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Review

Metabolomic approach to oral biofilm characterization—A future direction of biofilm research

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ABSTRACT

Research approaches to biofilm are stratified by a series of analyses: (1) microbial number and species; (2) microbial proteins, such as enzymes; and (3) microbial activity, such as metabolic activity. On the other hand, the hierarchical structure of biology includes the genome, proteome, and metabolome, in which the metabolome is the final output of biological function. Metabolome analysis is the comprehensive analysis of the metabolome, a new strategy for biological research in the 21st century. The stratified structure of biofilm research corresponds to the biological hierarchy, and the analysis of microbial activity, especially metabolic activity, is comparable to metabolome analysis; however, oral biofilm samples are too small to analyze the metabolome by conventional methods. Recently, a new device involving capillary electrophoresis (CE) and time-of-flight mass spectrometry (MS) has been developed, facilitating metabolomic investigation of the central carbon metabolic pathways (i.e., the EMP pathway, pentose phosphate pathway, and TCA cycle) in oral biofilm. Using CE–MS, we analyzed metabolome profiles of oral biofilm after oral rinsing with glucose *in vivo* and evaluated the effects of oral rinsing with fluoride and xylitol on the metabolome profiles of oral biofilm. The results were somewhat consistent with previous *in vitro* data obtained from single bacterial strains, namely, *Streptococcus* and *Actinomyces*; however, new information describing the metabolic properties of oral biofilm was also obtained. This metabolomic approach will reveal the functional characteristics of oral biofilm *in vivo*, potentially providing new insights into the nature of oral biofilm in health and disease.

Contents

1. Metabolome analysis: a new research approach to oral biofilm

The term "oral biofilm," also often referred to as dental plaque or the oral microbial community, refers to the complex of microorganisms in the oral environment and their nonmicrobial surroundings. Research approaches to oral biofilm are stratified as analyses of (1) microbial composition (recently termed the "microbiome"), (2) microenvironments, and (3) functions of the microbial community. Among them, the functions of the microbial community, such as metabolic activity, are crucial final outputs, because they directly relate to the pathogenicity of oral diseases (e.g., organic acids to dental caries, short fatty acids and sulfides to periodontal diseases, and sulfides and ammonia to halitosis) [1].

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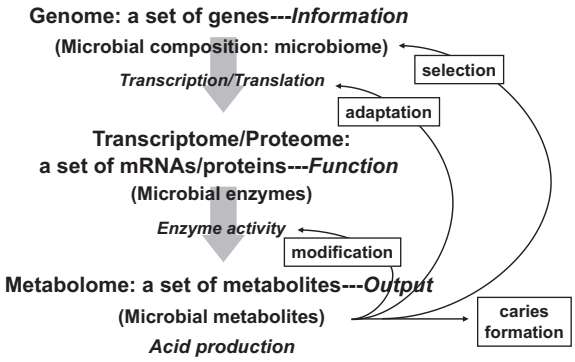

Fig. 1. Biological hierarchy from genome to transcriptome/proteome and metabolome, relevant to research strategies in cariogenic oral biofilm.

On the other hand, the presence of a biological hierarchy, beginning with the genome and culminating in the metabolome, has been proposed (Fig. 1). The genome is a set of genes that represents a series of information describing gene function. This set of genes can be transcribed into a set of messenger RNAs; these make up the transcriptome. The transcriptome is then translated into a set of proteins, or the proteome. The transcriptome and proteome represent the potential to function, that is, metabolic reactions catalyzed by enzyme activities. Then, a set of metabolites that make up the so-called metabolome is produced. The metabolome is the predominant final output of the biological hierarchy, and metabolome analysis or metabolomics is a new strategy for biological research in the 21st century.

The biological hierarchy seems to correspond to the stages of research into oral biofilm (Fig. 1). The genome is equivalent to the microbial composition (microbiome), and the transcriptome or proteome is like a list of microbial enzymes. The metabolome is the primary final output of the metabolism of oral biofilm and is also a crucial final output of oral biofilm, which directly relates to pathogenicity.

2. Metabolic activity: the final output of the biological hierarchy, which influences upper layers of the hierarchy

Furthermore, microbial metabolites or the metabolome can influence the upper layers of the biological hierarchy [1–3]. For example, in supragingival plaque biofilm, the final output is acid production, and this is directly associated with dental caries formation (Fig. 1).

The acids produced by supragingival plaque cause environmental acidification and subsequently influence microbial enzyme activities (acid-induced modifications of enzymatic activity) [4,5]. Transcription and translation are also regulated by environmental acidification (acid-induced adaptation through the induction of proteins/enzymes) [6–8]. In addition, the acidified environment causes a shift in microbial composition through microbial selection over acid stress (acid-induced selection of microorganisms). This cycle will continue as long as environmental acidification persists as a driving force.

Acid produced through microbial metabolism is a direct factor contributing to the development of dental caries. Furthermore, the acidic environment established by microbial metabolism is also an indirect factor contributing to dental caries, since caries activity can be enhanced through the acid-induced modification of enzymatic activity, adaptation of enzymes, and selection of microbial composition. Therefore, in order to elucidate the cariogenicity of supragingival plaque biofilm, it is not enough to analyze microbial composition (microbiome) (i.e., answer the question, "Who are they?"). Instead, elucidation of the metabolism of supragingival plaque biofilm *in vivo* (i.e., answer the question, "What are they doing there?") will be more crucial [9].

3. The central carbon metabolism in oral biofilm, derived from previous studies using a single bacterial species *in vitro*

The central carbon metabolism starts with classic glycolysis, the Embden–Meyerhof–Parnas (EMP) pathway, in which 1 molecule of glucose is degraded into 2 molecules of pyruvate, and under anaerobic conditions, pyruvate can further be degraded into lactate by the catalysis of lactate dehydrogenase and formate and acetate by pyruvate formate-lyase catalysis (*A* in Fig. 2). These pathways are shared by oral species of *Streptococcus*, *Actinomyces*, and *Lactobacillus*. In the presence of oxygen, pyruvate can be converted to acetate by the catalysis of pyruvate dehydrogenase or pyruvate oxidase in *Streptococcus* and *Lactobacillus* (*B* in Fig. 2), while lactate can be converted to acetate by the combination of lactate dehydrogenase and pyruvate oxidase in *Actinomyces* (*C* in Fig. 2). In the presence of bicarbonate, which is one of the salivary components, phosphoenolpyruvate can be converted to succinate with bicarbonate assimilation by phosphoenolpyruvate carboxylase and/or phosphoenolpyruvate carboxykinase, using part of the tricarboxylic acid (TCA) cycle (*D* in Fig. 2). This pathway is shared by *Actinomyces* [10–12].

These metabolic pathways have been elucidated using a single bacterial strain; however, although supragingival plaque biofilm consists mainly of oral species of *Streptococcus*, *Actinomyces*, and *Lactobacillus* [13,14], it is not clear whether these pathways function in supragingival plaque *in vivo*. In addition, there is no information on the metabolic function of the pentose-phosphate pathway and the rest of the TCA cycle in oral biofilm. However, the amount of supragingival plaque sampled from the oral cavity is too small to perform a metabolism study using conventional biochemical methods. In order to overcome this difficulty, metabolome analysis is an excellent candidate approach.

4. Metabolome analysis of oral biofilm using a combination of capillary electrophoresis and time-of-flight mass spectrometry (CE–MS)

Metabolome analysis is the comprehensive identification and quantification of metabolites in biological systems and is one of the most powerful approaches for metabolism research. Capillary electrophoresis (CE) combined with time-of-flight mass spectrometry (MS), or CE–MS, has been proven suitable to separate and quantify metabolites involved in the central carbon metabolism, including the EMP pathway, pentose-phosphate pathway, and TCA cycle [9,12,15,16]. CE is an excellent separator of ionized small molecules, such as metabolic intermediates, most of which are polar and ionic small molecules, while MS is an excellent technique for analysis of molecular mass. Using this system, it was possible to analyze as little as 10 mg of supragingival plaque and to identify and quantify most metabolic intermediates of the central carbon metabolism, as well as xylitol 5-phosphate formed by bacterial phosphorylation of xylitol (*E* in Fig. 2).

Table 1 shows the first metabolomic analysis of human supragingival plaque before and after a glucose rinse *in vivo* [12]. Five volunteers (periodontally healthy, with 0.40 ± 0.45 decayed teeth, not taking any antibiotics) were recruited to accumulate plaque by refraining from tooth brushing overnight. After confirming that the volunteers had not consumed any food for at least 2 h, supragingival plaque covering half the dentition of each volunteer was collected using sterilized

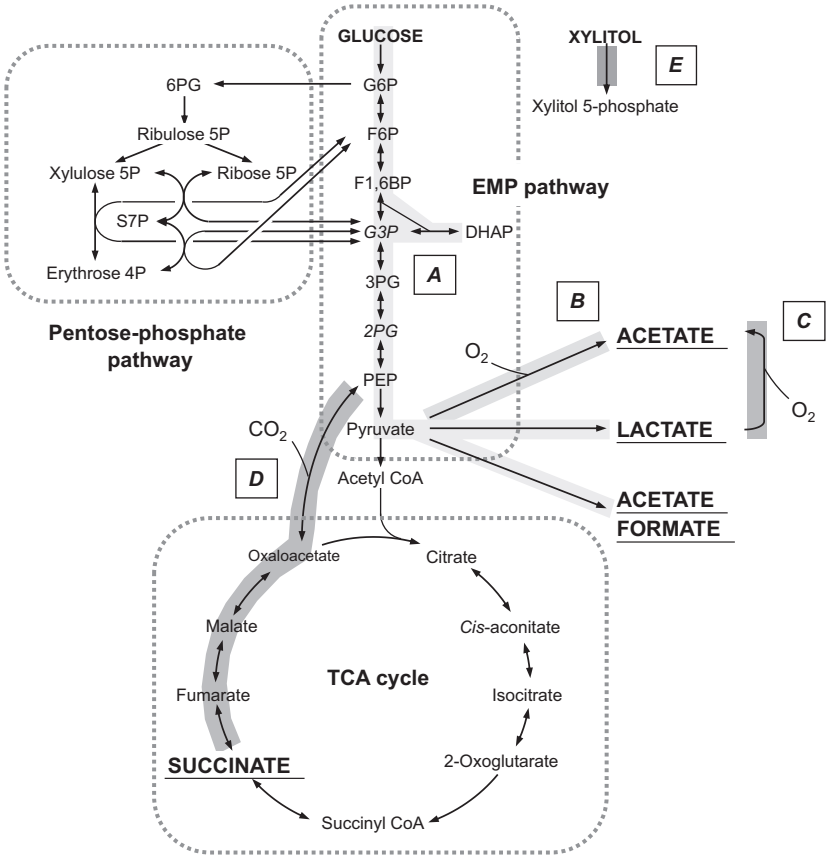

Fig. 2. The central carbon metabolism pathways, including the EMP pathway, pentose-phosphate pathway, and TCA cycle. *A*, metabolic pathways shared by oral *Streptococcus*, *Actinomyces*, and *Lactobacillus* (Glucose → Lactate, Acetate and Formate); B, metabolic pathway shared by oral *Streptococcus*, *Actinomyces*, and *Lactobacillus* under aerobic conditions (Pyruvate → Acetate); *C*, metabolic pathway shared by oral *Actinomyces* (Lactate → Acetate); *D*, metabolic pathways shared by oral *Actinomyces* in the presence of bicarbonate (PEP → Succinate); E, xylitol phosphorylation by *S. mutans*. G6P, glucose 6-phosphate; F6P, fructose 6-phosphate; F1,6BP, fructose 1,6-bisphosphate; DHAP, dihydroxyacetone phosphate; G3P, glyceraldehyde 3-phosphate; 2PG, 2-phosphoglycerate; 3PG, 3-phosphoglycerate; PEP, phosphoenolpyruvate; 6PG, 6-phosphogluconate; Ribulose 5P, ribulose 5-phosphate; Ribose 5P, ribose 5-phosphate; S7P, sedoheptulose 7-phosphate; E4P, erythrose 4-phosphate. Metabolites in italics were not detectable by the CE–MS system.

toothpicks. Consequently, the volunteers were asked to rinse with 10 mL of 10% glucose for 60 s, and 10 min after rinsing, supragingival plaque was collected from the rest of the dentition of each volunteer. Plaque samples were immediately immersed in ice-cold methanol after collection, and metabolites extracted from samples were analyzed by CE–MS. Analytical conditions for CE–MS are described in detail in the previous report [12].

This analysis demonstrated that the CE–MS method was capable of the comprehensive identification and quantification of metabolites in the central carbon metabolism, contained in a small amount of supragingival plaque. The results showed that supragingival plaque contained all the targeted metabolites in the central carbon metabolism, with no detection of erythrose 4-phosphate in the pentose-phosphate pathway and low detection of *cis*-aconitate and isocitrate in the TCA cycle (Table 1). After the glucose rinse, glucose 6-phosphate, fructose 6-phosphate, dihydroxyacetone phosphate, and pyruvate in the EMP pathway, as well as ribulose 5-phosphate and sedoheptulose 7-phosphate in the pentose-phosphate pathway increased significantly. There were also many variations in the levels of metabolic intermediates after the glucose rinse, although these were not statistically significant. Lactate also increased after the glucose rinse (Table 1). In addition, metabolome profiles found in supragingival plaque *in vivo* were basically similar to those obtained from *in vitro* metabolic experiments of representative oral bacteria, *Streptococcus mutans*, *Streptococcus sanguinis*, *Actinomyces naeslundii*, and *Actinomcyes oris* [12].

These results revealed that the EMP pathway, pentose-phosphate pathway, and TCA cycle function in supragingival plaque microbiota *in vivo*. Upon glycolytic activation, metabolites in the pentose-phosphate pathway also increased, indicating an associated regulation between the EMP and pentose-phosphate pathways. Furthermore, these metabolic pathways seemed to be the integration of metabolic pathways of representative plaque bacteria, namely, *Streptococcus* and *Actinomyces* [12], reflecting cohabitation of these bacteria in the supragingival plaque biofilm [13,14]. These results suggest that bacterial metabolic studies *in vitro* could be valid for the assessment of *in vivo* bacterial metabolic phenomena if the experimental conditions were well designed and that metabolomics approaches to microbiota, such as dental plaque, may give insight into the entire metabolic system and its mutual regulation within the microbiota.

5. Effects of xylitol and fluoride on the metabolome profile of oral biofilm *in vivo*

Fluoride and xylitol have been used worldwide as representative caries preventive reagents. Fluoride inhibits demineralization and promotes remineralization of the tooth surface [17,18] and is also known to inhibit bacterial acid production *in vitro* [19–21] and plaque acid production *in vivo* [22,23]. Previous *in vitro* studies, using oral streptococci, revealed that fluoride inhibits enolase, an enzyme in the EMP pathway [24–27]; however, the

Table 1
Metabolome profiles before and after rinsing with 10 mL of 10% glucose for 60 s in supragingival plaque *in vivo* (nmol/mg of wet weight of plaque) (modified from). Takahashi et al., 2010 [12]

Metabolite	Before glucose rinse	After glucose rinse
EMP pathway		
Glucose 6-phosphate	0.133 ± 0.002	0.442 ± 0.087*
Fructose 6-phosphate	0.033 ± 0.006	0.108 ± 0.025*
Fructose 1,6-bisphosphate	0.024 ± 0.010	0.099 ± 0.083
Dihydroxyacetone phosphate	0.037 ± 0.004	0.074 ± 0.011*
3-Phsophoglycerate	0.245 ± 0.165	0.218 ± 0.159
Phosphoenolpyruvate	0.094 ± 0.035	0.062 ± 0.037
Pyruvate	0.588 ± 0.461	4.236 ± 2.731**
Pentose phosphate pathway		
6-Phosphogluconate	0.008 ± 0.003	0.033 ± 0.017
Ribulose 5-phosphate	0.029 ± 0.014	0.054 ± 0.021*
Ribose 5-phosphate	0.011 ± 0.007	0.036 ± 0.028
Sedoheptulose 7-phosphate	0.058 ± 0.019	0.143 ± 0.041*
Erythrose 4-phosphate	nd	nd
TCA cycle		
Acetyl-CoA	0.020 ± 0.006	0.045 ± 0.019
Citrate	0.038 ± 0.031	0.017 ± 0.006
cis-Aconitate	0.002 ± 0.003	0.000 ± 0.009
Isocitrate	0.001 ± 0.002	nd
2-Oxoglutarate	0.013 ± 0.013	0.023 ± 0.012
Succinyl CoA	0.011 ± 0.018	0.019 ± 0.022
Succinate	1.834 ± 1.320	1.650 ± 1.001
Fumarate	0.034 ± 0.039	0.019 ± 0.019
Malate	0.105 ± 0.059	0.074 ± 0.044
Lactate	1.737 ± 0.823	13.124 ± 12.712*

EMP pathway, Embden–Meyerhof–Parnas pathway; TCA cycle, tricarboxylic acid cycle; nd, not detected; Succinyl CoA, succinyl coenzyme A.

* Significantly different from the amount of metabolites before glucose rinse ($p < 0.002$ by t test with Bonferroni correction).

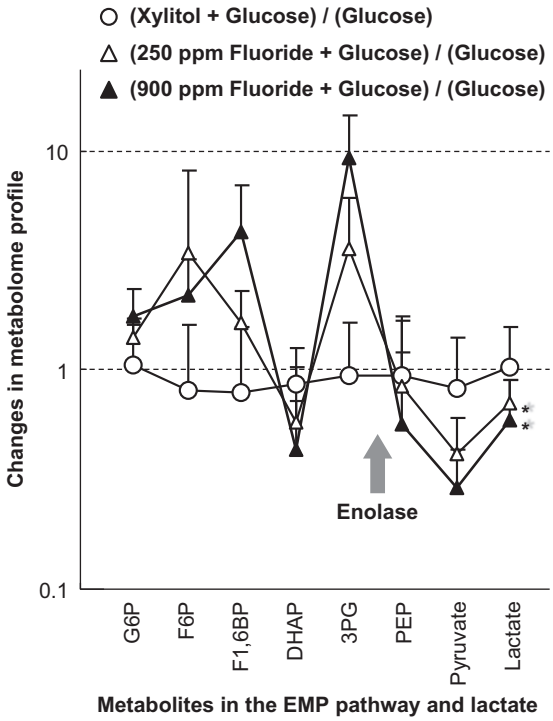

Fig. 3. Changes in metabolome profiles in the EMP pathway and lactate after rinsing with a glucose–xylitol mixture or sodium fluoride plus glucose (modified from Takahashi and Washio, 2011 [34]). Changes are expressed as the ratio of the amount (nmol/mg wet weight of plaque) of metabolites and lactate after rinsing with a glucose–xylitol mixture or fluoride (225 or 900 ppm F^-) plus glucose to that of metabolites after rinsing with glucose. *, Significantly different from the amount of metabolites present after rinsing with glucose ($p < 0.017$ by t-test with Bonferroni correction).

mechanism through which fluoride exerts its inhibitory activity has not been confirmed in oral biofilm, which consists of multiple species of bacteria *in vivo*.

On the other hand, xylitol, a nonfermentative sugar alcohol, does not cause dental caries, similarly to other sugar alcohols. Xylitol is known to repress the production of acid from glucose by *S. mutans* through the inhibition of glycolytic enzymes by xylitol 5-phosphate, an enzyme produced from xylitol through a constitutive phosphoenolpyruvate–fructose phosphotransferase system (*E* in Fig. 2) [28–31]. Furthermore, xylitol 5-phosphate is dephosphorylated and returned to xylitol, resulting in the formation of a "futile cycle," an energy-wasting cycle, and subsequent repression of *S. mutans* growth [32,33]. Although it is true that xylitol does not cause dental caries, the mechanism through which xylitol exerts its inhibitory has not been confirmed in oral biofilm *in vivo*.

Seven volunteers (periodontally healthy, with 0.14 ± 0.38 decayed teeth, not taking any antibiotics) were recruited to accumulate plaque, and supragingival plaque covering half the dentition of each volunteer was collected as described in Section 4. Subsequently, the volunteers were asked to rinse with 10 mL of 10% glucose, a 10% glucose-10% xylitol mixture, or sodium fluoride (225 or 900 ppm F^-) for 60 s. After 10 min, volunteers who had rinsed with glucose or the glucose–xylitol mixture were subjected to supragingival plaque sampling from the rest of the dentition. The volunteers who had rinsed with sodium fluoride were asked to rinse with 10 mL of 10% glucose for 60 s, and after 10 min, supragingival plaque was collected from the rest of the dentition of each of these volunteers as well. Plaque samples were treated and analyzed as described in Section 4, except for xylitol 5-phosphate [34].

Fig. 3 shows the effects of xylitol and fluoride on glucose metabolism in supragingival plaque *in vivo*. The effects of these dental washes were evaluated by determining changes in the amounts of metabolites and lactate in supragingival plaque. Changes were expressed as the ratio of the amount of metabolites after rinsing with the glucose–xylitol mixture or fluoride plus glucose to the amount of metabolites after rinsing with glucose.

When xylitol was added to glucose, there was no significant change in the metabolite profile or lactate production in supragingival plaque *in vivo* (Fig. 3). Xylitol 5-phosphate was detected at a significant level only after rinsing with the glucose–xylitol mixture [34], as previously reported [28], supporting that xylitol 5-phosphate is produced *via* a bacterial phosphoenolpyruvate-dependent phosphotransferase system [28,30,31]. However, the presence of xylitol 5-phosphate does not seem to influence glucose fermentation by supragingival plaque *in vivo*, although it is unknown whether such xylitol 5-phosphate formation may be involved in the "futile cycle." This may be because dental plaque covering the clinically healthy tooth surface contains only a small number of mutans streptococci [35,36] or because xylitol 5-phosphate is not as effective *in vivo* as *in vitro*. It seems that the role of xylitol in caries prevention is as a nonfermentative sugar substitute, since no lactate production from xylitol and no effect of xylitol on the metabolome profile were observed.

On the other hand, when fluoride was added to the oral cavity before glucose rinsing, 3-phosphoglycerate accumulated, suggesting that fluoride inhibited this metabolic step, catalyzed by the glycolytic enzyme enolase, which catalyzes the production of phosphoenolpyruvate (arrow in Fig. 3) [34]. Lactate production was also decreased significantly. This phenomenon is similar to the well-known fluoride-based inhibition of enolase, which has been demonstrated through *in vitro* experiments in *S. mutans* and *S. sanguinis* [25,27].

In the presence of fluoride, an increase in glucose 6-phosphate, fructose 6-phosphate, and fructose 1,6-bisphosphate and a decrease in dihydroxyacetone phosphate were also observed (Fig. 3), indicating the inhibition of aldolase, glyceraldehyde 3-phosphate dehydrogenase, and/or phosphoglycerate kinase in the EMP pathway, although there are no reports demonstrating this type of inhibition in either *S. mutans* or *S. sanguinis* [25,27]. Further studies are needed to clarify this phenomenon by investigating other plaque bacteria.

These series of results suggest the favorable potential of metabolome analysis in the investigation of the mechanisms through which medicinal agents (such as fluoride) and dietary ingredients (such as xylitol) influence the metabolic aspects of oral biofilm.

6. Conclusions: metabolomic approaches may answer the questions, "What are they doing?", "How are they controlled?", and more

In oral biofilm research, the analysis of microbial composition (the microbiome) is still the main paradigm, and, along with the progress of metagenomic technology, microbial analysis will eventually provide a comprehensive answer to the question, "Who are they?" However, most oral microorganism-mediated diseases, such as dental caries, periodontal disease, and oral malodor, are initiated and promoted by microbial metabolic activity, not only by the presence of specific microorganisms; for example, dental caries occurs under acidic conditions established by bacterial acid production, irrespective of the bacterial species. Thus, strategies to prevent these diseases do not necessarily need to involve killing or eliminating these microorganisms, but rather controlling their metabolic activity and consequently their growth and pathogenicity. Metabolome analysis, capable of analyzing the metabolic characteristics of oral biofilm, such as metabolic pathways and metabolic regulatory mechanisms *in vivo*, may answer the questions, "What are they (the microbiota) doing?" and "How are they controlled?" As described in this review, metabolic approaches to dental caries have been developed; however, those for periodontitis and oral malodor have not yet been established, probably due to difficulties in the identification of metabolites relevant to metabolic pathways, such as amino acids and amino sugars. Further development of analytical techniques and metabolite databases, as well as basic research for microbial metabolic pathway, is needed.

It has also become more important to understand the whole physiological function of oral biofilm, e.g., how the balance of oral biofilm is maintained (symbiosis) and how the balance shifts toward the pathogenic condition (dysbiosis) [1–3,37]. Metabolome analysis is a powerful tool to probe the metabolic characteristics of the entire oral biofilm, and this may provide new insights into the nature of oral biofilm in health and disease. Furthermore, metabolome analysis may enable us not only to characterize the metabolic properties of oral biofilm *in vivo*, but also to elucidate drug efficacy *in vivo*, from classical cariostatic fluoride to new-generation oral disease-preventive agents, and to identify new biomarkers relevant to oral diseases.

These considerations may lead us to the hypothesis that oral biofilm consists of a tremendous number of microorganisms, but functions as one organism or a as multicellular system. This is a realistic, reasonable hypothesis because it is known that microorganisms present in oral biofilm live together through intermicroorganism communication, including co-adhesion/aggregation, metabolic interactions, bacteriocin release, quorum sensing, gene exchange, etc. [38–41], and cooperate and compete through adaptation to and selection by environmental alteration, such as nutrition, pH, and reduction–oxidation potential, and even through modification of the environment [1–3,10,37,42–44]. Recently, it has been suggested that humans are a human-microbiota hybrid, where host tissue and symbiotic microorganisms function together and influence each other, a so-called "superorganism" [45]. With this concept, the metabolomic approach may open a new era in the research of symbiotic microbiota, including oral biofilm, and metabolome analysis is just the beginning.

Conflict of interest

The authors declare no potential conflicts of interest with respect to the authorship and/or publication of this review article.

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