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Interferon Gamma Response in Human Saliva Following Exposure to the Oral Probiotic *Streptococcus salivarius* BLIS K12

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Abstract

Streptococcus salivarius BLIS K12 is a probiotic strain developed for application to the oral cavity. The strain was originally characterised for its in vitro antibacterial activity against the prominent oral pathogen *Streptococcus pyogenes*. More recent research has expanded its applications to include reducing halitosis, preventing otitis media and protecting against virus infections of the respiratory tract. A potential mechanism for this anti-viral activity could be the stimulation of salivary interferon gamma (IFN-γ) production in the oral cavity. The aim of this study was to investigate whether the ingestion of and oral cavity colonisation by *S. salivarius* BLIS K12 is associated with enhancement of IFN-γ levels in saliva. Application of ELISA demonstrated that consumption of *S. salivarius* BLIS K12 effected an increase in salivary IFN-γ, and this response was more consistent with use of viable cells than following ingestion of heat-killed *S. salivarius* BLIS K12. Interestingly, those subjects who more successfully colonised with *S. salivarius* BLIS K12 did not experience a relatively larger increase in their IFN-γ levels, indicating that the observed IFN-γ response occurs independently of colonisation efficacy. In summary, the consumption of *S. salivarius* BLIS K12 increases salivary levels of IFN-γ, an effect that may contribute to protection of the host against certain virus infections.

Keywords *Streptococcus salivarius* BLIS K12 · Inteferon gamma · Oral probiotic · Virus · Immune response

Introduction

Probiotic bacteria have been widely investigated for their health benefits for the gut. These benefits result from a range of mechanisms including (a) colonisation of target host tissue sites, resulting in exclusion of potential pathogens by out-competing them for attachment sites and key nutrients, i.e. 'crowding out'; (b) production of inhibitory molecules such as bacteriocins [1]; (c) production of beneficial metabolites and enzymes; (d) enhancement of the epithelial barrier [2] and (e) modulation of the host's immune system and indigenous microbiota [3, 4]. The oral cavity probiotic strain *Streptococcus salivarius* BLIS K12 also exhibits many comparable traits. Originally, this strain was identified as a potential probiotic because of its particularly strong bacteriocin-mediated anti-competitor activity against *Streptococcus pyogenes* [5]. Subsequent studies [6] defined additional probiotic colonisation capability [7] and 'crowding out' of *Candida albicans* [8]. More recently, there has also been renewed interest in the possibility that beneficial modulation of the host immune system following the consumption of *S. salivarius* BLIS K12 may occur, and the preliminary evaluation of this prospect constitutes the primary focus of the present study.

A common approach to assessing the beneficial attributes of a prospective gut probiotic is to carry out a systemic immune response evaluation. In line with this, *S. salivarius* BLIS K12 was similarly assessed [9]. The outcome of this study was that increased levels of T regulatory cells (T regs) and IL-10 were detected in samples taken 24 h following the ingestion of *S. salivarius* BLIS K12 [9]. Both these shifts are consistent with the evoking of a peripheral anti-inflammatory response.

Mechanistically, *S. salivarius* BLIS K12 has been shown to downregulate the NF-KB pathway in bronchial epithelial cells resulting in suppressed inflammation [10]. The NF-KB pathway controls DNA transcription, cytokine production and cell survival. This research also demonstrated that a decrease in secretion of the proinflammatory cytokine IL-8, together with active stimulation of beneficial pathways, including the generation of type I and type II interferon, occurred in response to exposure to

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S. salivarius BLIS K12 [10]. More recent studies using human gingival fibroblasts have shown that *S. salivarius* BLIS K12 does not induce IL-6 and IL-8 in addition to reducing the induction of inflammatory cytokines by pathogenic bacteria [11]. Certain viruses have evolved the ability to modulate the NF-KB pathway, which aids their evasion of human immune responses by enhancing their replication and reducing host cell survival [12]. In balance, these previous studies support the hypothesis that the consumption of *S. salivarius* BLIS K12 may limit viral infectivity through beneficial immune modulation.

Clinically, there is a growing body of evidence that supports the application of *S. salivarius* BLIS K12 probiotic for the reduction of viral infections. Several clinical trials have shown that use of *S. salivarius* BLIS K12 reduces the occurrence of virus mediated upper and lower respiratory tract infections [13–16]. Furthermore, some users of *S. salivarius* BLIS K12 have anecdotally reported that dosing with this probiotic at the first sign of flu-like symptoms can sometimes appear to halt further progression of these infections.

Previous preliminary studies have demonstrated that *S. salivarius* BLIS K12 can elicit an IFN-γ respose [17], thereby providing a putative mechanism for the observed apparent antiviral activities in human subjects. The type II interferon, IFN-γ, is secreted by natural killer cells (NKs) and antigen-presenting cells (APCs) as part of the early host defence against pathogens. Its main role in the innate immune system is the activation of macrophages and indeed it was originally called macrophage stimulating factor [18]. IFN-γ ‘primes’ macrophages by enhancing their responsiveness to inflammatory stimuli, for example, Toll-like receptor ligands (TLR), type I IFNs and tumour necrosis factor (TNF). IFN-γ directly activates macrophages via the Jak-STAT pathway [19] also inducing the production of nitric oxide synthetase (iNOS), which can directly impact on virus viability [20, 21].

A probiotic capable of reducing inflammation in the local immune system while also upregulating (‘priming’) macrophage pathways may potentially function to protect the host from symptomatic infection following exposure to pathogens. The objective of the present study was to evaluate the intrinsic and extrinsic host cell responses occurring in human subjects following their consumption of *S. salivarius* BLIS K12. More specifically, the focus of this study was to assess the dynamics and amplitude of changes to the salivary levels of IFN-γ following exposure to various doses of *S. salivarius* BLIS K12.

Materials and Methods

Dosage Regimens

The participants were students at the University of Otago, Dunedin, NZ. Four different dosing regimens were utilised: a low dose of four lozenges containing 1.1×10^9 c.f.u. *S. salivarius* BLIS K12 per lozenge (4.4×10^9 c.f.u. total), a standard dose of 12 lozenges at 1.1×10^9 c.f.u. *S. salivarius* BLIS K12 per lozenge (1.32×10^{10} c.f.u.) and a placebo dose of 12 lozenges.

Comparison of IFN-γ Responses to Live and Dead Cells of *S. salivarius* BLIS K12

Lozenges containing 1.1×10^9 c.f.u. *S. salivarius* BLIS K12 per lozenge were sterilised by gamma irradiation. Four gamma-irradiated lozenges were then given to each of eight individuals. Another seven subjects were given four lozenges, each containing 1.1×10^9 c.f.u. of viable *S. salivarius* BLIS K12. Saliva was collected from all 15 individuals prior to dosing and after 24 h, and IFN-γ levels were detected.

Saliva Collection and Preparation

Ethical approval was obtained from the Health and Disability Ethics Committee LRS/09/02/002/AM03. All participants gave their informed consent. One millilitre of unstimulated saliva was collected from each subject prior to their consumption of lozenges/nutritional drinks. A follow-up saliva specimen was also collected from the subjects 24 h after consumption of the lozenges. Storage of the saliva specimens was at −80 °C until required for analysis. Saliva was centrifuged for 5 min at $10{,}000 \times g$ and the supernatant collected for testing. IFN-γ levels were detected using the Human IFN-γ ELISA MAX Deluxe Set by BioLegend. One hundred microlitres of saliva supernatant was added to each well. Standards were also added to wells. ELISA was conducted according to the manufacturer’s protocol. A standard curve was run with each assay. Plates were read on an iMark Microplate Absorbance Reader at 450 and 570 nm. Absorbance values at 570 nm were subtracted from values at 450 nm to remove optical imperfections. A standard curve was created for every plate/assay and IFN-γ levels calculated based off this standard curve equation: human IFN-γ (pg/mL) $= x$ (absorbance) $- y$. The minimum detectable dose of IFN-γ was determined to be 1.0 pg/mL.

Colonisation Efficacy

Whole saliva was diluted twice in 2-fold serial dilutions using sterile dH_2O. Fifty microlitres of sample was spiral plated onto Mitis Salivarius agar plates using a Spiral Biotech Autoplate 4000 Spiral Plater. Plates were read using a Qcounter to obtain a colony-forming-unit-per-millilitre count. Colonies having morphology similar to *S. salivarius* BLIS K12 (i.e. medium-large, blue, mucoid colonies) were picked for simultaneous antagonism testing as previously described [22]. Colonies were picked into human blood agar pre-seeded

with lawn culture inoculums of the BLIS-indicator bacteria I1 (*Micrococcus luteus T-18*) and I3 (*Streptococcus constellatus T-29*) [23]. *S. salivarius* BLIS K12 served as a positive control. The plates were incubated at 37 °C in 5% CO_2 in air atmosphere for 24 h. Colonies strongly inhibiting the growth of both I1 and I3 were recorded as putative *S. salivarius* BLIS K12. Fifty colonies were picked from the Mitis Salivarius agar of each participant's pre-saliva sample and post-saliva sample. The count of *S. salivarius* BLIS K12-like colonies was recorded as a number out of 50, and this figure was used to estimate the percentage of *S. salivarius* that were *S. salivarius* BLIS K12. The count of *S. salivarius* BLIS K12 24 h after consumption of the lozenges was taken to represent the level of oral cavity colonisation obtained by *S. salivarius* BLIS K12.

Statistical Analysis

The data was analysed by the Mann–Whitney or Kruskal–Wallis statistical test unless otherwise stated.

Results

Initially, 63 subjects were utilised to evaluate whether there was a difference in salivary levels of IFN-γ 24 h following the ingestion of different doses of *S. salivarius* BLIS K12. In this trial, 13 subjects consumed placebo, 21 subjects consumed a low dose (4.4×10^9 c.f.u. of *S. salivarius* BLIS K12) and 16 subjects consumed a standard dose (1.32×10^{10} c.f.u. of *S. salivarius* BLIS K12). The participants' saliva was tested for IFN-γ levels in samples collected just prior to the ingestion of the lozenges, and then 24 h later. Most (94%) of the subjects had elevated IFN-γ levels 24 h after consuming the standard dose of *S. salivarius* BLIS K12 (Fig. 1A). Approximately half (52%) of the subjects taking the low dose demonstrated increased IFN-γ (Fig. 1A). A significantly greater increase in IFN-γ levels was detected in subjects who consumed the standard dose of probiotic when compared with those taking placebo lozenges (Fig. 1B).

S. salivarius BLIS K12 colonisation levels at 24 h were assessed in saliva samples from 11 placebo, 13 low dose and 15 standard dose subjects. Most subjects given the standard dose of *S. salivarius* BLIS K12 were colonised at 24 h (Fig. 2A). Half of the subjects taking the low dose had *S. salivarius* BLIS K12 at 24 h. The level of colonisation achieved by subjects receiving either low or standard doses of the probiotic cells broadly correlated with the dosage levels. Overall, it appears that at all dosage levels, there was no significant difference in IFN-γ levels regardless of whether colonisation had occurred (Fig. 2B–D). Finally, the question of whether the consumption of lozenges containing only non-viable *S. salivarius* BLIS K12 could also evoke an elevation in salivary levels of IFN-γ was addressed. IFN-γ levels at 24 h were assessed in eight individuals given gamma-irradiated lozenges and seven who were given the low dose of 4.4×10^9 *S. salivarius* BLIS K12. Although the data trend indicated that viable cells may have a somewhat stronger stimulating effect on salivary levels of IFN-γ than dead cells (Fig. 3), the difference in the responses of the two groups was not statistically significant.

Discussion

The purpose of this study was to evaluate the influence of ingestion of the probiotic bacterium *S. salivarius* BLIS K12 on salivary levels of IFN-γ. The rationale for this study was the growing evidence base demonstrating a reduction of viral respiratory infections in subjects taking courses of this

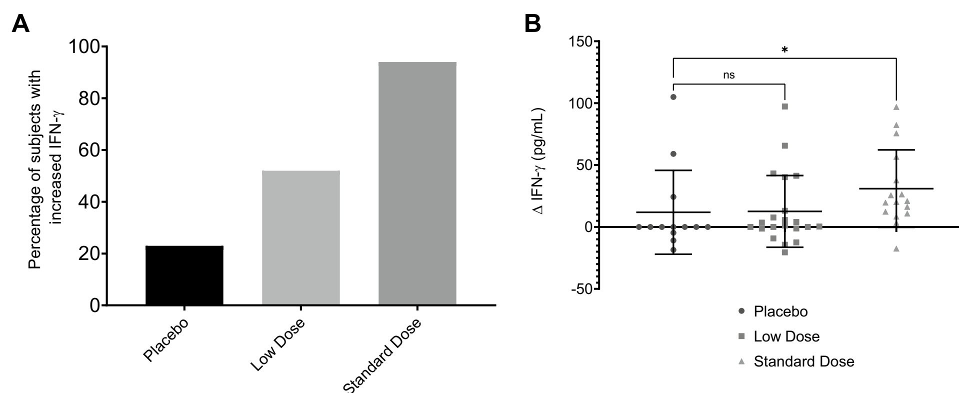

Fig. 1 **A** Percentage of individuals with stimulated (increased) IFN-γ levels in saliva 24 h after the consumption of either placebo, low dose or standard dose of *S. salivarius* BLIS K12. **B** Change in IFN-γ levels (pg/mL) in saliva samples 24 h following the consumption of either placebo, low dose or standard dose of *S. salivarius* BLIS K12. Kruskal–Wallis test compared each sample to the placebo dose. *$p < 0.05$. Error bars represent standard deviation. **A**, **B** Placebo $n = 13$, low dose $n = 21$, standard dose $n = 16$

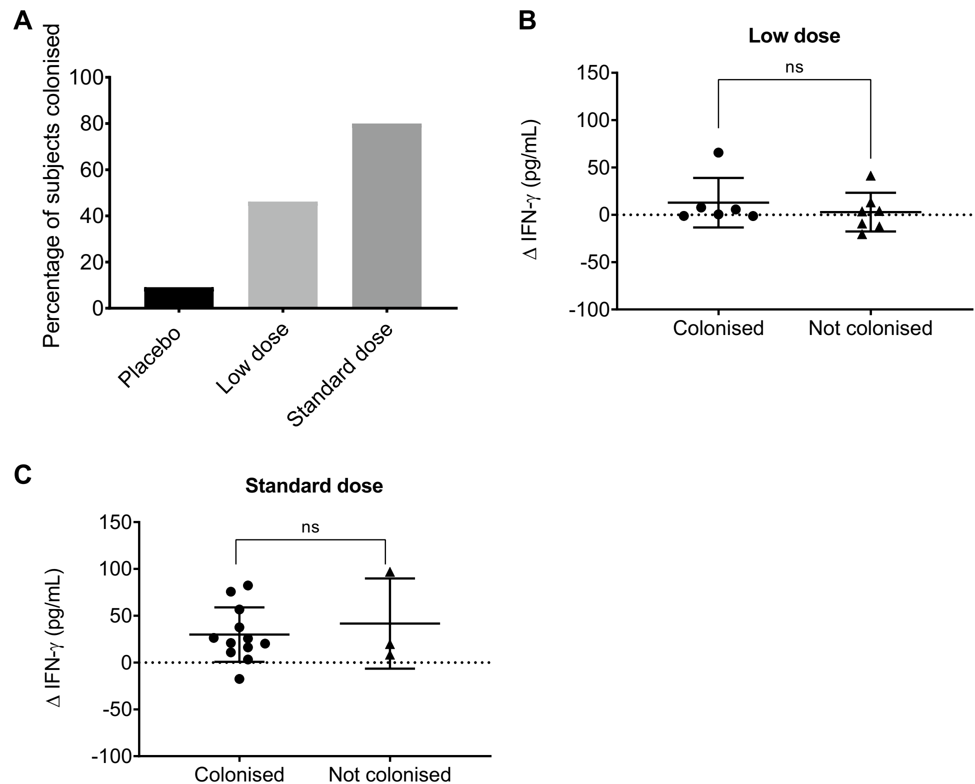

Fig. 2 Summary percentage of colonisation of *S. salivarius* BLIS K12 with different doses and influence of colonisation on salivary IFN-γ levels. Subjects: placebo $n=11$, low dose $n=13$, and standard dose $n=15$. **A** Percentage of subjects with *S. salivarius* BLIS K12 present in their saliva 24 h after consuming different doses of *S. salivarius* BLIS K12. **B**–**D** Mean change in IFN-γ levels (pg/mL) with subjects divided by colonisation status 24 h post-consumption of *S. salivarius* BLIS K12 **B** low dose **C** standard dose. Error bars represent standard deviation. Mann–Whitney test performed for (**B**) and (**C**) graphs and no significant differences were observed

probiotic. Previous preliminary studies had indicated that ingestion of *S. salivarius* BLIS K12 can evoke an elevation of salivary IFN-γ [17] and the present trials focused upon documenting salivary levels of IFN-γ following oral cavity exposure of the host to different doses of viable *S. salivarius* BLIS K12 cells and also to gamma-irradiated non-viable cells. IFN-γ is secreted as a key component of the early host defence against infection. Its principal role is the activation of macrophages, effectively priming the immune system response to infection [18]. Although IFN-γ is largely produced by NK cells it can also be formed by T and B cells [24, 25]. *S. salivarius* BLIS K12 has been previously shown to stimulate NK cells in vivo to produce IFN-γ, thereby providing preliminary support for the current hypothesis [25]. In the present study, it was found that consumption of 1.32×10^{10} c.f.u. of BLIS K12 yielded the highest levels of salivary IFN-γ. A secondary objective of the present study was to evaluate whether the magnitude of the IFN-γ response correlated with the level of colonisation by *S. salivarius* BLIS K12. No statistically significant difference was detected in IFN-γ levels in individuals who either did or did not colonise. One interpretation to account for this observation is that the IFN-γ increase is a response to the large influx of *S. salivarius* BLIS K12 at the time of ingestion rather than to the presence of the probiotic cells as components of the host's oral microbiota. In other words, the present observations indicate that the IFN-γ response generated by *S. salivarius* BLIS K12 is colonisation independent and that individuals consuming the probiotic may receive the immuno-stimulatory benefit even if colonisation is not achieved.

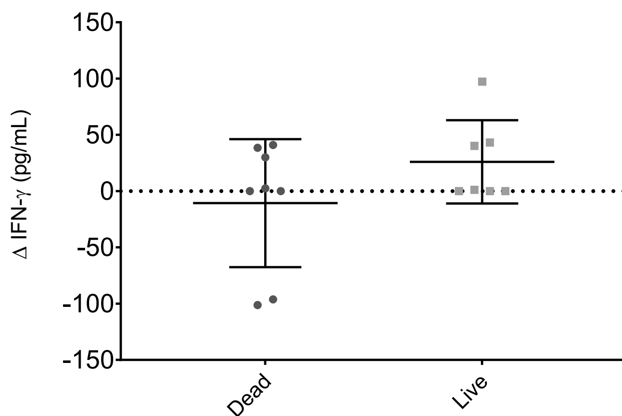

Fig. 3 Mean shift in IFN-γ levels (pg/mL) in individuals' saliva samples 24 h post-consumption of either gamma-irradiated BLIS K12 (dead cells) or $6.68 \times 10.^{9}$ BLIS K12 cells (live cells). Mann–Whitney test showed no significant difference. Error bars represent standard deviation. Dead cells $n=8$ and live cells $n=7$

An additional objective of this study was to assess whether differences in IFN-γ responses were evident on exposure to viable and non-viable cells. No statistically significant differences were detected. Nevertheless, there did

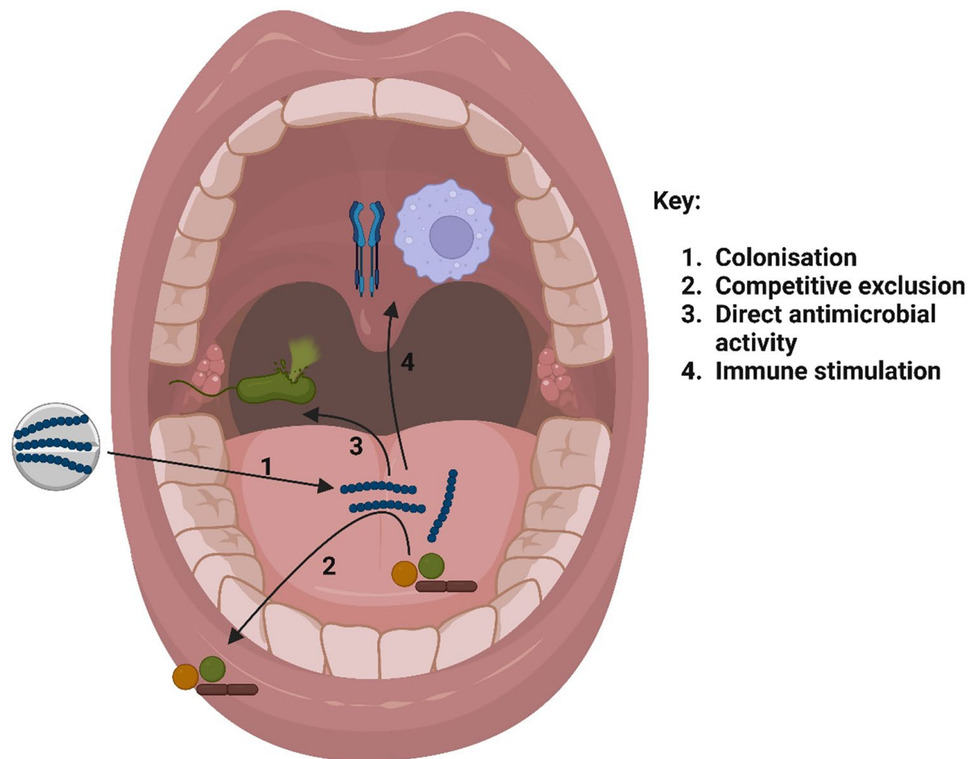

Fig. 4 Known mechanisms of action of *S. salivarius* BLIS K12 in the oral cavity. Created with BioRender.com

seem to be a trend of higher responses to viable cells. It is pertinent to note that all commercially prepared BLIS K12 lozenges will contain a number of non-viable cells as a consequence of the compression forces that they are subjected to during lozenge manufacture.

The present study has shown that the consumption of *S. salivarius* BLIS K12 can evoke a potentially beneficial immunological response in the oral cavity. These observations complement the previously demonstrated systemic immune anti-inflammatory benefits [9]. Taken together, it can be seen that the immunologically based benefits to the host are wide ranging and these augment the documented capability of *S. salivarius* BLIS K12 to reduce the occurrence of streptococcal infections using direct bacteriocin-mediated antibacterial activity and competitive exclusion [7, 26–30] (Fig. 4). In summary, this study has demonstrated that the oral probiotic *Streptococcus salivarius* BLIS K12 can increase IFN-γ levels in human saliva within 24 h of consumption. An important potential consequence of this is the potential for application as a short-term cross-protective ('priming') activity against viral infections initiating within the oral cavity. Indeed, the practical implications are considerable for example, for application as a short-term anti-viral intervention to be used by individuals undertaking travel or potentially encountering an increased risk of exposure to respiratory viruses. This currently observed salivary IFN-γ response to BLIS K12 may be the mechanism underlying clinical studies that have reported that daily consumption of BLIS K12 provides protection against viral infection [13, 14]. Clearly, follow-up adequately controlled clinical investigations are now indicated.

Author Contribution G.A.L. helped design the study, and ran the clinical trial and laboratory analysis. L.K.H. wrote the main manuscript text. J.R.T. contributed to the planning and assessment of results. J.D.F.H. designed the project and reviewed the results. All authors reviewed and contributed to writing.

Data Availability The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics Health and Disability Ethics Committee LRS/09/02/002/AM03.

Conflict of Interest G.A.L., L.K.H., J.R.T. and J.D.F.H are/or were employees of Blis Technologies at the time of this research.

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