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Study of apoptotic deletion mediated by *Bifidobacterium longum* with construction of recombinant strains for Serpin encoding gene and phenotypes comparison in a pig cell model.

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Part one: Introduction.

Chapter 1: Summary

The first part of the research project of the Co-Advisorship Ph.D Thesis was aimed to select the best *Bifidobacterium longum* strains suitable to set the basis of our study. We were looking for strains with the abilities to colonize the intestinal mucosa and with good adhesion capacities, so that we can test these strains to investigate their ability to induce apoptosis in "damaged" intestinal cells. Adhesion and apoptosis are the two process that we want to study to better understand the role of an adhesion protein that we have previously identified and that have top scores homologies with the recent serpin encoding gene identified in *B. longum* by Nestlè researchers. *Bifidobacterium longum* is a probiotic, known for its beneficial effects to the human gut and even for its immunomodulatory and antitumor activities. Recently, many studies have stressed out the intimate relation between probiotic bacteria and the GIT mucosa and their influence on human cellular homeostasis. We focused on the apoptotic deletion of cancer cells induced by *B. longum*. This has been valued *in vitro,* performing the incubation of three *B.longum* strains with enterocyte-like Caco-2 cells, to evidence DNA fragmentation, a cornerstone of apoptosis. The three strains tested were defined for their adhesion properties using adhesion and autoaggregation assays. These features are considered necessary to select a probiotic strain. The three strains named B12, B18 and B2990 resulted respectively: "strong adherent", "adherent" and "non adherent". Then, bacteria were incubated with Caco-2 cells to investigate apoptotic deletion. Cocultures of Caco-2 cells with *B. longum* resulted positive in DNA fragmentation test, only when adherent strains were used (B12 and B18). These results indicate

that the interaction with adherent *B. longum* can induce apoptotic deletion of Caco-2 cells, suggesting a role in cellular homeostasis of the gastrointestinal tract and in restoring the ecology of damaged colon tissues. These results were used to keep on researching and the strains tested were used as recipient of recombinant techniques aimed to originate new *B.longum* strains with enhanced capacity of apoptotic induction in "damaged" intestinal cells. To achieve this new goal it was decided to clone the serpin encoding gene of *B. longum,* so that we can understand its role in adhesion and apoptosis induction. *Bifidobacterium longum* has immunostimulant activity that *in vitro* can lead to apoptotic response of Caco-2 cell line. It secretes a hypothetical eukaryotic type serpin protein, which could be involved in this kind of deletion of damaged cells. We had previously characterised a protein that has homologies with the hypothetical serpin of *B. longum* (DD087853). In order to create *Bifidobacterium* serpin transformants, a *B. longum* cosmid library was screened with a PCR protocol using specific primers for serpin gene. After fragment extraction, the insert named S1 was sub-cloned into pRM2, an *Escherichia coli - Bifidobacterium* shuttle vector, to construct pRM3. Several protocols for *B. longum* transformation were performed and the best efficiency was obtained using MRS medium and raffinose. Finally bacterial cell supernatants were tested in a dot-blot assay to detect antigens presence against anti-antitrypsin polyclonal antibody. The best signal was produced by one starin that has been renamed *B. longum* BLKS 7. Our research study was aimed to generate transformants able to over express serpin encoding gene, so that we can have the tools for a further study on bacterial apoptotic induction of Caco-2 cell line.

After that we have originated new trasformants the next step to do was to test transformants abilities when exposed to an intestinal cell model. In fact, this part of the project was achieved in the Department of Biochemistry of the Medical Faculty of the University of Maribor, guest of the abroad supervisor of the Co-Advisorship

Doctoral Thesis: Prof. Avrelija Cencic. In this study we examined the probiotic ability of some bacterial strains using intestinal cells from a 6 years old pig. The use of intestinal mammalian cells is essential to study this symbiosis and a functional cell model mimics a polarised epithelium in which enterocytes are separated by tight junctions.
In this list of strains we have included the *Bifidobacterium longum* BKS7 transformant strain that we have previously originated; in order to compare its abilities. *B. longum* B12 wild type and *B. longum* BKS7 transformant and eight *Lactobacillus* strains of different sources were co-cultured with porcine small intestine epithelial cells (PSI C1) and porcine blood monocytes (PoM2) in Transwell filter inserts. The strains, including *Lb. gasseri*, *Lb. fermentum*, *Lb. reuterii*, *Lb. plantarum* and unidentified *Lactobacillus* from kenyan maasai milk and tanzanian coffee, were assayed for activation of cell lines, measuring nitric oxide by Griess reaction, H_20_2 by tetramethylbenzidine reaction and O_2^- by cytochrome C reduction. Cytotoxic effect by crystal violet staining and induction on metabolic activity by MTT cell proliferation assay were tested too. Transepithelial electrical resistance (TER) of polarised PSI C1 was measured during 48 hours co-culture. TER, used to observe epithelium permeability, decrease during pathogenesis and tissue becomes permeable to ion passive flow lowering epithelial barrier function. Probiotics can prevent or restore increased permeability. Lastly, dot-blot was achieved against Interleukin-6 of treated cells supernatants. The metabolic activity of PoM2 and PSI C1 increased slightly after co-culture not affecting mitochondrial functions. No strain was cytotoxic over PSI C1 and PoM2 and no cell activation was observed, as measured by the release of NO_2, H_20_2 and O_2^- by PoM2 and PSI C1. During co-culture TER of polarised PSI C1 was two-fold higher comparing with constant TER (~3000 Ω) of untreated cells. TER raise generated by bacteria maintains a low permeability of the epithelium. During

treatment Interleukin-6 was detected in cell supernatants at several time points, confirming immunostimulant activity. All results were obtained using *Lactobacillus paracasei* Shirota e *Carnobacterium divergens* as controls.

Chapter 2: Biology of *Bifidobacterium* spp.

2. 1: Taxonomy

Classification and taxonomy of bifidobacteria have been a source of controversy since their discovery more than a hundred years ago. In 1899, Tissier isolated Gram-positive, anaerobic bacteria with a hitherto unknown Y-shaped morphology from the faeces of breast-fed infants, which he termed *Bacillus bifidus communis* (lat. bifidus: cleft, divided; Tissier, 1900). Shortly after the introduction of the *Lactobacillaceae* in 1917, the Tissier strains were integrated in this new family by Holland (1920) and renamed to *Lactobacillus bifidus*. In 1924, Orla-Jensen suggested the new genus *Bifidobacterium* for the representatives of the species based on morphological, cultural, and biochemical investigations. However, the former nomenclature prevailed, and only fifity years later the bifidobacteria officially obtained their deserved status as a separate genus by the publication of the VIIIth edition of Bergey's Manual of Determinative Bacteriology (Buchanan

and Gibbons, 1974). This reclassification was the consequence of sugar fermentation studies (Dehnert, 1957; Reuter, 1963), of the discovery of the bifidobacterial hexose catabolism (see below) as well as of analyses of the genomic G+C content, in which bifidobacteria strongly differ from the lactic acid bacteria. Today, the genus *Bifidobacterium* is included in the newly established class *Actinobacteria*, the Gram-positive bacteria with a high G+C content in their genome (Schleifer and Ludwig, 1995; Stackebrandt *et al.*,1997). Currently approximately 30 species are differentiated, but as the flood of new publications referring to this topic shows (Cai *et al.*, 2000; Dong *et al.*, 2000; Hoyles *et al.*, 2002; Jiang and Dong, 2002; Sakata *et al.*, 2002; Ventura and Zink, 2002; Zhu *et al.*, 2003), the *Bifidobacterium* taxonomy will stay in a constant state of flux at least in the near future. Bifidobacteria can shortly be characterised as Gram-positive, non sporeforming, non motile, catalase negative, fermentative rods exhibiting pleomorphism, the cells being short or long, irregular, often curved, bifid or multiple-branched. The G+C content of their DNA varies from 55 to 67% (Biavati and Mattarelli, 2001). They are generally regarded as strictly anaerobic, yet some species possess a considerable oxygen tolerance (Meile *et al.*, 1997). They grow optimally between 37 and 41°C at pH values of 6.5 to 7, with the exception of the newly described *B. thermacidophilum*, which is able to grow at up to 49.5°C and at pH 4 (Dong *et al.*, 2000). Along with *Bacteroides*, *Eubacterium*, *Clostridium*, *Lactobacillus*, *Fusobacterium*, *Peptococcus*, *Peptostreptococcus*, *Enterococcus*, *Staphylococcus*, *Veillonella*, and *Enterobacteriaceae* species, bifidobacteria belong to the predominant bacterial groups within the intestinal microflora of humans (Savage, 1977; Mitsuoka, 1992), representing up to 15% of the cultivable bacteria of this ecosystem in adults and being the main pioneer colonisers in newborns (Marteau *et al.*, 2001; Tuohy *et al.*, 2001). Bifidobacteria are also widespread in the gastrointestinal tract of animals such as calves, lambs,

pigs, chickens, rabbits, rats, and even honeybees. Besides, they can be isolated from the human vagina, from the human oral cavity, and from sewage. In general, *Bifidobacterium* species are specific for either humans or animals, whereby the occurrence of the same species in suckling calves and breast-fed infants is the exception (Biavati and Mattarelli, 2001).

2. 2: Morphology

The group of bacteria that belong to the genus *Bifidobacterium* presents a rod-shape morphology, Gram positive, non motile and non sporeforming. This group of rod shaped bacteria have an irregular shape that lenght on average between 2 and 5 μm and presents swelling ends with club or slice form and with one ore more branches, but even more regular and cocci shape are not so rare. This type of polymorphism is generally species-specific, so that even the kind of morphology can be used for identification of bacteria. However, even in one single species it is possible to have different cells with different shapes; this is particularly due to growth conditions. Stress conditions as low pH, extreme temperatures or nitrogen starvation can trigger morphological alterations of bacterial cells that swell. When *Bifidobacterium bifidum* var. *pensylvanicus* is cultivated in a media without N-acethylglucosamine, the main component of glycane, assumes a club shape. When bifidobacteria are cultivated in a poor medium cells present the typical "Y" shape, but as soon as some aminoacids like, alanine, glutamic acid, serine ad aspartic acid, are added, cells tend to assume a rod shape. Probably, these aminoacids are implicated in peptidoglycan formation so that this pleomorphysm is due more to an altered synthesis than to degenerative process (Husain *et al.*, 1972). Even Ca^{++} ions

content can influence bacterial morphology; a low content causes “Y” shapes, while an high content causes rod shapes (Kojima *et al.*, 1970).

2. 3: Membrane and Cell Wall

Since1970 cell wall composition and peptidoglycan structure of Bifidobacteria have been studied and it has been sentenced that, like all Gram+ bacteria, cell wall is set up by a single homogenous layer of peptidoglycan , assembled from N-acethylmuramic acid (NAM) and N-acethylglutamic acid (NAG) with glycosidic β-1,4 bonds. A peptidic chain formed by four aminoacids (L-alanine, D-glutamate, L-lysine or L-ornithine and D-alanine) is bond to NAM carboxyl termini, instead this region represents the conservative region of *Bifidobacterium* genus. The variable region is set up by peptidic chains that link the conservative tetrapeptides between two peptidoglycan chain. The sequence of this aminoacidic bonds differs at interspecific level. Peptidoglycan is not the sole component of cell wall of Bifidobacteria, instead there are high contents of polysaccharides as, glucose and galactose linked to ramnose and high contents of teichoic acids (TA) and lipoteichoic acids (LTA). Teichoic acids are acid polysaccharides and represent the 10-15% of cell wall weight. These polymers can contains up to thirty glycerol-phosphate or ribitol-phosphate molecules linked to phosphate bonds. Sugars as, glucose or galactose, or aminoacids as, D-alanine, can be linked to hydrogen oxide ions of the alcohol and figure as antigenic determinants at serotype level. They are bound to 6-OH^- group in NAM structure of peptidoglycan. Lipoteichoic acids are teichoic acids generally set up by glycerol-phosphate chains, including a fatty acid that is used to bound to membrane lipids. LTAs get through cell wall and extend outside (Veerkamp *et al.* 1983). LTAs and TAs have structural functions,

contributing to the strength and the flexibility of cell wall and are immunogenic. Moreover can contribute to cations assumption because repeated phosphate groups make molecules strongly acid and negatively charged, thus facilitating cations bonds (Ca^{++}, K^{+}, Mg^{++}) and assumption. Further studies demonstrate that these lipoteichoic acids, single or associated with proteins, are responsible for the surface hydrophobicity of Bifidobacteria (Op den Camp *et al.* 1985). Cytoplasmatic membrane of *Bifidobacterium bifidum* has been exhaustively studied. Looks like a 9 nm depth structure set up by a double lipidic layer in which is inserted a single proteic layer. Membrane lipids are exclusively phospholipidsas, phosphathydil-glycerol, diphosphathydil-glycerol and polyglycerol-phospholipids (Exterkate *et al.,* 1971). Instead, myristic acid, palmitic acid, stearic acid and oleic acid are the most representative fatty acids of these membrane phospholipids (Ballongue, 1993). Membrane proteins are non-glycosilated and can be integrated or peripheric. Inside cytoplasma, plasmatic membrane generates several invaginations, called mesosomes, that differs at intraspecific level based on growth conditions (Greenwalt and Whiteside, 1975).

2. 4: Metabolism

2. 4. 1: Fermentation of simple charbohydrates

In the context of the use of bifidobacteria as probiotics and their role as target organisms for prebiotic substances the biochemical and genetic background of the bifidobacterial carbohydrate catabolism is of increasing interest. The fermentation ability of simple and complex carbohydrates varies widely within the genus (Biavati and Mattarelli, 2001) and was therefore a preferred method for species

identification (Mitsuoka, 1969) before less time-consuming molecular methods were introduced. Poly- and oligosaccharides are degraded by extra- and/or intracellular enzymes to their monomers before being further processed by the intermediary metabolism. The catabolism of hexoses in *Bifidobacterium* species follows a characteristic pathway known as the fructose 6-phosphate (F6P) or bifid(us) shunt as shown in figure 2. 1 (Scardovi, 1964; Scardovi and Trovatelli, 1965; De Vries *et al.*, 1967).

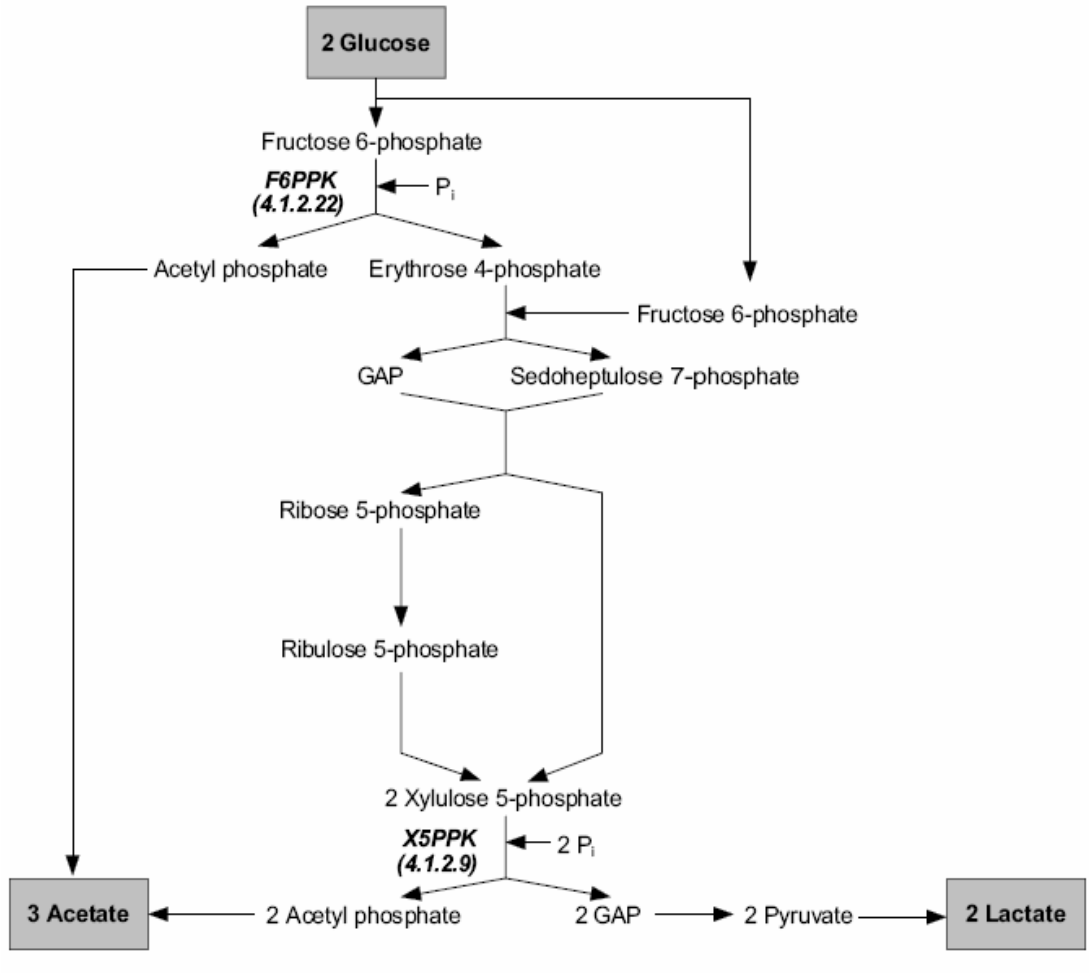

Fig. 2. 1: The fructose 6-phosphate or bifidus shunt as described by Scardovi and Torvatelli (1965) and De Vries *et al.*(1967). F6PPK and X5PPK, fructose 6-phosphate and xylulose 5-phosphate phosphoketolase, respectively.

The key enzyme of this pathway is fructose 6-phosphate phosphoketolase (F6PPK), which cleaves the hexose phosphate into erythrose 4-phosphate and acetyl phosphate. By the subsequent action of transaldolase and transketolase, pentose phosphates are formed from the tetrose phosphate and fructose 6-phosphate, which are then split by xylulose 5-phosphate phosphoketolase (X5PPK) into acetyl phosphate and glyceraldehydes 3-phosphoate, finally ending up in acetic and L(+)-lactic acids (Figure 2. 2). The apparently exclusive occurrence of F6PPK in *Bifidobacteriaceae* led to the common use of its activity as a definitive identification criterion for this family (Biavati and Mattarelli, 2001). The most important differences between the F6P and the phosphoketolase shunts are the lack of carbon dioxide production by bifidobacteria (except during gluconate degradation) and the ratio of the two primary fermentation products. While heterofermentative lactic acid bacteria produce at most equal amounts of acetate and lactate as their fermentation end products, the theoretical ratio of 3:2 (acetate/lactate) is the result of the F6P pathway. They explained it by the phosphoroclastic split of a part of the pyruvate into formic and acetic acids and the partial reduction of the latter to ethanol. The enzyme responsible for this reaction has never been purified or characterised; however, a gene coding for a putative pyruvate formate lyase is represented in the genome of *B. longum* bv. *longum*.

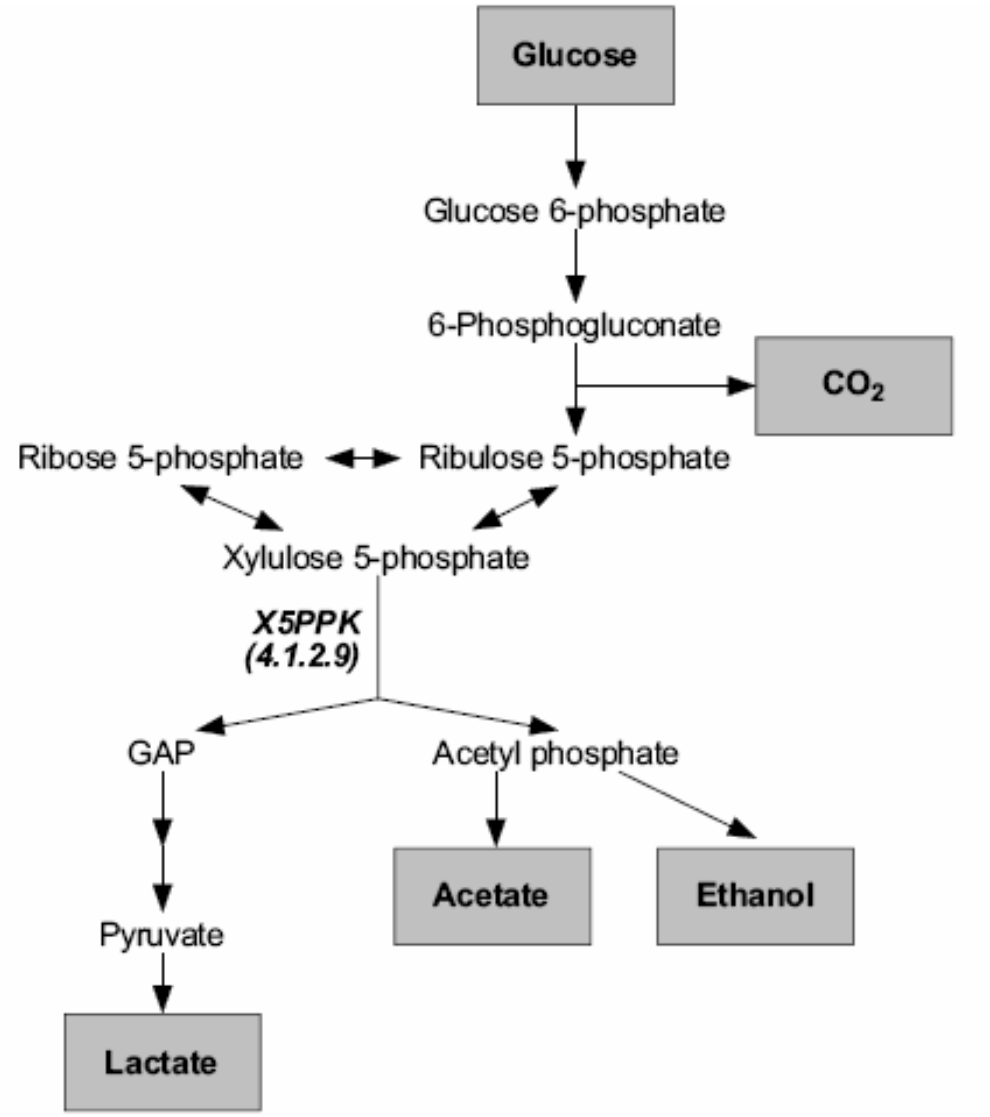

Fig. 2. 2: Phosphoketolase shunt of obligatory and facultative heterofermentative lactic acid bacteria. X5PPK, xylulose 5-phosphate phosphoketolase

The energy gained from glucose fermentation through the F6P shunt is 2.5 mol of ATP per mol of substrate and thus exceeds the yield obtained by homolactic (2 mol of ATP) or heterolactic (1 to 2 mol of ATP) fermentation (Buckel, 1999). A high yield of ATP does not necessarily have to be favourable; it is rather a high rate of ATP synthesis which allows bacteria to successfully outcompete others having a lower metabolic activity (Unden, 1998). Pathways other than the F6P shunt have

never been proven to exist in bifidobacteria, and glycolytic enzymes as well as those of the oxidative branch of the pentose phosphate pathway are generally assumed to be absent (Schlegel, 1992). Glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase (Scardovi and Trovatelli, 1969) as well as aldolase (Scardovi *et al.*, 1969) were reported to occur in several strains of animal origin. The physiological role of these enzymes in bifidobacteria remains unclear. Another glycolysis enzyme, phosphofructokinase, was found to be necessary as a supplier of fructose 1,6-bisphosphate, which was revealed to function as an essential activator of lactate dehydrogenase in *B. bifidum* (De Vries *et al.*, 1967).

2. 4. 2: Glucosidases and complex charbohydrates

Bifidobacteria have the capacity to metabolise even complex carbohydrates and this ability cover a fundamental role for bacterial growth, thus simple sugurs are rare to be found in the tract of colon, because this kind of sugars are firstly metabolised by saccharolytic species that habit the colon and are easily absorbed by the small intestine. For this reason the main source of degradable carbohydrates in the colon at disposition of bifidobacteria are complex carbohydrates. To this group belongs all that diet fibres as, cellulose, emicellulose, pectic substances, and all that mucines that protect the intestinal mucosa as, glycoproteins with fucose, esosamine and salicylic acids. Unfortunately, the aspect of metabolic processes that degrade these polymers known as fructo-oligosaccharides (FOS) is still not so clear. FOS are recognise as molecules with glucose and fructose residues linked by β-1,2 or β-1,6 bonds. These carbohydrates are not fermented by intestinal mucosa enzymes, but are fermented by intestinal bacterial species, particularly by bifidobacteria. Many studies have been reported about selection of bifidobacterial strains that can better use these polymers. Once these bacteria are inoculed in the host with their

colonization and persistence produce precursors that can be used by those bacteria incapable to ferment complex sugars (Scardovi V., 1969). In particular, some studies have revealed that porcine gastric mucine is preferably degraded by bifidobacterial species as, *B. bifidum*, *B. infantis* and *B. longum* Scardovi V., 1986). Bifidobacteria can ferment several different sugars, some like lactose, galactose or saccharose are fermented by the most of the species, but others like sorbitol or mannitol are metabolised just by few species. In some cases, this could be due to the fact that most species do not posses the specific sugar phosphotrasferase system (PTS). Once sugars get trough cell membrane, oligosaccharides and disaccharides are hydrolysed by specific glycosidases, while complex polysaccharides as amylase, amylopectin, xylan or arabic gum are degraded by specific extracellular enzymatic systems.

2. 5: Physiology

2. 5. 1: Temperatures and Ph

The origin of the different species of bifidobacteria reflect the different optimal temperature needed for their growth. Species of human origin prefer a range of temperature between 37° and 41° C and cannot stand temperatures higher than 45° C. In the most of the species of animal origin growth is possible even at more than 45° C, while it has never been demonstrate that these bacteria can live at temperature lower than 25° C. When bacteria are stored at 4° C, cultures are viable up to 1 month; when are harvested at -80° C, their full viability can be prolonged for several years, but when are lyophilised the cultures can be recovered even after 30 years. Bifidobacteria are acid tolerant microrganisms, but not acidophil, so they

grow in medium with a pH range between 5 and 7 preferring a restrict pH range between 6.5 and 7. Growth inhibition is obtained at pHs lower than 5 and higher than 8 (Scardovi V., 1986). Sometimes pH is used as selection tool in the medium culture.

2. 5. 2: Respiration

Bifidobacteria are anaerobe microrganisms, but oxygen sensibility differs from different species and different strains. There are strains less strictly anaerobe and there are mutant strains facultative anaerobe. The mechanisms that are implicated in oxygen tolerance are not well known. Anyway, bifidobacteria are catalase negative microrganisms, but among the aero-tolerant species *B. indicum* and *B. asteroides* become catalase-positive if grown in the presence of oxygen, respectively with or without the addiction of hemin. It has been hypothesed that atmospheric oxygen can interfere by two different mechanisms: helping redox potential, in this case the increasing oxygen is not lethal but can stop the growth of some strains, or producing H_2O_2, inhibitor of fructose-6-phosphate phosphoketolase, the key enzyme for sugar metabolism in bifidobacteria. Oxygen tolerance can be interpreted as an eventual capacity to degrade hydrogen peroxide (due to the slightly catalase activity as shown for *B. indicum* and *B. asteroides*) or to prevent its formation (due to the presence of NADH oxydase) (Scardovi V., 1986).

2. 5. 3: Antibiotic Resistance

Antibiotic resistance studies applied to bifidobacteria are necessary to outline selective treatments against pathogen microrganisms that do not affect bifidobacterial cells already present inside the gastro-intestinal tract. Antibiotics

can be use added to culture medium to discriminate bacterial species and isolate bifidobacteria in a heterogenic sample (*e.g.* stool sample). After several researches it has been stated that: the most of bifidobacterial species is resistant to many antibiotics and in particular to nalidixic acid, gentamycin, metronidazide, neomycin, polymyxin b and streptomycin, but the sensibility to this antibiotics differs among different species. Ampicillin, bacitracin, cloramphenicol, clindamycin, herythromycin, cincomycin, nitrofuralon, oleandomycin, penicillin G and vancomycin strongly inhibits the most of bifidobacterial species. Tetracycline resistance vary from species to species and from strains to strains (Scardovi V., 1986). For the isolation and identification of bifidobacterial species in dairy products, the use of dicloxacillin has been particularly efficient. As it has been described, the addition of dicloxacillin in a concentration of 2 pg/ml to the culture medium can discriminate all lactobacilli and streptococci from resistant bifidobacteria.

Chapter 3: Ecology of *Bifidobacterium* spp.

3. 1: Habitat

The group of species that has been isolated so far comes from different sources and habits different niches. Human origin bifidobacteria are mainly colon resident, in fact it is indicative that their presence is massively reported in fecal samples of

healthy humans and in the newborns. Species have been isolated from the oral cavity (*B. denticolens, B. dentium and B. inopitatum*) and even from vagina (*B. bifidum, B. breve, B. catenulatum, B. infantis and B. longum*), were represent a part of a consortium formed by other lactic acid bacteria (*e.g. Lactobacillus* spp.). Bifidobacteria of animal origin are mainly reported from mammal source. Presence of these bacteria has been highlighted in fecal samples from: rat, dog, cattle, pig, chicken, rabbit, while three species has been isolated from bees intestine. Some species seem to be host-specific, for example *B. magnum*, *B. suis* and *B. pullorum* have been specifically isolated in rabbit, pig and chicken, respectively. *B. dentium* is constantly associated with dental caries. *B. minimum* and *B. subtile* have been isolated from sewage and these two new species are the sole reported to habits non-living ecological niches (Table 3. 1).

Species	**Habitat**
B. adolescentis	Adult human; bovine, sewage.
B. animalis	Rat, chicken, cattle, rabbit and sewage
B. angulatum	Sewage and adult human
B. asteroides	Bees
B. bifidum	Adult human, newborn, cattle and human vagina
B. boum	Bovine and weaning pig
B. breve,	Newborn, cattle, human vagina, sewage
B. catenulatum	Newborn, cattle, human vagina, sewage
B. choerinum	Weaning pig and sewage
B. coryneforme	*Apis mellifera*
B. cuniculi	Rabbit
B. dentium	Adult human, oral caries, dental abscess, vagina
B. denticolens aka *Parascardovia denticolens*	Oral cavity
B. gallinarum	Chicken cecum tract
B. globosum	Weaning pig, cattle, rat, rabbit and sewage
B. infantis	Newborn, cattle, human vagina
B. inopitatum aka *Scardovia inopinata*	Oral cavity
B. longum	Newborn, cattle, human vagina, sewage
B. lactis	Newborn
B. magnum	Rabbit
B. merycicum	Bovine
B. minimum	Sewage
B. pseudocatenulatum	Newborn, cattle and sewage
B. pseudolongum	Pig, chicken, rabbit, cattle, rat, guinea pig
B. pollorum	Chicken
B. ruminantium	Bovine
B. saeculare	Rabbit
B. subtile	Sewage
B. suis	Weaning pig

Tab. 3. 1: Datas were obtained from Scardovi (1974), Lauer (1977) and (Watanabe 1995).

3. 2: Gastro-intestinal tract

The main habitat of bifidobacteria species is the gastro-intestinal tract of mammals. They represent an important part of human fecal flora both in the newborns and in the adults. The most of bifidobacterial species habits the colon, coexisting with a lot of other different strictly anaerobe microrganisms. During prenatal life the foetus lives in an aseptic habitat and just at the time of birth starts to get colonised by the mother fecal and vaginal microbiota. After weaning a natural and gradual change happens in the microbiota intestine; while, bifidobacteria decrease in number, others anaerobes like *Bacteroides, Peptostreptococcus, Eubacterium, Fusobacterium* and clostridia notably increase. Several studies have outlined that a progressive colonization of the intestinal tract is an important prerequisite to development immune system tolerance, about which loss could generate allergies and intolerancies (Figure 3. 1; Table 3. 2).

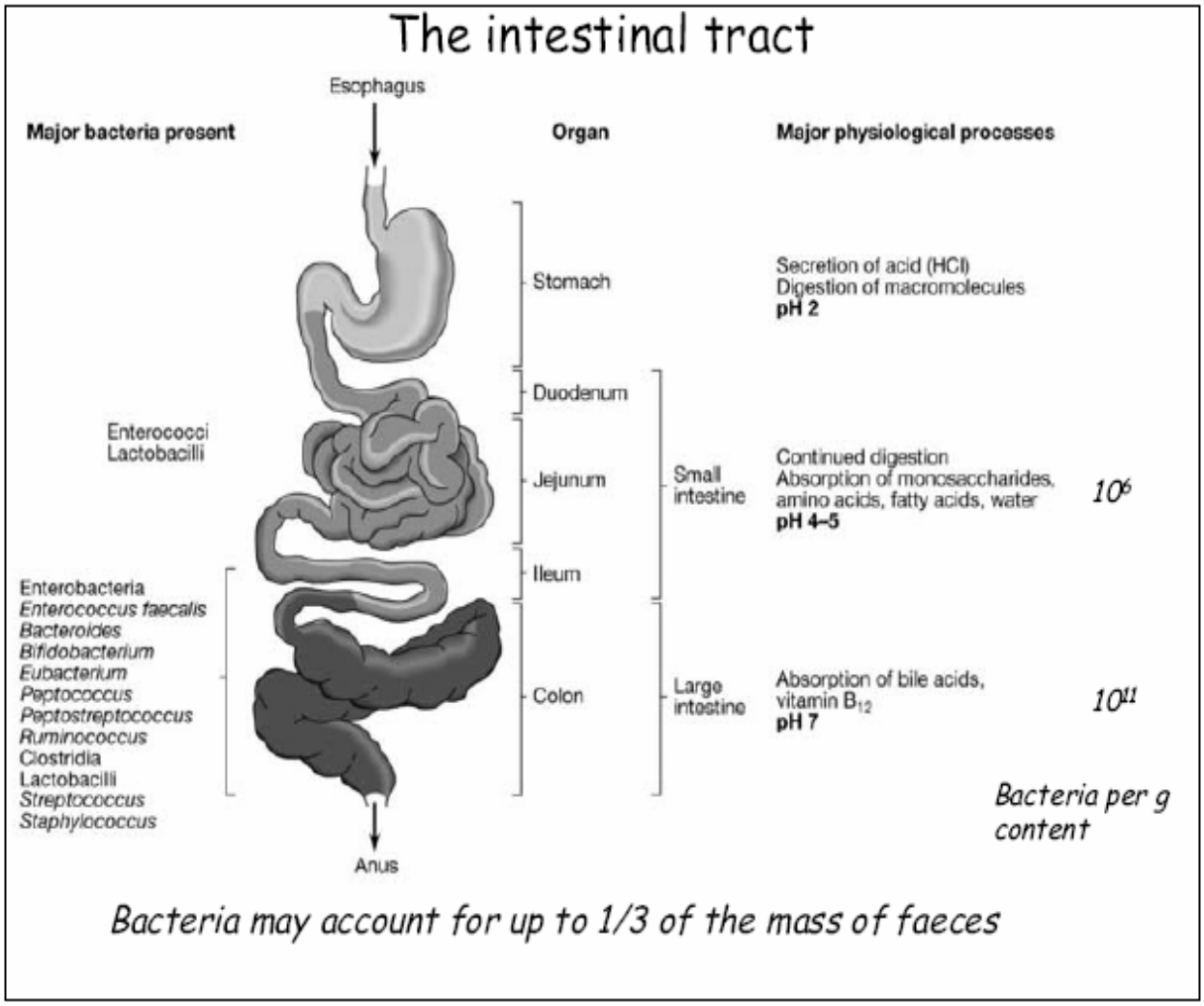

Fig. 3. 1: The gastrointestinal tract ecosystem showing the microbiota composition.

Gastrointestinal bacterial species	Stomach (pH 2 – 2.4)	Jejunum (pH 7.5)	Ileum (pH 6)	Colon (pH 5.5 – 7.5)
Total concentration of bacterial species	0 – 3	0 – 6	3 – 7	10 - 12
Aerobes and Facoltative Anaerobes				
Enterobacter spp.	0 – 2	0 – 3	2 – 5	4 – 10
Streptococcus spp.	0 - 3	0 – 4	2 – 6	5 – 10
Staphylococcus spp.	0 – 2	0 – 3	2 – 5	4 - 7
Lactobacillus spp.	0 – 3	0 – 4	2 – 5	6 - 10
Strictly Anaerobes				
Bacteroides spp.	Rare	0 – 2	3 – 6	9 – 11
Bifidobacterium spp.	Rare	0 – 3	3 – 7	8 – 10
Peptococcus spp.	Rare	0 – 3	3 – 4	8 – 10
Clostridium spp.	Rare	Rare	3 – 4	6 – 10
Fusobacterium spp.	Rare	Rare	Rare	9 – 10
Eubacterium spp.	Rare	Rare	3 – 5	9 - 10
Veillonella spp.	Rare	0 – 2	3 - 4	3 – 5

Tab. 3. 2: Most common species in the GIT microbiota of healthy humans.

Bifidobacteria skips from being the main bacterial genre in infants during weaning, to being the third or forth main bacterial group once the subject is adult. At the age of 65 the number of bifidobacterial species cells strongly decrease up for the increasing of lactobacilli, clostridia, streptococci and *E. coli* species. Moreover bifidobacterial species vary during host life cycle; in infants are present *B infantis,*

B. longum, B. bifidum and *B. breve*, while after weaning the most important species are *B. catenulatum*, *B. longum* and *B. pseudocatenulatum* (Table 3. 3). The intestinal microbiota maintains itself in a constant and sensitive equilibrium, thus stress, diet changes, pharmaceutical therapies, antibiotic treatments can degenerate so far this balance up to the vanish of some species.

Population	**Main species**	**Minor species**
Breast-fed newborns	*B. longum* *B. infantis* *B. breve*	*B. bifidum*
Bottle-fed newborns	*B. adolescentis*	*B.bifidum*
Infants	*B. infantis* *B. breve* *B. longum*	*B. adolescentis* *B. catenulatum*
Adults	*B. adolescentis* *B. longum* *B. catenulatum*	*B. breve* *B. bifidum*
Elders	*B. adolescentis* *B. longum*	*B. catenulatum*

Tab. 3. 3: Bifidobacteria isolated from stool samples of healthy humans

3. 3: Mucosa interactions

Microrganisms can be found moving free in a solution just during a transitory stage of their life, instead, particularly bacteria, they are found associated in a complex

community, as a biofilm, which permit the adhesion to surface. Studies have demonstrate that is more probable that bacteria tend to adhere when are in the log phase of growth. Inside the community of the biofilm, a single bacterial cell have many advantages; for example: it is protected from host phagocytosis and from toxic components released by host metabolism and it is anchored to the matrix, thus resisting to peristaltic movements. In nature, bacterial biofilms can be found practically in every environment and so even in mammals gastrointestinal tract. Biofilm development can take days and weeks and starts from a reversible absorption stage, passing to a non reversible absorption stage, then to cell division and bacterial growth stage and to the stage of polymers production and biofilms establishment and concludes with the of aggregation of others bacteria to the growing community (Figure 3. 2). From the first moment that there is an interface contact can be distinguish two kinds of interactions, from microrganisms and microrganisms and from microrganisms and surface. These interactions can be site specific, as the link ligand/receptor , or even non specific, as the hydrophobic interactions between adhesion proteins and lipoteichoic acids or the process of co-aggregation that results in adhesion between bacterial cells. Gastrointestinal tract is a very complex habitat where bacteria are exposed not only to molecules produced by other microrganisms, but even to that molecules produced by epithelial cells. of the mucosa..

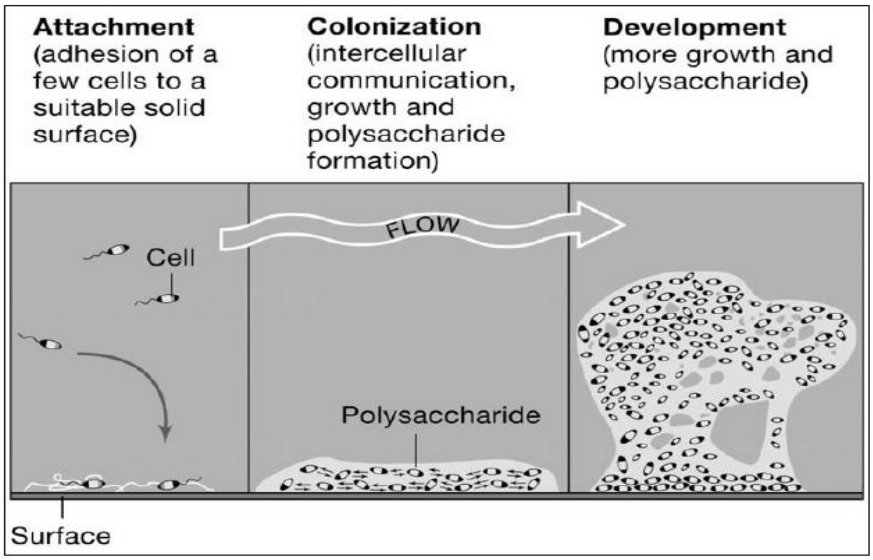

Fig. 3. 2: Schematic representation of biofilm formation

3. 3. 1: Adhesion

Bacterial adhesion to different matrix is a fundamental strategy for bacterial growth and niche's colonisation. Considering the gastrointestinal tract, there is to know that adhesion process of bacterial cells is a complex and multipartite phenomenon, which include the secretion of growth inhibitors, as bacteriocin, organic acid or reactive oxygen species, physiological changes and a non specific activation of the host immune system. The several steps required for adhesion are strongly influenced by several external biotic and non biotic factors. The first step consists in the approach of bacterial cells to the matrix to be colonised. This is possible due to the active or passive motility of bacteria, as the presence of pili or flagella or thanks to sedimentation and Brownian motion. In any case, a bacteria first to adhere to a biotic matrix has to overcome an energetic barrier (Gibbs energy), of which intensity is due to combined action of electrostatic forces and

electrodynamic forces, as Van der Waals force. Electrostatic force is always attractive, because are dependent of the ionic double layer electric charge, that form between two negative charged surfaces. Rather, electrodynamic forces are attractive, once from one or from both surfaces are secreted or are produced molecules that increase the matrix area. These two kind of molecules are able to alter the surface hydrophobicity charge; in fact, an elevated hydrophobicity rate results in a loss of Gibbs energy, permitting more easily to bacteria to reach the surface to be colonised. Once bacteria have overcome Gibbs energy and approach the surface they hit it and attach to it. If the period in which the interface remain linked is short, we can talk of adhesion, while if the period is prolonged in time, we can talk of attachment. Adhesion is encouraged by smooth surfaces and concern just non specific, short ranged and relatively weak forces. Instead, attachment is promoted by non regular surfaces and concern specific and non specific forces that improve a strong bond between bacterial ligands and eukaryotic receptors. Attachment lead off out and out colonisation due to the establishment of strong bonds between the interface. During colonisation bacteria are growing and produce exopolymers that are used to extend outside forming a fibre matrix that lead to the formation of microcolonies and biofilms.

3. 3. 2: Proteins involved in adhesion

These molecules are of different kinds and vary from simple carbohydrates to complex proteins. Specificity of interaction is modulated by proteins known as adhesins that interact with the different components of extracellular matrix as fibronectin and collagen. In the class of adhesins are included adhesins and lectins. The firsts are highly conserved proteins, in which the mutation even of a single aminoacid lead to a change in bond affinity with sugars present in receptor's active

site; while lectins are proteins capable to bind sugars specifically or able to mediate the link between different sugars. Receptors that interact with proteins are equally important in adhesion mechanism and are characterised by the presence of different sugars or for the presence of sialic acid. The toll-related proteins, of which until to date about 12 have been identified, are highly conserved through evolution. The TLRs are expressed on both enterocyte and immune cells. They recognize specific microbial components through leucine rich region domains (LRRs), such as surface determinants, lipopolysaccharide (LPS) of Gram-negative bacteria (TLR2 and TLR4) and unmethylated CpG DNA sequences (TLR9). Their activation induce the production of T-helper 1 (Th1) cytokines through a processn dependent on NF-κB activation (Miettinen *et al.*, 2000). LPS and the Lipid A fraction of LPS is recognized by TLR4. In humans the D299G mutation, affecting the LRR domain of TLR4, is associated with a blunted response to inhalated LPS. An increased expression of this receptor in the epithelial cells of patients with inflammatory bowel diseases has been related to changes in the intestinal flora (Cario and Podolski, 2000). In pathogenesis, bacterial adhesion has been particularly studied, because is one of the stages that precede infection. (Table 3. 4).

Adhesion factors	Description	Action
Adhesins	Surface proteins	Bind bacteria to a specific surface
Receptors	Complementary macromolecule binding site	Bind eukaryotic cells to specific adhesins or ligands
Lectins	Surface protein	Bind to sugars
Ligands	Surface molecule	Bind to a specific receptor
Mucous	Mucopolysaccharide layer of glycosaminoglycans	Cover the surface of mammals epithelial cells
Pili	Chain of oligomeric pilin proteins	Bind bacteria to a specific surface
Fimbriae	Filamentous protein	Permit bacteria to bind to a specific surface
Glycocalyx	Surface polysaccharides	Involvement in adhesion
Capsule	Surface polysaccharides layer	Mediation of bacterial attachment
Lipoplysaccharides (LPS)	Cell wall components	Mediation of bacterial specific adhesion
Teichoic and lipoteichoic acids	Cell wall component	Involvement in specific adhesion

Tab. 3. 4: Some factors involved in microrganisms-host interactions with descriptions and modes of action.

3. 4: Probiotic role

3. 4. 1: Probiotic overview

The Greek meaning of the word probiotic is "for life." One early formal definition was that of Parker (1974): "Organisms and substances which contribute to intestinal microbial balance." However, this was subsequently refined by Fuller (1989) whose revised definition is "A live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance." This version emphasizes the need for the supplement to be composed of viable microorganisms and is the most widely used and accepted definition. Probiotics, therefore, aim to produce a beneficial effect on the host by administration of viable microorganisms such as those found in traditional yogurts and other fermented foods, as well as powders, tablets, liquid suspensions and lyophilized forms in capsules. Monocultures as well as mixed species (up to nine) can be used in individual products. Primarily, these are lactic acid–excreting bacteria such as lactobacilli, streptococci, lactococci or bifidobacteria, although some yeasts and other fungi are also used. Metchnikoff (1907) first developed the concept of what we now know as probiotics at the beginning of this century. His hypothesis was that the complex microbiota of the colon was having an adverse effect on the host through what he termed the "autointoxication effect." As such, he believed that modification of the activity of the colonic microflora could occur through the ingestion of soured milks.

The theory was developed after he observed that Bulgarian peasants consumed large quantities of such milks and exhibited longevity. Metchnikoff isolated the bacteria responsible and used them in human feeding trials. After Metchnikoff's death, Rettger and colleagues became interested in the mechanism of the probiotic effects and researched the use of intestine-derived species (Rettger *et al.* 1935). The field then took a number of scientific progressions to reach today's situation in which live microbial feed additions are ubiquitous.

3. 4. 2: Immuno-stimulation

When the antigen gets into the body by an oral route, the systemic immune stimulation is produced by the cytokines released by lymphoid cells associated with the mucosa, which interact with the antigen. This response may be measured through in vitro assays by determining the cellular activity (e.g., the phagocytic activity) or the products released during the cellular interactions; examples are cytokines, using special cellular lines, such as the mono Mac 6 human macrophage cell line; human epithelioid carcinoma, cell transfected with cDNA for IL4 and IL5**;** and Caco-2 human colon carcinoma cell line. Then, the systemic immune response may be determined by measuring the nonspecific immune response through phagocytic activity of the peritoneal macrophages, which were stimulated by interleukins, and the specific immune response may be evaluated by measuring the activation grade of T-lymphocytes by assays of delayed-type hypersensitivity or by antibody level increased from T-lymphocytes and B-lymphocytes activation through cytokines. However, great activation of the immune system in a healthy host cannot be conveniently obtained, because the immune system is always in equilibrium with other systems (*e.g.*, the nervous and endocrine systems); a general activation of the immune system by constant antigen stimulation could produce

negative effects on the host, including autoimmunity. A circumstance that would require immune system stimulation is primarily immunosuppression from antitumor therapies, but never when this immunosuppression is caused by a therapy for autoimmune illness. Lactic acid bacteria might benefit the host in some situations, such as to prevent enteric infections or to act as immunomodulatory agents in other processes. The therapeutic use of lactic acid bacteria must consider the effects of these bacteria on the intestinal microenvironment, especially the microflora, which are responsible for oral tolerance (Perdigon *et al*., 1995).

3. 4. 3: Colon cancer

Cancer of the colon is one of the leading causes of cancer morbidity and mortality among men and women in the Western countries, including the United States (Parker *et al.* 1997). Epidemiologic studies suggest that increased consumption of fruits and vegetables and high total dietary fiber reduce the risk of development of colon cancer (Howe *et al.* 1992). Human metabolic and laboratory animal model studies indicate that beneficial effects of dietary fiber in relation to colon cancer development depend on the composition and physical properties of the fiber (Reddy *et al.* 1992; 1995). Among the types of dietary fiber, inulin and oligofructose, which occur in common food stuffs such as chicory, leeks, garlic, onion, artichoke and asparagus at high levels, are ß (2→1)D fructans. They are fermented by colonic microflora and behave as soluble fibers (Gibson and Roberfroid 1995). It is of great interest that they selectively stimulate the growth of bifidobacteria at the expense of bacteriodes, clostridia or coliforms, which are maintained at low levels (Gibson and Roberfroid 1995, Gibson *et al*. 1995). Bacterial fermentation of these prebiotics produces short-chain fatty acids (SCFA) in the colon, including a small amount of butyric acid (Campbell *et al*. 1997 , Gibson and Roberfroid 1995), which has been

shown to increase apoptosis in the colon (Hague *et al.* 1993). Of special interest are the beneficial effects of certain lactic acid–producing enterobacterial food supplements, probiotics, in the prevention of cancer (Hitchins and McDonough 1989). The lactic cultures, which are primarily used for fermentation of milk and other dairy products, have also been shown to possess antimutagenic and anticarcinogenic properties (Lidbeck *et al*. 1992). Colorectal cancer is now widely accepted to be the result of an accumulation of mutations in specific genes controlling cell division, apoptosis and DNA repair. There is also a wealth of evidence that dietary factors, including dietary fat and fibre, influence the development of colorectal cancer. However, until recently, there has been little understanding of how these dietary factors and genetic factors interact. It is generally believed that this interaction is mediated in part by events occurring in the lumen of the large bowel. IQ, a heteroxyclic aromatic amine produced from food pyrolysis, was first isolated from broiled fish. Subsequently, it was isolated from a variety of broiled or cooked fish and meat (Kasai *et al*. 1980). Because IQ induces colon tumors in male and female rats and mammary tumors in female rats, and bacterial cultures that ferment milk possess anticarcinogenic properties, the possibility exists that these bacterial cultures may prevent IQ-induced carcinogenesis. Accordingly the inhibitory effect of lyophilized cultures of *B. longum* on IQ-induced carcinogenesis was investigated in male and female F344 rats (Reddy and Rivenson 1993). The results indicated that lyophilized cultures of *B. longum* significantly inhibited the IQ-induced incidence (percentage of animals with tumors) of colon tumors (100% inhibition) and multiplicity of colon tumors (tumors per animal) in male rats. In diet intervention studies, Gibson *et al*. (1995) demonstrated that dietary administration of oligofructose or inulin significantly increased fecal bifidobacteria, whereas bacteriodes, clostridia and fusobacteria and/or gram-positive cocci were decreased on total fecal bacterial count. These

bifidobacteria, colonizing at the expense of enteropathogens, may bind the ultimate carcinogen by physically removing it via feces. The colonizing cells of bifidobacteria also produce lactic acid, thereby lowering the intestinal pH to create a bacteriocidal environment for putative enteropathogens such as *E. coli* and *Clostridium perfringens*, thus developing a favorable microenvironment. This favorable microenvironment may also involve the modulation of bacterial enzymes such as ß-glucuronidase that can convert procarcinogens to proximate carcinogens (Kulkarni and Reddy 1994). Polyamines play an essential role in cell proliferation and differentiation and participate in macromolecular synthesis. Ornithine decarboxylase (ODC) is the first and rate-limiting enzyme of this crucial polyamine biosynthetic pathway. Elevated levels of ODC activity have been reported in neoplastic human colons vs. normal-appearing colonic mucosa (Porter *et al*. 1987, Singh *et al.* 1992). Studies demonstrate that the colon tumor inhibitory property of lyophilized cultures of *B. longum* was associated with the inhibition of colonic mucosal cell proliferation and with suppression of ODC activity in the colonic mucosa and tumors compared with that in control diet. Aberrant crypt foci (ACF), which are recognized as early preneoplastic lesions in the colon, have consistently been observed in experimentally induced colon carcinogenesis in laboratory animals and in the colonic mucosa of patients with colon cancer (McLellan *et al*. 1991). ACF also express mutations in the *APC* gene and *ras* oncogene that are involved in colon cancer development (Vivona *et al.* 1993). Aberrant crypts are putative precursor lesions from which adenomas and carcinomas may develop in the colon. Several inhibitors of ACF formation have been shown to reduce the incidence of colon tumors in laboratory animals (Wargovich *et al*. 1996), suggesting that ACF induction can be used to evaluate novel agents for their potential chemopreventive properties against colon cancer. Studies were also conducted to evaluate the inhibitory properties of lyophilized cultures of *B. longum*

against induced colonic ACF development (Kulkarni and Reddy 1994). The results indicate that dietary administration of lyophilized cultures of *B. longum* significantly inhibited the total ACF formation and crypt multiplicity. The study results provide evidence for potential colon tumor-inhibitory properties of *B. longum*. *Ras* activation represents one of the earliest and most frequently occurring genetic alterations associated with human cancers, especially the cancer of the colon (Barbacid 1990). Elevated levels of *ras*-p21 have been correlated with increased cell proliferation, histologic grade, nuclear anaplasia and degree of undifferentiation (Kotsinas *et al*. 1993). Dietary *B. longum* cultures significantly suppressed the expression of total and mutated *ras* p-21 in the colonic mucosa and tumors compared with the control diet. After all these informations this chapter conclude stating that dietary administration of prebiotics such as oligofructose, inulin and lyophilized cultures of *B. longum* inhibits the formation of preneoplastic lesions in the colon. In addition, dietary administration of lyophilized cultures of *B. longum* suppressed colon and mammary carcinogenesis in the laboratory animal models. Inhibition of colon carcinogenesis by lyophilized cultures of *B. longum* is associated with the modulation of colonic cell proliferation and colonic mucosal and tumor ODC and *ras* p-21. Further studies are required to investigate the efficacy of prebiotics in combination with probiotics on the inhibition of colon tumors. Although pre- and probiotics comprise a diverse group with different modes of action, their ability to inhibit colon carcinogenesis may be important to the development of potential nutritional and related food supplements against colon cancer.

3. 5: Apoptosis

3. 5. 1: Cell Death Overview

That cell death is a completely normal process in living organisms was already discovered by scientists more than 100 years ago. The German scientist Carl Vogt was first to describe the principle of apoptosis in 1842. In 1885, anatomist Walther Flemming delivered a more precise description of the process of programmed cell death. However, it was not until 1965 that the topic was resurrected. Apoptosis (*apo* - from, *ptosis* – falling) was distinguished from traumatic cell death by Wyllie, Kerr and Cormack in a publication of 1972, originally using for the first time the term " *apoptosis* " to describe the phenomenon of natural cell death. In Greek, apoptosis means "dropping off" of petals or leaves from plants or trees. Cormack reintroduced the term for medical use as it had a medical meaning for the Greeks over two thousand years before. Hippocrates used the term to mean "the falling off of the bones". Galen extended its meaning to "the dropping of the scabs". Cormack was no doubt aware of this usage when he suggested the name.Programmed cell death is an integral part of both plant and animal tissue development. Development of an organ or tissue is often preceded by the extensive division and differentiation of a particular cell, the resultant mass is then "pruned" into the correct form by apoptosis. Unlike cellular death caused by injury, apoptosis results in cell shrinkage and fragmentation. This allows the cells to be efficiently phagocytosed and their components reused without releasing potentially harmful intracellular substances (such as hydrolytic enzymes, for example) into the surrounding tissue. Apoptosis can occur when a cell is damaged beyond repair, infected with a virus, or undergoing stress conditions such as starvation. DNA damage from ionizing radiation or toxic chemicals can also induce apoptosis via the actions of the tumor-

suppressing gene *p53*. The "decision" for apoptosis can come from the cell itself, from the surrounding tissue, or from a cell that is part of the immune system. In these cases apoptosis functions to remove the damaged cell, preventing it from sapping further nutrients from the organism, or to prevent the spread of viral infection. Apoptosis also plays a role in preventing cancer; if a cell is unable to undergo apoptosis, due to mutation or biochemical inhibition, it can continue dividing and develop into a tumor. For example, infection by papillomavirus causes a viral gene to interfere with the cell's p53 protein, an important member of the apoptotic pathway. This interference in the apoptotic capability of the cell plays a critical role in the development of cervical cancer. Although many pathways and signals lead to apoptosis, there is only one mechanism that actually causes the death of the cell in this process; after the appropriate stimulus has been received by the cell and the necessary controls exerted, a cell will undergo the organized degradation of cellular organelles by activated proteolytic caspases. A cell undergoing apoptosis shows a characteristic morphology that can be observed with a microscope. First the cell undergoes shrinkage and rounding due to the breakdown of the proteinaceous cytoskeleton by caspases. Then the cytoplasm appears dense, and the organelles appear tightly packed. Subsequently chromatin undergoes condensation into compact patches against the nuclear envelope in a process known as pyknosis, a hallmark of apoptosis (Susin *et al.,* 2000; Kihlmark *et al.*, 2001). In the next stage the nuclear envelope becomes discontinuous and the DNA inside it is fragmented in a process referred to as karyorrhexis. The nucleus breaks into several discrete *chromatin bodies* or *nucleosomal units* due to the degradation of DNA (Figure 3. 3) (Nagata S., 2000).

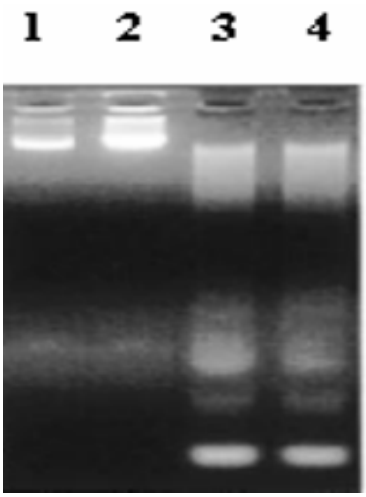

Fig. 3. 3: Oligonucleosomal DNA fragmentation assessed after exposure of control DNA to Apoptosis-inducing factor (AIF) and to Caspase-activated DNAse (CAD). 1) AIF alone . 2) CAD alone. 3) DNA after sequential exposure to AIF. 4) DNA after sequential exposure to CAD. (Susin *et al.*, 2000).

Then the cell membrane shows irregular buds known as blebs and finally the cell breaks apart into several vesicles called *apoptotic bodies*, which are then phagocytosed. Apoptosis progresses quickly and its products are quickly removed, making it difficult to detect or visualize. During karyorrhexis, endonuclease activation leaves short DNA fragments, regularly spaced in size. These give a characteristic "laddered" appearance on agar gel after electrophoresis. Tests for DNA laddering differentiate apoptosis from ischemic or toxic cell death (Iwata *et al.*, 1994).

3. 5. 2: Apoptotic signals

The process of apoptosis is controlled by a diverse range of cell signals, which may originate either extracellularly (*extrinsic inducers*) or intracellularly (*intrinsic inducers*). Extracellular signals may include hormones, growth factors, nitric oxide (Brüne B., 2003) or cytokines, and therefore must either cross the plasma membrane or transduce to effect a response. These signals may positively or negatively induce apoptosis; in this context the binding and subsequent initiation of apoptosis by a molecule is termed positive, whereas the active repression of apoptosis by a molecule is termed negative. Intracellular apoptotic signaling is a response initiated by a cell in response to stress, and may ultimately result in cell suicide. The binding of nuclear receptors by glucocorticoids, heat, radiation, nutrient deprivation, viral infection, and hypoxia are all factors that can lead to the release of intracellular apoptotic signals by a damaged cell. A number of cellular components, such as poly ADP ribose polymerase, may also help regulate apoptosis (Chiarugi and Moscowitz, 2002). Before the actual process of cell death is carried out by enzymes, apoptotic signals must be connected to the actual death pathway by way of regulatory proteins. This step allows apoptotic signals to either culminate in cell death, or be aborted should the cell no longer need to die. Several proteins are involved, however two main methods of achieving regulation have been identified; targeting mitochondria functionality, or directly transducing the signal via *adapter proteins* to the apoptotic mechanisms. The whole preparation process requires energy and functioning cell machinery. Even the mitochondria are exploited by some apoptotic pathways. Apoptotic proteins that target mitochondria affect them in different ways; they may cause mitochondrial swelling through the formation of membrane pores, or they may increase the permeability of the mitochondrial membrane and cause apoptotic effectors to leak out (Figure 3. 4).

There is also a growing body of evidence that indicates that nitric oxide (NO) is able to induce apoptosis by helping to dissipate the membrane potential of mitochondria and therefore make it more permeable (Brüne B., 2003).

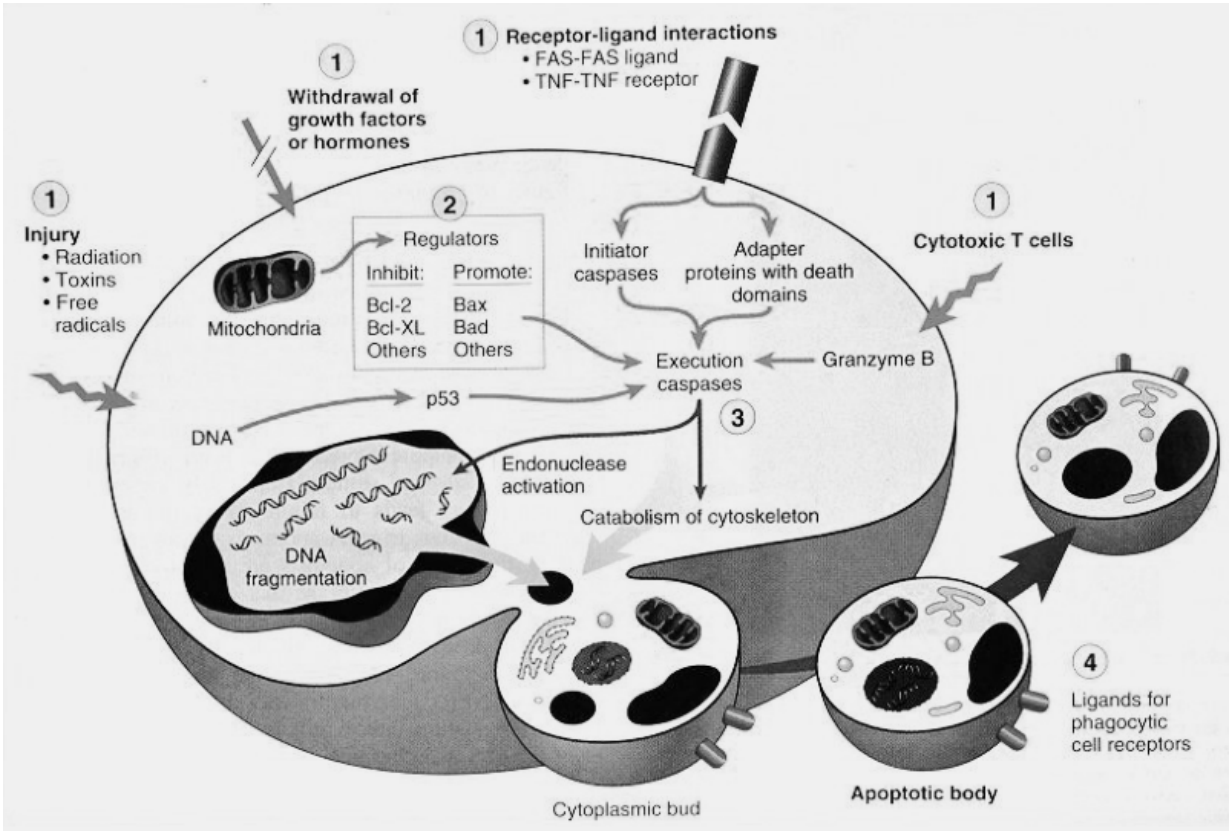

Fig. 3. 4: Signal induction and transduction in programmed cell death (Hengartner, 2000).

3. 5. 3: Apoptotic deletion

Apoptosis is an important regulatory process in the protection against the development of cancer. Apoptosis provides an innate cellular defense against oncogenesis by processes that include removal of cells with genomic instability that have developed during oncogenesis and by deletion of cells suffering DNA insult

from genotoxic agents such as carcinogens (Thomson C. B., 1995; Potten and Grant, 1998) . Upregulation or facilitation of apoptosis during initiation events might increase the elimination of mutated cells that might otherwise Among the major genera of colonic bacteria, bifidobacteria and lactobacilli are thought to have beneficial effects on the human host (Orrhage and Nord, 2000). These probiotic bacteria were shown previously to exert some cancer protective effects *in vitro* and in vivo (Singh *et al.*, 1997; Wollowski *et al.*, 2001). The precise mechanisms by which certain probiotic bacteria exert their antitumorigenic influence are uncertain, but might involve modifying gut pH and increasing the net production rate of SCFA (mainly acetate, propionate, and butyrate) (Sakata *et al.,* 1999), antagonizing pathogens through production of antimicrobial and antibacterial compounds (such as bacteriocins, cytokines and butyrate), stimulating immunomodulatory cells (Rolfe R. D., 2000), or competing with pathogens for available nutrients, receptors, and growth factors (Fuller and Gibson, 1997). Prebiotics are nondigestible dietary components that pass through the digestive tract to the colon. Thus, prebiotics are a potential substrate for fermentation by the microbiota and are capable of stimulating proliferation and/or activity of endogenous desirable bacteria (Gibson and Roberfroid, 1995). Prebiotics shown to stimulate the acute apoptotic response to a genotoxic carcinogen (AARGC) in the rat colon. The AARGC might regulate mutational load in the colon and eliminate DNAdamaged cells that might otherwise progress to malignancy, thereby exerting a protective effect at the early stages in the onset of cancer. Oligosaccharides also suppress azoxymethane (AOM)-induced preneoplastic aberrant crypt foci (ACF) and colon carcinogenesis in the rat colon (Rowland *et al.*, 1998; Femia *et al.,* 2002) as does wheat bran (McIntosh *et al.*, 1996; Reddy *et al.*, 2000). Prebiotics may exert their cancer protective effects via modulation of fermentative events, possibly by increasing SCFA production or by altering gut microbiota toward a more beneficial

composition. It was suggested that a combination of a probiotic and a prebiotic, termed synbiotics, might be more active than either a probiotic or prebiotic alone (Roberfroid M. B., 1998). This idea was supported by Rowland *et al*. (Rowland *et al.*, 19982) who showed that administration of the prebiotic inulin with the probiotic *Bifidobacterium longum* to rats resulted in additive effects, with a more potent inhibition of AOM-induced ACF than administration of either inulin or *B. longum* separately. These findings raise the possibility that probiotics, prebiotics,or synbiotic combinations thereof might be protective against colorectal cancer by regulating the consequences of carcinogen-induced damage to colonic epithelial cells. Thus, we aimed to determine whether supplementation with either the probiotic bacteria *Lactobacillus acidophilus* and *B. lactis* alone or in combination with a moderate amount of RS could influence the acute apoptotic response to a genotoxic carcinogen (AARGC) and fermentative events in the colon rats. In conclusion, the synbiotic combination of RS and *B. lactis* significantly facilitated the apoptotic response to a genotoxic carcinogen in the distal colon of rats. It appears likely that ingested RS acts as a metabolic substrate, thus creating the right conditions for *B. lactis* to exert its proapoptotic action. Because the synbiotic combination of these agents facilitates the apoptotic response to DNA damage by a cancer initiator in the colon of rats, it warrants further study for its capacity to protect against colorectal cancer.

Chapter 4: Genomic of *Bifidobacterium longum*.

4. 1: *B. longum* genome

4. 1. 1: Genome overview.

The whole sequence of the genome of *Bifidobacterium longum* NCC2705 has a 2,256,646-bp, 60% G+C chromosomal replicon containing 4 nearly identical *rrn* operons, 57 tRNAs, 16 intact insertion sequence (IS) elements, as well as possible prophage and integrated plasmid remnants (Figure 4. 1). Eight of the IS elements constitute new members of five known families, including one rare IS607-type. The genome harbored 14 integrase_recombinase genes, 9 of which were organized in 3 unusual and nearly identical structures and are included in a phage integrase family. More than 1,730 probable coding regions comprising 86% of the genome have been analysed. A specific or general function was assigned to 1,225 (71%) of them; 1,346 (78%) were attributed to a COG family. No functional description could be assigned to 505 genes (9%). Of these, 389 (22%) had no similarity to any predicted protein in public databases indicating either that they were either specific

to *B. longum* or that they remain to be identified in other genomes (Schell *et al.*, 2002). When the best BLASTP hits for each predicted protein were parsed by organism, it has been found that 34% of the ORFs had best hits from *Streptomyces coelicolor*, 9.3% from *M.tuberculosis*, and 3.8% from *Corynebacterium glutamicum*. This finding confirms assignment of *B. longum* to the *Actimomycetales* group. Surprisingly, a disparate number of best hits were found in genomes of phylogenetically distant genera that include GIT inhabitants. For example, 5.3% of the best hits were from *Clostridia*, 4.0% from *Streptococcus*, and 1.9% from *Escherichia coli*, whereas 0.7% were found in any other single prokaryotic genome.

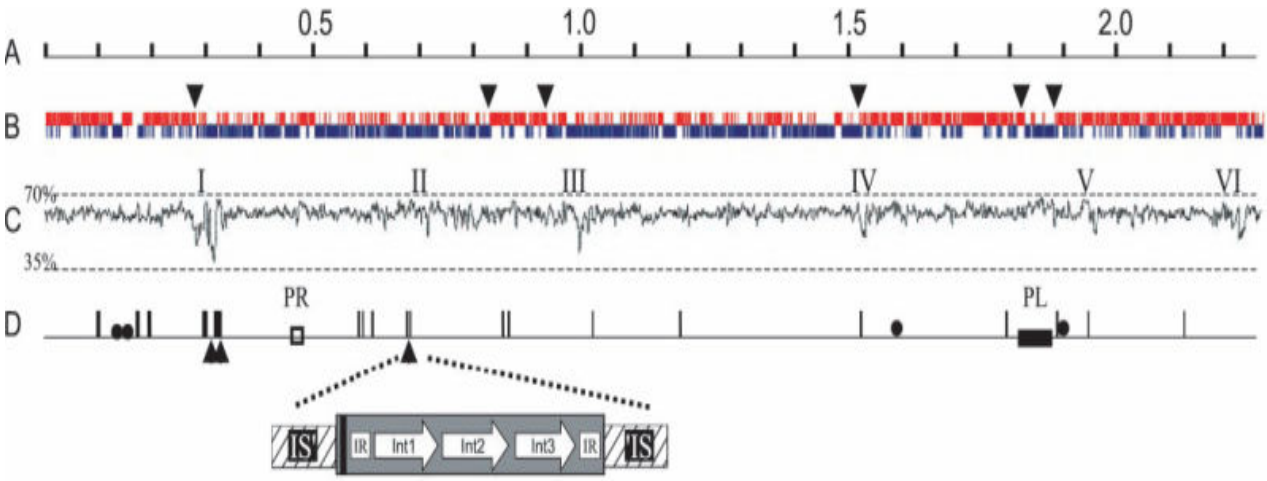

Fig. 4. 1: Linear map of the *B. longum* chromosome. (*A*) Scale in Mb. (*B*) Coding regions by strand. Upper and lower lines represent plus and minus strand ORFs, respectively. Arrows indicate transition points in cumulative GC skew from ORILOC. (*C*) G+C content with scale (window 1,000). Roman numerals mark regions where G+C is 2.5 SD units below average. (*D*) Intact (two IS*3*-type, five IS*21*-type, three IS*30*-type, five IS*256*-type, and one IS*607*-type) as well as partial IS elements are represented by vertical lines; boxes mark possible prophage remnant (PR) and integrated plasmid (PL). Filled circles represent rRNA operons.

Positions of the 3 nearly identical copies of the potentially new type of mobile genetic element in *B. longum* are indicated by triangles and an expanded view of one shown. The three different integrases (Int) are represented by arrows; the interrupted IS*3*-type element containing them is hatched. Black bar, 20-bp palindrome; IR, 97-bp perfect inverted repeat (Schell *et al*., 2002).

4. 1. 2: Genomic Adaptation of sugar metabolism.

Bifidobacteria colonize the lower GIT, an environment poor in mono- and disaccharides because they are consumed by the host and microflora in the upper GIT. Although past work showed that *B. longum* utilizes a variety of plant-derived dietary fibers, such as arabinogalactans and gums (Salyers *et al.*, 1977; Crociani *et al.*, 1994), the genome sequence suggests that this ability is much more extensive than previously anticipated, reflecting its adaptation to a special colonic niche. The genome contains a plethora of predicted proteins assigned to COGs in the carbohydrate transportmetabolism category, 8.5% of the total predicted proteins.This is 30% more than *E. coli*, *Enterococcus faecium*, *L. lactis*, *B. halodurans*, and *B. subtilis*. Numerous assignments were to COGs related to oligosaccharide hydrolysis and uptake such asthat to L-arabinofuranosidases, to glucosidase-related glycosidases, to glycosyl hydrolase family, to sugar permeases, and that related to solute binding proteins. *B. longum* has 40 predicted glycosyl hydrolases whose predicted substrates cover a wide range of di-, tri-, and higher order oligo saccharides, including 2 xylanases, 9 arabinosidases, 2 galactosidases, neopullanase, isomaltase, maltase, inulinase (fructofuranosidase), 4 galactosidases, 3 glucosidases, 3 hexosaminidases, and 3 mannosidases. To take full advantage of these enzymes and minimize crossfeeding of competitors, *B. longum* has 8 high-affinity MalEFG-type oligosaccharide transporters (Cabral *et al.*, 2001), more than in any other published prokaryotic genome. These likely help *B. longum* compete

for uptake of structurally diverse oligosaccharides released from plant fibers. Interestingly, many of the glycosyl hydrolases and oligosaccharide transporters are organized in 7 clusters that display a conserved modular architecture (Figure 4. 2). Each cluster consists of 5 predicted arabinosidases and a rare β-galactosidase homolog implying that its function is to release arabinose and galactose from internalized fragments of arabinogalactans and arabinoxylans. These fragments are probably generated by extracellular enzymes such as endoarabinosidase BL0183 and endoxylanase BL1544 acting on larger, hemicellulosic plant fibers in the GIT. The extent of *B. longum*'s metabolic adaptation to the GIT environment is further highlighted by cluster 6. In addition to the MalEFG-type oligosaccharide transporter and a glycosyl hydrolase, this cluster contains three α-mannosidases and an endo-NAc glucosaminidase, which are more commonly found in eukaryotes, where they remove the *N*-linked Man8-NAcGlc2 chains of glycoproteins (Cabral *et al.*, 2001). Cluster 6 may function in catabolism of galactomannan-rich plant gums, that among major GIT inhabitants, are fermented only by *B. longum* (Salyers *et al.*, 1977) or more interestingly, in catabolism of glycans, glycoconjugates, or glycoproteins produced by epithelial cells of the colon. In support of this Hooper *et al.* (Hooper *et al.*, 1999) showed *in vivo* that the GIT commensal *Bacteroides thetaiotamicron* induced mouse intestinal epithelium to produce specific glycans (oligosaccharides), which were metabolized by specialized bacterial enzymes for its own nutritional benefit, possibly enhancing colonization.

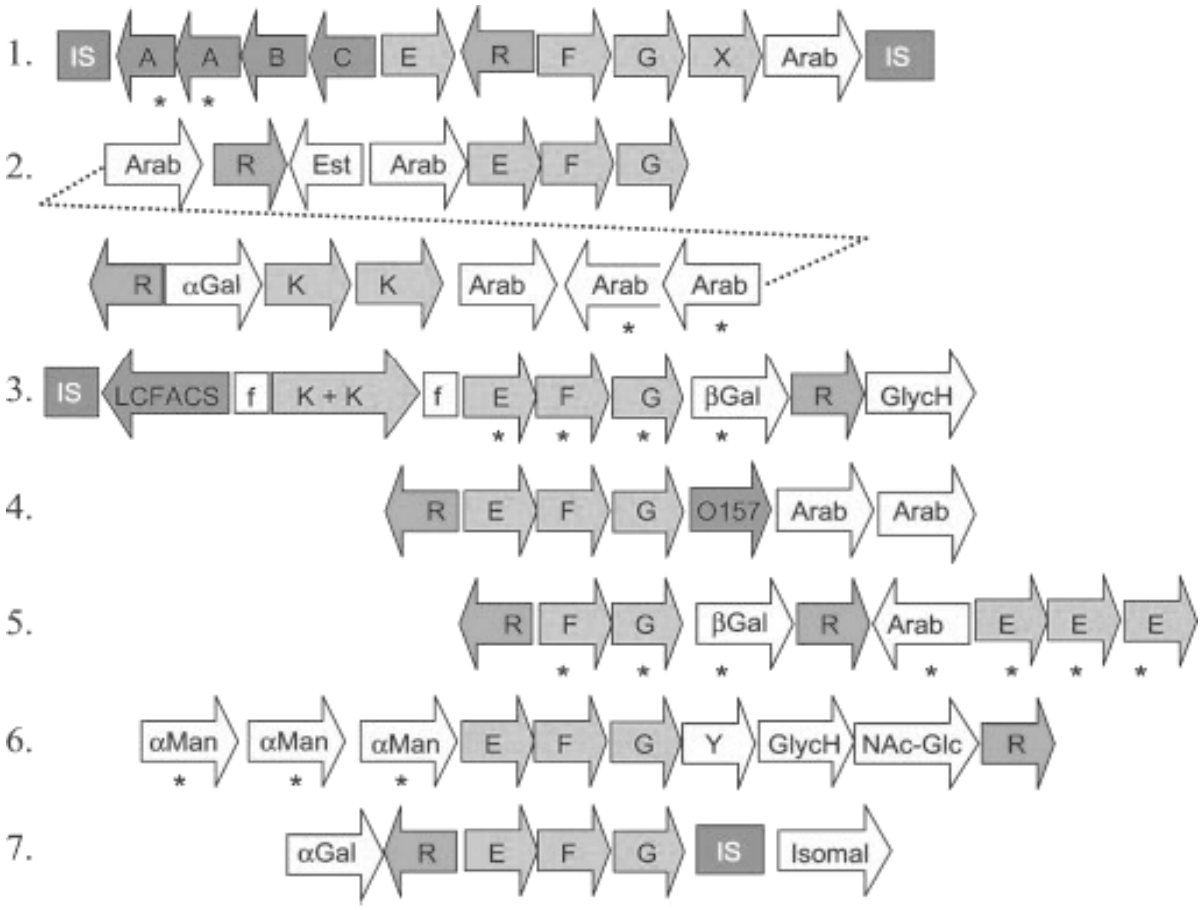

Fig. 4. 2: Oligosaccharide utilization gene clusters. Genes are represented by arrows. IS, insertion sequence; F and G, MalF-type and MalG-type permease subunits of ABC transporter, respectively; E and K, MalE-type solute binding protein and MalK-type ATP-binding protein of ABC transporter, respectively; R, LacI-type repressor; Arab, arabinosidase; β-Gal, β-galactosidase; α-Man, α-mannosidase; β-Gal, β-galactosidase; GlycH, glycosyl hydrolase of unknown specificity; Isomal, isomaltase; NAc-Glc, *N*-acetyl glucosamindase; O157, ORF with homolog only in *E. coli* O157; X and Y, unique hypothetical proteins; LCFACS, long chain fatty acyl CoA synthetase; Est, possible xylan esterase; A, *L. lactis* phage infection protein homolog; B, oxidoreductase; C, phosphoglycerate mutase; f, fragment of AraE permease. Asterisks mark recent gene duplications (Schell *et al.*, 2002).

4. 1. 3: Extracellular Components.

Extracytoplasmic proteins and structures play critical roles in establishing and maintaining interactions between a microbe and its environment. In bifidobacteria these could mediate important functions affecting the host, such as adhesion, nutrient availability, immune system modulation, or pathogen inhibition. Among 200 analysed proteins, 59 are apparently surface-associated lipoproteins, including 26 solute binding proteins of ABC transporter systems. In agreement with the extensive ability of *B. longum* to scavenge nutrients from extracellular polymers, several enzymes for polymer fragmentation were predicted to be secreted: 2 endoxylanases, 2 endoarabinosidases, an arabinogalactan β-galactosidase, a *N*-acetyl β-glucosaminidase. Additionally, two putative secreted proteins displayed a clear Gram-positive cell-surface anchor motif and are very large and possibly involved in attachment to or degradation of xylan hemicellulose.The most intriguing protein with a cell-surface anchor motif is 30% identical to the major component of the type 2 glycopropotein-binding fimbriae of the oral cavity inhabitant *Actinomyces naeslundii* (Yeung iet al., 1998). Fimbriae have never been described or proposed for bifidobacteria, yet they are of great potential significance because they are cell-surface filamentsthat can mediate microbial adhesion to and colonization of epithelial, mucosal, or other host cell surfaces (Kubiet *et al.*, 200). These additional cell-surface components include a sequence 31% identical to a fimbrial-associated sortase-like protein of *A. naeslundii* (Li *et al.*, 1999), a 262-kDa protein with a repetitive glycine-rich sequence characteristic of some Rickettsial cell-surface proteins, and a predicted prepilin peptidase with 35% identity to the product of *orfC*, the fourth gene in the fimbrial biogenesis operon of *A. naeslundii*. Of all predicted secreted proteins, BL0108 is most remarkable because it displays

identity to proteins from the serpin family of protease inhibitors found predominantly in mammals. In 100 genomes searched, there are prokaryotic homologs of BL0108 only in the heterocyst-forming cyanobacterium *Nostoc* sp.However, unlike what is found in *B. longum*, this serpin homolog is not predicted to be secreted and is adjacent to a gene encoding a probable target protease. In eukaryotes, serpins control key steps in physiological regulatory cascades by inhibiting specific proteases (Silvermann *et al.*, 2001). In some cases serpins play important roles in immune system evasion during pathogenesis, as in the case of a myxoma virus serpin that modulates host inflammatory response (Macen *et al.*, 1993).

4. 2: Serpins

4. 2. 1: Serpins at a glance

Serine proteinase inhibitors (Serpins) comprise a diverse group of proteins that form a superfamily including more than 100 members. The majority of Serpins act as protease inhibitors and are involved in the regulation of several proteinase-activated physiological processes, important for the individual, such as blood clotting, complement mediated lysis, the immune response, glomerulonephritis, pain sensing, inflammation, pancreatitis, cancer, regulating fertilization, bacterial infection and viral maturation. Though the primary function of Serpins appears to be neutralizing serine proteinase activity, these polypeptides have also been found to play a role in extracellular matrix remodelling and cell migration. Examples for Serpins include, alpha 1-antitrypsin, antithrombin III, plasminogen activator inhibitor 1 (PAI-1) or plasminogen activator inhibitor 2. The Serpins known so far have been the subject of intensive Research and they all seem to have a common

characteristic loop, termed the reactive site loop (RSL), extending from the surface of the molecule containing the recognition sequence for the active site of the cognate serine protease. The specificity of each inhibitor is considered to be determined primarily by the identity of the amino acid that is immediately amino-terminal to the site of potential cleavage of the inhibitor by the serine protease. This amino acid, known as the Pi site residue, is considered to form an acyl bond with the serine in the active site of the serine protease. The Serpins seem to act as "suicide inhibitors" forming a 1:1 stochiometric complex with the target proteinase, thus blocking their activity. According to recent data it has been indicated that the inhibitor is cleaved in the reactive center and that the complex is most likely trapped as a covalent acyl-enzyme complex. Numerous signaling pathways in higher organisms, such as apoptosis, inflammation, blood clotting, and others, involve proteolytic events as mediators of signal initiation, transmission, and termination. Substrate specificity of the involved proteases and a tight regulation of their activation and inhibition are essential regulatory mechanisms of temporal and spatial control in proteolytic signaling. Serpins (serine protease inhibitors) represent a large class of polypeptide serine protease inhibitors that are involved in regulation of a wide spectrum of protease-mediated processes (Gettins P. G., 2002; Silverman *et al.*, 2001). Serpins are widely distributed in higher eukaryotic organisms and are also found in some viruses where they appear to modulate virus-host interactions and viral infectivity (Gettins P. G., 20021). Thirty-four serpins identified in the human genome belong to nine different phylogenetic clades in the currently adopted serpin classification (Silverman *et al.*, 2001). Notably, some members of the serpin superfamily, such as ovalbumin, angiotensin, and others (Hunt and Dayhoff, 1980; Doolittle R. F., 1983), do not act as protease inhibitors but rather perform non-inhibitory biological functions despite a clear evolutionary relationship and an almost identical native fold. These non-inhibitory members of

the serpin superfamily appear to be incapable of the structural rearrangement required for protease inhibition (Wright *et al.*, 1990). Recently serpins were identified in bacteria and archea expanding their presence to all major domains of life (Schell *et al.*, 2002). This observation suggests that the unique serpin fold and the associated inhibitory mechanism may have originated at the early stages of evolution. Very little is currently known about the function of serpins in prokaryotes (Roberts *et al.*, 2004;). The sequence-based analysis suggests that prokaryotic members of the serpin superfamily are functional protease inhibitors, and a serpin from *Thermobifida fusca*, thermopin, was shown to inhibit chymotrypsin albeit by means of an unconventional cleavage site (Irving *et al.*, 2003). Thermopin is, however, unusual because of its ability to function at elevated temperature, whereas conventional serpins are prone to polymerization at higher temperature due to the metastable nature of their native fold (Fulton *et al*., 2005).

Class	Type	Action	Name	Research
Inhibitory	Proteases inhibitors	Control of proteolytic cascades	Antithrombin	Antithrombin deficiency results in thrombosis
		Coagulation and inflammation	Antitrypsin	Antitrypsin deficiency causes emphysema
Non-inhibitory	Hormone carriage proteins	Hormone binding	Cortisol binding globulin	Prevent metastasis in breast and prostate
	Tumor suppressor genes	Apoptosis induction	Maspin	Inhibits motility and invasion in Caco-2 cells in culture

Tab 4. 1: Serine proteases inhibitors in human plasma.

4. 2. 2: Serpins in Bifidobacterium longum

Serpin was identified in the genome of *Bifidobacterium longum* NCC2705, an infant-derived strain of bifidobacteria (Schell *et al.*, 2002). The sequencing of the *B. longum* genome revealed a genetic makeup that reflected a remarkable adaptation of this organism to the GIT environment. Intriguingly, the genome-wide search for predicted secreted proteins identified a serpin-like molecule encoded by

this microorganism (Schell *et al.*, 2002). To explore the physiological role of this *B. longum* protein in the GIT environment and its possible function in host-bacterium cross-talk, it has been performed a biochemical characterization of this hypothetical serpin. Despite the extensive knowledge about the role of serpins in regulation of the protease-mediated processes in higher multicellular eukaryotes, very little is currently known about their function in prokaryotes. The presence of serpins in bacteria and archea implies that this protein superfamily is likely to have originated before the separation of the major branches of life. However, serpins are rather sparsely present among the prokaryotes indicating that they are either easily lost when not essential for survival or alternatively that they may have been acquired by prokaryotes as the result of horizontal gene transfer. Most of the serpins from unicellular organisms are distantly related to the serpins from higher organisms and do not belong to any of the existing serpin clades. This also applies to the *B. longum* serpin, which is an orphan in the current serpin classification. These observations suggest that if unicellular serpins were acquired by horizontal gene transfer, such events would have occurred independently for different serpins, and the original multicellular donors of these genes are not present in the existing genomic databases (Roberts *et al.*, 2004). *B. longum* serpin inhibits elastase-like serine proteases following conventional cleavage of the P1-P1'. When combined with the primary sequence analysis of the unicellular serpins (Roberts *et al.*, 2004) the conventional inhibitory activity of the *B. longum* serpin suggests that the majority of serpins identified in unicellular genomes are inhibitory proteins with the inhibition mechanism identical to that of the inhibitory serpins in higher multicellular eukaryotes. Recent results indicate that the inhibitory function of serpins either evolved very early in the course of evolution or (in the case of the horizontal gene transfer scenario) that the inhibitory function of the serpins was predominant in the multicellular organims by the time horizontal gene transfers

occurred. The *B. longum* NCC2705 serpin is an efficient inhibitor of pancreatic elastase and neutrophil elastase, which appears relevant given that bifidobacteria are likely to be in contact with both of these enzymes in the gastrointestinal tract. Pancreatic elastase is a digestive enzyme secreted by the acinar cells in the pancreas, and unlike other pancreatic enzymes it is very stable during the passage through the intestine as its activity can readily be detected in the feces (Sziegoleit *et al.*, 1989). Protease inhibitors produced by bacteria may act to protect them against exogenous proteases and to provide an important competitive advantage. For example, secreted protease inhibitors identified in *Bacillus brevis* and *Privotella intermedia* are thought to protect these organisms against external proteolytic attack (Shiga *et al.*, 1995; grenier D., 1994), while the periplasmic protease inhibitor of *E. coli* ecotin was shown to protect these bacteria against neutrophil elastase (Eggers *et al.*, 2004). The healthy commensal flora required for normal intestinal function involves a delicate homeostatic balance on the mucosal surfaces of the gastrointestinal tract manifested in the apparent tolerance of the immune system toward commensal microorganisms, whereas pathological species are met with a vigorous and destructive immune response (Sansonetti P. J., 2004). Given the reported immunomodulatory properties of bifidobacteria, *B. longum* serpin inhibition of human neutrophil elastase is of particular interest, because the release of neutrophil elastase and other granule proteases by activated neutrophils at the sites of intestinal inflammation represent an important mechanism of innate immunity (Burg and Pillinger, 2001; Reeves *et al.*, 2002). Massive recruitment and activation of neutrophils in the intestine are triggered during intestinal inflammation caused by pathogenic bacteria but can also be a result of a pathological immune activation as observed in the inflammatory bowel disease. Identification of a novel neutrophil elastase inhibitor produced by the commensal bacterium *B. longum* suggests an intriguing possibility that the release of a neutrophil elastase inhibitor at

the sites of intestinal inflammation may be beneficial for reducing the deleterious effects of the HNE (human neutrophil elastase) activity, similar to the way the pathological tissue damage associated with the excessive HNE activity is attenuated by α_1-antitrypsin, the physiological HNE inhibitor in the blood plasma. Further investigation of the *B. longum* adaptation mechanisms to the mammalian GIT is required for better understanding of the potential role of the *B. longum* serpin in the interaction of this bacterial species with its host.

Chapter 5: Intestinal cell model

5. 1: Establishment of a pig cell model

5. 1. 1: Overview

To study the influence of protective and probiotic bacteria on their interactions The functional mammalian cell models to be established will mimic the intestinal tract of pigs. Cell lines will be used for the functional cell model. The most common cells in the intestinal epithelium are enterocytes. They are polarised cells with a distinct apical and basolateral cytoplasmatic membrane. Enterocytes are separated from one another by junctions, which form a tight epithelial barrier. Good *in vitro* cell models must satisfy two basic requirements: retention of tissue characteristics

to support interpretation of results for the *in vivo* situation and availability and easy handling for high-throughput testing. Cell culture models play a pivotal role in laboratory research of various fields. Appart of existance of many various cell lines, there is a substantial lack of good intestinal cell culture models. In spite of the fact, that human colon tumorigenic cell lines, like Caco-2, are widely used as a model of a small intestine, such cell lines do not represent the normal small intestine of the man. It is accepted, that pig is much better animal model than rodents in human research , therefore we have established a functional cell model of an adult pig small intestine, consistent of two pig intestinal epithelial cell lines (PSI and CLAB) and macrophages from the pig peripheral blood (PoMac). All newly established cell lines were spontaneously transformed to the continous cell line and characterised by morphological, immunochemical and functional characteristics. Results obtained proved, that the established functional cell model of the pig small intestine can be successfully used in various studies involving the small intestinal tract, as well as in studies of local or systemic immunity (Cencic *et al.*, 2006).

5. 1. 2: Epithelial cells

Are the most common epithelial cells in the intestinal epithelium, responsible for majority of the absorption of nutrients and drugs. They are polarised cells with distinct apical and basolateral cytoplasmatic membrane. Enterocytes are separated among themselves by tight junctions and form tight epithelial barrier (Figure 5. 1). Transport of signalling molecules or pathogens themselves across the intestinal epithelial barrier towards underlying mucosal immune system is a complex and dynamic process incuding various functional pathways (transcellular and paracellular transport, transport via carriers and endocytosis) (Balimane *et al.*, 2000).

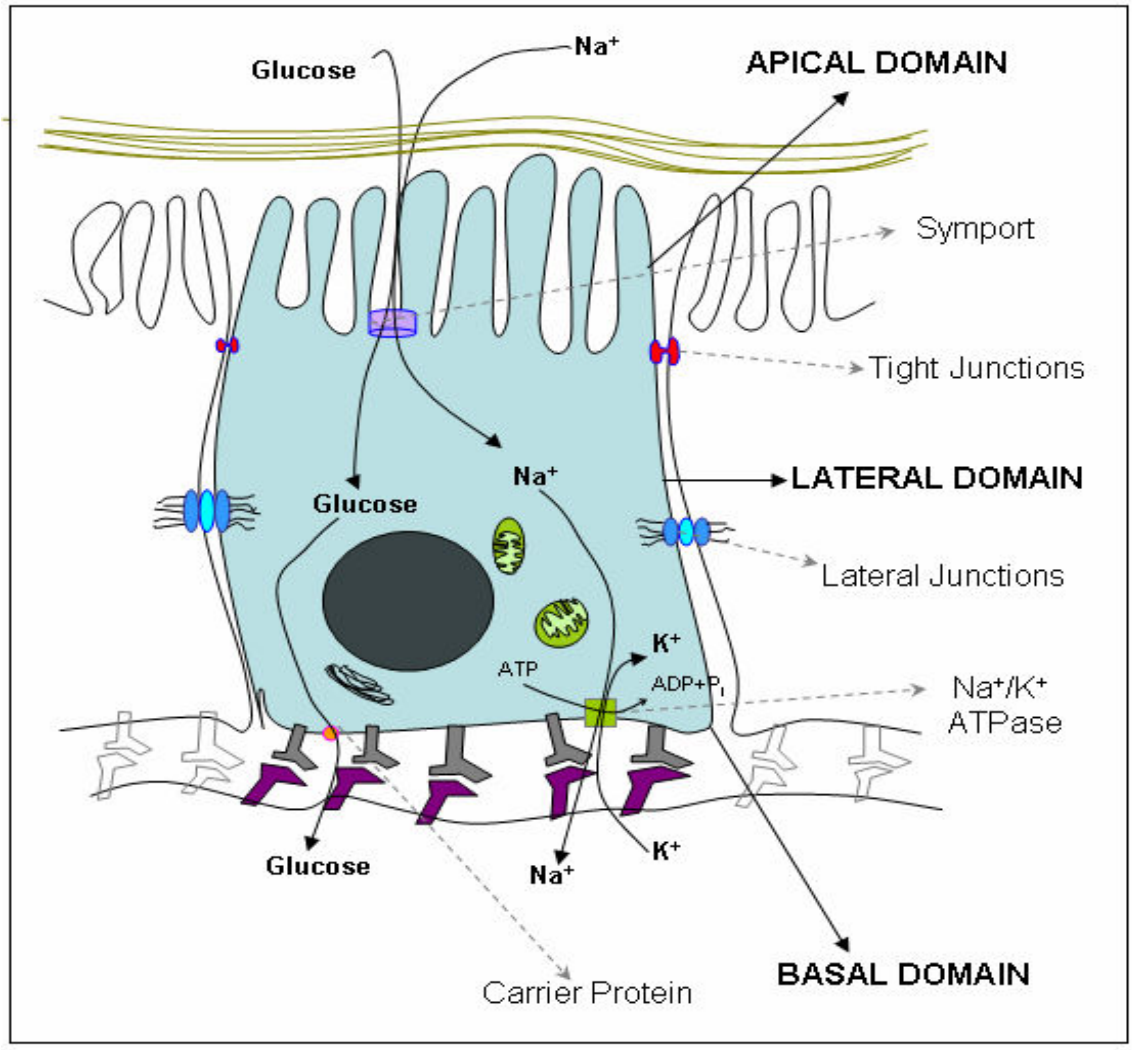

Fig. 5. 1. Enterocyte epithelial cell representation outlining cell polarity with three domains. (Original drawing of the author).

5. 1. 2: Monocyte cells

Monocytes play important roles in both innate and adaptive immune responses, killing microbial pathogens and tumour cells, and exerting immunoregulatory functions through cytokine production and processing and presentation of antigens

to lymphocytes. These cells arise from haemopoietic precursors in the bone marrow, and after 2–3 days in the blood migrate into different tissues, where they undergo further differentiation to become tissue-resident macrophages (Gordon *et al.*,1988). Under specific culture conditions *in vitro*, monocytes can differentiate into macrophages in the presence of serum or macrophage colony-stimulating factor (Chapuis *et al*., 1997; Palucka *et al*., 1998) or into dendritic cells when cultured with granulocyte–macrophage colony-stimulating factor and interleukin-4 (IL-4) (Sallusto and Lanzavecchia, 1994; Banchereau *et al.*, 2000). Although the heterogeneity of monocytes is not as obvious as that displayed by macrophages, in the human several monocyte subpopulations differing in phenotype and functional capabilities have been reported. Ziegler-Heitbrock *et al*. identified a minor population of $CD14^+$ $CD16^+$ monocytes that represents around 10% of the total monocyte population and exhibits proinflammatory features, producing high amounts of tumour necrosis factor-α (TNF-α) but no or little IL-10, while the major population of $CD14^+$ $CD16^-$ monocytes produces both types of cytokines (Passlick *et al.*, 1989; Ziegler-Heitbrock H.W., 1996). Moreover, within the $CD14^+$ $CD16^+$ monocytes, the M-$DC8^+$ marker identifies a subset with capacity to differentiate *in vitro* into potent dendritic cells (de Baey *et al.*, 2001). Two monocyte subsets have been characterized by Grage-Griebenow *et al*. based on the expression of CD64 (Grage-Griebenow *et al.*, 1993). Monocytes lacking CD64, comprise less than 10% of the whole monocyte population and display higher capacity for antigen presentation than the predominant $CD64^+$ monocytes which show higher phagocytic activity and produce more reactive oxygen intermediates. Simultaneous analysis of CD16 and CD64 allows a further division into four phenotypically and functionally different subsets. Out of them, $CD64^+$ $CD16^+$ monocytes exhibit dendritic-cell-like high antigen-presenting capacity, while $CD64^-$ $CD16^-$

monocytes resemble the plasmacytoid dendritic cell blood precursors with high interferon-α (IFN-α) producing capacity (Grage-Griebenow *et al.*, 2000 and 2001).

5. 1. 3: Functional polarity

The intestinal epithelium, the site of interface with probiotics as well as other luminal constituents, conducts several functions required for intestinal homeostasis. Among these, the ability to form a barrier to oppose permeation of solutes into the lamina propria, and to regulate fluid and electrolyte transport, have been studied extensively. Alterations in both barrier and transport functions are common sequelae of a variety of digestive disorders, and may underlie diarrhoeal symptoms. Moreover, both transport and barrier functions, and epithelial turnover and differentiation, are known to be regulated in part by signal transduction events originating from the epidermal growth factor receptor (EGFr) (Buret *et al.,* 1999). EGFr activation has been shown to cause redistribution of actin filaments in the apical zone of epithelia and to evoke changes in tight junctions and the cytoskeleton that are associated with an increase in transepithelial resistance (TER) and hence barrier function (Galan *et al.*, 1992; Resta-Lenert and Barrett, 2005). Unregulated inflammation of the gastrointestinal tract (GIT), *e.g.*, during active periods of inflammatory bowel diseases, causes an increased permeability of the epithelium, leading to deterioration of the epithelial barrier function (Madsen *et al.*, 2001). Consequently, luminal bacteria and luminal substances (e.g., bile salts and gastric or pancreatic enzymes) are allowed access into the underlying tissue and bloodstream of the host. Several studies have focused on the molecular mechanisms responsible for increased epithelial permeability (Nusrat, *et al.,* 2001; Otte and Podolsky, 2004) and have observed that it is linked to weakening of the tight junctions. Tight junctions establish a polarity of the epithelial cell layer by

forming a seal between adjacent epithelial cells, thereby separating the luminal compartment from the basolateral surface (Schneeberger and Lynch, 2004). Treatment with probiotic bacteria may prevent or reverse increased permeability of the epithelium and act antagonistically towards pathogens (Otte and Podolsky, 2004; Resta-Lenert and Barrett, 2003). A method for attaining the permeability in vitro includes the measurement of electrical physical resistance to determine the transepithelial electrical resistance (TER).

5. 2: Interaction with probiotic bacteria

5. 2. 1: ROS and Nitric oxide production

Whereas reactive oxygen species (ROS) are classically thought of as cytotoxic and mutagenic or as inducers of oxidative stress, recent evidence suggests that ROS play a role in signal transduction. ROS are implicated in stimulation or inhibition of cell proliferation, apoptosis, and cell senescence (Burdon R., 1995; Gansauge *et al.*, 1997). Whereas activated phagocytes produce very high levels of O_2^- and H_2O_2 that participate in host defence, many other cell types also generate ROS, albeit generally at lower levels (Burdon R., 1995; Cross and Jones 1991; Szatrowski and Nathan, 1991). The function of non-phagocytic ROS generation is unclear, but in some cases correlates with cell proliferation and activation of growth-related signalling pathways. Intriguingly, significant ROS generation is seen in cell lines derived from human cancers (Szatrowski and Nathan, 1991).The enzymatic origin

of ROS in phagocytes is established, but its origin in non-phagocytic cells is less clear. The phagocyte respiratory burst oxidase, an NADPH-dependent multicomponent enzyme, generates O_2^-, with secondary generation of H_2O_2 (Babior B. M., 1995). Whereas some ROS in non-phagocytes is of mitochondrial origin (Williams *et al.*, 1998), ROS production in many cells is blocked by inhibitors of the phagocyte NADPH oxidase.

5. 2. 2: Immune response

It has been previously reported that the bifidobacteria can enhance host immunological functions via the activation of macrophages and lymphocytes (Hatcher and Lambrecht, 1993; Sekine *et al*., 1994; Kirjavainen *et al*., 1999), antibody synthesis (Link-Amster *et al*., 1994; Fukushima *et al.,* 1999), and the proliferation of T- and B-cells (Takahashi *et al.,* 1993; Kang *et al.,* 1994). The intake of bifidobacteria has been demonstrated to enhance resistance against infection by pathogenic organisms (Sasaki *et al*., 1994; Nomoto, 2005), and may also help in the prevention of cancer (Rafter, 1995; Sekine *et al*., 1995). These immunopotentiating activities have been suggested to be mediated via interactions occurring between immune cells and intact bacterial cells or their components, including peptidoglycan, teichoic acid, and/or cellfree extract (Ouwehand *et al*., 1999; Amrouche *et al*., 2006). Activated macrophages evidence the capacity to induce the production of several cytokines which perform a pivotal function in a variety of immune responses. A variety of studies have demonstrated that viable or heat-killed *Lactobacillus* and *Bifidobacterium* species, as well as certain of their cell components, are capable of stimulating the production of hydrogen peroxide, nitric oxide, and cytokines such as interleukin (IL)-6 and tumor necrosis factor (TNF)-α in macrophage cell lines (Miettinen *et al.,* 1996; Park *et al.,* 1999; Tejada-

Simon and Pestka, 1999; Cross *et al*., 2004). Antigen-presenting cells (APCs), such as monocytes, macrophages, and dendritic cells (DCs), are responsible for detecting microbes and presenting their antigenic structures to T cells, thus eliciting acquired immune responses. In addition, monocytes and macrophages kill microorganisms by phagocytosis and produce proinflammatory cytokines. DCs are more potent APCs with a special ability to prime naïve T lymphocytes to novel protein antigens. Upon activation initiated by signals from microorganisms or from other cells in the tissue, DCs migrate to the regional lymph nodes. During this process, they undergo maturation, up-regulate costimulatory molecules, and produce cytokines, which make them qualified to activate naïve T cells. Monocytes and DCs recognize conserved motifs in bacteria through Toll-like receptors (TLRs), along with other pattern recognition receptors. The triggering of APCs through TLRs initiates a signal transduction cascade that culminates in the activation of transcription factors, such as NF-κB, which leads to secretion of cytokines and expression of costimulatory molecules. Furthermore, CD14 plays an essential role in the response to microbial components from both gram-positive and gram-negative bacteria (Karlsson *et al.*, 2004). Purified components of microbes have been used to study the specificity of individual TLRs. The gram-negative bacterial compound lipopolysaccharide (LPS) is recognized by TLR4 (Hoshino *et al.*, 1999; Schroder *et al.*, 2003). TLR2 recognizes many different microbial compounds, including peptidoglycan and lipoproteins from both gram-positive and gram-negative bacterial cell walls. However, the survival and colonization of orally-administered probiotic bacteria in the gastrointestinal environment is generally poor, as these bacteria are susceptible to low acidic pH in the stomach and to bile acid and pancreatic juice within the small intestine (Marteau *et al*., 1992; Clark and Martin, 1994). Presently, there is no clear understanding regarding the molecular or cellular basis for immunomodulation by bifidobacteria forced to withstand gastrointestinal

conditions. The objective of the current study was to compare the *in vitro* effects of undigested whole cells and enzymatically-digested bifidobacteria on the induction of nitric oxide and IL-1β, IL-6, IL-12, and TNF-α in a RAW 264.7 murine macrophage cell line.

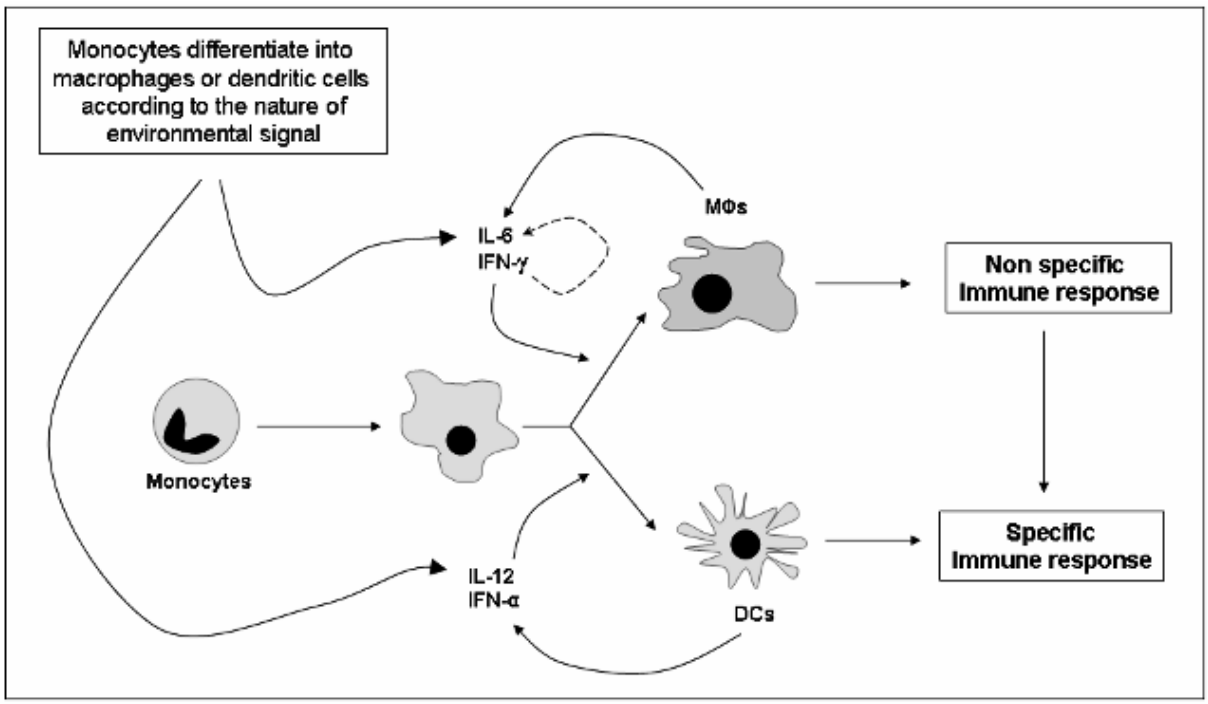

Fig. 5. 4: Schematic representation of cytokines sketching monocytes differentiation. (Original draw of the author).

Part two: Materials and Methods

Chapter 6: Materials and Methods, section I

6. 1: Bacterial strains

All isolates studied were *B. longum*. Strains B12, B18 and B2990 were obtained from the culture collection of the Microbiology area of the Department of Agroenvironmental Science and Technology (University of Bologna, Italy). The bacteria were cultured in TPY broth (Tryptone, Peptone, Yeast) (Scardovi, 1986; Modesto *et al.,* 2003) in anaerobic jars at 37°C (Merck, Darmstadt, Germany). Bacteria were harvested by centrifugation at 2500 rpm for 15 min at 20°C. After washing in phosphate-buffered saline (PBS) pH 7.4, at 25°C, bacterial cells were resuspended in the same buffer. Cell counts in the bacterial suspensions were estimated by optical density at 600 nm absorbance (Pharmacia Biotech, Piscataway, N); the O.D.600 aimed was 0.8, that match a final concentration of $10^8 \div 10^9$ cells/ml.

6. 2: Cell line

Caco-2 (human colon adenocarcinoma) cells were from American Type and Tissue Collection (ATCC). Caco-2 cells were routinely grown in DMEM Dulbecco's modified Eagle's medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% (w/v) heat-inactivated (30 min, 56°C) fetal bovine serum (FBS) (Cambrex, Baltimore, MD), 1% (w/v) L-glutamine (Cambrex) and 100U/mL penicillin and 100µg/mL streptomycin. Cells were cultured in 25-cm^2 FALCON flasks in an

anaerobic jar at 37°C in an atmosphere with a 5% concentration of CO_2, generated by a special catalyst (GenBox, Biomerieux, Rhône, France). They were refed every day with 5 ml of fresh medium and were subcultured serially when approximately 80% confluent. For all the assays reported, monolayers of Caco-2 cells were prepared in a Falcon 6-wells plates (BD Bioscience, Bedford, MA) with 2 x 10^5 cells/cm2, incubated at 37° C in an atmosphere with a 5% concentration of CO_2 . The cultures were used after 15 days of post confluent growth (Figure 6.1).

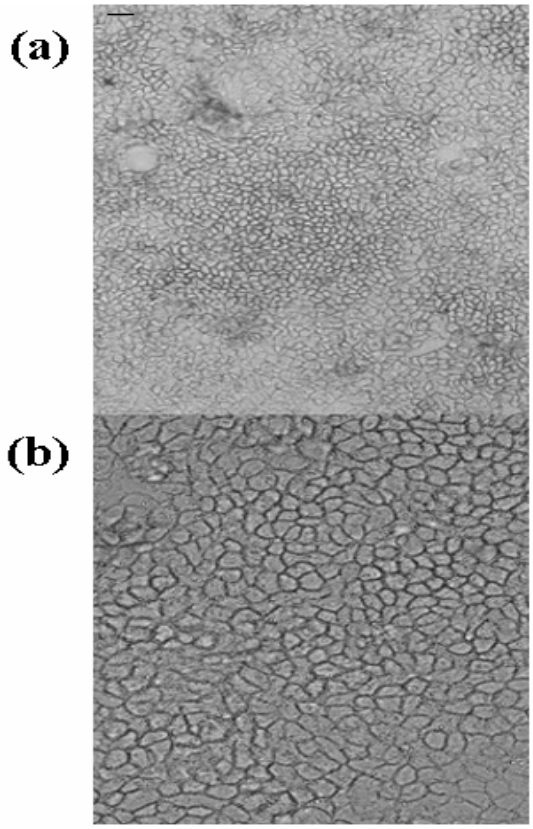

Fig. 6. 1: a) Monolayer of Caco-2 cell line after 15 days of post confluent growth observed with a light microscope under 1000x and b) 4000x magnification. Cells were grown in DMEM medium (Sigma-Aldrich) with 10% (w/v) FBS, 1% (w/v) L-glutamine and antibiotics. Cells were cultured in 25-cm^2 FALCON flasks in an anaerobic jar at 37° C with a 5% CO_2. Bar indicates

6. 3: Adhesion

6. 3. 1: Adhesion assay on Caco-2 cell line.

Adhesion assays were carried out following a previously described protocol (Del Re *et al.,* 2000). The cell monolayers were washed twice with phosphate-buffered saline (PBS) before the adhesion test. Bacterial strains were grown for 72 h at 37° C in (TPY) agar plates, colonies were collected in TPY broth, centrifuged and resuspended in DMEM at a concentration of 10^8 bacteria/ml. Aliquots of this suspension (2,5 ml) were added to the tissue culture dishes and incubated for 2 h at 37° C with 5% of CO_2 concentration. After incubation, the monolayers were washed five times with PBS, fixed with methanol, Gram stained and counted, using 20 randomized microscopic fields per dish. Adhesion was measured as number of bacterial cells adhering to monolayers of Caco-2 cells. Bacterial strains were designed as non adhesive (*Adh*-), when fewer than five bacteria adhered to 100 cells; adhesive (*Adh*), when from six to 40 bacteria adhered to 100 cells and strongly adhesive (*Adh*+), when more than 40 bacteria adhered to 100 cells.

6. 3. 2: Aggregation assays

Aggregation assays were performed with a previously described protocol (Del Re *et al.,* 2000). Bacteria were grown at 37° C for 24 h in TPY broth in anaerobic jars. The cells were harvested by centrifugation and resuspended in PBS to 0.5 optical density (O.D.) units at 600 nm. Two ml bacterial suspension were placed in each tube and centrifuged. The cells were then resuspended in their culture supernatant fluids. After incubation at 37° C for 2 h in anaerobic jars, 1 ml of the upper suspension was transferred to another tube and the O.D. at 600 nm measured. Aggregation was expressed as 1 - (O.D.600 upper suspension/O.D.600 total

bacterial suspension) x 100. The strains were defined as strongly autoaggregating (*Agg+*), when displayed an high aggregation percentage (80%) and aggregate immediately, forming a precipitate and resulting in a clear solution; non autoaggregating (*Agg-*), when were unable to autoaggregate (10%) and produce constant turbidity; and autoaggregating (*Agg+/-*), when showed a percentage around 50% and its suspension has both turbidity and precipitate.

6. 4: Apoptosis experiments

6. 4. 1: Apoptotic induction

The cultures of *B. longum* were harvested by centrifugation after a 24 hours growth. After washing in PBS, the supernatants were discarded, the pellets resuspended in the same buffer and directly seeded onto the Caco-2 layer at a density of 10^6-10^8 bacteria/ml. The induction of apoptosis was performed within 3 hours of incubation time, at 37° C in anaerobic jars with a 5% CO2 concentration. Then, the samples were washed in gentle shaking for five times with PBS pH 7,4 at 25° C to remove all bacterial cells; and finally treated with trypsin-EDTA x 4Na solution (Sigma-Aldrich, St. Louis) to release Caco-2 cells. Trypsin was inactivated with 500 µl of DMEM supplemented with FBS 10% (w/v). The suspensions were recovered and assayed.

6. 4. 2: DNA purification and analysis

DNA purification was carried out using a DNA purification kit (Roche Diagnostics GmbH, Mannheim, Germany), according to manufacturer instructions. The eluted DNA, 10 µg (wt/v), was collected and stored at -20°C or live assayed. The purified DNA was used to perform a standard gel electrophoresis on a 1% (w/v) TBE (Tris-

Borate EDTA solution) low melting agarose gel (Bio-Rad, Richmond, CA), buffered with 1x TBE solution, for two hours at 85 V. The DNA pattern was visualized with an UV transilluminator (Bio-Rad) and photographed (Nikon, Tokyo, Japan). The purified DNA of Caco-2 treated with campothecin (Roche), an apoptotic inducer that led to a typical DNA ladder, and the purified DNA of Caco-2 cells incubated for 3 hours with the sole PBS solution, were used as positive and negative controls of DNA fragmentation, respectively.

Chapter 7: Materials and Methods, section II

7. 1: Bacterial strains

Bifidobacterium longum strains B12, B18 and B2577 were obtained from the culture collection of Microbiology area of Department of Agroenvironmental Science and Technology (University of Bologna, Italy). Lyophilised bacteria were recovered in TPY (Tryptone, Peptone, Yeast) broth (Scardovi, 1986) in anaerobic jars at 37 °C (Merck, Darmstadt, Germany) and cultured in MRS (Man-Rugosa-Sharpe) medium (Oxoid Ltd., Hampshire, U.K.) supplemented with 0.05 % (w/v) L-cysteine-HCl. $MgCl_2$ (100 mM), $CaCl_2$ (10 mM), and the appropriate sugars were added to MRS to regenerate bacteria after electro-transformation protocol. *Escherichia coli* XL-1 and SOLR strains were purchased from the supplier (Stratagene, La Jolla, CA.) and were routinely grown in LB (Luria-Bertani) medium, at 37 °C under agitation. Tetracycline (12.5 μg/ml), ampicillin or spectinomycin (50 or 75 μg/ml) were added when necessary, as well as X-Gal (100 μg/ml) and IPTG (10 μg/ml), or 10 mM $MgSO_4$ and maltose (0.2 % w/v) (Table 7. 1).

7. 2: Screening for serpin gene

7. 2. 1: Genomic library

To determine the titer of the library XL1-Blue MRF' cells were diluted to an OD_{600} of 0.5 with sterile 10 mM MgSO4. and the following components were mixed: 1 µl of the library aliquot and 200 µl of XL1-Blue MRF' cells at an OD600 of 0.5 and 1 µl of a 1:10 dilution of the library aliquot and 200 µl of XL1-Blue MRF' cells at the same OD600. For amplified library titering, the amplified phage stock was first dilute in SM buffer by the following amounts: 1:10,000, 1:100,000, 1:1,000,000. 1 µl of each dilution was added to 200 µl of host cells. The phage and the bacteria were incubated at 37°C for 15 minutes to allow the phage to attach to the cells. Then the following components were added: 2–3 ml of NZY top agar (melted and cooled to ~48°C), 15 µl of 0.5M IPTG (in water), 50 µl of X-gal [250 mg/ml (in DMF)]. Everything was immediately plated onto dry, prewarmed NZY agar plates and incubated at 37°C. Plaques were visible after 12 hours. Background blue plaques were approximately < 1× 105 pfu/µg of arms, while white recombinant plaques were 10–100-fold above the background.

7. 2. 2: Sib-selection

In order to amplify serpin-encoding gene, a genomic library of *B. longum* B12 was screened following a non radioactive method previously described (King, 2000). Library DNA was used as template in a PCR reaction with 0.5 units of HerculaseII-TopFusion enzyme (Stratagene), 250 µM of each dNTP and 34 pmol of both *S1f*

and *M13uni* primers (Table 1). The PCR profile was as follow: 96°C for 2 min followed by 30 cycles (each cycle: 95 °C for 20 s, 53 °C for 20 s, 72 °C for 40 s) and a final extension at 72 °C for 3 min. PCR screening was repeated for three cycles and positive phages lastly obtained were recovered to be *in vivo* excised with fresh M13 phages co-infecting *E. coli* SOLR strain, according to the supplier's instruction (*in vivo* excision protocol Stratagene).

```
   1 atgagcgagc aactgatgga acagtaccgg ttgcgcggac aacgcaaatg ccgtaacgct
  61 tgtatcgccg ccatcgtgac agtagtgctt gtccttgccg tcgccggcgg cgtatggtgg
 121 acggccggcg atggcagcgc attggttcgc aatatgttca agccgaaggc cacgcctgcc
 181 acgcagccgg tagtcaacag caccgcaacc ttcgcctacc gcaccgcacc ggaattcctg
 241 gcgatggaag ccggcgaccg aggcaccggc aatgtgaact actctcctgc ttcgatgtgg
 301 atggcgttgg ccatcgccgc gcagggcgcc aatggcacga cccgctcgca actgaacgaa
 361 ctgctgggct ccggttcgct gaccgatagc gactaccaat cgctgctaag ttcgatcaac
 421 gggcaatatt cgggggcgaa atccgagatg agcgccgcga actcgctgtg gattgatgac
 481 gactactctc ttgccagcga ttaccaatcc accgtcaaga agatgttcga ggccgaagtc
 541 accacgttac cgttcgacga tcaggccgcc gccaagatgt ccgattggat tgccaagcat
 601 acgaatggtt cgctcaagcc gaagatcacg ctgcgtgacc gtgaagtcct gtccatcatc
 661 aacaccgtct atgcggatgg ccgctggaag gatccgttcg aagagcagtc caccggcaac
 721 ggcaccttcc acggcgaagc cggagatgct caggtgccga tgatgcacca gaccttcagc
 781 caaatggctt acggacatga tgagtacaac acttggcagc gggtggagat tccgttcgac
 841 aacggcggca atctggccat cgtgctgccg gccgaagggc atttcgacga gttggccggc
 901 gatgccgaga agctcagttg ggcgttcggt acatgctcga cggcatccct tggcgagggc
 961 gcaatgggtt gcgccgcgga cagtatgccc ggctggggcg tctccgtcaa ctcggtcatg
1021 gtgaacgtca cgctaccgcg attcaccatc gacagcatgt tcgactcgga agccaccatc
1081 aaggcattcg aaaaactggg ggtgaccgat gcgttcagtg caggcgacgc cgacttcacc
1141 aagatgatcg acaccggttc gcacggcgag aacctgtata tcggctcgat tctgcaaggc
1201 acgcgcatcg aggtgaacga agccggcgcc aaggccatgt ccttcaccaa ggtcggcgca
1261 gactccgtta gcgcgccggt ggacaacgtc gagttcacgg tggatcgccc atttctgtat
1321 tcgtacgtca ccccggacgg cataccatta ttcatcggtg cggtgcgcaa cctcggcgga
1381 gtcggtggag aaaactga
```

Fig. 7. 1: Bifidobacterium longum Serpin gene sequence: accession number: DD87853. (Shiffrin *et al.,* 2004*).*

7. 2. 3: In vivo excision

The ExAssist helper phage was supplied in 7% dimethylsulfoxide (DMSO) and stored at -80°C. The helper phage was stored for short periods of time at -20°C or

4°C. The helper phage prior to each use was titered of approximately 10^{10} pfu/ml. Positive phages lastly obtained were recovered to be *in vivo* excised with fresh M13 phages co-infecting *E. coli* SOLR strain, according to the supplier's instruction (*in vivo* excision protocol Stratagene). To determine the titer [in plaque-forming units per ml (pfu/ml)], use the following formula:

$$\left[\frac{\text{Number of plaques (pfu)} \times \text{dilution factor}}{\text{Volume plated (µl)}}\right] \times 1000\ \mu l / ml$$

where the volume plated (in microliters) refers to the volume of the helper phage solution added to the cells. The titer of the supernatant were included between 7.5×10^{10} and 1.0×10^{12} pfu/ml for ExAssist helper phage or between 1.0×10^{11} and 1.0×10^{12} pfu/ml for VCSM13 helper phage.

7. 3: Cloning

7. 3. 1: Fragment rescue and analysis

PBluescript plasmid from positive clones was rescued and purified with columns (Nucleo Spin Plasmid, MN, Macherey Nagel GmbH & Co. Kg. Duren, Germany). To amplify serpin encoding gene, the same plasmid was used as template DNA in a PCR reaction with 1.25 units of Pfu DNA Polymerase (Fermentas GmbH, St. Leon Rot, Germany), using 250 µM of each dNTP and 36 pmol of both *S1fBamHI* and *M13uni* primers (Table 1).The PCR profile was as follow: 96 °C for 1 min followed by 26 cycles (each cycle: 95 °C for 30 s, 53 °C for 30 s, 72 °C for 2 min and 30 s)

and a final extension at 72 °C for 5 min. PCR products were run on a 1.8 % TopVision™ Low Melting agarose gel (Fermentas) and the appropriate band, approximately 0.9 kb, was isolated and purified with columns (Nucleo Spin Gene, MN).

Strains, plasmids, oligonucleotides	**Description**	**Source of reference**
Bifidobacterium longum		
B12	Probiotic strain (wild type)	(Scardovi 1986)
B18	Probiotic strain (wild type)	(Scardovi 1986)
B2577	B2577 strain harbouring pRM2	(Missich *et al*., 1994)
B112	B12 strain harbouring pRM3	This work
B118	B18 strain harbouring pRM3	This work
Escherichia coli		
XL-1 Blue MRF'	Cloning host	
SOLR	*In vivo* excision protocol	(Bullock *et al*., 1987) Stratagene
Plasmids		
pUC18Serpin	pUC18 with S1f/M13 insert	(Missich *et al*., 1994)
pRM2	pGEM-5Z with pMB1 of B2577 Sp^r	This work
pRM3	pRM2 with *S1f/M13* insert	This work
Oligonucleotides		
S1f	5'-CAACTGAACGAACTGCTGG	This work
S1r	5'-AGACGGTGTTGATGATGGAC	This work
M13uni	5'-TGTAAAACGACGGCCAGT	MWG, Ebersberg, DE
S1f-BamHI	5'-GGTCTAGACAACTGAACGAACTG	This work

Tab. 7. 1: Strains, plasmids and oligonucleotides used in this study.

7. 3. 2: Vector construction

Serpin fragment was digested with BamHI and EcoRI enzymes (Fermentas) and purified, then ligated with purified and digested pRM2 or with pUC18 (Fermentas), generating respectively pRM3 and pUC18Serpin as positive control. Before ligation both plasmids were incubated with 1.2 units of alkaline phosphatase (Fermentas) to avoid plasmid self-ligation. Ligation was executed by 2 units of T4 DNA ligase (Fermentas) with approximately 86 ng of vector and 262 ng of insert as according to the supplier's instruction; its success was confirmed by 1.2 % agarose gel electrophoresis. General recombinant DNA techniques were performed by standard procedures (Sambrook *et al.,* 1989).

7. 4 Transformation

7. 4. 1: Escherichia coli

The product of ligation was then integrated chemically in *E. coli* XL-1 competent cells using TransformAid™ Bacterial Transformation Kit according to the supplier's instruction (Fermentas). Transformants were selected after plating in LB agar supplemented with 50 µg/ml ampicillin, 100 µg/ml X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (20 mg/ml in N,N-dimethylformamide) and 10 µg/ml of 0.1 M IPTG (isopropyl-β-D-thiogalactopyranoside) for detection of ampicillin resistance and β-galactosidase activity. Transformation efficiency (expressed as the number of transformants/µg of plasmid DNA) for *E. coli* XL-1

cells with pRM3 plasmid ranged 1 x 10^6 transformants/μg of pRM3. Plasmid DNAs of 20 blue recombinant clones were isolated and screened to confirm plasmid integrity via a colony PCR with Pfu enzyme (Fermentas).

7. 4. 2: Bifidobacterium longum

Bifidobacterium longum BuSc B12 and BuSc B18 (Scardovi, 1986) were electro-transformed with purified pRM3 plasmid DNA. All experiments were carried out with a Bio-Rad Gene Pulser apparatus equipped with a Pulser Control nit (Bio-Rad Laboratories, Hercules, CA) at a capacitance of 25 μF, using cuvettes with 0.2 cm inter-electrode distance. The use of different sugars supplementing MRS medium was executed to obtain the best electroporation efficiency. A stationary phase (18 h) culture of recipient strain grown in MRS medium, eventually enriched with one of the following sugars: raffinose 0.8 mol/l, glucose 0.7 mol/l, lactose 0.5 mol/l was inoculated (5 % inoculum) with 120 ml of same medium. *B. longum* strains were harvested at early-log phase (0.5 x 10^9 CFU/ml), washed with Na-phosphate buffer 5 mmol/l pH 7, resuspended in NaMR buffer (Na_2PO_4 5 mmol/l, $MgCl_2$ 1 mmol/l, raffinose 0.3 mmol/l, pH 4.8), then aliquoted and stored at – 135 °C. Cell suspension (80 μl) was mixed with 0.25 μg of plasmid DNA, cooled 5 min on ice, transferred into an electrocuvette and rapidly pulsed for a time constant between 3.8 and 4.3 ms. The apparatus was set at 200 Ω resistance and at voltages not exceeding 12.5 kV/cm. Cells were immediately recovered with 800 μl of MRS medium supplemented with sugars, including with 0.5 mmol/l ribose. After 4 hours of growth in regeneration broth, different aliquots were plated onto MRS agar with the right sugar and 50 μg/ml ampicillin and 75 μg/ml spectinomycin. The plates were incubated routinely for 72 h. Finally *B. longum* transformants colonies for

ampicillin and spectinomycin resistance were passed via replica plating method to MRS agar plates with X-gal and IPTG.

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7. 5: Serpin detection

7. 5. 1 PCR analysis

Several transformants blue colonies were screened using their plasmid DNAs as template in a PCR reaction with primers *S1f* and *S1r*. To confirm biochemical screening of *E. coli* XL-1 and *B. longum* B112 and B118 transformants, a colony PCR was performed using 1 unit of Pfu DNA Polymerase (Fermentas), 250 μM of each dNTP and 34 pmol for both *S1f* and *M13uni* primers (Table 1). In order to confirm the integrity of cloned Serpin gene in pRM3 from positive transformants *B. longum*, 10 ng of rescued pRM3 were used as template in a PCR reaction with 0.5 units of HerculaseII-TopFusion enzyme (Stratagene), 250 μM of each dNTP and 33 pmol of both *S1f* and *S1r* primers.

7. 5. 2 Dot-blot assay

Dot-Blot technique was used to detect serpin antigens in bacterial cell supernatants of B112 and B118 *B. longum* transformants strains. The protocol was achieved with a Bio-Rad Dot-Blot apparatus (Bio-Rad Laboratories, Richmond, CA) integrated with a vacuum pump (Millipore Corporation, Bedford, MA), following standard procedures (Sambrook *et al.,* 1989). Goat Anti-antitrypsin Polyclonal Antibody (Novus Biologicals, Littleton, CO) diluted 1:5000 in Blocking Solution and IgG anti-rabbit peroxidase conjugated (Sigma-Aldrich, St.Louis, MO) diluted

1:3000 in Blocking solution were used respectively as primary and secondary antibodies. A 0.2 μ nitrocellulose membrane (Pierce, Rockford, IL) was used for immunochemical reactions with "Supersignal West-Pico Chemiluminescent substrate" by BioPierce (Pierce) as substrate solution. TBS (1x) and TTBS (1x) solutions were used to wash membranes in several protocol steps. To avoid false positive signals, one sample was incubated with the sole secondary antibody. Signals were developed and fixed on a Kodak film.

Chapter 8: Materials and Methods, section III

8. 1: Probiotic bacteria

Twelve probiotic strains of bacteria were examined. The most of them were isolated from commercial cheese (PCA142, PCA185, PCK103, PCS20, PCS22, PCS25 and PCS26), two were isolated from *Kule naoto* that is a Kenyan Maasai milk, obtained from cattle and fermented in cattle's blood (PCK40 and PCK49), one from tanzanian coffee fermentation (PCK87), another was isolated from human intestine (PCA244), while PCK66 was isolated from Turkish *Salgam* that is a fermented juice of turnip and carrot. The twelve probiotic strains (tables 8.1 and 8.2) were previously been tested for their activity against five different new wide spread food-borne pathogens as *Saccharomyces cerevisiae*, *Escherichia coli* STEC (Shigella toxin-like *E. coli*), *Listeria monocytogenes*, *campylobacter jejuni* and

Penicillium nordicum producing ochratoxin, by some partners of Pathogen Combat, a project of the 6th Framework Programme of the European Commision, including: BFEL (Bundesministerium fur Ernahrung, Landwirtschaft und Verbraucherschutz), University of Bologna, Agricultural University of Athens and Danisco A/S. Strains were maintained at -80ºC in 20% (v/v) glycerol (Merck, Darmstadt, Germany) and were propagated anaerobically in MRS broth (Merck) for 24 h at 37° C using 3 litres anaerobic jars supplied with CO_2 generators (Anaerocult, Merck).

PathogenCombat Strains	Putative Species	Source of origin	Antagonistic activity				
			P. nordicum[1]	*S. cerevisiae*[2]	*L.. mono.*[3*]	*E. coli*[3]	*C. jej*[4]
Pca 142	*Lb. fermentum*	Kasseri cheese	+	0	NA	NA	0
Pca 185	*Lb. gasseri*	Feta cheese	+	0	NA	NA	0
Pca 244	*Lb. reuteri*	Adult intestine	(+)	0	NA	NA	0
Pck 40	*Lb. paracasei*	Kule Naoto	+	0	NA	NA	0
Pck 49	*Lb. acidophilus*	Kule Naoto	+	0	NA	NA	0
Pck 66	*Lb. brevis*	Ethiopian coffee	+	0	NA	NA	0
Pck 87	*Lb. plantarum*	Tanzanian coffee	+	0	NA	NA	0
Pck 103	*Lb. plantarum*	Turkish salgam	(+)	0	NA	NA	1/3
Pcs 20	*Lb. casei*	Local cheese	+	0	NA	NA	0/3
Pcs 22	*Lb. plantarum*	Local cheese	+	0	NA	NA	0/3
Pcs 25	*Lb. plantarum*	Local cheese	+	0	NA	NA	0/3
Pcs 26	*Lb. pentosus*	Local cheese	+	0	NA	NA	0/3

Tab. 8. 1: List of the strains tested, regarding the Working Programme 9 of EU6FP PathogenCombat integrated project. All strains were used to evaluate their potential probiotic abilities on a porcine small intestine cell model and to use as comparison to *B. longum* wild type strain and BLSK 7 transformant strain. 1) Tested by BFEL, DE. 2) Tested by Danisco S/A, DK. 3) Tested by AUA, GR. 4) Tested by BU, I. *) *L. mono.* stands for *L. monocytogenes*. *C. jej.* stands for *Campylobacter jejuni* Symbol references: "+" = strong effect; "(+)" = weak effect; "0" = no effect; "NA" = not assayed (end of 2006); "0/3" = weak effect; "1/3" = average effect.

8. 2: Pig intestinal cell model

8. 2. 1: Epithelial cells

The intestinal epithelial cell line PSI C1 (Porcine Small Intestine clone 1) (figure 8.1) and CLAB (figure 8.2) (table 8.2) were grown in advanced Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich, Grand Island, USA), supplemented with 5% foetal calf serum (Cambrex, Verviers, Belgium), L-glutamine (2 mmol/L, Sigma), penicillin (100 units/ml, Sigma) and streptomycin (1mg/ml, Fluka, Buchs, CH). Cell lines were routinely grown in 75 cm^2 culture flasks (Nunc, Roskilde, DK) at 37° C in a humidified atmosphere of 5% CO_2 and 95% air until a confluent monolayer was obtained. Culture medium was changed routinely. To obtain polarized monolayers, PSI C1 cells were seeded on Transwell® filter inserts (0.4 µm pore size, 12-mm, Corning Incorporated, Corning, NY) placed into 12 well plates (diameter: 22.1 mm; Corning Incorporated) at a density of 1×10^5 cells/cm^2. A volume of 500 µl cell growth medium was added to the inner chamber (apical compartment) and 1500 µl to the outer chamber (basolateral compartment). Functional polarity was developed when electrical resistance between apical and basolateral surface of the monolayers was >3000 Ω/cm^2 for PSI C1 cells measured as described below. For experiments including PSI C1 cells in the apical compartment and pig blood monocyte cells in the basolateral compartment, PSI C1 cells were seeded as described above and once polarized were placed into 12 well plates (diameter 22.1 mm; Corning Incorporated) containing confluent monolayers of monocytes in the basolateral compartment.

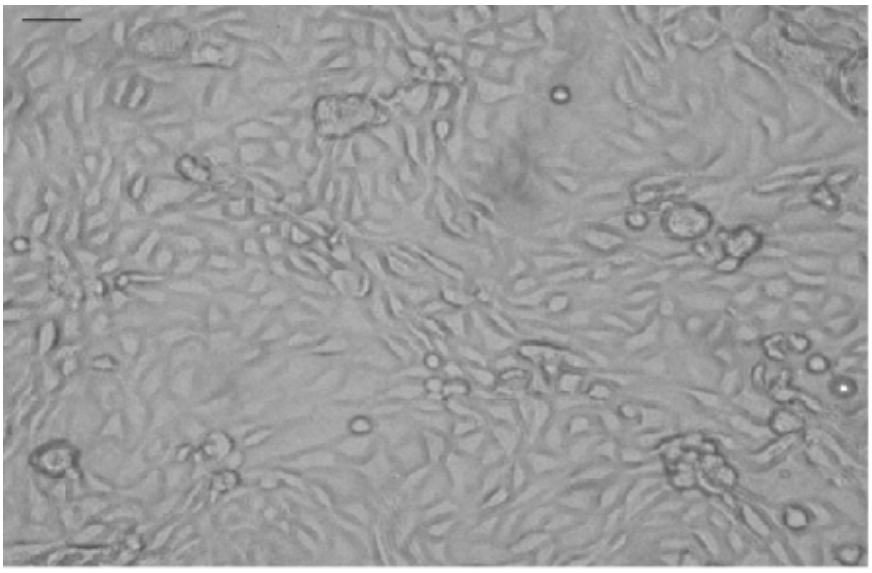

Fig. 8. 1: PSI c1 porcine small intestine epithelial clone 1 cells in a polarised monolayer after 3 days of growth at 37° C in a humidified atmosphere (5% CO_2 / 95% air) cultured in DMEM in a 25 cm^2 flask. Bar stands for 100 µm.

Clab cell line has been tested even in a functional cell model, but without testing its electric resistance. Cells were seeded as mentioned above on Transwell filter insert (Corning, Inc.) and placed into 12 well plates at a density of 1×10^5 cells/cm^2. A volume of 500 µl cell growth medium was added to the inner chamber (apical compartment) and 1500 µl to the outer chamber (basolateral compartment). Moreover Clab and PSI c1 cell lines, in regard to all the assays to achieve, were previously tested in a single cell system in order to have data to compare. Cells were separately seeded in 96 well plates at concentration 1×10^5 viable cells/ml and incubated in a humidified atmosphere of 5% CO_2 and 95% air for 24 h at 37° C to obtain a confluent monolayer. Before use, monolayers were washed twice with 100 µl DMEM without phenol red and supplements.

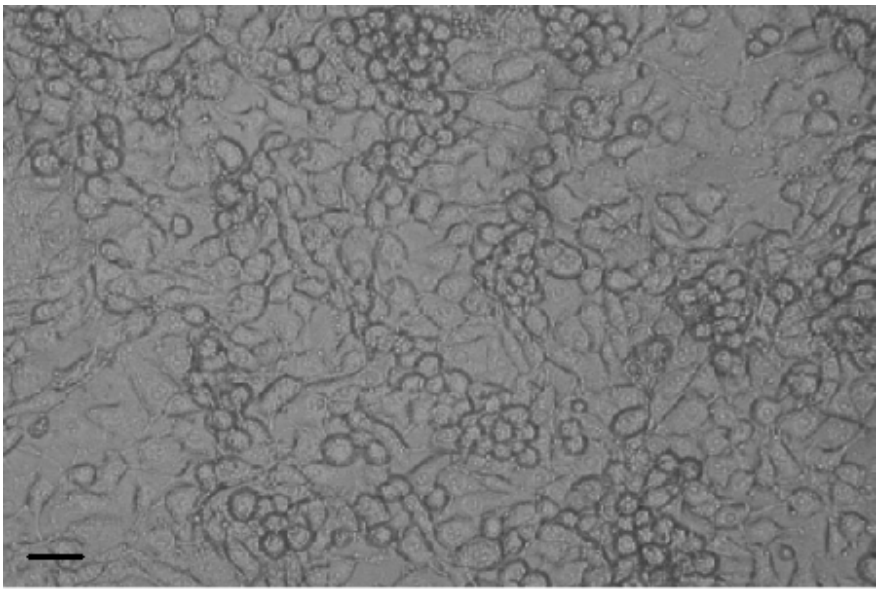

Fig. 8. 2: Clab porcine small intestine epithelial cells in a polarised monolayer after 3 days of growth at 37° C in a humidified atmosphere (5% CO_2 / 95% air) cultured in DMEM in a 25 cm^2 flask. Bar stands for 100 μm.

8. 2. 2: Monocyte cells

Monocytes/macrophages from pig blood (PoM2) (Porcine Monocytes clone 2) (figure 8.3) were grown in advanced Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich, Grand Island, USA), supplemented with 5% fetal calf serum (Cambrex, Verviers, Belgium), L-glutamine (2 mmol/L, Sigma), penicillin (100 units/ml, Sigma) and streptomycin (1mg/ml, Fluka, Buchs, Switzerland). Cells were routinely grown in 75 cm^2 culture flasks (Nunc, Roskilde, Dk) at 37° C in a humidified atmosphere of 5% CO_2 and 95% air until a confluent monolayer was obtained. Culture medium was changed routinely. For experiments including PSI C1 cells in the apical compartment and PoM2 cells in the basolateral compartment, a confluent monolayer of PoM2 was obtained and cells were seeded on 12 wells plates (basolateral compartment) at a density of 1×10^5 cells/ml and grown as

described above. PoM2 cell line has been tested even in a functional cell model, but without testing its electric resistance. Cells were seeded as mentioned above on Transwell filter insert (Corning, Inc.) and placed into 12 well plates at a density of 1×10^5 cells/cm^2. A volume of 500 µl cell growth medium was added to the inner chamber (apical compartment) and 1500 µl to the outer chamber (basolateral compartment). Moreover, PoM2 cells were previously tested in regard to all the assays to achieve, in a single cell system, in order to have data to compare. Cells were separately seeded in 96 well plates at concentration 1×10^5 viable cells/ml and incubated in a humidified atmosphere of 5% CO_2 and 95% air for 24 h at 37° C to obtain a confluent monolayer. Before use, monolayers were washed twice with 100 µl DMEM without phenol red and supplements.

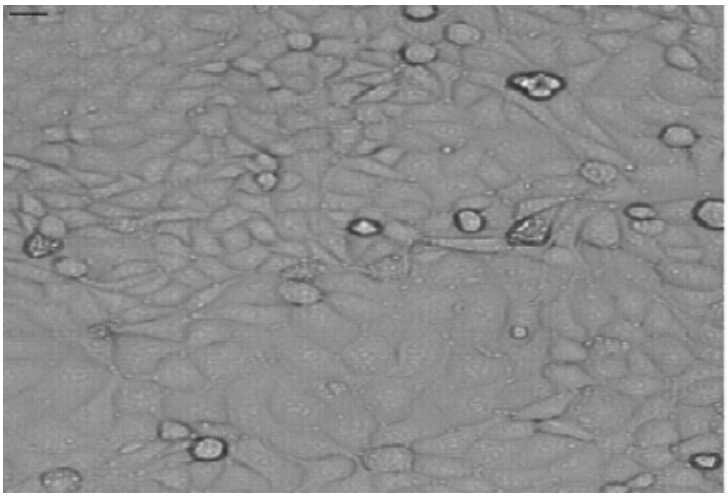

Fig. 8. 3: PoM 2 porcine blood monocyte cells forming a polarised monolayer after 3 days of growth at 37° C in a humidified atmosphere (5% CO_2 / 95% air) cultured in DMEM in a 25 cm^2 flask. Bar stands for 100 µm.

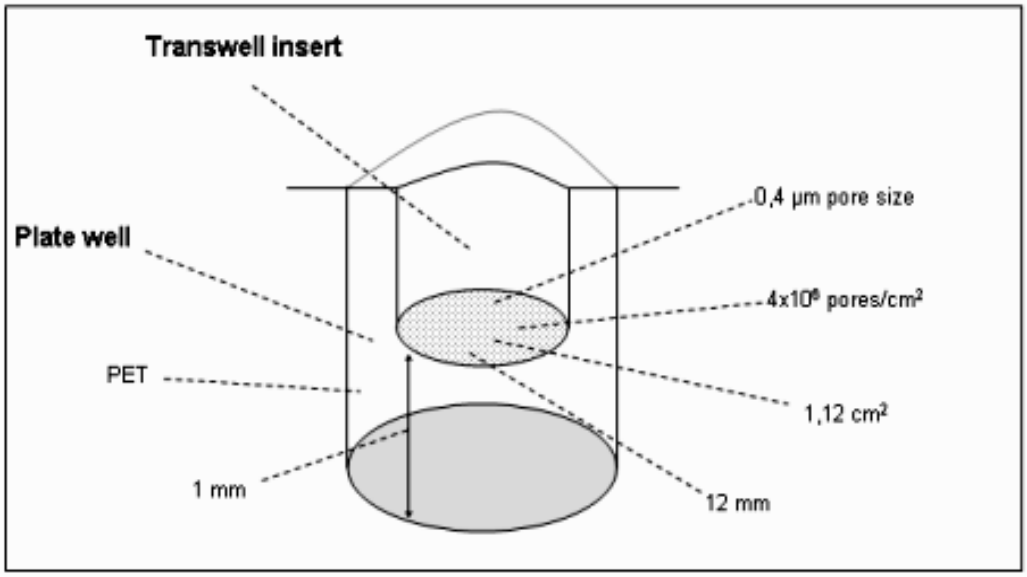

Fig. 8. 4: Schematic representation of a transwell insert of a twelve well plate (Original draw of the author).

Bacterial strains and Cell lines	Description	Source of reference
Cell lines		
• Clab	Porcine Small intestine Epithelial cells	Gradijnski and Cencic, 2006
• PoM2	Porcine Blood Monocyte cells	Gradijnski and Cencic, 2006
• PsI c1	Porcine Small intestine Epithelial cells	Gradijnski and Cencic, 2006
Bifidobacterium longum		
• BuSc 112	*B. longum* wild type	Scardovi V., 1979
• BuSc BSK7	BuSc 112 harbouring pRM3s vector	Nissen L., 2007
Lactobacillus species		
• Pca 142	*Lb. fermentum*	PathogenCombat, 2006
• Pca 185	*Lb. gasseri*	PathogenCombat, 2006
• Pca 244	*Lb. reuteri*	PathogenCombat, 2006
• Pck 40	*Lb. paracasei*	PathogenCombat, 2006
• Pck 49	*Lb. acidophilus*	PathogenCombat, 2006
• Pck 66	*Lb. brevis*	PathogenCombat, 2006
• Pck 87	*Lb. plantarum*	PathogenCombat, 2006
• Pck 103	*Lb. plantarum*	PathogenCombat, 2006
• Pcs 20	*Lb. casei*	PathogenCombat, 2006
• Pcs 25	*Lb. pentosus*	PathogenCombat, 2006
Unidentified species		
• Pcs 22	LAB strain isolated from cheese	PathogenCombat, 2006
• Pcs 25	LAB strain isolated from cheese	PathogenCombat, 2006
Control species		
• BFE 403	*Carnobacterium divergens*	BFEL, DE..
• Shirota	*Lb. paracasei*	Yakult, J.
• F19	*Lb. paracasei*	Ljungh Å., 2002
• 120021	*Lb. reuteri*	PathogenCombat, 2006

Tab. 8. 2: List of bacterial strains and cell lines used in this part of the study to evaluate probiotic abilities on a porcine small intestine cell model and to use as comparison to *B. longum* wild type strain and BLSK 7 transformant strain. Bacteria and cells, except *B. longum* strains, are regarded to the WP9 of EU6FP PathogenCombat.

8. 2. 3: Cell count

To carry out all experiments the number of cells in 1 ml of cell culture were counted. In order to achieve the count, 900 µl of 0.1 % trypan blue and 100 µl of cell culture were added to a 1.5 ml fresh tube. After cell resuspension, 100 µl of the mix were transferred to a hematocytometer and visualised with an inverted light microscope at magnification 100 x and cell number was counted in 25 squares, applying this formula:

$$\text{Cells / ml of culture} = \text{Counted cell number x } (10^6 / 25)$$

8. 2. 4: Functional polarity

After that cell cultures have reached a plateau in TER values and the monolayers are polarized and established, the functional pig cell models are ready to assay the effect of probiotic strains co-cultures. Bacterial strains were resuspended in cell growth medium without phenol red and supplements. The bacterial suspension (500 µl) was added to the apical compartment at a concentration of 1×10^7 cells/ml and incubated at 37° C in a humidified atmosphere of 5% CO_2 and 95% air. Cell culture medium without addition of bacteria was used as control. The effect of the probiotic treatment on cell polarity was evaluated by measurements of the transepithelial electrical resistance (TER) using Millicell-ERS Electrical Resistance System (Millipore, Bedford, MA) (Figure 8. 5 and 8. 6).

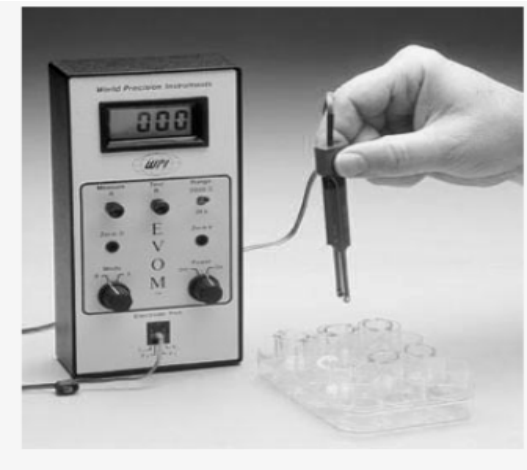

Fig. 8. 5: Picture of a device with its electrode chopstick similar to the Millicell-ERS Electrical Resistance System (Millipore, Bedford, MA) used in this work to measure electric resistances across cell line at the basolateral and at the apical compartment of 12 well plate Transwell inserts (Corning, Inc.).

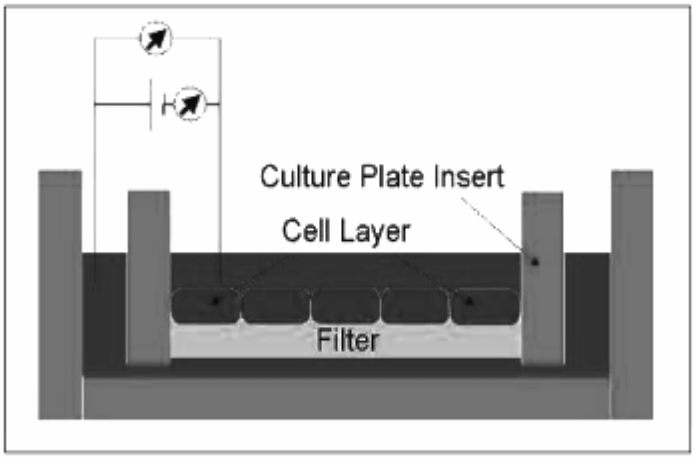

Fig. 8. 6: Schematic representation of a transwell well when is used with a Millicellers.

TER values were measured at different time points during the establishment of polarised monolayers (Figure 8. 7) and subsequently during the 72 hours period of co-culture with probiotic bacteria. The net value of TER (Ω/cm^2) was corrected for background resistance by subtracting the contribution of the cell free filter and the medium (110 Ω/cm^2). TER was measured before the addition of the bacteria (t=0) and then at various time intervals. TER of monolayers without bacteria added represented the control for each experiment.

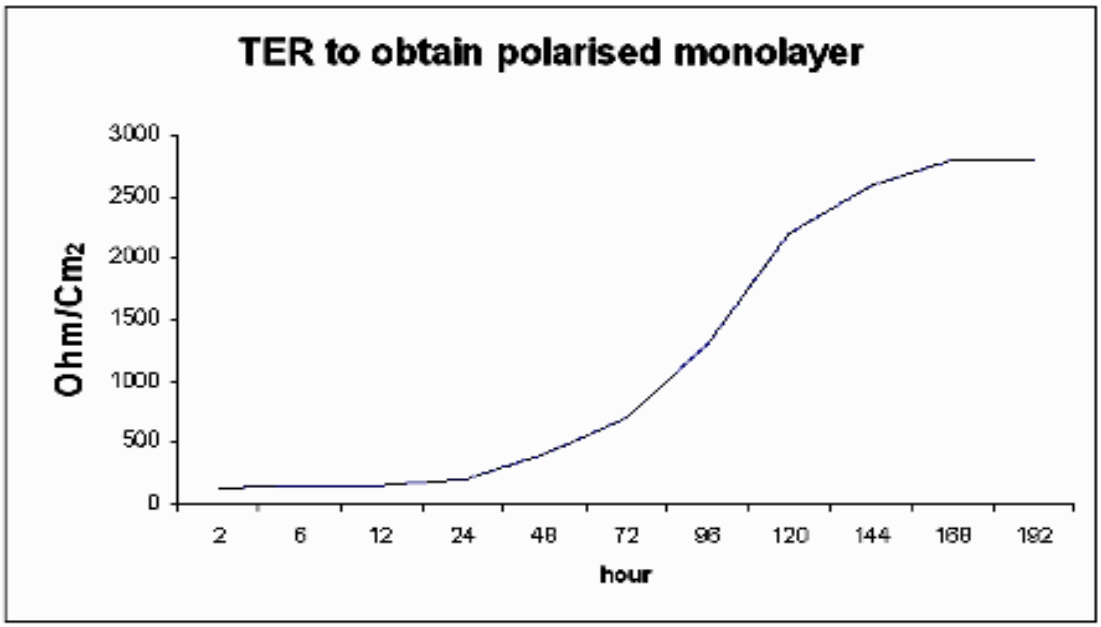

Fig. 8. 7: Trans Epithelial Electric Resistance of PSI clone1 cells cultured, starting from a density of 1×10^5 cells/cm^2, on Transwell® filter inserts (0.4 µm pore size, 12-mm, Corning Incorporated, Corning, NY) placed into 12 well plate (diameter: 22.1 mm; Corning Incorporated). Cells were cultured in DMEM at 37° C in a humidified atmosphere (5% CO_2 / 95% air).

8. 3: Effects of probiotics interaction

8. 3. 1: Adhesion

Adhesion ability of probiotics was assayed on the three different cell monolayers, using a functional cell model. When cells were ready, probiotics were resuspended as usually and seeded ($1x10^7$ cells/ml) onto monolayers formed in the apical compartment and incubated for 90 minutes in humidified atmosphere of 5% CO_2 and 95% air. Then, plates were washed twice in PBS and monolayers were trypsinized. Trypsinization was stopped by addition to each insert of 60 µl of cell growth medium without phenol red supplemented with 10% FBS. Cell suspensions were transferred and diluted five times, then streaked onto MRS agar Petri dishes and incubated up to 72 hours when bacterial colonies became visible. All strains assayed have shown to be able to adhere to monolayers, when compared to *Lb. paracasei* F19 as described in chapter 11.

8. 3. 2: Citotoxicity

Cytotoxic activity exerted by bacteria on cell monolayers after treatment was measured after 24 hours of co-incubation of cell monolayers and bacteria ($1x10^7$ bacteria/ ml) at 37° C in atmosphere of 5% CO_2. Following incubation, cells were carefully rinsed with PBS to remove excess of the bacteria and the plates were stained with Crystal violet in ethanol, rinsed with water, and destained with 10% (v/v) acetic acid. The absorbance at 595 nm was measured with a microplate reader (Multiscan).

8. 3. 3: Nitric oxide production

In 1879, Griess, a German chemist developed a reagent for the detection of nitrite in solution (Griess P., 1879). The reagent, an acid solution of sulfanilic acid and alpha naphthylamine, undergoes adiazotization reaction with nitrites to form a red azo dye. The test was originally intended for assessing bacterial contamination in municipal water supplies based on the principle that nitrate present in sewage will be reduced to nitrite by the action of bacteria (Shaus and Griess, 1956). Griess first described a colorimetric assay to measure the levels of nitrite (NO_2^-) in aqueous solutions over 100 years ago. Modifications to the Griess method have been published in more recent years (Green, *et al.*, 1982; Pollock, *et al.*, 1991), a fluorometric assay procedure has been published that is 50-100 times as sensitive as the Griess colorimetric assay (Figure 8. 8). The bacterial pellet was resuspended in cell growth media without phenol red and supplements in indicated concentrations and added to washed (2X) confluent monolayers of PSI C1, PoM2 or CLAB as described above. Monolayers treated with the bacterial suspension were incubated for 24 h or 72 h at 37° C in a humidified atmosphere of 5% CO_2 and 95% air. The NO concentration was determined measuring released NO, according with the modified Griess reaction (Sigma). Briefly, 50µl supernatant was transferred into each well of a 96-well plate and added 50 µl modified Griess reagent and incubated 15 min at room temperature. Optical density was measured at 540 nm by use of microplate reader (Multiscan)..

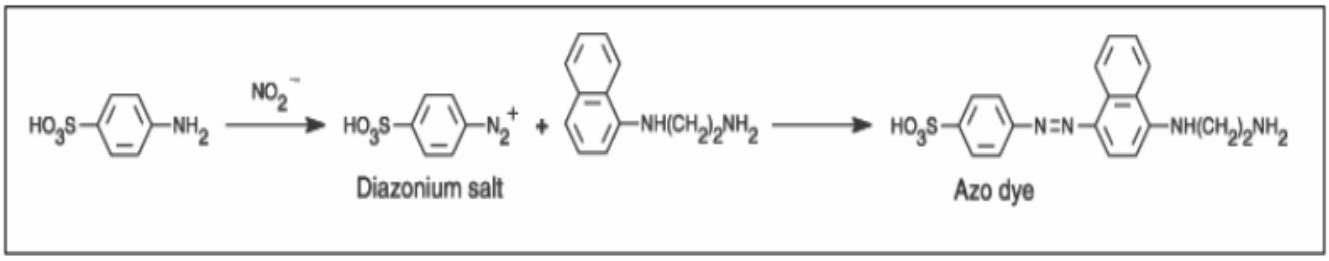

Fig. 8. 8: Schematic representation of the Modified Griess reaction used to quantify nitric oxide cells production with a spectrophotometer set at λ 540 nm.

8. 3. 4: Hydrogen peroxide production

The bacterial pellet was resuspended in cell growth media without phenol red and supplements in indicated concentrations and added to washed (2X) confluent monolayers of PSI C1, PoM2 or CLAB as described above. Monolayers treated with the bacterial suspension were incubated for 24 h or 48 h at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. The release of H_2O_2 was determined transferring 50µl of supernatant in a new 96-well plate and by the addition of 50 µl 0,01 % peroxidase and 100 µl TMB (Figure 8. 9) solution, diluted with distillated water (1:1). After 15 min incubation at room temperature the reaction was stopped by addition of 50µl 2mM H_3PO_4. Optical density was measured at 450 nm by use of microplate reader (Multiscan).

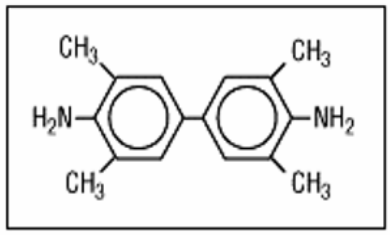

Fig. 8. 9: Tetramethylbenzidine (TMB) used to quantify H_2O_2 cells production with a spectrophotometer set at λ 452 nm.

8. 3. 5: Extracellular 0_2^- production

The bacterial pellet was resuspended in cell growth media without phenol red and supplements in indicated concentrations and added to washed (2X) confluent monolayers of PSI C1, PoM2 or CLAB as described above. Monolayers treated with the bacterial suspension were incubated for 24 h or 48 h at 37° C in a humidified atmosphere of 5% CO_2 and 95% air. The production of extra cellular O_2^- was measured by superoxide inhibitable cytochrome C reduction (Iacobini *et al.,* 1997). Briefly, supernatant was transferred into a 96-well plate and added 50 µl Cytochrome C solution (80µM) and incubated 1h at room temperature. Optical density was measured at 450 nm by use of microplate reader (Multiscan).

8. 3. 6: MTT assay

Measurement of cell viability and proliferation forms the basis for numerous in vitro assays of a cell population's response to external factors. The reduction of tetrazolium salts is now widely accepted as a reliable way to examine cell proliferation. The yellow tetrazolium MTT [3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide] is reduced by metabolically active cells (Figure 8. 10), in part by the action of dehydrogenase enzymes, to generate reducing equivalents such as NADH and NADPH. The resulting intracellular purple

formazan can be solubilised and quantified by spectrophotometric means. The MTT Cell Proliferation Assay measures the cell proliferation rate and conversely, when metabolic events lead to apoptosis or necrosis, the reduction in cell viability. The number of assay steps has been minimized as much as possible to expedite sample processing. The MTT Reagent yields low background absorbance values in the absence of cells. For each cell type the linear relationship between cell number and signal produced is established, thus allowing an accurate quantification of changes in the rate of cell proliferation (Ferrari *et al.,* 1990). The metabolic activity of PSI C1, CLAB and PoM2 was measured by the MTT cell proliferation Assay (ATCC). The protocol is based on tetrazolium MTT reduction by metabolically active cells that result in the intracellular purple formazan, which can be solubilised and quantified by a spectrophotometer at 650 nm.

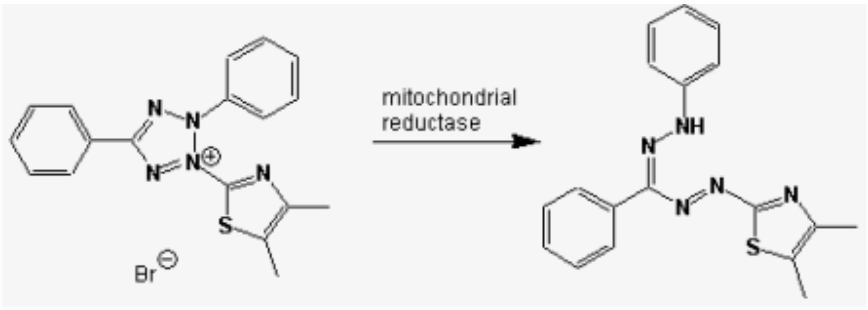

Fig. 8. 10: Schematic representation of tetrazolium MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) reduction used to quantify metabolically active cells with a spectrophotometer set at λ 650 nm.

Bacterial pellet was resuspended in cell growth media without phenol red and supplements in indicated concentrations and seeded onto washed (2 X) confluent monolayers of PSI C1, PoM2 or CLAB. Cell's monolayers with the added probiotic suspension were incubated for 24 h or 48 h at 37° C in a humidified

atmosphere of 5% CO_2 and 95% air. According to the manufacturer instructions a solution of MTT in PBS was added to each well and further incubated for 75 min. Solubilization of formazan was achieved after addition of 0.04% HCl in isopropanol. The optical density was measured at 650 nm using a microplate reader (Multiscan).

8. 3. 7: Immune response

Dot-Blot techniques were used to detect Interleukin-6 (IL-6) and Interferon-γ (IFN-γ) in PSI C1 and PoM2 cell supernatants grown in a functional cell model after probiotic treatment, working with a Bio-Rad Dot-Blot apparatus (Bio-Rad), integrated with a vacuum pump (Millipore) and following standard procedures. To test a possible cytokine expression in the culture supernatant, primary antibodies against polyclonal rabbit anti-pig INF-γ (INRA, Jouy-en-Josas, France) and against Interleukin-6 polyclonal rabbit anti-human IL-6 (SIGMA) were used, once diluted 1:500 in blocking solution. IgG anti-rabbit peroxidase conjugated (SIGMA), diluted 1:3000 in Blocking solution, was used as secondary antibody. A 0.2 μ Nitrocellulose membrane (Pierce, Rockford, IL) was used for the immuno-chemical reaction, while as substrate solution that fix the signals was used the "Supersignal West-Pico Chemiluminescent substrate" by Bio Pierce (Pierce). TBS (1x) and TTBS (1x) solutions were used to wash membranes in several protocol steps. The signals on the membrane to be exposed on a Kodak film were developed and fixed with Kodak developer and fixer solutions. *Lb. paracasei* F19 was used as positive control, while supernatants of monolayers not treated with bacteria were used as negative control. To avoid false positive reactions, all samples were even tested against the sole secondary antibody anti-rabbit.

Part three: Results and Discussions

Chapter 9: Results and Discussions, section I

9. 1: Adhesion and Autoaggregation

Autoaggregation of bifidobacterial cells and their adhesion to the epithelial mucosa are basic processes that get started to the colonization of the intestinal niches. To select the ideal strains to investigate the induction of apoptotic deletion of damaged cells, we have adapted the screening methods of our previous works (Del Re *et al.,* 1998; 2000). Among the several strains tested from our culture collection we have chosen three strains and defined their adhesion patterns (Table 9.1). B12 strain suggests being able of a long term presence in the mucosa due to the abilities to adhere to Caco-2 cells and to autoaggregate and can be designated as: "strongly adhesive" (*Adh*+) and "autoaggregating" (*Agg*+/-). B18 strain suggests an intermediate capability of a long term presence in the mucosa and can be designated as: "non-adhesive" (*Adh*-) and "strongly autoaggregating" (*Agg*+). B2990 strain demonstrates to be "non-adhesive" (*Adh*-) and "non-autoaggregating" (*Agg*-) (Figure 9. 1). The different phenotypes mentioned were significant to demonstrate the apoptotic deletion of Caco-2 cells. Indeed, the capabilities of Autoaggregation and adhesion are necessary to *B. longum* to establish a connection to the mucosa, to survive through the intestinal bacterial clearance and the competition for binding sites and to successfully colonize its niche.

Strains of *Bifidobacterium longum*	**Adhesion ability (%)**	**Autoaggregation ability (%)**	**Pattern description**
B. longum BuSc B12	68 ± 0.21	55 ± 0.26	*Adh*+ and *Agg*+/-
B. longum BuSc B18	3 ± 0.11	75 ± 0.17	*Adh*- and *Agg*+
B. longum BuSc B2990	2 ± 0.2	4 ± 0.22	*Adh*- and *Agg*-

Table 9. 1: Strains description and abilities. *Adh*+) strongly adhesive. *Adh*-) non adhesive. *Agg*+) strongly autoaggregating. *Agg*-) non autoaggregating. *Agg*+/-) autoaggregating. Adhesion is expressed as mean ± S.E. of bifidobacterial adhering cells x 100 Caco-2 cells. Autoaggregation is expressed as mean ± S.E. as 1 - (O.D. upper suspension / O.D. total bacterial suspension) x 100. Results are representative of three independent experiments following the previous methods introduced by Del Re B. *et al* 2000.

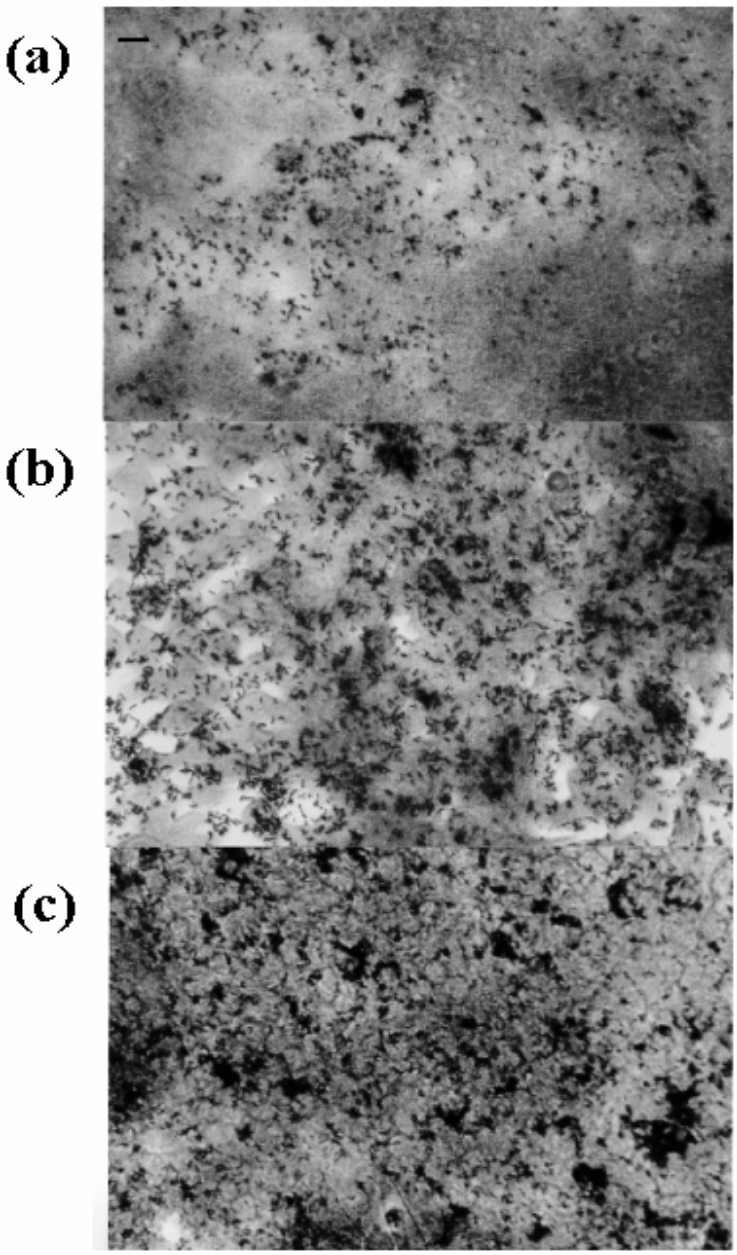

Figure. 9. 1: Co-cultures of Gram stained Bifidobacteria and Caco-2 cell line photographed under light microscope, visualised at 1000x magnification. Pictures were taken after a single wash in PBS to remove non adherent bacteria. A) *Bifidobacterium longum* B2990 strain adhering on Caco-2 cells monolayer. B) *B. longum* B18 strain adhering on Caco-2 cells monolayer. C) *B. longum* B12 strain adhering on Caco-2 cells monolayer. Bar indicates 1 mm.

9. 2: Apoptosis detection

Caco-2 cells were grown until differentiation and not unless the 80% of confluence was reached. Bifidobacterial strains were taken in their exponential phase of growth to achieve the test. After three hours of incubation of bacteria with Caco-2 cells, we isolated eukaryotic DNA and performed a DNA ladder assay (Figure 9. 2). The DNA samples from Caco-2, incubated with B12 and B18 *B. longum* strains as co-cultures, have undergone fragmentation. These two strains were designated as adherent and capable of a long term presence in the colon mucosa. The DNA sample from Caco-2, which did not show fragmentation, was the one treated with the B2990 strain known as *Adh- / Agg-,* capable of very weak adhesion and autoaggregation.

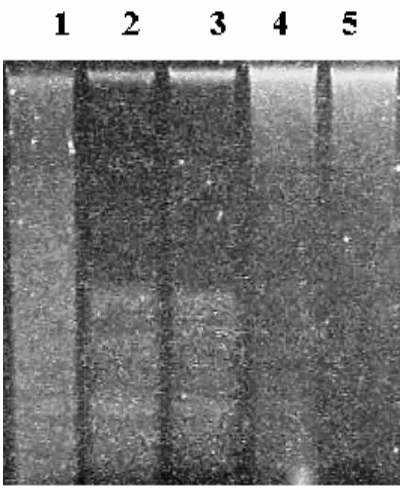

Fig. 9. 2: DNA ladder results. Ethidium bromide stained TBE 1% agarose gel. Lane 1) Caco-2 cells treated with campothecin used as positive control. Lane 2) Caco-2 cells induced by *Bifidobacterium longum* B12 showing DNA fragmentation. Lane 3) Caco-2 cells, induced by *B. longum* B18 shows DNA fragmentation. Lane 4) Caco-2 cells induced by *B. Longum* B2990 showing a DNA smear and no DNA fragmentation. Lane 5) Caco-2 cells incubated with PBS only, used as negative control. Results are representative of three independent experiments.

9. 3: Discussion

In this preliminary investigation we have observed a relationship between the adhesion properties of the *B. longum* strains and the ability to induce apoptotic deletion of the human enterocyte-like Caco-2 cell line. Our observations confirms precedent hypothesis, made isolating a 160 kDa cell wall associated protein from one of the most adherent *B. longum* strain of our collection. This protein could be involved in adhesion mechanisms (Ferrarini *et al*., 2003) and have homologies (data not shown) with eukaryotic serpins (serine proteases inhibitors). It is known that probiotics and in particular bifidobacteria exert direct activity on the colon

epithelia, regulating the tissue homeostasis. Indeed, it is reported that the commensal microflora can stimulate regeneration, differentiation and proliferation of the colon tissue. Our results could be somehow due to inducers of apoptosis such as proteins of the serpin superfamily. Indeed, in the full genome sequence of *B. longum* is reported an eukaryotic type serpin (Schell *et al.,* 2002), which has recently been characterized (Ivanov *et al.,* 2006) In eukaryotes, serpins controls key steps in physiological regulatory cascades, such as inflammation and apoptosis, by inhibiting specific proteases (Silvermann *et al.,* 2001). In some cases, serpins plays important roles in immune system evasion during pathogenesis (Macen *et al.,* 1993). Colonization is the only way how prokaryotes can interact with eukaryotic plasma membrane and establish a communication between. With a long lasting interaction, Bifidobacteria could module the human immune system or take part in cellular homeostasis, restoring the ecology of a damaged GIT tissue as the apoptotic deletion of Caco-2 cells suggests.

Chapter 10: Results and Discussions, section II

10. 1: DNA Manipulation

10. 1. 1: Sib selection screening

The gene of interest was screened in the library of *B. longum* genome via a non radioactive protocol, using 0.5 units of HerculaseII-TopFusion enzyme (Stratagene), 250 µM of each dNTP and 34 pmol of both *S1f* and *M13uni* primers (Table 1). The PCR profile was as follow: 96°C for 2 min followed by 30 cycles (each cycle: 95 °C for 20 s, 53 °C for 20 s, 72 °C for 30 s) and a final extension at 72 °C for 3 min. After three rounds of screening the positive clones were picked and assayed (Figure 10. 1).

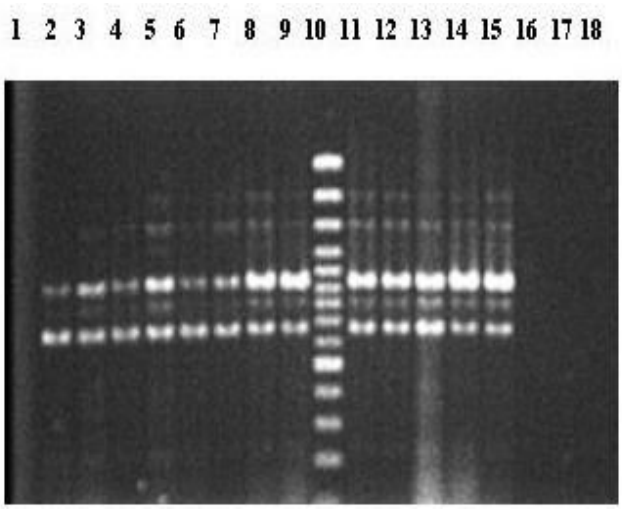

Fig. 10. 1: PCR electrophoretogram of non-radioactive library screening via a sib-selection protocol, using Herculase II enzyme. 1) and 16-18) plaques negative to serpin gene internal fragment. 2-9) and 11-15) plaques positive to serpin gene internal fragment. 10) DNA Ladder 20 Kb.

10. 1. 2: Vector construction

Plasmids rescue and oligonucleotides purification permitted to construct the vector for *E.coli* and *B. longum* transformations. Manipulated inserts and original inserts were firstly charge for an electrophoretic run on a 1.8 % low melting agarose gel and than quantify and purity checked with a UVis spectrophotometer The the 0.9 Kb insert fragment was manipulated to introduce BamHI sticky fingers and to be cloned in pRM2 and in pUC18, generating respectively pRM3 () and pUC18Serpin (Figures 10. 2 and 10. 3).

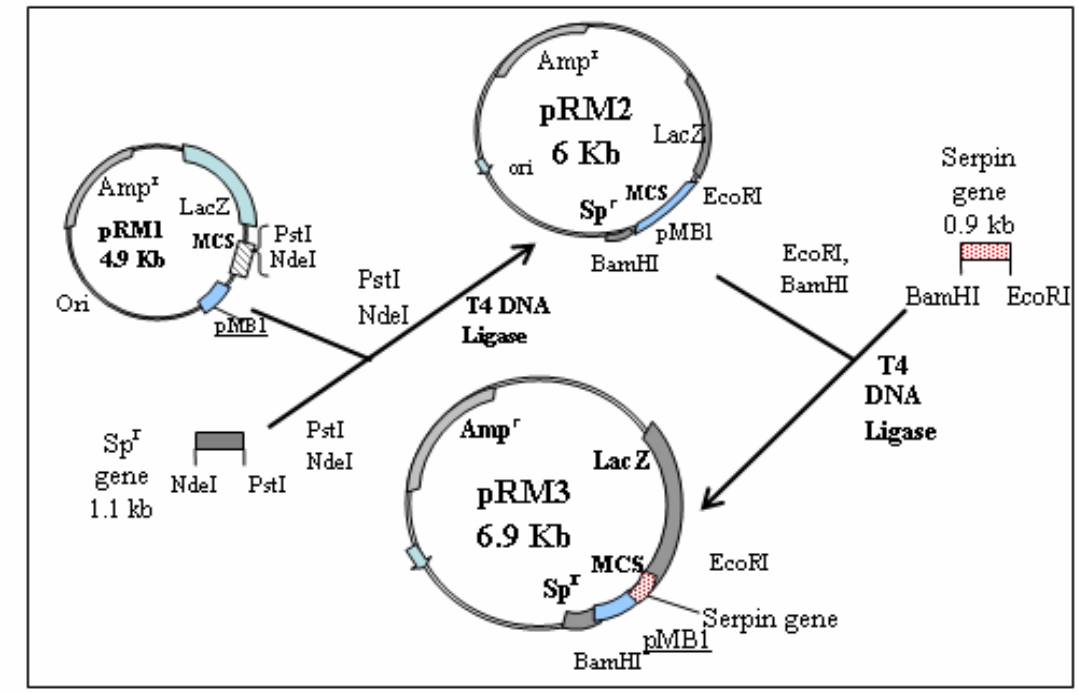

Fig. 10. 2: Construction of pRM3. The first recombinant plasmid was constructed as previously described (Missich *et al.,* 1994). The construction of pRM3 was concluded digesting plasmid and serpin insert and ligating both product with T4 DNA Ligase. Restriction sites and enzymes used are shown in the picture.

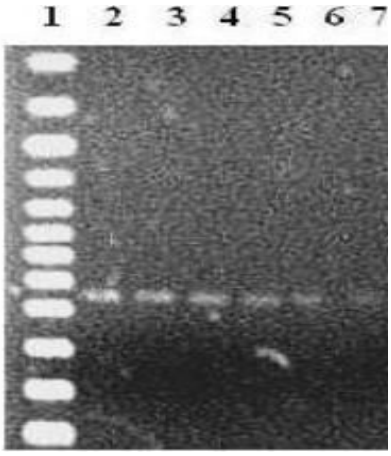

Fig. 10. 3: Insert check. 1) Gene Ruler DNA Ladder Mix (Fermentas). 2, 3 and 4) S1r/M13uni fragment (0,9Kb). 5, 6 and 7) XbaI-S1r/M13 fragment (0,9Kb), both at different dilutions.

10. 2: Transformation

10. 2. 1: Escherichia coli

E. coli XL1 strain was made competent and chemically transformed with both new plasmids. XL1 transformants were selected for ampicillin and α-complementation assay and finally with PCR using 1 unit of Pfu DNA Polymerase (Fermentas), 250 µM of each dNTP and 34 pmol for both *S1f* and *M13uni* primers (Table 1). The PCR profile was as follow: 96 °C for 1 min followed by 30 cycles (each cycle: 95 °C for 30 s, 53 °C for 30 s, 72 °C for 2 min) and a final extension at 72 °C for 3 min (Figures 10. 4).

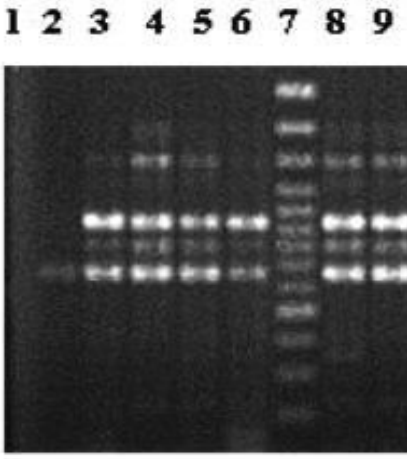

Fig. 10. 4: Pfu PCR electrophoretogram *of E. coli* screening with and *S1r/M13uni* primers pair. 3, 4, 5, 6, 8 and 9) Positive *E. coli* transformants colonies screened via PCR. Seeking for 0,9 Kb fragment. 7) Gene Ruler DNA Ladder Mix (Fermentas).

10. 2. 2: Bifidobacterium longum

Two clones were chosen, pRM3 and pUC18Serpin were purified, while *B. longum* B12 and B18 strain were made competent to be electro-transformed with both plasmids using a Gene-Pulser apparatus (BioRad Laboratories, Ca) . Several protocols for *B. longum* transformation were performed to obtain best efficiency. The best one tested was the one made with MRS medium and raffinose, which led to 3.3 x 10^4 transformants/μg DNA (Table 10. 1). Transformants were finally screened for ampicillin, for spectinomycin and for α-complementation and via a colony PCR with Pfu DNA polymerase.

Strains transformed	**Sugar supplement to MRS medium**	**Transformation efficiency (transformants/μg DNA)**
Bifidobacterium longum B112 + pRM2 *S1f/M13* insert	Glucose 0.7 mol/l	1.2×10^3
	Lactose 0.5 mol/l	2.2×10^3
	Ribose 0.5 mol/l	1.1×10^2
	Raffinose 0.8 mol/l	2.7×10^4
Bifidobacterium longum B118 + pRM2 *S1f/M13* insert	Glucose 0.7 mol/l	1×10^3
	Lactose 0.5 mol/l	2.3×10^3
	Ribose 0.5 mol/l	5.6×10^1
	Raffinose 0.8 mol/l	3.3×10^4

Tab. 10. 1: Transformation efficiency in *Bifidobacterium longum* B112 and B118 with pRM3 using different sugars (NaMR buffer, 12.5 kV/cm, 100/200 Ω and 25 μF). Results are from at least three independent experiments.

10. 3: Serpin detection

10. 3. 1: PCR analysis

To confirm biochemical screening of *B. longum* B112 and B118 transformants, a colony PCR was performed using 1 unit of Pfu DNA Polymerase (Fermentas), 250 µM of each dNTP and 34 pmol for both *S1f* and *M13uni* primers (Table 1). The PCR profile was as follow: 96 °C for 7 min followed by 30 cycles (each cycle: 95 °C for 30 s, 53 °C for 30 s, 72 °C for 2 min) and a final extension at 72 °C for 3 min (Figure 10.5).

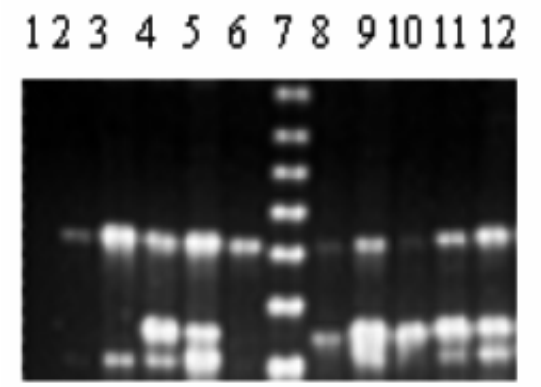

Fig. 10. 5: Pfu PCR electrophoretogram of *B. longum* transformants seeking for 0,9 Kb fragment. 3, 4, 5, 9, 11 and 12) Putatively positive *B. longum* transformants colonies screened via PCR with *S1r/M13uni* primers pair.. 7) Gene Ruler DNA Ladder Mix (Fermentas)

In order to confirm the integrity of cloned Serpin gene in pRM3 from positive transformants *B. longum*, 10 ng of rescued pRM3 were used as template in a PCR reaction with 0.5 units of HerculaseII-TopFusion enzyme (Stratagene), 250 µM of each dNTP and 33 pmol of both *S1f* and *S1r* primers. The PCR profile was as follow: 98 °C for 1 min followed by 26 cycles (each cycle: 95 °C for 20 s, 55 °C for 20 s and 72 °C for 20 s) and a final extension at 72 °C for 2 min. All PCR protocols were performed in a 25 µl reaction with a Biometra T-Gradient thermocycler (Biometra GmbH, Goettingen, Germany) (Figure 10. 6).

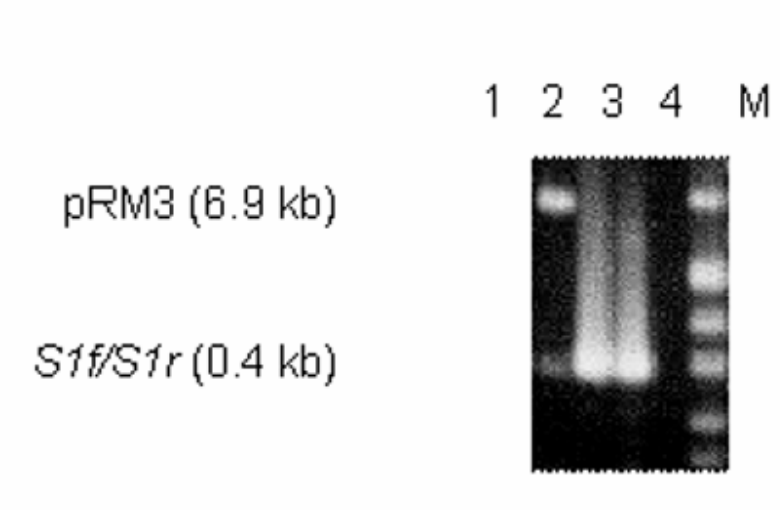

Fig. 10. 6: PCR electrophoretogram of pRM3 prepared from transformants *B. longum* strains. Amplification of serpin encoding gene *S1f/S1r* internal fragment, using pRM3 as template DNA. M: Gene Ruler DNA Ladder Mix (Fermentas); lane 1: pRM3; lane2: *S1f/S1r* amplicons from B112 transformant strain; lane3: *S1f/S1r* amplicons from B118 transformant strain; lane 4: negative control.

10. 3. 2: Dot-blot assay

Supernatants of B112 and B118 *B. longum* strains and B12 wild type strain were used in a dot-blot assay to detect presence of antigens to goat anti-antitrypsin polyclonal antibody. Signal comparison with positive antitrypsin control shows that both *B. longum* B112 and B118 produce a signal twice stronger than *B. longum* B12 wild type strain, confirming the expression of cloned serpin encoding gene (Figure 10. 7).

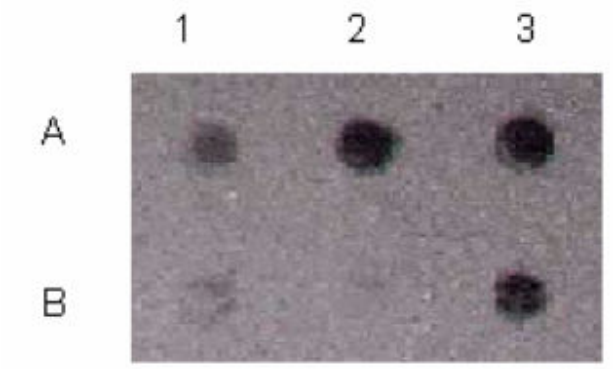

Fig. 10. 7: Dot Blot assay against goat anti-antitrypsin in bacterial cells supernatant of transformants *Bifidobacterium longum* B112, B118 and B12 *B. longum* wild-type. Row A, lane 1: *B. longum* B12 wild type strain; lane 2: *B. longum* B112 (harbouring pRM3); lane 3) *B. longum* B118 (harbouring pRM3). Row B, lane 1: control with the sole secondary antibody; lane 2) negative control (no supernatant); lane 3) positive control anti-antitrypsin.

10. 5: Discussion

Aim of this work was to clone a surface protein encoding gene, showing high homologies with the serpin encoding gene of *B. longum* (Ivanov *et al.,* 2006) and presenting the highly conserved domain of serine inhibitors. The presence of a specific serpin gene in *B. longum* genome had recently passionate several scientists, because until this issue prokaryotic serpin genes were present just in pathogen bacteria and used to take role in their pathogenesis, especially during inflammation stages. Otherwise is well known the probiotic nature of *B. longum* that in its symbiosis with the gut never originates any kind of damage to the host, but contributes to the body well being, even strengthening the host immune system. The course to know which is the serpin role in *B. longum* gut symbiosis is open and we focused our study on some strains that are capable of a strong adhesion to Caco-2 cell monolayer and high co-aggregation level, which are profiles of their host colonisation capability. Moreover these bacterial strains used to express high amount of the original surface protein, now compared with the serpin gene. The *B. longum* strains chosen for electro-transformation are able to induce apoptosis in damaged cells; in facts, we had previously tested their induction of apoptotic deletion of adenocarcinoma Caco-2 cell line (Nissen *et al.,* 2006). After the screening of *B. longum* DNA library in lambda phages, the excision of positive phages and the isolation and clean up of the appropriate fragment, our gene was subcloned in pRM2, a RCR plasmid, that act as an *E. coli* - *B. longum* shuttle vector (Missich *et al.,* 1994), to give pRM3. Unfortunately, the electroporation efficiency in *B. longum* always used to be very low and so had to be improved. *Bifidobacterium* species are difficult to transform by known techniques. These microorganisms are called recalcitrant (Guglielmetti *et al.,* 2007). Transformation is strenuous and the genetic material needs to be highly pure. Until now the highest

level of transformation in *B. longum* was obtained with a shuttle vector of pBLES100 series that produces 1.6 x 10^4 transformants/µg DNA (Nakamura *et al.,* 2002). In previous works the best efficiency of transformation obtained with the pMB1 replicon-based plasmid series was lower than 1 x 10^3 transformants/µg DNA (Missich *et al.*, 1994). To achieve better results, different electroporation protocols were performed leading to the maximum efficiency with 3.3 x 10^4 transformants/µg DNA. The clones obtained were stored lyophilised in several replicates. These bacteria will be used to test apoptotic induction in normal intestinal epithelial cells and blood monocytes and in tumor intestinal cell lines in the way to monitor and better understand serpin role in the elimination of bad cells and in restoring damaged ecology.

Chapter 11 Results and Discussion, section III

11. 1: Adhesion and Cytotoxicity

11. 1. 1: Adhesion to epithelial cells

Almost all bacteria tested have shown to have adhesive properties to PSI C1 intestinal epithelial cell line, even the strains that exhibit a weaker effect, still remain in part adhered to epithelial monolayers. In fact, when these bacteria are co-cultured with PSI c1 for 90 minutes at 37° C in a humidified atmosphere of 5% CO_2 and 95% air using DMEM without Phenol red, FBS and antibiotics, show to resist to cell line medium and micro-ecosystem in a percentage referred to the initial concentration of the inoculum. The range of % of adhesion is between 73 % of Pck 87 and 39 % of Pck 66; so even Pck 66, that have a weak effect, still remain in part attached to epithelial surface (Table 11. 1)

Strains	Adhesion % after 90 minutes	Description
Bifidobacterium longum		
• BuSc 112	59 ± 0.26	*Adh* +
• BuSc BSK7	53 ± 0.17	*Adh* +
Lactobacillus species		
• Pca 142	49 ± 0.15	*Adh*
• Pca 185	41 ± 0.23	*Adh*
• Pca 244	63 ± 0.11	*Adh* +
• Pck 40	65 ± 0.17	*Adh* ++
• Pck 49	41 ± 0.11	*Adh*
• Pck 66	39 ± 0.09	*Adh*
• Pck 87	73 ± 0.25	*Adh* ++
• Pck 103	66 ± 0.10	*Adh* ++
• Pcs 20	55 ± 0.13	*Adh* +
• Pcs 25	49 ± 0.18	*Adh*
Unidentified species		
• Pcs 22	65 ± 0.21	*Adh* +
• Pcs 26	69 ± 0.16	*Adh* +

Tab. 11.1: Strains adhesion abilities on PSI c1. *Adh*++) strong adhesive. *Adh*+) adhesive *Adh*) weak adhesive. Adhesion was expressed by the mean ratio (%, ± S.E.) of the concentration in the inoculum to the that in the suspension after 90 minutes of co-culture with PSI c1 epithelial cell line. Bacterial cells after this time were washed, resuspended at several dilutions and streaked on MRS agar plate, then incubated routinely up to 72 hours to determine CFU/ml. Results are representative of three independent experiments.

11. 1. 2: MTT assay

The metabolic activity of PoM2, PSI C1 and CLAB was investigated after co-culturing with probiotic strains. *Lb. casei* Shirota and *Carnobacterium divergens* BFE403 were used as positive and negative control, respectively, while cell lines without added bacteria were used as control well. Absorbance values that are lower than the control cells indicate a reduction in the rate of cell proliferation. Conversely a higher absorbance rate indicates an increase in cell proliferation.When PSI C1 and CLAB were grown independently in single cell systems using 96-well plates the metabolic activity of both cell lines increased slightly after exposure to 1×10^6 bacteria/ml. Instead, when the co-culture with probiotics is made testing PoM2 in the same conditions, a loss in metabolic activity is detected (figure 11. 1). But, when PoM2 are cultured coupled with PSI C1 in 12 Transwell plate, there is no loss in monocytes metabolic activity, because PoM2 are not directly in contact with bacterial cells (figure 11. 2). This result can be related to what happen *in vivo*, where monocytes rarely come in strict contact with commensal probiotic bacteria. No significant difference was observed in between the probiotics strains, but in comparison with *Lb. paracasei* Shirota, Pck 66, Pck 87 and in minority, Pcs 20 can lead cells to a reduction in metabolic activity. Anyway in comparison with the control well with no bacteria added, there are little differences in both increase and loss of metabolic activity, thus in any cases there are no changes in normal metabolic activity of cell lines, even considering that rarely an increase in proliferation may be offset by cell death. This result confirms that the bacteria tested are not harmful for the cell lines used for the assay.

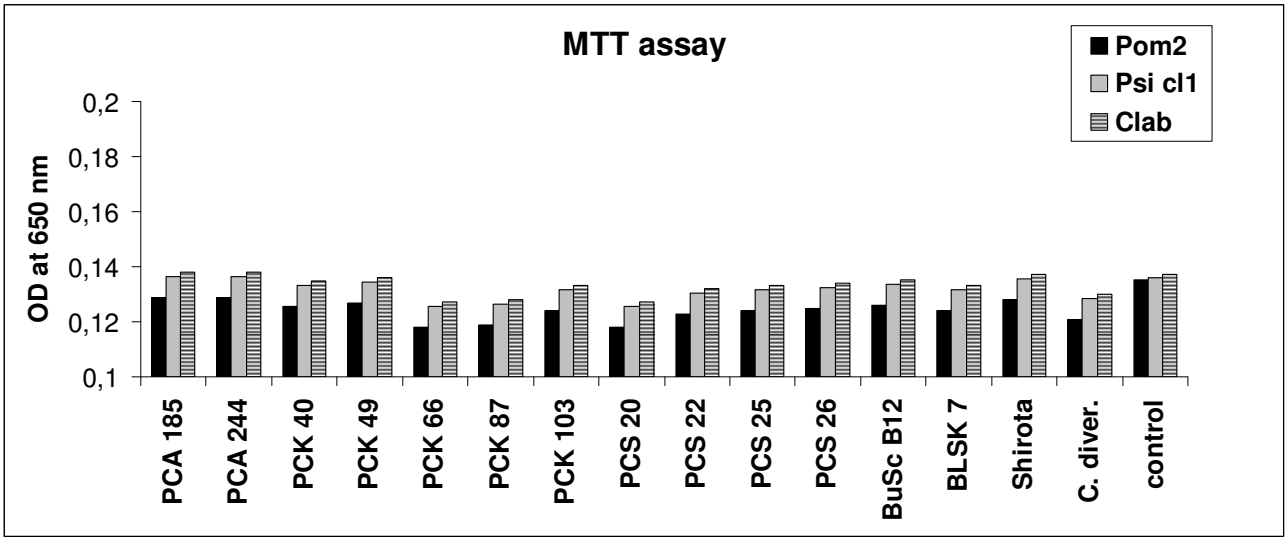

Fig. 11. 1: MTT Proliferation assay of different cell lines cultured in 96 well plates, after 24 hours exposure to probiotic bacteria. Yellow tetrazolium MTT was added at each well and incubated for 75 minutes. reaction was achieved with 0.04 % HCl in isopropanol. The optical density was measured with a multiplate reader set at 650 nm. Absorbance values that are lower than the control cells indicate a reduction in the rate of cell proliferation. Conversely a higher absorbance rate indicates an increase in cell proliferation. ±S.E <0.05. Three replicates were performed

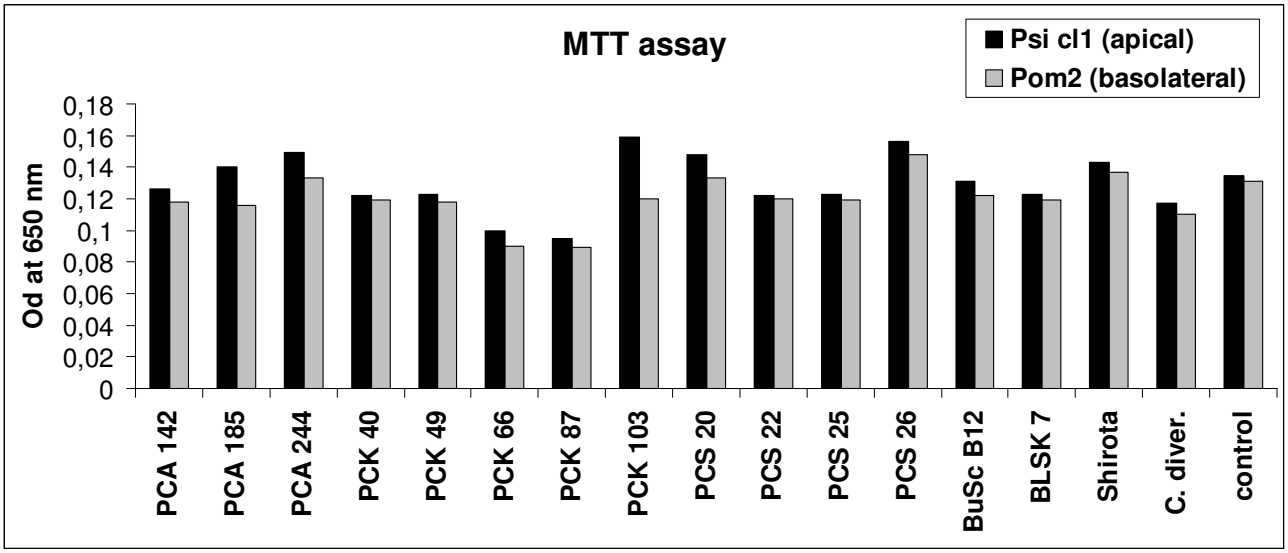

Fig. 11. 2: MTT Proliferation assay of PSI C1 and PoM2 coupled cultured in12 Transwell plates, after 24 hours exposure to probiotic bacteria. Yellow tetrazolium MTT was added at each well and incubated for 75 minutes. reaction was achieved with 0.04 % HCl in isopropanol. The optical density was measured with a multiplate reader set at 650 nm. Absorbance values that are lower than the control cells indicate a reduction in the rate of cell proliferation. Conversely a higher absorbance rate indicates an increase in cell proliferation. ±S.E <0.05. Three replicates were performed.

11. 1. 3: Cytotoxicity

Cytotoxicity of bacterial supernatants on PoM2, PSI C1 and CLAB monolayers was investigated after 24h co-culturing cells with the probiotic strains. A sole control of monolayers with no bacteria tratment was used as control. Absorbance was measured at 450 nm, and the results were expressed by the mean ratios (%, ± SD) of absorbances in treated wells to those in control wells All results are compared with this control and, as shown in figure , there are no significant citotoxic activity exerted by probiotics on epithelial cell lines (PSI C1 and CLAB). For these two cell lines the reduction in viability rarely overcomes the value of 10

% and in that cases are included the assays made with Pck 49, Pck 66 and Pck 87. Thus, this result indicates that the three last mentioned bacterial strains are the most aggressive, when compared to the others of the list and can generate a slight stress condition for cell lines. Otherwise, when pig blood monocytes (PoM 2) are exposed to a concentration at 10^7 bacterial cells/ml for 24 hours in routine incubation conditions, there is a loss of viability in monocyte cells, in fact absorbance measured at 595 nm is slightly higher compared to that of epithelial cell lines, meaning that bacterial co-cultures stress monocyte cells. These results are arguable considering that *in vivo* there are few possibilities of a contact between known probiotic bacteria and blood monocyte cells (Figure 11. 3).

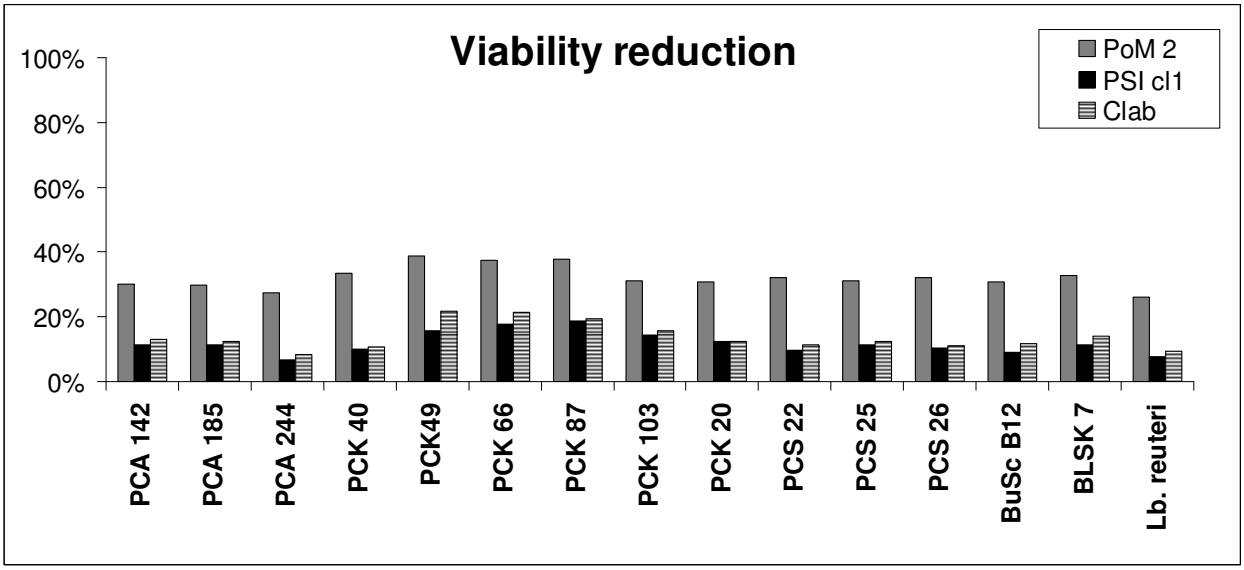

Fig. 11. 3: Citotoxicity effect of probiotic bacterial exposure to the different cell lines expressed as viability reduction in %. Cells were cultured, seeded at 6 x 10^6 cells/well, in single cell systems using 96 well plates and, when monolayers got confluent, were exposed to 1x10^7 bacteria/ ml, then co-coltured for 24 hours at 37° C in atmosphere of 5% CO_2. Cells were stained with crystal violet and destained with acetic acid 10% (w/v) and the optical density measured with a spectrophotometer set at 595 nm. Three replicates were performed and the results were expressed by the mean ratios (%, ± SD) of absorbances in treated wells to those in control wells

11. 2: ROS and Nitric Oxide production

11. 2. 1: Hydrogen peroxide production

The assays needed to measure hydrogen peroxide release by cell lines after exposure to the examined probiotic strains at 1 x 10^7 bacteria/ml, was conducted both in a single cell system, using 96 well plates, and in a double cell system, using

12 Transwell plate. *Lb. reuteri* and *Car. divergens* were taken as positive and negative control, respectively. Three replicates were performed. Absorbance at 450 nm was measured, and the results were expressed by the mean ratios (%, ± SD) of absorbances in treated wells to those in control wells. As seen in figures 11.4 and 11.5 no significant production of hydrogen peroxide was detected in both the systems tested; thus, just strains Pck 49, Pck 66 and Pck 87 shows to induce cell lines to produce more than the 10 % of amount of hydrogen peroxide, when this is related to the control well, where cells are not exposed to probiotics. Anyway, even Pck 87 results seems in contrast, since the activity shown in the assay with the double cell system lowers in the assay with single cell system. These results could be due to the original source of habitat of these strains that are found associated to process of strict fermentation in topic and changing ecosystems, as coffee fermentation and in Kule Naoto fermented milk. Moreover, BuSc B12 and BLSK 7 do not manifest to induce cell lines to produce antagonistic hydrogen peroxide, as demonstrate the results, included between 4 and 10 % of hydrogen peroxide release, that are so close related to that of the average of all tested bacteria. In conclusion, it can be stated that these bacterial strains do not induce cell lines to produce relevant amounts of hydrogen peroxide, thus not reaching a toxic level, but remaining at that level of hydrogen peroxide that is known to be beneficial for host eukaryotic cells, contributing to a good cell tenor.

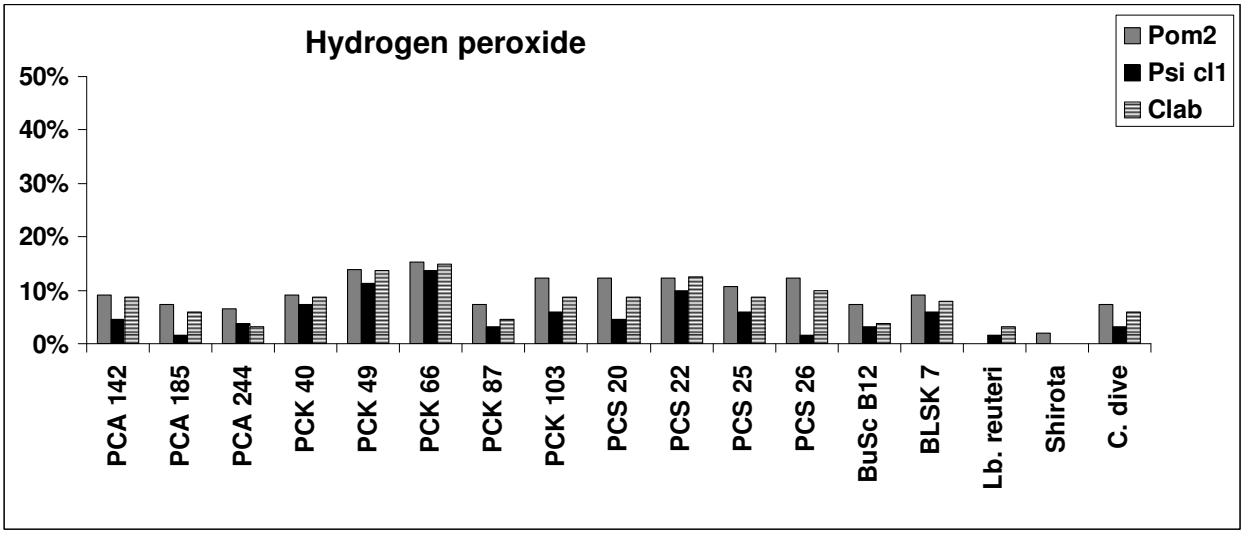

Fig. 11. 4: Effect of tested bacterial strains on H_2O_2 release by the different cell lines cultured independently in a single cell system for 24 hours. Cell were seeded in a 96 well plate at 1 x 10^5 cells/ml and then exposed to 1 x 10^6 cells/ml of probiotic culture and incubated at 37° C in atmosphere of 5% CO_2. Then, the amount of released H_2O_2 in cell supernatant was measured by addition of peroxidase and TMB solution. Reaction was stopped with H_3PO_4 as described under chapter 8. Three replicates were performed. Absorbance at 450 nm was measured, and the results were expressed by the mean ratios (%, ± SD) of absorbance in treated wells to that in control wells.

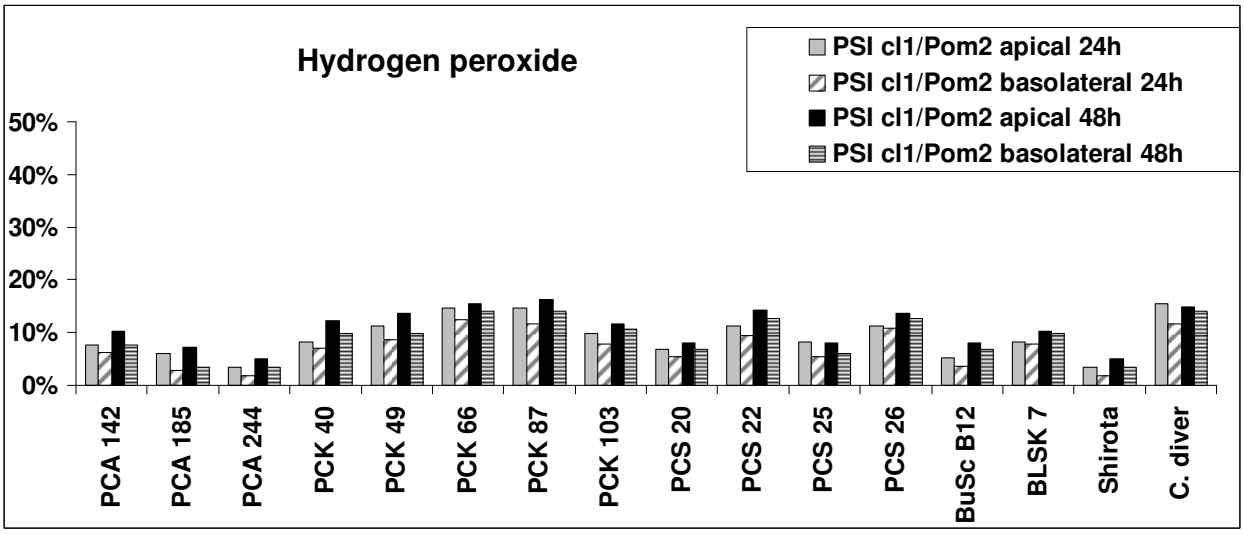

Fig. 11. 5: Effect of tested bacterial strains on H_2O_2 release by PSI C1 and PoM2 cultured in a double cell system for 24 and 48 hours. Cells were seeded in a 12 Transwell plate at 1 x 10^5 cell/ml density in both compartment and then exposed to 1 x 10^6 cells/ml of probiotic culture and incubated at 37° C in atmosphere of 5% CO_2. Then, the amount of released H_2O_2 in cell supernatant was measured by addition of peroxidase and TMB solution. Reaction was stopped with H_3PO_4 as described under chapter 8. Three replicates were performed. Absorbance at 450 nm was measured, and the results were expressed, by the mean ratios (%, ± SD) of absorbances in treated wells to those in control wells.

11. 2. 2: Extracellular superoxide production

The production of extra cellular O_2^- was measured by superoxide inhibitable cytochrome C reduction (Iacobini et al., 1997). Briefly, supernatants were used to react with 80µM Cytochrome C solution and were incubated 1h at room temperature. Optical density was measured at 450 nm by use of microplate reader (Multiscan). *Lb. paracasei* F19 and *Car. divergens* were taken as positive and negative control, respectively. Three replicates were performed. Absorbance at 550 nm was measured, and the results were expressed by the mean ratios (%, ± SD) of

absorbancies in treated wells to those in control wells. The assay permit to evaluate the activation of PoM2 monocytes after exposure to the examined probiotic strains at a concentration of $1x10^6$ cells/ml, compared to monocytes without bacteria added, as control well. The production of extracellular superoxide was measured in PoM2 cultured coupled with PSI C1 in 12 Transwell plates or in a single cell system using 96 well plates. Furthermore, superoxide production was tested even on PSI C1 and Clab epithelial cell lines in a single cell systems using 96 well plates to compare the signals. As seen in figure 11.6, no significant production of extracellular superoxide was observed when compared to monolayers not co-cultured with bacteria, in any of the culturing methods used.

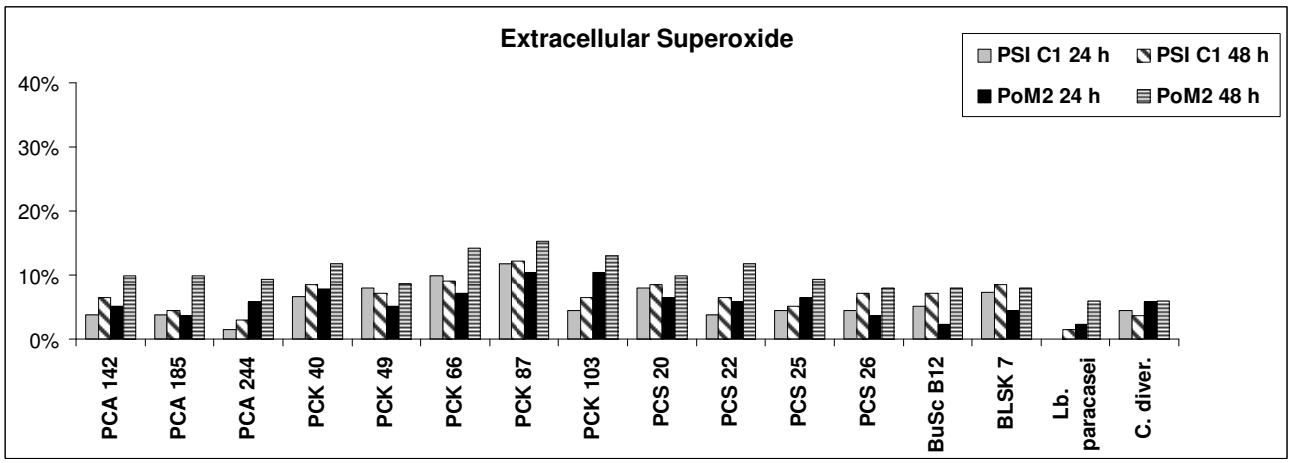

Fig. 11. 6: Superoxide production exerted by PSI C1 and PoM2 cultured in a double cell system for 24 and 48 hours, after exposure to probiotic bacteria. Cells were seeded in a 12 Transwell plate at 1 x 10^5 cell/ml density in both compartment and then exposed to 1 x 10^6 cells/ml of probiotic culture and incubated at 37° C in atmosphere of 5% CO_2. Three replicates were performed. Absorbance at 550 nm was measured, and the results were expressed by the mean ratios (%, ± SD) of absorbances in treated wells to those in control wells.

11. 2. 3: Nitric oxide production

The effect of nitric oxide production by cell lines after exposure to the examined probiotic strains at 1 x 10^6 bacteria/ml, was conducted both in a single cell system, using 96 well plates, and in a double cell system, using 12 Transwell plate. *Lb. paracasei* F19 and *Car. divergens* were taken as positive and negative control, respectively. Three replicates were performed. Absorbance at 540 nm was measured, and the results were expressed by the mean ratios (%, ± SD) of absorbances in treated wells to those in control wells. As seen in figure 11.7 and in figure 11.8 no significant production of NO was observed when compared to control wells in both single cell system and double cell model. In particular the accumulation of nitric oxide was very low when the bacterial strains were tested on PoM2, in comparison to the accumulation of nitric oxide in control well and compared to the values of tested epithelial cell lines. This result is in line with the natural deficiency of nitric oxide production by monocytes, that uses to accumulate this molecule in very low amount or when is strongly induced or stressed. Even in the double cell model including PSI C1 in the apical compartment and PoM2 in the basolateral compartment, there are evident differences between the two culture compartments. Epithelial cells can produce nitric oxide in response to stress factors, but even to improve cell tenor and to stimulate cell proliferation. The first type of accumulation is done at high nitric oxide concentration, while the second type is related to a low nitric oxide concentration, that is in line with the results obtained for this test. Omitting the results obtained with PoM2 monocytes, due to cell natural behave, and thus, considering the effect of bacterial strains on nitric oxide accumulation just in PSI C1 and Clab epithelial cells, there are no significant difference was observed in between the probiotics strains. In comparison with *Lb. paracasei* F19, Pck 66, Pck 87 and Pcs 26 can lead cells to an slightly higher nitric

oxide production in the range of the 10%. Anyway in comparison with the control well with no bacteria added, there are little differences that are all included in a range that never overcome the value of 0,4 mMol of nitric oxide concentration. This result confirms that the bacteria tested are not harmful for the cell lines used in the assay, but could be beneficial, contributing cells to produce a non toxic level of nitric oxide .

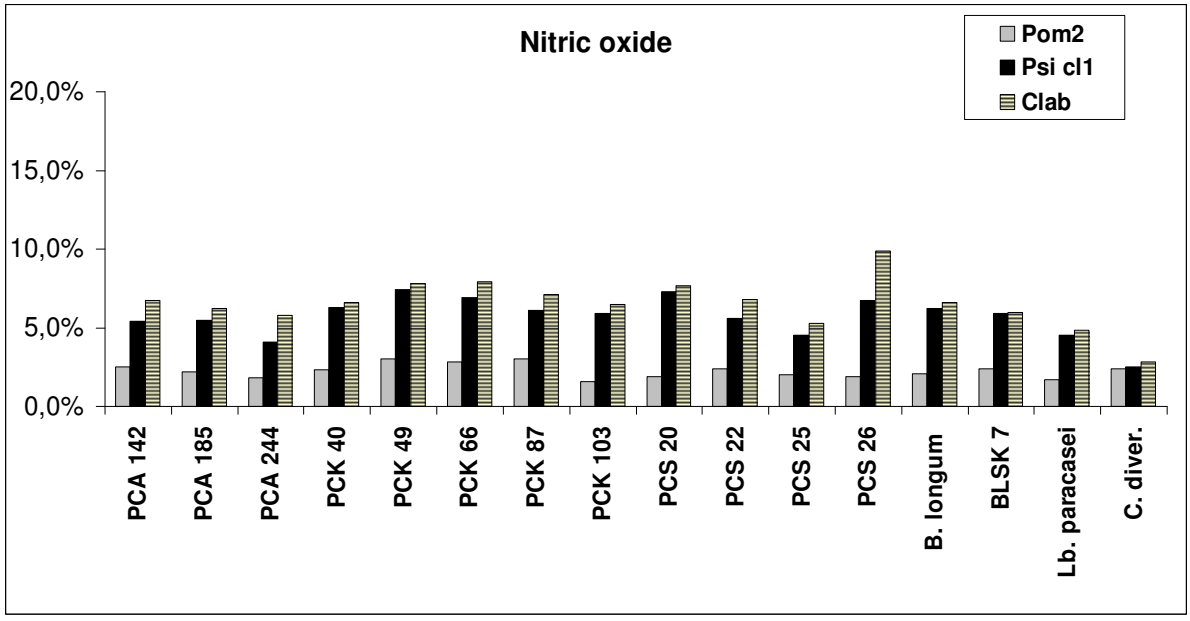

Fig. 11. 7: Effect of tested bacterial strains on Nitric oxide accumulation by all cell lines cultured independently in 96 well plates. Once monolayer were confluent, cell lines were exposed for 24 to 1 x 10^6 bacteria / ml at 37° C in atmosphere of 5% CO_2. Following incubation, realised NO in cell supernatant was measured by addition of Griess reagent as described under chapter 8. Absorbance values were measured with a multiplate reader at 550 nm and mMols of nitric oxide accumulation were extrapolated using a standard curve made with sodium nitrate. The results were expressed by the mean ratios (%, ± SD) of absorbances in treated wells to those in control wells

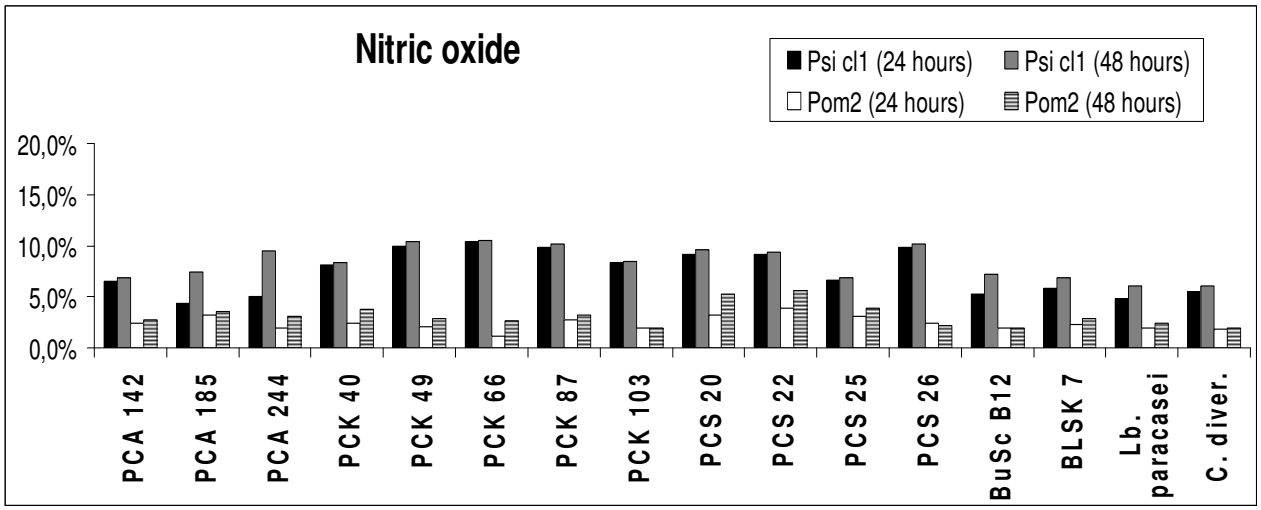

Fig. 11. 8: Effect of tested bacterial strains on Nitric oxide accumulation by PSI c1 and PoM2 cell lines cultured coupled in 12 Transwell plates. Once monolayer were polarised, cell lines were exposed for 24 and 48 hours to 1 x 10^6 bacteria/ ml at 37° C in atmosphere of 5% CO_2. Following incubation, realised NO in cell supernatant was measured by addition of Griess reagent as described under chapter 8. Absorbance values were measured with a multiplate reader at 550 nm and mMols of nitric oxide accumulation were extrapolated using a standard curve made with sodium nitrate.The results were expressed by the mean ratios (%, ± SD) of absorbances in treated wells to those in control wells.

11. 3: Transepithelial Electric Resistance

As shown in Figure 11.9, the probiotic strains examined, used at a concentration of 1x10^7 bacteria/ml, increased TER of polarized monolayers of PSI C1 from approximately 4000 ohm to >6000 ohm, but after 48 hours of co-culture TER starts dramatically decreasing to control value, maybe due to bacterial starvation and death that affect monolayers structure. No significant difference was observed

between the strains. The TER of PSI C1 monolayers with no bacteria added were constant (~3000 Ω) within 72 h. This means that the increase in TER generated by cells co-cultured with bacteria can maintain a low permeability of the epithelium to passive ion flux and avoid the deterioration of its barrier function not just against pathogens.

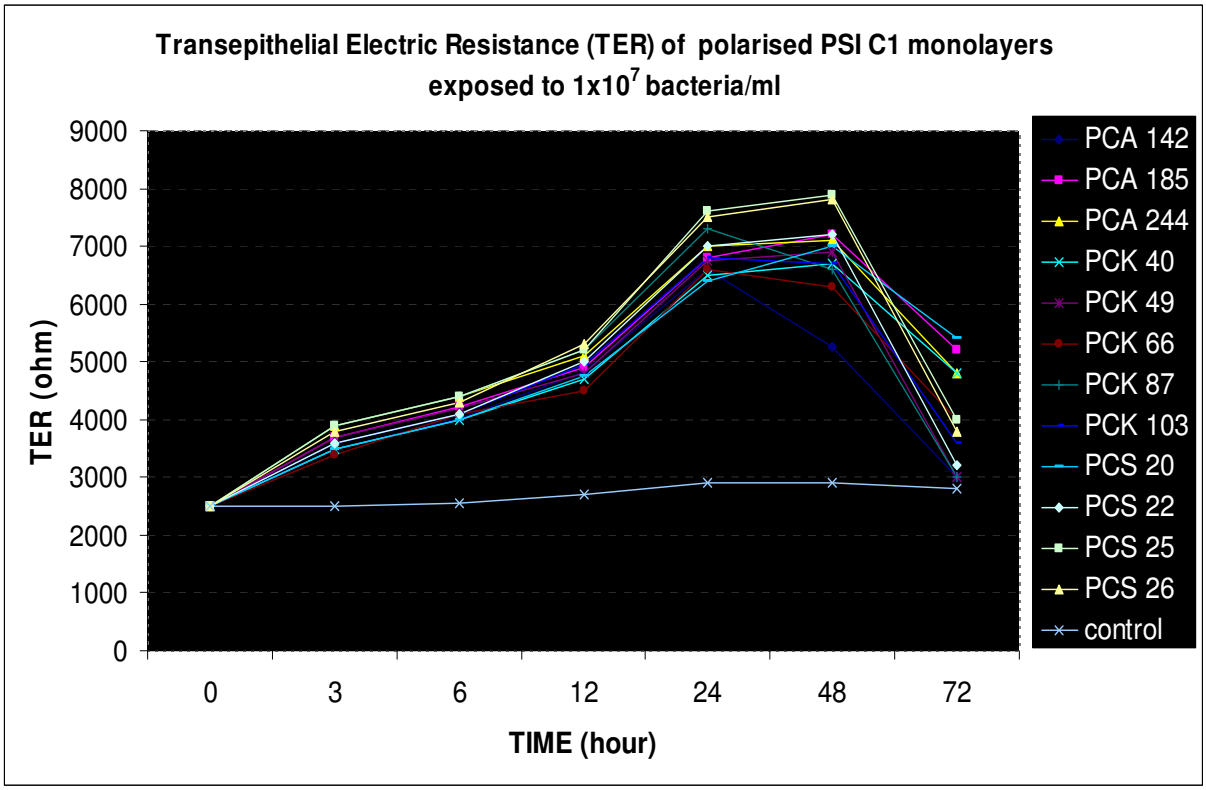

Fig. 11. 9: Transepithelial electrical resistance (TER) of polarised PSI C1 cell lines seeded at 1 x 10^6 in 12 Transwell plates grew until monolayers polarization and then exposed in co-cultures to 1×10^7 bacteria/ml. A sole control with no bacteria added was used as control well. ±S.E <0.05. Three replicates were performed.

11. 4: Immune Response

Dot-Blot was achieved against antibodies IFN-γ and IL-6 to detect the presence of specific antigens in culture supernatant after 24 and 48 hours exposure to bacteria. PoM2 and PSI C1 were tested alone or in combination, having PSI C1 in the apical compartment and PoM2 in the basolateral. PSI C1 alone was tested against IL-6, while PoM2 with PSI C1 was assayed against IFN-γ. As figure 11.10 shows, the best reactions obtained are that ones of cells treated for 48 hours. Interleukin-6 detection is more clear than IFN-γ detection; this could be due to the long lasting interaction of bacteria and cells of the 48 h treatment compared to the 24 h treatment. It can be supposed that after 24 hours the immune response is still unstable and not so specific, even because some positive signals disappear in the 48 hours treatment. Anyway, almost all the samples have shown reactivity against IL-6 antibody; this issue is easy to understand and confirm the well-known immunostimulating abilities of probiotics. In fact, while interferon-γ is a cytokine secreted by animal cells to challenge pathogens and tumours, Interleukin-6 is a pro-inflammatory cytokine secreted by animal cells to stimulate immune response to several traumas.

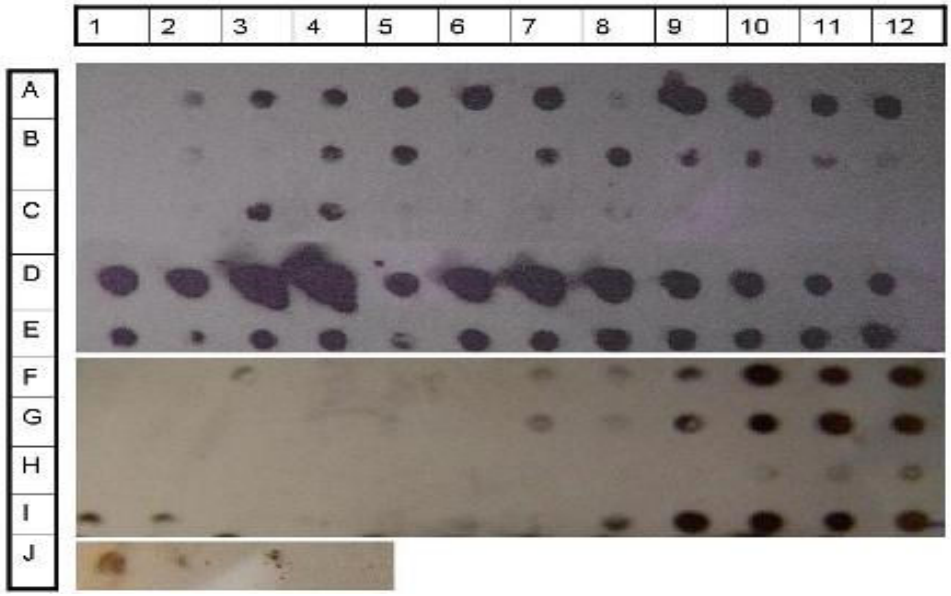

Fig. 11. 10: Dot-Blot experiments for the detection of antigens in culture supernatants for IL-6 antibody in PSI C1 and for IFN-γ antibody in PoM2. Three replicates were performed. Each row corresponds to each bacterial strain used in the treatment, as follow: row 1) PCA142, row 2) PCA185, row 3) PCA244, row 4) PCK40, row 5) PCK49, row 6) PCK66, row 7) PCK87, row 8) PCK103, row 9) PCS20, row 10) PCS22, row 11) PCS25, row 12) PCS26. Il-6 detection. Line A) 24 h PSI C1 apical compartment. Line B) 24 h PSI C1 basolateral compartment. Line D) 48 h PSI C1 apical compartment. Line E) PSI C1 48 h basolateral compartment. IFN-γ detection. Line F) 24 h PSI C1/PoM2 apical compartment. Line G) 24h PSI C1/PoM2 basolateral compartment. Line H) 48 h PSI C1/PoM2 apical compartment. Line I) 48 h PSI C1/PoM2 apical compartment. Controls are located in line C, row 3 and 4, Il-6 and IFN-γ, respectively. Controls in line J, rows 1, 2, 3 and 4 are respectively: J1) Il-6 detection: 24 h PSI C1 not treated, J2) Il-6 detection: 48h PSI C1 not treated, J3) IFN-γ detection: 24 h PoM2 not treated, J4) IFN-γ detection: 48 h PoM2 not treated.

11. 5: Discussion

The metabolic activity of PoM2, PSI C1 and CLAB increased slightly after direct exposure to a concentration of 1×10^7 bacteria/ml of any of the twelve probiotic strains tested and do not affect mitochondrial functions. Probiotic treatments manifested no cytotoxicity over PSI C1, CLAB and Pom2 monolayers, when compared to free-bacteria monolayers. In addition 1×10^7 bacteria/ml increased the transepithelial electrical resistance of polarized monolayers of PSI C1 and thereby strengthened the epithelial barrier function (Figure 11.11). These results indicate that the examined probiotics are not harmful when exposed to an healthy intestine. Furthermore, no cell activation was observed after exposure probiotics as measured by the release of NO, H_20_2 and O_2^- with PoM2, CLAB or PSI C1 monolayers grown in plastic or with PoM2 and PSI C1 grown in a functional cell model, even if three strains of the PCS series showed in both culturing system an high H_2O_2 production. Anyway, as seen in the TER experyment, seems that probiotics bacteria can last no longer than 72 hours when are co-cultured with cells in a functional cell model. This could be due to starvation when bacteria are firstly seeded at concentration of 1 x 10^7 bacteria/ml. Examinations of the immunostimulating effect of probiotics on PSI C1and PoM2 showed that almost all strains induce cells to secrete Il-6.

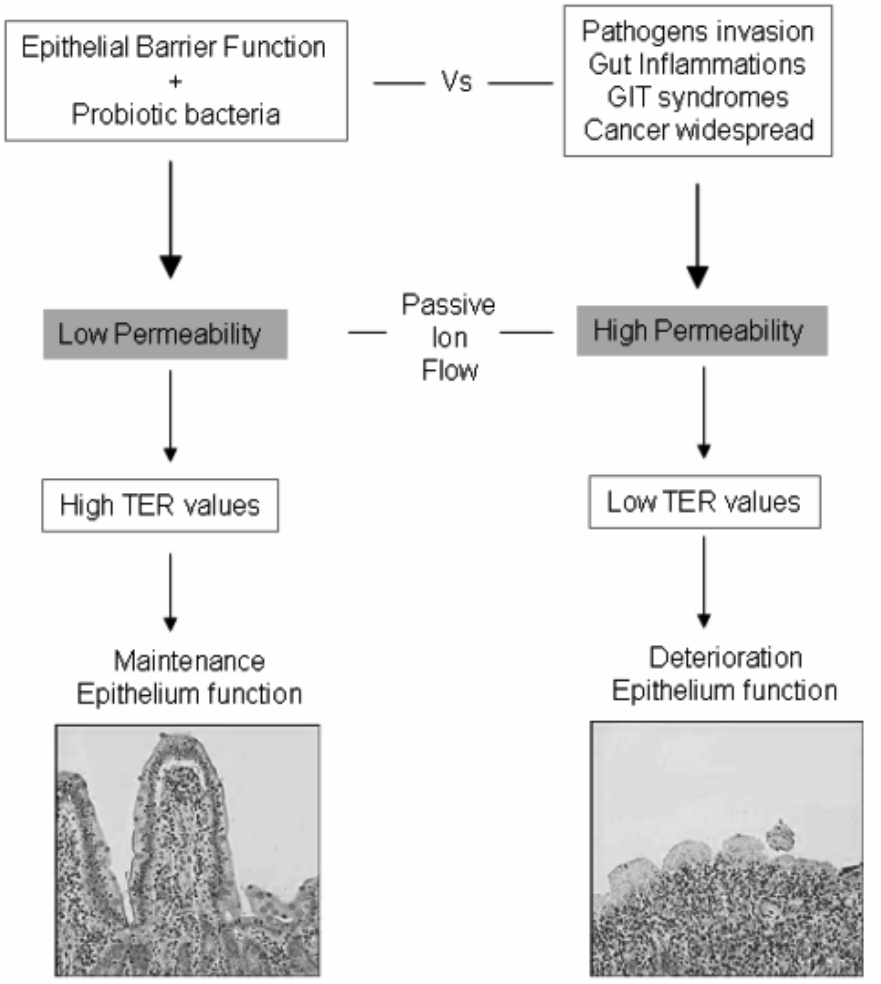

Fig. 11. 11. Schematic representation of TER applications (Original draw of the author).

Part four: Conclusions, Credits and References

Chapter 12: Conclusions

In conclusion we can state that both the list of putative probiotic bacteria and our new transformant strain of *B. longum* are not harmful when exposed to intestinal cells and could be selected as probiotics, because can strengthen epithelial barrier function and stimulate nonspecific immunity of intestinal cells on a pig cell model. Indeed, we have found out that none of the strains tested that have good adhesion abilities presents citotoxicity to the intestinal cells and that non of the strains tested can induce cell lines to produce high level of ROS, neither NO_2. Moreover we have assayed even the capacity of producing certain citokynes that are correlated with immune response. The detection of Interleukin-6 was assayed in all our samples, including *B.longum* transformant BKS 7 strain, this result indicates that these bacteria can induce a non specific immune response in the intestinal cells. In fact, when we assayed the presence of Interferon-gamma in cells supernatant after bacterial exposure, we have no positive signals, that means that there is no activation of a specific immune response, thus confirming that these bacteria are not recognize as pathogen by the intestinal cells and are certainly not harmful for intestinal cells. The most important result is the measure of Trans Epithelial Electric Resistance that have shown how the intestinal barrier function get strengthen when cells are exposed to bacteria, due to a reduction of the epithelium permeability. We have now a new strain of *B. longum* that will be used for further studies above the mechanism of apoptotic induction to “damaged cells” and above

the process of “restoring ecology”. This strain will be the basis to originate new transformant strains for Serpin encoding gene that must have better performance and shall be used one day even in clinical cases as in “gene therapy” for cancer treatment and prevention.

Chapter 13: Credits

PathogenCombat is an integrated project under the EU 6th Framework Programme. The project started 1st of April 2005 and will run til April 2010. PathogenCombat consistes of 44 partners in Europe and Australia. Of these 24 partners are research institutions and organisations, 17 Partners are SMEs and 3 Partners are industrial Partners. Food safety is of fundamental importance to the European consumer, the food industry and the economy. The impact on trade and competitiveness is very substantial. Despite significant investment, the incidence of food derived disease still increases in the EU. PathogenCombat attacks this pan-European problem through a holistic, multidisciplinary approach towards threats from new/emerging pathogens in the entire food chain. A number of advanced platforms will be developed to investigate the survival and virulence expression of pathogens in feed

and food, and on contact surfaces in the food chain including the intestinal tract of farm animals. The platforms, of which several are used for the first time in food safety studies, comprise bioimaging, laser tweezers, phage display/convergent evolution, functional mammalian cell models, functional genomics and microarrays. New/emerging foodborne bacteria, yeast, filamentous fungi and viruses are targeted for milk and dairy products, ruminants, poultry and pigs and their meat products. The overall and specific objectives of PathogenCombat can briefly be described as follows: production of safe food with none or acceptably low levels of pathogens. Determination of factors in the food chain, which enable the viability, persistence and virulence of pathogens. Detection and prediction of the occurrence and virulence of pathogens in the food chain with molecular biology based culture independent techniques and microarrays. Determination of host-pathogen interaction with functional cell model replacing the use of experimental animals. Prevention of pathogen transmission along the food chain by new processing technologies and systems, protective cultures and new information on host-pathogen interaction. Application in the food chain/SMEs of PathogenCombat deliverables. Pathogen control throughout the food chain by new mathematical models. Food Safety Management System, which incorporates the deliverables of PathogenCombat. Small and medium Enterprises (SME) Network including dissemination of knowledge, dissemination of results and training of SMEs and consumer awareness of food safety.

Chapter 14: References

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