Pathogenic Biofilm Removal Potential of Wild-Type Lacticaseibacillus Rhamnosus Strains

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Article

Pathogenic Biofilm Removal Potential of Wild-Type *Lacticaseibacillus rhamnosus* Strains

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Abstract: The emergence of antimicrobial resistance remains one of the greatest public health concerns. Biofilm formation has been postulated as a mechanism of microbial pathogens to resist antimicrobial agents. Lactic Acid Bacteria (LAB) and their metabolites have been proposed to combat bacterial biofilms, due to their antimicrobial activity. In this vein, the aim of the present study was to investigate the biofilm removal potential of cell-free supernatant (CFS) of five wild-type *Lacticaseibacillus rhamnosus* LAB strains, isolated from Greek natural products, in comparison to the commercial available *L. rhamnosus* GG strain, formed by common foodborne pathogens (*Salmonella* Enteritidis, *Salmonella* Typhimurium, *Escherichia coli*, *Listeria monocytogenes*, and *Staphylococcus aureus*). Biofilm removal activity of LAB was assessed on two-days mature biofilm using a microtiter plate-based procedure. Both non-neutralized and neutralized CFSs removed biofilms in a concentration-dependent manner. Biofilm removal activity of the non-neutralized CFSs was significantly higher compared to the neutralized CFSs, as expected, ranging 40 – 90% and 10 – 78%, respectively. Biofilm removal efficiency of *L. rhamnosus* OLXAL-3 was signifanctly higher among the wild-type *L. rhamnosus* strains tested (20 – 100% *v/v*). In conclusion, our results suggested the great potential of application of wild-type *Lacticaseibacillus rhamnosus* strains CFSs as effective natural agents against pathogenic bacterial biofilms.

Keywords: biofilm removal; *L. rhamonsus*; cell free supernatant; foodborne pathogen

1. Introduction

The rising anti-microbial resistance of pathogens consists a worldwide risk to human health and thus efficient antimicrobial alternatives are required urgently[1]. Bacterial biofilms, formed by the polymeric metabolites secreted by microbes, are one of the main resistance mechanisms that bacteria utilize to survive against various stresses, including antibiotics, disinfectants, and host defenses. The use of Lactic Acid Bacteria (LAB) to combat bacterial biofilms is a rapidly growing trend. Among others, it is documented that LAB produce several bioactive molecules, such as organic acids, alcohols, carbon dioxide, diacetyl, hydrogen peroxide and bacteriocins [2,3], many of which exert powerful antimicrobial activity [2,4]. Generally, most of these compounds are secreted during cultivation in a broth medium following the proliferation of bacteria cells, known as a supernatant. Hence, it has been observed that the LAB culture supernatant acts efficiently against bacterial biofilms [5–8]. Thus, probiotics and the antimicrobial agents produced by probiotics have recently been proposed as potential candidates for controlling bacterial biofilm formation against foodborne pathogens [9–11].

Bacterial biofilm formation is a consequence of the accumulation and non-reversible attachment of bacterial cells on a biological or non-biological surface, as well as of a body of extracellular polymers (Extracellular Polymeric Substance – ESP) or glycocalyx that is secreted by the same microorganisms [12,13].

In the food industry, microbial biofilms have been found in dairy products, fish and poultry, as well as in the production lines, e.g. of ready-to-eat manufacturing plants [14]. Members of the species *Salmonella* have been reported to form biofilms on a variety of surfaces and equipment, while *Bacillus*

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cereus, *Escherichia coli*, *Shigella* sp. and *Staphylococcus aureus* have been isolated during the production process of dairy products [15]. Furthermore, biofilm formation by *E. coli* was observed and isolated from surfaces during production of cattle derived meat products [14]. Frequently observed species, related to biofilm formation, are staphylococci, *Enterobacteriaceae* and the foodborne pathogen *Listeria monocytogenes*. Therefore, addressing and controlling the formation of microbial biofilms from foodborne pathogens is a challenge for the food industry, where the need to produce safe products is of paramount importance.

Hence, the aim of the present study was to assess biofilm removal potential of cell-free supernatant (CFS) of 5 LAB strains belonging to *Lacticaseibacillus rhamnosus* species, isolated from traditional Greek foods, formed by common foodborne pathogens.

2. Materials and Methods

2.1. Microbial strains

Five wild-type lactic acid bacteria, whose techonological and functional properties have been described by a previous study [16], isolated from Greek natural products, were used as shown in Table 1. All strains were grown in de Man, Rogosa and Sharpe broth (MRS, Condalab, Spain) at 37°C for 24 h. The commercial *Lacticaseibacillus rhamnosus* GG served as a reference strain, since its activity against pathogenic biofilm formation is reported in literature [17].

Table 1. Wild-type Lactic Acid Bacteria (LAB) used in the present study.

Isolate code	Bacterial Species	Source of isolation
GG (ATCC 53103)	*Lacticaseibacillus rhamnosus*	Human intestines
OLXAL-1	*Lacticaseibacillus rhamnosus*	Olive (fruit)
OLXAL-2	*Lacticaseibacillus rhamnosus*	Olive (fruit)
OLXAL-3	*Lacticaseibacillus rhamnosus*	Olive (fruit)
OLXAL-4	*Lacticaseibacillus rhamnosus*	Olive (fruit)
CHTH-2	*Lacticaseibacillus rhamnosus*	Feta-type cheese

Salmonella enterica subsp. *enterica* ser. Enteritidis FMCC B56 PT4 (kindly provided by Prof. Nychas G.J.E., Agricultural University of Athens, Athens, Greece), *Salmonella enterica* subsp. *enterica* ser. Typhimurium DSMZ 554, *Escherichia coli* ATCC 25922, *Listeria monocytogenes* NCTC 10527 serotype 4b and *Staphylococcus aureus* ATCC 25923 were grown in Brain Heart Infusion (BHI) broth (Condalab, Spain) at 37°C for 24 h.

2.2. Preparation of LAB CFS (Cell Free Supernatants)

An overnight bacterial culture of each isolated wild-type LAB strain (10^9 cfu/mL) was centrifuged at 8500 rpm, 4°C for 15 minutes and the supernatant was collected. In order to study the neutralized CFS (pH 7) against bacterial biofilm removal, part of the collected CFS was neutralized using 5M NaOH solution using a pH-meter (WTW Ph 330i, WTW, Germany). All CFS were then sterilized through filtration (0.22μm, Merck, Darmstadt, Germany) (cell free supernatant-CFS).

2.3. Biofilm removal activity

Biofilm removal activity was determined using 24-well polystyrene microtiter plates, according to Koohestani et al. [18] with some modifications. In each well 1800μL Brain Heart Infusion broth and 200μL bacterial suspension after two ten-fold dilutions to obtain a final concentration of 10^6 logcfu/mL were poured. For the establishment of the bacterial biofilms, the microplates were incubated at 37°C for 48h. After incubation, the free (planktonic) cells were discarded and the wells were washed with ¼ Ringer's (2.25 g/L NaCl, 0.105 g/L KCl, 0.12 g/L $CaCl_2H_{12}O_6$ and 0.05 g/L $NaHCO_3$) sterilized solution to remove any weakly-attached cells. In the next step, CFS (neutralized and non-neutralized) of all LAB strains were gently added to the wells at concentration 100%, 80%,

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60%, 40% or 20% and left undisturbed for 1 h at ambient temperature. Then, the CFS were vigorously decanted and the wells were washed again with ¼ Ringer's sterilized solution. The remaining attached bacterial biofilms were stabilized by the addition of 2000μL of methanol solution and allowed to dry for 5 min. Cell staining was performed using 1% w/v crystal violet (Sigma-Aldrich, St. Louis, USA) for 30 min followed by a final wash with distilled H_2O, in order to remove any excess of the staining solution. The remaining crystal violet at the bottom of the wells was diluted in ethanol/acetone solution (Chem-Lab NV, Belgium) at a ratio of 80/20 for 15 min. The solutions were then transferred into clear bottom 96-well microplates to determine the optical absorbance at 540nm. Wells containing only BHI broth and bacterial suspension without CFSs were used as negative and positive controls, respectively.

The estimation of the reduction percentage of biofilms exposed to the different CFSs was calculated according to following equation:

$$\text{Reduction percentage} = \frac{(C-B)-(\mathrm{T}-\mathrm{B})}{(\mathrm{C}-\mathrm{B})} \text{ x } 100, \quad (1)$$

where, C are the OD values of negative control wells, B are the OD values of positive control wells and T are the OD values of CFS treated wells at 540nm.

2.4. Statistical analysis

All experiments were performed at least in quadruple and the results were analyzed for statistical significance with analysis of variance (ANOVA). Duncan's test was used to determine significant differences [Coefficients, ANOVA tables, and significance ($p < 0.05$) were computed using Statistica for Windows, v.12.5 (StatSoft, Tulsa, USA)].

3. Results and Discussion

The effect of neutralized and non-neutralized CFSs resulted in significant high removal percentages of biofilms formed by the bacterial species *S.* Enteritidis, *S.* Typhimurium, *E. coli*, *L. monocytogenes* and *S. aureus* (Figures 1 and 2). The removal percentage decreased with decreasing CFSs concentrations (from 100 to 20%). The non-neutralized CFSs were significantly more effective at removing the bacterial biofilms ($p < 0.05$), than the neutralized CFSs.

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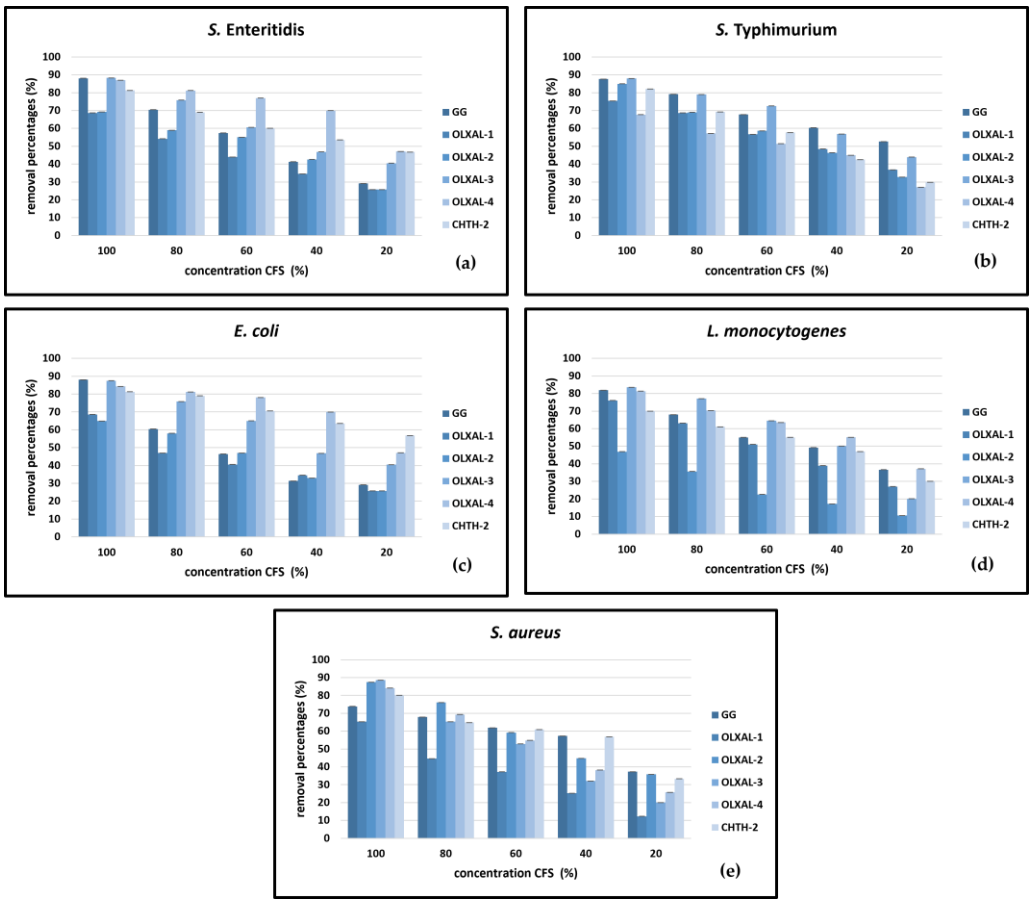

Figure 1. Biofilm removal activity of non-neutralized CFSs of five wild-type *L. rhamnosus* strains in comparison to *L. rhamnosus* GG against (**a**) *S.* Enteritidis, (**b**) *S.* Typhimurium, (**c**) *E. coli*, (**d**) *L. monocytogenes* and (**e**) *S. aureus*.

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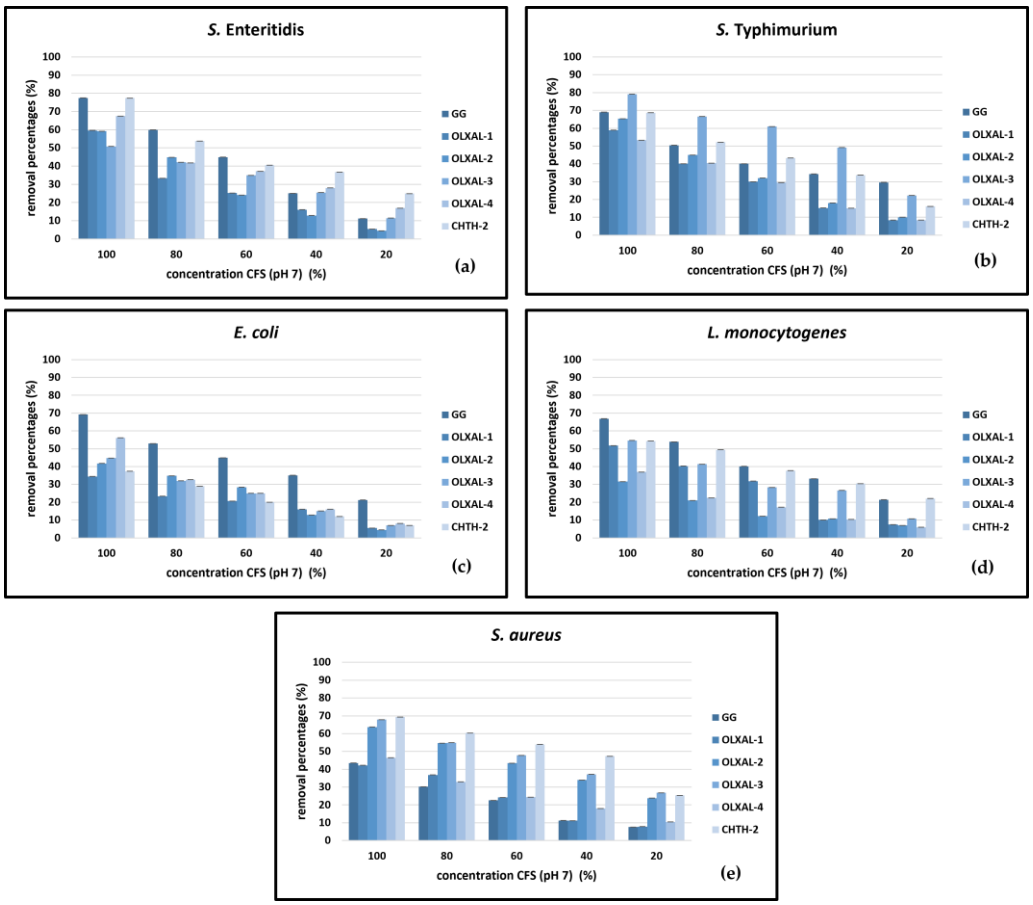

Figure 2. Biofilm removal activity of neutralized (pH 7) CFSs of five wild-type *L. rhamnosus* strains in comparison to *L. rhamnosus* GG against (**a**) *S.* Enteritidis, (**b**) *S.* Typhimurium, (**c**) *E. coli*, (**d**) *L. monocytogenes* and (**e**) *S. aureus*.

The non-neutralized CFSs with the greatest activity against *S.* Enteritidis bacterial biofilms were those of *L. rhamnosus* OLXAL-3 and *L. rhamnosus* OLXAL-4 strains (88% and 89%, respectively, $p < 0.05$), while for *S.* Tyhpimurium biofilms the most effective CFS was of *L. rhamnosus* OLXAL-3 strain (88%, $p < 0.05$) (Figures 1a and 1b). Likewise, the neutralized CFSs with the greatest activity against *S.* Enteritidis bacterial biofilms were *L. rhamnosus* CHTH-2 (78%, $p < 0.05$) and for *S.* Tyhpimurium of *L. rhamnosus* OLXAL-3 (79%, $p < 0.05$) (Figures 2a and 2b).

The above results are in agreement with previous research by Divyashree et al. [19], in which the effect of non-neutralized CFS of *Lactobacillus casei* MYSRD 108 and *Lactobacillus plantarum* MYSRD 71 against *Salmonella paratyphi* bacterial biofilms at 15% *v/v* CFS concentration was studied, reporting a removal activity over 75% by the non-neutralized *Lactobacillus casei* MYSRD 108 CFS and 81% by the non-neutralized *Lactobacillus plantarum* MYSRD 71 CFS of *S. paratyphi* biofilms, respectively. As expected, neutralized CFSs had significantly lower activity. The greater biofilm removal activity of the non-neutralized CFSs of the two strains was due to the presence of organic acids, according to Divyashree et al. [19]. In another study [20], the non-neutralized CFS of the probiotic strain *Weissella confusa* WM36 resulted in the removal of 95.68% of *Salmonella typhi* biofilm at 20% *v/v*, while the non-neutralized CFS of *Weissella viridescens* WM33 removed 66.46% of *Salmonella* Typhimurium biofilms at 15% *v/v*. Similarly, Tazehabadi et al. [21] studied the activity of *Bacillus subtilis* KATMIRA 1933 and *Bacillus amyloliquefaciens* B-1895 CFS against *Salmonella enterica* subsp. *enterica* serovar Hadar, *Salmonella enterica* subsp. *enterica* serovar Enteritidis phage type 4 and *Salmonella enterica* subsp. *enterica* serovar Thompson biofilms. The CFS *of Bacillus subtilis* KATMIRA 1933 removed 51.1, 48.3 and 56.9% of the biofilms formed by the *Salmonella* species studied, while the corresponding removal percentages observed by *Bacillus amyloliquefaciens* B-1895 CFS were 30.4, 28.6 and 35.5%, respectively.

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The activity against biofilms was not associated with low pH and organic acids production, as the pH of CFS was approximately 5.85 for both strains, but with the production of subtilisin peptides.

The highest removal activity against *E. coli* bacterial biofilms was noted by non-neutralized *L. rhamnosus* OLXAL-3 CFS (87%, $p < 0.05$), while the CFSs of *L. rhamnosus* OLXAL-3 and *L. rhamnosus* OLXAL-4 strains were most effective against *L. monocytogenes* biofilms (84% and 82% removal percentage, respectively, $p < 0.05$) (Figures 1c and 1d). On the other hand, the most efficient removal of *E. coli* and *L. monocytogenes* biofilms was noted for *L. rhamnosus* GG neutralized CFS (67% and 69%, respectively, $p < 0.05$) (Figures 2c and 2d).

Abdelhamid et al. [22] studied the activity of non-neutralized CFSs of six probiotic bacteria of the genera *Bifidobacterium* and *Lacticaseibacillus* against biofilms of multi-resistant *E. coli* WW1 and IC2 strains. The CFSs of *B. longum* and *L. plantarum* removed 57.94% and 64.57% of *E. coli* IC2 and *E. coli* WW1 biofilms, respectively. Furthermore, the skim milk CFS fermented by *L. helveticus* or *L. rhamnosus*, separately, removed 31.52 and 17.68% of *E. coli* IC2 biofilms, respectively, while the corresponding removal percentages recorded by *B. longum* or *L. helveticus* CFS were 70.81 and 69.49%. In a similar study published by Apiwatsiri et al. [23], neutralized and non-neutralized CFSs of *Lactobacillus plantarum* 22F, 25F and *Pediococcus acidilactici* 72N strains showed significant removal activity against bacterial biofilm strains of *E. coli* resistant to the antibiotic colistin.

According to the study by Shao et al. [24], non-neutralized CFS of three *Leuconostoc mesenteroides* strains removed effectively *L. monocytogenes* biofilms. *Leuconostoc* species produce bacteriocins, such as mesentericin Y105 produced by *Leuconostoc mesenteroides* spp. *mesenteroides*, leucocin A-UAL 187, which is produced by *Leuconostoc gelidum*, carnosin 44A produced by *Leuconostoc carnosum* and leuconocin S, which is produced *by Leuconostoc paramesenteroides* [25]. Bacteriocins of *Leuconostoc* species possess significantly high removal activity against *Listeria* biofilms. Moreover, Moradi et al. [26] studied the effect of *L. acidophilus* LA5 and *L. casei* 431 CFS on *L. monocytogenes* biofilms. A greater biofilm removal activity was noted for *L. acidophilus* CFS and it was found that *L. acidophilus* CFS contains 1.8% lauric acid, which is considered a possible surfactant, yet the mechanism of action is not fully understood. According to Moradi et al. [26] and in agreement with our results, the action of LAB CFSs in the removal of bacterial biofilms was strain-specific and was due to the presence of compounds, such as exopolysaccharides, organic acids and surfactants.

Regarding the removal activity of non-neutralized CFSs against *S. aureus* bacterial biofilms, the highest removal percentages were observed by *L. rhamnosus* OLXAL-2 and *L. rhamnosus* OLXAL-3 strains (87% and 89%, respectively, $p < 0.05$) (Figure 1e). On the other hand, the neutralized *L. rhamnosus* CHTH-2 CFS was the most effective on removing *S. aureus* bacterial biofilms (70%, $p < 0.05$) (Figure 2e).

In a recent study, Koohestani et al. [18] explored the removal activity of *L. acidophilus* LA5 and *L. casei* 431 CFSs against *S. aureus* bacterial biofilms. In accordance to our findings, their results highlighted the significant removal activity of both CFSs tested, in a concentration-dependent manner. Of note, the subsequent neutralization of both *Lactobacillus* CFSs resulted in reduced activity, in agreement to our results, as well as to previous reports [27].

4. Conclusions

A strong and concentration-dependent removal activity of *S.* Enteritidis, *S.* Typhimurium, *E. coli, L. monocytogenes* and *S. aureus* biofilms was observed by the CFSs of the wild-type *L. rhamnosus* strains studied. However, their effectiveness was reduced after neutralization, but still remained significant. Thus, more research is still required to identify the presence of other microbial metabolites, such as bacteriocins, hydrogen peroxide, diacetyl, etc., and clarify their effect and mechanism of action in biofilm removal.

Since the food industry is susceptible to the risks associated by biofilm formation and biofilms not only have an impact on food manufacturing operations, but also pose a wide public health risk through the contamination of food products, a better understanding of the mechanisms of their formation in the food chain is essential for developing prevention and control measures.

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

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