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Influences on the antimicrobial activity of surface-adsorbed nisin

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The efficacy of the antimicrobial peptide nisin was examined after adsorption to silica surfaces. Three protocols were used to evaluate nisin's activity against adhered cells of *Listeria monocytogenes*: bioassay using *Pediococcus pentosaceous* FBB 61-2 as the sensitive indicator strain; visualization and enumeration of cells by microscopic image analysis; and viability of adhered cells as determined by Iodonitrotetrazolium violet uptake and crystallization. The activity of adsorbed nisin was highly dependent upon conditions of adsorption. The highest antimicrobial activity of adsorbed nisin occurred with high concentrations of nisin (1.0 mg ml^{-1}) and brief contact times (1 h) on surfaces of low hydrophobicity. Sequential adsorption of a second protein (β-lactoglobulin or bovine serum albumin) onto surfaces consistently resulted in decreased nisin activity. These data provide direction for the development of applications to limit microbial attachment on food contact surfaces through the use of adsorbed antimicrobial peptides.

Keywords: nisin; *Listeria monocytogenes*; bacteriocin; protein adsorption

Introduction

Listeria monocytogenes is a ubiquitous pathogen that has been implicated in several outbreaks of foodborne illness [4,16,24]. These cells are capable of adhering to inert surfaces [22,28], and under favorable conditions can proliferate and form hazardous biofilms [7]. Since nisin can inhibit the growth of *L. monocytogenes* [5,20,21], adsorption of nisin onto food processing surfaces may provide an effective solution to the problem of Listeriae contamination.

Nisin is a small bacteriocin (M.W. = 3552) synthesized by *Lactococcus lactis* subsp *lactis*, which has proven effective as an inhibitor of Gram-positive spoilage bacteria in foods [23,31]. Nisin was affirmed GRAS (generally recognized as safe) by FDA in 1988 [15], and is now used as a biopreservative in 57 countries around the world [23]. After adsorption to silica surfaces, nisin can retain antimicrobial activity [8,12], but the specifics of the protein–surface interactions have not been fully explored. In general, charged amino acids are located on the exterior of a protein, and can affect adsorption through attractive or repulsive electrostatic interactions [18]. Intrinsic stability, in addition to hydrophilic and hydrophobic regions on a protein's surface, can influence its conformation at an interface [27]. Consequently, even minor alterations in the primary structure can result in major changes in interfacial activity [26]. The size and shape of a protein can also be important factors, since large molecules can form more non-covalent bonds with a surface, making desorption of the protein less likely [30]. The intrinsic properties of each protein, therefore, have a major influence on adsorption kinetics, post-adsorptive behavior, and equilibria.

Numerous studies have detailed the adsorption of protein from single-component solutions at an interface, however there are fewer references for research involving sequential and competitive adsorption. The introduction of a second protein to a previously adsorbed film results in some degree of displacement if the second protein has a more favorable electrostatic interaction with the surface [3,34].

Nisin adsorbed to food contact surfaces may have the potential to control pathogenic organisms such as *L. monocytogenes* in our food supply. The purpose of this study was to evaluate the effect of different adsorption conditions on the antimicrobial activity of adsorbed nisin.

Materials and methods

Bacterial culture

Listeria monocytogenes Scott A was originally obtained from C Donnelly, University of Vermont. The culture was maintained at 25° C in BHI broth (Difco Laboratories, Detroit, MI, USA). It was prepared for inoculation by subculturing it into a protein-free medium consisting of 21 g L^{-1} of RPMI 1640 (Sigma, St Louis, MO, USA), 20 g L^{-1} of casamino acids (Difco Laboratories, Detroit, MI, USA), and supplemented with 10 g L^{-1} of glucose. These cultures were grown at 25° C with transfers every 24 h for two days prior to the start of each experiment. Inoculation density was approximately 10^9 cells ml^{-1} as enumerated on BHI agar.

Preparation of hydrophobic surfaces

Polished silicon wafers (1-0-0 orientation, resistivity = 0.4–0.7Ω cm^{-1}, phosphorus doped) were obtained from Wacker Siltronics (Portland, OR, USA). Each surface was circular with an area of 1 cm^2. These have proven to be

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ideal for electro-optical investigation of protein adsorption [25,35] due to the optical flatness, smoothness, and specularity of their surfaces. Protein films which form on silica surfaces are the outcome of true adsorption events, rather than simple entrapment caused by surface irregularities. Silica surfaces were chemically modified to mimic hydrophilic and hydrophobic properties of food contact material. The surfaces were treated with dichlorodimethylsilane (0.01 or 0.1% DDS) in xylene to create two surface types of different hydrophobicities according to the procedure of Krisdhasima *et al* [25]. This process was employed since bacterial adhesion is significantly influenced by the hydrophobicity of the surface [1,29].

Protein solutions

Nisin (lots no NP 26/2 and NP 72) with an activity of 5×10^7 U g^{-1} was obtained from Aplin and Barrett (Dorset, UK). Nisin solutions were prepared by first dissolving 0.1 mg ml^{-1} of nisin in a monobasic phosphate buffer (0.01 M), and then adding sufficient dibasic phosphate buffer (0.01 M) to achieve a final pH of 7. Solutions of bovine β-lactoglobulin (β-Lg) and bovine serum albumin (BSA) (both from Sigma Chemical Co, St Louis, MO, USA), were prepared by dissolving 1 mg ml^{-1} of β-Lg, or 1.81 mg ml^{-1} of BSA in sodium phosphate buffer (0.01 M; pH 7). The choice of β-Lg and BSA for sequential adsorption studies stemmed from earlier research [2] showing that adhesion of *L. monocytogenes* to silica surfaces was greatest when β-Lg was present, and lowest with BSA. The concentrations of nisin and BSA were prepared to be equimolar with 1 mg ml^{-1} of β-Lg, so that these data could be compared with previous adsorption studies [2] involving milk proteins.

Adsorption protocol

To prepare a film of adsorbed nisin, silanized silica surfaces were immersed in solubilized nisin (0.1 mg ml^{-1}) for 1 or 8 h at 25° C. Surfaces were rinsed in 20 ml of pH 7 phosphate buffer (0.01 M) to remove non-adsorbed nisin, and then transferred to phosphate buffer for 5, 10, or 15 h at 25° C. For sequential adsorption, surfaces with preadsorbed nisin films were placed into solutions of BSA or β-Lg for 1 or 8 h before being transferred to buffer. Care was taken during all transfers to prevent the surfaces from drying.

Bioassay for nisin activity

Determination of activity levels for adsorbed nisin was based on a bioassay procedure [11], with slight modifications to accommodate solid samples [8]. Bioassay plates were prepared by autoclaving MRS medium (Difco, Detroit, MI, USA) at 121° C for 15 min, and then inoculating the tempered medium with 0.1% *Pediococcus pentosaceous* FBB 61-2 as the sensitive indicator strain. The media were poured into petri plates to a depth of 5 mm, and stored at 4° C until needed. The activity of adsorbed nisin was determined by placing each disk face down on the media. Each bioassay plate included standards that were prepared by placing 10 μl of a known nisin concentration onto a silanized disk. All samples were tested in triplicate. Bioassay plates containing experimental samples were held at 4° C for 24 h, and then incubated at 37° C for outgrowth of the indicator. Nisin activity was quantified on each disk by measuring the width of the zone of inhibition. The $\log_{10}$ concentration of each nisin control (U ml^{-1}) was plotted against the square of the corresponding zone width to obtain a regression line ($r > 0.98$) for calculating nisin activities of the samples.

Enumeration of adhered bacteria

Surfaces with adsorbed nisin were transferred to individual petri dishes containing cells of a 24-h culture of *L. monocytogenes* grown in protein-free liquid media. Cells were allowed to adsorb to each surface for 3 h before being rinsed as described previously [2], and prepared for microscopic image analysis. Wet surfaces were placed on a slide beneath a coverslip, and scanned at 100× with an oil immersion lens until 10 representative fields from each surface had been recorded. Images were obtained via a Cohu camera (Cohu Inc, San Diego, CA, USA) mounted on an Epistar incident light microscope (AO Scientific Instruments, Buffalo, NY, USA) and processed by a Visionplus-AT board (Imaging Technology Inc, Bedford, MA, USA). Cells were enumerated by Image-Pro Plus (Silver Spring, MD, USA) processing software which also contained a descriptive statistics program.

Cell activity

Iodonitrotetrazolium violet (INT) (Sigma, St Louis, MO, USA) was prepared as a 0.4% (wt/vol) solution in distilled water. This solution was then added to a *L. monocytogenes* surface film on silica surfaces at a ratio of 1 ml INT to 10 ml culture, and held for 1 h at 25° C. During this time the 2-[4-iodophenyl]-3-[4-nitrophenyl]-5-phenyltetrazolium chloride was reduced to iodonitrotetrazolium formazan by the electron transport system of actively respiring cells [38]. The optically dense formazan crystals within respiring cells were observed using brightfield microscopy. Cells which did not contain formazan crystals after 1 h were considered to be inactive or non-viable.

Results and discussion

Activity of adsorbed nisin

The activity of adsorbed nisin films decreased when placed in buffer for 5, 10, or 15 h, regardless of the original nisin solution concentration (0.1 or 1.0 mg ml^{-1}), the adsorption time (1 or 8 h), or the hydrophobicity of the surface (Table 1). For nisin concentrations of 0.1 and 1.0 mg ml^{-1}, the activities were initially similar; however, after 5, 10, or 15 h in buffer, the films from 1.0 mg ml^{-1} of nisin retained significantly more activity. The adsorption time also had an effect on the activity of the nisin film. Surfaces exposed to nisin for 8 h generally had less activity at both concentrations tested, than those exposed to nisin for only 1 h. The activity of adsorbed nisin was initially highest on surfaces of low hydrophobicity. This was true for both adsorption times (1 or 8 h), as well as for both concentrations (0.1 or 1.0 mg ml^{-1}). However, after incubation in buffer for 5, 10, or 15 h, the two surface types had activities which were not significantly different.

Table 1 Activity of adsorbed nisin films made from low (0.1 mg ml^{-1}) and high (1.0 mg ml^{-1}) concentrations of nisin after incubation in buffer for 0, 5, 10, or 15 h

Adsorption time	Surface type	Film age (h)	Nisin activity (U cm^{-2})	
			(0.1 mg ml^{-1})	(1.0 mg ml^{-1})
1 h	HL[a]	0	31.0[b]	30[c]
		5	10.0	19
		10	8.3	16
		15	5.3	17
	HB[d]	0	29.0	26
		5	13.0	20
		10	8.0	17
		15	6.3	18
8 h	HL	0	26.0	27
		5	13.0	17
		10	7.6	12
		15	7.0	12
	HB	0	23.0	25
		5	10.0	17
		10	5.6	14
		15	3.6	14

[a]HL are surfaces of low hydrophobicity
[b]Standard error of the mean was 1.6 U cm^{-2} for low nisin concentrations (0.1 mg ml^{-1})
[c]Standard error of the mean was 3.8 U cm^{-2} for high nisin concentrations (1.0 mg ml^{-1})
[d]HB are highly hydrophobic surfaces

Adhesion of L. monocytogenes *to adsorbed nisin films*

The concentration of nisin used to produce the adsorbed film influenced the number of *L. monocytogenes* cells which adhered. Surfaces immersed in nisin concentrations of 1.0 mg ml^{-1} had fewer cells attached than surfaces exposed to 0.1 mg ml^{-1}, even when incubated for 5, 10, or 15 h in buffer before being exposed to bacteria (Figure 1). This was consistently true, regardless of adsorption time (1 or 8 h) and surface hydrophobicity. The adsorption time also affected the number of cells which were able to adhere, as shown in Figure 2. There were generally fewer cells attached to 1-h films than to 8-h films, independent of nisin concentration and surface type. Hydrophobicity of the surface also influenced the number of cells which adhered. Highly hydrophobic surfaces typically had more cell adhesion than surfaces of lesser hydrophobicity (Figure 3), regardless of nisin concentration and adsorption time.

L. monocytogenes *on adsorbed nisin films*

The respiratory ability of *L. monocytogenes* cells attached to adsorbed nisin films was highly dependent upon the concentration of nisin (0.1 or 1.0 mg ml^{-1}) used during adsorption. High concentrations of nisin typically resulted in fewer respiring cells. The adsorption time (1 or 8 h) did not significantly affect the viability of attached cells. Prolonged exposure of nisin films to buffer increased the number of respiring cells on surfaces of both low and high hydrophobicities (Figure 4), however in general, more cells were active on surfaces of low hydrophobicity.

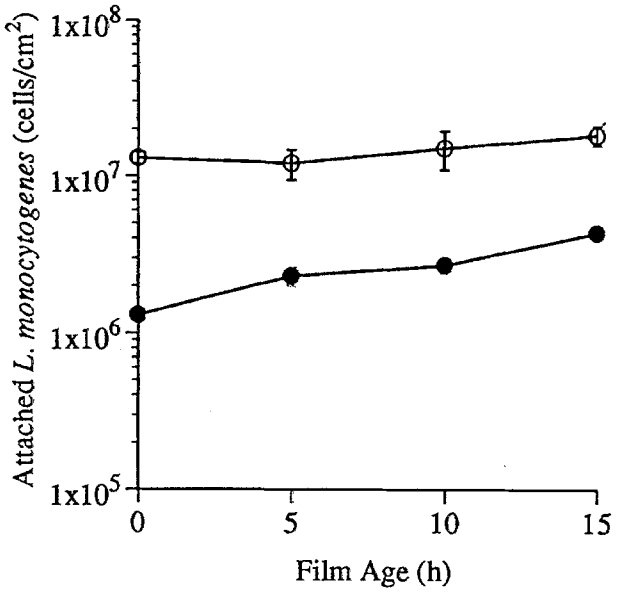

Figure 1 The relationship between the age of adsorbed nisin films on hydrophobic surfaces, and the attachment of *L. monocytogenes* cells following 1 h adsorption with 0.1 mg ml^{-1} (○), and 1.0 mg ml^{-1} (●) of nisin. Error bars represent the standard error of the mean

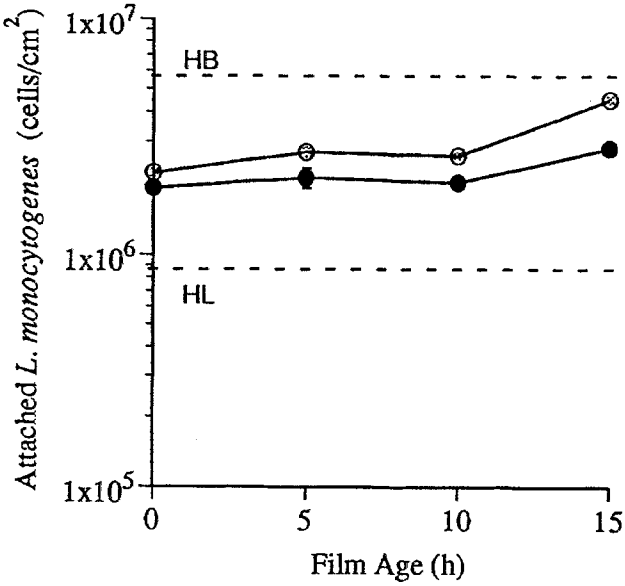

Figure 2 The relationship between the age of adsorbed nisin films on hydrophobic surfaces and the attachment of *L. monocytogenes* cells following adsorption of nisin (0.1 mg ml^{-1}) for 1 (●) or 8 (○) h. Dotted lines represent the number of cells attached to protein-free surfaces of high (HB) and low (HL) hydrophobic character. Error bars represent the standard error of the mean

Antimicrobial effectiveness of adsorbed nisin

The antimicrobial activity of nisin in these cases was possibly influenced by its conformation on the surface. Nisin is considered an amphiphilic molecule, however it is known to possess sections which are predominantly hydrophobic, and would likely experience a larger change in confor-

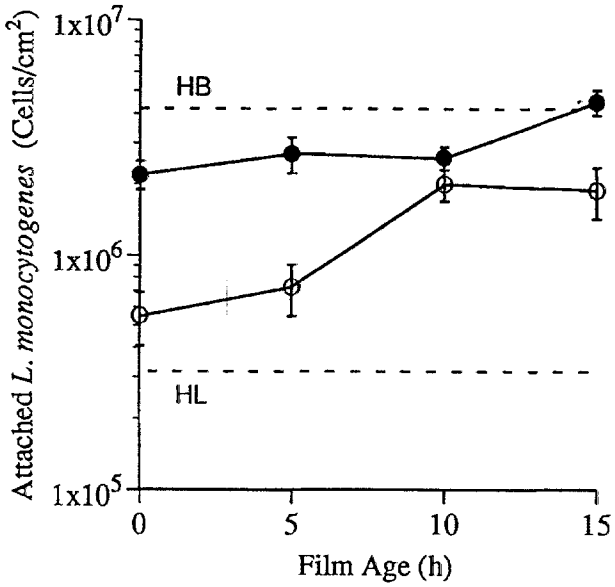

Figure 3 The relationship between the age of adsorbed nisin films, and the attachment of *L. monocytogenes* cells to surfaces of low (○) and high (●) hydrophobicity following adsorption of nisin (0.1 mg ml^{-1}; 8 h). Dotted lines represent the number of cells attached to protein-free surfaces of high (HB) and low (HL) hydrophobic character. Error bars represent the standard error of the mean

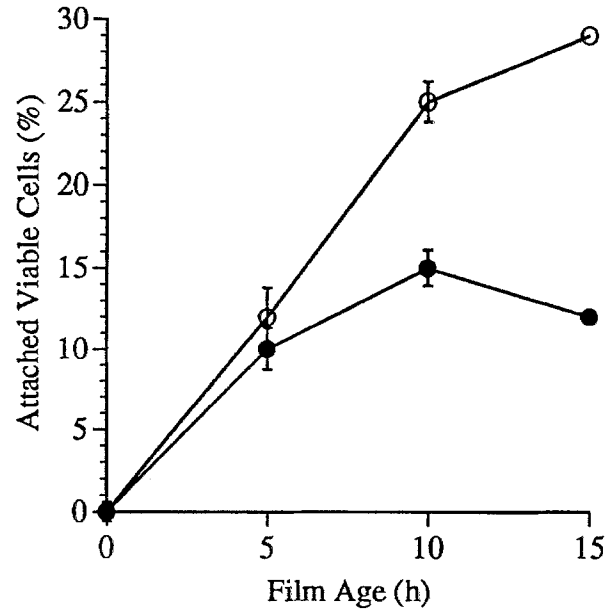

Figure 4 The influence of film age on cell viability, following adsorption of nisin (1.0 mg ml^{-1}; 8 h) on surfaces of low hydrophobicity (○) and highly hydrophobic surfaces (●). Error bars represent the standard error of the mean

mation when adsorbing to a hydrophobic surface than when adsorbing to a more hydrophilic one, if such structural alteration enhanced surface–protein hydrophobic association [25,33]. If greater molecular distortion does occur when nisin adsorbs to a hydrophobic surface, then a lower effectiveness of nisin might be expected, which was observed. The activity of nisin (by bioassay) decreased significantly on highly hydrophobic surfaces, and the number of attached cells increased, as compared to surfaces of less hydrophobic character, where the conformational change of nisin may be smaller.

The cell surface of *L. monocytogenes* is generally believed to be hydrophilic [1,13,29]. The low solid–water interfacial energy associated with hydrophilic solid surfaces would likely inhibit significant adhesion of hydrophilic bacteria [9]. This was confirmed experimentally by the decreased numbers of *L. monocytogenes* cells which were able to adhere to clean hydrophilic surfaces. Viability studies, however, revealed that more cells were alive on surfaces of low hydrophobicity, despite the higher levels of nisin activity registered by bioassay, and the overall lower number of adhered cells on these surfaces.

The exact mechanism of nisin's antimicrobial action is not completely understood, but it is known that nisin disrupts the cytoplasmic membrane of susceptible bacteria causing leakage of intracellular components, and subsequent cell death [23,32]. It is thought that when nisin adsorbs to a microorganism, the hydrophobic sections of the nisin molecule insert into the hydrophobic membrane of the bacterium [19]. A lethal ion channel is formed when additional nisin molecules associate with the embedded nisin molecule in a barrel-stave mechanism [6]. Barrel staves represent individual peptide molecules positioned with their hydrophilic amino acids predominantly on the inside, and their hydrophobic residues facing outward. The resulting structure would be stabilized by hydrophobic association with the hydrophobic environment inside the cell membrane. Additionally, this conformation would orient the hydrophilic sections of the nisin molecule to form a hydrophilic pore, thereby allowing ions to escape from within the cell.

This model may help explain the surface influence on nisin effectiveness that was observed in this study. It is possible that nisin molecules associate to form incomplete barrel-stave structures when brought together on a crowded hydrophobic surface, with the exterior of the staves oriented toward the surface. On hydrophilic surfaces however, the individual nisin molecules would be more inclined to associate hydrophobically with each other, thus preventing the premature assembly of a surface-bound barrel-stave structure. Our data showed lower activity results and higher cell counts when nisin was adsorbed to hydrophobic surfaces, possibly indicating that the self-assembled barrel-stave fragments could not easily desorb and embed themselves in the bacterial membrane. The nisin adsorbed to hydrophilic surfaces, however, would be more freely available to enter the susceptible microorganism and begin its multi-molecular assembly within the bacterial membrane. The greater nisin activities and fewer attached cells observed for surfaces of lower hydrophobicity give some support to this hypothesis.

Elution of adsorbed nisin by BSA and β-Lg

When nisin is adsorbed to silanized silica surfaces it can retain substantial amounts of antimicrobial activity. To be of value on food contact surfaces, however, adsorbed nisin must be able to withstand elution by other proteins. For this study, adsorbed nisin was tested against two well-characterized milk proteins, bovine serum albumin (BSA)

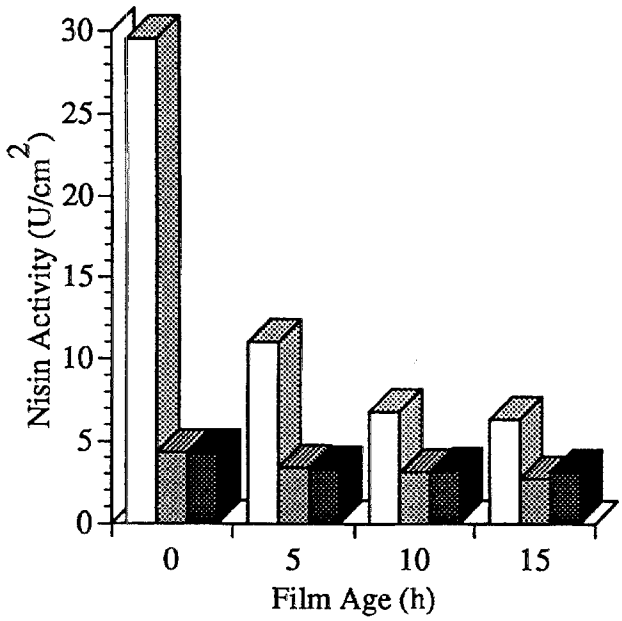

Figure 5 Activity of adsorbed nisin films (0.1 mg ml^{-1}; 1 h) on hydrophobic surfaces following sequential adsorption of BSA (gray bars) or β-Lg (black bars). White bars represent nisin films without the addition of a second protein

and β-lactoglobulin (β-Lg). BSA has a molecular weight of 66000, while β-Lg is much smaller (M.W. = 18400), and usually exists as a dimer at pH 7 [37]. Both are globular proteins which carry a negative charge at pH 7.

The activity of adsorbed nisin decreased substantially when either BSA or β-Lg was allowed to sequentially adsorb (Figure 5). This was true for both surfaces of low and high hydrophobicity. The results for surfaces following 8 h of nisin adsorption were similar, though slightly lower than those following adsorption for 1 h. The viability/respiratory activity of *L. monocytogenes* cells was also significantly affected by sequential adsorption of BSA or β-Lg (Figure 6). An increase in viable counts occurred whenever BSA was added, regardless of surface hydrophobicity, or adsorption time. Viable counts on surfaces sequentially adsorbed with β-Lg also increased with film age, however unlike BSA, cells initially were not viable, and the difference between sequentially adsorbed β-Lg and single component nisin was not statistically significant.

Despite a decrease in nisin activity after addition of a second protein, no increase in the number of attached cells was observed on either β-Lg (Figure 7) or BSA (Figure 8). In fact, the number of adhered cells after sequential adsorption more closely resembled the number found on single-component β-Lg or BSA films, than on single-component nisin films. These results are consistent with data obtained

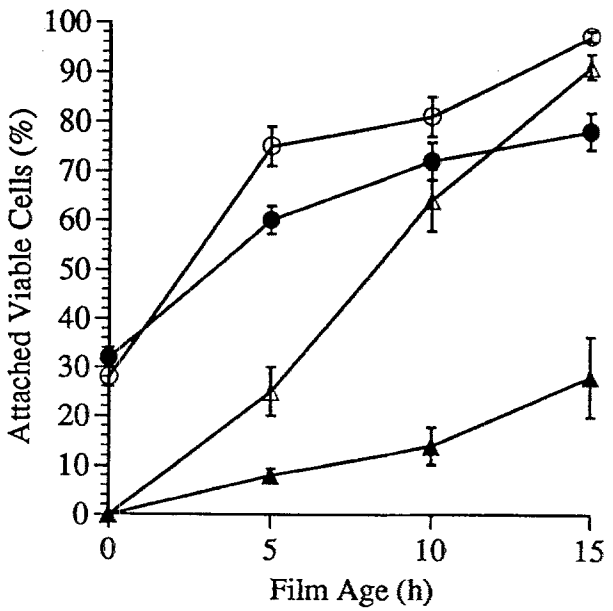

Figure 6 Effect of film age on the viability of *L. monocytogenes* cells attached to adsorbed nisin films (0.1 mg ml^{-1}; 1 h), following sequential adsorption of BSA (●) and β-Lg (▲) on highly hydrophobic surfaces, and BSA (○) and β-Lg (△) on surfaces of lesser hydrophobicity. Error bars represent the standard error of the mean

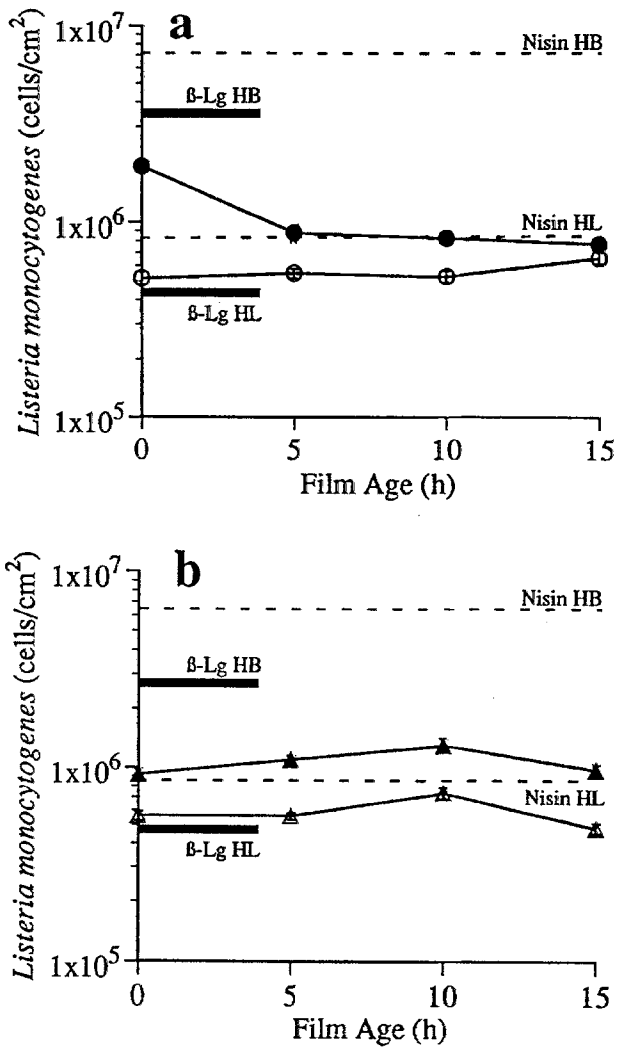

Figure 7 The effect of film age on the number of *L. monocytogenes* cells which attach to nisin films (0.1 mg ml^{-1}) following sequential adsorption of β-Lg for a) 1 h on surfaces of low (○) and high (●) hydrophobicity, and b) 8 h on surfaces of low (△) and high (▲) hydrophobicity. Also represented are single component films of nisin (dashed lines) and β-Lg (heavy lines) on surfaces of high (HB) and low (HL) hydrophobicity. Error bars represent the standard error of the mean

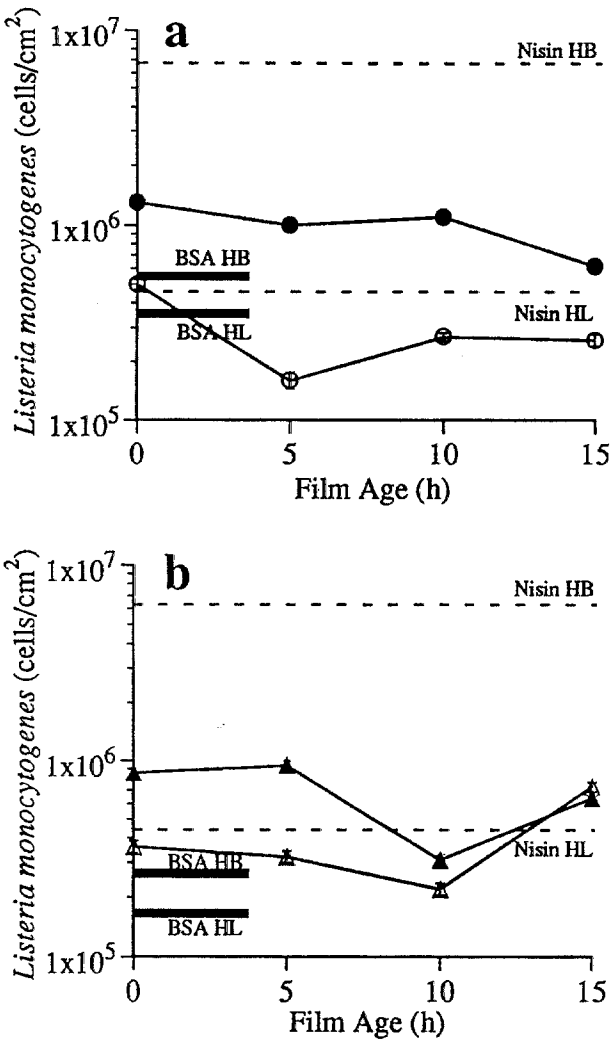

Figure 8 The effect of film age on the number of *L. monocytogenes* cells which attach to nisin films (0.1 mg ml^{-1}) following sequential adsorption of BSA for a) 1 h on surfaces of low (○) and high (●) hydrophobicity, and b) 8 h on surfaces of low (△) and high (▲) hydrophobicity. Also represented are single component films of nisin (dashed lines) and BSA (heavy lines) on surfaces of high (HB) and low (HL) hydrophobicity. Error bars represent the standard error of the mean

by Al-Makhlafi *et al* [2] during adsorption studies with BSA and β-Lg. The absolute number of adhered cells for β-Lg varied between the two studies due to minor procedural variations between researchers. A comparison of β-Lg and BSA, sequentially adsorbed to nisin films, demonstrated that fewer cells adhered when BSA was the second protein than when β-Lg was used. This effect has been observed previously [2], and is thought to be due to the difference in structure of these two proteins. The passivating quality of BSA on surfaces in preventing the attachment of cells has been well documented [14,17,36], as has the ability of β-Lg to promote cellular adhesion [2]. BSA was less likely to desorb from surfaces than β-Lg [2], thus producing fewer protein-free areas. This would explain the higher cell adhesion on β-Lg-covered surfaces, since more cells adhere to bare surfaces than on surfaces with adsorbed protein [2]. Additionally, β-Lg has an average hydrophobicity which is 10% higher than that of BSA [10], and hydrophobicity is correlated with cellular attachment.

This study has shown that the effectiveness of adsorbed nisin depends upon the conditions under which it was adsorbed. More antimicrobial activity was retained when nisin adsorbed to surfaces of lesser hydrophobic character, especially with high nisin concentrations, and brief contact times. However, when milk proteins were allowed to adsorb sequentially, nisin showed limited capacity to withstand elution. Further studies will be required to understand fully the complex events which occur when a second protein is sequentially introduced to an interface containing an adsorbed nisin film. This research was intended to provide direction on optimal use of antimicrobial proteins as an active barrier to microbial adhesion and biofilm formation.

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