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In vitro Inhibition of Clinical Isolates of Otitis Media Pathogens by the Probiotic *Streptococcus salivarius* BLIS K12

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Abstract
Otitis media is a common childhood infection, frequently requiring antibiotics. With high rates of antibiotic prescribing and increasing antibiotic resistance, new strategies in otitis media prevention and treatment are needed. The aim of this study was to assess the in vitro inhibitory activity *Streptococcus salivarius* BLIS K12 against otitis media pathogens. Efficacy of the bacteriocin activity of *S. salivarius* BLIS K12 against the otitis media isolates was assessed using the deferred antagonism test. Overall, 48% of pathogenic isolates exhibited some growth inhibition by *S. salivarius* BLIS K12. *S. salivarius* BLIS K12 can inhibit the *in vitro* growth of the most common pathogens.

Keywords Otitis media · Recurrent otitis media · Otitis media pathogens · Probiotics · *S. salivarius* BLIS K12 · In vitro inhibition

Introduction

Acute otitis media (AOM) is the most common bacterial infection occurring in pre-school aged children [1, 2]. A proportion of children go on to develop recurrent AOM or persistent middle ear effusions, with a significant impact on the affected families [3, 4]. The three key otopathogens in young children have been shown to be *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Moraxella catarrhalis* [5, 6].

To date, vaccines have had only a modest impact on AOM. There are increasing concerns regarding overuse of antibiotics for treatment of childhood AOM [7], along with concerns regarding antibiotic resistance as a consequence of high antibiotic consumption amongst children in New Zealand [8]. Accordingly, alternative prevention and/or treatment options have been sought including exploring a possible role for probiotics in the prevention of AOM [9–11].

Although there are many postulated mechanisms by which a probiotic could prevent infection [12], potentially the most effective of these is via the relatively specific probiotic mediated bacteriocin interference with the growth of the potential pathogens at the site of infection. Bacteriocins are proteinaceous substances produced by bacteria that display inter- and intra-species antagonism. The widely used probiotic bacterium, *Streptococcus salivarius* K12 (BLIS K12™), was originally isolated from the oral cavity of a healthy child and subsequently has been shown to produce the bacteriocins salivaricin A, salivaricin B and salivaricin MPS [13]. It has been suggested that bacteriocin-producing probiotics could potentially be used to prevent otitis media infections in children on the basis of their demonstrable inhibition of otitis media pathogens in vitro [14].

However, prior to the selection of a candidate anti-AOM probiotic for efficacy in clinical trials, it is essential to first evaluate their in vitro inhibitory capabilities against isolates of known AOM pathogens.

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Aim

The aim of this study was to assess the in vitro inhibitory activity of the commercial probiotic strain *Streptococcus salivarius* BLIS K12 against isolates of recognised pathogenic bacteria recovered from the middle ear fluid samples of subjects experiencing otitis media.

Methods

Bacterial isolates from middle ear fluid samples obtained in previous research by Mills and colleagues in two batches, from May to November 2011 and January to December 2014 [5, 15]. The samples were obtained via tympanocentesis intraoperatively from otitis media prone children undergoing elective ventilation tube insertion. Representative isolates were identified and then stored in a medium containing skim milk, tryptone, glucose and glycerin (STGG) at − 70 °C. The recovered species included *Haemophilus influenzae* (non-typeable), *Streptococcus pneumoniae*, *Moraxella catarrhalis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Streptococcus pyogenes*, *Staphylococcus lugdunensis*, *Alloiococcus otitidis*, *Turicella otitidis* and yeast. These were tested for in vitro sensitivity to *S. salivarius* BLIS K12.

The efficacy of the bacteriocin activity of *S. salivarius* BLIS K12 against the otitis media isolates was assessed using the deferred antagonism test originally devised by Tagg and Bannister [16]. The *H. influenzae* isolates were maintained on Haemophilus testing medium (HMT) agar (BD™). The other isolates were grown on calcium carbonate–supplemented human blood agar (hBaCa).

Every experimental batch of deferred antagonism testing using the BLIS K12 strain was controlled by incorporating a set of nine so-called standard indicator bacteria on one of the test agar plates. This was done to qualitatively assess the amount and spectrum of the bacteriocin activity that had been deposited by BLIS K12 in the agar under the current test conditions. The observation of strong (+++) inhibition of all nine indicators by *S. salivarius* K12 on this control plate was used as an internal control of the efficacy of the assay for evaluating the activity spectrum of BLIS K12. Deferred antagonism testing was repeated for any test strains displaying equivocal (+/−) susceptibility to BLIS K12 until a consensus + or − outcome was established.

The results were independently interpreted and recorded by two of the primary investigators. Any discrepancies were discussed to reach consensus. For the purposes of this study, inhibition was quantified as outlined by Tagg and Bannister as "−", "+", "++", "+++" and "++++", according to the width of the inhibition zone together with the recording of any apparently resistant growth within the inhibition zone.

Results

Of the set of 172 frozen isolates from the original OMIVI trial, 107 (62%) were recoverable by subculture (Table 1; Fig. 1).

The inhibitor profile given by *S. salivarius* BLIS K12 against a set of standard indicator strains showed that salivaricin A and salivaricin B were both being produced on HTM agar as well as on hBaCa. Interestingly, however, the growth of standard indicator I3 (a *Streptococcus constellatus* strain) appeared to be relatively more florid on HTM agar (Supplementary material Fig. 1)

Overall, 51 (48%) of the 107 isolates exhibited some growth inhibition by *S. salivarius* BLIS K12. The breakdown of degree of inhibition in these isolates was the following: 20 "++++", 16 "+++", 5 "++" and 10 "+" (Table 2).

All 9 (100%) *S. pneumoniae* were strongly inhibited by S. *salivarius* BLIS K12. Other species exhibiting uniformly strong susceptibility to BLIS K12 were *T. otitidis*, *Corynebacterium* sp. and *A. otitidis*.

The susceptibility of *M. catarrhalis* to *S. salivarius* BLIS K12 appeared more variable with 16 of the 31 isolates appearing to be insensitive under these test conditions. Ten isolates exhibited "+" susceptibility, and 5 were assessed as "++" sensitive.

None of the 34 non-typeable *H. influenzae* appeared sensitive to *S. salivarius* BLIS K12 when tested on HMT agar.

Table 1 OMIVI trial otopathogens overall survival

Organism	Survived	Trackable isolates	Percentage
A. *otitidis*	13	35	37.1%
T. *otitidis*	10	15	66.7%
P. *aeruginosa*	3	3	100.0%
Yeast	1	1	100.0%
S. *aureus*	4	6	66.7%
S. *pneumoniae*	9	11	81.8%
M. *catarrhalis*	31	36	86.1%
Corynebacterium spp.	1	7	14.3%
H. *influenzae*	34	56	60.7%
S. *pyogenes*	1	1	100.0%
S. *lugdunensis*	0	1	0.0%
Total Isolate Survival	107	172	62.2%

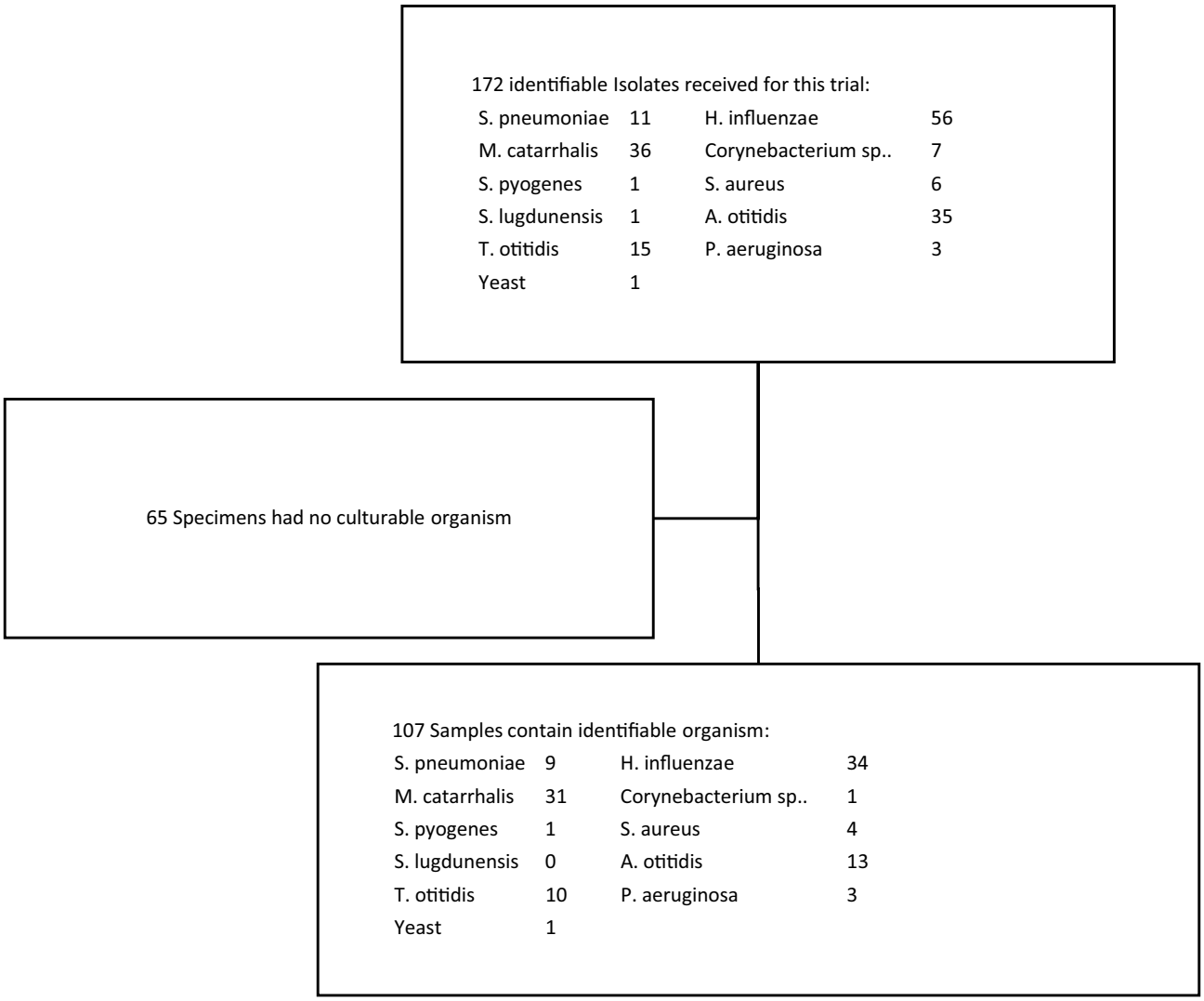

Fig. 1 Flowchart for middle ear effusion isolates

Discussion

This study is the first to test the in vitro inhibitory activity of probiotic strain *S. salivarius* BLIS K12 against isolates of recognised pathogenic species isolated from children with clinically documented ear infections. This is an important step in the development of a strategy for use of a probiotic strain to prevent AOM. Santagati and colleagues

Table 2 Range of inhibition scores from *S. salivarius* K12 deferred antagonism testing against otitis media pathogen isolates

Organism	–	Percent	+	Percent	++	Percent	+++	Percent	++++	Percent	Total
A. otitidis	0	0%	0	0%	0	0%	0	0%	13	100%	100%
T. otitidis	0	0%	0	0%	0	0%	8	80%	2	20%	100%
S. aureus	3	75%	0	0%	0	0%	1	25%	0	0%	25%
S. pneumoniae	0	0%	0	0%	0	0%	5	56%	4	44%	100%
M. catarrhalis	16	52%	10	32%	5	16%	0	0%	0	0%	48%
Corynebacterium spp.	0	0%	0	0%	0	0%	0	0%	1	100%	100%
Non-typeable H. influenzae	34	100%	0	0%	0	0%	0	0%	0	0%	0%
S. pyogenes	0	0%	0	0%	0	0%	1	100%	0	0%	100%

have in 2012 demonstrated that the BLIS activity of a probiotic candidate strain of *S. salivarius* inhibited the growth of potential pathogens isolated from the oral cavities of healthy children [17]. The present study adds to this by testing the in vitro inhibitory activity of *S. salivarius* BLIS K12 against bacteria from the middle ear fluid of children obtained during surgery for recurrent AOM or OME.

Immunisation against *S. pneumoniae* has not been an effective strategy for reducing the incidence of AOM [18, 19]. Furthermore, the promise [20] of conjugate vaccination to reduce carriage and disease from antibiotic-resistant *S. pneumoniae* immunisation has not borne out due to serotype replacement, ongoing antibiotic selection pressure and emergence/spread of antibiotic-resistant clones [21, 22]. This creates further motivation to explore the potential role of other therapies including probiotics as a way of reducing the need for repeated antibiotic courses in otitis-prone children.

Therefore, it is not unexpected that, in all of the *S. pneumoniae*, isolates were strongly inhibited by *S. salivarius* BLIS K12. *S. pneumoniae* has high carriage rate in otitis-prone children and is the most common aetiological agent of AOM [23]. There was also inhibition of other Gram-positive bacteria evaluated in the study, including *Corynebacterium* sp., *A. otitidis and T. otitidis*. The role of these species in otopathogenicity remains uncertain, as they are known to be common commensals of the ear canal. Nonetheless, their pathogenic potential has been supported by studies in which these organisms have been isolated via sterile tympanocentesis [24, 25]. On the basis of the present studies, it appears that *S. salivarius* BLIS K12 has the appropriate inhibitory capability to be effective as a probiotic for the prevention of AOM due to gram-positive organisms.

Bacteriocins typically kill bacteria which are closely related to the producing strain, whilst the producing strain usually shows immunity to its own bacteriocins [26]. It is not unexpected that the BLIS produced by *S. salivarius* K12 does not appear to inhibit the growth of the Gram-negative bacterium *H. influenzae*. However, *S. salivarius* BLIS K12 did display some in vitro growth inhibition of *M. catarrhalis* isolates, an observation not previously reported.

One of the strengths of the study is that all the bacteria tested were isolates from middle ear fluid of children with documented ear disease. It is likely that they were all involved in the pathogenesis of the children's ear infections, and therefore, any inhibition demonstrated by the BLIS K12 probiotic has potential to be clinically relevant. The main limitation of our study is the in vitro nature of the testing. The environment in which the BLIS production and bacterial interference has been evaluated does not replicate the in vivo environment of the active disease process. Furthermore, since the *H. influenzae* failed to grow on hBaCa agar, a different test medium (HMT agar) was used and it is not known how much BLIS production was obtained on this growth medium. In vitro BLIS production is known to be strongly dependent on the culture medium composition [27, 28].

Conclusion

This study demonstrates for the first time that a commercially available probiotic strain *S. salivarius* BLIS K12 can inhibit the in vitro growth of the most common pathogens causing AOM. Given the high incidence and prevalence of AOM in children, the use of probiotics could potentially have an impact on one of most common infections in children.

Supplementary Information The online version contains supplementary material available at (https://doi.org/10.1007/s12602-020-09719-7).

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Compliance with Ethical Standards

Ethical Approval The study was approved by the Health and Disability Ethics Committee, New Zealand. Ethics approval number—NTX/11/04/029/AM02.

Informed Consent Participants in the original OMIVI studies provided written consent for storage of their sample for later research.

Conflict of Interest Dr. J Hale, Professor J Tagg, Dr. R Jain and A. Voss are paid employees of Blis Technologies. None of the other authors has any conflict of interest to declare.

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