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Lactobacilli Have a Niche in the Human Nose

Highlights

- Lactic acid bacteria are enriched in the healthy human nose and nasopharynx
- *Lactobacillus casei* AMBR2 is functionally adapted to the upper respiratory tract
- *L. casei* AMBR2 has antimicrobial and immunomodulatory properties
- Live *L. casei* AMBR2 is safe for intranasal application in healthy humans

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In Brief

De Boeck et al. show that lactobacilli are decreased in patients with chronic rhinosinusitis and enriched in healthy controls. They isolate one strain adapted to the nasopharyngeal mucosa that shows beneficial probiotic effects and safety in the form of a nasal spray in healthy volunteers.

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Lactobacilli Have a Niche in the Human Nose

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SUMMARY

Although an increasing number of beneficial microbiome members are characterized for the human gut and vagina, beneficial microbes are underexplored for the human upper respiratory tract (URT). In this study, we demonstrate that taxa from the beneficial *Lactobacillus* genus complex are more prevalent in the healthy URT than in patients with chronic rhinosinusitis (CRS). Several URT-specific isolates are cultured, characterized, and further explored for their genetic and functional properties related to adaptation to the URT. Catalase genes are found in the identified lactobacilli, which is a unique feature within this mostly facultative anaerobic genus. Moreover, one of our isolated strains, *Lactobacillus casei* AMBR2, contains fimbriae that enable strong adherence to URT epithelium, inhibit the growth and virulence of several URT pathogens, and successfully colonize nasal epithelium of healthy volunteers. This study thus demonstrates that specific lactobacilli are adapted to the URT and could have a beneficial keystone function in this habitat.

INTRODUCTION

The upper respiratory tract (URT) is a crucial barrier with the external airborne environment. It is home to an important part of the commensal microbiota, which is essential for URT homeostasis and its optimal functioning (reviewed in Man et al., 2017). Although the development of next-generation sequencing techniques has revealed which bacteria are present in the different URT niches (De Boeck et al., 2017; Ramakrishnan et al., 2013; Stearns et al., 2015), most studies are observational without functional exploration of the role of specific bacterial members in URT health and disease. A new understanding of the beneficial functions of these commensals is required for the design of targeted approaches to modulate the bacteria in the URT and to prevent or treat symptoms. For example, chronic rhinosinusitis (CRS) affects more than 11% of the European population and causes a major burden on public health (Fokkens et al., 2020). The disease is often treated with antibiotics, but their effectiveness is questionable because the involvement of specific pathogenic bacteria as disease modulators of CRS is still controversial (Hellings et al., 2017). Moreover, if the disease and/or its persistence is rather the result of polymicrobial microbiome disturbances than single pathogens, alternative strategies such as microbiome modulation should be evaluated. One approach could consist of the addition of probiotics, i.e., live micro-organisms that, when administered in adequate amounts, confer a health benefit on the host (Hill et al., 2014). Although probiotics have been widely explored in gastrointestinal applications, studies on their importance, habitat adaptation, and use for topical applications outside the gut, for instance in the URT, are still very limited.

Here, we investigated whether lactobacilli, which are important taxa in several habitats of the human body, could also be important for the URT. We started by sequencing and phylogenetic placement of the amplicon sequence variants (ASVs) of the *16S rRNA* V4 gene region of URT samples taken from 100 healthy volunteers and 225 patients with CRS. We then aimed to cultivate lactobacilli from different healthy URT samples and implemented a combination of genomic and functional *in vitro* and *in vivo* analyses to explore their habitat adaptation to the human URT and their potential as URT probiotic. The colonization capacity of one nasopharynx-isolated strain of the lactic acid

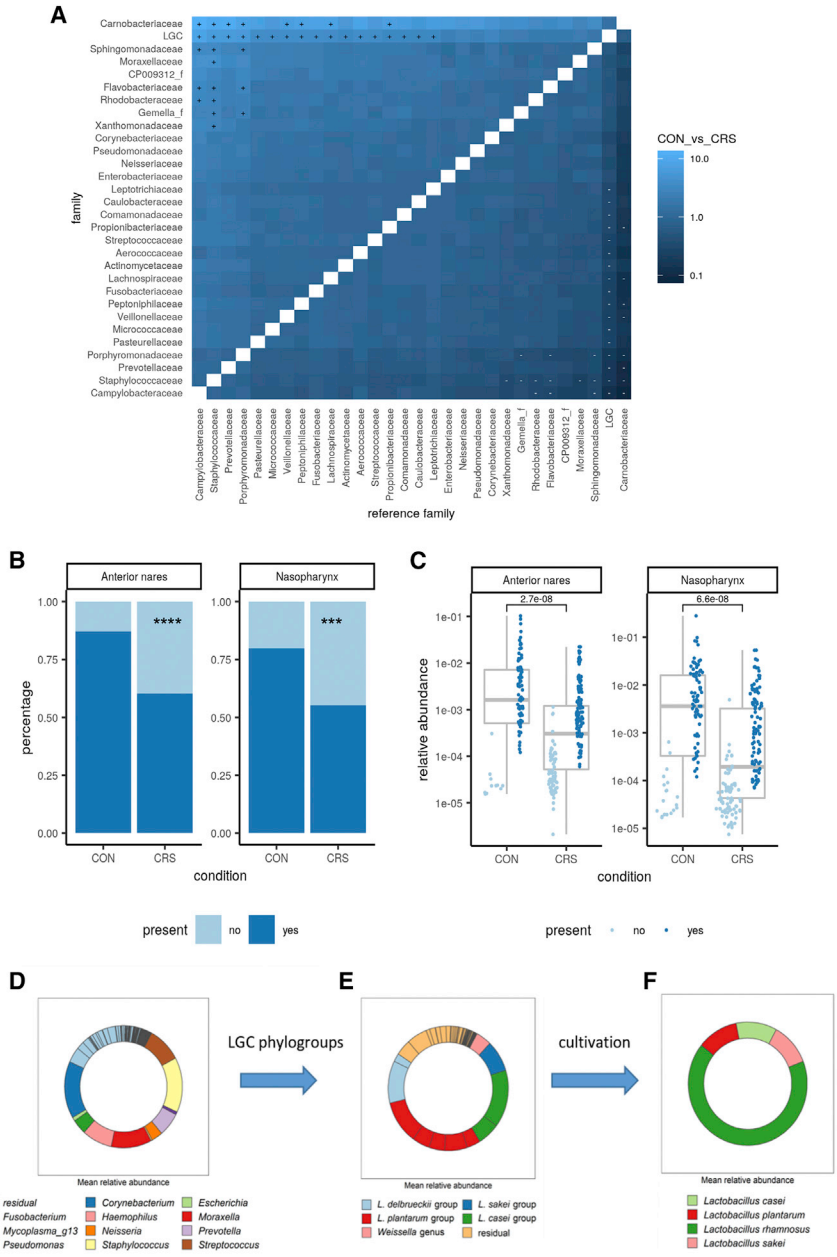

Figure 1. Prevalence and Abundance of Lactobacilli in the URT of Healthy Controls and CRS Patients

(A) Compositional differential abundance of the 30 most prevalent families between the healthy control and CRS samples in the anterior nares. Each tile shows an estimate of the differential abundance of a bacterial family on the y axis, relative to a family on the x axis. A plus/minus sign means that the family is significantly more/less abundant in healthy controls compared with CRS patients, with a false discovery rate of 10%. See also Figure S1 for the nasopharynx.

(B) Prevalence of taxa from the *Lactobacillus* genus complex (LGC) in the anterior nares (left) and in the nasopharynx (right) from healthy controls and CRS patients. Asterisks represent statistically significant differences between the niches (***$p < 0.001$, ****$p < 0.0001$).

(C) Relative abundance of taxa from the LGC upon presence in the anterior nares (left) and nasopharynx samples (right) from healthy controls and CRS patients. p values (Wilcoxon rank-sum tests) are indicated on the plot. Pseudocounts of 1 were added to all samples to allow visualization on the log scale. Samples where the LGC was found to be present are represented by dark blue points, whereas samples where the LGC was absent are colored light blue.

(D) Top 11 of the most abundant bacterial genera in nasopharynx samples of healthy controls and CRS patients. The LGC is part of the residual, ranked at number 21.

(E) Zoom in on the LGC phylogroups (Duar et al., 2017) in nasopharynx samples of healthy controls and CRS patients.

(F) *Lactobacillus* species isolated via selective cultivation and identified up to species level after whole-genome sequencing and comparative genomics.

bacteria (LAB), genetically and functionally shown to be adapted to several biotic and abiotic factors of this underexplored human body site, was then evaluated in healthy volunteers.

RESULTS

Lactobacillus Genus Complex Is Reduced in the URT Microbiome of CRS Patients

The *Lactobacillus* genus complex (LGC) includes important beneficial taxa in different human body niches (Heeney et al., 2018; Lebeer et al., 2018; Petrova et al., 2015). The bacteria belonging to this genus are known to be rod shaped, fermentative bacteria producing lactic acid from sugar fermentation under anaerobic or micro-aerophilic conditions. This lactic acid production is a hallmark of their beneficial functions, because it inhibits the growth of pathogens. In addition, also their long history of safe use and their beneficial immunomodulatory, metabolic, and epithelial cell interactions often underlie the beneficial roles of lactobacilli (Lebeer et al., 2008). To investigate whether the LGC could also be associated with URT health, we first compared the abundance of bacterial taxa at the family level between CRS patients versus healthy controls using a compositional data analysis approach. In brief, we calculated the pairwise relative abundance log ratios between all families and compared these between the two groups of samples. We did this for the 30 most prevalent families, resulting in 30 comparisons per family. Compared with CRS patients, two families were significantly more abundant in the anterior nares of healthy controls with respect to a large number of reference families (Figure 1A): *Carnobacteriaceae* and the LGC or lactobacilli. Similar results were obtained for the nasopharynx

(Figure S1). The LGC was almost 1.5-fold enriched in the healthy controls based on their prevalence, both in the anterior nares and the nasopharynx (Figure 1B). In the anterior nares, taxa from the LGC were found in 87% of the healthy control samples, as opposed to 60% in CRS (Fisher's exact test, $p = 0.000007$). A similar trend was observed in the nasopharynx, with 80% in the healthy controls versus 55% in CRS patients (Fischer's exact test, $p = 0.00006$). Also, the median relative abundance of ASVs from the LGC was more than 3-fold higher in the anterior nares (Wilcoxon rank-sum test, $p < 10e{-}7$) of healthy controls compared with those of CRS patients and more than 10-fold higher in the nasopharynx (Wilcoxon rank-sum test; $p < 10e{-}7$) (Figure 1C). Although the URT is dominated by other bacterial genera (Figure 1D), the LGC thus represents a minor, yet non-negligible genus in the URT, which is clearly enriched in healthy volunteers based on prevalence, relative abundance, and compositional analyses.

The most abundant LGC ASVs in the nasopharyngeal samples over the entire dataset were classified within the *Lactobacillus casei* group (new genus name *Lacticaseibacillus*) (Zheng et al., 2020) according to the EZBioCloud *16S rRNA* database (Yoon et al., 2017) and mean relative abundance of 0.16%, the *Lactobacillus delbrueckii* group (*Lactobacillus* genus *strictu sensu* according to Zheng et al., 2020) (*Lactobacillus iners*, 0.12%), the *Lactobacillus plantarum* group (new genus name *Lactiplantibacillus*) (0.12%), and the *Lactobacillus sakei* group (new genus name *Lactilactobacillus* sakei) (0.09%) (Figure 1E). For clarification, this manuscript still uses the old taxonomy names, because the reclassification was publicly available only after acceptance of this manuscript. However, a list of the new *Lactobacillus* species names according to the reclassification by Zheng et al. (2020) can be found in Data S1. Especially the detection of a *L. iners* ASV (in 21% of the samples) was surprising, because this species has been mainly associated with the human vagina (Gajer et al., 2012; Petrova et al., 2017), although we recently also found it on the human facial skin (Lebeer et al., 2018; Ross et al., 2017). Of interest, the same ASVs of lactobacilli were also most abundant, be it at lower relative abundances (<0.1%), in the anterior nares, which is a more oxygen-rich habitat and thus less favorable for fermentation.

Subsequently, we aimed to cultivate these LGC species from URT samples from our healthy subjects. The isolation of lactobacilli from URT samples turned out to be challenging, because of the high abundance of fast-growing aerobes such as staphylococci, even on selective culture media and conditions for lactobacilli (Kandler and Weiss, 1986). Additionally, it is possible that the targeted lactobacilli have undergone unidentified adaptations to the URT habitat, making it more challenging to grow them under standard growth conditions. From the 100 healthy volunteers sampled, nine isolates were obtained, whole-genome sequenced. and classified at species level as *L. rhamnosus*, *L. casei*, *L. sakei*, and *L. plantarum.* Of note, these taxa were also found to be among the most abundant *Lactobacillus* ASVs based on the microbiome profiles (Figure 1E). Six *L. rhamnosus* strains were isolated (*L. rhamnosus* strains AMBR1, AMBR3, AMBR4, AMBR5, AMBR6, and AMBR7), one *L. casei* (strain AMBR2), one *L. sakei* (strain AMBR8), and one *L. plantarum* (strain AMBR9) (Figure 1F). To study the genetic relatedness of the isolated strains and track the possible sources of these lactobacilli, we constructed a phylogenetic tree of the genomic assemblies together with the publicly available genome sequences from Sun et al. (2015) (Figure S2). In addition, pairwise average nucleotide identity (ANI) values were calculated for all genomes under study. High pairwise ANI values were obtained for *L. rhamnosus* AMBR1, AMBR5, AMBR6, and AMBR7 with the model gastrointestinal probiotic *L. rhamnosus* GG (GCA_003353455, GCA_000026505, GCA_000011045; 99.82%–99.99%), for *L. rhamnosus* AMBR3 and AMBR4 with type strain *L. rhamnosus* DSM20021 (GCA_001435405; 99.72%–99.99%), and for *L. plantarum* AMBR9 with *L. plantarum* CMPG5300 (GCA_000762955; 99.96%–99.98%) (Malik et al., 2014). Furthermore, variant calling of these genome sequences resulted in a low number of single-nucleotide polymorphisms. These isolates thus appeared to be highly similar or even identical to commercially available probiotics that probably originate from oral consumption, because the oral cavity and nasopharynx are connected. However, *L. casei* AMBR2 (maximum [max.] ANI value of 96.97%) and *L. sakei* AMBR8 (max. ANI value of 98.41%) showed max. ANI values lower than 99%, indicating that these isolates are more distinct from the publicly available genome sequences of mainly food-related lactobacilli and might be more adapted to the URT.

Adaptation of URT Lactobacilli to Oxidative Stress and Nasal Epithelial Adherence

To examine the adaptation capacity to typical conditions of the URT, such as high air flow and oxidative stress (De Rudder et al., 2018), the AMBR genomes were first screened for the presence of genes encoding catalases, superoxide dismutase, and related enzymes, known to be important for oxidative stress tolerance of bacteria in the respiratory tract (Eason and Fan, 2014). Catalase enzymes are uncommon in lactic acid-fermenting bacteria (Weinberg, 1997). Here, we found a gene encoding a heme- and a manganese-dependent catalase in isolate *L. casei* AMBR2 (as reported in Wuyts et al., 2017), and a heme-dependent catalase gene in *L. sakei* AMBR8 and *L. plantarum* AMBR9. Experimental validation showed that *L. casei* AMBR2 and *L. plantarum* AMBR9 indeed exhibit catalase activity under respiratory conditions, although this was not observed for *L. sakei* AMBR8 (Table S1). Further screening of the AMBR8 genome revealed the presence of inverted repeats in the leader sequence of the catalase gene, implying possible phase variation (van der Woude and Bäumler, 2004) (Figure S3). *L. casei* AMBR2 showed catalase activity both under aerobic and respiratory conditions (Table S1). Further screening of the evolutionary history of these enzymes in the whole LGC revealed that the heme-dependent catalase was found in all LGC taxa associated above with the URT in our 16S analysis, albeit with varying copy numbers (Figure 2, left). The phylogenomic analysis also indicated that a heme-dependent catalase was already present in their common ancestor, and thus encoded by a core gene in these phylogenetic groups. The manganese-dependent catalase appeared to be less conserved and was found as a core gene only in *L. casei*, *Lactobacillus kunkeei* (new genus name *Apilactobacillus kunkeei*), and *Lactobacillus lindneri* (new genus name *Fructilactobacillus lindneri*) (Figure 2, right). The two latter

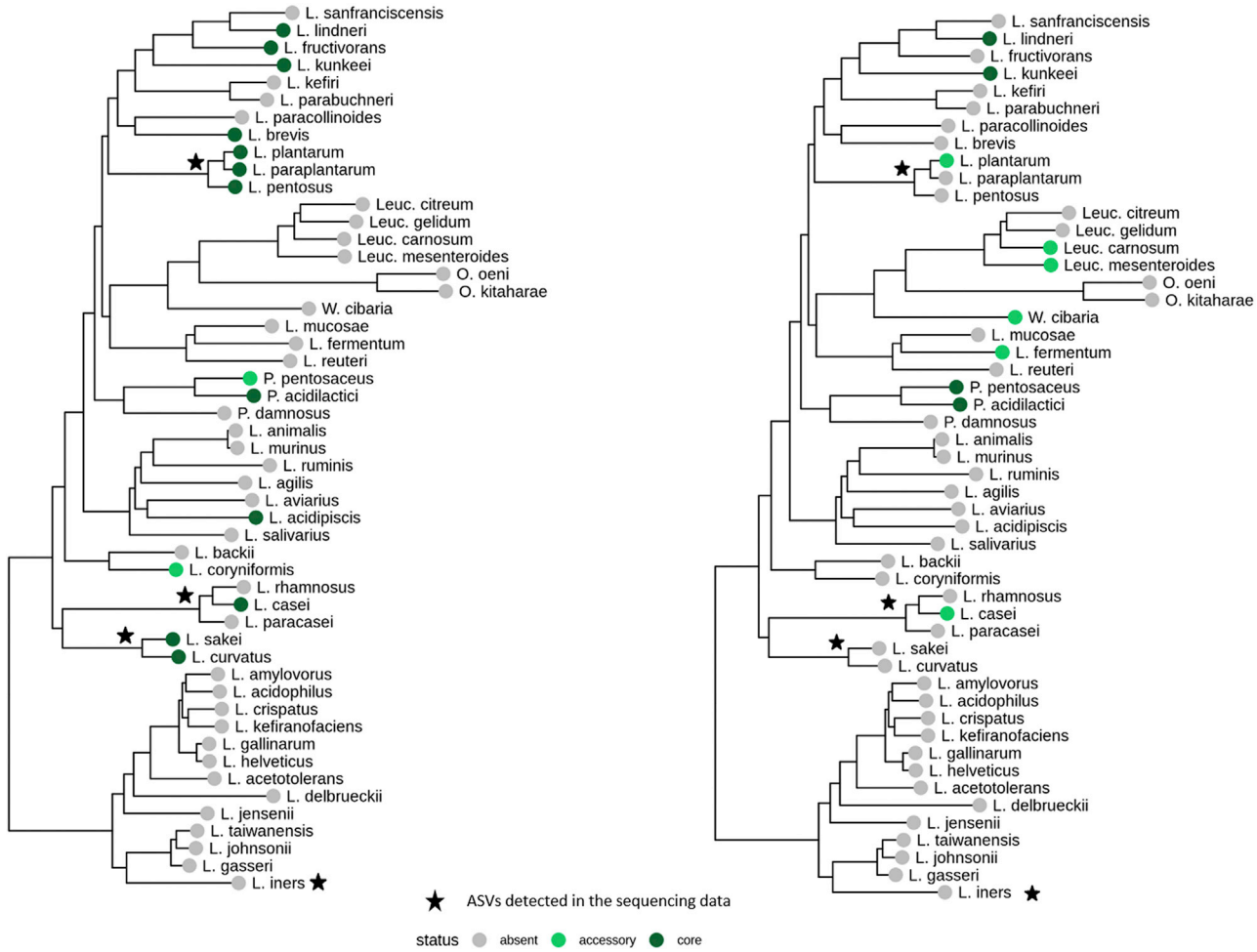

Figure 2. Adaptation of URT Lactobacilli to Oxidative Stress

(Left) Phylogenomic screening for heme-dependent catalase in the evolutionary history of the LGC based on a profile Hidden Markov Model (HMM) database constructed for the catalase gene families.

(Right) Phylogenomic screening for manganese-dependent catalase in the evolutionary history of the LGC. Given a species with n genomes, a gene was considered core for that species if it was found in n-1 or n genomes and accessory if it was found in 1 to n-2 genomes.

species are known to be adapted to insects, i.e., habitats that are not directly related to the human URT, but where also considerable oxidative stress could be envisaged (Anderson et al., 2018; Oliveira et al., 2011).

Because the capacity to tolerate oxidative stress has been linked to the growth capacity of LAB (Zotta et al., 2017), and because this is a key property for later development as a probiotic, we also explored the growth capacity, production rate, and cell dry weight of the AMBR isolates via batch fermentations (500 mL) in de Man, Rogosa, and Sharpe (MRS) medium at 37°C. The most oxidative stress-tolerant *L. casei* AMBR2 turned out to be one of the most robust strains in comparison with the other AMBR strains and the model strain *L. rhamnosus* GG. AMBR2 showed a desired growth capacity of more than 2×10^9 colony-forming units (CFU)/mL after 48 h (Figure S4A), a production rate of more than 75% (Figure S4B), and a cell dry weight of more than 1.5 g/L medium (Figure S4C).

Another adaptation to the URT is a sufficient high adherence capacity to nasal epithelium to prevent rapid nasal clearance. Therefore, we screened the genomes of our isolates for genes related to adhesive capacities. A SecA2/SecY2 secretion system with two large glycosylated putative surface adhesion proteins (*srr1* and *srr2* gene) was found in the draft genome sequence of *L. casei* AMBR2 (Wuyts et al., 2017) (see also Figure S5 for a schematic model for srr glycoprotein biogenesis) (Couvigny et al., 2017). Based on the gene sequences, the molecular weights of the two serine-rich repeat proteins were estimated at 290 and 738 kDa, which was confirmed via SDS-PAGE (Figure S6). In the closely related *L. casei* DSM20178, both *srr* genes and the secretion system were also identified. Therefore, we hypothesize that this strain might have similar adhesive structures on its cell surface as *L. casei* AMBR2 (Wuyts et al., 2017). In addition, the type strain *L. casei* ATCC393 and *L. casei* JCM1134 have the *srr2* gene, but no SecA2/SecY2 secretion system,

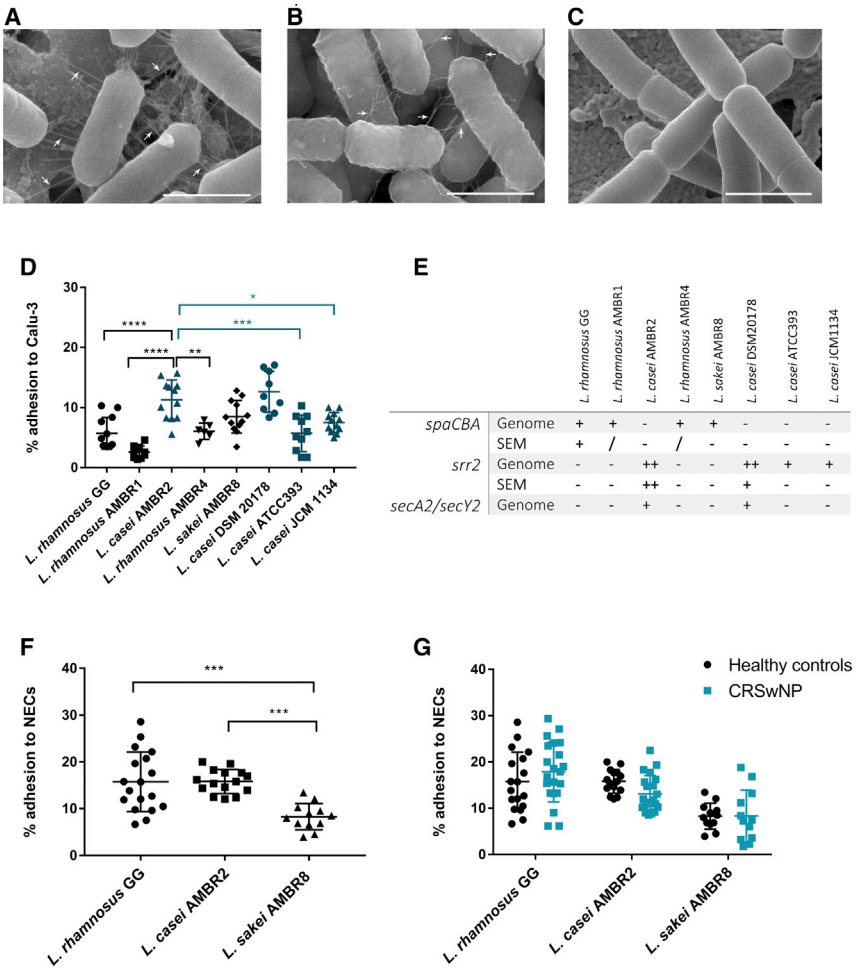

Figure 3. Adherence Properties to Overcome Nasal Clearance

(A) Scanning electron microscopy (SEM) of the URT isolate *L. casei* AMBR2 cell surface shows long, spike-like fimbriae. See also Figure S7 for SEM of closely related *L. casei* strains.

(B) For comparison, visualization of the SpaCBA pili on the cell surface of *L. rhamnosus* GG using SEM.

(C) SEM of the URT isolate *L. sakei* AMBR8 cell surface indicating no fimbriae structures present on the bacterial cell surface under the tested conditions. Scale bars, 1 μm.

(D) Overnight-grown cultures of *L. rhamnosus* AMBR1, *L. casei* AMBR2, *L. rhamnosus* AMBR4, *L. sakei* AMBR8, and the model strain *L. rhamnosus* GG were co-incubated with Calu-3 cells for 1 h in a final concentration of 10^8 CFUs/mL. In addition, three closely related *L. casei* strains, i.e., *L. casei* DSM20178, *L. casei* ACC393, and *L. casei* JCM1134, were analyzed as well to compare adherence of these strains with *L. casei* AMBR2. The proportion of adhered bacteria, expressed as a percentage, was determined. Significant differences compared with *L. casei* AMBR2 are indicated with an asterisk (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$). Data of three biological repetitions are represented as mean ± standard deviation (SD).

(E) Overview of predicted or confirmed fimbriae structures based on the *spaCBA*, *srr2*, and *secA2/secY2* gene cluster in the tested strains based on their genome sequence and SEM.

(F and G) Overnight-grown *L. casei* AMBR2, *L. sakei* AMBR8, and *L. rhamnosus* GG were co-incubated with primary NECs of healthy control donors (n = 6) (F) or CRSwNP patients (n = 6) (G) for 1 h in a final concentration of 10^8 CFUs/mL. The proportion of adhered bacteria, expressed as a percentage, was determined.

Significant differences are indicated with an asterisk (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$).

Data are represented as mean ± SD. One-way ANOVA and multiple t tests were used to determine statistical significance in GraphPad Prism. The p value was corrected for multiple comparisons using the Holm-Sidak's multiple comparisons test (D, F, and G). See also Table S2.

suggesting that these strains might not be able to express fimbriae structures. These findings were confirmed by scanning electron microscopy (SEM) analysis. SEM showed the presence of long, spike-like appendages on the cell surface of *L. casei* AMBR2, which stretched out with lengths ranging up to 2 μm long. They are referred to as fimbriae based on this initial visualization of their morphology (Figure 3A). Also, *L. casei* DSM20178 showed fimbriae structures on SEM images, although they were less prominent compared with *L. casei* AMBR2 (Figure S7A). *L. casei* ATCC393 and *L. casei* JCM1134 did not show fimbriae structures on SEM (Figures S7B and S7C). The fimbriae observed in *L. casei* AMBR2 clearly differed from the well-described SpaCBA pili of model intestinal probiotic *L. rhamnosus* GG, which are more limp and shorter in length ranging between 0.1 and 1 μm (Figure 3B; Table S2). Phylogenomic analysis of the *srr* genes that potentially express this phenotype in *L. casei* AMBR2 suggested these *srr* genes to be unique for these *L. casei* strains, because it was not found in the other isolated LGC AMBR strains isolated here or other species of lactobacilli analyzed earlier (Wuyts et al., 2017). In contrast, all *L. rhamnosus* AMBR strains had the *spaCBA* gene cluster present in their genomes, encoding the SpaCBA pili, as described for *L. rhamnosus* GG (Kankainen et al., 2009). Also, *L. sakei* AMBR8 showed the *spaCBA* gene cluster in its genome, but SEM revealed that *L. sakei* AMBR8 did not express fimbriae on its cell surface under the tested conditions (Figure 3C). Exploration of this gene cluster revealed the presence of inverted repeats in the leader sequence of the *spaCBA* cluster, possibly resulting in abolished expression, as also observed for the catalase gene (Figure S3). Finally, we experimentally validated the adhesion capacity of *L. rhamnosus* AMBR1, *L. casei* AMBR2, *L. rhamnosus* AMBR4, *L. sakei* AMBR8, the well-known probiotic *L. rhamnosus* GG, and the three closely related *L. casei*, DSM20178, ATCC393, and JCM1134, to the respiratory tract epithelial Calu-3 cell line and human primary nasal epithelial cells (NECs). The latter cell type was used for confirmation, because it has previously been shown to better represent the nasal epithelial cellular responses, morphology, biochemical characteristics, and donor variation than immortalized cell lines (Martens et al., 2018). Primary cells from both healthy control donors (n = 6)

and CRS patients with nasal polyps (CRSwNP) (n = 6) were included, because we rationalized that adhesion to diseased or unstable epithelium could be different due to, for instance, the expression of different receptors (Boita et al., 2016). In Calu-3 cells, adhesion of *L. casei* AMBR2 was significantly higher (11.3% ± 3.2%) compared with *L. rhamnosus* GG (5.7% ± 2.7%; $p < 0.0001$), *L. rhamnosus* AMBR1 (2.6% ± 1%; $p < 0.0001$), and *L. rhamnosus* AMBR4 (6% ± 1.4%; $p < 0.001$) (Figure 3D). In comparison with the other tested *L. casei* strains, adherence of *L. casei* AMBR2 was similar to that of *L. casei* DSM20178 (12.6% ± 3.4%), in accordance with our findings based on their genomes and SEM that both strains express fimbriae-like Srr2/SecA2/SecY2-dependent structures (Figure 3E). Adherence of *L. casei* ATCC393 (5.7% ± 3%) and *L. casei* JCM1134 (7.5% ± 1.6%) was significantly lower compared with *L. casei* AMBR2, in agreement with their genome-lacking genes encoding SecA2/SecY2. Because *L. casei* AMBR2 and *L. sakei* AMBR8 showed the best adhesion capacity to the Calu-3 cells of our URT isolates, these strains were further investigated in the primary NECs. *L. casei* AMBR2 exhibited a superior capacity ($p = 0.0002$) to adhere to NECs from controls compared with *L. sakei* AMBR8, with adhesion percentages of 15.8% and 8.3%, respectively (Figure 3F). Finally, adherence of the three tested strains was not influenced by disease status, because adherence was similar in primary cells from healthy controls and patient-derived cells (CRSwNP) (Figure 3G).

L. casei AMBR2 Has Antimicrobial and Immunomodulatory Properties against Important URT Pathobionts

Next, we performed a more in-depth analysis of *L. casei* AMBR2 and *L. sakei* AMBR8 to better evaluate their adaptability in terms of ecological interactions with dominant competing URT pathogens and tolerability by the nasal epithelium. Key URT pathogens are *Staphylococcus aureus*, *Haemophilus influenzae*, and *Moraxella catarrhalis*, which are all able to take a dominant niche as pathobionts in the URT upon infection. The capacity to prevent overgrowth of these pathogens can thus be considered as a keystone beneficial function. *S. aureus* and *H. influenzae* are often indicated as CRS pathobionts (Bachert et al., 2010; De Boeck et al., 2019; Hoggard et al., 2017). *M. catarrhalis* tends to be a marker for acute sinusitis (Brook et al., 2006; Murphy and Parameswaran, 2009), although it is also detected in the sinuses of CRS patients (De Boeck et al., 2019; Chalermwatanachai et al., 2018; Hoggard et al., 2017). Analysis of direct, antimicrobial effects of *L. casei* AMBR2 against these URT pathobionts via spot assays revealed growth inhibition for all three (Figure 4A), with the largest activity against *M. catarrhalis*. In contrast, the pathobionts showed no growth inhibition against themselves. Subsequently, we showed that respiratory epithelial cells did tolerate *L. casei* AMBR2 and *L. sakei* AMBR8 well, because they responded much less with production of interleukin-8 (IL-8), IL-1β, and tumor necrosis factor (TNF) to both lactobacilli in comparison with the tested pathogens, similarly to model probiotic *L. rhamnosus* GG (Figure 4B). IL-1β, TNF, and IL-8 are known to have important pro-inflammatory action in CRS (Cervin and Wallwork, 2007; Tomassen et al., 2016), and IL-8 and IL-1β are in addition important for neutrophil recruitment (Suzuki and Ikeda, 2002). No signs of virulence by invasion or destruction of the cells were observed for the tested lactobacilli. No increase in the inflammatory response was observed for any of the investigated pathogens upon co-culture with our selected *Lactobacillus* strains (Figures 4C–4E; see Table S3 for statistical analysis). We rather observed anti-inflammatory effects: *L. casei* AMBR2, but not *L. sakei* AMBR8, was able to significantly decrease the IL-8 ($p = 0.02$) and IL-1β ($p = 0.01$) expression induced by *M. catarrhalis* (Figure 4C). For *H. influenzae*, a decreasing tendency in the inflammatory response was also observed in co-culture with *L. casei* AMBR2 (Figure 4D). The anti-inflammatory action of *L. casei* AMBR2 was the least pronounced against *S. aureus* (Figure 4E), probably because the inflammation induced by this pathogen is significantly higher compared with *M. catarrhalis* and *H. influenzae*. Co-culture of *L. sakei* AMBR8 with *H. influenzae* showed an increasing tendency of TNF induction. Although this was not statistically significant, AMBR2 was preferred over AMBR8 for further analyses.

L. casei AMBR2 Temporarily Colonizes the Nasal Cavity of Healthy Volunteers without Adverse Effects

Finally, we aimed to validate our *in vitro* and *ex vivo* findings *in vivo*. Animal models are often an important intermediate step between *in vitro* characterization and human trials. However, most animal models have a different URT anatomy and physiology compared with humans, making the translation highly challenging. Therefore, because lactobacilli have a long history of safe and frequent use, especially for strains from the *L. casei* group (Sanders et al., 2010), and a qualified presumption of safety state (QPS) in Europe (Koutsoumanis et al., 2019), we aimed to validate our findings in humans, after some additional analyses. First, considering that the presence of transferable antibiotic resistance markers is an undesired characteristic for any microbe introduced in humans as potential probiotic or other microbiome therapeutic (EFSA Panel on Additives and Products or Substances used in Animal Feed, 2012), we performed a thorough *in silico*-based analysis of the genomes, confirming that chromosomal and plasmid antibiotic resistance genes were not present, except for a tetracycline resistance gene in *L. rhamnosus* AMBR1, AMBR5, and AMBR6 (Figure 5). Minimal inhibitory concentration (MIC) testing *in vitro* according to the European Food Safety Authority (EFSA) guidelines (EFSA Panel on Additives and Products or Substances used in Animal Feed, 2012) showed that all AMBR strains were susceptible to tetracycline (Figure 5). Some strains did show limited *in vitro* resistance against certain antibiotics (Figure 5), although known antibiotic resistance genes were not found in the genomes of these isolates. Intrinsic phenotypic antibiotic resistance is generally not considered a safety issue, because it cannot be horizontally transferred, but it should be kept to a minimum (EFSA Panel on Additives and Products or Substances used in Animal Feed, 2012). *L. casei* AMBR2 was the only isolated strain that displayed no antibiotic resistance based on *in silico* and *in vitro* phenotypic analysis (Figure 5), and thus was the most appropriate candidate for human testing. Furthermore, genomic screening against the Virulence Factor Database (VFDB) (Liu et al., 2019) also did not

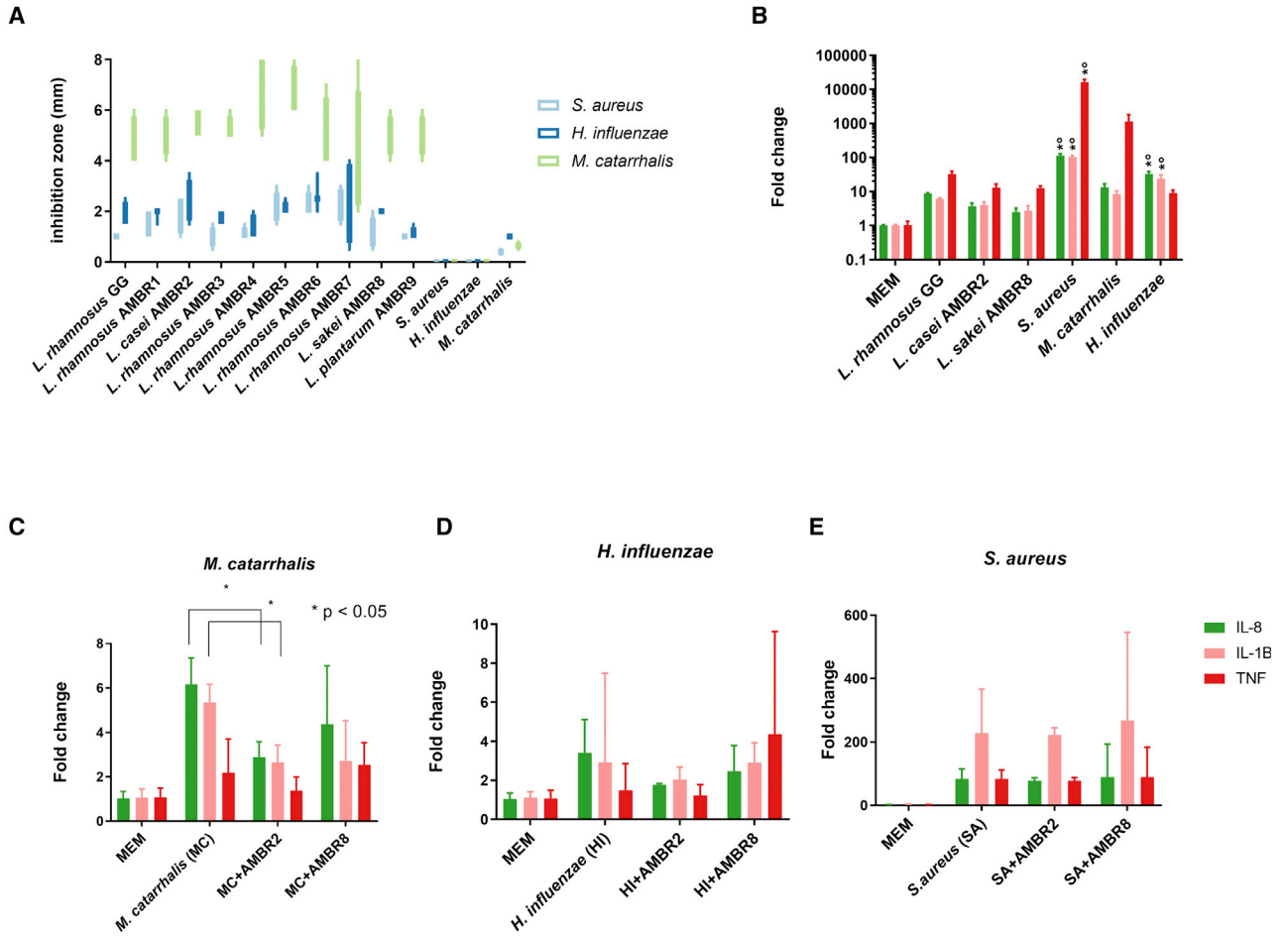

Figure 4. Antimicrobial and Immunomodulatory Functions for URT Lactobacilli

(A) Agar-based antimicrobial screening of the interaction between live lactobacilli and *S. aureus*, *M. catarrhalis*, and *H. influenzae*. Data are expressed as means ± SDs.

(B) Lack of induction of an inflammatory response in respiratory epithelial cells. Induction of IL-8, IL-1β, and TNF mRNA by *L. rhamnosus* GG, *L. casei* AMBR2, *L. sakei* AMBR8, *S. aureus*, *M. catarrhalis*, and *H. influenzae* in Calu-3 cells. Bacteria were incubated with the cells for 4 h in a final concentration of 10^8 CFUs/mL. MEM refers to the condition of cells alone without any bacteria. The presented data are representative of three independent experiments, with each experiment three biological repetitions. 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 of the immunomodulation of the pathobionts with *L. casei* AMBR2 are depicted with asterisks (*) and for *L. sakei* AMBR8 with degree signs (°). More details about the significance and other statistical results can be found in Table S3. Data are expressed as mean ± range (B) and means ± SDs (C–E).

(C) Beneficial homeostatic immunomodulatory function of URT lactobacilli. Induction of IL-8, IL-1β, and TNF mRNA by *M. catarrhalis* upon co-incubation with lactobacilli in Calu-3 cells.

(D) Induction of IL-8, IL-1β, and TNF mRNA by *H. influenzae* upon co-incubation with lactobacilli in Calu-3 cells.

(E) Induction of IL-8, IL-1β, and TNF mRNA by *S. aureus* upon co-incubation with lactobacilli in Calu-3 cells.

One-way ANOVA and multiple t tests were used to determine statistical significance in GraphPad Prism. The p value was corrected for multiple comparisons using the Holm-Sidak's multiple comparisons test (C–E). Results represent three biological repetitions (C–E).

reveal the presence of potentially harmful virulence factors or toxins.

Based on the safety assessment resulting from our detailed *in silico* and *in vitro* analysis, and given earlier described literature on the safe administration of related bacteria (LGC taxa and *Streptococcus salivarius*) in the nose of healthy and even diseased adult individuals (Mårtensson et al., 2016, 2017; Santagati et al., 2015), we received approval from the ethical committee (B300201835709) to explore the in-human adaptation and effects of *L. casei* AMBR2. Therefore, we set up an open-label exploratory study in 20 healthy volunteers, who administered spray-dried *L. casei* AMBR2 powder in the nose twice daily for 2 weeks. In brief, bacteria were spray dried and nasal-spray formulations were prepared, as described in the Method Details section. Participants resolved the powder daily in physiological water prior to use and administered at least 10^8 CFUs/puff in the nostril. Tables S4 and S5 give an overview of participant demographics and probiotic consumption of the participants,

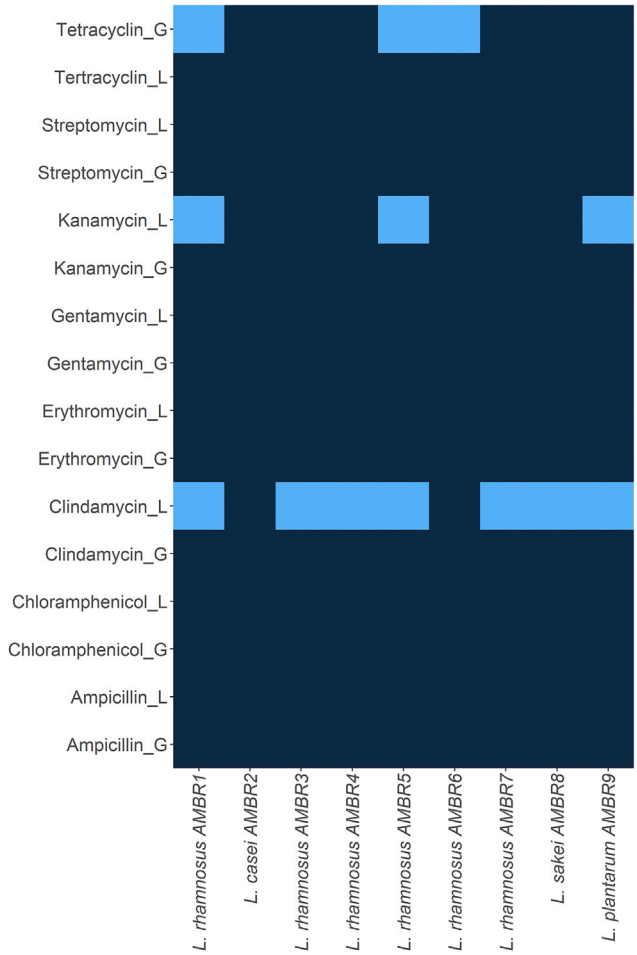

Figure 5. Antibiotic Resistance *In Silico* and *In Vitro* for All Isolated AMBR Strains

G, genome analysis; L, *in vitro* testing via MIC assay; dark blue, no hit/susceptible to antibiotic; light blue, hit/not sensitive to antibiotic.

respectively. The primary outcome measure of this study was to evaluate the fitness of *L. casei* AMBR2 in the healthy nasopharynx and its detection by DNA- and culture-based analysis after nasal administration. We determined that temporary URT colonization of sufficient numbers of *L. casei* AMBR2, preferably above 15 min, is an important milestone toward the development of a novel nasal probiotic to prevent immediate nasal clearance. Additionally, this *in vivo* persistence also gave us an idea about the ecological relevance and adaptation potential of lactobacilli in the human nose. In parallel, the modulation of the URT microbiome was followed during the study period. The second outcome measure was to evaluate the safety and tolerability of two doses of 10^8 CFUs/day of nasal *L. casei* AMBR2.

The persistence capacity of *L. casei* AMBR2 in the URT was investigated based on selective cultivation, *16S rRNA* V4 sequencing, and qPCR analyses with strain-specific primers, targeting the *srr2* gene (putative fimbriae) for *L. casei* AMBR2 of nasopharyngeal swabs. These were sampled at four time points (Figure 6A; Table S6). *L. casei* AMBR2 could be cultivated from all samples at T1 (5 min after first administration), in 73% of the samples at T2 (within 10–16 h after the last administration at the end of the 14-day treatment), and in none of the samples at T3 (2 weeks after the last administration). qPCR targeting of the *srr2* gene of *L. casei* AMBR2 (Figure 6B) yielded a similar trend in colonization potential. Based on a standard curve, the amount of *L. casei* AMBR2 was estimated to vary between 4×10^6 and 4×10^8 CFUs per nasopharyngeal swab at T1. At the second consultation (T2), the amount had decreased and varied between 1.5×10^3 and 5×10^7 CFUs. At T3, the strain could still be detected in two participants ($\approx 10^3$ CFUs). A similar trend was observed in the sequencing profiles. Of interest, the participants of this study did not contain the specific *L. casei* AMBR2 strain in their nose at T0 (start of the study), but other strains were present, including *L. casei/paracasei/zeae*, *L. iners*, and *L. curvatus/sakei*. A significant increase in the relative abundance of the LGC indicating different species based on their V4 sequence was observed at T1 ($p < 0.0001$), which was associated with a temporary decrease in the relative abundance of the other genera, some of which harbored some potential URT pathobionts such as *C. tuberculostearicum*, *H. influenzae/aegyptius*, and *S. aureus* (Figure 6C). At T2, the relative abundance of the LGC was decreased compared with the previous time point, although high relative abundances were still detected in several samples. For other genera, such as *Corynebacterium*, *Staphylococcus*, and *Streptococcus*, an initial drop in relative abundances was observed at T1, but their abundances started to increase again at T2, indicating no apparent side effects on the supposedly healthy URT microbiome profiles (Figure 6C). Of interest, the effects of *L. casei* AMBR2 on the relative abundance of the genus *Dolosigranulum* seemed to be long-lasting compared with the other genera. *Dolosigranulum*, a member of the *Carnobacteriaceae* and the LAB, has been linked to healthy respiratory conditions based on several microbiome studies (Biesbroek et al., 2014; De Boeck et al., 2019; Laufer et al., 2011). It is thus plausible that lactobacilli and *Dolosigranulum* have mutually exclusive beneficial functions as LAB in the nose and nasopharynx, but this remains to be explored in future work.

In addition to the temporary colonization capacity, the safety and tolerability of *L. casei* AMBR2 upon nasal administration were evaluated via reported symptoms, physical examination of the URT by the responsible ENT (ears, nose, and throat) specialist, and measurement of markers in the blood of the participants. First, the participant questionnaire revealed only minor side effects, of which a blocked nose, runny nose, and sneezing were most often reported (Figure 6D). However, none of the participants experienced one of these side effects for the entire duration of the study, and several effects were reported only once or twice over the entire study. In addition, these side effects are often reported when using other nasal sprays as well (Ganesh et al., 2017). Furthermore, C-reactive protein (CRP) levels in the blood were normal in 15/20 of the volunteers, and only slightly elevated in 5/20 participants (Table S7), which is in accordance with our data on the low inflammatory reaction toward *L. casei* AMBR2 in the Calu-3 cells (Figure 4B). This is in agreement with the fact that the selected strain is adapted to and tolerated by the human nose, because no overt response upon administration of the strain was observed. The few participants

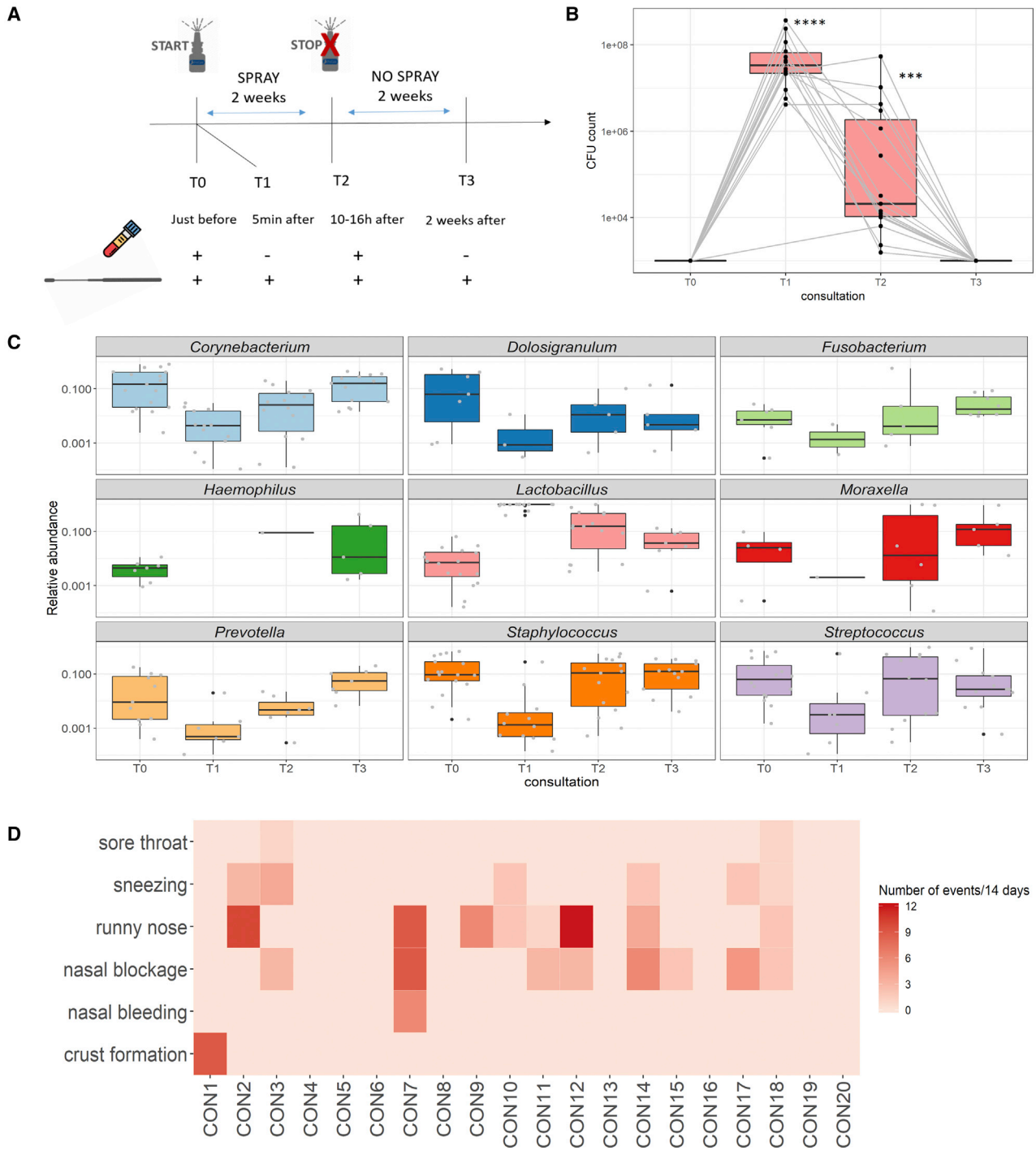

Figure 6. In-Human Potential of URT Lactobacilli as Beneficial URT Taxa

(A) Schematic overview of the study setup with the different samples collected at the different time points. Volunteers administered the *L. casei* AMBR2 nasal spray twice a day for 2 weeks. Time points were the start of the study (T0), 5 min after first administration (T1), within 10–16 h after the last administration at the end of the 14-day treatment (T2), and 2 weeks after the last administration (T3). A nasopharyngeal swab was collected at each time point, and a blood sample was collected at the start of the study and after the 2 weeks of nasal administration.

(B) Quantitative estimation of the amount of *L. casei* AMBR2 in the nasopharynx after nasal administration at T0, T1, T2, and T3 determined via dedicated qPCR targeting the *srr2* gene of *L. casei* AMBR2. The detection limit was estimated to be at 10^3 CFUs. Significant differences are indicated with an asterisk (***$p < 0.001$, ****$p < 0.0001$).

(legend continued on next page)

in whom some mildly elevated CRP levels were found did not report any signs of infection themselves, and one participant already displayed elevated CRP levels prior to the start of the study (Table S7). In addition, no signs of acute inflammation were observed upon physical examination of the upper airways. Furthermore, blood samples were cultivated on selective MRS agar plates to investigate the presence of *L. casei* AMBR2 in the blood. Samples from all participants were negative, indicating that translocation to the blood was below detection limit in these volunteers.

DISCUSSION

Lactobacilli have been described as important beneficial species of the gastrointestinal tract (Heeney et al., 2018), the vagina (Petrova et al., 2015), and the skin (Lebeer et al., 2018) of humans, many animals, and fermented foods, because of their antimicrobial and/or immunomodulatory capacities. They have consequently been investigated for their health-promoting properties in several of these habitats. In contrast, although there exist some occasional reports that detected lactobacilli in the URT (Abreu et al., 2012; Bogaert et al., 2011; Jensen et al., 2013; Ling et al., 2013; Stearns et al., 2015), their importance as commensals in the URT and their ability to colonize and function in this habitat have not been explored. In this study, we integrated a dedicated comparative microbiome profiling of the taxa in health and CRS disease, with the cultivation of URT-specific strains from healthy individuals as potential probiotics for the URT. Several LAB (*Lactobacillaceae* and *Carnobacteriaceae*) were clearly more prevalent in healthy individuals. Multiple isolates were cultivated from the most abundant taxa of lactobacilli, putatively classified as *L. rhamnosus*, *L. casei*, *L. sakei*, and *L. plantarum* species. Because these isolated lactobacilli could originate from fermented foods or commercial probiotics via the oronasopharynx, we carefully analyzed their genetic and functional adaptation capacity to the URT. We rationalized that oxidative-stress tolerance and adherent properties are important features for lactobacilli in order to be able to adapt and function in this habitat. One of our isolates, *L. casei* AMBR2, showed the presence of a functional heme- and manganese-dependent catalase, as well as a SecA2/SecY2 gene cluster encoding two putative glycosylated large-surface adhesin proteins. SEM analysis revealed the presence of fimbriae-like structures on the *L. casei* AMBR2 surface. These fimbriae structures might explain why AMBR2 adhered well to the respiratory epithelium, irrespective of health or disease status of the primary NECs. Adherence is believed to be an important feature to promote beneficial actions by probiotics to exert their beneficial effects, as demonstrated, for instance, for the model *L. rhamnosus* GG with its intestinal mucus-specific *SpaCBA* pili (Lebeer et al., 2012; Vargas García et al., 2015). An additional advantage of adherent strains, in particular for nasal applications, lies in their higher colonization capacity, because they would otherwise be rapidly eliminated by nasal clearance (Pandya and Tiwari, 2006). We further substantiated the multifactorial beneficial mode of action of *L. casei* AMBR2 in the URT, by demonstrating its anti-pathogenic effects through growth inhibition of URT pathobionts *in vitro*, and its capacity to reduce inflammatory responses of airway epithelial cells in co-culture with pathobionts.

The capacity of *L. casei* AMBR2 to withstand nasal clearance and at least temporary colonize the nose was then investigated in an open-label exploratory study in healthy participants. We could detect *L. casei* AMBR2 in 60%–95% of the nasopharynx samples, with MiSeq or qPCR, respectively, of the 20 healthy volunteers up to 10–16 h after the last administration of a 2-week treatment, and still in 10% (qPCR) to 35% (MiSeq) of the samples studied 2 weeks after the last dose. By way of comparison, *Streptococcus salivarius* 24SMB, which in another study was used in a nasal spray for 3 days, colonized the nasopharynx in 55% of the volunteers for up to 6 days after the last dose, without any adverse effects (Santagati et al., 2015). However, in the latter study, colonization of the applied *Streptococcus* strain was promoted by giving the participants an antibiotic treatment before the start of the study (Santagati et al., 2015). We did not adopt such an approach in healthy volunteers because of the increasing reports on long-term consequences and side effects of such antibiotic treatments (Langdon et al., 2016). In another study, in which the effect of a single dose of 13 honeybee LAB in a honey-based formulation was investigated in healthy volunteers, none of the applied LAB were detected in the nose 2 weeks posttreatment based on cultivation (Mårtensson et al., 2016). The same group investigated the effects of these honeybee LAB upon nasal administration in the URT of CRS patients without nasal polyps (CRSsNP) and demonstrated that application of these lactobacilli for 2 weeks was safe, but no beneficial effects on symptom severity nor the microbiota were observed in the patients (Mårtensson et al., 2017). This suggests that lactobacilli originating from another environment might have less potential for application in the human nose.

In conclusion, lactobacilli have been widely studied in the human gut and vagina, but knowledge on their role and beneficial functions in the human URT is limited. A thorough analysis of habitat-specific LGC species with a niche and adaptation potential to the URT environment had not been performed to date. Our study showed that *L. casei* AMBR2, which was isolated from the URT and shown to have superior properties in terms of oxidative-stress tolerance and fimbriae structures, was able to adapt to and colonize the human nasopharynx and was well tolerated. This highlights the importance of *Lactobacillus* taxa in the URT and the potential of *L. casei* AMBR2 as a probiotic for the URT.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

(C) Relative abundances focusing on the dominant URT genera determined at the different time points of the study, determined via V4 *16S rRNA* MiSeq sequencing.

(D) Reported side effects during the 14-day nasal treatment with *L. casei* AMBR2. Reported side effects included sore throat, sneezing, runny nose, nasal blockage, nasal bleeding, and crust formation. The number of complaints per side effect over the 14-day treatment period is visualized for each participant. See also Table S6.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.celrep.2020.107674.

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AUTHOR CONTRIBUTIONS

Conceived and designed the study: S.L., A.V., F.K., O.M.V., P.W.H., and T.V.d.W. Designed the experiments: I.D.B., C.N.A., M.F.L.v.d.B., and S.L. The experiments were performed by I.D.B., C.N.A., K.M., C.D.R., K.J., M.L., D.V., I.S., E.C., and T.E. Experimental work related to SEM was done in collaboration with S.T. and J.-P.T. Data analysis and interpretation were done by I.D.B., C.N.A., M.F.L.v.d.B., K.M., C.D.R., M.L., K.J., and I.S., and bioinformatics analysis and writing of the scripts by S. Wuyts and S. Wittouck. P.W.H., A.V.V., and O.M.V. were responsible for the clinical assessments and interventions. I.D.B. and S.L. wrote the first draft, and all authors participated in the revisions and read the final version.

DECLARATION OF INTERESTS

A patent application (PCT/EP2018/057497) was filed on March 23, 2018, related to this work.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and Virus Strains		
Lactobacillus rhamnosus GG	ATCC	ATCC53103
Lactobacillus rhamnosus AMBR1	LAMB Lab of Applied Microbiology and Biotechnology, University of Antwerp, Belgium.	PRJEB32716
Lactobacillus casei AMBR2	LAMB BCCM/LMG P-30039	PRJEB21025
Lactobacillus rhamnosus AMBR3	LAMB	PRJEB32716
Lactobacillus rhamnosus AMBR4	LAMB	PRJEB32716
Lactobacillus rhamnosus AMBR5	LAMB	PRJEB32716
Lactobacillus rhamnosus AMBR6	LAMB	PRJEB32716
Lactobacillus rhamnosus AMBR7	LAMB	PRJEB32716
Lactobacillus sakei AMBR8	LAMB	PRJEB32716
Lactobacillus plantarum AMBR9	LAMB	PRJEB32716
Haemophilus influenzae	ATCC	ATCC49247
Moraxella catarrhalis	ATCC	ATCC25238
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
Hemin	BioXtra, Sigma Aldrich	51280-1G
NAD	VWR	53-84-9
Glucose	Sigma Aldrich	G8270-1KG
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
Vancomycin	CELLPURE	0242.3
Menaquinone	Sigma Aldrich	V9378-250MG
0.1% hexetidine (Hextril®)	Famar Orléans	N/A
Pronase	Sigma Aldrich	10165921001
DMEM-F12	GIBCO	11330032
Ultroser G	Pall Life Sciences	15950-017
Dynabeads™ CD45	Thermofisher Scientific	***11153D***
Dynabeads™ CD15	Thermofisher Scientific	***11137D***
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
Critical Commercial Assays		
NucleoSpin 96 Tissue kit	Machery-Nagel	MN 740609.50
Nextera XT DNA Sample Preparation kit	Illumina	TG-131-1096

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
QIAamp Powerfecal DNA kit	QIAGEN	12830-50
Agencourt AMPure XP	Beckman Coulter	A63881
RNeasy Mini kit	QIAGEN	74104
NuPAGE® Novex® 3-8% Tris-Acetate gels	Invitrogen	EA0375PK2
Deposited Data		
Lactobacillus rhamnosus AMBR1	This paper	PRJEB32716
Lactobacillus casei AMBR2	(Wuyts et al., 2017)	PRJEB21025
Lactobacillus rhamnosus AMBR3	This paper	PRJEB32716
Lactobacillus rhamnosus AMBR4	This paper	PRJEB32716
Lactobacillus rhamnosus AMBR5	This paper	PRJEB32716
Lactobacillus rhamnosus AMBR6	This paper	PRJEB32716
Lactobacillus rhamnosus AMBR7	This paper	PRJEB32716
Lactobacillus sakei AMBR8	This paper	PRJEB32716
Lactobacillus plantarum AMBR9	This paper	PRJEB32716
DNA-Seq data study B300201524257	(De Boeck et al., 2017, 2019)	PRJEB23057, PRJEB30316
DNA-Seq data study B300201835709	This paper	PRJEB32716
Experimental Models: Cell Lines		
Calu-3	ATCC	HTB-55
Primary Nasal Epithelial Cells	University Hospitals Leuven	N/A
Oligonucleotides		
16S rRNA gene (27F) AGAGTTTGATCMTGGCTCAG	(Lane, 1991)	N/A
16S rRNA gene (1492R)GGTTACCTTGTTACGACTT	(Turner et al., 1999)	N/A
Srr2 (2759F) CCCGGGCCGTTACGTTGCAGGCAAAA	This study	N/A
Srr2 (2841R)ACTAGTTAATTGGTCAGTCGGTGCCC	This study	N/A
CYC1-F (qPCR)CATGTCCCAGATAGCCAAGGA	(Moretti et al., 2019)	N/A
CYC1-R (qPCR)CTTGTGCCGCTTTATGGTGTAG	(Moretti et al., 2019)	N/A
ATP5B-F (qPCR)GCAGGAAAGAATTA CCACTACCAAG	(Moretti et al., 2019)	N/A
ATP5B-R (qPCR)TGGTAGCATCCAAATGGGCAA	(Moretti et al., 2019)	N/A
IL1β-F (qPCR)TTGCTCAAGTGTCTGAAGCAGC	(Moretti et al., 2019)	N/A
IL1β-R (qPCR)CAAGTCATCCTCATTGCCACTG	(Moretti et al., 2019)	N/A
IL8-F (qPCR)TGGCAGCCTTCCTGATTTCT	(Moretti et al., 2019)	N/A
IL8-R (qPCR)TTAGCACTCCTTGGCAAAACTG	(Moretti et al., 2019)	N/A
TNF-F (qPCR)CCTCTGATGGCACCACCAG	(Moretti et al., 2019)	N/A
TNF-R (qPCR)TCTTCTCGAACCCCGAGTGA	(Moretti et al., 2019)	N/A
Software and Algorithms		
GraphPad Prism	GraphPad Software	https://www.graphpad.com/
DADA2, version 1.6.0	https://doi.org/10.1038/nmeth.3869	https://benjjneb.github.io/dada2/index.html
R version 3.4.4 (R Foundation for Statistical Computing, 2018)	(R Core Team, 2018)	https://www.r-project.org/
Tidyamplicons	(SWittouck/tidyamplicons: Functions to Manipulate and Visualize Amplicon Abundance Data)	https://github.com/Swittouck/tidyamplicons
SPAdes 3.12.0	(Bankevich et al., 2012)	http://cab.spbu.ru/software/spades/
checkM	(Parks et al., 2015)	https://ecogenomics.github.io/CheckM/
Prokka 1.12	(Seemann, 2014)	https://github.com/tseemann/prokka
fastANI	(Jain et al., 2018)	https://github.com/ParBLiSS/FastANI

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Blastp	(Altschul et al., 1990; Camacho et al., 2009)	https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins
snippy 4.3.6	N/A	https://github.com/tseemann/snippy
OrthoFinder	(Emms and Kelly, 2015)	https://github.com/davidemms/OrthoFinder
Mafft	(Katoh and Standley, 2013)	https://mafft.cbrc.jp/alignment/software/
RAxML	(Stamatakis, 2014)	https://cme.h-its.org/exelixis/web/software/raxml/
qbase^{+}	(Vandesompele et al., 2002)	https://www.qbaseplus.com/
Virulence Factor Database (VFDB)	(Liu et al., 2019)	http://www.mgc.ac.cn/VFs/
Other		
MiSeq Desktop sequencer	Illumina	(M00984, Illumina)
Spray drier B-290, Büchi	Büchi	B-290, Büchi
Synergy HTX multi-mode reader	Biotek	N/A
Leica EM CPD030	Leica Microsystems	CPD030
Leica EM Ace 600 coater	Leica Microsystems	EM Ace600
Quanta Scanning Electron Microscopy	ThermoFisher Scientific	Quanta FEG250 SEM system
Take3	Biotek	N/A
StepOne Plus Real-Time PCR System (v.2.0)	Applied Biosystems	N/A

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, PRJEB30316, and PRJEB32716. All AMBR genomes were deposited under accession number PRJEB32716 and PRJEB21025 for *L. casei* AMBR2. The R code generated during this study can be found on GitHub at https://github.com/Swittouck/urt_crs

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Human subjects

For the microbiome comparison, human subjects, aged between 18 and 65 years, were recruited and sampled according to study B300201524257 (ClinicalTrials.gov Identifier: NCT02933983). Nasopharyngeal samples were obtained from 100 healthy adult participants (39% male, average age 34 years) and 225 adult CRS patients (63% male, average age 42 years). The informed consent was obtained from all participants prior to sampling.

For the pilot study testing the fit-for-purpose *L. casei* AMBR2 nasal spray, 20 healthy adult male/female volunteers, aged between 18 and 65 years, were recruited and enrolled in the study (40% male, average age 36.7 years). Exclusion criteria were participants taking corticosteroid medication and participants suffering from chronic airway diseases. This study was approved by the Ethics Committee of the Antwerp University Hospital/University of Antwerp (registration number B300201835709, registered 5 March 2018, ClinicalTrials.gov Identifier: NCT03587545). The informed consent was obtained from all participants prior to sampling.

Microbe strains and culture

Microbial strains used are listed in the Key Resources Table. *Lactobacillus* strains were grown at 37°C without agitation in de Man, Rogosa and Sharpe (MRS) broth (Difco, Erembodegem, Belgium). *Moraxella catarrhalis* ATCC25238 was inoculated in Mueller Hinton (MH) broth (LabM Limited) and cultured aerobically at 37°C, *Staphylococcus aureus* was grown at 37°C without agitation in MH broth and *Haemophilus influenzae* ATCC49247 was grown at 37°C with 5% CO_2 in MH broth enriched with 0.5% of yeast extract, 15 mg/L

hemin and 15 mg/L NAD (Sigma Aldrich). Solid media contained 1.5% (v/w) agar. For antimicrobial assays, the media of the tested pathogens was supplemented with glucose (5 g/L) (Sigma Aldrich).

Cell culture

The human bronchial epithelial cell line Calu-3 ATCC® HTB-55™ (ATCC) was cultured at 37°C with 5% CO_2 and 90% relative humidity in 75 cm^2 cell tissue flasks containing 20 mL Minimal Essential Medium (MEM) (Life Technologies, Ghent, Belgium) supplemented with 10% heat inactivated fetal calf serum (FCS) (Hyclone) and penicillin-streptomycin (100 U/mL) (Life Technologies). Every three or four days, the culture medium was changed and cells reaching 70%–80% confluency were reseeded at a 1:2 split ratio using a 0.25% trypsin-EDTA solution (Life Technologies). Calu-3 cells were seeded in 12-well or 24-well culture plates (Cellstar, Diegem, Belgium) for adhesion and immunomodulation experiments respectively, at a density of 3×10^5 cells/cm^2 (1.1×10^6 cells/mL). Approximately one week after seeding, confluent monolayers were obtained. One day before an experiment, medium was replaced to MEM without any supplements.

Primary cell cultures

Inferior turbinates or polyps were used for isolation of nasal epithelial cells (NECs) at the University Hospitals of Leuven after patients gave informed consent. The inferior turbinate was collected from non-allergic, non-smoking, non-asthmatic healthy controls during aesthetic and/or functional rhinoplasty. Polyps were collected during FESS from non-allergic, non-smoking CRSwNP patients. A highly purified NEC population was obtained, as reported previously (Bobic et al., 2010). Tissue was washed in sterile saline and enzymatically digested in 0.1% Pronase (Protease XIV, Sigma) solution in DMEM-F12 culture medium supplemented with 100 U/mL penicillin, 100 mg/mL streptomycin, and 2% Ultroser G (Pall Life Sciences, Zaventem, Belgium). After overnight incubation at 4°C while shaking, the protease reaction was stopped by the addition of fetal calf serum (FCS) (10%). Cells were washed in culture medium and pelleted by means of centrifugation for 5 min at 100 x g. Cells were then resuspended in 10 mL of culture medium and incubated in a plastic culture flask for 1 h at 37°C to remove fibroblasts. The cell suspension was mixed with 2×10^7 prewashed CD45 and CD15 magnetic beads (Dynabeads; Invitrogen, Merelbeke, Belgium), and epithelial cells were purified by means of negative selection, according to the manufacturer's instructions. Cell purity was verified by using cytospin preparations and was found to be 98% or higher. Freshly isolated NECs were seeded in 12-well culture plates (Cellstar) at a density of 5×10^5 cells/mL. One day before an experiment, medium was replaced to DMEM-F12 without any supplements.

METHOD DETAILS

Large-scale microbiome comparison: study design, sample collection and Illumina MiSeq 16S rRNA amplicon sequencing

For microbiome comparison, nasopharyngeal samples were processed and sequenced as previously described (De Boeck et al., 2017, 2019). Briefly, dual-index paired-end sequencing was performed on the V4 region of the *16S rRNA* gene using a MiSeq Desktop sequencer (M00984, Illumina). Processing and quality control of the reads were performed for each run separately using the R package DADA2, version 1.6.0. Briefly, this entailed quality filtering of the reads, dereplication, denoising, removal of chimeras and read classification. All data handling and visualization was performed in R version 3.4.4 (R Core Team, 2018) using the tidyverse set of packages and the in-house package tidyamplicons (https://github.com/Swittouck/tidyamplicons).

The compositional analysis of differential abundance between CRS and control samples was inspired by the ANCOM method (Mandal et al., 2015). Read counts were first aggregated on the family level (the families *Lactobacillaceae* and *Leuconostocaceae* were merged into a single family representing the LGC). For each combination of a family and a reference family, the logratio of their relative abundances was calculated per sample and a Wilcoxon rank-sum test was executed to assess differential abundance between the healthy controls and CRS patients. Significant differences were then determined from the Wilcoxon p values by capping the false discovery rate at 10% with the method of Benjamini and Yekutieli (2001). To quantify the differential abundances, we used the two-sample Hodges–Lehmann estimator (the median of all pairwise differences between the samples). The R code of these analyses can be found on GitHub at https://github.com/Swittouck/urt_crs.

Intervention study with L. casei AMBR2 nasal spray

The *L. casei* AMBR2 was spray dried in a co-current system with a two-fluid nozzle (orifice diameter 1.4mm) under aseptic conditions, without addition of excipients, at an initial inlet temperature of 135°C, feed (bacterial suspension) flow rate of 7.5 mL/min, air flow rate of approx. 32.5 m^3/h, spray flow rate of 831 L/h and constant outlet temperature of 55°C. Briefly, bacterial cells were grown to the stationary phase, harvested, and dried using a laboratory-scale air spray dryer (B-290, Büchi, Flawil, Switzerland) (Broeckx et al., 2017). Spray-dried powder was collected from a single cyclone separator continuously throughout the process. Quality of the product was investigated via the total microbial aerobic count (TAMC), total combined yeasts/molds count (TYMC) according to the European Pharmacopoeia (PH. Eur. 2.6.12) (no more than 10^2 CFU/g), and by evaluating *S. aureus* and *Pseudomonas aeruginosa* absence (absent in 1 g). Nasal spray formulations were prepared by adding 30 mg of spray-dried *L. casei* AMBR2 powder containing 10^{11} CFU/g of live bacteria at the moment of production in a specific glass bottle with nasal spray applicator (Pharma Pack, Wilrijk, Belgium) in aseptic conditions. During the two weeks of the study, participants had to resuspend the powder in a 3 mL flush syringe

with sterile physiological water (BD PosiFlush TM SP Syringe, 0.9% sodium chloride) and shaking immediately before application was required. The packed product was stored at 4°C and the stability of the product was tested after 2, 4, and 6 weeks to ensure that the final concentration of administered bacteria was at least 10^8 CFU per puff. All participants administered the spray twice a day for 14 days, by one puff in each nostril. Participants were followed up for an entire month with three visits to the ENT outpatients' clinic. Nasopharyngeal samples of the study participants were collected by the responsible ENT specialist at the start of the study, 5 min after the first administration, after two weeks of spraying (within 10-16 h after the last administration), and finally after an additional two weeks without intervention. To detect the presence of *L. casei* AMBR2 in the nasopharyngeal samples, qPCR with specific primers for the *srr2* gene of AMBR2 was performed (Key Resources Table). Blood samples for assessing inflammatory markers and complete blood count analysis were collected at the start of the study and after two weeks. Participants had to fill in a questionnaire to report side effects, including sneezing, runny nose, blocked nose, and bleeding nose. Dual-index paired-end sequencing of the nasopharyngeal samples was performed on the V4 region of the *16S rRNA* gene on a MiSeq Desktop sequencer (M00984, Illumina), as described above.

Isolation and whole genome sequencing of Lactobacillus isolates from the healthy URT

Nasopharyngeal swabs from healthy volunteers were cultivated in MRS broth with 20 mg/mL vancomycin to promote the growth of *Lactobacillus* species. Of note, special culture conditions relevant to the URT, such as decreasing the growth temperature to 34°C (*in vivo* nasopharynx temperature), and addition of menaquinone (1 μg/mL) and hemin (2.5 μg/mL) did not increase the isolation frequency. Grown cultures were plated on MRS agar and single colonies were further identified with PCR and Sanger sequencing of the *16S rRNA* gene (27F-1492R). *Lactobacillus*-positive colonies were stored at −80°C in 25% v/v glycerol. Total DNA was extracted for whole genome sequencing using the NucleoSpin 96 Tissue kit (Machery-Nagel, Düren, Germany) with additional lysis step using 20 mg/mL lysozyme (Sigma-Aldrich, St. Louis, MO, USA) and 100 U/mL mutanolysin (Sigma Aldrich). Whole genome sequencing was performed with the Nextera XT DNA Sample Preparation kit (Illumina, San Diego, CA), followed by sequencing with the Illumina MiSeq platform (2 × 300 cycles) at the Center of Medical Genetics Antwerp (University of Antwerp).

Genome analysis

To improve phylogenetic analysis of the isolated AMBR genomes, all genomes that were used in the study of Sun et al. were retrieved from the National Center for Biotechnology Information (NCBI) Assembly database and included in this study as reference strains (Sun et al., 2015). Assembly was performed with SPAdes 3.12.0 (Bankevich et al., 2012). The completeness of the genomes was evaluated with checkM (Parks et al., 2015) and all genomes with a completeness lower than 95% were discarded. Prokka 1.12 (Seemann, 2014) was used to predict and annotate genes for all genome sequences. A phylogenetic tree was built using single-copy core orthogroups as described in Wuyts et al., 2019). Furthermore, ANI was calculated using fastANI (Jain et al., 2018). The presence of catalase and superoxide dismutase (SOD) genes was screened for using the same approach as described in Wuyts et al. (2017). *SpaCBA* and *srr2* genes presence was assessed with a BLAST search at protein level using blastp (Altschul et al., 1990; Camacho et al., 2009) with the protein sequences of the genes of interest as query and the protein sequences encoded by the genomes as reference. Finally, variant calling was performed using snippy 4.3.6. All genomes were uploaded under accession number PRJEB32716 and PRJEB21025 for *L. casei* AMBR2 .

For the presence/absence assessment of heme- and manganese-dependent catalase genes across the *Lactobacillus* genus complex (LGC), the gene families of the in-house "lactobacillus evolutionary genomics" (legen) pipeline, version 2, were used. Briefly, in this pipeline, the pangenome of the LGC was determined in three major steps. First, genomes were prepared: all publicly available LGC genomes were downloaded from GenBank, they were filtered based on estimated completeness and contamination and their core genes were extracted. Second, a *de novo* species-level taxonomy was constructed for these genomes by clustering them based on pairwise genome core nucleotide identities. *De novo* species were named based on comparisons to published species (Wittouck et al., 2019). Only species were retained with five or more genomes available and for species with more than 50 genomes, the 50 highest quality genomes were selected. Finally, the pangenome of the remaining genomes was determined in the following, hierarchical way. The pangenome of each of the species was inferred separately using the pangenome tool OrthoFinder (Emms and Kelly, 2015). Next, these species-level pangenomes were collapsed by only selecting a single representative sequence for each species-level gene family. Then, the collapsed species-level pangenomes were subjected to another round of gene family inference, also using OrthoFinder. Inflating the resulting LGC-level gene families with the species-level families again then yielded the pangenome of the entire LGC. To identify the gene families corresponding to the heme- and manganese-dependent catalases, a profile HMM database was constructed for the gene families. The sequence of an experimentally confirmed heme-dependent catalase as well as that of a manganese-dependent catalase (Table S8) were then searched against this database and the best-scoring profile (gene family) was selected for each one.

To infer a phylogeny of LGC species, a supermatrix was first constructed from nucleotide sequences of single-copy core genes, using mafft (Katoh and Standley, 2013). The tree was then inferred with RAxML (Stamatakis, 2014), with the General Time Reversible (GTR) model and the "-f a" option, which performs rapid bootstrapping first and then goes through multiple stages of optimizing the highest- likelihood trees of the previous stage, to ultimately end up with a single best tree.

Spot antimicrobial assay with live lactobacilli

The antimicrobial activity of isolated lactobacilli and *L. rhamnosus* GG against *S. aureus*, *M. catarrhalis,* and *H. influenzae* was explored by standard antimicrobial tests, as described in van den Broek et al. (2018). Briefly, 2 μL of each *Lactobacillus* culture was spotted on a standard agar plate (1.5% w/v) containing medium of the pathogen supplemented with glucose (5 g/L). These plates were incubated for 48 h at 37°C. After incubation, 450 μL, 45 μL and 300 μL of an overnight culture of *M. catarrhalis*, *S. aureus* and *H. influenza*, respectively, were inoculated in soft agar (0.5% w/v) and poured over the plates with *Lactobacillus* spots. The plates were incubated overnight according to the appropriate growth conditions of the tested pathogens, after which time the inhibition zones were measured. 0.1% hexetidine (Hextril®, Famar Orléans, Orléans, France) was used as a positive control while a spot of the pathogenic bacterium itself served as a negative control.

Minimal inhibitory concentration (MIC) assay with isolated Lactobacillus species

The MIC values of several antimicrobials, as listed by EFSA (EFSA Panel on Biological Hazards (BIOHAZ), 2013), were determined based on an earlier described procedure (Nizet et al., 2001). Lactobacilli were grown in MRS medium as described above. A 96-well plate was filled to obtain a total volume of 100 μL at a final concentration of 5×10^5 CFU/mL of the lactobacilli combined with an adequate concentration of the active molecule. The microplate was incubated overnight at 37°C and the OD at 600 nm was measured using a Synergy HTX multi-mode reader (Biotek, Drogenbos, Belgium).

Scanning electron microscopy

Scanning electron microscopy (SEM) was used to visualize the presence or absence of pili or fimbriae on the bacterial surfaces, as described in Wuyts et al., 2019). Briefly, grown bacteria were spotted on a gold-coated membrane (approximately 10^7 CFU per membrane) and fixed with 2.5% (m/v) glutaraldehyde in 0.1 M sodium cacodylate buffer (2.5% glutaraldehyde, 0.1 M sodium cacodylate, 0.05% $CaCl_2.2H_2O$ at pH 7.4) by gentle shaking for 1 h at room temperature (RT), followed by a further overnight fixation at 4°C. Bacteria were then rinsed 3x20 min and left overnight in cacodylate buffer (containing 7.5% (m/v) saccharose). Subsequently, bacteria were dehydrated in a series of ethanol solutions in ascending concentrations and critical point dried in a Leica EM CPD030 (Leica Microsystems Belgium, Diegem, Belgium). The membranes were mounted on a stub and coated with 5 nm of carbon in a Leica EM Ace 600 coater (Leica Microsystems Belgium). SEM imaging was performed with a Quanta FEG250 SEM system (Thermo Fisher, Asse, Belgium) at the Electron Microscopy for Material Science group (EMAT, University of Antwerp).

Adherence assays to human airway Calu-3 epithelial cells and primary nasal epithelial cells

Experiments to assess the adhesion of bacterial strains to Calu-3 cells were carried out according to the methods described in Lebeer et al. (Lebeer et al., 2012). One mL of the bacterial suspensions at a concentration of 1×10^8 CFU/mL was added to tissue culture plates containing Calu-3 cells or primary NECs. Bacteria were incubated with the human cells for one h at 37°C to allow adherence. After incubation, cells were rinsed once with prewarmed PBS. To detach the cells, 300 μL of trypsin (0.25%) was added to the cells for 10 min at 37°C. When the cells had been detached, 700 μL PBS was added and serial dilutions were plated out on solid MRS medium for *Lactobacillus*. The percentage of bacterial adhesion was calculated by comparing the total number of colonies counted after adhesion with the number of cells in the bacterial suspension originally added to the cells.

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

One mL of the bacterial suspensions at a concentration of 1×10^8 CFU/mL was added to tissue culture plates containing Calu-3 cells. Bacteria were incubated for four hours at 37°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 with the RNeasy Mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol and stored at −80°C. One mg of isolated RNA, quantified with Take3 (Biotek), was used for cDNA synthesis using Readyscript® cDNA synthesis mix (Sigma Aldrich). After geNorm analysis in qbase$^+$ (Vandesompele et al., 2002), cytochrome *c*-1 (CYC1) and ATP synthase subunit beta (ATP5B) were selected as reference genes. The expression of these reference genes with the expression of IL-8, IL-1β, and TNF was quantified by RT-qPCR on a StepOne Plus Real-Time PCR System (v.2.0; Applied Biosystems, Foster City, California, United States). Primers were tested for their efficiencies between 90%–110%. All primers were designed on the basis of published sequences (Moretti et al., 2019) and chemically synthesized by Integrated DNA Technologies (IDT, Leuven, Belgium) (Key Resources Table). Each cDNA sample was amplified in duplicate with PowerSYBR® Green PCR Master Mix (Applied Biosystems) in a total volume of 20 μL with 0.15 μM 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 were included for each run.

Surface proteome extraction

To extract the surface proteins from *L. casei* AMBR2, an overnight culture (300 mL) was pelleted and washed with PBS. The cell pellet was dissolved in PBS (30 mL) and the surface proteins were loosened by subsequent sonication (20 min; 2'' on, 2'' off, 20%), freezing and thawing. This cycle was repeated twice. Afterward, the cells were removed by cycles of centrifugation at increasing speed (4000 x g, 4500 x g, 4750 x g, each 15 min, 4°C). The protein fraction in the supernatant was precipitated by overnight incubation in two

volumes of ethanol and centrifugation (> 8000 x g, 30 min, 4°C). The obtained fraction was separated on NuPAGE® Novex® 3%–8% Tris-Acetate gels (Life Technologies).

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. For adherence assays and qPCR data, one-way ANOVA and multiple t tests were used to determine statistical significance in GraphPad Prism. The p value was corrected for multiple comparisons using the Holm-Sidak method in GraphPad Prism. For the prevalence and abundance of lactobacilli in the URT of healthy controls and CRS patients (Figure 1), Wilcoxon rank-sum test was executed to assess differential abundance between the healthy controls and CRS patients. Significant differences were then determined from the Wilcoxon p values by capping the false discovery rate at 10% with the method of Benjamini and Yekutieli (Benjamini and Yekutieli, 2001). p values for comparison of prevalence and mean relative abundance between heathy controls and CRS patients were determined with a Fischer's exact test and Wilcoxon rank-sum test, respectively.