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Recovery of *Enterococcus faecalis* from cheese in the oral cavity of healthy subjects

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Introduction: Enterococci are rarely found in the healthy human oral cavity, yet they are strongly associated with filled root canals. The origin of these enterococci remains unknown. Our hypothesis is that they are transient food-born colonizers under healthy conditions. This pilot study reinvestigated the prevalence of enterococci in the oral cavity of healthy volunteers, screened cheese samples for enterococci and investigated colonization of the oral cavity after ingestion of an enterocci-positive cheese.
Method: Concentrated oral rinse samples were collected from a cohort of 50 dental students and proved negative for viable enterococci. Twenty cheese samples were obtained from local supermarkets. Enterococci were cultured and identified using standard methods.
Results: Viable enterococci were detected in one of five specimens of Swiss Tilsiter, three of five samples of French soft cheese, one of five Mozzarella samples and one of five Feta samples. Eight volunteers from the cohort consumed 10 g of a cheese with high *Enterococcus faecalis* load. Oral rinse samples were collected before and 1, 10 and 100 min after cheese ingestion. One minute after ingestion, a median of 5480 *E. faecalis* colony-forming units was recovered from the oral rinse samples. Bacterial counts were reduced after 10 min, had dropped after 100 min to levels that were significantly ($P < 0.005$) different from the 1-min and 10-min scores and were below the detection limit after 1 week.
Conclusions: These findings suggest that colonization of the healthy oral cavity by enterococci is transitional, but at the same time add weight to our hypothesis that enterococcal root canal infections could be food-borne.

Key words: colonization; enterococcus; food; oral cavity; root canal treatment

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Enterococci are nosocomial pathogens, which become increasingly resistant to common antimicrobial agents, thus posing a growing threat to hospitalized patients (14). In dentistry, enterococci, especially *Enterococcus faecalis* strains, have been associated with caries lesions (2), chronic periodontitis (18) and, most frequently, failed root canal treatments, i.e. persistent apical periodontitis in root-filled teeth. Roughly 50% of culture-positive endodontic retreatment cases test positive for *E. faecalis* (13, 16). *E. faecalis* appears to occur frequently, albeit in low numbers, in primary root canal infections, especially in teeth with coronal leakage (11). Enterococci can survive in mono- and mixed cultures in root canal systems (6) and are hard to eliminate once present (5). In addition to *E. faecalis*, other *Enterococcus* species, such as *E. faecium* and *E. casseliflavus*, have been identified in necrotic human root canal systems (7).

The source of this infection, however, is unclear. *E. faecalis* was not found in the oral cavity of young infants (3). It was detected in oral rinse samples from only 11 of 100 patients receiving endodontic treatment and from one of 100 dental students with no history of endodontic treatment (20). A thorough analysis of over 2500 clones containing 16S ribosomal RNA genes from DNA samples obtained from five healthy individuals did not yield any enterococci-specific DNA sequences (1). It would thus appear that neither *E. faecalis* nor other species of the *Enterococcus* genus are common oral colonizers (21). Interestingly, viable

enterococci are frequently found in fermented food for raw consumption, such as cheese and meat, as well as in vegetables and olives (8). *E. faecium* is the main species found in meats, while *E. faecalis* predominates in cheese (8). The original enterococcal habitat is the mammalian, especially human, gastrointestinal tract. However, enterococci are able to colonize diverse niches because of their exceptional capacity to grow in different environments, such as soil and surface waters (9). While enterococci have been used as components of cheese starter cultures, they mostly occur as contaminants in fermented foods, and might add to the complex taste of certain meats and cheeses (9). Thus far, few if any pathogenic effects of enterococci from food have been reported (12). Based on the ubiquitous occurrence of enterococci in food products, it can be speculated that these may act as sources of transient oral colonization. In niches such as the unsealed necrotic root canal, the conditions may favor the survival of enterococci, and a long-standing local infection may become established (23).

The aim of this study was to address the following hypotheses:

1 Viable enterococci occur frequently and in high numbers in widely distributed cheese,
2 Young subjects with good oral hygiene and no caries or inflammatory oral diseases do not harbor enterococci in their oral cavities, and
3 The rate of clearance of cheese-derived enterococci from the oral cavity is such that infection of predilection sites cannot be excluded.

Four types of widely available cheese, Tilsiter, Mozzarella, Feta and French soft cheese were obtained from local supermarkets in Zürich, Switzerland, and assessed for their content of viable enterococci. In the same city, the occurrence of enterococci in the oral cavity of a cohort of 50 healthy dental students with good oral hygiene was evaluated. Subsequently, eight of these students volunteered to eat a cheese with a known load of *E. faecalis* cells, and *E. faecalis* recovery from the oral cavity was assessed over time.

Materials and methods

Ethics

The protocol of the current study was approved by the institutional ethics committee. Informed consent was obtained from all individuals participating in this investigation.

Occurrence of enterococci in cheese samples

Samples from cheeses belonging to the four cheese groups: Tilsiter, Mozzarella, Feta and French soft cheese, were assessed for their content of viable enterococci. Five individual brands per group were randomly bought from local supermarkets between August and September 2005 in Zürich, Switzerland. To exclude contamination from the surface, a 1-g piece was cut from the body of each cheese using sterile instruments. Both this sampling and all the subsequent laboratory procedures were exercised under aseptic conditions in a microbiological safety cabinet (SFE.120 EN, SKAN AG, Basel, Switzerland) using sterile materials. Cheese pieces were homogenized for 1 min in 10 ml of a 2% sodium citrate buffer using a tissue homogenizer (Ultra-Turrax T8, IKA-Werke, GmbH & Co., Staufen, Germany). Subsequently, 10-fold serial dilutions were prepared in 2% sodium citrate down to 10^{-5}. Fifty microliters of each dilution were plated on kanamycin aesculin azide agar (KAAA; Merck, Darmstadt, Germany) using a spiral diluter (Spiral Systems, Inc., Cincinnati, OH) and incubated at 42°C in a 10% carbon dioxide atmosphere (4). Colonies were counted after 3 days of incubation (17). Enterococcal colonies were presumptively identified by the blackening of the agar as a result of their hydrolysis of esculin. Representative colonies of all morphotypes were picked up and subcultured on Columbia blood agar (Oxoid, Basingstoke, UK) incubated as described above. Using a phase-contrast microscope (Leitz Dialux 22, Leica, Basel, Switzerland) at 1000× magnification, gram-positive coccoid bacteria growing in strings were identified. Subsequently, a catalase test was performed and gram-positive, catalase-negative cocci growing in strings were adjusted to an optical density of 1 at 550 nm, as assessed in a cuvette of 1-mm light path in a spectrophotometer (U 2001, Hitachi, VWR International AG, Dietikon, Switzerland), and were identified to species level using a biochemical test kit (rapid ID 32 STREP, bioMérieux, La Balme les Grottes, France) according to the instructions of the manufacturer.

Occurrence of enterococci in oral rinse samples of healthy young subjects

Participants were selected from dental students at the University of Zürich. They comprised 24 male and 26 female participants, aged 21–35 years (mean = 24 years). Subjects had to be fully dentate (except for orthodontically extracted teeth and/or wisdom teeth), have fewer than nine restored teeth (mean was 2.5) and no more than one endodontically treated tooth per individual (mean was 0.08), have good oral hygiene, and show no periodontitis, no caries and normal salivary flow rates. None of the subjects took any antibiotics before or during the experiments. Concentrated oral rinse samples (19) were obtained from all individuals. Participants were asked to thoroughly rinse the mouth for 60 s with 10 ml sterile distilled water. The oral rinse samples were collected in 50-ml Falcon polypropylene tubes (Becton Dickinson Labware, Franklin Lakes, NJ) and centrifuged at 3000 ***g*** for 10 min. Supernatants were discarded and the pellets were re-suspended in 1 ml sterile water and vortexed. Aliquots of 50 µl were plated on KAAA. All samples were processed within 1 h. Enterococci were identified as described above.

Recovery of *E. faecalis* from Brie de Meaux from oral cavities over time

Eight dental students (four male and four female, aged 23–33 years) from the above cohort volunteered for this part of the study. A concentrated oral rinse sample was obtained from each volunteer before the experiments. Subsequently, volunteers were asked to brush their teeth using a conventional manual toothbrush with flat trim (Meridol, GABA International AG, Münchenstein, Switzerland) and toothpaste (Elmex, GABA). After tooth brushing, the test persons rinsed their mouths with copious amounts of water. Subsequently, 10 g of a Brie de Meaux containing 4.8×10^4 *E. faecalis* cells/g was consumed on a piece of whole-wheat bread. Before consumption, and at 1-, 10- and 100-min intervals, oral rinse samples were subsequently obtained and analyzed as described above. Volunteers refrained from eating or drinking between cheese consumption and the harvesting of the oral rinse samples. To avoid bias caused by rinsing, assessments of *E. faecalis* recovery at different time-points were repeated on different days using aliquots of the same cheese, which had been stored at −25°C and thawed at 4°C overnight before the experiments. The intervals between experiments were 7 days, during which the volunteers maintained their normal diet and oral hygiene habits. The consistency of *E. faecalis* load in this cheese was confirmed on different experimental days by colony counts on KAAA.

Data presentation and analysis

Results are reported as colony-forming units (CFU) per gram of cheese and as CFU/oral rinse sample in the oral rinse samples. Theoretical detection limits were 100 CFU/g in the cheese specimens and 20 CFU/oral rinse sample in the oral rinse samples. The oral rinse sample data were skewed so non-parametric statistics were applied to compare *E. faecalis* recovery after cheese ingestion between different time-points: two-way analysis of ranks (Friedman test) was followed by Wilcoxon tests for individual comparisons. Bonferroni's correction was applied for multiple comparisons. The α-type error was set at < 0.05.

Results

None of the oral rinse samples from the 50 dental students contained viable enterococci. On the other hand, several of the cheese brands harbored *Enterococcus* species, namely *E. faecalis*, *E. faecium*, *E. gallinarum*, *E. casseliflavus* and/or *E. durans*. Overall, one of five of the Swiss Tilsiter, three of five of the French soft cheese, one of five of the Mozzarella and one of five of the Feta samples contained detectable enterococci (Table 1).

Among the student cohort, eight participants volunteered to eat 10 g of a Brie de Meaux containing a mean of 4.8×10^4 *E. faecalis* CFU/g (SD on different experimental days was $\pm 1.8 \times 10^4$ CFU/g). Before ingestion, none of the oral rinse samples contained any detectable enterococci. After 1 min, a median of 5480 CFU was recovered (range: 1220–31,600). This number did not change significantly when oral rinse samples were obtained from the same participants 10 min after ingestion of the same cheese on a different experimental day (median: 1410; range: 20–6800; $P = 0.06$, Wilcoxon, Bonferroni). After 100 min, the median CFU had dropped to 100 (range: 0–620). This drop was significant compared to *E. faecalis* recovery after both 1 min ($P < 0.005$) and 10 min ($P < 0.05$). A graphic overview of *E. faecalis* recovery after cheese ingestion is depicted in Fig. 1. After 100 min, negative cultures were obtained from two of the eight students. The 1-, 10- and 100-min experiments were conducted at 1-week intervals. The complete absence of viable enterococci in oral rinse samples before the cheese ingestion on the second and third experimental days indicated that no viable enterococci from the cheese had remained in the oral cavity of the volunteers.

Table 1. Enterococci growing on kanamycin aesculin azide agar after 3 days at 42°C recovered from random cheese samples bought in Zürich in August/September 2005

Type of cheese	Brand name	Milk type	*Enterococcus* species	CFU/g
Swiss Tilsiter	Surchoix	Cow, raw	*E. faecium*	2.8×10^4
			E. faecalis	200–2000
	Surchoix	Cow, raw		$<10^2$
	Doux, Globus	Cow, pasteurized		$<10^2$
	Doux, Coop	Cow, pasteurized		$<10^2$
	Goldinger	Cow, raw		$<10^2$
French soft cheese	Brie de Meaux	Cow, raw	*E. faecalis*	3.6×10^6
			E. faecium	3600
	Sternenberger Brie	Cow, raw	*E. gallinarum*	400
			E. casseliflavus	600
	Bio Camembert	Cow, raw	*E. faecalis*	1600
	Artisan Camembert	Cow, raw		$<10^2$
	M-Budget Brie	Cow, pasteurized		$<10^2$
Mozzarella	Campana, Jelmoli	Buffalo (70°C)	*E. faecium*	200
			E. durans	2800
	Campana, Globus	Buffalo (70°C)		$<10^2$
	Oberländer	Cow, pasteurized		$<10^2$
	Galbani Santa Lucia	Cow, pasteurized		$<10^2$
	Bio	Cow, pasteurized		$<10^2$
Feta	Feta, Globus	Sheep, pasteurized	*E. durans*	400
	Xenia	Sheep, pasteurized		$<10^2$
	Original Thessaloniki	Sheep, pasteurized		$<10^2$
	Faltigberg	Sheep, pasteurized		$<10^2$
	Hotos Manouri	Sheep		$<10^2$

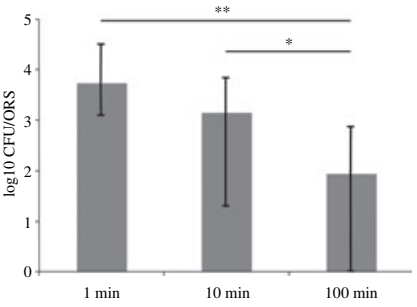

Fig. 1. Recovery over time of viable *Enterococcus faecalis* cells from the oral cavities of eight healthy volunteers after ingestion of a Brie de Meaux sample. Values given in CFU/oral rinse sample; columns represent median values, error bars represent ranges. Horizontal bars indicate significant differences between different time-points at $*P < 0.05$ and $**P < 0.005$ levels (Friedman test followed by Wilcoxon tests with Bonferroni correction).

Discussion

In a cohort of healthy dental students ($n = 50$) of the University of Zürich with good oral hygiene, no viable enterococci were recovered from the oral cavities. Cheese bought in the same town, on the other hand, frequently harbored culturable enterococci. After ingestion of Brie de Meaux containing a substantial number of viable *E. faecalis* cells, enterococci were recovered in decreasing numbers over time for 100 min from the oral cavities of eight volunteers who had ingested the Brie and then refrained from further eating or drinking. After 1 week of no intervention, normal diet and normal oral hygiene, none of the volunteers harbored any enterococci in their oral cavities. Consequently, all three hypotheses of the study were confirmed.

The results presented here are in line with reported recoveries of enterococci from healthy individuals with good hygiene habits (20), and the occurrence of enterococci in cheese (8). To the authors' knowledge, however, the present study is the first to establish a link between enterococci in food and their occurrence in the oral cavity. Oral rinse samples were used for the oral cavity screening because these detect more culturable enterococci than tongue dorsum or gingival sulcus samples (22). However, the method used does not allow for differentiation between different oral sites. Furthermore, the long-term fate of the enterococci from the cheese remains unclear. Our results suggest that after a certain period of time (over 100 min), no viable enterococci would have been recovered from oral rinse samples. However, only one cheese was tested, and it cannot be excluded that low numbers of *E. faecalis* cells might have survived. Different enterococci species and *E. faecalis* strains express a wide range of virulence factors (15), and their retention in the oral cavity may thus vary. Nevertheless, the current results are in

agreement with caries studies in gnotobiotic rats, in which enterococci could be maintained in monoculture but did not survive in the oral cavity of conventional rats (10). These results allow no conclusions to be drawn on what happens when enterococci reach niches that support their survival. It would appear that enterococci can survive and thrive in unsealed root canals in patients undergoing root canal treatment (23). Furthermore, patients with periodontitis appear to harbor more *E. faecalis* cells than counterparts with a healthy periodontium (22). This could be explained by the fact that periodontally compromised patients may have poorer oral hygiene compared to individuals with a healthy periodontium, and more spaces for food retention. In this context, it would be of interest whether plaque could retain the organisms for a longer period of time. This could not be investigated with the current cohort.

Future studies should aim at the identification of virulence factors expressed by food-derived enterococci that may favor their colonization of the oral cavity and/or promote inflammatory host reactions such as apical periodontitis. Furthermore, resistance profiles to the antiseptic agents used in dentistry should be assessed in enterococci isolated from food. Also, the possibility of oral–faecal contamination by enterococci should be explored using serological methods or DNA matching, and the likelihood of that route of infection should be compared to the food pathway.

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