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RESEARCH ARTICLE

Development of a live biotherapeutic throat spray with lactobacilli targeting respiratory viral infections

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Abstract

Respiratory viruses such as influenza viruses, respiratory syncytial virus (RSV), and coronaviruses initiate infection at the mucosal surfaces of the upper respiratory tract (URT), where the resident respiratory microbiome has an important gatekeeper function. In contrast to gut-targeting administration of beneficial bacteria against respiratory viral disease, topical URT administration of probiotics is currently underexplored, especially for the prevention and/or treatment of viral infections. Here, we report the formulation of a throat spray with live lactobacilli exhibiting several in vitro mechanisms of action against respiratory viral infections, including induction of interferon regulatory pathways and direct inhibition of respiratory viruses. Rational selection of *Lactobacillaceae* strains was based on previously documented beneficial properties, up-scaling and industrial production characteristics, clinical safety parameters, and potential antiviral and immunostimulatory efficacy in the URT demonstrated in this study. Using a three-step selection strategy, three strains were selected and further tested in vitro antiviral assays and in formulations: *Lacticaseibacillus casei* AMBR2 as a promising endogenous candidate URT probiotic with previously reported barrier-enhancing and anti-pathogenic properties and the two well-studied model strains *Lacticaseibacillus rhamnosus* GG and *Lactiplantibacillus plantarum* WCFS1 that display immunomodulatory capacities. The three strains and their combination significantly reduced the cytopathogenic effects of RSV, influenza A/H1N1 and B viruses, and HCoV-229E coronavirus in co-culture models with bacteria, virus, and host cells. Subsequently, these strains were formulated in a throat spray and human monocytes were employed to confirm the formulation process did not reduce the interferon regulatory pathway-inducing capacity. Administration of the throat spray in healthy volunteers revealed that the lactobacilli were capable of temporary colonization of the throat in a metabolically active form. Thus, the developed spray with live lactobacilli will be further explored in the clinic as a potential broad-acting live biotherapeutic strategy against respiratory viral diseases.

Irina Spacova and Ilke De Boeck contributed equally to this work.

INTRODUCTION

Viral respiratory tract infections (RTIs) result in a significant health and economic burden, as highlighted by the coronavirus disease 2019 (COVID-19) pandemic (Dong et al., 2020). Despite high prevalence of viral RTIs, few prevention or treatment options besides symptom relief are available to primary care patients. Nevertheless, RTIs can have drastic health consequences after the initial infection in the upper respiratory tract (URT), as several viruses such as influenza viruses, respiratory syncytial virus (RSV), human parainfluenza virus (HPIV), and human coronaviruses (e.g. HCoV-229E and severe acute respiratory syndrome coronavirus 2 [SARS-CoV-2]) can potentially trigger airway tissue disruption, severe inflammation (Berri et al., 2014; Blanco-Melo et al., 2020), and/or bacterial superinfection and subsequent pneumonia (Ruuskanen et al., 2011; Zhou et al., 2020). Severe disease caused by viruses is often accompanied by reduced type I and III interferon (IFN) production and/or overproduction of pro-inflammatory mediators (Berri et al., 2014; Blanco-Melo et al., 2020; Jérôme et al., 2020). Type I and III IFNs play an important role for innate immunity at mucosal barrier surfaces, such as the respiratory epithelial barrier, where they provide first-line antiviral defence mechanisms (Stanifer et al., 2020). Of note, reduced type I IFN–stimulated gene (ISG) expression can result from the presence of type I IFN autoantibodies, which are in turn associated with severe disease courses in COVID-19 patients (van der Wijst et al., 2021).

The respiratory mucosal surfaces targeted by viruses also harbour a microbiome consisting of resident microorganisms which have important multifactorial gatekeeper functions, including direct inhibition of incoming pathogens, maintaining epithelial barrier function, and immune homeostasis (Man et al., 2017). Importantly, the stability of the core URT microbiome can be compromised during viral RTIs, for instance as observed with influenza (Kaul et al., 2020). This can facilitate pathobiont overgrowth, as a result of virus-induced damage and immune dysfunction (DeMuri et al., 2017; Morris et al., 2017). Direct supplementation of beneficial bacteria from the URT microbiome could act on different stages of viral disease, for instance via enhancement of the airway epithelial barrier, direct inhibition of pathogens or stimulation of the immune system (Figure 1A), as we have recently reviewed in Spacova et al. (2021). Stimulation of antiviral immunity is one of the key mechanisms reported in animal models of respiratory viral disease upon topical administration of beneficial bacteria directly to the airways (Harata et al., 2010; Kumova et al., 2019).

Traditional oral supplementation with well-studied probiotic strains such as *Lacticaseibacillus rhamnosus* GG can significantly reduce the clinical incidence of virus-associated RTIs (Kumpu et al., 2012; Luoto et al., 2014). Of note, certain probiotic mechanisms of action are strain-specific so that multi-strain formulations are a promising approach to limit non-responder subpopulations to ensure clinical efficacy (Grumet et al., 2020). For example, a commercially available chewable containing a combination of four probiotic strains of lactobacilli and bifidobacteria and vitamin C has been shown to significantly prevent URT infection symptoms in children (Garaiova et al., 2021). However, the current number of clinical trials exploring URT microbiome modulation as a strategy against viral RTIs is limited. This is at least in part due to the challenge of selecting appropriate beneficial strains for the URT and the biotechnical difficulty of formulating live microbes with a long shelf life in a liquid form (Spacova et al., 2021).

We have recently isolated probiotic candidates *Lacticaseibacillus casei* AMBR2 (Wuyts et al., 2017) and *Streptococcus salivarius* (Jörissen et al., 2021) from the nasopharyngeal microbiome of healthy humans that are capable of respiratory pathogen inhibition. Especially *L. casei* AMBR2 is a promising candidate live biotherapeutic product (LBP) against respiratory diseases, including viral infections, due to its capacity to inhibit the growth and inflammatory characteristics of URT pathogens such as *Staphylococcus aureus* and promote epithelial barrier function (De Boeck et al., 2020; De Rudder et al., 2020; Martens et al., 2021). Furthermore, *L. casei* is on the qualified presumption of the safety (QPS) list in Europe (EFSA BIOHAZ Panel et al., 2022), and *L. casei* AMBR2 administration in a fit-for-purpose nasal spray formulation did not lead to any apparent side effects in 20 healthy volunteers (De Boeck et al., 2020). Importantly, this strain is well-adapted to the human URT, capable of adhering to the URT epithelium and can temporarily colonize the URT in healthy volunteers (De Boeck et al., 2020).

Currently, the rational implementation of selected lactic acid bacteria in high-dose URT-targeting, virus-inhibiting, and immune-active formulations is a major challenge that must be overcome before optimal clinical studies can be designed. In this study, we use several key evaluation criteria for beneficial lactobacilli strains as candidates for URT application based on not only their functionality against respiratory viral disease but also considering their strain safety and applicability in the formulation of interest (Figure 1B). Of note, similar criteria have previously allowed us to develop a multi-strain probiotic formulation for topical skin application against acne (Lebeer et al., 2022). Our goal was to develop a safe and readily applicable live biotherapeutic formulation for the URT with several lactobacilli that together are potentially capable of providing multifactorial activity against respiratory

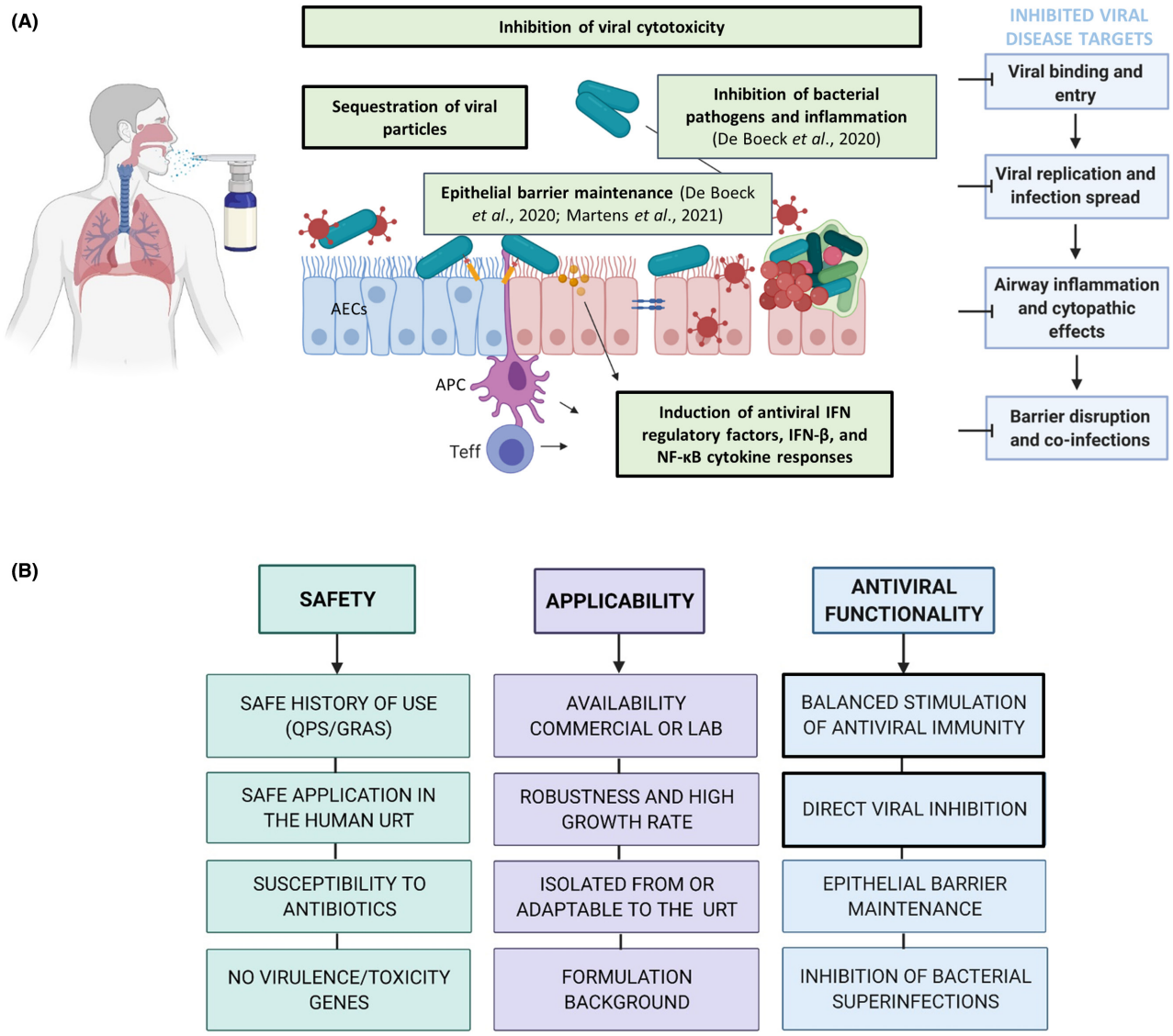

FIGURE 1 (A) Mechanisms through which topically applied beneficial lactobacilli can act against respiratory viral disease, and (B) Rationale for selection of *Lactobacillaceae* strains for topical application against respiratory viral disease. The modes of action experimentally explored in this study are indicated in bold frames. AECs, airway epithelial cells; APC, antigen-presenting cell; IFN, interferon; Teff, T effector cell (based on Spacova et al., 2021).

viruses. We used a three-step decision process based on theoretical strain selection, experimental in vitro testing, and formulation. First, the initial theoretical beneficial strain selection highlighted *L. casei* AMBR2 as a promising URT strain of choice for this study based on previous research (De Boeck et al., 2020), and additional *Lactobacillaceae* strains listed in Table 1 were considered to complement *L. casei* AMBR2. Second, subsequent selective in vitro experiments were conducted to assess the immunostimulatory capacity and anti-viral activity of the *Lactobacillaceae* strains. Third, the formulation of the three selected strains in a throat spray was performed taking into consideration their viability, spray taste/colour, and retention of the immunostimulatory ability in the final spray formulation. Subsequent application of the throat spray with the selected *Lactobacillaceae* was tested in human volunteers to explore temporary retention of all three strains in the URT microbiome.

EXPERIMENTAL PROCEDURES

Bacterial strains and culture

Bacterial strains used in this study and their properties are listed in Table 1. *Lactobacillaceae* strains were grown statically at 37°C in de Man, Rogosa and Sharpe (MRS) broth (Difco). *Escherichia coli* DH5α was cultured aerobically at 37°C in Luria-Bertani (LB) broth (Laboratories, 1986). For co-incubation with viruses or human cells, bacterial pellets were obtained

TABLE 1 Bacterial strains used in this study and their properties.

Strain	Origin	Relevant properties	References
Lacticaseibacillus casei AMBR2	Human URT isolate	URT probiotic candidate with immunomodulatory and anti-pathogenic action against URT pathobionts and the associated inflammation. Has epithelial barrier-promoting properties. Demonstrated safety and colonization upon administration in a nasal spray in healthy volunteers	De Boeck et al. (2020), Martens et al. (2021)
Lacticaseibacillus rhamnosus GG (ATCC 53103)	Human gastrointestinal tract isolate	Model oral probiotic strain with anti-pathogenic, barrier-enhancing, and immunomodulating properties. Topical URT application in mouse models of influenza led to immune stimulation of antiviral interferon and cytokine pathways. Significantly reduced the symptoms and/or incidence of virus-associated RTIs in clinical trials upon oral application	Harata et al. (2010), Kumova et al. (2019), Kumpu et al. (2012), Luoto et al. (2014)
Lactiplantibacillus plantarum WCFS1	Human saliva isolate	Model oral probiotic strain with anti-pathogenic, barrier-enhancing, and immunomodulating properties demonstrated in the gastrointestinal tract	van Baarlen et al. (2009), van den Nieuwboer et al. (2016)
Lactiplantibacillus pentosus KCA1	Human vaginal isolate	Well-characterized probiotic strain linked with microbiome modulation upon oral and topical skin application	Anukam et al. (2013), Onwuliri et al. (2021)
Limosilactobacillus reuteri RC14	Human vaginal isolate	Vaginal microbiome modulation upon topical application together with *L. rhamnosus* GR-1	Reid et al. (2003)
Lacticaseibacillus paracasei Immunitas (DN-114001)	Origin unclear	Commercial probiotic strain from Actimel Immunitas or Defensis, leads to a decrease in symptoms and duration of respiratory tract infections in clinical trials with children and the elderly	Guillemard et al. (2010), Merenstein et al. (2010) Taxonomy cfr. Wuyts et al. (2017)
Lacticaseibacillus paracasei Shirota	Human gastrointestinal tract isolate	Commercial probiotic strain from the Yakult® product (Yakult Ltd.), ameliorates influenza virus infection in a mouse model	Hori et al. (2002) Taxonomy cfr. Wuyts et al. (2017)
Lacticaseibacillus rhamnosus B442	USDA Agriculture Research Service (NRRL) Culture Collection	Proposed probiotic strain for use in ice cream active against enteric pathogens	Muyyarikkandy and Amalaradjou (2017), Pankiewicz et al. (2020)
Lacticaseibacillus rhamnosus LC705	Dairy strain	Commercial probiotic used by companies such as Valio, administered as part of probiotic mixtures in yogurt	Saxelin et al. (2010)
Lactobacillus acidophilus LMG 8151	Isolate from commercial acidophilus milk	Commercial probiotic strain, deposited by the Culture Collection University of Göteborg (CCUG), Department of Clinical Bacteriology, Institute of Clinical Bacteriology, Immunology, and Virology under number CCUG 12853	Pot et al. (1993)
Latilactobacillus sakei AMBR8	Healthy human URT isolate	Strain isolated from and potentially adapted to the human URT (e.g., adherence to airway epithelial cells) with immunomodulatory and anti-pathogenic action against URT pathobionts	De Boeck et al. (2020)
Lactiplantibacillus plantarum AMBR9	Healthy human URT isolate	Strain isolated from and potentially adapted to the human URT with anti-pathogenic action against URT pathobionts	De Boeck et al. (2020)

Abbreviations: NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; URT, upper respiratory tract.

by centrifugation at 2000*g* for 10 min and washed in sterile phosphate-buffered saline (PBS). After resuspension in corresponding media (for experiments with human cells) or PBS (for antiviral activity experiments), UV inactivation of bacteria was achieved in a biosafety level 2 cabinet using four 15-min rounds of UV irradiation followed by vortexing. Bacteria were plated out to confirm inactivation.

NF-κB and IRF induction in THP1-Dual monocytes

THP1-Dual monocytes (Invivogen) were maintained in RPMI 1640 (ThermoFisher Scientific) medium with 10% Fetal Calf Serum (FCS), 25 mM HEPES, and 2 mM L-glutamine at 37°C, 5% CO_2. For experiments with bacteria, THP1-Dual cells were seeded in a 96-well plate at a concentration of 10^5 cells/well. Bacteria were added to the cells at 10^6 CFU/well for live bacteria from cultures, 10^7 CFU/well for UV-inactivated bacteria from cultures, and 10^8 CFU/well for powdered bacteria. Spray was added at a 1:20 dilution. The plate was incubated for 24 hours at 37°C and 5% CO_2. Induction of NF-κB was assessed based on SEAP reporter activity at 405 nm with the Synergy HTX Plate Reader (BioTek) after the addition of a para-nitrophenylphosphate (pNPP) buffer. Induction of IRF was assessed based on luciferase reporter luminescence activity with the Synergy HTX Plate Reader (BioTek) after the addition of the QUANTI-Luc™ (InvivoGen) buffer. Poly (I:C) with Lipofectamine 2000 (Invitrogen) at 50 μg/ml for IRF induction or lipopolysaccharides (LPS) from *E. coli* (Sigma) at 20 ng/ml for NF-κB induction were used as positive controls.

Cell lines and viruses for antiviral assays

Human liver carcinoma cell line Huh7 (CLS Cell Lines Service), human epidermoid carcinoma HEp-2 (ATCC CCL-23), and Madin–Darby canine kidney (MDCK) cells, a gift from Dr. M. Matrosovich (Marburg, Germany), were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco Life Technologies) supplemented with 8% heat-inactivated fetal bovine serum (HyClone; GE Healthcare Life Sciences) and maintained at 37°C under 5% CO_2.

Human coronavirus (HCoV-229E) and respiratory syncytial virus (RSV Long) were purchased from ATCC (VR-740 and VR-26, respectively). The human influenza virus strains used are as follows: A/Ned/378/05 (A/H1N1 subtype) and B/Ned/537/05 (B/Yamagata lineage), clinical isolates generously donated by Prof. R. Fouchier (Rotterdam, The Netherlands), and A/HK/7/87 (A/H3N2 subtype) was obtained from Prof. J. Neyts (KU Leuven, Belgium).

Assessment of the antiviral activity of selected strains against HCoV-229E, RSV, and influenza viruses in human cells

Bacterial strains were prepared as described above with UV-inactivation. The antiviral evaluation of UV-inactivated bacteria against HCoV-229E was performed by seeding Huh7 cells into 384-well plates. After 24 h at 37°C, 5-fold serial dilutions of the bacteria (or PBS as a negative control) were added to the cells immediately prior to infection with HCoV-229E at 30 $CCID_{50}$ (50% cell culture infective doses) per well. At 3 days post-infection, the virus-induced cytopathogenic effect was measured colorimetrically by the formazan-based 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) cell viability assay (CellTiter 96 AQueous One Solution Cell Proliferation Assay from Promega, Madison, WI), and the antiviral activity was expressed as the 50% effective concentration (EC_{50}). In parallel, the 50% cytotoxic concentration (CC_{50}) was derived from mock-infected cells.

The evaluation of viral inhibition by UV-inactivated bacteria after pre-incubation with influenza viruses (A/H1N1 A/Ned/378/05, A/H3N2 A/HK/7/87 and B/Ned/537/05), respiratory syncytial virus, and human coronavirus (HCoV-229E) was performed by seeding MDCK, Hep-2, or Huh7 cells into 384-well dishes, and the plates were incubated for 24 h at 35°C (MDCK) or 37°C (HEp-2 and Huh7). One hour prior to infection, aliquots of UV-inactivated bacteria in PBS (or PBS alone as a negative control) were centrifuged, and the pellets were resuspended in an equal volume of infection medium containing the viruses diluted to yield 30 $CCID_{50}$ per well at infection. The mixtures were incubated at 37°C, 5% CO_2 for 1 h, and subsequently centrifuged (3000 *g* for 15 min at room temperature). The viruses containing supernatants were used for infection of the respective virus host cells. At 3 days (HCoV-229E) or 4 days post-infection (influenza and RSV), the virus-induced cytopathogenic effect was measured colorimetrically by the formazan-based MTS assay, and the antiviral activity was expressed as the % viability relative to the uninfected control. In parallel, the % viability was measured in mock-infected MDCK, HEp-2, or Huh7 cells to assess possible cytotoxic effects.

Formulation of the microbiome throat spray and assessment of bacterial viability

The live biotherapeutic spray consisted of freeze-dried *L. casei* AMBR2, *L. plantarum* WCFS1, and *L. rhamnosus* GG, in a ratio of 5:3:2, respectively. Viability of the powders from the individual strains was assessed at 4°C and 25°C every 4 weeks over a time period of 6 months via resuspension of the powders in PBS and plating out serial

dilutions on MRS agar. The amount of CFU/g powder was evaluated compared to the start concentration. For the mixture of the powders or the final spray formulation in a sunflower oil suspension with Aerosil, viability was assessed at 4, 15, and 25°C over a period of 6 months. Every 4 weeks, powders (after suspension in PBS) or spray were plated out in serial dilutions on MRS agar to measure the amount of CFU/g powder or spray.

Evaluation of lactobacilli retention in the throat of healthy participants

To evaluate whether the bacteria in the spray are able to temporarily colonize the throat, 12 healthy male and female adult participants were asked to use the spray and collect swabs of the throat at the start, after 30 min, and after 2 h of spray administration. The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Committee of Medical Ethics UZA/UAntwerpen (B3002021000018; approved 25th of January 2021; ClinicalTrials.gov Identifier NCT04793997). Informed consent was obtained from all participants involved in this study prior to inclusion. Samples were registered and stored at the Biobank Antwerpen, Antwerp, Belgium; ID: BE 71030031000.

At each time point, two throat swabs were collected with eNAT™ swabs for microbial DNA extraction using the PowerFecal DNA isolation kit (Qiagen), and in PBS for lactobacilli cultivation on MRS agar. Dual-index paired-end sequencing of the throat samples was performed on the V4 region of the *16S rRNA* gene on a MiSeq Desktop sequencer (M00984; Illumina), as previously described (De Boeck et al., 2017, 2019). For qPCR analysis, strain-specific primers for *L. casei* AMBR2, *L. plantarum* WCFS1, and *L. rhamnosus* GG were designed (Table 2). Initially, a standard curve for each strain was made to estimate the Ct ~ CFU ratio. The abundance of the corresponding specific *L. casei* AMBR2, *L. plantarum* WCFS1 and *L. rhamnosus* GG genes as indicated in Table 2 was quantified by RT-qPCR on a StepOne Plus Real-Time PCR System to estimate the CFU of each strain in the samples (v.2.0; Applied Biosystems). Each DNA sample was amplified 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.

Throat swabs were collected by swabbing along the back of the throat and both tonsils and cultivated after resuspension in 1 ml PBS and plating out serial dilutions on MRS agar. Plates were incubated for 2 days at 37°C. Single colonies were identified with PCR and Sanger sequencing of the full *16S rRNA* gene using the 27F (5′-AGA GTT TGA TCM TGG CTC AG-3′) and 1492R (5′-GGT TAC CTT GTT ACG ACT T-3′) primers.

Statistical data analysis

Data from in vitro assays were analysed in GraphPad Prism version 9.2.0. Normality testing was performed on data from in vitro assays with bacteria using the Kolmogorov–Smirnov test followed by one-way ANOVA with Dunnett's multiple comparisons test or the Kruskal–Wallis test with Dunn's multiple comparisons test. Differences were considered significant at $p < 0.05$.

RESULTS

Screening for interferon regulatory pathway- and NF-κB-inducing *Lactobacillaceae* strains

Using a three-step decision process based on theoretical strain selection, in vitro testing for immunostimulatory and antiviral properties, and formulation properties, we aimed to select *Lactobacillaceae* strains for formulation in an antiviral throat spray considering

TABLE 2 qPCR primers used in this study.

Gene and primer sequence	Target species	Reference
Srr2 (2759F) 5′-CCCGGGCCGTTACGTTGCAGGCAAAA-3′	*L. casei* AMBR2	De Boeck et al. (2020)
Srr2 (2841R) 5′-ACTAGTTAATTGGTCAGTCGGTGCCC-3′	*L. casei* AMBR2	De Boeck et al. (2020)
FM179322.1_443 (LGG_443_F) 5′-CGTAGCTCTTTGCGTCATCT-3′	*L. rhamnosus* GG	This study
FM179322.1_443 (LGG_443_R) 5′-CGCATTGTATGCAGCCTTATTC-3′	*L. rhamnosus* GG	This study
AL935263.2_413 (WCSF1_413_F) 5′-GCCACAACACTTCAGCAATAC-3′	*L. plantarum* WCFS1	This study
AL935263.2_413 (WCSF1_413_R) 5′-GTGCCATACACCCTGGTAAG-3′	*L. plantarum* WCFS1	This study

safety, applicability, and functionality criteria as depicted in Figure 1B. In the first step representing theoretical strain selection, the key endogenous URT strain *L. casei* AMBR2 was pre-selected based on previous research regarding its anti-pathogenic action, URT epithelial barrier promotion, and URT adaptation (De Boeck et al., 2020; De Rudder et al., 2020; Martens et al., 2021). A selection of additional *Lactobacillaceae* strains (outlined in Table 1) to complement *L. casei* AMBR2 was made based on documented safety, prior human or animal use, annotated genome information, and functional properties such as the capacity to inhibit bacterial and fungal pathogens. These strains included the robust, easy-to-formulate, and widely researched oral probiotic strains *Lacticaseibacillus rhamnosus* GG and *Lactiplantibacillus plantarum* WCFS1 known for their clinical safety.

In the second step involving in vitro testing, the selected lactobacilli were first tested for their ability to stimulate antiviral immune pathways (cfr. Figure 1A), thus providing additional modes of action potentially applicable against viral disease. The selection of 12 lactobacilli strains described in Table 1 was evaluated for their capacity to activate the interferon regulatory factor (IRF) and nuclear factor kappa B (NF-κB) immune signalling pathways in human THP1-Dual monocytes involved in antiviral responses. *L. plantarum* WCFS1 was superior to all other tested strains in activating the IRF pathway key for antiviral defences (Figure 2A), as well as NF-κB (Figure 2B). Out of all tested strains, only *L. rhamnosus* GG and *Lactiplantibacillus pentosus* KCA1 also significantly induced both IRF and NF-κB, although to a lesser degree than *L. plantarum* WCFS1. Of note, IRF induction was shown to be strain-specific with, for example, *L. rhamnosus* GG stimulating a stronger IRF and NF-κB activation than the other *L. rhamnosus* strains tested here, underlying the importance of appropriate bacterial strain selection.

Based on these results and considering the criteria in Figure 1B, the salivary isolate *L. plantarum* WCFS1 was selected to complement the less immunostimulatory *L. casei* AMBR2 to achieve potential multifactorial action against viral disease in the URT. Furthermore, we also chose to include *L. rhamnosus* GG, as it significantly induced both IRF and NF-κB in our assay, previously demonstrated local immunostimulatory activity in animal models of viral infection upon topical application in the URT (Harata et al., 2010; Kumova et al., 2019), and also significantly reduced the symptoms and/or incidence of virus-associated RTIs in clinical trials upon oral application (Kumpu et al., 2012; Luoto et al., 2014). Considering the URT applicability selection criteria outlined in Figure 1B, *L. pentosus* KCA1 (the only other tested strain capable of significantly inducing both IRF and NF-κB) was deemed less applicable in the URT due its vaginal origin and less safety/clinical and formulation data available. By contrast, both *L. plantarum* WCFS1 and *L. rhamnosus* GG are among the best-studied beneficial lactobacilli with demonstrated safety even upon high-dose application in humans (Segers & Lebeer, 2014; van den Nieuwboer et al., 2016), and both species naturally occur in the healthy human URT, unlike *L. pentosus* (De Boeck et al., 2020).

Based on these data and previous knowledge on their activity (Table 1), *L. casei* AMBR2, *L. plantarum* WCFS1, and *L. rhamnosus* GG were thus selected as strains potentially covering all key modes of action against viral diseases (outlined in Figure 1A). Hence, a product containing a combination of strains could be more efficient due to multifactorial action, providing that the strains do not inhibit each other's activity. Indeed, combining the three strains in equal ratios also resulted in significant induction of IRF and NF-κB pathways in human monocytes, comparable to the levels observed when *L. rhamnosus* GG and *L. plantarum* WCFS1 were used separately (Figure 2C–F). Of note, the NF-κB stimulation by lactobacilli was significantly lower than that of the LPS-containing Gram-negative *Escherichia coli* DH5α control laboratory strain (Figure 2C,E). To assess whether the bacteria had to be metabolically active to induce immunostimulatory effects, ultraviolet (UV)-inactivated strains were used (Figure 2E,F). Both UV-inactivated *L. plantarum* WCFS1 and *L. rhamnosus* GG, as well as the mix of the three strains, still significantly induced the IRF and NF-κB pathways, although to a lesser extent than their viable counterparts (Figure 2E,F).

Direct antiviral activity of selected *Lactobacillaceae* strains against respiratory viruses

In addition to establishing immunostimulatory activity on host cells, we aimed to assess the direct inhibitory activity of the three selected bacterial strains on respiratory viral particles. Probiotic screening for direct antiviral activity is not routinely implemented because of the complexity of working with tripartite systems consisting of bacteria, viruses, and host cells. Here, we implemented such innovative assays for common respiratory viruses. The inhibitory effects of the three selected *Lactobacillaceae* strains were evaluated against RSV, influenza A/H3N2, A/H1N1 and B viruses, and human coronavirus strain 229 (HCoV-229E), all representing viruses that are a common cause of RTIs (Ieven et al., 2018). HCoV-229E generally causes mild symptoms, but it has several features in common with SARS-CoV-2, such as homologous epitopes of the spike protein, and is suitable for high-throughput screening due to biosafety level 2 (Mateus et al., 2020). To avoid bacterial metabolites interfering with the assay read-outs, the lactobacilli were UV-inactivated for the assays, retaining the capacity of bacterial cell surface

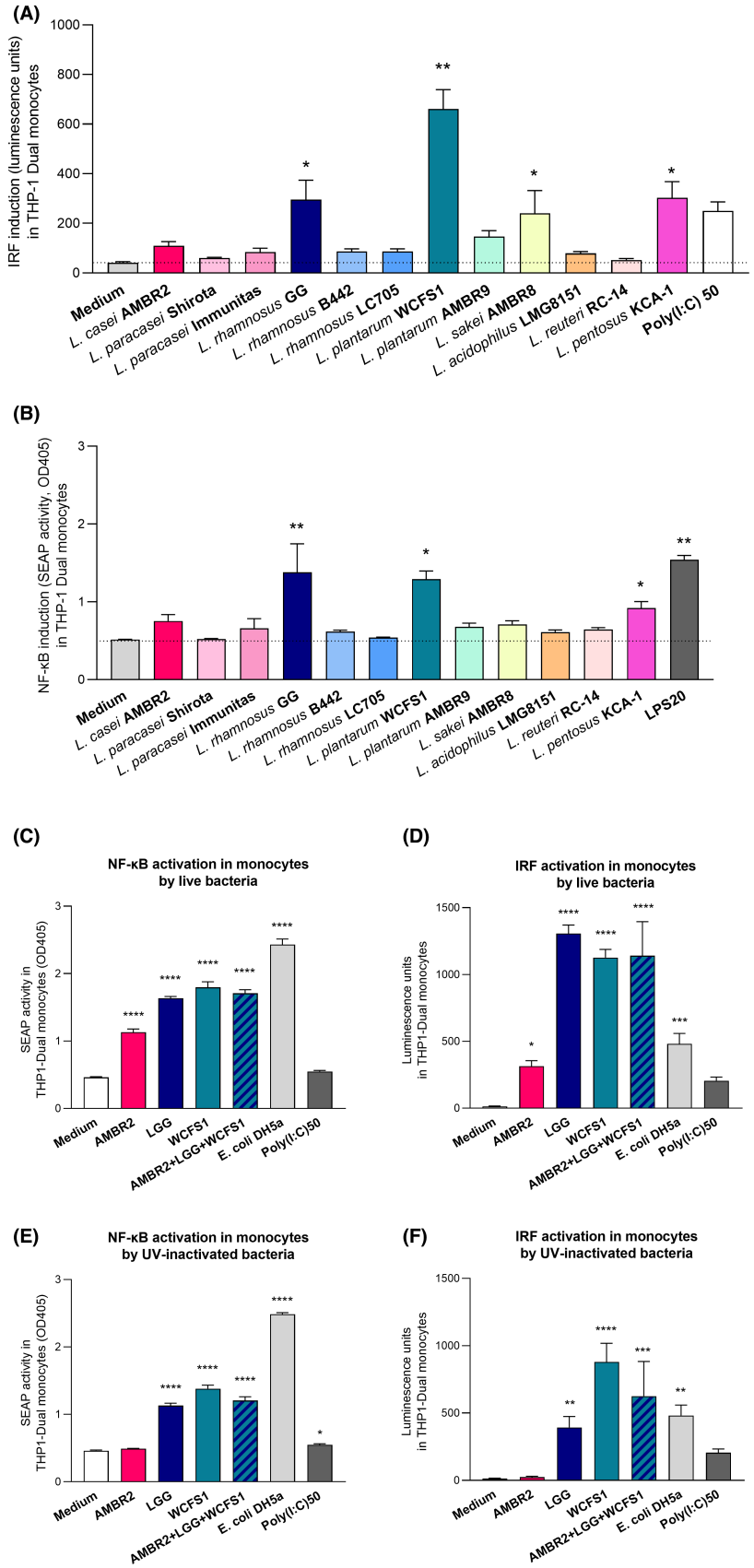

FIGURE 2 (A) Stimulation of interferon regulatory factors (IRFs) and (B) nuclear factor (NF)-κB by selected *Lactobacillaceae* strains in human monocytes, and (C–F) *L. casei* AMBR2, *L. rhamnosus* GG, *L. plantarum* WCFS1, and their combination AMBR2/WCFS1/LGG activate immune pathways involved in antiviral responses. Live *L. casei* AMBR2, *L. rhamnosus* GG and *L. plantarum* WCFS1 and their combination induce (C) nuclear factor (NF)-κB and (D) interferon regulatory factors (IRFs) in human THP1-Dual monocytes upon co-incubation. UV-inactivated *L. rhamnosus* GG and *L. plantarum* WCFS1 and their combination with *L. casei* AMBR2 also induce (E) NF-κB and (F) IRFs in human THP1-Dual monocytes. The medium condition represents the cells as such and serves as a baseline, while Poly(I:C) at 50 μg/ml with Lipofectamine (Poly(I:C)50) serves as the control IRF inducer and LPS (lipopolysaccharide) at 20 ng/ml (LPS20) and LPS-producing *E. coli* DH5α serve as the control NF-κB inducer. Data are depicted as mean ± SD per condition. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, and $****p < 0.0001$ as determined by a One-way ANOVA test followed by Dunnett's multiple comparisons test compared to the medium condition.

molecules to sequester or block viral particles (Malik et al., 2016).

First, we established the concentration of bacteria that had to be simultaneously added with the HCoV-229E virus to inhibit the virus-induced cytopathic effect in human Huh7 cells (non-respiratory cell line susceptible to HCoV-229E) by 50% (antiviral EC50) (Figure 3A). *L. casei* AMBR2 and *L. rhamnosus* GG showed the strongest effect, with respective concentrations of 2.57×10^6 colony-forming units (CFU)/ml and 5.06×10^6 CFU/ml required for 50% viral inhibition, which was lower than the required concentration of 1.26×10^7 CFU/ml for the non-probiotic *E. coli* DH5α strain. For *L. plantarum* WCFS1, the required concentration for 50% viral inhibition was 1.17×10^7 CFU/ml. Of note, Huh7 cells might not fully reflect the immune responses of the human respiratory tract. Therefore, it was also important to investigate the direct interactions of bacteria with viruses using a two-step experimental set-up not involving contact between bacteria and human cells.

Therefore, we also evaluated a set-up where the three bacterial strains or their combination in equal ratios were first pre-incubated with HCoV-229E, RSV, or influenza viruses, allowing trapping and/or inactivation of virus particles, and afterward unbound virus particles were added to human cells. HCoV-229E (Figure 3B) and RSV (Figure 3C) pre-incubated with *L. casei* AMBR2, *L. rhamnosus* GG, *L. plantarum* WCFS1, or their combination significantly lost the capacity to reduce human cell viability compared to virus pre-incubation with phosphate-buffered saline (PBS) which served as an inactive control. For example, the percentage of viable Huh7 cells infected with HCoV-229E was on average 2.9% for the PBS condition, and 34%, 30%, and 18% for *L. casei* AMBR2, *L. rhamnosus* GG, and *L. plantarum* WCFS1 conditions, respectively. Using the combination of the three strains also resulted in 18% of viable Huh7 cells, similar to *L. plantarum* WCFS1 only. Pre-incubation with *L. rhamnosus* GG or a combination of the three lactobacilli also significantly reduced the cytopathic effects induced by the common influenza A/H1N1 virus compared to PBS (Figure 2D). A reduction of cytopathic effects of influenza B was also observed after pre-incubation of *L. rhamnosus* GG (Figure 2F), while the effects of the tested lactobacilli on the cytopathic effects of influenza A/H3N2 virus were less pronounced (Figure 2E). The pre-incubation of the control laboratory strain *E. coli* DH5α with the tested viruses did not improve the viability of human cells, while the cell viability in all lactobacilli conditions was significantly higher than that of *E. coli* DH5α. Thus, based on previous research, and the immunostimulation and/or potential antiviral activity of *L. casei* AMBR2, *L. rhamnosus* GG, and *L. plantarum* WCFS1 observed in this study, in addition to their other beneficial properties (Table 1), the three strains represented excellent candidates to evaluate their combination in a throat spray formulation with potential multifactorial action against respiratory viral disease.

Formulation of viable *Lactobacillaceae* strains in a throat microbiome-targeting spray

L. casei AMBR2, *L. rhamnosus* GG, and *L. plantarum* WCFS1 were formulated into an oral/throat microbiome-targeting spray, based on a combination of bacterial powders in a sunflower oil suspension with Aerosil. In contrast, most probiotic sprays for the URT currently available consist of a bacterial suspension in saline or PBS (Andaloro et al., 2019). Aerosil (pure silicon dioxide) is used in pharmaceutical formulations as a thickening agent. In the final formulation, Aerosil was combined with oil for several reasons, including stability, organoleptic properties, and retention properties. The expected retention increase of the resulting suspension was based on the higher viscosity and the inherent aspects of an oil suspension, slowing down the impact from enzymatic degradation from saliva components.

As we demonstrated in the previous results section (Figure 2), live bacteria can be more efficient at NF-κB and IRF induction in vitro than UV-inactivated bacteria. Live lactobacilli produce compounds such as lactic acid as their main metabolite for which direct antiviral activity has been previously shown (Lange-Starke et al., 2014). First, the viability of each bacterial strain in freeze-dried powder form was evaluated at 4 and 25°C (Figure 4A). For all three strains, the viability at 4°C remained stable over time. For *L. casei* AMBR2 and *L. plantarum* WCFS1, no log reductions were observed, with 2.79×10^{10} CFU/g powder and 3.56×10^{11} at start for AMBR2 and WCFS1, respectively, to 2.21×10^{10} CFU/g powder and 3.15×10^{11} CFU/g powder after 26 weeks. For *L. rhamnosus* GG, the viability decreased from 1.18×10^{11} CFU/g powder to 7.15×10^{10} CFU/g. At room temperature (25°C), *L. plantarum* WCFS1 seemed most stable, with 1 log reduction at 26 week (5.84×10^{10}), while this was 8.57×10^9 for *L. rhamnosus* GG and 2.02×10^7 for *L. casei* AMBR2.

Based on functional in vitro data, the results for single-strain powder viability at 25°C showing more pronounced reduction in viable *L. casei* AMBR2 and our previous formulation experience pointing to a tendency of *L. rhamnosus* GG to induce discoloration, *L. casei* AMBR2, *L. plantarum* WCFS1, and *L. rhamnosus* GG concentrations at a ratio of 5:3:2 were chosen as a good ratio to counter stability effects in the final formulation during long-term storage at room temperature, which reflected the intended storage conditions. Next, the viability of the combined bacterial strains in powder form (Figure 4B) and in the throat spray formulation

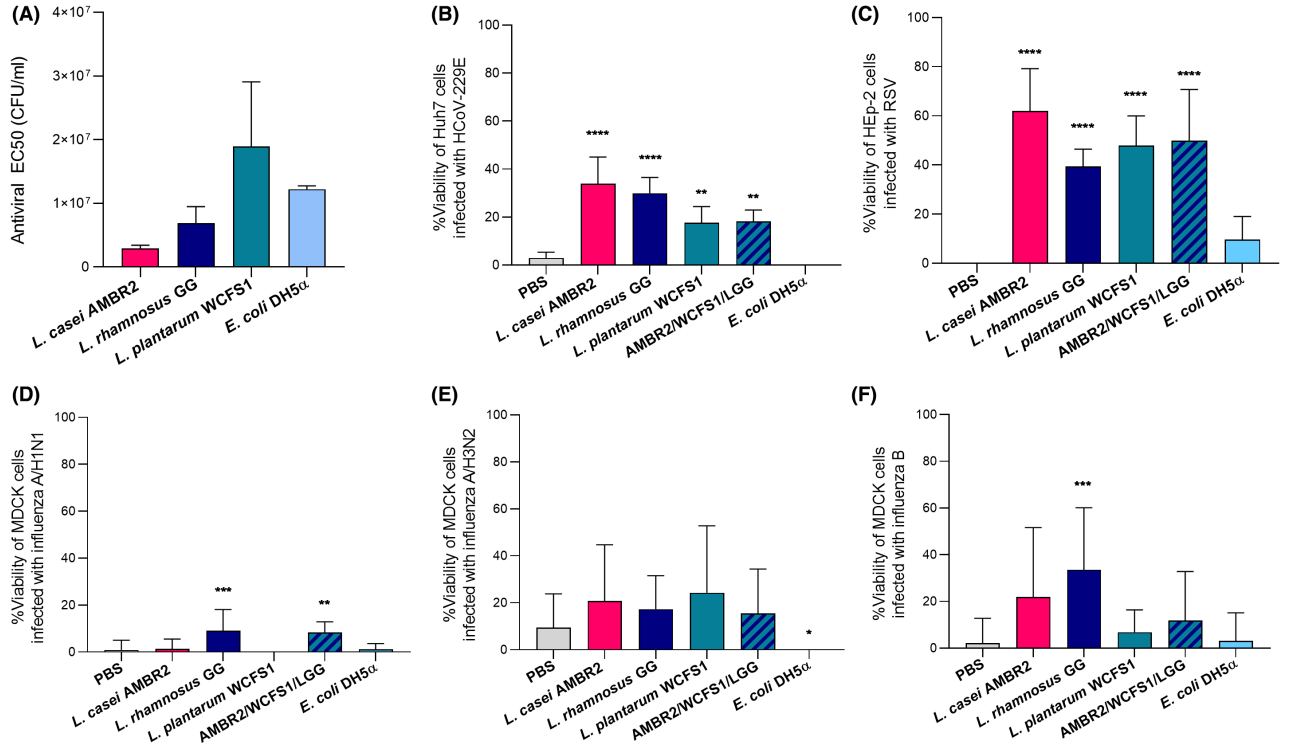

FIGURE 3 *L. casei* AMBR2, *L. rhamnosus* GG, *L. plantarum* WCFS1, and their combination AMBR2/WCFS1/LGG decrease cytopathogenic effects of HCoV-229E, RSV, and influenza A/H1N1, A/H3N2 and B viruses in vitro. *L. casei* AMBR2, *L. rhamnosus* GG, and *L. plantarum* WCFS1 that were added together with the HCoV-229E virus (A) or pre-incubated with HCoV-229E (B), RSV (C), influenza A/H1N1 (D), A/H3N2 (E) or influenza B (F) could inhibit the cytopathic effects induced in human cells. Phosphate-buffered saline (PBS) serves as the control that does not affect viral infection, *E. coli* DH5α represents a control laboratory strain. Data depicted as mean ± SD per condition. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, and $****p < 0.0001$ as determined by a One-way ANOVA test followed by Dunnett's multiple comparisons test compared to the medium or PBS conditions. CFU, colony-forming units; EC50, 50% Effective concentration producing 50% inhibition of virus-induced cytopathic effects, as determined by measuring the cell viability with the colorimetric MTS cell viability assay.

(Figure 4C) was evaluated at 4, 15, and 25°C. For the mixed powders (Figure 4B), viability decreased slightly from 2.09×10^{11} CFU/g at the start to 1.11×10^{11}, 4.51×10^{10}, and 8.01×10^{9} CFU/g at 26 weeks of storage at 4, 15, and 25°C, respectively. For the spray formulation (Figure 4C), viability starting with 3.78×10^{9} CFU/g spray remained stable at 4 and 15°C at 26 weeks. At 25°C, a 2 log reduction was observed (3.3×10^{7} CFU/g) at 26 weeks.

We subsequently confirmed the retention of immunostimulatory activity in human monocytes of the strains and their combination in powder form (Figure 4D,E), and in the spray formulation in oil (Figure 4F,G). All single strains in powder form and their combination were still capable of significant IRF and NF-κB induction (Figure 4D,E) at a dose of 10^{8} CFU/ml, which corresponds to the *L. casei* AMBR2 concentration per puff previously tested in healthy volunteers (De Boeck et al., 2020). The immunostimulatory action of the throat spray formulation with the three strains in an oil suspension was also compared to a placebo oil formulation without lactobacilli. The throat spray formulation with *L. casei* AMBR2, *L. plantarum* WCFS1, and *L. rhamnosus* GG at respective ratios of 5:3:2 in oil significantly induced IRF and NF-κB in human monocytes (Figure 4F,G). While the placebo formulation also induced NF-κB, albeit to a lower degree than the spray formulation with lactobacilli, it did not significantly affect IRF.

Evaluation of lactobacilli retention in the throats of healthy volunteers

Finally, using a longitudinal placebo-controlled sampling set-up, we evaluated the retention of the lactobacilli administered in the formulated spray in the throat of 12 healthy volunteers. Presence of the live applied *Lactobacillaceae* strains was assessed via cultivation and quantitative polymerase chain reaction (qPCR), and overall retention of lactobacilli was quantified via 16S rRNA amplicon sequencing at the DNA level (Figure 4A). The volunteers used the verum spray at the start of the study by spraying two puffs containing approximately 9.5×10^{8} CFU of lactobacilli, or the placebo spray not containing lactobacilli, and throat swabs were collected at the baseline, after 30 min, and after 2 h (Figure 5A).

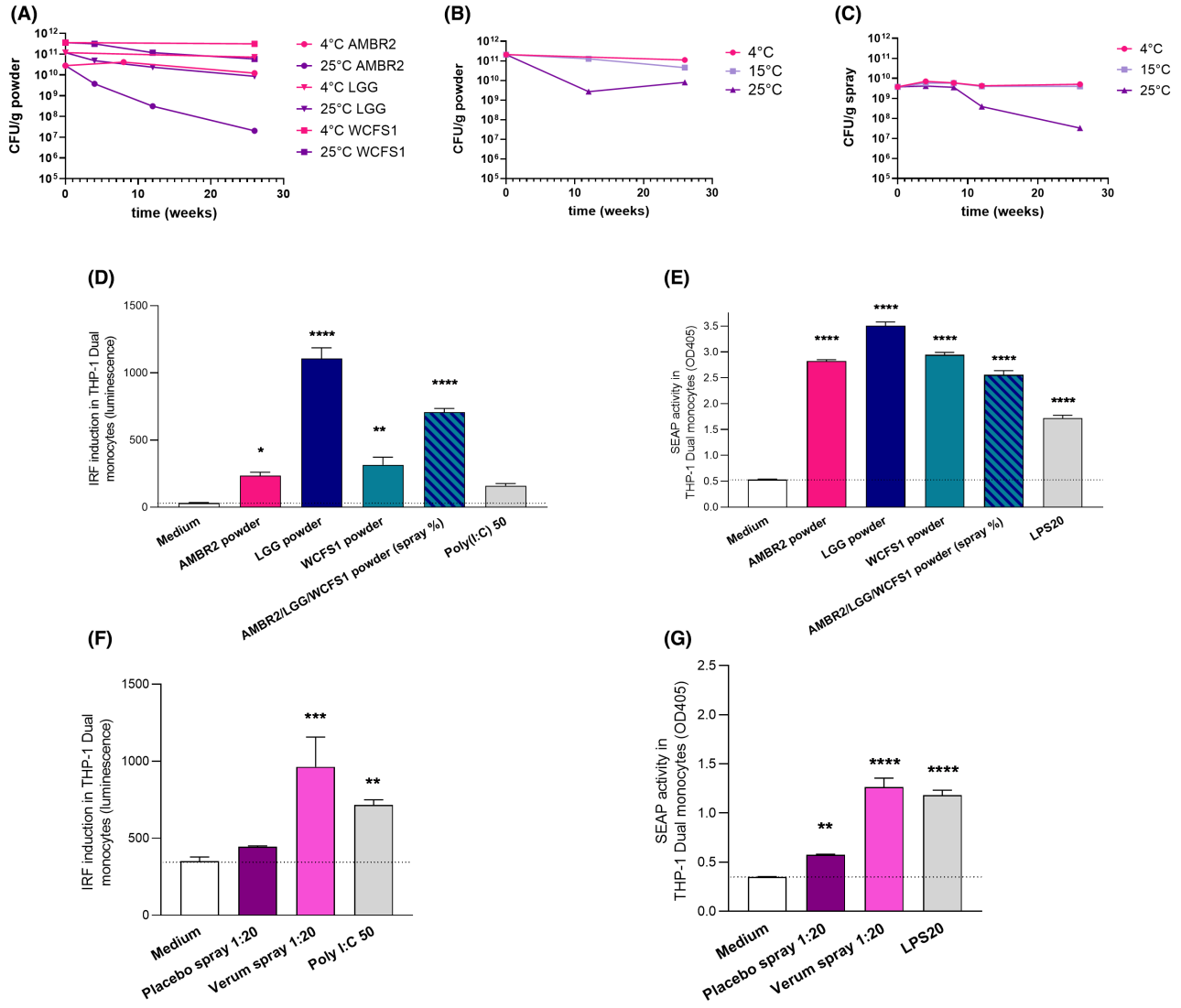

FIGURE 4 Viability of individual and combined *L. casei* AMBR2, *L. plantarum* WCFS1, and *L. rhamnosus* GG in (A, B) powder or (C) the throat spray formulation; Immunostimulatory activity of the powders (D, E) and the placebo and verum spray formulation without or with lactobacilli, respectively (F, G). The viability of the strain combination was evaluated at different storage temperatures over time; 4, 15, and 25°C. CFU, colony-forming units. The medium condition represents the cells as such and serves as a baseline, Poly(I:C) at 50 μg/ml with Lipofectamine (Poly(I:C)50) serves as control IRF inducer (expressed as luminescence units) and LPS at 20 ng/ml (LPS20) serves as control NF-κB inducer (expressed as SEAP activity units). Data are depicted as mean ± SD per condition. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$ as determined by a One-way ANOVA test followed by Dunnett's multiple comparisons test compared to the medium condition (dotted line).

Microbiome analysis of the throat swabs showed that the dominant bacterial genera in the throat belong to canonical throat commensals, such as *Prevotella*, *Veillonella*, and *Streptococcus* species. A principal coordinate analysis (PCoA) plot based on the microbiome data across all time points did not reveal a clear clustering per treatment group at any of the tested time points (Figure 5B). A clear difference in relative abundances of *Lactobacillaceae* amplicon sequence variants (ASVs) was observed between the placebo and verum spray groups, especially 30 min after the spray was used (Figure 5C). Three *Lactobacillus* ASVs, corresponding to the administered strains, were detected in the verum group 30 min after spray administration. *Lactobacillus* ASV 1 (*L. rhamnosus*) was detected in all six participants in the verum group, while *Lactobacillus* ASV 3 (*L. casei*) and *Lactobacillus* ASV 7 (*L. plantarum*) were detected in five out of six participants. In the placebo group, these *Lactobacillus* ASV were not detected, except for one participant that had low endogenous relative abundances of the *Lactobacillus casei* ASV after 30 min. After 2 h, five out of six participants in the verum group still had detectable *Lactobacillus* ASVs.

To confirm and quantify the high abundances of the administered strains observed by sequencing DNA derived from samples in the verum group after bacterial administration, we aimed to estimate the CFU/ml

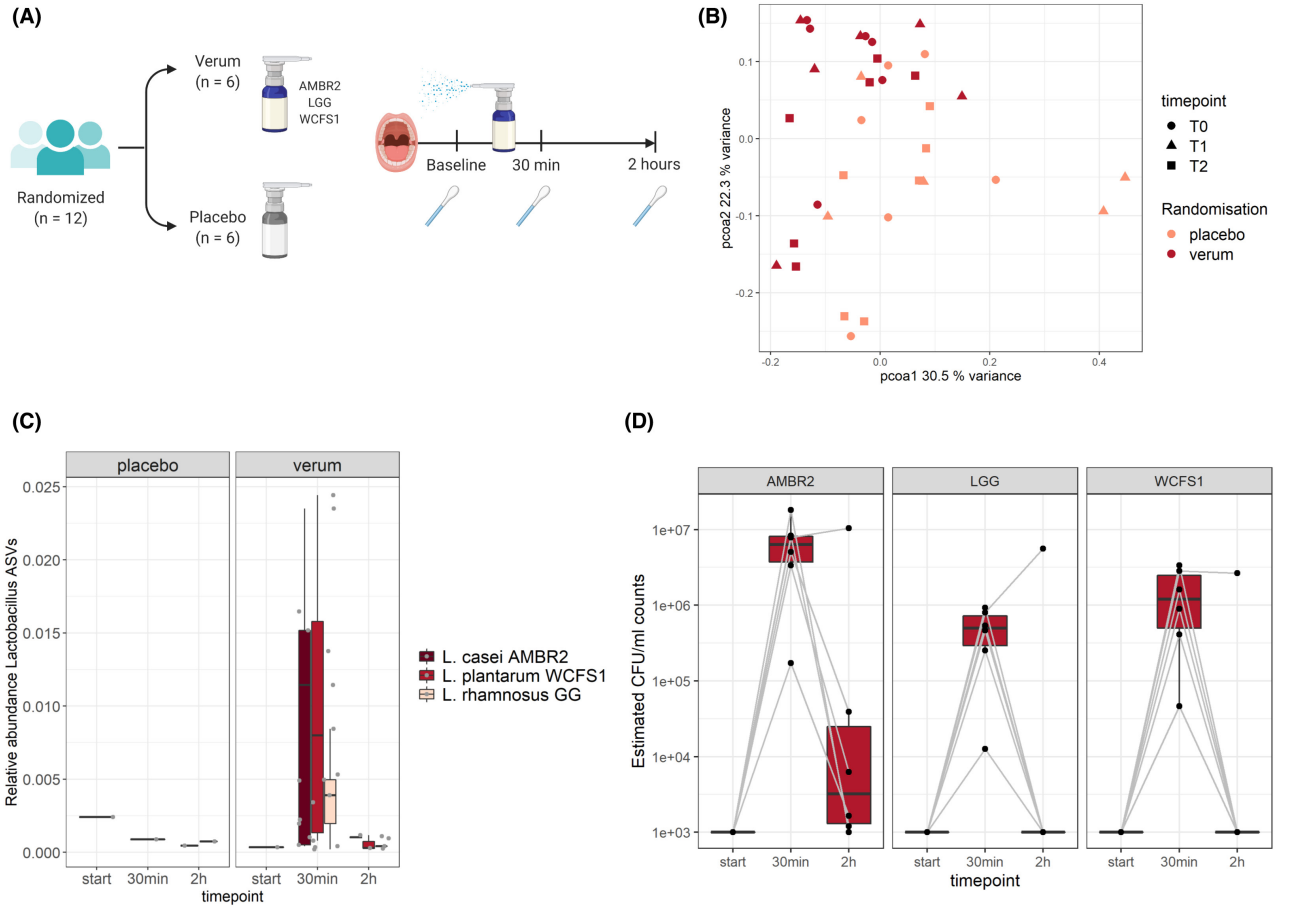

FIGURE 5 Evaluation of lactobacilli retention within the throat microbiome of healthy volunteers after spray application. (A) Study set-up; (B) PCoA plot of throat microbiome data based on microbiome analysis via 16S rRNA amplicon sequencing; (C) Relative abundance of the administered lactobacilli in throat swabs based on microbiome analysis via 16S rRNA amplicon sequencing; (D) Relative abundance of the administered lactobacilli in throat swabs based on qPCR analysis. Throat swabs were collected at baseline/start (T0), 30 min (T1), and 2 h (T2) after the throat spray was used. The presence of *L. casei* AMBR2, *L. rhamnosus* GG, and *L. plantarum* WCFS1 was evaluated via 16S rRNA amplicon sequencing (relative abundances) in panel C. At 30 min and 2 h, and qPCR with species-specific primers was used to estimate the CFU/ml counts in the verum group in panel D. Based on the standard curve, the detection limit was estimated to be at 10^3 CFU/ml.

counts based on targeted qPCR (Figure 5D). In line with the sequencing data, after 30 min, the estimated CFU counts for *L. rhamnosus* GG in the verum group were between 1.26×10^4 and 9.24×10^5 CFU/ml. For *L. casei* AMBR2, estimated CFU/ml counts ranged from 1.72×10^5 to 1.8×10^7 CFU/ml and for *L. plantarum* WCFS1 from 4.63×10^4 CFU–3.36×10^6 CFU. After 2 h, the amount of detected lactobacilli decreased. *L. rhamnosus* GG and *L. plantarum* WCFS1 were not detected anymore except in one participant. *L. casei* AMBR2, on the other hand, which was administered in the highest ratio of 50% in the spray, was still detected in five of the six participants, with a median CFU/ml count of 4×10^3 CFU/ml.

In addition to analysing the DNA of the bacteria, we also cultivated throat swabs to evaluate whether the administered lactobacilli were still viable. Cultured throat swabs from the verum group demonstrated colony morphologies typical for the three administered *Lactobacilaceae* strains, and the species identity was confirmed via colony PCR and sequencing of the *16S rRNA* gene, confirming that the species corresponding to the ones administered with the spray are detected in the throat via their DNA and can remain viable.

DISCUSSION

Topical microbiome therapeutics can act on different stages of viral infection by multiple modes of action, which can potentially prevent a broad range of viral diseases (Spacova et al., 2021). Here, we designed and implemented a dedicated pipeline to select lactobacilli for a topical throat spray against viral RTIs (Figure 5A). We demonstrated the immunostimulatory and antiviral effects of selected lactobacilli and also considered their safety, applicability, and previously reported beneficial properties such as barrier enhancement and antipathogenic effects, important during viral RTIs. Starting from this screening, we formulated *L. casei* AMBR2, *L.*

rhamnosus GG, and *L. plantarum* WCFS1 in a microbiome throat spray and confirmed their immunostimulatory activity in the spray formulation and the temporary colonization of the administered strains in throats of healthy volunteers.

An important property of the selected *Lactobacilaceae* strains was the activation of IFN regulatory pathways and NF-κB that could promote antiviral immune defences upon topical application of the selected lactobacilli in the URT, based on the documented key role of these pathways (Hiscott, 2007). Our results highlight that different lactobacilli isolates have strikingly variable capacities for NF-κB and IFN regulatory pathway stimulation, both at the species and strain level, highlighting that thorough screening is required to select the optimal strain for each application. Specifically, we have identified *L. plantarum* WCFS1 and *L. rhamnosus* GG as two promising strains leading to significant IFN regulatory pathway induction both in viable and, to a lesser degree, UV-inactivated form. This indicates their highest potential not only as live probiotics but also as postbiotics because of retained activity in inanimate forms (Salminen et al., 2021). Activation of proteins belonging to the IRF family has been described to play a key role for induction of in type I IFN genes necessary for antiviral defences in immune cells (Tailor et al., 2006). The strong induction of the IFN pathway by *L. plantarum* WCFS1 is in line with earlier observations demonstrating especially strong activation by this strain of the antiviral IFIT1 (Interferon Induced Protein With Tetratricopeptide Repeats 1) gene in differentiated human monocytes (Gutierrez-Merino et al., 2020). Similarly, intranasal supplementation with *L. rhamnosus* GG was previously shown to increase survival in mouse models of influenza infection by stimulating transcription of protective type I IFN genes in the neonatal airways (Kumova et al., 2019). These immunostimulatory effects could be extrapolated to humans, as in the duodenum of healthy participants, *L. plantarum* WCFS1 induced the NF-κB pathway linked to immune tolerance (van Baarlen et al., 2009), while consumption of *L. rhamnosus* GG up-regulated IFN-induced genes and cytokine-encoding genes (van Baarlen et al., 2011).

Importantly, we demonstrated that the combination of the selected lactobacilli retained their immunostimulatory capacity not only when cultured in laboratory conditions but also as part of the final throat spray formulation. These results are in line with our observation that the viability of *L. casei* AMBR2, *L. rhamnosus* GG, and *L. plantarum* WCFS1 is maintained after freeze-drying of the bacteria and resuspension in oil. The use of oil in the final spray formulation, in combination with the established adherence capacity to the respiratory epithelium of the bacterial strains themselves (De Boeck et al., 2020; Pretzer et al., 2005; van den Broek et al., 2018), ensures their temporary retention in the throat. To the best of our knowledge, other oral sprays with probiotics have not yet used oil, but instead suspended the bacteria in saline (Andaloro et al., 2019). In this study, retention of the administered lactobacilli was confirmed in vivo in healthy volunteers. We found that 30 minutes after spray administration, live *L. casei* AMBR2, *L. rhamnosus* GG, and *L. plantarum* WCFS1 and their DNA were detected in the throat. A contact time of 30 minutes has previously been shown to be sufficient for immune response initiation in vitro, including NF-κB induction by bacterial lipopolysaccharides in RAW 264.7 macrophages (Hobbs et al., 2018) and in human cell lines by interleukin 1β and tumour necrosis factor alpha (Confalone et al., 2010). The spray was successfully tested in healthy volunteers, which opens possibilities for its preventive use. However, the dynamics of bacterial retention in a disturbed throat environment during an active viral RTI could be different; therefore, the spray should also be tested in volunteers with viral RTIs to better assess its therapeutic potential. Promisingly, our experiments here already established the delivery of a dose of live cells at their target site in healthy volunteers comparable to the amount of cells that was capable to counteract viral infection in vitro.

Our formulation ensures that the throat is the main target for bacterial interaction, in contrast to other studies on oral bacteriotherapy where the formulation is not tailored to URT applications, with most of the bacteria ending up in the gut (Ceccarelli et al., 2021). This is important because studies in animal models show that applying immunomodulatory lactobacilli at the site of respiratory infection or inflammation, rather than in the gastrointestinal tract, promotes a more direct and efficient contact with specific cells of the URT immune system (Pellaton et al., 2012; Spacova et al., 2019; Yang et al., 2017; Youn et al., 2012).

Indeed, in addition to the important immunostimulatory effects on host cells, our in vitro results also demonstrate direct inhibitory activity of *L. casei* AMBR2, *L. rhamnosus* GG, and *L. plantarum* WCFS1 on HCoV-229E, RSV, and influenza. While production of L- and D-lactic acid has previously been shown to reduce influenza A virus A/WSN/33 (H1N1) titers in vitro (Lange-Starke et al., 2014), reduction of viral cytopathogenic effects upon direct contact with metabolically inactive lactobacilli has not yet been explored in detail, especially not for promising URT isolates such as *L. casei* AMBR2. Since the lactic acid bacterium *Enterococcus faecium* NCIMB 10415 is capable of direct adsorptive trapping of swine influenza H1N1 and H3N2 viral particles (Wang et al., 2013), we hypothesize that *L. casei* AMBR2, *L. rhamnosus* GG, and *L. plantarum* WCFS1 are also capable of sequestering HCoV-229E, RSV, and influenza viral particles and directly reducing their infectivity and transmission.

In combination with previous results, our comprehensive in vitro testing substantiated the selection of *L. casei* AMBR2, *L. rhamnosus* GG, and *L. plantarum*

WCFS1 as three promising strains potentially capable of immunostimulatory and antiviral activity against viral respiratory diseases when combined in a URT spray (Figure 5B). In our previous research, the potential of *L. casei* AMBR2 as a topical probiotic for the respiratory tract is already documented in several previous studies (De Boeck et al., 2021; De Rudder et al., 2020; Martens et al., 2021), including its safety in healthy volunteers and in mouse models of respiratory inflammation. An important feature of this strain in the context of viral disease is its strong ability to enhance the airway epithelial barrier, as well as its adaptation to the unique URT environment, including catalase genes and adherence to the airway epithelium (De Boeck et al., 2021; De Rudder et al., 2020; Martens et al., 2021; Wuyts et al., 2017). Probiotics such as *L. casei* AMBR2 and *L. rhamnosus* GG also inhibit URT pathobionts that can increase the morbidity and mortality of viral respiratory diseases, including *Haemophilus influenzae*, *S. aureus*, and *Moraxella catarrhalis* (De Boeck et al., 2020; Morris et al., 2017; van den Broek et al., 2018), and *L. rhamnosus* GG has been shown to be safe in numerous RTI clinical trials (Lehtoranta et al., 2014). These features, in combination with the newly reported antiviral activity in this study, pave the way for a more directed topical use of the developed *L. casei* AMBR2, *L. rhamnosus* GG, and *L. plantarum* WCFS1 throat spray against respiratory viral disease. From a topical application perspective, our in vitro results suggest that live bacteria might result in more efficient immunostimulatory action than inactivated bacteria. However, because inactivated bacteria were also capable of immunostimulation, from a mechanistic perspective, we propose that surface molecules of inactivated lactobacilli play a key role in immunostimulatory interactions with host cells and direct antiviral activity, allowing their potential use as inactivated postbiotics in vulnerable populations. While our study mainly focused on in vitro selection of bacterial strains in monocytes and permissive cell lines, follow-up experiments in respiratory epithelial cell lines should further elucidate the spray's antiviral mechanisms of action in the airways, and dedicated clinical studies are needed to substantiate the effectiveness of the multispecies spray against viral diseases in vivo.

CONCLUSIONS

The development of rationally selected, mechanistically substantiated sprays with probiotic lactobacilli and their employment in clinical trials is long overdue, especially in the context of viral RTI. Based on their safety, applicability in the airways, antiviral action, and stimulation of antiviral immunity, *L. casei* AMBR2, *L. rhamnosus* GG, and *L. plantarum* WCFS1 were selected as promising strains, followed by optimal formulation for topical application in the URT in a throat spray. Temporary throat colonization by the administered lactobacilli was demonstrated. The spray might be a stepping stone for prevention and treatment of viral respiratory tract infections with locally applied beneficial bacteria, and first positive results have already been obtained upon its evaluation in COVID-19 primary care patients (De Boeck et al., 2022).

AUTHOR CONTRIBUTIONS

Irina Spacova: Conceptualization (lead); data curation (lead); formal analysis (lead); investigation (lead); methodology (lead); project administration (lead); validation (lead); visualization (lead); writing – original draft (lead); writing – review and editing (lead). **Ilke De Boeck:** Conceptualization (lead); data curation (lead); formal analysis (lead); investigation (lead); methodology (lead); project administration (lead); validation (lead); visualization (lead); writing – original draft (lead); writing – review and editing (lead). **Eline Cauwenberghs:** Data curation (equal); formal analysis (equal); investigation (equal); methodology (lead); validation (equal); visualization (equal); writing – review and editing (supporting). **Lize Delanghe:** Investigation (supporting); methodology (supporting); validation (supporting); writing – review and editing (supporting). **Peter A. Bron:** Conceptualization (supporting); investigation (supporting); writing – review and editing (equal). **Tim Henkens:** Conceptualization (supporting); data curation (supporting); investigation (supporting); methodology (supporting); project administration (supporting); validation (supporting); writing – review and editing (supporting). **Alix Simons:** Formal analysis (supporting); investigation (supporting); methodology (supporting); validation (supporting); visualization (supporting); writing – review and editing (supporting). **Imane Gamgami:** Investigation (supporting); methodology (supporting); validation (supporting); writing – review and editing (supporting). **Leentje Persoons:** Formal analysis (equal); investigation (equal); methodology (equal); writing – review and editing (supporting). **Ingmar Claes:** Conceptualization (lead); data curation (supporting); funding acquisition (supporting); methodology (supporting); project administration (supporting); resources (lead); writing – review and editing (equal). **Marianne F. L. van den Broek:** Conceptualization (supporting); writing – review and editing (supporting). **Dominique Schols:** Funding acquisition (equal); resources (equal); writing – review and editing (supporting). **Peter Delputte:** Conceptualization (supporting); resources (supporting); writing – review and editing (supporting). **Samuel Coenen:** Conceptualization (equal); writing – review and editing (supporting). **Veronique Verhoeven:** Conceptualization (equal); writing – review and editing (supporting). **Sarah Lebeer:** Conceptualization (lead); funding acquisition (lead); investigation (lead); methodology (lead); project administration (lead); resources (lead); writing – original draft (lead); writing – review and editing (lead).

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CONFLICT OF INTEREST

A patent application BE2021/5643 (priority patent application filed on 12/08/2021) titled "Sprayable formulation comprising viable and/or stable bacteria" has been filed based on the results of this work. TH, AS, IG and IC are employees of YUN NV. SL is the chairperson of the scientific advisory board of YUN NV. PAB is a consultant for multiple companies in the food and health industry, but they were not involved in this manuscript. Other authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The datasets generated and analysed during the current study are available in the European Nucleotide Archive (ENA) under accession number PRJEB49183.

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