The nasal mutualist *Dolosigranulum pigrum* AMBR11 supports homeostasis via multiple mechanisms

**Highlights**

- Habitat mining reveals that *Dolosigranulum pigrum* prefers the human nasal cavity
- *D. pigrum* AMBR11 has immunomodulatory and nasal barrier-enhancing capacities
- *D. pigrum* AMBR11 exerts antimicrobial activity against *S. aureus*
The nasal mutualist *Dolosigranulum pigrum* AMBR11 supports homeostasis via multiple mechanisms

Ilke De Boeck,1 Stijn Wittouck,1 Katleen Martens,1,2 Irina Spacova,1 Eline Cauwenberghs,1 Camille Nina Allonsius,1 Jennifer Jörissen,1 Sander Wuyts,1 Wannes Van Beeck,1 Jelle Dillen,1 Peter A. Bron,1 Brecht Steelant,1 Peter W. Hellings,1,2 Olivier M. Vanderveken,1,5 and Sarah Lebeer1,6,*

**SUMMARY**

Comparing the nasal microbiome of healthy individuals and chronic rhinosinusitis (CRS) patients revealed *Dolosigranulum pigrum* as a species clearly associated with nasal health, although isolates obtained from healthy individuals are scarce. In this study, we explored the properties of this understudied lactic acid bacterium by integrating comparative genomics, habitat mining, cultivation, and functional characterization of interaction capacities. Mining 10,000 samples from the Earth Microbiome Project of 17 habitat types revealed that *Dolosigranulum* is mainly associated with the human nasal cavity. *D. pigrum* AMBR11 isolated from the nose of a healthy individual exerted antimicrobial activity against *Staphylococcus aureus*, decreased proinflammatory cytokine production in airway epithelial cells, and *Galleria mellonella* larvae mortality induced by this important nasal pathobiont. Furthermore, the strain protected the nasal barrier function in a mouse model using interleukin-4 as disruptive cytokine. Hence, *D. pigrum* AMBR11 is a mutualist with high potential as topical live biotherapeutic product.

**INTRODUCTION**

The microbiome of the upper respiratory tract (URT) has an important gatekeeper function by forming a barrier to potential pathogens and modulating immune responses (Man et al., 2017). The current knowledge on the URT microbiome is mainly based on association studies mapping the occurrence and relative abundance of different bacterial taxa by using culture-independent methods (Gan et al., 2021; Hasegawa et al., 2016; Lappan et al., 2018; Lauffer et al., 2011). These studies have so far primarily revealed that many potential pathogens such as *Streptococcus pneumoniae*, *Moraxella catarrhalis*, *Haemophilus influenzae*, and *Staphylococcus aureus* are often present and prevalent in healthy subjects, implying that the host context and other resident microbiota must play a key role in many URT pathologies. Indeed, it becomes increasingly clear that disorders such as chronic rhinosinusitis (CRS) are not caused by an overgrowth of specific pathogens. For example, we could only link 1 to 2% of the variance in human nasal microbiome profiles to CRS versus health as a predictor in our previous microbiome comparison based on amplicon sequencing of the V4 region of the 16S rRNA gene (De Boeck et al., 2019). However, one of our most striking findings was the identification of potential beneficial taxa of the order Lactobacillales as being more prevalent and abundant in the healthy subjects compared to the CRS patients. This order of lactic acid bacteria (LAB) encompasses taxa from the family of the Lactobacillaceae, including well-characterized lactobacilli (De Boeck et al., 2020), and from the family of Carnobacteriaceae (Lawson and Caldwell, 2014), especially the species *Dolosigranulum pigrum* (De Boeck et al., 2019). Compared to (nasal) lactobacilli that could be cultured and studied in lab, animal and even in human trials (De Boeck et al., 2020; Martens et al., 2021; De Rudder et al., 2020), nasal Carnobacteriaceae turned out to be more difficult to culture and study at functional level. *D. pigrum* was only first described in 1993 as a Gram-positive, catalase-negative bacterium and potentially opportunistic pathogen (Aguirre et al., 1993). Since then, *Dolosigranulum* has been isolated and studied mainly in the context of disease (Laclaire and Facklam, 2000). However, their pathogenic nature is questionable and it is likely that most isolates were coincidently cultivated from some specific infection cases. In addition, several more recent URT comparative microbiome studies have identified this species as being a potentially beneficial taxon (Gan et al., 2019; Lappan et al., 2018). The study of Lappan and colleagues for instance found that the nasopharyngeal microbiome of healthy children had higher abundances of *Dolosigranulum* compared to children with recurrent acute otitis media (Lappan et al., 2018).

*1Department of Bioscience Engineering, University of Antwerp, Groenenborgerlaan 171, 2020 Antwerp, Belgium
2KU Leuven Department of Microbiology, Immunology and Transplantation, Allergy and Clinical Immunology Research Unit, Herestraat 49, 3000 Leuven, Belgium
3Clinical Department of Otorhinolaryngology, Head and Neck Surgery, University Hospitals Leuven, Herestraat 49, 3000 Leuven, Belgium
4Faculty of Medicine and Health Sciences, Translational Neurosciences, University of Antwerp, Universiteitsplein 1, 2610 Antwerp, Belgium
5ENT, Head and Neck Surgery and Communication Disorders, Antwerp University Hospital, 2650 Edegem, Belgium
6Department of Bioscience Engineering, University of Antwerp, Groenenborgerlaan 171, 2020 Antwerp, Belgium

*Correspondence: sarah.lebeer@uantwerpen.be
https://doi.org/10.1016/j.isci.2021.102978

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Similar results were described in healthy adults compared to CRS in patients with nasal polyps (Gan et al., 2019). Nasal colonization with Dolosigranulum has also been associated with a decreased abundance of the important pathobiont S. aureus in adults (Brugger et al., 2020; Escapa et al., 2018; Yan et al., 2013) and in young children (Biesbroek et al., 2014a, 2014b). Unfortunately, most associative microbiota profiling studies lack subsequent functional characterization of individual species. A notable exception is a study by Brugger and colleagues, which reported on in vitro antimicrobial effects of D. pigrum strains against S. aureus (Brugger et al., 2020). Moreover, an inhibition of S. pneumoniae was also observed but this required a combination of D. pigrum and a nasal Corynebacterium. However, most D. pigrum strains used in this study were isolated in the context of disease or infection, and characterization was merely focused on its antimicrobial activity, so that the function and activity beyond its antimicrobial effects as beneficial mutualist remained to be substantiated. Indeed, for a microbe to be considered as a commensal or potential symbiont, its activities should go beyond mere effects on the microbial ecosystem, and direct beneficial effects such as barrier enhancing capacities or immunomodulatory role for the host should also be documented (Eloe-Fadrosh and Rasko, 2013).

In this study, a microbiome comparison of URT samples from 225 CRS patients and 100 healthy controls pointed at the genus Dolosigranulum as the taxon most clearly associated with health in terms of prevalence and abundance. Data mining of public microbiome data substantiated the human nose as the major habitat for this genus, of which D. pigrum is the only currently known species. Subsequently, to mechanistically validate the association of D. pigrum with a healthy URT detected at the microbiome level, we experimentally explored its anti-pathogenic, anti-inflammatory and barrier-promoting beneficial properties. Specific isolates were cultured from healthy noses of healthy individuals and assessed for anti-inflammatory characteristics in respiratory epithelial cells upon coculture with S. aureus, because this is one of the most important pathobionts in the human airways and associated with chronic airway disease (Derycke et al., 2010; Teufelberger et al., 2019). The interaction with the host was further substantiated in animal model studies, revealing that D. pigrum AMBR11 could decrease mortality of Galleria mellonella larvae after S. aureus injection and reduce IL-4-induced nasal barrier dysfunction in mice.

RESULTS

Dolosigranulum pigrum is metabolically active and more prevalent in the healthy URT

We recently established association between URT health and lactobacilli (De Boeck et al., 2020). This triggered us to revisit our observational microbiome study of healthy controls versus CRS patients (De Boeck et al., 2019) with full focus on taxa belonging to the Carnobacteriaceae, because they were even more strongly linked to health based on a compositional differential abundance analysis (De Boeck et al., 2020), but not yet explored at functional level. Only one abundant genus of Carnobacteriaceae was detected, namely Dolosigranulum. The genus Dolosigranulum was detected in the anterior nares of 81% versus only 54% in CRS patients (p < 0.0001). For the nasopharynx, the prevalence of this genus was 78% in healthy adults versus 42% of the CRS patients (p < 0.0001) (Figure 1B). The median relative abundance of Dolosigranulum in the anterior nares was also 3-fold higher in the control group compared to the CRS group (Figure 1C). In the nasopharynx, no significant difference in fold change was observed. As DNA-based technologies, such as the above, cannot discriminate between live and dead bacteria, RNA-based 16S rRNA gene sequencing was also performed on a subset of healthy control samples (Figure 1A). The RNA approach was able to detect this D. pigrum ASV with similar relative abundances (30% and 37% for DNA versus RNA sequencing in anterior nares, and 19% versus 15% for DNA versus RNA in nasopharynx samples, respectively) (Figure 1D), indicating that D. pigrum is not only more abundant in healthy individuals than in CRS patients, but also an active member of the healthy URT bacterial community.

Comparative genomics of healthy donor-isolated Dolosigranulum pigrum

To isolate D. pigrum strains for subsequent functional analyses on potential beneficial properties, samples derived from healthy nasal swabs were cultivated. After 2–3 days of growth, single colonies were used for full length 16S rRNA gene based identification, revealing that larger colonies, typically representing faster growing strains, mainly belonged to Staphylococcus, Streptococcus, and Corynebacterium species. By contrast, two of the smaller-sized colonies were identified as D. pigrum, in line with earlier observations that members of the Carnobacteriaceae display slow growth under laboratory conditions (Afzal et al., 2010). These isolates were designated as Dolosigranulum pigrum AMBR11 and AMBR12 and deposited in the Belgian Culture collection of Microbes (LMG P-31124 and LMG P-31154, respectively). Subsequently,
total DNA of pure cultures was extracted and subjected to whole genome sequencing. *D. pigrum* AMBR11 and AMBR12 were then revealed to harbor genome sizes of 1.88 and 1.90 Mb with GC contents of 39.6% and 39.7%, respectively. These genome features are highly comparable to what was observed earlier for related strains isolated from a disease context (Brugger et al., 2020), where an average genome size of 1.86 Mb was established for the previous 11 strains evaluated. The genome sizes for *Lactobacillales* species with a postulated free-living (e.g. Lentilactobacillus kefiri, 2.4 Mb) or nomadic (e.g. Lacticaseibacillus rhamnosus, 2.8 Mb) lifestyle are typically larger (Figure S1) (Duar et al., 2017). This relatively small genome size suggests a host-adapted lifestyle, in line with other host-adapted members of the lactic acid bacteria, such as *Lactobacillus gasseri* (1.8 Mb). Pairwise-genome comparison revealed average nucleotide identity (ANI) values of 97.7% for both strains with the type strain *D. pigrum* LMG15126, confirming that the isolated strains are appropriately classified as the species *D. pigrum*.

Figure 1. *Dolosigranulum* in the anterior nares and nasopharynx of 100 healthy participants and 225 CRS patients 
(A) Overview of the study groups and collected samples for both sequencing approaches. 
(B and C) 16S V4 rRNA URT microbiome sequencing analyzed for the prevalence (B) and relative abundance (C) of the *Dolosigranulum* ASV in 100 healthy controls versus 225 CRS patients. The means per location and condition are indicated with a black dot. ****p < 0.0001 (Fisher exact test). 
(D) Relative abundance of *Dolosigranulum* in a subset of anterior nares and nasopharynx samples (n = 45) with DNA versus RNA-based 16S rRNA sequencing. p values for comparison of mean relative abundance between DNA versus RNA sequencing were determined using Wilcoxon rank-sum test; n.s. = not significant.
Subsequently, we employed a phylogenomics approach to assess potential clustering of *D. pigrum* strains by body site or disease status, as well as the position of the species within the *Lactobacillales*. This order contains many taxa associated with the human host or with beneficial properties as probiotics (Zheng et al., 2020), but also pathogenic taxa such as *Streptococcus pneumoniae* (Du Toit et al., 2014). Initially, a phylogenetic tree of the order *Lactobacillales* was made, confirming that *Dolosigranulum* is an integral member of the *Lactobacillales* (Figure 2A). However, within this order, *D. pigrum* is only distantly related to well-documented beneficial genera for human health, such as the *Lactobacillus crispatus*, *Lactobacillus gasseri*, *Lactiplantibacillus*, *Latilactobacillus*, *Streptococcus*, *Enterococcus*, *Lactococcus*, *Leuconostoc* and *Pediococcus*.

![Phylogenetic tree](image)

**Figure 2. Comparative genomics of *D. pigrum***

(A) Maximum likelihood phylogenetic tree of *Lactobacillales* inferred on a concatenated alignment of the amino acid sequences of 389 (almost) single-copy core genes. One representative genome per species of *Carnobacteriaceae* was used, and one genome per genus of non-*Carnobacteriaceae*. D = Dolosigranulum pigrum, P = genera that contain documented probiotic strains from *Lactobacillus, Limosilactobacillus, Lacticaseibacillus, Ligilactobacillus, Lactiplantibacillus, Latilactobacillus, Streptococcus, Enterococcus, Lactococcus, Leuconostoc* and *Pediococcus*.

(B) Maximum likelihood phylogenetic tree of *Dolosigranulum pigrum* strains inferred on a concatenated alignment of the nucleotide sequences of the top 1,000 most single-copy core genes. Branches with a SH-aLRT (Shimodaira-Hasegawa approximate likelihood ratio test) support value of ≥ 80% and bootstrap support of ≥ 95% were considered trustworthy and were marked with a filled dot; other branches were marked with an open dot. Healthy isolates are written in bold, other strains were isolated in the context of disease. The two *D. pigrum* strains isolated in this study are indicated with an asterisk. See also Figures S1 and S2.
**Habitat mining of Dolosigranulum pigrum reveals a preference for the human nasal cavity**

We next aimed to explore the habitat range of Dolosigranulum. Because the genus was so distinctly detected in our samples of the anterior nares and nasopharynx (Figure 1), we first explored its presence and relative abundance in other habitats of the human body based on publicly available shotgun sequencing data (Pasolli et al., 2017). In total, 8,184 samples from six different body sites were analyzed, i.e., nasal cavity (n = 93), oral cavity (n = 701), skin (n = 512), stool (n = 6,784), vagina (n = 86), and breastmilk (n = 8). Dolosigranulum was detected in 38 nasal cavity samples (41%), 75 skin samples (15%), one milk sample (12.5%), 36 stool samples (0.53%), one oral cavity sample (0.14%), and not in vaginal samples (Figure 3A). The relative abundance of Dolosigranulum in the nasal cavity was 18% on average, far exceeding the relative abundances found for the other human body sites, e.g. on average 1.28% for skin samples, which had the second highest relative abundances (Figure 3B), reiterating our 16S-based analysis of nasopharynx and anterior nare samples of healthy subjects and CRS patients described above (Figure 1).

Besides the human body, we explored other potential habitats of D. pigrum. Its prevalence and relative abundance was mined in 17 habitat types including free-living environments (e.g. water, sediment, and soil) and host-associated environments (3 different plant and 5 different animal environments) using publicly available microbiome data from the Earth Microbiome Project (EMP), where the largest subset of 10,000 samples was used (Thompson et al., 2017). Dolosigranulum was generally not detected in the environmental samples analyzed (including water, soil, and plant samples), but was present (defined as > 1 reads per 10,000 reads or 0.01% relative abundance) in animal samples (Figure 3C). We therefore further evaluated its prevalence in 43 animal host species based on the EMP dataset, including rodents (Rattus norvegicus), fish (Ictalurus punctatus, Cynnis carpio, Catostomus), birds (Gila robusta), and primates (Pygathrix nemaeus, Nasalis larvatus), but its relative abundance was <1% (Figure 3D). In contrast, in humans and dogs (Canis lupus familiaris), Dolosigranulum was found in high relative abundances in some samples, up to almost 50% (Figure 3D). Interestingly, the samples from dogs with high relative abundances were all annotated as nasal secretions (Gilbert et al., 2014). Thus, our extensive data mining of more than 10,000 publicly available microbiome samples revealed that Dolosigranulum is almost exclusively found in the nasal cavity. Considering its relatively small genome size and its clear association with the human nose as habitat, it is likely that this species has a specific function in this ecosystem.
Antimicrobial properties of *D. pigrum* AMBR11 against *S. aureus*

As a first function screened for, we functionally assessed the potential antimicrobial effects of our healthy isolate *D. pigrum* AMBR11 against *Staphylococcus aureus*, a major URT pathobiont in CRS and also in other chronic airway diseases (Martina et al., 2019; Teufelberger et al., 2019). The AMBR11 isolate was selected for further functional analysis because this strain displayed better growth characteristics than isolate AMBR12 and showed more unique accessory genes (242 compared to 12 for AMBR12). The antimicrobial activity of *D. pigrum* AMBR11 was profiled and benchmarked against the *D. pigrum* type strain LMG15126 (isolated from a human spinal cord autopsy) and against the established effects of model probiotic lactic acid bacteria, namely, the commercial gut probiotic strain *L. rhamnosus* GG (van den Broek et al., 2018) and...
the URT-adapted probiotic candidate L. casei AMBR2 (De Boeck et al., 2020). The two Lacticaseibacillus and two D. pigrum strains tested were able to partially inhibit the growth of S. aureus ATCC29213. D. pigrum displayed the highest activity when expressed per colony-forming unit (CFU) (Figure 4A). Because lactic acid is a hallmark metabolite of lactic acid bacteria (De Keersmaecker et al., 2006), lactic acid production of D. pigrum AMBR11 and the type strain were also compared to L. rhamnosus GG and L. casei AMBR2 after overnight incubation (Figure 4B). In agreement with their classification as LAB within the order Lactobacillales (Figure 2B), D. pigrum AMBR11 and LMG15126 cells produced comparable L-lactic acid levels per CFU to the lactobacilli tested, while only low levels of D-lactic acid were found. This suggests that L-lactic acid and the consequential local low-pH environment could be one of the drivers of the observed S. aureus inhibition by AMBR11, while at the same time it is likely that other, currently unknown factors might explain the superior inhibitory effect observed for D. pigrum.

With the established S. aureus growth-inhibition properties of strain AMBR11 in vitro, we set out to investigate the actual capacity of our strain to reduce S. aureus infection in an animal model employing Galleria mellonella. In this model, the survival percentage of larvae was investigated when D. pigrum AMBR11 and S. aureus ATCC29213 were injected individually, as well as in coculture (Figure 4C). Negative controls (PBS or D. pigrum injection) resulted in high survival rates of the larvae (up to 100%). By contrast, S. aureus...
Figure 5. The effect of *D. pigrum* AMBR11 on the airway epithelial barrier in vitro and in vivo

(A–C) Induction of IL-8 (A), IL-1β (B), and TNF-α (C) mRNA by *S. aureus* as such or upon co-incubation with URT isolate *D. pigrum* AMBR11 or type strain *D. pigrum* LMG15126 with the Calu-3 cells. Bacteria were incubated with the cells for 4 hours in a final concentration of 10⁸ CFU/mL. The presented data are representative of two independent experiments. Data are expressed as means ± standard deviations. Significant differences between all tested conditions were evaluated with one-way ANOVA and multiple t tests in GraphPad Prism. The p value was corrected for multiple comparisons using the Holm-Sidak’s multiple comparisons test. Significant differences compared to *S. aureus* are indicated with an asterisks:**p < 0.01, ***p < 0.001, ****p < 0.0001. Significant differences between all tested conditions are shown in Table S1.

(D) Evaluation of TEER to measure epithelial barrier integrity for 6 hours after addition of *D. pigrum* AMBR11 and *S. aureus* on primary NECs from CRSwNP patients (n = 4). TEER values are expressed as relative percentages against the initial TEER. Bacterial strains were added in a concentration of 10⁷ CFU/mL and TEER was measured every 2 hours. Cells as such were used as negative control. Results are expressed as the mean with standard deviation.

(E) Murine model of IL-4-induced barrier disruption. Mice were endonasally pre-treated two times with *D. pigrum* AMBR11 (10⁷ CFU/ml). 24 hours later, IL-4 was applied three times with one hour interval. 24 hours after the last IL-4 application, FD4 was applied endonasally to evaluate mucosal permeability in the serum of the treated mice. Data are presented as mean ± standard deviation. The Mann Whitney test in Graphpad prism was used for statistical differences.
infection resulted in death of the vast majority of the larvae. The survival percentages of larvae exposed to S. aureus in combination with D. pigrum AMBR11 concentration was substantially improved (p < 0.05), demonstrating its in vivo protective effect.

Anti-inflammatory and barrier-enhancing capacity of D. pigrum AMBR11

Since microbe-microbe effects are insufficient to characterize the mutualistic nature of a microbe, we also studied phenotypes reflecting microbe-host interactions. First, we assessed whether D. pigrum AMBR11, besides the direct antimicrobial activity established above, could also reduce the typical pro-inflammatory cytokine profile induced by S. aureus ATCC29213 (Szkardakiewicz et al., 2012). Possible anti-inflammatory capacities of D. pigrum AMBR11 and type strain D. pigrum LMG15126 were evaluated by assessing their capacity to reduce inflammation induced by S. aureus ATCC29213 in Calu-3 respiratory epithelial cells. This inflammation was reflected by a significantly higher expression of mRNA for the pro-inflammatory markers interleukin (IL)-8, IL-1β, and TNF-α in Calu-3 cells stimulated with S. aureus ATCC29213 compared to D. pigrum AMBR11 and D. pigrum LMG15126 (Figures 5A–5C). Importantly, the expression of both interleukin (IL)-8 and IL-1β was significantly decreased when S. aureus ATCC29213 was co-incubated with D. pigrum AMBR11 or LMG15126, showing that D. pigrum AMBR11 and LMG15126 exerted dampening effects on the inflammation induced by S. aureus.

Subsequently, we studied the host interaction capacity of D. pigrum with the nasal barrier, because restoration of the epithelial barrier can be considered a beneficial homeostasis-promoting property for a nasal probiotic or symbiont (Hellings and Steelant, 2020). Pathogens such as S. aureus are known to disrupt the nasal epithelial barrier, especially via their enterotoxins (Martens et al., 2021) but also other mechanisms have been described— for instance, via serine protease-like proteins (Chandrabalal et al., 2021). Here, we confirmed that administration of live S. aureus on primary NECs from CRS donors was able to disturb the nasal epithelial barrier, reflected via a decrease in relative TEER; however, live D. pigrum AMBR11 did not affect the barrier (Figure 5D). Barrier disruption is also observed independent of S. aureus infection for CRS and also for other URT diseases, such as asthma (Xiao et al., 2011) and allergic rhinitis (Steelant et al., 2016), as a side effect of air pollution (Zhao et al., 2018). Here, we evaluated the barrier-enhancing capacity of D. pigrum in a murine model of IL-4-induced nasal barrier dysfunction (Steelant et al., 2018). Mice were pre-treated twice endonasally with D. pigrum AMBR11 prior to IL-4 application, an agent known to disrupt the epithelial barrier (Steelant et al., 2016). This is reflected by a significant increase in fluorescein isothiocyanate-dextran (FD4) passage for the positive control (PBS + IL-4). Pre-treatment with D. pigrum AMBR11 prevented this IL-4-induced increase in FD4 passage (Figure 5E, p < 0.001). D. pigrum AMBR11 without prior IL-4 treatment did not show significant effects on FD4 passage.

Finally, we explored the activation of some important Toll-like receptors (TLR) involved in epithelial barrier functioning for D. pigrum AMBR11. The results were compared against the D. pigrum type strain and URT pathobiont S. aureus. D. pigrum AMBR11 mainly activated the TLR2/TLR6 heterodimer at a significantly higher level (p < 0.0001) compared to S. aureus (Figure 5F). In contrast, stimulation of TLR2/TLR1 by D. pigrum was significantly lower compared to the pathobiont S. aureus (Figure 5G), showing that D. pigrum rather exploits TLR2/6 interaction mechanisms with host cells.

DISCUSSION

Lactic acid bacteria are among the most studied probiotic bacteria worldwide, mostly for gut and urogenital health. D. pigrum is an underexplored lactic acid bacterium, with the notable exception of some recent microbiome sequencing studies in which this species was associated with healthy airway conditions (Biesbroek et al., 2014b; De Boeck et al., 2019; Bosch et al., 2016; Brugger et al., 2020; Gan et al., 2021). Here, we
gained fundamental insights into the lifestyle and beneficial host interaction capacity of *Dolosigranulum* with a combination of isolation of strains from healthy subjects, comparative genomics, microbiome habitat mining, and functional characterization of antimicrobial and host interactions.

Despite its high abundance in the URT at DNA and RNA level, *Dolosigranulum* cultivation and isolation turned to be more complex than initially anticipated because of frequent cross-contamination with fast-growing species such as *S. aureus* and – in hindsight – also due to its narrow host and habitat range. The latter is also reflected in the small genome sizes of 1.88 Mb and 1.9 Mb of the URT-isolated *D. pigrum* AMBR11 and AMBR12, isolated in this study, and is in agreement with other *D. pigrum* genome sizes (Brugger et al., 2020). This small genome size is related to the postulated host-adapted features, as is typical for commensals and symbionts that often have undergone and are still undergoing evolutionary events that lead to genomic decay and loss of functions that are redundant in the host (Kirchberger et al., 2020). Also in the Lactobacillaceae, a reduced genome size is strongly correlated with obligate host-adaptation (Duar et al., 2017). For instance, the genome of *L. iners* is only 1.27 Mb, reflecting strong adaptation to the human vagina (Macklaim et al., 2011). In addition to a smaller genome size, a reduction in GC content has been associated with host-adaptation in lactobacilli; for instance, 32.5% (*L. iners*), 38.6% (*L. reuteri*), and 43.5% (*Ligilactobacillus ruminus*) (Duar et al., 2017). Here, a GC content of 39.6% was observed in *D. pigrum* AMBR11. As additional level of our ecological and functional characterization, we mined public data of *D. pigrum* in 17 different habitats including more than 10,000 environmental, plant, and animal samples, revealing that *D. pigrum* is highly associated with animal samples and mainly with the human host. Further exploration of this host-adapted nature pointed at an association with the human respiratory tract and nasal cavity. Taken together, our microbiome, genomic, and functional data suggest that *Dolosigranulum* has a mutualistic role in the human nose, justifying further functional research into its role as a beneficial member of the URT.

A first main function of *Dolosigranulum* appears to be inhibiting overgrowth of pathogens. This has already been previously suggested by Brugger et al. (Brugger et al., 2020), but was further refined here in an in vivo model for our isolate *D. pigrum* AMBR11 from a healthy donor. This health-associated strain showed a clear inhibitory effect on the growth of *S. aureus* based on microbe-microbe assays and in the invertebrate Galleria model, because the virulent effects of *S. aureus* in the larvae was dampened upon co-administration of *D. pigrum* AMBR11. This antimicrobial effect is likely related to its lactic acid production, which was in the range of other members of the Lactobacillaceae (van den Broek et al., 2018). Previous research has shown that lactic acid is also able to inhibit the growth of other important URT pathobionts, such as *Moraxella catarrhalis* (van den Broek et al., 2018) and other Gram-negatives (Alakomi et al., 2000). However, other antimicrobial compounds such as bacteriocins (Perez et al., 2014) cannot be ruled out at this stage, as already suggested based on previous analysis on *D. pigrum* strains (Brugger et al., 2020). To distinguish the activity of lactic acid in *D. pigrum* AMBR11 compared to bacteriocins or other antimicrobials, the purification and further identification of such molecules is of interest for future research, for instance, via mass spectrometry methods. Pathogen-inhibiting characteristics for *D. pigrum* strains studied from more disease context were also described by Brugger and colleagues (Brugger et al., 2020) and are in line with what was observed here. Furthermore, the same research group also performed a recent species-level reanalysis of a pediatric nostril microbiota dataset and adult nostril samples and found a negative association between relative abundances of *S. aureus* and *D. pigrum* based on an analysis of composition of microbiomes of microbiome data from pediatric nostril samples.

As another function in addition to these previously suggested antimicrobial effects, we observed anti-inflammatory effects important for *D. pigrum* potentially to maintain homeostasis. Indeed, the inflammatory response against *S. aureus* in the airway epithelial Calu-3 cells was diminished upon coculture with our strain *D. pigrum* AMBR11. This mechanism is in line with the fact that *D. pigrum* was previously associated with URT health and lower inflammation grades based on microbiome studies (Biesbroek et al., 2014b; Gan et al., 2021; Lappan et al., 2018). For example, in young children, *D. pigrum* was found to be significantly higher in the control group compared to children prone to recurrent acute otitis media based on 16S rRNA gene sequencing (Lappan et al., 2018). In adults, a depletion of *Dolosigranulum* was found in the middle meatus of CRS patients with nasal polyps compared to a control group (Gan et al., 2019). In these patients, an important role for *S. aureus* and its enterotoxins was established in the disease pathology and associated inflammation, further adding support for a negative association between *Dolosigranulum*
and *S. aureus*. Our experiments complement these earlier microbiome association studies and extend these with data on the anti-inflammatory potential of *D. pigrum* against *S. aureus*-induced inflammatory responses.

Furthermore, we could also show that the host interaction and nasal homeostasis-promoting capacity of *D. pigrum* involves the nasal epithelial barrier function. Barrier-enhancing effects in gut and skin epithelial cells are well documented for several LAB strains (Anderson et al., 2010; O’Neill et al., 2013; Orlando et al., 2014). For the nose, disruptions in epithelial barrier integrity have been described for chronic airway diseases such as CRS and allergic rhinitis, with among others, an important role for *S. aureus* because of its enterotoxins (Martens et al., 2021). Here, it is thus of interest that we found a protective effective of *D. pigrum* AMBR11 in a murine model of IL-4-induced barrier disruption. To the best of our knowledge, documentation on such barrier-enhancing effects on the URT epithelium is scarce. Recently, we have also found barrier-protective effects for the URT isolate *L. casei* AMBR2 (Martens et al., 2021). Notably, compared to *D. pigrum*, *L. casei* has a weaker association with the nose as its main habitat based on its lower prevalence and relative abundance in the URT (De Boeck et al., 2020) and its association with more habitats as nomadic species (Duar et al., 2017). Both immune signaling and pathogen killing likely play a role in the beneficial action of *D. pigrum*; however, understanding the detailed role of each of these mechanisms would require detailed dedicated research at the molecular level. TLR activation by *D. pigrum* was also explored in the present study, as these receptors are involved in epithelial barrier functioning, with TLR2/6 activation generally linked to barrier-enhancing and anti-inflammatory signaling effects; however, TLR2/1 is rather involved in inflammation and barrier-disruptive effects (Martens et al., 2021, 2021; Yuki et al., 2011). We observed that *D. pigrum* strongly activated the TLR2/TLR6 heterodimer, even stronger than *L. casei* AMBR2 (Martens et al., 2021) and *S. aureus*, the latter mainly inducing TLR1/6.

Taken together, the data on the anti-inflammatory and barrier-enhancing properties of the health-associated strain *D. pigrum* AMBR11 are important to substantiate a role for Dolosigranulum as nasal mutualist. The previous documented antimicrobial properties of *D. pigrum* (Brugger et al., 2020) were insufficient to establish such role, because several pathogens such as Candida albicans and *S. aureus* can overgrow their habitat by having antimicrobial properties against the resident microbiota, in addition to having toxic or barrier-disruptive effects such as invading target cells. Whether the previously described antimicrobial Dolosigranulum strains derived from a diseased context (Brugger et al., 2020) are also capable of preventing barrier disruption by pathogenic species and effectively protecting against *S. aureus* infection *in vivo* remains to be established, considering that anti-inflammatory properties are highly variable at the strain level (Lebeer et al., 2018; Lee et al., 2013), but also that isolation from a disease context does not imply a causative role in disease. Nevertheless, from a safety perspective, one could argue that *D. pigrum* AMBR11 isolated from a healthy person would represent a more appropriate candidate for development into a topical next-generation probiotic or live-biotherapeutic product. Such formulation might be of interest to a broad population, because Dolosigranulum is detected in high abundances, with relative abundance up to 50% observed in certain individuals, either adults (De Boeck et al., 2019; Brugger et al., 2020; Escapa et al., 2018; Gan et al., 2021; Yan et al., 2013) or children (Biesbroek et al., 2014a, 2014b). A broad range of patients suffering from diverse URT diseases, including CRS in adults or otitis media in children, might benefit from *D. pigrum* AMBR11 supplementation, but this of course requires extensive safety and efficacy evaluation in follow-up clinical trials.

**Limitations of the study**

One limitation of this study is that the suggested *D. pigrum* AMBR11 mechanisms of action responsible for its observed beneficial effects require more detailed investigation to unravel their exact contribution to nasal homeostasis. The genomic analysis of *D. pigrum* and the different mechanisms proposed in our work pave the way for more dedicated experimental design, and subsequent studies can, for instance, focus on molecular insights that can be obtained via purification of antimicrobial molecules produced by Dolosigranulum. Alternatively, the possibility to construct gene deletion mutants could be investigated, allowing direct comparison of such constructed mutant and its parental strain for relevant phenotypes, thereby directly establishing the relevance of the functionality encoded by the disrupted gene to nasal health. In addition, the exact role of Dolosigranulum on the airway epithelial barrier is an interesting topic for follow-up research to generate more mechanistic insights on, for instance, the tight junction proteins that are involved. Finally, the main focus here was on one isolate, *D. pigrum* AMBR11. The isolation of other
healthy isolates and the comparison of their activity with D. pigrum AMBR11 will also help to further understand the role of this microbe in airway health.

**STAR+METHODS**

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**SUPPLEMENTAL INFORMATION**

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2021.102978.

**ACKNOWLEDGMENTS**

This research was funded by a grant from the Flanders Innovation and Entrepreneurship Agency [IWT-SBO ProCure project (IWT/50052)], by the personal grants of I.D.B. 1517916N and S.W. 11A0618N, and by the European Research Council grant of S.L. (42/FA0/0500/8330). The authors want to thank the entire research group ENdEMIC of the University of Antwerp. They also want to thank the entire ENT department of the Antwerp University Hospital and University Hospitals of Leuven, the Center of Medical Genetics, and all volunteers that participated. Finally, the authors want to thank all partners of the IWT-SBO ProCure project. Graphical abstract created with BioRender.com.

**AUTHOR CONTRIBUTIONS**

Conceived and designed the study: IDB, SL. Designed the experiments: IDB, CA, IS, SL. The experiments were performed by IDB, CA, KM, and EC. Data analysis and interpretation was done by IDB, CA, KM, BS, IS, EC, JJ and PAB. Bioinformatics analysis and writing of the scripts by SaW, StW, WvB, and JD. PH and OV were responsible for the clinical assessments and interventions. IDB and SL wrote the first draft, PAB revised the manuscript and all authors read the final version.

**DECLARATION OF INTERESTS**

A patent application (PCT/EP2019/086763) has been filed on 20 December 2019 related to this work. P.B. is a consultant for multiple companies in the food and health industry, but they were not involved in this manuscript. The remaining authors have no conflicts of interest to declare.
INCLUSION AND DIVERSITY

We worked to ensure gender balance in the recruitment of human subjects. We worked to ensure that the study questionnaires were prepared in an inclusive way.

Received: May 7, 2021
Revised: July 28, 2021
Accepted: August 10, 2021
Published: September 24, 2021

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**STAR METHODS**

**KEY RESOURCE TABLE**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Bacterial strains** |        |            |
| Lactobacillus rhamnosus GG | ATCC | ATCC53103  |
| Lactobacillus casei AMBR2 | LAMB BCCM/LMG P-30039 | ENA: Study PRJEB21025, accession GCA_900185125 |
| Dolosigranulum pigrum AMBR11 | LAMB BCCM/LMG P-31124 | ENA: Study PRJEB32716, accession GCA_901830375 |
| Dolosigranulum pigrum AMBR12 | LAMB BCCM/LMG P-31154 | ENA: Study PRJEB32716, accession GCA_905071805 |
| Dolosigranulum pigrum LMG15126 | | NZ_AGEF0000000.1 |
| Staphylococcus aureus | ATCC | ATCC29213  |
| **Biological Samples** |        |            |
| adult human nasopharyngeal swabs | University of Antwerp, University Hospitals of Leuven | N/A |
| **Chemicals, Peptides, and Recombinant Proteins** |        |            |
| de Man, Rogosa and Sharpe (MRS) | Difco | BD288210  |
| Mueller Hinton | LabM limited | LAB039-A |
| Brain Hart Infusion (BHI) | International Medical Products | 8532134 |
| Tween 80 (polysorbate) | Carl Roth Belgium | 9139.1 |
| Sheep blood defibrinated | bioTRADING | BTSG100 |
| Tryptic Soy Agar | Merck Life Science B.V. (Sigma) | 22091-500G |
| Minimal Essential Medium (MEM) | Life Technologies | 31095-029 |
| Fetal Calf Serum (FCS) | HyClone | 12350273 |
| Penicillin-streptomycin (100U/mL) | Gibco | 15140122 |
| Trypsin EDTA (0.25%) | Gibco | 25200072 |
| Dulbecco’s Modified Eagle Medium (DMEM) | Life Technologies | 31053028 |
| Lysozyme | Sigma Aldrich | L6876-5G |
| mutanolysin (100 U/mL) | Sigma Aldrich | M9901-10KU |
| PBS | Gibco | 14040-091 |
| Readyscript cDNA synthesis mix | Sigma Aldrich | RDRT-500RXN |
| PowerSYBR® Green PCR Master Mix | Applied Biosystems | 13266519 |
| 0.1% hexetidine (Hextril®) | Famar Orléans | N/A |
| **Critical Commercial Assays** |        |            |
| NucleoSpin 96 Tissue kit | Machery-Nagel | MN 740609.50 |
| Nextera XT DNA Sample Preparation kit | Illumina | TG-131-1096 |
| QIAamp Powerfcal DNA kit | Qiagen | 12830-50 |
| Agencourt AMPure XP | Beckman Coulter | A63881 |
| RNeasy Mini kit | Qiagen | 74104 |
| Roche Yellow line kit D/L lactic acid | Roche | 11112821035 |
| **Deposited Data** |        |            |
| Lactobacillus casei AMBR2 | (Wuyts et al., 2017) | ENA: Study PRJEB21025, accession GCA_900185125 |
| Dolosigranulum pigrum AMBR11 | This paper | ENA: Study PRJEB32716, accession GCA_901830375 |
| Dolosigranulum pigrum AMBR12 | This paper | ENA: Study PRJEB32716, accession GCA_905071805 |
| DNA-Seq data study B300201524257 | (De Boeck et al., 2017, 2019) | ENA: PRJEB23057, PRJEB30316 |

(Continued on next page)
### REAGENT or RESOURCE SOURCE IDENTIFIER

#### Experimental Models: Cell Lines

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Calu-3 | ATCC | HTB-55 |
| HEK-Blue™ hTLR2-TLR1 | Invivogen | Cat code: hkb-hlrl21 |
| HEK-Blue™ hTLR2-TLR6 | Invivogen | Cat code: hkb-hlrl26 |

#### Experimental models: Organisms/strains

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Galleria mellonella | Anaconda reptiles (Kontich, Belgium) | N/A |
| BALB/c mice | Envigo (Horst, The Netherlands) | N/A |

#### Oligonucleotides

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| 16S rRNA gene (27F) | AGAGTTTGATCMTGGCTCAG | (Lane, 1991) |
| 16S rRNA gene (1492R) | GGTACCTTGTTAGCAGGTAG | (Turner et al., 1999) |
| CYC1-F (qPCR) | CATGCCAGATAGCCAAGGA | (Moretti et al., 2019) |
| CYC1-R (qPCR) | CTTGTGCGCCGTTTATGGTGTAG | (Moretti et al., 2019) |
| ATP5B-F (qPCR) | GCAGGAAAGAATTACCACCTACCAAG | (Moretti et al., 2019) |
| ATP5B-R (qPCR) | TGGTAGCATCCAATAATGGGCAA | (Moretti et al., 2019) |
| IL1β-F (qPCR) | TGGCTCAAGTGTCTGAAGCAGC | (Moretti et al., 2019) |
| IL1β-R (qPCR) | CAAGTCATCCTCATTGCCACTG | (Moretti et al., 2019) |
| IL8-F (qPCR) | TGGCACCTCCTTGATTCTTCTTCT | (Moretti et al., 2019) |
| IL8-R (qPCR) | TTAGCAGCTCTTGGCAAAACTG | (Moretti et al., 2019) |
| TNF-F (qPCR) | CCTCTGATGGCAGGTAGG | (Moretti et al., 2019) |
| TNF-R (qPCR) | CCTCCTGACCCGAGTGA | (Moretti et al., 2019) |

#### Software and Algorithms

| SOFTWARE | SOURCE | IDENTIFIER |
|----------|--------|------------|
| GraphPad Prism | GraphPad Software | https://www.graphpad.com/ |
| DADA2, version 1.6.0 | https://doi.org/10.13032/2883.52 | https://github.com/benjneb/dada2/index.html |
| R version 3.6.3 | R Core Team (2020) | https://www.r-project.org/ |
| Tidyamplicons | Wittouck, 2020 | github.com/swittouck/tidyamplicons |
| Phyloseq | https://doi.org/10.1371/journal.pone.0061217 | https://github.com/joey711/phyloseq |
| Biom python package | McDonald et al., 2012 | https://biom-format.org/documentation/biom_format.html |
| Genome Taxonomy Database (GTDB) | Parks et al., 2020 | https://gtdb.ecogenomic.org/ |
| Prodigal v2.6.3 | Hyatt et al., 2010 | https://github.com/hyattpd/Prodigal/releases |
| Progenomics version 13b9be1 | Wittouck et al., 2019 | https://github.com/swittouck/SCARAP |
| trimal v1.4.rev15 | Capella-Gutie´ rrez et al., 2009 | https://github.com/scapella/trimal |
| IQTREE v1.6.11 | Hoang et al., 2018; Kalyaanamoorthy et al., 2017; Nguyen et al., 2015 | http://www.iqtree.org/ |
**RESOURCE AVAILABILITY**

**Lead contact**

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Sarah Lebeer (sarah.lebeer@uantwerpen.be).

**Materials availability**

All unique/stable reagents generated in this study are available from the Lead Contact with a completed Materials Transfer Agreement.

**Data and code availability**

- All sequencing data were deposited in ENA (https://www.ebi.ac.uk/ena) under accession numbers PRJEB23057 and PRJEB30316.
- *D. pigrum* AMBR11 and *D. pigrum* AMBR12 were deposited in ENA under accession number GCA_901830375 and GCA_905071805.
- All scripts for the EMP data exploration can be found at https://github.com/swittouck/dolosigranulum_pigrum.
- All scripts for the phylogenomic and pangenome analyses can be found at https://github.com/swittouck/dolosigranulum_pigrum.

**EXPERIMENTAL MODEL AND SUBJECT DETAILS**

**Human subjects**

Nasopharyngeal swabs from human subjects, aged between 18 and 65 years, were collected in a standardized way by the responsible ear, nose, and throat (ENT) specialist from healthy participants (n = 100, 39% male, average age 34 years) and CRS patients (n = 225, 63% male, average age 42 years) at the University of Antwerp, the Antwerp University Hospital and the University Hospital of Leuven between 2015 and 2018, as previously described (De Boeck et al., 2017, 2019). The study was approved by the Ethics Committee of the Antwerp University Hospital/University of Antwerp (B300201524257).
Written informed consent was obtained from all participants prior to sampling.

**Microbial strains and culturing methodology**

Microbial strains used are listed in the key resources table. *Dolosigranulum* strains were grown at 37°C under shaking conditions in BHI broth, supplemented with 0.5% Tween 80. Lactobacilli were grown at 37°C without agitation in de Man, Rogosa and Sharpe (MRS) broth (Difco). *S. aureus* was grown at 37°C without agitation in Mueller Hinton (MH) broth (LabM Limited).

**Cell lines**

The Calu-3 ATCC® HTB-55™ (purchased from ATCC) cell line was cultured at 37°C with 5% CO₂ and 90% relative humidity in 75 cm² cell tissue flasks containing 20 mL Minimal Essential Medium (MEM) (Life technologies) supplemented with 10% heat inactivated fetal calf serum (FCS) (HyClone) and penicillin-streptomycin (100 U/mL) (Life technologies). Every three to four days, the culture medium was changed and cells were reseeded when 70–80% confluency was reached at a 1:2 split ratio using a 0.25% trypsin-EDTA solution (Life Technologies). Calu-3 cells were seeded at a density of 3 × 10⁵ cells/cm² (1.1 × 10⁶ cells/mL) in 12- or 24-well culture plates (Cellstar) for adhesion and immunomodulation experiments, respectively. Approximately one week after seeding, confluent monolayers were obtained. One day before each experiment, medium was replaced to MEM without any supplements.

HEK-Blue™ hTLR2-TLR1 and the HEK-Blue™ hTLR2-TLR6 reporter cell line (Invivogen) were cultured at 37°C with 5% CO₂ and 90% relative humidity in 75 cm² cell tissue flasks containing 20 mL in Dulbecco’s Modified Eagle Medium (DMEM). Prior to an experiment, cells were seeded in a 96-well plate at a concentration of 2.5 × 10⁵ cells/well and incubated for 48 hours at 37°C with 5% CO₂.

**Galleria mellonella**

*G. mellonella* were purchased from Anaconda reptiles (Kontich, Belgium) in their final larval stage. Upon arrival, the larvae were stored at 4°C and used within 7 days. Fifteen randomly selected larvae were used per group.

**In vivo animal model**

Male BALB/c mice (6–8 weeks) were obtained from Envigo (Horst, The Netherlands) and were kept under conventional conditions at the Animal facility of KU Leuven. Experimental procedures were approved by the Ethical Committee for Animal Research at the KU Leuven (P150/2017).

**METHOD DETAILS**

**Microbiome study and Illumina MiSeq 16S rRNA amplicon sequencing**

Samples were processed, sequenced and analyzed as previously described (De Boeck et al., 2017). Briefly, dual-index paired-end sequencing was performed on the V4 region of the 16S rRNA gene on a MiSeq Desktop sequencer (M00984, Illumina) at the Centre of Medical Genetics, University of Antwerp, Belgium. After sequencing, raw sequencing reads were filtered and denoised using DADA2 (v 1.1.6). Reads with more than 2 expected errors were removed. All sequencing data were deposited in ENA under accession numbers PRJEB23057 and PRJEB30316.

**Analysis of public data sets for Dolosigranulum**

Processed OTU-table and sample metadata from the Human Microbiome Project (HMPv35) (Consortium et al., 2012) and the shotgun metagenomic datasets were retrieved using the MicrobeDS R package and curatedMetagenomics R package (Pasolli et al., 2017), respectively. All data was loaded, processed and visualized in the R-environment using Phyloseq to evaluate the prevalence and relative abundance of *Dolosigranulum*.

For the exploration of *Dolosigranulum* presence and relative abundance in various environmental, plant, and animal samples, the following version of the Earth Microbiome Project data was downloaded (Gilbert et al., 2014): EMP release 1, subset of 10,000 samples, rarefied to 10,000 reads per sample and classified with release 123 of the SILVA 16S reference database. Relative abundance data and taxon metadata (e.g., taxonomic classification) were downloaded as a biom file, while sample metadata (e.g., sampling
location) was downloaded as a tsv file. The relative abundance data and taxon metadata were extracted from the biom file using the biom python package (McDonald et al., 2012). Abundance data, taxon metadata and sample metadata were then integrated and visualized using tidyamplicons version 0.2.0 (github.com/swittouck/tidyamplicons).

**Phylogenomic and pangenome analyses**

For the Lactobacillales dataset, one reference genome per species of Carnobacteriaceae and one reference genome per genus of non-Carnobacteriaceae was selected using the GTDB (Parks et al., 2020). Selected genomes were then downloaded from GenBank and genes were predicted with Prodigal v2.6.3 (Hyatt et al., 2010). The pan genome was inferred with Progenomics version 13b9be1 (Wittouck et al., 2019) and genes present in more than 95% of genomes were considered core. A concatenated alignment of the amino acid sequences of the core genes was then built and trimmed v1.4.rev15 (Capella-Gutierrez et al., 2009) was used to remove columns where more than 5% of the genomes had a gap. Finally, a maximum likelihood phylogenetic tree was inferred on the trimmed alignment with IQTREE v1.6.11 (Hoang et al., 2018; Kalyaanamoorthy et al., 2017; Nguyen et al., 2015), using the LG + F + I + G4 model for substitution rates, amino acid frequencies and among-site heterogeneity. The tree was visualized with ggtree v2.0.2 (Yu et al., 2017). Basic file parsing and data exploration and visualization were performed with R v3.6.3 (R Core Team, 2020) and the packages tidyverse v1.3.0 (Wickham et al., 2019), tidygenomes v0.1.3 (Wittouck, 2020b), ggtree v2.0.2 (Yu et al., 2017) and phangorn v2.5.5 (Schliep et al., 2017).

For the D. pigrum dataset, the same pipeline was used, with the following changes. All genomes annotated by the GTDB as D. pigrum were selected (including D. pigrum AMBR11), and MAGs reconstructed by Nayfach et al. (2020) identified as D. pigrum were added. The occurrence of all genes in these genomes and the completeness values of the genomes were jointly estimated by a log likelihood optimization procedure. Genes were considered core if their occurrence as estimated by this procedure was 99% or greater. Other changes with respect to the Lactobacillales tree were that the top 1,000 single-copy core genes were selected for tree inference, nucleotide sequences instead of amino acid sequences were aligned and alignment positions with a gap in more than 50% of the genomes were trimmed. The GTR + F + I + G4 model was used for tree inference.

**Isolation and whole genome sequencing of Dolosigranulum pigrum AMBR11 and AMBR12 from the healthy URT**

Nasopharyngeal swabs from healthy volunteers were cultivated in liquid brain heart infusion (BHI) broth (International Medical Products), supplemented with 0.5% (v/v) Tween 80 (polysorbate) at 37°C to promote growth of Dolosigranulum species. Grown cultures were stored at −80°C in 25% v/v glycerol until further identification. Next, bacterial stocks were cultivated on tryptic soy agar (TSA) supplemented with 5% sheep blood (bioTRADING). Single colonies were further identified by PCR and Sanger sequencing further identification. Next, bacterial stocks were cultivated on tryptic soy agar (TSA) supplemented with 0.5% (v/v) Tween 80 (polysorbate) at 37°C. The pangenome was inferred with Progenomics version 13b9be1 (Wittouck et al., 2019) and genes present in more than 95% of genomes were considered core. A concatenated alignment of the amino acid sequences of the core genes was then built and trimmed v1.4.rev15 (Capella-Gutierrez et al., 2009) was used to remove columns where more than 5% of the genomes had a gap. Finally, a maximum likelihood phylogenetic tree was inferred on the trimmed alignment with IQTREE v1.6.11 (Hoang et al., 2018; Kalyaanamoorthy et al., 2017; Nguyen et al., 2015), using the LG + F + I + G4 model for substitution rates, amino acid frequencies and among-site heterogeneity. The tree was visualized with ggtree v2.0.2 (Yu et al., 2017). Basic file parsing and data exploration and visualization were performed with R v3.6.3 (R Core Team, 2020) and the packages tidyverse v1.3.0 (Wickham et al., 2019), tidygenomes v0.1.3 (Wittouck, 2020b), ggtree v2.0.2 (Yu et al., 2017) and phangorn v2.5.5 (Schliep et al., 2017).

Time course analysis of the antimicrobial activity against S. aureus and determination of lactic acid concentration in supernatant

A time course analysis of the antimicrobial activity against S. aureus was performed as described previously (De Keersmaecker et al., 2006) with minor modifications. Briefly, an overnight culture of S. aureus was added to the wells of a microtitreplate in a 100-fold dilution and supplemented with cell-free supernatant (CFS) of the LAB under study (i.e., Lacticaseibacillus and Dolosigranulum species). CFS was obtained by centrifugation of a LAB overnight culture grown for 10 min at 2000 g at 4°C. Afterwards, the CFS was filter sterilized (0.20 µm cellulose acetate) and the concentration of lactic acid in CFS was measured with the commercially available Roche Yellow line kit (Roche, Basel, Swiss). Hexetidine (0.1%) was used as positive control. MRS brought to pH 4.3 and BHI +0.5% Tween were used as negative controls for Lacticaseibacillus...
and Dolosigranulum, respectively. S. aureus was grown for 30 h, and the optical density (OD) was measured every 30 min at 595 nm using a Synergy HTX multi-mode reader (Biotek, Drogenbos, Belgium). Each condition was measured at least in triplicate and the average OD was calculated.

**Induction of cytokine gene expression in human Calu-3 cells at mRNA level**

One mL of the bacterial suspensions at a concentration of \(10^8\) CFU/mL in MEM was added to tissue culture plates containing Calu-3 cells. Bacteria were incubated for four hours with the cells at \(37^\circ\)C with \(5\%\) CO\(_2\) and \(90\%\) relative humidity to induce cytokine gene expression. After incubation, cells were rinsed three times with prewarmed PBS. MEM was used as negative control. RNA was extracted using the commercially available RNeasy Mini kit (Qiagen), according to the manufacturer’s protocol and stored at \(-80^\circ\)C. 1 mg of isolated RNA, quantified with Take3 (Biotek), was used for cDNA synthesis using ReadyScript\textsuperscript® cDNA synthesis mix (Sigma Aldrich). Expression of the reference genes CYC-1 and ATP5B and the expression of genes for cytokines IL-8, IL-1\(\beta\), and TNF-\(\alpha\) was quantified by RT-qPCR on a StepOne Plus Real-Time PCR System. Primers were designed on the basis of published sequences (Moretti et al., 2019) and chemically synthesized by Integrated DNA Technologies (IDT) (key resources table). Each cDNA sample was amplified in duplicate with PowerSYBR\textsuperscript® Green PCR Master Mix (Applied Biosystems) in a total volume of 20 \(\mu\)L with 0.15 \(\mu\)M of each primer, 40 ng of cDNA and nuclease-free water. Data are presented as the ratio of the amount of cytokine mRNA to the amount of reference mRNA. Non-template controls (PCR grade water) were included for each run and could not have a Ct value below 35, which was used as threshold for the cytokine analysis. Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines were followed (Bustin et al., 2009).

**HEK-Blue\textsuperscript™ hTLR2/6 and hTLR2/1 induction experiments**

The ability of bacterial strains to stimulate the TLR2 dimers of TLR2/1 and TLR2/6 was estimated through the use of the HEK-Blue\textsuperscript™ hTLR2-TLR1 and the HEK-Blue\textsuperscript™ hTLR2-TLR6 reporter cell line (Invivogen). These cells produce secreted embryonic alkaline phosphatase in response to TLR2-TLR1 or TLR2-TLR6 stimulation. Bacterial strains were brought to a final concentration of \(10^7\) CFU/mL in DMEM and UV-inactivated by a four-time exposure to UV for 15 min. Afterwards, UV-inactivated cells were resuspended and diluted to a final concentration of 50 ng/mL, and used as a positive control. The cells were incubated with the UV-inactivated strains for 24 h at \(37^\circ\)C, 5\% CO\(_2\). The embryonic alkaline phosphatase secreted by the cells was quantified by adding 50 \(\mu\)L of supernatant of each well (in duplicate) to 100 \(\mu\)L of substrate solution (1.5 mg/mL pNPP, 150 mM Tris-HCl, 150 mM NaCl and 7.5 mM MgCl\(_2\) at pH 9.5). After 20 min of incubation shielded from light, absorbance was measured at 405 nm using a Synergy HTX multi-mode reader.

**Galleria mellonella survival assay**

To evaluate the safety and tolerability of D. pigrum AMBR11, the larvae were injected in their last prolegs with 10 \(\mu\)L of bacterial solution at different concentrations using a Hamilton syringe (Hamilton Company). Two control groups were used, one injected with PBS (10 \(\mu\)L) and one without injections to control for general viability. The larvae were kept on petridishes at \(37^\circ\)C and monitored daily for survival.

**In vivo effect of D. pigrum AMBR11 in a murine model of IL-4-induced barrier dysfunction**

Mice (n = 6 per condition) were pretreated endonasally with 20 \(\mu\)L of D. pigrum AMBR11 in PBS at a concentration of \(10^7\) CFU/mL, 48h and 24h prior to IL-4 application. Next, with one-hours interval, mice received three times 50 \(\mu\)L of IL-4 (250 ng) or PBS. 24 hours after the last nasal application, 20 \(\mu\)L FD4 (50 mg/mL PBS, Sigma-Aldrich) was applied endonasally for the evaluation of mucosal permeability. One hour after FD4 application, mice were sacrificed through intraperitoneal injection of Doletal (Vétoquinol S.A., Lure, France). Serum and nasal mucosa were collected for further analysis. Levels of FD4 were determined in the serum by a fluorescence reader (FLUOstar Omega).

**QUANTIFICATION AND STATISTICAL ANALYSIS**

Statistical details of experiments can be found in the figure legends, including the statistical tests used, exact value of \(n\), the definition of center, and dispersion and precision measures. Statistical analysis of microbiome data was performed in R using Fisher’s exact test for prevalence and Wilcoxon rank-sum test for relative abundance. In vitro and in vivo experimental data were plotted in GraphPad Prism 7.00 and are
represented as mean values ± standard deviation. One-way ANOVA and t test (for parametric data)/Mann Whitney test (for non-parametric data) were used in GraphPad Prism 8.4.3 for respectively multiple and pairwise statistical comparisons. The p value was corrected for multiple comparisons using the Holm-Sidak method in GraphPad Prism. The survival curves for Galleria mellonella were plotted, and statistical analysis was performed via a GraphPad Prism 8.4.3 using the de Log rank (Mantel-Cox) test. Where applicable, this test was preceded by the Bonferroni correction for multiple comparisons.