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Impact of prebiotics and probiotics on gut microbiota and human health

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Summary

Abstract

The human gut microbiota is recognized as a key factor in human health, so the search for active molecules or other strategies to improve its balance is an emerging field of interest. Probiotic strains belonging to *Bifidobacterium* and *Lactobacillus* genera exhibit health-promoting effects stimulating the immune system and controlling microbiota composition. Prebiotics could be employed to support probiotics in the harsh intestinal environment. Indeed, a prebiotic molecule is not digestible by human enzymes but is available for peculiar bacteria. Prebiotics and probiotics could be administered together as synbiotics, in the expectation of the enhancement of probiotics survival and growth thanks to prebiotics, to obtain the maximum benefit for human health.

Hence, the PhD project aims to characterize new probiotic strains able to balance the gut microbiota and improving human health, as well as to identify new prebiotic molecules, to be tested through *in vivo* and *in vitro* models. At least one synbiotic will be designed and its beneficial effects evaluated.

Several strains of *Lactobacillus* and *Bifidobacterium* were identified and characterized for their probiotic properties. *Lactobacillus plantarum* PBS067 raised the attention for the strongest antimicrobial activity, due to the production of a bacteriocin-like molecule.

Then, the fermentation ability of the probiotics was tested on commercial inulin and fructooligosaccharides (FOS), differing for the degree of polymerization. The strains preferentially used short- and medium-chain FOS as growth substrate. Therefore, the designed synbiotic formulation consisted of *L. plantarum* PBS067, *Bifidobacterium animalis* subsp. *lactis* PBS075 and *Lactobacillus acidophilus* PBS066 as probiotics, plus the FOS (DP~10 and DP~3-5) as prebiotics.

To test the efficacy of the formulation, a clinical study was developed on healthy elderly subjects assigned to receive the prebiotic formulation, or the synbiotic, or the placebo once daily for 28 days. The study duration was 56 days and at

the baseline, after 28 days of administration period, and after 28 days of follow-up, the composition of the gut microbiota and the levels of selected biomarkers were recorded. Analysing the microbiota taxonomic profiles, an enrichment of the community, linked to a significant change of specific bacterial genera and/or combination of specific taxa contributing to a positive metabolic shifting, favoring the short-chain fatty acids (SCFAs) producers was evidenced after the synbiotic intervention. Meanwhile, the results on the healthy state suggested the synbiotic effectiveness in reducing the incidence of common infectious disease (CID) symptoms, linked to a positive stimulation of the gut immune system.

Therefore, a new possible synbiotic was designed and tested through *in vitro* methodologies. An extract from *Grifola frondosa* (Maitake) mushroom was assessed for the prebiotic potential. Interestingly, the growth of the probiotics, also combined as a probiotic consortium, was comparable to those on FOS with low DP, so the mushroom was considered as a prebiotic. The metabolites produced after Maitake fermentation were collected and analyzed via gas-chromatography mass-spectrometry (GC-MSD) analysis. After their administration to healthy and cancerogenic human intestinal cell lines, a decrease in tumoral cell viability was evidence, as well as a protective effect from oxidative stress.

To implement the model, an *in vitro* reconstructed intestinal microbial community in batch fermentation, under anaerobic conditions was developed and validated mimicking the previously used synbiotic intervention. The growth of the community strains and of the probiotics was monitored as the increase of optical density at 600 nm and through the analysis of the metabolites for a maximum of 48 hours fermentation, while the modulations of single strain abundances were assessed through qPCR with species-specific primers. Subsequently, Maitake extract was used as prebiotic, instead of FOS. Interestingly, the most modulated bacteria were *Escherichia coli* and *L. plantarum*. The detected metabolites were propionic, lactic and valeric acid, all resulting from the prebiotic fermentation.

Riassunto

Il microbiota intestinale è ormai riconosciuto come un fattore chiave per la salute umana; quindi, la ricerca di molecole attive o altre strategie per migliorare il suo equilibrio è un campo di interesse sempre crescente. I ceppi di batteri probiotici appartenenti ai generi *Bifidobacterium* e *Lactobacillus* mostrano effetti benefici sulla salute, grazie alla stimolazione del sistema immunitario e al controllo della composizione del microbiota. I prebiotici possono essere impiegati per supportare la crescita dei batteri probiotici nel complesso ecosistema intestinale. Infatti, una molecola prebiotica non è digeribile dagli enzimi intestinali umani, rimanendo quindi disponibile per la crescita di specifici batteri benefici. Prebiotici e probiotici possono anche essere somministrati insieme come synbiotici, con l'obiettivo di potenziare la sopravvivenza e la crescita dei probiotici grazie ai prebiotici, per ottenere il massimo beneficio per la salute dell'ospite.

Il progetto di dottorato ha come obiettivi la caratterizzazione di nuovi ceppi di batteri probiotici in grado di bilanciare il microbiota intestinale e migliorare la salute umana, nonché di identificare nuove molecole prebiotiche, da testare attraverso modelli sia *in vivo* che *in vitro*. Almeno un synbiotico sarà progettato e i suoi eventuali effetti benefici valutati.

Diversi ceppi appartenenti ai generi *Lactobacillus* e *Bifidobacterium* sono stati identificati e caratterizzati per le loro proprietà probiotiche. Tra questi, *Lactobacillus plantarum* PBS067 ha mostrato la più forte attività antimicrobica, grazie alla produzione di una molecola batteriocine-like.

Inoltre, la capacità di fermentazione dei probiotici è stata testata su inulina e fruttooligosaccaridi (FOS) commerciali, che differiscono per il grado di polimerizzazione. I ceppi hanno utilizzato preferenzialmente FOS a catena corta e media come substrato di crescita. Pertanto, la formulazione synbiotica progettata consisteva in *L. plantarum* PBS067, *Bifidobacterium animalis* subsp. *lactis* PBS075 e *Lactobacillus acidophilus* PBS066 come probiotici, più i FOS (DP~10 e DP~3-5) come prebiotici.

Per testare l'efficacia della formulazione, è stato sviluppato uno studio condotto su soggetti anziani sani assegnati a ricevere la formulazione prebiotica, o il synbiotico, o il placebo una volta al giorno per 28 giorni. La durata dello studio è stata di 56 giorni e al tempo iniziale, dopo 28 giorni di somministrazione e dopo 28 giorni di follow-up, sono stati registrati la composizione del microbiota intestinale e i livelli di biomarcatori selezionati. Dall'analisi dei profili tassonomici del microbiota dei soggetti che hanno ricevuto il synbiotico è emerso un arricchimento della comunità, legato ad un significativo cambiamento di specifici generi batterici e/o combinazione di taxa specifici che contribuiscono ad uno spostamento metabolico positivo, favorendo i produttori di acidi grassi a catena corta (SCFAs). Inoltre, i risultati sullo stato di salute hanno suggerito l'efficacia della formulazione nel ridurre l'incidenza dei sintomi comuni delle malattie infettive, legati a una stimolazione positiva del sistema immunitario intestinale.

Pertanto, un nuovo possibile synbiotico è stato progettato e testato attraverso metodologie *in vitro*. Un estratto derivante dal fungo *Grifola frondosa* (Maitake) è stato valutato per il potenziale prebiotico. La crescita dei probiotici, anche combinati come consorzio, era paragonabile a quella su FOS con DP basso; quindi, il fungo è stato considerato un prebiotico. I metaboliti prodotti dopo la fermentazione di Maitake sono stati raccolti e analizzati tramite gascromatografia con spettrometria di massa (GC-MSD). Dopo la loro somministrazione a linee cellulari intestinali umane sane e tumorali, è stata evidenziata una diminuzione della vitalità nelle cellule tumorali, nonché un effetto protettivo dallo stress ossidativo.

Per implementare il modello, è stata sviluppata e validata una comunità microbica intestinale ricostruita *in vitro*, imitando l'intervento con il synbiotico precedentemente utilizzato. La crescita batterica è stata monitorata come aumento della densità ottica a 600 nm e attraverso l'analisi dei metaboliti prodotti, per un massimo di 48 ore di fermentazione. La modulazione della presenza di singoli ceppi è stata valutata attraverso qPCR con primer specie-specifici. Successivamente, l'estratto di Maitake è stato utilizzato come

prebiotico invece dei FOS. I ceppi più modulati sono risultati essere *Escherichia coli* e *L. plantarum*. I metaboliti rilevati erano acido propionico, lattico e valerico, tutti derivanti dalla fermentazione prebiotica.

Preface

Nowadays, it is established the presence of diverse community of microorganisms which live in symbiosis with the human beings. The composition of these different communities has evolved to carry out several specific tasks that could benefit the host, as well as to survive and thrive in sites that provide to the microorganisms a suitable nutrient-filled habitat. The symbionts could be mutualist, commensals, or pathogens (Reid et al., 2011). In particular, attention is given to the bacteria living within the gastrointestinal tract, known as gut microbiota. Outstanding properties of the microbial community are functionality and resilience (Swanson et al., 2020), therefore its balance is recognized as a key factor for the human health. Indeed, if the eubiotic state is maintained, it could influence the immune system in a positive way, leading to a right activation, and to the strengthening of the intestinal barrier. Whereas, in a dysbiotic state, an inflammatory, pathogenic, or metabolic condition could be established (Swanson et al., 2020), leading to diseases such as inflammatory bowel disease, colorectal cancer, and metabolic syndrome (Reid et al., 2011, Swanson et al., 2020). In this scenario, various strategies have been introduced to modulate the composition and/or the function of the gut microbiota, as fecal microbiota transplants, the use of non-digestible molecules such as prebiotics, and the administration of beneficial probiotic strains (Swanson et al., 2020). The principal rationale for the use of pre- and pro- biotics is to help in the restoration and in the maintenance of the homeostasis. Currently, an effective employed strategy is the combination of prebiotics supporting probiotic bacteria strains in a unique formulation, known as synbiotic. However, the existence of more than 1,000 prevalent species, and the high interpersonal variation within the human population in terms of genetics, environment, and habits, create an intricate context, where the number of know host-microbe interactions has grown rapidly over the past decades, with many aspects still unclear (Elzinga et al., 2019). Nevertheless, the composition of the microbiota is being more effectively

deciphered, leading the scientist to think about a reductionist approach in which both host and microbiome are simplified to the extent that experimental variables can be controlled and manipulated. (Elzinga et al., 2019). Although the models are still dawn, these systems could represent the future research, not only for ethical and financial reasons but also from a scientific perspective.

Chapter 1:
Introduction

1.1 Ecology of the human gastrointestinal tract

The gastrointestinal tract is the main part of the digestive system and represents one of the largest interfaces (between 250 and 400 m^2) between the host, environmental factors, and antigens in the human body (Thursby et al., 2017). In this compartment the enzymatic decomposition of food, the absorption of nutrients and water, and the excretion of non-digestible food parts occur. Also, several bacteria, archaea and eukaryote colonizing the GI tract participate to the digestive processes. As a whole, the microorganisms are known as the "human gut microbiota", which coevolved with the host to form an intricate and principally beneficial relationship with the host. This ecological community of symbiont is characterized by commensal (benefiting themselves but not the host), mutualist (benefiting themselves and the host), and pathogenic (benefiting themselves by harming the host) microorganisms (Reid et al., 2011, The Human Microbiome Project 2012, Thursby et al., 2017; Reid et al., 2011). Therefore, it is a high complex ecosystem, which mediates numerous interactions from both chemical and nutritional point of view. The number of microorganisms inhabiting the gastrointestinal tract has been estimated to exceed 10^{14}. A wide range of food-associated bacteria are found in the esophagus, from 10^1 to 10^3 CFU/mL in the stomach, 10^7 CFU/mL in the jejenum, more than 10^9 CFU/g in the terminal part of the ileum, and about 10^{11} CFU/g in the distal part of the colon (Figure 1.1) (Thursby et al., 2017).

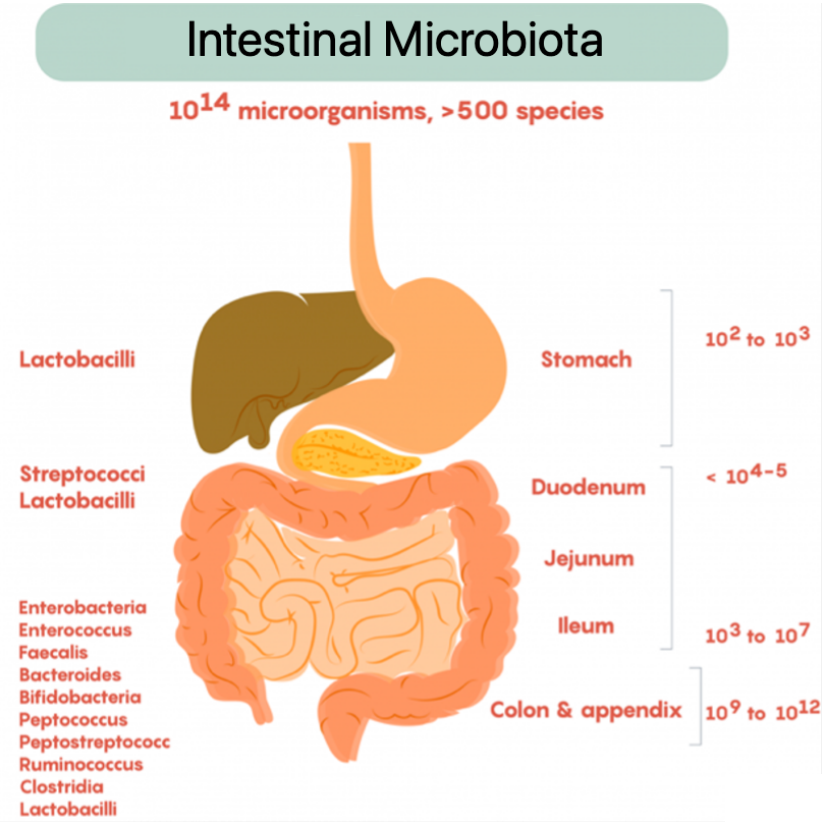

Figure 1.1. Representation of the gastrointestinal tract and associated microbiota.

In the last decade, the possibility to discover and characterize this large variety of bacteria is possible thanks to the advent of culture-independent approaches, such as the high-throughput and low-cost sequencing methods. Indeed, previously, most of the knowledge about the gut bacteria arise from labor-intensive culture-based methods, which however do not allow the growth of all the strains present in the intestine. Therefore, to explore the gut microbiota composition, a simple well-known approach consists in targeting the bacterial 16S ribosomal RNA (rRNA) gene, since it is present in all bacteria and archaea, and it contains nine high variable regions (from V1 to V9) which allow the identification of the species. A more precise approach could be provided by the whole-genome shotgun metagenomics, because of the higher resolution and sensibility respect to the 16S sequencing (Thursby et al., 2017). Combining the

sequence deposited in the biggest gene collections (MetaHit and the Human Microbiome Project) emerge that 2172 species isolated from human beings were identified, and 386 are strictly anaerobic. They are classified into 12 different phyla, of which 93.5% belong to Firmicutes (46-58% of total bacteria), Bacteroidetes (in particular, the *Bacteroides-Prevotella* group represent 10-30%), Actinobacteria (in particular, *Bifidobacterium* accounts for 4.4-4.8%), Proteobacteria, and Verrucomicrobia, which are the top 5 phyla representing the gut microbiome (Thursby et al., 2017; Nissen et al., 2020). 9,879,896 genes of the gut community were sequenced and identified, highlighting a functional redundancy. Indeed, the INRA researchers discover the presence of a core microbiota composed by a little number of species shared by all the subjects. This is probably the phylogenetic nucleus of the human gut microbiome (Tap et al., 2009). Indeed, *Bacteroides*, *Bifidobacterium*, *Streptococcus*, *Enterococcus*, *Clostridium*, *Lactobacillus*, and *Ruminococcus* could be identified as the predominant genera in the intestinal lumen; while *Clostridium*, *Akkermansia*, and again *Lactobacillus* and *Enterococcus* predominate in the mucosa associated with the surface (Frank et al., 2007; Eckburg et al.,2005).

Furthermore, the literature points out the presence of individual-specific gut microbiota, suggesting that the composition is shaped by environmental factors such as diet, diseases, host genetics, and the first environments with which the newborn came into contact (Ouwehand et al., 1999). The development of the microbiota is generally believed to begin from birth, and to change during the lifetime. Indeed, at birth, the gastrointestinal (GI) tract is sterile, but is rapidly colonized by microorganisms coming from the environment. Therefore, the mode of delivery appears to affect the microbiota composition. For example, the vaginally delivered infants acquired a bacterial community similar to their own mother's vaginal microbiota, dominated by *Lactobacillus* spp., *Prevotella* spp. or *Sneathis* spp., reflecting the contact with the vaginal environment, respect to the infants delivered by C-section, which reflects a community like the one on the skin surfaces of the mother, dominated by *Staphylococcus* spp.,

Corynebacterium spp., and *Propionibacterium* spp. (Thursby et al., 2017; Reid et al., 2011). In general, in early stages of development, the microbiota presents a poor biodiversity, and it is dominated by facultative anaerobic bacteria belonging principally to the Proteobacteria phylum. These strains reduce the redox potential of the intestine, creating the optimal environment for anaerobic microorganisms, such as Bifidobacteria, which can colonize the intestinal tract (Butel et al., 2014). They account for more than 91% of the total microbial population in breastfed babies, while they account for about 75% in artificially breastfeed babies (Beerens et al., 1991) because of the presence of *N*-acetylated sugars in breast milk and *trans*-galactosylate oligosaccharides in infant formulas (Tannock, 1997). With weaning, solid foods are introduced into the diet of the infant, so the relative amount of Bifidobacteria decreases significantly to stand at a 15% of the total cultivable microorganisms in adulthood. Finally, during old age, their number decreases further (Mitsuoka, 1990). Indeed, in individuals over the age of 65 the microbial community shifts, with an increase abundance of the Bacteroidetes and Clostridium cluster IV, in contrast with younger subjects where cluster XIVa is more prevalent (Thursby et al., 2017). Interestingly, the centenarians present an increase in the abundance of facultative anaerobes, such as *Escherichia coli*, and a re-organization of the profile of the butyrate producer strains, as *Faecalibacterium prausnitzii*. Overall, the capacity of the microbiota to carry out metabolic processes, such as short-chain fatty acids (SCFAs) production and amylolysis, is reduced in the elderly, whilst proteolytic activity is increased. Given the growing evidence for the role of SCFAs as key metabolic and immune mediators, it was suggested that the decrease in SCFAs may encourage the inflammageing process in the intestine of aged people (Thursby et al., 2017). For example, one factor influencing the process is the increased serum level of the inflammatory cytokine interleukin-6 (IL-6) linked to a weaker intestinal barrier, so gastrointestinal function is impaired. The dysfunction occurs mainly in the esophagus and in the colon, with

a decrease in peristaltic pressure and motility, which can be the cause of constipation (Clements et al., 2018).

Ultimately, only a small number of species belonging to the *Lactobacillus* genus turns out to be an autochthonous component of the intestine. Indeed, most of them come from exogenous sources, such as water and food. Although they are found throughout the gastrointestinal tract in different concentrations depending on anatomical and physiological conditions, Lactobacilli do not persist within the gastrointestinal ecosystem and are traceable only for a short time after their intake. Nevertheless, they sustain positive effects for the host (Figure 1.2.).

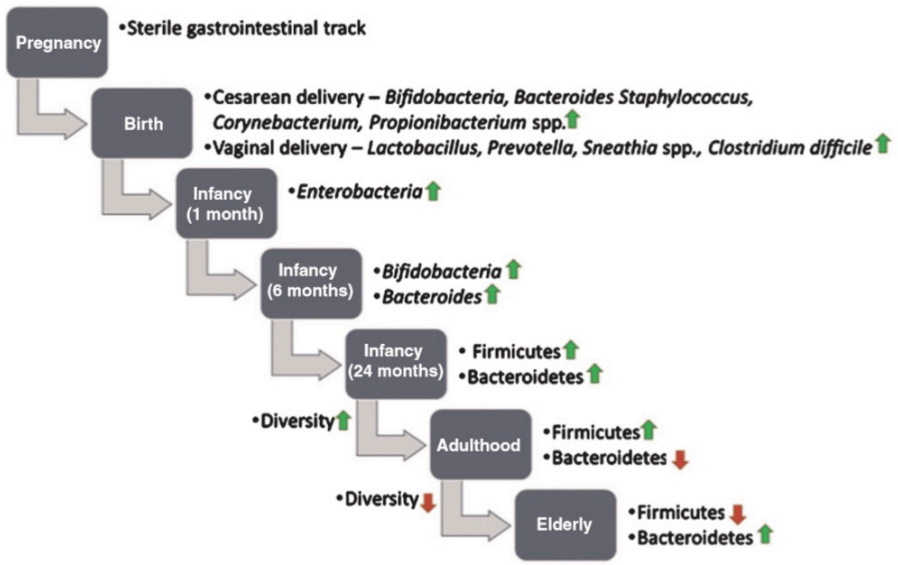

Figure 1.2. Scheme of the development of the human intestinal microbiota (Owehand et al., 1999)

Interestingly, several metagenomic studies evidence that diet plays a key role in the colonization and in the composition of the gut microbiota (Milani et al., 2016). For example, today it is known that bacteria belonging to the genus Clostridium group XIVa, are more present in the fecal microbiota of omnivores and less in the microbiota of vegetarians. Otherwise, a Western-type diet, characterized by few fibers and high fat intake, leads to a reduction of the bacteria responsible of

the fiber degradation, such as the ones belonging to the genera *Prevotella*, *Succinivibrio*, *Treponema* and *Bifidobacterium*. Finally, it has been highlighted that a predominantly meat-based diet involves the increase of bile-tolerant bacteria (such as *Alistipes*, *Bilophila* and *Bacteroides*) to the disadvantage of bacteria involved in the metabolism of plant polysaccharides (such as the one belonging to Firmicutes phylum) (Milani et al., 2016). Conversely, the intake of fiber with food promotes the growth and the activity of microorganisms producing butyrate and stimulates the beneficial populations of Bifidobacteria and Lactobacilli. Regarding the influence of fats taken with food, clinical studies show the direct modification of the intestinal microbiota occurring through the modulation of the composition and secretion of bile acids. In particular, it has been observed that the genus *Bacteroides* correlates positively with the intake of saturated fatty acids, while the genus *Prevotella* is only weakly associated with fats. Indeed, the polyunsaturated fats affect the adhesion of intestinal bacteria to the surface of the mucous membrane (Milani et al., 2016).

1.2 Role of the gut microbiota and its related metabolites in human health

Thanks to its large genomic content and to the metabolic functions, the gut microbiota provides numerous functions that affect host health.

An important one is the *de novo* synthesis of indispensable substances, such as vitamins of B and K groups, riboflavin, biotin, nicotinic acid, panthotenoic acid, pyridoxin, and thiamine (Hill, 1997). For example, lactic acid bacteria are key organisms in the production of vitamin B12, which cannot be synthetized by either animals, plants, or fungi. Instead, Bifidobacteria members are the main producers of folate, that is a B group vitamin involved also in DNA synthesis and repair. Then, vitamin K is involved in blood clotting.

The human gut microbiota forms also a physical protective barrier against potentially pathogenic bacteria. Indeed, in addition to physically hindering the achievement of the intestinal epithelium, through the formation of biofilms, there

are also mechanisms such as competition for nutrients and adhesion sites, the production of antimicrobial substances (AMPs) and the ability of immunomodulation (Ouwehand et al., 1999). Indeed, the GI microbiota stimulates the production of AMPs such as cathelicidins, C-type lectins, and defensins by the host Paneth cells via a PRR-mediated mechanism (Hooper et al., 2010). Another induced defense mechanism is the release of mucosal IgA (SIgA). These antibodies are anchored in the outer layer of the colonic mucus through combined interaction with mucins and gut bacteria, providing immune protection against pathogens and maintaining a mutually beneficial relationship with commensals (Rogier et al., 2014).

Moreover, several studies have shown the crucial role of microorganisms that colonize the human intestine first to stimulate the maturation of the immune system of the digestive tract (Saarela et al., 2000) (Figure 1.3.).

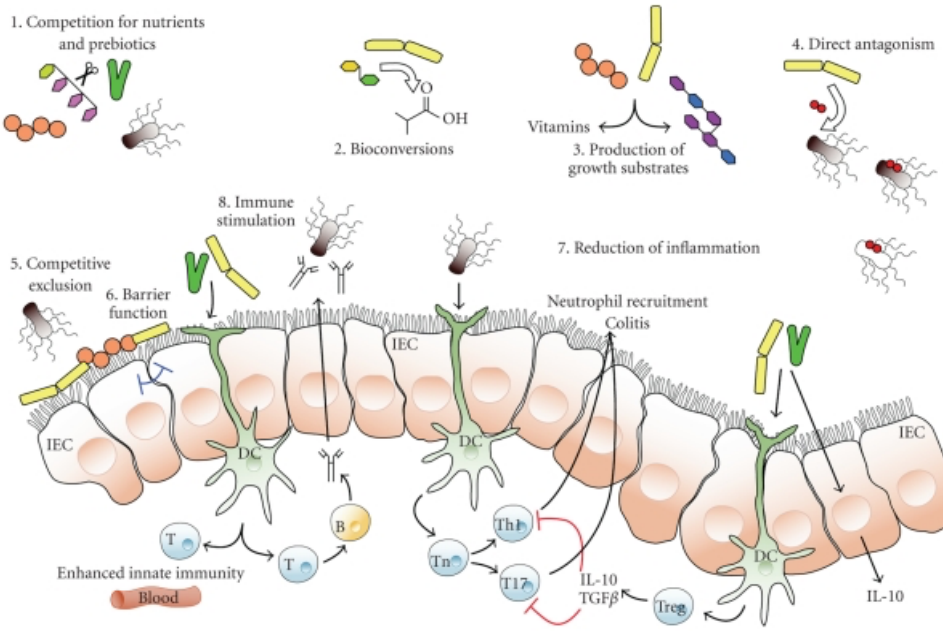

Figure 1.3. Illustration of known or hypothesized mechanisms of gut microbiota action. (1) direct competition for nutrient substrates. (2) bioconversion of nutrients into fermentation products with inhibitory properties, such as organic acids. (3) production of other growth substrates such as exopolysaccharides (EPS) or vitamins. (4) antagonism directed against pathogens through the release of antimicrobial metabolites. (5) exclusive competition for epithelium anchorage sites. (6) potentially of the gut barrier effect. (7) reduction of inflammation and (8) stimulation of the innate immune response. IEC: intestinal epithelial cells. DC: dendritic cells. T: T cells

Nevertheless, the most important function of the gut microbiota is probably the breakdown of substances that our digestive system is not able to dismantle, such as plant cell-wall polysaccharides, oligosaccharides, cellulose, and resistant starches. Indeed, many bacteria inhabiting the colon are involved in the degradation of dietary fibers, that are complex mixtures of plant polysaccharides. Several strains are specialist, attacking specific bonds in specific types of polymers thanks to the presence in their genomes of carbohydrate-active enzymes (Musso et al., 2010; Baxter et al., 2019). However, only a limited number of bacteria can degrade any resistant polysaccharide introduced with diet or as dietary supplement. Indeed, the major metabolic routes are the Embden-Meyerod-Parnad pathway (or glycolysis, for six-carbon sugars) and the pentose-phosphate pathway (for five-carbon

sugars), which converts the monosaccharides into phosphoenolpyruvate (PEP). Then PEP is converted into fermentation products such as organic acids or alcohols (den Besten et al., 2013). Therefore, within the gut ecosystem, a sort of hierarchy could be established based on the fermentative capacities. Indeed, the primary degraders depolymerize specific polysaccharides to mono-, di-, and oligo- saccharides, that could be fermented by the same bacteria, producing acidic end products such as acetate and lactate (SCFAs). However, most of the resistant starch degraders are not among the butyrate producers (Baxter et al., 2019). Instead, the secondary fermenters capture the degradation and fermentation products of the primary degraders, and metabolized them into new molecules, which could be also SCFAs. Anyhow, if the primary degraders consume all the prebiotic molecules efficiently, only a fraction of the carbon and energy may become available for the secondary fermenters (Baxter et al., 2019).

In general, SCFAs are waste products for the microbial community, required to balance the redox equivalent production in the anaerobic environment of the gut (den Besten et al., 2013). They are composed of saturated aliphatic organic acids, from one to six carbon atoms. The predominant in the gut environment are propionate (C3), butyrate (C4), and acetate (C2), in a proportion of 1:1:3 (Louis et al., 2014). Depending on the diet, the total concentration of these metabolites decreases from 70 to 140 mM in the proximal colon, to 20 to 70 mM in the distal colon (den Besten et al., 2013). The 95% of the SCFAs are quickly absorbed by the intestinal epithelial cells, where they regulate cellular processes such as differentiation, proliferation, and apoptosis (Correa-Oliveira et al., 2016). In general, SCFAs play a role in regulating the immune system and the inflammatory response, influencing the production of cytokines. For example, they stimulate the production of IL-18, which has a role in maintaining and repairing the epithelial integrity (Correa-Oliveira et al., 2016).

In the human gut, propionate is mainly produced by Bacteroidetes via the succinate or the propanediol pathway, depending on the nature of the sugar. It

can be formed via a primitive electron transfer chain, or by the reduction of lactate to propionate (via the acrylate pathway). Both pathways reduce the additional NADH, with comparison to the fermentation to lactate (den Besten et al., 2013). Another pathway is the acrylate one, in which lactate is converted through the activity of the lactoyl-CoA dehydratase and other reactions. It seems that only strains belonging to Veillonellaceae and Lachnospiraceae families could use this route (Rios-Covian et al., 2016). An important propionate producer from mucin degradation is *Akkermansia muciniphila*. Propionate is primarily absorbed by the liver, where it can activate gluconeogenesis. The ratio of propionate to acetate is important because propionate can inhibit the conversion of acetate to cholesterol and fat (Oliphant et al., 2019). Furthermore, propionate acts on β-cell functions and attenuates the reward-based eating behavior via striatal pathways (Thursby et al., 2017).

Instead, butyrate is produced from carbohydrates via the glycolysis and the acetoacetyl-CoA, that is reduced to butyryl-CoA (den Besten et al., 2013). It is important for maintaining health thanks to the regulation of the immune system leading to anti-inflammatory effects. Furthermore, it is an important energy source for the colonocytes. Indeed, a change in the gradient of butyrate from the lumen to the crypt seems to control the intestinal epithelial turnover, and its homeostasis by promoting the colonocyte proliferation at the bottom of the crypts, whilst increasing apoptosis of the cells closer to the lumen (Donohoe et al., 2012). To improve the presence of butyrate in the gut environment, it could be useful to identify butyrogenic configurations of the microbiota, because several different bacteria or their combination could be involved in the multistep process of secondary metabolites production (Baxter et al., 2019). The most abundant butyrate producers in the human gut microbiota are *Eubacterium rectale* and *F. prausnitzii*. Interestingly, strains belonging to *Roseburia* genus and some *F. prausnitzii* could be cross-fed by *Bifidobacterium* spp.. Also *Eubacterium hallii*, *Anaerostipes caccae*, or *Anaerostipes hadrus* could produce butyrate thanks to the acetate and lactate produced by the Bifidobacteria via the

Bifid shunt (see chapter 1.4.1.2 "*The genus Bifidobacterium*") (Baxter et al., 2019).

Actually, acetate is the most abundant SCFA in the colon and compose more than half of the total SCFAs detected in feces (Rios-Covian et al., 2016). Two metabolic routes have been described for the acetate production by the gut microbiota. One is used by most of the enteric bacteria, which ferment CHO (Rios-Covian et al., 2016). While it is produced by one-third of the gut anaerobic bacteria by hydrolysis of acetyl-CoA or from CO_2 via the Wood-Ljungdahl pathway, in which CO_2 is reduced to CO and converted to acetyl-CoA thanks to a methyl group and CoASH (den Besten et al., 2013). It is released into peripheral tissues, driving a lipogenic activity in the liver (Thursby et al., 2017).

1.3 Dysbiosis and strategies to rebalance the intestinal ecosystem

When the mutualistic relationship between the members of the microbiota, the metabolic products, and the host's immune system is compromised, the microbial ecosystem fails, and the condition is called "dysbiosis". Generally, in a dysbiotic ecosystem, potentially pathogenic bacteria take over at the expense of potentially beneficial bacteria. Dysbiosis does not occur in a single form but can be of several types, depending on the cause. The "deficiency dysbiosis" is the condition characterized by an overall reduction in beneficial bacterial species (such as Lactobacilli and/or Bifidobacteria), which can occur because of unhealthy diets or antibiotic therapies (Rodrigues et al., 2017) and may be associated with food intolerances (intolerance to milk or meat) due to lack of specific digestive enzymes (Gagliardi et al., 2018).

Instead, the "putrefactive dysbiosis" is characterized by an increase in putrefactive bacteria (especially the *Bacteroides*), generally deriving from a diet rich in fat and meat and low in fiber, whose metabolism products can be ammonia, amines and phenols that cause problems not only to the gastrointestinal tract, but to the whole organism. This type of dysbiosis is

characterized by an increase in bacterial growth in the small intestine due to the reduction of gastric acid production, leading to an excess of the fermentative activity of bacteria. The individuals which suffer of this form of dysbiosis are generally intolerant to gluten and carbohydrates (Gagliardi et al., 2018).

Actually, the “susceptibility dysbiosis” is associated with genetic causes that lead the immune system to respond abnormally to the intestinal microbiota. In this case there is a reduction in probiotic bacteria and an increase in pathogenic bacteria, linked to an alteration in intestinal motility, causing intestinal inflammation (Gagliardi et al., 2018).

Finally, the "fungal dysbiosis", is caused by a diet rich in sugars and poor in fibers. It is also characterized by the increase in *Candida* spp. or other species of fungi naturally present in the intestinal microbiota (Gagliardi et al., 2018).

On the other hand, the term "eubiosis" refers to a good balance and functioning of the intestinal microbiota. This condition is characterized by a preponderance of potentially beneficial species, belonging mainly to two phyla Firmicutes and Bacteroides, while potentially pathogenic species, such as some members of the phylum Proteobacteria, are present in low percentage (Iebba et al., 2013).

Over the past 15 years, the concept of a balanced microbiota has attracted the interest of many academic and industrial scientists and has become a popular research topic in nutrition and in biomedical fields. The application of new methods has improved the knowledge of the composition of the intestinal microbiota in terms of variety, classification, identity and relative concentrations of genera or species of microorganisms, as well as in terms of properties and interactions between microorganisms and with intestinal epithelial cells. This led the International Scientific Association for Probiotics and Prebiotics-ISAPP (Swanson et al., 2020) to propose the concept of "normobiosis" to characterize a normal gut microbiota, in which the genera and the species of microorganisms with potential health benefits predominate in number over potentially harmful ones. Instead, the "dysbiosis" characterizes an intestinal microbiota in which one or a few potentially harmful genera/species dominate, creating a situation prone

to diseases (Roberfroid et al., 2010). Several therapeutic strategies have been developed to restore intestinal eubiosis. Currently, the main strategy and the most widely used includes the administration of probiotic bacteria to counteract pathogenic bacteria and promote the rebalancing of the microbial community. Other strategies could be the administration of prebiotics (i.e., nutrients that are preferentially or exclusively metabolized by probiotic bacteria) that promote the growth of probiotic bacteria; and the administration of combinations of probiotics and prebiotics (the so-called "synbiotics"). The aim is to eliminate harmful bacteria with the most favorable ones to restore eubiosis.
The components of the synbiotics are discussed in detail in the next chapters.

1.4 Probiotics

According to the official FAO and WHO definition, probiotics are "live organisms that, administered in adequate quantities, bring a benefit to the health of the host" (Food and Agriculture Organization of the United Nations, World Healt Organization, 2001). The FAO/WHO document refers to non-pathogenic microorganisms present in food, or added to foods themselves, and excludes biotherapeutic agents and beneficial microorganisms not used in food. This underlining is very important for the safety of using vital bacteria.
The term probiotic is a relatively new word deriving from the Greek: "pro-bios", that is "for-life". Currently, it is used to indicate bacteria associated with beneficial effects for humans and animals. However, the concept of "beneficial bacteria" was shelved during the era of antibiotics and vaccinations. Indeed, the term "probiotic" was then proposed in 1965 by Lilly and Stillwell as an antithesis to the word "antibiotic", to identify a substance of microbial origin capable of stimulating the growth of another microorganism (Lilly et al., 1965; FAO/WHO 2001). A more recent, but probably not the last definition is "live microorganisms, which when consumed in adequate amounts, confer a health effect on the host" (Guarner et al., 1998; FAO/WHO 2001).

1.4.1 Taxonomy of Probiotic Strains

Nowadays, the members of the genera *Lactobacillus* and *Bifidobacterium* are mainly used, but not exclusively, as probiotic microorganisms in the food field. In addition to the wide range of fermented milks now present in supermarkets, the demand and therefore the search for new foods as possible vectors of Lactobacilli and Bifidobacteria has increased. Interestingly, many of the species found in fermented foods are identical or share physiological traits with species that play a key role in the health of the gastrointestinal tract (Marco et al., 2017).

1.4.1.1 The Lactobacillus genus

The *Lactobacillus* genus was proposed by Beijerinck in 1901. It is polyphyletic, and it comprehends Gram-positive, fermentative, acid tolerant, facultatively anaerobic or microaerophilic, non-sporeforming, coconut-shaped or rod-shaped bacteria (Bull et al., 2013). The genus is classified in the phylum Firmicutes, class *Bacilli*, order *Lactobacillales*, family *Lactobacillaceae*, which contains the genera *Lactobacillus, Paralactobacillus* and *Pediococcus* (Zheng et al., 2020). The genus is composed of 253 species, for which the names are validly published (Kong et al., 2020) and is the largest group of lactic acid bacteria (LAB) (Zheng et al., 2020, Bull et al., 2013).

In humans they are present as symbiont organisms in the vagina, where they are the 97-98% of the normal microbial flora, and in the gastrointestinal tract, where they form a small part of the human gut microbiota. Generally, Lactobacilli are ubiquitous and non-pathogenic. The size of their genome varies between 1.23 Mpb and 4.91 Mbps (Stefanovic et al., 2017). The content in guanine (G) and cytosine (C) is less than 53%. The comparative genomics studies of Lactobacilli confirm the trend seen in the genomes of other lactic acid bacteria, namely a loss of ancestral genes and a reduction in the size of genomes, as well as an acquisition of genes by horizontal gene transfer to adapt to the habitat (Stefanovic et al., 2017). For example, they lack catalase and oxidase, but their genomes encode the superoxide dismutase (SOD) and other mechanisms to

protect themselves from reactive oxygen species (Kong et al., 2020). The *Lactobacillus* are chemoorganotrophic organisms capable of degrading different types of carbohydrates and related molecules, thanks to the phosphorylation at the substrate level and the lactic fermentation. They produce lactic acid by fermentation of sugars, but also acetic acid, ethanol, carbon dioxide and other secondary compounds. Based on the type of fermentation used, Lactobacilli can be distinguished into homofermentative or heterofermentative, depending on whether they have the enzymes 1,6-fructose diphosphatase and phosphoketolase. In species carrying out homolactic fermentation, more than 90% of the fermentation products is lactic acid. On the other hand, in species carrying out heterolactic fermentation, there are compound secretions with a 1:1:1 ratio of lactic acid, carbon dioxide and ethanol.

The competition between the different strains of Lactobacilli determines the prevailing content of lactic acid. Secondary metabolites are aldehydes, ketones, and sulfur compounds (Figure 1.4 and Figure 1.5).

Lactobacilli live in environments very rich in nutrients and have developed few biosynthetic pathways. Instead, their genomes encode for proteolytic systems to derive amino acids from the degradation of proteins and peptides. These pathways are composed of enzymes that are located outside the cytoplasmic membrane, intracellular transport systems, and peptidases.

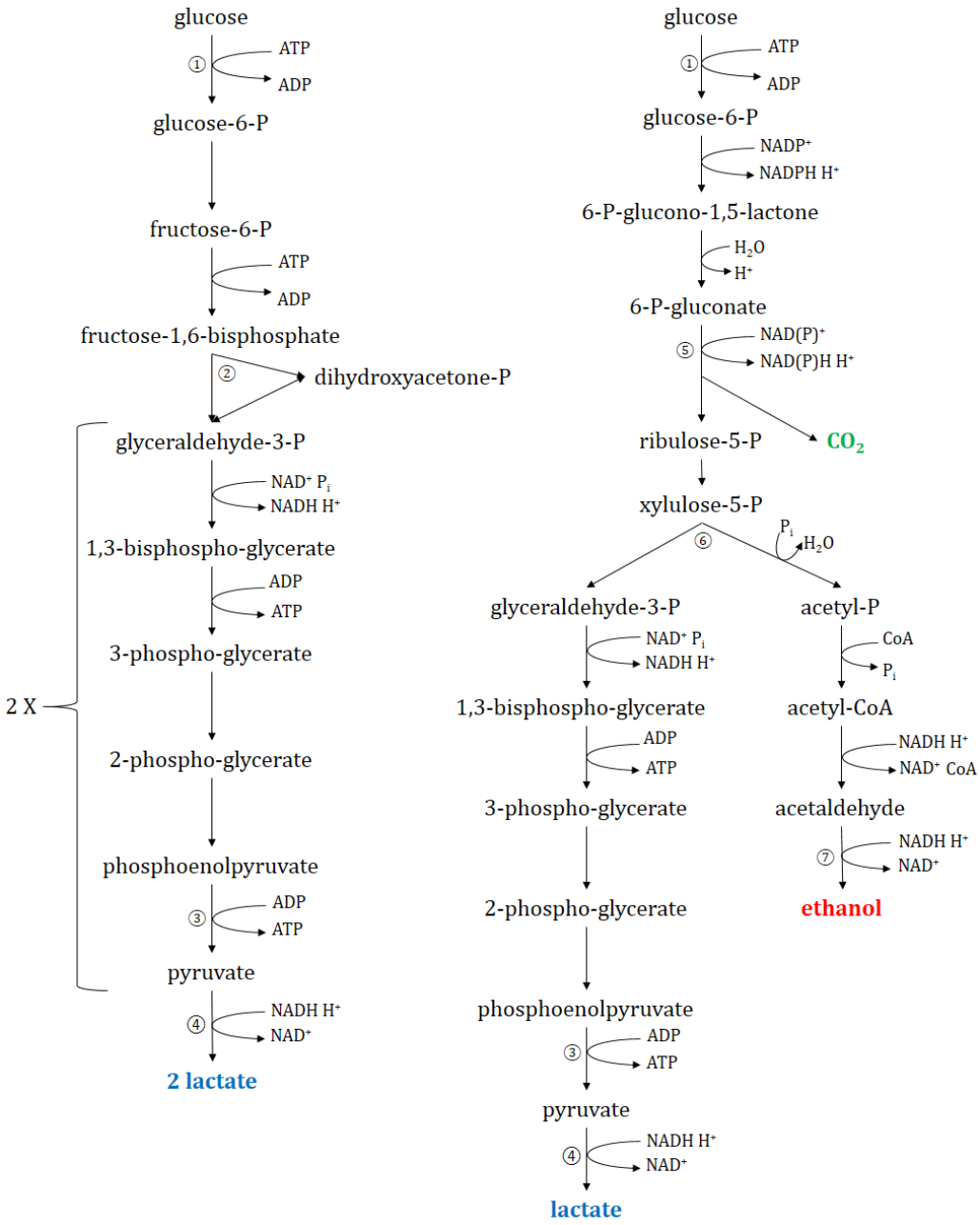

Figure 1.4. Homolactic fermentation (EMP pathway) and **Figure 1.5.** Heterolactic fermentation (6PG/PK pathway). Highlighted enzymes: 1) Hesokinase (EC 2.7.1.2) 2) Fructose-1,6-bisphosphate aldolase (EC 4.1.2.13) 3) Pyruvate kinase (EC 2.7.1.40) 4) Lactate dehydrogenase (EC 1.1.1.27) 5) 6-P-gluconate dehydrogenase (EC 1.1.1.3) 6) Phosphoketolase (EC 4.1.2.9) 7) Alcohol dehydrogenase (EC 1.1.1.1) (Salminen & Von Wright, 2011).

1.4.1.2 The Bifidobacterium genus

Bifidobacterium is a genus of Gram-positive, anaerobic, non-mobile, pleomorphic stick-shaped, capsule-free and asporiginal bacteria. At least 34 species have been described, with some different biotypes and subspecies. They colonize the gastrointestinal tract, the vagina, and the mouth of mammals. In the adults of the Northern Europe, they are the 4.4-4.3% of the total fecal microbiota (Van den Broek et al., 2008).

The oxygen tolerance is different between species and between strains of the same species (De Vries et al., 1968). The oxygen toxicity is linked to the sensitivity against hydrogen peroxide, produced under aerobiosis conditions (Mattarelli et al., 2002). Indeed, some strains are so sensitive to oxygen that they do not tolerate it even in small quantities. The sensitivity to oxygen varies considerably depending on the strain, above all in presence of carbon dioxide (Sonomoto et al., 2011). In general, Bifidobacteria do not exhibit catalase activity, but O_2-tolerant species have other mechanisms that defend them against ROS.

Bacteria belonging to the genus *Bifidobacterium* have a high content of G + C in DNA, between 55% and 67% and are phylogenetically distinct from true lactic acid bacteria because in these the content of G + C is usually less than 55% (Mitsuoka, 1990; Schleifer et al., 1995).

Bifidobacteria are chemoorganotrophs and have a fermentative energy metabolism: they ferment carbohydrates with acid production, but not gas. The metabolism of hexoses occurs accordingly to a characteristic metabolic pathway called "fructose shunt-6-phosphate" (Figure 1.6). The key enzyme of the metabolic pathway is fructose-6-phosphate phosphoketolase which, starting from two glucose molecules, splits the fructose-6-phosphate molecule into erythrose-4-phosphate and acetyl phosphate, which is then converted into acetate by the acetate kinase. The action of transaldolase and transketolase on the two pentose molecules that are generated leads to the production of three molecules of acetic acid and two of L-lactic acid. At the end of the fermentation

process, the yield is then acetate: lactate = 1.5 : 1. However, this balance can be altered due to the phosphoroclastic cleavage of a part of pyruvate into formic and acetic acid and the reduction of the latter to ethanol (De Vries et al., 1968). Different species of Bifidobacteria placed under the same conditions produce different amounts of acetic acid, lactic acid, ethanol, and formic acid. This is due to the influence of the quality and quantity of carbon sources present as a growth substrate.

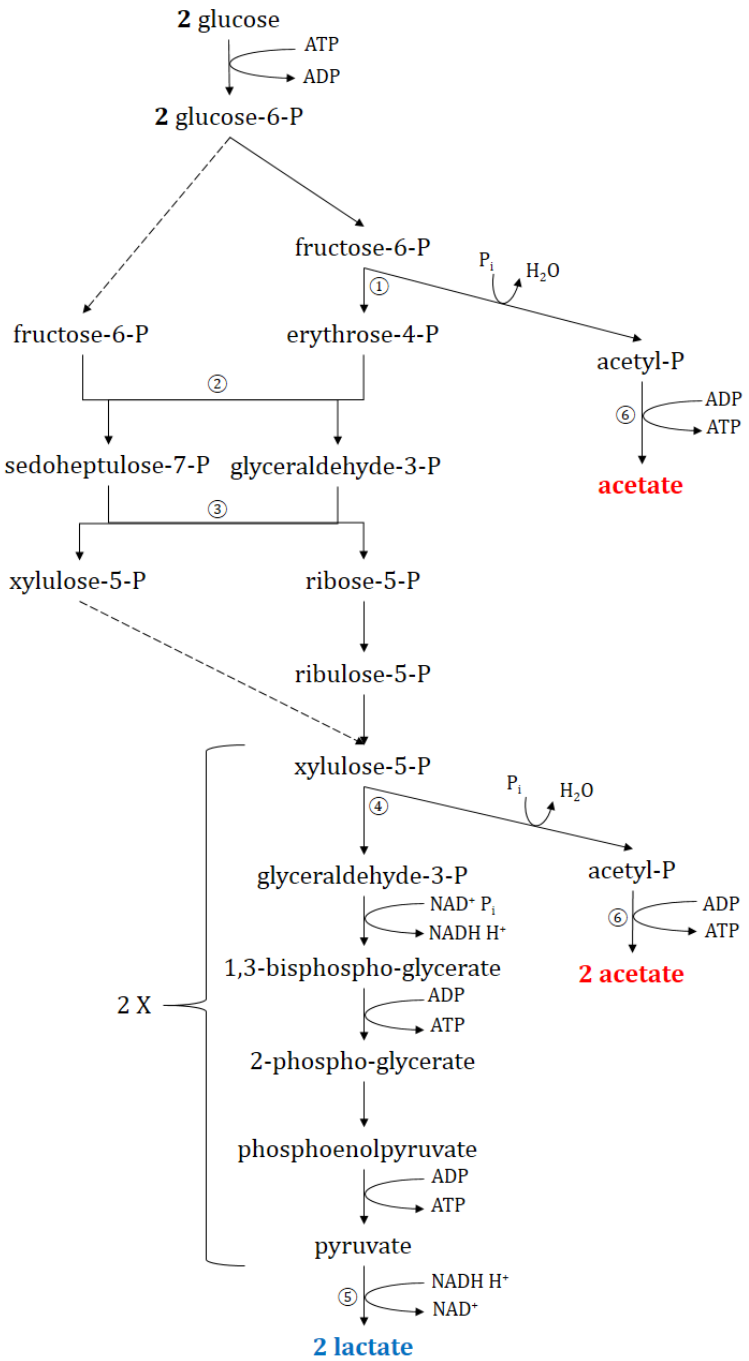

Figure 1.6. Bifidobacterium shunt, adapted from: (Pokusaeva et al., 2011). Highlighted enzymes: 1) Fructose-6-P-phosphoketolase (EC 4.1.2.22) 2) Transaldolase (EC 2.2.1.2) 3) Transketolase (EC 2.2.1.1) 4) Phosphochaeclease (EC 4.1.2.9) 5) Lactate dehydrogenase (EC 1.1.1.27) 6) Acetate kinase (EC 2.7.2.1).

1.4.2 Functional characteristics of probiotics

The selection of new probiotic strains is a big challenge, both for science and industry. To use a microorganism as a probiotic in pharmaceutical or food preparations, certain characteristics are needed, linked not only to the beneficial effect exerted on the host, but also to the practical purposes of large-scale production (Saarela et al., 2000; Holzapfel et al., 2002; Klein et al., 2000; Reuter et al., 2002). From a legislative point of view, the safety and non-pathogenicity of the new strain are essential. The theoretical criteria on which the selection of probiotic microorganisms is based concern the safety of the strain, the functional requirements, and the technological aspects.

1.4.2.1 Strain safety

The assessment of the safety of microbial strains proposed as probiotics has always been a staple for regulatory authorities. The bacteria that will be used in humans should preferably be of human origin and isolated from the gastrointestinal tract of healthy individuals. In addition, they must not have harmful effects, must not possess enzymes with toxic action and must never have been associated with diseases such as infectious endocarditis or disorders in the intestine. As for the metabolites released, probiotics must not produce gases such as hydrogen, methane, and sulfuric acid. During the analysis for the evaluation of new probiotic strains, the interactions between strains, microbiota, and intestinal mucosa of the host are also deepened (Saarela et al., 2000).

Antibiotic resistance, cited also in the Ministry's standard, can be intrinsic or acquired. The first is a capacity that manifests itself naturally, therefore it is an intrinsically characteristic of the strain, and it has minimal potential to be transmitted horizontally. While the second type derives either from a gene mutation or from the acquisition of exogenous DNA from other bacteria, even phylogenetically very distant. Horizontal gene transfer occurs thanks to mobile elements such as conjugative plasmids, transposons, integrins, and bacteriophages. It can be mediated by mechanisms such as natural

transformation, which involves the intake and incorporation of free DNA into the extracellular environment; or such as conjugation, which is a mechanism of DNA transfer between cells that are in contact; and like transduction, which consists of a gene transfer mediated by bacteriophages. Conjugation is thought to be the most common model since many resistance genes are found in plasmids. Moreover, this mechanism can also occur between different genus strains and species. For example, Lactobacilli show different resistances to antibiotics, some of which are intrinsic to the strain and rarely acquired (Mathur et al., 2005).

1.4.2.2 Functional requirements

To use a microorganism as a probiotic in pharmaceutical or food preparations, functional requirements must be verified by *in vitro* studies and possibly confirmed by human models.

RESISTANCE TO ACID pH AND BILE SALTS

A strain could be called “probiotic” if it overcomes the stomach and the duodenum, and it reaches the intestine with vitality. Therefore, the first functional characteristic that determines the selection of probiotics is the stability under acidic pH conditions and in the presence of bile salts. Indeed, the secretion of gastric acids in the stomach is a primary defense mechanism against most of the ingested microorganisms (Dunne et al., 2001; Santini et al., 2010).

To resist to this stress, the Lactobacilli use several strategies, such as maintaining intracellular pH homeostasis, repairing damaged proteins, and changing the cell wall. In addition, in some strains the acid resistance is positively affected by a preliminary exposure of the cells to moderately acidic environments. The molecular basis of the mechanism is still unclear. Nevertheless, recent proteomics studies identified some chaperonins, which are proteins involved in protein synthesis and in cell wall biogenesis, that can be considered potential markers of acid resistance and sensitivity (Hamon et al., 2014; Papadimitriou et al., 2016).

Furthermore, probiotics must remain viable in presence of bile salts, which are produced by the liver from cholesterol, and then poured by the gallbladder into the duodenum. Due to their cleansing properties, bile salts can alter the conformation of the cell membrane, also causing the denaturation of the proteins inserted in it. Indeed, bile salts can insert themselves between membrane lipids, thus changing the integrity and the permeability of the cell. In addition, they induce the production of oxygen free radicals, which alter the secondary structure of RNA, and generate damage in the DNA sequence. To defend themselves, bacteria transformed or improved some transporters for bile to allow the leakage of the salts from the cell milieu. Other bacteria change the composition of the wall, integrating cholesterol, which changes the fluidity, the potential, and the elasticity. Other strains produce various hydrolases that drive the deconjugation reaction of bile salts (Papadimitriou et al., 2016). The amino acids which are released after the reaction can be used as sources of carbon and nitrogen for the survival of the bacteria. Many studies have shown that the expression of bile hydrolase is inducible by adding conjugated bile salts in the culture (Dunne et al., 2001; Li et al., 2012). Temperature, pH, and other environmental factors may cause increased sensitivity or increased resistance, indicating that this is more a phenotypic response to the environment rather than a genetic mutation.

ADHESION TO THE INTESTINAL MUCOSA

The ability to adhere to the intestinal mucosa is essential for the colonization of the gastrointestinal tract. *In vitro* studies of microbial adhesion to the human intestinal epithelium show that this is a strain-specific feature. Indeed, different strains showed very different resistance times within the intestine. The probiotic genus which adhesive capacity has been most evaluated is Lactobacilli. For example, *L. casei* Fyos, *Lactobacillus acidophilus* 1 and *Lactobacillus rhamnosus* GG are the strains known for their great adhesive properties (Tuomola et al., 1998).

The adhesion mechanism of Lactobacilli, as well as of Bifidobacteria, includes passive forces, electrostatic interactions, hydrophilic forces, specific structures (such as lipoteichoic acids) and external components covered with lectin. The crosstalk between lactic acid bacteria and epithelial cells influences the expression of genes involved in many important gastrointestinal functions, such as nutrient intake, metabolism of xenobiotic molecules, angiogenesis, and post-natal intestinal cell maturation. In addition, the probiotics adhesion to host cells increases the antagonistic activity against pathogens, thanks to the competition for the adhesion to the substrate, and to the stimulation of the immune response exerted by them, as well as to the production of protective mucus. Probiotics with adhesion properties remain longer in the gastrointestinal tract, increasing the possibility of prolonging the benefits of their metabolic and immunomodulating activity. However, it is better that their presence is maintained only for the duration of the administration period, to not alter the balance of the microbiota, if it is in an eubiotic state (Saarela et al., 2000; Servin et al., 2004).

VITAMIN PRODUCTION

The human beings, like all the other mammals, are not able to produce vitamins by themselves. Therefore, they must take them from exogenous sources, as food, by the intestinal route. Many microorganisms that compose the gut microbiota can produce vitamins and release them into their surroundings. The vitamins produced by the intestinal symbionts are vitamin K and those water-soluble of B group (biotin, nicotinic acid, folate, riboflavin, thiamine, pyridoxine, pantotheic acid and cobalamin) (Burgess et al., 2009).

For example, *Lactobacillus reuteri* CRL1098 can produce cobalamin and its biosynthetic pathway has been fully identified and characterized (Taranto et al., 2003). Many strains are also producers of folic acid. Therefore, they are exploited by the food industry: i.e., *Lactococcus lactis* and *Streptococcus thermophilus* have been selected for their ability to enrich dairy products with

folate (Leblanc et al., 2007). This is important because the vitamins taken with the diet are absorbed in the proximal part of the small intestine, while the uptake of the microbial ones occurs mainly by the colon cells, helping the balance of the levels of systemic vitamins and the homeostasis of vitamins in the epithelial cells (Rossi et al., 2011; Capozzi et al., 2012).

ANTIMICROBIAL ACTIVITY

Several studies demonstrate the effectiveness of the use of probiotics as therapeutic agents against infections of the gastrointestinal tract. Antimicrobial activity can be exerted by both Lactobacilli and Bifidobacteria, through various mechanisms (Figure 1.7):

- Co-aggregation: it is the assembly of microbial communities into distinct, interlinked structures. For example, in the vagina, the lactobacilli could aggregate with each other and restore the homeostasis (Reid et al., 2011).
- Competition with pathogens: it can be a competition for limiting nutrients and/or for adhesion to the mucous membrane and to the intestinal epithelial cells. In addition, both genera can produce antimicrobial substances capable of stimulating mucosal immunity. Through the phenomenon known as "competitive exclusion" probiotics can adhere to the intestinal mucosa and consequently prevent the adhesion of pathogenic bacteria to the same site (Both et al., 2011).
- Ability to lower the pH: probiotic bacteria, especially the strains belonging to the genus *Lactobacillus*, produce acetic acid, lactic acid, and propionic acid that lower the local pH, leading to the growth inhibition of a wide range of pathogenic Gram-negative bacteria (Both et al., 2011). Indeed, the pH lowering makes the outer membrane more permeable, allowing the penetration of other antimicrobial substances produced by the host and thus increasing the sensitivity of the pathogen to those molecules (Alakomi et al., 2000).

- Production of hydrogen peroxide: it can be considered a non-specific defense mechanism. The H_2O_2 produced in particular by Lactobacilli is able to inhibit the growth of know human pathogens, such as *Staphylococcus aureus*. It can have either bacteriostatic or bactericidal effect depending on the concentration (Both et al., 2011).
- Production of antimicrobial molecules: biosurfactants are surface active compounds that are synthesized also by probiotics and may have a role in the restoration and in the maintenance of the microbial homeostasis thanks to the double property of surfactant and antimicrobial molecules. For example, the vaginal Lactobacilli could produce biosurfactants that are composed by proteins, lipids, and carbohydrates that help to displace the uropathogenic *E. coli*, *Enterococcus faecalis*, and *Gardenerella vaginals*. The positive effects appear even with a few Lactobacilli, suggesting that the biosurfactants are released and spread in the vaginal mucosa, altering the surface tension, and thus inhibiting the hydrophobic pathogens (De Giani et al., 2021b; Reid et al., 2011). Nevertheless, the antimicrobial molecules par excellence are bacteriocins. They are peptides and proteins synthesized by ribosomes and varying both in structure, microbial target, and mechanism of action. Generally, bacteriocins have a narrow killing spectrum and their mode of action include interfering with cell wall structure and biosynthesis, forming pores in the target bacterial membrane and permeabilizing membranes (Reid et al., 2011). They are produced by several probiotic lactic acid bacteria, belonging to the genera *Lactobacillus*, *Bifidobacterium*, *Lactococcus*, and *Enterococcus*. These strains could probably sense the bacterial dynamics of their niche and produce the molecules to retain a competitive position (Reid et al., 2011).

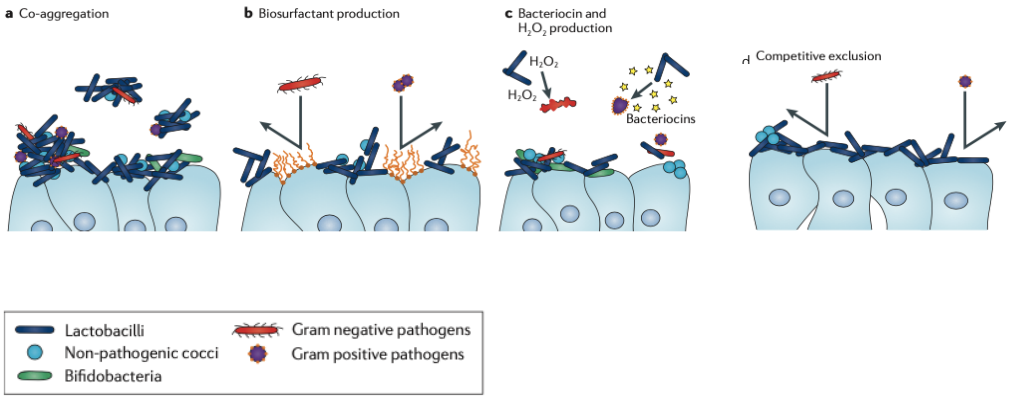

Figure 1.7. Competition mechanisms of probiotic bacteria in presence of pathogenic strains (adapted from Reid et al., 2011).

IMMUNE SYSTEM RESPONSE STIMULATION

The intestinal membrane offered by mucus and epithelial cells plays an important role in the exclusion and in the elimination of potentially dangerous antigens and microorganisms. At the same time, it promotes the selective absorption of nutrients (Both et al., 2011). It is known that a wide variety of pathogenic antigens and microorganisms manage to pass through the membrane, stimulating the immune response and in some cases causing diseases (Kailasapathy et al., 2000). Many studies show that probiotics can modulate the immune system both at specific response level, through the production of antibodies and cytokines, and through the proliferation of lymphocytes; and at non-specific response level, through the activation of natural killer (NK) cells. For example, administration of *Lactobacillus paracasei* has been shown to induce an increase in the production of circulating antibodies against *Pseudomonas aeruginosa*. Furthermore, a diet enriched with *L. rhamnosus* HN001 increases the number of natural killer cells in men, while the fermented milk of *L. casei* Shirota increases the cytotoxic activity of the same natural killers (Both et al., 2011). Specifically, the activation of neutrophils and NK could be closely connected with the anticancer and anti-inflammatory properties of probiotics.

The recognition of receptors on the surface of intestinal epithelial cells by probiotic bacteria can activate the signal that leads to the synthesis of cytokines, which simulate the function of lymphocytes in the mucous membrane. In addition, they can decrease the secretion of pro-inflammatory cytokines and increase the synthesis of the anti-inflammatory ones (Plaza-Diaz et al., 2014). These abilities allow probiotics to be considered an important biological alternative to the therapeutic drugs used today in the treatment of intestinal disorders.

1.4.3 Technological aspects

Nowadays, the maintaining of good health, leading to longevity has become an important issue. Certain foods containing bioactive compounds, that are matrices having a positive effect on human health and are classified as functional foods or nutraceuticals (Mishra et al., 2018). These novel foods, also containing probiotics, are part of the world market. However, till date, Japan is the only country that has formulated a specific regulatory approval process for the functional foods, coining the term “Foods for Specified Health Use (FOSHU)” (Mishra et al., 2018). The production, which began about twenty years ago, is still in increasing development. To promote the consumption of these products, the food industries are constantly looking for new probiotic strains, but also prebiotics and nutraceuticals, to enhance the life span and the quality of human beings. However, the new components must be added as not to alter the sensory properties of the final product in terms of color, flavor, and aroma. Furthermore, to preserve vitality of the microorganisms, innovative technologies such as immobilization, nanotechnology and encapsulation could be used (Mishra et al., 2018). Among the encapsulation techniques, polysaccharide matrices, such as alginate or starch, and covered with coating polymers of various kinds, such as proteins, chitosan, cellulose, and its derivatives, could be employed. This technology, known as “microencapsulation”, preserves probiotics from moisture and increases their stability during processing and storage of food or

supplements (Riaz et al., 2013). There are various techniques used for microencapsulation in probiotic foods, such as the extrusion technique (Iravani et al., 2015); the emulsion method (Mortazavian et al., 2007; Mortazavian et al., 2006); the freeze-drying (Carvalho et al., 2004); the spray-drying technique, that is useful for developing water-insoluble dry microcapsule preparation with small and controlled particle size (Picot et al., 2003; Groboillot et al., 1994). Lastly, the immobilization of probiotics which protects against physicochemical changes, such as pH, improved substrate utilization, temperature, higher productivity and efficiency, higher cell densities and cell loads, sterility and faster fermentation and maturation rates (Mitropoulou et al., 2013). Also, the appropriate choice of packaging material and the best storage conditions can also help to preserve the quality of functional products containing probiotics (Saarela et al., 2000).

1.4.4 Therapeutic potential of probiotics

Probiotics have long been used mainly as supplements to restore the intestinal imbalances, resulted from dysfunctions, nevertheless they can be used to reduce the risk of specific pathologies and to mitigate objective symptoms. In addition to the prevention of intestinal infections, probiotic bacteria have other, more specific therapeutic potentials.

1.4.4.1 Restoration and protection towards antibiotic-associated infectious diarrhea

Probiotics are effective in treating antibiotic-associated infectious diarrhea in adults, and in reducing the effects of infectious diarrhea in children, especially in Western countries, where Rotavirus infection is the main cause. For the latter pathology, the strains *L. rhamnosus* GG, *L. reuteri*, *L. casei* and *Saccharomyces boulardii* (Kopp-Hoolihan, 2001) are considered effective, as well as *L. acidophilus* e *Bifidobacterium longum* (Lee et al., 2015).

The Antibiotic-Associated Diarrhea (AAD) is the result of an imbalance in the microbiota present in the colon because of antibiotic therapy. The alteration of

the microbiota causes changes in carbohydrate metabolism, with reduced absorption of short-chain fatty acids, which lead to osmotic diarrhea. The other cause of diarrhea resulting from antibiotic therapy is the proliferation of potentially pathogenic microorganisms, such as *Clostridium difficile*. In this case, specific treatment with *L. rhamnosus* GG may reduce the incidence and severity of AAD. In addition, the administration of lactobacilli strains confirmed the possibility of preventing and treating diarrhea induced by other pathogens, such as *E. coli*, *Salmonella* spp., or *Shigella* spp. (Kailasapathy et al., 2000).

1.4.4.2 Antitumoral effects

The anticancer action of probiotics could be explained by:

- Inhibition of carcinogens and/or pro-carcinogens
- Inhibition of bacteria that convert pro-carcinogenic substances into carcinogens
- Activation of the immune response
- Lowering intestinal pH, which reduces microbial activity

The biochemical consequences of the action of probiotic bacteria include the reduction of fecal enzymes that convert pro-carcinogens into carcinogenic substances within the gastrointestinal system. For example, *L. acidophilus* and *Bifidobacterium* spp. may reduce the level of enzymes such as β-glucuronidase, nitrogen reductase and nitro reductase, responsible for catalyzing the conversion of carcinogenic amines (Kailasapathy et al., 2000). The consumption of probiotic bacteria, in association with oligosaccharides, could promote bacterial growth in the colon and increase the production of short-chain fatty acids such as butyrate. Furthermore, it is known that some bacteriocins secreted by probiotic bacteria such as *Lactobacillus plantarum* C11 and *Pediococcus acidilactici* PAC 1.0, have an antitumor effect, tested *in vitro* on various cell lines (Kaur et al., 2015).

1.4.4.3 Cholesterol lowering potential

Several studies on animal models have shown the effectiveness of *L. acidophilus* to reduce blood cholesterol levels by the deconjugation of bile acids into free bile salts, which are excreted much more quickly from the intestinal tract. In this way, the synthesis of new bile acids from cholesterol can reduce the total concentration of cholesterol in the body (Kailasapathy et al., 2000). More recent studies show that *P. acidilactici* LAB4 and *L. plantarum* LAB12, for example, can be used to combat hypercholesterolemia, as they are able to directly assimilate cholesterol from the surrounding medium (Lim et al., 2017).

1.4.4.4 Helping in the overcoming of lactose intolerance

Lactose intolerance is a problem that affects about 75% of the world's population and it is caused by the insufficient activity of several lactases at the level of the intestinal mucosa. This leads to abdominal discomfort of various magnitudes. Therefore, individuals with such intolerance are forced to reduce the consumption of dairy products. This in turn, exposes them to the risk of being deficient in certain nutrients, in particular calcium. (Kopp-Hoolihaan, 2001). Several studies show that appropriate lactic bacterial strains, in adequate quantities, can alleviate the symptom of lactose intolerance. These probiotic bacteria include *L. acidophilus*, *L. bulgaricus* and Bifidobacteria, which produce the β-D-galactosidase enzyme, allowing the digestion of lactose (Kailasapathy et al., 2000). Therefore, the consumption of lactic products containing adequate amounts and species of lactic bacteria could represent a good solution for lactose intolerant individuals to restore the introduction of essential nutrients for the diet (Kopp-Hoolihaan, 2001).

1.4.4.5 Reduction of the inflammatory or allergic response

In the recent years, in industrialized countries, there has been a significant increase in autoimmune and allergic diseases. The phenomenon is so relevant that it has led to the postulation of the "hygiene hypothesis", according to which

too high levels of cleanliness, which lead to a different or lack of exposure of children in the first months of life to microorganisms and other infectious agents, could lead to disturbances in the development of an adequate immune response and to an altered composition of the intestinal microbiota (Clark et al., 2016). This last hypothesis is supported by the observation of a different gut microbiota profile in children with allergies, in which there is a prevalence of fecal coliforms and *S. aureus*, while the number of Lactobacilli is reduced. Probiotics, i.e., *L. rhamnosus* GG, can relieve the symptoms of food allergies thanks to the breakdown of proteins, such as milk caseins, into small peptides and amino acids, and reduce the chance of developing atopic dermatitis or reducing its severity (Kopp-Hoolihaan, 2001). This is confirmed by recent clinical trials, which have shown that administering certain probiotic strains before and after the prenatal life of the baby significantly reduces the risk of developing atopic dermatitis (O' Neill et al., 2016).

It is also reported that several studies have highlighted the potential of probiotics in improving bacterial infections of the urinary tract and of the female genital tract. Indeed, the consumption of probiotics is shown to be able to reduce the recurrence of infection with *Candida albicans* and bacterial vaginosis.

Finally, it is stated that these bacteria bring moderate decreases in blood pressure and that they can eradicate infections of *Helicobacter pylori*, normally associated with gastritis, peptic ulcers tumor in the stomach.

1.5 Prebiotics

Gibson and Roberfroid were the first to introduce the prebiotic concept in 1995: “A prebiotic is a non-digestible food ingredient that positively affects the host by selectively stimulating the growth and/or the activity of one or a limited number of bacteria in the colon and thus improves the health of the host” (Gibson et al., 1995). However, the concept evolved during time. Finally, in 2008, ISAPP elaborated the latest and most current definition of prebiotic: "a food prebiotic is a selectively fermented ingredient that causes specific changes in the composition and/or in the activity of the gastrointestinal microbiota, thus conferring benefits on the health of the host." (Swanson et al., 2020).

Generally, prebiotics are carbohydrates that are not hydrolyzed by the host's hydrolytic enzymes and can act as fermentable carbon sources for the colon's saccharolytic microbiota. Indeed, the two main types of anaerobic fermentation that take place in the intestine are the saccharolytic and the proteolytic. The main end products of carbohydrate metabolism are SCFAs, that can be further metabolized systemically or locally to provide energy to the host. Whereas, the final products of protein fermentation are phenolic compounds, amines, and ammonia, which are toxic to the host. The proximal colon is essentially a site of saccharolytic fermentation, while in the distal part there is a depletion of available carbohydrates and increased protein metabolism.

1.5.1 General considerations about prebiotics

Despite the various definitions, the scientific community agrees that to be a prebiotic, a substance must have the following properties:

- Resistance to gastric juices, hydrolysis by human enzymes and absorption in the upper part of the gastrointestinal tract (Thammarutwasik et al., 2009). It is not necessary for the prebiotic to be completely non-digestible, but it must ensure that a significant amount of the product is bioavailable in the large intestine as a fermentable substrate (Gibson et al., 2004).

- Ability to be fermented in the large intestine by the intestinal microbiota, especially by bacteria belonging to *Bifidobacterium* and *Lactobacillus* genera (Kolida et al., 2002; Gibson et al., 2004). The ability of the gut strains to use and then ferment the potential prebiotic substrate must be demonstrated with experiments developed both *in vitro* and *in vivo*.
- Selective stimulation of the development and/or activity of intestinal bacteria that contribute positively to the health of the host. The selective ability to induce the increase of beneficial like Bifidobacteria, Lactobacilli and other bacterial genera including *Roseburia*, *Eubacterium*, *Faecalibacterium* and *Akkermansia*, must be demonstrated to evaluate the entire balance of the intestinal microflora following the intake of the prebiotic. In addition, the prebiotic must not favor possible pathogenic bacteria that cause gastrointestinal diseases, such as *Clostridium perfringens* (Gibson et al., 2004; Kolida et al., 2002)

The aim of the administration of prebiotics as food supplements is the increment of autochthonous beneficial bacteria and the stimulation of their metabolic functions due to prebiotics fermentation, leading to a reduction of putrefactive or of potential pathogenic microorganisms (Campbell et al., 1997; Rycroft et al., 2001). Therefore, most prebiotics are carbohydrates, because they are molecules that fulfill the above-mentioned criteria, however the definition does not exclude other molecules that could be used for the same purpose. Currently, the most employed are fructooligosaccharides (FOS), galactoolisaccharides (GOS), xylooigosaccharides (XOS), and resistant starches (RS). Interestingly, most of the prebiotic sources are natural. Indeed, several fruits and vegetables contain oligosaccharides such as FOS. Examples are artichoke, onion, garlic, banana, asparagus, leeks, Jerusalem artichoke, and chicory. However, the natural levels of the prebiotic molecules in these foods are too low to exert a significant effect, hence the ever-increasing demand to develop foods enriched with these natural prebiotic molecules (Gibson et al., 2004).

1.5.2 Classification of prebiotic carbohydrates

The primary classification of dietary carbohydrates is by molecular size, as determined by the degree of polymerization (DP), the type of linkage between the molecules (α or non-α) and the character of the individual monomer. The chemical approach categorizes the carbohydrates into three main groups: sugars (1<DP<2), oligosaccharides or short-chain polysaccharides (3<DP<9), and polysaccharides (DP>10) (Table 1.1) (Cummings et al., 2007).

Class (DP[a])	*Subgroup*	*Principal components*
Sugars (1–2)	Monosaccharides	Glucose, fructose, galactose
	Disaccharides	Sucrose, lactose, maltose, trehalose
	Polyols (sugar alcohols)	Sorbitol, mannitol, lactitol, xylitol, erythritol, isomalt, maltitol
Oligosaccharides (3–9) (short-chain carbohydrates)	Malto-oligosaccharides (α-glucans)	Maltodextrins
	Non-α-glucan oligosaccharides	Raffinose, stachyose, fructo and galacto oligosaccharides, polydextrose, inulin
Polysaccharides (⩾10)	Starch (α-glucans)	Amylose, amylopectin, modified starches
	Non-starch polysaccharides (NSPs)	Cellulose, hemicellulose, pectin, arabinoxylans, β-glucan, glucomannans, plant gums and mucilages, hydrocolloids

[a]Degree of polymerization or number of monomeric (single sugar) units.

Table 1.1. The major dietary carbohydrates (Cummings et al., 2007).

MONOSACCHARIDE COMPOSITION

Recognized prebiotics consist mainly of monosaccharides such as glucose, galactose, xylose, and fructose. The most employed oligosaccharides are fructooligosaccharides (composed of fructose molecules), galactooligosaccharides (compose of galactose and glucose), and xyloligosaccharides (composed by the xylan fraction of plant fibers). Finally, the polysaccharides are inulin (basically fructose and glucose molecules, with different degree of polymerization), starch (composed by amylose and

amylopectin), hemicellulose (based on xylan molecules) and pectins (composed by galacturonic acid, derived from galactose) (Moore, 2011).

GLYCOSIDIC BONDS

The link between monosaccharide residues is a crucial factor in determining both the selectivity of fermentation and the digestibility in the small intestine. For example, the fermentation of FOS is selective due to a β-fructofuranosidase in Bifidobacteria given the presence of β-glycosidic bonds (1-2) (Moore, 2011). However, there are several combinations of α- and β- linkages in different positions, such as 1→2, 1→3, 1→4, and 1→6 (Lahtinen et al., 2010).

DEGREE OF POLYMERIZATION (DP)

The degree of polymerization is the number of repetitive units present in the structure of a polymer. The DP of a homopolymer is equal to the ratio between the molecular weight of the polymer and the molecular weight of the individual repeating units, which are all the same. Most of the current used prebiotics have a relatively low DP, except for inulin. Indeed, inulin has the highest molecular weight and can have a degree of polymerization of up to 60 (Moore, 2011). This is important because oligosaccharides should be hydrolyzed by cell-associated bacterial glycosidases before the absorption of the resulting monosaccharides. Therefore, it is reasonable to assume that the longer is the oligosaccharide, the slower is the fermentation. Therefore, the prebiotic effect will turn out to be more effective throughout the colon. For example, long-chain inulin may exert a prebiotic effect in more distal colon regions than the low-polymerization FOS, because they can be more rapidly fermented in the saccharolytic proximal gut (Gibson et al., 2004).

1.5.3 Most employed prebiotic carbohydrates

Current interest is focused on oligosaccharides with low molecular weight because they can be used as carbon sources for bacteria in the large intestine. The prebiotics of natural origin most used today, and their description is described below.

FRUCTOOLIGOSACCHARIDES (FOS) AND INULIN

Fructooligosaccharides (FOS) are linear fructose oligosaccharides composed by D-glucose monomer linked to α-(1→2) to two or more β-(2→1)-linked D-fructosyl units. Inulin consists of fructose molecules linked by fructosyl-fructose linkages β-(2→1) and a final glucose molecule linked by fructosyl-glucose linkage β-(2→1) (Figure 1.8). These two types of carbohydrates can escape the human gastrointestinal digestion because of the β-(2→1) bond. However, once in the colon, the β-fructosidase-producing bacteria can hydrolyze it and growth (Moore, 2011). For example, members of the Prevotellaceae family depolymerize the molecules through the enzyme β-fructofuranosidase (β-Fru) that hydrolyzes the terminal units of fructose and acts as an invertase against sucrose. Therefore, these polysaccharides are often included in synbiotic formulations containing probiotic bacteria to promote their rapid growth in the intestinal environment (Peshev et al., 2014).

From the chemical point of view FOS and inulin differ exclusively in the degree of polymerization. FOS are molecules with a degree of polymerization between 2 and 10, with and average DP of 4; while inulin is a polymer whose degree of polymerization can reach 60, with an average DP of around 10 to 12 (Moore, 2011). This is because inulin is a polysaccharide used by plants as a storage nutrient. Then, by the action of the enzyme inulase, the resulting hydrolysis produces fructose. FOS can be produced from sucrose by fructosyltransferase that produces oligomers of increasing length, or by controlled hydrolysis of inulin extracted from plants. Indeed, inulin is found in over 3,600 fruits and vegetables,

especially those of the Cichorium family, e.g., chicory, artichoke, banana, large onion, and garlic. It is generally found also in some fungi.

Inulin and FOS can be dissolved in hot water (Tanya et al., 2002) at about 80°C (Ren et al., 2020), but are almost insoluble in cold water and alcohol (Wang, 1993). They are quite stable, have no undesirable sensory properties and are sweet to the taste. Therefore, they have been used in the food industry to improve the sensory and physical properties of some products. For example, inulin is used as a sweetener especially by diabetics. Furthermore, FOS and inulin help to preserve freshness and moisture in cakes and the physical stability of drinks. (Thammarutwasik et al., 2009).

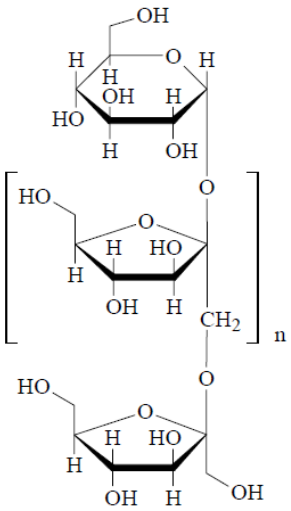

Figure 1.8. General structure of FOS (n = 3-10) and inulin (n = 11-60) (Moore, 2011).

GALACTOOLIGOSACCHARIDES (GOS)

Galactooligosaccharides (GOS) generally comprise a chain of galactose units, arising though consecutive transgalactosylation reaction, with terminal glucose unit. Typically, GOS consists of between 2 to 5 β-(1→6) galactopyranosyl monomers linked to a terminal glucopyranosyl residue by an α-(1→4) glycosidic bond (Figure 1.9). Therefore, the degree of polymerization and the chain length

of GOS can vary markedly (Moore, 2011). The GOS are also produced through enzymatic conversion of lactose with β-galactosidase. They are found in bovine milk, in breast milk and in yogurt.

Also GOSs are not digestible by salivary and intestinal enzymes, so they can pass into the large intestine intact (Jeurink et al., 2013). However, they can be hydrolyzed by the microorganisms in the large intestine, leading to the production of acetic acid, propionic acid, and butyric acid, and gases, such as H_2, CH_4 and CO_2. Indeed, GOS mimic the oligosaccharides found naturally in human milk, therefore they selectively stimulate the beneficial bacteria, primarly bifidobacterial (Moore, 2011). Furthermore, they can ameliorate the absorption of calcium and magnesium (Chonan et al., 1995; Yanahira et al., 1997). They can also exert a protective role in the prevention of colorectal cancer (Wijnand et al., 1999), regulating the activities of various bacterial enzymes, e.g., β-glucuronidase and nitroreductase involved in the production of toxins and carcinogens (Rowland et al., 1993; Sako et al., 1999), and reducing the production of dangerous compounds, such as ammonia, indole, and p-cresol, which stimulate cancer expansion (Sako et al.,1999).

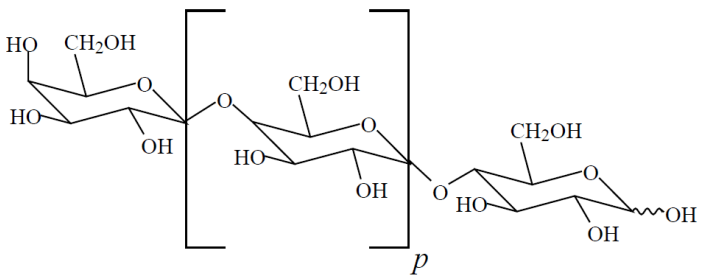

Figure 1.9. General structure of GOS (Moore, 2011).

XYLOOLIGOSACCHARIDES (XOS)

Xyloligosaccharides (XOS) are formed by 2-6 monomer units of xylose bound by β-(1→4) bonds. Their C5 (where C is a quantity of carbon atoms in each monomer) structure is fundamentally different from other prebiotics, which are based upon C6 sugars. They are produced from the xylan fraction of plant fiber (Figure 1.10). XOS are hydrolyzed by β-xylosidases to single units of xylose (Vernazza et al., 2006).

Also xylooligossaccharides could act as prebiotic within the digestive tract. Several clinical trials have been conducted with XOS, demonstrating a variety of health benefits, including improvements in blood sugars and lipids, digestive improvement, laxation, and beneficial changes to immune markers. These health benefits have typically been observed at 1–4 g/d, a lower dose than required for prebiotics such as FOS and inulin (Peshev et al., 2014).

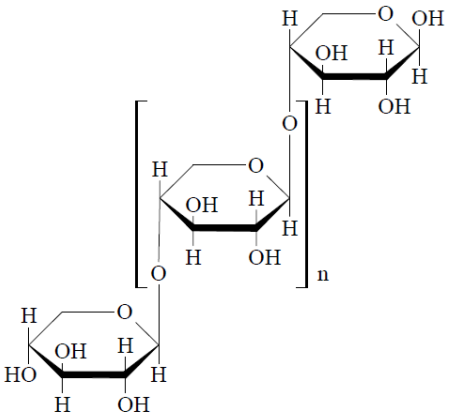

Figure 1.10. General structure of XOS.

RESISTANT STARCH (RS)

Starches are found in many common foods, such as grains, cereals, vegetables, legumes, seed, and nuts. However, not all of them are hydrolyzed to D-glucose and then absorbed in the small intestine but could be fermented in the colon by the resident microbiota (Cummings et al., 2007; Fuentes-Zaragoza et al., 2011). Indeed, starch is resistant to enzymatic digestion due to the size of starch-

containing fragments, the structure and conformation of intact starch granules, and the formation of retrograded crystallites caused by chemical modifications (Fuentes-Zaragoza et al., 2011). Like the other starches, RS are considered polysaccharides in which several monosaccharides (glucose) are linked together by either α-D-(1→4) and/or α-D-(1→6) linkages. The two fundamental structural components are amylose and amylopectin. Amylose consists of a mostly linear polymer with glucose monomers linked by α-D-(1→4) bonds, while amylopectin is a larger, highly branched molecule that consists of glucose monomers liked by both α-D-(1→4) and α-D-(1→6) (Moore, 2011) (Figure 1.11). Generally, the molecular weight is relatively low, ranging from 1.2 to 500 kDa.

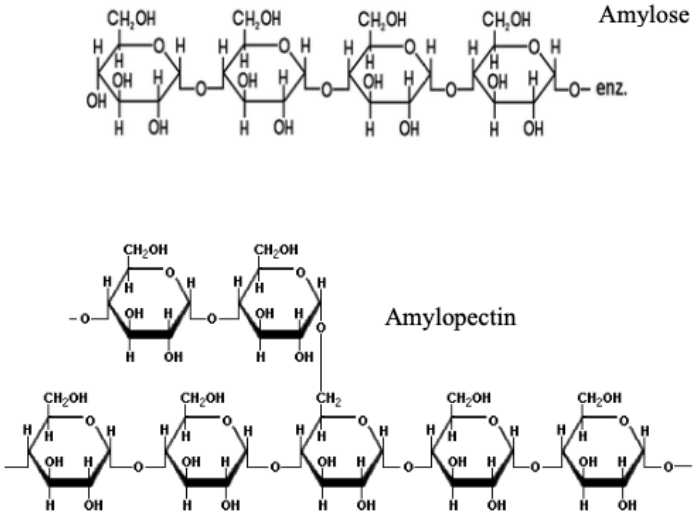

Figure 1.11. General structure of amylose and amylopectin, principal components of RS (Moore, 2011).

RS are classified as an incomplete *in vitro* digestion of starches in food products that have undergone cooking and cooling. Recently this definition has been expanded to include starch and starch degradation products that resist small intestinal digestion and enter the large bowel in healthy humans (Moore, 2011). Due to these characteristics, RS comprised several different types and materials. Furthermore, RS is classified into five general subtypes named form RS1 to RS5. RS1 includes physically inaccessible starch, locked within the cell

walls and in the food matrices, preventing the amylolysis. Instead, RS2 counts native starch granules from plants slowly hydrolysable by R-amylases. RS3 are formed after cooking and appear like crystalline nongranular starch. Therefore, are thermostable. RS4 are chemically modified starches used by food manufacturers to alter the functional characteristics of the starch. Finally, RS5 is the most interesting from the nutritional point of view because is a polysaccharide that consist of water-insoluble, linear poly-α-1,4-glucan, non-susceptible to degradation by α-amylases, promoting the SCFAs production in the colon, which could be boosted combining FOS with RS (Fuentes-Zaragoza et al., 2011).

1.5.4 Application as nutraceuticals and as functional foods

The health benefits exerted by prebiotics can be obtained by adding them into products obtaining functional foods with beneficial properties on one or more functions of the body, as well as adequate nutritional effects (Diplock et al., 1999); or nutraceuticals, that are formed by concentrated phytocomplexes (i.e., active substances extracted from vegetal matrices) with pharmacological effect in addition to the nutritional value (Santini et al., 2017).

Several food products could be enriched with prebiotics, for example functional drinks or breakfast cereals with added dietary fibers increasing the efficiency of the digestive system and improving the growth of probiotics; or baby food A study reports that the addition of inulin, FOS and GOS individually or in combination to baby food increase the number of Bifidobacteria and Lactobacilli in the baby's digestive system by 31 to 59% of the total intestinal microbiota during the first six weeks, like breastfeeding (Thammarutwasik et al., 2009). Or dairy products improving the calcium absorption.

In this context, nowadays, a healthy ageing process has received more and more attention. According to the United Nations report on "World Ageing" (DESA, UN, 2002), a person who is at least 60 years of age can be considered elderly. However, this classification can vary in the world, in fact the World Health

Organization (WHO) establishes that in the African population the onset of old age corresponds to the age of 55 years (MDS Project, WHO, 2002). In any case, it has been shown that around the age of 55-60 years, the counts of fecal Bifidobacteria considerably decrease with respect to those of the young population. The minor number of Bifidobacteria could then result in a reduction in the ability to resist the colonization of the gut environment by potential pathogens. Therefore, prebiotics can potentially be used to restore the balance of gut microbiota in the elderly population and, at the same time, provide indirect anti-pathogenic protection. For example, in the study of Walton et al. (2012), to compensate for the reduction in Bifidobacteria associated with aging, subjects aged 50 years and older were treated with prebiotic GOS. The number of Bifidobacteria increases with the treatment, linked to increased levels of Lactobacilli and butyrate. In another study (Gibson et al., 2004), the effect of a mixture of GOS on immune system markers and on the produced metabolites in the elderly (aged 65 to 80 years) was examined. The treatment caused an increase in *Bacteroides* and Bifidobacteria, correlated with an increase in lactic acid in the stool. Recently, also synbiotics have been tested to improve age-related changes in the gut microbiota. Indeed, a synbiotic composed by the probiotic *B. longum* and an inulin-based prebiotic was administered to elderly volunteers (Macfarlane et al., 2013). Results showed that Bifidobacteria counts were higher with respect to the basal levels, as well as the members of the phyla Actinobacteria and Firmicutes, while Proteobacteria were reduced. The treatment with the synbiotic also caused an increase in butyrate production and a reduction in pro-inflammatory responses. The interesting findings further support the link between diet, microbiota, metabolism, and inflammation in the elderly (Quigley, 2013), that could explain why probiotics in combination with prebiotics have been successfully used in the elderly for the treatment of respiratory and gastrointestinal infections and the improvement of vaccine responses (Bischoff, 2016).

1.6 Synbiotics

An alternative possibility to modulate or re-balance the intestinal microflora is the use of synbiotics. The term is formed from the Greek prefix *syn*, meaning "together" and the suffix *biotic*, meaning "pertaining to life". Indeed, a synbiotic is composed by probiotics and prebiotics. Twenty-five years ago, when the synbiotic concept was used for the first time, the idea that selectively fermentable non-digestible ingredients (the prebiotics) could be mixed directly with probiotic bacteria was chimerical. Indeed, the term synbiotic was poorly associated to a formulation of probiotics and prebiotics that beneficially affect the host (Gibson et al., 1995). However, even today there is confusion in using the term, maybe because of the original definition itself and because of the suffix "-biotics", which is include in words like postbiotics and pharmacobiotics. Therefore, in May 2019 the International Scientific Association for Probiotics and Prebiotics (ISAPP) gave clarity and guidance regarding the appropriate use of the term synbiotic (Swanson et al., 2020). The definition was updated to "a mixture comprising living microorganisms and substrate(s) selectively utilized by host microorganisms that confers a health benefit on the host". The host microorganisms are both autochthonous (resident or colonizing the host) and allochthonous (such as probiotics given by external formulations) microorganisms. Either can use as carbon and energy source the supply prebiotic, even if the administered probiotics could be present in the gut environment transiently. Besides, any combination must be tested to confirm that the health benefits are conferred by the formulation with comparison to a placebo. If not, the product should not be called synbiotic.

1.6.1 Essential characteristics of a synbiotic formulation

The common usage of the term synbiotic comprises formulations that mix probiotics and prebiotics, even if they could not have any great co-dependent function. Nevertheless, the single components could independently promote health benefits for the host. Indeed, a combination could be considered a

synbiotic if each component fulfils the probiotic and the prebiotic definitions (Swanson et al., 2020). The ISAPP categorized the synbiotic into two categories: the functional (or complementary) and the synergistic (Figure 1.12). The characteristics of each formulation are discussed below.

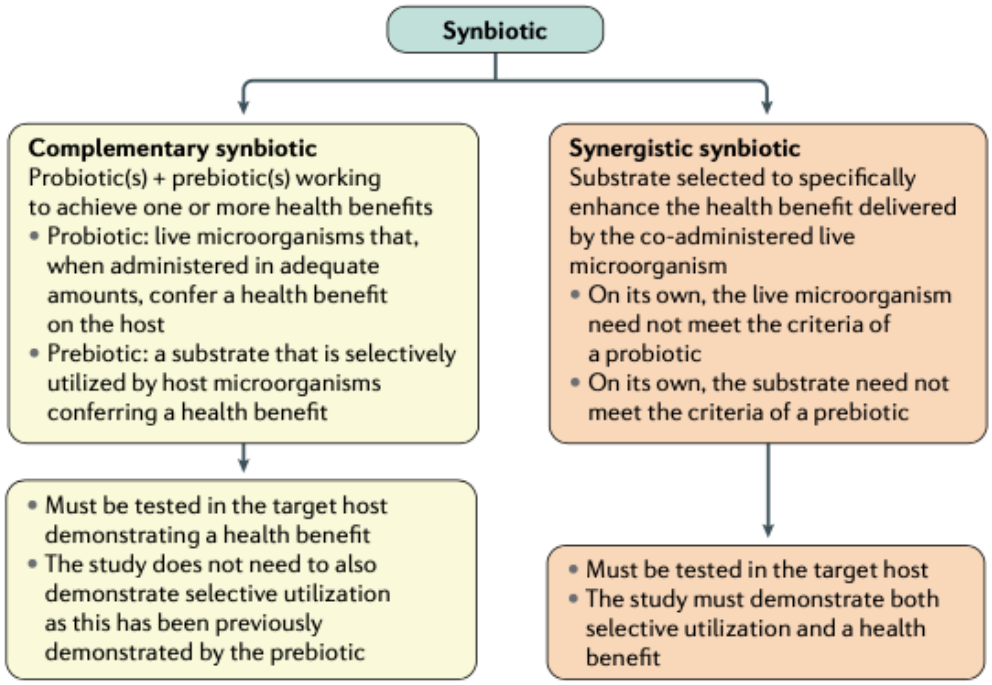

Figure 1.12. Synbiotic categories (Swanson et al., 2020).

FUNCTIONAL SYNBIOTIC

A functional synbiotic could be designed at doses below those at which the probiotic or the prebiotic could independently exert health benefits. Furthermore, the selected microorganism might not fulfil all the probiotic functions if administered alone, but it could exert them if it is used at high dosage, leading to ecological effects in the gut ecosystem (such as competition) in presence of the suitable substrate. Similarly, the source of carbon and energy, even at high doses, might not provide benefits to the host itself, but could do it selectively stimulating the microorganisms that can enhance the beneficial effects for the

host. Therefore, formulations designed in this way comprise a live microorganism and a substrate that depend on the presence of one another, and their function is closely related (Figure 1.13) (Swanson et al. 2020). To study the efficacy, a two-arm parallel or crossover study could be suitable. Indeed, the aim is to demonstrate that the combination is better than the placebo group, leading to relevant health benefits.

SYNERGISTIC SYNBIOTIC

A synergistic formulation is a synbiotic in which the substrate is designed to be selectively utilized by the co-administered microorganisms. In this kind of formulation, the probiotic is selected based on its ability to exert health benefits, and the prebiotic is chosen to principally support the growth or the activity of the selected probiotic (Kolida et al., 2011). Therefore, the simultaneous administration of probiotics and a substrate that they selectively metabolize offers to the administered strains a greater chance of colonization and survival in the host colon, enhancing or prolonging their beneficial effects.

However, the substrate could potentially enrich other beneficial members of the gut microbiota, but the main target is the microorganism comprised in the formulation. For these reasons, design and demonstrate the efficacy of a synergistic synbiotic is experimentally challenging (Figure 1.13). Indeed, the studies that compare the synergistic synbiotic to a control condition can provide supportive evidence that do not constitute the primary evidence confirming the synergistic mechanism. They study must consider as controls a group treated with the prebiotic alone, one treated with the probiotics alone, and another one treated with the placebo formula. Doing so, it could be possible to demonstrate that the combined effect is better than the estimated effect of each component administered separately, and better than the placebo both on the healthy status and on the gut microbiota composition of the subjects.

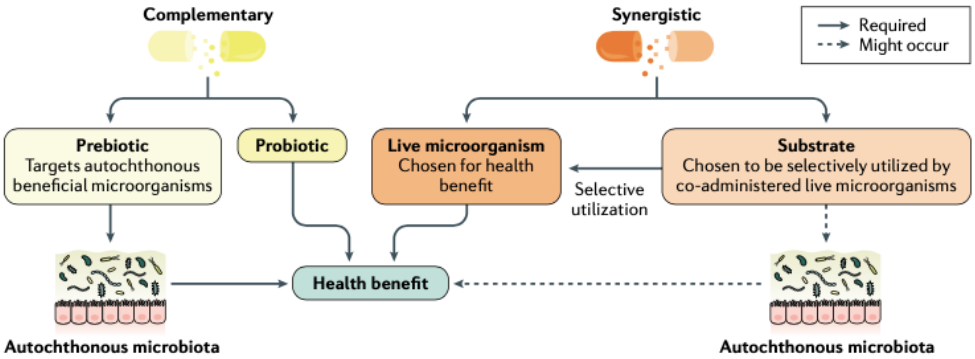

Figure 1.13. Design and mechanisms of action of functional and synergistc synbiotics (Swanson et al., 2020).

1.6.2 Examples of tested synbiotic formulations

There are several evidence supporting the positive impact of synbiotics on the intestinal microflora (Casiraghi et al., 2007; Rastall et al., 2002). The most widely used and already commercially available synbiotics concern mixtures of *Lactobacillus*, *Bifidobacterium*, and *Streptococcus* strains, combined with GOS, inulin or FOS (Swanson et al., 2020). However, the used prebiotic dose varies considerably, from the lowest 100 mg to the highest 10-15 g per day. For example, in the double-blind clinical study conducted by Sommacal et al., (2015), 100 mg of FOS were combined with *L. acidophilus*, *L. rhamnosus*, *L. casei*, and *Bifidobacterium bifidum*. They administered the synbiotic 4 days before and 10 days after the surgery for periampullary neoplasms. The results showed a lower number of postoperative infections, a shorter duration of antibiotic therapy, fewer non-infectious complications, shorter length of hospital stay, and reduced mortality than in patients receiving the placebo formulation. In this case, the 100 mg/day of FOS could not provide a prebiotic effect on the co-administered probiotic strains but could be sufficient to stimulate a related strain within the gut microbiota.

Instead, in another example (Costabile et al., 2017), it is described a synbiotic formulation composed by *L. rhamnosus* GG-PB12 or *L. rhamnosus* GG

combined with 8g of soluble corn fiber, tested thorough a 3 weeks prospective, double blind, randomized, cross-over study on healthy elderly subjects. The results showed that both the synbiotics were effective in stimulate the immune response and enhance in a positive way the gut microbiota community.
The paragon highlights that the appropriate dose, duration, and composition of a synbiotic formulation are specific to the context, including a plethora of factors such as the host targeted microbiota, and the environmental factors that could affect the treatment.

1.7 Methods to study probiotic, prebiotic, and synbiotic efficacy

Nowadays, the impact of the composition and modulation of the gut microbiota on human health is one of the most studied fields in biology and medicine. A deep understanding is now possible thanks to multi-omics technologies, that provide tools necessary to study community-wide, high-throughput assessment of the complex microbial ecosystem. Therefore, it is now possible to establish the mechanisms by which the gut microbiota interacts with the host and vice-versa. For example, it is known that the peculiar composition of the microbiota could predispose the individual to certain intestinal as well as systemic diseases, such as obesity and diabetes. However, it is still difficult to establish a cause-and-effect relationship in humans through *in vivo* studies. However, although the gut microbiota in healthy subjects is generally resistant to the interventions with pre-, pro- biotics, and synbiotics, they potentially have all the capacity to alter the microbiota in some extent (Krumbeck et al., 2018).
To try to overcome these problems, several *in vitro* gut fermentation models were introduced in nutrition and applied microbiology research. They mimic the complexity of the digestion stages, comprising the gut microbiota. Indeed, the most competitive models include a complex gut microbiota, small working volumes, distinct interconnected compartments, and rigorous bio-chemical and ecological settings. The principal aim is the study of the shift of the core microbial

groups and selected species with the related metabolites, testing their diversity, richness, and abundance over time. Furthermore, it is possible to follow the digestion of a compound, which metabolic pathways are activated, and the microbial metabolites produced (Nissen et al., 2020). Therefore, these new models could be applied if the researcher's aim is the study of the microbial community. The latest models comprised also modules with *in vitro* cell lines, to test the effects of the produced metabolites and have an indication of the possible effects on the host (Elzinga et al., 2019; von Martels et al., 2017; Marzorati et al., 2014). In the following paragraphs the methods and the problems connected to each type of studies are discussed.

1.7.1 In vivo studies

One of the first problem that must be faced for an *in vivo* study is the selection of the exclusion criteria. Indeed, in literature is reported that antibiotics, diet, body mass index, pregnancy, and ethnicity influence somehow the gut microbiota composition. Some of them are even considered exclusion criteria, such as the use of systemic antibiotics, antifungals, antivirals, or antiparasitic within the six months of sampling, as listed by the NIH Human Microbiome Project. However, the six months window is arbitrary, and in some studies a shorter window was used (Goodrich et al., 2014).

The second problem regards the setting of the study, in particular the choice of the control groups. Indeed, the factors impacting the gut microbiota should be balanced across the experimental groups. However, the selection of the subjects could be limited by the population available for the study. To work around this issue, in time-series studies the individuals can be treated as their own control, collecting baseline samples, and then treated samples. Furthermore, the Human Microbiome Project (HMP) would provide a healthy reference set to be used for the comparison for future studies. However, the adopted protocols must be compatible, the effect size (i.e., the difference between controls and subjects) of the study very large, the technical effects such as PCR primer choice and DNA

extraction method considered, because they can greatly outweigh the biological effect. Therefore, most of the studies require a carefully matched control group and cannot used the HMP as reference (Goodrich et al., 2014).

Accordingly, there are several a priori consideration that the scientist must ponder before starting a clinical study. For example, the effect size, which depends on the number of samples; the method to collect the metadata, i.e., all the information about the sample and the experimental procedures that must be recorder to help the subsequent data analysis; if the sujects have to be re-sample over time or sample more individuals only once; and if collect time-series data (Goodrich et al., 2014).

Furthermore, there are also technical questions to consider, such as the obtaining and storing the samples, because they can impact the DNA yields and quality. Also, the DNA extraction protocols, because different types can result in different diversity profile, due to the resistance of certain cell type to mechanical or chemical lysis methods. Fortunately, the wide use of commercial kits increases the possibility for consistency between studies. Then another issue regards the amplification of the selected 16S regions. Indeed, the choice of the region is dramatically important because the phylogenetic information varies along the length of the 16S rRNA gene. Another important factor is the subsequent sequence data analysis, which presents bioinformatical, statistical, and computational challenges (Goodrich et al., 2014).

Even if a study is planned in every detail, uncontrollable problems may arise, like a non-responder phenotype. For example, a bifidogenic response or other changes in the microbiota following a prebiotic supplementation occurs in some subjects, but not in others. The response to a treatment not only depends on the functional and taxonomic composition of the subject gut microbiota, but also to abiotic factors, such as the own digestive enzymes, the stomach and intestinal pH, the transit time in the intestine (Krumbeck et al., 2018). In this scenario, the use of synbiotics is favorable, because the probiotic has a niche opportunity as selectively fermentable prebiotic, and so it has a competitive fitness, that could

advantage it and significantly increase the persistence in the gut environment (Krumbeck et al., 2018).

1.7.2 In vitro gut microbiota models

The *in vitro* models of intestinal gut microbiota permit to explore the influence on the intestinal community of different factors such as dietary compounds, microbial pathogens, bioactive molecules, pharmaceutical and toxic substances. The intrinsic challenge is the cultivation of the more representative human gut microbiota under regulated environmental conditions, and to study its metabolism and shift during time. Some issues described for the *in vivo* studies must be faced also in the *in vitro* models, such as the DNA extraction from the microbial community, the choice of which 16S region amplify, and the bioinformatic analysis. Furthermore, most of the models lack the host feedback because there are not present epithelial and immune cells (Nissen et al., 2020). Different models were developed by several researcher groups around the world, basing on similar rationales. The simplest, most versatile, and accessible models are based on batch fermentation. In this approach, the environment is closed, static and anaerobic, and could be used for short time stimulations. However, they poorly mimic the *in vivo* condition, which is somewhat greater in the dynamic fermentation models (DFM). The DFMs could be used for longer experiments and permit to test a more complex ecosystem, considering different niches. In fact, essential nutrients are constantly refilled, and the milieu is kept. One example is the Reading Model of the University of Reading (Gibson et al., 1988), composed by three connecting vessels.

Increasing the complexity, the TIM-2 is a patented DFM of the Netherlands Organization for Applied Research and simulates the whole human gut in four vessels. It could be connected to TIM-1, that simulates the stomach and the small intestine conditions. Interestingly, the model was used to evaluate potential differences between microbiota from lean and obese subjects and

clarify the production of SCFAs after administration of indigestible carbohydrates or prebiotic molecules (Aguirre et al., 2014).

Further increasing complexity is reached by the Simulator of the Human Intestinal Microbiota Ecosystem (SHIME), patented by the ProDigest and the University of Ghent. Indeed, five reactors are connected in series to reproduce the whole gastrointestinal tract, from the gastric to the distal colon. The first two vessels mimic the stomach and the small bowel. Then the subsequent three mimic the three sections of the colon. The microbial inoculum used derive from healthy donors and 14 days of acclimatation are needed before any experiment. Therefore, this system is time and labor consuming, not cost accessible, and not suitable for all labs. Nevertheless, it has the highest *in vivo* resemblance among the *in vitro* models (Nissen et al., 2020). Interestingly, the model was further implemented with a distinct segment for mucous microbes (M-SHIME), that interact with the host epithelium and generate the immune system response.

Similarly, the Food Science Research Institute of Madrid developed the SIMulator gastro-intestinal (SIMGI), that is composed by five unit and works similar to the SHIME, and fecal microbiota is used as inoculum (Barroso et al., 2014; Tamargo et al., 2018). Respect to SHIME, SIMGI is more cost accessible, logistically flexible and operator friendly. However, is time consuming and not so realistic and reproducible, due to less strict environmental parameters.

A problem in common with all the models so far discussed is the use of a fecal inoculum. Indeed, this approach does not consider the different skills and strategies used by the bacteria to colonize and resist in the host intestinal epithelium. Furthermore, numerous strains can overcome others (for example thanks to be more competitive in the intestinal environment), so less aggressive bacteria may become uncompetitive, leading to an unrealistic *in vitro* model. To overcome the problem, the ETH of Zurich developed the PolyFermentor intestinal model (PolyFermS), which employs an immobilization process of the fecal bacteria, trapping the microbiota through encapsulation. This allows an

experimental design until 38 days in proximal colon conditions (Berner et al., 2013; Poeker et al., 2018; Nissen et al., 2020).

However, some doubts regarding the reproducibility and reliability remains, so "synthetic" microbial consortium begin to be developed. For example, Schape et al. (2019) proposed the extended basic human gastrointestinal microbiota (SIHUMIx), that is a mock community composed of *A. caccae*, *Bacteroides thetaiotaomicron*, *B. longum*, *Blautia producta*, *Clostridium butyricum*, *Clostridium ramosum*, *E. coli* K-12, and *L. plantarum*. These strains drive the main metabolic events in the real gut environment. Instead, Gutierrez et al. (2019) used a synthetic consortium composed by 14 gut bacteria to evaluate the utilization of prebiotic inulin in batch bioreactors, monitoring substrate consumption, microbial composition, and metabolite production. Therefore, the synthetic microbiota could facilitate the assessment of the influence of the tested molecule or bacteria on the composition and function of the microbiota, helping in the identification of keystone species, redundant functions and conditions that contribute to the stability of the community. Indeed, redundancy is a peculiar characteristic of the gut microbiome, and its reduction in terms of microbial strains could be useful for the design of bacterial consortia with specific metabolic properties, even if the synthetic communities are less stable over long time experiments respect to the complex models (Guitierrez et al., 2019; Nissen et al., 2020).

1.7.3 In vitro intestinal cell models

Among the *in vitro* models, a distinction must be made between models focused on the gut microbiota (as discussed above) and models design to realistically represent the human host. Obviously, the goal is the combination of the two approaches into one model.

The simplest model mimicking the human gut is the two-dimensional (2D) model, principally composed by intestinal or colorectal, or other type of cell lines, where the interaction with the immune system or other tissue is not possible. An

evolution of the model is the transwell coculture model, in which bacteria, mucosal immune cells, and intestinal epithelial cells are put together, allowing a more comprehensive study. Indeed, if an apical anaerobic compartment is implemented, anaerobic bacteria can be cocultured with the intestinal cell line, allowing the direct study of the host-microbe interactions. However, the employed cell lines are principally tumor-derived, so the epithelial characteristics are affected; moreover, they lacked the tissue-specific context and the organization in crypts and villi (Elzinga et al., 2019). To overcome these problems, gut organoids were developed. These are self-organizing, three-dimensional (3D) epithelial structure that derived from intestinal stem cells or human pluripotent stem cells. In the organoids, the structure is very closed, so the establishment of a hypoxic environment (from 5% to 15% luminal oxygen) is possible in the core lumen because it is enclosed with epithelial cells and mucus layer (Elzinga et al., 2019).

The model so far discussed present a common limitation: the static nature of the system, that reduce their applicability for research purpose. Therefore, more advance *in vitro* systems were designed and developed, such as the organ-on-a-chip, that are microfluidic dynamic devices where shear stress and gut peristalsis are simulated by continuous medium flow and stretching/relaxing of the membrane, respectively. Therefore, also the organs spatiotemporal chemical gradients and the mechanical forces are mimicked thanks to the employment of different cells. The aim is the maximal reconstruction *in vitro* of the structural tissue arrangements and the functional complexity present in real organs (Elzinga et al., 2019). The literature reports only one device where the intestinal bacterium *L. rhamnosus* GG was successfully cultured (Kim et al., 2012). The device is composed by two channels, mimicking the gut lumen and a blood vessel, separated by a membrane coated with extracellular matrix and the CACO-2 cell line.

Finally, a new frontier is represented by electrospun structures as scaffolds for microbial adhesion and growth. In the work of Biagini et al. (2020) a stable 3D *in*

vitro model of the common inhabitants of the human gut microbiota (*E. coli*, *E. faecalis*, *Clostridium innocuum*, *Bacteroides fragilis*, and *C. albicans*) was developed using an electrospun natural polymer-based scaffold. The authors suggest a possible implementation of the model with the coculture on intestinal cell lines.

Irrespectively of the type and complexity of the utilized model, the cellular processes cannot be easily validated *in vivo* in human subjects. Nevertheless, the models can increase the understanding of the molecular mechanisms at the base of the interaction with bacteria and their metabolites, eliminating potentially confounding factors present *in vivo* (Elzinga et al., 2019).

1.8 Scope of the thesis work

The aims of the PhD project are the characterization of the probiotic properties of *Lactobacillus* and *Bifidobacterium* strains and the definition of the prebiotic potential of commercial and natural products, capable to sustain the growth of the selected probiotic strains. Then, the set-up of a pre- and pro- biotic combination, known as synbiotic, designed with the most powerful probiotic for the human health and the suitable prebiotic fiber. The efficacy of the synbiotic is evaluated through a clinical study on healthy and elderly subjects, evaluating the possible effects on the general health state of the subject and the modulation of their gut microbiota. Finally, the conception of *in vitro* alternative models for testing new synbiotic formulation, comprehensive of both synthetic gut microbiota consortia and *in vitro* intestinal cellular models.

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Chapter 2:

Assessment of the capacity of selected probiotic strains to utilize inulins and fructooligosaccharides with different degree of polymerization as carbon and energy sources, and deeper investigation of peculiar probiotic traits

This chapter focuses on the characterization of the capacity of selected probiotic strains belonging to the *Lactobacillus* and *Bifidobacterium* genera to grow on commercial inulins and fructooligosaccharides (FOS) with different degree of polymerization. The growth was monitored spectrophotometrically and compared with the one on a reference substrate. Then, the probiotic traits of *L. plantarum* LP were deeply investigated, in particular its antimicrobial activity due to the production of a bacteriocin-like molecule. Overall, the results let to design a possible synbiotic formulation.

2.1 Introduction

Inulin-type fructans are the most studied and used prebiotics (Waqas et al., 2019). They consist of repetitive fructosyl moiety chain, linked by β-(2,1) bonds with terminating glucosyl moieties. Inulin molecules are found in a variety of vegetables and are characterized by different degree of polymerization (DP) which is associated to their different functional and therapeutic features, such as preventive and physioprotective effects as lowering of blood cholesterol or glucose level by reducing lipogenesis, and antioxidant effects (Waqas et al., 2019; Mensink et al., 2015; Rondanelli et al., 2019; Niziol-Lukaszewska et al., 2018; Salekzamani et al., 2019). Moreover, these inulin-type fructans selectively stimulate the growth of *Bifidobacterium* and *Lactobacillus* spp. in the human colon and, as a result of their fermentation, short-chain fatty acids (SCFAs) can be produced. However, most of the bacterial strains were reported to degrade only short-chain fructans (fructooligosaccharide with DP<10), while limited number of probiotics has the capacity to metabolize long chain inulin-type fructans (Rossi et al., 2005; Falony et al., 2008). Strains with this ability are very important, because long-chain inulins, used as carbon source for growth, affect the production of short-chain fatty acids. Recently, it was also demonstrated that long-chain inulins of DP comprised between 10 and 60 exert higher immune response in human than short-chain inulins of DP<25 (De Vos et al., 2018).

Therefore, the first goal of this study was the evaluation of the capacity of commercial inulins and FOS with different degree of polymerization to sustain the growth of selected strains belonging to different *Lactobacillus* and *Bifidobacterium* species, previously characterized as probiotics (Presti et al., 2015).

In this context, among the probiotic characteristics of the strains, the production of substances with antagonistic activity against potential pathogens is an important capability, considering the struggle within the gastrointestinal tract. It could be exerted also by bacteriocin molecules, which are interesting in the biotechnological field because of their several applications, like natural food preservatives or novel therapeutic agents to complement conventional antibiotics (Trivedi et al., 2013). Indeed, bacteriocins are ribosomally synthesized antimicrobial peptides or complex proteins secreted by various Gram-positive and Gram-negative bacteria (Desriac et al., 2010). Based on the biochemical properties, they could be divided into three groups (Klaenhammer, 1993): Class I is the lantibiotics family, like nisin from *L. lactis*; Class II is the small non-modified peptides, heat and pH resistant, like Pediocin PA-1/Ach from *P. acidilactici*; Class III is the larger heat-labile proteins. Class II is subdivided into four groups and class IIA bacteriocins are characterized by YGNGV consensus sequence near their N-terminal (Porto et al., 2017) and are known as “pediocin-like” peptides. Generally, their synthesis requires four genes, encoding a pre-bacteriocin, an ABC-transporter protein, an immunity protein, and an accessory protein, regulated by a quorum sensing mechanism (da Silva Sabo et al., 2014; Diep et al., 2009). These proteins and peptides are known for their antimicrobial properties against some potential pathogens like *E. coli*, *S. aureus* (Ahmad et al., 2017), and *Bacillus* spp. (Zhao et al., 2016). It is known that bacteriocins can kill target bacteria by membrane permeabilization or by binding to a specific membrane protein called “bacteriocin receptor”, where the interaction between peptide and receptor protein leads to membrane leakage and cell death (Oppegard et al., 2016). This mechanism explains both the

extreme potency of many bacteriocins and their narrow inhibition spectra. However, it is not clear the nature of the interaction between bacteriocins and receptors, and the interaction between bacteriocin and intestinal cells. Indeed, some authors reported that bacteriocins can exert some beneficial effect on human host, because of their interaction with the intestinal epithelia, leading also to an anti-proliferative effect on human colon cancer (Lee et al., 2008; Ma et al., 2010; You et al., 2004). Ma et al. (2010) speculated that the activity is mediated through the inhibition of the Epidermal Growth Factor Receptor (EGFR) kinase activity. Yan et al. (2011) described the effect of a bacteria-derive soluble protein p40 from *L. rhamnosus* GG, that was able to prevent cytokine induced apoptosis in intestinal epithelial cell lines through the regulation of the activation of Akt. Nevertheless, probiotic effects are strain dependent and different species might have different mechanisms of action (Dimitrovski et al., 2014).

During the characterization of the selected probiotic strains (Presti et al., 2015), the antimicrobial potential was investigated in pH-neutralized and non-neutralized cell-free supernatants from all the probiotic bacteria and the direct growth of the potential pathogens on the LABs. The results let to speculate that bacteriocins can be produced, especially from *L. plantarum* PBS067. So, the second aim of the present work was to screen the ability of *L. plantarum* PBS067 to produce bacteriocins, purify and characterize them, then evaluate the ability to inhibit potential pathogens growth. Finally, to test if they can have effects on healthy and cancer intestinal epithelial cell lines.

2.2 Materials and methods

2.2.1 Bacterial strains, media, and substrates

The bacterial strains used in this study are listed in Table 2.1. The *Lactobacillus* and *Bifidobacterium* strains were previously selected and characterized for their probiotic properties (Presti et al., 2015). They were provided from a private collection of the company Roelmi Hpc (Origgio, Italy), while *E. coli* ATCC 25922,

S. aureus ATCC 6538, *P. aeruginosa* ATCC 9027, and *E. faecalis* ATCC 2922 derived from ATCC collection and were used as potential pathogen strains.

Probiotic bacteria were activated by growing them on MRS broth (Condalab.) supplemented with 0.03% L-cystein (Sigma) for 48 hours, at 37°C, under anaerobic conditions using Anaerocult GasPack System (Merck). Microbial cells were then transferred at 1% v/v inoculation concentration in MRS broth medium followed by incubation for 24 hours prior to use. A modified MRS medium (mMRS), as described by Watson et al., 2012, without glucose and supplemented with 0.03% L-cysteine, was used for the growth trials in presence of inulins and FOS. The pH of the medium was adjusted to 6.8 before sterilization (121°C for 20 minutes). Long-chain inulin (30<DP<60, Sigma), Inulin (DP~25, Sigma), medium FOS (DP~10, Farcoderma), and FOS (3<DP<5, Farcoderma) were added to mMRS as the sole carbon source (at a final concentration of 2%, w/v). In all cases, the listed sugars were sterilized through membrane filtration using Millex Syringe Filter Units (pore size, 0.45 µm; Merck Millipore) and added aseptically to the sterile mMRS medium.

The potential pathogen strains were growth in Luria-Bertani medium (LB) agar, modified by Lennox known as LD (Lennox, 1995), at 37 °C in aerobiosis.

Strain	Source
Lactobacillus acidophilus PBS066 (formerly DSM 24936)	Human
Lactobacillus fermentum PBS073 (formerly DSM 25176)	Human
Lactobacillus plantarum PBS067 (formerly DSM 24937)	Human
Lactobacillus reuteri PBS072 (formerly DSM 25175)	Human
Lactobacillus rhamnosus PBS070 (formerly DSM 25568)	Human
Bifidobacterium animalis spp. *lactis* PBS075 (formerly DSM 25566)	Human
Bifidobacterium bifidum LMG P-29508	Human
Bifidobacterium longum spp. *infantis* LMG P-29639	Human
Bifidobacterium longum spp. *longum* PBS108 (formerly DSM 25174)	Human

Table 2.1. Bacterial strains used in the study

2.2.2 Growth experiments for the evaluation of the prebiotic effect on Lactobacillus and Bifidobacterium strains

All probiotic bacteria cited in Table 2.1 were inoculated into MRS plus agar 48 hours prior to use. Inulins, and fructooligosaccarides were added individually to the mMRS before inoculation to give a final concentration of 2% w/v. Sterile tubes containing 10 mL of mMRS broth medium were inoculated with around 100 µL of a solution of each bacterial strain to obtain an initial Optical Density (OD) at 600 nm of 0.1, then mixed, capped, and introduced into anaerobic jars. They were incubated at 37°C. Samples were removed at 48 hours to measure the OD_{600nm}.

2.2.3 Screening for antimicrobial activity

The antimicrobial activity was determined by the agar well diffusion assay (AWDA) for Lactobacilli according to the protocol of Santini et al. (2010) with some modifications. Overnight MRS cultures were centrifuged at 7000 rpm at 4°C for 15 min. The pHs of the supernatants were measured and recorded. Then, supernatants that were naturally at pH 4 were collected and filtered through 0.22-µm pore filter membranes to remove any residual bacterial cell (CFS, Cell Free Supernatant). The CFS antimicrobial activity was measured against *E. coli*, *P. aeruginosa*, *E. faecalis*, and *S. aureus*. The antagonist strains were inoculated into LD medium and let to grow until the OD_{600nm} was 0.5, corresponding to around 10^7 CFU/mL. 2.5% v/v of each culture was inoculated into 20 mL of LD agar and the plates were allowed to solidify. Four wells of 8 mm in diameter were made on each agar plate with a sterile glass cylinder. 50 µL and 100 µL of *L. plantarum* PBS067 CFS was dispensed into each well; neutralized-acidified PBS067 CFS, not-inoculated and acid not-inoculated MRS (100 µL) were used as controls. Plates were incubated overnight at 37°C in aerobiosis. The growth inhibition haloes were measured.

The same method was used to test the antimicrobial activity of the concentrated crude extract and the purified bacteriocin-like compound against *E. coli*, and *S. aureus* as representative strains of Gram-negative and Gram-positive bacteria, respectively.

2.2.4 Purification of the bacteriocin-like compound from culture medium of L. plantarum

Adsorption/desorption method (step 1)

A modification of Yang et al. (1992) extraction method, based on the hydrophobicity and the charge of the compounds secreted by the cells, was used to identify bacteriocin-like compounds from *L. plantarum* PBS067 cultures.

First, the strain was pre-cultured in 15 mL of MRS medium at 37°C overnight, in anaerobic conditions. Then, the pre-culture was inoculated in four flasks each

containing 300 mL of MRS medium with an initial OD_{600nm} of 0.01. The flasks were incubated at 37°C, for 16 h in microaerophilic conditions. Final cultures had an OD_{600nm} between 3 and 4, which corresponded to the initial latency period.

The established method allowed to partially purify cell secreted compounds based on the pH of the extraction medium promoting the adsorption/desorption of these molecules from the lactic acid bacteria membrane. Therefore, in the step 1 the cultures were neutralized to pH 6.5 with NaOH 1.5M and let to stand for 30 min at room temperature. Then, they were centrifuged at 7000 rpm at 4°C for 10 min. After the cells had been washed with a phosphate buffer (0.1M pH 6.5), they were resuspended in 60 mL of NaCl 0.1M at pH 2 and mixed with a magnetic stirrer for 1 h at 4°C. Cell suspension was then centrifuged at 16,000*g* for 30 min at 4°C. This fraction of the supernatant containing the bacteriocin-like molecule (called crude extract) was used for further analysis and characterization.

An aliquot of this fraction containing the bacteriocin-like molecule was ten-fold concentrated and used for AWDA (see above) to assess its antimicrobial activity using 100 µL of the concentrated crude extract. 100 µL of CFS (see above) and 100 µL of NaCl 0.1M at pH 2 were used as positive and negative controls, respectively. The growth inhibition zones around the wells were measured.

Before proceeding with the purification, the pH of the fraction was increased up to 10-11 with NaOH 1.5M, and then the solution was lyophilized.

Organic phase extraction (step 2)

The lyophilized crude extract (deriving from 300 mL of supernatant) was extracted with 10 mL of *n*-butanol under agitation, at room temperature for 20 h. This step was repeated twice. Then, the butanol extracts were filtered through a paper filter and the solvent was evaporated. The residue was resuspended in 1 mL of methanol and analyzed in SEC-HPLC.

Size exclusion chromatography (SEC) HPLC (step 3)

SEC analyses were performed with a Waters 600E delivery system equipped with Waters 486 UV-Vis detector and a Phenogel 5 100 A, Phenomenex, 300x4.6 mm column eluted in isocratic conditions with methanol at a flow rate of 0.3 mL/min. The eluted compounds were detected at 254 nm and their retention times were compared with the retention time of the standard nisin, the model bacteriocin from *E. lactis*. In these conditions, the retention time of nisin was 11.8 min. An aliquot of the eluted compound containing the bacteriocin-like molecule was ten-fold concentrated and used for AWDA to assess its antimicrobial activity.

2.2.5 Characterization of the bacteriocin-like plantaricin P1053 by ESI-full mass spectrometry (MS)

The eluted compound containing the bacteriocin-like molecule was analyzed by the electrospray ionization ESI-full mass spectrometry. The analysis was performed on LCQ Fleet ion trap mass spectrometer. The MS was operated in positive ionization mode acquiring spectra in the *m/z* range of 200 to 2000.

2.2.6 Effects of proteinase K enzyme on the stability of the purified bacteriocin-like compound and total protein concentration

To evaluate the purified bacteriocin-like molecule sensitivity to the proteinase K (pH 7.5; Sigma Aldrich), the bacteriocin-like compound was treated with 1 mg/mL (final concentration) of proteinase K (ratio 1:5) at its optimal pH. After 3 h of incubation at 37°C, the reaction was stopped at 4°C. Subsequently, the sample was adjusted to pH 2 using 6M HCl and assayed for antimicrobial activity. The bacteriocin-like molecule at the original pH (pH 2) without any heat or enzyme treatments was used as the control sample. The agar well diffusion

assay was carried out to test the remaining activity against the indicator strain *E. coli* ATCC 25922 (Santini et al., 2010).

The total protein concentration was assessed on the partial purified and purified bacteriocin-like molecule extracts using a Pierce BCA protein assay kit (Thermo Fisher Scientific) according to the manufacturer's protocol. Protein concentrations were calculated from the standard curve by 20 mg/mL bovine albumin serum.

2.2.7 Maintenance and growth of cell lines for in vitro tests

CCD841 (ATCC CRL-1790) healthy colon cell line (Thompson et al., 1985) were grown in EMEM medium supplemented with heat-inactivated 10% FBS, 2mM l-glutamine, 1% non-essential amino acids, 100 U/mL penicillin and 100 µg/mL streptomycin and maintained at 37°C in a humidified 5% CO_2 incubator.

E705 colon cancer cell line (supplied by IRCCS Foundation, Cancer National Institute) (Mozzi et al., 2015) was grown in DMEM medium supplemented with heat-inactivated 10% FBS, 2mM l-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin and maintained at 37°C in a humidified 5% CO_2 incubator.

All the reagents for cell culture were supplied by Euro-Clone (EuroClone S.p.A).

2.2.8 Viability assay

Cell viability was assessed using an *in vitro* MTT based toxicology assay kit (Sigma). Cells were seeded in a 96-well micro titer plates at a density of $8x10^4$ cells/100 µL and incubated overnight. The attached cells were treated with a range of the bacteriocin-like molecule (formerly plantaricin P1053) up to 1 µg/mL. After 48 h of treatment, MTT test was performed according to the manufacturer's protocol and absorption was measured at 570 nm using a micro plate reader to assay the effect of plantaricin P1053 on healthy CCD841 cells and colon cancer E705 cells. Results were expressed as mean values ± SD of three determinations.

2.2.9 Protein extraction from treated cell lines and analysis

After treatment with plantaricin P1053 at 0 min, 30 min, 1 h, 3 h and 24 h, cells were washed with ice-cold PBS and lysed in RIPA buffer, containing protease and phosphatase inhibitors and 1mM PMSF. After lysis on ice, homogenates were obtained by passing crude extracts five times through a blunt 20-gauge needle fitted on a syringe and subsequently centrifuging them at 14,000 rpm for 30 min at 4°C. Supernatants were analyzed for protein content by the BCA protein assay (Smith et al., 1985).

SDS-Page and western blot were carried out by standard procedures. Thirty micrograms of proteins were separated on 10% acryl-amide/bis-acrylamide SDS-PAGE, transferred onto a nitrocellulose membrane (Millipore, Billerica, MA), probed with the appropriate antibodies and visualized using ECL detection system (Millipore). Protein levels were quantified by densitometry of immunoblots using ScionImage software (Scion Corp., Fredrick, MD). The following primary antibodies (all purchased by Cell signaling Technology, Danvers, MA) were used: anti-EGFR (dilution 1:1000), anti-phospho-EGFR (Tyr 1068; dilution 1:1000), anti-p44/42 MAPK (Erk1/2; dilution 1:1000), anti-phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204; dilution 1:1000), anti-Akt (dilution 1:1000), anti-phosho-Akt (Ser473; dilution 1:1000) and anti-GAPDH (dilution 1:10,000). IgG HRP-conjugated secondary antibodies (purchased by Cell Signaling Technology) were diluted 1:10,000.

2.2.10 Statistical analysis

Experiments were performed in triplicate and results were presented as mean values ± standard deviation. The statistical relevance was assessed by *t*-Student's test. The significance was defined as *** p-value <0.001, ** p-value <0.01 and * p-value <0.05.

2.3 Results

2.3.1 Prebiotic effect of commercial inulins and FOS

To prove that commercial inulin and FOS are effectively fermented by the *Bifidobacterium* and *Lactobacillus* strains originally isolated from the human colon (Table 2.1), we have studied the effect of their addition to the probiotic cultures, starting with an OD_{600nm} of 0.1 and by measuring the final OD_{600nm} of the cultures after 48 hours of anaerobic fermentation. Figure 2.1 shows that not all the strains were able to considerably ferment long-chain inulin of 30<DP<60, but only *L. plantarum* PBS067, *L. reuteri* PBS072, *B. lactis* PSB075 (*p*-value <0.01 comparing the growth on mMRS as CTR with respect to the growth on the considered substrate), and *L. rhamnosus* PBS070 (*p*-value <0.05). A similar response was observed testing inulin with an average degree of polymerization around 25. Indeed, only *L. acidophilus* PBS066, *B. infantis* LMG P-29639 (*p*-value <0.05), and again *B. lactis* PBS075 (*p*-value <0.01) could utilized as carbon and energy source this kind of sugar. As expected, the simpler the molecules become, the more bacteria were able to use them for growth. Actually, all the tested strains except *L. rhamnosus* PBS070 and *L. reuteri* PBS072 could growth on FOS with DP~10. Interestingly, the highest differences respect to the control were showed by two Bifidobacteria (*B. bifidum* LMG P-29508 and *B. infantis* LMG P-29639, *p*-value <0.001). Finally, the highest OD_{600nm} was obtained when FOS with the lowest DP were used as growth substrate. All the strains could growth until an optical density of around 3 and 4, except again for *L. rhamnosus* PSB070 and *L. reuteri* PBS072 (Figure 2.1).

Therefore, these findings show no significant differences among the tested strains, belonging to two different genera and different species, in the ability to ferment also long chain inulins.

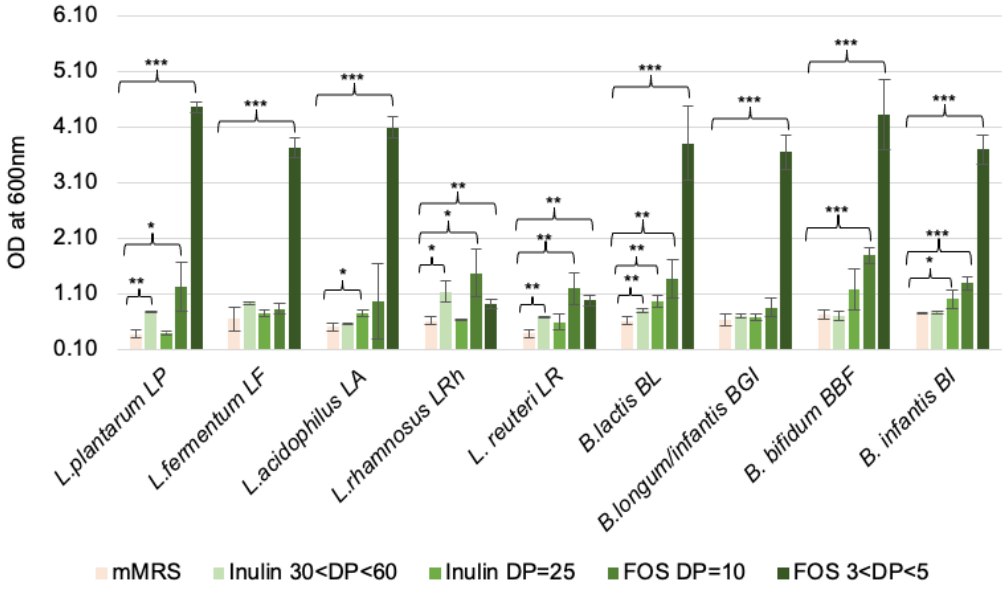

Figure 2.1. Growth of *Lactobacillus* and *Bifidobacterium* strains on mMRS (CTR), Inulin (30<DP<60 or DP~25), and FOS (DP~10 or 3<DP<5). Data represent the mean of three independent experiments, each performed in duplicate. Data are presented as mean ± standard deviation. Statistically significant differences were tested with t-Student's test: *** *p*-value <0.001, ** *p*-value <0.01 and * *p*-value <0.05.

2.3.2 Identification of antimicrobial activity of L. plantarum strain PBS067

L. plantarum PBS067 was grown until reaching the stationary phase of the cells and the cell free supernatant (CFS) was used in the antimicrobial activity through the agar well diffusion assay. A halo of growth inhibition of the antagonist bacteria was observed and measured (Table 2.2). Results showed an inhibition halo with a diameter of 18 mm ± 0.2 for *E. coli* and 20 mm ± 0.2 for *S. aureus* as representative of Gram-negative and Gram-positive bacteria, respectively.

Antagonist bacteria	**Inhibition halo by *L. plantarum* (mm)**	
	50 µL[a]	**100 µL[a]**
Escherichia coli ATCC 25922	18 ± 0.2	35 ± 0.2
Enterococcus faecalis ATCC 2922	10 ± 0.5	20 ± 0.5
Pseudomonas aeruginosa ATCC 9027	20 ± 0.5	40 ± 0.5
Staphylococcus aureus ATCC 6538	20 ± 0.2	40 ± 0.2

Table 2.2. Dimension of the growth halo inhibition measured by CFS from *L. plantarum* against the antagonist bacteria. The measures of the inhibition zone are expressed in mm.
[a] The concentration of the solution containing the plantaricin P1053 was of 200 µg/mL.

Results were compared to the acidified non-inoculated medium as negative control, to exclude that the inhibition was caused by the low pH of the medium after the growth. Moreover, obtained data showed that the antimicrobial activity was also maintained when the supernatant was neutralized and then brought again to acid pH, as reported as an example for *E. coli* in Figure 2.2. For this reason, it was hypothesized that the strain PBS067 was able to produce bioactive bacteriocin-like compounds inhibiting the growth of the tested antagonist bacteria. As the bacteriocin-like compounds produced by lactobacilli are strain-specific and with a peculiar kind of biological activity, it was decided to isolate and characterize the bioactive compounds produced by *L. plantarum* PBS067 strain and to test their range of antimicrobial activity.

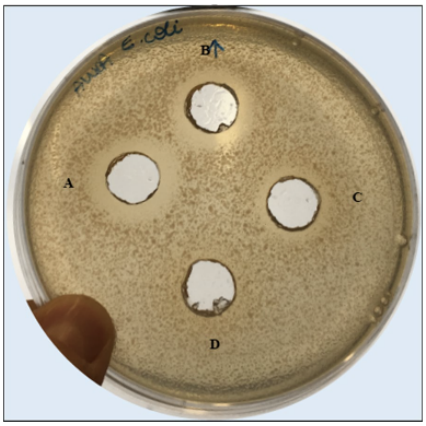

Figure 2.2. The antimicrobial activity of the CFS from *L. plantarum* was determined as halo of growth inhibition of *E. coli* ATCC 25922 through the AWDA. The figure shows the inhibition zones produced by CFS (A), by neutralized-acidified CFS (B), not inoculated MRS at pH 4 (C), and not inoculated MRS (D) against *E. coli*.

2.3.4 Purification of the bacteriocin-like compound from L. plantarum strain PBS067

Bacteriocin-like compounds were isolated from CFS of *L. plantarum* PBS067 culture using the modified method described by Yang et al. (1992) based on the hydrophobicity and the charge of the compounds secreted by cells. The established method allowed to extract and partially purify the bacteriocin-like molecules in a fraction called “crude extract”. The crude extract was screened for the antimicrobial activity using the agar well diffusion assay to verify that this property was maintained after this extraction phase (Figure 2.3A, step 1). Results were compared to NaCl 0.1M at pH 2 as negative control and CFS as positive control. The assay showed an increase in specific antimicrobial activity in this fraction (crude extract) measured through the dimension of the inhibition halo. The activity was not shown at neutral and alkaline pH, but it was recovered when the pH was lowered to 4, the lactobacilli physiological pH.

Then, the lyophilized crude extract from *L. plantarum* supernatant was submitted to another step of purification performed with *n*-butyl alcohol. After the antimicrobial activity of the partially purified extract deriving from this second

step was demonstrated, the obtained extract was analyzed and characterized (Figure 2.3B, step 2).

The extract deriving from *n*-butanol extraction was first analyzed in HPLC by Size Exclusion Chromatography (SEC). The UV/visible absorption spectra of the sample revealed a maximum absorbance-wavelength at 254 nm. Results from SEC-HPLC analysis showed a main peak at 254 nm with a retention time of 14.5 min whose eluate maintained the antimicrobial activity (Figure 2.3C, step 3). As a preliminary characterization, the purified extract was compared with the only reference standard available, the 3354 Da nisin from *L. lactis*. Based on the comparison of the retention times, the main peak of the extract had a molecular weight lower than 3354 Da. On this basis, the ESI mass spectrometry analysis of the purified extract will be used to thoroughly characterize the bacteriocin-like compound and determine the molecular weight.

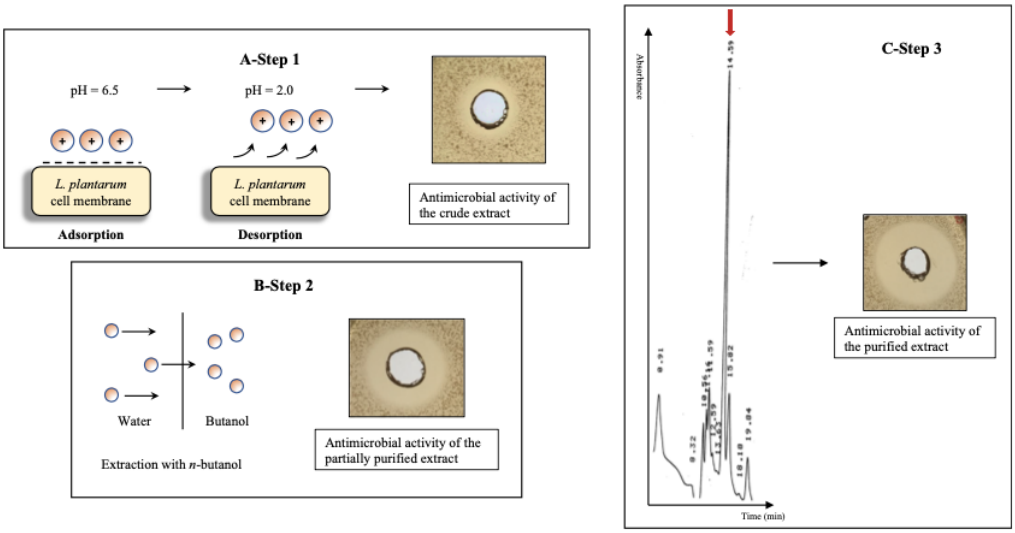

Figure 2.3. Step-by-step purification and antimicrobial activity of the bacteriocin-like compound. The antimicrobial activity of the purified extract was verified through agar well diffusion assay. Step 1 of purification and inhibition halo produced by the crude extract obtained after adsorption/desorption method (A). Step 2 of purification and antimicrobial activity of the partial purified extract through an extraction with *n*-butyl alcohol (B). Step 3 of purification and antimicrobial activity of the eluted bacteriocin-like compound (C). All the samples were tested against *E. coli* ATCC 25922.

2.3.5 Effects of proteinase K on the stability of the purified bacteriocin-like compound and total protein concentration

The sensitivity of the purified bacteriocin-like molecule to the proteinase K was evaluated after 3 h of incubation at 37°C with the hydrolytic enzyme. Results indicated that the treatment with proteinase K showed the expected effect on the purified bacteriocin-like molecule; in fact, the inhibitory activity of the purified bacteriocin-like molecule against *E. coli* strain ATCC 25922 was completely lost (Figure 2.4) after the treatment.

The crude extract and the purified bacteriocin-like molecule were positive to BCA protein assay. In particular, the total protein concentration of the crude extract was 200 µg/mL, while the corresponding expected concentration was 3900 µg/mL based on the initial dry weight of the supernatant. After the purification of the bacteriocin-like molecule, the total protein concentration was 4000 µg/mL, while the corresponding calculated concentration was 4140 µg/mL based on the lyophilized amount. Results demonstrated that the bacteriocin-like compound was a protein-like molecule and that after purification a 100% enrichment of proteins was determined.

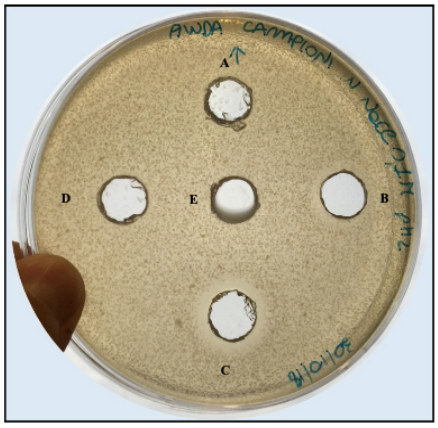

Figure 2.4. The antimicrobial activity of the purified bacteriocin-like compound was determined as a halo of growth inhibition of *E. coli* ATCC 25922 through AWDA. The figure shows the lack of inhibition zone produced by the treatment of the bacteriocin-like compound by proteinase K (A); the inhibition halo produced by the no-treated bacteriocin-like compound after incubation at 37°C and pH 7.5 (the proteinase K optimal pH) (B); the inhibition zone produced by untreated bacteriocin-like compound at pH2 (its optimal pH), as a positive control of the test (C); the lack of inhibition halo produced by negative control NaCl 0.1M pH 7.5 (buffer of the reaction with proteinase K) (D); and the lack of the inhibition halo produced by the negative control NaCl 0.1M pH2 (buffer for bacteriocin-like molecule activity) (E).

2.3.6 Characterization of the bacteriocin-like compound by ESI mass spectrometry

An ESI-full MS analysis was performed on the enriched fraction of the isolated bacteriocin-like compound. This analysis revealed that the extract contained a main compound with a molecular weight of 1053 Da (Figure 2.5) (from here named plantaricin P1053), smaller than the most important plantaricins described in literature, as for example plantaricin C19 (3.8 kDa) from *L. plantarum* C19 (Atrih et al., 2001), but with a molecular weight similar to those of JLA-9 from *L. plantarum* JLA-9 (Zhao et al., 2016). The weight is in agreement with the results obtained by SEC-HPLC chromatography.

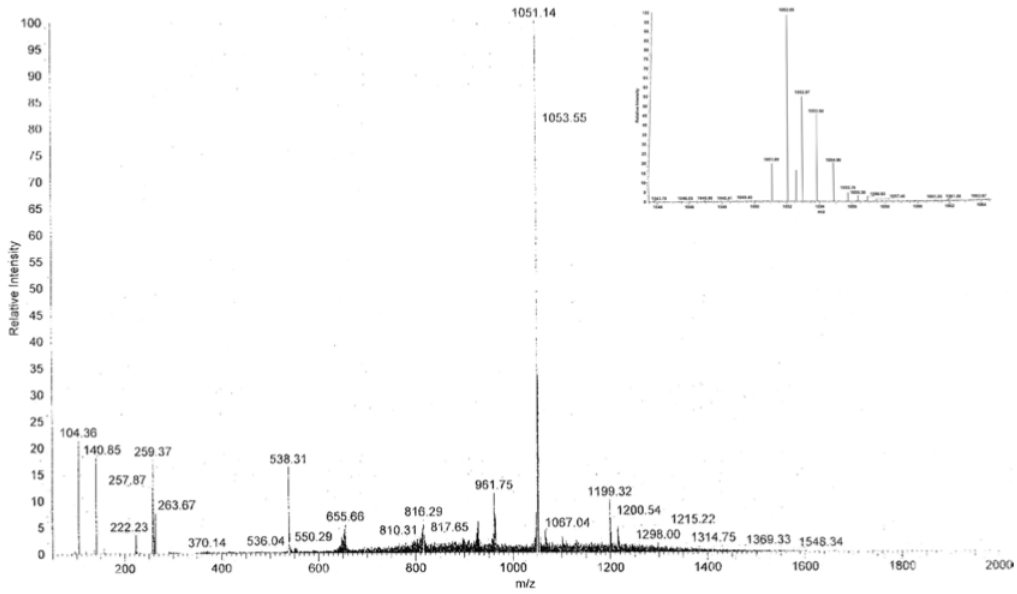

Figure 2.5. Analysis of the bacteriocin-like compound by mass spectrometry. Full scan mass spectrum of the extract in positive ESI ion detection mode with one main fragment showing *m/z*=1053.05 Da. ITMS + cHESI E full MS (1.16 e4). The area around M+ is enlarged in the insert.

2.3.7 Antimicrobial spectrum activity of plantaricin P1053

Antimicrobial spectrum activity of the purified plantaricin P1053 was determined by the measure of the halo inhibition of the growth against *E. coli* and *S. aureus* as representative of Gram-negative and Gram-positive antagonist bacteria, respectively. Results are showed in Figure 2.6. Plantaricin P1053 exhibited a notable antimicrobial activity against both *E. coli* and *S. aureus* with a growth inhibition zone of 2.5 cm and 3.0 cm, respectively. The inhibition spectrum of plantaricin P1053 appeared to be relatively wide because of the activity against both Gram-negative and Gram-positive bacteria.

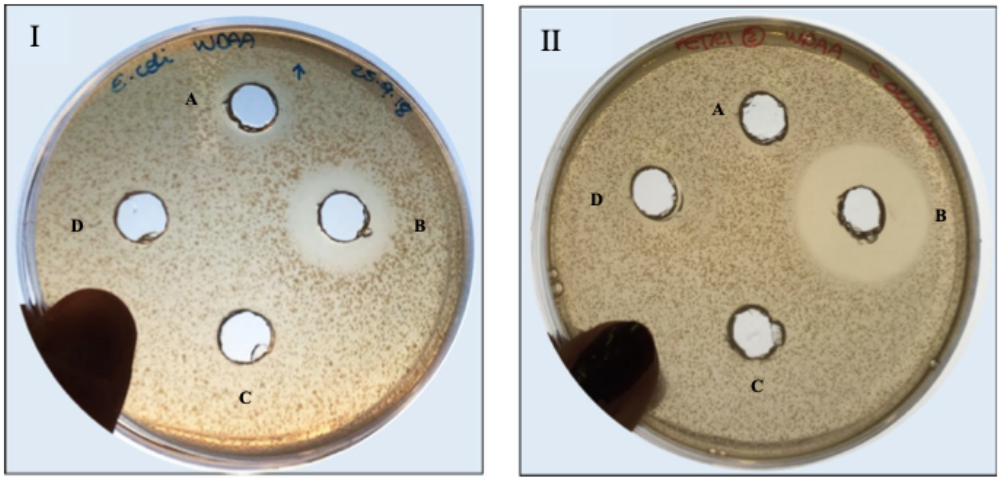

Figure 2.6. The antimicrobial spectrum of plantaricin P1053 was verified through the inhibition growth halo in the AWDA. The first panel shows the inhibition haloes against *E. coli* ATCC 25922 and the second panel against *S. aureus* ATCC 6538 produced by plantaricin P1053 at neutral pH (A), by the plantaricin P1053 at pH2 (B), by the NaCl 0.1M pH2 (C), and by NaCl 0.1M pH6.5 (D) as negative controls.

In addition, the minimum inhibition growth concentration (MIC) of plantaricin P1053 against these bacteria was determined and data are reported in Table 2.3. Results show that the MIC values are 7.8 µg/mL for *E. coli* strain ATCC 25922 and 31 µg/mL for *S. aureus* strain ATCC 6538, respectively.

These data indicated an antimicrobial activity similar to those of the plantaricin JLA-9, which presented a MIC of 16 µg/mL against *S. aureus* and 64 µg/mL against *E. coli* strain ATCC 25922.

Plantaricin P1053 (µg/mL)	***E. coli* ATCC 25922 growth inhibition (%)**	***S. aureus* ATCC 6538 growth inhibition (%)**
250.00	65.74	89.55
125.00	62.70	91.19
62.50	64.12	96.02
31.25	62.95	69.25
15.62	66.25	0.00
7.81	68.85	0.00
3.90	14.41	0.00

Table 2.3. Minimum inhibitory concentration (MIC) of the growth measured by plantaricin P1053 isolated from L. plantarum PBS067 against *E. coli* ATCC 25922 and *S. aureus* ATCC 6538.

2.3.8 In vitro test of plantaricin P1053 on healthy colon CCD841 cell line

To verify that plantaricin P1053 could have beneficial effects also on human intestinal epithelial cells, initially the viability of the healthy colorectal CCD841 cell line was verified in the presence of the obtained plantaricin. To this end, the viability of CCD841 cells treated with 1 µg/mL of plantaricin P1053 was assayed by MTT test. Results reported in Figure 2.7 show an increase in CCD841 cell viability of about 20%. It appears that plantaricin P1053 acts on healthy cells as previously demonstrated for a few bacteriocins isolated from lactobacilli (Wang et al., 2014; Tao et al., 2005). So, it was decided to investigate whether the

isolated bacteriocin-like molecule was able to influence some pathways in healthy human cell lines.

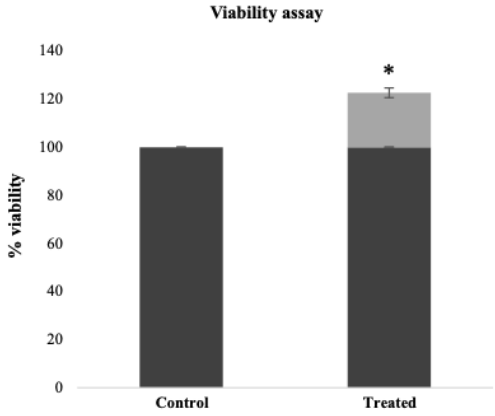

Figure 2.7. Evaluation of the cell viability of CCD841 cell line after the treatment with P1053 though MTT test. Buffer NaCl 0.1M pH2 was used as control and it did not have any effect on cell viability. Values are represented as mean ± SD. Statistical analysis were performed using Student's t-test. * *p*-value < 0.05

To evaluate a possible role for plantaricin P1053 in the complex pathway triggered by EGFR activation in healthy cell lines, cells were treated with 1 µg/mL of plantaricin P1053 for different times (0 min, 30 min, 1 h, 3 h and 24 h). CCD841 cells were employed because they represent the wild type cell line (healthy cells) for all the proteins involved in EGFR pathway (Akt and Erk proteins are not mutated).

Western blot analysis showed an activation of EGFR pathway in the healthy human cell line CCD841. Plantaricin P1053 administration to CCD841 cells led to an increase of phospho-Akt as an early response (30 min-3 h) and a significant decrease as a late response (24 h). Erk showed a peak in its phosphorylation level after 30 min of exposure to the bacteriocin-like compound, that decreased gradually from 1 to 3 h after the administration, becoming completely unphosphorylated (ND) after 24 h (Figure 2.8).

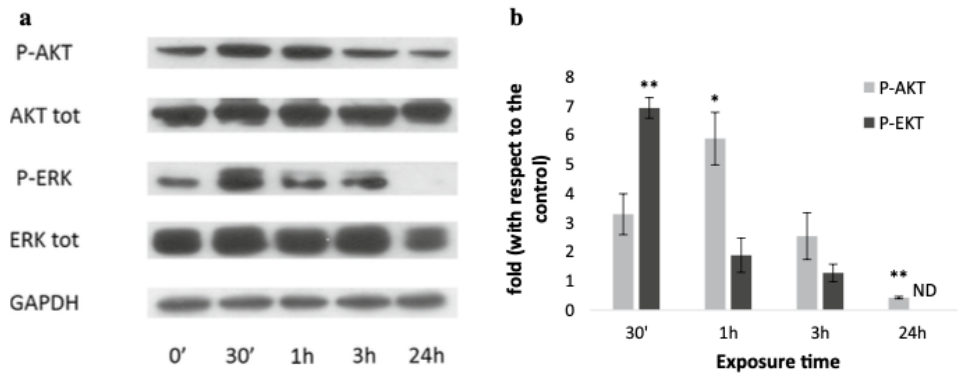

Figure 2.8. Representative western blotting analysis performed on crude extract using anti-P-AKT, anti-AKT, anti-P-ERK1/2, anti-ERK1/2, and anti-GAPDH antibodies. GAPDH was used as control. The experiments were performed in triplicate. (A) densitometric analysis performed with Scion Image Software. Values are expressed by comparing the data obtained after the treatment with plantaricin P1053 with those obtained from 0 minutes of exposition to the compound. (B) values are represented as mean ± SD. ND, no protein phosphorylated is detected. * *p*-value<0.05, ** *p*-value<0.01

2.3.9 In vitro test of plantaricin P1053 on colon cancer E705 cell line

A possible beneficial effect of plantaricin P1053 on cancerogenic epithelial intestinal cells was also evaluated. A viability test of E705 cells treated with different concentrations of plantaricin P1053 was performed. Results showed a significant inhibitory effect, near 30%, on E705 cells proliferation in a concentration dependent manner. The higher concentration of plantaricin 1053 (1 µg/mL) evidenced the higher inhibitory effect, as reported in Figure 2.9.

Also in this case a possible role for plantaricin P1053 in the complex pathway triggered by EGFR activation in cancerogenic cell lines, was evaluated. For this, cells were treated in the same manner with plantaricin P1053 for different times (0 min, 30 min, 1 h, 3 h and 24 h). By Western blot analysis no phosphorylation of Akt or Erk was detected, although both proteins were detected in their unphosphorylated forms (data not shown).

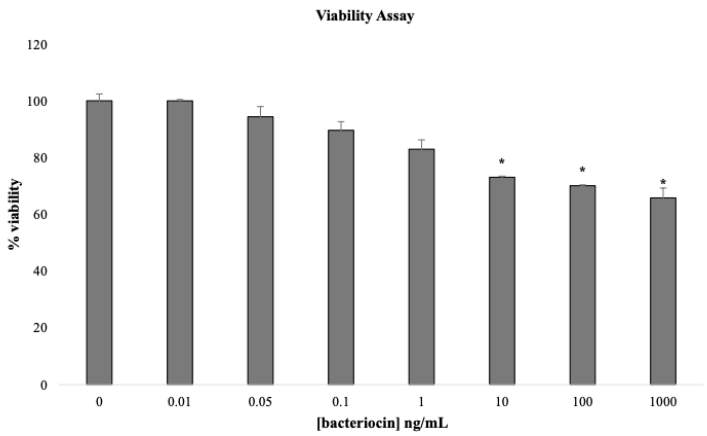

Figure 2.9. MTT test was performed on cancerogenic E705 cell line. Buffer NaCl 0.1M pH2 was used as control and it did not have any effect on cell viability (100% viability). Values are represented as mean ± SD. Statistical analysis were performed using Student's t-test. * *p*-value < 0.05

2.4 Discussion

Inulin polymers, especially of plant origin, are a subject of interest in many food research programs, for their low food caloric value and their dietary fiber effects. They are a polydisperse β-2,1 fructans with varying degree of polymerization which determines their physicochemical properties and thus their functional characteristics (Waqas et al., 2019; Mensink et al., 2015).

The prebiotic effect of inulin has been well proven (Waqas et al., 2019; Rubel et al., 2014). It lies in stimulating the growth of beneficial bacteria in the intestine. Among those, bifidobacteria and lactobacilli are the most known (Morrison et al., 2016). In the first part of the work, to assess the prebiotic effect of several commercial inulins and FOS, the ability of various microbial strains belonging to *Bifidobacterium* and *Lactobacillus* genera to grow on them has been investigated *in vitro*. All tested strains were able to grow on the selected substrates (Figure 2.1). However, of the five tested *Lactobacillus* species, *L. rhamnosus* PBS070 and *L. reuteri* PBS072 grew not so well on FOS with the smallest DP, whereas all the other strain, comprising Bifidobacteria, could use very well these small molecules as carbon and energy sources. A similar behavior was observed on FOS with DP around 10. Interestingly, several tested

strains could growth also on the biggest molecules, such as inulin with a degree of polymerization ranging from 25 to 60.

These results suggest that all these strains present metabolic pathways of degrading both higher DP inulin and FOS.

In literature is reported that *Lactobacillus* strains possess two alternative pathways for the metabolism of sucrose and higher FOS through intracellular hydrolysis. In contrast, an extracellular hydrolysis of sucrose, oligo- and poly-saccharide is less frequently found in this genus. For instance, among several *Lactobacillus* strains, only *L. reuteri* SD2112 presented extracellular enzymes able to degrade sucrose, FOS and polysaccharides (Ganzle et al., 2012). The degradation of inulin by extracellular enzymes is not the only reported mechanism. For instance, *L. delbrueckii* TU-1 and *L. delbrueckii* JCM 1002 hydrolyze inulin without possessing an extracellular *β*-fructosidase (Tsujikawa et al., 2013). Since these two strains fail to degrade fructose, the authors suggested the presence of a transporting system in which larger molecules of inulin are more preferably taken up into the cells where their hydrolysis occurs.

In our fermentation experiments, *Lactobacillus* strains show a similar growth behavior towards inulin and FOS with respect to the literature mentioned above, suggesting the presence of different sugars metabolic mechanisms.

Unlike Lactobacilli, in which sugar metabolism is mostly restricted to intracellular enzymes, Bifidobacteria have been reported to maintain a more extensive tool set for extracellular hydrolysis and transport of complex carbohydrates sugars (Sela et al., 2008; Van den Broek et al., 2008). The obtained growth results of the four *Bifidobacterium* strains on FOS and inulin are in accordance with previous reports, where it was demonstrated that the majority of Bifidobacteria can degrade FOS and inulin by extracellular fructosidases, whose induction is dependent on the type of sugar used as growth substrate (Van den Broek et al., 2008). For example, *Bifidobacterium adolescentis* ALB 1 activated extracellular fructosidases when grown on inulin rather than FOS (Rossi et al., 2005).

In conclusion, the first part of this study represents a first step to develop synbiotic formulations by combining inulins and FOS characterized by various degree of polymerization with different *Lactobacillus* and *Bifidobacterium* species for further studies, including *in vitro* gut simulation models and *in vivo* trials.

However, to design a powerful synbiotic, it is important to consider the probiotic characteristics. Among them, the antimicrobial activity is crucial for competing in the intestinal environment. Therefore, the second part of this work describes the isolation and characterization of a bacteriocin-like compound produced by *L. plantarum* strain PBS067, that shows an antimicrobial activity against potential human pathogens and that affects both normal and cancerogenic human intestinal cells. The probiotic strain, isolated from healthy patients, was able to produce the maximum amount of a bacteriocin-like compound at the onset of the stationary phase of the growth, during an incubation for 16 h at 37°C at pH 4. This result is quite similar to that showed by Messi et al. (2001), who reported that *L. plantarum* 35d started to produce bacteriocin during the late logarithmic growth phase. Plantaricin 35d reached the maximum concentration after 19 h of incubation (stationary phase), at pH 4.

Preliminary experiments evidenced that the cell free supernatant (CFS) of *L. plantarum* PBS067 culture had an inhibition of the antagonist bacteria growth, as *E. coli* and *S. aureus*. Thus, a purification procedure of the CFS metabolites was performed. Based on the pH dependence, it was decided to use the adsorption/desorption method as the first step in the purification procedure (Yang et al., 1992). The second step of purification consisted of the extraction with a solvent as *n*-butanol, and the last consisted of the elution in SEC-HPLC. At each purification step, the antibacterial activity was tested and a comparison of the activity of the acidified purified compound with respect to the crude CFS, was verified. Results showed an increase of the antimicrobial activity along the purification procedure. The purified compound from supernatant of *L. plantarum* strain PBS067 was then characterized. An enzymatic hydrolytic activity by

proteinase K was demonstrated on the isolated compound. The mass analysis by ESI-full MS determined a molecular mass of 1053 Da for the compound (plantaricin P1053). Because of its bactericidal activity, its behavior based on the hydrophobicity and the charge of the bioactive compound secreted by the cells, pH resistance and low molecular mass, plantaricin P1053 could be classified as a small bacteriocin-like presumably belonging to Class II, according to the definition given by Klaenhammer (1993). The data are in line with the results obtained by Zhao et al., where for the first time a plantaricin with a low molecular weight of 1044 Da able to act against *Bacillus* spp. was isolated from *L. plantarum* strain JLA-9 (Zhao et al., 2016) and obtained by Zhu et al. that described the identification of the plantaricin ZJ008 with a MW of 1335 Da able to act against Gram-positive and Gram-negative bacteria (Zhu et al., 2014).

Interestingly, this second parte of the work demonstrates not only that the bacteriocin-like compound isolated from *L. plantarum* PBS067 showed a broad range of antimicrobial activity towards Gram-positive and Gram-negative bacteria with a good MIC, but also its activity towards human cells, extending its role from a bacteriocin to a multifunctional factor that has effect both on normal and cancer cells. In fact, in this work plantaricin P1053 effect were investigated towards human cells using both healthy and cancerogenic cell lines. Unlike other authors, like Dimitrovski et al. (2014) who used the supernatant of the broth culture, in this study only the bacteriocin-like molecule resuspended in NaCl buffer was administered. The plantaricin P1053 increased the vitality of healthy CCD841 cell line. These results led to speculate that this bacteriocin-like compound could act on pathways involved in cell survival and proliferation, such as the epidermal growth factor receptor (EGFR) pathway. Therefore, this pathway was investigated because it has been reported by Wang et al. (2014) that a *L. rhamnosus* GG-derived soluble protein, p40, is able to activate EGFR and its downstream target Akt in intestinal epithelial cells, leading to an inhibition of apoptosis and a preservation of the barrier function by an upregulation of mucin production. Moreover, Tao et al. (2005) reported that a soluble factor from

the probiotic strain *L. rhamnosus* GG could rapidly activate the MAPKs. The activation of EGFR, Akt and Erk was therefore investigated after plantaricin P1053 administration at different times. This compound can rapidly activate, within 30 min, Akt and Erk in healthy intestinal cells. The phosphorylation of Akt leads to an anti-apoptotic effect, while Erk activation is pro-proliferative. It is important to note that Akt and Erk activation is finely controlled, with a decrease in their phosphorylation level till a nearly switch off 24 h after treatment.

Concerning the effect of plantaricin P1053 on cancerogenic epithelial cell lines, a significant loss of viability was observed, near the 30%, of E705 cells in a concentration dependent manner. These data are in line with those of Dimitrovski et al. on different cancerogenic cell lines (Dimitrovski et al., 2014), although they used only the supernatant obtained from *L. plantarum* cultures. SDS-PAGE electrophoresis showed no Akt and Erk activation in cancerogenic E705 cells, leading to think that the difference in the vitality between healthy and cancer cells is due to EGFR downstream targets activation in the former. Moreover, the experiments show that plantaricin P1053 can reduce E705 cancer cells viability, although the mechanism involved is still not elucidated. At the same time, its effect on healthy intestinal cells is an increase in viability, due to a transient phosphorylation of both Akt and Erk which is achieved with a different timing and is readily switched off in 24 h.

In conclusion, in this second part of the work, it was demonstrated that the antimicrobial activity of *L. plantarum* PBS067 is mediated by the plantaricin P1053 action, that was active against both representative Gram-positive and Gram-negative bacteria. The compound can also affect the host cells through an enhancing of healthy cells and a reduction of cancer cells viability. Although some molecular mechanisms must be elucidated in further studies, plantaricin P1053 could represent one of the first multifunctional bacteriocin-like compound on human epithelial intestinal cells.

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Chapter 3:

Effects of inulin-based prebiotics alone or in combination with probiotics on human gut microbiota and on the markers of the immune system: a randomized, double-blind, placebo-controlled study on healthy elderly subjects

This chapter focuses on the design and the *in vivo* test of a synbiotic formulation composed by two *Lactobacillus* and one *Bifidobacterium* stains as probiotic and FOS (DP~10 and 3<DP<5) as prebiotic. The study duration was 56 days, and the healthy and elderly subjects were divided into three groups (the synbiotic, the prebiotic, and the placebo) to monitor at the baseline, after 28 days of administration period, and after 28 days of follow-up period the composition of the gut microbiota and the levels of selected immune biomarkers. High throughput technologies and qPCR with species specific primers were employed to monitor the gut microbiota of the subjects in terms of richness and modulation (at different taxonomic levels). Diaries filled everyday by the subjects were used to monitor the common infectious diseases symptoms, while immunological tests were used to evaluate the levels of fecal and salivary markers. The results suggest the effectiveness of this synbiotic formulation in the preservation of the healthy status of the elderly subjects in terms of eubiotic microbiota and well-being.

3.1 Introduction

The gastrointestinal tract is the central district of the human body inhabited by the microbial communities playing a fundamental role in many physiological processes, like absorption of nutrients, protection against ingested pathogens, and the maintenance of good health (Soenen et al., 2016; Zmora et al., 2019) through also the communication with the brain (Stower, 2019). Linked to microbial diversity, a variety of metabolic capabilities are associated to the gastrointestinal tract (Tandon et al., 2019), as well as an important role in supporting the efficiency of the immune system, and in the modulation of host physiology and metabolism (Jin et al., 2017).

However, gut microbiota together with gut functions change during lifetime (Woodmansey, 2007). Up to date, elderly is considered the last phase of life, usually associated with deterioration. The age-related decline implies some alterations in the host physiology, in the reactivity of the immune system as well

as in the composition of the gut microbiota (Finlay et al., 2019; Clements et al., 2018). Indeed, the intestinal microbiota is a very complex ecosystem (Mariat et al., 2009), whose members play an important role despite the interpersonal variability (Salazar et al., 2017). Considering elderly people, some studies underlined that Bifidobacteria decline (Bartosch et al., 2005), and others showed a reduction of *Bacteroides*, *Clostridia*, and *Lactobacilli* populations (Woodmansey, 2007). Likewise, the mucosal membranes covering gastrointestinal tract are continuously exposed to pathogenic microorganisms and the consequent immune system response suffers a progressive age-related decline. This immune-senescence phenomenon can be evaluated by different biological markers (Kotani et al., 2017). Faecal calprotectin and β-defensin2 are promising markers for the assessment of immune system activation caused by bacterial products, especially in relation to age (Guigoz et al., 2008). While secretory IgA, which are important in the maintenance of gut microbiota homeostasis and the protection against pathogens, could be used as indicators of the immune system senescence at respiratory tract level (Kolibab et al., 2005). In this district, also the total antioxidant capacity (TAC) can be used as a biomarker for oxidative stress (Maciejczyk et al., 2019).

Nowadays, it is universally accepted that a healthy gut microbiota contributes to an overall state of well-being which is crucial during the process of aging. In this context, it is considered worthwhile that an enrichment in bacterial population that can ferment dietary fibers can be beneficial (Salazar et al., 2017). Therefore, a growing interest in recovering or maintaining the gut microbiota in a healthy state through adapted diets or thanks to new formulations composed of selected probiotics, prebiotics, or synbiotics is emerging. Indeed, in literature are reported several studies about the role of dietary fibers in shaping the intestinal microbiota and in the modulation of the dominant species, indicating that a diversified microbiota could be favorable for the host (Clements et al., 2018; Kato et al., 2014). Moreover, other recent studies report the use of synbiotic formulations (Ouwehand et al., 2009; Macfarlane et al., 2013; Huang et al., 2018), that can

modulate the intestinal ecosystems and thus ameliorate the function of the gut barrier and the immune response (Ouwehand et al., 2009). However, few papers described beneficial effects in terms of changes of metabolism and composition of gut microbiota and immune parameters in elderly people after synbiotic administration, and none of them reports the effect of either the prebiotic or the probiotic alone with respect to the synbiotic effect (Salazar et al., 2017). The majority describes that the effect could be influenced by the selected probiotic strains included in the formulate, and by the kind of selected prebiotics, in some cases also associated with a dose-response effect (Tandon et al., 2019; Krumbeck et al., 2018a; Krumbeck et al., 2018b). Only the studies of Macfarlane et al. and Ouwehand et al. on healthy elderly subjects reported the improvement of some markers of the intestinal microbiota composition associated with gastrointestinal tract functions (e.g., an increase of butyrate production deriving from the increase of Actinobacteria and Firmicutes and a reduction of the pro-inflammatory response) after the intake of different synbiotic formulations (Macfarlane et al., 2013; Ouwehand et al., 2009).

Considering all these aspects, it is very important to collect more data regarding the effectiveness of bacteria genera among probiotics (for example *Bifidobacterium* and *Lactobacillus*) and the efficacy related to the fibers (respect to the synbiotics), and the correlation between the gut microbiota composition and the healthy status of elderly people (WHO, 1995).

In this context, this study aimed to evaluate the effects of a new synbiotic formulation on both the composition of gut microbiota and some biomarkers of the immune system in elderly subjects. Therefore, a randomized, double-blind, three-arm parallel, placebo-controlled, clinical study was carried out by administering the synbiotic formulation composed of two *Lactobacillus* strains (*L. plantarum* PBS067 and *L. acidophilus* PBS066) and one *Bifidobacterium* strain (*B. animalis* spp. *lactis* BL050), and two types of fructans (FOS with a degree of polymerization 3<DP<5 and FOS with DP around 10). The treatment period was of 28 days (T28), then a subsequent follow-up period of other 28

days (T56). At every time-point the gut microbial composition and modulation, the variability of fecal β-defensin2 and calprotectin, and salivary IgA and total antioxidant capacity (TAC) were evaluated, as well as the Common Infectious Disease incidence (CID) as markers of well-being condition of all the enrolled elderly subjects. This study design allowed us to evaluate the efficacy of the synbiotic treatment with respect to the group with the prebiotics alone and with respect to the placebo group.

3.2 Materials and methods

3.2.1 Study design

A randomized, double-blind, three-arm parallel, placebo-controlled study was carried out in accordance with the Declaration of Helsinki and the Good Clinical Practice guideline E6. The study protocol and the informed consent form were approved by the "Independent Ethical committee for Non-Pharmacological Clinical studies (Voghera, Italy)". The study took place at Complife Italia S.r.l. (Italy) facilities. 75 subjects were enrolled and divided into three groups (A, B, D). All subjects provided written informed consent before initiation of any study-related procedures. The 75 subjects were randomly assigned to receive one mixture of prebiotic formulation (A) or synbiotic formulation (B) or placebo (D) once daily (the first day was T0) for 28 days (T28); then the subjects attended 28 days of follow-up period (28 days from the last ingestion of the tested products) (T56).

The tested formulations consisted of food supplements in form of sticks (Farcoderma s.r.l., Italy) containing *Lactobacillus* and *Bifidobacterium* probiotic strains (Presti et al., 2015; De Giani et al., 2019) and/or prebiotics (Table S1 and Table S2) as follows:

A. Prebiotic formulation (Group 1): 50 mg short-chain FOS (fructo-oligosaccharides with a degree of polymerization between 3-5) and 50 mg medium-chain FOS (with a degree of polymerization of 10) (for a total of 100 mg of fibers), and common excipients used in formulations

B. Probiotic and prebiotic synbiotic formulation (Group 2): $1x10^9$ CFU *L. acidophilus* PBS066 (30 mg as lyophilized), $1x10^9$ CFU *L. plantarum* PBS067 (12 mg as lyophilized), $1x10^9$ CFU *B. animalis* spp. *lactis* BL050 (30 mg as lyophilized) (all the strains are from the private collection of Roelmi HPC, Italy), plus 50 mg short-chain FOS (fructo-oligosaccharides with a degree of polymerization between 3-5) and 50 mg medium-chain FOS (with a degree of polymerization of 10) (for a total of 100 mg of fibers), and common excipients used in formulations

D. Placebo formulation (Group 3): The same formulation without prebiotics and probiotics included in the other two formulations.

The study flow and the schedule of the assessment chart are reported in Figure 3.1. Indications about the ingestion of the food supplements include their ingestion away from meals, in a glass of no-sparkling water, once a day for 28 days.

A questionnaire and an explanation of the protocol of the study were given to the subjects. Symptom's questionnaire was performed as an interview with the enrolled subjects at the time-points of the study.

Each subject collected fecal samples (at least 4g) in a sterile plastic feces container at home and kept them at 4°C for a maximum of 3 hours until the reaching the laboratory, where fecal samples were frozen immediately at -20°C. Salivary samples were collected directly at the medical center into sterile plastic tubes informing the subjects to refrain from eating, drinking, using tobacco products, or chewing gum for at least 30 minutes before the collection time.

Salivary samples for immunological parameters (IgA level and TAC) and stool samples for fecal microbiota analysis and immunological parameters (Calprotectin and β-defensin2) were obtained at the beginning of the treatment (T0) and at the end of the intake (T28) for a total period of 28 days followed by a follow-up period of other 28 days (T56).

The study was deposited as clinical study with the following registration number: ISRCTN37538805.

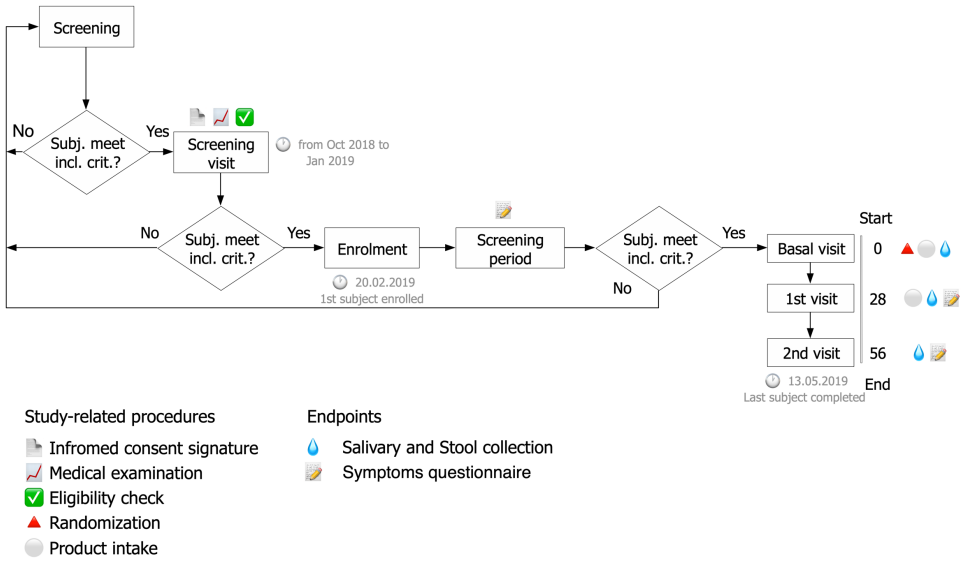


Figure 3.1. Study flow and schedule of assessment chart depicting the overall study design.

3.2.2 Subjects of the study

Eligible subjects were all Caucasian healthy elderly male and female with mean±SD age of 69.5±4.9 years. They were enrolled according to specific inclusion/exclusion criteria. Inclusion criteria were: i) to be healthy free-living both gender elderly aged from 65 to 75 years old having a 18.5-24.99 Body Mass Index (BMI), ii) to able to comply with all the trial procedures, iii) to be inoculated with an influenza vaccine, iv) to be intended not to vary the normal daily routine (i.e. lifestyle, physical activity, etc.), v) to be intended not to alter their usual diet or fluid intake and to follow the proposed dietary supplement for all the study period, vi) to be intended not to use products likely to interfere with the testing formulations and to use only the testing product during all the study period, vii) to be aware of the study procedures and to have signed an informed consent form.

Exclusion criteria were: i) to have contraindications to influenza vaccine, ii) to be undergoing treatment related to immune system modulation in the previous 4 weeks, or therapy for immune-suppressants for more than 2 weeks, or stopped

it less than 3 months before the study, iii) to have received influenza vaccination less than one year before the study, iv) to be under a current antibiotic administration, v) to have a known history of chronic medical condition, such as congenital heart disease, liver or kidney disease, or immune deficiency, vi) to have received a probiotic treatment in the previous 6 months, vii) to have used fiber products within the last 6 weeks, viii) to have severe concurrent diseases, ix) to report drug or alcohol abuse.

3.2.3 Randomization

Subjects were assigned to treatment arms using a computer-generated PASS 11 statistical software (version 11.0.8 for Windows; PASS, LLC, Kaysville, UT, USA) restricted randomization list ("Efron's biased coin" algorithm) as reported in Mezzasalma et al. (Mezzasalma et al., 2016). Subjects were randomized in a 1:1:1 (A, B, D) ratio. The software was running on Windows Server 2008 R2 Standard SP1 64 Edition (Microsoft, USA). Subjects, investigators, and collaborators were kept blind to product assignment. The randomization list was stored in a safe place by the *in-site* study director.

3.2.4 Assessment of clinical effects

3.2.4.1 Endpoints

Subjects were evaluated three times during the study: at the beginning (the baseline), 28 days from the starting point, and 28 days after the intervention period to assess the post-administration effects. The primary efficacy endpoint was the evaluation of the proportion of participants whose CID, of the level of fecal calprotectin, fecal β-defensin2, salivary IgA, and salivary TAC, and of the composition of gut microbiota after formulation supplementations. The secondary efficacy endpoint was the maintenance of the positive effects obtained 28 days after the last formulation intakes.

3.2.4.2 Symptom's questionnaire

The participants were asked to complete questionnaires on general wellbeing and bowel habit daily.

The Common Infectious Disease (CID) symptoms of subjects were recorded (Lefevre et al., 2015). The questions regarded symptoms of GI and upper/lower respiratory tract infections (cough, hoarseness, sore throat, itchy throat, rhinorrhea, sneezing, nasal obstructions, fatigue, headache, myalgia, nausea, vomiting) and diarrhea. The intensity of each symptom of GI and upper/lower respiratory tract infections was rated on a 4-point-scale (from 0: no symptom to 3: severe symptom). All symptoms were analyzed by the investigator who determined if they complied with the diagnosis of a CID or not. For the analysis of the mean number of days with CID and the frequency of CID, all the subjects were considered and the value "0" was applied to subjects without CID.

Compliance in consumption of the formulates was verified at each visit of the study. Compliance was satisfactory up to missing 2 doses during the 28 days of product intake, and each individual result did not exceed such limit.

3.2.4.3 Assessment of fecal calprotectin

Fecal calprotectin was measured using the PhiCal ELISA kit (Immuno-diagnostic, Germany) according to the manufacturer's instructions. The values were estimated in relation to the concentration of the calibration curve and resulted in a detection limit of 2 ng/mL.

Calprotectin values minor than 45 µg/g stool were considered as normal values (Klinberg et al., 2019).

3.2.4.4 Assessment of fecal β-defensin2

Fecal β-defensin2 (HβD-2) was measured by enzyme-linked immunosorbent assay according to manufacturer's instructions (Immuno-diagnostic kit, Germany). The declared detection limit was 0.01 ng/mL (Schwiertz et al., 2009; Kabeerdoss et al., 2011).

3.2.4.5 Assessment of salivary IgA

Saliva samples were collected, immediately provided to the laboratory, and centrifuged (at 3,000 rpm for 15 min). Then, the supernatants were immediately frozen and stored at -20 °C (Lefevre et al., 2015). Salivary IgA concentration was measured in all participants by a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Elabsciences, USA) with a detection range of 1.56-100 ng/mL. Experiments were carried out according to the manufacturer's protocol, and samples were diluted when necessary.

3.2.4.6 Assessment of salivary TAC

Salivary samples were evaluated by Ferric Reducing Antioxidant Parameter (FRAP) assay, as described by Benzie and Strain (Benzie et al., 1996). Ferric to ferrous ion reduction at pH 3.6 causes a colored ferrous-2,4,6-tripyridyl-s-triazine (TPTZ) complex. The absorbance at 595 nm of each sample was recorded after 4 min of incubation at room temperature. The absorbance values were compared to a Fe(II) standard curve. Final results are expressed as Fe(II) μM (Karlik et al., 2015).

3.2.5 Fecal sample collection

Fecal samples were collected from subjects at T0, T28, and T56. Fresh fecal samples were homogenized by vortex, mixing of the fecal mass, and separated into aliquots to be stored at -20°C. An aliquot of 1g was collected into Stool Nucleic Acid Collection and Preservation Tubes (Norgen Biotek Corp., Canada) (Kim et al., 2019) until the subsequent analysis.

3.2.6 Characteristics of the probiotic strains

In this study, two *Lactobacillus* spp. strains and one *Bifidobacterium* spp. strain, supplied by the private collection of Roelmi HPC (Italy), were selected for the preparation of the formulations. Table S1 describes the characteristics of each strain, in terms of antimicrobial activity and growth capacity on the selected FOS.

3.2.7 DNA extraction

To set up standard curves of DNA extracted from the probiotic strains, microbial cultures were performed in De Man, Rogosa and Sharp medium (MRS) (Condalab) and incubated at 37°C for 24 hours in anaerobic conditions using anaerobic atmosphere generation bags for microbiology (Sigma-Aldrich). For *B. animalis* spp. *lactis* a supplementation of 0.3 g/L of L-cysteine hydrochloride monohydrate (Sigma-Aldrich) was included in the growth medium (c-MRS).

DNA from microbial cultures was extracted by Ultraclean Microbial DNA Isolation Kit (MoBIO Laboratories, Italy). A total of 1.8 mL of 10^9 CFU/mL culture was used for obtaining genomic DNA following the protocol reported by Mezzasalma et al. (Mezzasalma et al., 2016).

DNA extraction from stool samples in preservation tubes was performed from 400 µL of the sample dissolved in the preservation buffer, using Stool Nucleic Acid Isolation Kit (Norgen Biotek Corp., Canada) following the protocol provided by the manufacturer with some modifications. Briefly, 400 µL of the sample dissolved in the preservation buffer were added to 600 µL of Lysis Buffer, vortexed for 5 minutes, and centrifuged for 4 minutes at room temperature. For the final elution of the nucleic acid sample, 50 µL and then other 50 µL (total volume of 100 µL of final sample) of Elution Buffer were used.

Both DNA extracted from probiotic cultures and DNA from stool samples were utilized to perform qPCR. DNA from stool samples was also used for the microbiome sequencing through Illumina MiSeq platform (Panek et al., 2018).

3.2.8 Fecal microbiology analysis by quantitative PCR

qPCR reactions were conducted using PCR Real-Time StepOne Plus (Applied Biosystems) and the PowerUp SYBR Green Master Mix (Applied Biosystems). Species-specific primer sets were developed by the authors in a previous work (Manfredi, 2015) and reported in Table S3. Reactions were carried out in a 10 µL qPCR mix containing 5.6 µL of PowerUp SYBR Green Master Mix, the Forward (10µM) and the Reverse primer (10µM) and 4.4 µL of DNA template,

according to the following qPCR program: 10 minutes 95°C and 40 cycles of 15 seconds 95°C and 1 minute 60°C (followed by a dissociation step) (Manfredi, 2015).

For each strain, standard curves were constructed using DNA extracted from microbial cultures using ten-fold dilutions ranging from 10^8 CFU/mL to 10 CFU/mL. Each DNA sample both from feces and from culture dilution was analyzed in triplicate.

Resulted data on the quantification were expressed as mean values ± SEM. The average slope and *y*-intercept of each standard curve of *L. plantarum* PBS067, *L. acidophilus* PBS066, and *B. animalis* susp. *lactis* BL050 were determined by regression analysis and used to calculate the fecal cell number for each bacterial target. Samples with resultant CFU/mL of the considered probiotic bacteria over the fixed upper bound of 10^8 CFU/mL per g of feces were excluded from the data processing, because the values exceed the maximum of the standard curve. For samples with Ct values around the lower bound of the detection limit, the corresponding CFU/mL were considered 10 (the minimum value obtained from the standard curve). Finally, if the Ct value was “undetermined”, 0 CFU/mL of that probiotic bacteria were considered in data analysis.

3.2.9 Gut microbiome community characterization by Next Generation Sequencing

Fecal nucleic acid concentration and purity were estimated spectrophotometrically (NanoDrop One Microvolume UV-Vis Spectrophotometer, ThermoFisher Scientific).

5 ng of NanoDrop quantified DNA was used to amplifying V3-V4 region of 16S rDNA. Amplicon sequencing (Illumina MiSeq platform v3 kit producing 300-bp paired-end sequences, requires a load between 6 and 20pM) was performed at Biodiversa S.r.l. (Italy). The V3-V4 region of 16S rDNA gene was amplified using primer pair Pro341F (5'-CCTACGGGNBGCASCAG-3') and Pro805R (5'-GACTACNVGGGTATCTAATCC-3’) (Takahashi et al., 2014). All the generated

sequences were submitted to the European Nucleotide Archive (ENA) Database through the Bioproject N. PRJEB38178 with the name ena-STUDY-University of Milano-Bicocca-06-05-2020-00:03:05:422-646.

3.2.10 Microbial composition and community structure analysis

The raw paired-end FASTQ reads were imported into the Quantitative Insights Into Microbial Ecology 2 program (QIIME2, ver. 2019.4.) and de-multiplexed native plugin (Bolyen et al., 2019). To perform quality filter, trim, denoise, mergepairs, and chimera removal of the data, the Divisive Amplicon Denoising Algorithm 2 (DADA2) (Callahan et al., 2016) was used. The taxonomic assignment of the ASV (Amplicon Sequence Variant) was carried out using the feature-classifier2 plugin (Bokulich et al., 2018) implemented in QIIME2 against the SILVA SSU non-redundant database (132 release), adopting a consensus confidence threshold of 0.6. Multibar plots were generated with the QIIME2 dedicated plugin taxa.

To estimate the effect of the synbiotic intake on the overall structure of the gut microbial communities of the participants, the Faith phylogenetic index (Faith PD) was calculated (Faith, 2016).

Pairwise difference tests implemented in q2-longitudinal were used to test whether alpha diversity (Faith PD) changed significantly between the intervals of the three (successive/non-successive) different time-points (Bokulich et al., 2018).

Unweighted and weighted UniFrac distance were used to estimate community dissimilarity (Lozupone et al., 2006). For the relationship between community dissimilarity and synbiotic intake, non-metric multidimensional scaling analyses (NMDS), canonical correspondence analysis (CCA), analysis of similarities (ANOSIM), and multi response permutation procedure (MRPP) were performed. To quantify the relative rate of change over time in the three groups we calculated a “microbial maturity” index from a regression model trained on feature data.

Microbiota-by-age z-scores (MAZ) were calculated as described in Subramanian et al. (Subramanian et al., 2014), using the following formulae: Microbial maturity (MM) = predicted microbiota "age "(time-points) − median microbiota age of the control group (D) of a similar age. MAZ = MM/S.D. of predicted microbiota "age" of the treatment control group at a similar age.

The volatility plot obtained, computes maturity index z-scores (MAZ) to compare relative "maturity" between each group, as described by Subramanian et al. (Subramanian et al., 2014). We use the calculated MAZ scores as input metrics (dependent variables) in linear mixed effects models to test if considering the trend of the single sequence variants ASV was possible to discriminate the three groups.

3.2.11 Statistical methods

The Wilcoxon signed-rank test was used for pairwise comparison between time-points within a treatment group for qPCR data, as data were not normally distributed. The Mann-Whitney test was used for comparison between treatment groups because data were not normally distributed. R version 3.5.1 was employed. Differences were considered statistically significant at *p*-value <0.05 (*) and *p*-value <0.01 (**).

The number of days related to CID symptoms were represented as the average number of days±SEM. Data regarding the markers of the immune system including fecal β-defensin2, salivary IgA, and salivary TAC were expressed as mean values±SEM. Since data did not distribute normally, two tailed Mann-Whitney or Wilcoxon test were used for pairwise comparison (Lefevre et al., 2015). R version 3.5.1 was employed. Statistically significant differences were considered as *p*-value <0.05 (*) and *p*-value <0.01 (**) (Zhang et al., 2019; Nakagawa et al., 2007).

Regarding fecal calprotectin, the number of subjects with marker levels below the normal value of 45 µg/g stool were recorded and the results were expressed as percentage of subjects.

3.3 Results

3.3.1 Gut microbiota profile of the subjects during the treatment

For each subject, faecal samples were collected, and DNA extracted for the gut microbiota analysis. The V3-V4 region of 16Sr RNA sequencing of the gut microbiota resulted in a total 24,952,554 reads. After DADA2 filtering step, a total of 16,813,759 reads with an average of 70,350±57,098 reads for each sample was obtained, resulting in 14,839 ASVs. A total of 16 phyla, 24 classes, 38 orders, 69 families, and 215 genera were identified. Analysis of taxonomic profiles indicated a dominance of bacteria belonging to the following five phyla: Firmicutes, Bacteroidetes, Proteobacteria, Verrucomicrobia, and Actinobacteria (Figure 3.2).

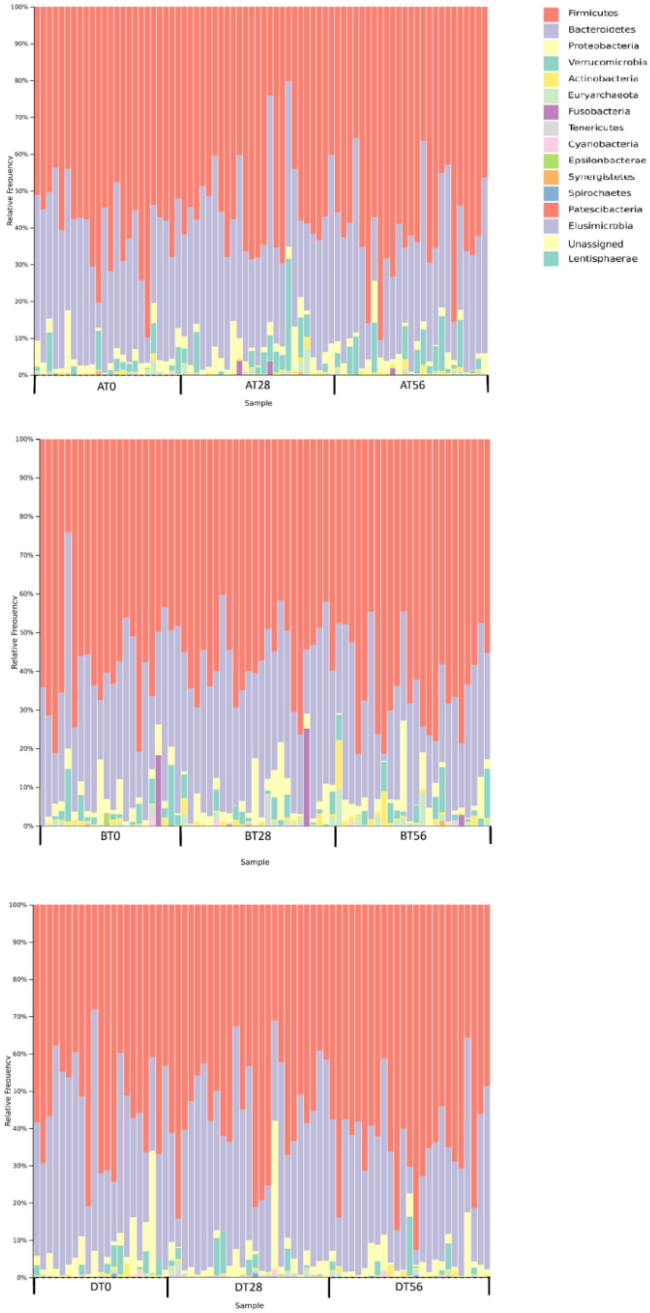


Figure 3.2. Composition of gut microbiota in elderly subjects. The relative abundance of bacterial ASVs at phylum level in every subject belonging to Prebiotic (A), Synbiotic (B) or Placebo (D) groups is shown by taxa bar plots. Each bar represents a subject and the different colored boxes represent a specific taxon (the percentage of relative abundance) within the sample. The legend shows the taxa from the most abundant (top) to the less abundant (bottom).

Moreover, considering the three groups in terms of relative abundances across the different time-points of the study, the most represented ASVs are assigned to *Bacteroides, Faecalibacterium, Prevotella, Alistipes, Subdoligranulum, Akkermansia, Roseburia, Blautia, Lachnospira,* and *Phascolarctobacterium*. Although present in a no consistent manner, even the ASVs belonging to *Ruminococcus, Eubacterium*, *Veionella, Dorea, Butyricicoccus, Lactobacillus,* and *Bifidobacterium* were observed.

To evaluate the core microbiome of the three groups after the treatment (T28), the ASVs were compared in a Venn Diagram. The results showed that 59% of ASVs were shared by the three groups, while the A, B, and D groups showed unique ASVs percentages of 6%, 7%, and 11%, respectively (Figure 3.3). A similar distribution was maintained at the end of the follow-up period (T56).

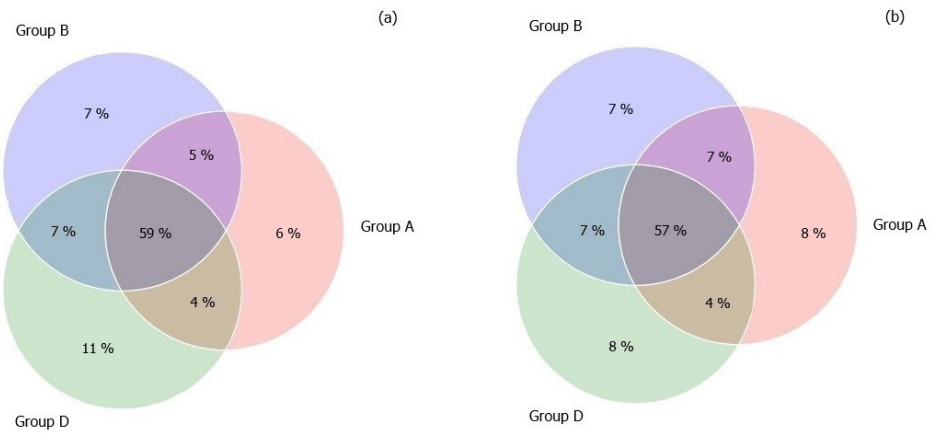


Figure 3.3. Venn diagram illustrating the percentage of ASVs that were shared or not by the gut microbiota of the three study-groups: Prebiotic A group, Synbiotic B group, or Placebo D group. Panel A represents the T28, and panel B represents the T56.

3.3.2 Gut microbiota diversity of the elderly subjects

Bacterial diversity of the three groups (*alpha*-diversity) was used to estimate the changes of biodiversity in the intervals of the three (successive/non-successive) time-points within each treatment-group. The pairwise difference test showed that there were no significant differences in Faith PD value in the interval between T0 and T28 for the three treatment groups. While both synbiotic (B) and prebiotic (A) groups showed a significant increase between the initial and the final time-points (T0-T56) (*p*-value of 0.045 and 0.02, respectively). No significant increases were evidenced in the interval between T28 and T56, except for the placebo group (D) (Table S4).

To analyze the variation of the biodiversity among the three groups in the different time-points intervals, the pairwise group comparison test (employing Mann-Whitney test at two levels of significance) was applied. The Mann-Whitney test showed a significant variation between A *vs* D and B *vs* D groups after the administration period (T0-T28) (Figure 3.4A). Indeed, the boxplot showing the difference in Faith PD value of the interval T0-T28, highlights a significant difference of A and B groups with respect to D group (A *vs* D U-value = 149 with *p*-value 0.02; B *vs* D U-value = 301 with *p*-value 0.04). Considering the difference in Faith PD value between the baseline and the end of the follow-up (T0-T56), no significant difference was evidenced between A *vs* D group and B *vs* D (*p*-value >0.05) (Figure 3.4B). Likewise, Figure 3.4C representing the interval T28-T56, shows no significant difference from the comparison of A *vs* D groups and B *vs* D groups (*p*-value >0.05). Overall, these results indicated that the synbiotic treatment led to a higher biodiversity variation after 28 days of administration and the biodiversity is maintained until the end of the follow-up (T56).

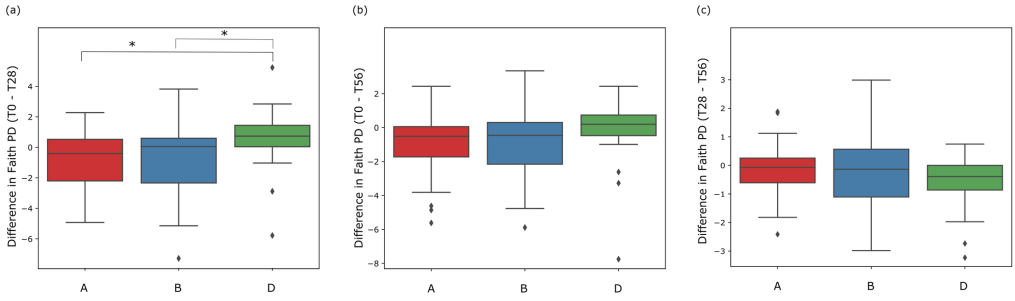


Figure 4. Comparison of the variation of the gut microbiota biodiversity by Faith PD value between Prebiotic A group, Synbiotic B group, and Placebo D group during the following intervals of the study: the first 28 days of administration (T0-T28) (A), during the 56 days of the study (T0-T56) (B), and during the follow-up period (T28-T56) (C). Statistical differences between treatment groups were calculated using Mann-Whitney test (* *p*-value <0.05).

To thoroughly evaluate the variation of microbiota biodiversity between groups, the analysis of microbiota maturity (measured as MAZ score) was performed. To assess how MAZ score changed over time in each group a volatility graph was plotted (Figure 3.5), depicting as trend lines the pattern observed for all the subjects of each group in the various time-points. It shows a similar behaviour for both the prebiotic (A group) and the synbiotic (B group) treatments, while the placebo (D group) shows a different and stable trend. The calculated MAZ scores indicated an increase of the community richness in A and B groups with respect to D group after the intake (T28). At the final time-point, there was a slight regression even if the MAZ score value remains higher in the synbiotic group compared to the prebiotic group. In general, the maturity analysis shows that the intake of synbiotic (B group) causes a variation in the structure of the microbial communities mainly at time T28.

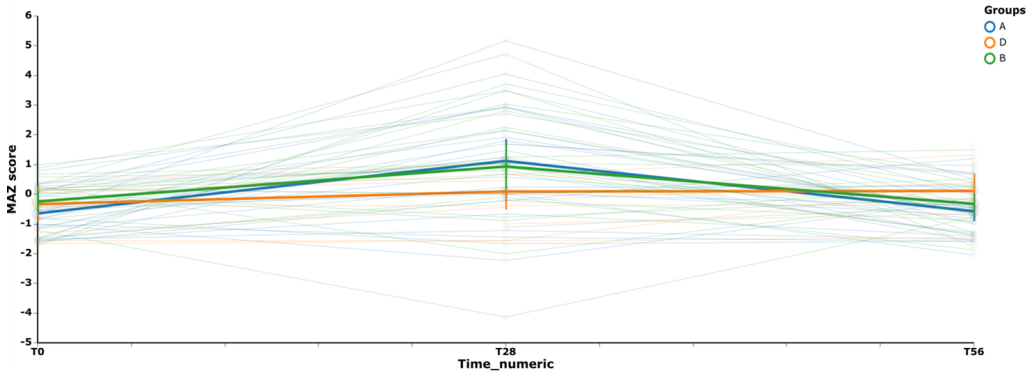


Figure 5. Volatility plot representing the trend of variation of the gut microbiota of the three treatment-groups. It represents the degree of change between selected time-points (T0, T28, and T56) and the resilience of the subject microbiota after the treatment with Prebiotics (A), Synbiotic (B) or Placebo (D). Thick curves are colored according to MAZ median-values, and the vertical bars represent error bars.

To appreciate the variation in microbiota biodiversity, a supervised learning regression was used to identify the most important ASVs able to promote a microbial shift in the treated groups across time. The analysis reveals that 50 different ASVs participated to discriminate the groups and that 12 ASVs belonging to *Bacteroides, Alistipes, Blautia, Lachnospira, Roseburia, Faecalibacterium*, and *Subdoligranulum* (*p*-value <0.05) were the most abundant (Figure 3.6). Among the ASVs that mostly contributed to the variation of the community biodiversity, the ASVs belonging to *Bacteroides* (ASVs 1819 and 6420) and *Subdoligranulum*_5538 showed a high variation in the A group at T56; while those belonging to *Blautia*_2926*, Lachnospira*_1103*, Lachnospiraceae*_4093, and *Roseburia*_6213 showed a lower frequency of variation at both T28 and T56. *Faecalibacterium* (ASVs 7470 and 7065) showed instability, reverting at time T56. The synbiotic treatment, instead, influenced *Faecalibacterium* (ASVs 7470 and 7065) to a highly stable frequency for all the duration of the study. Regarding the other ASVs mostly contributing to the variation in B group, *Alistipes*_4404, *Bacteroides*_2258, and *Subdoligranulum*_5538 showed a frequency varying especially during the administration period. Concerning the ASVs frequency in the D group, no significant variation during the time of treatment was observed.

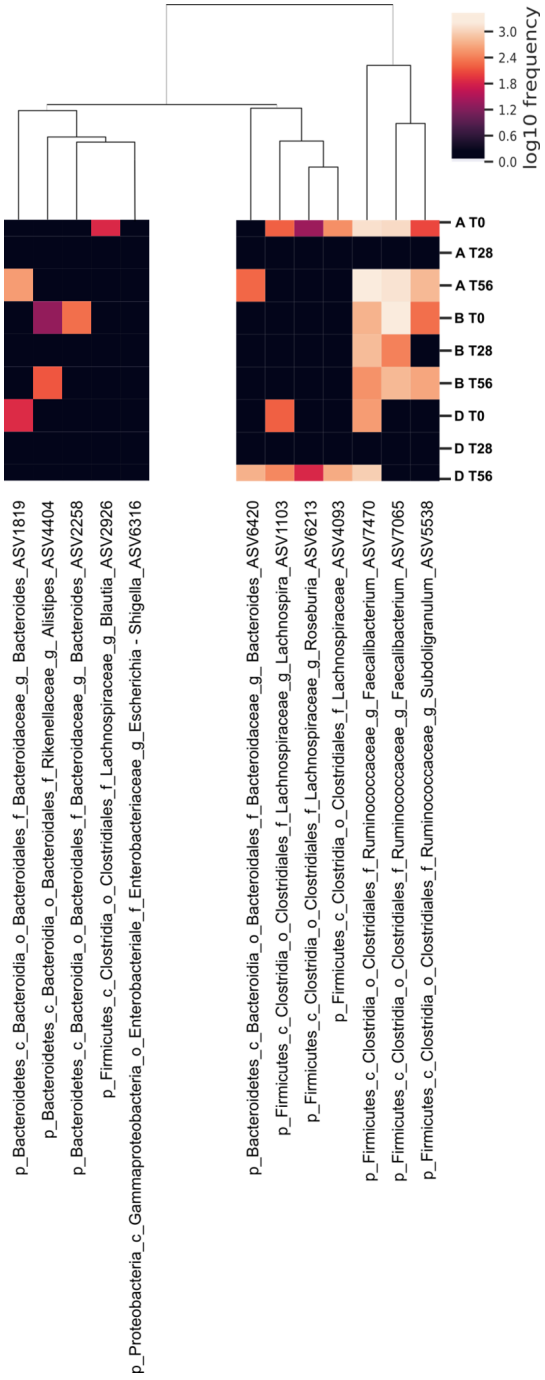


Figure 3.6. Heat map of ASVs variation of gut microbiota of elderly subjects of each treatment-group over time. In the logarithmic scale, color intensity indicates the abundance of each ASV feature. On the bottom of the heat map, the ASVs that varied for the three treatment-groups (Prebiotic A group, Synbiotic B group, Placebo D group) during the three time-points (T0 baseline, T28 time-point corresponding to the end of the treatment period, T56 time-point corresponding to the end of the follow-up period). On the top, the dendrogram of similarity between all the samples is represented.

3.3.3 Effects of the synbiotic intake on the most relevant taxa

Often the bacteria with an immuno-modulatory role and producing several beneficial effects on the host are not the most abundant within the human intestinal microbiota (Tandon et al., 2019). In this contest, another objective of the study was to evaluate the effects of the synbiotic intake on the gut microbial taxa that are known to have an impact on various aspects of gut metabolism. The significant biodiversity variation of the gut microbiota community within synbiotic group with respect to the other two treatment-groups in the intervals of the three (successive/non-successive) time-points (employing Mann-Whitney test at two levels of significance) was evaluated. Among the ASVs that varied significantly, *Akkermansia*, *Bifidobacterium*, *Blautia*, *Faecalibacterium*, *Prevotella*, *Roseburia*, and *Ruminococcus* were considered for their high rate of variation and for their relevance in inducing gut metabolism changes (El Hage et al., 2017) (Figure 3.7).

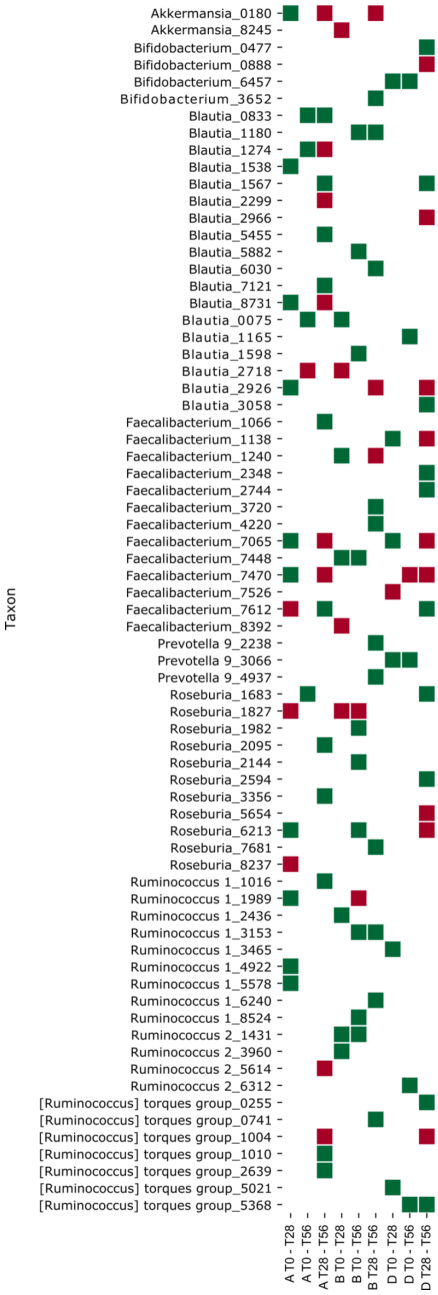


Figure 3.7. Heat map representing the significant biodiversity variation of ASVs assigned to the following bacterial genera: *Akkermansia*, *Bifidobacterium*, *Blautia*, *Faecalibacterium*, *Prevotella*, *Roseburia*, and *Ruminococcus* for the three treatment-groups (Prebiotic A group, Synbiotic B group, and Placebo D group) during the three intervals of time-points (T0-T28, T0-T56, and T28-T56). Mann-Whitney test at two levels of significance was applied. The red squares depict a negative variation, the green ones depict a positive variation.

Summing the number of ASVs that showed a significant relative abundance variation at different intervals of time, a differential bar-plot was generated. Positive cumulative variation (ASVs with positive abundance variation) is depicted with green bars and negative cumulative variation (ASVs with negative abundance variation) with red bars (Figure 3.8). The synbiotic treatment generated an increase of the ASVs number with a positive abundance variation (green bars) during all the intervals of time (T0-T28, T0-T56, and T28-T56) (Figure 8); and it generated a decrease of the ASVs number with a negative abundance variation (red bars). This can be appreciated by observing the specific positive variation (green squares) of the following ASVs: *Blautia*_0075, *Faecalibacterium*_1240, *Faecalibacterium*_7448, *Ruminococcus* 1_2436, *Ruminococcus* 2_1431, and *Ruminococcus* 2_3960 reported in the heat map of Figure 7 (T0-T28). During the follow-up period, the synbiotic treatment highlighted an increase of specific features (*Bifidobacterium*_3652, *Prevotella* 9_2238, and _4937) that in the prebiotic were not induced, although the placebo modulated diversely these two taxa. On the contrary, both the prebiotic and the placebo treatments induced a decrease of the ASVs number with a positive abundance variation during the two intervals T0-T28 and T0-T56. During the follow-up period (T28-T56), an increase of the ASVs number with a negative abundance variation is evidenced in the prebiotic and in the placebo groups. Nevertheless, the prebiotic intake induced a counterbalanced effect between T28 and T56, indeed the ASVs variation is comparable both in positive and in negative directions. Likewise, the D group demonstrated to have a similar response (Figure 3.8).

Results depicted the credence to the observation that the intake of the synbiotic promotes a significant change of specific bacterial genera and/or combination of specific bacterial taxa of the gut microbiota contributing to the metabolic shifting, i.e., SCFAs producers.

Figure 3.8. Bar plot representing the cumulative variation numbers of ASVs corresponding to seven bacterial genera (*Akkermansia*, *Bifidobacterium*, *Blautia*, *Faecalibacterium*, *Prevotella*, *Roseburia*, and *Ruminococcus*) for each treatment-group (Prebiotic A group, Synbiotic B group, and Placebo D group) during the three intervals of time-points (T0-T28, T0-T56, and T28-T56). The red squares depict a negative variation, the green ones depict a positive variation.

3.3.4 Effects of the synbiotic intake on Lactobacillus spp. and Bifidobacterium spp.

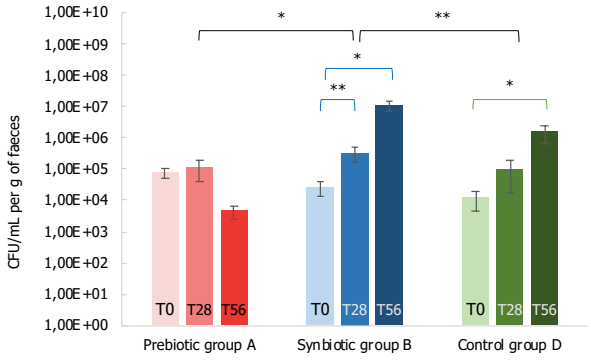
Around 30 ASVs belonging to *Lactobacillus* and 70 ASVs assigned to *Bifidobacterium* were observed. Interestingly, the number of *Bifidobacterium* ASVs increased after the synbiotic intake (T28), and a similar effect was evidenced by the prebiotic treatment towards both *Lactobacillus* and *Bifidobacterium* at T28. Instead, in the placebo group, a stable number of bacteria belonging to these genera with small physiological fluctuations was observed for the entire study. To specifically quantify the number of bacteria belonging to the same species of the probiotics administered within the synbiotic i.e., *L. plantarum, L. acidophilus*, and *B. animalis* subsp. *lactis*, the total DNA extracted from faecal samples (A, B, and D groups) was used to perform a qPCR analysis with species-specific primers (after sample collection at the times T0, T28, and T56). The qPCR analysis demonstrated that at time T0 in all three

subject groups the average of the total cell amount of *L*. *plantarum*, *L*. *acidophilus*, and *B*. *lactis* was around 10^4 CFU/mL per g, 10^5 CFU/mL per g, and 10^6 CFU/mL per g, respectively (Figure 3.9).

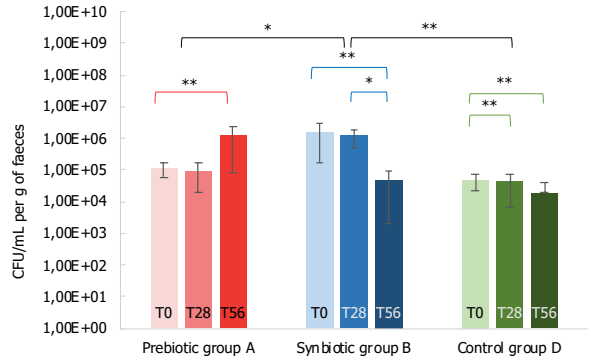
In general, an increasing trend in the amount of these probiotic bacteria was observed in the treatment with the synbiotic (B group). Specifically, at time T28, a significantly higher amount of *L*. *plantarum* and *B*. *lactis* (p-value < 0.01) with respect to time T0 was evidenced in B group; besides, at the same time-point, *L*. *plantarum* and *B. lactis* resulted statistically higher compared to both A and D groups (p-value <0.01: comparing *L. plantarum* B *vs* D, and *B*. *lactis* B *vs* D; p-value <0.05 comparing *L. plantarum* A *vs* B and *B. lactis* A *vs* B, respectively (Figure 9, panel A and C). The amount of *L*. *acidophilus* was stable and statistically higher with respect to both A (p-value <0.05) and D (p-value <0.01) groups at T28 (Figure 9 panel B). At time T56 different trends were detected. The amount of *L*. *plantarum* in B group resulted significantly higher with two orders of magnitude compared to T28 (p-value <0.05 T0 *vs* T56). Instead, the amount of *L*. *acidophilus* and *B. lactis* decreased. Nonetheless, at T56 the total amount of *B*. *lactis* is comparable to the level of A group.

Overall, these results indicate that the synbiotic contributed to enhance the *Lactobacillus* and *Bifidobacterium* species within the gut microbial community after their ingestion for 28 days. Meanwhile, the persistence of the synbiotic strains in the gut system likely needs a longer period of administration.

Panel A



Panel B



Panel C

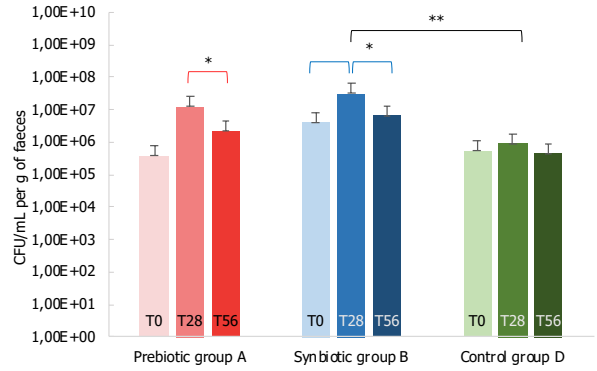


Figure 3.9. Quantification of cell numbers of *L. plantarum* (Panel A), *L. acidophilus* (Panel B), and *B. animalis* spp. *lactis* (Panel C) in fecal samples by species-specific qPCR at different time-points (T0, T28, and T56). Data are expressed as the mean values of CFU/mL per g of feces ± SEM. The Wilcoxon rank-signed test was applied for the comparisons within each treatment group, highlighted in red, blue, and green color for Prebiotic A, Synbiotic B, and Placebo D groups, respectively. The Mann-Whitney test was applied for the comparisons between groups, reported in black color (* *p*-value <0.05 and ** *p*-value <0.01).

3.3.5 Impact of synbiotic intake on common infectious disease incidence

The incidence of Common Infectious Disease (CID) of the subjects was monitored all over the study period. No subjects were drop-out or lost in the follow-up period and no adverse effects that are related to the administration of the treatments were recorded. The treatment efficacy in terms of CID symptom amelioration was evaluated both as the number of subjects that recorded at least one symptom of CID throughout the whole study (T0-T56), during the uptake period (T0-T28), and during the follow-up period (T28-T56), and as the average of days with one or more CID episodes.

Collected data highlighted that during both the uptake and the follow-up period (T0-T28 and T28-T56), B group experienced a minor percentage of subjects (36% and 12%) that had at least one CID episode with respect to A (48% and 20%) and D (56% and 20%) groups. Besides, considering the total period of study (T0-T56), it was observed the same pattern: the percentage of subjects that had at least one CID episode was minor in the synbiotic group (48%) with respect to placebo (76%) and prebiotic (68%) groups. Moreover, a lower percentage of B subjects presented the persistence of CID (20%) compared to the other two treated groups (A 24% and D 32%). Overall, these data indicated the synbiotic effectiveness in reducing the CID symptoms incidence.

Considering the average number of days linked to CID, 5±2 days was the average for B group (3±1 during the administration period and 2±1 during the follow-up period) and it was statistically lower than the average of the prebiotic (p-value <0.05) and the control (p-value <0.01) groups (Figure 3.10). Curiously, the average day number of the follow-up period of B group was significantly reduced (p-value <0.05) with respect to D group, suggesting the persistence of the positive effect in the synbiotic group.

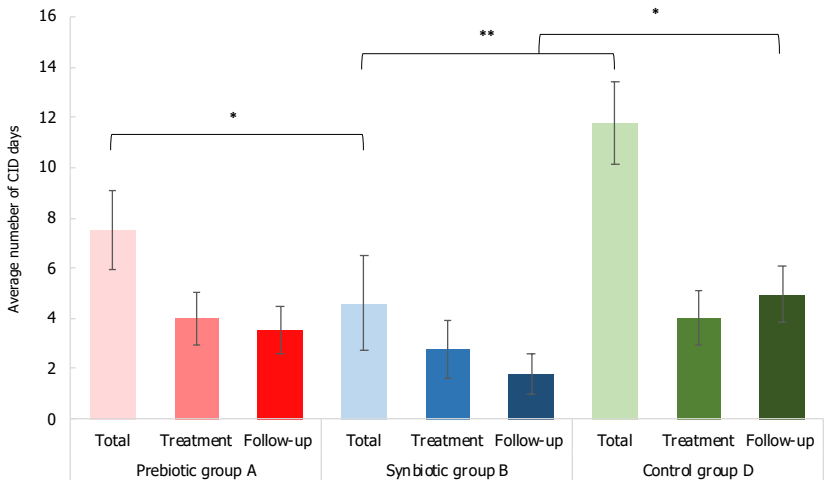


Figure 3.10. Average number of days related to CID symptoms that affected the elderly subjects at the different intervals of the three time-points (T0-T56, T0-T28, and T28-T56) for Prebiotic A group, Synbiotic B group, and Placebo D group. Data are expressed as mean ± SEM. The Mann-Whitney test was applied (* *p*-value <0.05 and ** *p*-value < 0.01).

3.3.6 Analysis of the levels of the immunological markers

The activity of the immune system was monitored in two facing districts, the gastrointestinal system, and the respiratory tract, measuring the faecal calprotectin, the faecal β-defensin2, the salivary IgA, and the salivary TAC.

Regarding faecal markers, Figure 3.11 shows the subject percentage presenting the faecal calprotectin level below 45 µg/g that is considered physiological for elderly people (Klinberg et al., 2019). At the beginning of the study, 88% of B subjects had normal levels of this faecal marker that after the synbiotic intake shifted in terms of number of subjects to 96% (T28) and 100% (T56) showing calprotectin level below 45 µg/g. Despite few unusual marker values, the calprotectin level of prebiotic and placebo group was stable during the study, presenting during the follow-up period 84% and 92% of subjects with calprotectin level below 45 µg/g, respectively.

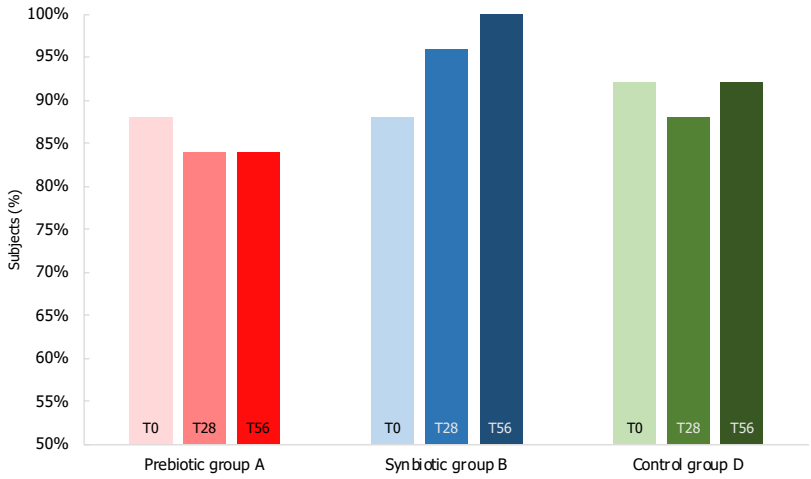


Figure 3.11. Percentage of subjects with levels of fecal calprotectin below the normal value of 45 µg/g. Levels were measured at T0, T28, and T56 for Prebiotic A Group, Synbiotic B Group, and Placebo D Group.

Data on the faecal β-defensin2 levels showed that the amount increased in all three groups (Figure 3.12). In particular, the synbiotic enhanced significantly (*p*-value <0.05) the β-defensin2 levels with around 22% increase with respect to the beginning of the treatment (T0-T56). In addition, the levels of B group at the end of the follow-up period were statistically higher compared to both A (*p*-value <0.05) and D (*p*-value <0.05) groups. Although the placebo subjects evidenced an increase at the end of the administration (*p*-value <0.05), these values were not comparable to the ones of the synbiotic. Even the prebiotic group presented a significant shift of these marker levels (*p*-value <0.05) just at the first endpoint (25% increase with respect to the beginning of the treatment).

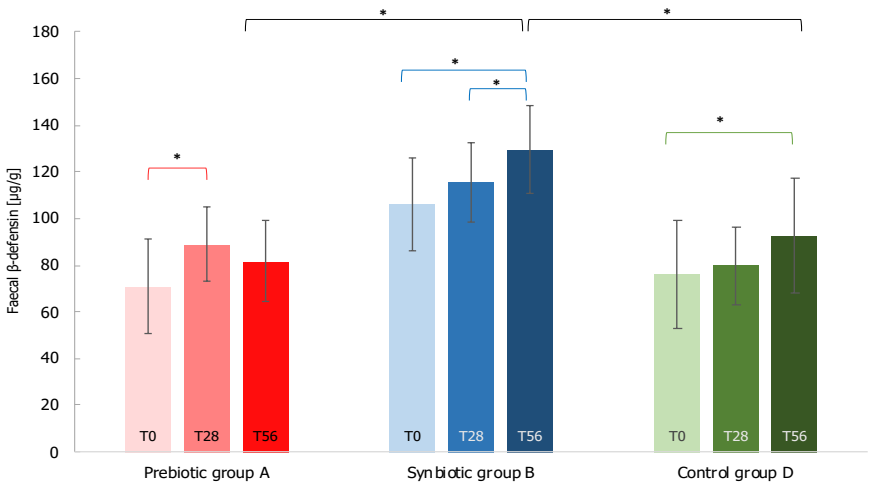


Figure 3.12. Fecal β-Defensin2 levels measured at T0, T28, and for Prebiotic A Group, Synbiotic B Group, and Placebo D Group. Values are expressed as mean ± SEM. The Wilcoxon rank-signed test was applied for the comparisons within each treatment group, highlighted in red, blue, and green color for Prebiotic A, Synbiotic B, and Placebo D groups, respectively. The Mann-Whitney test was applied for the comparisons between groups, reported in black color (* *p*-value <0.05).

The elderly immune system associated with the respiratory tract is often characterized by the decrease of IgA and the increase of reactive oxygen species contributing to a state of senescence.

Considering the salivary IgA values (Figure 3.13), the levels increased only for the synbiotic group (2% at T28 and 8% at T56 with respect to the beginning of the study), in line with the levels registered in elderly people (Jafarzadeh et al., 2010). For A and D groups, the levels decreased similarly at T28 (around 4.5%) and T56 prebiotic group decreased much more than the placebo group.

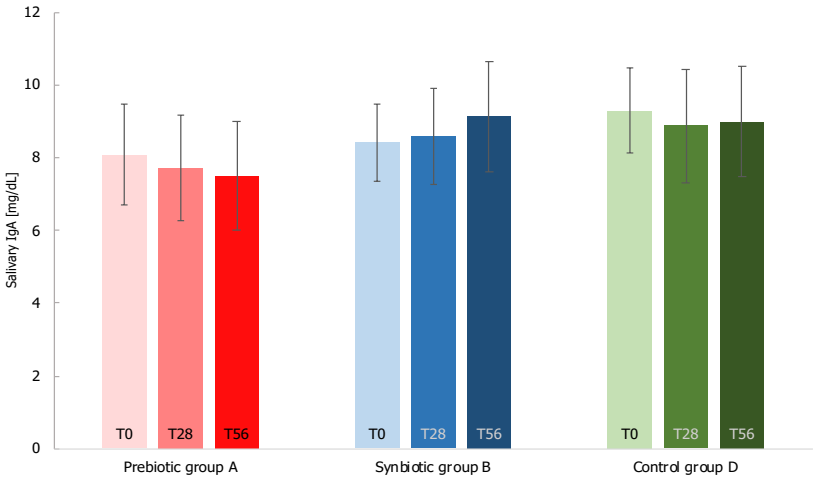


Figure 3.13. Salivary IgA levels measured at T0, T28, and T56 for Prebiotic A Group, Synbiotic B Group, and Placebo D Group. Values are expressed as mean ± SEM. The Wilcoxon rank-signed test and the Mann-Whitney test were applied, and no significant differences were observed.

Finally, the TAC parameter did not show important shifts after the treatments, except for the synbiotic intake that resulted in a significant increase of TAC levels between T0-T28 (*p*-value <0.05) (Figure 3.14). Indeed, A and D groups presented stable TAC levels with an average of around 523 µmol considering both T28 and T56.

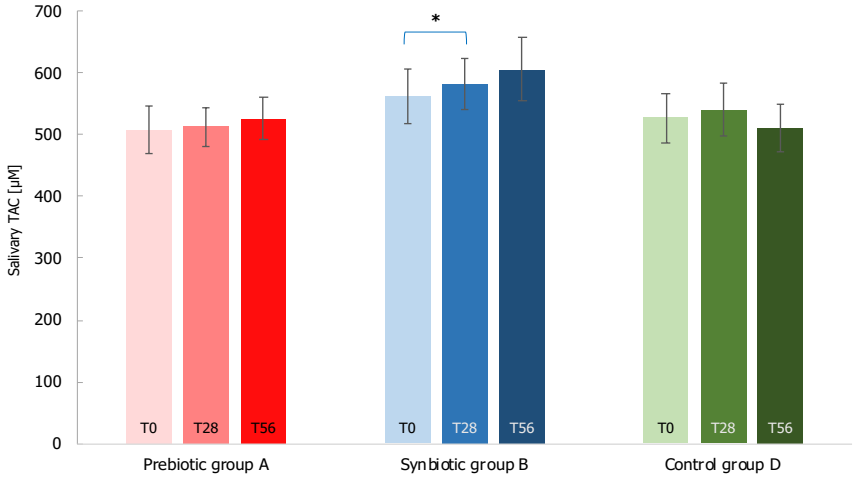


Figure 3.14. Salivary Total Antioxidant Capacity (TAC) measure at T0, T28, and T56 for Prebiotic A Group, Synbiotic B Group, and Placebo D Group. Values are represented as mean ± SEM. Statistical differences were calculated using Wilcoxon rank-signed test (* *p*-value <0.05). The statistical comparison within the synbiotic treatment group is highlighted in blue color.

3.3 Discussion

It was considered an experimental design of a clinical study including the synbiotic treatment (B group) with respect to the group with the prebiotics alone (A group) and with respect to the placebo group (D group). The clinical study aimed to evaluate the effect of the new synbiotic on both the gut microbiota and the immune system of healthy elderly subjects. To our knowledge, no other study reports the elucidation of the synbiotic effects on both healthy and elderly population, associating the microbial gut composition to the markers of health-promoting properties, and including techniques that allowed the quantification of the probiotic strains. Moreover, in literature, the experimental design rarely comprised prebiotic alone and synbiotic intake. Results related to the gut microbiota biodiversity obtained in this study indicated that the synbiotic treatment led to a higher biodiversity variation after 28 days of treatment with respect to the placebo group and the biodiversity is maintained until the end of the follow-up period (T56). Interestingly, the taxa that mostly contributed to the variation of biodiversity after the synbiotic treatment (B group) are different in comparison with the prebiotic alone (A group) during the time. This is in line with most of the literature data (Costabile et al., 2017; Lin et al., 2014), in which it is reported that the composition of the gut microbiota is principally modulated by the synbiotic intake (Krumbeck et al., 2018a). Many papers report that the effect of these treatments on the gut microbiota composition was remarkably specific, promoting mainly the increase of bifidobacteria and specific beneficial taxa, and promoting the decrease of a limited number of opportunistic pathogens (Salazar et al., 2017; Tandon et al., 2019). In this study, a slight biodiversity variation of probiotic strains (as Lactobacilli and Bifidobacteria) was observed probably due to the small amount of prebiotics administered to the subjects of A and B group, although the amount of the selected prebiotics was chosen to comply with the protocol of the clinical trial development. Nevertheless, it is clearly established that resident members of gut microbiota can utilize the administered prebiotics,

competing with the supplied probiotic strains for the substrate (Krumbeck et al., 2018b).

The analysis of the whole gut microbial community provided insights on the ecological and functional impact of the synbiotic administration, through the variation of biodiversity composition. After synbiotic administration (T0-T28), *Blautia*, *Faecalibacterium*, and *Ruminococcus* genera had a positive cumulative variation, that demonstrate the efficacy of the synbiotic intake to fight their normal decrease in the microbiota of elderly subjects (Costabile et al., 2017). Moreover, during the follow-up period, the synbiotic administration highlighted an increase variation of specific features including *Bifidobacterium* and *Prevotella* that are associated with probiotic effect and fiber intake, respectively (Tandon et al., 2019; Kovatcheva-Datchary et al., 2015). A similar change of bacteria profile in the gut microbiota in the elderly subjects after the synbiotic consumption was observed by Gao et al. and Costabile et al. (Gao et al., 2019; Costabile et al., 2017). These bacteria are considered beneficial gut microorganisms and candidates to produce SCFAs (Zhang et al., 2015). Interestingly, the observed ecological gut microbiota shift could indicate an amelioration in terms of metabolic functions. Indeed, *Faecalibacterium* taxa possess anti-inflammatory property as well as actively contribute to the intestinal health and the maintenance of gut homeostasis (Quevrain et al., 2016), as also *Roseburia* genus (David et al., 2014). *Akkermansia* is also known to promote immune modulation, healthy metabolic homeostasis, and protect against inflammation (El Hage et al., 2017; Biagi et al., 2016). The presence of *Akkermansia* is often positively correlated to a high metagenome richness and specifically to *Ruminococcus* and *Bifidobacterium* taxa (Derrien et al., 2017). Additionally, *Faecalibacterium*, *Roseburia*, and *Ruminococcus* are considered as butyrate producing genera (Tandon et al., 2019; Krumbeck et al., 2018a), *Blautia* and some species of *Akkermansia* are known as candidates for the SCFAs production (Zhang et al., 2015), whereas *Prevotella* genus is acknowledged as long-chain fatty acids producer (Zhao et al., 2018).

As a primary outcome of our study, calprotectin and β-defensin2 as faecal biomarkers to follow inflammation in the gastrointestinal tract were analysed. Calprotectin is a calcium binding protein found principally in neutrophils and released in faeces upon accumulation and activation at the site of inflammation. It is also known that faecal calprotectin levels can change with age. Indeed, few healthy subjects of the study, especially belonging to B group, showed high level of calprotectin. Thus, the possible positive effect of synbiotic treatment was slightly detectable. Nevertheless, at the end of the study period (T56), all the subjects (100%) administered with synbiotic exhibited normal calprotectin levels. On the contrary, in the presence of gut dysbiosis and disorders (IBS, Chron's diseases, etc.), the levels of calprotectin usually are high and several times higher than the values of the healthy elderly at the end of our study (Klinberg et al., 2019). Likewise, the increase of human β-defensin2 secretion was observed in healthy adult volunteers after administration of Lactobacilli strains (Mondel et al., 2009; Ghadimi et al., 2011). In these papers, the authors report that only a few strains favour the effect of the β-defensins, and they suppose that the difference among the strains is in the ability to induce the defensin expression correlated to the presence of genes encoding glycosylated cell surface structures. Our data confirmed literature data (Kabeerdoss et al., 2011; Costabile et al., 2017), suggesting a correlation between the effects on the microbiota of the subjects of B group and the immunological tested biomarkers with respect to the placebo group. Indeed, an increase of the levels of the β-defensin2 and a reduction of the faecal calprotectin during the synbiotic treatment was obtained, associated to an increase of healthy microbiota in terms of diversity, stability, and resilience. Moreover, the positive stimulation of the innate gut immune system by the synbiotic treatment, for example observed with β-defensin2 increase, can be associated with the intestinal cell reaction and the increase of beneficial molecule produced by specific bacterial genera such as *Akkermansia*, *Prevotella,* and *Bifidobacterium*. This is further supported by literature, indeed *Akkermansia* can exhibit potential anti-inflammatory responses

and can potentially modulate the resident gut microbiota and, together with *Bifidobacterium,* was a health-associated genus well-known for promoting immune modulation and protection against inflammation (Biagi et al. 2016; Derrien et al., 2017).

Because it is known that also respiratory infections are dominant causes of diseases or mortality in the elderly for the association of aging and decline of the innate and adaptive immune system, also salivary IgA, and salivary total antioxidant capacity (TAC) were analysed as indices of the respiratory tract. IgAs have an important role in the maintenance of gut microbiota homeostasis and in the protection of gastrointestinal and respiratory tracts against pathogens (Kolibab et al., 2005). In this study, no significant responses deriving from the IgA markers were detected, except a slight increase after the synbiotic intake, as it was also observed by Kotani et al. (2017).

Regarding the total antioxidant capacity as a biomarker used to investigate oxidative stress and the imbalance between reactive oxygen species (ROS) production and antioxidant defence of the organism, the obtained values suggested a positive effect after the administration of the synbiotic formulation (B group) for the period of treatment. Indeed, this salivary homeostasis occurs because of aging and can be counterbalanced using synbiotics (Maciejczyk et al., 2019).

Finally, when the systemic inflammatory disorders increased for a different CID susceptibility in the subjects, the CID incidence was reduced after the treatment with the synbiotic formulate, similarly to Lefevre et al. (2015), that observed a reduction of CID incidence in elderly due to probiotic intake.

In conclusion, our study strengthened literature data regarding the effects of a new synbiotic formulation on the gut microbiota biodiversity associated with biological markers for immune system (especially correlated to β-defensin2) with respect to the placebo treatment, leading to an amelioration of the declining of the health of elderly. However, this study is characterized by several limitations. First of all, the study included a number not so large of subjects, because of the

difficulty to enrol eligible elderly subjects without severe concurrent diseases. A second criticism was the employment of immune biomarkers in the absence of clinical outcomes. This led to a difficulty in the interpretation of the biological significance of minor immunomodulatory effects (Costabile et al., 2017). Nevertheless, we chose calprotectin, β-defensin2, salivary IgA, and TAC biomarkers, because they are stable and ideal for non-hospitalized patients. Third, the minimum fiber concentration suggested for a functional synbiotic (Swanson et al., 2020) was used in the study, that in theory could be sufficient to stimulate the cognate gut microorganisms. However, the synbiotic formulation was unconventionally combined with two *Lactobacillus* and one *Bifidobacterium* probiotic strains.

In conclusion, with the increased life expectancy worldwide, the development of strategies aimed to reduce the imbalance of the gut microbial community and the immune system in elderly is one of the major challenges in the field of nutraceuticals and probiotic supplementations. This study extensively demonstrates, from the gut microbiota to the clinical and metabolic point of view, that formulations with probiotics and prebiotics as nutritional supplementation can help to maintain a healthy status by re-equilibrating the gut microbiota of the elderly population.

3.4 References

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3.5 Supplementary materials

Probiotic strain	Deposit number	Antimicrobial activity *vs*	Growth capacity on inulin-type fructans (different Degree of Polimerization DP)
Lactobacillus plantarum PBS067	DSM 24937	*C. albicans; E. faecalis; P. aeruginosa; S. aureus; E. coli.* Production of bacteriocin-like compound (P1053) active against *S. aureus* and *E. coli*	FOS 3<DP<5; FOS DP~10; Inulin 30<DP<60
Lactobacillus acidophilus PBS066	DSM 24936	*C. albicans; E. faecalis; P.aeruginosa; S. aureus; E.coli*	FOS 3<DP<5; Inulin~25
Bifidobacterium animalis subsp. *lactis* BL050 (formerly PBS075)	DSM 25566	*E. faecalis; P. aeruginosa; E.coli*	FOS 3<DP<5; FOS DP~10; Inulin DP~25; Inulin 30<DP<60

Table S1. List of the lactic acid bacteria strains used in this study, deposit number and their most relevant antimicrobial characteristics (described in Presti et al., 2015 and De Giani et al., 2019).

Fiber selected for the study	Description
FOS	50 mg of fructo-oligosaccharides with a degree of polymerization between 3 and 5, from Farcoderma s.r.l. (Italy)
Inulin 90%	50 mg of inulin from chicory with a degree of polymerization around 10, from Farcoderma s.r.l. (Italy)

Table S2. Composition and characteristics of prebiotics employed in the administered formulations used in this study.

Probiotic	Primer code	Sequence (5'→ 3')	DNA region	Amplified length (bp)
L. plantarum	Lpl2F LpL2R	CATTGGAACCGAACCAGTTG CGGTGTTCTCGGTTTCATTATG	16S/23S IS	203 bp
L. acidophilus	Lacid2F Lacid2R	GGGCAAATCACGAACGAGTA CTTTGTTTTCGTTCGCTTCA	pre16S	132 bp
B. animalis subp. *lactis*	AnimF AnimR	GCACGGTTTTGTGGCTGG GACCTGGGGGACACACTG	pre 16S	171 bp

Table S3. List of the species-specific primers used in this study for qPCR analysis (adjusted from Mezzasalma et al., 2015).

Group	W (Wilcoxon signed-rank test)	*p*-value	FDR *p*-value
T0-T28			
A	93.0	0.103	0.155
B	81.0	0.230	0.230
D	66.0	0.085	0.155
T0-T56			
A	69.0	0.020	0.061
B	58.0	0.045	0.068
D	100.0	0.590	0.590
T28-T56			
A	136.0	0.476	0.692
B	125.0	0.692	0.692
D	50.0	0.002	0.007

Table S4. Pairwise difference test on the *alpha*-diversity of A, B, and D groups in the different intervals of time (T0-T28, T0-T56, and T28-T56) using Wilcoxon signed-rank test.

Chapter 4:

Prebiotic effect of Maitake extract on a probiotic consortium, its action after microbial fermentation on colorectal cell lines, and its ability to modulate a synthetic in vitro reconstructed gut microbiota

This chapter focuses on the characterization of an extract from *Grifola frondosa* mushroom (known as Maitake) and on its prebiotic potential, tested on probiotic strains belonging to the *Lactobacillus* and *Bifidobacterium* genera, also combined as a probiotic consortium. The growth was monitored spectrophotometrically and compared with the one on a reference substrate. Then, the metabolites derived from the Maitake fermentation were analyzed and administered to healthy and tumoral colorectal cell lines. The results evidence the possibility to administer the Maitake extract as a new nutraceutical, with beneficial effects both for probiotics and the host. Furthermore, an *in vitro* reconstructed gut microbiota community was validated and used to test the modulation after a synbiotic intervention with Maitake extract as prebiotic.

4.1 Introduction

The nutritional and medicinal effects of mushrooms are recognized worldwide, in particular in Asian, Northern and Central American countries. Indeed, over 270 fungal species are identified for their biological activities such as anti-inflammatory, antimicrobial, and antioxidant properties (Sharifi-Rad et al., 2020). Nevertheless, an important role has always been recognized in the preservation of the healthy state of the gastrointestinal tract (Yamin et al., 2012). Accordingly, nowadays mushrooms are used not only in the pharmaceutical industry but also in the nutraceutical and cosmeceutical ones. This is due to the high protein and low-fat contents, as well as to the presence of several vitamins, and minerals (Sharif-Rad et al., 2020), but most of all it is due to the presence of important functional components such as dietary fibers, chitin, and glucans (Rodrigo et al., 2021). In particular, glucans are polysaccharides composed of chemically heterogeneous glucose molecules, classified in α- or β-glucans depending on the glycosidic linkage (Mallard et al., 2019). β-glucans are known for their beneficial effects in lowering blood pressure, reducing glycemia, acting as antitumoral and antioxidant agents. Furthermore, they could be used by the

intestinal microflora as prebiotics. Therefore, they are currently the most requested functional food (Yamin et al., 2012).

Among the mushrooms with health booster properties, there is *Grifola frondosa* (knows as Maitake, Hen of the wood, or Signorina mushroom) (He et al., 2017). It belongs to the Polyporaceae family (Pan et al., 2018), and it is characterized by caps with smoky brown color. Generally, it grows in temperate regions, such as Japan, Europe, northeastern states of America, and subtropical regions at high elevations (He et al., 2017). Regarding the chemical composition, Maitake is characterized by 3.8% water-soluble polysaccharides; among them, 13.2% corresponds to (1→3,1→6)-β-D-glucans (He et al., 2017). Typically, the water-soluble polysaccharides could be divided into two subpopulations based on the molecular weight, i.e., 722.7 kDa and 19.6 kDa (Su et al., 2016).

Other components are starch, oligofructose, fructooligosaccharides (FOS), lactulose, galactomannan, polydextrose, and dextrin (Jayachandran et al., 2017). Because of these characteristics, researchers are trying to combine the possible beneficial effects of the mushroom with the helpful action of probiotics. Generally, this type of fermentation is applied to several food ingredients because probiotics can enhance the nutritional value of food (Yang et al., 2015), and support positive health effects, as immune modulation, and maintenance of a state of eubiosis in the context of the gut microbiota composition. Furthermore, mushrooms themselves could improve the antioxidant condition through the modulation of the gut microbiota (Jayachandran et al., 2017). As a consequence, an important role in the status of the intestinal mucosal epithelial cells could be played by the released bacterial secondary metabolites generated by mushrooms fermentation, in particular the short-chain fatty acids (SCFAs) (Shuwen et al., 2019), including acetate, propionate, butyrate, and valerate (Kilner et al., 2016). In the presence of a medium-rich fiber diet, the SCFAs concentration in the intestine is around 10 mmol/L, so the intestinal epithelial cells are constantly exposed to these metabolites, mediating the crosstalk between the microbiota and the host (Zeng et al., 2017). It is known that a fiber-

rich diet could also prevent the development of colorectal cancer (CRC). Interestingly, especially the SCFA butyrate could act as an antitumoral agent, through the modulation of several transduction pathways, including the cellular proliferation one (Shuwen et al., 2019). For example, butyrate inhibited the proliferation of a colorectal tumoral cell line decreasing the presence of the phosphorylated extracellular-regulated kinase 1/2 (p-ERK 1/2), which is classified as a survival signal (Zeng et al., 2017).

In this study we analyzed a commercially available Maitake (*Grifola frondosa*) dried extract, characterized for its β-glucan content. Based on the specifics, we tested the prebiotic property on several probiotic strains, comprising both *Lactobacillus* and *Bifidobacterium* genera. Then, we studied a powerful probiotic consortium that, in the presence of Maitake preparation, released SCFAs. The fermentation products were then tested for the effects on the vitality of both healthy and tumoral colorectal cell lines. Then, the rescue of the viability of a cell line after induced stresses was evaluated. Finally, the possible modulation due to Maitake and probiotic administration of an *in vitro* reconstructed gut microbiota consortium was evaluated.

4.2 Materials and methods

4.2.1 Preparation of the Maitake extract

The Maitake (*Grifola frondosa* Dicks. Gray) extract was obtained from Amita HC Italia S.r.l. (Solaro, Italy). The original mushrooms came from China, and they were wild at the time of the manual collection (from August to November). Then, the sporophorum part was selected for the extraction of the polysaccharides.

The commercial Maitake dried extract was prepared as follows. The Maitake sporophora were first ground and weighted. Then, to have an extract enriched in polysaccharides, the obtained material was resuspended in a ethanol:water (20:80 ratio) solution and the separation was allowed. After overnight incubation, the precipitate was collected and then dried at 50°C to eliminate the ethanol.

Finally, the obtained extract was blended and sieved, to have a brownish fine powder characterized by a particle size less than 180 μm.

4.2.2 Characterization of the Maitake extract

4.2.2.1 Determination of the starch molecules content

The starch molecules content was estimated through the Megazyme kit K-TSHK (Megazyme Inc. IL. USA) as described by the manufacturer's instruction.

A starting weight of 100 mg of Maitake extract powder was used for the measurement. Then, 0.2 mL of aqueous ethanol (80% v/v) were added, and then stirred using a magnetic stirrer. 2 mL of 2M KOH were added and the solution was stirred for 20 minutes in an ice-water bath. Then 8 mL of 1.2M sodium acetate buffer (pH 3.8) were added, together with 0.1 mL of thermostable α-amylase (from Megazyme kit) and 0.1 mL of amyloglucosidase (20U, from Megazyme kit). All the contents were stirred and then incubated at 50˚C for 30 minutes. The obtained solution was centrifuges at 3000 rpm for 10 minutes and 0.1 mL of the supernatant were analyzed.

The calculations were made through Mega-Calc sheet (Megazyme Inc. IL. USA).

4.2.2.2 Determination of α- and β-glucans content

α- and β-glucans content was estimated through the Megazyme kit K-YBGL (Megazyme Inc. IL. USA) as described by the manufacturer's instruction. A starting weight of 100 mg of Maitake extract powder was used for the measurement. Then, 2 mL of ice-cold 2M KOH were added, and then stirred using a magnetic stirrer at 4°C for 20 minutes. 1.2M sodium acetate buffer was added, and then amyloglucosidase (1630 U/mL) plus invertase (500 U/mL) (200μL) (from Megazyme kit). All the contents were mixed and then incubated at 40˚C for 30 minutes. The obtained solution was centrifuged at 2000 rpm for 10 minutes and 0.1 mL of the supernatant was analyzed for the glucose presence. For the total glucan measurement, 100 mg of Maitake extract powder were used. 2 mL of ice-cold 12M sulfuric acid was added and then stirred. The

tubes were then placed at 4°C for 2 hours in agitation. Then 10 mL of water were added to each sample, which was placed in a hot water bath (100°C) for 2 hours. After cooling at room temperature, 6 mL of 10M KOH were added and the content well mixed. Then, the volume was adjusted to 100 mL with 200mM sodium acetate buffer at pH 5. 100 µL of the sample were incubated with 100 µL of a mixture of exo-1,3-β-glucanase (20 U/mL) plus β-glucosidase (4 U/mL) at 40˚C for 60 min, and the glucose was determined with GOPOD reagent (all the reagents used were in the Megazyme kit). Absorbance was measured at 510 nm. A 0.1 mL aliquot of 1 mg/mL glucose standard solution was incubated in triplicate with GOPOD reagent; and 0.1 mL of acetate buffer (200 mM, pH 5) was incubated with 3.0 mL of GOPOD reagent as blank sample. Finally, the β-glucan content was determined by subtracting the α-glucan content from the total glucan content.

The calculations were made through Mega-Calc sheet (Megazyme Inc. IL. USA).

4.2.2.3 Determination of polyphenol content

The total phenolic content of the Maitake extract was measured using the Folin-Ciocalteau phenol assay described by Singleton et al., 1999. A standard solution of Gallic Acid (GA), ranging from 0 to 100 µg/mL was used for the calibration. The GA solutions were prepared in 80% methanol, and the absorbance values were measured at 765 nm. For the quantification, 0.5 mL of Folin-Ciocalteau phenol reagent (1:10 dilution) and 1 mL of distilled water were added to 100 µL of mushroom sample. The solutions were mixed and incubated at room temperature for 1 min. Then, 1.5 mL of 20% sodium carbonate (Na_2CO_3) solution were added to the sample, and mixed. After the incubation for 120 min, absorbance was recorded at 765 nm against blank sample. Results were expressed as mg of Gallic Acid Equivalent (GAE)/g of Maitake powder. All the measures were done in triplicate.

4.2.2.4 Determination of protein content

The protein content of Maitake extract was determined according to Bradford method (Bradford et al., 1976). A calibration curve using bovine serum albumin as a standard was performed to determine the protein concentration of the extract.

4.2.2.5 Determination of fructans and reducing sugars content

The quantification of fructans in the Maitake extract powder was developed using the fructan assay procedure kit Megazyme (McCleary et al., 2000), accordingly to the manufacturer's instruction. The fructans concentration was calculated considering the fructose, glucose, and sucrose contents in the mushroom extract before and after the hydrolysis with fructanase. The samples were treated with a specific sucrase/maltase enzyme to completely hydrolyze saccharides to D-glucose and D-fructose. The reference values of the samples were determined by a direct analysis of D-glucose plus D-fructose using the hexokinase/phosphoglucose isomerase/glucose 6-phosphate dehydrogenase analytical procedure. The amount of NADPH formed in this reaction is stechiometric with the amount of D-glucose plus D-fructose. NADPH formation is measured by the increase in absorbance at 340 nm. The fructan content of the samples was determined after hydrolyzation to D-fructose and D-glucose by endo- and exo- inulinases, and then D-fructose and D-glucose content was measured as described above. The fructan content was determined by subtracting absorbance values of the reference from those of the sample. Before, each enzymatic assay samples were heated for 30 min at 50°C to ensure sample complete dissolution.

The calculations were made through Mega-Calc sheet (Megazyme Inc. IL. USA).

4.2.3 Bacterial strains and culture conditions

The bacterial strains used in this study are reported in Table 4.1. The *Lactobacillus* and *Bifidobacterium* strains were provided by a private collection

of the company Roelmi HPC (Origgio, Italy) and were previously selected and characterized for the probiotic features by Presti et al. 2015. Instead, the basal community strains were supplied by BEI Resources (USA).

The probiotic strains were routinely grown on MRS broth (Condalab.) supplemented with 0.03% L-cysteine (Sigma) for 48 hours. Instead, the basal community strains were normally grown on RCM broth (Condalab.), supplemented with 0.03% L-cysteine (Sigma) and 0.01 g/L of hemin (Sigma) (Gutierrez et al., 2019) for 72 hours. All the strains growth at 37°C, under anaerobic conditions using Anaerocult GasPack System (Merck, Germany).

A modified MRS medium (mMRS), as described by Watson et al. (2012), without glucose and supplemented with 0.03% L-cysteine, was used for the growth trials. The pH of the medium was adjusted to 6.8 before sterilization (121°C for 20 minutes). Maitake extract was added to mMRS at the concentration of 2% w/v as carbon source.

Strain	Source	Abbreviation
Lactobacillus acidophilus PBS066 (formerly DSM 24936)	Human	LA
Lactobacillus fermentum PBS073 (formerly DSM 25176)	Human	LF
Lactobacillus plantarum PBS067 (formerly DSM 24937)	Human	LP
Lactobacillus reuteri PBS072 (formerly DSM 25175)	Human	LR
Lactobacillus rhamnosus PBS070 (formerly DSM 25568)	Human	LRh
Bifidobacterium animalis spp. *lactis* PBS075 (formerly DSM 25566)	Human	BL
Bifidobacterium longum spp. *infantis* LMG P-29639	Human	BI
Bifidobacterium longum spp. *longum* PBS108 (formerly DSM 25174)	Human	BLg
Bacteroides cellulosilyticus CL02T12C19	Human	BC
Clostridium symbiosum WAL-14673	Human	CS
Clostridium orbiscindes 1_3_50AFAA (also referred to as *Flavonifractor plautii*)	Human	CO
Escherichia coli ATCC 25922	Human	EC

Table 4.1. Bacterial strains used in the study

4.2.4 Growth experiments with single probiotic strains on Maitake extract

The probiotic bacteria described in Table 1 were pre-inoculated in MRS broth for 48 hours, at 37°C in anaerobic condition before the setup of the prebiotic experiment. Maitake extract powder was added to mMRS to a final concentration of 2% w/v, and then together sterilized before inoculation.

1 mL of sterile mMRS or sterile mMRS plus Maitake extract were added to every well of a sterile multi-well (24 wells, SPL Lifesciences). Then each well was inoculated with a proper volume of each probiotic pre-inoculum (around 20 µL), to have a final optical density (OD) at 600 nm of 0.1. Subsequently, the plates were capped, and incubated in an anaerobic jar at 37°C for 48 hours. At the end of the experiment, the OD at 600 nm was measured.

4.2.5 Growth experiments with mixed probiotic strains as consortium on Maitake extract

The probiotic bacteria described in Table 1 were pre-inoculated in MRS broth for 48 hours at 37°C in anaerobic condition before the setup of the experiment. Maitake extract powder was added to mMRS to a final concentration of 2% w/v, and then together sterilized before inoculation.

The consortium was prepared in a sterile tube, mixing the selected probiotic to have an OD at 600 nm of 0.1. After the homogenization, the proper volume of the consortium (around 200 µL) was inoculated in sterile tubes (Falcon) with a final volume of 10 mL. Each tube contained only mMRS as control, or mMRS plus Maitake extract. After the inoculum, the tubes were placed in an anaerobic jar, and then incubated for 48 hours at 37°C. The growth was evaluated as optical density at 600 nm.

4.2.6 Growth experiments with basal community strains and probiotics in batch fermentation

The basal community strains described in Table 1 were pre-inoculated in RCM broth for 72 hours at 37°C in anaerobic condition before the setup of the experiment. Instead, *L. plantarum* PBS067, *L. acidophilus* PBS066, and *B. animalis* spp. *lactis* PBS075 (described in Table 1) were pre-inoculated in MRS broth for 48 hours at 37°C in anaerobic condition.

FOS (with 3<DP<5 and DP~10, mixed 1:1; Farcoderma s.r.l, Italy) or Maitake extract powder were added to mMRS to a final concentration of 2% w/v, and then together sterilized before inoculation.

400mL of sterile mMRS, or sterile mMRS plus FOS, or sterile mMRS plus Maitake extract were added to a batch reactor and allowed to become anaerobic blowing nitrogen for at least 8 hours. Then the medium was inoculated with a proper volume of each basal community strain or probiotic strain pre-inocula (around 3 mL), to have an OD_{600nm} of 0.1 for each strain as starting cultures. Subsequently, the reactor was closed, set at 37°C, and nitrogen was blown until the end of the experiment. The OD_{600nm}, the modulation of the single strain abundance (through qPCR analysis), and the production of SCFAs were recorder at time 0 hours (T0), 8 hours (T8), 16 hours (T16), 24 hours (T24), 32 hours (T32), and 48 hours (T48).

4.2.7 Extraction and characterization of fermentation secondary metabolites

4.2.7.1 Extraction of the metabolites

The possible produced short-chain fatty acids (SCFAs) after FOS or Maitake fermentation were extracted from inoculated and uninoculated media at the end of the fermentation using ethyl-acetate (anhydrous, 99.8%, Sigma). Tubes containing 10 mL of broth culture were centrifuged at 7000 rpm for 10 minutes at room temperature, to separate the cell pellet from the supernatant. In a glass

tube (Colaver), 5 mL of the supernatant were acidified up to pH 2 with HCl 6M. Then, 5 mL of ethyl-acetate were added, followed by 20 minutes of strong manual agitation. The obtained suspension was centrifuged at 4000 rpm for 20 minutes, and the organic phase was withdrawn, and conserved in a new glass tube. Other 5 mL of ethyl-acetate were added to the remaining broth, followed by 5 minutes of strong manual agitation. The suspension was again centrifuged, and the organic phase collected and pooled with the one obtained in the first extraction.

4.2.7.2 Analysis of the extracted metabolites

The extracted organic phase was submitted to derivatization with N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA, Sigma) before the Gas Chromatography (GC) injection. The samples and BSTFA were mixed in ratio 3:1 and the reaction allowed at 60°C for 20 minutes. After the cooling down of the temperature, SCFAs were analyzed by a GC-MSD instrument, using a Technologies 6890 N Network GC System, interfaced with 5973 Network Mass Selective Detector (MSD) (Agilent Technologies). A ZB-5MS capillary column was used (5% diphenyl-95% dimethylpolysiloxane 60 m x 0.25 mm, 0.25 µm; Alltech). Analyses were developed in split-less injection mode, using helium at 99.99% as carrier gas (Sapio). The program for the hoven was set as 65°C for 2 minutes, then 5°C min^{-1} to 110°C, then 12°C min^{-1} to 260°C, holding this temperature for 10 minutes. Electron impact ionization spectra were obtained at 70 eV, with recording of specific mass spectra at 73, 75, 117, 129, 132, 145, 159, 171, 173, 187, 201, 215, 229, 243, and 257 m/z. All the analyses were carried out in triplicate.

The registered mass spectra were compared with those of the library of National Institute of Standards and Technology (NIST) of the instrument.

4.2.9 DNA extraction from the synthetic in vitro reconstructed gut microbiota

To set up standard curves of DNA extracted from the probiotic and the basal community strains, microbial cultures were performed in MRS or RCM medium (Condalab, Italy), respectively; and incubated at 37°C for 24 or 48 hours in anaerobic conditions using anaerobic atmosphere generation bags for microbiology (Sigma-Aldrich). For the probiotic strains a supplementation of 0.3 g/L of L-cysteine hydrochloride monohydrate (Sigma-Aldrich) was included in the growth medium (c-MRS), while for the basal community strains, also 0.01 g/L of hemin (Sigma) were added.

DNA from probiotic bacteria cultures was extracted by Ultraclean Microbial DNA Isolation Kit (MoBIO Laboratories, Italy). A total of 1.8 mL of 10^9 CFU/mL culture was used for obtaining genomic DNA following the protocol reported by Mezzasalma et al. (Mezzasalma et al., 2016).

DNA from the strain composing the basal community was extracted using Stool Nucleic Acid Isolation Kit (Norgen Biotek Corp., Canada) following the protocol provided by the manufacturer with some modifications. Briefly, to 400 µL of the bacterial culture were added to 600 µL of Lysis Buffer, vortexed for 5 minutes, and centrifuged for 4 minutes at room temperature. For the final elution of the nucleic acid sample, 50 µL and then other 50 µL (total volume of 100 µL of final sample) of Elution Buffer were used. A total of 10^8 CFU/mL culture was used for obtaining the genomic DNA.

Both DNA extracted from probiotic and basal community strain cultures were utilized to perform qPCR.

4.2.10 Monitoring of the modulation of the strain abundances of the in vitro reconstructed gut microbiota through qPCR

qPCR reactions were conducted using PCR Real-Time StepOne Plus (Applied Biosystems) and the PowerUp SYBR Green Master Mix (Applied Biosystems). Some species-specific primer sets were developed by the authors in a previous work (Manfredi, 2015), other are designed for the experiment. All are reported in Table S6. Reactions were carried out in a 10 µL qPCR mix containing 5.6 µL of PowerUp SYBR Green Master Mix, the Forward (10µM) and the Reverse primer (10µM) and 4.4 µL of DNA template. Different qPCR programs were employed. For DNA amplification of *L. plantarum*, *L. acidophilus*, *B. animalis* spp. *lactis*, and *E. coli*, the program was: 10 minutes 95°C and 40 cycles of 15 seconds 95°C and 1 minute 60°C (followed by a dissociation step) (Manfredi, 2015). For *C. symbiosum* and *C. orbiscindens* the program was: 10 seconds 95°C, followed by 40 cycles of 5 seconds 95°C and 30 seconds 60°C (followed by a dissociation step) (adapted from Ogita et al., 2020). Finally, to amplify the DNA from *B. cellulosilyticus* 5 minutes 95°C, followed by 40 cycles of 10 seconds 95°C, 20 seconds 65°C, and 15 seconds at 72°C (followed by a dissociation step) (adapted from Christensen et al., 2020).

For each strain, standard curves were constructed using DNA extracted from microbial cultures using ten-fold dilutions ranging from 10^8 or 10^7 CFU/mL to 10 CFU/mL. Each DNA sample was analyzed in triplicate.

Resulted data on the quantification were expressed as mean values ± SEM. The average slope and *y*-intercept of each standard curve were determined by regression analysis and used to calculate the cell number for each bacterial target.

4.2.11 Maintenance and growth of cell lines for in vitro tests

The healthy mucosa cell line CCD841 (ATCC CRL-1790) and the colon cancer cell line CACO-2 (ATCC HTB-37) were grown in EMEM medium supplemented with heat-inactivated 10% FBS, 2mM l-glutamine, 1% nonessential amino acids, 100 U/mL penicillin, 100 µg/mL streptomycin and maintained at 37°C in a humidified 5% CO_2 incubator; the colon cancer cell line HT-29 (ATCC HTB-38) was grown in DMEM medium supplemented with heat-inactivated 10% FBS, 2mM l-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin and maintained at 37°C in a humidified 5% CO_2 incubator.

ATCC cell lines were validated by short tandem repeat profiles that are generated by simultaneous amplification of multiple short tandem repeat loci and amelogenin (for gender identification).

All the reagents for cell cultures were supplied by EuroClone (EuroClone S.p.A, Italy).

4.2.12 Cell viability assay and test with stress agents

Cells were seeded in a 96-well microtiter plates at a density of $1x10^4$ cells/well and incubated for 24 h.

To evaluate the effect of the extracts on CCD841, CACO-2 and HT-29 viability, the cells were treated for 24 h with 0.25, 0.5 and 1 mg/ml for each extract.

HT-29 cell line was treated with stressor compounds, H_2O_2 (0-8mM) or SDS (0-0.1%), for 24 h to determine the concentration responsible for about 50% reduction in cell viability. To evaluate a potential role of the extract in cell viability rescue, cells were pre-treated for 1 hour with 0.25, 0.5 and 1 mg/ml for each extract, and then 1mM H_2O_2 or 0.0075% SDS were added to the cells with a 24 hours endpoint. Following the treatment, cell viability was assessed using an in vitro MTT based toxicology assay kit (Sigma, USA): the medium was replaced with a complete medium without phenol red, and 10 µL of 5 mg/mL MTT (3-(4,5-dimethylthiazol-2)-2,5-diphenyltetrazolium bromide) solution was added to each well; after 4 hours of incubation, formed formazan crystals were solubilized with

10% Triton-X-100 in acidic isopropanol (0.1N HCl) and absorbance was measured at 570 nm using a micro plate reader. Results were expressed as mean values ± ES of at least three independent experiments.

4.2.13 Statistical analysis

Regarding the bacterial growths, all the experiments were performed in triplicate and results were presented as mean values ± standard deviation. The statistical relevance of the results was assessed by t-Student's test. The significance was defined as *p*-value <0.01 or *p*-value <0.05.

Regarding the experiments with the intestinal cell lines, all the experiments were performed in triplicate. The results were shown as mean value of vitality % ± standard error. Statistical differences were calculated using Dunnett's multiple comparisons test: * *p*-value <0.05, ** *p*-value <0.01, *** *p*-value <0.001.

4.3 Results

4.3.1 Characterization of the principal components of Maitake extract

The determination of the main components of the Maitake extract was evaluated by enzymatic assays using Megazyme methods as described in materials and methods. The major components of the mushroom preparation are starch (around 50% w/w), and glucans (around 25% w/w, comprising α- and β-glucans). Interestingly, the presence of α-glucans is very low with respect to the β-ones, which are dominant in the extract (around 6.2% *vs* 18.8%, respectively). Furthermore, Maitake extract was also analyzed for its content of polyphenols and protein by chemical reactions. Each extract preparation contained at least 1.9% w/w of polyphenols and 0.02% w/w of proteins. Other components are sugars; indeed the extract is characterized by 1.2% w/w of fructans and 3.6% w/w of free reducing sugars. The content in glucans, fructans, free reducing sugars, starch and polyphenols and protein is listed in Table 4.2.

	Maitake extract (%)
Starch	49.5
Total glucans	25.0
Aplha-glucans	6.2
Beta-glucans	18.8
Proteins	0.02
Polyphenols	1.9
Fructans	1.2
Reducing sugars	3.6

Table 4.2. Composition of the Maitake extract used in this study.

4.3.2 Prebiotic potential of Maitake extract on Lactobacillus and Bifidobacterium strains

The possible prebiotic capability of Maitake extract at a concentration of 2% w/v was evaluated through *in vitro* growth assays employing *Lactobacillus* and *Bifidobacterium* strains, originally isolated from the human colon (Presti et al., 2015). The initial OD_{600nm} of each culture was 0.1, then it was recorded at the end of the experiment, after 48 hours of anaerobic fermentation (Figure 4.1). The medium containing all except the extract was used as a control. As shown in figure 1, the mushroom preparation could be considered as a prebiotic substrate for the probiotic bacteria, because all the tested strains were able to ferment it and the difference between the growth in the control condition and the growth on the prebiotic is statistically significant (p-value <0.01). All the *Lactobacillus* strains showed high growth values (p-value <0.01). Among them, *L. fermentum* and *L. reuteri* showed the most positive responses. While, regarding the Bifidobacteria members, also in this case all the strains showed an important growth increase on Maitake extract (p-value <0.01), in particular *B. lactis*. The results highlight the prebiotic characteristic of the mushroom formulation.

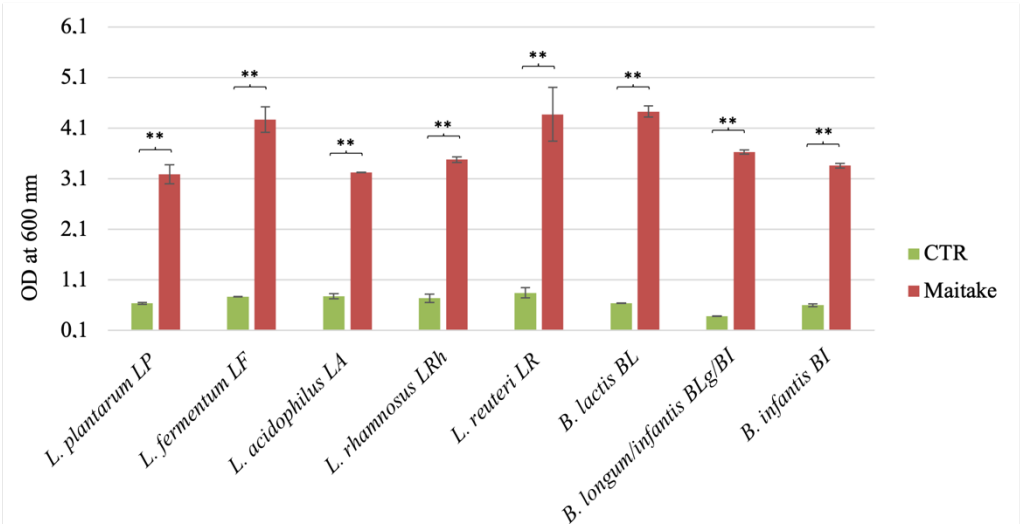


Figure 4.1. The figure represents the growth of the selected *Lactobacillus* and *Bifidobacterium* strains in presence of CTR and Maitake extract at a concentration of 2% (w/v). Values are represented as mean value of OD at 600nm ± standard deviation. Statistical differences were calculated using t-Student's test: ** *p*-value <0.01.

4.3.3 Growth of the probiotic consortium on Maitake extract

To enhance the possible beneficial effects for the human host attributable both to probiotics and prebiotics, a possible combination of the bacteria was studied. All the probiotic strains previously used were inoculated at OD_{600nm} equal to 0.1, making sure to have a homogeneous suspension of all the considered bacteria. The growth capacity was evaluated on the same conditions used for the experiment with single strains, i.e., control medium and Maitake extract 2% w/v. After 48 hours of anaerobic growth at 37°C, the optical density was measured (Figure 4.2). Interestingly, the final OD on the mushroom preparation had a mean value of 3.7±0.21. The prebiotic potential of Maitake extract is confirmed also in this kind of experiment, because of the very significant difference between the growth on the sole medium and the one on the medium added with Maitake extract (*p*-value <0.01).

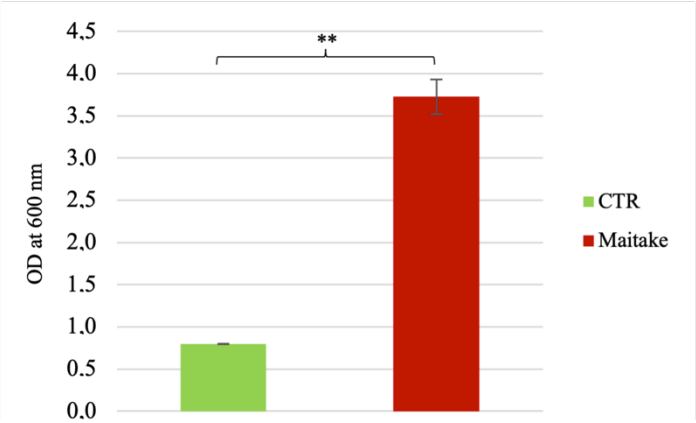


Figure 4.2. The figure represents the growth of the probiotic consortium in presence of CTR and Maitake extract at a concentration of 2% (w/v). Values are represented as mean value of OD at 600nm ± standard deviation. Statistical differences were calculated using t-Student's test: ** *p*-value <0.01.

4.3.4 Extraction and characterization of probiotic consortium secondary metabolites

The production of potentially beneficial secondary metabolites derived from the fermentation of complex carbohydrates by probiotics is well documented. They are recognized as short-chain fatty acids (SCFAs) and as branched-chain fatty acids (BCFAs) (Krautkramer et al., 2021).

To investigate which compounds are produced by the probiotic combination after the Maitake extract fermentation, a liquid-liquid extraction with ethyl-acetate of the broth culture after 48 hours of anaerobic fermentation was initially conducted. Then, a gas-chromatography analysis was performed, and the chromatograms were interpreted by mass spectrometry. Each peak was compared to the example present in the NIST library.

In comparison with the control condition, samples deriving from fermented Maitake extract presented several additional peaks (Figure 4.3). The highest peak, at a retention time of 8.2 min, is referred to lactic acid. The second most important peak is at 12.2 min of retention time, and it is assigned to valeric acid. The third highest peak is the one referred to succinic acid (retention time of 13.6 min). Other detected molecules were the SCFA butyrate (retention time of 10.8 min), and hydrocinnamic acid (retention time of 16.8 min) which is not a bacterial

secondary metabolite, but a Maitake component (Anwar et al., 2018) probably released thanks to probiotic digestion, and the cinnamic acid (retention time of 19.9 min).

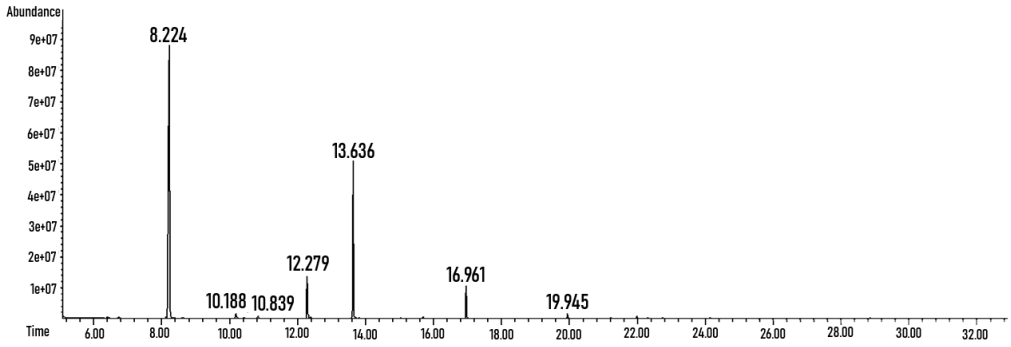


Figure 4.3. The Figure represents the chromatogram obtained after GC-MSD analysis. The probiotic consortium was incubated for 48 hours on Maitake extract at a concentration of 2% (w/v). The cultural broth was extracted and then analyzed in GC-MSD. The intermediate metabolites identified are reported in the graph.

4.3.5 Differential effects of extracted secondary metabolites on cell viability

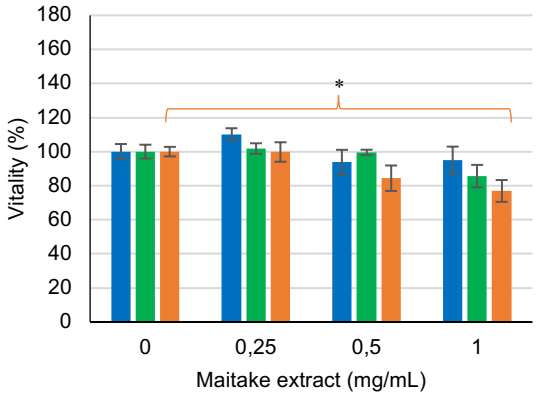
To understand the possible role of the previously characterized extracted secondary metabolites, viability assays on both healthy and tumoral cell lines were developed.

The healthy mucosa cell line CCD841 was not affected by the treatment with the metabolites (figure 4.4), except for the fermented Maitake extract, responsible for a 20% reduction in cell viability at the highest concentration analysed (figure 4.4B).

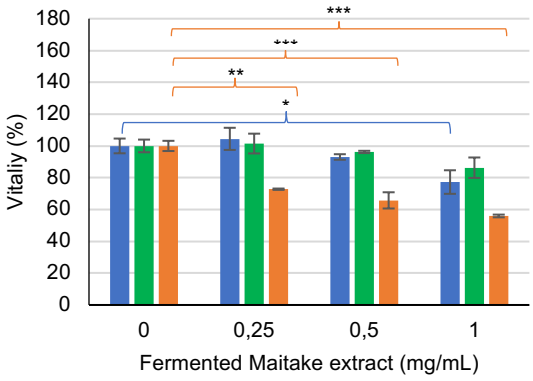
Regarding the two colorectal cancer cell lines, no variation in vitality was observed in the HT-29 cell line (Figure 4.4), while the CACO-2 cell line behaved quite differently. In fact, in this cell line, the viability showed about 20% reduction at a Maitake extract concentration of 1 mg/mL (figure 4.4A), as well as after exposure to fermented probiotic basal medium extract (figure 4.4C). The treatment with the Maitake-probiotic fermented extract showed a significant

dose-dependent decrease in cell viability already detectable at the lowest dose (Figure 4.4B).

Panel A



Panel B



Panel C

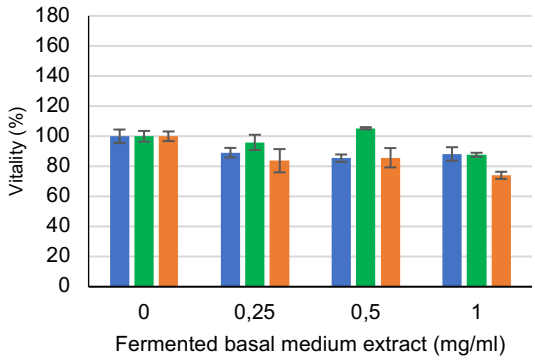


Figure 4.4. The figure represents the viability of the three different cell lines in presence of Maitake extract (A), fermented Maitake extract (B), and fermented control medium extract (C). Values are represented as mean value of vitality % ± standard error. Statistical differences were calculated using Dunnett's multiple comparisons test: * p-value <0.05, ** p-value <0.01, *** p-value <0.001. In Blu CCD841, in Green HT-29, and in Orange CACO-2 cell lines

4.3.6 Protective effect of Maitake-probiotic fermented extract on HT-29 cell line

Since the HT-29 cell line represents a valuable and complementary tool for the study of food digestion and the effect of food components on the gut (Martínez-Maqueda et al., 2015), the evaluation of a potential protective effect exerted by the extracts in stress conditions was conducted on this cell line.

Firstly, the cell survival rate in the presence of several stressors at different concentrations was investigated (data not shown). In the presence of either 1mM H_2O_2 or 0.0075% SDS, cell viability compared to control was found to be 42.97±5.49% and 56.13±3.07%, respectively. Subsequently, the co-treatment with the stressor compounds and the different extracts at the established concentrations demonstrated how only the Maitake-probiotic fermented one protects the cells from H_2O_2 challenge (Figure 4.5A), but not from SDS stress (Figure 4.5B). Furthermore, the protective effect was evidenced only at the low-middle doses (Figure 4.5).

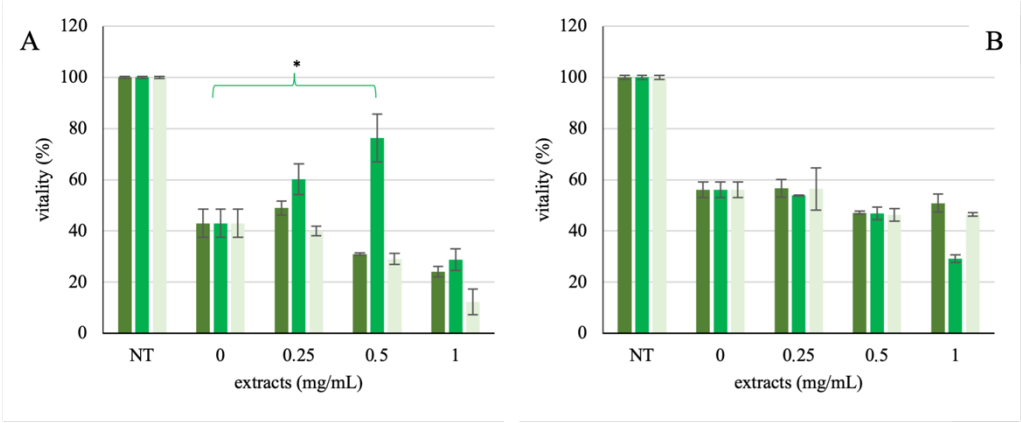


Figure 4.5. The figure represents the viability rescue of the HT-29 cell line stressed with H_2O_2 (A) or SDS (B) in presence of Maitake extract (Dark green), probiotic-fermented Maitake extract (Green), and fermented basal medium extract (Light green). Values are represented as mean value of vitality % ± standard error. Statistical differences were calculated using Dunnett's multiple comparisons test: * *p*-value <0.05.

4.3.7 Validation of the synthetic in vitro reconstructed gut microbiota

The *in vitro* gut microbiota seems to be the future for the study of the prebiotic potential of new molecules. In literature are reported different setup, based on different technologies (Elzinga et al., 2019; Nissen et al. 2020). As Gutierrez et al. (2019), we reconstruct a synthetic gut microbiota community composed by the strains reported in Table 1. To validate the model, the synbiotic intervention (described in chapter 3) was used. Therefore, to the basal mMRS medium, 2% of FOS were added as prebiotcs, and then *L. plantarum* PBS067 *L. acidophilus* PBS066 and *B. animalis* spp. *lactis* PBS065 as probiotics. At different time-points, the growth was monitored as $OD_{600nm,}$ and growth curves were plotted. As shown by figure 4.6, probiotic bacteria reached high OD values after 16 hours of fermentation, then they were quite stable. The highest growth was reached mimicking the synbiotic intervention after 32 hours of fermentation, maybe due to the utilization of the FOS by the probiotics. Finally, FOS are not so fermented by the basal community strains. Therefore, we can speculate that the FOS acted as prebiotic, because of the enhancement of the growth of the probiotics.

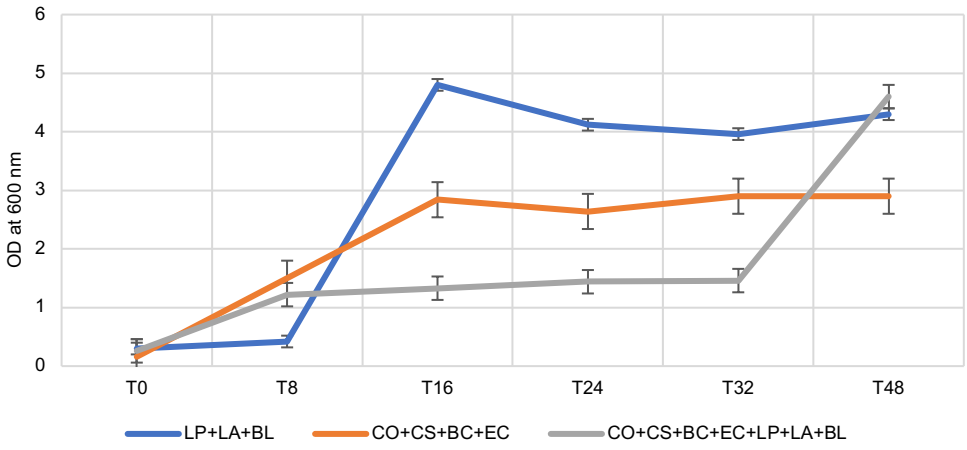


Figure 4.6. The figure represents the growth profile of only probiotic strains on FOS 2%, only basal community strains on FOS 2%, and basal community strains + synbiotic intervention. Data are represented as mean OD_{600nm} values ± standard deviation.

To further validate the model and to deep understand the modulation of the microbial community, qPCR analysis with species-specific primers were carried out. As showed in figure 4.7, at the end of the experiment (T48) the community was stable, while the highest OD value was principally due to the exponential growth of *B. animalis* spp. *lactis*, that reached the value of 10^{12} CFU/mL. The modulations represented as percentages considering the whole community are reported in the supplemental material section (Figure S1).

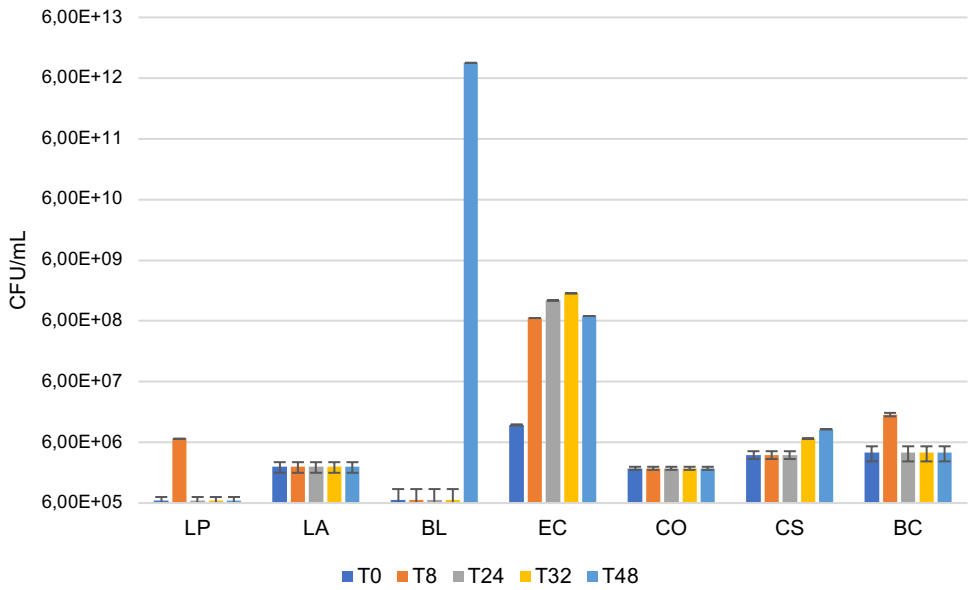


Figure 4.7. Quantification of cell numbers of probiotic and basal community strains in the batch fermentation model of synthetic gut microbiota by species-specific qPCR at different time-points (T0, T8, T24, T32 and T48). Data are expressed as the mean values of CFU/mL ± SEM.

Finally, to have a further confirmation of the goodness of the model, the secondary metabolites deriving from FOS fermentation were extracted and identified thanks to the comparison with the NIST library. As showed by figure 4.8, two main pecks were detected, corresponding to the SCFAs propionic acid or lactic acid (retention time of 8.2 min) and succinic acid (retention time of 13.6 min).

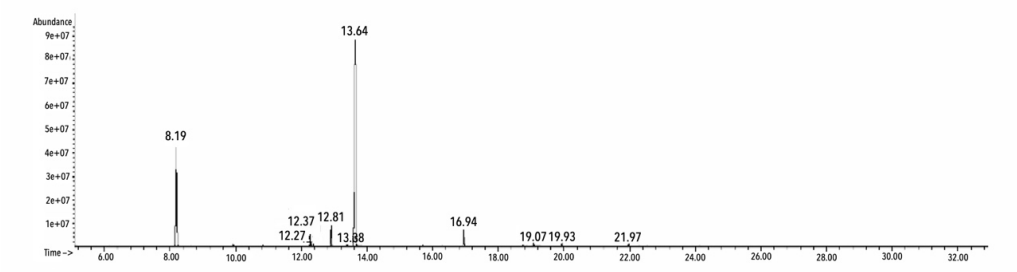


Figure 4.8. The Figure represents the chromatogram obtained after GC-MSD analysis. The intervention with the synbiotic was mimicked for 48 hours of incubation with the basal community. The broth culture was extracted and then analyzed in GC-MSD. The extracted metabolites identified are reported in the graph.

4.3.8 Synthetic in vitro gut microbiota modulation by Maitake extract

The same experiment conducted for the validation of the *in vitro* model was performed using Maitake extract at 2% w/v instead of FOS. The synbiotic intervention was mimicked using the same probiotic stains.

Figure 4.9 shows the growth profiles, that are quite similar. Nevertheless, the basal community strains alone reached a higher OD value with respect to the probiotics, maybe due to the presence of simple sugars and starches (Table 4.2).

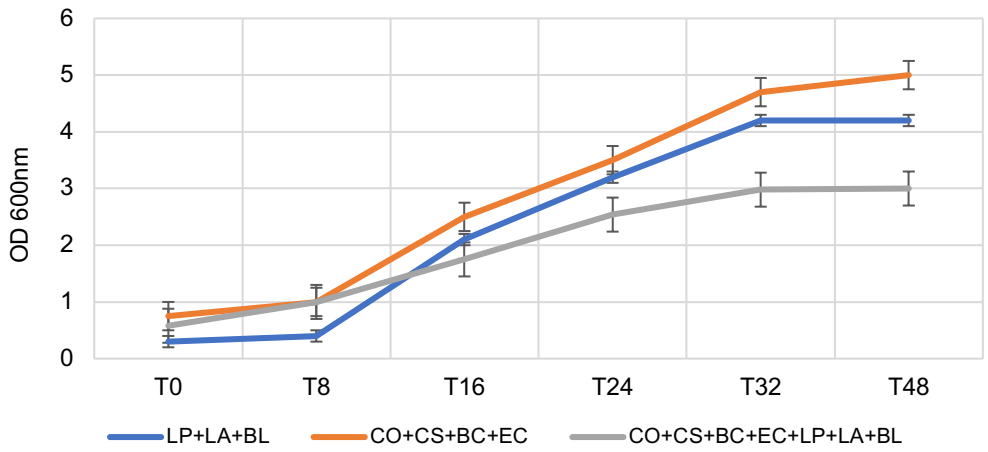


Figure 4.9. The figure represents the growth profile of only probiotic strains on Maitake extract 2%, only basal community strains on Maitake extract 2%, and basal community strains + synbiotic intervention with Maitake extract. Data are represented as mean OD_{600nm} values ± standard deviation.

Looking at the single modulation of the strains, the basal community strains showed a stable trend, except for *E. coli*, that reached a value of 10^9 CFU/mL at the end of the fermentation period. Nevertheless, the prebiotic potential of Maitake extract was confirmed sustaining *L. plantarum* growth until around 10^8 CFU/mL (Figure 4.10). The modulations represented as percentages considering the whole community are reported in the supplemental material section (Figure S2).

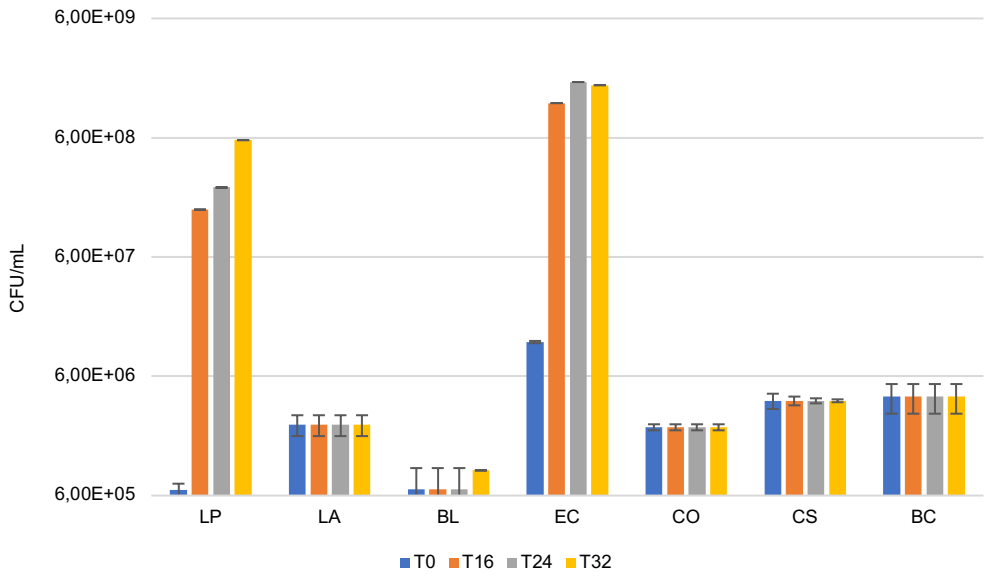


Figure 4.10. Quantification of cell numbers of probiotic and basal community strains in the batch fermentation model of synthetic gut microbiota by species-specific qPCR at different time-points (T0, T16, T24, and T32). Data are expressed as the mean values of CFU/mL ± SEM.

Then, the secondary metabolites produced after Maitake fermentation in presence of the basal community strains and of the probiotic bacteria were extracted and characterized. The chromatogram in figure 4.11 showed at a retention time of 8.2 min a peck assigned to propionic acid or lactic acid, both SCFAs; at 12.92 min quinoline, that could be a component of the Maitake extract. Finally, at a retention time of 13.64 min succinic acid, a bacterial secondary metabolite.

Therefore, the results showed that the synthetic gut microbiota community could be used as a tool to test the efficacy of a pre- and pro- biotic intervention, due to the possibility to monitor the modulation of the community and the production of secondary metabolites, such as SCFAs.

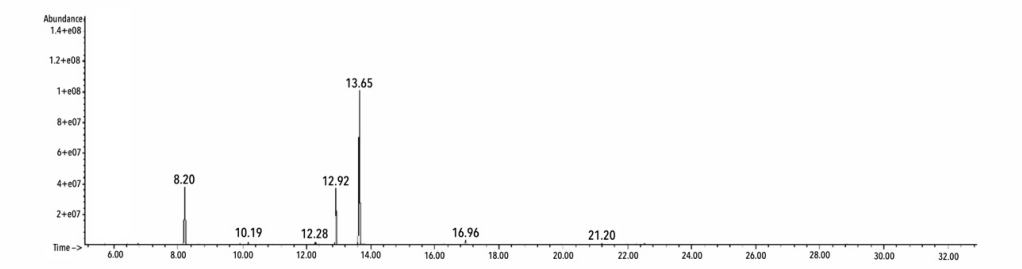


Figure 4.11. The Figure represents the chromatogram obtained after GC-MSD analysis. The intervention with the synbiotic was mimicked for 32 hours of incubation with the basal community. The broth culture was extracted and then analyzed in GC-MSD. The extracted metabolites identified are reported in the graph.

4.4 Discussion

Extracts deriving from vegetables naturally contain polysaccharides, that are characterized by different chemical compositions (Barbosa et al., 2021). These molecules can be extracted and employed as nutraceuticals, leading to several beneficial effects for the host and its gut microbiome. In particular, mushrooms are known for their high content of polysaccharides with antioxidant and immunomodulatory potential (Barbosa et al., 2021). Among the most interesting polysaccharides, there are glucans, which are a group of chemically heterogeneous glucose molecules, classified as α- or β-glucans based on the glycosidic linkage (Mallard et al., 2019). The Maitake extract tested in this study contains around 25% of total glucans, in line with Mallard et al., 2019 study. Interestingly, in the Maitake extract analyzed in this study, the presence of α-glucans is very low with respect to the β- ones (around 6.2% *vs* 18.8%, respectively), and this is in accordance with the literature. Indeed, high levels of α-glucans are not naturally present in mushrooms (Mallard et al., 2019). Moreover, β-glucans are not cleaved by the mammalian digestive enzymes (Rossi et al., 2018), so they can reach the intestine and be used as carbon and energy sources, as well as metabolized to other compounds by the resident microbiota. Therefore, to be considered a prebiotic, the Maitake preparation has to benefit the host also by the selective stimulation of the growth of a limited

number of bacteria in the colon (Gibson and Roberfroid, 1995), i.e., the probiotics. For this reason, we tested the potential prebiotic effect on different *Lactobacillus* and *Bifidobacteria* strains, previously characterized for their probiotic features (Presti et al., 2015). All the tested strains were able to ferment the Maitake preparation at a concentration of 2% w/v, reaching a statistically significant growth difference with respect to the control. The maximum growth values on Maitake are similar to the ones of known prebiotics, i.e., inulin or fructooligosaccharides (FOS). For example, the growth values of the same probiotic strains on commercial FOS with a degree of polymerization comprised between 3 and 5 at a concentration of 2% w/v (Zeaiter et al., 2019; see also chapter 2) are similar to the ones obtained in this study, after 48 hours of Maitake extract fermentation at the same concentration, that are around an optical density at 600 nm of 4. Therefore, the Maitake extract could be considered a prebiotic molecule, in line with literature data. Indeed, Nowak et al., 2018 studied the possible prebiotic effect of polysaccharides extracted from 53 wild-growing different mushrooms on *L. acidophilus* (used as reference strain) and *L. rhamnosus*. The extracted fungal molecules could stimulate the Lactobacilli growth more than commercially available prebiotics, like inulin and FOS from chicory. Also, Bifidobacteria could benefit from the presence of β-glucans in the growth medium, as reported by Lam et al., 2013 in the reconstruction of the catabolic pathway of *B. longum* subsp. *infantis* strain.

Generally, probiotics are provided as a unique individual strain. However, multi-strain formulations could boost the beneficial effects for the host, compared to the single-strain products (Belguesmia et al., 2019). For this reason, some probiotic strains (5 different *Lactobacillus* and 3 different Bifidobacteria) were combined and tested, confirming again the prebiotic potential of the Maitake preparation. Furthermore, the metabolites deriving from the fermentation of the mushroom preparation by the consortium were characterized for their possible impact on the host intestinal cells. This is a new concept reported in literature as “metabiotics” (Lam et al., 2013), i.e., signaling molecules with a determined

chemical structure that can optimize host-specific physiological functions and regulate metabolic and behavior reactions (Shenderov et al., 2012). Indeed, the production of potentially beneficial secondary metabolites deriving from probiotics fermentation on different carbon sources as complex carbohydrates, is well known (Krautkramer et al., 2021). These released molecules are commonly recognized as short-chain fatty acids (SCFAs) and include acetate, propionate, and butyrate, which comprise more than 95% of the total SCFA pool. Less abundant released metabolites are the branched-chain fatty acids (BCFAs), like isobutyrate, 2-methylbutyrate, isovalerate, lactate, and succinate (intermediates of the propionate), which can also drive several effects on the host (Krautkramer et al., 2021). From the consortium broth culture, lactic acid, valeric acid and butyrate were obtained after liquid extraction. In a gut microbiome ecosystem, the bacteria that catabolize specific molecules to simpler ones, which could be fermented and result in acidic molecules, as acetate or lactate, are classified as primary degraders. Then, the secondary fermenters could use the secondary metabolites from the primary degraders to produce other end products, like butyrate (Baxter et al., 2019). Among the community, Bifidobacteria are interesting because they promote butyrate release by other community members due to the production of acetate and lactate through the characteristic Bifid shunt (Baxter et al., 2019). Nevertheless, the microbiota could ferment proteins, which represent the 0.02% w/w of the Maitake extract, resulting in BCFAs production (Krautkramer et al., 2021), and it could liberate and modify bioactive polyphenols, as the detected cinnamic and hydrocinnamic acids are. These molecules drive a physiological response in the host despite the low availability thanks to the metabolism of the original complex molecules by the gut microbiota (Krautkramer et al., 2021).

In general, in literature, the beneficial effects for the host of all these secondary metabolites are largely discussed. Indeed, lactic acid promotes the balance of intestinal pH (Belguesmia et al., 2019); while butyrate also modulates the immune system, as well as the strength the epithelial barrier, and could be

protective against colorectal cancer (Baxter et al., 2019). Therefore, drive the microbiota through a beneficial composition could be important for the host's health. However, considering the intestinal system, the first barrier of the human body in contact with the lumen is composed by epithelial cells. Therefore, the extracted and characterized metabolites after the probiotic fermentation of the Maitake preparation were tested on different healthy and tumoral colorectal cell lines. Among the extract only the probiotic fermented-Maitake one caused a significative dose-dependent reduction in CACO-2 cell line, with the HT-29 and CCD841 not affected at 0.25 and 0.5 mg/mL doses; while at the highest concentration used also the healthy one showed a slight reduction. These results are in accordance with the paper by Zeng et al. (2017), in which the authors demonstrated the inhibitory effect of butyrate on cancerous cell lines. The different responses of HT-29 and CACO-2 to the Maitake-probiotic fermented extract treatment could be due to a different mutational status on the downstream EGFR target BRAF. In fact, HT-29 cells present a hyperactivating mutation in BRAF, responsible for constitutive ERK1/2 phosphorylation, as shown in the paper by Bovio et al. (2020).

Consequently, the human colon adenocarcinoma cell line HT-29 was selected for further studies. Indeed, the cell line is a good model for studies on food digestion and bioavailability because the cells can form a monolayer characterized by tight junctions and the typical apical brush border, representative of mature intestinal cells (Martínez-Maqueda et al., 2015). Thus, a possible protective effect due to the probiotic secondary metabolites in different stress conditions was evaluated. As results showed (figure 5), the protection is principally against the H_2O_2 challenge, because HT-29 vitality recovers up to about 80%. This is important, because the formation of reactive oxygen species (ROS) is common in human organs, as a result of the oxidative processes, however, they are precursors of systemic cells and tissue damage (Anwar et al., 2018). Generally, the human body has an endogenous defence

system against these free radicals, however, it can be supported and potentiate by supplemented antioxidants (Anwar et al., 2018).

Finally, the validated synthetic *in vitro* gut microbiota could be used as a screening tool for the prebiotic potential of new molecules and new natural extracts. First, the model was validated mimicking the synbiotic intervention described in chapter 3. As expected, in the presence of probiotic bacteria, a high growth was registered, as well as the presence of lactic acid as secondary metabolite. Regarding single strains modulation, also the important growth of *B. animalis* spp. *lactis* was hypnotized, because of the bifidogenic effect of FOS. Instead, regarding the experiment on Maitake extract, the greater growth of the basal community than the probiotic strains was quite surprising. A reason to explain the data could be the presence of starches (Table 1), that could not be used by the probiotics. Nevertheless, the probiotic strains reached the same OD value registered in the experiment of the probiotic consortium (Figure 2), suggesting that the model worked also in this kind of experiment.

In literature there are few models based on selected basal community strains challenged with potential prebiotics and probiotic strains. For example, Gutierrez et al., 2019 studied the dependency of the reconstructed community in presence or absence of selected bacteria, removing one strain at time from the community. They emphasized that the exponential phase of community growth is between 4 and 6 hours of incubation, and that the stationary phase is reached between 12 and 14 hours. However, in our experiment we saw that from 8 hours surely the growth has begun, especially on FOS, while the onset of the stationary phase could be around 32 hours of fermentation. This may be due to the presence of fewer strains than Gutierrez et al. Furthermore, it is reported that *C. symbiosum*, which is a producer of butyrate, appears to be the most sensitive to the lack of primary degraders, but it is stimulated by the presence of *E. coli*, *B. adolescentis*, *Bacteroides dorei* and *L. plantarum*. This is also found in our experiment, as in the conditions in which all seven strains are present, the greatest growth of *C. symbiosum* on FOS is noted. The growth was detectable also at the level of

metabolite analysis, as butyrate is present (with a retention time of 12 minutes, low peaks in figure 8). Furthermore, Gutierrez et al. pointed out that the dominant species were *Ruminococcus gnavus*, *E. coli*, *B. dorei*, *Lachnoclostridium symbiosum* and *L. plantarum*. Surely in our experiment *E. coli* was dominant in both conditions tested, while *L. plantarum* was among the dominant species only on Maitake extract. Then, *C. symbiosum* is the third dominant strain in the presence of FOS (Figure S1).

Unlike Gutierrez et al., who did not see a dominance of *B. adolescentis* in the presence of inulin (and considered it a contradictory result as Bifidobacteria are good users of that substrate), in our experiment *B. lactis* resulted as the most abundant strain in the presence of FOS, so it could be in line with the expected results.

Flavonifrator plautii, i.e., *C. orbiscindens*, was always little grown, because it probably had special requirements. This also resulted from our tests, because it grew only in the presence of *B. cellulosilyticus* and *C. symbiosum* (Figure S1 and S2).

Regarding the produced secondary metabolites, always comparing with Gutierrez et al., it is correct to find the production of propionate in the presence of *B. cellulosilyticus*, as it is indicated in the paper as a producer of the metabolite. In addition, *E. coli* and *L. symbiosum* may indirectly contribute to the presence of propionate. *L. symbiosum* can also produce butyrate from acetate and lactate. So, a cross-feeding mechanism could be hypothesized.

F. plautii was defined as not important for the fitness of the consortium by the same authors.

Then, the presence of *L. plantarum* could correlate with the presence of lactic acid, as *L. plantarum* has an optional heterofermentative metabolism and produces large amounts of lactic acid under anaerobic conditions.

In conclusion, the study demonstrates the prebiotic properties of Maitake extract and its bioactive compounds. These molecules are used as carbon and energy sources by different bacteria strains belonging to *Lactobacillus* and

Bifidobacterium genera. The combination of the eight probiotic strains and their fermentation of the mushroom preparation leads to the production of beneficial secondary metabolites, that have positive effects on colorectal cell lines. In particular, they promote the recovery of cell viability after the stress induced by ROS species. Furthermore, the synthetic *in vitro* gut microbiota consortium provided further information about the modulation of the community in terms of growth profiles, abundances, produced possible beneficial secondary metabolites, and interactions among the bacterial strains.

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4.6 Supplementary material

Bacterial strain	Primer code	Sequence (5'→ 3')	DNA region	Reference
L. plantarum	Lpl2F LpL2R	CATTGGAACCGAACCAGTTG CGGTGTTCTCGGTTTCATTATG	16S/23S IS	Mezzasalma et al., 2016
L. acidophilus	Lacid2F Lacid2R	GGGCAAATCACGAACGAGTA CTTTGTTTTCGTTCGCTTCA	pre16S	Mezzasalma et al., 2016
B. animalis subp. *lactis*	AnimF AnimR	GCACGGTTTTGTGGCTGG GACCTGGGGGACACACTG	pre 16S	Mezzasalma et al., 2016
B. celluloilyticus	BC_F BC_R	ATTTGTGGACGCTACTGTTATTCGT ACGACGCCACTTCGGAATACG	*rpoB*	Christensen et al., 2020
C. orbiscindens	CO_F CO_R	TGAGTAACGCGTGAGGAACC TCGTCGGGTACCGTCATTTG	16S	Oogita et al., 2020
C. symbiosum	CS_F CS_R	GTGAGATGATGTGCCAGGC TACCGGTTGCTTCGTCGATT	*2-hydroxyglutaryl-CoA dehydratase*	Xie et al., 2017
E. coli	EC_F EC_R	CATGCCGCGTGTATGAAGAA CGGGTAACGTCAATGAGCAAA	16S	Huijsdens et al., 2002

Table S6.. List of the species-specific primers used in this study for qPCR analysis.

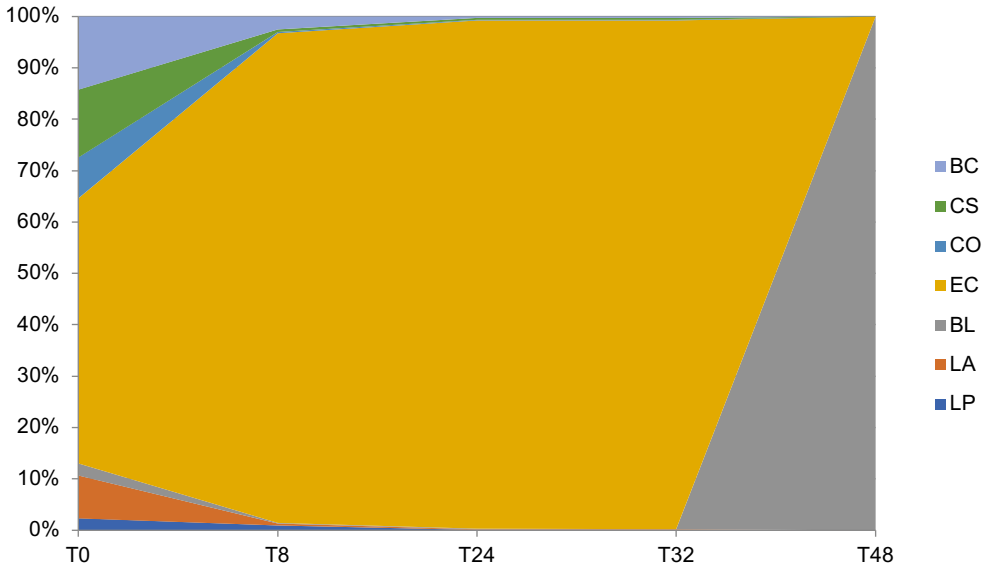

Figure S1. Modulation of single strain abundances in presence of FOS during time represented as CFU/mL % with respect to total CFU/mL %.

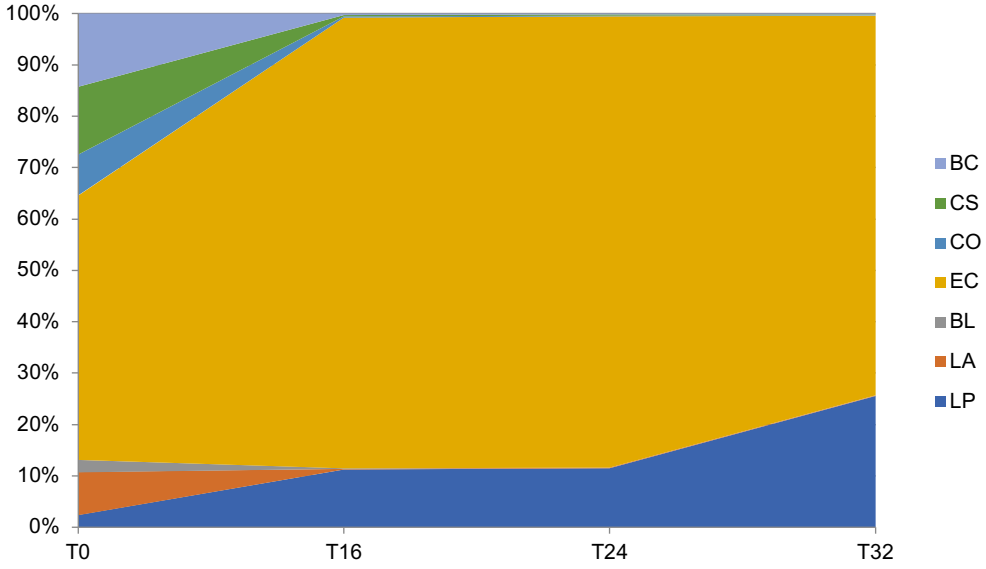

Figure S2. Modulation of single strain abundances in presence of Maitake extract during time represented as CFU/mL % with respect to total CFU/mL %.

Chapter 5:
Conclusions and future perspectives

The main purpose of this PhD project was the development of a method to study how to combine new emerging prebiotic molecules with probiotic bacteria, to obtain formulations leading to maximum benefits for the human host. This has been achieved both thanks to *in vivo* tests and thanks to established or innovative *in vitro* techniques.

Nowadays, the human gut microbiota is increasingly recognized as a fundamental element in human health, because of its role in the metabolism of ingested food or drugs, and its direct connection with the immune system, regulating the response in a positive way. In this context, the search for active molecules or other strategies to improve its balance is an emerging field of interest, because it is established that dysbiotic configurations of the intestinal microbial population could be the starting points or the boosting agents of several pathologies, such as Chron's disease or irritable bowel syndrome (IBS). It is now known that probiotic bacteria belonging to *Bifidobacterium* and *Lactobacillus* genera exhibit health-promoting effects, such as the stimulation of the immune system and the control of the microbiota composition. Instead, prebiotics are molecule not digestible by human enzymes but available for peculiar bacteria, i.e., the probiotics, sustaining their growth and fermentation abilities in the harsh intestinal environment. So, prebiotics and probiotics could be administered together in a unique formulation known as synbiotic, in the expectation of the enhancement of probiotics survival and growth thanks to prebiotics, to obtain the maximum benefit for human health.

A first screening of the fermentation ability of the probiotic strains of our collection was performed on commercial inulin and fructooligosaccharides (FOS) differing in the degree of polymerization (DP). Indeed, this kind of polysaccharides are among the most studied and used prebiotics, also because of their other beneficial effects for the host in addition to the fermentability by the probiotic bacteria. In particular, long-chain inulins are among the molecules that stimulate most the production of the beneficial secondary metabolites short-chain fatty acids (SCFAs), but they are also more difficult to be used by bacteria

as carbon and energy sources. Interestingly, among the tested strains, *L. plantarum* PBS067, *L. rhamnosus* PBS070, *L. reuteri* PBS072, and *B. animalis* spp. *lactis* PBS075 showed the capacity to ferment long-chain inulin (30<DP<60). While *L. acidophilus* PBS066, *B. animalis* spp. *lactis* PBS075, and *B. longum* spp. *infantis* LMG P-29639 proved to be capable to utilized inulin with DP around 25. The simpler the molecules become, the more fermentable were for the bacteria; indeed, almost all the bacteria metabolized medium-chain FOS (DP around 10), and the littlest tested FOS (3<DP<5), obtaining very high growth values.

The detailed characterization of the important antimicrobial capacity of *L. plantarum* PBS067 revealed the capacity of the strain to produce a bacteriocin-like molecule, called plantaricin P1053 because of its molecular weight. The antimicrobial protein could inhibit both Gram-positive and Gram-negative bacterial growth, as demonstrated by the AWDA on *S. aureus* and *E. coli* as examples of potential pathogen strains. Furthermore, plantaricin P1053 exerted positive effects on healthy intestinal cell lines and decreased the cell viability of a tumoral cell line (De Giani et al., 2019)

Overall, these results, combined with the general previously obtained information about the probiotic capabilities of the strains of our collection (Presti et al., 2015) led to the design of a functional synbiotic formulation composed by *L. plantarum* PBS067, *L. acidophilus* PBS066, and *B. animalis* spp. *lactis* PBS075 as probiotic bacteria, and medium and little FOS (DP around 10, and 3<DP<5) as prebiotics. The aim was the reduction of the adverse changes of the gut microbiota induced by aging. Actually, the intestinal bacterial population changes during the lifetime, generally deflecting towards a dysbiotic configuration, characterized by less amounts of beneficial bacteria, boosting the inflammaging phenomenon. Therefore, a randomized, doubled-blind, three-arm parallel, placebo-controlled study was developed enrolling healthy elderly subjects, that were divided into three groups: the prebiotic group (receiving only the prebiotic FOS), the synbiotic group (receiving the synbiotic formulation), and

the placebo group. The study duration was 56 days, and at the baseline (T0), after 28 days of administration period (T28), and after 28 days of follow-up period (T56), the composition of the gut microbiota of each subject and the levels of selected fecal (calprotectin and β-defensin2) and salivary (IgA and total antioxidant capacity TAC) biomarkers were evaluated. Also, the impact on the general health state of the subjects was evaluated through the monitoring of the common infectious diseases (CID) symptoms incidence throughout the study period. Interestingly, the results showed that the synbiotic formulation was effective in the enrichment of the intestinal microbial population of the subjects treated with it. Looking at the *beta*-diversity and at the maturity of the microbiota among the study groups during time, it was evident that the synbiotic led to a higher biodiversity variation linked to a variation in the structure of the community after 28 days of administration, that is maintained until the end of the follow-up. To deep understand this variation in microbiota biodiversity, a supervised learning regression was used to identify the most important amplicon sequence variants (ASVs) promoting the microbial shift across time. Intriguingly, the ASVs assigned to *Faecalibacterium*, *Alistipes*, *Bacteroides*, and *Subdoligranulum* showed a frequency varying especially during the synbiotic intake period. Furthermore, the evaluation of the effects of the synbiotic administration on the gut microbial taxa known to have an impact on various aspects of gut metabolism pointed out also the significant variation of ASVs belonging to *Akkermansia*, *Bifidobacterium*, *Blautia*, *Prevotella*, *Roseburia*, and *Ruminococcus* genera. These species are known for the positive impact on the production of the SCFAs, as acetic acid, propionate, and butyrate, that control the functional state of the gut epithelial barrier. Furthermore, looking specifically at the variation of the administered probiotic bacteria, the synbiotic contributed to enhance the *Lactobacillus* and *Bifidobacterium* species within the gut microbial community after the ingestion for 28 days. Meanwhile, the persistence of the synbiotic strains in the gut system likely needs a longer period of administration.

Regarding the wellbeing of the elderly subjects, the collected data indicated the synbiotic was effective in reducing the CID symptoms incidence, especially with respect to the placebo group, considering both the number of subjects that experienced almost one CID during the study period or its timepoints, and the average number of days with CID (during all the study period and its intermediate timepoints).

Considering the biomarkers indices of the activity of the immune system, the most beneficial results were linked to the positive effects on the intestinal response. Indeed, considering faecal calprotectin level below 45 μg/g as physiological for elderly people, after the synbiotic administration the 96% (T28) and 100% (T56) of the subjects showed calprotectin level below the threshold value. Furthermore, the synbiotic enhanced significantly the β-defensin2 levels at the end of the study with respect to the beginning of the treatment (T0-T56), indicating a positive response to the bacterial administration of the cells present in the intestinal epithelium.

Concerning the respiratory tract, the synbiotic intake was not as effective as in the intestinal environment, because the IgA levels were not significantly changed by the treatment. Nevertheless, the antioxidant status of these treated subjects enhanced, as showed by the increased in TAC values.

In conclusion, this study demonstrates, from the gut microbiota to the clinical and metabolic point of view, that formulations with probiotics and prebiotics as nutritional supplementation can help in maintaining a healthy status by re-equilibrating the gut microbiota, in particular of the elderly people.

Inspired by the limitations of this *in vivo* study, the last aim of the project was the development of a new synbiotic formulation based on different prebiotic molecules of natural origin, to be tested in *in vitro* models of intestinal colorectal cell lines and synthetic gut microbiota community. This experimental setup is challenging, because the idea is the simulation of what happens in the gastrointestinal tract, considering both chemical, microbiological, and then physiological aspects impacting the host health.

After an initial screening, *Grifola frondosa* (Maitake) mushroom extract was selected as a possible prebiotic molecule, because of the presence of β-glucans, that are polysaccharides indigestible by the human enzymes and known for beneficial effects on the human immune system. Interestingly, from the principal component analysis of the extract, around 20% w/w correspond to β-glucans. Furthermore, after the administration of the Maitake extract to the probiotic strains of our collection, the results showed that the mushroom preparation could be considered as a prebiotic substrate, because all the tested strains were able to ferment it and the differences between the growths in the control condition and the growth on the prebiotic is statistically significant. So, to enhance the beneficial effect for the host, a probiotic consortium was design (composed by 5 *Lactobacillus* and 3 *Bifidobacterium* strains) and challenged for the fermentation ability of Maitake extract. Also in this kind of experiment, the prebiotic capacity of the extract was confirmed, because of the very significant difference between the growth on the sole medium and the one on the medium added with Maitake extract.

From the literature is known that the production of potentially beneficial secondary metabolites (SCFAs and BCFAs) derived from the fermentation of complex carbohydrates by probiotics. So, their production by the probiotic consortium after Maitake extract fermentation was tested. Lactic, succinic, and valeric acid, and butyrate were detected after their extraction from the broth culture and their analysis through GC-MSD. Also the Maitake polyphenolic component cinnamic acid was individuated, with its biotransformed molecule hydrocinnamic acid. This observation could be important in the light of the intestinal ecosystem, where also polyphenols have a role on the other bacteria of the intestinal community and on the intestinal cells. Therefore, viability assays on both healthy (CCD841) and tumoral (CACO-2 and HT-29) cell lines were developed to understand the possible role of the previously characterized extracted secondary metabolites. Interestingly, the effects were principally on the CACO-2 cell line, which showed about 20% reduction at the maximum

Maitake extract concentration of 1 mg/mL. Furthermore, the effect was expressed in a dose-dependent manner, already detectable at the lowest dose. So, a possible protective effect exerted by the probiotic fermented Maitake extract was supposed. The HT-29 cell line was fist challenged with a stressor compound, and then treated with the probiotic fermented mushroom extract. The co-treatment with the stressor compounds and the extract demonstrated that it protects the cells from H_2O_2 challenge, but not from SDS stress. Furthermore, the protective effect was evidenced only at the low-middle doses, comparable to the physiological ones (De Giani et al., 2021a).

Finally, to test the possible gut microbiota modulation, a synthetic reconstructed *in vitro* gut microbiota was designed and then validated using the synbiotic formulation previously tested on healthy elderly subjects. The platform allowed to follow the growth profiles, the modulation of each strain present in the community, and the production of the secondary metabolites of interest. Therefore, the effect of the Maitake extract on the synthetic consortium were evaluated. Curiously, the basal community could growth more than the probiotics on the mushroom extracts, meanwhile the beneficial strains reached the same OD_{600nm} value of the probiotic consortium. Looking at the single strain abundances, the highest growth values were achieved by *E. coli* ATCC 25922 and *L. plantarum* PBS067, confirming the prebiotic potential of the Maitake extract and a possible control of the growth of other strains exerted by the probiotics. Finally, among the detected metabolites, there are some SCFAs.

Overall, this PhD project highlighted the potential applications of probiotic-based formulation, enriched with prebiotic products of natural origin, in the nutraceutical field. Furthermore, it allowed to setup a preliminary *in vitro* platform to screen the effects on the gut microbiota and on the intestinal cell lines of bacteria and their metabolites, and of molecules of different nature.

Future perspectives of this project regard the improvement of the *in vitro* gut microbiota platform, enriching the bacterial community with other commensal bacteria, until the use of a faecal inoculum, to have a gut microbiota more and

more similar to the real one. Then, the addition of an enzymatic gastrointestinal digestion step, to simulate the gastrointestinal reactions, before the arriving of the molecules to the intestinal bacteria. Finally, the improvement of the *in vitro* models for the interaction with the host, resulting in improved information about how the microbiota and the cellular response (both epithelial and immunological) takes place.

Scientific contributions

Research papers

Published:

De Giani A., Bovio F., Forcella M., et al. Prebiotic effect of Maitake extract on a probiotic consortium and its action after microbial fermentation on colorectal cell lines. *Foods* **10**: 2536, 2021a. doi: 10.3390/foods10112536

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D'Orazio G., **De Giani A**., Zampolli J., et al. PVP-co-DMAEMA as novel polymeric coating material for probiotic supplementation delivery. *Macromolecular chemistry & Physics* **220(21)**, 2019. doi: 10.1002/macp.201900291

Submitted:

Sandionigi A. & **De Giani A.**, Tursi F., Michelotti A., et al. A multistrain probiotic formulation enhanced resistance to common infectious diseases in flu-vaccinated health free-living elderly subjects, modulating gut microbiota diversity. *Paper submitted to BioMed Research International (submission number 3860896)*

De Giani A., Sandionigi A., Zampolli J., et al. Effect of inulin-based prebiotics alone or in combination with probiotics on human gut microbiota and markers of immune system: a randomized, double-blind, placebo controlled study in healthy elderly. *Paper submitted to PlosOne (submission number PONE-D-21-31494)*

Conferences contributions

De Giani A., Sandionigi A., Zampolli J., Michelotti A., Tursi F., Labra M., Di Gennaro P. Clinical study on healthy and elderly subjects: effects of prebiotics and probiotics on human gut microbiota and related immune system. Poster presentation at 12th International Symposium on Gut Microbiology (INRAE-Rowett) Virtual Venue, 13-15 October 2021

De Giani A., Sandionigi A., Zampolli J., Michelotti A., Tursi F., Labra M., Di Gennaro P. Effects of Prebiotics and Probiotics on Human Gut Microbiota and Related Immune System: A Study on Healthy and Elderly Subjects. Poster presentation at 14th international scientific conference on prebiotics, probiotics, gut microbiota, and health-IPC2020, Praga, 11 November 2020

De Giani A., Labra M., Michelotti A., Carlomagno F., Di Gennaro P. Effect of prebiotics and probiotics on human gut microbiota of elderly people. Poster presentation at Fifth NeuroMI-Food for Brain: promoting health and preventing diseases, International Meeting, Università degli studi di Milano-Bicocca, Milano, 20-22 November 2019

De Giani A., Zeaiter Z., Labra M., Regonesi M.E., Di Gennaro P. The probiotic potential of new fructooligosaccharides on probiotic strains and their possible synergistic effect on human gut health. Poster presentation at XXXIII Congresso della Società Italiana di Microbiologia Generale e Biotecnologie Microbiche (SIMGBM), Università di Firenze, Firenze, 19-22 June 2019

De Giani A., Bovio F., Di Gennaro P. Characterization of probiotic strains for their role as antimicrobial agents and their possible effect on epithelia intestinal cell lines. Poster presentation at MicrobiotaMI 2018, International Congress, Università degli studi di Milano-Bicocca, Milano, 5-7 November 2018

De Giani A., Boccarusso M., Di Gennaro P. Characterization of new probiotic strains for their role as antimicrobial agents. Poster presentation at XXXII Congresso della Società Italiana di Microbiologia Generale e Biotecnologie Microbiche (SIMGBM), Palermo, 17-20 September 2017

Annex

De Giani et al., 2021a

De Giani et al., 2021b

De Giani et al., 2019

D'Orazio et al., 2019

foods

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Article

Prebiotic Effect of Maitake Extract on a Probiotic Consortium and Its Action after Microbial Fermentation on Colorectal Cell Lines

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Abstract: Maitake (*Grifola frondosa*) is a medicinal mushroom known for its peculiar biological activities due to the presence of functional components, including dietary fibers and glucans, that can improve human health through the modulation of the gut microbiota. In this paper, a Maitake ethanol/water extract was prepared and characterized through enzymatic and chemical assays. The prebiotic potential of the extract was evaluated by the growth of some probiotic strains and of a selected probiotic consortium. The results revealed the prebiotic properties due to the stimulation of the growth of the probiotic strains, also in consortium, leading to the production of SCFAs, including lactic, succinic, and valeric acid analyzed via GC-MSD. Then, their beneficials effect were employed in evaluating the vitality of three different healthy and tumoral colorectal cell lines (CCD841, CACO-2, and HT-29) and the viability rescue after co-exposure to different stressor agents and the probiotic consortium secondary metabolites. These metabolites exerted positive effects on colorectal cell lines, in particular in protection from reactive oxygen species.

Keywords: prebiotics; mushroom; Maitake; probiotics; functional food; nutraceuticals

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1. Introduction

The nutritional and medicinal effects of mushrooms are recognized worldwide, in particular in Asian and northern and Central American countries. Indeed, over 270 fungal species are identified for their biological activities, such as anti-inflammatory, antimicrobial, and antioxidant properties [1]. Nevertheless, an important role has always been recognized in the preservation of the healthy state of the gastrointestinal tract [2]. Accordingly, nowadays, mushrooms are used not only in the pharmaceutical industry but also in the nutraceutical and cosmeceutical industries [1]. This is due to their high protein and low-fat contents, as well as to the presence of several vitamins, and minerals [1], but most of all, it is due to the presence of important functional components such as dietary fibers, chitin, and glucans [3]. In particular, glucans are polysaccharides composed of chemically heterogeneous glucose molecules, classified in α- or β-glucans depending on the glycosidic linkage [4]. β-glucans are known for their beneficial effects in lowering blood pressure, reducing glycemia, and acting as antitumoral and antioxidant agents [5]. Furthermore, they could be used by the intestinal microbiota as prebiotics [2], defined as "non-digestible dietary food ingredients that, when passing through the colon, will benefit the host by selectively stimulating the growth and/or activity of one or a limited number of

beneficial bacteria in situ" [6]. Therefore, they are currently the most sought after functional food [2].

Among the mushrooms with health booster properties, there is *Grifola frondosa* (knows as Maitake, Hen of the wood, or Signorina mushroom) [7]. It belongs to the Polyporaceae family [8], and it is characterized by caps with a smoky brown color [7]. Generally, it grows in temperate regions, such as Japan, Europe, northeastern states of America, and subtropical regions at high elevations [7]. Regarding the chemical composition, Maitake is characterized by 3.8% water-soluble polysaccharides; among them, 13.2% correspond to (1 → 3,1 → 6)-β-D-glucans [7]. Typically, the water-soluble polysaccharides can be divided into two subpopulations based on the molecular weight, i.e., 722.7 kDa and 19.6 kDa [9].

Other components are starch, oligofructose, fructooligosaccharides (FOS), lactulose, galactomannan, polydextrose, and dextrin [10]. Because of these characteristics, researchers are trying to combine the possible beneficial effects of the mushroom with the helpful action of probiotics (i.e., live microorganisms that confer a health benefit to the host when administered in adequate amounts [11]). Generally, this type of fermentation is applied to several food ingredients because probiotics can enhance the nutritional value of food [5] and support positive health effects, such as immune modulation and the maintenance of a state of eubiosis in the context of the gut microbiota composition. Furthermore, mushrooms themselves could improve the antioxidant condition through the modulation of the gut microbiota [10]. As a consequence, an important role in the status of intestinal mucosal epithelial cells could be played by the released bacterial secondary metabolites generated by mushrooms' fermentation, in particular, short-chain fatty acids (SCFAs) [12], including acetate, propionate, butyrate, and valerate [13]. In the presence of a medium-rich fiber diet, SCFA concentration in the intestine is around 10 mmol/L, so the intestinal epithelial cells are constantly exposed to these metabolites, mediating the crosstalk between the microbiota and the host [14]. It is known that a fiber-rich diet could also prevent the development of colorectal cancer (CRC). Interestingly, the SCFA butyrate could especially act as an antitumoral agent through the modulation of several transduction pathways, including the cellular proliferation pathway [12]. For example, butyrate inhibited the proliferation of a colorectal tumoral cell line, decreasing the presence of the phosphorylated extracellular-regulated kinase 1/2 (p-ERK 1/2), which is classified as a survival signal [14].

In this study, we analyzed a commercially available Maitake (*Grifola frondosa*) dried extract, characterized for its β-glucan content. Based on the specifics, we tested the prebiotic property on several probiotic strains, comprising both *Lactobacillus* and *Bifidobacterium* genera. Then, we studied a powerful probiotic consortium that, in the presence of Maitake preparation, released SCFAs. The fermentation products were then tested for their effects on the vitality of both healthy and tumoral colorectal cell lines. Finally, the rescue of the viability of a cell line after induced stresses was evaluated.

2. Materials and Methods

2.1. Preparation of the Maitake Extract

The Maitake (*Grifola frondosa* Dicks. Gray) extract was obtained from Amita HC Italia S.r.l. (Solaro, MI). The original mushrooms came from China, and they were wild at the time of the manual collection (from August to November). Then, the sporophorum part was selected for the extraction of polysaccharides.

The commercial Maitake dried extract was prepared as follows. The Maitake sporophorums were first ground and weighted. Then, to obtain an extract enriched in polysaccharides, the obtained material was resuspended in a solution of ethanol:water (20:80 ratio), and the separation was allowed. After overnight incubation, the precipitate was collected and then dried at 50 °C to eliminate the ethanol. Finally, the obtained extract was blended and sieved to create a brownish fine powder characterized by a particle size lower than 180 μm.

2.2. Characterization of the Maitake Extract

2.2.1. Determination of Starch Molecules Content

The starch molecules content was estimated through the Megazyme kit K-TSHK (Megazyme Inc., Chicago, IL, USA) as described by the manufacturer's instructions.

A starting weight of 100 mg of Maitake extract powder was used for the measurement. Then, 0.2 mL of aqueous ethanol (80% *v*/*v*) was added and then stirred using a magnetic stirrer. An amount of 2 mL of 2 M KOH was added, and the solution was stirred for 20 min in an ice-water bath. Then, 8 mL of 1.2 M sodium acetate buffer (pH 3.8) was added, together with 0.1 mL of thermostable α-amylase (from Megazyme kit) and 0.1 mL of amyloglucosidase (20 U, from Megazyme kit). All the contents were stirred and then incubated at 50 °C for 30 min. The obtained solution was centrifuged at 3000 rpm for 10 min, and 0.1 mL of the supernatant was analyzed.

2.2.2. Determination of α- and β-Glucans Content

α- and β-glucans content was estimated through the Megazyme kit K-YBGL (Megazyme Inc., Chicago, IL, USA) as described by the manufacturer's instruction. A starting weight of 100 mg of Maitake extract powder was used for the measurement. Then, 2 mL of ice-cold 2 M KOH was added and then stirred using a magnetic stirrer at 4 °C for 20 min. In total, 1.2 M sodium acetate buffer was added, and then amyloglucosidase (1630 U/mL) plus invertase (500 U/mL) (200 μL) (from Megazyme kit) was added. All the contents were mixed and then incubated at 40 °C for 30 min. The obtained solution was centrifuged at 2000 rpm for 10 min and 0.1 mL of the supernatant was analyzed for the glucose presence. For the total glucan measurement, 100 mg of Maitake extract powder was used. An amount of 2 mL of ice-cold 12 M sulfuric acid was added and then stirred. The tubes were then placed at 4 °C for 2 h in agitation. Then, 10 mL of water was added to each sample, which was placed in a hot water bath (100 °C) for 2 h. After cooling at room temperature, 6 mL of 10 M KOH was added, and the content was mixed well. Then, the volume was adjusted to 100 mL with 200 mM sodium acetate buffer at pH 5. In total, 100 μL of the sample was incubated with 100 μL of a mixture of exo-1,3-β-glucanase (20 U/mL) plus β-glucosidase (4 U/mL) at 40 °C for 60 min, and the glucose was determined with GOPOD reagent (all of the reagents used were in the Megazyme kit). Absorbance was measured at 510 nm. A 0.1 mL aliquot of 1 mg/mL glucose standard solution was incubated in triplicate with GOPOD reagent; 0.1 mL of acetate buffer (200 mM, pH 5) was also incubated with 3.0 mL of GOPOD reagent as a blank sample. Finally, the β-glucan content was determined by subtracting the α-glucan content from the total glucan content.

The calculations were made through Mega-Calc sheet (Megazyme Inc., Chicago, IL, USA).

2.2.3. Determination of Polyphenols Content

The total phenolic content of the Maitake extract was measured using the Folin–Ciocalteau phenol assay previously described [15]. A standard solution of Gallic Acid (GA) ranging from 0 to 100 μg/mL was used for the calibration. The GA solutions were prepared in 80% methanol (Sigma, Milano, Italy), and the absorbance values were measured at 765 nm. For the quantification, 0.5 mL of Folin-Ciocalteau phenol reagent (1:10 dilution) and 1 mL of distilled water were added to 100 μL of mushroom sample. The solutions were mixed and incubated at room temperature for 1 min. Then, 1.5 mL of 20% sodium carbonate (Na_2CO_3) solution was added to the sample and mixed. After the incubation for 120 min, absorbance was recorded at 765 nm against the blank sample. Results were expressed as mg of Gallic Acid Equivalent (GAE)/g of Maitake powder. All the measurements were made in triplicate.

2.2.4. Determination of Protein Content

The protein content of Maitake extract was determined according to the Bradford method [16]. A calibration curve using bovine serum albumin as a standard was performed to determine the protein concentration of the extract.

2.2.5. Determination of Fructans and Reducing Sugars Content

The quantification of fructans in the Maitake extract powder was developed using the fructan assay procedure kit Megazyme [17] in accordance with the manufacturer's instructions. The fructans concentration was calculated considering the fructose, glucose, and sucrose contents in the mushroom extract before and after the hydrolysis with fructanase. The samples were treated with a specific sucrase/maltase enzyme to completely hydrolyze saccharides to D-glucose and D-fructose. The reference values of the samples were determined by a direct analysis of D-glucose plus D-fructose using the hexokinase/phosphoglucose isomerase/glucose 6-phosphate dehydrogenase analytical procedure. The amount of NADPH formed in this reaction is stechiometric with the amount of D-glucose plus D-fructose. NADPH formation is measured by the increase in absorbance at 340 nm. The fructan content of the samples was determined after hydrolyzation to D-fructose and D-glucose by endo- and exo-inulinases, and then D-fructose and D-glucose content was measured as described above. The fructan content was determined by subtracting absorbance values of the reference from those of the sample. Beforehand, each enzymatic assay sample was heated for 30 min at 50 °C to ensure sample complete dissolution.

2.3. Bacterial Strains and Culture Conditions

The bacterial strains used in this study are reported in Table 1. The strains provided by a private collection of the company Roelmi HPC (Origgio, Italy) were previously selected and characterized for the probiotic features by [18]. The probiotic strains were routinely grown on MRS broth (Conda Lab, Madrid, Spain) supplemented with 0.03% L-cysteine (Sigma-Aldrich, Milano, Italy) for 48 h, at 37 °C, under anaerobic conditions using an Anaerocult GasPack System (Merck, Darmstadt, Germany). A modified MRS medium (mMRS), as described by [19], without glucose and supplemented with 0.03% L-cysteine, was used for the growth trials. The pH of the medium was adjusted to 6.8 before sterilization (121 °C for 20 min). Maitake extract was added to mMRS at the concentration of 2% *w*/*v* as a carbon source.

Table 1. Bacterial strains used in this study.

Strain	Source
Lactobacillus acidophilus LMG P-29512 (formerly DSM 24936)	Human
Lactobacillus fermentum DSM 25176	Human
Lactobacillus plantarum DSM 24937	Human
Lactobacillus reuteri DSM 25175	Human
Lactobacillus rhamnosus LMG P-29513 (formerly DSM 25568)	Human
Bifidobacterium animalis spp. *lactis* LMG P-29510 (formerly DSM 25566)	Human
Bifidobacterium longum spp. *longum* DSM 25174	Human
Bifidobacterium longum spp. *infantis* LMG P-29639	Human

2.4. Growth Experiment with Single Probiotic Strains on Maitake Preparation

The probiotic bacteria described in Table 1 were pre-inoculated in MRS broth for 48 h, at 37 °C in anaerobic conditions before the setup of the prebiotic experiment. Maitake extract powder was added to mMRS and then sterilized together before inoculation, to a final concentration of 2% *w*/*v*.

In total, 1 mL of sterile mMRS or sterile mMRS + Maitake preparation was added to every well of a sterile multi-well (24 wells, SPL Lifesciences, Korea, Gyeonggi-do). Then, each well was inoculated with a proper volume of each probiotic pre-inoculum (around 20 μL), to achieve a final optical density (OD) at 600 nm of 0.1. Subsequently, the plates were capped and incubated in an anaerobic jar at 37 °C for 48 h. At the end of the experiment, the OD at 600 nm was measured.

2.5. Growth Experiment with Mixed Probiotic Strains as Consortium on Maitake Preparation

The probiotic bacteria described in Table 1 were pre-inoculated in MRS broth for 48 h at 37 °C in anaerobic conditions before the setup of the experiment. Maitake extract powder was added to mMRS and then sterilized together before inoculation, to a final concentration of 2% *w*/*v*.

The consortium was prepared in a sterile tube, mixing the selected probiotic in order to achieve an OD at 600 nm of 0.1. After the homogenization, the proper volume of the consortium (around 200 μL) was inoculated in sterile tubes (BD, Milano, Italia) with a final volume of 10 mL. Each tube contained only mMRS as a control or mMRS plus Maitake extract. After the inoculum, the tubes were placed in an anaerobic jar and then incubated for 48 h at 37 °C. The growth was evaluated as optical density at 600 nm.

2.6. Extraction and Characterization of Probiotics Secondary Metabolites

2.6.1. Extraction of the Metabolites

The possible produced short-chain fatty acids (SCFAs) after Maitake fermentation were extracted from inoculated and uninoculated tubes at the end of the fermentation using ethyl-acetate (anhydrous, 99.8%, Sigma-Aldrich, Milano, Italy). The tubes were centrifuged at 7000 rpm for 10 min at room temperature to separate the pellet from the supernatant. In a glass tube (Colaver, Vimodrone, MI, Italy), 5 mL of the supernatant was acidified up to pH 2 with HCl 6 M. Then, 5 mL of ethyl-acetate was added, followed by 20 min of strong manual agitation. The obtained suspension was centrifuged at 4000 rpm for 20 min, and the organic phase was withdrawn and conserved in a new glass tube. Another 5 mL of ethyl-acetate were added to the remaining broth, followed by 5 min of strong manual agitation. The suspension was again centrifuged, and the organic phase collected and pooled with the one obtained in the first extraction.

2.6.2. Analysis of the Extracted Metabolites

The extracted organic phase was submitted to derivatization with BSTFA (Sigma-Aldrich, Milano, Italy) before the gas chromatography (GC) injection. The samples and BSTFA were mixed in a ratio of 3:1 and the reaction took place at 60 °C for 20 min. After the temperature cooled down, SCFAs were analyzed with a GC-MSD instrument, using a Technologies 6890 N Network GC System, interfaced with a 5973 Network Mass Selective Detector (MSD) (Agilent Technologies, Santa Clara, CA, USA). A ZB-5MS capillary column was used (5% diphenyl-95% dimethylpolysiloxane 60 m × 0.25 mm, 0.25 μm; Alltech, Lexington, KY, USA). Analyses were developed in the splitless injection mode, using helium at 99.99% as carrier gas (Sapio, Treviglio, Bergamo). The program for the oven was set at 65 °C for 2 min, then 5 °C min^{-1} to 110 °C, then 12 °C min^{-1} to 260 °C, holding this temperature for 10 min. Electron impact ionization spectra were obtained at 70 eV, with recording of specific mass spectra at 73, 75, 117, 129, 132, 145, 159, 171, 173, 187, 201, 215, 229, 243, and 257 *m*/*z*. All the analyses were carried out in triplicate.

The registered mass spectra were compared with those of the library of National Institute of Standards and Technology (NIST) of the instrument.

2.7. Maintenance and Growth of Cell Lines for In Vitro Tests

The healthy mucosa cell line CCD841 (ATCC® CRL-1790™) and the colon cancer cell line CACO-2 (ATCC® HTB-37™) were grown in EMEM medium supplemented with heat-

inactivated 10% FBS, 2 mM L-glutamine, 1% nonessential amino acids, 100 U/mL of penicillin, and 100 μg/mL of streptomycin and maintained at 37 °C in a humidified 5% CO_2 incubator; the colon cancer cell line HT-29 (ATCC® HTB-38™) was grown in DMEM medium supplemented with heat-inactivated 10% FBS, 2 mM L-glutamine, 100 U/mL of penicillin, and 100 μg/mL of streptomycin and maintained at 37 °C in a humidified 5% CO_2 incubator.

ATCC® cell lines were validated by short tandem repeat profiles that were generated by the simultaneous amplification of multiple short tandem repeat loci and amelogenin (for gender identification).

All the reagents for cell cultures were supplied by EuroClone (EuroClone S.p.A, Pero, MI, Italy).

2.8. Cell Viability Assay and Test with Stress Agents

Cells were seeded in 96-well microtiter plates at a density of 1×10^4 cells/well and incubated for 24 h.

To evaluate the effect of the extracts on CCD841, CACO-2 and HT-29 viability, the cells were treated for 24 h with 0.25, 0.5, and 1 mg/mL for each extract.

HT-29 cell line was treated with stressor compounds, H_2O_2 (0–8 mM), or SDS (0–0.1%), for 24 h in order to determine the concentration responsible for an about 50% reduction in cell viability. To evaluate a potential role of the extract in cell viability rescue, cells were pre-treated for 1 h with 0.25, 0.5, and 1 mg/mL for each extract, and then 1 mM H_2O_2 or 0.0075% SDS was added to the cells with a 24 h endpoint. Following the treatment, cell viability was assessed using an in vitro MTT-based toxicology assay kit (Sigma, St. Louis, MO, USA): the medium was replaced with a complete medium without phenol red, and 10 μL of 5 mg/mL MTT (3-(4,5-dimethylthiazol-2)-2,5-diphenyltetrazolium bromide) solution was added to each well; after 4 h of incubation, formed formazan crystals were solubilized with 10% Triton-X-100 in acidic isopropanol (0.1 N HCl) and absorbance was measured at 570 nm using a micro plate reader. The results were expressed as mean values ± ES of at least three independent experiments.

2.9. Statistical Analysis

Regarding the growth of probiotics, all the experiments were performed in triplicate and results were presented as mean values ± standard deviation. The statistical relevance of the results was assessed by a *t*-Student's test. The significance was defined as p-value < 0.01 or p-value < 0.05.

Regarding the experiments with the intestinal cell lines, all the experiments were performed in triplicate. The results were shown as the mean value of vitality % ± standard error. Statistical differences were calculated using Dunnett's multiple comparisons test: * p-value < 0.05, ** p-value < 0.01, *** p-value < 0.001.

3. Results

3.1. Characterization of the Principal Components of Maitake Extract

The determination of the main components of the Maitake extract was evaluated with enzymatic assays using Megazyme methods, as described in the Materials and Methods Section. The major components of the mushroom preparation are starch (around 50% *w*/*w*), and glucans (around 25% *w*/*w*, comprising α- and β-glucans). Interestingly, the presence of α-glucans is very low with respect to the β-ones, which are dominant in the extract (around 6.2% vs. 18.8%, respectively). Furthermore, Maitake extract was also analyzed for its content of polyphenols and protein by chemical reactions. Each extract preparation contained at least 1.9% *w*/*w* of polyphenols and 0.02% *w*/*w* of proteins. Other components are sugars; indeed, the extract is characterized by 1.2% *w*/*w* of fructans and 3.6% *w*/*w* of free reducing sugars. The contents of glucans, fructans, free reducing sugars, starch, polyphenols and protein are listed in Table 2.

Table 2. Composition of Maitake extract used in this study.

	Maitake Extract (%)
Starch	49.5
Total glucans	25.0
alpha-Glucans	6.2
beta-Glucans	18.8
Proteins	0.02
Polyphenols	1.9
Fructans	1.2
Reducing sugars	3.6

3.2. Prebiotic Potential of Maitake Preparation on Lactobacillus and Bifidobacterium Strains

The possible prebiotic capability of Maitake extract at a concentration of 2% *w/v* was evaluated through *in vitro* growth assays employing *Lactobacillus* and *Bifidobacterium* strains, originally isolated from the human colon [18]. The initial OD_{600nm} of each culture was 0.1; then, it was recorded at the end of the experiment, after 48 h of anaerobic fermentation (Figure 1). The medium containing all components except the extract was used as a control. As shown in Figure 1, the mushroom preparation could be considered as a prebiotic substrate for the probiotic bacteria, because all the tested strains were able to ferment it and the difference between the growth in the control condition and the growth on the prebiotic was statistically significant (p-value < 0.01). Among the *Lactobacillus* strains, *L. fermentum*, *L. rhamnosus*, and *L. reuteri* showed the most positive responses (p-value < 0.05), while, regarding the Bifidobacteria members, all the strains showed an important growth increase in Maitake extract, in particular *B. longum/infantis* and *B. lactis* (p-value < 0.01). The results highlight the prebiotic characteristic of the mushroom formulation.

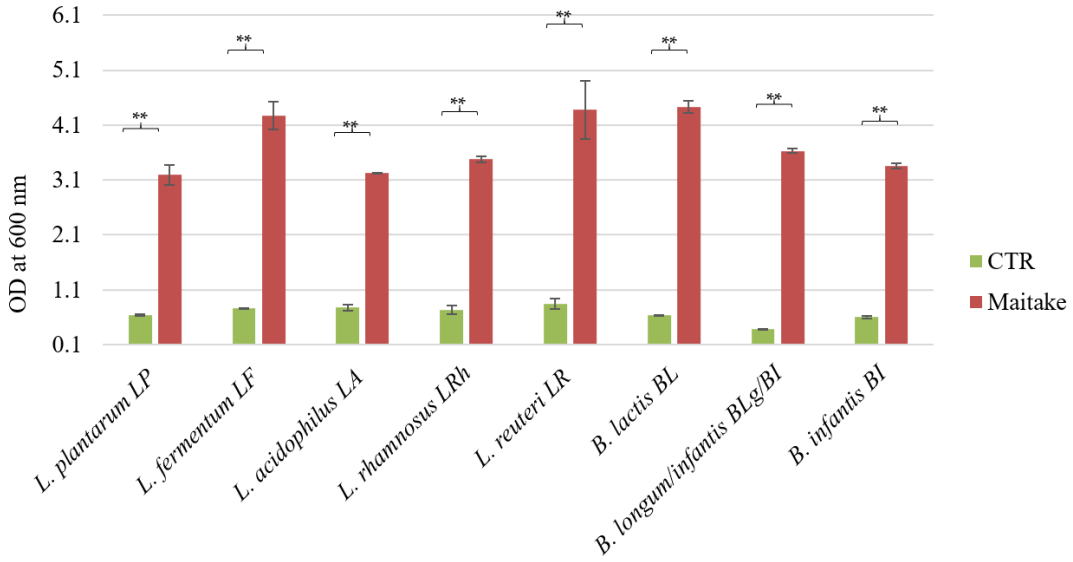


Figure 1. Prebiotic potential of Maitake extract on *Lactobacillus* and *Bifidobacterium* strains. The Figure represents the growth of the selected *Lactobacillus* and *Bifidobacterium* strains in presence of CTR medium and Maitake extract at a concentration of 2% *w/v*. Values are represented as mean value of OD at 600 nm ± standard deviation. Statistical differences were calculated using *t*-Student's test: ** p-value < 0.01.

3.3. Growth of the Probiotic Consortium on Maitake Preparation

To enhance the possible beneficial effects for the human host attributable both to probiotics and prebiotics, a possible combination of the bacteria was studied. All the probiotic strains previously used were inoculated at OD_{600nm} equal to 0.1, making sure to have a

homogeneous suspension of all the considered bacteria. The growth capacity was evaluated on the same conditions used for the experiment with single strains, i.e., control medium and Maitake extract 2% *w*/*v*. After 48 h of anaerobic growth at 37 °C, the optical density was measured (Figure 2). Interestingly, the final OD on the mushroom preparation had a mean value of 3.7 ± 0.21. The prebiotic potential of Maitake preparation is also confirmed in this kind of experiment, because of the very significant difference between the growth on the sole medium and the one on the medium added with Maitake extract (*p*-value < 0.01).

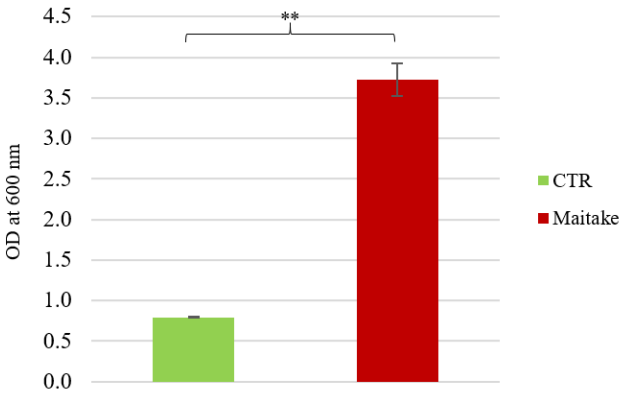


Figure 2. Prebiotic potential of Maitake extract on probiotic consortium. The figure represents the growth of consortium of the selected probiotic strains in presence of CTR medium and Maitake extract at a concentration of 2% *w*/*v*. Values are represented as mean value of OD at 600 nm ± standard deviation. Statistical differences were calculated using *t*-Student's test: ** *p*-value < 0.01.

3.4. Extraction and Characterization of Probiotic Consortium Secondary Metabolites

The production of potentially beneficial secondary metabolites derived from the fermentation of complex carbohydrates by probiotics is well documented. They are recognized as short-chain fatty acids (SCFAs) and as branched-chain fatty acids (BCFAs) [20].

To investigate which compounds are produced by the probiotic combination after the Maitake extract fermentation, a liquid–liquid extraction with ethyl-acetate of the broth culture after 48 h of anaerobic fermentation was initially conducted. Then, a gas chromatography analysis was performed, and the chromatograms were interpreted using mass spectrometry. Each peak was compared to the example present in the NIST library.

In comparison with the control condition, samples deriving from fermented Maitake extract presented several additional peaks (Figure 3). The highest peak, at a retention time of 8.2 min, refers to lactic acid. The second most important peak is at 12.2 min of retention time, and it is assigned to valeric acid. The third highest peak refers to succinic acid (retention time of 13.6 min). Other detected molecules were the SCFA butyrate (retention time of 10.8 min) and hydrocinnamic acid (retention time of 16.8 min), which is not a bacterial secondary metabolite, but a Maitake component [21] probably released due to probiotic digestion, and cinnamic acid (retention time of 19.9 min).

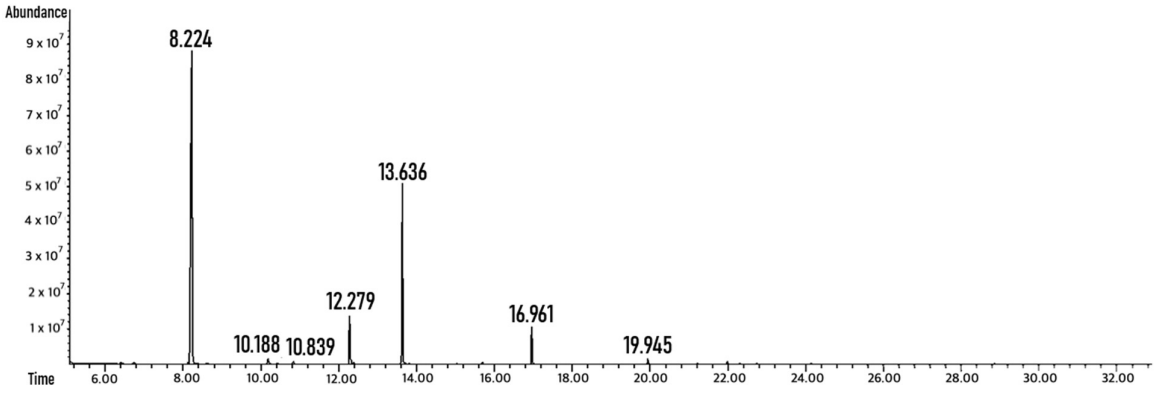


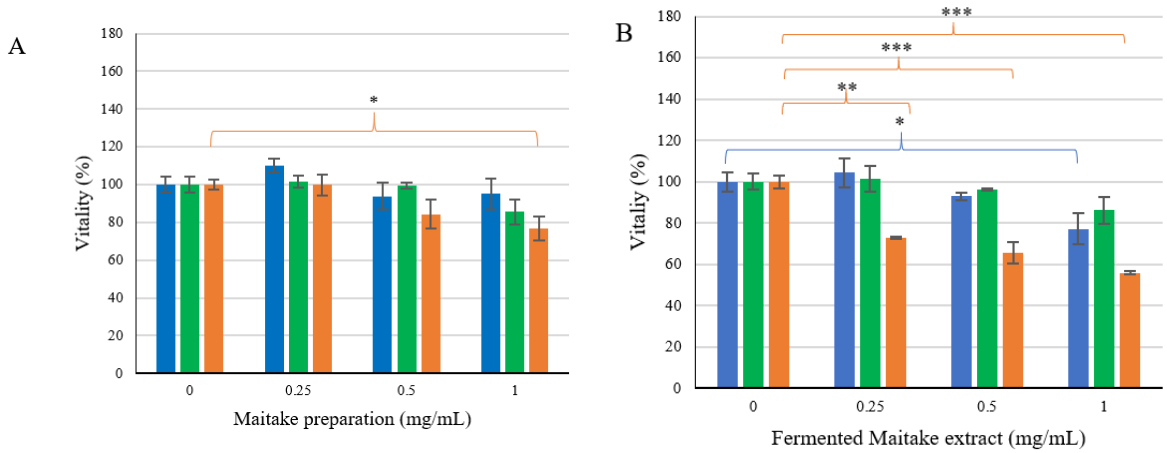
Figure 3. Probiotic consortium secondary metabolites analysis by GC-MSD after growth on Maitake extract. The figure represents the chromatogram obtained after GC-MSD analysis. The probiotic consortium was incubated for 48 h on Maitake extract at a concentration of 2% *w/v*. The cultural broth was extracted and then analyzed in GC-MSD. The intermediate metabolites identified are reported in the graph.

3.5. Differential Effects of Extracted Secondary Metabolites on Cell Viability

To understand the possible role of the previously characterized extracted secondary metabolites, viability assays on both healthy and tumoral cell lines were developed.

The healthy mucosa cell line CCD841 was not affected by the treatment with the metabolites (Figure 4), with the exception of the fermented Maitake extract, responsible for a 20% reduction in cell viability at the highest concentration analyzed (Figure 4B).

Regarding the two colorectal cancer cell lines, no variation in vitality was observed in the HT-29 cell line (Figure 4), while the CACO-2 cell line behaved quite differently. In fact, in this cell line, the viability showed an about 20% reduction at a Maitake extract concentration of 1 mg/mL (Figure 4A), as well as after exposure to fermented probiotic basal medium extract (Figure 4C). The treatment with the Maitake–probiotic fermented extract showed a significant dose-dependent decrease in cell viability already detectable at the lowest dose (Figure 4B).



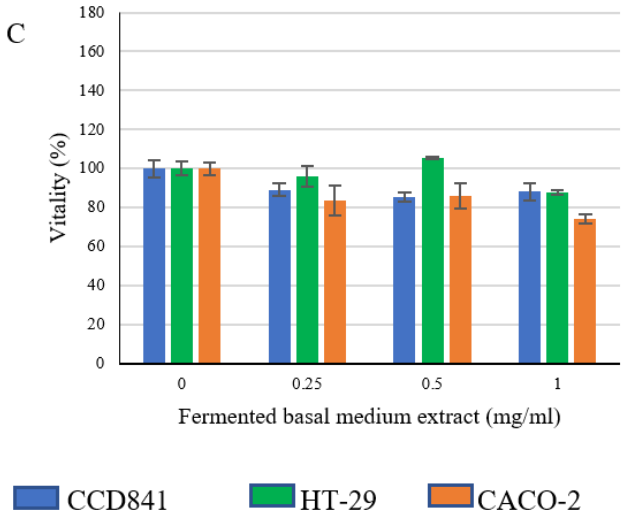


Figure 4. Differential effects of extracted secondary metabolites on cell viability. The figure represents the viability of the three different cell lines in presence of Maitake extract (**A**), fermented Maitake extract (**B**), and fermented control medium extract (**C**). Values are represented as mean value of vitality % ± standard error. Statistical differences were calculated using Dunnett's multiple comparisons test: * p-value < 0.05, ** p-value < 0.01, *** p-value < 0.001.

3.6. Protective Effect of Maitake-Probiotic Fermented Extract on HT-29 Cell Line

Since the HT-29 cell line represents a valuable and complementary tool for the study of food digestion and the effect of food components on the gut [22], the evaluation of a potential protective effect exerted by the extracts in stress conditions was conducted on this cell line.

Firstly, the cell survival rate in the presence of several stressors at different concentrations was investigated (data not shown). In the presence of either 1 mM H_2O_2 or 0.0075% SDS, cell viability compared to control was found to be 42.97 ± 5.49% and 56.13 ± 3.07%, respectively. Subsequently, co-treatment with the stressor compounds and the different extracts at the established concentrations demonstrated how only the Maitake–probiotic fermented extract protects the cells from H_2O_2 challenge (Figure 5A), but not from SDS stress (Figure 5B). Furthermore, the protective effect was only shown in the low–middle doses (Figure 5).

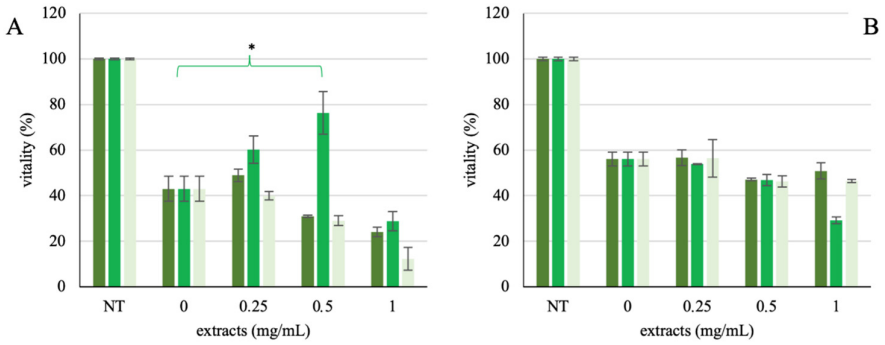


Figure 5. Vitality rescue of HT-29 cell line co-treated with stressor compounds and different extracts. The figure represents the viability rescue of the HT-29 cell line stressed with H_2O_2 (**A**) or SDS (**B**) in presence of Maitake preparation ( ), fermented Maitake water extract ( ), and fermented basal medium extract ( ). Values are represented as mean value of vitality % ± standard error. Statistical differences were calculated using Dunnett's multiple comparisons test: * p-value < 0.05.

4. Discussion

Extracts deriving from vegetables naturally contain polysaccharides that are characterized by different chemical compositions [23]. These molecules can be extracted and employed as nutraceuticals, leading to several beneficial effects for the host and its gut microbiota. In particular, mushrooms are known for their high content of polysaccharides with antioxidant and immunomodulatory potential [23]. Among the most interesting polysaccharides, there are glucans, which are a group of chemically heterogeneous glucose molecules, classified as α- or β-glucans based on the glycosidic linkage [4].

The Maitake extract preparation tested in this study contains around 25% of total glucans, in line with the study of [4]. Interestingly, in the Maitake water extract analyzed in this study, the presence of α-glucans is very low with respect to the β- ones (around 6.2% vs. 18.8%, respectively), and this is in accordance with the literature. Indeed, high levels of α-glucans are not naturally present in mushrooms [4]. Moreover, β-glucans are not cleaved by mammalian digestive enzymes [24], so they can reach the intestine and be used as carbon and energy sources, as well as metabolized to other compounds by the resident microbiota. Therefore, to be considered a prebiotic, the Maitake preparation also has to benefit the host by the selective stimulation of the growth of a limited number of bacteria in the colon [6], i.e., the probiotics. For this reason, we tested the potential prebiotic effect on different *Lactobacillus* and *Bifidobacterium* strains previously characterized for their probiotic features [18]. All the tested strains were able to ferment the Maitake preparation at a concentration of 2% *w*/*v*, reaching a statistically significant growth difference with respect to the control. The best growth results were obtained with *L. fermentum* LF, *L. reuteri* LR, and *B. animalis* spp. *lactis* BL. The maximum growth values on Maitake preparation are similar to the ones of known prebiotics, i.e., inulin or fructooligosaccharides (FOS). For example, the growth values of the same probiotic strains on commercial FOS with a degree of polymerization comprised between 3 and 5 at a concentration of 2% *w*/*v* [25] are similar to the ones obtained in this study, after 48 h of Maitake preparation fermentation at the same concentration, that are around an optical density at 600 nm of 4.

Therefore, the Maitake preparation could be considered a prebiotic molecule, in line with the literature data. Indeed, [26] studied the possible prebiotic effect of polysaccharides extracted from 53 different wild-growing mushrooms on *Lactobacillus acidophilus* (used as reference strain) and *Lactobacillus rhamnosus*. The extracted fungal molecules could stimulate the Lactobacilli growth more than commercially available prebiotics, such as inulin and FOS from chicory. Additionally, Bifidobacteria could benefit from the presence of β-glucans in the growth medium, as reported by [27] in the reconstruction of the catabolic pathway of *B. longum* subspecies *infantis* strain.

Generally, probiotics are provided as a unique individual strain. However, multistrain formulations could boost the beneficial effects for the host compared to single-strain products [28]. For this reason, some probiotic strains (five different *Lactobacillus* and three different Bifidobacteria) were combined and tested, again confirming the prebiotic potential of the Maitake preparation.

Furthermore, the metabolites derived from the fermentation of the mushroom preparation by the consortium were characterized for their possible impact on the host intestinal cells. This is a new concept reported in the literature as "metabiotics" [27], i.e., signaling molecules with a determined chemical structure that can optimize host-specific physiological functions and regulate metabolic and behavior reactions [29]. Indeed, the production of potentially beneficial secondary metabolites deriving from probiotics' fermentation on different carbon sources as complex carbohydrates, is well known [20]. These released molecules are commonly recognized as short-chain fatty acids (SCFAs) and include acetate, propionate, and butyrate, which comprise more than 95% of the total SCFA pool. Less abundant released metabolites are branched-chain fatty acids (BCFAs), such as isobutyrate, 2-methylbutyrate, isovalerate, lactate, and succinate (intermediates of the propionate), which can also have several effects on the host [20]. From the consortium broth culture, lactic acid, valeric acid and butyrate were obtained after liquid extraction.

In a gut microbiota ecosystem, the bacteria that catabolize specific molecules to simpler ones, which could be fermented and result in acidic molecules, such as acetate or lactate, are classified as primary degraders. Then, the secondary fermenters could use the secondary metabolites from the primary degraders to produce other end products, such as butyrate [30]. Among the community, Bifidobacteria are interesting because they promote butyrate release by other community members due to the production of acetate and lactate through the characteristic Bifid shunt [30]. Nevertheless, the microbiota could ferment proteins, which represent 0.02% *w*/*w* of the Maitake preparation, resulting in BCFA production [20], and it could liberate and modify bioactive polyphenols, as the detected cinnamic and hydrocinnamic acids are. These molecules create a physiological response in the host despite the low availability due to the metabolism of the original complex molecules by the gut microbiota [20].

In general, in the literature, the beneficial effects for the host of all these secondary metabolites are largely discussed. Indeed, lactic acid promotes the balance of intestinal pH [28], while butyrate also modulates the immune system, as well as the strength of the epithelial barrier, and could be protective against colorectal cancer [30]. Therefore, creating microbiota through a beneficial composition could be important for the host's health. However, considering the intestinal system, the first barrier of the human body in contact with the lumen is composed of epithelial cells.

Therefore, the extracted and characterized metabolites after the probiotic fermentation of the Maitake preparation were tested on different healthy and tumoral colorectal cell lines. Among the extracts, only the Maitake–probiotic fermented extract caused a significative dose-dependent reduction in the CACO-2 cell line, with the HT-29 and CCD841 not being affected at 0.25 and 0.5 mg/mL doses, while at the highest concentration used, the healthy one also showed a slight reduction. These results are in accordance with the paper by [14], in which the authors demonstrated the inhibitory effect of butyrate on cancerous cell lines. The different responses of HT-29 and CACO-2 to the Maitake–probiotic fermented extract treatment could be due to a different mutational status on the downstream EGFR target BRAF. In fact, HT-29 cells present a hyperactivating mutation in BRAF, responsible for constitutive ERK1/2 phosphorylation, as shown in the paper by [31]. Consequently, the human colon adenocarcinoma cell line, HT-29, was selected for further studies. Indeed, the cell line is a good model for studies on food digestion and bioavailability because the cells can form a monolayer characterized by tight junctions and the typical apical brush border, representative of mature intestinal cells [22]. Thus, a possible protective effect due to the probiotic secondary metabolites in different stress conditions was evaluated. As the results showed (Figure 5), the protection is principally against the H_2O_2 challenge, because HT-29 vitality recovers by up to about 80%. This is important, because the formation of reactive oxygen species (ROS) is common in human organs, as a result of the oxidative processes; however, they are precursors of systemic cells and tissue damage [21]. Generally, the human body has an endogenous defense system against these free radicals; however, it can be supported and potentiated by supplemented antioxidants [21].

In conclusion, this study demonstrates the prebiotic properties of Maitake extract and its bioactive compounds. These molecules are used as carbon and energy sources by different bacteria strains belonging to *Lactobacillus* and *Bifidobacterium* genera. The combination of the eight probiotic strains and their fermentation of the mushroom preparation leads to the production of beneficial secondary metabolites that have positive effects on colorectal cell lines. In particular, they promote the recovery of cell viability after the stress induced by ROS species.

Therefore, in the midst of the pandemic caused by COVID-19, eating healthy foods that reinforce our gut microbiota and have intrinsically functional properties could be a way to prevent and improve our defenses [23]. The optimization of the composition of the

probiotic consortium and the choice of the bioactive natural extracts to obtain new synbiotic formulations with a powerful antioxidant potential on the intestinal cells and a beneficial modulatory potential on host gut microbiota could be valuable alternative supports.

5. Conclusions

In conclusion, the results revealed the prebiotic properties of Maitake extract due to the stimulation of the growth of the probiotic strains, also in consortium, leading to the production of SCFAs, including lactic, succinic, and valeric acid. These metabolites exerted positive effects on colorectal cell lines, in particular protecting from reactive oxygen species. These data indicate that prebiotics from Maitake could be suitable for use in food applications and could be combined with probiotics in synbiotic formulations.

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REVIEW
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Recent Trends on Biosurfactants With Antimicrobial Activity Produced by Bacteria Associated With Human Health: Different Perspectives on Their Properties, Challenges, and Potential Applications

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The attention towards the bacteria associated with human health is growing more and more, above all regarding the bacteria that inhabit the niches offered by the human body, i.e., the gastrointestinal tract, skin, vaginal environment, and lungs. Among the secondary metabolites released by microorganisms associated with human health, little consideration is given to the biosurfactants, molecules with both hydrophobic and hydrophilic nature. Their role in the complex human environment is not only the mere biosurfactant function, but they could also control the microbiota through the *quorum sensing* system and the antimicrobial activity. These functions protect them and, accordingly, the human body principally from microbial and fungal pathogens. Consequently, nowadays, biosurfactants are emerging as promising bioactive molecules due to their very different structures, biological functions, low toxicity, higher biodegradability, and versatility. Therefore, this review provides a comprehensive perspective of biosurfactants with antimicrobial activity produced by bacteria associated with the human body and related to everything human beings are in contact with, e.g., food, beverages, and food-waste dumping sites. For the first time, the role of an "*-omic*" approach is highlighted to predict gene products for biosurfactant production, and an overview of the available gene sequences is reported. Besides, antimicrobial biosurfactants' features, challenges, and potential applications in the biomedical, food, and nutraceutical industries are discussed.

Keywords: human microbiota, human health, bioactive molecules, biosurfactants, antimicrobial activity, *-omic* approach

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INTRODUCTION

Innumerable symbiotic, pathogenic, and commensal microbes colonized the human body collectively acknowledged as human microbiota (Malla et al., 2019).

Nowadays, interest in the human microbiota, the relative metabolites, and its effects on the host is rapidly growing. In 2010, searching "*microbiota*" within the PubMed database (the free

search system accessing the MEDLINE database of references and abstracts about life science and biomedical studies) accounted for only 1,068 items. In 2020, 14,342 items were published and collected under the same keyword (**Figure 1**). This significant explosion is because of the recent awareness about the essential functions carried out by human-associated microorganisms and the importance of their secondary metabolites for host physiology. Indeed, both bacteria and their metabolites have a role in host metabolism, immune system's reactivity, neuronal development, and well-being (Zmora et al., 2019).

The human microbiota is composed of 10–100 trillion bacteria living in symbiosis with us (Ursell et al., 2012), mostly belonging to four phyla: Actinobacteria (36.6%), Firmicutes (34.3%), Proteobacteria (11.9%), and Bacteroidetes (9.5%) (Reid et al., 2011). Microbial communities inhabit any accessible area of a host body; nevertheless, the niches with stable communities in humans include the respiratory system, nasal and oral cavities, skin, vagina, and urinary tract, and gastrointestinal (GI) system (Barton et al., 2019). Among the body sites, the intestine receives more attention because it harbors several interesting bacteria, like lactic acid bacteria (LAB). For instance, members of the *Lactobacillus* genus are the most prominent LAB to promote human health due to their ability to produce several bioactive molecules, such as hydrogen peroxide (H_2O_2), short-chain fatty acids (SCFAs), and bacteriocins (Satpute et al., 2016).

In general, human-associated bacteria can also produce surface-active compounds that are useful molecules for biomedical and biotechnological applications. Biosurfactants (BSs) are surface-active compounds characterized by a double nature, both hydrophilic (polar) and hydrophobic (non-polar) (Ghasemi et al., 2019). These molecules can have a role in the maintenance of microbial homeostasis, primarily in the oral cavity and vagina (Reid et al., 2011). BSs can be industrially employed in different fields because of their properties—high biodegradability, environmentally and eco-friendly—several have low toxicity against human hosts and resistance at extreme conditions of pH and temperature maintaining their activity (Santos et al., 2016).

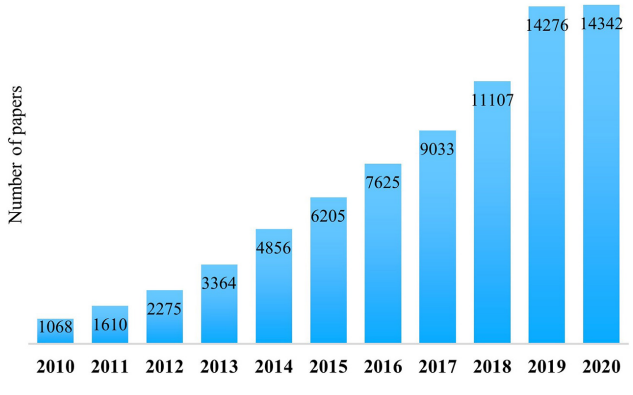

FIGURE 1 | Number of published papers in PubMed with "microbiota" as a keyword, considering a 10-year period (from 2010 to 2020).

Besides, surface-active compounds can exhibit different activities such as antiadhesive, antiviral, anticancer, anti-HIV, anti-inflammatory immune-modulatory, and antimicrobial activities (Banat et al., 2000; Seydlová and Svobodová, 2008; Jahan et al., 2020).

Additionally, they can be produced from the bacterial fermentation of natural waste products, promoting low-cost bioprocesses (Ghasemi et al., 2019).

A large number of reviews have detailed the promising applications of BS molecules from different bacteria to resolve biomedical problems or for food preservation (Fariq and Saeed, 2016; Naughton et al., 2019; Satpute et al., 2019; Ribeiro et al., 2020). Few review papers inquire into the human body sites where BSs are produced as well as the diverse bioactivities that they harbor (beyond the surfactant capacity) (Chen et al., 2017). Among the other activities, no articles report a collection of surface-active compounds with an antimicrobial activity deriving from bacteria associated with human health. Furthermore, no reviews associated antimicrobial BSs with an "*-omic*" approach to unveil the biosynthetic genes that may encode pathways capable of producing specialized metabolites.

This review focuses on the antimicrobial BSs principally produced by bacteria associated with human health; specifically, we refer to the human microbiota and food-associated bacteria (including bacteria derived from food and beverage or environmental sites related to food such as rhizosphere of agricultural fields or kitchen waste dumping sites). In detail, we will discuss antimicrobial BS structures and chemical properties in relation to the exerted activities, especially the antimicrobial activity. Besides, this work represents the first attempt to correlate the antimicrobial BSs with the genes harbored by the bacteria possibly involved in the production of antimicrobial BSs. Moreover, BS features will be correlated to the possible beneficial effects for the host, and the potential industrial and biotechnological applications.

BIOSURFACTANT PRODUCED BY BACTERIA ASSOCIATED WITH HUMAN HEALTH

The stability and the correct balance of the microbial communities of the different human body sites are strictly correlated with the surface-active compounds that they produce. Ding and Schloss (2014) reported that there are correlations between communities placed in different body sites, like the oral cavity and the vagina, but also between stool samples and the oral cavity. Therefore, they affirmed that bacteria could pass through the gut and the intestine and share some ecological environments. The most unstable microbiota belongs to the oral cavity, while the most stable are placed in stool and vagina. Accordingly, the release of bioactive molecules including BSs could be crucial for the maintenance of a niche. For example, the health status of the vaginal environment is associated with a stable community of lactobacilli. However, *Lactobacillus* strains with a BS-producing activity are not prevalent in the vaginal environment, and BS molecules could disseminate in the milieu

and change the surface tension (ST) to block the pathogens (Banat et al., 2010).

Members of the *Lactobacillus* genus can produce BSs composed principally of protein, polysaccharide, and phosphate in different ratios (Brzozowski et al., 2011), and they are mainly classified as glycolipids or glycolipoproteins (Fracchia et al., 2010; Morais et al., 2017; **Supplementary Table S1**). The molecules have also an antimicrobial effect against several common potential pathogenic bacteria such as *Neisseria gonorrhoeae* (Foschi et al., 2017), *Escherichia coli*, *Staphylococcus saprophyticus*, *Enterobacter aerogenes*, and *Klebsiella pneumoniae* and an antifungal activity against *Candida albicans* (Morais et al., 2017). Nevertheless, lactobacilli are present in other compartments of the human body, i.e., the skin, oral cavity, intestine, and gut.

The GI tract usually possesses a stable microbiota community, but several BS-producing bacteria were isolated from food, and only their intake allows them to inhabit or transit in it. For instance, *Lactobacillus paracasei* ssp. *paracasei* A20 was isolated from Portuguese dairy plants; and the produced cell-free BS has potent antimicrobial and antiadhesive activities against several bacteria and fungi (Gudina et al., 2010). Also, *Lactobacillus acidophilus*, *Lactobacillus pentosus*, and *Lactobacillus fermentum* isolated from dairy products, breast milk, fermented shrimps, and fruits in Malaysia produce cell-free BSs with an antimicrobial activity (Abdalsadiq and Zaiton, 2018). Among the other BS producers, *Pediococcus dextrinicus* SHU1593 (re-classified as *Lactobacillus* in Haakensen et al., 2009) produces a cell-bound lipoprotein BS with an antimicrobial activity against *Bacillus cereus*, *E. aerogenes*, and *Salmonella typhimurium* (Ghasemi et al., 2019; **Supplementary Table 1**).

Concerning the oral cavity, Merghni et al. (2017) suggested the use of the cell-associated BS molecules produced by *Lactobacillus casei* LBI and *L. casei* American Type Culture Collection (ATCC) 393 for the prevention of oral diseases since their antimicrobial and antibiofilm activities against *Staphylococcus aureus* (**Supplementary Table 1**).

The ability to produce antimicrobial BSs is evidenced not only for bacteria belonging to *Lactobacillus* genus but also for other inhabitants of different sites of the human body. Among them, *Pseudomonas aeruginosa* is a notable producer of BSs; and for example, *P. aeruginosa* ATCC 10145 is able to produce a cell-free rhamnolipid BS with antimicrobial and antifungal activities (El-Sheshtawy and Doheim, 2014; **Supplementary Table 1**).

In the literature, the major class of antimicrobial BSs from bacteria associated with human health is lipopeptides, glycolipids, glycopeptides, and glycolipoproteins (Abdalsadiq et al., 2018; Abdalsadiq and Zaiton, 2018; Hippolyte et al., 2018; Emmanuel et al., 2019). About 16 research papers described lactobacilli antimicrobial BS production, 10 of which are characterized as cell-free BSs and seven as cell-associated BSs (Cornea et al., 2016) describe two kinds of molecules, one released in the medium and one bound to the cell. Among the cell-free BSs, five described only the nature of the released molecules including small glycolipids as in the case of the molecule released by *L. acidophilus* NCIM 2903 (Satpute et al., 2018) and lipopeptides, i.e., the one produced by a *Lactobacillus* strain isolated from homemade curd (Emmanuel et al., 2019). Instead, the cell-associated antimicrobial BSs are more complex and characterized by several constituents. For example, many papers described glycolipoproteins (Morais et al., 2017; Hippolyte et al., 2018; Satpute et al., 2019; **Supplementary Table 1**). However, details about conformations, or molecular weight, or mechanisms of action are still unknown.

PREDICTION OF BIOSURFACTANT WITH AN ANTIMICROBIAL ACTIVITY BY AN "*-omic*" APPROACH

Several research studies are underway to find new BSs and antimicrobial compounds that can be used for biotechnological and medical applications or to fight against resistant pathogens. However, the efforts often lack the understanding of the molecular mechanisms that are behind their production, since the primary approaches usually involve several cultivation conditions and experimental assays before understanding which kind of secondary metabolites are produced by a microorganism (Gomaa, 2013; Hippolyte et al., 2018; Emmanuel et al., 2019).

The advent of the so-called "*-omic era*," in other words, in the last two decades when research studies were mainly undertaken on a genome-wide scale, changed the perspective (Zampolli et al., 2018). Together with the development of new bioinformatics tools, the amount of available bacterial genome sequences that allow the reconstruction of biosynthetic gene clusters (BGCs) that may encode pathways capable of producing specialized metabolites has been growing (Ceniceros et al., 2017). Therefore, the search for new antimicrobial compounds and BS molecules could begin with the exploration of a strain genome.

Referring only to the number of papers describing antimicrobial BSs produced by bacteria associated with human health (including human microbiota and bacteria from food, food-waste dumping sites, and agricultural soil for food production), few so far have exploited an "*-omic*" approach and studied the genetic determinants (**Table 1**). Considering only these articles, until now, the genera associated with biosynthetic genes and BGCs are the following: *Serratia*, *Bacillus*, *Pseudomonas*, and *Lactobacillus* (**Figure 2**).

The most common species of *Serratia* genus is *Serratia marcescens*, whose members are often opportunistic pathogens associated with nosocomial infections, such as the urinary and respiratory tracts, surgical wound, and bloodstream infections (Khanna et al., 2013). On the contrary, environmental *Serratia* strains including food-associated bacteria are non-pathogenic strains (Sandner-Miranda et al., 2018); for this reason, this category is included in the considered antimicrobial BS producers related to humans.

Biosurfactants produced by members of the *Serratia* genus are low-molecular-weight, non-ionic lipopeptides with an antimicrobial activity comprising serrawettin W1, W2, W3, and stephensiolides A to K (cyclic lipopeptides characterized by a macrolactone ring) (Clements et al., 2019a). The investigation into the mechanisms involved in the biosynthesis of serrawettin W1, serrawettin W2, and stephensiolides revealed *swrW*, *swrA*, and *sphA* genes, respectively (**Figure 2A**). In the open reading

TABLE 1 | List of available genes involved in the biosynthesis of biosurfactant with antimicrobial activity produced by bacteria associated with human health.

Gene	Product	Strain	References
swrW, *swrA*, *sphA* (NRPS family)	Non-ribosomal serrawettin W1 synthetase (*swrW*), serrawettin W2 synthetase (*swrA*), stephensiolides (*sphA*)	*Serratia marcescens*	Clements et al., 2019a,b
sfp	Putative surfactin transcriptional terminator (*sfp*)	*Bacillus amyloliquefaciens*, *Bacillus thuringiensis*, *Bacillus subtilis*	Perez et al., 2017; Isa et al., 2020
srfAA, *srfAB*, *srfAC*, and *srfAD* (NRPS family)	Surfactin synthase subunit 1, 2, and 3 (*srfAA*, *srfAB*, *srfAC* surfactin synthase thioesterase subunit *srfAD*)	*Bacillus pumilus* SF214	Saggese et al., 2018
rhlA, *rhlB*, and *rhlC*	Chain A of a rhamnosyl transferase (*rhlA*), chain B of the enzyme (*rhlB*), rhamnosyl transferase 2 (*rhlC*)	*Pseudomonas aeruginosa* CR1	Sood et al., 2020
Type II polyketide synthase genes (PKS)	Ketasynthase domain: PKS condensation, β-ketoacyl-[ACP] synthase II, Ab hydrolase	*Lactobacillus reuteri*	Vazquez, 2013
npsA (NRPS family)	Unknown molecule with 5289 amino acid length	*Lactobacillus plantarum* WCFS1 and *L. plantarum* RI-515	Vazquez, 2013

NRPS, non-ribosomal peptide synthetase; PKS, polyketide synthase.

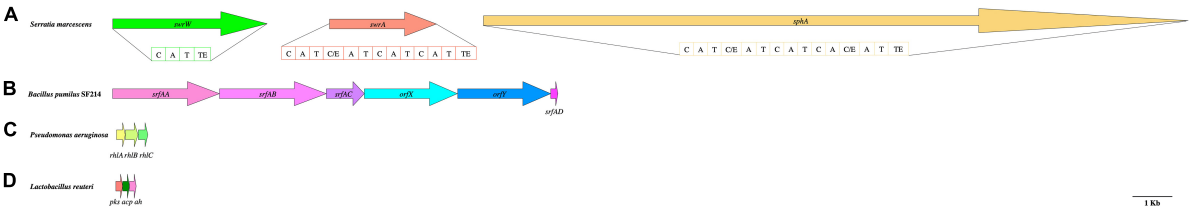

FIGURE 2 | Gene clusters involved in the biosynthesis of biosurfactant with antimicrobial activity. **(A)** Serrawettin genes from *Serratia marcescens*—*swrW*, *swrA*, and *sphA*—encoding, respectively, for serrawettin W1, serrawettin W2, and stephensiolide. The gene domains are as follows: C, condensation; A, adenylation; T, thiolation; TE, thioesterase. **(B)** *srf* operon [non-ribosomal peptide synthetase (NRPS) family] from *Bacillus pumilus* SF214—*srfAA*, *srfAB*, and *srfAC*—encoding, respectively, for surfactin synthase subunit 1, 2, and *orfX*; *orfY* encoding for uncharacterized genes; and *srfAD* encoding for surfactin synthase thioesterase. **(C)** Rhamnolipid genes from *Pseudomonas aeruginosa* CR1—*rhlA*, *rhlB*, and *rhlC*—encoding, respectively, for chain A of a rhamnosyl transferase, chain B of the enzyme, and rhamnosyl transferase 2. **(D)** Type II polyketide synthase genes (PKS) from *Lactobacillus reuteri*—*pks*, *acp*, and *ah* genes—encoding for PKS condensation, β-ketoacyl-[ACP] synthase II, and Ab hydrolase, respectively.

frame (ORF), modules consist of condensation, adenylation, thiolation, and thioesterase domains (Clements et al., 2019a; **Figure 2A**). These discoveries have been already exploited by Clements et al. (2019b) for the screening of 22 BS-producing bacteria isolated from municipal wastewater treatment plants for the biosynthesis of serrawettin A. They used a primer set designed for the identification of *swrA* gene and *swrW* gene, encoding for the non-ribosomal serrawettin W2 synthetase and serrawettin W1 synthetase, respectively.

The same approach has also been applied to discover new antimicrobial BSs in members of the *Bacillus* genus. For instance, *Bacillus subtilis* produces a lipopeptide BS known as surfactin, which is characterized as a cyclic heptapeptide linked to a β-hydroxy fatty acid (Perez et al., 2017). The surfactin transcriptional terminator is encoded by *sfp* gene, which can be employed as a marker sequence (Perez et al., 2017; Isa et al., 2020). Besides, the *srfA* operon can be used for the prediction of surfactin production by members of the *Bacillus* genus (Kanmani et al., 2013). For example, Saggese et al. (2018) searched for *srfAA*, *srfAB*, *srfAC*, and *srfAD* genes, encoding respectively for surfactin synthase subunit 1, 2, 3, and surfactin synthase thioesterase (**Figure 2B**).

Interestingly, both *srw* genes of *Serratia* and *srfA* genes of *Bacillus* are considered part of the non-ribosomal peptide synthetase (NRPS) family (Saggese et al., 2018; Clements et al., 2019a), comprising multi-modular enzyme complexes essential for the synthesis of secondary metabolites, such as antibiotics (Singh et al., 2017).

Another gene cluster useful as a marker sequence for the search of BSs with an antimicrobial activity is the *rhl* operon discovered in members of the *Pseudomonas* genus, encoding for rhamnolipid production. The gene cluster comprises the following: *rhlA* gene encoding for chain A of a rhamnosyl transferase, utilizing ACP-βhydroxy-acids and producing a fatty acid dimer; *rhlB* gene encoding for chain B of the same enzyme, using the fatty acid dimer and TDP-L-rhamnose as the substrate to catalyze the formation of mono-rhamnolipids; and *rhlC* gene encoding for a rhamnosyl transferase 2 that produces di-rhamnolipids from mono-rhamnolipids and a rhamnose moiety (Sood et al., 2020; **Figure 2C**). It is interesting to note that *rhlAB* operon is under the control of factors related to the *quorum sensing* system. Consequently, it undergoes transcriptional and post-transcriptional regulations; and the transcription depends on the environment (Reis et al., 2011). It is noteworthy to underline that Sood et al. (2020) used *in silico* analysis to predict rhamnolipid biosynthetic pathways before the extraction of the BS from *Pseudomonas* sp. CR1.

Type II polyketide synthase genes (PKS) is a well-characterized gene family involved in the production of antimicrobial BS molecules. The gene products are enzymes for the biosynthesis of

polyketides composed of various domains; and they are generally grouped with the NRPSs because of the complex biosynthetic machinery and the production of secondary metabolites with an antimicrobial activity (Singh et al., 2017). Most of the KS domains in PKS genes are found in Actinobacteria synthetizing BSs and antimicrobial molecules (Selvin et al., 2016). Nevertheless, the work of Vazquez (2013) described a *Lactobacillus reuteri* strain harboring a single PKS gene cluster including the following gene products: PKS condensation (*pks*), β-ketoacyl-[ACP] synthase II (ACP is acyl carrier protein) (*acp*), and Ab hydrolase (*ah*) (**Figure 2D**).

Despite the several massive sequencing projects regarding bacteria of *Lactobacillus* genus or intestinal related genera, little is known about the genes encoding for the BS production potential. Vazquez (2013) screened *in silico* the surlactin BS genes from 173 *Lactobacillus* species, indicating with the termed "surlactin" every kind of BS produced by lactobacilli, regardless of the chemical nature or if the molecules are cell-bound or released in the medium. In general, they conclude that several strains of *Lactobacillus plantarum*, *Lactobacillus inners*, *L. reuteri*, and *Lactobacillus brevis* have the potential to produce surlactin sharing high identity with surfactin biosynthetic pathway (principally from *Bacillus* species; this could be expected because *Bacillus* and *Lactobacillus* genera belong to the Bacillaceae family). Indeed, they found that lactobacilli harbor both NRPS and PKS genes. Specifically, *L. plantarum* WCFS1 and RI-515 showed NRPS genes that comprise *npsA* gene encoding for an unknown 5,289-amino acid length chain that is a good candidate as a marker sequence for surlactin production, while the abovementioned *L. reuteri* harbors the PKS genes. Unfortunately, NRPS and PKS systems are connected not only with BS synthesis but also with other secondary metabolites such as antibiotics and bacteriocins (Lin et al., 2015; Zhang et al., 2019). Such overlap of possible transcriptional functions can lead to the prediction of the ability to produce different secondary metabolites.

CLASSIFICATION OF BIOSURFACTANT COMPOUNDS FROM BACTERIA ASSOCIATED WITH HUMAN HEALTH

The antimicrobial BSs derived from microorganisms associated with human health can be categorized into two main classes: cell-associated and cell-released BSs (**Supplementary Table 1**).

Literature survey illustrates that the bacteria producing antimicrobial BSs associated with human health mostly belong to the phyla Firmicutes and Proteobacteria. Currently, the bacteria genera grouped within cell-associated BS class fall in the genera *Lactobacillus* and *Pediococcus*, while the cell-released BS class includes few strains of *Lactobacillus*, *Pseudomonas*, *Bacillus*, and *Enterobacter* genera (**Figure 3**).

Cell-Associated Biosurfactants

The antimicrobial BSs from the *Lactobacillus* genus are generally cell-associated, probably for their intrinsic characteristics. The chemical analysis of characterized cell-associated BSs shows that they are high-molecular-weight molecules composed mainly of proteins, fatty acids, and sugars with different percentages.

Satpute et al. (2019) isolated a glycolipoprotein-type BS from *Lactobacillus* acidophilus NCIM 2903 with the ability to reduce the ST of 45 mN m^{-1} (from 71 to 26 mN m^{-1}) and a critical micelle concentration (CMC) equal to 23.6 mg ml^{-1}. The antibacterial and antiadhesive properties of the glycolipoprotein were detected at a concentration of 25 mg ml^{-1} that was able to inhibit *Escherichia coli* NCIM 2065 and *Proteus vulgaris* NCIM 2027 growth of more than 30% and *Bacillus subtilis* MTCC 2423 growth of 26%. The action was not so potent against *Pseudomonas putida* MTCC 2467 (14% of growth inhibition). The stronger antiadhesive effect was against two Gram-positive bacteria as shown by 81% and 79% of inhibition of *Staphylococcus aureus* NCIM 2079 and *B. subtilis* MTCC 2423, respectively. Moreover, the cell-associated glycolipoprotein showed antiadhesive and antibiofilm potential against *P. vulgaris* NCIM 2027 and *S. aureus* NCIM 2079.

Similarly, antimicrobial BSs from *Lactobacillus jensenii* P_{6A} and *Lactobacillus gasseri* P_{65} isolated from vaginal fluids of healthy women after 72 h of fermentation yield of 0.27 and 0.42 g L^{-1}, respectively (Morais et al., 2017). The BSs produced by *L. jensenii* P_{6A} and *L. gasseri* P_{65} reduced the water-surface tension of 28.8 mN m^{-1} (from 72 to 43.2 mN m^{-1}) and 29.5 mN m^{-1} (from 72 to 42.5 mN m^{-1}) with comparable CMC values of 7.1 and 8.58 mg ml^{-1}, respectively. They also shared a similar chemical composition: P_{6A} molecule was composed of 51.49% of carbohydrates, 15.17% of proteins, and 29.45% of lipids, while P_{65} was composed of 38.61% of carbohydrates, 9.81% of proteins, and 49.53% of lipids. Considering the last constituent category, only 14-methypentandecanoic acid, a 16-carbon fatty acid, was present in both BSs. This molecule was the main fatty acid present in *L. jensenii* P_{6A} BS, representing 69% of the lipid fraction, while eicosanoic acid (47.43% of the lipid fraction) characterized *L. gasseri* P_{65} BS. Furthermore, galactose, glucose, and ribose were present in both the molecules in different percentages, even if rhamnose is peculiar of *L. jensenii* P_{6A}. The antimicrobial activity against different potential human urogenital tract pathogens showed similar minimum inhibitory concentration (MIC) values for the two isolated BSs: 16 μg ml^{-1} for *E. coli* and 128 μg ml^{-1} for *Klebsiella pneumoniae*, *Enterobacter aerogenes*, and *Staphylococcus saprophyticus*. Interesting is the antifungal activity against the potential vaginal pathogen *Candida albicans* ATCC 18804, which was inhibited at a concentration of 16 μg ml^{-1}. Furthermore, the biomolecules exhibited an antibiofilm activity showing the best result against *E. aerogenes* (its biofilm was disrupted for 64%).

Sambanthamoorthy et al. (2014), Cornea et al. (2016), and Merghni et al. (2017) reported the production of cell-associated antimicrobial BSs from different *Lactobacillus* strains, without any structural characterization. *Lactobacillus casei* produces two cell-associated BSs, named BS-B1 and BS-Z9 with an antioxidant activity (Merghni et al., 2017). At a concentration of 5.0 mg ml^{-1}, BS-B1 and BS-Z9 BSs showed 74.6 and 77.3% of α-diphenyl-β-picrylhydrazyl (DPPH) radical scavenging activity, respectively. Furthermore, the antiproliferative potential on human epithelial cells HEp-2 after 48 h showed calculated values

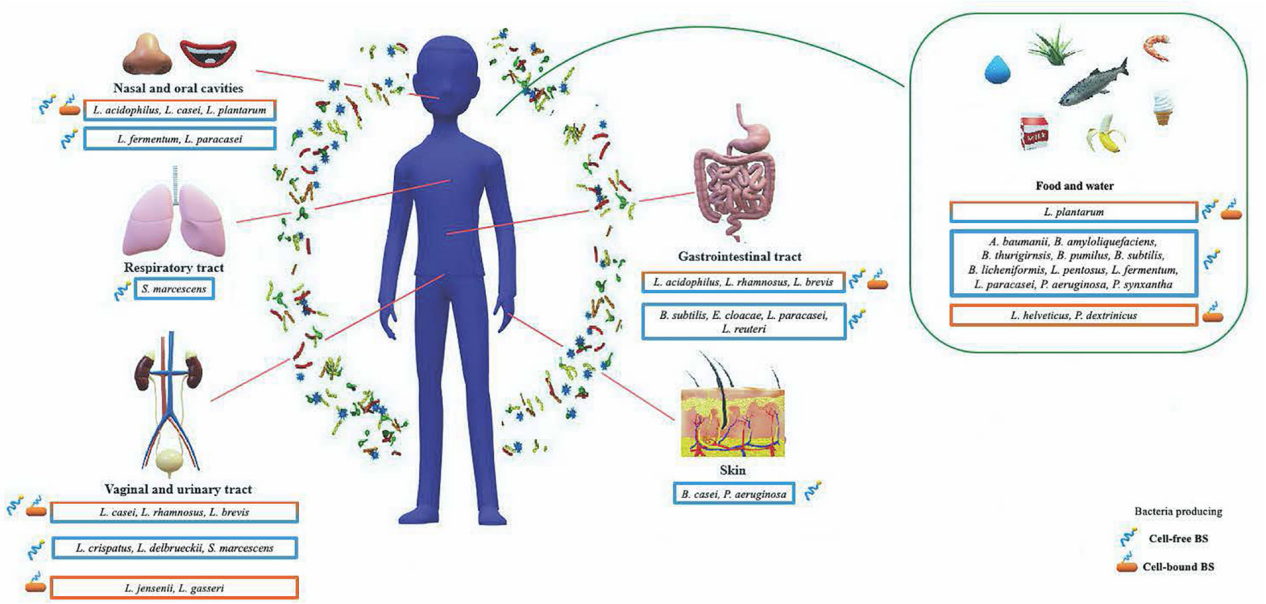

FIGURE 3 | Schematic representation of biosurfactants with antimicrobial activity produced by microorganisms associated with human health according to the human body districts. Bacteria enclosed in an orange box produce biosurfactants (BSs) with antimicrobial activity belonging to the cell-bound class; bacteria enclosed in a light blue box produce BSs with antimicrobial activity released in the surrounding environment; bacteria enclosed in an orange-light blue box produce BSs with antimicrobial activity belonging to both classes.

of IC_{50} that ranged from 109.1 to 129.7 mg ml^{-1}. Moreover, the antiproliferative effect of BSs was directly proportional to its concentration; indeed, at the maximum BS-B1 and BS-Z9 concentration of 200 mg ml^{-1}, the antiproliferative levels were 67.19% and 66.72%, respectively. The antimicrobial and antiadhesive activities were evaluated only against *S. aureus* strains (the reference strain ATCC 6538 and the oral strains 9P and 29P). BS-LZ9 showed an antibacterial effect against the ATCC 6538 strain, while BS-B1 was effective against the oral pathogens 9P and 29P, showing IC_{50} values of 1.92 and 2.16 mg ml^{-1}, respectively. However, the ATCC strain was more susceptible to the displacing (80.87–84.86% of inhibition) rather than 29P strain (48.74–68.84%) at a concentration of 12.5 mg ml^{-1}. The antiadhesive capacity was maintained at the lowest concentration (1.56 mg ml^{-1}) since both BSs inhibit the adhesion of *S. aureus* ATCC 6538 and 9P of almost 50%. The antibiofilm potential was effective also on pre-formed biofilms.

Cornea et al. (2016) isolated *Lactobacillus plantarum* L26, *L. plantarum* L35, and *Lactobacillus brevis* L61 from Romanian traditional fermented food for the ability to produce BSs. Their antimicrobial and antifungal activities were evaluated against microorganisms having a role in food contamination or spoilage. All the extracted cell-bound BSs could inhibit *E. coli* growth, while a limited inhibition effect was observed by the L61 strain BS against *Bacillus cereus*. No effect was evidenced against *S. aureus*, yeast, and fungi. However, the BSs were able to inhibit the mycotoxigenic fungi sporulation, without affecting the mycelial growth that was justified assuming a non-optimal BS concentration. Finally, Sambanthamoorthy et al. (2014) focused on *L. jensenii* and *Lactobacillus rhamnosus* able to inhibit clinical multidrug-resistant (MDR) strains of *Acinetobacter baumannii* (AB5075 and AB5711), *E. coli* EC433, and *S. aureus* strains [methicillin-resistant *S. aureus* (MRSA), clinical isolate 243, and UAMS-1]. Their crude BS extracts were effective against the MDR pathogens at a concentration of 50 mg ml^{-1}. The best inhibition was due to *L. rhamnosus* molecule whose activity suppressed *A. baumannii* growth of 96–97%, *S. aureus* UAMS-1 and MRSA strains between 80 and 93%, and *E. coli* of 72–85%. These BSs also showed antibiofilm potential in a range of 25 to 50 mg ml^{-1}. At the maximum concentration, *A. baumannii* and *E. coli* could not produce biofilm, while the ability was unpaired at lower concentrations for *S. aureus*. In order to use these biomolecules for biomedical applications, the cytotoxicity was tested against human A549 lung epithelial cells with different BS concentrations (from 25 to 200 mg ml^{-1}) for 24 h. Both biomolecules were a little bit toxic at the maximum concentration, while they were safe from 25 to 100 mg ml^{-1}.

Among other bacteria belonging to the Firmicutes phylum, an antimicrobial cell-bound BS was isolated from the lactic acid bacterium *Pediococcus dextrinicus* SHU1593 (Ghasemi et al., 2019). BS yield of 0.7 g L^{-1} was obtained when the strain grew on three substrates including a modified MRS medium (where glucose was replaced by lactose and Tween 80 was not present) and two low-cost materials such as molasses and date syrup. The BS solution at a CMC of 2.7 mg ml^{-1} showed the minimum ST of 39.01 mN m^{-1}. The chemical characterization pointed out its lipoprotein composition with around 52% (w/w) and 47% of lipid and proteins content, respectively

(the remaining 1% corresponds to sugars, but the authors attributed this portion to the precipitated culture medium). The predominant fatty acids were oleic (60.28%), palmitic (25.08%), stearic (7.43%), and lauric (4.60%) acids. The BS composition was not dependent on the growth media; thus, they all had similar nature, which was comparable with lipopeptides produced by members of *Bacillus* genus. At a concentration of 25 mg ml^{-1}, *Pediococcus* BS inhibited *E. coli*, *E. aerogenes*, and *Pseudomonas aeruginosa* growth. However, the biomolecule was not active against Gram-positive bacteria such as *B. cereus* and *S. aureus*. Nevertheless, an important antiadhesive activity was evidenced against *B. cereus* (70.5%), *P. aeruginosa* (61.84%), and *Salmonella typhimurium* (58.69%).

Biosurfactants Released by Bacteria in the Surrounding Environment

Most of the BSs with an antimicrobial activity produced by bacteria associated with human health are molecules in the class of BSs released in the surrounding environment belonging to Firmicutes and Proteobacteria phyla (**Supplementary Table 1**). Understanding their characteristics, mechanisms of action, and their release might help to elucidate the relationships between bacteria–bacteria and bacteria–environment. Consequently, this knowledge brings along the advantage of the high biotechnological potential. To date, the human-associated antimicrobial BSs described in the literature are included in the following chemical types: lipopeptides, glycolipids, glycoproteins, and glycolipoproteins.

Lipopeptide Biosurfactants

A proteinaceous nature is widely common among the molecules with an antimicrobial activity. For example, several antibiotics or bacteriocins have a protein nature, as well as the antimicrobial molecules released by the human body or by other cells.

Therefore, the importance of studying the protein domain through a genome-based or molecular approach for the prediction of the antimicrobial potential is clear, as evidenced by NRPS and PKS gene families (see section "Prediction of Biosurfactant With an Antimicrobial Activity by An "*-omic*" Approach").

Some BSs with a proteinaceous nature have an antimicrobial potential against Gram-negative and Gram-positive bacteria, and several have also antifungal activities principally against *Candida* spp. Numerous authors reported the potential of cell-released BSs as antimicrobial, antifungal, and antibiofilm agents (Gudina et al., 2010; Gomaa, 2013; Perez et al., 2017). Among members of *Lactobacillus* genus, only two research studies report strains able to release antimicrobial lipopeptide BSs (Abdalsadiq et al., 2018; Emmanuel et al., 2019).

The first study reports the bioactive potential of the lipopeptide fraction compared with the glycolipid fraction isolated from the cell cultures of *L. acidophilus* and *Lactobacillus pentosus* against several antagonists such as *Proteus mirabilis*, *S. aureus*, *Streptococcus pneumoniae*, *K. pneumoniae*, and *C. albicans* (Abdalsadiq et al., 2018). The inhibition measured by the agar well diffusion assay (AWDA) on the extracted BS resulted in haloes ranging from 14.00 mm (against *K. pneumoniae*) to 44.00 mm (against *S. aureus*). The quantification of the MIC revealed that the lipopeptide fraction had a stronger antimicrobial effect at lower MIC values ranging from 7.81 $\mu g\ ml^{-1}$ (against *P. mirabilis*) to 62.5 $\mu g\ ml^{-1}$ (against *K. pneumoniae*), whereas glycolipid fraction from 15.6 to 62.5 $\mu g\ ml^{-1}$. Furthermore, the antiadhesive activity against all pathogens showed inhibition percentages ranging from 65% (against *P. mirabilis* at a concentration of 250 $\mu g\ ml^{-1}$) to 93% (against *K. pneumoniae* at a concentration of 250 $\mu g\ ml^{-1}$) depending on the concentration of the lipopeptide fraction, while the antiadhesive activity of the glycolipid fraction produced a smaller percentage of inhibition (from 45 to 72.7%). Finally, the antibiofilm capacity of the molecules was demonstrated at a concentration of 250 $\mu g\ ml^{-1}$, whose effectiveness was up to 100%. Specifically, the lipopeptide fraction evidenced the maximum antibiofilm percentage against *K. pneumoniae* and *P. mirabilis* and the lowest against *S. aureus* (85%).

Another example of antimicrobial lipopeptide BS produced by lactobacilli is the one from a *Lactobacillus* sp. strain isolated from homemade curd, yielding 3.21 g L^{-1} (Emmanuel et al., 2019). It was characterized by the presence of alkene, alkyne groups, and conjugated diene and an emulsification index (E_{24}) of 58.1%. The antimicrobial and antibiofilm activities of the BS were tested only against those of *E. coli*. The first showed a comparable antimicrobial activity with respect to sodium dodecyl sulfate (SDS), and the BS inhibited the biofilm of *E. coli*. Indeed, after 6 h, the number of *E. coli* cells forming the biofilm was lower as the lipopeptide concentration increased.

Undoubtedly, one of the most well-known lipopeptide BS producers are members of the *Bacillus* genus (Zhao et al., 2017). Several strains are employed as probiotics because of the formation of spores that survive in extreme conditions, such as low gastric pH. Once in the intestine, spores can germinate; thus, *Bacillus* strains grow and re-sporulate, exerting an antimicrobial activity and other beneficial effects. However, nowadays, the use of *Bacillus* species as probiotics is disputed due to the capability of transferring genes for the antimicrobial resistance to the microbial population. Furthermore, the production of enterotoxins and biogenic amines by *Bacillus* strains is reported (Lee et al., 2019).

Nevertheless, helpful metabolites such as the antimicrobial lipopeptide BSs can be produced by members of the genus. For instance, a miscellaneous of surfactin lipopeptides was isolated from *B. subtilis* and *Bacillus amyloliquefaciens* supernatants after 24 h of fermentation on Malaysian fermented food: soybean known as *tempeh* showed a maximum surfactin yield at 84.08 mg L^{-1} and cassava *tapai* the lowest at 26.9 mg L^{-1} (Isa et al., 2020). However, the antimicrobial activity of the soybean BSs was not effective against *S. aureus*, *S. pneumoniae*, *Serratia marcescens*, and *S. typhimurium*, while the BSs from *tapai* inhibited the growth of both Gram-positive and Gram-negative tested bacteria. The most antimicrobial surfactins were produced by the growth on fish sauce *budu* showing a great inhibition halo against *B. cereus* (MIC 10 mg L^{-1}) and *S. pneumoniae* (MIC 25 mg L^{-1}) and a moderate one against *Listeria monocytogenes* (MIC 25 mg L^{-1}), *S. aureus* (MIC 25 mg L^{-1}), *K. pneumoniae* (MIC 25 mg L^{-1}), and *S. marcescens* (MIC 50 mg L^{-1}). Interestingly,

Isa et al. (2020) confirmed the surfactin-producing ability of the strains through the detection of the *sfp* marker gene as well as the genus affiliation.

Ultimately, Schlusselhuber et al. (2018) reported *Pseudomonas* strain UCMA 17988, isolated from raw cow milk, for its ability to produce lipopeptide BS, although *Pseudomonas* spp. are famous for rhamnolipid production (Pornsunthorntawee et al., 2010). The maximum yield of 47.6 mg L^{-1} was obtained after 4 days of cultivation. Interestingly, four molecules were identified differing at 14 Da, which suggested the presence of several lipopeptide isoforms. The hypothesis was confirmed by analyzing the differences due to the fatty acid chain: the major isoform was at 1,409 *m*/*z*, and the three other isoforms were detected at 1,381, 1,395, and 1,423 *m*/*z*. Therefore, the lipopeptide BSs were called "milksin" A, B, C, and D. The antimicrobial activity of the major isoform was observed against *S. aureus* CIP 53.154 with MIC of 0.5 mg ml^{-1}, against *L. monocytogenes* WSLC 1685, and *Salmonella enterica* Newport CIP 105629 with MIC of 1 mg ml^{-1}. Also, the antifungal activity was observed against strains representative of fungal groups: *Mucor hiemalis* CBS 201.65, *Aspergillus niger* CMPG 814, and *Cladosporium herbarum* CMPG 38 showed a major MIC of 20 mg ml^{-1}, and *Penicillium expansum* CMPG 136 showed a MIC equal to 20 mg ml^{-1}.

Glycolipid Biosurfactants

Glycolipids are complex molecules composed of a carbohydrate moiety and a lipid fraction. Although *Pseudomonas* spp. are the most prominent strains reported in the literature as glycolipid BS producers, also Gram-positive bacteria provide the same type of compounds that are released in the environment, for example, microorganisms grouped in the *Lactobacillus* genus.

Lactobacillus acidophilus NCIM 2903 is reported to produce a glycolipid type BS in 72 h of fermentation (obtaining 1.5 g L^{-1}) (Satpute et al., 2018). Indeed, its chemical characterization revealed the following principal functional groups: hydrocarbon, OH stretching, ester bonds, and sugars. The CMC was 625 μg ml^{-1}, which corresponds to a reduction of the ST from 72 to 27 mN m^{-1}. At the CMC value, the glycolipid inhibited 87% growth of *S. aureus* NCIM, 85% of *P. aeruginosa* MTCC 2297, 82% of *B. subtilis* MTCC 2423, 80% of *E. coli* NCIM 2065, 70% of *P. putida* MTCC 2467, and *P. vulgaris* of NCIM 2027. Satpute et al. (2018) utilized an innovative approach mimicking the biofilm microenvironment through microfluidic strategies to evaluate the antibiofilm property that showed no biofilm in the presence of the BS.

Also, *Lactobacillus helveticus* M5, isolated from yogurt, releases a glycolipid, characterized by a cycle aliphatic structure of the lipidic moiety when cultivated on lactose (5.5 g L^{-1} yield in 120 h) (Kadhum and Haydar, 2020). It displayed an E_{24} of 75.3% and a reduction of the ST until 33.2 mN m^{-1}. Its antimicrobial bioactivity was prevalently against Gram-positive bacteria than Gram-negative bacteria, showing an inhibition halo ranging from 15 to 31 mm against *S. aureus* and from 12 to 29 mm against *P. aeruginosa* at a concentration of between 20 and 100 mg ml^{-1}. Thus, the authors speculated that the glycolipid could interfere with the peptidoglycan layer of the Gram-positive bacteria, leading to dysfunctions of the cell wall. Besides, the glycolipid acted as an antiadhesive agent at a concentration of 50 mg ml^{-1}, inhibiting 78% and 74.5% of the adhesion of *S. aureus* and *P. aeruginosa*, respectively.

Among the gut commensal bacteria, *Enterobacter cloacae* B14 produced a glycolipid-like molecule releasing 39.8 mg BS (g cell dry weight)$^{-1}$ when yeast extract is used as a substrate. Its antimicrobial action was more pronounced against Gram-positive bacteria (inhibition haloes 20.7–26.7 mm against *B. cereus*, *B. subtilis*, and *S. aureus*) with respect to the Gram-negative bacteria (9.7–17 mm against *E. coli*, *P. aeruginosa*, and *S. marcescens*). Interestingly, the BS was more effective than the commonly used antibiotic tetracycline against *B. subtilis* (respectively 22 vs. 20 mm of growth inhibition), and the BS inhibited the growth of the tetracycline-resistant strain *S. marcescens* (Ekprasert et al., 2020).

As already mentioned, *P. aeruginosa* is the most studied bacterium for rhamnolipid production. These molecules are formed by a rhamnose moiety linked to an aliphatic variable chain with a BS property. Different rhamnolipids exhibit an antimicrobial activity, such as the ones released by *P. aeruginosa* CR1 (Sood et al., 2020; Wahib et al., 2020).

Pseudomonas aeruginosa CR1 BS showed considerable antimicrobial and emulsification activities; indeed, the E_{24} was 53%, and ST decreased until 35 mN m^{-1} (Sood et al., 2020). It was recovered after the strain grew on both Luria Bertani (LB) broth supplemented with glycerol and basal medium enriched with rice bran oil, showing a maximum production of 10 g L^{-1}. The chemical analyses revealed that *P. aeruginosa* strain CR1 produced only mono-rhamnolipids and that no di-rhamnolipids were detected. These data were confirmed by genome analyses showing the lack of *rhlC* gene coding for the rhamnosyl transferase responsible for di-rhamnolipid synthesis (**Figure 2C** and **Table 1**).

Wahib et al. (2020) evaluated *P. aeruginosa* strain, isolated from a clinical source, for its capacity to release 20.04 g L^{-1} of antimicrobial BS when grown on glycerol medium. The BS was characterized as a mixture of mono- and di-rhamnolipids with E_{24} of 88.18%. Interestingly, at a concentration of 0.5 or 1 g ml^{-1}, its rhamnolipids could inhibit *E. coli*, *K. pneumoniae*, and *S. aureus* growth, showing the maximum antimicrobial effect against *S. aureus*.

Glycoprotein Biosurfactants

Intriguingly, from literature retrieval, glycoproteins with antimicrobial and BS features are produced only by the *Lactobacillus* genus. Mouafo et al. (2018) investigated the potential of three *Lactobacillus* strains (*Lactobacillus delbrueckii* N2, *Lactobacillus cellobiosus* TM1, and *L. plantarum* G88) to produce BSs during growth on sugar cane molasses or glycerol. Their yields ranged between 2.43 and 3.03 g L^{-1} on sugar cane molasses (with E_{24} ranging between 49.89 and 81%) and from 2.32 to 2.82 g L^{-1} on glycerol (with E_{24} ranging from 41.81 to 61.81%). The molecules produced from the growth on glycerol were composed of a bigger fraction of lipids with respect to the BS obtained on sugar cane molasses. This suggested that

lactobacilli could direct the glycerol in the lipolytic pathway and gluconeogenesis, consequently generating more lipids. The growth of *L. cellobiosus* TM1 and *L. delbrueckii* N2 on sugar cane molasses led to producing glycoproteins without a lipid fraction. The measured protein and sugar content were, respectively, 52.93 g/100 g MS and 27.10 g/100 g MS for *L. cellobiosus* TM1-BS, and 63.64 g/100 g MS and 51.13 g/100 g MS for *L. delbrueckii* N2-BS. Since the presence of sugars is independent of the carbon source (sugar cane molasses or glycerol), the authors speculated that the hydrophilic substrates were broken down in glycolytic pathway intermediates, such as glucose-6-phosphate, which is the precursor carbohydrate found in the BS composition. The antimicrobial effect indicated that Gram-positive bacteria were more sensitive than Gram-negative. As an example, *Bacillus* sp. BC1 growth was the most affected by the action of *L. delbrueckii* N2 glycolipid BS showing 57.5 mm of inhibition zone.

Glycolipoprotein Biosurfactants

Likewise, the production of glycolipoprotein BS was recorded only from two *Lactobacillus* strains, *L. plantarum* G88 and *Lactobacillus paracasei* subsp. *tolerans* N2 (Hippolyte et al., 2018; Mouafo et al., 2018), although these complex molecules are often cell-bound because of their big dimensions (see the section "Cell-Associated Biosurfactants").

Briefly, *L. plantarum* G88 growth on sugar cane molasses produced a molecule characterized by 8.96 g/100 g MS proteins, 51.13 g/100 g MS sugars, and 39.60 g/100 g MS lipids (Mouafo et al., 2018). Distinguishing an antimicrobial activity from that of *E. coli* E6, *P. putida* PSJ1 and PSV1, and *Salmonella* sp. SL2 was evidenced by the diameter of their inhibition haloes of 32.00, 32.00, 32.00, and 41 mm, respectively.

Curiously, Hippolyte et al. (2018) exploited *L. paracasei* subsp. *tolerans* N2's ability to release bioactive compounds during growth on sugar cane molasses to evaluate the optimization of the production of an antimicrobial BS through a mathematical model. The model outputs were the predicted production yield and two values indicating the BS properties: the diameter of growth inhibition, a measure of the antimicrobial potential, and the ST related to the surfactant effect. After the fermentation under the optimal conditions (temperature between 33°C and 34°C, sugar cane molasses concentration ranging from 5.49 to 6.35%), they obtained an active BS with an experimental ST around 37.02 mN m^{-1}, which was comparable with the predicted value (36.65 mN m^{-1}). The best glycolipoprotein production conditions for the highest antimicrobial activity comprised the lowest percentages of molasses (5.49%) and the lowest temperature (33°C). The measured inhibition halo against *P. putida* PSJ1 was 63.89 mm, which was comparable with the predicted one (62.07 mm). Then, the antimicrobial activity was assessed against other bacteria: *P. aeruginosa* PSB2, *Salmonella* sp. SL2, *E. coli* MTCC 118, *Bacillus* sp. BC1, and *S. aureus* STP1. *S. aureus* and *Bacillus* were the most sensitive bacteria to the glycolipoprotein with a MIC of 3.2 mg ml^{-1}, while *Salmonella* and *E. coli* were the less sensitive with a MIC of 12.80 mg ml^{-1}. Subsequently, a partial chemical characterization revealed that the main constituents were proteins, sugars, and lipids (63.64 g/100 g DM, 35.26 g/100 g DM, and 1.10 g/100 g DM, respectively), suggesting a glycolipoproteins nature.

Other Cell-Released Biosurfactants

Some antimicrobial BSs related to bacteria associated with human health were described for their bioactive properties without an exhaustive chemical elucidation; or in few cases, the assembled chemical features make them part of new BS categories.

Although not characterized in-depth, the following examples showed the importance of BS properties for clinical, health-related, and nutrition problems and future applicative developments.

Foschi et al. (2017) focused the attention on the anti-gonococcal potential of *Lactobacillus* strains isolated from healthy premenopausal women. They principally belong to three different species: *Lactobacillus crispatus*, *L. gasseri*, and *Lactobacillus vaginalis* among which *L. crispatus* strains showed the best anti-*Neisseria gonorrhea* effect. In fact, their supernatant was able to eradicate *N. gonorrhea* viability after 7 and 60 min, while *L. crispatus* and *L. gasseri* species were capable only after 60 min. The most effective was produced by *L. crispatus* BC1, also possessing a potent BS property. The characterization of the molecules released in the supernatants indicated that their molecular weight was more than 10 kDa.

The BS extracted from *Pseudomonas synxantha* NAK1 stands out for its interesting biomedical application (Mukherjee et al., 2014). Indeed, the strain, isolated from *Mycobacterium smegmatis* plate, inhibits the *Mycobacterium* growth, which is a non-pathogenic bacterium model for the study of tuberculosis caused by *Mycobacterium tuberculosis* (Yamada et al., 2018). *P. synxantha* NAK1 cultivation generated metabolites that were preliminarily characterized as a 15-carbon aliphatic chain with intermediate oxygen and a terminal allyl bond with surfactant properties. The antimicrobial potential against other bacteria was thoroughly elucidated. The activity was very low against *E. coli* DH5α and *P. aeruginosa* AKS9 (MIC 200 μg ml^{-1}); moderate against *B. subtilis*, *Shigella sonnei* NK4010, and *S. typhimurium* B10827 (MIC 100 μg ml^{-1}); high against *S. aureus* ATCC 25923, *M. tuberculosis* $H_{37}Rv$, and BGC (MIC 50 μg ml^{-1}); and, finally, very high against two *M. tuberculosis* strains (mc^2155 and $H_{37}Ra$, MIC 25 μg ml^{-1}). Therefore, this kind of secondary metabolite produced by *P. synxantha* NAK1 could be useful as an anti-tubercular agent against the mycobacteria pathogens.

Within the Proteobacteria phylum, other *Pseudomonas* strains revealed promising antimicrobial BSs. *P. aeruginosa* ATCC 10145 provides up to 1 g L^{-1} of BS, characterized by an ST lowering capacity of 40 mN m^{-1} (from 72 to 32 mN m^{-1}). The BS has also antimicrobial and antifungal activities showing an effect against *Sarcina lutea*, *Micrococcus luteus*, and *Bacillus pumilus*; and among the fungi, the effect was against *Penicillium chrysogenum* and *C. albicans* (El-Sheshtawy and Doheim, 2014).

Among the first paper published regarding the *Lactobacillus* genus within the considered decade (2010–2020), Gudina et al. (2010) described a BS extracted from *L. paracasei* ssp. *paracasei* A20, which was isolated from Portuguese dairy plant. The extracted molecule was tested against

18 microorganisms, including species associated with the oral cavity, pathogenic bacteria, yeasts, and skin-associated pathogenic fungi. The antimicrobial potential was observed vs. all the strains, and the growth inhibition was observed for around 67% of the microorganisms at 50 mg ml^{-1}. Only the cariogenic *Streptococcus mutans* strains NS and HG985, *P. aeruginosa*, the yeast *Malassezia* sp., and the fungi *Trichophyton mentagrophytes* and *Trichophyton rubrum* were non-sensible to the BS molecule. Regarding the antiadhesive capacity, a BS concentration of 50 mg ml^{-1} inhibited the non-pathogenic *Lactobacillus reuteri* and *L. casei* of 77.6–78.8% and 56.5–63.8%, respectively.

The work of Gomaa (2013) described 10 *Lactobacillus* strains isolated from Egyptian dairy products among which *L. paracasei* produced a BS with an antimicrobial activity against *C. albicans*, *S. aureus*, and *Staphylococcus epidermidis*. Therefore, the authors compared this capacity with that of *L. paracasei* A20. Results showed that the novel extracted BSs demonstrated more potent antiadhesive compounds with respect to A20 strain. However, the best antiadhesive potential was attributable to *Lactobacillus fermentum* bioactive molecule (84.69% of inhibition) (Gomaa, 2013).

Other BS molecules were produced by strains isolated from food matrices. Two *L. plantarum* strains, called L26 and L35, and *L. brevis* strain L61, isolated from a Romanian traditional fermented food, produced BSs with an antimicrobial effect only against *E. coli* (Cornea et al., 2016).

Another example of BS from food derivatives is the screening of BS-producing capacity of bacteria isolated from dairy products, breast milk, fermented shrimps, and fruits (Abdalsadiq and Zaiton, 2018). Among 160 bacteria and 70 randomly selected to test the BS activity, only 20 cell-free supernatants were positive to drop collapse test and oil spreading assay. Furthermore, only six of the isolates were able to reduce the water-surface tension, leading to an average reduction from 72.22 to 37.21 mN m^{-1}. The antibacterial activity was evidenced only for nine cell-free supernatants. Among them, the isolate *L. acidophilus* Fm1 was the most effective because it could inhibit the growth of *Pseudomonas fluorescence* (33.4 mm of zone inhibition), *S. typhimurium* (30.4 mm), *P. aeruginosa* ATCC 2785 (29.7 mm), *P. aeruginosa* 14T28 (25.5 mm), and *E. coli* (20.2 mm) (Abdalsadiq and Zaiton, 2018).

Moreover, among the four *L. plantarum* strains (Is2, Is9, Is12, and Is13) isolated from plantain wine (*Mbamvu*, or banana wine), a typical African fermented beverage, one isolate showed interesting BS and antimicrobial features. It was able to strongly inhibit the growth of selected pathogens, such as *E. coli* (3.3 cm of growth inhibition halo), *Shigella flexneri* (4.2 cm), *Salmonella* sp. (3.3 cm), *P. aeruginosa* (3.5 cm), and *S. aureus* (4 cm) (Moukala et al., 2019).

The last research studies represent an important description of the properties of bacterial bioactive compounds related to food and beverage fermented matrices as beneficial products for people's health and ultimately to raise knowledge about nutritional issues (Parthasarathi and Subha, 2018).

APPLICATION OF BACTERIA-DERIVED BIOSURFACTANTS WITH ANTIMICROBIAL ACTIVITY

The attention of the scientific community on BS antimicrobial compounds is rapidly growing because of their intrinsic characteristics of BSs and antimicrobial agents, the interest towards the producer strains, and their low organismal and environmental impact.

The intrinsic surface-active capacity of these compounds can be evaluated by drop-collapsing method and oil displacement tests and quantified by a tensiometer that measures the reduction of water-surface tension (Walter et al., 2010). In general, the most efficient surfactant molecules are those able to reduce the water-surface tension from 72 to around 30 mN m^{-1} compared with a standard at a defined condition (Saimmai et al., 2020). One of the most powerful antimicrobial BSs from bacteria associated with human health is the glycolipid from *Lactobacillus acidophilus* NCIM 2903 since it reduced the ST to 27 mN m^{-1} (Satpute et al., 2018). Another effective BS is produced by *Bacillus subtilis* VSG4, which showed a minimum ST value of 27.2 mN m^{-1} at pH 7 (Giri et al., 2019). BSs produced by members of *Lactobacillus* genus can be also considered as effective surfactants compared with synthetic ones (Santos et al., 2016). For instance, the BS released by *Lactobacillus paracasei* subsp. *tolerans* N2 lowered the ST to 37.85 mN m^{-1} (Hippolyte et al., 2018), while the glycolipoproteins from *Lactobacillus jensenii* P_{6A} and *Lactobacillus gasseri* P_{65} reduced the water-surface tension to 43.2 and 42.5 mN m^{-1}, respectively (Morais et al., 2017).

Considering the other bioactive functions of these compounds deriving from bacteria associated with human health, some authors suggest that the antimicrobial BSs produced by *Lactobacillus* spp. could be employed in the prevention or treatment of hospital-acquired infections. Indeed, these new antimicrobial BS agents showed an antagonist effect against bacteria causing infections and diseases in the urinary, vaginal, and GI tracts, as well as in the skin (Gudina et al., 2010); a very low cytotoxic effect on human lung epithelial cells; and antimicrobial, antiadhesive, and antibiofilm capacity against clinical MDR strains (Sambanthamoorthy et al., 2014). Additionally, they can be used on various surfaces of biomedical devices as antimicrobial, antiadhesive, and antibiofilm agents (Satpute et al., 2019; Kadhum and Haydar, 2020) or for controlling bacterial overgrowth in the food and nutraceutical industry (Cornea et al., 2016; Hippolyte et al., 2018; Mouafo et al., 2018; Emmanuel et al., 2019; Moukala et al., 2019; Isa et al., 2020; **Supplementary Table 1**).

Consequently, the number of potential applications in different fields, especially in biomedical, food safety, and nutraceutical sectors, is currently increasing.

Moreover, nowadays, the interest in greenways of producing add-value compounds is rapidly growing, in this specific case, the attention is towards BSs with an antimicrobial activity.

Therefore, it is fundamental to underline the importance of the valorization of by-products, residues, and wastes through biological processes to avoid the loss of other useful resources for

new business models (Hippolyte et al., 2018; Mouafo et al., 2018; Singh et al., 2018; Deseo et al., 2020).

Biosurfactants derived from human-associated bacteria that utilize by-products or cheap substrates could have a great impact on biotechnological and industrial levels. For instance, various members of the *Lactobacillus* genus are able to produce valuable antimicrobial BS molecules during the fermentation of sugar cane molasses, which are products with generally recognized as safe (GRAS) status of the steam process of the sugar cane mill industry (Hippolyte et al., 2018; Mouafo et al., 2018; Deseo et al., 2020). Consequently, the need for more research studies and in-depth investigations on novel bioactive compounds with a high rate of production for other potential applications is still high.

Biomedical Applications

Nowadays, the resistance to antimicrobial substances represents a big challenge to face, above all in the hospital environment. Indeed, the use of the BSs as alternatives to conventional antibiotics is a promising answer to this issue that can be developed by *Lactobacillus* BSs (Gudina et al., 2010; Hippolyte et al., 2018). These bioactive compounds are considerably efficient against potential pathogens responsible for diseases and infections in the urinary, vaginal, and GI tracts, as well as in the skin. The BSs produced by *L. jensenii* P_{6A} and *L. gasseri* P_{65} can have a role as antibiotic agents for the vaginal compartment, because of their significant antimicrobial activities against *Escherichia coli* and *Candida albicans* and their antiadhesive potential against *E. coli*, *Staphylococcus saprophyticus*, and *Enterobacter aerogenes* (Morais et al., 2017).

The need for "antibiotic-free" strategies vs. genital diseases, such as gonorrhea, can be accomplished by safe bioactive compounds. For example, Foschi et al. (2017) suggested that the BS isolated from *Lactobacillus crispatus* BC1 is capable of killing *Neisseria gonorrhoeae* after a short contact period. This novel isolated compound could be used in the prevention of *N. gonorrhoeae* infections in women since the disturbing pathogen had developed antimicrobial resistance.

In the field of oral diseases and their prevention, the BS-LBI and BS-LZ9 BSs from *Lactobacillus casei* LBI could be employed for their antibiofilm capacity that was demonstrated against two *Staphylococcus aureus* strains isolated from the oral cavity of Tunisian patients. Moreover, the BS molecules tested on human epithelial cell line HEp-2 showed an antiproliferative effect (Merghni et al., 2017).

Of particular interest is the BS coating agents of surfaces, such as catheters or other instruments for biomedical support. Satpute et al. (2019) extracted a glycolipoprotein-type BS from the cell surface of *L. acidophilus* NCIM 2903 that showed antibiofilm and antiadhesive activities on polydimethylsiloxane-based contact lens surfaces. This kind of study deals with the problem of the failure of implants due to the colonization of biofilm-forming microorganisms, often resistant to antibiotics. In this context, the glycolipid released by *Lactobacillus helveticus* M5 with the antimicrobial and antiadhesive actions against *Pseudomonas aeruginosa* and *S. aureus* could be also very useful as a coating agent (Kadhum and Haydar, 2020).

In line with these discoveries, the antibiofilm, antiadhesive, and antimicrobial BSs extracted from *L. jensenii* and *Lactobacillus rhamnosus* support the employment of BSs on abiotic surfaces as a medical coating instrument to combat microbial colonization. It could be also speculated to use them in a topical application since both the molecules resulted in low cytotoxicity on human A549 lung epithelial cells (Sambanthamoorthy et al., 2014).

The studies conducted by Gomaa (2013), Mukherjee et al. (2014), Abdalsadiq et al. (2018), Abdalsadiq and Zaiton (2018), Satpute et al. (2018), Clements et al. (2019a), Emmanuel et al. (2019), Ghasemi et al. (2019), and Giri et al. (2019) provided new diverse antimicrobial BSs deriving from different bacteria with possible employment in the biomedical field. Their relevance is linked to the field as adjuvants in immunology, drug delivery system, therapeutic agents, gene deliveries, antimicrobial, antiadhesive, and antibiofilm agents (Saimmai et al., 2020).

Application in Food and Nutraceutical Industries

In the field of the food industry, the most important factor is the quality of the products, which is strictly connected to their provenience, maintenance, and storage and the product safety for the health of the consumers (Nalini et al., 2020). Therefore, texture, consistency, aroma, taste, and safety had a role in the perception of the food and the assignment of excellence. In this context, surfactants deriving from microorganisms are more advantageous than chemical ones, because they are less toxic, biodegradable, and eco-friendly (Nalini et al., 2020).

Among the producer strains, lactobacilli are interesting because they usually have the GRAS status, and they are also naturally present in the food products.

One of the principal applications of BSs in the food industry is as bio-emulsifiers. The emulsifying properties of BSs from *Lactobacillus* strains were evaluated and exploited against edible oils such as sunflower and olive oil (Cornea et al., 2016; Emmanuel et al., 2019). Besides, the antimicrobial activity of *Lactobacillus* sp. strain from homemade curd BS was effective against *E. coli* (Emmanuel et al., 2019), and one of the lactobacilli derived from Romanian traditional fermented food BSs was effective also against *Bacillus cereus*, as a food pathogen (Cornea et al., 2016).

In the same context, Hippolyte et al. (2018) and Mouafo et al. (2018) isolated, respectively, *L. paracasei* subsp. *tolerans* N2 (capable of producing an antimicrobial BS from traditional fermented milk known in Cameroon as "*pendidam*") and *Lactobacillus delbrueckii* N2, *Lactobacillus cellobiosus* TM1, and *Lactobacillus plantarum* G88 for their fermenting ability of sugar cane molasses and the production of antimicrobial BSs that generate stable emulsions for at least 72 h at room temperature. These properties lead to the hypothesis that the molecules can form and stabilize emulsions and could be suitable as bio-preservatives.

It is important to underline that it is preferable to use non-pathogenic organisms in a bioprocess, such as in the case of the

antimicrobial BS production from *Pediococcus dextrinicus* strain SHU1593 (Ghasemi et al., 2019).

Moreover, the isolated bioactive molecule should be defined as safe to utilize as antimicrobial agents in products that come in contact with humans, such as food and beverage products. For this reason, it is fundamental to verify this requirement, for instance, through the evaluation of the viability of a cell line in the presence of a bioactive compound under analysis (Basit et al., 2018). Another important requirement for bioactive compounds with surfactant and emulsification features is the antimicrobial activity for the biocontrol of pathogenic and food spoilage bacteria as suggested by Giri et al. (2019). Indeed, *B. subtilis* VSG4 and *Bacillus licheniformis* VS16 BSs showed stable emulsification and appropriate ST values in pH values, respectively, ranging from 4 to 10 and from 5 to 9 and in the presence of different temperatures, from 20°C to 90/100°C for 30 min. Besides, they exert their antimicrobial potential against both Gram-positive and Gram-negative bacteria (Giri et al., 2019).

Intriguingly, Moukala et al. (2019) and Isa et al. (2020) raised the attention on the local fermented food beverages as a source of bioactive molecules together with the intrinsic bioactive substances such as polyphenols, flavonoids, and carotenoids. The BSs produced by *L. plantarum* strains and *P. aeruginosa* strain, isolated, respectively, from banana wine and Malaysian fermented foods, could have a role not only in the food industry but also in the health state of the local population. BSs can be also important molecules for the nutraceutical industry to stabilize the formulation thanks to the emulsification and stabilization properties, and the antiadhesive and antimicrobial capacity.

CONCLUSION AND FUTURE PERSPECTIVES

Bacteria associated with human health are capable of producing antimicrobial BSs with great biomedical potential, useful in the food industry and generally beneficial for human health. For example, the employment of the antimicrobial and antibiofilm molecules could prevent hospital-acquired infections. Furthermore, molecules with antibiofilm properties could be utilized in the eradication and preservation of urogenital infections in addition to or as replacements of conventional antibiotics.

In the field of the BS industry, the search for novel strains able to ferment by-products or able to use renewable/cheaper substrates is very important and constantly increasing. Moreover, the application of molecules derived from GRAS bacteria that are already defined as safe for contact with human bodies is fruitful for the food industry.

Generally, some bioactive compounds can be employed for environmental application, thus improving the ecosystem from which our food derives and the habitat we are in contact with.

An important goal that still needs to be reached is the elucidation of the chemical features of the already extracted but not yet characterized BSs, to enhance the opportunities for possible therapeutic approaches. Furthermore, deeper knowledge about the encoding genes for the BS production or insight into the mechanisms involved in the production process could be interesting for the prediction of this capability, to better set up a development and the scale-up of possible industrial projects.

AUTHOR CONTRIBUTIONS

AD conceived the review and developed the whole manuscript by writing the different text parts. JZ wrote the section "Prediction of Biosurfactant With an Antimicrobial Activity by an "*-omic*" Approach" and revised the whole manuscript by providing useful suggestions. PD helped in shaping the manuscript. All the authors provided critical feedback and contributed to the final manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.655150/full#supplementary-material

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Conflict of Interest: The 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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ORIGINAL ARTICLE

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Identification of a bacteriocin-like compound from *Lactobacillus plantarum* with antimicrobial activity and effects on normal and cancerogenic human intestinal cells

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Abstract

In this paper, we demonstrate that the antimicrobial activity of *L. plantarum* PBS067 strain against antagonist microorganisms was mediated by the production of a bacteriocin-like compound secreted at the stationary phase of the growth. The novel bacteriocin-like compound, designed plantaricin P1053, was identified by using sorption–desorption method, butanol extraction and SEC-HPLC. The molecular mass of plantaricin P1053 was shown to be 1053 Da by ESI-MS analysis. Plantaricin P1053 exhibited a broad-spectrum antimicrobial activity against Gram-positive bacteria as *S. aureus* and Gram-negative bacteria as *E. coli*. In addition to the antimicrobial activity, the isolated bacteriocin-like compound showed effects on normal and cancerogenic epithelial intestinal cell lines through an enhancing of viability of healthy cells and a proliferation reduction of cancer cells. Moreover, in this paper we demonstrate that the isolated bacteriocin-like compound acts on healthy cells through the epidermal growth factor receptor (EGFR) pathways. In conclusion, plantaricin P1053 isolated from *L. plantarum* PBS067 strain could represent one of the first multifunctional bacteriocin-like compound acting on human epithelial intestinal cells.

Keywords: *Lactobacillus plantarum*, Bacteriocin-like compounds, Antimicrobial activity, Healthy cells, Anti-tumoral activity

Introduction

Probiotics are recognized as live microorganisms that confer a health benefit to the host when administered in adequate amounts (FAO/WHO 2001). These bacteria exert health-promoting properties, including the effects on specific tissues, particularly on the intestine. Most of the mechanisms involve the modification of the gut microbiota because of the immune-modulating attitude on the intestinal tissues (Valeur et al. 2004) or the release of essential micronutrients in the guts, such as group B vitamins (LeBlanc et al. 2011) and the production of substances such as organic acids or bacteriocins with antagonistic activity against potential pathogens (Servin 2004). Among the antimicrobial substances produced by these bacteria, bacteriocins are interesting because of their several applications, like natural food preservatives or novel therapeutic agents to complement conventional antibiotics (Trivedi et al. 2013). The biochemical characterization can be an important goal to understand their mode of action. In fact, bacteriocins are ribosomally synthesized antimicrobial peptides or complex proteins secreted by various Gram-positive and Gram-negative bacteria (Desriac et al. 2010). Based on biochemical properties, bacteriocins have been divided into three groups: family of lantibiotics (Class I), small non-modified peptides resistant to heat and pH (Class II), larger heat-labile

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proteins (Class III) (Klaenhammer 1993; Porto et al. 2017). Class II is subdivided into four sub-groups. One of the four sub-groups is the Class IIA that contains bacteriocins known as "pediocin-like" peptides whose synthesis requires four genes encoding proteins regulated by a quorum sensing mechanism (Da Silva et al. 2014; Diep et al. 2009). These proteins and peptides are known for their antimicrobial properties against some potential pathogens like *Escherichia coli*, *Staphylococcus aureus* and *Bacillus* spp. (Ahmad et al. 2017; Zhao et al. 2016). Many of these bacteriocins are produced from *L. plantarum* strains. Some of them have been studied, such as *L. plantarum* JLA-9 (Zhao et al. 2016), *L. plantarum* ZJ5 (Song et al. 2014), *L. plantarum* ZJ008 (Zhu et al. 2014). The bacteriocins isolated from these strains have the peculiarity to have a very low molecular weight around 1000–3000 Da.

The mechanism of action of bacteriocins is not well understood, but in literature it is reported that they can kill target bacteria by membrane permeabilization or by binding to a specific membrane protein called "bacteriocin receptor", where the interaction between peptide and receptor protein leads to membrane leakage and cell death (Oppegard et al. 2016). This mechanism explains both the extreme potency of many bacteriocin-like compounds and their narrow inhibition spectra. However, we still lack knowledge about the nature of the interaction between these compounds and receptors, and also the interaction between these compounds and intestinal human cells. Some authors reported that bacteriocins can exert some beneficial effects on humans, because of their interaction with the intestinal epithelia. It is reported that metabolites from probiotic cultures showed an anti-proliferative effect on human colon cancer (do Lee et al. 2008; Ma et al. 2010; You et al. 2004). Moreover, Ma et al. (2010) speculated that the activity is mediated through the inhibition of Epidermal Growth Factor Receptor (EGFR) kinase activity. Also Yan et al. (2011) described the effect of a bacteria-derived soluble protein p40 from *Lactobacillus rhamnosus* GG, which was able to prevent cytokine induced apoptosis in intestinal epithelial cell lines through the regulation of the activation of Akt. Nevertheless, probiotic effects are strain dependent and different species might have different mechanisms of action (Dimitrovski et al. 2014).

In a previous work (Presti et al. 2015), strains of *Lactobacillus* spp. and *Bifidobacterium* spp. were characterized for their probiotic properties, among which there was the antimicrobial activity. Previous results of cell-free supernatants at neutral and acidic pH from all selected probiotic strains and the direct growth inhibition of the potential pathogens on the LABs, made us speculate that bacteriocin-like compounds can be produced, especially from *Lactobacillus plantarum* strain PBS067, showing a strong antimicrobial activity.

The aim of this work was to demonstrate that the antimicrobial activity exhibited from *L. plantarum* strain PBS067 against antagonist microorganisms was mediated by a bacteriocin-like compound, produced and secreted in the medium during the growth of the strain. The purified compound was described as likely belonging to an uncommon group of bacteriocins (Class II). The novelty of the work is that plantaricin P1053 revealed to possess both an antimicrobial activity against pathogenic bacteria and an effect on host cells through an enhancement of healthy cells viability and a reduction of cancerogenic intestinal cells viability.

Materials and methods

Strains and culture conditions

Lactobacillus plantarum strain PBS067 (Presti et al. 2015) (deposited at DSMZ culture collection as DSM 24937), isolated from the feces of healthy humans, was supplied from a private collection (Principium Europe S.r.l., now Roelmi Hpc). *L. plantarum* PBS067 was selected for this study for its ability to exhibit a strong antimicrobial activity against different human pathogens as *E. coli*, *P. aeruginosa*, *S. aureus*, *E. faecium* after 24 h of incubation at 37 °C. Unless otherwise specified, *L. plantarum* was cultured in deMan, Rogosa and Sharpe (MRS) medium. The cultures were incubated at 37 °C under anaerobic conditions using anaerobic atmosphere generation bags (Anaerogen, Oxoid).

As antagonistic microorganisms for antimicrobial activity assay, *E. coli* ATCC 25922, *Staphylococcus aureus* ATCC 6538, *Pseudomonas aeruginosa* ATCC 9027, and *Enterococcus faecalis* ATCC 2922 were employed. The cultures were performed in Luria–Bertani medium (LB) agar, modified by Lennox known as LD (Lennox 1995), at 37 °C in aerobiosis.

Screening for antimicrobial activity

The antimicrobial activity was determined by the well diffusion agar assay (WDAA) for lactobacilli according to the protocol of Santini et al. (2010) with some modifications. Overnight MRS cultures were centrifuged at 7000 rpm at 4 °C for 15 min. The pHs of the supernatants were measured and recorded. The supernatants that were naturally at pH 4 were collected and filtered through 0.22-μm pore filter membranes to remove any residual bacterial cell (CFS, Cell Free Supernatant). The CFS antimicrobial activity was measured against *E. coli, P. aeruginosa, E. faecalis*, *S. aureus* bacteria. In particular, the antagonist strains were inoculated into LD medium and let to grow until the optical density at 600 nm (OD_{600}) was 0.5, corresponding to around 10^7 CFU/mL.

2.5% (v/v) of the culture was inoculated into 20 mL of LD agar and the plates were allowed to solidify. Four wells of 8 mm in diameter were made on each agar plate with a sterile glass cylinder. 50 µL and 100 µL of *L. plantarum* PBS067 culture supernatant (CFS) was dispensed into each well; neutralized-acidified PBS067 CFS, not-inoculated and acid not-inoculated MRS (100 µL) were used as controls. Plates were incubated overnight at 37 °C in aerobiosis. The growth inhibition haloes were measured.

The same method was used to test the antimicrobial activity of the concentrated crude extract and the purified bacteriocin-like compound against *E. coli*, and *S. aureus* as representative strains of Gram-negative and Gram-positive bacteria, respectively.

Purification of the bacteriocin-like compound from culture medium of *L. plantarum*

Adsorption/desorption method (step 1)

A modification of Yang et al. (1992) extraction method, based on the hydrophobicity and the charge of the compounds secreted by the cells, was used in order to identify bacteriocin-like compounds from *L. plantarum* strain PBS067 cultures.

First, the strain was pre-cultured in 15 mL of MRS medium at 37 °C overnight, in anaerobic conditions. Then, the pre-culture was inoculated in four flasks each containing 300 mL of MRS medium with an initial 0.01 OD_{600}. The flasks were incubated at 37 °C, for 16 h, in microaerophilic conditions. Final cultures had an OD_{600} between 3 and 4, which corresponded to the initial latency period.

The established method allowed to partially purify cell secreted compounds on the basis of the pH of extraction medium promoting the adsorption/desorption of these molecules from the lactic acid bacteria membrane. Therefore, in the step 1 the cultures were neutralized to pH 6.5 with NaOH 1.5 M and let to stand for 30 min at room temperature. Then, they were centrifuged at 7000 rpm at 4 °C for 10 min. After the cells had been washed with a phosphate buffer (0.1 M pH 6.5), they were resuspended in 60 mL of NaCl 0.1 M at pH 2 and mixed with a magnetic stirrer for 1 h at 4 °C. Cell suspension was then centrifuged at 16,000*g* for 30 min at 4 °C. This fraction of the supernatant containing the bacteriocin-like molecule (called crude extract) was used for further analysis and characterization.

An aliquot of this fraction containing the bacteriocin-like molecule was tenfold concentrated and used for WDAA (see above) in order to assess its antimicrobial activity using 100 µL of the concentrated crude extract. 100 µL of cell free supernatant (CFS) (see above) and 100 µL of NaCl 0.1 M at pH 2 were used as positive and negative controls, respectively. The growth inhibition zones around the wells were measured.

Before proceeding with the purification, the pH of the fraction was increased up to 10–11 with NaOH 1.5 M, and then the solution was lyophilized.

Organic phase extraction (step 2)

The lyophilized crude extract (deriving from 300 mL of supernatant) was extracted with 10 mL of *n*-butanol under agitation, at room temperature for 20 h. This step was repeated twice. Then, the butanol extracts were filtered through a paper filter and the solvent was evaporated. The residue was resuspended in 1 mL of methanol and analysed in SEC-HPLC.

Size exclusion chromatography (SEC) HPLC (step 3)

SEC analyses were performed with a Waters 600E delivery system equipped with Waters 486 UV–Vis detector and a Phenogel 5 100 A, Phenomenex, 300 × 4.6 mm column eluted in isocratic conditions with methanol at a flow rate of 0.3 mL/min. The eluted compounds were detected at 254 nm and their retention times were compared with the retention time of the standard nisin, the model bacteriocin from *Enterococcus lactis*. In these conditions, the retention time of nisin was 11.8 min. An aliquot of the eluted compound containing the bacteriocin-like molecule was tenfold concentrated and used for WDAA in order to assess its antimicrobial activity.

Characterization of the bacteriocin-like plantaricin P1053 by ESI-full mass spectrometry (MS)

The eluted compound containing the bacteriocin-like molecule was analysed by the electrospray ionization ESI-full mass spectrometry. The analysis was performed on LCQ Fleet ion trap mass spectrometer. The MS was operated in positive ionization mode acquiring spectra in the *m/z* range of 200 to 2000.

Effects of proteinase K enzyme on the stability of the purified bacteriocin-like compound and total protein concentration

In order to evaluate the purified bacteriocin-like molecule sensitivity to the proteinase K (pH 7.5; Sigma Aldrich, Italy), the bacteriocin-like compound was treated with 1 mg/mL (final concentration) proteinase K (ratio 1:5) at its optimal pH. After 3 h of incubation at 37 °C, the reaction was stopped at 4 °C. Subsequently, the sample was adjusted to pH 2 using 6 M HCl and assayed for antimicrobial activity. The bacteriocin-like molecule at the original pH (pH 2) without any heat or enzyme treatments was used as the control sample. The agar well diffusion assay was carried out to test the remaining

activity against the indicator strain *E. coli* ATCC25922 (Santini et al. 2010).

The total protein concentration was assessed on the partial purified and purified bacteriocin-like molecule extracts using a Pierce BCA protein assay kit (Thermo Fisher Scientific, Italy) according to the manufacturer's protocol. Protein concentrations were calculated from the standard curve by 20 mg/mL bovine albumin serum.

Maintenance and growth of cell lines for in vitro tests

CCD 841 (ATCC CRL-1790) healthy colon cell line (Thompson et al. 1985) were grown in EMEM medium supplemented with heat-inactivated 10% FBS, 2 mM L-glutamine, 1% non-essential amino acids, 100 U/mL penicillin and 100 µg/mL streptomycin and maintained at 37 °C in a humidified 5% CO_2 incubator.

E705 colon cancer cell line (supplied by IRCCS Foundation, Cancer National Institute) (Mozzi et al. 2015) was grown in DMEM medium supplemented with heat-inactivated 10% FBS, 2 mM L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin and maintained at 37 °C in a humidified 5% CO_2 incubator.

All the reagents for cell culture were supplied by EuroClone (EuroClone S.p.A, Pero (MI), Italy).

Viability assay

Cell viability was assessed using an in vitro MTT based toxicology assay kit (Sigma). Cells were seeded in a 96-well micro titer plates at a density of 8×10^4 cells/100 µL and incubated overnight. The attached cells were treated with a range of plantaricin P1053 up to 1 µg/mL. After 48 h of treatment, MTT test was performed according to the manufacturer's protocol and absorption was measured at 570 nm using a micro plate reader to assay the effect of plantaricin P1053 on healthy CCD 841 cells and colon cancer E705 cells. Results were expressed as mean values ± SD of three determinations.

Protein extraction from treated cell lines and analysis

After treatment with plantaricin P1053 at 0 min, 30 min, 1 h, 3 h and 24 h, cells were washed with ice-cold PBS and lysed in RIPA buffer, containing protease and phosphatase inhibitors and 1 mM PMSF. After lysis on ice, homogenates were obtained by passing crude extracts five times through a blunt 20-gauge needle fitted on a syringe and subsequently centrifuging them at 14,000 rpm for 30 min at 4 °C. Supernatants were analyzed for protein content by the BCA protein assay (Smith et al. 1985).

SDS-Page and western blot were carried out by standard procedures. Thirty micrograms of proteins were separated on 10% acryl-amide/bis-acrylamide SDS-PAGE, transferred onto a nitrocellulose membrane (Millipore, Billerica, MA), probed with the appropriate antibodies and visualized using ECL detection system (Millipore). Protein levels were quantified by densitometry of immunoblots using ScionImage software (Scion Corp., Fredrick, MD). We used the following primary antibodies (all purchased by Cell signaling Technology, Danvers, MA): anti-EGFR (dilution 1:1000), anti-phospho-EGFR (Tyr 1068; dilution 1:1000), anti-p44/42 MAPK (Erk1/2; dilution 1:1000), anti-phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204; dilution 1:1000), anti-Akt (dilution 1:1000), anti-phosho-Akt (Ser473; dilution 1:1000) and anti-GAPDH (dilution 1:10,000). IgG HRP-conjugated secondary antibodies (purchased by Cell Signaling Technology) were diluted 1:10,000.

Statistical analysis

Experiments were performed in triplicate and results were presented as mean values ± standard deviation. The statistical relevance was assessed by Student's t test. The significance was defined as p value < 0.05.

Results

Identification of antimicrobial activity of *L. plantarum* strain PBS067

Lactobacillus plantarum strain PBS067 was grown until reaching the stationary phase of the cells and the cell free supernatant (CFS) was used in the antimicrobial activity through the well diffusion agar assay. A halo of growth inhibition of the antagonist bacteria was observed and measured (Table 1). Results showed a halo of inhibition with a diameter of 18 mm ± 0.2 for *E. coli* and 20 mm ± 0.2 for *S. aureus* as representative Gram-negative and Gram-positive bacteria, respectively.

Results were compared to the acidified non-inoculated medium as negative control, in order to exclude that the inhibition was caused by the low pH of the medium after the growth. Moreover, obtained data showed that the antimicrobial activity was also maintained when the

Table 1 Dimension of the growth halo inhibition measured by CFS from *L. plantarum* PBS067 against the antagonist bacteria

Antagonist bacteria	Halo inhibition by *L. plantarum* PBS067 (mm)	
	50 µL[a]	100 µL[a]
Escherichia coli ATCC 25922	18 ± 0.2	35 ± 0.2
Enterococcus faecalis ATCC 2922	10 ± 0.5	20 ± 0.5
Pseudomonas aeruginosa ATCC 9027	20 ± 0.5	40 ± 0.5
Staphylococcus aureus ATCC 6538	20 ± 0.2	40 ± 0.2

The measures of the inhibition zone are expressed in mm

[a] The concentration of the solution containing the plantaricin P1053 was of 200 µg/mL

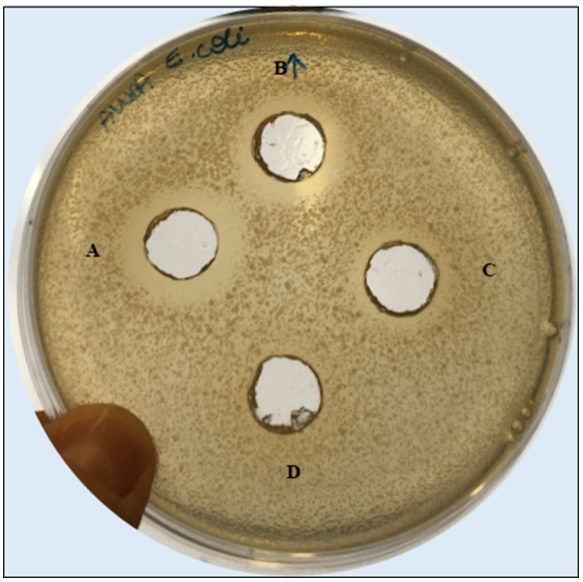

Fig. 1 Antimicrobial activity of *Lactobacillus plantarum* PBS067 cell free supernatant. The antimicrobial activity of *L. plantarum* PBS607 cell free supernatant (CFS) was determined as halo of growth inhibition of *E. coli* ATCC 25922 through the agar well diffusion assay. The figure shows the inhibition zones produced by CFS (A), by neutralized-acidified CFS (B), not inoculated MRS at pH 4 (C), not inoculated MRS (D) against *E. coli* ATCC 25922

supernatant was neutralized and then brought again to acid pH, as reported as an example for *E. coli* in Fig. 1. For this reason, we hypothesized that the strain PBS067 was able to produce bioactive bacteriocin-like compounds inhibiting the growth of the tested antagonist bacteria. As the bacteriocin-like compounds produced by lactobacilli are strain-specific and with a peculiar kind of biological activity, we decided to isolate and characterize the bioactive compounds produced by *L. plantarum* PBS067 strain and to test their range of antimicrobial activity.

Purification of the bacteriocin-like compound from *L. plantarum* strain PBS067

Bacteriocin-like compounds were isolated from cell free supernatant (CFS) of *L. plantarum* PBS067 culture using the modified method described by Yang et al. (1992) based on the hydrophobicity and the charge of the compounds secreted by cells. The established method allowed to extract and partially purify the bacteriocin-like molecules in a fraction called crude extract. The crude extract was screened for the antimicrobial activity using the agar well diffusion assay to verify that this property was maintained after this extraction phase (Fig. 2A, step 1). Results were compared to NaCl 0.1 M at pH 2 as negative control and CFS as positive control. The assay showed an increase in specific antimicrobial activity in this fraction (crude

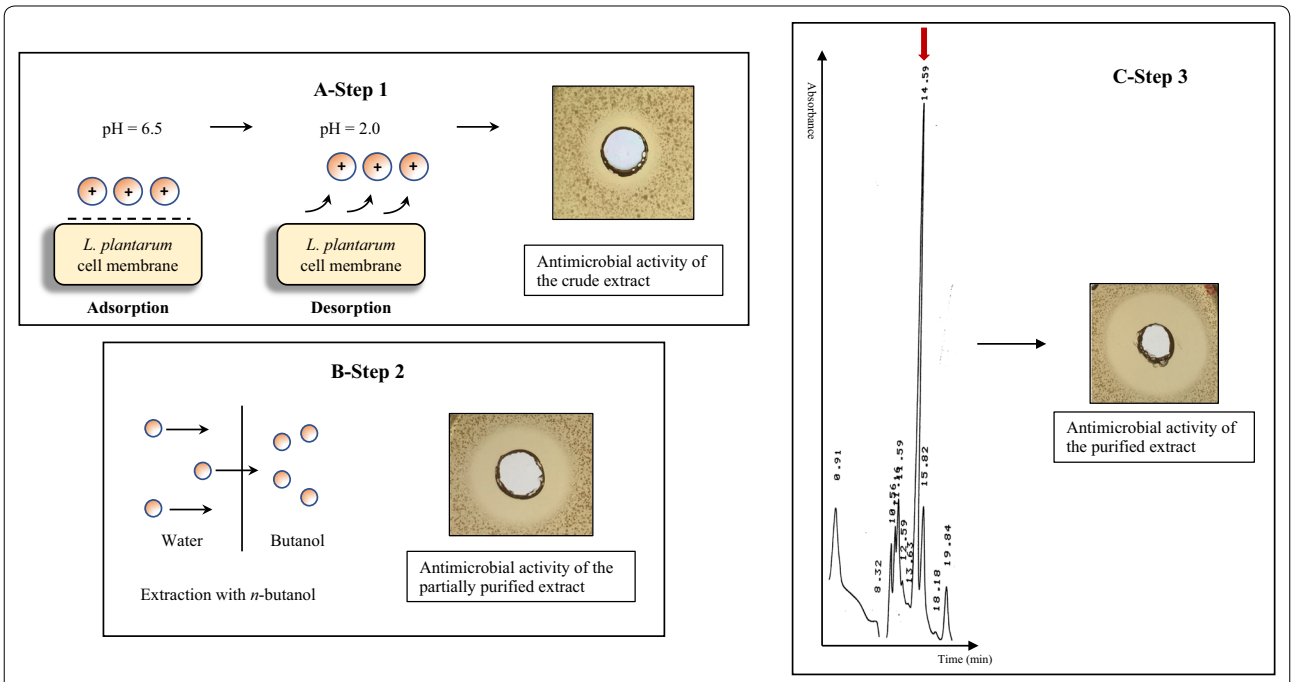

Fig. 2 Step-by-step purification and antimicrobial activity of the bacteriocin-like compound. The antimicrobial activity of the purified extract was verified through agar well diffusion assay. Step 1 of purification and inhibition halo produced by the crude extract obtained after adsorption/desorption method (A), step 2 of purification and antimicrobial activity of the partial purified extract through an extraction with *n*-butyl alcohol (B), step 3 of purification and the growth inhibition of the eluted bacteriocin-like compound (C). All samples were tested against *E. coli* ATCC 25922

extract) measured through the dimension of the inhibition halo. The activity was not shown at neutral and alkaline pH, but it was recovered when the pH was lowered to 4, the lactobacilli physiological pH.

Then, the lyophilized crude extract from *L. plantarum* supernatant was submitted to another step of purification performed with *n*-butyl alcohol. After the antimicrobial activity of the partially purified extract deriving from this second step was demonstrated, the obtained extract was analyzed and characterized (Fig. 2B, step 2).

The extract deriving from *n*-butanol extraction was first analyzed in HPLC by Size Exclusion Chromatography (SEC). The UV/visible absorption spectra of the sample revealed a maximum absorbance–wavelength at 254 nm. Results from SEC-HPLC analysis showed a main peak at 254 nm with a retention time of 14.5 min whose eluate maintained the antimicrobial activity (Fig. 2C, step 3). As a preliminary characterization, the purified extract was compared with the only reference standard available, the 3354.0 Da nisin from *Lactococcus lactis*. Based on the comparison of the retention times, the main peak of the extract had a molecular weight lower than 3354.0 Da. On this basis, the ESI mass spectrometry analysis of the purified extract will be used to thoroughly characterize the bacteriocin-like compound and determine the molecular weight.

Effects of proteinase K on the stability of the purified bacteriocin-like compound and total protein concentration

The sensitivity of the purified bacteriocin-like molecule to the proteinase K was evaluated after 3 h of incubation at 37 °C with the hydrolytic enzyme. Results indicated that the treatment with proteinase K showed the expected effect on the purified bacteriocin-like molecule; in fact, the inhibitory activity of the purified bacteriocin-like molecule against *E. coli* strain ATCC25922 was completely lost (Fig. 3) after the treatment.

The crude extract and the purified bacteriocin-like molecule were positive to BCA protein assay. In particular, the total protein concentration of the crude extract was 200 µg/mL, while the corresponding expected concentration was 3900 µg/mL based on the initial dry weight of the supernatant. After the purification of the bacteriocin-like molecule, the total protein concentration was 4000 µg/mL, while the corresponding calculated concentration was 4140 µg/mL based on the lyophilized amount. Results demonstrated that the bacteriocin-like compound was a protein-like molecule and that after purification a 100% enrichment of proteins was determined.

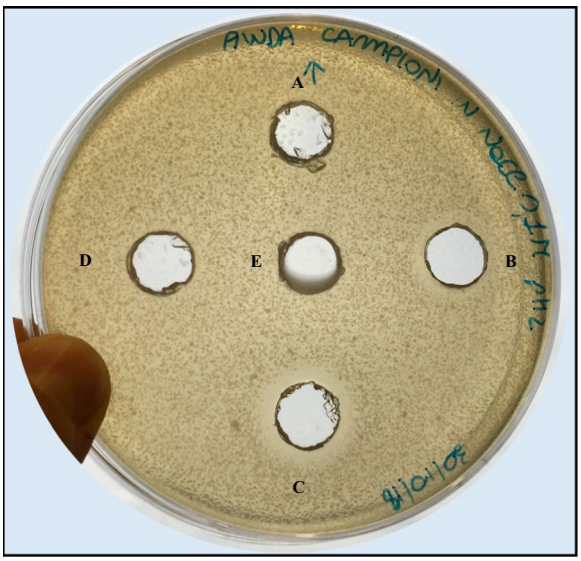

Fig. 3 Effect of proteinase K on the purified bacteriocin-like compound. The antimicrobial activity of the purified bacteriocin-like compound was determined as halo of growth inhibition of *E. coli* ATCC 25922 through the agar well diffusion assay. The Figure shows the lack of inhibition zone produced by the treatment of the bacteriocin-like compound by proteinase K (A); the inhibition halo produced by the no-treated bacteriocin-like compound after incubation at 37 °C and pH 7.5 (the proteinase K optimal pH) (B); the inhibition zone produced by the untreated bacteriocin-like compound at pH 2 (its optimal pH), as a positive control of the test (C); the lack of inhibition halo produced by the negative control NaCl 0.1 M pH 7.5 (buffer of the reaction with proteinase K) (D); and the lack of inhibition halo produced by the negative control NaCl 0.1M pH 2 (buffer for bacteriocin-like molecule activity) (E)

Characterization of the bacteriocin-like compound by ESI mass spectrometry

We decided to perform an ESI-full MS analysis on the enriched fraction of our bacteriocin-like compound. This analysis revealed that the extract contained a main compound with a molecular weight of 1053 Da (Fig. 4) (from here named plantaricin P1053); smaller than the most important plantaricins described in literature as for example plantaricin C19 (3.8 kDa) from *L. plantarum* C19 (Atrih et al. 2001), but with a molecular weight similar to those of JLA-9 from *L. plantarum* JLA-9 (Zhao et al. 2016). The weight is in agreement with the results obtained by SEC-HPLC chromatography.

Antimicrobial spectrum activity of plantaricin P1053

Antimicrobial spectrum activity of the purified plantaricin P1053 was determined by the measure of the halo inhibition of the growth against *E. coli* and *S. aureus* as representative of Gram− and Gram+ antagonist bacteria, respectively. Results are showed in Fig. 5. Plantaricin P1053 exhibited a notable antimicrobial activity against both *E. coli* and *S. aureus* with a growth inhibition zone of 2.5 cm and 3.0 cm, respectively. The inhibition

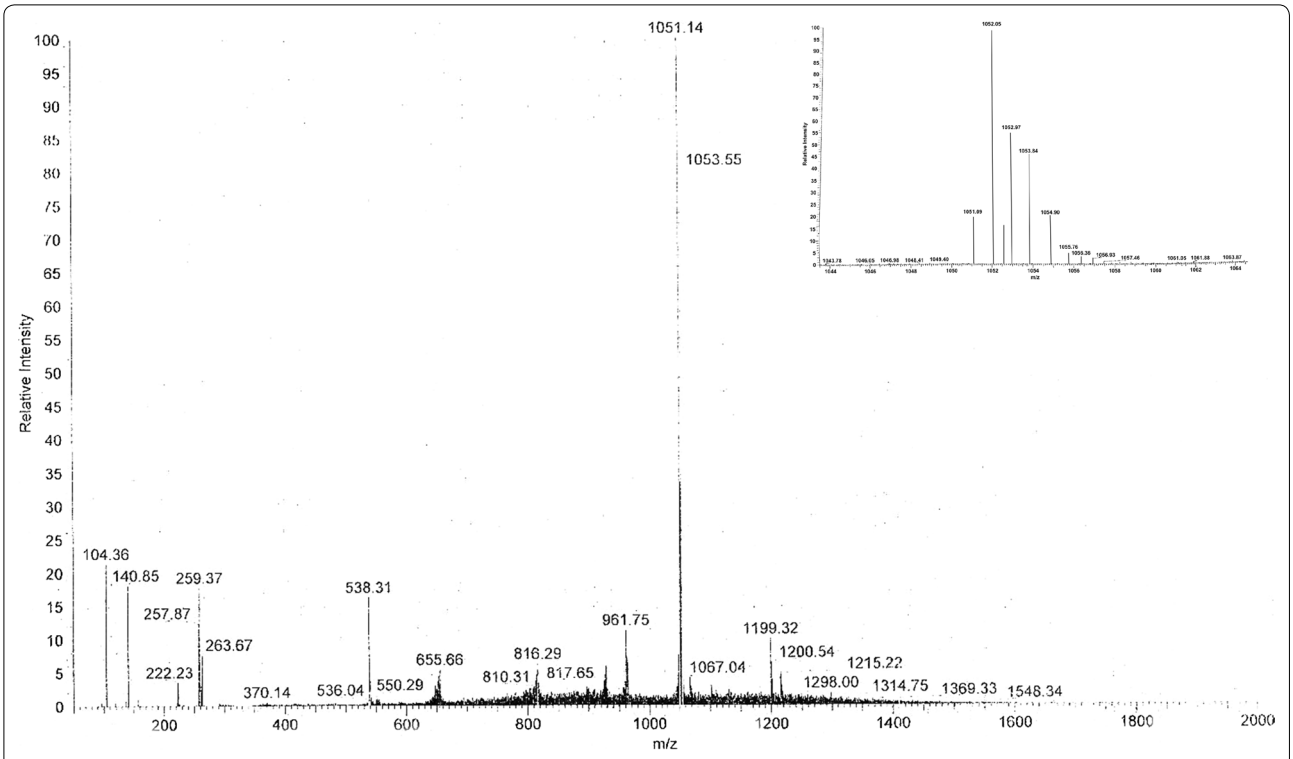

Fig. 4 Analysis of the bacteriocin-like compound by mass spectrometry. Full scan mass spectrum of the extract in positive ESI ion detection mode with one main fragment showing *m/z* = 1053.05 Da. ITMS + c HESI E full MS (1.16 e4). The area around the M+ is enlarged in the insert

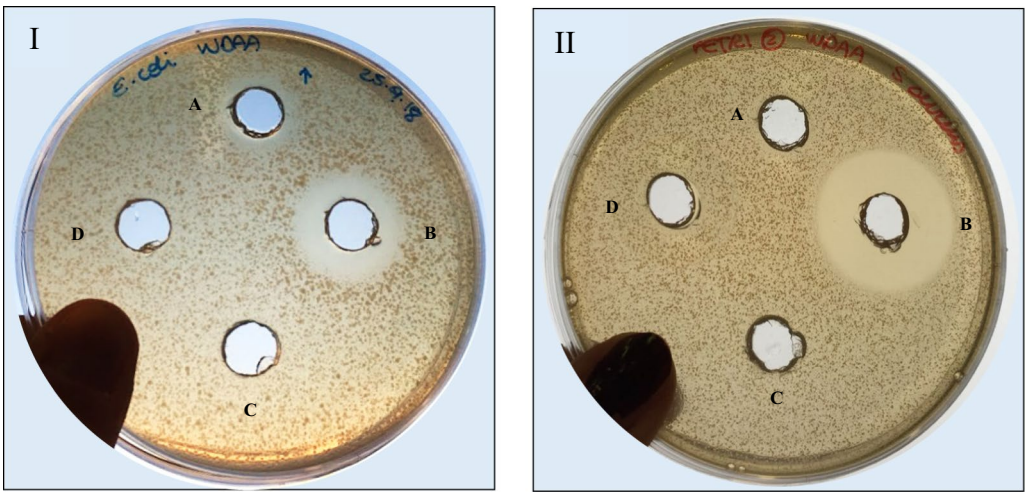

Fig. 5 Antimicrobial spectrum activity of plantaricin P1053. The antimicrobial spectrum activity of the plantaricin P1053 was verified through the inhibition growth halo in the agar well diffusion assay. The first panel shows the inhibition haloes against *E. coli* ATCC 25922 and the second panel against *S. aureus* ATCC 6538 produced by the plantaricin P1053 at neutral pH (A), by the plantaricin P1053 at pH 2 (B), by the NaCl 0.1 M at pH 2 (C) and by NaCl 0.1 M at pH 6.5 (D) as negative controls

spectrum of plantaricin P1053 appeared to be relatively wide because of the activity against both Gram-negative and Gram-positive bacteria.

In addition, the minimum inhibition growth concentration (MIC) of plantaricin P1053 against these bacteria was determined and data are reported in Table 2. Results

Table 2 Minimum inhibitory concentration (MIC) of the growth measured by plantaricin P1053 isolated from *L. plantarum* strain PBS067 against *E. coli* ATCC 25922 and *S. aureus* ATCC 6538

Plantaricin P1053 (μg/mL)	*E. coli* ATCC 25922 growth inhibition (%)	*S. aureus* ATCC 6538 growth inhibition (%)
250.00	65.74	89.55
125.00	62.70	91.29
62.50	64.12	96.02
31.25	62.95	69.25
15.62	66.25	0.00
7.81	68.85	0.00
3.90	14.41	0.00

show that the MIC values are 7.8 μg/mL for *E. coli* strain ATCC25922 and 31 μg/mL for *S. aureus* strain ATCC 6538, respectively.

These data indicated an antimicrobial activity similar to those of the plantaricin JLA-9, which presented a MIC of 16 μg/mL against *S. aureus* and 64 μg/mL against *E. coli* strain ATCC25922.

In vitro test of plantaricin P1053 on healthy colon CCD 841 cell line

In order to verify that plantaricin P1053 could have beneficial effects also on human intestinal epithelial cells, we initially verified the viability of the healthy colon CCD 841 cell line in the presence of the obtained plantaricin. To this end, the viability of CCD 841 cells treated with 1 μg/mL of plantaricin P1053 was assayed by MTT test. Results reported in Fig. 6 show an increase in CCD 841 cell viability of about 20%. It appears that plantaricin P1053 acts on healthy cells as previously demonstrated for a few bacteriocins isolated from lactobacilli (Wang et al. 2014; Tao et al. 2005). So, we decided to investigate whether the isolated bacteriocin-like molecule was able to influence some pathways in healthy human cell lines.

To evaluate a possible role for plantaricin P1053 in the complex pathway triggered by EGFR activation in healthy cell lines, cells were treated with 1 μg/mL of plantaricin P1053 for different times (0 min, 30 min, 1 h, 3 h and 24 h). We decided to use CCD 841 cells because they represent the wild type cell line (healthy cells) for all the proteins involved in EGFR pathway (Akt and Erk proteins are not mutated).

Western blot analysis showed an activation of EGFR pathway in the healthy human cell line CCD 841. Plantaricin P1053 administration to CCD 841 cells led to an increase of phospho-Akt as an early response (30 min–3 h) and a significant decrease as a late response (24 h). Erk showed a peak in its phosphorylation level after 30 min of exposure to the bacteriocin-like compound, that decreased gradually from 1 to 3 h after the administration, becoming completely unphosphorylated (ND) after 24 h (Fig. 7).

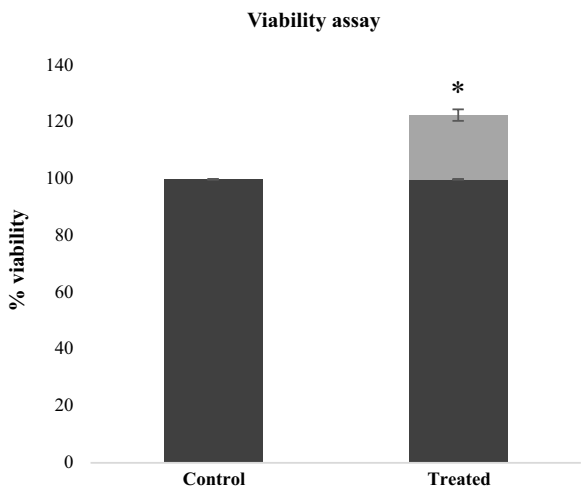

Fig. 6 Evaluation of cell viability on normal CCD 841 cell line after treatment with plantaricin P1053. MTT test was performed on normal CCD 841 cell line. Buffer NaCl 0.1 M pH 2 was used as control and it did not have any effect on cell viability. Values are presented as mean ± SD. Statistical analyses were performed using the Student's t-test. *$p < 0.05$

In vitro test of plantaricin P1053 on colon cancer E705 cell line

A possible beneficial effect of plantaricin P1053 on cancerogenic epithelial intestinal cells was also evaluated. A viability test of E705 cells treated with different concentrations of plantaricin P1053 was performed. Results showed a significant inhibitory effect, near 30%, on E705 cells proliferation in a concentration dependent manner. The higher concentration of plantaricin 1053 (1 μg/mL) evidenced the higher inhibitory effect, as reported in Fig. 8.

Also in this case a possible role for plantaricin P1053 in the complex pathway triggered by EGFR activation in cancerogenic cell lines, was evaluated. For this, cells were treated in the same manner with plantaricin P1053 for different times (0 min, 30 min, 1 h, 3 h and 24 h). By Western blot analysis no phosphorylation of Akt or Erk was detected, although both proteins were detected in their unphosphorylated forms (data not shown).

Discussion

This work describes the isolation and characterization of a bacteriocin-like compound produced by *Lactobacillus plantarum* strain PBS067 that shows an antimicrobial activity against potential human pathogens and that

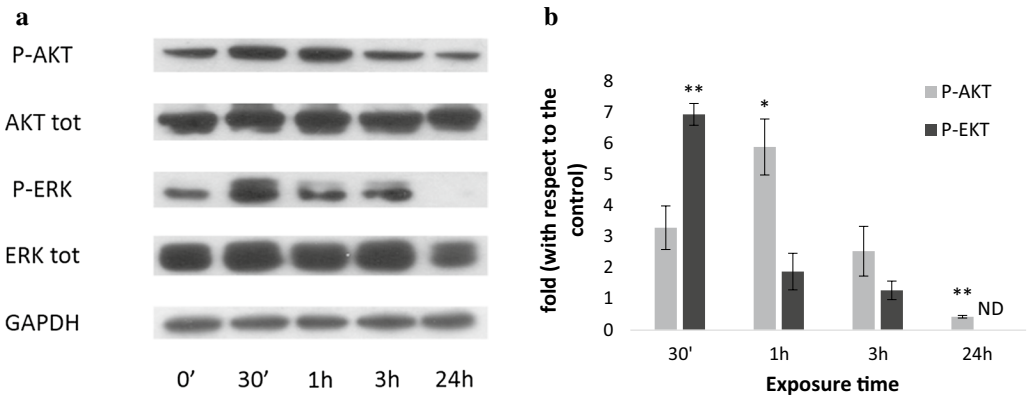

Fig. 7 Western blotting analysis of EGFR pathway activation after exposure to plantaricin P1053 in CCD 841 cell line. Representative western blotting analyses performed on crude extracts, using anti-P-AKT, anti-AKT, anti-P-ERK1/2, anti-ERK1/2 and anti-GAPDH antibodies. GAPDH was used as a control. The experiments were performed in triplicate (**a**). Densitometric analysis performed with Scion Image Software. Values are expressed by comparing the data obtained after the treatment with plantaricin P1053 with those obtained from 0′ of exposition to the compound (**b**). Values are presented as mean ± SD. ND, no protein phosphorylation was detected

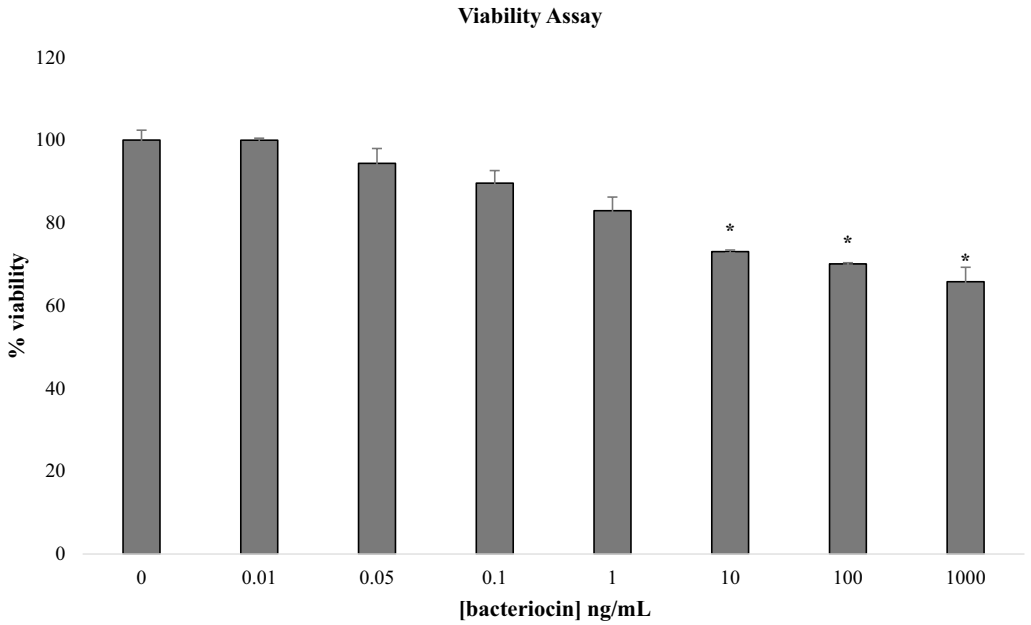

Fig. 8 Evaluation of cell viability on E705 cancer colon cell line after treatment with plantaricin P1053. MTT test was performed on cancerogenic E705 cell line. Buffer NaCl 0.1 M pH 2 was used as control and it did not have any effect on cell viability (100% viability). Values are presented as mean ± SD. Statistical analyses were performed using the Student's t-test. *$p < 0.05$

affects both normal and cancerogenic human intestinal cells. The probiotic strain, isolated from healthy patients, was able to produce the maximum amount of a bacteriocin-like compound at the onset of the stationary phase of the growth, during an incubation for 16 h at 37 °C at pH 4. This result is quite similar to that showed by Messi et al. (2001), who reported that *L. plantarum* 35d started to produce bacteriocin during the late logarithmic growth phase. Plantaricin 35d reached the maximum concentration after 19 h of incubation (stationary phase), at pH 4.

Preliminary experiments evidenced that the cell free supernatant (CFS) of *Lactobacillus plantarum* PBS067 culture had an inhibition of the antagonist bacteria growth, as *E. coli* and *S. aureus*. Thus, a purification procedure of the CFS metabolites was performed.

On the basis of the pH dependence, we decided to use the adsorption/desorption method as the first step in the purification procedure (Yang et al. 1992). The second step of purification consisted of the extraction with a solvent like *n*-butanol, and the last consisted of the elution in SEC-HPLC. At each purification step, the antibacterial activity was tested and a comparison of the activity of the acidified purified compound in respect to the crude CFS, was verified. Results showed an increase of the antimicrobial activity along the purification procedure.

The purified compound from supernatant of *L. plantarum* strain PBS067 was then characterized. An enzymatic hydrolytic activity by proteinase K was demonstrated on the isolated compound.

The mass analysis by ESI-full MS determined a molecular mass of 1053 Da for the compound (plantaricin P1053). Because of its bactericidal activity, its behavior based on the hydrophobicity and the charge of the bioactive compound secreted by the cells, pH resistance and low molecular mass, plantaricin P1053 could be classified as a small bacteriocin-like presumably belonging to Class II, according to the definition given by Klaenhammer (1993).

Our data are in line with the results obtained by Zhao et al., where for the first time a plantaricin with a low molecular weight of 1044 Da able to act against *Bacillus* spp. was isolated from *L. plantarum* strain JLA-9 (Zhao et al. 2016) and obtained by Zhu et al. that described the identification of the plantaricin ZJ008 with a MW of 1335 Da able to act against Gram-positive and Gram-negative bacteria (Zhu et al. 2014).

Interestingly, this paper demonstrates not only that the bacteriocin-like compound isolated from *L. plantarum* PBS067 showed a broad range of antimicrobial activity towards Gram-positive and Gram-negative bacteria with a good MIC, but also its activity towards human cells, extending its role from a bacteriocin to a multifunctional factor that has effect both on normal and cancer cells.

In fact, in this work we investigated also plantaricin P1053 effect towards human cells using both healthy and cancerogenic cell lines. Plantaricin P1053 was tested on human intestinal epithelial cells. Unlike other authors, like Dimitrovski et al. (2014) who used the supernatant of the broth culture, in this study we administered only the bacteriocin-like molecule resuspended in NaCl buffer. The plantaricin P1053 increased the vitality of healthy CCD 841 cell line. These results made us speculate that this bacteriocin-like compound could act on pathways involved in cell survival and proliferation, such as the epidermal growth factor receptor (EGFR) pathway. We have decided to investigate this pathway because it has been reported by Wang et al. (2014) that a *Lactobacillus rhamnosus* GG-derived soluble protein, p40, is able to activate EGFR and its downstream target Akt in intestinal epithelial cells, leading to an inhibition of apoptosis and a preservation of the barrier function by an upregulation of mucin production. Moreover, Tao et al. (2005) reported that a soluble factor from the probiotic strain *Lactobacillus rhamnosus* GG could rapidly activate the MAPKs. The activation of EGFR, Akt and Erk was therefore investigated after plantaricin P1053 administration at different times. This compound is able to rapidly activate, within 30 min, Akt and Erk in healthy intestinal cells. The phosphorylation of Akt leads to an anti-apoptotic effect, while Erk activation is pro-proliferative. It is important to note that Akt and Erk activation is finely controlled, with a decrease in their phosphorylation level till a nearly switch off 24 h after treatment.

Concerning the effect of plantaricin P1053 on cancerogenic epithelial cell lines, we observed a significant loss of viability, near the 30%, of E705 cells in a concentration dependent manner. These data are in line with those of Dimitrovski et al. on different cancerogenic cell lines (Dimitrovski et al. 2014), although they used only the supernatant obtained from *L. plantarum* cultures.

SDS-PAGE electrophoresis showed no Akt and Erk activation in cancerogenic E705 cells, leading us to think that the difference in the vitality between healthy and cancer cells is due to EGFR downstream targets activation in the former. Moreover, our experiments show that plantaricin P1053 is able to reduce E705 cancer cells viability, although the mechanism involved is still not elucidated; at the same time its effect on healthy intestinal cells is an increase in viability, due to a transient phosphorylation of both Akt and Erk which is achieved with a different timing and is readily switched off in 24 h.

In conclusion, in this paper we have demonstrated that the antimicrobial activity of *L. plantarum* PBS067 is mediated by the plantaricin P1053 action. The novelty of the paper is that in addition to exhibiting an antimicrobial activity against both representative Gram-positive and Gram-negative bacteria, this compound can also affect the host cells through an enhancing of healthy cells and a reduction of cancer cells viability. Although some molecular mechanisms must be elucidated in further studies, plantaricin P1053 could represent one of the first multifunctional bacteriocin-like compound on human epithelial intestinal cells.

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Authors' contributions

PDG and PF planned the experiments. ADG and FB performed experiments. GS performed chemical analysis. PDG and MF analysed and interpreted the data. PDG and GS wrote the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
All data generated during this study are included in this article and its additional files.

Ethics approval and consent to participate
All procedures performed in this study were compliance with Ethical Standards. This article does not contain any studies with human participants or animals performed by any of the authors.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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PVP-co-DMAEMA as Novel Polymeric Coating Material for Probiotic Supplements Delivery

*Giuseppe D'Orazio, Alessandra De Giani, Jessica Zampolli, Zahraa Zeaiter, Patrizia Di Gennaro, and Barbara La Ferla**

The paper investigates the use of poly(1-vinylpyrrolidone-co-2-dimethylaminoethylmetacrilate) (PVP-co-DMAEMA) as a novel coating agent in the preparation of alginate-based microcapsule for the supplement delivery of probiotics. Probiotic *Lactobacillus plantarum* is used to study the viability of the encapsulated bacterium when exposed to conditions simulating the gastrointestinal tract and industrial process. Results demonstrate that the PVP-co-DMAEMA polymer constitutes a pH-responsive coating suitable for probiotic supplement delivery.

1. Introduction

Probiotics have been frequently associated with a positive effect on maintaining microbial gut balance and on combating harmful pathogens and diseases when administered in a sufficient amount.[1–5] Their stability and viability are a prerequisite for their functionality in the host and could be hindered during probiotic manufacturing and storage by adverse conditions including, high temperature, oxygen, acidity, and humidity.[6] Furthermore, probiotics are subject to harsh physiological stimuli, such as low pH, bile salts, osmotic, and oxidative stresses, through the passage in the gastrointestinal tract (GI) and could lose their viability before reaching the host intestine where they should be released.[7] In this respect, encapsulation techniques are commonly applied to limit the loss of viability as probiotic pass in the gastric tract[8]; nevertheless, there is still the necessity to find new biocompatible polymers with improved properties, to be used in probiotics and supplements delivery.

Microcapsules based on alginate core have been widely applied in probiotic delivery systems.[9] This polymer is used as encapsulating material due to its mild gelling conditions. However, the limitation of using alginate as sole encapsulating agent is due to the inability of this polymer to withstand low pH encountered in the stomach. To overcome this limitation, multiple coating techniques able to sequential removal in different physiochemical conditions have already been proposed by us[10,11] and others.[12,13]

We recently developed a probiotic formulation containing *L. plantarum*, encapsulated in alginate-glucose core and chitosan coating microcapsule.[10] The chitosan coat layer was used to protect alginate core in low gastric pH, while alginate-glucose and calcium were used as matrix that entraps the probiotic because of the mild gelling conditions of this polymer. It gels in the presence of Ca^{2+} while it disassembles following Ca^{2+} release in the intestine, allowing probiotics delivery. We showed that this microcapsule protects *L. plantarum* in simulated gastric juice (SGJ) condition, heat, and osmotic stresses, and that glucose increased the resistance of the microcapsule to SGJ (**Figure 1**).

Despite the good performance of chitosan as a coating layer, the possible development of complications like allergy and immunomodulatory effects in some patients by this natural polymer[14] prompt us to search for alternatives. Therefore, in this work, we employed a synthetic co-polymer poly(1-vinylpyrrolidone-co-2-dimethylaminoethylmetacrilate) (PVP-co-DMAEMA) as a coating agent in microcapsule preparation.

2. Results and Discussion

We investigated a synthetic polymer because of its great versatility in terms of chemical structures while providing safe and cost-effective alternative. In particular, we used the cationic polymer PVP-co-DMAEMA, a biocompatible and not toxic polymer that was previously tested in human cells in vitro as a potential gene delivery system in gene therapies.[15] In addition, poly(1-vinylpyrrolidone) (PVP) is widely used as binder in tablet formulations, as particle surface stabilizer in nanoparticle synthesis and as cryo-protectant for adipose tissue-derived stem cells (hADSC).[16] In this study, we designed microcapsules composed of 2% alginate, 2% glucose, calcium ions, and 1% freeze-dried bacteria core and PVP-co-DMAEMA coating. The microcapsules were prepared in a two-step process in which a core alginate structure containing the bacteria and the additive glucose, is assembled using an emulsion-gelation method (Table S1, Supporting Information).

The polymer coating is added in the second step adjusting the procedure previously used for chitosan[10] and briefly

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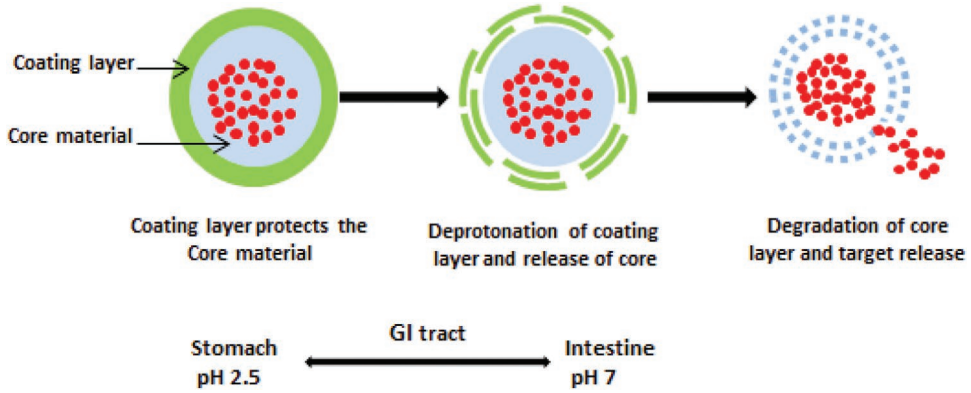

Figure 1. Coated microcapsule protection mechanism and release.

consisting of the immersion of the fresh alginate beads in a solution of PVP-co-DMAEMA followed by centrifugation. The microcapsules where characterized through fluorescent microscopy (**Figure 2**) and showed a dimension ranging from 100 to 200 µm.

We hypothesize that the poly-cationic PVP-co-DMAEMA could act as a pH-responsive coating polymer, being able to graft the anionic alginate microcapsules and act as a protective layer preventing the acidic degradation in the stomach; when the microcapsules reach the intestine the increase in the pH to around 7.0 would cause the release of the polymer coating due to the deprotonation of the amino groups, and the alginate core, exposed to the calcium-chelating phosphates of the intestine, would dissolve delivering the *L. plantarum* strain.

In order to confirm our hypothesis, we first tested in vitro the tolerance of coated and uncoated microcapsules on SGJ.

We found that the incubation of PVP-co-DMAEMA-coated alginate-glucose microcapsules in SGJ at pH 2.5 for 2 h did not affect the initial cell count of *L. plantarum* after exposure to high acid conditions (**Figure 3**a, Table S2, Supporting Information), fact that suggests the ability of the coating to conserve the integrity of the microcapsules or at least to avoid the acid condition to reach the microorganism. However, in the absence of PVP-co-DMAEMA coating, the viable cell count measured in colony forming unit (CFU) of *L. plantarum* in alginate-glucose microcapsules decreased of 1 log cycle under similar conditions. Once proved the ability of the novel polymer to allow the survival through the simulated gastric condition, we investigated the performance of the microcapsules in simulated industrial conditions. These studies are particularly significant for the eventual insertion of probiotics in food supplements and functional food. In this context, we tested the tolerance of PVP-co-DMAEMA-coated and uncoated alginate-glucose microcapsules under osmotic stress caused by immersion in a glucose-fructose syrup, in 70% isoglucose, and heating at 50 °C up to 8 h. We found that the survival of *L. plantarum* in osmotic stress after 24 h incubation at room temperature slightly decreases one log cycle of CFU/g when inserted in the PVP-co-DMAEMA coated microcapsules (Figure 3b, Table S3, Supporting Information), while a substantial decrease of more than six log cycles occurred in the absence of the coating. These results are indicative of the efficacy of the PVP-co-DMAEMA coating layer in conferring high protection to the probiotic from osmotic stress. Another important industrial stress condition is represented by heat treatment. To test the efficacy of the new coating, the microcapsules were incubated at 50 °C in water and the cell viability was monitored at different time laps (Figure 3c, Table S4, Supporting Information). Results suggest that the PVP-co-DMAEMA coating is able to protect the probiotics in the first 30 min with a slight decrease of 1 log cycle of CFU/g compared

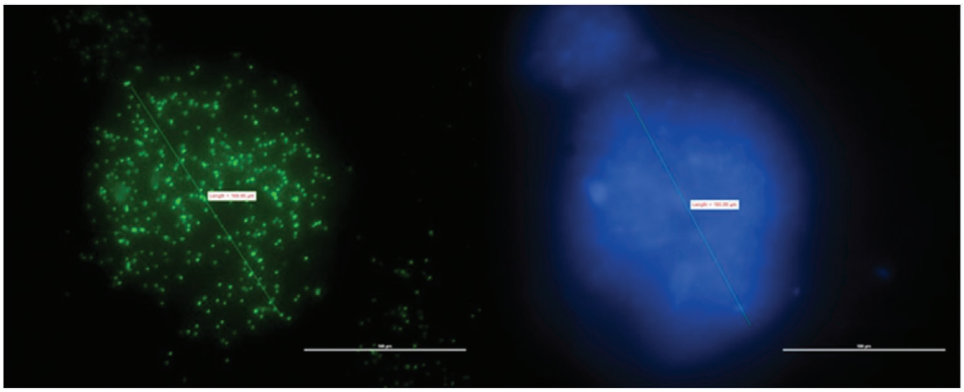

Figure 2. Fluorescence microscopy analysis after the addition of SYBR Green stain (spotting in green live bacterial cells) (left) and after the addition of calcofluor white stain (spotting in blue PVP-co-DMAEMA layer) (right) evidencing a PVP-co-DMAEMA-coated alginate-glucose microcapsule with a dimension ranging from 100 to 200 µm.

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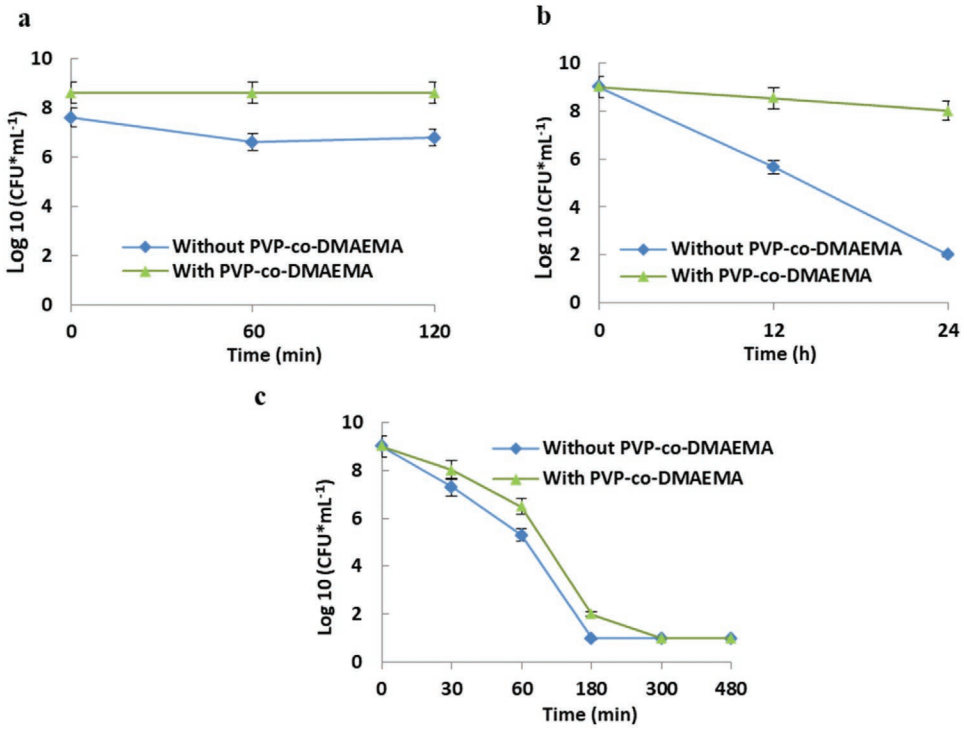

Figure 3. Residual viability and kinetic of resistance of PVP-co-DMAEMA coated and uncoated alginate–glucose microencapsulated samples of *L. plantarum*: a) after 2 h incubation in simulated gastric juice; b) after treatment with isoglucose for 24 h; c) after heat treatment at 50 °C up to 8 h.

to the 2 log cycle of the uncoated microcapsules; when heat stress is further prolonged the cell survival is seriously compromised with a decrease of 3 log cycle of CFU/g at 60 min, and reaching a residual survival of 1 log cycle of CFU/g at 180 min. Therefore, PVP-co-DMAEMA coating layer confers high protection of *L. plantarum* from osmotic stress while being efficacious when subjected to high temperature only for a limited time lap.

3. Conclusion

In conclusion, we designed a microcapsule based on PVP-co-DMAEMA coating and alginate-glucose core for the encapsulation of probiotics. The novel polymer coating confers protection to the probiotic bacterium *L. plantarum* in simulated gastric juice, and in industrial conditions such as osmotic stress and heating. These results show that PVP-co-DMAEMA protection is comparable to chitosan, highlighting the possibility to employ this polymer as an efficient and safe substitute of chitosan for probiotic delivery.

4. Experimental Section

Alginate Microencapsulation (core preparation): The encapsulation of the freeze-dried probiotic in alginate and glucose matrix was conducted according to the emulsion method described by Sultana et al. 2000.[17] Briefly, 50 mg of the freeze-dried strain was suspended in 5 mL of a mix composed by 2% alginate solution (2% w/v, Sigma-Aldrich) and 2% D-glucose (2% w/v, Sigma-Aldrich). The blend was gently poured into 25 mL of seed oil containing 0.02% V/V of Tween 80 (Sigma-Aldrich) and stirred vigorously till it was emulsified. Subsequently, 25 mL of 0.1 M calcium chloride solution (Sigma-Aldrich) was dropped to the emulsion, maintaining a vigorous agitation of the final mix. The latter was then allowed to stand for 30 min at room temperature for the settlement of the calcium alginate beads on the bottom of the aqueous layer, then was conserved at 4 °C overnight for beads-hardening. Subsequently, the oil phase layer was extruded, and the beads were collected by centrifugation (7500 rpm, 20 min, 4 °C), washed twice with a 0.9% saline solution containing 5% V/V glycerol (Sigma-Aldrich) and once with deionized water. The collected beads were stored at 4 °C or directly used for PVP-co-DMAEMA coating preparation.

PVP-co-DMAEMA Coating: The alginate beads were coated by PVP-co-DMAEMA layer according to the method provided by Krasaekoopt et al. (2004).[13] Briefly, 10 g of PVP-co-DMAEMA at 20% (SigmaAldrich) were suspended in 100 mL acetate buffer to obtain a final solution of PVP-co-DMAEMA (2% w/v). The pH was adjusted to 6. 3–4 g of fresh alginate beads were immersed into 20 mL of the PVP-co-DMAEMA (2% w/v) solution and mechanically mixed at 300–500 rpm for 20 min to achieve homogeneously coating. Then the suspension was centrifuged at 7500 rpm for 20 min, the supernatant was discarded, and the beads were collected and washed twice with deionized water. The microcapsules were weighed and stored at 4 °C (Table S1, Supporting Information).

Fluorescence Microscopy of Microencapsulated Bacteria: The fluorescence microscopy analysis showed the entrapped probiotic strain inside the microcapsule. Samples of microencapsulated bacteria were stained by calcofluor white stain (Sigma-Aldrich) according to the description of the technical datasheet for the polymer PVP-co-DMAEMA coating (reading at Em λ 475 nm) and SYBR Green (Sigma-Aldrich) for bacteria (reading at Em λ 520 nm). The prepared samples were analyzed by fluorescence microscopy with a Nikon Eclipse E600 microscope equipped with a Leica DC350F charge-coupled device camera and controlled by the Leica FW400 Software.

Viability of the Probiotic: The number of viable entrapped *Lactobacillus plantarum* cells in the PVP-co-DMAEMA-coated and uncoated alginate microcapsules was determined using the following. 1 g of fresh beads

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(PVP-co-DMAEMA-coated and uncoated) was suspended in 10 mL of phosphate buffer solution (0.1 M, pH 7.3) and homogenized by stirring mechanically for 30 min to ensure a complete solubilization of the beads. A serial dilution of microbial suspension was performed and plated on cMRS agar medium; after two over-nights incubation at 37 °C the cell viability was determined by counting the CFU/mL of bacteria before and after passage through the different stress conditions. The determinations were performed in triplicate and the means of three independent experiments were calculated.

Viability of the Probiotic after Treatment in Simulated Gastric Juice (SGJ): The tolerance of *L. plantarum* entrapped in PVP-co-DMAEMA-coated and uncoated alginate microcapsules on simulated gastric juice (SGJ) was determined by adding the samples into simulated gastric juice (SGJ), 0.9% saline solution of pH 2.5. 1 g of fresh microcapsules was suspended in 10 mL of SGJ and incubated at 37 °C for up to 120 min. Samples were taken at regular time intervals (0, 60, and 120 min), centrifuged (7500 rpm, 20 min, 4 °C), washed twice with deionized water and handled for the enumeration of the viable cells by plate count method on cMRS plates. The determinations were performed in triplicate and the means of three independent experiments were calculated.

Viability of the Probiotic after Osmotic Stress Treatment: The osmotic stress caused by sugar-based food ingredient, isoglucose was used to study the ability of PVP-co-DMAEMA -coated alginate microcapsules to tolerate osmotic stress. For each type of the microcapsules, 1 g was added to 10 mL of high-fructose corn syrup (HFCS) or isoglucose solution and incubated for 24 h at room temperature. At time (0 and 24 h), samples were taken, centrifuged, solubilized, and homogenized in phosphate buffer (PBS) and eventually determined for cell viability by plate count method on cMRS plates. The determinations were performed in triplicate and the means of three independent experiments were calculated.

Viability of the Probiotic after Heat Treatment: Heat tolerance of PVP-co-DMAEMA-coated and uncoated alginate microcapsules was determined by heating samples at 50 °C in water bath. For each type of the microcapsules, 200–300 mg (containing almost 2–3 mg of probiotic strain) was inoculated in 1 mL of water and incubated at 50 °C for up to 8 h. At regular time intervals (0, 30, 60, 180, 300, and 480 min), samples were taken, centrifuged, solubilized, and homogenized in phosphate buffer (PBS) and eventually determined for cell viability by plate count method on cMRS plates. The determinations were performed in triplicate and the means of three independent experiments were calculated.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

microencapsulation, pH responsiveness, polymer coating, probiotics

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