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Comparative Screening of Probiotic-Derived Formulations for Inhibition of Biofilm Formation by Milking Equipment Isolates and Reference Strains

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Abstract

Biofilms in dairy production lines, including milking equipment, can contribute to persistent microbial contamination. This exploratory study screened 32 formulations derived from *Lactobacillus acidophilus*, *Lactobacillus helveticus*, *Lacticaseibacillus rhamnosus*, and *Saccharomyces cerevisiae* to identify candidates for further biofilm-control studies. Nine bacterial targets, including isolates from milking equipment and reference strains, were tested individually. The attached biomass was measured using crystal-violet staining, with De Man–Rogosa–Sharpe (MRS) controls adjusted to pH 3.8 and 6.8. Planktonic growth was assessed after exposure to native-pH cell-free supernatants (CFSs). The screening comprised one independent experiment with three technical replicates per formulation target combination. All four native-pH CFSs inhibited attached-biomass formation by the six strong biofilm-forming targets, with displayed inhibition values of 71–100% and no or low formulation-only staining. The three lactobacillus-derived CFSs also generally reduced planktonic turbidity to near-background levels, whereas the effect of *S. cerevisiae* CFS on planktonic growth varied among targets. *L. acidophilus* pH-adjusted CFS and *S. cerevisiae* heat-inactivated whole-culture preparation also retained broad antibiofilm activity. Meanwhile, several other cell-containing or pH-adjusted formulations showed target-dependent effects or produced substantial surface-associated material. Native-pH CFSs were the most consistent candidates for independent confirmation and further testing on equipment-relevant surfaces.

Keywords: antibiofilm activity; cell-free supernatant; commercial probiotics; inactivated cell supernatant; *Pseudomonas aeruginosa* ATCC 27853; *Staphylococcus aureus* ATCC 25923

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Academic Editor: Analía Inés Etcheverría

Received: 31 July 2026
Revised: 26 October 2026
Accepted: 27 October 2026
Published: 30 October 2026

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1. Introduction

Biofilms on surfaces that contact milk can contribute to microbial persistence and recurrent contamination in dairy production. Their extracellular matrix and surface-associated growth can reduce the effectiveness of cleaning and disinfection [1,2]. Milking equipment is a particularly relevant environment. Biofilms sampled directly from milking machines contain diverse bacterial communities, including organisms of concern for milk quality and hygiene [3]. This diversity makes activity against different bacterial targets an important consideration when selecting preparations for further development.

Microorganisms used as probiotics can produce compounds with antimicrobial or antibiofilm activity, including organic acids, bacteriocins, and biosurfactants [4]. Their cell-free supernatants contain soluble cultivation products, whereas whole-culture preparations

also contain microbial cells. Studies of lactic acid bacteria-derived preparations against dairy-associated bacteria have shown that activity varies with the producing culture, preparation type, and target organism [5]. It remains unclear how formulations derived from the commercial cultures selected for this study affect biofilm formation by bacteria recovered specifically from milking equipment, and which formulation types combine activity across these targets with little surface-associated material of their own.

Formulation pH is another consideration. Comparisons of native-pH and pH-adjusted lactic acid bacteria-derived preparations indicate that acidity can contribute to their antimicrobial and antibiofilm activity [5]. In a study of established *P. aeruginosa* biofilms, HCl controls adjusted to pH 4 or 5 did not reproduce the biofilm removal effect of *L. acidophilus* cell-free filtrate [6]. This result illustrates why low pH alone cannot be assumed to explain every response, although the experiment endpoint differs from the biofilm formation screen used here. Therefore, we compared formulations at their native and adjusted pH and included pH-adjusted medium controls. For the intended equipment application, inhibitory activity was considered alongside the surface-associated material produced by each formulation.

This exploratory study aimed to screen 32 formulations derived from four commercially available microbial cultures (*Lactobacillus acidophilus* THT 030101, *Lactobacillus helveticus* THT 031102, *Lacticaseibacillus rhamnosus* GG, and *Saccharomyces cerevisiae* DSM 33148) for their effects on biofilm formation by nine individually tested bacterial targets, comprising milking equipment isolates and reference strains. The formulations included cell-free supernatants, preparations containing viable or heat-inactivated cells, and pH-adjusted variants. We sought to identify formulations that inhibited attached-biomass formation across several targets without producing substantial surface-associated material or increasing total attached biomass in the tested cultures.

2. Materials and Methods

2.1. Probiotics

Two probiotic dietary supplements and two commercially available lyophilized lactic acid bacterial cultures were used to prepare the formulations. The microorganisms and their commercial sources were:

1. *Lactobacillus acidophilus* THT 030101 (THT s.a., Isnes, Belgium);
2. *Lactobacillus helveticus* THT 031102 (THT s.a., Isnes, Belgium);
3. *Lacticaseibacillus rhamnosus* GG (dsm-firmenich, Kaiseraugst, Switzerland);
4. *Saccharomyces cerevisiae* DSM 33148 (Longevience AB, Örebro, Sweden).

Initially, 0.1 g of each commercial product was inoculated into 45 mL of MRS (Oxoid, Thermo Fisher Scientific, Basingstoke, UK) broth. The cultures were incubated at 37 °C at 150 rpm for 12 h, except *L. acidophilus* and *S. cerevisiae*, which were incubated for 24 h, until the late exponential phase. Cultivation was performed in an RTS-8+ multichannel bioreactor (Biosan, Riga, Latvia), which enabled real-time monitoring of culture growth. The cultures were then washed twice with distilled water and reinoculated into fresh MRS broth at an approximate pellet-to-broth ratio of 1:10. Following reinoculation, the OD_{600} measured using RTS-8+, was 4.0 for *L. helveticus*, 8.0 for *L. rhamnosus*, 3.0 for *S. cerevisiae*, and 3.5 for *L. acidophilus*. Fresh MRS broth was added to adjust the starting culture to an OD_{600} from 0.4 to 0.6. This adjustment standardized the optical density range, but did not equalize viable cell counts across the four cultures (Table 1). The cultures were then incubated at 37 °C at 150 rpm for 42 h to obtain the cultures used to prepare the probiotic-derived formulations.

Table 1. Characteristics of the probiotic-derived formulations.

No.	Formulation	Probiotics	pH	CV Signal *	Colony-Forming Units, mL^{-1}
1	LCS	*L. acidophilus*	3.89	moderate	7.33×10^7
2	LCS	*L. helveticus*	3.97	moderate	5.30×10^4
3	LCS	*L. rhamnosus*	3.93	high	5.53×10^7
4	LCS	*S. cerevisiae*	5.32	low	4.20×10^8
5	LCS-N	*L. acidophilus*	6.80	low	7.33×10^7
6	LCS-N	*L. helveticus*	6.75	low	5.30×10^4
7	LCS-N	*L. rhamnosus*	6.74	high	5.53×10^7
8	LCS-N	*S. cerevisiae*	6.77	moderate	4.20×10^8
9	ICS	*L. acidophilus*	3.9	moderate	ND
10	ICS	*L. helveticus*	3.99	not detectable	ND
11	ICS	*L. rhamnosus*	3.95	low	ND
12	ICS	*S. cerevisiae*	5.31	low	ND
13	ICS-N	*L. acidophilus*	6.89	not detectable	ND
14	ICS-N	*L. helveticus*	6.74	moderate	ND
15	ICS-N	*L. rhamnosus*	6.78	high	ND
16	ICS-N	*S. cerevisiae*	6.76	not detectable	ND
17	CFS	*L. acidophilus*	3.92	not detectable	ND
18	CFS	*L. helveticus*	3.99	low	ND
19	CFS	*L. rhamnosus*	3.93	low	ND
20	CFS	*S. cerevisiae*	5.26	not detectable	ND
21	CFS-N	*L. acidophilus*	6.78	not detectable	ND
22	CFS-N	*L. helveticus*	6.76	not detectable	ND
23	CFS-N	*L. rhamnosus*	6.76	high	ND
24	CFS-N	*S. cerevisiae*	6.75	not detectable	ND
25	LC	*L. acidophilus*	4.34	moderate	1.69×10^8
26	LC	*L. helveticus*	4.65	high	1.30×10^5
27	LC	*L. rhamnosus*	4.62	high	3.46×10^8
28	LC	*S. cerevisiae*	5.27	moderate	8.07×10^8
29	IC	*L. acidophilus*	4.77	high	ND
30	IC	*L. helveticus*	5.09	moderate	ND
31	IC	*L. rhamnosus*	4.99	low	ND
32	IC	*S. cerevisiae*	6.09	high	ND
			pH controls		
33	MRS + HCl	-	3.80	not detectable	ND
34	MRS + NaOH	-	6.80	moderate	ND

LCS—whole-culture preparation containing viable cells at the native pH; LCS-N—whole-culture preparation containing viable cells at an adjusted pH; ICS—heat-inactivated whole-culture preparation at the native pH; ICS-N—heat-inactivated whole-culture preparation, at an adjusted pH; CFS—cell-free supernatant at the native pH; CFS-N—cell-free supernatant at an adjusted pH; IC—heat-inactivated cells at the native pH; LC—viable probiotic cells at the native pH; ND—not detected (<20 CFU mL^{-1}). * The crystal violet (CV) signal represents the total attached biomass measured in formulation control wells (formulation only) using a crystal violet microtiter plate assay. The cut-off optical density (OD_c) was calculated as the mean OD of the MRS blank control plus three standard deviations, following the method described by [7]. The CV signal of the formulation controls was subsequently assigned to one of four adapted categories: not detectable, low, moderate, or high, with a signal classified as not detectable when the OD of the formulation control did not exceed OD_c, indicating that no CV-stainable material attached to the well was detected against the background of the sterile MRS control under the assay conditions.

Culture purity was assessed by streaking 10 µL of each culture onto Tryptic soy agar (TSA; Liofilchem, Roseto degli Abruzzi, Italy) and MRS agar (Oxoid, Thermo Fisher Scientific, Basingstoke, UK). Following aerobic incubation at 37 °C for 48 h, the plates were examined for uniform colony morphology and the absence of morphologically distinct colonies. Representative colonies were identified to the species level using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (VITEK MS MALDI-TOF,

bioMerieux, Marcy-l'Étoile, France) at the Research Laboratory of Biotechnology, the Latvia University of Life Sciences and Technologies (LBTU) (Jelgava, Latvia), using the VITEK MS Knowledge Base, Version V3.2 for clinical use (bioMerieux, Marcy-l'Étoile, France).

After 42 h of cultivation, three cultures had OD600 values of 3.55–3.94, whereas *L. rhamnosus* had an OD_{600} of 6.68 ± 0.04. The *L. rhamnosus* culture was, therefore, diluted with fresh MRS broth to an OD of approximately 4.0. The pH, viable cell counts, and formulation-only crystal violet signals of the probiotic-derived preparations are reported in Table 1.

2.2. Probiotic-Derived Formulations

Eight formulations were prepared from each of the four probiotic cultures to evaluate the effects of viable cells (LCs), inactivated cells (ICs), cell-free supernatants (CFSs), and whole-culture preparations containing either viable cells (LCSs) or inactivated cells (ICSs). Formulations containing culture supernatants were tested at their native pH (generally 3.89–3.99; 5.26–5.32 for *S. cerevisiae*). The pH of the CFS, LCS, and ICS preparations was also adjusted to approximately 6.8, yielding CFS-N, LCS-N, and ICS-N, respectively (Table 1). These comparisons were intended to help assess the contribution of acidity to the observed activity. The formulation abbreviations were adapted from Falqueto et al.'s study to reflect preparations used in this study [5].

The formulations were prepared as follows:

LCS and LCS-N: Two 10 mL aliquots of each whole culture were collected. One was retained at its native pH (LCS), and the other was adjusted to approximately pH 6.8 with 1 M NaOH (LCS-N).

ICS and ICS-N: Two 10 mL aliquots of each whole culture were heated in a water bath at 85 °C for 25 min and cooled to room temperature. One was retained at its native pH (ICS), and the other was adjusted to approximately 6.8 to obtain ICS-N. The viable cell concentration of the culture before filtration is reported in Table 1 under LCS.

CFS and CFS-N: Two 20 mL aliquots of each whole culture were centrifuged at 4 °C at 4000 rpm for 10 min. The supernatants were passed through sterile 25 mm, 0.22 µm polyethersulfone filters (Avantor, Radnor, PA, USA). One filtered supernatant was retained at its native pH (CFS), and the other was adjusted to approximately 6.8 (CFS-N). The viable cell concentration of the culture before filtration is reported in Table 1 under LCS.

LC and IC: Following centrifugation and removal of the supernatant, the cell biomass was washed twice with distilled water. The washed cells were resuspended in 10 mL of distilled water and homogenized via vortexing. One aliquot was heated at 85 °C for 25 min (IC), while the other was left untreated (LC).

The absence of detectable microorganisms in the cell-free supernatants and heat-inactivated preparations was assessed by inoculating five 10 µL aliquots of each sample onto Columbia blood agar (CBA) (Liofilchem, Roseto degli Abruzzi, Italy) and MRS agar. The plates were incubated aerobically at 37 °C for 72 h, after which they were examined for microbial growth. No growth was detected on either medium. A total volume of 50 µL per sample was examined on each medium, corresponding to a nominal detection limit of 20 CFU mL^{-1}. Viable bacterial counts in the live probiotic formulations were determined using the microdrop plate counting technique described by Naghili et al. [8]. Serial tenfold dilutions ranging from 10^{-4} to 10^{-11} were prepared, and five 10 µL aliquots of each dilution were inoculated onto MRS agar. The plates were incubated aerobically at 37 °C for 48–72 h. Colonies were counted at countable dilutions, and viable bacterial concentrations were expressed as colony-forming units per milliliter (CFU mL^{-1}).

2.3. Dairy Spoilage and Pathogenic Bacteria

Bacteria tested in this study were isolated from the internal surfaces of milking equipment at a dairy farm in Latvia. During two sampling events, the same 11 sites were sampled on both occasions, yielding 137 morphologically distinct isolates. Isolates were identified using VITEK MS MALDI-TOF mass spectrometry. The most frequently identified genera, in descending order, were *Aerococcus*, *Staphylococcus*, *Bacillus*, *Enterococcus*, *Citrobacter*, *Pseudomonas*, and *Klebsiella*. The predominant species within these genera were then selected, and one isolate of each species was chosen at random. For *Staphylococcus*, two isolates were selected at random: one representing coagulase-positive staphylococci (*S. aureus*), and the other representing coagulase-negative staphylococci (*S. haemolyticus*). The biofilm-forming capacity of the selected isolates was assessed in three independent experiments using the crystal violet assay. After excluding the non-biofilm-forming isolates (one *Aerococcus viridans* and one *Citrobacter freundii* isolate), the following six isolates were retained for further testing:

1. *Enterococcus faecalis* V2-11;
2. *Staphylococcus aureus* V2-7;
3. *Staphylococcus haemolyticus* V2-8;
4. *Bacillus subtilis* VM;
5. *Pseudomonas aeruginosa* V2-3;
6. *Klebsiella ornithinolytica* V2-4.

Additionally, three reference strains widely used in biofilm and antibiofilm studies were included:

1. *Staphylococcus aureus* ATCC 25923 (Microbiologics, St. Cloud, MN, USA);
2. *Pseudomonas aeruginosa* ATCC 27853 (TCS Biosciences, Botolph Claydon, UK);
3. *Bacillus subtilis* ATCC 6633 (Thermo Fisher Scientific, Lenexa, KS, USA).

2.4. Biofilm-Forming Capacity

The biofilm-forming capacity of the selected bacteria was evaluated using the crystal violet assay. The bacteria were streaked onto TSA and incubated at 37 °C for 16–18 h. One or more isolated colonies were then inoculated into the tryptone soy broth with 1% glucose (TSBG; Oxoid, Thermo Fisher Scientific, Basingstoke, UK) for Gram-positive bacteria or Luria–Bertani medium (LB, Liofilchem, Roseto degli Abruzzi, Italy) for Gram-negative bacteria. Cultures were incubated at 37 °C with shaking at 200 rpm for 2–3.5 h until their turbidity reached approximately 0.5 McFarland.

The cultures were diluted 1:10 in fresh medium, and 200 µL of each diluted culture was transferred to wells of sterile flat-bottom 96-well microplates (Thermo Fisher Scientific, Roskilde, Denmark). The plates were incubated at 37 °C for 48 h with gentle agitation at 50 rpm. Before removing the culture, optical density at 620 nm (OD_{620}) was measured using a HiPo MPP-96 microplate photometer (Biosan, Riga, Latvia) to confirm planktonic growth in the untreated bacterial controls and the absence of growth in the negative controls, thereby verifying assay's validity. The planktonic phase was removed by inverting the plate and gently tapping out the contents of the wells. The wells were then rinsed three times with 200 µL of 0.9% saline. The remaining biofilms were fixed with 200 µL of methanol (Merck KGaA, Darmstadt, Germany) for 15 min. The methanol was removed via pipetting, and the plate was left uncovered until completely dry. The biofilms were then stained with 190 µL of 0.1% crystal violet for 15 min. The unbound dye was removed via pipetting, and the biofilms were washed three times with 200 µL of distilled water. Crystal violet bound to the biofilms was extracted with 200 µL of 96.2% ethanol (Kalsnavas Elevators, Jaunkalsnava, Latvia). The OD values of the biofilms were measured at a wavelength of

570 nm. Biofilm-forming capacity was assessed by calculating the cut-off optical density (ODc), which was calculated as follows:

$$OD_c = OD_{NC} + 3 \times SD_{NC} \quad (1)$$

where OD_{NC} represents the average OD of the negative control, and SD_{NC} represents the standard deviation of the negative control. Depending on the OD values, the isolates were classified as non-biofilm producers ($OD \leq ODc$); weak biofilm producers ($OD_c < OD \leq 2{\times}OD_c$); moderate biofilm producers ($2{\times}OD_c < OD \leq 4{\times}OD_c$); or strong biofilm producers ($4{\times}OD_c < OD$), according to Stepanovic et al. [7]. Isolates whose OD_{570} values fell on different sides of the weak/moderate cut-off in assessments were described as weak/moderate biofilm formers. These isolates were presented with the weak biofilm formers, without calculating percentage inhibition.

2.5. Biofilm Inhibition Assay

The effects of the probiotic-derived formulations on biofilm formation were assessed using a crystal violet microplate assay. Bacterial precultures were prepared as described in Section 2.4. Their mean concentrations were 4.12×10^7 CFU mL^{-1} in the experiment testing *L. helveticus*- and *L. rhamnosus*-derived formulations and 2.30×10^7 mL^{-1} in the experiment testing *L. acidophilus*- and *S. cerevisiae*-derived formulations. The precultures were diluted 1:10 in fresh medium. Each well then received 100 µL of diluted bacterial culture and 100 µL of formulation, yielding a final bacterial dilution of 1:20 relative to the preculture. The corresponding mean initial bacterial concentrations in the test wells were approximately 2.06×10^6 CFU mL^{-1} and 1.15×10^6 CFU mL^{-1}, respectively. Concentrations for individual bacterial cultures are provided in the Supplementary Table S1.

Each assay included a negative control containing 200 µL of sterile LB or TSBG broth. The untreated bacterial control contained 100 µL of diluted bacterial culture and 100 µL of the corresponding fresh bacterial growth medium. Two medium controls each contained 100 µL of the bacterial culture and 100 µL MRS of broth adjusted either to pH 3.80 with 1 M HCl or to pH 6.80 with 1 M NaOH. Both adjusted MRS media and all 32 probiotic-derived formulations were also tested without bacteria to measure their background crystal violet signals. Each formulation was tested against nine bacterial targets in three technical replicates per formulation target combination. Bacteria-free medium controls were tested in 12 replicates. The complete allocation of samples and controls is provided in the Supplementary Material S4.

The plates were incubated, stained, and measured as described in Section 2.4. For each treated culture, the OD_{570} of the corresponding bacteria-free formulation control was subtracted from the measured OD_{570}. The untreated bacterial control was corrected using its corresponding bacteria-free blank. The absorbance of the untreated positive control was corrected using its corresponding bacteria-free blank. Percentage inhibition relative to the untreated bacterial control was calculated using an equation adapted from Falqueto et al. [5]:

$$\text{Biofilm inhibition}(\%) = \left(1 - \frac{OD_t}{OD_c}\right) \times 100 \quad (2)$$

where OD_t represents the background-corrected OD_{570} of the treated culture, and OD_c represents the background-corrected OD_{570} of the untreated positive control.

Negative corrected OD_{570} values were retained in the calculations and could therefore yield inhibition estimates exceeding 100%. Values above 100% were capped only for display in the heatmap; uncapped values are available in the Supplementary Material S4.

For Figures 1 and 2, the OD_{570} of the corresponding bacteria-free formulation control was subtracted from the OD_{570} of each formulation-bacterium well. The untreated bacterial control was corrected using its bacteria-free medium blank. These corrected values were used to calculate percentage inhibition for the six strong biofilm forming targets according to Equation (2). For the weak or weak/moderate targets shown in Figure 3, only sterile bacterial growth medium blank was subtracted from formulation-treated wells. The corresponding bacteria-free formulation control signal was not subtracted. Percentage inhibition was not calculated for these targets.

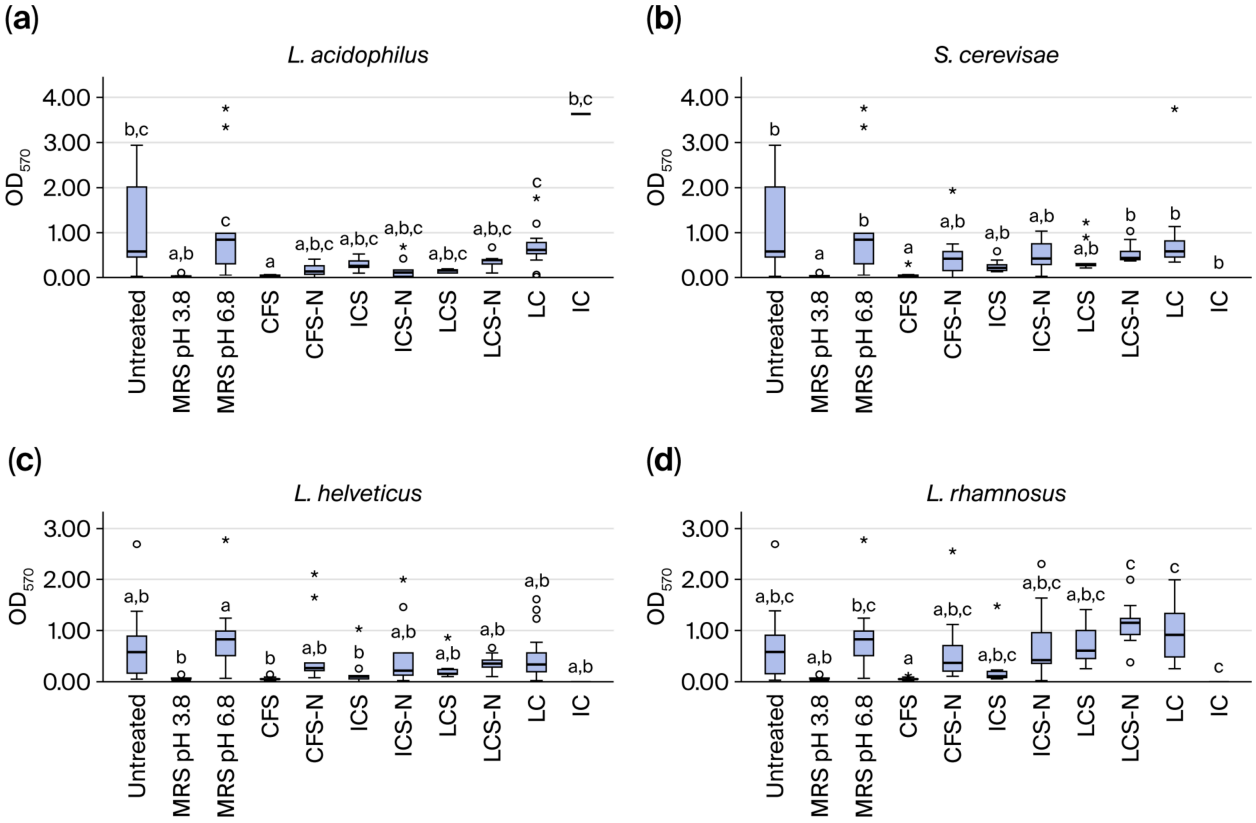

Figure 1. Effects of probiotic-derived formulations on attached biomass across nine individually tested bacterial targets. Formulations were derived from (**a**) *L. acidophilus*, (**b**) *S. cerevisiae*, (**c**) *L. helveticus*, and (**d**) *L. rhamnosus* cultures. Each panel includes eight formulations derived from the corresponding probiotic culture, giving 32 formulations in total. Biofilm-associated biomass was quantified by optical density (OD) in three technical replicates for each formulation–bacterial target combination. Technical replicates were averaged before analysis; thus, each box summarizes nine mean OD values, one for each bacterial target. The nine targets served as blocks in the exploratory Friedman comparison within each panel. The center line indicates the median, the box indicates the interquartile range (IQR), and the whiskers indicate the most extreme values within 1.5 × IQR. Asterisks (*) and circles (○) indicate extreme and mild outliers, respectively. Differences among formulations within each panel were assessed using the Friedman test, followed by Bonferroni-adjusted pairwise comparisons. Groups sharing at least one letter are not significantly different, whereas groups with no letters in common differ significantly (adjusted $p < 0.05$). Letter annotations are comparable only within individual panels. CFS—cell-free supernatant at the native pH; CFS-N—cell-free supernatant at an adjusted pH; ICS—heat-inactivated whole-culture preparation at the native pH; ICS-N—heat-inactivated whole-culture preparation at an adjusted pH; LCS—whole-culture preparation containing viable cells at the native pH; LCS-N—whole-culture preparation containing viable cells at an adjusted pH; LC—viable probiotic cells at the native pH; IC—heat-inactivated cells at the native pH.

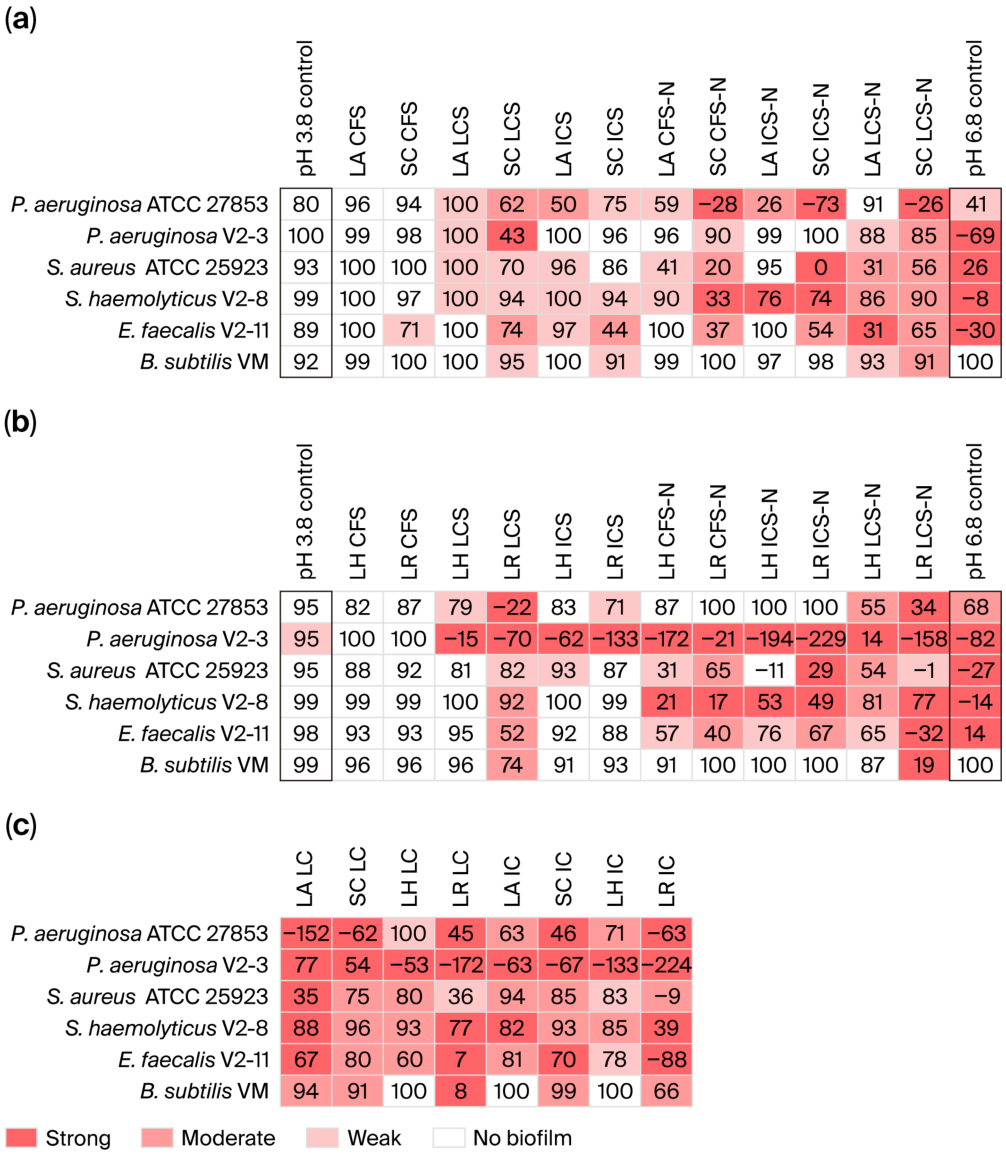

Figure 2. Target-specific biofilm inhibition and residual biofilm category across six strong biofilm-forming bacterial targets. Numbers indicate mean biofilm inhibition (%) calculated from formulation-background-corrected OD_{570} values. Cell shading indicates the residual biofilm category determined from the corresponding corrected OD_{570}: white—corrected OD_{570} at or below the biofilm-detection cutoff; light pink—weak; medium pink—moderate; and dark pink—strong. Panels show (**a**) *L. acidophilus* (LA)- and *S. cerevisiae* (SC)-derived formulations with the corresponding pH controls; (**b**) *L. helveticus* (LH)- and *L. rhamnosus* (LR)-derived formulations with the corresponding pH controls; and (**c**) viable and heat-inactivated cell suspensions. Each cell represents the mean of three technical wells from one screening experiment. Values exceeding 100% are displayed as 100%; uncapped values are available in the Supplementary Material S4. Negative inhibition values were retained and indicate that the background-corrected attached-biomass OD_{570} exceeded the untreated-control value. Inhibition estimates reaching or exceeding 100% do not demonstrate complete elimination of the target biofilm. Color categories and numerical inhibition values represent related but distinct summaries of the same corrected OD_{570} measurements. CFS—cell-free supernatant at the native pH; CFS-N—cell-free supernatant at an adjusted pH; ICS—heat-inactivated whole-culture preparation at the native pH; ICS-N—heat-inactivated whole-culture preparation at an adjusted pH; LCS—whole-culture preparation containing viable cells at the native pH; LCS-N—whole-culture preparation containing viable cells at an adjusted pH; LC—viable probiotic cells at the native pH; and IC—heat-inactivated cells at the native pH.

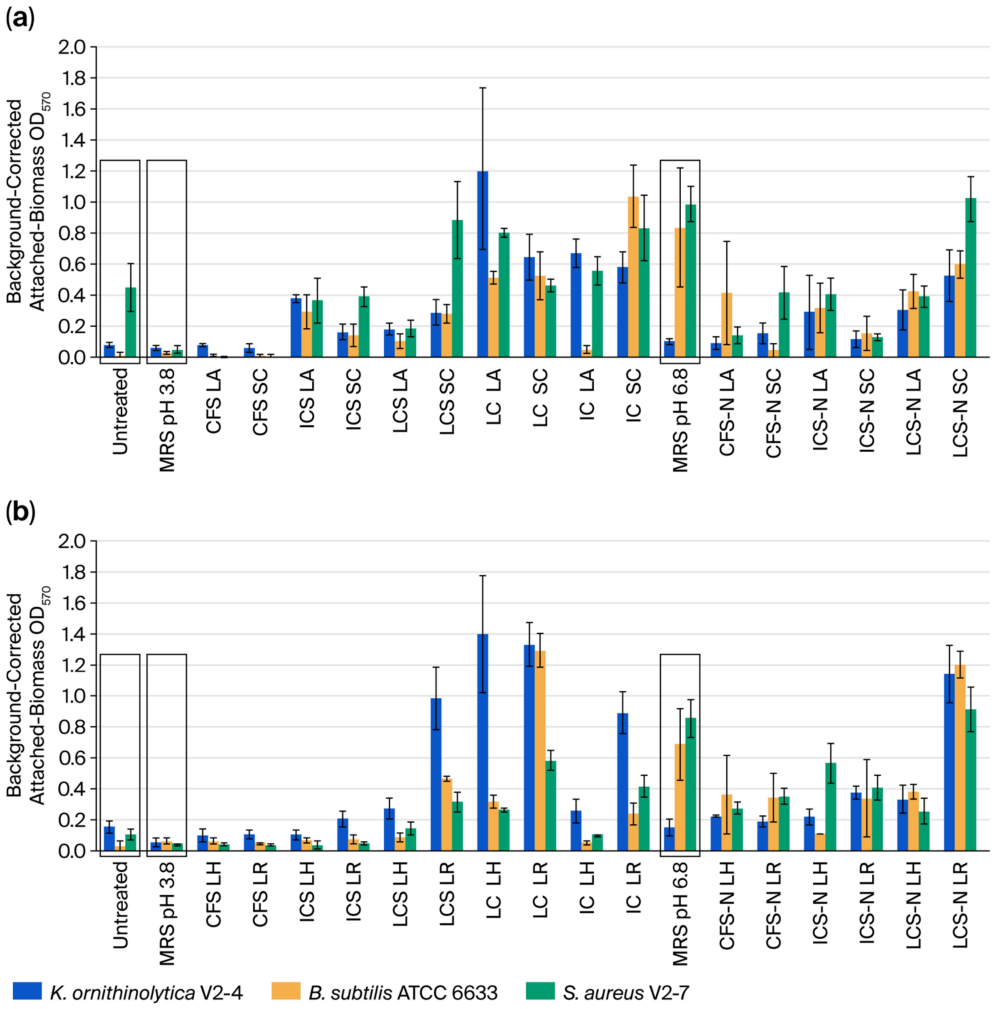

Figure 3. Background-corrected attached-biomass OD_{570} of weak or weak/moderate biofilm-forming bacterial targets exposed to probiotic-derived formulations. The displayed OD_{570} values were corrected using the corresponding sterile bacterial growth medium blank; bacteria free formulation control signals were not subtracted. Panels show formulations derived from (**a**) *L. acidophilus* (LA) and *S. cerevisiae* (SC) and (**b**) *L. helveticus* (LH) and *L. rhamnosus* (LR). Bars represent the mean and SD of three technical replicates from one screening experiment. The displayed OD_{570} values were corrected for assay background; bacteria-free formulation controls were not subtracted. Percentage inhibition was not reported because the low OD_{570} values of the untreated controls produced unstable relative values. Untreated bacterial cultures and the corresponding pH-adjusted media served as controls and are outlined by boxes in the figure. CFS—cell-free supernatant at the native pH; CFS-N—cell-free supernatant at an adjusted pH; ICS—heat-inactivated whole-culture preparation at the native pH; ICS-N—heat-inactivated whole-culture preparation at an adjusted pH; LCS—whole-culture preparation containing viable cells at the native pH; LCS-N—whole-culture preparation containing viable cells at an adjusted pH; LC—viable probiotic cells at the native pH; IC—heat-inactivated cells at the native pH.

2.6. Assessment of Planktonic Growth and Recovery of Cultivable Bacteria After CFS Exposure

Planktonic bacterial turbidity following exposure to cell-free supernatants was assessed after incubation by measuring optical density at 620 nm (OD_{620}) with the HiPo MPP-96 microplate photometer (Biosan, Riga, Latvia). Because the tested CFS preparations did not contain detectable viable probiotic cells, OD_{620} was used as an optical measure of turbidity associated with the target bacteria. Untreated bacterial cultures and bacterial cultures containing MRS broth adjusted to pH 3.8 were included as controls. Each CFS bacterium combination and the corresponding controls were tested in three technical replicate wells. To determine whether cultivable bacteria remained after exposure, samples from the microplate

wells were examined using a modified qualitative drop-plating procedure. Following incubation, 10 µL was collected from each of the three replicate wells representing the same CFS bacterium combination. The three aliquots were pooled to obtain a total volume of 30 µL and mixed with 270 µL of sterile 0.9% NaCl solution, resulting in a 1:10 dilution. From each diluted pooled sample, two 10 µL drops were plated onto TSA. The plates were incubated at 37 °C for 12–48 h, depending on the colony development rate of the tested bacterium. The plating results were evaluated qualitatively as growth detected or no growth recovered. The two drops were plating replicates of one pooled sample. The plating results were used only to complement the OD_{620} measurements and were not used to calculate viable bacterial counts.

2.7. Statistical Analysis

Each formulation-bacterial target combination was tested in three technical replicates. For the overall comparison shown in Figure 1, technical replicates were averaged, and formulations were compared separately for each probiotic culture using the Friedman test, with the nine bacterial targets serving as blocks. Pairwise comparisons were performed with Bonferroni correction (adjusted $p < 0.05$). These exploratory comparisons do not establish reproducibility across independently prepared cultures or formulation batches. All other results were analyzed descriptively. Statistical analyses were performed using IBM SPSS Statistics, version 32.0.0.0 (134) (IBM Corp., Armonk, NY, USA), and figures were created using Microsoft Excel, version 2013 (Microsoft Corporation, Redmond, WA, USA) or IBM SPSS Statistics.

3. Results

3.1. Characteristics of the Probiotic-Derived Formulations and Target Bacteria

No morphologically distinct colonies were observed in the four recovered cultures under the conditions used, and representative colonies were identified to the species level by MALDI-TOF MS. Eight formulations were prepared for each of the four probiotic sources, totaling thirty-two formulations. Twelve of the thirty-two formulations contained viable probiotic microorganisms; twelve contained non-viable (inactivated) probiotic microorganisms; and eight formulations did not contain microbial cells. Bacteria-free formulation controls differed in their surface-associated CV signals (Table 1). Most cell-free supernatant (CFS) preparations produced no detectable or only low signals, whereas several cell-containing formulations produced moderate or high signals. Formulations producing moderate or high formulation-only CV signals were considered unsuitable for prioritization because they generate substantial attached material in the absence of target bacteria. None of the heat-inactivated formulations or CFSs formed colonies after 72 h of incubation on CBA and MRS agar, indicating viable microorganism levels below 20 CFU mL^{-1}.

The observed levels of biofilm formation capacity were as follows: *K. ornithinolytica* V2-4 and *B. subtilis* ATCC 6633 were weak biofilm producers, *S. aureus* V2-7 was a weak/moderate biofilm producer; and *S. aureus* ATCC 25923, *S. haemolyticus* V2-8, *P. aeruginosa* V2-3, *P. aeruginosa* ATCC 27853, *B. subtilis* VM, and *Enterococcus faecalis* V2-11 were strong biofilm producers. Both *B. subtilis* isolates produced a pellicle at the air–liquid interface; however, it was not included in the measurements, as the OD was determined only for the plate-attached biomass.

3.2. Screening of Antibiofilm Activity Across Bacterial Targets

Figure 1 provides an overall screening of all 32 probiotic-derived formulations across the nine individually tested bacterial targets.

Among the formulation types, native-pH CFS preparations consistently showed the lowest or among the lowest background-corrected attached-biomass OD_{570} distributions in all four panels. In contrast, pH-adjusted and cell-containing formulations showed wider and generally higher distributions, indicating greater target-dependent variability. Native-pH CFS, ICS, and LCS generally showed lower median OD_{570} values than their corresponding pH-adjusted formulations. This tendency was most pronounced for the *L. rhamnosus*-derived preparations for which the CFS resulted in significantly lower biofilm OD values than LCS-N, LC, and IC. However, the corresponding native-pH and pH-adjusted pairs generally shared statistical-group letters; therefore, the observed pattern should be interpreted as an overall descriptive tendency rather than a consistently significant pairwise effect of pH adjustment. Overall, the broad distributions and outlying values demonstrate that attached-biomass responses depended on the specific combination of bacterial target, formulation type, and formulation-producing culture.

3.3. Target-Dependent Antibiofilm Activity of the Formulations

The effect varied depending on both the formulation and the bacterial target tested. Among the six strong biofilm-forming bacteria, all four native-pH CFS preparations produced positive inhibition, whereas cell-containing and pH-adjusted formulations showed more variable activity, including negative inhibition values for individual targets (Figure 2). For the weak or weak/moderate biofilm producers, responses were evaluated using background-corrected OD_{570} rather than percentage inhibition because their low untreated-control signals produced unstable relative values.

Across the six strong biofilm-forming targets, inhibition by the native-pH CFS preparations ranged from 71% to 100%. The lowest value within this formulation type was observed for *S. cerevisiae* CFS against *E. faecalis* V2-11. In contrast, several cell-containing and pH-adjusted formulations showed strongly target-dependent responses. The greatest variability was observed for *P. aeruginosa* V2-3, for which several *L. helveticus*- and *L. rhamnosus*-derived LCS, ICS, and pH-adjusted formulations produced negative inhibition values. Negative values were also observed for selected formulations against *P. aeruginosa* ATCC 27853.

Viable and heat-inactivated cell suspensions showed similarly heterogeneous profiles. Negative inhibition values occurred predominantly against the two *P. aeruginosa* targets, although *L. rhamnosus* IC also produced negative values, indicating that the background-corrected attached-biomass OD_{570} exceeded the corresponding untreated-control value; they do not, by themselves, establish the stimulation of biofilm formation by the target bacterium.

For the weak biofilm-forming targets, native-pH CFS preparations were associated with consistently low background-corrected OD_{570} values (Figure 3). Higher and more variable attached-biomass signals occurred with selected viable-cell-containing and pH-adjusted preparations, with the most pronounced increases observed among some *L. rhamnosus*-derived formulations. Because the crystal violet assay measures total surface-associated material, these higher values were interpreted as increased total attached-biomass signals rather than as confirmed stimulation of the target bacterium's biofilm.

The percentage inhibition of attached biomass in strong biofilm-forming bacterial targets exposed to probiotic-derived formulations is reported in Supplementary Material Figure S1.

Taken together, the target-specific analysis identified native-pH CFS as the most uniform formulation type under the present screening conditions, while the activity of cell-containing and pH-adjusted preparations depended more strongly on the bacterial target. The high activity of the pH 3.8 medium control should be considered when interpreting the contribution of formulation acidity.

3.4. Effects of Native-pH CFS on Planktonic Bacterial Growth

Planktonic growth after 48 h of exposure to native-pH CFS was assessed by measuring the OD_{620} (Figure 4).

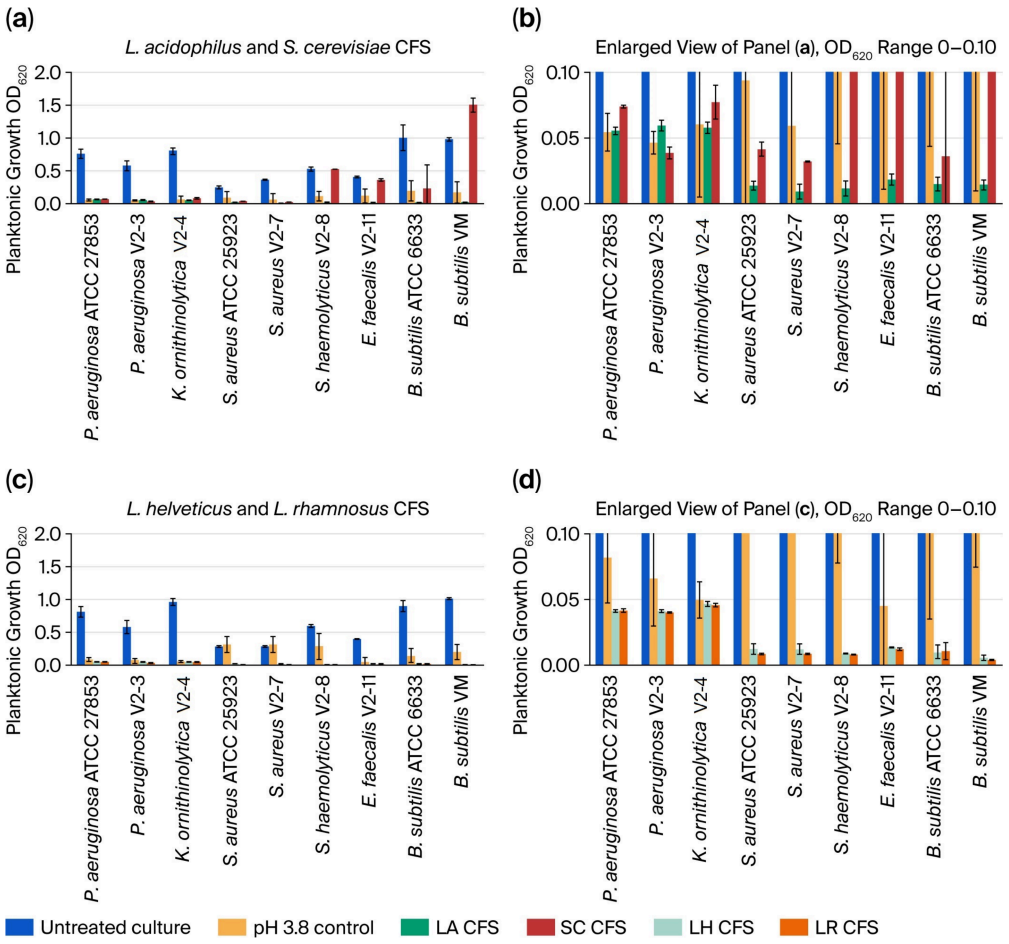

Figure 4. Effects of native-pH cell-free supernatants on planktonic bacterial growth after 48 h of incubation. Panels (**a**,**c**) show responses to *L. acidophilus*- and *S. cerevisiae*-derived CFSs and to *L. helveticus*- and *L. rhamnosus*-derived CFSs, respectively. Panels (**b**,**d**) show enlarged views of the OD_{620} range (0–0.10); values exceeding this range are shown in full in panels (**a**,**c**), respectively. Bars represent the mean OD_{620} ± SD of three technical replicates from one screening experiment. The untreated bacterial culture and acidified MRS medium (pH 3.8) served as controls.

The CFSs derived from *L. acidophilus*, *L. helveticus*, and *L. rhamnosus* reduced OD_{620} to near-background levels for most targets (Figure 4). The pH 3.8 MRS control also yielded lower OD_{620} values for several targets, but its effect on *S. aureus* ATCC 25923 was less pronounced than that of the CFS preparations. Responses to *S. cerevisiae* CFS varied among targets: OD_{620} remained close to the untreated value for *S. haemolyticus* V2-8 and was higher than the untreated value for *B. subtilis* VM. In the qualitative plating assay, no growth was recovered after exposure to the three lactobacillus-derived CFSs, whereas growth remained detectable after exposure to *S. cerevisiae* CFS and the acidified MRS control.

The OD_{620} findings were complemented by cultivation of aliquots from the test wells on TSA. No bacterial growth was recovered after exposure to the CFSs derived from *L. acidophilus*, *L. helveticus*, and *L. rhamnosus*. Meanwhile, bacterial growth remained detectable after exposure to *S. cerevisiae* CFS and the acidified MRS control.

3.5. Integrated Prioritization of Formulations for Further Investigation

The formulations were prioritized by integrating their activity profiles across the six strong biofilm-forming bacterial targets with the residual biofilm category and the formulation-only CV signal (Figure 2; Tables 1 and 2). Detailed descriptive measures and the complete classification criteria are provided in the Supplementary Materials, Table S2.

Table 2. Integrated prioritization of probiotic-derived formulations for further investigations.

Priority Group	Classification	Formulations	Main Reason for Assignment
Group 1	Broad-spectrum formulations with consistently high inhibitory activity	LA CFS, LH CFS, LR CFS, SC CFS	Positive inhibition across all six targets, broad high-level activity, and no detectable or low formulation-only signal
Group 2	Promising broad-spectrum formulations with minor limitations	LA CFS-N, SC ICS	Broad activity with minor limitations in consistency or residual biofilm suppression
Group 3	Formulations with conditional inhibitory potential	LA ICS-N, LA LCS-N, SC LCS, LH LCS-N	Positive inhibition across all six targets, but less uniform activity or less favorable residual-biofilm profiles
Group 4	Low-priority formulations	Remaining 22 formulations; see Table S2	Negative or insufficiently broad inhibition, high variability, unfavorable residual biofilm, or moderate/high formulation-only CV signal

LA—*L. acidophilus*; SC—*S. cerevisiae*; LH—*L. helveticus*; LR—*L. rhamnosus;* CFS—cell-free supernatant at the native pH; CFS-N—cell-free supernatant at an adjusted pH; ICS—heat-inactivated whole-culture preparation at the native pH; LCS—whole-culture preparation containing viable cells at the native pH; LCS-N—whole-culture preparation containing viable cells at an adjusted pH.

LA CFS, LH CFS, LR CFS, and SC CFS were assigned to Group 1 because they showed positive inhibition across all six targets, broad high-level activity, and no detectable or only low formulation-only CV signals.

LA CFS-N and SC ICS were assigned to Group 2 because they retained broad inhibitory activity but showed minor limitations in consistency or residual biofilm suppression.

SC LCS, LA ICS-N, LH LCS-N, and LA LCS-N were assigned to Group 3. These formulations showed positive inhibition across all six targets but had less uniform activity or less favorable residual-biofilm profiles.

The remaining formulations were assigned to Group 4 because of negative or insufficiently broad inhibition, marked target-dependent variability, unfavorable residual biofilm categories, or moderate-to-high formulation-only CV signals. LA LCS and LA ICS were assigned to Group 4 despite high activity against several targets because their bacteria-free formulation controls produced moderate CV signals.

This classification represents an application-oriented screening framework for candidate selection and should not be interpreted as a statistically validated ranking of formulation efficacy.

Overall, the integrated classification identified the four native-pH CFS preparations as the most consistent candidates across the tested panel, while SC ICS and selected pH-adjusted formulations retained sufficient activity to warrant conditional further investigations.

4. Discussion

The antibiofilm activity of probiotic microorganisms and probiotic-derived preparations can vary with both the producing strains and the bacterial target [5,9]. Consideration of strain- and target-dependent activity is particularly important when developing antimicrobial preparations for food production environments, including milking systems, where diverse microorganisms are present. Some can persist on equipment surfaces as biofilms [3].

This study, therefore, examined preparations derived from four commercially available cultures: *Lactobacillus acidophilus* THT 030101, *Lactobacillus helveticus* THT 031102, *Lacticaseibacillus rhamnosus* GG, and *Saccharomyces cerevisiae* DSM 33148. The screening served two purposes: to assess their effects on biofilm formation by dairy-associated pathogenic and spoilage bacteria, and to compare formulation types to identify candidates with activity across several targets. Evidence on the antibiofilm activity of preparations derived from these particular cultures remains limited. Although the cell-free supernatant derived from *L. rhamnosus* GG has been studied previously [10], those studies did not establish how the formulations examined here perform against the dairy-associated bacterial targets.

We tested nine bacterial targets individually to identify both broadly active formulations and target-specific responses. Such variations have been observed in studies of probiotic-derived exopolysaccharides and cell-free supernatants tested against different microorganisms [5,11,12]. Testing several targets was, therefore, relevant to selecting preparations for further evaluation in dairy production environments, where a single target would provide only a limited picture of their activity.

For this intended application, a formulation with a low attached-biomass signal (which is characterized by CV signal) and broad activity against different bacterial targets is preferable to a narrowly active formulation. Conversely, a formulation was considered unsuitable for prioritization if it produced a substantial crystal-violet-stainable deposit in bacteria-free controls or was associated with increased total attached biomass in any tested bacterial culture. Because crystal violet quantifies total surface-associated material, the assay cannot determine whether an increased signal originated from the target bacterium, probiotic cells, inactivated cells, extracellular material, or their combination. Formulation-only controls help account for formulation-derived staining, although their subtraction does not distinguish target bacterial biomass from other attached material in mixed cultures. Inhibition estimates reaching or exceeding 100% indicate a corrected CV signal at or below zero and should not be interpreted as proof of complete elimination of the target bacterium's biofilm. Such observations, therefore, identify an application-relevant exclusion signal but do not demonstrate stimulation of a specific pathogen's biofilm without species-specific confirmation.

Overall, the four native-pH cell-free supernatants (CFSs) showed the most consistent inhibition across the six strong biofilm-forming targets and produced no or only low formulation-only CV signals (Figures 1 and 2; Tables 1 and 2). Their displayed inhibition values ranged from 71% to 100% across these targets; values above 100% were capped at 100% for the integrated ranking. Strong antibiofilm activity of probiotic-derived CFS has also been reported against *S. aureus* and *P. aeruginosa* [13,14]. For the three lactobacillus-derived CFS preparations, inhibition of attached biomass generally coincided with near-background planktonic-growth OD_{620} values and no detectable growth on subsequent qualitative plating (Figure 4). *S. cerevisiae* CFS also inhibited attached biomass across all six strong biofilm formers; however, it did not suppress planktonic growth as consistently, and growth remained detectable after exposure. Its native pH was 5.26, compared with 3.92–3.99 for the lactobacillus-derived CFSs (Table 1). This pH difference may have contributed to the distinct planktonic growth responses. However, the pH 3.8 MRS control was not matched to *S. cerevisiae* CFS (pH 5.26), so this comparison cannot isolate the contribution of acidity to that supernatant's activity.

Thus, a low biofilm CV signal alone does not provide evidence that bacterial growth or cultivability was eliminated. To compare the efficacy of CFS from the four producer cultures against individual targets more precisely, the selected preparations should be tested at lower concentrations in independent experiments, with biofilm, planktonic growth, and viable count endpoints assessed in parallel.

The other formulations showed less uniform responses. In particular, some pH-adjusted and cell-containing preparations yielded higher formulation-only CV signals, and *L. rhamnosus*-derived formulations included several pronounced attached-biomass responses. The bacteria-free MRS control adjusted to pH 6.8 also produced a moderate CV signal (Table 1). Therefore, elevated formulation-only signals after pH adjustment cannot automatically be attributed to microbial cells or fermentation products alone. Cell suspensions without fermentation medium (LC and IC) generally produced higher formulation-only CV signals than preparations containing fermentation medium (Table 1). Thus, the apparent benefit of retaining microbial cells must be considered alongside the material they leave on the tested surface.

The integrated ranking placed *L. acidophilus* LCS and ICS in the lowest priority group despite their activity against several targets because their bacteria-free controls produced moderate CV signals (Tables 1 and 2). Their inhibition profiles still justify mechanistic study under different exposure conditions, but the current results do not support prioritizing them for direct applications to milking equipment. In addition to native-pH CFS, *L. acidophilus* CFS-N and *S. cerevisiae* ICS retained sufficiently broad activity to merit further investigation, with the limitations identified by the integrated prioritization criteria (Table 2; Table S2).

Giordani et al. found that exopolysaccharides from vaginal lactobacilli promoted biofilm formation by beneficial lactobacilli while inhibiting biofilms of opportunistic pathogens [11]. This illustrates that surface-associated material can have implications in different applications. In the displayed inhibition analysis, *L. acidophilus* LCS reached the 100% reporting cap for all six strong targets; however, its formulation-only wells had a moderate CV signal, and the assay cannot identify the material remaining on the surface. Because crystal violet cannot identify the organisms or components contributing to attached material, these results do not establish whether target cells remained on the surface. This formulation is, therefore, unsuitable for prioritizing milking equipment based on the present criteria; however, its inhibitory activity may warrant investigation in a setting where surface association is acceptable.

Cultivation conditions may also contribute to differences between formulations. Squarzanti et al.'s study compared supernatants from lactic acid bacteria grown in De Man–Rogosa–Sharpe (MRS) and alternative broth. They found that the *Lactobacillus johnsonii* supernatant produced in the alternative broth reduced *S. aureus* biofilm viability more strongly; the media also yielded different supernatant acid and protein profiles [15]. In our study, all four probiotic cultures were grown in MRS broth, but their supernatants were not chemically profiled. We therefore cannot determine whether differences in metabolite production contributed to the variation observed between our formulations.

The results varied with the type of formulation and its acidity. Native-pH preparations containing fermentation medium generally showed lower attached-biomass signals than their pH-adjusted counterparts, and the pH 3.8 MRS control inhibited several targets (Figures 1 and 2). However, native-pH and adjusted pairs generally shared statistical-group letters in the overall analysis across the nine bacterial targets (Figure 1), while Figure 2 shows that their responses varied by target. Selected pH-adjusted formulations, including CFS-N and cell-containing preparations, retained activity against multiple targets. These findings indicate that acidic conditions contributed to inhibition but do not explain every observed response. Because the acidic medium control was also active and the supernatants

were not chemically characterized, inhibition by the native-pH CFS cannot be assigned to specific fermentation products. Other metabolites could nevertheless act together with organic acids, potentially contributing to the observed inhibition. Vitale et al. reported that the growth-inhibitory activity of *Limosilactobacillus reuteri* CFS against *S. aureus* could not be explained by its lactic acid content alone, suggesting a contribution from other compounds [16]. Potential synergistic interactions were not assessed in the present study. Concentration–response experiments with pH-matched controls, combined with chemical characterization of the CFS, would help distinguish the contributions of acidity and other supernatant components [6].

The responses of the weak biofilm-forming targets require particular caution. Their low untreated-control OD_{570} values made percentage inhibition sensitive to small absolute changes. Therefore, we present the background-corrected OD_{570} values for these targets rather than unstable relative inhibition estimates (Figure 3). Percentage inhibition was reserved for the six strong biofilm producers, for which the untreated controls supported a more interpretable comparison. This reporting choice reflects assay sensitivity and does not imply that baseline biofilm strength determines susceptibility to the preparations.

Direct comparisons with published inhibition percentages are limited by differences in producer strains, target strains, formulation concentrations, incubation conditions, and biofilm assays. Kim et al., for example, reported the activity of CFSs from other *S. cerevisiae* isolates against *S. aureus* biofilms [17], whereas our *S. cerevisiae* preparations showed more variable target-dependent responses. In our screening, the pH-adjusted CFS showed limited activity against *S. aureus* ATCC 25923 relative to the native-pH CFS (Figure 2). Falqueto et al. evaluated CFSs, inactivated cells, and combined extracts from lactic acid bacteria against dairy-associated organisms, offering a closer comparison of formulation types, although their producer and target strains differed from ours [5]. Such differences prevent a direct ranking of strains across studies.

The screen was conducted as one independent experiment with three technical replicates per formulation–bacterium combination. Technical wells describe variation within the assay but do not estimate variation among independent experiments or formulation batches. The candidate ranking and statistical comparisons are, therefore, exploratory. Direct quantitative comparisons between the two formulation-testing series should be interpreted cautiously because their mean initial target inocula differed. Furthermore, the findings concern the prevention of attached biomass under the tested conditions, not the eradication of mature biofilms.

The next step is to confirm the selected candidates using independently prepared cultures and batches, additional isolates of relevant species, and concentration–response experiments. Quantitative recovery of cultivable cells and measurements of biofilm-associated viability would complement CV staining. Comparing pre-exposure with treatment of established biofilms would distinguish prevention from removal. Further testing on stainless steel and elastomer surfaces under relevant exposure, rinsing, and repeated-cleaning conditions is needed before claiming practical efficacy or material compatibility. The present results prioritize native-pH CFS for these studies while retaining selected non-CFS formulations for focused evaluation [2].

5. Conclusions

In this exploratory screening, the four cell-free supernatants at their native pH showed the most consistent reduction in attached biomass across the six strong biofilm-forming bacterial targets. Responses to other preparations varied more by formulation and target. Some pH-adjusted formulations retained activity against several targets, indicating that the observed responses were not explained solely by the formulations' native acidity.

The screening also identified a practical consideration for selecting candidates for further study: some cell containing preparations produced crystal-violet-stainable material even in the absence of a bacterial target. Their inhibitory responses must therefore be considered alongside the material they may leave on a surface. Planktonic growth measurements further showed that reduced attached biomass did not always coincide with reduced bacterial turbidity.

These findings prioritize native-pH cell-free supernatants while identifying selected other formulations for focused study. The screen provides a basis for choosing candidates to confirm in independent experiments and test on materials used in milking equipment.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation12110511/s1, Table S1: Bacterial concentrations in precultures and biofilm inhibition assay wells; Table S2: Integrated classification of the formulations; Figure S1: Strong formers, biofilm inhibition by formulations; Supplementary Material S4: The complete allocation of samples and controls.

Author Contributions: Conceptualization, G.G.; methodology, G.G.; writing—original draft preparation, G.G.; writing—review and editing, A.V.; visualization, G.G.; sample collection and bacteria isolation, identification, S.V., V.D., G.G.; biofilm assay, G.G.; supervision, A.V.; technical support, A.B. and V.A. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Activity 1.1.1.9 "Post-doctoral Research" of the Specific Objective 1.1.1 "Strengthening research and innovative capacities and introduction of advanced technologies in the common R&D system" of the European Union's Cohesion Policy Programme for 2021–2027 research application No 1.1.1.9/LZP/1/24/162 (1.1.1.9/1/24/I/001) "Development of postbiotics' compositions including the evaluation of its bioactivity against pathogenic bacteria and their produced biofilms in the dairy industry".

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The original contributions presented in this study are included in the article/Supplementary Materials. Further inquiries can be directed to the corresponding author.

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

Abbreviations

The following abbreviations are used in this manuscript:

CBA	Columbia blood agar
CFS	Cell-free supernatant, native pH
CFS-N	Cell-free supernatant, adjusted pH
CV	Crystal violet (signal)
IC	Inactivated cells, native pH
ICS	Whole culture containing inactivated cells and supernatant, native pH
ICS-N	Whole culture containing inactivated cells and supernatant, adjusted pH
LC	Viable cells, native pH
LCS	Whole culture containing viable cells and supernatant, native pH
LCS-N	Whole culture containing viable cells and supernatant, adjusted pH
LB	Luria–Bertani medium
MRS	De Man–Rogosa–Sharpe (agar or broth)
TSA	Tryptic soy agar
TSBG	Tryptone soy broth with 1% glucose

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