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THE PROBIOTIC-HERBAL SYNERGY: A NOVEL APPROACH TO CONTROL FOOD BORNE PATHOGENS

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ABSTRACT

Foodborne pathogens remain a major public health concern due to increasing antimicrobial resistance and recurrent outbreaks. Natural therapeutic alternatives such as probiotics and medicinal herbs have gained attention because of their antimicrobial, antioxidant, and immunomodulatory properties. However, limited studies evaluate their combined synergistic antibacterial potential. The present study assessed the antibacterial activity of four aqueous herbal extracts, *Withania somnifera* (Ashwagandha), *Cinnamomum verum* (Cinnamon), *Syzygium aromaticum* (Clove), and *Rubia cordifolia* (Manjishtha) along with cell-free supernatants (CFS) and partially purified bacteriocins from selected probiotic strains against six foodborne pathogens including *Escherichia coli*, *Salmonella typhi*, *Shigella flexnerii*, *Klebsiella aeruginosa*, *Salmonella typhi* A, and *Salmonella typhi* B. Antibacterial activity was determined using agar well diffusion assay, and zones of inhibition were expressed as Mean ± SD (mm). Comparative evaluation demonstrated that individual herbal extracts and probiotic CFS exhibited moderate inhibitory effects, whereas combination treatments significantly enhanced zones of inhibition across most test pathogens. Synergistic interactions were particularly evident against *E. coli*, *Salmonella typhi*, and *Shigella*, indicating amplified bacteriostatic activity when treatments were combined.

KEYWORDS: Probiotic–herbal synergy; Agar well diffusion assay; Foodborne pathogens,

Bacteriocin; Cell-free supernatant; Antibacterial activity; Medicinal herbs; Antimicrobial resistance.

1. INTRODUCTION

Foodborne infections continue to pose a serious global public health challenge, contributing significantly to morbidity, mortality, and economic burden. Pathogenic bacteria such as *Escherichia coli*, *Salmonella typhi*, *Shigella flexnerii*, and *Klebsiella aeruginosa* are frequently implicated in outbreaks associated with contaminated food and water.[5,11] The emergence of multidrug-resistant strains has further complicated treatment strategies, highlighting the urgent need for safe, natural, and sustainable antimicrobial alternatives to conventional antibiotics.[4,7,9]

Probiotics have gained attention as promising biological agents due to their ability to inhibit pathogenic microorganisms through multiple mechanisms. They produce organic acids, hydrogen peroxide, short-chain fatty acids, and bacteriocins that reduce environmental pH, disrupt bacterial cell membranes, and interfere with pathogen adhesion and colonization.[6, 13,18] In addition, probiotics enhance host immune responses and contribute to maintenance of microbial balance.[12]

Medicinal herbs represent another important source of natural antimicrobial compounds. Bioactive phytochemicals such as alkaloids, flavonoids, tannins, and essential oils exert antibacterial effects by destabilizing cell membranes, denaturing proteins, and inducing oxidative stress in pathogens.[2] Compounds such as cinnamaldehyde from *Cinnamomum verum* and eugenol from *Syzygium aromaticum* are particularly known for their broad-spectrum antimicrobial activity.[17,10,1]

When combined, probiotics and herbal extracts may generate synergistic antibacterial effects by targeting multiple cellular pathways simultaneously. This combined approach has the potential to enhance antimicrobial efficacy while reducing the risk of resistance development.[3,15,19] Therefore, the present study was undertaken to systematically evaluate the antibacterial and synergistic potential of selected probiotic strains and medicinal herbal extracts against common foodborne pathogens using standardized microbiological assays.[3,4]

2. MATERIALS AND METHODS

2.1 Test Pathogens

Clinical isolates of selected foodborne pathogens were used in the present study. The test organisms included *Escherichia coli*, *Salmonella typhi*, *Salmonella typhi A*, *Salmonella typhi B*, *Shigella flexnerii*, and *Klebsiella aeruginosa*. These pathogens are commonly associated with foodborne outbreaks.[5,11]

All bacterial cultures were maintained on nutrient agar slants and subcultured prior to experimentation to ensure viability and purity. For antibacterial assays, inocula were prepared by suspending freshly grown colonies in sterile saline and adjusting the turbidity to 0.5 McFarland standard to standardize the microbial load across all experimental plates, following standard antimicrobial susceptibility testing procedures.[8,18]

2.2 Probiotic Strains

The probiotic strains evaluated in this study included *Lactobacillus rhamnosus* (LGG), *Bifidobacterium longum*, *Saccharomyces boulardii*, and *Bacillus coagulans*. These genera are widely reported for their antimicrobial metabolite production and probiotic efficacy.[6,13,16]

Pure isolates were cultured in their respective media, namely de Man, Rogosa and Sharpe (MRS) broth for lactic acid bacteria and Yeast Peptone Dextrose (YPD) broth for yeast strains, and incubated at 37°C for 24 hours. Following incubation, the cultures were centrifuged at appropriate speed to pellet microbial cells, and the supernatant was carefully collected. The obtained supernatant was filtered to remove any remaining cells, yielding the cell-free supernatant (CFS), as described in probiotic antimicrobial evaluation studies.[3,18]

2.3 Herbal Extract Preparation

Aqueous extracts of *Withania somnifera* (Ashwagandha), *Cinnamomum verum* (Cinnamon), *Syzygium aromaticum* (Clove), and *Rubia cordifolia* (Manjishtha) were prepared using the water extraction method. Briefly, powdered plant material was mixed with sterile distilled water and kept under continuous agitation for 24 hours to facilitate extraction of bioactive phytochemicals, following previously reported phytochemical extraction methods.[2,14]

The mixture was then filtered through Whatman No. 1 filter paper to remove particulate matter. The clear filtrates were collected and stored at 4°C until further use in antibacterial assays.

2.4 Antibacterial Assay

Antibacterial activity was assessed using the agar well diffusion method on Mueller–Hinton agar plates. Sterile 8 mm cork borers were used to create uniform wells in the agar medium. Each plate was evenly inoculated with standardized test cultures adjusted to 0.5 McFarland turbidity. Wells were loaded with individual herbal extracts, individual probiotic cell-free supernatants, combinations of herbal extracts with CFS (1:1 v/v), and combinations of herbal extracts with partially purified bacteriocin. The plates were incubated at 37°C for 24 hours. After incubation, zones of inhibition were measured in millimeters along two perpendicular axes, and the mean values were calculated, as described in antimicrobial and synergy evaluation studies.[3,8,18]

2.5 Statistical Analysis

All experiments were performed in duplicate to ensure reproducibility and accuracy of results. Data were expressed as Mean ± Standard Deviation. Statistical comparisons between treatments and test pathogens were performed using **two-way analysis of variance (two-way ANOVA)** to evaluate the effect of different treatments and microbial species, as applied in antimicrobial synergy research[8] A p-value of less than 0.05 was considered statistically significant.

3. RESULTS

3.1 Antibacterial Activity of Aqueous Herbal Extracts

The antibacterial activity of aqueous herbal extracts was evaluated against six foodborne pathogens using the agar well diffusion assay. The results are summarized in Table 1 and Graph 1.

Among the four herbal extracts tested, *Syzygium aromaticum* (Clove) demonstrated the highest antibacterial activity against all test pathogens. The maximum zone of inhibition was observed against *Shigella flexnerii* (17.5 ± 1.1 mm), followed by *Klebsiella aeruginosa* (17 ± 0.82 mm), *Salmonella typhi B* (16.5 ± 1.26 mm), and *Salmonella typhi A* (16.2 ± 1.2 mm). Moderate inhibition was observed against *Salmonella typhi* (13 ± 0.8 mm) and *Escherichia coli* (11.5 ± 0.58 mm).

Cinnamomum verum exhibited moderate antibacterial activity across pathogens, with inhibition zones ranging between 9.5 ± 1.29 mm and 12.7 ± 0.96 mm. *Rubia cordifolia* demonstrated comparable inhibitory effects, particularly against *Salmonella typhi* (14.5 ±

1.29 mm) and *Salmonella typhi A* (14 ± 0.82 mm). In contrast, *Withania somnifera* showed negligible antibacterial activity under the tested conditions, with no measurable zones of inhibition recorded.

Two-way ANOVA analysis revealed a statistically significant effect of both treatment type and pathogen species on antibacterial activity ($p < 0.001$), confirming extract-dependent variability in inhibition patterns across different test organisms.

3.2 Antibacterial Activity of Probiotic Cell-Free Supernatants (CFS)

The antibacterial potential of probiotic CFS was evaluated against the selected pathogens, and the results are presented in Table 2 and Graph 2.

Lactobacillus rhamnosus (LGG) CFS demonstrated consistent antibacterial activity across most test organisms, with the highest inhibition observed against *Salmonella typhi A* (15 ± 2.19 mm). Moderate inhibition was recorded against *Shigella flexnerii* (13.75 ± 0.96 mm) and *Salmonella typhi B* (14 ± 1.63 mm).

CFS of *Bifidobacterium longum* exhibited comparable inhibition, particularly against *Salmonella typhi B* (13.25 ± 1.2 mm). Limited inhibition was observed for *Bacillus coagulans* and *Saccharomyces boulardii* under individual treatment conditions.

Two-way ANOVA indicated a significant influence of probiotic strain and pathogen type on antibacterial activity ($p < 0.01$), demonstrating strain-dependent inhibitory potential across different test organisms.

3.3 Synergistic Antibacterial Activity of Herbal Extracts and Probiotic CFS

To evaluate synergistic effects, combinations of herbal extracts and probiotic CFS (1:1 v/v) were tested. The findings are presented in Tables 3 and 4 and Graph 3.

Combination treatments generally exhibited enhanced zones of inhibition compared to individual applications. The most pronounced synergistic effect was observed with Clove + probiotic CFS combinations. For instance, Clove + *Bifidobacterium longum* showed inhibition of 16 ± 1.15 mm against *Salmonella typhi B* and 15.5 ± 1.19 mm against *Shigella flexnerii*. Similarly, Clove + LGG demonstrated increased inhibition against *Escherichia coli* (14 ± 1.6 mm) and *Salmonella typhi* (14.5 ± 1.28 mm). In most cases, combination treatments showed larger inhibition zones than individual herbal extracts or probiotic CFS alone. Two-

way ANOVA confirmed a highly significant interaction effect between treatment combinations and pathogen species ($p < 0.0001$), indicating strong synergistic enhancement of antibacterial activity.

3.4 Synergistic Antibacterial Activity of Partially Purified Bacteriocin with Herbal Extracts

Partially purified bacteriocins extracted from *Lactobacillus rhamnosus* and *Bifidobacterium longum* were further evaluated in combination with herbal extracts. The results are shown in Table 5 and Graph 4.

Enhanced antibacterial activity was observed in bacteriocin–herb combinations compared to bacteriocin alone. The highest inhibition was recorded for Clove + LGG bacteriocin against *Shigella flexnerii* (18 ± 0.82 mm) and *Salmonella typhi B* (17.5 ± 0.45 mm). Comparable enhancement was observed for Clove + *Bifidobacterium longum* bacteriocin combinations. Across all tested pathogens, bacteriocin– herb combinations produced significantly larger zones of inhibition than individual treatments. Two-way ANOVA revealed a highly significant interaction between bacteriocin treatment and pathogen species ($p < 0.0001$), confirming potentiation effects across different microbial strains.

Table 1

Antibacterial activity of aqueous extract of Ashwagandha, Clove, Cinnamon & Manjishtha				
Test Cultures	**Diameter of zone of inhibition (mm)**			
	Ashwagandha extract	**Clove extract**	**Cinnamon extract**	**Manjishtha extract**
E.coli	-	11.50 ± 0.58	10 ± 0.8	9.25 ± 0.5
Klebsiella	-	17 ± 0.82	11.7 ± 0.9	11.5 ± 1.2
Salmonella typhi	-	13 ± 0.8	9.6 ± 0. 75	**14.5 ± 1.29**
Salmonella typhi A	-	16.2 ± 1.2	10.25 ± 1.2	14 ± 0.82
Salmonella typhi B	-	16.5 ± 1.26	9.50 ± 1.29	13.50 ± 1.00
Shigella	-	**17.5 ± 1.1**	**12.7 ± 0.96**	14 ± 0.82

Table 2

Antibacterial activity of CFS of probiotics

Test Cultures	Diameter of zone of inhibition (mm)			
	LGG rhamnosus CFS	Bifidobacterium longum CFS	Bacillus coagulans CFS	Saccharomyces boulardii CFS
E.coli	11 ± 2.4	11 ± 1.8	-	-
Klebsiella	12.5 ± 1.29	11.50 ± 1.29	-	-
Salmonella typhi	12 ± 0.82	10 ± 0.82	-	9.75 ± 0.50
Salmonella typhi A	**15 ± 2.19**	11 ± 0.82	9.2 ± 0.8	-
Salmonella typhi B	14 ± 1.63	**13.25 ± 1.2**	-	-
Shigella	13.75 ± 0.96	12.7 ± 0.96	-	-

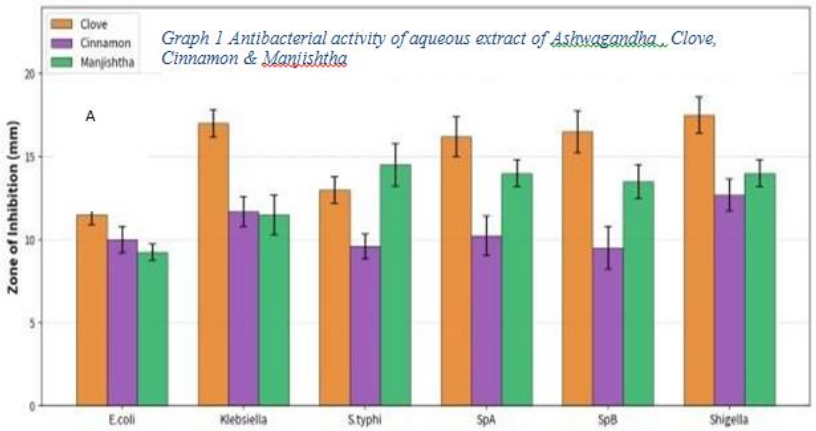
Table 3

	Antibacterial activity of Herbal extract + CFS of probiotics				
	Diameter of zone of inhibition (mm)				
Cultures	Herbs	LGG rhamnosus	Bifidobacterium longum	Bacillus coagulans	Saccharomyces boulardii
E.coli	Ashwagandha	9.75 ± 0.96	10.50 ± 1.2	9.50 ± 0.58	10.25 ± 1.5
	Clove	**14 ± 1.6**	**15 ± 1.4**	**13.5 ± 1.2**	**14.5 ± 1.2**
	Cinnamon	9.75 ± 0.50	10.25 ± 1.5	10.75± .95	10 ± 0.82
	Manjishtha	10 ± 0.82	12 ± 1.1	11 ± 1.15	11 ± 1.2
Klebsiella	Ashwagandha	9.50 ± 0.58	10.25 ± 0.96	9.75 ± 0.50	10 ± 0.82
	Clove	**12 ± 1.3**	**13 ± 1.4**	**12.5±1.29**	**13.5 ± 1.26**
	Cinnamon	9.75 ± 0.50	10.5 ± 1.29	10 ± 0.82	10.2 ± 0.96
	Manjishtha	9 ± 0.82	10± 1.1	9.50 ± 0.58	10.2 ± 1.3
Salmonella typhi	Ashwagandha	9.50 ± 0.58	10.25 ± 0.96	9.75 ± 0.5	10 ± 0.82
	Clove	**14.5± 1.28**	**13.5 1.2**	**13 ± 1.13**	**14 ± 1.83**
	Cinnamon	9.75 ± 0.50	10.5 ± 1.2	10.00 ± 0.82	10.2 ± 0.96
	Manjishtha	10 ± 0.82	11 ± 1.13	10.50 ± 0.58	11 ± 1.8
Salmonella typhi A	Ashwagandha	10.75 ± 0.96	11.5 ± 1.2	11 ± 0.82	11.2± 0.96
	Clove	11 0.82	12 1.15	11.5 0.58	11.75 0.96
	Cinnamon	11 ± 0.82	12 ± 1.1	11.± 0.58	11.75 ± 0.96
	Manjishtha	**15.50 ± 1.2**	**13.50± 1.28**	**12 ± 0.82**	**13 ± 1.4**
Salmonella	Ashwagandha				

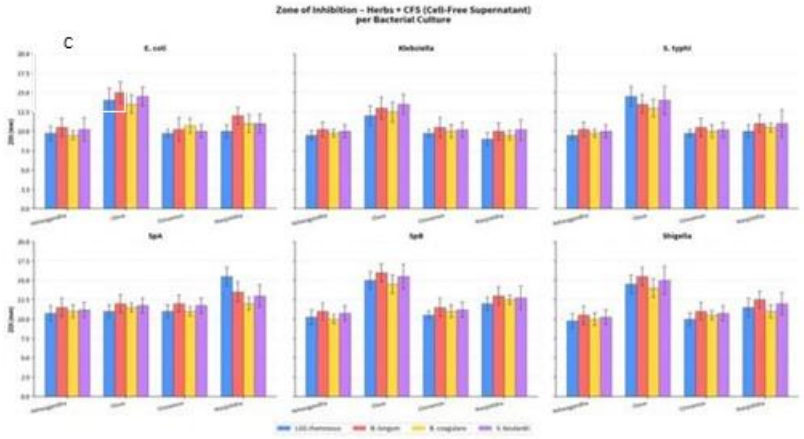
typhi B		10.25 ± 0.96	11 ± 1.1	10 ± 0.58	10.75 ± 0.96
	Clove	**15 ± 1.12**	**16 ± 1.15**	**14.5 ± 1.2**	**15.5 ± 1.6**
	Cinnamon	10.5 ± 0.5	11.5 ± 1.2	11 ± 0.82	11.2 ± 0.97
	Manjishtha	12 ± 0.82	13 ± 1.15	12.5 ± 0.58	12.75 ± 1.5
Shigella	Ashwagandha	9.75 ± 0.96	10.50 ± 1.2	10 ± 0.82	10.25 ± 0.96
	Clove	**14.5 ± 1.2**	**15.5± 1.19**	**14 ± 1.2**	**15 ± 1.8**
	Cinnamon	10 ± 0.82	11 ± 1.15	10.5 ± 0.58	10.75 ± 0.96
	Manjishtha	11.5 ± 1.2	12.5 ± 1.1	11 ± 0.82	12 ± 1.4

Table 4

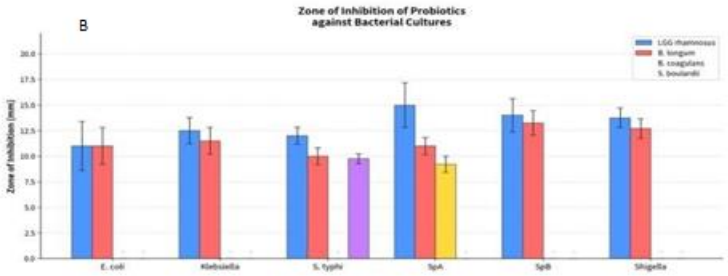
	Antibacterial activity of Herbal extract + Bacteriocin				
Diameter of zone of inhibition (mm)					
Cultures	**Bacteriocin**	**Ashwagandha**	**Clove**	**Cinnamon**	**Manjishtha**
E.coli	*LGG rhamnosus*	12.5 ± 0.58	**16.5 ± 0.58**	11.5 ± 0.3	13.5 ± 0.58
	Bifidobacterium longum	11.75 ± 0.50	**15.5 ± 0.58**	11.25 ± 0.5	12.7 ± 0.3
Klebsiella	*LGG rhamnosus*	13.5 ± 0.58	**16.7 ± 0.5**	12.5 ± 0.4	13.5 ± 0.58
	Bifidobacterium longum	12.5 ± 0.54	**16.5 ± 0.86**	11.5 ± 0.8	13.5 ± 0.59
S.typhi	*LGG rhamnosus*	13 ± 0.5	**16.25 ± 0.4**	12 ± 0.55	14 ± 0.82
	Bifidobacterium longum	13 ± 0.4	**15.5 ± 0.58**	11.25 ± 0.50	12.75 ± 0.50
SpA	*LGG rhamnosus*	15.7 ± 1.2	**17.25 ± 0.36**	12.75 ± 0.50	15.5 ± 0.58
	Bifidobacterium longum	12 ± 0.2	**16.25 ± 0.50**	12 ± 0.58	14.5 ± 0.58
SpB	*LGG rhamnosus*	14.5 ± 0.5	**17.5 ± 0.45**	12.5 ± 0.58	14.5 ± 0.58
	Bifidobacterium longum	13.2 ± 0.45	**16 ± 0.82**	11.75 ± 0.5	13.5 ± 0.58
Shigella	*LGG rhamnosus*	14.3 ± 0.52	**18 ± 0.82**	13.5 ± 0.5	15.25 ± 0.50
	Bifidobacterium longum	13.52 ± 0.50	**16.75 ± 0.50**	12 ± 0.86	14 ± 0.82



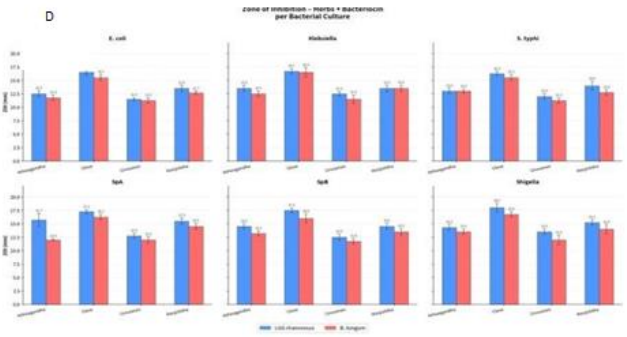
Graph 2- Antibacterial activity of A) aqueous herbal extracts; B) CFS of probiotics



Graph 3- Antibacterial activity of CFS of probiotics



Graph 4- Antibacterial activity of Synergy of CFS-aqueous herbal extract



4. DISCUSSION

The present study evaluated the antibacterial activity of aqueous herbal extracts, probiotic cell-free supernatants (CFS), and their synergistic combinations against selected foodborne pathogens including *Escherichia coli*, *Salmonella typhi*, *Salmonella typhi A*, *Salmonella typhi B*, *Shigella flexnerii*, and *Klebsiella aeruginosa*. The findings demonstrate that while individual herbal extracts and probiotic metabolites exhibited measurable inhibitory effects, their combination significantly enhanced antibacterial activity. These observations align with the growing body of literature suggesting that probiotic–herbal synergy represents a multi-

targeted antimicrobial strategy capable of overcoming pathogen resistance mechanisms.

With respect to the antibacterial activity of aqueous herbal extracts, previous studies have consistently reported that medicinal plants such as *Syzygium aromaticum* and *Cinnamomum verum* exhibit broad- spectrum antimicrobial properties due to the presence of bioactive compounds like eugenol and cinnamaldehyde. Ahmed et al. (2014) described the role of phenolic compounds and essential oils in destabilizing microbial membranes and inhibiting enzyme systems. Similarly, Ahamad (2024) highlighted the strong antibacterial potential of clove against Gram-negative bacteria, attributing its efficacy to eugenol-mediated membrane disruption. Ranasinghe et al. (2014) further reported that cinnamaldehyde interferes with bacterial metabolic pathways and quorum sensing mechanisms. In the present study, *Syzygium aromaticum* demonstrated the highest antibacterial activity across all tested pathogens, particularly against *Shigella flexnerii* and *Klebsiella aeruginosa*, which is consistent with the findings of Ahamad (2024) and Ahmed et al. (2014). However, *Withania somnifera* exhibited negligible antibacterial activity in aqueous form. This difference may be attributed to extraction methodology, as Mikulska et al. (2023) have reported that alcoholic extracts of Ashwagandha demonstrate stronger antimicrobial activity than aqueous extracts due to enhanced solubility of withanolides. Therefore, the comparatively lower activity observed in the present study may be linked to the use of water as the extraction solvent, which may not efficiently extract hydrophobic bioactive compounds.

Regarding the antibacterial activity of probiotic cell-free supernatants, literature indicates that probiotic strains inhibit pathogens through the production of organic acids, bacteriocins, and hydrogen peroxide. Rishi et al. (2011) demonstrated that *Lactobacillus plantarum* cell-free extract effectively inhibited *Salmonella Typhimurium* via acidification and bacteriocin activity. Zhou et al. (2016) further explained that probiotic metabolites disrupt membrane integrity and suppress virulence factor expression. Additionally, Jasim et al. (2022) reported enhanced immune and antimicrobial responses in fish supplemented with probiotic and cinnamon combinations, supporting the antimicrobial potential of probiotic metabolites. In the present study, *Lactobacillus rhamnosus* CFS exhibited consistent inhibitory activity, particularly against *Salmonella typhi A* and *Shigella flexnerii*, which aligns with previous findings that lactic acid bacteria produce strong antimicrobial metabolites. The comparatively moderate inhibition observed with *Bacillus coagulans* and *Saccharomyces*

boulardii may reflect strain-specific variability in metabolite production, as probiotic efficacy is widely known to be strain dependent (Darbandi et al., 2022). Variations in metabolite concentration, growth conditions, and pH of the supernatant may also have influenced inhibitory potential.

In evaluating the synergistic antibacterial activity of herbal extracts combined with probiotic CFS, previous studies have reported enhanced inhibition when plant extracts are used in conjunction with probiotic metabolites. Aminnezhad et al. (2015) demonstrated synergistic interactions between probiotic cell-free supernatants and antimicrobial agents against *Pseudomonas aeruginosa*, indicating that combined treatments enhance membrane permeability and antimicrobial penetration. Moradi et al. (2020) further reported that probiotics and therapeutic plants exhibit complementary mechanisms that amplify antibacterial efficacy. Similarly, Parihar et al. (2025) observed increased inhibition of *Staphylococcus aureus* when essential oils were combined with probiotic strains. The present study revealed significantly increased zones of inhibition in Clove + CFS combinations across multiple pathogens, particularly against *Salmonella typhi B* and *Shigella flexnerii*. This synergistic enhancement can be explained by the acidic microenvironment created by probiotic metabolites, which may facilitate increased penetration of phytochemicals such as eugenol into bacterial cells. Additionally, plant phenolics may increase membrane permeability, thereby enhancing bacteriocin diffusion and activity. The highly significant interaction observed in two-way ANOVA supports the presence of a true synergistic effect rather than a simple additive response. Furthermore, bacteriocin–herb combinations demonstrated the strongest antibacterial activity among all tested treatments. Literature suggests that bacteriocins function by forming pores in bacterial membranes and binding to cell wall precursors such as lipid II (Zhou et al., 2016). When combined with plant- derived essential oils, which destabilize lipid bilayers, the antimicrobial spectrum is broadened and efficacy is amplified. Aminnezhad et al. (2015) reported enhanced antibacterial outcomes when probiotic-derived antimicrobials were combined with other agents. Similarly, Kim et al. (2020) demonstrated synergistic antibacterial effects when probiotic lactic acid bacteria were combined with *Curcuma longa* extract. In agreement with these findings, the present study showed that Clove combined with bacteriocin from *Lactobacillus rhamnosus* produced the highest zones of inhibition, particularly against *Shigella flexnerii* and *Salmonella typhi B.* The increased efficacy observed in bacteriocin–herb combinations compared to crude CFS treatments likely reflects the higher concentration of active antimicrobial peptides, along with

membrane-disrupting phytochemicals that facilitate bacteriocin access to intracellular targets.

Overall, the findings of the present investigation corroborate existing evidence that probiotic–herbal synergy enhances antimicrobial activity through multi- targeted mechanisms involving acidification, membrane destabilization, oxidative stress induction, and inhibition of metabolic pathways. Variations between the present results and some reported studies may be attributed to differences in extraction methods, probiotic strains, pathogen susceptibility profiles, and experimental design. Nevertheless, the strong synergistic patterns observed across multiple pathogens highlight the therapeutic and food safety potential of probiotic–herbal combinations as sustainable alternatives to conventional antibiotics in the context of rising antimicrobial resistance.

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