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Staphylococcus aureus adheres to human intestinal mucus but can be displaced by certain lactic acid bacteria

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There is increasing evidence that *Staphylococcus aureus* may colonize the intestinal tract, especially among hospitalized patients. As *Staph. aureus* has been found to be associated with certain gastrointestinal diseases, it has become important to study whether this bacterium can colonize the intestinal tract and if so, whether it is possible to prevent colonization. Adhesion is the first step in colonization; this study shows that *Staph. aureus* adheres to mucus from resected human intestinal tissue. Certain lactic acid bacteria (LAB), mainly commercial probiotics, were able to reduce adhesion and viability of adherent *Staph. aureus*. In displacement assays the amount of adherent *Staph. aureus* in human intestinal mucus was reduced 39–44 % by *Lactobacillus rhamnosus* GG, *Lactococcus lactis* subsp. *lactis* and *Propionibacterium freudenreichii* subsp. *shermanii*. Moreover, adherent *Lactobacillus reuteri*, *Lc. lactis* and *P. freudenreichii* reduced viability of adherent *Staph. aureus* by 27–36 %, depending on the strain, after 2 h incubation. This was probably due to the production of organic acids and hydrogen peroxide and possibly in the case of *L. reuteri* to the production of reuterin. This study shows for the first time that *Staph. aureus* can adhere to human intestinal mucus and adherent bacteria can be displaced and killed by certain LAB strains via *in situ* production of antimicrobial substances.

INTRODUCTION

Staphylococcus aureus is an opportunistic pathogen causing a broad range of nosocomial and community-acquired infections. Diseases caused by this bacterium can range from skin infections to foodborne illnesses and severe infections such as endocarditis, osteomyelitis and sepsis (Lowy, 1998). The nasal carriage of *Staph. aureus* is common, 20–50 % of the population (Cespedes *et al.*, 2005), but also intestinal carriage appears to be increased among hospitalized patients (Dupeyron *et al.*, 2001; Ray *et al.*, 2003; Rimland & Roberson, 1986; Squier *et al.*, 2002) and infants (Lindberg *et al.*, 2000). Lindberg *et al.* (2000) showed that over 75 % of Swedish infants have *Staph. aureus* in their stools while Bjorksten *et al.* (2001) showed that 65 % of infants have these bacteria in their stools. Towards adulthood the intestinal carriage of *Staph. aureus* decreases due to increased complexity of the adult microbiota and so-called 'colonization resistance', meaning that the indigenous intestinal microbiota provides protection against colonization of the gastrointestinal tract by exogenous micro-organisms (Lindberg *et al.*, 2004; van der Waaij *et al.*, 1971).

As the microbiota covering the intestinal epithelium has a protective role in preventing colonization of ingested bacteria, certain bacterial strains belonging to the healthy intestinal microbiota can be isolated and used as probiotics. Probiotics are 'live micro-organisms which when administered in adequate amounts confer a health benefit on the host' (WHO, 2001). There are several reports showing that specific probiotic strains protect against gastrointestinal infections (Gorbach *et al.*, 1987; Saavedra *et al.*, 1994; Vanderhoof *et al.*, 1999). Different mechanisms for this have been suggested, such as overall reduction of the gut pH, a direct antagonism against pathogens (production of antimicrobial components such as hydrogen peroxide and bacteriocins), competition for the same binding sites as pathogens, stimulation of the immune system and competition for nutrients (Collins & Gibson, 1999).

The aim of the present study was to assess whether *Staph. aureus* can adhere to healthy human colonic mucus and whether adhesion and viability of potentially adherent *Staph. aureus* can be reduced by specific lactic acid bacteria;

Abbreviation: LAB, lactic acid bacteria.

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a preliminary investigation was made of the possible mechanisms for such effects.

METHODS

Bacterial strains and growth conditions. The *Staphylococcus aureus* strains used were RN4220, which is derived from the strain 8325-4 (Kreiswirth *et al.*, 1983), and a bioluminescent variant of the same strain, *Staph. aureus* RN4220/pAT19 (Vesterlund *et al.*, 2004). *Salmonella enterica* serovar Typhimurium ATCC 14028 was used as a negative control in adhesion assays, as in previous experiments it has exhibited low adhesion (Vesterlund *et al.*, 2005). The 11 strains of lactic acid bacteria (LAB) used are listed in Table 1. All bacterial stocks were stored at −86 °C in 40 % (v/v) glycerol. *Staph. aureus* and *Sal. enterica* serovar Typhimurium were plated first and subsequently cultured by inoculating one colony into Luria–Bertani broth (LB; yeast extract and tryptone were from Pronadisa). When adhesion was measured, 10 µl ml^{-1} of [5′-^{3}H]thymidine (16·7 Ci $mmol^{-1}$; 618 GBq $mmol^{-1}$) was added to the cultures to metabolically radiolabel the bacteria. In the case of the bioluminescent *Staph. aureus* strain, broth and plates were supplemented with 10 µg erythromycin ml^{-1}. *Staph. aureus* and *Sal. enterica* serovar Typhimurium cultures were grown for 16 h without agitation at 30 °C to reach stationary growth phase. LAB strains were grown in de Man, Rogosa and Sharpe (MRS) broth (Oxoid) and they were inoculated directly as a 0·5 % inoculum from the glycerol stocks. When adhesion kinetics was measured, the LAB cultures were supplemented with 10 µl [5′-^{3}H]thymidine ml^{-1}. In the case of *Lb. reuteri* 40 mM glycerol was added as a substrate for production of reuterin in the culture broth (Talarico *et al.*, 1988). LAB were grown in anaerobic conditions for 20 h at 37 °C (except for *Lc. lactis* subsp. *lactis* and *P. freudenreichii* subsp. *shermanii* JS, which were grown for 2 days at 30 °C) in order to reach the late exponential growth phase. All bacterial strains were harvested by centrifugation and washed twice with 1 ml phosphate-buffered saline (PBS; pH 7·2). The OD_{600} of the bacterial suspensions was adjusted with PBS to 0·5 ± 0·02, corresponding to $0{\cdot}5 \times 10^8$ c.f.u. ml^{-1} for *Lb. acidophilus* La5, 1–2×10^8 c.f.u. ml^{-1} for *Lb. casei* Shirota, *Lb. johnsonii* LA1, *Lb. rhamnosus* GG and *Lc. lactis* subsp. *lactis* and 2–4×10^8 c.f.u. ml^{-1} for the remaining strains. Although the number of added bacteria varied from $0{\cdot}5 \times 10^8$ to 4×10^8 c.f.u. ml^{-1}, the bacterial concentrations used led to a linear relationship between added and bound bacteria and thus constant adhesion percentages. Moreover, the saturation level, when the numbers of added bacteria are too high, leading to underestimation of the percentage bound bacteria, was not reached.

Human intestinal mucus. Resected human intestinal tissue was used as a source of mucus. The use of resected tissue was approved by the joint ethical committee of the University of Turku and the Turku University Central Hospital and informed written consent was obtained from the patient. The tissue sample used in this study was from ascending colon and obtained from a colorectal cancer patient from the healthy area adjacent to the tumour. The intestinal material was processed as described earlier (Ouwehand *et al.*, 2002). In short, resected material was collected on ice within 20 min and processed immediately by washing gently with PBS containing 0·01 % gelatin. Mucus was collected by gently scraping with a rubber spatula into a small amount of HEPES-Hanks buffer (10 mmol HEPES l^{-1}; pH 7·4) and centrifuged (13 000 ***g***, 10 min). After measurement of the protein content, the mucus was stored at −20 °C. In adhesion assays the mucus was diluted to a protein concentration of 0·5 mg ml^{-1} with HEPES-Hanks buffer. Mucus was passively immobilized on a polystyrene microtitre plate (Maxisorp, Nunc; and in bioluminescence measurements B&W Isoplate 1450-581, PerkinElmer) as a volume of 100 µl by incubating overnight at 4 °C (Ouwehand *et al.*, 2003).

Adhesion assay. After overnight incubation the mucus-coated microtitre plate wells were washed three times with 250 µl HEPES-Hanks buffer. Then radiolabelled *Staph. aureus* bacteria were added to the wells in a volume of 100 µl (in competition assays in a volume of 50 µl, i.e. 50 µl of *Staph. aureus* incubated alone or together with 50 µl of LAB). Four parallel wells were used in each experiment. Bacteria were allowed to adhere for 1 h at 37 °C and the wells were washed three times with 250 µl HEPES-Hanks buffer to remove the nonadherent bacteria. In exclusion assays LAB were incubated first with the mucus, then washed away and followed by incubation with radiolabelled *Staph. aureus*. Similarly in displacement assays radiolabelled *Staph. aureus* was incubated first with the mucus, then washed away and followed by incubation with LAB. The bacteria bound to mucus were released and lysed with 1 % SDS/0·1 M NaOH by incubation at 60 °C, followed by measurement of radioactivity by liquid scintillation. *Sal. enterica* serovar Typhimurium was

Table 1. Lactic acid bacteria used in the study

Bacterium	Source
Lactobacillus acidophilus La5*	Chr. Hansen, Hørsholm, Denmark
Lactobacillus casei Shirota†	Yakult, Tokyo, Japan
Lactobacillus johnsonii LA1†	Nestlé, Lausanne, Switzerland
Lactobacillus paracasei-33‡	Uni-President Enterprises Corp., Tainan Hsien, Taiwan
Lactobacillus plantarum†	American Type Culture Collection (ATCC 8014)
Lactobacillus reuteri ING1‡	Ingmanfoods, Söderkulla, Finland
Lactobacillus rhamnosus GG†	Valio, Helsinki, Finland
Lactococcus lactis subsp. *lactis*†	Valio, Helsinki, Finland
Enterococcus faecium†	Arla Foods, Viby, Denmark
E. faecium SF68‡	Oriola, Espoo, Finland
Propionibacterium freudenreichii subsp. *shermanii* JS†	Valio, Helsinki, Finland

*A generous gift from Dr B. Grenov, Christian Hansen A/S, Hørsholm, Denmark.
†A generous gift from Dr M. Saxelin, Valio Ltd, Helsinki, Finland.
‡Isolated from a commercial product containing the strain.

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used as a negative control in adhesion assays. The adhesion ratio (%) of bacteria was calculated by comparing the radioactivity of the adhered bacteria to that of the added bacteria.

Viability of adherent bacteria. The bioluminescent indicator strain has been used earlier in the screening of antimicrobial substances produced by LAB against *Staph. aureus* (Vesterlund *et al.*, 2004). In short, this indicator strain allowed stable light production since it harboured *luxAB* genes responsible for light production as well as *luxCDE* genes responsible for the production of the substrate (long-chain fatty aldehyde) for the reaction. The effect of adherent LAB on the viability of adherent *Staph. aureus* was determined in a competition assay. This ensured that the number of adherent *Staph. aureus* was the same regardless of the presence or absence of LAB. After adhesion and washings, the wells were covered either with HEPES-Hanks or with LB supplemented with 1 % glucose. HEPES-Hanks was used as it is used in adhesion assays, whereas LB supplemented with glucose allows the effect of available nutrients on viability to be observed. Results were calculated after 2 h incubation by comparing the viability of the sample to the viability of the adherent *Staph. aureus* incubated without LAB.

Antimicrobial substances produced by LAB. The production of antimicrobial substances by those strains which were able to reduce viability of *Staph. aureus* was studied. A newly developed assay was used for this purpose (Vesterlund *et al.*, 2004). This assay allows detection of organic acids, hydrogen peroxide or bacteriocins produced by LAB. In short, LAB were grown as described above and the culture supernatants were collected by centrifugation, filter-sterilized (0·22 μm pore size) and supplemented with erythromycin. Erythromycin was used as the used indicator strain carries an erythromycin resistance marker. When the production of hydrogen peroxide and bacteriocins was determined, the supernatants were neutralized to pH 7·2 with 4 M NaOH and phosphate buffer (pH 7·2; 0·1 M phosphate final concentration). To determine possible production of hydrogen peroxide by LAB, the supernatants were treated with catalase; to determine possible effects of bacteriocins, the supernatants were treated with proteinase K (both enzymes were purchased from Sigma and used at a concentration of 1 mg ml^{-1}). MRS was used as a negative control and nisin (10 IU ml^{-1}) as a positive control in the assay and they were treated in a similar way as supernatants.

Determination of maximum number of adhered bacteria on mucus and dissociation constants of bacteria

Theory. Michaelis–Menten-type dissociation kinetic models have been used to describe adhesion kinetics of bacteria (Lee *et al.*, 2000). Briefly, the equation:

$$\text{Bacterial cell } (x) + \text{mucus } (e) \underset{k-1}{\overset{k+1}{\longleftrightarrow}} \text{Bacterium}-\text{mucus complex } (e_x)$$

is in equilibrium. When the concentration of free bacterial cells is $(x-e_x)$, the dissociation constant (k_d) for the process can be written as $k_d=(k-1)/(k+1)=(x-e_x)x/e_x$. Rearrangement of the equation gives the concentration of the bacterium–mucus complex: $e_x=e\cdot x/(k_d+x)$. When x is very much larger than k_d, e_x approaches e. As a result the maximum value of e_x is obtained when mucus is saturated with bacteria as e_m, which may be written as $e_x=e_m\cdot x/(k_d+x)$. This equation can be further rearranged to give a linear relationship:

$$1/e_x = 1/e_m + k_d/(e_m\cdot x) \quad (1)$$

Hence, plots of $1/e_x$ against $1/x$ give straight lines, in which the intercepts on the ordinate give the values of $1/e_m$ and those on the abscissa give the values of $-1/k_d$.

Assay. The adhesion assay was performed with twofold dilution series from each bacterium and followed the protocol described above.

Statistical analysis. Pair-wise Student's *t*-test was used to determine the significance ($P<0·05$) of differences between the control and the samples. Results shown are from three or four independent experiments.

RESULTS

Adhesion of bacteria to human intestinal mucus and effect of LAB on adhesion ability of *Staph. aureus*

Among the tested LAB, three strains showed relatively high adhesion: for *Lb. rhamsosus* GG, *Lc. lactis* subsp. *lactis* and *P. freudenreichii* subsp. *shermanii* JS the adhesion ratios were 11·5 %, 10·1 % and 11·3 %, respectively (Table 2). *Staph. aureus* showed similar adhesion as *Lb. acidophilus* La5, 4·4 % and 4·0 %, respectively (Table 2), and showed higher adhesion ($P<0·05$) than the rest of the tested strains. Three of the LAB strains, *Lb. casei* Shirota, *Lb. paracasei*-33 and *E. faecium* adhered poorly, expressing similar adhesion as the negative *Salmonella* control (0·4 %).

When the effect of LAB on the adhesion ability of *Staph. aureus* was tested in displacement, exclusion and competition assays, statistically significant effects ($P<0·05$) of certain LAB were seen only in the displacement assay. Interestingly, the same strains that expressed high adhesion ratios were also able to displace *Staph. aureus* from mucus: *Lb. rhamnosus* GG reduced the amount of adherent *Staph. aureus* by 44 %, *Lc. lactis* by 41 % and *P. freudenreichii* by 39 % after 1 h (Table 3). Furthermore, a trend of reduced adhesion of *Staph. aureus* was seen with *Lb. rhamnosus* in competition

Table 2. Adhesion (%) of bacteria to human intestinal mucus

Results shown are means ± SD of three independent experiments (in the case of *Staph. aureus*, mean ± SD of six independent experiments).

Bacterium	Adhesion (%)
Lb. acidophilus La5	4·0 ± 1·5
Lb. casei Shirota	0·4 ± 0·1
Lb. johnsonii LA1	2·6 ± 0·6
Lb. paracasei-33	0·5 ± 0·1
Lb. plantarum	1·7 ± 0·7
Lb. reuteri ING1	1·1 ± 0·2
Lb. rhamnosus GG	11·5 ± 3·1
Lc. lactis subsp. *lactis*	10·1 ± 2·4
E. faecium	0·3 ± 0·1
E. faecium SF68	1·1 ± 0·1
P. freudenreichii subsp. *shermanii* JS	11·3 ± 3·6
Staph. aureus RN4220	4·4 ± 1·2
Sal. enterica serovar Typhimurium (negative control)	0·4 ± 0·1

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Table 3. Effect of LAB on adhesion ability of *Staph. aureus*

The results (means ± SD of four independent experiments) are represented as percentages compared to adhesion of *Staph. aureus* without LAB (taken as 100 %).

LAB	Displacement	Exclusion	Competition
Lb. acidophilus La5	74·0 ± 22·7	126·6 ± 35·6	96·7 ± 47·4
Lb. casei Shirota	85·2 ± 16·3	110·8 ± 30·7	87·9 ± 21·1
Lb. johnsonii LA1	69·7 ± 13·2	109·9 ± 33·4	91·2 ± 35·1
Lb. paracasei-33	73·5 ± 21·7	112·1 ± 14·5	96·5 ± 20·1
Lb. plantarum	72·8 ± 12·5	111·4 ± 23·1	82·4 ± 35·4
Lb. reuteri ING1	81·1 ± 22·0	109·0 ± 32·7	93·5 ± 18·9
Lb. rhamnosus GG	56·1 ± 9·7*	97·0 ± 16·9	84·9 ± 14·0
Lc. lactis subsp. *lactis*	59·2 ± 13·6*	86·3 ± 25·8	80·4 ± 36·4
E. faecium	74·9 ± 24·3	103·0 ± 23·0	71·5 ± 19·9
E. faecium SF68	78·5 ± 36·7	85·9 ± 31·0	62·9 ± 17·0
P. freudenreichii subsp. *shermanii* JS	60·7 ± 13·5*	79·0 ± 26·0	94·7 ± 28·6

*These LAB significantly reduce adhesion of *Staph. aureus* ($P < 0{\cdot}05$).

(15 %; $P = 0{\cdot}24$), with *Lc. lactis* in exclusion (14 %; $P = 0{\cdot}21$) and in competition (20 %; $P = 0{\cdot}32$), and with *P. freudenreichii* in exclusion (21 %; $P = 0{\cdot}23$) after 1 h, but statistical significance was not reached due to relatively high variation between experiments.

Maximum number of adhered bacteria on mucus and dissociation constants of bacteria

When the reciprocal concentrations of adhered bacteria were plotted against the reciprocal concentrations of the added bacteria, in all cases a linear relationship was observed. With two tested bacteria, *Lb. casei* Shirota (See Fig. 1) and *P. freudenreichii* subsp. *shermanii* JS, two linear regions were observed. This may mean that two binding mechanisms are involved for these bacteria, one for a high bacterial concentration (lower affinity and thus probably non-specific adhesion when the adhesion sites are masked due to a high number of bacteria; forces such as van der Waals and hydrophobic interactions included) and one for a lower concentration, which implies higher affinity and thus probably specific adhesion. Thus at low bacterial concentration, the adhesion of bacterial cells on mucosa involves the maximum number of adhesion sites, and at high bacterial concentration there is self-competition for the adhesion and thus minimum numbers of adhesion sites are involved (Lee *et al.*, 2000).

The linear relationships of the most adhesive strains, *Lb. rhamnosus*, *Lc. lactis*, *P. freudenreichii* and *Staph. aureus*, are shown in Fig. 2. By using equation 1, the maximum number of adhered bacteria on mucus (e_m) and dissociation constants (k_d) for each strain were calculated, and are summarized in Table 4. The values were calculated as c.f.u. per well, which compares a mucus area of 0·1 cm^2. As shown in Table 4, among the 12 bacterial strains tested, *Lb. plantarum*

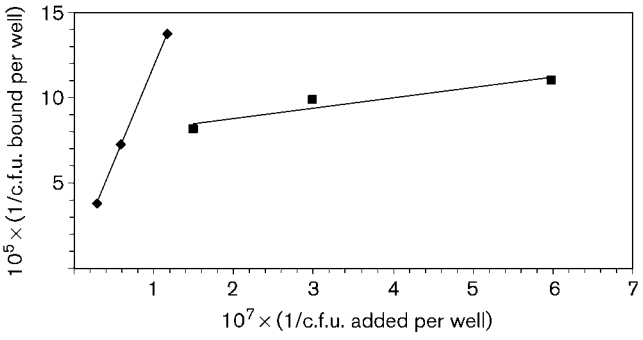

Fig. 1. Adhesion kinetics of *Lb. casei* Shirota. The lines indicate the linear fit according to the least-squares method. ■, Low bacterial concentration; ◆, high bacterial concentration.

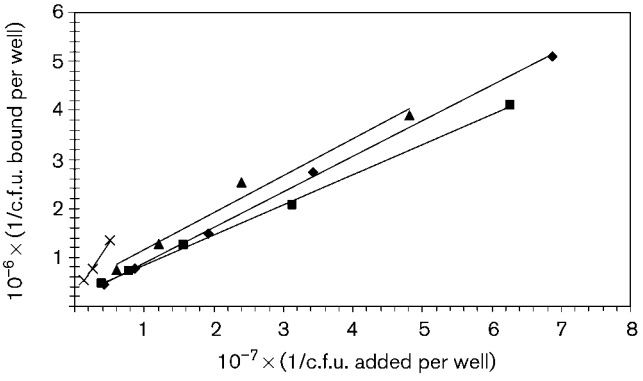

Fig. 2. Double-reciprocal representation of the adhesion of *Lb. rhamnosus*, *Lc. lactis*, *P. freudenreichii* and *Staph. aureus* to human intestinal mucus. The lines indicate the linear fit according to the least-squares method. ■, *Lb. rhamnosus*; ◆, *Lc. lactis*; ▲, *P. freudenreichii*; ×, *Staph. aureus*.

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Table 4. Maximum number of adhered bacteria (e_m) on human intestinal mucus and dissociation constant (k_d) of bacteria

Results shown are means ± SD of three or four independent experiments. Low bacterial concentration: number of added bacteria $<7{\cdot}7\times10^6$ c.f.u. per well for *Lb. casei* and $<5{\cdot}6\times10^5$ c.f.u. per well for *P. freudenreichii*.

Bacterium	Maximum no. of adhered bacteria (e_m) (c.f.u. per well)	Dissociation constant, k_d (c.f.u. per well)
Lb. acidophilus La5	$2{\cdot}0\times10^5$†	$5{\cdot}5\times10^6$‡
Lb. casei Shirota		
High concn	$3{\cdot}7\times10^5$‡	$3{\cdot}0\times10^8$*
Low concn	$1{\cdot}8\times10^4$‡	$1{\cdot}2\times10^6$‡
Lb. johnsonii LA1	$3{\cdot}6\times10^5$†	$8{\cdot}3\times10^6$‡
Lb. paracasei-33	$8{\cdot}4\times10^4$†	$5{\cdot}1\times10^7$
Lb. plantarum	$1{\cdot}4\times10^7$	$1{\cdot}2\times10^9$
Lb. reuteri ING1	$1{\cdot}2\times10^6$†	$3{\cdot}8\times10^8$
Lb. rhamnosus GG	$3{\cdot}7\times10^5$†	$1{\cdot}2\times10^6$‡
Lc. lactis subsp. *lactis*	$6{\cdot}7\times10^6$*	$5{\cdot}1\times10^7$†
E. faecium	$2{\cdot}7\times10^5$†	$8{\cdot}1\times10^7$
E. faecium SF68	$3{\cdot}7\times10^5$†	$2{\cdot}2\times10^7$†
P. freudenreichii subsp. *shermanii* JS		
High concn	$3{\cdot}1\times10^6$	$2{\cdot}4\times10^7$†
Low concn	$1{\cdot}5\times10^5$†	$6{\cdot}3\times10^5$‡
Staph. aureus RN4220	$3{\cdot}3\times10^6$	$7{\cdot}7\times10^7$

*Significantly higher compared to *Staph. aureus* ($P<0{\cdot}05$).
†Significantly lower compared to *Staph. aureus* ($P<0{\cdot}05$).
‡Significantly lower compared to *Staph. aureus* ($P<0{\cdot}001$).

had the highest amount of adhered bacteria on mucus ($1{\cdot}4\times10^7$ c.f.u. per well) and the e_m was 170 times higher compared to *Lb. paracasei*-33, which had the lowest e_m ($8{\cdot}4\times10^4$ c.f.u. per well). However, *Lb. plantarum* showed an adhesion ratio of only 1·7 % (Table 2); this is likely to be due to its having the highest k_d among the tested strains, $1{\cdot}2\times10^9$ c.f.u. per well (Table 5), implying low affinity for adhesion to mucus. *Staph. aureus* had the third highest e_m among the tested strains and this also explained its relatively high adhesion ability (4·4 %; Table 2). The e_m for *Staph. aureus* was $3{\cdot}3\times10^6$ c.f.u. per well and only *Lb. plantarum* ($1{\cdot}4\times10^7$ c.f.u. per well) and *Lc. lactis* subsp. *lactis* ($6{\cdot}7\times10^6$ c.f.u. per well) had a higher e_m. However, the k_d of *Staph. aureus* was relatively high, $7{\cdot}7\times10^7$ c.f.u. per well, indicating that *Staph. aureus* dissociates from mucus more easily compared to six tested LAB: *Lb. acidophilus* La5 ($5{\cdot}5\times10^6$ c.f.u. per well; $P<0{\cdot}001$), *Lb. johnsonii* LJ1 ($8{\cdot}3\times10^6$ c.f.u. per well; $P<0{\cdot}001$), *Lb. rhamnosus* ($1{\cdot}2\times10^6$ c.f.u. per well; $P<0{\cdot}001$), *Lc. lactis* ($5{\cdot}1\times10^7$ c.f.u. per well; $P<0{\cdot}05$), *E. faecium* SF68 ($2{\cdot}2\times10^7$ c.f.u. per well; $P<0{\cdot}05$) and *P. freudenreichii* ($2{\cdot}4\times10^7$ c.f.u. per well; $P<0{\cdot}05$). With lower bacterial concentrations, *Lb. casei* Shirota and *P. freudenreichii* also showed tighter binding ($P<0{\cdot}001$) to mucus compared to *Staph. aureus*: $1{\cdot}2\times10^6$ c.f.u. per well, $6{\cdot}3\times10^5$ c.f.u. per well and $7{\cdot}7\times10^7$ c.f.u. per well, respectively. However, these lower bacterial

Table 5. Effect of adherent LAB on the viability of adherent *Staph. aureus* after 2 h

The results (means ± SD of three independent experiments) are represented as percentages compared to viability of adherent *Staph. aureus* without LAB (taken as 100 %).

LAB	HEPES-Hanks	LB with 1 % glucose
Lb. acidophilus La5	93·0 ± 1·3*	98·1 ± 13·3
Lb. casei Shirota	106·1 ± 9·0	106·9 ± 8·7
Lb. johnsonii LA1	100·8 ± 8·9	87·1 ± 7·8
Lb. paracasei-33	101·2 ± 7·5	111·9 ± 5·2
Lb. plantarum	98·4 ± 4·5	85·4 ± 15·4
Lb. reuteri ING1	98·3 ± 4·7	72·7 ± 10·0*
Lb. rhamnosus GG	105·8 ± 7·7	76·9 ± 14·6
Lc. lactis subsp. *lactis*	97·8 ± 8·7	64·4 ± 3·6*
E. faecium	97·0 ± 5·4	89·2 ± 8·1
E. faecium SF68	98·2 ± 10·3	86·9 ± 7·8
P. freudenreichii subsp. *shermanii* JS	99·6 ± 7·3	72·7 ± 7·6*

*These LAB significantly reduce viability of *Staph. aureus* ($P<0{\cdot}05$).

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concentrations were not used in displacement, exclusion and competition assays (Table 3).

Effect of adherent LAB on the viability of adherent *Staph. aureus*

The effect of adherent LAB on the viability of adherent *Staph. aureus* was determined by using a sensitive reporter system based on a bioluminescent *Staph. aureus* indicator strain. We have previously shown that bioluminescence emission correlates closely with the viability of *Staph. aureus* (Vesterlund *et al.*, 2004). This has been proven also with several other bacterial strains (Beard *et al.*, 2002; Rocchetta *et al.*, 2001; Unge *et al.*, 1999). When bacteria were allowed to adhere to the mucus, the non-bound bacteria were washed away and the microtitre plate wells were covered with HEPES-Hanks, *Lb. acidophilus* La5 was able to reduce the viability of *Staph. aureus* (Table 5). However, when the wells were filled with culture medium, *Lb. acidophilus* had no effect on *Staph. aureus*. Although most of the strains were able to reduce viability of *Staph. aureus* in the presence of culture medium, a statistically significant reduction (27–36 %; $P<0{\cdot}05$) was obtained with *Lb. reuteri*, *Lc. lactis* and *P. freudenreichii*.

Antimicrobial substances produced by LAB

Supernatants of *Lb. reuteri*, *Lc. lactis* and *P. freudenreichii* were collected, neutralized and treated with catalase or proteinase K. Proteinase K treatment did not cause recovery of bioluminescence when compared to non-proteinase-treated but neutralized supernatant, indicating that LAB were not producing bacteriocins against *Staph. aureus*. However, either catalase treatment or neutralization caused recovery, indicating that hydrogen peroxide and organic acids had antimicrobial activity against *Staph. aureus*.

DISCUSSION

The number of both community-acquired and hospital-acquired staphylococcal infections has increased steadily (Kielian *et al.*, 2001). Treatment of these infections has become difficult due to emergence of antibiotic-resistant strains, and new agents to treat and especially prevent staphylococcal infections are needed. The possible intestinal carriage of *Staph. aureus* may have negative health effects; for example during antibiotic treatment it can lead to the overgrowth of bacteria in the intestine and thus antibiotic-associated diarrhoea (Ackermann *et al.*, 2005; Boyce & Havill, 2005). Also association of *Staph. aureus* with inflammatory bowel disease has been suggested, as lumen-derived *Staph. aureus* superantigens have been shown to elicit inflammation in a mouse model (Lu *et al.*, 2003). Moreover, there is accumulating evidence that the colon may serve as a reservoir of antibiotic-resistance genes (Salyers *et al.*, 2004), for example vancomycin-resistant *Staph. aureus* (VRSA) (Ray *et al.*, 2003). Although it is unclear whether *Staph. aureus* belongs to the normal human colon microbiota, it seems that at least among hospitalized patients colonization is possible (Donskey, 2004). The hypothesis of intestinal colonization is also supported by a recent study showing that the caecal mucus layer may provide an important niche for intestinal colonization by *Staph. aureus* (Gries *et al.*, 2005).

As *Staph. aureus* has been found to adhere to nasal mucin (Shuter *et al.*, 1996), we hypothesized here that adhesion to intestinal mucus, of which the main components are mucins, would be possible as well. Moreover, in earlier studies several bacteria have been found to adhere to intestinal mucin oligosaccharides (Moncada *et al.*, 2003). In the present study we used a model based on human intestinal mucus obtained from resected colonic tissue to assess whether *Staph. aureus* adheres to mucus. Human cell-lines Caco-2 and HT-29 do not produce mucus and the mucus-producing cell line HT-29-MTX (Lesuffleur *et al.*, 1990) produces mainly mucins with gastric immunoreactivity (MUC3 and MUC5C) and only few mucins with colonic immunoreactivity (MUC2 and MUC4) (Lesuffleur *et al.*, 1993). Intestinal epithelial cells offer an important model for studying adhesion of bacteria to intestinal areas without a mucus layer, such as Peyer's patches, or areas where the mucus is eroded due to disease, but they can not be used as models for adhesion to mucus. Another advantage of the use of mucus is that the colon's own mucosa-associated microbiota is present and its effect on adhesion is also taken into account. A drawback is the availability of the mucus and also the need to process it immediately.

Here we show for the first time that *Staph. aureus* can adhere to human colonic mucus but can be displaced by specific LAB. *Lb. rhamnosus* GG, *Lc. lactis* subsp. *lactis* and *P. freudenreichii* subsp. *shermanii* were able to displace *Staph. aureus* from human colonic mucus by 39–44 %. Interestingly, the displacement capability was restricted to the LAB with relatively high adhesion ability, *Lb. rhamnosus* GG, *Lc. lactis* subsp. *lactis* and *P. freudenreichii* subsp. *shermanii* JS, with adhesion ratios of 11·5 %, 10·1 % and 11·3 %, respectively (Table 2). Mathematical modelling including determination of the maximum number of adhered bacteria on mucus (e_m) and the binding affinity (k_d) to mucus as well as measurement of viability of adherent *Staph. aureus* were used to explain the mechanism of displacement. *Staph. aureus* showed the third highest e_m among the tested bacteria. Only *Lb. plantarum* and *Lc. lactis* had higher e_m values. This also explained the relatively high binding of *Staph. aureus* to mucus. However, the binding affinity of *Staph. aureus* to mucus was only moderate ($7{\cdot}7\times10^7$ c.f.u. per well; Table 4), and the highest affinity to mucus was obtained with *Lb. rhamosus* GG ($1{\cdot}2\times10^6$ c.f.u. per well). This indicates that *Staph. aureus* can be outcompeted by probiotics which have higher affinity to the mucus. This is likely to explain why *Lb. rhamnosus*, *Lc. lactis* and *P. freudenreichii* displaced *Staph. aureus* from mucus. Similarly under *in vivo* conditions, *Staph. aureus* would probably be washed out more easily from the intestinal mucus surface than for example *Lb. rhamnosus* GG. However, in competition

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assays, LAB showing higher affinity than *Staph. aureus* to mucus were not able to reduce its adhesion. This may have been due to the amounts of bacteria used: in displacement the adherent pathogens were covered with LAB and outnumbered whereas in competition the amounts of bacteria were similar. In exclusion assays there was no effect of LAB on adhesion of *Staph. aureus*, indicating that the bacteria do not use same adhesion receptors.

When viability of adherent *Staph. aureus* was measured in the presence of adherent LAB, the LAB had an effect only when nutrients were available. Adherent *Lb. reuteri*, *Lc. lactis* and *P. freudenreichii* significantly reduced the viability of *Staph. aureus* by 27–36 % within 2 h. The reduction of viability was not due to competition for nutrients (which were present in excess) but rather to the *in situ* production of organic acids and hydrogen peroxide, and in the case of *Lb. reuteri* possibly reuterin (Arques *et al.*, 2004; Vesterlund *et al.*, 2004). Uehara *et al.* (2001) showed that colonization of meticillin-resistant *Staph. aureus* (MRSA) in the oral cavities of newborns was inhibited by the viridans group of streptococci, and that this was probably due to the production of hydrogen peroxide by these streptococci. However, it is unclear whether LAB can produce antimicrobial substances against *Staph. aureus in vivo*. It is also possible that the hydrogen peroxide produced is degraded by the metabolism of other bacteria (Ryan & Kleinberg, 1995).

The emergence of antibiotic resistance among *Staph. aureus* strains and possibly increased intestinal colonization of these bacteria require alternative methods for prevention and treatment of staphylococcal diseases. Our results show that *Staph. aureus* adheres to human colonic mucus and that certain LAB could have antiadhesive and antimicrobial effects against this bacterium. However, it remains for further studies to show that other virulent *Staph. aureus* strains can adhere to colonic mucus *in vitro* and *in vivo*, and to show that LAB have antiadhesive and antimicrobial effects against *Staph. aureus* also *in vivo*.

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