CZECH UNIVERSITY OF LIFE SCIENCES
Faculty of Agrobiology, Food and Natural Resources (FAFNR)

Department of Microbiology, Nutrition and Dietetics

Cell culture-based model for the evaluation of adhesive properties of probiotic bacteria

Bachelor Thesis

Author: Vasiliki Theodorou

Supervisor: doc. Ing. Jaroslav Havlík, Ph.D.

Declaration

I declare that the Bachelor Thesis "Cell culture-based model for the evaluation of adhesive properties of probiotic bacteria" is my own work and all the sources I cited in it are listed in Bibliography.

Prague 15. 4. 2016 Signature _____

Acknowledgements:

I would never have been able to finish my dissertation without the guidance of my supervisor, advisors, support from my family, and help from friends.

I would like to express my deepest gratitude to my supervisor, Doc. Ing. Jaroslav Havlík, Ph.D, for giving me the opportunity to stay and complete my thesis in the Czech University of Life Sciences. I would have never been able to participate in this research without his help, and his excellent guidance.

I am also grateful to my laboratory mentor, Ing. Ivo Doskočil, for providing me with all the necessary facilities for the research, and an excellent and congenial working environment.

I wish to express sincere and special thanks to my advisor, Ing. Veronika Jarošová. Without her continuous encouragement, support and dedicated involvement in every step throughout the process, this paper would have never been accomplished.

I would also like to thank my parents, who have always been on my side, for supporting me in every possible aspect of my life throughout all these years, and for believing in me.

Finally, I would like to thank my dearest and special friends, always willing to help me overcome my stress, cheering me up and stood by me through the good times and the challenging times. .

Cell culture-based model for the evaluation of adhesive properties of probiotic bacteria

Summary

Probiotic microorganisms, defined as living microorganisms which when administered in adequate amounts confer a health benefit to the host, and their adhesion and colonization of intestinal epithelium, are critical factors in maintaining probiotic efficacy. Polyphenols are a large and heterogeneous group of phytochemicals in plant-based foods, such as tea, coffee, wine, cocoa, cereal grains, soy, fruits and berries. In the last decade, there has been much interest in the health benefits of dietary plant polyphenols that arise from their potential ability to promote adhesion of probiotic bacteria to the human intestinal epithelium.

The purpose of this study was to investigate the effect of four polyphenols: isoquercetrin, phloretin, procyanidin B2 and rutin on the adhesion ability of two potentially probiotic strains (*Lactobacillus casei, Lactobacillus gasseri*) to *in vitro* human intestinal epithelial model consisting of Caco-2 and mucus-secreting HT29-MTX co-culture.

The adhesion of *Lactobacillus casei* after treating the co-culture cell lines with isoquercetrin, phloretin, and rutin was increased by 49.76, 72.97, 63.66 % respectively, whereas procyanidin B2 inhibited the adhesion 20.25% compared with the control sample. The adhesion of *Lactobacillus gasseri* after treatment of the co-culture with isoquercetrin, phloretin, procyanidin B2 and rutin was increased by 35.45, 31.28, 45.69, 25.01 % respectively compared with the control sample.

Keywords: microbial adherence, cell culture models, polyphenols, Caco-2, HT29-MTX cancer cell lines

Table of contents

Table of Figures

1 Introduction

Probiotic microorganisms are defined as live microorganisms which, when administered in adequate amounts, confer a health benefit on the host (FAO/WHO, 2001). Lactobacilli, other lactic acid bacteria (LAB) and bifidobacteria are the most common colonizers of the human intestinal mucosa and coexist with the host. Many members of these groups exert additional probiotic properties and provide health benefits to the host (Bermudez-Brito et al., 2013). In order to provide beneficial health effect to the host, probiotic bacteria must survive in adequate amounts the passage through the gastrointestinal tract along with its barriers: acid, bile and gastrointestinal enzymes, and finally adhere and colonize in the intestinal epithelium. Indeed, those functional properties of probiotic microorganisms such as gastrointestinal tolerance and adhesion to intestinal epithelium are critical factors in maintaining probiotic efficacy (Ranadheera et al., 2014).

Nowadays, one of the most important scientific targets is the development of functional foods and substances which can promote a healthy microbial balance and gut wellness (Ranadheera et al., 2014). Over the last twenty years, polyphenols have been studied for their potential involvement in many areas including cancer, cardiovascular problems, inflammation and microbial diseases, like peptic ulcer (Li et al., 2014; Farzaei, Abdollahi & Rahimi, 2015). Polyphenols are naturally occurring compounds found largely in the fruits, vegetables, edible and wild flowers, tea, cereals and beverages. Fruits like grapes, apples, pears, cherries and berries contains up to 200–300 mg polyphenols per 100 grams fresh weight, and the products manufactured from these fruits, also contain polyphenols in significant amounts. They are secondary metabolites of plants and are generally involved in defense against ultraviolet radiation or aggression by pathogens. In food, those substances may contribute to the bitterness, astringency, color, flavor, odor and oxidative stability (Pandy & Rizvi, 2009). Polyphenols could be divided into different groups by the number of phenol rings that they contain and the basis of structural elements that bind these rings, which were classified into several sub-classes, such as the phenolic acids, flavonoids, stilbenes and lignans.

In this study, we attempted to examine the adhesion of two lactobacilli strains to human epithelial intestinal cell lines (co-culture Caco-2 and HT29-MTX) after treatment with four polyphenols: isoquercetrin, phloretin, procyanidin B2 and rutin.

2 Objectives of work

2.1 Objectives of work

The objective of work is to determine the effect of selective polyphenols on the adherence of lactobacilli strains in culture cells model *in vitro.*

2.2 Hypothesis

Cell culture models aid in predicting the adherence properties of bacteria to intestinal epithelium. The adherence is said to be critical property determining the bacterial ability to colonize digestive tract and might be useful in re-establishing bacterial communities in clinical application such as bacterial disbalances following the antibiotic use.

3 Literature overview

3.1 Gastrointestinal System

The digestive system includes two major groups of organs; the gastrointestinal (GI) tract and the accessory digestive organs. The GI tract, or alimentary (nourishment) canal, is a continuous epithelium-lined tube that extends from the mouth to the anus (Desesso & Jacobson 2001), and serves as an interface between the body and the external environment (Schneeman 2002). Organs of the gastrointestinal tract include the mouth, most of the pharynx, esophagus, stomach, small intestine, and large intestine. The accessory organs include the teeth, tongue, salivary glands, liver, gallbladder, and pancreas. Teeth aid in the physical breakdown of food, and the tongue assists in chewing and swallowing. The other accessory digestive organs never come into direct contact with food. They produce or store secretions that flow into the GI tract through ducts; the secretions aid in the chemical breakdown of food (Tortora & Derrickson, 2009)

3.1.1 Description

The functions of the oral cavity are the prehension of food, mastication of the food material, and swallowing of the material while protecting from inhalation of the foodstuffs (Reece et al., 2015). Digestion begins at the mouth which is the entry of the GI tract, and ingested material is physically broken down and mixed with saliva by chewing (mastication). In addition to carrying enzymes that initiate the breakdown of carbohydrates and fats, saliva also lubricates the ingested material to aid in swallowing. The pharynx and esophagus are muscular structures that serve for the transfer of ingested material to aid in swallowing (deglutition). When this material (masticated food) arrives at the stomach, muscular contractions mix it with secreted enzymes to form chyme (molecular fragments of proteins and polysaccharides, droplets of fat, and salt, water, and various other small molecules ingested in the food), a semifluid mixture of solutes, emulsion particles and suspended material. The procedure continuous as the chyme is then released from the stomach to the small intestine via the pylorus, a muscular ring that separates the two. The small intestine is the major site for digestion and the absorption of nutrients, water and electrolytes. It is divided along its length into three unequally sized portions: the duodenum, the jejunum and the ileum. Most absorption occurs in the duodenum and the proximal half of the jejunum (Desesso & Jacobson 2001). The large intestine, which is about 1.5 m (5 ft) long and 6.5 cm

(2.5 in.) in diameter, extends from the ileum to the anus. Structurally, the four major regions of the large intestine are the cecum, colon, rectum, and anal canal. It is the terminal portion of the GI tract. The overall functions of the large intestine are the completion of absorption, the production of certain vitamins, the formation of feces, and the expulsion of feces from the body (Tortora & Derrickson, 2009).

3.1.2 Histology

The inner lining of the digestive tract that starts from the stomach and continuous to the anus is a mucous membrane called mucosa, and consists of a layer of epithelium in direct contact with the contents of the GI tract, a layer of connective tissue called the lamina propria, and a thin layer of smooth muscle the muscularis mucosae.

The type of epithelium that lines the stomach and intestines is simple columnar epithelium, and has two main functions: secretion and absorption. In between the epithelial cells there are exocrine cells (Goblet cells) that secrete mucus and fluid into the lumen of the tract, and several types of endocrine cells (enteroendocrine cells), that secrete hormones into the blood.

Just below the epithelium is a layer of connective tissue, the lamina propria, through which pass small blood vessels, nerve fibers, and lymphatic ducts (Vander et al., 2001)

A thin layer of smooth muscle fibers called the muscularis mucosae separates the lamina propria from underlying tissues. Movements of the muscularis mucosa ensure that all absorptive cells are fully exposed to the contents of the GI tract.

The submucosa is a thick connective tissue layer that lies beneath the mucosa, containing nerves, small glands and many blood and lymphatic vessels that receive absorbed food molecules (Tortora & Derrickson, 2009).

3.1.3 Intestinal epithelial tissue

We can identify four major ways in which the epithelial tissue functions (Rizzo, 2001):

1. It protects underlying tissues. The epithelial tissue of the GI tract protects the underlying tissue from abrasion as food moves through the tract.
2. It absorbs. In the lining of the intestine nutrients from our digested food enter blood capillaries and get carried to the cells of our body.
3. It secretes. All glands are made of epithelial tissue; the endocrine glands secrete hormones, the mucous glands secrete mucus, and our intestinal tract contains cells that

secrete digestive enzymes in addition to the pancreas and the liver, which secrete the major portions of digestive enzymes.

4. It excretes. Sweat glands excrete waste products such as urea.

When epithelial tissue has a protective or absorbing function, it is found in sheets covering a surface, like the skin or intestinal lining. When it has a secreting function, the cells grow from the surface into the underlying tissues to form glandular structures. The cells are very tightly packed together and thus this tissue is not as easily penetrated as other tissues.

Epithelial cells are connected to underlying tissues by a specialized membrane called the basement membrane. It is very important because it acts as an anchor for the attached side of the epithelial cells and it provides protection for other underlying tissue like connective tissue (Rizzo, 2001).

As we mentioned before, according to the classification based on arrangement and shape, the intestinal epithelium is simple columnar epithelium. Simple columnar epithelium is a single layer of tall, thin cells. These large cells contain organelles that enable them to perform complex functions. Moreover, many of these cells are ciliated (microvilli). For example, the simple columnar epithelium of the small intestine produces and releases digestive enzymes that complete the process of digesting food. The columnar cells then absorb the digested foods by active transport, facilitated diffusion, or simple diffusion (Marieb & Hoehn, 2001)

According to the classification based on the function of the epithelial tissue, we must refer to the mucous membrane which lines the digestive, respiratory, urinary, and reproductive tracts. It lines all body cavities that open to the outside. It is usually ciliated (has microvilli). Its most obvious function is to produce mucus. It secretes enzymes for the digestion of food and nutrients before absorption. Mucous membrane protects, absorbs nutrients, and secretes mucus, enzymes, and bile salts.

Moreover, there is also the glandular epithelium which forms glands. Glands are involutions of epithelial cells specialized for synthesizing special compounds. The body has two types of multicellular glands. Exocrine glands have excretory ducts that lead the secreted material from the gland to the surface of a lumen or the skin. Endocrine glands are the second type of multicellular glands in the body. They are ductless and secrete hormones; examples are the thyroid and pituitary glands. Goblet cells are glands that secrete mucus and they are located among the epithelial cells that make up mucous membranes (Rizzo, 2001).

To conclude, the mucosa of the small intestine is simple columnar epithelium with four major cell types (Marieb & Hoehn, 2001):

- Absorptive cells, which have microvilli, produce digestive enzymes, and absorb digested food.
- Goblet cells, which produce a protective mucus.
- Granular cells (Paneth's cells), which may help protect the intestinal epithelium from bacteria.
- Endocrine cells, which produce regulatory hormones. The epithelial cells are produced within tubular glands of the mucosa, called intestinal glands (crypts of Lieberkühn), at the base of the villi. Granular and endocrine cells are located in the bottom of the glands.

3.2 Intestinal cell models

Model systems are fundamental steps in studying the impact of exogenous factors on the composition and/or activity of the gut microbiota. The most commonly used are small animal models and *in vitro* simulations, both of which have generated valuable data, yet both of which are anomalous to the human GI tract (Tuohy & Del Rio, 2014). Ideally, cell models should be similar to the *in vivo* conditions; however, in most *in vitro* experimental models, epithelial cells are cultivated as monolayers, in which the establishment of functional epithelial features is not achieved. To overcome this problem, co-culture experiments with probiotics, dendritic cells and intestinal epithelial cells attempt to resemble the complex and dynamic interactions that exist *in vivo* between the intestinal epithelium and bacteria on the luminal side and between the epithelium and the underlying immune system on the basolateral side (Bermudez-Brito et al., 2013).

All multicellular organisms with an organized intestine carry an intestinal microbiota, and it is well known that lactobacilli, other lactic acid bacteria (LAB) and bifidobacteria have been abundant colonisers of the human intestinal mucosa and coexist with the host. Some members of these groups have additional probiotic properties that provide health benefits to the host via the regulation of immune system and other physiological functions.

3.2.1 *In vitro* models

Even though human clinical trials are the most efficient tool for establishing probiotic functionality, the use of *in vitro* models is necessary to select the most promising strains for these trials. Many *in vitro* studies evaluate the adhesion ability of potential probiotic

bacteria and their interactions with pathogens at the intestinal epithelial interface. The aim of these studies is to understand the immunomodulatory effects of different bacterial strains on *in vitro* cell models, together with evaluating whether the strain-dependent characteristics of commensal bacteria make them appropriate strains for the prevention and treatment of diseases. A wide variety of cells are used as *in vitro* models for probiotic evaluation (Bermudez-Brito et al., 2013).

Three of the most widely used commercially available human cell lines are Caco-2, T84 and HT-29, all of which were isolated from colon adenocarcinomas, express the features of enterocytes and are useful for attachment and mechanistic studies. In the differentiated state, these cell lines mimic the typical characteristics of the human intestinal epithelium. The HT29-MTX is a cell line obtained from HT29 cells adapted to methotrexate(19), which differentiate into goblet cells and secrete mucin (Bermudez-Brito et al., 2013).

For the study of bacterial adhesion to intestinal epithelial cells in particular, Caco-2, HT-29 and HT29-MTX are commonly used. According to Ouwehand and Salminen (2003), due to the difference in cellular structure bacteria exhibit different adhesion to the three types of epithelial cells, and adhesion appears to be higher to HT-29 MTX cells than to HT-29 or Caco-2 cells. Moreover, adhesion of *Salmonella* to HT29-MTX cell model was significantly higher, than to Caco-2 and HT-29 cell models (Gagnon et al., 2013). Data of the same study showed that the HT29-MTX cell model is occasionally more amenable to infection than the Caco-2 cell model and suggested that *Salmonella* penetrates the protective mucus layer and subverts the mucus to enhance invasion. Coppa et al. (2006), based on the fact that breast-fed children have a lower incidence of acute gastroenteritis compared with the bottle-fed ones, proved in their study that human milk oligosaccharides are one of the most important defensive factors because they inhibited the adhesion of three common pathogenic strains that were studied to Caco-2 cells. Specifically, they came to a result that oligosaccharides (as a whole) were effective in inhibiting the adhesion of *Vibrio cholerae* and *Escherichia coli* O119 to Caco-2 cells but not of *Salmonella fyris.* The adhesion of *Salmonella fyris* was inhibited by both the acidic and neutral fraction of oligosaccharides. They based their results on the fact that, the adhesion of a bacterium to the host cell includes binding to receptors present on the surface of cell membranes (of the host cells) made up of oligosaccharidic residues of glycoproteins and glycolipids, and the adhesion to such receptors can be reduced or inhibited by the presence of free

oligosaccharides with a structure analogous to that of cell receptors. Moreover, in the research of Ranadheera et al. (2014), the adhesion ability of *Lactobacillus acidophilus* LA-5, *Bifidobacterium animalis* subsp. *lactis* BB-12 and novel potential probiotic *Propionibacterium jensenii* 702, alone and in various co-culture combinations in fermented goat's milk, was evaluated. The results were that all three probiotics either alone or in combinations with fermented goat's milk were able to adhere to Caco-2 cells, and although there were significant differences among the rates of adhesion of the various probiotic bacteria, substantial numbers of each probiotic were able to attach to the Caco-2 cell layers. Attachment of probiotics to Caco-2 cell layers was further confirmed by scanning through electron microscopy, from which it was noted that the Caco-2 cell layer surface was clearly not fully populated with bacterial cells. Where bacterial cells were attached, there was evidence of considerable clumping in most cases, and autoaggregation (adherence of bacteria which belong to the same strain to each other) and or co-aggregation (adherence of bacteria of two or more different species to each other) abilities of probiotics may have had an effect on adhesion behaviour. In the study of Laparra and Sanz (2009), the adhesion of probiotic: *Lactobacillus rhamnosus* GG and *Bifidobacterium animalis* subsp. *lactis* Bb12, commensal: *Bifidobacterium* animalis IATAA2 and *Bifidobacterium* bifidum IATA-ES2 and potentially pathogenic bacteria: *Escherichia coli* and *Listeria monocytogenes* was determined, with or without mucin (type II which is predominantly expressed in the colon), and different configurations of Caco-2 and/or HT29-MTX cell cultures. The results obtained, showed that the adhesion percentages of probiotic and commensal bacteria were significantly higher than those of potentially pathogenic strains. Also that in general, probiotic bacterial adhesion percentages to Caco-2 cell monolayers were remarkably lower than those to mucin and more similar to commensals and pathogens such as E. coli and L. monocytogenes, respectively. These results seem to suggest the unspecific adhesion of probiotic bacteria to mucin, although the implication of mucus-binding elements similar to those identified and characterized in *Lactobacillus* cannot be ruled out. Finally, the adhesion ability of the different bacterial strains to independent cultures of the HT29-MTX cells was markedly lower than that to Caco–2 cell cultures. In another study where *Lactobacillus salivarius* and *Lactobacillus plantarum* were investigated *in vitro* to examine their ability to competitively exclude Staphylococcus aureus (pathogen that can colonize human and animal intestinal tracts, causing certain gastrointestinal diseases), and the results showed that all *Lactobacillus* strains at any of the concentrations tested resulted in significant reductions in the attachment of S. aureus to Caco-2 cells (Ren et al. 2012). In the study of Hurst (2014), blackcurrant

(*Ribes nigrum*) juices were studied in relation to their chemical composition, antioxidant ability and effects on the proliferation of *Salmonella* and Lactobacilli species and their adhesion to a gut epithelium model. Blackcurrants are berry fruit which contain many polyphenolic compounds, with anthocyanins being the prevalent of the flavonoids. Anthocyanins are well known for their protective effect against cardiovascular disease, cancer and in general for contributing to human health and well-being. Indeed, in this study it was proved from the analysis of pure anthocyanins that they significantly inhibited the adhesion of *Salmonella enterica* serovar Typhimurium to Caco-2 cells, but also all the different brands of juices examined inhibited as well the adhesion (and the proliferation) of *Salmonella enterica* serovar, with their effect being concentration dependent, and had no significant effect on the adhesion of *Lactobacillus rhamnosus* to Caco-2 cells, instead they enhanced its proliferation. In another study, the adhesion of 12 different *Lactobacillus* strains using Caco-2 cell line as an in vitro model for intestinal epithelium was investigated, and among the strains tested *Lactobacillus casei* (Fyos®) was the most adhesive strain, with the adhesion not being significantly different from the adhesion of *Lactobacillus acidophilus* (LC1®), *Lactobacillus rhamnosus* LC-705 and *Lactobacillus* GG (ATCC 53103), and the number of bacteria bound to Caco-2 cell cultures was directly related to the number of bacteria added (Salminen & Tuomola 1998). Furthermore, Forestier et al. (2001), investigated the probiotic activities of a human isolate of *Lactobacillus casei* subsp. *rhamnosus* strain (Lcr35) and by using intestinal Caco-2 cell line as an *in vitro* model, demonstrated that this strain exhibited adhesive properties. At the same study, the inhibitory effects of Lcr35 organisms on the adherence of three pathogens, enteropathogenic *Escherichia coli*, enterotoxigenic *E. coli* and *Klebsiella pneumoniae*, were determined. As far as adhesive properties are concerned, when the pathogens and the probiotic were tested individually the level of adhesion of the pathogens was at least ten times higher than that of *Lactobacillus casei* subsp. *Rhamnosus.* However, the adherence of the three pathogens was decreased by addition of Lcr35, regardless of whether the Lcr35 was added before, during or after the incubation with the pathogen. Gopal et al. (2001), determined the adhesion and colonization properties of three probiotic strains namely, *Lactobacillus rhamnosus* DR20, *Lactobacillus acidophilus* HN017, and *Bifidobacterium lactis* DR10 *in vitro*, using the differentiated human intestinal cell-lines including HT-29, Caco-2, and HT29-MTX. Also in the same study, the inhibitory effect of adhering strains against the intestinal cell monolayer colonization by a known enterotoxigenic strain of *Escherichia coli* (strain O157:H7), was investigated. All three probiotic strains showed strong adhesion with the cell-lines used, and the adhesion index

of all three of them was 2-3 times greater with the mucus-secreting HT-29 MTX cell line. Concerning the enterotoxic *Escherichia coli* strains, it is well known that they are a major cause of bacterial diarrhoea in humans, especially in infants and in travelers to developing countries. According to the results of the same study, pre-treatment of *Escherichia coli* O157:H7 with 2.5-fold concentrated cell-free culture supernatants from *Lactobacillus acidophilus* HN017, *Lactobacillus rhamnosus* DR20 and *Bifidobacterium lactis* DR10 reduced the culturable *E. coli* numbers and also reduced the invasiveness and cell association charcteristics of this toxic strain.

Even though tissue culture cells are often used to assess the adhesiveness of micro-organisms under study, the mucus layer covering the mucosal enterocytes is also an important potential site for colonization. Adhesion to the intestinal mucosa has been suggested to enhance the ability to stimulate the immune system and also, adhesion of lactobacilli to damaged gastric mucosa has been shown to stimulate healing of the tissue (Ouwehand et al., 1999), thus the investigation of the adhesion of probiotics (and pathogens) to human intestinal mucus is a very important aspect included in the *in vitro* models tested for adhesion. In the study of Ouwehand et al. (1999), adhesion to human intestinal mucus of a human faecal isolate, probiotic, dairy and type culture strains was determined and the variation in adhesion between the strains was ranging from 3% (*Lactobacillus casei* 01) to 43% (*Lactobacillus rhamnosus* GG) adhesion of the applied cells. Also, two of the tested dairy strains; *Lactobacillus bulgaricus* and *Lactococcus lactis* ssp. *cremoris*, were found to adhere well. In another research, potential new probiotic strains *Lactobacillus brevis* PEL1, *Lactobacillus reuteri* ING1, *Lactobacillus rhamnosus* VTT E-800 and *Lactobacillus rhamnosus* LC-705 were tested for their adhesion properties using the human intestinal mucus model and simulation of gastric and food processing conditions was provided by exposure to acid, pepsin and milk. *Lactobacillus rhamnosus* E-800, *Lactobacillus reuteri* ING1 and Lactobacillus GG expressed high adhesion to mucus, since more than 30% of the added bacteria adhered, while *Lactobacillus brevis* PEL1 had intermediate adhesion. Moreover, according to the results, pepsin treatment was found to significantly reduce the adhesion of all tested strains, the adhesion of *Lactobacillus brevis* PEL1 *and Lactobacillus reuteri* ING1 was significantly reduced by exposure to low pH, and the exposure of the bacteria to milk before adhesion reduced the adhesion of all tested strains (Ouwehand et al., 2001).

3.3 Intestinal microbiota

It is well known that humans travel with a heavy luggage made up of approximately 10^{14} prokaryotic organisms, mostly bacteria but also viruses and fungi. That is, every one of us has about ten micro-organisms per each "own" eukaryotic cell. This extra weight is distributed in well-defined areas: the skin, the conjunctiva, the vagina, the upper respiratory tract, and especially the GI tract (Sanchez de Medina et al., 2013).

The microbial content of the GI tract changes along its length, ranging from a narrow diversity and low numbers of microbes in the stomach to a wide diversity and high numbers in the colon (Power et al., 2014). In the stomach, gastric acid secretion has a result of a not stable pH raging from as low as 2 to neutral depending on meal times and dietary intake, with numbers of viable cells limited to between 10^2 and 10^4 CFU/mL (Tuohy & Del Rio, 2014). Even though there are some microorganisms that can tolerate the acidic environment and survive passage through the stomach, like certain *Salmonella* and *Shigella* species, in general the low acid conditions of the stomach provide an important barrier to pathogens ingested with food and water. In the intestine, bacterial numbers and diversity are limited by a fast transit time and digestive secretions such as bile acids. In the lower reaches of the small gut (ileum), the movement of gut contents slows and sizeable microbial populations are observed (about 10^6 CFU/ml). The colonic microflora is extremely complex, being made up of more than 500 different species (Steer et al., 2000).

3.3.1 Factors influencing the composition of the microbiota

There are many factors affecting the composition of the gut microbiota like genetics of the host and geography, age, diet, medication and disease.

3.3.1.1 Age

The human microbiota is established during birth or/and shortly afterwards, and the intestine becomes inhabited by a population that is characterized by instability. At birth the gut is sterile and as the infant is exposed to bacteria in its environment, the birth canal, maternal faecal bacteria and other sources, the colonization process begins (Edwards & Parrett 2002). There are also other factors influencing the microbiota like gestational age, hospitalization of the infant, antibiotic use and infant feeding (Power et al., 2014). Several studies have shown that the flora of the breast-fed infant is dominated by *Bifidobacterium*

and *Ruminococcus*, whereas colonization by *Escherichia coli*, *Clostridium difficile*, *Bacteroides fragilis* group bacteria and lactobacilli being significantly lower than those observed in exclusively formula−fed infants, which leads to the conclusion that even though formulas provide a safe, nutritious and healthy food for growth and development, they cannot replicate the bioactive and immunomodulatory properties of breast milk (Mountzouris, Mccartney & Gibson 2002). During adulthood the composition of the intestinal microbiota is relatively stable, but this relative stability is reduced in old age.

3.3.1.2 Diet

Diet is one of the most important external factors, related to both human disease risk and gut microbiota function. The microbiota, depending on dietary composition (nutrient availability), can produce either harmful metabolites related to human disease or beneficial compounds that protect against host disease. It is estimated that 20-60 g of dietary carbohydrates (resistant starch, plant cell wall polysaccharides and non-digestible oligosaccharides), that escape digestion in the upper gut, reach the colon on a daily basis (Power et al., 2014). The intestinal microbiota is predominantly fermentative, with the fermentation of dietary carbohydrates being the one to provide energy and carbon sources for fermentative species themselves and supporting a complex food web in which the end product of one microorganism is the growth substrate for another and of course, the human host. Even though fermentation of amino acids is energetically less favourable than carbohydrate fermentation, some microorganisms will ferment amino acids either from endogenous or dietary protein releasing end products which are potentially harmful to human health and have been linked to diseases such as cardiovascular disease and colon cancer. Most of the dietary fat is absorbed in the upper gut, yet some can reach the colon. Bacteria are poor utilizers of lipids under anaerobic conditions and they cannot receive enough energy from the conversion of dietary lipids into either beneficial or harmful fatty acids. To conclude, it is important to mention that even though high-fat, low-fibre, diets provide to the host even more energy than the amount that he actually needs, they contribute to the establishment of aberrant microbiota profiles shown to increase the risk of metabolic and autoimmune disease (Tuohy & Del Rio, 2014).

3.3.1.3 Antibiotics

Different types of antibiotics have different types of action mechanisms and as a result, different effects on the human microbiota. In addition, individual human responses may be different. Bifidobacteria are typically susceptible to the majority of clinically relevant

antibiotics such as penicillins (Maukonen & Saarela 2015). In general, antibiotic treatment leads to a decrease in the diversity of the microbiota and studies have shown that there are some members of the microbial community quite resilient and can resemble the pre-treatment state in a matter of days or weeks, as well as other members failing to return to pre-treatment levels and these may even be lost from the community indefinitely (Power et al., 2014).

3.4 Probiotics

3.4.1 History-Current definition of Probiotics

It is well known that fermentation is one of the oldest methods of preserving foods. By 6000 BC, cheese was being made from cow's and goat's milk in China, and fermented products such as kefir, koumiss, leben, and dahi were also used therapeutically long before the existence of microorganisms was discovered by Leeuwenhoek in 1683. Louis Pasteur isolated lactic acid bacteria from milk in 1857 (Makinen et al., 2012), but it was in 1907 that the concept of probiotics was born after Elie Metchnikoff's publication of the book entitled *The Prolongation of Life*. In this book, Metchnikoff suggested that people should consume fermented milk containing lactobacilli to prolong their lives (Maity & Maity, 2009).

During this time Henry Tissier, a French paediatrician, observed that children with diarrhoea had in their stools a low number of bacteria characterized by a peculiar, Y shaped morphology, whereas these "bifid" bacteria were abundant in healthy children. He suggested that these bacteria could be used as a treatment to patients with diarrhoea to help them restore a healthy gut flora (FAO/WHO, 2001).

Metchnikoff and Tissier were the first to make scientific suggestions about the probiotic use of bacteria, even if the term "probiotic" was coined by Kollath in 1953, which he defined as "active substances that are essential for a healthy development of life". In 1989 Fuller, redefined the word as "a live microbial feed supplement which beneficially affects the host animal by improving its intestinal balance". In 1992 a quite similar definition was proposed by Havenaar and Huis in 't Veld , "a viable mono or mixed culture of bacteria which, when applied to animal or man, beneficially affects the host by improving the properties of the indigenous flora" (FAO/WHO, 2001). Nowadays, the most accepted definition of the term, is the one provided by the 2001 joint FAO/WHO expert consultation that probiotics are "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host" (Gil et al., 2013).

3.4.2 Taxonomy and Selection

The human body contains diverse groups of commensal microbiota which regulate intestinal epithelial development and function and any interruption of these interactions may result in disease conditions. The beneficial effects of the gut microbiota are attributed to probiotics (Gaudana et al., 2010). Most probiotic organisms are lactobacilli and bifidobacteria, representatives of which are normal inhabitants of the human gut (Wohlgemuth et al., 2010).

3.4.2.1 The genus *Lactobacillus*

Most of the microorganisms isolated from fermented products belong to the *Lactobacillus* (Figure 1) genus. It is a fact that lactic acid bacteria (LAB) and other microorganisms (including some species of bifidobacteria) are well known and have been used for centuries in fermentation of dairy products. Spontaneous milk fermentation has a long history in different regions of Mongolia or Africa, and the use of beneficial microorganisms in fermented dairy products has been practiced for many generations. These traditional fermented milks contain complex composition of LAB species and therefore provide a useful source of probiotic strains (Gil et al. 2013). Lactobacilli are Gram-positive, microaerophilic, catalase negative microorganisms, and according to *Taxonomic Outline of the Prokaryotes* (Felis et al., 2007), the genus *Lactobacillus* belongs to the phylum *Firmicutes*, class *Bacilli*, order *Lactobacillales*, family *Lactobacillaceae*.

3.4.2.2 The genus *Bifidobacterium*

Bifidobacteria are widely distributed among living organisms that provide their offspring with parental care such as mammals, birds and social insects, and there are no bifidobacteria that have been isolated so far from other animals such as reptiles and fish. Therefore, an important reason of their ecological distribution may be due to direct transmission of bifidobacterial cells from parent/carer to offspring. Bifidobacteria are common inhabitants of the mammalian gut, but are also found in three other ecological niches: human blood *(Bifidobacterium scardovii*), sewage (e.g., *Bifidobacterium minimum* and *Bifidobacterium thermacidophilum*) and food products (e.g., *Bifidobacterium animalis subsp. lactis*) (Tuohy & Del Rio, 2014). Bifidobacteria are Gram-positive anaerobic catalase negative, (with some exceptions) microorganisms, and According to the *Taxonomic Outline of the Prokaryotes* (Felis et al., 2007), the genus *Bifidobacterium* belongs to the phylum *Actinobacteria*, class *Actinobacteria*, subclass *Actinobacteridae*, order *Bifidobacteriales*, family *Bifidobacteriaceae.*

3.4.2.3 Selection

To be considered a probiotic, a strain should be able to colonize the GI tract and promote host health through its metabolic activities (Genove et al. 2013). Specifically, the theoretical basis for selection of probiotic microorganisms (Figure 2) includes safety, functional (survival, adherence, colonisation, antimicrobial production, immune stimulation, antigenotoxic activity and prevention of pathogens) and technological aspects (growth in milk, stability, viability in processes) (Saarela, Mogensen & Fonde. 2000).

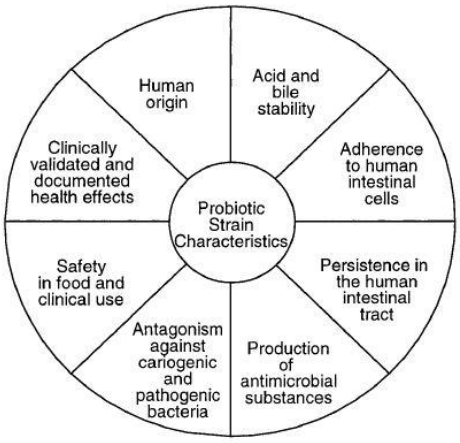

Figure 1: The theoretical basis for selection of probiotic microorganisms (Saarela et al., 2000)

3.4.3 Mechanisms of action

The mechanisms of action of probiotics have not been clearly understood and more studies are needed to prove them, however there are many results from in vitro experiments and animal models ((Daliri & Lee 2015) which show that probiotics are involved in production of antibacterial substances, improvement of the barrier functions of gut mucosa, competitive exclusion of pathogenic bacteria and modulation of host immune functions (Amara & Shibl 2015). Specifically, concerning the inhibition of pathogenic bacteria, one way is the production of bacteriocins. In detail, these are proteins or protein complexes, produced by certain strains of bacteria, which can have antagonistic action against species that are closely related to the producer bacterium (Fooks & Gibson, 2002). Although probiotic

strains may produce bacteriocins, their role in the pathogen inhibition in vivo can only be limited, since traditional bacteriocins have an inhibitory effect only against closely related species such as other *Lactobacillus* or on sporefomers such as *Bacillus* or *Clostridium*. However, low molecular weight metabolites (such as hydrogen peroxide, lactic and acetic acid, and other aroma compounds) and secondary metabolites may be more important since they show wide inhibitory spectrum against many harmful organism like *Salmonella*, *Escherichia coli*, *Clostridium*, and *Helicobacter* (Saarela et al. 2000). As a matter of fact, lactic acid lowers the local pH and inhibits the growth of bacteria sensitive to acidic conditions (Wohlgemuth et al., 2010). Concerning the competitive exclusion of pathogenic bacteria, it relies on binding to the same receptor sites on the epithelial surface by probiotic and pathogenic bacteria. Regarding the immunomudalotory properties, gut associated lymphoid tissue may have contact with adhesive probiotic strains and their components and therefore adhesion is one way of provoking immune effects. Many human studies have shown that probiotic bacteria can have positive effects on the immune system of their host (Saarela et al. 2000), like for example the regulation of pro- and anti-inflammatory cytokine production by direct interactions with immune cells (Wohlgemuth et al., 2010). For the activation of those mechanisms, probiotic bacteria need first to survive the passage through the GI tract, which includes the low pH and antimicrobial action of pepsin in the stomach as well as the bile and gastric enzymes, and then to adhere and colonize the epithelium.

One of the most important factors which influences their viability and colonization is the food matrix. According to Lee and Puong (2002), carbohydrates have been shown to inhibit adhesion of bacteria to the intestinal cell surface, and the results of their study proved that the eight carbohydrates tested, inhibited the adhesion of *Lactobacillus casei* Shirota to the Caco-2 intestinal cell line, and the adhesion of *Lactobacillus rhamnosus* GG to Caco-2 cells was affected by only one of the carbohydrates tested. Moreover, the adhesion of *Lactobacillus acidophilus* LA-5 *Bifidobacterium animalis* subsp. *lactis* BB-12 *Propionibacterium jensenii* 702 using goat's milk ice cream, plain and fruit yoghurts was evaluated in another study, and the results obtained showed that compared to the initial cells, the proportion of cells of each probiotic strain that were found to adhere to Caco-2 cell layers were relatively low in each carrier food type and the number of viable bacteria that were able to attach to the Caco-2 cells were 10^5-10^6 cfu/g. In the same study it was proved that fruit yoghurt improved the adhesion of all three probiotics and the adhesion was higher in the fruit yoghurt than the ice cream (Ranadheera et al. 2012). Also, according to the study of Volstatova et al. (2015),

acid−hydrolized milk had an effect on reducing the adhesion *Lactobacillus gasseri* R and *Lactobacillus plantarum* S2. All these studies clearly indicate the potential importance of the food matrix as a factor influencing probiotic colonization of the gut (Ranadheera et al. 2012), but it is necessary also to add that according to Isolauri, Salminen, and Gueimonde (2011) who isolated *Lactobacillus rhamnosus* GG from specific probiotic products, all the isolates tested showed an ability to adhere to human colonic mucus that did not vary significantly, whereas pathogen exlusion by inhibition and competition varied significantly among the different *Lactobacillus rhamnosus* isolates. Specifically, this study concluded that, since the isolates tested were chosen to be from different products and origins, apart from the food matrix, the manufacturing process (industrial production) has a significant impact on the strain properties. For example, Iaconelli et al. (2015), came to a result that the type of the drying process (air-drying, freeze-drying and spray-drying) have an impact on viability and functionality on three types of probiotic bacteria: *Bifidobacterium bifidum, Lactobacillus plantarum* and *Lactobacillus zeae*. Some of the results presented in their study are that the freeze drying process without protective agents, gave the best result for cultivability, enzymatic activity and cell integrity but caused the greatest growth retardation, as well as that adherence can be stimulated (air-drying) or inhibited (spray-drying) by drying process.

Another very important factor that affects pathogen inhibition, maintenance of microbial balance, immunomodulation, and enhancement of the epithelial barrier function is the diversity of the cell surface of Lactobacilli and their ability to express specific surface components or secrete certain compounds, in response to the host environment. It is well known that they have developed responses and adaptations to survive environmental stress factors during their transit through the GI tract, like low pH, bile acids and starvation stress, by the expression or suppression of genes which alter cellular process like cell division, membrane composition and DNA metabolism, and finally adhere to the epithelium and exclude other pathogens. For example, components of the surface wall like mucus binding proteins (adhesins, S-layer proteins) and polysaccharides play major roles in the adherence of Lactobacilli to the intestinal epithelium. Also, studies have shown direct interaction between *Lactobacillus kefir* S-layer proteins and *Salmonella* surface adhesins. Pre−treatment of *Salmonella* with purified S-layer proteins has been shown to protect two human intestinal epithelial cells , parental Caco-2 and the TC-7 clone from *Salmonella* invasion (Sengupta et al. 2013).

3.4.4 Clinical and medical aspects of probiotics

Probiotics have gained growing popularity in the past two decades because of their beneficial health effects backed by abundant scientific evidences, and some of the clinical effects are demonstrated in Table 1. The proposed favourable effects of probiotics on human health include amelioration of gastrointestinal health, improvement of lactose intolerance, and reduced risk of various other gut- and metabolism-associated maladies. Various probiotic strains, especially Lactobacilli and Bifidobacteria, are now commercially accessible for human use. However, the characteristics of probiotic strains and their function, efficacy, and safety in relation to the gastrointestinal health and environment remains to be fully elucidated, and therefore needs to be further explored.

Many studies have proved that there is a connection between the consumption of probiotics and cholesterol assimilation. Two of the main lipoproteins that transport cholesterol are the low-density lipoprotein- (LDL) and high-density lipoprotein (HDL)-cholesterol. During hypercholesterolaemia, higher concentrations of LDL and lower concentrations of HDL is usually found in blood and the excess can accumulate on the walls of the arteries, and together with plaques, this leads to the narrowing of the arteries. There are many risks associated with hypercholesterolaemia, ranging from coronary artery disease to heart attack and stroke, causing morbidity and mortality (Venema & Do Carmo, 2015). Many clinical trials examined the effects of probiotics on LDL-C in order to explore their potential as a therapeutic agent. A total of 26 clinical studies and two meta- analyses were reviewed and of the probiotics examined, *Lactobacillus reuteri* NCIMB 30242 was found to best meet therapeutic lifestyle change dietary requirements by significantly reducing LDL-C and total cholesterol, improving other coronary heart disease risk factors such as inflammatory biomarkers, and being generally recognized as safe. Thus, concluded that *Lactobacillus reuteri* NCIMB30242 is a viable candidate for both future dietary studies and as a potential option for inclusion in dietary recommendations in patients with hypercholesterolemia (Mizock 2015). According to Maity and Maity (2009), A Dutch trial involving 30 healthy men also found that consuming yoghurt fermented with *Lactobacillus. acidophilus* cultures for several weeks decreased both total and LDL cholesterol levels by 4.4 and 5.4 % respectively compared with controls, and an 1998 study by Taranto *et al.*, identified that supplementation with *L. reuteri* CRL 1098 (104 cells/day) for 7 days in Swiss albino hypercholesterolaemic mice increased the ratio of HDL to LDL by 20% relative to control

mice (Venema & Do Carmo, 2015). Guardamagna et al. (2014), made a study, which was at the same time a first clinical experience, to evaluate the effects of a probiotic formulation containing three *Bifidobacterium* strains (*Bifidobacterium animalis* subspecies *lactis* MB 2409, *Bifidobacterium bifidum* MB 109B, and *Bifidobacterium longum* subspecies *longum* BL04) on lipid profiles in children affected by primary dyslipidaemia, and concluded that the administration of properly selected probiotics was mildly effective in improving the lipid profilein children affected by primary dyslipidaemia when compared with placebo group. All these studies suggest that probiotics can have a positive influence on blood lipids which are commonly elevated in obesity, metabolic syndrome and cardiovascular disease, however more studies and clinical trials are needed to examine all the factors related to those diseases. For example, Ivey et al. (2015), in their study with the aim to determine the effect of *Lactobacillus acidophilus* La5 and *Bifidobacterium animalis* subsp *lactis* Bb12, provided in either yoghurt or capsule form, on home blood pressure and serum lipid profile, came up with no evidence that blood pressure, heart rate, or lipid concentrations were altered.

There is an increase in the number of studies providing the potential for probiotic microorganisms to modulate the immune response and prevent onset of allergic diseases. Most of the clinical studies until now are still limited and include pregnant women and their new-borns. A study which included 159 expectant mothers with either a first-degree relative or partner with atopic disease and groups were randomised and given *Lactobacillus rhamosus* GG or placebo prenatally, continued through breast-feeding and given to the infant for the first six months after birth, concluded that the incidence of atopic dermatitis in the probiotic group was 23% compared with 46% in the placebo. These results suggest that a modulation in the microflora and an increase in immune-modulatory cytokines in both the mother and infants lead to a reduction in the potential of the infant for atopic dermatitis (Furrie 2005). In other clinical studies with infants allergic to cow's milk In other clinical studies with infants allergic to cow's milk, atopic dermatitis was alleviated by ingestion of probiotic strains *Lactobacillus rhamnosus* GG and *Bifidobacterium lactis* BB-12 (Joint FAO/WHO Expert Consultation, 2001).

Another very important health aspect of probiotics is related to colorectal cancer (CRC). CRC is the third most common cancer and the fourth leading cause of cancer death worldwide and its incidence keeps increasing not just in all Western countries, but also in the developing ones. CRC is mainly influenced by environmental factors such as, diet and dietary habits, physical inactivity, consumption of tobacco and other occupational hazards. Food carcinogens produced during cooking at elevated temperatures and air pollution seem to be potential risk

factors for CRC and other cancer types (Ambalam et al., 2016). As far as diet is concerned, it appears that a high intake of animal fat (like red and processed meat) and a low intake of fibre and fish play a role in the pathogenesis of CRC. The colonic microbiota is involved in the aetiology of CRC and many studies have indicated that there is a difference in the composition of gut microbiota in CRC patients from healthy controls. Bacteria that have been found to be more abundant in stools of patients with CRC include anaerobes such as *Bacteroides* and *Clostridium* species, but also *Enterococcus, Escherichia, Shigella, Klebsiella, Streptococcus* and *Peptostreptococcus* have as well been reported to be present in increased quantities (Mizock 2015). Thus, probiotics may alter intestinal microbiota composition and reduce colonic adenocarcinoma and apart from studies including animal models, there are many others that proved that *Lactobacillus paracasei* subsp. *paracasei* LC01 (LC01) consumption in healthy young adults significantly decreased *Escherichia coli* and increased *Lactobacillus*, *Bifidobacterium*, and *Roseburia intestinalis* population and that four-week commercial yogurt consumption supplemented with *Bifidobacterium animalis* subsp. *lactis* (BB-12) and *Lactobacillus acidophilus* (LA-5) significantly increased the faecal numbers of Bifidobacteria and Lactobacilli and decreased counts of faecal Enterococci.

Inflammatory bowel diseases (IBD), including Crohn's disease (CD) and ulcerative colitis (UC), are characterized by chronic intestinal inflammation resulting from the interaction among genetic factors, environmental factors (antigens derived from commensal bacteria) and the immune system (Cammarota et al. 2015), but the exact aetiology of these disorders is not clear. CD can affect any part of the GI tract but the most commonly affected parts are the lower ileum and colon, and it is characterized by discontinuous inflammation of the epithelial lining and deep ulcers. UC affects only the colon and rectum and involves continuous mucosal inflammation and superficial ulcers. The clinical symptoms of IBD are abdominal pain, diarrhoea, rectal bleeding, malaise and weight loss (Power et al., 2014). The fact that there is an increase in the incidence and prevalence of IBD over the past two years in the developed countries (Western Europe and North America) can be related to the diet, since many studies associate high fat and simple sugars intake with IBD and others which show that diets with eliminated or refined carbohydrates resulted to a positive reaction of more than 50% of patients with CD (Steer et al., 2000), due in part to effects on the intestinal microbiome (Mizock 2015). Indeed, several studies have proved that there are differences in the composition (and function) of the gut microbiota of patients with IBD compared to healthy subjects, like low amount of Bifidobacteria in patients with CD (Maity & Maity 2009), but at the same time some changes in the composition of the microbiota are

similar between the UC and CD patients. However, it is still unclear if these shifts cause the disease or arise due to the changes in the gut environment that result from the disease (Power et al., 2014). All these observations suggest the possibility of preventing or treating IBD by manipulating the local microenvironment, and therefore increasing evidence supports the potential therapeutic role of probiotics in IBD for the microbial balance to be restored. Even though there is currently no strong evidence from clinical studies to support the use of probiotics as maintenance therapy for UC or CD and more future studies are needed (Tamboli & Caucheteux 2003), there are many encouraging results obtained from studies that probiotic therapy was used in several animal models with experimental colitis as the administration of *Lactobacillus* sp. has been shown to significantly reduce the inflammation in rats and mice (Amadini et al. 2002).

Table 1: Clinical effects of some probiotic and yoghurt strains (Saarela, Mogensen, & Fonde, 2000)

Strain	**Clinical Effect**
***Lactobacillus rhamnosus* GG (ATCC 53103)**	Lowering faecal enzyme activities, reduction of antibiotic-associated diarrhoea in children, treatment and prevention of rotavirus and acute diarrhoea in children, treatment of relapsing Clostridium difficile diarrhoea, immune response modulation, alleviation of atopic dermatitis symptoms in children
***Lactobacillus johnsonii* (*acidophilus*) LJ-1 (La-1)**	Modulation of intestinal flora, immune enhancement, adjuvant in Helicobacter pylori treatment
***Bifidobacterium lactis* Bb-12**	Prevention of traveller's diarrhoea, treatment of viral diarrhoea including rotavirus diarrhoea, modulation of intestinal flora, improvement of constipation, modulation of immune response, alleviation of atopic dermatitis symptoms in children

Lactobacillus reuteri **(BioGaia Biologics)**	Shortening of rotavirus diarrhoea in children, treatment of acute diarrhoea in children, safe and well-tolerated in HIV-positive adult subjects
Lactobacillus casei **Shirota**	Modulation of intestinal flora, lowering faecal enzyme activities, positive effects on superficial bladder cancer and cervical cancer, no influence on the immune system of healthy subjects
Lactobacillus plantarum **DSM9843 (299v)**	Modulation of intestinal flora, increase in faecal short-chain fatty acid content
Saccharomyces boulardii	Prevention of antibiotic-associated diarrhoea, treatment of Clostridium difficile colitis, prevention of diarrhoea in critically ill tube-fed patients
Yoghurt strains (*Streptococcus thermophilus*, and/or *L. delbrueckii* subsp *bulgaricus*)	No effect on rotavirus diarrhoea, no immune enhancing effect during rotavirus diarrhoea, no effect on faecal enzymes, weak effect on respiratory burst activity of blood leukocytes but not on overall phagocytic activity in healthy adults

4 Material and Methods

4.1 Bacterial Strains

The two lactobacilli strains used, *Lactobacillus casei* and *Lactobacillus gasseri*, were obtained from the collection of the Department of Microbiology, Nutrition and Dietetics, Faculty of Agrobiology, Czech University of Life Sciences.

4.2 Preparation of polyphenolic compounds

The polyphenols phloretin, procyanidin B2, isoquercitrin and rutin were obtained from Extrasynthese (Genay Cedex, FR). The solutions of each polyphenols compounds were prepared by appropriate dilution in PBS for the final concentration of 25 μg ml^{-1}.

4.3 Cell Cultures

The adhesion ability of the two lactobacilli strains in the presence of four polyphenols (isoquercetrin, phloretin, procyanidin B2 and rutin), was assessed by the use of the human epithelial intestinal cell lines Caco-2 (colorectal adenocarcinoma) and HT29-MTX (mucin producing). The two cell lines were grown in Dulbecco's modified Eagles medium (DMEM) supplemented with 10% foetal bovine serum (FBS), 1% nonessential amino acids, 100 U ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin. The cell lines were maintained at 37°C in a humidified atmosphere containing 5% (v/v) CO_2 and 95% air. The medium was changed every two days, and the cells were sub-cultured at 80% confluence every week (Volstatova et al., 2015).

4.4 Bacterial Suspension

The two lactobacilli strains were grown anaerobically on Man, Rogosa, and Sharpe (MRS) broth (Oxoid) at 37°C for 24 h, diluted in Dulbecco's phosphate-buffered saline (DPBS; Sigma-Aldrich). Bacteria were centrifuged (2 000 × g, 10 min); the pellet was washed twice with phosphate-buffered saline (PBS; pH 7.0). The bacterial suspension was diluted in PBS to a final concentration of 2 × 108 CFU ml^{-1} by measuring the optical density at 420 nm (Volstatova et al., 2015).

4.5 Adhesion Assays

Combined co-culture Caco-2/HT29-MTX in ratio 9:1 was used as the adhesion model and the cell lines (before the adhesion assays) were seeded in 24-well culture plates at concentration

of 3.6 × 104 cells per well (Caco-2) and 4 × 103 cells per well (HT29-MTX) and grown 14 ± 1 days past confluence at 37°C in a humidified atmosphere containing 5% CO2 and 95% air. The culture medium was changed every two days and the cell layers were washed with DPBS to remove the antibiotics from the original cell media. Bacterial suspension of 100 µl volume was added to previously washed cell monolayers. After that, 4 different types of polyphenols (concentration of 25 µg ml^{-1}) were added along with the bacterial suspensions at a ratio 10:1 (bacteria/eukaryotic cell). As a control wells was added 100 µl of PBS. For each strain, controls and treated wells were set in triplicate. Then, the plates were incubated at 37°C for 1 h under 5% CO2. After the incubation period, supernatants were removed and the cell layers were softly washed three times with Dulbecco's PBS to remove non-attached bacteria. In the end the cell layers were trypsinized by addition of 300 µl 1% Triton-X100 (Sigma-Aldrich) per well for 3 min followed by addition of 700 µl PBS. The remaining suspensions with viable adhered bacteria were diluted and plated on MRS agar (Oxoid) in Petri dishes. Bacterial counts were determined after aerobic incubation for 48 h at 37°C (Volstatova et al., 2015).

5 Results

5.1 Concentration of polyphenols

Caco-2, HT29-MTX and co-culture cells were treated with eight different concentrations of polyphenols (5-500 μg/mL) for 72h. The effect on cell viability was assayed by the MTT method. Finally, the concentration considered appropriate for all the polyphenols used to treat Caco-2, HT29-MTX and the co-culture (Caco-2 and HT29-MTX) cell lines was 25 μg/mL.

5.2 Bacteria adhesion assay

5.2.1 Adhesion of *Lactobacillus casei*

As it is demonstrated in Figure 3, all the polyphenols except procyanidin B2 provided the adhesion of *Lactobacillus casei* to the co-culture (Caco-2 and HT29-MTX). Specifically, after treatment of the co-culture with isoquercetrin, the adhesion was increased by 49.76% compared with the control sample. Respectively, for phloretin it was 72.97% and for rutin 63.66%. It can be easily observed that treatment with phloretin had the most significant effect on the adhesion of *Lactobacillus casei* to the co-culture, followed by rutin and after that isoquercetrin. The only polyphenol which significantly inhibited the adhesion of *Lactobacillus* was procyanidin B2 (20.25% compared with the control sample).

5.2.2 Adhesion of *Lactobacillus gasseri*

As it is demonstrated in Figure 4, all the polyphenols provided the adhesion of *Lactobacillus gasseri* to the co-culture (Caco-2 and HT29-MTX). Specifically, after treatment of the co−culture with isoquercetrin, phloretin, procyanidin B2 and rutin the adhesion was increased by 35.45%, 31.28%, 45.69%, 25.01% respectively compared with the control sample. It can be easily observed that treatment with procyanidin B2 had the most significant effect on the adhesion of *Lactobacillus gasseri* to the co-culture (followed by isoquercetrin, phloretin and rutin) which is interesting because comparing to the results of adhesion of *Lactobacillus* casei, it was the only polyphenol which significantly inhibited the adhesion. It is also important to observe that the percentages of adhesion *Lactobacillus* casei for all the polyphenols (apart from procyanidin B2) are significantly higher than those demonstrated for *Lactobacillus gasseri*.

Table 2: Treatment of the co-culture (Caco-2 and HT29-MTX) cell lines with four different polyphenols and percentage of adhesion of *Lactobacillus casei* compared with the control sample

Treatment	**%**
Control	100.00
Isoquercetrin	149.76
Phloretin	172.97
Procyanidin B2	80.25
Rutin	163.66

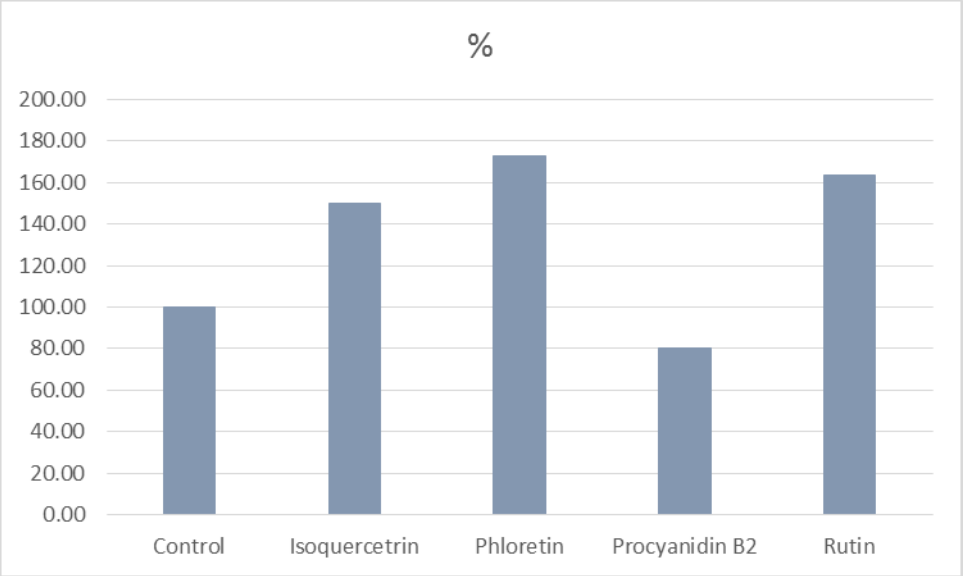

Figure 2: Adhesion of *Lactobacillus casei* to the co-culture (Caco-2 and HT29-MTX) cell lines after treatment with each polyphenol

Table 3: Treatment of the co-culture (Caco-2 and HT29-MTX) cell lines with four different polyphenols and percentage of adhesion of Lactobacillus gasseri compared with the control sample

Treatment	**%**
Control	100.00
Isoquercetrin	135.45
Phloretin	131.28
Procyanidin B2	145.69
Rutin	125.01

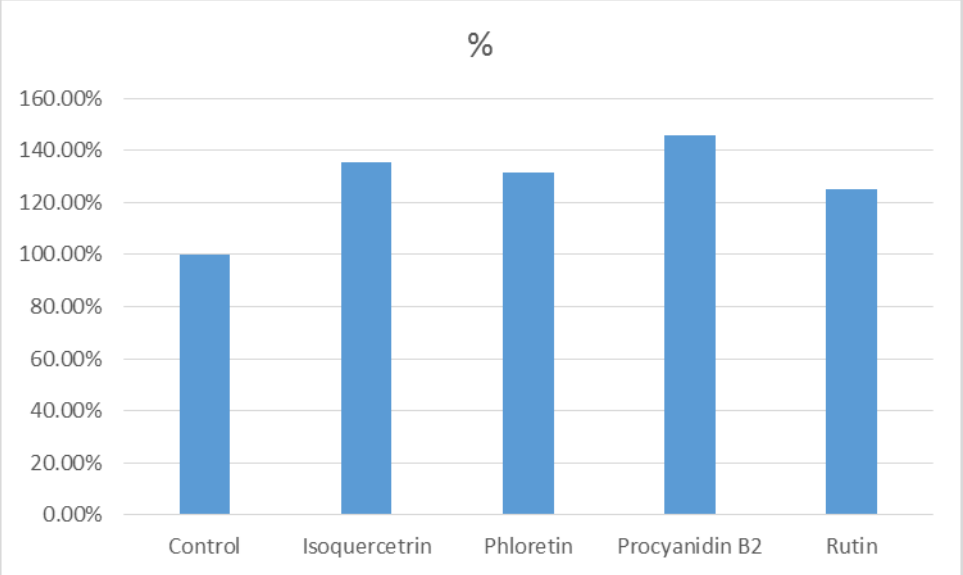

Figure 3: Adhesion of *Lactobacillus gasseri* to the co-culture (Caco-2 and HT29-MTX) cell lines after treatment with each polyphenol

6 Discussion

In this study we tried to investigate the effect of four polyphenols: isoquercetrin, phloretin, procyanidin B2 and rutin on the adhesion ability of two potentially probiotic strains (*Lactobacillus casei*, *Lactobacillus gasseri*) to *in vitro* human intestinal epithelial model consisting of Caco-2 and mucus-secreting HT29-MTX co-culture.

Phenolic compounds, as an important category of phytochemicals, exist in plants and are found largely in the fruits, vegetables, edible and wild flowers, tea, cereals and beverages and have been considered to have high antioxidant ability (Li et al., 2014). Accordingly, phenolic compounds have attracted increasing attention as potential agents for preventing and treating many oxidative stress-related diseases, such as cardiovascular diseases, cancer, ageing, diabetes mellitus and neurodegenerative diseases. For example Mediterranean diets are associated with reduced risk of cardiovascular disease due to adequate intake of olive oil and red wine, which contained high contents of polyphenols (Li et al., 2014).

Phloretin is abundantly present in the peel of apple and in strawberries. It occurs in different glycosidic forms, such as naringin dihydrochalcone, phlorizin, and phloretin-4-*O*-glucoside, in the different parts of the plants, and they contribute to various physiological properties of the plants, as well as to their color. Phloretin and its glycosides have been determined to have beneficial biological activities (Pandey et al., 2013).

Studies have proved that phloretin has inhibitory activity against glucose cotransporter, antioxidant activity, and activity to suppress the tumour necrosis factor alpha-induced inflammatory response, ameliorate inflammation of the colon, positively affect body weight loss, modulate Ca^{2+} activated K^{+} channels, and increase endothelial nitric oxide production, which might help to protect against atherosclerosis. Importantly, phloretin has other biological functions, like anticarcinogenic and estrogenic activities and inhibition of cardiovascular disease (Pandey et al., 2013). In another study, phloretin reduced RANKL−stimulated resorptive activity in osteoclasts via retarding differentiation. In addition, phloretin promoted osteoclast apoptosis and inhibited estrogen deficiency-induced osteoclastogenic resorption and in a more recent study, it was proved that phloretin manipulated protein kinase-signaling components responsible for the osteoclast cytoskeleton organization, which may display favorable effects in combating resorptive bone diseases (Lee et al., 2015).

In our current study, one of the four polyphenols examined for promoting adhesion of some probiotic strains (*Lactobacillus casei*, *Lactobacillus gasseri*) to *in vitro* human intestinal

epithelial model consisting of Caco-2 and mucus-secreting HT29-MTX co-culture, was phloretin. The adhesion of *Lactobacillus casei* to the co-culture (Caco-2 and HT29-MTX) cell lines after treatment with phloretin was increased by 72.97% compared with the control sample, which is the highest percentage, comparing to the other polyphenols used for the specific strain. The Adhesion of *Lactobacillus gasseri* to the co-culture (Caco-2 and HT29-MTX) cell lines after treatment with phloretin was increased by 31.28% compared with the control sample, which is not the highest percentage but still significant.

Proanthocyanidins (condensed tannins), the oligomeric forms of flavan-3-ols, are among the most widespread polyphenols in plants and also in the human diet. Procyanidins are the commonest type of proanthocyanidin and procyanidin-rich beverages and foods include cocoa, grapes, apples, strawberries, and red wine (Stoupi et al., 2010). In our study, the adhesion of *Lactobacillus casei* to the co-culture (Caco-2 and HT29-MTX) cell lines after treatment with procyanidin B2 was 20.25% less than the control sample, and it was only polyphenol tested exhibiting this result and only to this specific strain. The adhesion of *Lactobacillus gasseri* to the co-culture (Caco-2 and HT29-MTX) cell lines after treatment with procyanidin B2 was increased by 45.69% compared with the control sample, which is the highest percentage exhibited to this strain from all the polyphenols tested. According to those results we attempt to say that the adhesion ability is related to the strain used.

Rutin is a glycoside composed of quercetin and the disaccharide rutinose, exists in relatively large amounts in bracken ferns, red grapes, buckwheat, apple, and various teas, and it is also the major effective components of Flos sophorae, the dried flowers or buds of Chinese scholar tree *Sophora japonica* L. (Lu, Wang, Lin, & Zhang, 2012). Also, other studies have proved that it has been reported to possess cytoprotective and gastroprotective effects in animal models of gastroduodenal ulcer, mucosal ulceration and necrosis (Farzaei et al., 2015). According to our study, the adhesion of *Lactobacillus casei* to the co-culture (Caco-2 and HT29-MTX) cell lines after treatment with rutin was increased by 63.66% compared with the control sample, which is the second highest percentage, comparing to the other polyphenols used for the specific strain. The adhesion of *Lactobacillus gasseri* to the co−culture (Caco-2 and HT29-MTX) cell lines after treatment with rutin was increased by 25.01% compared with the control sample, which is the lowest percentage of all the polyphenols tested but still significant.

Isoquercitrin is a kind of flavonoid widely distributed in plantage. As a derivative of rutin, the structural difference between them is only a rhanmosidase. Related to rutin, isoquercitrin has been found to increase blood flow and perhaps be a cure for such maladies as varicose

veins, hemorrhoids, and possible use for arterial flow as well. It also has been shown to have anti-irritation properties as well. A significant amount of studies have shown its possibilities in increased brain functions due to increased blood flow and might be useful in the treatment of progressive Alzheimer's disease. While a certain experiment has showed that rutin has no exhibit anticancer activity (Lu, Wang, Lin, & Zhang, 2012). In our current study, the adhesion of *Lactobacillus casei* to the co-culture (Caco-2 and HT29-MTX) cell lines after treatment with isoquercitrin was increased by 49.76% compared with the control sample, which is the lowest percentage, comparing to the other polyphenols used for the specific strain. The adhesion of *Lactobacillus gasseri* to the co-culture (Caco-2 and HT29-MTX) cell lines after treatment with phloretin was increased by 35.45% compared with the control sample, which is not the highest percentage but still significant.

As it was mentioned before, the percentages of adhesion *Lactobacillus casei* for all the polyphenols (apart from procyanidin B2) are significantly higher than those demonstrated for *Lactobacillus gasseri*. If we take other studies into consideration, we can attempt to say that there is somehow a similar pattern in the effect of different polyphenols tested on gut microbiota (pathogenic and potential probiotic) but this cannot be a statement and more research is needed to come to a final result. For example, in the study of Parkar et al. (2014), the different berry fruit juices tested (in relation to their chemical composition, antioxidant ability and effects on the proliferation of Salmonella and Lactobacilli species and their adhesion to a gut epithelium model), inhibited the proliferation of *Salmonella enterica* serovar Typhimurium (Gram negative) and its adhesion to gut epithelial cells *in vitro* and enhanced the proliferation of *Lactobacillus rhamnosus.* According to Parkar et al. (2008), the Gram positive enteropathogen *Staphylococcus aureus* was the most sensitive to the polyphenols tested (caffeic acid, chlorogenic acid, ocoumaric acid, p-coumaric acid, catechin, epicatechin, phloridzin, rutin naringenin, daidzein, genistein and quercetin) while the Gram positive probiotic *Lactobacillus rhamnosus* was less sensitive to the same polyphenols, requiring a minimum inhibitory concentration and although all the polyphenols tested demonstrated an inhibitory effect on the adhesion of the pathogen *Staphylococcus typhimurium* at doses of 30 μg/ml and more, they had little inhibitory effect on the adhesion of lactobacilli, and three compounds increased the adhesion of lactobacilli to Caco-2 cells. In the study of Cheng et al. (2006), Growth of certain pathogenic bacteria such as *Clostridium perfringens*, *Clostridium difficile* and *Bacteroides* spp. was significantly repressed by tea phenolics and their derivatives, while commensal anaerobes like *Clostridium* spp., *Bifidobacterium* spp. and probiotics such as *Lactobacillus* sp. were less severely affected. Polyphenols have been

demonstrated potential antibacterial, antifungal and antiviral activities and among most of the studies, the antimicrobial effects of polyphenolic compounds were assessed against both Gram-negative (*Salmonella*) and Gram-positive bacteria (*Listeria monocytogenes*). The destabilization of the outer membrane of Gram-negative microorganisms, as well as interactions with the cell membrane might be one of the specific mechanisms behind the antibacterial action (Li et al., 2014). More research needs to be done to study and understand the mechanisms of interaction that can be related to each specific strain.

.

7 Conclusion

The objective of our work, which was to determine the effect of selective polyphenols on the adherence of lactobacilli strains in culture cells model *in vitro*, was accomplished.

Our results demonstrated that the polyphenols isoquercetrin, phloretin, procyanidin B2 and rutin have the potential to alter gut microbiota by modifying adhesion of selected probiotic *Lactobacillus* spp. strains to intestinal cells. The addition of the polyphenols to the assay promoted (except procyanidin B2 which inhibited the adhesion of *Lactobacillus casei*) the adhesion in both used strains of lactobacilli. Consequently, the consumption of food and drinks which are rich in polyphenols could affect the intestinal microbiota and improve microbiota imbalances. Further studies on the effect of polyphenols on the adhesion ability and viability of other bacteria will help to better understand their interaction with gut microbiota. Determining how these components contribute to probiotic action could lead to improved and more effective probiotic formulas and specific dietary recommendations for consumer's health.

8 Bibliography

1. Amadini, G. et al., 2002. - Role in inflammatory bowel disease.
2. Amara, A.A. & Shibl, A., 2015. Role of Probiotics in health improvement , infection control and disease treatment and management. *Saudi Pharmaceutical Journal*, 23(2), pp.107–114.
3. Ambalam, P. et al., 2016. Probiotics, prebiotics and colorectal cancer prevention. *Best Practice & Research Clinical Gastroenterology*, 30(1), pp.119–131.
4. Anon, 2001. Health and nutritional properties of probiotics in food including powder milk with live lactic acid bacteria. *Report of a Joint FAO/WHO Expert Consultation on Evaluation of Health and Nutritional Properties of Probiotics in Food Including Powder Milk with Live Lactic Acid Bacteria, Amerian Córdoba Park Hotel*.
5. Bermudez-Brito, M. et al., 2013. In vitro cell and tissue models for studying host–microbe interactions: a review. *British Journal of Nutrition*, 109(S2), pp.S27–S34.
6. Cammarota, G. et al., 2015. Pharmacology & Therapeutics The involvement of gut microbiota in in fl ammatory bowel disease pathogenesis : Potential for therapy. *Pharmacology and Therapeutics*, 149, pp.191–212.
7. Coppa, G. V et al., 2006. Human Milk Oligosaccharides Inhibit the Adhesion to Caco-2 Cells of Diarrheal Pathogens : Escherichia coli , Vibrio cholerae , and Salmonella fyris. , 59(3).
8. Daliri, E.B. & Lee, B.H., 2015. New perspectives on probiotics in health and disease. *Food Science and Human Wellness*, 4(2), pp.56–65.
9. DeSesso, J.M. & Jacobson, C.F., 2001. Anatomical and physiological parameters affecting gastrointestinal absorption in humans and rats. *Food and Chemical Toxicology*, 39(3), pp.209–228.
10. Edwards, C.A. & Parrett, A.M., 2002. Intestinal flora during the first months of life : new perspectives.
11. Farzaei, M.H., Abdollahi, M. & Rahimi, R., 2015. Role of dietary polyphenols in the management of peptic ulcer. *World journal of gastroenterology: WJG*, 21(21), p.6499.
12. Felis, G.E. & Dellaglio, F., 2007. Taxonomy of lactobacilli and bifidobacteria. *Current issues in intestinal microbiology*, 8(2), p.44.
13. Fooks, L.J. & Gibson, G.R., 2002. Probiotics as modulators of the gut flora. *British Journal of Nutrition*, 88(S1), pp.s39–s49.
14. Forestier, C. et al., 2001. Probiotic activities of Lactobacillus casei rhamnosus: in vitro

adherence to< space> intestinal cells and antimicrobial properties. *Research in Microbiology*, 2(152), pp.167–173.

15. Furrie, E., 2005. Probiotics and allergy. , pp.465–469.
16. Gagnon, M. et al., 2013. Comparison of the Caco-2, HT-29 and the mucus-secreting HT29-MTX intestinal cell models to investigate Salmonella adhesion and invasion. *Journal of microbiological methods*, 94(3), pp.274–279.
17. Gaudana, S.B., Dhanani, A.S. & Bagchi, T., 2010. Probiotic attributes of Lactobacillus strains isolated from food and of human origin. , pp.1620–1628.
18. Genove, S. et al., 2013. Isolation , identification and characterisation of three novel probiotic strains (Lactobacillus paracasei CNCM I-4034 , Bifidobacterium breve CNCM I-4035 and Lactobacillus rhamnosus CNCM I-4036) from the faeces of exclusively breast-fed infants British .
19. Gil, A. et al., 2013. Sources , isolation , characterisation and evaluation of probiotics.
20. Gopal, P.K. et al., 2001. In vitro adherence properties of Lactobacillus rhamnosus DR20 and Bifidobacterium lactis DR10 strains and their antagonistic activity against an enterotoxigenic Escherichia coli. , pp.207–216.
21. Guardamagna, O. et al., 2014. Bifidobacteria supplementation: effects on plasma lipid profiles in dyslipidemic children. *Nutrition*, 30(7-8), p.831.
22. Cheng, H. et al., 2006. Effect of tea phenolics and their aromatic fecal bacterial metabolites on intestinal microbiota. , 157, pp.876–884.
23. Iaconelli, C. et al., 2015. Drying process strongly affects probiotics viability and functionalities. *Journal of Biotechnology*, 214, pp.17–26.
24. Isolauri, E., Salminen, S. & Gueimonde, M., 2011. Manufacturing process influences properties of probiotic bacteria. , pp.887–894.
25. Laparra, J.M. & Sanz, Y., 2009. Comparison of in vitro models to study bacterial adhesion to the intestinal epithelium. *Letters in Applied Microbiology*, 49(6), pp.695–701.
26. Lee, E.-J. et al., 2015. Inhibition of Osteoclast Activation by Phloretin through Disturbing αvβ3 Integrin-c-Src Pathway. *BioMed research international*, 2015.
27. Lee, Y.-K. & Puong, K.-Y., 2002. Competition for adhesion between probiotics and human gastrointestinal pathogens in the presence of carbohydrate. *British Journal of Nutrition*, 88(S1), pp.S101–S108.
28. Li, A. et al., 2014. Resources and Biological Activities of Natural Polyphenols. , pp.6020–6047.

29. Maity, T.K. & Maity, A.K., 2009. Probiotics and human health: Synoptic review. *African Journal of Food, Agriculture, Nutrition and Development*, 9(8).
30. Makinen, K., Berger, B. & Ananta, E., 2012. Science and technology for the mastership of probiotic applications in food products. *Journal of Biotechnology*, 162(4), pp.356–365.
31. Marieb E.N, Hoehn K.N., 2001. Anatomy and physiology. 4th Edition. Pearson. ISBN-13: 978-0321616401
32. Maukonen, J. & Saarela, M., 2015. Conference on " Diet , gut microbiology and human health " Symposium 3 : Diet and gut metabolism : linking microbiota to bene fi cial products of fermentation Human gut microbiota : does diet matter ? Proceedings of the Nutrition Society Proceedings of th. , (August 2014), pp.23–36.
33. de Medina, F.S. et al., 2013. Host–microbe interactions: the difficult yet peaceful coexistence of the microbiota and the intestinal mucosa. *British Journal of Nutrition*, 109(S2), pp.S12–S20.
34. Mizock, B.A., 2015. Disease-a-Month Probiotics. , 61, pp.259–290.
35. Mountzouris, K.C., Mccartney, A.L. & Gibson, G.R., 2002. Review article Intestinal microflora of human infants and current trends for its nutritional modulation. , pp.405–420.
36. Ouwehand, A.C. et al., 1999. Adhesion of probiotic micro-organisms to intestinal mucus. *International Dairy Journal*, 9(9), pp.623–630.
37. Ouwehand, A.C. et al., 2001. Assessment of adhesion properties of novel probiotic strains to human intestinal mucus. *International journal of food microbiology*, 64(1), pp.119–126.
38. Ouwehand, A.C. & Salminen, S., 2003. In vitro adhesion assays for probiotics and their in vivo relevance: a review. *Microbial ecology in health and disease*, 15(4), pp.175–184.
39. Pandey, K.B. & Rizvi, S.I., 2009. Plant polyphenols as dietary antioxidants in human health and disease. *Oxidative medicine and cellular longevity*, 2(5), pp.270–278.
40. Pandey, R.P. et al., 2013. Enzymatic synthesis of novel phloretin glucosides. *Applied and environmental microbiology*, 79(11), pp.3516–3521.
41. Parkar, S.G. et al., 2014. In vitro studies of modulation of pathogenic and probiotic bacterial proliferation and adhesion to intestinal cells by blackcurrant juices. *Journal of Functional Foods*, 8, pp.35–44. Available at: http://www.sciencedirect.com/science/article/pii/S1756464614000711 [Accessed

April 14, 2016].

42. Parkar, S.G., Stevenson, D.E. & Skinner, M.A., 2008. The potential influence of fruit polyphenols on colonic microflora and human gut health. *International journal of food microbiology*, 124(3), pp.295–298.
43. Power, S.E. et al., 2014. Review Article Intestinal microbiota , diet and health. , pp.387–402.
44. Ranadheera, C.S. et al., 2014. Effect of dairy probiotic combinations on in vitro gastrointestinal tolerance, intestinal epithelial cell adhesion and cytokine secretion. *Journal of Functional Foods*, 8, pp.18–25. Available at: http://www.sciencedirect.com/science/article/pii/S1756464614000723 [Accessed April 8, 2016].
45. Ranadheera, C.S. et al., 2012. In vitro analysis of gastrointestinal tolerance and intestinal cell adhesion of probiotics in goat ' s milk ice cream and yogurt. *FRIN*, 49(2), pp.619–625.
46. Reece, W.O. et al., 2015. *Dukes' physiology of domestic animals*, Am Vet Med Assoc.
47. Ren, D. et al., 2012. Inhibition of Staphylococcus aureus adherence to Caco-2 cells by lactobacilli and cell surface properties that influence attachment. *Anaerobe*, 18(5), pp.508–515.
48. Rizzo, D. C. 2001, Delmar's Fundamentals of Anatomy & Physiology. New York: Thomson Learning. ISBN: 0-7668-0498-4
49. Saarela, M., Mogensen, G. & Fonde, R., 2000. Probiotic bacteria : safety , functional and technological properties. , 84, pp.197–215.
50. Salminen, S.J. & Tuomola, E.M., 1998. Adhesion of some probiotic and dairy Lactobacillus strains to Caco-2 cell cultures. , 41, pp.45–51.
51. Sengupta, R. et al., 2013. The Role of Cell Surface Architecture of Lactobacilli in Host-Microbe Interactions in the Gastrointestinal Tract. , 2013.
52. Schneeman, B.O., 2002. Gastrointestinal physiology and functions. , pp.159–163.
53. Steer, T. et al., 2000. Perspectives on the role of the human gut microbiota and its modulation by pro- and prebiotics. , 44, pp.229–254.
54. Stoupi, S. et al., 2010. In vivo bioavailability, absorption, excretion, and pharmacokinetics of [14C] procyanidin B2 in male rats. *Drug Metabolism and Disposition*, 38(2), pp.287–291.
55. Tamboli, C.P. & Caucheteux, C., 2003. Probiotics in inflammatory bowel disease : a critical review. , 17(5), pp.805–820.

56. Tortora, G. J, Derrickson B., 2009. Principles of anatomy and physiology. 12th Edition. ISBN: 978-0-470-08471-7
57. Tuohy, K. & Del Rio, D., 2014. *Diet-microbe interactions in the gut: effects on human health and disease*, Academic Press.
58. Vander A.J, Sherman J.H, Luciano D.S., 2001. Human physiology: The mechanism of body function. 8th Edition. McGraw Hill. ISBN-13: 978-0072908015
59. Venema, K. & do Carmo, A.P., 2015. *Probiotics and Prebiotics: Current Research and Future Trends*, Caister Academic Press.
60. Volštátová, T. et al., 2015. Effect Of Hydrolyzed Milk On The Adhesion Of Lactobacilli To Intestinal Cells*. *Scientia agriculturae bohemica*, 46(1), pp.21–25.
61. Wohlgemuth, S., Loh, G. & Blaut, M., 2010. Recent developments and perspectives in the investigation of probiotic effects. *International Journal of Medical Microbiology*, 300(1), pp.3–10.