Effect of Bacteriocins Pediocin PD-1, Plantaricin 423, and Nisin on Biofilms of *Oenococcus oeni* on a Stainless Steel Surface

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The effect of pediocin PD-1, plantaricin 423, and nisin was tested against established biofilms formed by a commercial starter culture of *Oenococcus oeni* on stainless steel slides and planktonic (free-living) cells. The percentage viable and nonviable cells were determined by using a viability probe and an epifluorescence microscope with image analysis. Pediocin PD-1 (3000 AU/mL) removed all cells in a biofilm of *O. oeni* that formed in acidic grape medium after five hours. Plantaricin 423 (3000 AU/mL) and nisin (3000 AU/mL) killed all viable cells in the biofilm. However, approximately 42 and 49% of the original cells on the stainless steel surface remained attached, with the majority staining nonviable when treated with the latter two peptides, respectively. In a modified Chardonnay must, all cells (viable and nonviable) of *O. oeni* in the biofilm were removed after one hour when treated with pediocin PD-1 (3000 AU/mL). In similar experiments, treatment with plantaricin 423 and nisin resulted in undetectable numbers of viable cells in the biofilm. Approximately 36 and 43% of the original cells on the stainless steel surface remained attached, with the majority staining nonviable after five hours of treatment with the latter two peptides, respectively. After five hours of treatment with the respective antimicrobial peptides (3000 AU/mL), the planktonic cell numbers of *O. oeni* decreased from 1.3×10^{10} cfu/mL to undetectable numbers of viable cells in acidic grape medium. The same experiment performed in modified Chardonnay must yielded no detectable cells of *O. oeni*.

Key words: Pediocin PD-1, plantaricin 423, nisin, *Oenococcus oeni*, planktonic cells, biofilms, removal

Malolactic bacteria occur throughout the winemaking process, but generally only commence growth after primary (alcoholic) fermentation [28]. Malolactic fermentation (MLF) is considered important for the deacidification, flavor modification and microbial stability of wine, particularly in wines produced from grapes grown in cool climates which often have a high acid (tartrate plus malate) content. MLF is also desired in some white and red wines produced from grapes grown in warmer climates, since it often introduces favorable organoleptic compounds [16]. As MLF is a delicate process, it is important to control the presence of naturally occurring malolactic bacteria.

Many wines, including Chardonnay, are fermented in stainless steel tanks before maturation in oak vats. Apart from this, the must and wine is pumped through stainless steel pipelines and valves. Adherence of malolactic bacteria to stainless steel surfaces may have a pronounced affect on the ability of malolactic bacteria to survive during the primary (alcoholic) fermentation and conduct MLF. Furthermore, if biofilms of malolactic bacteria are not effectively destroyed, they may contaminate wine in which MLF is undesirable or even lead to the development of bacteriophages, which may cause stuck or sluggish MLF in wines dependent on a secondary fermentation.

Numerous papers have been published regarding biofilm formation by food-borne pathogens and bacteria that, after adhesion to stainless steel surfaces, are a source of contamination for food products (reviewed by Kumar and Anand [26] and Zottola and Sasahara [42]). Previously, most research in this field focused on the adhesion [2,3,27], growth and development [12,41], and chemical removal [10,24] of bacteria from stainless steel surfaces. Although several reports mentioned the effect of antimicrobial peptides and antibacterial agents on biofilms [1,23], they focused primarily on the effect these compounds have on biofilms of medical importance. Jayaraman et al. [21], however, reported on the effect of in situ production of gramicidin S by an established biofilm of *Bacillus brevis* before colonization of the sulfate-reducing bacterium *Desulfovovibrio vulgaris* as a method to inhibit the growth of the latter bacterium on stainless steel surfaces. Bower et al. [5] reported on the antimicrobial activity of surface-adsorbed nisin to food contact surfaces to control pathogenic organisms [20].

Little is known regarding the effect bacteriocins have on stainless steel-adhered malolactic biofilm communities; hence, there are no reports on the use of bacteriocins to control these biofilms in the wine industry. This study was aimed at determining whether a commercial starter culture of *Oenococcus oeni*

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is able to form biofilms on stainless steel, and, if so, whether bacteriocins could be used to prevent biofilm formation.

Materials and Methods

Bacterial strains and culture conditions. *Pediococcus damnosus* NCFB 1832 was grown in De Man Rogosa and Sharpe (MRS) broth (Oxoid, Hampshire, England) for 55 hr at 30°C. The production of pediocin PD-1 was optimized by supplementing MRS broth (Oxoid) with bacteriological peptone (1.7%, w/v), $MnSO_4$ (0.014%, w/v), and Tween 80 (3%, v/v) and adjusting of the pH to 6.7 with 2N NaOH.

Lactobacillus plantarum 423 was cultured in MRS broth (Oxoid) for 15 hr at 30°C. Optimal plantaricin 423 production was obtained in the latter medium, supplemented with tryptone, $MnSO_4$, and Tween 80 and adjusted to pH 5.8, as described by Verellen et al. [39]. A strain of *Oenococcus oeni,* isolated from a commercial starter pack (Lallemand, Saint-Simon, France), was cultured in acidic grape broth [9] at 26°C.

Preparation of bacteriocins. Cell-free supernatants containing pediocin PD-1 and plantaricin 423 were obtained by centrifuging 2 L of each culture at 9800 × g for 10 min at 8°C. These cell-free supernatants and a 1.3% (w/v) suspension of nisin (1×10^6 IU/g, Aplin & Barrett Ltd., Trowbridge, Wilts, England) were tested for antibacterial activity against metabolically active cells of *O. oeni* embedded in acidic grape soft agar (0.75% w/v agar), according to the spot-on-lawn technique [29] and expressed as AU (activity units)/mL. Based on the activity levels obtained, the supernatants of the three bacteriocins were diluted with sterile distilled water to represent equal activity levels (AU/mL). These supernatants were then individually freeze-dried and the lyophilisate of each redissolved in 10 mL sterile distilled water.

Effect of pediocin PD-1, plantaricin 423, and nisin on biofilms formed by *O. oeni*. Stainless steel (type 304, grade 2B finish) was cut into 75 × 25 × 2 mm slides. The slides were wiped with 96% (v/v) ethanol, rinsed five consecutive times in distilled water, submerged in distilled water, and autoclaved. The slides were then aseptically transferred to a 250-mL screw-cap bottle containing 240 mL acidic grape medium and inoculated with *O. oeni* (1%, v/v, $OD_{600} = 0.6$). After 9 days of static incubation at 26°C, the slides were aseptically removed from the bottle, rinsed for 5 sec with sterile distilled water to remove nonadherent bacteria, stained with 300 µL of the Live/Dead *Bac*Light viability probe (Molecular Probes Inc., Eugene, OR), and left for 15 min in the dark at room temperature. Care was taken not to disturb the slides.

In a separate, but similar experiment, pediocin PD-1, plantaricin 423, and nisin suspensions were added to a 9-day-old culture of *O. oeni* to obtain final activity levels of 100, 500, 1000, 2000, and 3000 AU/mL. After periods of 1 hr, 3 hr, and 5 hr of incubation at 26°C, the stainless steel slides were aseptically removed and studied under the microscope. Slides incubated in the absence of bacteriocins served as control.

Images of the biofilms on the slides were captured using a high-performance CCD camera (Cohu Inc., San Diego, CA) mounted on a Nikon Eclipse E400 epifluorescence microscope, equipped with an ×60/1.4 Dic H oil objective and filters. A minimum of 20 images were selected at random on each disk and the percentage viable and nonviable cells per optical field calculated. Corrections were made for spectral overlap and background fluorescence and the images analyzed with Scion Image software (U.S. National Institutes of Health; http://rsb.info.nih.gov/nih-image).

Effect of pediocin PD-1, plantaricin 423, and nisin on planktonic cells of *O. oeni*. Cells of *O. oeni* were grown for 9 days at 26°C to an optical density of approximately 1.1 (at 600 nm) in acidic grape broth (240 mL in a 250-mL screw-cap bottle), containing sterile stainless steel slides, as described before. The three bacteriocins were then added to the respective cultures to represent final concentrations of 100, 500, 1000, 2000, and 3000 AU/mL. Cultures to which no bacteriocins were added served as control. Stirring was kept to the minimum to maximize contact of the bacteriocin to the planktonic cells. Care was taken to avoid disturbance of biofilm formation on the slides. After 10 min, 30 min, 1 hr, 3 hr, and 5 hr, 100 mL was withdrawn from each culture, diluted in sterile saline, plated out onto acid grape agar, incubated at 26°C and the number of viable cells determined after 14 days at 26°C.

Effect of bacteriocins on biofilm formation and planktonic cells of *O. oeni* in a modified Chardonnay grape must medium. Chardonnay must (2 L) was centrifuged at 9800 × g for 10 min at 8°C and the supernatant supplemented with 5% (w/v) yeast extract (Biolab, Midrand, South Africa) to stimulate bacterial growth. The must was then divided into 240-mL volumes and dispensed into 250-mL screw-cap bottles. The pH was adjusted to 4.5 with 2N NaOH and heated to boiling point. Sterile stainless steel slides were added to each of the three volumes and inoculated with *O. oeni* and incubated at 26°C for 9 days. The 9-day-old cultures then each received 3000 AU/mL of a specific bacteriocin. The effect of the bacteriocins on biofilm formation and the planktonic cells was recorded as described previously.

Replication of experiments. All experiments were done in duplicate with a minimum of three repeats for each fermentation. Viable and nonviable cell counts obtained in the biofilms and planktonic cultures for all three repeats of each fermentation did not vary by more than 10%.

Results and Discussion

Growth and attachment of *O. oeni* to stainless steel slides. The planktonic cell density of *O. oeni* increased from 0.6 (OD_{600}) to approximately 1.25 after 9 days at 26ºC. During this time the cells gradually adhered to the stainless steel surfaces to form mature and evenly dispersed biofilms. Approximately 75 and 86% of the surface areas of the slides submerged in acidic grape medium and in Chardonnay must, respectively, were covered with cells. Cells that emitted a green fluorescence had intact cell membranes and were viable; whereas cells that fluoresced red were in a bactericidal or bacteriostatic state. Based on these observations, only a small percentage of the cells in the biofilms (5 to 7% of the slide surface areas), whether generated in acidic grape broth or Chardonnay must, were nonviable.

Effect of bacteriocins on mature biofilms. The addition of pediocin PD-1 to a 9-day-old biofilm of *O. oeni* resulted in a drastic decrease in viable cell numbers, as depicted in the differences recorded between the viable cell numbers in the control biofilm (no pediocin PD-1 added) versus the viable cell numbers recorded on the slides in the presence of the bacteriocin (Table 1, Figure 1A). In general the number of viable cells decreased as incubation in the presence of pediocin PD-1 increased from 1 to 5 hr. After 1 hr in the presence of 100 AU mL^{-1} pediocin PD-1, less than 3% viable cells of the total number of cells in the biofilm were detected, and after an additional 4 hr of incubation, less than 0.1% viable cells of the total number of cells in the biofilm were detected. The effect of pediocin PD-1 on the viable cells in the biofilm was more pronounced as the concentration increased to 500 AU/mL and above.

The nonviable cells in the biofilm increased from approximately 5% (control biofilm with no pediocin PD-1) to approximately 52% after 1 hr in the presence of 100 AU/mL pediocin PD-1 (Table 1, Figure 1A). The number of nonviable cells remained approximately the same after 5 hr in the presence of 100 AU/mL pediocin PD-1. However, at activity levels of 500 AU/mL and above, pediocin PD-1 resulted in a more efficient removal of the cells in the biofilm (from approximately 52% cell coverage at 100 AU/mL to no adherent cells detected after 5 hr at 3000 AU/mL).

In the presence of plantaricin 423, the number of viable cells in the biofilm decreased from approximately 70% coverage (control) to approximately 9% after 1 hr and approximately 4% after 5 hr at 100 AU/mL (Table 1, Figure 1B). As expected, few viable cells were recorded after 1 hr in the presence of 2000 AU/mL plantaricin 423, and low levels of viable cells were detected after longer incubation at this concentration or at

Table 1 Percent coverage of *O. oeni* cells on stainless steel slides submerged in acidic grape broth and treated with bacteriocins.[a]

Bacteriocin concn (AU/mL)	Contact time (hr)	Viability[b]	Pediocin PD-1	Plantaricin 423	Nisin
100	1	V	2.33	8.90	5.47
	1	NV	52.17	65.06	60.54
	3	V	1.00	5.02	2.63
	3	NV	53.37	66.25	63.99
	5	V	0.10	3.98	0.54
	5	NV	52.79	68.65	67.98
500	1	V	1.01	5.04	1.90
	1	NV	35.85	66.35	66.70
	3	V	0.76	2.69	1.26
	3	NV	28.11	64.55	68.15
	5	V	0.05	1.65	0.50
	5	NV	21.36	62.47	70.33
1000	1	V	0.34	2.00	0.48
	1	NV	15.02	57.65	68.48
	3	V	0.12	0.81	0.32
	3	NV	12.55	55.52	67.35
	5	V	0.08	0.14	0.30
	5	NV	9.98	53.99	65.05
2000	1	V	0.08	0.54	0.13
	1	NV	4.11	52.39	62.13
	3	V	0.01	0.09	0.05
	3	NV	2.57	48.00	59.65
	5	V	0	0	0.02
	5	NV	1.01	46.83	54.65
3000	1	V	0	0	0.01
	1	NV	0.42	44.65	55.66
	3	V	0	0	0
	3	NV	0.04	42.66	53.10
	5	V	0	0	0
	5	NV	0	41.98	49.45
Control viable			69.52	69.52	69.52
Control nonviable			5.57	5.57	5.57

[a]Each value represents an average cell count of 20 microscopic fields per treatment, with a minimum of three repeats per treatment.
[b] V = viable; NV = nonviable.

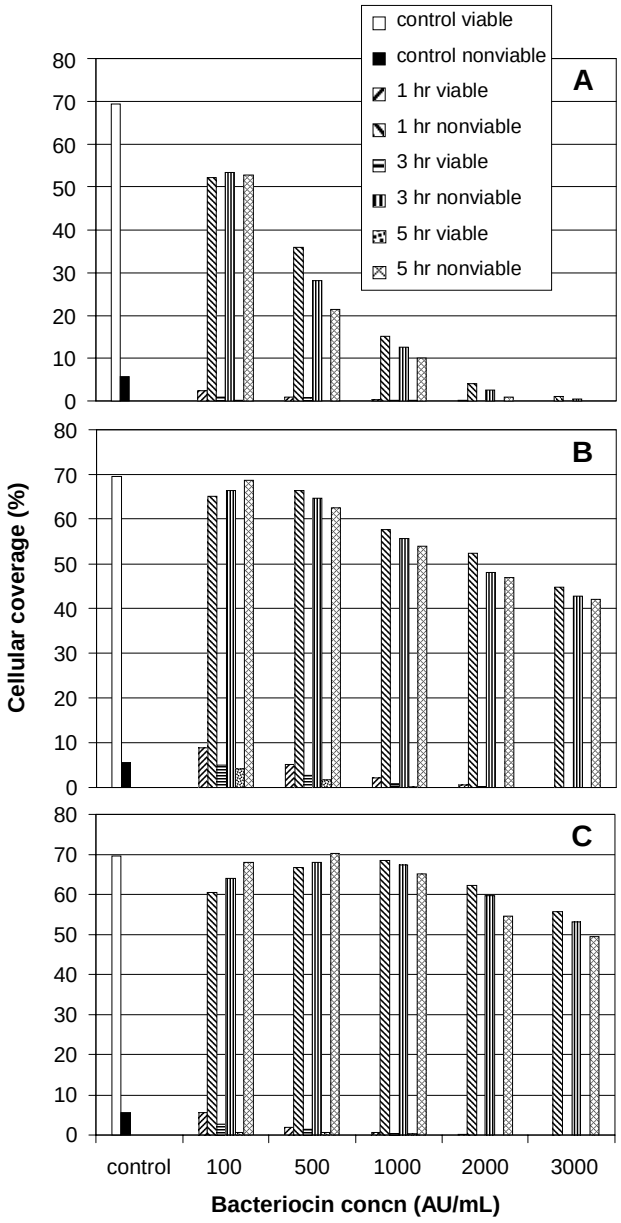

Figure 1 Effect of different concentrations of pediocin PD-1 (**A**), plantaricin 423 (**B**), and nisin (**C**) on an established biofilm of *O. oeni* in acidic grape medium at specific time intervals.

higher levels of the bacteriocin. No drastic changes (less than 4% coverage of cells based on an average of three repeats in duplicate experiments) were recorded in the number of nonviable cells after 5 hr in the presence of 100 AU/mL plantaricin 423 (Table 1, Figure 1B). However, a slight but consistent decrease in the number of cells was observed at higher levels of plantaricin 423 or when the biofilm remained for longer in contact with the same concentration of the bacteriocin. After 5 hr of treatment with plantaricin 423 (3000 AU/mL), approximately 42% of the original cells remained in the biofilm with the majority staining nonviable.

The biofilm treated with nisin produced approximately the same pattern of viable cell decrease as recorded for pediocin PD-1 and plantaricin 423 (Table 1, Figure 1A-C). However, the percent coverage of nonviable cells in the biofilm increased at 100 and 500 AU/mL nisin as the contact time increased from 1 to 5 hr, respectively (Table 1, Figure 1C). A decrease in cells was observed only at higher activity levels of nisin (1000 AU/mL and above). After 5 hr of treatment with nisin (3000 AU/mL), approximately 49% of the original cells remained in the biofilm with the majority staining nonviable.

Effect of bacteriocins on planktonic cells. After 9 days of incubation at 26°C, the planktonic cells of *O. oeni* in the control medium (in the absence of bacteriocins) reached 1.4×10^{10} cfu/mL. An increase in the activity levels and contact time of each bacteriocin resulted in a generally linear reduction in planktonic cell counts (Figure 2A-C). Pediocin PD-1 (3000 AU/mL) reduced the planktonic cell numbers from 1.4×10^{10} cfu/mL to 1.8×10^{2} cfu/mL after 5 hr of contact (Figure 2A). This correlates to a reduction of eight log cycles (approximately 80%) in viable cell numbers. Similar results were recorded after 3 hr with 3000 AU/mL plantaricin 423 (Figure 2B) and 5 hr with 1000 AU/mL Nisin (Figure 2C).

Effect of pediocin PD-1, plantaricin 423, and nisin on biofilms and planktonic cells of *O. oeni* in Chardonnay must. The coverage of viable cells decreased from approximately 80% (control) to no adherent viable cells detected after 1 hr of incubation in the presence of 3000 AU/mL pediocin PD-1, plantaricin 423, and nisin (Table 2, Figure 3). In the presence of pediocin PD-1, no adherent nonviable cells were detected within 1 hr (Table 2, Figure 3). However, after 1 hr in the presence of plantaricin 423 and nisin, the number of adherent nonviable cells detected were approximately 38 and 48%, respectively, followed by a slight reduction after 3 and 5 hr (Table 2, Figure 3).

The planktonic cells in the control medium (in the absence of bacteriocins) reached 1.3×10^{10} cfu/mL (Figure 4). Pediocin PD-1 reduced the planktonic cell numbers to undetectable cfu after 5 hr of contact; whereas plantaricin 423 and nisin yielded the same result after 3 hr and 1 hr, respectively (Figure 4).

Concluded from the results obtained in the present study, the three bacteriocins effectively removed cells of *O. oeni* from a mature biofilm. Pediocin PD-1 proved to be the most effective, whether tested on biofilms formed in acidic grape broth or in Chardonnay must. Microbial cells in biofilms are known to be as much as 1000 times more resistant to biocides [7,25,32,40]. In cases of extensive biofouling, thick biofilms

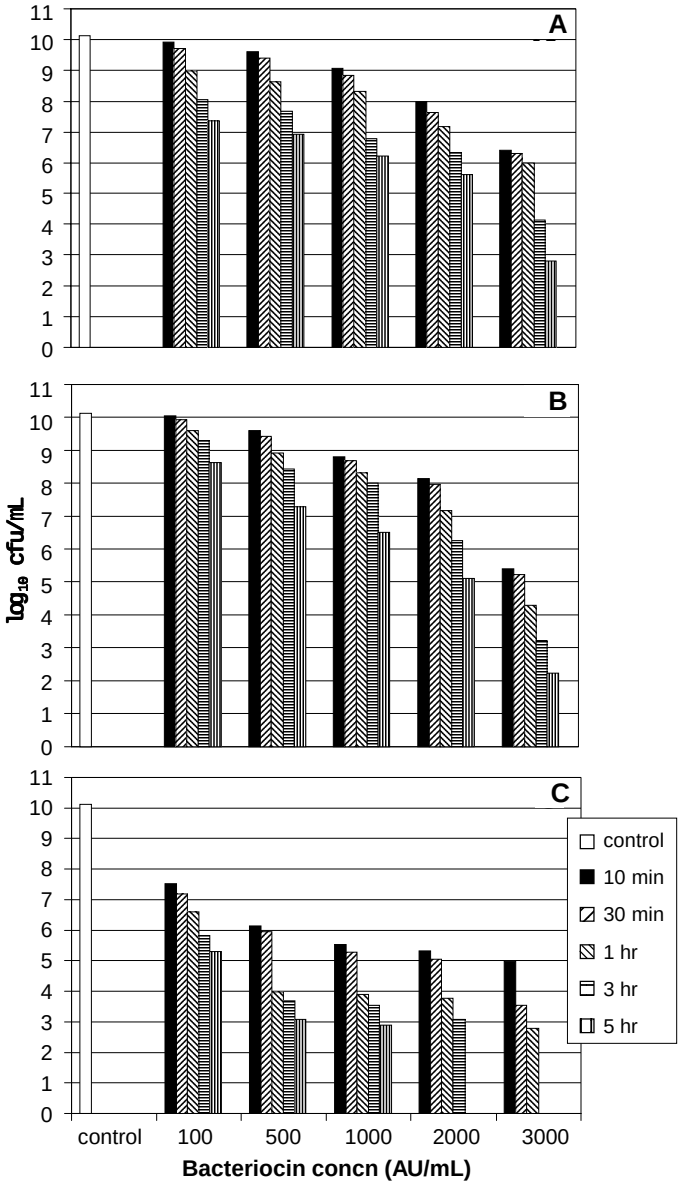

Figure 2 Effect of different concentrations of pediocin PD-1 (**A**), plantaricin 423 (**B**), and nisin (**C**) on planktonic cells of *O. oeni* at specific time intervals.

Table 2 Percent coverage of *O. oeni* cells on stainless steel slides submerged in modified grape must and treated with bacteriocins.[a]

Bacteriocin concn (AU/mL)	Contact time (hr)	Viability[b]	Pediocin PD-1	Plantaricin 423	Nisin
3000	1	V	0	0	0
	1	NV	0.01	37.54	47.98
	3	V	0	0	0
	3	NV	0	36.09	45.79
	5	V	0	0	0
	5	NV	0	35.68	42.79
Control viable			78.78	78.78	78.78
Control nonviable			7.04	7.04	7.04

[a]Each value represents an average cell count of 20 microscopic fields per treatment, with a minimum of three repeats per treatment.
[b]V = viable; NV = nonviable.

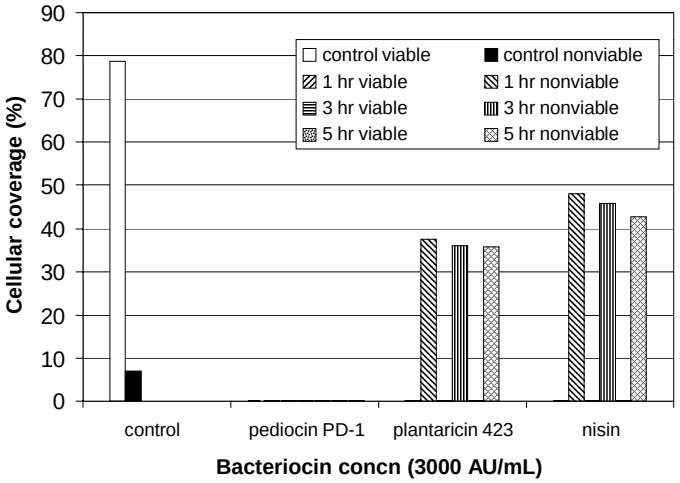

Figure 3 The effect of 3000 AU/mL of pediocin PD-1, plantaricin 423, and nisin on an established biofilm of *O. oeni* in a modified Chardonnay must medium at specific time intervals.

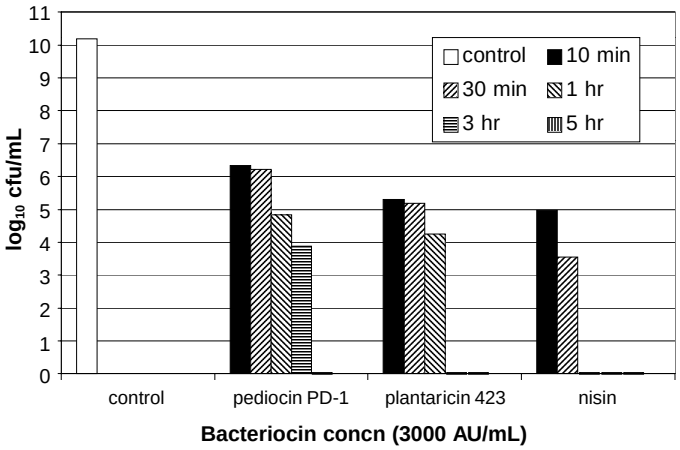

Figure 4 Effect of pediocin PD-1, plantaricin 423, and nisin (3000 AU/mL) on planktonic cells of *O. oeni* in a modified Chardonnay must medium at specific time intervals.

are formed that may include many metabolically dormant cells with altered growth rates and physiology, resulting in increased resistance to antimicrobial agents [11,14,31].

It is well known that biofilms on food-contact surfaces show increased resistance when treated with conventional sanitizers such as acid anionic biocides and quaternary ammonium compounds [13,17,26]. The reduced efficacy of antimicrobial agents is likely due to the ineffective penetration of biofilms [19] or variation in environmental conditions on the contact surface [26]. In some studies where disinfectants proved to be effective, a rapid re-formation of biofilms was reported [22].

Resistance of biofilms to antimicrobial agents is attributed to the combined mechanisms and varied properties associated with the biofilm, including reduced diffusion, physiological changes due to reduced growth rates, the production of enzymes degrading antimicrobial substances, and often exopolysaccharide (EPS) matrixes [10,24,26]. Although EPS may act as a diffusion barrier, molecular sieve, and adsorbent [6], the resistance to antimicrobial compounds is lost as soon as the 3-dimensional structure of the biofilm is disrupted [18,36]. As many strains of *O. oeni* are known to produce EPS [37], this is a key consideration when selecting an antimicrobial agent to prevent biofilm formation.

Plantaricin 423 is homologous to the class IIA pediocins PA1/AcH [38]. Pediocins PA-1/AcH and the lantibiotic nisin form ion-permeable channels in the cytoplasmic membrane of susceptible cells, resulting in an increase in membrane permeability, disturbing the membrane potential, and causing an efflux of intracellular low-molecular-mass compounds [34]. Ultimately, the biosynthesis of macromolecules and energy production is inhibited, resulting in cell death. In some cases cell lysis has been observed for pediocin AcH [4]. Cells treated with nisin over an extended period often undergo autolysis [30]. However, the inhibition of cell wall biosynthesis is a comparatively slow process. Thus, pore formation is considered the primary mode of action of nisin and pediocins PA-1/AcH [33,35].

The mechanism of action of pediocin PD-1 remains to be elucidated and is the subject of current investigation. Preliminary results reveal that a low concentration of pediocin PD-1 induces complete lysis of sensitive cells of *O. oeni*. Cell lysis is only observed at high concentrations of nisin and plantaricin 423. Dislodgement of cells of *O. oeni* in a biofilm, when treated with pediocin PD-1, may be due to cell lysis. This may explain why pediocin PD-1 superseded the other two bacteriocins tested.

The bacteriocins included in this study are also active against other lactic acid and malolactic bacteria, including certain food-spoilage bacteria [8,15,39]. Use of antimicrobial peptides to control the formation of biofilms offers a promising alternative to conventional treatment strategies, especially in the wine industry where implementation of chemical disinfectants, including SO_2, is becoming more restricted. Similar studies have to be conducted to determine the effect of pediocin PD-1, plantaricin 423, and nisin in other food and beverage environments.

Conclusions

The three bacteriocins included in this study, pediocin PD-1, plantaricin 423, and nisin, successfully killed all viable cells in an established biofilm of *O. oeni* that formed in acidic grape medium. Apart from being the most effective against viable cells of *O. oeni* in the biofilm, pediocin PD-1 also proved to be the most effective in the removal of nonviable cells from the stainless steel surfaces. Similar results were obtained in Chardonnay must. Based on results obtained in this study, it is safe to assume that all cells of *O. oeni* (viable and nonviable) will be removed from these stainless steel surfaces after 5 hr at a concentration of 3000 AU/mL pediocin PD-1. These results also suggest that, of the three bacteriocins evaluated, pediocin PD-1 would be the best choice to prevent potential re-formation of malolactic biofilms on stainless steel surfaces. The application of bacteriocins in the control of bacterial biofilm formation could be one of the answers to a safer and environmentally friendlier method of sanitation.

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