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**Beyond Live Probiotics: Biochemical and Functional Profiling of
Postbiotics from *Limosilactobacillus reuteri* DSM 17938 and
Bifidobacterium longum subsp. *longum* BG-L47®**

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“G. d’Annunzio” University of Chieti-Pescara

Department of Innovative Technologies in Clinical
Medicine and Dentistry
XXXVIII PhD cycle

**Beyond Live Probiotics: Biochemical and Functional
Profiling of Postbiotics from *Limosilactobacillus reuteri*
DSM 17938 and *Bifidobacterium longum* subsp. *longum*
BG-L47[®]**

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Background

The term “gut microbiota” refers to the collection of microorganisms that inhabit the gastrointestinal tract of humans and animals. This complex and dynamic community is essential for human health since it contributes to nutrient metabolism, immune modulation and several physiological functions, influencing different human organs even distant each other. Certain naturally occurring members of this ecosystem are capable of modulating the gut microbiota and also contributing to the immune modulation, thus helping in the maintenance of homeostasis, better referred to as eubiosis. These beneficial microbes can be used as probiotics, that are widely known as beneficial microorganisms providing health benefits when administered in adequate amount and have been extensively studied over the years to better understand the molecular basis of their success.

For this reason, a complementary area of study is the one about postbiotics, that, as the word itself suggests, are all the metabolites, compounds and cellular structures released by the bacteria in the culture media/environment. Postbiotic products include the effectors of the bacterial cross-talk as well as the interactions between bacteria and host cells. Their composition varies depending on the available substrate and growth conditions; thus, their complexity make them a challenging feature to be analysed. Among them, membrane vesicles, spherical bilayer structures, have gained particular interest given their promising potential in human health.

The studies reported in this thesis aim to characterize one type of postbiotics, namely cell free supernatant and extracellular membrane vesicles, of *Limosilactobacillus reuteri* DSM 17938 and *Bifidobacterium longum* subsp. *longum* BG-L47[®]. The former is a widely commercialized strain with proven efficacy in ameliorating gastrointestinal diseases, especially in infants; the latter is a new probiotic strain, not yet on the market. The characterization in terms of composition and bioactivity provides new evidence to clarify the mechanisms behind both the postbiotics and probiotics activity. Furthermore, these findings allow to design future experimental set up to evaluate clinical applications of postbiotics.

List of publications

Paper I

Vitale I., Spano M., Puca V., Carradori S., Cesa S., **Marinacci B.**, Sisto F., Roos S., Grompone G., Grande R. (2023) Antibiofilm Activity and NMR-Based Metabolomic Characterization of Cell-Free Supernatant of *Limosilactobacillus reuteri* DSM 17938. Front Microbiol 14. <https://doi.org/10.3389/FMICB.2023.1128275>

Paper II

Marinacci B.[†], D'Ambrosio C.[†], Vitale I., Di Sotto A., Cairone F., Spano M., Carradori S., Scaloni A., Gullì M., Puca V., Francati S., Matuozzo M., Ermann Lundberg L., Grompone G., Roos S., Grande R. (2025) Biochemical and Functional Properties of Vesicles From Planktonic and Biofilm Phenotypes of *Limosilactobacillus reuteri* DSM 17938. Sci Rep 15:18889. <https://doi.org/10.1038/s41598-025-03823-w>

Paper III

Marinacci B. et al. *Bifidobacterium longum* BG-L47[®]: *in vitro* Antimicrobial Activity of the Cell-Free Supernatant and *in vitro* and *in vivo* evaluation of the Immunomodulatory Effect of the Membrane Vesicles (Manuscript in progress)

1. Probiotics

1.1 History and definition

Since ancient times, fermentation has been known as a successful method of preserving foods, even if the key microscopic players of this process were not known. Evidence of the consumption of fermented products traces back to 7000 BC, in China, where, presumably, one of the first fermented beverages was produced. 3500 years BC, the Egyptians used to drink “Laban Rayad” and “Laban Khed”, two fermented milks that are still offered today (Ozen and Dinleyici, 2015). Other historical sources documented that even Sumerians and other populations of east Asia participated in spreading the tradition of fermented foods around Europe (Vasiljevic and Shah, 2008) culminating with Plinio, the Roman historian who, in AD 76, exhorted to eat fermented dairy products as a remedy for gastrointestinal diseases (Brandelli, 2022). Therefore, over time people have started to appreciate the health benefits of food fermentation but only at the beginning of the 20th century scientists began to truly investigate its microbiological aspects. In 1890, Ernst Moro, an Australian physician, isolated *Lactobacillus acidophilus* for the first time. Nine years later, the French paediatrician Henry Tissier (Pasteur Institute) observed the dominance of bifidobacteria in the gut microbiota of breastfed newborns becoming the first to describe the genus *Bifidobacterium*, highlighting its clinical benefit in the treatment of infant diarrhoea (ISAPP board of directors, 2022). Subsequently, thanks to Elie Metchnikoff, another scientist from the Pasteur Institute, the concept of “probiotics” was postulated. His thesis referred to the idea of the “intestinal auto-intoxication”, which is driven by the proteolytic metabolism of microbes colonizing the human gut and is correlated to aging. Metchnikoff suggested that the production of toxic compounds could be counteracted by replacing harmful microorganisms with beneficial ones. For this purpose, he proposed the consumption of sour milk containing the “Bulgarian Bacillus”, currently known as *Lactobacillus delbrueckii* subsp. *bulgaricus* (World Gastroenterology Organization, 2023). Meanwhile, in 1917 in Germany, Professor Alfred Nissle isolated the first non-LAB probiotic, namely *Escherichia coli* strain Nissle 1917, from the faeces of a soldier not affected by enterocolitis during World War I (Ozen and Dinleyici, 2015). As the exploration proceeded, in 1935 the first fermented probiotic milk beverage was

commercialized by Yakult as a result of the discovery of the Japanese microbiologist Minoru Shirota who isolated and characterized the *Lacticaseibacillus paracasei* subsp. *paracasei* strain *Shirota* (World Gastroenterology Organization, 2023).

According to the available information, the word “probiotic” was used for the first time in 1953 by the German bacteriologist Werner Kollath who described the use of organic and inorganic supplement to heal malnourished patients. Over the years, many definitions have been proposed; one of the latest was suggested by the English Professor Roy Fuller who stated that probiotics are “live microbial feed supplements which beneficially affect the host animal by improving its intestinal microbial balance” (ISAPP board of directors, 2022). In 2001, experts from the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) described “probiotics” as “live microorganisms which when administered in adequate amounts confer a health benefit on the host”. This definition has been accepted worldwide and confirmed by the expert consensus document of the International Scientific Association for Probiotics and Prebiotics (ISAPP) which, in 2014, clarified the proper application and range of the term “probiotic” (Hill et al., 2014).

1.2 Characteristics and selection criteria

The FAO/WHO definition clarifies that the health benefit is a mandatory characteristic of a probiotic, to be defined as such. Therefore, it is necessary to underline that the association of the word “probiotics” and “fermented food” is only eligible when the live microorganisms inside the product have proven positive effects on the host. Otherwise, these two terms have to be used independently (Brandelli, 2022). Considering that each probiotic strain has specific properties, the concept of “core” benefits has been introduced by the ISAPP experts. Among the features shared by most probiotics there are the capability to support the gut microbiota, improve the function of the digestive tract and sustain the immune system as well as the potential to competitively exclude pathogens (Hill et al., 2014). Beyond these, there are additional species- and strain-specific effects validating the recommendation of a particular strain for each different clinical issue (Hill et al., 2014; Sanders et al., 2018). The beneficial effects of a probiotic have to be proved at least by one clinical trial, preferably following certain experimental

criteria that allow to define the correlation between the probiotic and the outcome, the specificity of the association and the dose-related effect (Hill et al., 2014; Binda et al., 2020). Generally, the recommended dose corresponds to $10^{10} - 10^9$ Colony Forming Units (CFU) per day, even though some probiotics strain could be effective at lower dosage (10^8 CFU/day) (Brandelli, 2022).

Selecting a new probiotic is a multi-step process which necessarily includes complying some required safety criteria (**Table 1**). One of the most relevant elements is the absence of virulence factors and/or acquired antimicrobial resistance genes that, if detected, cannot be located within transposable elements within the genome or on plasmids. In particular, probiotic strains must be qualified as “Generally Recognized as Safe” (GRAS) by the United States Food and Drug Administration (FDA) and, at the species level, have to be included in the “Qualified Presumption of Safety” (QPS) list of the European Food Safety Authority (EFSA). The GRAS and QPS certification guarantee a proper identification and a documented history of safe use (Binda et al., 2020). Additionally, new probiotics should have several functional and technological characteristics such as resistance to low pH and bile salts – thus the ability to survive in the gastrointestinal tract – genetic stability, good viability after the downstream processes and appropriate survival rate in the final product (Markowiak and Śliżewska, 2017).

Selection criteria	Required properties
Safety	• History of safe use • Precise diagnostic identification (phenotype and genotype traits) • Absence of data regarding an association with infective disease • No adverse effects • Absence of genes responsible for antibiotic resistance localised in non-stable elements
Functionality	• Competitiveness with respect to the microbiota inhabiting the intestinal ecosystem • Ability to survive and maintain the metabolic activity, and to grow in the target site • Resistance to bile salts and enzymes • Resistance to low pH in the stomach • Competitiveness with respect to microbial species inhabiting the intestinal ecosystem (including closely related species) • Antagonistic activity towards pathogens • Resistance to bacteriocins and acids produced by the endogenic intestinal microbiota • Adherence and ability to colonise some particular sites within the host organism, and an appropriate survival rate in the gastrointestinal system
Technological usability	• Easy production of high biomass amounts and high productivity of cultures • Viability and stability of the desired properties of probiotic bacteria during the fixing process (freezing, freeze-drying), preparation, and distribution of probiotic products • high storage survival rate in finished products (in aerobic and micro-aerophilic conditions) • Guarantee of desired sensory properties of finished products (food industry) • Genetic stability • Resistance to bacteriophages

Table 1. Selection criteria of probiotic strains. Adapted from (Markowiak and Śliżewska, 2017) under the Creative Commons Attribution License (CC BY)

1.3 Lactobacilli

The term lactobacilli refers to a group of bacteria that once included 261 species of Gram-positive, facultatively anaerobic and non-spore-forming microorganisms. Their name is closely related to one of their essential characteristics which is the production of lactic acid after carbohydrate fermentation. However, other end-products can be released, such as ethanol, acetate and carbon dioxide, due to the existence of both homofermentative and heterofermentative lactobacilli.

These bacteria are pervasive as they are strongly adapted to nutrient-rich environments, in fact they can be found in humans but also in animals, plants, soil and fermented foods (Mejía-Caballero and Marco, 2025). However, down to the strain level, specialist or generalist lactobacilli can be identified considering the niche breadth, adaptability and distribution (Xu et al., 2022). In the human host, lactobacilli are subdominant members of the gut microbiota during infancy, while, in adulthood, they represent only a minor component (0.01%) of faecal bacteria. Despite what was previously thought, lactobacilli are not part of the intestinal core microbiome of adults and most of them may be considered only transient members within the human gut, where they can be found due to the widespread consumption of fermented foods. On the other hand, the vaginal tract represents a body district with a high prevalence (>70%) of lactobacilli, constituting the 50% of the total bacterial population. A healthy vaginal microbiota is typically dominated by *Lactobacillus crispatus*, *Lactobacillus gasseri* and *Lactobacillus jensenii* that, thanks to the production of lactic acid, can counteract the growth and adhesion of pathogens. Additional anatomical sites where lactobacilli are distributed are oral cavity (*L. casei*, *L. fermentum*, *L. gasseri*, *L. plantarum*, *L. rhamnosus* and *L. salivarius*), respiratory tract (*L. delbrueckii*, *L. iners*, *L. plantarum*, *L. rhamnosus* and *L. sakei*) and skin (*L. crispatus*, *L. gasseri*, *L. iners* and *L. jensenii*) (Mejía-Caballero and Marco, 2025).

The broad variety of habitats occupied by the former genus *Lactobacillus* directly correlates with their high genotypic and phenotypic diversity which encouraged Zheng

and colleagues to propose a reclassification in 2020 that led to the designation of 25 genera in total based on shared phylogenetic, ecological and metabolic properties (Zheng et al., 2020). Among the new genera, the *Limosilactobacillus* is the one of particular interest for this thesis.

1.3.1 *Limosilactobacillus reuteri*

Limosilactobacillus refers to the word “limosus” which means “slimy”, perfectly describing a defining property of this genus which comprises heterofermentative bacteria that can produce exopolysaccharides from sucrose metabolism. With the exception of some species that cannot ferment glucose, the majority of them ferment a wide variety of carbohydrates. Another key feature of this genus is acid resistance which is mediated by several enzymes such as urease, glutaminase, glutamate decarboxylase and arginine deiminase (Zheng et al., 2020). Undoubtedly, *Limosilactobacillus reuteri* is one of the most well-studied species of this genus as well as one of the most widely dispensed probiotics. It is named after the German microbiologist Gerhard Reuter who isolated this bacterium in 1962 from the small intestine of healthy patients (Lee et al., 2025). Recently, Li and colleagues conducted a phylogenomic analysis of *L. reuteri* concluding that rodents and birds are the ancestral hosts of this species because the lineages isolated from those vertebrates have maintained a long-term evolutionary relationship with them. According to the same authors lineages correlated to humans seemed to be lacking in adaptation traits and showed a more dynamic transmission (Li et al., 2023). However, several studies have defined *L. reuteri* as autochthonous to humans, as historical data showed that it was frequently detected in human gut and because it shared a symbiotic long-term relationship with the host (Walter, 2008; Oh et al., 2009; Walter et al., 2011).

1.3.2 Health-promoting properties of *Limosilactobacillus reuteri*

There is a wide range of health-promoting properties that can be addressed to *L. reuteri* but, as stated above, it has to be considered that probiotics have strain-specific effects. Regarding the disorders of the gastrointestinal tract (GIT), *L. reuteri* DSM 17938

is one of the world's most studied strains and is widely used to ameliorate the symptomatology of colicky infants. Authors proved that the probiotic administration reduced the daily crying time and was associated with increased levels of mRNA FoxP3 expression, which is a marker of regulatory T cells, thus resulting in an anti-inflammatory action. The positive modulation of CC-chemokine receptor 7, which is involved in the recruitment of immune cells, was also observed (Savino et al., 2018, 2019). Of note, one of the key mechanisms related to the amelioration of infantile colics associated with *L. reuteri* DSM 17938 is the region-specific modulation of gut motility – inhibitory effect in the jejunum and stimulatory effect in the colon – combined with the inhibition of the nociceptor TRPV1 which results in the reduction of visceral pain signalling (Wu et al., 2013; Perez-Burgos et al., 2015). Moreover, this probiotic strain is also effective in preserving intestinal epithelial integrity due to the upregulation of tight-junction proteins occurring alongside the reduction of pro-inflammatory cytokines levels (Karimi et al., 2018). Additional clinical trials, in 2014, demonstrated that *L. reuteri* DSM 17938 is also suitable for children and adults. The probiotic lowered the risk of contracting both diarrhoea and respiratory tract infection in children aged 6-36 months and reduced the frequency and duration of symptoms in subjects who were already diseased (Gutierrez-Castrellon et al., 2014). Regarding adults with chronic constipation, a 4 week supplementation of DSM 17938 helped in restoring a correct frequency of evacuations (Ojetti et al., 2014). As extensively reviewed by Abuqwider et al in 2022 (Abuqwider et al., 2022), *L. reuteri* DSM 17938 has also proven efficacy in alleviating liver failure reducing inflammation in the same organ. A preclinical investigation in a D-galactosamine-induced liver failure model demonstrated that pretreatment with this probiotic mitigated the injury in rats, reversing the upregulation of pro-inflammatory genes and secreted factors in the liver, while restoring the gut microbiota perturbation (Jiang et al., 2021).

When dysbiosis occurs, the establishment of an inflammatory environment can lead to the disruption of the intestinal mucosal barrier, potentially resulting in the spread of pathogens, the so-called “leaky gut” (Lee et al., 2025). Daily administration (for 14 days) of *L. reuteri* I5007 to newborn male piglets was effective in increasing the abundance of tight junction proteins such as claudin-1, occludin and zonula occluden-1. Similarly, *in vitro*, pretreatment with *L. reuteri* prevented the downregulation of the

aforementioned proteins in porcine intestinal epithelial cell lines challenged with LPS, supporting the hypothesis that it could have a protective role in prevention or treatment of tight junctions issues (Yang et al., 2015).

One of the most promising properties of probiotics is competitive exclusion of pathogens which directly implicate adhesion capacity. Singh and colleagues, in 2017, investigated this trait in eight probiotic *L. reuteri* strains isolated from infant faeces. They carried out *in vitro* experiments using Caco-2 cells and 4 pathogenic microorganisms, namely *Escherichia coli* ATCC 25922, *Salmonella typhi* NCDC113, *Listeria monocytogenes* ATCC53135 and *Enterococcus faecalis* NCDC115. They found out that all the probiotics adhered to cell monolayers, probably thanks to surface proteins, and most of them, with different degrees of efficiency, excluded *E. coli*. Regarding the other pathogens, an heterogeneous exclusion ability was observed, being some strains more effective than others (Singh et al., 2017). The colonization capacity has also been proved for another strain that is *L. reuteri* ATCC 55730 (mother strain of the aforementioned DSM 17938). In an open clinical investigation, 10 healthy volunteers and 9 volunteers with ileostomy were enrolled to receive 4×10^8 CFU/day for about 1 month. Biopsies taken from the gastric corpus or ileum were used to confirm the presence of *L. reuteri* by using fluorescence in situ hybridization (FISH). Additionally, immunohistochemical analysis revealed a significant increase of CD4-, CD20- and CD68-positive cells in different GI sections. Thus, the study not only confirmed the colonization capacity but also highlighted the *in situ* immunomodulatory effect of the strain (Valeur et al., 2004).

As extensively discussed by the scientific community, the interplay between the microbiota and human GIT is not limited to this body district but has an impact on the whole organism. Indeed, the gut microbiota is considered an “invisible organ” and its imbalance may lead to the dysregulation of the overall health (O’Hara and Shanahan, 2006; Li et al., 2020). Therefore, it is reasonable suppose that also probiotics may contribute to the broader picture. In this context, there has been an increasing interest in the so-called “Gut-Brain Axis”, another research field where *L. reuteri* has gained interest (Abuqwider et al., 2022). Interesting results were obtained in 2023, by Lu and colleagues in a pre-clinical study. It is known that acute or chronic inflammatory state of the mother – related to diseases or external factors – negatively impacts the neurodevelopment of children, therefore, they used maternal immune activation models, obtained by exposing

pregnant mice to LPS. *L. reuteri* ATCC PTA 6475 was supplemented at birth and throughout the entire lactation period resulting in rescued blood-brain barrier (BBB) deficits and improved spatial learning later in life. The proposed mechanism was the *L. reuteri*-mediated modulation of offspring's microbiota and metabolic profile (Lu et al., 2023). The same *L. reuteri* strain showed promising outcomes in a pilot double-blind randomized placebo-controlled trial involving children with autism spectrum disorder (ASD). Compared to placebo, the probiotic administration improved social functioning and adaptive social functioning of children while improving social behaviour in BTBR mice (idiopathic ASD mouse model) (Mazzone et al., 2024).

1.4 Bifidobacteria

The discovery of the first member of bifidobacteria dates back to 1899 when the French paediatrician Henry Tissier isolated an anaerobic bacterium from the faeces of a healthy breast-fed infant. This microorganism had a peculiar morphology, a bifurcated shape, therefore it was named *Bacillus bifidus* (Hidalgo-Cantabrana et al., 2017). The *Bifidobacterium* genus comprises Gram-positive, anaerobic, nonmotile, nonsporulating bacteria characterized by a high G+C genome content. To date, 82 species and 12 subspecies have been recognized (Duranti et al., 2020). Few years ago, a core-genome based analysis identified 10 phylogenetic groups that could be assigned to a specific ecological niche. *B. adolescentis*, *B. longum*, *B. pseudolongum* and *B. bifidum* are typically found in the GIT of humans, *B. pullorum*, *B. asteroides* and *B. tissieri* are present in the microbiota of birds, insects and tamarins, respectively, while the others are widely distributed among different animal species (Lugli et al., 2019). A peculiar trait of bifidobacteria is the consumption of monosaccharides via the fructose-6-phosphate pathway which produces acetate, lactate and ethanol (Hidalgo-Cantabrana et al., 2017). Several species have specific gene clusters dedicated to human milk oligosaccharides (HMOs) metabolism, thus they are essential for the breast milk digestion in infants (Bocchio et al., 2024). Unsurprisingly, bifidobacteria are the pioneer colonizers of infants: the primary source of bacteria is the microbiota of the vaginal mucosa and, subsequently, the intake of human milk sustains the permanence of these microorganisms. *B. bifidum*, *B. longum* subsp. *infantis* and *B. breve*, species specialized in HMOs utilization, were

found to be the predominant in infant gut (Hidalgo-Cantabrana et al., 2017; Turrone et al., 2018). After weaning, with the introduction of a more complex diet, there is a shift of the microbiota composition characterized by an increased complexity: the relative abundance of *Bifidobacterium* decreases while *Bacteroides* spp. increase as well as different genera belonging to the phylum Firmicutes (*Clostridia*, *Faecalibacterium*, *Ruminococcus*, *Veillonella*) (Martino et al., 2022). However, bifidobacteria do not entirely disappear. *B. adolescentis*, *B. catenulatum*, *B. longum* subsp. *longum* are typical species isolated from the adulthood microbiota and constitute approximately the 4% of the total microbial community (Turrone et al., 2018; Duranti et al., 2020). Of note, despite numerous studies have reported a significant reduction of bifidobacteria in elderly people as a common trait of ageing, other research has demonstrated a surprising increase of these bacteria in centenarians allowing to speculate a possible correlation between *Bifidobacterium* species and longevity (Ku et al., 2024).

1.4.1 *Bifidobacterium longum*

Bifidobacterium longum belongs to the group of mutualistic bacteria of the human gut microbiota and is among the species which have received the QPS certification, thus it is widely used as probiotic (Arbolea et al., 2016). The *B. longum* species is further differentiated in 4 subspecies, namely *Bifidobacterium longum* subsp. *longum*, *Bifidobacterium longum* subsp. *infantis*, *Bifidobacterium longum* subsp. *suis*, and *Bifidobacterium longum* subsp. *suillum*, all expressing a different pool of genes encoding for enzymes involved in carbohydrate metabolism (Xiao et al., 2025). Many studies have reported that *B. longum* is one of the first colonizers of infants' gut, acquired via vertical transmission, but it remains detectable in the GIT throughout adult life as well. Its survival within the human gut could be guaranteed by the production of exopolysaccharides (EPS) which seem to increase co-aggregation and, consequently, resistance to bile salts and low pH (Alp and Aslim, 2010). At the same time, pili and surface proteins allow the adherence to the intestinal mucosa where *B. longum* exerts a relevant immunoregulatory activity through extracellular solute-binding proteins and serpin. The latter is a serine protease inhibitor which can inactivate eukaryotic pro-inflammatory proteases, such as human neutrophil and pancreatic elastase, contributing to the preservation of gut homeostasis.

Moreover, colonization is driven by the enzymes involved in carbohydrates metabolism that are responsible for the *B. longum* adaptation ability to different niches and confer a competitive advantage over the other members of the gut microbiota (Ruiz et al., 2017; Xiao et al., 2025).

1.4.2 Health-promoting properties of *Bifidobacterium longum*

As described above for *Limosilactobacillus reuteri*, gut health is one of the major areas of application for *B. longum* probiotic strains. A common, uncomfortable condition that could be improved by *B. longum* administration is constipation. *B. longum* BB536 was tested on 24 constipated adults and resulted to be effective in increasing the bowel movement frequency, although differences in individual responses were observed. Authors speculated that variation between responders and non-responders may be attributed to the composition of the gut microbiota and, consequently, to the corresponding metabolome (Nakamura et al., 2022). Similar results were obtained in a murine model by Wang and colleagues who proved that the increased production of acetic acid in faeces and the reduction of aquaporin 8 expression in the colon are two of the mechanisms involved in the enhancement of intestinal motility and in the regulation of electrolytes dispersion. The same authors also correlated the success of *B. longum* supplementation with the positive influence that it had on the modulation of the gut microbiota and, in turn, on the production of beneficial metabolites such as acetate and propionate (Wang et al., 2022). An additional mechanism involved in constipation improvement is the upregulation of excitatory gastrointestinal peptides such as motilin and gastrin, combined with the downregulation of the inhibitory ones (e.g. vasoactive intestinal peptide) (Zhou et al., 2023).

A severe disorder of the GIT, characterized by persistent inflammation, is Inflammatory Bowel Disease (IBD). Affected patients suffer from abdominal pain, diarrhoea and fever, while blood can be detected in their faeces together with extraintestinal manifestations. Most importantly, there is a high risk of bowel and colorectal cancer occurrence. Two different conditions, classified under the term “IBD”, are Crohn’s disease and ulcerative colitis, both of which have shown a significant increase in industrialised countries over the past 30 years (Strayer and Rubin, 2015). The common

therapy includes immunosuppressive drugs and antibiotics, correlated to a plethora of adverse effects. Numerous studies have proved the promising potential of *B. longum* supplementation in IBD treatment or prevention. A mechanism that seems quite common among *B. longum* strains is related to the expression of *Sir2* genes that regulate the FOXO-dependent transcription resulting in the reduction of reactive oxygen species (ROS) (Guo et al., 2017; Huang et al., 2020). In addition, some strains can directly produce and release antioxidant enzymes such as NADH oxidase, NADH peroxidase, catalase and superoxide dismutase (Yao et al., 2021). Besides antioxidant activity, *in vitro* experiments have confirmed that *B. longum* may also reduce the levels of inflammatory cytokines (e.g. IL-8 and TNF- α) and transcription factors (e.g. NF- κ B) in intestinal epithelial cell models (e.g. HT-29 and Caco-2 cells) (MacPherson et al., 2017; Kim et al., 2018; In Kim et al., 2019); this findings were confirmed in *in vivo* models as well as human clinical trials. *B. longum* 5^{1A} treatment, concomitant to disease induction, significantly reduced IL-1 β levels in dextran sodium sulphate (DSS) mouse model, preserved intestinal permeability and decreased colitis clinical score (Abrantes et al., 2020). Several human *B. longum* isolates lowered TNF- α , IL-6, IL-1 β and Lipocalin-2 levels in DSS-induced colitis in mice and, overall, demonstrated to be able to ameliorate the disease outcome modulating multiple parameters (Singh et al., 2020). In clinical trials, ulcerative colitis patients treated with *B. longum* BB536 displayed a clinical remission and, similarly, *B. longum* ES1 reduced pro-inflammatory cytokines and improved the integrity of the intestinal barrier in IBS-D patients (Tamaki et al., 2016; Caviglia et al., 2020).

In 2016, *Bifidobacterium longum* 1714 was first described as a “psychobiotic” thanks to a repeated measures placebo-controlled study conducted on 22 healthy male volunteers. Using validated psychometric and neuropsychological tests, authors proved that probiotics intake reduced both physiological and psychological responses to acute stress, as well as self-reported stress, improving hippocampus-dependent visuospatial memory (Allen et al., 2016). Since then, many studies have confirmed the role of this bacterial species in the gut-brain axis. A probiotic product containing *Bifidobacterium bifidum* BGN4 and *Bifidobacterium longum* BORI was consumed by healthy elderly (>65 years) volunteers and the data obtained from cognitive tests, stool samples 16S rRNA sequencing and serum markers measurements revealed the beneficial effects on mental

well-being. Two mechanisms associated to this outcome were the modulation of microbiota composition and increased levels of brain-derived neurotrophic factor (BDNF), which is involved in learning, memory and stress (Kim et al., 2021). BDNF has also a pivotal role in Alzheimer's disease (AD) and its probiotics-mediate regulation may help restoring the cognitive function of patients (Hsu et al., 2023).

Early bifidobacterial colonization of infant gut is crucial for a correct immune maturation because these bacteria produce immunoregulatory metabolites (e.g. SCFAs) that shape lymphocytes' differentiation. In addition, a growing body of literature has proved that the *Bifidobacterium* peak during the first months of life protects against the further emergence of allergies (Donald and Finlay, 2023). As stated before, *B. longum* strains are among these pioneer bacteria but their immunomodulatory potential has been also demonstrated across various age groups. For example, Wu and colleagues demonstrated that the administration of *B. longum* BB536 to newborns regulated the IFN- γ /IL-4 secretion resulting in a positive modulation of Th1 cells' function while, in a previous study, the same strain exhibited a protective effect on natural killer cells in elderly patients (Akatsu et al., 2013; Wu et al., 2016). *B. longum* immunomodulatory effects can also be appreciated in non-intestinal diseases, such as allergies. In a four-week clinical trial, a mixture of *Bifidobacterium longum* IM55 and *Lactobacillus plantarum* IM76 was orally administered to patients with permanent allergic rhinitis and, at the end of the study, authors found that symptoms were significantly improved in treated group compared to placebo. Moreover, reduction of eosinophils count and allergen specific Ig-E, together with the increase of IL-10 levels, were detected (Kang et al., 2020). Recently, a promising application of a symbiotic mixture for skin barrier dysfunction has also been assessed. *B. longum* KAAC 91563, combined with galactooligosaccharides (GOS), improved the condition of skin barrier in atopic dermatitis murine model lowering the expression of pro-inflammatory Th2 cytokines and upregulating epidermal barrier proteins (Kim et al., 2022).

2. Postbiotics

2.1 Definition

The International Scientific Association of Probiotics and Prebiotics (ISAPP) published, in 2021, a consensus statement to clarify the term “postbiotic” and promote a responsible use of the term, not only in scientific publications but also on product labels. According to the panel of experts, a postbiotic is a “preparation of inanimate microorganisms and/or their components that confers a health benefit on the host”. The adjective “inanimate” highlights that microorganisms are no longer vital, but the remaining biomass has retained part of its function. The definition also includes the word “components” suggesting that structures, such as pili and cell wall fragments, derived from bacterial killing, are part of the preparation together with metabolites and byproducts released during the growth. However, the authors specified that purified metabolites or mixture that do not include cellular biomass cannot be considered postbiotic preparations (Salminen et al., 2021).

Despite the purpose of the ISAPP members was to present a definition that could have been widely accepted, many scientists have raised concerns about it and there is still disagreement among the scientific community. Aguilar-Toalá and colleagues have been the first to publish a commentary on the previously published consensus statement in which they explain the reasons of their different opinion and offer alternative perspectives. The core issue of the debate was that the newest definition did not align with the prevailing usage of the term in existing literature, the latter referring to “any factor resulting from the metabolic activity of a probiotic or any released molecule capable of conferring beneficial effects to the host in a direct or indirect way”. Considering the screening of published papers on the topic, they concluded that the definition should be revised given that “postbiotics are well-defined mixtures of (or single) molecules with demonstrated benefit for the host (which would encompass health benefits) and do not include inactivated cells” (Aguilar-Toalá et al., 2021). Currently, the debate is still open, since many scientific papers and reviews published after 2021 have reported different definitions of postbiotics that are often classified as metabolites released by probiotics

during their growth, thus including SCFAs, exopolysaccharides, enzymes, proteins, cell wall fragments but also cell free supernatant (CFS) and extracellular membrane vesicles (MVs) (González-Lozano et al., 2022; Thorakkattu et al., 2022; Liu et al., 2024). This latter concept will be the one adopted in this thesis.

Beyond the issue of terminology, a defining characteristic of postbiotics is the capability to promote host health, which have been extensively studied as a result of deepening the understanding of probiotics interaction with biological systems. Nonetheless, postbiotics are not only recommended for their beneficial properties, but also because they represent a safer alternative when the supplementation of live bacteria is not recommended, e.g. immunocompromised patients. From a practical perspective, postbiotics are more stable for pharmaceutical use and have also a better potential as functional ingredients in foods. Currently there is a lack of a universal regulation about postbiotic-containing products, and, considering that most postbiotics are an heterogeneous mix of components, it can be inferred that the bacteria, as well as the substrate and growing conditions, must be characterised in order to standardize the production (Thorakkattu et al., 2022; Meena et al., 2025). To date, postbiotics predominantly derive from probiotic strains belonging to lactobacilli and *Bifidobacterium* spp. (Brandelli, 2022)

2.2 Source and isolation of postbiotics

In humans, the primary source of bioactive components, potentially being part of a postbiotic, is the gut microbiota which naturally ferments dietary fibers and non-digestible compounds. The products of this metabolism are known to exert a protective effects against gastrointestinal as well as extra-intestinal diseases and, most importantly, are key effectors of the cross-talk between the bacterial community, thus participating in the maintenance of the eubiosis (Ozma et al., 2022; Maiuolo et al., 2024). The pool of metabolites and complex structures that bacteria release within the GIT is extremely complex and unique to each individual. On the contrary, under laboratory- and/or industrial-scale conditions the production of postbiotics can be performed from a specific bacterial strain and by using standardized protocols which guarantee the quality of the final product. Fermentation media, temperature, pH values and atmospheric conditions

are the key parameters that must be fixed. Then, depending on the requirements of the final product, extracellular metabolites and/or structures can be separated via centrifugation and filtration; on the other hand, if structural components of the cells must be saved, different inactivation processes (e.g. heat treatment) can be adopted to ensure the total bacterial killing (Hernández-Granados and Franco-Robles, 2020; Bueno et al., 2025).

2.2.2 Short Chain Fatty Acids

Short chain fatty acids (SCFAs) are produced by intestinal bacteria after the fermentation of non-digestible fibers. Acetate, propionate and butyrate are the most abundant with an estimated molar ratio of 60:20:20 in the colon. This ratio tends to vary in faecal samples of healthy individuals while the concentrations of propionate and butyrate are quite similar, corresponding to 20 mM and 15 mM, respectively. Lower SCFAs levels can also be detected in liver or blood suggesting putative systemic functions. Among the butyrate producers there are bacteria belonging to the phylum Firmicutes (e.g. *Faecalibacterium prausnitzii*, *Clostridium leptum*, *Eubacterium rectale* and *Roseburia* spp.) while *Bifidobacterium* and *Akkermansia muciniphila* provide propionate and acetate (Parada Venegas et al., 2019). SCFAs, are essential metabolic sources for intestinal epithelial cells and represent about the 10% of the daily caloric requirements of the host itself. Due to the close relationship with host nutrition, it is thought that they can be involved in the development of metabolic disorders such as obesity, insulin resistance and diabetes, especially when dysbiosis of the GIT microbiota occurs (LeBlanc et al., 2017). Moreover, SCFAs are among the key effectors of cross-feeding strategies among the bacterial community (Zhang et al., 2023).

Lactobacilli are not among the primary SCFAs producers, however some strains can release acetate and propionate. The production of a particular type of SCFA is strain-dependent, because it strictly correlates with the metabolic pathway of the species, but it is also driven by the presence of electron acceptors that can induce a shift of the energetic reactions that mainly result in acetate production (Hatti-Kaul et al., 2018; Nami et al., 2025). *Limosilactobacillus reuteri* DSM 17938 was found to produce acetate, especially when an electron acceptor or a prebiotic was supplied (Calvigioni et al., 2023; Rao et al.,

2023; De Bruyn et al., 2024). Similarly, *Bifidobacterium breve* CKDB002 and *Bifidobacterium longum* CKDB004 supplementation resulted effective in restoring the SCFAs profile in a murine model of non-alcoholic fatty liver disease. Acetate, propionate and butyrate significantly increased in the treated group and it can be supposed that this outcome is the results of both direct production of SCFAs and induction of SCFAs-producing bacteria (Yoon et al., 2023).

2.2.3 Exopolysaccharides

Other by-products of bacterial metabolism are exopolysaccharides (EPS), extracellular macromolecules that lay on the external surface of the cells preventing desiccation, phagocytosis, bacteriophage attacks and other stresses. They are constituted by repeating units of sugar and, depending on the type of monomers, are classified as homopolysaccharides and heteropolysaccharides. EPS produced by lactic acid bacteria are particularly important in food production because they confer peculiar organoleptic properties while exhibiting high thermal stability; at the same time, many studies have proved their potential for therapeutic applications having demonstrated their immunomodulatory, antioxidant, antibacterial and anti-inflammatory activity (Yoon et al., 2023). EPS from *L. reuteri* DSM 17938 and *L. reuteri* L26 Biocenol™ resulted to be effective in hampering enterotoxigenic *E. coli* adhesion to intestinal epithelial cells and modulated the expression of several cytokines revealing a strong anti-inflammatory effect combined with a protective role in epithelial barrier maintenance (Kšonžeková et al., 2016). A recent study, while exploring *L. reuteri* DSM 17938 metabolic conversion of lyoprotectant components during formulation, confirmed that the EPS from this strain partly contribute to an immunomodulatory effect increasing the IL-6 and IL-10 secretion in peripheral blood mononuclear cells (PBMCs) (Ermann Lundberg et al., 2024a). The protective effect was also confirmed for EPS from *Lactobacillus casei* 01 that preserved intestinal cells from DNA damage and demonstrated a significant antiproliferative effect on colon cancer cells (HT-29 cell line) (Liu et al., 2011). Interestingly, *B. longum* W11 induced an immune response in human peripheral blood mononuclear cells, modulating IL-6 and IL-10 at different concentrations, under both basal and activated conditions (Inturri et al., 2018). Moreover, another study proved that EPS from *B. longum* BCRC

14634 could interfere with pathogens division, therefore may have a potential application as preservative in food-spoilage but also be used to implement the host's defence against infections (Wu et al., 2010).

2.2.4 Extracellular Membrane Vesicles

Extracellular membrane vesicles (MVs) are nanoscale bilayer structures that are released from both pathogenic and probiotic bacteria and are involved in multiple functions such as molecules transportation, bacterial cross-talk, modulation of the gut microbiota, biofilm formation and regulation of host immune responses (Krzyżek et al., 2023; Puca et al., 2024; Xie et al., 2024). The biogenesis of MVs derived from Gram-positive bacteria is less characterized than in Gram-negative counterparts, however different mechanisms have been proposed. The expression of endolysins, encoded by prophages, can trigger the formation of vesicles, as demonstrated for *Lactococcus lactis* and *Lacticaseibacillus casei* (da Silva Barreira et al., 2022; Liu et al., 2022). When peptidoglycan hydrolysis occurs, as a consequence of exogenous endolysins or antibiotics, a portion of the cell can protrude through the holes, thus the vesicles are released. Due to the lack of membrane integrity, some cells die during the process that is named “bubbling cell death” (Toyofuku et al., 2023). Another possible mechanism, observed in *Staphylococcus aureus*, is vesicle “blebbing”: rearrangements of the cytoplasmic membrane are induced by phenol-soluble modulins, surfactant-like peptides, followed by peptidoglycan weakening promoted by autolysins that allow the release in the extracellular environment (Wang et al., 2018). Vesicles production occurs under physiological conditions but can be over-stimulated under stressful conditions such as oxidative and pH stress, nutrient limitations and antibiotic exposure (Abraham et al., 2025). Extracellular membrane vesicles can have a heterogeneous composition depending on the biogenesis mechanisms and the cultivation medium/ecological niche of bacterial growth. The vesicle content may include cytoplasmatic or membrane proteins, autolysins, metabolites and nucleic acids (Figure 1). Moreover, evidence suggests that the phenotype of the producer microorganism – planktonic or biofilm phenotype – can lead to distinct MVs that have thoroughly different content, and hence properties (Krzyżek et al., 2023; Toyofuku et al., 2023; Puca et al., 2024).

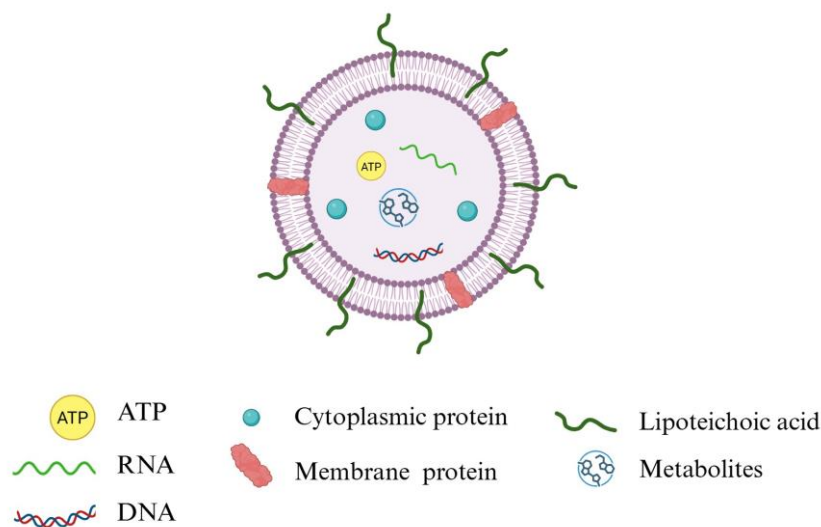


Figure 1. Schematic representation of Gram-positive membrane vesicles (Created with BioRender.com).

Probiotic-derived membrane vesicles are gaining increasing interest due to the discover of their potential bioactivity that is not only limited to the intestine but also involve systemic health benefits. *L. reuteri* DSM 17938 can release both planktonic (pMVs) and biofilm (bMVs) membrane vesicles, as first described in 2017 (Grande et al., 2017). Later, MVs produced by the same strain were characterized in terms of anti-inflammatory activity; they demonstrated to be effective in reducing IFN- γ and IL-17A in a monocyte dependent manner (Mata Forsberg et al., 2019). In 2020, West and colleagues demonstrated that *L. reuteri* DSM 17938 MVs were the key effectors of the modulation of gut motility that, as reported in the first chapter, was previously attributed to the bacterial cells (West et al., 2020). Similarly, it was also demonstrated that these vesicles can antagonize the TRPV1 receptor, mimicking the effect of live bacteria. In addition, their immunomodulatory activity was demonstrated in PBMCs measuring IL-6 and IL-1 β levels (Pang et al., 2022). Interestingly, in a recent publication, same authors demonstrated that the aforementioned properties of *L. reuteri* DSM 17938 MVs were enhanced when the bacterium was co-cultured with BG-L47[®], supporting the hypothesis that strains combination could be a strategy to obtain better-performing postbiotics (Ermann Lundberg et al., 2024b). Depending on the species and strain, the extracellular vesicles could also display antimicrobial or antiviral effects, given that they can deliver bacteriocins or other antimicrobial peptides (Ñahui Palomino et al., 2019; Dean et al.,

2020). Similar properties were also observed with respect to *B. longum* membrane vesicles that, inducing IL-10 secretion in murine splenocytes and dendritic cells-CD4⁺ T co-culture, showed anti-inflammatory activity. The latter could be related to the expression of ABC transporters and extracellular proteins that were previously shown to have a role in *B. longum*-mediated inflammation suppression (Mandelbaum et al., 2023). To further support the hypothesis that *B. longum* MVs can directly modulate immune cells, a recently published study demonstrated this effect in a murine model of ulcerative colitis. Vesicles significantly ameliorated intestinal barrier damage and exerted an anti-inflammatory effect even in pseudo-germ-free animals, suggesting that the activity did not depend on microbiota regulation (Nie et al., 2025).

2.2.5 Cell Free Supernatant

At the end of the probiotic fermentation in broth medium bacterial cells can be removed via centrifugation followed by filtration, thus the remaining liquid is the cell free supernatant (CFS) that encompass all the metabolites, as well as MVs, released during microbial growth. CFS can be considered a heterogeneous mixture which includes all the aforementioned postbiotics, with acetic and lactic acid being the most abundant (Thorakkattu et al., 2022). A large body of evidence supports the potential of CFS as a novel antimicrobial strategy, while the use of different probiotic strains expands its application, since strain, substrate, and growth conditions result in distinct CFS content (Mani-López et al., 2022).

The *L. reuteri* DSM 17938 CFS showed greater antimicrobial efficacy against Gram-negative pathogens compared to Gram-positive ones. Most likely, this selectivity is related to lactic acid which was proven to permeabilize the membrane of Gram-negative bacteria favouring the transfer of other compounds within the target cell. Indeed, the antimicrobial activity was more likely attributable to a synergy of different compounds rather than a single effector (Maccelli et al., 2020). In a dendritic cells model, the same CFS promoted the tolerogenic and mucosal-like phenotype, downregulating specific sets of genes and cytokines, all involved in gut homeostasis maintenance (Haileselassie et al., 2016). Interesting results were also obtained for different lactobacilli strains whose CFS demonstrated strong antibacterial activity versus clinical isolates of *Pseudomonas*

aeruginosa: the study confirmed that the efficacy was pH dependent but also varied depending on the producer probiotic strain. Small organic acids were supposed to be the active components (Pompilio et al., 2024). Comparable outcomes were obtained testing supernatants isolated from bifidobacteria. The CFS from *Bifidobacterium breve* CNCM I-4035 was effective at reducing the levels of pro-inflammatory cytokines and restored TGF- β in intestinal dendritic cells challenged with *Salmonella typhi*. In addition, a modulation of the TLR signalling pathway was detected (Haileselassie et al., 2016). At the same time, antibacterial and antioxidant properties were described in breast milk isolates of *B. breve* and *B. infantis*. Their CFS inhibited gut-related pathogens and showed strong antioxidant activity (Haileselassie et al., 2016).

2.3 Potential applications

All the aforementioned properties of postbiotics make them excellent candidates for innovative applications in both food and pharmaceutical industries. Postbiotics are naturally contained in fermented foods but they can also be added in adequate amounts in functional ones, especially when the use of probiotics is not recommended (Thorakkattu et al., 2022). As extensively demonstrated, one of the principal areas of use is the preservation of dairy products due to their antimicrobial properties: the CFS of different probiotic lactobacilli demonstrated a great efficacy in food spoilage prevention, thus enhancing the shelf life (Khani et al., 2025). Moreover, the confirmed health-promoting properties make postbiotics excellent candidates for pharmaceutical applications. One of the most exciting possibilities to explore is their potential use as alternative therapeutic agents in the context of antimicrobial resistance; their efficacy is not only related to pathogens inhibition but also correlates with various mechanisms such as modulation of the gut microbiota, stimulation of the immune system and strengthening the intestinal barrier. Nevertheless, there are still numerous regulatory issues that must be addressed, as well as the need for protocol standardization aiming to obtain a postbiotic product with a consistent and reproducible composition (Tomičić et al., 2025).

3. Aim and general framework of the study

This doctoral research was conducted within the framework of a collaboration with the Swedish probiotic company BioGaia which offered the opportunity to work with two probiotic strains owned by the industrial partner: *Limosilactobacillus reuteri* DSM 17938 and *Bifidobacterium longum* subsp. *longum* BG-L47[®] (trademark of BioGaia AB, deposited in the Leibniz Institute German Collection of Microorganisms and Cell Cultures (DSMZ) collection as DSM 32947). *L. reuteri* DSM 17938 is one of the most extensively studied probiotics and its efficacy, as well as safety, has been documented by over 200 clinical studies. Currently, this strain is commercialized as probiotic supplement for both babies and adults. Meanwhile, *B. longum* BG-L47[®] has not been released on the market yet but it is a new promising strain.

The aim of this study was the characterization of the two bacterial strains in terms of bioactivity of their postbiotics. Notably, even if the efficacy of these probiotics is documented by clinical evidence, the underlying mechanisms of action of their postbiotics open new avenues for future investigations. Postbiotics are essential effectors of several healthful effects on the host because they are naturally released within the ecological niche where the bacteria are located and interact with cells or other microorganisms. Thus, their characterization in terms of chemical nature and/or composition via metabolomic and proteomic approaches is a key step in the understanding of their functions. It is important to highlight that, depending on cultivation parameters, such as substrate, incubation time, temperature, pH or oxidative stress, the composition and the amount of postbiotics can vary. This aspect is crucial whether the aim is to reproduce the *in vivo* conditions but also to standardize a protocol with the purpose of purifying postbiotics to be used in clinical applications. One of the main advantages of postbiotics is that they can be administered to at-risk populations, such as immunocompromised or elderly people, for whom probiotics consumption is not always recommended. Furthermore, depending on the delivery system, postbiotics products can have better bioavailability, thus enhanced efficacy compared to probiotics. Lastly, a longer shelf life together with ease of storage and handling make them promising alternatives.

In Paper I and II of this thesis, CFS and MVs isolated from *L. reuteri* DSM 17938 have been characterized for antibiofilm activity and potential effect on cancerous and noncancerous cells, respectively. To better interpret the observed outcomes metabolomic and proteomic analysis have been carried out. In Paper III the CFS of *B. longum* BG-L47[®] have been tested for its antimicrobial activity and the immunomodulatory properties of MVs have been evaluated in human cell models and *in vivo*, using *Galleria mellonella* larvae. Overall, these findings aim to expand the knowledge about these probiotic strains and encourage investigation to develop innovative therapeutical strategies.

4. Integrated discussion of the experimental findings

L. reuteri DSM 17938 is a probiotic strain that has been extensively studied but continue to be the subject of numerous research. In 2020 the antimicrobial activity of its CFS was investigated versus clinically relevant pathogens and the lowest values of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) were obtained towards Gram-negative bacteria (Maccelli et al., 2020). Starting from these findings, the purpose of the current work was to continue the characterization of the CFS to further explore whether it was effective in inhibiting biofilm formation or eradicating mature biofilms (Paper I). At the same time, the antimicrobial potential of the CFS isolated from *B. longum* BG-L47[®] was evaluated versus the same bacterial species.

As described in the previous chapter, the CFS is a mixture of heterogeneous components, thus proteomic and metabolomic approaches represent useful techniques to narrow the pool of candidates and identify the most promising molecules. Nevertheless, the CFS also contains complex structures, namely extracellular membrane vesicles, that have gained ever-increasing interest over the years. *L. reuteri* DSM 17938 and *B. longum* BG-L47[®] can release vesicles when they grow in a planktonic phase (pMVs) as well as when they form biofilm (bMVs) (Paper II and III).

4.1 General experimental strategies

CFS and MVs were isolated from *L. reuteri* DSM 17938 (Paper I and II) and *B. longum* BG-L47[®] (Paper III) using a standardized protocol, as previously described (Maccelli et al., 2020). The antibiofilm activity of *L. reuteri* DSM 17938 CFS (Paper I) and the antimicrobial activity of *B. longum* BG-L47[®] CFS (Paper III) were tested against *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Streptococcus mutans* UA 159, *Staphylococcus aureus* ATCC 29213 and *Fusobacterium nucleatum* ATCC 25586. CFU count, metabolic and biofilm-related assays were used to quantify the efficacy of the CFS. All the preparations isolated from the two probiotic strains were analysed using Nuclear Magnetic Resonance (NMR) to obtain a chemical profile (Paper

I, II and III). In addition, the proteomic profile of MVs from *L. reuteri* DSM 17938 was characterised in Paper II. Colorimetric analyses were carried out on *L. reuteri* DSM 17938 samples (Paper I and II) to detect potential degradation process, thus obtaining preliminary information on the stability of these postbiotic products.

The cytotoxicity of *L. reuteri* DSM 17938 MVs was investigated *in vitro* in different human cancer cell lines to evaluate a potential antiproliferative effect. In parallel, noncancerous cell lines and a *Galleria mellonella* larvae model were used to assess their tolerability (Paper II). Human monocytes/macrophages were used to investigate the immunomodulatory capacity of *B. longum* BG-L47[®] MVs. The encouraging results obtained from pMV were a starting point for the subsequent experiments in *G. mellonella*, thus vesicles were used to pre-treat the larvae prior to infection. The immunophenotypic profile of haemocytes was investigated considering the modulation of human CD markers (Paper III).

The following sections are dedicated to the discussion of the key findings, whereas detailed experimental protocols are reported in the corresponding papers.

4.2 Antimicrobial and antibiofilm activity of probiotic CFS

The antimicrobial and antibiofilm activities observed across the two studies revealed that both probiotics can release active metabolites in the culture medium. The results obtained from *B. longum* BG-L47[®] revealed that, overall, the CFS was more effective versus Gram-negative compared to Gram-positive pathogens (Paper III). The detection of lactate and acetate as the most representative metabolites in CFS suggested that these organic acids could be two of the key effectors of the antimicrobial activity. Indeed, it is proved that high concentrations of lactic acid are the primary driven of pH reduction that is recognised as one of the principal mechanisms of the antagonistic activity (Tejero-Sariñena et al., 2012). However, as reported in Paper III, the complete CFS (SurP) showed the same antimicrobial potential with respect to the fractionated sample (SurE 10K). The NMR analysis confirmed a lower amount of organic acids in the latter, thus it is reasonable to suppose that these compounds are not the only ones participating in the bioactivity of the CFS. As previously demonstrated for other probiotic strains, non-acidic substances, such as hydrogen peroxide or bacteriocins, may also be involved (Tejero-

Sariñena et al., 2012). This hypothesis is also confirmed in Paper I. Except for *S. mutans* UA 159, the CFS isolated from *L. reuteri* DSM 17938 was effective in eradicating the mature biofilm of the tested pathogens. The contribution of lactic acid was evaluated in a complementary assay using *S. aureus* as reference strain and the results clearly demonstrated that this organic acid is not the only effector of the inhibitory activity. Furthermore, both CFS and SurE 10K showed the same antibiofilm activity versus *E. coli* and *S. aureus* but this trend was not confirmed for *P. aeruginosa*. *P. aeruginosa* biofilm was significantly eradicated by the complete CFS (SurP) but not by the SurE 10K. The activity was restored when a different fraction, namely SurE 30K, was tested. First, these findings suggest that the CFS contains multiple components responsible for the antimicrobial activity and that they are species-specific. On the other hand, in the case of *Pseudomonas*, the functional compounds have a size higher than 30 kDa. Undoubtedly, understanding the mechanisms behind the antibiofilm activity is a challenging task given the complexity of the biofilm itself. Proteinaceous compounds, such as bacteriocins, may also play a role since it has been demonstrated that they can successfully prevent biofilm formation by creating pores in the cell membrane and inhibiting peptidoglycan synthesis. Moreover, their combination with EPS seemed even more effective (Tejero-Sariñena et al., 2012). Interestingly, purified nisin A was found to promote the killing of both *S. aureus* planktonic and biofilm cells showing a significantly higher activity compared to other antimicrobial peptides (Tejero-Sariñena et al., 2012). These findings strengthen the hypothesis that the antimicrobial and antibiofilm potential of the CFS is strictly species-specific since different probiotic bacteria, when grown on the same substrate under identical conditions, produce different metabolites in different proportions. Furthermore, a specificity towards pathogens can also be addressed.

4.3 Proteomic and metabolomic characterization of MVs

As previously reported, *L. reuteri* DSM 17938 MVs did not show antimicrobial potential versus the tested microorganisms (Maccelli et al., 2020). Metabolomic and proteomic analysis were performed to obtain a comprehensive characterization of their composition. These analyses provided complementary information allowing to elucidate functional properties and potential applications. bMVS showed a higher concentration of

each detected metabolite compared to pMVs and organic acids resulted to be the most abundant in both samples (Paper II). Same analysis was performed on *B. longum* BG-L47[®] MVs with opposite results. Organic acids remained the most abundant metabolites but were markedly reduced compared to *L. reuteri* DSM 17938 samples. In addition, not all metabolites were shared among the analysed samples.

The predominant fraction of proteins identified in *L. reuteri* DSM 17938 consisted of cytoplasmic proteins while membrane and secreted proteins accounted for lower proportions. No proteins were exclusively found in one phenotype (pMVs or bMVs) compared to the other; conversely, all of them were shared although some were significantly more abundant in pMVs than in bMVs, and vice versa. Interestingly, part of the identified proteins was involved in metabolic processes, such as lactose, galactose and fructose permease or fructokinase. The detection of these enzymes in MVs suggests that, once released in the environment, they may confer metabolic benefits to other bacterial cells or host cells (Krzyżek et al., 2023). MVs isolated from *L. reuteri* DSM 17938 encompassed several proteins identified as “moonlighting”, in other words highly conserved cytoplasmatic proteins that show a completely different biological activity when released or attached to the bacterial surface. For some of them, such as EF-Tu, GroES and GroEL, one of the most intriguing observations is their involvement in immune and inflammatory responses as well as adhesion. These findings are consistent with other studies proving the importance of these proteins in the binding to the intestinal mucosa: the interaction can trigger the modulation of immune cells as well as counteract the adhesion of enteropathogens resulting in a competitive exclusion (Granato et al., 2004; Dhanani and Bagchi, 2013). The proteomic analysis, combined with the metabolomic profile, indicated that MVs are actively involved in numerous functions outside the producer bacteria, thus it can be stated that they are long-distance effectors of many probiotic properties. These findings correlated with the proteomic analysis given that, as reported above, several proteins expressed on the vesicles were classified as immunoregulatory.

4.4 Safety and immunomodulatory activity of MVs

Across the different studies, MVs isolated from the two probiotic strains, demonstrated to be well tolerated by human cells and in the *in vivo* model of *Galleria mellonella*. In Paper II, these larvae displayed 100% of survival after MVs treatment proving that vesicles were safe to use at least at the maximum concentration tested in the experiment. This result was confirmed in human cell lines given that the treatment of noncancerous cholangiocytes (H69 cells) with different MVs concentration did not affect the cell viability over time. Similarly, tolerability was confirmed in RAW 264.7 macrophages since vesicles did not alter the neutral red uptake of these cells. Interestingly, *L. reuteri* DSM 17938 MVs showed immunomodulatory properties on the same cell line. In macrophages stimulated with LPS, thus under pro-inflammatory conditions, both pMV and bMV were effective in restoring the neutral-red uptake. Again, these outcomes regarding vesicles safety are in line with several published studies which confirmed the absence of toxicity of probiotics-derived vesicles both in *in vitro* and *in vivo* models (Kang et al., 2013; Tong et al., 2021; Yue et al., 2025). Under the same experimental conditions, *L. reuteri* DSM 17938 MVs demonstrated only a weak anticancer effect because were not capable of significantly reduce the viability of pancreatic, biliary, liver and mammary cancer cells (Paper II).

The vesicle-mediated immunomodulation was deeply investigated in Paper III. *B. longum* BG-L47[®] pMV resulted to be the most effective in modulating monocytes and macrophages under both basal and pro-inflammatory conditions. On the other hand, bMV were effective only on LPS-stimulated macrophages. Interestingly, bMV and pMV showed a dose-dependent effect on CD163 modulation, which is a M2 macrophage marker and it is related to an anti-inflammatory phenotype involved in inflammation resolution and tissue repair. However, pMV confirmed to perform better than bMV. These findings correlate well with a recent publication demonstrating the potential application of bifidobacteria extracellular vesicles in the treatment of ulcerative colitis. MVs isolated from a *B. longum* strain showed anti-inflammatory properties and appeared to be involved in multiple mechanisms, such as: improvement of intestinal barrier integrity; inhibition of M1-macrophages polarization; enhancement of anti-inflammatory Treg cells; modulation of cytokines release (Nie et al., 2025). The immunomodulatory capacity of MVs released from other *Bifidobacterium* species was also proved *in vitro*,

confirming that the anti-inflammatory potential may be considered a shared features of certain bifidobacteria (López et al., 2012; Jeon et al., 2025).

B. longum BG-L47[®] pMV_s showed promising potential in the *G. mellonella* larvae model (Paper III). This animal model is particularly used for infection studies and drug screening due to the convenient cost-effectiveness and ease of use. Moreover, there are no ethical concerns because insect models do not require ethical approval. These larvae do not possess an adaptive immune system, but their innate system shows extensive similarities to that of humans. For this reason, *G. mellonella* is considered a valuable model that allow to carry out preliminary experiments without involving mammals. The protocol used in this work refers to a previous publication in which it was proved that haemocytes isolated from this animal shared markers typically expressed on human immune cells, thus they can be identified to evaluate how the system reacts to different challenges (Gallorini et al., 2024). The pre-treatment of the larvae with the highest dose of pMV_s partially restored larval survival after the infection with *S. aureus* or *P. aeruginosa*, but the surprising results were demonstrated in the modulation of CD markers. pMV_s appeared to act synergistically with antibiotic in the modulation of CD200 and CD200R, the ligand and its receptors, respectively. CD200-CD200R is known as a key immune-checkpoint, crucial in regulating immune homeostasis under physiological conditions. Its activation inhibit the massive release of pro-inflammatory cytokines and the excessive mast cells degranulation (Nip et al., 2023). Interestingly, the modulation of CD200 was also evaluated in a murine model after *L. reuteri* DSM 17938 feeding: increased levels of this marker after the probiotic supplementation in stressed newborns was correlated with reduced microglial inflammation (Park et al., 2021). The better outcomes were obtained when larvae were challenged with *S. aureus* while, in case of *P. aeruginosa* infection, vesicles displayed a milder modulation of the CD markers promoting the assumption that, depending on the intrinsic characteristics of the pathogen, the pre-treatment could be sufficient or not. It should also be noted that the *P. aeruginosa* strain used in this experiment was a highly virulent one. However, the outcomes suggested a potential application of MV_s in infection protection motivating the setup of more efficacious protocols (e.g. repeated MV_s administration before infection).

5. Conclusions and future perspectives

Lactobacilli and bifidobacteria represent the main subject of probiotic research with several strains having been commercially available for a long time. Their safety and health-promoting properties have been widely certified by the scientific community, yet interest in this topic continues unabated. As extensively described, the interactions between probiotics and the host are far from being merely basic and limited to a certain niche. Indeed, they are complex relationships regulated by a multitude of heterogeneous molecules, compounds and cellular structures. Key effectors of probiotic activity are all the metabolites and components released by the bacteria in the environment, one of the categories included in the term “postbiotics”. The scientific papers, constituting the core of this thesis, highlight the use of different techniques to characterize the postbiotics from both chemical and functional perspectives, providing a comprehensive understanding of their role in the health benefits promoted by *L. reuteri* DSM 17938 and *B. longum* BG-L47®.

Overall, the results indicate that the CFS of these two probiotics strains has promising antimicrobial and antibiofilm activity and could potentially become an innovative strategy to apply in infection treatment and prevention. At the same time, membrane vesicles show excellent tolerability in the proposed models and, considering evidence reported in literature, it is reasonable to suppose their safety in more complex systems. MVs, particularly the pMV_s derived from *B. longum* BG-L47®, have promising immunomodulatory properties and this preliminary data open to wider investigations. Once considered that their metabolomic and proteomic content varies according to the bacterial growth conditions, it is not unreasonable to consider new substrates to obtain better-performing postbiotics. Moreover, the chemical and functional characterization of these preparations allow to comprehend which could be the mechanisms behind the probiotic effects.

Abbreviations

LAB	Lactic Acid Bacteria
FAO	Food and Agriculture Organization of the United Nations
WHO	World Health Organization
ISAPP	International Scientific Association for Probiotics and Prebiotics
CFU	Colony Forming Units
GRAS	Generally Recognized as Safe
FDA	United States Food and Drug Administration
EFSA	European Food Safety Authority
QPS	Qualified Presumption of Safety
GIT	Gastrointestinal Tract
FISH	Fluorescence In Situ Hybridization
BBB	Blood-Brain Barrier
ASD	Autism Spectrum Disorder
HMOs	Human Milk Oligosaccharides
EPS	Exopolysaccharides
IBD	Inflammatory Bowel Disease
ROS	Reactive Oxygen Species
DSS	Dextran Sodium Sulphate
BDNF	Brain-Derived Neurotrophic Factor
AD	Alzheimer's Disease
GOS	Galactooligosaccharide
CFS	Cell Free Supernatant
MVs	Extracellular Membrane Vesicles
SCFAs	Short Chain Fatty Acids

EPS	Exopolysaccharides
MIC	Minimum Inhibitory Concentration
MBC	Minimum Bactericidal Concentration
NMR	Nuclear Magnetic Resonance
CD	Cluster of Differentiation
NTA	Nanoparticle Tracking Analysis
DLS	Dynamic Light Scattering

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Antibiofilm activity and NMR-based metabolomic characterization of cell-free supernatant of *Limosilactobacillus reuteri* DSM 17938

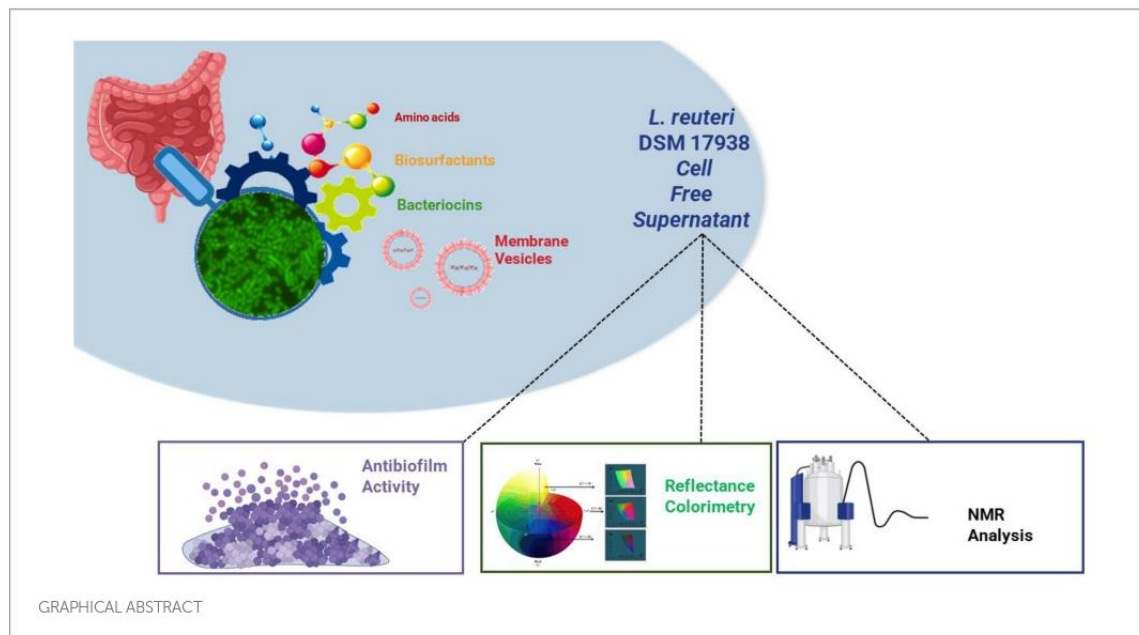
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The microbial biofilm has been defined as a "key virulence factor" for a multitude of microorganisms associated with chronic infections. Its multifactorial nature and variability, as well as an increase in antimicrobial resistance, suggest the need to identify new compounds as alternatives to the commonly used antimicrobials. The aim of this study was to assess the antibiofilm activity of cell-free supernatant (CFS) and its sub-fractions (SurE 10K with a molecular weight <10kDa and SurE with a molecular weight <30kDa), produced by *Limosilactobacillus reuteri* DSM 17938, vs. biofilm-producing bacterial species. The minimum inhibitory biofilm concentration (MBIC) and the minimum biofilm eradication concentration (MBEC) were determined via three different methods and an NMR metabolomic analysis of CFS and SurE 10K was performed to identify and quantify several compounds. Finally, the storage stability of these postbiotics was evaluated by a colorimetric assay by analyzing changes in the CIEL*a*b parameters. The CFS showed a promising antibiofilm activity against the biofilm developed by clinically relevant microorganisms. The NMR of CFS and SurE 10K identifies and quantifies several compounds, mainly organic acids and amino acids, with lactate being the most abundant metabolite in all the analyzed samples. The CFS and SurE 10K were characterized by a similar qualitative profile, with the exception of formate and glycine detected only in the CFS. Finally, the CIEL*a*b parameters assess the better conditions to analyze and use these matrices for the correct preservation of bioactive compounds.

KEYWORDS

Lactobacillus reuteri, *Limosilactobacillus reuteri*, cell-free supernatant, NMR, color analysis, antibiofilm activity, probiotics, postbiotics



Introduction

The microbial biofilm represents a microbial strategy of cooperation in which the microorganisms aggregate, communicate, and defend themselves thanks to the extracellular polymeric substances (EPS) that constitute a protective barrier (Hall-Stoodley et al., 2004; Flemming et al., 2016). It has always been believed that the formation of biofilm began following the adhesion of “pioneer microorganisms” on a biotic or abiotic surface; therefore, biofilms are defined as “manifestations of microbial life, not only growing on surfaces, but developing at any solid–liquid, liquid–liquid, liquid–gas, and solid–gas interfaces” (Flemming et al., 2021). The EPS matrix is very heterogeneous and complex and depends on many environmental factors as well as on bacterial strains, culture conditions, and biofilm age (Allesen-Holm et al., 2006; Grande et al., 2014). It can be constituted from polysaccharides, proteins, lipids, insoluble components such as amyloids, fimbriae, pili, flagella, and nucleic acids, in particular extracellular DNA (eDNA), released as free components or associated with extracellular vesicles (Hall-Stoodley et al., 2004; Grande et al., 2015). Regarding the eDNA, we showed in our previous work that it can play a different role in membrane vesicles, depending on the bacterial phenotype (Grande et al., 2015, 2017).

Koo et al. (2017) recognized microbial biofilm formation as a “key virulence factor” for numerous microorganisms responsible for chronic infections. The tolerance expressed by biofilms against traditional antimicrobial drugs as well as the host immune defenses makes them difficult to eradicate, contributing to the spread of the antibiotic resistance phenomenon (Grande et al., 2021). A wide range of microorganisms that possess different characteristics (Gram-positive and Gram-negative, motile and non-motile, aerobic, anerobic, and microaerophilic) are biofilm producers, and many of them are opportunistic pathogens associated with hospital-acquired infections. Based on the consideration that biofilm infections can be derived from dysbiosis, the characterization of natural compounds produced by probiotic strains could represent a new goal to achieve (Grande et al., 2020). The cell-free supernatant (CFS) produced by

many probiotic strains (e.g., *Limosilactobacillus reuteri*) contains many bioactive compounds such as biosurfactants, bacteriocins, and antimicrobial peptides, free or delivered by extracellular vesicles, with inhibitory effects on the growth of the pathogen (Alakomi et al., 2000; Aminnezhad et al., 2015; Grande et al., 2017; Maccelli et al., 2020). However, each probiotic strain produces its own compounds. In a previous report, we demonstrated that *Limosilactobacillus reuteri* DSM 17938 CFS has antimicrobial activity vs. both Gram-negative and Gram-positive bacteria, while membrane vesicles of planktonic phenotype (pMV) and biofilm phenotype (bMV) were not active (Maccelli et al., 2020). The CFS and its subfraction SurE 10 K, which contains molecules or compounds with a molecular size lower than 10 kDa, were tested at different concentrations against reference and clinical strains of *Escherichia coli*, *Pseudomonas aeruginosa*, *Fusobacterium nucleatum*, *Staphylococcus aureus*, and *Streptococcus mutans* characterized by different antimicrobial susceptibility patterns. The results demonstrated that the CFS contained antimicrobial compounds, in addition to the well-known reuterin, showing greater activity against Gram-negative bacteria than Gram-positive bacteria, and the efficacy was related to the species rather than to the individual strains (Maccelli et al., 2020). Moreover, the data obtained supported the hypothesis that the antimicrobial effect could be associated with synergistic activity between several compounds contained in the CFS, as previously demonstrated by other authors (Poppi et al., 2015).

On the basis of the results previously obtained and considering the clinical relevance of microbial biofilms, the first aim of this study was to evaluate the antibiofilm activity of the CFS and some of its fractions (SurE 10 K and 30 K). We chose clinically relevant species that form biofilms to test the capability of the CFS to both inhibit biofilm formation and eradicate mature biofilms. Successively, to better comprehend the potential of this probiotic, we aimed at improving our previous mass spectrometry-based knowledge of the metabolic content by means of qualitative and quantitative NMR analyses. Given that the chemical composition of the entire set of metabolites of a probiotic is very complex, an untargeted metabolite profile characterization of this matrix can be useful to identify

the main metabolites of the considered system and potentially correlate them with the biological activity. In this context, NMR spectroscopy represents a very powerful tool for this kind of analysis since this methodology can be used to obtain a chemical profile of a biological system (Bianchi et al., 2020). Finally, we monitored the stability of microbial products with an affordable and fast color analysis assay. Previous studies on cyanobacteria monitored biofilm formation and how pigment production was affected by the environment (Sanmartín et al., 2010, 2012). Our previous studies demonstrated how color could be useful to monitor the shelf-life of different systems, providing useful information about oxidative stability and, more generally, about browning, which represents the main modification induced by aging on the biological matrices (Cesa et al., 2015; Menghini et al., 2021). In this view, a preliminary shelf-life stability study to monitor the chemical composition of CFS was performed by CIEL*a*b colorimetric analysis for a period of 4 weeks. The workflow described earlier is shown in Figure 1.

Materials and methods

Bacterial strains and culture conditions

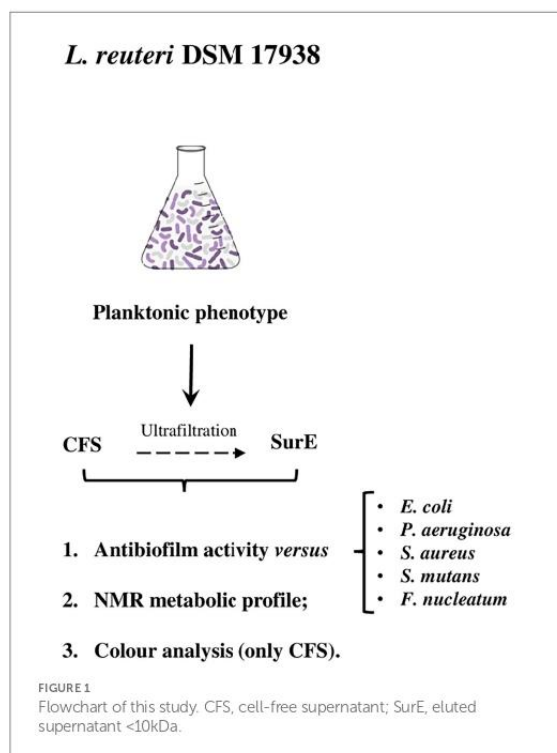
Limosilactobacillus reuteri DSM 17938 (Rosander et al., 2008), provided by BioGaia AB (Stockholm, Sweden), was used in the study. The bacteria were plated on DeMan, Rogosa, and Sharpe agar (MRSA; Oxoid Limited, Hampshire, United Kingdom), and incubated at 37°C for 24 h in an anaerobic atmosphere (Anaerogen Pak Jar, Oxoid Ltd.). The CFS and its fractions, SurE 10 K and 30 K, were obtained following the procedure described by Maccelli et al. (2020). Regarding the other

bacterial strains used, different media and growth conditions were used on the basis of the species.

Biofilm formation assay

The biofilms were developed on 96-well flat-bottomed polystyrene microtitre plates (Eppendorf, Hamburg, Germany) by using different media and growth conditions depending on the species. The OD₆₀₀ of each inoculum was read and adjusted to reach a final concentration in each well of 1.0×10^5 CFU/mL. More details are given as follows:

- *Escherichia coli* ATCC 25922 was grown in Mueller Hinton Broth (MHB; Oxoid Limited, Hampshire, United Kingdom), and the biofilm was developed in MHB for 24 h of incubation at 37°C in static and aerobic conditions;
- *Pseudomonas aeruginosa* ATCC 27853 growth and biofilm formation were conducted in Luria Bertani Broth (LB; Oxoid Limited, Hampshire, United Kingdom) for 24 h of incubation at 37°C in static and aerobic conditions;
- *Streptococcus mutans* UA 159 was grown in Brain Heart Infusion (BHI; Oxoid Limited, Hampshire, United Kingdom), and the biofilm was developed in BHI + 1% of sucrose (BHIS) for 24 h of incubation at 37°C in static and anaerobic conditions;
- *Staphylococcus aureus* ATCC 29213 was grown in Tryptic Soy Broth (TSB; Oxoid Limited, Hampshire, United Kingdom), and the biofilm was developed in TSB + 1% of glucose (TSBG) for 24 h of incubation at 37°C in static and aerobic conditions;
- *Fusobacterium nucleatum* ATCC 25586 growth and biofilm formation were conducted in BHI for 48 h of incubation at 37°C in static and anaerobic conditions.



Determination of CFS and SurE 10K minimum biofilm eradication concentration

The MIC and MBC of the CFS and SurE 10K vs. *P. aeruginosa* ATCC 27853, *F. nucleatum* ATCC 25586, *E. coli* ATCC 25922, *S. aureus* ATCC 29213, and *S. mutans* UA159 were previously determined (Maccelli et al., 2020).

Regarding the determination of the Minimum Biofilm Eradication Concentration (MBEC), the biofilms were developed depending on the bacterial species. The MBEC was defined as the lowest concentration, which completely eradicated bacterial biofilms developed in 96-well flat-bottomed polystyrene microtiter plates. At the end of incubation, the biofilms were washed with phosphate-buffered saline (PBS), and the CFS and SurE 10K were added to the pre-formed biofilms at concentrations corresponding to 1×MIC, 2×MIC, 3×MIC, and 4×MIC of each test sample, except for the *F. nucleatum* biofilm that was treated with 2×MIC, 3×MIC, 4×MIC, and 5×MIC. Controls consist of (i) non-treated biofilms and (ii) biofilms with the addition of MRS broth (the medium in which CFS and SurE are resuspended). The microtiter plates were then incubated at 37°C for the time of incubation chosen on the basis of bacterial species under static conditions. The eradication effects of CFS and SurE 10K were measured using three different methods, namely Colony Forming Units (CFU) counting; metabolic assays such as alamarBlue® (AB) (Thermo Fisher Scientific, Waltham, MA, United States) or XTT (sodium 3'-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis-(4-methoxy-6-nitro) benzene sulfonic acid hydrate; Cell Proliferation Kit II XTT, Roche Diagnostic, Mannheim, Germany); and Crystal Violet (CV) assay.

Cell viability evaluation through colony-forming unit count

The CFU enumeration was performed to evaluate the bacterial cell viability in the treated and non-treated biofilms with different concentrations of CFS and SurE 10 K. A volume of 100 μ L of the sample was taken from each well and used for CFU counts. Serial dilutions of the stock were performed in PBS (pH 7.2) and plated on the suitable agar media at 37°C for 18–24 h. The same evaluation was performed in the case of the AB assay due to its non-toxic nature.

Cell viability assays

The MBEC was confirmed using the XTT metabolic assay and/or AB assay according to the manufacturer's recommendations (Zengin et al., 2018; Maccelli et al., 2020). Given that we noticed that some components or supplements present in the media that were used interfered with the dyes present in the kits, it was decided to use both kits and compare the data obtained.

The absorbances corresponding to 490 nm for XTT and 570 and 600 nm for AB were then read with a microplate reader (Synergy H1 Multi-Mode Reader, BioTek, Winooski, VT, United States). The XTT and Alamar Blue MBEC were defined as the lowest concentrations of the sample, resulting in a detectable colorimetric change in the assays. The colorimetric change is a direct correlation with the metabolic activity of the biofilm. Two independent experiments were performed in triplicate.

Crystal violet assay

The Crystal Violet assay was used to stain the biofilm biomass. The treated and non-treated biofilms were rinsed with 100 μ L of PBS and fixed for 1 h at 60°C. Then, 100 μ L of crystal violet at 0.1% diluted in MilliQ water was added for 5 min to *P. aeruginosa*, *S. mutans*, *E. coli*, and *S. aureus* pre-formed biofilms. Crystal violet at 0.5% diluted in MilliQ water was added for 2 min to pre-formed biofilm of *F. nucleatum*. The biofilms were washed with 200 μ L of MilliQ water and dried at room temperature (RT) for 30 min. In the end, 100 μ L of 33% acid acetic was added to each well for 10 min and absorbance was read at 590 nm.

Live/dead assay

The qualitative analysis of the CFS-treated and untreated biofilms developed by the different bacterial species was performed using live/dead staining (BacLight kit; Thermo Fisher Scientific, Waltham, MA, United States) as indicated by the manufacturers and fluorescent microscopy evaluation with a Leica DMR Fluorescent Microscope (Leica Microsystem, Wetzlar, Germany). Two experiments were performed in triplicate.

Determination of SurE 30K minimum biofilm eradication concentration vs. *Pseudomonas aeruginosa* ATCC 27853

SurE 30 K was obtained *via* ultrafiltration of CFS with Amicon Ultra-15 30 K (30000 MWCO) Centrifugal Filter Devices (Merck KGaA, Darmstadt, Germany). The MIC of SurE 30 K was determined as for SurE 10 K (data

not shown; Maccelli et al., 2020). After determining the MIC, a *P. aeruginosa* biofilm was developed in LB, and after 24 h, the mature biofilm was treated with SurE 30 K at 1 $\times$ MIC, 2 $\times$ MIC, 3 $\times$ MIC, and 4 $\times$ MIC. The MBEC was evaluated with an AB assay, CFU counting, and CV assay.

Minimum biofilm inhibitory concentration of CFS and SurE 10K

To determine the MBIC, the CFS and SurE 10 K were diluted into each well until reaching a concentration of half of the Minimum Inhibitory Concentration (MIC). The MIC values of *L. reuteri* CFS and SurE 10 K vs. the bacterial species used were previously described (Maccelli et al., 2020). Then, the OD₆₀₀ of each inoculum was read and adjusted to reach a final concentration in each well of 1.0×10^5 CFU/mL. The plates were incubated at 37°C for 24 h for *P. aeruginosa*, *S. mutans*, *S. aureus*, and *E. coli* and for 48 h for *F. nucleatum*. The valuation of the MBIC was determined by both CFU counts and metabolic assays previously described.

Determination of minimum inhibitory concentration of CFS and SurE 10K vs. *Lactobacillus rhamnosus* ATCC 53103, *Lactobacillus paracasei* CNCM I-1572, and *Lactobacillus acidophilus* LA14 ATCC SD 5212

The minimum inhibitory concentration was determined using the broth microdilution assay in 96-well polystyrene microtiter plates and CFU counting. Briefly, *Lactobacillus rhamnosus*, *Lactobacillus paracasei*, and *Lactobacillus acidophilus* were grown in MRSB for 17 h at 37°C under shaking conditions at 125 rpm. The overnight cultures were resuspended until reaching an optical density of 0.1 nm (OD₆₀₀) that corresponds to 10^7 CFU/mL and then diluted to 10^5 CFU/mL in the 96-well plate. CFS and SurE 10 K were tested from a maximum concentration of 88 μ L/100 μ L to a minimum concentration of 5.5 μ L/100 μ L. The plates were incubated in static and anaerobic conditions for 24 h at 37°C. The MIC was defined as the lowest concentration without visible growth, and it was determined both visually and *via* CFU counting. Controls consisted of MRSB media without the addition of CFS. Two independent experiments were performed in triplicate.

Determination of MIC and MBC values of lactic acid vs. *Staphylococcus aureus*

The antimicrobial activity of lactic acid (Puraq Bioquímica SRL, Spain) was tested vs. *Staphylococcus aureus* ATCC 29213 *via* the broth microdilution method in 96-well plates according to the CLSI guidelines and confirmed by XTT assay, following the methods previously described (Maccelli et al., 2020). Lactic acid was diluted in MHB and tested in the range of 0.05–6.4 μ L/100 μ L.

Statistical analysis

The differences in the means of the results between untreated and treated bacterial strains were evaluated by one-way ANOVA (GraphPad Software, San Diego, CA, United States) and Dunnett's multiple

comparison test. Media supplemented with MRS broth at the maximum concentration of CFS tested was used as the control. The probability value of $p \leq 0.05$ was considered significantly different. Analysis of cytotoxicity data (expressed as mean $\pm$ standard error) was performed by the GraphPad Prism TM 6.00 software (GraphPad Software, San Diego, CA, United States).

NMR-based qualitative and quantitative analyses

A volume of 1 mL of each sample was lyophilized and then dissolved in 750 μ L of 200 mM phosphate buffer/D₂O, containing 1.4 mM TSP (3-(trimethylsilyl)propionic acid sodium salt) as internal standard. Finally, 700 μ L of the solution was transferred into a 5 mm NMR tube. NMR analyses were carried out on a Jeol JNM-ECZ 600R operating at the proton frequency of 600.17 MHz and equipped with a Jeol 5 mm FG/RO DIGITAL AUTOTUNE probe. ¹H NMR experiments were carried out by using the following parameters: 298 K, 256 scans, residual water signal suppression with a presaturation pulse, 7.7 s relaxation delay, 90° pulse of 8.3 μ s, 64 k data points, and 9,000 Hz spectral width. ¹H spectra were referenced to methyl group signals of TSP ($\delta_{\text{H}} = 0.00$ ppm) in D₂O. Homonuclear ¹H-¹H TOCSY experiment was acquired with 96 scans, 8 k data points in *f*₂ and 128 in *f*₁, 50 ms mixing time, 2 s relaxation delay, and 9,000 Hz spectral width in both dimensions. Heteronuclear ¹H-¹³C HSQC experiment was acquired with 88 scans, 8 k data points in *f*₂ and 256 in *f*₁, 3 s relaxation delay, and a spectral width of 9,000 Hz and 33,000 Hz for *f*₂ and *f*₁, respectively. ¹H-¹³C HMBC experiment was acquired with 64 scans, 8 k data points in *f*₂ and 256 in *f*₁, 2.8 s relaxation delay, and a spectral width of 9,000 Hz and 39,000 Hz for *f*₂ and *f*₁, respectively. Spectra processing and signal integration were carried out with the JEOL Delta software (v5.3.1). For each metabolite, a characteristic signal was considered and integrated, normalizing the area to those of the methyl group signals of TSP, set to 100. Results were expressed as μ g/mL of sample.

Colorimetric analysis

The samples were analyzed at the initial time (*t*⁰) for their color character, with a colorimeter X-Rite MetaVue™ (X-Rite, Prato, Italy), equipped with full spectrum LED illuminant and an observer angle of 45°/0° imaging spectrophotometer. Samples that were stored in a refrigerator (4°C) were analyzed again in the same conditions after 2 (*t*^{2w}) and 4 weeks (*t*^{4w}). Cylindrical coordinates *C*_{ab}^{*} and *h*_{ab} and the color distances (ΔE) were calculated according to the reference samples at *t*⁰ sample, as known by the literature (Cairone et al., 2020).

Results

Determination of the minimum biofilm eradication concentration

The ability of factors secreted by *L. reuteri* DSM 17938 to eradicate biofilms produced by different pathogenic bacteria was evaluated by using three different methods to obtain more reliable results. Two

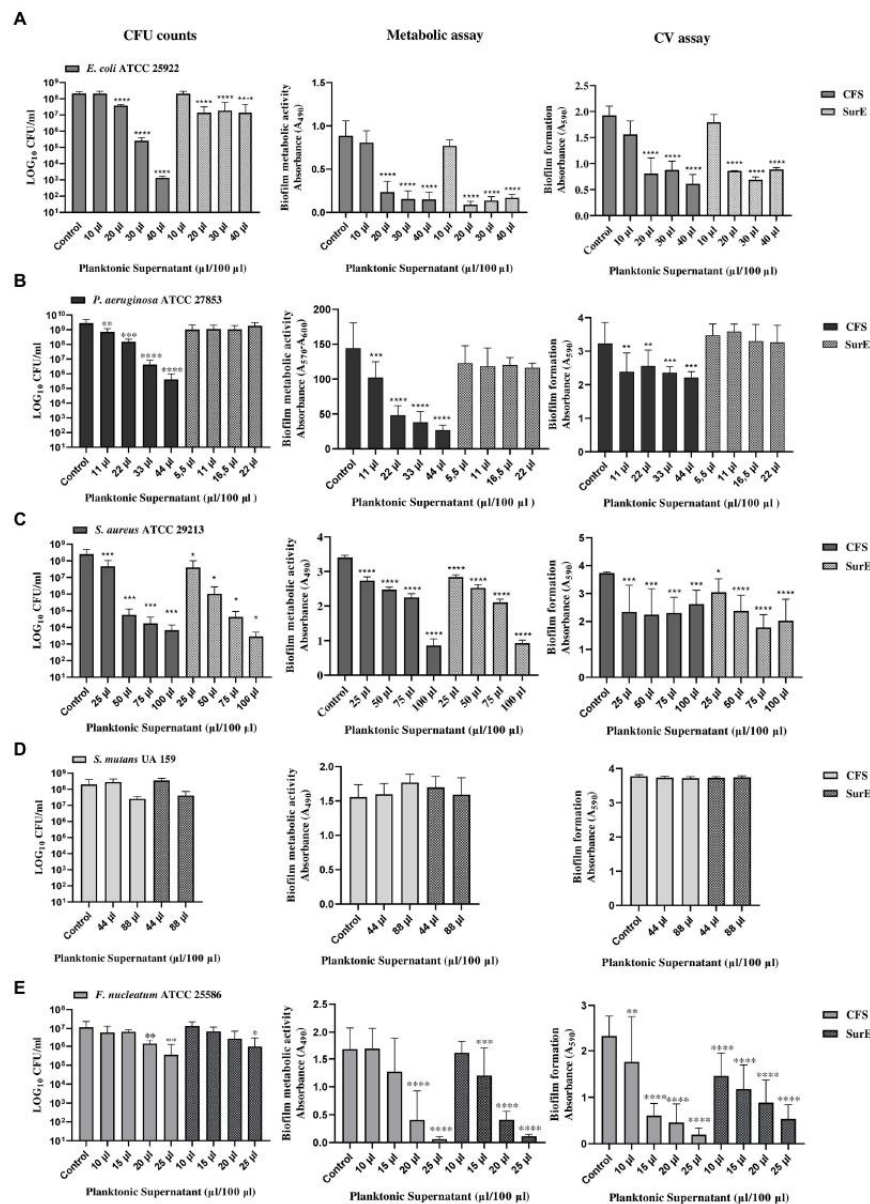
independent experiments, performed in triplicate, have been carried out for each method. The methods used were: CFU counting, biofilm metabolic activity, and crystal violet (CV) assay, followed by fluorescent microscopy qualitative analysis (data not shown), which represented an additional confirmation. The exact correspondence of the results obtained guarantees us the accuracy and reproducibility of the data. The CFS showed the capability to eradicate pre-formed biofilms developed by *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *S. aureus* ATCC 29213, and *F. nucleatum* ATCC 25585 (Figure 2). In particular, the effect of the CFS at 2×MIC (corresponding to 20 μ L/100 μ L) on the eradication of *E. coli* pre-formed biofilm was confirmed by the significant reduction in CFU counts and metabolic activity, as well as a statistically significant reduction in the biofilm biomass, as shown in Figure 2A. While the effect of CFS on the eradication of *P. aeruginosa* and *S. aureus* mature biofilms was detected at 2×MIC or at MIC (corresponding to 11 μ L/100 μ L and 25 μ L/100 μ L, respectively; Figures 2B,C). On the contrary, CFS was not capable of eradicating the biofilm developed by *S. mutans* UA 159 (Figure 2C). Regarding the SurE 10K, it showed a value of MBEC comparable to those of CFS vs. *E. coli* and *S. aureus* (see Figures 2A,C). On the contrary, it did not work against *P. aeruginosa*, suggesting that the molecules responsible for the inhibitory activity are contained in the CFS fraction (Figure 2B). To demonstrate this, we performed an intermediate fractionation by using 30 K columns. The results obtained demonstrated that SurE 30 K is capable of eradicating *P. aeruginosa* mature biofilm at 3×MIC (corresponding to 33 μ L/100 μ L; Figure 3). Finally, SurE 10 K, as previously demonstrated for CFS, did not eradicate *S. mutans* biofilm (Figure 2D). Regarding the antibiofilm activity of the CFS against the *F. nucleatum* ATCC 27853 biofilm, the results showed a statistical reduction at 4×MIC for CFS and 5×MIC for SurE 10K, demonstrating a more powerful eradication ability of the CFS.

SurE 30K MIC and MBEC vs. *Pseudomonas aeruginosa* ATCC 27853

Since SurE 10 K did not show any antibiofilm effect toward *P. aeruginosa*, it was decided to test SurE ultrafiltrated through a 30 kDa (SurE 30 K) filter to check if the 10 kDa filtration did not allow the passage into the eluted sample of the compound(s) responsible for the antibiofilm activity. SurE 30 K showed a MIC of 11 μ L/100 μ L toward *P. aeruginosa*, the same as in CFS. The MBEC value corresponded to 33 μ L/100 μ L (3×MIC; Figure 3).

Determination of the minimum biofilm inhibitory concentration

We next evaluated the ability of the secreted factors to inhibit biofilm formation. In particular, the capability of the CFS and SurE 10 K to inhibit the biofilm formation of the species previously mentioned was determined by the evaluation of the Minimum Biofilm Inhibitory Concentration (MBIC) by using, as previously mentioned, the CFU counting, biofilm metabolic activity, and CV assay. Both the CFS and SurE 10 K were not capable of inhibiting biofilm formation in any of the microorganisms tested. No significant reduction in the number of CFUs, biofilm metabolic activity, or biomass of the biofilm was detected (Supplementary Figure S1).



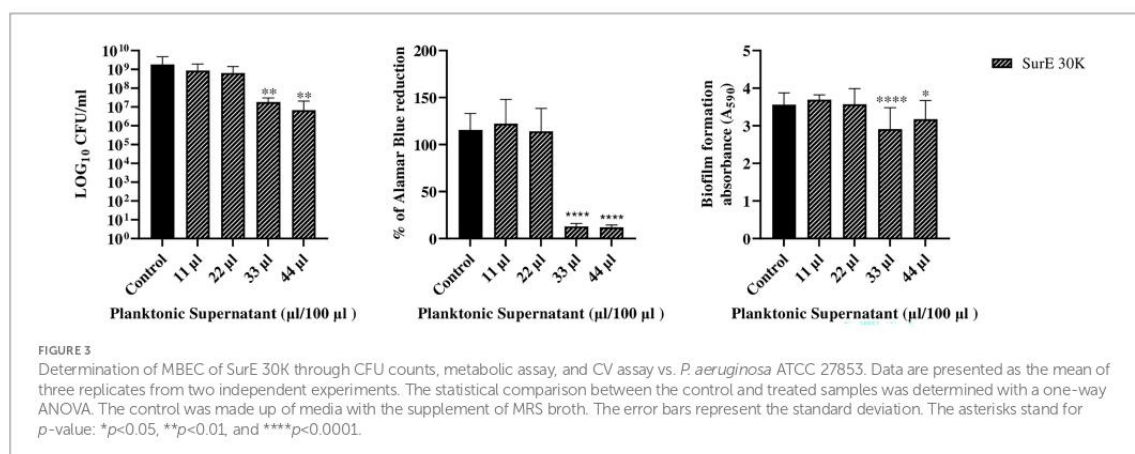


TABLE 1 Evaluation of the MIC of CFS and SurE 10K, obtained by *L. reuteri* DSM 17938, toward three probiotic strains.

Bacterial strains	CFS MIC (µL/100µL)	SurE 10K MIC (µL/100µL)
<i>L. rhamnosus</i> ATCC 53103	>88	>88
<i>L. paracasei</i> CNCM 1-1572	>88	>88
<i>L. acidophilus</i> LA14 ATCC SD 5212	88	88

TABLE 2 Quantitative results obtained from the NMR analysis of samples subtracted from their blank (MRSB).

Metabolite	CFS	SurE 10K
Lactate	6746.00	8427.49
Formate	28.49	–
Leucine	236.04	308.93
Isoleucine	92.08	114.03
Valine	158.56	213.97
Alanine	380.62	523.70
Glycinebetaine	63.33	75.71
Glycine	550.09	–
Tyrosine	29.19	38.09
Phenylalanine	113.28	151.04
Tryptophan	3.20	8.38
Choline	10.72	15.55

Results are expressed as µg/ml of sample. “–” means “not detected.”

NMR-based metabolomic profile

The NMR analysis of the considered samples (including MRS broth as the blank) allows identifying two organic acids (lactate and formate), nine amino acids (alanine, valine, glycinebetaine, isoleucine, leucine, glycine, phenylalanine, tyrosine, and tryptophan), and choline by means of 2D experiments and literature data (Di Matteo et al., 2021; Spano et al., 2021) as reported in Supplementary Table S1 and Supplementary Figures S2–S6. Comparing the NMR results obtained in Table 2, lactate and formate were identified among organic acids, whereas leucine, isoleucine, valine, alanine, glycine, tyrosine, phenylalanine, tyrosine, and tryptophan were revealed among amino acids. Choline and glycinebetaine were also present. Formate and glycine were detected in CFS samples only.

Determination of the MIC of lactic acid vs. *Staphylococcus aureus*

Due to the well-known antimicrobial activity of lactic acid-producing bacteria and the high content of this organic acid detected by quantitative NMR analyses (Table 2), lactic acid MIC was detected by unaided eye at 0.2 µL/100 µL and was also confirmed by XTT assay (Figure 4A). The minimum bactericidal concentration of lactic acid was determined at 0.4 µL/100 µL through the CFU count (Figure 4B).

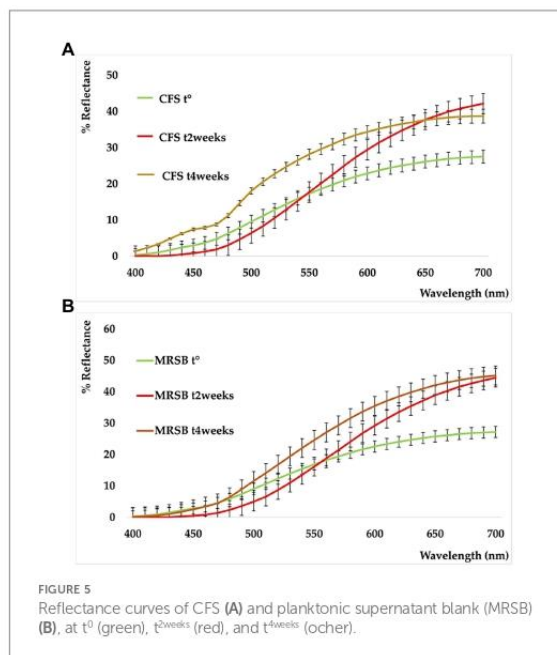
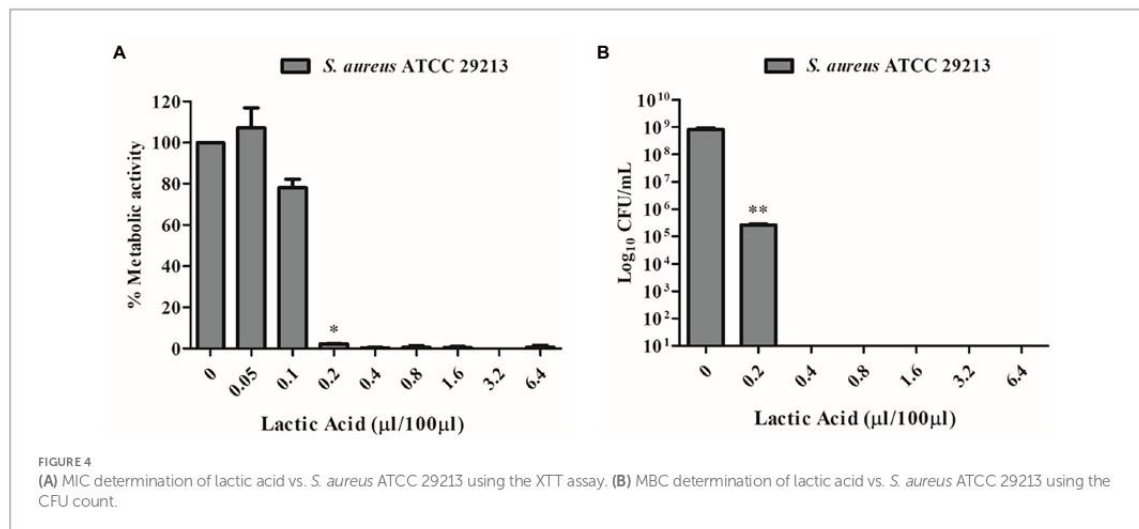
Stability studies using reflectance colorimetry

The shelf-life of the samples was monitored after 2 and 4 weeks of storage at 4°C. All the detected color parameters are reported in

Supplementary Table S2. Data showed the color differences existing among samples, both in terms of elapsed time and different analyzed samples. This is also shown by the sample reflectance curves reported in Figure 5. In the CFS (A), the variation with respect to t^0 is particularly marked at t^{2w} and assumes the mean of a powerful whitening. This trend, however, is well delineated also in the medium used as reference (MRSB, B) at t^{2w} , so that the difference between the two systems remains low ($\Delta E = 4.87$, CFS t^{2w} vs. MRSB t^{2w}). On the contrary, an evident change is denoted after 4 weeks because the color of the supernatant is turning toward blue and green rather than increasing in red and yellow as in the medium ($\Delta E = 18.56$ CFS t^{4w} vs. MRSB t^{4w}).

Discussion

Probiotics are considered an effective approach for treating infections and inhibiting the spread of antibiotic resistance. In particular, lactobacilli are involved in the process of biofilm formation on oral surfaces, exerting anti-adhesive activity against different pathogens given that they interfere with adhesion and microbial cells'



co-aggregation *via* the secretion of antagonistic substances such as bacteriocins, organic acids, enzymes, and biosurfactants (Söderling et al., 2011; Barzegari et al., 2020).

Different articles have shown that probiotics like *Limosilactobacillus fermentum* and *Lactiplantibacillus plantarum* and some of their derivatives possess an antibiofilm effect on *P. aeruginosa* (Sharma et al., 2018; Shokri et al., 2018). In particular, Sharma et al. demonstrated that postbiotics, produced by *L. fermentum*, can reduce quorum sensing signals needed for biofilm formation, confirming results from previous studies (Kanmani et al., 2011; Bulgasem et al., 2015; Sharma et al., 2018). Here, we demonstrated that CFS from *L. reuteri* can eradicate *P. aeruginosa* biofilm at 1 × MIC, even if its 10 kDa filtered fraction is

not able to exert any eradication activity, suggesting that the molecules/compounds responsible for its action, have a molecular weight higher than 10 kDa. As previously mentioned, the antimicrobial activity of CFS is probably due to the synergistic effect of different molecules/compounds (Maccelli et al., 2020). These data confirm our hypothesis, according to which, in this case, the antibiofilm activity is associated with several compounds, some of them with a size higher than 30 kDa, potentially involved either in the disintegration or in the permeabilization of the biofilm EPS matrix, facilitating the penetration of other components with antimicrobial activity. Regarding this hypothesis, Sharma et al. proposed that some bacteriocins from different probiotics are able to create pores through the *P. aeruginosa* cell membrane, leading to a change in cell membrane integrity and inducing cell death; similarly, some bacteriocins produced by *Streptococcus pyogenes* FF22 are able to disrupt the membrane potential of a *S. aureus* biofilm by forming short-lived pores that allow the passage of ions, leading to a major ATP efflux from biofilms which is correlated with the antimicrobial activity of bacteriocins (Okuda et al., 2013; Sharma et al., 2018). Tunçer et al. also showed very interesting results related to the action of CFS from *S. salivarius* M18. The authors demonstrated that CFS increases the lipid unsaturation index of *P. aeruginosa* and decreases the content of cellular polysaccharides, factors that have a crucial role in the formation of *P. aeruginosa* biofilm (Tunçer and Karaçam, 2020).

Ghane et al. showed that CFS produced from different probiotics, in particular, *Lactobacillus rhamnosus* and *Lactobacillus paracasei*, had a strong uropathogenic *E. coli* (UPEC) biofilm inhibitory effect (Ghane et al., 2020). On the contrary, Al-Dhabi et al. showed that *L. reuteri* LR12 has an inhibition rate of 53% on *E. coli* biofilm, a 68% inhibition rate against *P. aeruginosa* biofilm, and a 100% inhibition rate vs. *S. aureus* biofilm (Al-Dhabi et al., 2020). These data support our results, although the efficacy is known to be strain-specific. In fact, we demonstrated a more powerful eradication by *L. reuteri* DSM 17938 CFS against *S. aureus* and *P. aeruginosa* biofilms (1 × MIC), with respect to *E. coli*, where the MBEC value was higher (2 × MIC). In contrast to our findings, Melo et al. showed that *L. fermentum* TCUESC01 can reduce the formation of *S. aureus* biofilm under sub-inhibitory concentrations, at 50% of the MIC, confirming that the

inhibition growth ability of probiotics differs among strains (Keller et al., 2011; Melo et al., 2016).

Concerning *S. mutans*, our findings showed both that the MIC value is higher (44 µL/100 µL) when compared to other bacterial strains, including *F. nucleatum* (5 µL/100 µL), and that CFS does not have any biofilm eradication action until $2 \times$ MIC concentration. It is important to take into consideration that the biofilm was developed in BHI supplemented with 1% of sucrose and that *S. mutans* uses sucrose from dietary sources for the synthesis of EPS, which act as a scaffold and contribute to its pathogenicity and antimicrobial resistance (Yang et al., 2019). We never supplemented MRSB media with glycerol, which is required by *L. reuteri* to produce reuterin (Maccelli et al., 2020). When considering the action of reuterin, it is important to consider that the amount of glycerol in the plaque is very low, therefore it may not be so important for oral health (Söderling et al., 2011). Yang et al. demonstrated that *L. reuteri* AN417 CFS is able to reduce *S. mutans* biofilm formation in the absence of reuterin. The authors developed *S. mutans* biofilm in the absence of sucrose, which was capable of increasing its pathogenicity, and water-insoluble glucan, derived from sucrose metabolism, and involved in *S. mutans* biofilm formation (Hasibul et al., 2018; Yang et al., 2021). The results in the literature are controversial, but it is known that considerable genotypic and phenotypic differences exist between *S. mutans* strains (Senpuku et al., 2018).

Besides reuterin, *L. reuteri* strains can produce different antimicrobial substances, including lactic acid, acetic acid, and ethanol. Data have evidenced the importance of the acidic environment in the interactions between *L. reuteri* and other microorganisms, in particular with the effect of lactic acid (Geraldo et al., 2020). To ensure that lactic acid was not the only player responsible for the antimicrobial activity despite its high content in both *L. reuteri* CFS and SurE 10 K, we evaluated the MIC of lactic acid against *S. aureus*. The results showed a MIC value of 0.2 µL/100 µL. Conversely, the MIC of *L. reuteri* CFS was 25 µL/100 µL, demonstrating that the inhibitory activity was not associated with the action of lactic acid alone but was derived from a synergistic effect among different molecules or compounds present in the supernatant and characterized by a molecular size lower than 10 kDa. In fact, 0.025 mL of CFS or SurE 10 K should contain 0.16 mg and 0.21 mg of lactic acid, respectively, based on the amounts reported in Table 2, whereas 0.2 µL of lactic acid corresponds to 0.24 mg (lactic acid density = 1,209 mg/mL). It is well-known that probiotics can produce some bacteriocin-like compounds that have been claimed to be more active at acid pH than at neutral or alkaline pH (Li et al., 2015); therefore, we could also hypothesize the presence of such compounds in CFS. Further studies are needed to better understand which molecules could contribute to the efficacy of CFS.

With regards to *F. nucleatum*, some articles in the literature have shown that some probiotics like *Bifidobacterium lactis* and *Bifidobacterium infantis* are able to cause an antagonistic effect toward periodontopathogens like *F. nucleatum* (Valdez et al., 2021). *Fusobacterium nucleatum* has the ability to co-aggregate with other bacteria, and this may be due to its long rod morphology that acts as a bridge in dental plaque (Kolenbrander and London, 1993). Moreover, *F. nucleatum* is able to reduce oxygen levels, contributing to the growth of less aerotolerant and more pathogenic bacterial species like *Porphyromonas gingivalis* (Bradshaw and Marsh, 1998; Llama-Palacios et al., 2020).

Many studies have demonstrated the efficacy of CFS and probiotic cells on *F. nucleatum* growth. Yang et al., for example, showed that the CFS produced by *L. reuteri* AN 417 was very effective against *P. gingivalis* growth but less effective on *F. nucleatum* and *S. mutans* (Yang et al., 2021). With regards to the antibiofilm activity toward *F. nucleatum*, a limited number of details are reported. Jiang et al. proved that *S. mutans* and *F. nucleatum* have a poorer ability to grow in mono-species biofilms and they demonstrated better abilities of adhesion and reproduction in dual-and/or multispecies biofilms (Jiang et al., 2016). In multi-species biofilms, neighbors can take advantage of the productivity of the community, in particular in the oral cavity, in which they develop dental plaque (Elias and Banin, 2012). In the end, the evaluation of the effect of *L. reuteri* DSM 17938 vs. other probiotic strains showed promising results given that the CFS did not demonstrate any inhibitory activity toward the probiotic strains tested but only toward pathogens. The results reported represent the first contribution to the evaluation of the antibiofilm activity of CFS produced by *L. reuteri* DMS 17938 vs. *F. nucleatum* mono-species biofilm. The capability of *L. reuteri* DSM 17938 CFS to inhibit *F. nucleatum* growth and eradicate its biofilm could represent a strategy to prevent the formation of polymicrobial dental plaque, avoiding the development of biofilm-related pathologies in the oral cavity. Further studies should be performed in order to determine the activity of CFS in multi-species biofilms, especially from species of clinical relevance that colonize the oral cavity, such as *S. mutans* and *P. gingivalis*.

The application of NMR spectroscopy for the metabolomic analysis of these matrices was effective for the identification and quantification of primary and secondary metabolites, giving important information with regards to the potential role of some of them in the observed anti-biofilm activity. Only one article regarding the NMR analysis of *Lactobacillus* vesicles has been found (Ñahui Palomino et al., 2019). In respect to the assignments present, a further identification was observed in this work by detecting citrate, malate, and glycinebetaine. The latter is an osmoprotectant metabolite that usually improves adaptation to environmental salt stresses, especially for those bacteria living in the mammalian gut (e.g., *C. difficile*), where salinity could hamper bacterial growth (Michel et al., 2022). This is the first report revealing the presence of this compound in probiotic species as well. Generally, Sur E 10 K was richer in terms of all detected metabolites, despite the fact that formate and glycine were present only in the CFS. Lactic acid was the most abundant metabolite. Among amino acids, we found the following substances in the order of decreasing amounts: glycine > alanine > leucine > valine > phenylalanine. All these compounds were also detected via untargeted metabolomic profiling by means of high-resolution fourier transform ion cyclotron resonance mass spectrometry (FT-ICR MS) coupled with electrospray ionization source (ESI; Maccelli et al., 2020), but without any knowledge of the content.

To assess the stability of CFS and Sur E 10 K, we performed a fast, affordable, and non-destructive analysis based on reflectance colorimetry. Besides a well-known role in the consumer's compliance toward a product, color could play a main role in assessing differences and peculiarities among the commodity class, as well as give information related to stability and allow the evaluation of their shelf-life. As previously explained, this is of particular significance in the case of rapidly evolving biological systems. In fact, color is influenced and quickly modified by humidity, temperature, oxygen content, and metal presence, apart from many other factors correlated to the nature of the analyzed matrix, its solid or liquid state, the presence of

enzymes, the molecular composition, and light exposure (Cesa et al., 2017).

To the best of our knowledge, the tristimulus colorimetric analysis was applied for the first time to evaluate the color characters, the differences, and the stability of CFS derived from *L. reuteri*. Only some articles are available in the literature which deal with the color onset in cyanobacteria forming biofilm (Sanmartín et al., 2010, 2012), while some other articles deal with the color evaluation of fermented foods in which lactobacilli were grown (Pelicano et al., 2003; Antunes et al., 2013; Zhu et al., 2020; Wang et al., 2021). In previous studies, the change of color in milk was evaluated in relation to the microorganism growth based on time and temperature (Ziyaina et al., 2019), and the ΔE scale, as proposed by Limbo and Piergiovanni (2006), was used to discriminate colors. The results confirmed that these natural matrices can be subjected to degradation phenomena after 2 and 4 weeks of storage and that they must be conserved at temperatures below 4°C to preserve the bioactive components.

The results indicated that CFS and its sub-fractions from *L. reuteri* DSM 17938 have a promising potential for the eradication of biofilms produced by clinically relevant species that are biofilm producers and responsible for nosocomial and oral infections. The potential of CFS should be thoroughly investigated in order to understand novel inhibitory compounds as new antibiofilm agents. The NMR metabolomic profile discriminated specific metabolites, which, along with MS data, can be considered the fingerprint of these natural matrices. Their stability tested spectrophotometrically for up to 4 weeks also gave new insights concerning storage time within the performance of biological experiments.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author.

Author contributions

RG and SCa designed the project. RG and SCa designed the experiments, discussed the results, and drafted the manuscript. IV, VP, BM, MS, SCe, and FS performed the experiment consisting of the isolation of CFS, evaluation of the antibiofilm activity, and analysis of metabolomic/color profiles. RG, SCa, GG, and SR drafted the final

editing of the manuscript and critically revised it. All authors contributed to the article and approved the submitted version.

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Conflict of interest

SR and GG are currently employed by the company BioGaia AB. RG and SCa have received a research grant from Company BioGaia which partially supported the project.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2023.1128275/full#supplementary-material>

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OPEN Biochemical and functional properties of vesicles from planktonic and biofilm phenotypes of *Limosilactobacillus reuteri* DSM 17938

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Limosilactobacillus reuteri DSM 17938 is among the world's most studied probiotic strains and has been shown to provide several health benefits for the host. We have previously shown that the cell-free supernatant of *L. reuteri* DSM 17938 possesses antimicrobial activity and contains several bioactive compounds. Furthermore, the strain was shown to be a biofilm producer that releases both planktonic and biofilm Membrane Vesicles (MVs). In this study, membrane vesicles isolated from planktonic (pMVs) and biofilm (bMVs) phenotypes were comparatively investigated for their toxicity, ability to kill cancer as well as non-cancer cell lines and modulate phagocytosis in murine macrophages. Neither pMVs nor bMVs showed any in vivo toxicity in a *Galleria mellonella* model, and weakly affected cancer and noncancerous cell viability after both short- and long-term treatments. However, they were able to affect phagocytosis in lipopolysaccharide challenged RAW 264.7 macrophages, suggesting possible immunomodulatory properties. NMR-based metabolomic analysis of pMVs and bMVs identified and quantified engulfed compounds, mainly organic acids and amino acids, with lactate being the most abundant molecule in both vesicle types. bMVs contained higher concentrations of all measured metabolites compared to pMVs. Proteomic analysis of pMVs and bMVs described equivalent protein cargos, emphasizing quantitative compositional differences that presumably reflect the physiological state of each parent bacterial phenotype. Through the assignment of molecules possibly acting as mediators of immune/inflammatory responses in the host and/or modulating known beneficial effects of *L. reuteri*, important signaling functions of these vesicles were suggested. Finally, storage stability of MVs up to four weeks was established.

Keywords *Lactobacillus reuteri*, *Limosilactobacillus reuteri*, Extracellular membrane vesicles, Proteomics, Metabolomics, Cytotoxicity, Probiotics, Postbiotics, Immunomodulatory properties

Nowadays, the beneficial effects of probiotics on human health have been widely recognized. Probiotics can exert their function in different ways, for instance by modulating the immune system or affecting the microbiota by e.g. counteracting intestinal pathobionts and pathogens. In this context, different studies have proven pathogens inhibition and disease mitigating properties of probiotics^{1,2}. The health-promoting effects of probiotics are

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strain-specific and can be mediated both through direct interactions with host cells and through the release of functional bacterial products^{2,3}. Among these, several metabolites and proteins (e.g. lactic acid, bacteriocins, quorum sensing molecules and enzymes) are known to have antioxidant, immunomodulatory and antibacterial properties and seem to exert antiproliferative activity on cancer cells^{3,4}. Membrane Vesicles (MVs) are also included in the Cell-Free Supernatant (CFS) of probiotics, thus their characterization is essential to better describe how bacteria interact with the host and the surrounding environment^{5,6}. All these active components can be part of postbiotic products that, according to the definition recently proposed by the International Scientific Association for Probiotics and Prebiotics, is “a preparation of inanimate microorganisms and/or their components that confers a health benefit on the host”⁷. Consequently, many scientists included the CFS of probiotic bacteria in postbiotic formulations whose composition varies according to the growth conditions and is unique for each bacterial strain^{8–10}.

More than 15 years of research recognizes the efficacy of *Limosilactobacillus reuteri* DSM 17938 in promoting beneficial effects on human health. For instance, Savino and coworkers have demonstrated that the administration of *L. reuteri* DSM 17938 alleviates symptoms in breastfed colicky infants, with a significant reduction of the daily crying time^{11,12}. A clinical trial also proved that a four-weeks supplementation of *L. reuteri* DSM 17938 promotes an increase of bowel movements in adults with chronic constipations, helping to increment the frequency of evacuations per weeks in treated patients¹³. Furthermore, *L. reuteri* DSM 17938 daily administration has been shown to reduce frequency and duration of diarrheal episodes in Mexican day school children aged 6–36 months. The trial has also evidenced that the supplementation of this probiotic strain could be related to the amelioration of other diseases, such as respiratory tract infections¹⁴.

As described above, probiotic properties can be promoted by released bacterial compounds. In this context, our previous works have already proved that the CFS of *L. reuteri* DSM 17938 exhibits both antimicrobial and antibiofilm activity against Gram-positive and Gram-negative bacteria^{15,16}. We showed that the CFS of *L. reuteri* DSM 17938 is directly involved in counteracting clinically relevant pathogens, and this activity can be related to the synergy between various CFS compounds. In our effort to further characterize the secretome of this bacterium, we have focused on *L. reuteri* DSM 17938 membrane vesicles. These lipid bilayer particles are released by bacteria and are loaded with macromolecules such as proteins, glycolipids, lipopolysaccharide (LPS) and nucleic acids; they can differ in dimension, biogenesis and composition depending on the producing strain¹⁷. MVs have been proposed as a system of communication between bacteria and with the host, involved in quorum sensing, biofilm formation and in the modulation of different biological processes⁸. As mentioned, MVs are also involved in microorganism-host interactions, and several authors have recently suggested that MVs released by probiotics may be of importance for the probiotic action^{18–20}. Accordingly, probiotic or postbiotic products containing MVs could potentially be used to reduce the risk for or ameliorate a wide range of diseases, such as chronic inflammatory conditions, neurological disorders, infections and cancer¹⁹.

Depending on its physiological state and the environmental conditions, *L. reuteri* DSM 17938 can have a planktonic or biofilm phenotype, both of which also produce MVs. In this work we have characterized and highlighted differences between planktonic membrane vesicles (pMVs) and biofilm membrane vesicles (bMVs). After isolation of the MVs, they were first comparatively assayed for in vivo toxicological properties in a *Galleria mellonella* model. Thereafter, the ability of pMVs and bMVs to inhibit various human gastro-intestinal cancer cell lines, including extrahepatic cholangiocarcinoma, pancreas adenocarcinoma, hepatoma and triple negative cancer, was evaluated. Tolerability of the MVs was also comparatively assessed in noncancerous cells. In addition, the ability of the MVs to modulate phagocytic properties of murine macrophages, both in the absence and presence of LPS from *Escherichia coli* O111:B4, was evaluated in terms of neutral red dye uptake²¹. Finally, the metabolomic and proteomic cargos of pMVs and bMVs were comparatively investigated to identify possible metabolites and proteins associated with the functional properties (Fig. 1).

Results and discussion

The workflow of the present study is shown in Fig. 1.

L. reuteri DSM 17938 MVs isolation and quantification

L. reuteri DSM 17938 pMVs and bMVs were isolated as previously described and further characterized for their physicochemical properties^{15,22}. The average size and concentration of nanoparticles were investigated via Nanoparticle Tracking Analysis (NTA), and pMVs had an average diameter of 85 ± 4 nm and a concentration of 1.83×10^{11} particles/mL, while the bMVs had an average diameter of 145 ± 2 nm and a concentration of 7.87×10^{10} particles/mL. A Transmission Electron Microscopy (TEM) analysis was performed in order to assess the morphology and the integrity of both MVs phenotypes (Fig. 2).

MVs from *L. reuteri* DSM 17938 are well tolerated by *G. mellonella* larvae

Freshly prepared *L. reuteri* DSM 17938 pMVs and bMVs were comparatively assayed for their in vivo toxicological properties in the *G. mellonella* in vivo model. All larvae were confirmed alive 4 days after treatment. As shown in Fig. 3A, no larvae died after receiving *L. reuteri*-derived MVs and the survival curves of the treated groups were comparable with that of the controls. At the same time, all larvae treated with DMSO died after the injection. These outcomes indicated no toxicity of MVs in *G. mellonella* at the concentration tested. In Fig. 3D–E–F–G, images of the larvae are shown at day 4 after treatment. The larvae appeared responsive to touch and were able to correct themselves when rolled onto their back; in addition, no color change was detected. Similar results were obtained in the *L. reuteri* DSM 17938 treated groups. As shown in Fig. 3B, no larvae died at 4 days post-infection. On the contrary, the infection performed with different concentrations of *Staphylococcus aureus* ATCC 43300 showed a decrease of larval survival over time, confirming previously published data (Fig. 3C)²³.

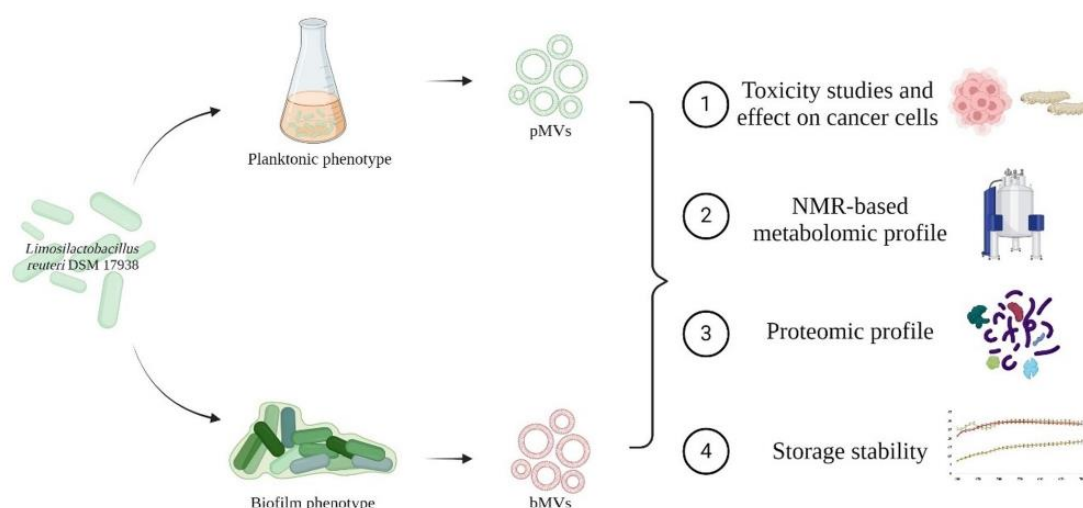


Fig. 1. Flowchart of this study. Membrane vesicles of the planktonic phenotype, pMV; membrane vesicles of biofilm phenotype, bMV.

These preliminary results highlight the safety of both pMV and bMV at a dosage corresponding to 10^8 MVs/larva which represents the highest dosage injectable.

Cytotoxicity studies

MVs effects on the viability of human cancer and noncancerous cells

The cytotoxicity of freshly prepared *L. reuteri* DSM 17938 pMV and bMV (concentration range of 1×10^5 to 1×10^9 MVs/mL in a 1X Dulbecco's Phosphate-Buffered Saline (PBS) solution) was investigated in different human cancer cell lines to highlight a possible antiproliferative activity; similar experiments were accomplished in noncancerous cells to test the tolerability of the treatment. The effect of the treatments was compared to PBS and doxorubicin (10 μ g/mL), included as vehicle and positive controls, respectively. Doxorubicin is widely recognized as a standard cytotoxic agent with multifaceted mechanisms of action, including interference with DNA replication and repair, induction of oxidative stress, thus disrupting key processes involved in cell growth and proliferation²⁴.

According to the ISO 10,993-5:2009 for in vitro cytotoxicity studies, a higher than 30% inhibition of cell viability with respect to the vehicle control was considered as a biologically significant cytotoxicity (toxicity axis in Figs. 4, 5, 6 and 7)²⁵.

After 24 h exposure, both pMV and bMV were nontoxic up to the concentration of 1×10^7 MVs/mL in Bx-PC3 pancreas adenocarcinoma cells. In the same cells, bMV produced a 10 to 20% inhibition of cancer cell viability starting from 1×10^8 MVs/mL, while pMV showed early cytotoxicity signs only at the highest concentration of 1×10^9 MVs/mL (Fig. 4A). Similarly, both pMV and bMV were noncytotoxic to Mz-ChA-1 extrahepatic cholangiocarcinoma and HepG2 hepatoma cells up to the highest concentration (Fig. 4C and E); conversely about a 10% reduction of cell viability was produced in MDA-MB-468 triple negative cancer cells by the two highest concentrations of pMV and only the highest concentration of bMV (Fig. 4G). When the exposure was extended up to 72 h, no cytotoxicity signs were highlighted almost in all the tested cells, except for the highest concentration of pMV and bMV in Bx-PC3 cells and that of pMV in MDA-MB-468 cells, which induced about 15% inhibition in cell viability (Figs. 4B, D, F and H).

In H69 cholangiocytes, both pMV and bMV showed weak cytotoxic effects after 24 h exposure, which were not observed after 72 h (Fig. 5). Indeed, the treatments usually resulted in less than 10% reduction in cell viability compared to the control, except for the two highest concentrations, where pMV and bMV induced 14% and 18% cytotoxicity, respectively (Fig. 5A). This evidence suggests that MVs were generally well-tolerated by noncancerous cells, with a slight lowering in cell viability that cannot be attributed to a biologically significant cytotoxic effect. The latter results confirmed the in vivo toxicity data for pMV and bMV evaluated in the *G. mellonella* model.

Emerging results highlight that probiotic bacteria may exhibit anti-cancer properties, owing to their ability to regulate inflammation, cell proliferation and apoptosis through the release of anti-cancer mediators^{19,26-37}. Among them, bacterial membrane vesicles have been found to be involved in both the pathogenesis and prevention of diseases such as cancers^{38,39}. Despite the cancer promoting abilities highlighted for MVs from *Bacteroides fragilis* (colorectal cancer), *Fusobacterium nucleatum* (colorectal and breast cancer) and *Helicobacter pylori* (gastric cancer), several probiotics have been found to be tumour-suppressive³⁹. Among them, Gurunathan and coworkers reported that *Bacillus licheniformis* nanovesicles induced a 50% inhibition in cell viability and

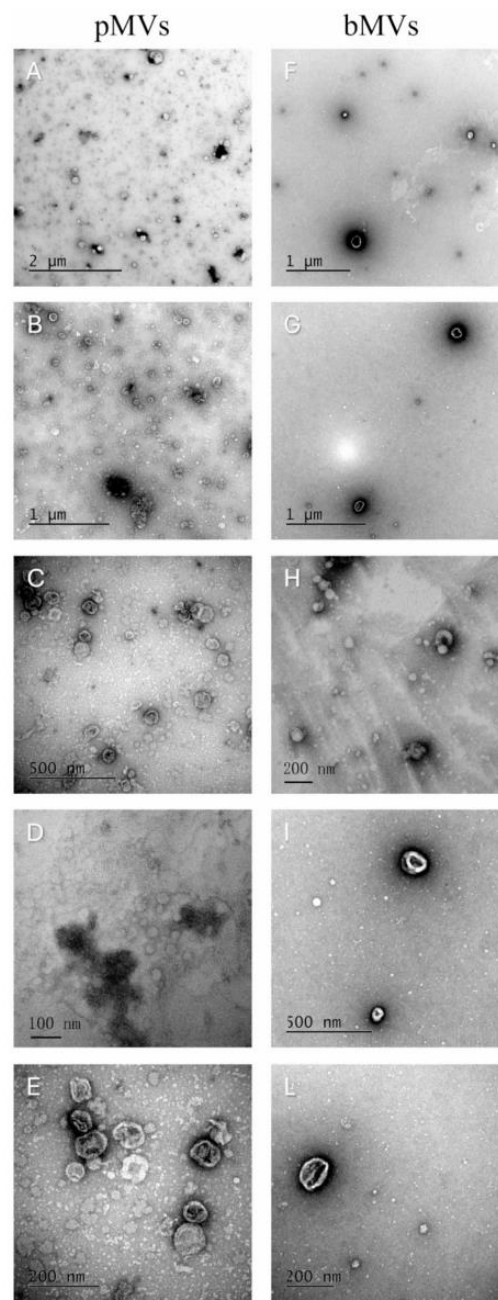


Fig. 2. Images of pMV (A–E) and bMV (F–L) obtained after negative staining and TEM analysis. (A) pMV, scale bar 2 μm , magnification 13,000 $\times$. (B) pMV, scale bar 1 μm , magnification 23,000 $\times$. (C) pMV, scale bar 500 nm, magnification 49,000 $\times$. (D) pMV, scale bar 100 nm, magnification 73,000 $\times$. (E) pMV, scale bar 200 nm, magnification 98,000 $\times$. (F) bMV, scale bar 1 μm , magnification 18,500 $\times$. (G) bMV, scale bar 1 μm , magnification 23,000 $\times$. (H) bMV, scale bar 200 nm, magnification 36,000 $\times$. (I) bMV, scale bar 500 nm, magnification 49,000 $\times$. (L) bMV, scale bar 200 nm, magnification 68,000 $\times$.

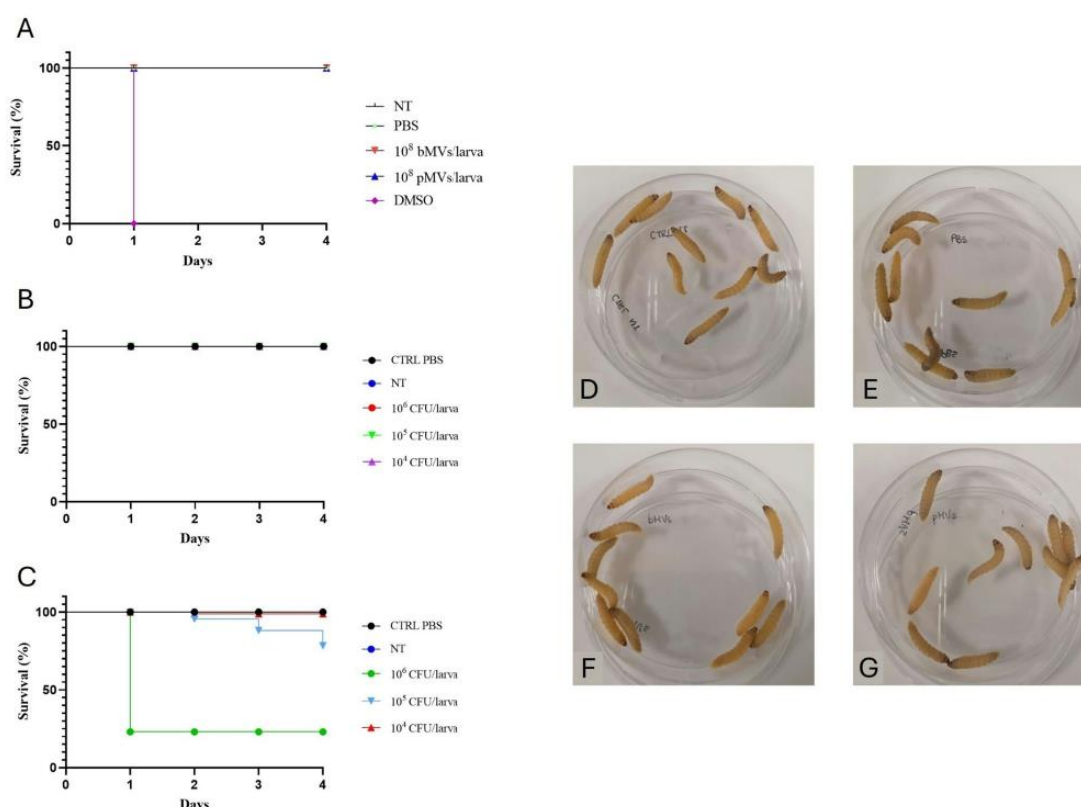


Fig. 3. (A) Effect of freshly prepared *L. reuteri* DSM 17938 MVs on *G. mellonella* survival (Kaplan-Meier survival curve) and *G. mellonella* larvae at day 4 after treatment with freshly prepared *L. reuteri* DSM 17938 MVs. (B) Effect of *L. reuteri* DSM 17938 on *G. mellonella* survival. (C) Effect of *S. aureus* ATCC 43300 on *G. mellonella* survival. (D) Non-treated larvae; (E) PBS-treated larvae; (F) bMV's-treated larvae; (G) pMV's-treated larvae.

proliferation in MDA-MB-231 and A549 cells⁴⁰. Keyani and colleagues highlighted up to a 60% reduction in cell viability of colorectal cancer cells by *Lactobacillus rhamnosus* GG MVs⁴¹, while Behzadi and coworkers reported about a 20% cytotoxicity and pro-apoptotic effects of MVs derived from *Lactobacillus rhamnosus* GG in liver cancer HepG2 cells⁴². An and Ha showed that MVs from *Lactobacillus plantarum* (recently renamed as *Lactiplantibacillus plantarum*) induced a 20% inhibition of cell viability in the human HCT116 colon adenocarcinoma cell line with almost a 60% inhibition in the derived 5-fluorouracil resistant HCT116 cell line⁴³. The marked effect in resistant cells was ascribed to a reduction in the glucose metabolism, associated with a downregulation of pyruvate dehydrogenase kinase 2 expression, suggesting a possible interest in *L. plantarum* MVs to overcome chemoresistance⁴⁴. Accordingly, MVs from *L. plantarum* (Bio-67374) were able to block the proliferation and decrease the glycolytic metabolic reprogramming of human colon cancer cell lines by modulating the SIRT5/p53 axis⁴⁵. Moreover, MVs from *Lactobacillus paracasei* PC-H1 exhibited about a 20% inhibition in cell viability of colorectal cancer cells and in xenograft mice; this effect was associated with a downregulation of the PDK1/AKT/Bcl-2 signaling pathway and a decrease in HIF-1 α -mediated glycolysis⁴³. Recently, the MVs from a *Lentilactobacillus buchneri* strain isolated from an Iranian yogurt were able to inhibit the cell viability of human gastric and colon cancer cell lines (AGS and HT-29, respectively), likely by inducing a cell cycle arrest in G0/G1 phase, apoptosis, and block of cell migration⁴⁶. Although it is known that MVs may deliver diverse bioactive agents, including bioactive proteins, nucleic acids, lipids and metabolites, the true compounds involved in the highlighted anticancer properties have not been clarified.

Under our experimental conditions, pMV's and bMV's from *L. reuteri* DSM 17938 caused up to a 20% decrease in the viability of pancreatic and mammary cancer cells. Specifically, bMV's were more efficacious against pancreatic cancer cells, whereas pMV's showed greater efficacy against mammary cancer cells. These results agree with previous evidence, highlighting anti-proliferative and pro-apoptotic effects of *L. reuteri* strains in diverse cancers^{27–29,36,37}; more recently, Yi et al. found that the extracellular vesicles from *L. reuteri* possessed

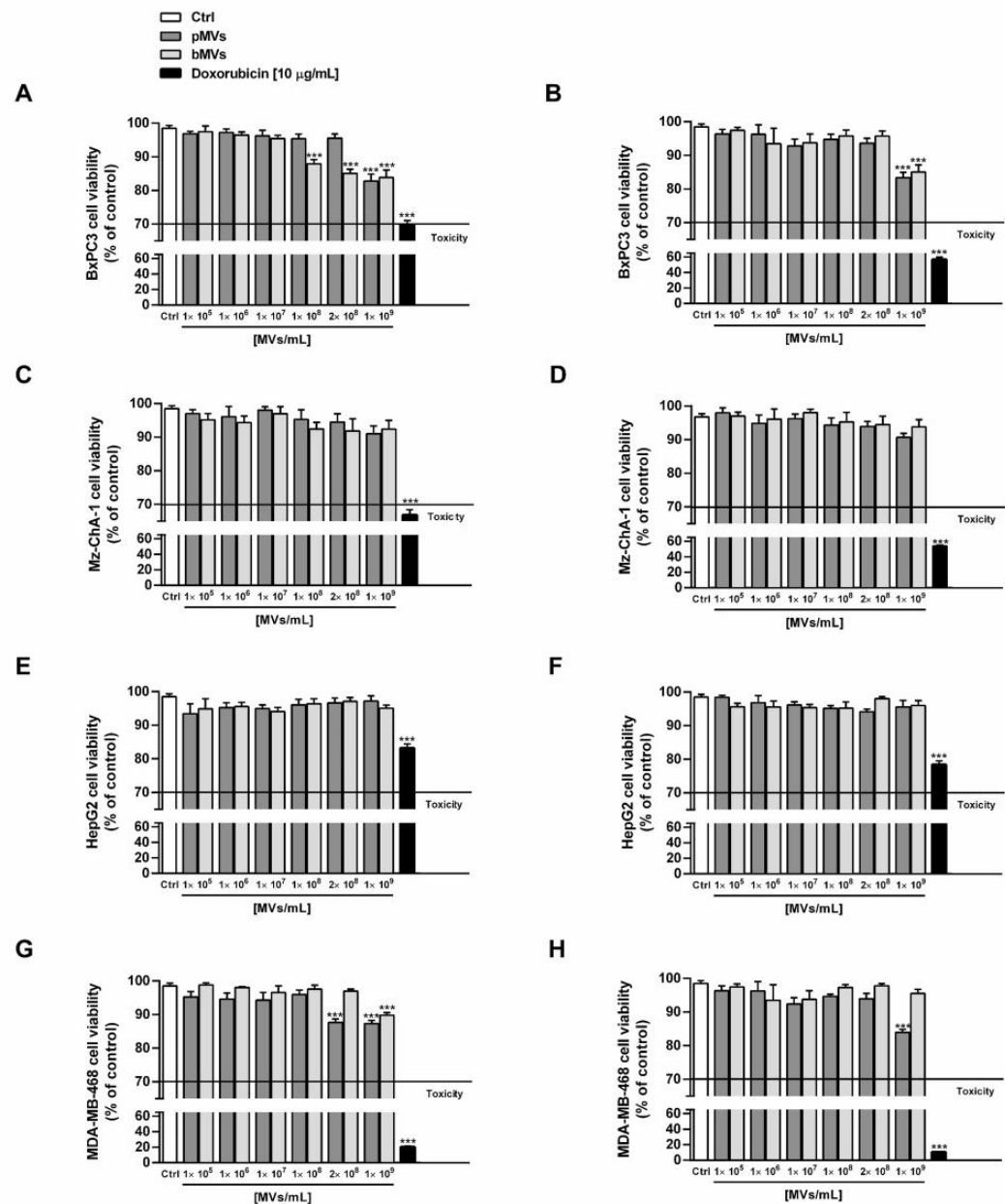


Fig. 4. Effect of freshly prepared *L. reuteri* DSM 17938 pMV and bMV and doxorubicin (black bar, 10 µg/mL) on human cancer cell viability after 24 h (A, C, E, G) and 72 h (B, D, F, H) exposure. In the negative control (Ctrl), the cells were treated with a 1X PBS solution. Bars represent the mean ± standard error of at least six measurements from two independent experiments. A, B. Bx-PC3 pancreas adenocarcinoma cancer cells. C, D. Mz-ChA-1 cholangiocarcinoma cells. E, F. HepG2 hepatoma cells. G, H. MDA-MB-468 triple negative cancer cells. *** $p < 0.001$ vs. Ctrl (ANOVA + Dunnett's Multiple comparison post-test).

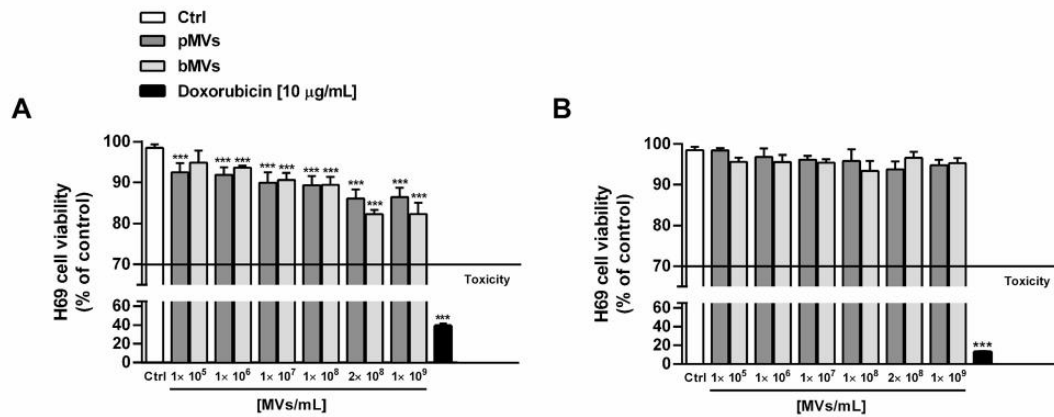


Fig. 5. Effect of freshly prepared *L. reuteri* DSM 17938 pMVs and bMVs and doxorubicin (black bar, 10 µg/mL) on the cell viability of noncancerous H69 cholangiocytes after 24 h (A) and 72 h (B) exposure. In the negative control (Ctrl), the cells were treated with a 1X PBS solution. Bars represent the mean $\pm$ standard error of at least six measurements from two independent experiments. *** $p < 0.001$ vs. Ctrl (ANOVA + Dunnett's Multiple comparison post-test).

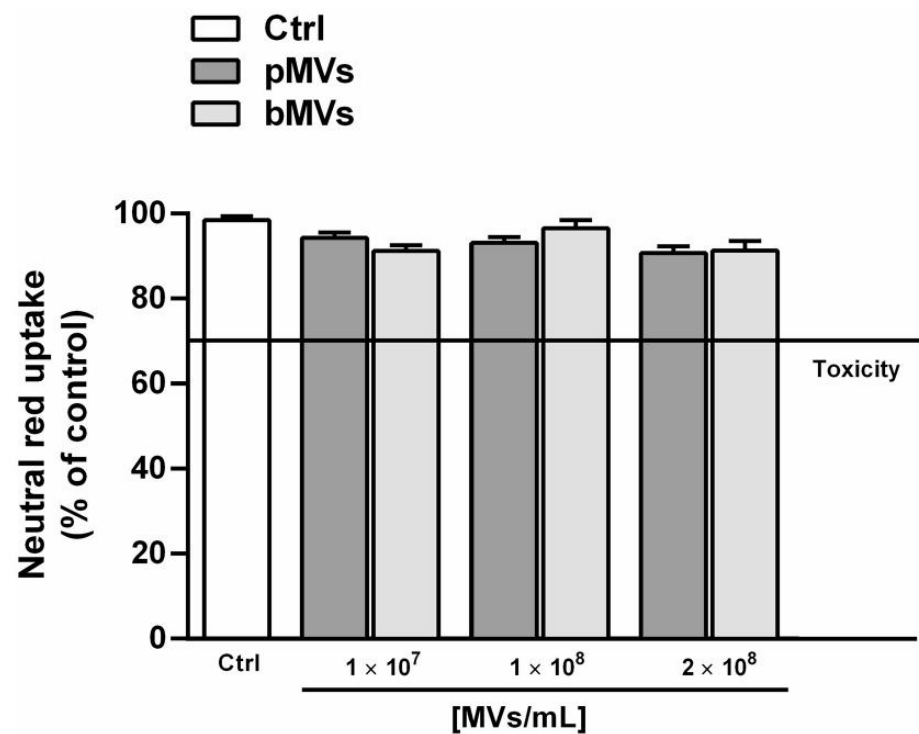


Fig. 6. Effect of freshly prepared *L. reuteri* DSM 17938 pMVs and bMVs on the neutral red dye uptake by murine RAW 264.7 macrophages. In the negative control (Ctrl), the cells were treated with a 1X PBS solution. Bars represent the mean $\pm$ standard error of at least six measurements from two independent experiments.

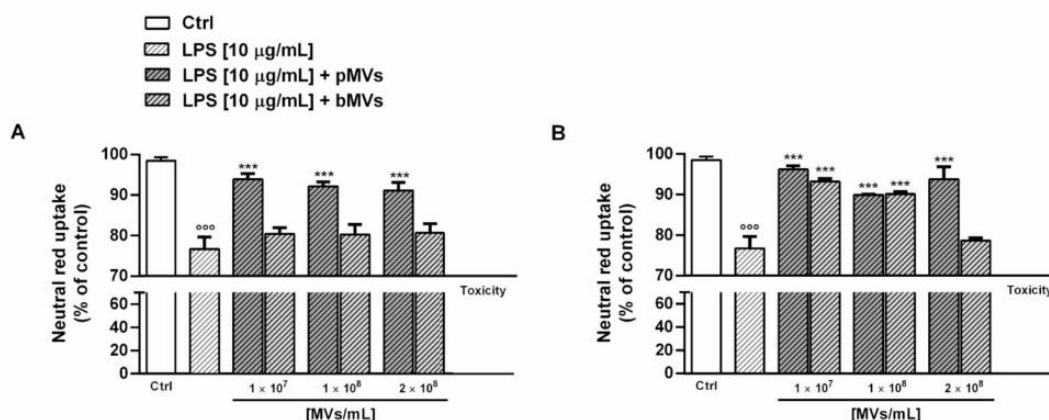


Fig. 7. Effect of pre- (A) and co-treatment (B) of freshly prepared *L. reuteri* DSM 17938 pMV and bMV on the neutral red dye uptake by LPS-induced murine RAW 264.7 macrophages. In the negative control (Ctrl), the cells were treated with a 1X PBS solution. Bars represent the mean $\pm$ standard error of at least six measurements from two independent experiments. *** $p < 0.001$ vs. Ctrl (t-Student test); ** $p < 0.001$ vs. LPS (ANOVA + Dunnett's Multiple comparison post-test).

antiproliferative, antimigration and proapoptotic effects in A549 cells, as well as antitumor activity in a xenograft mouse model⁴⁷.

Interestingly, we found that both types of MVs were weakly cytotoxic in noncancerous cholangiocytes only after a 24 h exposure, without effects after 72 h treatment, likely as a consequence of a cell recovery. Accordingly, Kahouli and colleagues reported well tolerability of *L. reuteri* NCIMB 701,359 in CRL-1831 epithelial normal colon cells³⁷. Based on the available data, we cannot rule out the possibility that MVs selectively affect critical targets for cancer cell survival. Further studies are needed to investigate these specific mechanisms and to clarify this issue.

Our findings also suggest that pMV and bMV from *L. reuteri* DSM 17938 possess distinct chemical and physical features that may account for the observed, albeit subtle, differences in their toxicity toward cancer cells. Both types of MVs from *L. reuteri* DSM 17938 were found to be a source of diverse organic acids, especially lactate, citrate and succinate, and amino acids, such as alanine and glycine, with higher amounts in bMV than pMV. Among them, organic acids have attracted great attention as both oncometabolites and regulators of cell cycle and cancer cell metabolism and survival^{48–50}. In this respect, both in vitro and in vivo studies showed that citrate possessed promising antitumor properties, being able to disrupt cancer metabolism, enhance apoptosis, neutralize tumor microenvironment (TME) acidity, and reduce tumor growth⁴⁹. However, conflicting evidence has been reported for lactate and succinate, being both potentially able to either interfere with tumor cell metabolism and promote tumor growth. For instance, high intracellular lactate levels may induce lactylation and increased expression of genes involved in angiogenesis, immune evasion, and metastasis⁵¹; moreover, the compound may control the cell cycle and proliferation⁵². Similarly, the intracellular accumulation of succinate seems to promote cancer growth and progression, through the activation of genes involved in angiogenesis, glycolysis, and adaptation to hypoxia⁵³. On the other hand, succinic acid exhibited antiproliferative properties and proapoptotic effects in various cancer models, among which breast, renal and lung cancer⁵⁴. Based on these findings, these compounds may contribute to the cell viability modulation by MVs from *L. reuteri* DSM 17938 in pancreatic and breast cancer cells. Their higher amounts in bMV with respect to pMV could explain the higher potency in pancreatic cells. Differences in the cancer cytotoxicity potency of pMV and bMV may also arise from their different size, which could influence their uptake efficiency by host cells. The diameter of bMV from *L. reuteri* DSM 17938, approximately double that of pMV, is similar to that reported for MVs from *L. buchneri* and *L. paracasei* PC-H1, which inhibited the viability of gastric adenocarcinoma and colon cancer cell lines⁴³; however, the true role of vesicle size in their bioactivities is unknown. Altogether, the obtained results suggest a potential ability of pMV and bMV to control cell viability and proliferation, potentially inducing a cell cycle arrest or apoptotic cell death, as previously reported for MVs from other lactic acid bacteria^{41,43}, although the specific underlying mechanisms require further exploration.

MVs effects on the uptake by RAW 264.7 murine macrophages

The effect of freshly prepared *L. reuteri* DSM 17938 pMV and bMV on the uptake by RAW 264.7 murine macrophages was evaluated by the Neutral Red Uptake (NRU) assay, a well-established method for assessing cell viability, wherein viable cells incorporate the neutral red dye, and internalize it inside lysosomes; thus, cytotoxicity arises because of the hindered dye uptake induced by the treatments⁵⁵. In the case of macrophages,

this assay may provide preliminary insights into their uptake capabilities, which are crucial for their immune defense function.

Under the experimental conditions used, the 24 h exposure to both pMV and bMV did not affect NRU until the highest concentration tested (Fig. 6); conversely, the tested MVs restored the basal NRU after a 24 h stimulation by LPS from *Escherichia coli* O111:B4 (10 µg/mL). Indeed, despite a 20% reduction induced by LPS, NRU was more than 90% in the presence of pMV under both pre- and co-treatments (Fig. 7A and B) and in the presence of bMV under co-treatment (Fig. 7B).

Although more targeted studies are needed to confirm the effects of *L. reuteri* DSM 17938 pMV and bMV on the phagocytic activity of murine macrophages, our results suggest possible immunomodulatory properties of the vesicles, as also reported in other studies^{56,57}.

In this respect, Kim and coworkers showed that MVs from *L. plantarum* APsulloc 331261 induced the secretion of the anti-inflammatory and immunomodulatory cytokines in human skin organ cultures and stimulated the transition of HP1 monocytes to the anti-inflammatory and immunomodulatory M2b macrophages⁵⁸. In addition, *L. plantarum* Q7-derived extracellular vesicles have also been shown to regulate intestinal microbiota and ameliorate inflammation in a mouse model of ulcerative colitis⁵⁹. Moreover, a dedicated study demonstrated that *L. reuteri* BBC3-derived MVs maintained the intestinal immune homeostasis against LPS-induced inflammatory responses in broilers⁶⁰. Others have also demonstrated the ability of *L. reuteri* to regulate the immune system in humans and animals^{61,62}.

Hu and colleagues proposed that MVs released by *L. reuteri* BBC3 may inhibit pro-inflammatory mediators produced by activated inflammatory cells and activate innate immune cells to produce immunoregulatory cytokines, ultimately leading to the development of regulatory T cells with anti-inflammatory activities⁶⁰.

In accordance with these findings, we found that pMV and bMV from *L. reuteri* DSM 17938 counteracted the pro-inflammatory stimulation induced by LPS by restoring the basal NRU abilities. LPS is known to polarize macrophages into M1 phenotype, which triggers an inflammatory response and releases pro-inflammatory factors⁶³. Under our experimental conditions, pMV restored macrophage NRU impaired by LPS both in the pre- and co-treatment protocols, while bMV only under co-treatment, suggesting that pMV may either exert a preventive or early-stage effect, potentially by modulating cellular pathways or environments before exposure to LPS, and directly counteract the LPS injury. Similarly, the MVs derived from *L. reuteri* DSM 17938 were found to be able to dampen the inflammatory response induced by *Staphylococcus aureus* in peripheral blood mononuclear cells⁵⁶. Among the identified metabolites, lactate is recognized as an immune modulator, influencing the function of immune cells within the tissue microenvironment, both in normal and pathological conditions. For instance, it reduced the release of proinflammatory cytokines by LPS-stimulated RAW 264.7 macrophages; however, it also induced the expression of inflammatory genes in human monocyte-derived macrophages⁶⁴. Moreover, both succinate and citrate play a pivotal role in modulating inflammation and influencing immunity⁶⁵. Particularly, in LPS-stimulated macrophages as well as in cancer models, succinate accumulation activates a cascade that leads to exacerbation of inflammation and enhanced tumor survival and growth; moreover, a citrate increase has been also found, suggesting its involvement in the activation of inflammatory response. Choi et al. also showed that citrate was able to counteract the pro-inflammatory effects induced by LPS, through a modulation of oxidative stress and inflammatory factors⁶⁶. These findings suggest that the effects of *L. reuteri* DSM 17938 MVs in RAW 264.7 macrophages may be due to the contribution of diverse metabolites, among which organic acids; however, the possible involvement of other unidentified compounds, which may also explain the different potency of pMV and bMV in the pre-treatment, cannot be excluded.

Altogether this evidence suggests an interest in *L. reuteri* DSM 17938 MVs as immunoregulatory and anti-inflammatory factors; nevertheless, additional and more targeted evaluations are needed to gain a deeper understanding of the mechanisms underpinning these effects, to assess their in vivo impact, and to explore their potential implications for human health.

NMR-based metabolomic analysis

To date, the metabolite characterization of *L. reuteri* vesicles has not been reported, as most of the attention has been focused on the corresponding protein content¹⁸. In this paper, we present the first metabolite mapping and characterization of *L. reuteri* vesicles, using NMR. A similar approach was reported for assessing the metabolites within MVs from *Lactobacillus crispatus* and *Lactobacillus gasseri*⁶⁷.

By applying NMR spectroscopy on freshly prepared *L. reuteri* DSM 17938 vesicle samples, using 2D experiments and literature data^{68,69}, we identified six organic acids (acetate, lactate, malate, citrate, formate and succinate), nine amino acids (alanine, valine, glycine betaine, isoleucine, leucine, glycine, phenylalanine, tyrosine and tryptophan) and choline (Table S1). All the listed metabolites were identified and quantified in bMV, whereas tyrosine and tryptophan were not detected in pMV. From a quantitative point of view for each metabolite, bMV were characterized by a higher concentration with respect to pMV. Organic acids represented the most abundant class of compounds in vesicle samples, with lactate being the most abundant acid, measured at concentrations of 713 µg/mL and 183 µg/mL in bMV and pMV, respectively (Table 1). SCFAs represent a crucial point in the continuous cross-feeding and cross-talking among the diverse inhabitants of the microbiota because of their positive effects on the intestinal barrier function, anti-inflammatory properties, and the contribution of daily energy needs⁷⁰. Moreover, the presence of high amounts of lactate is an expected result and represents a positive factor considering the potential use of these vesicles for pharmaceutical purposes. In fact, the synergistic antibacterial activity of lactic acid in presence of other biologically active metabolites has been largely demonstrated⁷¹ and recently D-lactate produced by *Lactobacillus* was shown to regulate gut homeostasis in mice reducing liver fibrosis⁷². It is noteworthy to underline that, up to now, no studies regarding an NMR-based metabolite comparison among planktonic and biofilm MVs have been carried out. Considering the rich

Metabolite	pMVs	bMVs
Lactate	183.02	713.74
Acetate	12.85	19.07
Succinate	14.73	51.99
Citrate	23.88	80.77
Malate	6.63	23.08
Formate	0.45	0.83
Leucine	5.96	20.61
Isoleucine	2.76	11.41
Valine	4.49	15.85
Alanine	18.78	46.60
Glycinebetaine	1.60	5.79
Glycine	15.02	54.20
Tyrosine	–	1.67
Phenylalanine	1.49	9.41
Tryptophan	–	1.56
Choline	0.08	0.33

Table 1. Quantitative results obtained from NMR analysis of freshly prepared *L. reuteri* DSM 17938 vesicle samples subtracted from their blank (PBS). Results are reported as µg/ml of sample. “–” means “not detected” or “not assigned”.

information here obtained, the application of metabolite profile analysis to compare different vesicle groups can be of great interest to better understand their potential activities and uses.

Regarding amino acids, alanine and glycine were measured as the most abundant metabolites. The presence of this class of compounds in the analyzed vesicles can represent an important starting point regarding the potential application of MVs for pharmaceutical purposes. In particular, putative antiviral effect of *L. crispatus* and *L. gasseri* extracellular vesicles have been reported⁶⁷; the authors statistically correlated the above-mentioned biological activity to the high content of some amino acids, including methionine, hypoxanthine, asparagine, glutamate, and glycine.

Proteomic analysis of MVs

To highlight possible functional properties of *L. reuteri* DSM 17938 pMVs and bMVs, freshly prepared vesicles were separately extracted to recover identical amounts of proteins⁷³, which were subjected to proteomic analysis according to the Tandem Mass Tag (TMT)-based approach⁷⁴. Resulting mass spectrometric data were subjected to database search against a *L. reuteri* repository (UniProtKB) leading to the identification of common 296 proteins in pMVs and bMVs, among which 87 showed a fold change ≥ 1.5 depending on the vesicle type. No specific proteins were identified in pMVs that were not assigned in bMVs, and vice versa. Quantitative proteomic data are reported in **Supplementary Table S2**.

Regarding the nature of all the proteins present in pMVs and bMVs, results generally confirmed previous observations on lysed *L. reuteri* vesicles^{60,75}, with variations possibly depending on the different proteomic technologies used in this and other studies. A PSORT3.0-based prediction⁷⁶ of the subcellular localization of the identified proteins showed that 61.5% of them were predicted to be cytoplasmic proteins, 18.6% membrane components, 2.4% secreted proteins, while 17.9% had an unknown localization. The high percentage of cytoplasmic proteins here identified in vesicles was not surprising based on the methodological approach and according to previous studies on other Gram-positive and -negative bacteria^{60,75,77–79}, and may derive from a passive molecular packaging phenomenon during membrane vesicle formation. According to the distribution of biological functions analyzed by GO annotation, most of the identified *L. reuteri* proteins were classified as metabolic and proteolytic enzymes, DNA/RNA-binding proteins, membrane components, chaperone and ribosomal proteins, which are involved in bacterial metabolic, molecular transport, transcription and translation, signaling and stress response pathways (Table 2). In contrast, Pang and colleagues⁵⁶ used a surface shaving-based method and described a significantly greater proportion of proteins of DSM 17938-derived MVs that were predicted to be secreted. We found that several cytosolic proteins identified in *L. reuteri* pMVs and bMVs were previously reported as “moonlighting”^{80–82}, which are highly conserved cytoplasmic proteins that exhibit a different biological function when released or attached to the bacterial cell wall. This is in line with the results of the previously mentioned surface shaving study, in which the authors described several moonlighting proteins to be localized on the surface of the membrane vesicles of *L. reuteri* DSM 17938⁵⁶.

Notably, some of the bacterial proteins identified in pMVs and bMVs were already reported being vesicle mediators of immune/inflammatory responses in the host or associated with beneficial effects of probiotic organisms; they included ABC-type antimicrobial peptide transport system (ATPase component), elongation factor Tu (EF-Tu), chaperonins GroEL and GroES, enolase and mucus adhesion promoting protein (MapA), the latter also being named collagen-binding protein (CnBP)⁵⁶. The ABC-type antimicrobial peptide transport system belongs to the group of transporters potentially involved in bacteriocin/antimicrobial peptide export, self-immunity and resistance, and it was described influencing the local microbial ecology^{75,83}. Moonlighting

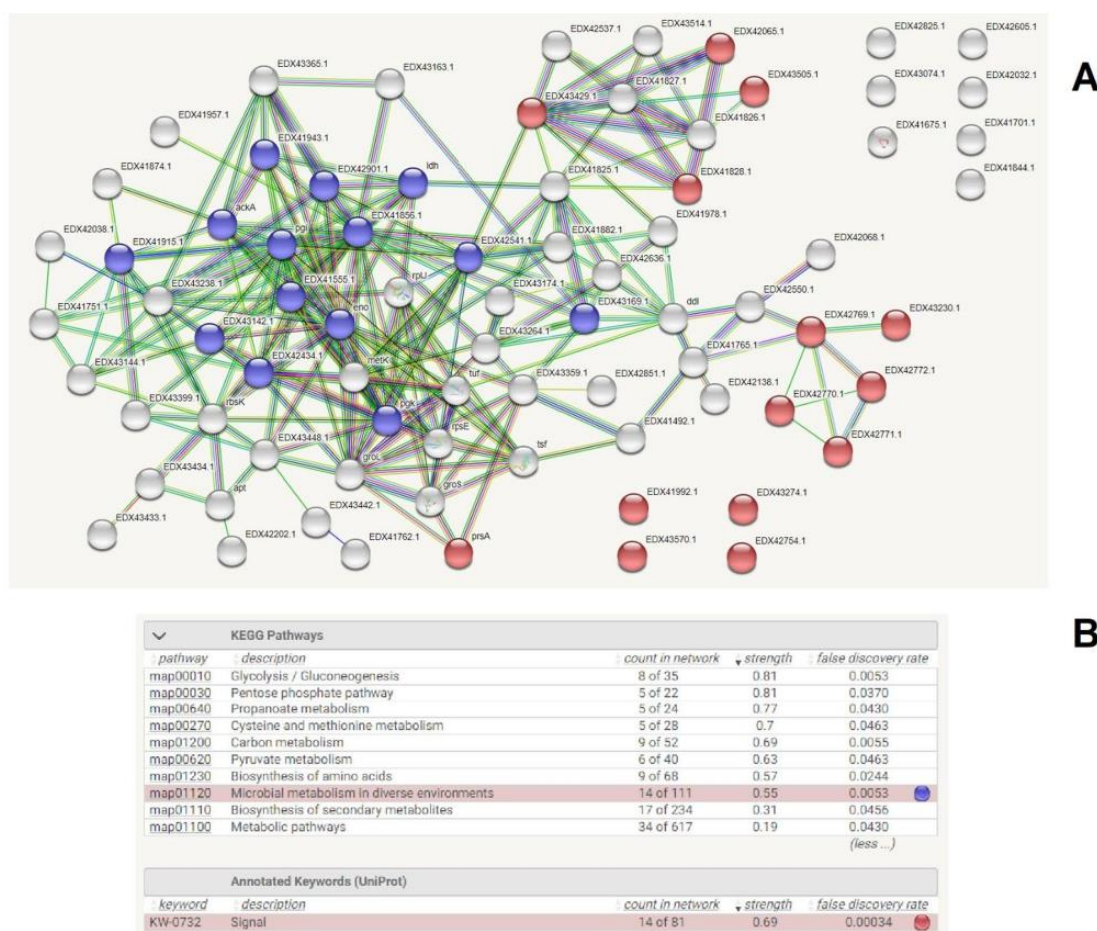
KEGG pathways				
Pathway	Description	Count in network	Strength	FDR
Map00261	Monobactam biosynthesis	4 of 4	0.94	0.0393
Map00020	Citrate cycle (TCA)	5 of 7	0.80	0.0388
Map00010	Glycolysis/Gluconeogenesis	18 of 35	0.65	4.14e-05
Map00030	Pentose phosphate pathway	11 of 22	0.64	0.0030
Map01200	Carbon metabolism	23 of 52	0.59	1.50e-05
Map00190	Oxidative phosphorylation	8 of 18	0.59	0.0305
Map00620	Pyruvate metabolism	17 of 40	0.57	0.00041
Map00270	Cysteine and methionine metabolism	11 of 28	0.54	0.0132
Map00640	Propanoate metabolism	9 of 24	0.52	0.0388
Map01120	Microbial metabolism in diverse environments	40 of 111	0.50	3.36e-07
Map01230	Biosynthesis of amino acids	24 of 68	0.49	0.00014
Map03010	Ribosome	19 of 59	0.45	0.0026
Map01110	Biosynthesis of secondary metabolites	57 of 234	0.33	7.10e-06
Map01100	Metabolic pathways	116 of 617	0.22	3.36e-07

Table 2. Functional analysis of whole proteins identified in freshly prepared *L. reuteri* DSM 17938 pMV and bMV. Proteins identified within the UniProtKB *L. reuteri* repository (296 proteins in number) were subjected to functional enrichment based on KEGG database. Top-14 enriched pathways are reported. Shown is the pathway code, the corresponding description, count in network, strength and false discovery rate (FDR).

protein EF-Tu has previously been identified in vesicles of various pathogenic and probiotic bacteria^{77,84–90}. As a cytoplasmic factor, EF-Tu is involved in protein translation; when expressed on the extracellular surface of bacteria, it has shown alternative functions including the stimulation of immune responses and promotion of adhesion and invasion⁸⁷. It was speculated that EF-Tu acts as an immunogenic factor that triggers antibody responses^{88,89} and as a binding effector to macrophages through its interaction with fibronectin⁹⁰. Some studies have also demonstrated that EF-Tu mediates the attachment of lactobacilli to mucins and intestinal cells, participating in the regulation of gut homeostasis by evoking a proinflammatory response in HT-29 cells⁹¹. The same capability of binding to mammalian fibronectin was also reported for another bacterial moonlighting protein, namely enolase⁸⁰. When exposed on the bacterial membrane, moonlighting chaperones GroES and GroEL have been shown to act like adhesins sustaining the microorganism binding to the host tissues, also exerting a stimulation of the immune system^{92,93}. In addition, GroEL was demonstrated to induce tolerogenic dendritic cells and mediate TLR-2 dependent immunoregulatory effects^{94,95}. Finally, MapA/CnBP was shown to participate in the binding of *L. reuteri* to collagen, mucus and Caco-2 cells^{96,97} and it was recently detected on *L. reuteri* DSM 17938 MVs⁵⁶. Taken together, these findings confirmed that pMV and bMV carry multiple immunoregulatory proteins, as previously suggested by other researchers^{57,60}, potentially explaining their effect on regulating phagocytosis in LPS-induced RAW 264.7 macrophages (Fig. 7).

Quantitative proteomics recognized 87 proteins that showed a fold change ≥ 1.5 in pMV and bMV, consistent with the notion that vesicle content may differ depending on the bacterial phenotype, the mode of growth, and presumably the age of the broth culture⁹⁸. Fifty-nine proteins showed higher levels in pMV, while twenty-eight had a higher concentration in bMV. Among the proteins over-represented in pMV, worth mentioning are some of the above-discussed mediators of immune/inflammatory responses, already associated with beneficial interactions of probiotics, namely EF-Tu and MapA. Additional proteins worth to mention include: (i) four Penicillin-Binding Proteins (PBPs) and peptidoglycan hydrolases, which are involved in cell wall biogenesis⁹⁹ and have been suggested to have a possible role in vesicle formation^{56,100}; (ii) universal stress proteins and thiol peroxidase that are involved in the general response of bacteria to abiotic stresses. In pMV, augmented levels were also observed for proteins involved in bacterial cell division/elongation, like DivIVA domain protein¹⁰¹, and carbohydrate metabolism, such as lactose and galactose permease, fructose permease, fructokinase, beta-phosphoglucosyltransferase, UDP-glucose 4-epimerase, pyruvate kinase, and two phosphoglycerate kinase isoforms. Various proteases with different substrate specificity were also highly represented in pMV, namely carboxypeptidase A0 A1 C2GEW9, zinc metalloprotease rseP, peptidase M23 family, dipeptidase PepV and aminopeptidase PepN. Some of the above-mentioned components have already been reported to contribute to the growth of bacteria in their planktonic state^{102,103}.

In the group of proteins more abundant in bMV, worth mentioning are the above-reported moonlighting proteins associated with the binding to other molecules/cells and/or the mediation of host immune response, namely GroEL, GroES and enolase. GroEL was already demonstrated to play a key role in the lactobacilli colonization of organic surfaces and biofilm development^{104,105}. A similar function was also ascribed to enolase, which was demonstrated highly affecting the ability of *L. plantarum* to produce biofilm¹⁰⁶. In the same functional context, several metabolic enzymes with augmented levels in bMV are also worth mentioning, namely 6-phosphogluconate dehydrogenase (PGDH), ornithine transcarbamylase, guanosine monophosphate reductase (GMPR), inosine 5'-monophosphate dehydrogenase (IMPDH), L-lactate dehydrogenase (LDH) and aldehyde dehydrogenase (ALDH) (NAD) family protein. PGDH converts 6-phosphogluconate to ribulose 5-phosphate within the pentose phosphate pathway¹⁰⁷, which was recently shown to impact metabolism, energy and



nucleotide output in *S. aureus*, strongly affecting biofilm formation and resistance to stress stimuli¹⁰⁸. Similarly, ornithine transcarbamylase has recently been demonstrated to participate in the degradation of arginine in *Staphylococcus epidermidis*¹⁰⁹ by catalyzing the formation of ornithine and carbamoyl phosphate¹¹⁰; arginine has a key role in the regulation of cell growth and biofilm formation, as shown in *Streptococcus gordonii*¹¹¹. GMPT and IMPDH are involved in the synthesis of adenine-guanine nucleotides and have been described to greatly affect bacterial proliferation^{112,113}. LDH usually catalyzes the conversion of pyruvic acid to lactic acid¹¹⁴, but it has also been shown to promote the production of phenyl lactic acid, having antimicrobial activity against fungi and bacteria¹¹⁵. Finally, ALDH has already been described to be involved in stress responses in both pathogenic and non-pathogenic bacteria^{116,117}. Based on these proteomic observations and the measured levels of extracellular DNA (eDNA) present on the surface of *L. reuteri* DSM 17938 bMVs²², which has already been demonstrated to be involved in cell aggregation and biofilm formation in *Helicobacter pylori*^{118,119}, we hypothesize that the above-reported vesicular proteins, eDNA and ultimately the bMVs may act as synergistic effectors of microbial adhesion and aggregation, promoting biofilm development and sustaining the corresponding phenotype.

STRING analysis of differentially represented proteins in *L. reuteri* pMVs and bMVs allowed the prediction of a functional protein association map, which was characterized by a ramified network including 77 knots and linking together 66 components, plus 11 non-associated species (Fig. 8A). The involvement of the most differentially represented proteins in this network emphasized the occurrence of a functional assembly bridging different components from specific deregulated metabolic pathways/molecular processes possibly associated with physiological differences of the bacterium having its planktonic or biofilm phenotype. Functional analysis of the components reported in Fig. 8A (according to the KEGG metabolic pathway classification) highlighted selective enrichment of proteins involved in glycolysis, metabolism of sulfur-containing amino acids, pyruvate metabolism, biosynthesis of amino acids and microbial metabolism in different environments (Fig. 8B). Functional analysis of the same proteins, according to annotated keywords in UniProtKB database, highlighted a selective enrichment of bacterial components involved in signaling.

Fig. 8. STRING analysis of differentially represented proteins present in *L. reuteri* DSM 17938 pMV_s and bMV_s. Panel (A). Protein interaction network of differentially represented proteins (87 in number) identified in freshly prepared *L. reuteri* pMV_s and bMV_s. Functional protein associations were based on the corresponding data recorded in the STRING database. Medium-confidence interactions (0.4) are shown. Proteins involved in microbial metabolism in diverse environments and bacterial signaling are shown in blue and red, respectively. ackA, acetate kinase; EDX43163.1, aldo/keto reductase; EDX43448.1, malate dehydrogenase; EDX41751.1, ROK family protein; EDX42770.1, hypothetical protein LOC688242; prsA, ppic-type peptidyl-prolyl cis-trans isomerase; EDX43142.1, 6-phosphogluconate dehydrogenase, decarboxylating; EDX41882.1, putative aspartate aminotransferase; eno, phosphopyruvate hydratase; EDX41874.1, Udp-glucose 4-epimerase; EDX41675.1, histone-like DNA-binding protein wrapping DNA to stabilize it; EDX43505.1, substrate-binding region of ABC-type glycine betaine transport system; EDX42754.1, mannosyl-glycoprotein endo- β -N-acetylglucosamidase; rplJ, ribosomal protein L10; EDX41915.1, phosphoglucomutase/phosphomannomutase alpha/beta/alpha domain I; EDX43238.1, alcohol dehydrogenase GroES domain protein; EDX41957.1, acyltransferase 3; EDX43169.1, cysteine synthase/cystathionine beta-synthase family protein; EDX42851.1, mannosyl-glycoprotein endo-beta-N-acetylglucosamidase; EDX41492.1, membrane-associated zinc metallopeptidase M50; EDX41826.1, polar amino acid ABC transporter, inner membrane subunit; EDX43399.1, beta-phosphoglucomutase; EDX41844.1, putative transcriptional regulator, GntR family; EDX42772.1, peptidase M23; EDX42605.1, histidine triad (HIT) protein; tsf, translation elongation factor ts; EDX42550.1, penicillin-binding protein transpeptidase; EDX42636.1, dipeptidase M20; groS, chaperonin cpn10; EDX43274.1, protein of unknown function DUF1002; EDX41827.1, ABC-type polar amino acid transport system, ATPase component; EDX43144.1, PTS system, glucose subfamily, IIA subunit; EDX43264.1, peptidase M1 membrane alanine aminopeptidase; metK, methionine adenosyltransferase; EDX43570.1, conserved hypothetical protein; pgi, glucose-6-phosphate isomerase; EDX43174.1, peptidase M13 neprilysin; EDX42202.1, conserved hypothetical protein; EDX42038.1, conserved hypothetical protein; EDX42068.1, conserved hypothetical protein; EDX42901.1, alcohol dehydrogenase GroES domain protein; EDX43434.1, inosine/uridine-preferring nucleoside hydrolase; EDX42541.1, glutamine synthase; apt, adenine phosphoribosyltransferase; rbsK, ribokinase; EDX43429.1, ABC-type amino acid transport system, permease and periplasmic component - ionotropic glutamate receptor; EDX43433.1, sodium-dependent nucleoside transporter; EDX42434.1, ribulose-phosphate 3-epimerase family protein; EDX42771.1, hypothetical protein; EDX41825.1, cystathionine gamma-synthase; tuf, translation elongation factor tu; EDX42032.1, universal stress protein; ddl, D-alanine/D-alanine ligase; EDX42537.1, nlpA lipoprotein family protein; EDX41856.1, iron-containing alcohol dehydrogenase family protein; EDX43442.1, membrane protein of unknown function UCP033111; EDX41992.1, conserved hypothetical secreted protein; EDX41978.1, ornithine carbamoyltransferase; EDX41828.1, bacterial solute-binding protein 3 family protein; EDX43074.1, ferritin dps family protein; EDX41701.1, protein of unknown function DUF322; EDX43359.1, peptidase S1 and S6 chymotrypsin/Hap; EDX43514.1, nlpA lipoprotein family protein; EDX41943.1, phosphate acetyltransferase; rpsE, ribosomal protein S5; groL, chaperonin GroEL; EDX43230.1, peptidoglycan-binding LysM; EDX42138.1, cell division initiation protein; EDX43365.1, 1,3-propanediol dehydrogenase; EDX41765.1, penicillin-binding protein, 1 A family; EDX42825.1, redoxin domain protein; pgk, phosphoglycerate kinase family protein; EDX41762.1, conserved hypothetical protein; EDX42769.1, hypothetical protein; EDX41555.1, pyruvate kinase family protein; EDX42065.1, glutamine ABC transporter substrate binding component; ldh, L-lactate dehydrogenase. Panel (B). Functional analysis of differently represented proteins identified in freshly prepared *L. reuteri* pMV_s and bMV_s. Differentially represented proteins (87 in number) were subjected to functional enrichment based on KEGG pathway database. The top-10 pathways are reported. Shown is the pathway code, the corresponding description, count in network, strength and false discovery rate (FDR).

Preliminary vesicle stability studies using reflectance colorimetry

Organoleptic properties of a mixture or a dispersed system are often subjected to rapid physicochemical changes, which are related to different deep modification or degradation processes. Color analysis was recently applied to different systems with the aim to study the corresponding changes over time in relation to product quality and stability¹⁶. In this context, different review studies have explained how CIEL*a*b* color space can be used to monitor microbiological and physicochemical parameters, so that color results can directly be used as prediction parameters to evaluate the shelf-life of a product^{120–122}. Above-mentioned color changes were often associated with microbial proteolytic and lipolytic activities, as well as with independent Maillard reaction and/or oxidation processes.

On this basis, a rapid and inexpensive technique, such as colorimetric analysis, was here used to evaluate the shelf-life of *L. reuteri* DSM 17938 MV_s samples. To this purpose, freshly prepared pMV_s and bMV_s were monitored for their color properties after 0, 2 and 4 weeks of storage at 4 °C (Fig. 9A and B, respectively); corresponding blank samples were also analyzed as control (Fig. 9C). All the recorded color parameters are reported in **Supplementary Table S3**. The data showed color differences among samples, both in terms of elapsed time and of different analyzed samples. pMV_s remained quite similar and faintly greyish color after 2 weeks (pMV_s at t^{2weeks}), either with respect to their initial condition (pMV_s at t⁰) and the initial status of the medium (PBS at t⁰); in the latter case, the calculated color difference ΔE value was 1.82. Conversely, they showed an evident browning after 4 weeks (pMV_s at t^{4weeks}), with a darker and more yellow color, and a ΔE value of 16.41. This color difference can be considered even more significant, as the medium after 4 weeks (PBS at t^{4weeks}) showed an opposite trend of bleaching, already visible after 2 weeks (PBS at t^{2weeks}). Conversely, bMV_s both after 2 and 4 weeks (bMV_s at t^{2weeks} and t^{4weeks}) showed a very little color difference either with respect to their initial

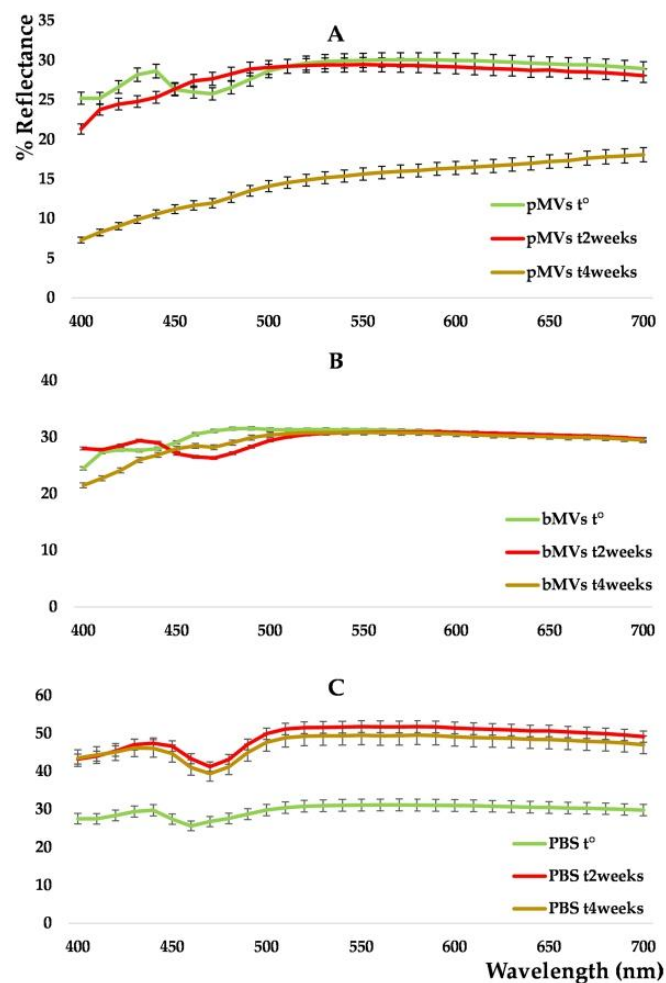


Fig. 9. Reflectance curves of *L. reuteri* DSM 17938 related to membrane vesicles of the planktonic phenotype (pMVs; Panel A), membrane vesicles of biofilm phenotype (bMVs; Panel B) and control samples (PBS; Panel C) after 0 (t^0 , green), 2 (t^{2weeks} , red) and 4 (t^{4weeks} , yellow) weeks of storage at 4 °C. The measurements were carried out at least in triplicate, and the results are reported as mean values standard deviation.

condition (bMVs at t^0) ($\Delta E = 2.24$ and 2.13 , respectively), and to the medium after 0 weeks (PBS at t^0), with $\Delta E = 0.33$ and 1.45 , respectively, thus denoting better stability over time. This trend was confirmed by the reflectance spectra where a general overlapping of the curves was observed only for bMVs (Fig. 9B). To our knowledge, no studies have been reported in the literature, where CIEL*a*b* parameters were used to assess stability of membrane vesicles. In addition, colorimetric analysis can also be utilized to monitor turbidity, as it assesses both light absorption and reflectance in a sample. This property is closely related to the turbidity of the suspension and can provide indirect insights into changes in the concentration or stability of suspended components, such as membrane vesicles. While the absence of significant ΔE variations suggests potential stability of MVs over time, we recognize that colorimetric analysis alone cannot fully confirm vesicle integrity. Without positive control or additional size/concentration data, these results should be considered preliminary and a limitation of this study. However, the use of CIEL*a*b* parameters has been previously validated in literature for shelf-life evaluation in various biological and pharmaceutical systems, supporting its value as a rapid screening tool. Recent studies support the application of colorimeters and spectrophotometers in assessing turbidity alongside color to evaluate physical stability in solutions with dispersed particles^{123,124}. The observed variations in ΔE between pMVs and bMVs align with the distinct metabolomic and proteomic profiles identified through NMR and proteomic analyses. Specifically, the greater color stability of bMVs may reflect their higher concentrations of metabolites and structurally significant proteins, contributing to increased resistance to degradation or aggregation over

time. Conversely, pMVs, characterized by lower metabolite content and differing protein compositions, may be more susceptible to physicochemical changes, influencing the recorded color variations.

Conclusions

This study describes the biochemical characterization and a preliminary functional analysis of pMVs and bMVs isolated from *L. reuteri* DSM 17938. Considering their possible application in postbiotics formulation, the experiments in the *G. mellonella* model allowed to obtain preliminary results regarding the in vivo toxicity of vesicles. The data obtained in this work demonstrated that *L. reuteri* DSM 17938 pMVs and bMVs did not show toxicity at the tested concentration, corresponding to 10^8 MVs/larva. As stated above, the maximum dosage is strictly dependent on the initial count of the samples, evaluated via NTA analysis. Authors recognize that this could be a limitation of the experimental design here used and consider the possibility to further investigate the effect of higher doses over *G. mellonella*. However, this first screening is a valuable starting point to deepen the understanding of the in vivo effect of MVs.

Despite the antiproliferative properties described for other lactobacilli postbiotics in colon cancer cells^{3,4}, *L. reuteri* DSM 17938 MVs only weakly affected the viability of pancreatic, biliary, liver and mammary cancer cells, as well as that of noncancerous cholangiocytes, after both short and long-term treatments. This suggests a low in vitro anticancer power of the MVs, at least in the tested cell models; further studies could be carried out to clarify their cytotoxic effect in other experimental models, especially in colorectal cancer cells. Moreover, under our experimental conditions, pMVs and bMVs were both able to affect phagocytosis in LPS-induced RAW 264.7 macrophages, emphasizing possible immunomodulatory characteristics. Our results confirmed recent observations on *L. reuteri* MVs as extracellular structures able to maintain the intestinal immune homeostasis against LPS-induced inflammatory responses in broilers⁶⁰. NMR-based metabolomic analysis of pMVs and bMVs demonstrated the occurrence of engulfed organic acids and amino acids, whereas bMVs contained the highest concentrations. Proteomic analysis of pMVs and bMVs provided a molecular snapshot of the corresponding bacterial phenotype, highlighting molecular processes and metabolic pathways variably represented in each microorganism physiological state. At the same time, proteomics highlighted specific components possibly exerting signaling functions toward their cellular targets, and molecules acting as mediators of immune/inflammatory responses in the host. The vesicle-mediated character of the delivery system associated with pMVs and bMVs ensures that the latter molecules could reach target cells protected from the degradation by exogenous enzymes. Differential analysis of pMVs and bMVs expanded knowledge deriving from previous studies on MVs from *L. reuteri*^{60,75}, and demonstrated significant quantitative composition differences between protein cargos, whether secreted from the bacterial planktonic or biofilm phenotype. This original information correlates well with other dedicated studies on other Gram-positive bacteria^{102,103,125,126}. At the same time, authors recognize that, due to the protocol used for the MVs isolation, further analysis should be taken into account regarding the vesicles isolated from the biofilm phenotype: the presence of the EPS biofilm matrix can promote the formation of vesicles aggregates which can be retained during the filtration process leading to underestimated results, thus, the authors reserve the right to carry out additional investigations to improve the isolation method and disrupt possible aggregates.

The above reported results indicate that *L. reuteri* pMVs and bMVs have a promising potential as postbiotics modulating the immune/inflammatory responses in the host. Accordingly, novel dedicated studies are encouraged to ascertain the functional properties of *L. reuteri* pMVs and bMVs, with the aim to definitively support their use in nutraceutical and food industries.

Materials and methods

Bacterial strains and culture conditions

L. reuteri DSM 17938 was kindly provided by BioGaia AB (Stockholm, Sweden) and used in the study. The bacteria were plated on DeMan, Rogosa, and Sharpe agar (MRSA; Oxoid Limited, Hampshire, UK), and incubated at 37 °C, for 24 h, in an anaerobic atmosphere (Anaerogen Pak Jar, Oxoid Ltd.).

MVs isolation and *L. reuteri* DSM 17,938 MVs characterization and quantification

L. reuteri DSM 17938 pMVs and bMVs, were isolated from the planktonic and biofilm bacterial phenotypes as previously described²². Briefly, *L. reuteri* DSM 17938 was incubated anaerobically overnight in MRSB under shaking conditions. After incubation, the broth culture was diluted in fresh medium to obtain an optical density (OD_{600}) of 0.10 corresponding to 10^7 CFU/ml, transferred into 90 mm diameter TC-treated Petri dishes (Nunc™ Cell Culture/Petri Dishes, Thermo Fisher Scientific) and incubated at 37 °C anaerobically for 24 h, without shaking. After incubation, the supernatant of each petri dish, containing non-adherent cells, was collected, while the biofilm was washed with PBS, then scraped and harvested in fresh PBS. Planktonic and biofilm suspensions thus obtained were centrifuged for 20 min at $4,000 \times g$ at 4 °C and subsequently filtered with 0.22 µm cellulose membrane filters (Corning, New York, NY, USA) to ensure getting a Cell-Free Supernatant (CFS). To isolate vesicles, both planktonic and biofilm CFS were centrifuged at 50,000 rpm for 2 h at 4 °C using a Beckman Coulter Optima XL-100 K ultracentrifuge (Beckman Coulter, USA), washed with PBS and ultra-centrifuged for the second time at the same conditions. Planktonic and biofilm MVs pellets (pMVs and bMVs) were resuspended in fresh PBS and characterized and quantified by using Nanoparticle Tracking Analysis (NTA). After isolation, they were resuspended in PBS and directly tracked with the NanoSight NS300 system (NanoSight™ technology, Malvern, UK). The analysis was performed at the ALFATEST laboratory (Milan, Italy), according to the company's standard operating procedure (Dilution factor – 1:200; laser – 488 nm; camera level – 16; syringe pump speed – 80; detection threshold – 4). To observe both pMVs and bMVs, the vesicles suspension was distributed on a formvar-carbon-coated grid (Electron Microscopy Sciences, Hatfield, United Kingdom), and

negatively stained with phosphotungstic acid solution (1% v/v). Samples were then analyzed with a Talos L120 C-G2 TEM (Thermo Fisher Scientific).

Evaluation of the toxicity of *L. reuteri* DSM 17938 membrane vesicles over *G. mellonella*

MVs were tested over the *G. mellonella* wax moth model to evaluate the corresponding in vivo toxicity. *G. mellonella* larvae were kindly provided by Prof. Maria Luisa Dindo (Department of Agricultural and Food Science, University of Bologna) and stored in dark, at 20 °C²³. Larvae weighing within the range of 200–250 mg were used for the experiment. Each larva was injected in the third left proleg using a Hamilton syringe (Hamilton, Nevada, USA) and received a total volume of 10 µL of pMVs and bMVs. Both pMVs and bMVs were resuspended in phosphate buffer saline (PBS). Each group consisted of 10 larvae, and the groups included: (i) pMVs-treated larvae; (ii) bMVs-treated larvae; (iii) larvae injected with PBS; (iv) untreated larvae (not injected). Considering the initial MVs count, larvae received the highest achievable dose of vesicles corresponding to 10⁸ MVs/head. Another group, consisting of larvae injected with 100% DMSO, was included in order to validate the injection procedure and to mimic the outcome of a toxic effect (positive control) due to the fact that kills the larvae few minutes after the injection. As an additional positive control, larvae were infected with *Staphylococcus aureus* ATCC 43300 (10⁶, 10⁵ and 10⁴ CFU/larva), a pathogen that allows to observe the reduction of survival through time. Lastly, larvae were also challenged with *L. reuteri* DSM 17938 (10⁶, 10⁵ and 10⁴ CFU/larva) to compare probiotic with pathogen. After the injection, larvae were housed in petri dishes, at 37 °C, and were monitored daily for 4 days to score mortality. Two independent experiments were performed.

Cell cultures

Mz-ChA-1, HepG2 and H69 cells were kindly provided by Prof. G. Alpini (Indiana University School of Medicine, Indianapolis, IN) and Prof. R. Mancinelli (Dept. of Anatomical, Histological, Forensic and Orthopedic Sciences, Sapienza University of Rome). Bx-PC3, HepG2, MDA-MB-468 and RAW 264.7 cells were purchased from Interlab Cell Line Collection (IRCCS San Martino Polidinic Hospital, Genova, Italy). The cells were cultured under standard conditions (37 °C and 5% CO₂ atmosphere), in the recommended media¹⁵, which were renewed twice a week. To evaluate cytotoxicity and phagocytic abilities, the cells were allowed to grow into a 96-well microplate (2 × 10⁴ cells/well) for 24 h after seeding, and then subjected to the treatment with progressive dilutions of pMVs and bMVs for further 24 h and 72 h.

After treatments, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was performed to determine the cytotoxic effects of the treatments. Briefly, 10 µL of 5 mg/mL MTT solution was added to each well, and the plate was incubated at 37 °C for 80 min. Thereafter, the culture medium was discarded, and 200 µL of DMSO was added to each well to dissolve the formazan crystals, directly correlated with cell viability, whose absorbance was measured at 595 nm using a microplate reader (Epoch Microplate Spectrophotometer, BioTek).

Phagocytic abilities of RAW 264.7 macrophages were evaluated in terms of capacity to incorporate and accumulate neutral red (NR) dye within the cells. To this end, NR dye (50 µg/mL) was added to cells for 30 min; then, cells were washed twice in PBS to remove any residual dye and incubated at 4 °C with a 1:1 mixture of ethanol and glacial acetic acid (200 µL/well) for further 120 min to enable the release of NR dye taken up by lysosomes. Thereafter, the neutral red absorbance was measured at 515 nm by an Epoch microplate spectrophotometer (BioTek). The ability of MVs to modulate neutral red uptake by RAW 264.7 cells was assessed under both basal conditions and following the LPS stimulation. To this end, two different protocols were applied: a pre-treatment of 24 h with MVs followed by a 24 h of LPS stimulation, and a co-treatment in which MVs and LPS were administered simultaneously.

To ensure reliable results, the experiments were performed in triplicate for each experiment, with at least two independent experiments conducted. The reduction in cell viability induced by the treatment was evaluated by comparison with the number of viable cells in the vehicle control (1X PBS). A treatment was considered cytotoxic when the cell viability was less than 70% with respect to the control²⁵. Data are expressed as mean ± SE (*n* = 6). A concentration–response curve was obtained using Hill equation, according to previous methods¹²⁷. A significant lowering (*p* < 0.05) in cell viability by treatments with respect to the vehicle control was evaluated by one-way analysis of variance (one-way ANOVA), followed by Dunnett's multiple comparison post-test. Statistical analysis was performed by GraphPad Prism™ (Version 5.00) software (GraphPad Software, Inc., San Diego, CA, USA).

NMR-based metabolomic analysis

One mL of each sample was lyophilized and dissolved in 750 µL of 200 mM phosphate buffer/D₂O, containing 1.4 mM 3-(trimethylsilyl)propionic acid sodium salt (TSP) as internal standard. To lyse vesicles, each solution was sonicated at room temperature for 15 min and centrifuged for 15 min (6000 × *g*, at 20 °C); finally, 700 µL of each solution were transferred into a 5 mm NMR tube. Analyses were carried out on a Jeol JNM-ECZ 600R equipped with a Jeol 5 mm FG/RO DIGITAL AUTOTUNE probe. Mono-dimensional ¹H and bi-dimensional ¹H-¹H TOCSY, ¹H-¹³C HSQC and ¹H-¹³C HMBC NMR experiments were carried out using the previously reported conditions.

Proteomic analysis

Pellets from pMVs and bMVs were solved with the appropriate amount of lysis buffer (8 M urea, 1% w/v SDS, containing protease inhibitor cocktail). Lysis was performed by sonication on ice for 2 min, and then the samples were kept on ice for additional 30 min. Supernatants were recovered by centrifugation at 6000 × *g* for 15 min, at 4 °C. For quantitative proteomics, protein concentration of samples was determined using the BCA Protein assay kit (Thermo Fisher Scientific), according to manufacturer's instructions.

An aliquot of each protein sample (100 µg) was adjusted to a 100 µL final volume with 100 mM TEAB, and then reduced with 5 µL of 200 mM tris (2-carboxyethylphosphine), for 60 min, at 55 °C. Protein samples were then alkylated by adding 5 µL of 375 mM iodoacetamide for 30 min, at 25 °C, in the dark. Alkylated proteins were then precipitated by addition of 6 vol. of cold acetone, pelleted by centrifugation at 8000 × g, for 10 min, at 4 °C, and air-dried. Each sample was digested with freshly prepared trypsin (ratio of enzyme to protein 1:50) in 100 mM TEAB, at 37 °C, overnight. Resulting peptides from each protein sample were labelled with the TMT Label Reagent Set (Thermo-Fisher Scientific, USA) according to the matching pMV6-TMT6-126, bMV6-TMT6-127, at 25 °C, according to manufacturer's instructions. After 1 h of reaction, 8 µL of 5% w/v hydroxylamine was added in each tube and mixed for 15 min to quench the derivatization reaction. For a set of comparative experiments, tagged peptide mixtures were mixed in equal molar ratios (1:1) and vacuum-dried under rotation. Then, pooled TMT-labelled peptide mixtures were suspended in 0.1% trifluoroacetic acid and fractionated by using the Pierce™ High pH Reversed-Phase Peptide fractionation kit (Thermo-Fisher Scientific) to remove unbound TMT reagents and reduce sample complexity, according to manufacturer's instructions. After fractionation, eight fractions of TMT-labelled peptides were collected, vacuum-dried and finally reconstituted in 0.1% formic acid for subsequent mass spectrometric analysis.

TMT-labelled peptide fractions were analyzed on a nanoLC-ESI-Q-Orbitrap-MS/MS platform consisting of an UltiMate 3000 HPLC RSLC nano system (Dionex, USA) coupled to a Q-ExactivePlus mass spectrometer through a Nanoflex ion source (Thermo Fisher Scientific). Peptides were loaded on an Acclaim PepMap TM RSLC C18 column (150 mm × 75 µm ID, 2 µm particles, 100 Å pore size) (Thermo-Fisher Scientific), and eluted with a gradient of solvent B (19.92/80/0.08 v/v/v water/acetonitrile/formic acid) in solvent A (99.9/0.1 v/v water/formic acid), at a flowrate of 300 nL/min. The gradient of solvent B started at 5%, increased to 60% over 125 min, raised to 95% over 1 min, remained at 95% for 8 min, and finally returned to 5% in 1 min, with a column equilibrating step of 20 min before the subsequent chromatographic run. The mass spectrometer operated in data-dependent mode, using a full scan (m/z range 375–1500, nominal resolution of 70,000), followed by MS/MS scans of the 10 most abundant ions. MS/MS spectra were acquired in a scan m/z range 110–2000, using a normalized collision energy of 32%, an automatic gain control target of 100,000, a maximum ion target of 120 ms, and a resolution of 17,500. A dynamic exclusion value of 30 s was also used. Quadruplicate analysis of each sample was performed.

All MS and MS/MS raw data files per sample were merged for protein identification and relative protein quantification into ProteomeDiscoverer vs. 2.4 software (Thermo Scientific), enabling the database search by Mascot algorithm v. 2.4.2 (Matrix Science, UK) using the following criteria: *L. reuteri* UniProtKB protein database (72,034 protein sequences, 06/2022) including the most common protein contaminants; carbamidomethylation of Cys and TMT6plex modification of lysine and peptide N-terminus as fixed modifications; oxidation at Met, deamidation at Asn and Gln, pyroglutamate formation at Gln as variable modifications. Peptide mass tolerance was set to ±10 ppm and fragment mass tolerance to ±0.02 Da. Proteolytic enzyme and maximum number of missed cleavages were set to trypsin and 2, respectively. Protein candidates were considered confidently identified when they were assigned based on at least two sequenced peptides having an individual Mascot Score ≥25. For quantification, ratios of TMT reporter ion intensities in the MS/MS spectra from raw datasets were used to calculate fold changes between samples. Results were filtered to 1% false discovery rate (FDR). Proteomic data were deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD035326.

Protein cell localization, functional and interaction analysis was performed with PSort3.0 software, Kyoto Encyclopedia of Genes and Genomes (KEGG) database^{128,129} and STRING database¹³⁰ resources, respectively.

Colorimetric analysis

pMVs and bMVs samples in PBS at the standard concentration of 1.83×10^{11} particles/mL and 7.87×10^{10} particles/mL respectively, were analyzed, such as, for their color character with a X-Rite MetaVue™ colorimeter as previously described¹³¹. Measurements were performed under the same experimental conditions on freshly prepared vesicle samples (t^0), and then after 2 weeks (t^{2weeks}) and 4 weeks (t^{4weeks}) after their storage at 4 °C. After 4 weeks, pMVs samples presented traces of mold and the experiment was stopped. Control samples contained only PBS. Cylindrical coordinates C^*_{ab} , h_{ab} and the color distances (ΔE) were calculated with respect to the corresponding data of the control samples at the initial time (t^0), as previously reported¹³². The analysis was performed on two independent samples, each measured in triplicate.

Data availability

Data is provided within the text and supplementary information files.

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Author contributions

R.G. and S.C. designed the project, R.G., S.C., A.D.S., A.S. designed the experiments, discussed the results and drafted the paper. B.M., C.D.A., I.V., V.P., M.G., M.S., M.M., S.F. and F.C. performed the experiment consisting in the isolation of MVs, evaluation of the corresponding toxic and cytotoxic activity, proteomic analysis, analysis of metabolic/color profiles and cell toxicity. R.G., S.C., G.G., S.R., L.E.L. and A.S. drafted the final editing of paper and critically revised the paper.

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Declarations

Competing interests

Authors L.E.L., S.R. and G.G. are currently employed by the company BioGaia AB. Authors R.G. and S.C. have received a research grant from Company BioGaia that supported the project. The remaining authors declare no competing interests.

Additional information

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Paper III

***Bifidobacterium longum* BG-L47®: *in vitro* Antimicrobial Activity of the Cell-Free Supernatant and *in vitro* and *in vivo* evaluation of the Immunomodulatory Effect of the Membrane Vesicles**

Marinacci B. et al. 2026

Introduction

Bifidobacteria were first characterized in 1899 by the French pediatrician Henry Tissier who isolated them from faeces of healthy breast-fed infants (Hidalgo-Cantabrana et al., 2017). In addition to the gastrointestinal tract (GIT) of mammals and non-mammals, Bifidobacteria can be found in sewage and in the blood and oral cavity of humans; the ability to adapt to different ecological niches is strictly species-specific and correlates with the unique pool of genes involved in carbohydrate degradation (Milani et al., 2017; Alessandri et al., 2019). In fact, the catabolism of monosaccharides is a peculiarity of these microorganisms that guides the colonization steps in human host (Hidalgo-Cantabrana et al., 2017). Many studies support the hypothesis that those belonging to the *Bifidobacterium* genus are among the first bacterial species to colonize infants' gut where their growth is supported by a plethora of bifidogenic molecules delivered with breast milk; subsequently, after weaning, the introduction of different carbohydrates and complex substrates induces a shift in the microbiota composition characterized by lower but stable levels of bifidobacteria over time (Solís et al., 2010; Di Gioia et al., 2014; Milani et al., 2017; Turroni et al., 2018). Besides the mother's gut microbiota composition, there are four additional factors influencing the early colonization, to wit: gestation duration; delivery mode (natural or C-section); type of feeding (breast or formula milk); living environment (Milani et al., 2017). Full-term and breast-fed infants show a healthier gut microbiota characterized by the predominance of bifidobacteria and low diversity in the first three months of life; conversely, it has been proved that formula-fed babies develop a microbiota characterized by *Clostridiales*, *Veillonella* and *Lachnospiraceae* over bifidobacteria while *Enterobacteriaceae* are more likely to be found in faeces of preterm infants (Donald and Finlay, 2023).

One of the main benefits of the intestinal colonization by bifidobacteria is their glycans-metabolizing capability which guarantees the degradation of both diet- and host-derived carbohydrates, such as fructans, cellulose, mucin and Human Milk Oligosaccharides (HMOs); the breakdown products released after this metabolic conversion are used by host enterocytes but also by other microorganisms of the resident microbiota, confirming the role of bifidobacteria in cross-feeding strategies (Alessandri et al., 2021). Furthermore, the degradation of HMOs leads to increased acetate production which is known for its protective effect on the gut barrier function, especially when infection occurs (Fukuda et al., 2012). Several

studies suggest that acetate and other Short-Chain Fatty Acids (SCFAs) represent key effectors in the foetal immune development and that the balance of their level throughout life can have a massive impact on human health. Unsurprisingly, one of the hallmarks which correlates with the maturation of an efficient immune system is the high abundance of *Bifidobacterium* during the first three months of life that, as stated before, is associated with an healthy gut microbiota (Donald and Finlay, 2023; Fusco et al., 2023). Other immunomodulatory factors related to bifidobacteria are the surface-associated exopolysaccharides (EPSs), in detail heteropolysaccharides, that can vary not only at the species level but also within strains of the same species, thus conferring them unique properties (Milani et al., 2017). For example, Schiavi and colleagues demonstrated in 2018 that the purified exopolysaccharide from *B. longum* 35624 modulated *in vitro* the response of human monocyte-derived dendritic cells lowering the release of proinflammatory factors and, at the same time, exerted a protective effect in a respiratory allergy murine model (Schiavi et al., 2018). Similarly, López et al., analysing three different *Bifidobacterium* species and a total of eighteen strains, proved that each purified EPS induced the release of a different cytokine pattern from peripheral blood mononuclear cells resulting in distinguished immunomodulatory effects (López et al., 2012).

Bifidobacteria can also release complex lipidic structures, known as extracellular Membrane Vesicles (MVs), that currently are a major area of interest within the field of microbiology since they could be both probiotics- and pathogens-related (Krzyżek et al., 2023; Marinacci et al., 2023; Puca et al., 2024). Nishiyama et al., in 2020, identified several putative mucin-binding proteins in *B. longum* NCC2705 MVs suggesting that those nanostructures might be involved in the colonization of the GIT (Nishiyama et al., 2020). Meanwhile, MVs have been attributed anti-inflammatory properties. Mandelbaum et al. reported that *B. longum* AO44-derived vesicles induced the release of IL-10 from specific pathogen-free murine splenocytes and, as a consequence, triggered the activation of CD4 and CD8 cells (Mandelbaum et al., 2023). Similarly, Nie and colleagues recently proved the anti-inflammatory potential of *B. longum* NSP001 MVs in ulcerative colitis demonstrating that they can downregulate the M1-type macrophages differentiation and reduce the secretion of cytokines, such as IL-6 and TNF- α ; this observation was confirmed *in vivo*, in DSS-induced colitis murine model, where all the clinical parameters – body weight, disease activity index and colon length – improved after vesicle treatment (Nie et al., 2025). On the contrary, Morishita et al. showed that a subcutaneous administration of *B. longum* MVs promoted an increase in TNF- α concentration in a ddY mouse model, highlighting a possible application of vesicles as immunoadjuvant. Thus, this effect was not obtained after an intravenous administration. Moreover, different outcomes were associated with *Lactiplantibacillus plantarum* WCFS1 membrane vesicles leading the authors to conclude that the properties of a vesicle preparation are not only dependent on the administration route but are also strictly related to the bacterial species (Morishita et al., 2023). This observation encourage to consider that the properties of MVs could also vary among strains of the same species giving the different cargo, in terms of protein and nucleic acid content, that they have (Luo et al., 2023).

Thanks to the aforementioned properties, nowadays several strains have been recognized as probiotic *Bifidobacteria* and are currently commercialized (Chichlowski et al., 2020; Sabaté and Iglicki, 2022). In this work we have investigated the *Bifidobacterium longum* subsp. *longum* BG-L47®. As previously reported by Ermann Lundberg and colleagues, who first described *B. longum* BG-L47® in 2024, the safety and tolerability of this new probiotic strain is supported by sequencing analysis and clinical studies: collected data proved that BG-L47® is well tolerated at a dosage of up to 10¹⁰ CFU/day in healthy adults and no significant adverse events occurred (Ermann Lundberg et al., 2024). The aim of the present study is to characterize *B. longum* BG-L47® in terms of antimicrobial and immunomodulatory activity. It is well known that many probiotic bacteria can release a plethora of different bioactive compounds in the culture medium (Maccelli et al., 2020; Vitale et al., 2023), thus the Cell Free Supernatant (CFS) of BG-L47® has been tested versus Gram + and Gram – clinically relevant pathogens, in order to evaluate its effectiveness in counteracting microbial infections. At the same time, the putative immunomodulatory effect of the MVs released by this bacterium in its planktonic phenotype has been tested *in vivo* using the innovative animal model *Galleria mellonella* (Gallorini et al., 2024). Finally, both CFS and MVs have been characterised via NMR and GC-MS analysis in order to identify their composition in terms of metabolites and volatile chemicals content.

Materials and methods

Bacterial strains and culture conditions

Bifidobacterium longum BG-L47®, kindly provided by BioGaia AB (Stockholm, Sweden), was used in this study. The strain was plated on DeMan, Rogosa, and Sharpe Agar (MRSA; Oxoid Limited, Basingstoke, Hampshire, UK) and incubated at 37 °C for 24 h in an anaerobic atmosphere (Anaerogen Pak Jar, Oxoid Ltd.).

Different media and growth conditions were selected for the different bacterial species used in this work. In particular:

- *Escherichia coli* ATCC 25922 (*E. coli*), *Staphylococcus aureus* ATCC 43300 (*S. aureus*), and *Pseudomonas aeruginosa* ATCC 27853 (*P. aeruginosa*) were cultured on Tryptic Soy Agar (TSA) for 24 h in aerobic conditions;
- *Fusobacterium nucleatum* ATCC 25586 (*F. nucleatum*) was cultured on Fastidious Anaerobe Agar (FAA; Lab M, Heywood, UK) supplemented with 5% (v/v) of defibrinated horse sterile blood (Oxoid Ltd.) (FAAS) for 48 h in anaerobiosis (Anaerogen Pak Jar, Oxoid Ltd.);
- *Streptococcus mutans* UA 159 (*S. mutans*) was cultured on TSA agar supplemented with 5% (v/v) of defibrinated horse sterile blood (Oxoid Ltd.) (TSAS) for 24 h at 5% CO₂.

***B. longum* Membrane Vesicles (MVs) isolation**

MVs from the planktonic (pMVs) and biofilm (bMVs) phenotype were isolated according to the protocol previously described (Grande et al., 2017). Briefly, an overnight culture was diluted in fresh medium until reaching 10^7 CFU/ml, dispensed into 90 mm Petri dishes (Corning Incorporated, New York, NY, USA) and incubated for 24 hours at 37°C in anaerobic atmosphere. After incubation, non-adherent cells were harvested, while the biofilms, adhered to Petri dishes surface, were scraped and transferred to 26 ml of Dulbecco's Phosphate Buffer (dPBS). The collected supernatants from planktonic and biofilm cultures were then centrifuged for 20 min, $4000 \times g$ at 4°C. After centrifugation, supernatants were filtered through a 0.22 μ m filter and dispensed into polycarbonate ultracentrifuge tubes (Beckman Coulter, United States). The MVs were isolated using a Beckman coulter Optima XL 100K ultracentrifuge (Beckman coulter, United States) at 50000 rpm, for 2 h at 4°C. After the first step, the pellets were washed with dPBS and ultra-centrifuged for a second time (50000 rpm, 2 h at 4°C). The pellets were then dissolved in 500 μ L dPBS or H₂O, filtered through 0.22 μ m cellulose membrane filters (Corning, New York, NY, USA) and stored at 4°C until use. To ensure the sterility of MVs pellets, a proper amount of MVs were plated onto Mueller Hinton Agar (MHA, Oxoid). The amount of MVs in the pellet was evaluated through Nanoparticles Tracking Analysis (NTA) (Alfatest, Milano).

Production of *B. longum* Planktonic Supernatant (SurP) and Eluted Supernatant (SurE 10K)

B. longum was inoculated in MRSB supplemented with 1 mg/ml of L-cysteine (L-Cysteine hydrochloride, Sigma Aldrich) (MRSB-Lcys) and incubated overnight at 37°C in anaerobic atmosphere under shaking at 125 rpm for 17 hours. After incubation, bacterial culture was diluted into MRSB-Lcys until reaching an optical density (OD₆₀₀) of 0.30 corresponding to 10^7 CFU/ml and it was incubated for 24 hours at 37°C. The Cell Free Supernatant (CFS) indicated as Planktonic Supernatant (SurP), was obtained after centrifugation for 20 minutes at 4°C and $4000 \times g$ and subsequently filtered through a 0.22 μ m cellulose membrane filters (Corning, New York, NY, USA). To separate MVs from other free components, the SurP was concentrated by using Amicon® Ultra-15 10K (10,000 MWCO) Centrifugal Filter Devices (Merck KGaA, Darmstadt, Germany) according to the manufacturer's recommendations. Briefly, 15 mL of the SurP were centrifuged for 40 min at $4000 \times g$ at 25°C. The eluted SurP was called Eluted Supernatant 10K (SurE 10K). SurE 10K was then filtered through a cellulose membrane filter of 0.22 μ m pore size (Corning, New York, USA) and tested for the antimicrobial activity.

Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of Planktonic Supernatant and Eluted Supernatant

The Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) were determined using the broth microdilution method into 96-well polystyrene microtiter plates.

The results were determined by Colony Forming Units (CFU) and confirmed by the hydro soluble formazan XTT assay, as previously demonstrated (Maccelli *et al*, 2020).

In particular:

- *E. coli*, *S. aureus*, and *P. aeruginosa* were grown in Mueller Hinton Broth 2 (MHB 2) (Oxoid) for 16 h at 37 °C under shaking conditions at 125 rpm. The overnight broth cultures were resuspended in MHB2 until reaching an optical density (OD₅₅₀) of 0.4 corresponding to $2-5 \times 10^8$ Colony-Forming Units (CFU)/mL (SPARK[®] Microplate Reader, Tecan, Switzerland). The broth cultures were then diluted in MHB 2 to $2-5 \times 10^5$ CFU/mL per well. The 96-well plates were incubated for 24 hours, at 37°C, in anaerobic conditions.
- *F. nucleatum* was grown in Fastidious Anaerobe Broth (FAB; Lab M, Heywood, UK) for 18 h at 37 °C under shaking conditions at 125 rpm in anaerobiosis condition. The overnight broth cultures were resuspended in FAB to an optical density at 600 nm (OD₆₀₀) of 0.15 corresponding to $2-5 \times 10^8$ CFU/mL, diluted in FAB to $2-5 \times 10^5$ CFU/mL per well. The 96-well plate was incubated for 48 hours, at 37°C, in anaerobic conditions.
- *S. mutans* was grown in Brain Heart Infusion Broth (BHI, Oxoid Ltd.) for 17 h at 37 °C at 5% CO₂. The overnight broth cultures were resuspended to an OD₆₀₀ of 0.35 corresponding to $2-5 \times 10^8$ CFU/mL and then diluted in BHI to $2-5 \times 10^5$ CFU/mL per well. The 96-well plate was then incubated for 24 hours, at 37°C, in 5% CO₂ atmosphere.

After the incubation period, the MIC was defined as the lowest concentration without visible growth. The MBC was defined as the lowest concentration that gave a reduction of CFU with 99.9% compared to the initial inoculum. Two controls were used and they consisted of (i) bacterial broth cultures grown in the appropriate media and (ii) bacterial broth cultures grown with the addition of the maximum concentration of MRSB-Lcys (the media in which the SurP is resuspended).

Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of pMV's and bMV's

The MIC of bMV's and pMV's against bacterial strains were evaluated as mentioned before. The number of MV's isolated by the two phenotypes of *B. longum* BG-L47[®] was determined with NTA, as mentioned above. The pMV's and bMV's were added to the broth cultures from 1×10^7 until 1×10^{11} MV's per well. The results were evaluated with XTT assay and CFU counts.

XTT Metabolic Assay

The MIC and MBC were confirmed by using XTT metabolic assay (Cell Proliferation Kit II XTT, Roche Diagnosis, Mannheim, Germany) according to the manufacturer's recommendations, as previously described (Pérez et al., 2010). The colorimetric assay is based on the cleavage of the yellow tetrazolium salt XTT, added to the sample, to form an orange formazan dye by metabolic active cells. The conversion occurs in the presence of viable cells. The formazan production can be determined by using a plate reader. In brief, the XTT labeling reagent and the electron-coupling reagent were thawed in a water bath at 37 °C and mixed to obtain a clear solution, immediately before use. To confirm the MIC values evaluated by eye, in each well, containing the different broth cultures plus different concentrations of SurP or SurE 10K, 50 µL of the XTT labeling mixture were added to each sample (final XTT concentration 0.3 mg/mL). The microplates containing bacterial species were then incubated for 2 h in the proper atmosphere conditions previously described. The absorbance was then read at 490 nm using a microplate reader (SPARK® Microplate Reader, Tecan, Switzerland). The MIC in the microplate reader was defined as the lowest concentration of SurP, SurE 10K or MVs that caused a significative colorimetric change of the XTT assay in respect to the MRSB-Lcys control.

CFU counts

The MIC and MBC were determined through CFU counts. Briefly, after the incubation period of the XTT assay, 100 µL were taken from the well and they were used for CFU counts. Serial dilutions of the stock were performed in dPBS and plated on the following agar:

- *P. aeruginosa*, *S. aureus* and *E. coli* were plated onto Mueller Hinton Agar (MHA);
- *F. nucleatum* onto FAAS;
- *S. mutans* onto TSAS.

The plates were incubated at 37°C for the suitable period and atmosphere, as previously mentioned.

Cell culture

Undifferentiated human monocytes (CRL-9855™) were purchased from ATCC® and cultured in RPMI 1640 (Merck, Darmstadt, Germany) at 37 °C and 5% CO₂. The growth medium was supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco, ThermoFisher Scientific, Waltham, MA, USA), 1% penicillin/streptomycin (EuroClone S.p.a., Milan, Italy) and 1% sodium pyruvate (Merck, Darmstadt, Germany). Monocytes were differentiated into macrophage-like cells by stimulation with 100 ng/mL of PMA (phorbol-12-myristate-13-acetate, purchased from Merck, Darmstadt, Germany, stock solution 1 mM in DMSO) in complete RPMI for 48 h at 37 °C and 5% CO₂.

Cell metabolic activity (MTS assay)

Undifferentiated monocytes were seeded (5×10^3 cells/well) in 96 multi-well culture plates (Corning™ Falcon™, Glendale, USA) and differentiated as described previously in this section. Then, the differentiation medium was discarded after 48 h and replaced with a fresh one containing complete RPMI (untreated cells = CTRL) or bMVs and pMVs at decreasing concentrations. 5×10^3 /well of undifferentiated monocytes were exposed to vesicles at the same concentrations in parallel.

To induce inflammation, monocytes or macrophages were stimulated with LPS 0.5 µg/mL (lipopolysaccharide from *E. coli*, purchased from Merck, Darmstadt, Germany, stock solution 1 mg/mL in water) and further exposed to vesicles.

At the established time point (24 h), the incubation medium was harvested for further analyses and complete RPMI containing 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) at a concentration of 0.5 mg/mL was added to each well. Cells were incubated for 4 h at 37 °C and 5% CO₂. After that, absorbance was measured at 490 nm using a spectrophotometer (Multiscan GO, Thermo Fisher Scientific, Waltham, MA, USA). The percentage of metabolically active cells in treated cultures was calculated, setting the untreated control to 100%.

Galleria mellonella pre-treated with pMVs and infected with *Pseudomonas aeruginosa* ATCC 39324 and *Staphylococcus aureus* ATCC 43300

In order to investigate the effect of *B. longum* BG-L47® on the modulation of haemocytes from larvae, we pre-treated *G. mellonella* larvae with vesicles and then we infected them with two pathogenic reference strains, namely *Staphylococcus aureus* ATCC 43300 and *Pseudomonas aeruginosa* ATCC 39324. In addition, antibiotic treatment was also administered two hours post infection.

Galleria mellonella larvae were bred at the Department of Agricultural and Food Science (DISTAL) of the University of Bologna (Italy). Only the larvae in their sixth-instar stage (about 2 cm long) weighing 200-250 mg were used in this experiment. Larvae were pre-treated with 10 µL of *B. longum* BG-L47® planktonic MVs (pMVs) at a dosage corresponding to 10^9 MVs/larva. After 24 hours they were infected with 10^6 CFU/larva of *S. aureus* ATCC 43300 and 10 CFU/larva of *P. aeruginosa* ATCC 39324. 2 hours post infection, they were treated with vancomycin and levofloxacin, respectively. The antibiotic doses administered in our experiments have been chosen referring to the human dosage as reported by Hill et al. and Adamson et al (Hill et al., 2014b; Adamson et al., 2015). We also included lower doses of antibiotic to further investigate the application of vesicles as a combined therapeutic strategy. All the treatment and control groups are reported in Figure 5. 24 h post-infection, larvae were sacrificed and their haemolymph was extracted in order to evaluate the modulation of the CD markers expressed on their immune cells (haemocytes): 20 µL of the haemolymph pool collected from 2 larvae belonging to the same experimental groups were mixed with 100 µL of an anticoagulant solution prepared according to García-Carnero et al. (García-Carnero et al., 2020). For each group, a total of 4 larvae were sacrificed.

Haemocytes culture

Isolated haemocytes from untreated larvae or pre-treated with pMV₁ and afterwards infected with *S. aureus* or pre-treated with pMV₁ and afterwards infected with *S. aureus* and exposed to 12.5 mg/mL of vancomycin, were placed in RPMI 1640 (Merck, Darmstadt, Germany) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% penicillin/streptomycin, and 1% sodium pyruvate (all from Gibco, Invitrogen, Life Technologies, Carlsbad, CA, USA) at 37 °C and 5% CO₂. Haemocytes were afterwards observed by means of a phase-contrast microscope equipped with a camera (Leica). Images were acquired and analysed by the Leica Application Suite LAS EZ version 3.4 (Leica, Wetzlar, Germany) after 24 h.

Immunophenotyping of undifferentiated monocytes, *in vitro* differentiated macrophages and haemocytes isolated from *G. mellonella*

The expression of surface markers (CDs) was analysed using flow cytometry as previously described (Gallorini et al., 2024). Briefly, after the exposure times (24 and 48 h), differentiated macrophages by PMA (1.5 x 10⁴ cells/well in 6 well plates) and isolated haemocytes from larvae were incubated with fluorochrome-conjugated anti-human mouse monoclonal antibodies: CD14-FITC, CD44-FITC, CD80-PE, CD163-PE, CD200-PE and CD200R-PE (all purchased by BD Biosciences, MA, USA). Relative fluorescence emissions of gated cells by forward and side scatter properties (FSC/SSC) were analyzed using the CytExpert Software (Beckman Coulter) and results were expressed as the percentage of positive cells for each CD marker. Individual values obtained from independent experiments (n = 6) were summarized as means and standard deviations (Gallorini et al., 2024).

Statistical Analysis

The differences in the means of the results between untreated and treated bacterial strains were evaluated by one way ANOVA (GraphPad Software, San Diego, CA, USA) and Dunnet's and Tukey's multiple comparison test. Media supplemented with MRSB-Lcys at the maximum concentration of CFS tested was used as the control in the statistical analysis. The probability value of $p \leq 0.05$ was considered significantly different.

Results

Determination of MIC and MBC of *B. longum* SurP and Sure 10K

The MIC of SurP and Sure 10K were determined by broth microdilution method. It was shown that both SurP and Sure 10K inhibited the growth of *P. aeruginosa* ATCC 27853 and *E. coli* ATCC 25922 at 15 µL/100 µL. Regarding *S. aureus* 43300 the MIC was of 20 µL/100 µL and the MBC value was of 40 µL/100 µL. The MIC value against *F. nucleatum* ATCC 25586 was of 10 µL/100 µL and the MBC value of 20 µL/100 µL. The highest value of MIC detected was against *S. mutans* UA 159 towards which the MIC value

was of 60 µL/100 µL. The data obtained demonstrated a greater activity towards Gram-negative bacteria than towards Gram-positive bacteria, as previously reported for *L. reuteri* DSM 17938 CFS (Maccelli et al., 2020).

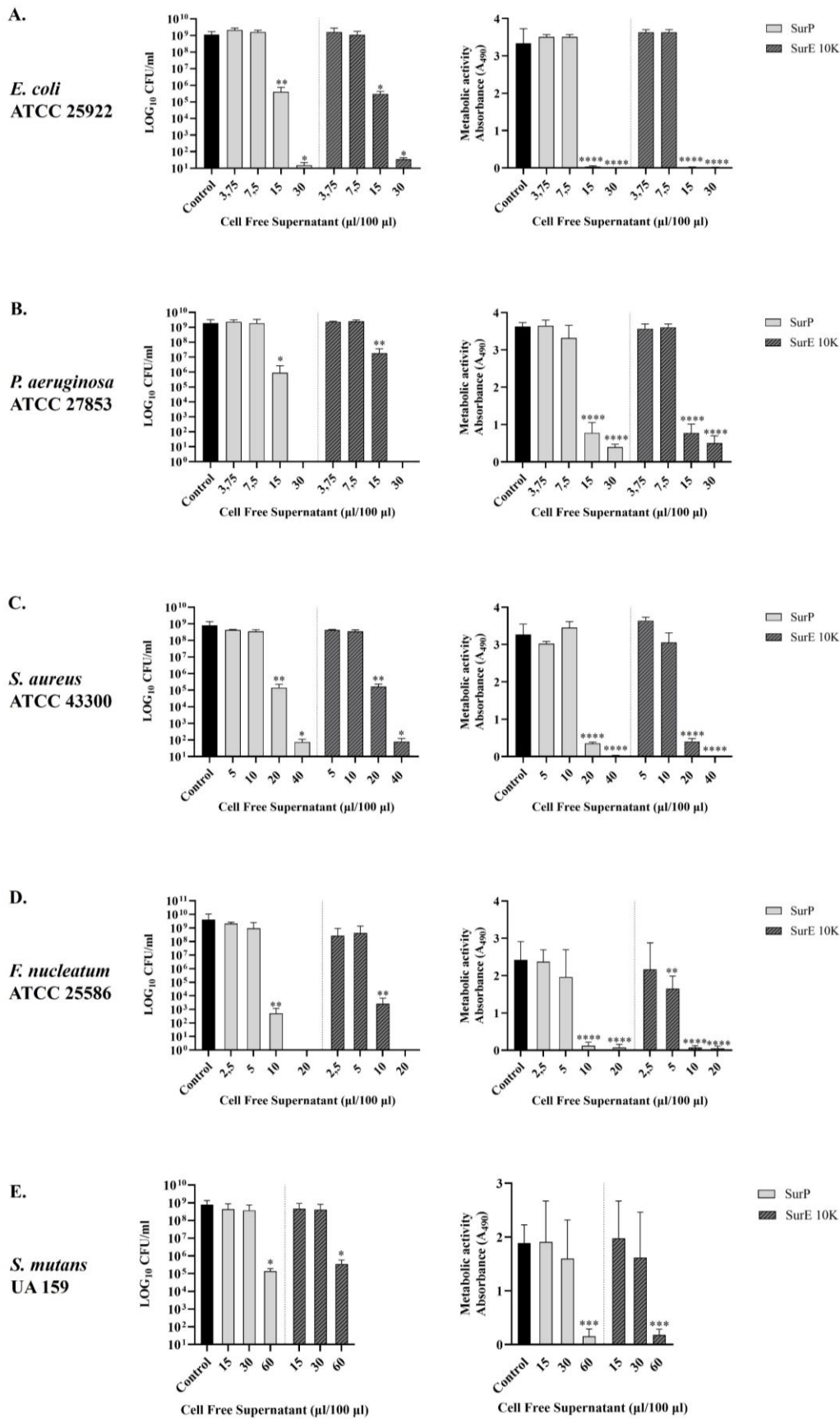


Figure 1. Determination of MIC of SurP and SurE 10K through CFU counts and XTT assay versus *E. coli* ATCC 25922 (A), *P. aeruginosa* ATCC 27853 (B), *S. aureus* ATCC 43300 (C), *F. nucleatum* ATCC

25586 (**D**) and *S. mutans* UA 159 (**E**). Data are presented as the mean of three replicates of two independent experiments. The statistical comparison between control and treated samples was determined with one way ANOVA. The control was composed by media with the supplement of MRSB. The error bars stand for standard deviation. The asterisks stand for p-value: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Bacterial Strains	SurP MIC ($\mu\text{L}/100 \mu\text{L}$)	SurP MBC ($\mu\text{L}/100 \mu\text{L}$)	SurE MIC ($\mu\text{L}/100 \mu\text{L}$)	SurE MBC ($\mu\text{L}/100 \mu\text{L}$)
<i>E. coli</i> ATCC 25922	15	30	15	30
<i>P. aeruginosa</i> ATCC 27853	15	30	15	30
<i>S. aureus</i> ATCC 43300	20	40	20	40
<i>F. nucleatum</i> ATCC 25586	10	20	10	20
<i>S. mutans</i> UA 159	60	>60	60	>60

Table 1: Evaluation of MIC and MBC of *B. longum* SurP and SurE 10K through CFU counts and XTT assay versus *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *S. aureus* ATCC 43300, *F. nucleatum* ATCC 25586 and *S. mutans* UA 159.

NTA results

To determine the concentration of MVs, obtained through ultracentrifugation was used NTA. The results obtained are reported in the table below (Table X). The biofilm MVs appear to be slightly bigger in respect to the planktonic MVs.

	Concentration (MV/ml)	Diameter (nm)	Mode (nm)
pMV s	$5,39 \times 10^{11} \pm 2,98 \times 10^{11}$	$61,25 \pm 2,42$	$55,27 \pm 1,48$
bMV s	$1,85 \times 10^{10} \pm 1,63 \times 10^{10}$	$74,02 \pm 5,14$	$62,6 \pm 12,36$

Table 2: The table reports the average results of counts, diameter and diameter mode of MVs obtained through ultracentrifugation, both from the planktonic and biofilm phenotype.

Determination of MIC and MBC of *B. longum* bMV and pMV

By adding different amounts of pMV and bMV (from 10^7 to 10^{11} MVs/well) it was demonstrated that MVs, isolated from *B. longum* BG-L47®, did not exert any antimicrobial activity versus all the bacterial species tested (Data not shown).

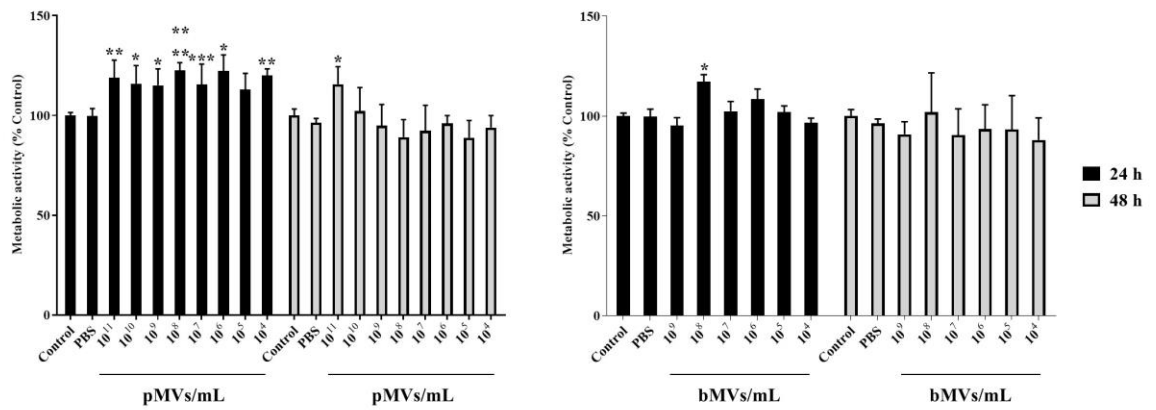
Effect on the cell metabolic activity of undifferentiated monocytes and macrophages of pMV and bMV under basal conditions

To verify the biocompatibility of both pMV and bMV and to investigate their effectiveness on the immunostimulation of both monocytes and macrophages, samples were administered at decreasing concentration on cells under basal conditions. At the established times of exposure, the cell metabolic activity was measured (Figure 2). Undifferentiated monocytes are highly stimulated by pMV, mainly at 24 h. After 48 h, only the highest concentration administered was still effective in increasing cell metabolic activity (Fig. 2A). As for bMV, they seem not significantly effective (Fig. 2A). In parallel, exposing macrophages to pMV revealed a significant immunostimulation, with percentages of cell metabolic activity being increased at almost all the concentrations tested (Fig. 2B).

pMV and bMV showed biocompatibility at all the concentration tested and the cell metabolic activity of both cell types was significantly lower than the one of the controls (untreated cultures). Moreover, when exposing cells to PBS, it was highlighted that the vehicle of MV does not affect cell metabolic activity.

When monocytes or macrophages were exposed to LPS (pro-inflammatory conditions), their metabolic activity increased as a sign of immunostimulation (Fig. 3). LPS-stimulated monocytes exposed to MV increased their metabolic activity after 48 h compared to LPS alone (Fig. 3A) whereas bMV were ineffective. In parallel, both pMV and bMV were extremely effective in decreasing cell metabolic activity of LPS-stimulated cells in comparison with LPS alone, and this effect was dose-dependent after 24 h of exposure.

A.



B.

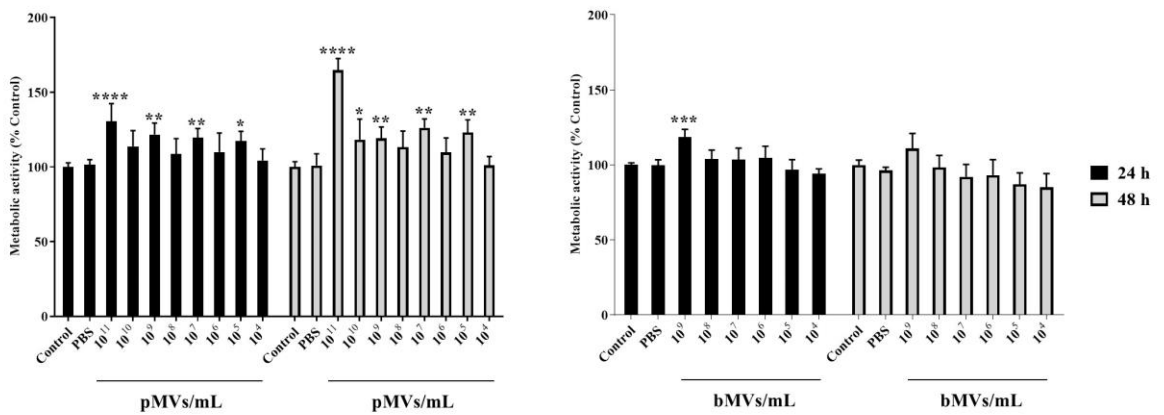
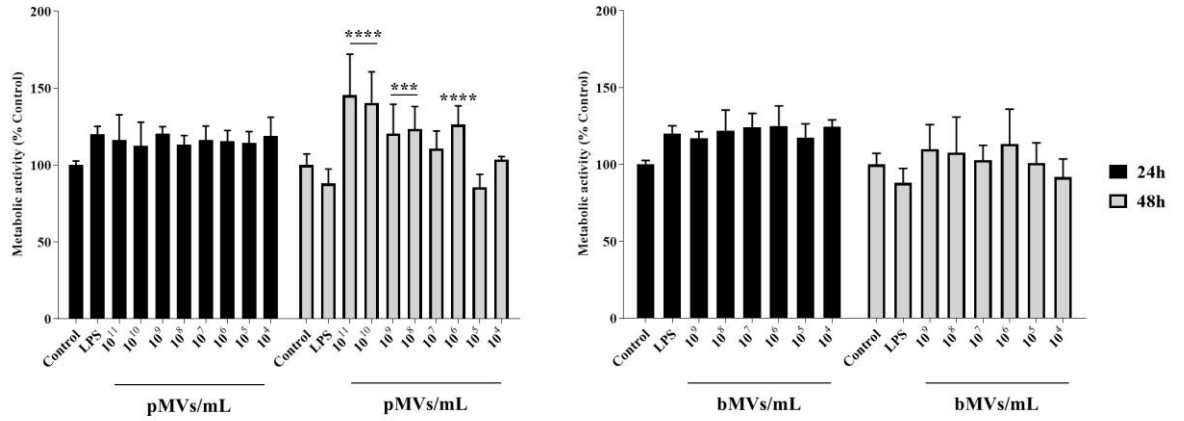


Figure 2: Cell metabolic activity of undifferentiated monocytes (A) and macrophages (B) under basal conditions exposed to decreasing concentrations of pMV and bMV after 24 and 48 h. Bar graphs represent percentages of metabolically active cells normalized on the untreated control set as the 100% (Control). PBS = cells exposed to PBS only (vehicle). *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001 between test samples and control.

A.



B.

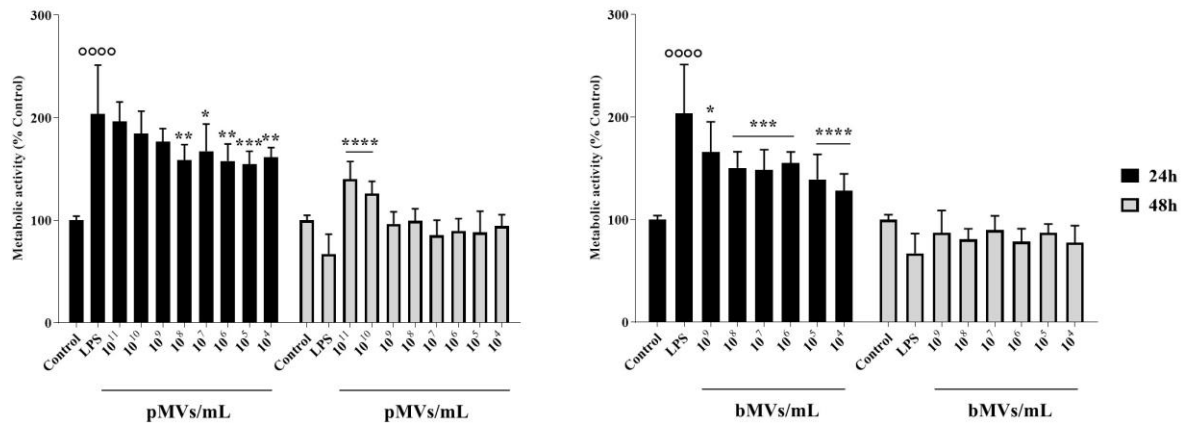


Figure 3: Cell metabolic activity of undifferentiated monocytes (**A**) and macrophages (**B**) under pro-inflammatory conditions exposed to decreasing concentrations of pMVs and bMVs after 24 and 48 h. Bar graphs represent percentages of metabolically active cells normalized on the untreated control set as the 100% (Control). PBS = cells exposed to PBS only (vehicle). LPS = cells exposed to 0.5 μ g/mL of LPS. $^{\circ\circ\circ}$ p<0.0001 between LPS only and control; *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001 between test samples and LPS.

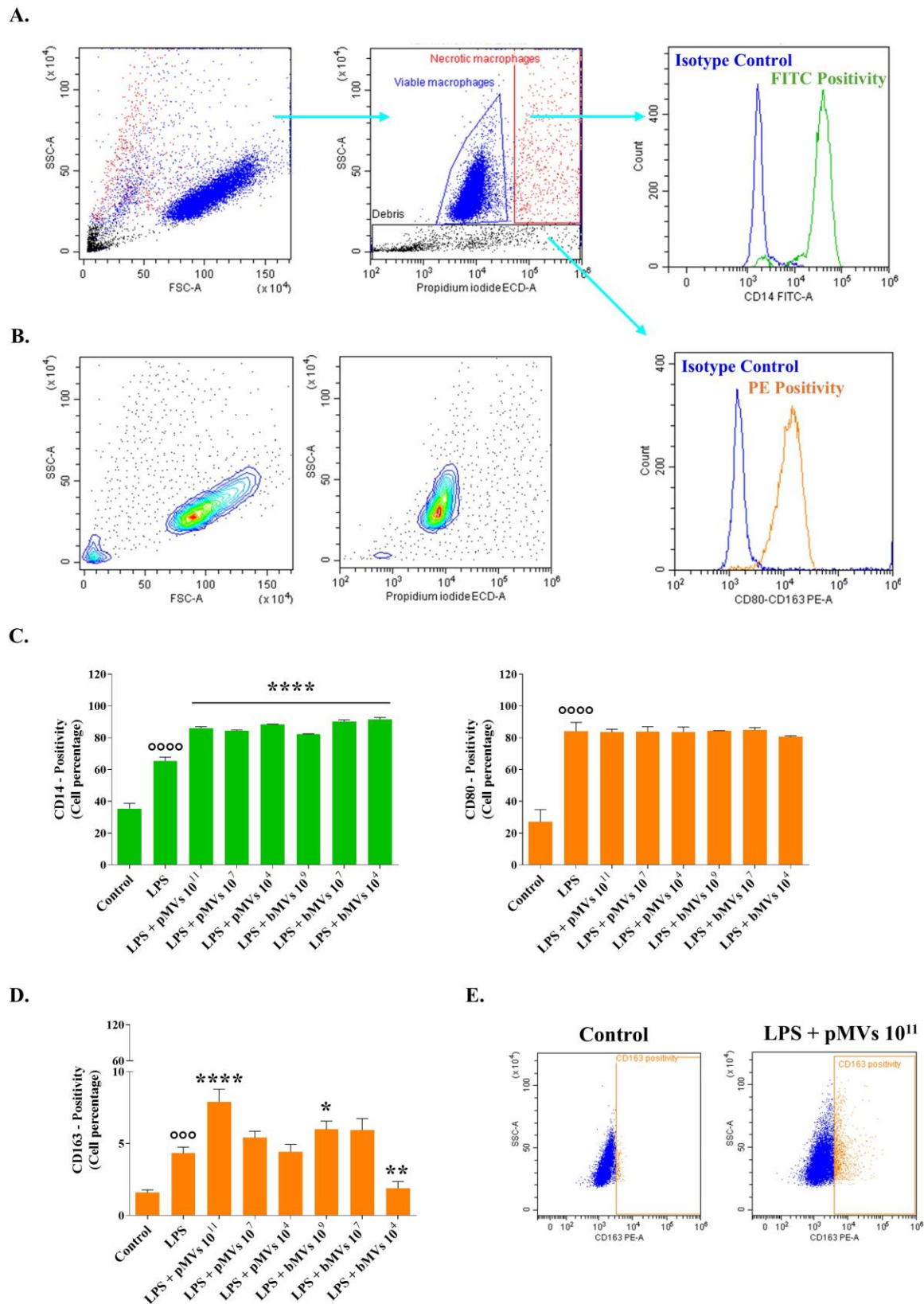


Figure 4: Immunophenotypic profile of LPS-stimulated macrophages in the presence of pMV and bMV at selected concentrations after 24 h. **A)** Gating strategy for the immunophenotype analysis performed by flow cytometry. Side scatter/Forward scatter (SSC/FSC) dot plots represent morphological parameters of unstained macrophages. Cells were incubated with anti-human clusters of designation (CDs)-fluorochrome-conjugated and propidium iodide (PI) in parallel to assess the necrotic cell population. PI-positive cells (red

gate) stained positive were excluded from analyses. The Count/FITC (fluorescein) or PE (phycoerythrin) histograms represent fluorescent emissions proportional to fluorochrome-conjugated antibodies. Right-shifted cells in the FITC and PE gates were considered positive for the marker analyzed. Data were expressed as the percentage of positive cells. **B)** Density dot plots highlight a high number of cells analyzed. **C)** The bar graph shows percentages of cells stained positive for CD14-FITC and CD80-PE and **D)** for CD163-PE. **E)** SSC/PE dot plots generated by flow cytometry related to CD163. $^{\circ\circ\circ}p<0.0001$ between LPS only and control; $*p<0.05$, $**p<0.01$ and $****p<0.0001$ between test samples and LPS.

Effect of pMV's pre-treatment on *G. mellonella* haemocytes

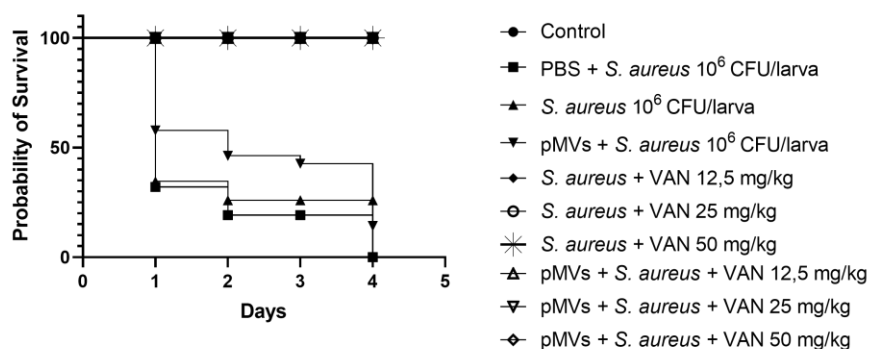
The survival of *G. mellonella* larvae infected by *S. aureus* and *P. aeruginosa*, pre-treated with pMV's and exposed to antibiotics was evaluated (Figure 5) and the modulation of CD14, CD44, CD200 and CD200R was measured in isolated haemocytes to investigate the effects of pMV's in the immunomodulation of the *G. mellonella* system. Firstly, surface markers were detected on haemocytes injected three times with PBS to assess whether injections themselves might influence the immune response (Figure 6D). As shown, only CD14 was significantly modulated, being the mean fluorescent intensity doubled compared to control. Next, the same CD markers were measured on haemocytes infected with *S. aureus* (Figure 6E). As expected, cells from infected larvae showed dramatically increased CD14 expression and CD44 expression, as a sign of immunostimulation. In parallel, CD200 was slightly but significantly upregulated while CD200R was not modulated compared to untreated samples. When larvae were pre-treated with pMV's, the expression of CD14 was significantly reduced to levels comparable to the control samples. CD44 and CD200 were weakly downregulated by the pMV's pre-treatment. When increasing concentrations of vancomycin were injected without the pMV's pre-treatment, CD14 was downregulated to the same extent of samples only pre-treated with pMV's and the same trend was registered for CD44. Moreover, the effect was not dose dependent. In parallel, CD200 was significantly downregulated compared to the control, *S. aureus*-infected larvae and *S. aureus*-infected larvae pre-treated with pMV's while CD200R was upregulated. Likewise, the effect was not dose-dependent from the concentration of vancomycin. Finally, when larvae were pre-treated with pMV's, infected with *S. aureus* and exposed to increasing concentration of vancomycin, CD14 downregulation was even more amplified compared to MV's pre-treatment alone or vancomycin alone. In parallel, also CD44 and CD200 expression decreased while CD200R was upregulated. Again, these effects were independent from the concentration of vancomycin.

Figure 6F shows the immunophenotypic profile of larvae infected with *P. aeruginosa*, pre-treated or not with pMV's and exposed to levofloxacin. Again, haemocytes isolated from infected larvae display a significant CD14 upregulation but not a CD44. Likewise, CD200 and CD200R were not modulated. Haemocytes from larvae pre-treated with pMV's showed a significant CD14 down-regulation compared to infected larvae without pre-treatment. Once again, CD44, CD200 nor CD200R were modulated. When

levofloxacin was administered, a significant dose-dependent decrease of CD14 was registered compared to larvae only infected. The CD44 trend fluctuated, being generally increased with respect to infected haemocytes, but displaying the absolute high amount with levofloxacin 25 mg/Kg. In parallel, there was a clear inverse dose-dependent effect on CD200 expression and a significant direct dose-dependent effect for CD200R expression. When the pMV's pre-treatment was injected and the levofloxacin was administered, CD14 remained stably downregulated. More in details in the presence of pMV's and levofloxacin 2.5 mg/Kg, CD14 expression was lower than the one found on haemocytes in the presence of levofloxacin 5 mg/Kg only. In parallel, the pMV's pre-treatment and the exposure to levofloxacin 2.5 mg/Kg caused a downregulation of CD44 that was lower than the effect of the antibiotic at 20 mg/Kg. The same trend was registered for CD200 and CD200R, being decreased and increased, respectively.

To confirm that the morphology of haemocytes was not affected by the process of isolation, cells were cultured for 24 h (Figure 7). In the control sample, cells were in large numbers, their membranes were not damaged, and they were round-shaped and fluctuating, being coherent with data in literature. When haemocytes are isolated from infected larvae, they were decreased in number, more adherent to plastics and polarized, as a sign of their resemblance to macrophage cells. Finally, when larvae were pre-treated with pMV's and exposed to antibiotics, haemocytes were more proliferating and fluctuating and their initial morphology seemed to be restored.

A



B

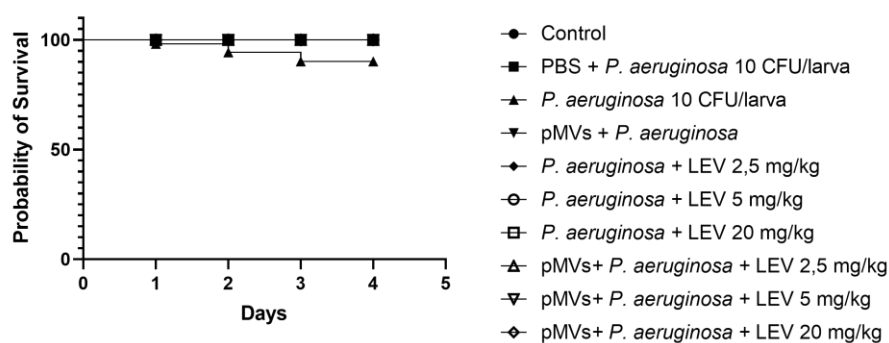


Figure 5: Effect of pre-treatment, infection and antibiotic administration in *G. mellonella* larvae A) *S. aureus* ATCC 43300 infection; B) *P. aeruginosa* ATCC 39324 infection.

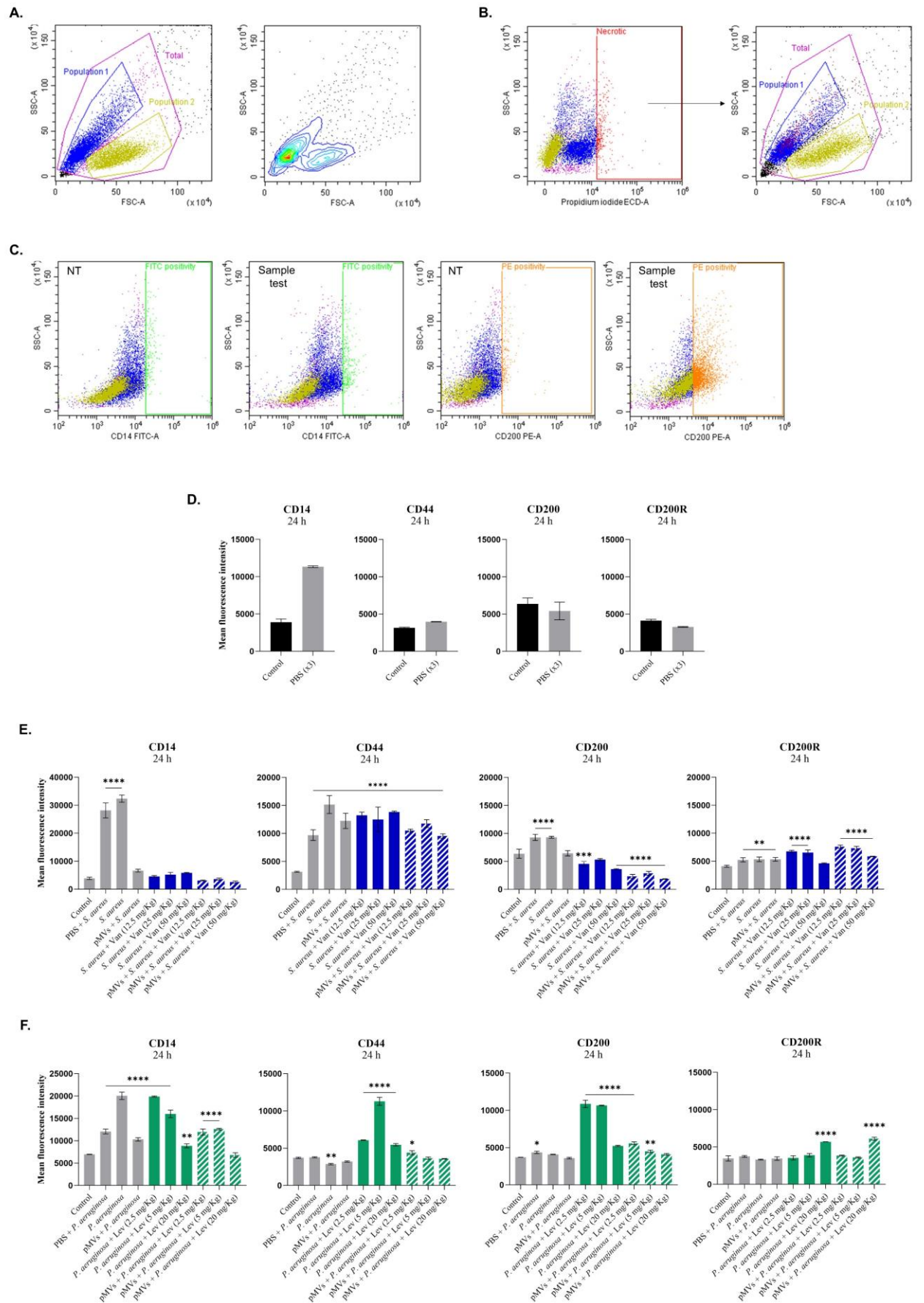


Figure 6: Immunophenotypic profile of isolated haemocytes in the presence of pMV at selected concentrations after 24 h. A, B) Gating strategy for immunophenotyping: dot plots (FSC/SSC) display

populations gated for their morphological parameters (FSC/SSC). Cells stained positive for propidium iodide (necrotic population) were excluded from further analyses. C) Cells were afterwards stained by selected fluorochrome-conjugated monoclonal antibodies (FITC or PE) and the right-shift of fluorescence was measured as the percentage of cells entering the positivity gate, afterwards translated as the mean fluorescent intensity. D) Bar graphs show the immunophenotypic profile of haemocytes from larvae injected three times with PBS compared to the untreated ones (Control). E, F) Bar graphs display the immunophenotypic profile of isolated haemocytes from larvae in the various experimental conditions.

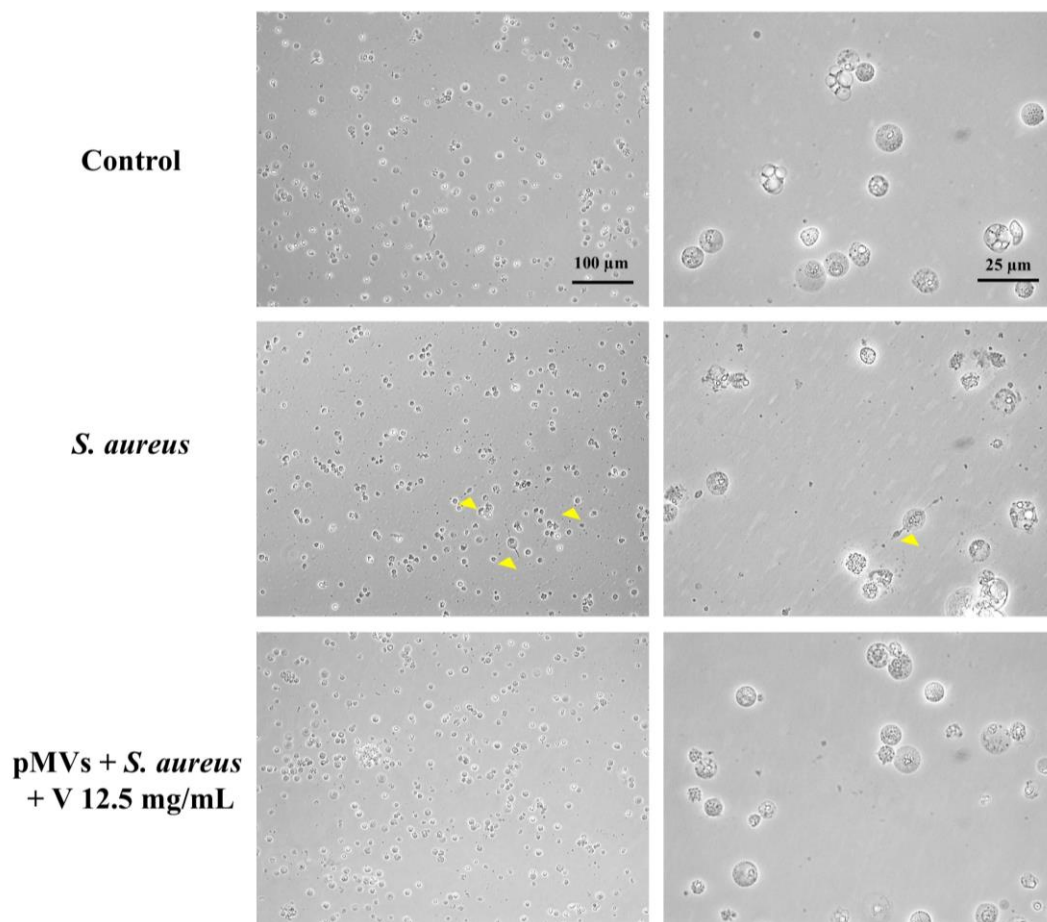


Figure 7: Microscopic images of haemocytes infected by *S. aureus* or pre-incubated with pMVs and afterwards infected with *S. aureus* and treated with vancomycin at 12.5 mg/mL after 24 h of incubation. Relevant differences in cell morphology in the same cell population are highlighted by yellow arrows. Magnification 10x (1 cm = 100 µm) and 40x (1 cm = 25 µm).

Discussion and conclusion

As their definition suggests, probiotics are widely known for their beneficial effects on human host, being their activity associated with several mechanisms, e.g. gut microbiota modulation, pathogens exclusion, immune regulation and preservation of the intestinal mucosa (Sanders et al., 2018). Some of these mechanisms are shared by the majority of probiotics but, in parallel, peculiar features are not widespread and are identified as species- or strain-specific. The latter are relevant to distinguish which

probiotic is the best candidate when a certain health effect is needed: each strain exhibits a distinct efficacy, thus it could be more or less effective than others, depending on the area of application (Hill et al., 2014a; Sanders et al., 2018). Bifidobacteria are among the most well studied colonizer of the infants intestine, specific species seem to be frequently detected during adulthood and, interestingly, others may be also linked to longevity (Hidalgo-Cantabrana et al., 2017; Ku et al., 2024).

Multiple studies have demonstrated that different *Bifidobacterium* spp. possess antimicrobial activity. Cheikhyyoussef et al. demonstrated that the CFS of *B. infantis* was effective towards multiple targets such as *S. aureus*, *Bacillus subtilis* and *B. cereus*, *E. coli*, *Clostridium butyricum* and *Shigella dysenteriae*. In this study authors speculated that the bactericidal effect was due to a bacteriocin-like inhibitory substance released by the probiotic (Cheikhyyoussef et al., 2009). Similarly, *B. breve* and *B. bifidum* showed promising results in counteracting *Clostridioides difficile* and *Streptococcus agalactiae*, respectively. In both cases, the antimicrobial potential was likely attributable to the synergism of several metabolites released in the culture medium, thus preserved in the CFS; the main mechanism proposed was the destruction of bacterial membranes followed by the alteration of metabolism and the induction of oxidative stress (Yang and Yang, 2019; Ramezani et al., 2025). Moreover, it was also documented that other *Bifidobacterium* spp., *B. longum* included, were effective against methicillin-resistant *S. aureus* (MRSA) suggesting their potential application as alternative treatments (Nashaat AL-Saadi, 2016). In line with these findings, in the present work we characterized for the first time *Bifidobacterium longum* subsp. *longum* BG-L47® in terms of antimicrobial potential. The CFS was tested against clinically relevant pathogens demonstrating its effectiveness in inhibiting both Gram-positive and Gram-negative targets. As reported in the results section, it can be stated that, overall, *B. longum* BG-L47® was more effective versus Gram-negative microorganisms since the lowest MIC values were recorded for *E. coli* and *P. aeruginosa* (15 µL/100 µL) and *F. nucleatum* (10 µL/100 µL). The report obtained from the metabolomic analysis allowed us to speculate on which compounds could be responsible for the antimicrobial activity; organic acids, like lactate and acetate, were the most abundant in both SurP and SurE 10K and may be possible candidates. Notably, their antimicrobial activity has long been extensively documented in literature (Lee et al., 2002; Wang et al., 2015) and in the context of lactic acid bacteria (LAB) characterization. Ogawa et al. proposed lactic acid as the effector of *L. casei* and *L. acidophilus* bactericidal activity on Shiga toxin-producing *E. coli* O157:H7 (Ogawa et al., 2002) and its antimicrobial activity against common gastrointestinal pathogens has also been confirmed for other LAB strains and bifidobacteria (Tejero-Sariñena et al., 2012; Wang et al., 2024). One of the key mechanisms of action hypothesized for organic acids is the permeabilization of the outer membrane of Gram-negative bacteria which leads to cell disruption (Alakomi et al., 2000) but, as demonstrated by Fukuda et al. in a murine model, they can also indirectly protect against infections by modulating the response of epithelial cells – anti-inflammatory and anti-apoptotic effects – and preventing the spread of Shiga toxin in bloodstream (Fukuda et al., 2011). On the other hand, the detection of amino acids in ¹H-NMR spectra might be related to the presence of proteins or peptides that could participate in

conferring specific properties to the CFS (Fuochi et al., 2019). Moreover, several amino acids are already known for their potential antimicrobial activity, thus their contribution cannot be excluded. For instance, glycine, which was one of the most representative in our SurP sample, has been shown to have antimicrobial activity because it could interfere with enzymes involved in peptidoglycan synthesis. Authors demonstrated that glycine had a synergistic effect with β -lactam antibiotics that led to the restoration of multidrug resistant (MDR) bacteria susceptibility to these drugs (Giordano and Barnini, 2024). As for the results obtained in this study, further experiments are needed to better understand which components of the CFS are the most effective: pH neutralization, protease and heat treatment are some examples of possible analysis that may help in designing the screening.

As described above, the CFS of *B. longum* BG-L47® also encompasses complex structures, namely extracellular membrane vesicles (MVs). We isolated MVs from both planktonic and biofilm phenotype and tested them on human cell lines and in the *G. mellonella* model to investigate a putative immunomodulatory activity. Nowadays bacterial vesicles – especially those derived from probiotics - are a hot topic and the remarkable interest of the scientific community is demonstrated by the increasing number of publications on this subject (Krzyżek et al., 2023; Marinacci et al., 2023; Puca et al., 2024). It has been widely demonstrated that probiotic membrane vesicles can participate in the crosstalk between bacteria belonging to the gut microbiota, but they can also modulate communication with the human host. Many studies, using Caco-2 cells model, have proved that probiotic MVs preserve the integrity of the intestinal barrier by modulating the expression of tight junction proteins, thus they can strongly regulate intestinal permeability (Chen et al., 2024). Another MVs mechanism of action is the interaction with toll-like receptors (TLRs) expressed by both epithelial cells and immune cells. The interaction with the latter is crucial for the well-known immune regulation mediated by MVs. Hu and colleagues demonstrated that vesicles released by the probiotic *E. coli* Nissle 1917 could be internalized by RAW 254.7 macrophages triggering anti-inflammatory responses (Hu et al., 2020). Similarly, another study reported that *L. mucosae*-derived MVs had anti-diarrheal macrophage-dependent effects in a murine model, confirming the importance of the interaction with immune cells. Same authors detected *L. mucosae* MVs in the cytoplasm of epithelial cells *in vitro*, suggesting that they can also be internalized and probably activate intracellular pathways (Li et al., 2023). In this study *B. longum* BG-L47® MVs were tested on monocytes and macrophages under both basal and pro-inflammatory conditions to evaluate their biocompatibility and their potential immunomodulatory effect. Under basal conditions, only pMVs proved to be effective in a positive modulation of monocytes and macrophages inducing an increase of their metabolic activity. When cells were exposed to LPS the pMVs treatment seemed to induce an immunostimulation on monocytes after 48h, while the reduction of the metabolic activity in macrophages, compared to the LPS-stimulated control, suggested that pMVs had an anti-inflammatory effect. These findings were further confirmed by flow-cytometry analysis which demonstrated that *B. longum* MVs had a dose-depending effect on CD163 modulation on macrophages. CD163 is a typical marker of M2-cells, associated with an anti-inflammatory

phenotype typically related to resolution of inflammation and tissue repair. Similar effects have already been reported for probiotics. In 2022, Jia et al. demonstrated that *L. johnsonii* supplementation could ameliorate colitis in mice thanks to the upregulation of CD206⁺ macrophages (M2-phenotype) both *in vitro* and *in vivo* (Jia et al., 2022). Recently, another study, confirmed that the MVs delivered by the same bacterial species had outcomes comparable to those of live bacteria administration and relied on macrophages polarization (Tao et al., 2025). According to another study, *B. longum* AO44 MVs showed equivalent effects; the stimulation of antigen-presenting cells and CD4⁺ T cells with vesicles led to a significant increase in IL-10 levels, therefore authors associated MVs treatment with an anti-inflammatory activity (Mandelbaum et al., 2023).

Given the evidence on monocytes and macrophages we tested pMV_s, which resulted to be the most effective, *in vitro*. *G. mellonella* larvae were pre-treated with vesicles, subsequently infected with *S. aureus* or *P. aeruginosa* and finally treated with antibiotics. One of the most interesting outcomes was the ability of pMV_s to modulate the CD200-CD200R checkpoint which is known for its importance in regulating immune homeostasis since its activation inhibits the massive release of pro-inflammatory cytokines (Jeon et al., 2025). Considering the modulation of CD markers observed in *G. mellonella*, we hypothesize that *B. longum* BG-L47® MVs could be considered as effectors of a positive interaction between the bacterium and the host immune system. Surely, further investigations are needed to better characterize vesicle mechanisms of action, identify the molecular patterns and validate alternative protocols to evaluate possible clinical applications.

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