applied microbiology

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Antibacterial Activity of a Probiotic and Synbiotic Suspension Combined with Inulin Against *Helicobacter pylori* in an In Vitro Model

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Abstract

Helicobacter pylori is a highly prevalent pathogen associated with chronic gastritis, peptic ulcers, and gastric cancer. Treatment is increasingly challenging due to antibiotic resistance and adverse effects that can reduce adherence. These limitations have encouraged the exploration of complementary strategies. This study evaluated the in vitro antibacterial activity of selected probiotic strains and synbiotic formulations containing inulin against clinical isolates of *H. pylori*. Isolates obtained from gastric biopsies were identified by MALDI-TOF. Four probiotic strains (*Lacticaseibacillus casei*, *Lacticaseibacillus rhamnosus*, *Limosilactobacillus fermentum*, and *Streptococcus thermophilus*) were tested individually and as a mixed culture, both alone and combined with inulin. Antibacterial activity was assessed using the agar well diffusion method under microaerophilic conditions after 72 h of incubation at 37 °C. Variable inhibitory effects were observed, with *L. fermentum* (8.08 ± 1.98 mm) and the probiotic mixture (7.92 ± 0.90 mm) showing greater activity, while *S. thermophilus* exhibited limited inhibition. The addition of low-dose inulin (3 mg/mL) was associated with increased inhibition by the probiotic mixture (9.58 ± 1.51 mm), whereas higher concentrations did not enhance this effect. These findings indicate that certain probiotic and synbiotic formulations exhibit in vitro activity against *H. pylori* and warrant further investigation as complementary approaches.

Keywords: probiotics; prebiotics; inulin; *Helicobacter pylori*

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Academic Editors: Francesco Broccolo and Filomena Nazzaro

Received: 12 February 2026
Revised: 18 March 2026
Accepted: 19 March 2026
Published: 25 March 2026

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1. Introduction

Helicobacter pylori (*H. pylori*) currently infects more than half of the global population. This Gram-negative, spiral-shaped, microaerophilic bacterium has been recognized as a major human pathogen since its discovery by Warren and Marshall in 1982 [1]. Despite its small size, it has a significant impact on public health. The high proportion of asymptomatic infections leads to substantial underreporting, contributing to its status as one of the most common and widespread bacterial infections worldwide [2].

The infection is primarily acquired during childhood, typically through the ingestion of contaminated water or food, and can persist throughout life if not properly treated. Many individuals remain asymptomatic; however, *H. pylori* infection can cause chronic

gastritis and, in more severe cases, progress to peptic ulcer disease, functional dyspepsia, or gastric cancer [2,3].

Conventional treatment consists of a combination of antibiotics and a proton pump inhibitor. Although generally effective, prolonged use promotes bacterial resistance and can lead to adverse effects, including alterations of the gut microbiota that facilitate the colonization of opportunistic microorganisms and the onset of secondary infections [2,4]. These limitations have driven the search for safer and more effective complementary therapeutic strategies.

Among the most promising alternatives are synbiotics, which combine probiotics and prebiotics. Probiotics are live microorganisms that, when administered in adequate amounts, confer health benefits to the host, while prebiotics are non-digestible compounds that selectively stimulate the growth of beneficial intestinal bacteria [5,6].

Several studies have shown that different probiotic strains can inhibit the growth of *H. pylori* in both in vitro and in vivo models. The most commonly used species belong to the genera *Bifidobacterium*, *Saccharomyces*, and *Lactobacillus*, either alone or in combination with conventional therapy. Experimental protocols vary widely in terms of strains, culture conditions, and analytical methods, reflecting the broad scope of research approaches [7–12].

Described mechanisms of action for probiotic strains include the production of lactic acid, short-chain fatty acids, hydrogen peroxide, and bacteriocins, which can reduce gastric pH, inhibit bacterial adhesion, decrease urease activity in *H. pylori*, and modulate the host immune response [13,14].

Prebiotics are defined as "substrates that are selectively utilized by host microorganisms, conferring a health benefit." [15]. Inulin is a fructooligosaccharide found in vegetables such as asparagus, artichokes, onions, and bananas, and has been studied for its ability to promote the growth of beneficial bacteria such as *Bifidobacterium* spp., *Lactobacillus* spp., *Bacteroides* spp., *Enterococcus faecalis*, and *Enterococcus faecium* [16]. These effects are associated with improvements in intestinal motility, reductions in fecal acidity, enhanced mineral absorption, and the regulation of inflammation and insulin sensitivity [16–19].

The present study aimed to evaluate the antibacterial activity of a probiotic suspension composed of *Lacticaseibacillus casei*, *Limosilactobacillus fermentum*, *Streptococcus thermophilus*, and *Lacticaseibacillus rhamnosus*, as well as a synbiotic formulation containing inulin, against *H. pylori*. The goal was to obtain supporting evidence for their potential as complementary therapies and to provide a foundation for the development of safer, more effective, and less aggressive treatment options for affected patients.

2. Materials and Methods

An in vitro experimental study was conducted to evaluate the antibacterial activity of probiotic suspensions (individual and mixed) and a synbiotic formulation (probiotics + inulin) against *H. pylori*. The study was carried out at the Research Laboratory of the Universidad de Ciencias Medicas (UCIMED), Costa Rica. Antibacterial activity was quantified using the agar well diffusion method by measuring inhibition zones (mm).

2.1. Bacterial Strains Used in the Study

Urease-positive gastric biopsy samples were transported in brain heart infusion (BHI) broth (Neogen, Lansing, MI, USA), mechanically macerated with a #20 needle in sterile saline solution (0.85%NaCl) (SSS) (prepared in the laboratory), and subsequently inoculated onto *H. pylori* agar (Liofilchem, Roseto degli Abruzzi, Italy), Columbia agar supplemented with 5% sheep blood (bioMérieux SA, Marcy-l'Étoile, France) [20], and liquid media including BHI, Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, St. Louis, MO,

USA) supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich), and DMEM with 10% FBS and factor XII. Additionally, laboratory-formulated media containing agar, meat extract (HiMedia Laboratories, Mumbai, India), yeast extract, NaCl (Labquimar, San Jose, Costa Rica), defibrinated rabbit blood, FBS, and hemin (Sigma-Aldrich) were used [21–24].

Inoculated media were incubated for 72 h under microaerophilic conditions (CampyGen, Thermo Scientific, Basingstoke, Hampshire, UK) [20] at 37 °C. Characteristic colonies were confirmed using oxidase and catalase tests and identified by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (Argos Biolab, San Jose, Costa Rica). Once identified, a cryopreservation stock was prepared using 1 mL of BHI broth supplemented with 20% glycerol and 10% FBS (Sigma-Aldrich) [25,26]. The suspensions were transferred to cryovials and stored at −70 °C.

Lyophilized probiotics were rehydrated using the Microbiologics (St. Cloud, MN, USA) Kwik-Stik method and inoculated onto Columbia agar with 5% sheep blood. *Lacticaseibacillus rhamnosus* (ATCC 7469), *Limosilactobacillus fermentum* (ATCC 9338), and *Lacticaseibacillus casei* (ATCC 393) were incubated for 48 h, while *Streptococcus thermophilus* (ATCC 19258) was incubated for 24 h, all at 37 °C in 5% CO_2. Cryopreserved stocks were prepared using the same procedure employed for *H. pylori*.

2.2. Preparation of Inulin Solutions and Probiotic Suspensions

Prebiotic inulin (Now, 227 g) solutions were prepared at concentrations of 3, 6, and 16 mg/mL in SSS. Solutions were sonicated for 15 min and sterilized by filtration through 0.2-µm membranes. Individual probiotic suspensions were adjusted to 0.5 McFarland (1.5×10^8 CFU/mL) using SSS. The probiotic mixture was prepared by combining equal volumes of each probiotic suspension adjusted to 0.5 McFarland in a sterile tube. Therefore, each strain contributed equally to the final mixture, with an approximate concentration of 0.38×10^8 CFU/mL per strain in the mixture.

2.3. Antibacterial Activity Assay

Antibacterial activity was evaluated using the agar well diffusion method with minor modifications [27,28]. Mueller–Hinton agar supplemented with 5% sheep blood (Liofilchem) [20] was uniformly inoculated with *H. pylori* adjusted to McFarland 1 (3.0×10^8 CFU/mL) using a sterile swab. Once the inoculation was completed, wells were made in the agar using the base of sterile micropipette tips (approximately 6 mm in diameter). Probiotic suspensions (individual and mixed) were added to wells in volumes of 20, 50, and 70 µL. To evaluate the effect of inulin and potential synergism, wells were inoculated with 50 µL of probiotic suspensions and 20 µL of inulin solutions at 3, 6, or 16 mg/mL.

Each assay was performed using four wells per plate as technical replicates. The study was conducted using a pooled *H. pylori* inoculum prepared from the two successfully recovered clinical isolates. Therefore, the reported inhibition zones correspond to repeated technical measurements of the same pooled inoculum.

To validate the assays, different experimental controls were included using the same culture medium as in the main tests. As a positive control, *H. pylori* adjusted to 3.0×10^8 CFU/mL was used, with one well containing 20 µL of amoxicillin (≥900 µg mg^{-1} potency, catalog number A8523, Sigma-Aldrich) at a concentration of 1.3 mg/mL. The negative control consisted of *H. pylori*, with one well containing 20 µL of SSS, which was also used as the diluent for the probiotic suspensions. To verify the viability of the probiotic strains under the assay conditions, a probiotic growth control was included, in which each strain was incubated under the same conditions but in the absence of *H. pylori*. Additionally, in the assays involving inulin, a specific control was incorporated containing

wells with 20 µL of inulin at concentrations of 3, 6, and 16 mg/mL without probiotics, to evaluate its innocuity and to determine whether inulin alone exhibited any intrinsic antibacterial effect.

All plates were incubated for 72 h under microaerophilic conditions (CampyGen) [20] at 37 °C. Inhibition zones were measured in millimeters (mm), subtracting the well diameter (6 mm).

2.4. Hemolysis Test

During standardization, a change in medium color was observed. The probiotic genera used are typically reported to be γ-hemolytic (non-hemolytic), so hemolysis was experimentally evaluated [29–33].

Hemolytic activity of the probiotic strains was evaluated using blood-supplemented agar media. A well-isolated colony from each probiotic strain grown on the corresponding culture medium was streaked directly onto Mueller–Hinton agar supplemented with 5% sheep blood, Columbia agar with 5% sheep blood, and tryptic soy agar supplemented with 5% human blood (prepared in the laboratory). *S. pyogenes* (β-hemolysis) and *E. faecalis* (α-hemolysis) served as positive controls [34,35]; plates were incubated with 5% CO_2 at 37 °C for 24–48 h. After incubation, the plates were visually examined for hemolytic activity surrounding the bacterial growth. Hemolysis patterns were interpreted as follows: α-hemolysis was defined as a greenish discoloration surrounding the colonies caused by partial hemoglobin degradation; β-hemolysis as a clear zone indicating complete lysis of red blood cells; and γ-hemolysis as the absence of hemolytic activity [35].

2.5. Statistical Analysis

All antibacterial activity assays were performed in 12 technical replicates. The experiments were conducted using the pooled *H. pylori* culture described above. and the inhibition zones represent the average measurements ± standard deviation obtained from repeated technical assays. Differences among groups were analyzed using one-way ANOVA followed by Tukey's honestly significant difference (HSD) test. Statistical analyses were performed using Microsoft Excel (Microsoft 365, Microsoft Corporation, Redmond, WA, USA) with the Data Analysis ToolPak add-in. A p-value < 0.05 was considered statistically significant.

3. Results

The raw data supporting the results of this study are provided in the Supplementary Materials (Supplementary File S1).

3.1. Isolation and Cryopreservation of Strains

H. pylori was successfully isolated and identified by MALDI-TOF in two of the nine gastric biopsies processed. Because of the low bacterial recovery obtained from these isolates, the two cultures were pooled to obtain sufficient inoculum for the subsequent assays. When the laboratory-formulated culture media were used, bacterial colonies became visible earlier than in commercial media. In several cases, colonies were observed before 48 h of incubation and were sufficiently developed for manipulation after approximately 3 days. In contrast, when commercial media were used, visible growth generally required around 3 days of incubation and optimal colony development for subsequent procedures was typically achieved after 4 days.

The strains remained viable after eight months of cryopreservation at −70 °C, although a longer recovery time after thawing was observed.

3.2. Evaluation of Antibacterial Activity

Assessment of antibacterial activity using the agar well diffusion method revealed that the applied volume significantly influenced (Table 1) the size of the inhibition zones for most of the individual strains and the mixed culture (one-way ANOVA). In the case of *L. rhamnosus*, no significant differences were observed between any of the volumes.

Table 1. *p*-values obtained from one-way ANOVA comparing the inhibition zones produced by different probiotic strains at the evaluated volumes against *H. pylori* using the agar well diffusion assay.

Probiotic	*p* Value
L. casei	0.0008
L. fermentum	1.70×10^{-9}
S. thermophilus	0.010
L. rhamnosus	0.057
Mixture	7.84×10^{-6}

Figure 1 shows the relationship between the applied volume (20, 50, and 70 µL) and the average diameter of the inhibition zone. The highest antibacterial activity was consistently observed with the probiotic mixture of the four strains, followed by *L. fermentum*.

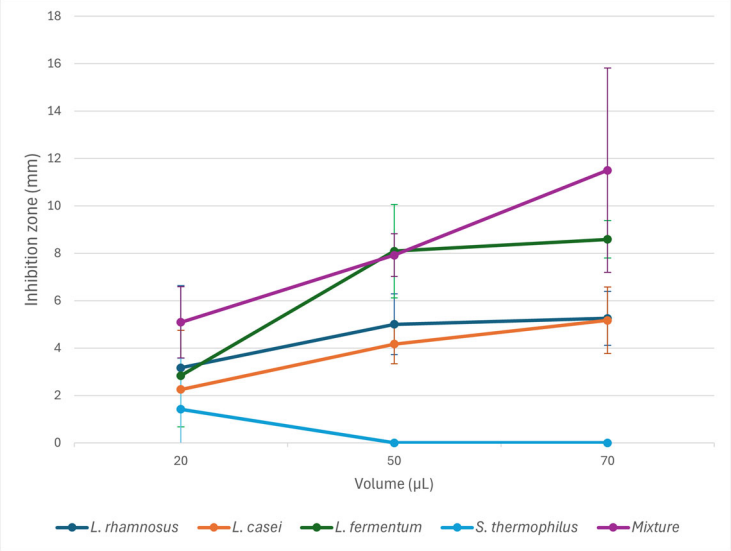


Figure 1. Mean inhibition zone diameters (mm) produced by individual probiotic strains (*L. rhamnosus*, *L. casei*, *L. fermentum*, and *S. thermophilus*) and a probiotic mixture against *H. pylori* as a function of the applied volume (20, 50, and 70 µL), evaluated using the agar well diffusion assay. Points represent mean values and error bars indicate ± SD.

At the tested volume of 50 µL, *Limosilactobacillus fermentum* and the probiotic mixture produced mean inhibition zones of (8.08 ± 1.98 mm) and (7.92 ± 0.90 mm), respectively, surpassing *L. casei* (4.17 ± 0.83 mm), *L. rhamnosus* (5.00 ± 1.28 mm), and *S. thermophilus* (0.00 ± 0.00 mm).

As shown in Table 2, in the case of the probiotic mixture, significant differences ($p = 7.8 \times 10^{-6}$) were detected among all of the test volumes. For *L. fermentum*, *L. casei*, and *S. thermophilus*, no significant differences were observed between the 50 and 70 µL volumes. *S. thermophilus* exhibited antibacterial activity only at the 20 µL volume. Tukey's HSD test was used to determine significant differences between the applied volumes.

Table 2. Tukey's HSD post hoc test comparing inhibition zones produced by different probiotic strains at the evaluated volumes against *H. pylori* in the agar well diffusion assay. Bold values indicate statistically significant differences ($p < 0.05$).

Probiotic	Volumes (µL)	Mean Difference	HSD
L. casei	20 vs. 50	**1.92**	1.73
	50 vs. 70	1.00	1.73
	20 vs. 70	**2.92**	1.73
L. fermentum	20 vs. 50	**5.25**	1.77
	50 vs. 70	0.5	1.77
	20 vs. 70	**5.75**	1.77
S. thermophilus	20 vs. 50	**1.42**	1.25
	50 vs. 70	0.00	1.25
	20 vs. 70	**1.42**	1.25
Mixture	20 vs. 50	**2.83**	2.71
	50 vs. 70	**3.58**	2.71
	20 vs. 70	**6.42**	2.71

As shown in Figure 2, the probiotic mixture and *L. fermentum* suspensions exhibited the greatest antibacterial activity, whereas *S. thermophilus* showed no detectable antibacterial activity at this volume.

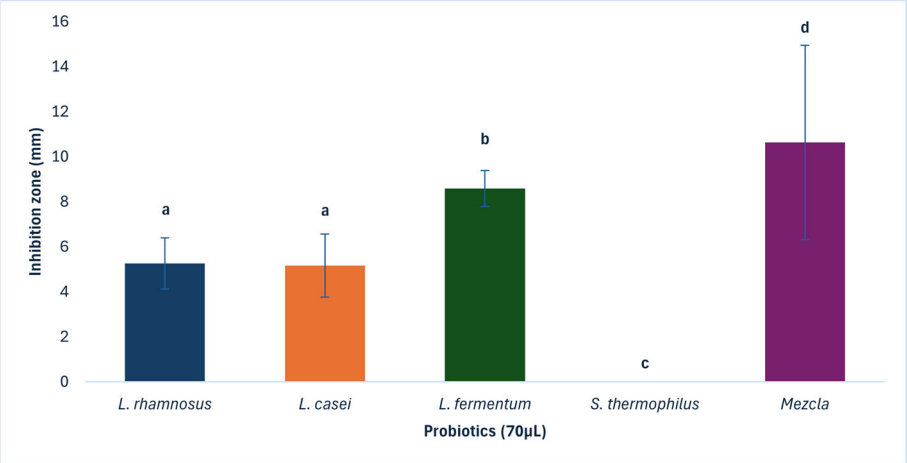


Figure 2. Mean inhibition zone diameter (mm) produced by individual probiotic strains (*L. rhamnosus*, *L. casei*, *L. fermentum*, and *S. thermophilus*) and a probiotic mixture against *H. pylori*, evaluated using the agar well diffusion assay with 70 µL of each suspension. Data are presented as mean ± SD. Different letters above the bars indicate statistically significant differences between groups ($p < 0.05$).

As shown in Table 3, all of the evaluated strains displayed a significant difference ($p < 0.05$) in their Tukey's HSD test results, with the exception of *L. rhamnosus* and *L. casei*.

As shown in Figure 3, a decrease in antibacterial activity was observed for the probiotic strains *L. rhamnosus* and *L. fermentum* when inulin was added at 6 mg/mL. One-way ANOVA revealed significant differences between the groups with and without inulin for both strains. The effect was highly significant for *L. rhamnosus* ($p = 2.25 \times 10^{-10}$) and also significant for *L. fermentum* ($p = 0.0024$). Inulin alone did not exhibit antibacterial activity under the tested conditions.

Table 3. Tukey's HSD post hoc test comparing inhibition zones produced by different probiotic strains at a volume of 70 µL against *H. pylori* in the agar well diffusion assay. Bold values indicate statistically significant differences ($p < 0.05$).

Probiotic Groups	Mean Difference	HSD
L. rhamnosus vs. *L. casei*	0.08	2.48
L. rhamnosus vs. *L. fermentum*	**3.33**	
L. rhamnosus vs. *S. thermophilus*	**5.25**	
L. rhamnosus vs. Mixture	**6.25**	
L. casei vs. *L. fermentum*	**3.42**	
L. casei vs. *S. thermophilus*	**5.17**	
L. casei vs. Mixture	**6.33**	
L. fermentum vs. *S. thermophilus*	**8.58**	
L. fermentum vs. Mixture	**2.92**	
S. thermophilus vs. Mixture	**11.50**	

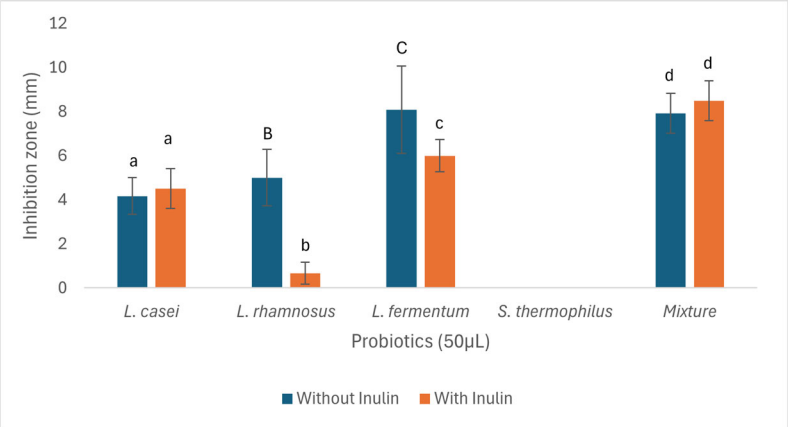


Figure 3. Effect of inulin (6 mg/mL) on the antibacterial activity of individual probiotic strains and the probiotic mixture against *H. pylori*, evaluated using the agar well diffusion assay. Inhibition zone diameters (mm) were measured after applying 50 µL of probiotic suspensions with or without inulin. Bars represent mean ± SD. Within each probiotic group, different uppercase letters represent statistically significant differences ($p < 0.05$). Letters were assigned per strain, and comparisons apply only within each strain (with vs. without inulin), not between strains.

All probiotic–inulin combinations, except *S. thermophilus*, exhibited some degree of antibacterial activity against *H. pylori*, with variations in activity depending on the probiotic strain used and the inulin concentration, as shown in Figure 4. A significant decrease ($p < 0.05$) in antibacterial activity was observed for *L. rhamnosus* and *L. fermentum* when inulin was added. In *L. rhamnosus*, the inhibition zones decreased from 5.00 ± 1.28 mm without inulin to 0.67 ± 0.49 mm with inulin, while in *L. fermentum*, they decreased from 8.08 ± 1.98 mm to 6.00 ± 0.74 mm.

The results presented in Figure 4 confirm those presented in Figure 3, namely that *L. fermentum* and *L. rhamnosus* are negatively affected by the addition of inulin. In addition, the probiotic mixture exhibited an increase in antibacterial activity at a concentration of 3 mg/mL with inhibition zones increasing from (7.92 ± 0.90 mm) to (9.58 ± 1.51 mm). This finding was confirmed through one-way ANOVA and Tukey's HSD test, which demonstrated that there was a significant difference ($p = 0.018$) between the probiotic mixture without inulin (0 mg/mL) and the mixture with inulin at 3 mg/mL.

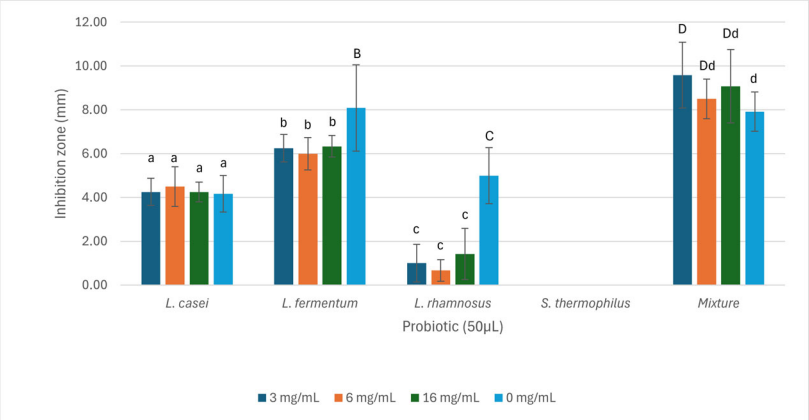


Figure 4. Mean inhibition zone diameters (mm) produced by the individual probiotic strains and the probiotic mixture against *H. pylori*, evaluated using the agar well diffusion assay. Probiotic suspensions were tested in the presence of inulin at different concentrations (3, 6, and 16 mg/mL) and in suspensions without inulin (0 mg/mL). Results are expressed as mean ± SD. Different letters indicate statistically significant differences between groups ($p < 0.05$). Letters were assigned per strain, and comparisons apply only within each strain (with vs. without inulin), not between strains.

3.3. Hemolysis Test

In the main assays, a change in the color of the medium around the probiotic inoculation site was observed (Figure 5).

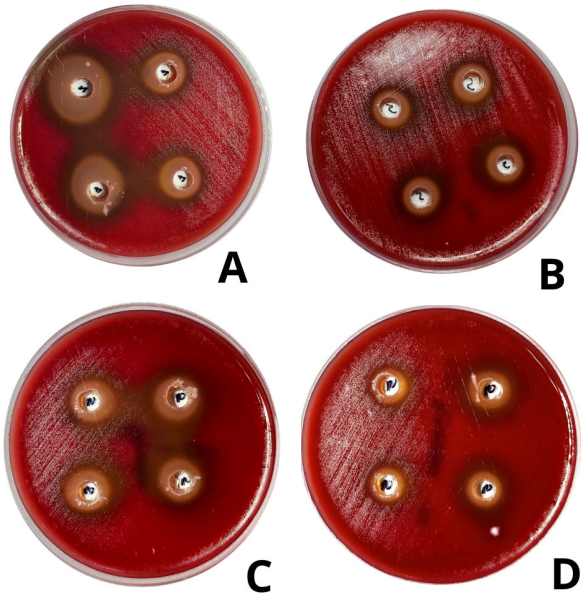


Figure 5. Presence of an inhibition zone and color change in the medium. Panels (**A**) and (**B**) show the probiotic strain mixture, (**C**) corresponds to *Lacticaseibacillus rhamnosus*, and (**D**) corresponds to *Limosilactobacillus fermentum*.

A visible color change in the culture medium was observed during the growth of the probiotic strains. This qualitative observation was consistent across two independent replicates for each strain. The most noticeable discoloration was observed in the probiotic mixture, followed by *L. fermentum* and *L. rhamnosus*, whereas *L. casei* showed a less pronounced change and *S. thermophilus* exhibited minimal alteration of the medium.

To further investigate this observation, the probiotic strains were cultured on different blood-supplemented media, including Mueller–Hinton agar with 5% sheep blood, Columbia agar with 5% sheep blood, and tryptic soy agar supplemented with 5% human blood. No visible discoloration was observed when tryptic soy agar containing human

blood was used. In contrast, cultures grown on media containing 5% sheep blood showed a greenish halo surrounding bacterial growth, consistent with α-hemolysis. This observation was reproducible in both experimental replicates. Because the probiotic strains used are generally considered non-hemolytic, this complementary validation assay was performed.

4. Discussion

The present study evaluated the inhibitory activity of several probiotic strains against *H. pylori* using an agar well diffusion model and explored the potential influence of a synbiotic combination with inulin. The results showed that the majority of the tested probiotic strains exhibited inhibitory activity against *H. pylori*, although differences in the magnitude of inhibition were observed among strains and between individual probiotics and the probiotic mixture.

The recovery of *H. pylori* initially presented difficulties, despite the use of enrichment and selective media. The incorporation of liquid media such as BHI, DMEM supplemented with 10% FBS, and DMEM supplemented with 10% FBS plus factor XII favored bacterial growth [36]. The solid media formulated in the laboratory also improved bacterial recovery, particularly for cryopreserved samples, reducing incubation times, and increasing colony yield.

Reducing the time between biopsy extraction and processing to less than 6 h significantly increased the microorganism's viability and the likelihood of successful isolation, indicating that transport time is a determining factor because of the environmental lability of the bacterium [37]. Other factors that may affect its growth include recent antibiotic use by the patient from whom the sample was obtained, low bacterial load, and sample contamination [38].

In the assays performed, the probiotic strains were adjusted to a concentration of 1.5×10^8 CFU/mL, while *H. pylori* was inoculated at a concentration of 3.0×10^8 CFU/mL. Despite this difference, notable inhibition zones were still observed; for example, *L. fermentum* showed clear inhibitory activity at the highest tested volume. This finding may suggest that under conditions of equal bacterial concentration, the inhibition zones could be even larger, indicating a volume-dependent antibacterial effect.

Figure 1 shows that *Limosilactobacillus fermentum* and the probiotic mixture produced the largest inhibition zones, while *L. rhamnosus* and *L. casei* showed moderate inhibitory activity. In contrast, *S. thermophilus* did not exhibit detectable inhibition against *H. pylori* under the conditions tested. These results suggest that the antibacterial activity depends primarily on the intrinsic properties of each probiotic strain rather than on the applied volume.

These findings are consistent with those reported in other in vitro studies, where different *L. fermentum* strains (particularly *L. fermentum* UCO-979C) demonstrated strong anti-*H. pylori* activity, inhibiting bacterial growth and adhesion in both cellular and animal models, in addition to actively modulating the host immune response [39–41]. In another study [42] using the same *L. fermentum* strain employed in the present research, the authors reported that its antibacterial effect may be attributed to the production of lactic acid. The same study also demonstrated that this probiotic strain can inhibit *H. pylori* adhesion to human gastric epithelial cells [42].

One-way ANOVA demonstrated significant differences among the applied volumes for most of the evaluated probiotics, except for *L. rhamnosus*. Subsequently, Tukey's HSD test (Table 2) revealed that the magnitude of the inhibitory effect depended on the specific probiotic strain. For *L. casei*, significant differences were observed between the lowest evaluated volume and the higher volumes, while no significant differences were detected between the two highest volumes. A similar pattern was observed for *L. fermentum*, where inhibition at the lowest volume was significantly smaller than that observed at higher

volumes, with no significant differences between the two largest volumes, suggesting that the antibacterial effect may stabilize beyond a certain volume threshold. In contrast, the probiotic mixture showed significant differences ($p = 7.8 \times 10^{-6}$) among all evaluated volumes, indicating a proportional response to the applied amount and suggesting a possible synergistic effect resulting from the combination of strains.

As shown in Figure 2, *L. fermentum* and the probiotic mixture generated the largest inhibition zones compared with the other individual strains. In contrast, *L. rhamnosus* and *L. casei* produced smaller inhibition zones, while *S. thermophilus* did not exhibit detectable antibacterial activity under the tested conditions. The results of Tukey's HSD test (Table 3) confirmed that *L. rhamnosus* and *L. casei* did not differ significantly from each other, indicating similar antibacterial effects. However, *L. fermentum* and the probiotic mixture produced significantly larger inhibition zones, further supporting the hypothesis of a synergistic effect that may enhance antibacterial activity beyond the individual contribution of each strain.

To the best of our knowledge, no previous studies have been reported using the same probiotic combination employed in this work. However, the results of this study suggest a high potential for this formulation and justify further research to confirm the observed synergistic effect.

S. thermophilus showed the lowest individual antibacterial activity, as evident in Figure 1. This finding is consistent with the results reported in a similar in vitro study conducted by Saracino et al. [43], in which the efficacy of five probiotic strains against *H. pylori* was evaluated, including *S. thermophilus*. In that study, the bacterium inhibited only ~18% of the *H. pylori* strains tested, and its bactericidal activity was the weakest among the strains analyzed. Its low efficacy was attributed to its limited ability to acidify the medium, resulting in a higher pH in its supernatant, which could explain its reduced effect in the agar well diffusion assay [43].

Although this strain showed low direct antibacterial activity against *H. pylori* in our in vitro model, it is important to note that previous studies have demonstrated beneficial effects of its use when evaluated through different methodologies [44].

In a study by Marcial et al. [44], the *S. thermophilus* CRL1190 strain was shown to significantly reduce *H. pylori* adhesion to a gastric adenocarcinoma epithelial cell line (termed AGS) under controlled co-incubation and pre-incubation conditions. However, when antibacterial activity was evaluated using an agar diffusion assay (similar to the one employed in our study), no inhibition halos were observed. This suggests that the anti-*H. pylori* effect of this strain is not necessarily mediated by antimicrobial substances, but rather by mechanisms of competitive exclusion and modulation of the host inflammatory response.

The mean inhibition zone produced by *L. casei* at a volume of 70 µL in our assay was (5.17 ± 1.40 mm), a result consistent with those reported in other in vitro studies [42,45,46]. These studies attribute this effect to the production of lactic acid, which decreases pH and neutralizes the activity of the urease enzyme. Additionally, a recent investigation [46] identified a particularly interesting mechanism in *L. casei*, in which it induces irreversible inhibition of *H. pylori* motility through alterations in bacterial cell morphology. This effect transforms the helical cells into coccoid forms, accompanied by the loss of the flagellins FlaA and FlaB, which are essential for motility.

L. rhamnosus showed the ability to inhibit the growth of *H. pylori*, producing inhibition zones similar to those obtained with *L. casei*, as shown in Figure 2. According to the literature [42], this effect is associated with the production of lactic acid, which leads to a decrease in pH and, consequently, a reduction in the activity of the urease enzyme, a key factor for *H. pylori* adaptation to the acidic environment. It is noteworthy that these findings were obtained using the same strain employed in our study. Another study [47]

demonstrated that the cell-free supernatant of *L. rhamnosus* JB3 inhibits *H. pylori* adhesion and entry into gastric cells by interfering with the formation of lipid rafts, structures essential for the delivery of virulence factors such as *CagA* and *VacA*. Additionally, this treatment reduced the expression of virulence genes and inflammatory cytokines, indicating both antibacterial and immunomodulatory effects.

It is important to note that the agar well diffusion assay used in this study provides an initial indication of inhibitory activity but does not allow differentiation between inhibition caused by antimicrobial compounds and that resulting from environmental changes such as acidification of the medium. Probiotic bacteria can produce various metabolites, including organic acids, hydrogen peroxide, and bacteriocins [20,42,46], which may contribute to the observed inhibition zones. In addition, this assay does not distinguish between bacteriostatic and bactericidal effects. Therefore, future studies should include additional controls, such as cell-free supernatants and pH-neutralized preparations, as well as complementary antimicrobial assays that quantify bacterial viability, to better elucidate the mechanisms and nature of the observed inhibition.

The antibacterial activity observed in the probiotic–inulin combinations did not significantly exceed that of the individual strains, as shown in Figure 3, a significant decrease in antibacterial activity was observed in *L. rhamnosus* and *L. fermentum* when inulin was added. These results suggest that, under these conditions, the addition of the prebiotic may negatively interfere with the antibacterial activity of these strains. Previous studies have indicated that synergy between probiotics and prebiotics is not universal and depends on both the probiotic species and the specific strain used [48]. In the probiotic mixture, a significant increase in antibacterial activity was observed with 3 mg/mL of inulin. This finding suggests a possible synergistic effect between the components.

An important variable that may affect the results is the atmospheric condition under which the study is conducted, particularly for enabling inulin fermentation. In a study by Renye et al. [48], some *L. casei* strains and other probiotics required anaerobic environments for the fermentation of fructooligosaccharides and inulin, although this was not necessary for all strains.

In in vitro studies conducted by Gomaa et al. [49], the combination of probiotics with inulin generated inhibition zones against *Salmonella heidelberg* in agar well diffusion assays performed under anaerobic conditions. Similarly, Mandal et al. [50] evaluated the growth of probiotics in broth with and without inulin under anaerobic atmospheres, where inulin supported bacterial growth. An important finding from that study was that some *Lactobacillus* spp. strains lost or showed reduced antibacterial activity when cultured in inulin-supplemented broth, while they retained inhibitory activity against indicator strains when grown in conventional Man–Rogosa–Sharpe (MRS) medium [50]. This observation is consistent with the behavior of the strains evaluated in our study, specifically *L. rhamnosus* and *L. fermentum*.

At the clinical level, Şirvan et al. [17] demonstrated that synbiotic therapy with inulin improved *H. pylori* eradication rates and reduced the adverse effects of conventional treatment, such as diarrhea, nausea, vomiting, and epigastric pain, thereby increasing treatment tolerance and adherence, which translates into improved therapeutic outcomes.

Other studies have reported contradictory results, in which the incorporation of inulin and probiotics did not demonstrate any effect on *H. pylori* eradication. For example, in the study by Ustundag et al. [51], the addition of inulin and *Bifidobacterium lactis* B94 did not increase the eradication rate or reduce symptoms. By contrast, the results reported by Islek et al. [52], who used the same probiotic strain and inulin, showed a reduction in adverse effects and an indirect increase in eradication rates due to improved adherence to treatment.

Considering the role of prebiotic fermentation in probiotic activity, a limitation of the experimental design relates to the synbiotic assay, where inulin was added directly to the wells together with the probiotic suspensions without a prior fermentation step. In our case, pre-incubation was not performed to maintain the same experimental conditions used in the antibacterial activity assay without inulin, with a known concentration, allowing for a proper comparison of the data.

Since prebiotics such as inulin require time to be metabolized by probiotic bacteria into active metabolites [50,53], the experimental design may not fully reflect the metabolic interactions that occur in vivo. Consequently, the observed reduction in inhibitory activity in the presence of inulin should be interpreted cautiously. Future experiments incorporating pre-incubation of probiotics in inulin-supplemented media could provide a more accurate evaluation of potential synbiotic effects.

These results suggest that future analyses exploring the interaction between the probiotic mixture evaluated in this study and inulin could be of great interest for additional clinical or in vitro research.

Although the agar well diffusion assay provides a useful initial approach to evaluate antibacterial activity, it does not fully reproduce the complex microenvironment in which *H. pylori* colonizes the human stomach. In vivo, this bacterium primarily resides within the gastric mucosal layer, where the presence of mucins, the acidic gastric pH gradient, and interactions with epithelial and immune cells significantly influence bacterial survival and microbial competition [3,39,54]. These host-related factors can modulate both the activity of probiotic microorganisms and the susceptibility of *H. pylori*. Therefore, while the inhibitory effects observed in this in vitro model suggest a potential antagonistic interaction between the evaluated probiotics and *H. pylori*, further studies using more physiologically relevant models, such as gastric epithelial cell cultures, mucus-containing systems, or in vivo models, are necessary to better understand how these interactions occur within the gastric mucosal environment.

During the development of the project, a change in medium coloration was observed in the growth controls, occurring only in plates inoculated with the probiotics and primarily in media supplemented with sheep blood. This observation may reflect an interaction between probiotic metabolites and the components of the medium rather than true hemolytic activity.

As shown in Figure 5, the color change was more pronounced in the plates inoculated with the probiotic mixture than in those inoculated with the individual strains, which correlates with the fact that the mixture also demonstrated the greatest antibacterial activity. This may be associated with an increased release of anti-*H. pylori* metabolites when the strains are combined, along with a greater interaction with the components of the medium. As mentioned previously, the probiotics evaluated can produce lactic acid [42,45,46], acidification of the medium may alter erythrocyte integrity in blood agar and produce an apparent hemolytic-like discoloration that is primarily attributable to pH changes rather than to hemolytic factors. This hypothesis requires experimental verification through pH measurements and supernatant analysis, as well as additional in vitro hemolysis assessment.

The probiotic strains were inoculated onto three different media. A change in coloration was observed in two of the media, specifically those supplemented with sheep blood, while no change was observed in the medium containing human blood. This assay was performed under the atmospheric and temperature conditions recommended by the ATCC [55–58], with the aim of determining whether the change in the medium was due to the microaerophilic incubation conditions or to the intrinsic properties of the probiotic strains. The literature [59–61] reports cases in which certain strains vary their hemolytic

behavior depending on the type of blood used in the medium, and this was observed in the tests performed; however, such behavior is not expected in probiotic strains.

Taken together, these findings indicate that the information obtained in the present study is not sufficient to confirm the safety of the evaluated strains or confirm hemolytic activity. Further studies are therefore necessary to perform a more comprehensive safety assessment before these microorganisms can be considered for use in human studies or in products intended for human consumption.

5. Conclusions

This study demonstrated that the probiotic strains *L. rhamnosus*, *L. casei*, *L. fermentum*, and *S. thermophilus*, individually and in combination, exhibit antibacterial activity against *H. pylori* under in vitro conditions. Among the evaluated treatments, the probiotic mixture showed the most consistent inhibitory effect, suggesting that the combined use of probiotic strains may enhance antibacterial activity. The results further suggest that the presence of the prebiotic inulin may enhance the inhibitory effect of the probiotic mixture, highlighting the potential relevance of synbiotic strategies. However, since inulin was added simultaneously with the probiotic suspensions and no pre-incubation step was performed, the experimental design does not fully reflect the temporal dynamics of prebiotic fermentation. Consequently, the effects observed in the presence of inulin should be interpreted with caution.

Overall, these results highlight the potential of selected probiotic formulations as complementary strategies for the control of *H. pylori*. Further studies are required to better understand the mechanisms involved and to evaluate the effectiveness and safety of these combinations in more complex experimental models and clinical settings.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/applmicrobiol6040048/s1.

Author Contributions: Conceptualization, H.P.G. and G.G.B.; methodology, H.P.G., G.G.B. and J.B.J.; software, H.P.G. and G.G.B.; validation, V.B.M., M.M.G. and J.B.J.; formal analysis, H.P.G., G.G.B. and J.B.J.; investigation, H.P.G. and G.G.B.; resources, H.P.G. and G.G.B.; data curation, H.P.G. and G.G.B.; writing—original draft preparation, H.P.G. and G.G.B.; writing—review and editing, V.B.M. and M.M.G.; visualization, H.P.G. and G.G.B.; supervision, V.B.M. and M.M.G.; project administration, H.P.G., G.G.B. and V.B.M.; funding acquisition, H.P.G. and G.G.B. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: This study, conducted as in vitro experimental research, did not require approval from a local Ethics Committee or Institutional Review Board (IRB) according to national and institutional regulations. The research was reviewed and approved at the institutional level by the Research Commission of Universidad de Ciencias Medicas (UCIMED), in compliance with Costa Rican data protection regulations (Law No. 8968, Personal Data Protection Law). All procedures involving human-derived material were conducted in accordance with the principles of the Declaration of Helsinki.

Informed Consent Statement: Written informed consent has been obtained from the patients to publish this paper.

Data Availability Statement: The raw data supporting the conclusions of this article are available in the Supplementary Materials (Supplementary File S1).

Acknowledgments: The authors would like to express their sincere gratitude to Lauren Valerio and Verónica González from Gastrobene Digestive Center for their valuable collaboration in providing

the gastric biopsy samples that made this study possible. We thank Edanz (https://jp.edanz.com/ac, accessed on 15 December 2026) for editing a draft of this manuscript.

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

Abbreviations

The following abbreviations are used in this manuscript:

BHI	Brain heart infusion
DMEM	Dulbecco's modified Eagle's medium
MALDI-TOF	Matrix-assisted laser desorption/ionization time-of-flight
SSS	Sterile saline solution
FBS	Fetal bovine serum
HSD	Honestly significant difference
AGS	Adenocarcinoma gastric stomach
MRS	Man–Rogosa–Sharpe
ATCC	American type culture collection

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