Journal of
Applied Microbiology

Journal of Applied Microbiology ISSN 1364-5072

ORIGINAL ARTICLE

A spontaneous lactate dehydrogenase deficient mutant of *Streptococcus rattus* for use as a probiotic in the prevention of dental caries

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Keywords
dental caries, lactate dehydrogenase, probiotic, *Streptococcus rattus*

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2008/1300: received 25 July 2008, revised 26 February 2009 and accepted 19 March 2009

doi:10.1111/j.1365-2672.2009.04333.x

Abstract

Aims: To study the ability of daily applications of *Streptococcus rattus* strain JH145 to affect the numbers of an implanted *Streptococcus mutans* strain in a rat model.

Methods and Results: A spontaneous L(+)-lactate dehydrogenase (LDH)-deficient mutant of *Streptococcus rattus*, JH146, was isolated by screening on selective medium and compared with a previously isolated spontaneous LDH deficient strain, JH145. Both strains were shown to have single base pair deletion mutations in the structural gene (*ldh*) for LDH, and reversion frequencies were approximately the same. Animals treated once daily with $\geq 10^6$ CFU (colony forming units) of JH145 showed a statistically significant decrease in the proportion of implanted *S. mutans* to total cultivable bacteria in oral swab samples. The rate of decrease in *S. mutans* levels was dose-dependent. No adverse effects were observed by in-life observation of treated animals, and histopathological, haematological and blood chemistry analyses were unremarkable.

Conclusions: The results presented indicate that daily application of JH145, a naturally occurring LDH-deficient variant of *S. rattus*, can compete with *S. mutans* for its habitat on the tooth surface.

Significance and Impact of the Study: *S. rattus* JH145 has potential as a probiotic for use in the prevention of dental caries.

Introduction

Probiotics are defined as live micro-organisms, which, when administered in adequate amounts, confer a health benefit on the host (Pineiro and Stanton 2007). To date, there is no definitive evidence that altering the microbiota of a healthy human adult is beneficial, but probiotics that alter the normal commensal microbiota of healthy farm animals are widely and routinely used to prevent certain conditions (Walker and Buckley 2006). Similarly, there are a number of conceivable scenarios in which adjusting the microbiota of healthy human subjects could have a beneficial prophylactic effect. For example, reducing the levels of the oral bacterium *Streptococcus mutans* with a probiotic strain of bacterium that does not produce enamel-eroding acid could reduce the risk for tooth decay in otherwise healthy people.

Several oral bacteria, including *S. mutans* and the *Lactobacillus* species, have been implicated at various stages of the dental caries process (ten Cate and van Loveren 1992; Ozaki *et al.* 1994; Featherstone 2000). In fact, there is considerable evidence compiled over the past 50 years implicating *S. mutans* as the principal etiologic agent of dental caries (Tanzer *et al.* 2001; Kuramitsu 2003; Caufield *et al.* 2005). The efficient production of lactic acid via glycolytic metabolism of dietary sugar (Hillman 1978; Johnson *et al.* 1980; Hillman *et al.* 2000), as well as the ability to convert sucrose to adherent glucan molecules that promote the attachment of these organisms to teeth (Gibbons and Nygaard 1968), were

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shown to be keys to this micro-organism's pathogenic potential. Mutations in the structural gene (*ldh*) for lactate dehydrogenase (LDH) can dramatically reduce the cariogenicity of *S. mutans* (Johnson *et al.* 1980).

One approach to understanding the interactions among the oral microflora, the environment, and the host is based on the ecological plaque hypothesis (Marsh 1991, 2003). This theory proposes that disease is because of a change in local environmental conditions, which disturbs the natural balance that exists between plaque and the host, leading to an increase in organisms that can cause disease. For example, the frequent exposure of plaque to low pH leads to an inhibition of acid-sensitive species and the outgrowth of organisms that are less sensitive to low pH, such as *S. mutans* and lactobacillus species (Marsh 1994). One interesting strategy, which would be compatible with the ecological plaque hypothesis, is a probiotic approach to change the oral environment toward health by the deliberate implantation of specific oral streptococci that naturally lack the ability to produce lactic acid to encourage the shift from a pathogenic to a nonpathogenic plaque or biofilm (Hillman 2002).

In addition to *S. mutans*, there are six other species of viridans streptococci that are closely related and which may be found naturally on the teeth of humans and other mammals (Coykendall 1989). These include *S. rattus*, *S. cricetus*, *S. sobrinus*, *S. ferus*, *S. macacae*, and *S. downei*. *Streptococcus mutans* is by far the most common member of this group in humans, but *S. sobrinus*, *S. cricetus* and *S. rattus* are also found.

A spontaneous mutant of the human *S. rattus* isolate BHT-2 was reported which lacked LDH activity (Johnson *et al.* 1980). Like the LDH-deficient mutant of *S. mutans*, this mutant had significantly diminished cariogenic potential. Instead of L(+)-LDH, metabolism of pyruvate derived from glycolysis appeared to proceed via the pyruvate-formate lyase and pyruvate dehydrogenase pathways (Hillman *et al.* 1987). The result was that significant amounts of substrate were converted to neutral end-products, principally ethanol and acetoin. The strain behaved identically to its parent with regard to every other phenotypic property examined.

Because of the similarity between *S. mutans* and *S. rattus*, it is interesting to speculate that the two may compete with each other for binding sites, nutrients and in other unknown regards for the same niche in the plaque biofilm. If so, a naturally occurring, lactic acid-deficient mutant of *S. rattus* could be used as a probiotic to reduce the levels of *S. mutans* in plaque, and thereby promote dental health. The present study used an animal model to test the efficacy and safety of an LDH-deficient *S. rattus* strain for use as a probiotic to reduce the numbers of *S. mutans* in the mouth and thereby decrease the risk of dental caries.

Materials and methods

Bacteria and reagents

The wild-type *S. mutans* strain NG8 has been described previously (Lee *et al.* 1989; Gutierrez *et al.* 1996). *Streptococcus rattus* strain JH145 is a spontaneous LDH-deficient mutant of BHT-2 previously reported (Johnson *et al.* 1980). JH146 was isolated in this study using the same methods, which involved growing an overnight culture of BHT-2 in Todd-Hewitt broth (Becton, Dickinson and Co., Sparks, MD, USA). The cells were washed with phosphate buffered saline (PBS) and diluted in PBS to give 12×10^4 CFU ml^{-1}. One hundred microlitre samples were spread on glucose tetrazolium medium plates (Lederberg 1948), which were incubated in candle jars for 72 h. Red colonies that appeared were picked and purified on the screening medium. Single colonies were tested for LDH deficiency by determining the terminal pH of saturated cultures grown in semi-defined medium (SDM; 0·06 mol l^{-1} KH_2PO_4, 0·04 mol l^{-1} K_2HPO_4, 0·01 mol l^{-1} NH_4Cl, 2·5% yeast extract) supplemented with 1% glucose, fructose, galactose, mannitol or sorbitol as carbon sources, and using BHT-2 and JH145 cultures as controls. When appropriate, culture liquors were quantitatively tested for lactic acid using the lactic acid concentration kit from Sigma (St. Louis, MO, USA). L(+)-LDH activity was measured in crude cell-free extracts as previously described (Hillman 1978).

Determination of reversion frequencies

Fifty replicate cultures of JH145 and JH146 were started by dilution of overnight cultures into 10 ml of SDM containing a growth limiting (0·35%) amount of glucose. The initial cell concentration, as measured by viable cell counts, was $\sim 10^3$ CFU ml^{-1}. The cultures were incubated for 72 h at 37°C to allow complete consumption of the carbohydrate. The concentrations of lactic acid in each culture were measured after removal of cells by centrifugation at 10 000 ***g*** for 15 min.

Effect of JH145 on NG8 in a rat model

Thirty-six male and 36 female weanling Sprague Dawley rats were obtained from Charles River Laboratories (Wilmington, MA, USA). The animals were caged separately and fed fluoride-containing tap water and (non-cariogenic) Purina rat chow (St Louis, MO, USA) *ad libitum*. In this conventional rat model, the animals were

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screened for the presence of indigenous *S. mutans* (Johnson *et al.* 1980) in plaque and saliva samples just prior to infection with strain NG8. Failure to observe morphologically characteristic colonies of *S. mutans* on the mitis-salivarius selective media was taken as evidence that the animals lacked a naturally occurring strain of *S. mutans*. Beginning at 27 days of age, each animal was infected for three consecutive days with 1×10^9 CFU of *S. mutans* strain NG8 in 100 μl of PBS. The rats were sampled once per week for one month to ensure uniformly good colonization of NG-8. Equal numbers of male and female rats were randomly assigned to each of six groups. Groups 1 to 5 were treated by delivering 100 μl of JH145 to give 10^9, 10^8, 10^7, 10^6, and 10^5 CFU respectively. JH145 doses were prepared by harvesting overnight SDM-glucose cultures by centrifugation and resuspending the cells in resuspension medium (SDM/30% glycerol) to the appropriate cell concentration. The doses were aliquoted and stored at −80°C until used. Animals in group 6 were sham treated with SDM/30% glycerol. The treatment was performed once per day, Monday through Friday, for 26 weeks. Oral swab samples for determination of NG8, JH145, and total cultivable bacteria were obtained on Monday morning prior to treatment with JH145. NG8 and JH145 in oral swab samples from rats were selected on S/S medium containing bacitracin and the pH indicator 2,3,5-triphenyltetrazolium chloride (Lederberg 1948), and, following 3 days incubation in candle jars at 37°C, NG8 and JH145 were differentiated as white and red colonies respectively. At the completion of the treatment period, all 12 animals in group 1 (treated with 10^9 CFU JH145/day) and three male and three female animals in group 6 (control) were euthanized with sodium pentobarbitol and necropsied. Blood was collected for analysis. Haematology (white blood cells, red blood cells, haemoglobin, haematocrit, mean corpuscular volume, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration, platelets, mean platelet volume, neutrophils, lymphocytes, monocytes, eosinophils and basophils), blood chemistry (albumin, alkaline phosphate, alkaline aminotransferase, aspartate aminotransferase, blood urea nitrogen, calcium, cholesterol, chloride, creatine kinase, creatinine, enzymatic carbon dioxide, glucose, potassium, sodium, phosphorus, total bilirubin, total protein, and triglycerides) and visual examination of organs and tissues (brain, spinal cord, thyroid gland, parathyroid gland, oesophagus, trachea, aorta, lung, heart, mandibular salivary gland, mandibular lymph node, kidney, tongue, adrenal gland, liver, spleen, stomach, duodenum, pancreas, jejunum, ileum, cecum, colon, rectum, mesenteric lymph node, uterus, cervix, ovary, vagina, testes, urinary bladder, skin, and eye) were performed by TherImmune Research Corporation (Gaithersburg, MD, USA). Oral samples from animals in group 4 (treated with 10^6 CFU JH145/day) and the remaining animals in group 6 (control) were obtained for an additional 8 weeks to determine post-treatment effects of JH145 on levels of NG8.

Statistical analyses

The animals' microbiological data were submitted to a group (6: 10^9, 10^8, 10^7, 10^6, 10^5, control) × Time (8: Baseline, weeks 6–8, weeks 9–11, weeks 12–14, weeks 15–17, weeks 18–20, weeks 21–23, weeks 24–26) Multivariate repeated measures analysis of variance (MANOVA). A second analysis was conducted to compare the 8-week period following the cessation of treatment in group 4 relative to control. The Omnibus analysis included a group (2: 10^6, control) × Time (2: weeks 27–30, weeks 31–34) repeated measures MANOVA. The Wilks' Lambda statistic was used for analyses with three or more within-animal levels. Planned comparisons were conducted between each treatment group and the control group for significant differences. The family-wise alpha level was set at $P = 0.05$.

Results

Screening of *c.* 5×10^5 individual colonies of BHT-2 led to the isolation of seven colonies with bright red coloration on glucose tetrazolium medium. Cultures of these in SDM medium containing 1% glucose revealed that one, JH146, produced essentially no detectable lactic acid. The same result was observed when fructose, lactose and galactose were used as the carbon source. Cell-free extracts of JH146 contained no detectable L(+)-LDH activity. JH146 thus appeared to be phenotypically identical to JH145, the previously reported spontaneous LDH-deficient mutant of BHT-2 (Johnson *et al.* 1980).

The genetic defects in JH145 and JH146 were determined by comparing the sequences of their *ldh* structural genes to that of BHT-2. Both JH145 and JH146 were found to have single base pair deletions (Fig. 1). The deletion in JH145 occurred in the middle of the gene and the shift in reading frame leads to a premature stop codon 158 bp downstream of the mutation. The deletion in JH146 occurred in a run of 3 adenines 134–136 bp before the gene's stop codon, and the shift in reading frame leads to a premature stop codon just 8–10 bp downstream of the mutation.

Because the reversion frequencies of the LDH mutations in JH145 and JH146 could not be directly measured, the relative stability of their mutations was measured by determining the concentration of lactic acid in 50 independent cultures of each strain. Each culture was started from 1×10^9 cells and allowed to incubate at

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Start →

ATGACTGCAACTAAACAACATAAAAAAGTCATCCTTGTCGGTGATGGTGCTGTA
G
GATCATCTTACGCCTTCGCCCTTGTTAACCAAGGAATCGCTCAAGAACTCGGAAT
TATTGAAATTCCTCAGCTGTTTGAAAAGGCTGTTGGAGACGCTCTTGACCTTAGC
CATGCACTTGCCTTCACTTCACCAAAGAAAATTTACGCTGCTAAATATGAAGACT
GTGCGGATGCTG**[A]**CCTTGTTGTCATTACTGCAGGTGCACCTCAAAAACCAGGT
GAAACTCGTCTTGACCTTGTCGGTAAAAACCTTGCAATCAACAAATCTATCGTTA
CACAAGTGGTTGAATCAGGCTTTAAGGGAATCTTCTTGGTTGCTGCCAACCCAGT
TGACATCT**TGA**CTTATTCAACATGGAAATTCTCAGGTTTCCCTAAAGAACGCGT
CATTGGTTCTGGTACATCTCTTGATACTGCTCGTTTCCGTCAAGCTCTTGCTGAAA
AAATCGGGGTTGATGCTCGGTCAGTCCACGCCTATATCATGGGTGAACACGGTG
ATTCAGAATTTGCCGTTTGGTCTCATGCCAATGTAGCTGGTGTTAAATTAGAACA
ATGGCTGCAAGACAACCGTGATGTTGATGCTGAAGGTCTCGTAAAACTGTTTGTA
TCTGTTCGTGATGCTGCTTATTCAATCATCAACAAAAAAGGTGCTACTTTCTATGG
TATCGCTGTCGCCCTTGCCCGTATCACTAAAGCAATCTTGGATGACGAAAACGCC
GTTCTTCCGCTTTCAGTTTTCCAATCAGGCCAATACGAAGGTGTTGAAGATGTCTT
CATCGGACAGCCGGCCATCGTTGGTGCACACGGTATCGTTCGTCCAGTAA**{A}**TA
TTCCGT**TAA**ATGATGCTGAACTGCAAAAAATGCAGGCTTCTGCTAAACAGCTGA
AAGCAATCATTGACGAAGCTTTCTCAAATGAAGAATTTGCTGCTGCTGCAGCTCG
TAACCTAAACAATAAAAAATCCA**TAA**
End

Figure 1 Location of Mutations and Premature Stop Codons in JH145 and JH146. Complete sequence of the LDH structural gene (*ldh*) in the parent strain, BHT-2, showing the locations of the deletion mutations in JH145 [A] and JH146 {A} and the positions of the first downstream stop codon created by the frameshifts (underlined).

37°C to enable complete metabolism of the limited glucose supplied. The average lactic acid concentrations for JH145 and JH146 cultures (not including 'jackpot' cultures) were identical (3·125 mmol l^{-1}). The average of the lactic acid concentrations for the 3 jackpot JH145 cultures was 13·66 ± 1·37 mmol l^{-1} and for the two jackpot JH146 cultures was 31·75 ± 0·35 mmol l^{-1}). Based on these results, we chose to proceed with JH145 for the following studies.

Seventy-two Sprague Dawley rats were infected for three consecutive days with 100 μl of *S. mutans* strain NG-8 containing 10^{10} CFU per ml and then sampled once per week for one month to ensure its stable colonization in the oral microflora. Table 1 provides weekly mean group values (n = 72) for total bacterial counts, NG8 counts, and the ratio of NG8 to total counts, and demonstrates that the NG8 strain achieved a stable colonization within a one-month period following infection of the animals with the *S. mutans* strain NG8. The rats were randomly distributed into six groups and treated with JH145 by oral infusion once per day, Monday through Friday, for 26 weeks. The concentration of the dose for each group ranged from 10^5 to 10^9 CFU. Animals in the control group were sham treated.

Oral plaque/saliva samples were taken from each rat with a cotton swab weekly and assayed to determine the numbers of JH145, NG8, and the total bacteria present. Table 2 provides microbiological cell counts for total bacteria, NG8 and JH145, and demonstrates that JH145 regularly appeared in the oral swab samples during treatment. Considerable variability occurred in bacterial counts in the swab samples and so the proportion of NG8 to total cultivable bacteria was determined. As the extent of the effect of JH145 on levels of NG8 was,

Table 1 Mean ± SD (n = 72) for NG8 and total cultivable bacteria, and the ratio of NG8 to total bacteria, as a function of time during 4-week baseline phase of study

	Week 1	Week 2	Week 3	Week 4
Total	2·47 ± 3·52	1·81 ± 2·33	11·60 ± 11·25	0·47 ± 0·54
NG8	1·82 ± 3·19	0·99 ± 1·22	6·25 ± 9·41	0·28 ± 0·41
NG8/total*	0·630 ± 0·245	0·574 ± 0·242	0·533 ± 0·262	0·562 ± 0·265

All mean ± SD count values × 10^5 colony forming units/oral swap sample.
*No significant difference in ratio between any two time points.

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Table 2 Means ± SD (n = 12) of 3-week subsets for NG8, JH145, and total cultivable bacteria of treated groups 1 and 4 relative to the control group 6 as a function of time

Group*	Baseline	6–8	9–11	12–14	15–17	18–20	21–23	24–26
1: Total	3·81 ± 7·20	12·45 ± 13·80	9·56 ± 7·28	9·22 ± 7·53	5·18 ± 5·41	7·04 ± 6·98	5·63 ± 5·01	4·49 ± 5·14
NG8	2·23 ± 5·79	5·69 ± 9·67	4·14 ± 5·67	3·34 ± 3·53	2·13 ± 2·71	3·12 ± 3·68	2·44 ± 2·79	2·12 ± 2·31
JH145	0	3·17 ± 5·62	2·64 ± 4·05	3·23 ± 4·48	0·96 ± 1·83	2·14 ± 2·28	1·37 ± 1·49	0·92 ± 1·04
4: Total	4·13 ± 5·10	6·81 ± 6·42	9·46 ± 11·69	6·55 ± 5·19	6·01 ± 6·95	5·55 ± 6·92	4·13 ± 2·99	3·34 ± 3·51
NG8	2·32 ± 3·32	2·67 ± 2·72	4·00 ± 12·05	2·67 ± 2·93	3·10 ± 4·94	3·54 ± 5·89	1·72 ± 1·36	1·27 ± 2·04
JH145	0	1·94 ± 3·09	1·35 ± 3·07	0·88 ± 1·51	0·97 ± 2·45	0·65 ± 0·93	0·62 ± 0·82	0·52 ± 1·27
6: Total	2·42 ± 3·13	10·46 ± 8·01	10·26 ± 8·37	6·93 ± 7·57	6·60 ± 4·07	6·67 ± 4·94	5·82 ± 7·08	3·22 ± 2·51
NG8	1·44 ± 1·85	6·22 ± 5·97	6·79 ± 6·03	3·61 ± 3·52	3·92 ± 3·05	4·15 ± 3·71	3·35 ± 3·48	1·91 ± 1·90
JH145	0	0	0	0	0	0	0	0

Time points (in 3-week subsets) (all mean ± SD count values × 10^5 colony forming units/oral swap sample)

logically, time-dependent, this interaction was analysed by breaking down the weekly group mean values for total bacteria counts, NG8 and JH145 by using the calculated mean for 3-week subsets. The proportion of NG8 to total cultivable bacteria and JH145 to total cultivable bacteria were also determined. Table 3 contains a summary determination of whether statistically significant differences occurred in the proportions of NG8 to total cultivable bacteria in the treatment groups relative to the control group as a function of time. Because of the random assignment of animals to the six groups, comparison of baseline data yielded no significant group effect. However, from the 9–11 week subset forward, there were significant group differences in the determined proportions of NG8 relative to the control for the higher (from 10^6 to 10^9 CFU of JH145) levels of JH145 treatment. Animals dosed with 10^5 CFU of JH145 did not demonstrate a significant difference in the proportion of NG8 to total bacteria throughout the entire course of the study. For illustrative purposes, Fig. 2 shows a comparison of the control group *vs* group 1 (oral dosing of JH145 at 10^9 CFU) and its effect on the levels of *S. mutans* NG8 as a function of time. As clearly evidenced by the trendlines, a significant decline in the proportion of NG8 to total cultivable bacteria was demonstrated within 3–6 weeks following the onset of treatment.

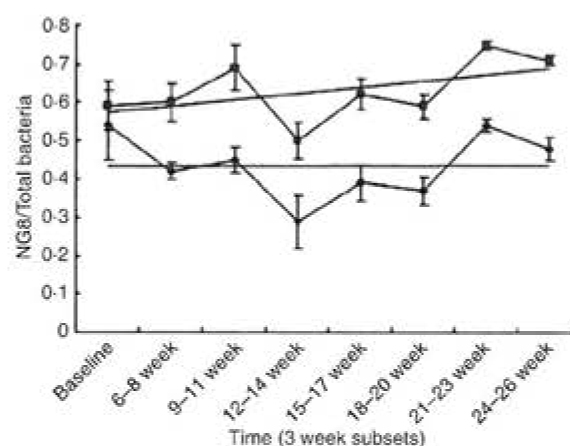

Figure 2 Effect of Oral Dosing of JH145 on the Proportions of *Streptococcus mutans* NG8 as a Function of Time. The mean (± SD) proportions of NG8 to total cultivable bacteria and trendlines are shown for group 1 (dosed daily with 10^9 colony forming units of JH145, n = 12) and group 6 (control group, n = 12) during baseline and 26-week treatment period. (–◆–, Group 1) and (–■–, Group 6)

After treatment for 26 weeks, animals in group 4 were analysed for post-treatment effect of JH145 on NG8 levels over an 8 week period. Table 4 contains post-treatment microbial data for group 4 (n = 12) and the control group (n = 5). The post-treatment analysis did not yield

Table 3 Statistical comparison of differences in means of 3-week subsets in NG8 proportions of treated groups relative to the control group as a function of time

Group*	Baseline	6–8	9–11	12–14	15–17	18–20	21–23	24–26	27–30	31–34
1	–	–	+	+	+	+	+	+		
2	–	+	+	+	+	+	+	+		
3	–	–	+	–	+	+	+	+	+	
4	–	–	+	+	+	–	+	+	+	–
5	–	–	–	–	–	–	–	–		

Time points (in 3-week subsets); post-treatment

+, $P < 0.05$ relative to control; –, $P \geq 0.05$ relative to control.

*n = 12 for each group.

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Table 4 Mean ± SD for NG8, JH145 and total cultivable bacteria, and the ratios of NG8 to total bacteria and JH145 to total bacteria, for group 4 (10^6 CFU of JH145, n = 12) and group 6 (control, n = 5) as a function of time during the post-treatment phase of study

Group	Week 24–26	Week 27–30	Week 31–34
4: Total	4·95 ± 7·29	8·89 ± 6·12	8·76 ± 10·99
NG8	2·35 ± 3·61	4·71 ± 5·01	5·51 ± 10·23
JH145	0·93 ± 2·86	1·12 ± 2·16	0
NG8/total	0·436 ± 0·177	0·481 ± 0·142	0·595 ± 0·328
JH145/total	0·144 ± 0·139	0·073 ± 0·127	0
6: Total	7·86 ± 7·02	18·76 ± 7·05	7·44 ± 5·24
NG8	6·17 ± 6·21	12·93 ± 4·06	4·81 ± 2·45
JH145	0	0	0
NG8/total	0·655 ± 0·208	0·684 ± 0·173	0·643 ± 0·184
JH145/total	0	0	0

All mean ± SD count values × 10^5 colony forming units/oral swap sample.

a significant effect. This lack of significance was likely because of the small sample size comprising the control group (n = 5). Although speculative, *post hoc* tests conducted to compare possible group effects at each time point yielded a significant effect at Week 27–30 where the control group sample was larger (n = 11), t (21) = 2·6, P = 0.015, revealing decreased proportion in group 4 relative to control. No such group effect was observed during Week 31–34.

The ratio of JH145 to total bacteria reflected the number of CFU administered, and was, at most, 0·35 of the total cultivable flora in group 1 animals receiving 10^9 CFU JH145/day. The number of total cultivable bacteria did not change significantly over the time course of the experiment, regardless of treatment.

No signs of adverse physical or behavioural effects of treatment were noted during daily observations of the animals throughout the study. After 26 weeks of treatment, all 12 animals receiving 10^9 CFU of JH145 and six control animals were euthanized. Necropsy, histopathological examination of haematoxylin and eosin stained tissues, haematological and blood chemistry testing of treated animals failed to reveal any significant differences from control animals.

Discussion

Naturally occurring *S. rattus* mutants that do not produce lactic acid and which, therefore are poorly cariogenic (Johnson *et al.* 1980), were isolated by screening colonies of the parent strain, BHT-2, grown on medium containing the pH indicator 1,3,5-triphenyltetrazolium chloride. On this medium, absence of acid production by a colony enables oxidation of tetrazolium to an insoluble formazan, which stains the colony bright red (Bochner and Savageau 1977). JH145, previously reported (Johnson *et al.* 1980), and JH146 reported here, had identical phenotypes, which included virtual absence of measurable L(+)-LDH activity, normal consumption of sugars and polyols (mannitol and sorbitol), and growth rates indistinguishable from their parent strain, BHT-2.

We have reported that LDH deficiency is lethal in most strains of *S. mutans* tested, probably because of the accumulation of toxic intermediates or to NAD-NADH imbalance (Hillman *et al.* 1994, 1996, 2000). *Streptococcus rattus* appears able to survive LDH deficiency as a result of having more active alternate pathways for pyruvate dissimilation, principally pyruvate-formate lyase and pyruvate dehydrogenase (Hillman *et al.* 1987). JH145 was previously shown to produce significant amounts of ethanol and acetoin under both aerobic and anaerobic conditions. It is also this production of neutral glycolytic end-products that renders LDH-deficient mutants of *S. rattus* less cariogenic than the wild-type.

Sequencing confirmed that LDH deficiency in both JH145 and JH146 was because of single base pair deletion mutations. The positions of the mutations differed. Also, there was a difference in the number of base pairs following the frameshift mutations before a stop codon was reached. In the case of JH145, there were 158 base pairs to the first premature stop codon and in the case of JH146 there were only 8–10 (depending on which of the run of three adenines was deleted). Because of this difference, it was felt that JH145 might be more prone than JH146 to second site mutations that restored some or all LDH activity. Because the *ldh*⁻ phenotype does not permit direct selection of revertants, we used lactic acid concentration as an indirect measure of reversion. We found that the frequency of jackpot cultures was virtually the same in both strains, and chose JH145 for further study.

Streptococcus rattus is a very close relative of *S. mutans* and, at one time was classified as a subspecies of *S. mutans* (Bratthall and Kohler 1976). Thus, it was hypothesized that *S. rattus* can compete with indigenous, decay-causing *S. mutans* for the same binding sites on the tooth surface, nutrients and other essential resources. An experimental animal model demonstrated that this is the case. Although the variability associated with sampling the oral flora is inevitably great as performed in this study, the proportion of NG8 to total cultivable bacteria remained essentially constant in the control group throughout the study, indicating the validity of this measure. JH145 was shown to have a dose dependent effect on the proportion of NG8 in oral swab samples. A significant and persistent decline in the proportion was observed at the higher (10^9 and 10^8 CFU) dose levels within 3 to 6 weeks following the onset of treatment. Lower doses (10^7 and 10^6 CFU) achieved significant effects 9 to 12 weeks following

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treatment onset. The lowest dose (10^5 CFU) did not produce a significant effect throughout the 26-week time-course of this study. The effect achieved by the higher doses did not diminish over time, indicating that NG8 did not become resistant to the treatment. This is the result expected based on the hypothesis that JH145 negatively interacts with *S. mutans* at a number of different points important to colonization.

The 4-week data subset immediately following the cessation of treatment indicated that animals treated with 10^6 CFU of JH145 retained a lower proportion of NG8 to total bacteria than control animals. This effect was eliminated in the second 4-week subset following cessation of treatment, indicating that the depression in the levels of NG8 caused by JH145 and that the effects of the treatment were reversible. The proportion of JH145 to total bacteria in oral swab samples correlated with the number of JH145 introduced in each group and constituted, at most, 35% of the total flora in animals receiving 10^9 CFU day^{-1} (data not shown). JH145 could be found in group 4 animals even 8 weeks following cessation of treatment, which correlated with the observed slow rise back to baseline of NG8.

Chronic exposure to JH145 appears to be safe within the framework of this experimental model. No in-life observational or clinical laboratory tests revealed any adverse side effect during treatment for 6 months at the highest (10^9) dose level. With the exception of the implanted *S. mutans*, no attempt was made to quantify the members of the indigenous oral flora. Thus, the effect of daily JH145 treatment on the oral ecology cannot be ascertained beyond its effect on the implanted strain of *S. mutans* and the observation that the total number of bacteria recovered from animals did not change with treatment.

The level of *S. mutans* in saliva, and hence on tooth surfaces, is a well-established risk factor for dental caries (Hardie 1992; Ruiz Miravet *et al.* 2007). The results of the study presented here suggest the possibility that reduction of *S. mutans* levels by daily administration of JH145 would decrease the risk of dental caries. Consumption of probiotic micro-organisms has long been accepted as a rational approach for the maintenance of gastrointestinal health and for enhancing the immune system. The use of JH145 for the maintenance of dental health is a logical extension of this strategy, and we propose to further test the safety and efficacy of JH145 as a probiotic for this application.

Acknowledgements

The authors thank Pamela Christopher and Liqun Song for their technical assistance. A portion of this work was supported by NIDCR grant DE04529.

Conflict of interest statement

The authors, J.D. Hillman, E. McDonell, T. Cramm and R.T. Zahradnik are employees of Oragenics Inc., the distributors of ProBiora3.

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