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Different Patterns of Virulence Genes in *Streptococcus mutans* and *Streptococcus sobrinus* Originating from Estonian Toddlers—Mothers Cohort

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Abstract: Aims: Mutans streptococci include *Streptococcus mutans* and *Streptococcus sobrinus*, which can cause tooth decay. The current study aimed to compare their virulence genes with each other and to correlate them with the clinical data of patients. Materials and methods: Altogether 21 *S. mutans* and 19 *S. sobrinus* strains were investigated, originating from 24 children (age 2.7 ± 0.4 years) and 13 mothers (27.3 ± 3.7). The PCR method was applied to detect 11 virulence genes. Caries indices (dmf, decayed/missing/filled; DMFT, decayed/missing/filled teeth) and SM score (Mutans streptococci amount in saliva) were recorded. Results: Most of the *S. mutans* strains harbored all the virulence genes studied, while *S. sobrinus* had significantly fewer genes. The genes *gbpA*, *gbpB*, *wapA* and *ftf* were present in all isolates of *S. sobrinus*, the *spaP*, *gtfB*, *vicR*, *SMU.1037c* and *SMU.105* genes were present in 41–88% of the isolates, while *gtfD* and *SMU.104* genes were absent in *S. sobrinus* strains studied. A positive correlation appeared between the biofilm-related *vicR* and polysaccharide-production-related *gtfD* genes. In contrast, another polysaccharide-production-related *gtfB* gene was present in some cases in strains lacking the *vicR* or *gtfD* gene. Positive association was found between the presence of adhesion-related *spaP* gene in pediatric-derived *S. sobrinus* strains and an increase in SM score. Conclusions: Differences exist between the two common species of mutans streptococci: strains of *S. mutans* have more virulence genes than that of *S. sobrinus*, both crucial and virulence enhancing. Deeper research is needed to clarify the mechanisms behind the increased cariogenicity in cohabitation.

Keywords: mutans streptococci; virulence gene; dental caries; *Streptococcus mutans*; *Streptococcus sobrinus*

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1. Introduction

Dental caries is a common disease in both adults and children—about 70–90% of the world's population is affected by caries [1,2]. The prevalence of dental caries in toddlers varies within Europe, ranging from 8% in Finland [3] to 56% in Poland [4]. A study conducted in Estonia revealed that caries occurred in 29% of 3-year-old children, 72% in 6-year-old children and 68% in 12-year-olds [5].

The oral cavity contains highly diverse normal microbiota, which performs various useful functions. In total, about 1000 different species of microorganisms have been found in the oral cavity [6], of which about 50–100 species reside in the mouth of one person. Streptococci make up about 50% of the oral microbiota [7,8]. The development of dental caries is associated with numerous factors, such as oral cleaning and eating habits, salivation and dentition, but it is also significantly associated with changes in the composition of the oral microbiota, in particular, the excessive proliferation of mutans streptococci (MS). The most common species among MS in humans are *Streptococcus mutans* and *Streptococcus sobrinus* [1].

The environmental conditions in the mouth and focal dental caries are complex and constantly changing. This highlights the remarkable adaptability of MS, which is attributable to a wide range of virulence factors, including biofilm formation and adhesion, polysaccharide production, and carbohydrate cleavage with acid production [8,9]. A biofilm is an aggregate of bacteria attached to a surface, usually covered with a matrix of exopolysaccharides [10]. Bacteria living in biofilms are significantly more tolerant to antibiotics and biocides [11]. Biofilm formation by MS is associated with the genes *wapA* [12] and *vicR*, and the latter helps the bacterium detect environmental changes and respond to stress conditions [13]. Glycan-binding proteins (Gbps—*gbpA* and *gbpB*), glycosyltransferases (GTF—*gtfB* and *gtfD*) [14], as well as a surface protein called antigen I/II (coded by *spaP*, also known as pac, P1 and Ag I/II) are involved in MS adhesion [15]. Gbps and GTF-producing genes are closely related because *S. mutans* synthesizes glycans from sucrose (a substrate for GTFs) using glycosyltransferases [14]. The function of *spaP* is to mediate *S. mutans* adhesion to saliva-coated tooth surfaces [16]. In addition, streptococci have the ability to produce extracellular polysaccharides (EPS) from sucrose [17], involving *gtfB*, *gtfD*, and *ftf* genes [1,18]. EPS are important components in biofilm [19] and contribute to the cariogenicity, stress tolerance and antimicrobial resistance of *S. mutans* [20]. EPS also serves to provide a supply of substrates for the bacterium to promote adhesion and aggregation between microorganisms, and to increase the thickness and density of plaque [21].

Another important virulence factor for MS is acidogenicity [1]. MSs ferment carbohydrates and produce organic acids, especially lactic acid, by changing the pH of the external environment [22]. The environment of the dental plaque becomes acidic, resulting in the demineralization of tooth enamel and later dentin. Caries bacteria themselves are acid tolerant [2,23].

Although caries and its causes have been studied for decades, there are few studies comparing the virulence genes of both major caries pathogens and associating them with oral health indicators. Therefore, this investigation aimed to compare the cariogenicity-related virulence genes and clinical impact of *S. mutans* and *S. sobrinus* isolated from 2- to 4-year-old children and their mothers.

2. Materials and Methods

2.1. Bacterial Strains

Forty MS strains were included in the study, including 21 *S. mutans* and 19 *S. sobrinus*. The strains originated from a former study where the oral health of mothers and children was assessed [24], with permission of the Ethics Committee for Human Research of the University of Tartu (protocol no. 166/T-7). The strain donors included 24 children (aged 24 to 41 months) and 13 mothers (aged 22 to 31 years) (Table 1). All donors had dental caries. Background data included the DMF index [25] and SM score measured using the commercial kit Dentocult SM Strip mutans (Orion Diagnostica Oy, Espoo, Finland). The strains are stored in the HUMB collection (Human Microbial Biobank) at the University of Tartu (http://eemb.ut.ee/humb, accessed on 3 November 2022).

Table 1. Clinical parameters (mean ± SD) of strain donors.

Donors	Sex (%)		Age (Years)	dmf (0–20)	DMFS (0–128)	DMFT (0–32)	SM Score (0–3)	DAS (4–20)
	F	M						
mother (n = 13)	100	0	27.3 ± 3.7	-	20.8 ± 14.62	11.20 ± 5.18	2.23 ± 0.93	11.8 ± 3.61
child (n = 24)	62.5	37.5	2.7 ± 0.4	0.46 ± 0.83	-	-	1.46 ± 1.22	-

dmf—decayed/missing/filled; DMFS—decayed/missing/filled surface; DMFT—decayed/missing/filled teeth; SM—Strip mutans (Orion Diagnostica); DAS—dental anxiety scale.

2.2. Molecular Methods

A QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) was used to isolate DNA.

To determine if all mutans streptococci strains were different, genotyping was performed by pulsed field gel electrophoresis (Supplementary Figure S1). Based on the genotyping data, 38 bacterial strains were included in the analysis.

The PCR method was used to reveal virulence genes. The known virulence genes of MS are presented in Table 2; of them, 11 virulence genes were selected for the study: *gbpA*, *gbpB* and *spaP* genes, responsible for adhesion; *wapA* and *vicR*, involved in biofilm formation; *ftf*, *gtfB* and *gtfD*, involved in the production of the polysaccharides needed for biofilm; *SMU.104* and *SMU.105*, responsible for acid production, and *SMU.1037c* contributing to acid tolerance.

Table 2. Virulence mechanisms and their genes in mutans streptococci (adapted from [26]).

Virulence Mechanism	Genes *
Adhesion to tooth enamel	sucrose-dependent adhesion: ***gbpA***, ***gbpB***, ***ftf***, ***vicR***, ***wapA***
	sucrose-independent adhesion: ***spaP***
Biofilm formation	*atlA*, ***ftf***, *SMU.609*, ***vicR***, ***wapA***
Production of polysaccharides	*gtfA*, ***gtfB***, *gtfC*, ***gtfD***, ***ftf***, ***vicR***
Decomposition of carbohydrates with acid production	*mipB*, ***SMU.104***, ***SMU.105***, *sorA*
Acid tolerance	*comD*, ***SMU.1037c***

* The genes that were selected for the current study are indicated as bold.

More details of molecular methods are presented in Supplementary Table S1. The primers and the most suitable primer annealing temperatures are presented in Supplementary Table S2.

2.3. Statistical Analysis

Data were stored and analyzed in MS Excel software. Spearman's rs correlation test ($p < 0.05$) was used to find the assocation of virulence genes with caries markers ($p < 0.05$). Pearson's Chi square test ($p < 0.05$) was used to find differences between the groups.

3. Results

Most of the investigated *S. mutans* strains had all the virulence genes studied, both essential and virulence-enhancing (Figure 1). Only the *SMU.104* gene, which is involved in acid production, was absent in a quarter of the strains of this species. In contrast, several virulence genes were significantly less represented in the *S. sobrinus* strains: *gbpA*, *gbpB*, *wapA* and *ftf* genes were present in all strains, and *spaP*, *gtfB*, *vicR*, *SMU.1037c* and *SMU.105* genes in 41–88% strains, while *gtfD* and *SMU.104* genes were not found in the *S. sobrinus* strains. There was a statistically significant difference between the two species for the genes *spaP*, *vicR*, *gtfD* and *SMU.104* ($p < 0.01$). A correlation appeared between the *vicR* and *gtfD* genes ($r^2 = 0.574$; $p < 0.01$), the latter of which was absent from strains lacking the *vicR* gene. In contrast, the *gtfB* gene was present in some cases in strains lacking the *vicR* or *gtfD* gene (*vicR* and *gtfB* $r^2 = 0.328$; $p < 0.05$ and *gtfD* and *gtfB* $r^2 = 0.325$; $p < 0.05$).

Tables 3 and 4 show correlations between clinical parameters and virulence genes. We found a positive association between the *spaP* gene and the SM score in children ($r^2 = 0.643$, $p = 0.033$).

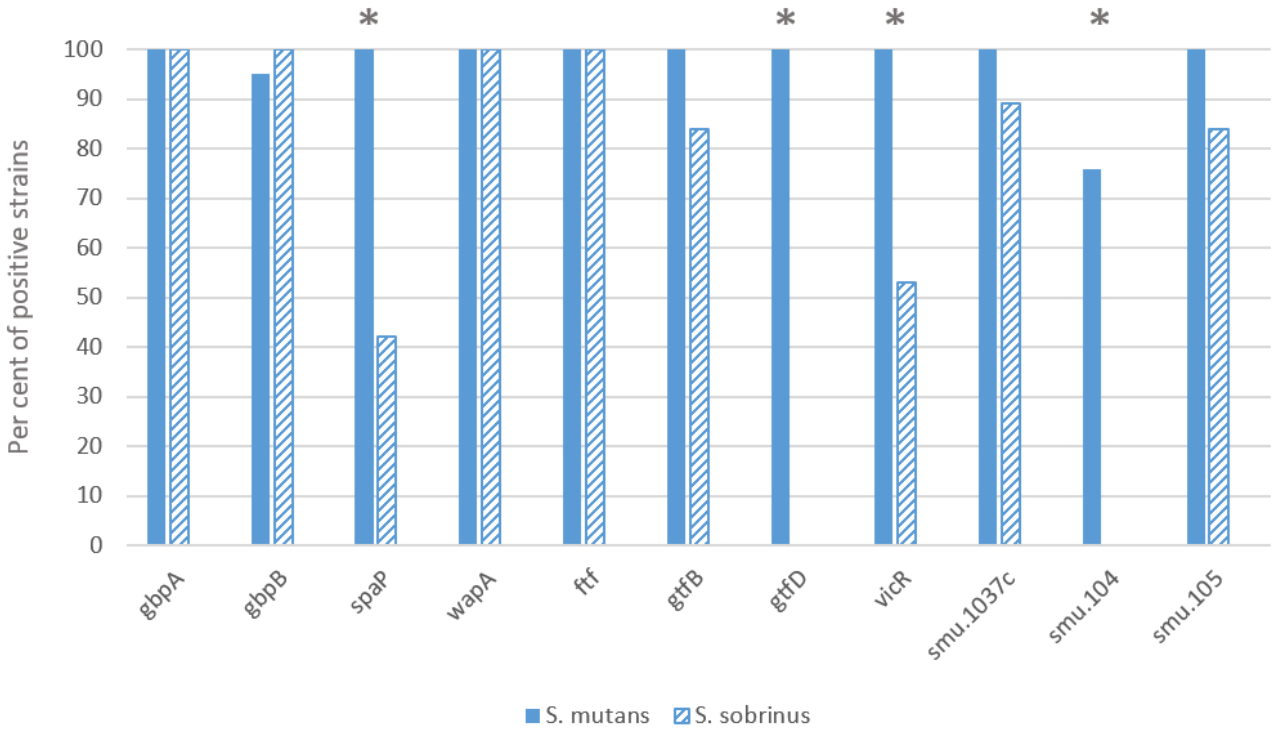

Figure 1. Distribution of virulence genes between two mutans streptococci species. Pearson chi-square test was applied, the asterisk indicates difference $p < 0.01$.

Table 3. Correlation between SM score and virulence genes between mutans streptococci and mothers/children. The level of each person's SM score (0–3) is compared to the specific gene present/absent in their bacterial strain. Results indicating positive correlation and statistical significance ($p < 0.05$) are marked in bold.

Gene	Correlation of SM Scores									
	S. mutans and *S. sobrinus*		*S. mutans*		*S. sobrinus*					
	(*n* = 38)		(*n* = 21)		(*n* = 17)		Children (*n* = 11)		Mothers (*n* = 6)	
	r^2	*p*-Value	r^2	*p*-Value	r^2	*p*-Value	r^2	*p*-Value	r^2	*p*-Value
gbpA	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
gbpB	0	NS (1)	−0.057	NS (0.805)	n/a	n/a	n/a	n/a	n/a	n/a
spaP	0.117	NS (0.796)	n/a	n/a	0.404	NS (0.108)	**0.643**	***p* = 0.033**	0	NS (1)
wapA	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
ftf	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
gtfB	0.019	NS (0.912)	n/a	n/a	0.101	NS (0.699)	0	NS (1)	0.447	NS (0.374)
gtfD	−0.149	NS (0.372)	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
vicR	−0.295	NS (0.072)	n/a	n/a	−0.398	NS (0.113)	−0.428	NS (0.189)	−0.333	NS (0.512)
smu.1037c	0.023	NS (0.894)	n/a	n/a	0.059	NS (0.819)	0	NS (1)	n/a	n/a
smu.104	−0.163	NS (0.329)	−0.095	NS (0.681)	n/a	n/a	n/a	n/a	n/a	n/a
smu.105	−0.281	NS (0.088)	n/a	n/a	−0.398	NS (0.114)	−0.371	NS (0.262)	−0.447	NS (0.374)

Spearman's r^2 correlation; SM score—Strip mutans score (Orion Diagnostica); n/a—not available; NS—not significant.

Table 4. Relationship between DMFT index and virulence genes between mutans streptococci in mothers and dmf index and virulence genes between mutans streptococci in children. Each mother's DMFT level (0–32)/children's dmf level (0–20) is compared to the specific gene present/absent in the bacterial strain.

Gene	Correlation of DMFT/dmf Scores											
	S. mutans and *S. sobrinus*				*S. mutans*				*S. sobrinus*			
	Mothers (DMFT) (n = 14)		Children (dmf) (n = 24)		Mothers (DMFT) (n = 8)		Children (dmf) (n = 13)		Mothers (DMFT) (n = 6)		Children (dmf) (n = 11)	
	r^2	*p*-Value	r^2	*p*-Value	r^2	*p*-Value	r^2	*p*-Value	r^2	*p*-Value	r^2	*p*-Value
gbpA	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
gbpB	−0.313	NS (0.276)	n/a	n/a	−0.417	NS (0.304)	n/a	n/a	n/a	n/a	n/a	n/a
spaP	−0.198	NS (0.497)	0.157	NS (0.465)	n/a	n/a	n/a	n/a	−0.533	NS (0.276)	0.086	NS (0.802)
wapA	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
ftf	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
gtfB	0.035	NS (0.906)	0.173	NS (0.419)	n/a	n/a	n/a	n/a	0.135	NS (0.799)	0.221	NS (0.514)
gtfD	0	NS (1)	0.176	NS (0.411)	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
vicR	0.197	NS (0.501)	0.108	NS (0.616)	n/a	n/a	n/a	n/a	0.402	NS (0.429)	0	NS (1)
smu.1037c	n/a	n/a	−0.101	NS (0.639)	n/a	n/a	n/a	n/a	n/a	n/a	−0.332	NS (0.319)
smu.104	0	NS (1)	0.121	NS (0.572)	0	NS (1)	−0.03	NS (0.921)	n/a	n/a	n/a	n/a
smu.105	−0.035	NS (0.906)	0.118	NS (0.578)	n/a	n/a	n/a	n/a	−0.135	NS (0.799)	0.148	NS (0.664)

Spearman's r^2 correlation; DMFT—decayed/missing/filled (permanent) teeth; dmf—decayed/missing/filled (primary teeth); n/a—not available; NS—not significant.

4. Discussion

This study confirmed that both SM species have multiple virulence genes but differences detected between the species and strains. Only the adhesion genes *gbpA*, *gbpB*, *ftf* and *wapA* were present in all investigated strains. Significantly more virulence genes were found in *S. mutans* than in *S. sobrinus*. Analysis also showed a positive association between the presence of *spaP* in *S. sobrinus* and an increase in the SM score in children.

All of the investigated genes play important roles in the physiology of MS. If the genes are turned off, the strain may be less resistant to environmental conditions, as changes may occur in the formation and structure of the biofilm [12,27,28], in adhesion to the tooth surface [29–31] or a decrease in EPS production [21,32]. Current research revealed that most of the *S. mutans* strains carried all the virulence genes studied, both essential and virulence-enhancing, related to biofilm formation, adhesion, acid production and tolerance, and interactions with the environment. In contrast, several virulence genes occurred less frequently in *S. sobrinus* strains. Previous studies have also shown some differences between these species. For example, de Soet et al. [33] and Igarashi et al. [34] found that *S. sobrinus* has a higher acid production capacity than *S. mutans*. In addition, previous research [34,35] has shown that *S. sobrinus* is rich in GTF-producing genes, and the most important is *gtfB*. In our study, this gene was found in 82% of the *S. sobrinus* strains studied, but the *gtfD* gene was completely absent in our strains. Other genes not studied in this work may compensate for the production of different glycotransferases.

Vic genes regulate the expression of several other virulence-related genes (*gtfB*, *gtfC*, *gtfD*, *ftf*, and *gbpB*) by acting on their promoter regions. The null-mutation of *vicR* is

probably lethal to *S. mutans* [13] and is therefore present in all strains studied. In a study of Zhuang et al. [36], 121 *vicR* genes were isolated and purified from *S. mutans* isolated from the children with and without caries and were found to be conserved in all isolates. Although *vicR* has been found to be essential for *S. mutans*, this may be different for *S. sobrinus*, which corresponds to the current study, where nearly half of the *S. sobrinus* strains lacked this gene. The prevalence of *vicR* varies among streptococci while the cause is not yet clear [13,37]. The *VicR*K signaling system is known to affect GTF expression in *S. mutans*. In the absence of the *vicR*K system in mutant strains of *S. mutans*, a decrease in *gtfD* gene expression and an increase in *gtfB* gene expression were observed [13,38]. The current study did not examine the expression level but revealed a positive correlation between the presence of the *vicR* and *gtfD* genes.

The *SMU.1037c* gene was found in 88% of *S. sobrinus* strains, and it helps to adapt the bacterium to changing environmental conditions. Conrads et al. [35] found that *S. sobrinus* lacked the *SMU.1037c* and TCS-7 systems, but they compared only two strains of this species. The *SMU.104* encodes the protein α-glucosidase glycosyl hydrolase, whose biological role is involved in carbohydrate metabolism and whose molecular function is involved in the hydrolysis of glycosyl bonds [39]. According to Banas [40], acid production varies between MS strains. *SMU.104* and *SMU.105* genes are not necessarily essential for the bacterium but may increase the competitive advantage over other strains by contributing to faster acid production. In our study, a quarter of the *S. mutans* strains and all *S. sobrinus* strains lacked the *SMU.104* gene.

S. mutans and *S. sobrinus* strains may have different mechanisms of perception and response, and because they often symbiotically coexist in the biofilm, *S. sobrinus* may not need to have all the genes. The main virulence traits of *S. mutans* are controlled or modulated by quorum sensing and thus depend on its own cell number but maybe also on cell numbers of cohabitants [35]. Although the genes selected for the study are important in the development of virulence, not all genes required for virulence were identified in the study. On the other hand, MS may have more virulence genes that were overlooked in this study.

Caries markers of children in our study were similar to a previous Estonian study where caries was diagnosed in 42% of the children at the age of 41 months, and the average dmft index was 1.6 ± 2.5 [41] as well as to studies conducted in other countries—1.64 ± 3.84 in Greece, 1.25 ± 2.47 in China [42,43]. DMFT index in mothers was also nearly similar to studies conducted worldwide (11.02 ± 6.3 in Brasil, 14.45 in the Philippines) [44,45]. Here, the clinical markers of the strain donors with the genetic information of the strains were compared. Although the *spaP* gene was detected to a much lesser extent in *S. sobrinus* than in *S. mutans*, a positive relationship between this gene and SM score in children was observed. This may be a prerequisite for further increased cariogenicity in these children. Because *spaP* is responsible for adhesion, bacteria with this gene may have a better ability to attach to the tooth and, ultimately, colonize the tooth surface better [30,46]. Our result corresponds to some previous studies showing that children with caries tend to carry *spaP*-positive mutans streptococci [45,47].

The other correlations between the virulence genes and clinical markers were not statistically significant. It should be taken into consideration that one person may have both *S. mutans* and *S. sobrinus* in the mouth, and they have been found to increase cariogenicity when living together [48–51]. At the same time, the set of strains was not large in our study, and the donor group was heterogeneous, which can be considered a limitation of the study. On the other hand, scarce studies have been performed on *S. sobrinus* so far, and only a few strains have been investigated in these studies; therefore, our study can be used as reference work for further studies.

5. Conclusions

Differences exist between the virulence gene patterns of the mutans streptococci: strains of *S. mutans* have more virulence genes than that of *S. sobrinus*, both crucial and

virulence enhancing. The clinical significance of different virulence genes needs further investigation. Deeper research is needed to clarify the mechanisms behind the increased cariogenicity in cohabitation.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/microbiolres13040065/s1, Table S1: Details of molecular methods; Table S2: Primers of virulence genes studied; Figure S1: A—Genotyped *S. sobrinus* strains. M1—SmaI enzyme; all strains are identified by their last three digits from their HUMB code; strains with similar gene patterns of mother and child are marked in blue (child 011-HUMB_13011, 012-HUMB_13012 and mother 105-HUMB_13105). Strain 018 is similar to latter strains, but the strain has been isolated from another individual. Strains 038 and 039 are isolated from the same caries site and also have the same gene pattern. The gene pattern of the previous strains are also similar to strain 104, but it is also isolated from another individual. Among the strains were three pairs of bacterial strains from the same individuals. Since the two pairs of strains also had a similar gene pattern, the two replicates were removed from further analysis. Two strains of *S. sobrinus* with different gene patterns were isolated from a third person, so both strains were included in the analysis. Thus, 37 human information but 38 bacterial strains were included into the study. B—Genotyped *S. mutans* strains. M2—ApaI enzyme; all strains are identified by their last three digits from their HUMB code.

Author Contributions: Conceptualization, R.M. and J.Š.; methodology, J.Š. and G.M.; software, M.J. and J.Š.; formal analysis, G.M., J.Š., M.J., J.O. and R.M.; investigation, G.M. and J.Š.; resources, R.M. and J.O.; data curation, J.Š., M.J. and R.M.; writing—original draft preparation, G.M.; writing—review and editing, J.Š., M.J., J.O. and R.M.; visualization, G.M. and R.M.; supervision, J.Š., M.J. and R.M.; project administration, R.M.; funding acquisition, R.M. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The strains originated from a former study where the oral health of mothers and children was assessed [24], with permission of the Ethics Committee for Human Research of the University of Tartu (protocol no. 166/T-7).

Data Availability Statement: All the data used to support the findings of this study are available from the corresponding author upon request.

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Conflicts of Interest: The authors report no conflict of interest regarding the publication of this paper.

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