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Evaluation of safety and human tolerance of the oral probiotic *Streptococcus salivarius* K12: A randomized, placebo-controlled, double-blind study

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ABSTRACT

Streptococcus salivarius is naturally a predominant member of the human oropharynx and the commercial probiotic strain K12 has been consumed for more than a decade. The present study examines the health responses of human volunteers to oral ingestion of high doses of *S. salivarius* K12.

A randomized group of 53 subjects received a dose of 1×10^{10} cfu *S. salivarius* K12 ($N = 25$) or placebo ($N = 28$) for 28 days, followed by a 28-day wash out period. Blood, urine and saliva samples were collected at baseline and following treatment and analyzed, while the oral and gastrointestinal tolerance of the subjects to the dosing regimen was determined by use of questionnaires. Adverse events (AE)s were recorded for both groups.

No statistically significant differences between the probiotic and placebo treated groups were detected in either the blood clinical chemistry or hematology results ($P > 0.05$). The questionnaire responses of the subjects indicated that both treatments were well tolerated. The frequency and intensity of AEs was similar in the two groups.

This data demonstrates that the daily ingestion of *S. salivarius* K12 over a 28-day period does not adversely affect the human host and supports the safety of its oral delivery in a food-based carrier.

1. Introduction

Until recently, the usage of probiotics by humans has almost exclusively focused on the derivation of benefits through the ingestion of microbes originally obtained from intestinal sources. However, with the more widespread acceptance of the potential for probiotic intervention to also effect heath benefits for non-intestinal body sites, there has come the increased application of effector strains of species that are indigenous to alternative target tissues, in order to obtain more specific and enduring benefits. Addressing safety concerns presents a major obstacle to the probiotic application of microorganisms that do not already have an extensive history of use in traditional fermented products, even if they are widely considered to be common and innocuous inhabitants of the normal human indigenous microbiota. The species *Streptococcus salivarius* is best known as a numerically-predominant member of the human oral and nasopharyngeal indigenous microbiota. However, it is also common in human breast milk and has been detected in various non-pasteurized indigenous fermented milk products (Callon et al., 2004; Martin et al., 2007; Ongol and Asano, 2009; Pesic-Mikulec, 2005). Heightened interest in the probiotic potential of *S. salivarius* stems from (i) its numerical predominance in the oropharynx, (ii) the production by some strains of a particularly diverse array of anti-competitor molecules [bacteriocins and bacteriocin-like inhibitory substances (BLIS)] and (iii) demonstrations of its beneficial application to the relief or control of various upper respiratory tract ailments including strep sore throat, otitis media and halitosis for which strain *S. salivarius* K12 has been applied (Burton et al., 2006a; Dierksen and Tagg, 2000; Wescombe et al., 2009).

The safety testing of chemicals is typically performed with use of well-substantiated animal toxicity models. However, for the safety assessment of probiotics, validated animal models do not exist, and hazard characterization of a probiotic microorganism must be determined through the application of well-designed randomized placebo-controlled studies in healthy human subjects (Shane et al., 2010; Vankerckhoven et al., 2008). The safety criteria for probiotics must also include affirmation that the organism is not routinely implicated in non-opportunistic infections and that it does not exchange virulence or antibiotic resistance determinants with other organisms (Shane et al., 2010; WHO, 2002). The

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first safety study of *S. salivarius* K12 (Burton et al., 2006b) assessed its propensity to cause disease according to (i) its content of known virulence factors and antibiotic resistance determinants, (ii) its genetic and biochemical stability over time and under various propagation conditions, and (iii) whether any proliferation of deleterious organisms occurs when *S. salivarius* K12 is administered to humans. A second safety study reported toxicological and predicted-genotoxicity analyses of strain K12 and observations of adverse events (AE)s occurring either acutely or for up to a month following the dosing of animals. Also documented was the natural occurrence of strain K12-like bacteriocin-producing *S. salivarius* in humans (Burton et al., 2010). The current study focuses specifically upon the interaction of *S. salivarius* K12 with humans and more specifically it assesses the propensity of strain K12 to effect health parameters and occurrence of AE's when taken at 10 times the conventionally recommended dose.

While products containing *S. salivarius* K12 have now been sold for more than a decade with no verified product related AEs reported, there have been no controlled human safety studies conducted to specifically document the lack of occurrence of AEs. In practice this is not unusual, since most studies of probiotics have to date been predominantly efficacy-focused, with only a secondary emphasis on safety.

The aim of the present study was to evaluate the probiotic *S. salivarius* K12 for its safety and tolerability when ingested in high doses by a group of closely-monitored human subjects. Included was sequential documentation of the clinical chemistry, hematology and urinalysis parameters of the K12-treated subjects to determine whether the values obtained extended beyond normal limits or differed significantly from the findings in the placebo group. Additionally, the number, duration and intensity of AEs reported was closely monitored, as well as the perception of quality of life through questionnaires focused particularly upon the subjects' oral and gastrointestinal health.

2. Materials and methods

2.1. Study design

The objective of the study was to assess the safety and potential impact on health parameters, oral and intestinal tolerance of human subjects to *S. salivarius* K12. The double-blinded, placebo-controlled study included 56 male and female subjects randomized into two treatment groups (probiotic or placebo). The subjects were screened to determine whether they met the inclusion criteria 7 days prior to commencement of the 28-day test period, followed by further monitoring at 56 days, following a 28-day wash out period. A variety of biological samples including blood, urine and saliva were taken at various time points from the participants for analysis and described later in this section. The study monitor performed regular monitoring visits to the clinical research facility. The Copernicus Group Independent Review Board, NC, reviewed and approved the protocol and informed consent documents, in accordance with US Food and Drug Administration guidelines, ICH Harmonized Tripartite Guideline, Guideline For Good Clinical Practice E6(R1). Study design assistance was provided by Cantox (Missussagua, ON) and the study was audited by Clinical Development Associates, Inc. (Richmond, VA) and conducted under cGCP compliance consistent with the principles in the declaration of Helsinki at National Clinical Research Inc (NCR, Richmond, VA).

2.2. Subject inclusion

Strict study entry criteria were assessed with regards to the physical and physiological health status of the subjects prior to enrollment into the study. Healthy male and non-pregnant female volunteers, between the ages of 18 and 60, with a body mass index in the range of 18.5–35 kg/m^2 and no known food allergies or intolerances were eligible for recruitment. All clinical chemistry, hematology and urinalysis parameters and vital signs (blood pressure, respiratory rate, temperature, heart rate) were required to be within clinically acceptable ranges.

Subjects were excluded if they had used probiotic food products (e.g., yogurt, acidophilus milk, or other food products containing the word probiotic in the labeling) within the previous month or at any time during the trial. Usage of traditional fermented foods like regular yogurt, cured meats and cheeses was acceptable. Certain subjects were excluded if it was considered on the basis of either their medical history, their responses to the oral and gastrointestinal tolerance questionnaires or their baseline physical examination that they may potentially have some underlying compromised health condition. Examples of these conditions include subjects with unstable metabolic diseases/disorders, heart failure or a history of endocarditis, immune-compromisation or prior gastrointestinal surgery. Also excluded were individuals using antibiotics in the 3 months prior to study initiation, or at any time during the study, frequent users of alcohol or over the counter laxatives and consumers of herbal-based supplements (but not vitamin supplements) or prescription drugs that may potentially have influenced the biomarkers measured in the study.

2.3. Examination of physical condition and vital signs

Subject evaluations were performed at initial screening (week-1), baseline (week 0), the end of the 28-day treatment period (week 4) and at the final recovery visit (week 8). Documented demographic characteristics of the study population included medical and surgical histories, alcohol and tobacco use and methods of contraception (for females of childbearing age). Abbreviated physical examinations were conducted at baseline and at the end of treatment. Parameters recorded included the subjects' vital signs (heart rate, systolic and diastolic blood pressure, respiratory rate and temperature) as well as any detectable abnormalities of the skin, eyes, ears, nose and throat, heart, lungs, abdomen, musculoskeletal system, lymph nodes and nervous system. Vital signs were obtained at each examination session. The subjects' dietary compliance and use of alcohol was also regularly assessed throughout the study period. The oral cavity was inspected at each visit for abnormalities of the pharynx, tongue, teeth, gums and oral mucosa to document any adverse or unusual changes that may potentially have been associated with consumption of the test material.

2.4. Preparation of test material

The *S. salivarius* K12 cultures were grown in an ISO 22000 (ISO standards for food safety) quality-accredited facility. The *S. salivarius* K12 powder was blended with the commonly utilized food grade carriers, trehalose and maltodextrin and flavorings and then packaged into sachets under GMP conditions. Each sachet contained 1.1×10^{10} cfu *S. salivarius* K12, which is 10 times the usual recommended dose. The placebo differed only in containing no K12 powder. The active and placebo preparations were identical in appearance and taste. Cell counts were obtained by an independent laboratory (Cawthron Institute, Nelson, NZ) just prior to commencement of the study. At the end of the study any unopened sachets were returned and tested to re-establish the identity and cell count of the test organism.

2.5. Subject randomization and dosing

Allocation concealment was performed by an off-site statistician who generated active and placebo numerical sequences. Subjects who met the inclusion criteria were randomly allocated to the placebo or probiotic treatment group in a 1:1 ratio irrespective of sex. The participants were instructed to, each day at breakfast, consume the entire contents of a sachet after mixing into a half glass (4 oz.) of cold water. The taking of each dose throughout the 28-day treatment period was self-recorded in a diary. Compliance was monitored by contacting each subject by telephone two weeks after delivery of the sachets and by the return of all unused sachets at the end of the treatment period (compliance was considered acceptable if $\geqslant$80% of the doses had been taken).

2.6. Analysis of samples

2.6.1. Blood

Fasting blood samples were obtained by venipuncture both at the screening visit and upon completion of treatment (week 4). If any abnormalities were detected at the week 4 visit, additional samples were obtained at the final recovery visit (week 8) for re-testing. The blood was sent to LabCorp (Burlington, NC) for analysis of serum chemistry and liver function tests (glucose, BUN, creatinine, sodium, potassium, chloride, calcium, albumin, globulin, bilirubin, alkaline phosphatase, aspartate aminotransferase (AST) alanine aminotransferase (ALT) and hematology: white blood cell (WBC) count, red blood cell (RBC) count, hemoglobin, hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentrations (MCHC), platelets, neutrophils, lymphocytes, monocytes, eosinophils and basophils.

2.6.2. Urine

Urine samples were evaluated for clarity, color, specific gravity, pH, protein, occult blood, and leukocyte esterase. Urine pregnancy testing for women of childbearing potential was also undertaken (beta-hCG) at baseline and at the end of treatment.

2.6.3. Saliva

Non-stimulated saliva samples (1 mL; all subjects) were obtained at weeks 0, 4 and 8. The samples were snap-frozen at −20 °C and then stored at −70 °C.

2.7. Quantitative PCR detection of S. salivarius K12

Total DNA was extracted from each 1 mL saliva from those in the probiotic group aliquot using the PureLink™ genomic DNA kit (Invitrogen) as per the manufacturer's instructions for gram-positive bacteria. DNA was eluted from the column in 100 μl of elution buffer. A 1/10 dilution was made of each DNA extract (to reduce the effect of PCR inhibitors) before use in a specific real time PCR assay to quantitate the number of K12 colony-forming units (cfu)/mL of saliva. The standard curve was made using a dilution series of DNA extracted from a culture of *S. salivarius* K12 for which the corresponding count as cfu/mL was determined by plating onto Columbia Agar Base (CAB) agar. Real-time PCR was run on a Fast 7500 machine (Applied Biosystems) using a standard amplification protocol and with the *S. salivarius* K12-specific primers, Sea1 midL: CGG CAA AAC CAA AGC TAG AG and Sea1 midR: ACG TGG TTT TTG GGG GTT AG. These primers target part of the gene sharing homology with surface exclusion protein Seal on the megaplasmid of strain K12 (Hyink et al., 2007). These primers have been validated against megaplasmid containing *S. salivarius*, K12 seeded and normal saliva samples."

2.8. Oral and gastrointestinal tolerance monitoring

Assessment of oral and gastrointestinal health using self-reported questionnaires was performed at weeks 0, 4 and 8. Assessment of the condition of the teeth, mouth, gums and freshness of the breath was based upon a 10-point severity visual analog scale (VAS). General gastrointestinal health, including the presence and severity of pain, bloating, gastric reflux, nausea, vomiting, diarrhea, and gas/flatulence was alsorated also rated using a 10-point severity VAS. No oral and gastrointestinal health VAS questionnaires specific to healthy subjects and consistent with the objectives of the present study could be identified in the literature, but the adopted questionnaires were modeled upon some used in previous studies (CDC, 2009; Treiber et al., 2004) and these were approved by an Independent Ethics Board.

2.9. Adverse events monitoring

Documentation of any surgical, dental or medical procedures and the concomitant use of medications were collected throughout the study. AEs were recorded throughout the study at each clinic visit and by follow up telephone call at two weeks after commencing treatment. An AE is defined as any adverse health-related occurrence (sign, symptom, illness, abnormal laboratory value, or other medical event) experienced by a person participating in a clinical study, whether or not it is considered directly related to the prescribed treatment, and which either appears or worsens during the course of the study, or within a specified period of time following completion of treatment. Information describing the AE collected for assessment and documentation included the following: date of onset/resolution, intensity (a decrease or increase of the intensity), seriousness and causal relationship to study treatment or study conduct (as determined by the physician). Any AE that occurred was followed to resolution or until determined to be clinically insignificant by the physician.

2.10. Study monitoring, data collection and statistical analysis

The study was monitored regularly for regulatory compliance, protocol adherence and completeness of data collection by a clinical research organization, Clinical Development Associates, Inc. (Richmond, VA). The data were collected in case report forms and verified with source documents at the clinic. Data on each case report form was entered into a Microsoft Access database and then transferred into SAS version 9.2 (SAS Institute, Cary, NC) for statistical analysis. All data management and statistical analyses were performed by Clinical Development Associates, Inc. (Richmond, VA).

Demographic and baseline data were analyzed by treatment group. Differences between the treatment groups were assessed by *t*-tests for continuous variables and by chi square tests for categorical variables. Changes from baseline in each response on the oral and gastrointestinal health 10-point VAS questionnaires was analyzed by an analysis of covariance that included the effects of treatment group and baseline assessment as a covariate. AEs were summarized according to the first occurrence of each AE; hence subjects with multiple occurrences of the same event were only counted once. AEs were also summarized according to their relationship to treatment (possible, probable, or highly probable), the incidence of serious AEs, and the intensity. Changes from baseline in vital signs and in each laboratory variable were analyzed by an analysis of covariance that included the effects of treatment group and baseline assessment as a covariate.

3. Results

3.1. Study population

Of 67 volunteers initially screened for eligibility, 56 healthy subjects were subsequently enrolled in the study from October 26–December 14, 2010 (27 subjects were randomized to the probiotic group [5 males and 22 females, $P = 0.035$] and 29 to the placebo group [13 males and 16 females, $P > 0.05$]). The numbers of subjects completing the study were 25 (probiotic group) and 28 (placebo group) (Fig. 1). Progress of all participants was tracked through the study during enrollment, allocation, follow up and analysis phases and was based on the CONSORT reporting method (Schulz et al., 2010). The subjects were 20–60 years of age with an average of 39.7 for the probiotic and 37.2 for the controls ($P > 0.05$). The average height, weight, BMI, race, and history of tobacco and alcohol use was not statistically significantly different between the two groups ($P > 0.05$).

3.2. Analysis of oral and gastrointestinal health questionnaires

The subjects were asked to assess their oral health through a series of questionnaires at baseline, end of treatment and at the end of the washout period. The questionnaire results indicated that the perception by the participants was that regardless of whether they were taking the probiotic or placebo there was no significant change in the condition of their teeth, the freshness of their breath or the extent of gum bleeding at the end of either the treatment phase or wash out phase when compared to their baseline values ($P > 0.05$). The only question answered showing a marginally significant difference was tooth sensitivity, which was reduced for the probiotic group when compared to the placebo group at the end of the treatment period (−0.39 AU compared to baseline, $P = 0.080$) and then was further reduced at the end of the washout period (−0.79 AU compared to baseline $P = 0.013$). Reanalysis accounting for sex distribution resulted in a non-significant result ($P > 0.05$).

The subjects were asked to assess their own gastrointestinal health through a series of questionnaires at baseline, end of treatment and at the end of the washout period. The results for abdominal pain complaints, bloating, gastric reflux, nausea, vomiting, diarrhea and flatulence were not significantly different between the two treatment groups at the various assessment points throughout the study ($P > 0.05$). There was however, a significant difference ($P = 0.006$) between the probiotic and placebo groups after the treatment period because those in the placebo group felt that their general gastrointestinal health had declined over the period, whereas the average response for those in the probiotic group had remained relatively stable.

3.3. Adverse events

No serious AEs were observed in either the probiotic or placebo group. Thirty-seven percent of the subjects in the probiotic group, compared to the 51.7% of subjects in the placebo group, had at least one non-serious AE recorded during the treatment or washout periods. The type of AE appeared to be relatively evenly distributed in low numbers among the 18 categories that were coded (data not shown). Table 1 shows the severity of all AEs by intensity during the entire study. No AEs were reported as severe in intensity in the probiotic group. Two AEs of severe intensity (but not serious) were recorded for subjects in the placebo group, but overall the small numbers of AEs recorded by subjects in the probiotic and placebo groups were mostly of a mild nature. One AE was not coded in the probiotic group and was due to lumbar pain from an accidental fall. Only one of the severe AEs (in the placebo group) occurred during the treatment phase (data not shown). Table 2 shows the summary of all AE occurring during the treatment phase of the study for both groups. Table 3 summarizes all of the AEs potentially relating to treatment, (i.e. AE considered possibly, probably or highly probably related to treatment) and Table 4 documents the intensity of these AEs. The number of AEs coinciding

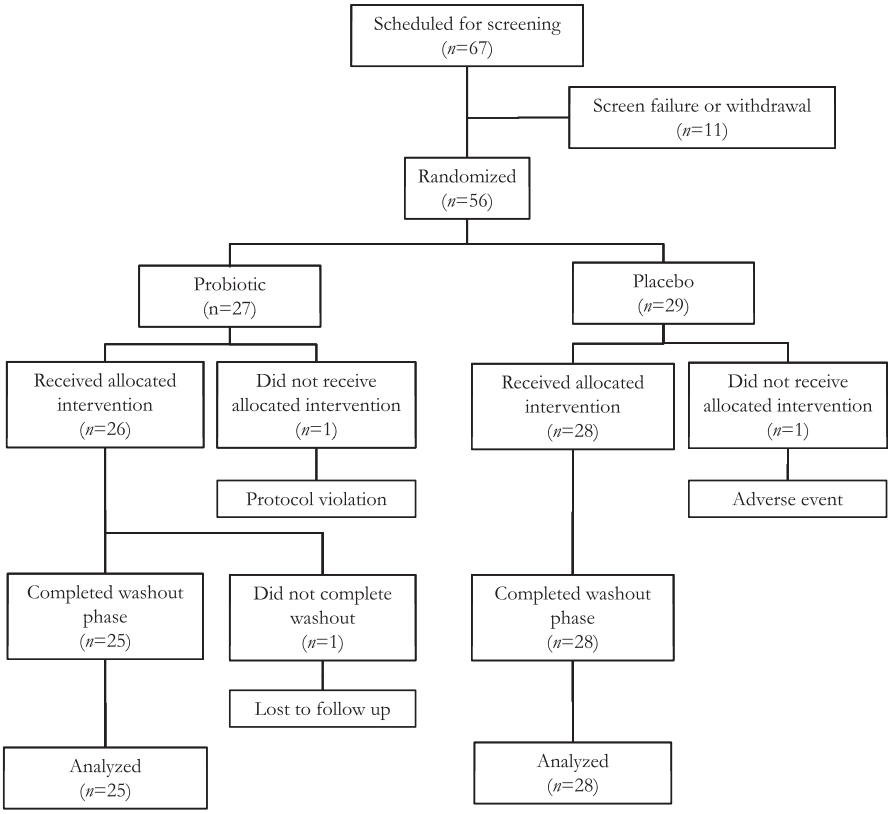

Fig. 1. Flow chart of the study subjects accounted through the study.

Table 1
Summary of all adverse events by intensity.

Adverse event	Probiotic (*N* = 27)			Placebo (*N* = 29)		
	Mild	Mod.	Severe	Mild	Mod.	Severe
Abdominal pain	2	0	0	0	0	0
Anxiety	0	0	0	0	1	0
Appetite increased	1	0	0	0	0	0
Asthma	0	0	0	0	0	1
Cough increased	0	0	0	1	0	0
Dizziness	0	0	0	0	1	0
Dyspepsia	1	0	0	2	0	0
Flatulence	1	0	0	0	0	0
Gastroenteritis	0	0	0	0	2	0
Headache	0	1	0	0	0	1
Herpes simplex	1	0	0	0	0	0
Infection	3	0	0	3	1	0
Myalgia	0	0	0	1	0	0
Nausea	1	0	0	1	0	0
Rhinitis	1	0	0	3	0	0
Vaginitis	1	0	0	2	0	0
Vomiting	0	0	0	0	1	0
Not coded	1	0	0	0	0	0
Total	13	1	0	13	6	2

N = Number of subjects.

Table 2
Summary of adverse events – treatment phase

Adverse event	Probiotic (*N* = 27)	Placebo (*N* = 29)
Number with at least one AE	8 (29.6%)	11 (37.9%)
Abdominal pain	2 (7.4%)	0 (0%)
Anxiety	0 (0%)	1 (3.4%)
Appetite increased	1 (3.7%)	0 (0%)
Asthma	0 (0%)	1 (3.4%)
Dizziness	0 (0%)	1 (3.4%)
Dyspepsia	1 (3.7%)	1 (3.4%)
Flatulence	1 (3.7%)	0 (0%)
Gastroenteritis	0 (0%)	2 (6.9%)
Headache	1 (3.7%)	1 (3.4%)
Herpes simplex	1 (3.7%)	0 (0%)
Infection	3 (11.1%)	2 (6.9%)
Myalgia	0 (0%)	1 (3.4%)
Rhinitis	0 (0%)	3 (10.3%)
Vaginitis	0 (0%)	2 (6.9%)
Vomiting	0 (0%)	1 (3.4%)
Not coded	1 (3.7%)	0 (0%)

N = Number of subjects.

with consumption of either the probiotic or placebo appeared low and the number of these AEs considered possibly attributable to the treatments was very low (Table 3). During the washout phase, there were also a limited number of AEs reported, but these were almost exclusively described as mild (data not shown) and these reactions were considered unlikely to be treatment-related.

3.4. Vital signs changes

There were no statistically significant changes ($P > 0.05$) between the baseline and end of treatment vital signs measurements (heart rate, systolic BP, diastolic BP, respiration rate or oral temperature) for the subjects receiving either the probiotic or placebo treatment (Table 5).

3.5. Effect on clinical hematology, chemistry and urinalysis parameters

Comparison of the end of treatment hematology results to the baseline values in the two groups of subjects showed that there was no statistically significant difference ($P > 0.05$) for WBC, RBC, hemoglobin, hematocrit, MCV, MCH, MCHC, platelets, neutrophils, lymphocytes, monocytes, EOS and basophils. Comparisons are presented in Table 6.

Table 3
Summary of all adverse events related to treatment.[a]

Adverse event	Probiotic (*N* = 27)	Placebo (*N* = 29)
Number with at least one related AE	2 (7.4%)	2 (6.9%)
Anxiety	0 (0%)	1 (3.4%)
Dizziness	0 (0%)	1 (3.4%)
Dyspepsia	1 (3.7%)	1 (3.4%)
Flatulence	1 (3.7%)	0 (0%)

N = Number of subjects.
[a] Events considered possible, probable, or highly probable related to treatment.

Table 4
Summary of adverse events by intensity – treatment phase.

Adverse event	Probiotic (*N* = 27)			Placebo (*N* = 29)		
	Mild	Mod.	Severe	Mild	Mod.	Severe
Abdominal pain	2	0	0	0	0	0
Anxiety	0	0	0	0	1	0
Appetite increased	1	0	0	0	0	0
Asthma	0	0	0	0	0	1
Dizziness	0	0	0	0	1	0
Dyspepsia	1	0	0	1	0	0
Flatulence	1	0	0	0	0	0
Gastroenteritis	0	0	0	0	2	0
Headache	0	1	0	0	0	1
Herpes simplex	1	0	0	0	0	0
Infection	3	0	0	2	0	0
Myalgia	0	0	0	1	0	0
Rhinitis	0	0	0	3	0	0
Vaginitis	0	0	0	2	0	0
Vomiting	0	0	0	0	1	0
Not coded	1	0	0	0	0	0
Total	10	1	0	9	5	2

N = Number of subjects.

Table 5
Baseline and change from baseline in vital signs.

Assessment		Probiotic (*N* = 25)	Placebo (*N* = 28)
Heart rate (bpm)	Baseline mean ± std	71.0 ± 10.2	71.2 ± 9.7
	Change at end of treatment		
	Mean[a]	1.0	1.8
	P-value	0.72	
	Difference (95% CI)	0.8 (−3.8, 5.5)	
Systolic BP (mm Hg)	Baseline mean ± std	117.1 ± 11.0	118.0 ± 10.7
	Change at end of treatment		
	Mean[a]	0.5	2.0
	P-value	0.640	
	Difference (95% CI)	1.5 (−4.9, 7.9)	
Diastolic BP (mm Hg)	Baseline mean ± std	75.9 ± 6.6	74.6 ± 7.8
	Change at end of treatment		
	Mean[a]	2.2	2.1
	P-value	0.976	
	Difference (95% CI)	0.1 (−3.5, 3.6)	
Respiration rate (bpm)	Baseline mean ± std	14.7 ± 1.8	13.8 ± 1.7
	Change at end of treatment		
	Mean[a]	−0.5	−0.1
	P-value	0.506	
	Difference (95% CI)	−0.4 (−1.5, 0.7)	
Oral temp. (°F)	Baseline mean ± std	98.0 ± 0.5	98.0 ± 0.5
	Change at end of treatment		
	Mean[a]	0.2	0.2
	P-value	0.949	
	Difference (95% CI)	0.0 (−0.35, 0.33)	

N = Number of subjects.
[a] Adjusted for baseline.

The end of treatment chemistry results of the two groups when compared to the baseline values also showed no statistically significant differences ($P > 0.05$) for glucose, BUN, creatinine, sodium, potassium, chloride, calcium, albumin, globulin, bilirubin, alkaline phosphatase, AST or ALT. Comparisons are presented in Table 7.

In the urine, there was a significant difference in specific gravity ($P < 0.05$) between the probiotic and placebo groups, with the placebo group showing a small increase (0.006 AU, Table 8). However, both values were well within normal limits for healthy humans (1.005–1.030, LabCorp). The difference in urine pH and other values were not statistically different ($P > 0.05$).

3.6. Quantitative PCR (qPCR) detection of S. salivarius K12 levels in saliva

At baseline, the mean qPCR-derivedcell count for K12-like bacteria in the subjects' saliva specimens was 1.84×10^3 cfu/ml (Table 9). Approximately half of the saliva samples had estimates of K12-like bacteria that were at least a logarithmic unit lower than the mean value (data not shown). These estimates are at the lower level of the detection limit of the qPCR system (approximately 1×10^3 cfu/ml of saliva) and can be considered negligible or the background associated with a complex clinical sample. On the day after taking their last K12 dose the average salivary count of K12-like bacteria had increased to 5.24×10^3 cfu/ml and in some subjects the estimates were as high as 6.19×10^4cfu/ml. By the end of the washout period the mean count of K12-like bacteria had declined to 1.61×10^3 cfu/ml, a value similar to baseline levels.

4. Discussion

Streptococcus salivarius is a predominant human commensal of the oropharynx and can constitute a large proportion of the total bacterial population inhabiting this region (Kononen et al., 2002). It is also predominant in human breast milk (Dalidowitz, 2005; Heikkila and Saris, 2003). The persistent natural exposure of humans to large numbers of indigenous *S. salivarius* from just after birth until death supports the proposition that re-exposure to this bacterium from external sources is likely to be non-hazardous, whether effected by natural transmission from other human hosts or by exposure to probiotic preparations (Favier et al., 2002; Kononen et al., 2002; Park et al., 2005; Preza et al., 2009). This study supports the view that *S. salivarius* K12 is not pathogenic or toxicologically active when regularly ingested in large numbers over a period of 28 days. By use of qPCR it was shown that, following its delivery in the described format strain, K12 is cleared from the oral cavity by natural processes during the course of a 28-day wash out period.

Clinical studies are usually powered in the design stage of planning such that they will adequately measure a desired change in clinical endpoint at a predetermined level of statistical confidence. In most cases, a study will have one or two primary endpoints, which makes selection of sample size relatively easy to estimate. In this case however, the primary endpoint is safety, which potentially involves a very large number of clinical chemistry, hematology and other endpoints, thereby complicating the calculation of sample size. Interestingly, there are relatively few human studies specifically detailing clinical chemistry or hematology endpoints

Table 6
Baseline and change from baseline in hematology data.

Laboratory test		Probiotic ($N = 25$)	Placebo ($N = 28$)
WBC	Baseline mean ± std	7.12 ± 1.90	6.53 ± 1.86
	Change at end of treatment		
	Mean[a]	−0.25	−0.07
	P-value	0.586	
	Difference (95% CI)	−0.19 (−0.88, 0.50)	
RBC	Baseline mean ± std	4.58 ± 0.37	4.72 ± 0.47
	Change at end of treatment		
	Mean[a]	−0.01	−0.02
	P-value	0.811	
	Difference (95% CI)	0.01 (−0.09, 0.11)	
Hemoglobin	Baseline mean ± std	13.9 ± 1.2	13.9 ± 1.4
	Change at end of treatment		
	Mean[a]	0.0	−0.05
	P-value	0.790	
	Difference (95% CI)	0.05 (−0.3, 0.4)	
Hematocrit	Baseline mean ± std	41.9 ± 3.0	42.1 ± 3.6
	Change at end of treatment		
	Mean[a]	−0.4	−0.7
	P-value	0.541	
	Difference (95% CI)	0.3 (−0.7, 1.4)	
MCV	Baseline mean ± std	91.7 ± 5.9	89.5 ± 5.3
	Change at end of treatment		
	Mean[a]	−0.8	−1.2
	P-value	0.221	
	Difference (95% CI)	0.4 (−0.3, 1.1)	
MCH	Baseline mean ± std	30.5 ± 2.4	29.6 ± 2.1
	Change at end of treatment		
	Mean[a]	0.0	0.0
	P-value	0.853	
	Difference (95% CI)	0.0 (−0.3, 0.3)	
MCHC	Baseline mean ± std	33.2 ± 1.1	33.1 ± 0.8
	Change at end of treatment		
	Mean[a]	0.3	0.4
	P-value	0.520	
	Difference (95% CI)	−0.1 (−0.5, 0.3)	
Platelets	Baseline mean ± std	291.5 ± 57.9	279.1 ± 65.8
	Change at end of treatment		
	Mean[a]	1.9	5.0
	P-value	0.731	
	Difference (95% CI)	−3.1 (−21.2, 14.9)	
Neutrophils	Baseline mean ± std	58.8 ± 9.0	57.5 ± 8.2
	Change at end of treatment		
	Mean[a]	−2.7	−2.7
	P-value	0.993	
	Difference (95% CI)	0.0 (−3.4, 3.4)	
Lymphs	Baseline mean ± std	30.6 ± 8.7	31.4 ± 7.5
	Change at end of treatment		
	Mean[a]	2.2	2.3
	P-value	0.966	
	Difference (95% CI)	−0.1 (−3.0, 3.1)	
Monocytes	Baseline mean ± std	7.68 ± 1.97	8.54 ± 2.12
	Change at end of treatment		
	Mean[a]	0.14	0.13
	P-value	0.973	
	Difference (95% CI)	0.01 (−0.63, 0.66)	
Eos	Baseline mean ± std	2.36 ± 1.93	2.00 ± 0.82
	Change at end of treatment		
	Mean[a]	0.42	0.34
	P-value	0.814	
	Difference (95% CI)	0.08 (−0.59, 0.66)	
Baso	Baseline mean ± std	0.56 ± 0.58	0.46 ± 0.51
	Change at end of treatment		
	Mean[a]	−0.02	0.05
	P-value	0.570	
	Difference (95% CI)	−0.07 (−0.32, 0.18)	

N = Number of subjects.
[a] Adjusted for baseline.

and we are not aware of any validated reports documenting the minimum numbers of subjects needed to measure the clinically and statistically-significant changes in a wide variety of parameters in a safety study.

Toxicity studies conducted under controlled conditions using in-bred strains of rodents, may not require large numbers of animals (Festing and Altman, 2002). Unfortunately, these models are not validated for probiotics (Shane et al., 2010) as the bacterial

Table 7
Baseline and change from baseline in chemistry data.

Laboratory test		Probiotic (*N* = 25)	Placebo (*N* = 28)
Glucose	Baseline mean ± std	89.1 ± 9.0	91.5 ± 9.3
	Change at end of treatment		
	Mean[a]	2.4	1.6
	P-value	0.742	
	Difference (95% CI)	0.8 (−3.9, 5.5)	
BUN	Baseline mean ± std	12.3 ± 2.8	12.3 ± 3.9
	Change at end of treatment		
	Mean[a]	0.8	0.6
	P-value	0.794	
	Difference (95% CI)	0.2 (−1.3, 1.7)	
Creatinine	Baseline mean ± std	0.82 ± 0.15	0.85 ± 0.15
	Change at end of treatment		
	Mean[a]	0.00	0.01
	P-value	0.587	
	Difference (95% CI)	−0.01 (−0.05, 0.03)	
Sodium	Baseline mean ± std	139.0 ± 2.0	139.8 ± 2.4
	Change at end of treatment		
	Mean[a]	−0.1	−0.2
	P-value	0.867	
	Difference (95% CI)	0.1 (−1.0, 1.1)	
Potassium	Baseline mean ± std	4.14 ± 0.25	4.10 ± 0.31
	Change at end of treatment		
	Mean[a]	−0.11	−0.03
	P-value	0.337	
	Difference (95% CI)	−0.08 (−0.24, 0.08)	
Chloride	Baseline mean ± std	102.8 ± 2.8	102.9 ± 1.7
	Change at end of treatment		
	Mean[a]	−0.72	−0.29
	P-value	0.450	
	Difference (95% CI)	−0.43 (−1.57, 0.71)	
Calcium	Baseline mean ± std	9.3 ± 0.4	9.4 ± 0.3
	Change at end of treatment		
	Mean[a]	−0.12	−0.15
	P-value	0.680	
	Difference (95% CI)	0.03 (−0.11, 0.17)	
Albumin	Baseline mean ± std	4.38 ± 0.34	4.36 ± 0.25
	Change at end of treatment		
	Mean[a]	−0.05	−0.07
	P-value	0.740	
	Difference (95% CI)	0.02 (−0.10, 0.13)	
Globulin	Baseline mean ± std	2.63 ± 0.34	2.73 ± 0.39
	Change at end of treatment		
	Mean[a]	0.01	−0.02
	P-value	0.571	
	Difference (95% CI)	0.03 (−0.07, 0.13)	
Bilirubin	Baseline mean ± std	0.49 ± 0.2	0.49 ± 0.22
	Change at end of treatment		
	Mean[a]	0.00	−0.04
	P-value	0.436	
	Difference (95% CI)	0.04 (−0.06, 0.14)	
AlkPhos	Baseline mean ± std	59.4 ± 12.6	66.4 ± 16.0
	Change at end of treatment		
	Mean[a]	2.0	1.4
	P-value	0.843	
	Difference (95% CI)	0.5 (−4.8, 5.9)	
AST	Baseline mean ± std	20.9 ± 5.5	21.0 ± 9.8
	Change at end of treatment		
	Mean[a]	0.6	0.7
	P-value	0.962	
	Difference (95% CI)	−0.1 (−3.9, 3.7)	
ALT	Baseline mean ± std	19.0 ± 7.3	20.3 ± 13.1
	Change at end of treatment		
	Mean[a]	−0.3	0.7
	P-value	0.555	
	Difference (95% CI)	−1.0 (−4.4, 2.4)	

N = Number of subjects.
[a] Adjusted for baseline.

strains used as probiotics are often host species-specific. Adults randomly recruited from the general population are more genetically heterogeneous than the subjects used in an animal model. Although the use of human subjects is of course consistent with the proposed specific application of the probiotic, it is more difficult to detect significant changes in clinical endpoints because the expected level of variance in humans will be greater than in in-bred animals. Few previous studies provide specific guidance concerning the planning of clinical safety studies. Wolf and colleagues (Wolf et al., 1998) assessed the safety of a *Lactobacillus*

Table 8
Baseline and change from baseline in urinalysis data.

Laboratory test		Probiotic (N = 25)	Placebo (N = 28)
Specific gravity	Baseline mean ± std	1.015 ± 0.009	1.016 ± 0.007
	Change at end of treatment		
	Mean[a]	0.001	0.006
	P-value	0.041	
	Difference (95% CI)	−0.005 (−0.009, −0.0002)	
pH	Baseline mean ± std	5.60 ± 0.79	5.52 ± 0.76
	Change at end of treatment		
	Mean[a]	−0.29	−0.20
	P-value	0.536	
	Difference (95% CI)	−0.09 (−0.39, 0.20)	

N = Number of subjects.
[a] Adjusted for baseline.

Table 9
Quantification of *S. salivarius* K12 in saliva samples from subjects that received the probiotic treatment.

Treatment phase	Meancfu/ml
Baseline phase ± std	1.8×10^3 (1.7×10^3)
Mean at end treatment phase ± std	5.2×10^3 (1.3×10^4)
Mean at end of washout phase ± std	1.6×10^3 (1.6×10^3)

reuteri probiotic strain in an HIV-infected population by analyzing various standard toxicological markers. They had earlier conducted a pilot study (Wolf et al., 1995) to assist with the derivation of the number of subjects required to see a difference in the levels of probiotic cells recovered from stool samples, but the calculations did not factor in other clinical end points or other biological markers of safety. A conservative approach led them to recruit 36 subjects to be given either probiotic or control preparations. However, given their HIV status this cannot be considered a normal population and disease marker progression may have been aberrant. In a more recent study (Wind et al., 2010) incorporating 17 test (probiotic-utilizing) subjects and 17 controls, clinical significance was claimed, but no details were given of how this was calculated. In the present study, 53 subjects were randomized into either probiotic or placebo groups. This relatively larger sample size when compared to other previously published studies was to increase the sensitivity of the study in order to heighten the chances of detecting any clinically-significant changes in the wide range of defined safety parameters.

The length of previously reported probiotic safety studies has been around 21 days, with the active dosing phase often followed by a similar period for washout (Wind et al., 2010; Wolf et al., 1995, 1998). Shane et al. (2010), when discussing the optimal length of such studies, indicated that there is not a priori preferred exposure time because this will depend upon the disease that the probiotic treatment is targeting; for example, diarrhea may only last a few days and there may be little point testing beyond the usual period of use. One typical application of *S. salivarius* K12 (when attempting to achieve oral colonization) is for as little as three days, but smaller individual doses are also sometimes taken on a daily basis for extended time periods (months) either for colonization maintenance or in supplements that may also contain additional conventional probiotic strains of lactobacilli or bifido bacteria.

In this study the examination of the subjects for their vital signs and physical condition showed no significant difference between those taking probiotic and placebo, and this was supported by examination of the content of the questionnaires completed by the subjects. Validated quality of life questionnaires such as the IBS-QOL questionnaire used with subjects specifically suffering irritable bowel syndrome (Drossman et al., 2000) do not exist in the literature for monitoring subjects taking probiotics. It can be anticipated that as the use of probiotics broadens beyond a primary focus on those of intestinal origins the type of study required to evaluate the occurrence of AE will also change to reflect the differences in targeting. The development in the present study of a specific set of questionnaires incorporating an oral focus, was an attempt to provide a more robust and qualitative monitoring of AEs reflective of strain K12's natural habitat and the target tissues that this probiotic was considered most likely to interact with and impact upon.

5. Conclusion

A single daily dose of strain K12 ten times its conventional daily dose taken over 28 days was used to demonstrate safety and tolerability in this study. Previous toxicology studies in animals did not indicate any dose-dependent effects of multiple dosing (Burton et al., 2010). Multiple dose studies can be useful to show that an adverse effect is treatment-related, with the intensity increasing in direct proportion to dose. However, the self-reporting by subjects on the occurrences of AEs and the documentation in questionnaires relating to their oral and gastrointestinal health combined with the results of blood chemistry, hematology and other clinical assessments clearly show that *S. salivarius* K12 is well tolerated by humans, even when taken in doses considered to be 10-fold the usual recommended daily intake.

Conflict of Interest

Authors J.P. Burton, P.A. Wescombe and J.R. Tagg are employees of BLIS Technologies Ltd.

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References

Burton, J., Chilcott, C., Wescombe, P., Tagg, J., 2010. Extended safety data for the oral cavity probiotic *Streptococcus salivarius* K12. Probiot. Antimicro. Prot. 2, 135–144.

Burton, J.P., Chilcott, C.N., Moore, C.J., Speiser, G., Tagg, J.R., 2006a. A preliminary study of the effect of probiotic *Streptococcus salivarius* K12 on oral malodour parameters. J. Appl. Microbiol. 100, 754–764.

Burton, J.P., Wescombe, P.A., Moore, C.J., Chilcott, C.N., Tagg, J.R., 2006b. Safety assessment of the oral cavity probiotic *Streptococcus salivarius* K12. Appl. Environ. Microbiol. 72, 3050–3053.

Callon, C., Millet, L., Montel, M.C., 2004. Diversity of lactic acid bacteria isolated from AOC Salers cheese. J. Dairy Res. 71, 231–244.

CDC, 2009. National Health and Nutrition Examination Survey 2007–2008. Documentation, Codebook, and Frequencies. Oral Health.

Dalidowitz, C., 2005. Fortified breast milk safety. J. Am. Diet. Assoc. 105, 1572–1573.

Dierksen, K.P., Tagg, J., 2000. The influence of indigenous bacteriocin-producing *Streptococcus salivarius* on the acquisition of *Streptococcus pyogenes* by primary school children in Dunedin, New Zealand. Streptococ. Streptococ. Dis. Enter. New Millen., 81–85.

Drossman, D., Patrick, D., Whitehead, W., Toner, B., Diamant, N., Hu, Y., 2000. Further validation of the IBS-QOL: a disease specific quality of life questionnaire. Am. J. Gastroenterol. 95, 999–1007.

Favier, C.F., Vaughan, E.E., De Vos, W.M., Akkermans, A.D., 2002. Molecular monitoring of succession of bacterial communities in human neonates. Appl. Environ. Microbiol. 68, 219–226.

Festing, M.F., Altman, D.G., 2002. Guidelines for the design and statistical analysis of experiments using laboratory animals. ILAR J. 43, 244–258.

Heikkila, M.P., Saris, P.E., 2003. Inhibition of *Staphylococcus aureus* by the commensal bacteria of human milk. J. Appl. Microbiol. 95, 471–478.

Hyink, O., Wescombe, P.A., Upton, M., Ragland, N., Burton, J.P., Tagg, J.R., 2007. Salivaricin A2 and the novel lantibiotic salivaricin B are encoded at adjacent loci on a 190-kilobase transmissible megaplasmid in the oral probiotic strain *Streptococcus salivarius* K12. Appl. Environ. Microbiol. 73, 1107–1113.

Kononen, E., Jousimies-Somer, H., Bryk, A., Kilp, T., Kilian, M., 2002. Establishment of streptococci in the upper respiratory tract: longitudinal changes in the mouth and nasopharynx up to 2 years of age. J. Med. Microbiol. 51, 723–730.

Martin, R., Heilig, H.G., Zoetendal, E.G., Jimenez, E., Fernandez, L., Smidt, H., Rodriguez, J.M., 2007. Cultivation-independent assessment of the bacterial diversity of breast milk among healthy women. Res. Microbiol. 158, 31–37.

Ongol, M.P., Asano, K., 2009. Main microorganisms involved in the fermentation of Ugandan ghee. Int. J. Food Microbiol. 133, 268–291.

Park, H.K., Shim, S.S., Kim, S.Y., Park, J.H., Park, S.E., Kim, H.J., Kang, B.C., Kim, C.M., 2005. Molecular analysis of colonized bacteria in a human newborn infant gut. J. Microbiol. 43, 345–353.

Pesic-Mikulec, D., 2005. Microbiological study of fresh white cheese. Appl. Ecol. Environ. Res. 4, 129–134.

Preza, D., Olsen, I., Willumsen, T., Grinde, B., Paster, B.J., 2009. Diversity and site-specificity of the oral microflora in the elderly. Eur. J. Clin. Microbiol. Infect. Dis. 28, 1033–1040.

Schulz, K.F., Altman, D.G., Moher, D., Group, C., 2010. CONSORT 2010 statement: updated guidelines for reporting parallel group randomized trials. Ann. Intern. Med. 152, 726–732.

Shane, A.L., Cabana, M.D., Vidry, S., Merenstein, D., Hummelen, R., Ellis, C.L., Heimbach, J.T., Hempel, S., Lynch, S.V., Sanders, M.E., Tancredi, D.J., 2010. Guide to designing, conducting, publishing and communicating results of clinical studies involving probiotic applications in human participants. Gut. Microbes. 1, 243–253.

Treiber, G., Schwabe, M., Ammon, S., Walker, S., Klotz, U., Malfertheiner, P., 2004. Dyspeptic symptoms associated with *Helicobacter pylori* infection are influenced by strain and host specific factors. Aliment. Pharmacol. Ther. 19, 219–231.

Vankerckhoven, V., Huys, G., Vancanneyt, M., Snauwaert, C., Swings, J., Klare, I., Witte, W., Van Autgaerden, T., Chapelle, S., Lammens, C., Goossens, H., 2008. Genotypic diversity, antimicrobial resistance, and virulence factors of human isolates and probiotic cultures constituting two intraspecific groups of *Enterococcus faecium* isolates. Appl. Environ. Microbiol. 74, 4247–4255.

Wescombe, P.A., Heng, N.C., Burton, J.P., Chilcott, C.N., Tagg, J.R., 2009. Streptococcal bacteriocins and the case for *Streptococcus salivarius* as model oral probiotics. Future Microbiol. 4, 819–835.

WHO, 2002. Guidelines for the Evaluation of Probiotics in Food Joint FAO/WHO Working Group Report on Drafting Guidelines for the Evaluation of Probiotics in Food. Food and Agriculture Organization of the United Nations World Health Organisation, London, Canada.

Wind, R.D., Tolboom, H., Klare, I., Huys, G., Knol, J., 2010. Tolerance and safety of the potentially probiotic strain *Lactobacillus rhamnosus* PRSF-L477: a randomised, double-blind placebo-controlled trial in healthy volunteers. Br. J. Nutr. 104, 1806–1816.

Wolf, B.W., Garleb, K.A., Ataya, D.G., Casas, I.A., 1995. Safety and tolerance of *Lactobacillus reuteri* in healthy adult male subjects. Microb. Ecol. Health Dis. 8, 41–50.

Wolf, B.W., Wheeler, K.B., Ataya, D.G., Garleb, K.A., 1998. Safety and tolerance of *Lactobacillus reuteri* supplementation to a population infected with the human immunodeficiency virus. Food Chem. Toxicol. 36, 1085–1094.