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Aciduric microbial taxa including *Scardovia wiggsiae* and *Bifidobacterium* spp. in caries and caries free subjects

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ABSTRACT

Actinobacteria came into focus of being potential caries-associated pathogens and could, together with the established *Streptococcus mutans* and lactobacilli thus function as caries indicator species. Here we analyzed the role and diagnostic predictive value of the acidogenic–aciduric species *Scardovia wiggsiae* and *Bifidobacterium dentium* together with *S. mutans*, lactobacilli and bifidobacteria in biofilm of non-cavitated (n = 20) and cavitated (n = 6) caries lesions versus controls (n = 30). For the genus *Bifidobacterium* and for *B. dentium* new sets of primers were designed. Based on real-time quantitative PCR and confirmed by DNA sequencing we found a higher prevalence (61.5%) of *S. wiggsiae* in caries lesions than in controls (40%). However, among the controls we found three individuals with both the highest absolute and relative *S. wiggsiae* numbers. Testing for *S. mutans* revealed the same prevalence as *S. wiggsiae* in caries lesions (61.5%) but in controls its prevalence was only 10%. *B. dentium* was never found in healthy plaque but in 30.8% of clinical cases, with the highest numbers in cavitated lesions. The *Bifidobacterium*-genus specific PCR had less discriminative power as more control samples were positive. We calculated the relative abundances and applied receiver operating characteristic analyses. The top results of specificity (93% and 87%) and sensitivity (100% and 88%) were found when the constraint set was "*Lactobacillus* relative abundance ≥0.02%" and "two aciduric species with a relative abundance of each ≥0.007%". Combinatory measurement of several aciduric taxa may be useful to reveal caries activity or even to predict caries progression.

1. Introduction

Dental caries is caused by an interaction between acidogenic bacteria, a carbohydrate substrate which is frequently sucrose, and host susceptibility. The acidogenic–aciduric bacterial species *Streptococcus mutans* in combination with various lactobacilli (both *Firmicutes*) is recognized to be involved in cariogenic processes including early childhood caries, white spot lesions, cavitated lesions, or carious dentin [1]. Over time, its attributed role changed from pathogen (specific plaque hypothesis [2]) to enhancer (active role) and/or indicator (passive role) of a sugar-triggered cariogenic vicious circle (extended caries ecological hypothesis [3,4]) and the discussion still goes on [5].

As a matter of fact, *S. mutans* is detected in a few caries-free and found absent in several caries-active individuals impairing its caries indicatory potential.

Searching for complementary caries indicators we carefully analyzed facts related to the cariogenicity of several candidates and excluded the following taxa giving a motivation and references in brackets: *Actinomyces* sp. (low discriminative power on genus level, in high numbers in every healthy subject [6,7]), *Campylobacter* sp. and *Neisseria* sp. (both utilize organic acids serving as an acid sink [8]), *Prevotella* sp. (might reflect deep anaerobic carious lesion rather than direct contribution to caries but this is discussed controversially [6,9]), *Propionibacterium* sp. (low abundance, utilizes organic acids serving as an acid sink [8]), *Rothia dentocariosa* (relevant in a few caries patients, few publications about its cariogenic role [10]), *Selenomonas* sp. (ambiguous role [8]), *Slackia exigua* (interesting, but very limited data [11]), *Streptococcus salivarius* (relevant in caries but cross contamination with saliva reduces discriminative power [12]), other non-mutans-streptococci (ambiguous role, low discriminative power), and yeasts (no significance [13]), leaving *Scardovia wiggsiae*, *Bifidobacterium* spp., and

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especially the species *Bifidobacterium dentium* as candidate indicators. *Atopobium* spp. [6,9,14,15] and *Streptococcus sobrinus* (for review see [16]) have a proven impact in caries but were not included in this study.

The primary purpose of this pilot study was to measure – restricted by the complexity of an oral sample and without claiming clinical relevance – the absolute and relative abundances of the *Actinobacteria*-taxa *S. wiggsiae*, *Bifidobacterium* spp. (as genus and *B. dentium* as a species) in 26 adults with (test group) and 30 adults without (control group) symptoms of tooth decay. Abundances were determined by q-PCR and results compared to *Firmicutes*-taxa *S. mutans* and *Lactobacillus* spp. The first hypothesis of this study was that the acidogenic and aciduric microbiota differ between attached biofilm from adults with carious lesions and caries-free controls. The second hypothesis was that a combinatory measurement of several aciduric taxa may be useful to reveal caries activity.

2. Materials, subjects and methods

2.1. Bacterial strains, media and growth conditions

S. mutans UA159 was grown under standard conditions on Columbia blood agar (Oxoid, Wesel) aerobically, with 10% CO_2 at 37 °C. All *Lactobacillus* strains except *Lactobacillus paracasei* DSM 20020 were provided by Susanne Kneist, Biologisches Forschungslabor, Jena (*Lactobacillus fermentum* NP 11/60/5, NP 13/54; *Lactobacillus gasseri* NP 9/21/1, D 156; *L. paracasei* E 17/91; NP 15/67/1, D 375; *L. paracasei* ssp. *paracasei* D 131, D 599; *L. paracasei* ssp. *tolerans* D 110, D 318; *L. plantarum* NP 15/70/1, D 567; and *Lactobacillus paraplantarum* E 25/126). For testing the *in silico* designed *Bifidobacterium* spp. specific primers we used *B. dentium* DSM 20436, *Bifidobacterium breve* DSM 20091, *Bifidobacterium bifidum* DSM 20456, and *Bifidobacterium adolescentis* DSM 20083. All Bifidobacteria, *S. wiggsiae* DSM 22547, and lactobacilli were grown anaerobically at 37 °C on Columbia blood agar (Oxoid, Wesel) using the GasPak System (BD, Sparks, Maryland, USA). For standardized cell suspensions reference bacteria (*B. dentium* DSM 20436, *L. paracasei* DSM 20020, *S. wiggsiae* DSM 22547, and *S. mutans* UA159) were harvested from agar plates and suspended in Brain–Heart Infusion (BHI) broth. The cell number was determined by serial dilution/plating combined with counting cells per aggregate in a Neubauer chamber. The resulting cell number was found to be between 1.4×10^8 (*B. dentium*) and 1.6×10^9 (*S. mutans*) per ml suspension resulting in 100 μl DNA extract. DNA extracts were serially diluted to produce PCR-standards.

2.2. PCR primer design

Primers and amplification conditions for *S. wiggsiae*, *S. mutans*, lactobacilli, and total *Bacteria* were used as published [11,17–19]. We developed two new sets of 16S based primers addressing all bifidobacteria (Bifgsp) or *B. dentium* (Bifdent). All primers and PCR conditions as used in this study are listed in Table 1. Respective Bifidobacteria sequences for primer design were obtained from the National Centre of Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/) and the Ribosomal Database Project (RDP, http://rdp.cme.msu.edu/). For *Bifidobacterium* spp. it was found to be difficult – if not impossible – to develop 16S based PCR primers covering all currently known 43 species and excluding closely related genera (such as *Scardovia*, *Parascardovia*, and *Gardnerella*). Especially the *Bifidobacterium magnum/cuniculi/pseudolongum*-cluster (isolates from animal faeces) could not be included as they are too distantly related. As it was our intention to certainly include all oral bifidobacterial species (which are *Bifidobacterium animalis*, *B. breve*, *B. dentium*, *Bifidobacterium longum*, *Bifidobacterium scardovia*, and *Bifidobacterium subtile* according to the HOMD database) we designed the primers Bifgsp636F and Bifgsp857R. These primers cover all oral *Bifidobacterium* species, most of the other *Bifidobacterium* species known so far and have still 1–2 mismatches with their phylogenetically nearest neighbors *Scardovia inopinata*, *S. wiggsiae*, *Parascardovia denticolens*, and *Gardnerella vaginalis*.

2.3. Subjects and sample collection

Twenty-six adult test subjects (aged 18–78, mean 37.7, m 9, f 17) with cavitated (P1–P6) or non-cavitated (white spot lesions in most cases, P7–P26) dental caries and thirty gender-matched caries-free controls (21–33, mean 23.7, m 11, f 19) were recruited from the Clinic of Operative Dentistry, Periodontology and Preventive Dentistry, RWTH Aachen, Germany. The recruitment was in accordance with the guidelines of the Ethics Committee of the RWTH University Hospital, Aachen. All patients and volunteers were informed and gave their written consent before examination. Those who had taken antibiotics within the last three months were not included. After removing the top biofilm layer, the test samples were prepared by the aid of sterile spoon excavators or by a round burr at slow speed in a hand piece and samples collected with mini brush tips (applicator tips 60667198, Dentsply DeTrey, Konstanz, Germany). As controls interdental plaque was collected from a single reference site (regio 24–25) by an interdental brush stick (Dontodent, Cologne, Germany).

The mini- and interdental brush tips/sticks (capacity for about 10^8 to 10^9 cells or about 1–2 mg wet-weight) were cut under sterile conditions at 1 cm from the end and samples collected with 1.5 ml Eppendorf reaction tubes. A volume of 200 μl DNase free bidistilled water and 4–5 glass beads (diameter 1 mm) were added. After intense vortexing the cell suspensions were transferred to new reaction tubes and cells recovered by centrifugation at 7000 rcf for three minutes. Using a *S. mutans* standard suspension (1.6×10^9) the recovery rate of brush tips/sticks was found to be about 50%. For washing, a volume of 200 μl sterile bidistilled water was added to the pellets, vortexed and cells were again pelleted by centrifugation at 10,600 rcf for two minutes, followed by subsequent discard of the supernatant. After addition of 20 μl of a lysozyme/mutanolysin solution (0.3 mg lysozyme plus 10 U mutanolysin) to the pellet a 30-min incubation step at 37 °C preceded DNA extraction and purification using the QIAamp DNA Mini kit ("tissue protocol", Qiagen, Hilden, Germany) according to the manufacturer's instructions.

2.4. PCR experiments

One μl of purified DNA (from standards and from samples) was used as template for real-time quantitative PCR (q-PCR) on the Light Cycler 2.0 system (Roche, Mannheim). The total number of bacterial genome equivalents per μl DNA extract (referred to as "bacterial cell counts") was measured according to a modified protocol by Nadkarni et al., 2002 [18] using *S. wiggsiae* DNA as standard. Accordingly, cell counts of *Bifidobacterium* spp., *B. dentium*, *Lactobacillus* spp., *S. mutans*, and *S. wiggsiae* were measured using the corresponding reference strains mentioned above as standards and applying the primers and protocols listed in Table 1. The identity of amplicons was verified by spot check sequencing using the Applied Biosystems 310 DNA sequencer (Applied Biosystems, Foster City, USA). Thus, we assume the specificity for our species-specific PCRs to be very high. However, for the genus specific PCRs the resulting sequences could not be resolved in all cases, but at least grave cross-reactions were excluded. Due to the relative homogeneity within the genus *Lactobacillus* and to our