# Tween 80 and its derivative Oleic acid promotes the growth of *Corynebacterium accolens* and inhibits *Staphylococcus aureus* clinical isolates

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**Running title:**
Tween 80 promotes *C. accolens* growth

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## Introduction

In health, the human nasal cavity is colonized by a wide variety of commensal bacteria and pathobionts and is dominated by Actinobacteria (mainly *Corynebacteriaceae*) and Firmicutes (mainly *Staphylococcaceae*).[1, 2] In the context of chronic rhinosinusitis (CRS) however, an imbalance or dysbiosis occurs that is characterised by a decreased relative abundance of Actinobacteria and an overgrowth of pathogens such as *Staphylococcus aureus*.[3]

The fatty acid Tween 80 is commonly used as an excipient in nasal formulations to promote the solubilisation of the active drug [4] and is approved by the US Food and Drug Administration (FDA) at a maximum concentration of 0.5%. [5] It is considered to be well tolerated when delivered to mucosal, intradermal, and intravenous sites.[6] Bacteria belonging to the genus *Corynebacterium* can degrade Tween 80 and use the degradation products (polyoxyethylenic acids and oleic acid) as building blocks to synthesize novel glycolipids that become part of their cell envelope.[7] In contrast, fatty acids can also exhibit concentration-dependent anti-microbial activity against pathogens by interfering with cell membrane permeability.[8]

These unique properties of fatty acid containing formulations and the notion that Tween 80 is already FDA approved for use in nasal sprays raise the possibility that Tween 80 may have the potential to be used as a prebiotic in topical nasal formulations.

In this study, we aimed to evaluate the dose-dependent activity of Tween 80 and its derivative, oleic acid, on the growth of *Corynebacteriae, S. aureus* and bacteria frequently found in the human nasal cavity using *in-vitro* analysis.

**Materials and Methods**

Twenty two nasal clinical isolates (CIs) including C. accolens (n=4), C. *propinquum* (n=3), C. *pseudodiptheric*um (n=3), *S. epidermidis* (n=4), *S. aureus* (n=4) and *P. aeruginosa* (n=4) and *S. aureus* ATCC25923 (ATCC, Manassas, Virginia, USA) were used in this study (Human Research Ethics Committee approval number HREC/15/TQEH/132). Tween 80 and oleic acid (Sigma-Aldrich, USA) were diluted at various concentrations in nutrient broth (NB) or brain heart infusion (BHI) (Oxoid, Basingstoke, United Kingdom), incubated with bacteria for 24 hours followed by determining optical density to determine the minimum inhibitory concentration (MIC). The effects of Tween 80 and oleic acid on the formation of biofilms and on established biofilms were then evaluated by growing *S. aureus* CI8 and ATCC25923 for 48 hours in Tryptone-soya broth (TSB) containing Tween 80 or oleic acid or by adding both compounds to preformed 48-hour biofilms, each time followed by measuring the viability using an Alamar Blue assay (FLUOstar OPTIMA, Germany), LIVE/DEAD™ BacLight ™ staining (Invitrogen Bacterial Viability Kit, USA) and confocal laser scanning microscopy (Carl ZEISS 16.0, Germany). One-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test using GraphPad Prism version 8.0 (GraphPad Software California, U.S.A) was used for statistical analysis unless specified otherwise. Details of materials and methods are found in this article's online repository.

**Results**

In nutrient-poor media (NB, representing the sinonasal environment), the mean growth of *C. accolens* and *C. pseudodiptheriticum* was significantly increased by Tween 80 at low concentrations (up to 1.6 fold in the presence of 0.0625-0.125% (v/v) for C. *pseudodiptheriticum* and up to 1.9 fold in the presence of 0.03125-0.0625% (v/v) for *C. accolens*) after 24 hours exposure compared to untreated growth controls ($p<0.05$). Higher 1% concentrations of Tween 80 (and 0.5% for *S. aureus* and *P. aeruginosa*) significantly reduced the growth of all bacteria tested (Fig. 1A). Furthermore, the mean growth of *C. accolens* (but no other strains) significantly increased following 24 hours oleic acid treatments at 0.0625-0.25% concentrations compared to untreated control (up to 3 fold, $p<0.01$) (Fig. 1B). Similarly, in nutrient-rich media (BHI, used in laboratory conditions), addition of Tween 80 and oleic acid significantly stimulated *C. accolens* growth (at 0.03125-0.5% concentration for Tween 80 and 0.125-1% (v/v) for oleic acid) compared to untreated control (Fig. 1C and 1D). Adding Tween 80 or oleic acid at the start of biofilm formation, followed by 48 hours incubation, resulted in a significant reduction in biofilm viability for both *S. aureus* CI8 and ATCC25923 compared to control for various concentrations (between 0.125-1%) of Tween 80 and oleic acid) ($P<0.05$) (Fig. 2A). This was also seen using LIVE/DEAD staining where Tween 80 at 0.0315-0.125% (but not oleic acid) reduced the formation of 48-hour biofilms compared with untreated control (Fig. 2B). Tween 80 and oleic acid did not reduce the viability of established *S. aureus* biofilms (results not shown).

## Discussion

This study showed that Tween 80 at FDA approved concentrations of 0.5% and below promoted planktonic *C. accolens* growth and reciprocally reduced the viability of *S. aureus* planktonic cells and of newly forming *S. aureus* biofilms. *C. accolens* is considered a benign lipid-requiring commensal species that can degrade human skin triacylglycerols thereby producing free fatty acids that interfere with the growth of pathogens such as *S. pneumoniae*.[9] Such host-microbe-microbe interactions are thought to help shape the human microbiome. [9] A recent international sinonasal microbiome study showed *Corynebacterium* to be the most prevalent genus present in >75% of CRS patients and controls with a significant reduction in its relative abundance in CRS patients compared to controls.[2] The high prevalence of *Corynebacterium* lends itself to the possibility of manipulating that existing microbiome towards homeostasis by promoting its growth. Whilst further research is needed to validate our findings in the *in vivo* setting, they support the prebiotic potential of low Tween 80 concentrations to be used in nasal rinse solutions potentially promoting microbiome homeostasis in the context of CRS.

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FIGURE 1A and 1B. The effect of Tween 80 (A) and Oleic acid (B) on planktonic growth (OD595) of different bacterial strains after 24 hours in nutrient- poor growth media (Nutrient Broth, NB). Data represent the mean ± SEM of each bacterial strain; C. accolens (n=4), C. propinquum (n=3), C. pseudodiptheriticum (n=3), S. epidermidis (n=4), S. aureus (n=4) and P. aeruginosa (n=4). The experiments were conducted for different treatment concentrations in at least 6 replicates. *P<0.05 ** P<0.01, *** P<0.001, ****P<0.0001, One-way ANOVA NS, not significant; SEM, standard error of the mean.

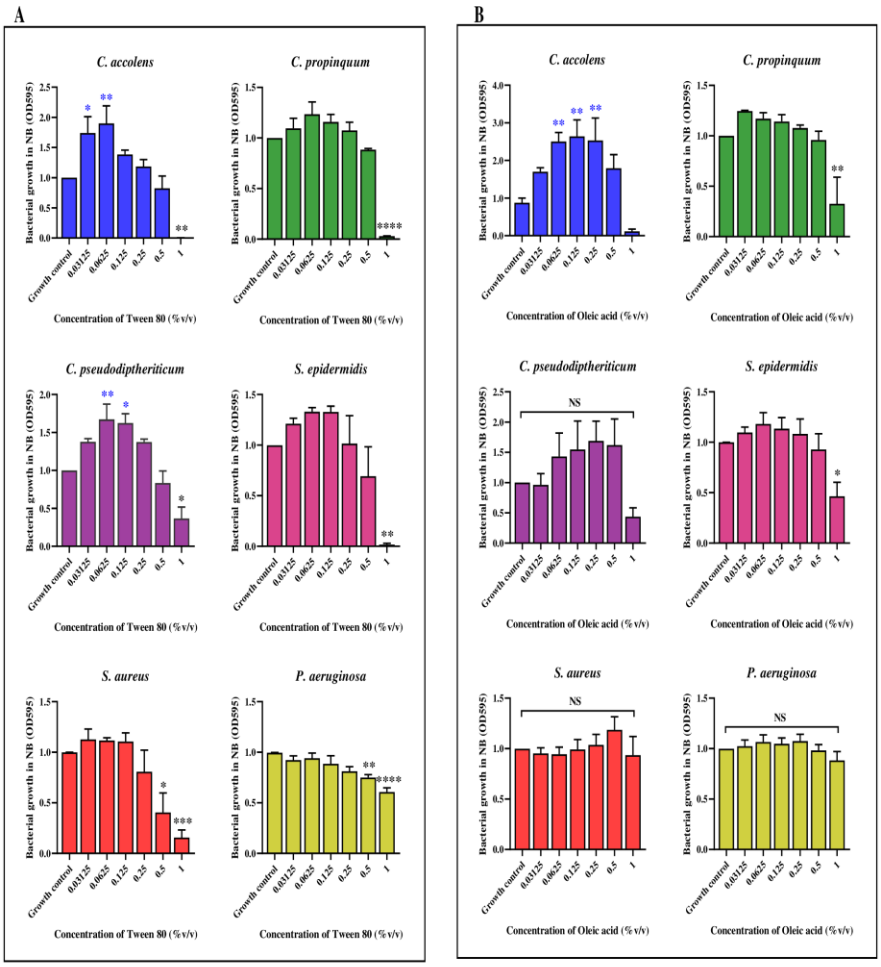

FIGURE 1C and 1D. The effect of Tween 80 (A) and Oleic acid (B) on planktonic growth (OD595) of different bacterial strains after 24 hours in nutrient- rich growth media (Brain-Heart Infusion, BHI). Data represent the mean ± SEM of each bacterial strain; C. accolens (n=4), C. propinquum (n=3), C. pseudodiptheriticum (n=3), S. epidermidis (n=4), S. aureus (n=4) and P. aeruginosa (n=4). The experiments were conducted for different treatment concentrations in at least 6 replicates. *P<0.05 ** P<0.01, *** P<0.001, ****P<0.0001, One-way ANOVA NS, not significant; SEM, standard error of the mean

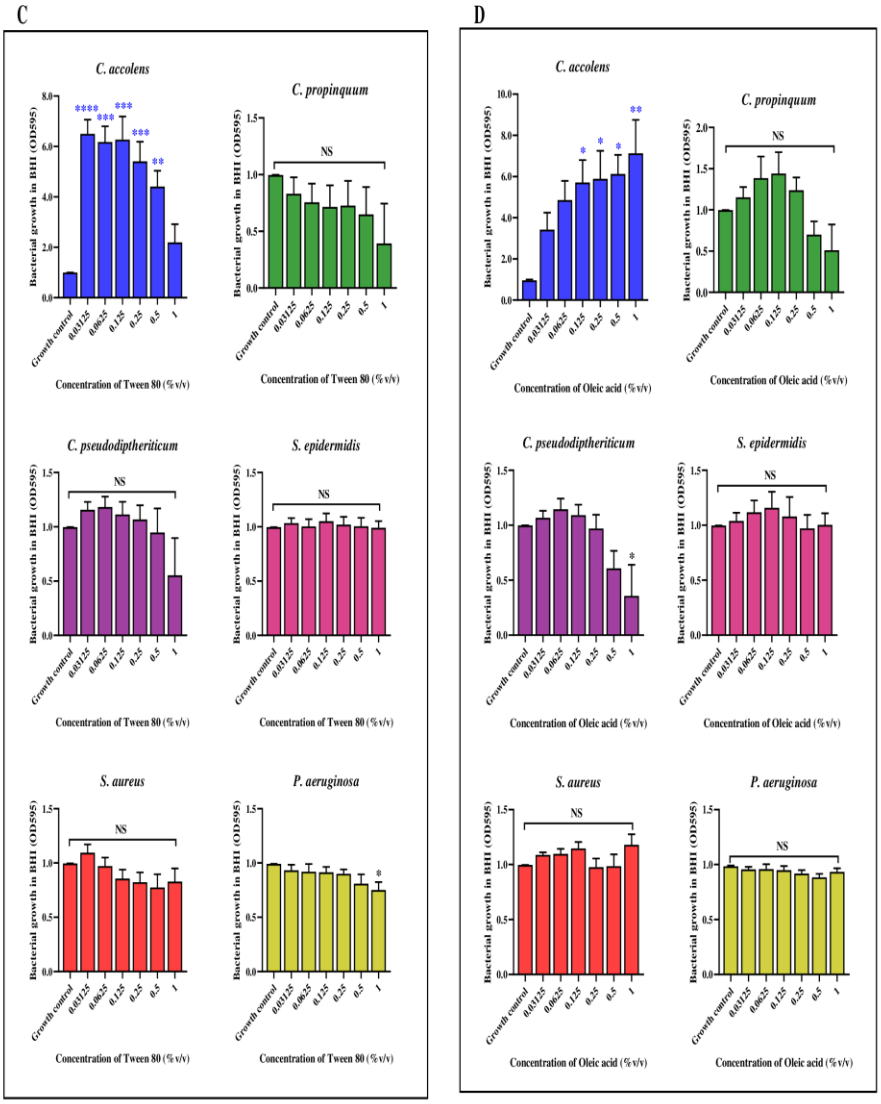

FIGURE 2A. Treatment of Tween 80 and Oleic acid at concentrations of 0.03125% -1% (v/v) or maximum growth control and its effect on biofilm viability of S. aureus clinical isolate (S. aureus CI8) and laboratory reference strain (S. aureus ATCC25923). The experiments were conducted in each strain at least three times. Data represents the mean + SEM of the three replicates. *P<0.05 ** P<0.01, *** P<0.001, ****P<0.0001, One-way ANOVA; NS, not significant; SEM, standard error of the mean.

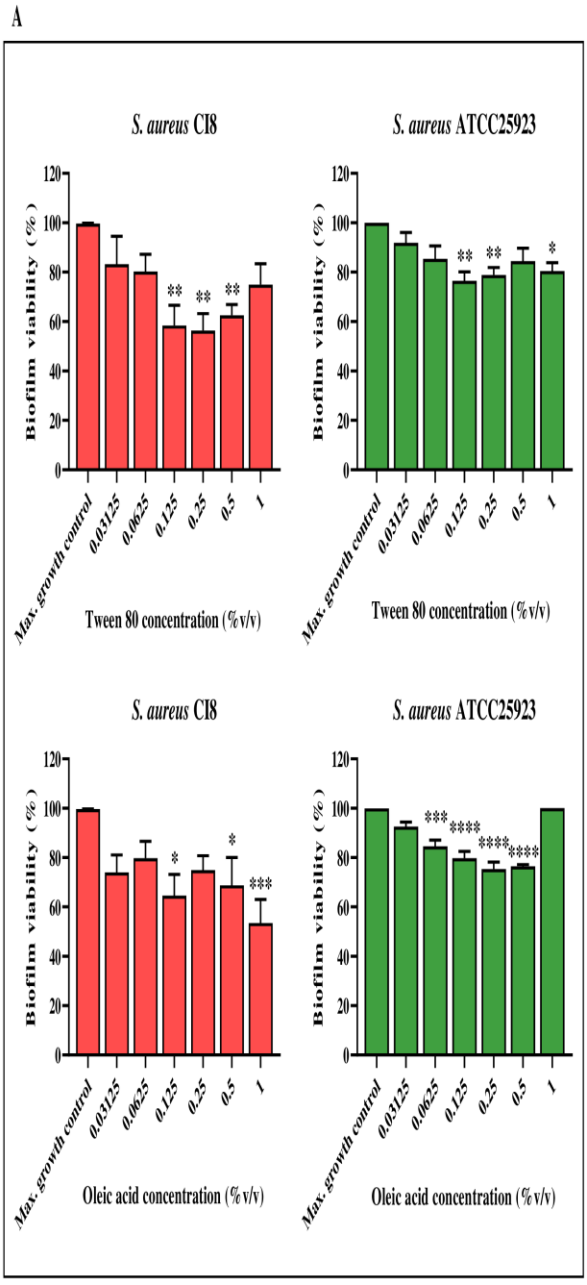

FIGURE 2B. LIVE/DEAD staining and CLSM visualization of S. aureus CI8 biofilms treated with Tween 80 or oleic acid in various concentrations. The green colour represents live cells whereas red colour represents dead cells. Data was presented by calculating the mean + SEM of the fluorescent intensity value of at least three microscopic images obtained from three replicate experiments. (SA, S. aureus; T80, Tween80; OA, Oleic acid; *statistically significant, *P<0.05, ***P<0.001; NS, not significant; SEM, Standard error of the mean).