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PhD thesis

Adhesion properties to human cell lines and other features of probiotic interest in *Lactobacillus rhamnosus* and *Akkermansia muciniphila* strains

Coordinator of the PhD Course: Prof. Giuseppe Maiorano
Supervisor: Prof. Elena Sorrentino
Co-Supervisor: Prof. Mariantonietta Succi

PhD Student: Autilia Cozzolino
Matr:155927

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Summary

Abstract

In recent years, the market for probiotics has evolved rapidly, thanks mainly to more interest that consumers give to those foods that are marketed primarily for their health benefits. Although many potential benefits of probiotics on human health have been hypothesized, only for few strains there is scientific evidence that demonstrate their beneficial effect. For this reason, it is necessary intensive research aimed at investigating new probiotic strains, their mechanisms of action, and their health properties. The aim of this study is to investigate new potential probiotic strains and to test criteria for their screening.

Eight *Lactobacillus rhamnosus* strains of different origin (infant faeces or cheese), and *Akkermansia muciniphila* of human origin, were tested in this study.

In the first step of this research, the interactions between lactobacilli and prebiotics were investigated, considering eight different strains belonging to *Lb. rhamnosus* and different prebiotics often found in commercial symbiotic products, such as FOS, lactulose, inulin, mannitol, and sorbitol. The survival and the growth kinetic parameters of *Lb. rhamnosus* strains cultivated in presence of the different prebiotics as a unique carbon source were evaluated. Moreover, the influence of pre-cultivation in the presence of different prebiotics on the cell surface properties of strains (hydrophobicity, auto-aggregation, co-aggregation) was estimated. Results showed that the conbination of some prebiotics with specific strains can be a stress factor that significantly affects the growth of these strains. In detail, most of the prebiotics used as unique carbon source caused a growth delay compared with glucose, as evidenced by increased values of the lag phase and decreased values of the μmax. Moreover, the results showed significant differences in the cell surface properties (hydrophobicity, auto- and co-aggregation) in the different combinations of strain and prebiotics. Therefore when formulating combinations of prebiotics with probiotics, growth should not be the only parameter taken in to account, since cell surface properties essential to the probiotic function of the strain are also affected.

The second part of the research assessed the safety and some probiotic characteristics of the *Lb. rhamnosus* and *A. muciniphila* strains for use in humans. Therefore, it was important to test whether the strains could survive in the acidic environment of the stomach, multiply in the intestine even in the presence of bile salts, and be able to

adhere to intestinal cells. Therefore, experiments were performed to evaluate the survival at the GI transit, and the adhesion features, such as auto-aggregation, hydrophobicity and adhesion to human cell lines of the tested strains. Another objective of this step of the study was to evaluate whether aggregation capacity and hydrophobicity can be used as a preliminary and rapid screening of the adhesion properties of strains.

The results of survival to simulated GI transit evidenced significant differences in the responses of the strains to the stresses encountered. In addition, most *Lb. rhamnosus* strains have shown a greater sensitivity to exposure to low pH and bile salts, compared to *A. muciniphila*. Furthermore, this study demonstrates that the exposure to the low pH of the stomach inhibits the survival of *Lb. rhamnosus* strains to bile-salt induced stress.

The results of the adhesion tests to Caco-2, HT29, and MiaPaca-2 cell lines have shown that in the strains tested adhesion is a strain-specific trait, little or not at all influenced by the isolation matrix. The intensity of adhesion to the Caco-2 cell line was the highest using an inoculum of up to 10^5 CFU/mL in *Lb. rhamnosus* strains. While a similar adhesion was observed for *A. muciniphila* in all cell lines.

The results obtained indicate, with sufficient degree of reliability, that the characteristics of hydrophobicity and auto-aggregation of the strains do not correlate to their ability to adhere to intestinal tissues.

Riassunto

Negli ultimi anni, il mercato dei probiotici si è evoluto rapidamente, grazie al crescente interesse dei consumatori nei confronti dei prodotti funzionali. Sebbene siano molti i benefici dei probiotici sulla salute umana ad oggi ipotizzati, solo per pochi ceppi l'effetto benefico è stato dimostrato attraverso studi *in vivo*. Il presente lavoro, inserendosi nel complesso filone della probiosi, ha inteso individuare nuovi ceppi potenzialmente probiotici definendone anche nuovi criteri di selezione.

A tal proposito sono stati individuati 8 ceppi di diversa origine appartenenti a *Lactobacillus rhamnosus* ed un ceppo di *Akkermansia muciniphila* di origine umana.

Nelle prime fasi della ricerca, sono state studiate le interazioni tra lattobacilli e differenti sostanze prebiotiche spesso impiegate in prodotti simbiotici commerciali, quali FOS, lattulosio, inulina, mannitolo e sorbitolo. In particolare è stata valutata la capacità di crescita dei lattobacilli e l'influenza della pre-coltivazione con i diversi prebiotici sulle caratteristiche della superficie cellulare dei ceppi (idrofobicità, auto-aggregazione, co-aggregazione). I risultati hanno mostrato che le combinazioni lattobacillo-prebiotico influenzano significativamente il comportamento dei ceppi condizionandone la capacità di sviluppo, le proprietà della superficie cellulare nonché l'attività antagonista. Pertanto, si evince in maniera chiara che nella progettazione di un prodotto simbiotico occorre indagare il rapporto tra il ceppo di interesse salutistico e la sostanza prebiotica valutando non solo la capacità di sviluppo ma anche l'espressione dei principali caratteri probiotici.

Nel successivo periodo di dottorato l'attenzione è stata rivolta alla valutazione di alcuni caratteri funzionali dei ceppi di *Lb. rhamnosus*, confrontandoli con quelli esibiti dal ceppo tipo di *A. muciniphila*, ritenuto un probiotico emergente.

I benefici dei probiotici sono strettamente connessi all'abilità di questi batteri di superare le condizioni avverse incontrate nel tratto gastro-intestinale e di permanere nell'intestino, che rappresenta il sito target dove esplicano i loro effetti positivi. Pertanto, sono stati condotti esperimenti per valutare la sopravvivenza al transito GI e le caratteristiche di adesione, come l'auto-aggregazione, l'idrofobicità e l'adesione a linee cellulari umane. Inoltre, è stata valuta la correlazione tra le proprietà della superficie cellulare (auto-aggregazione e idrofobicità) e l'adesione a linee cellulari. Dall'analisi dei dati ottenuti è stato riscontrato che i ceppi esibiscono una differente sensibilità alle condizioni di stress simulato nel passaggio gastrointestinale. La maggior parte dei ceppi

di *Lb. rhamnosus* ha mostrato una maggiore sensibilità agli stress rispetto al ceppo riferibile a *A. muciniphila*. Inoltre, è emerso che la sopravvivenza dei ceppi di *Lb. rhamnosus* è fortemente inibita in presenza di sali biliari come conseguenza del danno determinato dalla precedente esposizione a pH bassi.

I risultati dei test di adesione a linee cellulari Caco2, HT29 e MiaPaca2 hanno evidenziato che tutti i ceppi saggiati hanno un comportamento ceppo-specifico, poco o per nulla influenzato dalla matrice di isolamento.

Per quanto riguarda l'intensità di adesione, i ceppi di *Lb. rhamnosus* hanno mostrato una percentuale di adesione più elevata alle cariche microbiche comprese tra 3 e 5 log CFU/mL solo nel caso della linea cellulare Caco-2, mentre *A. muciniphila* ha evidenziato il medesimo comportamento su tutte le linee cellulari utilizzate.

Infine, è importante sottolineare che i risultati ottenuti indicano, con sufficiente grado di affidabilità, che le caratteristiche di idrofobicità e auto-aggregazione dei ceppi non consentono di presagire la loro capacità di aderire ai tessuti intestinali.

Chapter 1

1.1 The microbiota

The famous statement by Hippocrates “all disease begins in the gut” not only seems to be supported by many evidences but is becoming increasingly important nowadays. With these words the father of the modern medicine suggested the essential role played by gut and diet in many vital homeostatic functions of the human body (Savel & Munro, 2014).

In the recent decade, our understanding of the role of the human gut microbiome has been revolutionized by enormous advances in investigative methods (especially high throughput DNA sequencing). Based on this knowledge, the gut microbiome is concerned as the most densely populated and diverse microbial consortium (Konturek *et al.*, 2015).

The human microbiota is a dynamic ecosystem established after birth and composed by all the microorganisms living on the human body surface or inside our body in naturally symbiotic relationship within it. (van Best *et al.*, 2015).

The intestinal microbiota has the highest microbial diversity of the human body, with more than 1000 different bacterial species mainly belonging to *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, and *Proteobacteria*, these four microbial *phyla* account for 98% of the intestinal microbiota (D'Argenio & Salvatore, 2015).

Microbial colonization is a process that begins at birth, when the baby encounters microorganisms from the urogenital tract of the mother. After birth, the neonatal intestine becomes rapidly colonized by maternal and environmental microorganisms and colonization continues during lactation, increasing complexity and microbial diversity (Koenig *et al.*, 2011). The microbiota composition is influenced by many factors such as: the type of diet, the body temperature, the use of drugs, the quantity of ingested food and other physiological characteristics; accordingly, it is subject to variations in relation to the changes that occur in the life of an individual.

Any portion of the gastrointestinal tract is colonized by specific bacteria that adapt to local conditions. The number of bacterial cells present in the gastrointestinal tract of a

mammal shows a continuum increasing, varying from 10^3 bacteria/g in the stomach and duodenum, to 10^4- 10^7 in jejunum and ileum, to over 10^{12} cells/g in the cecum and in the colon (Figure 1.1).

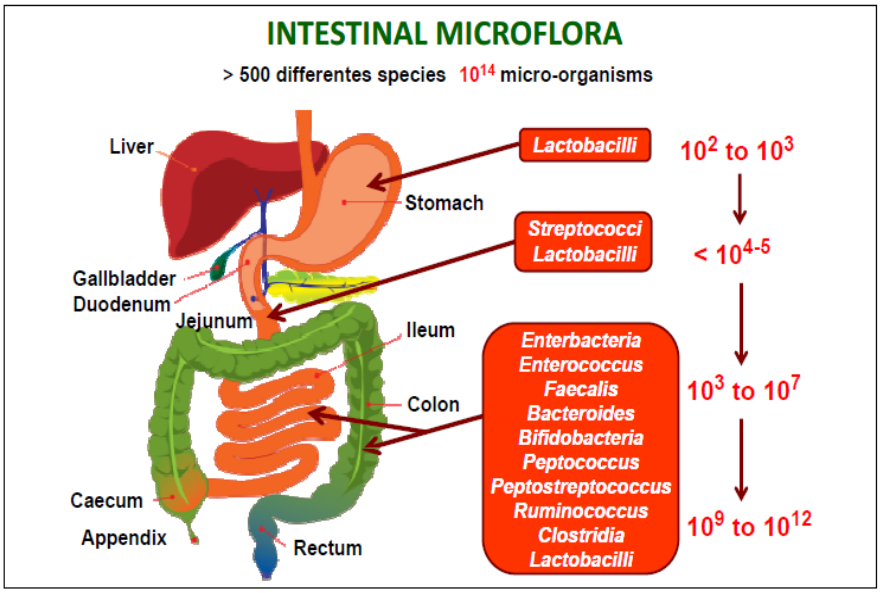

Figure 1.1 Spatial and longitudinal variations in microbial numbers and composition across the length of the gastrointestinal tract (Konturek *et al.*, 2015).

The oral cavity due to its characteristics of temperature, pH and nutrient availability is a favorable environment for the growth of many microorganisms. Bacteria are the main inhabitants of the oral cavity. In healthy adults, most species belong to the bacterial *phyla Firmicutes*, *Proteobacteria*, *Actinobacteria*, *Bacteroidetes*, and *Fusobacteria* (Human Microbiome Project Consortium 2012). In addition, archaea, protozoa, viruses, and fungi are present. Of the 700+ species of identified oral bacteria, a healthy person is colonized by 100 - 200+ microbial species (Rosier *et al.*, 2018).

Thick mucus layer, acidic gastric juice and peristaltic movement in the stomach have raised the dogma that "the stomach is a sterile organ" (Massarrat *et al.*, 2016). However, the dogma quickly changed after the discovery of *Helicobacter pylori* (Marshall & Warren, 1984). The detection of the bacterial genera such as *Streptococcus*, *Lactobacillus*, *Propionibacterium*, and *Staphylococcus* in the stomach of healthy individuals revealed that the human stomach could serve as a specialized niche

for certain microorganisms despite the occurrence of acidic pH and digestive enzymes (Delgado *et al*., 2013).

Recently, the use of culture-independent molecular technologies has revealed that five microbial genera, other than *H. pylori*, reside in the stomach: *Neisseria, Haemophilus, Prevotella, Streptococcus*, and *Porphyromonas* (Iizasa *et al*., 2015; Massarrat *et al*., 2016).

Most of the bacteria reside in the lower part of the digestive system, especially in the large intestine, also because in the most proximal tract bile and pancreatic secretions are toxic or not favorable for the growth of most microorganisms. Although there is considerable diversity in the intestinal microbiota, a few *phyla* predominate: *Bacteroidetes*, *Firmicutes*, *Actinobacteria*, *Proteobacteria*, *Verrucomicrobia*, *Fusobacteria*, and a limited number of Archaea, mainly methanogens. In addition, despite the consistency of these major phyla, their relative proportions and the species present can vary dramatically between individuals (Shortt *et al*., 2018).

These microbial populations are normally not pathogenic and play a major role in the breakdown, absorption, and metabolism of key dietary constituents, contributing, for example, to the breakdown of indigestible substances, the biosynthesis of vitamin and bioactive compounds, the degradation of potentially harmful substances and allergenic proteins (Kailasapathy & Chin, 2000; Shortt *et al*., 2018).

Microbial richness, intended as high bacterial diversity, is usually considered an indicator of a healthy status and makes the host less prone to a number of diseases. Low richness is associated with several life-style related non-communicable diseases such as obesity, metabolic syndrome, immune-related, and inflammatory diseases (Jordan *et al.*, 2015).

The number and diversity of bacterial species within an individual's gastrointestinal tract remain relatively constant throughout life, as mentioned previously, but it is possible to stimulate the proliferation of specific microorganisms with beneficial health effects by manipulating the host diet (Petschow *et al.*, 2013). More recently, groups of bacterial families have been classified into enterotypes based on their functions (Sun & Chang, 2014). This classification is based on metabolism of dietary components and ability to tolerate and metabolize drugs which should help to further understand the role of enteric microbiota in health and disease.

1.2 Development and Composition

Microbial colonization of the human gut begins at birth. The infant's intestines are sterile or contain a very low level of microbes at birth, but the GIT is quickly colonized during and after delivery. As a neonate passes through the birth canal, he or she is exposed to the microbial population of the mother's vagina. This process influences the development of an infant's intestinal microbiota, which shows similarities to the vaginal microbiota of his or her mother. Infants who were delivered through cesarean section showed reduced microbial numbers in the gut at 1 month when compared with those who were delivered vaginally, although these differences do not remain detectable at 6 months of age (Bull & Plummer, 2014). Breast milk contains living bacteria in a concentration of 10^2 to 10^4 CFU/mL, prebiotic nutrients and bioactive components, playing an important role in the establishment of the neonatal microbiota. During the first year, the intestinal tract of the baby passes by a condition of sterility to a very dense colonization (Ballard *et al.*, 2013; Fernandez *et al.*, 2013).

It is believed that the initial gut colonization is instrumental in shaping the composition of the adult's gut microbiota. This fact was demonstrated by Ley *et al*. (2005), who observed that the gut microbiota of newly born mice was closely related to that of their mothers, suggesting kinship as an important factor in determining the composition of the gut microbiota.

The infant's gut microbiota undergoes a succession of changes that are related with a shift from breast- or formula-feeding to weaning and the introduction of solid food. In fact, weaning and solid food introduction increase the diversity of the microbiota and therefore of the microbiome that functionally matures with a decrease in the relative abundance of genes involved in the degradation and utilization of lactose and an enrichment of genes involved in the degradation of other carbohydrates (Subramanian *et al.*, 2015).

At two years of age, the baby intestinal microbiota is almost stabilized. Despite the relative similarities of the gut microbiota in mothers and their offspring, microbial succession in the GIT is also influenced by numerous external and internal, host-related factors. External factors include the microbial contamination of the immediate environment, the type of food eaten, and feeding habits, in addition to the composition of the maternal microbiota. Also, dietary and temperature-related stresses can influence the succession of microbes. Internal factors include, but are not limited to, intestinal pH;

microbial interactions; body temperature; physiological factors, such as peristalsis; bile salts; host secretions and immune responses; drug therapy; and bacterial mucosal receptors (Matthew *et al.*, 2014).

1.3. Functions of the Gut Microbiota

1.3.1. Metabolism.

The microbiota collectively encodes more than 3.3 million non-redundant genes, exceeding the number encoded by the human host genome by 150-fold, consequently, it can carry out a variety of metabolic functions that humans are unable to do or are able to do only partially. Gene diversity in the microbial community provides various enzymes and biochemical pathways that are distinct from the host's own constitutive resources. Intestinal bacteria can produce a variety of vitamins, all the essential and non-essential amino acids and carry out the biotransformation of the bile (Vyas & Ranganathan, 2012). In addition, the microbiome provides the vital biochemical pathways for the fermentation of non- digestible carbohydrates that is a major source of energy in the colon. Non-digestible carbohydrates include large polysaccharides (resistant starches, cellulose, hemicellulose, pectin, and gums), some oligosaccharides that escape digestion, and unabsorbed sugars and alcohols; and host-derived mucins (Bull & Plummer, 2014). This function results in the recovery of energy and absorbable substrates for the human host and a supply of energy and nutrients for bacterial growth and proliferation (Guarner & Malagelada, 2003).

1.3.2 Host Protection and Immune-system Development

Many intestinal bacteria produce antimicrobial compounds and compete for nutrients and sites of attachment in the gut lining, thereby preventing colonization by pathogens. This action is known as the barrier or competitive-exclusion effect (Bull & Plummer, 2014).

Different mechanisms have been implicated in the barrier effect. One mechanism is, *in vitro*, nonpathogenic bacteria compete for attachment sites in the brush border of intestinal epithelial cells, preventing the attachment and subsequent entry of

enteroinvasive pathogens into the epithelial cells. Furthermore, commensal bacteria compete for nutrient availability in ecological niches and maintain their collective habitat by administering and consuming all resources (Guarner & Malagelada, 2003). Finally, bacteria can inhibit the growth of their competitors by producing antimicrobial compounds such as bacteriocins, and the ability to synthesize these substances is widely distributed among gastrointestinal bacteria (Drissi *et al*., 2015).

The intestinal epithelium is the main interface between the immune system and the external environment. The development of the host immune system is affected by continuous and dynamic interactions with the intestinal microbiota and its metabolites. Bacteria are integral to the early development of the gut-mucosal immune system, both in terms of its physical components and its function, and continue to play a role later in life in its operation. The cells of the intestinal epithelium avert threats from pathogens by signaling to the innate immune system through specific receptors that recognize and bind to specific molecules associated with bacteria, leading to the production of a host's immune response and the release of protective peptides, cytokines, and white blood cells (Vyas & Ranganathan, 2012). The result can be a protective response to commensal bacteria, an inflammatory response to pathogenic organisms, or a trigger for a host's cell death.

Exposure to intestinal bacteria is also implicated in the prevention of allergies (i.e., a disproportionate reaction of the immune system to non-harmful antigens). Allergic infants and young children have been found to have a different composition of intestinal bacteria than those who do not develop allergies. It is hypothesized that the intestinal microbiota stimulates the immune system and trains it to respond proportionately to all antigens (Bull & Plummer, 2014). An altered composition of intestinal microbiota in early life can lead to an inadequately trained immune system that can, and often does, overreact to antigens (Björkstén *et al*., 2001).

1.3.3 The Gut–Brain Axis

The gut–brain axis is a communication system that integrates neural, hormonal, and immunological signaling between the gut and the brain, offering the intestinal microbiota and its metabolites a potential route through which to access the brain (Collins *et al.*, 2012). This communication system is bidirectional, established pathways of communication include the autonomic nervous system (ANS), the enteric nervous

system (ENS), the neuroendocrine system, and the immune system (Bull & Plummer, 2014).

The communication system enables the brain to command gastrointestinal functions, such as peristalsis and mucin production, and immune functions (Mayer *et al.*, 2011).

In fact, stress has been shown to influence the integrity of the gut epithelium and to alter peristalsis, secretions, and mucin production, thereby altering the habitat of the intestinal microbiota and promoting changes in microbial composition and/or metabolism (Collins *et al.*, 2012).

In the last years, neuroscientists are taking notice of these novel reports that highlight also the 'bottom-up' influence of microbes themselves; in fact, several studies show that commensal bacteria are important to CNS function (Zhou & Foster, 2015). Different studies suggest that gut microbiota is an important player in how the body influences the brain, contribute to normal healthy homeostasis, and influence risk of disease, including anxiety and mood disorders (Clarke *et al.*,2012; Heijtz *et al.*, 2011).

1.3.4. Gut Microbiota in Disease

Associations have been established between human intestinal microbiota and a seemingly ever-increasing number of intestinal and extra-intestinal disorders. Intestinal disorders include inflammatory bowel disease, irritable bowel syndrome (IBS), and coeliac disease, while extra-intestinal disorders include allergy, asthma, metabolic syndrome, cardiovascular disease, type 2 diabetes and obesity.

In many of these conditions, the mechanisms leading to disease development involve the pivotal mutualistic relationship between the colonic microbiota, their metabolic products, and the host immune system. The establishment of a 'healthy' relationship early in life appears to be critical to maintaining intestinal homeostasis.

Based on evidence to date, we can assess the potential to positively modulate the composition of the colonic microbiota and ameliorate disease activity through bacterial intervention (Carding *et al.*, 2015).

1.3.4.1. Irritable Bowel Syndrome

Irritable bowel syndrome (IBS) is defined as a group of functional bowel disorders in which abdominal discomfort or pain is associated with defecation or a change in bowel

habits and with features of disordered defecation. The symptoms can greatly weaken patients' quality of life and account for notable economical costs for society.

IBS is thought to affect approximately 10% to 20% of adults and adolescents worldwide (Longstreth *et al.,* 2006). The exact cause of IBS is unknown and is thought to be multifactorial. Genetic factors, motor dysfunction of the GIT, visceral hypersensitivity, infection, inflammation, and immunity as well as psychopathological factors are thought to play roles in its development (Ghoshal *et al.,* 2012). Together with these factors, variation in the gut microbiota is thought to be complicit in the low-grade intestinal inflammation associated with the syndrome (Guinane & Cotter, 2013). In the healthy gut, the intestinal microbiota either have direct bactericidal effects or can prevent the adherence of pathogenic bacteria to the wall of the GIT (Kellow *et al.,* 2006).

1.3.4.2. Inflammatory Bowel Disease

Inflammatory bowel disease (IBD) is a chronic, relapsing and remitting disease, with both ulcerative colitis (UC) and Crohn's disease (CD) causing significant morbidity. The precise etiology of IBD is unclear, however, its development, progression and phenotype are multifactorial, with genetics and environment playing a role (Nagalingam & Lynch, 2012). There is increasing evidence supporting a microbial influence in the pathogenesis of IBD resulting from an inappropriate immune response towards the components of the commensal microbiota (DuPont & DuPont, 2011).

In particular, CD is characterized by a cobblestone-like pattern of inflammation (i.e., affected regions interrupted by healthy tissue), which can occur anywhere along the length of the GIT (Bull & Plummer 2014). It is also typified by ulcerations that may span the entirety of the intestinal wall, resulting in fissures that may perforate the intestinal wall and impact other organs such as the kidney or uterus (Nagalingan *et al.,* 2012).

UC typically manifests as contiguous inflammation involving only the surface layers of the intestinal wall. It is primarily localized in the colon and most commonly originates at the rectum (Baumgart & Sandborn, 2012). Given the role of the gastrointestinal microbiota in driving inflammation in IBD, treatments that manipulate the microbiota

have been investigated including the use of probiotics and prebiotics, with variable evidence for their efficacy.

1.3.4.3. Systemic Metabolic Diseases

Systemic metabolic diseases include obesity and type 2 diabetes. Both obesity and diabetes are characterized by a state of chronic low-grade inflammation with abnormal expression and production of multiple inflammatory mediators such as tumor necrosis factor and interleukins. Recent studies have shown a relationship between the composition of the intestinal microbiota and metabolic diseases like obesity and diabetes (Everard & Cani, 2013).

Early indications that the gut microbiota is involved in obesity came when metabolically obese mice, with a mutation in the leptin gene, were shown to have a significantly different microbiota compared with mice without the mutation (Ley *et al.*, 2005).

Recently, research has indicated that the risks related to the development of type 2 diabetes may also involve the composition of the intestinal microbiota. The gut microbiota of participants with type 2 diabetes displayed only a limited deviation from the non-diabetic control group, although a decline in butyrate-producing bacteria that may be metabolically beneficial was observed (Qin *et al.*, 2012). This observation suggests that a state of functional dysbiosis, rather than any specific microbial species, could have a direct association with the pathophysiology of type 2 diabetes.

1.3.4.4. Atopic Eczema and Other Allergic Disease

Allergic diseases, specifically those driven by type 1 hypersensitization—atopic eczema, atopic asthma, rhinitis—and type 1 food allergies have risen globally in incidence over the past 50 years, with the developed world now showing an incidence at 20% of the population, providing a considerable proportion of overall disease burden (Okada *et al.*, 2010). Atopic sensitization occurs primarily in the first 2 years of life and can persist through a lifetime, with the expression of allergic disease typically beginning with eczema (0-2 y), asthma (>5 y), and rhinitis (>8 y) in what is referred to as the *atopic march* (Shen *et al*., 2013)

The causes of atopic eczema are potentially numerous and are not well understood, although the method of birth and a mutation in a particular human gene involved in

skin-barrier function are known to be implicated (Williams & Grindlay, 2010). Characterization of the gut microbiota of sufferers of atopic eczema showed that infants at 1 month of age with the disease had a significantly lower microbial diversity, particularly with regard to the Bacteroidetes phylum, compared with infants without atopic eczema (Abrahamsson *et al.*, 2012).

Several epidemiological studies and experimental research suggest that microbial stimulation of the immune system influences the development of tolerance to innocuous allergens (Penders *et al.*, 2007). The gastrointestinal microbiota composition may be of particular interest, as it provides an early and major source of immune stimulation and seems to be a prerequisite for the development of oral tolerance. Although most studies indicated an association between the gut microbiota composition and atopic sensitization or symptoms, no specific harmful or protective microbes can be identified yet (Penders *et al.*, 2007). To gain more insight into the role of the gut microbiota in the etiology of atopy, large-scale prospective birth cohort studies using molecular methods to study the gut microbiota are needed.

1.4. Manipulating the gut microbiota through diet

Changes in the intestinal microbiota can occur when the diet is changed, but the healthy microbiota is resilient to changes through dietary interventions, meaning that homeostatic reactions restore the original community composition (Valdes *et al.*, 2018). Dietary amounts of protein, saturated and unsaturated fats, carbohydrates, and dietary fiber influence the abundance of different types of bacteria in the gut (Valdes *et al.*, 2018).

The microbiota can also be modified by adding live micro-organisms to food. Although, there are concerns that most microbial supplements are unable to establish themselves in the gut and fail to exert an effect on the resident community (Kristensen *et al.*, 2016; Walter *et al.*, 2018). However, probiotics can affect health independently of the gut microbiota through direct effects on the host; for example, through immune modulation or the production of bioactive compounds (Valdes *et al.*, 2018).

Emerging trends in probiotic treatment include: i) the use of new microorganisms and new formulations, which combine probiotics and prebiotics, symbiotics (Plovier *et al.*, 2017); ii) personalized approaches based on the individual characteristics of the intestinal microbiota (Chua *et al.*, 2017).

Given the current gaps in knowledge, many other clinical trials using prebiotics or probiotics or fecal microbiota transplantation are needed to assess changes in intestinal microbiota composition and health effects.

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Chapter 2

2.1 Probiotics

The word “probiotic” was coined from the Greek, “προ” plus “βιοτος” meaning literally “for life”. The term came into common use after 1980. The introduction of the concept is generally attributed to Nobel recipient Élie Metchnikoff. He suggested in 1907 that "the dependence of the intestinal microbes on the food makes it possible to adopt measures to modify the flora in our bodies and to replace the harmful microbes by useful microbes" (Metchnikoff, 1907). He observed that some rural communities in Europe, who used to drink fermented milk, had a relatively long life, and that milk fermented by lactic acid bacteria (LAB), inhibited the growth of proteolytic bacteria due to the low pH value. Since that time, probiotics have become a multibillion-dollar industry, helped by their categorization as “dietary supplements,” which are not subject to stringent evaluation by the US Food and Drug Administration (FDA).

The rationale for the use of probiotics is based on the gastrointestinal and genitourinary regulatory role played by the commensal microbiota and the need to restore the microbial ecosystem after disturbances due to disease or antibiotics. The current definition, formulated in 2002 by FAO (Food and Agriculture Organization of the United Nations) and WHO (World Health Organization) working group experts, states that probiotics are “live strains of strictly selected microorganisms which, when administered in adequate amounts, confer a health benefit on the host” (FAO & WHO, 2002). The definition was maintained by the International Scientific Association for Probiotics and Prebiotics (ISAPP) in 2014 (Hill *et al*., 2014).

To be defined as probiotics, microorganisms must fulfil specific requisites. The criteria for the selection and assessment of probiotic microorganisms are the result of the collaboration between research institutions and universities with food industries. Markowiak *et al.*, (2017), according to WHO, FAO, and EFSA suggestions, have identified the safety and functionality criteria, including the technological usefulness, of potential probiotic strains, as listed in Table 2.1

Table 2.1 Selection criteria of probiotic strains (Markowiak, *et al.*, 2017)

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

Probiotic characteristics are not associated with the genus or species of a microorganism, but with few and especially specific strains belonging to a particular species (Hill *et al.*, 2014). The safety of a probiotic strain is defined by its origin, the absence of association with pathogenic cultures, and its antibiotic resistance profile. Functional aspects define the survival of probiotic in the gastrointestinal tract and its immunomodulatory effect. Probiotic strains must meet the requirements associated with the technology of their production, which means they must be able to survive and maintain their properties throughout storage and distribution processes (Reale *et al.*, 2015). Probiotics must also have documented pro-health effects consistent with the characteristics of the strain present in a marketed product.

The strains with beneficial properties, potential probiotics, most frequently belong to the genera *Bifidobacterium* and *Lactobacillus*. However, it must be considered that the probiotic potential of different bacterial strains, even within the same species, differs. Different strains of the same species are always unique, and may have different areas of adherence (site-specific), specific immunological effects, and their action on healthy versus an inflamed mucosal milieu may be different (Soccol *et al*., 2010).

2.1.1. Mechanism of action

Probiotics have numerous advantageous functions in human organisms. Their main advantage is the effect on the development of the microbiota inhabiting the organism ensuring proper balance between pathogens and the bacteria that are necessary for the normal functions of the organism (Oelschlaeger, 2010).

The mechanism of probiotic action is not completely understood but molecular and genetic studies have determined the basic mechanisms underlying their beneficial effects, these involve four mechanisms:

- antagonism through the production of antimicrobial substances;
- competition with pathogens for adhesion to the epithelium and for nutrients;
- stimulation of immunity;
- inactivation and removal of microbial toxins.

The first mechanism is directly associated with its effect on pathogens. *Lactobacilli* and *Bifidobacteria* can produce antimicrobial compounds such as: low-molecular-weight substances (e.g., lactic acid, acetic acid, carbon dioxide, diacetyl, acetaldehyde,

hydroperoxide and short-chain fatty acids) and bacteriocins (Oelschlaeger, *et al.*, 2010). Probiotic bacteria can, also, produce so-called deconjugated bile acids which are derivatives of bile salts. Deconjugated bile acids show a stronger antimicrobial activity compared to the bile salts synthesized by the host organism. How probiotics protect themselves from these ‘‘self-made’’ metabolites or if they are resistant to deconjugated bile acids, remains to be elucidated (Bermudez-Brito *et al*., 2012).

The second mechanism is competition with pathogen microorganisms. In fact, probiotics may be able to adhere to epithelial cells, thus blocking pathogens. This mechanism exerts an important effect on the host health condition. Moreover, the adhesion of probiotic microorganisms to epithelial cells may trigger a signalling cascade, leading to immunological modulation (Markowiak *et al.*, 2017). Probiotic strains can adhere specifically or non-specifically. Specific adhesion is when an adhesion on the bacterial cell bind to a receptor on the epithelial cell, which is often defined as a lock and key function. Non-specific adhesion is a general phenomenon mediated by hydrophobic or electrostatic interaction. Non-specific adhesion may not be of significance in the colonization of epithelia in vivo, but may be important in the colonization of luminal contents. For example, non-specific adhesion may enhance substrate uptake and thus enhance growth. Because probiotic bacteria are able to adhere to epithelial cells in cell culture assays, blocking the adherence of pathogens, it is extrapolated that this is important for the probiotic effect in the host. The anti-adhesive effect could be the result of competition between the probiotic and the pathogen for the same receptor; the induction by probiotics of (increased) mucin production (competitive exclusion); or the degradation of carbohydrate receptors by secreted proteins when they establish biofilms (Oelschlaeger, 2010).

In addition to producing anti-pathogenic bioactive compounds that directly affect pathogens, and to competing with pathogens for receptor sites, and nutrients, probiotics also stimulate host anti-pathogenic defense pathways, such as stimulating or activating the pathway involved in the production of defensins that are cationic anti-microbial peptides produced in several cell types (Kerry *et al*., 2018).

The last probiotic effect is based on actions leading to inactivation of toxins and help with the removal of toxins from the body. Help in detoxification, by probiotics, can take place by adsorption (some strains can bind toxins to their cell wall and reduce the intestinal absorption of toxins), but can also result from the degradation of toxins by microorganisms (Markowiak *et al.*, 2017; McCormick, 2013).

2.1.2. *Lactobacillus* genus

The genus *Lactobacillus* is the largest group of lactic acid bacteria (LAB), to date, 182 species have been described in the genus. Taxonomically, this genus is diverse and it contains at least 12 separate phylogenetic groups.

The genus *Lactobacillus* is one of the most important taxa involved in food microbiology and human nutrition, and probably the most used as a probiotic in various foods, especially fermented meat and dairy products.

Many species belonging to this genus are essential in the production of fermented foods and are used as starter and protective cultures. This genus includes a high number of GRAS species (Generally Recognized As Safe) and with qualified presumption of safety (QPS). Furthermore, some strains of human origin are exploited as probiotics (Goh *et al.*, 2009).

Species of the genus *Lactobacillus* are non-sporeforming, catalase negative (although some strains can produce pseudocatalase), obligate saccharolytic rods or coccobacilli characterized by a low content of GC of the genome although the upper limit of the content of GC reaches 59.2 mol% (Salvetti *et al.*, 2014).

The growth temperature ranges from 2 to 53 °C, and can grow in a pH range between 3 and 8. The optimal growth temperature and pH are usually 30-40 °C and 5.5-6, 2, respectively.

They have complex nutritional requirements in terms of amino acids, peptides, vitamins, salts, fatty acids or fatty acid esters, and are found in rich habitats where substrates containing carbohydrates are available as food (dairy products, cereal products, meat and fish products , beer, wine, fruit and fruit juices, pickled vegetables, sauerkraut, ensilage and sourdough); they are part of the normal flora of the mouth, of the gastrointestinal tract and of the human genital tracts of many animals (Hammes & Hertel, 2009).

The taxonomy of lactobacilli has always been based on phenotypic properties as carbohydrate fermentation models, resistance to different NaCl concentrations, growth in different soils at defined temperatures or pH range and antibiotic resistance, extended to cell wall composition, fatty acids cell phones, isoprenoids quinones and other cell characteristics (Hammes & Hertel, 2009).

Originally, the lactobacilli were grouped according to their growth temperature and the fermentation of hexoses (Orla-Jensen., 1919) and subsequently based on their

homo/heteropherence potential (Kandler *et al.*, 1986; Carr *et al.*, 2002). The subdivision of the genus *Lactobacillus* has been revisited by Pot *et al.* (1994), but the accepted "modern" definition is that given by Hammes and Vogel (1995) and Hammes and Hertel (2009) which divides lactobacilli as obligatory homofermentative, heterogeneous optional and obligate heterofermentative, based on the type of fermented sugars and fermentation products. Homofermentative lactobacilli (commonly referred to as metabolic group A) ferment almost exclusively (> 85%) of lactic acid via the Embden-Meyerhof-Parnas (EMP) or glycolysis pathway; pentose and gluconate are not fermented. The optional heterofermentative species (metabolic group B) ferment hexose in lactic acid by EMP and can degrade pentose and gluconates through an inducible phosphoketolase, an enzyme of the pentose phosphate (PP) pathway, with consequent production of acetic acid, ethanol and acidic form under glucose limitation. Finally, the obligatory heterofermentative lactobacilli (metabolic group C) possess FDB aldolase, but not phosphoketolase, and metabolize pentose and hexoses exclusively via the phosphogluconate pathway (corresponding to the first part of the PP) and produce lactic acid, ethanol (or acetic acid) and CO_2 (Hammes & Hertel, 2009).

The description of new species in the last 20 years has led to a progressive review of the genus with the recognition of a growing number of variable phylogenetic groups (Hammes *et al.*, 2003; Felis *et al.*, 2007). Although the analysis of the 16S rRNA gene sequence contributed to the development of a more exhaustive taxonomy for lactobacilli, it became evident that there is only a small correlation between the traditional classification based on metabolic properties and phylogenetic correlation (Hammes & Hertel, 2009).

2.1.3. *Lactobacillus rhamnosus*

Lactobacillus rhamnosus is a LAB that colonizes diverse habitats, including the gastrointestinal tract, plant materials, and food (Reale *et al.*, 2015). *Lb. rhamnosus* is a Gram-positive, non-spore-forming, catalase-negative, facultative anaerobic or generally microaerophilic bacteria species. These bacteria appear in a rod form and they can form small chains of bacilli. Without any doubt, the strain GG (ATCC 53103) is the most extensively studied probiotic with several hundreds of citations and twelve significant clinical results. *Lb. rhamnosus* GG (LGG) was isolated, for the first time, from faecal samples of healthy individuals by the two researchers Sherwood Gorbach and Barry

Goldin (1989), from which it derives its name composed of the letters GG. It has been identified as a powerful probiotic because it was able to resistance to acids and bile, to optimal growth capacity and to adhere to the intestinal epithelium. For these reasons, it is one of the most studied probiotic strains and it is used in many pharmaceutical formulas or fermented milk-based preparations. Its beneficial effects have been evaluated widely in clinical trials and in human intervention studies, through which this strain was found to be effective against diarrhea and, in particular, it was able to repress the growth and development of pathogens that are present in the human intestine. For example, *Lb. rhamnosus* GG affects immunomodulation by increased expression of mucin-secreting cells and IgA that repress rotavirus development. *Lb. rhamnosus* GG is also able to decrease the risk of prolongation of diarrhea caused by *Clostridium difficile* in individuals that took antibiotics. Other *in vitro* studies of *Lb. rhamnosus* GG showed inhibition of the adhesion or infection of additional intestinal pathogens such as *Salmonella enterica* subsp. enteric serovar typhimurium, *Shigella sonei*, *Pseudomonas*, *Staphylococcus* and *Streptococcus*. Aditionally, *Lb. rhamnosus* GG showed excellent adhesion capacity *in vitro* and *in vivo* studies. It is thought that adhesion is mediated by appendages similar to fimbriae, also called "pili", these are thin protrusions of proteic nature present on the cell surface. These characteristics distinguish *Lb. rhamnosus* GG from other strains belonging to the *Lb. rhamnosus* species, from which it also differs due to its ability to persist longer and in greater concentration in the intestine.

2.2. Prebiotics

The first definition of prebiotic was given by Gibson and Roberfroid (1995) as “a non digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health”. A food ingredient must fulfil three criteria to be considered a prebiotic:

- neither be hydrolysed nor absorbed in the upper part of the gastrointestinal tract;
- be a selective substrate for one or a limited number of potentially beneficial bacteria commensal to the colon, which are stimulated to grow and/or are metabolically activated;

- consequently, be able to alter the colonic microflora towards a healthier composition, for example, by increasing the number of saccharolytic species and reducing putrefactive microorganisms such as a saccharolytic clostridia and *Enterobacteriaceae* (Salminen *et al.*, 1998; Collins *et al.*, 1999).

Amongst food ingredients, non digestible carbohydrates (oligo- and polysaccharides), some peptides and proteins, and certain lipids (both ethers and esters) are candidate prebiotics (Mitsuoka *et al.*, 2014). Because of their chemical structure, these compounds are not absorbed in the upper part of the gastrointestinal tract or hydrolyzed by human digestive enzymes. Such ingredients could be called "colonic foods", i.e. foods entering the colon and serving as substrates for endogenous colon bacteria, thus indirectly providing the host with energy, metabolic substrates and essential micronutrients. Non-digestible carbohydrates include miscellaneous compounds such as resistant starch, non-starch poly saccharides such as plant cell wall polysaccharides, hemicellulose, pectins, gums, and non-digestible oligosaccharides. However, even though they can all be classified as colonic foods, not all are prebiotics. Indeed, for most of these substances, the process of colonic fermentation is rather nonspecific. When these compounds are ingested they stimulate, in the colon, the growth and/or metabolic activity of different bacterial species, including species that are both potentially harmful and beneficial (Wang & Gibson, 1993). Consequently, they lack the necessary metabolic selectivity for one or a limited number of beneficial bacteria such as lactobacilli and bifidobacteria which is a critical criterion for classification as a prebiotic. In fact, prebiotics by definition, act on the intestinal flora and improve the balance of the flora by enhancing the growth of beneficial intestinal bacteria and/or inhibiting the growth of harmful ones. The effects of prebiotics depend on the type and concentration of the prebiotic, and on the concentration of bacteria in the intestine of the host, however no simple dose-effect relationship exists. Moreover, different prebiotics will stimulate the growth of different indigenous gut bacteria. Prebiotics have enormous potential for modifying the gut microbiota, but these modifications occur at the level of individual strains and species and are not easily predicted a priori. Furthermore, the gut environment, especially pH, plays a key role in determining the outcome of interspecies competition.

Prebiotic substances, as evidenced by in vitro and in vivo studies, are disaccharides (lactulose), oligosaccharides (such as FOS), polysaccharides (such as inulin or cellulose) and polyols (such as mannitol or sorbitol).

2.3. Hydrophobicity, auto-aggregation and co-aggregation activity

Successful probiotic bacteria must reach the intestine and adhere to the intestinal wall before they can exert their beneficial effects (de Champs *et al.*, 2003, Salminen *et al.*, 1996). The adherent ability plays an important role in colonization therefore, and consequently this property of bacteria has been considered as a potential probiotic marker along with other desirable attributes for screening of novel probiotic lactobacilli (Santarmaki *et al.*, 2017). As it is difficult to investigate bacterial adherence *in vivo*, an interest has been drawn in the development of *in vitro* models for preliminary screening of potentially adherent strains. The adhesion process involves the properties of three partners: the bacterial surface, the human cell surface, and the chemical composition of the suspending medium (Hori *et al.*, 2010). Therefore, the physical and chemical characteristics of the microbial cell surface as the hydrophobicity (MATH) measurements and auto-aggregation (Botes *et al.*, 2008) as well as adhesion to human cells capacity evaluation could give information on the ability of a strain to interact with its environment (Duary *et al.*, 2011).

Microbial cell surface hydrophobicity is without any doubt the most studied property of the cell surface with regard to their adhesion to surfaces. In fact, microbial adhesion to hydrocarbons (MATH) such as hexadecane, octane, xylene or toluene, and other hydrophobic ligands is often considered as a measure for microbial cell surface hydrophobicity (Marin *et al.*, 1997) although it has been argued several times that adhesion-based cell surface hydrophobicity assays essentially probe an interplay of all physical-chemical and structural factors involved in microbial adhesion rather than one single factor, i.e. the cell surface hydrophobicity.

The origin of microbial cell surface hydrophobicity determined by MATH-like assays involves a comparison of the organism preference for the aqueous phase and the hydrocarbon phase, similar to other assays based on adhesion to hydrophobic ligands. Therefore, organisms adhering to the hydrocarbon or any other hydrophobic ligand either do so because of their affinity for the hydrocarbon or their dislike for water.

Neglecting the role of electrostatic interactions and structural cell surface features the affinity of microorganisms for hydrocarbons is solely determined by Lifshitz–van der Waals forces due to the inability of the hydrocarbon surface to exert acid–base interactions. In contrast, the affinity of microorganisms for water is largely determined by acid–base interactions. Nearly all microbial cell surfaces are able to exert acid–base interactions, due to their predominantly electron-donating character. Bellon-Fontaine *et al.* (1996) elegantly demonstrated that acid–base interactions are essentially the origin of microbial cell surface hydrophobicity by measuring microbial adhesion to hexadecane and hexane, both unable to exert acid–base interactions, to chloroform, a slight electron-accepting organic solvent and to diethylether, a slightly electron-donating organic solvent. Electron-donating strains adhered significantly better in a MATH-like assay to chloroform than to hexadecane, due to attractive acid–base interactions, while they adhered less to diethylether than to hexane, due repulsive acid–base interactions.

Previous studies indicated a correlation between adhesion ability and hydrophobicity of some lactobacilli (Wadstroum *et al.*, 1987; Xu *et al.*, 2009). Moreover, it was reported that adhesion ability, auto-aggregation and co-aggregation of LAB strains were significantly related (Del Re *et al.*, 2000). In fact, the ability of probiotic bacteria to form cellular aggregates is considered a desirable characteristic, as they can potentially inhibit adherence of pathogenic bacteria to intestinal mucosa either by forming a barrier via self-aggregation or co-aggregation with commensal organisms on the intestinal mucosa or by direct co-aggregation with the pathogens to facilitate clearance (Bujnakova *et al.*, 2002; Schachtsiek *et al.*, 2004). In addition, studies have suggested aggregation as an important mechanism for genetic exchange, adhesion, and colonization in the host environments, as well as immunomodulation of colonic mucosa (Cesena *et al.*, 2001; Voltan *et al.*, 2007).

In general, the mechanism of cellular aggregation in probiotic lactobacilli remains unclear, although it appears that the aggregation phenomenon is a result of complex interactions among components on the cell surface, such as proteins, glycoproteins, teichoic or lipoteichoic acids, and secreted factors.

Researches had found that some lactic acid bacteria can prevent adherence of pathogenic bacteria to intestinal mucosa either by forming a barrier via auto-aggregation or by co-aggregation with the pathogens (Collado *et al.*, 2008). In fact, the probiotic cells bind pathogenic cells determining a clumping effect that limits the

interaction of pathogens with the surfaces of the host and facilitates the excretion of pathogens by biological fluids (saliva, faeces, etc). Hence, the co-aggregation properties of probiotics with pathogens are of major importance for both food preservation and therapeutic impact of food on intestinal microbiota. Probiotics may also inhibit pathogen adhesion not by interacting with the epithelial cells themselves but by forming aggregates with the pathogens in the planktonic phase, thereby preventing them from associating closely with the cell surface (Golowczyz *et al.*, 2007).

2.4. Research aim

New probiotics and prebiotics are continually required for the purpose of modulating the gut microbiota, given the importance that it has on human health. The use of prebiotics can beneficially affect the host by improving the survival and implantation of live microbial strains in the gastrointestinal tract, by selectively stimulating growth and/or by activating the metabolism of one or a limited number of health-promoting bacteria, and thus improving host welfare. Moreover, in the last years, great attention has been given to the use of certain prebiotics in order to ensure better delivery of probiotics. This approach is of great interest, since in this way prebiotics could be used for combined purposes: i) for their historical role as non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth or/and activity of one/or a limited number of bacteria in the colon; and ii) as protective agents for probiotics against various environmental stresses.

The following section of the study aimed to determine the ability of *Lb. rhamnosus* strains to grow in the presence of different prebiotics (polyols and FOS). In the first step of the research, we worked with combinations of Lactobacilli/prebiotics, considering eight different strains belonging to the *Lb. rhamnosus* species, including *Lb. rhamnosus* GG and different prebiotics often found in commercial symbiotic products, such as, lactulose, FOS (fructo-oligosaccharides), mannitol and sorbitol. Additionally, we evaluated, the effect of prebiotic substances on physicochemical properties of bacterial cell surface (auto-aggregation, co-aggregation and hydrophobicity). Furthermore, the antimicrobial activity of *Lb. rhamnosus* species in the presence of prebiotics against *E. coli* DMS 5698, was tested. Since antimicrobial ability is relevant in probiotic strains.

2.5. Materials and Methods

2.5.1. Bacterial Strains and Prebiotics

The bacterial strains used in this study are listed in Table 2.2 and include: two strains (*Lb. rhamnosus* AT194 and AT195), previously isolated from Parmigiano Reggiano cheese (Coppola *et al.*, 2005), one strain (*Lb. rhamnosus* 39) previously isolated from Caciocavallo cheese (Coppola *et al.*, 2003) and four strains (*Lb. rhamnosus* FL2, FL3, FL4 and FLC5) previously isolated from infant faeces. These strains were obtained from the Food Microbiology Culture Collection of the DiAAA (Dept. of Agricultural, Environmental and Food Science, University of Molise). In addition, they include the commercial strain *Lactobacillus rhamnosus* GG previously isolated from a pharmaceutical preparation (Valio LTD, Helsinki, Finland), as described by Succi *et al.*, (2014).

Table 2.2 Probiotic strain used in this study

Strains	Sources of isolation
Lb. rhamnosus GG	Dicoflor, Dicofarm Italy
Lb. rhamnosus FL2	Infant faeces
Lb. rhamnosus FL3	Infant faeces
Lb. rhamnosus FL4	Infant faeces
Lb. rhamnosus FLC5	Infant faeces
Lb. rhamnosus AT194	Parmesan Cheese
Lb. rhamnosus AT195	Parmesan Cheese
Lb. rhamnosus 39	Caciocavallo Cheese
E. coli DMS 5698	Origin unknown

Lb. rhamnosus strains were anaerobically cultivated in MRS broth (Oxoid Ltd., Hampshire, UK) supplemented with 0.05% cysteine-HCl at 37 °C for 24 h, conducted in a GasPak anaerobicall system with AnaeroGen (Oxoid). All strains were kept at -20°C in 30% glycerol. D-sorbitol (extra pure for microbiology), D-mannitol (≥99.0% purity) and lactulose (≥ 98.0% purity), FOS (≥99.0% purity) were purchased from

Sigma-Aldrich (St. Louis, MO, USA); each prebiotic was dissolved in sterile distilled water in order to obtain a 25% (w/v) stock solution.

Moreover, E. coli strain was cultivated in Mueller-Hinton broth (MH; Oxoid Ltd., Hampshire, UK) at 37 °C for 24 h.

2.5.2. Growth of *Lb. rhamnosus* in presence of prebiotics

Lb. rhamnosus strains grown in MRS broth at 37 °C were taken in the mid-exponential phase and centrifuged at 7.500 rpm for 15 min at 4 °C (Centrifuge Eppendorf, 5804R). The pellet was washed 2 times with 1X phosphate buffer (1X PBS), and was re-suspended to 1% in Erlenmeyer flasks containing 500 mL of sterile modified MRS (final pH 6.2) prepared following the standard formula omitting glucose and citrate. The MRS was amended with 10 g/L (final concentration) of each filter-sterilized prebiotic (Filter Unit Red 0.22-µm pore size; Schleider & Schuell, Dassel, Germany). This test was performed two twice.

1) Preliminary test: four *Lb. rhamnosus* strains were used. Two strains (*Lb. rhamnosus* AT194 and AT195), previously isolated from Parmigiano Reggiano cheese; the type strain DSM20021 that was provided by the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany); and the commercial strain *Lb. rhamnosus* GG that was previously isolated from a pharmaceutical preparation (Valio LTD, Helsinki, Finland), as described by Succi *et al.* (2014). Four prebiotics were tested: inulin, lactulose, D-sorbitol and D-mannitol (Sigma-Aldrich, Italy). The growth of strains (1% inoculum) was performed in MRS broth (control) or in modified MRS broth without glucose (MOD) and with each prebiotic as shown in Table 2.3.

Table 2.3 Growth media amended with different carbon sources used in this study.

MEDIUM	ABBREVATION
MRS broth	MRS
MRS broth without glucose	MOD
Modified MRS broth without glucose + Inulin	MOD+ I
Modified MRS broth without glucose + Lactulose	MOD+ L
Modified MRS broth without glucose + D-Sorbitol	MOD+ S
Modified MRS broth without glucose + D-Mannitol	MOD+ M

2) Second test: eight *Lb. rhamnosus* strains, shown in table 2.2, were used.
Four prebiotics were tested: fructo-oligosaccharides (FOS), lactulose, D-sorbitol and D-mannitol (Sigma-Aldrich).
The growth of strains (1% inoculum) was performed in MRS broth (control) or in modified MRS broth with glucose omitted (MOD) and added with each prebiotic as shown in the Table 2.4

Table 2.4 Growth media added of different carbon sources used in this study.

MEDIUM	ABBREVATION
MRS broth	MRS
MRS broth without glucose	MOD
Modified MRS broth without glucose + FOS	MOD+ FOS
Modified MRS broth without glucose + Lactulose	MOD+ L
Modified MRS broth without glucose + D-Sorbitol	MOD+ S
Modified MRS broth without glucose + D-Mannitol	MOD+ M

The growth was assessed by plate counts on MRS agar (Oxoid) at regular time intervals. The growth kinetic parameters were estimated with the D-model of (Baranyi & Roberts, 1994) using the excel addin DMFit v.3 (Baranyi & Le Marc, 1996). In detail, maximum specific grow rate (μmax), lag phase, initial load values (y_0) and final load values (y_end) were evaluated. Moreover, the load increase was evaluated as reported equation 2.1.

$$\Delta\_Y = y\_end - y\_0 \qquad \text{(Equation 2.1)}$$

Three independent experiments were performed and the results were reported as averages.

2.5.3 Physical-chemical properties of bacterial cell surface

2.5.3.1. Hydrophobicity assays

Determination of cell surface hydrophobicity was evaluated based on the ability of the bacteria to adhere to hydrocarbons. Microbial adhesion to hydrocarbons (MATH) assay was performed according to the procedure described by Collado *et al.* (2008) with appropriate modifications, using xylene, to simultaneously estimate the hydrophobicity and electronic-accepting and electron-donating characteristics of bacterial membranes. The *Lb. rhamnosus* strains were grown overnight at 37 °C in MRS broth, in MOD and in MOD with each prebiotic. Cultures were collected by centrifugation (8000 rpm for 10 min at 4°C) from the early logarithmic growth phase, washed twice and re-suspended in a physiological solution (PS) to an optical density of approx. 0.5 (OD 580). The test hydrocarbon (Xylene, 2mL) was added to test tubes containing 3 mL of washed cells. The tubes were vortexed for 5 min, and left to stand for 60 min for the separation of the two phases, then the OD 580 of the aqueous phase was measured using a spectrophotometer (Bio-spectrometer basic, Eppendorf, Italy). Hydrophobicity was calculated as the percentage decreases in the optical density of the initial bacterial suspension due to cells partitioning into a hydrocarbon layer. Cell surface hydrophobicity in terms of percent was calculated using the equation 2.2:

$$\% \text{ Hydrophobicity} = \left(\frac{O.D._{before\ mixing} - O.D._{after\ mixing}}{O.D._{before\ mixing}}\right) \times 100 \qquad \text{Equation } 2.2$$

where $O.D._{\text{before mixing}}$ and $O.D._{\text{after mixing}}$ are the absorbance before and after extraction with xylene, respectively.

2.5.3.2. Auto-aggregation assays

Bacteria were grown at 37 °C for 24 h in MRS broth. Four mL of bacterial suspension was placed in each tube and centrifuged at 8000 rpm for 10 min at 4 °C. The bacterial pellets were suspended in PBS to 0.5 optical density (OD) units at 580 nm to standardize the number of bacteria (10^8 CFU/mL). Auto-aggregation was measured at 5 and 24 hours, after which the OD 580 nm of 1mL of the upper suspension was measured. The percentage of auto-aggregation was calculated using the formula (Del Re *et al.*, 2000):

$$\text{Auto-aggregation } \% = 1 - \left(\frac{O.D._{\text{upper suspension}}}{O.D._{\text{total suspension}}}\right) \times 100 \qquad \text{Equation 2.3}$$

where $O.D._{\text{upper suspension}}$ and $O.D._{\text{total bacterial}}$ are the absorbances detected after 5 and 24 h and T0 respectively.

2.5.3.3. Co-aggregation assays

Bacteria were grown at 37°C for 24 h in MRS broth and in MOD whit each prebiotic. Bacterial suspensions were prepared as described for MATH. Equal volumes of cells (500 µL) of the different probiotic and pathogen strains (*E. coli* DH5α) were mixed and incubated at 37°C without agitation. Absorbance (580 nm) of the mix and of each bacterial suspension was monitored during 5 and 24 h of incubation. The percentage of co-aggregation was calculated using the formula of Handley *et al*. (1987) equation 2.4:

$$\text{Co-aggregation } \% = \frac{(\text{Apat.} + \text{Aprob.})/2 - (\text{Amix})}{(\text{Apat.} + \text{Aprob.})/2} \times 100 \qquad \text{Equation 2.4}$$

where Apat and Aprob represent the absorbances detected after 5 and 24 h of *Lb. rhamnosus* strains and *E. coli* respectively, and Amix represents the absorbance of the mixture.

2.5.4. Antimicrobial activity

The antimicrobial activity of the *Lb. rhamnosus* strains (producers) against *E. coli* DMS 5698, used as the indicator strain, was evaluated using the spot on the lawn technique (Tremonte *et al.*, 2017). In detail, Petri plates (Ø 9 cm) containing 10 mL of MRS agar or MOD agar with 1% prebiotic substances, were inoculated (by spot) with three aliquots (10 µL) of an overnight culture in MRS broth of each *Lb. rhamnosus* strain, and incubated at 37 °C for 24 h. A maximum of three strains was spotted per plate. After incubation, the plates were covered with 7 mL of MH (Muller-Hinton) soft agar (0.7% agar) and MH soft agar with 1% of the different prebiotics inoculated with the indicator strain at a final concentration of about 10^7 CFU/mL. After incubation at 37 °C for 24h, plates were checked for inhibition zones, and the presence of a distinguishable halo around the spots was considered as positive antagonistic effect. A calibrated-densitometer (GS-800, Bio-Rad, Hermles CA, USA) was used for imaging acquisition and Adobe Photoshop CS4 Extended software was used for the measurement of clearing zones. Antimicrobial activity was expressed in mm and the results are expressed as the mean ± standard deviation. Each experiment was carried out in triplicate.

2.6. Results and Discussion

2.6.1. Effect of prebiotics on the growth of *Lb. rhamnosus*

Growth kinetics of *Lb. rhamnosus* in presence of glucose or prebiotics, obtained from the preliminary test, are illustrated in Figure 2.1. The four assayed strains showed similar parameters when cultivated in presence of glucose (MRS, control condition - CC), with no significant differences ($P > 0.05$) in the maximum specific growth rate values (μmax). In CC, the lowest lag phase was appreciated for *Lb. rhamnosus* GG. Conversely, the statistical analysis highlighted that the different prebiotics affected the growth parameters of tested strains. In detail, all prebiotics caused an increase in the lag phase and a decrease in the μmax, with mannitol showing intermediate μmax values between those registered in CC and those detected with the other assayed prebiotics. Moreover, strains showed different growth capabilities when cultivated with mannitol. In fact, AT195, AT194, and GG had Δ_y values comprised in the range 4.5–5.1, that is, about 2-fold higher than that exhibited by the type strain DSM20021 (about 2.3). As far as the other prebiotics are concerned, sorbitol allowed the growth of only the strains GG and AT195, while lactulose supported the growth of only AT195. A moderate growth in the presence of inulin was detected for strain AT194 only. The results of this preliminary test are published in the article: "Pre-cultivation with selected prebiotics enhances the survival and the stress response of *Lactobacillus rhamnosus* strains in simulated gastrointestinal transit" (Succi *et al*., 2017).

Preliminary test results highlighted that the ability to grow in the presence of different prebiotics as the sole carbon source is a strain-dependent capability.

Based on this interesting conclusion, the experiment was repeated by increasing the number of *Lb. rhamnosus* tested. Furthermore, to verify whether the origin may affect the ability of the strain to use different prebiotics as a carbon source, among the *Lb. rhamnosus* strains tested in this second experiment strains isolated from infant feces were also included.

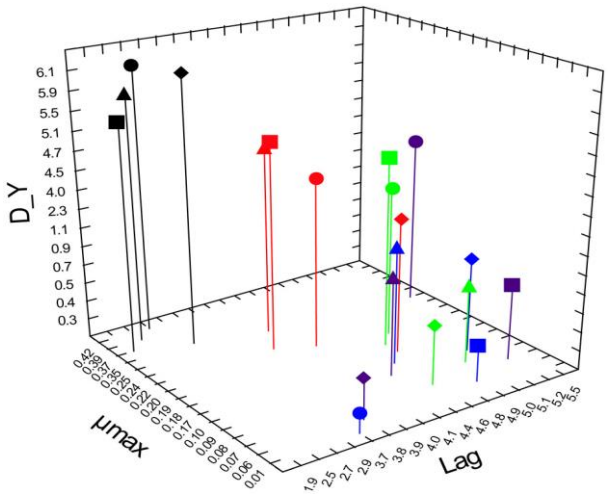

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Figure 2.1 3D Scatterplot representing growth kinetic parameters (Lag phase, Lag; maximum specific growth rate, µmax; increase in microbial load, D_Y) of four *Lb. rhamnosus* strains. AT194 (triangle), AT195 (circle), GG (square) and DSM20021 (diamond) were cultivated in presence of glucose (black), inulin (blue), lactulose (purple), mannitol (red), or sorbitol (green).

In addition, based on the results obtained in the preliminary test, inulin was not further tested, because all *Lb. rhamnosus* strains previously tested were not able to metabolize it. In the second test inulin was replaced with FOS, therefore the in the second test the prebiotics tested were FOS, lactulose, D-sorbitol and D-mannitol.

In the second test the ability to develop in the presence of different prebiotic substances by the strains GG, FL2, FL3, FL4, FLC5, AT194, AT195 and 39, belonging to the species *Lb. rhamnosus*, was studied by evaluating the growth of strains on MRS agar and subsequent modelling of the experimental points through a dynamic model of Baranyi and Roberts (1994). The growth curves are shown in Figures 2.2.a-h, while the kinetic parameters are shown in Tables 2.5.a-h.

Figure 2.2.a shows the growth curves of *Lb. rhamnosus* GG in the different cultivation substrates. From the growth curves, it is evident that the development of *Lb. rhamnosus* GG is conditioned by the carbon source present in the culture substrate. As expected, in the absence of carbon sources (MOD), the strain has shown a limited growth capacity, a similar behavior has been observed in the presence of lactulose (MOD + L). On the contrary, in the presence of mannitol (MOD + M) and FOS (MOD + FOS) as the only carbon sources, GG grew well; while in the presence of sorbitol (MOD + S) it showed

an intermediate growth capacity. However, it should be noted that the analysis of the kinetic parameters (Table 2.5.a) indicate different situation, in fact, the lag phase in the presence of mannitol (2.26 h) was longer than that detected with glucose (1.43 h), while the lag phase recorded in the presence of FOS (1.19 h) and lactulose (1.15 h) was shorter. Moreover, the maximum growth rate in mannitol was lower than that detected in the presence of glucose (MRS), which was comparable to those recorded in the presence of FOS and lactulose. So, *Lb. rhamnosus* GG, although able to use mannitol as a carbon source in the absence of glucose, requires an adaptation phase which results in a prolongation of the lag phase and a reduction of the maximum growth rate. Different results are obtained in the presence of FOS where both the lag phase and the μmax are similar to those recorded in the presence of glucose.

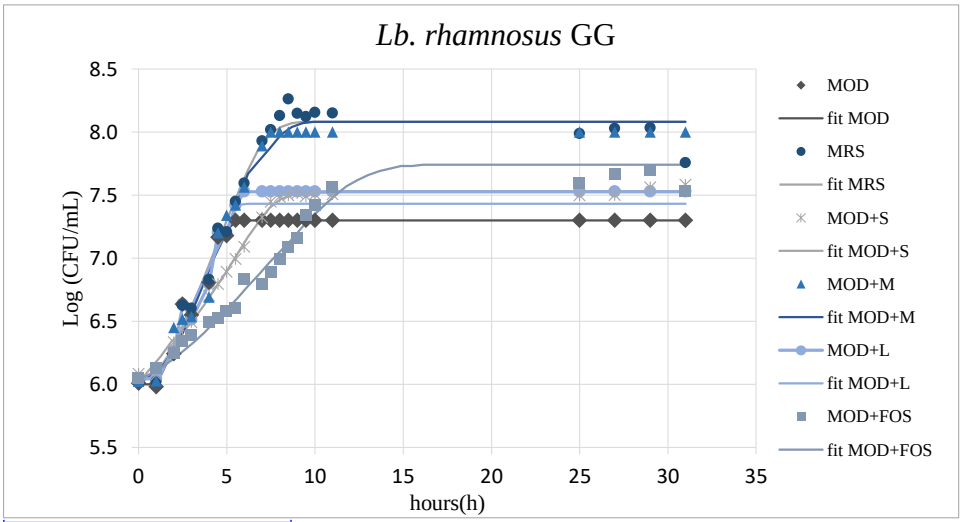

Figure 2.2.a Growth of *Lb. rhamnosus* GG in different carbon sources MOD (MRS broth without glucose), MRS broth, MOD+M (MRS broth without glucose + D-mannitol), MOD+S (MRS broth without glucose + D-sorbitol), MOD+L (MRS broth without glucose + Lactulose) and MOD+FOS (MRS broth without glucose + Fructo-oligosaccharides).

Commento [UdMO2]: Fai attenzione devi modificare i titoli di tutti i grafici

Table 2.5.a Kinetic parameters related to the growth of *Lb. rhamnosus* GG in different carbon sources MOD (MRS broth without glucose), MRS broth, MOD+M (MRS broth without glucose + D-mannitol), MOD+S (MRS broth without glucose + D-sorbitol), MOD+L (MRS broth without glucose + Lactulose) and MOD+FOS (MRS broth without glucose + Fructo-oligosaccharides**).**

Curve	y0 (LogCFU/mL)	Lag (h)	yEnd (LogCFU/mL)	µmax (h-1)	R^2	SE of FIT
Lb. rhamnosus GG MOD	6.00 ± 0.04	1.06±0.23	7.20 ± 0.15	0.31±0.02	0.98	0.06
Lb. rhamnosus GG MRS	6.01 ± 0.10	1.43±0.49	8.08 ± 0.04	0.36±0.03	0.97	0.12
Lb. rhamnosus GG MOD+M	6.33 ± 0.03	2.26±0.31	7.72 ± 0.02	0.22±0.01	0.99	0.05
Lb. rhamnosus GG MOD+S	6.27 ± 0.02	2.22±0.16	7.52 ± 0.02	0.23±0.01	1.00	0.03
Lb. rhamnosus GG MOD +Fos	6.01 ± 0.06	1.19±0.32	7.83 ± 0.02	0.32±0.02	0.99	0.07
Lb. rhamnosus GG MOD+L	6.04 ± 0.05	1.15±0.24	5.94 ± 0.17	0.31±0.02	0.99	0.07

As found for the previous strain, *Lb. rhamnosus* FL2 in the presence of glucose had an optimal development, similar behavior was found in the presence of all the prebiotics used, in fact, the final charge levels obtained are comparable to those recorded in the presence of glucose (figure 2.2.b.). From the analysis of the kinetic parameters (Table 2.5.b) it is clear that even the maximum growth rate recorded in the presence of all prebiotics had similar values to those observed in the presence of glucose. In contrast, the lag phase was different depending on the carbon source, it was longer than glucose alone (0.85 h), in the presence of mannitol (2.41 h) and FOS (1.56 h). In the presence of sorbitol, the strain FL2 had a lag phase equal to that detected in glucose while in the presence of lactulose the lag phase was shorter (0.29 h). So, *Lb. rhamnosus* FL2 can metabolize all the prebiotics tested, and only in the presence of mannitol and FOS requires an adaptation phase that results in a prolongation of the lag phase.

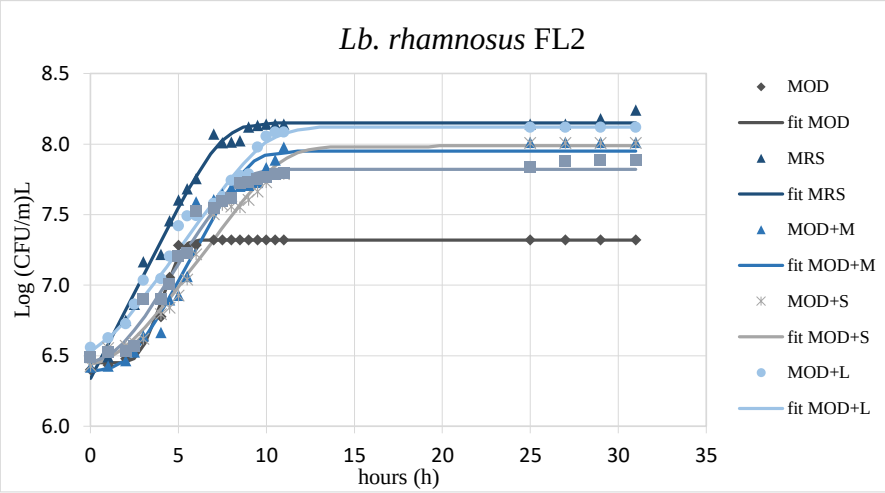

Figure 2.2.b Growth of *Lb. rhamnosus* FL2 in different carbon sources MOD (MRS broth without glucose), MRS broth, MOD+M (MRS broth without glucose + D-mannitol), MOD+S (MRS broth without glucose + D-sorbitol), MOD+L (MRS broth without glucose + Lactulose) and MOD+FOS (MRS broth without glucose + Fructo-oligosaccharides).

Table 2.5.b Kinetic parameters related to the growth of *Lb. rhamnosus* FL2 in different carbon sources MOD (MRS broth without glucose), MRS broth, MOD+M (MRS broth without glucose + D-mannitol), MOD+S (MRS broth without glucose + D-sorbitol), MOD+L (MRS broth without glucose + Lactulose) and MOD+FOS (MRS broth without glucose + Fructo-oligosaccharides**).**

Curve	y0 (LogCFU/mL)	Lag (h)	yEnd (LogCFU/mL)	µmax (h-1)	R^2	SE of FIT
Lb. rhamnosus FL2 MOD	6.45 ± 0.02	2.76±0.17	7.32 ± 0.01	0.35±0.03	0.99	0.06
Lb. rhamnosus FL2 MRS	6.44 ± 0.05	0.85±0.33	8.13 ± 0.02	0.27±0.02	0.99	0.09
Lb. rhamnosus FL2 MOD+M	6.40 ± 0.08	2.41±0.60	7.92 ± 0.05	0.24±0.03	0.97	0.09
Lb. rhamnosus FL2 MOD+S	6.34 ± 0.08	0.85±0.73	7.79 ± 0.04	0.17±0.01	0.97	0.07
Lb. rhamnosus FL2 MOD +Fos	6.45 ± 0.06	1.56±0.49	7.82 ± 0.03	0.20±0.01	0.98	0.06
Lb. rhamnosus FL2 MOD+L	6.53 ± 0.06	0.29±0.53	8.13 ± 0.03	0.16±0.01	0.99	0.06

The behavior of *Lb. rhamnosus* FL3 is shown in Figure 2.2.c, this strain showed low growth in the presence of sorbitol, while it exhibited a good ability to use mannitol, lactulose and FOS. However, from the analysis of the kinetic parameters, shown in table 2.5.c, in the presence of lactulose, the lag phase is equal to 2.22 h, a value higher than that recorded in the presence of glucose (1.76 h).

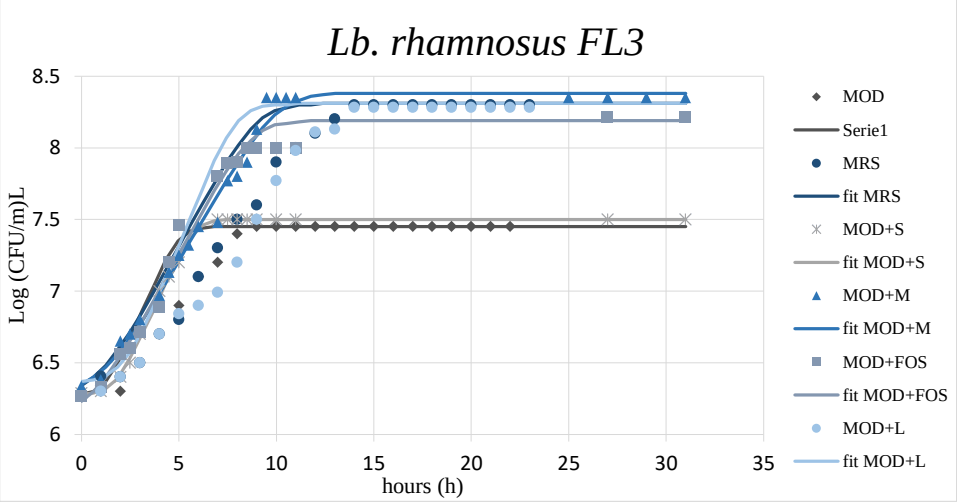

Figure 2.2.c Growth of *Lb. rhamnosus* FL3 in different carbon sources MOD (MRS broth without glucose), MRS broth, MOD+M (MRS broth without glucose + D-mannitol), MOD+S (MRS broth without glucose + D-sorbitol), MOD+L (MRS broth without glucose + Lactulose) and MOD+FOS (MRS broth without glucose + Fructo-oligosaccharides).

Table 2.5.c Kinetic parameters related to the growth of *Lb. rhamnosus* FL3 in different carbon sources MOD (MRS broth without glucose), MRS broth, MOD+M (MRS broth without glucose + D-mannitol), MOD+S (MRS broth without glucose + D-sorbitol), MOD+L (MRS broth without glucose + Lactulose) and MOD+FOS (MRS broth without glucose + Fructo-oligosaccharides**).**

curve	y0 (LogCFU/mL)	Lag (h)	yEnd (LogCFU/mL)	µmax (h-1)	R^2	SE of FIT
Lb. rhamnosus FL3 MOD	6.30 ± 0.02	1.11±0.10	7.43 ± 0.07	0.28±0.01	1.00	0.02
Lb. rhamnosus FL3 MRS	6.37 ± 0.03	1.76±0.17	8.30 ± 0.01	0.34±0.01	1.00	0.04
Lb. rhamnosus FL3 MOD+M	6.35 ± 0.06	1.33±0.43	8.38 ± 0.04	0.23±0.01	0.99	0.08
Lb. rhamnosus FL3 MOD+S	6.27 ± 0.01	1.71±0.11	7.50 ± 0.01	0.30±0.01	1.00	0.02
Lb. rhamnosus FL3 MOD +Fos	6.25 ± 0.08	1.22±0.47	8.09 ± 0.04	0.27±0.02	0.98	0.09
Lb. rhamnosus FL3 MOD+L	6.37 ± 0.05	2.22±0.30	8.31 ± 0.03	0.34±0.02	0.99	0.08

On the other hand, with the remaining carbon sources, latency times were shorter compared to glucose. Regarding the maximum growth rate, the values obtained are mostly comparable with each other. Hence, *Lb. rhamnosus* FL3 showed good growth not only in glucose but also in the presence of mannitol, lactulose and FOS, and only in the presence of lactulose an adaption phase is necessary.

The behaviour of *Lb. rhamnosus* FL4 is shown in figure 2.2.d. It has a good capacity to use lactulose whereas in the presence of mannitol and FOS the growth of this strain is very low, even lower than that detected in MOD. The kinetic parameters of FL4 (Table 2.5.d) confirm what was previously reported. *Lb. rhamnosus* FL4 can only use lactulose.

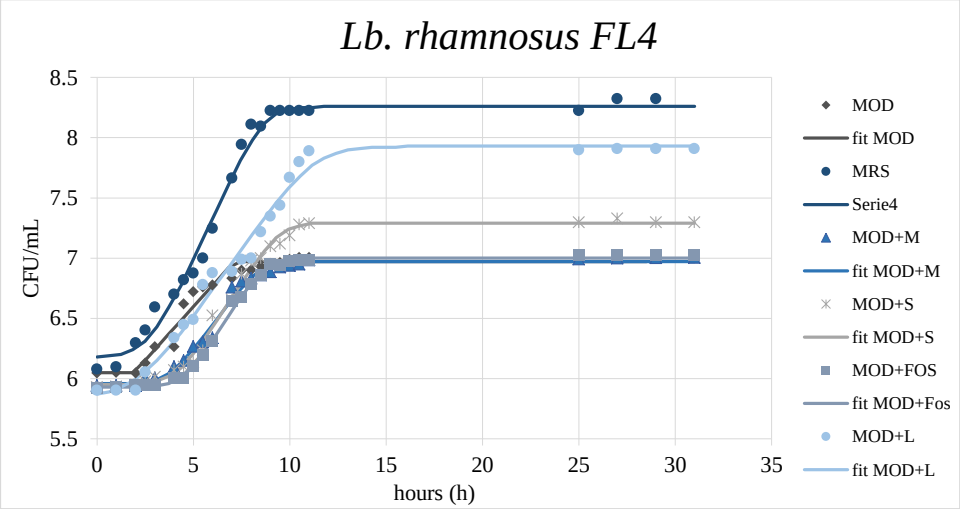

Figure 2.2.d Growth of *Lb. rhamnosus* FL4 in different carbon sources MOD (MRS broth without glucose), MRS broth, MOD+M (MRS broth without glucose + D-mannitol), MOD+S (MRS broth without glucose + D-sorbitol), MOD+L (MRS broth without glucose + Lactulose) and MOD+FOS (MRS broth without glucose + Fructo-oligosaccharides).

able 2.5.d Kinetic parameters related to the growth of *Lb. rhamnosus* FL4 in different carbon sources MOD (MRS broth without glucose), MRS broth, MOD+M (MRS broth without glucose + D-mannitol), MOD+S (MRS broth without glucose + D-sorbitol), MOD+L (MRS broth without glucose + Lactulose) and MOD+FOS (MRS broth without glucose + Fructo-oligosaccharides)

curve	**y0 (LogCFU/mL)**	**Lag (h)**	**yEnd (LogCFU/mL)**	**µmax (h-1)**	**R^2**	**SE of FIT**
*Lb.rhamnosus*FL4 MOD	6.05 ± 0.05	1.89±0.372	7.12 ± 0.29	0.18±0.01	0.96	0.07
Lb. rhamnosus FL4 MRS	6.18 ± 0.06	2.70 ± 0.32	8.26 ± 0.03	0.35±0.02	0.99	0.09
Lb. rhamnosus FL4 MOD+M	5.95 ± 0.02	3.70 ± 0.25	6.97 ± 0.02	0.21±0.01	0.99	0.05
Lb. rhamnosus FL4 MOD+S	5.94 ± 0.02	4.07 ± 0.21	7.29 ± 0.02	0.25±0.01	0.99	0.05
Lb. rhamnosus FL4 MOD +Fos	5.93 ± 0.01	4.40 ± 0.13	7.00 ± 0.01	0.24±0.01	1.00	0.03
Lb. rhamnosus FL4 MOD+L	5.87 ± 0.06	2.23 ± 0.48	7.93 ± 0.04	0.23±0.01	0.99	0.09

Lb. rhamnosus FLC5 showed a good ability to use all prebiotics (figure 2.2.e). The analysis of the kinetic parameters (Table 2.5.e) shows a similar behaviour in the presence of the various carbon sources used. *Lb. rhamnosus* FLC5 is, therefore, able to effectively use all prebiotics tested in this experiment.

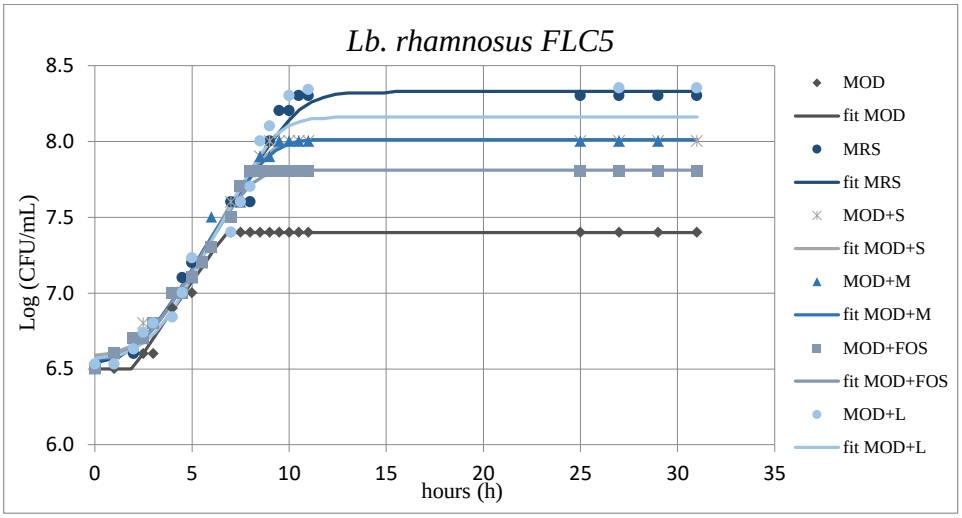

Figure 2.2.e Growth of *Lb. rhamnosus* FLC5 in different carbon sources MOD (MRS broth without glucose), MRS broth, MOD+M (MRS broth without glucose + D-mannitol), MOD+S (MRS broth without glucose + D-sorbitol), MOD+L (MRS broth without glucose + Lactulose) and MOD+FOS (MRS broth without glucose + Fructo-oligosaccharides).

Table 2.5.e. Kinetic parameters related to the growth of *Lb. rhamnosus* FLC5 in different carbon sources MOD(MRS broth without glucose), MRS broth, MOD+M (MRS broth without glucose + D-mannitol), MOD+S (MRS broth without glucose + D-sorbitol), MOD+L (MRS broth without glucose + Lactulose) and MOD+FOS (MRS broth without glucose + Fructo-oligosaccharides**).**

curve	y0 (LogCFU/mL)	Lag (h)	yEnd (LogCFU/mL)	µmax (h-1)	R^2	SE of FIT
Lb. rhamnosus FLC5 MOD	6.50 ± 0.03	1.92±0.19	6.74 ± 0.16	0.19±0.01	0.99	0.04
Lb. rhamnosus FLC5 MRS	6.54 ± 0.05	2.27±0.40	8.32 ± 0.03	0.22±0.01	0.99	0.07
Lb. rhamnosus FLC5 MOD+M	6.56 ± 0.04	2.19±0.32	8.01 ± 0.02	0.21±0.01	0.99	0.05
Lb. rhamnosus FLC5 MOD+S	6.59 ± 0.03	2.52±0.27	8.02 ± 0.02	0.22±0.01	0.99	0.05
Lb. rhamnosus FLC5 MOD +Fos	6.57 ± 0.03	2.32±0.28	7.81 ± 0.02	0.21±0.01	0.99	0.05
Lb. rhamnosus FLC5 MOD+L	6.56 ± 0.06	2.76±0.48	8.38 ± 0.06	0.23±0.02	0.98	0.09

The results related to *Lb. rhamnosus* AT194 are shown in figure 2.2.f. The analysis of the growth curves showed that its development in the presence of mannitol was similar to that observed in the presence of glucose while in the presence of other prebiotics the development of the strain was lower. The analysis of the kinetic parameters shown in

table 2.5.f. shows that the longest lag phase was recorded in the presence of lactulose, moreover, in the presence of this prebiotic the lowest μmax was also observed. Hence *Lb. rhamnosus* AT194 is able to use only mannitol effectively while its development is delayed in the presence of lactulose.

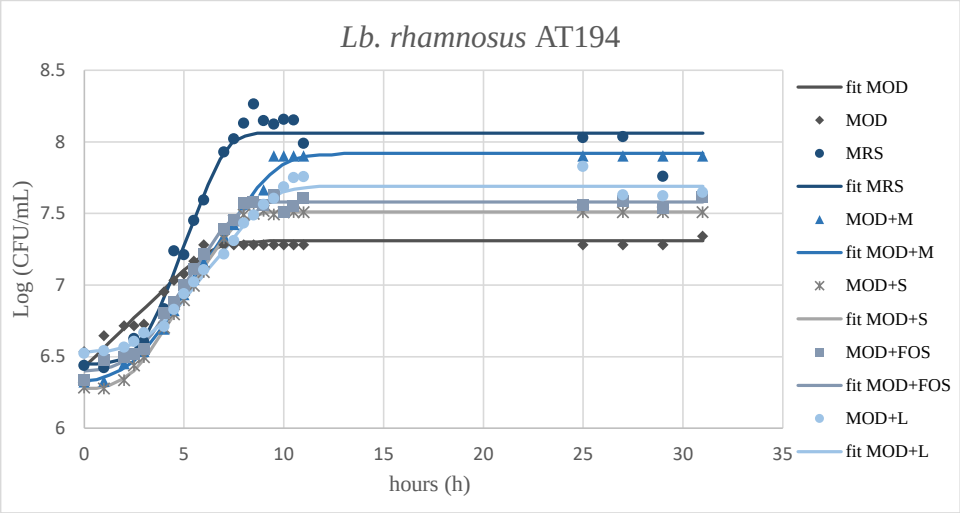

Figure 2.2.f Growth of *Lb. rhamnosus* AT194 in different carbon sources MOD(MRS broth without glucose), MRS broth, MOD+M (MRS broth without glucose + D-mannitol), MOD+S (MRS broth without glucose + D-sorbitol), MOD+L (MRS broth without glucose + Lactulose) and MOD+FOS (MRS broth without glucose + Fructooligosaccharides).

Table 2.5.f Kinetic parameters related to the growth of *Lb. rhamnosus* AT194 in different carbon sources MOD(MRS broth without glucose), MRS broth, MOD+M (MRS broth without glucose + D-mannitol), MOD+S (MRS broth without glucose + D-sorbitol), MOD+L (MRS broth without glucose + Lactulose) and MOD+FOS (MRS broth without glucose + Fructooligosaccharides**).**

curve	y0 (LogCFU/mL)	Lag (h)	yEnd (LogCFU/mL)	μmax (h-1)	R^2	SE of FIT
Lb. rhamnosus AT194 MOD	6.43 ± 0.03	/	7.30 ± 0.01	0.14±0.01	0.97	0.05
Lb. rhamnosus AT194 MRS	6.45 ± 0.07	2.82±0.43	8.06 ± 0.05	0.36±0.03	0.97	0.12
Lb. rhamnosus AT194 MOD+M	6.33 ± 0.03	2.29±0.22	7.92 ± 0.02	0.22±0.01	1.00	0.04
Lb. rhamnosus AT194 MOD+S	6.28 ± 0.01	2.24±0.12	7.51 ± 0.01	0.23±0.01	1.00	0.02
Lb. rhamnosus AT194 MOD +Fos	6.40 ± 0.03	2.29±0.26	7.58 ± 0.01	0.22±0.01	0.99	0.04
Lb. rhamnosus AT194 MOD+L	6.53 ± 0.04	3.06±0.41	7.68 ± 0.02	0.18±0.01	0.98	0.06

Figure 2.2.g shows the results of growth in the presence of prebiotics of *Lb. rhamnosus* AT195. This strain showed that in the presence of lactulose and mannitol the growth trends are comparable to those observed in glucose, while, the lowest level of growth was observed in the presence of FOS. The analysis of the kinetic parameters reported in

table 2.5.g confirms what has been reported, highlighting that strain AT195 recorded in the presence of mannitol, a lag phase (1.93 h) shorter than in glucose (2.60 h), even if with a lower μmax. To summarize, *Lb. rhamnosus* AT195 showed good growth capacity in the presence of lactulose and mannitol.

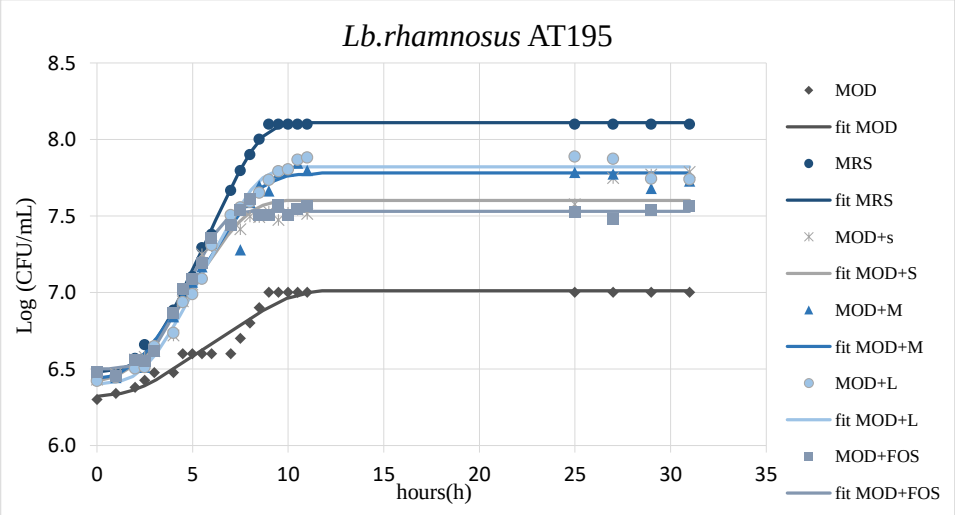

Figure 2.2.g Growth of *Lb. rhamnosus* AT195 in different carbon sources MOD (MRS broth without glucose), MRS broth, MOD+M (MRS broth without glucose + D-mannitol), MOD+S (MRS broth without glucose + D-sorbitol), MOD+L (MRS broth without glucose + Lactulose) and MOD+FOS (MRS broth without glucose + Fructo-oligosaccharides).

Table 2.5.g Kinetic parameters related to the growth of *Lb. rhamnosus* AT195 in different carbon sources MOD (MRS broth without glucose), MRS broth, MOD+M (MRS broth without glucose + D-mannitol), MOD+S (MRS broth without glucose + D-sorbitol), MOD+L (MRS broth without glucose + Lactulose) and MOD+FOS (MRS broth without glucose + Fructo-oligosaccharides**).**

curve	y0 (LogCFU/mL)	Lag (h)	yEnd (LogCFU/mL)	μmax (h-1)	R^2	SE of FIT
Lb. rhamnosus AT195 MOD	6.32 ± 0.04	1.98±0.84	7.01 ± 0.03	0.09±0.01	0.95	0.06
Lb. rhamnosus AT195 MRS	6.48 ± 0.03	2.60±0.18	8.11 ± 0.01	0.28±0.01	1.00	0.04
Lb. rhamnosus AT195 MOD+M	6.44 ± 0.06	1.93±0.50	7.78 ± 0.03	0.20±0.02	0.98	0.08
Lb. rhamnosus AT195 MOD+S	6.34 ± 0.07	/	7.72 ± 0.06	0.13±0.01	0.93	0.13
Lb. rhamnosus AT195 MOD +Fos	6.50 ± 0.03	2.67±0.30	7.53 ± 0.01	0.26±0.02	0.98	0.05
Lb. rhamnosus AT195 MOD+L	6.40 ± 0.04	2.49±0.34	7.82 ± 0.02	0.24±0.01	0.98	0.06

Regarding *Lb. rhamnosus* 39, the results are shown in Figure 2.2.h. Strain 39 showed good growth capacity in the presence of mannitol with values comparable to those obtained in glucose. From the analysis of the kinetic parameters reported in table 2.5.h

we can see that strain 39 showed a longer lag phase in the presence of lactulose compared to the other conditions, but with a slightly higher μmax.

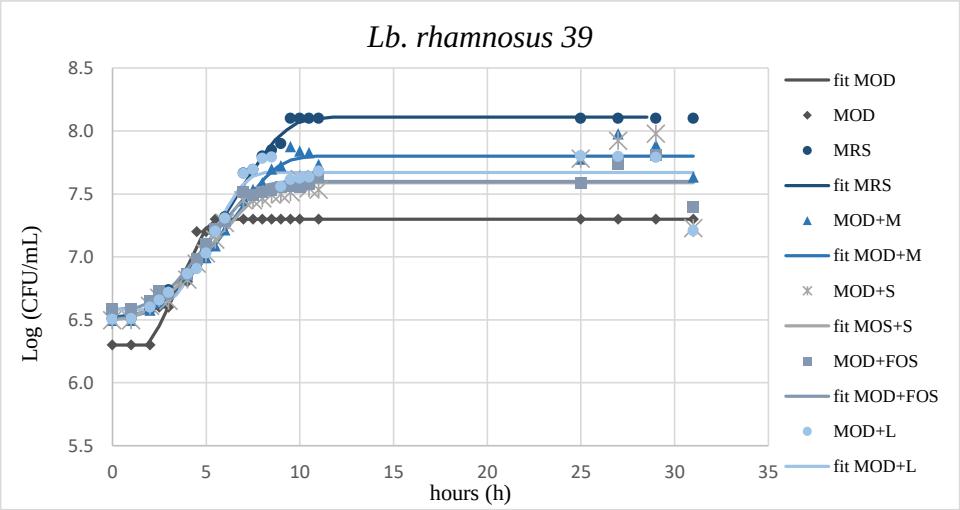

Figure 2.2.h Growth of *Lb. rhamnosus* 39 in different carbon sources MOD (MRS broth without glucose), MRS broth, MOD+M (MRS broth without glucose + D-mannitol), MOD+S (MRS broth without glucose + D-sorbitol), MOD+L (MRS broth without glucose + Lactulose) and MOD+FOS (MRS broth without glucose + Fructo-oligosaccharides).

Table 2.5.h Kinetic parameters related to the growth of *Lb. rhamnosus* 39 in different carbon sources MOD (MRS broth without glucose), MRS broth, MOD+M (MRS broth without glucose + D-mannitol), MOD+S (MRS broth without glucose + D-sorbitol), MOD+L (MRS broth without glucose + Lactulose) and MOD+FOS (MRS broth without glucose + Fructo-oligosaccharides)

curve	y0 (LogCFU/mL)	Lag (h)	yEnd (LogCFU/mL)	μmax (h-1)	R^2	SE of FIT
Lb. rhamnosus 39 MOD	6.32 ± 0.04	1.98±0.84	7.01 ± 0.03	0.09±0.01	0.95	0.06
Lb. rhamnosus 39 MRS	6.48 ± 0.03	2.60±0.18	8.11 ± 0.01	0.28±0.01	1.00	0.04
Lb. rhamnosus 39 MOD+M	6.44 ± 0.06	1.93±0.50	7.78 ± 0.03	0.20±0.02	0.98	0.08
Lb. rhamnosus 39 MOD+S	6.34 ± 0.07	2.21±1.26	7.72 ± 0.06	0.13±0.01	0.93	0.13
Lb. rhamnosus 39 MOD +Fos	6.50 ± 0.03	2.67±0.30	7.53 ± 0.01	0.26±0.02	0.98	0.05
Lb. rhamnosus 39 MOD+L	6.40 ± 0.04	2.49±0.34	7.82 ± 0.02	0.24±0.01	0.91	0.06

Analysis of the data on growth parameters as reported in figure 2.3 highlighted that different *Lb. rhamnosus* strains were able to growth, albeit with different intensity, only in the presence of some prebiotic substances while in the presence of others no growth has been observed. This result confirms what was already found in the preliminary test,

the ability to growth in the presence of different prebiotics as the sole source of carbon is a strain-specific characteristic.

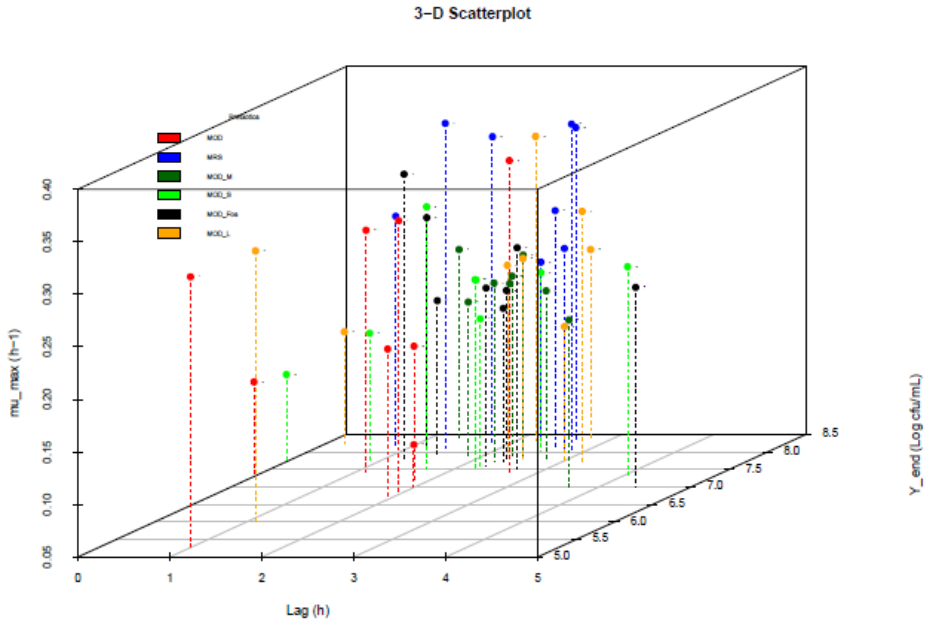

Figure 2.33D Scatterplot representing growth kinetic parameters (Lag phase, Lag; maximum specific growth rate, µmax; increase in microbial load, D_Y) of eight Lb. rhamnosus strains were cultivated in presence of glucose (blue), lactulose (yellow), mannitol (dark green), or sorbitol (green), Fos (black)and whitaout glucose or prebiotics (red).

Commento [UdMO3]: Aggiusta la didascalia della figura … corsivo, spazi ecc.

Furthermore, it emerged that sorbitol, although described in the literature (Hammes *et al*., 1992) as an assimilable substrate for this species, it is used only by the commercial probiotic strain *Lb. rhamnosus* GG.

A growth comparable to that expressed in the presence of glucose was detected when the strains were cultured in the presence of lactulose. In fact, in the presence of lactulose, only *Lb. rhamnosus* GG showed a low growth.

Finally, it should be pointed out that *Lb. rhamnosus* of human origin GG, FL2, FL3, FL4 and FLC5 showed, in general, higher growth rates, in the presence of prebiotics, compared to those observed for AT194, AT195 and 39 isolates from ripened cheeses.

2.6.2. Hydrophobicity and Aggregation activity in presence of prebiotics

Based on the evidence of the previous experiment, i.e. that the type of carbon source is able to influence the development of the *Lb. rhamnosus* strains, in this study, there we carried out other tests to verify if prebiotics are able to affect other characteristics of bacteria. In particular, some features, considered important for the selection of a probiotic such as hydrophobicity, auto-aggregation, co-aggregation and antimicrobial activity were evaluated in the presence of various prebiotics.

Surface hydrophobicity was determined, using MATH assays, to test for possible correlation between this physical-chemical property and the ability to adhere to the intestinal mucus as suggested by Ngwai *et al.*, 2006. Hydrophobicity and surface charge of bacteria may differ between species, as a function of the physiological state of cells and composition of suspension media (Abdulla *et al*., 2014).

In this study, significant differences in the adhesion values were observed depending on the different strains and prebiotics (Figure 2.4). In detail, *Lb. rhamnosus* GG, 39, FL2, FL3, and FL4 showed greater hydrophobicity than the other strains, expressed as the percentage of cells that are transferred to the xylene phase. In addition, sorbitol had a higher hydrophobicity in all strains compared to glucose. *Lb. rhamnosus* GG showed maximum hydrophobicity in the presence of sorbitol (55%) and mannitol (51%). *Lb. rhamnosus* FL3 showed maximum hydrophobicity when cultivated in the presence of sorbitol (77.9%). The strain *Lb. rhamnosus* 39 displayed the highest hydrophobicity in the presence of FOS (91.0%) and mannitol (90.4%). Whilst, *Lb. rhamnosus* FL2 and FL4 showed the maximum percentage of adhesion in the presence of FOS (54.1% and 54.3% respectively). Finally, the AT194, AT195 and FLC5 strains showed a very low or zero hydrophobicity in all the assayed conditions.

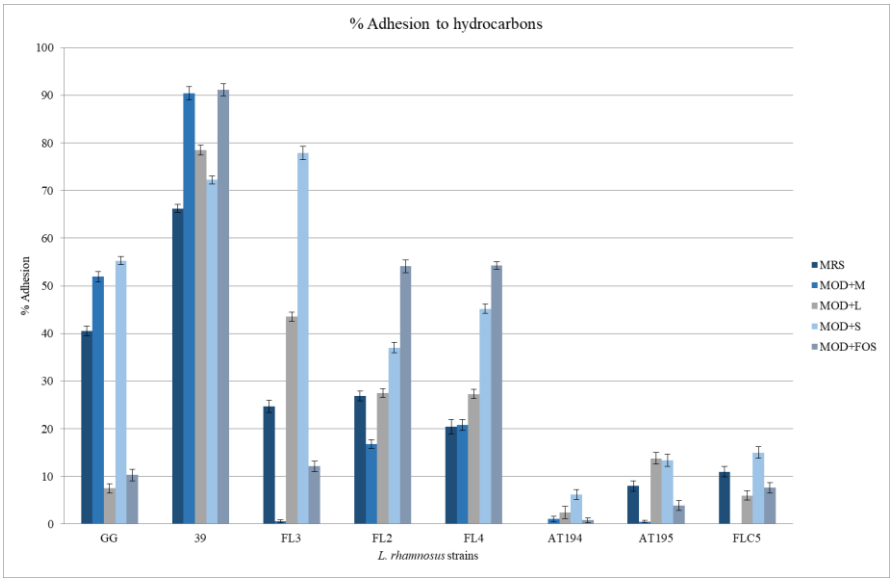

Figure 2.4 Adhesion to hydrocarbons of probiotic with different prebiotic as measured using the MATH assay.

Nikolic *et al.* (2010) reported that lactobacilli with high hydrophobicity also showed high auto-aggregation ability. In addition, Martin *et al.* (2005) hypothesized that lactobacilli with aggregation ability and hydrophobic cell surface could have more chance for adhesion to intestinal cells. For these reasons and based on previous results, strains GG, 39, and FL3 were selected to evaluate their auto-aggregation and co-aggregation properties. These three strains were selected because they showed, in the presence of sorbitol the highest hydrophobicity compared to the other prebiotics tested and glucose. Sorbitol was chosen to investigate whether it positively influenced other characteristics of the cell surface, such as auto-aggregation and co-aggregation.

The results of auto-aggregation assays are reported in table 2.6. The percentage of auto-aggregation for the selected strains was always higher at 24 h than at 5 h in agreement with results obtained by Collado *et al.* (2008). *Lb. rhamnosus* GG and *Lb. rhamnosus* 39 showed higher auto-aggregation ability in comparison to *Lb. rhamnosus* FL3. Strains GG and 39 showed the highest percentage of auto-aggregation in presence of glucose (MRS) and mannitol, while strain FL3 showed the highest percentage in presence of glucose and lactulose.

Table 2.6 Auto-aggregation percentages for *Lb. rhamnosus* strains

	Lb. rhamnosus GG		*Lb. rhamnosus* 39		*Lb. rhamnosus* FL3	
	%Autag.5h	**%Autag.24h**	**%Autag.5h**	**%Autag.24h**	**%Autag.5h**	**%Autag.24h**
MRS	30.1 ± 1.0	65.1 ± 4.9	33.4 ± 2.3	67.7 ± 1.0	29.7 ± 1.0	39.5 ± 1.5
MOD+M	24.4 ± 5.3	54.5 ± 6.0	27.7 ± 1.1	60.2 ± 6.4	19.0 ± 2.0	19.8 ± 3.2
MOD+S	23.1 ± 3.4	45.6 ± 2.8	26.4 ± 2.1	40.8 ± 3.2	11.5 ± 1.0	16.1 ± 1.5
MOD+L	23.3 ± 1.5	46.3 ± 1.2	29.5 ± 1.0	57.8 ± 4.1	25.1 ± 1.0	28.9 ± 1.4
MOD+FOS	24.3 ± 2.7	52.6 ± 4.5	29.0 ± 1.2	45.0 ± 3.2	2.0 ± 0.2	10.1 ± 1.1

Aggregation plays an important role in the formation of biofilms. In most cases, aggregation ability is related to cell adherence properties, also involving their ability to survive and persist in GIT (Ferreira *et al*., 2011).

In this study, no correlations were found between auto-aggregation and hydrophobicity of the tested strains, this result agrees with what was observed by Vlková *et al.* (2008) for bifidobacteria and by Tuo *et al.* (2013) for lactobacilli.

The results of auto-aggregation were partially confirmed by the co-aggregation test with *E. coli* DMS 5698 (Table 2.7). The highest percentages of co-aggregation with *E. coli* for all selected strains were detected in the presence of glucose (MRS). *Lb. rhamnosus* GG showed a good percentage of co-aggregation also in the presence of mannitol.

Table 2.7 Co-aggregation percentages for *Lb. rhamnosus* strains in presence of different prebiotic

	Lb. rhamnosus GG + *E. coli*		*Lb. rhamnosus* 39 + *E. coli*		*Lb. rhamnosus* FL3 + *E. coli*	
	%Coagr.5h	**%Coagr.24h**	**%Coagr.5h**	**%Coagr.24h**	**%Coagr.5h**	**%Coagr.24h**
MRS	35.7 ± 8.1	60.4 ± 2.9	37.5 ± 0.6	58.7 ± 0.3	32.6 ± 4.9	46,4 ± 0.2
MOD+M	32.1 ± 4.3	60.3 ± 2.1	24.4 ± 1.3	34.2 ± 2.8	19.9 ± 2.1	24.0 ± 1.1
MOD+S	22.6 ± 1.3	54.9 ± 1.8	21.3 ± 1.2	11.8 ± 0.9	14.5 ± 1.9	17.3 ± 2.8
MOD+L	19.9 ± 1.1	47.8 ± 1.3	31.6 ± 3.1	35.8 ± 1.0	23.4 ± 1.3	26.9 ± 2.3
MOD+FOS	22.4 ± 5.3	44.3 ± 2.3	20.9 ± 1.3	27.3 ± 0.3	17.1 ± 3.3	19.8 ± 1.2

Co-aggregation is an important feature for probiotics, because it may play a crucial role in eliminating pathogens from the GIT (Todorov *et al*., 2008). *Lactobacillus* strains can form a barrier that prevents colonization by pathogens through co-aggregation (Ferreira

et al., 2011). Moreover, co-aggregation with a potential pathogen allows the probiotic strain to produce antimicrobial substances in very close proximity to them, which may inhibit the growth of pathogenic strains in the gastrointestinal tract (Tuo *et al.*, 2013).
Auto-aggregation and co-aggregation are fundamental in the formation of biofilms that protect the host from colonization by pathogens (Ocaña & Nader-Macias, 2002).
On these basis, several authors suggest that auto-aggregation properties together with co-aggregation ability with potential pathogens can be used for preliminary selection of probiotic bacteria (Collado *et al.*, 2008; Ferreira *et al.*, 2011; Vlková *et al.*, 2008).

2.6.3 Antimicrobial activity

The results of the spot-on-the-lawn method to evaluate the antimicrobial activity of the *Lb. rhamnosus* strains against *E. coli* DMS 5698 are shown in Table 2.8. *E. coli* was chosen as it is a natural component of the human intestinal bacterial population.
All the strains tested inhibited *E. coli* DMS 5698 on MRS, and none on MOD. While different results were observed with other media, based on the prebiotic used (Table 2.8).

Table 2.8 Antimicrobial activity of *Lb. rhamnosus* strains against *E. coli* DMS 5698, in presence of different prebiotic.

strains	**MOD**	**MRS**	**MOD+M**	**MOD+S**	**MOD+L**	**MOD+FOS**
Lb. rhamnosus GG	/	15.4 ± 0.3	16.3 ± 1.5	/	/	/
Lb. rhamnosus FL2	/	16.6 ± 0.8	18.2 ± 1.2	/	19.3 ± 0.9	/
Lb. rhamnosus FL3	/	15.9 ± 0.4	16.2 ± 0.9	/	18.3 ± 0.8	/
Lb. rhamnosus FL4	/	16.9 ± 0.9	17.6 ± 1.4	/	16.1 ± 1.4	/
Lb. rhamnosus FLC5	/	16.7 ± 0.7	16.6 ± 1.0	/	18.1 ± 0.9	/
Lb. rhamnosus AT194	/	16.3 ± 1.1	15.1 ± 0.6	/	17.2 ± 1.1	/
Lb. rhamnosus AT195	/	14.6 ± 0.5	14.1 ± 0.9	/	17.1 ± 0.4	/
Lb. rhamnosus 39	/	16.8 ± 0.6	13.9 ± 1.5	/	17.9 ± 1.2	/

All strains cultivated in mannitol could inhibit the growth of *E. coli* DMS 5698, and the antimicrobial activity in the presence of this prebiotic did not show significant differences compared to that detected in MRS. In presence of lactulose, the *Lb. rhamnosus* strains, generally, showed a higher inhibition activity (halos larger) respect

those cultivated in glucose and mannitol, except of *Lb. rhamnosus* GG, that was not able to inhibit the growth of *E. coli* in the presence of lactulose.

On the contrary, the *Lb. rhamnosus* strains cultivated in the presence of sorbitol and FOS did not show antimicrobial activity against the indicator strain, as observed in the medium without carbon source (MOD).

These data show that the antimicrobial activity as well as the growth of *Lb. rhamnosus* are strongly related to the carbon source.

2.7. Conclusion

The importance of understanding at a molecular level the functional diversity within individual bacterial species that are considered for probiotic applications has only recently been appreciated. Bearing in mind their potential use in food and/or probiotic applications, we studied the differences within strains of the species *Lb. rhamnosus* using both food and commensal isolates. The outcome of this study agrees with previous studies showing a wide phenotypic diversity among strains of *Lb. rhamnosus* (Douillard *et al.*, 2013; Succi *et al.*, 2005; Succi *et al.*, 2017; Vancanneyt *et al.*, 2006). In this field, several researche has been made on different combinations of prebiotics/probiotics, all the studies were planned to evaluate the growth, survival and performances of probiotics in presence of prebiotics. For instance, Liong and Shah (2005) tested the effect of different prebiotics, including mannitol, sorbitol and inulin, on the cholesterol removal ability of *Lb. acidophilus* ATCC 4962, showing the best results in presence of mannitol and inulin. Yeo and Liong (2010) evaluated different probiotic lactobacilli and bifidobacteria for their viability in soymilk supplemented with mannitol and sorbitol, and they found out a significant improvement of soymilk features in the presence of prebiotics. Several other studies were produced to describe the effect of previous carbon sources on different probiotic strains belonging to *Lactobacillus* and *Bifidobacterium* (Miremadi & Shah, 2012; Sawangwan *et al.*, 2015).

On the other hand, only a few studies are available on the combination of *Lb. rhamnosus* strains with lactulose, mannitol, FOS or sorbitol, whilst there are many studies on the synbiotic mixture of probiotic *Lb. rhamnosus* strains and inulin. Noteworthy, Douillard *et al.*, (2013) confirmed mannitol and sorbitol as fermentable substrates for *Lb. rhamnosus* strains, including *Lb. rhamnosus* GG, these data were partially confirmed in our study, where mannitol and sorbitol were metabolised with average values. Instead, lactulose was found by Kontula *et al.* (1999) as a prebiotic hardly fermented by *Lb. rhamnosus* strains, but this result wasn't confirmed in this study, in fact, a growth comparable to that expressed in the presence of glucose was detected when the tested strains were grown in the presence of lactulose.

The results obtained in this study, documented that the prebiotics influenced the antimicrobial activity, hydrophobicity, auto-aggregation and co-aggregation of *Lb. rhamnosus* strains. Results of antimicrobial activity showed that all the strains tested

inhibited E. coli DMS 5698 on MRS, and none on MOD. While different results were observed with the other media, based on the prebiotic used.

Results showed a great heterogeneity for adhesion to hydrocarbons (assessed with xylene) among probiotic strains tested. In addition, no correlations were found between hydrophobicity and auto-aggregation abilities of the strains.

Finally, the obtained data support the hypothesis that the co-aggregation abilities are related to auto-aggregation properties. These parameters have been correlated with adhesion, which is known to be a prerequisite for intestinal colonization. Consequently, the ability to aggregate and co-aggregate are desirable properties for probiotics in health-promoting foods.

In conclusion, it is possible to state that probiotic or potentially probiotic strains belonging to *Lb. rhamnosus* are not always able to metabolize prebiotic compounds. This capacity is not attributable to the species but it is a specific character of the strain that is expressed in particular conditions. The data highlights the importance of establishing clear selection criteria for probiotic microorganisms which, if used in combination with prebiotics, should be tested beforehand in order not only, to ascertain their capacity for development in the presence of these substances, but also, to assess their hydrophobicity, auto-aggregation, and co-aggregation.

Results of this part of the study added new information regarding the influence of specific prebiotics on growth and some probiotic features of *Lb. rhamnosus* strains. This fact could open a new strand in the search for strategies to design synbiotic products.

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Chapter 3

3.1 *Akkermansia muciniphila:* new potential probiotic strain

Akkermansia muciniphila is an oval shaped, gram-negative, strictly anaerobic, representative of the phylum Verrucomicrobia, that constitutes 3–5% of the gut microbial community in both human and other mammalian species (Everard *et al.*, 2013; Geerlings *et al.*, 2018).

It was firstly isolated from a human fecal sample in 2004 (Derrien *et al.*, 2004), and interest in this microorganism has increased during time due to its ability to release enzymes into the intestinal tract that help regulate mucin, a major component of the mucous layer that resides on the surface on the gastrointestinal mucosa (Corazziari *et al.*, 2009). In fact, *A. muciniphila* is able to degrade human mucin providing competitive exclusion against pathogenic mucus degraders and may have a gate-keeping and signaling function (Belzer & De Vos, 2012).To gain a competitive advantage, *A. muciniphila* has evolved to metabolize the complex glycans provided by the mucus layer as its sole carbon and nitrogen source (Van Passel *et al.*, 2011; Derrien *et al.*, 2017) in fact, an efficient colonization of *A. muciniphila,* was observed with the highest numbers in the cecum, where most of the mucin is produced (Derrien *et al.*, 2011). Thus, the core activity of *A. muciniphila* seems to be to degrade mucus using the many mucolytic enzymes encoded in its genome (Van Passel *et al.*, 2011). Mucous degradation by *A. muciniphila* leads to the production of metabolites such as acetic acid, propionic acid and oligosaccharides, wich may play a role in metabolic health or inflammatory host status (Ottman *et al.*, 2017).

Furthermore, it has been shown that *A. muciniphila* is abundant in biopsy of healthy subjects, but reduced in those of patients with inflammatory bowel disease (IBD), obesity and other related diseases (Derrien *et al.*, 2017). Because of its low presence in people affected by various intestinal disorders and its link with the well-being of the mucus layer, *A. muciniphila* was suggested as a biomarker for the state of health, as it could indicate the progress of diseases (Berry *et al*., 2013; Swidsinski *et al.*, 2011).

Experiments of murine and human models of association studies support the hypothesis that *A. muciniphila* is able to modulate energy homeostasis, glucose metabolism, inflammation linked to obesity and even diabetes mellitus, although the mechanisms

involved here are not yet well understood (Cani & Everard, 2014; Everard *et al.*, 2013; Shin *et al.*, 2014; Swidsinski *et al.*, 2011; Van Passel *et al.*, 2011). In fact, it has been recently observed that the abundance of *A. muciniphila* decreased during obesity and diabetes (Le Chatelier *et al.*, 2013; Everard *et al.*, 2013) and that a high abundance is significantly associated with the improvement of cardiometabolic parameters in subjects with obesity undergoing caloric restriction. Other studies have shown that the daily administration of *A. muciniphila* can counteract the development of obesity and dysfunction of the high fat intestinal barrier (HFD) (Everard *et al.*, 2013). However, the mechanisms underlying these effects are not yet clear.

A. muciniphila has been identified in human milk samples immediately after delivery (colostrum), and at 1 and 6 months. *A. muciniphila* cell counts in breast milk were measured after conducting qPCR, revealing that *A. muciniphila* was higher in abundance in overweight than normal weight mothers (Collado et al., 2012).

3.2. Antibiotic susceptibility

Antibiotics play a role in the health industry to fight bacterial infections, however, their overuse has contributed to the emergence of resistant bacteria. Antibiotic resistance can cause significant danger for many people with common bacterial infections, that were once easily treated with antibiotics. Antibiotic resistance is the phenomenon by which a bacterium is resistant to the activity of an antimicrobial drug. Over the years this phenomenon has become increasingly important, especially for those bacteria whose sensitivity to certain drugs seemed undisputed. This aspect has already been studied extensively in foodborne pathogenic bacteria (Rahimi *et al.*, 2010; Gousia *et al.*, 2011), but also commensal bacteria, commonly present in food, have received particular attention as potential vehicles of the spread of antibiotic resistance determinants, which could be transferred to pathogenic bacteria by horizontal gene transfer (Snary *et al.*, 2004). Genes that confer resistance to tetracycline, erythromycin and vancomycin were detected in *Lactococcus lactis*, *Enterococcus* spp. and, recently, in *Lactobacillus* species isolated from fermented meat and dairy products (Mathur & Singh, 2005); this explains the importance of systematically testing strains for the presence of these genes before using them as probiotics or as starter cultures in food.

In fact, one of the most important selection criteria for bacterial strains intended for use in the food industry is their safety. In Europe, according to the QPS guidelines, the nature of any antibiotic resistance determinant present in a candidate microorganism should be assessed before approval of the status of QPS.

Resistance to a given antibiotic can be intrinsic or acquired. Intrinsic resistance of a bacterial species or genus (intrinsic or natural resistance) translates into the ability of an organism to survive in the presence of an antimicrobial agent due to intrinsic characteristics. Intrinsic resistance it is not horizontally transferable and does not represent any risk in non-pathogenic bacteria. Instead, acquired resistance to antimicrobial agents may arise or by bacterial genome mutations or through the acquisition of additional genes that encode for a resistance mechanism. Moreover, acquired resistance could be distributed horizontally between bacteria.

Currently, it is generally accepted that the possibility of transfer is related to the genetic basis of the resistance mechanism, i.e. if the resistance is intrinsic, acquired as a result of a chromosomal mutation or acquired by horizontal gene transfer (Gueimonde *et al.*, 2013).

Therefore, antibiotic resistance by itself is not a safety problem; it becomes such only when there is a risk of resistance transfer. The probiotics belonging to species included in the QPS list have excellent safety records and the harmful effects caused as a consequence of their ingestion are very poor (Gouriet *et al.*, 2015). However, a safety assessment should be made and it starts with an accurate identification of the strain and an *in vitro* assessment of the potential risks such as antibiotic resistance.

3.3 Survival at the gastrointestinal transit

The human gastrointestinal tract (GIT) is colonized by a vast and diverse consortium of microbes (microbiota) necessary for its proper functioning. These microorganisms colonize specific regions and niches in the GIT and have evolved together with their host. A balanced and complex microbiota is necessary for normal digestion and to maintain homeostasis of the intestinal ecosystem (Widodo *et al.*, 2014).

The ability to survive to stressful conditions of GIT, such as low pH and bile salts, is an important criterion for the selection of probiotics strains. For this reason, the analysis of probiotics in multi-compartmental models *in vitro* that simulate the physical-chemical

conditions of the human GIT is a prerequisite for subsequent *in vivo* experiments. The development and implementation of these systems are strongly encouraged by the FAO and the WHO (2002).

The various models proposed, simulate the phenomena that occur during digestion, from filling to gradual emptying of the stomach. Lysozyme and chewing stress are the first obstacle to the oral tract. After mastication, the first barrier that bacteria must overcome is the low pH of the stomach with values ranging from 2 to 6. In particular, on a full stomach, the bacteria ingested together with the food matrix are subjected to pH values of 5.0-6.0, but on an empty stomach the bacteria are subjected to pH starting from 2 (Nagata *et al.*, 2009). The exposure of bacteria to acidic environments, modifies the motive power of the proton through the membrane, favouring an accumulation of protons inside the cell (Corcoran *et al.*, 2008). The emptying of the gastric sac is an event that takes place gradually, in tandem with the digestion of food. The empty liquids from the stomach are faster than the solids and generally the food stays in the stomach between 1 and 2 hours, while the transit time through the small intestine takes from 1 to 4 hours.

The adverse conditions of the small intestine are caused by the presence of bile and pancreatin, the pH is around 8.0. The bile salts secreted in the duodenum emulsify and solubilize lipids and fat-soluble vitamins (Begley *et al.*, 2005). A concentration of 0.15 - 0.3% of bile salts was considered as a suitable concentration to select probiotic bacteria for human use (Huang & Adams, 2004; Schillinger *et al.*, 2005). Bacterial cells have various defensive mechanisms to resist hostile environments (Van de Guchte *et al.*, 2002; Mills *et al.*, 2011). Some bifidobacterial species have demonstrated the adoption of different strategies to cope with the gastrointestinal stress, including exposure to digestive enzymes, acidic pH, defensins, and antimicrobial peptides (Sharma *et al*., 2018). *Lb. rhamnosus* GG also possesses a large repertoire of mechanisms for responding to stress conditions. For example, the acid stress response included changes in central metabolism and in specific responses, such as the induction of proton-trans-locating ATPase, a membrane transporter used for increasing intracellular pH (Vinusha *et al*., 2018).

3.4. Adhesion

Adhesion or adherence is defined as the measurable union between a bacterium and a substratum. A bacterium is said to have adhered to a substratum when energy is required to separate the bacterium from the substratum (Ofek *et al*., 1994).

Adhesion to the intestinal mucosa is considered one of the main criteria for the selection of potential probiotics, as it may increase their persistence in the intestine and thus allows the probiotic to exert its effects (Kolida *et al*., 2006). The adhesion of bacteria to the intestinal cells is a complex process that involves specific and nonspecific mechanisms. In fact, some factors contribute to the nonspecific interaction with the host tissues, such as cell surface hydrophobicity and auto-aggregation (Kos *et al*., 2003, Ferreira *et al*., 2011) and other factors contribute to the specific interaction such as intelipoteichoic acids and cell surface proteins (Garcia-Cayuela *et al*., 2014).

Adhesion is also known to be a prerequisite for colonization and infection of the gastrointestinal tract by many pathogens (Scaletsky *et al*., 2002). An example is Enterohemorrhagic *Escherichia coli* (EHEC), a human pathogen that enters the intestinal tract as a result of food contamination and causes hemorrhagic colitis and hemolytic uremic syndrome (HUS) (Kim *et al*., 2009).

It has been shown that certain lactobacilli possess surface adhesins similar to those on pathogens (Neeser *et al*., 2000) and thus they may interfere with pathogen adhesion on the gut mucosa. So, the ability of probiotic bacteria to adhere to the intestinal surface is an important factor for competition to pathogens (Gueimonde *et al*., 2006). A probiotic should be able to compete with a pathogen for the binding sites, nutrients, production of antimicrobial substances and immune-stimulating compounds.

A possible mechanism for bacterial adherence and colonization of the host involves the binding of microbial cell-surface molecules to the protective mucus layer covering the epithelial cells of the GIT.

The first physical barrier to host-cell stimulation by bacteria in the gut, is represented by the mucus layer bound to gastrointestinal epithelia, that it is comprised mainly of mucin (mucus glycoprotein). The mucins are composed of glycoprotein monomers linked through disulfide bridges, that act as a protective barrier for the host against harmful antigens and promote luminal motility. The adhesion to mucus layer is, therefore, the first requirement for probiotic organisms to interact with host cells. The

thickness of the human intestinal tract mucus layer is variable. Generally, it is greater starting from the small intestine, where the intestinal microbiota is more abundant, and it is thinner in the rectum (Van Tassell & Miller, 2011).

Several cell surface proteins with highly repetitive structures have been reported to bind to epithelial cells and/or mucus. These include the mucus-binding proteins from *Lb. reuteri* (Båth *et al*., 2005), *Lb. acidophilus* (Buck *et al*., 2005) and *Lb. plantarum* (Boekhorst *et al*., 2006).

An example is provided by the distribution of mucus-binding proteins (MUB) encoded by Lactobacillales-specific clusters of orthologous protein coding genes (LaCOG) in *Lactobacillus* genomes (Yanagihara *et al*., 2017; Etzold *et al*., 2014). These Mub-repeat-containing proteins are most abundant in lactobacilli of the GIT, strongly suggesting that the Mub repeat is a functional unit that could fulfill an important function in host-microbe interactions.

Another group of proteins identified as putative adhesins in some *Lactobacillus* is the S-layer proteins. *Lactobacillus* species, such as *Lb. brevis*, *Lb. helveticus*, *Lb. acidophilus*, *Lb. crispatus*, *Lb. amylovorus* and *Lb. gallinarum* possess surface-layer proteins (Slps) (Johnson *et al*., 2017). S-layers are crystalline arrays of proteinaceous subunits located at the outermost part of the cell wall. Due to the high number of Slp subunits required to cover the entire cell surface, S-layer proteins represent approximately 10% of the total cellular proteins (Åvall-Jääskeläinen & Palva, 2005). Slps of several lactobacilli, including *Lb. crispatus* and *Lb. acidophilus*, whose ability to bind to host epithelial cells is decreased after removal or disruption of the S-layer proteins (Frece *et al*., 2005, Buck *et al*., 2005) have been shown to confer tissue adherence and competitive exclusion of pathogens such as *Escherichia coli* and *Salmonella*.

The ability to colonize the gastrointestinal tract is, therefore, a requirement recognized for many lactobacilli and bifidobacteria, but it is also interesting for emerging microbial species, which are found in the intestines of healthy individuals, such as *A. muciniphila*.

3.4.1 Cell lines used in vitro model adhesion

Due to obvious difficulties in performing *in vivo* studies, preliminary studies of potentially adherent strains are mainly based on in vitro adhesion assays. Currently,

there is not an *in vitro* adhesion standard protocol, for this reason the results are highly variable (Laparra & Sanz, 2010).

Good *in vitro* cell models must satisfy two basic requirements: retention of tissue characteristics to support the interpretation of results for the *in vivo* situation and availability and easy handling for high-throughput testing. Human intestinal cell models are widely used to study host-enteric pathogen interactions, with different cell lines exhibiting specific characteristics and functions in the gut epithelium. The human intestinal cell lines Caco-2 and HT29, are considered two of the best representations of the *in vivo* environment and they can be grown in culture to form a homogeneous polar monolayer of mature enterocytes resembling the tissue of the small intestine Both cell lines were isolated from human colon adenocarcinoma (Cencic & Langerholc, 2010).

The Caco-2 cell line forms polarized monolayers in culture and differentiates into cells with high homology to enterocytes in the intestinal epithelium. Caco-2 cells are capable to initiate spontaneous differentiation and reach confluence under normal culture conditions (e.g., the presence of glucose and serum) (Fossati *et al*., 2008). Over a period of 20 - 30 days of post-confluent culture, Caco-2 cells gradually acquire a morphological polarity comparable with those of mature intestinal absorbing cells.

HT29 cells in culture, although essentially undifferentiated, are heterogeneous in that they contain a small proportion (i.e. < 5%) of mucus-secreting cells and columnar absorptive cells (Huet *et al*., 1995). The HT29 cells were used recently in a rotating wall vessel-derived 3-D organotypic model of human intestinal epithelium to better approximate the parental tissue and provide important insights into microbial pathogenesis of *S. typhimurium* (Radtke *et al*., 2010, De Weirdt *et al*., 2012)

3.4.2. Biofilm

The idea that bacteria grow preferentially on surfaces has come to the fore whenever microbiologists have used direct methods to examine natural populations of these organisms growing in real ecosystems. Around 1670, Antonie van Leuwenhoek used his primitive but effective microscope to describe aggregates of '*animalcules*' that he had scraped from human tooth surfaces. More than 250 years later, in 1934, Claude Zobell examined natural marine populations by direct microscopy, and concluded that these bacteria are attracted to the surfaces to which they sometimes adhere, to form sessile populations. However, only a very small percentage of microbiologists chose to

examine their samples directly, using microscopy and they discovered that bacteria grow differently after the adhesion to a surface and start biofilm formation.

The bacterial cell exhibits two types of growth modalities, namely planktonic cell and sessile aggregate which is known as biofilm. Biofilm is an association of microorganisms in which cells attach to each other on a living or non-living surface enclosed within a self-produced matrix of extracellular polymeric substance (Hall *et al*., 2004).

Biofilm formation is a very complex process, in which the cells of microorganisms are transformed from a planktonic to a sessile growth mode (Okada *et al*., 2005). It is a multi-step process that begins with attachment to a surface, thus the formation of micro-colonies which leads to the formation of the three-dimensional structure and finally ends with the maturation followed by detachment. During biofilm formation, many species of bacteria are able to communicate with each other through a specific mechanism called quorum sensing that is a system of stimuli for coordinating the different gene expression. It has also been suggested that biofilm formation depends on the expression of specific genes that guide the establishment of biofilms (Okada *et al*., 2005; Sauer *et al*., 2004). The biofilm formation process takes place through a series of events that lead to adaptation under different nutritional and environmental conditions (Hentzer *et al*., 2005; Rivera *et al*., 2007).

Biofilm formation appears to be an ancient, universal, and fundamental survival mechanism that provides microorganisms with critical advantages, including greater access to nutritional resources, enhanced organism interactions, and greater environmental stability. Interactions of microorganisms in close spatial juxtaposition within the biofilm matrix facilitate metabolic cooperation and genetic exchanges. Furthermore, microbial biofilms can thrive in extreme or hostile environments where individual microorganisms would have difficulty not only growing but also surviving (Dang & Lovell, 2016).

Biofilms show an increased survival and resistance to environmental and chemical stressors mainly, but not only, by the protection conferred by the extracellular polysaccharide matrix In biofilms, bacterial cells exhibit 10 to 1,000 times less susceptibility to specific antimicrobial agents compared with their planktonic counterparts. This reduced susceptibility is caused by a combination of different factors, namely: (i) a poor antibiotic penetration into the polysaccharide matrix; (ii) the arbitrary

presence of cells showing a resistant phenotype (known as “persisters”); and (iii) the presence of either non-growing cells or cells that triggered stress responses under unfavorable chemical conditions within the biofilm matrix (Balcázar *et al*., 2015).

3.5. Research aim

Some authors stated thst *A. muciniphila* exerts a range of biological activities positively related to the human health, so that, it has been proposed as a new potential probiotic species (Gomez Gallego *et al.,* 2016). Currently, the species is not included by EFSA in QPS list (2018 update). However, all the knowledge acquired on this species provides grounds for proposing that *A. muciniphila* is added to this list.

According to the WHO, FAO, and EFSA recommendations, probiotic strains must meet the criteria of safety and functionality, including technological usefulness. For these reasons, the research activities, reported in this chapter, have been undertaken to evaluate the safety and some characteristics of a strain of *A. muciniphila* of human origin and the strains of *Lb. rhamnosus* strains already investigated in the previous chapter. The experiments carried out were: antibiotic resistance, survival to gastrointestinal stress, and biofilm formation and growth in co-culture. All these abilities are considered important for the screening of probiotic strains (Kos *et al*., 2003; Tamang *et al.*, 2009).

Furthermore, this study aimed to asses the correlation between cell surface characteristics and adhesion. Some authors have hypothesized a correlation between the adhesion capacity and physical-chemical properties of bacterial cell surfaces, such as hydrophobicity and auto-aggregation (Nikolic *et al*., 2010; Xu *et al*., 2009). These tests have become popular because they are cheaper and faster. However, aside from them we performed adhesion tests on in vitro grown cell-lines. to evaluate if physical-chemical properties do indeed correlate to adhesion.

3.6. Materials and Methods

3.6.1. Antimicrobial Susceptibility Test

The antibiotic susceptibility of the 8 *Lb. rhamnosus* strains and the *A. muciniphila* strain was performed with the Etest (Epsilometer test) gradient technology (Biomerieux, Marcy-l'Etoile, France). The Etest gradient technology is based on a combination of dilution concepts and diffusion principles for sensitivity testing. The system includes a predefined antibiotic gradient that is used to determine the Minimum Inhibitory Concentration (MIC), in µg/mL, of various antimicrobial agents against microorganisms tested on agar medium.

In the present study, the Etest strips of chloramphenicol, clindamycin, ampicillin, gentamicin, tetracycline, streptomycin, kanamycin and erythromycin were used in a concentration range of 0,016 – 256 µg/mL. MRS agar plates (for *Lb. rhamnosus*) and BHI agar plates (for *A. muciniphila*) were inoculated with the bacterial suspension prepared as follows. The inocula were prepared emulsifying with a sterile saline solution several well-isolated colonies from an overnight agar plate culture in an appropriate medium. The bacterial cell density of suspensions was adjusted to match the McFarland turbidity standard 0.5 using saline solution and spectrophotometer.

The inoculated plates were left drying for 15-20 min to allow the absorption of excess moisture. After drying the surfaces of the plates, Etest®- strips of tested antibiotics were applied directly onto the surface. The plates were incubated under anaerobic conditions at 37 ºC overnight. Results were read per manufacturer instructions.

3.6.2. Co-culture *Lb. rhamnosus* and *A. muciniphila*

The experiments were performed in triplicate; three independent cultures of each bacterium were analyzed following the protocols described by Ruiz *et al.* (2009) with some modifications.

Lb. rhamnosus strains were anaerobically grown in MRS broth supplemented with 0.05% cysteine-HCl at 37 °C for 24 h, conducted in a GasPak anaerobic system with AnaeroGen (Oxoid). Instead *A. muciniphila* DSM 22959 was anaerobically grown in BHI broth (Oxoid) at 37°C prior to their use. Before to prepare co-culture mixture, both species (*Lb. rhamnosus* with *A. muciniphila*) were grown overnight at 37 °C in BHI broth. Cells were collected by centrifugation (8000 rpm for 10 min at 4°C) from the

early logarithmic growth phase, washed twice and re-suspended in a saline solution to an optical density (OD 600) of approx. 0.5 that corresponds at 10^8 CFU/mL. For growth in co-culture assessment, each strain was inoculated at 10^3 CFU/mL.

These cultures were used to inoculate (1% v/v) one tube containing 50 mL of fresh medium preheated at 37 °C. In order to promote the bacteria to mix and to allow the interchange of secreted molecules present in the supernatants, the solution was vigorously vortexed and incubated anaerobically at 37 °C. At time 0 and after 3, 6 and 24 h of incubation microbial count was performed. *Lb. rhamnosus* strains were counted in MRS agar added with kanamycin at 37 °C in anaerobiosis. *A. muciniphila* was counted on BHI agar added with chloramphenicol at 37 °C in anaerobiosis.

3.6.3. Survival in the gastrointestinal transit

The experiments were performed in triplicate; three independent cultures of each bacterium were analyzed, following the protocols described by de Palencia *et al.* (2008) and Succi *et al.* (2014; 2017) with some modifications. The *Lb. rhamnosus* strains and *A. muciniphila* DSM 22959 were grown overnight at 37 °C in MRS broth, and in BHI broth, respectively. Then cultures (225 mL) were centrifugated (10.000 rpm for 10 minutes) and the pellets were resuspended in the same volume of saline solution. To simulate the *in vivo* dilution of saliva, 50 mL of a sterile electrolyte solution (NaCl 6.2 g/L, KCl 2.2 g/L, $CaCl_2$ 0.22 g/L and $NaHCO_3$ 1.2 g/L) with lysozyme (Sigma-Aldrich) to a final concentration of 0.01% was added. This solution was added to each cell suspension then it was divided into 9 bottles with 30 mL, and incubated for 2 min at 37 °C, after incubation microbial count was performed, in MRS agar for *Lb. rhamnosus* strains and in BHI agar for *A. muciniphila* strain at 37 °C in anaerobiosis.

To simulate the gastric environment, 20 mL of electrolyte solution containing 0.3% pepsin (final concentration) (Sigma-Aldrich) at pH 2.0, 2.5, and 3.0 was added to the cell suspension, and incubated at 37 °C. Aliquots of the suspension were collected after 30, 60 and 90 minutes of incubation, for each pH, and used for microbial count, performed as described above.

To simulate intestinal stress, oxygen was replaced by nitrogen to obtain an anaerobic atmosphere and the pH was adjusted to 5.0 with a saturated sodium bicarbonate solution (8 g of sodium bicarbonate in 100 mL of distilled water, sterilized at 121 °C for 15 minutes); 4.4 mL of a sterile electrolyte solution containing 0.45% porcine bile extract

and 0.1% pancreatin (final concentration, both from Sigma-Aldrich) were added. Thus, the pH was adjusted to 6.3 and slowly increased to 7.5 until the end of the test. After 4 h (end of intestine simulation) aliquots of each strain were collected used for microbial count, carried out as previously described.

The gastric and gastrointestinal solutions were freshly prepared daily and the whole study was performed at 37 °C.

3.6.4. Adhesion assay

For the adhesion test, all strains were analyzed beforehand by viable counts and spectrophotometer, using absorbance measurements at 580 nm, in order to standardize the microbial concentrations to be used in adhesion assays.

As an example, the growth curves, the absorbance values and the correlations between the growth values and the absorbance are shown for two of the tested strains (Figure 3.1.).

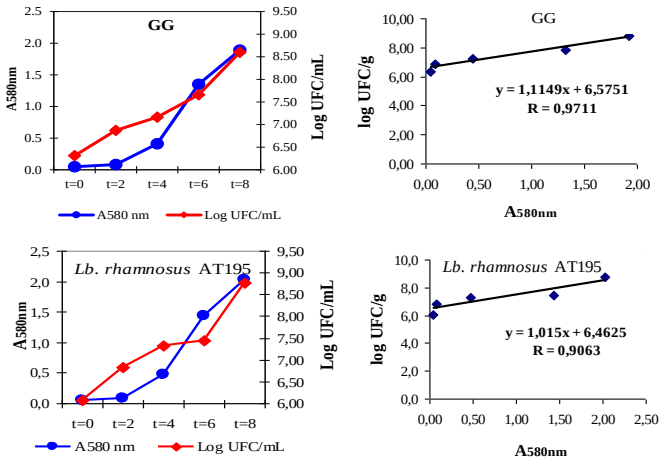


Figure 3.1 Microbial counts determined from spectrophotometric analysis at 580 nm and from plate count of *Lb. rhamnosus* GG and *Lb. rhamnosus* AT195 strains (left graphs). Correlation between the data obtained from the spectrophotometric analysis and the data obtained from viable counts of *Lb. rhamnosus* GG and *Lb. rhamnosus* AT195 (right graphs).

The following cell lines were tested: Caco-2 ATCC HTB37 (human colon adenocarcinoma), HT29 ATCC HTB37 (human colon adenocarcinoma) and MIA-PaCa2 ATCC CRL-1420 (human pancreas adenocarcinoma), all purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). The MIA-PaCa2 cell line differs in origin to colonocytes and thus express different antigens that those

expressed by the Caco-2 and HT29 cell lines. Therefore, the Mia-PaCa2 cell line have used to verify the specificity of adhesion to intestinal cell lines.

The cells were cultured in Dulbecco's modified Eagle's minimal essential medium (DMEM; HyClone Laboratories Inc., Logan, UT, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; HyClone), L-glutamine (2 mmol L^{-1}) (Sigma-Aldrich) without penicillin and streptomycin (Biological Industries, Kibbutz Beth Haemek, Israel). The incubation was carried out at 37 °C, 95% (v/v) humidified air and 5% (v/v) CO_2. Caco-2, HT29 and MIA PaCa-2 cells were seeded at 10^5 cells mL^{-1} (pre-confluence) in 8 well tissue culture plates and fully differentiated at 16 days (post-confluence) (Del Re *et al*., 2000). For the adhesion assessment, each tested strain was inoculated at c.a. 10^4, 10^5, 10^6, 10^7 and 10^8 CFU/mL into Caco-2, HT29 and MIA PaCa-2 cells grown in late post-confluence. The inoculated 8-well plates were incubated for 2 h at 37°C. After incubation, the non-adherent bacteria were removed by washing two times with PBS. The monolayers of cells were treated with 0.05% trypsin-EDTA. To enumerate the adherent bacteria, cell lysates were serially diluted with sterile saline solution and enumerated on appropriate medium. The adhesion percentage was calculated as follows (Valeriano *et al.*, 2014):

$$\% \text{ adhesion} = \left(\frac{(CFU/mL)_t}{(CFU/mL)_0} \right) \times 100 \qquad \text{Equation 3.1}$$

where, $(CFU/mL)_t$ was the number of adherent bacteria while $(CFU/mL)_0$ was the number of bacterial cells initially added.

3.6.5. Cell surface hydrophobicity

Microbial adhesion to solvent (MATH) assay was performed according to the procedure described in paragraph 2.5.3.1 with appropriate modifications. In this test MATH assay was performed using xylene and toluene. The test was carried out on *Lb. rhamnosus* and *A. muciniphila* strains adding toluene or xylene to test tubes containing 3 mL of a cell suspension prepared as previously described. The tubes were vortexed for 2 min, and left to stand for 15, 30, and 60 min for separation of the two phases and the OD 580 of the aqueous phase was measured. It is important to underline that the contact time was chosen following different protocols, such as Abdulla *et al.*, 2014; Collado *et al.*, 2008; Draksler *et al.*, 2004; Xu et al., 2009.

Hydrophobicity was calculated as the percentage using Equation 2.2.

3.6.6. Auto-aggregation assay

Auto-aggregation ability of the tested strains of *Lb. rhamnosus* and *A. muciniphila* was investigated according to the procedure described in paragraph 2.5.3.2 with appropriate modifications. Auto-aggregation was measured at 1, 2 and 5 h, after which the OD at 580 nm of the upper suspension was measured. Aggregation percentage was calculated using Equation 2.3.

3.6.7. Biofilm formation

Lb. rhamnosus strains were grown overnight at 37 °C in MRS broth and in TSB broth and the *A. muciniphila* strain was grown overnight at 37 °C in BHI broth and in TSB broth. After incubation, the strains were harvested by centrifugation at 8000 rpm for 10 min at 4 °C, washed twice with PBS and resuspended in different media. The media used in this test were tryptic soy broth (TSB) without glucose, TSB (Oxoid) supplement with 0,25%, 1%, and 2,5% D-glucose and MRS.

The quantitative assay for biofilm formation was performed according to the method described by Stepanović *et al.* (2000), with some modification.

Three wells of sterile 96-well flat-bottomed plastic tissue culture plates with a lid were filled with 200 µL of bacterial suspension each. Negative control were wells contained different broth without bacterial suspension. The plates were covered and incubated anaerobically for 24 h at 37 °C. The content of each well was eliminated, and washed three times with sterile saline solution. The non-adherent bacteria were removed by vigorously shaking the plates. The remaining attached bacteria were fixed with 200 µL of 99% methanol (Sigma–Aldrich), and after 15 min plates were removed and left to dry. The plates were colored for 5 min with 0,2 mL of 2% crystal violet (Liofilchem, Italy). Excess crystal violet was rinsed off by placing the plate under running tap water.

After the plates were air dried, the adherent cells were resuspended in 160 µL of 33% (v/v) glacial acetic acid. The OD of each well was measured at 580 nm by using an automated PerkinElmer 1420 Multilabel Counter.

All strains were classified into the following categories: non-adherent (0), weakly (+), moderately (++), or strongly adherent (+++), based upon the OD of bacterial films. We defined the cut-off OD (ODC) for this test as three standard deviations above the mean OD of the negative control (Christensen *et al.*, 1985). Strains were classified as follows:

$OD \leq OD_C$	non-adherent
$OD_C < OD \leq OD_C$	weakly adherent
$2 X OD_C < OD \leq 4X OD_C$	moderately adherent
$4 X OD_C < OD$	strongly adherent

All tests were carried out three times and the results were averaged.

3.6.8. Statistical analysis

All previously reported analyzes were performed in triplicate and the results were reported as mean and standard deviation.

Data processing was conducted using the Kruskal-Wallis statistical test, to evaluate significant differences ($p < 0.05$) relative to the adhesion capability on the three cell lines.

the data obtained the tests conducted in the presence of hydrocarbons, were processed using the Mann-WhitneyRank Sum Test statistical method. The differences in median values were considered significant for P-value <0.05.

The correlation between hydrophobicity and adhesion capacity to cell lines was evaluated using non-parametric statistical tests. In this regard, the correlation coefficient of Spearman (ρ) was calculated. Also, the correlation between auto-aggregation and adhesion capability was evaluated using the same statistical test.

Statistical analysis of the data was performed with the Systat software (Version 13).

3.7 Results and Discussion

3.7.1. Antimicrobial Susceptibility

The antibiotics tested (chloramphenicol, clindamycin, ampicillin, gentamicin, tetracycline, streptomycin and erythromycin) were selected on the basis of the EFSA document regarding bacteria of human importance (Efsa, 2012). The results obtained of antibiotic susceptibility test are shown in Table 3.1.

As reported also by other authors (Coppola *et al*., 2005) it is evident that the susceptibility of *Lb. rhamnosus* to the antimicrobial agents is strain-dependent. Moreover, the differences between *Lb. rhamnosus* and *A. muciniphila* were more remarkable for all of the tested antimicrobial agents. *Lb. rhamnosus* strains were susceptible to most antibiotics. In fact, *A. muciniphila* seemed to be susceptible to the ampicillin, tetracycline, gentamicin and kanamycin and resistant to clindamycin, streptomycin, chloramphenicol and erythromycin (Table 3.1). On the contrary, all *Lb. rhamnosus* strains tested seem to be resistant to kanamycin. Moreover *Lb. rhamnosus* FL3 showed to be resistant also to streptomycin, while *Lb. rhamnosus* FL4, FLC5 and AT195 showed a low sensitivity to this antibiotic with values equal to cut-off value. Furthermore, *Lb. rhamnosus* FLC5 showed, also, a low sensitivity to gentamycin.

On the basis of these results, kanamycin and chloramphenicol were selected to evaluate the growth of *Lb. rhamnosus* and *A. muciniphila* in co-culture. In fact, *A. muciniphila* showed a high sensitivity to kanamycin while all tested strains of *Lb. rhamnosus* were resistant to this antibiotic, in contrast, *Lb. rhamnosus* strains were susceptible to chloramphenicol while *Akkermansia* was resistant.

Table 3.1 Antimicrobial susceptibility of *Lb. rhamnosus* and *A. muciniphila* strains against chloranphenicol, ampicillin, clindamycin, tetracycline, gentamicin, streptomycin, kanamycin and eritromycin.

ANTIMICROBIAL AGENT (MIC ug/mL)								
Strains	Chloramphenicol	Ampicillin	*Clindamycin*	*Tetracycline*	*Gentamicin*	*Streptomycin*	*Kanamycin*	*Erythromycin*
Lb .rhamnosus GG	3	1.5	0.5	0.5	8	24	256	0.064
Lb. rhamnosus FL2	2	0.75	0.25	0.75	12	16	256	0.064
Lb.rhamnosusFL3	1.5	1	0.19	0.75	12	48	256	0.047
Lb.rhamnosusFL4	1.5	0.5	0.25	0.75	8	32	256	0.032
Lb.rhamnosusFLC5	3	0.75	0.094	0.5	16	32	256	0.032
Lb. rhamnosusAT194	3	0.5	0.5	0.75	6	6	96	0.094
Lb.rhamnosusAT195	3	0.5	1.5	0.5	4	32	192	0.047
Lb.rhamnosus 39	1.5	0.19	0.25	0.25	4	3	256	0.094
A. muciniphila	256	2	256	0.75	4	128	12	64
Cut- off	4	4	1	8	16	32	64	1

3.7.2. Co-culture of *A. muciniphila* and *Lb. rhamnosus*

Co-culture is an experimental model of rather simple culture, which allows the study of interactions between different species, similar to what would happen in vivo, where the different micro-organisms interact with each other, exchanging chemical signals, metabolites, etc.

In this trial, an *in vitro* model was set up to evaluate the interactions between the *A. muciniphila* DSM 22959 and *Lb. rhamnosus* strains. The aim of this test was to establish whether the two species, both present in the intestine, were negatively or positively influencing eachother, or if instead they did not influence each other.

As positive controls, broth cultures were prepared with *A. muciniphila* and *Lb. rhamnosus* grown individually.

Figure 3.2 shows the results obtained by the growth in co-culture. After 24 h the positive control of *A. muciniphila* showed a final count equal to 8.8 Log CFU/mL.

The positive control of *Lb. rhamnosus* GG after 24 hours reached 9.5 Log CFU/mL, and in co-culture reached 9.6 Log CFU/mL, while *A. muciniphila* reached 8.4 Log CFU/mL, so co-culture did not interfere with the growth of the two strains. Also in the case of co-culture *Lb. rhamnosus* FL2 and *A. muciniphila* there are no substantial differences, in detail, the positive control of *Lb. rhamnosus* FL2 after 24 hours reached 7.7 Log CFU/mL in co-culture the same strain showed a final count equal to 8.1 Log CFU/mL, while *A. muciniphila* reached 8.9 log CFU/mL.

Similar behavior was found for the co-culture of *Lb. rhamnosus* FL4 and *A. muciniphila*, in fact, the positive control of *Lb. rhamnosus* FL4 showed a count after 24 hours equal to 8.2 Log CFU/mL, in co-culture this strain showed a slightly higher count (8.6 log CFU/mL), whereas *A. muciniphila* reached a count of 8.5 log CFU/mL. Thus, the *Lb. rhamnosus* FL4 seems positively influenced by *A. muciniphila*, even if not significantly.

For the *Lb. rhamnosus* FL3 significant differences are observed, in fact, the positive control after 24 hours reached 7.8 log CFU/mL while the same strain in co-culture reached a value of 9.1 log CFU/mL. The value reached by *A. muciniphila* in co-culture was equal to its positive control. In this case a positive influence of *A. muciniphila* on the *Lb. rhamnosus* FL3 strain is evident. In fact, this strain, in co-culture, reached a count higher than 1.3 log with respect to its positive control. On the contrary, the strain of *A. muciniphila* was not influenced by the presence of the FL3 strain. Also *Lb. rhamnosus* AT195 showed significant differences between positive control and co-culture, in fact, at

24 hours the control had a count of 7.2 log CFU/mL, while the co-culture strain reached a value of 8.7 log CFU/mL. *A. muciniphila* in co-culture reached 8.6 log CFU/mL. Once again, the *Lb. rhamnosus* AT195 was positively influenced by *A. muciniphila*, which, however, was not influenced by the presence of the AT195 strain.

The opposite behavior was found for the co-culture of *Lb. rhamnosus* FLC5 and *A. muciniphila*, since the positive control of *Lb. rhamnosus* FLC5 after 24 hours was equal to 9.31 log CFU/mL, while the same strain in co-culture had a count of 7.7 log CFU/mL, therefore, in co-culture, there was a lower growth. *A. muciniphila* in co-culture after 24 hours showed growth equal to 8.9 log CFU/mL, value in line with its positive control. In this case the growth of the *Lb. rhamnosus* FLC5 was not favored by the presence of *A. muciniphila* while this was not influenced by the presence of the strain FLC5.

For the *Lb. rhamnosus* AT194 strain, no substantial differences were found between control and co-culture, in fact the positive control after 24 hours showed a growth equal to 8.6 log CFU/mL value in line with that recorded in co-culture (8.8 log CFU/mL). *A. muciniphila* in co-culture reached a count of 8.7 log CFU/mL. For both strains the values recorded in co-culture did not differ significantly from those recorded for their positive controls.

Finally, in the case of *Lb. rhamnosus* 39 the positive control after 24 hours showed a final count equal to 7 log CFU/mL, while in co-culture it reached 7.5 log CFU/mL. *A. muciniphila* after 24 hours in co-culture showed growth equal to 9.4 log CFU/mL. From this it emerges that *Lb. rhamnosus* 39 and *A. muciniphila* in co-culture showed a slight mutual synergy, confirmed by a count increase of 0.5 and 0.6 log CFU/mL, respectively.

From the results obtained it is possible to state that the strain of *A. muciniphila* DSM 22959 was not negatively influenced by the presence of the strains of *Lb. rhamnosus*.

The behavior of the *Lb. rhamnosus* strains was diversified: for some strains (FL3 and AT195) the stimulating effect exerted by *A. muciniphila* was very evident, in fact, in co-culture these strains reached significantly higher counts than those achieved when they were grown alone; for other strains (39 and FL4) this effect was less; finally other strains (GG, FL2 and AT194) were not influenced by *A. muciniphila*, only *Lb. rhamnosus* FLC5 showed a negative influence.

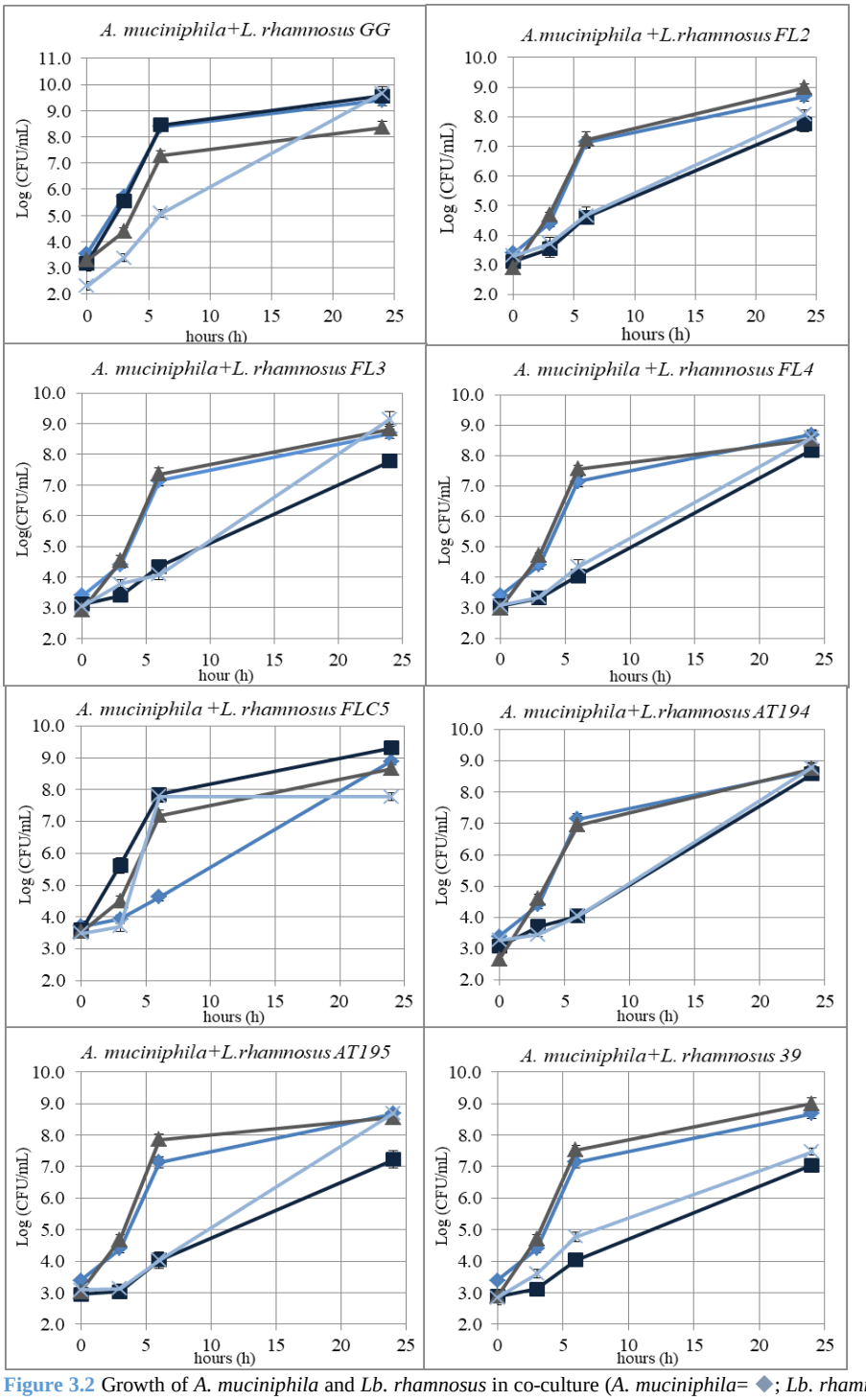


Figure 3.2 Growth of *A. muciniphila* and *Lb. rhamnosus* in co-culture (*A. muciniphila*= ◆; *Lb. rhamnosus* = ■; *A. muciniphila* in co-culture = ▲; *Lb. rhamnosus* in co-culture X)

3.7.3. Survival at simulated gastrointestinal transit

It is well known that the ability of probiotic microorganisms to provide health benefits is strictly related to the amount of viable bacteria able to reach the large intestine. So the ability of probiotic microorganisms to survive to the stresses encountered during GI transit is an important requirement to reach the colon at adequate levels. Therefore, the ability to survive at the GI transit of the strain *A. muciniphila* DSM 22959 and of the *Lb. rhamnosus* strains was evaluated by an *in vitro* simulation test. To reproduce the various conditions that bacteria can find in the stomach, particularly when it is empty and when it is full, the simulation of the gastric passage was performed using 9 different combinations of pH (2, 2.5 and 3) and permanence time/contact (30, 60 and 90 minutes).

Table 3.2 Table shows the explanation of the abbreviations in the following graphs

Abbreviation	Tract	pH	time (minutes)	During intestinal tract	Abbreviation	pH	time (h)
S 2-30	Stomach	2	30	→	I 2-30	5/7	4
S 2-60	Stomach	2	60	→	I 2-60	5/7	4
S 2-90	Stomach	2	90	→	I 2-90	5/7	4
S 2.5-30	Stomach	2.5	30	→	I 2.5-30	5/7	4
S 2.5-60	Stomach	2.5	60	→	I 2.5-60	5/7	4
S 2.5-90	Stomach	2.5	90	→	I 2.5-90	5/7	4
S 3-30	Stomach	3	30	→	I 3-30	5/7	4
S 3-60	Stomach	3	60	→	I 3-60	5/7	4
S 3-90	Stomach	3	90	→	I 3-90	5/7	4

The results of *Lb. rhamnosus* GG survival at the simulation of the GI transit, are shown in Figure 3.3.A. The permanence in the electrolyte solution with added lysozyme for about 2 minutes, to simulate the oral passage, did not cause significant effects on the viability of the strain, the same result was observed for all the other strains studied. Different results were recorded after simulation of the gastric passage as a function of the different

pH/permanence time combinations. At pH 3 the viability of the GG strain was not affected, while, at pH 2 and 2.5 a similar behavior was observed, with the vitality of the strain decreasing as a function of permanence time; the greater the time, the greater the decrease in vitality was observed. In detail, after 30 minutes of permanence in the gastric solution, similar results to the initial ones were recorded, whereas after 60 minutes a decrease of about 2 log was observed; this decrease in vitality was much more evident after 90 minutes (about 4 log CFU/mL). The intestinal simulation, which lasted 4 hours, recorded a decrease in vital cells for all the conditions tested and were confirmed on final counts similar to each other. This result is in agreement with other authors (Sumeri et *al*., 2010), and it is probably due to the combined action of acid stress, digestive enzymes (pepsin and pancreatin) and bile salts. Despite the significant reduction in viability observed during the GI transit simulation, the final counts were around 5 log CFU/mL, demonstrating a relatively good resistance of the GG strain to GI stress.

The results of survival at the GI transit of the *Lb. rhamnosus* FL2, isolated from human feces, are shown in Figure 3.3.B. This strain showed a much greater sensitivity to gastric stress than the GG strain. In fact, in all the tested conditions there was a strong decrease in cell viability, only for the sample at pH 3 after 30 minutes there was no significant decrease in the viability. During the passage in the intestinal environment the number of viable cells was further lowered, settling on a final count for all the tested conditions of about 2 log CFU/mL. Also, in this case, the combined action of acid stress and intestinal stress was observed on the viability of the FL2 strain which at the end of intestinal transit was on very low counts, showing a low resistance of the strain to gastrointestinal stress.

Data on the *Lb. rhamnosus* FL3, isolated from human feces, are shown in Figure 3.3.C. This strain showed good resistance to gastric stress, only after 60 and 90 minutes at pH 2 a decrease of about 5 log CFU/mL was recorded. In the other conditions, only a slight decrease in the vital counts was observed. The simulation in the intestinal environment had highlighted a situation similar to those previously, with the viable counts that were further lowered compared to those observed after the gastric passage. It should be noted the anomalous behaviour of the FL3 strain which, at the end of transit, registered a significantly lower vitality in the cells exposed to pH 3 compared to those exposed to lower pH (pH 2 and 2.5).

The *Lb. rhamnosus* FL4 (isolated from human samples) showed a high sensitivity to gastric stress (Figure 3.3.D). In fact, in all the tested conditions there was a strong decrease in the viability of the cells, comprised between almost 6 log CFU/mL for the pH condition

2/90 min and about 3.5 log CFU/mL for the pH 3/30 min. During the simulated intestinal passage, the number of viable cells was further lowered, particularly the cells exposed to pH 2 during the gastric simulation were unimportant after intestinal passage. For this reason, it is possible to state that the *Lb. rhamnosus* FL4 after passing the gastric pH 2 environment was strongly injured and is not able to survive to the additional stress it encountered in the intestinal environment.

Figure 3.3.E shows the results related to the simulation of the GI passage of the *Lb. rhamnosus* FLC5 strain, isolated from human feces. Also, the FLC5 strain, like the previous one, showed a high sensitivity to gastric stress by recording in all the tested conditions a decrease in the vitality of the cells, more accentuated at pH 2 and 2.5. After the intestinal simulation, the strain showed a further decrease in viability reaching the final count of about 3 log CFU/mL for all the assayed conditions, showing a poor resistance to gastrointestinal stress.

Results related to the *Lb. rhamnosus* AT194, isolated from Parmigiano Reggiano cheese, are shown in Figure 3.3.F. This strain showed good resistance to simulation of gastric stress at pH 3, while at pH 2 and 2.5 it showed good survival only when exposure lasted 30 minutes, while an exposure of 60 and 90 minutes caused a decrease in vitality even if with different intensities. Also this strain showed a further reduction of the counts during the simulation of the intestinal passage, only the cells exposed to pH 3 for 30 minutes stood on counts comparable to those recorded for the GG strain, while for the other conditions the vital counts were decidedly lower.

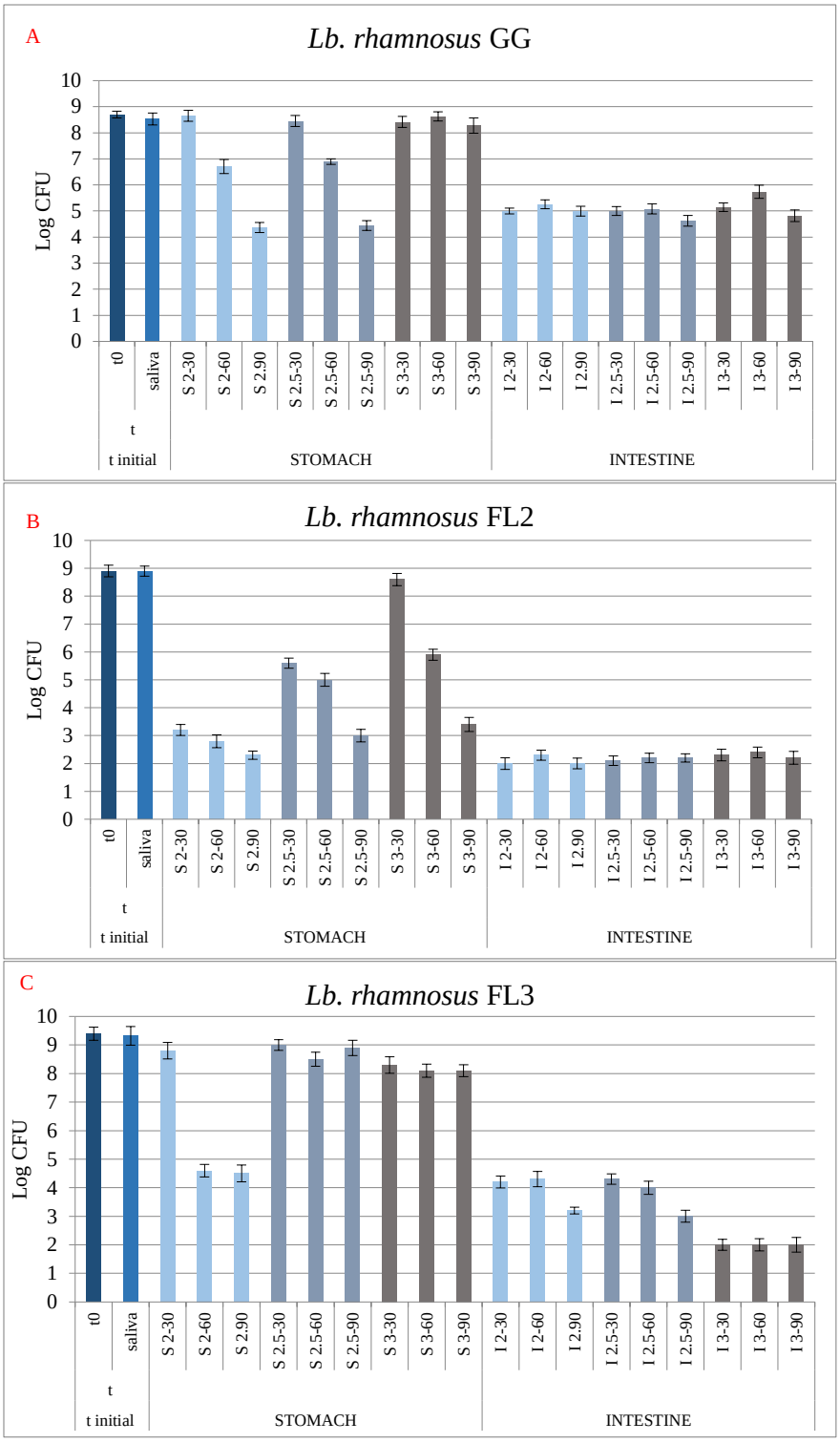
Data on the simulation of the gastrointestinal transit of the *Lb. rhamnosus* AT195, isolated from Parmigiano Reggiano cheese, are shown in Figure 3.3.G. This strain showed a behavior very similar to that observed for the *Lb. rhamnosus* FL4. In detail, it was shown to be sensitive to both gastric and intestinal stress. Also, for the AT195 strain the exposure to pH 2 during the gastric simulation caused a loss of vitality such that the charges are irrelevant after the simulation of intestinal transit. The *Lb. rhamnosus* AT195, however, showed a greater sensitivity than the FL4 strain in the other conditions tested, showing slightly lower vital charges after simulation of intestinal transit.

Figure 3.3.H shows the data related to the *Lb. rhamnosus* 39, isolated from caciocavallo cheese. The strain 39 showed a differentiated sensitivity to gastric stress. In all the conditions tested, the cell vitality decreased, except in the conditions pH 2.5 and 3 for 30 minutes, for which the registered counts were similar to the initial ones. Moreover, the exposure to pH 2 caused a drastic reduction in the vitality of the strain which for the

contact times 30 and 60 minutes stood at about 2.7 log CFU/mL, while for the contact time 90 minutes they were less than 1 log CFU/mL. Furthermore, strain 39 showed a higher sensitivity than the other *Lb. rhamnosus* strains also tested for intestinal conditions, in fact, after the simulation of intestinal transit the counts were always irrelevant.

Finally, the results of the survival tests of the gastrointestinal transit simulation of the *A. muciniphila* DSM 22959 strain are shown in Figure 3.3.I. From the results obtained it is possible to state that the behaviour of *A. muciniphila* DSM 22959 is profoundly different from that of the 8 strains of *Lb. rhamnosus*. The strain was sensitive to the simulation of gastric transit, a decrease in vitality of about 2 log CFU / mL for all the tested combinations was recorded. After 4 hours of simulation of the intestinal environment it was possible to detect, for all conditions, an increase in count above 1 log CFU/mL, recording values between 8 log CFU/mL (pH 2 for 90 minutes) and 8, 5 log CFU/mL (pH 3 for 30 minutes). *A. muciniphila* DSM 22959 showed a decrease in vitality caused by stress induced by gastric conditions, also observed by other authors (Van der Ark et *al*., 2017), the subsequent passage in the intestinal environment, instead, encouraged an increase in vitality reaching final values of about 8 log CFU/mL.

The behaviour similar to that of *A. muciniphila* DSM 22959 during the simulated transit has already been observed by other authors for lactic acid bacteria (Faye *et al.*, 2012; Possemiers *et al.*, 2010; Succi *et al*., 2017). Some authors hypothesized that a part of the bacterial population, when exposed to gastric stress, enters into a so-called viable but not cultivable state (VBNC) (Possemiers *et al.*, 2010). In the intestine, in the presence of less hostile conditions, some VBNC cells could return to a cultivable form, thus explaining the increased vitality observed after simulation of intestinal transit (Faye *et al.*, 2012; De Palencia, *et al.*, 2008). Furthermore, Fernández de Palencia *et al.* (2008) have observed that the conditions of gastric stress, due to the low pH, cause the formation of aggregates of cells that can give rise to a single colony in the vital plate count, resulting in an underestimation of viable cells. On the contrary, these aggregations tend to disappear when the pH conditions become more permissive as those encountered during intestinal transit. Furthermore, Pierre et *al*., (2016) observed a positive effect of circulating primary bile acids on the *A. muciniphila* abundance in mice, while Van der Ark et *al.* (2017) found that the addition of 0.1%, 0.5%, and 1% porcine bile extract resulted in increased growth of *A. muciniphila* in comparison to the medium that did not contain bile.


A
Lb. rhamnosus GG
Log CFU
0
1
2
3
4
5
6
7
8
9
10
t0
saliva
S 2-30
S 2-60
S 2.90
S 2.5-30
S 2.5-60
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S 3-30
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S 3-90
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I 3-90
t
t initial
STOMACH
INTESTINE
B
Lb. rhamnosus FL2
Log CFU
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1
2
3
4
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7
8
9
10
t0
saliva
S 2-30
S 2-60
S 2.90
S 2.5-30
S 2.5-60
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S 3-90
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I 3-30
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I 3-90
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t initial
STOMACH
INTESTINE
C
Lb. rhamnosus FL3
Log CFU
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t0
saliva
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S 2-60
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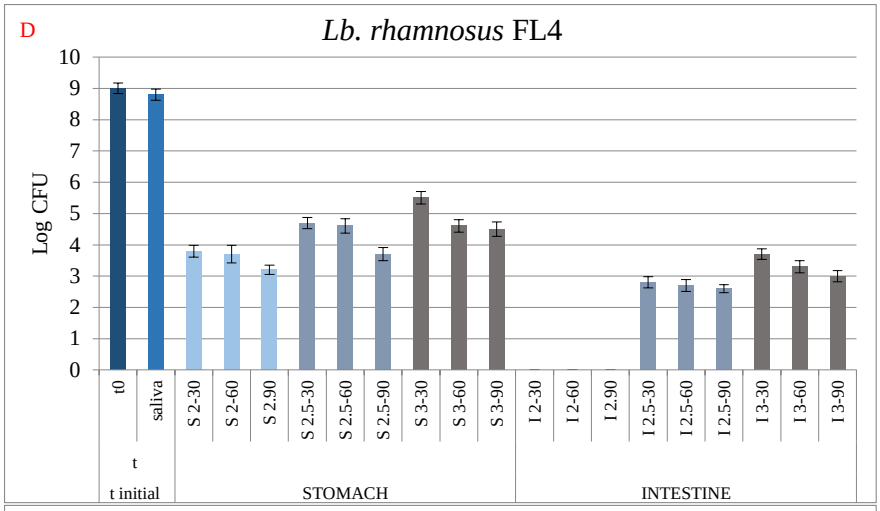
t initial
STOMACH
INTESTINE


D
Lb. rhamnosus FL4
Log CFU
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t0
saliva
S 2-30
S 2-60
S 2.90
S 2.5-30
S 2.5-60
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S 3-30
S 3-60
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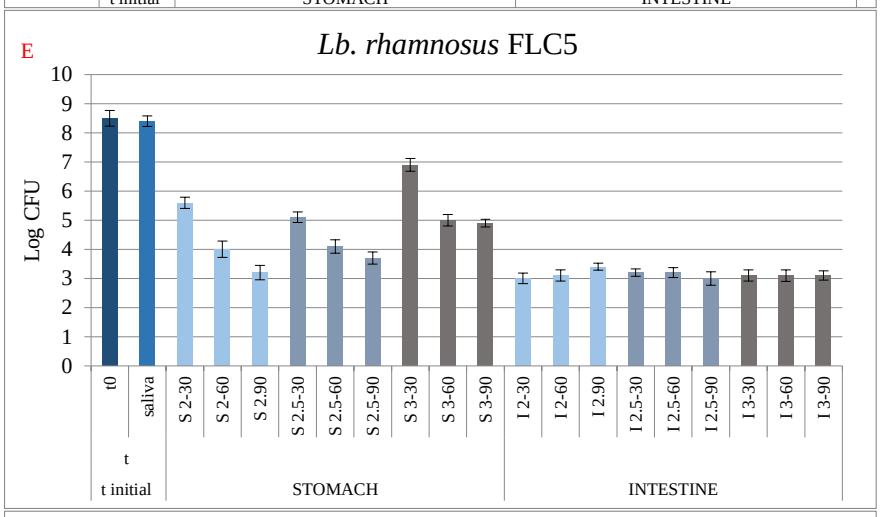
E
Lb. rhamnosus FLC5
Log CFU
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4
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7
8
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10
t0
saliva
S 2-30
S 2-60
S 2.90
S 2.5-30
S 2.5-60
S 2.5-90
S 3-30
S 3-60
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t initial
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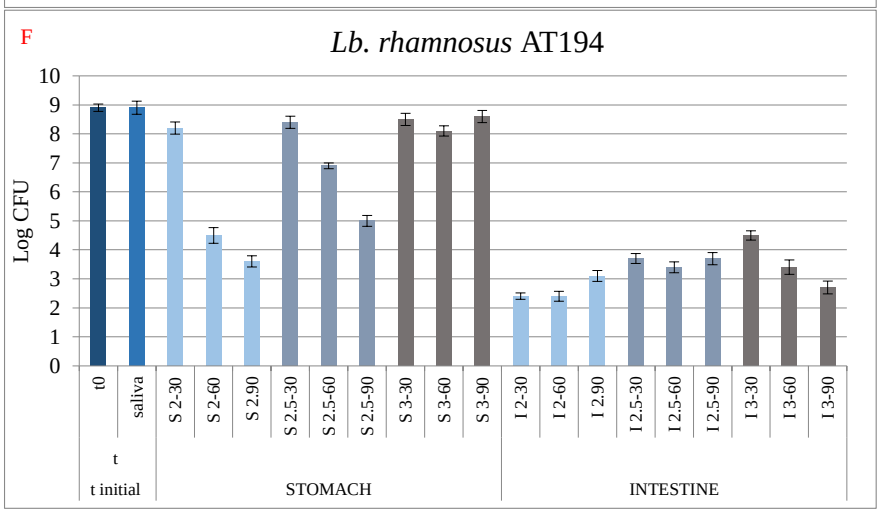
INTESTINE


F
Lb. rhamnosus AT194
Log CFU
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3
4
5
6
7
8
9
10
t0
saliva
S 2-30
S 2-60
S 2.90
S 2.5-30
S 2.5-60
S 2.5-90
S 3-30
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t initial
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INTESTINE

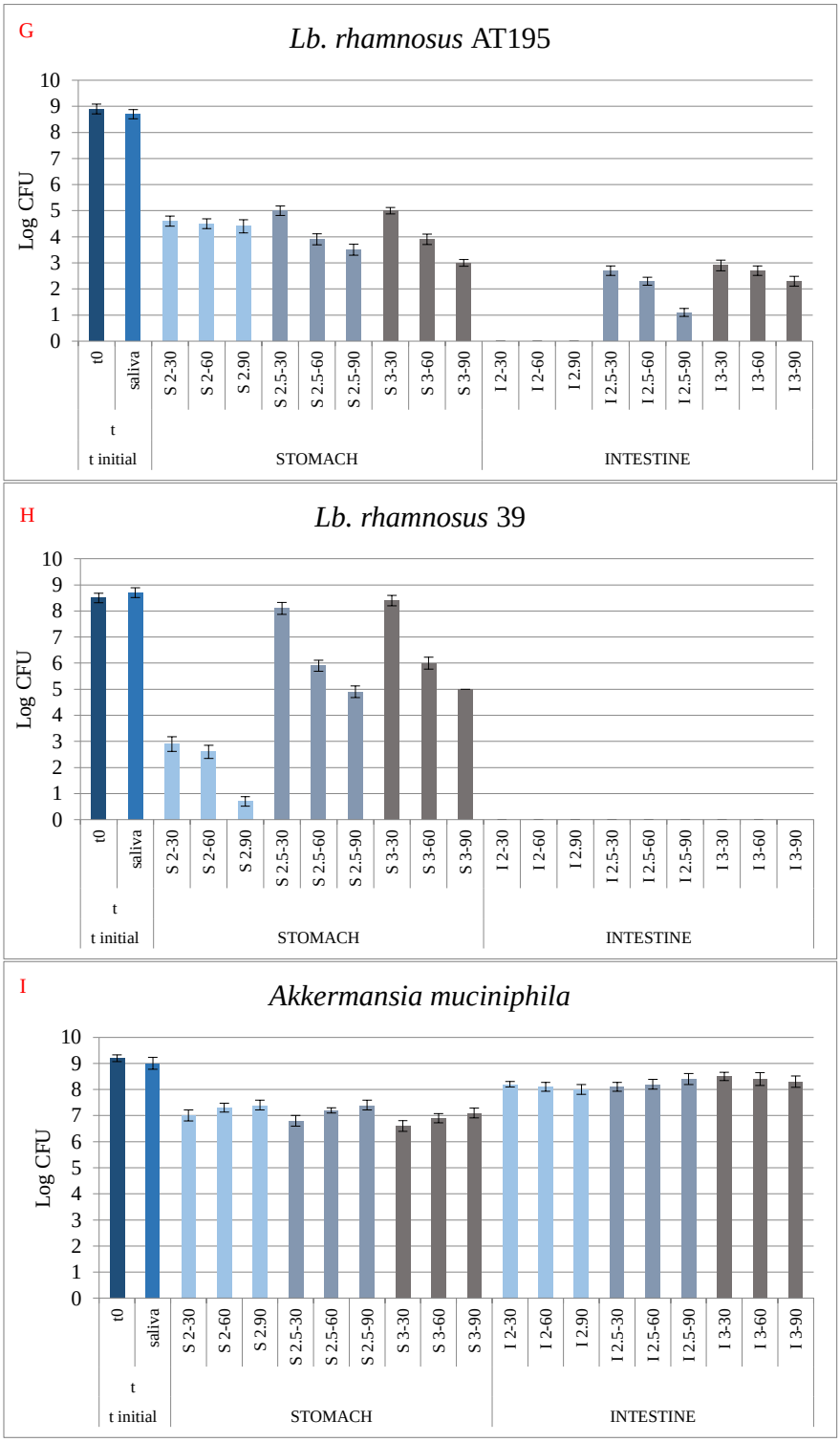

Figure 3.3 Survival at the gastrointestinal transit of *A. muciniphila* and 8 *Lb. rhamnosus*

3.7.4. *In vitro* adhesion to different cell lines of human origin

The intestinal epithelium consists of a thick layer of cells that have opposite functions. On one hand, they represent a physical barrier between the intestinal contents and the rest of the body, on the other they represent the point of contact with the external environment, ensuring the essential nutrient absorption and production, at the same time, defense substances, such as mucus, anti-microbial peptides, and cytokines with defense and regulating properties of the immune system. These functions are complex and influenced by many factors, including motility, the interaction with the molecules introduced by the diet and the digestive processes that bring various substances such as bile salts and enzymes to the intestine (Verhoeckx *et al*., 2015). For these reasons, the *in vivo* study of the host-microbiota interaction turns out to be complex, even if the knowledge of the mutual influences between human intestinal microbiota, digestion and health has increased significantly in recent years as a consequence of the evolution of investigative techniques, especially of biomolecular ones. Moreover, in the last decades, numerous models have been developed to simulate the behavior of the intestinal epithelium and the interactions with the diet molecules and with the microorganisms. Although these models cannot fully reflect what happens *in vivo*, they are often the only way to obtain useful information for understanding certain biological processes. For example, to study probiotic bacteria adhesion to the intestinal mucosa, one of the preferentially used models is represented by cell lines such as Caco-2 and HT29. These cell lines derive from colon adenocarcinoma. One of the main advantages in their use is that these cells spontaneously differentiate into mono-layer and possess the peculiar properties of the absorbent enterocytes with brush border typical of small intestine cells. Some bacteria have surface proteins and other interaction factors able to mediate adhesion with intestinal mucosal cells (Valeriano *et al.*, 2014). Literature data indicate that these features vary from species to species and, within the same species, from strain to strain. These findings were confirmed by the results obtained in the context of this thesis. In fact, as shown in Figure 3.4, the strains of *Lb. rhamnosus* tested showed different adhesion capacities, and also different from those showed by *A. muciniphila*. In particular, the reported data in Figure 3.4 suggest that all the tested strains are able to adhere to the three tested cell lines, with more marked differences only for the FLC5 and 39 strains, for which the adhesion to Caco-2, for the former, and to HT29, for the latter, were higher and lower, respectively, than that found with the other analyzed cell lines.

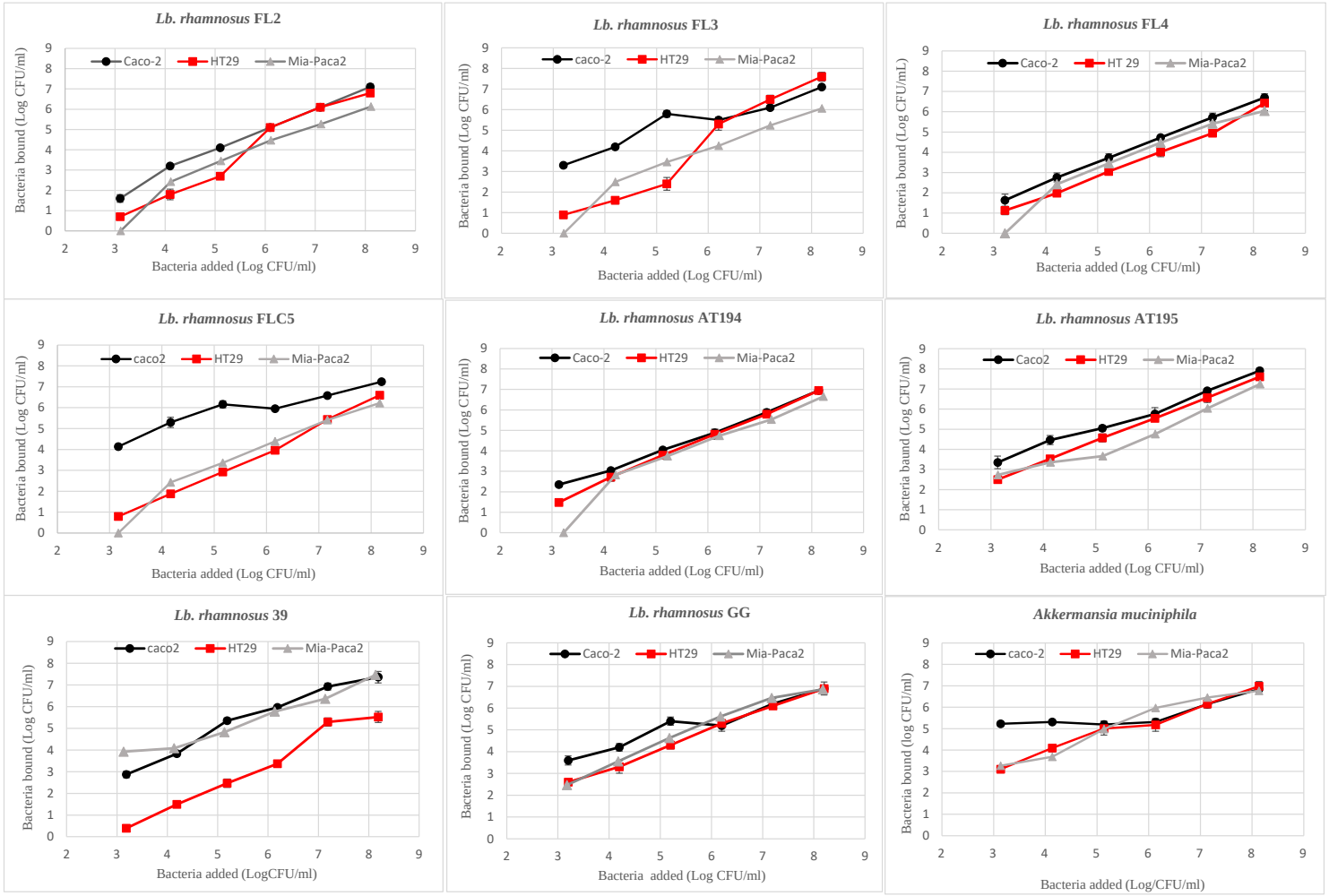

Figure 3.4 Adhesion of *Lb. rhamnosus* and *A. muciniphila* strains to Caco-2, HT29 and Mia-Paca 2.

A more in-depth data analysis provided a greater body of information about the adhesion capability of the microorganisms tested in the present study. In fact, expressing the result as adhesion percentage, calculated as the number of adherent bacteria compared to the initial number of added bacteria, significant differences were found not only with respect to the tested strain, but also with respect to the initial charge added and the type of cell line (Figure 3.5). Specifically, the strains *Lb. rhamnosus* FL3, FLC5, AT195, 39 and GG showed high adhesion capability to the Caco-2 cell line, while the FL2, FL4 and AT194 strains showed very low adhesion capability to all cell lines. These first data indicate, as previously reported, the strain-specific character of the adhesion capability. Furthermore, the highest adhesion percentages were recorded when the microorganisms were added to the Caco-2 up to 5 Log CFU/mL.

The use of high charges (6, 7 and 8 Log CFU/mL) caused a decrease in the percentage of adherent bacteria, although in other studies (Tuomola & Salminen, 1998) a positive correlation between the number of bacteria added and the number of bacteria attached to Caco-2 was found. It could be hypothesized that the results observed in this study may be due to the saturation of the Caco-2 binding sites in the presence of high concentrations of microorganisms, with the inversion of the adhesion curves with high concentration of bacteria added due to several factors such as the lack of the physical space necessary for cells, the accumulation of secondary metabolites derived from the bacterial metabolism, the acidification of the culture medium and the saturation of adhesion sites of the cell lines.

A. muciniphila showed a behaviour similar to that depicted for some *Lb. rhamnosus* strains in the case of Caco-2 cell lines, but a higher ability to adhere to HT29 and MiaPaca-2 cell lines was also observed.

The data obtained on the adhesion capability of the tested strains offer a valuable point of consideration taking into account of the ability of these microorganisms to overcome the gastroenteric barrier. In fact, the results of several *in vitro* studies on survival capability of *Lb. rhamnosus* at the typical conditions of the gastrointestinal tract (Succi *et al.*, 2014; Succi *et al.*, 2017) indicate that this bacterium is strongly injured, especially from the low pH and from gastric enzymes, arriving in the intestine with loads lower than 4 Log CFU/g even when the initial charges exceed 9 Log CFU/g. Consequently, the intake of high probiotic charges, strongly recommended by the reference literature (Kechagia *et al.*, 2013), plays a fundamental role so that these microorganisms can reach the intestine in such quantities as to be able to adhere to the intestinal mucosa without causing an excessive acidification. With regard to the other cell lines, *A. muciniphila* showed high

adhesion capability also on HT29 cell line, in contrast with almost all the *Lb. rhamnosus* tested strains. Regarding the MiaPaca2 cell line, a strain-specific adhesion dependent on the initial load of added bacteria was highlighted. In particular, only *Lb. rhamnosus* 39 and *A. muciniphila* have shown excellent percentages of adhesion when added with loads of 3-4 Log CFU/mL.

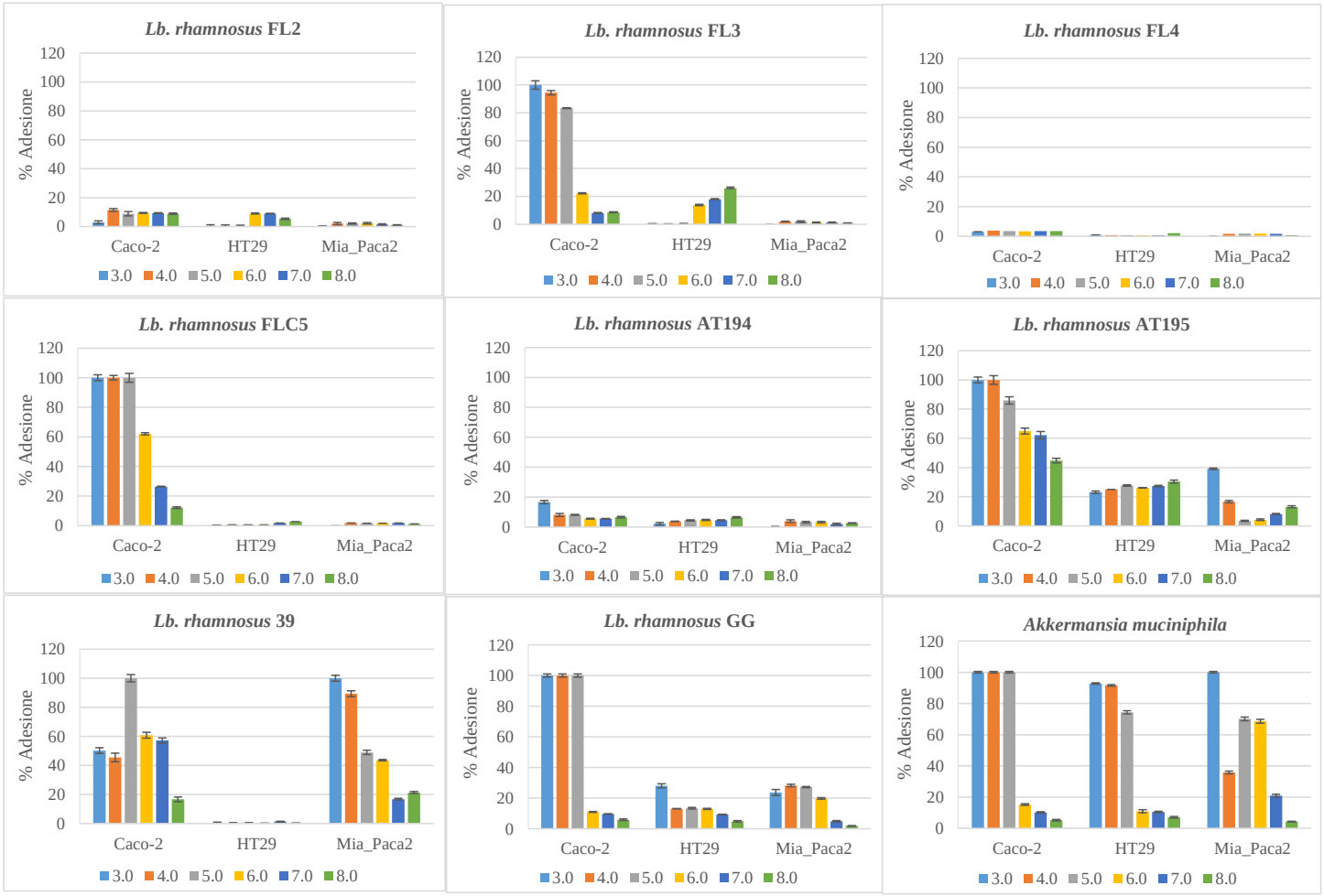

Figure 3.5 Adhesion, expressed as a percentage, of *Lb. rhamonsus* and *A. muciniphila* strains to Caco2, HT29 and MiaPaca2 cell lines. The color of the bars represents the initial charge of bacteria adding on the cells (from 3 to 8 Log CFU/mL).

The statistical analysis of the adhesion capability of tested bacteria to different cell lines was evaluated at two different microbial concentrations. Specifically, the comparison was made between 10^5 CFU/mL and 10^8 CFU/mL bacteria added, since the results previously exposed made it possible to ascertain a negative correlation between microbial load and the adhesion percentage, with values of maximum adhesion shown in the presence of charges up to 10^5 CFU/mL and minimum values measured with charges equal to 10^8 CFU/mL. From Figure 3.6 A, concerning bacteria added to 10^5 CFU/mL, it is clear that the adhesion was significantly influenced by the cell line used. In fact, the highest percentage of adhesion was recorded on Caco-2 cell line. In this case the median was 85.9, a significantly ($p < 0.05$) higher value than that recorded for HT29 (8.0) and MiaPaca-2 (33) cells. Figure 3.6 B shows data on the adhesion capability of bacterial cells added at a concentration of 10^8 CFU/mL to Caco-2, HT29 and MiaPaca-2 cell lines. The graph shows, once again, a greater adhesion on Caco-2 cells, but in this case significant differences ($p < 0.05$) were obtained only between Caco-2 and MiaPaca-2 cell lines. Furthermore, the lower adhesion capability was recorded on MiaPaca-2 cells. In fact, the median values were 8.9, 3.5 and 1.9 for Caco-2, HT29 and MiaPaca-2, respectively.

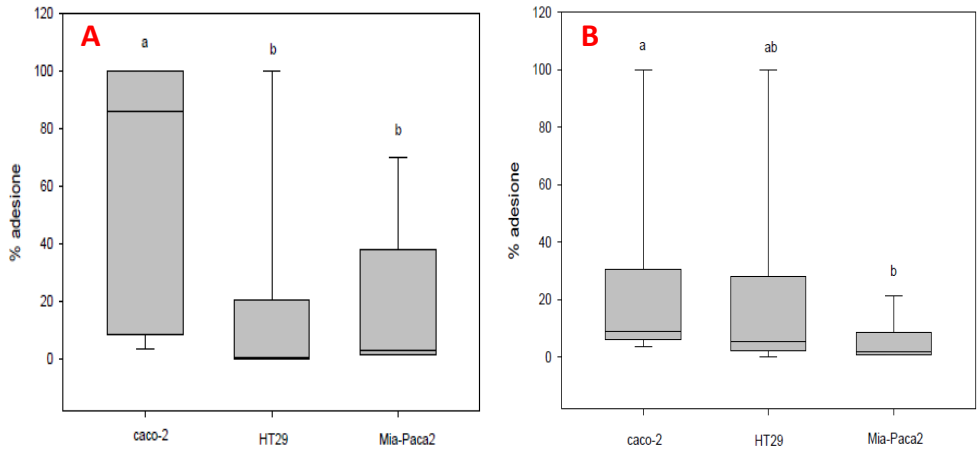

Figure 3.6 Statistical analysis of the adhesion capability of different cell lines evaluated at two different microbial concentrations (A, 10^5 CFU/mL, B, 10^8 CFU/mL). The result is reported as a percentage ratio between adherent bacterial cells and inoculated bacterial cells.

It is interesting to note that the MiaPaca-2 cell line, although non-specific for microbial adhesion tests (remember that it is a line derived from pancreatic adenocarcinoma), had

supported, in the case of bacteria added at concentration equal to 5 log CFU/mL, a greater adhesion respect to the HT29 line. These data, together with the others described above, indicate that adhesion mechanisms depend not only on the bacterial strain used, but also on the epithelial cell capability to allow the adhesion process.

3.7.5. Auto-aggregation

In order to manifest beneficial effects, probiotic bacteria need to achieve an adequate mass through aggregation. Consequently, the ability of probiotics to aggregate is a desirable property. In addition, auto-aggregation ability is considered to be predictive of the adhesive capability of probiotic strains. For this purpose, the auto-aggregation ability of tested strains was determined at different time (1, 2, and 5 hours). Results are shown in Table 3.3. In general, the auto-aggregation ability increased after 2-5 h of incubation in comparison with 1 h of incubation

Table 3.3 Auto-aggregation percentages of *Lb. rhamnosus* and *A. muciniphila strains* evaluated after incubation of 1, 2, and 5 hours.

	%auto-aggregation		
	1h	2h	5h
Lb. rhamnosus GG	4.67 ± 0.12	5.19 ± 0.13	6.57 ± 0.11
Lb. rhamnosus FL2	0.65 ± 0.05	5.40 ± 0.15	5.73 ± 0.09
Lb. rhamnosus FL3	1.51 ± 0.09	9.20 ± 0.16	9.53 ± 0.11
Lb. rhamnosus FL4	0.48 ± 0.09	6.90 ± 0.18	7.70 ± 0.22
Lb. rhamnosus FLC5	0.18 ± 0.05	1.40 ± 0.07	1.75 ± 0.05
Lb. rhamnosus AT194	1.08 ± 0.07	4.68 ± 0.18	4.86 ± 0.07
Lb. rhamnosus AT195	1.70 ± 0.06	3.59 ± 0.12	3.78 ± 0.07
Lb. rhamnosus 39	1.26 ± 0.04	2.51 ± 0.11	22.44 ± 0.43
Akkermansia muciniphila	3.59 ± 0.09	28.70 ± 0.69	30.94 ± 0.36

In particular *A. muciniphila* (30.94%) and *Lb. rhamnosus* 39 (22.44%) showed the highest percentages of aggregation after 5 h of incubation. The other tested strains showed less percentages, which varied from 1.75 % to 9.53%.

3.7.6 Adhesion of bacterial cells to liquid hydrocarbons

As previously stated, the adherence to the intestinal epithelial cells is an important prerequisite for colonization of probiotic strains in the gastrointestinal tract. The hydrophobic properties of the bacterial surface are considered the main factors

underlying the adhesion and biofilm formation both on animated and inanimate surfaces (Doyle & Rosenberg, 1990). In particular, the hydrophobicity is most likely due to complex interactions between positive and negative charges, between hydrophobic and hydrophilic components characterizing the bacterial surface (Abdulla *et al.,* 2014). Studies conducted on the chemical components of the bacterial surface have shown that the presence of glico-protein on the surface is responsible for greater hydrophobicity, while hydrophilic surfaces are associated with the presence of polysaccharides (Perez *et al.*, 1998; Rojas and Conway, 1996). Different techniques, such as adhesion to hydrocarbons, aggregation with salt solutions and adhesion to nitrocellulose filters, have been proposed to measure the hydrophobicity of bacterial cells (Donlon & Colleran, 1993).

Furthermore, different studies have indicated that the surface hydrophobicity of different bacteria, including *Lb. rhamnosus* GG, is a good indicator for assessing the adhesion capability of potential probiotic strains. For example, Xu *et al.* (2009) reported a high correlation between in vitro adhesion to Caco-2 cells and hydrophobicity assessed with xylene for different probiotic bacterial strains. However, other authors have shown a weak relationship between hydrophobicity and adherence to intestinal cell lines (Shillinger *et al.,* 2005). Collado *et al.* (2008) have instead found that hydrophobicity is related to auto-aggregation properties, since strains of lactobacilli with high adhesion in the presence of hydrocarbons have shown high capability of auto-aggregation. Starting from these considerations, in the present study the hydrophobicity of tested strains was evaluated as well as its correlation to adhesion on cell lines.

The results of the hydrophobicity in the presence of xylene and toluene are reported in Table 3.4. In particular, 3 different contact times have been adopted considering different protocols acquired from the literature (Abdulla *et al.*, 2014; Collado *et al.*, 2008; Draksler *et al.,* 2004; Xu et al., 2009).

The strains showed different hydrophobic characteristics. In particular, *Lb. rhamnosus* 39 and GG recorded higher levels of hydrophobicity compared to the other tested strains. A low hydrophobicity has instead distinguished the FLC5 and AT194 strains. The remaining *Lb. rhamnosus* strains showed intermediate hydrophobicity values. *A. muciniphila* exhibited medium-high hydrophobicity characteristics.

Table 3.4 Adhesion (%) to xylene and toluene of *Lb. rhamnosus* and *A. muciniphila* strains evaluated after 15, 30 and 60 minutes (contact time).

		% Hydrophobicity	
	Contact time	Xylene	Toluene
Lb. rhamnosus GG	15 min	55.9 (±0.3)	48.2 (±0.4)
	30 min	64.0 (±0.6)	48.5 (±0.3)
	60 min	64.0 (±0.4)	49.4 (±0.6)
Lb. rhamnosus FL2	15 min	16.5 (±0.3)	19.3 (±0.3)
	30 min	18.0 (±0.4)	21.3 (±0.3)
	60 min	20.9 (±0.5)	30.2 (±0.3)
Lb. rhamnosus FL3	15 min	21.1 (±0.4)	25.8 (±0.3)
	30 min	22.0 (±0.8)	25.8 (±0.5)
	60 min	23.2 (±0.3)	26.2 (±0.3)
Lb. rhamnosus FL4	15 min	25.3 (±0.3)	14.9 (±0.4)
	30 min	26.5 (±0.5)	23.4 (±0.6)
	60 min	28.8 (±0.6)	25.0 (±0.7)
Lb. rhamnosus FLC5	15 min	6.6 (±0.3)	10.0 (±0.6)
	30 min	6.6 (±0.4)	10.0 (±0.4)
	60 min	13.6 (±0.3)	12.6 (±0.4)
Lb. rhamnosus AT194	15 min	2.0 (±0.4)	1.3 (±0.3)
	30 min	2.6 (±0.4)	1.3 (±0.5)
	60 min	6.2 (±0.5)	4.4 (±0.6)
Lb. rhamnosus AT195	15 min	7.3 (±0.6)	10.1 (±0.5)
	30 min	16.9 (±0.3)	11.2 (±0.5)
	60 min	21.7 (±0.3)	12.4 (±0.2)
Lb. rhamnosus 39	15 min	84.8 (±0.5)	93.0 (±0.6)
	30 min	86.9 (±0.7)	93.4 (±0.3)
	60 min	95.4 (±0.6)	93.9 (±0.4)
A. muciniphila	15 min	6.9 (±0.5)	25.6 (±0.4)
	30 min	38.0 (±0.6)	40.7 (±0.6)
	60 min	39.4 (±0.3)	43.7 (±0.4)

The comparison with the results in the literature can be partially done only for *Lb. rhamnosus* GG, for which Collado *et al* (2008) reported a hydrophobicity percentage

with xylene in perfect agreement with that recorded in the present study. Other Authors (Xu *et al.*, 2009) found a 50% of hydrophobicity in the presence of xylene, but for a contact time of only 5 minutes, while Deepica *et al.* (2009) found hydrophobicity percentages for *Lb. rhamnosus* GG equal to about 80% using hexadecane and a contact time of 20 minutes.

The hydrophobicity of the strains expressed in presence of xylene was compared with the hydrophobicity of the strains expressed in the presence of toluene (Figure 3.7).

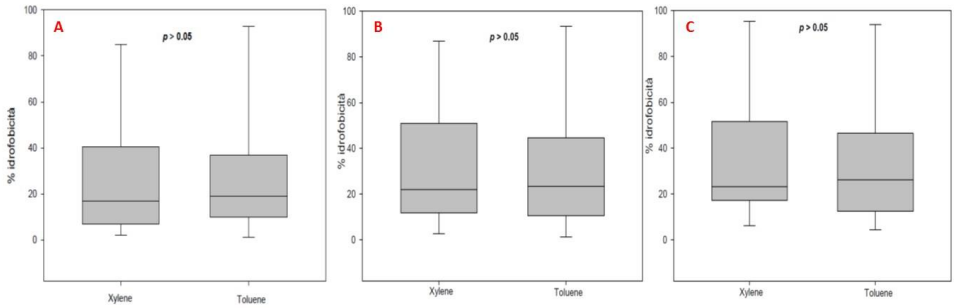

Figure 3.7 Statistical analysis of the percentage of hydrophobicity to xylene and toluene evaluated after 15 (A), 30 (B), and 60 (C) minutes.

The graphs show that the use of xylene or toluene did not significant influence the hydrophobicity of the strains under study in all contact times analyzed. In fact, the median values of the xylene-treated strains were substantially equal ($p > 0.05$) to the median values of the toluene-treated strains at all contact times, 15 min (Fig. 4.6A), 30 min (Fig. 4.6B) and 60 min (Fig. 4.6C).

3.7.7. Correlation between hydrophobicity and adhesion capability

The assessment of surface hydrophobicity through adhesion to different hydrocarbons is still extensively implemented to evaluate the prerequisites of probiotic potential lactic bacteria (Santiago-López *et al.*, 2018). The results reported in the previous paragraph showed a strong heterogeneity among the tested strains in the adhesion tests with the use of xylene and toluene, while no significant differences emerged from the comparison of the hydrophobicity data obtained in the presence of the two hydrocarbons tested. Several authors have noted the presence of a correlation between the adhesion capability of potentially probiotic bacteria and their surface hydrophobicity measured through the use of hydrocarbons (Del Re *et al.*, 2000). Other

Authors (Vinderola *et al.*, 2004) did not find significant relationships. In this study we did not find a significant correlation between the hydrophobicity and the adhesion percentage in the three tested cell lines (Figure 3.8), except for the adhesion on HT29 cells with addition of 10^8 CFU/mL and the hydrophobicity with xylene, contact time 15 min (HT29, ρ = -0.627, p-value = 0.07, Figure 3.8.B), for which a negative correlation was detected, and for adhesion on MiaPaca-2 with addition of 10^5 CFU/mL and hydrophobicity with xylene, contact time 60 min (MiaPaca2, ρ = 0.668, p-value = 0.05, Figure 3.8.C), for which a positive correlation was detected.

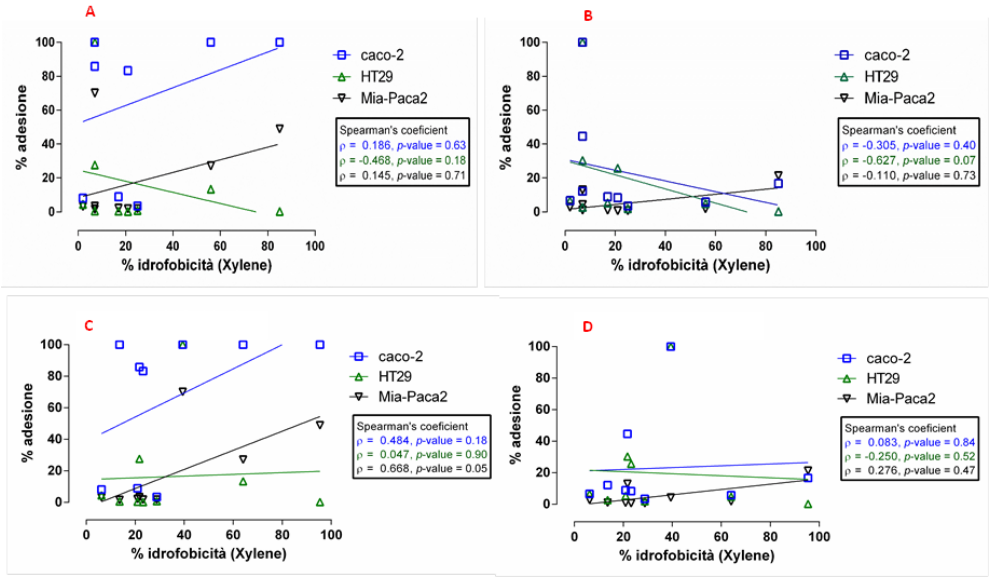

Figure 3.8 Correlation between percentage of adhesion to Caco2, HT29 and MiaPaca2 cell lines and percentage of hydrophobicity with xylene (A, correlation between adhesion percentage with addition of 10^5 CFU/ mL and percentage of hydrophobicity with xylene, contact time 15min; B, correlation between percentage of adhesion with addition of 10^8 CFU/ mL and percentage of hydrophobicity with xylene, contact time 15min; C, correlation between adhesion percentage with addition of 10^5 CFU/ mL and the percentage of hydrophobicity with xylene, contact time 60 min; D, correlation between adhesion percentage with addition of 10^8 CFU/ mL and percentage of hydrophobicity with xylene, contact time 60 min).

These results demonstrated a lack of consistent correlation between hydrophobicity and bacterial adhesion to the cell lines. In one instance out of twelve, there was a positive correlation which could be considered sporous. The lack of correlation was such that in another instance there was even a negative correlation. We conclude that even though hydrophobicity is one of the chemical-physical properties that facilitates the first contact between microorganisms and host cells it is not a determinant factor. Adhesion

is a complex emerging property that also involves surface proteins and lipoteichoic acids (Shillinger *et al.*, 2005)

3.7.8. Correlation between auto-aggregation and adhesion capability

Auto-aggregation has been correlated with adhesion, which is known to be a prerequisite for colonization of the gastrointestinal tract by many microorganisms (Collado *et al.*, 2008). In most cases, aggregation ability is related to cell adherence properties (Del Re *et al.*, 2000).

In this study there we did not find a significant correlation between the auto-aggregation and the adhesion percentage in the three tested cell lines (Figure 3.9), except for the adhesion on HT29 cells with addition of 10^8 CFU/mL and the auto-aggregation at 5h (HT29, = 0.033, p-value = 0.95, Figure 3.9.C), for which a positive correlation was detected, and for adhesion on MiaPaca-2 with addition of 10^8 CFU/mL and auto-aggregation at 5 h (MiaPaca2, = 0.075, p-value = 0.85, Figure 3.9.C), for which a positive correlation was detected.

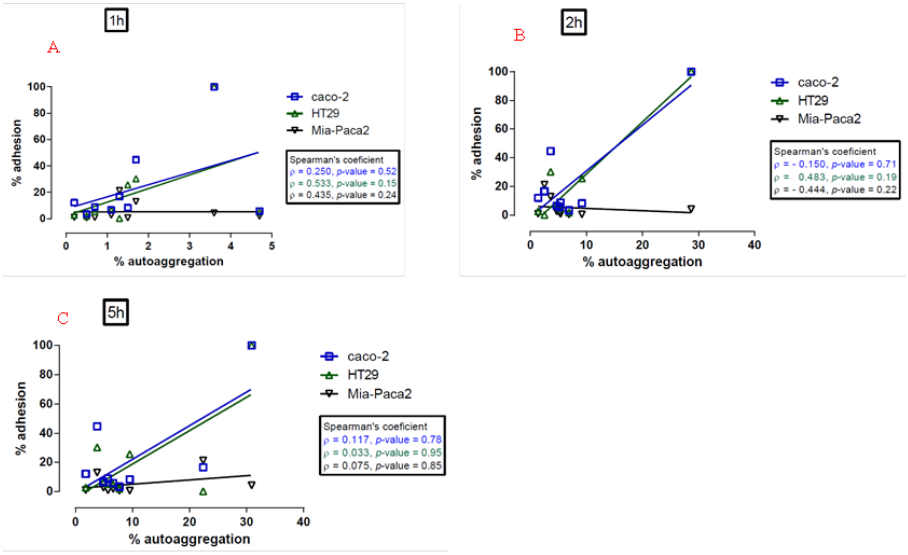

Figure 3.9 Correlation between percentage of adhesion to Caco2, HT29 and MiaPaca2 cell lines and percentage of auto-aggregation at 1h, 2h and 5 h (A, correlation between adhesion percentage with addition of 10^8 CFU/mL and percentage of auto-aggregation at 1h; B, correlation between percentage of adhesion with addition of 10^8 CFU/mL and percentage of auto-aggregation at 2 h C, correlation between adhesion percentage with addition of 10^8 CFU/mL and the percentage of auto-aggregation at 5h).

3.7.9. Biofilm

The experiments performed in this study allowed to measure the rate of adherence and subsequent biofilm formation of tested bacteria. Results of the microtiter-plate tests are summarized in Table 3.5.

Table 3.5 Biofilm production of *Lb. rhamnosus* and *A. muciniphila* strains Strains were classified as follows: OD ≤ ODC=non-adherent (N.A.); ODC < OD ≤ ODC=weakly adherent (W.A.); 2 X ODC <OD ≤ 4X ODC=moderately adherent (M.A.); 4 X ODC <OD=strongly adherent (S.A.).

strains	TSB 0%	TSB0,25%	TSB 1%	TSB 2,5%	MRS
Lb. rhamnosus GG	S. A.	S. A.	S. A.	M. A.	S. A.
Lb. rhamnosus FL2	N. A.	W. A.	N. A.	N. A.	N. A.
Lb. rhamnosus FL3	N. A.	W. A.	N. A.	N. A.	N. A.
Lb. rhamnosus FL4	N. A.	N. A.	N. A.	N. A.	N. A.
Lb. rhamnosus FLC5	N. A.	N. A.	N. A.	N. A.	W. A.
Lb. rhamnosus AT194	N. A.	N. A.	N. A.	N. A.	N. A.
Lb. rhamnosus AT195	N. A.	N. A.	N. A.	N. A.	N. A.
Lb. rhamnosus 39	N. A.	N. A.	N. A.	N. A.	W. A.
A. muciniphila	W. A.	W. A.	W. A.	W. A.	W. A.

According to the literature, the quantitative microtiter-plate assay eliminates subjectivity in reading of obtained results (Deighton & Balkau, 1990). In this study, the most of strains were classified as non-adherent, which showed that they were not able to form a biofilm. Except for *Lb. rhamnosus* GG which was found to be strongly adherent, i.e. capable of forming biofilms. Furthermore, the results showed that the presence of glucose did not affect the adherence, and therefore, the biofilm formation ability in most of the strains.

3.8. Conclusion

In this study, some possible interactions between probiotic strains and the intestinal epithelial cells were evaluated through *in vitro* tests. The results of the adhesion tests to Caco-2 and HT29 cell lines (widely used for this type of evaluation) and MiaPaca-2 have shown that this character is strain-specific in *Lb. rhamnosus*, little or not at all influenced by the isolation matrix (food or human). These data significantly enrich the decalogue of selection procedures for bacterial strains to be used as potential probiotics demonstrating that, at least regarding the adhesion capability/colonization of the large intestine, the origin of the strain has a completely secondary relevance. The intensity of adhesion to the Caco-2 cell line had a higher percentage in the presence of microbial concentrations of up to 10^5 CFU/mL, charges perfectly in line with those found in the intestine after simulated gastro-enteric transit.

A. muciniphila has shown a behavior similar to that described above for *Lb. rhamnosus* strains, with an excellent adhesion capability not only to Caco-2 cells, but also to HT29 and MiaPaca-2.

Finally, it is important to underline that the obtained results indicate with sufficient degree of reliability that the use of hydrocarbons can provide indications on hydrophobicity characteristics of bacterial surfaces wheras not on the adhesion capability to intestinal tissues.

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Final considerations

The objective of this thesis is to contribute to the identification of new potential probiotic strains and to the further development of the screening criteria used in their identification.

In the first part of this work we focused on the differences within strains of the species *Lb. rhamnosus* isolated from different food matrices and feces. Additionally, we worked with combinations of *Lactobacilli* with different prebiotics. We considering eight different strains belonging to the *Lb. rhamnosus* species, including *Lb. rhamnosus* GG and different prebiotics often found in commercial formulas, such as, Lactulose, Fos (fructooligosaccharides), Mannitol and Sorbitol. This was planned to evaluate the growth, survival and different performances (hydrophobicity, auto-aggregation, co-aggregation and antimicrobial activity) of probiotics in presence of prebiotics. The outcome of this study is in agreement with previous studies showing a wide phenotypic diversity among strains of Lb. rhamnosus (Douillard, *et al.*, 2013; Vancanneyt, *et al.*, 2006, Succi, *et al.*, 2005 , Succi, *et al.*, 2017). Our data confirmed mannitol and sorbitol as fermentable substrates for *Lb. rhamnosus* strains, and it indicated that lactulose is a new fermentable substance for *Lb. rhamnosus* GG. Moreover, the results obtained in this study, confirmed that the prebiotics used influenced hydrophobicity, auto-aggregation, and co-aggregation of *Lb. rhamnosus* strains. These results showed a great heterogeneity for adhesion to hydrocarbons (assessed with xylene) among the probiotic strains tested, also, the strains with higher adhesion to hydrocarbons showed high auto-aggregation abilities. Finally, the data obtained supports the hypothesis that co-aggregation is related to auto-aggregation.

In the second part of this work, we evaluated strain safety by testing their antibiotic resistance, furthermore we assessed the efficacy of the bacterial strains, such as *Lb. rhamnosus* and *A. muciniphila*, for their use in humans. To this end is fundamental that the strains can survive in the acidic environment of the stomach, to multiply in the intestine even in the presence of bile salts, and to adhere to an intestinal cell line. Once demonstrated that a probiotic can survive GI transit and influence the GI tract flora it is important to determine its probiotic characteristics.

The Results of survival to gastrointestinal tract evidenced a significant difference on how the strains survived the typical stresses of the gastrointestinal tract. Some *L. rhamnosus* strains showed more sensitivity to exposure to low pH and bile salts, in

contrast to strains, like *A. muciniphila*, that showed excellent performance. Moreover, this study verifies that tolerance to bile stress is a critical factor for survival of *Lb. rhamnosus* strains in the gastrointestinal tract and that bacterial species respond differently to the bile in GITS, demonstrated by death rates that differed by several orders of magnitude. This agrees with the research of Sumeri *et al.*, 2010, where *A. muciniphila* decreased its counts after passing through the stomach (pH2; 2.5 and 3) followed by an increase in the intestinal tract.

The trend of *A. muciniphila* detected during the GI simulated transit was already observed in other studies (Faye, *et al.*, 2012; Possemiers *et al.*, 2010). Some Authors hypothesized that a part of the bacterial population, when exposed to acid gastric stress, enters a so-called viable but non-cultivable (VBNC) state (Possemiers *et al.*, 2010). Under more permissive intestinal conditions, some VBNC cells could return into a cultivable form, explaining the observed viability increase (Faye *et al.*, 2012; de Palencia, *et al.*, 2008). Moreover, de Palencia *et al.* (2008) assumed that the GI-stress conditions provoke the formation of bacterial cell aggregates which could give rise to a single colony on a culture plate, resulting in an underestimation of viable cells. On the contrary, these aggregations could disperse during the intestinal transit.

The results of the adhesion tests to Caco-2 and HT-29 cell lines (widely used by the scientific community for this type of evaluations) and MiaPaca-2 have shown that *Lb. rhamnosus* has strain-specific behaviors, not likely influenced by the matrix from which they were isolation (food or human intestine). This data showed that he origin of the strain has a marginal relevance, at least regarding the adhesion capability / colonization of the large intestine. Regarding the intensity of adhesion to the Caco-2 cell line, a higher percentage of adhesion was found at a starting microbial load of up to 10^5 CFU/mL, which is perfectly in line with those found after simulated gastro-enteric transit.

A. muciniphila showed an excellent adhesion capability both to Caco-2 cells and to HT-29, with values of up to 100% and independent from the microbial load used.

Moreover, Bacterial aggregation and hydrophobicity assays of *Lb rhamnosus* and *A. muciniphila* strains were performed to assess a correlation with their adhesion abilities using Caco-2, HT29 and Mia-Paca 2 cell lines in-vitro models. Aggregation ability has been suggested to be an important property of many bacterial strains used as probiotics, which plays an important role in the formation of biofilms to protect the host from colonization by pathogens (An *et al.*, 2000). Previous research found that bacteria with

aggregation abilities and hydrophobic cell surfaces were more capable to adhere to intestinal cells (Oca e Macias, 2001). Interestingly, the present study did not find a correlation between hydrophobicity/adhesion and auto-aggregation/adhesion as demonstrated by the statistical analysis. This is not in accordance to the research of other authors that have shown a weak relationship between hydrophobicity and adherence to intestinal cell lines. Instead Shillinger *et al.*, 2005 and Collado *et al.*, 2008 found that hydrophobicity is related to self-aggregation properties, since strains of lactobacilli with high adhesion in the presence of hydrocarbons have shown high capability of auto-aggregation.

The different probiotic-related characteristics evaluated do not always correlate and are very strain specific. To date the studied characteristics such as adhesion, survival to GI, auto-aggregation and hydrophobicity are taken together to characterize bacterial strains. Those considered to have high rates of GI survival along with adhesion capacities are usually considered suitable probiotics. However, this choice precludes strains that could have superior probiotic value if they had better GI survival.

Lb. rhamnosus AT195 and *Lb. rhamnosus* 39 are strains that fended poorly in the GI passage survival test but out-did the rest in the adhesion tests. We propose that their incapacity to survive well the intestinal pH should not be considered as a definitive limitation. We are experimenting with their microencapsulation, which would enable them to survive successfully and in sufficient numbers the GI passage. Through the microencapsulation of *Lb. rhamnosus* AT195 and *Lb. rhamnosus* 39 we might obtain an optimum probiotic with the capacity to survive the GI passage and prolifically adhere to the intestinal epithelium. An additional future assay beyond the scope of the present study is to replicate the adhesion assays on the HT-29MTX cell line. This cell line is characterized by being induced to produce mucus, and therefore it might be a more suitable model for the intestine epithelium in the adhesion assay.

A very important outcome of our project was the discovery that *A.* muciniphila. promoted the increased growth of *Lactobacillus sp.* when co-cultured. This is an interesting emerging property that merits more research. To further investigate what seems to be a growth adjuvant property of *A. muciniphila.* on *Lactobacillus sp*, we recommend performing new adhesion tests, such as the ones performed with in-vitro intestinal cell cultures along with GI passage tests using combinations of *A. muciniphila.* and *Lactobacillus sp.*

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