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Probiotic nasal spray development by spray drying

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Abstract

The upper respiratory tract (URT) is the main entrance point for many viral and bacterial pathogens, and URT infections are among the most common infections in the world. Recent evidences by our own group and others imply the importance of lactobacilli as gatekeepers of a healthy URT. However, the benefits of putting health-promoting microbes or potential probiotics, such as these URT lactobacilli, in function of URT disease control and prevention is underestimated, among others because of the absence of adequate formulation modalities. Therefore, this study entails important aspects in probiotic nasal spray development with a novel URT-derived probiotic strain by spray drying. We report quantitative and qualitative analysis of several spray-dried formulations, i.e. powders for reconstitution, based on disaccharide or sugar alcohol combinations with a polymer, including their long-term stability. Four formulations with the highest survival of $>10^9$ (Colony Forming Units) CFU/g after 28 weeks were further examined upon reconstitution which confirmed sufficiency of one bottle/dosage form during 7 days and rheological properties of shear-thinning. Tests also demonstrated maintained viability and cell morphology overall upon spraying through a nasal spray bottle in all 4 formulations. Lastly, application suitability in terms of high adherence to Calu-3 cells and antimicrobial activity against common URT pathogens was demonstrated and was not impacted neither by powder production process nor by spraying of reconstituted powder through a nasal spray device.

1. Introduction

The human body is a host to 10-100 trillions of microbes, predominantly bacteria, that are collectively defined as the human microbiome, essential for our well-being [1]. Especially after the Human Microbiome project started, it has become widely accepted that these microbes are related to a spectrum of health effects while a disbalance in their compositions, also called dysbiosis, is linked with various different diseases including upper respiratory tract (URT) diseases [1]. The microbiome of the URT, as reviewed by van den Broek et al. [2], is subject to variations from the first day of life depending on different environmental factors such as, delivery mode, feeding and attending day care. An imbalance of this microbiome has been associated with several URT diseases, such as otitis media (OM) [3,4], chronic rhinosinusitis (CRS) [5–8], and acute sinusitis in children [9,10]. Probiotics, ' live microorganisms that, when administered in adequate amounts, confer a health benefit on the host', on the other hand can provide beneficial effects to the host and restore or prevent this disbalance [11]. However, nasal application of probiotics and formulation strategies to target the URT remain unexplored, although it potentially offers practical advantages such as non-invasive mode of delivery, good adverse effect profile and thus better patient compliance [12,13].

Based on a Cochrane review including 13 randomised clinical trials (RCTs), probiotics were found to be better than placebo regarding the number of participants experiencing episodes of acute upper respiratory tract infections (URTIs) highlighting their importance in prevention of URTIs [14]. On the other hand, two RCTs in patients with CRS conducted so far, one with oral application of *Lacticaseibacillus rhamnosus* R0011 (former *Lactobacillus rhamnosus* [15]) [16], and one with nasal spray administration of mixture of honeybee lactobacilli [17] showed good tolerance of such probiotic interventions but little to no benefits in comparison to placebo. In contrast, Habermann et al. [18] in multicentre placebo-controlled study in 157 patients with oral application of *Enterococcus faecalis* demonstrated a reduction of frequency of acute

exacerbations of CRS. Moreover, RCT with oral administration of *Limosilactobacillus reuteri* ATCC 55730 (former *Lactobacillus reuteri* [15]) improved healthiness in Tetra Pak workers in Sweden by reducing the frequency of sick-leaves caused by respiratory or gastrointestinal problems [19]. Nevertheless, these rather limited scientific evidences do imply a probiotic potential for prevention/treatment of URTIs, but also a need for better strain selection and proper administration, considering that most of the studies were conducted with gastrointestinal strains via oral delivery, such as *L. rhamnosus* GG (LGG), hence not adapted to the unique environment of the URT, or with strains that are not even human-derived. Therefore, our previous works [7,20,21] and the current work are focused on human isolates originating from the healthy URT, as we observed that lactobacilli were more abundant in the URT of healthy individuals in comparison to URT niches of people suffering from CRS [22].

Spray drying showed a significant potential for viability and functionality maintenance of newly isolated human derived URT strain *Lacticaseibacillus casei* AMBR2 and was a better choice for this particular strain than traditional freeze-drying (data not published) [20]. Martens et al. [21] demonstrated a nasal epithelial barrier dysfunction restoration in CRS *in vivo* by spray-dried powders of *L. casei* AMBR2. Moreover, with the aid of spray drying in a proof-of-concept study, we showed how promising and safe nasal administration of live *L. casei* AMBR2 cells were in healthy volunteers [7]. Therefore, it was further considered as an attractive route for a nasal formulation where bacterial cells would be enclosed in a glassy matrix formed by excipients. This encapsulation would enable a longer viability, e.g. several months or couple of years, and a better recovery after reconstitution. In addition, this formulation development would allow more robust procedures and easier scale-up of production to meet the needs of future research on probiotic effects in a much larger group of patients suffering from CRS where data on probiotic efficacy is the most limited.

Hence, the current study was dedicated to finding an optimal probiotic formulation for nasal delivery with respect to nasal niche specificities. Firstly, different combinations of excipients (sugars, sugar alcohols and polymers) were screened for their capacity for preserving the viability of *L. casei* AMBR2 after spray drying and during shelf-life. Next, the most promising formulations were selected and tested for stability. Also, rheological properties upon the resuspension of spray-dried powders were examined. The main goal was to simulate an actual use of a nasal spray, allowing to determine the maximum time-period of use of a single spray bottle. Furthermore, the impact of spraying of reconstituted spray-dried probiotic powder through a nasal spray bottle was tested in relation to viability, morphology changes and functionality of respective formulations. The functionality of chosen formulations was evaluated via antimicrobial assays against common URT pathogens as well as adherence of formulations in respiratory Calu-3 cell lines.

2. Materials and methods

2.1 Cultivation of bacterial strains

Cultivation of *L. casei* AMBR2 (LMG P-30039, Belgian Coordinated Collections of Microorganisms) was performed in the same manner as documented in our previous works [20,23]. Briefly, cells were maintained as 25% (v/v) glycerol stock cultures at -80 °C and afterwards cultivated until stationary phase. Afterwards cells were washed with phosphate buffered saline (PBS) [composed of 0.3 g/l $NaH_2PO_4.2H_2O$ (Carl Roth, Mühlburg, Germany), 1.54 g/l $Na_2HPO_4.2H_2O$ (Merck, Darmstadt, Germany) and 8.2g/l of NaCl (Merck Darmstadt, Germany) with pH of 7.2] and harvested by centrifugation (Sigma 3-16PK, Sigma Zentrifugen, Germany) at 3983 x g and 20 °C for 12 minutes and prepared for further processing. The cell numbers after cultivation were approximately 2 x 10^9 (Colony Forming Units) CFU/mL.

2.2 Media composition and spray drying

In purpose of nasal formulation development different excipients were used: lactose (L) (Sigma-Aldrich, Germany), trehalose (T) (kindly provided by Nagase, Germany), sucrose (S) (Calbiochem, USA), xanthan gum (XG) (Carl Roth, Mühlburg, Germany), hydroxypropylmethylcellulose (HPMC) (Alfa Aesar, Thermo-Fisher, Germany), gum arabic (GA) (Carl Roth, Mühlburg, Germany), xylitol (X) (Xlear Inc., Utah, USA), isomalt (I) (kindly provided by Beneo, Mannheim, Germany). The disaccharides and sugar alcohols were used in a concentration of 2.5% (w/V) whereas polymers/gums were used in a 1% (w/V) except for XG. XG was used in 0.4% (w/V), which was previously established as sufficient for obtaining a homogenous solution/suspension in demineralised water. L and T were tested as such as a reference, while the protection capacity of solely S was examined in a different context (data not published). Sugar alcohols, X and I, when used as such, did not result in a free flowing dry powder. Therefore, combinations of disaccharides or sugar alcohols with polymers were tested in above mentioned concentrations. The combinations that resulted in sufficient powder amounts were: sucrose and xanthan gum (SXG), isomalt and xanthan gum (IXG), trehalose and HPMC (TH), lactose and HPMC (LH), xylitol and HPMC (XH), trehalose and GA (TA), lactose and GA (LA). These formulations were prepared by polymer swelling in demineralised water followed by a 24h-hydration, after which sugars/sugar alcohols were dissolved in such prepared polymer, followed by inoculating of centrifuged bacteria.

Laboratory-scale spray drying by B-290 spray-dryer (Büchi, Flawil, Switzerland) in an open mode was performed with 7.5 mL/min feed flow rate, 536 L/h spray flow rate (atomisation) and 32.5 m3/h gas flow rate, as previously optimised [20]. The outlet temperature was kept constant at 55 °C while the heat exposure time, i.e. process length duration, was maximum 15 minutes, with regular powder harvesting underneath a single cyclone separator.

2.3 Shelf-life stability

Spray-dried powders were packed in Eppendorf tubes (VWR International Europe, Leuven, Belgium), sealed with Parafilm®. The tubes were further packed in heat-sealed aluminium bags (Daklapack, Kortrijk, Belgium). Constant storage conditions were 4-8 °C and ambient relative humidity (RH). Viability in all respected formulations was re-evaluated after periods of 4 and 28 weeks.

2.4 Viability enumeration

Viability counts before and after spray drying, and after shelf-life, reconstitution and spray tests were obtained via ten-fold serial dilutions, of reconstituted spray-dried powders or fresh cells, inoculated onto de Man Rogosa and Sharpe (MRS) agar plates (Carl Roth, Mühlburg, Germany) in triplicate. Reconstitution in all tested samples was done in 1:100 ratio of spray-dried powder vs. resuspension media (demineralised water, PBS, saline). MRS plates were subsequently incubated at 37 °C for 48h and bacterial colonies were afterwards counted. Results are expressed in CFU/g as mean value ± standard deviation.

2.5 Morphology

All tested formulations were visualised by scanning electron microscopy with Quanta FEG250 SEM system (Thermo Fisher, Asse, Belgium). The powders of respective formulations were mounted on SEM stubs using conductive carbon tapes. The stubs were sputter-coated with gold (10 nm) and imaged at a voltage of 5 kV.

Dry particle size in produced powders was measured using the laser diffraction technique (Malvern 3000, Malvern Instruments Ltd., Malvern, UK) in dry conditions in a small sample device. The measurement principle is based on the Mie theory of light scattering thus results are reported as a volume equivalent sphere diameter.

2.6 Water content of powders/formulations

Karl-Fisher Titration device (Karl-Fisher Titrino Plus, Metrohm, Germany) was used to determine the water content in all powders. Analyses were carried out at room temperature with Aqualine Composite 5 (Fisher Scientific, UK) with constant stirring.

2.7 Differential scanning calorimetry

All spray-dried formulations were subjected to thermal analysis using calibrated Discovery DSC25 equipment from TA Instrument (New Castle, DE, USA). Powder samples (5-10 mg) were analysed in Tzero hermetic aluminium pans under 50 mL/min nitrogen gas purge in the modulated temperature mode. All samples were heated from -40 to 270 °C at a 10 °C/min heating rate with a modulation of 1.6 °C/min. Thermogram evaluations were done by TA Instruments TRIOS v5.0.0 software.

2.8 Powder yield

Yield after drying was calculated as previously reported in our works, i.e. as the ratio of the amount of the dried powder vs. amount of total solids in the bacterial suspension subjected to spray drying.

$$Yield\ (\%) = \frac{weight\ of\ spray\ dried\ powder\ (g)}{weight\ of\ solids\ before\ drying\ (g)}\ x\ 100$$

2.9 Stability after re-suspension of spray-dried powders

Several formulations were selected, spray dried, re-suspended and tested for viability in a wet form during storage at refrigerated conditions (4-8 °C) and ambient relative humidity for 7 days in Eppendorf tubes (VWR International Europe, Leuven, Belgium). Sample portions were taken at several time points, 6 h, 24 h, 48 h, 120 h and 168 h. These samples were diluted serially and plated out on MRS agar plates, incubated and enumerated as described (section 2.4).

2.10 pH measurements

Measurements of pH of reconstituted spray-dried powders were performed by a pH meter (HI5221, Hanna Instruments).

2.11 Rheology

Rheological measurements were performed using a rotational viscosimeter (Anton Paar MCR 102, Belgium) with a parallel plate method (50mm diameter) with gap size of 1mm. Measurements were performed with increasing shear rates from 0,1 s^{-1} to 100 s^{-1} at 20 °C.

2.12 Osmolality measurements

The osmolalities of the chosen formulations were measured using an advanced Micro Osmometer (Model 3320 Advanced Instruments Inc, Norwood, MA, US) by the freezing-point method. The measurements were performed in triplicate (on 20-µL aliquots) and mean values used for analysis. Samples with an osmolality >1200 mOsm/kg were diluted for measurement. Clinitrol™ 290 was used as reference solution (Advanced Instruments Inc, Norwood, MA, US).

2.13 Spray tests and flow-cytometric analysis

Spray tests were conducted with selected spray-dried formulations by spraying them upon complete reconstitution through a nasal bottle with 100 µL spray volume per puff (Pharma Pack, Wilrijk, Belgium). Sample portions were serially diluted and plated out on MRS agar plates, incubated and enumerated as described (section 2.4). The resulting viability numbers were further compared with the numbers obtained in the same reconstituted formulations before spraying. Additionally, flow cytometry analysis with LIVE/DEAD™ kit (Syto 9 3.34 mM and Propidium Iodide-PI 20 mM, Invitrogen by ThermoFisher, Oregon, US) was done to determine whether any cell damage occurred after spray drying, reconstitution and spraying through the described spray bottle. The analysis was performed on fresh cells as a control,

spray-dried and reconstituted chosen formulations before and after spraying through the nasal spray bottle. Staining procedure was done as already reported in Jokicevic et al. [20]. Dyed bacterial suspensions were analysed by Attune NxT Acoustic Focusing Cytometer (Model AFC2, Thermo Fisher Scientific, Woodlands, Singapore). The data analysis was performed in 10000 events and with Attune NxT software version 3.1.2.

2.14 Adherence assay to human airway Calu-3 epithelial cells

Experiments to assess adhesion behaviour of chosen spray-dried formulations after reconstitution and before and after spraying through the nasal spray bottle were carried out in the same manner described and standardised by De Boeck et al. [7]. The human bronchial epithelial cell line Calu-3 ATCC® HTB-55™ (purchased from ATCC) was used. The cells were incubated with 0.5 mL rehydrated spray-dried powder with a concentration of 2 x 10^8 CFU/mL, both before and after spraying through the nasal spray bottle ((2 x 10^8 CFU/mL in Minimal Essential Medium without fetal calf serum (Life technologies, Ghent, Belgium)) for 1h at 37 °C, 5% CO_2, 100% humidity. After the incubation, cells were rinsed once with pre-warmed phosphate buffered saline (PBS). To detach the cells, 175 µL of trypsin (0.25%) was added to the cells for 10 minutes at 37 °C. Afterwards, 325 µL PBS was added and appropriate serial dilutions were plated out on solid MRS and evaluated after 48 h of incubation at 37 °C. The adherence percentage was calculated by comparing the total number of colonies counted after adhesion with the number of cells in the bacterial suspension originally added to the cells.

2.15 Antimicrobial tests

The antimicrobial activity of selected formulations and fresh cells of *L. casei* AMBR2 against URT pathogens, *Staphylococcus aureus*, *Moraxella catarrhalis* and *Haemophilus influenzae* was tested by standard antimicrobial tests, as documented previously by van den Broek et al. [24]. Briefly, 2 µL of each reconstituted spray-dried formulation, both before and after spraying

through the spray bottle, and 2 µL of fresh AMBR2 culture were spotted on a standard agar plate (1.5% w/v) containing medium of the pathogen supplemented with glucose (5 g/L). These plates were incubated for 48 h at 37 °C. After incubation, 450 µL, 45 µL and 300 µL of *M. catarrhalis*, *S. aureus* and *H. influenzae*, respectively, were inoculated in soft agar (0.5% w/v) and poured over the plates with spots. Hexetidine (antibiotic) was used as a positive control. The plates were incubated overnight according to the growth conditions of the tested pathogens, after which the inhibition zones were measured. Zones of inhibition were measured as halos surrounding inoculated samples – distances between the edges of grown samples and edges of the formed halos (Supplement 1).

2.16 Statistical analysis

Statistical analysis was performed with SPSS 26 software (IBM statistics, New York, USA) using one-way ANOVA-test and Tukey's multiple comparisons test at a significance level of α=0.05. Data are expressed as means ± standard deviation.

3. Results

3.1 Shelf-life stability and formulation characteristics after spray drying towards formulation selection

Different combinations of excipients were screened with the goal to preserve the cell viability of *L. casei* AMBR2 in a powder form after spray drying and during a time period of 7 months of shelf-life. Sugars, lactose and trehalose, were used as a reference, while sucrose was documented in a separate concept and not further considered here due to the nature of related powders consisting of partially merged particles without free-flowing characteristics. Sugar alcohols, isomalt and xylitol, were here described only in formulations that yielded a powder form and in substantial amounts. Viability enumeration after spray drying revealed a significant improvement in viability outcomes when T, IXG, LH, TH were used as

protectants/encapsulation material, in comparison with AMBR2 cells spray-dried as such (unprotected cells) [20,23] (~0.4 - 0.5 log reduction) (Fig.1 (A)). Viability improvements were also noticeable in formulations SXG, TA, LA, although not statistically significant, while L resulted in the same viability as unprotected cells, and XH in a significantly lower viability counts than unprotected cells (Fig. 1 (A)). However, viability recorded immediately after processing – spray drying does not by default imply high shelf-life viability outcomes [25]. Therefore, all powders were maintained under refrigerated conditions, owing to the metabolic activity and promoted growth of *L. casei* AMBR2 at 25 °C, and re-evaluated for viability after 4 and 28 weeks. This evaluation clearly discriminated formulations with the highest viability after 28 weeks, approx. 5×10^9 CFU/g and higher, in T, SXG, IXG, TH and LH encapsulation matrix (Fig. 1 (B)). Other formulations, L, TA, LA, XH resulted in a steep viability decline during storage. Interestingly, both, L and T, demonstrated a visual change in powder properties, gradual re-crystallisation, as a consequence of storage conditions at ambient RH and small particle size, much more prominent in L formulations impacting significantly its viability outcomes. The highest viability of approx. 3×10^{10} CFU/g, i.e. cell loss of only 0.2 - 0.3 log, was achieved in TH formulation after storage. SEM observations (Fig. 1 (C)) revealed the particle structure in all tested formulations. L and T formulations resulted in spherical particles of different sizes with noticeable small indentations causing "golf-ball" like appearance of certain particles. SXG and IXG powders consisted of mainly spherical particles with slightly shrivelled surfaces, where larger particles seemed to consist of layered patches, whereas some particles clearly showed imprints of bacterial cells on the outer surface. TH and LH formulations consisted of irregularly shaped particles of different sizes with deeply wrinkled surfaces showing high indentations. LA and TA powders consisted of spherical just lightly dented particles of different sizes, while XH powders exhibited particles visually comparable to formulations also containing HPMC, TH and LH.

Table 1 depicts dry powder characteristics such as yield that was above 50% in all formulations except SXG and IXG, dry particle size that varied from 3 to 11.5 μm and water content that was below 4% in all samples as suggested by literature [26]. Notably, a glass transition temperature (Tg) was present in all samples except XG. This corresponded to literature findings for respective formulations, although formulations containing GA, LA and TA, had an increase in Tg for 10 °C in comparison to Tgs when L and T were used as such. Taken all these parameters into consideration, our data showed that LH, TH, SXG and IXG were most suitable to maintain formulation characteristics and provide stable shelf-life of AMBR2 after spray drying.

3.2 Stability after reconstitution of the spray-dried powder

Since spray-dried nasal spray formulations need to be re-suspended prior use, we also evaluated the stability after reconstitution. In Fig. 2 (A) loss of viability of *L. casei* AMBR2 in TH, LH, SXG, IXG formulations upon reconstitution and refrigerated storage for 7 days (168 h) is illustrated. The visible loss of cell viability, as expected, started after 48 h but was maintained above 10^9 CFU/g in all formulations during the period of testing. This viability evolution was followed by changes in pH from 6.2 at T0 to 5.4 - 5.5 at T168 h. Rheological behaviour (Fig. 2 (B)) of all samples may be regarded as shear-thinning to thixotropic, i.e. decrease of viscosity with an increase in shear rate with reversible viscosity, although more notable in SXG and IXG reconstituted powders. SXG and IXG also demonstrated higher apparent viscosity than TH and LH formulations. Rheological behaviour was maintained throughout the examined period of 7 days (Supplement 2). Osmolality measurements at 0h (Table 2) of all four formulations indicated hypo-osmotic suspensions in demineralised water and isotonic suspensions in saline as anticipated.

3.3 Spray tests

Spraying/dispersion of the re-suspended powder through the nasal spray bottle did not lead to significant viability changes in any of the formulations (Fig. 3 (A)). Flow-cytometric analysis using fluorescent dyes clearly enabled differentiation between viable cells with intact membrane, dead cells, and so called “cells with a slightly damaged membrane”, i.e. damaged cells. Fig. 3 (B) depicts representative flow charts of fresh cells (a), cells after spray drying within formulations (TH, LH, SXG, IXG) (b) and cells after spray drying with mentioned formulations and subsequent spraying/dispersing through the nasal spray bottle (c). Fluorescence intensity was the highest in population of fresh, untreated cells, that were detected solely within Syto9 quadrant indicating a complete absence of damage and cell integrity. Next, the fluorescence intensity decreased slightly in cells subjected to spray drying followed by a slight shift in side and forward scatter and an appearance of a very small population of dead cells and cells in between PI and Syto9 quadrants classified as damaged cells. Here the population of live undamaged cells varied from 85% - 90% and higher out of 10000 analysed events. Spraying through the nasal bottle had an impact on cell morphology as indicated in further fluorescence peak broadening, presence of small populations of dead and damaged cells, a drop in count as visible in Fig. 3 (B) (c). This resulted in still high percentage, 75% - 85%, of live undamaged cells on average.

3.4 Functionality tests

In addition to the viability and cell morphology tests described in section 3.3, adherence and antimicrobial tests were done with four formulations TH, LH, SXG, IXG in order to test the impact of shear effects of dispersing through the spray bottle and impact of processing overall. Experiments in Calu-3 cell line resulted in an unchanged high adherence percentage (>10%) in TH and LH formulations in comparison to the adhesion of fresh cells of *L. casei* AMBR2 (used as a control and also reported in our previous works [7,20]) independently of the dispersion

through the nasal spray bottle (Fig. 4 (A)). On the other hand, SXG and IXG, both sprayed/dispersed and non-sprayed, had a significantly lower adherence (up to 5%) in comparison to fresh cells, unprotected spray-dried cells [20] and TH and LH.

Analysis of antimicrobial effects of *L. casei* AMBR2 against URT pathobionts, *S. aureus*, *M. catarrhalis*, and *H. influenzae* via spot assay revealed growth inhibition of all three pathogens. This was independent of the formulation tested and the impact of dispersing through the nasal spray. This effect was the greatest against *H. influenzae* and the weakest against *S. aureus* (Fig. 4 (B)). The drop in antimicrobial activity in comparison to fresh, non-dried cells of AMBR2 was recorded in the assay with *S. aureus* (SXGS, IXG, IXGS), also with *M. catarrhalis* where all tested samples led to lower inhibition zones compared to the fresh cells, while the assay with *H. influenzae* did not result in major differences.

4. Discussion

Although presence of lactobacilli in the URT of healthy children and adults as well as seasonal variations of their abundance have been discovered [7], topical applications of probiotics containing lactobacilli remain unexplored for prevention or ease of symptoms of URT infections [7,27,28]. Therefore, in this study we aimed to evaluate the possibility of nasal spray formulation development by using excipients/protectants with an adequate safety profile and stability across a broad range of pH for the intended way of administration in purpose of cell microencapsulation. This research expands on our previous findings of feasibility of the use of spray drying for viability and functionality preservation of human-derived probiotics – novel URT lactobacilli [20]. Microencapsulation, here by spray drying, is an effective method that mainly acts as a physical barrier against adverse environmental, processing, storage and or intestinal conditions probiotics undergo [29–31]. Disaccharides, such as lactose and trehalose, were documented by several authors as a microencapsulation tool with high probiotic viability retention during spray drying [32–34], explained by the hypothesis of water molecules

replacement inside membrane's lipid bilayer by these molecules and thus cell membrane stabilisation [35]. However, according to literature and our observations lactose and trehalose powders are hygroscopic especially at room temperature and ambient RH requiring special packaging solutions. Additionally, their combinations with polymers and other excipients in the same purpose have been unreported, as well as the use of sugar alcohols. Among sugar alcohols - polyols, mainly mannitol has been investigated as a protectant in freeze drying and spray drying formulation of probiotics [32,36]. Isomalt possess very good thermal stability without changes in structure when melted and is non-hygroscopic which could explain its good protective capacities [37], while xylitol has been demonstrated to exert bacteriostatic and bactericidal effects towards spoilage microorganisms [38] and cariogenic *Streptococcus mutans* [39] that might have also caused poor survival of AMBR2 during spray drying observed in this work, although such effects were not documented in fresh cell suspensions containing it. The rationale for polymer use is a potential to improve adhesion capacity of formulations and powder properties, maintain a more stable glassy matrix with embedded cells, and allow powder manipulations in terms of particle size, flowability, etc. Here one of the expected benefits of the polymer use was the additional stabilisation in terms of amorphisation of disaccharide and sugar alcohol molecules and/or film formation around bacterial cells towards better viability outcomes after spray drying and during storage. This was indeed observed in all our experiments and was very prominent when HPMC was used (Fig. 1 (A) and 1 (B)). Yet, it must be noted, that thermal analysis indicated only T, TH, IXG and TA formulations as completely amorphous systems with completely absent melting peaks of crystalline sugar molecules, although Tg's were recorded in all samples except XH (Table 1). Food grade polymers, such as maltodextrin, alginate, pea proteins, and gums, gum arabic and xanthan gum, are also regarded as excellent materials for probiotic encapsulation due to their non-toxicity, biocompatibility, and gel forming abilities [34,40]. Maltodextrin, xanthan gum and gum arabic

are also commonly considered for non-dairy probiotic formulations [32,40] and drug formulations in general, as film formers, viscosity-increasing, thickening and stabilising agents, commonly found in oral and topical drug dosage forms [37,41]. Arepally et al. [40] with *L. acidophilus* demonstrated good protection capabilities of increasing gum arabic concentrations, up to 10%, yet shelf-life behaviour was not monitored in this concept. Moreover, Liu et al. [42] showed good storage stability up to 16 weeks of *L. casei* microcapsules containing gum arabic in alignment with the highest Tg's of these formulations. Based on our results, formulations with gum arabic indeed had highest Tg's (Table 1), but that did not result in such effectiveness of this polymer to retain high shelf-life viabilities (Fig. 1 (B)). A possible explanation could be the low concentration used (1% w/v) in comparison with the mentioned studies, although our preliminary viability tests indicated it as suitable and sufficient. Xanthan gum-based probiotic encapsulation matrices have been reported in freeze drying [43,44], hot-melt extrusion [45] and novel method - pneumatic atomisation [42]. Comparably, our results illustrate the benefits of matrices with low xanthan gum amounts, such as SXG and IXG formulations, in relation to viability preservation (Fig. 1 (A) and 1 (B)). Intriguingly, in the work of Yonekura et al. [25] HPMC as a co-encapsulant of *L. acidophilus* NCIMB 701748 during spray drying was not superior to alginate and during room temperature storage for 35 days yielded higher losses than both alginate and chitosan. On the other hand, our results showed the best protective capacity of disaccharide – HMPC matrices with *L. casei* AMBR2, although monitored at refrigerated conditions due to the strain-related properties explained above. These experiments resulted in the choice of 4 formulations TH, LH, SXG and IXG for further nasal spray considerations.

By monitoring viability of spray-dried and re-suspended formulations during 7 days we elucidated the potential of the use of a single spray bottle longer than a single application. The slight decrease of viability throughout the tested period was expected as watery bacterial

suspensions are bulky and unstable due to inevitable promotion of metabolic processes, thus the efforts for maintaining bacteria in a dry form [32]. Nevertheless, the counts of viable bacteria remained higher than 10^{10} CFU/g after 5 days and >10^9 CFU/g after 7 days which corresponds to the dosage range for probiotic nasal sprays used in studies [17,46], assuming powder re-suspension in 1:100 ratio (Fig. 2 (A)). According to literature, pH of a healthy nasal mucosa is 5.5 – 6.5 [47]. This criterion in potential nasal sprays was met when water and isotonic saline were used as re-suspending agents, and importantly, it was also maintained during the 7 days of the monitoring after reconstitution. Of note, considering that after 7 days, pH of all samples was on the lower border (although 4.5 - 7.4 is tolerated for nasal sprays), owing to production of compatible solutes by bacteria, one spray bottle with reconstituted powder should not be re-used longer than the mentioned period to avoid irritations. Moreover, although our current and previous research does not imply significant changes in bacterial viability enumeration when re-suspending agents are varied, osmolality measurements do play an important role. Isotonic preparations are a prerequisite in order to avoid mucociliary clearance more often than physiological (15-30 minutes). For the intended ratio of powder vs. re-suspending agent, isotonic saline provides the needed osmo-balance (Table 2). Nasal sprays are commonly formulated to exhibit rheological behaviour described as thixotropic or at least with shear-thinning phenomena in order to be successfully sprayed but also with inhibited particle sedimentation in resting [48]. Considering that such effects were observed in repeated measurements over 7 days all our formulations correspond to guidelines (Fig. 2 (B) and Supplement 2).

Furthermore, special focus was on the influence of spraying through a nasal spray bottle in terms of impacts on viability and morphology of AMBR2 cells (Fig. 3) and functionality via *in vitro* simulated application assays (Fig. 4). This is of special importance, as spraying – additional partitioning of reconstituted bacteria into small droplets can potentially cause cell

433 and extracellular effectors' damage caused by shear stress. Importantly, viability enumeration
434 showed no such indications. On the contrary, flow-cytometric analysis did reveal small changes
435 in morphology and count of AMBR2 cells, besides morphology changes after spray drying.
436 These changes can be indicated mainly as changes in cell complexity, as no major cell viability
437 losses were recorded neither by plate counting nor flow analysis. Of note, morphology changes
438 after spray drying by using any of the matrices (LH, TH, SXG, IXG) were much less
439 pronounced than when unprotected cells were subjected to spray drying implying good
440 protective capacities of the chosen excipients. However, drying is inevitably followed by
441 disruptions of membrane integrity mainly caused by the loss of water molecules [49], whereas
442 further spraying poses a shear, mechanical stress to the membrane, extracellular molecules and
443 chain occurrence within lactobacilli cell population.

444 Lastly, adhesion to epithelial cells and antimicrobial properties of probiotics are considered as
445 one of the most important action mechanism hosts (humans, animals, etc.) can benefit from
446 [50,51]. High adherence to Calu-3 cells, greater than 10%, observed in TH and LH
447 formulations is in agreement with our previous results and is considered as an adaptation
448 mechanism to URT [7,20] (Fig. 4 (A)). Moreover, the adherence recorded is believed to be
449 linked to the strain itself owing to the spike-like appendages, i.e. pili or fimbriae, present on
450 the cell surface of *L. casei* AMBR2 and considering the immediate release of cells from
451 formulated matrices [7,52]. Intriguingly, SXG and IXG exhibited significantly reduced
452 adherence potential (Fig. 4 (A)). Possible reason could be the higher apparent viscosity of these
453 formulations upon reconstitution due to faster thickening of XG than HPMC that by keeping
454 bacterial cells in a stable suspension – inhibiting sedimentation, potentially led to a limited
455 contact of bacterial cells and Calu-3 cells. Another explanation could be that spray drying with
456 XG caused a higher shear stress and damage to pili/fimbriae that could not be noticed in other
457 tests. Yet again, the higher shear stress could possibly be linked with higher beginning viscosity

prior spray drying of SXG and IXG formulations. Contrary to findings of Zhou M. and Donovan D. M. [53], Pennington et al. [54] and Gavini et al. [55] that increasing viscosity by using bio-adhesive polymers can prolong therapeutic activity of a nasal spray and slow down the mucocilliary clearance, our study resulted in either no impact when HPMC was used or the negative impact of more viscous samples when XG was used. Future research could address this phenomenon more in detail by varying sample viscosity more significantly and/or by testing multiple different polymeric agents in *in vivo* models or volunteers. Antimicrobial assays against common URT pathogens that act as opportunistic pathogens in the URT during infection, albeit variable among pathogen strains and formulations tested, implied that formulation and processing did not alter this beneficial feature of the strain. This unique characteristic might derive from secretion of antimicrobial substances such as lactic acid, bacteriocins, H_2O_2 [24,56,57], although in another study authors, Allonsius et al. [58] described the involvement of other molecular mechanisms of *L. rhamnosus* GG against *Candida albicans*. More research is needed to identify the effector molecules responsible for the described effects of the chosen strain. Importantly, spraying through the nasal spray bottle did not influence any of the examined probiotic functionality parameters.

5. Conclusions

In this work, we have successfully elucidated potential probiotic nasal spray formulations leading to the successful application of *L. casei* AMBR2 in respiratory cell lines and in URT pathogen competition in antimicrobial assays. Our results decipher important aspects to specifically target URT application with a bacterial strain with demonstrated URT niche adaptation and tackle major potential formulation hurdles in an economical manner. Moreover, this study highlights the importance of the careful examination of probiotic functionality after manufacturing separately from viability outcomes as it can be not only strain-dependent but also formulation and process-dependent. Lastly, this study paves the way for novel microbial-

based dried biotherapeuthics targeting nasal cavity that could ease the battle against infectious diseases and fast-evolving antibiotic resistance and could be of practical importance even for vaccine technologies as strains as the one used in this study are often considered as adjuvants due to the immunological effects.

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7. Declarations of interest

None.

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Table 1. Dry powder characteristics of tested formulations.

Table 2. Osmolality measurements of re-suspended formulations in water and in saline containing *L. casei* AMBR2.

Fig. 1. Viability outcomes after spray drying of *L. casei* AMBR2 within 9 different formulations. The horizontal line represents viability of spray-dried unprotected cells and stars (*) represent the statistically significant changes in viability in relation to the unprotected cells ($p>0.05$) (A). Refrigerated shelf-life viability (at 4-8 °C) of different formulations at ambient RH for 4 and 28 weeks where -6 represents the limit of viability detection (B). SEM micrographs of spray-dried formulations (L (a), T (b), SXG (c), IXG (d), TH (e), LH (f), TA (g), LA (h) and XH (i)) where white arrows indicate imprints of bacterial cells on formed particles (C).

Fig. 2. Reduction in cell viability of TH, LH, SXG and IXG formulations upon powder reconstitution and refrigerated storage for 7 days (A). Rheological behaviour of tested / chosen formulations after storage for 7 days (B).

Fig. 3. Comparison of reduction in viability of *L. casei* AMBR2 after spray drying and reconstitution (TH, LH, SXG, IXG) and after spraying through the nasal spray bottle (THS, LHS, SXGS, IXGS) (A). Flow-cytometric analysis of *L. casei* AMBR2 cells before spray drying (a), after spray drying and reconstitution (b) and after spray drying, reconstitution and spraying through the nasal spray bottle (c) (B).

Fig. 4. Adherence of *L. casei* AMBR2 in spray-dried, reconstituted formulations – TH, LH, SXG and IXG, and adherence of the same formulations spray-dried reconstituted and sprayed

through the nasal spray bottle – THS, LHS, SXGS, IXGS. Stars (*) indicate the size of statistical significance in relation to the adherence of fresh cells of AMBR2 ($p<0.05$) (A). Antimicrobial activity of reconstituted formulations of AMBR2 before and after spraying through the nasal spray bottle against *S. aureus*, *M. catarrhalis* and *H. influenza* depicted as zones of inhibitions (B).

Supplement 1. Antipathogen tests and inhibition zone determination in *S. aureus* (A), *M. catarrhalis* (B) and *H. influenzae* (C).

Supplement 2. Rheological behaviour of reconstituted spray-dried powders of TH, LH, SXG, IXG after 7 days of refrigerated storage.

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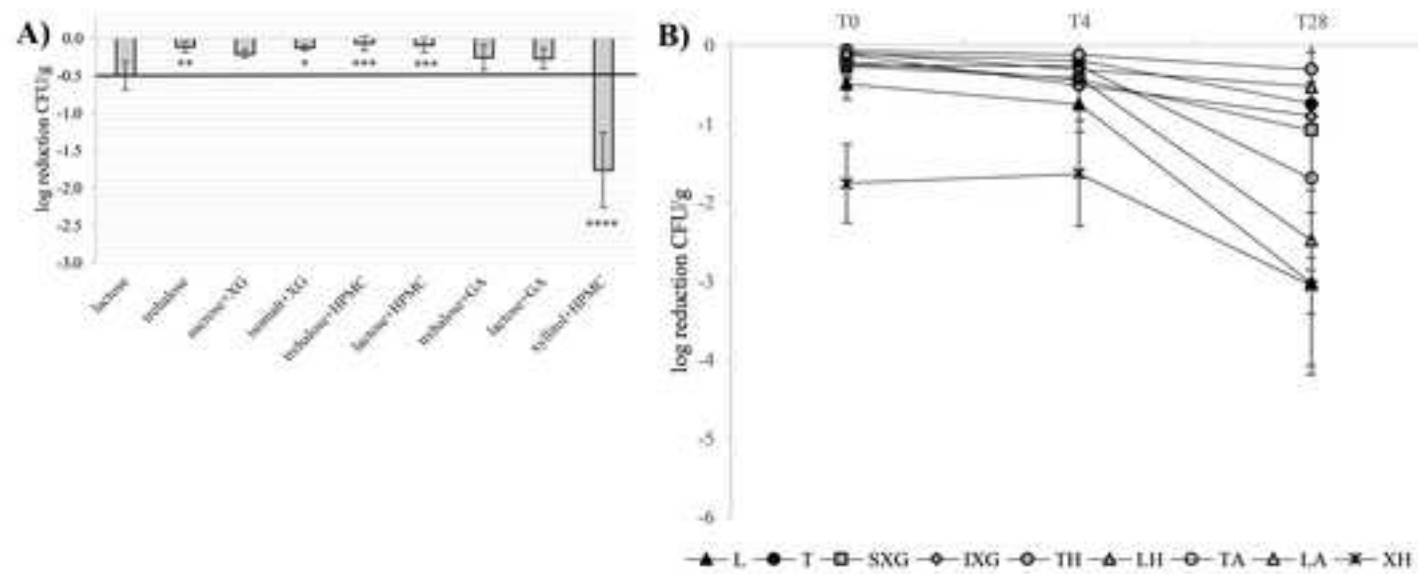

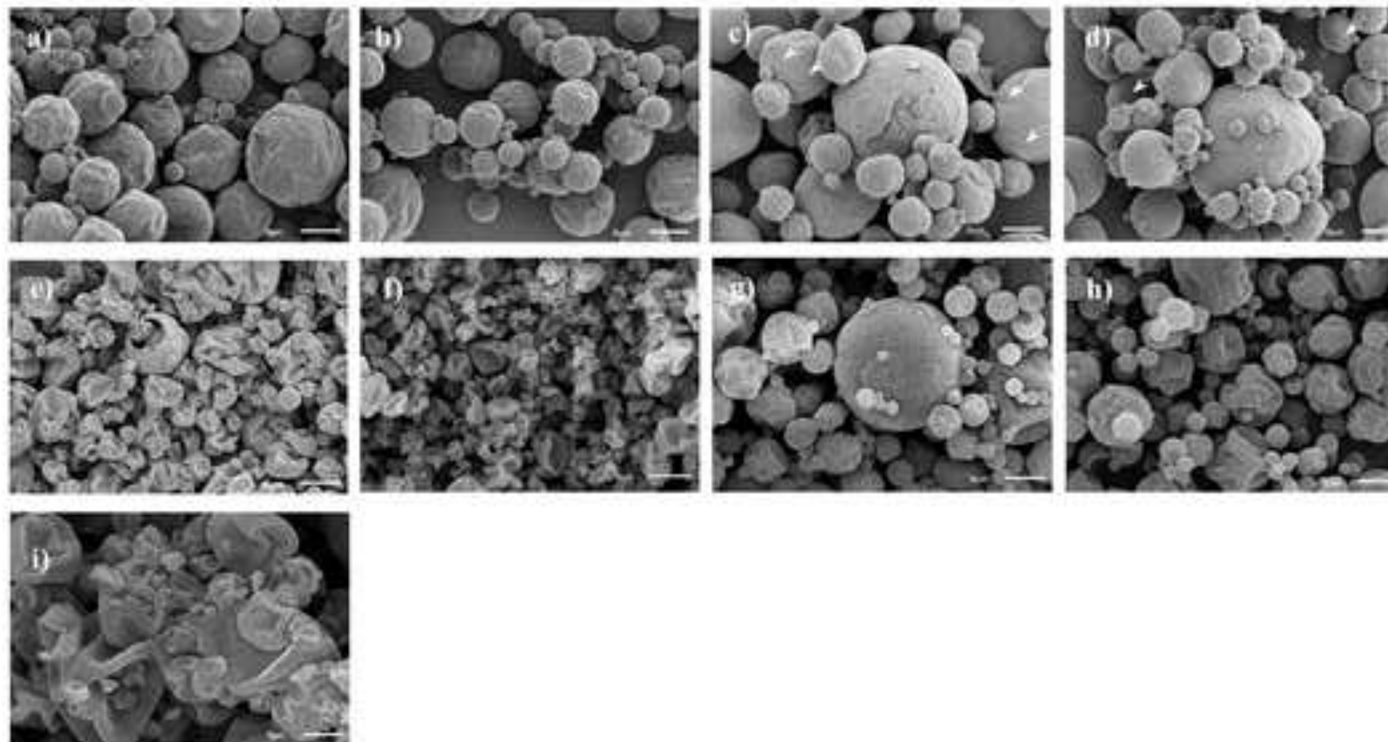

Figure

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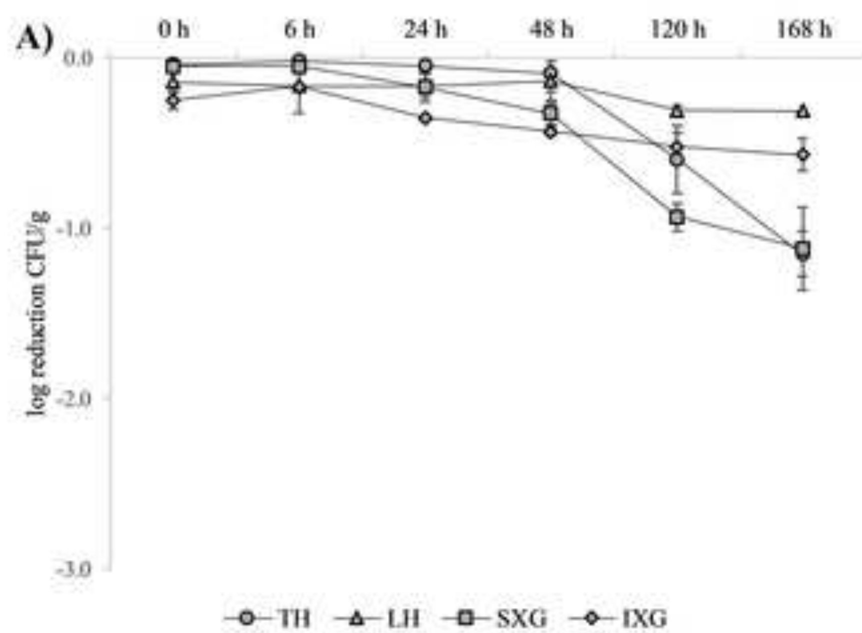

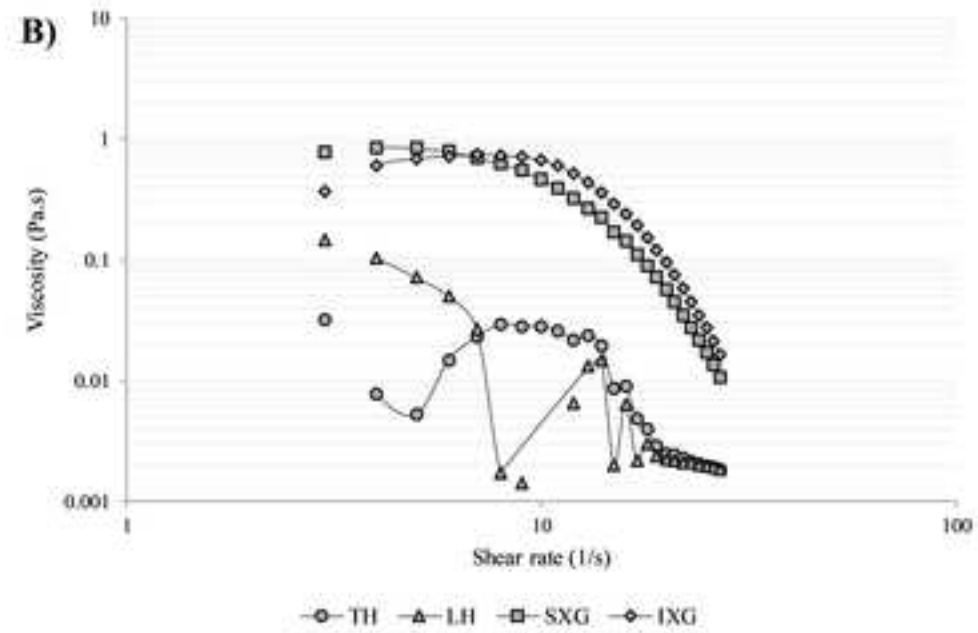

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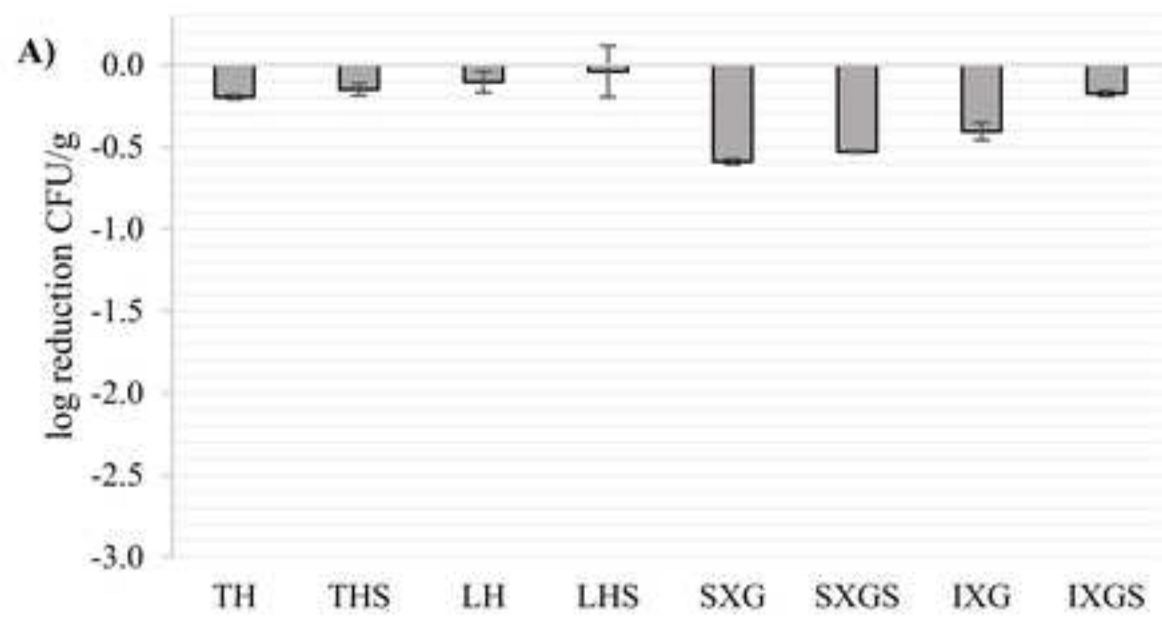
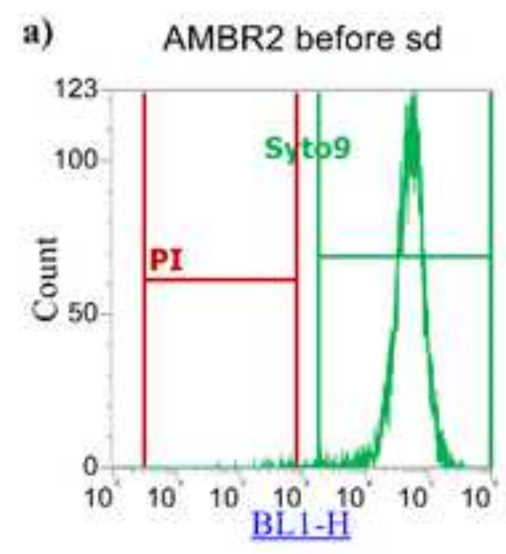
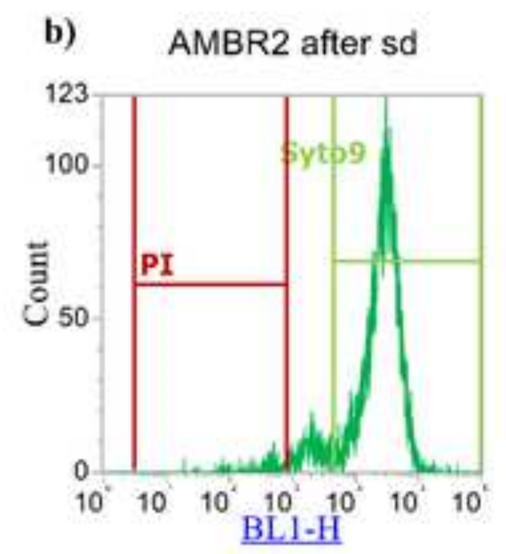
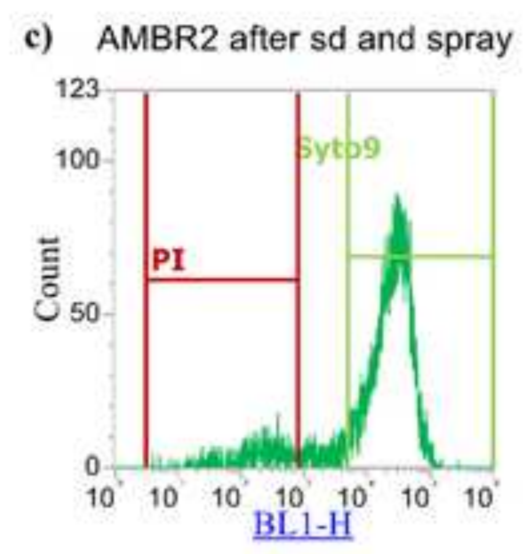


B)







Figure

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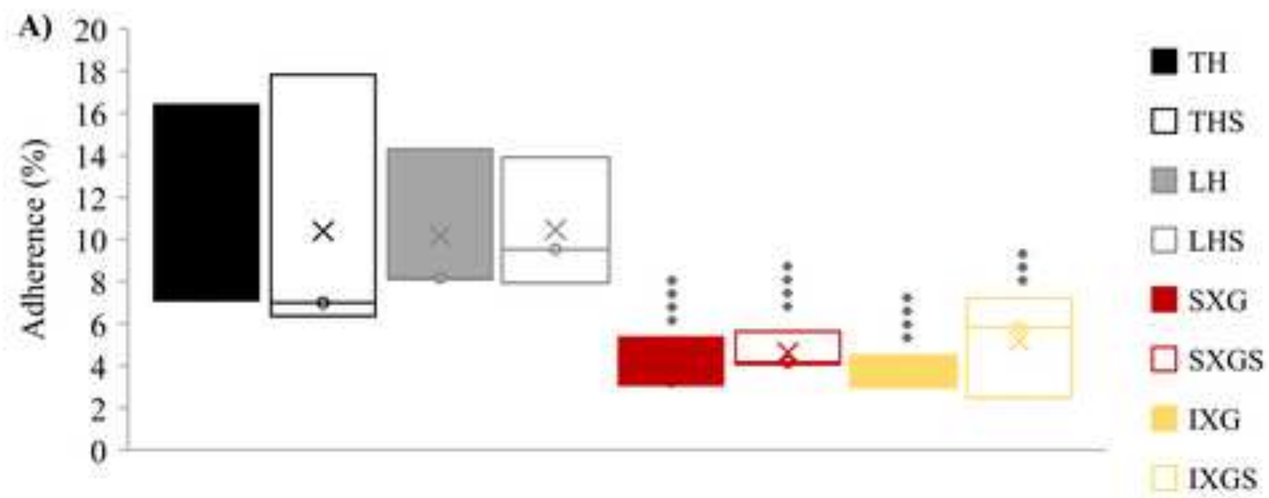

B)
Zones of inhibition (mm)
9
8
7
6
5
4
3
2
1
0
FRESH
TH
THS
LH
LHS
SXG
SXGS
IXG
IXGS
S. aureus
M. catarrhalis
H. influenzae

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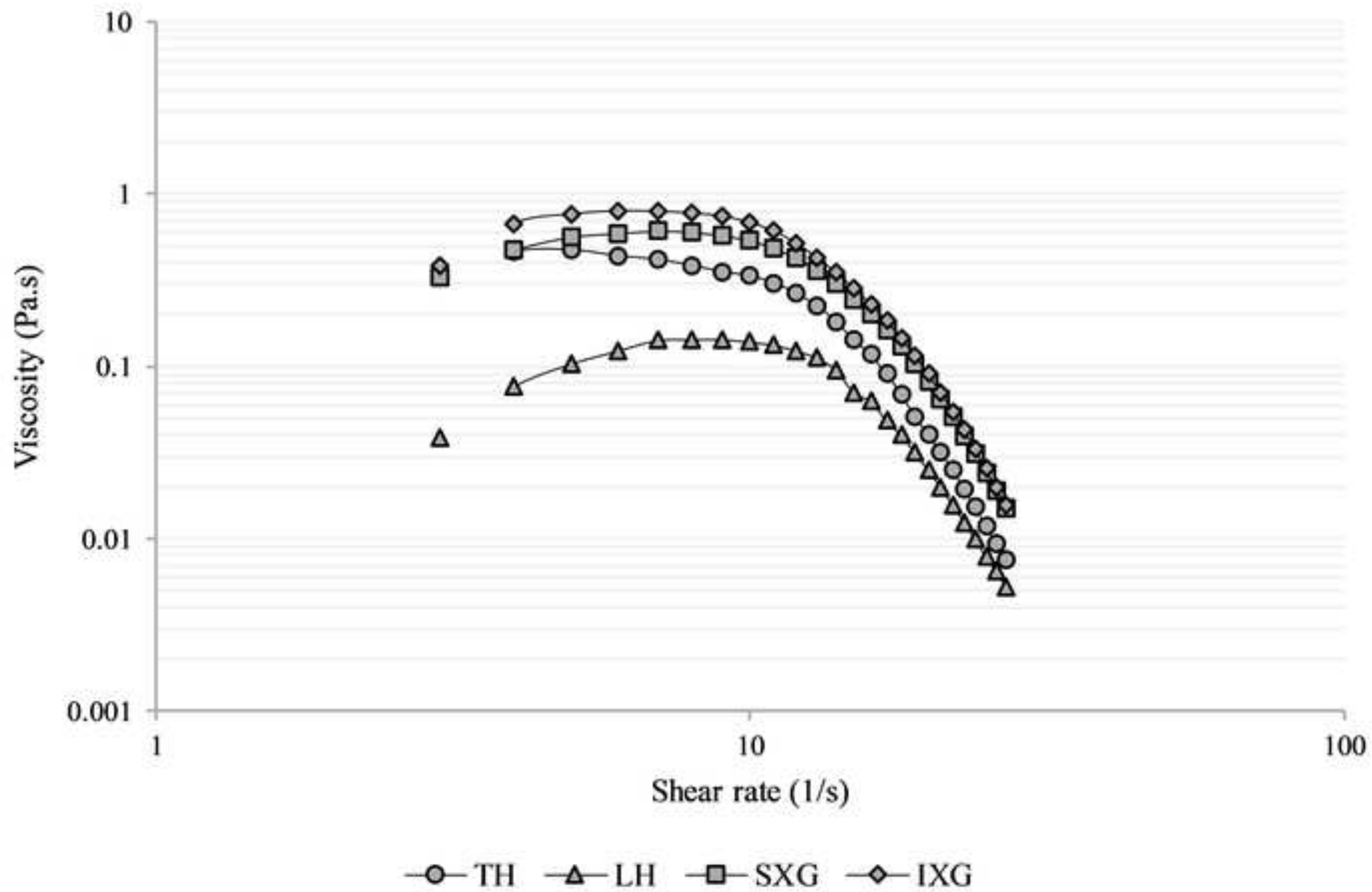

Table 2

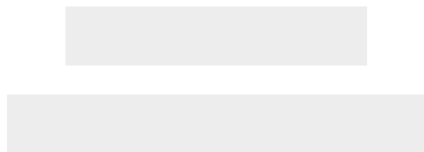

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Table
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