



The Behavior of *Staphylococcus aureus* Dual-Species Biofilms Treated with Bacteriophage phiIPLA-RODI Depends on the Accompanying Microorganism

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ABSTRACT The use of bacteriophages as antimicrobials against pathogenic bacteria offers a promising alternative to traditional antibiotics and disinfectants. Significantly, phages may help to remove biofilms, which are notoriously resistant to commonly used eradication methods. However, the successful development of novel antibiofilm strategies must take into account that real-life biofilms usually consist of mixed-species populations. Within this context, this study aimed to explore the effectiveness of bacteriophage-based sanitation procedures for removing polymicrobial biofilms from food industry surfaces. We treated dual-species biofilms formed by the food pathogenic bacterium *Staphylococcus aureus* in combination with *Lactobacillus plantarum*, *Enterococcus faecium*, or *Lactobacillus pentosus* with the staphylococcal phage phiIPLA-RODI. Our results suggest that the impact of bacteriophage treatment on *S. aureus* mixed-species biofilms varies depending on the accompanying species and the infection conditions. For instance, short treatments (4 h) with a phage suspension under nutrient-limiting conditions reduced the number of *S. aureus* cells in 5-h biofilms by ~ 1 log unit without releasing the nonsusceptible species. In contrast, longer infection periods (18 h) with no nutrient limitation increased the killing of *S. aureus* cells by the phage (decrease of up to 2.9 log units). However, in some cases, these conditions promoted the growth of the accompanying species. For example, the *L. plantarum* cell count in the treated sample was up to 2.3 log units higher than that in the untreated control. Furthermore, phage propagation inside dual-species biofilms also depended greatly on the accompanying species, with the highest rate detected in biofilms formed by *S. aureus*-*L. pentosus*. Scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM) also showed changes in the three-dimensional structures of the mixed-species biofilms after phage treatment. Altogether, the results presented here highlight the need to study the impact of phage therapy on microbial communities that reflect a more realistic setting.

IMPORTANCE Biofilms represent a major source of contamination in industrial and hospital settings. Therefore, developing efficient strategies to combat bacterial biofilms is of the utmost importance from medical and economic perspectives. Bacteriophages have shown potential as novel antibiofilm agents, but further research is still required to fully understand the interactions between phages and biofilm-embedded bacteria. The results presented in this study contribute to achieving a better understanding of such interactions in a more realistic context, considering that most biofilms in the environment consist of mixed-species populations.

KEYWORDS *Staphylococcus aureus*, biofilms, phage

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Biofilms are microbial communities attached to biotic or abiotic surfaces (1), whose structure is maintained by an extracellular matrix (2). Biofilms can be composed of single or multiple species, the latter scenario being the most common in both natural and man-made environments. The presence of different microorganisms renders the structure of the biofilm very complex, which has important implications for biofilm eradication and removal. Indeed, multispecies interactions in mixed biofilms are known to affect resistance to disinfectants (3). For instance, the presence of a curli-producing *Escherichia coli* strain was found to protect *Salmonella enterica* serovar Typhimurium biofilm cells from chlorine (4). Additionally, the matrix of mixed-species biofilms often presents greater biochemical complexity compared with that of the matrix of single-species communities (5). The presence of several microorganisms may also alter the spatial structure of the biofilm, as the different species tend to distribute according to their particular requirements along the gradients of nutrients, oxygen, and metabolites (6). Finally, quorum-sensing signals from one microorganism may interfere with the signaling of other species, thereby altering their gene expression and physiology (7).

In the food industry, biofilms are an important source of food contamination (8), posing a serious risk for the health of consumers. Indeed, biofilm-related contamination of equipment has been estimated to cause 59% of foodborne disease outbreaks (9). For this reason, efforts to find new biofilm control strategies have increased considerably over the last decade. Recently developed methods include the use of antibacterial surfaces (10), biofilm detachment procedures (11), and matrix degradation techniques (12). Within this context, numerous studies have explored the potential use of bacteriophages and phage-encoded proteins as antibiofilm agents. Phages, due to their antimicrobial activity, have been proposed as natural weapons for the control of pathogenic bacteria (“phage therapy”) in clinical, veterinary, food safety, and environmental settings (13, 14). Moreover, numerous studies have already shown the ability of phages to remove biofilms formed by a variety of bacteria, including staphylococcal species (15–18). Additionally, phage-encoded proteins, such as endolysins and exopolysaccharide depolymerases, can also be used for biofilm removal (19–21). Nevertheless, most studies available to date have focused on single-species communities, while only limited work has tackled the elimination of polymicrobial biofilms with phage-based products (22–26).

The Gram-positive bacterium *Staphylococcus aureus* is a major cause of foodborne illness due to its ability to synthesize enterotoxins. Indeed, staphylococcal food poisoning represented 7.5% of all outbreaks reported in the European Union in 2014 (27). Some studies described a high incidence of *S. aureus* on food industry surfaces (28, 29), where this bacterium often forms complex communities with other pathogenic and spoilage bacteria (30). In a previous work, we described the isolation and characterization of the myophage philPLA-RODI. Notably, this lytic phage has a wide host range among staphylococcal species, including *S. aureus* and *S. epidermidis*. Thus, exposure of 24-h biofilms to phage philPLA-RODI resulted in a 2-log decrease in the number of *S. aureus* cells in single-species biofilms and 4 log units in *S. aureus*-*S. epidermidis* dual-species biofilms (15).

The aim of this study was to assess the efficacy of bacteriophage philPLA-RODI against dual-species biofilms formed by *S. aureus* with strains of *Lactobacillus plantarum*, *Enterococcus faecium*, and *Lactobacillus pentosus*. All these accompanying bacteria have been isolated from food processing surfaces and are not sensitive to this phage. In this framework, we investigated the impact of different phage concentrations and incubation conditions on the behavior of *S. aureus* and the accompanying species. Additionally, we analyzed the ability of philPLA-RODI to propagate inside the dual-species biofilms. These data may help in the design of improved phage-based disinfection products for the food industry.

RESULTS

***S. aureus* forms biofilms with strains of *L. plantarum*, *L. pentosus*, and *E. faecium*.** Before performing the phage-infection experiments, we examined the ability of *S.*

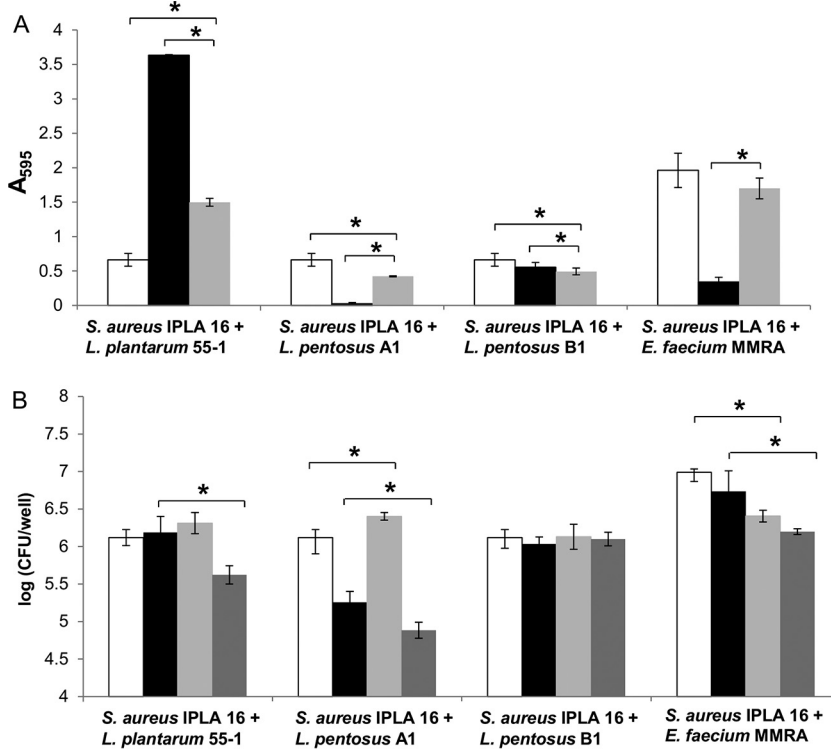


FIG 1 Biofilms formed by *S. aureus* IPLA16 with *L. plantarum* 55-1, *E. faecium* MMRA, *L. pentosus* A1, and *L. pentosus* B1 after incubating 5 h at 32°C or 37°C. (A) Biomass of monospecies biofilms formed by *S. aureus* IPLA16 (white bars), accompanying species (black bars), and dual-species (gray bars). (B) Numbers of viable cells in monospecies (white bars, *S. aureus* IPLA16; black bars, accompanying species) and dual-species (light gray bars, *S. aureus* IPLA16; dark gray bars, accompanying species) biofilms. Means and standard deviations were calculated for three biological replicates. Data from dual-species biofilms were compared to those from single-species biofilms with a two-tailed Student's *t* test. *, *P* < 0.05.

aureus IPLA16 to establish biofilms in the presence of other microorganisms. To do that, four strains belonging to three different species (*L. plantarum* 55-1, *L. pentosus* A1, *L. pentosus* B1, and *E. faecium* MMRA) were selected based on previous data demonstrating their presence in biofilm samples from food industry surfaces (16). These strains were grown in mono- and dual-species biofilms and, after incubating for 5 h, the total biomass and viable cell counts were determined (Fig. 1). The data confirmed the formation of dual-species biofilms in all four cases. However, there were some differences in the behavior of *S. aureus* IPLA16 depending on the accompanying species. For instance, the total biomass of dual-species biofilms formed by *S. aureus* IPLA16 and *L. plantarum* 55-1 decreased 58% compared with that in *L. plantarum* 55-1 monospecies biofilms and increased 100% compared with that in *S. aureus* monospecies biofilms (Fig. 1A). In contrast, the biomass values of dual-species biofilms formed by *S. aureus* with *L. pentosus* A1 or *L. pentosus* B1 were slightly smaller compared with that of *S. aureus* IPLA16 monospecies biofilms (Fig. 1A). It must also be noted that, while *L. pentosus* B1 formed biofilms even in the absence of *S. aureus*, *L. pentosus* A1 showed negligible attached biomass levels in monocultures (Fig. 1A). Despite the absence of biofilm-forming capacity, *L. pentosus* A1 cells adhered to the well surface, as determined by viable cell counts of monospecies and dual-species biofilms involving this strain (Fig. 1B). Nonetheless, the number of *L. pentosus* A1 cells in dual-species biofilms was approximately 1.6 log units smaller than that of *S. aureus* IPLA16 cells (Fig. 1B). Finally, the biomass of dual-species biofilms formed by *S. aureus* IPLA16 and *E. faecium* MMRA was not significantly different than that of *S. aureus* IPLA16 monospecies biofilms (Fig. 1A). In contrast, the biomass of the *E. faecium* MMRA single-culture biofilm was significantly smaller than that of the mixed biofilm (Fig. 1A). Both species, *S. aureus*

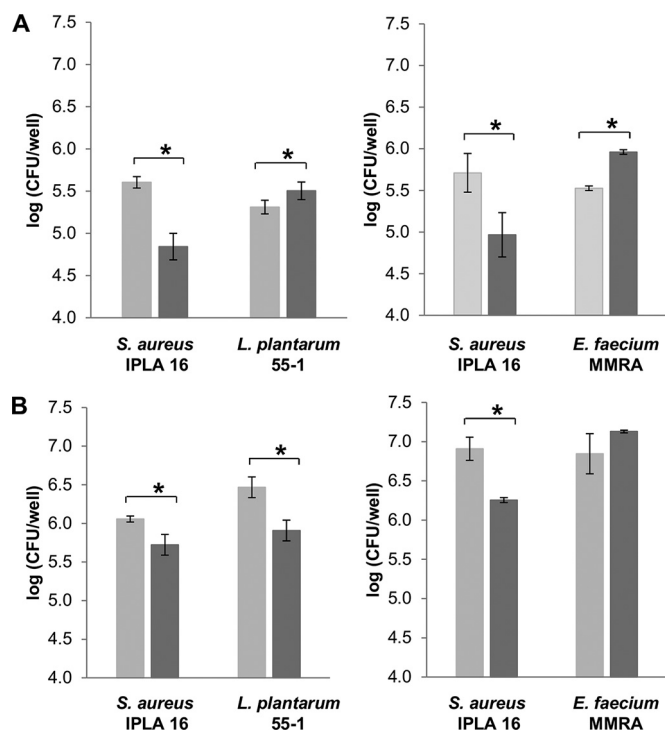


FIG 2 Effect of phage phiIPLA-RODI on 5 h (A) and 24 h (B) biofilms formed by *S. aureus* IPLA16-*L. plantarum* 55-1 and *S. aureus* IPLA16-*E. faecium* MMRA after 4 h of treatment with 10^9 PFU/well in SM buffer. Values represent the means \pm standard deviations of bacterial counts performed in triplicate. Light gray and dark gray bars correspond to control and treated samples, respectively. *, $P < 0.05$.

and *E. faecium*, showed small (<1 log unit) but significant decreases in cell counts in the mixed-species biofilm compared to those of the respective single cultures (Fig. 1B).

Treatment of *S. aureus* dual-species biofilms with phage phiIPLA-RODI under nutrient-limiting conditions. Once we characterized the dual-species biofilm models, we investigated the ability of bacteriophage phiIPLA-RODI to attack these structures. To do that, we established 5-h and 24-h dual-species biofilms formed by *S. aureus* IPLA16 with *L. plantarum* 55-1 or *E. faecium* MMRA and then treated them for 4 h with increasing concentrations of phage phiIPLA-RODI diluted in SM buffer (10^7 , 10^8 , and 10^9 PFU/well). These phage concentrations correspond, respectively, to multiplicity of infection (MOI) values of 10, 100, and 1,000 in 5-h biofilms and to MOIs of 1, 10, and 100 in 24-h biofilms. MOIs were calculated considering the number of cells present in the biofilms at the time of treatment.

The exposures of the mixed-species biofilms to phage concentrations of 10^7 and 10^8 PFU/well did not significantly affect bacterial cell counts or biomass (data not shown). In contrast, the treatment of 5-h biofilms with 10^9 PFU/well decreased the biomass of *S. aureus* IPLA16-*L. plantarum* 55-1 and *S. aureus* IPLA16-*E. faecium* MMRA biofilms by 31% and 67%, respectively (data not shown). In addition, the *S. aureus* IPLA16 cell counts were reduced by 0.8 and 0.7 log units, respectively (Fig. 2A). Similar results were obtained after treatments of 24-h biofilms. Thus, exposing *S. aureus* IPLA16-*L. plantarum* 55-1 and *S. aureus* IPLA16-*E. faecium* MMRA biofilms to the phage reduced the biomass by 18% and 63%, respectively (data not shown), and decreased viable *S. aureus* cell counts by 0.4 and 0.6 log units, respectively (Fig. 2B).

Regarding the accompanying species, phage treatments of 5-h biofilms slightly but significantly increased viable cell counts of *E. faecium* MMRA and *L. plantarum* 55-1 (Fig. 2A). However, in 24-h biofilms, the *L. plantarum* 55-1 cell count was only slightly decreased, while the *E. faecium* MMRA cell count was not affected by phage treatment (Fig. 2B).

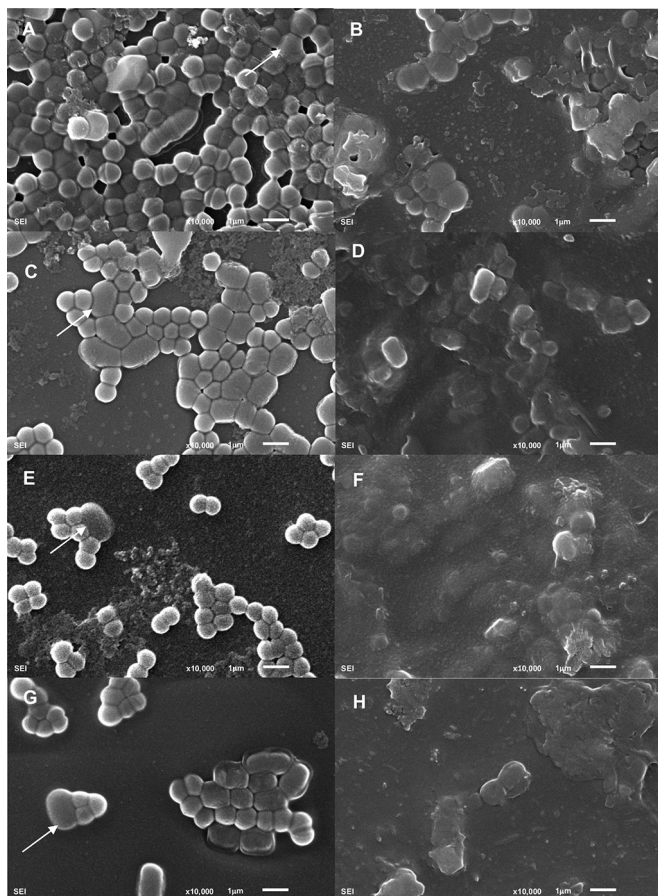


FIG 3 Scanning electron micrographs of *S. aureus* dual-species biofilms. Images correspond to 5-h biofilms formed by *S. aureus* IPLA16 with *E. faecium* MMRA (A and B), *L. plantarum* 55-1 (C and D), *L. pentosus* A1 (E and F), or *L. pentosus* B1 (G and H) following a 4-h treatment with SM buffer (left) or phlPLA-RODI (right). White arrows indicate cells from the accompanying species.

Analysis of dual-species biofilms by scanning and confocal microscopy. The effect of phage phlPLA-RODI on dual-species biofilms was also evaluated by microscopy (Fig. 3 and 4; see also Fig. S1 in the supplemental material). Images obtained by scanning electron microscopy of control biofilms treated with SM buffer showed well-organized cells belonging to both species attached to the coverslip surface (Fig. 3). Extracellular material was observed in all dual-species biofilms, although it was more evident in the sample corresponding to *S. aureus* IPLA16-*L. plantarum* 55-1 (Fig. 3C). The biofilm formed by *S. aureus* IPLA16 and *L. pentosus* A1 (Fig. 3E) contained fewer apparent cells of the accompanying species than did the other three dual-species biofilms (Fig. 3A, C, and G). This was consistent with the viable cell count results (Fig. 1B). Additionally, the biofilms formed by *S. aureus* IPLA16-*L. pentosus* A1 (Fig. 3E) and *S. aureus* IPLA16-*L. pentosus* B1 (Fig. 3G) were less dense than the other two (Fig. 3A and C). Similarly, the dual-species biofilms involving the two *L. pentosus* strains displayed the lowest biomass values in polystyrene (Fig. 1A). All four dual-species biofilms treated with phlPLA-RODI contained lower numbers of cells than their respective control samples (Fig. 3B, D, F, and H). Also, the remaining cells appeared embedded in amorphous material, which likely resulted from *S. aureus* cell lysis (Fig. 3B, D, F, and H).

Similar results were observed by confocal laser scanning microscopy (CLSM). In this case, biofilms were dyed with SYTO 9, which stains cells of all species, and wheat germ agglutinin (WGA) Alexa Fluor 647 to detect the presence of the extracellular polysaccharide poly-beta-1,6-*N*-acetylglucosamine (PIA/PNAG). Each of the untreated biofilm samples exhibited microcolony-like groups of bacteria (Fig. 4A, C, E, and G), although

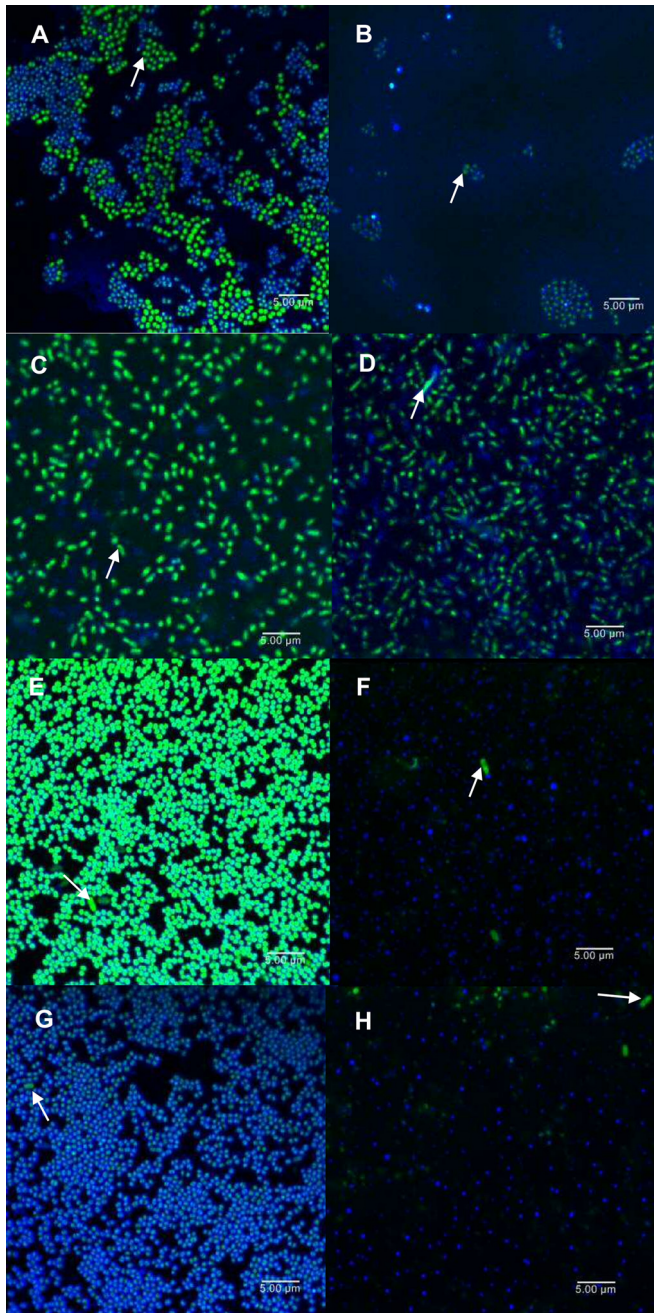


FIG 4 CLSM images of *S. aureus* IPLA16 and *E. faecium* MMRA (A and B), *L. plantarum* 55-1 (C and D), *L. pentosus* A1 (E and F), or *L. pentosus* B1 (G and H) dual-species biofilms. Images correspond to 5-h biofilms untreated (A, C, E, and G) or treated with phlPLA-RODI for 4 h (B, D, F, and H). All samples were stained with SYTO 9 (green), which dyes cells of all species, and WGA Alexa Fluor 647 (blue), which stains the extracellular polysaccharide in the biofilm matrix. White arrows indicate cells from the accompanying species.

these structures were organized differently depending on the accompanying species. Moreover, staining of PIA/PNAG revealed the presence of extracellular material in all mixed-species biofilms, particularly surrounding the *S. aureus* cells (Fig. 4A, C, E, and G). Phage treatment disaggregated the biofilm structures and decreased the amount of *S. aureus* intact cells (Fig. 4; see also Fig. S1). Regarding the accompanying species, the numbers of cells were not apparently changed in the cases of the two *L. pentosus* strains (Fig. 4F and H). In contrast, there were fewer *E. faecium* and more *L. plantarum*

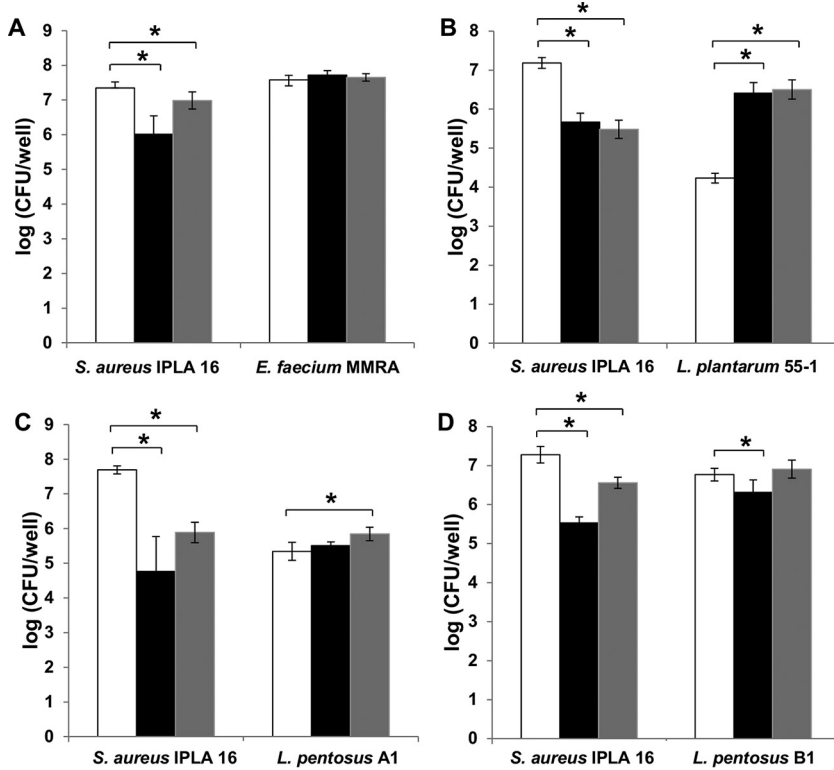


FIG 5 Bacterial counts of 5-h biofilms formed by *S. aureus* IPLA16 with other species under proliferation conditions and treated with phage phiIPLA-RODI. Values represent the means \pm standard deviations of two independent biological replicates. The accompanying species were *E. faecium* MMRA (A), *L. plantarum* (B), *L. pentosus* A1 (C), and *L. pentosus* B1 (D). White bars, untreated controls; black bars, samples treated with 10^9 PFU/well; gray bars, samples treated with 10^6 PFU/well. *, $P < 0.05$.

cells evident in the treated biofilms (Fig. 4B and D). Treatment with phage phiIPLA-RODI also altered the three-dimensional structures of the mixed biofilms (see Fig. S1). Biofilms formed by *S. aureus* IPLA16-*E. faecium* MMRA, *S. aureus* IPLA16-*L. pentosus* A1, and *S. aureus* IPLA16-*L. pentosus* B1 were flatter and less organized compared with the biofilms of the untreated samples (see Fig. S1B, F, and H). Phage treatment also reduced the thickness of *S. aureus* IPLA16-*L. plantarum* 55-1 biofilms (see Fig. S1D).

Infection of *S. aureus* dual-species biofilms by phiIPLA-RODI impacts the accompanying species. In addition to infection experiments using SM buffer as a diluting agent, we assessed the efficacy of the phage when applied in a rich growth medium. Our rationale was that efficient phage propagation, which relies on the availability of nutrients to sustain active growth of the host, may improve biofilm removal. To test this hypothesis, we infected dual-species biofilms formed by *S. aureus* IPLA16 and the different accompanying species with phiIPLA-RODI (10^9 and 10^6 PFU/well, corresponding to MOIs of 1000 and 1, respectively) diluted in tryptic soy broth supplemented with glucose (TSBG). We determined the total adhered biomass and viable cell counts after incubating the biofilms with the phage for 18 h at 37°C or 32°C. The biomass of *S. aureus* IPLA16-*E. faecium* MMRA biofilms treated with 10^9 PFU/well and 10^6 PFU/well decreased by 61% and 21%, respectively (see Fig. S2). Similarly, viable cell counts of *S. aureus* decreased by 1.3 and 0.4 log units after treatments with high and low phage concentrations, respectively. However, the number of viable *E. faecium* MMRA cells was not affected by phage treatment (Fig. 5A).

Unexpectedly, the total biomass of biofilms formed by *S. aureus* IPLA16 and *L. plantarum* 55-1 was 120% higher after treatment with 10^9 PFU/well compared with that of the untreated control (see Fig. S2). Regarding viable counts, treatments with each of the phage concentrations reduced the viable cell counts for *S. aureus* IPLA16 (about 2 log units) and increased the counts for *L. plantarum* 55-1 (about 2.3 log units) (Fig. 5B).

Finally, the biomass of 5-h biofilms formed by *S. aureus* IPLA16 and *L. pentosus* A1 decreased 86% with phiIPLA-RODI infection, while no significant difference was observed in *S. aureus* IPLA16-*L. pentosus* B1 biofilms (see Fig. S2). Viable cell counts of *S. aureus* IPLA16 in *S. aureus* IPLA16-*L. pentosus* A1 biofilms decreased 2.9 and 1.8 log units after treatment with 10^9 and 10^6 PFU/well, respectively (Fig. 5C). A lesser impact was observed in *S. aureus* IPLA16-*L. pentosus* B1 biofilms, in which cell counts were reduced 1.7 and 0.7 log units following treatment with 10^9 and 10^6 PFU/well, respectively (Fig. 5D). A slight increase in the *L. pentosus* A1 population (approximately 0.5 log units) was detected after treatment with 10^6 PFU/well (Fig. 5C). In the case of *L. pentosus* B1, treatment with 10^6 PFU/well did not change the cell number, whereas a slightly lower count was observed for the samples treated with 10^9 PFU/well.

Propagation of phiIPLA-RODI in *S. aureus* mixed biofilms depends on the accompanying species. To maximize the efficacy of phages as antibiofilm agents, it is important to determine if the viral particles can reach the target bacteria and propagate inside the biofilms. Here, we estimated propagation by titrating the number of phages in the planktonic phase and in the biofilm, differentiating between viral particles associated with cells (infectious centers) and those present as free virions.

The titration of phiIPLA-RODI isolated from dual-species biofilms challenged with 10^9 PFU/well showed the same trends for all species combinations. Thus, the planktonic phase had the highest number of viral particles, as the number of free virions was 0.5 to 1 log unit higher than that of infective centers. Biofilms contained a smaller viral load and most phage particles were free in the extracellular matrix. Consequently, the cell-associated fraction corresponding to the biofilm had the lowest phage titer. The total phage titer per well was approximately 10^9 PFU/well for each of the dual-species biofilms, which is equal to that of the starting inoculum. However, there were differences in the abilities of phiIPLA-RODI to multiply inside the biofilms depending on the accompanying species. Indeed, the numbers of free and cell-associated viral particles in dual-species biofilms formed by *S. aureus* IPLA16 with *E. faecium* MMRA and *L. pentosus* A1 were approximately 1 log unit higher than in those formed by *S. aureus* IPLA16 with *L. plantarum* 55-1 and with *L. pentosus* B1 (Fig. 6 A to D).

The propagation of phiIPLA-RODI in *S. aureus* dual-species biofilms treated with 10^6 PFU/well showed different patterns depending on the accompanying strain. In the case of biofilms involving the three lactobacillus strains, the trend was fairly similar to the one described for samples treated with 10^9 PFU/well. Indeed, phage titers obtained in samples from the planktonic phase were higher than those from biofilms (Fig. 6 B-D). Also, both phases (planktonic and biofilm) contained higher numbers of free phage particles than cell-associated virions (Fig. 6 B-D). All three dual-species biofilms formed by *S. aureus* with lactobacilli and treated with 10^6 PFU/well showed significant increases in phage particles compared with that of the starting inoculum. Thus, the calculated *P* values were 0.001, 3.15×10^{-5} , and 0.001 for biofilms formed by *S. aureus* with *L. plantarum*, with *L. pentosus* A1, and with *L. pentosus* B1, respectively. Interestingly, the total phage titer (biofilm and planktonic phase) corresponding to *S. aureus*-*L. plantarum* biofilms (7.1 log PFU/well) was significantly lower than those estimated for sessile communities formed by *S. aureus*-*L. pentosus* A1 and *S. aureus*-*L. pentosus* B1 (8.5 and 8.4 log PFU/well, respectively). The respective *P* values were 3.97×10^{-4} and 4.40×10^{-4} for comparisons of *S. aureus*-*L. plantarum* with *S. aureus*-*L. pentosus* A1 and *S. aureus*-*L. pentosus* B1 biofilms treated with 10^6 PFU/well. Unexpectedly, similar numbers of phages were obtained in the planktonic and biofilm phases of communities formed by *S. aureus* and *E. faecium* that were treated with 10^6 PFU/well. Moreover, the numbers of infective centers and free particles in the planktonic phase were similar. However, the phage titer inside the biofilm structure was slightly higher than in the planktonic phase, and there were more phage particles (about 10^6 log units) free in the extracellular matrix than there were associated with host cells (Fig. 6A). Interestingly, the phage titer in *S. aureus*-*E. faecium* biofilms treated with 10^6 PFU/well was not significantly increased compared with the starting inoculum (*P* value = 0.93).

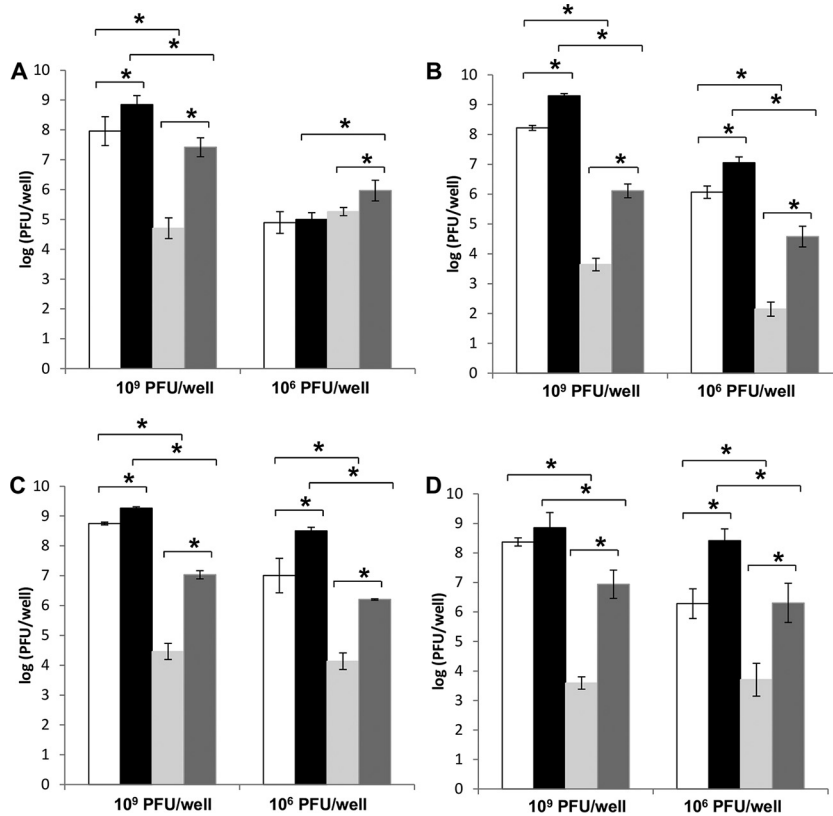


FIG 6 Propagation of phage phiIPLA-RODI on 5-h dual-species biofilms of *S. aureus* IPLA16 with other bacteria treated for 18 h with 10^9 PFU/well or 10^6 PFU/well. The accompanying species tested were *E. faecium* MMRA (A), *L. plantarum* 55-1 (B), *L. pentosus* A1 (C), and *L. pentosus* B1 (D). White bars, phages present in the planktonic phase associated with cells; black bars, phages present as free virions in the planktonic phase; light gray bars, infectious centers in the adhered (biofilm) phase; dark gray bars, free phages present in the adhered phase. These results represent the means \pm standard deviations of three replicates. The following data sets were compared by statistical analyses: free phages versus cell-associated phages (biofilm), free phages versus cell-associated phages (planktonic phase), biofilm versus planktonic phase (free phages), and biofilm versus planktonic phase (cell-associated phages). *, $P < 0.05$.

DISCUSSION

Recently, a number of articles reported the potential of bacteriophages for the control of bacterial biofilms. However, most of these studies have focused on mono-species cultures, which do not reflect the majority of biofilms found in nature. This study addressed the use of phage phiIPLA-RODI to control dual-species biofilms composed of *S. aureus* together with other species from food-related environments (*L. plantarum*, *L. pentosus*, and *E. faecium*). None of the selected microorganisms was susceptible to phiIPLA-RODI infection, and importantly, all four strains formed stable dual-species biofilms with *S. aureus*. In most cases, the numbers of cells of *S. aureus* and the accompanying species were fairly similar, except for with *L. pentosus* A1. This might be due to the poor adhesion to polystyrene and glass displayed by strain A1, which was observed even in the absence of *S. aureus*. Interestingly, there were fewer cells of *L. plantarum* in dual-species biofilms compared with in monocultures. In this case, it appears that *S. aureus* might exert a negative impact on the presence of this bacterium.

In a previous study performed in our laboratory, we described that treatment with phiIPLA-RODI reduced the number *S. aureus* IPLA16 cells by 2.4 log units in mono-species biofilms and by 4.2 log units in dual-species biofilms formed with the phage-sensitive strain *S. epidermidis* LO5081 (15). Thus, the presence of a second species, also susceptible to the phage, seemed to enhance the efficacy of phage treatment. In contrast, the results of this study show lower efficacy rates for short phage treatment of mixed-species biofilms performed under nutrient-limiting conditions. Indeed, cell

counts of *S. aureus* IPLA16 remained below 1 log unit for dual-species biofilms formed with nonsensitive bacteria, such as *L. plantarum* and *E. faecium*, regardless of the biofilm maturation stage or the phage concentration used. Therefore, it appears that the presence of cells from a nonsensitive species in the biofilm may hinder the ability of the phage to reach and lyse the target cells. It is well known that mixed-species biofilms frequently display a higher resistance to antibiotics than do monospecies biofilms (31, 32). This phenomenon may involve multiple mechanisms, including interspecies signaling, spatial distribution of physiologically different bacteria, and interference from the matrix (3). Similarly, the low efficacy of phages to infect dual-species biofilms might be due to the protection of the sensitive host by the nonsensitive species (33, 34), coaggregation of biofilm communities (35), limited penetration of phages (36, 37), or changes in the availability of phage receptors on the cell surface of the host species (38).

Despite the low efficacy from applying short phage treatments to dual-species cultures, microscopy revealed changes in the structures of treated biofilms compared with those of their respective controls. Indeed, all mixed-species biofilms displayed decreased numbers of intact *S. aureus* cells and disaggregated three-dimensional structures of the biofilms. However, a possibility is that the use of a different substrate for these experiments (glass instead of polystyrene) influenced the final results. Therefore, it may be more effective to apply the phage suspension to biofilms formed on glass coverslips than to those formed on polystyrene wells.

With regard to the treatment duration, previous studies reported that biofilm removal with phage-based products does not require incubation times longer than 5 h (25, 39). However, our results indicate that the control of biofilms consisting of several species might benefit from longer incubation times. Additionally, it appears that treatment is more effective under conditions that allow active growth of the host. Nonetheless, it must be considered that incubation under nutrient-rich conditions may also affect the proliferation of the accompanying species. This effect would be undesirable when the accompanying species are pathogenic or spoilage bacteria, especially if they are good biofilm producers. Conversely, there may be a synergistic effect of interspecies competition between the phage and the nonsensitive bacteria during active growth, thereby favoring elimination of the target pathogen. This may also limit phage-resistance acquisition, which generally confers a loss of competitive ability of the bacteria (30). This seemed to be the case, for example, of dual-species biofilms formed by *E. coli* and *Pseudomonas aeruginosa* (22). Here, there were differences in the behaviors of the four accompanying strains studied following antistaphylococcal phage treatments. Thus, the reduction in the number of *S. aureus* IPLA16 cells due to phage infection led to an increase in the number of *L. plantarum* cells. Also, *S. aureus*-*L. plantarum* biofilms displayed increased biomass after phage treatment. These results were not surprising given the inhibitory effect of *S. aureus* over *L. plantarum* that was observed in the preliminary experiments. As mentioned previously, the number of viable *L. plantarum* 55-1 cells in dual-species biofilms formed with *S. aureus* was significantly lower than in the *L. plantarum* single-species community. In contrast, partial elimination of the *S. aureus* population by phage treatment seems to enable the *L. plantarum* population to develop in a manner similar to that of the single-species biofilm. This is reflected in the higher *L. plantarum* cell number and increased adhered biomass. Additionally, growth of *L. plantarum* may be facilitated by the greater availability of nutrients resulting from phage-induced bacterial lysis or the reduced growth of *S. aureus* IPLA16. Nevertheless, the interactions between bacterial species in a biofilm are very complex and not always easy to explain. Although phage predation has been shown to promote biofilm formation in certain bacteria growing in monospecies biofilms (40), our results suggest that the increase in biomass in *S. aureus*-*L. plantarum* cocultures is due to increases in the accompanying species rather than inducing the surviving *S. aureus* cells to form a biofilm.

With regard to the other three strains studied, namely, *L. pentosus* A1, *L. pentosus* B1, and *E. faecium* MMRA, no major increases in cell counts were observed after treatments

with phiPLA-RODI. Nevertheless, comparing the cell counts obtained here with previous results of biofilm formation growth curves (unpublished data) indicates that the populations in treated and control samples reached the maximum cell numbers for the aforementioned strains. Interestingly, phage treatment did not release cells from the accompanying species to the planktonic phase. Similar results were described for biofilms formed by *Pseudomonas fluorescens* and *Staphylococcus lentus* under static conditions. However, under dynamic conditions, phage Φ IBB-PF7A caused the release of the nonsusceptible *S. lentus* cells (25).

Even though it is widely recognized that bacteriophages can replicate inside biofilms, data concerning phage propagation in multiple species communities are scarce. The phage life cycle inside the biofilm requires attachment to bacterial cells embedded in an extracellular matrix and, after production of the phage progeny, the diffusion of new phages to other areas of the biofilm to reach new susceptible cells (41). Depending on the bacterial species, the metabolic and physicochemical conditions of the biofilm (matrix density, number of cells, and metabolic state) can be quite different. Phage propagation in the four dual-species biofilms studied here was evaluated using two different phage concentrations. Not surprisingly, the phage titer in the planktonic phase was considerably higher than the number of phages inside the adhered phase for all dual-species biofilms. Moreover, our results showed that dual-species biofilms support phage multiplication, since a significant percentage of phage particles was isolated as cell-associated infectious centers. Overall, it seems that the adsorption rate of phages to host cells is favored under planktonic conditions compared to that in a biofilm environment. One explanation for this phenomenon is that the production and migration of phage particles in biofilms are limited considerably by extracellular material (42). Interestingly, phage propagation in the biofilm phase was lower in *S. aureus*-*L. plantarum* biofilms than in the dual species biofilms involving either of the two *L. pentosus* strains. As mentioned previously, *L. plantarum* forms biofilms better than the *L. pentosus* strains and is known to produce extracellular material. Therefore, it is possible that the greater complexity of the extracellular matrix in the *S. aureus*-*L. plantarum* biofilms hinders phage propagation in the biofilm. Nonetheless, some studies seem to indicate that phage penetration into biofilms is not completely blocked by the extracellular matrix. For instance, it has been shown that c2 phage can diffuse into biofilms through water-filled channels, although some phage particles may interact with their specific binding sites on bacteria and are immobilized there (37). However, the interaction of phages with extracellular components cannot be completely discarded (22, 43). In addition to the role played by the matrix, the lower infectivity detected in biofilms may also be due to the heterogeneity of these communities with regard to the structure and metabolic state of the host cells. Thus, phage infection may be more prevalent in metabolically active cells located on the biofilm surface than in the slow-growing cells located in the inner layers of the biofilm (1, 41). Overall, we conclude that successful propagation of phages in mixed-species biofilms depends on the accompanying nonsusceptible species. This is likely due to the differences in the composition of the extracellular matrix, which can affect the ability of the viral particles to reach susceptible cells.

In conclusion, this study provides evidence that phage infection in dual-species biofilms is a complex process likely subject to population dynamics. Hence, phage treatment may require the use of phage cocktails or phages combined with other antimicrobials to target the accompanying species. Overall, our results contribute to the understanding of interspecies interactions in the context of developing phage-based strategies for the control of mixed-species biofilms.

MATERIALS AND METHODS

Bacterial strains, bacteriophage, and growth conditions. All of the bacterial strains used in this study are listed in Table 1. The staphylococcal strains and *E. faecium* MMRA were routinely grown in TSB (tryptic soy broth; Scharlau, Barcelona, Spain) at 37°C with shaking or on TSB plates containing 2% (wt/vol) bacteriological agar (TSA). *L. plantarum* 55-1 (kindly supplied by R. Jiménez-Díaz, IG-CSIC, Sevilla, Spain) and the exopolysaccharide producers *L. pentosus* A1 (EPS A and EPS B) and *L. pentosus* B1 (EPS B)

TABLE 1 Bacterial strains used in this study

| Species | Strain | Origin | Reference |
|--------------------------------|--------|--------------------------------|-----------|
| <i>Staphylococcus aureus</i> | IPLA1 | Dairy industry surface | 30 |
| <i>S. aureus</i> | IPLA16 | Meat industry surface | 30 |
| <i>Enterococcus faecium</i> | MMRA | Dairy product | 46 |
| <i>Lactobacillus plantarum</i> | 55-1 | Natural fermentation of olives | 47 |
| <i>L. pentosus</i> | LPS26 | Natural fermentation of olives | 44 |
| <i>L. pentosus</i> | A1 | Derivative from LPS26 | 44 |
| <i>L. pentosus</i> | B1 | Derivative from LPS26 | 44 |

were grown in MRS (Scharlab S.L., Spain) broth or agar at 32°C (44). For selective growth of the different species, the following media were used: Baird-Parker agar (BP) for *S. aureus*, Kenner Fecal (KF) agar for *E. faecium*, and MRS agar plates for the other three strains. Bacteriophage phiIPLA-RODI was propagated on *S. aureus* IPLA1 as previously described (15).

Biofilm formation. Overnight cultures of the different strains were diluted to 10⁶ CFU/ml into fresh TSB supplemented with 0.25% glucose (TSBG). Mixtures of 100 µl aliquots from each single culture and 100 µl of TSBG or mixtures of two strains (100 µl of each strain) were poured into each well of 96-well microtiter plates (TC Microwell 96U with lid, Nunclon D SI; Thermo Scientific, Nunc, Madrid, Spain). These plates were incubated for 5 or 24 h at 37°C for *S. aureus-E. faecium* biofilms or at 32°C for *S. aureus-L. plantarum* and *S. aureus-L. pentosus* biofilms. After biofilm formation, the planktonic phase was removed and wells were washed twice with phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, and 2 mM KH₂PO₄, pH 7.4). The adhered cells were collected by scratching the bottom of the well with a sterile swab and then suspended in 9 ml of PBS by vigorously vortexing the sample for 1 min. Serial dilutions of these suspensions were plated on selective medium for bacterial counting. Alternatively, the total biomass adhered to the well was determined by crystal violet (0.1%, wt/vol) staining as described previously (20). Each experiment was repeated at least three times.

Infection of established biofilms with phage phiIPLA-RODI. To perform short infection period experiments, biofilms were grown for 5 h or 24 h and washed with PBS. Then, 100 µl of SM buffer and 100 µl of phiIPLA-RODI were added to each well (10⁷, 10⁸, or 10⁹ PFU/well). A volume of 200 µl of SM buffer was added to the control wells. The plates were incubated for 4 h at 32°C or 37°C depending on the accompanying species. To test the effect of long infection periods under nutrient-rich conditions, biofilms were established for 5 h, washed with TSBG, and incubated with phage diluted in TSBG (10⁹ PFU/well or 10⁶ PFU/well). TSBG was added to the control wells. Biofilms were treated for 18 h at 32°C or 37°C depending on the species. Afterwards, the planktonic phase was collected and the wells were washed twice with PBS. The number of cells attached to the well surface and the total adhered biomass were determined as described above.

The titrations of “free” and “cell-associated” phages (or infective centers) in the planktonic phase and the biofilm were performed as follows. First, the planktonic phase was taken directly from the wells and adhered cells were collected, after washing, by scratching the wells as indicated above. Then, all samples were centrifuged at 16,100 × *g* for 5 min to separate the supernatant containing the “free” viral particles from the pellets containing the “cell-associated” phages. Both supernatants and pellets were stored at 4°C until further processing. To determine the titer of “free” phages, the supernatants were filtered (0.45 µm; VWR, Spain), diluted in SM buffer, and plated on the reference strain *S. aureus* IPLA1 by the double-layer technique (45). Prior to titrating the infective centers, the pellets from planktonic and biofilm samples were resuspended in PBS. Then, 50 µl of chloroform was added to each sample to lyse the bacterial cells and release the viral particles. After vortexing, the cell debris was separated further by centrifugation. The resulting supernatants were then titrated by the double-layer technique.

Microscopy techniques. For microscopy analyses, biofilms were formed on borosilicate glass coverslips (18-mm diameter; VWR International, UK) previously placed into the wells of a 12-well microtiter plate (Thermo Scientific, Nunc, Madrid, Spain). To prepare the inoculum for each biofilms, overnight cultures of the different strains were diluted to 10⁶ CFU/ml into fresh TSB supplemented with 0.25% glucose (TSBG). Mixtures of 1 ml aliquots from each single culture and 1 ml of TSBG or mixtures of two strains (1 ml of each strain) were poured into each coverslip-containing well. These plates were incubated for 5 h at 37°C for *S. aureus-E. faecium* biofilms or at 32°C for *S. aureus-L. plantarum* and *S. aureus-L. pentosus* biofilms. The planktonic phase was then removed and the established biofilms were treated with phage phiIPLA-RODI (10⁹ PFU/ml) or SM buffer for 4 h. After treatment, the coverslips were washed twice with PBS and dried for 20 h at 25°C.

For scanning electron microscopy (SEM), the samples were subsequently covered in gold (gold sputter coating) and examined using a scanning electron microscope (JSM-6610LV; JEOL Ltd., Tokyo, Japan).

For confocal laser scanning microscopy (CLSM), samples were washed twice with PBS and stained with SYTO 9 from the Live/Dead BacLight kit (Invitrogen AG, Basel, Switzerland) and wheat germ agglutinin (WGA) Alexa Fluor 647 conjugate (Life Technologies, Oregon, USA) following the manufacturer's instructions. SYTO 9 and the WGA Alexa Fluor 647 conjugate stain bacterial cells of all species and the exopolysaccharide PIA/PNAG, respectively. Images were acquired with a confocal scanning laser microscope (DMi8; Leica Microsystems) using a 100× oil objective.

Statistical analysis. All experiments were performed with at least three biological replicates and the means ± standard deviations were obtained and further analyzed to establish significant differences.

Statistical analyses were performed by a two-tailed Student's *t* test and significance was considered at a *P* of <0.05.

SUPPLEMENTAL MATERIAL

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TEXT S1, PDF file, 0.3 MB.

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