points. (A) Untreated control HCT cells (NH) (black arrows) represent normal morphology with intact cell structure and clearly visible nuclei. (B) HCT cells treated with *Lactobacillus* postbiotics (LPH) for 24 h showed typical apoptotic features, including cellular shrinkage and membrane blebbing (red arrows). (C) After 48 h, LPH-treated cells continued to display apoptotic morphology, characterized by enhanced cellular contraction and membrane blebbing (red arrows). (D) Micrographs obtained after 72 h of LPH treatment revealed persistent apoptotic characteristics, including cell shrinkage and prominent membrane blebs (red arrows). (E) At 24 h, the combined treatment with *Lactobacillus* postbiotics and carrageenan/soy protein (LPCS) induced pronounced morphological alterations and markedly increased cell death (red arrows). (F) At 48 h, LPCS treatment resulted in dramatic cytotoxic effects, with substantially elevated levels of cell death (red arrows). (G) After 72 h, LPCS treatment produced the most pronounced cytotoxic response, with extensive apoptotic morphology and a markedly higher incidence of cell death (red arrows). The scale bar is 100 μm .

2.2. Time-Dependent Effects of Lactobacillus Postbiotics and Combination Therapy on HCT Cell Viability

At 24 h post-treatment, comparative analysis of HCT cell viability showed significant reductions in both treated groups relative to the untreated cells. The combination therapy, comprising *Lactobacillus* postbiotics, carrageenan, and soy protein, induced a markedly greater decrease in viability ($\approx 73\text{--}76\%$; $p < 0.0001$) compared with both the control and the postbiotics-only group, and resulted in a further significant reduction relative to *Lactobacillus* postbiotics alone ($p < 0.001$). Treatment with *Lactobacillus* postbiotics alone produced a moderate but significant reduction in viability ($\approx 84\text{--}90\%$; $p < 0.001$), indicating clear cytotoxic activity (Figure 2A). After 48 h, untreated cells maintained high viability, whereas treated groups showed a clear time-dependent decline. The combination treatment continued to exert a stronger cytotoxic effect, reducing viability to approximately 73% compared with the control ($p < 0.0001$) and remaining significantly more effective than postbiotics alone ($p < 0.001$). In contrast, cells treated with *Lactobacillus* postbiotics alone showed a moderate yet significant reduction in viability ($\approx 90\%$; $p < 0.05$), with a slight recovery compared with 24 h values (Figure 2B). At 72 h, untreated HCT cells still exhibited high viability, whereas the combination therapy produced the most pronounced and sustained reduction in cell viability compared with both the control ($p < 0.001$) and postbiotics-only treatment ($p < 0.0001$). Postbiotic monotherapy also significantly suppressed cell viability relative to the control with levels comparable to those observed at 48 h ($p < 0.001$) (Figure 2C).

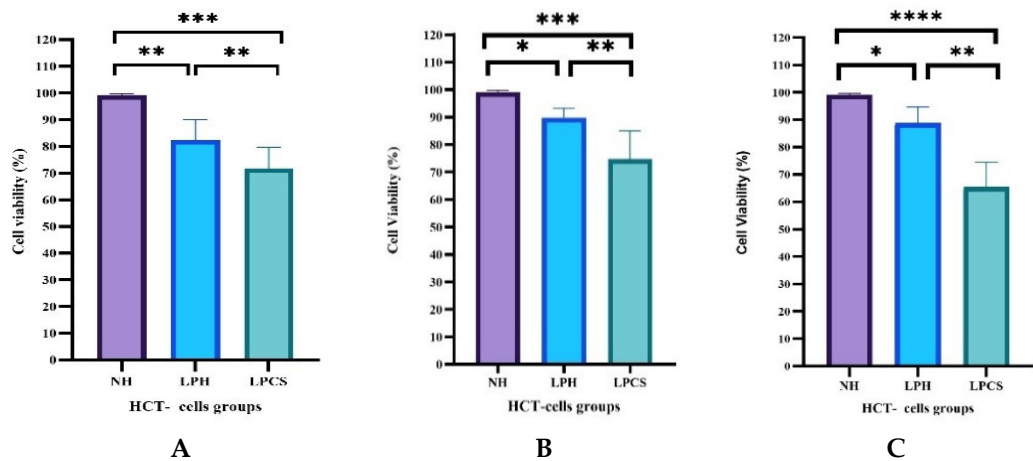


Figure 2. Effect of different treatments on HCT cell viability, (a) after 24 h, (b) 48h, (c) 72h. The untreated control group is designated as “NH,” while “LPH” represents cells treated with *Lactobacillus postbiotics*, and “LCSH” indicates cells receiving the combination therapy, including *Lactobacillus postbiotics*, carrageenan, and soy protein, indicating enhanced anticancer activity of the combined formulation. Data are presented as mean ± SD. Statistical significance between groups is indicated by * $p < 0.01$, ** $p < 0.001$, *** $p < 0.0002$, **** $p < 0.0001$.

2.3. Time-Dependent Progression of Apoptosis and Necrosis in HCT Cells

Analysis of cell death pathways demonstrated a clear time-dependent response to the applied treatments. Early apoptosis analysis showed that, at 24 h, HCT cells treated with the combination of postbiotics, carrageenan, and soy protein displayed significantly lower early apoptosis than the postbiotics-only group ($p < 0.001$) and did not differ from untreated cells. In contrast, HCT cells treated with *Lactobacillus postbiotics* alone exhibited a significant increase in early apoptotic activity compared with the untreated control ($p < 0.0002$). (Figure 3A). At 48 h, the combination treatment significantly increased early-stage apoptosis relative to both the untreated control and the postbiotics-only group ($p < 0.0002$ for each comparison), whereas no significant difference was observed between postbiotics-treated and untreated cells (Figure 3B). After 72 h, early apoptotic activity remained significantly elevated in the combination group compared with the control ($p < 0.0002$), while no significant difference was detected between the two treatment groups; notably, postbiotics alone also significantly increased early apoptosis relative to untreated cells at this point ($p < 0.0002$) (Figure 3C).

Late apoptosis analysis further confirmed a time-dependent treatment effect. At 24 h, both *Lactobacillus postbiotics* alone and the combination treatment significantly increased late apoptotic cells compared with the untreated control ($p < 0.0001$), with no significant difference between the two treatment groups (Figure 3D). After 48 h, late apoptosis remained minimal in untreated cells, whereas both treatments significantly elevated apoptotic levels relative to the control. Notably, late apoptosis was significantly higher in the postbiotics-only group than in the combination group ($p < 0.0002$), although both effects remained significant compared with untreated cells ($p < 0.0001$ and $p < 0.001$, respectively) (Figure 3E). By 72 h, the combination treatment induced the highest level of late apoptosis, which was significantly greater than that observed in both the untreated control and the postbiotics-only group ($p < 0.0001$ for both comparisons). Postbiotics alone also significantly increased late apoptosis compared with the control ($p < 0.001$) (Figure 3F).

Necrosis analysis revealed a pronounced time-dependent pattern. At 24 h, the combination treatment significantly increased the proportion of necrotic HCT cells compared with both the untreated control and the postbiotics-only group ($p < 0.0002$), whereas no significant difference was observed between untreated cells and those treated with postbiotics alone (Figure 3G). After 48 h, untreated cells continued to exhibit minimal necrosis, while both treatments significantly enhanced necrotic cell death relative to the control ($p < 0.0001$). Necrosis was significantly greater in the postbiotics-only group than in the combination group ($p < 0.0001$), indicating distinct cytotoxic profiles (Figure 3H). At 72 h, the combination treatment produced the highest level of necrosis,

significantly exceeding that of both the untreated control ($p < 0.001$) and the postbiotics-only group ($p < 0.0001$). Treatment with *Lactobacillus* postbiotics alone also resulted in a sustained and significant increase in necrotic cells compared with the control ($p < 0.0001$) (Figure 3I).

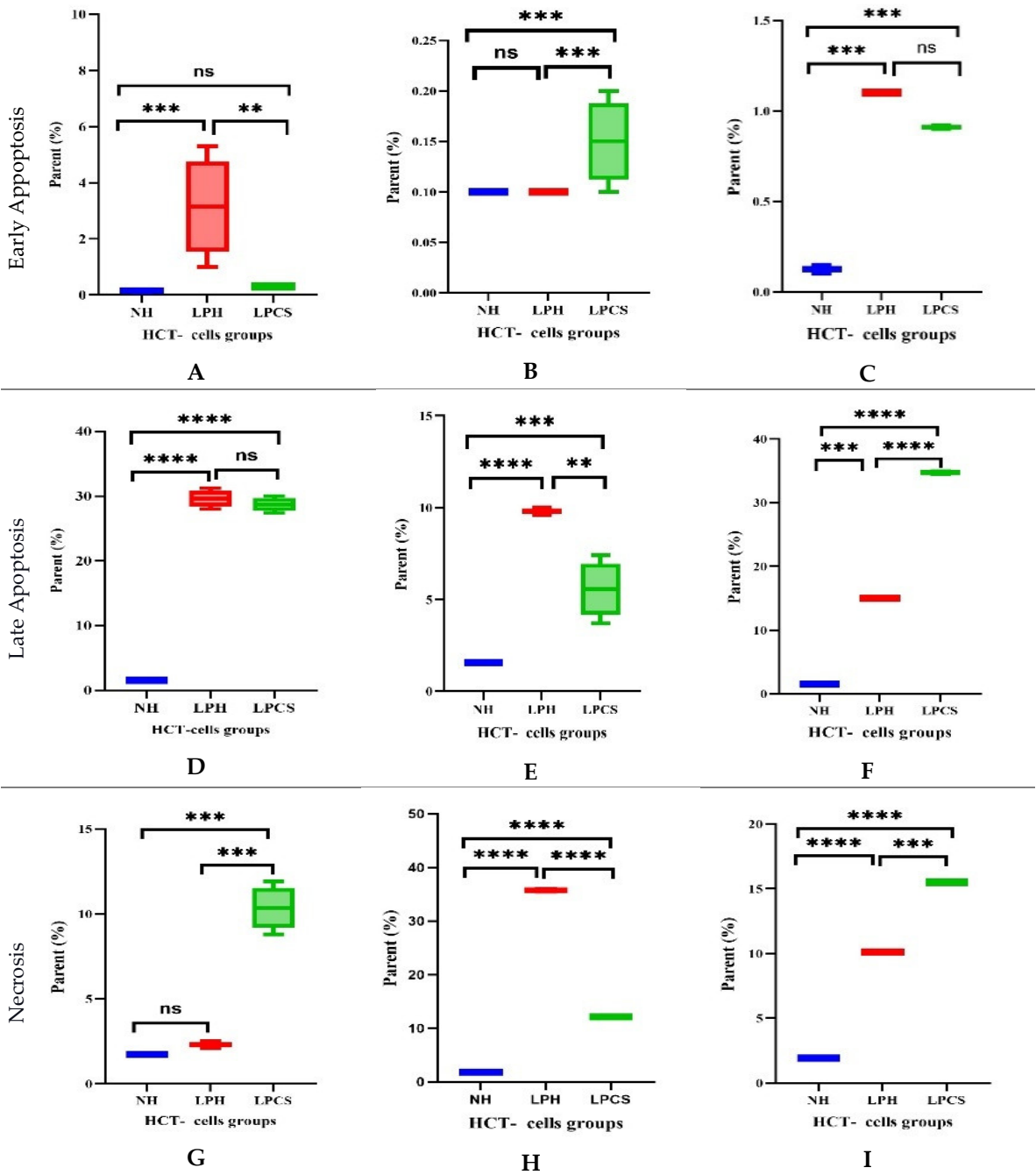


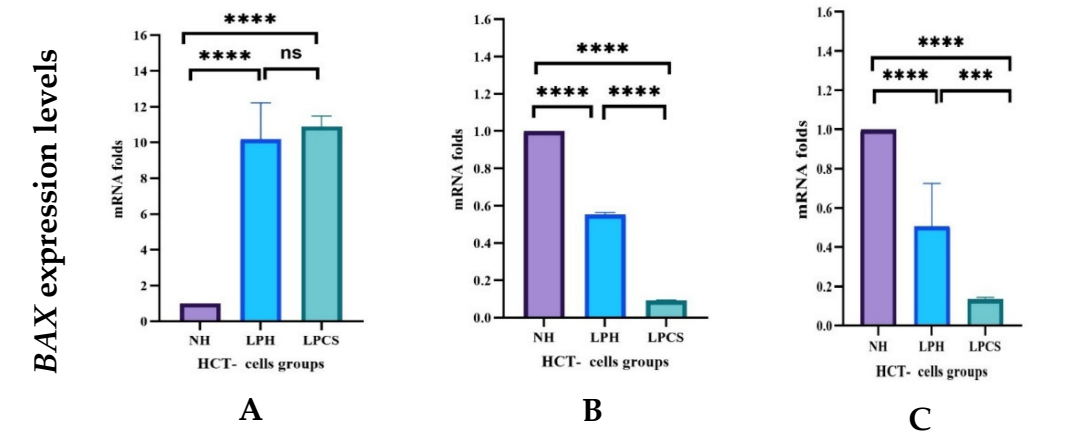
Figure 3. Time-dependent effects of *Lactobacillus* postbiotics and combination treatment on apoptotic and necrotic responses in HCT cells. Panels (a–c) illustrate early apoptosis at 24, 48, and 72 h, respectively; panels (d–f) show late apoptosis at the corresponding time points; and panels (g–i) depict necrosis after 24, 48, and 72 h of treatment. The untreated control group is denoted as NH, LPH represents cells treated with *Lactobacillus* postbiotics alone, and LPSH indicates cells treated with the combination of *Lactobacillus* postbiotics, carrageenan, and soy protein. The combination treatment demonstrates enhanced anticancer activity through increased induction of apoptotic and necrotic cell death. Data is expressed as mean $\pm$ SD. Statistical significance is indicated as $p < 0.05$, $*p < 0.01$, $**p < 0.001$, $***p < 0.0002$, and $****p < 0.0001$.

2.4. Time-Dependent Apoptotic Gene Expression Responses in HCT Cells

2.4.1. Levels of Apoptotic Regulatory Genes

Gene expression profiling revealed a pronounced time-dependent modulation of the apoptotic markers *BAX* and *BCL2* in response to the different treatments. At 24 h, the combination of *Lactobacillus* postbiotics, carrageenan, and soy protein significantly upregulated *BAX* expression (10.8-fold) compared with untreated control cells ($p < 0.0001$), exceeding the 9.2-fold increase observed with postbiotics alone ($p < 0.0001$). However, the difference between the two treatment groups was not statistically significant (Figure 4A). At 48 h, *BAX* expression was markedly suppressed in cells treated with the combination therapy compared with both untreated controls and postbiotics-only treatment ($p < 0.0001$ for both comparisons), while postbiotics alone also significantly reduced *BAX* levels relative to the control ($p < 0.0001$) (Figure 4B). By 72 h, the combination regimen produced the strongest inhibitory effect on *BAX* expression, which was significantly lower than that in both untreated and postbiotics-treated cells ($p < 0.0001$ and $p < 0.0002$, respectively). Nevertheless, *BAX* expression was significantly suppressed in the postbiotics-only group compared to the untreated control cells ($p < 0.0001$) (Figure 4C).

In parallel, *BCL2* expression displayed distinct temporal changes. After 24 h, *BCL2* mRNA levels were significantly upregulated in both treatment groups, with a greater increase observed in the combination group (2.03-fold), while the postbiotics-only group (1.47-fold) compared to untreated control cells ($p < 0.0002$ and $p < 0.001$, respectively). *BCL2* mRNA levels were significantly upregulated in the postbiotics-only group compared to the untreated control group ($p < 0.05$) (Figure 4D). At 48 h, *BCL2* expression was significantly reduced in the combination-treated cells compared with both untreated controls and postbiotics-treated cells ($p = 0.001$ for each comparison), whereas *BCL2* levels in the postbiotics-only group remained comparable to those of the control (Figure 4E). After 72 h, the combination treatment induced the most pronounced suppression of *BCL2* expression, which was significantly lower than that observed in both untreated and postbiotics-only groups ($p = 0.0000$ for each comparison). In contrast, treatment with *Lactobacillus* postbiotics alone resulted in only a non-significant increase in *BCL2* expression at this time point (Figure 4F).



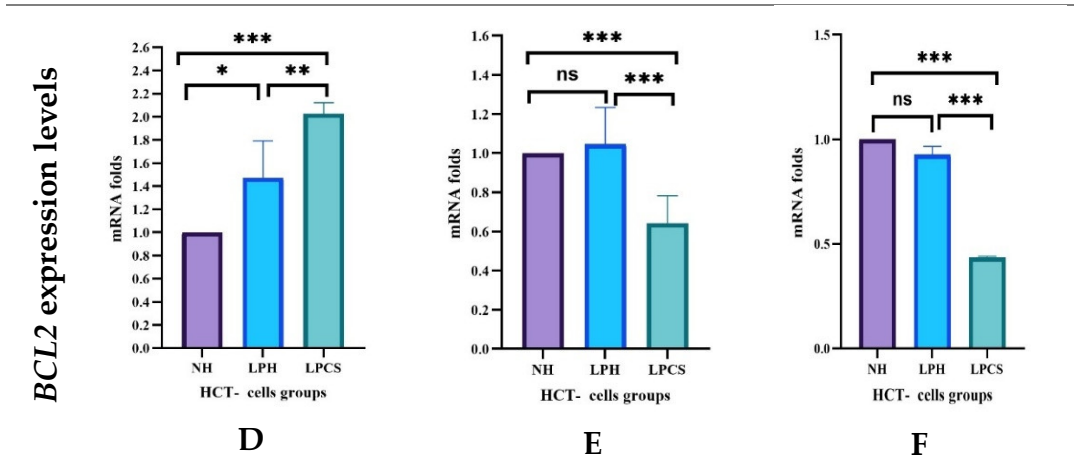


Figure 4. displays the comparative mRNA expression patterns of mitochondrial regulatory-related genes in HCT cells after exposure to various treatments. Panels (A–C) illustrate BAX expression at 24, 48, and 72 h, respectively; panels (D–F) show BCL2 expression at the corresponding time points. The untreated control group is denoted as NH, LPH represents cells treated with Lactobacillus postbiotics alone, and LCSH indicates cells treated with the combination of Lactobacillus postbiotics, carrageenan, and soy protein. The combination treatment demonstrates enhanced anticancer activity through increased induction of apoptotic and necrotic cell death. Data is expressed as mean $\pm$ SD. Statistical significance is indicated as $p < 0.05$, $*p < 0.01$, $**p < 0.001$, and $***p < 0.0002$, $****p < 0.0001$, ns (non-significant).

2.4.2. Levels of Inflammatory Gene Expression

Analysis of *NF- κ B* gene expression revealed a dynamic, time-dependent response to the applied treatments. At 24 h, both the combination treatment and Lactobacillus postbiotics alone significantly downregulated *NF- κ B* expression compared with untreated control cells ($p < 0.0001$ for both), with no significant difference observed between the two treatment groups (Figure 5A). After 48 h, cells treated with the combination formulation exhibited a marked upregulation of *NF- κ B* expression (3.35-fold) relative to both untreated controls and the postbiotics-only group ($p = 0.0001$ for each comparison). In contrast, *NF- κ B* mRNA levels in the postbiotics-treated group remained significantly lower than those in untreated cells ($p < 0.05$) (Figure 5B). By 72 h, *NF- κ B* expression was significantly reduced in both treatment groups compared with the untreated control ($p < 0.0001$), and no significant difference was detected between the combination and postbiotics-only treatments (Figure 5C).

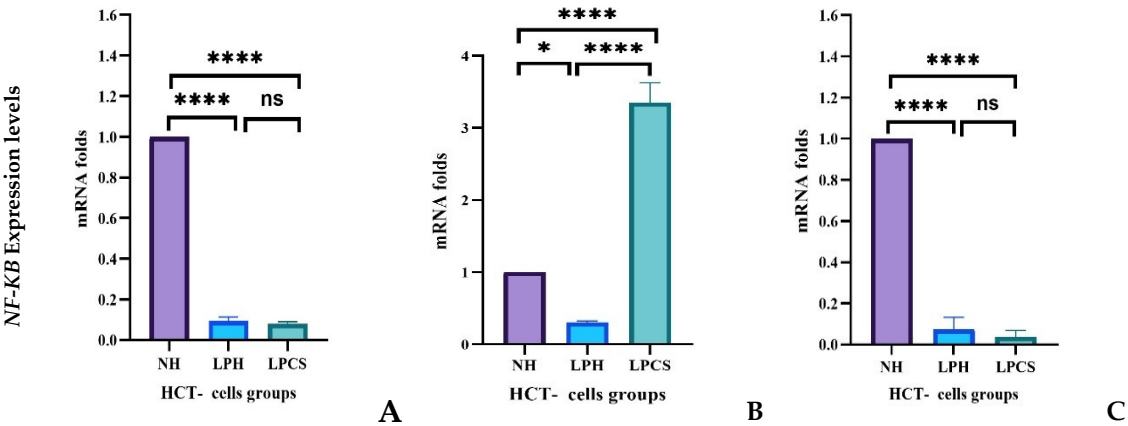


Figure 5. displays the comparative mRNA expression patterns of the *NF- κ B* gene in HCT cells after exposure to various treatments. Panels (a–c) illustrate *NF- κ B* expression at 24, 48, and 72 h. The untreated control group is denoted as NH, LPH represents cells treated with Lactobacillus postbiotics alone, and LCSH indicates cells treated with the combination of Lactobacillus postbiotics, carrageenan, and soy protein. The combination treatment demonstrates enhanced anticancer activity through increased induction of apoptotic and necrotic cell death. Data is expressed as mean $\pm$ SD. Statistical significance is indicated as $p < 0.05$, $*p < 0.01$, and $****p < 0.0001$, ns (non-significant).

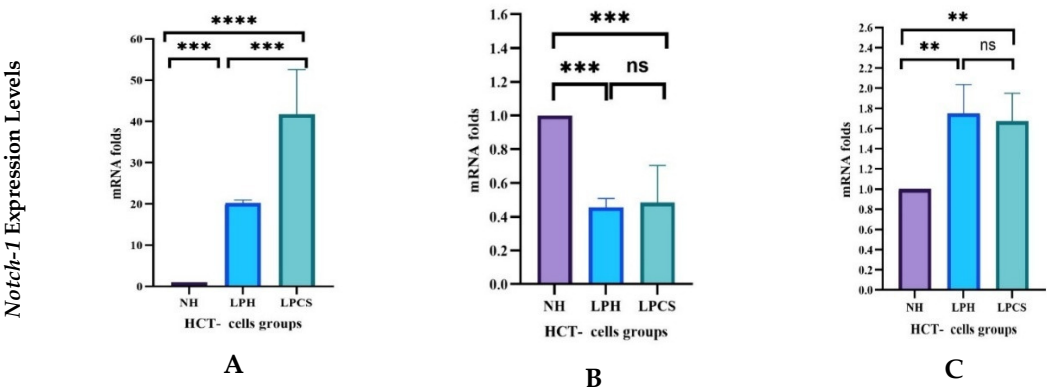
2.4.3. Levels of Notch Pathway Gene Expressions

Notch-1 expression exhibited distinct time-dependent changes in response to the treatments. At 24 h, *Notch-1* mRNA levels were significantly elevated in the combination-treated group compared with both the postbiotics-only and untreated control groups ($p < 0.0002$ and $p < 0.0001$, respectively). Treatment with *Lactobacillus* postbiotics alone also resulted in a significant increase relative to control cells ($p < 0.001$) (Figure 6A). At 48 h, *Notch-1* expression was significantly reduced in both treatment groups compared with the control ($p < 0.0002$ for each), with no significant difference between the two treatments (Figure 6B). By 72 h, *Notch-1* levels were again significantly increased in both the combination and postbiotics-only groups ($p < 0.001$ for each comparison), although the difference between treatments was not statistically significant (Figure 6C).

Notch-2 expression also demonstrated clear temporal regulation. At 24 h, *Notch-2* mRNA levels were significantly upregulated in both treatment groups compared with untreated controls ($p < 0.0001$ for each), with significantly greater induction observed in the combination-treated group ($p < 0.0001$) (Figure 6D). At 48 h, *Notch-2* expression remained significantly elevated in the combination group relative to control cells ($p < 0.0001$), whereas the increase observed with postbiotics alone was not significant. *Notch-2* levels were also significantly higher in the combination group than in the postbiotics-treated group ($p < 0.0001$) (Figure 6E). After 72 h, *Notch-2* expression was significantly increased in both treatment groups compared with controls ($p < 0.0001$ for each), with no significant difference between treatments (Figure 6F).

JAG-1 expression was significantly and differentially modulated over time. At 24 h, both treatments induced significant upregulation of *JAG-1* compared with untreated cells ($p < 0.0001$ for each), although expression levels were significantly lower in the combination group than in the postbiotics-only group ($p < 0.001$) (Figure 6G). At 48 h, *JAG-1* expression remained significantly elevated in both treatment groups relative to controls ($p < 0.0001$ for each), with a pronounced reduction observed in the combination-treated group compared with postbiotics alone ($p < 0.0001$) (Figure 6H). By 72 h, *JAG-1* expression was significantly increased in the combination-treated group compared with both untreated controls ($p < 0.05$) and the postbiotics-only group ($p < 0.0001$). In contrast, postbiotics treatment alone resulted in significant downregulation of *JAG-1* relative to control cells ($p < 0.0001$) (Figure 6I).

HES-1 expression also showed significant time-dependent changes. At 24 h, *HES-1* mRNA levels were significantly upregulated in both treatment groups compared with untreated controls ($p < 0.0001$ for each), with a greater increase observed in the postbiotics-only group ($p < 0.001$) (Figure 6J). At 48 h, *HES-1* expression was significantly reduced in both treatment groups relative to controls ($p < 0.0001$ for each), with more pronounced suppression in the combination-treated group ($p < 0.001$) (Figure 6K). This trend persisted at 72 h, where *HES-1* levels remained significantly downregulated in both treatment groups compared with controls ($p < 0.0001$), and suppression was significantly greater following combination therapy than postbiotics alone ($p < 0.0001$) (Figure 6L).



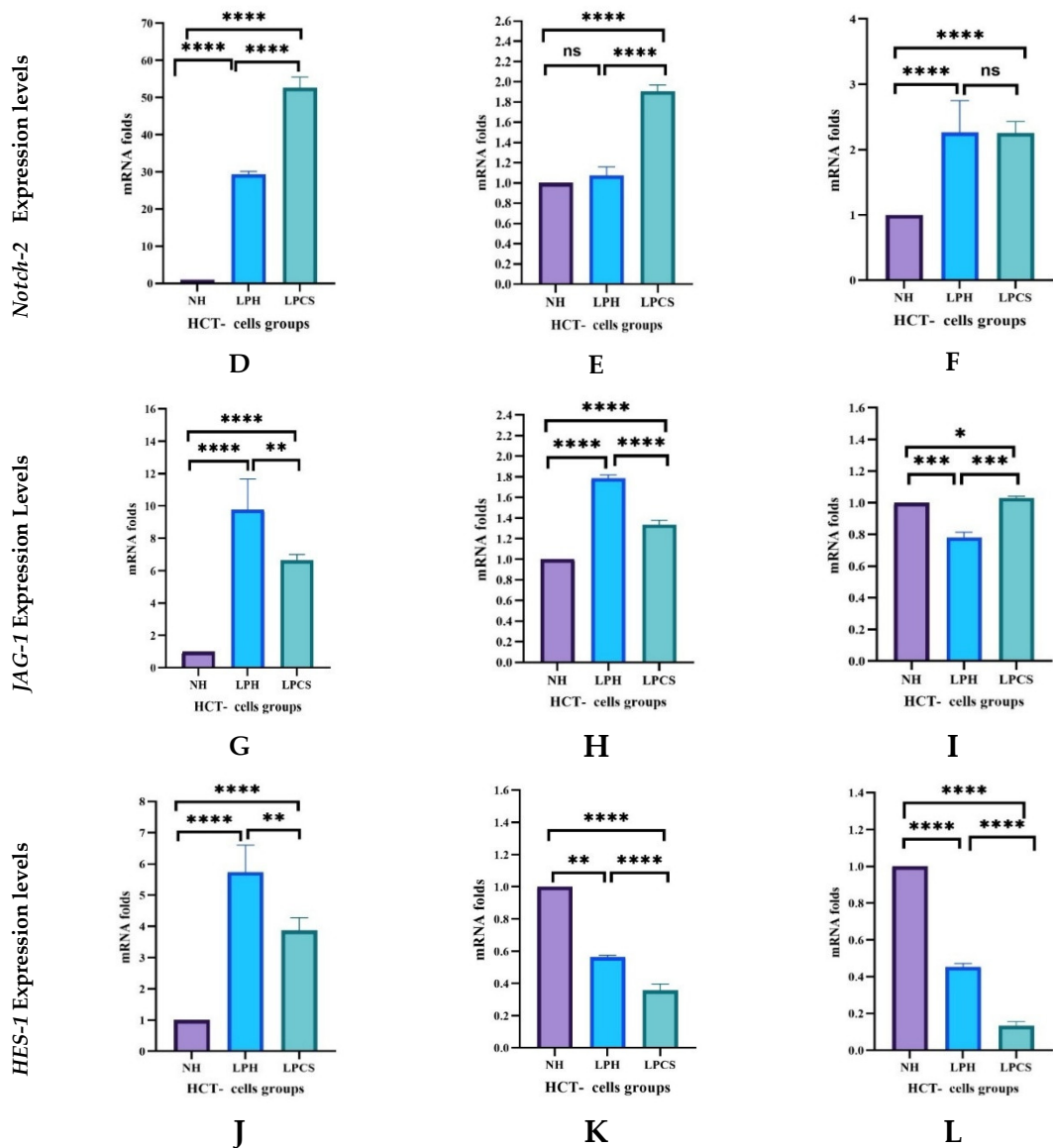


Figure 6. displays the comparative mRNA expression patterns of Notch pathway-related genes in HCT cells after exposure to various treatments. *Panels (A-C) illustrate Notch-1 expression at 24, 48, and 72 h, respectively; panels (D-F) show Notch-2 expression at 24, 48, and 72 h, respectively; panels (G-I) show JAG-1 expression at 24, 48, and 72 h; panels (J-L) show HES-1 expression at the corresponding time points.* The untreated control group is denoted as NH, LPH represents cells treated with *Lactobacillus* postbiotics alone, and LCSH indicates cells treated with the combination of *Lactobacillus* postbiotics, carrageenan, and soy protein. The combination treatment demonstrates enhanced anticancer activity through increased induction of apoptotic and necrotic cell death. Data is expressed as mean ± SD. Statistical significance is indicated as $p < 0.05$, $*p < 0.01$, $**p < 0.001$, and $***p < 0.0002$, $****p < 0.0001$, ns (non-significant).

2.4.4. Levels of CXCR4 Receptor Gene Expression

The expression of CXCR4 exhibited distinct and time-dependent modulation in response to the applied treatments.

Similarly, CXCR4 expressions were significantly modulated by the treatments over time. At 24 h, CXCR4 mRNA levels were significantly elevated in both the combination and postbiotics-only groups compared with untreated controls ($p < 0.0002$ for each), with a significantly higher expression in the combination group than in the postbiotics-only group ($p < 0.01$) (Figure 7A). After 48 h, CXCR4 expression remained significantly increased in both treatment groups compared with the control ($p < 0.0002$ and $p < 0.001$ for each group, respectively). However, a modest but statistically significant reduction was observed in the combination group relative to the postbiotics-only group ($p < 0.001$)

(Figure 7B). By 72 h, the combination treatment markedly suppressed CXCR4 expression compared with both untreated controls and postbiotics-only treatment ($p < 0.0001$ and $p < 0.001$, respectively), whereas Lactobacillus postbiotics alone did not significantly alter CXCR4 expression relative to untreated cells (Figure 7C).

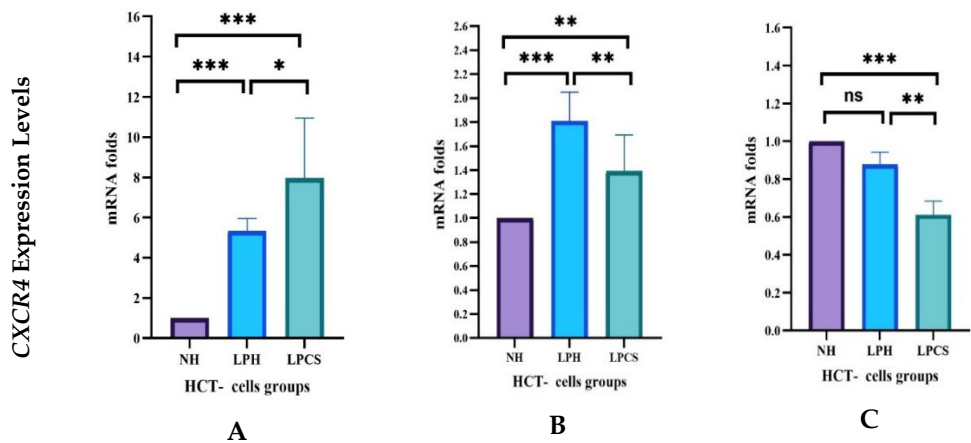


Figure 7. displays the comparative mRNA expression patterns of the metastatic marker gene in HCT cells after exposure to various treatments. Panels (A-C) show CXCR4 expression at the corresponding time points. The untreated control group is denoted as NH, LPH represents cells treated with Lactobacillus postbiotics alone, and LPCSH indicates cells treated with the combination of Lactobacillus postbiotics, carrageenan, and soy protein. The combination treatment demonstrates enhanced anticancer activity through increased induction of apoptotic and necrotic cell death. Data is expressed as mean $\pm$ SD. Statistical significance is indicated as $p < 0.05$, $*p < 0.01$, $**p < 0.001$, $***p < 0.0002$, and ns (non-significant).

2.5. Detailed Analysis of Gene Expression Correlations Post-Treatment

2.5.1. Temporal Analysis of Gene Regulatory Networks in Lactobacillus, Carrageenan, and Soy Protein Combination Treatment

Correlation analysis at 24 h following the combination treatment revealed distinct interaction patterns among apoptosis, inflammation, and Notch-related genes (Figure 9). A strong negative correlation was observed between BAX and NF- κ B ($r = -0.62$). BCL2 exhibited weak to moderate correlations with the analyzed genes. Notably, NF- κ B showed consistent negative correlations with Notch-1, JAG-1, Notch-2, and HES-1, with correlation coefficients ranging from -0.58 to -0.85 . A strong positive correlation was detected between Notch-1 and HES-1 ($r = 0.91$). In contrast, CXCR4 demonstrated strong negative correlations with Notch-1 ($r = -0.99$) and HES-1 ($r = -0.96$), while a positive correlation was observed between CXCR4 and NF- κ B ($r = 0.68$) (Figure 8A).

At 48 h, correlation analysis demonstrated altered association patterns among the examined genes (Figure 8B). Strong positive correlations were observed between BAX and JAG-1 ($r = 1.00$) and between BAX and Notch-2 ($r = 1.00$). Moderate positive correlations were also identified between BAX and BCL2 ($r = 0.56$) as well as NF- κ B ($r = 0.35$). BCL2 showed strong positive correlations with Notch-1 ($r = 0.85$), HES-1 ($r = 0.98$), and CXCR4 ($r = 0.87$), alongside a negative correlation with NF- κ B ($r = -0.59$). In contrast, NF- κ B displayed strong negative correlations with Notch-1 ($r = -0.92$), HES-1 ($r = -0.74$), and CXCR4 ($r = -0.91$). Strong positive correlations were observed between Notch-1 and HES-1 ($r = 0.94$) and between Notch-1 and CXCR4 ($r = 1.00$). Both JAG-1 and Notch-2 showed moderate positive correlations with HES-1 ($r = 0.46$), while HES-1 was strongly positively correlated with CXCR4 ($r = 0.95$) (Figure 8B).

At 72 h, correlation analysis revealed further shifts in gene association patterns (Figure 8C). Strong positive correlations were detected between BAX and NF- κ B ($r = 1.00$), BAX and HES-1 ($r = 0.96$), and BAX and CXCR4 ($r = 0.98$). Similarly, NF- κ B exhibited strong positive correlations with HES-1 ($r = 0.98$) and CXCR4 ($r = 0.99$). In contrast, Notch-1 showed strong negative correlations with

BAX ($r = -0.82$), *NF- κ B* ($r = -0.86$), *HES-1* ($r = -0.95$), and *CXCR4* ($r = -0.92$), while maintaining a strong positive correlation with *Notch-2* ($r = 0.97$). *JAG-1* displayed a strong negative correlation with *BCL2* ($r = -0.99$) and moderate positive correlations with *BAX* ($r = 0.46$) and *NF- κ B* ($r = 0.39$). Additionally, *Notch-2* showed negative correlations with *BAX* ($r = -0.64$), *NF- κ B* ($r = -0.70$), *HES-1* ($r = -0.84$), and *CXCR4* ($r = -0.79$). A perfect positive correlation was observed between *HES-1* and *CXCR4* ($r = 1.00$) (Figure 8C).

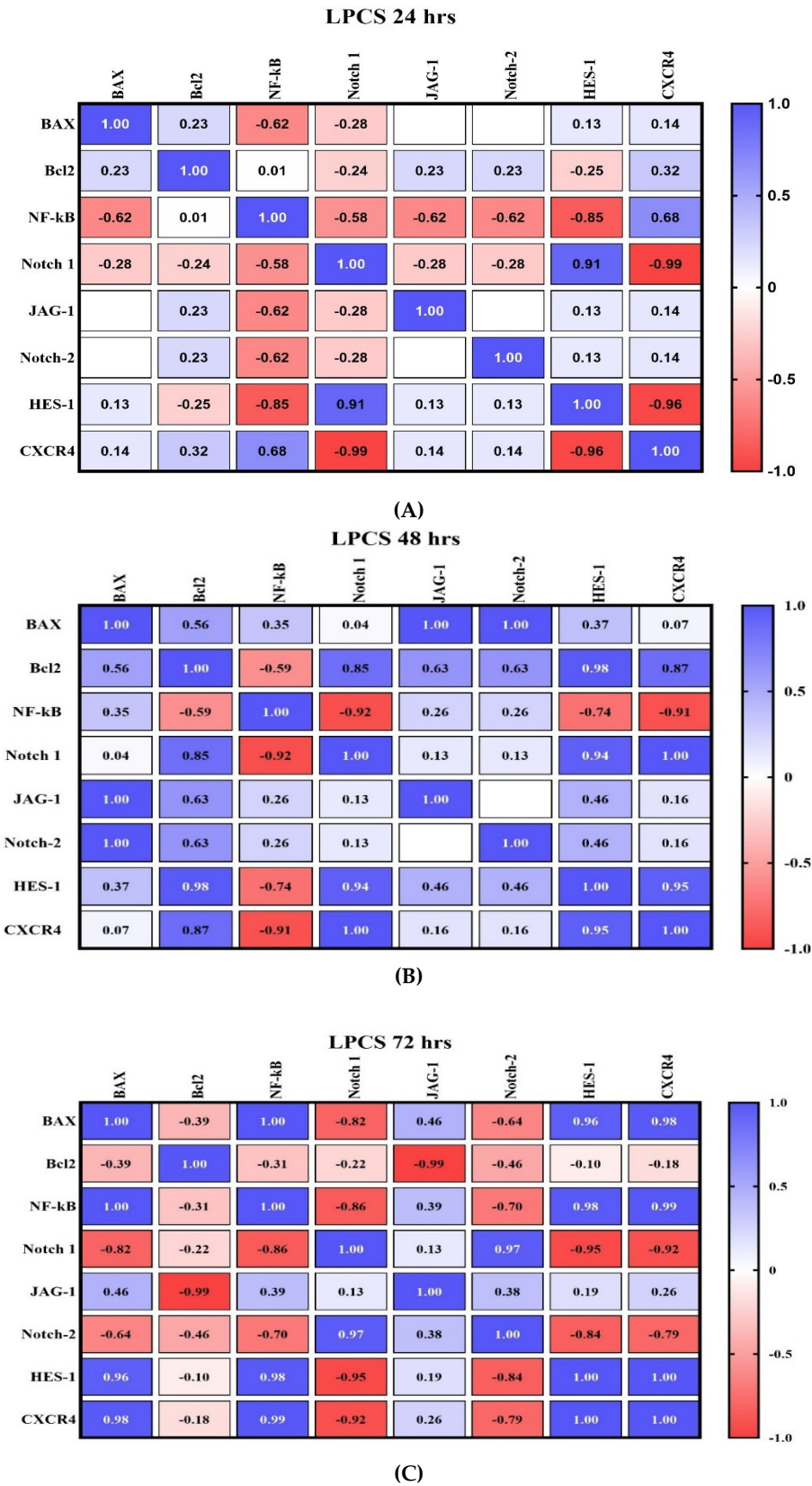


Figure 8. Correlation heatmaps of gene expression profiles in HCT cells following Lactobacillus, Carrageenan, and Soy Protein Combination Treatment (LPCS) at different time points. The matrices display the pairwise correlation coefficients (*r*) between apoptotic markers (BAX, BCL2), signaling regulators (NF- κB, Notch-1, Notch-2, JAG-1, HES-1), and the metastatic marker CXCR4 at (A) 24 h, (B) 48 h, and (C) 72 h post-treatment. The color scale represents the strength and direction of the correlation, ranging from dark red (strong negative correlation, (*r* = -1.0) to dark blue (strong positive correlation, (*r* = 1.0), with white indicating no correlation (*r* = 0). Values inside the cells denote the specific Pearson correlation coefficient for each gene pair.

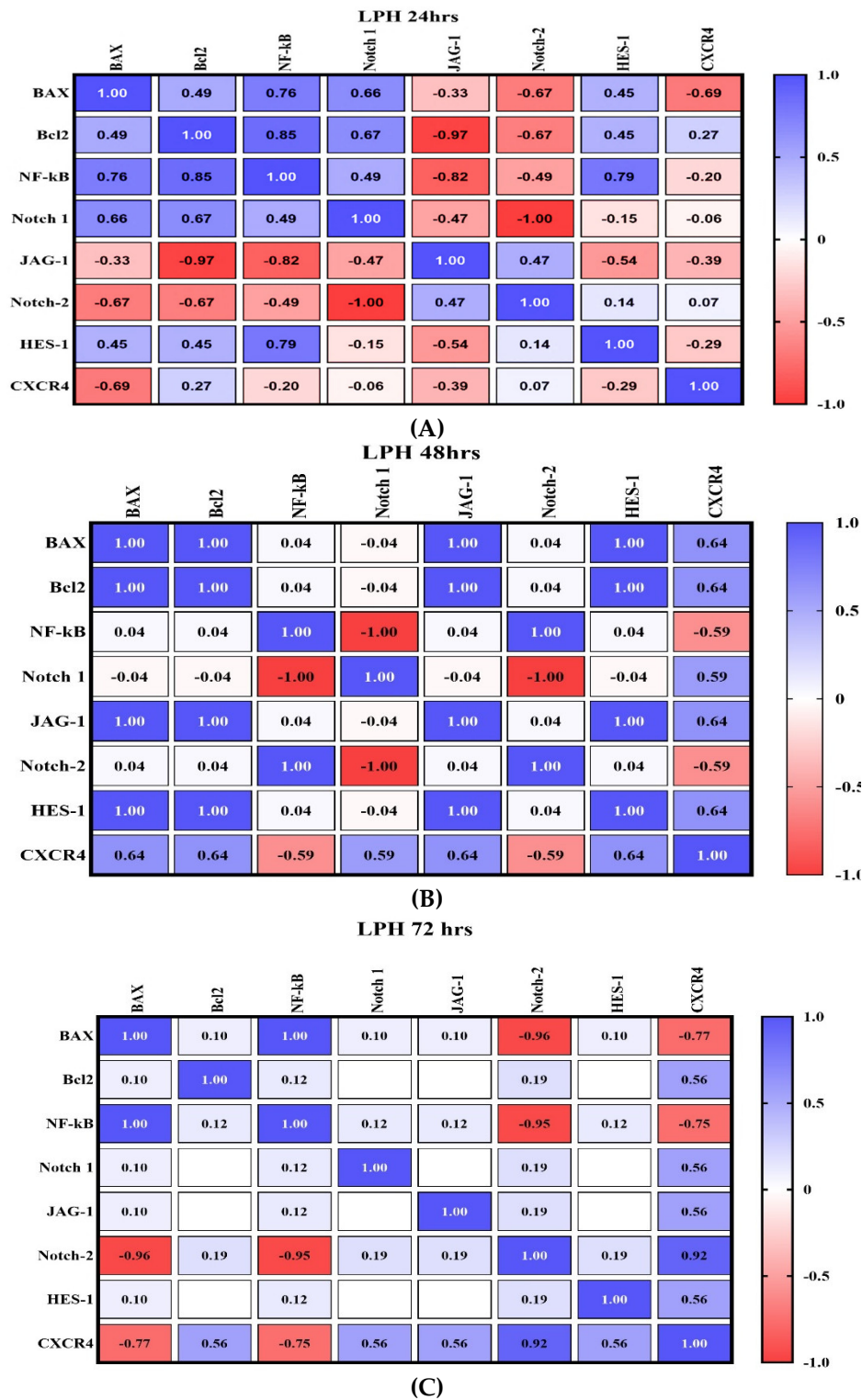


Figure 9. Correlation heatmaps of gene expression profiles in Lactobacillus cells treated with postbiotics (LPH) at different time points. The matrices display the pairwise correlation coefficients (*r*) between apoptotic markers (BAX,

BCL2), signaling regulators (*NF- κ B*, *Notch-1*, *Notch-2*, *JAG-1*, *HES-1*), and the metastatic marker *CXCR4* at (A) 24 h, (B) 48 h, and (C) 72 h post-treatment. The color scale represents the strength and direction of the correlation, ranging from dark red (strong negative correlation, $r = -1.0$) to dark blue (strong positive correlation, $r = 1.0$), with white indicating no correlation ($r = 0$). Values inside the cells denote the specific Pearson correlation coefficient for each gene pair.

2.5.2. Temporal Analysis of Gene Regulatory Networks in *Lactobacillus* Postbiotic-Treated Cells

The temporal evolution of gene correlation patterns revealed distinct regulatory shifts at 24, 48, and 72 h post-treatment (Figure 9).

At 24 h, the initial response was characterized by a dynamic and competitive regulatory landscape. A defining feature of this early phase was a striking “isoform switch” within the Notch signaling pathway, evidenced by a perfect inverse correlation between *Notch-1* and *Notch-2* ($r = -1.00$). Additionally, *JAG-1* acted as a potent negative regulator of survival, displaying profound inverse correlations with both *BCL2* ($r = -0.97$) and *NF- κ B* ($r = -0.82$). Notably, *CXCR4* expression at this stage was inversely associated with the initiation of apoptosis, as indicated by its negative correlation with *BAX* ($r = -0.69$) (Figure 9A).

At 48 hours: A strong, positively correlated cluster formed, including *BAX*, *BCL2*, *JAG-1*, and *HES-1*, all of which showed perfect co-expression ($r = 1.00$). Conversely, the antagonistic polarity within the Notch pathway persisted; *Notch-1* maintained a perfect negative correlation ($r = -1.00$) with the *Notch-2* / *NF- κ B* axis, while these two markers were perfectly co-regulated ($r = 1.00$). Additionally, *CXCR4* expression shifted to moderately align with the apoptotic *BAX/BCL2* cluster ($r = 0.64$) and negatively correlated with the *NF- κ B* / *Notch-2* survival pathway ($r = -0.59$) (Figure 9B).

At 72 hours: A synchronized stress-response pathway was identified between *BAX* and *NF- κ B* ($r = 1.00$). In contrast, *Notch-2* and *CXCR4* formed a strong, closely correlated cluster ($r = 0.92$) linked to metastatic potential. These two signaling pathways showed a significant inverse relationship: the *Notch-2* / *CXCR4* cluster was strongly negatively correlated with the *BAX/NF- κ B* axis (r values ranging from -0.75 to -0.96). Meanwhile, markers such as *BCL2*, *Notch-1*, *JAG-1*, and *HES-1* demonstrated moderate correlations with *CXCR4* ($r = 0.56$) but were disconnected from the *BAX/NF- κ B* response. (Figure 9C).

3. Discussion

In this study, the combination of *Lactobacillus* postbiotics with a carrageenan/soy protein blend exhibited the most pronounced and sustained anticancer activity in HCT colorectal cancer cells, with maximal efficacy observed at 72 h. The combined treatment induced severe and progressive morphological damage, including marked cell shrinkage, extensive apoptotic features, and pronounced cell detachment, together with the highest levels of late apoptosis and necrosis, indicating a clear shift from reversible cellular stress to irreversible cell death [5]. Importantly, the cytotoxic response followed a distinct time-dependent trajectory. Although early apoptotic signaling was minimal at 24 h in the combination group, a significant decrease in cell viability was already observed at this time point, indicating early growth inhibition and cellular stress that likely began before this checkpoint [28]. Further observations revealed that, by 48 h, early apoptotic characteristics increased markedly in parallel with additional loss of viability and worsening morphological relapses, representing a critical transition from apoptotic priming to commitment [29]. This process culminated at 72 h in dominant late apoptosis and necrosis, consistent with cumulative cytotoxic pressure and terminal engagement of cell death pathways [30]. This stepwise progression is biologically plausible, as postbiotic metabolites, such as organic acids and bacteriocin-like peptides, are known to disrupt mitochondrial function and cellular energy metabolism [31]. Meanwhile, the carrageenan/soy protein matrix may enhance membrane perturbation, oxidative stress modulation, and caspase-dependent apoptotic signaling, thereby amplifying cellular damage during prolonged exposure [32]. In contrast, *Lactobacillus* postbiotics alone exerted their strongest effects at the early 24 h time point, characterized by a significant increase in early apoptosis consistent with rapid initiation of intrinsic apoptotic signaling reported for short-chain fatty acids and related postbiotic

compounds [33]. However, these effects were not sustained at later time points, as evidenced by partial recovery of cell viability and reduced dominance of terminal death markers, suggesting activation of adaptive or pro-survival mechanisms under sublethal stress conditions [34]. Collectively, these findings indicate that while postbiotics alone primarily trigger early apoptotic responses, their combination with carrageenan and soy protein markedly enhances both the magnitude and durability of cytotoxic effects, driving a sustained progression toward late apoptosis and necrosis. This highlights the therapeutic potential of multi-component postbiotic formulations to overcome adaptive resistance and achieve prolonged and irreversible anticancer activity in colorectal cancer models, supporting their further development as rational, network-targeting strategies in cancer therapy.

In the present study, gene expression analysis demonstrated a clear time-dependent modulation of the apoptotic regulators *BAX* and *BCL2*, with the combination of *Lactobacillus* postbiotics, carrageenan, and soy protein exerting the most pronounced effects, particularly at 72 h. At 24 h, the combination significantly upregulated *BAX*, indicating early activation of pro-apoptotic signaling comparable to postbiotics alone [35], while the concurrent transient increase in *BCL2* suggested an initial adaptive survival response to cellular stress [36]. Also, by 48 h, the combination treatment induced a coordinated suppression of both *BAX* and *BCL2* relative to controls and postbiotics alone, reflecting a shift from early transcriptional stress responses toward commitment to apoptosis, likely governed by post-transcriptional and execution-phase mechanisms [37]. At 72 h, the combination produced the strongest modulation, with profound downregulation of *BCL2* and *BAX*, consistent with loss of anti-apoptotic protection, irreversible apoptotic commitment, and progression to late apoptosis and necrosis [4]. In contrast, *Lactobacillus* postbiotics alone showed their most effective response at 24 h through significant *BAX* upregulation but failed to sustain suppression of *BCL2* at later checkpoints, indicating a less durable apoptotic response [38]. Overall, these current findings highlight that while current postbiotics alone primarily trigger early apoptotic signaling, their combination with carrageenan and soy protein more effectively disrupts apoptotic balance over time, resulting in sustained and irreversible cancer cell death.

Analysis of the current *NF- κ B* gene expression levels revealed a clear time-dependent response across all checkpoints (24, 48, and 72 h) and treatment groups. In the present study, at 24 h, both the combination of *Lactobacillus* postbiotics with carrageenan and soy protein and postbiotics alone significantly downregulated *NF- κ B* expression compared with untreated cells, indicating early suppression of pro-survival and inflammatory signaling, lowering the apoptotic threshold, and limiting stress-adaptation capacity [39], with no significant difference between the current treated groups. By hour 48, the present combination treatment uniquely induced a significant upregulation of *NF- κ B* relative to both untreated and postbiotics-only groups, suggesting a transient compensatory or stress-adaptation response [40], whereas *NF- κ B* expression in the postbiotics-only group remained suppressed; however, this short-lived activation did not indicate treatment failure, as it was not sustained. In the present study, by 72 h, *NF- κ B* expression was again significantly downregulated in both treatment groups compared with untreated controls, coinciding with maximal cytotoxicity and dominant late apoptotic outcomes [41] under the combination treatment. In contrast, although postbiotics alone maintained *NF- κ B* suppression at intermediate time points, this was insufficient to produce durable apoptotic or cytotoxic effects. Overall, the present results indicate that while *Lactobacillus* postbiotics alone primarily mediate early and sustained *NF- κ B* inhibition, their combination with carrageenan and soy protein induces a dynamic regulatory pattern characterized by early suppression, transient activation, and final inhibition of *NF- κ B* signaling, which likely underlies the superior and sustained anticancer efficacy of the combined formulation during prolonged exposure.

The present findings demonstrate a complex, time-dependent modulation of the Notch signaling pathway in response to treatment, with the combination of *Lactobacillus* postbiotics, carrageenan, and soy protein exerting the most pronounced and sustained regulatory effects. In the current combination-treated group, *Notch-1* and *Notch-2* expressions were significantly upregulated at 24 h,

indicating early activation of Notch signaling [42], a response that has been reported in colorectal cancer cells undergoing stress adaptation or differentiation-associated signaling [13]. Such early activation may reflect an initial cellular attempt to maintain survival or plasticity under treatment-induced stress [43]. However, by 48 h, both Notch receptors were significantly suppressed relative to untreated cells, coinciding with increased cytotoxic pressure and suggesting disruption of Notch-dependent survival mechanisms [44]. This present mid-phase suppression is particularly relevant, as persistent Notch signaling in colorectal cancer is associated with enhanced proliferation, stemness, and resistance to apoptosis [11] [15]. In the present study, at 72 h, *Notch-1* and *Notch-2* expression were again elevated in the combination group, likely reflecting late-stage transcriptional dysregulation or compensatory signaling in cells undergoing terminal stress rather than effective pathway reactivation [45]. Moreover, the regulation of the Notch ligand *JAG-1* further highlights the distinctive impact of the current combination treatment. Although *JAG-1* was initially upregulated at 24 h, its expression was significantly lower than that observed with postbiotics alone, and this reduction became more pronounced at 48 h, indicating effective attenuation of ligand-mediated Notch activation [46] during the critical mid-treatment phase. By 72 h, *JAG-1* expression level was significantly increased in the combination group compared with both controls and postbiotics alone, which may reflect aberrant ligand expression in cells undergoing irreversible damage [47]. Importantly, downstream signaling was strongly affected, as evidenced by significant suppression of *HES-1* at 48 and 72 h, with inhibition being more pronounced in the current combination group. Given that *HES-1* is a key transcriptional effector that maintains proliferation and inhibits differentiation and apoptosis [48], its sustained suppression supports effective blockade of functional Notch signaling and aligns with the enhanced apoptotic outcomes observed under combination treatment [13]. In contrast, *Lactobacillus* postbiotics alone exerted their most prominent effects during the early 24 h time point, where significant upregulation of *Notch-1*, *Notch-2*, *JAG-1*, and *HES-1* was observed relative to untreated cells. This pattern is consistent with a transient activation of Notch signaling, potentially reflecting a stress-response or survival mechanism triggered by postbiotic metabolites [15]. At 48 h, postbiotics alone suppressed *Notch-1* and *HES-1* but did not significantly reduce *Notch-2* expression, indicating incomplete inhibition of the pathway [49]. By 72 h, while *Notch-1* and *Notch-2* expression remained elevated, *JAG-1* was significantly downregulated, and *HES-1* suppression persisted, suggesting partial and inconsistent disruption of Notch signaling that may limit long-term cytotoxic efficacy [50]. Overall, analysis across all checkpoints demonstrates that while both treatments modulate the Notch pathway in a time-dependent manner, the combination of *Lactobacillus* postbiotics with carrageenan and soy protein induces a more coordinated and sustained disruption of Notch signaling. Early receptor activation is followed by mid-phase suppression of receptors, ligands, and downstream effectors, culminating in pronounced inhibition of *HES-1*, a key mediator of Notch-driven tumor maintenance. This dynamic yet ultimately suppressive regulation of the Notch axis likely contributes to the superior and sustained anticancer efficacy of the combined formulation compared with postbiotics alone.

The results demonstrate a clear time-dependent modulation of *CXCR4* expression, with the combination of *Lactobacillus* postbiotics, carrageenan, and soy protein exerting the most pronounced regulatory effect during prolonged exposure. In the combination-treated cells, *CXCR4* expression was significantly upregulated at 24 h, likely reflecting an early stress- or chemokine-responsive adaptation, as *CXCR4* is rapidly inducible under inflammatory and oxidative conditions [51] and is commonly activated as a transient survival response in colorectal cancer cells [52]. At 48 h, *CXCR4* levels remained elevated relative to untreated controls but were significantly lower than those observed with postbiotics alone, indicating the initiation of effective suppression of *CXCR4*-mediated signaling [53]. Notably, by 72 h, the combination treatment induced a marked downregulation of *CXCR4* compared with both untreated and postbiotics-only groups, suggesting robust inhibition of the *CXCR4* axis. Given the established role of *CXCR4* in promoting colorectal cancer cell survival, invasion, angiogenesis, and metastatic dissemination through CXCL12 signaling, its sustained late-stage suppression is biologically significant and likely contributes to the enhanced cytotoxic and anti-

migratory effects observed under combination treatment [54]. In contrast, *Lactobacillus* postbiotics alone produced a transient increase in CXCR4 expression at 24 h that persisted at 48 h, but failed to suppress CXCR4 at 72 h, indicating maintenance of CXCR4-dependent signaling over time [53]. Overall, while both treatments initially induced CXCR4 expression, only the combined formulation achieved progressive and durable suppression by the final checkpoint, highlighting its superior capacity to disrupt CXCR4-mediated pro-tumorigenic pathways.

Temporal gene–gene correlation analysis revealed pronounced time-dependent regulatory rewiring, with the combination treatment producing the most coordinated and biologically decisive effects across all time points. At 24 h, the combined formulation induced a highly dynamic adaptive state characterized by strong antagonism within the Notch pathway, evidenced by an inverse relationship between *Notch-1* and *Notch-2*, together with negative correlations between *JAG-1* and the survival regulators *BCL-2* and *NF- κ B*, indicating early suppression of pro-survival and inflammatory signaling [55]. During this early phase, CXCR4 showed inverse association with *BAX* and alignment with *NF- κ B*, suggesting a transient chemokine-driven stress adaptation response that may support short-term resistance [56]. By 48 h, sustained combination exposure triggered substantial network reorganization, marked by the emergence of a tightly co-regulated module involving *BAX*, *BCL-2*, *JAG-1*, and *HES-1*, reflecting a transitional state toward apoptotic commitment in which apoptotic pressure coexisted with compensatory survival attempts [57]. Antagonistic polarity within the Notch pathway persisted, with *Notch-1* remaining inversely associated with the *Notch-2* /*NF- κ B* survival axis, while CXCR4 progressively disengaged from inflammatory signaling and aligned more closely with the apoptotic network. At 72 h, the regulatory architecture underwent decisive polarization, with a dominant stress-apoptotic axis centered on *BAX* and *NF- κ B* emerging, accompanied by collapse of *BCL-2*-mediated protection and functional disengagement of Notch- and CXCR4-dependent survival and migratory pathways [58]. This stepwise transition from early adaptive stress responses to terminal apoptotic signaling provides strong mechanistic support for the superior and sustained anticancer efficacy of the combination treatment through coordinated dismantling of survival, inflammatory, and resistance networks.

In contrast, *Lactobacillus* postbiotics alone elicited an early but limited and reversible regulatory response that failed to progress toward stable apoptotic commitment. At 24 h, postbiotics induced modest perturbations in apoptotic and inflammatory signaling, consistent with transient metabolic and mitochondrial stress; however, gene–gene interactions remained fragmented and did not form tightly co-regulated apoptotic clusters. By 48 h, most correlations weakened or reverted toward baseline, indicating activation of adaptive and compensatory survival mechanisms [59], including partial restoration of *NF- κ B*- and Notch-associated signaling. At the same time, apoptotic markers failed to maintain coordinated engagement [60]. At 72 h, the regulatory network remained dispersed, with no evidence of dominant stress-apoptotic axis formation or network collapse, consistent with sublethal stress adaptation and reduced terminal apoptosis [34]. Collectively, these findings indicate that postbiotics alone induce only transient apoptotic priming, whereas the combination treatment uniquely enforces sustained network-level disruption and irreversible apoptotic signaling, highlighting the mechanistic basis for its enhanced anticancer efficacy.

4. Materials and Methods

4.1. Preparation of *Lactobacillus acidophilus* Postbiotics and Treatment Formulations

Commercial Saudi yogurt obtained from Jeddah was used as a source of *Lactobacillus acidophilus*. Following sterilization (121 °C, 15 min), bacterial isolates were cultured on de Man, Rogosa, and Sharpe (MRS) agar at 37 °C for 72 h. Selected colonies were subsequently expanded in MRS broth and incubated at 37 °C for 2–3 days under 5% CO₂ with gentle agitation (100 rpm) to enhance bacterial growth. Cultures were centrifuged at 5000×g for 20 min at 4 °C, and the resulting cell-free supernatant was collected and lyophilized. The pH of the *Lactobacillus* postbiotic supernatant

ranged from approximately 4.2 to 4.6 [61]. The freeze-dried postbiotic preparations were stored at -20 °C until further use.

κ-Carrageenan (Sigma-Aldrich, CAS No. 11114-20-8) and commercial soy protein (iHerb®) were used in combination treatments. Both compounds were diluted in culture medium and sterilized by filtration through 0.22 μm membrane filters. Working solutions were prepared at final amounts of 0.025 mg carrageenan and 0.005 mg soy protein per 100 μL of DMEM, corresponding to treatments applied to 5 × 10³ HCT cells.

4.2. HCT-116 Cell Maintenance, Treatment, and Sample Harvesting

Human colorectal carcinoma HCT-116 cells (ATCC® CCL-247™) were cultured in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and antibiotics under standard conditions (37 °C, 5% CO₂). Cells were routinely maintained and passed at approximately 70% confluence using conventional cell dissociation procedures, with cell viability assessed before experimentation. The experimental design included three groups: untreated control cells (NH), cells treated with *Lactobacillus acidophilus* postbiotics (LPH), and cells treated with a combination of *Lactobacillus* postbiotics, κ-carrageenan, and soy protein (LCSH). Following treatment for 24, 48, or 72 h, cells were harvested by centrifugation, culture supernatants were removed, and cell pellets were collected for downstream RNA analyses (Table 1).

Table 1. Experimental treatment conditions for HCT-116 cell groups (NH, lactobacillus postbiotics, and LCSH) in gene expression analysis.

Comparative Item	Untreated Control group	Lactobacillus postbiotics group	Lactobacillus postbiotics, κ-Carrageenan, and soy protein group
Number of HCT cells in each flask	4 × 10 ⁶		
Treatment concentration	Untreated	50 mg postbiotic /10 ml growth media	50 mg postbiotic + 25 mg carrageenan + 5 mg soy protein/10 ml growth media

4.3. Evaluation of Cytotoxicity and Apoptosis in HCT-116 Cells

The cytotoxic and apoptotic effects of the different treatments on HCT-116 cells were assessed at 24, 48, and 72 h using complementary approaches, including an MTT-based cell viability assay and flow cytometric analysis of apoptosis.

4.3.1. MTT Cell Viability Assay

HCT-116 cells were seeded in 96-well plates at a density of 5 × 10³ cells per well and allowed to adhere for 24 h. Cells were then either left untreated (UHC) or exposed to the carrageenan/soy protein formulation (CS-HCT) and incubated at 37 °C for the indicated time points. At each endpoint, the culture medium was replaced with fresh medium containing 10 μL of MTT solution (12 mM) and incubated for an additional 4 h. Subsequently, 25 μL of medium was removed, and 50 μL of dimethyl sulfoxide (DMSO) was added to dissolve the formazan crystals. Absorbance was measured at 540 nm using a microplate reader, and cell viability was expressed relative to untreated controls.

4.3.2. Flow Cytometric Analysis of Apoptosis

For apoptosis analysis, HCT-116 cells were seeded in T-25 flasks at a density of 2 × 10⁶ cells per flask and treated as described above. After incubation for 24, 48, or 72 h, cells were harvested, washed twice with phosphate-buffered saline (PBS), and resuspended in 100 μL of 1× binding buffer. Cells were stained with 5 μL of FITC-conjugated Annexin V and 5 μL of propidium iodide (PI) using the Annexin V-FITC Apoptosis Detection Kit II (Cat. No. 51-6710AK), followed by incubation for 5 min

in the dark. After dilution with 300 μ L of 1 $\times$ binding buffer, apoptotic populations were quantified by flow cytometry (Beckman Coulter, USA).

4.4. Quantitative Gene Expression Analysis

HCT-116 cells (6×10^6 cells per flask) were treated and harvested at 24, 48, and 72 h. Total RNA was isolated using the QIAGEN RNeasy® Midi Kit according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized using the Im-Prom-II™ Reverse Transcription System. Quantitative Real-Time PCR (qPCR) was performed using BioFact™ SYBR Green Master Mix and gene-specific primers (Supplementary Data, Table A1). Gene expression levels were normalized to GAPDH and calculated using the $2^{-\Delta\Delta C_t}$ method [62].

4.5. Statistical Analysis and Data Visualization

Statistical analyses were performed using GraphPad Prism (version 10). One-way analysis of variance (ANOVA) was applied to assess differences within control groups and between treated and untreated cells, with statistical significance set at $p < 0.05$. To enhance data processing, correlation analysis, and visualization, Python-based libraries, including NumPy, pandas, and seaborn, were employed. NumPy was used for numerical computations, pandas for structured data handling, and seaborn for graphical representation, enabling efficient and transparent data analysis consistent with contemporary research standards.

5. Conclusions

This study demonstrates that combining *Lactobacillus* postbiotics with a carrageenan/soy protein blend produces a more potent and sustained anticancer effect in HCT colorectal cancer cells than postbiotics alone. The combined treatment induced clear time-dependent progression from early cellular stress and growth inhibition to irreversible late apoptosis and necrosis at 72 h, supported by coordinated modulation of apoptotic regulators, dynamic suppression of *NF- κ B* signaling, and sustained disruption of the *Notch* and *CXCR4* pathways. Temporal gene–gene correlation analysis further revealed that the combination treatment drives network-level collapse of survival and adaptive signaling, whereas postbiotics alone elicited only transient apoptotic priming followed by partial recovery of pro-survival mechanisms. Collectively, these findings indicate that multi-component postbiotic formulations can overcome adaptive resistance by enforcing durable, system-wide disruption of cancer cell survival pathways, supporting their further development as promising therapeutic strategies for colorectal cancer.

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Conflicts of Interest: The authors declare no conflicts of interest.

Appendix A

Table A1. Oligonucleotide primers were employed for gene expression studies, using GAPDH as the reference gene.

Primer Name	Primer sequence	Primer length
<i>Notch-1 Forward</i>	GAC ATC ACG GAT CAT ATG GA	20
<i>Notch-1 Reverse</i>	CTC GCA TTG ACC ATT CAA AC	20
<i>Notch-2 Forward</i>	GAT GCC ACC TGA ACA ACT GC	20
<i>Notch-2 _ Reverse</i>	TGA CAA CAG CAA CAG CAA GG	20
<i>Jadded-1_ Forward</i>	AGC GAC CTG TGT GGA TGA G	19
<i>Jadded-1_ Reverse</i>	GGC TGG AGA CTG GAA GAC C	19
<i>HES-1 Forward</i>	CCA GTT TGC TTT CCT CAT TCC	21
<i>HES-1 Reverse</i>	TCT TCT CTC CCA GTA TTC AAG TTCC	25
<i>BAX Forward</i>	CCT GTG CAC CAA GGT GCC GGA ACT	24
<i>BAX_ Reverse</i>	CCA CCC TGG TCT TGG ATC CAG CCC	24
<i>BCL-2 Forward</i>	TTG TGG CCT TCT TTG AGT TCG GTG	24
<i>BCL-2_ Reverse</i>	GGT GCC GGT TCA GGT ACT CAG TCA	24
<i>NF-κB Forward</i>	ATC CCA TCT TTG ACA ATC GTG C	22
<i>NF-κB Reverse</i>	CTG GTC CCG TGA AAT ACA CCT C	22
<i>CXCR-4 Forward</i>	TTC TAC CCC AAT GAC TTG TG	20
<i>CXCR-4 Reverse</i>	ATG TAG TAA GGC AGC CAA CA	20
<i>GAPDH Forward</i>	GCA CCG TCA AGG CTG AGA AC	20
<i>GAPDH Reverse</i>	TGG TGA AGA CGC CAG TGG A	19

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