In Vivo Colonization with Candidate Oral Probiotics Attenuates Colonization and Virulence of Streptococcus mutans

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ABSTRACT

A collection of 113 Streptococcus strains from supragingival dental plaque of caries-free individuals were recently tested in vitro for direct antagonism of the dental caries pathogen Streptococcus mutans, and for their capacity for arginine catabolism via the arginine deiminase system (ADS). To advance their evaluation as potential probiotics, twelve strains of commensal oral streptococci with various antagonistic and ADS potentials were assessed in a mouse model for oral (i.e., oral mucosal pellicles and saliva) and dental colonization under four diets (healthy or high-sucrose, with or without prebiotic arginine). Colonization by autochthonous bacteria was also monitored. One strain failed to colonize, whereas oral colonization by the other eleven strains varied by 3 log units. Dental colonization was high for five strains regardless of diet, six strains increased colonization with at least one high-sucrose diet, and added dietary arginine decreased dental colonization of two strains. Streptococcus sp. A12 (high in vitro ADS activity and antagonism) and two engineered mutants lacking the ADS (∆arcADS) or pyruvate oxidase-mediated H₂O₂ production (∆spxB) were tested for competition against S. mutans UA159. A12 wild type and ∆arcADS colonized only transiently, whereas ∆spxB persisted, but without altering oral or dental colonization by S. mutans. In testing four additional candidates, S. sanguinis BCC23 markedly attenuated S. mutans’ oral and dental colonization, enhanced colonization of autochthonous bacteria, and decreased severity of smooth surface caries under highly cariogenic conditions. Results demonstrate the utility of the mouse model to evaluate potential probiotics, revealing little correlation between in vitro antagonism and competitiveness against S. mutans in vivo.

IMPORTANCE
Our results demonstrate *in vivo* testing of potential oral probiotics can be accomplished and can yield information to facilitate the ultimate design and optimization of novel anti-caries probiotics. We show human oral commensals associated with dental health are an important source of potential probiotics that may be used to colonize patients under dietary conditions of highly varying cariogenicity. Assessment of competitiveness against dental caries pathogen *Streptococcus mutans* and impact on caries identified strains or genetic elements for further study. Results also uncovered strains that enhanced oral and dental colonization by autochthonous bacteria when challenged with *S. mutans*, suggesting cooperative interactions for future elucidation. Distinguishing a rare strain that effectively compete with *S. mutans* under conditions that promote caries further validates our systematic approach to more critically evaluate probiotics for use in humans.

**INTRODUCTION**

Dental caries remains a highly prevalent disease and global health problem. Caries results from repetitive and/or prolonged demineralization of tooth enamel driven by exposure to low pH from organic acids produced by acidogenic oral bacteria during fermentation of dietary carbohydrates (1). Counterbalancing acid production and demineralization are alkalinization of oral biofilms by certain commensal bacteria, which in addition to salivary buffering and delivery of supersaturated calcium and phosphate ions, promotes enamel remineralization (1).

Furthermore, dental caries is a complex polymicrobial biofilm disease associated with competitive interplay between exopolysaccharide matrix-forming and acidogenic opportunistic pathogens with health-associated oral commensal species (1). With increasing exposure to dietary carbohydrates, such as sucrose (i.e., table sugar) or the high fructose corn syrups used in
many soft drinks and processed foods, the progressive production by cariogenic pathogens of organic acids and of a surrounding insoluble extracellular matrix composed of specific glucose polysaccharides (i.e., glucans) results in a microbial community shift that favors colonization of tooth biofilms by acidogenic pathogens at the expense of non-pathogenic and less acid-tolerant (aciduric) commensals (1). However, a number of commensals in dental plaque are associated with the health of dental surfaces, including several Streptococcus species (i.e., S. gordonii, S. sanguinis) (2-4). In vitro, commensals can combat cariogenic pathogens, such as the acidogenic and aciduric human caries pathogen S. mutans, by hindering its growth and viability through one or more mechanisms that include production of H₂O₂, the secretion of bacteriocins and other antimicrobial compounds, and interference with intracellular signaling pathways (1, 5-8). In addition, many oral commensals produce ammonia, either through the action of urease enzymes on urea, which is present at millimolar levels in saliva, or by catabolism of arginine to produce ornithine, ATP and CO₂ plus two molecules of ammonia by the three-enzyme arginine deiminase system (ADS) (9, 10). ATP generation is thus beneficial bioenergetically to ADS-containing commensals. Micromolar levels of arginine are present in ductal saliva, and arginine is abundant in salivary peptides and proteins (11). Generated ammonia counteracts acids from acidogenic bacteria to promote dental plaque pH homeostasis and tooth remineralization, while assisting less-aciduric commensals to survive, grow and compete against S. mutans (9, 10, 12). Collective evidence strongly supports a positive correlation between ammonia generation by commensals within dental biofilms and a lower incidence and severity of caries (13, 14). Also, delivery of 1.5% arginine as a prebiotic within dentifrices was shown in clinical studies to reduce caries onset (15) and decrease the increments of decayed, missing and filled (DMF) teeth and DMF surfaces (16, 17).
A strategy gaining acceptance to support the health of dental tissues is use of probiotics; administering bacterial strains to the oral cavity that are beneficial to the growth and maintenance of healthy biofilms, but that further suppress colonization and virulence mechanisms of cariogenic pathogens (18). Current evidence of probiotics to prevent dental caries primarily incorporate conventional strains used in treating gastrointestinal disorders (e.g., Lactobacillus ssp. and Bifidobacterium ssp), but with inconsistent results in pilot clinical studies based mostly on salivary levels of mutans streptococci, although a handful evaluated caries incidence (19, 20). Furthermore, oral colonization by probiotics are frequently transient and limited (19, 21). Although there is abundant in vitro physiological and molecular data of competition between a cariogenic pathogen and an oral commensal (1, 5-8) including potential probiotic strains (5, 18, 22-24), there are very few reports testing oral commensals as probiotics (24-27) even though these species are naturally adapted to colonization of specific oral sites, including dental biofilms (28).

A putative probiotic strain must contend in vivo with bacteriostatic, bactericidal and clearance mechanisms of saliva (29), and further cope with the intermittent availability of host dietary components for its own metabolism. A probiotic also must contend with symbiotic and competitive interactions with the widely diverse non-pathogenic and pathogenic microbial inhabitants of the soft tissues of the oral cavity and dental biofilms (5). In vivo models are therefore key to further advance caries research by providing a framework to assess a putative probiotic in its interactions with the host, in addition to commensal and pathogenic oral species. Mice have been used in caries studies (30-37) and are susceptible to colonization by human commensal streptococci (38-42). Knockout mice additionally allow interrogation of the impact
of host factors, such as specific salivary constituents, on colonization by commensals and the
induction of caries by oral pathogens (43-45).

To identify and test putative probiotic strains in the prevention and treatment of caries we
have taken a systematic approach, targeting commensal streptococci which represent an
abundant genera found in healthy dental plaque (28). First, we recently isolated 113
*Streptococcus* strains representing ten species from supragingival dental plaque of individuals
free of clinical lifelong dental caries. Each strain was tested *in vitro* for two specific phenotypes:
Production of ammonia from arginine catabolism by the ADS, and direct antagonism of colony
growth by pathogenic *S. mutans* UA159 (18), a highly virulent strain that displays great stress
tolerance compared to other clinical isolates of mutans streptococci (46). In this study, twelve
potential probiotic strains representing seven species of streptococci with various capacities to
express the ADS and antagonize *S. mutans* were further evaluated for oral and dental
colonization, *in vivo*, using a previously established mouse caries model, but with extensive
modifications (43-45). Modifications included incorporation of strain-specific qPCR assays to
examine colonization by each strain of inoculated human oral streptococci, and a newly
developed qPCR assay to evaluate colonization by the population of murine autochthonous
bacteria. Novel healthy diets of varying cariogenicity, with and without addition of the prebiotic
arginine, were incorporated to identify strains that colonize well under each dietary condition.
The competitiveness against *S. mutans* of select human commensal strains and otherwise-
isogenic deletion mutants were then examined, *in vivo*, and the impact on smooth surface and
sulcal caries assessed. Collective results highlight the importance of systematically evaluating
candidate probiotic strains, *in vivo*, to more critically evaluate attractive probiotic candidates and
functional genetic elements for further study. More specifically, we identify a strain that under
highly cariogenic conditions promotes colonization by dental autochthonous bacteria, attenuates colonization by *S. mutans* and decreased severity of smooth surface caries.

RESULTS

Colonization by Twelve Human Oral Commensals

Colonization by twelve strains of human commensal streptococci with various levels of ADS and *S. mutans* antagonism, as assessed by zone of inhibition (18) (see Fig. S1), were initially tested, *in vivo*, using a mouse model, as described in Materials and Methods. Our initial goal was to compare among these twelve strains their recoveries from dental biofilms and from recurrent oral swabs. As demonstrated later in Results, oral swabs provide a measure of colonization within saliva and the oral mucosal pellicle (47), thus representing potential reservoirs for subsequent colonization of dental biofilms. Recoveries of bacteria released from swabs or after sonication of molar teeth to disperse bacteria from dental biofilms were determined by strain-specific qPCR assays of genomic DNA, thus avoiding genomic integration of an antibiotic resistance cassette, which could unpredictably alter the behavior of a strain *in vivo*. Also, estimates of recovered total bacteria were determined using a novel qPCR assay targeting conserved regions of the ubiquitous single copy gene, *rpsL* (30S ribosomal protein S12) (48), rather than by CFU on blood agar plates (44, 45); the latter may overlook bacteria rendered non-viable during molar sonication. Subtraction of recovered genomes of inoculated strains from total recovered genomes thus estimates the population of recovered murine autochthonous bacteria. Colonization was compared among two primary diets; a diet representative of the average healthy American diet with 11.5% added sugar as sucrose (49) (average diet) and a highly cariogenic diet containing 37.5% sucrose, plus providing mice with 4% (w/v) sucrose in
their drinking water, *ad libitum* (high-sucrose diet). We reasoned that a probiotic must effectively colonize the oral soft and hard tissues irrespective of the cariogenicity of an individual’s diet, as diets will vary among humans, and from day-to-day for a given individual. Two other diets were created by addition of 1.5% arginine to each primary diet to determine whether arginine provided as a prebiotic influence’s colonization by a candidate probiotic strain. An increase in colonization with added dietary arginine would suggest that some minimum level of ADS activity may be required for a strain to more effectively counteract acids produced by members of the autochthonous bacterial population, and may therefore require arginine as a prebiotic to be competitive against *S. mutans*. Conversely, decreased colonization with added arginine indicates that simultaneous use of arginine as a prebiotic may be contraindicated. Diets were based on the nutritionally balanced diet, AINS-93G (50), rather than the commonly used cariogenic diet, Diet 2000, which is nutritionally deficient in vitamins and minerals that likely influence its cariogenic properties (51). Constituents of each diet are given in Table 1.

**Oral colonization:** Two of the twelve strains were each examined in one of six separate experiments. Shown in Fig. 1A is a timeline for each experiment. As shown in Fig. 1B, a striking outcome of the colonization results is the nearly complete inability of *S. cristatus* A52 to colonize the oral cavity and molar biofilms, whereas all other strains colonized, albeit to varying extents. Oral colonization, as assessed from oral swabs, ranged from $10^2$ genomes for *S. mitis* BCC15 to $10^5$ genomes for BCC32 and A12. There was only a single example in which oral colonization was significantly increased by added arginine (i.e., *S. gordonii* BCC32), whereas *S. mitis* BCC08 displayed decreased colonization. Though, in both cases, these differences were observed only with the high-sucrose diets and were inconsistent among swabs at experimental days 10 and 20. Interestingly, *S. gordonii* BCC32 has very high ADS activity compared to
nearly undetectable ADS activity in *S. mitis* BCC08 (Fig. 1S). However, oral colonization by all other strains with either similar or higher ADS activity were not impacted by arginine. Collective results suggest oral colonization is mostly independent of added dietary arginine and a strain’s ADS activity.

The only example where increased sucrose showed a trend towards impacting oral colonization was for A12, where recoveries from oral swabs were consistently at least 5-fold higher in the high-sucrose diet versus the average diet at day 10 and day 20. In only a few cases did oral colonization under the same diet change significantly from experimental day 10 to day 20, suggesting oral colonization mostly achieved steady-state levels by day 10.

**Dental colonization:** Five of the twelve strains exhibited relatively high levels of dental colonization with all four diets, ranging from about $10^5$ to slightly above $10^6$ genomes (i.e., *S. sanguinis* BCA8, *S. sanguinis* BCC23, *S. mitis* BCC45, *S. mitis* BCC08, *S. mitis* BCA12). Only *S. cristatus* BCA6 exhibited a significant increase in dental colonization with both high-sucrose diets, whereas six strains displayed increased colonization with at least one of the high-sucrose diets (i.e., *S. intermedius* A3, A12, *S. cristatus* BCA6, *S. gordonii* BCC32, *S. mitis* BCC15 and *S. oralis* subsp. *dentisani* BCA1). Interestingly, *S. intermedius* A3 stood out by exhibiting 4-log higher molar colonization with the high-sucrose diet versus the average diet. *S. intermedius* A3 and *S. oralis* subsp. *dentisani* BCA1 displayed significantly lower dental colonization with addition of arginine to the high-sucrose diet, and a trend towards lower colonization when arginine was added to the average diet. None of the other strains exhibited a significant increase in molar colonization when arginine was added to either diet.

**Murine autochthonous bacteria:** Murine autochthonous bacteria recovered from oral swabs were in nearly all cases consistent among the four diets and between experimental day 10
and day 20, suggesting resident bacteria are at steady-state levels by experimental day 10, regardless of diet. With respect to colonization of molars, mouse commensals also displayed few and relatively minor differences between diets, especially when comparing a high-sucrose diet to its respective average diet. A notable exception were mice inoculated with *S. intermedius* A3 in which recoveries were extremely high from mice fed the average and high-sucrose diets, but were dramatically reduced by addition of arginine to each diet, mirroring in large part colonization by *S. intermedius* A3. Furthermore, mouse commensals recovered from dental biofilms of mice inoculated with *S. cristatus* A52, which failed to colonize, were consistent across diets (10^6 to 2 x 10^6 genomes) and not significantly higher than that seen with mice challenged with the other eleven colonizing strains of human commensals (10^5 to 10^6 genomes).

These results suggest the total population of resident dental bacteria were only moderately impacted when mice were infected with a human commensal, regardless of diet. In all cases, mice fed each of the four diets gained equivalent body weights during the experiments (Fig. S2A), an indication that the overall health of mice was not compromised by a colonizing human strain or diet.

**Examination of *Streptococcus* sp. A12 and mutant derivatives thereof.**

The novel strain *Streptococcus* sp. A12, a relative of *Streptococcus australis* based on comprehensive phylogenomic analyses (9), has been studied *in vitro* due to its high levels of antagonism against *S. mutans* and ADS activity (Fig. S1) (5, 7, 9, 52). One mechanism used by A12 to inhibit growth of *S. mutans, in vitro*, is H$_2$O$_2$ production through pyruvate oxidase, encoded by *spxB* (9). Also, production of ammonia from arginine via the ADS is predicted to help counteract oral biofilm acidification by *S. mutans* to promote pH homeostasis, *in vivo*, thus...
creating an environment less favorable for the emergence of aciduric organisms. We therefore
set out to determine whether A12 interferes with colonization by *S. mutans* or colonization of
autochthonous bacteria, *in vivo*, and if either outcome was affected by elimination of the ADS or
pyruvate oxidase, using mutant strains ΔarcADS, lacking ADS activity or ΔspxB, respectively.

*Colonization:* Before testing A12 and its mutant strains, we first determined whether
each mutant colonizes the mouse oral cavity and dentition. Ultimately, we wanted to examine
A12 and its mutants under strongly cariogenic conditions to construct a rigorous test of each
strain’s colonization and potential competitiveness against *S. mutans*, which would provide
insights into the relative contribution of antagonism and pH moderation to competitive fitness.
We therefore examined colonization with mice fed the high-sucrose diet with 1.5% arginine and
4% sucrose water. In subsequent competition experiments, added arginine was then posited to
further amplify any deficits in competition associated with deletion of the ADS. In these initial
colonization experiments, we used the same timeline for colonization as in the previous set of
experiments with the twelve strains of human commensals (Fig. 2A). As shown in Fig. 2B, oral
colonization by the ADS mutant was not significantly different than A12 WT. However, dental
colonization by the ADS-deficient mutant was approximately 80% less than A12 WT, but still
sufficient for further testing. In contrast, both oral and dental colonization of A12 ΔspxB were
similar to A12 WT. Oral and dental colonization of mouse commensals were not significantly
different between the WT and each mutant group. The mean increase in body weights between
groups were also not significantly different (Fig. S2B).

*Colonization by S. mutans:* In testing human commensals in competition experiments,
our strategy was to first establish colonization by the commensal, followed by oral inoculations
with *S. mutans* UA159 and monitoring of colonization. We reasoned that first establishing the
human commensal likely reflects future clinical applications of probiotics in which patients at high caries risk would first undergo comprehensive removal of supragingival dental biofilms, followed by administration of a probiotic immediately thereafter, and subsequent self-administration of probiotic at periodic intervals after brushing of teeth and/or use of an oral antiseptic. Because our initial oral colonization results indicated human oral commensals appeared to reach a steady-state by experimental day 10 (swab 1) or earlier, we scheduled subsequent inoculations with *S. mutans* to start at experimental day 7. A concern with this strategy was that mice would not be inoculated with *S. mutans* until 6.5 to 7 weeks of age. It is established that colonization of rodents by *S. mutans* and subsequent development of dental caries is greatest when inoculated before weaning at age 21 days, when tooth eruption is in its early stages, and that colonization and caries then declines markedly with age (43, 53). In contrast, BALB/c mice greater than 8 weeks of age were able to be colonized with *S. mutans* after antibiotic suppression of the oral microbiota (41, 42) and to induce measurable caries (54). It was thus necessary to first establish in our model how well *S. mutans* colonized and induced caries, but to also determine whether colonization and induction of caries by *S. mutans* decreased when inoculations were initiated at experimental day 7, compared to day 0. We used the high-sucrose diet with 4% sucrose water and extended the experiment to seven weeks after the first inoculation with *S. mutans* to compare caries levels with those of prior experiments in which mice were challenged with *S. mutans* before weaning and fed a highly cariogenic diet for seven weeks (43, 53).

Shown in Fig. 3A is the experimental timeline. Mice were divided into two groups, with one group on experimental day 0 receiving the first of five-daily inoculation of *S. mutans*, and the second group five daily inoculations without added bacteria (mock inoculations). On day 7,
five daily inoculations were initiated. Fig. 3B shows that oral colonization by \textit{S. mutans} in both cases soon reached a steady-state of slightly less than $10^5$ genomes, whereas murine autochthonous bacteria (mouse commensals) tended to increase more gradually, eventually reaching levels comparable to \textit{S. mutans}. Molar colonization levels were statistically equivalent between \textit{S. mutans} and murine autochthonous bacteria, both within and across the two conditions. Importantly, the incidence and severities of smooth surface and sulcal caries were highly similar after inoculating mice with \textit{S. mutans} UA159 on experimental day 7 compared to day 0 (Table 2), demonstrating the absence of an age-related attenuation on colonization and induction of caries by \textit{S. mutans}. Moreover, sulcal caries were comparable to those obtained in previous studies with mice inoculated prior to weaning and using Diet 2000 containing 56% sucrose with 5% sucrose water (43-45). There also were no differences in the mean increase in body weights between the two groups during the experiment (Fig. S2C).

\textit{Competition between \textit{S. mutans} and A12}: Having established that A12 mutants Δ\textit{arcADS} and Δ\textit{spxB} colonize mice and further validating the competition experimental protocol, we next examined whether A12 altered colonization of \textit{S. mutans} and/or the levels of autochthonous bacteria, and if deletion of ADS activity or of pyruvate oxidase affected the outcomes. As shown in Fig. 4A, the major events in the experimental timeline are the same as in the competition validation experiment, except the experiment was ended at experimental day 28, 21 days after starting \textit{S. mutans} inoculations, but with more frequent oral swabbing to monitor oral colonization. As explained above, mice were fed the high-sucrose diet with 1.5\% added arginine and 4\% sucrose water. A group in which initial inoculations were without added bacteria (mock inoculations) was included as a control group for \textit{S. mutans} alone. As shown in Fig. 4B, oral and dental colonization by \textit{S. mutans} in the mock group were each robust, whereas oral colonization
by murine commensals were lower but persistent. Similar to results of the validation experiment (Fig. 3B, Inoculated Days 7-11 group), dental colonization by mouse commensals was about 2 logs greater than its oral colonization and comparable to that of \textit{S. mutans}. There were distinct differences in colonization between the three strains of A12. First, A12 WT was undetectable in swabs at experimental day 27 and barely detectable a day later in dental biofilms. A12 ∆\textit{arcADS} appeared even less competitive against \textit{S. mutans}, as oral colonization was undetectable a week earlier than the WT and undetectable in molar biofilms. In stark contrast to A12 WT, oral colonization by \textit{S. A12 ∆spxB} was unexpectantly persistent, with recoveries comparable to its recovery from dental biofilms. Oral colonization by murine autochthonous bacteria in this group was also persistent and similar to the mock group, while recoveries of mouse commensals in both the A12 WT and A12 ∆\textit{arcADS} groups were erratic at times, but nonetheless comparable or slightly higher than in the mock group by experimental day 27, respectively. Importantly, none of the A12 strains had an impact on dental colonization by \textit{S. mutans}. Furthermore, mean increase in body weights between the four groups were not significantly different (Fig. S2D).

**Examination of the Competitiveness of Four Additional Human Commensal Streptococci**

We further assessed four additional human commensals in competition against \textit{S. mutans} (i.e., \textit{S. gordonii} BCC32, \textit{S. mitis} BCA12, \textit{S. sanguinis} BCA8 and \textit{S. sanguinis} BCC23) because they exhibited the highest levels of colonization of dental biofilms under all four test diets (Fig. 1B). As a group, these strains also demonstrated various levels of ADS activity and antagonism (Fig. S1). Because an effective probiotic is expected to function well even when a patient is consuming a meal high in cariogenic carbohydrates, mice were given the high-sucrose diet with added arginine and provided 4% sucrose water. The experimental timeline (Fig. 5A) and
inclusion of a mock group were the same as with the A12 strains. As shown in Fig. 5B, oral and
dental colonization by *S. mutans* in the mock group were at high levels compared to murine
autochthonous bacteria, although oral colonization of murine commensals was persistent.

Oral colonization by *S. gordonii* BCC32 progressively decreased to low levels, as did
murine autochthonous bacteria. Noteworthy, though, was the approximate 10-fold increase in
oral colonization of mouse commensals following initial inoculations with *S. gordonii* BCC32,
as well as the more than 3-fold decrease in *S. mutans* compared to the mock group at
experimental day 27. A moderate level of *S. gordonii* BCC32 was recovered from dental
biofilms. Compared to the mock group the recovery from dental biofilms of *S. mutans* was
unaltered, whereas autochthonous bacteria were enhanced. Importantly, the very low recovery
(about 500 genomes) of mouse commensals from swabs on day 27 (swab 5) compared to nearly
10^6 genomes recovered from mandibular molars on day 28, demonstrates oral swabs capture
bacteria primarily from non-dental biofilms, most likely from saliva, epithelial biofilms (i.e.,
mucosal pellicles) and papillary groves of the tongue.

Oral colonization of *S. mitis* BCA12, like *S. gordonii* BCC32, progressively decreased
with time. Conversely, oral colonization levels of murine autochthonous bacteria were
consistent and levels of *S. mutans* increased 4-fold at experimental day 27. Dental colonization
of *S. mitis* BCA12 was at a very low level, whereas dental colonization of *S. mutans* was
unaltered, compared to the mock group. However, recovery of mouse commensals from dental
biofilms was greater than in the mock group.

Unlike the two aforementioned strains, oral colonization by *S. sanguinis* BCA8 remained
markedly consistent after introduction of *S. mutans*, and it populated molar biofilms at a level
only about 4-fold lower than *S. mutans*. As for autochthonous bacteria, *S. sanguinis* BCA8 had a
positive impact on dental colonization of this population, and a transient positive effect on their oral colonization. Nevertheless, oral and dental colonization levels of \textit{S. mutans} mimicked those of the mock group.

The most striking results were seen with \textit{S. sanguinis} BCC23. Its oral colonization was stable, then increased during the final week. Its presence was associated with significant and consistently higher levels of murine autochthonous bacteria when compared to the mock group. Conversely, the levels of oral colonization of \textit{S. mutans} were depressed initially, but increased during the final week in conjunction with \textit{S. sanguinis} BCC23 and autochthonous bacteria. More importantly, dental colonization of \textit{S. mutans} was nearly 4-fold lower than in the mock group, and also markedly lower than autochthonous bacteria. Dental colonization by \textit{S. sanguinis} BCC23 was greater than $10^5$ genomes, equivalent to \textit{S. mutans}. Furthermore, mouse commensals in dental biofilms were significantly greater than in the mock group. There were also no differences in the mean increase in body weight between each of the five groups during the experiment (Fig. S2E).

Assessment of caries: Because \textit{S. sanguinis} BCC23 effectively competed with \textit{S. mutans} for colonization of molar biofilms, and all four groups enhanced dental colonization of autochthonous bacteria, we examined the mandibular molars of each of the groups to determine whether the incidence and severity of caries was impacted by a strain compared to the mock group. Bear in mind this experiment was designed to examine colonization, ending only three weeks after the first inoculation with \textit{S. mutans}, compared to seven weeks in a typical caries experiment. Thus, caries levels were expected to be relatively low. Nonetheless, as shown in Table 3, mice colonized with \textit{S. sanguinis} BCC23 demonstrated decreased severity of total smooth surface caries, due primarily to lower incidences in buccal and lingual lesions.
Colonization by *S. sanguinis* BCA8 and *S. gordonii* BCC32 was associated with a trend towards fewer lesions on buccal surfaces and decreased severity of lingual caries, respectively. However, all four groups exhibited sulcal caries similar to the mock group.

**Reproducibility between experiments**

There were two cases in which experimental conditions were repeated in separate experiments, providing an opportunity to assess the reproducibility of bacterial recoveries in the *in vivo* model. The first case was in two experiments testing colonization by WT A12, in which there was only a single significant difference; a 4.0-fold difference in the recovery of A12 at swab 2 (Fig. 6A). The second case included the two mock groups in competition experiments with *S. mutans*. There are two instances of significant differences between these two experiments; recovery of *S. mutans* at swab 4 (7.5-fold) and recovery of mouse commensals from mandibular molars (6.9-fold) (Fig. 6B). Overall, though, there was good reproducibility between experiments.

**DISCUSSION**

**Diet and Colonization by Human Commensal Streptococci and Autochthonous Bacteria**

*Oral and dental colonization in relation to dietary sucrose:* In the context of dental caries, dietary carbohydrates are a critical determinant of the oral microbiome and oral health, due in large part to the ability of *S. mutans* to rapidly utilize sucrose to produce a structural matrix of insoluble glucans that can greatly enhance the cariogenic potential of oral biofilms (1). The high cariogenicity of the high-sucrose diet used in this study was confirmed, as smooth surface and sulcal caries of mice inoculated with *S. mutans* alone were comparable to prior
experiments that incorporated Diet 2000, containing 56% sucrose and 5% sucrose water (43). In contrast, the average diet contained nearly 70% less sucrose, and mice were supplied with sterile drinking water without added sucrose. Omission of sucrose from the drinking water further reduces the cariogenicity of a diet, likely by decreasing the frequency of exposure of *S. mutans* to sucrose. For example, in a pilot study using our earlier mouse caries model with mice fed Diet 2000, the incidences of total smooth surface caries, total sulcal caries and recovery of *S. mutans* from molar sonicates were 84%, 62% and 49% less, respectively, in mice provided sterile water, compared to mice provided with 5% sucrose water (Culp, unpublished observations). Therefore, in light of the differences in cariogenicity between the average and high-sucrose diets, the ability of the great majority of candidate probiotics to colonize the oral cavity and dental biofilms at relatively moderate to high levels, regardless of the level of dietary sucrose, is considered a reflection of their adaptation to dental biofilms of caries-free individuals and is a highly desirable attribute, as diet will likely vary in cariogenicity among patients taking probiotics. In addition, colonization of non-dental sites in the oral cavity such as oral epithelium and within papillary groves of the tongue potentially creates a reservoir for persistent recolonization of dental biofilms. Of particular note was the inexplicable extreme increase in molar colonization by *S. intermedius* A3 with the high-sucrose diet compared to the average diet. Additional investigations are required explain this phenotype.

*Added dietary arginine and colonization:* Added dietary arginine had no consistent positive impact on either oral or dental colonization by nine strains, and was associated with decreased dental colonization of two strains. The absence of a positive impact suggests those strains with relatively high ADS activity (e.g., *S. gordonii* BCC32, *S. sanguinis* BCC23 and A12) are sufficiently aciduric or do not require increased ADS activity because base production via
urease from salivary urea combined with the ADS utilizing arginine supplied via salivary
peptides and dietary proteins, is enough to counteract any acids produced by the autochthonous
bacterial population. Conversely, colonizing strains not impacted by arginine, but with very low
ADS activity (e.g., *S. sanguinis* BCA8, *S. mitis* BCA12 and *S. mitis* BCC08), may be sufficiently
aciduric. Interestingly, *S. intermedius* A3 with very high ADS activity was negatively impacted
by arginine. Added arginine may have increased environmental pH to levels substantially above
neutrality (e.g., 8.0), levels where many human streptococci display poor growth (10), thus
explaining the extreme decrease in molar colonization by both *S. intermedius* A3 and murine
autochthonous bacteria with added dietary arginine.

*Murine autochthonous oral bacteria:* Murine autochthonous oral bacteria recovered
rapidly after antibiotic treatment as observed in each mock inoculation group of the two
competitive colonization experiments, where recoveries of mouse commensals on day -1 were no
more than 2-fold lower compared to day 6. Furthermore, the inability of *S. cristatus* A52 to
colonize mice demonstrates inoculated strains must compete with murine autochthonous
bacteria. Similar levels of either oral or molar colonization by autochthonous bacteria across the
four diets indicates added sucrose or arginine do not alone impact the total population of mouse
commensals. The presence of a significant population of mouse resident flora upon introduction
of human oral commensal strains, combined with establishment of eleven of the twelve strains
under different dietary conditions, again speaks to the adaptation of these strains to the oral
environment.

Examining specific strains and isogenic mutants in competition against *S. mutans*
A12 and ADS in colonization and competition: The poor competitiveness of A12 WT against *S. mutans*, *in vivo*, was unanticipated. Of the twelve strains, A12 has very high ADS activity and the highest level of antagonism, *in vitro*, and was recently shown to have multiple strategies to antagonize *S. mutans*, such as inhibition of *comX*-inducing peptide signaling (5). Nevertheless, the more rapid decrease in oral colonization by A12 ∆arcADS versus the WT when in competition with *S. mutans* may be due to decreased competitiveness against *S. mutans*, as the mutant displayed similar oral colonization as A12 WT in the absence of *S. mutans*. In contrast, molar colonization by A12 ∆arcADS in the absence of *S. mutans* was significantly less than the WT, indicating ADS contributes to A12’s competitiveness against autochthonous bacteria in dental biofilms under cariogenic conditions. Perhaps one or more members of the population of mouse commensals are sufficiently acidogenic that A12’s ADS is required to help support dental colonization. Although A12 ∆arcADS failed to colonize molar biofilms in the presence of *S. mutans*, it is unclear whether ADS activity exerts any additional competitiveness for A12 WT against *S. mutans* in addition to competitiveness against autochthonous bacteria.

A12 pyruvate oxidase: Surprisingly, A12 ∆spxB persisted orally at levels significantly greater than A12 WT, but did not interfere with oral and dental colonization by *S. mutans*. In a dual-species biofilm model of A12 and *S. mutans*, both species formed microcolonies adjacent to each other without any detectable integration, with a decrease in both biomass and biofilm maximal thickness compared to biofilms of *S. mutans* alone (52). It would thus be interesting to determine in dental biofilms, *in vivo*, whether A12 ∆spxB is localized adjacent to *S. mutans* and if the ADS is required for its dental and persistent oral colonization.

A closer look at the combined results of all three strains of A12 provides a putative explanation for persistent oral colonization of A12 ∆spxB. First, the total population of mouse...
oral commensals was not impacted when *S. mutans* was introduced in the mock group, suggesting autochthonous bacteria readily adapt to introduction of *S. mutans*. However, addition of an A12 strain expressing pyruvate oxidase activity (i.e., A12 WT and A12 ΔarcADS) appears to initiate microbial interactions that are detrimental to A12 and negatively affect autochthonous bacteria, but allow the commensal population to eventually recover. A similar scenario may also explain the poor colonization of dental biofilms by A12. Additional studies are thus warranted to determine how production of H₂O₂ by A12 alters competitive mechanisms of oral autochthonous commensals when *S. mutans* is entered into the environment. In a broader context, the results call for more thorough investigation of the influence of H₂O₂ production by commensal streptococci on the establishment, persistence and virulence of oral pathogens and pathobionts (55).

**Interrogation of four additional human commensal streptococci:** Interestingly, the total population of mouse commensals within dental biofilms when mice were challenged with *S. mutans* was enhanced in the presence of each of the four strains, indicating these strains promote the resilience and competitiveness of dental biofilm residents. Further investigations of cooperative mechanisms beneficial to autochthonous bacteria may elucidate additional approaches to help prevent caries. With respect to *S. gordonii* BCC32, its inability to affect dental colonization by *S. mutans* is consistent with previous results in rats with *S. gordonii* Challis CH1, which failed to alter molar colonization by *S. mutans* or the incidence of caries, despite showing antagonism against *S. mutans*, *in vitro* (56). Only *S. sanguinis* BCC23 and *S. sanguinis* BCA8 demonstrated persistent oral colonization and were recovered at high levels from dental biofilms. More importantly, *S. sanguinis* BCC23 attenuated *S. mutans*’ colonization of molar biofilms and showed promise in lowering smooth surface caries. Interestingly, colonization of newly acquired teeth in children by *S. sanguinis* precedes and also delays
colonization by *S. mutans*, suggesting an inhibitory effect on *S. mutans* (57). Furthermore, within cavitated lesions, in which *S. mutans* accounts for up to 55% of the microbiota, *S. sanguinis* persists (4). Thus, other oral strains of *S. sanguinis* may represent an important source of probiotic strains. BCC23 is only the second oral commensal strain with demonstrated competitiveness against *S. mutans* under such highly cariogenic conditions, *in vivo* (58). Limited clinical studies of potential probiotics incorporating oral streptococci have reported lower caries development in children (27), decreased levels of *S. mutans* in either saliva (25, 26) or dental plaque (59), demonstrating the potential for effective anti-caries probiotics based on strains of oral commensal streptococci. *S. sanguinis* BCC23 is therefore a highly attractive probiotic candidate and can serve as an important tool to elucidate competitive mechanisms that hinder the establishment, persistence, and virulence of *S. mutans*. For example, because *S. sanguinis* BCC23 has relatively high ADS activity, it would be of interest to determine the effects on competitiveness of added dietary arginine and of deletion of ADS activity.

Antagonism in vitro does not correlate with competitiveness *in vivo*

A closer look at antagonism, *in vitro*, of all five strains examined for competitiveness against *S. mutans* shows that antagonism levels of A12 and *S. gordonii* BCC32 are about 40-fold and 2-fold greater, respectively, than that for *S. sanguinis* BCC23. *S. mitis* BCA12 and *S. sanguinis* BCA8 have only slightly lower levels than *S. sanguinis* BCC23 (see Fig. S3). Furthermore, the failure of strains other than *S. sanguinis* BCC23 to affect dental colonization by *S. mutans* is not related to their ability to colonize dental biofilms, as each strain colonized molar teeth at levels higher or slightly lower than *S. sanguinis* BCC23 when mice were fed the same diet (Fig. S3). These combined results strongly suggest antagonism against *S. mutans, in vitro,*
which is highly dependent on growth conditions, does not correlate with competitiveness in vivo, at least under the conditions referenced herein, and further warrant that caution should be exercised when extrapolating in vitro phenotypes to the in vivo environment. In vivo testing of competitiveness of a candidate probiotic thus represents an important discriminating assessment to identify strains for further study, especially in light of the phenotypic heterogeneity displayed by oral streptococci is not species specific, nor always reflected by genotype (18). Initial identification in an animal model of strains such as S. sanguinis BCC23 that colonize, persist and compete against S. mutans can further reduce unnecessary clinical studies that test strains based solely on in vitro data. However, because functional genomics of commensal strains such as A12 are identifying previously unrecognized genetic elements that function in competitiveness, in vitro, genes shown to impact competitive fitness may eventually serve as biomarkers to better recognize beneficial organisms in vivo (5). Such efforts will nevertheless require in vivo examination of isogenic mutants.

Collective results demonstrate human oral commensals strongly associated with dental health are generally well adapted to colonize both the soft and hard tissues of mice under highly cariogenic and healthier dietary conditions, and identifies a highly attractive probiotic candidate, S. sanguinis BCC23. Health-associated dental isolates from humans thus represents a source of putative probiotic strains with the potential to colonize dental and oral biofilms of patients, regardless of diet. Results further demonstrate the in vivo model is sensitive and reproducible, representing a reliable platform to rigorously test putative probiotic strains to colonize soft and hard tissues, to compete against severe challenge with a highly virulent pathogen, to support autochthonous commensals and to reduce the incidence of caries. The model is also amenable to interrogations of key molecular mechanisms responsible for competitiveness against S. mutans.
and persistent colonization of epithelial and/or dental biofilms. The model is further amenable to explore the effectiveness of the dose and frequency of administration of a probiotic or prebiotic. We therefore demonstrate the utility of *in vivo* assessments to more stringently evaluate the oral fitness of candidate strains to help facilitate the rational design and optimization of novel probiotic strategies to target microbial ecology in protection of supra-gingival dental surfaces.

**MATERIALS and METHODS**

*Procedures with mice:* The mouse model is a modification of a previously described mouse caries model (44). All procedures with solutions and samples were performed under BSL2 conditions and mice were kept under ABSL2 conditions. Briefly, inbred 3-week-old female SPF BALB/cJ mice (The Jackson Laboratory, Bar Harbor, ME) were placed in pairs in sterile cages. Two days later, mice were given drinking water containing 0.8 mg/ml sulphamethoxazole/0.16 mg/ml trimethoprim for a total of 10 days to suppress indigenous oral bacteria, followed by a 3-day washout period with sterile drinking water. On the following day (designated experimental day 0) mice were placed on one of four diets (see Results) and inoculated daily for five successive days with 50 µl of 1.5% (wt/vol) carboxymethylcellulose in saliva buffer (50 mM KCl, 1.0 mM KPO₄, 0.35 mM K₂HPO₄, 1.0 mM CaCl₂ 2H₂O, 0.1 mM MgCl₂ 6H₂O, pH 6.5) containing approximately 1x10⁹ cells of the indicated strain grown to an OD₆⁰⁰ between 0.55 to 0.70 or inoculated with 1.5% (wt/vol) carboxymethylcellulose alone (mock inoculations). In competition experiments, mice also underwent on experimental day 7 five consecutive daily inoculations with approximately 1x10⁹ cells of *S. mutans* UA159. At the end of each experiment mice were euthanized by CO₂ asphyxiation followed by cervical dislocation. The protocol was reviewed and approved by the Institutional Animal Care and Use
Committee at University of Florida (IACUC protocol #201810470). More detailed information regarding the caging and feeding of mice and the preparation and delivery of inoculants is given in Supplementary Material, page 8.

**Bacterial strains and growth conditions:** Low-passage *S. mutans* UA159 and human commensal streptococcal isolates were initially grown in BHI broth (Brain Heart Infusion broth powder, 3.7% containing 0.2% glucose; Difco Laboratories, Detroit, MI) at 37°C in a 5% CO₂ aerobic environment to an optical density at 600 nm (OD₆₀₀) of 0.5, then glycerol added to 25% (v/v) and 40 aliquots of 1 ml frozen at -75°C. For A12 mutants the BHI contained 1 mg/ml kanamycin. Each frozen aliquot was used either to grow cells in BHI for isolation of genomic DNA for qPCR standards or to prepare a single inoculant.

**Oral swabs:** Oral swabs were taken at indicated intervals using HydraFlock® 6” Sterile Micro Ultrafine Flock swabs (Puritan Medical Products, Guilford, ME). Swab tips were vortexed (3 times for 5 seconds) in 1 ml sterile PBS, the tips removed and 200 µl added of ice-cold PBS containing approximately 5 x 10⁸ depurinated cells of laboratory strain *S. mitis* UF2 (see below). The tube was then vortexed 5 seconds and centrifuged (10,000 x g, 10 min at 4 °C) to pellet recovered cells. Cell pellets were then processed for DNA isolation using the DNeasy UltraClean Microbial kit (Qiagen Inc., Germantown, MD) as per manufacturer’s instructions. More detailed information regarding swabbing mice and recovery of bacteria is given in Supplementary Material, page 9.

**Preparation of depurinated cells:** In preliminary experiments, employment of a high concentration of depurinated cells was found to greatly enhance quantitative pelleting and recovery of low cell numbers and subsequent DNA, thus increasing the sensitivity of qPCR assays. The cells walls of depurinated cells remain intact and therefore at high concentrations act
as a carrier to help limit non-specific binding and promote pelleting of recovered bacteria.

Purine bases in genomic DNA are lost by depurination, producing apurinic sites and rendering DNA undetectable in all qPCR assays. To prepare depurinated cells, a 200 ml culture (OD$_{600} = 0.5$) of laboratory strain *S. mitis* UF2 in BHI were pelleted (4 x 50 ml at 10,000 x g x 7 min at 4 °C) and each pellet resuspended in 11 ml sterile ice-cold PBS. Cell were pooled and again centrifuged. The pellet was resuspended in 35 ml of 0.2 N HCl and placed in a 70°C water bath for 90 min with vortexing (5 x 2 s) every 15 min. Cells were then pelleted as before and the 90 min incubation in fresh 0.2 N HCl repeated. Cells were washed 3 times with 30 ml sterile ice-cold PBS. Before the third centrifugation the cell concentration was estimated from OD$_{600}$ and the subsequent cell pellet resuspended in sterile ice-cold PBS to a concentration of approximately 2.5 x 10$^9$ cells/ml, then aliquoted and stored at -75°C.

**Dental colonization:** To assess dental colonization, the left and right halves of each mandible were aseptically extracted by first breaking the fibrous symphysis at the rostral midline, then gripping one incisor and pulling the left or right half of mandibular bone away from the temporal mandibular joint and nearly all associated soft tissue. Then, under a dissecting microscope, any remaining extraneous soft tissue near the molar teeth was removed by scraping with a scalpel followed by removal of bone approximately 2 mm anterior and posterior to the three molar teeth. Molar teeth with remaining underlying bone were sonicated on ice in 1 ml sterile PBS, pH 7.4, in siliconized 2 ml microcentrifuge tubes. Molar teeth with remaining bone were then aseptically removed using sterile forceps. Approximately 5 x 10$^8$ depurinated cells of laboratory strain *S. mitis* UF2 were then added, the tube vortexed 5 sec and centrifuged (10,000 x g, 10 min at 4 °C). Cell pellets were then processed for DNA isolation as described above for
swabs. More detailed information regarding recovery of bacteria from mandibular molars is given in Supplementary Material, page 9.

Quantitative PCR: Quantitative PCR was used to estimate total recovered bacterial genomes and recovered genomes of inoculated strains in each DNA sample. DNA isolation using the DNeasy UltraClean Microbial kit (Qiagen) resulted in 50 µl of DNA that was diluted to 125 µl with nuclease free water resulting in DNA in 4 mM Tris-HCl, pH 8.0. Samples from swabs were stored at -75 °C in aliquots. Samples from molars were treated similarly, but further diluted 10-fold with 4 mM Tris-HCl, pH 8.0 to eliminate interference in qPCR assays caused by unknown components in the samples, then aliquoted and stored at -75 °C. Each qPCR assay included 9 µl of diluted DNA. Resultant genome numbers from the average of triplicates were then multiplied by either 13.89 (for swab DNA) or 138.89 (for mandibular DNA) to calculate total recovered genomes in each sample. To estimate total recovered bacteria, degenerate primers were used to PCR amplify conserved regions of the ubiquitous single-copy gene, rpsL (30S ribosomal protein S12) (48). Recovery of mouse commensals was then estimated by subtracting recovered genomes of inoculated strains from total recovered bacterial genomes.

Primers and qPCR conditions used are given in Supplementary Table S1. Standard curves were derived from DNA samples isolated from each strain grown to mid-exponential phase in BHI. S. mutans UA159 was used as standard for rpsL assays. Efficiencies, slopes and r² values for standard curves were greater than 90%, -3.205 and 0.978, respectively. Statistical comparisons of colonization between groups were by one-way ANOVA with Tukey’s multiple comparisons test. More detailed information regarding development of qPCR assays is given in Supplementary Material, pages 9-10.
Caries scoring: Smooth surface and sulcal caries of mandibular and maxillary molars were scored by a single calibrated examiner using Larson's modification of the Keyes' scoring system, as described previously (44). The linear evaluations of carious enamel involvement are expressed as E, while severities of carious lesions, based on degree of dentin involvement, are expressed as Ds (dentin exposed) and Dm (3/4 of the dentin affected). To stabilize variances, caries scores were expressed as proportions of their maximum possible values (124 for smooth surface caries and 56 for sulcal surface caries) and then the arcsine of the square root of the proportions calculated, as described previously (43). Transformed scores were compared by analysis of variance with the Tukey’s multiple comparisons test using Prism v8.1 (GraphPad Software, San Diego, CA). More detailed information regarding preparing jaws for caries scoring is given in Supplementary Material, page 10.

Construction of A12 mutant strains: Construction and characterization of A12 ΔspxB was described previously (9). The mutant strain, A12 ΔarcADS, was constructed in a similar manner by double-crossover recombination using linear DNA assembled through a Gibson assembly kit (New England BioLabs, Beverly, MA). Briefly, primers were designed to PCR amplify two DNA fragments flanking the coding sequences of five genes within the ADS operon from the 5'-end of arcA to the 3'-end of arcT and containing at least 27 bases of sequence that overlapped with the termini of the nonpolar resistance cassette in pALH124 (60) (see Fig. S4). The two flanking DNA fragments and the kanamycin resistance cassette were mixed in equimolar concentrations in a single isothermal ligation reaction. Overnight cultures of A12 were inoculated into fresh BHI cultures, and 0.5 µg of the ligated DNA products were used to transform A12 in BHI using 50 nM A12 sCSP to induce competence. After 3 h of incubation, cells were plated onto BHI agar with 1 mg/ml kanamycin and isolated colonies picked for PCR.
verification. PCR products from positive transformants underwent DNA sequencing to ensure correct insertion and the absence of mutations in the flanking regions used for homologous recombination. Primer sequences are listed in Table S3.

**ADS activity:** A12 ΔarcADS, was confirmed for the inability to express ADS by monitoring citrulline production from arginine using protocols detailed previously (7). Briefly, mid-log phase cultures in TY medium containing 25 mM galactose, with or without 10 mM arginine, were permeabilized using toluene-acetone. An aliquot was assessed for protein using the Pierce (Waltham, MA, USA) bicinchoninic acid protein assay kit, then ADS activity determined and normalized to protein. Assays were performed in biological duplicates and repeated two independent times with wild type A12 as positive control.

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D. J. Culp contributed to conception and experimental design of the study, data analysis, interpretation of results, and drafted and critically revised the manuscript. R. A. Burne contributed to the experimental design, data interpretation and critically reviewed the manuscript. William Hull, Matthew J. Bremgartner, Todd A. Atherly and Kacey N. Christian
 helped developed and carried out qPCR protocols. Kacey N. Christian, Mary Killeen, Madeline R. Dupuis and Alexander C. Schultz carried out procedures associated with feeding, inoculating and swabbing of mice, in isolation of DNA, in aseptic extraction of mandibles and their sonication. William Hull and Alexander C. Schultz also embedded mandibles and maxillary molars and carried out hemisectioning for sulcal scoring. Mary Killeen and Madeline R. Dupuis also performed caries scoring after undergoing calibration under the direction of Dr. Culp. Brinta Chakraborty and Kyulim Lee constructed the A12 mutants, performed phenotypic and genotypic characterizations of each strain and prepared initial aliquots of each strain used in subsequent procedures to isolate genomic DNA and to prepared inoculants. Deneen S. Wang, Verisha Afzal and Timmy Chen performed DNA isolations and assisted with feeding and inoculating mice. All authors gave final approval and agree to be accountable for all aspects of the work.

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**FIGURE LEGENDS**

**Fig. 1.** Colonization of the oral cavity and mandibular molars of mice by isolated human oral commensal strains when fed one of four different diets. Diets include the average diet (Ave), average diet + 1.5% arginine (Ave + Arg), high-sucrose diet + 4% sucrose water (Hi Suc) and the high-sucrose diet + 1.5% arginine with 4% sucrose water (Hi Suc + Arg). A. Timeline of key events in the experiment. B. Results for each indicated inoculated strain and mouse oral commensals expressed as genomes recovered as determined by qPCR (mean ± SE, n = 10 mice per group). Results are from six experiments, each testing a pair of human commensal strains. Each experimental pair of strains is presented side-by-side. Oral swabs 1 (open bars), oral swabs 2 (gray bars) and sonicates of mandibular molars (black bars) each taken at times indicated in panel A. *, p ≤ 0.05 by one-way ANOVA with Tukey’s multiple comparisons test. †, p ≤ 0.05 versus the same diet in swab 1, by one-way ANOVA with Tukey’s multiple comparisons test.
Fig. 2. Comparison of colonization of the oral cavity and mandibular molars of mice by 
*Streptococcus* sp. A12 wild type (WT) or the indicated A12 mutant strain. A. Timeline of key events 
in the experiment. B. Colonization results from two separate experiments for each indicated 
inoculated strain and mouse oral commensals from oral swabs 1 and 2 taken at the times indicated in 
A, and from sonicates of mandibular molars. Each experiment included a A12 WT control group. 
Mice were fed the high-sucrose diet plus 1.5% arginine with 4% sucrose water. Results are mean ± 
SE (n = 10 mice per group) of recovered genomes estimated by qPCR. Statistical comparisons by 
one-way ANOVA with Tukey’s multiple comparisons test. *, p ≤ 0.05 versus S. A12 WT.

Fig. 3. Comparison of *S. mutans* UA159 colonization when inoculated starting on day 0 versus day 
7. A. Timeline of major experimental events. B. Colonization by *S. mutans* and mouse oral 
commensals from mandibular molars and from oral swabs at the indicated number of days relative to 
the first inoculation of *S. mutans* UA159, as determined by qPCR. Mice were fed the high-sucrose 
diet with 4% sucrose water. Results are from a single experiment and expressed as mean ± SE of 
recovered genomes (n = 20 per group). *, p ≤ 0.05 versus the previous swab or an earlier swab as 
indicated by its number (e.g., 1 for Swab 1). #, p ≤ 0.05 versus *S. mutans* swab day 48 of Day 7 
group, and mouse commensals in all swabs of Day 7 group and all swabs but day 29 of Day 0 group. 
Statistical comparisons by one-way ANOVA with Tukey’s multiple comparisons test.

Fig. 4. Comparison of colonization of the oral cavity and mandibular molars of mice by 
*Streptococcus* sp. A12 wild type (WT) or the indicated A12 mutant in competition with *S. mutans* 
UA159. A. Timeline of key events in the experiment. B. Results are from a single experiment that 
included each indicated inoculated A12 strain (closed triangles, solid lines), *S. mutans* UA159 (open
circles, dashed lines) and mouse oral commensals (closed squares, dotted lines) from oral swabs 1-5 taken at the times indicated in A and from sonicates of mandibular molars (M). Mice were fed the high-sucrose diet plus 1.5% arginine with 4% sucrose water. Results are mean ± SE (n = 14 mice per group) of recovered genomes estimated by qPCR. *, p ≤ 0.05 versus the previous swab or an earlier swab as indicated by its number (e.g., 2 for Swab 2). M, p ≤ 0.05 versus the same point in the mock group. W, p ≤ 0.05 versus A12 wild type group. Statistical comparisons by one-way ANOVA with Tukey’s multiple comparisons test.

Fig. 5. Comparison of colonization of the oral cavity and mandibular molars of mice by human oral commensal strains in competition with S. mutans UA159. A. Timeline of key events in the experiment. B. Results are from a single experiment that included each indicated inoculated human commensal strain (closed triangles, solid lines), S. mutans UA159 (open circles, dashed lines) and mouse oral commensals (closed squares, dotted lines) from oral swabs 1-5 taken at the times indicated in A and from sonicates of mandibular molars (M). Mice were fed the high-sucrose diet plus 1.5% arginine with 4% sucrose water. Results are mean ± SE (n = 14 mice per group) of recovered genomes estimated by qPCR. *, p ≤ 0.05 versus the previous swab or an earlier swab as indicated by its number (e.g., 2 for Swab 2). M, p ≤ 0.05 versus the same point in the mock group. Statistical comparisons by one-way ANOVA with Tukey’s multiple comparisons test.

Fig. 6. Comparisons between separate identical experiments of colonization of the oral cavity and mandibular molars of mice. A. Comparisons between the two experiments (1 & 2) in Fig. 2B of colonization by A12 wild type (WT) and murine autochthonous commensals (Mouse 40 circles, dashed lines) and mouse oral commensals (closed squares, dotted lines) from oral swabs 1-5 taken at the times indicated in A and from sonicates of mandibular molars (M). Mice were fed the high-sucrose diet plus 1.5% arginine with 4% sucrose water. Results are mean ± SE (n = 14 mice per group) of recovered genomes estimated by qPCR. *, p ≤ 0.05 versus the previous swab or an earlier swab as indicated by its number (e.g., 2 for Swab 2). M, p ≤ 0.05 versus the same point in the mock group. W, p ≤ 0.05 versus A12 wild type group. Statistical comparisons by one-way ANOVA with Tukey’s multiple comparisons test.
Commensals). B. Comparisons between the two mock groups in competition experiments of Fig. 4B (1) and Fig. 5B (2) that included colonization by *S. mutans* and mouse commensals. Results are mean ± SE (A, n = 10 mice per group; B, n = 14) of recovered genomes estimated by qPCR. Statistical comparisons between swabs at a given point are by one-way ANOVA with Sidak’s multiple comparisons test. Statistical comparisons between recoveries from mandibles are by the two-tailed unpaired t test. *, p ≤ 0.05 versus same point in the alternate experiment.
Table 1. Experimental Diets (% Total Dry Wight).

| Ingredients      | High-Sucrose Diet | Average Diet | High-Sucrose Diet + Arg | Average Diet + Arg |
|------------------|-------------------|--------------|-------------------------|--------------------|
| Sucrose          | 37.5              | 11.5         | 37.5                    | 11.5               |
| Corn Starch      | 24.0              | 24.0         | 24.0                    | 24.0               |
| Casein           | 20.0              | 20.0         | 20.0                    | 20.0               |
| Maltodextrin     | 3.2               | 29.2         | 1.7                     | 27.7               |
| Arginine         | -                 | -            | 1.50                    | 1.50               |
| Total Protein    | 17.7              | 17.7         | 19.2                    | 19.2               |
| Total Carbohydrates | 62.1         | 60.8         | 60.7                    | 59.4               |
| Total Fat        | 7.2               | 7.2          | 7.2                     | 7.2                |
| Envigo Cat #     | TD.160810         | TD.160809    | TD.160812               | TD.160811          |
| Water Additive   | 4% sucrose        | -            | 4% sucrose              | -                  |

Diets are modifications of Envigo’s AIN-93G purified diet in which corn starch is decreased 40% and replaced with maltodextrin or sucrose. All diets contain the following ingredients in addition to those listed above (% total dry wt.): 7% soybean oil, 5% cellulose, 3.5% complex mixture of minerals without sodium fluoride, 1.5% complex vitamin mixture (AIN-93-VX; Cat. #94047), 0.3% added L-cystine to balance amino acid contents, 0.25% choline bitartrate, 14 µg/g tert-butylhydroquinone as antioxidant. The vitamin mixture accounts for 1.5% of sucrose in each diet. In the diets high in sucrose, mice also were also provided with 4% sterile sucrose water ad libitum.
TABLE 2. Development of caries and their severities on molars of BalbC/J mice on the high caries diet and inoculated with *S. mutans* UA159 on either experimental days 0-4 or experimental days 7-11 (with mock inoculations on days 0-4).

| SMOOTH SURFACES | Day 0   | Day 7   |
|-----------------|--------|--------|
| Total - E       | 3.42 (0.29) | 3.80 (0.37) |
| Total - Ds      | 1.79 (0.15) | 2.10 (0.16) |
| Total - Dm      | 1.21 (0.16) | 1.00 (0.19) |
| Buccal E        | 0.32 (0.11) | 0.50 (0.22) |
| Buccal Ds       | 0.00 (0.00) | 0.05 (0.05) |
| Buccal Dm       | 0.00 (0.00) | 0.00 (0.00) |
| Lingual E       | 2.10 (0.13) | 2.55 (0.20) |
| Lingual Ds      | 1.79 (0.15) | 2.05 (0.11) |
| Lingual Dm      | 1.21 (0.16) | 1.00 (0.19) |
| Proximal E      | 1.00 (0.20) | 0.75 (0.22) |
| Proximal Ds     | 0.00 (0.00) | 0.00 (0.00) |
| Proximal Dm     | 0.00 (0.00) | 0.00 (0.00) |

| SULCAL SURFACES | Day 0   | Day 7   |
|-----------------|--------|--------|
| Total - E       | 15.10 (0.75) | 16.60 (0.60) |
| Total - Ds      | 8.47 (0.93)  | 8.70 (0.62)  |
| Total - Dm      | 0.26 (0.10)  | 0.45 (0.13)  |

Values are means (SE) of Larson’s modified Keyes’ scores from a single experiment. Total smooth surface caries is sum of buccal, lingual and proximal caries. E (enamel affected), Ds (dentin exposed) and Dm (3/4 of the dentin affected). No differences (p > 0.10; n = 20) between day 7 versus day 0 scores by ANOVA with the Tukey’s multiple comparisons test.
TABLE 3. Development of caries and their severities on mandibular molars of mice in the competition experiment.

| SMOOTH SURFACES | Mock | S. sanguinis BCC23 | S. sanguinis BCA8 | S. gordonii BCC32 | S. mitis BCA12 |
|------------------|------|---------------------|-------------------|-------------------|---------------|
| Total - E        | 3.74 (0.96) | 1.86 (0.31) | 3.00 (0.68) | 2.64 (0.44) | 3.36 (0.58) |
| Total - Ds       | 1.07 (0.44) | 0.14 (0.14)* | 0.43 (0.20) | 0.43 (0.17) | 0.64 (0.23) |
| Total - Dm       | 0.14 (0.10) | 0.00 (0.00) | 0.00 (0.00) | 0.00 (0.00) | 0.00 (0.00) |
| Buccal E         | 2.07 (0.69) | 0.43 (0.17)* | 1.71 (0.34) | 0.93 (0.38)† | 1.43 (0.51) |
| Buccal Ds        | 0.29 (0.29) | 0.07 (0.07) | 0.14 (0.10) | 0.00 (0.00) | 0.21 (0.21) |
| Buccal Dm        | 0.07 (0.07) | 0.00 (0.00) | 0.00 (0.00) | 0.00 (0.00) | 0.00 (0.00) |
| Lingual E        | 1.50 (0.23) | 1.43 (0.20) | 1.14 (0.33) | 1.71 (0.19) | 1.93 (0.20) |
| Lingual Ds       | 0.79 (0.24) | 0.07 (0.07)* | 0.29 (0.16)† | 0.43 (0.17) | 0.43 (0.14) |
| Lingual Dm       | 0.07 (0.07) | 0.00 (0.00) | 0.00 (0.00) | 0.00 (0.00) | 0.00 (0.00) |
| Proximal E       | 0.14 (0.14) | 0.00 (0.00) | 0.14 (0.14) | 0.00 (0.00) | 0.00 (0.00) |
| Proximal Ds      | 0.00 (0.00) | 0.00 (0.00) | 0.00 (0.00) | 0.00 (0.00) | 0.00 (0.00) |
| Proximal Dm      | 0.00 (0.00) | 0.00 (0.00) | 0.00 (0.00) | 0.00 (0.00) | 0.00 (0.00) |
| SULCAL SURFACES |      |                    |                   |                   |               |
| Total - E        | 7.86 (1.40) | 8.00 (0.56) | 8.93 (0.77) | 5.86 (0.90) | 8.00 (0.97) |
| Total - Ds       | 1.50 (0.53) | 0.57 (0.17) | 1.36 (0.41) | 1.00 (0.38) | 1.57 (0.40) |
| Total - Dm       | 0.14 (0.10) | 0.00 (0.00) | 0.07 (0.07) | 0.07 (0.07) | 0.07 (0.07) |

Values are means (SE) of caries and their severities as described in Table 3. Comparisons by ANOVA with Tukey’s multiple comparisons test. *, P ≤ 0.05 versus mock; †, P ≤ 0.10 versus mock. n = 14 for each group.
Fig. 1, Page 1

A. Timeline of experimental design:
- Mice Arrive (ASL-Sale)
- Start Diet
- 5 Daily Inoculations
- Experimental Strain
- Euthanize

Genomes Recovered

B. Bar graphs showing the recovery of various bacterial species:
- S. A12
- S. intermedius A3
- S. cristatus A52
- S. mitis BCC15
- S. mitis BCC45

Genomes Recovered

Mouse Commensals

* denotes significant difference
† denotes trend towards significance
Fig. 1. Colonization of the oral cavity and mandibular molars of mice by isolated human oral commensal strains when fed one of four different diets. Diets include the average diet (Ave), average diet + 1.5% arginine (Ave + Arg), high sucrose diet + 4% sucrose water (Hi Suc) and the high sucrose diet + 1.5% arginine with 4% sucrose water (Hi Suc + Arg). A. Timeline of key events in the experiment. B. Results for each indicated inoculated strain and mouse oral commensals expressed as genomes recovered as determined by qPCR (mean ± SE, n = 10 mice per group). Results are from six experiments, each testing a pair of human commensal strains. Each experimental pair of strains is presented side-by-side. Oral swabs 1 (open bars), oral swabs 2 (gray bars) and sonicates of mandibular molars (black bars) each taken at times indicated in panel A. *, p ≤ 0.05 by one-way ANOVA with Tukey’s multiple comparisons test. †, p ≤ 0.05 versus the same diet in swab 1, by one-way ANOVA with Tukey’s multiple comparisons test.
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Mice were fed the high-sucrose diet plus 1.5% arginine with 4% sucrose water. Results are mean ± SE (n = 10 mice per group) of recovered genomes estimated by qPCR. Statistical comparisons by one-way ANOVA with Tukey’s multiple comparisons test. *, p ≤ 0.05 versus *S. A12* WT.
Fig. 3. Comparison of *S. mutans* UA159 colonization when inoculated starting on day 0 versus day 7. A. Timeline of major experimental events. Colonization by *S. mutans* and mouse oral commensals from mandibular molars and from oral swabs at the indicated number of days relative to the first inoculation of *S. mutans* UA159, as determined by qPCR. Mice were fed the high-sucrose diet with 4% sucrose water. Results are from a single experiment and expressed as mean ± SE of recovered genomes (n = 20 per group). *, p ≤ 0.05 versus the previous swab or an earlier swab as indicated by its number (e.g., 1 for Swab 1). #, p ≤ 0.05 versus *S. mutans* swab day 48 of Day 7 group, and mouse commensals in all swabs of Day 7 group and all swabs but day 29 of Day 0 group. Statistical comparisons by one-way ANOVA with Tukey’s multiple comparisons test.
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