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# *Corynebacterium* and *Dolosigranulum*: future probiotic candidates for upper respiratory tract infections

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**The presence of the bacterial genera *Corynebacterium* and *Dolosigranulum* has consistently been associated with a healthy upper respiratory tract (URT). Commonly occurring together in the nasopharynx of healthy children, the role of these commensal organisms in nasopharyngeal health is unknown, as few studies have sought to determine whether they actively contribute to maintaining a healthy state. We recently identified *Corynebacterium pseudodiphtheriticum* and *Dolosigranulum pigrum* as the major nasopharyngeal species associated with resistance to recurrent ear infections, via 16S rRNA gene sequencing and metagenomics. Using *in vitro* bacterial interference assays, we observed a reduction in the growth of *Moraxella catarrhalis* – one of the three major otopathogens – in the presence of *C. pseudodiphtheriticum*. Further *in vitro* and *in vivo* studies of the interactions between commensal *C. pseudodiphtheriticum* and *D. pigrum* strains, URT pathogens, and the human host will help to clarify their role in**

**nasopharyngeal health. If they play a protective role, these organisms are promising candidates for the development of a probiotic therapy for the treatment or prevention of URT diseases in children.**

Human microbiome research has traditionally focused on differences in the composition of the microbial community between healthy and disease states. This research has naturally progressed into 'mining the microbiota for therapeutics'; the process of identifying members of the microbiota that contribute to health and developing them into therapeutic agents, either in the form of probiotics (live, beneficial microorganisms) or isolated antimicrobial compounds. Several examples mirroring this process have already been demonstrated, where defined bacterial mixtures have successfully treated[1] or prevented[2] infection, or the production of an antimicrobial substance by a commensal organism inhibits a pathogenic close relative[3,4].

## Markers of a healthy nasopharynx

In the microbiome of the upper respiratory tract (URT), the genera *Corynebacterium* and *Dolosigranulum* have been consistently associated with health, particularly in children (Table 1). In these studies, they are frequently reported to co-occur, their presence corresponds with breastfeeding and a lower risk of respiratory infection, and they appear to be negatively impacted by antibiotic use. This suggests that these genera are important characteristics of a healthy nasopharynx. It may be hypothesised that the absence or reduction (by antibiotic use) of *Corynebacterium* and *Dolosigranulum* from the URT microbiota results in respiratory pathogens preferentially colonising the nasopharynx and an increased susceptibility to respiratory disease.

While it is clear that *Corynebacterium* and *Dolosigranulum* are strongly associated with health, little work has been done *in vitro* to characterise how they contribute to health. Do they compete with respiratory pathogens for nutrients and space, or prevent infection via bacterial antagonism? No previous studies appear to have directly tested *D. pigrum* for such activity. Of those that have investigated *Corynebacterium*, one early study reported *C. pseudodiphtheriticum* binding to pharyngeal cells, competitively excluding *M. catarrhalis*; though no zone of inhibition was observed on blood agar[18]. A more recent study reported that inoculating *C. pseudodiphtheriticum* strain 090104 into mice improved resistance to infection by respiratory syncytial virus and secondary pneumococcal infection, where non-viable 090104 had a weaker effect[19]. Recently, the commensal also demonstrated inhibition of *S. aureus* by exploiting its own virulence components[20]. Other *Corynebacterium* species have shown similar promising antimicrobial activity; *C. propinquum* uses siderophores to restrict the availability of iron to coagulase-negative staphylococci[21], and *C. accolens* inhibits *S. pneumoniae* via antimicrobial free fatty acids, which *C. accolens* requires for growth[10].

## *C. pseudodiphtheriticum* and *D. pigrum* in resistance to recurrent ear infections

Recently, we used 16S rRNA gene sequencing to characterise the nasopharyngeal microbiomes of children with recurrent ear infections (recurrent acute otitis media; rAOM), and healthy rAOM-resistant children[17]. The relative abundance of *Corynebacterium* and *Dolosigranulum* was significantly higher in the rAOM-resistant children, concordant with previous studies comparing the nasopharyngeal microbiota of children with and without OM[6,7,13]. Metagenomics further revealed that *C. pseudodiphtheriticum* and *D. pigrum* were the dominant species in the healthy rAOM-resistant nasopharynx with *C. propinquum* and *C. accolens* present to a lesser extent[22]. To investigate whether these commensal organisms have a protective role in the nasopharynx interfering with the growth of pathogens, we conducted bacterial interference assays[22]. *C. pseudodiphtheriticum* and *D. pigrum* were assessed for inhibitory activity against the major otopathogen species (non-typeable *Haemophilus influenzae* – NTHi, *Streptococcus pneumoniae* and *Moraxella catarrhalis*); two strains of each commensal species and seven strains of each otopathogen were tested, including mixtures of both commensals together. Growing the pathogens adjacent to the commensals on agar revealed that neither commensal affected the growth of *S. pneumoniae* or NTHi, but all seven *M. catarrhalis* strains were inhibited by *C. pseudodiphtheriticum* (Figure 1). This effect was not influenced by the presence of *D. pigrum*.

To attempt to identify the production of an antimicrobial compound by *C. pseudodiphtheriticum* as a potential mechanism, we inoculated filter paper discs with cell-free *C. pseudodiphtheriticum* broth culture and cell lysates for a disc diffusion assay on *M. catarrhalis*. No inhibition was observed (Figure 2)[22]. It is possible that the inhibitory effect of *C. pseudodiphtheriticum* is not mediated by an antimicrobial substance, for example by competitive acquisition of nutrients. However, it is also likely that such a substance is produced, but was not successfully extracted from *C. pseudodiphtheriticum* culture in a functional state or at sufficient concentrations to produce a zone of inhibition, or its production requires a trigger (like the presence of *M. catarrhalis*). This preliminary work demonstrates that *C. pseudodiphtheriticum* inhibits the growth of *M. catarrhalis*; while this is the least common of the major otopathogens, it may be an important one to target

Table 1. Studies observing the presence of *Corynebacterium* and *Dolosigranulum* in the URT microbiota and their association with a healthy state.

| Reference | Major *Corynebacterium* and *Dolosigranulum* findings | Subjects |
|---|---|---|
| Bogaert *et al.* (2011)[5] | • Both identified as common genera in the nasopharynx of healthy children | 96 healthy children |
| Laufer *et al.* (2011)[6] | • Both genera associated with decreased risk of pneumococcal colonisation and diagnosis of otitis media (OM) | 108 children with upper respiratory tract infection symptoms |
| Pettigrew *et al.* (2012)[7] | • Both genera associated with decreased risk of acute OM (AOM) when *Streptococcus* and *Haemophilus* abundance was low and no antibiotics used in the previous 6 months<br>• Levels of *Dolosigranulum* were lower in children receiving antibiotics (mostly β-lactams) in the previous 6 months | 73 healthy children, 95 children with upper respiratory infection (URI), and 72 children with both URI and AOM |
| Biesbroek *et al.* (2014)[8] | • Increased presence and abundance of both genera in breastfed children at 6 weeks of age<br>• *Dolosigranulum* negatively associated with wheezing and number of respiratory tract infections<br>• *Corynebacterium* operational taxonomic unit highly homologous to *C. pseudodiphtheriticum* and *C. propinquum* | 101 breastfed infants and 101 formula-fed infants |
| Biesbroek *et al.* (2014)[9] | • Both genera associated with breastfeeding and lower rates of respiratory infections<br>• Infants at 1.5 months of age dominated by *Corynebacterium* and *Dolosigranulum* shifted to *Moraxella* and *Dolosigranulum* at 6 months of age. These profiles were more stable than those dominated by *Haemophilus* or *Streptococcus* | 60 healthy children |
| Bomar *et al.* (2016)[10] | • Both genera associated with the absence of *S. pneumoniae* colonisation<br>• Demonstrated *in vitro* that *C. accolens* inhibits *S. pneumoniae* with antipneumococcal free fatty acids | 9 children colonised with *S. pneumoniae* and 18 children without |
| Prevaes *et al.* (2016)[11] | • Both genera associated with healthy infants compared to those with cystic fibrosis<br>• Antibiotic treatment (predominantly co-trimoxazole) related to a reduction in *Corynebacterium* and *Dolosigranulum* in both groups | 20 infants with cystic fibrosis and 45 healthy infants |
| Bosch *et al.* (2017)[12] | • Prolonged reduction of *Corynebacterium* and *Dolosigranulum* associated with a higher number of respiratory tract infections<br>• Both genera significantly reduced in abundance after antibiotic usage | 112 healthy infants |
| Chonmaitree *et al.* (2017)[13] | • *Corynebacterium* found to be associated with infants without AOM<br>• Antibiotics (most commonly amoxicillin) did not affect otopathogen genera but significantly decreased *Corynebacterium* and *Dolosigranulum* | 65 infants with AOM and 74 infants without AOM |
| Hasegawa *et al.* (2017)[14] | • Both genera associated with low likelihood of severe bronchiolitis | 40 hospitalised infants with bronchiolitis and 110 healthy infants |
| Kelly *et al.* (2017)[15] | • Both genera associated with healthy children compared to those with pneumonia<br>• HIV-infected children with pneumonia had a near complete absence of *Dolosigranulum* | 204 children with pneumonia (14 with HIV infection) and 60 healthy children |
| Copeland *et al.* (2018)[16] | • Both genera associated with healthy adults compared to those with chronic rhinosinusitis | 21 adults with chronic rhinosinusitis vs 12 healthy adults |
| Lappan *et al.* (2018)[17] | • Both genera associated with children resistant to recurrent AOM (rAOM) | 86 children with rAOM and 98 rAOM-resistant children |

given its apparent synergistic interactions with NTHi and *S. pneumoniae*[23,24]. However, the mechanism of interference, the role of *D. pigrum* and whether *Corynebacterium* species can inhibit URT pathogens at a clinically useful degree is yet unknown.

## Towards a potential probiotic therapy using *Corynebacterium* and *Dolosigranulum*

The consistent correlation between *Corynebacterium, Dolosigranulum* and upper respiratory health presents a very promising avenue of research towards development of probiotic therapies for the URT. The recent studies demonstrating antimicrobial activity by *C. accolens* against *S. pneumoniae*[10], *C. pseudodiphtheriticum* against both *S. aureus*[20] and *M. catarrhalis*[22], and *C. propinquum* against coagulase-negative staphylococci[21] suggest it is possible that multiple *Corynebacterium* species are beneficial to the URT, acting via different mechanisms against different pathogens. The role of *D. pigrum* in these interactions remains unknown, however it has been hypothesised that the production of lactic acid by

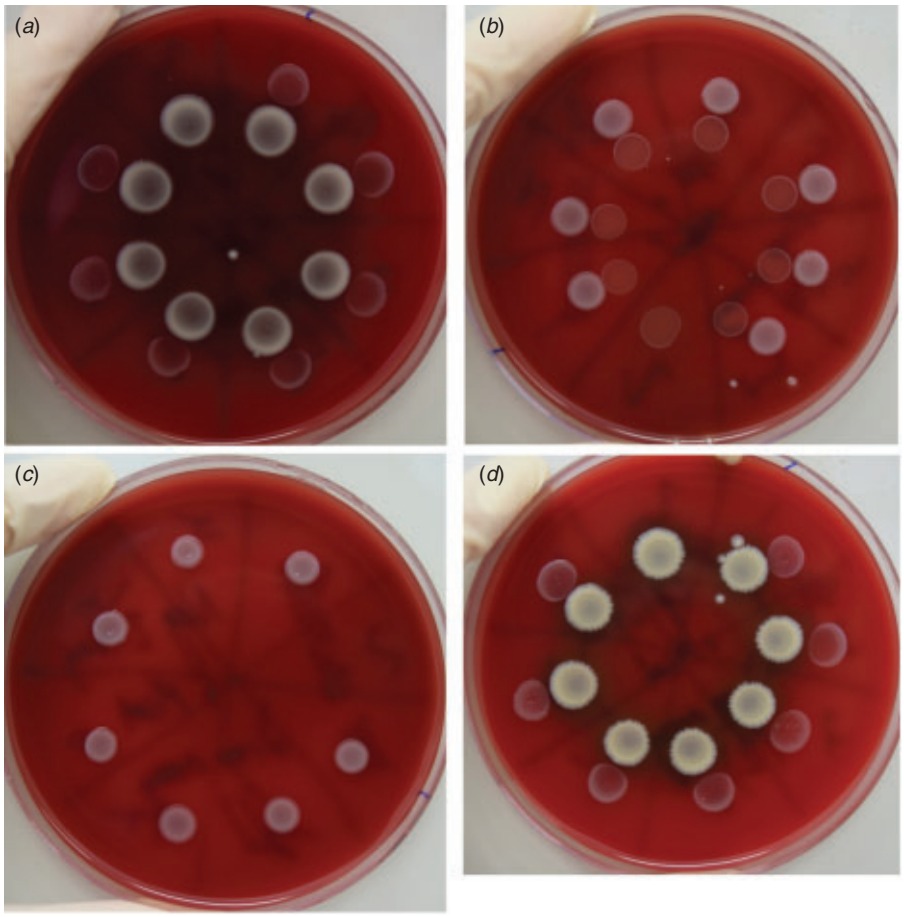


Figure 1. *C. pseudodiphtheriticum* inhibits *M. catarrhalis* on blood agar. The growth of *M. catarrhalis* (outer drops on all images) is inhibited by (*a*) *C. pseudodiphtheriticum* (inner drops) but not by (*b*) *D. pigrum* or (*c*) broth alone (negative control). The inhibitory effect is the same with (*d*) *C. pseudodiphtheriticum* and *D. pigrum* together.

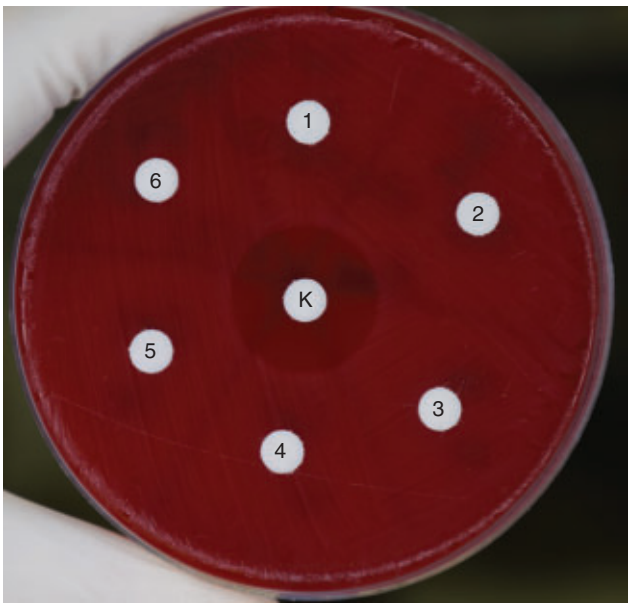


Figure 2. Cell-free extracts from *C. pseudodiphtheriticum* do not inhibit *M. catarrhalis*. The central disc contains 30 µg kanamycin (positive control). There are no zones of inhibition around the four other discs containing cell-free *C. pseudodiphtheriticum* extracts from either strain of the commensal (broth supernatants on discs 1 and 2; cell lysates on discs 4 and 5) or the two negative controls (negative broth on disc 3, negative lysate on disc 6).

*D. pigrum* lowers the pH of the local environment, selecting for the growth of *Corynebacterium* species[25]. This is highly plausible, as several species of *Lactobacillus* inhibit *M. catarrhalis* via lactic acid[26], which also exhibits antimicrobial and anti-inflammatory properties in the human vagina[27]. A niche-specific probiotic therapy containing multiple species with complementary effects may be an effective approach for the URT, especially for restoration after antibiotic use, which has been demonstrated to reduce *Corynebacterium* and *Dolosigranulum* populations (Table 1). Previous clinical trials using nasal spray probiotics for children with rAOM have suggested that pre-treatment with antibiotics allows re-establishment of the healthy microbiota, preventing the re-emergence of pathogens[28,29].

To fully investigate the possibility that these commensal organisms work synergistically to provide resistance to respiratory pathogens, and the potential for this to be translated into a therapy, there is an array of possibilities for future research. First, it remains likely that *C. pseudodiphtheriticum* produces an antimicrobial substance that inhibits *M. catarrhalis*. To isolate it, a range of commensal nasopharyngeal strains could be screened for optimal antimicrobial

activity, and optimisation of culture conditions or enrichment of proteins may be required, similar to the approach taken by Latham *et al.* (2017) for the isolation of an antimicrobial substance from *Haemophilus haemolyticus*[4]. The commensal *Corynebacterium* genomes are not well characterised; a very recent sequencing effort by Stubbendieck *et al.* (2019) has improved the available genomic information for *C. pseudodiphtheriticum* and *C. propinquum*[21], but these draft genome assemblies are still incomplete as of August 2019 (NCBI Genome Assembly and Annotation reports). There is now one strain of *D. pigrum* with a complete assembly (83VPs-KB5) available. Mining the genomes of multiple commensal strains for putative antimicrobial genes may provide further evidence for protective factors. Additionally, epithelial cell models have previously been successful in understanding the competition between *H. haemolyticus* and NTHi in the URT[30] and would also be useful to obtain a deeper insight into the competitive mechanisms and host responses, to see if colonisation with these organisms influences susceptibility to later infection with *S. pneumoniae, M. catarrhalis* or *S. aureus*.

Further research into the potential of *Corynebacterium* and *Dolosigranulum* to compete with respiratory pathogens, alone or in synergy, will provide a strong direction for the use of animal models and clinical trials in the development of a probiotic therapy, which has the potential to treat or prevent a range of upper respiratory illnesses in children.

## Conflicts of interest

The authors declare no conflicts of interest.

## Acknowledgements

We thank Kyra Cottrell, Hanna Sidjabat, Janessa Pickering and Barbara Chang for helpful discussion on the bacterial interference assays. We also thank Lea-Ann Kirkham, Caitlyn Granland, Deborah Lehmann and Jacinta Bowman for the provision of the otopathogen strains, and Paul Healy for the provision of the *C. pseudodiphtheriticum* and *D. pigrum* strains. This research was supported by the Telethon New Childrens' Hospital Research Fund.

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## Biographies

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# Pathogen adaptation to vaccination: the Australian *Bordetella pertussis* story


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**Whooping cough (pertussis) is a highly contagious vaccine preventable respiratory disease caused by the Gram-negative bacterium *Bordetella pertussis*. Despite high level vaccination coverage over the past 20 years, Australia has one of the highest per capita burdens of pertussis globally. One of the primary factors associated with the re-emergence of pertussis is pathogen adaptation of *B. pertussis* to the current acellular vaccines used. This article will focus on the genomic and proteomic changes that have occurred in the Australian *B. pertussis* population, the significance of these adaptive changes on fitness in a vaccinated environment and what we can do to reduce the significant burden of pertussis in the future.**


## The rising incidence of *B. pertussis* in Australia

Pertussis vaccinations were first introduced in Australia in 1953 using a whole cell vaccine (WCV), which contained dead *B. pertussis* cells. This led to a dramatic reduction in the number of pertussis notifications from 767 cases per 100 000 in the 1930s