Oral Microbiota Display Profound Differential Metabolic Kinetics and Community Shifts upon Incubation with Sucrose, Trehalose, Kojibiose, and Xylitol

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ABSTRACT This study compares the metabolic properties of kojibiose, trehalose, sucrose, and xylitol upon incubation with representative oral bacteria as monocultures or synthetic communities or with human salivary bacteria in a defined medium. Compared to sucrose and trehalose, kojibiose resisted metabolism during a 48-h incubation with monocultures, except for *Actinomyces viscosus*. Incubations with *Lactobacillus*-based communities, as well as salivary bacteria, displayed kojibiose metabolism, yet to a lesser extent than sucrose and trehalose. Concurring with our *in vitro* findings, screening for carbohydrate-active enzymes revealed that only *Lactobacillus* spp. and *A. viscosus* possess enzymes from glycohydrolase (GH) families GH65 and GH15, respectively, which are associated with kojibiose metabolism. Donor-dependent differences in salivary microbiome composition were noted, and differences in pH drop during incubation indicated different rates of sugar metabolism. However, functional analysis indicated that lactate, acetate, and formate evenly dominated the metabolic profile for all sugars except for xylitol. 16S rRNA gene sequencing analysis and α -diversity markers revealed that a significant shift of the microbiome community by sugars was more pronounced in sucrose and trehalose than in kojibiose and xylitol. In *Streptococcus* spp., a taxon linked to cariogenesis dominated in sucrose (mean $\pm$ standard deviation, $91.8 \pm 6.4\%$) and trehalose ($55.9 \pm 38.6\%$), representing a high diversity loss. In contrast, *Streptococcus* ($5.1 \pm 3.7\%$) was less abundant in kojibiose, which instead was dominated by *Veillonella* ($26.8 \pm 19.6\%$), while for xylitol, *Neisseria* ($29.4 \pm 19.1\%$) was most abundant. Overall, kojibiose and xylitol incubations stimulated cariogenic species less yet closely maintained an abundance of key phyla and genera of the salivary microbiome, suggesting that kojibiose has low cariogenic properties.

IMPORTANCE This study provides a detailed scientific insight on the metabolism of a rare disaccharide, kojibiose, whose mass production has recently been made possible. While the resistance of kojibiose was established with monocultures, delayed utilization of kojibiose was observed with communities containing lactobacilli and *A. viscosus* as well as with complex communities of bacteria from human saliva. Kojibiose is, therefore, less metabolizable than sucrose and trehalose. Moreover, although conventional sugars cause distinct shifts in salivary microbial communities, our study has revealed that kojibiose is able to closely maintain the salivary microbiome composition, suggesting its low cariogenic properties. This study furthermore underscores the importance and relevance of microbial culture and *ex vivo* mixed cultures to study cariogenicity and substrate utilization; this is in sharp contrast with tests that solely rely on monocultures such as *Streptococcus mutans*, which clearly fail to capture complex interactions between oral microbiota.

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Dental caries is one of the major noncommunicable diseases caused by oral bacteria and is estimated to affect nearly 2.5 billion people globally (1, 2). The mouth harbors a highly diverse bacterial population (3) residing in an organized matrix of polymicrobial biofilms, also known as plaque, on the tongue, teeth, and subgingival surfaces with diverse metabolic capacities (4). Their metabolic interactions are largely influenced by the nutrient supply from food and saliva, biofilm community structure, and redox potential that ranges from aerobic to microaerophilic and even anaerobic conditions (5).

Given the short transition time food has in the mouth, the oral bacteria primarily derive their carbon from free sugars and fermentable carbohydrates (6, 7). Sucrose is the most widely used sugar in many food applications owing to its superior technological and sensorial properties (8). However, the production of organic acids by saccharolytic bacteria from fermentable sugars, including sucrose, can lower the pH below the critical level of 5.5, resulting in demineralization of the tooth enamel (caries) (4, 6, 9). The susceptibility to developing caries can occur at any age, though the side effects are cumulative throughout one's lifetime. A healthy oral site at nearly neutral pH is dominated by a microbial community of several genera: *Actinomyces*, nonmutans *Streptococcus*, *Porphyromonas*, *Bifidobacterium*, *Fusobacterium*, *Neisseria*, and *Veillonella* (10, 11). During caries development, *Actinomyces* and nonmutans streptococci utilize fermentable sugars to initiate cariogenic activities (12) before successor mutans streptococci colonize the acidic sites to further promote cariogenicity (11). The successive drop in pH selectively increases the dominance of aciduric bacteria, including *Veillonella*, *Neisseria*, and *Streptococcus*, at about a pH of 5.5 (10). In mature caries, *Lactobacillus*, *Propionibacterium*, and streptococci become relatively abundant (10, 12).

When evaluating product cariogenicity, much focus has been placed on mutans streptococci, particularly *Streptococcus mutans*; however, these microbes offer a narrow representation of the complex community and their metabolic interaction in the development and progression of caries. Furthermore, the advancements in DNA sequencing technologies have precisely and efficiently enabled the identification of which microorganisms are present. Nonetheless, what these microbes functionally do has not been well elucidated; therefore, it is crucial to monitor organic acids in order to functionally understand the contribution of the oral microbiome to the development of caries.

Even though many rare sugars are suggested as potential sucrose alternatives, their evaluation and application are hindered by their low quantities in nature and the associated high cost of production (13). A rare sugar that has recently gained research interest is kojibiose, a disaccharide of α -1,2-linked glucose units whose properties are still under active evaluation. Interestingly, upon screening several sugars with *S. mutans*, Hodoniczky et al. (14) suggested that kojibiose is less cariogenic. Noteworthy, enzyme engineering has most recently made it possible to produce kojibiose in an efficient and scalable manner (15). Like kojibiose, trehalose is a disaccharide of α -1,1-linked glucose, and it is commercially produced from starch (16) at a reasonable price (\$8/kg on Amazon).

To assess the potential application of kojibiose and trehalose as dietary sugar alternatives to ameliorate/avoid caries, this study endeavored to evaluate the metabolic potential of these sugars using oral bacteria as monocultures, synthetic communities, and as complex communities from human saliva in a defined carbon-limited medium. Furthermore, the genomic potential of representative strains to metabolize kojibiose and trehalose was screened on the Carbohydrate-Active enZymes (CAZy) database, while 16S rRNA amplicon sequencing was used to decode the potential impact sugars have on the saliva microbial community.

RESULTS

pH change and organic acid generation by oral bacteria monocultures. pH plays a critical role in the caries process, and it is generally accepted that a pH below 5.5 promotes the demineralization of tooth enamel (6). Our first approach was to investigate if monocultures of representative oral bacteria are able to metabolize the disaccharides kojibiose and trehalose as potential sucrose alternatives in a carbon-limited medium. We observed that the pH decrease for sucrose- and trehalose-amended incubations (Fig. S1A in the supplemental material) was significant ($P < 0.001$) yet not sufficient to drop below the critical value of 5.5 for most of the strains. *Prevotella intermedia* exhibited a faster fermentation in both sucrose and trehalose incubations, resulting in a pH drop below 5.5. In addition, incubations for sucrose with *Streptococcus sobrinus* ($\text{pH } 5.2 \pm 0.1$) and *Actinomyces viscosus* ($\text{pH } 5.2 \pm 0.0$) also resulted in a pH drop to below 5.5 by 48 h. Concomitant to the drop in pH, more organic acids were generated from sucrose (7.6 ± 5.7 mM) and trehalose (6.5 ± 5.6 mM) (Fig. S1B), although the pH drop in *A. viscosus* did not directly correspond with the respective increase in organic acid. Even though most streptococcal strains in this study produced high levels of organic acids from sucrose and trehalose, *Streptococcus mitis* and *Streptococcus salivarius*, however, generated low levels from trehalose. On the other hand, low but comparable amounts of organic acid were produced from kojibiose (0.85 ± 0.4 mM) and xylitol (0.81 ± 1.0 mM); this kept the pH above the critical value across all the strains. Interestingly, only *A. viscosus* and *P. intermedia*, respectively, exhibited potential to metabolize kojibiose and xylitol (Fig. S2). Notably, *Lactobacillus fermentum*, *Lactobacillus gasseri*, *Fusobacterium nucleatum*, *Porphyromonas gingivalis*, *Parvimonas micra*, *Actinomyces naeslundii*, *Veillonella parvula*, and *Aggregatibacter actinomycetemcomitans* failed to significantly grow as monocultures in all the sugars.

pH change and organic acid generation by synthetic communities of oral bacteria. Our second approach entailed incubating sugars with four synthetic communities, namely (i) a *Streptococcus* population (7 strains), (ii) a *Lactobacillus* population (4 strains), (iii) a population of other endogenous strict oral anaerobes (8 strains), and (iv) a community comprising all 19 strains together. We found pH decreases to be dependent on time, microbial composition, and supplemented sugar ($P < 0.05$) (Fig. 1A). For trehalose and sucrose incubations, the pH remained above the critical level within the first 24 h but dropped below 5.5 for all four microbial communities at 48 h. The levels of organic acids generated from sucrose (14.0 ± 6.3 mM) and trehalose (15.2 ± 7.1 mM) by the four bacterial communities were high and comparable (Fig. 1B). Of note, the community of *Lactobacillus* showed metabolic preference for trehalose over sucrose.

For kojibiose, the pH remained fairly constant across the four communities, although a small drop (not below the critical value) was noted for the incubation with *Lactobacillus* population ($\text{pH } 5.7 \pm 0.1$) and the community comprising all 19 strains ($\text{pH } 6.1 \pm 0.0$) at 48 h (Fig. 1A). Correspondingly, we observed an increase in the amounts of organic acids (Fig. 1B) for the *Lactobacillus* community (18.2 ± 0.4 mM) and the community comprising all 19 strains together (13.9 ± 0.7 mM). The community only comprising *Streptococcus* strains (1.2 ± 0.1 mM) generated the smallest amount of organic acid followed by strict anaerobic strains (4.4 ± 0.5 mM). Only the community comprising strict anaerobes exhibited potential to metabolize xylitol. Overall, the organic acids generated by the *Streptococcus* and *Lactobacillus* communities were largely dominated by lactate, while strict anaerobes and all communities exhibited a similar mixed acid profile of acetate, formate, and lactate (Fig. 1B).

Sugar metabolism by the *in vivo*-derived microbiota from human saliva. We incubated sugars with salivary microbiota from human origin of 11 different individuals in order to investigate sugar metabolism under more representative conditions for the oral cavity and to capture interindividual variability. The salivary cell counts of donors were found to vary between 2.6×10^6 and 3.6×10^8 cells/ml of saliva (Fig. 2A) and demonstrated a weak correlation ($r = 0.16$; $P = 0.01$) with the total organic acid con-

FIG 1 Change in pH (A) and levels and profile of organic acids generated (B) from sugars by four synthetic communities. The communities assembled were *Lactobacillus* alone (LA) (4 strains), *Streptococcus* alone (ST), strict anaerobes (8 strains) other than *Lactobacillus* or *Streptococcus* strains (SA), and the community comprising all 19 strains (TO) (46). CO is a sugar medium without bacterial inoculation. Data points represent the mean of three biological replicates.

centrations. Incubations with salivary bacteria revealed a drop in pH dependent on the donor ($P < 0.0001$), sugar ($P < 0.0001$), and time ($P < 0.008$) (Fig. 2B). For sucrose (pH 4.1 ± 0.1), the drop reached its lowest level across all the donors within the first 24 h, corresponding well with the observed high levels of organic acids generated at 24 h (25.6 ± 4.2 mM) and 48 h (30.1 ± 1.8 mM) (Fig. 2C). Despite trehalose being metabolized to a lesser degree than sucrose, a similarly low pH (pH 5.0 ± 0.7) was recorded across all the donors, coinciding with the large amounts of organic acids at both incubation times. In contrast, the pH for kojibiose at 24 h remained higher and was even comparable to the pH levels obtained for xylitol incubations. Nevertheless, upon extended incubation, the pH for kojibiose dropped to pH 5.4 ± 0.8 , which was lower than for xylitol (pH 6.8 ± 0.0).

Lactic acid was the predominant organic acid for incubations with sucrose and trehalose despite trehalose also showing high levels of propionic acid compared to other sugars ($P < 0.001$). A mixed organic acid profile was observed for kojibiose dominated by acetic acid. For xylitol, low levels, which were predominantly acetic acid, were observed.

Sugar-hydrolyzing capabilities of the oral bacteria. Having observed that *A. viscosus* and communities containing *Lactobacillus* were able to generate organic acids from kojibiose, we became interested in the genomic potential of our strains to utilize sugars, particularly kojibiose. We screened the CAZy database for relevant carbohydrate-hydrolyzing enzymes. The strains manifested versatile carbohydrate-utilizing capabilities based on the number and type of functional enzyme families (Table S1). They manifest pathways that can utilize both monosaccharides (glucose, rhamnose,

FIG 3 Relative abundance of the top 20 genera constituting >0.1% and “other genera” accounting for all other genera constituting <0.1% of the oral bacteria in the inoculum and sugars at 24 and 48 h of incubation. The letters D1 to E2 represent donors.

of the total phyla, while “*Bacteria* unclassified,” *Spirochaetes*, and *Synergistetes* were present in low abundance. At the genus level (Fig. 3; Table S2), *Streptococcus* ($19.4 \pm 9.2\%$), *Veillonella* ($14.9 \pm 8.5\%$), “*Enterobacteriaceae* unclassified” ($12.8 \pm 12.2\%$), and *Neisseria* ($12.0 \pm 13.2\%$) accounted for nearly 60% of the total genera. *Prevotella* ($5.9 \pm 2.9\%$), *Burkholderia* ($5.4 \pm 7.9\%$), *Leptotrichia* ($2.8 \pm 3.9\%$), “SR1 unclassified” ($2.4 \pm 3.8\%$), and *Rothia* ($2.4 \pm 3.5\%$) were present in saliva at more than 2% relative abundance. Additionally, the other 32 genera even had a relative abundance between 0.2 to <2%.

Sugar significantly lowers the initial diversity of salivary bacteria. Further analysis was conducted to explore the microbial community structure for each sugar and how the structure was associated with the observed metabolic function. The α -diversity measurements, Shannon, Pielou’s evenness, and Simpson indices (Fig. 4A), revealed that the high microbial diversity of the inoculum was lowered ($P < 0.001$) by the sugars. For time comparison, 24 and 48 h had similar diversity despite lowering the diversity of the inoculum (Fig. 4B). Furthermore, a small interindividual variation in bacterial community composition among the donors was observed, although donor E1 exhibited a lower diversity than some donors (Fig. S3C). Diversity loss by sugars was more pronounced in sucrose and trehalose than in kojibiose and xylitol. Sucrose and trehalose shifted the community to over 70% *Firmicutes* at the phylum level (Table 1 and Fig. S3B), which was nearly completely dominated by *Streptococcus* (sucrose at $91.8 \pm 6.4\%$ and trehalose at $55.9 \pm 38.6\%$) at the genus level (Fig. 3 and Table S2). An increase of *Veillonella* ($20.7 \pm 22.3\%$) was also observed in trehalose. On the other hand, *Neisseria* was markedly reduced in sucrose ($0.7 \pm 1.4\%$) and trehalose ($4.0 \pm 7.8\%$). Similarly, *Prevotella* was reduced in sucrose ($0.3 \pm 0.2\%$) and trehalose ($1.6 \pm 1.9\%$). Contrastingly, kojibiose ($35.7 \pm 19.5\%$) and xylitol ($33.3 \pm 14.0\%$) incubations fairly maintained *Firmicutes* levels at the same level as in the inoculum ($38.7 \pm 14.1\%$). At the genus level, kojibiose interestingly favored *Veillonella* ($26.8 \pm 19.6\%$) as opposed to *Streptococcus*, which instead was lowered by 14%, while *Granulicatella* significantly increased in xylitol ($11.5 \pm 8.7\%$) (Fig. 4C; Table S2). In addition, the level of *Bacteroidetes* in kojibiose ($9.1 \pm 23.0\%$) was comparatively not altered, but a 7% increase was observed in xylitol. The increase in the *Fusobacteria* by 12% in kojibiose was largely contributed to by the

FIG 4 (A, B) Pairwise comparison of different α -diversity indices: Simpson, Shannon, and Pielou's evenness in salivary bacteria in inoculum and sugars (A) and Shannon index in inoculum (0 h) and incubation times 24 and 48 h (B). The log relative normalized abundance distribution based on DESeq2 test of the top five most differentially abundant genera between inoculum and sugars with their corresponding P values. (C) Order of importance is represented by the numerals and the bars in the upper panel. (D) Constrained canonical analysis of principal coordinates (CAP) based on Bray-Curtis distance showing the association between bacterial community structure and the treatment variables. The association significance test was based on adonis function in the vegan package ($P = 0.001$). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

proliferation of *Leptotrichia* (from $2.8 \pm 3.9\%$ to $15.1 \pm 19.3\%$). *Burkholderia* was reduced in all other sugars except in kojibiose ($6.1 \pm 6.3\%$), which maintained the levels as in the inoculum ($5.4 \pm 7.9\%$). The proportion of *Neisseria* in xylitol ($29.4 \pm 19.1\%$) was more than double that in other sugars. SR1 unclassified, *Rothia*, and *Aggregatibacter* were reduced to $<0.2\%$ in all sugar incubations despite having $>1.5\%$ relative abun-

dance in the saliva inoculum (Table S2). In contrast, some genera, including *Bifidobacterium* and *Chryseobacterium*, increased in relative abundance in xylitol, whereas *Stenotrophomonas* and *Haemophilus* expanded in both kojibiose and xylitol (Table S2).

Despite the overall loss and gain in abundance of some phyla (Fig. S3) and genera (Fig. 3), kojibiose and xylitol closely maintained the abundance of the core phyla and genera as in the inoculum in all donors except D8 and E1. Beta diversity analysis through Bray-Curtis distance showed that the community structure between the sugars was significantly ($P < 0.001$) dispersed (Fig. 4B). Sucrose particularly clustered well together, while a close clustering near the inoculum was observed with xylitol and kojibiose. Trehalose, on the other hand, clustered at both coordinates, demonstrating a nonhomogeneity of dispersion. A constrained ordination based on the Bray-Curtis method revealed that sucrose was more associated with lactate, while trehalose was associated with both lactate and acetate. On the other hand, kojibiose was associated with both acetate and propionate. The permutational multivariate analysis of variance (PERMANOVA) test confirmed the significance ($P < 0.001$) of the association between the community compositions with the variables, pH, and organic acids.

DISCUSSION

Sugar fermentation in the oral cavity is a major contributing factor to the development of dental caries and is modulated by sugar fermentability, pH in the oral cavity, and the metabolic capacity of the oral microbiota (6, 9, 17, 18).

In this study, we demonstrate that trehalose does have comparable metabolic properties to sucrose, bringing about profound shifts in metabolic profiles and microbiome composition. In sharp contrast, we found kojibiose to be a disaccharide that resists oral microbial metabolism much more than sucrose and trehalose. We provide evidence at different levels of complexity with monocultures, synthetic microbial communities, and *in vivo*-derived human oral microbiota.

The interindividual variation in bacterial cell density of saliva inoculum in this study was comparable to those in an earlier study which monitored bacterial cell density over time (1.5×10^7 to 5.1×10^8 cells/ml) using flow cytometry (19). The abundance of typical phyla and genera in the saliva microbiome, though varied between individuals, was in agreement with the literature (20–22). Incubation with sucrose and trehalose shifted the salivary microbiota to an abundance of more than 70% *Firmicutes* at the phylum level, largely dominated by the *Streptococcus* genus that accounted for over 90% in sucrose and 50% in trehalose. This suggested increased acidogenic activities, as was also evidenced by the pH drop below the critical level occasioned by the large amounts of organic acids. The *Streptococcus* genus plays a critical role in caries development as a primary colonizer of the teeth surfaces owing to its ability to generate extracellular polysaccharides from sugars and acidogenic activities (11). The successive drop in pH during sugar fermentation reduces microbial diversity and selectively increases the relative abundance of aciduric bacteria, including *Streptococcus*, *Veillonella*, *Neisseria*, *Lactobacillus*, and *Propionibacterium* (10, 12, 23). Consistent with our findings, clinical observations of patients with dental caries have also confirmed the dominance of *Streptococcus* (23, 24), underlining the importance of this genus in sugar metabolism and dental caries.

In a starvation state, some genera, including *Klebsiella* and *Neisseria*, can remain transcriptionally active and increase in abundance at the expense of *Streptococcus* species (25). Consistent with this study, *Neisseria* levels in kojibiose were maintained as in the inoculum but more than doubled in xylitol with a concomitant decrease in the abundance of *Streptococcus* in both sugars. This illustrates that these sugars were not readily available for *Streptococcus* metabolism.

Since kojibiose, on the other hand, was slowly metabolized, it is clear that other saccharolytic bacteria other than *Streptococcus* species were actively involved in its metabolism but might have first required enzyme induction (5). Plausible genera with such potential include *Neisseria*, which was the fourth most relatively abundant genus

in the inoculum. In the oral cavity, *Neisseria sicca*, *Neisseria subflava*, and *Neisseria mucosa* are normal microbiota of the oro- and nasopharynx (26, 27), and based on the CAZy database, these *Neisseria* species possess the GH65 family of enzymes with kojibiose-hydrolyzing activity (28).

Similar to our study, an increase in relative abundance of *Leptotrichia* in kojibiose with a concomitant decrease in *Streptococcus* species has also been reported (29). *Leptotrichia* is a normal oral bacterium, and its role in the oral cavity is unclear. It is nonetheless been shown that *Leptotrichia* is versatile in sugar metabolism, producing lactate as the main organic acid (30); however, this is very species and sugar dependent (31). Some *Leptotrichia* species, for example, *Leptotrichia goodfellowii*, have the GH65 family of enzymes, while others do not, demonstrating the variability in sugar metabolism in this genus. Notably, *Granulicatella*, belonging to the *Firmicutes* phylum, increased in xylitol and kojibiose. The bacterial *Granulicatella* genus is a fastidious microorganism with complex nutritional requirements (32), and its increase in these sugars is intriguing. Limited information on this genus exists, with no single species represented in the CAZy database, and therefore, its metabolic characterization requires more clarification.

We also observed a near-complete shift of microbial profile in kojibiose and xylitol with genera that were relatively low in abundance in the donor E1 and D8 inocula. In the donor D8 inoculum, the *Neisseria* genus was the most abundant and could have contributed to the initial kojibiose breakdown. While it remains unclear why the relatively low-abundance genera, *Haemophilus* and *Stenotrophomonas*, rapidly became abundant in kojibiose incubations in the D8 inoculum, they are normal oral microbiota of the oro- and nasopharynx (26, 27). Some species, including *Stenotrophomonas maltophilia*, have the GH15 family of enzymes, which exhibit flexibility in hydrolyzing α -1,2 bonds (kojibiose). The inoculum from donor E1, which was predominantly composed of *Prevotella*, did not demonstrate significant metabolic activity, as evidenced by the organic acid and pH in both kojibiose and xylitol in this study. Our monoculture incubations revealed that *P. intermedia* was unable to utilize kojibiose but was the only strain with the ability to produce significant amounts of organic acid in xylitol, illustrating their potential to utilize xylitol.

Overall, the oral microbiome represents a diverse metabolic potential, and our study highlights the importance of microbiome diversity and strain specificity in substrate utilization and that substrates can have a profound shift in the microbial community and modulate health.

Most oral bacteria as monocultures and synthetic communities easily fermented sucrose and trehalose based on pH drop and a relatively high and similar profile of organic acids, suggesting that most oral bacteria have active hydrolases to metabolize sucrose and trehalose. Indeed, our genomic screening predicted that most oral bacteria contain genes encoding GH13 enzymes, which represent the dominant family found in the genome of all our strains except in *P. micra* and *V. parvula*. GH13 constitutes the largest family of enzymes comprising trehalase and other hydrolases bearing activity on α -1,1, α -1,2, α -1,3, and α -1,5 glycosidic linkages (28). Additionally, *Streptococcus*, *Prevotella*, and *Lactobacillus* possess GH32, a group of enzymes that includes invertase with fructose-containing, carbohydrate-hydrolyzing capabilities. Moreover, *Lactobacillus* harbors GH65, an enzyme family that contains trehalase and trehalose phosphorylase (28). This enzyme machinery can explain the capabilities of oral bacteria to utilize sugars of diverse linkages, particularly trehalose and sucrose, as has been confirmed by our experimental data. Consistent with our findings, Hodonczyky et al. previously showed that *S. mutans* was able to ferment trehalose to below the critical pH within a few hours of incubation (14). Neta et al. (33) had also reported a pH drop below the critical level in sucrose and trehalose within a few minutes when incubated with *S. mutans* and *S. sobrinus*, although the drop was considerably faster in sucrose. Despite this rapid drop in pH, trehalose is reported to have low cariogenic properties in comparison to sucrose in humans and rats owing to the low production of insoluble glucans (33). In contrast, kojibiose was poorly fermented compared to both trehalose

and sucrose by oral bacteria, suggesting its low metabolic property. This was acknowledged by high pH levels above the threshold of 5.5 and small amounts of organic acids formed by the monocultures, synthetic communities, and salivary incubations except for donor D8 in the first 24 h. The pH drop in kojibiose in D8 could be attributed to the high formate levels (pK_a , 3.75).

Remarkably, we observed that only *A. viscosus* as a monoculture could significantly generate organic acids from kojibiose at extended incubation. Furthermore, a metabolic synergy was observed when kojibiose was incubated with synthetic communities consisting of only *A. viscosus* or *Lactobacillus* strains or communities comprising both. The metabolic synergy does not solely depend on phylogenetic diversity but also on metabolic potential of the microbes and their interactions. Our data revealed that communities consisting only of *Lactobacillus* strains had a 30% increased production of organic acids over communities comprising both *A. viscosus* and *Lactobacillus* strains. Microbial community interactions can both be synergistic or competitive depending on the substrate structure and metabolic potential of the individual bacteria in the community (34).

We also confirmed that *Lactobacillus* strains have genes that can encode the GH65 family of hydrolyzing enzymes, which includes kojibiose phosphorylase (28) and can explain why the *Lactobacillus* community generated significant amounts of organic acid from kojibiose. As for *A. viscosus*, in addition to having GH13 and GH32, it has GH15 but not GH65 found in *Lactobacillus* (28). GH15 are extracellular enzymes that hydrolyze the nonreducing end residues of α -glucosides and present a form of flexibility that is not only able to hydrolyze an α -1,4-glycosidic bond but to a lower degree also α -1,6-, α -1,3-, and α -1,2-bonds (35). This suggests that *A. viscosus* exhibited this flexibility in order to hydrolyze the α -1,2 glycosidic linkage of kojibiose. Kojibiose is considerably therefore difficult to metabolize by the oral bacteria. In support of our findings, Hodoniczky et al. (14), in an attempt to classify the influence of sugar structure on cariogenicity, also observed that bonds of α -1,2 and α -1,3 were difficult to ferment by the *S. mutans* compared to α -1,1. Moreover, a partial resistance of kojibiose to enzymatic hydrolysis has also been reported in germ-free rats (36) and intestinal enzymes (37), further revealing that kojibiose is difficult to metabolize. In this study, however, we observed a low abundance of *Lactobacillus* in the saliva, confirming that other genera actively metabolized kojibiose. Taken together, the development of dental caries requires a polymicrobial interaction (7, 11); our observations clearly highlight that functional bacterial interaction is too important to ignore and future research should employ phylogenetic diversity and interaction in testing substrate metabolism.

Interestingly, based on the organic acid levels and change in pH, we also observed that *P. intermedia* was as robust as *Streptococcus* strains in metabolizing sucrose and trehalose and, much to our surprise, also metabolizing xylitol but not kojibiose. Genomically, *P. intermedia*, apart from having genes that can encode the GH13 family of enzymes, also has GH97. The GH97 family of enzymes shows activity on α -1,6, α -1,3, and α -1,2, as well as α -1,4 linkages. These enzymes accord *P. intermedia* metabolic versatility to utilize sucrose and trehalose and not kojibiose, further illustrating metabolic resistance of kojibiose across most oral bacteria. Xylitol, on the other hand, is thought to be nonfermentable (38). However, its fermentation has only been demonstrated with lactobacilli after >15 days of incubation (39). Some of our strains were not able to grow in our medium, while *L. fermentum* and *L. gasseri* exhibited poor growth, as observed from the high pH values and small amount of organic acids. Previous studies have, in fact, reported the inability of some oral bacteria to grow as monocultures, particularly in minimal medium, and can only have significant growth in cocultures (40–42). This could be indicative of dependence on other carbon forms and/or cross-feeding interactions with other species as has been noted (4, 5, 43). One such known cross-feeding pattern involves the conversion of lactate mainly generated by primary sugar degraders *Streptococcus*, *Actinomyces*, and *Lactobacillus* (9, 11) to propionate by the *Veillonella* genus (10, 44). Concordant with our study, we observed a

TABLE 2 Bacterial strains used in this study

Genus/type	Strain
<i>Streptococcus</i>	<i>S. mutans</i> ATCC 25175
	<i>S. sobrinus</i> ATCC 33478
	<i>S. gordonii</i> ATCC 49818
	<i>S. mitis</i> ATCC 49456
	<i>Streptococcus sanguinis</i> LMG 14657
	<i>S. salivarius</i> TOVE-R
	<i>Streptococcus oralis</i> KU Leuven, Belgium
<i>Lactobacillus</i>	<i>Lactobacillus casei</i> ATCC 393
	<i>L. fermentum</i> ATCC 9338
	<i>Lactobacillus rhamnosus</i> ATCC 7469
	<i>L. gasseri</i> DSM 20243
Strict anaerobes	<i>A. actinomycetemcomitans</i> ATCC 43718
	<i>F. nucleatum</i> ATCC 10953
	<i>P. gingivalis</i> ATCC 33277
	<i>P. intermedia</i> ATCC 25611
	<i>A. naeslundii</i> ATCC 51655
	<i>A. viscosus</i> ATCC 15987
	<i>P. micra</i> ATCC 33270
	<i>V. parvula</i> DSM 2008

coinciding increase in abundance of *Veillonella* in kojibiose (11%) and trehalose (6%) incubations with propionate levels.

In conclusion, our *in vitro* model has offered a high-throughput screening of the metabolic capabilities of several oral strains and complex salivary bacteria on sugar in a carbon-limited medium. Our study has yet not addressed other factors that could modulate sugar metabolism in the host oral cavity and hence the caries process, such as host dietary background and interaction with the saliva. The flow of saliva constantly supplies glycoproteins as a carbon source, proteins, and immune cells and dilutes the organic acids. Future *in vivo* tests, therefore, would be important in corroborating these findings. Fundamentally, our results provide insight that sugar type can drive and/or define the structure of the microbial community, which, by extension, can impact host health. However, on the basis of the ease of metabolism by the oral bacteria, it is very unlikely that kojibiose will substantially promote the development of dental caries as much as sucrose and trehalose.

MATERIALS AND METHODS

Selection and culturing of representative oral strains. In total, 19 oral bacterial strains (Table 2) were selected for this study, including seven streptococcal strains that are known to be first colonizers of dental plaque. Four *Lactobacillus* strains are well evidenced for acid production and are found in both the healthy oral cavity and mature caries. Eight strict anaerobes are thought to be either commensals or oral pathogens, including *F. nucleatum*, which is suggested to be a backbone in dental plaque (40).

Bacterial culture conditions. Streptococci and lactobacilli were first grown on brain heart infusion (BHI) agar plates, while strict anaerobes were grown on blood agar supplemented with 5% horse blood (Oxoid, Hampshire, UK), 5 mg/ml of hemin (Sigma-Aldrich, Belgium), and 1 mg/ml of menadione (Sigma-Aldrich, Belgium). The plates were then incubated at 37°C in anaerobic jars containing AnaeroGen bags (Oxoid, Hampshire, UK). Grown colonies of streptococcal and *Lactobacillus* strains were subcultured twice in BHI medium (Sigma, Overijse, Belgium) in tubes, while the strict anaerobes in Hungate tubes (flushed with 10% CO₂ and 90% N₂ gas mixture) were then incubated at 37°C for 24 to 48 h accordingly.

Collection and preparation of donor saliva. To evaluate interindividual variability in sugar metabolism, bacteria from human saliva was used. Saliva contains pooled bacteria from distinct oral surfaces, including plaque, throat, buccal mucosa, and the tongue dorsum and therefore possesses considerable microbial diversity (45). Ethical clearance was approved by the Medical Ethical Committee of Ghent University with reference number BE6702018363. With informed consent, saliva was collected as has been previously described (46) from 11 healthy donors (8 women and 3 men) aged between 22 and 45 years with no medications and antibiotic use in the preceding 3 months. At least 2 h after eating or brushing teeth, the oral cavity was flushed with drinking water before collection.

Preparation of a chemically defined carbon-limited medium. In order to determine the metabolic potential of oral strains on sugar, a carbon-limited medium in which the sugar of interest was the only carbon source was made. A modified unbuffered medium was made as described previously (10, 47). Chemicals were purchased from Sigma (Bornem, Belgium) unless otherwise specified, and the compo-

sition per liter included 0.8 g $(\text{NH}_4)_2\text{SO}_4$, 0.6 g NaCl, 0.16 g $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.01 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 5.0 g KH_2PO_4 , 0.3 g L-cysteine hydrochloride, 0.3 g peptone water, and 0.018 g phenol red indicator. The media pH was adjusted to 7 and autoclaved, and then 20 ml of $50\times$ filter-sterilized vitamin mix (pH 7) was added, which, per liter, consisted of 0.002 g folic acid, 0.002 g biotin (D-biotin), 0.005 g riboflavin, 0.0001 g vitamin B₁₂, 0.005 g thiamine hydrochloride monohydrate, 0.005 g nicotinic acid, 0.005 g D-pantothenic acid hemicalcium salt, 0.005 g *p*-aminobenzoic acid, 0.001 g pyridoxine hydrochloride, and 0.005 g thioctic acid. Before the experiment, 1% (wt/vol) of each test sugar, kojibiose (Biocatalysis and Enzyme Engineering Unit, Ghent University), trehalose (Cargill B.V, Netherlands), sucrose, and xylitol (ICN Bio-medicals Inc., USA) were filter sterilized and added as sole carbon sources.

Inoculation and incubation of oral bacteria. Prior to inoculation, bacterial cells and saliva were harvested and washed twice with prerduced phosphate-buffered saline (PBS; pH 7.2) by centrifugation at $6,000 \times g$ for 5 min to strip off carryover nutrients from BHI and saliva. Flow cytometry was used to determine the cell density of pure strains and saliva as previously described (48). Two-milliliter volumes of medium containing each respective sugar were aliquoted into 24-well plates (Costar, Corning Inc., USA) before inoculating with 100 μl in triplicate of each bacterial strain at a final cell density of 10^6 cells/ml in the incubation medium. Dental plaque is a polymicrobial community; hence, we assembled four synthetic communities in order to determine the metabolic interactions between oral bacteria. The communities comprised of either (i) *Streptococcus* alone (7 strains); before mixing, each strain was set at 10^7 cells/ml given its acidogenic properties; (ii) *Lactobacillus* alone (4 strains); (iii) strict anaerobes (8 strains), with each strain set at 10^8 cells/ml; and (iv) all communities, which were composed of all the 19 strains together. Given the number and rapid sugar metabolism, a 10 times lower concentration of streptococcal strains (7 strains) was made to slow their dominance and the rapid drop in pH. With respect to incubations with the salivary microbiota from human origin, a 2-fold dilution was made to obtain enough inoculum but not normalized between donors to maintain interindividual variability. All the incubations were done at 37°C for 24 and 48 h in a microaerophilic condition, which was achieved by first flushing the anaerobic jar (schuett-biotec, Germany) with 10% CO₂ and 90% N₂ before adding 6% oxygen.

pH monitoring and determination of organic acid profile. At the end of each incubation, the pH in each well was measured (Consort SP28X, Turnhout, Belgium). Subsequently, the samples were filtered (0.22 μm) into 1.5 ml Eppendorf tubes and stored at -20°C until organic acid analysis. The organic acids (C₁ to C₅) from sugars were determined accordingly (49) on a 930 Compact IC Flex (Metrohm, Switzerland) ion chromatography system with an in-line bicarbonate removal (Metrohm CO₂ suppressor).

In silico genomic screening of glycoside hydrolases of individual pure strains. To infer carbohydrate utilization potential of our strains, genes associated with the glycoside hydrolase family of the enzymes that are primarily important for the hydrolysis of carbohydrates were identified using the Carbohydrate-Active enZymes (CAZy) database (<http://www.cazy.org/>) (28).

Impact of sugar on the salivary microbiome. (i) FastPrep DNA extraction. After incubations, samples were scraped from the bottom of the 24-well plate, transferred into tubes, pelletized at $13,000 \times g$ for 10 min, and stored at -20°C until needed. The extraction of DNA was performed accordingly (50). Briefly, pellets were disrupted in the PowerLyzer (Mo Bio Laboratories, Carlsbad, CA, USA) at 2,000 rpm for 5 min in a 1-ml lysis buffer containing 100 mM Tris pH 8, 100 mM EDTA pH 8, 100 mM NaCl, 1% polyvinylpyrrolidone (PVP40), 2% sodium dodecyl sulfate (SDS), and 200 mg of 0.1-mm glass beads (Sartorius). The supernatant was recovered by centrifugation at $13,000 \times g$ for 5 min, and then DNA was extracted following phenol-chloroform extraction. One volume of ice-cold isopropyl alcohol and 0.1 volume 3 M sodium acetate were used to precipitate DNA for 1 h at -20°C before centrifugation at $18,000 \times g$, 4°C for 30 min. Air-dried DNA was resuspended in 50 μl Tris-EDTA (TE) buffer and then stored at -20°C until needed. The quality of the extracted DNA was assessed on a 1% agarose gel.

(ii) Amplicon sequencing. Hypervariable V3 to V4 regions of the 16S rRNA gene libraries were prepared as previously described (51) and sequenced on an Illumina MiSeq platform (Illumina, Hayward, California) with v3 chemistry using primers 341F and 785Rmod (52). MiSeq standard operating procedure (SOP) (53) was used for assembling reads and cleaning prior to taxonomic phylotype assignment based on a naive Bayesian classifier and expanded Human Oral Microbiome Database (eHOMD 16S rRNA RefSeq version 15.2) (54). The clustering of contigs into operational taxonomic units (OTUs) was set at 97% sequence similarity. Using the PhyloSeq package (55) in R (v3.6.3) (56), the data set was then filtered to only consider the OTUs with sequences of relative abundance of $>0.1\%$ in at least one sample (51) before resampling of all the samples to equal the smallest read size of 8,421 sequences. The microbiome R package (57) was used to identify core microbiome of the donor saliva with a minimum prevalence of 90% and detection threshold of 0.1% relative abundance.

Data analysis. Statistical analysis was performed in R (v3.6.3) with differences among means assessed using analysis of variance (ANOVA) and considered significant at $P < 0.05$. The relative abundances were computed using the PhyloSeq package. The α -diversity within the saliva inoculum and sugar incubations was quantified using the Pielou's evenness, Shannon, and Simpson diversity indices (58). Constrained ordination based on Bray-Curtis distance was used to explore how treatment variables were associated with the changes in the microbial composition at the genus level. The adonis function in the vegan library based on 999 permutations was employed for testing the significance of this association following PERMANOVA (58) (code courtesy of Denef Lab tutorial; <http://deneflab.github.io/MicrobeMiseg/>). To identify the genera that were differentially abundant in sugars and the inoculum, the DESeq2 package (59) was used. The random forest classifier was applied to identify the top five genera that were most differentially abundant in the microbial community.

Data availability. The supporting data for this study have been provided within this article and its supplemental material. Raw sequences and the associated metadata are available in the NCBI BioProject database under accession number [PRJNA601417](https://www.ncbi.nlm.nih.gov/bioproject/PRJNA601417).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.5 MB.

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We also declare that there are no conflicts of interest.

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