A: POLYMICROBIAL/MICROBIOME; **B:** MECHANISMS/REGULATION;

TITLE: Viable but non-culturable state: a strategy for *Staphylococcus aureus* survivable in dual-species biofilms with *Pseudomonas aeruginosa*

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While considerable research has focused on studying individual-species, we now face the challenge of determining how interspecies interactions alter bacterial behaviours and pathogenesis. Of particular interest is the ecological behaviour between *P. aeruginosa*-*S. aureus*, since microbial communities containing both pathogens can display enhanced virulence. Curiously, their interaction is often reported as competitive under laboratory conditions.

Here we investigate the interaction between *P. aeruginosa*-*S. aureus* in a biofilm co-culture model. The growth of 24- and 48h-old dual-species biofilms was monitored by CFU and flow-cytometry counting, and the gene expression profile inspected by qPCR.

Plating experiments showed that *S. aureus* growth significantly decreased from 24- up to 72-h of growth in dual-species biofilms with *P. aeruginosa*, suggesting a clear competitive advantage for *P. aeruginosa* after 24-h. The gene expression profile showed that *P. aeruginosa* PA14 significantly overexpressed several key virulence-related genes (*pqsE, rhlR, pvdE* and *las1*) after 24-h of growth with *S. aureus* ATCC 25923, comparatively with single-species biofilms, which could justify the rapid decline of *S. aureus* population after 24-h of co-culture with *P. aeruginosa*. Nevertheless, the isolated strain *P. aeruginosa* 362668 non-mucoid exhibited a reverse pattern by downregulated the expression of those genes during dual-species growth. As *P. aeruginosa* exoproducts in addition to cause *S. aureus* lysis or inhibition may also induce biofilm dispersion, the bulk-fluid of 48-h biofilms was analysed by culture counts and flow-cytometry. Interestingly, while no *S. aureus* cells were detected by CFU, flow cytometry data revealed the presence of *S. aureus* in high numbers (~6 Log). The metabolic activity of *S. aureus* cells was confirmed by measure the expression levels of *sodA* gene.

In conclusion, the presence of *S. aureus* in a viable but non-cultivable state within dual-species biofilms with *P. aeruginosa* underscores the impact of interspecies interactions on antibiotic efficacy in the context of polymicrobial infections.