fermentation

MDPI

Article

Cultivation of Lactic Acid Bacteria and Evaluation of the Antimicrobial Potential of Partially Purified Bacteriocin-like Inhibitory Substances against Cariogenic and Food Pathogens

Amanda Romana Santos da Silva [1], Pamela Oliveira de Souza de Azevedo [1], Attilio Converti [2,*] and Ricardo Pinheiro de Souza Oliveira [1]

[1] Department of Biochemical-Pharmaceutical Technology, Faculty of Pharmaceutical Sciences, University of São Paulo, Av. Prof. Lineu Prestes, 580, Bl 16, São Paulo 05508-900, Brazil
[2] Department of Civil, Chemical and Environmental Engineering, University of Genoa, Pole of Chemical Engineering, Via Opera Pia 15, 16145 Genoa, Italy
* Correspondence: converti@unige.it; Tel.: +39-010-3352593

Abstract: One of the major challenges in the pharmaceutical industry is the search for new antimicrobial compounds that can replace antibiotics. Lactic acid bacteria (LAB) can produce bacteriocin-like inhibitory substances (BLIS) that have a bacteriostatic or bactericidal effect against different bacterial genera, including those responsible for dental caries. Among the pathological processes of microbial etiology, the dental caries stands out, whose main pathogenic agent is the species *Streptococcus mutans*, present in about 80–90% of the oral cavity. In this context, this study aimed to produce and semi-purify BLIS from *Lactobacillus plantarum* ST16 Pa, *Bifidobacterium lactis* BL 04, *Lactococcus lactis* CECT-4434 and *Lactobacillus lactis* 27 as well as to assess their antimicrobial potential against important dental caries causing pathogens like *S. mutans* UA159, *Listeria innocua* 2711, *Carnobacterium maltaromaticum* CECT 4020, *Staphylococcus aureus* CECT 239, and *Escherichia coli* ATCC 25922. While BLIS from *L. plantarum* ST16 Pa and *L. lactis* CECT-4434 were able to inhibit the growth only of *S. mutans* UA159, that which was produced by *B. lactis* BL 04 did so against all bioindicator strains; therefore, this suggests that its application could be important in the control of cariogenic microorganisms.

Keywords: bacteriocins; lactic acid bacteria; purification; caries; control of pathogens; antimicrobial activity; food

check for updates

Citation: da Silva, A.R.S.; de Souza de Azevedo, P.O.; Converti, A.; de Souza Oliveira, R.P. Cultivation of Lactic Acid Bacteria and Evaluation of the Antimicrobial Potential of Partially Purified Bacteriocin-like Inhibitory Substances against Cariogenic and Food Pathogens. *Fermentation* **2022**, *8*, 400. https://doi.org/10.3390/fermentation8080400

Academic Editor: Chih Yao Hou

Received: 28 July 2022
Accepted: 15 August 2022
Published: 18 August 2022

1. Introduction

The human oral cavity is inhabited by hundreds of bacterial species that relate to each other and the host in a complex way, such as through competitive, cooperative, and parasitic relations, which can lead to oral diseases [1,2]. Oral diseases are one of the world's main problems that affect child and youth populations. The Global Burden of Disease estimated that oral diseases affect about 3.5 billion people worldwide, the most common being dental caries. Globally, it is estimated that 2.3 billion people suffer from caries in permanent teeth and more than 530 million children suffer from caries in deciduous teeth [3].

Streptococcus mutans is considered the main agent of caries, as it has peculiar properties such as the ability to colonize the dental surface through the adhesion and production of extracellular polysaccharides, which enables the formation of a thick biofilm. In addition, it has the ability to survive in acidic mediums and accumulate intracellular polysaccharides from the fermentation of glucose and other carbohydrates [4]. A carious injury begins with the establishment of a specific bacterial population, which is able to demineralize the enamel under a specifically modified environment in the oral cavity [5]. Different changes appear in the oral ecosystem leading to the proliferation of bacterial biofilm, composed especially by *S. mutans* cells.

Another important microorganism is *Staphylococcus aureus*, which can be found in the larynx, oral and nasal cavity, or in contaminated foods [6]. *S. aureus* is also the main vector of bacterial endocarditis because, using dental caries as a gateway to the blood system, it can reach the heart and produce a series of toxins, which can give children more risk and even be fatal [7].

The oral cavity is a dynamic ecosystem with permanent characteristics and a peculiar heterogeneous microbiota, whose imbalance leads to the beginning of major oral diseases such as periodontitis and dental caries [8]. Conventional treatment of these diseases involves the removal of bacterial plaque by mechanical means and therapy with antimicrobial drugs that may have limited efficacy due to resistance of microorganisms to antibiotics [9]. According to the World Health Organization (WHO) and the United Nations Food and Agriculture Organization (FAO), probiotics are "live microorganisms which when administered in adequate amounts confer a health benefit on the host" [10]. Traditionally, the main area of study of probiotics concerns the gastrointestinal tract, with the objective of preventing and/or treating diseases. Even though probiotics are widely used for the treatment of intestinal diseases, they have not yet been applied to treat oral diseases due to the scarcity of dental studies. Studies have already shown that some probiotics can increase the proportion of beneficial bacteria in the mouth, contributing to the prevention and therapy of oral diseases [11–13]. To be effective in the limitation or prevention of dental caries, probiotics must (a) adhere to and colonize dental surfaces by integrating themselves into the bacterial community constituting dental biofilm; (b) compete with cariogenic and pathogenic bacteria, antagonizing them, and inhibiting and/or delaying their proliferation; (c) metabolize sugars with low acid production to minimize tooth enamel erosion; (d) hinder the organization of extracellular matrix responsible for biofilm formation; (e) limit the production of cytotoxic products by pathogenic bacteria; and (f) beneficially alter the parameters influencing tooth plate formation (e.g., salivary components, buffering power, etc.) [11–15].

During the last decade, several researchers have demonstrated the beneficial use of probiotics in the oral cavity. For instance, bacteria belonging to the genus *Bifidobacterium* showed encouraging results in the control of ligature-induced periodontitis in mice by modulating the host response [16]. In humans, the ingestion of *B. animalis* subsp. *lactis* led to decreased IL-1β levels in the gingival crevicular fluid [17] and was successfully used as a complement to mechanical treatment of periodontitis [18]. Some *Lactobacillus* strains exhibited potential for dental caries prevention, mainly due to their possible inhibitor activity against cariogenic streptococci and the fact that they are considered safe for oral administration in humans [8].

Bacteriocins are low molecular weight peptides that have inhibitory activity against bacteria, protozoa, fungi, and viruses. Although bacteriocins are produced by several microorganisms, those produced by lactic acid bacteria (LABs) have been receiving greater attention because this group of bacteria is generally safe for human consumption [19,20]. The antimicrobial activity of bacteriocins varies according to the bacteria that produce them and the environment in which they grow, and may have a bacteriostatic or bactericidal effect. Although bacteriocins produced by probiotics are generally recognized as safe (GRAS), nisin is the only approved for use as a food preservative, whose activity analysis parameters, concentration in each type of food, and issues related to safety and commercial use by the food industry are very well defined by WHO/FAO [21].

The relevance of this work is justified in the high rates of caries on a world scale and the therapeutic potential of probiotics that showed promising results in major oral diseases such as periodontitis and dental caries [22,23]. However, doubts still remain about the best probiotic strains for each oral disease [24], as well as their dosage and frequency of administration. Studies dealing with the prevention of oral pathologies are mostly directed to diet control, salivation stimuli, mechanical removal of dental plaque, and remineralization of non-cavitated surfaces in which the process has already begun. Therefore, it is ignored by the main oral health promotion programs one of the most

important characteristics of oral microbiota—that is, the relationships among the different bacterial communities, especially the competitive ones [1].

In the context of the search for effective bacteriocins against cariogenic and food pathogens, the aim of this study was the identification of bacteriocin-producing LABs with the potential to contribute to the development of alternative methods for the control of oral biofilm and health promotion in the oral cavity.

2. Materials and Methods

2.1. Bacterial Strains

Different species of bacteriocin-producing LABs were used in this study, namely *Lactobacillus plantarum* ST16 Pa, *Pediococcus pentosaceus* ATCC 33316, *Lactococcus lactis* CECT-4434, *Bifidobacterium lactis* BL 04 (Danisco, Sassenage, France), *Lactobacillus sakei* subsp. *sakei* 2a, and *Lactobacillus lactis* 27 (S.T. Dupont, Paris, France), while *Streptococcus thermophilus* TA040 (S.T. Dupont, Paris, France) was used as a control.

As for the bioindicator strains, *Streptococcus mutans* UA159 (ATCC 700610) and *Escherichia coli* ATCC 25922 were purchased from the American Type Culture Collection, *Carnobacterium maltaromaticum* CECT 4020 and *Staphylococcus aureus* CECT 239 from Spanish Type Culture Collection (CECT), while *Listeria innocua* CLIST 2711 was kindly provided by the Instituto Oswaldo Cruz (Rio de Janeiro, Brazil).

2.2. Culture Conditions

P. pentosaceus ATCC 33316, *L. plantarum* ST16 Pa, *L. lactis* CECT-4434, *B. lactis* BL 04, *L. sakei* 2a, and *L. lactis* 27 were grown anaerobically in De Man, Rogosa, and Sharpe (MRS) medium (Difco Laboratories, Detroit, MI, USA) at 30 °C and pH 6.5 ± 0.2 for 16 h under 150 rpm orbital agitation (TE-424, Technical, Piracicaba, Brazil). For a purity check, cells from each culture were seeded in Petri plates containing MRS medium plus 2% agar and incubated overnight at 37 °C.

All bioindicator strains (1.0 mL), previously cryopreserved at −70 °C in the presence of 20% glycerol (*w*/*v*), were aerobically reactivated in 5.0 mL of Tryptic Soy Broth (TSB) (Difco Laboratories, Detroit, MI, USA) at 37 °C for 16 h without agitation. For purity check, cells from each culture were seeded in Petri plates containing TSB plus 2% agar and incubated overnight at 37 °C.

2.3. Co-Cultures

To detect possible synergistic effects, bacteriocin-producing strains (1.0 mL) were cultivated in co-cultures with each other and/or with *S. thermophilus* TA040 in 250 mL Erlenmeyer flasks containing 100 mL of MRS broth supplemented with 1% (*w*/*v*) L-cysteine and 0.04% (*w*/*v*) sodium thioglyolate (Difco).

2.4. Kinetic Study of Lactic Acid Bacteria Growth

The growth kinetics of LABs (*L. plantarum* 16 PA, *L. lactis* CECT4434, *B. lactis* BL04, and *L. lactis* 27) was investigated along 52 h cultures using the pour plate methodology. For this purpose, the initial concentration of each LAB was adjusted to an optical density at 600 nm ($OD_{600\,nm}$) of 0.8–0.9, which corresponded to 10^7–10^8 colony forming units (CFU) per mL. LABs were grown in sterile tubes containing 50 mL of MRS broth (Difco) at 37 °C, from which 100 µL samples were collected every 2 h. Afterwards, serial dilution (1:10) was performed using sterile distilled water, and all dilutions were seeded on the surface of 1% (*w*/*v*) MRS agar in triplicate. After plate incubation at 37 °C for 24 h, colonies were counted, and the results expressed in CFU/mL.

2.5. Acidification Kinetics

The acidification profile was monitored for 24 h by the CINAC system (Cinétique d'Acidification) software (Ysebaert, Frépillon, France). Both monocultures and co-cultures were kept in a water bath without agitation at 37 °C in 250 mL Schott flasks (Schott Duran,

Laborglas, São Paulo, Brazil) containing 200 mL of MRS medium (for LABs producing bacteriocin-like inhibitory substances (BLIS)) or M17 medium (for *S. thermophilus*). After the assessment of the acidification profile, the cultures were centrifuged at 4470× *g* and 4 °C for 15 min to obtain the cell-free supernatant (CFS) for later determination of bacteriocin antimicrobial activity.

2.6. Determination of Antimicrobial Activity by the Critical Dilution Technique

To prevent the action of organic acids, the CFSs had their pH adjusted to 6.0–6.5 by addition of 1.0 M NaOH. They were then heated at 80 °C for 10 min to inactivate proteases, and sterilized by filtration in membranes with 0.20 µm pore diameter (Millipore, Billerica, MA, USA).

The antimicrobial activity of each BLIS was evaluated against the bioindicator strains *L. innocua* CLIST 2711, *C. maltaromaticum* CECT 4020, *S. aureus* CECT 239 and *E. coli* ATCC 25922, and the results were expressed in arbitrary units per milliliter (AU/mL). After serial dilution of CFSs (1:2, 1:4 and 1:8, *v*/*v*) in 25 mM sodium phosphate buffer, pH 7.0, 10 µL of each dilution were applied on the surface of plates containing TSB agar (0.75%, *w*/*v*) previously inoculated with the selected bioindicator strain (~10^6 CFU/mL) and later incubated at 37 °C for 24 h. The first dilution in which there was no inhibition halo formation (*n*) was considered in the equation:

$$\text{AU/mL} = \frac{D^n \cdot 1000}{P} \quad (1)$$

where *D* is the dilution factor and *P* the volume of supernatant placed on the TSB agar medium.

2.7. Determination of Antimicrobial Activity against Cariogenic Microorganism

To evaluate the potential anticariogenic effect of BLIS produced by LABs at different stages of bacterial growth, BLIS activity was also determined as ability to inhibit the growth of the *S. mutans* UA159 strain by the micro-dilution method using a microplate reader [25,26]. The assays were performed on sterile 96-well plate (TPP, Trasadingen, Switzerland) containing M17 (Difco) medium supplemented with 1.0% (*w*/*v*) lactose (100 µL), an aliquot of overnight culture of this pathogen (50 µL), and BLIS solution (50 µL), after pH correction to 6.0–6.5 with 1.0 M NaOH, thermal treatment for 10 min at 80 °C, and filter sterilization (0.22 µm). The resulting suspension (200 µL) was well mixed, and the microplate was incubated inside a microplate reader (BioTek, Winooski, VT, USA) at 37 °C. *S. mutans* growth was determined automatically every hour for 24 h on the microplate reader at $OD_{600\,nm}$. BLIS activity was determined in triplicate.

2.8. Effect of Proteases, Solvents, Salts and Detergents on BLIS

To investigate the eventual protein nature of antimicrobial compounds, the CFSs were treated with 1.0 mg/mL tripsin, pepsin, or papain (Sigma-Aldrich, Saint Louis, MO, USA) for 2 h at 37 °C. A 10 µL aliquot of BLIS solution prepared as described in the Section 2.6 was poured in a Petri dish containing *L. innocua* 2711 as a bio-indicator strain. The same volume of 1.0 mg/mL enzyme solution was poured in the Petri dish in such a way that it would just partially cover BLIS [27]. The proteinaceous nature of BLIS was determined by the absence of inhibition halo against the bio-indicator strain.

To check BLIS stability, CFSs were treated in 1.0-mL conical tubes at 30 °C for 2 h with different organic solvents (acetonitrile and isopropanol), salts (sodium chloride and ammonium sulfate), and detergents (Triton X-100, ethylenediaminetetraacetic acid (EDTA), Tween 20, Tween 80, sodium dodecyl sulfate (SDS)) at a final concentration of 1.0% (*w*/*v*). In addition, BLIS was submitted to heat treatment at different temperatures, namely 30, 50, 70, and 90 °C for 1 h, or 120 °C for 15 min. After that, the BLIS stability was checked by the agar diffusion method against *L. innocua* 2711 [27].

2.9. BLIS Concentration by Precipitation with Ammonium Sulfate

After verification of protein nature of BLIS, they were concentrated by the salting-out technique. Precipitation was performed using 10 mL of each CFS by adding ammonium sulfate at six different concentrations (10, 20, 30, 40, 50, and 60% *w*/*v*). The antimicrobial activity of BLIS was then assessed to identify the optimal ammonium sulfate concentration to recover them. Afterwards, the tubes were vigorously mixed for 1 min and then incubated at 10 °C and 100 rpm for 1 h. After sample centrifugation at 4470× *g* and 4 °C for 30 min, each precipitate was resuspended with 10% (*v*/*v*) of the initial volume (20 mL) of 25 mM ammonium acetate solution (pH 6.5) (Labsynth Produtos para Laboratórios, Diadema, Brazil) [25], and sterilized by filtration through membranes with 0.22 µm pore diameter [27]. The antimicrobial activity of all concentrated BLIS was determined by the agar diffusion method.

3. Results and Discussion

3.1. Antimicrobial Activity of BLIS by the Critical Dilution Technique

The results of the antimicrobial activity of cell-free supernatants (CFSs) determined by the critical dilution technique showed that, among the BLIS-producing bacteria under investigation, *L. plantarum* ST16 PA and especially *B. lactis* BL 04 had the largest inhibitory effects against *L. innocua* 2711 (Table 1). Antimicrobials produced by *L. plantarum* ST16 Pa also showed efficiency in inhibiting different species of the genera *Enterococcus*, *Lactobacillus*, *Pseudomonas*, *Streptococcus*, *Staphylococcus,* and *Listeria* [19]. In a similar study, *B. lactis* CFS was able to inhibit both *L. monocytogenes* and *E. coli* ATCC 25922 growth, thus demonstrating its antimicrobial potential against both Gram-positive and Gram-negative bacteria [28].

Table 1. Antimicrobial activity, expressed in AU/mL, of bacteriocin-like inhibitory substances (BLIS) produced by the lactic acid bacteria (LAB) under investigation against different bioindicator strains.

BLIS-Producing LAB	**Bioindicator Strain**			
	***Listeria innocua* 2711**	***Carnobacterium maltaromaticum* CECT 4020**	***Staphylococcus aureus* CECT 239**	***Escherichia coli* ATCC 25922**
Lactobacillus plantarum ST16 Pa	3200	400	800	- [1]
Pediococcus pentosaceus ATCC 33316	-	-	-	-
Lactococcus lactis CECT-4434	800	-	-	-
Bifidobacterium lactis BL 04	6400	800	800	400
Lactobacillus sakei 2a	-	-	-	-
Lactobacillus lactis 27	1600	800	3200	-

[1] No antimicrobial activity.

When compared to the other BLIS-producing LABs, *L. lactis* 27 exhibited the highest antimicrobial activity against *S. aureus* CECT 239, contrary to *L. lactis* CECT-4434 that showed no activity against this pathogen. *P. pentosaceus* ATCC 33316 and *L. sakei* subsp. *sakei* 2a, although described in the literature as bacteriocin producers [29,30], did not exert any antimicrobial activity against the bioindicator strains tested in this study.

3.2. Kinetics of Lactic Bacteria Growth in Monocultures

The selected LABs showed quick initial growth, with start of the exponential phase after only 2 h of cultivation. The only exception to this was *L. plantarum* ST16 PA, which displayed a 6 h lag phase. Therefore, this strain suffered a reduction (4 log units) in cell concentration after 52 h of cultivation ($4.0 \pm 0.0 \times 10^5$ CFU/mL), compared to the beginning of the exponential phase ($3.2 \pm 0.0 \times 10^9$ CFU/mL), which was much larger than those observed for *L. lactis* CECT 4434, *L. lactis* 27 (1 log unit), and *B. lactis* BL04 (2 log units) (Figure 1).

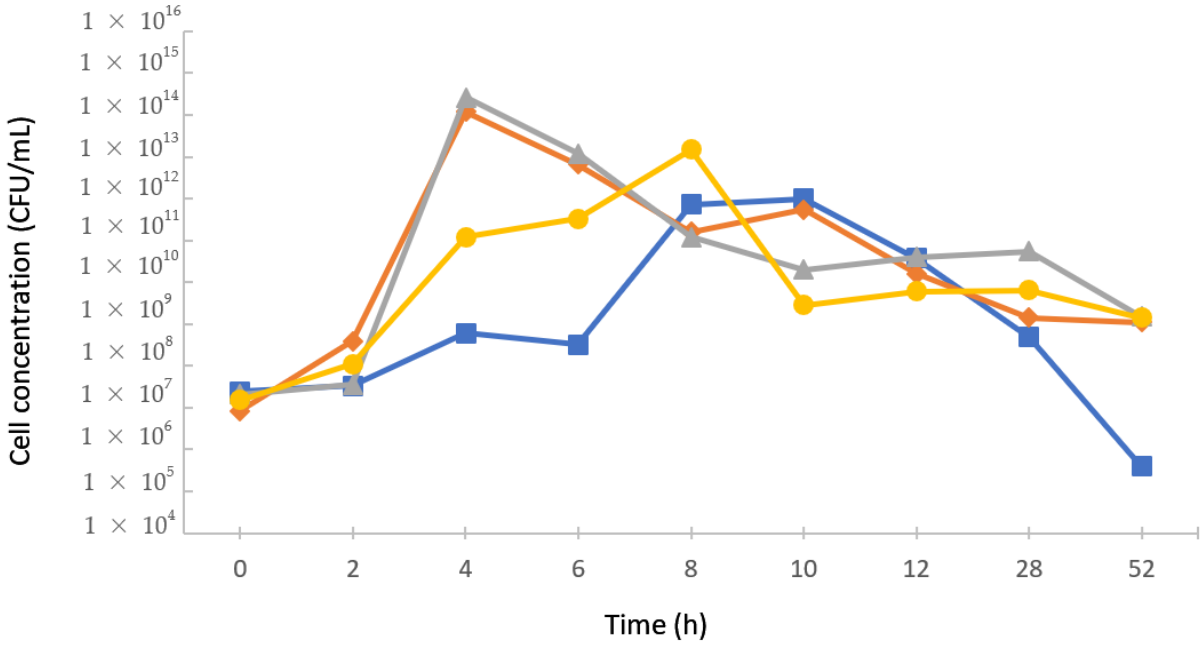

Figure 1. Cell concentration, expressed in colony forming units per milliliter (CFU/mL), of the lactic acid bacteria under investigation along 52-h cultivations. *Lactobacillus plantarum* STP16 A (■), *Lactococcus lactis* CECT-4434 (♦), *Bifidobacterium lactis* BL4 (▲), *Lactococcus lactis* 27 (●).

3.3. Antimicrobial Activity against Cariogenic Microorganism

To evaluate the antimicrobial activity against the cariogenic *S. mutans* UA159 strain by the micro-dilution method, only the BLIS-producing LABs that were successful against non-cariogenic bioindicator strains were selected. Although all the selected LABs demonstrated antimicrobial activity against *S. mutans* UA159, *L. plantarum* ST16 Pa and *L. lactis* CECT-4434 ensured the most significant and promising results (Table 2).

Table 2. Antimicrobial activity of cell-free supernatants (CFS) from cultures of lactic acid bateria (LABs) able to produce bacteriocin-like inhibitory substances (BLIS) against *Streptococcus mutans* UA159. Results were expressed by optical density ($OD_{600\ nm}$).

BLIS-Producing LAB	CFS [1]	CFS1 [2]	CFS2 [3]
Lactobacillus plantarum ST16 Pa	1.43	0.94	0.54
Lactococcus lactis CECT-4434	1.46	0.73	0.27
Bifidobacterium lactis BL 04	0.8	0.24	0.12
Lactobacillus lactis 27	1.29	0.64	0.32

[1] CFS = cell free supernatant; [2] CFS1 = 1:1/2 (*v*/*v*) diluted cell free supernatant; [3] CFS2 = 1:1 (*v*/*v*) diluted cell free supernatant.

Figure 2 shows that the addition of CFS containing the *B. lactis* BL 04 BLIS delayed the growth of *S. mutans* UA159 by approximately 13 h compared to its cultivation without CFS, and that this effect was directly proportional to BLIS concentration. However, the capability of the bioindicator strain to grow, although more slowly, even without any CFS dilution suggests that the BLIS effect was likely bacteriostatic rather than bactericidal. In addition, the effectiveness of this effect is demonstrated by the fact that it occurred even in the presence of the most diluted CFS (CFS2), leading to a reduction of no less than 40% of the final $OD_{600\ nm}$ compared to the culture without BLIS.

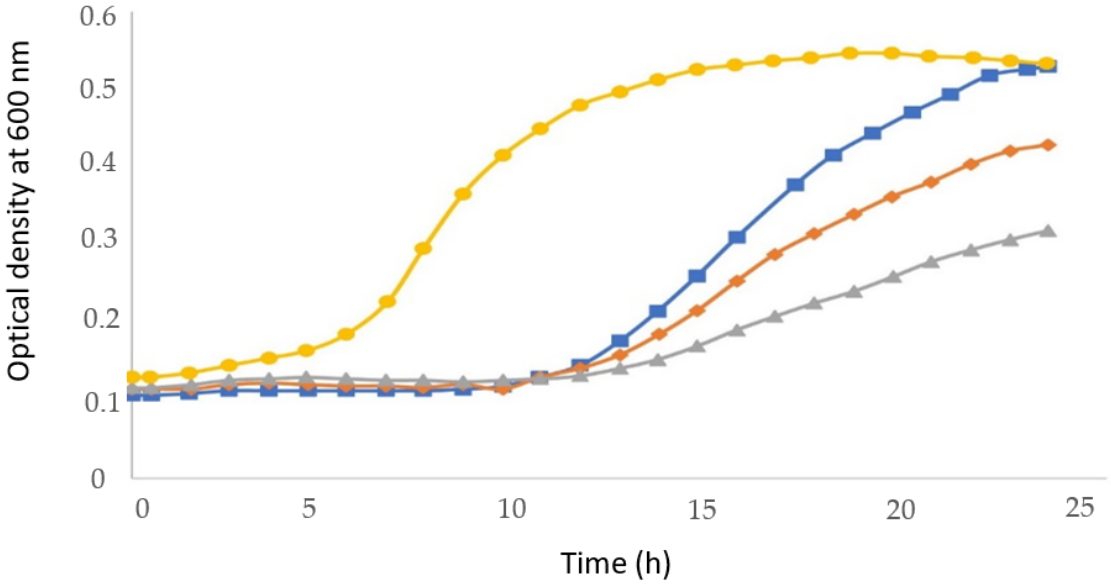

Figure 2. *Streptococcus mutans* UA159 growth curves in the absence (●) or in the presence of cell-free supernatant (CFS) from *Bifidobacterium lactis* BL 04 culture. CFS = CFS as such (▲), CFS1 = 1:1/2 (*v*/*v*) diluted CFS (♦), CFS2 = 1:1 (*v*/*v*) diluted CFS (■).

These results corroborate with the observations of a study in which the administration of *B. animalis* subs. *lactis* BB-12 and *L. rhamnosus* GG allowed to control *S. mutans* development, thus reducing oral plaque and gingival inflammation in healthy young adults [17]. Similarly, Bhalla et al. [31] observed a significant reduction in *S. mutans* counts in saliva after 1 h and 7 days of *B. animalis* subsp. *lactis* BB-12 administration, confirming the anticariogenic power of this LAB.

As Figure 3 shows, the addition of CFS from *L. plantarum* ST16 PA culture, either as such or diluted, had an even more pronounced effect on *S. mutans* growth. In particular, while the most diluted CFS (CFS2) delayed in just a few hours the onset of the exponential phase compared to the run without BLIS, the least diluted one (CFS1) caused a delay as long as 17 h, and the undiluted one completely suppressed growth.

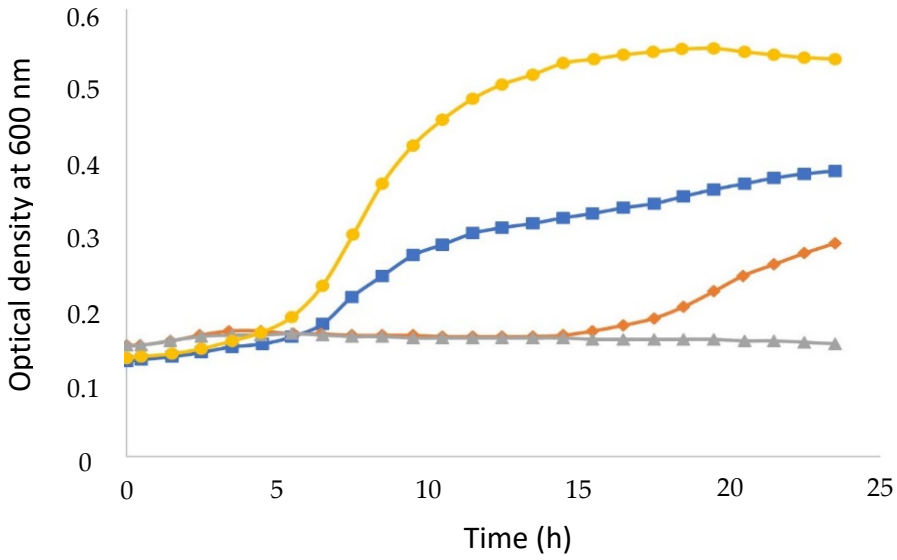

Figure 3. *Streptococcus mutans* UA159 growth curves in the absence (●) or in the presence of cell-free supernatant (CFS) from *Lactobacillus plantarum* ST16 Pa culture. CFS = CFS as such (▲), CFS1 = 1:1/2 (*v*/*v*) diluted CFS (♦), CFS2 = 1:1 (*v*/*v*) diluted CFS (■).

As a result, the reduction of $OD_{600\ nm}$ at the end of cultures (i.e., of the final concentration of *L. plantarum* ST16 Pa cells) was directly proportional to BLIS content in CFS. Similar results were reported against different species belonging to the genera *Enterobacter*, *Enterococcus*, *Lactobacillus*, *Pseudomonas*, *Staphylococcus*, and *Streptococcus* [32].

Figures 4 and 5 illustrate the effects on the growth of the same pathogen of CFSs from *L. lactis* CECT-4434 and *L. lactis* 27 cultures, respectively. As expected by the fact that the two strains belong to the same species, the two antimicrobials showed similarity in their peculiar inhibition profile, which was characterized by no significant delay in the onset of the exponential phase, reduction in $OD_{600\ nm}$ at the end of cultures proportional to BLIS content in CFS, and total growth suppression in the presence of undiluted CFSs.

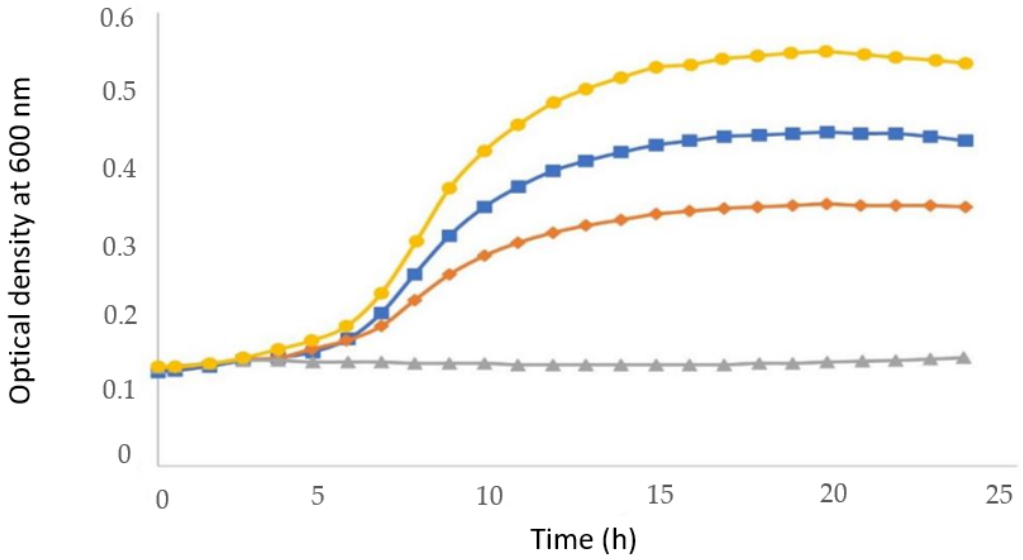

Figure 4. *Streptococcus mutans* UA159 growth curves in the absence (●) or in the presence of cell-free supernatant (CFS) from *Lactococcus lactis* CECT-4434 culture. CFS = CFS as such (▲), CFS1 = 1:1/2 (*v*/*v*) diluted CFS (♦), CFS2 = 1:1 (*v*/*v*) diluted CFS (■).

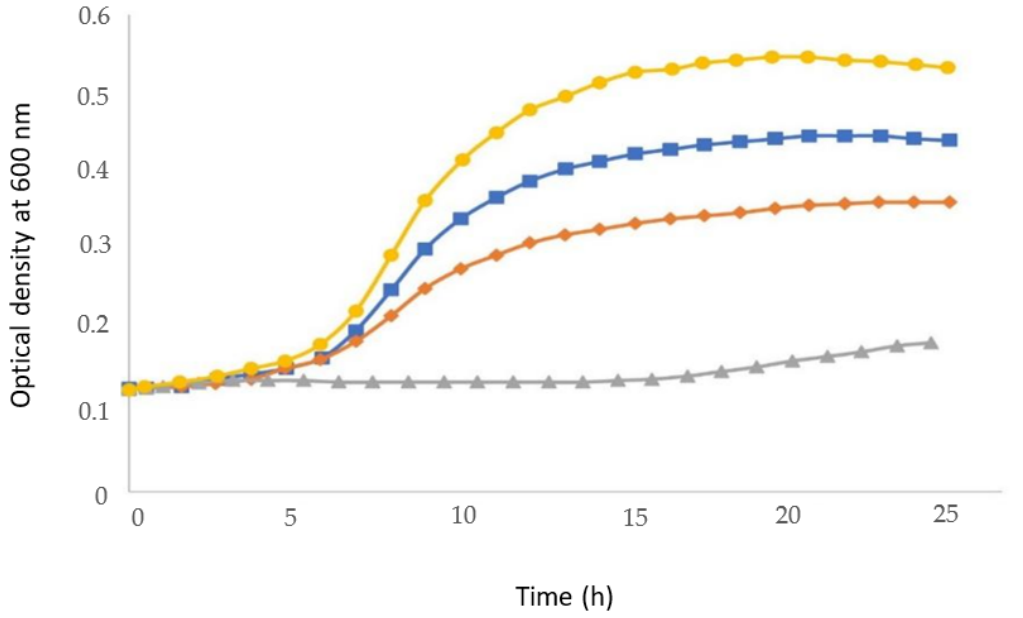

Figure 5. *Streptococcus mutans* UA159 growth curves in the absence (●) or in the presence of cell-free supernatant (CFS) from *Lactobacillus lactis* 27 culture. CFS = CFS as such (▲), CFS1 = 1:1/2 (*v*/*v*) diluted CFS (♦), CFS2 = 1:1 (*v*/*v*) diluted CFS (■).

In the study by Sudhir et al. [33], *L. acidophilus*, when administered in children for 30 days using curd as a vehicle, promoted significant reduction in *S. mutans* counts in saliva. The joint administration of *L. acidophilus* LA5 and *B. lactis* BB12 also significantly reduced the growth of *S. mutans* by 7 and 30 days after the ingestion of these probiotics by children [34]. However, after 6 months of discontinuation of probiotic administration, *S. mutans* counts returned to the level observed at the beginning of the study, which means that there was no effective colonization of the oral cavity by these probiotics and that they should be constantly administered.

Comparing the results of this section, one can infer that the almost complete suppression of *S. mutans* UA159 growth evident in Figures 2–4 may have been the result of a bactericidal effect of BLIS produced by *L. plantarum* ST16 Pa, *L. lactis* CECT-4434, and *L. lactis* 27 cultures, unlike the bacteriostatic one evidenced in Figure 1 for *B. lactis* BL 04 BLIS. In addition, the absence of any delay of the exponential phase of the pathogen growth in Figures 3 and 4 suggests that the bactericidal action of *L. lactis* CECT-4434 and *L. lactis* 27 BLIS may have taken longer than those of BLIS produced by *L. plantarum* ST16 Pa and *B. lactis* BL 04 BLIS. However, any attempts to relate these different inhibition profiles with possible action mechanisms would require additional research effort.

3.4. Preliminary Characterization of BLIS

To investigate the chemical nature of BLIS, each CFS that exhibited antimicrobial activity in previous tests was treated separately with 1.0 mg/mL papain, pepsin and trypsin, and its antimicrobial activity was again tested against *L. innocua* 2711. The results showed that all enzyme treatments resulted in loss of antimicrobial activity, indicating the protein nature of all BLIS and suggesting that they could be class II bacteriocins [32].

To evaluate the stability of the protein compounds responsible for antimicrobial activity, the CFSs of *B. lactis* BL 04 and *L. lactis* 27 underwent treatments with different organic solvents (acetonitrile and isopropanol), salts (sodium chloride and ammonium sulfate), and detergents (Triton 100-X, EDTA, Tween 20, Tween 20 and SDS) and tested for their inhibitory effect against *L. innocua* 2711 (Table 3).

Table 3. Results of stability tests carried out on *Bifidobacterium lactis* BL 04 and *Lactobacillus lactis* 27 cell-free supernatants (CFSs) after treatment in different organic solvents, salts and detergents. Values refer to the post-treatment ability of CFSs to inhibit the growth of the pathogenic strain *Listeria innocua* 2711 expressed as diameter of inhibition halos (mm).

Treatment Agent	*Bifidobacterium lactis* BL 04	*Lactobacillus lactis* 27
Acetonitrile	9.57 ± 0.09	9.41 ± 0.09
Isopropanol	11.29 ± 0.14	9.27 ± 0.08
Sodium chloride	11.45 ± 0.14	15.35 ± 0.13
Ammonium sulfate	7.81 ± 0.08	14.35 ± 0.12
Triton 100-X	15.07 ± 0.13	14.12 ± 0.12
EDTA	11.65 ± 0.11	15.92 ± 0.14
Tween 20	11.18 ± 0.10	13.54 ± 0.12
Tween 80	9.83 ± 0.09	12.35 ± 0.11
SDS	10.80 ± 0.10	8.23 ± 0.08
Water (control)	10.04 ± 0.10	8.85 ± 0.09

The inhibition halos generated by *B. lactis* BL 04 and *L. lactis* 27 CFSs after solvent treatment showed diameters (9.57–11.29 and 9.27–9.41 mm, respectively) close to those obtained using water as a control (10.04 and 8.85 mm, respectively), demonstrating that solvents did not influence the inhibitory effect of their respective BLIS. On the other hand, the *B. lactis* BL 04 BLIS suffered a small reduction in its antimicrobial effect after ammonium sulfate treatment (7.81 mm), while this same salt enhanced the effect of the *L. lactis* 27 one (14.35 mm). These results suggest that, despite the small decrease in antimicrobial activity observed in the former case, salting-out with ammonium sulfate could generally be a

viable methodology as a first step for BLIS concentration and partial purification without significant losses of their activities.

Pathogenic bacteria such as *E. coli* and members of the *Listeria* genus are known to be important vehicles of diseases transmitted by contaminated foods [35]. The CFS antimicrobial activity observed in this study corroborates the results of a recent study in which the *L. lactis* GH1 CFS proved to be able to inhibit the growth of *L. monocytogenes* ATCC 15313 (1.55 ± 0.14 mm) [36] as the likely result of the production of organic acids, with consequent pH reduction, hydrogen peroxide, bacteriocins, or BLIS [37]. The stability of *L. lactis* 27 BLIS in organic solvents observed in this study is in line with the complete retention of *L. lactis* strains' antimicrobial activity reported in mixture with 10% (*v*/*v*) acetone, benzene, chloroform, dimethylsulfoxide, ethanol, ethyl acetate, and methanol [38]. On the other hand, the small loss of antimicrobial activity of *B. lactis* BL 04 CFS caused by the ammonium sulfate addition can be attributed to some change in protein three-dimensional structure of BLIS, which commonly leads to a loss of functionality and the formation of potentially immunogenic aggregates.

3.5. BLIS Production in Co-Cultures

The results of monocultures showed that *B. lactis* BL 04 is the LAB, among those considered in this study, with the broadest spectrum of action against the tested bioindicator strains, including *L. innocua* 2711. Therefore, it was grown in co-culture with *S. thermophilus* TA040, which previously displayed synergism in co-culture with *L. rhamnosus* by significantly reducing the acidification time [39]. The results of the antimicrobial activity of the CFS of this co-culture against *L. innocua* 271 are gathered in Table 4 and compared to those of the respective monocultures.

Table 4. Antimicrobial activity (AU/mL) against *Listeria innocua* 2711 of the cell-free supernatants from monocultures and co-culture of *Bifidobacterium lactis* BL 04 and *Streptococcus thermophilus* TA040.

Culture	Antimicrobial Activity (AU/mL)
Bifidobacterium lactis BL 04	6400
Streptococcus thermophilus TA040	1600
Bifidobacterium lactis BL 04 + *Streptococcus thermophilus* TA040	12,800

The CFS from *S. thermophilus* TA040 monoculture showed low antimicrobial activity when using MRS broth supplemented with 1.0% (*w*/*v*) L-cysteine and 0.04% (*w*/*v*) sodium thioglycolate (1600 AU/mL), and even null antimicrobial activity when using the M17 medium, thus corroborating the absence of reports in the literature on antimicrobial activity of this strain. On the contrary, the antimicrobial activity of CFS from *B. lactis* BL 04 monoculture was remarkable (6400 AU/mL) and coincided with the one determined using the critical dilution technique. When this microorganism was cultivated in co-culture with *S. thermophilus* TA040, the CFS antimicrobial activity doubled (12,800 AU/mL) compared to its monoculture, proving the previously supposed synergism [39].

It is known that the acidification ability of probiotic strains is one of the most significant technological characteristics of starter cultures, particularly in the manufacture of milk-based products, because the pH reduction inhibits the growth of a large number of pathogenic microorganisms and/or microorganisms responsible for deterioration of fermented products, thus improving their storage properties [36].

As illustrated in Figure 6, the *B. lactis* BL 04 and *S. thermophilus* TA040 co-culture shortened the acidification time (i.e., the time to lower the pH from 5.8 to 4.3) by approximately 8 h compared to the *B. lactis* BL 04 monoculture (24 h), while *S. thermophilus* TA040 alone could not achieve this goal even at the end of cultivation.

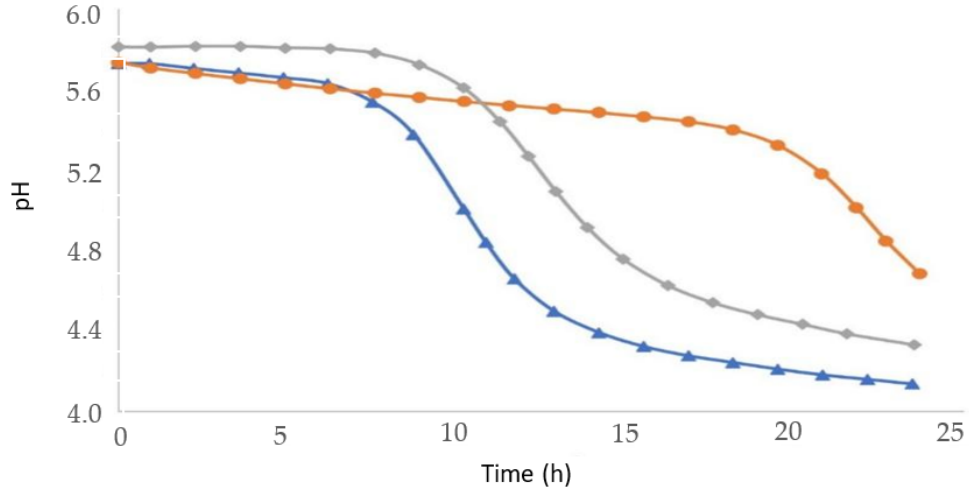

Figure 6. Acidification profiles of monocultures of *Streptococcus thermophilus* TA040 (●) and *Bifidobacterium lactis* BL 04 UA159 (♦) as well as of their co-culture (▲).

3.6. BLIS Concentration and Partial Purification by Salting out with Ammonium Sulfate

Table 5 shows the results of the antimicrobial activity of the CFSs from *L. lactis* 27 and *B. lactis* BL 04 monocultures after BLIS concentration with ammonium sulfate at different concentrations (10, 20, 30, 40, 50, 60% *w*/*v*).

Table 5. Antimicrobial activity (AU/mL) against *Listeria innocua* 2711 of the cell-free supernatants from *Bifidobacterium lactis* BL 04 and *Lactobacillus lactis* 27 monocultures after precipitation with ammonium sulfate at different concentrations (% *w*/*v*).

Ammonium Sulfate Concentration (% *w*/*v*)	***Bifidobacterium lactis* BL 04**	***Lactobacillus lactis* 27**
10	3200	800
20	6400	3200
30	12,800	3200
40	25,600	3200
50	51,200	6400
60	102,400	6400

In both cases the strongest antimicrobial activity was obtained using the largest dosage of ammonium sulfate (60%, *w*/*v*), but that of *B. lactis* BL 04 BLIS (102,400 AU/mL) was nothing less than 15 times higher than that of *L. lactis* 27 BLIS. These results suggest that the BLIS produced by this bifidobacterium was insensitive to possible changes induced by the salting out process in its protein three-dimensional structure and may be successfully exploited to hinder the development of pathogens.

4. Conclusions

The results of this study showed that, among the selected lactic acid bacteria (LABs), *Lactobacillus plantarum* ST16 Pa, *Bifidobacterium lactis* BL 04, *Lactococcus lactis* CECT-4434, and *Lactobacillus lactis* 27 were able to produce bacteriocin-like substances (BLIS), unlike *Pediococcus pentosaceus* ATCC 33316 and *Lactobacillus sakei* 2a. Among them, *B. lactis* BL 04 was the LAB with the largest spectrum of antimicrobial activity, being able to inhibit the growth of *Listeria innocua* 2711, *Carnobacterium maltaromaticum* CECT 4020, *Staphylococcus aureus* CECT 239, and *Escherichia coli* ATCC 25922. On the other hand, *L. plantarum* ST16 Pa and *L. lactis* CECT-4434 showed the greatest antimicrobial effect against the cariogenic pathogen *Streptococcus mutans* UA159. *B. lactis* BL 04 antimicrobial activity was two times higher than those of the other LABs, and the acidification time was significantly reduced

when this bacterium was cultivated in co-culture with *Streptococcus thermophilus* TA040, which suggests a possible synergistic effect between these two microorganisms.

The antimicrobial activities of *L. lactis* 27 and *B. lactis* BL 04 BLIS after ammonium sulfate precipitation increased proportionally to the concentration of this salt up to 60% *w*/*v*. However, the activity of latter (102,400 AU/mL) was 15 times higher than the one of former, suggesting that the *B. lactis* BL 04 BLIS may have been insensitive to possible three-dimensional changes induced by the salting-out process. The results of this study also indicate the potential application of *B. lactis* BL 04 BLIS, produced either in monoculture or in co-culture with *S. thermophilus* TA040, to prevent the development of pathogens.

Author Contributions: Conceptualization, A.R.S.d.S. and R.P.d.S.O.; methodology, A.R.S.d.S. and P.O.d.S.d.A.; validation, P.O.d.S.d.A., R.P.d.S.O. and A.C.; formal analysis, P.O.d.S.d.A., R.P.d.S.O. and A.C.; investigation, A.R.S.d.S.; resources, R.P.d.S.O.; data curation, P.O.d.S.d.A. and A.C.; writing—original draft preparation, A.R.S.d.S.; writing—review and editing, P.O.d.S.d.A., R.P.d.S.O. and A.C.; visualization, P.O.d.S.d.A. and A.C.; supervision, R.P.d.S.O.; project administration, R.P.d.S.O.; funding acquisition, R.P.d.S.O. All authors have read and agreed to the published version of the manuscript.

Funding: The authors are grateful for the financial support of São Paulo Research Foundation (FAPESP) [grant 2018/25511-1] and the National Council for Scientific and Technological Development (CNPq) [grants 870258/1997-4; 312923/2020-1; 408783/2021-4].

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

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

References

1. Belda-Ferre, P.; Alcaraz, L.D.; Cabrera-Rubio, R.; Romero, H.; Simón-Soro, A.; Pignatelli, M.; Mira, A. The oral metagenome in health and disease. *ISME J.* **2012**, *6*, 46–56. [CrossRef] [PubMed]
2. Marsh, P.D.; Zaura, E. Dental biofilm: Ecological interactions in health and disease. *J. Clin. Periodontol.* **2017**, *44*, S12–S22. [CrossRef] [PubMed]
3. GBD 2017 Disease and Injury Incidence and Prevalence Collaborators. Global, regional, and national incidence, prevalence, and years lived with disability for 354 diseases and injuries for 195 countries and territories, 1990–2017: A systematic analysis for the Global Burden of Disease Study 2017. *Lancet* **2018**, *392*, 1789–1858. [CrossRef]
4. Gálvez, A.; Abriouel, H.; López, R.L.; Ben Omar, N. Bacteriocin-based strategies for food biopreservation. *Int. J. Food Microbiol.* **2007**, *120*, 51–70. [CrossRef] [PubMed]
5. Lata, S.; Varghese, N.O.; Varughese, J.M. Remineralization potential of fluoride and amorphous calcium phosphate-casein phospho peptide on enamel lesions: An in vitro comparative evaluation. *J. Conserv. Dent.* **2010**, *13*, 42–46. [CrossRef]
6. Rubaba, M.; Muhamma, H.; Amin, N.S.; Deog-Hwan, O. Biosensors for rapid and sensitive detection of *Staphylococcus aureus* in food. *Biosens. Bioelectron.* **2018**, *105*, 49–57. [CrossRef]
7. Mealey, B.L. Influence of periodontal infections on systemic health. *Periodontol. 2000* **1999**, *21*, 197–209. [CrossRef]
8. Willis, J.R.; Gabaldón, T. The human oral microbiome in health and disease: From sequences to ecosystems. *Microorganisms* **2020**, *8*, 308. [CrossRef]
9. Haque, M.; Sartelli, M.; Haque, S.Z. Dental infection and resistance-global health consequences. *Dent. J.* **2019**, *7*, 22. [CrossRef]
10. Food and Agriculture Organization (FAO) of the United Nations Food Safety and Quality: Probiotics. Available online: http://www.fao.org/food/food-safety-quality/a-z-index/probiotics/en/ (accessed on 24 July 2022).
11. Sabatini, S.; Lauritano, D.; Candotto, V.; Silvestre, F.J.; Nardi, G.M. Oral probiotics in the management of gingivitis in diabetic patients: A double blinded randomized controlled study. *J. Biol. Regul. Homeost. Agents.* **2017**, *31*, 197–202.
12. Tekce, M.; Ince, G.; Gursoy, H.; Dirikan Ipci, S.; Cakar, G.; Kadir, T.; Yilmaz, S. Clinical and microbiological effects of probiotic lozenges in the treatment of chronic periodontitis: A 1-year follow-up study. *J. Clin. Periodontol.* **2015**, *42*, 363–372. [CrossRef] [PubMed]
13. Keller, M.K.; Brandsborg, E.; Holmstrom, K.; Twetman, S. Effect of tablets containing probiotic candidate strains on gingival inflammation and composition of the salivary microbiome: A randomised controlled trial. *Benef. Microbes.* **2018**, *9*, 487–494. [CrossRef] [PubMed]

14. Nadkerny, P.V.; Ravishankar, P.L.; Pramod, V.; Agarwal, L.A.; Bhandari, S. A comparative evaluation of the efficacy of probiotic and chlorhexidine mouthrinses on clinical inflammatory parameters of gingivitis: A randomized controlled clinical study. *J. Indian Soc. Periodontol.* **2015**, *19*, 633–639. [CrossRef] [PubMed]
15. Grusovin, M.G.; Bossini, S.; Calza, S.; Cappa, V.; Garzetti, G.; Scotti, E.; Gherlone, E.F.; Mensi, M. Clinical efficacy of *Lactobacillus reuteri*-containing lozenges in the supportive therapy of generalized periodontitis stage III and IV, grade C: 1-year results of a double-blind randomized placebo-controlled pilot study. *Clin. Oral Investig.* **2020**, *24*, 2015–2024. [CrossRef] [PubMed]
16. Oliveira, L.F.; Salvador, S.L.; Silva, P.H.; Furlaneto, F.A.; Figueiredo, L.; Casarin, R.; Ervolino, E.; Palioto, D.B.; Souza, S.L.S.; Taba, M., Jr.; et al. Benefits of *Bifidobacterium animalis* subsp. *lactis* probiotic in experimental periodontitis. *J. Periodontol.* **2017**, *88*, 197–208. [CrossRef]
17. Toiviainen, A.; Jalasvuori, H.; Lahti, E.; Gursoy, U.; Salminen, S.; Fontana, M.; Flannagan, S.; Eckert, G.; Kakaras, A.; Paster, B.; et al. Impact of orally administered lozenges with *Lactobacillus rhamnosus* GG and *Bifidobacterium animalis* subsp. *lactis* BB-12 on the number of salivary mutans streptococci, amount of plaque, gingival inflammation and the oral microbiome in healthy adults. *Clin. Oral Investig.* **2015**, *19*, 77–83. [CrossRef]
18. Invernici, M.M.; Salvador, S.L.; Silva, P.H.F.; Soares, M.S.M.; Casarin, R.; Palioto, D.B.; Souza, S.L.S.; Taba, M., Jr.; Navaes, A.B., Jr.; Furlaneto, F.A.C.; et al. Effects of *Bifidobacterium* probiotic on the treatment of chronic periodontitis: A randomized clinical trial. *J. Clin. Periodontol.* **2018**, *45*, 1198–1210. [CrossRef]
19. Sabo, S.S.; Vitolo, M.; Domínguez González, J.M.; Oliveira, R.P.S. Overview of *Lactobacillus plantarum* as a promising bacteriocins producer among lactic acid bacteria. *Food Res. Int.* **2014**, *64*, 527–536. [CrossRef]
20. Balciunas, E.M.; Al Arni, S.; Converti, A.; Leblanc, J.G.; Oliveira, R.P.S. Production of bacteriocin-like inhibitory substances (BLIS) by *Bifidobacterium lactis* using whey as a substrate. *Int. J. Dairy Technol.* **2016**, *69*, 236–242. [CrossRef]
21. Porto, M.C.W.; Kuniyoshi, T.M.; Azevedo, P.O.S.; Vitolo, M.; Oliveira, R.P.S. *Pediococcus* spp.: An important genus of lactic acid bacteria and pediocin producers. *Biotechnol. Adv.* **2017**, *35*, 365–374. [CrossRef] [PubMed]
22. Seminario-Amez, M.; Lopez-Lopez, J.; Estrugo-Devesa, A.; Ayuso-Montero, R.; Jane-Salas, E. Probiotics and oral health: A systematic review. *Med. Oral Patol. Oral Cir. Bucal.* **2017**, *22*, e282–e288. [CrossRef] [PubMed]
23. Sivamaruthi, B.S.; Kesika, P.; Chaiyasut, C. A review of the role of probiotic supplementation in dental caries. *Probiotics Antimicrob. Proteins* **2020**, *12*, 1300–1309. [CrossRef] [PubMed]
24. Veiga, P.; Suez, J.; Derrien, M.; Elinav, E. Moving from probiotics to precision probiotics. *Nat. Microbiol.* **2020**, *5*, 878–880. [CrossRef]
25. Piazentin, A.C.M.; Mendonça, C.M.N.; Vallejo, M.; Mussato, S.I.; Oliveira, R.P.S. Bacteriocin-like inhibitory substances production by *Enterococcus faecium* 135 in co-culture with *Ligilactobacillus salivarius* and *Limosilactobacillus reuteri*. *Braz. J. Microbiol.* **2022**, *53*, 131–141. [CrossRef] [PubMed]
26. Pereira, W.A.; Piazentin, A.C.M.; Oliveira, R.C.; Mendonça, C.M.N.; Tabata, Y.A.; Mendes, M.A.; Fock, R.A.; Makiyama, E.N.; Corrêa, B.; Vallejo, M.; et al. Bacteriocinogenic probiotic bacteria isolated from an aquatic environment inhibit the growth of food and fish pathogens. *Sci. Rep.* **2022**, *12*, 5530. [CrossRef] [PubMed]
27. Todorov, S.D.; Dicks, L.M.T. Screening for bacteriocin-producing lactic acid bacteria from boza, a traditional cereal beverage from Bulgaria. *Process. Biochem.* **2006**, *41*, 11–19. [CrossRef]
28. Martinez, F.A.C.; Domínguez, J.M.; Converti, A.; Oliveira, R.P.S. Production of bacteriocin-like inhibitory substance by *Bifidobacterium lactis* in skim milk supplemented with additives. *J. Dairy Res.* **2015**, *82*, 350–355. [CrossRef]
29. Carvalho, K.G.; Bambirra, F.H.S.; Nicoli, J.R.; Oliveira, J.S.; Santos, A.M.C.; Bemquerer, M.P.; Miranda, A.; Franco, B.D.G.M. Characterization of multiple antilisterial peptides produced by sakacin P-producing *Lactobacillus sakei* subsp. *sakei* 2a. *Arch. Microbiol.* **2018**, *200*, 635–644. [CrossRef]
30. Maria, P.; Sofia, A. Pediocins: The bacteriocins of pediococci. Sources, production, properties and applications. *Microb. Cell Fact.* **2009**, *8*, 3. [CrossRef]
31. Bhalla, M.; Ingle, N.A.; Kaur, N.; Yadav, P. Mutans streptococci estimation in saliva before and after consumption of probiotic curd among school children. *J. Int. Soc. Prev. Community Dent.* **2015**, *5*, 31–34. [CrossRef]
32. Todorov, S.D.; Prévost, H.; Lebois, M.; Dousset, X.; LeBlanc, J.G.; Franco, B.D.G.M. Bacteriocinogenic *Lactobacillus plantarum* ST16Pa isolated from papaya (*Carica papaya*)-From isolation to application: Characterization of a bacteriocin. *Food Res. Int.* **2011**, *44*, 1351–1363. [CrossRef]
33. Sudhir, R.; Praveen, P.; Anantharaj, A.; Venkataraghavan, K. Assessment of the effect of probiotic curd consumption on salivary pH and *Streptococcus mutans* counts. *Niger. Med. J.* **2012**, *53*, 135–139. [CrossRef]
34. Ashwin, D.; Ke, V.; Taranath, M.; Ramagoni, N.K.; Nara, A.; Sarpangala, M. Effect of probiotic containing ice-cream on salivary mutans streptococci (SMS) levels in children of 6–12 years of age: A randomized controlled double blind study with six-months follow up. *J. Clin. Diagn. Res.* **2015**, *9*, ZC06–ZC09. [CrossRef] [PubMed]
35. Alegbeleye, O.O.; Singleton, I.; Sant'Ana, A.S. Sources and contamination routes of microbial pathogens to fresh produce during field cultivation: A review. *Food Microbiol.* **2018**, *73*, 177–208. [CrossRef] [PubMed]
36. Jawan, R.; Abbasiliasi, S.; Mustafa, S.; Kapri, M.R.; Halim, M.; Ariff, A.B. In vitro evaluation of potential probiotic strain *Lactococcus lactis* Gh1 and its bacteriocin-like inhibitory substances for potential use in the food industry. *Probiotics Antimicrob. Proteins* **2021**, *13*, 422–440. [CrossRef]

37. Nishant, T.; Sathish Kumar, D.; Arun Kumar, R.; Hima Bindu, K.; Raviteja, Y. Bacteriocin producing probiotic lactic acid bacteria. *J. Microb. Biochem. Technol.* **2011**, *3*, 121–124. [CrossRef]
38. Barman, S.; Ghosh, R.; Mandal, D.C. Production optimization of broad spectrum bacteriocin of three strains of *Lactococcus lactis* isolated from homemade buttermilk. *Ann. Agrar. Sci.* **2018**, *16*, 286–296. [CrossRef]
39. Oliveira, R.P.S.; Perego, P.; Nogueira, M.; Oliveira, D.; Converti, A. Effect of inulin on the growth and metabolism of a probiotic strain of *Lactobacillus rhamnosus* in co-culture with *Streptococcus thermophilus*. *LWT-Food Sci. Technol.* **2012**, *47*, 358–363. [CrossRef]