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Scienze degli alimenti

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Lactic acid bacteria and bioactive compounds: production in fermented foods and fate after digestion

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*A reader who has little knowledge of such matters may be surprised by my recommendation to absorb large quantities of microbes, as a general belief is that microbes are harmful. This belief is erroneous. There are many useful microbes, amongst which the lactic bacilli have an honourable place.*

**Il'ja Il'ič Mečnikov**

*Marveling at everything is the first step of reason towards discovery.*

**Louis Pasteur**

# Index

# Abstract and aim of the thesis

In every one of us, there is an impressive amount of microorganisms, some of them are dangerous, some are neutral while many others are more than helpful. Microorganisms reported to exert a positive effect in the host when administered in adequate amount are known as “probiotics” [1,2]. Lactic Acid Bacteria (LAB) are often part of these good ones, with several strains recognized as probiotics, or due to their long history of use and wide spread in the great majority of fermented foods. Fermented foods have remained a staple throughout history for good reason. The bacteria we ingest through food become part of our microbiomes, delivering several health benefits. Diets rich in fermented foods has immune benefits, reducing inflammation associated with disease and stress [3]. Consumer interest in fermented foods has been driven mostly by their suggested nutritional benefits, leading to renewed popularity of these foods worldwide. Health promoting effect of fermented foods vary, resulting from nutritive alteration of raw ingredients and the biosynthesis of bioactive compounds, modification of the human gut microbiota, and development and modification of the immune system. Considerable progress has been made towards understanding the function of individual microorganisms in fermented food production and the same is happening regarding their potential contribution to human health [4]. All this considered, the aim of this thesis was to study the ability of LAB to produce potentially bioactive compounds upon fermentation, which could convey health benefits to the consumer of fermented foods

# Chapter 1- Eating Fermented: Health Benefits of LAB-Fermented Foods

Since the born of the first human communities fermentation has always been exploited by mankind to preserve, make more attractive and confer positive features to food. It is not well established if the first fermented food was deriving from spoiled fruit or by milk carried in bags made with animal's stomach. What is known is that fermented food is with men since 10000 B.C. Since then the empiric knowledge have confered healing and salutistic properties to fermented food. In different countries fermented foods are used in traditional medicine as remedies for gastric and intestinal ailments. Science of probiotics has passed through different phases, from the studies on food carrying positive features, to the selection of probiotic microorganisms and use of pure culture. Nowadays probiotics science is exploring a new frontier, using traditional fermented foods to carry microorganisms not recognized as probiotics, but genetically close. This microorganisms are adapted to harsh enviroments and can carry positive features also after cellular death, like post-biotics and para-probiotics.

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**Abstract:** Lactic acid bacteria (LAB) are involved in producing a considerable number of fermented products consumed worldwide. Many of those LAB fermented foods are recognized as beneficial for human health due to probiotic LAB or their metabolites produced during food fermentation or after food digestion. In this review, we aim to gather and discuss available information on the health-related effects of LAB-fermented foods. In particular, we focused on the most widely consumed LAB-fermented foods such as yoghurt, kefir, cheese, and plant-based products such as sauerkrauts and kimchi.

**Keywords:** lactic acid bacteria; fermented foods; health benefits; bioactive compounds

## 1. Introduction

Lactic fermented foods have been consumed by humankind since ancient times for their organoleptic characteristics and prolonged shelf-life. Nowadays, the consumption of fermented foods is also driven by a health-related perspective. The market of fermented foods is nowadays touching all countries and shows an increasing trend [3]. Natural microflora of fermented foods is often composed mainly by LAB. LAB are Gram-positive, nonsporing, generally nonmotile, with complex nutritional requirements, depending on the presence of a fermentable carbohydrate for active growth. As an end product of this fermentation, LAB produce copious amounts of lactic acid alone (homofermentative) or together with acetic acid, ethanol, and carbon dioxide (heterofermentative) [4]. Genetic diversity and habitat variation are considerably wide within LAB. Therefore, no general limits for pH, $a_w$, temperature, or other parameters exist for the LAB, and the growth-limiting circumstances vary depending on the considered species (Table 1) [5]. LAB are responsible for a great diversification in the flavor and texture of fermented foods, and can be, in some circumstances, responsible for food spoilage [6]. They can also release an array of health-modulating compounds and signal molecules in the matrix during fermentation. These food-derived bacteria and their metabolites can interact with the intestinal microbiome and with the host itself like members of an orchestra playing a health symphony for the intestine and the organisms in general. Regular ingestion of fermented food can therefore contribute in many ways to homeostasis and organism functions. A link between health and the use of eating LAB-fermented foods has been hypothesized since Metchnikov's intuition that an increased life expectancy of balcanic population was attributable to the significant consumption of lactic fermented milk. Since then, many studies have led to the granting of the probiotic status to different LAB responsible for the fermentation of foods frequently associated with health benefits [7,8]. Many fermented foods are considered functional foods as they contain microorganisms, enhancing the overall health level of consumers [9]. Due to the positive effects exerted both by fermenting microorganisms and the products of their metabolism, LAB-fermented foods could represent a safe, unexpensive, and reliable tool in improving human health. This review highlights the health effects reported by the literature on LAB responsible for the fermentation of different foods, which could contribute to maintaining and promoting consumers' health.

**Table 1.** Food's LAB groups and characteristics.

| Families | | Genera Frequently Found in Foods | $CO_2$ from Glucose | Growth at 10 | Growth at 45 | Growth in 6.5% NaCl | Growth in 18% NaCl | Growth at pH 4.4 | Growth at pH 9.6 |
|---|---|---|---|---|---|---|---|---|---|
| *Carnobacteriaceae* | Rods | *Carnobacterium* | - | + | - | - | - | - | - |
| *Enterococcaceae* | Cocci | *Enterococcus* | - | + | + | + | - | + | + |
| | | *Tetragenococcus* | | | | | | | |
| *Streptococcaceae* | | *Streptococcus* | - | + | - | + | + | - | + |
| | | *Lactococcus* | - | - | +/- * | - | - | - | - |
| *Lactobacillaceae* | Cocci | *Leuconostoc* | + | + | - | +/- * | - | +/- * | - |
| | | *Oenococcus* | + | + | - | +/- * | - | +/- * | - |
| | | *Pediococcus* | - | + | +/- * | +/- * | - | + | - |
| | Rods | *Lactobacillus* | +/- * | +/- * | +/- * | +/- * | - | +/- * | - |
| | | *Lacticaseibacillus* | - | + | +/- * | +/- * | - | + | - |
| | | *Lactiplantibacillus* | - | + | +/- * | + | - | + | - |
| | | *Furfurilactibacillus* | + | + | - | + | +/- * | + | - |
| | | *Fructilactibacillus* | + | - | + | + | - | + | - |
| | | *Levilactobacillus* | + | + | - | + | +/- * | + | - |
| | | *Limosilactobacillus* | + | - | +/- * | +/- * | +/- * | + | +/- * |
| | | *Latilactobacillus* | - | +/- * | +/- * | + | +/- * | + | - |
| | | *Lentilactobacillus* | + | + | + | + | - | + | +/- * |
| | | *Weissella* | + | + | - | +/- * | - | +/- * | - |

* response may vary according to strains. "-" absence of the tract. "+" presence of the tract.

### *1.1. Lactic Acid Bacteria as Probiotics*

Despite the evolution of the probiotic concept, the term probiotic has been linked to bacteria beneficial for the host health since Elie Metchnikoff's observation that the regular consumption of dairy products fermented by LAB was associated with enhanced health and longevity in the elderly Bulgarian people [10]. Over the years, a considerable number of microorganisms was proposed as

probiotics, with health-improving abilities. Most known probiotics belong mainly to the LAB group and Bifidobacteriaceae, while for others such as the yeast *Saccharomyces boulardii,* or *Enteroccoccus spp*., the use as probiotic has been debated for long due to their intrinsic opportunistic nature and the possibility to transfer antimicrobial resistance genes [11,12]. In contrast to the traditional probiotics, non-conventional, native gut microbiota bacteria have rapidly attracted much more attention for promoting health and therapeutic purposes, leading to the concept of Next-Generation-Probiotics (NGP) [13,14]. Because of the development in microbial culturing techniques [15], in the metagenomics and genomics technologies involved in sequencing [16] and editing of bacterial genome [17,18], the range of microorganisms considered for their potential positive effects on hosts health has nowadays broadened up, involving non-LAB genera like *Actinobacteria* (*Akkermansia muciniphila* is among many others an emerging star in the field), *Bacteroidetes*, *Firmicutes*, and *Verrucomicrobia* [14,19]. Despite this, LAB are still the most used health-related bacteria in food production. Due to their long history of safe use, LAB have been listed either as Generally Regarded as Safe (GRAS) at the strain level by the United States Food and Drug Administration (FDA), or as Qualified Presumption of Safety (QPS) at the species level by the European Food Safety Authority (EFSA). Thus, they can be used as food or food supplements [13], and confer to the fermented food functional characteristics, entailing a vast arsenal of aces in the hole in granting benefits to human health [9].

### *1.2. Health Effects of Probiotics*

Health-related features ascribed to probiotic microorganisms are multifaceted. Probiotics are known for: (i) the production of valuable compounds, (ii) antagonist activity towards pathogenic bacteria, (iii) stimulation and regulation of immune response, and many other effects [2]. As they generally exert their effect starting from the intestine, probiotics should show: (i) good resistance to acids, and (ii) disaggregating effect of biliary salts, (iii) ability to colonize intestinal walls, (iv) compete for nutrients, and (v) remain alive in the harsh and selective conditions of Gastro-Intestinal (GI) tract [2]. By colonizing intestinal mucosa and interacting with the mucus layer, probiotics modulate immune response, improving defense to external attacks. Maintaining a constant presence in the gut, the immune system is stimulated, also leading to reduced severity of autoimmune aggressions and lowering allergic response, according to Rook and the “old friend theory” [4,5]. Indeed, in the colonic region, from the fermentation of digested material, they can produce antioxidants and anti-carcinogenic compounds, together with a series of molecules activating a signalling process between bacteria and intestinal epithelium [20]. This starts a cascade of effects that eliminate pathogenic and harmful microorganisms, thus creating a better environment and maintaining homeostasis [2]. To reach these goals, probiotics might be in a viable state and with an adequate amount [2]. However, some experimental evidence suggests a role exerted by non-viable or dead microbial cells in improving the health status of hosts, opening the door to the concepts of post-biotics and para-probiotics [6]. Post-biotics term is referred both to non-viable microorganisms present in the preparation and to soluble compounds released by probiotics after cellular lysis, comprising (i) short-chain fatty acids (SCFA), (ii) lactate, (iii) cellular

wall components, and (iv) peptides [21]. Conte et al. reported using post-biotics from different lactobacilli as treatment to reduce the entrance of gluten proteins in CaCo-2 cells of patients affected with celiac disease [21]. Para-probiotics comprise non-viable microorganisms and the entire microbial fraction released after cellular lysis [22]. Sugawara et al., in an intervention study, showed an improvement in intestinal environment and functions after 3 weeks of consumption of a para-probiotics beverage containing non-viable cells of *Lactobacillus gasseri* [22]. Both viable and non-viable (or part of) cells can interact particularly in the intestinal epithelium through the stimulation of intracellular signalling pathways [23]. Many of these features have been described in LAB, which can produce different compounds, like bioactive sequences of peptides, sugars polymers, and fatty acids involved in boosting human health [11]. LAB can also produce organic acids, bacteriocins, hydrogen peroxide ($H_2O_2$), and nitric oxide (NO), that are active against pathogens [12]. Furthermore, during fermentation in the intestinal lumen, LAB also produces SCFA. These acids can be produced also by other microorganisms, for example: acetate can be produced by *Akkermansia muciniphila, Bacteroidetes, Bifidobacterium* spp., and *Clostridium* spp.; propionate by *Veillonella parvula*, *Bacteroides eggerthii*, *Bacteroides fragilis*, *Ruminococcus bromii*, and *Eubacterium dolichum*; and butyrate by *Faecalibacterium prausnitzii*, *Clostridium leptum*, and *Eubacterium rectale* [24]. SCFA are involved in different processes, for example butyric acids furnish metabolic energy to colonocytes and is studied for its effect in avoiding the development of cancer cells [23]. Propionate enhances gluconeogenesis and helps maintain glucose homeostasis in the organisms by increasing the expression of leptin, an anorectic hormone, in adipocytes [25]. Acetate is involved in the lipogenesis and synthesis of cholesterol [26].

### *1.3. Health Effects of Foods Fermented by LAB*

In the past years, the consumption of probiotics was strongly recommended, and the involvement of positive microorganisms in the formulation of foods with a health claim was widespread. Nowadays, due to a more profound knowledge of the probiotics' health effects and the mechanism behind them, it is possible to broaden the range of microorganisms involved in the formulation of functional foods. In some cases, LAB that are part of the spontaneous microbial population of one food, drive the beneficial effects to the host without being recognized (yet) as probiotics [27–30]. Positive effects connected to fermented foods have been empirically known for centuries. In many cultures, fermented foods are heritage foods and an integral part of local traditions, probably because fermentation was the only way to preserve foods [30]. Nowadays, regular consumption of fermented foods, especially lactic-fermented ones, has been reported to improve the immune system, reducing the probability of developing morbidities [27] due to a constant communication between bacteria and host immune system. This communication changes the microbial composition of the intestine, maintaining under control pathogenic microflora and meanwhile supporting beneficial microbes populations [31].

Among fermented foods, dairy products have been mainly associated with beneficial effects. This is partly due to the significant number of proteins available in the substrate for cellular

duplication. During fermentation, because of acidification and microbial enzymes activity, proteins are denatured and lose their original conformation, releasing sequences of small peptides studied for their potential health-related effects. One of the most studied and regarded groups of bioactive peptides is represented by Angiontensin-1-Converting Enzyme (ACE) inhibitors. These bioactive peptides have been studied for their anti-hypertensive effect, and several guidelines suggest consuming fermented dairy products as a non-pharmacological way of controlling hypertension. Scientific evidence reported two main peptides as carriers of hypotensive effect: VPP (valine, proline, proline) and IPP (isoleucine, proline, proline) [29,31,32]. ACE inhibition occurs when ACE I is sequestered by the C-terminal sequence of ACE-inhibitors. In this way, ACE cannot convert angiotensin I in angiotensin II, a potent vasoconstrictor. Synthesis of angiotensin II leads also to degradation of bradykinin, a vasodilator; soaring blood vessels' constriction; and dramatically increasing blood pressure [32,33].

Furthermore, LABs can produce exopolysaccharides (EPS), long sugars polymers formed by repeated units of mono- or oligosaccharides, that are gaining a lot of attention from the scientific community, due to their technological role [34], but also for their promising health benefits [35]. EPS can be divided in two macro-categories depending on the sugars presents in the main chain: (i) Heteropolysaccharides (HePSs) are polymers of different monosaccharides; (ii) Homopolysaccharides (HoPSs) are polymers of one sugar, repeated many times. In the latter case, HoPSs can be divided into glucans or fructans depending on the sugar composing the polymer chain, glucose, and fructose, respectively. Production of HoPS takes place outside microbial cells, mediated by membrane enzymes that hydrolyse and reassemble the sugars in a new EPS chain. By contrast, HePSs synthesis is more complex, and the chain contains more than one sugar moiety, normally being glucose, galactose, and rhamnose. Still, in different LAB's EPS it is possible to find different sugars or other functional groups like acetyl and phosphate groups [36]. Normally, HePSs are associated with the modulation of host function, e.g., antioxidant effect or immune modulation, while HoPSs are associated with prebiotic properties, indicating how the conformation of these branched sugars and the monomeric composition influence the impact on the host [36,37]. The prebiotic effect exerted by LAB's EPS is the subject of particular interest, because of the production of SCFA, gasses, and organic acids involved in the inhibition of noxious bacteria and the improvement of host's metabolism [36]. EPS produced by LABs proved to be more effective in increasing the amount of Bifidobacteriaceae in the intestinal lumen with respect to inulin, the most used bifidogenic oligosaccharides. At the same time, an antagonist effect towards *Bacteroides* and Clostridia was shown. Gut microbiota is strongly affected by the presence of EPS in the intestinal lumen, especially by HoPSs, that result to be the most suitable substrates for fermentation, while HePSs are normally not fermentable, but their ability to modulate the immune system make them of capital importance in maintaining a general health status [37].

In fact, EPS are supposed to have antioxidant and immunomodulatory effects, as well as the ability to reduce cholesterol in the bloodstream and its absorption; anticancer and anti-diabetic effects are just some of the positive features that may be exerted. Furthermore, they also have a role in fighting the presence of harmful bacteria in the intestine, since they can disrupt biofilms, removing the protection of pathogenic microorganisms and exposing them to stresses and attacks. Different studies were carried out to explore these proposed effects for EPS. Still, it has to

be considered that many of these experiments were carried out in vitro or with animal models, missing the confirmation from clinical trials on humans [37]. Some studies on animals pointed out the anti-cholesterolemic effect of EPS. This effect is based on increasing the high-density lipoprotein (HDL) ratio: total cholesterol with reduction of lipidic deposits in the bloodstream, especially in the aorta. In other experiments, it was observed that bile acids were scavenged by EPS, reducing in this way the amount of cholesterol present in the blood. This can be due also to the utilization of blood cholesterol to synthesize new bile acids, which are subsequently employed in digestion processes. Results are of course promising, even if the mechanism through which EPS lowers cholesterol content in the blood is still not precisely known [37].

Health effects of food fermented by LAB (Figure 1) are known and have been studied for a long time. Despite this, we do not yet know all the mechanisms of action and the secondary effects of LAB and their derived compounds. For many years, literature have focused on health effects of bacteria isolated and recognized as probiotics, but more recent studies shed light on the beneficial effects of bacteria involved in food fermentation that are not considered probiotics due to the non-complete compliance to probiotics guidelines. As an example, LAB proved to be useful in homeostasis both directly in the gut and indirectly utilizing pathways' modifications that lead to an improvement of host health status [38,39].

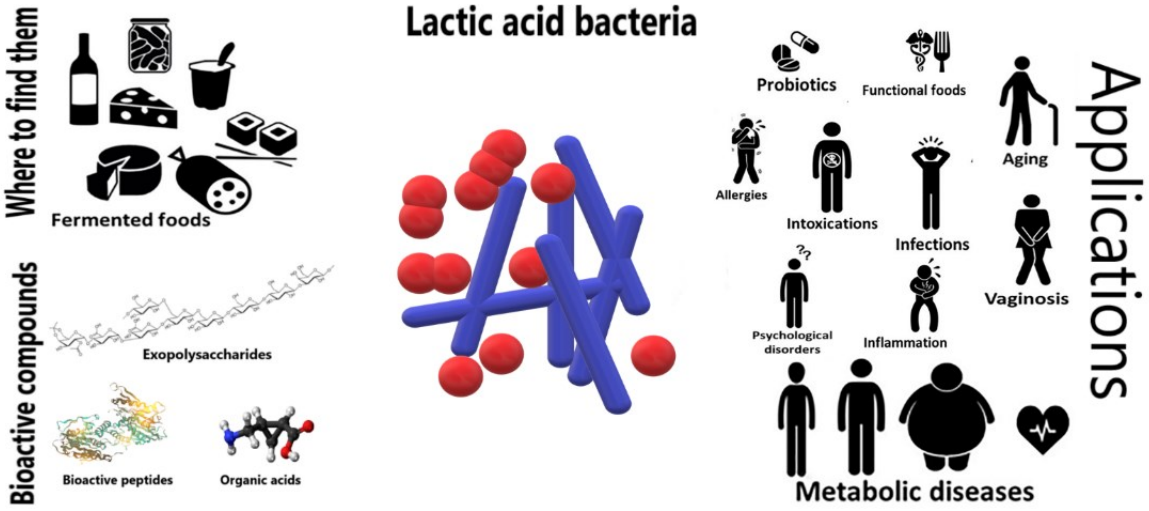


**Figure 1.** Scheme of LAB bioactive compounds and health-related effects, adapted with permission from [40] Copyright © 2018 George, Daniel, Thomas, Singer, Guilbaud, Tessier, Revol-Junelles, Borges and Foligné.

## 2. Health-Related Effects of Different LAB Fermented Foods

### *2.1. Fermented Dairy Products*

Milk is probably one of the first fermented food staples by mankind. Historically, the first fermentations happened accidentally due to unpasteurized milk's tendency to spontaneously ferment due to the high level of nutrients and microbes [41,42]. From a biochemical point of view,

fermentation is a complex combination of events. After lactose metabolism, different compounds are generated, such as: acids, ethanol, and carbon dioxide. The production of acids leads to a decrease of the pH, limiting the growth of negative microflora. Aroma compounds are also produced, increasing palatability and acceptance of foods and nutritional compounds like vitamins, minerals, bioactive molecules, and EPS [43]. Nowadays, after millennia of traditions and evolution of dairy art, fermented milk products represent about 20% of the total revenue generated by the fermented-foods markets all over the world. Production of fermented milks arose after 1950 when the demand for yoghurts and other similar products increased sensibly, attracting the attention of companies and consequently moving the production from a small-scale, in artisanal farms, to a mass production led by big multinationals [43]. Milks from different animals have become raw material for dairy fermentations. In fact, it is possible to find yoghurts, cheeses, and sour milks produced with cow milk, goat, sheep and horse milk as just examples in global markets. Even though dairy fermentations originally started from wild LAB present in milk, nowadays companies cannot rely anymore on spontaneous microflora, because of technological properties and possible health issues related to raw materials. For this reason, almost all industrially-fermented dairy products are produced with selected starters, or with back-slopping technique [43–45]. Fermented dairy products can be divided in different categories; in this review, for the sake of brevity, we focus only on fermented milks and cheese. Fermented milks are many and can be classified basing on: production techniques, the origin of milk, and other factors [46]. Since the variety of these products is humongous, considering traditional and industrial processes, novel fermented milks, and ones deeply rooted in archaic societies, we only consider the two most consumed and spread fermented milk products: yoghurt and kefir.

#### 2.1.1. *Yoghurt*

Due to its taste and versatility, yoghurt is one of the most consumed milk-derived products worldwide [47]. Like other dairy products, yoghurt is strongly recommended in diets, for its provided nutrients, like essential amino acids, and bioactive compounds, such as lactic acid, EPS, and liposoluble vitamins [41], which are otherwise rare and difficult to be introduced with the diet [48]. In a standard yoghurt's serving, it is possible to find many useful nutritional compounds like (i) vitamins and minerals in a rapidly absorbable form [49]; (ii) bioactive peptides with many health-modulating effects [30,50]; (iii) branched-chain amino acids (BCAA) positively correlated with muscle growth and body maintenance [51]; (iv) mono- and poly-unsaturated fatty acids vehiculating liposoluble vitamins (A, E, K, and D); and (v) conjugated linoleic acid (CLA), known for the anti-carcinogenic activity and apoptotic induction in cancerous cells, as reported by different papers, especially towards breast cancer in vivo and in vitro [47,52–55]. All the listed compounds, or precursors, are already present in milk, but the fermentation process is essential to liberate this vast amount of positive health-related compounds in the matrix. Fermentation of milk to produce yoghurt is carried out by two specific LAB: *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus,* even if other species can be added as a plus. Bacteria from yoghurt are

known for making part of the so-called transient microbiota, since they usually cannot colonize the intestine. Despite this, the health contribution of yoghurt microflora should not be underrated. Kousgard et al. reported a clinical trial on patients affected with pouchitis and treated with a fecal microbiota transplant. In that study, four out of four patients with pouchitis symptoms remission regularly consumed yoghurt, while only one out of five patients with relapse issues consumed yoghurt on a daily basis [56]. The remission effect could also be correlated to the presence of organic acids produced by microorganisms, which contribute to fighting pathogenic microorganisms and maintaining a safer gut environment. Several dietary guidelines suggest the implementation of yoghurt in a healthy diet daily, also for lactose-sensitive people, due to the ability of the contained LAB species to improve this sugar digestion [57]. Different scientific papers focused on the utilization of probiotic fortified yoghurts in the management of type 2 diabetes. At the same time, Barengolts et al. in a meta-analysis of randomized controlled trials demonstrated that consumption of yoghurt can improve management of diabetes complications, reporting no difference between effects exerted by conventional and probiotic fortified yoghurts [58]. Kong et al. reported the utilization of yoghurt in combination with fruits and caloric restriction to fight non-alcoholic-fatty-liver-disease (NAFLD). Their data showed the ability of the diet intervention to modify the gut microbiota. An intimate relationship between gut and liver is already well known to the scientific community. In fact, results from that research paper highlighted how modifications in gut microbial population can retard or even prevent the start of different chronic diseases, like NAFLD, among others [59]. Liu et al. in clinical tests on mice reported traditional yoghurt being able to modulate intestinal microflora, repairing and avoiding dysbiosis that can negatively affect brain functions and behaviour. In fact, in transgenic mice modified to develop Alzheimer disease' (AD) symptoms in early stage of life, yoghurt's supplementation reduces the deposition of myeloid-beta plaques in brain cortex and hippocampus, even though it is highly correlated with the onset and development of AD disease. It derives that gut microbiota modulation, operated by ingestion of yoghurt, and its microbiome can help in reducing the issues connected with AD and cognitive function [59,60]. Considering all these health-related effects, yoghurt reveals to be a cost-effective way to introduce in the diet countless health-boosting compounds, it helps in the management of non-communicable disease, and is negatively associated with all-cause mortality [61–63].

#### 2.1.2. *Kefir*

It is one of the first fermented milks. Traditional kefir owes its longevity in human diet and traditions to its peculiar organoleptic characteristics and to an unconscious association with health benefits and life prolongation [64]. Traditionally, kefir is made by the action of kefir grains, in which are comprised LAB, Acetic Acid Bacteria (AAB), and yeasts enveloped in a slimy matrix composed of EPS and proteins [65]. Kefir can be defined as a "natural complex probiotic" because of the interaction between many different microorganisms, and it is supposed to exert anticarcinogenic, immunomodulatory, antiallergenic, antidiabetic, antistress, and antiasthmatic effects [66–68]. Kefir microflora depends not only on the inoculum of the grains, but also on external factors (light, temperature, kefir grains/milk ratio, agitation…), which can influence organoleptic features as well as bioactivities, favouring the growth of specific strains, while a core population always exists [69].

Health-related effects of kefir can be ascribed to the presence of bacteria, but also to bioactivities [70]. It can, for example, modulate gut microbiota and increase *Lactobacillus* and *Bifidobacterium*, while decreasing *Bacteroidetes* level in the intestine of patients affected with metabolic syndrome, leading to improvements in fasting glycaemia, reduction of inflammation signals, and blood pressure [71]. Modifications of the gut microbiota exerted by kefir's bacteria are reported also by Yilmaz et al., who noticed in a randomized control trial that *Lentilactobacillus kefiri* LK9 was able to colonize the intestine of volunteers after 1 month of administration, resulting as present in faeces at $10^5$–$10^6$ Log CFU/g. *L. kefiri* is also reported to inhibit other microorganisms associated with the start of pro-inflammatory chain events and gastrointestinal illness [72]. Kim et al. investigated the effect of kefir in reducing the incidence of obesity, induced by a high-fat diet (HFD) and NAFLD. In their experiments, results show a decrease of 60% of incidence of obesity in mice concerning control group, showing that a 0.2 mL supplementation of kefir reduces the effects of HFD and related NAFLD. Also, blood cholesterol and systemic inflammation, both induced by a fat-rich diet, were reduced by kefir supplementation. The mechanism of action in the reduction of obesity and related problems seems to be exerted by the cooperation of three different factors: LAB, yeasts, and EPS. In fact, kefir-derived bacteria can influence the gut microbiota directly by colonizing gut epithelium and indirectly by modifying pH of the intestinal lumen and inducing expression of genes that codify for useful enzymes. Reduction of pH creates a harsh environment for pathogenic and undesired microorganisms, but not for LAB that are normally used in acidic environments. In the same experiments, Kim et al. concluded that the introduction of probiotics derived from natural kefir is able to up-regulate peroxisome proliferator-activate receptor. This system plays a central role in beta-oxidation and reveals to be a fundamental drug helping in fighting NALFD [73,74]. Many studies in recent years focused on anti-cancer abilities of fermented foods, and kefir is one of the most investigated since its health-boosting effects have been known from the dawn of time. Anti-cancer activities exerted by kefir are mediated by different compounds, like bioactive peptides, EPS, and sphingolipids. The mechanism of action of these compounds seems to be bound to modulation of signalling pathways and of cells' processes, e.g., cellular proliferation and apoptosis [66,75]. In a systematic review of the literature, Rafie et al. reported that according to the state of the art, the mechanism of the action exerted by kefir in inducing apoptosis is not fully understood yet, but it can be due to the formation of reactive oxygen species (ROS), mediated by peptides. The liberation of ROS in the cell creates damage and activates endonucleases that cleave DNA, creating an escalating apoptotic effect. ROS disrupt mitochondria, creating a cascade of events that leads the cells to death. This cascade effect seems extremely powerful since peptides from kefir are naturally positively charged, thus being electrochemically attracted by negatively charged components of cancerous cells. EPS contributes to apoptosis of cancerous cells, activating macrophages and T-lymphocyte. Moreover, regulation of genes expression seems to be involved in anti-tumour potential of kefir, as its consumption seems to up-regulate pro-apoptotic systems and down-regulate proliferations systems [76]. In their review, Rafie et al. reported the amount of kefir supplemented for the experiments, ranging from 200 µL to 5 mL, but, as all the listed experiments are in vitro on cancerous cells, the precise amount that has to be consumed to reach a positive effect needs to be further investigated [76]. Kefir was administered by Özcan et al. to postmenopausal women to improve quality of sleep and thus reduce mental disorders, like depression and stress

accumulation. It is well known that the gut–brain axis is a highway, and what affect the guts, reflects on the brain [77,78]. In this sense, the beneficial effect of kefir reducing harmful microflora, improving motility, and modulating immune function helps to reduce sleeps disorders, depression, stress, and anxiety, thus increasing the quality of life. In this study, patients were supplemented with 500 mL of kefir daily, to drink half in the morning and the rest in the evening. The ingested amount is considerably high, but it has to be taken into account that the experiments were conducted in Turkey, where kefir consumption is traditionally rooted in the population [79]. Kefir was administered also to ovariectomized mice to study the effect of kefir's peptide fraction on estrogenic deficiency-induced osteoporosis and evaluate in model systems prevention of menopausal osteoporosis. As already stated, in fact, kefir can modulate gut microbiota through different patterns, influencing many aspects of physiological processes like absorption of nutrients, hormone regulation, and metabolic processes. Moreover, through EPS of kefiran, kefir exerts a bifidogenic effect, increasing sensibly the amount *Bifidobacterium* in the guts, reducing the amount of pathogenic microflora (fungi, protozoa, viruses, and bacteria), due to the production of organic acids and bioactive peptides [80]. Modulation on the hosts exerted by kefir is also broadened by the promotion of fatty acids oxidation by increasing *Lactobacillaceae* population as well as *Kluyveromyces* spp. presence in the gut [73]. Kefir containing a natural probiotic, able to release SCFA in the media and the guts, contributes to bone formation and improves bone density [81]. Different studies focused on this topic both in animal and humans, confirming the effect of kefir in reducing bone loss, increasing bone density and elastic moduli of bones, and preventing fractures that may result in fatal ending for elderly persons. This effect is enhanced when combined with calcium-carbonate supplementation [82–84]. In the end, being so widespread, easy to use, and obtain, kefir looks like a treasure chest of positive effects for consumers.

#### 2.1.3. *Cheese*

Cheese is an umbrella term under which many products differentiated by production techniques, composition, environment, and microbial evolution find space. The combination of productive processes and microbiota are fundamental to differentiate products. For example, during fast ripening, the amount of lactose is reduced by microorganisms, leaving a reduced amount of lactose final product, making these cheeses a choice for lactose-sensitive individuals. On the other hand, during prolonged ripening, which can last for months and even years, lactose is completely consumed by LAB, making these cheeses an attractive source of dairy micro- and macro-nutrients for lactose-intolerant people. During the first stages of fermentation, bacteria consume carbohydrates, leaving just a fraction of indigestible oligosaccharides in the matrix that is proven to reach the intestine and exert prebiotics effect, stimulating positive microflora [85,86]. During early stages of ripening, lactose is rapidly degraded in lactate, by means of starter LAB. Lactate can then be metabolized by *Propionibacterium*, Clostridia, and Pediococci in propionate, butyrate, and formic acid, respectively [87]. Milk contains also citrate that is normally involved in LAB metabolism by citrate positive bacteria, mainly Lactococci [87]. Strains usually involved are *Lactobacillus lactis* ssp.

*lactis* biovar *diacetylactis*, and *Leuconostoc mesenteroides*, which produce acetate, diacetyl, 2-butanone, and 2,3-butanediol [87]. Another important metabolism of LAB during ripening is proteolysis, resulting in the release in the matrix of branched-chain amino acids such as leucine, iso leucine, and valine; aromatic amino acids such as tryptophan, phenylalanine, and tyrosine; and sulfur-containing amino acid such as methionine. Peptides and ammino acids in cheese are often in an interesting bioavailable form [87]. During ripening time, small peptides are released by the action of enzymes, residual rennet activity, and LAB. A part of these peptides can be metabolized by LAB [88], and is well known for bioactivities, such as opioids, ACE-inhibitors, and immuno-stimulating activities. Some other peptides vehiculate minerals to the intestine and peripheric organs via blood transport [89]. There is an expanding body of evidence concerning a negative correlation between intake of dairy products and development of hypertension [90]. This anti-hypertensive effect seems to be correlated to the presence of calcium and small peptides with ACE-inhibitors activity, like IPP or VPP peptides [91,92]. Ripening of cheese is positively correlated with these bioactive peptides, which are normally present in cryptic form inside caseins. In a double-blind study, Crippa et al. fed Grana Padano, a long ripened Italian cheese, to 30 patients with hypertension issues and reported a significant decrease in systolic and diastolic blood pressure after 2 months of administration of 35 grams of grated cheese per day. The decrease of blood pressure was in the order of −4.8/3.5 mmHg, which is interesting considering that a reduction of 3 mmHg can reduce the risk of heart attack and failure of about 13% [93]. In recent years, cheese-isolated probiotics have gained attention due to their ability to produce a variety of bioactive compounds like SCFA from the fermentation of non-digestible carbohydrates [94]; their antimicrobial effect towards pathogenic microflora; as well as their ability to improve immune response, reduce serum cholesterol level, and alleviate diarrheic symptoms [95]. Recently, literature focused on compounds with the ability to modulate mood [96,97]. One of the most studied mood-modulators is γ-aminobutyric acid (GABA). GABA is a non-protein amino acid derived from decarboxylation of glutamate [88] and is one of the main inhibitory neurotransmitters in the central nervous system of mammalians. Studies showed its involvement in managing stress, influencing behaviour and personality, and hypotensive and anti-diabetic properties [96,98]. Moreover, its effect was also noticed in preventing depression and helping in the treatment of alcoholism by activating specific receptors and increasing lymphocyte counts [88]. Strains able to produce GABA during fermentation of milk are *Lacticaseibacillus paracasei*, *Lentilactobacillus buchneri*, *L. delbrueckii* subsp. *bulgaricus*, *Lactiplantibacillus plantarum*, *Levilactobacillus brevis*, *Lacticaseibacillus rhamnosus*, and *Lactococcus lactis* [96,99]. Cheese seems to exert a protective effect towards these bacteria, due to the high fat content that protects bacteria and allows them to reach the intestine, where they can exert multiple positive effects [99]. Knowing this, the introduction of cheese, especially long ripened ones in the diet, allows the introduction of numerous positive compounds like bioactive peptides, minerals, liposoluble vitamins, organic acids, and other antimicrobial compounds, together with a positive and stress-resistant microflora (Table 2). Moreover, cheese can convey mood modulators to the hosts, helping in the management of stress and altered mood states.

**Table 2.** Health-related effects of fermented dairy products.

| Health Effects | Specific Effects | Fermented Food | Microorganisms | Reference |
|---|---|---|---|---|
| Reduce initiation and progression of cronic disease: | | food ingredients, including living microbial cells | *Lactobacillus* and *Lactococcus* genera | [100] |
| | Musculoskeletal disorders | | | |
| | Cardiovascular diseases | | | |
| | Mental health pathologies | | | |
| | Type 2 diabetes | | | |
| Production of Bioactive peptides: | | Milk-derived foods (Fermented milks, Cheese, yoghurt, kefir) | *Lactobacillus* and *Lactococcus* genera | [100] |
| | Satiety regulation | | | |
| | Antimicrobial | | | |
| | Anti-carcinogenic | | | |
| | Anti-thrombotic | | | |
| | Mineral absorption | | | |
| | Hypotensive | | | |
| | Anti-inflammatory | | | |
| | Stress relief | | | |
| | Aids relaxation and sleep | | | |
| | Reduces symptoms of psoriasis | | | |
| | ACE-inhibitors | | | |
| Amelioration of glucose metabolism | | LAB-fermented foods, especially fermented milks | GRAS Lactic acid bacteria | [101] |
| Amelioration of glucose intollerance symptoms | | LAB-fermented foods, especially fermented milks | GRAS Lactic acid bacteria | [101] |
| Reduce severity of infections | | LAB-fermented foods, especially fermented milks | GRAS Lactic acid bacteria | [101] |
| Reduce burden of IBS | | LAB-fermented foods, especially fermented milks | GRAS Lactic acid bacteria | [101] |
| Anti-anxiety effect | | LAB-fermented foods, especially fermented milks | GRAS Lactic acid bacteria | [101] |
| Reduction of serum cholesterol level | | | | [102] |

| | | | | |
|---|---|---|---|---|
| Production of B's group vitamines | | Fermented milks, Yoghurts, Fermented Soymilk, Kefir | *L. casei, Bifidobacterium infantis, L. plantarum*... | [102] |
| Production of GABA | | | | [102] |
| | Antidiabetic, blood pressure | Fermented milk, Fermented soy milk, Yoghurt | *L. casei* Shirota, *S. salivarius, L. plantarum, L. brevis* | |
| Production of conjugated linoleic acid | | | | [102] |
| | Cholesterol lowering | Cheddar cheese, Buffalo cheese, Fermented buffalo milk, Yoghurt | *L. lactis, L. rhamnosus, S. thermophilus, B. bifidum* | [102] |
| Exopolysaccharides production | | | | [102] |
| | Immunostimulatory | Yoghurt, Cheddar cheese, Turkish cheese, Kefir, Fermented ice-cream | *L. bulgaricus, L. mucosae, P. freudenreichii, L. lactis, B. longum* | |
| | Hypocholesterolemic | | | |
| | Microbiota modulation | | | |
| | Immune modulation | | | |
| Bacteriocines production | | Camembert/Semihard cheese, Cheddar, Yoghurt, Munster cheese | *L. lactis, L. acidophilus, P. acidilactici* | [102] |
| Alleviate constipation | | Yoghurt | *B. animalis* subsp *lactis* DN-173010, *L. casei* subsp Shirota | [103] |
| Reduce eczemas | | fermented milk | | [103] |
| Antibiotic-associated diarrhea | | Fermented drink, yoghurt | *Lactobacillus casei* DN-114001 | [103] |
| Prevention of pediatric diarrhea | | Fermented drink, yoghurt | *Lactobacillus casei* DN-114002 | [103] |
| Prevention and help healing from respiratory infections | | Fermented drink, yoghurt | *Lactobacillus casei* DN-114003 | [103] |
| Fights infections | | | | [103] |
| | *H. pylori* infection | Fermented oat gruel in fruit drink | *L. plantarum* 299v (DSM9843) | |
| | *Clostridium difficile* infection | Fermented drink | *L. acidophilus* CL1285 + *L. casei* Lbc80r + *L. rhamnosus* CLR2 | |
| Improves microbiota | | Yoghurt | *L. acidophilus* + *B. animalis* subsp *lactis* | [103] |

### *2.2. Vegetable Fermented Products*

Since ancient times, the fermentation of vegetables has also been practiced by mankind, as proved by a long history of traditional products spread all over the world. Vegetables are mainly fermented by LAB both spontaneously and by means of inoculum and back-slopping [104,105]. Among these lacto-fermented vegetables are fermented cabbage (kimchi and sauerkrauts), fermented leaf (gundruk) and pickles (cucumber, chillies, capers and others). Many of the positive features related to fermented vegetables are derived from the effects of acids and fermentation, which, as a consequence of fermentation, change their form to become more bioavailable, thus increasing their effect and elimination of anti-nutritional compounds [106]. In this review, we focus on the two main products derived from cabbage fermentation, representing a widely consumed staple in western and eastern areas of the world: sauerkrauts and kimchi. Fermentation of vegetables has as primary effect of increasing the shelf-life of food. Moreover, it allows to ameliorate the intake of nutrients like fiber, vitamin*s,* and minerals. This effect is particularly useful since it permits the introduction of these micronutrients in periods when vegetables are unavailable*.* In a recent review, Bousquet et al. tried to find a relation between decease due to COVID-19 and diet of populations, focusing on the consumption of sauerkrauts. From their data analysis emerged how in the areas where the consumption of sauerkrauts is higher the number of deaths is slightly lower. Data anyway do not seem to be correlated and many other factors and bias contribute to the obtained results, thus further studies are needed to confirm any link [107].

#### *2.2.1. Sauerkraut*

Sauerkrauts are the product of cabbage fermentation (*Brassica oleracea* var. *capitata*). Sauerkraut manufacturing can be carried out following spontaneous fermentation or fermentations guided by selected and specific bacteria [108]. During fermentation, the composition of the product changes and, at the end, aside from macronutrients, it is possible to find a good amount of fiber, vitamin C, organic acids (lactic, acetic, malic and succinic), SCFA (propionic acid), ethanol, and acetaldehyde. Due to the knowledge about bioactive compounds present in fermented foods, in recent years, many efforts were made to improve the general quality of fermented vegetables while creating a product rich in bioactive compounds. For example, the utilization of a nisin-resistant strain of *Leuconostoc mesenteroides* in combination with a nisin-producer strain of *L. lactis* allowed obtaining a product with a suppressed native microflora [108]. Also, *Leu. mesenteroides* in combination with *Pediococus dextrinicus* showed a good potentiality to produce bioactive enriched foods. *Latilactobacillus sakei* showed a predominance in this feature since its utilization in vegetable fermentations allows the obtaining of foods with three times the concentration of bioactive compounds concerning any other studied bacterial strains [108]. Standardization of the product is of course a feature researched by companies. Despite this effort to standardize the products, aiming to use only selected microorganisms, it has to be considered that a reduced microflora diversity could lead to products with decreased bioactivities and a lower release of post- and para-probiotics

in the final product [109–111]. Thus, aiming to obtain a safe and health-contributing product, it is important not to underestimate the potential contribution of the autochthonous microflora in the fermentation process [112]. Since many reports suggest that regular consumption of this product can lead to the intake of a considerable amount of healthy bacteria (>$10^6$ log CFU/g), recent studies have focused their attention on the isolation of LAB from sauerkrauts. Strains of *Lactiplantibacillus paraplantarum*, *L. brevis,* and others *Lactobacillus* strains isolated from sauerkrauts showed adhesion to Caco-2-cells and inhibitory activity towards pathogenic microorganisms [108]. Nielsen et al. [113] reported that the effect of sauerkraut consumption on irritable bowel syndrome (IBS) affected a patient and reported that consumption of sauerkrauts, both fresh and pasteurized, led to a reduction of symptoms after 6 weeks, with a change in microbial composition of faecal matters of participants. Also, the high presence of dietary fibers seems to be involved in alleviating IBS symptoms [113]. Cabbages are also rich in phytochemicals with multiple possible bioactivities, but these compounds, mainly glucosinolates, are normally not bioavailable in the fresh product. Hydrolysis of glucosinolates leads to release of isothiocyanates, thiocyanates, epithionitriles, nitriles, and indolic compounds, all recognized for their valuable health boosting activities. Like many other fermented foods, sauerkrauts show antitumoral properties, exerted by activating enzymes that eliminate xenobiotics and increasing apoptosis of cancerous cells [108]. Specifically, indole-3-carbinol (I3C) is deeply investigated since it was shown to exert inflammation-modulating effects, promote cells proliferation, and inhibit tumour invasion in different tissues [108]. The presence of vitamins and organic acids gives to sauerkraut a powerful antioxidant feature, but it is also related to reduced inflammation, atherothrombosis, and increased human system efficiency in neutralizing reactive oxygen species. Antioxidant activities are also connected to reduced oxidative damage at the expense of DNA, which can also be due to indolic compounds' ability to scavenge chemicals, avoiding damages to DNA and other structures [114,115]. Fermentation enriches sauerkraut with a group of enzymes called Mono Ammino Oxidase Inhibitors (MAOIs), inhibiting Mono Ammino Oxidase (MAOs), which are a family of enzymes involved in arising depressive states, anxiety, obsessive-compulsive disorder, and development of Parkinson's disease [116]. The administration of sauerkraut was also studied in fighting IBS. In a pilot study, Nielsen et al. fed 34 volunteers with pasteurized and unpasteurized sauerkraut to evaluate the reduction in abdominal discomfort and problematics bound to IBS. From the results, it emerged that administration for 6 weeks of unpasteurized sauerkraut and 8 weeks of pasteurized sauerkraut can sensibly reduce abdominal discomfort and negative effects of IBS. Despite the difference in number of live bacteria, this similarity in results can be due to the natural composition of sauerkrauts, rich in glucosinolates and complex carbohydrates, acting as fiber in the intestine. In this optic, fermentation of sauerkrauts leading to glucosinolates breakdown can increase the bioactivity of this fermented food. Also, cells breakdown and liberation in para-probiotics media can contribute to the health-related positive effects of fermented cabbages [113]. Further experiments in this field should consider unpasteurized cabbage to estimate precisely the effect of fermentation with respect to unfermented product [117]. When talking about sauerkraut, many sources refer to its potentiality as a source of fiber and healthy compounds, forgetting about the presence of an abundant and vital LAB microbiota, mainly deriving from spontaneous fermentations that select microorganisms with an increasingly harsh environment. These bacteria have increased possibilities to reach the gut and

colonize intestine walls, where they can exert positive effects modulating the microbiota and immune response. As mentioned above, during recent years, literature has explored the idea that microorganisms' viability is not mandatory to exert probiotic effects. Cell wall material, cytosol compounds, and genetic information released after cells death are enough to vehiculate positive features. In this new post- and para-biotic field, sauerkraut has found a niche, since industrial productions require a pasteurization step, resulting in the death of live cells but not hampering the beneficial effects of wild LAB. Sauerkrauts are one of the most studied fermented vegetables, in fact suggestions about the introduction of sauerkraut in the diet are easy to find in the literature, even though intervention studies and dietary supplementation with this fermented food are still lacking and further investigation is surely needed. The literature reveals that sauerkraut possess a vast array of health effects related to glucosinolate compounds and microbial contribution in terms of microbiome, para-, and post-biotics. Despite esethese incredibly appealing features, there is a possible presence of biogenic ammines [118], while paying attention to microbial populations, since some harmful bacteria can survive to harsh conditions that arise during fermentation.

### *2.2.2. Kimchi*

Kimchi is the most produced and consumed lacto-fermented vegetable of Korea and its national product. It is often made by natural fermentation of Napa cabbage and other ingredients like onion, garlic, chillies, and fish sauce; their addition is fundamental in helping to control pathogenic and harmful microorganisms, allowing the growth of the beneficial ones. It is mainly *Leu. mesenteroides* that creates the acidic and anaerobic environment adaptable to the growth of more acid-resistant bacteria like *L. brevis* and *L. plantarum*. Kimchi is considered a natural functional food for the high presence of dietary fibers, minerals, vitamins, capsaicin, organic acids, polyphenols, and fermentation by-products (organic acids, bacteriocins, and others). The presence of these compounds is reported in scientific literature to produce positive effects on the health of consumers. Presence of a wild and acid-resistant microbiota is connected to the lowering of pH in the intestinal lumen and in the faeces, which is connected to a better microbiota, with an increased count in LAB and *Bifidobacteria* and a lower level of harmful and pathogenic microorganisms. Kimchi is studied, especially in the most recent literature, for its ability to modulate gut microflora, and Park et al. studied the effect of kimchi to exert an anti-obesogenic effect on the microbiome, starting from the assumption that many factors can cause obesity, such as an unbalanced diet; genetic factors; and unhealthy gut microflora resulting in modifying energy intake and accumulation in the adipocytes, increasing obesogenic effect [119]. The intestinal microbiota is more than the sum of its part, it is an organism able to live in symbiosis between the same parts composing it and ourselves. Due to microbial diversity, long-term stability, ease of use, and domestic preparation, kimchi was taken into account to modify gut microbiota [117], helping pathologically obese subjects, normalizing their lipid levels and modulating their microbiome [120–122]. Results of these experiments highlighted that supplementation with kimchi in mice fed with an HFD cannot significantly decrease weight gain, with respect to mice fed with just an HFD, indicating that the

number of calories introduced is the main factor in weight gain [119]. The introduction of kimchi anyway showed a reduction in blood glucose, triglycerides, and high- and low-density lipoproteins with respect to mice fed with only HFD [123]. Even if the total weight gain was not significantly decreased by kimchi in HFD mice, other indexes like total body fat gain, liver weight, and adipocytes' dimensions and counts were lowered by the administration of kimchi. Also, gut microflora resulted modulated by the administration of kimchi, *Akkermansiaceae*, *Coriobacteriaceae*, and *Erysipelotrichaceae*, which are normally related to HFD and consequently to obese subjects, were lowered in mice fed with kimchi, while the abundance of *Muribaculaceae,* negatively correlated with obesity, increased in kimchi-fed mice [119]. Kimchi, due to the high presence of fibers and nutritional compounds was also studied as a solution to cope with prediabetics patients [124]. Prediabetics are subjects who have blood glucose higher than unaffected subjects, but not high enough to be considered properly diabetics, and are strongly subject to develop this issue later, due to unhealthy lifestyle and diet [124]. Fortunately, a change in dietary and lifestyle habits can slow, and in some case even stop, the progression of prediabetes into diabetes. In an intervention study, An et al. administered 100 grams of kimchi per meal to 21 prediabetic volunteers for 2 weeks, followed by a 4-week washout period. From anthropometric parameters after regular consumption of kimchi, it emerged that insulin sensitivity and resistance and blood pressure were positively affected by introduction of this fermented product. Also, the participants' body mass index (BMI) and weight decreased significatively, together with waist circumference, which is strongly bound to insulin resistance. The consumption of kimchi thus revealed to be a strong ally in fighting the onset of diabetes [124,125]. Being rich in anti-microbial compounds, produced mainly by an active and resistant positive microflora, kimchi is employed from centuries as "medicine" food and can be ascribed in the functional foods group. Functional foods are "foods or dietary components that may provide a health benefit beyond basic nutrition" [126]. Some studies focused on the utilization of kimchi to fight infections by *Helicobacter pylori*, which is a well-known contributor to the development of peptic and perforative ulcers and one of the recognized class I carcinogens [125,127]. The high level of antioxidants, vitamins, and the presence of other phytochemicals, together with the reduction of ingestion of other harmful products prove to be an effective, cheap, and easy way to control the development of *H. pylori* and help eradicate it. During *H. pylori* infection, the body reacts by increasing the expression of pro-inflammatory genes to fight the attack, but the maintenance of an inflammation state for too long in the body can lead to DNA damage through oxidative stress and disruption of cell life cycle. In this way, gastric and intestinal carcinogenesis is facilitated. Therefore, the introduction into the diet of a food possessing antioxidant and anti-inflammatory properties such as kimchi results as being useful. In the optic of cancer's prevention, kimchi has been employed as a tool to fight the development of colon-rectal colitis-associated cancer in patients chronically affected with IBS. From the experiments led by Han et al., it emerged that a special formulation of kimchi supplemented to mice, containing pear extracts and see tangle juice, prevents cancer formation by means of inflammasome reduction [128], resulting in anti-inflammatory and anti-oxidant effects, cytoprotective ability, and reduced proliferation of harmful microorganisms due to induction of apoptosis. In the same experiments, it emerged that the introduction of unfermented kimchi does not exert the same protective effect, accelerating the formation of cancers in the gut instead. This highlighted once more that the development of the

native microbiota of the vegetable represents the real game changer in health-related effects of fermented foods [128]. Ordinarily, to cope with inflammation and ulcers, anti-inflammatory drugs are prescribed, and this can lead to resistance phenomena and to reduction of positive microbiota sensitive to drugs. Ingestion of kimchi could be a strong ally, due to ease of use, stability, and of course due to the introduction of a series of nutritional compounds exerting a plethora of positive health-related effects. Kimchi can play an important role also in maintaining under control the degeneration of several chronic diseases like IBS, Crohn's disease, and infections due to external attacks or unhealthy eating habits [129–131]. However, it has to be considered that, as for other spontaneous fermented foods, kimchi contains a high level of salt involved in the formulation to control negative microflora. In some studies, the kimchi's supplementation was in fact limited to around 100 g of fermented food [124], matching nutritional suggestions for salt introduction to the diet, while other studies increased to 210 g per day the administration of kimchi, which provides a salt content higher than what the guidelines suggest [132]. All this considered, the introduction of kimchi as a regular meal or side-dish also in Western countries could help populations to control the development of gastro-intestinal issues (Table 3).

**Table 3.** Health-related effects of fermented vegetables products.

| Health Effect | Specific Effect | Fermented Food | Microorganisms | References |
|---|---|---|---|---|
| Antioxidant | Carotenoids modified by fermentation | Kimchi and Sauerkraut, Soybean, | *W. koreensis, L. brevis, Leu. gelidum* | [133] |
| Reduction of cronic diseases | Cardiovascular disease<br>Cancer<br>Diabetes<br>Alzheimer<br>Cataracts<br>Age-related functional declines | Tomato Juice, Leek, Carrots,<br>Fennels, Onions, Pomegranate<br>Pear juice, Pineapple juice, Apple,<br>Quince, Grape, Kiwifruit | *Leu. mesenteroides, L. plantarum, W. Confusa,*<br>*L. delbrueckii subsp. lactis, B. thermophilum* | [133] |
| Hypoglycemic | | | | [133] |
| Anti-inflammatory | | | | [133] |
| Hypolipidemic | | | | [133] |
| Immunomodulatory | | | | |

| | | | | |
|---|---|---|---|---|
| Anti-microbic | | | *Lactobacillus* and *Lactococcus* genera | [133,134] |
| | Eliminate *H. pylori* | | | [134] |
| Reduction of anti-nutritional compounds | | Fermented legumes and cereals | *Lb. plantarum* and other LAB | |
| Increase of nutritional density | | Every fermented vegetable | Generic LAB | |
| | Breakdown of complex carbohydrates | | | |
| | proteolysis | | | |
| Glucosinolates breakdown | | | | [133] |
| | Increase antioxidant activity | Brassica vegetables | Generic LAB | |
| | Ameliorate metabolic syndrome | Brassica vegetables | Generic LAB | |
| Production of SCFA | | Every fermented vegetable | Generic LAB | [133] |
| Anti obesogenic effect | | Every fermented vegetable | Generic LAB | |
| | Lower obesity incidence | | | |
| | Direct anti-obesogenic effect | | | |
| Prebiotic effect | Production of EPS | | *W.confusa and W. hellenica,Lactobacillus, Lactococcus, Leuconostoc, Pediococcus* and *Weissella*, | [133] |

## 3. Conclusions

The aim of this review was to highlight the ability of LAB involved in food's fermentation to exert beneficial effects on human health. Regular ingestion of foods fermented by LAB in the diet can be a great help, due to the introduction of bioactive compounds that are released during fermentation and become available during digestion. It is well established that the ingestion of LAB-fermented foods can modulate the gut microbiome in its functionality and response to stress and attacks, both due to the presence of health-related LAB species and their metabolites produced during fermentation. LAB's ability to produce bioactive peptides, vitamins, organic acids,

bacteriocins, signalling molecules (NO), and antimicrobial compounds ($H_2O_2$) plays a fundamental role in promoting and maintaining a health status in consumers of LAB-fermented products. Despite the need of a higher amount of in vivo studies on a wider population and considering also the possible interaction among different fermented foods contemporaneously introduced, the pieces of evidence reported in the literature so far suggest that higher ingestion of LAB-fermented foods in the diet, daily, could contribute to a healthy lifestyle and in the maintenance of organisms functions and health.

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# Chapter 2- Probiotics and Covid-19

Research is influenced by many external factors, both in positive and negative ways. During the last 2 years the COVID-19 pandemic had stricken hard our society with great impact on people's everyday life. During the first months of pandemic the world stopped aiming to preserve the weakest and sensible to this respiratory virus. Also scientific research, was severely influenced from the pandemic, with laboratories that were closed and researcher which were hampered to proceed with their studies. During this time also my research had to stop, and experiments were delayed sensibly. Despite the stop, the studies manage to go over and during the pandemic we were studying the literature, also at the light of the new discoveries of secondary infection caused by COVID-19. These secondary infections were reported affecting the guts of patients, causing severe diarrhea and other problematics able to aggravate an already threatening clinical situation. For this reason, my small contribute to the global research about COVID and its derived health issues was to search the literature for studies concerning respiratory viruses and a possible involvement of probiotic microorganisms in fighting the onset and reduce symptoms of pulmonary infections. Probiotics, besides to their multiple positive effects showed the ability to reduce damages caused by secondary infection in the intestine, and when administered via upper respiratory tracts contribute to fight the settlement of viruses and other pathogens. Delaying and in some case preventing the development of the disease, and its fatal ending that unfortunately every one of us has learned about.

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## Abstract

Coronavirus disease 2019 (COVID-19) has become pandemic very rapidly at the beginning of 2020. In the rush to possible therapeutic options, probiotics administration has been proposed mainly based on indirect observation. Some evidence of COVID-19 effects on intestinal microbiota dysbiosis has been shown and probiotics have been considered for their efficacy in the management of respiratory tract viral infections. These observations could be reinforced by the more and more evident existence of a lung-gut axis, suggesting the modulation of gut microbiota among the approaches to the COVID-19 prevention and treatment. As different possible roles of probiotics in this extremely severe illness have been contemplated, the aim of this work is to collect all the currently available information related to this topic, providing a starting point for future studies focusing on it.

Keywords: Covid-19, Probiotics, SARS-CoV2, Immunomodulation, Lactic Acid Bacteria, Gut microbiota

Introduction

## 1. Introduction

In December 2019 a viral outbreak referred to as COVID-19 [1] has been reported from Wuhan, China. The viral agent has been recognized as a zoonotic beta-coronavirus, named severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), similar to other SARS and MERS (Middle East Respiratory Syndrome) coronaviruses [2]. COVID-19 causes a severe acute respiratory syndrome (SARS) named specifically SARS-CoV-2 with a lethality ranging from 2% in China [3] to 12% in certain region of Italy [4]. Post-mortem analysis on a patient died by SARS-CoV-2 conducted by these authors on lung, liver and hearth tissue that had shown severe damages at lungs with oedema and desquamation, evident symptoms of breath complications and fatigue. Some patients with COVID-19 showed intestinal microbial dysbiosis with decreased probiotics such as *Lactobacillus* and *Bifidobacterium*, suggesting the needing to assess nutritional and gastrointestinal function for all patients [3]. Formerly, dysbiosis of the human gut microbiome has been linked to various health conditions, including respiratory tract infections (RTIs) through the gut-lung axis [5]. Many studies, conducted on a variety of participant, with different ages, provenience and social extraction have explored the correlation between the ingestion of probiotics or symbiotics, in different forms and the onset of diseases, with a particular attention to RTIs [6–9]. Nutritional support and application of prebiotics or probiotics were suggested also in COVID-19 infected patients, to regulate the balance of intestinal microbiota and reduce the risk of secondary infection due to bacterial translocation [3]. Other authors have speculated that COVID-19 may be related to the gut microbiota, since some evidence highlighted a secondary gut infection or dysfunction in patient with RTIs, probably due also to antibiotics which are not selective towards harmful bacteria. This suggest also a gut–lung crosstalk, and in some extent that the symptoms may be modulated by probiotics, altering in this way the gastrointestinal symptoms favourably and protecting also the respiratory

system [10]. Despite no treatment has been approved so far for the treatment or prevention of COVID-19 infections due to the lack of scientific evidences and clinical trials, an urgent need to find options to help these patients and preclude potential death is pushing the entire scientific community to focus on this topic [11]. As a possible role of probiotics in COVID-19 prevention and treatment has been contemplated [12], the aim of this work is to collect all the currently available information related to this topic, providing a starting point for future studies focusing on it.

## 2. Role of probiotics in health and diseases

Probiotics are "live microorganisms which when administered in adequate amounts confer health benefits to the host" [13]. Their use to enhance human health has been studied since long, formerly as food ingredients and later also as cultures preparations [14–16]. The application of probiotics has been primarily investigated for the prevention and treatment of gastrointestinal infections and diseases [17], but other possible effects have been studied such as nutritional effects, prevention and treatment of oral infections, diarrhoea caused by several factors, irritable bowel syndrome, inflammatory bowel diseases, *Helicobacter pylori* infections, allergic diseases, antitumor effects and reduction of serum cholesterol [18,19]. Due to the reported ability of several probiotic strains to improve mucosal immunity against pathogens, possible effects also in the prevention and treatment of RTIs have been suggested. However, the efficacy and safety of probiotics are strain, dose, disease and possibly host dependent. Furthermore, despite the huge increase of in vitro studies regarding probiotic strains, the needing for in vivo studies, followed by animal studies and clinical trials on human studies, has been underlined [20]. All this considered, as clinical data sustaining the use of probiotic in preventing COVID-19 are increasing, their use to reduce the burden and severity of this pandemic appears worthy of consideration [12].

## 3. Probiotic effects on immune responses

Boosting immune responses during the incubation and non-severe stages of Covid-19 infection, to eliminate the virus and preclude disease progression to severe stages, have been proposed as extremely important. In the gastrointestinal tract (GIT), known as one of the most microbiologically active ecosystems playing a crucial role in the working of the mucosal immune system, probiotics stimulate the immune system and induce a network of signals mediated by the whole bacteria or their cell wall structure [21]. Many probiotic effects are mediated through immune regulation, particularly through balance control of proinflammatory and anti-inflammatory cytokines [22]. Another important effect exerted by probiotics is to enforce and maintain the integrity of junction between enterocytes, in this way entrance of SARS-CoV2 is reduced, as well as the risk to develop COVID-19 [12]. During recent years, the connection between gut microbiota and general health have been demonstrated. Diet can modulate the functionality of the intestinal microbiome which uses nutrients from ingested foods, releases harmful or beneficial metabolites and regulates the immune system [23,24]. The gut mucosal surface is a principal site of entry of

pathogens into the human body but in healthy subjects, intestinal epithelium and its microbiota provide an efficient barrier to invading microorganisms [25]. Gut is involved in immunity as dendritic cells of the intestinal lumen are the first cells of the mucosal immune system to encounter commensal and pathogenic bacteria [26]. Dysbiosis, meaning imbalances in the composition and function of the intestinal microbes, is associated with various human diseases [27]. Thus, manipulation of the intestinal microbiota has been proposed as a potential alternative approach for maintaining health and preventing and/or treating diseases [28]. This can be done by stimulating beneficial bacteria colonizing the GIT through the diet [29] or by the administration of probiotics. Probiotics able to assist in restoring unbalanced microbiota and maintaining gut immune homeostasis have been defined also as immunobiotics, i.e. microorganisms which possess the ability of improve innate immune response. Some studies have shown that this stimulation is exerted by intra and extra cellular molecules like peptidoglycan, phospho-polysaccharides lipoteichoic acid or DNA. Specifically, the ability to modulate the innate immune system is attributed to membrane molecules of probiotics that can communicate and signaling with the epithelial cells of gut, exerting in this way the probiotic effect [30]. Modulation activity of immune system is also fundamental since it has been proven that an excessive immune response can cause as much damages as the pathogenic infection itself. Restoring gut microbiota has been shown to improve resistance to virus or pathogenic attacks also at the respiratory mucosa level [31,32]. In different trials, it has been demonstrated that probiotics, such as *L. rhamnosus* GG, can help improving intestinal and lung barrier and homeostasis, by increasing regulatory T cells, ameliorating anti-viral defense, and decrease pro-inflammatory cytokines in systemic and respiratory infections. These immunomodulatory benefits are especially important to individuals who have developed, or are at risk of developing, COVID-19 [33]. The gut and lungs are anatomically distinct, but potential anatomic communications and complex pathways involving their respective microbiota have reinforced the existence of a gut–lung axis, which can shape immune responses and interfere with the course of respiratory diseases. Probiotic strains could be used to manipulate these microbiota, offering new perspectives in the management of respiratory failures [34] which is one of the leading causes of death due to COVID-19 infection [35].

## 4. Probiotics used for prevention and treatment of respiratory tract infections

In recent years' probiotic strains have been increasingly considered as a powerful ally in fighting and prevent RTIs. Treatments with probiotics bacteria have been shown to reduce both upper and lower respiratory tracts infections [33]. Probiotic lactic acid bacteria (LAB) have been administered both directly in the respiratory tract or integrated in the diet to improve the immune response and fight viral infections [36]. In that study the author evaluates the effect of *L. rhamnosus* CRL1505 in modulating the immune response of malnourished mice towards inoculated *Streptococcus pneumoniae* [36]. Mice supplemented with *L. rhamnosus* CRL 1505 showed an ameliorated response to *S. pneumoniae* infections mediated by myeloid cells and lymphocytes B. In another study by Perdigón [37], mice infected with *S. pneumoniae* were administrated with 1 of 3

different probiotic LAB strains, *Lacticaseibacillus casei* CRL 431, *Lactococcus lactis* NZ9000, *L. rhamnosus* CRL1505 or a probiotic fermented yoghurt produced by *Lactobacillus delbrueckii* subsp. *bulgaricu*s CRL 423 and *Streptococcus termophilus* CRL 412. In all cases, several beneficial effects were recorded in treated mice such as the reduction of pathogen present in lungs and blood, an increase of neutrophil count in blood and higher level of IgA, known for the anti-pneumococcal activity, in the intestine and in the aerial ways [31,37,38]. Probiotic LAB have been used also as antiviral agent to fight or prevent respiratory infections both in human and animals, administrated locally or involved in food preparation [36,39]. They have been described for their ability to inhibit virus by directly interacting with them with a mechanism similar to phagocytosis. As an example, *Lactiplantibacillus plantarum* YU, isolated from fermented Japanese food, showed a very high interleukine-12-inducing activity in vitro, inducing activity in mouse peritoneal macrophages. The probiotic strain suppressed antigen-specific Immunoglobulin E production by activation of Th1 immune responses in mice and enhanced natural killer cell activity and IgA production in vitro, proving a protective effect against influenza A virus infection in vivo [40]. More recently lactobacilli isolated from healthy human noses have been shown to have probiotic effects in the form of nasal spray [41]. Other lactobacilli are able to avoid the attack of viral particles to mucosal cells, this open also the possibility of employment of probiotics in nasal spray to ameliorate immune system and avoid respiratory tract infections [33]. Further mechanisms that Lactobacillus species exert against respiratory viruses have been proposed such as the production of proteinaceous or non-proteinaceous inhibitors factor like $H_2O_2$, lactic acid and bacteriocins [39], being the mechanisms of action of these latter against viruses non fully understood [42]. A combination of *L. rhamnosus* GG and *Bifidobacterium animalis* subsp. *lactis* BB12 was shown to inhibit the incidence of diseases caused by respiratory viruses and needing for antibiotics of about 50% in the group who was administered with probiotics with respect to placebo group [43]. Influenza virus H1N1 titers in lungs of infected mice have been decreased by the oral daily administration of *L. plantarum* L-137, a strain with proinflammatory activity. Moreover, *L. rhamnosus* CRL 1505 had shown the ability to stimulate immune system by secretion of IFN-γ and IL in 3 weeks-old mice, reducing viral load in lungs tissue injuries after the challenge with respiratory syncytial virus, without the help of antibiotics [44,45]. The administration of probiotic fermented drinks and probiotics such as *L. casei* Shirota showed to increase antibody responses to influenza virus vaccination in the elderly and accelerate innate immune response of respiratory tract and protect against various respiratory infections in newborns, infants and children, groups at higher risks of respiratory infections [46,47]. Furthermore, probiotic oral administration has been shown to influence inflammatory cytokine production in the lungs, which has been linked with Covid-19 lethality [48].

## 5. Probiotics ACE inhibitory effect

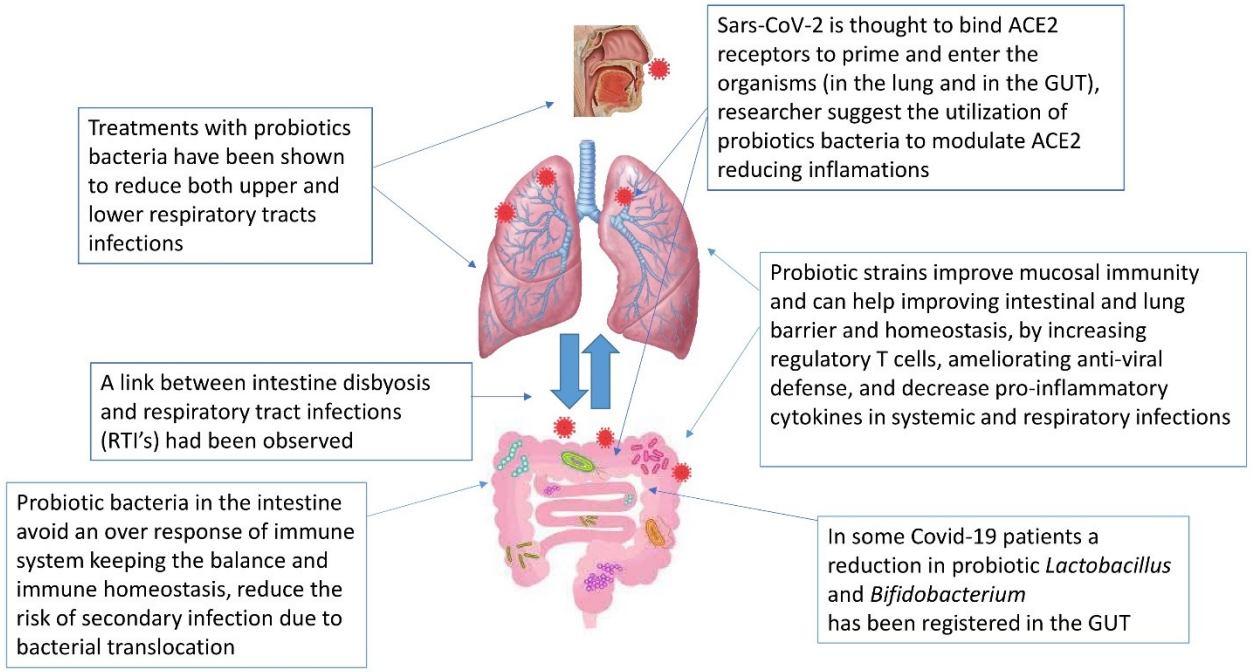
Similar to other SARS coronaviruses, SARS-CoV-2 is thought to bind, through its surface spikes proteins with the angiotensin-converting-enzyme 2 (ACE2) receptor for intracellular invasion and utilize cellular serine protease TMPRSS2 to prime and replicate in infected organisms [49–51]. On the other hand, the mechanism for acute lung injury during infection has been postulated to be mediated through activation of renin angiotensin system (RAS), in which ACE2 produces several

protective effects [52]. The expression of ACE2, which is expressed by epithelial cells of the lung, intestine, kidney, and blood vessels, is substantially increased in patients treated with ACE inhibitors [53]. For these reasons, some researchers suggested that ACE inhibitors might benefit patients with Covid-19 by reducing pulmonary inflammation [54], although others argued that ACE inhibitors might enhance viral entry by regulating ACE2 levels. Several probiotics, particularly LAB, have been reported as able to produce peptides with ACE inhibitory effect [55]. Indeed, some media sources have recently called for the administration of probiotic food and drugs, both prophylactically and in the context of suspected Covid-19. However, due to the still controversial debate on the effect of ACE-inhibitors in the Covid-19 progression [56], the potential role of probiotic in modulating ACE2 level is to be critically considered when they are proposed as an adjunctive therapeutic option [57]. Further, following the demonstration of a non-catalytic role for ACE2 in amino acid transport in the gut, a recent work speculated that a therapeutic effect of ACE2 can be mediated, in part, by its actions on the gastrointestinal tract and/or gut microbiome. This is consistent with emerging data supporting the existence of a link between the gut and lungs and suggesting that dysbiosis of the gut and lung microbiomes is associated with cardiopulmonary disease [52]. In this optic, a possible role of probiotic in shaping the evolving role for gut and lung microbiota in the onset of SARS-CoV2 infection' symptoms should be assessed.

## 6. Conclusion

On the basis of the available evidence, the possible benefits of probiotic administration in the framework of Covid-19 infection (Fig. 1), may be due, principally, to their effects on innate and adaptive immunity. Probiotic actions such as influence on cytokines production by intestinal epithelial cells, IgA secretion stimulation to improve mucosal immunity, activation of phagocytosis and macrophage production, modulation of levels and function of regulatory cells, and induction of dendritic cells maturation, likely affect systemic inflammation. Furthermore, increasing evidence supports a link between the gut and lungs, thus, further studies should be addressed to investigate a potential role of probiotic in attenuating COVID-19 either through immunomodulatory actions on systemic inflammation or by direct interaction with the lungs. However, not all probiotics are likely to be the same, thus a more targeted approach through the characterization of specific properties of probiotic bacteria at strain level during the development of potential application in COVID-19 and its comorbidities.

Fig1. Clues suggesting a possible application of probiotics in reducing burden and severity of Sars-CoV-2 infections

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# Chapter 3 - Wild *Lactobacillus casei* group strains: potentiality to ferment plant derived juices

Positive effects linked to fermentation carried out by LAB are many and well documented. Despite this, the studies on positive effects of fermented foods have particularly focused on specific LAB and their typical ecological niches. However, LAB are known to be able to adapt and evolve in changing ecosystems, and their capabilities may be exploited beyond their origin of isolation. In this optic, the ability of wild LAB strains selected from dairy substrates to ferment plant derived juice was investigated. The choice of vegetable juices as a fermentation substrate was guided by the increasing interest of the consumers for minimally processed food, that is well perceived and carries attributed positive features. The production of EPS was one of the main aspects considered, as it is well documented that LAB's EPS may have anti-cancer, immunomodulating, anti-oxidant, biofilm degrading and prebiotic effects, besides showing an important role in formulation of dairy and baking products, due to their contribution to rheology and structure of fermented foods.

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**Abstract:** Plant derived beverages have recently gained consumers' interest, particularly due to their intrinsic functional properties. They can also represent non-dairy carriers for probiotics and prebiotics, meeting the needs of lactose allergic/intolerant people and vegans. Direct fermentation of fruit and vegetables juices by probiotic lactic acid bacteria, could be a tool to increase safety, shelf-life, nutrient bioavailability and improve sensorial features of plant derived juices. This study aimed to screen wild Lactobacillus casei-group strains isolated from dairy matrices for probiotic features, such as acid and bile salts resistance, and test them for the potentiality to ferment celery and orange juices. Strains ability to produce exopolysaccharides (EPS) in situ was also checked. These evaluations were performed for the first time in fruit and vegetables matrices by means of an impedometric analysis, recently shown to be a suitable and rapid method to measure microorganisms' growth, acidification performances and EPS production. This study allowed to select three potentially probiotic *L. casei*-group wild strains able to ferment fruit and vegetable juices also producing EPS. These strains with three-in-one abilities could be used to produce new functional fermented plant derived juices.

**Keywords:** Wild *Lactobacillus casei*-group strains; plant derived juices fermentation; impedometric analysis; exopolysaccharides; probiotic

## 1. Introduction

Demand for plant derived products has increased in recent years due to their recognized health benefits [1–3] . Although recommendations suggest the consumption of fresh fruit over fruit juices and derivatives, sometimes, supplying the markets with fresh fruit can be tricky because of the high intrinsic perishability of fruit. In this optic non sweetened fruit and vegetable juices can be valid alternatives to whole fruits equivalents in meeting dietary requirements, improving cardiovascular health and lowering incidence of several chronic non-communicable diseases [4–6]. For this reason, the attention of industries has focused on producing juices or formulated beverages with nutritional properties, like richness in bioactive compounds and nutrient factors [7]. However industrial production of these kind of products requires a particular attention, in fact, to obtain safe juices with a prolonged shelf life, treatments are needed to stabilize them. Even though mild technologies are sometimes applied to these products [8–10], most frequently, thermal treatments are used [11,12]. These could modify juices' nutritional properties, by degrading micronutrients and lowering contents of vitamin C, provitamin A and other nutritional factors like antioxidants and phytochemicals [13]. A useful alternative able to maintain and/or improve the safety, nutritional, sensory and shelf life properties of fruits and vegetables, is lactic acid fermentation [14–18]. Traditionally, most fermented products were based on milk but fermentation of non-dairy matrices is gaining increasing attention [19] and it is possible thank to the ability of LAB to ferment also plant derived and mildly acid substrates such as fruit and vegetable juices [18,20]. Dairy fermentation is usually driven by starter LAB species such as *Streptococcus thermophilus*, *Leuconostoc mesenteroides*, *Lactococcus lactis* and *Lactobacillus delbrueckii* spp. *bulgaricus* [21,22]. However, species belonging *to Lactobacillus casei*-group are frequently used as adjunctive and/or secondary

starters to improve fermented products characteristics. Species belonging to this group are also well known for their probiotic traits [21,23]. Probiotics are defined as “live microorganisms which when administered in adequate amounts confer a health benefit to the host’’ [24]. Considering that plant derived juices have proved to be promising carriers for probiotics [25], direct fermentation of vegetables and fruit juices by probiotic LAB, could be a tool to increase safety, shelf-life, nutrient bioavailability and to improve sensorial features of plant derived juices [19]. Furthermore, consumer’s demand for non-dairy probiotic foods is constantly increasing due to drawbacks related to dairy foods such as allergy, lactose intolerance and cholesterol content, as well as revolution in living standards, eating habits (i.e. vegetarian and strict vegans) religious beliefs and augmented health awareness [21,25]. Finally, from a technological point of view, plant derived probiotic products could offer a direct prebiotic activity and may help to deliver the probiotic organisms to the target sites [21]. Prebiotics have been defined as a non-digestible food ingredient that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improve host health [26]. Among prebiotics, many exopolysaccharides (EPSs) from LAB have been already studied for their potential prebiotic activities[27,28]. EPS production seems to help the survival of probiotic bacteria during the gastrointestinal transit [29], suggesting the EPS production as an interesting property to be considered for the selection of putative probiotic strains. The aim of this study was to evaluate the ability of wild dairy *L. casei*-group strains to ferment plant derived substrates and to produce EPS in situ, in order to select the most suitable one to be used for the production of functional juices. This evaluation has been performed for the first time in these matrices by means of an impedometric analysis, recently shown to be a suitable and rapid method to measure the growth of microorganisms [30] and reveal EPS production [31].

## 2. Materials and Methods

### *2.1. Bacterial strains*

Fifty-three wild *L. casei* group strains previously isolated from dairy matrices and belonging to the microbial collection of the Department of Food and Drug of the University of Parma, were tested for their acid and bile salts resistance (Table 1). Ten out of the 53 analyzed strains, were tested for their ability to grow in non-dairy extracts: 4 *Lacticaseibacillus casei* (*Lc* 2233, *Lc* 2243 *Lc* 2404, *Lc* 2410); 1 *Lacticaseibacillus paracasei* (*Lp* 2306) and 5 *Lacticaseibacillus rhamnosus* (*Lr* 2216, *Lr* 2299, *Lr* 2325, *Lr* 2409, *Lr* 2462). Moreover, a commercial probiotic strain, *Lacticaseibacillus rhamnosus* GG (ATCC 53103), was used. All the strains were maintained as stock cultures at -80 °C in MRS broth (Oxoid, Basingstoke, UK) supplemented 20% (v/v) glycerol until use. Upon usage, bacteria were revitalized by inoculating 200 µL of thawed cultures in 6 mL of sterile MRS and incubated for 24 hours at 37°C in anaerobiosis condition. Overnight cultures were counted to verify the microbial cell load, washed with Ringer solution and properly diluted to reach an inoculum level of 8 log CFU/mL

*2.2. Determination of acid and bile salts resistance*

Resistance to increasing concentration of bile salts was measured for the 53 *L. casei*-group strains (Table 1) by streaking 10 µL of overnight cultures on MRS agar supplemented with 0.2 g/L and 0.4 g/L of bile salts and incubated at 37°C for 48 hours. Acid resistance was tested by streaking 10 µL of overnight cultures of strains on MRS agar adjusted at pH 2.5 with hydrochloride acid and incubated at 37°C for 48 hours. As a positive control, strains were grown on MRS plates. After 48 hours of incubation, growth was verified by visual inspection of plates. Results were reported in Table 1 as follows: (-) absence of growth, (+) low growth, (++) abundant growth

Table 1. Strains resistance to acid and bile salts. For each strain, the collection number, species and isolation matrix are given. Results are reported as absence of growth (-), low growth, (++) abundant growth. Strains written in bold were chosen for further experiments.

| **Strain** | **Species** | | **Isolation matrix** | | | |
|---|---|---|---|---|---|---|
| | | | | MRS HCl | MRS+bs 0,2 | MRS+bs 0,4 |
| **2233** | ***Lacticaseibacillus*** | ***casei*** | **Parmigiano Reggiano cheese** | **++** | **++** | **++** |
| **2243** | *Lacticaseibacillus* | ***casei*** | **Parmigiano Reggiano cheese** | **++** | **++** | **++** |
| **2322** | *Lacticaseibacillus* | *casei* | Parmigiano Reggiano cheese | ++ | ++ | ++ |
| **2326** | *Lacticaseibacillus* | *casei* | Parmigiano Reggiano cheese | ++ | ++ | ++ |
| **2333** | *Lacticaseibacillus* | *casei* | Parmigiano Reggiano cheese | ++ | + | + |
| **2337** | *Lacticaseibacillus* | *casei* | Parmigiano Reggiano cheese | - | - | - |
| **2404** | ***Lacticaseibacillus*** | ***casei*** | **Parmigiano Reggiano cheese** | **++** | **++** | **++** |
| **2405** | *Lacticaseibacillus* | *casei* | Parmigiano Reggiano cheese | ++ | ++ | ++ |
| **2406** | *Lacticaseibacillus* | *casei* | Parmigiano Reggiano cheese | ++ | - | - |
| **2407** | *Lacticaseibacillus* | *casei* | Parmigiano Reggiano cheese | ++ | ++ | - |
| **2410** | ***Lacticaseibacillus*** | ***casei*** | **Parmigiano Reggiano cheese** | **++** | **++** | **++** |
| **2413** | *Lacticaseibacillus* | *casei* | Parmigiano Reggiano cheese | ++ | - | + |
| **2092** | *Lacticaseibacillus* | *paracasei* | Grana Padano cheese | ++ | ++ | - |
| **2302** | *Lacticaseibacillus* | *paracasei* | Parmigiano Reggiano cheese | ++ | ++ | ++ |
| **2303** | *Lacticaseibacillus* | *paracasei* | Parmigiano Reggiano cheese | ++ | ++ | ++ |
| **2306** | ***Lacticaseibacillus*** | ***paracasei*** | **Parmigiano Reggiano cheese** | **++** | **++** | **++** |
| **2408** | *Lacticaseibacillus* | *paracasei* | Parmigiano Reggiano cheese | ++ | ++ | + |
| **1019** | *Lacticaseibacillus* | *rhamnosus* | Parmigiano Reggiano cheese | ++ | ++ | + |
| **1200** | *Lacticaseibacillus* | *rhamnosus* | Parmigiano Reggiano cheese | ++ | ++ | ++ |
| **1473** | *Lacticaseibacillus* | *rhamnosus* | Parmigiano Reggiano cheese | ++ | ++ | + |
| **1678** | *Lacticaseibacillus* | *rhamnosus* | Parmigiano Reggiano curd | - | - | - |
| **2118** | *Lacticaseibacillus* | *rhamnosus* | Grana Padano cheese | ++ | ++ | + |
| **2190** | *Lacticaseibacillus* | *rhamnosus* | Grana Padano cheese | ++ | ++ | + |

| | | | | | | |
|---|---|---|---|---|---|---|
| **2197** | *Lacticaseibacillus* | *rhamnosus* | Raw milk | ++ | + | + |
| **2203** | *Lacticaseibacillus* | *rhamnosus* | Raw milk | ++ | ++ | ++ |
| **2216** | ***Lacticaseibacillus*** | ***rhamnosus*** | **Raw milk** | **++** | **++** | **++** |
| **2222** | *Lacticaseibacillus* | *rhamnosus* | Raw milk | + | ++ | + |
| **2232** | *Lacticaseibacillus* | *rhamnosus* | Parmigiano Reggiano curd | ++ | ++ | + |
| **2240** | *Lacticaseibacillus* | *rhamnosus* | Parmigiano Reggiano cheese | ++ | ++ | ++ |
| **2246** | *Lacticaseibacillus* | *rhamnosus* | Parmigiano Reggiano cheese | + | ++ | + |
| **2247** | *Lacticaseibacillus* | *rhamnosus* | Parmigiano Reggiano cheese | + | + | + |
| **2298** | *Lacticaseibacillus* | *rhamnosus* | Parmigiano Reggiano cheese | ++ | ++ | ++ |
| **2299** | ***Lacticaseibacillus*** | ***rhamnosus*** | **Parmigiano Reggiano cheese** | **++** | **++** | **++** |
| **2300** | *Lacticaseibacillus* | *rhamnosus* | Parmigiano Reggiano cheese | ++ | ++ | ++ |
| **2310** | *Lacticaseibacillus* | *rhamnosus* | Parmigiano Reggiano cheese | ++ | ++ | ++ |
| **2323** | *Lacticaseibacillus* | *rhamnosus* | Parmigiano Reggiano cheese | ++ | ++ | ++ |
| **2325** | ***Lacticaseibacillus*** | ***rhamnosus*** | **Parmigiano Reggiano cheese** | **++** | **++** | **++** |
| **2334** | *Lacticaseibacillus* | *rhamnosus* | Parmigiano Reggiano cheese | ++ | ++ | + |
| **2335** | *Lacticaseibacillus* | *rhamnosus* | Parmigiano Reggiano cheese | ++ | ++ | ++ |
| **2336** | *Lacticaseibacillus* | *rhamnosus* | Parmigiano Reggiano cheese | ++ | ++ | ++ |
| **2352** | *Lacticaseibacillus* | *rhamnosus* | Grana Padano cheese | ++ | ++ | + |
| **2362** | *Lacticaseibacillus* | *rhamnosus* | Grana Padano cheese | ++ | ++ | + |
| **2400** | *Lacticaseibacillus* | *rhamnosus* | Grana Padano cheese | ++ | ++ | + |
| **2409** | ***Lacticaseibacillus*** | ***rhamnosus*** | **Parmigiano Reggiano cheese** | **++** | **++** | **++** |
| **2411** | *Lacticaseibacillus* | *rhamnosus* | Parmigiano Reggiano cheese | ++ | ++ | ++ |
| **2412** | *Lacticaseibacillus* | *rhamnosus* | Parmigiano Reggiano cheese | ++ | - | + |
| **2414** | *Lacticaseibacillus* | *rhamnosus* | Parmigiano Reggiano cheese | ++ | ++ | + |
| **2415** | *Lacticaseibacillus* | *rhamnosus* | Parmigiano Reggiano cheese | ++ | ++ | ++ |
| **2416** | *Lacticaseibacillus* | *rhamnosus* | Parmigiano Reggiano cheese | ++ | - | - |
| **2438** | *Lacticaseibacillus* | *rhamnosus* | Raw milk | - | - | - |
| **2462** | ***Lacticaseibacillus*** | ***rhamnosus*** | **Grana Padano cheese** | **++** | **++** | **++** |
| **2465** | *Lacticaseibacillus* | *rhamnosus* | Grana Padano cheese | ++ | ++ | + |
| **2466** | *Lacticaseibacillus* | *rhamnosus* | Grana Padano cheese | ++ | ++ | - |

### *2.3. Fruit and vegetables juices extraction, pH measurement and microbial enumeration*

Fresh, organic, and commercially matured orange, celery and red beet were purchased in a local market and quickly transported to laboratory to be processed and prepared for the experiments. Selected fruit and vegetables were cleaned and separated from peels and leaves not used to produce the extracts. Raw materials were then washed with cold water, rinsed with demineralized

water and allowed to dry for 30 min at room temperature. Raw vegetables were cut in pieces and the juices extracted with a domestic juice extractor (Juice art plus 110631, RGV, Italy).

pH of fresh juices was measured electrometrically, just after extraction, with a pH meter Beckman φ™ 300 series (Beckman Instruments, Inc. 4300 N. Harbor Blvd. Fullerton, CA 93835).

Microbial counts were evaluated on fresh juices just before inoculation and after 60 hours of fermentation. Ten-fold serial dilutions were prepared in 0.9 % Ringer solution (VWR chemicals, Radnor, Pennsylvania, USA) and spread onto MRS agar (De Man, Rogosa, Sharpe, Oxoid, Basingstoke, UK) for LAB count, YEDC (Yeast extract, dextrose, chloramphenicol agar, Lenexa, Kansas, USA) for yeasts and molds, PCA (Plate Count Agar, Oxoid, Basingstoke, UK) for total microbial count (TMC), VRBA (Violet red bile agar Oxoid, Basingstoke, UK) for *E. coli* and SSA (*Salmonella Shigella* agar Oxoid, Basingstoke, UK) for *Salmonella* spp. Plates were incubated at 37°C under aerobic condition for 24 hours (72 hours for YEDC). Colony forming unit were finally counted and expressed as Log CFU/ mL of fresh juice.

*2.4. Acidification ability and EPS production of LAB strains in MRS and juices*

To investigate strains ability to grow, acidify and produce EPS, impedance measurements were performed by means of BacTrac 4300® (Sylab, Generon, San Prospero, Italy) in fresh juices and with MRS as a control.

The strains were 10-fold diluted in sterile Ringer solution and used to inoculate at a 2% (v/v), 18 mL of MRS and 18 mL of fresh juices. Both were equally divided into 3 sterilized BacTrac 4300® measurement vials which were located inside the instrument and incubated at 37°C for 60 hours.

For the evaluation of strains' acidification ability, the M-values, which is the overall impedance variation of the media in the vials was measured. The M-value was recorded every 10 minutes for 60 h and shown as M%. This value is automatically calculated by the instrument as relative changes compared to a starting value. [30]. The resulting M% data were fitted to the Modified Gompertz equation to obtain the kinetic parameters Lag and yEnd, used to describe the performances of LAB both in MRS and juices [30]. Lag is described as an adjustment period and is measured in hours. The highest the value, the bigger the time that the cells need to adapt to the growth conditions. yEnd is the highest variation of impedance recorded and is interpreted as the maximum acidifying capacity of the strains [29].

For the evaluation of EPS production, both in MRS and juices, the E-values, which is the electrochemical double layer of the electrodes-electrolyte impedance, were measured every 10 minutes for 60 hours. As for M%, the measured E-values are shown as E% changes compared to a staring value [31]. As already described by Bancalari et al., the EPS production can be revealed by measuring the decreasing in E% values [31]. To this end, parameter ΔE% was calculated as the difference between the maximum value reached by E% and the value recorded after 60 h of incubation. The ΔE% values were calculated from triplicate experiments.

*2.5. Statistical analysis*

Results of impedometric measurements were statistically analyzed with a two-way ANOVA model performed using PRC GLM of SAS (SAS Inst. Inc., NC, USA), whereas SIMCA (Sartorius Stedim Data Analytics) software was used to create principal component analysis (PCA) biplot to get visual interpretation of the data analyzed.

# 3. Results

### *3.1. Resistance to bile salts and acid condition*

Results obtained after the screening of 53 strains analyzed for their acid and bile salts resistance, were reported in Table 1. Not all the tested strains resulted resistant to both acid and bile salts. *Lc* 2337, *Lr* 1678 and *Lr* 2438 were not able to grow in any of the stress condition, two strains *Lc* 2406 and *Lr* 2416 could grow in MRS with HCl but not in MRS with both concentrations of bile salts. Finally, five strains (*Lc* 2407, *Lr* 2412, *Lc* 2413, *Lr* 2466, *Lp* 2092) could grow both on acid MRS and MRS with bile salts at the lowest concentration (Table 1).

Taken together, these results showed that ten out of the 53 analyzed strains, highlighted with bold characters in Table 1, had a higher (++) resistance to all the tested stress conditions. Considered this, these strains were considered for the further analysis. Acid and bile salts resistance are two important traits that a microorganism should have to be considered as a probiotic able to explicate health benefits to the host gut [32–34]. In fact, in order to reach the gut, microorganisms must have the ability to pass through the human gastro-intestinal tract (GIT) characterized by an extremely low pH and the presence of toxic glycoconjugated bile salts [35,36].

It must be noted that these results can only suggest a potential probiotic activity of the chosen strains but are not enough to define them as probiotic.

### *3.2. Microbial enumeration of analyzed juices*

Extracted juices were analyzed immediately after the extraction and after 60 hours, by plate counting. Results showed that celery and orange juices had a neglectable initial TMC, 1.82±0.02 Log CFU/mL, while red beet juice presented a TMC of 5.93±0.03 Log CFU/mL. Due to this, red beet juice was not considered for the fermentation with LAB and further analysis. An initial high contamination level in fact would make impossible to obtain a stable and safe fermented juice, without a stabilization treatment (e.g. thermal treatment) able to achieve a sufficient reduction of microbial load [37,38] but also possibly decrease potential functional properties of the juice [8,10,11]. Celery and orange juices were then inoculated with the 10 selected LAB strains and *Lr* GG, and incubated for 60 hours at 37°C. At the end of fermentation, the TMC showed a small increase while no *E. coli* were detected in the samples. Yeasts and mold count decreased down to 0.7 log CFU/mL in orange juice and to 1 log CFU/mL in celery juice. *Salmonella* spp was not detected in any condition.

**Table 3.** Plant derived juices microbial cell load for yeast (YPD), lactic acid bacteria (MRS) total microbial count (TMC), *E. coli* (VRBA) and *Salmonella spp* (SSA) and pH of fermented and unfermented juices. Counts are expressed as log CFU/mL.

| T0 | TMC | MRS | YPD | VRBA | SSA | pH |
|---|---|---|---|---|---|---|
| Red beet | 5.93 ± 0.03 | 3.22 ± 0.01 | 4.43 ± 0.01 | 1.5 ± 0.1 | nd | 6.0 |
| Celery | 1.82 ± 0.01 | 1.30 ± 0.03 | 1.18 ± 0.02 | 0.8 ± 0.02 | nd | 5.8 |

| | | | | | | |
|---|---|---|---|---|---|---|
| Orange | 1.82 ± 0.02 | 1.48 ± 0.01 | 2.47 ± 0.01 | nd | nd | 4.0 |
| T60 | TMC | MRS | YPD | VRBA | SSA | pH |
| Celery | 4.5 ± 0.02 | 5.2 ± 0.1 | 1 ± 0.01 | nd | nd | 5.1 |
| Orange | 3.8 ± 0.1 | 2.6 ± 0.02 | 0.7 ± 0.01 | nd | nd | 3,9 |

Nd: not detected in 1 milliliter.

### *3.3. Acidification ability of LAB strains in fresh juices*

Impedometric analysis, performed on celery and orange juices inoculated with the ten most acid and bile salt resistant strains and *Lr.*GG, are shown in Table 3 as mean value ± SD of three replicated for each strain.

All the tested strains were able to grow in both juices giving a measurable variation of impedometric (M%) signal.

Nevertheless, the impedometric analysis revealed a diverse growth ability of the strains in juices as compared to the one measured in MRS. These differences were evaluated by observing both the yEnd values and the pH (Table 4).

These values, although lower than those recorded in MRS, confirmed that all the tested strains were able to duplicate, metabolize and acidify both in celery and orange juice despite the initial low pH values of 5.8 and 4 respectively. The greater decrease of pH was measured for strains growing in celery juice, resulting in higher ΔpH (difference between initial pH and after 60 hours' fermentation) values (Table 3)

In particular, the strains that caused the highest decrease of pH were *Lr* 2216*, Lr*2409 and *Lr* GG.

The low pH caused by the production of organic acids by LAB is known to act as an antimicrobial agent, making the environment not suitable for the development of pathogenic and spoilage microorganisms [39,40]. So in the case of celery juice, the higher acidification would ensure the safety of the product (Table 4), while in orange juice would be ensured anyway by the very low starting pH.

Regarding yEnd values, which is interpreted as the maximum acidifying capacity of the strains, they ranged from 4.25 to 8.66 in celery and from 4.28 to 10.22 in orange juice (Table 4). However, yEnd values are not related to ΔpH values. This is due to the fact that the impedometric technique does not depend only on pH variation but it measures the complex modification of the electrical conductivity of the medium in which LAB strains develop [41]. Despite the absence of a direct correlation between yEnd and pH values we found it interesting that *Lr* GG showed the greatest yEnd value and the lowest ΔpH. This may suggest that the metabolism of the strains in fresh juices is more complex than what can be observed by simply measuring pH decrease.

Lag values, indicating the time needed by microorganisms to adapt to the substrate, were less than 4 h for all the strains growing in celery juice, with a low variability (ΔLag 2.18 h) among the strains. In particular, the strain with the statistically lowest Lag value (1.82 h) was *Lr* GG and thus the fastest adapting in celery juice (Table 4).

The strains behavior in orange juice was more heterogeneous, with statistically significant differences among the strains. *Lc* 2243 was the slowest adapting strain (Lag 18.59 h) followed by *Lc* 2404 (Lag 15.19 h), and *Lr* 2325 (Lag 13.31 h). The fastest adapting strain in orange juice was again *Lr* GG, with a Lag value of 6.43 h, comparable only to *Lc* 2410. The variability of Lag values among strains was statistically higher (ΔLag 12.6 h) in orange juice, than in celery, suggesting the slowest adaptability of the strains to orange juice, probably due to its composition in terms of polyphenols, antioxidant, combined with the low pH [42].

**Table 4.** Results of the impedometric measurements reported as mean value ± SD, for each strains in both the substrates used.

| Species | Strains | Juice | Lag ± SD | yEnd ± SD | pH± SD | ΔpH |
|---|---|---|---|---|---|---|
| *Lb. casei* | *2233* | Celery | 3.88 $^{h}$ ± 0.15 | 4.75 $^{kijl}$ ± 0.25 | 4.5 $^{c}$± 0 | 1.4 |
| *Lb. casei* | *2243* | Celery | 4.00 $^{h}$ ± 0.30 | 5.02 $^{kijgh}$ ± 0.54 | 4.8$^{a}$± 0 | 1.1 |
| *Lb. casei* | *2404* | Celery | 3.46 $^{h}$ ± 0.43 | 4.99 $^{kijh}$ ± 0.12 | 4.7 $^{b}$± 0 | 1.3 |
| *Lb. casei* | *2410* | Celery | 3.55 $^{h}$ ± 0.09 | 5.10 $^{ijgh}$ ± 0.17 | 4.4 $^{d}$± 0 | 1.5 |
| *Lb. paracasei* | *2306* | Celery | 3.68 $^{h}$ ± 0.02 | 4.48 $^{kjl}$ ± 0.52 | 3.9 $^{h}$ ± 0 | 1.0 |
| *Lb. rhamnosus* | *2216* | Celery | 3.04 $^{h}$ ± 0.52 | 6.50 $^{e}$ ± 0.51 | 4.2 $^{f}$± 0 | 1.9 |
| *Lb. rhamnosus* | *2299* | Celery | 2.93 $^{ih}$ ± 0.44 | 5.16 $^{igh}$ ± 0.16 | 4 $^{g}$± 0 | 1.6 |
| *Lb. rhamnosus* | *2325* | Celery | 3.48 $^{h}$± 0.47 | 4.40 $^{kl}$ ± 0.53 | 4.4 $^{d}$± 0 | 1.4 |
| *Lb. rhamnosus* | *2409* | Celery | 3.59 $^{h}$ ± 0.18 | 5.46 $^{fgh}$ ± 0.47 | 4.3 $^{e}$± 0 | 1.8 |
| *Lb. rhamnosus* | *2462* | Celery | 3.44 $^{h}$ ± 0.19 | 4.25 $^{l}$ ± 0.22 | 4.7 $^{b}$± 0 | 1.1 |
| *Lb. rhamnosus* | *GG* | Celery | 1.82 $^{i}$ ± 0.08 | 8.66 $^{b}$ ± 0.03 | 3.9 $^{h}$± 0 | 1.9 |
| *Lb. casei* | *2233* | Orange | 7.82 $^{f}$ ± 0.24 | 7.46 $^{c}$ ± 0.32 | 3.9 $^{h}$± 0 | 0.3 |
| *Lb. casei* | *2243* | Orange | 18.59 $^{a}$ ± 0.42 | 6.43 $^{e}$ ± 0.51 | 3.8 $^{i}$± 0 | 0.2 |
| *Lb. casei* | *2404* | Orange | 15.19 $^{b}$ ± 0.85 | 5.41 $^{igh}$ ± 0.22 | 3.8 $^{i}$± 0 | 0.1 |
| *Lb. casei* | *2410* | Orange | 6.56 $^{g}$ ± 0.60 | 7.80 $^{c}$ ± 0.24 | 3.7 $^{j}$± 0 | 0.3 |
| *Lb. paracasei* | *2306* | Orange | 12.37 $^{c}$ ± 0.55 | 6.08 $^{fe}$ ± 0.21 | 3.7 $^{j}$ ± 0 | 0.2 |
| *Lb. rhamnosus* | *2216* | Orange | 8.40 $^{ef}$ ± 0.87 | 7.19 $^{dc}$ ± 0.06 | 3.9 $^{h}$± 0 | 0.3 |
| *Lb. rhamnosus* | *2299* | Orange | 10.43 $^{d}$ ± 0.40 | 4.28 $^{l}$ ± 0.26 | 3.7 $^{j}$± 0 | 0.1 |
| *Lb. rhamnosus* | *2325* | Orange | 13.31 $^{c}$ ± 1.86 | 6.64 $^{de}$ ± 0.74 | 3.7 $^{j}$± 0 | 0.3 |
| *Lb. rhamnosus* | *2409* | Orange | 9.53 $^{ed}$ ± 1.80 | 6.53 $^{de}$ ± 0.66 | 3.7 $^{j}$± 0 | 0.3 |
| *Lb. rhamnosus* | *2462* | Orange | 14.98 $^{b}$ ± 0.88 | 5.66 $^{fg}$ ± 0.55 | 3.7 $^{j}$± 0 | 0.3 |
| *Lb. rhamnosus* | *GG* | Orange | 6.43 $^{g}$ ± 0.81 | 10.22 $^{a}$ ± 0.51 | 3.7 $^{j}$± 0 | 0.3 |

$^{a-l}$ Different lowercase letters by column indicate the presence of significant differences according to ANOVA ($P<0.001$).

However, even if at a different extent, the ability of the tested strains to grow and acidify both celery and orange juice is in agreement with Amal Bakr Shori [43], who found that several lactobacilli have a very high tolerance to a plant derived acid environment, probably because of the high nutrient content that makes them an ideal substrate for probiotic strains growth [44].

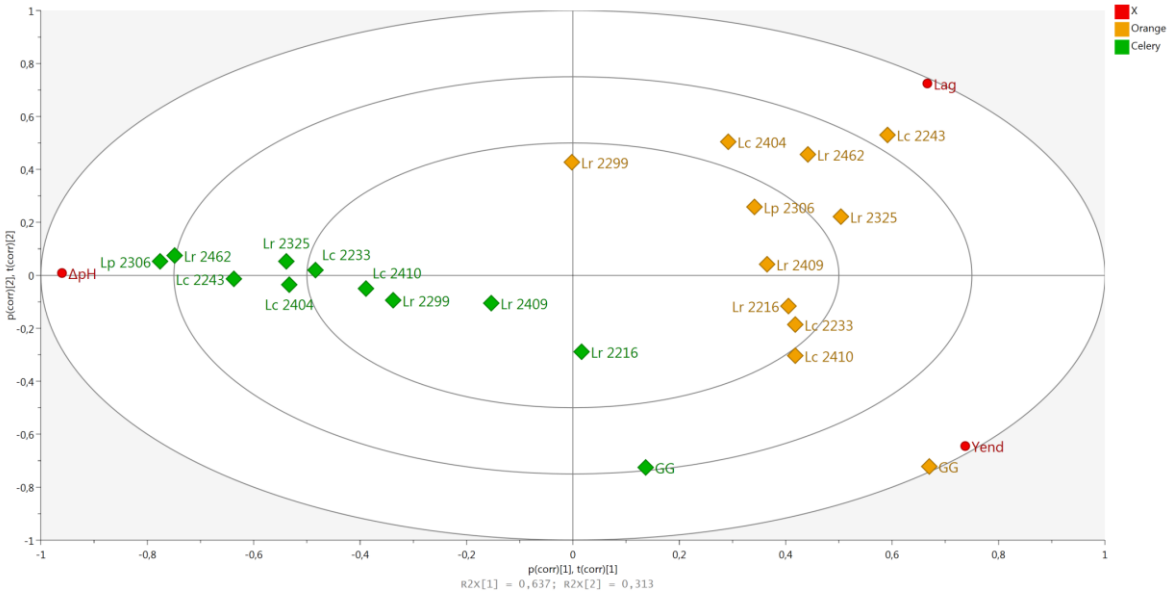


**Figure 2.** PCA score plot (t1 vs t2) of the first two PCs of the data set considered, against the loading plot (p1 vs p2). The variables Lag, yEnd and ΔpH are marked (red). The first component explains 64% of the variation, and the second component 33%. Observations are colored according to juices.

All the measured values (Lag, yEnd and pH) were then plotted together in a PCA biplot in order to get a better view of the behavior of the strains in the two plant derived juices (Figure 2). As it can be seen, the strains were well separated on the first component, accordingly to the matrix.

In particular, it can be noted that the highest Lag values were found for strains fermenting orange juice, while the highest ΔpH values were recorded for the strains fermenting celery juice.

Observing the biplot it can be noted that *Lr* GG is well separated from the other strains, showing the best adapting ability (lower Lag value) and also the best acidification performances (lowest pH value and higher yEnd).

Nevertheless, among the wild tested strains, *Lc* 2410, *Lc* 2233 was the best performing strains in orange juice, while *Lr* 2216 was the best one in both types of juices.

### *3.4. EPS production*

The potentiality of the strains to produce EPS during fermentation in juices, was investigated by using the capacitance value (E%) recorded by the BactTrac 4300® [31]. E%, that is the double layer capacitance of the electrodes/electrolyte interface, is strongly affected by any modification of the ionic layers in the vicinity of the electrode surface [31]. For this reason, this measurement is extremely sensitive to slight alteration of the surface of the electrodes-electrode impedance, and this is the reason why it was used to detect the EPS production.

In fact, in case of EPS production, their adhesion or placement in the electrodes nearby, slightly alters the interface impedance by blocking the registration of electrical impedance at the area of contact, causing the decrease of the capacitance values (E%) [31].

EPS production was thus calculated as ΔE% that is the difference between the maximum value reached by capacitance values recorded (E% max) and after 60 h of incubation. The ΔE% values were calculated from triplicate experiments. ΔE% was measured for all the strains also in MRS as a control.

**Table 5.** Mean value of the measured ΔE% value for each strain in MRS, Celery and Orange.

| Species | Strain | ΔE% MRS | ΔE% Celery | ΔE% Orange |
|---|---|---|---|---|
| *Lb. casei* | 2233 | 3.52±1.92 | 4.35±2.20 | 0.11±0.22 |
| *Lb. casei* | 2243 | 1.75±0.08 | 0.06±0.04 | 0.49±0.83 |
| *Lb. casei* | 2404 | 2.47±0.66 | 0.61±0.47 | 0.25±0.03 |
| *Lb. casei* | 2410 | 2.07±1.13 | 5.56±2.32 | 0.21±0.02 |
| *Lb. paracasei* | 2306 | 6.25±2.49 | 2.81±0.98 | 0.6±0.01 |
| *Lb. rhamnosus* | 2216 | 4.28±0.99 | 2.51±1.34 | 0.55±0.12 |
| *Lb. rhamnosus* | 2299 | 6.55±2.94 | 4.43±2.21 | 0.81±0.42 |
| *Lb. rhamnosus* | 2325 | 4.25±1.84 | 6.06±0.61 | 0.1±0.03 |
| *Lb. rhamnosus* | 2409 | 2.37±0.67 | 5.83±2.37 | 0.75±0.08 |
| *Lb. rhamnosus* | 2462 | 2.43±0.51 | 2.88±1.67 | 0.45±0.07 |
| *Lb. rhamnosus* | GG | 4.09±1.62 | 3.04±0.86 | 0.67±0.12 |

As only the strains with a ΔE% higher than 3 are considered EPS producers [31], we concluded that no strain was able to produce EPS in orange juice. In MRS 6 strains out of 11 showed a ΔE% value higher than 3 being therefore considered able to produce EPS. Four of this strains, 3 *Lb. rhamnosus* (*Lr* GG, *Lr* 2299, *Lr* 2325) and one *L. casei (Lr* 2233), were able to produce EPS also in celery juice. Interestingly, two strains, *Lr* 2409 and *Lc* 2410, were able to produce EPS in celery juice but not in MRS. The strain *Lr* 2216 that was the best performing strains in both juices, was able to produce EPS in MRS but not in the juices. This would make it a good candidate for plant derived juices fermentation, but would open questions on its potential probiotic effect as it has been demonstrated that EPS have a protective effect on probiotics transiting the GIT [45], protecting them from low pH and the high bile salt concentration [28].

# 4. Conclusions

Our results showed that *Lr* GG, a well-known commercial probiotic strain, showed the best ability, among the tested strains to ferment both orange and celery juice. On the other hand, we were able to select at least 3 wild strains (*Lr* 2299, *Lr* 2409 and *Lc* 2410) with good fermentation performances in both juices, also producing EPS in celery juice. These displayed characteristics open new perspectives for dairy isolates to be used as starter also in plant derived juices fermentation. Furthermore, the idea to add probiotic strains to drive the fermentation process can be a good strategy to obtain a probiotic product avoiding any thermal treatment and therefore maintaining all the intrinsic beneficial properties of the juices. In conclusion, the use of selected potentially probiotic strains with three-in-one abilities (good acidification performances, probiotic and EPS-producers) could be a valid tool to obtain a new functional fermented plant derived beverage.

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# Chapter 4- Feeding Lactic Acid Bacteria with different sugars: effect on EPS production, molecular characteristics and growth behaviour

After the pandemic we went back focusing on LAB and their ability to produce potentially bioactive compounds such as EPS. EPS are reported to have many bioactivities, according to producer microorganism and environmental conditions structural characteristics of the EPS can be different. Basing on molecular weight and conformation of the saccharides chain also the bioactivities exerted by the EPS is different. Bioactivities exerted by EPS are especially linked to positive effects in the guts. EPS are reported to improve immune response, have anti-inflammatory properties, that proved to help in fighting Irritable Bowel Disease and associated disturbs (e.g. Chron's disease and ulcerative colitis). They also exert other bioactivities that makes them particularly attractive for companies who want to obtain a claim for their products, including the consideration that this EPS can be produced by LAB *in situ* helping to maintain a cleaner label, fact that is particularly appreciated by consumers.

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Andrea Fuso and Vincenzo Castellone equally contributed to the work

## 1. Introduction

Lactic Acid Bacteria (LAB) are closely associated with humans since ancient times and all throughout history [1]. Nowadays LAB are receiving increasing attention due to their ability to ferment different matrices and to produce healthy compounds with an increased value. Amongst these, exopolysaccharides (EPS) are gaining interest due to their multiple effects. In fact, different LAB species are able to produce a wide variety of EPS with different structure, size, and thus with diverse functions [2,3]. Generally, EPS can be divided in two macro-categories, depending on the composition of the saccharidic chain: i) hetero-polysaccharides (HePS) and ii) homo-polysaccharides (HoPS). Tipically HoPS are composed of the same monosaccharide and show a linear and bigger structure (>$10^6$ Da) than HePS. These two types of polysaccharides differ also for the biosynthetic pathway, in fact HoPS are normally synthesized outside of the cells, where a specific enzyme gathers and assembles the monomeric residues of sugars (dextran and levan are two common examples) in a specific way [4]. HoPS do not present charge and are primarly associated with prebiotic effects [5–7]. HePS present 2 or more sugar moieties in a linear or branched chain. HePS are smaller ($10^4$-$10^6$ Da), may have non-carbohydrates residues in their composition, including charged groups and are primarily associated with a boosting effect towards host immune functions [8–10]. HePS synthetic pathway is more complex as compared to HoPS as they are normally synthesized inside the cells, and then carried outside the cell after the assembly of the polysaccharides chain. EPS are essential components of extracellular polymeric substances and it has been reported that they can be used by cells as a strategy to cope with lack of nutrients or harsh conditions, like pollution or presence of harmful substances [4]. In the last years EPS have gained a lot of attention for their promising features that can be exploited in improving food technological characteristics but also to increase the nutritional value [8,9]. Industrial production of EPS is mainly achieved by feeding LAB with industrial by-products and media rich in sucrose [10]. Nevertheless many studies have proven the ability of LAB of adapting to different media and change their behaviours according to the medium characteristics [11,12]. As an example, *L. delbrueckii* subsp. *bulgaricus* NCFB 2722 has proven to

produce higher amount of EPS when grown in media containing glucose or lactose with respect tu media containing fructose [11]. In a study Cheng et al. [13], measured growth and EPS' production of *L. plantarum* LPC-1 on media with glucose, sucrose and a mix of those sugars. Their results suggest the effect of sucrose in increasing EPS' production and their antioxidant activity [13]. Moreover, different recent studies highlighted EPS' prebiotic effect i.e., increasing the amount of desired microorganisms in the intestine, especially in the colonic tract. Amongst the 30 different species recognized as EPS-producers, the most known are: *Lacticaseibacillus paracasei, Lacticaseibacillus rhamnosus*, *Lactobacillus helveticus*, *Lactobacillus delbrueckii*, *Lactobacillus acidophilus*, *Latilactobacillus sakei*, *Lactiplantibacillus plantarum* [14–21]. Many of these EPS-producers were isolated from traditional food matrices, where they contribute to the improvement of the texture, mouthfeel and stability of fermented products. In the present study, three LAB strains belonging to the EPS's producer species and commonly found in dairy products, were considered. In particular, *L. delbrueckii* represents one of the most commonly used starter species in cheese and yoghurt [22], while *L. paracasei and L. rhamnosus* are known for their importance as non-starter strains in ripened cheeses and their potential health benefits [23,24].

Taking into consideration that the LAB's EPS production and structure have a great variability that depend on several variables, the aim of the present study was to monitor how and whether the variation of the carbon source may affect the growth behavior of the strains and their ability to produce different EPS.

## 2. Materials and methods

### *2.1 Bacterial strains, growth conditions and media*

Three wild LAB strains, namely *L. delbrueckii* 1932, *L. rhamnosus* 1019, and *L. paracasei* 2333, previously isolated from dairy matrices and belonging to the microbial collection of the Department of Food and Drug of the University of Parma, were tested. All the strains were maintained as stock cultures at -80°C in MRS broth (Oxoid, Basingstoke, UK) supplemented with 20% (v/v) glycerol until use. To have a fresh culture to be used, the bacteria were propagated twice (24 h at 37°C) in anaerobiosis (Fisher Scientific Italia, Rodano (MI), Italy) in MRS broth.
Before the use, the cultures were harvested by centrifugation (10.000g, 10 minutes at 4°C) and washed three times with Ringer's (Oxoid, Basingstoke, UK) solution to remove any residue of the media.
The washed cells were then transferred twice in modified MRS medium (mMRS) that was used as basic EPS production medium. mMRS was prepared according to Degeest et al. [25] as follows: 30 g $l^{-1}$ of peptone (Oxoid, Ltd., Basingstoke, United Kingdom), 12 g $l^{-1}$ of yeast extract (Merck, Darmstadt, Germany), 2 g $l^{-1}$ of K2HPO4 (Merck, Darmstadt, Germany), 5 g $l^{-1}$ of sodium acetate (Merck, Darmstadt, Germany), 2 g $l^{-1}$ of triammonium citrate (Merck, Darmstadt, Germany), 0.2 g $l^{-1}$ of $MgSO_4 \cdot 7H_2O$ (Sigma Aldrich Darmstadt, Germany); 0.038 g $l^{-1}$ of $MnSO_4 \cdot H_2O$ (Sigma Aldrich Darmstadt, Germany),and 1 ml $l^{-1}$ of Tween 80 (Biolofe, Monza, Italy). [25,26].
The carbon sources consisting of Sucrose (SUC), Glucose (GLU), Lactose (LAC), Fructose (FRU), and Maltose (MAL) (Merck, Darmstadt, Germany), were prepared as a concentrated water solution, sterilized separetely from the medium, and then properly added to each bottle of mMRS to a final concentration of 5%.

Final cells concentration after double propagation in mMRS was estimated by plate counting on MRS agar after 20 hours of incubation (data not shown). After washing, the cells were inoculated in 6 mL of the five different aliquots of mMRS broths prepared with the five different sugars (SUC, GLU, LAC, FRU and MAL) and cultured at 37°C in anaerobiosis.
After 20 hours of incubation the cells were diluted to a final concentration of 7 log CFU $ml^{-1}$ and used to inoculate: i) 200mL of media that were incubated in anaerobiosis at 37°C, then used for the chemical analysis and ii) 18 ml of media for growth behavior determination

### *2.2 Strains' growth behavior with different sugars*

To evaluate strains' growth behaviour with different sugars, 18 ml of each of the five different mMRS inoculated with each of the three strains were equally divided into three sterilized BacTrac 4300® measurement vials, which were located inside the instrument BacTrac 4300® Microbiological Analyzer system (Sylab, Austria) and incubated at 37°C for 30 hours. Two sets of analysis were carried out, and each sample was analysed in triplicate.
During the 30 h of incubation, the variation of both overall conductance (M%) and capacitance (E%) were recorded every 10 minutes. The E% data were collected and fitted to the modified Gompertz equation according to Bancalari et al. [27,28] to obtain the kinetic parameters Rate and yEnd that were used to describe the growth behaviour of the strains [27,28].

### *2.3 Impedance measurement for detection of EPS production.*

From the Bactrac measurments, also infomation about EPS production was collected for all the tested strains. In fact, the E% measured during the analysis has been used to estimate the ability of the strains to produce EPS using the different sugars provided. As previously reported by Bancalari and colleagues [28], the EPS production can be revealed by measuring the decrease in E% values. To this end, parameter ΔE% was calculated as the difference between the maximum value reached by E% and the value recorded after 25 hours of incubation. The ΔE% values were calculated from triplicate experiments.

### *2.4 EPS extraction and quantification*

Total EPS amount produced by the strains in 20 mL culture broth was determined by the AOAC official enzymatic-gravimetric method for dietary fibres [29]. The analysis was carried out in triplicate. Residual ash in extracted fibres was determined through mineralization at 550 °C for 5 h, while residual nitrogen was determined using a Kjeldahl system (DKL heating digestor and UDK 139 semiautomatic distillation unit, VELP SCIENTIFICA) and using 6.25 as nitrogen-to-protein conversion factor.
The same enzymatic-gravimetric method, with few modifications, was also used for the EPS extraction, in order to enable further analysis on their chemical structure. After the employment of heat-stable amylase, protease and amyloglucosidase, EPS were precipitated by adding four volumes of 96% ethanol, then the solution was centrifuged at 3900 rpm, at 4 °C for 30 minutes and the pellet was dried at 40 °C in an oven.

### *2.5 EPS monosaccharide composition and quantification analysis through Gas Chromatography-Mass Spectrometry (GC-MS)*

The EPS monosaccharide composition was investigated following two different protocols. The first was employed in order to detect neutral and acid sugars, following a method previously proposed by Xia et al. with some modifications [30]. Here, 10 mg of sample were dissolved in 3 mL of 2N trifluoroacetic acid (TFA) and hydrolysed at 110 °C for 2 hours. Then, an aliquot of the solution was withdrawn and put together with 125 µL of 1190 ppm phenyl-β-D-glucopyranoside, used as internal standard, and then evaporated by rotavapor. The obtained dried hydrolysate was washed with 1 mL of methanol to remove the residue of TFA and evaporated again. 1 mL of 0.5 M $NH_4OH$ was subsequently added to delactonize the eventually present acid sugar lactones in the sample, and again evaporated by rotavapor. Finally, the dried hydrolysate was dissolved in 800 µL of dimethylformamide (DMF) and 200 µL of N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA), the latter used as derivatizing agent. The reaction was held for 1 hour at 60 °C and then the derivatized sample was injected in gaschromatography.
The second protocol was used with the aim of detecting amino sugars by acid hydrolysis with hydrochloric acid. Briefly, 10 mg of sample were dissolved in 6 mL of 7 N HCl and kept for 4 hours at 110 °C. Later, an aliquot was added to 125 µL of 1190 ppm phenyl-β-D-glucopyranoside and they were together evaporated. As in the first method, 800 µL of DMF and 200 µL of BSTFA were subsequently added, the reaction was held at 60 °C for 1 h and the solution was ready to be injected.
GC-MS analysis of monosaccharides was performed with a 6890 N gas chromatograph coupled to a 5973 N mass selective detector (Agilent technologies, Santa Clara, CA). A SLB-5ms, 30 m × 0.25 mm, 0.25 µm thickness column (Supelco, Bellafonte, PA, USA) was used. The chromatogram was recorded in the scan mode (40–500 m/z) with a programmed temperature from 60 °C to 270 °C. The initial temperature was 60 °C, held for 2 minutes, then increased to 160 °C at a rate of 10 °C/min, held isothermal for 5 minutes, increased to 220 °C at a rate of 10 °C/min, kept for 5 minutes, increased to 270 °C at a rate of 20 °C/min and maintained for 5 minutes. Quantification was performed with a response factor, considering the area and concentration ratios between the internal standard (phenyl-β-D-glucopyranoside), and the following monosaccharides: D-glucose, D-fructose, D-galactose, D-mannose, D-rhamnose, D-ribose, D-xylose, D-fucose, D-galacturonic acid, D-glucuronic acid, D-glucosamine and D-galactosamine.

*2.6 Evaluation of EPS molecular weight through HPSEC-RID*

The molecular weight of EPS produced by the selected strains was investigated through high-performance size-exclusion chromatography (HPSEC), with an Agilent 1260 Infinity II LC system equipped with a refractive index detector (RID) (Agilent, Santa Clara, CA, USA). The EPS extracted from the culture broth (Paragraph 2.4), were dissolved in ultrapure water at a concentration of 10 mg/mL. Later, the solutions were filtered through a 0.45 µm membrane. A 50 mM NaCl aqueous solution was used as mobile phase at a flow rate of 1 mL/min and a PL aquagel-OH MIXED-M column, 7.5 x 300 mm, 8 µm (Agilent, Santa Clara, CA, USA) was employed to separate the different molecular weight fractions. The injection sample volume was set at 100 µL, column temperature 30 °C and RID temperature 35 °C. Standard pullulans having known molecular weight were used for the calibration curve.

*2.7 Statistical analysis*

All the calculated parameters (amount of EPS produced, relative percentage of monosaccharides, yEnd, rate, ΔE and percentage of various EPS fractions with different molecular weights) were

compared each other through Pearson correlation by employing IBM SPSS software version 21.0 (SPSS Inc., Chicago, IL, USA). Significant correlations were considered for values > 0.6 and < -0.6. Moreover, a one-way analysis of variance (ANOVA) with Tukey's post-hoc test was applied with the same software at a confidence level of 95 % (p-value = 0.05) in order to determine significant differences among the amounts of EPS produced in the various experiments.

# 3. Results and discussions

*3.1 Strains growth behavior with different sugars*

The instrument BacTrac 4300®, used to perform the impedometric analysis, allows the detection of bacterial activity in real time by measuring the decrease of the impedance in an alternating current (AC) field. In fact, during duplication, bacteria viable cells break down sugars into smaller molecules that makes the medium more conductive, decreasing the overall resistance and total impedance, and thus is used as a measure of bacteria metabolism [28]. During the incubation period, the instrument is able to register two specific impedance values for each single measurement: i) the conventional conductance value (M-value) that corresponds to the overall medium impedance, and ii) the capacitance value (E-value) which is the measure of electrochemical double layer impedance in the vicinity of the electrodes. Both these values, simultaneously recorded every 10 min, which are shown as relative changes compared to a starting value, are expressed as M% and E% and visualized as a capacitance or conductance curve [28]. At the end of the analysis all recorded capacitance data (E%) were used in 3 different ways: i) to be fitted by the Gompertz equation, according to Bancalari et el. [27], to obtain the kinetic parameter Rate and yEnd; ii) to build a graphical representation of the original capacitance curve (Fig. 1); iii) to calculate the ΔE parameter as index of EPS production [31].

From the Figure 1 it is possible to observe that the growth curves, obtained by plotting the impedometric data against time, are different from strain to strain. In particular, *L. delbrueckii bulgaricus* 1932 showed less variability in terms of growth behaviour when fed with the different sugars. On the other hand*, L. rhamnosus 1019* and *L. paracasei 2333* showed the greatest differences in growth behaviour depending on the sugar used. Especially, *L. rhamnosus 1019* (fig.1 A) showed a lower ability to grow when fed with maltose and sucrose, as it can be seen from the lower height of these two curves.

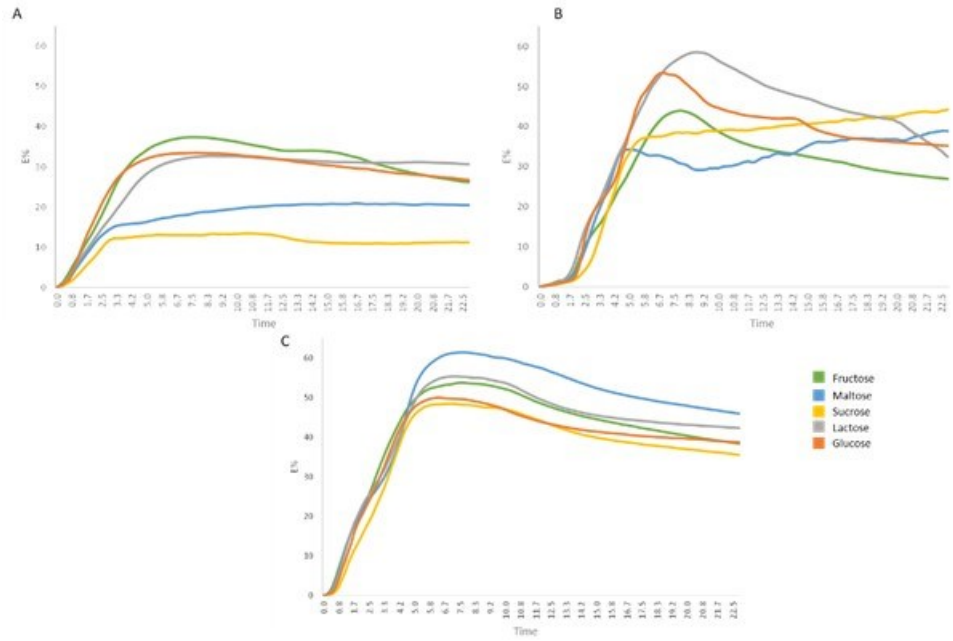


*Figure 1. The growth curves for each strain tested (A: L. rhamnosus 1019, B: L. paracasei 2333, C: L. delbrueckii 1932) are represented coloured according to the different sugars present in the medium during growth.*

Observing the different shape of conductimetric curves, the greatest differences appeared for strain *L. paracasei 2333* (Fig.1B) where the growth behaviours are very diverse depending on the sugar used. To quantify the differences, the E% data were fitted by the Gompertz equation, according to Bancalari et el. [27], and the kinetic parameter Rate and yEnd were used to describe the growth kinetics of the strains (Table 1). In particular, Rate value describes the acidification speed, thus the bigger the value, the fastest the acidification, while yEnd represents the highest variation of impedance recorded and is thus interpreted as the maximum acidifying capacity of the strain. Also in this case, the higher the value, the greatest the acidifying capacity [27].

| Strain | Rate | yEnd | ΔE |
|---|---|---|---|
| *L. paracasei* 2333 FRU | 8,84 | 33,46 | 16,95 |
| *L. paracasei* 2333 MAL | 12,58 | 33,60 | -8,21 |
| *L. paracasei* 2333 SUC | 11,30 | 40,18 | -5,75 |
| *L. paracasei* 2333 LAC | 11,25 | 46,70 | 25,19 |
| *L. paracasei* 2333 GLU | 12,26 | 41,27 | 16,20 |
| *L. rhamnosus* 1019 FRU | 8,16 | 32,47 | 11,19 |
| *L. rhamnosus* 1019 MAL | 2,49 | 19,58 | -1,77 |
| *L. rhamnosus* 1019 SUC | 4,30 | 11,54 | 1,71 |
| *L. rhamnosus* 1019 LAC | 5,95 | 31,16 | 1,90 |
| *L. rhamnosus* 1019 GLU | 8,89 | 30,25 | 6,72 |
| *L. delbrueckii* 1932 FRU | 11,34 | 45,92 | 15,27 |
| *L. delbrueckii* 1932 MAL | 10,59 | 53,59 | 15,42 |
| *L. delbrueckii* 1932 SUC | 12,94 | 41,61 | 12,59 |
| *L. delbrueckii* 1932 LAC | 10,36 | 47,66 | 12,79 |

| | | | |
|---|---|---|---|
| *L. delbrueckii* 1932 GLU | 11,30 | 43,11 | 10,65 |

*Table 1. Acidification speed (rate), Maximum acidifying capacity (yEnd) and ability to produce EPS (ΔE) of tested LAB*

Rate of the strains indicates their speed in acidification of the media, thus indirectly their ability to adapt grow. Growth of microorganisms is related to the ability of adaptying to the environment and to use the source of energy present in the medium. In this optic the different Rate values obtained for all the strains is an indication of the preference of these strains for different sugars.

*L. paracasei* 2333 showed similar ability to duplicate with the comparable Rate value for four of the five sugars, with a lower value when cultivated in presence of fructose (Table 1). However, its acidifying capacity (Yend) was very different varying from the lowest values when fed with fructose but also with maltose, to the highest value in presence of lactose (Table 1, fig 1b). Similarly, *L. delbrueckii* 1932 showed a very similar Rate value in presence of all sugars but a different acidifiction capacity ranging from a lower value when fed with sucrose and glucose, higher when fed with lactose and fructose, and very high in presence of maltose (Table 1, Figure 1c). For *L. rhamnosus* 1019 the lowest value of Rate and Yend were recorded measuring the difficulty of the strain to develop in al condition. However, the strain showed the best performace when fed with fructose and glucose.

### *3.2 EPS production with different carbon sources*

The ability of the strains to produce EPS was assessed both by impedometric analysis and by quantifying EPS through a gravimetric method. The gravimetric method allows a quantification of the EPS produced after bacteria growth.

The results of the quantification of EPS produced from each strain using different sugars (Fig. 2) show that the maximum quantity of EPS is not always reached by using sucrose as unique carbon source. Up to nowadays most of the literature available has reported the addition of sucrose to the growth media, as an effective method to maximize the EPS production by LAB [32].

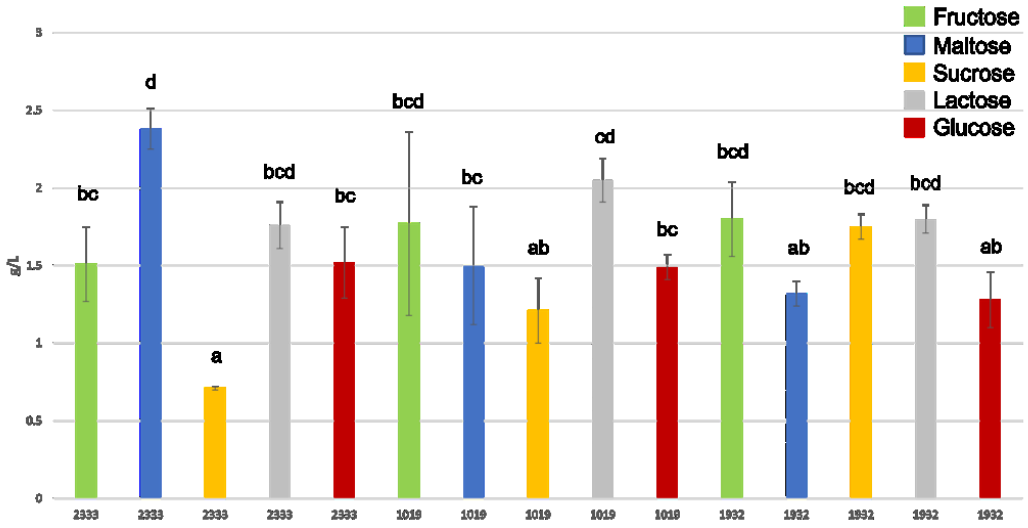


***Figure2:*** *Amount of EPS produced by tested microorganisms with different carbon sources, expressed as g $l^{-1}$. Different letters above the bars indicate significant differences ($p < 0,05$).*

Furthermore, it has been possible to observe a great variability both in terms of the amount of EPS produced by the different strains, but also at strain level. From figure 2 it is possible to observe that by using maltose, the strain *L. paracasei* 2333 turned out to be the best EPS producer in terms of quantity as compared to the other two strains in the same conditions. It is interesting to note that this strain showed the lowest EPS production when sucrose was the only carbon source used.

The same behaviour was observed for the strain *L. rhamnosus* 1019, that produced the lowest amount of EPS when grown in mMRS with sucrose, even though higher than *L. paracasei* 2333 grown with the same supplementation. On the contrary, the highest amount of EPS was quantified when *L. rhamnosus* 1019 was grown in mMrs with lactose. *L. delbrueckii bulgaricus* 1932 can produce good amounts of EPS starting from fructose, sucrose and lactose as carbon source. These results show that different strains have different behaviours when fed with different sugars also in EPS production, underlining the importance to consider the peculiarity and strain-specific attitudes when chosing the sugar to maximixe the EPS production.

The E% value obtained from the impedometric analysis, that is the measure of the double layer capacitance, was already used by Bancalari and colleagues [28] to detect the presence of released EPS during LAB growth [31]. From measured E% values, the parameter ΔE% was calculated, as the difference between the maximum value reached by E% and the value recorded after 25 h of incubation (Table 1). In a previous work [31] it was observed that strains causing a drop in ΔE value higher than 3 are able to create EPS. Since it waqs observed the presence of sticky and filamentous material covering the electrodes.
According with this type of measurement, *L. delbrueckii* 1932 seems to be able to produce EPS starting from all the sugars fed, with highest peak reached when fed with fructose and the lowest with glucose, in agreement with gravimetric method. Even though, its ability to produce EPS seems to be stable between all the sugars. Interestingly, the highest acidification ability, reached in maltose based media, is associated with one of the lowest acidifying speed, remarking how these two parameters are not directly linked to pH, but to the complex modification that take place in the media during bacterial growth.

*L. paracasei* 2333 shows good EPS production in media with fructose, lactose and glucose. While on the other hand, production of EPS is absent in media containing sucrose as carbon source, as confirmed also by gravimetric analysis. This result is interesting since the production of EPS is generally achieved feeding the microorganisms with sucrose, that in this case seems not supporting EPS formation [33]. On the other hand, the gravimetric method revealed the highest production of EPS in presence of maltose completely in disagreement with the conductimetric data. These different results should be due to the different molecular composition of the EPS that differently influenced the electric measurement.
Among the three tested microorganisms, *L. rhamnosus* 1019 showed the lowest acidifying ability and capacity, but the ability to produce EPS when fed with fructose and glucose is in agreement with gravimetric method. In the presence of the other three sugars, maltose, sucrose and lactose, in absence of the agreement between the two methods, it is possible to formulate the same hypothesis made for *L. paracasei* EPS.

### *3.3 EPS' monosaccharide composition*

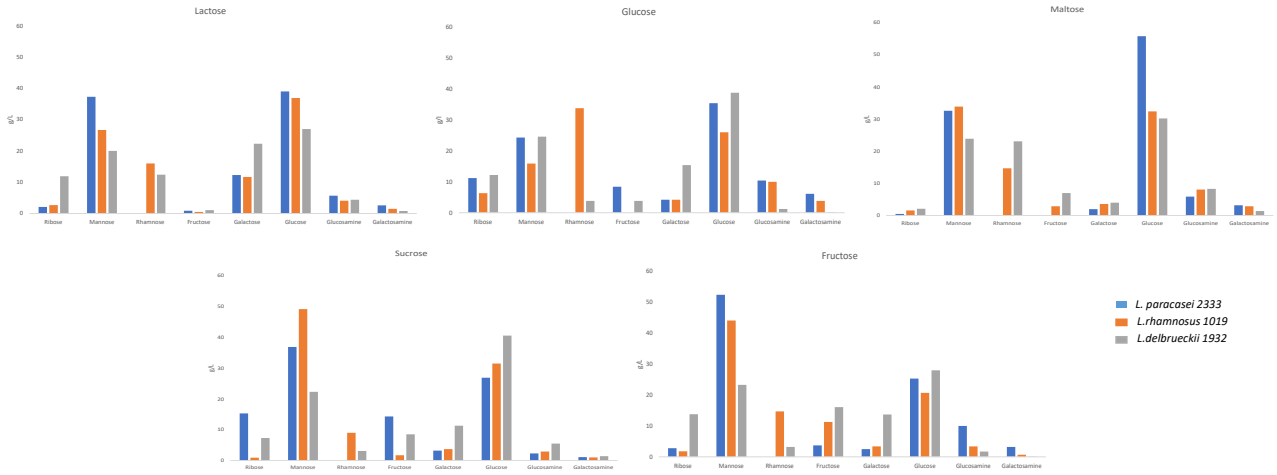


***Figure 3:*** *monosaccharide composition of EPS produced by three LAB strains fed with five different sugars*

The monosaccharide composition of EPS produced by the three selected strains fed with different sugars is reported in Figure 3. As can be observed by this figure, all the three strains were able to produce EPS containing glucose, mannose, galactose, fructose, ribose, glucosamine and galactosamine as principal sugars. Only *L. rhamnosus* 1019 and *L. delbrueckii bulgaricus* 1932 produced EPS containing also rhamnose. This seems to indicate that *L. paracasei* 2333, regardless of the carbon source it was fed with, was unable to produce and add rhamnose within the polysaccharide chain, probably due to the lack of gene clusters encoding for the production of this sugar [34]. This phenomenon can be also linked to the nature of EPS, that in contrast with what happen for protein synthesis is not directed by pre-established templates but it depends on individual cellular enzymes for digestion and synthesis of carbohydrates [16]. Rhamnose was observed to be uncommon in EPS produced by lactobacilli in Zeidan et al. [16] that reports the presence of this sugar only for some strains of *L. delbrueckii bulgaricus* and *L. rhamnosus,* in agreement with our results. Except for this clear difference, the other sugars were produced in similar, albeit variable, quantities. All the three strains produced EPS consisting mostly of glucose and mannose, regardless of the carbon source added to the growth medium. The sum of mannose and glucose, expressed as a relative percentage of total sugars, was found to range between 42 % and 88 %, with an average of 64 %, suggesting that despite the presence of other hexoses and pentoses, the EPS produced were mainly classifiable as glucomannans. In particular, mannose was found to be present in greater quantities when fructose was present as a carbon source, both as it is and when present within sucrose. This correlation is supported by the fact that mannose and fructose are metabolically close, with only one metabolic step between them [35]. In general, these results are quite in agreement with the literature, where glucose, galactose, mannose, rhamnose, glucosamine and galactosamine are reported to be always the most frequent monosaccharides in LAB's EPS [9]. However, in some cases, fructose [36] and ribose [37] have also been found as monosaccharides constituting the LAB-produced EPS chain. The relatively low quantities of glucose and mannose found in some samples are explained because of the simultaneous high quantity of other sugars, such as rhamnose, ribose, galactose and in few cases fructose. Rhamnose was found to be particularly present in the EPS produced by *L. rhamnosus* 1019, with average quantities that are twice as much as those found in the EPS made by *L. delbrueckii bulgaricus* 1932 (17.6% and 9%, respectively). As concerns ribose, it showed considerable variations within the polysaccharides produced by the same strain that have been fed with different carbon sources: *L. paracasei* 2333 originated amounts of ribose varying between 0.5 and 15.3%, *L. rhamnosus* 1019 between 0.9 and 6.3% and *L. delbrueckii bulgaricus* 1932 between 2.1 and 13.8%. The highest concentrations were almost always found when glucose was used in the culture broth. Fructose, on the other hand, was

variable between 0 and 14.3%, between 0 and 11.3% and between 1.1 and 16.1% within the EPS produced by *L. paracasei* 2333, *L. rhamnosus* 1019 and *L. delbrueckii bulgaricus* 1932 respectively, depending on the carbon source that was employed. Generally, it showed the highest concentrations when fructose itself was used as a carbon source. Galactose also showed considerable variability. It was particularly abundant when the EPS produced by *L. delbrueckii bulgaricus* 1932 was considered, with an average amount 2.5 times higher than that produced by the other two strains. Furthermore, also the carbon source influenced the quantities again: in fact, the largest amounts was found when the three strains were grown on lactose, which is the only galactose-containing carbon source among the selected ones, and the lowest quantities have been found when maltose was employed as feed. Finally, glucosamine and galactosamine were always found but in relatively low quantities, ranging between 1.2 and 10.4% and between 0.1 and 6.1%, respectively. The presence of amino sugars in the EPS chain is actually of great importance, because of their characteristic electric charge. In fact, when this latter is present on the EPS it may cause, depending on the ionic strength, an increase in the intramolecular repulsion forces and therefore a consequent increase in the hydrodynamic volume and intrinsic viscosity [38].

### *3.4 EPS molecular weight (Mw)*

The molecular weight of EPS produced in the various experiments was investigated by size-exclusion chromatography coupled with a refractive index detector (HPSEC-RID). Results are reported in Table 2.

| | | **% of total peak area** | | | | |
|---|---|---|---|---|---|---|
| **Strain** | **Carbon source** | Fraction 1 (> 500 KDa) | Fraction 2 (130 - 200 KDa) | Fraction 3 (40 - 65 KDa) | Fraction 4 (10 - 25 KDa) | Fraction 5 (< 10 KDa) |
| *L. paracasei* 2333 | Fructose | / | 7 | 38 | 56 | / |
| | Maltose | / | 5 | 42 | 53 | / |
| | Sucrose | / | 4 | 44 | 40 | 12 |
| | Lactose | / | 6 | 41 | 52 | / |
| | Glucose | / | 9 | 37 | 54 | / |
| *L. rhamnosus 1019* | Fructose | / | 10 | 42 | 47 | / |
| | Maltose | / | 6 | 36 | 57 | / |
| | Sucrose | 4 | 10 | 25 | 29 | 32 |
| | Lactose | 5 | 17 | 36 | 42 | / |
| | Glucose | / | 8 | 34 | 57 | / |
| *L. bulgaricus 1932* | Fructose | 16 | 4 | 61 | 19 | / |
| | Maltose | / | 11 | 32 | 48 | 9 |
| | Sucrose | 15 | 5 | 47 | 32 | / |

| | | | | | |
|---|---|---|---|---|---|
| Lactose | 18 | 3 | 24 | 20 | 36 |
| Glucose | 15 | 3 | 29 | 23 | 30 |

Table 2: molecular weight profile (expressed as relative percentages of total chromatographic area) of EPS produced by three LAB strains, fed with five different carbon sources

First it can be noted that all the selected strains gave rise to heterogeneous EPS, having different fractions of different Mw, as often happens for LAB-deriving HeEPS [39]. *L. paracasei* 2333 produced EPS that are always very similar to each other, regardless of the carbon source used. In particular, three different fractions having Mw between 10 and 200 KDa emerged in all the experiments, and always in very similar proportions. The highest Mw fraction (130 - 200 kDa) was actually produced in relatively small amounts, corresponding to 4-9% of the total EPS. On the contrary, the two most abundant fractions were the third and the fourth one, having smaller Mw variable in the range 10 - 65 KDa, which constituted 84-95% of the total. When sucrose was used as a carbon source, another very small fraction with Mw lower than 10 KDa was detected, and it was equal to 12% of the total EPS.

*L. rhamnosus* 1019, as well as *L. paracasei* 2333, produced EPS which were mainly represented by medium-Mw fractions, which together represent 78-94% of the total EPS in four out of five cases. The most peculiar case was found again when *L. rhamnosus* 1019 was fed with sucrose, since an abundant low-Mw fraction equal to 4 KDa was detected, representing 32% of the total. Furthermore, in two experiments, when sucrose and lactose were added as the only carbon source, even a high-Mw fraction (> 500 kDa) was detected, albeit in low quantities.

*L. delbrueckii bulgaricus* 1932 was the strain that behaved in the most different way compared to the others: although it also always produced EPS with abundant medium-Mw fractions, they were less abundant, and their sum was 43-80%. The presence of the high-Mw fraction was also detected, in quantities ranging between 15 and 18%, when the strain was grown on all the carbon source except maltose. Furthermore, when maltose, glucose and lactose were added to the growth medium, EPS produced by *L. delbrueckii bulgaricus* 1932 were characterized also by the smallest fraction (< 10 KDa) which represented 9, 30 or 36% of the total EPS.

From these results, it appears that the Mw profile of the EPS is mainly dependent on the bacterial strain. However, in several cases, it was found out that the carbon source has an influence on this feature too, although not always predictable and constant. This agrees with a study by Polak-Berecka and colleagues, in which a *L. rhamnosus* strain, fed with five different carbon sources, produced EPS having different Mw [40]. On the other hand, in that study, the absolute values of EPS molecular fractions were very far from those obtained in our work, confirming that this structural peculiarity is mainly related to the selected strain. In general, the Mw of LAB's He-EPS that are reported in the literature vary from $10^4$ to $10^6$ Da [41]. Our values fall within this range, with the only exception of fraction 5, having Mw < 10 KDa. Actually, it must be specified that many authors perform an ultrafiltration step (10 KDa cut-off) before analysing the Mw of the considered EPS [42], thus preventing the detection of that fraction. However, the presence of low-Mw EPS can be considered an element of further valorization for the producing strains, because they have been reported to be more effective in terms of antioxidant activity than high-Mw EPS [43]. Conversely, the high-Mw fractions found in some samples, and originated especially by *L. delbrueckii bulgaricus* 1932, may have an interesting potential for technological and functional activities of EPS related to the influence on viscosity. In fact, the positive correlation between Mw of EPS and induced viscosity is now well known [44], so as it is for the relation between an increase in viscosity and better cholesterol-lowering and antimicrobial properties [9].

### *3.5 Correlation of factors involved in EPS production*

| Correlations | EPS g/L | Ribose | Mannose | Rhamnose | Fructose | Galactose | Glucose | Glucosamine | Galactosamine | Rate | yEnd | ΔE | Fraction1 | Fraction2 | Fraction3 | Fraction4 | Fraction5 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|

| | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| EPS g/L | 1 | -0.382 | -0.180 | -0.032 | -0.346 | 0.230 | 0.503 | 0.076 | 0.126 | 0.122 | 0.05 | 0.05 | 0.145 | 0.151 | 0.229 | 0.084 | -0.369 |
| Ribose | -0.382 | 1 | -0.468 | -0.268 | ,549* | 0.464 | -0.218 | -0.340 | -0.191 | 0.497 | 0.484 | 0.095 | ,555* | -,534* | 0.229 | -,560* | 0.294 |
| Mannose | -0.180 | -0.468 | 1 | -0.371 | 0.002 | -0.489 | -0.168 | -0.071 | -0.038 | -0.392 | -0.504 | -0.096 | -0.463 | 0.127 | -0.038 | 0.243 | -0.009 |
| Rhamnose | -0.032 | -0.268 | -0.371 | 1 | -0.286 | -0.088 | -0.395 | 0.279 | -0.022 | -0.427 | -0.169 | -0.085 | -0.155 | 0.440 | -0.368 | 0.193 | -0.003 |
| Fructose | -0.346 | ,549* | 0.002 | -0.286 | 1 | -0.084 | -0.388 | -0.325 | -0.291 | 0.328 | 0.337 | 0.115 | 0.152 | -0.210 | ,672** | -0.283 | -0.195 |
| Galactose | 0.230 | 0.464 | -0.489 | -0.088 | -0.084 | 1 | 0.031 | -0.457 | -0.474 | 0.244 | 0.48 | 0.386 | ,850** | -0.311 | -0.081 | -,726** | 0.457 |
| Glucose | 0.503 | -0.218 | -0.168 | -0.395 | -0.388 | 0.031 | 1 | -0.024 | 0.215 | 0.319 | 0.032 | -0.271 | 0.034 | -0.105 | 0.057 | 0.079 | -0.115 |
| Glucosamine | 0.076 | -0.340 | -0.071 | 0.279 | -0.325 | -0.457 | -0.024 | 1 | ,848** | -0.044 | -0.044 | 0.205 | -,541* | 0.219 | -0.165 | ,778** | -0.472 |
| Galactosamine | 0.126 | -0.191 | -0.038 | -0.022 | -0.291 | -0.474 | 0.215 | ,848** | 1 | 0.061 | -0.14 | 0.047 | -,585* | 0.161 | -0.070 | ,762** | -0.484 |
| Rate | -0.141 | 0.334 | -0.150 | -0.284 | 0.292 | -0.314 | 0.170 | 0.336 | 0.369 | 1 | ,792** | 0.337 | -0.033 | -0.300 | 0.161 | 0.130 | -0.131 |
| yEnd | -0.189 | 0.400 | -0.389 | -0.083 | 0.488 | -0.017 | -0.051 | 0.250 | 0.089 | ,792** | 1 | ,563* | 0.140 | -0.225 | 0.258 | 0.003 | -0.190 |
| ΔE | -0.024 | 0.123 | -0.216 | -0.011 | 0.227 | 0.312 | -0.218 | 0.218 | -0.073 | 0.337 | ,563* | 1 | 0.309 | -0.024 | 0.047 | -0.147 | -0.030 |
| Fraction1 | 0.145 | ,555* | -0.463 | -0.155 | 0.152 | ,850** | 0.034 | -,541* | -,585* | 0.272 | 0.341 | 0.216 | 1 | -0.431 | 0.069 | -,893** | 0.483 |
| Fraction2 | 0.151 | -,534* | 0.127 | 0.440 | -0.210 | -0.311 | -0.105 | 0.219 | 0.161 | -0.459 | -0.304 | -0.045 | -0.431 | 1 | -0.203 | 0.342 | -0.272 |
| Fraction3 | 0.229 | 0.229 | -0.038 | -0.368 | ,672** | -0.081 | 0.057 | -0.165 | -0.070 | 0.373 | 0.25 | 0.084 | 0.069 | -0.203 | 1 | -0.015 | -,669** |
| Fraction4 | 0.084 | -,560* | 0.243 | 0.193 | -0.283 | -,726** | 0.079 | ,778** | ,762** | -0.173 | -0.232 | -0.08 | -,893** | 0.342 | -0.015 | 1 | -,667** |
| Fraction5 | -0.369 | 0.294 | -0.009 | -0.003 | -0.195 | 0.457 | -0.115 | -0.472 | -0.484 | -0.087 | -0.018 | -0.076 | 0.483 | -0.272 | -,669** | -,667** | 1 |

*Table 3. Correlation matrix of factors involved in EPS production and composition. "Fraction" represents different groups of EPS at different molecular mass: Fraction 1 (> 500 KDa), Fraction 2 (130 - 200 KDa), Fraction 3 (40 - 65 KDa), Fraction 4 (8 - 25 KDa), Fraction 5 (< 10 KDa).*

** Correlation is significant at a 0,05 level*

*** Correlation is significant at a 0,01 level*

In table 3 the correlation matrix among EPS chemical characteristics and production of EPS is presented. Coloured cells represent data with a negative (red) or postive (green) correlation score higher than 0,6, indicating a strong link between the examined variables. A positive correlation can be observed between fructose and EPS' fraction 3. As that fraction includes Mw ranging between 40 and 65 KDa, this result suggest that LAB produce medium to small size EPS when fructose is the available carbon source. On the other hand, fraction 1 is positively correlated with galactose. Fraction 1 comprises EPS with the highest Mw, therefore metabolic efforts to produce these EPS for microbial cells could be major and this would justify that they are generally less present in all the samples [4]. Correlation of high Mw EPS with galactose can be due to the sugar-storage function of EPS, that may be used by the microbial cell for further nutritional purposes [7,8]. Galactose is also negatively correlated with EPS' fraction 4. This fraction comprises EPS with a Mw between 8 and 25 KDa and is positively correlated with glucosamine and galactosamine. These correlations suggest the presence of HePS in this Mw range, both for the small dimensions and for the presence of amino groups in the chain [9,10].

# 4. Conclusions

Results reported in this work suggest new possibilities to exploit LAB for the production of different EPS. As showed by the data, it is possible to achieve the production of different EPS starting from the same microorganism by modulating the carbon source in the media. This opens new possibilities for the production of symbiotic products. Our data suggest the production of different EPS *in situ.* These EPS are known for their

prebiotic effect and can be used as a source of energy for the same microorganisms that produce them, but also from other positive microorganisms that are present in the guts. Moreover, it is known that apart from prebiotic effect EPS can exert other positive effects in the hosts' guts. Among the most known are counted, antioxidant, antimicrobial, immunomodulatory, anti-carcinogenic, biofilm disrupting effect and many others. Our results seem to open new opportunities in the formulation of products with increased health features, maintaining in the same a cleaner label, since the production of positive compounds is entrusted to the present microflora.

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# Chapter 5- Peptide profile of Parmigiano Reggiano cheese after simulated gastrointestinal digestion from quality drivers to functional compounds

Probably one of the first fermented foods consumed by mankind, dairy products are a literal cornucopia of macro- and micronutrients. Moreover, micronutrients are not only present in cheeses, fermented milks, yoghurts and so on, but also amount and relative proportions are good, improving their digestibility and absorbability. Among all dairy products, the greatest interest has recently been given to long ripened cheese. This interest is moved by many factors, as for example social, as they production and consume are deeply rooted in specific productive areas. Reasons can be dietary, due to high nutritional values of the cheese, or health related, since many studies during a large spawn of time focused on effects on overall health status of consumers of long ripened cheese. In fact, long ripened cheeses are often associated with high contents of fat and salt, both risk factors for cardiovascular health and development of non-communicable diseases. Despite this, dairy products in general and long ripened cheese particularly are associated with low incidence of heart attack and cardiovascular diseases. Fact that can be due to high presence of positive compounds like SCFA, proteins, vitamin, minerals and above everything of bioactive peptides. Bioactive peptides are the result of proteolysis that starting from casein is able to liberate in the cheese a paraphernalia of compounds with many positive effects, like anti-oxidant, anti-microbial, ACE-inhibitors, opioid and others. The presence and release of potentially bioactive peptides were thus investigated in a long ripened cheese such as Parmigiano Reggiano.

Castellone Vincenzo, Prandi Barbara, Bancalari Elena, Tedeschi Tullia, Gatti Monica and Bottari Benedetta- In preparation

# 1. Introduction

Fermented dairy products are consumed from the dawn of time, and nowadays are gaining even more attention due to their potential functional features. [1–3]. Scientific evidence is accumulating on the role of long ripened cheeses in conferring positive health effects to consumers. As an example, cheese consumption has been associated with a significantly reduced risk of blood hypertension, stroke, and coronary heart disease [4], as well as colorectal cancer [5]. During the manufacturing of long ripened cheese, many processes take place, from milk gathering to the time when the cheese is finally ready. Among all these processes, ripening is regarded as the most important and despite the static appearance of the cheese wheels in the ripening chamber, it is a deeply active period of radical biochemical changes in matrix. In particular, proteolysis is recognized as one of the most important event during cheese ripening, and the interrelationship between cheese microbiota and aging results in a specific peptide profile [6], that leads to

the valuable and recognizable characteristics of long-ripened cheeses, and where bioactive peptides (BP) have frequently been found [7–9]. BP are protein fragments produced from parent proteins, involved in carrying out various physiological functions, such as anti-microbial, antioxidant, antihypertensive, and ACE-inhibitory activities, mainly described for BP identified in cheeses [5,10–12]. Recently, some authors have investigated the presence of BP in Parmigiano Reggiano (PR) cheese [5,10,13–15]. PR is a protected designation of origin (PDO), raw milk, hard-cooked cheese, with a minimum ripening time of 12 months [16]. PR peptide profile and its evolution during ripening have been described in detail [6,11].

It is known that gastrointestinal digestion is a key element for determining biological activities of BP, that might be degraded or transformed in new sequences released from inactive or less active precursors by pepsin and pancreatic enzymes [12]. Lately, the effect of ripening and *in vitro* digestion on the evolution and fate of BP in PR were studied [10,12]. In this work we have exploited the complex sampling that we have already published in a previous work where we described the peptide profile of numerous PR samples, as a function of ripening time and microbial dynamics. Taking advantage of that unique and tightly controlled production and sampling set up, the present work was aimed at investigating the presence of BP in the cheese samples of different ripening time (from curd to 24 months) produced in different dairies and to evaluate the effect of simulated gastrointestinal digestion on both the PR peptide profile and the presence of BP.

# 2. Materials and methods

## *2.1 Sampling of cheeses*

The cheese samples were provided by the "Consorzio del Parmigiano-Reggiano" (Reggio Emilia, Italy) and obtained according to Bottari et al. [6] from six dairies (called A–F) located in the PR PDO production area. For each dairy, samples were taken from the acidified curd (48 h), after brining (1 month of aging) and after 6, 12, and 24 months. For dairies C, E, and F, samples were also taken at 2, 7, and 9 months. Samples were taken for each dairy at different ripening times from the same original wheel and from different wheels with the same ripening times (**Figure 1**). Sixty of the original collected samples were used in this study: 6 curd (48 h) samples, 6 samples of 1-months-old cheese, 3 samples of 2-months-old cheese, 24 samples of 6-months-old cheese, 3 samples of 7-months-old cheese, 3 samples of 9-months-old cheese, 12 samples of 12-months-old cheese and 3 samples of 24-months-old cheese. For each dairy (A–F) the samples were identified with the letter W followed by a number, indicating the sampled wheel, and a slash followed by a second number, indicating the stage of ripening (e.g.: AW1/0 corresponds to the dairy A, wheel 1, 0 months of ripening, i.e. the curd 48 hours after cheese making). The cheeses were produced according to EU PDO Regulation established by Article 11 of Regulation (EU) no. 1151/2012 [17]. The samples were obtained by coring, thus obtaining a cross section for each wheel. Each whole section was grated and mixed before analysis to have a representative sample of the entire wheel. Aliquots of the samples were kept at -20°C until digestion and subsequent analysis.

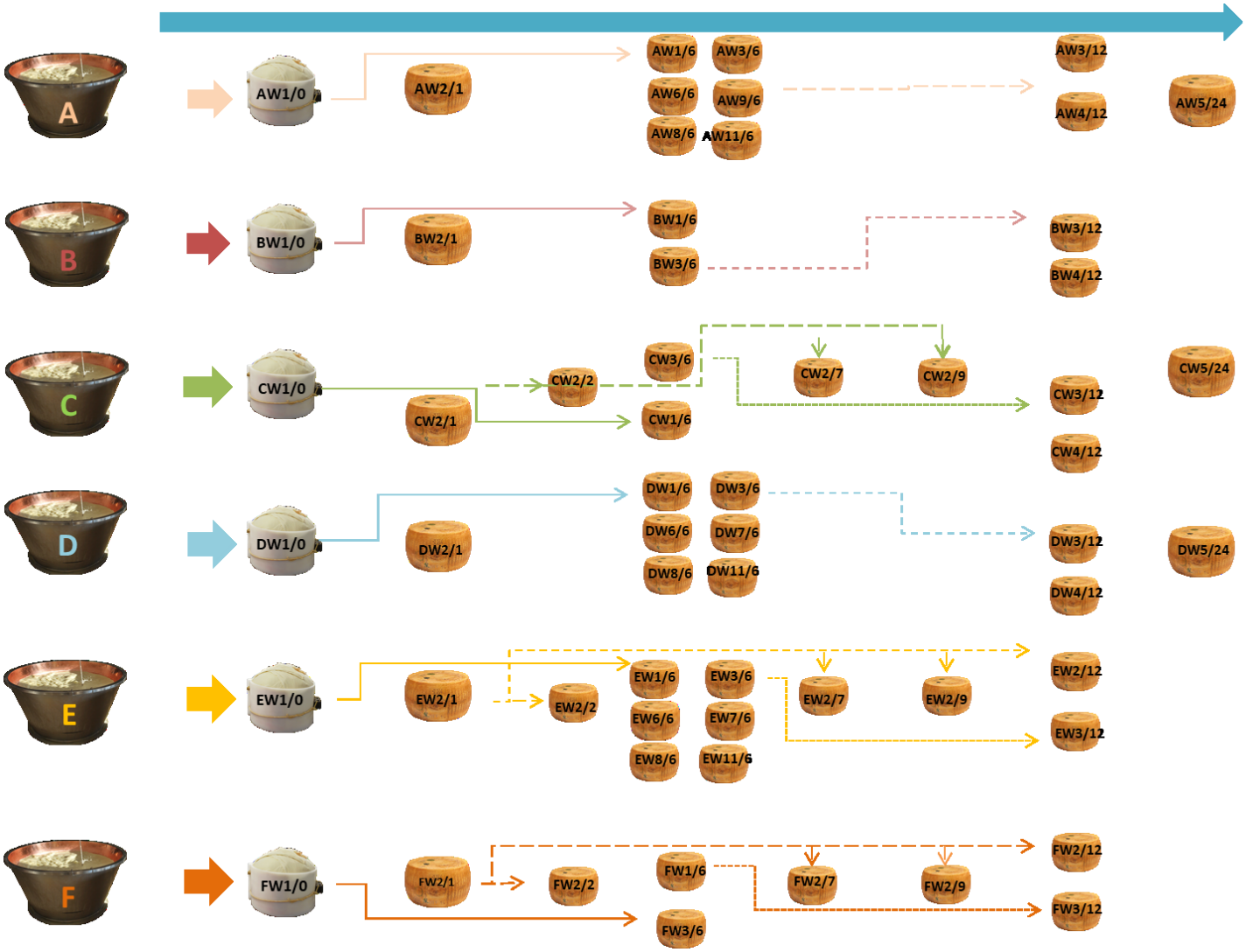


*Figure 1. Cheese sampling scheme.*

### *2.2 Simulated gastrointestinal digestion*

The simulated digestion of the samples was performed according to INFOGEST protocol [18] for *in vitro* gastrointestinal digestion. First, the stock solutions were prepared, as reported in Table 1. Simulated salivary

| Reagents | Amount to be weighted or measured | Final volume of the solution (in demineralized water) | Final concentration of the solution |
|---|---|---|---|
| KCl | 1.87 g | 50 mL | 37.3 g/L |
| $KH_2PO_4$ | 0.68 g | 10 mL | 68 g/L |
| $NaHCO_3$ | 8.40 g | 100 mL | 84 g/L |
| NaCl | 2.93 g | 25 mL | 117 g/L |
| $MgCl_2(H_2O)_6$ | 0.1525 g | 5 mL | 30.5 g/L |
| $(NH_4)_2CO_3$ | 0.0480 g | 1 mL | 48 g/L |
| HCl | 2.50 mL | 5 mL | 6 M |
| NaOH | 4.00 g | 100 mL | 1 M |
| $CaCl_2(H_2O)_2$ | 2.20 g | 50 mL | 44 g/L |

(SSF), gastric (SGF) and intestinal (SIF) fluids were then prepared by suitably mixing the solutions (Table 2).

*Table 1. Reagents and relative quantity to be dissolved for the different solutions*

| Solution | mL to be withdrawn for SSF | mL to be withdrawn for SGF | mL to be withdrawn for SIF |
|---|---|---|---|
| KCl | 15.1 | 6.9 | 6.8 |
| $KH_2PO_4$ | 3.7 | 0.9 | 0.8 |
| $NaHCO_3$ | 6.8 | 12.5 | 42.5 |
| NaCl | - | 11.8 | 9.6 |
| $MgCl_2(H_2O)_6$ | 0.5 | 0.4 | 1.1 |
| $(NH_4)_2CO_3$ | 0.06 | 0.5 | - |
| HCl 6M | 0.09 | 1.3 | 0.7 |

*Table 2. Solutions and volumes to be taken for the different digestive fluids.*

The solutions were then brought to 400 mL with demineralized water. In addition to the previous solutions, enzymatic solutions were prepared suitably dissolved in the corresponding digestive fluid (Table 3).

| Enzyme | Concentration needed | Final volume of enzyme solution | Digestive fluid to dissolve enzymes and bile |
|---|---|---|---|
| Amylase | 1500 U/mL | 5 mL | SSF |
| Pepsine | 25000 U/mL | 25 mL | SGF |
| Pancreatine | 800 U/mL | 50 mL | SIF |
| Bile | 160 mM | 25 mL | SIF |

*Table 2. Digestive enzyme, bile salts, and final concentration for the experiments.*

After the preparation of the solutions containing digestive fluids and enzymes, 2.5 grams of grated PR cheese are weighted, and in vitro digestion begins with the oral phase. For the oral phase we mixed the grated cheese with 1.75 mL of SSF, 250 µL of amylase solution, 12.5 µL of $CaCl_2$ and 487.5 µL of demineralized water. The samples were then homogenized for a few seconds with a vortex and incubated for 2 minutes at 37°C under constant stirring, to simulate chewing. After the oral phase, the gastric phase was continued by adding 3,75 mL of gastric solution, 800 µL of pepsin solution, 2.5 µL of $CaCl_2$, 0.3475 mL of demineralized water and sufficient HCl to the bolus to bring the pH to 3. Then the gastric phase was carried out in incubation for 2 hours at 37°C under constant stirring. Last phase is the intestinal phase, in which 5.5 mL of SIF, 2.5 mL of pancreatin and 1.25 mL of bile have been added to the chyme, along with 20 µL of $CaCl_2$ and enough NaOH to bring the pH to 7. The samples were then incubated at 37°C for an additional 2 hours. After incubation, digestion was stopped by bringing the samples to 95°C for 10 minutes. Samples were then centrifuged at 10000*g* for 10 min at 4°C and filtered with 0.45 µm sterile syringe filters to remove all the particulates. At last samples were spiked with 1 mM of (*L*,*L*)-phenylalanyl-phenylalanine (Phe-Phe) as internal standard to allow semiquantification. The samples were then frozen and held at -20°C until UHPLC/ESI-MS/MS analysis. Each sample was extracted and analyzed in triplicate

### *2.3 UHPLC/ESI-MS/MS analysis*

UHPLC/ESI-MS/MS analysis was performed on digested PR samples and corresponding undigested controls as described in [19]. Chromatographic separation was achieved using a reversed phase column (Aeris Peptide 1.7 µm XB-C18, 150 × 2.10 mm, Phenomenex, Torrance, CA) equipped with a Security Guard ULTRA Cartridge (C18-Peptide, ID 2.1 mm; Phenomenex, Torrance, CA, USA) in a UHPLC system (Dionex Ultimate 3000, Thermo Scientific, Waltham, MA, USA). Eluent A was $H_2O$ + 0.2% $CH_3CN$ + 0.1% HCOOH, eluent B was $CH_3CN$ + 0.2% $H_2O$ + 0.1% HCOOH. The flow was maintained at 0.2 mL/min and the applied gradient was: 0–7 min,

100% A; 7–50 min, 100% A to 50% A; 50–-52.6 min, 50% A; 52.6–53 min, 50% A to 0% A; 53–58.2 min, 0% A; 58.2–59 min, 0% A to 100% A; 59–72 min, 100% A. Total run time: 72 min; column temperature: 35°C; sample temperature: 10°C; injection volume: 2 µL for Full Scan analysis, 4 µL for Product Ion Scan analysis. Detection was achieved using a triple quadrupole TSQ Vantage (Thermo Scientific, Waltham, MA) using the following parameters: positive ion mode, acquisition time: 7–58.2 min (7 min of solvent delay was applied at the beginning of the chromatographic run), acquisition range: 100–1500 m/z, micro scans: 1, scan time: 0.50, Q1 PW: 0.70, spray voltage: 3200 V, capillary temperature: 250 °C, vaporizer temperature: 250 °C, sheath gas flow: 22 units. The samples were first analyzed in Full Scan mode, then in Product Ion Scan mode. The collision energies (CE) were calculated as CE=3.314+0.034×m/z [20]. The peptides were identified as reported in [21]. The peptide sequences were assigned based on the obtained tandem mass spectra. In short, the FindPept software (http://web.expasy.org/findpept/) was used to find the peptide sequences within the target proteins (UniprotKB accessions: P02666, P02662, P02663, P02668, P02754, P00711), whose molecular weight corresponded to the experimental data. Then, the Proteomics Toolkit software (http://db.systemsbiology.net/proteomicsToolkit/FragIonServlet.html) was used to check the correspondence between the theoretical MS/MS fragmentation and the obtained tandem MS spectra.

### *2.4 Identification of peptides with a reported bioactivity in PR samples before and after simulated digestion*

After UHPLC/ESI-MS/MS analysis, chromatograms were processed to identify all peptides present in the samples. The peptides from digested samples were semi quantified against an internal standard (Phe-Phe). After the identification of the peaks and the semi quantification, the Milk Bioactive Peptide Database (MBPB) was used to identify peptides reported as bioactive in the protein fractions [22]. Peptide sequences with 100% similarity to the recorded BP sequence were considered for the bioactivities. Thirty-four peptide sequences identified in a previous work in the same PR samples before digestion [6], and 105 peptide sequences identified in the present study in digested PR cheeses were analyzed.
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### *2.5 Statistical analysis*

Statistical analyses were carried out using IBM SPSS Statistics software (version 27.0, Armonk, NY, United States). Bivariate correlation was performed using Spearman's coefficients, with a two-tailed significance test, and pairwise case exclusion for missing values. Significance was fixed to a $p$ <0.05. The ANOVA followed by the Tukey HSD test were performed to detect statistical differences ($p \leq 0.05$) among microbial counts and biodiversity indices as a function of ripening time. SIMCA 16.0.1 (Sartorius Stedim Data Analytics, Göttingen, Germany) software was used to create principal component analysis (PCA) biplot to get a visual interpretation of the analysed data.

## 3. Results and discussion

### *3.1 Peptides resulting from simulated gastrointestinal digestion*

The peptide fraction of 60 PR cheeses collected from 6 different dairies (A-F) throughout the PR area, at different ripening times (0, 1, 2, 6, 7, 9, 12 and 24 months) was analysed after simulated digestion to detect peptides, and in particular BP, released after the passage in the first part of the GIT. After *in vitro* digestion, 105 different peptides were detected. Out of 105 identified peptides, 47 derive from α-S1-casein and 50 from β-casein. Regarding α-S1-casein the shortest peptides are composed by 2 amino acids and the longest by 14

amino acids, with an average length of 6 amino acids. On the other hand, the peptides deriving from β-casein are composed by peptides which the shortest dipeptides, and the longest have 16 amino acids, with an average length of 5 amino acids. Eight out of 105 of the detected peptides derived from α-S2-casein and whey proteins. Principal Component Analysis (PCA) was performed to investigate possible correlations among peptides detected after digestion and the ripening time of samples (Figure 2).

In the biplot, the samples and all the 105 detected peptides are reported, coloured by months of ripening. Samples tend to create a big cluster in the centre of the biplot, where samples from all the dairies of origin and ripening times are present. In particular, the 24 months ripened PR samples group together in this cluster. A second cluster can be observed at the bottom left side were some 6 months ripened samples from farm A and C and 1 sample of 12 months of ripening produced by dairy A cluster together. A third cluster contains samples of 6 months of ripening mainly from dairies D, E and 2 samples of 9 months of ripening, respectively from dairy C and E. Finally, a fourth cluster is present on the right-hand side which is characterized by most peptides and includes four samples of different ripening time. These data suggest that after digestion, samples share a common core of few peptides, while on the other hand, most peptides are widely distributed along the two components, and they do not characterize a specific ripening time. This is not surprisingly as during ripening the profile of cheese changes a lot because of microbial dynamics [6], with peptides of different size and sequence characterizing each time point. Thus, the peptides that after digestion are common to several ripening stages may have been released by both bigger proteins and already hydrolysed peptides with similar specific cleavage sites [23]. Cheeses of 6 months of ripening are those widely distributed along the two PCOs. It is known that the most relevant proteolytic transformations in PR cheese occur in the first 12 months [6,24] with a huge peptide evolution, thus peptides at this stage can present many different cleavage sites for digestive enzymes.

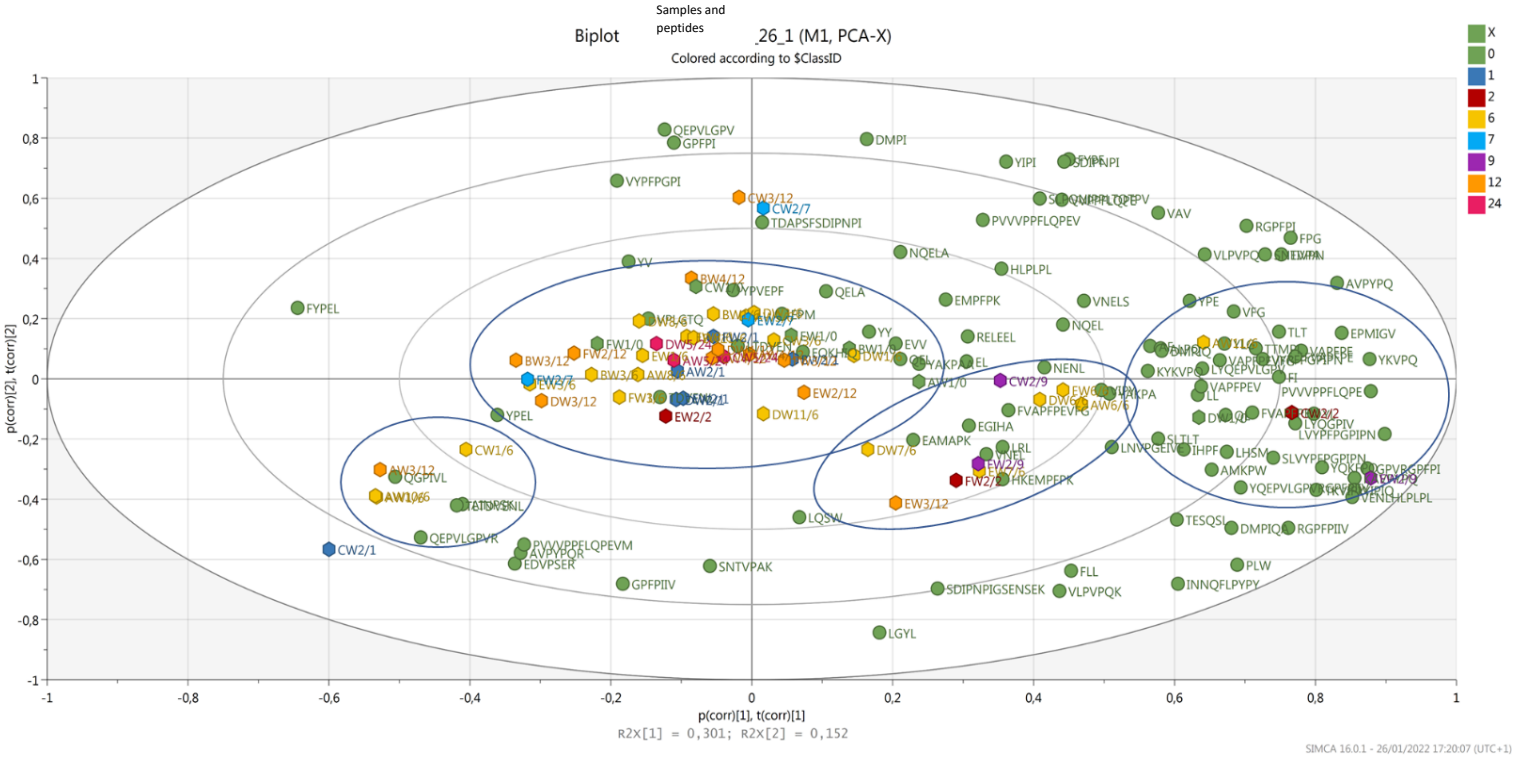


*Figure 2. Biplot of samples and all peptides detected. The samples are coloured by month of ripening.*

As the majority of identified peptides derive from α-S1-casein and β-casein, two separate PCA were run on data, to evaluate the distribution of the samples and peptides coming from the two native proteins according to ripening time.

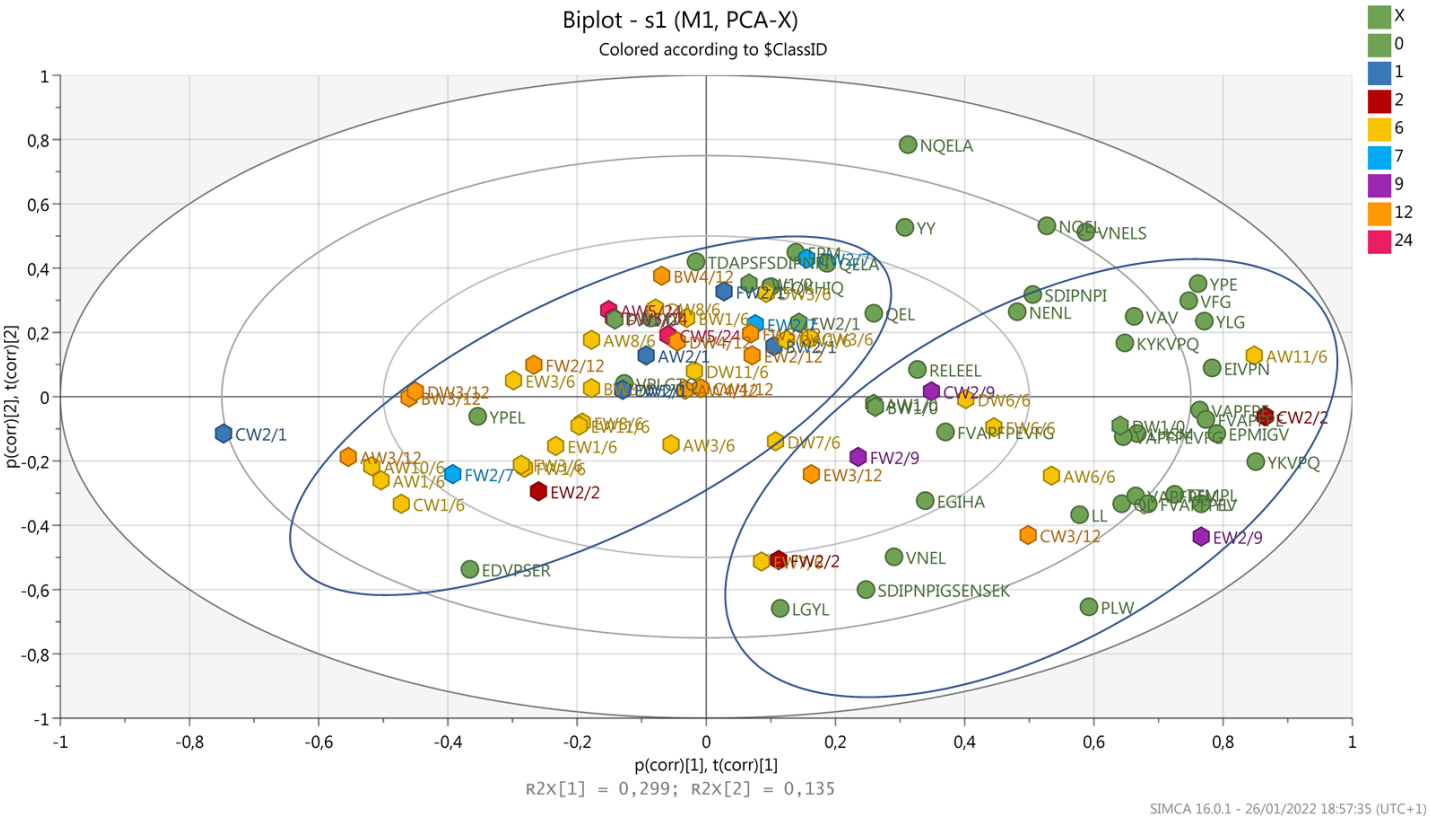


*Figure 3. Biplot of peptides derived from **α-S1-casein**. Samples are coloured for ripening time*

Few peptides released after digestion which were deriving from α-S1-casein (Figure 3) clustered in a group at the center of the biplot, where the great majority of samples are. This common core of peptides is not influenced neither by the area of production, nor by ripening time, as this cluster include samples from all dairies and different ripening times. Considering that the same samples clustered according to ripening time when analyzed before digestion [6], the result obtained in the present study confirm that the *in vitro* digestion greatly influences the peptide profile of PR cheese [15]. On the other hand, a small group of samples of 6, 9 and 12 months of ripening seems to be characterized by other peptides, clustering apart, at the right of the biplot. Most of these peptides contain a proline, that is known to increase the resistance to gastro-pancreatic proteases action [25].

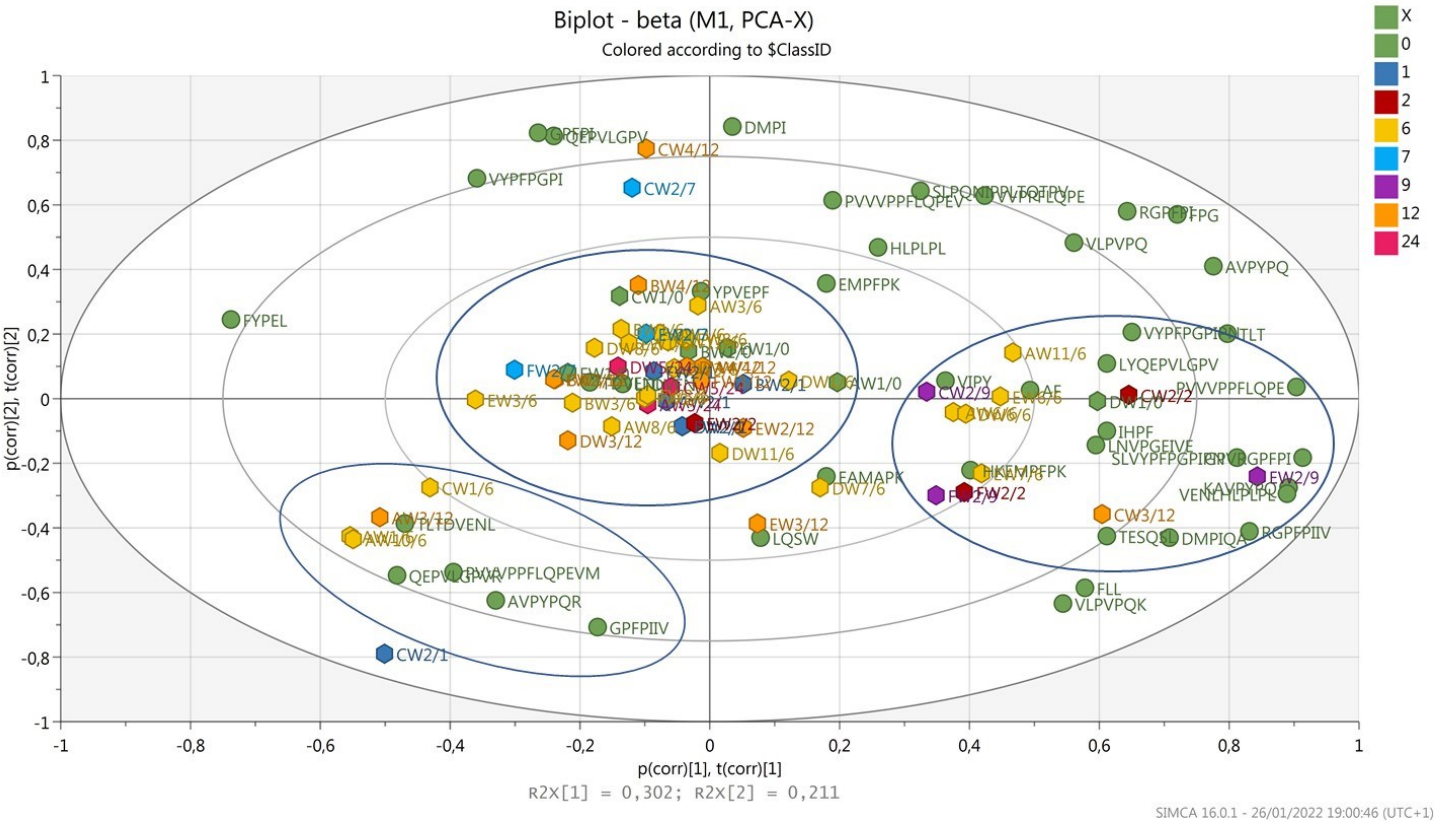


*Figure 4. Biplot of peptides derived from β-casein. Samples are coloured for ripening time.*

Figure 4 shows the biplot with the variance explained for the peptides released after digestion which are derived from β-casein. In this case the samples are grouped in three different clusters. Few peptides, namely LTDVEN, TDVENL and YPVEPF (β-casein) characterize one central cluster, where most samples are, meaning that those peptides are released from all cheese samples after digestion, independently from the ripening time and the dairy of origin. Interestingly, most of the 12-months ripened cheese and all the 24 months ripened samples group together in this cluster. YPVEPF is a casomorphin peptide, which is reported being resistant to digestion due to the presence on the cleavage sites of one histidine. This peptide has been recently reported as an antimicrobial peptide involved in bacterial ecosystem regulation [26], and described for its potential sleep-enhancing activity [27]. On the other hand, the other peptides show an increased resistance to cutting and cleavage, also because of their small dimensions [28,29]. Some interesting information was found in the literature concerning those peptides, as LTDVEN has been found to display features of dipeptidyl peptidase IV (DPP-IV) inhibitory activity [30], while TDVENL has been shown to suppress micellar cholesterol solubility which can induce a cholesterol-lowering action [31]. A second cluster, characterized by several peptides, is present on the right side of the biplot, where samples from 2 to 12

months of ripening group. Three samples deriving from the same cheesemaking (dairy C, same wheel sampled at 2, 9 and 12 months of ripening) are present in this cluster, suggesting that the peptide profile originating from the first biochemical events and evolving during ripening, show a common trend after digestion. Finally, 6 samples of 6 and 12 months of ripening, mainly from dairy A, group together in a third cluster at the bottom left side of the biplot. In a previous study conducted on the same sample set[6], the production in different dairies was not significantly responsible for peptide variability. In the present study, simulated gastrointestinal digestion seems to reveal the presence of few peptides common to 6- and 12-months old cheeses from dairy A.

### *3.2 Peptides with reported bioactivities*

The presence of potential BP was investigated for both undigested (Tab 4a) and digested cheese samples (Tab 4b). Analysing the 34 peptide sequences previously identified in the same PR cheeses before digestion [6], only 4 potentially BP were found, deriving 1 from α-S1-casein and 3 from β-casein. As shown in Tables 4a, the functionality of BP present in PR cheeses of different ripening times, can be multiple, with a single peptide expressing many bioactivities. In fact, 2 of these peptides are reported to exert more than one bioactivity, specifically the peptide RPKHPIKHQGLPQEVLNENLLRF deriving from α-S1-casein shows immunomodulatory and antimicrobial activities. However, this potentially bioactive sequence was found only in the peptide profile of PR curd samples. This is not surprising as the length of the peptide makes it easily susceptible to proteolysis in the further ripening stages. On the other hand, the peptides YQEPVLGPVRGPFPIIV, DKIHPF, and RELEEL respectively reported as immunomodulator/antithrombin/antimicrobial/ACE-inhibitor, ACE-inhibitor, and antioxidant, were found in almost all the considered ripening stages, albeit in different amount, with the latter particularly present in 6 months ripened cheeses (data not shown).

Regarding the analysis of potential BP after digestion, 20 potentially BP were detected. Out of these 20 potentially BP, 12 were derived from β-casein, 7 from α-S1-casein and 1 from α-S2-casein (Table 4b). Some of them, specifically 6 out of 20, seem to exert more than one functionality. Among the range of bioactivities reported for bioactive peptides, one of the most studied features is the ability to inhibit angiotensin–converting enzyme (ACE), preventing a sharp rise in blood pressure, and limiting the risk of heart failure and stroke. Eight BP known for their ACE inhibitory effect [32,33] were detected in the analysed cheese samples after digestion. 1 of the BP detected possess potential bradykinin-enhancing effect, which in combination with the ACE-inhibitory effect helps to maintain a regular blood pressure level [1]. 1 peptide is reported to exert antidiabetic effects [28]. Other known bioactivities attributed to BP found in the samples include opioid effect and delayed cognitive decline along with inhibition of prolyl-endopeptidase- and cathepsin B. These latter effects, combined, could be useful in delaying the progression of tumours [34,35] and fighting the onset and development of Parkinson's disease [36]. A potential beneficial effect of cheese consumption on cardiovascular health related to the presence of potential BP, or their release after digestion, had already been hypothesized by other authors [4,12,37] for PR [10,14]. Conversely, further studies to establish whether the absorption of these peptides in the body is sufficient to develop the bioactive effect are needed [37,38].

In our experiments, only 4 potentially BP were detected in undigested cheese samples, while among the 105 different peptides revealed by the analysis of digested cheese samples, 20 peptides showed at least one bioactivity as reported by the Milk Bioactive Peptides Database (MBDP). This is in agreement with the literature which reports a higher number of BP released after digestion than undigested food [39–41]. Digestion is a key step to free BP from their cryptic form and to increase the probability of having a positive effect exerted by the food components. Only one BP sequence, namely YQEPVLGPVRGPFPIIV, was found both before and after digestion meaning that neither digestive enzymes nor gastric acids can hydrolyse it.

Indeed, this multifunctional peptide is known to be resistant to digestion, due to a particular conformation [42]. Other BP found in undigested PR cheese does not resist simulated digestion, although some bioactive fragments found in digested cheese may result from larger bioactive sequences found in undigested PR cheese [10,43]. For example, the antioxidant peptide "EL" in digested PR cheese may derive from "RELEEL", a peptide with the same reported bioactivities, present in undigested cheese.

| Sequence | Protein | Position | Effect | Reference |
|---|---|---|---|---|
| RPKHPIKHQGLPQEVLNENLLRF | α-S1-casein | 16-38 | Antimicrobial/ Immunomodulatory | [44,45] |
| RELEEL | β-casein | 16-21 | Antioxidant | [46] |
| DKIHPF | β-casein | 62-67 | ACE-inhibitory | [47] |
| YQEPVLGPVRGPFPIIV | β-casein | 208-224 | Immunomodulatory/ Antithrombin/ Antimicrobial/ ACE-inhibitory | [42,44,48,49] |

*Table 4a. List of reported bioactive peptides present in undigested PR cheese samples.*

| Sequence | Protein | Position | Effect | Reference |
|---|---|---|---|---|
| QL | α-$S_1$-casein | 112-113 | Anti-diabetic | [28] |
| EAMAPK | β-casein | 115-120 | Antimicrobial | [50] |
| YY | α-$S_1$-casein | 180-181 | ACE-inhibitory | [51] |
| YLG | α-$S_1$-casein | 106-108 | Antioxidant/Improves cognitive decline | [52–54] |
| EL | α-$S_1$-casein | 54-55, 156-157, 163-164 | Antioxidant | [55] |
| HKEMPFPK | β-casein | 121-128 | Antimicrobial | [50] |
| YL | α-$S_1$-casein | 106-107, 109-110 | ACE-inhibitory | [56] |
| AVPYPQR | β-casein | 192-198 | Antioxidant/Antimicrobial/ACE-inhibitory | [57,58] |
| VLPVPQK | β-casein | 185-191 | Antioxidant/Antimicrobial/ACE-inhibitory/Wound healing/Osteoanabolic/Anti-apoptotic | [59–61] |
| AMKPW | α-$S_2$-casein | 204-208 | ACE-inhibitory | [62] |
| EMPFPK | β-casein | 123-128 | Increase MUC4 expression/ Bradykinin-Potentiating/ Antimicrobial/ACE-inhibitory | [50,63–65] |
| SDIPNPIGSENSEK | α-$S_1$-casein | 195-208 | Antimicrobial | [66] |
| VLPVPQ | β-casein | 185-190 | Inhibition of cholesterol solubility | [31] |
| YPEL | α-$S_1$-casein | 161-164 | Antioxidant | [55] |
| LNVPGEIVE | β-casein | 21-29 | ACE-inhibitory | [47] |
| YPVEPF | β-casein | 129-134 | Opioid/ Increase MUC4 expression/ DPP-IV Inhibitory/ Antioxidant/ Antimicrobial | [65,67–69] |
| GPFPI | β-casein | 218-222 | Cathepsin B Inhibitory | [70] |
| VYPFPGPIPN | β-casein | 74-83 | Antioxidant | [71] |
| VYPFPGPI | β-casein | 74-81 | Prolyl Endopeptidase-Inhibitory | [72] |
| YQEPVLGPVRGPFPIIV | β-casein | 208-224 | Immunomodulatory, Antithrombin, Antimicrobial, ACE-inhibitory, | [44,49] |

*Table 4b. List of the best-known bioactive peptides detected in PR cheese samples after digestion*

### *3.3 Semi-quantitative amounts of potentially BP in PR cheese*

Table 5 shows the semiquantitative data of peptides known as bioactive released during the digestion of different ripened PR cheese samples. From the data, it is possible to see different trends followed from the peptides. Some peptides have a constant increasing or decreasing trend according to ripening time, while others increase until reaching a plateau, then start to decrease during aging. It is also fundamental to consider the effect of digestion in releasing encrypted peptides that otherwise are locked in bigger and not active sequences [73].

| | QL | EAM APK | YY | YLG | EL | HKEM PFPK | YL | AVPYPQR | VLPVPQK | AMKPW | EMPFPK | SDIPNP IGSENSEK | VLPVPQ | YPEL | LNVP GEIVE | YPVEPF | GPFPI | VYPFPGPIPN | VYPFPGPI | YQEPVLGPVRGPFPI IV |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A0 | 0,35 | 0,06 | 0,32 | 1,00 | 0,04 | 4,57 | 3,18 | 0,23 | 2,86 | 9,47 | 4,57 | 0,49 | 6,79 | 0,47 | 0,45 | 3,22 | 3,60 | 12,51 | 0,77 | 0,38 |
| A1 | 0,21 | 0,01 | 0,21 | 0,90 | 0,05 | 1,69 | 2,17 | 0,06 | 0,75 | 5,62 | 3,72 | 0,26 | 3,27 | 0,77 | 0,21 | 3,61 | 3,05 | 8,68 | 0,60 | 0,02 |
| A6 | 0,56 | 0,17 | 0,63 | 1,48 | 0,57 | 0,00 | 2,63 | 0,98 | 4,24 | 5,98 | 4,82 | 1,32 | 9,35 | 0,46 | 0,09 | 0,28 | 4,61 | 13,03 | 0,67 | 0,97 |
| A12 | 0,23 | 0,05 | 0,21 | 0,84 | 0,53 | 0,00 | 1,87 | 0,92 | 2,18 | 3,73 | 4,53 | 1,01 | 4,60 | 1,08 | 0,00 | 1,69 | 4,16 | 9,78 | 0,51 | 0,02 |
| A24 | 0,23 | 0,12 | 0,63 | 1,01 | 0,55 | 0,00 | 1,92 | 0,04 | 0,76 | 4,18 | 3,53 | 0,40 | 3,21 | 0,64 | 0,01 | 0,00 | 3,11 | 9,13 | 0,46 | 0,02 |
| B0 | 0,31 | 0,00 | 0,80 | 1,33 | 0,05 | 0,00 | 3,21 | 1,36 | 4,27 | 10,97 | 6,96 | 1,07 | 9,67 | 1,67 | 0,06 | 6,82 | 7,35 | 9,37 | 1,21 | 0,74 |
| B1 | 0,28 | 0,23 | 0,50 | 0,96 | 0,05 | 0,00 | 2,64 | 0,09 | 1,43 | 6,62 | 5,08 | 0,35 | 5,80 | 0,91 | 0,29 | 5,08 | 4,04 | 11,32 | 0,61 | 0,04 |
| B6 | 0,29 | 0,12 | 0,75 | 1,05 | 0,28 | 0,00 | 1,93 | 0,00 | 0,28 | 4,46 | 5,35 | 0,41 | 5,51 | 1,62 | 0,05 | 4,83 | 5,24 | 9,27 | 0,77 | 0,02 |
| B12 | 0,20 | 0,03 | 0,41 | 0,62 | 0,32 | 0,00 | 1,20 | 0,03 | 0,20 | 2,72 | 6,48 | 0,12 | 6,21 | 1,32 | 0,02 | 5,17 | 6,33 | 7,14 | 0,61 | 0,02 |
| C0 | 0,12 | 0,00 | 0,70 | 0,92 | 0,04 | 0,00 | 2,21 | 0,00 | 0,00 | 6,77 | 6,43 | 0,12 | 5,44 | 0,00 | 0,04 | 5,73 | 6,04 | 11,39 | 1,12 | 0,02 |
| C1 | 0,00 | 0,23 | 0,09 | 0,04 | 0,01 | 4,50 | 1,02 | 5,28 | 7,31 | 7,79 | 4,79 | 1,70 | 0,33 | 0,66 | 0,00 | 0,00 | 0,16 | 0,71 | 0,02 | 0,50 |
| C2 | 0,47 | 0,00 | 0,55 | 1,70 | 0,06 | 8,44 | 3,75 | 0,37 | 5,23 | 18,89 | 6,89 | 1,29 | 8,34 | 0,00 | 0,00 | 0,00 | 4,21 | 21,28 | 0,53 | 0,85 |
| C6 | 0,22 | 0,00 | 0,75 | 1,21 | 0,45 | 0,00 | 3,83 | 3,66 | 9,30 | 11,26 | 13,29 | 2,32 | 12,34 | 2,60 | 0,01 | 0,00 | 11,26 | 21,58 | 1,89 | 0,49 |
| C7 | 0,37 | 0,08 | 0,83 | 1,58 | 1,03 | 0,00 | 1,62 | 0,00 | 0,00 | 3,00 | 10,58 | 0,08 | 9,34 | 2,06 | 0,02 | 7,58 | 10,91 | 9,43 | 1,34 | 0,02 |
| C9 | 0,29 | 0,14 | 0,53 | 1,65 | 0,58 | 0,00 | 2,95 | 0,21 | 5,12 | 6,71 | 6,64 | 0,87 | 6,62 | 0,94 | 0,15 | 7,10 | 4,64 | 12,37 | 0,50 | 0,17 |
| C12 | 0,41 | 0,04 | 0,10 | 1,26 | 0,73 | 0,28 | 2,64 | 0,43 | 5,71 | 4,94 | 5,69 | 1,19 | 9,41 | 0,30 | 0,07 | 4,78 | 6,95 | 10,15 | 0,84 | 0,61 |
| C24 | 0,37 | 0,09 | 0,73 | 1,12 | 1,10 | 0,00 | 2,10 | 0,83 | 4,36 | 3,87 | 7,07 | 0,64 | 9,06 | 1,29 | 0,02 | 6,15 | 5,36 | 6,80 | 0,70 | 0,24 |
| D0 | 0,49 | 0,00 | 0,54 | 1,77 | 0,06 | 7,57 | 3,93 | 0,33 | 5,38 | 16,29 | 6,72 | 1,06 | 8,55 | 0,97 | 0,61 | 6,59 | 3,92 | 15,73 | 0,50 | 0,61 |
| D1 | 0,23 | 0,04 | 0,51 | 0,87 | 0,03 | 3,03 | 2,33 | 0,09 | 1,67 | 5,15 | 4,69 | 0,32 | 4,63 | 0,89 | 0,24 | 3,90 | 3,58 | 7,91 | 0,46 | 0,03 |
| D6 | 0,29 | 0,09 | 0,63 | 1,42 | 0,45 | 0,76 | 2,24 | 0,24 | 3,03 | 5,72 | 4,97 | 0,68 | 5,61 | 0,98 | 0,11 | 3,12 | 3,93 | 10,55 | 0,53 | 0,28 |
| D12 | 0,16 | 0,00 | 0,36 | 0,62 | 0,39 | 0,00 | 1,09 | 0,04 | 2,02 | 1,33 | 3,39 | 0,66 | 3,42 | 0,59 | 0,03 | 2,73 | 5,33 | 8,65 | 0,64 | 0,09 |
| D24 | 0,21 | 0,14 | 0,65 | 1,11 | 0,58 | 0,00 | 0,14 | 0,00 | 0,79 | 2,54 | 5,03 | 0,22 | 7,41 | 1,19 | 0,03 | 5,50 | 6,20 | 6,89 | 0,41 | 0,03 |
| E0 | 0,45 | 0,13 | 0,75 | 0,97 | 0,04 | 0,00 | 3,00 | 0,00 | 0,00 | 0,00 | 4,49 | 0,19 | 4,27 | 1,26 | 0,20 | 4,52 | 3,87 | 11,80 | 0,85 | 0,01 |
| E1 | 0,17 | 0,08 | 0,43 | 0,80 | 0,04 | 1,80 | 1,99 | 0,00 | 1,56 | 0,17 | 3,32 | 0,20 | 4,52 | 1,20 | 0,10 | 4,14 | 3,25 | 7,88 | 0,83 | 0,01 |
| E2 | 0,24 | 0,00 | 0,00 | 0,00 | 0,00 | 3,57 | 2,64 | 0,06 | 2,73 | 5,45 | 5,17 | 0,55 | 5,11 | 1,19 | 0,18 | 4,88 | 3,68 | 7,64 | 0,47 | 0,03 |
| E6 | 0,35 | 0,06 | 0,28 | 0,54 | 0,15 | 2,27 | 2,65 | 0,17 | 3,06 | 6,65 | 6,16 | 0,72 | 5,62 | 1,43 | 0,11 | 4,62 | 4,90 | 11,57 | 0,67 | 0,17 |
| E7 | 0,36 | 0,26 | 0,97 | 1,49 | 0,46 | 0,00 | 2,19 | 0,07 | 0,00 | 3,53 | 3,02 | 0,33 | 8,81 | 1,80 | 0,04 | 6,30 | 8,12 | 10,58 | 0,90 | 0,03 |
| E9 | 0,52 | 0,42 | 0,46 | 2,55 | 1,05 | 0,00 | 3,94 | 0,27 | 10,85 | 9,04 | 6,11 | 2,71 | 11,14 | 1,11 | 0,25 | 0,00 | 2,99 | 17,24 | 0,32 | 1,38 |
| E12 | 0,46 | 0,15 | 0,50 | 1,28 | 0,59 | 0,00 | 1,98 | 0,22 | 3,24 | 6,11 | 5,35 | 1,94 | 6,35 | 1,01 | 0,04 | 3,59 | 3,35 | 8,39 | 0,40 | 0,43 |

| | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| F0 | 0,10 | 0,06 | 0,62 | 0,75 | 0,03 | 0,00 | 2,39 | 0,00 | 0,00 | 4,00 | 3,95 | 0,10 | 4,83 | 0,94 | 0,09 | 3,52 | 4,77 | 6,34 | 0,62 | 0,01 |
| F1 | 0,15 | 0,22 | 0,64 | 1,02 | 0,04 | 0,00 | 1,91 | 0,00 | 0,00 | 4,96 | 4,41 | 0,31 | 5,79 | 0,00 | 0,10 | 0,00 | 5,21 | 10,04 | 0,65 | 0,02 |
| F2 | 0,75 | 0,00 | 0,27 | 0,00 | 0,00 | 6,14 | 3,82 | 0,70 | 13,85 | 5,26 | 5,34 | 0,62 | 6,33 | 0,84 | 0,24 | 4,66 | 1,59 | 8,91 | 0,25 | 0,78 |
| F6 | 0,22 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 2,25 | 0,06 | 1,25 | 3,19 | 4,52 | 0,33 | 6,15 | 1,04 | 0,13 | 4,76 | 4,87 | 7,67 | 0,52 | 0,03 |
| F7 | 0,19 | 0,00 | 0,00 | 0,76 | 0,00 | 0,00 | 1,79 | 0,00 | 2,91 | 3,77 | 6,71 | 0,86 | 5,87 | 0,00 | 0,01 | 0,00 | 7,42 | 11,25 | 0,96 | 0,02 |
| F9 | 0,26 | 0,04 | 0,26 | 0,98 | 0,22 | 1,20 | 2,03 | 0,00 | 3,60 | 3,44 | 6,33 | 0,72 | 5,90 | 0,52 | 0,05 | 1,38 | 5,89 | 8,21 | 0,77 | 0,09 |
| F12 | 0,31 | 0,08 | 0,43 | 1,42 | 0,70 | 2,07 | 2,62 | 0,12 | 4,65 | 3,43 | 5,46 | 0,56 | 6,57 | 0,73 | 0,09 | 2,87 | 4,88 | 7,58 | 0,49 | 0,18 |

*Table 5. Relative quantification of potential BP in cheeses with different ripening times after simulated human digestion.The letters A to F represent the different dairies. The numbers represent the ripening time, with 0 representing the curd after 48h of moulding.*

| Pearson's correlation | QL | EAMAPK | YY | YLG | EL | HKEMPFPK | YL | AVPYPQR | VLPVPQK | AMKPW | EMPFPK | SDIPNPIGSENSEK | VLPVPQ | YPEL | LNVPGEIVE | YPVEPF | GPFPI | VYPFPGPIPN | VYPFPGPI | YQEPVLGPVRGPFPIIV |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ripening | -0,007 | 0,018 | -0,019 | 0,108 | ,581** | -,321* | -0,246 | -0,123 | -0,015 | -,375** | -0,006 | 0,043 | 0,107 | 0,022 | -,392** | 0,011 | 0,140 | -0,223 | -0,116 | -0,072 |
| ANOVA | QL | EAMAPK | YY | YLG | EL | HKEMPFPK | YL | AVPYPQR | VLPVPQK | AMKPW | EMPFPK | SDIPNPIGSENSEK | VLPVPQ | YPEL | LNVPGEIVE | YPVEPF | GPFPI | VYPFPGPIPN | VYPFPGPI | YQEPVLGPVRGPFPIIV |
| Sig | 0,573 | 0,026* | 0,346 | 0,263 | 0,00** | 0,002** | 0,01* | 0,762 | 0,157 | 0,019* | 0,645 | 0,353 | 0,378 | 0,811 | 0,013* | 0,707 | 0,040* | 0,037* | 0,028* | 0,460 |

*Table 6. Pearson correlation and ANOVA on BP and ripening time.*

To get a statistical value of these observation, semi-quantification data were analysed by Pearson's correlation and ANOVA. Results are reported in Table 6. Statistical analysis shows that peptide "EL" follows an increasing trend in digested samples according to ripening time. While on the contrary, "HKEMPFPK", "AMKPW" and "LNVPGEIVE" follow a decreasing trend in longer ripened cheeses after digestion. ANOVA analysis confirms that the presence of BP was statistically different among the samples for 9 out of 20 detected potentially BP.

Since PDO regulation allows the PR cheese to be sold only after minimum 12 months of ripening, differences among BP presence were then searched between 12- and 24-months ripened cheeses. Results suggest higher amounts (*p* value of 0,03) of potentially BP were found in 24 months ripened samples compared to 12 months ripened ones. This is in agreement with other authors [15] and can be explained by the higher proteolytic activity of lactic acid bacteria exerted in the longer ripened cheeses [6].

### *3.4 Frequency of identification of peptides with potential bioactivities*

Potential bioactivities of digested cheese samples resulting from BP profiles showed small variations based on ripening time (Figure 5). According to the graph, the main bioactivities possibly present at each ripening time are antimicrobial, ACE-inhibitor and antioxidant. These 3 bioactivities make up almost 60% of the total bioactivities reported for each single time point. These results agree with Solieri *et al.* [5], which reported ACE inhibitory and antimicrobial activities as the most present in PR samples. During digestion, peptides evolve and their relative quantity increases or decreases, giving rise to changes in the associated bioactivities as well. However, it is not always possible to obtain the maximum beneficial effect from the potential BP present in the PR cheese, since, as indicated by the EU PDO regulation, the PR cheese can only be sold after 12 months of ripening while some bioactivities have the highest peak before reach the minimum ripening time to be marketed (Figure 5).

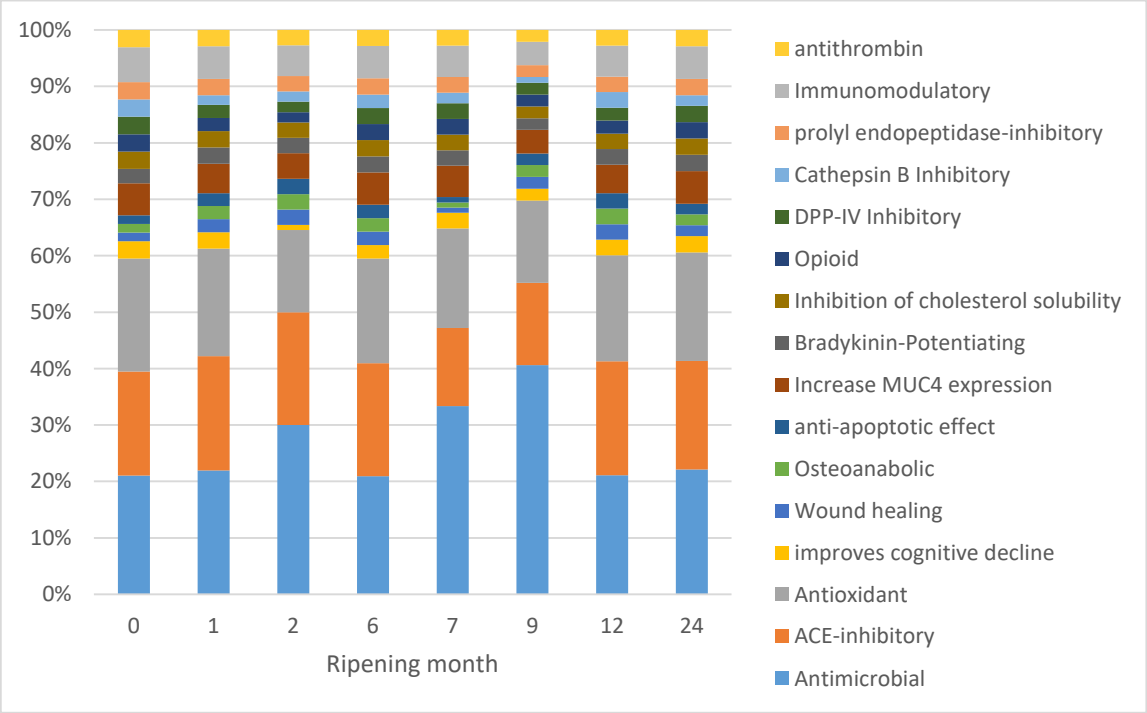


*Figure 5. Bioactivities reported for peptides found in PR cheese samples at different ripening times.*

The link between dairy intake and health benefits is not entirely clear. Many studies in the scientific literature try to evaluate the production of BP and their absorption in the human body. The big drawback to these

experiments is the difficulty in studying the effects *in vivo*. In fact, many of these works concern *in vitro*, *in silico* or animal approaches [74–76].

The regular introduction of dairy products into the diet is suggested by many nutritional guidelines for maintaining an healthy diet. The indications also suggest introducing dairy products, especially long-ripened ones, more than once a week. The consumption of long-ripened cheeses has long been debated due to possible negative effects, such as a high content of salt and saturated fats, well known to be linked to an increased risk of hearth failure and cardiovascular disease, together with other compounds with possible positive effects. Despite this, diets that involve a high consumption of cheese, such as the Mediterrenean and French diets, have been shown to reduce the risk of developing non-communicable diseases such as heart attacks and *angina pectoris* [4,77,78], suggesting that somehow the positive outcomes of a moderate but constant introduction to the diet of long-ripened cheeses may outweigh the risk posed by the high introduction of salt, saturated fat and cholesterol [4,79,80].

The study of the presence of BP in cheese must take into account not only the production, release and therefore the presence of peptides in the cheese, but also the mechanisms of release and absorption by the intestinal wall cells [12,81]. Since many studies in the literature reporting the positive effect of BP are conducted *in vitro*, the BP should be tested for their effect *in vivo.*

## 4. Conclusions

The complexity of enzymatic activities that occur during the production of long-ripened cheese has been widely described. This also applies to the PR cheese, which despite its restricted geographical area of production is known almost all over the world. Further steps have recently been taken in the literature regarding understanding what happens to this treasure of nutrients and possibly bioactive compounds after digestion. With the present study, conducted on a large number of PR cheese samples with different ripening times and produced by different dairies, we were able to expand what was previously known about the effect of gastrointestinal digestion on the peptide profile of cheese during ripenening. The presence of potential BP in samples before and after digestion was also investigated. Samples treated with simulated digestion were mostly characterized by peptide profiles that shared a common peptide core, regardless of ripening time and company of manufacture. On the other hand, some peptides were released during the digestion of specific samples. This has a double importance, both for the potential production of specific peptides of dairy origin, the release of which can be increased by digesting less ripened cheeses, and for the nutritional/functional aspect of PR cheese. At the required minimum ripening time of 12 months, the peptide profile after digestion is similar between different PR cheeses. The amount of potential BP increases with digestion, particularly for long-ripened PR cheeses. Any differences related to the production dairy could be considered to guide and predict proteolysis from the milk to cheese consumed.

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# Chapter 6- Fiber and Gut microbiota interactions: Location and production of SCFA and PAC related metabolites.

Study of bioactive compounds is fundamental to understand potential positive effects of fermented foods on human organism. Nevertheless, a part seems to miss, what happen when in the guts after the ingestion of food containing precursor of bioactive compounds? I tried to answer to this question during my abroad period, when I studied the formation and evolution during fecal fermentation of SCFA and phytochemicals. Gut microbiota starts to develop in the exact moment of birth, when it is delivered by the mother and evolve all through the life of the subject. Despite the evolutions and adaption processes, a resistant group of microorganisms known as core microbiome is still present in all the humans and remain constant during the life. In the guts the most dominant genera belong to *Bacteroidetes* and *Firmicutes*. Different amounts and relative distribution of these microorganisms in the colon can affect the overall health status of the colon and then of the organism itself. Low microbial diversity in the guts has been associated with inflammatory diseases, obesity, insulin resistance and others chronic diseases. Since it is known that complex carbohydrates, like fibers present in the fruit are a nourishment source for microorganisms and by fermenting dietary fiber gut microbiota can produce healthy compounds also for us, the study of fecal fermentations represented a further piece of the puzzle.

Castellone Vincenzo, Bottari Benedetta, Rubert, Josep (in preparation)

# 1. Introduction

Human gastrointestinal tract is an active site containing trillions microorganisms, bacteria, archaea, viruses, and fungi [1]. This living ensemble, recently described as a "superorganism" act as a stand-alone living being guided by an hive mind and have proved itself to have a great impact on the host' overall health status [2,3]. It is, in fact, well established the role of human microbiota in most of the fundamental vital functions of hosts. Gut microbiota (GM) is directly involved in maintaining the pillars of individual gut's health: avoid gastro-intestinal disorders, improve digestive efficiency, absorb food and derived components and maintain healthy, live and stable microbial communities in the guts [4]. Specifically, gut microbiota can ferment indigestible food component (e.g. fibers) to extract energy and positive compounds like short chain fatty acids (SCFA), gasses and signalling molecule [5,6]. In this optic, an healthy and active microflora in the intestine is needed in order to maintain the homeostasis and well-being of hosts [7]. Investigation on well-being of gut microflora leads to conclusion that one of the most suitable and efficient way to take care of gut microbiota is the regular introduction of dietary fibers. Dietary fibers, from different sources has been elected as one of the most effective and attractive way to modulate gut microflora, leading to the development of positive and desirable microflora. Dietary fibers can be a great help in the modulation of gut microflora. Influence of fiber on gut microbiota depend on regular introduction of dietary fiber can depend on different parameters like the molecular size, cell wall architecture, water solubility, polymerization degrees, side chains (presence and distribution) and cross-linking between different polymers [8,9]. Dietary fiber comprises resistant starch and other non-starchy polysaccharides. During the years scientific literatures have taken for granted the assumption that soluble fiber is fermented in the colon leading to production of healthy compounds and gas, while non-soluble fiber is inert and only increase fecal volume. Even though this assumption is supported by strong evidences and generally true, can mislead to wrong results. Solubility in vitro of fibers can be sensibly different by the solubility of the same fiber in the guts, since some links can be broken by digestive enzymes and enzymes on the brush borders of small intestine [9].

Among the trillions of microorganisms present at all level in the guts, the most interesting microorganisms for gut's health are saccharolytic bacteria due to their ability to ferment dietary fiber [10,11] producing short chain fatty acids (SCFA) [12]. SCFA have many positive effects, for example butyric acid act as an energy source for microbial cells and fight the development of cancers [5]. Propionate enhances gluconeogenesis and helps maintain glucose homeostasis in the organisms by increasing the expression of leptin, an anorectic hormone, in adipocytes [13]. Acetate is involved in the lipogenesis and synthesis of cholesterol [14,15]. Moreover, acetate and other SCFA play a pivotal role in modulating the gut brain axis. Potentiating, among others, the effects exerted by vagus nerve of recognizing between positive and harmful microorganisms [16]. SCFA in the guts are proven also to improve learning and cognitive function, as well as retard neurodegenerative damages [17]. The amount of carbohydrates introduced with diet and specific fermentation pathways of microorganisms present in the guts in the synthesis determine which and the amount of SCFA produced in the guts [18]. SCFA are mainly absorbed by epithelial cells of the colon. In fact only a 5-10% are excreted with feces. Moreover, SCFA acidify the feces and the colon, creating a stressing environment for pathogens, but also promoting elimination of ammonium with the feces,

improving the environment for LAB. These 2 mechanisms both contributes to reduction of harmful and pathogenic microorganisms in the guts, and in the meantime boosting positive and probiotic microorganisms [9].

Experiments aimed to study the fate of dietary compounds and of the fermentation processes lead by gut microflora should take in account several factors. The complexities, linked to ethical and economic reasons. *In vivo* studies on human are not always possible, can be influenced by external factors and also subjective compliance should be taken in account [9]. In this optic, *in vitro* models seems to be an interesting tool since it poses no ethical dilemmas and can precisely represent what happens in the colonic region. Methods for *in vitro* analysis can be static (fecal batch cultures) or dynamic (SHIME, TIM-1, DGM, HGS…). Each of these systems have advantages and disadvantages. In our experiments we decided to take the best of two different systems: SHIME and *In vitro* batch cultures. SHIME is a model that uses different vessels with controlled pH, temperature, atmosphere and feeding to mimic the entire gut environment, has the advantages of being very precise and representative. In our case we use these features to mimick the 3 different tracts of the colonic region, after a 2 weeks period of stabilization these microbiotas were withdrawn and used as inoculum for the in vitro batch cultures. *In vitro* batch cultures are used to measure capacity of human fecal microbiota to ferment different matrices in an anaerobic environment. Knowing the importance of dietary fiber in maintaining the health status of hosts, since polysaccharides extracted from plants and fruits are considered as a fundamental part of an healthy diet, for the boosting effect toward gut microorganisms [19] the combination of the two methods (SHIME and *in vitro* batch cultures) was used to study the effect of colonic fermentation on apples. Apples were chosen as representative of foods containing fibers and phytochemicals.

## 2. Materials and methods

### *2.1 Raw material digestion*

For our experiment we select Apple of the variety Renetta Canada and Pectine deriving from the same apples. Moreover, Procyanidin C1 was taken as positive control, to assess microbiota's fermentative processes. Apples were purchased fresh at a local market and rapidly digested following INFOGEST protocol [20]. For the *in vitro* digestion, the stock solutions were prepared, as reported in Table 1. Simulated salivary (SSF), gastric (SGF) and intestinal (SIF) fluids were then prepared by suitably mixing the solutions (Table 2).

| **Reagents** | **Amount to be weighted or measured** | **Final volume of the solution (in demineralized water)** | **Final concentration of the solution** |
|---|---|---|---|
| KCl | 1.87 g | 50 mL | 37.3 g/L |
| $KH_2PO_4$ | 0.68 g | 10 mL | 68 g/L |
| $NaHCO_3$ | 8.40 g | 100 mL | 84 g/L |
| NaCl | 2.93 g | 25 mL | 117 g/L |
| $MgCl_2(H_2O)_6$ | 0.1525 g | 5 mL | 30.5 g/L |
| $(NH_4)_2CO_3$ | 0.0480 g | 1 mL | 48 g/L |

| | | | |
|---|---|---|---|
| HCl | 2.50 mL | 5 mL | 6 M |
| NaOH | 4.00 g | 100 mL | 1 M |
| $CaCl_2(H_2O)_2$ | 2.20 g | 50 mL | 44 g/L |

*Table 1. Reagents and relative quantity to be dissolved for the different solutions.*

| **Reagents** | **Amount to be weighted or measured** | **Final volume of the solution (in demineralized water)** | **Final concentration of the solution** |
|---|---|---|---|
| KCl | 1.87 g | 50 mL | 37.3 g/L |
| $KH_2PO_4$ | 0.68 g | 10 mL | 68 g/L |
| $NaHCO_3$ | 8.40 g | 100 mL | 84 g/L |
| NaCl | 2.93 g | 25 mL | 117 g/L |
| $MgCl_2(H_2O)_6$ | 0.1525 g | 5 mL | 30.5 g/L |
| $(NH_4)_2CO_3$ | 0.0480 g | 1 mL | 48 g/L |
| HCl | 2.50 mL | 5 mL | 6 M |
| NaOH | 4.00 g | 100 mL | 1 M |
| $CaCl_2(H_2O)_2$ | 2.20 g | 50 mL | 44 g/L |

| **Solution** | **mL to be withdrawn for SSF** | **mL to be withdrawn for SGF** | **mL to be withdrawn for SIF** |
|---|---|---|---|
| KCl | 15.1 | 6.9 | 6.8 |
| $KH_2PO_4$ | 3.7 | 0.9 | 0.8 |
| $NaHCO_3$ | 6.8 | 12.5 | 42.5 |
| NaCl | - | 11.8 | 9.6 |
| $MgCl_2(H_2O)_6$ | 0.5 | 0.4 | 1.1 |
| $(NH_4)_2CO_3$ | 0.06 | 0.5 | - |
| HCl 6M | 0.09 | 1.3 | 0.7 |

*Table 2. Solutions and volumes to be taken for the different digestive fluids.*

The solutions were then brought to 400 mL with demineralized water. In addition to the previous solutions, enzymatic solutions were prepared suitably dissolved in the corresponding digestive fluid (Table 3).

| **Enzyme** | **Concentration needed** | **Final volume of enzyme solution** | **Digestive fluid to dissolve enzymes and bile** |
|---|---|---|---|
| Amylase | 1500 U/mL | 5 mL | SSF |
| Pepsine | 25000 U/mL | 25 mL | SGF |
| Pancreatine | 800 U/mL | 50 mL | SIF |
| Bile | 160 mM | 25 mL | SIF |

*Table 3. Digestive enzyme, bile salts, and final concentration for the experiments.*

After the preparation of the solutions containing digestive fluids and enzymes, 25 grams of apple and pectin cheese are weighted, and in vitro digestion begins with the oral phase. For the oral phase we mixed raw materials with 17.5 mL of SSF, 2.5 mL of amylase solution, 125 µL of $CaCl_2$ and 4,875 mL of demineralized water. The samples were then homogenized for a few seconds with a vortex and incubated for 2 minutes at 37°C under constant stirring, to simulate chewing. After the oral phase, the gastric phase was continued by adding 37.5 mL of gastric solution, 8 mL of pepsin solution, 25 µL of $CaCl_2$, 3.475 mL of demineralized water and sufficient HCl to the bolus to bring the pH to 3. Then the gastric phase was carried out in incubation for 2 hours at 37°C under constant stirring. Last phase is the intestinal phase, in which 55 mL of SIF, 25 mL of pancreatin and 12.5 mL of bile have been added to the chyme, along with 200 µL of $CaCl_2$ and enough NaOH to bring the pH to 7. The samples were then incubated at 37°C for an additional 2 hours. After incubation, digestion was stopped by snapping the samples in liquid nitrogen. Samples were then frozen and held at -20°C until further analysis. Each sample was extracted and analyzed in triplicate

### *2.2 Fecal batch cultures*

Before to start the fecal batch cultures we selected different microbiota starting from feces donation of three different donors (Table 4). To obtain a good approximation of the selected microbiota we used a SHIME. The SHIME is an apparatus able to *in vitro* replicate what happen in a human intestine. SHIME is formed by different double-jacket reactors that reproduce the environment of: stomach, proximal intestine and the three tracts of colon. For our experiments we select microbiota from the three tracts of colon: ascending, transversal and descending.

| | Donor 1 | Donor 2 | Donor 3 |
|---|---|---|---|
| BMI | 27 | 22 | 18,6 |
| Age | 27 | 35 | 26 |
| Smoke | No | No | No |
| Nationality | European | South-American | European |
| Gender | M | M | F |
| Probiotics | No | No | No |
| Antibiotics | No | No | No |
| IBS | No history | No history | No history |

*Table 4. Fecal donors characteristics*

SHIME simulate processes in the intestine, therefore it must be maintained in the same conditions. Therefore, the system was maintened in a constant stirring at 37°C, flushed everyday with nitrogen to maintain a strict anaerobic environment. pH was maintained at phisiological level by additon of HCl and NaOH when needed. Moreover, three times per day the system was fed with a synthetic media containing all the nutrients for microorganisms. The last three vessels were inoculated with fecal microbiota and during two weeks of adaption time the microbiota is selected, mainly by pH, until representative of one of the three colonic regions of interest. Microbiota from the SHIME was then used as inoculum for fecal batch cultures.

For the fecal batch cultures sterile bottles, capped with silicone lids, were filled with 43 mL of Buffered Colonic Medium (Table 5)

| Component | g/L |
|---|---|
| $K_2HPO_4$ | 5,22 |
| $KH_2PO_4$ | 16,32 |
| $NaHCO_3$ | 2 |
| Yeast extract | 2 |
| Mucine | 1 |
| L-Cysteine | 0,5 |
| Tween-80 | 2 mL |

*Table 5. Components and relative amount for 1000mL of BCM*

After the BCM, 20 mL of digested apple, pectin or Procyanidin C1 dissolved in sterile $H_20$ were added to the bottle to a final concentration of 1% of treatment per bottle. Bottles were then flushed with nitrogen to remove the oxygen and create strict anaerobiosis. The last passage was the inoculum of microbiota. 7 mL of each microbiota was inoculated in the bottles at 10% v/v. Inoculated bottles were then incubated at 37°C at constat agitation for 48 hours and samples were taken every 12 hours.

At the same time controls were prepared in the same, only substituting inoculum or treatment with the same amount of sterile water. Experimental design is reported in figure 1.

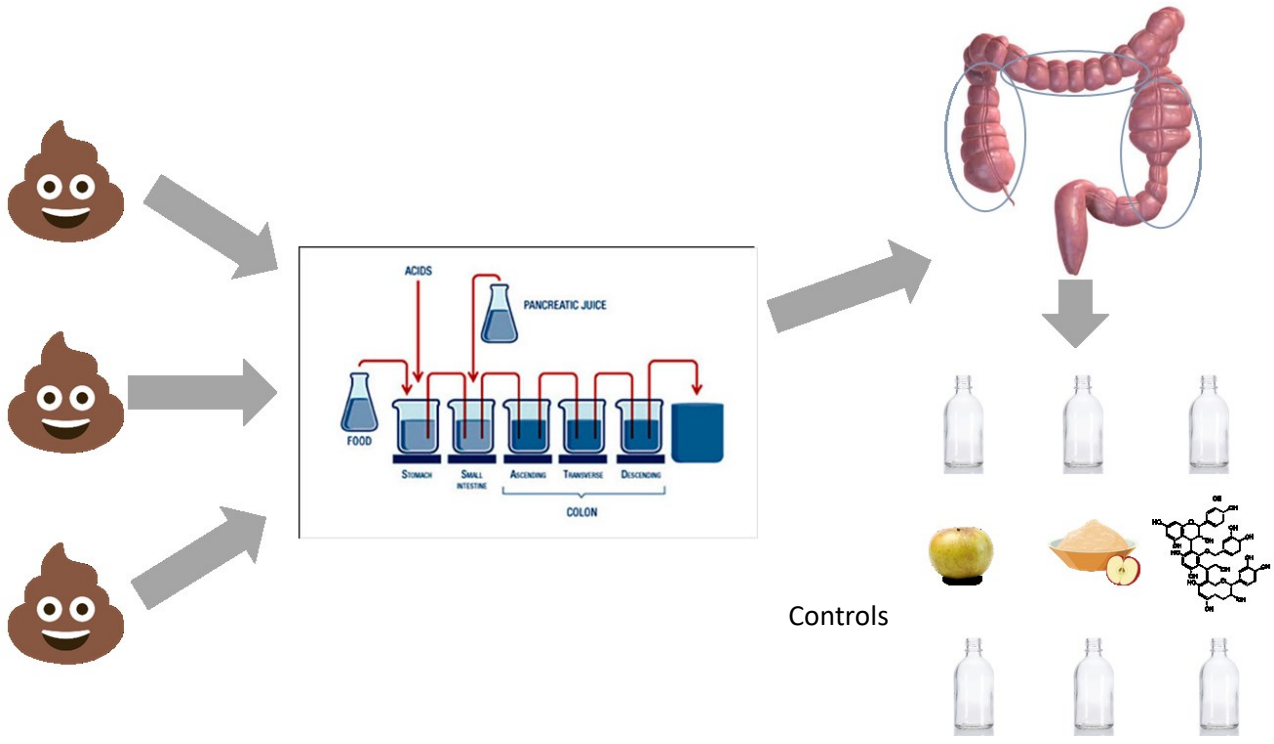



*Figure 3. Graphical abstract of the experiments*

*2.3 GC-MS analysis*

A GC–MS/MS system was used, consisting of Trace GC Ultra gas chromatograph (Thermo Fisher Scientific, San Jose, CA, USA), equipped with an autosampler PAL combi-xt autosampler (CTC, Zwingen, Switzerland) coupled to a TSQ Quantum XLS tandem mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA). A fused silica Stabilwax®-DA column (30m× 0.25mmi.d.×0.25 µm) (Restek Corporation, Bellefonte, USA) was used for the chromato-graphic separation. At the begin of the analysis, the injector and transfer line temperature were set to 250 °C, for all the samples. The surge pressure was set at 250 kPa, and the surge and the splitless time was preselected at 0.8 min. Helium (99.9995% purity) was used as carrier gas at a constant flow of 1.2 mL min−1. The oven temperature was programmed as follows: (i) initial temperature 40 °C, (ii) linearly raised at 10 °C/min to 200 °C and (iii) in the final step the temperature was ramped at 25 °C/min to 250 °C, kept up to 4 min (total run-time of22 min). The MS detection operated on full-scan mode (EI at 70 eV, ion source temperature at 250 °C, m/z values ranged from 40 to 300 Da and acquisition scan time 0.2 s) and multiple reaction monitoring (MRM) acquisition mode (Argon collision gas pressure of1.2 mTorr, a scan time 50 ms for each transition and time window of 1 min). The GC–MS data processing was performed using a qualitative and quantitative software package, XCALIBUR™ 2.2 software (Thermo Fisher Scientific, San Jose, CA, USA).

*2.4 LC-MS analysis*

Culture samples were centrifuged at 10.000g per 5 minutes, to separate the debris from the supernatant. Then the aliquots of samples were sonicated at 30°C for 5 minutes to destroy microbial cells and collect the cellular material. Samples were then briefly centrifugated for 2 minutes at 10.000 g to pellet the cellular walls and other debris, and the supernatant filtered with 0,22 µm cellulose filters. After preparation, samples were analyzed for the presence of phenolic compounds in native form and of the fermentation derived phenolic metabolites were carried out according to Dall'Asta et al [21]. According to this protocol a Waters 2695 Alliance separation module coupled with a Micromass Quattro Micro Api mass spectrometer fitted with an electrospray interface (ESI; Waters, Milford, MA, USA) was used. Separations was achieved using an Atlantis dC18-3 mm (2.1 x 150 mm) reverse-phase column (Waters).

## 3. Results and discussion

Results from our analysis were the first set of the experiments in the context of a wider project that is still running. The main goal of this study was to evaluate fermentative performances of different microbiota isolated from fecal donors and adapted to represent different colonic microbiota. Results of GC/MS and LC/MS are then to be framed in the optic of preliminary experiments, which were completed, while the second round of experiments has already started, aimed to evaluate experimental plan and to set up the analysis parameters.

3.1 *GC/MS analysis*

Production of acetic acid in the guts has many positive effects, due to its involvement in cholesterol metabolism and the lowering effect on colonic pH. From our experiments emerged that microorganisms isolated from descending colon are able to produce acetic acid even before the consumption of apple, as it is possible to see from the presence of acetic acid at the moment of the inoculum. During fecal fermentation bacteria of descending colon start to produce acetic acid already after the first hours after the inoculum reaching 2000 µM after 12 hours of fermentation and being significantly higher than the controls. Communities from ascending and transversal colon, did not show the ability to significantly produce acetic acid after fecal fermentation.

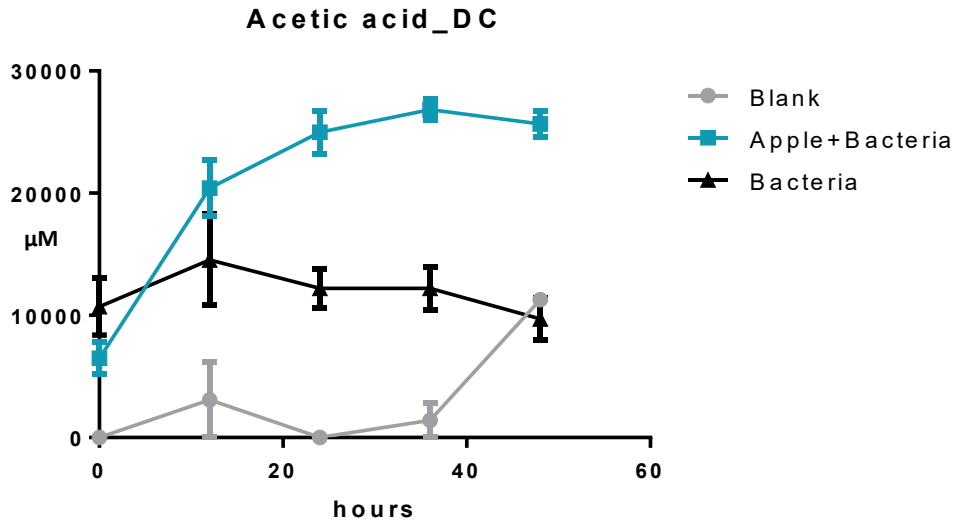



*Figure 4. Production of acetic acid by descending colon microbial communities*

| TIME 0 | Mean Diff. | Significant? | Summary | Adjusted P Value |
|---|---|---|---|---|
| Apple+Bacteria vs. Blank | 6499 | Yes | * | 0,0372 |
| Bacteria vs. Blank | 10718 | Yes | *** | 0,0009 |
| Bacteria vs. Apple+Bacteria | 4220 | No | ns | 0,1172 |
| TIME 12 | | | | |
| Apple+Bacteria vs. Blank | 17338 | Yes | **** | < 0.0001 |
| Bacteria vs. Blank | 11467 | Yes | *** | 0,0003 |
| Bacteria vs. Apple+Bacteria | -5871 | Yes | * | 0,0323 |
| TIME 24 | | | | |
| Apple+Bacteria vs. Blank | 24997 | Yes | **** | < 0.0001 |
| Bacteria vs. Blank | 12216 | Yes | **** | < 0.0001 |

| | | | | |
|---|---|---|---|---|
| Bacteria vs. Apple+Bacteria | -12781 | Yes | **** | < 0.0001 |
| TIME 36 | | | | |
| Apple+Bacteria vs. Blank | 25433 | Yes | **** | < 0.0001 |
| Bacteria vs. Blank | 10804 | Yes | *** | 0,0003 |
| Bacteria vs. Apple+Bacteria | -14629 | Yes | **** | < 0.0001 |
| TIME 48 | | | | |
| Apple+Bacteria vs. Blank | 14384 | Yes | **** | < 0.0001 |
| Bacteria vs. Blank | -1564 | No | ns | 0,5543 |
| Bacteria vs. Apple+Bacteria | -15948 | Yes | **** | < 0.0001 |

*Table 6. Significativity of production of acetic acid in descending colon*

Propionic acid seems to be more widely produced during intestinal transit since it is produced both in transversal and descending colon tracts. Production of propionic acid start in the transversal colon as it is possible to see in figure 3. After 36 hours of fecal fermentations the production of propionic acid increases significantly (Tab. 7), reaching about 1000 µM, remaining stable at 48 hours of fermentation. These data are encouraging and could be relevant since propionic acid is linked to homeostasis and support gut's health. Nevertheless, is important to notice that this production happens after 36 hours of fermentation in transversal colon, it has to be determined the real time of food and relative compounds stay in that tract and if the microorganisms are actually able to produce this SCFA.

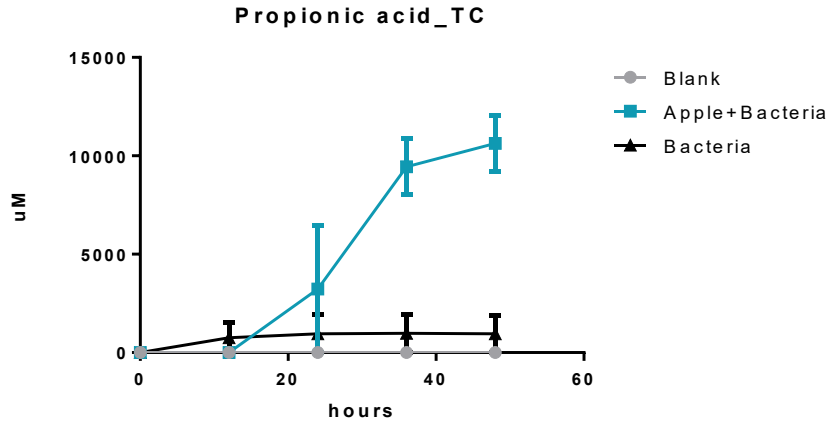



*Figure 5. Production of propionic acid by transversal colon microbial communities*

| TIME 0 | Mean Diff. | 95% CI of diff. | Significant? | Summary | Adjusted P Value |
|---|---|---|---|---|---|
| Apple+Bacteria vs. Blank | 0 | -3796 to 3796 | No | ns | > 0.9999 |
| Bacteria vs. Blank | 0 | -3796 to 3796 | No | ns | > 0.9999 |
| Bacteria vs. Apple+Bacteria | 0 | -3796 to 3796 | No | ns | > 0.9999 |
| TIME 12 | | | | | |
| Apple+Bacteria vs. Blank | 0 | -3796 to 3796 | No | ns | > 0.9999 |
| Bacteria vs. Blank | 758,7 | -3037 to 4554 | No | ns | 0,8753 |
| Bacteria vs. Apple+Bacteria | 758,7 | -3037 to 4554 | No | ns | 0,8753 |
| TIME 24 | | | | | |
| Apple+Bacteria vs. Blank | 3226 | -569.8 to 7021 | No | ns | 0,1079 |
| Bacteria vs. Blank | 956,7 | -2839 to 4752 | No | ns | 0,8096 |
| Bacteria vs. Apple+Bacteria | -2269 | -6065 to 1526 | No | ns | 0,3175 |
| TIME 36 | | | | | |
| Apple+Bacteria vs. Blank | 9448 | 5652 to 13244 | Yes | **** | < 0.0001 |
| Bacteria vs. Blank | 977 | -2819 to 4773 | No | ns | 0,8024 |
| Bacteria vs. Apple+Bacteria | -8471 | -12267 to -4676 | Yes | **** | < 0.0001 |
| TIME 48 | | | | | |
| Apple+Bacteria vs. Blank | 10630 | 6834 to 14425 | Yes | **** | < 0.0001 |
| Bacteria vs. Blank | 955,6 | -2840 to 4751 | No | ns | 0,81 |
| Bacteria vs. Apple+Bacteria | -9674 | -13470 to -5878 | Yes | **** | < 0.0001 |

*Table 7. Significativity of production of propionic acid in transversal colon*

In descending colon production of propionic acid proceeds, even if in this case the production of the compound is fully responsibility of descending colon communities, since the fermentation was static, and the fermentation bottles were not communicating. In this case we can notice that the production of propionate starts earlier, since already at 24 hour of fermentation the amount of this

SCFA is significantly higher with respect to controls. The production of propionate after 24 hours remains stable showing only negligible increase and reaching at the end the same amount reached in the transversal tract (Tab. 8; Fig. 4).

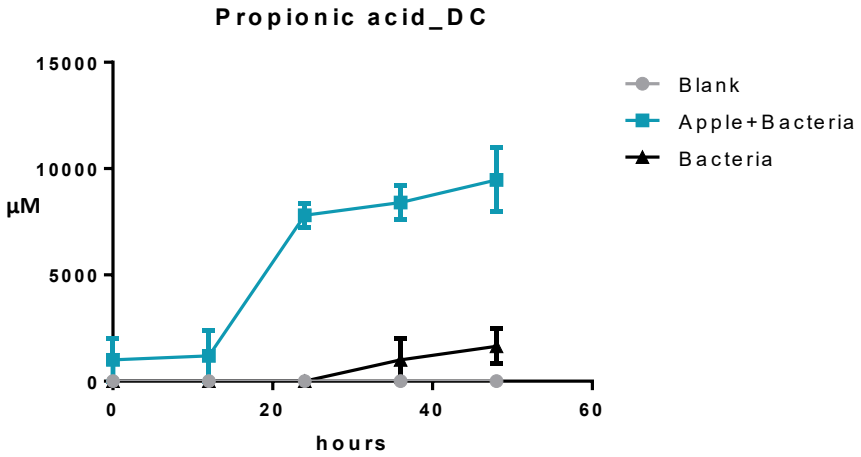



*Figura 6. Production of propionic acid by descending colon microbial communities*

| TIME 0 | Mean Diff. | 95% CI of diff. | Significant? | Summary | Adjusted P Value |
|---|---|---|---|---|---|
| Apple+Bacteria vs. Blank | 1011 | -1440 to 3463 | No | ns | 0,5719 |
| Bacteria vs. Blank | 0 | -2451 to 2451 | No | ns | > 0.9999 |
| Bacteria vs. Apple+Bacteria | -1011 | -3463 to 1440 | No | ns | 0,5719 |
| TIME 12 | | | | | |
| Apple+Bacteria vs. Blank | 1199 | -1253 to 3650 | No | ns | 0,4592 |
| Bacteria vs. Blank | 0 | -2451 to 2451 | No | ns | > 0.9999 |
| Bacteria vs. Apple+Bacteria | -1199 | -3650 to 1253 | No | ns | 0,4592 |
| TIME 24 | | | | | |
| Apple+Bacteria vs. Blank | 7803 | 5352 to 10254 | Yes | **** | < 0.0001 |
| Bacteria vs. Blank | 0 | -2451 to 2451 | No | ns | > 0.9999 |
| Bacteria vs. Apple+Bacteria | -7803 | -10254 to -5352 | Yes | **** | < 0.0001 |
| TIME 36 | | | | | |
| Apple+Bacteria vs. Blank | 8406 | 5955 to 10857 | Yes | **** | < 0.0001 |

| | | | | | |
|---|---|---|---|---|---|
| Bacteria vs. Blank | 1003 | -1448 to 3454 | No | ns | 0,5771 |
| Bacteria vs. Apple+Bacteria | -7403 | -9854 to -4952 | Yes | **** | < 0.0001 |
| TIME 48 | | | | | |
| Apple+Bacteria vs. Blank | 9471 | 7020 to 11922 | Yes | **** | < 0.0001 |
| Bacteria vs. Blank | 1644 | -807.4 to 4095 | No | ns | 0,2397 |
| Bacteria vs. Apple+Bacteria | -7827 | -10278 to -5376 | Yes | **** | < 0.0001 |

*Table 8. Significativity of production of propionic acid in descending colon*

### *3.2 LC/MS analysis*

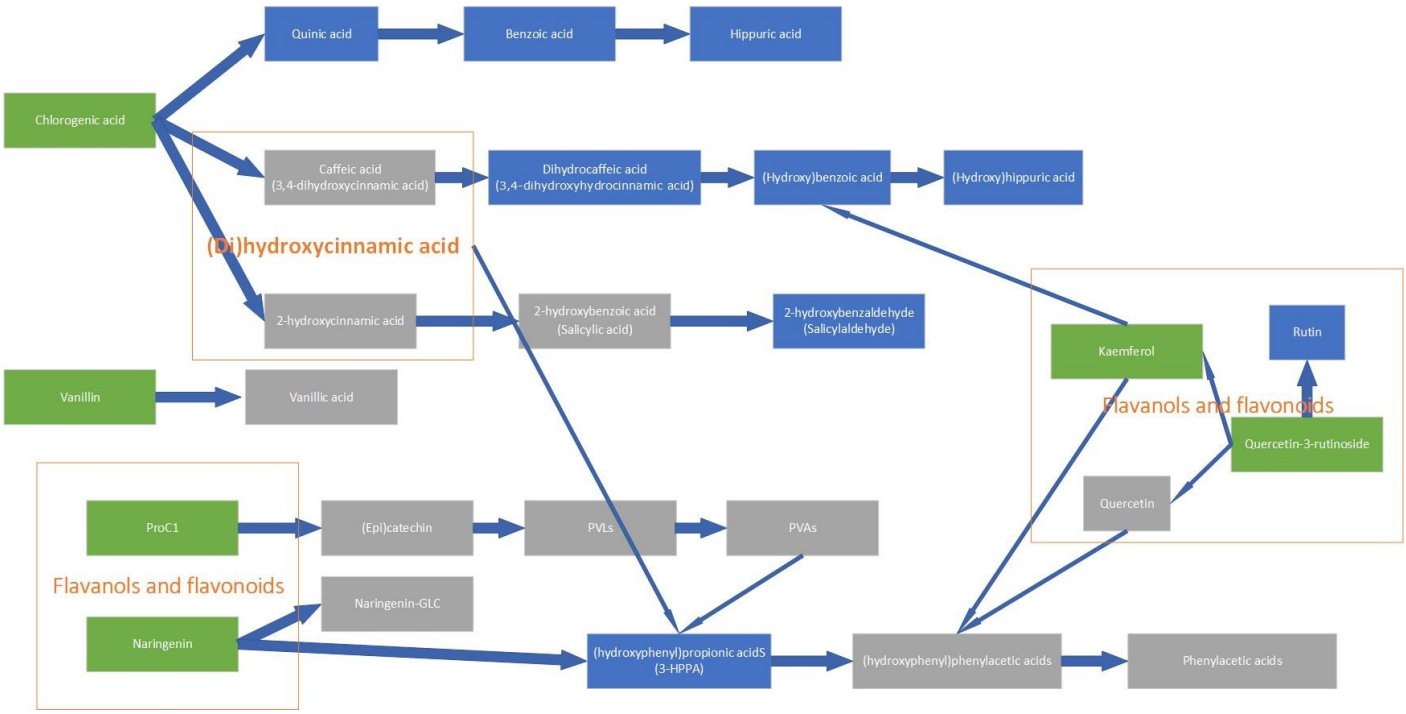



*Figura 7. Scheme of production of phenolic compounds during fecal fermentation*

In this scheme all the compounds originated by gut microbiota during fecal fermentation are presented. From preliminary analysis it was observed that there is an increased production of phenolic metabolite starting from digested apple than the production obtained by fermentation of pure procyanidin C1 and apple pectin. The most produced compounds are: cinnamic acids, benzoic acids, phenolic aldehydes, flavanols and flavonoids, and phenyl propionic acids.

#### *3.2.2 Cinnamic and benzoic acids*

Chlorogenic acid is reported to reach the colon almost untouched, since the absorption of this acid during digestion is small (≈ 30%) [22]. Even though once reached the intestine, chlorogenic acid is

transformed in its gut microbial metabolites. Metabolites that largely depend on individual gut microbiota, as well as their bioavailability and disponibility [22]. During fermentation chlorogenic acid is degraded in quinic acid, that through fermentation is metabolized again in benzoic acid being metabolized and at the end in hippuric acid for effect of microbial fermentation, all of these compounds were detected by our analysis. In figures 6 a,b,c,d are reported the amount expressed in ppm of the analysed compound and their evolution during fecal fermentation.

Chlorogenic acid (fig. 6a) is rapidly degraded by all the microbial communities, in fact in all the samples at 6 hours is completely disappeared. When the communities are supplemented with digested apples, is it possible to notice an increasing in the amount of this cinnamic acid. The trends are different for each colonic tracts, in the ascending colon after the first total disappear at 6 hours there is an increase at 12 hours of fermentations. This increasing and decreasing scheme is repeated during the time, since at 24 hours chlorogenic acid is not detected anymore, but is again present at 36 and then totally disappeared again at 48 hours. Different behaviour for chlorogenic acid in transversal colon that after the first drop at 6 hours, is produced by microorganisms and follow a constant increase until a peak at 36 hours, then is degraded, since at 48 hours is not revealed anymore. At the contrary, in descending colon, after the drop at 6 hours and an increase at 12 hours chlorogenic is not detected anymore. These results are partly in agree with what reported by Tomas-Barberan et al [22]. They report a complete degradation of chlorogenic acid at about 6 hours of fecal fermentation, and no signs of any other successive increase. Since they followed the fermentation for 24 hours, some differences are expected, even though in our samples a sensibly increase in the amount of chlorogenic acid in the samples is appreciable even at 12 hours. This difference can be due to many factors, one above all the differences between individuals, but also the diet can concur in these differences [22,23].

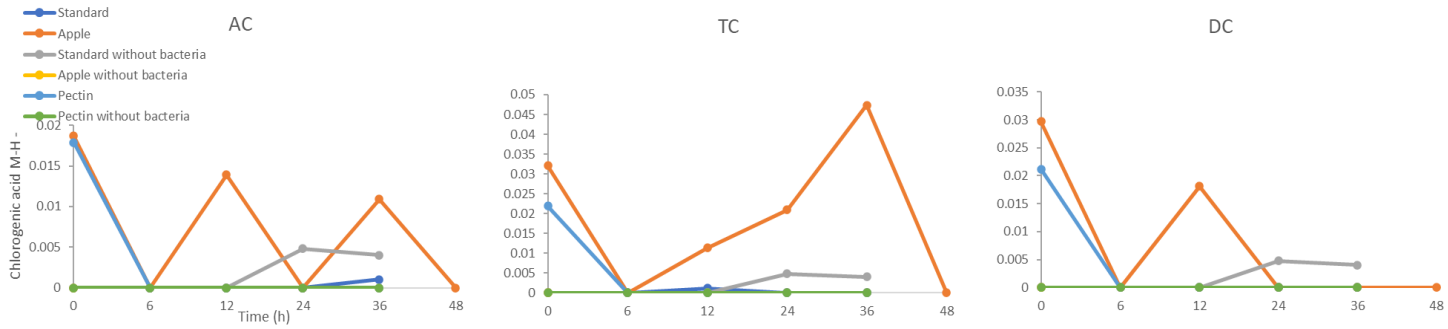
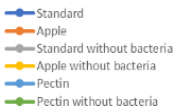



*Figure 6a. Chlorogenic acid concentration in ppm, in different intestinal tract, for different supplementations*

Quinic acid (Fig 6b) as well as cholorogenic acid was present in the digested material, but after 6 hours of fermentation is possible to notice an increase in the amount in all the colonic tracts. Since it is also a reaction product of fermentation on chlorogenic acid. Then, in the ascending colon, for samples inoculated with gut microbiota and supplemented with digested apple, quinic acid continues to decrease, until being not detected at 24 and 36 hours of fermentation, reaching then a peak at 48 hours. On the other hand, in transversal and descending colon quinic acid follows a common trend, even if the total amount in descending colon is higher. Quinic acid



reach a plateau at 6 hours of fermentation and then is degraded between 12 and 24 hours of fermentation, before to rise again at 48 hours of fermentation. Formation of quinic acid was reported by Parkar et al. [24] which observed in their experiments formation of quinic acid and caffeic acid starting from chlorogenic acid. Caffeic acid was not revealed by the analysis since it is reported to be quickly degraded to form other metabolites [25].

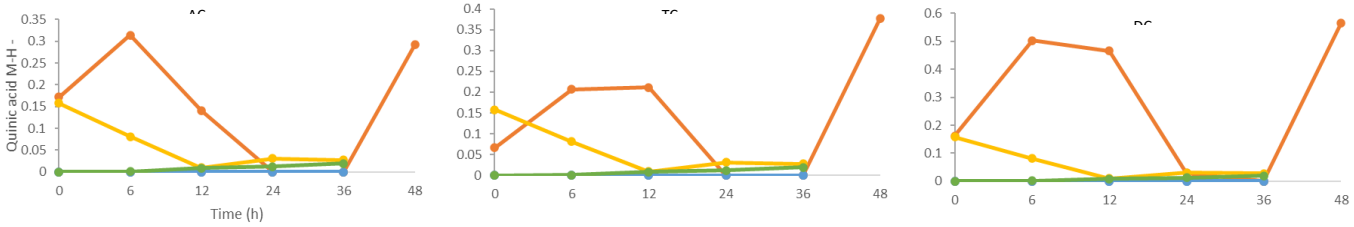



*Figure 6b. Quinic acid concentration in ppm, in different intestinal tract, for different supplementations*

Benzoic acid (fig. 6c) was not revealed in samples of digested apple, while on the contrary was present at time 0 in samples supplemented with pectin and proanthocyanidin C1, at least in transversal and descending colon. Indicating that it is produced by fermentation of anthocyanins present in the apples [26]. Despite it is not revealed in samples of digested apple at the moment of inoculum, after 12 hours of fermentation an appreciable amount of benzoic acid is produced in all the intestinal tracts. Then in ascending colon it follows an increase-decrease trend with two peaks at 12 and 36 hours and being not revealed at 24 and 48 hours of fermentation. In transversal colon benzoic acid is constantly produced until a peak at 36 hours of fermentation and it is rapidly degraded, resulting to be under the limit of detection at 48 hours. In descending colon after a small peak of production at 12 hours, benzoic acid is degraded, going under limit of detection and it is not produced anymore by this gut microbial community.

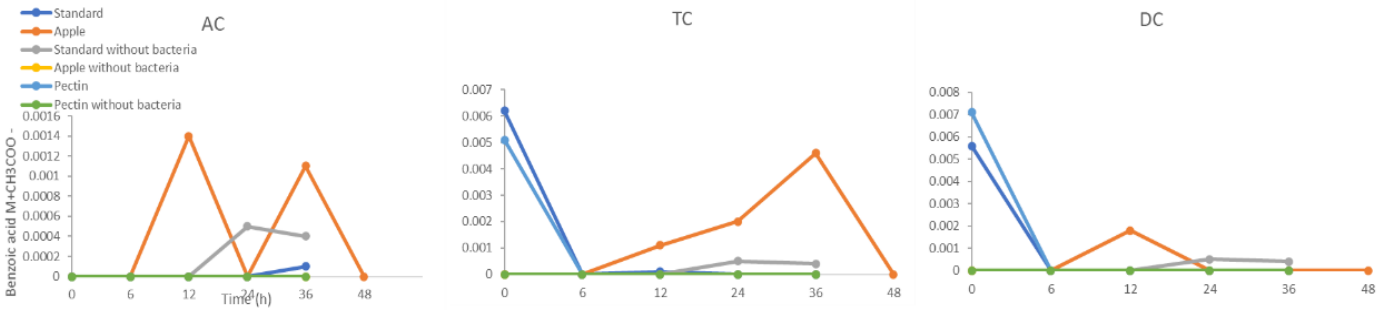



*Figure 6c. Benzoic acid concentration in ppm, in different intestinal tract, for different supplementations*

Hippuric acid (fig. 6d) was present at T0 in almost all the samples, except for the standards, where this metabolite is produced even without the help of bacteria [27]. On the other hand, in pectin without bacteria the amount remains stable all through the considered time. When digested apple is fed to ascending colon communities it remains stable untile 12 hours of fermentation, then is

degraded and the amount resulted not detectable, until the 48 hours when this acid is accumulated again and reached the same amount as 24 hours of fermentation. In transversal and descending colon hippuric acid is rapidly degraded and not detectable already at 6 hours of fermentation, remaining undetected for all the fermentations time. Exception for time 48 in descending colon where it reaches peak, similar to the starting point.

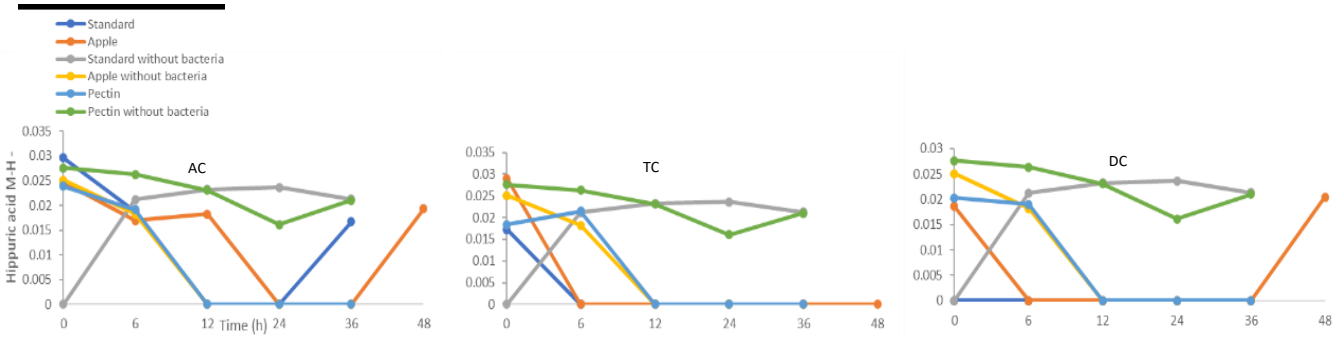



*Figure 6d. Hippuric acid concentration in ppm, in different intestinal tract, for different supplementations*

## 4. Conclusions

Introduction in the diet of food rich in phenolic compounds and fibers has proved its beneficial effects on host' health status, since the production of gut microbial metabolites can lead to formation of SCFA and small phenolic compounds. Both compounds can follow then many pathways to be absorbed by the human organism, but they can also be used by the gut microbiota as an energy source and to modify the environment creating a selective pressure and ensuring a competitive advantage on harmful microorganisms. The aim of this work was to evaluate the advantages and disadvantages of using two methods for the study of fecal fermentation carried out by different microbiota. Moreover, the keeping of cellular vitality of gut microbiota isolated from different was evaluated by measuring gut microbial metabolites all long the fermentation. In this optic this work, albeit preliminary, shows good results and can be used as a promising starting point for further studies of interaction between different intestinal microbiota and food component having strong link with healthy metabolites.

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# About the author

Vincenzo Castellone is born 27th November, in Porretta Terme near Bologna, in Italy. In 2009, was accepted to bachelor degree in Food technologies, University of Bologna. After the bachelor degree he achieved the MSc degrees with an abroad thesis carried at Lund University, Sweden. After the master degree he worked in food microbiology laboratory of University of Bologna for three years and in 2018 he passed the selection for the Doctoral School in Food Sciences at the University of Parma, under the supervision of professor Benedetta Bottari. During his Ph.D he dealth with different aspect of LAB fermented foods, with a particular focus on bioactive compounds produced during fermentation. As a part of his Ph.D he also carried out an abroad period of 6 months where he managed to perform fecal fermentations.

During the Ph.D experience Vincenzo also develop a personal interest in probiotic science and positive aspects of fermented foods. During this time time science communication and dissertation became one of his personal interest. In this thesis are reported results achieved during all the three years.

## Original papers

Probiotics and Covid-19. Bottari B., Castellone V., Neviani E. (2020) Internationa Journal of Food Science and Nutrition

Wild *Lactobacillus casei* group strains: potentiality to ferment plant derived juices. Elena Bancalari, Vincenzo Castellone, Benedetta Bottari and Monica Gatti. Foods 2020, 9, 314

ATP Bioluminescence for Rapid and Selective Detection of Bacteria and Yeasts in Wine. Saverio Monica, Elena Bancalari, Vincenzo Castellone, Jos Rijkx, Sarah Wirth, Anika Jahns and Benedetta Bottari

Eating fermented: health benefits of LAB- fermented foods
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## Communications

PACs and Gut microbiota interactions: Location and production of SCFA and PAC related metabolites. *1st telematic Workshop on the Developments in the Italian PhD Research on Food Science, Technology & Biotechnology*

## Poster presentations

Bioactive peptides in Parmigiano Reggiano cheese: time, microorganisms and gastro-intestinal digestion shaping a treasure. *IDF International Cheese Science and Technology Symposium*, June 7-11, 2021.

Wild lactic acid bacteria fermentation of plant food materials to produce functional ice cream. *5th International conference on microbial diversity 2019*, Catania, Italy, September 25-27, 2019

Bioactive compounds from lactic acid bacteria: beneficial effects of fermented foods. 24th *Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology* (University of Florence, Florence, September 11th – 13th, 2019).

Utilization of EPS from Lactic Acid Bacteria (LAB): a study on biodiversity. *Microbioss- Scuola per la conservazione e la caratterizzazione della biodiversità microbica d'interesse agroalimentare ed ambientale,* 8-11 maggio 2019, Sassari

## Partecipation to PhD school and workshops

24th Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology (University of Florence, Florence, Italy, September 11th – 13th, 2019)

*1st* Telematic Workshop on the Developments in the Italian PhD Research on Food Science, Technology & Biotechnology, September, 14th-15nd, 2021

*Microbioss- Scuola per la conservazione e la caratterizzazione della biodiversità microbica d'interesse agroalimentare ed ambientale,* 8-11 maggio 2019, Sassari


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