MICROBIOLOGY AUSTRALIA, 2021, 42, 116–119
https://doi.org/10.1071/MA21032

Exploiting the struggle for haem: a novel therapeutic approach against *Haemophilus influenzae*

Brianna Atto[A,C], *David Gell*[B] *and Stephen Tristram*[A]

[A]School of Health Sciences, University of Tasmania, Newnham Drive, Launceston, Tas. 7248, Australia
[B]School of Medicine, University of Tasmania, 17 Liverpool Street, Hobart, Tas. 7000, Australia
[C]Email: brianna.atto@utas.edu.au

Abstract. Over the past decade, nontypeable *Haemophilus influenzae* (NTHi) has gained recognition as a major opportunistic pathogen of the respiratory tract that imposes a substantial global burden of disease, owing to a high rate of morbidity and ensuing complications. Further amplifying the global impact of NTHi infections is the increasing spectrum and prevalence of antibiotic resistance, leading to higher rates of treatment failure with first- and second-line antibiotics regimes. The threat of antibiotic resistance was recognised by the World Health Organization in 2017, listing NTHi as a priority pathogen for which new therapies are urgently needed. Despite significant efforts, there are currently no effective vaccine strategies available that can slow the growing burden of NTHi disease. Consequently, alternative preventative or therapeutic approaches that do not rely on antibiotic susceptibility or stable vaccine targets are becoming more attractive. The nutritional dependency for haem at all stages of NTHi pathogenesis exposes a vulnerability that may be exploited for the development of such therapies. This article will discuss the therapeutic potential of strategies that limit NTHi access to this vital nutrient, with particular focus on a novel bacteriotherapeutic approach under development.

Received 1 July 2021, accepted 5 August 2021, published online 6 September 2021

NTHi is a major respiratory pathogen for which new therapies are needed

Nontypeable *Haemophilus influenzae* (NTHi) is a common coloniser of the upper respiratory tract in healthy children (20–80%) and adults (20–30%), the prevalence of which varies considerably across geographical regions[1–4]. However, in susceptible individuals, NTHi represents a major cause of opportunistic infections in the respiratory tract, namely acute otitis media and sinusitis in children, and lower respiratory tract infections in elderly individuals or those with chronic obstructive pulmonary disease[5]. Collectively, these infections and subsequent long-term health complications, such as hearing loss or decline in lung function, impart a significant global disease burden[5,6]. Further amplifying the global impact of NTHi infections is the rapidly expanding spectrum and prevalence of antibiotic resistance, leading to treatment failure with first- and second-line antibiotics[5,7]. The high morbidity and long-term antibiotic prescription associated with NTHi infections, collectively expose a substantial proportion of the population to antimicrobial agents, driving resistance to a broad-spectrum of antibiotics in the community[8,9]. The threat of antibiotic resistance was recognised by the World Health Organization in 2017, listing NTHi as a priority pathogen for which new therapies are urgently needed[10]. Owing to the high genetic heterogeneity and phase-variable expression of conserved antigen targets, there are currently no effective vaccine strategies available that can slow the growing burden of NTHi disease[11]. Consequently, novel preventative or therapeutic approaches that do not rely on antibiotic susceptibility or stable vaccine targets are becoming more attractive.

Haem-iron acquisition is a major determinant of NTHi pathogenesis

The pathogenesis of NTHi is largely dictated by interactions with host airway epithelia. Although the exact mechanisms are poorly understood, NTHi adhesion and colonisation of the host pharyngeal epithelium, followed by migration to privileged anatomical sites, is required to elicit an infection[12]. Survival and persistence at the site of infection is mediated by host-cell internalisation, formation of biofilms, or modulation of the immune response that protects bacterial populations from immune or antibiotic clearance[13–15]. In addition to being an essential growth requirement, access to iron-containing haem plays an important role in the ability of NTHi to perform these interactions and as such, the ability to sequester host-derived sources of haem is a key determinant of pathogenesis[16,17]. The consequence of NTHi haem starvation, either by disruption of acquisition mechanisms or by environmental restriction, has been demonstrated to attenuate virulence in animal models of invasive disease and otitis media[18–21]. Strategies that interrupt NTHi acquisition or utilisation of host-derived sources of haem may therefore have a significant impact on the ability of NTHi to cause disease.

A new therapeutic approach: exploitive competition for haem-iron

Recently, we discovered strains of the closely related commensal *Haemophilus haemolyticus* (Hh) that also inhabit the pharyngeal niche and secrete a novel haemophore (since named haemophilin; Hpl) that elicits potent inhibitory activity against NTHi[22,23].

Functional and proteomic investigation demonstrated that Hpl is a previously unrecognised haem uptake mechanism of Hh, which inhibits NTHi growth through exploitative competition for haem. We have since conducted several investigations *in vitro* and *in vivo* to test the NTHi-inhibitory capacity of Hpl-producing strains of Hh (Hh-Hpl$^+$) and propose their therapeutic utility as a respiratory probiotic.

In vitro *investigations*

In a broth co-culture system, NTHi strains were outcompeted by Hh-Hpl$^+$ and suffered a complete loss of fitness over subsequent generations[24]. Similarly, in tissue culture models of nasopharyngeal (D562) and lung epithelia (A549), Hh strains with high levels of *hpl* expression protected cell monolayers against adhesion and invasion by NTHi[25] (Figure 1). Significant inhibition of NTHi adherence and invasion was maintained when Hh-Hpl$^+$ treatment doses were 10–100-fold lower than the NTHi challenge. In both *in vitro* models, NTHi-inhibitory activity correlated with levels of *hpl* expression and Hpl protein quantified from competition media. The absence of NTHi-inhibitory activity in a *hpl* knockout or native non-producing strains confirmed that the inhibitory phenotype was mediated by the ability to produce Hpl.

In vivo *investigations*

Considering the NTHi-inhibitory activity *in vitro* we hypothesised that natural pharyngeal carriage of Hh strains with the *hpl* open reading frame would be associated with a lower prevalence and/or density of NTHi colonisation in healthy individuals. Real-time PCR was used to quantitatively compare the oropharyngeal carriage load of NTHi and Hh populations with the Hh-*hpl*$^+$ or Hh-*hpl*$^-$ genotype from 257 healthy adults in Australia. Compared to carriage of Hh-*hpl*$^-$ strains, adult (18–65 years) and elderly (>65 years) participants that were colonised with Hh-*hpl*$^+$ were 2.43 (95% CI, 1.95–2.61; $P < 0.0001$), or 2.67 times (95% CI, 2.63–2.70; $P = 0.0036$) less likely to carry NTHi, respectively. Colonisation with high densities of Hh-*hpl*$^+$ correlated with low NTHi carriage load and a 2.63-times (95% CI, 2.56–2.70, $P = 0.0112$) lower likelihood of acquiring/maintaining NTHi colonisation status between visits[26] (Figure 2).

Potential translation as a respiratory probiotic to prevent NTHi infections

The presence of healthy carriers of NTHi indicates that a complete eradication of NTHi is not necessary to prevent infection. Furthermore, higher NTHi pharyngeal carriage loads are correlated with an increased susceptibility to otitis media *in vivo*[27–30] and an increased severity of airway inflammation, exacerbations, and daily symptoms in chronic obstructive pulmonary disease[31,32]. Thus, even small reductions in NTHi carriage might have beneficial clinical outcomes. Using a model designed to predict the risk of otitis media in children

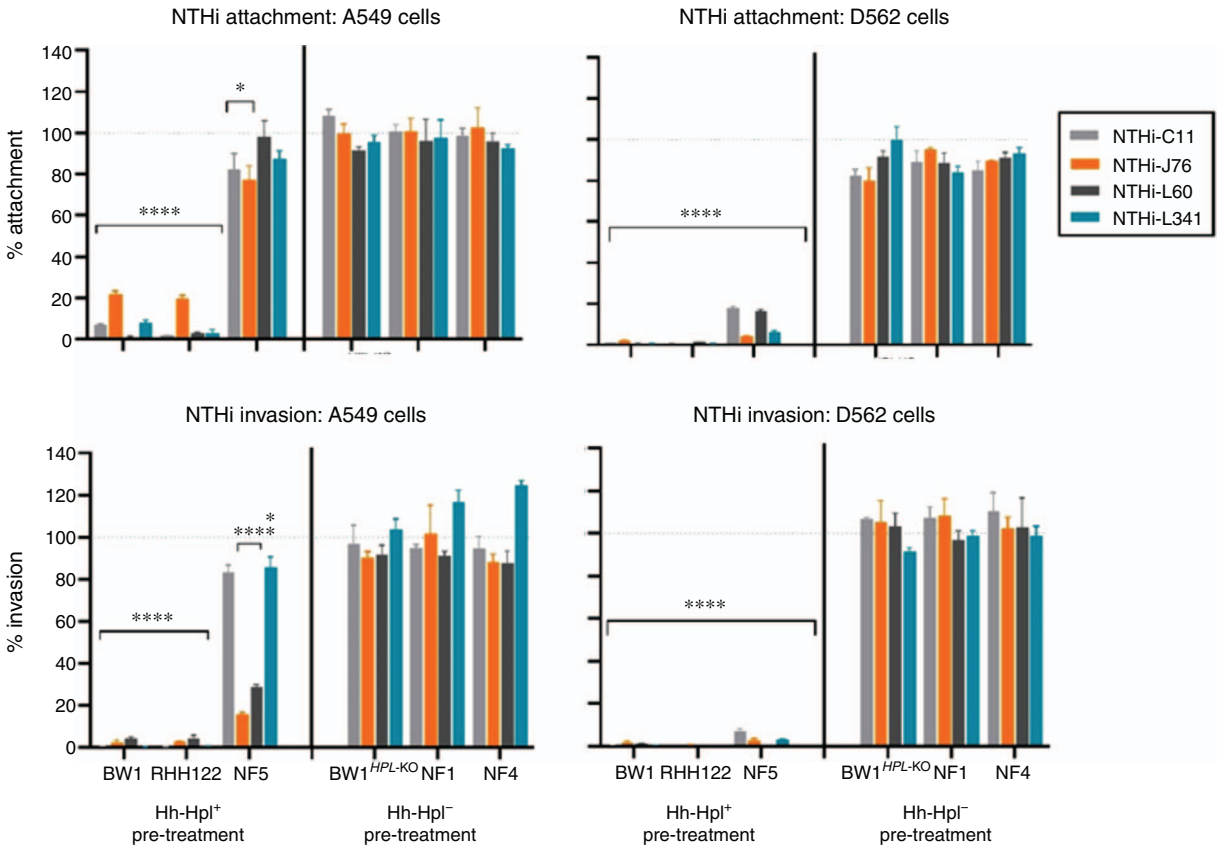

Figure 1. NTHi attachment and invasion of A549 and D652 cells post treatment with *Haemophilus haemolyticus* (Hh) strains (BW1, RHH122, NF5, NF5, NF1) or the *hpl* knockout (BW1$^{hpl\text{-}KO}$). The percent attachment of NTHi (compared to media control) to A549 (*a*) and D562 (*b*) cell monolayers post 4-h pre-treatment with Hpl-producing Hh (Hh-Hpl$^+$) or Hh strains that do not produce Hpl (Hh-Hpl$^-$). Percent of internalised NTHi (compared to media control) after exposure to A549 (*c*) and D562 (*d*) cell monolayers post 4-h pre-treatment with Hh-Hpl$^+$ or Hh-Hpl$^-$. Error bars represent the ±SEM (standard error of the mean) of three biological replicates, measured triplicate: *$P < 0.05$, ****$P < 0.0001$.

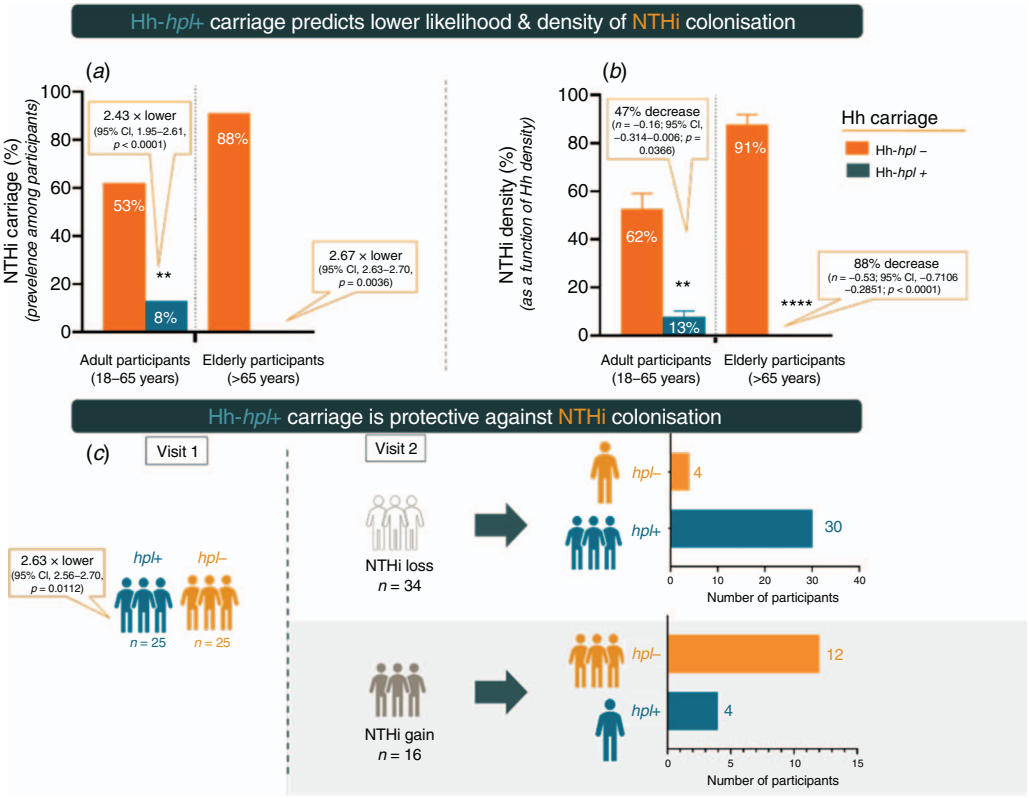

Figure 2. NTHi dominance in oropharyngeal swabs of healthy adult (18–65 years) or elderly (>65 years) participants co-colonised with Hh. NTHi oropharyngeal carriage prevalence (*a*) or proportion of NTHi (as a function of total Hh) (*b*) among participants concurrently carrying Hh strains that possess the *hpl* ORF (Hh-*hpl*$^+$) or do not possess the *hpl* ORF (Hh-*hpl*$^-$). Hh-*hpl*$^+$ (predominant) denotes instances where *hpl*$^+$ is the predominant Hh genotype (>0.5 of total Hh). NTHi colonisation status in participants carrying *hpl*$^+$ ($n = 25$) or *hpl*$^-$ ($n = 25$) strains of Hh on follow-up testing (visit 2) 2–6 months after their initial visit (visit 1). Error bars represent ±SEM (standard error of the mean); statistical significance was determined by simple logistic regression (*a*) or nonparametric Spearman correlation (*b*); $^{**}P < 0.005$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

based on NTHi pharyngeal carriage load[30], we could predict a ≈40% decrease in the risk of infection, provided the level of protection conferred by Hpl-producing Hh to model cell lines was preserved in the context of the respiratory tract. Hh also possesses favourable characteristics suited to probiotic applications; it has not been implicated as a causative agent of respiratory tract infection[33,34] and as a normal pharyngeal inhabitant, is able to thrive in the niche amongst other microbial inhabitants[35]. Additionally, probiotic-based therapies have a narrow spectrum of activity that do not damage host tissue, provoke collateral damage to the healthy microbiome or promote enrichment of resistant clones[36]; properties which make them an asset against the emergence of antibiotic resistance.

In conclusion, Hpl-producing Hh may be a promising respiratory probiotic candidate for the prevention of NTHi infections by inhibiting requisite pharyngeal colonisation.

Conflicts of interest

The authors declare no conflicts of interest.

Declaration of funding

The studies informing this work were funded by the Clifford Craig Medical Research Trust (CCF170, CCF192).

References

1. Chi, D.H. *et al.* (2003) Nhasopharyngeal reservoir of bacterial otitis media and sinusitis pathogens in adults during wellness and viral respiratory illness. *Am. J. Rhinol.* **17**, 209–214. doi:10.1177/194589240301700406
2. Greenberg, D. *et al.* (2004) Relative importance of nasopharyngeal versus oropharyngeal sampling for isolation of Streptococcus pneumoniae and *Haemophilus influenzae* from healthy and sick individuals varies with age. *J. Clin. Microbiol.* **42**, 4604–4609. doi:10.1128/JCM.42.10.4604-4609.2004
3. Mackenzie, G.A. *et al.* (2010) Epidemiology of nasopharyngeal carriage of respiratory bacterial pathogens in children and adults: cross-sectional surveys in a population with high rates of pneumococcal disease. *BMC Infect. Dis.* **10**, 304. doi:10.1186/1471-2334-10-304
4. Rawlings, B.A. *et al.* (2013) Bacterial pathogens in the nasopharynx, nasal cavity, and osteomeatal complex during wellness and viral infection. *Am. J. Rhinol. Allergy* **27**, 39–42. doi:10.2500/ajra.2013.27.3835
5. Van Eldere, J. *et al.* (2014) Non-typeable *Haemophilus influenzae*, an under-recognised pathogen. *Lancet Infect. Dis.* **14**, 1281–1292. doi:10.1016/S1473-3099(14)70734-0
6. Cerquetti, M. and Giufrè, M. (2016) Why we need a vaccine for non-typeable *Haemophilus influenzae*. *Hum. Vaccin. Immunother.* **12**, 2357–2361. doi:10.1080/21645515.2016.1174354
7. Tristram, S. *et al.* (2007) Antimicrobial resistance in *Haemophilus influenzae*. *Clin. Microbiol. Rev.* **20**, 368–389. doi:10.1128/CMR.00040-06
8. Venekamp, R.P. *et al.* (2015) Antibiotics for acute otitis media in children. *Cochrane Database Syst. Rev.* doi:10.1002/14651858.CD000219.pub4

9. Ito, M. *et al.* (2010) Clonal spread of β-lactamase-producing amoxicillin–clavulanate-resistant (BLPACR) strains of non-typeable *Haemophilus influenzae* among young children attending a day care in Japan. *Int. J. Pediatr. Otorhinolaryngol.* **74**, 901–906. doi:10.1016/j.ijporl.2010.05.008
10. World Health Organization (2017) Prioritization of pathogens to guide discovery, research and development of new antibiotics for drug-resistant bacterial infections, including tuberculosis.
11. Jalalvand, F. and Riesbeck, K. (2018) Update on non-typeable *Haemophilus influenzae*-mediated disease and vaccine development. *Expert Rev. Vaccines* **17**, 503–512. doi:10.1080/14760584.2018.1484286
12. Duell, B.L. *et al.* (2016) Host–pathogen interactions of nontypeable *Haemophilus influenzae*: from commensal to pathogen. *FEBS Lett.* **590**, 3840–3853. doi:10.1002/1873-3468.12351
13. Clementi, C.F. and Murphy, T.F. (2011) Non-typeable *Haemophilus influenzae* invasion and persistence in the human respiratory tract. *Front. Cell. Infect. Microbiol.* **1**, 1. doi:10.3389/fcimb.2011.00001
14. Goyal, M. *et al.* (2015) Cellular interaction of nontypeable *Haemophilus influenzae* triggers cytotoxicity of infected type II alveolar cells via apoptosis. *Pathog. Dis.* **73**, 1.
15. Moriyama, S. *et al.* (2009) Formation of biofilm by *Haemophilus influenzae* isolated from pediatric intractable otitis media. *Auris Nasus Larynx* **36**, 525–531. doi:10.1016/j.anl.2008.10.010
16. Harrison, A. *et al.* (2013) Ferric uptake regulator and its role in the pathogenesis of nontypeable *Haemophilus influenzae*. *Infect. Immun.* **81**, 1221–1233. doi:10.1128/IAI.01227-12
17. Rodríguez-Arce, I. *et al.* (2019) Moonlighting of *Haemophilus influenzae* heme acquisition systems contributes to the host airway-pathogen interplay in a coordinated manner. *Virulence* **10**, 315–333. doi:10.1080/21505594.2019.1596506
18. Morton, D.J. *et al.* (2004) Reduced severity of middle ear infection caused by nontypeable Haemophilus influenzae lacking the hemoglobin/hemoglobin–haptoglobin binding proteins (Hgp) in a chinchilla model of otitis media. *Microb. Pathog.* **36**, 25–33. doi:10.1016/j.micpath.2003.08.007
19. Morton, D.J. *et al.* (2009) The heme-binding protein (HbpA) of *Haemophilus influenzae* as a virulence determinant. *Int. J. Med. Microbiol.* **299**, 479–488. doi:10.1016/j.ijmm.2009.03.004
20. Seale, T.W. *et al.* (2006) Complex role of hemoglobin and hemoglobin-haptoglobin binding proteins in *Haemophilus influenzae* virulence in the infant rat model of invasive infection. *Infect. Immun.* **74**, 6213–6225. doi:10.1128/IAI.00744-06
21. Szelestey, B.R. *et al.* (2013) Haemophilus responses to nutritional immunity: epigenetic and morphological contribution to biofilm architecture, invasion, persistence and disease severity. *PLoS Pathog.* **9**, e1003709. doi:10.1371/journal.ppat.1003709
22. Latham, R.D. *et al.* (2017) An isolate of *Haemophilus haemolyticus* produces a bacteriocin-like substance that inhibits the growth of nontypeable *Haemophilus influenzae*. *Int. J. Antimicrob. Agents* **49**, 503–506. doi:10.1016/j.ijantimicag.2016.12.010
23. Latham, R.D. *et al.* (2020) A heme-binding protein produced by *Haemophilus haemolyticus* inhibits non-typeable Haemophilus influenzae. *Mol. Microbiol.* **113**, 381–398. doi:10.1111/mmi.14426
24. Atto, B. *et al.* (2020) *In vitro* anti-NTHi activity of haemophilin-producing strains of *Haemophilus haemolyticus*. *Pathogens* **9**, 243. doi:10.3390/pathogens9040243
25. Avadhanula, V. *et al.* (2006) Nontypeable *Haemophilus influenzae* adheres to intercellular adhesion molecule 1 (ICAM-1) on respiratory epithelial cells and upregulates ICAM-1 expression. *Infect. Immun.* **74**, 830–838. doi:10.1128/IAI.74.2.830-838.2006
26. Atto, B. *et al.* (2021) Oropharyngeal carriage of *hpl*-containing *Haemophilus haemolyticus* predicts lower prevalence and density of NTHi colonisation in healthy adults. *Pathogens* **10**, 577. doi:10.3390/pathogens10050577
27. de Gier, C. *et al.* (2019) PCV7-and PCV10-vaccinated otitis-prone children in New Zealand have similar pneumococcal and *Haemophilus influenzae* densities in their nasopharynx and middle ear. *Vaccines (Basel)* **7**, 14. doi:10.3390/vaccines7010014
28. Hill, A.T. *et al.* (2000) Association between airway bacterial load and markers of airway inflammation in patients with stable chronic bronchitis. *Am. J. Med.* **109**, 288–295. doi:10.1016/S0002-9343(00)00507-6
29. Hood, D. *et al.* (2016) A new model for non-typeable *Haemophilus influenzae* middle ear infection in the Junbo mutant mouse. *Dis. Model. Mech.* **9**, 69–79.
30. Smith-Vaughan, H. *et al.* (2006) Measuring nasal bacterial load and its association with otitis media. *BMC Ear Nose Throat Disord.* **6**, 10. doi:10.1186/1472-6815-6-10
31. Desai, H. *et al.* (2014) Bacterial colonization increases daily symptoms in patients with chronic obstructive pulmonary disease. *Ann. Am. Thorac. Soc.* **11**, 303–309. doi:10.1513/AnnalsATS.201310-350OC
32. Pfaffl, M.W. (2001) A new mathematical model for relative quantification in real-time RT–PCR. *Nucleic Acids Research* **29**, e45. doi:10.1093/nar/29.9.e45
33. Kirkham, L.-A.S. *et al.* (2010) Nasopharyngeal carriage of *Haemophilus haemolyticus* in otitis-prone and healthy children. *J. Clin. Microbiol.* **48**, 2557–2559. doi:10.1128/JCM.00069-10
34. Murphy, T.F. *et al.* (2007) *Haemophilus haemolyticus*: a human respiratory tract commensal to be distinguished from *Haemophilus influenzae*. *J. Infect. Dis.* **195**, 81–89. doi:10.1086/509824
35. De Boeck, I. *et al.* (2021) Lactic acid bacteria as probiotics for the nose? *Microb. Biotechnol.* **14**, 859–869. doi:10.1111/1751-7915.13759
36. Hols, P. *et al.* (2019) Mobilization of microbiota commensals and their bacteriocins for therapeutics. *Trends Microbiol.* **27**, 690–702. doi:10.1016/j.tim.2019.03.007

Biographies

Brianna Atto is a final year PhD candidate at the University of Tasmania. She is interested in the development of novel therapeutic strategies that overcome antibiotic resistance to treat and prevent infections. Her research is currently exploring the therapeutic value of upper respiratory commensals to tackle the growing disease burden of the major respiratory pathogen nontypeable *Haemophilus influenzae*.

David Gell is a protein chemist at the University of Tasmania with an interest in the role of haem-binding proteins at the interface of host-microbe interactions.

Stephen Tristram lectures in Medical Microbiology in the School of Health Sciences at the University of Tasmania and is an Associate Professor and Academic Lead of the Laboratory Medicine Programs. He has been researching antibiotic resistance and pathogenesis in *Haemophilus influenzae* for over 20 years.