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RESEARCH ARTICLE

Bacteriocin-producing oral streptococci and inhibition of respiratory pathogens

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Abstract

The use of bacteria as probiotics is in continuous development, thanks to their capacity to maintain or restore a host's natural microbiome by interference with and/or inhibition of other microorganisms mediated by antimicrobial peptide production such as bacteriocins. In the oral cavity, *Streptococcus salivarius*, a non-pathogenic and predominant oral species, is one of the major bacteriocin producers that is able to coexist in this environment and reduce the frequency of colonization of the main pathogens involved in upper respiratory tract infections. The aim of this study was to screen oral bacteria colonizing healthy children for their use as potential oral probiotics. Eighty-one α-hemolytic streptococci isolated from nasal and/or pharyngeal swabs of 31 healthy children aged between two and twelve years were isolated. Among them, 13 α-hemolytic streptococci were selected for their bacteriocin-like inhibitory activity against potential pathogens. These strains were tested for bacteriocin production and assayed for their capacity to adhere to HEp-2 cell lines. Our data showed that 13 bacteriocin producer strains were able to inhibit different gram-positive pathogens. Among them one strain, *S. salivarius* 24SMB, deposited as DSM 23307, was selected as a potential oral probiotic, thanks to its safety assessment, ability to inhibit *Streptococcus pneumoniae* and the absence of virulence and antibiotic resistance genes.

Introduction

The microorganisms that inhabit the human oral cavity have been designated as the oral microflora or more recently, as the oral microbiome; the microbiome is the ecological community of commensal, symbiotic, and pathogenic microorganisms that colonize different sites in the human body. The Human Microbiome Project states that an understanding of human health and disease is impossible without understanding the human microbiome (Dewhirst *et al.*, 2010). More than 700 bacterial species are present in the oral cavity and, maintaining the bacterial communities unaltered, has a significant impact on general health by either preventing or causing infections. It has been suggested that changes in the structure of this complex community could contribute to a shift in the balance of the resident microflora to a disease-associated species composition (Marsh, 1991; Aas *et al.*, 2005; Caglar *et al.*, 2005). Bacterial interference, such as antagonism, has a fundamental role in keeping the balance of the microbial ecology associated with the ability of bacterial species to interfere during surface colonization. This phenomenon represents an interesting mechanism of defense because of the capability of endogenous microflora to interfere or inhibit the growth of potential pathogens (Falagas *et al.*, 2008). Clinical evidence of bacterial interference in the treatment of halitosis and/or *Streptococcus pyogenes* infection has been reported by J. R. Tagg and co-workers, attributing this ability to the presence of *Streptococcus salivarius* K12 belonging to the normal commensal flora of the nasopharynx as it is a *sal*A bacteriocin producer strain able to interfere with *S. pyogenes* species (Burton *et al.*, 2006a, b; Power *et al.*, 2008). *Streptococcus salivarius,* a non-pathogenic species and predominant colonizer in the oral microbiome, is one of the major producers of a variety of bacteriocin-like inhibitory

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substances (BLISs), which are active against other microorganisms, reducing the frequency of colonization of the main pathogens involved in upper respiratory tract infections (URTIs) (Wescombe *et al.*, 2009). For this reason, *S. salivarius* is a good candidate for oral probiotics in humans. Probiotics are traditionally associated with gut health, in fact, many probiotics are used to prevent or treat several diseases mainly in the intestinal tract (Gareau *et al.*, 2010), and recently many studies have been involved in the development of oral probiotic applications. Many of them, now, have the GRAS (generally regarded as safe) status, a designation generally used by the Food and Drug Administration (FDA) to indicate that these products can be used without any demonstrable harm to consumers. Some streptococci have a GRAS status for their virtuous nature, and among these *S. salivarius*, even if it is not yet included in the GRAS status, is most closely related to *Streptococcus themophilus*, used by yogurt manufactures, than to other oral species in which the virtuous nature is controversial. (Food & Drug Administration, 2005; EFSA, 2005).

Oral probiotic applications of *S. salivarius* are commercially available: BLIS K12^{TM} Throat Guard that contains *S. salivarius* K12 characterized by strong BLIS activity against *S. pyogenes*, one of the major pathogens involved in bacterial pharyngitis (Wescombe *et al.*, 2009). There have been no reports of negative effects associated with the use of *S. salivarius* as an oral probiotic over the last few years. The use of safe commensal organisms able to interfere with pathogens as a sort of 'bacteria-therapy' may offer a valid alternative to antibiotics in the prevention or treatment of bacterial infections. This hypothesis led us to screen commensal bacteria species from healthy children to use them as possible pathogen-inhibitor agents. We collected 13 α-haemolytic streptococci from nasal and pharyngeal swabs and only one strain of *Streptococcus salivarius* 24SMB was selected as a potential oral probiotic for its characteristics of the following: potential safety for the host, potent capacity of adhesion to HEp-2 cells, and excellent inhibitory activity against *Streptococcus pneumoniae.*

Materials and methods

Isolation of bacteria from pharyngeal mucosa, bacterial strains, and culture conditions

Thirty-one swabs from healthy children taken during routine check-ups were analyzed for α-haemolytic strains. The children did not have URTIs.

The 31 nasal and/or pharyngeal swabs were plated directly onto Columbia Agar Base (Oxoid, Basingstoke, UK), plus 5% horse blood to determine a total microflora population and Mitis Salivarius agar (Difco Laboratories), a selective medium for streptococci, used for differentiation of the viridans strains. Cultures were incubated overnight at 37 °C in 5% CO_2 in air atmosphere. A total of 81 α-haemolytic streptococci were isolated and identified by API Strep and sequencing of 16S rRNA gene and the *sodA* gene encoding for superoxide dismutase and used for correct speciation (Santagati *et al.*, 2009; Teles *et al.*, 2011).

All strains were frozen at −70 °C in Brain heart infusion broth (Oxoid) with 20% glycerol.

Susceptibility testing

Tests for susceptibility to erythromycin, tetracycline, amoxicillin and penicillin were performed by the disc-diffusion test as recommended by EUCAST (http://www.eucast.org/clinical_breakpoints; European Committee on Antimicrobial Susceptibility Testing, 2011).

Test for BLIS production

Each morphologically distinct colony grown in Mitis Salivarius agar was tested for BLIS production using a deferred antagonism test on Columbia Agar Base (Oxoid) supplemented with 5% horse blood and 0.1% $CaCO_3$ (Tagg & Bannister, 1979). The test strain was inoculated diametrically across the test agar plate as a 1-cm wide streak. The visible growth of the test strain was removed using a glass slide, and the surface of the plate was sterilized by exposure to chloroform vapors for 30 min.

The agar surface was then aired to remove residual chloroform for 15 min. Then, Todd Hewitt broth cultures of the indicator strains, grown for 18 h at 37 °C, were streaked across the growth line of the original producer strain for BLIS production. The plates were incubated for 18 h at 37 °C and examined for interference zones with the indicator. The isolates that inhibited the growth of an indicator strain were considered to be inhibitory for that species.

The indicator strains were representative strains of URTIs including AOM pathogens: *S. pyogenes* group (*S. pyogenes* 2812A serotype M18, *S. pyogenes* Spy35370 serotype M1 and $F_2$22 serotype M2), *Haemophilus influenzae* 3ATF, *S. aureus* 10F, *Escherichia coli* 12I, *Pseudomonas aeruginosa* 115, *S. salivarius* ATCC13419, and *B. catarrhalis* 120, *S. pneumoniae* group including three not-typed clinical isolates of *S. pneumoniae* (11ATN, 22ATN and 148) and three *S. pneumoniae* serotype 19A (BT *S. pneumoniae;* CR *S. pneumoniae*; GC *S. pneumoniae*), which are responsible for cases of pediatric meningitis in Sicily, Italy. All *S. pneumoniae* used were resistance to erythromycin, clindamycin, and susceptibility to penicillin and ampicillin.

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All strains used as indicator strains in the deferred antagonism test were clinical strains except *S. salivarius* ATCC13419. The BLIS production was also tested using a deferred antagonism test on Trypticase Soy Yeast Extract Calcium agar (Trypticase Soy Broth; Oxoid) + 2% Yeast extract (Oxoid) + 1.5 agar (Oxoid) + 0.1% $CaCO_3$.

Pulsed-field-gel-electrophoresis and DNA hybridization

Total bacterial DNA was extracted in agarose plugs as described before (Santagati *et al.*, 2009). After digestion with the SacII enzyme (TaKaRa BIO), macro-restriction fragments were resolved in a 1% agarose gel using 0.5× tris-borate-ethylene diamine tetra-acetic acid buffer (BioRad) at 14 °C. The CHEF DRPFGE (BioRad) system was used, and switch and run times were 1″ to 15″ for 20 h, with a voltage gradient of 6 V cm^{-2}. The macrorestriction fragments were visualized by a blue-light transilluminator (Safe Imager Invitrogen) after staining with 1× SYBR Green (SYBR Safe DNA gel staining Invitrogen) in TBE0.5×. The macrorestriction fragments were transferred from the gel to a nylon Hybond N+ membrane, (Amersham International UK) in a downward direction using a Vacuum blotter 785 (BioRad) and denaturing solutions (NaOH 0.5 M/NaCl 1.5 M). DNA fragments were immobilized by UV radiation (Ultraviolet Crosslinker, Amersham). The hybridization assays with *sag*A, *smeZ-2*, *spe*B, *speC*, *speJ*, *speG*, *prtF*, and *sof* probes were performed using the 'ECL Direct Nucleic Acid Labeling and Detection System' (RPN 3000 Amersham), following the protocol provided with the kit. The probes were obtained by PCR from the *S. pyogenes* SF370 and *S. pyogenes* 2812A genome and purified with the QIAquick PCR purification kit (Qiagen) using the primers described in Table 1.

Table 1. Primers used in this study

Gene	Primer name	Primer sequence (5′-3′)	Amplicon size (bp)	Accession number
sivA	MS386	TGATTTAACGCAACATCATCATCAAC	640	GQ857551
	MS387*	CTTCATACATCAAATATCCATTCCTTG		
nisA	MS389	CAGGTGCATCACCACGCATTA	600	DQ146939
	MS390*	CAACCACTGAGTATCCAATCTTATACCC		
nisF	MS391	TGGAACAGTCTGTGGTTTATTAGGAG	560	U17255
	MS392*	TCACATTCCTCCATGCACAATCTTAA		
nsuB	MS394	ATCCTATGGTGAAGAGTATCGAGAT	774	DQ146939
	MS395*	CTAGCCCCTAGATACTTAGAACAACC		
mutII	MS396	GGGTGGTAATCGTTGGTGGCAAG	560	U40620
	MS397*	GCATATCGCCTACTACTTGAAGGCTG		
mutIII	MS398	TTGTGTCGCATGTCAGCGGC	664	AF207710
	MS399*	GGAGTTGATCTCGTGTTATAACGGGC		
*sag*A	MS380	ATGTTAAAATTTACTTCAAATATT TTA	300	CP000829
	MS381*	TTATTTACCTGGCGTATAACTTCCG		
smeZ-2	MS382	GTAATTGATTTTAAAACCAGTCATAAC	400	AF086626
	MS383*	GATACTTTCTTTATCTCTATATCCTAC		
*spe*B	MS384	GGCATGTCCGCCTACTTTACCGA	800	NC_007297
	MS385*	CAGGTGCACGAAGCGCAGAAG		
speC	MS410	TACTGATTTCTACTATTTCACCTATCATC	447	NC_002737
	MS411*	TCTGATTTTAAAGTCAATTTCCTGG		
speG	MS412	GCTATGGAAGTCAATTAGCTTATGCAG	488	NC_007297
	MS413*	CCGATGTATAACGCGATTCCGA		
speJ	MS414	CACTCCTTGTACTAGATGAGGTTGC	508	NC_007297
	MS415*	ACGCATACGAAATCATACCAGTAG		
prtF	MS400	CGGAGTATCAGTAGGACATGCGGA	882	NC_011375
	MS401*	CTCCCACCAACATTGCTTAATCCA		
sof	MS402	ATGCCTGGTTGGGTATCTTCGGT	406	NC_011375
	MS403*	AGAGAACAAAACGTTCTGCGCCTA		

*Reverse primers.

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Plasmid extraction and gene localization

For all bacteriocin producer strains, the presence of plasmids was investigated by Plasmid Midi Kit (Qiagen) according to the manufacturer's instructions, preceded by one lysis step with 20 mg mL^{-1} lysozyme solution and incubated at 37 °C for 30 min. In addition, the chromosomal versus plasmid localization was evaluated by the I-*Ceu*I method, as described previously (Liu *et al.*, 1993). *Streptococcus salivarius* K12 was used as positive control. Total genomic DNA was digested overnight with I-*Ceu*I and was subjected to pulsed-field gel electrophoresis (PFGE) as previously described. Southern hybridization was carried out using an *sbo*B gene and 16S rRNA gene obtained from 7SMB *S. mitis* and *S. salivarius* K12.

Detection of bacteriocin-encoding genes

Genes responsible for bacteriocin production (*sal*A, *sbo*B, *siv*A, *srt*A, *scn*A, *nis*A, *nis*F, *nsu*B, *mut*II, *mut*III, *srtF*, *lan*B, and *lan*C) were amplified by PCR using primers previously published (Hynes *et al.*, 1993; Karaya *et al.*, 2001; Upton *et al.*, 2001; Wescombe *et al.*, 2006; Wirawan *et al.*, 2006) and those designed for this study see Table 1. For *mef*(E) detection and PCR, we used previously published protocols (Santagati *et al.*, 2009).

In vitro safety assessment

To exclude the presence of potential virulence determinants, hemolytic activity and detection of virulence genes were assayed.

Hemolytic activity

The hemolytic ability of 24SMB was tested using: (1) horse blood in a base containing starch medium (Saunders & Ball, 1980); (2) TSA with 5% defibrinated sheep blood; and (3) Columbia Agar with 5% defibrinated sheep blood.

Detection of virulence genes

In *S. salivarius* 24SMB, the main streptococcal virulence genes, *sag*A (streptolysin S), *smeZ-2* (mitogenic exotoxin Z), *spe*B (pyrogenic exotoxin), *speC*, *speG* and *speJ* (exotoxin type C, G, J), *prtF*, (fibronectin-binding protein), and *sof* (serum opacity factor) were detected by PCR using the primers described in Table 1 and by hybridization with specific probes. *Streptococcus pyogenes* SF370 and *S. pyogenes* 2812A were used as positive control.

Sequencing and sequence analysis

All amplification products were purified by the 'QIAquick PCR gel extraction Kit' (Qiagen) and sequenced with a LICOR DNA 4000L sequencer. The DNA sequence was analyzed by the Gapped BLAST software (Altschul *et al.*, 1997).

Test of adhesion of *S. salivarius* on HEp-2 cell line

This method used the HEp-2 cell line (human, Caucasian, larynx, carcinoma, squamous cell), ATCC CCL 23. The bacteria were grown from 16 to 18 h in 5 mL of Todd Hewitt broth.

The density of all bacterial cultures was adjusted photometrically so that cultures contained approximately 10^5–10^6 CFU mL^{-1} prior to their use in the assay.

HEp-2 (ATCCCCL23) cells were maintained in Eagle's Minimal Essential Medium (EMEM; Invitrogen). The medium was supplemented with 10% fetal bovine serum (FBS), penicillin (100 IU mL^{-1}), and streptomycin (100 μg mL^{-1}). HEp-2 adherence assays were conducted as previously described (Benga *et al.*, 2004). The number of adherent bacteria was obtained by subtraction from the total number of CFU. This is expressed as percentage adherence. All experiments were performed in duplicate wells and repeated at least three times. In each experiment, wells containing only cells were used as controls. Bacterial adhesion to the HEp-2 cell layer was also performed on microscope cover glasses as previously described (Guglielmetti *et al.*, 2010). Briefly, approximately 2×10^8 cells resuspended in PBS were incubated with a monolayer of HEp-2 cells for 1 h at 37 °C. After washes with PBS, the cells were fixed with 3 mL of methanol and incubated for 8 min at room temperature. Cells, after the removal of methanol, were stained with 3 mL of Giemsa stain solution (1 : 20; Carlo Erba, Milan, Italy) for 30 min at room temperature. Wells were washed and then dried at 30 °C for 1 h. Adherent bacteria were examined microscopically (magnification ×100) in 20 random microscopic fields obtaining bacterial counts and averages.

Adhesion indexes (ADI; number of bacteria/100 Hep-2 cells); strong adhesion: ADI of > 2500; good adhesion: ADI of between 2500 and 500; weak adhesion: ADI of between 500 and 100; no adhesion, ADI of < 100 (Guglielmetti *et al.*, 2010).

Patent number

24SMB *S. salivarius* was patented (Pat. num: WO 2011/125086) and registered as DSM 23307.

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Results

Identification and molecular characterization of bacterial isolates

The averages of the total microflora population and oral streptococci obtained from 31 samples from healthy donors were approximately 10^6 and 10^2 CFU mL^{-1}, respectively, and a total of 81 α-hemolytic streptococci were isolated, among these only 13 were selected for their inhibitor activity against indicator strains (i.e. bacteriocin producers). These strains were identified by sequencing the 16S rRNA gene and the *sod*A genes, which are able to provide an accurate identification at the species level. The nucleotide sequence analysis identified the following strains: four *S. salivarius,* eight *S. mitis*, and only one *S. sanguis*.

Inhibitory activity and detection of bacteriocin genes

All α-haemolytic streptococci were tested for production of bacterial inhibitors by deferred antagonism against the indicator strains *S. pyogenes* group, *S. pneumoniae* group, *H. influenzae* 3ATF, *S. aureus* 10F, *E. coli* 121, *P. aeruginosa* 115, *S. salivarius* ATCC13419, *B. catarrhalis* 120. The indicator strains included the main pathogens responsible for URTIs. We found five *S. mitis* (5SMB, 6SMB, 8SMB, 10SMB, 11SMB) and four *S. salivarius* (1SMB, 2SMB, 24SMB, 4SMB) active against six *S. pneumoniae* strains (11ATN, 22ATN and 148 *S. pneumoniae* and BT, CR, GC *S. pneumoniae* serotype 19A); two strains: *S. sanguis* 13SMB and *S. mitis* 9SMB active against *B. catharralis* and two *S. mitis* strains (7SMB and 12SMB) showed a broad inhibitory activity against *S. pyogenes, S. pneumonie*, *S. aureus*, and *S. salivarius* (Table 2).

It is interesting to note that 24SMB BLIS activity assayed on TSYCa, using the same standard method, demonstrated a change in the inhibitory activity with respect to that obtained in blood agar-calcium: this strain is able to inhibit not only *S. pneumoniae* strains, but also three clinical isolates of *S. pyogenes* – 2812A, Spy35370 and $F_2$22 – belonging to serotype M18, M1, and M2 respectively.

All strains did not show any activity against *E. coli, P. aeruginosa*, and *H. influenzae*. In only three of the 13 strains were bacteriocin characterized at the molecular level: *sal*A in *S. mitis* 11SMB and *sbo*B in *S. mitis* 7SMB and 12SMB. In the last two strains, the *sbo*B gene was not associated with the *sal*A gene and it had a different location with respect to *sbo*B characterized in *S. salivarius* K12 (Hyink *et al.*, 2007) in which it was located in a transmissible megaplasmid; however, our strains were plasmid free demonstrated by the I-*Ceu*I analysis (data not shown).

All the other isolates, despite showing an evident inhibition of the indicator strains, did not possess any of the usual streptococcal bacteriocin genes such as *sal*A, *sbo*B, *siv*A, *srt*A, or *scn*A. These results led us to make an extensive search in Genbank to identify other bacteriocin genes predominant in other species such as *Lactococcus lactis, S. mutans*, and *S. uberis*. For this reason, we designed primers to detect the presence of *nis*A, *nis*F, *Nsu*B, *mut*II, *mut*III, *srtF*, *lan*B, and *lan*C genes. In all cases, we were unable to amplify any of the above described genes. The Columbia Agar containing $CaCO_3$

Table 2. Inhibitory activity of α-hemolytic streptococci by deferred antagonism tests and bacteriocin genes

Producer Strains	Putative bacteriocin genes	Inhibition of Indicator strains					
		S. pyogenes strains	*S. pneumoniae* strains	*H. influnzae* 3A-TF	*S. aureus* 10F	*S. salivarius* ATCC13419	*B. catarrhalis* 120
1SMB *S. salivarius*	nd	–	+	–	–	–	–
2SMB *S. salivarius*	nd	–	+	–	–	–	–
24 SMB *S. salivarius*	nd	–	+	–	–	–	–
4SMB *S. salivarius*	nd	–	+	–	–	–	–
5SMB *S. mitis*	nd	–	+	–	–	–	–
6SMB *S. mitis*	nd	–	+	–	–	–	–
7SMB *S. mitis*	*sbo*B	+	+	–	+	+	–
8SMB *S. mitis*	nd	–	+	–	–	–	–
9SMB *S. mitis*	nd	–	–	–	–	–	+
10SMB *S. mitis*	nd	–	+	–	–	–	–
11SMB *S. mitis*	*sal*A	–	+	–	–	–	–
12SMB *S. mitis*	*sbo*B	+	+	–	+	+	–
13SMB *S. sanguis*	nd	–	–	–	–	–	+

nd, not detected.

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excluded inhibitory activity mediated by non-specific metabolic production.

Safety assessment by antibiotic susceptibility, virulence genes, and metabolic profile

From among the 13 producer strains, we selected only the four *S. salivarius* strains because of their absence of pathogenic potential; for this reason, the safety assessment was performed only for these four strains. The disc-diffusion test for erythromycin, tetracycline, clindamycin, amoxicillin, and penicillin showed that only one strain, *S. salivarius* 24SMB (DSM 23307), was susceptible to all the antibiotics tested, while the other strains were macrolide resistant carrying the *mef*(E) gene, which is responsible for the M-phenotype of resistance, and 1SMB was also penicillin- and amoxicillin resistant. The presence of harmful enzymatic reactions for the human host and the presence of virulence genes were assessed in *S. salivarius* DSM 23307: it was found to have no hemolytic activity or harmful enzymatic activity. In addition, it also lacked the main streptococcal virulence genes, that is, streptolysin S, mitogenic exotoxin Z, pyrogenic toxin B, fibronectin-binding protein, serum opacity factor, and exotoxin type C, G, and J as demonstrated by the lack of PCR amplification and the absence of any hybridization with the corresponding probes. Figure 1 shows a representative gene example.

Test of adhesion of *Streptococcus salivarius* on HEp-2 cell line

Streptococcus salivarius 24SMB, *S. salivarius* K12, and one representative *S. salivarius* 4SMB were tested for their ability to adhere to the HEp-2 cell line. The results are expressed as percentage adherence comparing the initial inoculum, the initial cell count (10^6CFU mL^{-1}) and the cells that adhered to HEp-2 cells after extensive washing with PBS. We found that between 50% and 57% of *S. salivarius* DSM 23307 remained attached to the HEp-2 monolayer, a similar percentage (50–60%) for *S. salivarius* K12, while *S. salivarius* 4SMB showed the lowest percentage of adhesion (25–30%). Our result on HEp-2 cell line adhesion was confirmed by microscopic examination. The adhesion index (ADI; number of bacteria/HEp-2 cell) of *S. salivarius* 24SMB and *S. salivarius* K12 (used as positive control) showed a similar value of adhesion indicating good adhesion, on the contrary, *S. salivarius* 4SMB showed a weak adhesion Fig. 2. For this reason, *S. salivarius* 24SMB was patented and registered as DSM 23307.

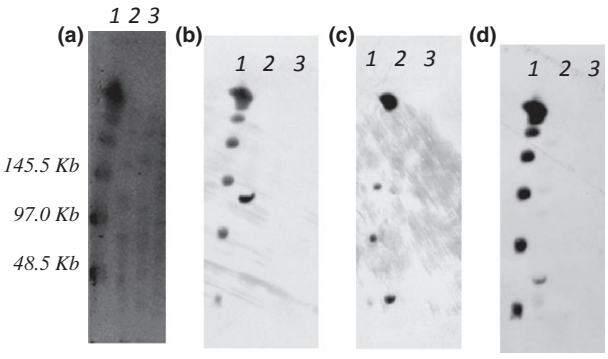

Fig. 1. Southern blots of SacII PFGE hybridized with several virulence genes as probes, Lane 1: λ DNA PFGE marker, Lane 2: *Streptococcus pyogenes* SF370; Lane 3: *Streptococcus salivarius* 24SMB. (a): Lanes containing SacII-PFGE from *S. pyogenes* SF370 and *S. salivarius* 24SMB; (b): hybridization with *spe*B probe; (c) hybridization with *smeZ* probe; (d) hybridization with *sagA* probe.

Discussion

The aim of this study was to characterize oral bacteria for use as potential probiotics in the prevention and/or treatment of URTIs including chronic and/or acute otitis media. We isolated 13 BLIS strains of oral streptococci, with only four strains belonging to the *S. salivarius* species. Among them, one strain, *S. salivarius* DSM 23307, isolated from nasal swabs, possessed the main characteristics that make it suitable to be used as a potential oral probiotic and was further characterized. It is well known that the α-hemolytic streptococci – such as *S. salivarius, S. mitis, S. mutans*, and *S. sanguis* – isolated from the human pharynx have been the target of many studies because of their ability to interfere with respiratory pathogens (Book, 1999; Roos *et al.*, 2000; Power *et al.*, 2008). They are predominant in the oral cavity, being the main producers of antimicrobial peptides such as bacteriocins and for this reason they could be good candidates for oral probiotics (Wescombe *et al.*, 2009; Guglielmetti *et al.*, 2010), even if some species such as *S. mitis* have been associated, in some cases, with infections, resulting in their exclusion for their potential pathogenicity (Johnston *et al.*, 2010). On the other hand, in the oral microbiome, *S. salivarius*, a primary and predominant colonizer of oral mucosal surfaces in humans, is characterized by low pathogenic potential and is able to persist as a dominant species in the oral cavity (Horz *et al.*, 2007).

The safety of probiotics has been the subject of active discussion and, to date, there have not been any clear general guidelines for all strains: *S. salivarius* is a typical example, in fact, this species, in other parts of the world but not in Europe, has been included in the GRAS status (Burton *et al.*, 2005, 2006a, b). For this reason, the safety assessment of each strain that could be used as a probiotic represents the fundamental step for a good commercial

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(a)

Species	Strain	AdI
Streptococcus salivarius	K12	1059
Streptococcus salivarius	24SMB	1362
Streptococcus salivarius	4SMB	500

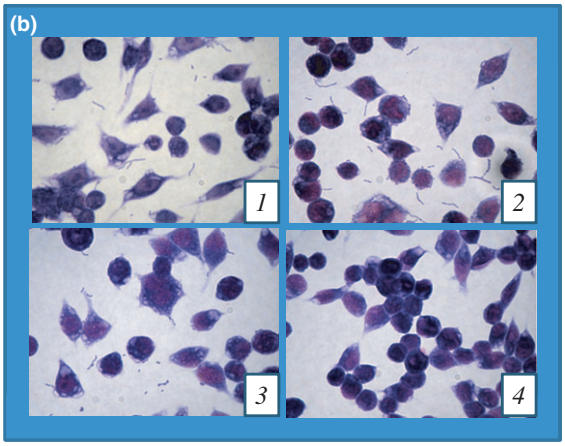

Fig. 2. Bacterial adhesion to HEP-2 cell layer. (a) Adhesion indexes (AdI; number of bacteria/100 FaDu cells). (b) Cell layers observed after Giemsa staining, using light microscopy. Bar, 8 μm. (1) *Streptococcus salivarius* K12; (2) *S. salivarius* 24SMB; (3) *S. salivarius* 4SMB; (4) Negative control.

product. The report of the FAO/WHO Working Group (Food and Drug Administration 2008) recommended the need to determine: (1) the genus and species of the probiotic strain; (2) antibiotic resistance patterns, in particular, for resistance genes associated with mobile elements; (3) virulence determinants; (4) metabolic activity that could be harmful for the host; and (5) hemolytic activity if the strain belongs to species that can have hemolytic potential.

Streptococcus salivarius, even if it does not have the GRAS status yet, is closely related to *Streptococcus thermophilus*, a species belonging to the *salivarius* group with major economic importance because of its wide use for production of yoghurt and cheese.

Many comparative genomic studies regarding taxonomy and phylogeny among dairy streptococci have demonstrated that *Streptococcus* spp. are clustered in two main groups: one comprising *S. macedonicus*, and *S. bovis* species and the other *S. thermophilus* and *S. salivarius*: the species in each group show strong similarities in the DNA sequence of the ribosomal locus (Facklam, 2002; Mora *et al.*, 2003). For all these reasons, *S. salivarius* has been commercialized for several years as an oral probiotic (BLISK12^{TM} Throat Guard) because of its safety and its excellent capability to interfere with other bacteria.

Streptococcus salivarius DSM 23307, characterized in this study, is sensitive to the main antibiotics used for the treatment of URTIs, does not possess dangerous enzymatic reactions, as demonstrated by its metabolic profile, and lacks the main streptococcal virulence genes, that is, *sag*A, *smeZ-2,* and *spe*B. All this is further proof of its virtuous nature. Moreover, a fundamental property of this strain is its strong BLIS activity against *S. pneumoniae* including virulent and multidrug resistance strains such as the most diffused serotypes circulating in our country involved in severe infections in children and adults (Resti *et al.*, 2010; Ansaldi *et al.*, 2011); furthermore, it does not interfere with other *S. salivarius* strains.

The BLIS activity of *S. salivarius* DSM 23307 is not associated with typical streptococcal bacteriocin genes such as *sal*A, *sbo*B, *srt*A, *scn*A, and *siv*A as demonstrated by PCR experiments, suggesting the presence of variant or different antimicrobial peptide genes. These molecular data correlated with its unusual inhibitory spectrum primarily oriented versus *S. pneumoniae* and only in particular growth conditions, that is, in TSYCa versus *S. pyogenes*.

The strong *in vitro* capacity to inhibit *S. pneumoniae* resembles the BLIS activity of the nisin inhibitory spectrum (Goldstein *et al.*, 1998), even if the presence of this gene was excluded.

Another essential characteristic of strains for use as bacterial replacement therapy is their capability to adhere to host tissues: the cells of *S. salivarius* DSM 23307 remained attached to the HEp-2 monolayer demonstrating a good adherence capacity.

In conclusion, in this study, we identified one strain as a potential oral probiotic, possessing desirable characteristics for bacteria-therapy: *S. salivarius* DSM 23307 possesses a strong activity against *S. pneumoniae* and is harmless to other *S. salivarius* strains, it is non-pathogenic for the host as demonstrated by safety assessment and it efficiently adheres to human larynx cells.

Further studies on *S. salivarius* DSM 23307 are ongoing both to completely characterize the antimicrobial peptides and to confirm its probiotic use in humans.

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