**Supplementary Material**

# ***Streptococcus mutans* adhesion force sensing in multi-species oral biofilms**

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**Supplementary Table 1** Zeta potentials measured in a calcium-phosphate buffer (1 mM $CaCl_2$, 2 mM potassium phosphate, 50 mM KCl, pH 6.8) of the three bacterial strains involved in this study, demonstrating similar zeta potentials of live and dead *S. oralis* J22 and *A. naeslundii* T14V-J1 (no significant difference at $P > 0.05$, Mann-Whitney test). ± signs represent standard deviations (SD) over triplicate experiments.

| **Strains** | **Live/dead** | **Zeta potential (mV)** |
|---|---|---|
| *S. mutans* UA159 | live | -2.8 ± 0.3 |
| *S. oralis* J22 | live | -8.4 ± 0.2 |
| | dead | -8.7 ± 0.5 |
| *A. naeslundii* T14V-J1 | live | -4.6 ± 0.3 |
| | dead | -5.3 ± 0.5 |

**Supplementary Table 2** Primer sequences of the genes used in this study.

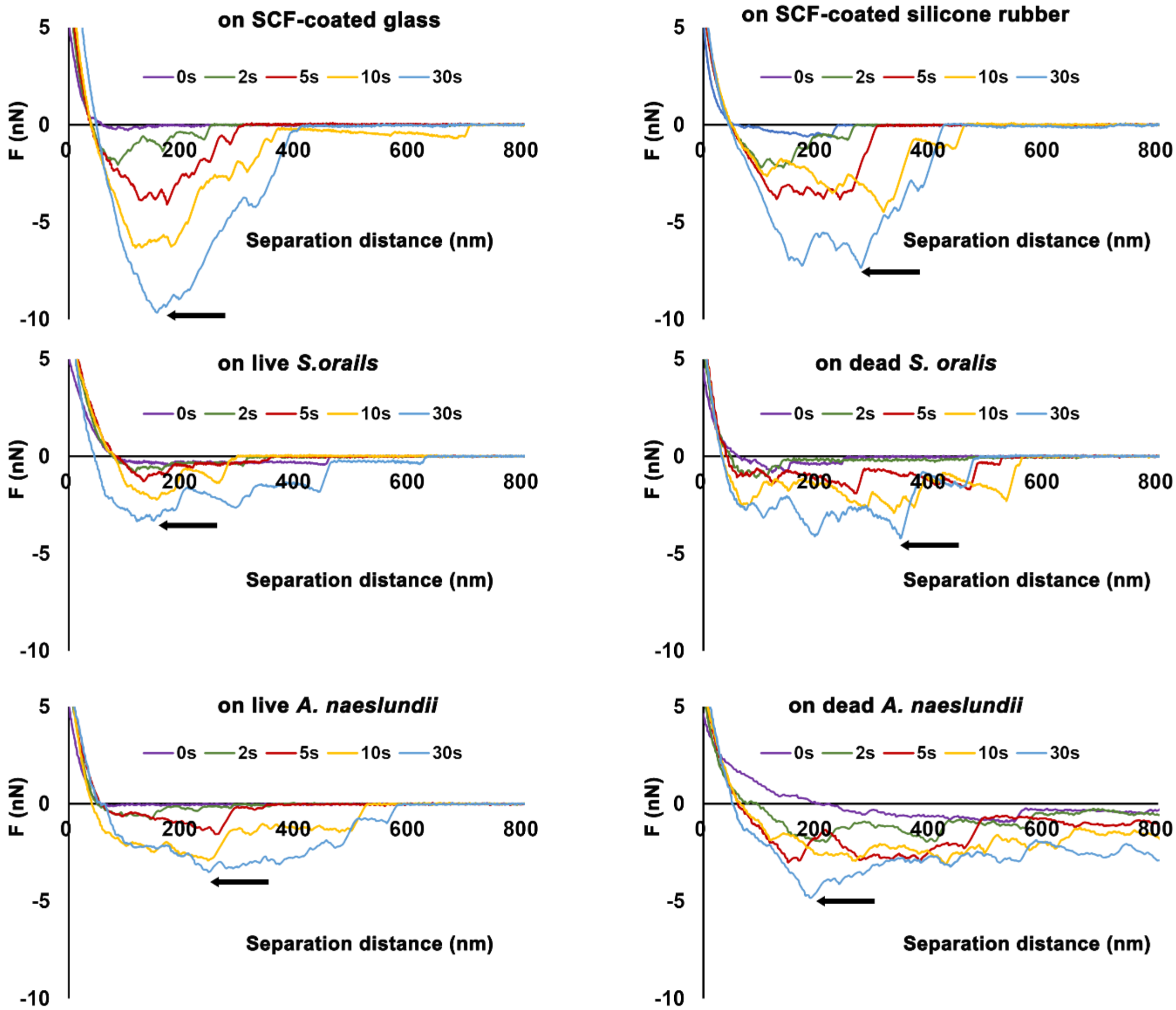
| Gene | Primer sequence (5′ to 3′) | |
|---|---|---|
| | forward | reverse |
| *16S rRNA* | ACCAGAAAGGGACGGCTAAC | TAGCCTTTTACTCCAGACTTTCCTG |
| *brpA* | GGAGGAGCTGCATCAGGATTC | AACTCCAGCACATCCAGCAAG |
| *gbpB* | ATACGATTCAAGGACAAGTAAG | TGACCCAAAGTAGCAGAC |
| *comDE* | ACAATTCCTTGAGTTCCATCCAAG | TGGTCTGCTGCCTGTTGC |

**Calculation of initial adhesion forces, characteristic bond maturation times and stationary adhesion forces**

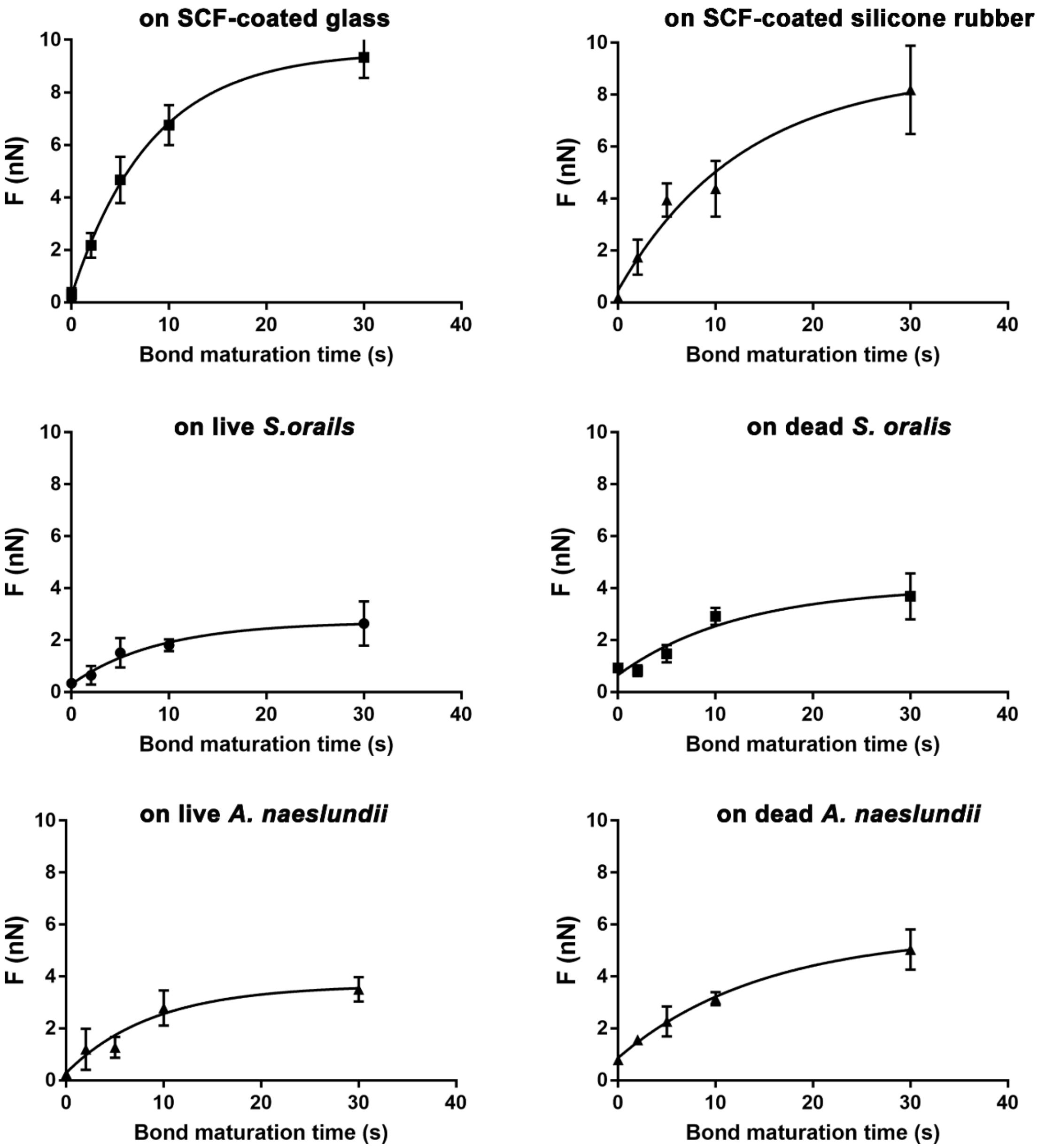
Adhesion forces have been described[1] to increase with contact time between the interacting according to an exponential function

$$F_t = F_0 + (F_{stationary} - F_0)(\exp(-\frac{t}{\tau})) \quad (1)$$

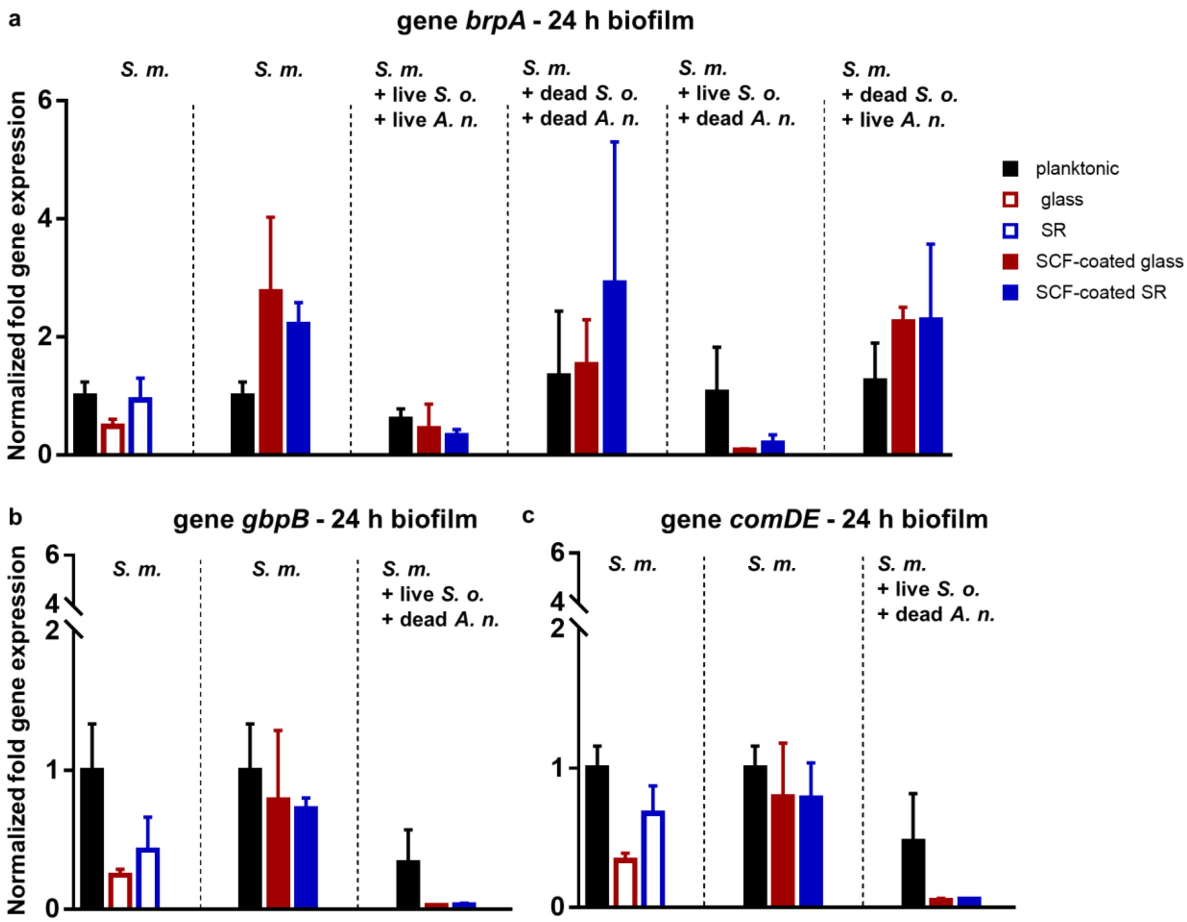
in which $F_t$ is the adhesion force after bond maturation time t, $F_0$ is the initial adhesion force at 0 s bond maturation time and $F_{stationary}$ indicates the stationary adhesion force reached after complete bond maturation, while $\tau$ is the characteristic time constant for bond maturation. Least-square fitting of $F_0$, $\tau$ and $F_{stationary}$ to the adhesion forces measured as a function of time was applied for their calculation.




**Supplementary Fig. 1** Examples of retraction force-distance curves taken after different bond maturation times for a *S. mutans* UA159 probe on SCF-coated glass (left) and SCF-coated silicone rubber (right) surfaces, on live *S. oralis* J22 and *A. naeslundii* T14V-J1 cell surfaces (left) and on dead *S. oralis* J22 and *A. naeslundii* T14V-J1 cell surfaces (right). The arrow points to the most-negative force value, taken as the adhesion force for a given curve.




**Supplementary Fig. 2** *S. mutans* UA159 adhesion forces as a function of bond maturation time on SCF-coated glass (left) and SCF-coated silicone rubber (right) surfaces, on live *S. oralis* J22 and *A. naeslundii* T14V-J1 cell surfaces (left) and on dead *S. oralis* J22 and *A. naeslundii* T14V-J1 cell surfaces (right). Error bars represent standard deviations (SD) over example measurements, each taken on five contact points with one *S. mutans* probe.



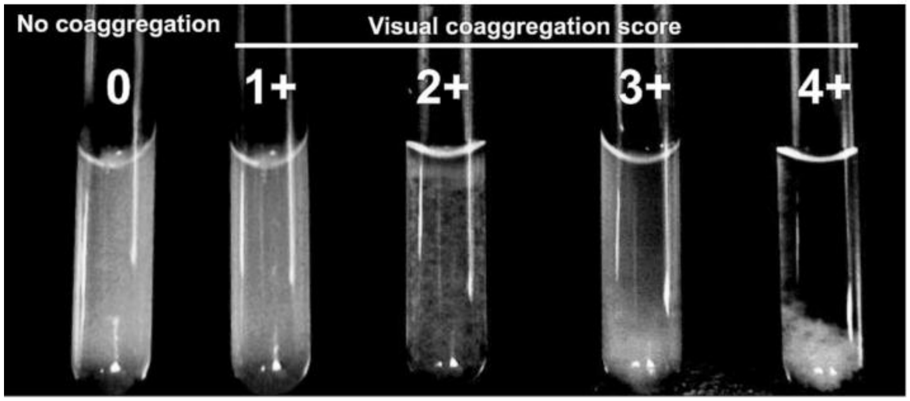

**Supplementary Fig. 3 Gene expression in 24 h *S. mutans* UA159 (*S. m.*) mono-species biofilms and different triple-species oral biofilms**. Biofilms comprised of *S. mutans* UA159 (*S. m.*) in combination with live or dead *S. oralis* J22 (*S. o.*) and *A. naeslundii* T14V-J1 (*A. n.*) on different substratum surfaces in the absence and presence of a salivary conditioning film.

**a** *brpA* gene expression

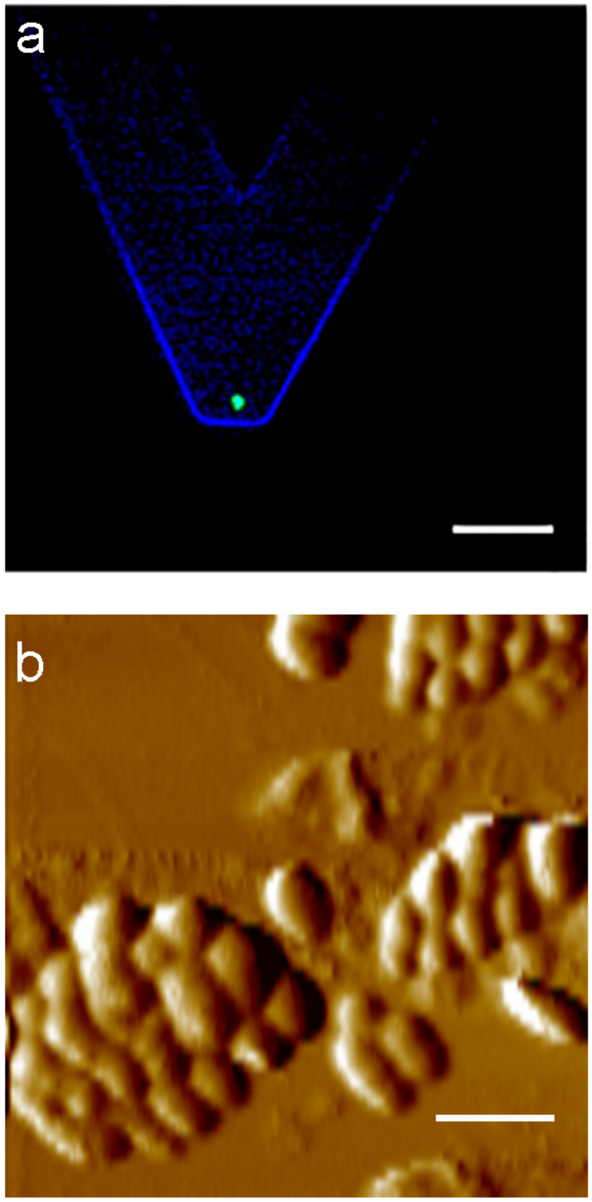
**b** *gbpB* gene expression

**c** *comDE* gene expression.

Error bars represent SD values over triplicate experiments with separately grown biofilms. Data on *brpA*, *gbpB* and *comDE* expressions in *S. mutans* UA159 mono-species biofilms on glass and silicone rubber surfaces in absence of a salivary conditioning film were taken from Wang et al.[2]




**Supplementary Fig. 4** Visual aggregation assay showing different coaggregation scores. Score 0: no change in turbidity and no visible co-aggregates; Score 1: weak coaggregation with dispersed aggregates in a turbid background; Score 2: clearly visible, small co-aggregates, not settling immediately; Score 3: large settling co-aggregates, leaving a slightly turbid suspension; Score 4: maximum coaggregation large co-aggregates settled immediately leaving a fully clear supernatant.(Taken from K. R. Min, M. N. Zimmer & A. H. Rickard (2010) Physicochemical parameters influencing coaggregation between the freshwater bacteria *Sphingomonas natatoria* 2.1 and *Micrococcus luteus* 2.13, Biofouling, 26:8, 931-940, DOI: 10.1080/08927014.2010.531128 and reprinted with permission from Taylor & Francis Ltd., https://pubmed.ncbi.nlm.nih.gov/21058055).




**Supplementary Fig. 5 Demonstration of single bacterial probe use.**

**a.** Fluorescence image of a single LIVE/DEAD stained *S. mutans* UA159 bacterium adhering on a tipless AFM cantilever. Green fluorescence demonstrates bacterial viability. Scale bar represents 20 μm.

**b.** Verification of the absence of double-contour lines upon imaging *S. oralis* adhering to a glass surface, using a *S. mutans* probe. Scale bar represents 2 μm.

## References

1. Busscher, H. J., Norde, W., Sharma, P. K. & Van der Mei, H. C. Interfacial re-arrangement in initial microbial adhesion to surfaces. *Curr. Opin. Colloid. Interf. Sci.* **15,** 510-517 (2010).
2. Wang, C., Hou, J., Van der Mei, H. C., Busscher, H. J. & Ren, Y. Emergent properties in *Streptococcus mutans* biofilms are controlled through adhesion force sensing by initial colonizers. *mBio* **10,** e01908-19 (2019).