ABSTRACT

Purpose: A defective epithelial barrier has been demonstrated in chronic rhinosinusitis with nasal polyps (CRSwNP). Lactobacilli are shown to restore epithelial barrier defects in gastrointestinal disorders, but their effect on the airway epithelial barrier is unknown. In this study, hence, we evaluated whether the nasopharyngeal isolates Lacticaseibacillus casei AMBR2 and Latilactobacillus sakei AMBR8 could restore nasal epithelial barrier integrity in CRSwNP.

Methods: Ex vivo trans-epithelial tissue resistance and fluorescein isothiocyanate-dextran 4 kDa (FD4) permeability of nasal mucosal explants were measured. The relative abundance of lactobacilli in the maxillary sinus of CRSwNP patients was analyzed by amplicon sequencing of the V4 region of the 16S rRNA gene. The effect of spray-dried L. casei AMBR2 and L. sakei AMBR8 on epithelial integrity was investigated in vitro in primary nasal epithelial cells (pNECs) from healthy controls and patients with CRSwNP as well as in vivo in a murine model of interleukin (IL)-4 induced barrier dysfunction. The activation of Toll-like receptor 2 (TLR2) was explored in vitro by using polyclonal antibodies.

Results: Patients with CRSwNP had a defective epithelial barrier which positively correlated with the relative abundance of lactobacilli-specific amplicons in the maxillary sinus. L. casei AMBR2, but not L. sakei AMBR8, increased the trans-epithelial electrical resistance (TEER) of pNECs from CRSwNP patients in a time-dependent manner. Treatment of epithelial cells with L. casei AMBR2 promoted the tight junction proteins occludin and zonula occludens-1 reorganization. Furthermore, L. casei AMBR2 prevented IL-4-induced nasal permeability in vivo.

Lacticaseibacillus casei AMBR2 Restores Airway Epithelial Integrity in Chronic Rhinosinusitis With Nasal Polyps

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**L. casei AMBR2 Restores Nasal Epithelial Barrier**

The nasal epithelial barrier is a pseudostratified epithelium that forms a physical and immunological barrier. The integrity of the nasal epithelium is maintained via intercellular junctions such as tight junctions (TJs), adherens junctions and desmosomes. TJ proteins regulate the mucosal transport of water, ions and certain molecules, and consist of different transmembrane proteins, including the claudin family and occludin, as well as cytoplasmic proteins such as the zonula occludens (ZO)-1, ZO-2 and ZO-3. Disturbance in the formation, expression or function of TJs can result in a temporal opening of the epithelium, facilitating the penetration of foreign molecules, such as allergens, to the submucosal region. Indeed, impaired epithelial barrier function as a result of a decreased expression of occludin and ZO-1 has been reported in different chronic airway diseases such as chronic rhinosinusitis (CRS). CRS is an immune disorder of the sinonasal mucosa, which is generally divided into two phenotypes: CRS with nasal polyps (CRSwNP) and CRS without nasal polyps (CRSsNP). Moreover, bacterial translocation of *Streptococcus pneumoniae* and *Haemophilus influenzae* across the epithelium is demonstrated in mice along with a decrease in claudin-7 and -10 expression by activating Toll-like receptor (TLR)-2 and TLR4 signaling, respectively. Similarly, incubation of human nasal epithelial cells with *Staphylococcus aureus* V8 protease disrupted epithelial integrity via ZO-1 delocalization. In contrast, certain beneficial bacteria, such as specific strains of lactobacilli, appear to have the capacity to restore a defective epithelial barrier both in vitro as in vivo, especially in the gastrointestinal tract. The lactobacilli encompasses the strains, species and genera of *Lactobacillaceae*, one of the best studied groups of beneficial bacteria, recently reclassified in 23 genera. *Lactobacillus plantarum* MB452 (new genus name *Lactiplantibacillus plantarum* MB452) has been shown to promote the expression of occludin and ZO-1. Abreu et al. demonstrated a reduction in the relative abundance of taxa belonging to the order of Lactobacillales in the maxillary sinus of CRS patients. Recently, we have also demonstrated that CRS patients have a reduced level of lactobacilli in the nose, with isolates such as *Lacticaseibacillus casei* AMBR2 having specific properties promoting its adaptation and survival in this human body site.

In addition to the effects on epithelial integrity, lactobacilli also induce other health effects in the host, including modulation of local and systemic host immune responses. Activation of TLRs present on the epithelium. Activation of TLRs can induce different signaling cascades that mount a proper immune response against the microorganisms detected. In vivo modulation of TLR9 expression by *Lactobacillus reuteri* ATCC 23272 (new taxonomy *Limosilactobacillus reuteri* ATCC 23272) resulted in reduced levels of interleukin (IL)-5 and IL-6 and attenuated allergic inflammation in the lungs of ovalbumin-sensitized mice.

**Conclusions:** A defective epithelial barrier in CRSwNP may be associated with a decrease in relative abundance of lactobacilli-specific amplicons. *L. casei AMBR2* would restore nasal epithelial integrity and can be a novel therapeutic strategy for CRSwNP.

**Keywords:** Lactobacillus casei; lactobacillus sakei; sinusitis; nasal polyps; epithelium; tight junctions; permeability; toll like receptor 2

**Disclosure**

There are no financial or other issues that might lead to conflict of interest.
receptor plays a pivotal role in maintaining immune homeostasis. More specifically, TLR2 either dimerizes with TLR1 or TLR6 to induce a pro-inflammatory or anti-inflammatory response, respectively. Changes in TLR expression have been reported in CRS patients. However, the interactions between lactobacilli and TLRs in the upper and/or lower airways are still underexplored.

The aim of the present study was to investigate the effect of the nasopharyngeal lactobacilli isolates *L. casei* AMBR2 and *Latilactobacillus sakei* AMBR8 on the epithelial barrier function of primary nasal epithelial cells (pNECs) of patients with CRSwNP and in a mouse model of IL-4 induced barrier dysfunction. Our results show that a decreased relative abundance of lactobacilli correlated with impaired epithelial barrier integrity in CRSwNP. Additionally, *L. casei* AMBR2 restores the nasal epithelial barrier in CRSwNP via TLR2 activation *in vitro*. Finally, *L. casei* AMBR2 prevents IL-4-induced barrier dysfunction in a mouse model.

**MATERIALS AND METHODS**

**Illumina MiSeq V4 16S rRNA amplicon sequencing to determine the relative abundance of lactobacilli**

This study was approved by the Institutional Review Board of University Hospitals of Leuven and Antwerp (study B300201524257). Nasal swabs from the maxillary sinus were obtained from CRS patients (n = 14) during functional endoscopic sinus surgery (FESS) as previously described previously. Samples were processed and analyzed by performing the dual-index paired-end sequencing on the V4 region of the 16S rRNA gene on the MiSeq Desktop sequencer (M00984; Illumina, Seoul, Korea) as previously described. The relative abundance of lactobacilli (family *Lactobacillaceae*) was determined at the genus level. The sequencing data were deposited in ENA under accession number PRJEB30316.

**Ussing chamber experiments for the evaluation of mucosal explant integrity**

Sinus tissues from 14 non-smoking CRSwNP patients were collected during FESS, of whom 36% had an allergy, 43% had concomitant asthma and 36% underwent a previous FESS. The inferior turbinate was collected from 7 non-allergic, non-smoking, non-asthmatic healthy controls during esthetic rhinoplasty, of whom 14% underwent a previous esthetic and/or function rhinoplasty. Biopsies, in duplicate, were mounted in Ussing chambers to evaluate epithelial integrity *ex vivo*. Briefly, trans-epithelial tissue resistance (TER) was recorded for 2 hours and the average of all time points was calculated and are presented as Ω × cm². Fluorescein isothiocyanate-dextran 4kDa (FD4, 1 mg/mL) (Sigma-Aldrich, St Louis, MO, USA) was added to the mucosal compartment. Serosal samples were collected every 30 minutes over 2 hours to evaluate fluorescence intensity with a fluorescence reader (FLUOstar Omega; BMG Labtech, Ortenberg, Germany). Patients’ demographics are be found in Table 1.

**Isolation of primary epithelial cells from nasal polyp tissue and inferior turbinate**

Nasal polyp tissue from patients with CRSwNP and inferior turbinate of healthy controls were used to isolate pNECs. A highly purified epithelial cell population was obtained as previously reported. Isolated pNECs were grown in bronchial epithelial basal medium (Lonza BioWhittaker, Basel, Switzerland) supplemented with the SingleQuot Kit in a T75 culture flask at 37°C. Once cells reached 75%–80% confluency, cells were detached and seeded on Transwell inserts (Greiner Bio-One, Vilvoorde, Belgium) at a concentration of 110,000 cells/transwell. After 5–7 days, a confluent monolayer was obtained and medium was removed.

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apically to allow further cell differentiation at air-liquid interphase (ALI). Then, pNECs were cultured in Dulbecco’s modified Eagle’s medium (DMEM/F12; Lonza BioWhittaker) supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin and 2% Ultroser G (Pall Life Sciences, Zaventem, Belgium). Medium was changed every other day.

**Trans-epithelial electrical resistance (TEER) and paracellular flux measurements**

The methodology for TEER and paracellular flux measurements of FD4 has previously been described. In brief, the epithelial integrity of ALI cultures was evaluated by measuring the TEER with an EVOM/Endohm (WPI, Sarasota, FL, USA) as described in the online data supplement. Paracellular flux measurements were made by applying FD4 (2 mg/mL) apically and measuring the FD4 intensity of the basolateral medium at different time points with a fluorescence reader (FLUOstar Omega; BMG LABTECH, Ortenberg, Germany). FD4 concentration was calculated and is expressed as pmols.

**Spray drying process**

*L. casei* AMBR2 and *L. sakei* AMBR8 were isolated from the nasopharynx of a healthy individual as part of the study B300201524257. The strains were grown at 37°C without agitation in the Man, Rogosa and Sharpe (MRS) broth (Difco, Erembodegem, Belgium). For spray-drying, the bacterial suspension was centrifuged at 3,983 × g for 12 minutes at 20°C after the strains reached the stationary phase. The pH of the supernatant was measured as quality control. The bacterial pellet was re-suspended to its original volume in phosphate-buffered saline (PBS) or demineralized water. PBS medium contained 0.3 g/L NaH₂PO₄.2H₂O, 1.54 g/L Na₂HPO₄.2H₂O and 8.2g/L Sodium chloride (NaCl), and the pH was adjusted to 7.2. Sodium chloride (NaCl) was purchased from Carl Roth (Mühlburg, Germany), sodium dihydrogen phosphate dihydrate (NaH₂PO₄.2H₂O) and disodium hydrogen phosphate dihydrate (Na₂HPO₄.2H₂O) of analytical grade were acquired from Merck (Darmstadt, Germany). Spray drying was done using a laboratory-scale spray dryer (B-290; Büchi, Flawil, Switzerland). A co-current spray dryer configuration was used, and the feed was sprayed into the heated drying chamber using a 2-fluid nozzle (orifice diameter 1.4 mm). The spray drying parameters applied during the experiments were: inlet temperature of 135°C, feed rate of 25% (7.5 mL/min), atomization–spray flow rate of 45 mm (831 L/h), airflow rate of approximately 32.5 m³/h and outlet temperature of 55°C. The spray-dried powder was collected from a single cyclone separator, and stored in Eppendorf tubes (VWR International Europe, Leuven, Belgium), sealed with Parafilm®, and kept at 4°C.

Viability after spray drying, expressed in CFU/g, was evaluated via plate counting. The spray-dried powder was re-suspended in an appropriate medium (i.e., PBS or demineralized water)

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**Table 1. Patient demographics**

| Characteristics               | Control | CRSwNP | P-value |
|-------------------------------|---------|--------|---------|
| No. of patients               | 7       | 14     |         |
| Mean age with SD (yr)         | 29 ± 11 | 44 ± 13| 0.0195  |
| Sex (male/female)             | 4/3     | 10/4   |         |
| Allergy (%)                   | 0       | 36     |         |
| Smoking (%)                   | 0       | 0      |         |
| Asthma (%)                    | 0       | 43     |         |
| Nasal polyps (%)              | 0       | 100    |         |
| Prior surgical history (%)    | 14      | 36     |         |

CRSwNP, chronic rhinosinusitis with nasal polyps; SD, standard deviation.
for several minutes at room temperature. Serial dilutions were prepared prior to plating onto MRS agar plates, followed by incubation at 37°C for 2 days.

**In vitro stimulation experiments with spray-dried *L. casei* AMBR2 and *L. sakei* AMBR8**

ALI cultures of healthy controls and CRSwNP patients were stimulated on day 21 with *L. casei* AMBR2 or *L. sakei* AMBR8 at 10⁵ CFU/mL, 10⁶ CFU/mL or 10⁷ CFU/mL, at the apical site for 6 hours. Human TLR2 polyclonal antibody (10 µg/mL; R&D Systems, Abingdon, UK) was added apically 2 hours prior to stimulation with *L. casei* AMBR2 (10⁷ CFU/mL) in ALI cultures of CRSwNP patients. At time points 0, 2, 4 and 6 hours, TEER was measured. The barrier restoring effects of *L. casei* AMBR2 were evaluated by adding IL-4 (10 ng/mL; R&D systems) 24 hours prior to stimulation with *L. casei* AMBR2 (10⁵ CFU/mL) in ALI cultures of CRSwNP patients. TEER was measured for 6 hours.

**TLR2-signaling assay**

Human TLR2+TLR6/NF-κB/SEAP reporter HEK293 cells (InvivoGen, San Diego, CA, USA) and human TLR2+TLR1/NF-κB/SEAP reporter HEK293 cells (InvivoGen) were grown according to the manufacturer’s recommendations. HEK cells were seeded on a 96 well plate when 70%–80% confluency was reached. After 48 hours, HEK cells were stimulated for 24 hours with spray-dried *L. casei* AMBR2 (10⁷ CFU/mL), Pam2CSK4 (1 ng/mL) or Pam3CSK4 (1 µg/L). The induction of TLR2 signaling was assessed by measuring the pNPP in the supernatant of stimulated HEK cells. Therefore, 50 µL of the supernatant from stimulated HEK cells was added onto a 96 well plate and 100 µL of pNPP solution was added. After 20 minutes, absorbance was measured at 450 nm.

**Quantitative reverse transcription polymerase chain reaction (RT-qPCR) for TJ expression**

The full methodology for mRNA isolation and RT-qPCR are described in the online data supplement. Primer sequences are found in Supplementary Table S1 in the online data supplement.

**Immunofluorescence staining of TJs**

ALI cultures were fixed with 4% paraformaldehyde (Sigma-Aldrich) after stimulation with *L. casei* AMBR2. Cultures were stained for occludin (mouse anti-occludin antibody; ThermoFisher, Waltham, MA, USA) which was detected with anti-mouse antibody Alexa Fluor 488 (ThermoFisher) and ZO-1 (rabbit: anti-ZO-1 antibody; ThermoFisher) which was detected with goat anti-rabbit antibody Alexa Fluor 555 (ThermoFisher). Finally, samples were stained with 4’-6-diamidino-2-phenylindole dihydrochloride (DAPI; ThermoFisher) and mounted on microscope slides. Images were recorded on a Zeiss LSM 780 – 8P Mai Tai HP DS with Z-stacking (12 slides with a thickness of 11 µm for each section were used for creating the Z stack) (Cell and Tissue Imaging Cluster, Supported by Hercules AKUL/11/37 and FWO G.0929.15 to Pieter Vanden Berghe, University of Leuven). Images were processed using ImageJ (Java; NIH, Bethesda, MD, USA). The immunostainings were performed on 3 controls and 3 CRSwNP patients. From these controls and patients, several regions of interest were chosen, and a representative image of the specific condition was used in the figures.
Evaluation of the effect of spray-dried \textit{L. casei} AMBR2 and \textit{L. sakei} AMBR8 in a mouse model of IL-4-induced barrier dysfunction

Male BALB/c mice (6–8 weeks) were obtained from Envigo (Horst, The Netherlands) and were kept under conventional conditions. Experimental procedures were approved by the Ethical Committee for Animal Research at the KU Leuven (P015/2017). Mice were pretreated twice with 20 µL of spray-dried \textit{L. casei} AMBR2 (10⁷ CFU/mL), spray-dried \textit{L. sakei} AMBR8 (10⁷ CFU/mL) or PBS, 48 and 24 hours prior to IL-4 application. Next, the mice received 50 µL of IL-4 (250 ng/application) or PBS 3 times, at 1-hour intervals. Twenty-four hours after the last nasal application, 20 µL of FD4 (50 mg/mL) was applied endonasally for the evaluation of mucosal permeability. One hour after FD4 application, mice were sacrificed with an intraperitoneal injection of Dolethal (Vétoquinol S.A., Lure, France). Serum and nasal mucosae were collected for further analysis. The Levels of FD4 were determined in the serum by a fluorescence reader (FLUOstar Omega).

Statistical analysis

Data were analyzed using Graphpad Prism 7 (Graphpad, La Jolla, CA, USA). Normality was tested using the Shapiro-Wilk normality test. Differences between the 2 groups were analyzed using an Mann-Whitney test. Data are presented as means ± standard error of mean or medians with interquartile range. Differences between multiple groups were analyzed using One-way analysis of variance (ANOVA) with \textit{post hoc} analysis. Two-way ANOVA with \textit{post hoc} analysis was used to evaluate the effect of stimulation over time. Spearmen \(\rho\) measurements were used to determine correlations. Values were considered significantly different when \(P < 0.05\).

RESULTS

Impaired barrier integrity in CRSwNP patients correlates with decreased relative abundance of lactobacilli

Previously, we have reported a lower relative abundance of lactobacilli in the anterior nares and nasopharynx of patients with CRSwNP and CRSsNP compared to healthy controls.\(^1\) Given the observation of an impaired epithelial barrier in CRS,\(^2\) we first investigated a possible correlation between nasal lactobacilli and nasal mucosal integrity. Therefore, mucosal biopsies were collected from 14 CRSwNP patients and 7 controls, and mucosal integrity was evaluated in Ussing chambers. A reduced TER, along with increased FD4 permeability, was found in the mucosal biopsy samples of CRSwNP patients compared to controls (Fig. 1A). Additionally, the relative abundance of lactobacilli in the maxillary sinuses of CRSwNP patients was determined by amplicon sequencing of V4 region of the 16S rRNA gene. The TER of the mucosal biopsy samples positively correlated with the relative abundance of lactobacilli (\(r = 0.6434, P < 0.05\)) in 12 CRSwNP patients, for which we obtained high-quality microbial profiles (Fig. 1B).

To further explore the effect of lactobacilli on barrier integrity, we profiled the mRNA expression of different TJ proteins, \(i.e.,\), occludin, ZO-1, claudin-1 and claudin-4, and correlated this with the relative abundance of lactobacilli of CRSwNP patients. We focused on these TJs, as their involvement in epithelial barrier dysfunction in CRSwNP has already been described.\(^3\) Interestingly, we observed an increased mRNA expression of claudin-4 in CRS patients compared to healthy controls (Fig. 1C). At the protein level, immunofluorescence staining for ZO-1 and occludin in ALI cultures of patients showed a decreased TJ architecture.

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**L. casei AMBR2 Restores Nasal Epithelial Barrier**

**Fig. 1.** Correlation between relative abundance of lactobacilli and mucosal integrity in patients with CRSwNP. (A) TER and FD4 permeability was measured on nasal biopsies collected from healthy controls and patients with CRSwNP. (B) Correlation between the relative abundance of lactobacilli and TER in patients with CRSwNP. (C) mRNA expression of claudin-4, claudin-1, occludin and ZO-1 in nasal biopsies of healthy controls (n = 5) and patients with CRSwNP (n = 10). Relative mRNA expression vs the housekeeping genes β-actin and β-2-microglobulin. (D) Representative immunofluorescence staining for occludin and ZO-1 in ALI cultures of healthy controls (n = 3) and CRSwNP patients (n = 3). White arrows indicate opening of the tight junctions (scale bar = 1 µm). (E) Correlation between the relative abundance of lactobacilli and TER in patients with CRSwNP. Furthermore, we observed a positive, albeit insignificant, correlation between the claudin-4 mRNA expression and the relative abundance of lactobacilli. *P < 0.05; †P < 0.01.

**L. casei AMBR2 increases TEER in patients with CRSwNP**

Since we observed an association between the relative abundance of lactobacilli and mucosal integrity in CRSwNP, we questioned whether lactobacilli could reconstitute a defective epithelial barrier. For this purpose, 2 spray-dried nasopharyngeal isolates, *L. casei* AMBR2 and *L. sakei* AMBR8, were used.**13** ALI cultures of pNECs of healthy controls and patients with CRSwNP were stimulated for 6 hours with different concentrations of *L. casei* AMBR2 or *L. sakei* AMBR8. In ALI cultures of patients with CRSwNP, *L. casei* AMBR2 (10^7 CFU/mL) significantly increased TEER (**Fig. 2A**). FD4 permeability was also decreased after stimulation with *L. casei* AMBR2 (10^7 CFU/mL), though it did not reach significance (**Fig. 2A**). *L. casei* AMBR2 had no effect on TEER or FD4 permeability in ALI cultures of healthy controls (**Fig. 2A**). The isolate *L. sakei* AMBR8 did not alter TEER nor FD4 passage in ALI cultures of CRSwNP patients (**Supplementary Fig. S1A and B**).
Next, we evaluated the effect of *L. casei* AMBR2 on the mRNA expression of TJJs in healthy controls and patients with CRSwNP. The effect of *L. sakei* AMBR8 on TJ expression and

Fig. 2. The effect of *L. casei* AMBR2 on nasal epithelial barrier function *in vitro*. Primary nasal epithelial cells from healthy controls and CRSwNP patients were grown at air-liquid interface for 21 days, and were stimulated with different concentrations of *L. casei* AMBR2 (both n = 6) for 6 hours. (A) Effect of different concentrations of *L. casei* AMBR2 on TEER and FD4 passage at time point six hours of epithelial cell cultures from healthy controls and patients with CRSwNP. (B) mRNA expression of occludin, ZO-1, claudin-1 and claudin-4 in nasal biopsies of healthy controls and patients with CRSwNP (both n = 5). Relative mRNA expression vs the housekeeping gene β-actin. (C) Representative immunofluorescence staining for occludin and ZO-1 in CRSwNP patients after treatment with *L. casei* AMBR2 (10⁷ CFU/mL), compared to controls. White arrow indicates disrupted tight junction formation (scale bar = 1 µm). In (A), data are presented as mean ± SEM and in (B) data are presented as median with interquartile range. Two-way ANOVA with post hoc analysis for TER measurements in (A). One-way ANOVA with post hoc analysis for FD4 measurements in A and B. CRSwNP, chronic rhinosinusitis with nasal polyps; TEER, trans-epithelial electrical resistance; FD4, fluorescein isothiocyanate–dextran 4 kDa; CFU, colony-forming unit; SEM, standard error of mean; ANOVA, analysis of variance; ZO-1, zonula occludens-1. *P < 0.05; †P < 0.01; ‡P < 0.001.
function was not investigated, as no effect on TEER and FD4 passage in ALI cultures of CRSwNP patients was found. RT-qPCR revealed a significant increase in the expression of claudin-1 and claudin-4 24 hours after stimulation of nasal explants from CRSwNP patients with $10^7$ CFU/mL *L. casei AMBR2* (Fig. 2B). At the protein level, immunofluorescence staining showed that *L. casei AMBR2* promoted reorganization of occludin and ZO-1 after 6 hours in ALI cultures of CRSwNP patients (Fig. 2C).

**L. casei AMBR2 restores nasal epithelial barrier integrity through activation of TLR2-TLR6**

Lactobacilli modulate host immune responses via activation of TLR2 signaling. Given that *L. casei AMBR2* reconstituted epithelial defects in CRSwNP, we questioned whether *L. casei AMBR2* activates TLR2 to restore epithelial defects. Using TLR2-TLR6-transfected HEK293 cells, we verified a dose-dependent activation of TLR2/6 with *L. casei AMBR2* (Fig. 3A), whereas TLR2-TLR1-transfected HEK293 cells were not activated (Supplementary Fig. S2). To validate

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**Fig. 3.** Role of TLR2 activation in *L. casei AMBR2*-induced epithelial barrier restoration in vitro. (A) Activation of TLR2:TLR6 receptor in transfected HEK293 cells by *L. casei AMBR2*. Pam2CSK4 and medium were used as positive and negative controls respectively. (B) Effect of 2 hours pre-treatment with human TLR2 polyclonal antibody before incubation with *L. casei AMBR2* for 6 hours in ALI cultures of CRSwNP patients (n = 9). (C) Representative immunostainings for occludin and ZO-1 in ALI cultures from CRSwNP patients. White arrows indicate opening of tight junctions (scale bar = 1 µm). Data are presented as mean ± SEM. Two-way ANOVA with post hoc analysis for (B).

TLR, Toll-like receptor; TEER, trans-epithelial electrical resistance; CRSwNP, chronic rhinosinusitis with nasal polyps; ALI, air-liquid interphase; ANOVA, analysis of variance; CFU, colony-forming unit; ZO-1, zonula occludens-1.

*P < 0.05; †P < 0.01; ‡P < 0.001; §P < 0.05.
the involvement of TLR2-activation in the *L. casei* AMBR2-mediated effect on epithelial integrity, ALI cultures from CRSwNP patients were pre-incubated with blocking anti-TLR2 polyclonal antibodies before stimulation with *L. casei* AMBR2 (10⁷ CFU/mL). Pre-treatment with anti-TLR2 antibodies prevented *L. casei* AMBR2-mediated increase in TEER (Fig. 3B). At the protein level, pre-treatment with anti-TLR2 prevented occludin and ZO-1 reorganization in ALI cultures of CRSwNP patients (Fig. 3C).

**L. casei** AMBR2 restores IL-4 induced barrier dysfunction

To confirm the barrier-restorative capacity of *L. casei* AMBR2 on barrier function, we explored whether *L. casei* AMBR2 could restore IL-4-induced barrier dysfunction *in vitro*. For this purpose, ALI cultures of controls and CRSwNP patients were stimulated with IL-4 for 24 hours. Next, *L. casei* AMBR2 (10⁷ CFU/mL) was applied apically and TEER was measured for 6 hours. *L. casei* AMBR2 prevented further IL-4-mediated disruption of epithelial barrier integrity in ALI cultures of controls and CRSwNP patients (Fig. 4A). Immunofluorescence staining confirmed that *L. casei* AMBR2 restored ZO-1 and occludin protein reorganization (Fig. 4B and C).

Finally, we evaluated the barrier preventive effect of *L. casei* AMBR2 in a mouse model of IL-4-induced barrier dysfunction.²⁸ Mice were pre-treated endonasally with *L. casei* AMBR2 (10⁷ CFU/mL) or *L. sakei* AMBR8 (10⁷ CFU/mL) as controls, 48 and 24 hours prior to IL-4 application. FD4 passage was evaluated 24 hours after the last IL-4 instillation (Fig. 4D). Pre-treatment with *L. casei* AMBR2 prevented IL-4-induced barrier dysfunction (Fig. 4E). *L. sakei* AMBR8 could not prevent IL-4-induced barrier dysfunction *in vivo* (Supplementary Fig. S3).

**DISCUSSION**

Although not always present in high amounts, lactobacilli are commensals of the upper respiratory tract¹²,¹³ and are able to restore epithelial barrier integrity in chronic inflammatory disorders of the gut.¹⁴ However, little is known about the involvement of lactobacilli in maintaining an intact epithelial barrier in the airways. In this study, we investigated the possible correlations between the relative abundance of lactobacilli and the nasal epithelium in patients with CRSwNP.

Our data showed that patients with CRSwNP had a defective nasal epithelial barrier compared to healthy controls and confirmed previous findings.² Furthermore, we observed that the relative abundance of lactobacilli in the maxillary sinuses positively correlated with mucosal integrity in these CRSwNP patients, suggesting that lactobacilli in the sinonasal cavity might play a role in maintaining an intact epithelial barrier, probably by modulating claudin-4 expression. In general, claudins are subdivided into 2 groups: pore-forming claudins like claudin-2 that increase epithelial permeability, and sealing claudins such as claudin-4 and claudin-5, which strengthen epithelial barrier function.²⁹,³⁰ Elevated claudin-4 expression might explain the strengthening of the epithelial barrier. Nevertheless, further research is warranted to confirm the involvement of lactobacilli in maintaining an intact epithelial barrier.

Considering the positive correlation between the relative abundance of lactobacilli and epithelial barrier function in CRSwNP, we next investigated whether administration of *L. casei* AMBR2 and *L. sakei* AMBR8 could improve barrier integrity. Our results showed that *L. casei*
AMBR2 significantly increased epithelial integrity in ALI cultures of CRSwNP, mainly via the reorganization of TJ proteins. Interestingly, the barrier-enhancing effect of lactobacilli was only found with *L. casei* AMBR2, but not with *L. sakei* AMBR8, emphasizing bacterial species- and strain-specific actions such as differences in cell membrane components and/or production of metabolites. Similar positive effects of lactobacilli were seen on intestinal epithelial integrity and TJ expression with *Lactiplantibacillus plantarum* MB452 and *Lacticaseibacillus rhamnosus* GG.\textsuperscript{11}

Of note, *L. casei* AMBR2 did not significantly alter epithelial integrity in ALI cultures of healthy individuals. We speculate that this discrepancy is related to a different inflammatory state of epithelial cells from healthy controls versus CRSwNP patients. More specifically, epithelial cells from healthy controls have an intact epithelial barrier,\textsuperscript{31} and have a lower expression of TLR2,\textsuperscript{21} and less production of pro-inflammatory cytokines IL-6 and IL-8.\textsuperscript{21}

**Fig. 4.** The effect of *L. casei* AMBR2 on IL-4 induced barrier dysfunction in vitro and in vivo. (A) Effect of *L. casei* AMBR2 with/without IL-4 on TEER of epithelial cell cultures of healthy controls (n = 5) and patients with CRSwNP patients (n = 6). (B, C) Representative immunostainings for occludin and ZO-1 from stimulated ALI cultures of healthy controls and patients with CRSwNP. White arrows indicate the opening of the tight junctions (scale bar = 1 µm). (D) Mice were endonasally pre-treated two times with *L. casei* AMBR2 (10\textsuperscript{7} CFU/mL). Twenty-four hours later, IL-4 was applied three times with one hour interval. Twenty-four hours after the last IL-4 application, FD4 was applied endonasally to evaluate mucosal permeability in the serum of the treated mice. (E) Mucosal permeability for FD4. Data are presented as mean ± SEM. Two-way ANOVA with post hoc analysis for (A). Mann-Whitney test for (D).

CRSwNP, chronic rhinosinusitis with nasal polyps; TEER, trans-epithelial electrical resistance; IL, interleukin; FD4, fluorescein isothiocyanate-dextran 4 kDa; SEM, standard error of mean; ANOVA, analysis of variance; CFU, colony-forming unit; PBS, phosphate-buffered saline; ZO-1, zonula occludens-1.

*\textsuperscript{*}P < 0.05; \textsuperscript{†}P < 0.01; \textsuperscript{‡}P < 0.001.
Lactobacilli interact with the epithelial barrier via TLRs to induce a proper immune response.\textsuperscript{32} As for, TLR2 has been studied in most detail.\textsuperscript{14} TLR2 signaling plays a pivotal role in maintaining immune homeostasis and an intact epithelial barrier, which depends on the dimerization with either TLR1 or TLR6.\textsuperscript{31-36} TLR2-TLR6 dimerization induces anti-inflammatory responses with the production of IL-10,\textsuperscript{18-20} whereas TLR2 and TLR1 dimerization results in a pro-inflammatory response with the production of pro-inflammatory cytokines such as TNF-\textgreek{a}, IL-6 and IL-8.\textsuperscript{18-20} As such, dysregulation of TLR2 function can lead to chronic inflammation. Indeed, in inflammatory bowel disease, distinctive changes in mucosal TLR expression in the intestinal epithelium have been demonstrated,\textsuperscript{38} with an inflammation-dependent induction of TLR2 and TLR4 expression in intestinal macrophages.\textsuperscript{38} In addition, changes in TLR expression have also been reported in CRS, which may be related to bacterial composition, diversity and abundance in the sinuses of these patients.\textsuperscript{21} We observed a dose-dependent effect of \textit{L. casei} AMBR2 on TLR2 expression. Blocking TLR2 activation prevented the \textit{L. casei} AMBR2-mediated restoration and reorganization of occludin and ZO-1 in ALI cultures of CRSwNP patients, suggesting a positive role for TLR2 in \textit{L. casei} AMBR2 barrier promoting function. Moreover, pretreatment with anti-TLR2 antibodies prevented \textit{L. casei} AMBR2 induced increase in TEER, confirming the involvement of TLR2 activation in the \textit{L. casei} AMBR2-mediated effect on epithelial integrity. Additionally, stimulation of TLR2-TLR6-transfected HEK cells with \textit{L. casei} AMBR2 suggests that activation of TLR2 by \textit{L. casei} AMBR2 leads to dimerization between TLR2 and TLR6, which is in line with the result of a previous study on the interactions between different lactobacilli and TLRs.\textsuperscript{27} Nevertheless, further research is warranted to confirm if \textit{L. casei} AMBR2 activates TLR2 directly by binding to the receptor or whether certain secreted metabolites are involved in TLR2 activation. We have preliminary data that \textit{L. casei} AMBR2 has pili structures, which favors direct interaction, though we momentarily cannot exclude the contribution of metabolites.

CRSwNP is typically characterized by a type 2 immune response with increased levels of IL-5, IL-4 and IL-13.\textsuperscript{39,40} We and others have shown that IL-4 disrupts epithelial barrier integrity via decreasing the expression of TJs both \textit{in vitro} as \textit{in vivo}.\textsuperscript{28} As such, we evaluated the effect of \textit{L. casei} AMBR2 on IL-4-induced barrier dysfunction. \textit{In vitro}, \textit{L. casei} AMBR2 restored IL-4-induced barrier dysfunction in ALI cultures of CRSwNP patients via reorganization of the TJ molecules occludin and ZO-1. \textit{In vivo}, pre-treatment with \textit{L. casei} AMBR2 prevented IL-4-induced increase in FD4 permeability. A possible mechanism could be that \textit{L. casei} AMBR2 counteracts the effect of IL-4 on RhoGTPases. RhoGTPases are identified as major regulators of the NF-\textkappa B signaling pathway and play a role in modulating the actin cytoskeleton.\textsuperscript{41,42} Activation of the RhoGTPase signaling can lead to actinomyosin contractility or direct modification of the TJ transmembrane proteins by inducing phosphorylation and thereby resulting in increased permeability.\textsuperscript{41} IL-4 up-regulates RhoA protein expression, which promotes TJ breakdown. \textit{L. casei} AMBR2, on the other hand, initiates the MyD88-dependent intracellular signaling pathway via TLR2. This pathway induces nuclear translocation of NF-\textkappa B to mediate several crucial cellular functions, including inflammatory cytokine production.\textsuperscript{14} There are some studies showing that TLR2 activation can modulate GTPases activity.\textsuperscript{43,44} As such, it is plausible that \textit{L. casei} AMBR2 stimulates TLR2 which might lead to downregulation of RhoGTPase and in turn reorganization of the TJs, which counteracts the effect of IL-4 on the TJs.

In summary, our study demonstrates for the first time a positive correlation between epithelial barrier function and the relative abundance of lactobacilli in CRSwNP, and that
Lactobacilli maintain epithelial integrity by modulating TJ expression. Additionally, the nasopharynx isolates *L. casei* AMBR2 promotes nasal epithelial barrier function in patients with CRSwNP by reorganizing the expression of TJs, which is TLR2-TLR6 signaling-dependent. As such, this study highlights the potential of *L. casei* AMBR2 as a novel treatment strategy for CRSwNP.

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**SUPPLEMENTARY MATERIALS**

**Supplementary Table S1**
Primer and probe sequences used for RT-qPCR

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**Supplementary Fig. S1**
Effect of *L. sakei* AMBR8 on epithelial integrity *in vitro*. (A) Effect of different concentrations of *L. sakei* AMBR8 (10⁶ and 10⁵ CFU/mL) on TEER of epithelial cell cultures from healthy controls and patients with CRSwNP (both n = 4). (B) Effect of *L. sakei* AMBR8 on FD4 passage at time point 6 hours in ALI cultures of healthy controls and CRSwNP patients. Two-way ANOVA with post hoc analysis. Data are presented as mean ± SEM.

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**Supplementary Fig. S2**
Role of TLR2 triggering in *L. casei* AMBR2 -induced epithelial barrier restoration *in vitro*. Effect of *L. casei* AMBR2 on TLR2/1 transfected reporter HEK cells. Pam3CSK4 was used as positive control. Pooled date from 2 experiments. Absorbance of pNPP was measured in the supernatant of HEK stimulated cells.

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Supplementary Fig. S3
The effect of L. sakei AMBR8 on IL-4 induced barrier dysfunction in vivo. Mice were endonasally pre-treated 2 times with L. sakei AMBR8. Twenty-four hours later, IL-4 was applied 3 times with 1 hour interval. Twenty-four hours after the last IL-4 application, FD4 was applied endonasally to evaluate mucosal permeability in the serum of the treated mice. Mucosal permeability for FD4. Data are presented as mean ± SEM. One-way ANOVA with post hoc analysis.
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