Microbial exopolysaccharides as postbiotics for the development of new functional foods

Giulia Bisson (bisson.giulia@spes.uniud.it)

Dept. of Agriculture Food Environmental and Animal Science, University of Udine, Italy

Tutor: Prof. Marilena Marino

This PhD project aims to isolate, produce, and characterize new exopolysaccharides (EPS) of microbial origin. The EPS will be chemically characterized, and bioactivity and technological properties will be evaluated. Finally, EPS will be added to a food product and the effect on the gut microbiota will be evaluated.

Esopolisaccaridi di origine microbica per lo sviluppo di nuovi alimenti funzionali

Lo scopo di questo progetto di ricerca è quello di isolare, produrre e caratterizzare nuovi esopolisaccaridi (EPS) di origine microbica. I polisaccaridi verranno caratterizzati da un punto di vista strutturale, tecnologico e in termini di bioattività. Infine, gli EPS verranno utilizzati nella formulazione di un prodotto alimentare e verrà valutato il loro effetto sul microbiota.

Keywords: Exopolysaccharides, lactic acid bacteria, chemical characterization, technological properties, bioactivities.

# **1. Introduction**

Following the PhD thesis project previously described (Bisson, 2021), this oral communication reports the main results of the following activities:

A1) Determining the chemical and morphological features of one EPS (EPS\_O) produced by *Leuconostoc mesenteroides* strain Lm\_O through NMR, FT-IR, and Scanning Electron Microscopy (SEM)

A2) Assessing the biological activities of EPS\_O (antimicrobial, antibiofilm, and antioxidant)

A3) Assessing the technological properties of EPS\_O (water holding capacity (WHC), oil holding capacity (OHC), solubility, rheology, …)

# **2. State of the art**

In recent years, there has been increasing interest in microbial EPSs due to their technological properties and potential health benefits. EPS are large polymers produced during fermentation by many microorganisms, including lactic acid bacteria (LAB) (Yang et al., 2023). They can be classified into homopolysaccharides (HoPS), composed of a single type of sugar, or heteropolysaccharides (HePS), consisting of repeated units of different sugars (Yildiz and Karatas, 2018). *Leuconostoc* spp. is a LAB genus known for its ability to produce HoPSs, such as α-glucans and β-fructans (e.g., dextran and levan) (Bisson et al., 2023; Taylan et al., 2019). Dextran is a versatile EPS made up of α-(1,6) linked glucose units with varying degrees of branching. This polymer is commonly used in the medical and food industries due to its water retention capacity, solubility, and unique rheological properties (Díaz-Montes, 2021). Moreover, dextran has shown to possess also interesting bioactivities (prebiotic, anti-inflammatory,…) (Zarour et al., 2017). *Leuconostoc* strains produce unique dextran with specific characteristics (Mw, degree of branching, …) which determines both the technological and biological properties. For this reason, the discovery of new EPSs with peculiar structures and bioactivities represents an interesting research field.

# **3. Materials and Methods**

## **3.1 EPS production**

*Leuc. mesenteroides* strain Lm\_O was isolated from Italian semi-hard cheese and identified by partial 16S rRNA gene amplification. The ability to produce EPS was qualitatively assessed on Mayeux Sandine ed Elliker (MSE) agar. EPS (named from here on EPS\_O) was produced in MRS-S broth (glucose replaced by sucrose 20 g/L) inoculated at 1% (v/v) with an overnight culture and incubated at 25 °C for 48 h in aerobic conditions. EPS\_O was then recovered following the method of Wang et al. (2019). After freeze-drying, EPS\_O yield was determined by gravimetry.

## **3.2 Structural characterization**

The UV-Vis spectrum of EPS\_O was acquired by Varian Cary 50 spectrophotometer (Agilent Technologies, Santa Clara, CA). Functional groups were analysed using an Alpha-P(ATR)-FTIR spectroscope (Bruker Optics, Milan, Italy) (4 cm-1 32 scans). EPS\_O was hydrolysed and monosaccharide composition was obtained using a 1260 Infinity HPLC system (Agilent Technologies) equipped with a quaternary pump autosampler, refractive index detector, and Ultra Amino column (250 mm, 4.6 mm internal diameter, 5 μm) (Restek S.r.l., Cernusco sul Naviglio, Italy). The mobile phase was an acetonitrile/MilliQ water mixture (70:30, v/v), and the flow rate was 1.0 mL/min. The NMR spectra were acquired using a Bruker Avance III 400 MHz digital NMR spectrometer (Bruker, Karlsruhe, Germany). NMR DOSY experiments were used to estimate the Mw of the EPS building a calibration curve using dextran standards (Mw 1.16×103 - 6.68×105 g/mol). The surface morphology was observed by an EVO 40 scanning electron microscope (Carl Zeiss, Jena, Germany) (SEM) equipped with energy dispersive x-ray spectroscopy (EDXS).

## **3.3 Antimicrobial and antibiofilm activity**

Antimicrobial activity was tested against *Staphylococcus aureus* DSA 226, *Salmonella enterica* spp. *arizonae* DSMZ 9386, *Escherichia coli* DSA 8048, *Listeria monocytogenes* Scott A, *Listeria monocytogenes* APC 154, *Listeria monocytogenes* 1325, and *Enterococcus faecium* DSMZ 2146 following the method described by Bisson et al. (2023). Antibiofilm activity was measured following the protocol by Stepanović et al., (2000) against *Listeria monocytogenes* 284 and *Pseudomonas fluorescens* DSA L22.

## **3.4 Antioxidant activity**

The antioxidant activity of EPS\_O (0.5, 1, 2, 4 mg/mL) was assessed using the 2-2-diphenyl 1-pycrilhaydrazyl (DPPH) assay (Li et al., 2022) and the 2,2’-azino-bis (3-ethylbenzothialzoline-6-sulfonic acid) (ABTS) assay (Re et al., 1999). Ascorbic acid was used as positive control.

## **3.5 Technological properties**

The Water Solubility Index (WSI) of EPS\_O (5% w/w) was determined following the method of Saravanan et al. (2016). WHC and OHC were assessed following the method of Gan et al., (2020) resuspending 50 mg of EPS\_O in 1 mL of water and oil, respectively. The thermal properties were evaluated using a TA4000 differential scanning calorimeter connected to GraphWare software TAT72.2/5 (Mettler-Toledo, Greifensee, Switzerland). The sample (2.5 ± 0.1 mg) was subjected to a heating ramp of 5 °C/min from 25 °C to 90 °C under a nitrogen flow maintaining the temperature for 1 min before cooling to 25 °C and finally heating from 25 °C to 120 °C. The glass transition temperature (Tg), the onset temperature (To), peak temperature (Tp), end-set temperature (Te), and transition enthalpy (ΔH) were determined using the software STARe ver.8.10 (Mettler-Toledo).

EPS\_O was dispersed in water at 5% (w/w) and its viscoelastic properties were evaluated using a Haake RheoStress 6000 controlled stress rheometer (Thermo Scientific) equipped with a Peltier system for temperature control and parallel plate geometry (35 mm diameter, 1.0 mm gap). The flow behavior was measured by recording shear stress values when shearing the samples at an increasing shear rate from 0.01 to 100 s-1. The linear viscoelastic region was detected by stress sweep tests performed from 0.1 to 100 Pa at a constant frequency of 1 Hz and 20 °C. A frequency sweep test was carried out at 20 °C between 0.1 and 10 Hz using a stress value included in the linear viscoelastic region. Storage (G’) and loss (G”) moduli were obtained.

## **3.6 Statistical analysis**

## All trials were carried out at least in duplicate. Values are reported as the means ± SD. Analysis of variance was performed to evaluate the significance of differences among means (*p*<0.05) by using R v.3.0.2 for Windows (The R foundation for statistical computing, Wien, Austria).

## **4. Results and Discussion**

## **4.1 Strain identification and EPS yield**

*Leuc. mesenteroides* strain Lm\_O formed mucous and slimy colonies on MSE agar plates. The yield of EPS\_O in MRS-S broth was 3.84 g/L.

## **4.2 Chemical characterization**

The UV-Vis spectrum didn’t show the presence of peaks at 260 and 280 nm, indicating the absence of contaminants (proteins or nucleic acids). Monosaccharide composition analysis evidenced that EPS\_O was constituted only of glucose units. FT-IR spectrum evidenced different peaks in the region between 3400 and 500 cm-1 (Fig 1a). The peak at 3000, 2000, 1600, 1640, 1149, and 1103 cm-1 corresponded to the stretching vibrations of O-H, C-H, water, C-O-C, and C-O groups respectively. The peak at 1000 cm-1 is due to the chain flexibility around the α (1→6) bond. The 1H NMR spectrum, in the anomeric region, presented two signals at 4.9 and 5.3 ppm, corresponding to the branching D-glucoside residues α (1→3) and α (1→6) respectively (Fig 1b). The HSQC evidenced the α anomeric nature of the principal signals of EPS\_O due to the presence of a cross peak at δH-1/δC-1 = 4.9/97.7 ppm. 13C – DEPTQ and HSQC spectra allowed the identification of the main resonance at 65.5 ppm (δH-6,6′ = 3.69, 3.92) as the C-6 carbon, and the 13C chemical shift demonstrated that the position is involved in the glycosidic linkage. The remaining signals in the range 69.5–73.4 ppm were assigned by homonuclear COSY and heteronuclear HSQC to the 1H and 13C bulk region, in agreement with previous data (Llamas-Arriba et al., 2019). The integration of 1H anomeric signals highlighted that EPS\_O has <5% of α (1→3) branching. The Mw of EPS\_O was estimated with DOSY, and it was estimated > 6.68×105 g/mol.

SEM images (Fig 1c) revealed that EPS\_O has a compact structure. This tool is useful for understanding the physical properties of macromolecules (Insulkar et al., 2018). This result differs from others reported in the literature for dextran produced by other *Leuconostoc* species, indicating the heterogeneity of dextran-type EPS that this genus can synthesize (Zhao et al., 2019; Zhou et al., 2018).

**Figure 1** *a) FT-IR spectrum, b) 1H-NMR spectrum, and c) SEM images at different magnifications (up to 2000x) of EPS\_O*

c)

b)

Immagine che contiene testo, diagramma, Diagramma, linea

Descrizione generata automaticamente****

a)

## **4.3 Antimicrobial and antibiofilm activity**

The effect of different concentrations of EPS\_O on the growth kinetic parameters of specific microbial foodborne targets is reported in Table 1. EPS\_O modified the growth kinetics parameters in different ways, depending on the pathogen tested. A statistically significant elongation of the lag phase was observed for *Salm. enterica* and *E. coli* in the presence of EPS\_O, and the maximum growth rate was also reduced for *Salm. enterica*, *E. coli,* and *L. monocytogenes* at both the concentration tested. Indeed, the main growth parameter affected by the presence of EPS\_O was the amplitude (i.e., the difference between the initial and the final OD). A statistically significant decrease in the amplitude was observed for almost all the pathogens tested (except for *E. coli*). The mechanisms underlying the antimicrobial effect of EPS are not clearly understood yet. It has been hypothesized that EPS could interfere with the cell membrane leading to its destruction, as well as cell division inhibition and DNA degradation could be responsible for the antimicrobial activity (He et al., 2010). Another speculation about the antimicrobial activity of dextran could be that due to its high Mw, the polymer could envelop the cells leading to an accumulation of secondary metabolites in the media, affecting the viability of the cells (Salachna et al., 2018). These results could open the door to the application of EPS as a new antimicrobial substance, whereas other studies are needed for understanding the mechanisms involved.

**Table 1** *Effect of EPS\_O on growth kinetic parameters of foodborne pathogens. For the same parameter and the same microorganism, asterisks indicate a significant difference (p< 0.05) with respect to the control*

## Immagine che contiene testo, numero, menu, Carattere Descrizione generata automaticamente

EPS\_O inhibited the biofilm formation by *L. monocytogenes* 284 (Fig 2), whereas no effect was observed against *P. fluorescens* L22 (data not shown). EPS\_O slowed down the formation of *L. monocytogenes* biofilm also at the lowest concentration tested (5 mg/mL). These data hold significance since the presence of *L. monocytogenes* biofilm is a relevant issue in the food industry due to the possible cross-contamination. Indeed, it is well known that listeriosis exhibits a high fatality rate. On the other hand, EPS\_O didn’t inhibit the biofilm formation of *P. fluorescens*, probably due to the different nature of the biofilm (Marino et al., 2018).

**Figure 2** *Biofilm formation (OD570) by L. monocytogenes 284 in the presence of EPS\_O*



## **4.4 Antioxidant activity**

The antioxidant activity was tested using two biochemical assays (DPPH and ABTS). In all cases ascorbic acid showed a stronger antioxidant activity. EPS\_O demonstrated a good DPPH radical scavenging ability which increased as the concentration increased reaching a maximum of 71.33±3.73 % at the highest concentration tested (4 mg/mL) (Fig 3a). On the other hand, EPS\_O demonstrated to exert a lower ABTS radical scavenging activity which reached the highest score (24.52±0.001 %) when EPS\_O concentrations was 2 mg/mL (Fig 3b). These results could be of interest since other studies on the antioxidant activity of dextran reported lower radical scavenging activity (Bisson et al., 2023). These different results could be attributed to the different Mw of the polymers, since it has been previously reported that the Mw of dextran strongly influenced its antioxidant properties (Soeiro et al., 2016).

**Figure 3** *Scavenging a) DPPH, b) ABTS activity of EPS\_O*



## **4.5 Technological properties**

The water solubility index (WSI), WHC, and OHC are important properties to be evaluated in considering the application of microbial EPS in food formulations. The solubility of EPS in water is strictly related to the length and degree of branching of the polymer and affects its dispersion and hydration. A high WHC and OHC are instead related to the ability of the molecule to hold water or oil influencing the technological performance of EPS and different food properties such as the retention of flavours and palatability. The WSI, WHC, and OHC of the EPS\_O from *Leuc. mesenteroides* Lm\_O were 99%, 784%, and 496%, respectively. Results confirmed the highly hydrophilic nature of the polysaccharide. Moreover, the polymer was extremely capable to retain water in its molecular structure, probably impacting its functional properties (emulsification, viscosity, gelation, …). The oil absorption capacity of EPS\_O also makes it suitable for foods where the retention of flavour is desired (e.g., bakery products). The thermal stability of EPS\_O was analyzed by DSC and results revealed that the melting peak was around 147 °C and the Tg at 83 °C, indicating the possible use of this polymer in foods where the use of high temperatures is required.

The potential application of EPSs depends also on their rheological properties. In this study, EPS\_O (5% w/w) showed a pseudoplastic behavior, as the viscosity decreased with the increase of the shear stress (Fig 4a). G’ was always higher than G’’ and frequency-dependent in the considered range indicating a weak gel behavior (Fig 4b).  All these characteristics could highlight the potential use of EPS\_O as a promising ingredient with specific technological properties for food applications.

**Figure 4** *Viscosity-shear rate profile (a) and rheological properties G’ and G’’ (b) of EPS\_O solution (5% w/w)*



# **5. Conclusions and Future Perspectives**

*Leuc. mesenteroides* Lm\_O produced a high Mw dextran that proved to exert exploitable bioactivities versus bacterial foodborne pathogens. EPS\_O inhibited the growth and the biofilm formation of some of the microbial targets, suggesting the possible application of this polymer as a new antimicrobial agent. Moreover, the results on the antioxidant activity of EPS\_O are encouraging, since a good DPPH radical scavenging activity was observed, suggesting its possible use as antioxidant in food products. EPSs are also known for their physicochemical attributes, improving the texture and palatability of foods. For this reason, is important to screen EPS with novel properties which can make these molecules of commercial value. The EPS analyzed in this study showed high WSI, WHC, and OHC, which make it a good candidate as texture improving agent in foods. Moreover, the dextran showed a non-Newtonian pseudoplastic behavior and it was able to form viscous solutions suggesting its possible use as a thickening and gelling agent.

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