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SHORT COMMUNICATION

Salivary mucins promote the coexistence of competing oral bacterial species

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Mucus forms a major ecological niche for microbiota in various locations throughout the human body such as the gastrointestinal tract, respiratory tract and oral cavity. The primary structural components of mucus are mucin glycoproteins, which crosslink to form a complex polymer network that surrounds microbes. Although the mucin matrix could create constraints that impact inhabiting microbes, little is understood about how this key environmental factor affects interspecies interactions. In this study, we develop an experimental model using gel-forming human salivary mucins to understand the influence of mucin on the viability of two competing species of oral bacteria. We use this dual-species model to show that mucins promote the coexistence of the two competing bacteria and that mucins shift cells from the mixed-species biofilm into the planktonic form. Taken together, these findings indicate that the mucus environment could influence bacterial viability by promoting a less competitive mode of growth.
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Mucus lines wet epithelia throughout the human body and is a major ecological niche for microbiota in the respiratory tract, gastrointestinal tract and oral cavity among other locations (Tabak, 1995; Worlitzsch *et al.*, 2002; Derrien *et al.*, 2010). The mucus layer is a three-dimensional hydrogel primarily composed of densely glycosylated polymers called mucins (Tabak *et al.*, 1982; Bansil and Turner, 2006). In the gel, mucin chains crosslink to form a network that surrounds microbes and, consequently, could create geometric and diffusive constraints for biotic and abiotic environmental factors. Little is known, however, about how these constraints influence microbial interactions, such as cell-cell communication and competition, among the vast number of organisms that live in mucus. In this study, we build upon our previous work showing that MUC5B mucins affect intraspecies interactions by promoting dispersal of bacteria and fungi (Caldara *et al.*, 2012; Kavanaugh *et al.*, 2014; Frenkel and Ribbeck, 2015). Here we develop an experimental model to probe the influence of gel-forming human salivary mucins on dual-species bacterial competition to understand how this matrix affects the viability of competing bacteria.

The dual-species model is composed of *Streptococcus sanguinis* JFP36 and *S. mutans* UA159, which compete in the oral cavity through the production of hydrogen peroxide and antimicrobial peptides called mutacins, respectively (Kreth *et al.*, 2005, 2008; Ge *et al.*, 2008; Senty Turner *et al.*, 2009). In this model, the two species were inoculated sequentially to more closely mimic the natural environment of the oral cavity where surfaces are generally coated by microbes before other species attempt to colonize. When *S. mutans* was the primary colonizer, and MUC5B mucins were not present in the growth medium, viability of the secondary colonizer (*S. sanguinis*) rapidly declined, suggesting that *S. mutans* outcompetes *S. sanguinis* (Figure 1A (I, II)). In contrast, when MUC5B was present in the growth medium, the total number of viable *S. sanguinis* cells increased by 18- and 88-fold after 4 and 5 h of co-culture, respectively, compared with the control without mucin (Figure 1A (II)). The same protective effect by MUC5B was observed when *S. sanguinis* was the primary colonizer. In this case, the addition of MUC5B to medium enhanced survival of both *S. sanguinis* and *S. mutans* (Figure 1B (I, II)). After 4 and 5 h of co-culture in the presence of MUC5B, the number of viable *S. sanguinis* cells increased by 9- and 94-fold, respectively, relative to the control (Figure 1B (I)). *S. mutans* CFU increased by 2-, 3-, and 7-fold at 4, 5 and 6 h, respectively (Figure 1B (II)). Of note is that, although MUC5B significantly enhanced *S. sanguinis* viability, there was an overall reduction in *S. sanguinis* CFU due to self-killing, which could be caused by increasing hydrogen peroxide concentrations. The same reduction in viability was

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observed when *S. sanguinis* was grown in monoculture, indicating that the killing was not due to *S. mutans* (Supplementary Figure 1). Methylcellulose, a gel-forming polymer that is commonly used to mimic the viscosity of mucus, did not have the same protective effect on *S. mutans* or *S. sanguinis* in both of these experimental models; the enhanced survival is likely not due to the addition of a polymer, which could increase viscosity or cause osmotic stress (Supplementary Figure 2) (Ivic *et al.*, 2002; Smith *et al.*, 2009). Together, these results show that MUC5B significantly enhances bacterial diversity by increasing survival of at least one bacterial population compared with the control without MUC5B (Figures 1C and D).

To better understand how MUC5B influences bacterial viability, we studied several aspects of *S. mutans* and *S. sanguinis* growth in the presence of MUC5B. First, we determined that the observed protective effect of MUC5B was not due to increased bacterial growth; MUC5B slightly reduces or has no effect on *S. mutans* and *S. sanguinis* growth rates (Figures 2A and B). In addition, *S. mutans* and *S. sanguinis* did not grow in PBS containing mucin, indicating that MUC5B is not used as a nutrient source under the conditions studied (Figures 2A and B). Another way MUC5B could increase bacterial survival is by altering the cells' mode of growth, which can influence interspecies competition and cell properties such as gene regulation and cell-cell communication (O'Toole and Kolter, 1998; Pratt and Kolter, 1998; Prigent-Combaret *et al.*, 1999; Kearns *et al.*, 2005; Oliveira *et al.*, 2015; Schluter *et al.*, 2015). Our data show that MUC5B efficiently reduces *S. mutans* and *S. sanguinis* biofilm formation in single-species cultures at 6 h and 9 h:

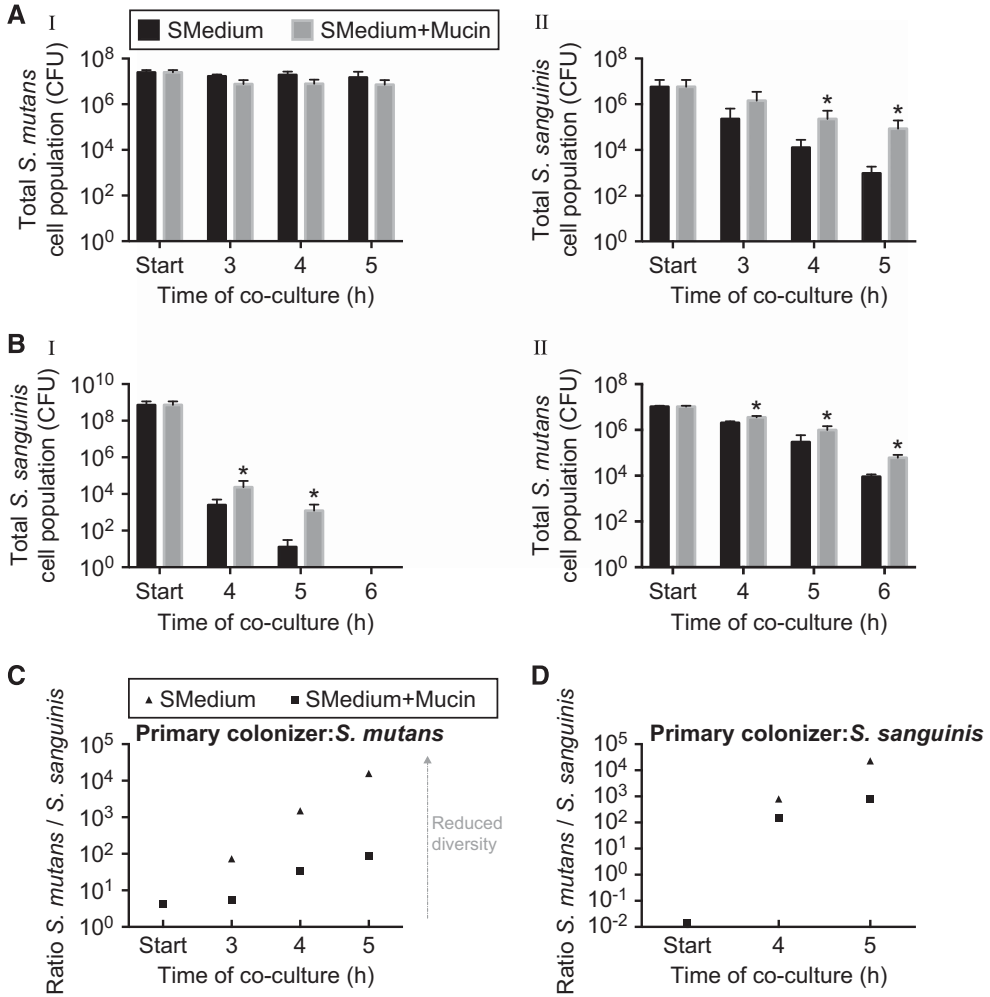

Figure 1 MUC5B promotes *S. mutans* and *S. sanguinis* coexistence, which leads to increased bacterial diversity. *S. mutans* and *S. sanguinis* viability in a dual-species experimental model containing control medium (half-strength BHI with 1% sucrose; SMedium) and control medium containing 0.4% MUC5B mucin. Viability was studied when *S. mutans* was the primary colonizer (**A** (I)) and *S. sanguinis* was the secondary colonizer (**A** (II)) and the reverse scenario where *S. sanguinis* was the primary colonizer (**B** (I)) and *S. mutans* was the secondary colonizer (**B** (II)). (**C, D**) the ratios of viable *S. mutans* and *S. sanguinis* when *S. mutans* was the primary colonizer (**c**) and when *S. sanguinis* was the primary colonizer (**D**) as an indication of species diversity. *, statistically significant increase relative to the control with half-strength BHI containing 1% sucrose determined by Student's *t*-test ($P < 0.05$). Experiments were performed in triplicate and error bars represent s.d. of CFU between replicates.

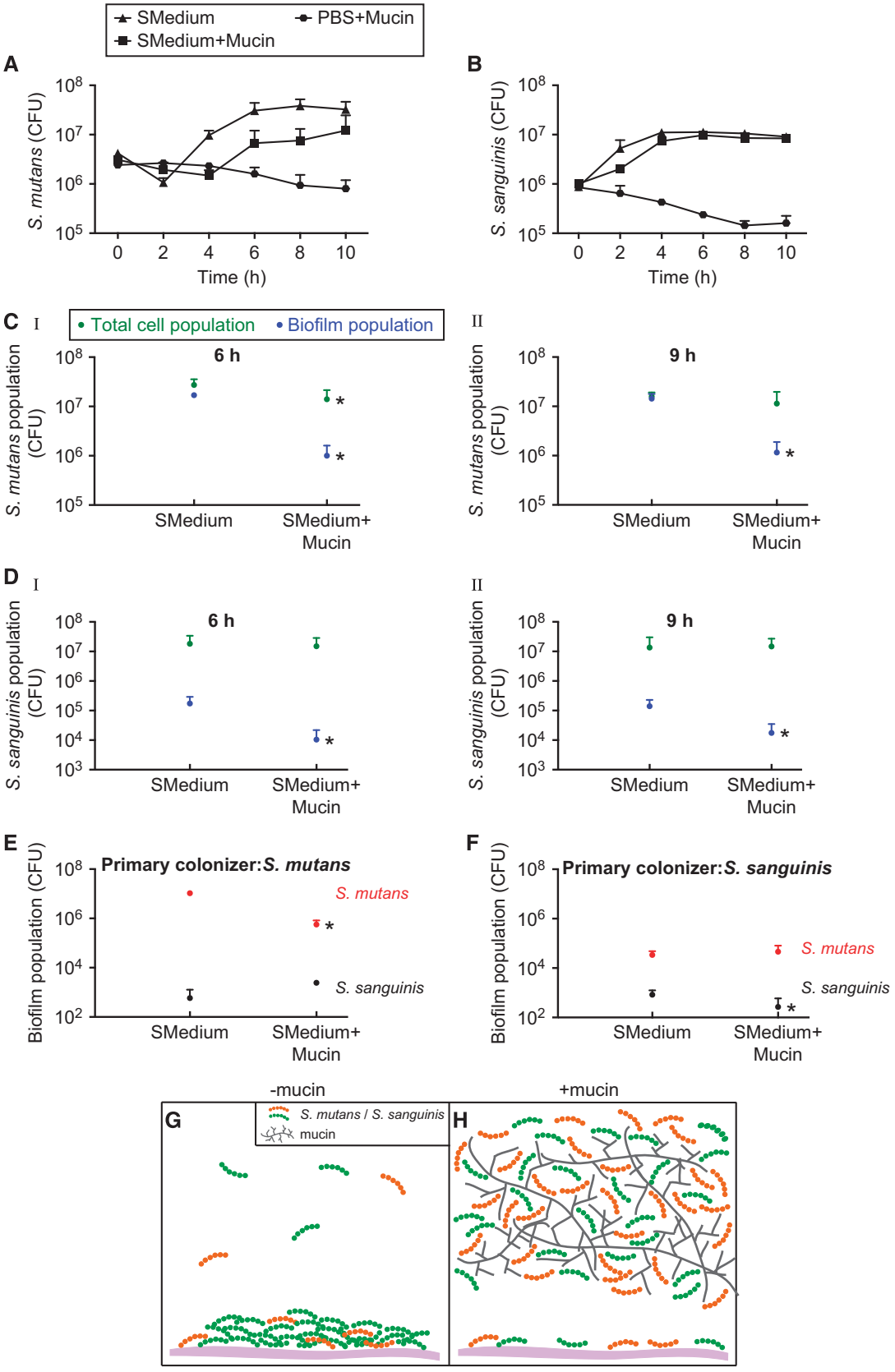
SMedium
SMedium+Mucin
PBS+Mucin
A
S. mutans (CFU)
Time (h)
B
S. sanguinis (CFU)
Time (h)
C
I
II
Total cell population
Biofilm population
6 h
9 h
S. mutans population (CFU)
SMedium
SMedium+ Mucin
D
S. sanguinis population (CFU)
E
Primary colonizer: S. mutans
Biofilm population (CFU)
S. mutans
S. sanguinis
F
Primary colonizer: S. sanguinis
-mucin
+mucin
G
H
S. mutans / S. sanguinis
mucin

Figure 2 MUC5B reduces *S. mutans* and *S. sanguinis* surface attachment by shifting cells into the planktonic state. *S. mutans* (**A**) and *S. sanguinis* (**B**) growth in control medium (half-strength BHI with 1% sucrose; SMedium), control medium containing 0.4% MUC5B mucin, and PBS containing 0.4% MUC5B mucin. (**C** (I, II)) *S. mutans* biofilm population and total cell population in mono-species cultures containing control medium and control medium with 0.4% MUC5B mucin at 6 h (**C** (I)) and 9 h (**C** (II)). (**D** (I, II)) *S. sanguinis* biofilm population and total cell population in mono-species cultures containing control medium and control medium with 0.4% MUC5B mucin at 6 h (**D** (I)) and 9 h (**D** (II)). (**E, F**) *S. mutans* and *S. sanguinis* biofilm formation in control medium and control medium containing 0.4% MUC5B mucin in a dual-species model after 4 h of co-culture when *S. mutans* was the primary colonizer (**E**) and when *S. sanguinis* was the primary colonizer (**F**). (**G, H**) schematic illustrating a summary of conclusions. In the absence of MUC5B (**G**), biofilm formation of *S. mutans* and *S. sanguinis* increases and bacterial coexistence decreases compared with biofilm formation and species coexistence in the presence of mucin (**H**). *, statistically significant decrease relative to the control with half-strength BHI containing 1% sucrose determined by Student's t test ($P<0.05$). Experiments were performed in triplicate and error bars represent s.d. of CFU between replicates.

S. mutans biofilm formation was reduced by 17- and 12-fold, and *S. sanguinis* biofilm formation decreased 16- and 8-fold compared with the control without mucin (Figures 2C (I, II) and D (I, II)). Strikingly, the total *S. mutans* and *S. sanguinis* cell populations were unchanged at all time points in the presence of MUC5B (except for a slight decrease at 6 h, but the number of cells in the biofilm at this time still account for only 13% of total cells) (Figures 2C (I, II) and D (I, II)). This result implies that, in the presence of MUC5B, the vast majority of cells shifted into the planktonic state. Because the single cell bacterial form can be less competitive than the surface-attached state, this movement of cells away from the biofilm could be a mechanism to reduce interspecies competition. After 4 h of co-culture in the dual-species model, we found that MUC5B also reduced biofilm formation of the primary colonizer in the mixed biofilm by 19-fold for *S. mutans* and 3-fold for *S. sanguinis* relative to the control without polymer (Figures 2E and F). As shown in Figures 1A (I) and B (I), the total cell population in each of these cases was unaffected (*S. mutans*) or increased (*S. sanguinis*) by MUC5B, indicating a decrease in the relative proportion of biofilm cells. In the case of the secondary colonizer, there was also an overall reduction in the proportion of biofilm cells for both *S. mutans* and *S. sanguinis*; the total number of viable cells increased in the presence of MUC5B (Figures 1A (II) and B (II)), yet there was only a slight increase or no change in biofilm formation in the presence of MUC5B relative to the control without polymer (Figures 2E and F). Taken together, these results indicate that MUC5B could enhance bacterial coexistence and, ultimately, bacterial diversity, by shifting competing species away from the biofilm and into the less competitive planktonic state.

In this work, we use a dual-species bacterial model containing human MUC5B salivary mucin to understand how this prevalent environmental factor influences bacterial viability. Our results show that: (1) MUC5B promotes *S. mutans* and *S. sanguinis* coexistence, and (2) MUC5B shifts cells from the biofilm into the planktonic state (Figures 2G and H). By promoting the single-cell (planktonic) state, MUC5B could alter cell-cell interactions, toxin production, or other mechanisms of competition. Although this model is not as complex as the oral cavity microbiome, these findings are among the first to indicate that mucus and its primary structural component, mucins, could influence bacterial survival in a multispecies environment. Further studies are needed, however, to understand if the observed increase in bacterial survival and reduction in surface colonization in the presence of mucin are due to an indirect influence of mucin, such as altered transport of secreted factors, or a direct impact on bacterial physiology, which could change gene regulation.

Conflict of Interest

The authors declare no conflict of interest.

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