UNIVERSITA' CATTOLICA DEL SACRO CUORE

PIACENZA

Scuola di Dottorato per il Sistema Agro-alimentare
Doctoral School on the Agro-Food System

cycle XXIII
S.S.D: AGR/17

Effects of *Streptococcus thermophilus* bacteria on rat gene expression profiles

Candidate: Fatima Chegdani

Matr. n.: 3611473

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- Muriel Thomas

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SUMMARY

<u>CHAPTER III:</u> "Genes induced *in vivo* by *Strepcoccus Thermophilus* using supressive subtractive hybridization (SSH) thecnology"

REFERENCES

CHAPTER IV: "Microaerray based indentification of colonic epithelium genes differentially expressed in the presence of *Streptococcus Thermophilus* bacteria."

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إهـــــــداء

أهدي هذا العمل إلى روح جدتي الحبيبة التي أفتقد حنانها كثيرا

إهداء خاص إلى المرأة المغتربة و التي تواجه الكثير من العقبات في بلاد الغربة و تتحمل كثيرا حتى تصل إلى النجاح

ثم أحب أن أشكر كل من علمني حرفًا و ساعدني حتى أصل بهذا العمل المتواضع إلى صورته النهائ

CHAPTER I

State of art

I) GENERAL INTRODUCTION

Associations between diet and chronic disease have long been recognised through epidemiological studies. New genomic technologies are now enabling us to find out more about the basis of these associations by studying the functional interactions of food with the genome at the molecular, cellular and systemic levels and, moreover, the ways in which individuals respond differently to different diets according to their individual genetic make-up.

These two areas, often differentiated as nutrigenomics and nutrigenetics respectively, are potentially of huge importance to the health care system and to the food industry, as well as to those seeking to understand the biology underlying normal homeostasis and disease.

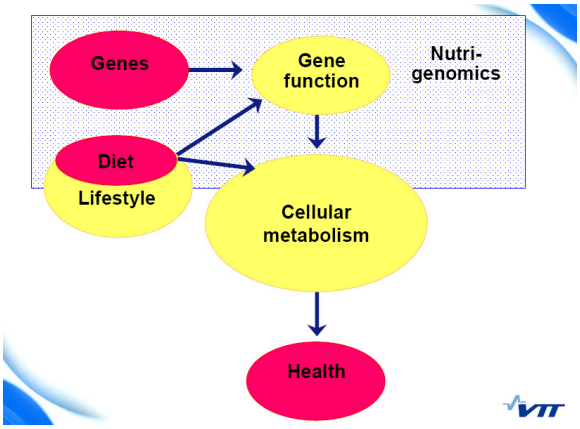
Nutrigenomics has emerged when new technologies, such as transcriptomics, proteomics, metabolomics, and epigenomics, have added more complex functional analysis to the basic sequence information provided by the Human Genome Project. Transcriptomics, for example, is a very valuable way of beginning understanding how cells and tissues are potentially influenced by diet. Three possible definitions for nutrigenomics can be used:

" … the application of high throughput genomics tools in nutrition research" (Müller & Kersten, 2003)

[nutrigenomics] *"… seeks to examine 'dietary signatures' in cells, tissues and organisms and to understand how nutrition influences homeostasis"* (Müller & Kersten, 2003)

*"… the interface between the nutritional environment and cellular/ genetic processes". (*Kaput & Rodriguez, 2004)

There are a number of other definitions, and several authors distinguish between nutrigenomics and nutrigenetics (as the study of how genotype determines optimal dietary requirements for health on an individual basis), while others prefer to retain the use of nutrigenomics as a more generic term (R.M. Debusk et al, 2005). No matter how it is defined, this science investigates the interplay between genes, and diet.



To understand how nutritional exposure and environment factors influence gene expression on a genomic scale, it is possible to group genes of interest for particular metabolic processes and capture information from all of these at once, to see how the cell is functioning at any given time or under certain conditions. This is now possible thanks to the commercial development of chips dedicated to the investigation of the expression of genes involved into particular metabolic or functional systems.

It's known that some of the development work about Nutrigenomics has focused on cardiovascular disease or type II diabetes mellitus, where various groups have identified potential diet-gene interactions (Mutation Research, 2007). However what about gut health? And Gene–diet–bacteria interactions?

The gastrointestinal barrier is a complex cellular structure, made up of four main components, which collectively act as a biological bouncer to protect the body from the entry of bacteria and antigens. The first are the intestinal epithelial cells that form a physical barrier and have absorptive functions. The second component is the mucus layer produced by goblet cells, which forms a chemical barrier covering the epithelium. The third component, the immunological barrier, is regulated by immune cells that deliver pathogens to the mucosal lymphoid tissue and dendritic cells that extend their arms through the tight junctions to capture luminal antigens directly. The fourth component, the microbiological barrier, consists of the commensal bacteria that limit the

colonisation of pathogens by competitively binding to the epithelium, competing for nutrients and producing antimicrobial compounds.

How these components interact to maintain the gastrointestinal tract is fundamental to the delicate balance between health and disease. Human gastrointestinal tract is inhabited by 10^{13} microbes more than 10 times the number of cells that make up the human body with representatives from 500 to 1,000 species. In adults, strict anaerobes outnumber facultative anaerobes by a factor of 100 to 1,000, so these are likely to have a profound influence on the function of the gastrointestinal tract. The identity of almost all of these microbes and their mechanism(s) of action remains largely unknown. It has been illustrated in a number of germ-free mouse experiments, for example, that these mice require a calorific intake higher than conventional mice to maintain their weight and also had less developed immune systems (i.e. less circulating immunoglobulin, less T cells, etc.). Colonisation of these animals by the commensal bacterium *Bacteroides thetaiotaomicron* assisted with the correct development of these processes by modulating the expression of host genes involved in nutrient absorption, glucose and fat metabolism and immune responses (Gordon et al, 2006). Colonisation by other commensal bacteria shaped the immunity and maturation of the intestinal tract, and molecules produced by the bacteria are believed to play a key role. Although the interactions between intestinal cells and pathogenic bacteria are only partially understood, recent studies have shown that pathogenic bacteria (E.coli) can modulate the cell cycle (Samba Louaka et al, Cell Microbiol, 2008), but in general their interactions with commensal bacteria have been poorly described.

Dirk Haller (Technical University of Munich) provided evidence that intestinal epithelial cell signalling and host-derived regulators, along with enteric bacteria, are critical components of chronic intestinal inflammation. This points to a sophisticated communication network between commensal bacteria and intestinal cells. The gastrointestinal tract is not a passive fermentation vessel, but in a state of active intercommunication with its resident microbial ecosystem and our environment. We cannot forget that perhaps one of the most important environmental factors that

influences this interaction is our diet. We have only begun to understand the delicate balance between commensal bacteria, the gastrointestinal tract they inhabit and our health and optimal wellness versus disease. It becomes apparent that we need to identify both our allies and potential foes in order to exploit this diverse ecosystem, and to understand how our diet interacts with two genomes; the microbial and our own.

In the 2 August 2005 issue of the *Proceedings of the National Academy of Sciences*, Ley and other researchers led by Jeffrey Gordon, director of the Center for Genome Sciences at Washington University, found that the gut microbiota of genetically obese mice contained a high percentage of bacteria from the phylum *Firmicutes* whereas their lean littermates had more bacteria from the phylum *Bacteroides*. In the 21/28 December 2006 issue of *Nature*, Ley reported the same observation in obese and lean human volunteers. She also found that the microbiota of obese people who lost weight through a low calorie diet shifted to look more like that of leaner people.

It's difficult to say which came first in some of these studies, obesity or the altered microbiota. In another study from the same Washington University laboratory, also published in the 21/28 December 2006 issue of *Nature*, germ-free mice were colonized with the microbiota of either obese mice or lean mice. Mice that received the obese microbiota gained a higher percentage of body fat than mice receiving the lean microbiota (47.0% versus 0.86%), suggesting that microbiota shifts may contribute to obesity onset. The researchers also found that the obese microbiota, which contained more genes involved in breaking down sugars, appeared to actually harvest more energy from the same diet than did the lean microbiota. The transplant experiments provided functional evidence that this difference may be biologically relevant. It's clear that dietary differences appear to influence our microbiota, consequently influence our genes and our phenotype.

The influence of diet and of some living bacteria on health has been known for more than a hundred years. During recent decades, knowledge of these influences has greatly increased. A relationship with specific food components has also emerged. Based on this new knowledge, we are now increasingly able to design new and healthier foods, thereby reducing the risk of developing a

number of chronic or infectious diseases. These foods are often called functional foods or designer foods. They are traditional foods modified in such a way that they have specific health benefits which are absent from non-modified products. The largest groups of functional foods are probiotic foods, prebiotic foods and synbiotic foods. These are foods targeted to improve the gut microbiota and, through this, human health.

Probiotics included in functional foods are defined as live microbial food ingredients that have a beneficial effect on the intestinal microbiota and on human health. The live microorganisms are most often bacteria from the genera *Lactobacillus* and *Bifidobacterium*. The first probiotic products were fermented dairy products but these now include many other types of foods, e.g. other dairy products, meat products, drinks and fermented foods in general. Prebiotics included in prebiotic foods are defined as food components, which avoid digestion by mammalian enzymes in the upper regions of the gastrointestinal tract, reach the colon in an intact state and stimulate the growth of specific, beneficial organisms of the gut microflora.

II) COLONIC EPITHELIUM AND MICROBIOTA

II-1) COLONIC EPITHELIUM

The intestinal epithelium is considered as a succession of different compartments with a specific dynamic structure. The gastrointestinal epithelium is an active self renewal layer and its turnover time is gradually prolonged toward the colonic epithelium where it arrives at a maximum (Alam et al, 1994). This ecosystem harbours also a large number of commensal micro-organisms that enables the gut to accomplish its physiological function (O'Keefe SJ , 2008). The important roles of commensal microbiota include protective effect maintained by specific bacterial species, regulation of epithelial cell proliferation and differentiation, production of essential mucosal nutrients, such as short-chain fatty acids, prevention of pathogenic bacteria colonization, and maturation and stimulation of intestinal immunity (Kelly et al, 2007).

II-1-1) MORPHOLOGICAL STRUCTURE AND RENEWAL OF THE COLONIC EPITHELIAL CELLS.

The colonic epithelium is organized in main structures and hierarchical compartments. The crypts are short invaginations of mucosal epithelium that provide protected pockets for special cellular functions. These finger-like invaginations into the underlying connective tissue of the *lamina propria* form the basic functional unit of the colonic epithelium. Studies based on labeling using tritiated thymidine have shown that the proliferation occurs in the lower part of the crypts (Falk et al 1998) (figure 1).

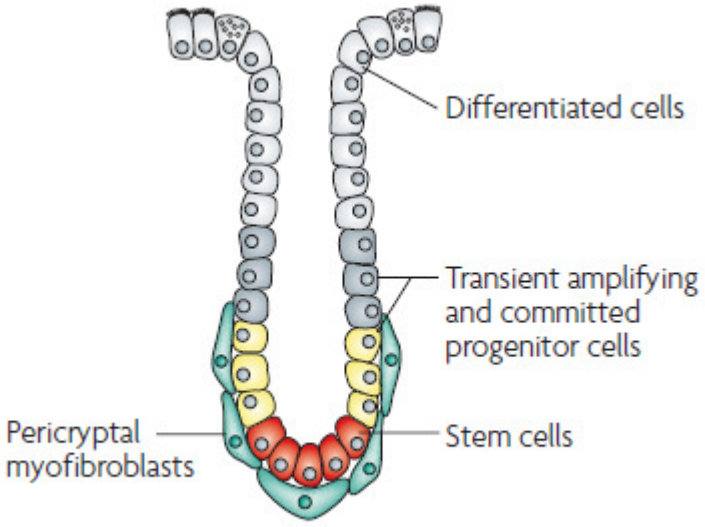


Figure 1: Colonic crypt organization, patterns of stem cell divisions, niche succession and clonal conversion. A diagrammatic representation of the colonic crypt. Stem cells are housed in the base of the crypts where they communicate with the niche cells — the pericryptal myofibroblasts, which are outside the crypt but communicate by cell signalling. Stem cells feed the transit-amplifying compartment, where most cell production occurs. This portionof the crypt is thought to also house the committed progenitor cells: cells committed to one or more cell lineages.

In mouse, the number of dividing cell has been estimated to one hundred for each colonic crypt per day (ten cells per hour). The rate of cell renewal is quite impressive. One cell division occurs, on average, every 5 min in each of the approximately 10^6 crypt in the adult mouse small intestine (Falk et al 1998). In humans, the cell number in small intestine crypts is larger than in mouse, the cell division time is approximately twice that of mouse, and the number of amplifying transit cell generations is believed to be greater (Potten et al, 1992; Potten et al, 1992; wrigh et al, 1984). Cells emerge from colonic crypt and are incorporated into surface epithelial cuffs that surround the orifice of each crypt (Table 1).

	Mouse	Human
Cells/Crypt	300-450	2250
Cell cycle	15-36h	36-96h
Stem cell cycle	>36h	>36h
Stem cell/crypt	1-4	?
Transit cell generations	5-9	5-9

Table 1: estimated values for colonic crypt cell populations. Data from Potten et al, 1992.

The cell migration is also remarkably well organized: several studies using chimeric mice have shown that crypt cells after proliferation migrate rapidly from the bottom of the crypt to the top (surface epithelium) where they are subsequently removed by exfoliation in the lumen or by apoptosis. This migration along the axis of the crypt is coupled with the cell differentiation process, characterized by some changes in cell function and structure. Figure2.

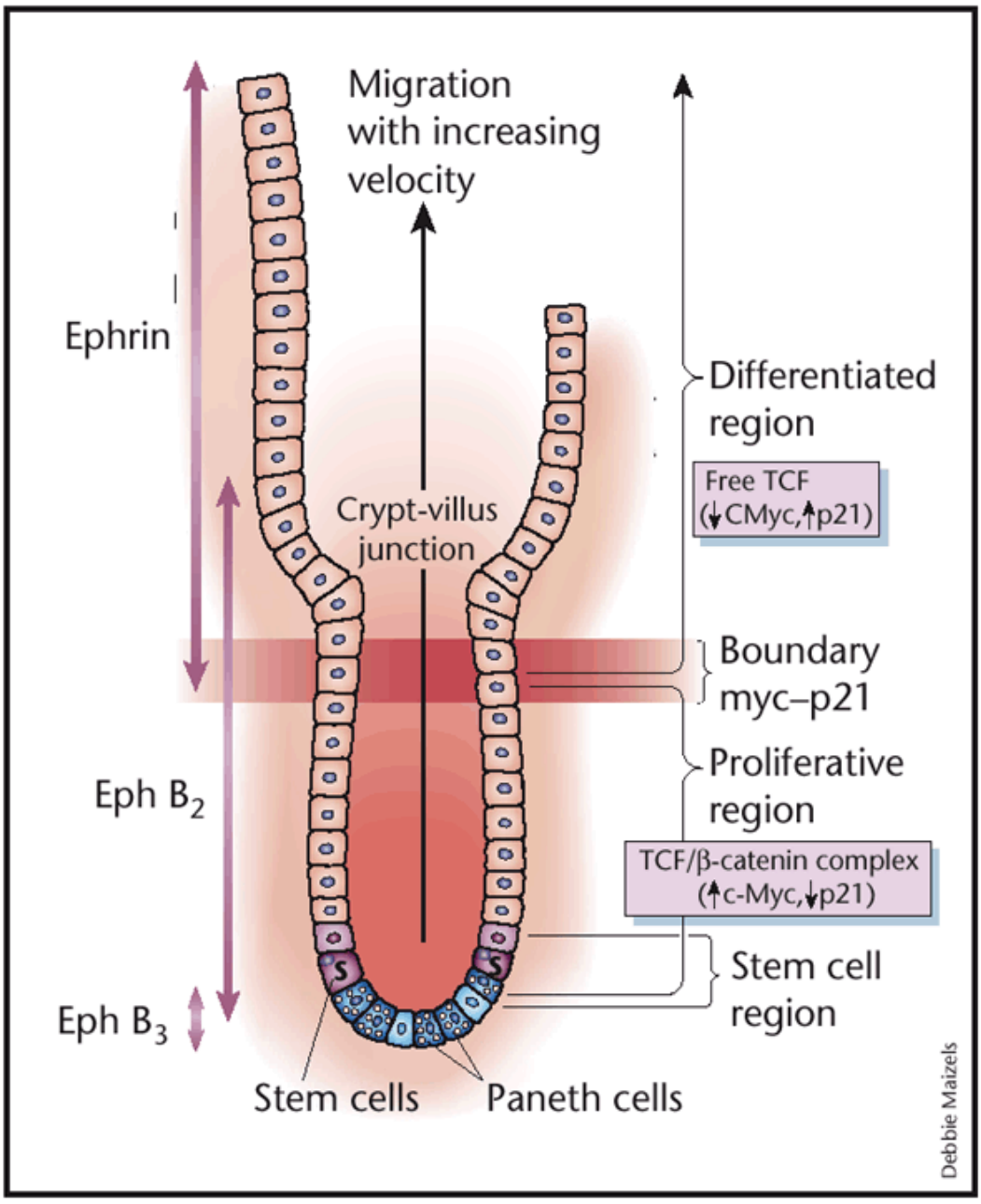
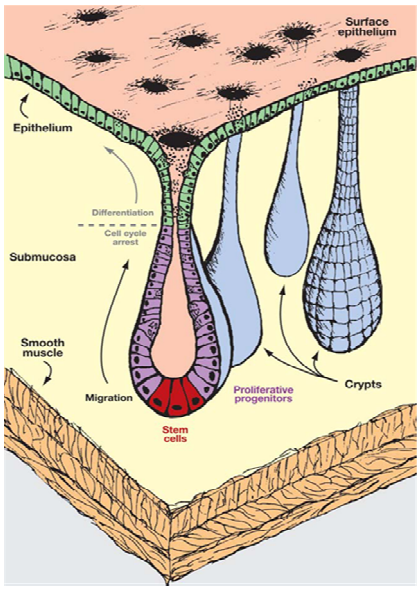


Figure 2: Colonic cells organization during the migration and differentiation process.

The surface of the epithelium is composed of sheets of epithelial cells that form the border of the planar region of the mucosa. During the process of differentiation cells migrate towards the surface of epithelium. The mature colon epithelium is mainly composed by three types of differentiated cells: the goblet cells, the colonocytes or intestinal absorptive cells, and endocrine cells (Figure3).

Colon structure
Sancho et al, 2004. Ann.Rev.Cell.Dev.Biol



Three types of differentiated cells :
-colonocytes : absorptive cells
-enteroendocrine : hormones secreting cells
-goblet cells : mucous secreting cells

Figure 3: organization of the three types of differentiated cells in the colonic epithelium.

- The goblet cells or mucus cells, principally found in midcrypt, secret the mucus that is integrant part of the intestine and have a protective function against aggression.
- The absorptive cells or colonocytes, found in the top of the crypt, represent 80% of all the cells. They have a major role in the transport of electrolytes and short fatty acids produced by colonic fermentations. The main function of the adult colon epithelium is the absorbption of water and salt. The formation of colonic villi occurs in embryonic proximal intestine, in humans these structures are flattened at birth (Pascal et al, 2003).

- Endocrines cells represent less than 3% of epithelial cells and are mainly located at the base of the crypt (Chang. w et all, 1971). They are specialized in the synthesis of peptides.

Several studies have established that the differentiated cell types of the colonic epithelium derive from stem cells sited at the base of crypt (Potten CS et al, 1997; Marshaman E et al, 2002). All cellular 'movements' are therefore towards the lumen (P. de Sanata Barbara et al, 2003). The control and balance of the different processes of proliferation, differentiation, migration and apoptosis is essential to normal gut morphology and function (Ruemmele FM et al,1998). Loss of the balance between proliferation and apoptosis leads to the development of cancer (Hanahan D et al, 2000).

II-1-2) MOLECULAR MECHANISM'S CONTROLLING COLONIC EPITHELIUM PATTERNING.

As previously described, the colonic epithelium is organized along the crypt axis, with stem cells in the bottom of crypt and terminally differentiated cells at the top of the crypt (Potten CS et al, 1990). Homeostasis of the colonic epithelium is maintained by molecular and cellular controls, witch involve different pathways (figure 4).

Recent studies have shown that Wnt pathway play a central role in the physiology of the intestine. Wnt factors are secreted by epithelial cells located in the bottom of the crypt (Gregorieff et al, 2005). Studies *in vivo* have indicated that proliferation of transient amplifying cells (AT) in the crypt is dependent on a continuous stimulation from the Wnt pathway. Van de wetering and his collaborators (2002) have demonstrated that the β-catenin transcription factor accumulated in the stem cells at crypt bottom and these cells respond to Wnt signalling.

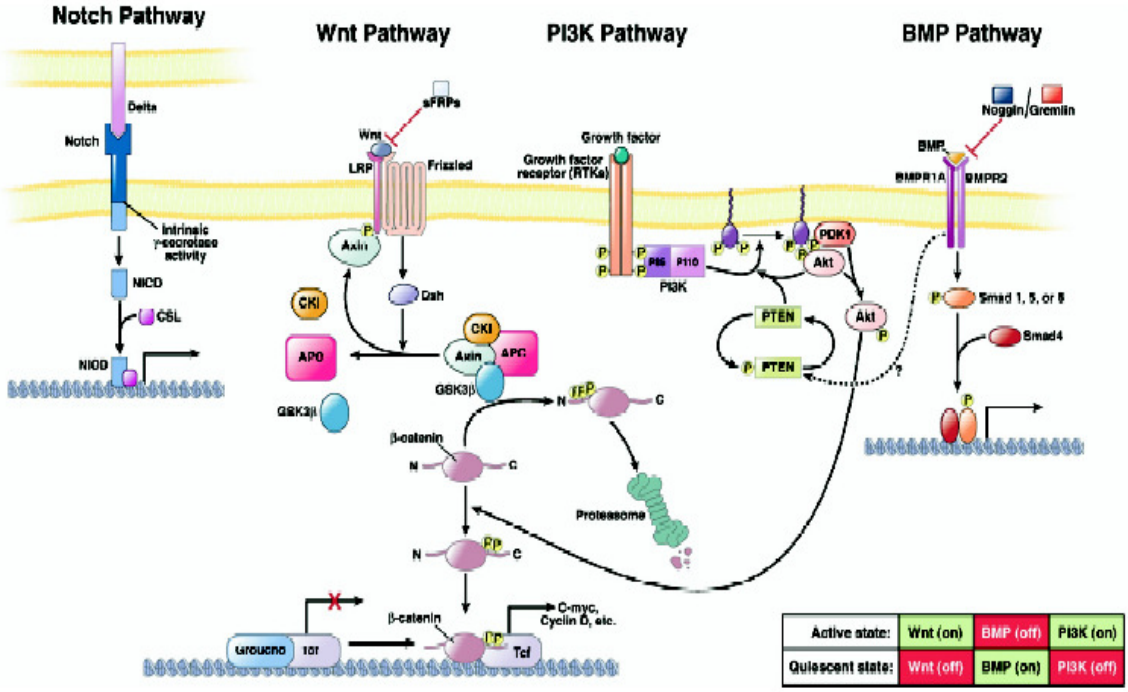


Figure 4: Signaling pathways within the crypt – ISC activation. Outline of Notch, Wnt, PI3K, and BMP pathways and their potential points of interaction. As mentioned within the review, most of the data regarding pathways involved in regulating ISC activation are based on +4 LRCs. Normally +4 LRCs are maintained in a quiescent state through canonical BMP signaling via the transcriptional effects of SMADs and/or possible regulation of PTEN and subsequent inhibition of PI3K signaling. In addition, Wnt inhibitors, such as secreted frizzled-related proteins (sFRPs), act to hinder Wnt mediated effects. Transient activation of +4 LRCs is coordinately regulated by Wnt pathway activation, expression of BMP antagonists, such as Noggin that abrogate BMP inhibition, as well as PI3K activity. Induction of the PI3K pathway results in Akt activation and subsequent C-terminal S552 phosphorylation of β-catenin which likely affects the nuclear activity of this molecule. Thus, Akt assists Wnt-induced β-catenin activation that promotes cell cycle entry and progression within these putative ISCs. Evidence also suggests that Notch pathway activation may be permissive for Wnt induced crypt cell proliferation.

Mice with removed TCF4, β-catenin, or overexpression of DKK-1 resulted in a complete loss of proliferation (Vakorinek et al, 1998; Pinto et al, 2003; Kuhnert et al, 2004). Whereas, mutation of APC gene, negative regulator of Wnt signalling, generated hyperproliferation of colonic epithelium and contributed to colon cancer (Harada et al, 1999; Romagnolo et al, 1999; Smits et al, 1999; Sanson et al, 2004; Andreu et al, 2005).

These results indicate that genes stimulated by β-catenin/ TCF4 complex are involved in the process of proliferation and are located in the crypt proliferative compartment, whereas genes inhibited by

the same complex are involved in the differentiation process and are expressed along the walls and at the top of the crypt.

The self-renewal of the colonic epithelium requires a highly coordinated regulation of the Wnt/β-catenin and Notch signalling pathways, to control the proliferation of multipotent stem cells. Nicolas Coant and collaborators have demonstrated that NADPH oxidase 1 (NOX1), highly expressed in colonic epithelial cells as a consequence of the presence of reactive oxygen species (ROS), is a pivotal determinant of cell proliferation and fate that integrates Wnt/β-catenin and Notch1 signals.

In addition, NOX1-deficient mice reveal a massive conversion of progenitor cells into post-mitotic goblet cells at the cost of colonocytes, due to the repression of the PI3K/AKT/Wnt/β-catenin and Notch1 signalling complexes. These results indicate that NOX1 controls the balance between goblet and absorptive cell types in the colon by coordinately modulating PI3K/AKT/Wnt/β-catenin and Notch1 signaling. This finding provides the molecular basis for the role of NOX1 in cell proliferation and post-mitotic differentiation (Nicolas Coant et al, 2010).

Barker et al (2008) have identified a new Wnt target gene, Lgr5, with a much more restricted expression within crypts. Lgr5 is expressed in the CBC cells. In mice Lgr5 knock out in the colon lead to 100% death.

II-2) COLONIC MICROBIOTA

There is growing evidence that several diseases related to the intestine and/or the immune system are associated with variations in the composition and consequently activity of the gut microbiota (Alverdy, J.C.et al, 2008; Backhed. F et al, 2005; Backhed, F.et al, 2004; Hooper, L.V et al, 2002; Frank, D.N. et al, 2006). Recent evidence also indicates that the bacterial conversion of foods produce a large number of compounds that may have beneficial (e.g. inulin); (Macfarlane, G.T.et al, 1995; Flint. H.J. et al, 2007) or deleterious effects on the host health, (Bingham. S.A et al,1999; van Nuenen, M.H., 2004 ; Blaut, M et al, 2007). Understanding the effects on health of specific food-

derived microbial metabolites is key to optimize the diet and aid re-establishment of a healthy homeostasis in patients, to increase the robustness of homeostatic control and to help healthy subjects to stay healthy and thus to properly target interventions aiming at modulating the microbiota. However, distinguishing the direct impact of the food component on host health from indirect effects through the microbial metabolites or the microbiota itself remains a major challenge.

II-2-1) AN ANIMAL MODEL FOR STUDYING THE FUNCTIONAL IMPACT OF MICROBIOTA ON COLONIC EPITELIUM.

The impact of colonic microbiota on human health and the interface role played by these microbiota between transiting nutrient and the host are fields that have yet to be explored.

Studies with gnotobiotic mice have shown that the enteric microbiota is not functionally isolated from the mucosa; in contrast, these bacteria influence epithelial metabolism, proliferation and survival, and barrier function (Hooper LV et al, 2001; Ismail AS et al, 2005). The small intestinal villi of the germ-free gut are relatively longer, whereas crypts are atrophic, show a slower turnover of the epithelial cells (Ha E.M. et al, 2005), and exhibit defective angiogenesis. Hooper et al (2001) reported robust transcriptional responses of gnotobiotic mice monocolonized with a single gut symbiont species (*Bacteroides thetaiotaomicron*). Jeffrey I., from Gordon's team, have proposed a simplified animal model to investigate the effect of commonly consumed live microbial strains varying in their ability to colonize the Intestinal Gut (Sonnenburg JL, Chen CT & Gordon JI 2006). The model is a Germ Free NMRI mice colonized by *Bacteroides thetaiotaomicron,* a prominent symbiotic member of human microbiota whose genome has been completely sequenced or by *Bifidobacterium longum,* is a minor member of gut microbiota commonly used as probiotic.

Results have shown that the presence of *Bifidobacterium longum* lead to an increase of degradation of target polysaccharides by *Bacteroides thetaiotaomicron* and induces host genes involved in the innate immunity. However, the *Bifidobacterium longum* induced diversification in the

carbohydrates accessed by *Bacteroides thetaiotaomicron* occurs independently of the host genotype.

In conclusion, this animal model provides an excellent instrument to investigate the impact of probiotic species on gut health, and illustrates both the generalities and specificities of the relationship between a host and a component of its microbiota.

II-2-2) COLONIC MICROBIOTA COMPOSITION

The human normal flora, or microbiota, is vast, both in its absolute quantitative mass and its qualitative diversity. An estimated 10^{13} intestinal microorganisms are distributed along the human intestinal tract and reach a remarkable density in the colon. The absolute complexity of the gut ecosystem has required the development of modern molecular biology techniques as most of the microbes present have not yet been cultured and are only known as a result of their detection via 16S ribosomal RNA (rRNA) or DNA (rDNA).

The number of different species comprised in the human gut microbiota is controversial, with many authors referring to estimates of 400-500 species based on culture studies, while more recent estimates are reaching into the thousands (Backhed. F et al, 2005, Rajilic-Stojanovic. M et al, 2007). A combination of sequence analysis of 16S rRNA genes and metagenomic libraries, and of fluorescent in situ hybridization targeting the 16S rRNA has shown that the most abundant bacterial groups in the human intestine belong to the phyla of the *Firmicutes* (including the large class of *Clostridia* and the lactic acid bacteria) and *Bacteroidetes,* which dominate the ecosystem, followed by *Actinobacteria* (including *Collinsella* and *Bifidobacterium* spp.), *Proteobacteria, and Archaea,* and finally bacteriophages, that out number bacteria by 10 to 1 (Table **2**; Eckburg, P.B et al, 2005; Zoetendal, E.G et al, 2006)

Major Phyla	Important Families	*Main Fermentation Products
Bacteroidetes	Bacteroides Prevotellae Prophyromonadaceae Rikenellaceae	acetate, propionate, succinate from carbohydrates
Firmicutes	Clostridiaceae Lactobacillaceae Leuconostocaceae Bacillaceae Streptococcaceae Eubacteriaceae Staphylococcaceae Peptococcaceae Peptostreptococcaceae	acetate, formate, L- and D-lactate, butyrate, succinate, propionate from carbohydrates; BCFAs, indoles, sulphides, phenols, amines, NH3, H2, CO2, CH4 from proteins and amino-acids
Actinobacteria	Bifidobacteriaceae Actinomycetaceae Coriobacteriaceae Corynebacteriaceae Propionibacteriaceae Micrococcaceae	lactate, acetate, formate from carbohydrates
Proteobacteria	Enterobacteraceae Oxalobacteriaceae Pseudomonadaceae Desulfovibrionaceae Helicobacteraceae	lactate, acetate, succinate, formate from carbohydrates; sulphide from sulphate, H2S, mercaptans
Euryarchaeota	Methanobacteriaceae	CH4
Fusobacteria	Fusobacteriaceae	acetate, butyrate, NH3, formate, lactate
Verrucomicrobia	Verrucomicrobiaceae	N.R
Lentisphaerae	Victivallaceae, e.g. Victivallis vadensis	acetate, ethanol, H2

Table 2. Main Phylogenetic Groups (Based on 16S rRNA) in the Human Gut Microbiota with Important Families and the Main Fermentation Products (Rajilic-Stojanovic, M et al, 2007; Louis, P. et al, 2007; Zoetendal, E.G et al, 2003]

**The fermentation end-products are only indicative of some cultured representatives of a family as the vast majority has not been isolated and researched; N.R. not reported.*

Microorganisms from the genera *Bifidobacterium* and *Lactobacillus* are the main members of the gut microbiota recognized as being beneficial for health (Boesten, R.J et al, 2008; Macfarlane, G.T et al, 2008). There is considerable circumstantial evidence that colonic bifidobacteria impact positively on the host via various mechanisms and consequently bifidobacteria are the target of prebiotic functional foods and supplements. A prebiotic is a nondigestible food ingredient that beneficially affects the host by selectively stimulating growth and/or modifying the metabolic activity of one or a limited number of bacterial species in the colon that have the potential to improve host health (Gibson, G.R et al, 1995). Furthermore, specific probiotic strains, often of the *Bifidobacterium* and *Lactobacillus* genera, may be ingested with the food or in supplements. Convincing evidence indicates that specific strains of these genera have a positive health effects on the host (Macfarlane, G.T et al, 2002).

In human gut the composition of microbiota is specific for different intestinal tracts. Remarkable differences in composition are also present between populations and individuals, even if at the functional level a rather high uniformity is present in the vast majority of individuals, after weaning (Gill. S.R et al, 2006; Kurokawa. K. et al, 2007). The gut microbiota of each individual is unique and it is influenced by a combination of factors, including the legacy acquired during birth, the genotype of the individual, host physiological status, lifestyle and diet (Zoetendal E.G et al, 2006; Li M.et al, 2008; Turnbaugh P.J., 2008). The assembly of the gut microbiota commences at birth. In fact, in the neonates, this assembly starts with *Escherichia coli, clostridium spp., Streptoccocus spp., Lactobacillus spp., Bacteroides spp., and Bifidobacterium spp*. Differences in the composition of the fecal flora are found between children from different geographic regions, reflecting, in part, the impact of the environment (sanitary conditions). The mode of delivery, feeding patterns, hospitalization, and antibiotic treatment are other factors known to affect the composition of the gut flora in children. Research published by Chana Palmer et al. in the 26 June 2007 edition of *PLoS Biology* showed that, at 1 year of age, infants started to converge toward a microbiota profile that looked more like the adult GI tract, particularly as they begin to eat solid foods. Once fully

developed in adulthood, the intestinal microbiota is thought to remain quite stable over months or years. Dietary modulation of the gut microbiota can dramatically alter the microbiota community and its activity, and consequently, nutrient bioavailability and metabolism.

II-2-3) COLONIC MICROBIOTA AND METABOLISM.

Transit of undigested and indigestible food components through the colon is slow, allowing for the development of diverse microbiota in the large intestine. Microbial metabolism in the colon is influenced by the amount and type of dietary compounds that survive small intestinal digestion. In general, microbial metabolites including digested dietary compounds absorbed by the gut as well as non-nutrient compounds produced by the microbiota are co-metabolized by host enzymes in the liver (Figure 5). These modified metabolites are returned to the colon by the bile and possibly by other secreted fluids for further metabolism and/or excretion. The commensal bacteria derived metabolites can have various effects on the host immune system (Jia W. et al, 2005).

The interaction of colonic microbial metabolites with the host is basically restricted to the extracellular metabolites (De Graaf A.A. et al, 2008). Whereas the microbial composition of an individual is unique and even varies considerably with time (Barcenilla A. et al, 2009), it is assumed that the microbiota as a whole performs a stable set of activities within a population, given the enormous functional overlap between microorganisms (De Graaf A.A. et al, 2008). In agreement with this, the inter-individual variation observed in faecal extracts from healthy volunteers has been found to originate mainly from variable metabolite concentrations rather than from variable metabolite compositions Jacobs *et al.* (Jacobs D.M. et al, 2008), suggesting that the core of gut microbes share general biochemical characteristics to metabolize different substrates through specific metabolic patterns (Mortensen P.B. et al, 1992). Nevertheless, there is substantial inter-individual variation between the gut microbiota of different individuals, and these global, regional and personal differences are likely to lead to significant differences in response to diet, that may impact on health (Zoetendal E.G. et al, 2006; Li M. et al, 2008).

In particular ‘non-digestible’ dietary carbohydrates, including polysaccharides, oligosaccharides, lignin and associated plant material, are the preferred energy source for colonic microbiota. They are metabolized to short chain fatty acids (SCFAs), primarily to acetate, propionate, and butyrate, which are considered to promote health (Wong J.M. et al, 2006; Elia M. et al, 2007).

Much of the non-digestible dietary carbohydrates are fermented in the proximal colon, where they influence proteolytic fermentation.

Dietary proteins can also reach the colon, and, in addition to endogenous secretions such as mucin, they provide other substrates for fermentation and deamination of amino-acids. These can result in a range of metabolites, including the branched-chain fatty acids (BCFAs) isobutyrate, isovalerate and isocaproate, and also indoles, sulphides, ammonium, phenols, histamine and oxaloacetate, amongst others. These putrefactive components are generally considered to be toxic and to cause adverse effects on the colonic epithelium. A strong causal relationship between proteolytic fermentation in the (distal) colon and the occurrence of colon cancer (Bingham S.A. et al, 1999) and inflammatory bowel disease (IBD) (Van Nuenen M.H. et al, 2004) is hypothesized.

In the colon, polyphenols are deconjugated by bacterial glycosidases, glucuronidases, and sulfatases and further fermented to a wide range of low-molecular-weight phenolic acids (Scalbert A. et al, 2002). Thus, the gut microbiota plays a key role in the bioavailability of polyphenols and has been shown to modulate the health promoting activity through transformation of diet components into more active derivatives (Scalbert A. et al, 2002; Fava F. et al, 2006).

In addition to food ingredients, which influence gut homeostasis via mediating metabolic or biochemical factors, such as intra-colonic pH, production of bile acid metabolites, and fermentative production of metabolites, disturbances of the gut ecology can be caused by a variety of factors, including antibiotics, environmental toxins, infections, stress, and genetic predisposition. In particular, the use of antibiotics is speculated to be linked to several disorders such as obesity, insulin resistance, diabetes, irritable bowel syndrome and diarrhea (Eckburg P.B. et al, 2005; Arvola T. et al, 1999; Mendall M.A. et al 1998). Modern microbiota-targeted therapies envisage

combinations of antibiotics, probiotics, prebiotics and perhaps laxatives to restore the homeostasis of gut ecology in the host (Jia W. et al, 2008).

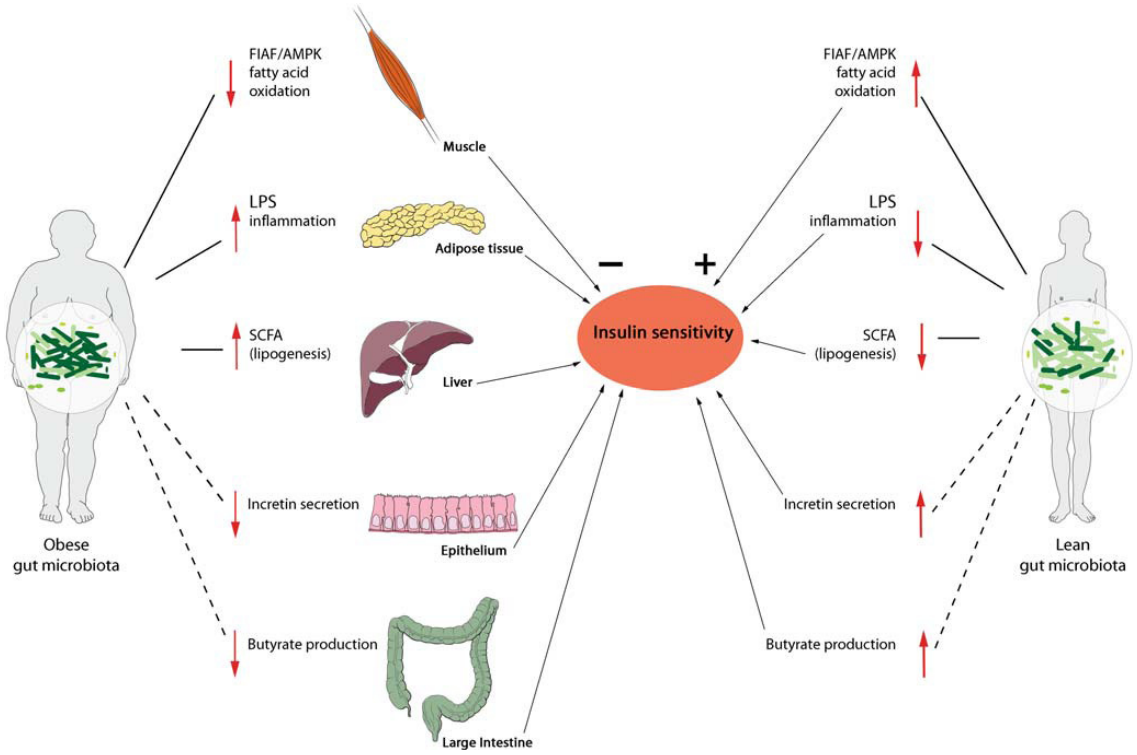


Figure 5: Possible links between the gut microbiota and metabolism. Continuous lines, likely pathway; dotted lines, putative pathway

II-2-4) DEFENSE BARRIER AND MUCOSAL IMMUNE SYSTEM.

The intestinal microbiota is an important constituent of the mucosal defense barrier. Resident bacteria serve as a first line of resistance to colonization by exogenous microbes and this helps in preventing the potential invasion of the intestinal mucosa by an incoming pathogen. This protective function is known as the barrier effect or colonization resistance (Geraldine et al, 2008). The protective function is that adherent nonpathogenic bacteria can often prevent attachment and subsequent entry of suspected pathogens into epithelial cells. In addition, recent studies have revealed further effects of butyrate on the intestinal barrier function (Peng et al, 2007). This barrier function, may be classified into physical and biological barrier functions.

The biological barrier includes a detoxification system formed by detoxification enzymes and efflux transporters (Benet L.Z. et al, 2001) which inhibits the invasion of the body by harmful compounds. Secretion of IgA antibodies against various pathogens (Wehkamp J. et al, 2007) can also be included in the biological barrier functions of IECs.

Furthermore, *in vitro* or animal studies have clearly found beneficial effects of butyrate against oxidative stress and on various components of the intestinal barrier (including mucus, epithelial permeability and migration), trefoil peptides (antimicrobial peptides) and heat shock proteins (Wong J.M. et al, 2006; Hamer H.M. et al, 2008), which contribute to improving the colonic barrier defense. At the molecular level, it is known that butyrate is a histone deacetylase inhibitor (HDAC), leading to changes in chromatin conformation and gene expression (Siavoshian S. et al, 2000). This mechanism is assumed to be crucial for the ability of butyrate to modulate the expression of numerous genes involved in colonic health (Daly K. et al, 2006; Segain J.P. et al, 2000). Apart from acting on epithelial cells, butyrate is also capable of interacting with immune cells. In fact a number of studies have shown that butyrate has anti-inflammatory properties in IFN-γ-stimulated macrophages *in vitro* (Park J.S. et al, 2007) through the interaction with the GPR43 transmembrane receptor recently identified on immune cells (Brown A.J. et al, 2003; Le Poul E. et al, 2003).

Another important trophic factor is the communication network that exists between the host and resident microflora at the mucosal interface, which channels the development of a competent immune system. Monteleone and Gordon (2005, 1997) have reported that there is a clear indication that microbial colonization of the gastrointestinal tract affects the composition of the gut asociated lymphoid tissue (GALT).

II-2-5) REGULATION OF COLONIC EPITHELIUM STRUCTURE

The microflora of the gut has a direct impact on the architecture and morphology of the digestive system (Falk P.G. et al, 1998) For example, the villi of the small intestine are longer; crypts are shorter and contain fewer cells in germfree mice than in age-matched conventional animals (Alam

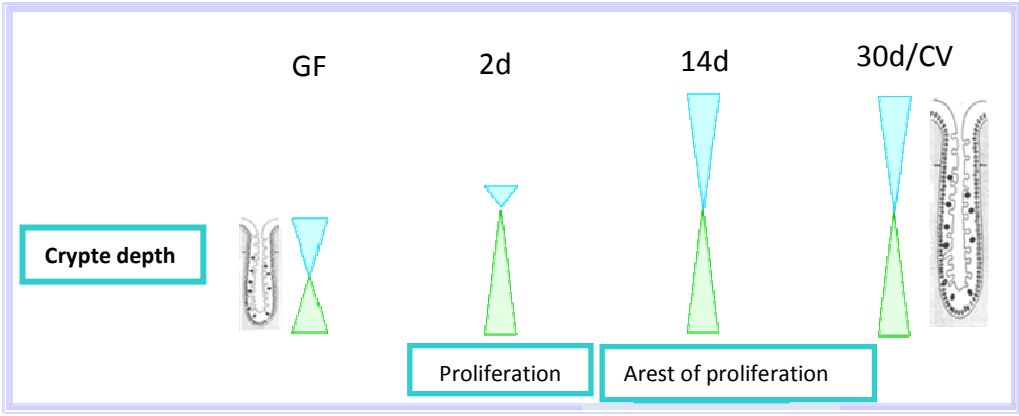
M. et al, 1994). The largest difference is in areas with the highest bacterial density. Since the microbiota are chiefly responsible for the degradation of mucus glycoproteins, the absence of the flora in germfree mice results in the accumulation of these components in the distal small bowel and proximal colon, with a corresponding enlargement. However, this enlargement can be easily reversed with monoassociation with *Peptostreptococcus micros* or conventionalization with flora from normal mice (Carlstedt-Duke B. et al, 1984). These important observations on the contribution of microflora to the architecture of gut have been possible primarily because of the use of animal models such as conventionalized or germ free mice. However, it is essential to remember that the pattern of colonization of the microbiota (mucosal adherent/non-adherent), epithelial cell renewal, cells/crypt and crypt/villus ratio in the gut of mice are different from humans (Falk P.G. et al, 1998). Nevertheless, these animal models have been instrumental in providing us insights into the contributions of the microbiota to gut morphology.

The structure and homeostasis of intestinal epithelium are the basis of the absorptive function of the intestine and are modulated throughout life by environmental factors. It is known that the surface epithelium and crypt depth increase after birth. A number of studies have shown that adult humans increased colonic crypt depth reflects a morphological adaptation that occurs following intestinal resection (Joly F. et al, 2009). In this context, some studies investigated the effect of the microbiota on the structure and homeostasis of the colonic epithelium. It has observed in vivo using gnotobiotic animals that the presence of microbiota leads to an increase in the crypts depth of the colonic epithelium. This trophic effect of microbiota was accompanied by a highly orchestrated induction of proteins involved in colonic epithelial cell cycle. The presence of a complex of microflora in the intestinal tract is a determinant factor of its ecosystem. The establishment of microbiota in the host induces an early hyper-proliferation phase that is then offset by a late phase of proliferation arrest and differentiation (Cherbuy C. et al, 2010) (figure 6). Recent

studies report that cell cycle proteins that are induced by the commensal microbiota have also been described as cellular targets of some pathogenic bacteria (Samba L. et al, 2008).

Effect of microbiota on the colonic epithelium

- 2 days after inoculaton: transitory proliferation pick
- 14 days after inoculation : crypt reaches the maximum development.



Cherbuy *et al*, 2010, Am. J. Physiol-gastrointestinal and liver,

Figure 6: this figure summarize the effect of microbiota on the colonic epithelium of Germ Free in comparison with conventional rats at 30days after 2 days and 14 days of microbiota inoculation. At 2 days its clear that proliferation has a transitory pick, at 14 days there is an arrest of the proliferation and the crypt reaches the maximum development.

BACTERIA AS THERAPEUTIC PROBIOTIC AGENTS?

III) PROBIOTIC EFFECT AND HEALT

III-1) PROBIOTIC DEFINITION AND HISTORY

Probiotics are living microorganisms which, upon ingestion in certain numbers, exert health benefits beyond inherent basic nutrition. All known probiotic bacteria belong to the group called lactic acid bacteria, which in this context includes the species *Lactococcus, Lactobacillus, Streptococcus, Leuconostoc, Pediococcus, Bifidobacterium and Enterococcus.* Among these, only some *Lactobacillus-, Bifidobacterium- and Enterococcus* strains are commercially available as probiotics in foods (Table 2), but other strains are also considered probiotics, e.g. *Lactococcus, Pediococcus, Streptococcus and Leuconostoc.*

The term probiotic was derived from the Greek, meaning "in favour of life." The Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) have stated that there is adequate scientific evidence to indicate that there is potential for probiotic foods to provide health benefits and that specific strains are safe for human use (Food and agriculture organization of the united Nations and World Health Organization, 2001). An expert panel commissioned by FAO and WHO defined probiotics as "Live microorganisms which when administered in adequate amounts confer a health benefit on the host." This is the definition that should be used, and probiotics should not be referred to as biotherapeutic agents (McFarland et al, 1995). Probiotics represents an expanding research area. A Medline search of the term probiotics illustrates the significant increase in research undertaken in this area during the past 5 years. There is a relatively large volume of literature that supports the use of probiotics to prevent or treat intestinal disorders.

The principle of using harmless bacteria for conquering pathogens has been recognized for many years. In fact, probiotics have been used for as long as people have eaten fermented foods. However, it was Metchnikoff at the turn of the century who first suggested that ingested bacteria could have a positive influence on the normal microbial flora of the intestinal tract (Metchnikoff 1907). He hypothesized that lactobacilli were important for human health and longevity, and promoted yogurt and other fermented foods as healthy.

The belief in the beneficial effects of probiotics is based on the knowledge that the intestinal microbiota can protect humans against infection and that disturbance of this microbiota can increase susceptibility to infection. Numerous in vivo and in vitro studies have shown that the normal intestinal microbiota is an extremely effective barrier against pathogenic and opportunistic microorganisms (Fuller 1991).

Probiotics are usually targeted for use in intestinal disorders in which specific factors (such as antibiotics, medication, diet or surgery) disrupt the normal microbiota of the gastrointestinal tract, making the host animal susceptible to disease. Examples of such diseases include antibiotic-induced diarrhea, pseudomembranous colitis and small bowel bacterial overgrowth. The goal of probiotic therapy is to increase the numbers and activities of those microorganisms suggested to possess health-promoting properties until such time that the normal flora can be reestablished. These diseases include traveler's diarrhea, *Helicobacter pylori* gastroenteritis and rotavirus diarrhea. Many microorganisms have been used or considered for use as probiotics. A probiotic preparation may contain one or several different strains of microorganisms. Because viable and biologically active microorganisms are usually required at the target site in the host, it is essential that the probiotic be able to withstand the host's natural barriers against ingested bacteria.

The most widely documented effects of lactobacilli in humans are stimulation of the immune system, prevention and reduction of the intensity and duration of diarrhea, and reduction of lactose intolerance (Wolvers et al., 2010). Lactobacilli also have other potential but less documented

beneficial effects such as vitamin B synthesis, improvement of mineral and nutrient absorption, degradation of antinutritional factors, or modulation of intestinal physiology and reduction of pain perception. Lactobacilli may thus be of benefit to health and help protect against some diseases. The beneficial effects of *Lactobacillus* and *Bifidobacterium* have been discussed for decades. Bacteria in these two genera resist gastric acid, bile salts and pancreatic enzymes, adhere to intestinal mucosa and readily colonize the intestinal tract. They are considered important components of the gastrointestinal microbiota and are relatively harmless.

There are many mechanisms by which probiotics enhance intestinal health, including stimulation of immunity, competition for limited nutrients, inhibition of epithelial and mucosal adherence, inhibition of epithelial invasion and production of antimicrobial substances. Probiotics can also improve host growth (Figure 7) providing growth factors that they were involved in intestinal maturation (Turpin et al, 2010).

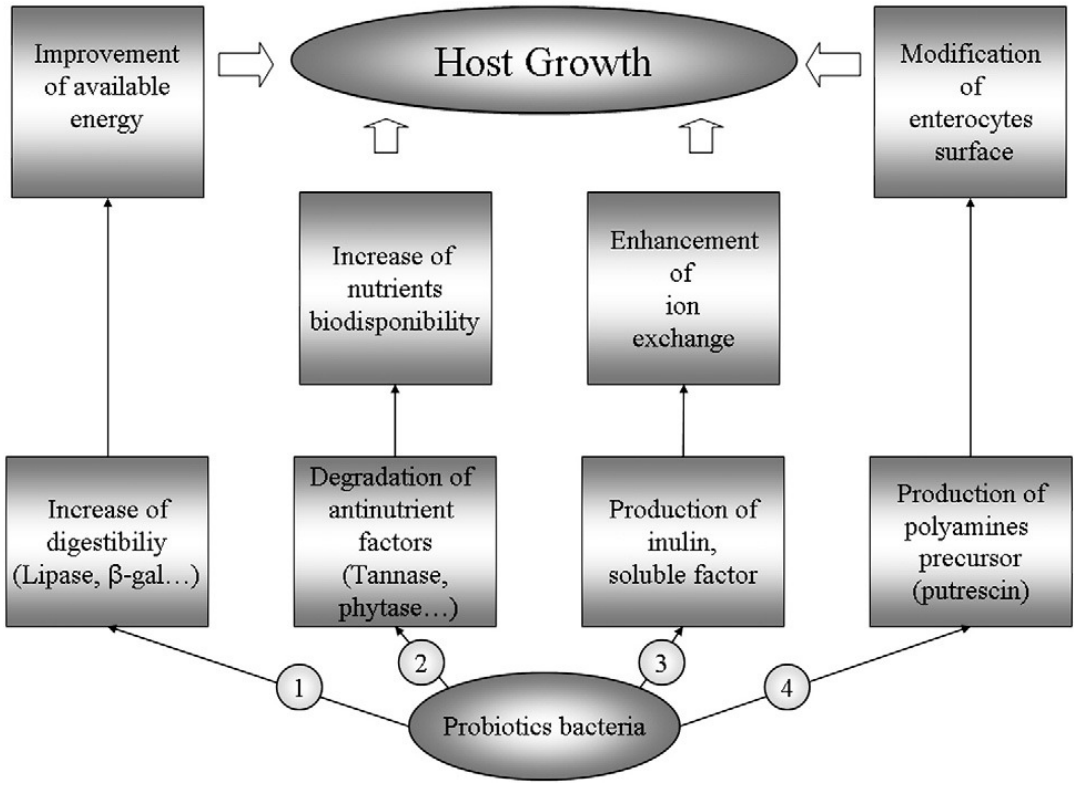


Figure 7: Probiotics can improve host growth by increasing biodisponibility of macronutrients (1), degradation of antinutrient factors (2), enhancement of mineral absorption (3), and production of growth factors (4). The other squares indicate the mechanism involved in host growth.

While this demonstrates the potential significance of this emerging field, much still remains to be done to standardize which strains actually fulfill the criteria of true probiotic microorganisms and must be undertaken additional investigations before their role in intestinal health can be delineated clearly . Turpin et al (2010 International Journal of Food Microbiology) have reviewed current knowledge of some probiotic functions including nutritional physiology, disease prevention, stimulation of the immune system and protection against pathogens in relation with the type of strain, the molecular determinants and the mechanisms involved, when the information is available. For the other functions. See also a number of recent reviews (Goh and Klaenhammer, 2009; Kleerebezem and Vaughan, 2009; Lebeer et al., 2008).

III-2) PROBIOTICS AND SURVIVAL OF GASTROINTESTINAL MICROFLORA

The benefit of probiotics is based on the survival of these bacterial or yeast cultures in thegastrointestinal tract and the resulting effects they might generate to overall health. Having established that some ingested probiotics can affect the composition and behavior of intestinal microflora, it is important to explore what is the factors that determine the survival of probiotics while in transit. Studies performed in vivo and in vitro show that about 10–30% of probiotics survive during gastrointestinal transient. This depends on a number of variables, including the type of probiotic.(Marteau P,1997 Pochart P,1992).. It was determined that the organisms most resistant to stomach acid were *B. bifidum* and *Lactobacillus bulgaricus*, with a half-life of _140 min. *Streptococcus thermophilum* and *L.acidophilus* had a half-life of _40 min.

Several other characteristics essential for probiotics are adherence to cells, gastric acid and bile stability, production of antimicrobial substances, and activity against pathogenic bacteria (Elmer GW et al, 1996).

Probiotics in the gut must be able to colonise and proliferate and exert a beneficial effect on the host They should also possess good organoleptic properties and be phage resistant (Mattila-Sandholm et

al, 2002). In conclusion To be a successful probiotic, the bacteria must fulfil some criteria for example :Be safe (e.g. of human origin and non-pathogenic), Be resistant to technologic processes and exert minimal sensory influence on the probiotic food, Be resistant to passage through the gastrointestinal tract (gastric acidity and bile acids), and Provide health benefits.

The probiotic organism must reach the intestines, in particular the colon, in sufficient numbers per serving in order to influence the microbiota significantly; often the figure 108 is considered minimal. From many clinical studies, our knowledge of the health benefits of probiotic foods is increasing rapidly, in particular the effects on constipation, diarrhoea, colon cancer, food pathogens, the immune system, cardiovascular diseases (serum cholesterol), lactose malabsorption and gastric ulcer (Table 3).

Reported effects	**Probiotic species**
Modulation of immune system	*L. acidophilus, L. casei, L. plantarum, L. delbrueckii, L. rhamnosus*
Balancing of gut microbiota	*L. acidophilus, L. casei, Bifidobacterium bifidum*
Reduced carcinogens (enzymes)	*L. acidophilus, L. casei, L. gasseri, L. delbrueckii*
Antitumour	*L. acidophilus, L. casei, L. gasseri, L. delbrueckii, L. plantarum, B. infantis, B. adolescentis, B. bifidum, B. longum*
Prevention of traveller's diarrhoea	*Saccharomyces spp., mixture of L. acidophilus, B. bifidum, Streptococcus thermophilus, L. bulgaricus*
Prevention of rotavirus diarrhoea	*L. rhamnosus, B. bifidum*
Prevention of C. difficile diarrhoea	*L. rhamnosus, S. spp.*
Prevention of other diarrhoea	*L. acidophilus, L. rhamnosus, B. bifidum*

Table 3 - Reported health effects of probiotic bacteria (reference Finn Holm, 2001)

III-3) IMPACT OF PROBIOTICS ON THE GUT HEALTH

III-3-1) INFLAMMATION (IBD)

In the search for probiotics to effectively treat certain inflammatory bowel diseases (IBD), it is essential to have appropriate selection tools and not rely solely on in vitro methods. The selected strains also need to be tested for safety, especially when the host's intestinal mucosa is damaged. For these purposes, a laboratory of the Institut Pasteur in Lille (France) has developed and tested a mouse model of TNBS-induced colitis (Daniel C et al, 2006).

Three species of *lactobacillus* (*L. plantarum Lp-115, L. salivarius Ls-33 and L. acidophilus NCFM*), previously selected in vitro for their probiotic potential and their persistence in the digestive tract, were administered to healthy mice and to mice presenting severe or very severe colitis. A non-probiotic strain of *L. paracasei subsp paracasei* (Lpp) isolated from the blood of a patient suffering from endocarditis was also used in the experiments. To evaluate inflammation, macroscopic scores (0 to 10) were determined using Wallace's criteria and myeloperoxidase activity was measured. The safety of the strains was assessed by selectively counting the bacteria present in the mesenteric lymph nodes, spleen, liver and kidneys. The animals' weights and mortality were also monitored.

Whatever the degree of severity of the colitis, only the *L. salivarius* strain showed a significant reduction in the inflammation score. These results were in contrast with those for the Lpp strain, responsible for Osler's disease, which exacerbated the colitis and spread to the other organs.

This result illustrates the importance of conducting in vivo tests before making any assumptions about the properties of a potential probiotic selected in vitro. In all cases, it is still essential to verify whether the strain also expresses its properties in humans. The study also shows that certain strains of probiotics can translocate in conditions of extreme inflammation. While these conditions probably cannot be transposed to human subjects, the authors nevertheless recommend choosing

probiotic strains whose safety profile has been assessed, especially if they are to be used for patients in a critical condition.

III-3-2) PROBIOTICS AND INTESTINAL BARRIER

ACTION OF VSL#3 STIMULATES MUCUS SECRETION IN THE RAT

The probiotic cocktail VSL#3 was assessed for its effects on the protective mucus layer of the intestine of the rat (Caballero et al, 2007). After 7 days' intragastric administration of the bacterial preparation, mucus secretion was measured by evaluating incorporation of tritiated glucosamine in the luminal glycoproteins of the animals' colons. Expression of mucin genes (muc2 in particular) in colonic cells was also quantified using amplification methods (RT-qPCR). The results show that the animals fed with the probiotic preparation produced a 60% increase in mucin secretion compared to the control animals. Expression of the muc2 gene was also increased by a factor of 5.

For more precise observation, the authors then put colonic epithelial cells(1) in culture in contact with the mixture of bacteria that make up VSL#3 and with each of the bacteria concerned, separately. Under these conditions, no mucin secretion was observed. However, both the VSL#3 culture medium and the media from each individually cultured species caused an increase in mucin secretion. Thus the culture media of each of the four *lactobacillus* species used in the probiotic cocktail prove as effective as the VSL#3 medium itself, while the three *bifidobacterium* species and *Streptococcus salivarius* show less marked stimulation abilities. Paradoxically, in the conditions of the experiment mucin secretion was not accompanied by an increase in the expression of the muc2 gene. Additional tests were conducted to try to identify the molecular factor in VSL#3 that is responsible for inducing mucin secretion. This factor proves to be resistant to temperature (15 mins boiling), to proteases and to DNase I. The authors conclude that it could be a lipoprotein or a polysaccharide.

AN ACTION MECHANISM OF ESCHERICHIA COLI NISSLE 1917

Escherichia coli Nissle 1917 (EcN) has been used as a probiotic strain for decades. This strain can prevent diarrhoea caused by infections due to pathogens and studies suggest it is effective in the treatment of certain inflammatory diseases of the digestive tract. However, few experimental studies have looked for the cellular and molecular mechanisms underlying the beneficial effects of this probiotic.

A German team (Zyrek AA et al, 2007) investigated the molecular response of epithelial cells in culture with or without the presence of EcN and/or an enteropathogenic strain of *E. coli* (EcEP). They particularly focused on the expression and cellular localization of two proteins: ZO-2, which interacts with the membrane proteins of the tight junction and so helps to maintain the cohesion of the epithelial cells; and an isoform of protein kinase C (PKCz) which, by phosphorylation, causes ZO-2 to detach from the protein complex of the tight junction, so destabilizing the epithelial barrier. When epithelial cells were incubated in the presence of EcN, the quantity of mRNA of ZO-2 and the protein's level of expression increased. ZO^{-2} also concentrated at the points of contact between cells, suggesting that the protein had associated with the tight junction complex. Conversely, when cells were in the presence of EcEP, the expression of ZO^{-2} declined and its subcellular distribution was significantly different. At the same time, the expression, activity and localization of PKCz showed the opposite trends to those of ZO^{-2}. When epithelial cells were incubated in the presence of both EcEP and EcN, the levels of expression and the localization of the proteins studied were comparable with those in the experiments with EcN alone. The experiments thus show that the probiotic neutralizes the deleterious effects of the pathogenic strain by preventing it from destabilizing the tight junction. Additional results further show that the probiotic is capable of restoring the integrity of the epithelial barrier in cells already infected with the bacterial pathogen.

III-3-3) PROBIOTICS AND IMMUNOMODULATION

Current probiotics are naturally occurring indigenous microbes that are aimed to restore lost bacteria or metabolic activities in colonized organs, restore a balanced immune response similar to that induced by the usual indigenous microbiota, or to suppress pathogenic microbes (Kalliomaki and Isolauri 2003). The scope of probiotics being used is narrow: several intestinal species predominate, including *Bifidobacterium* and *Lactobacillus* species, *Streptococcus thermophilus*, *Enterococcus* and *Bacillus* species, *E. coli* and yeasts, including *Sacharomyces boulardii*. Preliminary studies indicate that probiotics can affect innate immunity, as evidenced by oral tolerance, which cannot be achieved in germ-free animals (Sudo, Sawamura et al. 1997), is reduced by antibiotic use (Bashir, Louie et al. 2004), and can be restored by administering probiotics (Braat, van der Brande et al. 2004). Probiotics also protect against pathogens by strengthening the intestinal mucosal (immune) barrier (Figure8), decreasing pathogen adherence (Mack, Michail et al. 1999), or by production of acid or antibiotics inhibitory to pathogens (Gibson, Mc Cartney et al. 2005). More of scientific understanding of currently used probiotics is limited; their beneficial effects are usually low, with strong placebo effects, and a substantial lack of robustness across experiments. Modern era lifestyles are impacting human microbiome in ways we they are just beginning to elucidate. Its might need to have a probiotic in the future, but first, well-designed clinical trials are needed.

Rather than directly inhibiting the growth or viability of the pathogen, probiotics may compete for an ecological niche or, otherwise, create conditions that are unfavorable for the pathogen to take hold in the intestinal tract. There are many possible mechanisms for how pathogen exclusion may take place. First, several probiotics have been demonstrated to alter the ability of pathogens to adhere to or invade colonic epithelial cells in vitro, for example, evidences studies reported by (K. C. Johnson-Henry et al, 2007, R. R. Spurbeck et al, 2008). Second, probiotics could sequester essential nutrients from invading pathogens and impair their colonization ability. Third, probiotics may alter the gene expression program of pathogens in such a way as to inhibit the expression of

virulence functions (M. J. Medellin-Pe˜na et al, 2007). In the last, probiotics may create an unfavorable environment for pathogen colonization by altering pH, the mucus layer, and other factors in the local environment.

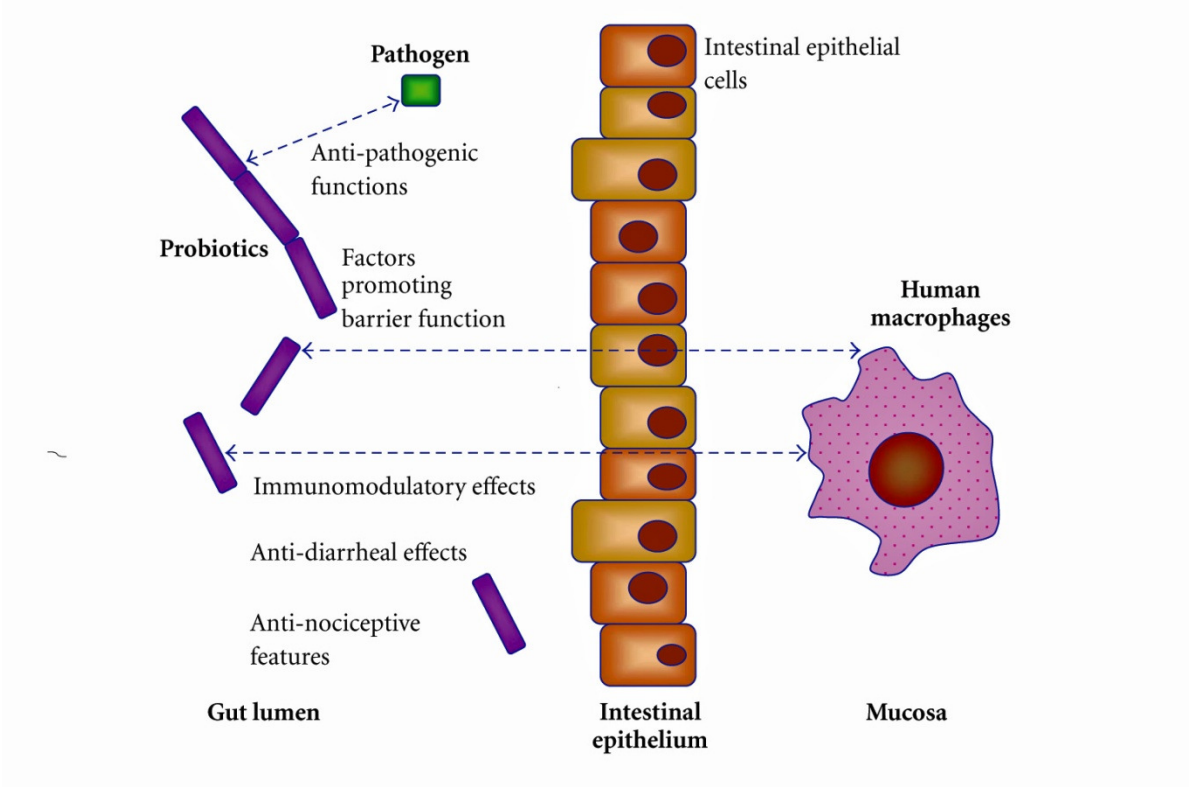


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Figure 8: *Probiotics and Beneficial Effects in the Intestine*. Depiction of the interactions between beneficial bacteria (left side), their secreted factors, pathogens, and the intestinal mucosa (right side). Potential beneficial effects of probiotics are listed. Only two host cell types are shown, intestinal epithelial cells and macrophages although other cell types including dendritic cells, lymphocytes, myofibroblasts, and neutrophils comprise the intestinal mucosa. The arrows indicate the release and possible distribution of secreted factors derived from probiotics. demonstrated.
(Figure reference, R. A. Britton and J. Versalovic; Hindawi Publishing Corporation Interdisciplinary Perspectives on Infectious Diseases Volume 2008, Article ID 290769, 10 pages doi:10.1155/2008/290769)

III-3-4) PROBIOTICS AND CANCER

Probiotics have been found by several researchers to decrease fecal concentrations of enzymes and secondary bile salts, and reduce absorption of harmful mutagens that may contribute to colon carcinogenesis (Rafter JJ et al, 1995)

Certain probiotics may protect the host from carcinogenesis activity: exemple: *L. acidophilus* and *L. casei* supplementation in humans helped to decrease levels of the enzymes involved in the

carcinogenesis, as shown (Hayatsu H et al, 1993;Lidbeck A et al,1992) In animal studies , administration of *Lactobacillus GG*.45, the suppressed the activity of enzymes involved in the tumurogenesis. Burns and Rowland(2000) suggested that increasing the amount of lactic acid bacteria in the colon decreases the ability of microbiota to produce carcinogens. A randomized, controlled study by Aso and Akazan57 of 48 Japanese patients demonstrated that the recurrence of bladder tumors was delayed with daily intake of *L. casei.*. In an animal model with dimethylhydrazine (DMH)-induced colon cancer, it was shown that *Lactobacillus* GG significantly reduced the incidence of colon tumors (Goldin BR et al, 1996) A review article by Vanderhoof (2001) describes a study using two carcinogens to test the effects of probiotics on the prevention of DNA damage in an animal model. Several probiotic strains were tested in this study, including *L. gasseri, L. confusus, S.thermophilus, B. breve, B. longum,* and *L. acidophilus.* All of the strains showed an antigenotoxic effect after MNNG administration (Vanderhoof JA et al, 2001). Similar studies have shown that pretreatment with *L. acidophilus, L. confusus, L. gasseri, B. longum,* and *B. breve* inhibited DNA damage from DMH, but that only one of four *S. thermophilus* strains and only one of three *L. delbrueckeii* strains were protective (Rafter JJ et al, 1995). In vitro data suggest that some lactobacilli may have a protective effect against the multi-step process leading to carcinogenesis (Figure 9) but they can also have adverse effects. Some lactobacilli can modulate enzymes involved in the xenobiotic pathway. Lb. fermentum I5007 has been shown to be able to increase the level of detoxifying glutathione S transferase (GST) in Caco-2 cells (Yang et al., 2007). Several mechanisms have been proposed as to how lactic acid bacteria may inhibit colon cancer; these include: enhancing the host's immune response, altering the metabolic activity of the intestinal microflora, binding and degrading carcinogens, producing antimutagenic compounds, and altering the physiochemical conditions in the colon (Hirayama K et al, 2000).

The result was a decrease in fecal pH and cell proliferative activity in the upper colon(Biasco G et al, 1991). The mechanisms of the links of probiotics to antitumor activity are not completely clear, but offer useful potential material for future cancer studies.

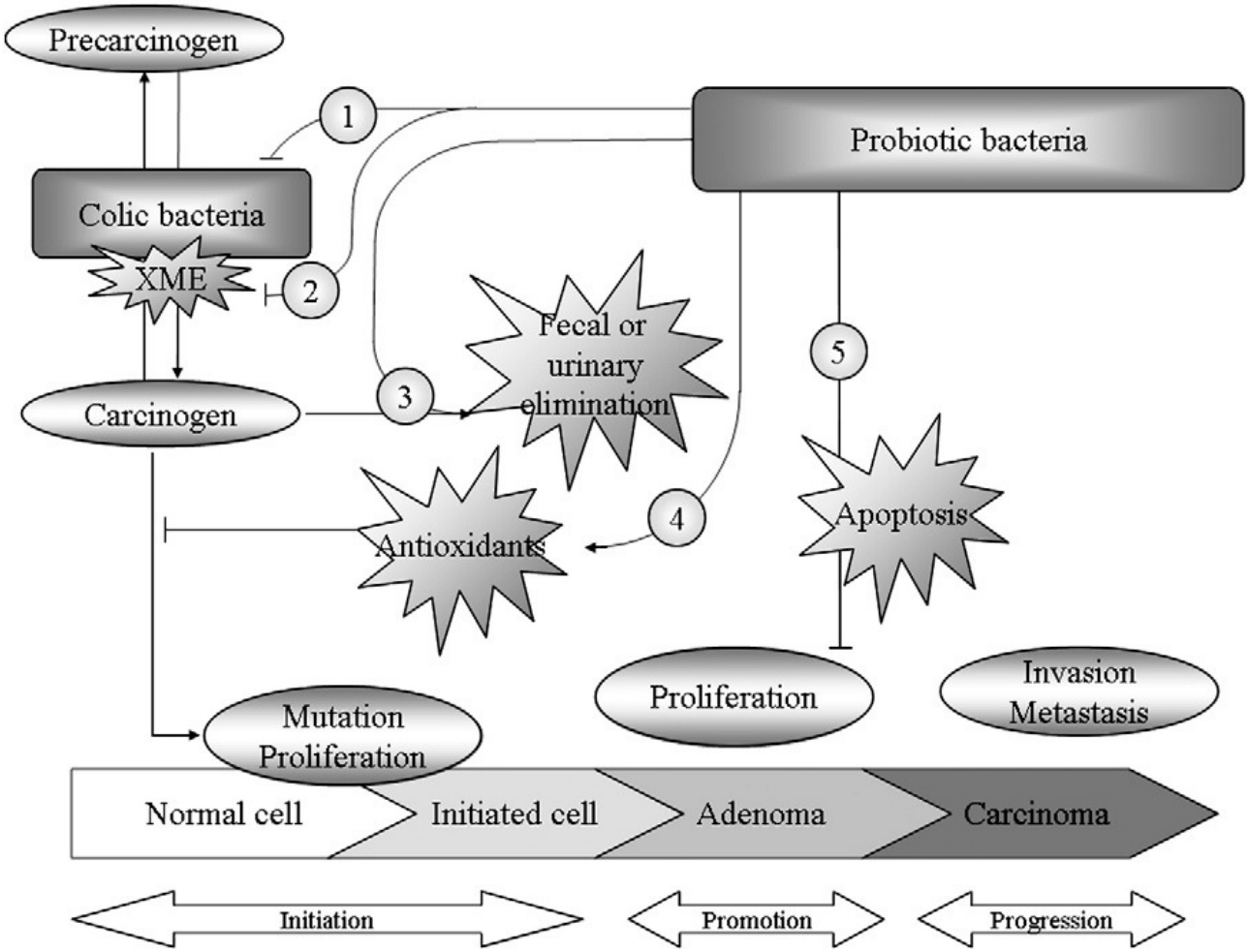

Figure 9: Carcinogenesis and action of probiotics: inhibition of enzymatic activities of colonic bacteria (1), modulation of xenobiotic metabolizing enzymes (XME) (2), enhancement of carcinogen elimination (3), prevention of mutation through antioxidant production (4), augmentation of apoptosis of abnormal cells (5) (Turpin et al, 2010).

III-3-5) OTHER EFFECTS OF PROBIOTICS

A LACTOBACILLUS STRAIN TO MAKE UP VITAMIN B DEFICIENCIES

A clinical study was performed by Mohammad MA et al (2006) to investigate the impact of a milk fermented by *Lactobacillus acidophilus* on the vitamin status of Egyptian children.

Two groups were formed, each with 12 subjects. For 6 weeks, one group (test group) received the probiotic fermented milk while the second group (control group) consumed an equivalent quantity of commercial yoghurt. Various measure of vitamin B12 and vitamin B9 in the start and in the end of experiment were performed. The results show that consuming the probiotic for 6 weeks, 1 child in 12 presented a vitamin B12 deficiency and only 1 was deficient in vitamin B9. Measurements of methylmalonic acid(biomarker for deficiency of vitamine B12 and B9) and total homocysteine

(biomarker for difecency of vitamine B12) in the blood or urine of group by *Lactobacillus acidophilus* also indicated a significant reduction in these two metabolites at the end of the protocol. Results indicate an improvement in the vitamin status of subjects consuming probiotic fermented milk.

The results of this study contrast with those of two earlier clinical trials which did not show any significant effects of probiotic products on vitamin status (Donaldson MS (2000); Elmadfa I, 2001). While the new results are encouraging, they need to be confirmed. The authors suggest several other parameters it seems to be important to take into account in this type of study, including the quantity and viability of the probiotic and the initial nutritional status of the subjects.

CHOLESTEROL

Forty-four adults with total serum cholesterol ≥ 4 mmol.L-1 were subjects in a 10-week clinical trial designed to assess the impact of capsules of Lactobacillus fermentum on their levels of low-density lipoproteins (LDL) (Simons LA et al, 2006). A non-significant reduction in LDL levels was observed in the patients treated with L. fermentum, but also in the placebo group.

Moreover, no variation was observed in total cholesterol, high-density lipoproteins (HDL) or triglycerides. Contrary to preliminary observations in animals, this study did not reveal any anticholesterolemic action by this strain of *L. fermentum* in humans.

Animal studies have demonstrated the efficacy of a range of LAB to be able to lower serum cholesterol levels, presumably by breaking down bile in the gut, thus inhibiting its reabsorption (which enters the blood as cholesterol). Some, but not all human trials have shown that dairy foods fermented with specific LAB can produce modest reductions in total and LDL cholesterol levels in those with normal levels to begin with, however trials in hyperlipidemic subjects are needed.(Sanders ME, 2000)

IV) YOGHURT AS FUNCTIONAL FOOD

Immunologist lie Metchnikoff, spoke highly about the possible health benefits of the lactic acid-bacteria (LAB). He wrote in his book,"*The Prolongation of Life",*that consumption of live bacteria, such as *Lactobacillus bulgaricus* and *Streptococcus thermophilus*, in the form of yogurt was beneficial for gastrointestinal health, as well as for health in general, and for longevity.

According to current scientific concepts, yoghurt cultures are probiotics if a beneficial physiological effect can be obtained by consumption of the live cultures and the benefit has been substantiated appropriately in human studies. All *S. thermophilus* and most *L. bulgaricus* strains have a high lactase activity (Sanders et al. 1996). It is well recognized that yoghurt consumption improves lactose digestion and eliminates symptoms of lactose intolerance. The physiological effects have been clearly demonstrated in a large number of human studies in which consumption of yoghurt (with live cultures) has been compared with consumption of a pasteurized product (with heat-killed bacteria) (Gilliland & Kim, 1984; Savaiano et al.1984; McDonough et al. 1987; Dewit et al. 1988; Lerebours et al. 1989; Pochart et al. 1989; Marteau et al. 1990; Varela-Moreiras et al. 1992; Rizkalla et al. 2000; Labayen et al. 2001; Pelletier et al. 2001).

All studies have shown better lactose digestion and absorption in subjects who consumed yoghurt with live cultures, as well as reduction of gastrointestinal symptoms. The benefit on lactose absorption was also demonstrated in healthy subjects without lactose mal digestion (Rizkalla et al. 2000). All these studies highlight the essentiality of live bacteria for the beneficial effect on lactose digestion (not excluding that other beneficial effects can be due to non-viable bacteria). There are no major scientific discrepancies on this issue fully established by human intervention studies.

The functional properties of yoghurt are consistent with further evidence obtained in important ancillary studies that confirmed viability and metabolic activity of yoghurt bacteria in the human intestine (Martini et al. 1987; Pochart et al. 1989; Marteau et al. 1990), as well as in in vivo animal models (Lick et al. 2001; Drouault et al. 2002). Yoghurt bacteria can also be detected in faeces of human subjects consuming yoghurt (Brigidi et al. 2003; Callegari et al. 2004).

Yoghurt is also being used in the management of acute diarrhea disorders, as recommended by World Health Organization (1995). This recommendation is based on the traditional approach in many countries all over the world, as well as on evidence gained in human intervention studies (Boudraa et al. 1990, 2001). Yoghurt feeding in children with acute watery diarrhoea decreased stool frequency and shortened the duration of diarrhea episodes (Boudraa et al. 2001). Other studies have addressed the role of yoghurt on the immune system. Yoghurt consumption may enhance the immune response particularly in immunocompromised populations, such as the elderly (Meydani & Ha, 2000). The role of yoghurt in the modulation of the immune system was further demonstrated by Van de Water et al. (1999) in a randomized controlled trial with human subjects. Long-term consumption of yoghurt, as compared with either the same product heat-treated after fermentation or exclusion of yoghurt products from the diet during the length of the study (1 year),was associated with a significant decrease in allergic symptoms.

V) GENERAL CHARACTERISTICS OF *STREPTOCCOCUS THERMOPHILUS*

Streptococcus thermophilus is a major dairy starter used in the manufacture of yoghurt and sheese. For cheese-making, *S. thermophilus* is used alone or in combination with several lactobacilli or mesophilic starters while in yoghurt it is always cocultured with *Lactobacillus delbrueckii* ssp. *Bulgaricus* (*L. bulgaricus*) (Tamime et al, 1999). *S. thermophilus* is closely related to *L. lactis*, but it is even more closely related to other streptococcal species including several pathogens (Mitchell, T. J.et al, 2003). Recently, the complete genome sequence of three strains became available (Bolotin, A. et al, 2004; Hols P. et al,2005; Makarova, K. et al, 2006). Comparison of *S. thermophilus* genome sequences with published genomes of streptococcal pathogens reveals that the most important determinants for pathogenicity are either absent or present as pseudogenes and that *S. thermophilus* has followed an evolutionary path divergent to that of pathogenic species due to its adaptation to milk (Bolotin, A. et al, 2004) *S. thermophilus* is highly adapted to grow on lactose, the main carbon source in milk and rapidly converts it into lactate during growth. Lactose is

transported into the cell by a lactose permease (Foucaud, C. et al, 1992). Lactose is efficiently transported into the cell and subsequently hydrolyzed by an intracellular βgalactosidase.. The milk is poor in free amino acids (AA) and short peptides (Desmazeaud, M. J. et al, 1990), therefore for optimal growth, *S. thermophilus* requires either hydrolysis of caseins followed by the internalization and the degradation of the resulting peptides (Hols, P. et al,2005). For many LAB including *S. thermophilus*, the hydrolysis of milk caseins (*i.e*. the amino acid supply) mostly depends on the activity of a cell-wall-anchored proteinase. Few strains of *S. thermophilus* possess this proteinase (Shahbal, S. et al, 1991); they grow rapidly in milk and reach up to 109 cell/mL, when they are proteinase-minus, they only reach a cell density of about 108 cell/mL (Letort, C. et al, 2002). For the internalization and degradation of peptides, *S. thermophilus* posses an efficient oligopeptide transport system (Garault, P. et al, 2002) and a set of intracellular peptidases (Rul, F. et al, 1997). Although, some strains of *S. thermophilus* are prototroph for amino acids, others are auxotrophs for given amino acids. The importance of amino acids biosynthesis for the growth in milk was established (Hols, P. et al, 2005) and the branched-chain amino acids biosynthesis pathway was identified as a key pathway for optimal growth of *S. thermophilus* in milk (Garault, P. et al, 2000).

Whitford, E.J. et al (2009) study has shown very positive results when *Streptococcus thermophilus* TH-4 was used to treat rats with mucositis caused by chemotherapy drugs. Rats responded to the treatment by showing a normalization of healthy cell function in the affected areas and a significant reduction of distress to the tissue of the intestines.As research continues to produce varied strains of Streptococcus thermophilus, the usefulness of this probiotic seems limitless. Its effects on digestive and overall health have already been widely documented. Its benefits to cancer patients undergoing chemotherapy are beginning to be understood. Streptococcus thermophilus is one of the many reasons probiotics are changing the health industry (U.S. Department of Energy Office of Science, 2009). Recent data indicate that strains related to *S.thermophilus* LMD-9 (Strain used in this study) are among the 57 bacteria species found in 90% of 124 European individuals (Qin J. et al, 2010). In comparison with the overall human intestinal microbiota, *S.thermophilus* is numerically non

dominant species with high variable levels (Qin J. et al, 2010; Mater D. et al, 2005). At birth, *Streptococcus* genus -with in some studies a precision at the level of *S. thermophilus* species- is among the first coloniser of the GIT, since it has been detected in infant faeces and breast milk (Moreau M. et al, 1986- Perez .P. F. et al, 2007). Thus, *Streptococcus*, as pioneer bacteria colonising a yet immature GIT, may impact the maturation and homeostasis of intestinal epithelium after birth. In order to understand the interplay between lactic acid bacteria and host, recent study (Françoise Rul et al, 2010;submitted) on the gnotobiotic rats colonized by *Streptococcus thermophilus* have shown that: - after progressive adaptation in GIT rat the main response *Streptococcus thermophilus* was the massive induction of the glycolysis pathway leading to formation of lactate in the caecum while in the colonic epithelium of the host there's an abundance of $p27^{kip1}$ Protein that is protein involved in the cell cycle arrest.

VI) AIM OF THESIS

The prospects of these work is to understand molecular mechanisms by which the microbiota influence homeostasis of the colonic epithelium using bacterial populations and simplified using strains probiotic lactic acid bacteria as *streptococcus thermophilus* strain LMD9.

The strategies that we have adopted to answer at this goal :

1) Generation of mono-associated rats with *Streptococcus thermophilus* by oral administration and the integrative study of the crosstalk established between the bacterium and the host. "Impact of the metabolic activity of S*treptococcus Thermophilus o*n the colon epithelium of gnotobiotic rats". (paper submitted /JBC).
2) Transcriptomic study using Supressive subtractive hybridization (SSH) technology to investigate genes differentially expressed in colonic epithelium rat in the presence of *Streptococcus thermophilus.*

"Genes induced in vivo by S*treptococcus Thermophilus* using supressive subtractive hybridization (SSH) thecnology"

3) Large-scale molecular analysis of colonic epithelium in mono-associated vs. germ free rats using a microarray technology. Therefore, microarrays may be used to link molecular events with physiological response and identify critical genes and biological pathways.

"Microaerray based indentification of colonic epithelium genes differentially expressed in the presence of S*treptococcus Thermophilus* bacteria"

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CHAPTER II

Impact of the metabolic activity of *Streptococcus Thermophilus* on the colon epithelium of gnotobiotic rats.

(PAPER)

IMPACT OF THE METABOLIC ACTIVITY OF *STREPTOCOCCUS THERMOPHILUS* ON THE COLON EPITHELIUM OF GNOTOBIOTIC RATS.

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ABSTRACT

The thermophilic lactic acid bacterium *Streptococcus thermophilus* is widely and traditionally used in dairy industry. Despite the vast level of consumption of *S. thermophilus* through yoghurt or probiotic functional food, very few data are available about its physiology in the gastro-intestinal tract (GIT). The objective of the present work was to explore both *S. thermophilus'* metabolic activity and host response *in vivo*. Our study profiles the protein expression of *S. thermophilus* after its adaptation to the GIT of gnotobiotic rats and describes the impact of *S. thermophilus* colonization on the colonic epithelium. *S. thermophilus* colonized progressively the GIT of germ-free (GF) rats to reach a stable population in 30 days (10^8 CFU/g faeces). This progressive colonisation suggested that *S. thermophilus* undergoes an adaptation process within GIT. Indeed, we showed that the main response of *S. thermophilus* in the rat's GIT was the massive induction of the glycolysis pathway leading to formation of lactate in the caecum. In the colonic epithelium, the abundance of $p27^{kip1}$ protein involved in the cell cycle arrest increased in the presence of *S. thermophilus* compared to GF rats. Based on different mono-associated rats harbouring two different strains of *S. thermophilus* (LMD-9 or LMG18311) or weak lactate producing commensal bacteria (*Bacteroides thetaiotaomicron* and *Ruminococcus gnavus*), we propose that lactate could be a signal produced by *S. thermophilus* inducing $p27^{kip1}$ in the colon.

I) INTRODUCTION

Streptoccocus thermophilus (S. thermophilus) belongs to the group of the thermophilic lactic acid bacteria and is traditionally and widely used as a starter in manufacturing dairy products (Emmental, Gruyere, Parmigiano, Mozarella, Yoghurt etc). Yoghurt, which results from the fermentation of milk by *S. thermophilus* and *Lactobacillus delbrueckii* sp. *bulgaricus (L. bulgaricus)*, fulfils the current specifications required to be recognized as a probiotic product (Guarner, F. et al, 2005). The health beneficial effect of yoghurt consumption is linked to the metabolic properties of *S. thermophilus* and *L. bulgaricus.* As such, it improves lactose digestion in the gastro-intestinal tract (GIT) through their lactose hydrolysing activity present in yoghurt and in the GIT, thus reducing symptoms of lactose intolerance (Lomer, M. C. et al,2008; Rabot, S. et al, 2010).

Yoghurt cultures were shown to induce other health benefits such as reduction of diarrhoea or allergic disorders as well as modulation of the immune system (Guarner, F. et al, 2005; Higashikawa, F. et al, 2010). *S. thermophilus* is also present at high concentration in VSL#3, a probiotic mixture of eight different bacterial strains, that possesses beneficial effects in several intestinal conditions (Pagnini, C et al, 2010; Preidis, GA. et al, 2009).

Recent data indicate that strains related to *S. thermophilus* LMD-9 are among the 57 bacteria species found in 90% of 124 European individuals (Qin, J. et al, 2010). In comparison with the overall human intestinal microbiota, *S. thermophilus* is numerically non dominant species with high variable levels (Qin, J. et al, 2010; Mater, D. D. et al, 2005). At birth, *Streptococcus* genus -with in some studies a precision at the level of *S. thermophilus* species- is among the first coloniser of the GIT, since it has been detected in infant faeces and breast milk (Moreau, M. C. et al, 1986; Perez, P. F. et al, 2007). Thus, *Streptococcus*, as pioneer bacteria colonising a yet immature GIT, may impact the maturation and homeostasis of intestinal epithelium after birth.

Convergent data show that intestinal bacteria modulates proliferation and differentiation processes of the intestinal epithelium (Alam, M. et al, 1994; Willing, B. P. et al, 2007), which is one of the most dynamic tissues of the whole organism. Moreover, we have recently demonstrated that microbiota increases colonic epithelium crypt depth in concordance with a well orchestrated modulation of the level of proteins involved in cycle life steps like PCNA, Bcl2, and $p21^{cip1}$, $p27^{kip1}$ (markers of proliferation, anti-apoptotic pathway and cell cycle arrest, respectively) (Cherbuy, C. et al, 2010). Despite its large utilisation and consumption, its probiotic-associated traits and its presence in the intestinal microbiota, the role of *S. thermophilus* in the gut is still largely unknown. A previous study (Drouault, S. et al, 2002) showed that, in mono-associated animals, *S. thermophilus* produced an active β-galactosidase in the gut which is responsible for lactose hydrolysis. Therefore, the aim of the present study was to expand our view of *S. thermophilus'* metabolic activity in the intestinal environment and to investigate its relationship with the host. To do this, we assessed the behaviour of 2 sequenced strains of *S. thermophilus*, LMD-9 and LMG18311, in gnotobiotic rats. We established the first global protein profile of *S. thermophilus* after its survival in GIT in mono-associated rats. We also described how *S. thermophilus* influences crypt depths and cell cycle-related proteins of the colonic epithelium. In view of our findings using different mono-associated models, we propose that lactate resulting from the adaptive metabolic activity of *S. thermophilus* may serve as a biological signal to communicate with host epithelium.

II) EXPERIMENTAL PROCEDURES

II-1) BACTERIAL STRAINS, MEDIA, AND CULTURE CONDITIONS.

The strains *S. thermophilus* LMD-9 (ATCC BAA-491, USA), *S. thermophilus* LMG18311 (BCCM collection, Belgium), *L. bulgaricus* ATCC11842 (USA), *Bacteroides thetaiotaomicron* (BtII8) and *Ruminococcus gnavus* (FRE1) were used. Stock cultures of *S. thermophilus* LMD-9, *S. thermophilus* LMG18311 and *L. bulgaricus* ATCC11842 were prepared in reconstituted 10%

(wt/vol) Nilac skim milk (NIZO, Ede, the Netherlands) as previously described (Herve-Jimenez, L. et al, 2002). *S. thermophilus* monocultures and *S. thermophilus*/*L. bulgaricus* co-cultures were obtained by inoculating Nilac milk with 10^6 CFU/ml of stock cultures of each species and incubated at 42°C until pH 5.4-5.5. One ml of culture was used for rat gavage and the remaining was N-liquid frozen and stored at -20°C until protein extraction. The cultures were enumerated *a posteriori* by plating appropriate dilutions on M17 agar lactose (10g/L) for *S. thermophilus* or on acidified MRS agar lactose (20g/L) (pH 5.2) for *L. bulgaricus*. After 16 h (*S. thermophilus*) or 24 h (*L. bulgaricus*) incubation at 42°C under anaerobiosis (Anaerocult A, Merck, Darmstadt, Germany), colonies were counted.

II-2) ANIMALS AND EXPERIMENTAL DESIGN**.**

All procedures were carried out according to European and French guidelines for the care and use of laboratory animals (permission 78-123, dedicated to MT).

A the age of 2 months, germ-free (GF) rats (male, Fisher 344) were inoculated either with *S. thermophilus* LMD-9 (Ino-LMD9, n=11), *S. thermophilus* LMG18311 (Ino-LMG18311, n=4), or with a mix of *S. thermophilus* and *L. bulgaricus* (Ino-LMD9+Lb, n=5) according to the following protocol: 1 ml of a culture of *S. thermophilus* in Nilac milk ($5x10^8$ *S. thermophilus*/ml) or 1 ml of a co-culture with *S. thermophilus* ($2x10^9$ *S. thermophilus*/ml) and *L. bulgaricus* ($8x10^7$ *L. bulgaricus*/ml) were transferred to GF rat by oral gavage. As a control, GF rats were also inoculated with 1 ml of sterile Nilac milk (without bacteria). GF, mono-and di-associated rats were housed in sterile Plexiglas isolators (Ingénia, Vitry-sur-Seine, France). Rats mono-associated with either *Bacteroides thetaiotaomicron* (Ino-*Bt*), or *Ruminococcus gnavus* (Ino-*Rg*) were described in (Cherbuy, C. et al, 2010). All groups of rats received the same standard diet (UAR), which was sterilized by gamma irradiation. Throughout the experiment, and two times a week, *S. thermophilus*

(and *L. bulgaricus* in the case of Ino-LMD9+Lb) was enumerated by plating serial dilutions of the faeces. All rats were euthanized at three months old, *i.e*, 30 days after inoculation.

At 9:00h, rats were anesthetized with isoflurane, and tissues were removed. The colon was immediately used either for cell isolation or for histological procedures. The luminal content of jejunum, ileum, caecum and faeces were diluted in 10 volume of M17, vigorously vortexed with sterile glass beads (3.5 mm diameter) and bacteria were enumerated. All results were expressed as log10 (CFU/gram or ml).

II-3) SCANNING ELECTRON MICROSCOPY.

Scanning electron microscopy (20) analyses were performed on the MIMA2 plateform (http://mima2.jouy.inra.fr/mima2) with 0.2g of caecal samples being suspended in 1 ml 0.1 M Tris buffer (pH 7.5) and mixed. Debris were removed by centrifugation at 1000g for 5 min at 4°C. Supernatant of caecal samples were centrifuge to recover bacteria, the pellets were suspended in 0.1 M Tris buffer (pH 7.5) and 2-time washed in the same buffer by centrifugation at 5000 g for 5 min at 4°C. The bacterial pellets were suspended and fixed in 200 µl glutaraldehyde 3% ruthenium red and stored at 4°C. Scanning electron microscopy was performed as previously reported (Joly, F. et al, 2010).

II-4) PROTEIN EXTRACTION**.**

Bacteria were isolated as follows. 500 ml of frozen milk cultures (3 replicates) were homogenized with an Ultra-turrax (Bioblock, Paris, France). The suspension was centrifuged at 8000 g for 10 min at 4°C and 3-time washed in Na phosphate buffer (5 mM) containing 1 mM EDTA, pH 7; bacteria were suspended in 2 mL Na phosphate buffer (20 mM, pH 7). Frozen faeces from 3 Ino-LMD9 rats were pooled and 12g of faeces were suspended in 25 ml Na phosphate buffer (20 mM, pH 6.4), homogenized with an Ultra-turrax and faecal debris were removed by 2 successive centrifugations

at 400g for 5 min at 4°C. The bacteria were then collected by centrifugation at 5000 g for 10 min at 4°C, 3-time washed with Na phosphate buffer (20 mM, pH 6.4) and the pellets were suspended in 2 mL of this buffer. Bacterial pellets from milk cultures and faeces were then submitted to a high-speed centrifugation on a Nycodenz density gradient as previously described (Roy, K. et al, 2008). After ultracentrifugation, the bacterial suspensions were washed with 14 ml Na phosphate buffer (20 mM, pH 6.4) and the bacteria were recovered by centrifugation at 5000 g for 10 min at 4°C.

The bacterial pellets were suspended in 0.5 mL of Na phosphate buffer (20 mM, pH6.4) containing protease inhibitor cocktail (Sigma-Aldrich, St Louis, Mo), 40 U/ml catalase and 10 mM tributylphosphine (Applied Biosystems). Cells were mechanically disrupted with a FP120 FastPrep cell disruptor (Bio 101 Systems, Qbiogen, Irvine, Canada) by two 30s cycles of homogenization at maximum speed (6.5) at 1 min interval at 4°C. Supernatants were collected by centrifugation at 5000 g, 15 min at 4°C and centrifuged (200000 g, 30 min, 4°C); they correspond to cytoplasmic proteins whereas the pellet was a fraction enriched with envelope-associated proteins. The protein concentrations were determined by the Coomassie protein assay reagent (Pierce, Rockford, Ill) using bovine serum albumin as a standard.

II-5) 1-DE COUPLED TO LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY (LC-MS/MS) ANALYSIS.

Proteins of the cell-envelope enriched fraction (4.7 µg) from faecal sample were separated and identified by one-dimensional (1D) electrophoresis (SDS-PAGE) coupled with liquid chromatography-tandem mass spectrometry as previously described (Gardan, R. et al, 2009).

Comparative bi-dimensional (2-DE) protein analysis and image analysis. A volume of cytosolic fraction corresponding to 250 µg of proteins was treated as previously described (Derzelle, S. et al, 2005). Fractions of three independent cultures in milk were analyzed. Fractions of two faecal batches were used, each batch resulting from the pool of faeces of three rats. Isoelectric focusing

was performed with 24cm 4-7 pH IPG strip and 12% SDS-PAGE was used for the second dimension. The gels were stained with BioSafe colloidal Coomassie blue (Biorad) for 1h and rinsed out with three successive washes in deionised water. Gels were digitized using an Epson Expression 1640XL scanner set (at 256 grey levels) controlled by the Silver Fast software and analyzed using the Progenesis SameSpot software (Nonlinear Dynamics). The relative volume of each spot was obtained from its intensity and normalized with the intensity of all spots. Only spot volume differences of at least twofold between the milk and fecal samples were further analyzed. 276 different protein spots on pH4-7 2-DE gels were detected.

MS analyses were performed using a Voyager-DE-STR (Applied Biosystems, Framingham, USA) on the PAPSSO proteomic plateform (http://pappso.inra.fr). The proteins were identified using MS-FIT (http://prospector.ucsf.edu).

II-6) WESTERN BLOT ANALYSIS**.**

Colonic epithelial cells were isolated from the whole colon according to the method described by Cherbuy *et al.* (Cherbuy, C. et al, 1995). Cell pellet was immediately used for protein extraction. Western blot analysis was performed as previously described (17) by using a denaturing (SDS)–polyacrylamide gel. Proteins were analysed using anti-PCNA (GeneTex; diluted 1/1000), anti-$p21^{cip1}$ (Oncogene; 1/200 or Santa Cruz Biotechnology; 1/200), anti- $p27^{kip1}$ (Santa Cruz Biotechnology; 1/500). Several loading controls were used, either GAPDH, Skp1 or cullin1 (Cherbuy, C. et al, 2010). Signals imprinted on autoradiography films were quantified by scanning densitometry of the autoradiograph using Biovision 1000 and logiciel bio1D (Vilber Lourmat, France).

II-7) DOSAGE OF D- AND L-LACTATES**.**

D- and L-lactates were measured in caecal contents and faeces of GF and mono-associated rats by 2X- diluting of 50-100 mg samples in 0.1 M triethanolamine buffer (pH 9.15). Samples were

centrifuged at 13 000 g for 5 min at 4°C, the supernatant were precipitated with trichloroacetic acid (10%) and centrifuged at 4500 g for 20 min at 4°C. The lactates were measured in the supernatants with the Biosentec D/L lactic acid enzymatic kits according to the manufacturer instructions (Biosentec, Toulouse, France).

II-8) HISTOLOGY AND IMMUNOHISTOCHEMISTRY.

Colon samples were cut into 2 cm sections, fixed in 4% paraformaldehyde (4 hours, room temperature), dehydrated and embedded in paraffin according to standard histological protocols. Five micrometer sections were mounted on SuperFrost® Plus slides. Slides were stained with hematoxylin and eosin for histological analysis. Crypt depths were analyzed with ImageJ. Only U shaped longitudinally cut crypts with open lumina along the crypt axis were evaluated. Results were the mean obtained by analysis at least 30 crypts per rat (n=2).

II-9) RNA ISOLATION AND QUANTITATIVE RT-PCR ANALYSIS.

Total RNAs were extracted from colon epithelial cells of 6 rats (2 GF and 4 mono-associated with *S. thermophilus* LMD-9 strain) by the guanidinium thiocyanate method (Chomczynski, P. et al, 1987). The RNA yield was quantified by spectrophotometer analysis and the RNA purity was determined by Agilent 2100 Bioanalyzer analysis using the RNA 6000 Nano Assay Kit. Reverse transcription was performed with 1 µg of total rat RNA using iScript™cDNA Synthesis Kit (BioRad). Quantitative RT-PCR (RT-qPCR) was performed on the Opticon Monitor 3 (BioRad) using specific primers and Master Mix (2X) Universal (KAPA SYBR® FAST qPCR Kits) KAPA BIOSYSTEMS. Primers were $^{5'}$GGCGGCAAGATGTCAAACGTG$^{3'}$ and $^{5'}$GGGCCGAAGAGGTTTCTG$^{3'}$. Primers were validated to ensure efficient amplification of a single product at 60°C (annealing temperature). Relative mRNA levels were calculated using the comparative $2^{-\Delta\Delta Ct}$ method. Comparisons were made with samples from epithelial cells harvested from GF and mono-associated colon rats (2 GF animals and 4 Ino-LMD9) using GAPDH (glyceraldehyde-3-phosphate dehydrogenase) as the reference gene and $p27^{kip1}$ gene as target gene.

II-10) STATISTICAL ANALYSIS**.**

Results are presented as means ± SE for the number of animals indicated. Comparisons of group data between different batches of rats were performed using one–way analysis of variance (ANOVA) followed by Tuckey's student range test where appropriate. Significance was a P value lower than 0.05. Statistical analysis was performed using the JMP® software (version 7, SAS institute INC).

III) RESULTS

III-1) PROGRESSIVE ADAPTATION OF *S. THERMOPHILUS* TO THE GIT.

After a single gavage, *S. thermophilus* LMD-9 progressively colonized the GIT of germ-free rats leading to Ino-LMD9 mono-associated rats (Figure 1A). The implantation of *S. thermophilus* LMD-9 occurred in a 3-way step: i) initiation phase with implantation of 10^5 CFU/g; ii) growth period between weeks 2 and 3 where it reached a population of 10^8 and iii) maintenance phase (post third week). Thus, four weeks were necessary to reach a level of 10^8 CFU/g faeces. At day 30, the population of S. *thermophilus* LMD-9 was higher in the distal digestive compartment with an increasing gradient of population from jejunum ($1.6x10^3$CFU/ml) to colon ($9.3x10^8$ CFU/ml) (Figure 1B). The progressive implantation curve was also observed with another strain of *S. thermophilus,* LMG18311 (Ino-LMG18311 rats). In Ino-LMG18311 rats, the final implantation of LGM18311 was similar to that obtained with LMD-9, with $2x10^8$ CFU/g (n=4). To mimic the levels present in yogurt, GF rats were inoculated with a co-culture containing *S. thermophilus* LMD-9 10^9 CFU/ml and *L. bulgaricus* 10^7 CFU/ml, which is similar to the relative proportion of both strains in yoghurt. 24 hours after gavage, *L. bulgaricus* was undetectable in faeces whereas the implantation curve of *S. thermophilus* LMD-9 was similar to that observed with Ino-LMD9 (data not shown). The progressive implantation of *S. thermophilus* suggested that an adaptation of *S. thermophilus* occurred in the GIT.

It has been previously shown that the morphology of *Lactobacillus sakei* changed after its survival through the GIT (Chiaramonte, F. et al, 2009). Thus, the global morphology of *S. thermophilus* recovered from caecum of Ino-LMD9 rats was observed by scanning electron microscopy (Figure 1C). In caecum, *S. thermophilus* exhibited the expected ovococcus-shape chains and was present as dividing cells with visible septa.

III-2) PROTEOMIC PROFILES OF *S. THERMOPHILUS* IN THE DIGESTIVE TRACT.

In the faeces of Ino-LMD9 rats, proteins of *S. thermophilus* were analysed by LC-MS/MS, listed (Supplemental data) and classified into different functional categories (Figure 2). This result showed that the main *S. thermophilus* LMD-9 proteins expressed in the GIT are involved in staple cellular functions as translation, carbon metabolism, nitrogen metabolism or nucleic base metabolism. This indicated that *S. thermophilus* LMD-9 was metabolically active in the rat GIT 30 days after inoculation.

Using a comparative proteomic analysis, we then identified the proteins that were differentially produced when *S. thermophilus* LMD-9 was grown in milk (inoculum) or when it was recovered from faeces of Ino-LMD9 rats (day 30). In both conditions, bacterial populations were similar, *i.e.*, 1 to $5x10^8$ CFU/ml (Figure 1). 52 proteins displayed different abundances between the 2 conditions: 11 were up-regulated (induction from 2 to 11 fold) and 41 were down-regulated (repression from 3 to 25 fold) in faeces compared to milk (Table 1).

Of the 11 up-regulated proteins, 3 are related to environmental signal responses: SodA (superoxide dismutase) and GroEL which are stress proteins and EF-Tu (Elongation Factor Tuf), which is a translation/adhesion-related protein. The remaining up-regulated 8 proteins in the gut played a role in carbon metabolism, specifically in the glycolytic pathway. We have also verified whether these proteins involved in glycolysis were more abundant in faeces of Ino-LMG18311 than in milk (data

not shown). Thus, proteins involved in glycolysis that are already expressed at high level in milk (24) were over-expressed after a survival in GIT.

The proteins down-regulated in faeces compared to milk were involved in protein synthesis, cell division (FtsZ), nucleotide biosynthesis and salvage (PyrG, PurC, PurL, GuaB), energy providing (F0-F1 ATP synthase AtpD) and iron metabolism (Dpr bacterioferritin). Overall, the proteins of the 2 strains of *S. thermophilus* that were over-expressed in the GIT compared to milk are devoted to glycolytic pathway.

III-3) PRODUCTION OF LACTATE BY *S. THERMOPHILUS* IN THE DIGESTIVE TRACT

Considering the preponderant abundance of enzymes involved in glycolysis, L-lactate concentration was measured in caecum of gnotobiotic rats. Although no lactate (either D- or L-) was found in GF rats, 13.6 mM±0.9 and 9.8 mM±2.5 L-lactate was detected respectively in caecum and faeces of Ino-LMD9 rats. Note that only the L -form was detected in caecum of Ino-LMD9 rats as expected due to the well known capacity of *S. thermophilus* to produce this isoform. This production of L-lactate did not induce any acidification of the caecal content of Ino-LMD9 rats (mean pH 7.7±0.08, n=11; versus pH: 7.7±0.2, n=6 GF). Acetate, propionate and butyrate levels in Ino-LMD9 (respectively 1.8 mM±0.7, <0.1 mM, <0.1 mM) were not statistically different from that obtained with GF rats (1.7 mM±1, <0.1 mM, <0.1 mM). Similar caecal concentration of lactate was measured in Ino-LMG18311 rats (10.02±3.0 mM) and in Ino-LMD9+Lb rats (9.91±0.99 mM); thus lactate was found in GIT of mono-associated rats with two different *S. thermophilus* strains.

III-4) CROSSTALK BETWEEN *S. THERMOPHILUS* AND THE COLONIC EPITHELIUM.

The colonic tissue of Ino-LMD9 rats was structured in crypts (Figure 4A); with 201±13 µm crypt depth mean and the detection of bifurcating crypts (noted with arrows in figure 3A). Considering these two parameters, the global histological trait of colonic epithelium in Ino-LMD9 rats was

similar to that we have previously observed in GF rats (Cherbuy, C. et al, 2010). When comparing the amount of colon proteins involved in cell cycle between Ino-LMD9 and GF rats, we noticed that PCNA, Bcl2, $p21^{cip}$ were similar between GF and Ino-LMD9 rats (Figure 3B) whereas $p27^{kip1}$ abundance was significantly higher (1.8 fold induction) in the presence of *S. thermophilus* LMD-9. The induction of $p27^{kip1}$ was also observed in Ino-LMG18311 (2 fold) and Ino-LMD9+Lb (1.5 fold) (Figure 4). In contrast to the protein level, the $p27^{kip1}$ mRNA amount was not statistically different between GF (mean ΔCt: 4.8±0.3, n=3) and Ino-LMD9 rats (ΔCt: 4.7 ±0.1, n=4). Thus, the induction of protein $p27^{kip1}$ was observed *in vivo* with 2 different strains of *S. thermophilus* and did not result from a parallel induction at mRNA level. It has been previously shown in epithelial keratinocyte cell line that $p27^{kip1}$ is induced by lactate *in vitro* (Hsiao, Y. P. et al, 2009). This suggests that lactate accumulates in GIT of mono-associated rats leading to the induction of $p27^{kip1}$ protein. In order to demonstrate the preponderant role of lactate we have tried to inactivate the *ldh* gene encoding the lactate deshydrogenase responsible for lactate production. We have failed to generate this mutant (data not shown), confirming previous unsuccessful attempts to obtain this probably lethal mutant (Hols, P. et al, 2009). However, in rats mono-associated with *Bacteroides tethaiotaomicron* (Ino-*Bt*) or *Ruminococcus gnavus* (Ino-*Rg*) (Cherbuy, C. et al, 2010) containing 0 and 3.2±0.7mM of caecal lactate, respectively, no $p27^{kip1}$ induction was observed in comparison with GF (Figure 4).

IV) DISCUSSION

In order to gain a better understanding of the behaviour of lactic acid bacteria within the intestinal environment and the complex bacterium/host crosstalk system, here we combined *in vivo* characterization of *S. thermophilus* metabolism and the resulting host response. This study led to novel insights of the interplay between *S. thermophilus* and the host through its major metabolite product, lactate.

The implantation of *S. thermophilus* in GF rats occurred progressively and this trend was observed with both strains studied, LMD-9 and LMG18311. These 2 strains differ in particular by the presence of a cell-wall protease PrtS and a sortase A1 present in LMD-9 strain. This suggests that the latter proteins were probably not essential for the kinetic of implantation of this bacterium. In contrast to *S. thermophilus*, the implantation was maximal in few days for *E. coli* (Alpert, C. et al, 2009), *R. gnavus* and *B. thetaiotaomicron* (Cherbuy, C. et al, 2010), *Lactobacillus casei* and *Bifidobacterium breve* (Shima, T. et al, 2008) and *Lactococcus lactis* (Roy, K. et al, 2008). This adaptive response of *S. thermophilus* was not accompanied by significant morphological changes contrarily to what was observed with *L. sakei* or *E. coli* (Chiaramonte, F. et al, 2009; Giraud, A. et al, 2008). The proteomic analysis of *S. thermophilus* before- (milk inoculum) and after GIT transit (faeces) shed light on its adaptive metabolic profile. Indeed, we showed that the main response to the passage of *S. thermophilus* through the rat GIT was the massive induction, at the maintenance phase, of the glycolysis pathway leading to formation of lactate in caecum. Here, we propose that *S. thermophilus* modulates $p27^{kip1}$ through production of lactate. This hypothesis stems from four observations: 1) the main *S. thermophilus* metabolic pathway enhanced in GIT compared to a milk culture was the glycolysis leading to production of lactate; 2) the increase of $p27^{kip1}$ level in colon was observed in the presence of two *S. thermophilus* strains producing equivalent amount of lactate; 3) the increase of $p27^{kip1}$ level in colon did not appear in the presence of non- or low lactate-producing bacteria (*B. thetaiotaomicron* and *R. gnavus*) and 4) $p27^{kip1}$ was induced *in vitro* by the presence of lactate (Hsiao, Y. P. et al, 2009). Obviously, our hypothesis does not exclude other underlying mechanisms involved in the interplay between *S. thermophilus* and the host. Thus, using other lactic acid bacteria it would be of interest to test if the induction of $p27^{kip1}$ systematically correlates with the high level of lactate production.

The increase in proteins abundance belonging to glycolysis in GIT compared to milk was unexpected for *S. thermophilus* as these proteins are already present at high levels in the milk (Derzelle, S. et al, 2005). The capacity for a bacterium to diversify its carbon sources seems to be

essential for the colonization in GF rodents (Roy, K. et al, 2008; Alpert, C. et al, 2009; Bron, P. A. et al, 2004; Marco, M. L. et al, 2009). *S. thermophilus* is particularly-well adapted to milk, in particular *via* the assimilation of lactose which is its preferential sugar and is present at high and non-limiting concentration in milk (4.5g L^{-1}). However, we showed here that *S. thermophilus* may develop an efficient ability to use other sources of saccharides present in GF rat (Sonnenburg, J. L. et al, 2005) since the rat diet did not contain lactose. This flexibility is rather unexpected since *S. thermophilus* has a small genome with a large proportion of non functional genes (10% of pseudo-genes), in particular genes involved in carbon utilization in line with the paucity of carbon sources in milk (Bolotin, A. et al, 2004). Therefore, it would be of interest to determine whether the implantation level and the adaptive responses of *S. thermophilus* may be improved by using mono-associated rats receiving a diet enriched with lactose, as previously suggested (Mater, D. D. et al, 2006). The use of mono-associated rats is an efficient tool to reveal the activity of bacterial proteins, especially those with dual functions. For example, EF-Tu is an elongation factor playing a key role in protein synthesis but also in the maintenance in the GIT. The up-regulation of EF-Tu was observed *in vivo* for *L. lactis* (Beganovic, J. et al, 2010), *Bifidobacterium* (Yuan, J. et al, 2008) and *in vitro* for *L. plantarum* (Ramiah, K. et al, 2007). While *S. thermophilus* reduced most of its translation and protein synthesis machinery, EF-Tu was the sole protein belonging to protein synthesis pathway that was increased at high level in digestive tract compared to milk (induction factor 12). We thus propose that in Ino-LMD9 rats, EF-Tu -that displays the characteristics of an adhesion factor (Dallo, S. F. et al, 2002)- is mainly involved in the maintenance of *S. thermophilus* rather than in the protein synthesis.

We have recently demonstrated that the colonisation of GF rats with a complex microbiota leads to increase in the absorptive surface by deepening crypts and splitting bifurcated crypts by crypt fission process (Cherbuy, C. et al, 2010). This trophic effect of microbiota is associated with a modulation of several cell-cycle related proteins. Along the colonisation with a complex microbiota,

we have previously described a precocious hyper-proliferative phase at the level of colonic epithelium which is counter-balanced by an induction of proteins restraining the proliferation and a decrease of anti-apoptotic proteins (Cherbuy, C. et al, 2010). In contrast to what was observed with a complex microbial population, the presence of *S. thermophilus* did not trigger deeper crypts and the division of bifurcating crypts. Thus, as we reported previously (Cherbuy, C. et al, 2010), a single strain is not sufficient to switch on proliferation and the associated greater absorptive surface. In contrast, the induction of cell cycle arrest proteins could be triggered by a single bacterium. Indeed *B. thetaiotaomicron* and *R. gnavus* increased $p21^{cip1}$ (Cherbuy, C. et al, 2010) while *S. thermophilus* increased $p27^{kip1}$. $p21^{cip1}$ and $p27^{kip1}$ are cyclin-dependent-kinases (CDKs) inhibitors and are preponderant cell cycle regulators in GIT (Besson, A. et al, 2008). In particular, $p27^{kip1}$ is involved in the capacity of epithelium and host to attenuate deleterious effects of environmental stimuli (Zheng, Y. et al, 2008). Thus we have shown that $p27^{kip1}$ is stabilized *in vivo* by a complex microbial population (Cherbuy, C. et al, 2010) and by lactate produced by *S. thermophilus* (Figures 3 and 4). In human healthy adults, no or low (0 to 2 mM) lactate is detected in fecal samples (Hove, H. et al,1995; Hove, H. et al, 1993), since the luminal lactate is absorbed by host and is also used as an energy source by other commensal bacteria (He, T. et al, 2008; Marquet, P. et al, 2009). However, increased fecal lactate has been associated with intestinal mal-absorption and colitis (Hove, H. et al, 1995). It could also be of interest to measure fecal lactate in humans with short bowel syndrome, since we have shown that their microbiota is deeply enriched in lactobacilli at the expense of others bacterial members (Joly, F. et al, 2010). Thus, the effect of lactate that we evidenced here by using a model of lactic acid bacteria in an experimental model may have significance in human digestive diseases. Since *S. thermophilus* is able to adapt its global metabolism to gut environment and to induce $p27^{kip1}$, this work provides new insights into the functional "panoply" of one of the two yoghurt bacteria. Finally, the fact that *S. thermophilus* emphasizes its carbohydrate metabolism in the digestive tract is in accord with the beneficial role of fermented milk consumption in improving lactose intolerance.

Table 1: Fold changes in protein abundance (2-DE) of *S. thermophilus* LMD-9 between faeces and late growth phase in milk.

Gene/locus	pI_{th}[a]	MW_{th}[b] (kDa)	Peptide number[c]	Sequence coverage (%)	Fold change	Protein identification
Higher level in TD						
						Carbohydrate metabolism
eno	4.67	47	22	63	3.06	Phosphoenolpyruvate carboxylase
gapA1	5.76	37.2	18	60	4.69	Glyceraldehyde-3-phosphate dehydrogenase
hdhL	5.09	34.4	11	41	2.97	Putative L-2-hydroxy isocaproate/malate/lactate dehydrogenase
malQ	5.2	57.6	13	28	2.39	4-alpha-glucanotransferase
pgk	4.86	42.2	17	54	3.02	3-phosphoglycerate kinase
pfkA	5.09	36.0	13	38	7.67	6-phosphofructokinase
pyk	5.21	56.6	17	49	3.01	Pyruvate kinase
ster_1876	4.81	31.5	9	47	6.05	Fructose/tagatose bisphosphate aldolase
						Stress
sodA	5.1	24.7	6	48	2.44	Superoxide dismutase (Mn)
groEL	4.7	56.9	35	69	2.46	Chaperonin GroEL (HSP60 family)
						Translation
tuf	4.9	43.9	15	45	11.76	GTPase - translation elongation factor
Lower level in TD						
						Transcription, translation
alaS	5.03	96.7	25	31	0.12	Alanyl-tRNA synthetase
asnS	4.93	51.1	27	51	0.1	Asparaginyl-tRNA synthetase
aspS	4.81	65.9	21	37	0.19	Aspartyl-tRNA synthetase
fus	4.88	76.6	37	62	0.25	Translation elongation factor (GTPase)
gltX	4.97	55.3	26	47	0.29	Glutamyl- and glutaminyl-tRNA synthetase
glyQ	4.91	34.9	9	33	0.33	Glycyl-tRNA synthetase, alpha subunit
glyS	5.05	78.5	22	38	0.09	Glycyl-tRNA synthetase, beta subunit
hisS	5.44	48.4	18	49	0.23	Histidyl-tRNA synthetase
leuS	4.99	94.4	13	17	0.11	Leucyl-tRNA synthetase
rplJ	5.36	17.5	7	43	0.33	Ribosomal protein L10
rplL	4.42	12.4	9	81	0.1	Ribosomal protein L7/L12
rpoB	5.02	29.2	13	50	0.33	DNA-directed RNA polymerase, beta subunit/140 kD subunit
rpsA	4.88	43.9	21	55	0.1	Ribosomal protein S1
rpsB	5.3	28.4	12	43	0.12	30S ribosomal protein S2
ster_1166	5.02	29.2	13	50	0.33	Putative translation factor
thrS	5.39	74.8	36	45	0.06	Threonyl-tRNA synthetase
tig	4.41	46.7	22	53	0.16	FKBP-type peptidyl-prolyl cis-trans isomerase (trigger factor)
tsf	4.75	37.4	15	60	0.04	Translation elongation factor Ts
						Nitrogen metabolism
						Amino acids biosynthesis
asnA	5.1	37.5	13	31	0.14	asparagine synthetase A
bcaT	4.9	37.5	9	30	0.22	branched chain amino acid aminotransferase
cysM1	5.2	32.3	15	51	0.31	cysteine synthase
lysA	4.81	46.4	10	38	0.17	diaminopimelate decarboxylase
metK	4.93	44.7	15	44	0.33	S-adenosylmethionine synthetase
ster_1751	4.87	50.1	11	32	0.16	glutamine synthetase

						Peptidases
pepC	4.85	50.4	9	17	0.16	aminopeptidase C
pepN	4.59	96.3	15	19	0.18	aminopeptidase N
						Nucleotide biosynthesis and salvage
pyrG	5.51	59.1	12	28	0.26	CTP synthase (UTP-ammonia lyase)
purC	5.00	27	22	80	0.25	phosphoribosylaminoimidazole – succinocarboxamide synthase
purL	4.93	136	19	19	0.05	phosphoribosylformylglycinamidine synthase II (FGAM synthetase)
guaB	5.31	52.9	13	29	0.07	IMP dehydrogenase/GMP reductase
						Cell division protein
ftsZ	4.6	46.5	17	43	0.38	Cell division GTPase
						Carbohydrate metabolism
gdhA	5.21	48.9	19	56	0.13	Glutamate dehydrogenase/leucine dehydrogenase
						Miscellaneous
atpD	4.73	50.9	11	32	0.14	F0F1-type ATP synthase, beta subunit
dpr	4.59	19.2	5	29	0.18	DNA-binding ferritin-like protein
murC	5.23	49.6	15	44	0.17	UDP-N-acetylmuramate-alanine ligase
panE	5.6	36.1	13	42	0.40	ketopantoate reductase
secA	5.22	96.4	28	41	0.09	Preprotein translocase subunit SecA
ster_0157	4.77	46.4	15	38	0.22	hypothetical protein
ster_1854	4.41	59.5	17	32	0.21	Predicted kinase related to dihydroxyacetone kinase
ster_1995	4.74	60.9	23	49	0.18	ATPase component of ABC transporter with duplicated ATPase domain
typA	4.9	68.7	21	38	0.09	Predicted membrane GTPase involved in stress response

a: theorical isoelectric point

b: theorical molecular weight

c: number of peptides identified

Figure 1

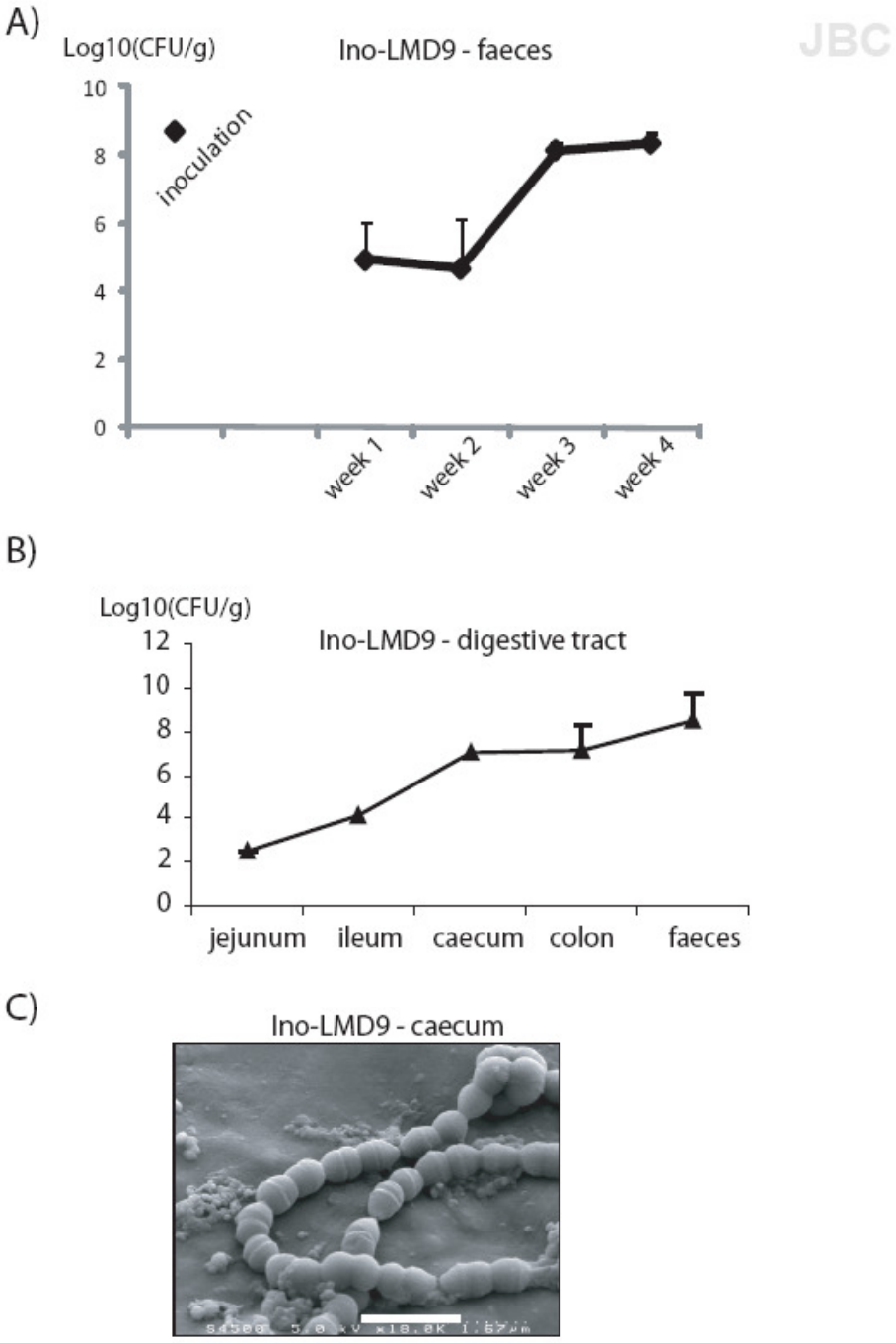

Figure 1. *S. thermophilus* in the digestive tract.
Enumeration of viable *S. thermophilus* isolated from faeces (A) and different rat digestive tract sections (B) of Ino-LMD9 rats. Faecal or luminal sample were diluted and immediately plated on culture medium M17+lactose. Colonies were numbered after 24h-anaerobic culture. Results are expressed on a log scale. n: number of rats used. (C) Snaps shot of *S. thermophilus* isolated from caecum of Ino-LMD9 rats obtained by scanning electron microscopy.

Figure 2

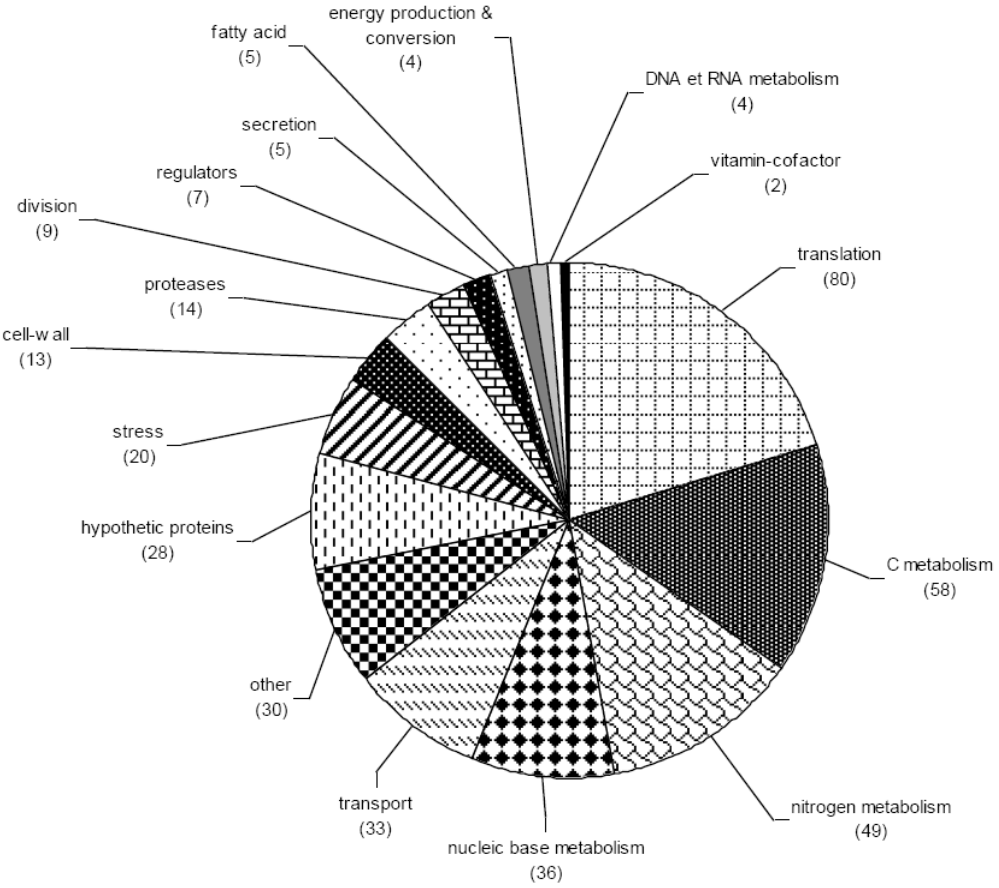

Figure 2. Functional distribution of the proteins of *S. thermophilus* LMD-9 isolated from rat faeces. Proteins from the envelope-enriched fraction were separated and identified by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). Transport, regulation: transporters and regulators, respectively, other than those involved in carbon metabolism, nitrogen metabolism, nucleic base metabolism. In brackets: number of proteins.

Figure 3

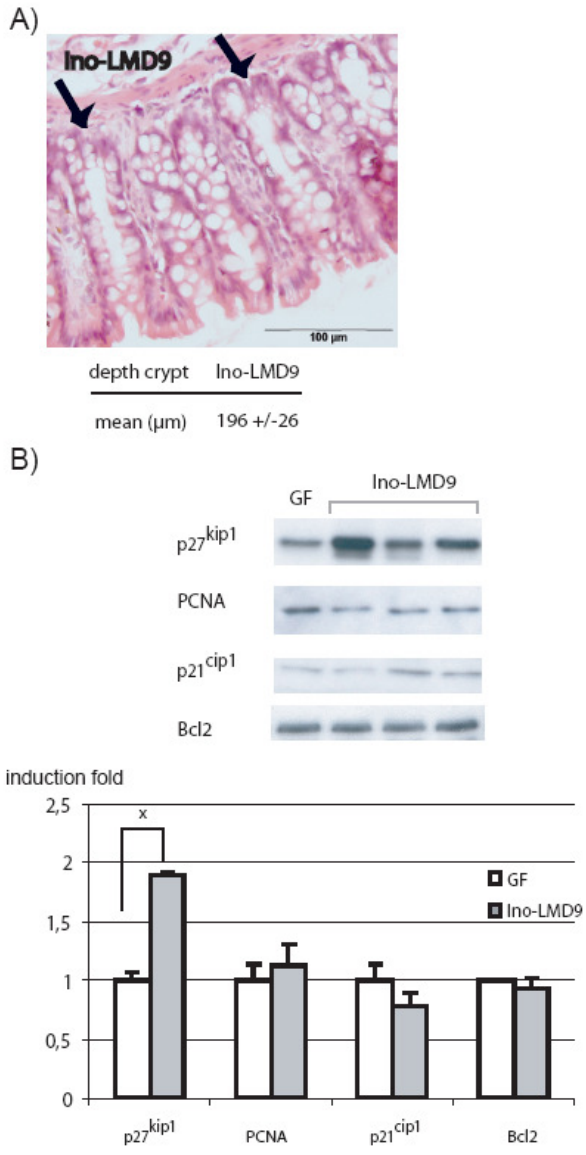

Figure 3: Histological and molecular analysis of the colonic epithelium of Ino-LMD9 rats.
A) Representative photomicrograph of colonic sections obtained from Ino-LMD9 rats stained with hematoxylin and eosin. Arrows indicated a bifurcating crypt in colonic epithelium. Crypt depth mean (µm) of Ino-LMD9 (2 rats, 30 measures/rat) was indicated. B) Western blot analysis and quantification of $p27^{kip1}$, PCNA, $p21^{cip1}$ and Bcl2 from colonic epithelial cells of GF and Ino-LMD9 rats. Autoradiography represents representative blots with Ino-LMD9 (n=3) and GF (n=1) rats. Densitometric analysis was means (± SE) obtained with 8 rats per group and values are expressed in fold induction considering the GF as 1. Statistical significantly differences ($p < 0.05$) between groups.

Figure 4

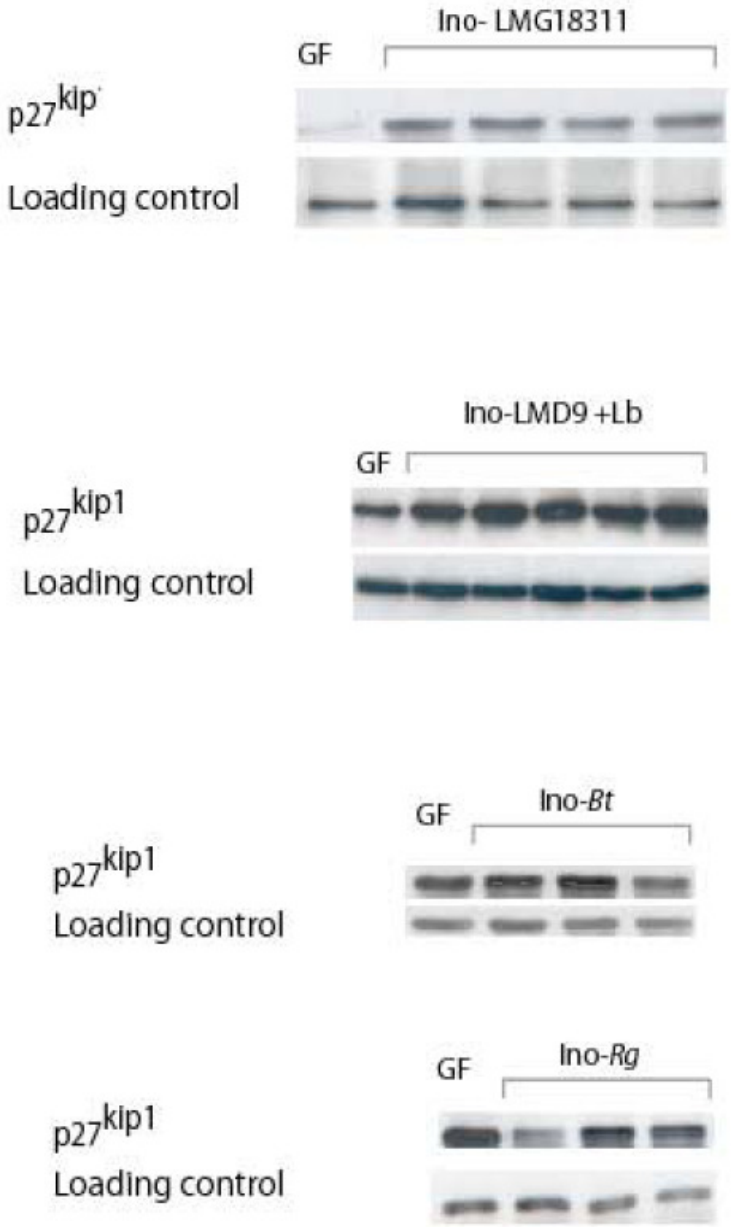

Figure 4: $p27^{kip1}$ detection in different mono-associated groups. Ino-LMD9+Lb rats have been inoculated with a mixture of *S. thermophilus* and *L. bulgaricus*. At the end of the experiment (30 days after the gavage), rats were only mono-associated with *S. thermophilus*, since *L. bulgaricus* did not efficiently implanted. Ino-LMG18311 (n=4), Ino-*Bt* (n=3) and Ino-*Rg* (n=3) were respectively harbouring *S. thermophilus* strain LMG18311, *B. thetaiotaomicron* and *R. gnavus*. Western blots were from colon cells.

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FOOTNOTES

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CHAPTER III

Genes induced *in vivo* by S*treptococcus Thermophilus* using supressive subtractive hybridization (SSH) thecnology

GENES INDUCED *IN VIVO* BY S*TREPTOCOCCUS THERMOPHILUS* USING SUPRESSIVE SUBTRACTIVE HYBRIDIZATION (SSH) THECNOLOGY

Fatima Chegdani

ABSTRACT

Streptococcus thermophilus is a lactic acid bacteria largely used by the food industry for production of yoghurt and cheese, among other products. In spite of its wide consumption, insufficient information is presently available on the molecular effects of its consumption at the level of intestinal cells. The present research aimed at studying the impact of *S. thermophilus* on colonic epithelial cells gene expression. In this study we have used Differential screening (Suppressive Subtractive Hybridization or SSH) methods to identify and study genes induced by *S. thermophilus* in colonic epithelial cells. The subtraction library was prepared subtracting mRNA between epithelial cells isolated from colonic mono-associated rats (inoculated with LMD9 strain of *S. thermophilus*) and germ-free rats (without bacteria). Eighty clones were sequenced after SSH. These clones correspond to genes whose expression in colonic epithelial cells is potentially activated in the presence of *S. thermophilus*. These "candidate" genes can be grouped into four Functional groups: 1) genes that play a role in cell-cycle arrest and induction of differentiation; 2) genes involved in cell-communication; 3) genes involved in calcium binding; 4) genes encoding growth factors. Tow candidate genes involved inthe regulation of cell cycle, Kruppel like factore and 14-3-3 σ, were selected and tested by RT-PCR , qRT-PCR to quantify mRNA and Western blot to analyze the related protein. The results of various tests confirmed that selected candidate genes involved in cell cycle and differentiation increase their expression in the presence of *S.thermophilus*.These results indicate that *S.thermophilus* modifies the expression of genes involved in essential intestinal functions. They, combined with the observation that this bacterial genus is present in women breast milk and in the bowel of infants (> 10^8/g faeces), suggest that *S. thermophylus* may play a role in the early on-setting of colon correct structure and function.

I) INTRODUCTION

The gastrointestinal tract contains within it a microenvironment of bacteria that influences the host Human/animal in many ways. This microbial ecosystem serves for a numerous important functions for its human and animal host, including education of the immune system (Gordon et al, 2005), regulation of angiogenesis (Stappenbeck et al, 2002), modification of the activity of the enteric nervous system, extraction and processing nutrients consumed in the diet (Ley RE et al, 2005, Bckhed F et al, 2004, Hooper et al, 2002), integrity of the intestinal mucosal barrier and modulation of proliferation and differentiation of its epithelial lineages (Alam et al, 1994, Thomas et al, 2009). It is clear that this list is not yet complete; as this field of study expands, we can discovering a new functions and relationships. In contrast the structure and homeostasis of intestinal epithelium are the basis of the absorptive function of the intestine and are modulated throughout life by environmental factors. At birth, it is known that the surface epithelium and crypt depth increase. Recent studies have shown that adult humans increased colonic crypt depth reflects a morphological adaptation that occurs following intestinal resection (Joly et al, Am J. Physiol, 2009). In this context, studies were concentrated to provide evidences of the effect of the microbiota on the structure and homeostasis of the colonic epithelium. Experimentally work in vivo using gnotobiotic animals that the presence of microbiota leads an increase in the depth of the crypts of the colonic epithelium. This trophic effect of microbiota accompanied by a highly orchestrated induction of proteins involved in cell cycle colonic epithelial cells. The establishment of microbiota in the host induces an early phase hyper-proliferation that is then offset by a late phase of proliferation arrest and differentiation (Cherbuy et al, 2010). the . Some cell cycle proteins that are induced by the commensal microbiota have also been described as cellular targets some pathogenic bacteria (Samba Louaka et al, Cell Microbiol, 2008). Studies of Germ free(GF) and colonized mice have revealed an important relationship between the gut and microbiota and have been particularly

illustrating the essential role of the gastrointestinal microbiota in normal gut development (Palmer et al, 2007). The prospects of these work is to understand molecular mechanisms by which the microbiota influence homeostasis intestinal epithelium using bacterial populations and simplified using strains probiotic lactic acid bacteria as *streptococcus thermophilus*. We have used for this study *S.thermophilus* bacteria first one because we have find a several papers which haves been reported that *S.thermophilus* among the first to colonise the intestinal tract in the birth (>108 /g faces), (Moreau et al, 1986) (Palmer et al, 2007) and her presence in the faces and in breast milk is recently described (Perez et al, 2007). So, *S.thermophilus* can have an impact on the maturation and homeostasis of intestinal epithelium after birth. The second one is to the importance and the use of *S.thermophilus* as probiotics in the manufacturing of some industrials product in particularly yoghurt and milk fermentation. This bacterium belongs to the group of the *thermophilic* lactic acid bacteria and is traditionally used in combination with others Lactobacillus for the manufacture of yogurt and so-called hard ‘‘cooked’’ cheeses (e.g., emmental, gruye`re, grana), at a relatively high process temperature (45°C) (Fox et *a*l, 1993, Tamime et *al*, 1980). *S. thermophilus* is a ‘‘generally recognised as safe species and over 1021 live cells are ingested annually by the human population. Recently, the complete genome sequence of certain yogurt strains (LMG 18311, CNRZ1066 and LMD9), (Bolotin et al, 2004).

For this study we have choice a Supressive Subtractive Hybridization (SSH) method as a differential screening to studies the impact of *S.thermophilus* on gene expression profile of the host (rat animal). Suppression subtractive hybridization (SSH) is a widely used method for separating DNA molecules that distinguish two closely related DNA samples. Two of the main SSH applications are cDNA subtraction and genomic DNA subtraction. To our knowledge, SSH is one of the most powerful and popular methods for generating subtracted cDNA or genomic DNA libraries. It is based primarily on a suppression polymerase chain reaction (PCR) technique and combines normalization and subtraction in a single procedure (Diatchenko et al, 1996). The

normalization step equalizes the abundance of DNA fragments within the target population, and the subtraction step excludes sequences that are common to the populations being compared. This dramatically increases the probability of obtaining low-abundance differentially expressed cDNAs or genomic DNA fragments and simplifies analysis of the subtracted library. SSH technique is applicable to many comparative and functional genetic studies for the identification of disease, developmental, tissue specific, or other differentially expressed genes, as well as for the recovery of genomic DNA fragments distinguishing the samples under comparison. The results have been validated using the quantitative RT-PCR and western blot analysis

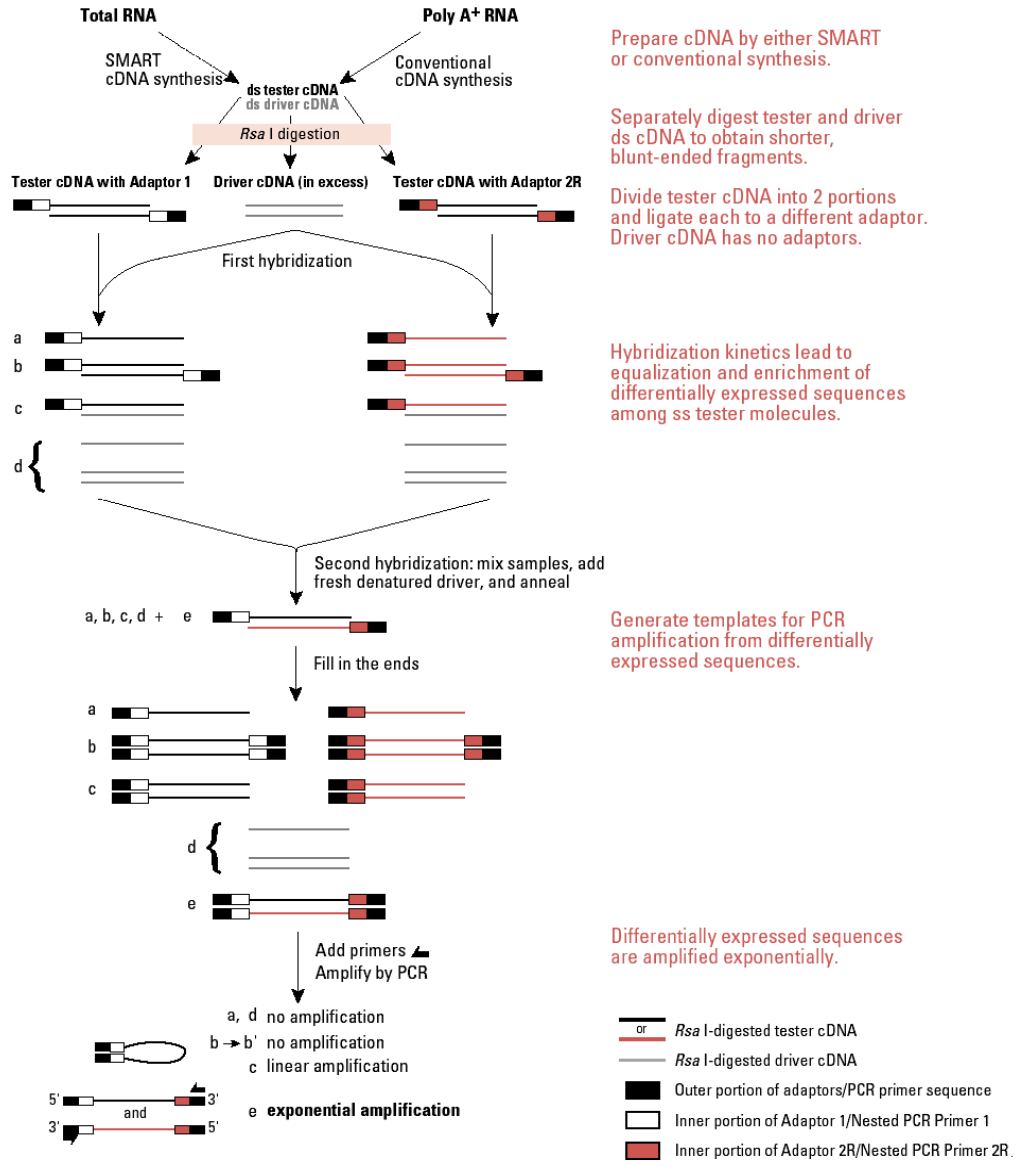


Figure PCR-Select cDNA subtraction technique.

II) MATERIAL AND METHOD.

II-1) ANIMALS

All procedures were carried out according to the European and French guidelines for the care and use of laboratory animals (permission 78-123, dedicated to MT). At the age of two months, three germ-free (GF) rats (male, Fisher 344) were inoculated by oral administration with one ml of *S.thermophilus* LMD9 (Ino-LMD9) in Nilac milk ($5x10^8$ *S.thermophilus*/ml) . As a control, three GF rats were also inoculated with one ml of sterile Nilac milk (without bacteria). GF, and mono-associated rats were housed in sterile Plexiglas isolators (Ingénia, Vitry-sur-Seine, France). All groups of rats received the same standard diet (UAR), which was sterilized by gamma irradiation. In addition, germfree rats received supplemental vitamin K in their drinking water. Twice a week during the experiment, *S. thermophilus* (Ino-LMD9) was enumerated by plating serial dilutions of the faeces. The feces of germfree rats were also checked weekly for the absence of microorganisms. All rats were scarified at three months of age, *i.e*, 30 days after inoculation. Rats were anesthetized with isoflurane aspiration, and the colons were removed rapidly and immediately used for cell isolation procedure.

II-2) CELL ISOLATION

Colonic cells originating from both groups of rats were isolated using a protocol previously described by Cherbuy et al (Cherbuy C et al, 1995&2004). Briefly: the colons removed were flushed clean, first with NaCI (9 g/L) and then with a Ca^{2+}- and Mg^{2+}-free Krebs'-Henseleit bicarbonate buffer (pH 7.4) containing 10 mmol/L HEPES, 5 mmol/L dithiothreitol, and 2.5 *glL* albumin and equilibrated against a mixture of O_2/CO_2 (19:1, vol/vol). Thereafter, the colons were perfused for 20 minutes (30 mL/min at 37°C) with the same buffer containing 10 mmol/L EDTA. Then, the colons were gently squeezed and the luminal fluid collected and centrifuged (150x*g* for 3 minutes) to harvest the cell fraction. An additional 15 minutes incubation was performed in a

shaking water bath (100 oscillations/min at 37°C) using the same buffer without EDTA and in the presence of hyaluronidase (4 g/L). The isolated cells were washed twice and resuspended in the same bicarbonate buffer medium (pH 7.4) without dithiothreitol and containing $CaC1_2$ (1.3 mmol/L), $MgCl_2$ (2 mmol/L), and 10 g/L albumin (incubation buffer). After two washes and centrifugations, the colonic cell pellet was immediately used for RNA and proteins extraction.

II-3) GENE EXPRESSION

II-3-1) RNA PURIFICATION

Total RNA was extracted from epithelial cells by the guanidinium thiocyanate method (Chomczynski P et al, 1987). The RNA yielded was quantified by spectrophotometric "Nanodrop " analysis and the RNA purity was determined based on the A260-A280 ratio. All RNA samples were analysed by agarose gel electrophoresis to check for integrity of 18S and 28S rRNA. Finally, the quality of the RNA was verified by Agilent 2100 Bioanalyzer analysis using the RNA 6000 Nano Assay Kit.

II-3-2) ASSESSMENT OF RNA QUALITY AND QUANTITY USING THE NANODROP

Total RNA was quantified by an ND-1000 Spectrophotometer (ND-1000 spectrophotometer; Nanodrop Technologies, Wilmington, DE) using the nucleic acid function which uses the absorbance at 260 nm to calculate concentration using Beer's law. The ratio of absorbance at 260 nm and 280 nm was used to assess purity of the RNA with a ratio of ~2 indicating sample purity. The 260/280 ratios for RNA samples were all in the acceptable range of 1.8-2.1.

II-3-3) ASSESSMENT OF RNA QUALITY USING GEL ELECTROPHORESIS.

When yields of RNA were adequate, gel electrophoresis was used to assess RNA quality.

5 ug of total RNA was loaded onto TAE agarose gel with ethidium bromide. Gels were electrophoresed for 20-30 minutes in Sub Cell-GT electrophoresis tanks (Biorad) at 40-120 volts at room temperature using power packs (PowerPAC-300, Biorad). Agarose gels were visualized under UV light (260 nm) using the UVP Dual Density transilluminator and photographed with the UVP Image Store 7500 system . Figure 2 showed total RNA gel for RNA extracted from animals used for this study .

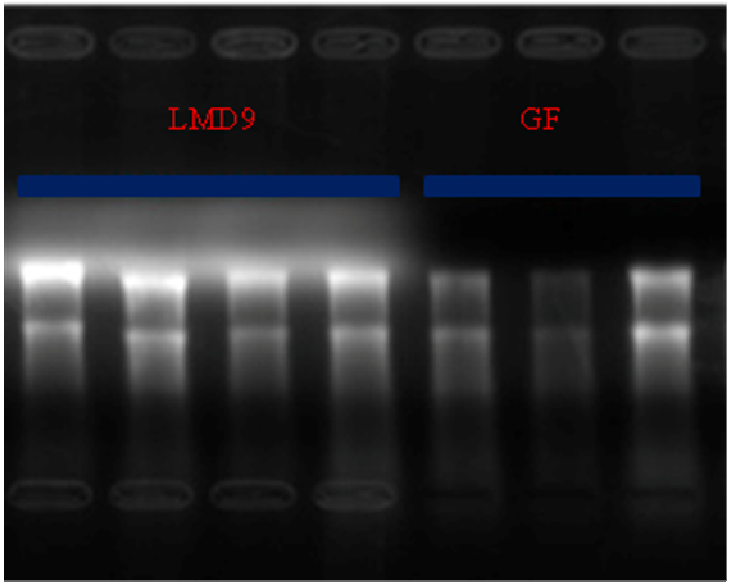


Figure 2:RNA gel with total RNA extracted from colonic epithelium cells of 3 mono-associated rats with *Streptococcus thermophilus* and 4 germ free rat : well presents the total RNA extracted of each sample.

II-3-4) ASSESSMENT OF RNA QUALITY USING THE BIOANALYZER

Quality of total RNA for microarray analysis was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies). Total RNA was used to generate an electropherogram (Figure 1) that allowed an estimate of RNA concentration and ribosomal ratio to be obtained. High quality RNA showed the following features on a electropherogram:

- clear 18S and 28S rRNA peaks
- A low small RNAs (5S, transfer RNA and miRNA) presence relative to the rRNA peaks

- A flat base line in the fast-migrating region

- A flat inter-rRNA peak regions

- A return to the baseline after the ribosomal RNA peaks

In addition to visual inspection of the electropherogram, integrated software generated an RNA integrity number (RIN) by analysis of the entire electrophoretic trace. This number provides a de facto standard for RNA integrity with an RIN >7 indicating a sample of sufficient quality for successive analysis.

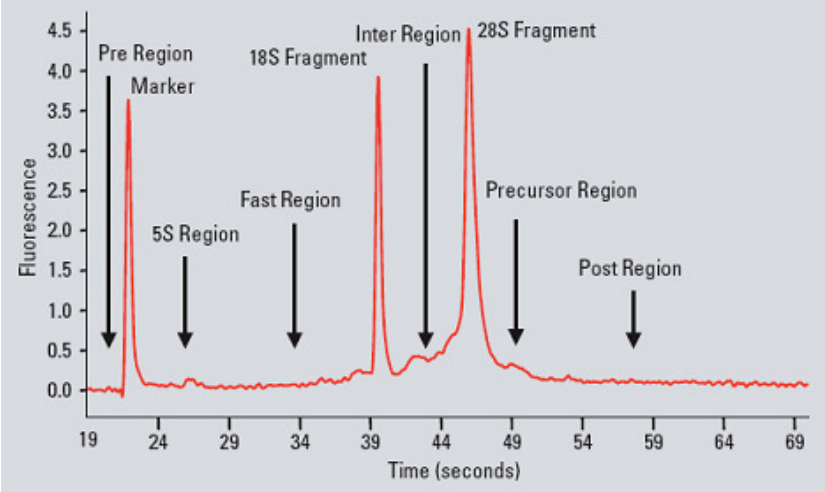


Figure 3: Features of a total RNA bioanalyzer electropherogram.

II-4) CONSTRUCTION OF SUBSTRACTED cDNA LIBRAIRIE USING SSH TECHNIQUE

II-4-1) DRIVER AND TESTER cDNA SYNTHESIS

mRNA purified from the total RNA, using commercial Kit (Pharmacia, Biotech), extractable from colonocytes of colonic epithelium was reverse transcribed and the cDNA was subjected to PCR using the PCR-Select cDNA Subtraction kit (Clontech Laboratories Inc., Palo Alto, CA). Protocols were followed according to the manufacturer's instructions. Briefly, about 100 ng of mRNA from both mono-associated LMD9 (driver) And Germ Free (tester) was denatured at 70°C for 2 min,

then reverse transcribed in the presence of, 10 µM of cDNA synthesis primer, 5X First-Strand Buffer, 10 mM each dNTPs, and 20 units/µl of AMV reverse transcriptase and incubated at 42°C for 1.5 h in an air incubator. The samples were placed on ice to terminate first-strand cDNA synthesis and immediately proceeded to Second-Strand cDNA Synthesis. First strand cDNA was incubated at 16°C thermal cycler for 2 h in the presence of 5X Second-Strand Buffer, 10 mM dNTP Mix and 20X Second-Strand Enzyme Cocktail. Additional incubation at 16°C thermal cycler for 30 min after added of 6 u of T4 DNA Polymerase. the reaction was stopped by adding 20X EDTA/Glycogen and the product was conserved at -20°C. The product was purified once with 100 ul of phenol:chloroform:isoamyl alcohol (25:24:1). After centrifugation, total of aqueous phase was removed and collected in a fresh 0.5-ml microcentrifuge tube with 100 ul chloroform:isoamyl alcohol (24:1). The mix was centrifuged and the top aqueous layer was collected and transfered in a fresh 0.5-ml microcentrifuge. Precipitation step was immediately preceded by adding 40 µl of 4 M NH4OAc and 300 µl of 95% ethanol after pellet recuperation a step of wash was computed using 500 ul of 80% ethanol. Finally the precipitate was Dissolved in 50 µl of sterile H2O. Both driver and tester cDNAs were then digested with 50 units of *Rsa*I in a 50 µl reaction mixture at 37°C for 3 h, and the reaction was stopped by adding 2.5 ul of 20X EDTA/Glycogen Mix. *Rsa*I digested driver and tester cDNA were extracted once with phenol:chloroform:isoamyl alcohol. The aqueous phase was removed, precipitated with ethanol, and the pellet was redissolved in 5.5 ul of of H2O. One microliter of *Rsa*I digested tester cDNA was diluted in 5 ul of sterile water, and 2 ul of the diluted tester was then ligated with 2 ul of adapter 1 and adapter 2R (10 uM) according to the guidelines provided in the PCR-Select cDNA subtraction kit (Clontech) in separate ligation reactions in a total volume of 10 ul at 16°C overnight using 400 units of T4 DNA ligase in 2 ul of 5X ligation buffer. The ligation was stopped by adding EDTA/glycogen and heated at 72°C for 5 min to inactivate the ligase. Samples were stored at - 20°C. A PCR-based ligation efficiency analysis to verify that at least 25% of the cDNAs had adaptors on both ends was performed according to the instructions detailed in the Clontech PCR-Select cDNA subtraction kit user manual.

II-4-2) SUPPRESSION SUBTRACTIVE HYBRIDIZATION (SSH)

SSH was performed using the Clontech PCR-Select cDNA subtraction kit. Briefly, 1.5 ul of driver cDNA was added to each of two tubes containing 1.5 ul of adapter 1 and adapter 2R-ligated tester cDNA in 1 ul of 4X hybridization buffer. The samples were denatured at 98°C for 1.5 min, and then allowed to anneal at 68°C for 8h. Following first hybridization, the two samples were combined in the presence of fresh excess denatured driver cDNA in 1 ul of 4X hybridization buffer. The samples were allowed to hybridize overnight at 68°C. The hybridized samples were diluted in 200 ul of dilution buffer (20 mM Hepes pH 8.3, 50 mM NaCl, and 0.2 mM EDTA), heated at 68°C for 7 min, and stored at -20°C.

II-4-3) PCR AMPLIFICATION OF SUBTRACTED PRODUCTS

Two PCR amplifications of the subtracted cDNAs were performed. The primary PCR contained 1 ul of diluted, subtracted cDNA, and 24 ul of the PCR master mixture prepared using the reagents provided in the kit. PCR was performed at 75°C for 5 min to extend the adaptors; 94°C for 25 sec; and 27 cycles at 94°C for 10 sec, 66°C for 30 sec, and 72°C for 1.5 min. The amplified products were diluted 10-fold in sterile deionized water. The diluted primary PCR product was used as template in a secondary nested PCR for 10 cycles at 94°C for 10 sec, 68°C for 30 sec, and

72°C for 1.5 min using two nested primers, 1 and 2R, provided in the kit. Primary and secondary PCR products were analyzed on a 2% agarose gel. A second PCR-based analysis was performed according to the instructions detailed in the Clontech PCR-Select cDNA subtraction kit user manual to test for the efficiency of subtraction.

II-4-4) CLONING AND ANALYSIS OF SUBTRACTED cDNA

Following PCR subtraction, the amplified products were cloned into the pCR II vector of the TA cloning kit (Invitrogen) and used to transform competent DH5a *Escherichia coli* cells. Colonies were grown for 16–18h at 37°C on Luria broth (LB) agar plates containing ampicillin, X-gal (5-

bromo 4-chloro 3-indoyl-b-D-galactopyranoside) and ampiciline antibiotic for blue/white colony selection. Plasmids were extracted and the inserts were subjected to DNA sequencing using a Big Dye terminator Cycle Sequencing Ready Reaction Kit and an automated DNA sequencer ABI PRISM 310. Basic Local Alignment Search Tool (BLAST) analysis from the National Center for Biotechnology Information was used to search GenBank for homologous nucleotide and protein sequences.

II-5) QUANTITATIVE RT-PCR ANALYSIS

Total RNA extracted from colon epithelial cells of 5 rats (2 germ free and 3 mon-associated with *streptococcus thermophilus* LMD strain) was used to cDNA synthesis.

Reverse transcription was performed with 1 µg of rat total RNA using iScript™cDNA Synthesis Kit (Bio-rad). Quantitative RT-PCR (RT_qPCR) was performed on the Opticon Monitor 3 (Bio-rad) using specific primers (table1), which were designated to have a PCR product that incorporates at least one exon-exon splice junction to allow a product obtained from the cDNA to be distinguished on electrophoresis from genomic DNA contamination, and Master Mix (2X) Universal (KAPA SYBR®FASTqPCR Kits) KAPA BIOSYSTEMS.

All primers sets were validated to ensure efficient amplification of a single product before use in assays. Relative mRNA levels were calculated using the comparative $2^{-\Delta\Delta Ct}$ method. Comparisons were made with samples from epithelial cells harvested from Germ Free and mono-associated colonic epithelium cells of rats (2 germ free animals and 3 mono-associated) using GAPDH (glyceraldehyde-3-phosphate dehydrogenase) as the reference genes. Expression levels were shown in the results.

Primer Name	Primer sequences	Product size
GAPDHL1	ATACTTGGCAGGTTTCTCCA	116pb
GAPDHR1	ATGGCCTTCCGTGTTCCTAC	
SNFL1	CCAGTAGAGCCGGAAAGGGT	151pb
SNFR1	CCTTGAGACCAATGCTGGAT	
KLF4L1	TTAAAAGTGCCTCCTGTGT	126pb
KLF4R1	GCCACCCACACTTGTGACTA	

Table 1: Gene specific primer sequences shown were used for real-time RT-PCR validation of the changes in gene expression in mono-associated rats with *Streptococcus thermophilus* revealed by SSH analyses. Real-time PCR based on SYBR green (Biorad) mediated detection was run on an Biorad Mini-Opticon. Primer sequences are named as L1and R1 representing forward and reverse primer sequences, respectively.

II-6) WESTERN BLOT ANALYSIS

Total proteins were extracted from colonocytes with RIPA buffer. and quantified by Lowry method using spectrophotometer (Spectra Fluor Plus; XFLUOR4 V4.11). Different amount of these proteins were loaded in a 10% SDS-polyacrylamide gel, after denaturation at 100°C for 5 min and migration at room temperature for 2h, and then electro-transferred to a PVDF membrane (Amersham Pharmacia Biotech; Saclay, France). Membrane transferred was pre-hybridized with5% milk/PBST 1X Overnight . Proteins were analysed using anti- p27kip1 (Santa Cruz Biotechnology; 1/500), anti 14-3-3 sigma (chemicon AB9742 ; 200X) and anti KLF4 (IMGENEX, IMG-6081A; 500X) Several loading controls were used, either GAPDH, Skp1 or cullin1 (Cherbuy C. et al, 2010). To check homogeneity of loading, all membranes were stripped and re-hybridized with an antibody against -tubulin (1:20000) (Sigma; L'Isle d'Abeau Chesnes, France). An anti-mouse antibody (Jackson Immuno Research Laboratories; West Grove, PA) conjugated with peroxydase was used at 1:12000 to detect immune complexes by chemiluminescence using the ECL+ detection system (Amersham Pharmacia Biotech; Orsay, France). Systematically, a protein ladder was loaded and the weight of the band was checked. Signals imprinted on autoradiography

films were quantified by scanning densitometry of the autoradiograph using Biovision 1000 and logiciel bio1D (Vilber Lourmat, France).

II-7) STATISTICAL ANALYSIS

II-7-1) GENE ONTOLOGY

Gene Ontology analysis of the 46 unique sequences was carried out using the BLAST2GO program(Conesa et al, 2005), a universal gene ontology annotation, visualization and analysis tool for functional genomics research http://www.blast2go.org/start_blast2go. This program is a Java application enabling gene ontology (GO) assignment, based on data mining of sequences for wich no GO annotation is currently available. FASTA-formatted sequences representing all the sequences generated by SSH were uploaded to the program and BlASTX carried out against the Swiss-Prot database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). GO terms associated with the hits (e^{-} ^{value} < $1X10^{e-5}$) were retrieved by the program, and queries were annotated based on hit similarity and GO evidence codes

II-7-2) QRT-PCR

Statistical analysis for qRT-PCR was computed using REST 2009 Software jointly developed by Dr. Michael W. Pfaffl (Chair of Physiology, Technical University Munich) and Qiagn.

REST 2009 Software applies a mathematic model that takes into account the different PCR efficiencies of the gene of interest and reference genes (Relative Expression Software Tool http://rest.gene-quantification.info). Compared to using a single reference gene, using multiple reference genes for normalization can improve the reliability of results (Randomization Tests http://ordination.okstate.edu/permute.htm).

III) RESULTS

III-1) GENERAL LIBRARY ANALYSIS

In order to find genes expressed in monoassociated rats after 30 days of colonization we constructed a SSH library. The tester library was prepared with germ free rats mono-associated with *Streptococcus termophilu*s from 3 months old. whereas the driver library was prepared with germ free rats 3 months old. cDNA sequencing from the SSH library resulted in Sequences obtained were filtered as low quality reads or as ribosomal RNA, polyA+ tails or vector sequences and 56 sequences were selected and analyzed .To identify unique sequences from all transcripts, the dataset was filtered in order to reduce redundancy of all mammalians, as well as of Rattus Norvegicus in particular. Identical or highly similar sequences (E-value > e^{-100}) where identified with a custom script employing Blast (Altschul et al. 1997; Gish 1996-2009) and the longest sequence among two or more similar sequences was selecte. After the filtering 46 unique sequences were considered for the successive analysis. We carried out direct nucleotide comparison (blastn), and only matches with high similarity were considered significant for labeling them as known genes. In summary, we found that 31 sequences had counterpart genes known in Rattus Norvegicus, whereas 15 were described as ESTs with no hit.

III-2) GENE ONTOLOGY ANALYSIS

Sequences resulted after filtering, blasting, mapping and annotation using Blast2GO program, were classified into the three ontology categories: "Molecular function", "Biological process" and "Cellular component" (Figure4:A, B and C, respectively). In the category "Biological process" , the most frequent activity was cellular process with 22 sequences, biological regulation with 18 sequences, response to stimulus with 17 sequences, signaling with 12 sequences and cell growth and development process with 11 sequences. "Molecular function" the most frequent was Binding with 29 sequences followed by catalytic activity with 6 sequences and transcription regulator with 2

sequences. Concerning the category "Cellular component", cellular and membrane enclosed lumen were the most frequent activities. Of all categories unknown sequences were not represented.

III-3) STRATIFIN (14-3-3Σ) AND KRUPPEL LIKE FACTOR 4 (KLF4) TOW PRIVILEGED GENES CANDIDATES.

A total of 46 transcripts differentially expressed were identified as unique sequences, 9 sequences have determinate functions. Tow genes of all transcripts were privileged. These gene have a main role in the cell cycle control and were involved in some different functions (Stier S. et al ,2005; G.Suske et al, 2005; J.Kaczynski et al, 2003; L.A Garrett-Sinha et al , 2003).

III-3-1) EXPRESSION OF KRUPPEL LIKE FACTOR 4 IN COLONIC EPITHELIUM IN GERM FREE RATS ASSOCIATED WITH *STREPRTOCOCCUS THERMOPHILUS* STRAIN BACTERIA (LMD9).

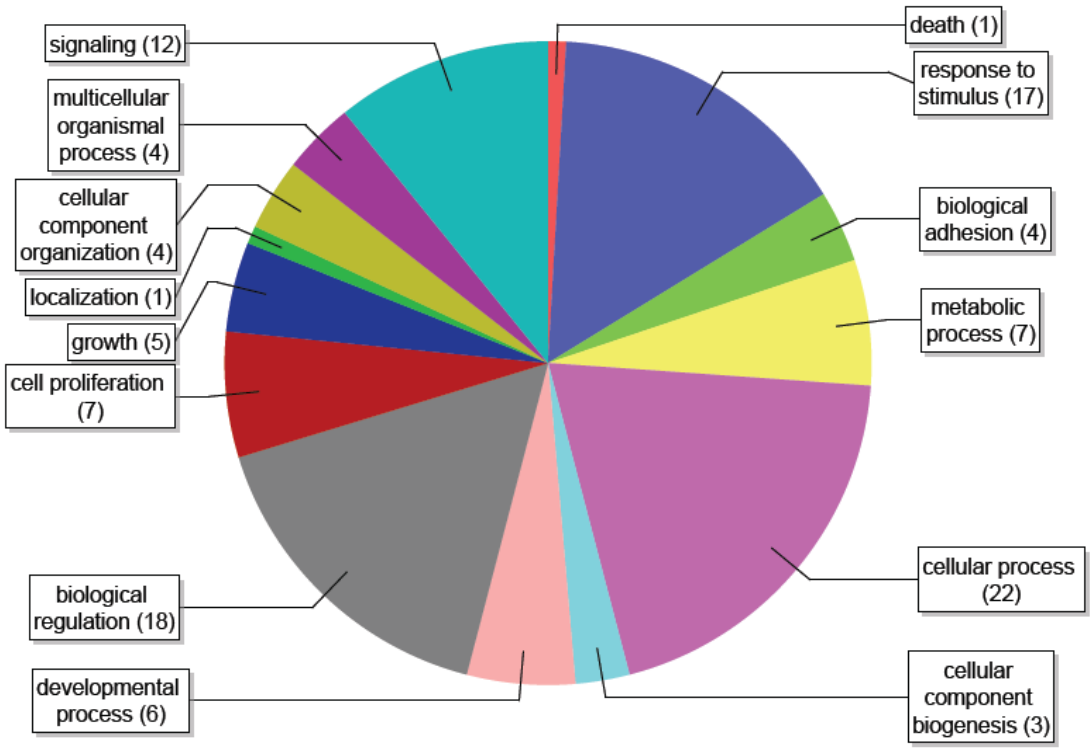
Klf4 gene was identified after analysis using SSH represented with 2 clones relatively to all of genes generated. To extend these data, we verified the expression of klf4 using conventional PCR at different cycles and we have noticed that klf4 expression were increased in the in colonic epithelium cells of mono-associated LMD9 rat at 30 cycles (figure 5) relatively to germ free and was quasi similar to Conventional rats (animals with complex flora). In order to give an evidence of our finding we have quantified relatively the expression of klf4 using the qRT-PCR (Figure 6). Klf4 resulted up regulated in sample group LMD9 in comparaison to control group by a mean factor of 1,26 (S.E. range is 1,008-1,524) and with *pvalue* =0,031. This results combined to the semi-quantitative data suggests that *Streptococcus thermophilus* influenced klf4 expression in the colonic epithelium cells. Additionally, and in order to give more evidence of our results we extended these data using western blot approach to analyze the related protein. Consistent with data generated of previous analysis the expression of klf4 protein was higher in the colonic epithelium cells of mono-associated LMD9 rats to the colonic epithelium cells of germ free (figure 7).

III-3-2) EXPRESSION OF STRATIFIN OR 14-3-3Σ IN COLONIC EPITHELIUM INGREM FREE RATS ASSOCIATED WITH *STREPRTOCOCCUS THERMOPHILUS* STRAIN BACTERIA (LMD9).

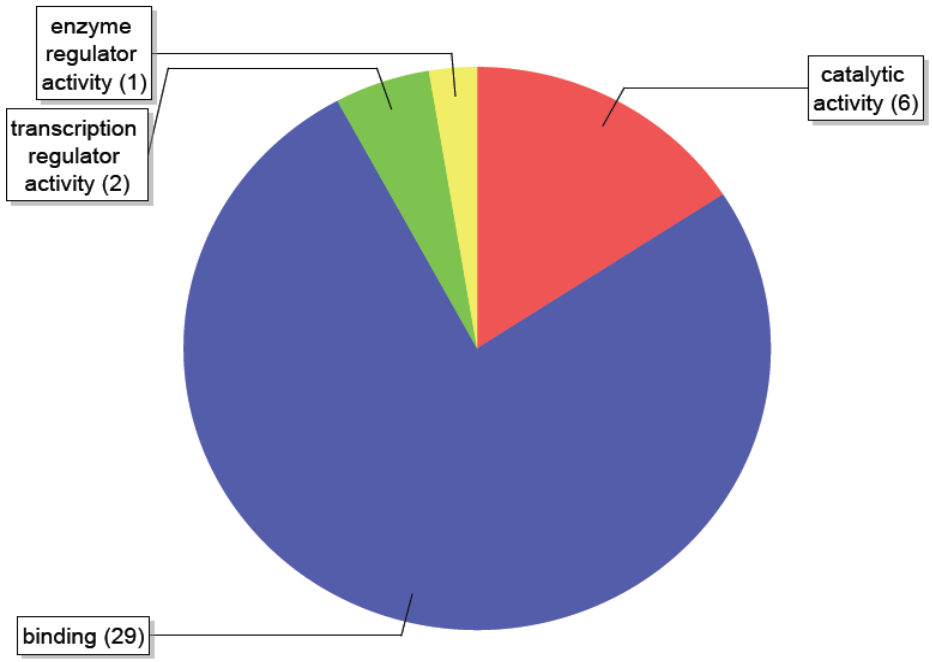
One of the most important genes that we have identified of the total sequences generated by SSH analysis was a stratifin gene called also 14-3-3σ. 14-3-3 σ protein is one of 14-3-3 family proteins that have many divers functions in a board range of organisms, including critical roles in signal transduction pathways and cell cycle regulation (Aitken A. et al, 1999; Fu H. et al, 2000; Wang W. et al, 1996). And the 14-3-3σ gene is one of seven isoform members of the 14-3-3 family (Aitken A. et al, 1995).

To validated the influence of *Streptococcus thermophilus* on 14-3-3σ expression we have quantified the mono-associated LMD9 mRNA using conventional RT-PCR (figure 5). Here we have noticed that 14-3-3σ expression was increased in mono-associated LMD9 in comparaison to Germ Free rats and this expression was quasi similar to the conventional rats at 30 cycles PCR. To have a robust confirmation we have tested the relative mRNA expression of 14-3-3σ using qRT-PCR (Figure 8) . significantly, the expression level of 14-3-3σ was increased in rats associated with *Streptococcus thermophilus* in comparaison to germ free rats by a mean factor of 1,96 (S.E. range is 0,951 - 4,317) and with *pvalue* =0,038. To further confirm the previous results we have quantified the expression of related protein using western blot analysis. We showed that 14-3-3σ protein was higher in the colonic epithelium cells of mono-associated LMD9 rats to germ free rats (figure 9).

A)biological_process Level 2



B) molecular_function Level 2



C) cellular_component Level 2

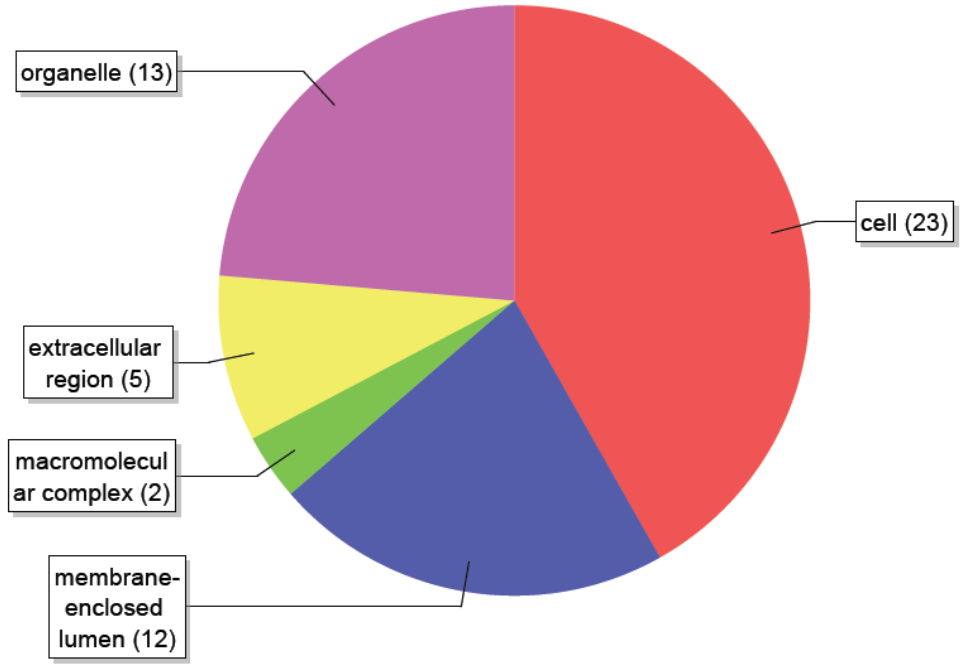


Figure 4:A-Distribution by biological processes, B-Molecular Functions, and C-Cellular Components involved of differentially expressed transcripts generated by SSH in the rat mono-associated with *Streptococcus thermophilus* LMD9 strain compared to Germ Free rat. Distribution was by Biological Processes, Molecular Functional, and Cellular Components among those sequences with associated GO terms according to the Blast2-GO software (level 2).

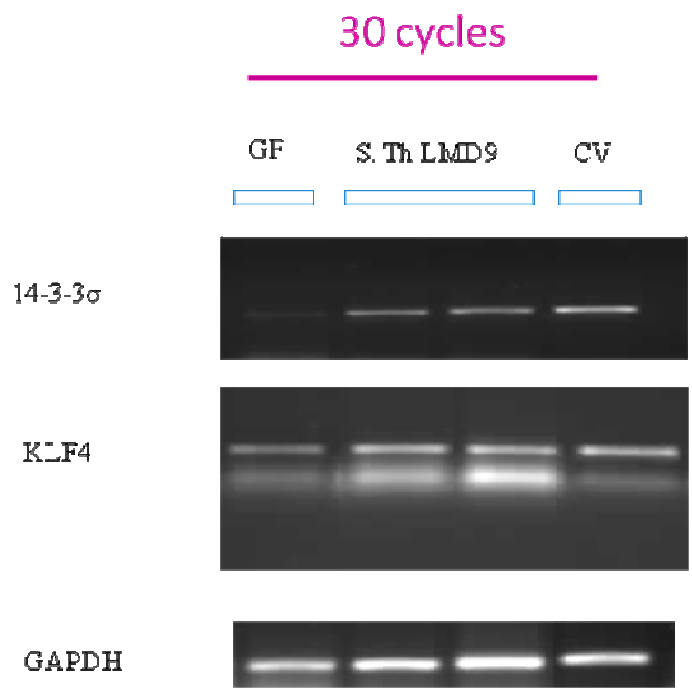


Figure 5: RT-PCR using specific primer to detect abundance of tow privilege genes 14-3-3σ and KLF 4 between 3 group of rat: Germ Free (GF), mono-associated (*S.th*) and conventional (CV). GAPDH is the endogenous control.

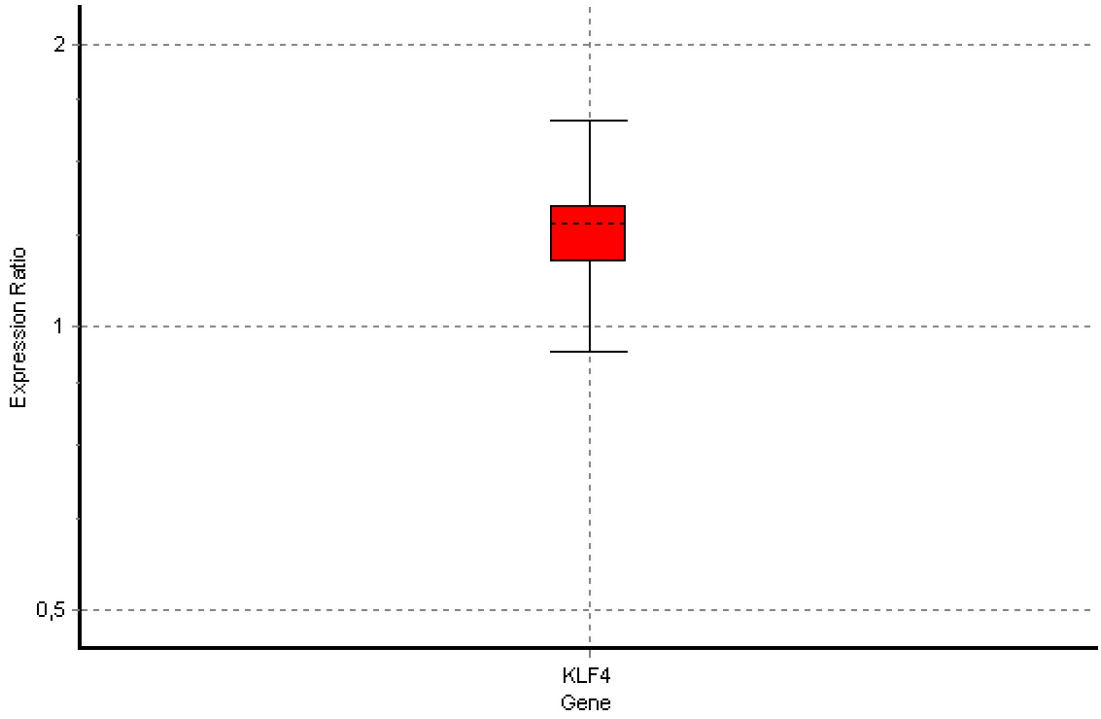


Figure 6: Boxes represents the interquartile range, or the middle 50% of observations. The dotted line represents the median gene expression. Whiskers represent the minimum and maximum observations. KLF4 is UP-regulated in sample group (in comparison to control group) by a mean factor of 1,265 (S.E. range is 1,008 - 1,524). KLF4 sample group is different to control group. P(H1)=0,031

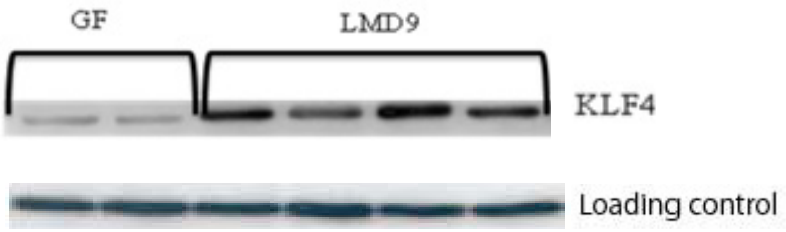


Figure 7: KLF4 detection in different mono-associated samples. Mono-associated LMD9 rats have been inoculated with *S. thermophilus*. Western blots were from colon cells.

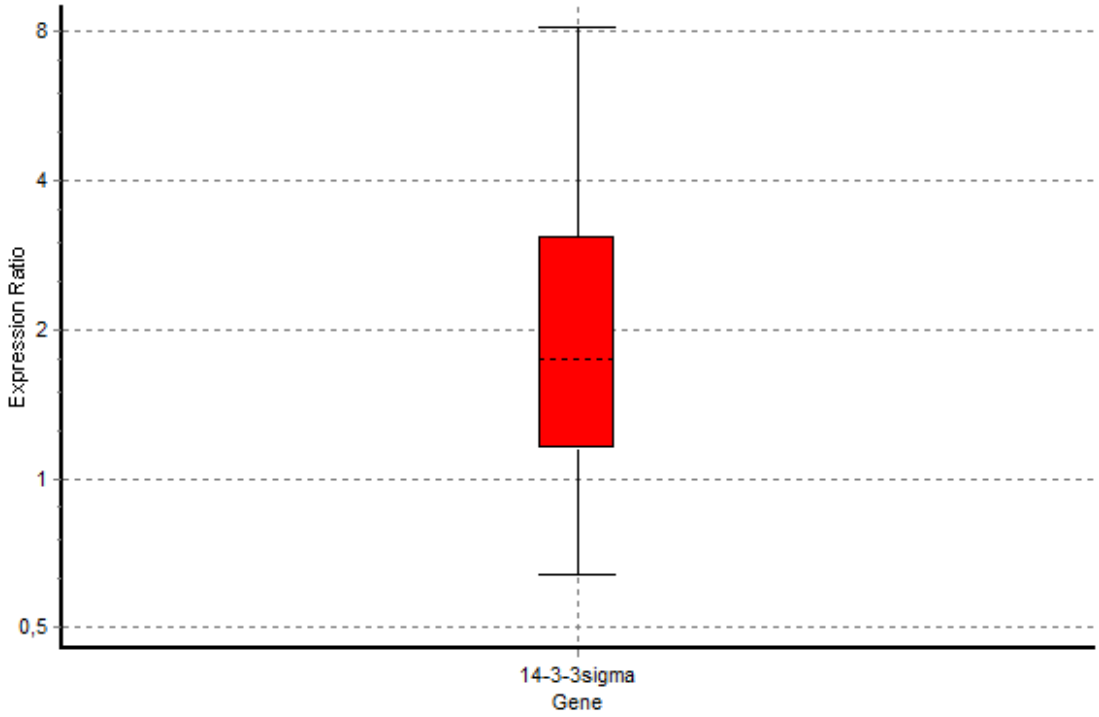


Figure 8: Boxes represents the interquartile range, or the middle 50% of observations. The dotted line represents the median gene expression. Whiskers represent the minimum and maximum observations. 14-3-3sigma is UP-regulated in sample group (in comparison to control group) by a mean factor of 1,963 (S.E. range is 0,951 - 4,317). 14-3-3sigma sample group is different to control group. P(H1)=0,038

Figure 9:14-3-3σ detection in different mono-associated LMD9 samples. Western blots were from colon cells.

IV) DISCUSSION

Streptococcus thermophilus is a lactic acid bacteria largely used by the food industry for production of yoghurt and cheese, among other products. In spite of its wide consumption, insufficient information is presently available on the molecular effects of its consumption at the level of intestinal cells. The research we discuss in this work aimed at studying the impact of *S. thermophilus* on colonic epithelial cells gene expression. In this study we have used a suppression subtractive hybridization to identify and study genes induced by *S. thermophilus* in colonic epithelial cells. The subtraction library was prepared subtracting mRNA between epithelial cells isolated from colonic mono-associated rats (inoculated with LMD9 strain of *S. thermophilus*) and germ-free rats (without bacteria).46 clones were sequenced after SSH. Thirty of these clones correspond to genes whose expression in colonic epithelial cells is potentially activated in the presence of *S.thermophilus.* Candidate genes were grouped into essential Functional groups: genes that play a role in cell-cycle arrest and regulation of proliferation and differentiation, genes involved in the pathway of cell-communication, genes involved in binding and genes involved in regulation of biological process. We have focused our interest on two candidatesthat were kruppel like factor 4 and 14-3-3σ involved in cell cycle. These two candidates genes were tested by RT-PCR to quantify mRNA and Western blot to analyze the related proteins in mono-associated animals versus germ-free animals and conventional animals. The results confirmed that selected candidate genes increase their expression in the presence of *S.thermophilus*.

Krupel like factore 4(klf4) was identified initially as a gene whose expression accompanies growth arrest (Shields JM et al, 1996). Further, it's *in vivo* pattern of expression mirrors that seen *in vitro* (Chen X. et al, 2003). Thus klf4 mRNA is found primarily in terminally differentiated and mitotically inert epithelial cells of the intestine (Shields JM et al, 1996, Chen et al, 2003) and epidermis (Garrett-Shinha LA. et al, 1996; Segre JA. et al, 1999). Chen et al,(Chen et al, 2001), have reported in a recent work using inducible RKO cell line that the inducible expression of klf4

result in an arrest in the cell cycle at the transition phase between G1 and S, and that this arrest is accompagnied by the activation of $p21^{WAF1/Cip1}$ a potent suppressor of cell proliferation (El-Deiry WS. et al, 1993). The role of klf4 in regulating differentiation of epithelial tissues was demonstrated by gene targeting experiments that confirmed the effect of klf4 on terminal differentiation of two epithelial cell types, epidermal keratinocytes (Segre JA. et all, 1999) and colonic goblet cells (Katz JP. et al, 2002) . Increased expression in vivo of klf4 in differentiated cell compartments, of the villi of the small intestine and near the surface of the mouse colonic crypts, was demonstrated by M. Flandez (Flandez M. et al, 2008).Recent study computed by Rull et al, untitled impact of the metabolic activity of *Streptococcus thermophilus* on the colon epithelium of gnotobiotic rats (Rull et al, 2010 submetted), have demonstrated that *S.thermophilus* increased $p27^{kip1}$ $p27^{kip1}$ is cyclin-dependent-kinases (CDKs) inhibitor and is preponderant cell cycle regulators in gut intestinal tract (Besson A. et al,2008). Our present study give in part an evidence to the previous observations and confirmed that *Streptococcus thermophilus* has a beneficially role to enhance and maintain colonic homeostasis. The present study has demonstrated that 14-3-3σ was another important gene differentially expressed. 14-3-3σ called also stratifin, an inhibitor of G2/M progression of the cell cycle following DNA damage (Hermeking H. et al, 1997; Chan TA. et al, 1999). Expression of the 14-3-3σ was activated by klf4 (Chen X. et al, 2003). However, our results of all experiments (Semi-quantitative RT-PCR, quantification of mRNA expression by quantitative PCR, and western blot analysis) demonstrated that it was a coordination between induction of klf4 and 14-3-3σ. Additionally, recent study reported by Yang et al, (Yang H. et al, 2006), demonstrated that 14-3-3σ mediated cell cycle arrest concurred with $p27^{Kip1}$ up-regulation and Akt inactivation.

In summary, using differential gene expression, we identified groups of genes that are differentially expressed upon the presence of Streptococcus thermophilus in a colonic epithelium cells. Functional analysis of all of transcripts generated by SSH reveal that they may mediate some and

most of the previously reported functions of klf4 and 14-3-3σ that were arrest of cell proliferation and promotion of differentiation process consequently maturation of colonic epithelium cells. Gene ontology has provide other most process involved by all of the generated transcripts, including transport, binding and trafficking, that were involved to maintain the right function of colonic epithelium.

These results indicate that *S. thermophilus* modifies the expression of genes involved in essential intestinal functions. They, combined with the observation that this bacterial genus is present in women breast milk and in the bowel of infants suggest that *S. thermophylus* may play a role in the early on-setting of colon correct structure and function.

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CHAPTER IV

Microaerray based indentification of colonic epithelium genes differentially expressed in the presence of *Streptococcus Thermophilus* bacteria

MICROAERRAY BASED INDENTIFICATION OF COLONIC EPITHELIUM GENES DIFFERENTIALLY EXPRESSED IN THE PRESENCE OF *STREPTOCOCCUS THERMOPHILUS* BACTERIA

Fatima Chegdani

ABSTRACT

Probiotics are deliberately ingested preparations of live bacterial species that confer health benefits on the host**.** However, the scientific evidence is still scarce and the mechanisms by which probiotics influence the host organism are only beginning to be explored. The initial step in the characterization of mechanism of action for functional food products is the identification of a specific interaction between the active component of this food and an effect in the host organism that is potentially beneficial for health. One approach to investigate these interactions is to map the changes in transcription profile of the host organism caused by nutrient intake. Recent development of expression profiling by the use of microarray technology has made it possible to monitor the expression of thousands of genes simultaneously, allowing systematic analysis of complex biological processes and offering an advantage of reducing bias in data collection, compared with the candidate gene-based approaches. In the present study , we have studied regulation of intestinal gene expression of the host organism after colonization for 30 days by probiotic bacteria strain. Oligonucleotide microarrays, using the advanced Combimatrix technology, were applied to compare global transcriptional profiles in the colonic epithelium rats receiving *streptococcus thermophilus* LMD9 with the control group of rat receiving a placebo product. The effect of *Streptococcus Thermophilus* on the colonic epithelium gene expression has been analyzed by the context of networks of interacting proteins to convert a complex dataset to biological understanding. We showed that *Streptococcus thermophilus* caused important changes on the transcriptional. profile of the host. This bacteria evoked a complex response in the gut, reflected by differential regulation of a number of genes involved in essential biological functions such as metabolic activity, transport activity, development and cell growth, which are essential of a healthy colonic functionality. In conclusion differential expression of an array of genes described in this report evokes novel hypothesis of possible interactions between the *Streptococcus thermophilus* bacteria and the host organism and warrants further studies to evaluate the functional significance of these transcriptional changes on the functionality of the host.

I) INTRODUCTION

Progress in molecular biology has provided methods to rapidly and comprehensively analyse genes and their products in a massively paralleled manner. The first high-throughput techniques developed were gene-sequencing methods, resulting in a great number of available genome sequences of different organisms. The comprehensive studies of active and regulated genes through transcriptomic techniques, such as microarrays, have promoted the study of molecular disease mechanisms. The understanding of the molecular basis of health is an even greater challenge, as a health status is less defined than a disease state. Nevertheless, global gene expression analysis begins to contribute also in this field. The analysis of gene expression deepens the understanding of regulatory networks, helps identifying diagnostic and prognostic biomarkers as well as potential targets for medical and nutritional intervention. Moreover, transcriptomic studies have improved the understanding of the complex interaction between genetic and environmental factors, such as lifestyle and nutrition (Hocquette, 2005) and have enabled the assessment of nutritional interventions at global gene expression level.

I-1) MICROARRAY TECHNOLOGY, ANALYSES AND INTERPRETATION OF DATA

Microarray experiments are, in principle and practice, extensions of hybridization-based methods that have been used for decades to identify and quantify nucleic acids in biological samples (E.M. Southern et al, 1975; F.C. Kafatos et al, 1979). Microarray technology utilizes gene-specific probes that represent individual genes which are arrayed on an inert substrate. Several types of microarray plat- forms are available (G. Hardiman et al,2004; F.E. Ahmed et al, 2006), but currently the most commonly used arrays are manufactured by companies such as Agilent (P.K. Wolber et al, 2006) or Affymetrix (D.D. Dalma-Weiszhausz et al, 2006). More recently, other arraying technologies have emerged, which offer the design of custom arrays at a lower cost, such as Combimatrix (modified semiconductor technology), MetriGenix (flow-through chip four-dimensional microarrays),

SuperArray (printed oligonucleotides or cDNAs on nylon matrix), or Illumina (probe-coated small beads deposited on the array surface). With this choice of custom arrays at hand, scientists can now design high-density arrays for virtually any gene set of any organism, provided sequence data are available. Studies conducted with customized array have already shown the benefit of complementing whole genome experiments by expression analysis of gene subsets (Comelli et al., 2002; Dougherty and Geschwind, 2002; Morris et al., 2005). The experimental procedure involves extraction of RNA from biological samples, followed by labeling with a detectable marker (typically a fluorescent dye). After hybridization to the array and subsequent washing, an image of the array is acquired by determining the extent of hybridization to each gene-specific probe (J. Quackenbush et al, 2006; M.B. Eisen et al, 1999). The data are then normalized to facilitate the comparison between the experimental samples. Next, a set of objective criteria is applied, for example the elimination of genes with minimal variance between the samples. The most common aim of transcriptome analysis is to find genes that are differentially expressed between the various experimental samples. Although early microarray papers used a simple 'fold change' approach to generate lists of differentially expressed genes, most analyses now rely on more sound statistical tests to identify differences in expression between groups (D.B. Allison et al, 2006). Unfortunately, there are no standards at the level of data filtering, which is often done according to personal preference and experience. This leads to discrepancies and prevents a high degree of reproducibility. It should also be emphasized that statistical significance is not the same as biological significance. Moreover, the classical approach of treating genes as independent entities is increasingly being criticized. The main reasons are first the arbitrariness of the chosen cut-off (e.g. false discovery rate, fold change) and second the disregards of the broader context in which gene products function. Although by design this approach will enable the identification of genes that show large changes in gene expression, it might not reveal small yet coordinated changes in gene expression in a set of related genes, which is often the case in nutrigenomics research. In response, testing for gene classes is becoming increasingly popular. Gene classes are traditionally based on

Gene Ontology categories (M. Ashburner et al, 2000), but recently also relationships based on e.g. metabolic pathways or signal transduction routes are taken into account (A. Subramanian et al, 2005). Gene class testing thus improves the identification of affected biological processes in microarray data sets, promoting greater understanding of the underlying mechanisms driving the observed differences between samples.

I-1-1) COMBIMATRIX MICROARRAY TECHNOLOGY

Application of oligonucleotide microarrays in different areas of molecular biology, bio-defense, and infectious disease monitoring has been rapidly growing during the last decade (Burton et al., 2005; Dobbin et al., 2005; Irizarry et al., 2005; Larkin et al., 2005). Commonly, microarray manufacturing is based on spotting of pre-synthesized oligonucleotides, inkjet depositing technologies (Hughes et al., 2001), and light-directed synthesis processes (Tan et al., 2003). Measurements of signal intensities of bio-components bound to the manufactured arrays are performed using fluorescent detection (laser scanning or imaging with a CCD camera) (Shi et al., 2005). CombiMatrix core technology is based on a specially modified semiconductor adapted for biological applications, which contains arrays of platinum microelectrodes. The semiconductor logic circuitry directs digitally controlled simultaneous synthesis of different oligonucleotides at thousands of electrodes in response to a computer software program. To perform a synthetic step, a microelectrode is activated to selectively generate acid by means of an electrochemical reaction. The generated acid in turned protects the growing oligonucleotide chain activating it for binding of the next nucleotide. Since a different oligonucleotide can be synthesized at each microelectrode, this technology enables one to design a microarray of any desired configuration. CombiMatrix has previously demonstrated feasibility of the Combi Matrix Custom Array 90 microarray platform for electrochemical detection in oligonucleotide hybridization assays and multiplexed immunoassays (Dill et al., 2004).

I-2) STATISTICS FOR MICROARRAY DATA ANALYSIS

Microarray technology takes advantage of hybridization properties of nucleic acid and uses complementary molecules attached to a solid surface, referred to as probe*s*, to measure the quantity of specific nucleic acid transcripts of interest that are present in a sample, referred to as the *target*. The molecules in the target are labeled, and a specialized scanner is used to measure the amount of hybridized target at each probe, which is reported as an intensity. Various manufacturers provide a large assortment of different platforms. Most manufacturers, realizing the effects of optical noise and non-specific binding, include features in their arrays to directly measure these effects. The raw or *probe-level* data are the intensities read for each of these components. In practice, various sources of variation need to be accounted for, and these data are heavily manipulated before one obtains the genomic-level measurements that most biologists and clinicians use in their research. This procedure is commonly referred to as preprocessing. The different platforms can be divided into two main classes that are differentiated by the type of data they produce. The *high-density oligonucleotide array* platforms produce one set of probe-level data per microarray with some probes designed to measure specific binding and others to measure non-specific binding. The two-color spotted platforms produce two sets of probe-level data per microarray (the red and green channels), and local background noise levels are measured from areas in the glass slide not containing probe. Despite the differences among the different platforms, there are some tasks that are common to all microarray technology.

I-2-1) PREPROCESSING OF MICROARRAY DATA

Preprocessing can be divided into 6 tasks: image analysis, data import, background adjustment, normalization, summarization, and quality assessment.

Image analysis: permits us to convert the pixel intensities in the scanned images into probe-level data. Flexible data import methods are needed because data come in different formats and are often

scattered across a number of files or database tables from which they need to be extracted and organized.

Background adjustment: is essential because part of the measured probe intensities are due to non-specific hybridization and the noise in the optical detection system. Observed intensities need to be adjusted to give accurate measurements of specific hybridization.

Normalization: Without proper normalization, it is impossible to compare measurements from different array hybridizations due to many obscuring sources of variation. These include different efficiencies of reverse transcription, labeling, or hybridization reactions, physical problems with the arrays, reagent batch effects, and laboratory conditions. For each gene, the background adjusted and normalized intensities need to be summarized into one quantity that estimates an amount proportional to the amount of RNA transcript.

Quality assessment: is an important procedure that detects divergent measurements beyond the acceptable level of random fluctuations. These data are usually flagged and not used, or down weighted, in subsequent statistical analyses. The complex nature of microarray data and data formats makes it necessary to have flexible and efficient statistical methodology and software.

I-2-2) DIFFERENTIAL GENE EXPRESSION

Differential expression analysis of microarray data is fraught with many classical statistical issues, such as appropriate test statistics, replicate structure, sample size, outlier detection and statistical significance of results. The original and simplest approach to identifying differentially expressed genes was to use a fold change criteria; selecting cutoff was something of an ad-hoc procedure; a 2-fold change was however thought as being a suitable cutoff. This selection process is however, completely biased towards individual genes with large fold changes and completely disregards the fact that groups of related genes showing smaller deviations could be just as important and also does not allow for assessment of significance of expression differences in the presence of biological

and experimental Biological experiments are continuously generating more and more data. It has become nearly impossible to analyze a biological experiment without statistics and bioinformatics. Bioconductor is an open-source / open-development set of tools which can be widely employed in a number of genetic and biomedical settings. Bioconductor was started in the Fall of 2001. The core maintainers and hosts of the Bioconductor websites are located at Fred Hutchinson Cancer Research Center. A new version of Bioconductor is released twice-anually and is linked to the release of R (http://cran.at.r-project.org). R is a comprehensive statistical environment and programming language for professional data analysis and graphical display. The associated BioConductor project provides many additional R packages for statistical data analysis in different life science areas, such as tools for microarray, sequence and genome analysis.

There are a number of statistical tests available that can be applied to assess differential expression between populations of microarray data, such as the *t*-test, which can be used to assess the statistical probability that, given the number of samples available, the true expression levels for a given gene differ in the overall populations. In such an analysis, the number of samples is invariably far less than the number of genes which are being investigated. The number of genes could run into tens of thousands, but the number of arrays used will, due to overall cost or rarity of tissue samples, rarely exceed thirty, thus creating a multiple testing problem. For example, on an array of 25,000 genes, if even 5% are misinterpreted as being differentially expressed, or "false positives", then »1,250 genes will be construed as being differentially expressed when they are in fact not. There are a number of solutions available to the problem of false positives which result from the large number of variables in a statistical test; they include False Discovery Rate (FDR) developed by Benjamini and Hochberg (1995) , or the more stringent Bonferroni Method which controls the family-wise error rate. These and other methods can be applied to address the problem of false positives in microarray gene expression analysis. One of most functions available in Bioconductors was LIMMA package to calculate differential expression of GeneChip, dual dye and single dye data, as the same principals can be applied to all of these data types.

Limma "Linear model"

Limma uses linear models to analyze designed microarray experiments (Yang and Speed, 2003; Smyth, 2004). This approach allows very general experiments to be analyzed nearly as easily as a simple replicated. Limma provides functions topTable and decideTests, which summarize the results of the linear model, perform hypothesis tests and adjust the *p*-values for multiple testing. Results include (log) fold changes, standard errors, *t*-statistics, and *p*-values. The basic statistic used for significance analysis is the moderated *t*-statistic, which is computed for each probe and for each contrast. This has the same interpretation as an ordinary *t*-statistic except that the standard errors have been moderated across genes, i.e., shrunk toward a common value, using a simple Bayesian model. This has the effect of borrowing information from the ensemble of genes to aid with inference about each individual gene (Smyth, 2004). Moderated *t*-statistics lead to *p*-values in the same way that ordinary *t*-statistics do except that the degrees of freedom are increased, reflecting the greater reliability associated with the smoothed standard errors. A number of summary statistics are presented by topTable for the top genes and the selected contrast. The most popular form of adjustment is "fdr", which is Benjamini and Hochberg's method to control the false discovery rate (Benjamini and Hochberg, 1995). The meaning of "fdr" adjusted *p*-values is as follows. If all genes with *p*-value below a threshold, say 0.05, are selected as differentially expressed, then the expected proportion of false discoveries in the selected group is controlled to be less than the threshold value, in this case 5%. The *B*-statistic (lods or B) is the log-odds that the gene is differentially expressed (Smyth, 2004). A *B*-statistic of zero corresponds to a 50-50 chance that the gene is differentially expressed. The *B*-statistic is automatically adjusted for multiple testing by assuming that 1% of the genes, or some other percentage specified by the user in the call to eBayes, are expected to be differentially expressed. The *p*-values and *B*-statistics will normally rank genes in the same order. The eBayes function computes one more useful statistic. The moderated *F*-statistic (F) combines the *t*-statistics for all the contrasts into an overall test of significance for that gene. The *F*-statistic tests

whether any of the contrasts are non-zero for that gene, i.e., whether that gene is differentially expressed on any contrast.

I-3) FUNCTIONAL ANALYSIS

The relationships between genes and their involvement in specific cellular functions can be better characterized. However, owing to large number of gens and to the small number of samples, there are many many statistical problems associated with microarray data(Audic S. et al, 1997; Eisen MB. et al, 1998), which mackes the detection of differential gene expression a challenging task. One of the main problems is the huge amount of data generated by microarray technology. Consequently , algorithms such as Ingenuity Pathway Analysis, LSGraph, Cognia Molecular, Metacore, ecct…, were developed to analyse and understand complex biological systems, distinguishing functional categories of genes and association and interaction between genes.

Ingenuity Pathway Analysis.

Ingenuity Systems is a leading provider of information solutions for the exploration, interpretation, and analysis of life science information. Ingenuity's products and services have one common goal to help life science researchers generate maximum value from all types of biological and chemical knowledge. Ingenuity offers a broad range of flexible solutions that can be tailored to the needs of its clients, including academic and therapeutic area researchers, computational biologists and informatics departments, and suppliers in the life sciences industry. All Ingenuity products leverage the Ingenuity knowledge base, the largest database of its kind, which houses biological and chemical relationships extracted from the scientific literature. Today, Ingenuity's solutions are used by thousands of researchers at hundreds of leading pharmaceutical, biotechnology, and academic research institutions worldwide. Ingenuity was founded in 1998 and is headquartered in Redwood City, California with offices in Germany, Switzerland, France, the United Kingdom, Australia, and Japan.

IPA is an all-in-one, web-based application that enables people to analyze data derived from gene expression and SNP microarrays, metabolomics and proteomics experiments, and smaller-scale experiments that generate gene lists. With IPA researchers can search for targeted information on genes, proteins, chemicals, and drugs, and build interactive models of the experimental systems. IPA's data analysis and search capabilities help to understand the significance the data, specific target, or candidate biomarker in the context of larger biological or chemical systems, backed by the Ingenuity knowledge base of highly structured, detail-rich biological and chemical findings.

II) AIM OF MICROARRAY STUDY

During the last decades the role of the diet in development, as well as in prevention and management, of many diseases has been subjected to intense research. The term 'functional food'

has been adapted to denote foods that may provide a health benefit beyond basic nutrition (Saris et al. 1998). The oldest and probably best-known functional food products are health-promoting bacteria or probiotics, defined as live microbial dietary supplements that beneficially affect consumers through their effects in the intestinal tract (Roberfroid,2000). At present, probiotics, most often belonging to the species *Streptococcus thermophilus* , are almost exclusively consumed as fermented dairy products, such as yoghurt or cheese . Several health-related effects associated with the intake of probiotics have been reported in different animal models as well as in human studies (Roberfroid,2000; Turpin et al, 2010). However, the scientific evidence is still scarce and the mechanisms by which probiotics influence the host organism are only beginning to be explored. The initial step in the characterization of mechanism of action for functional food products is the identification of a specific interaction between the active component of this food and an effect in the host organism that is potentially beneficial for health. One approach to investigate these interactions is to map the changes in transcription profile of the host organism caused by nutrient intake. Recent development of expression profiling by the use of microarray technology has made it possible to

monitor the expression of thousands of genes simultaneously, allowing systematic analysis of complex biological processes and offering an advantage of reducing bias in data collection, compared with the candidate gene-based approaches. In the present section , we have studied regulation of intestinal gene expression of the host organism after colonization for 30 days by probiotic bacteria strain. Oligonucleotide microarrays were applied to compare global transcriptional profiles in the colonic epithelium rats receiving *streptococcus thermophilus* LMD9 with the control group of rat receiving a placebo product.

III) MATERIAL AND METHODS

III-1) ANIMALS AND EXPERIMENTAL DESIGN.

All procedures were carried out according to the European and French guidelines for the care and use of laboratory animals (permission 78-123, dedicated to MT). At the age of two months, three germ-free (GF) rats (male, Fisher 344) were inoculated by oral administration with one ml of *S.thermophilus* LMD9 (Ino-LMD9) in Nilac milk (5x108 *S.thermophilus*/ml) . As a control, three GF rats were also inoculated with one ml of sterile Nilac milk (without bacteria). GF, and mono-associated rats were housed in sterile Plexiglas isolators (Ingénia, Vitry-sur-Seine, France). All groups of rats received the same standard diet (UAR), which was sterilized by gamma irradiation. In addition, germfree rats received supplemental vitamin K in their drinking water. Twice a week during the experiment, *S. thermophilus* (Ino-LMD9) was enumerated by plating serial dilutions of the faeces. The feces of germfree rats were also checked weekly for the absence of microorganisms. All rats were euthanized at three months of age, *i.e*, 30 days after inoculation. At 9:00h, rats were anesthetized with isoflurane, and the colons were removed rapidly and immediately used for cell isolation procedure.

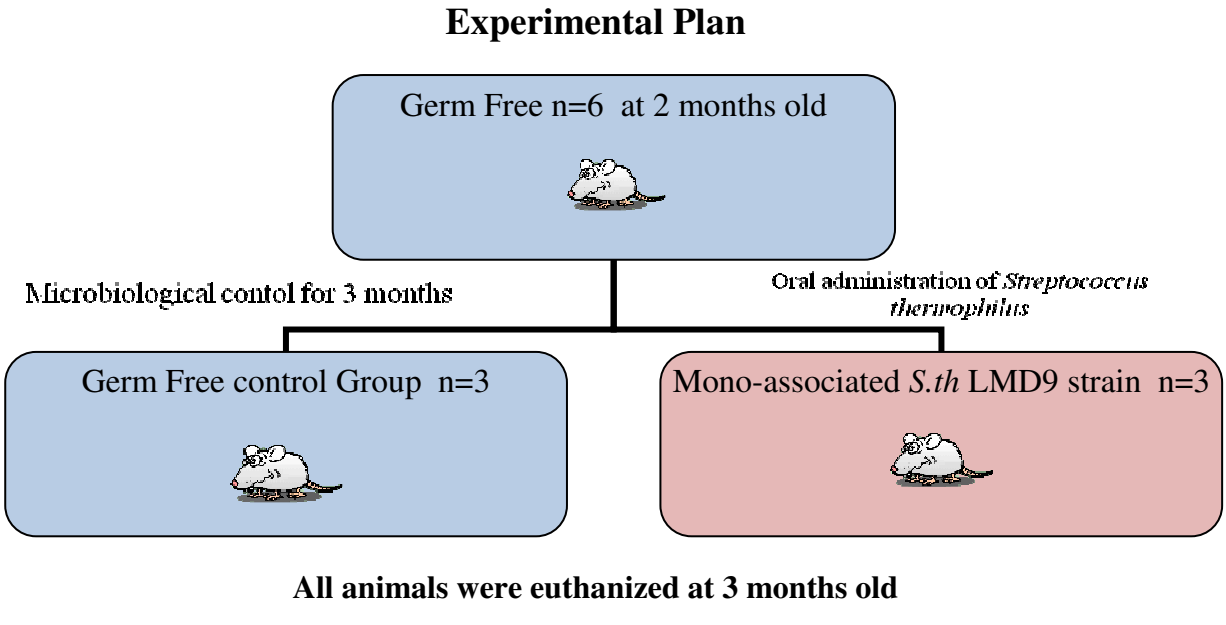


Figure 1: Schematic diagram summarizing the number of animal and groups used for the experiment.

III-2) CELL ISOLATION PROCEDURES

Colonic cells originating from both groups of rats were isolated using a protocol previously described by Cherbuy *et al* (Cherbuy C *et al*, 1995&2004). Briefly: the colons removed were flushed clean, first with NaCI (9 g/L) and then with a Ca^{2+}- and Mg^{2+}-free Krebs'-Henseleit bicarbonate buffer (pH 7.4) containing 10 mmol/L HEPES, 5 mmol/L dithiothreitol, and 2.5 *glL* albumin and equilibrated against a mixture of O_2/CO_2 (19:1, vol/vol). Thereafter, the colons were perfused for 20 minutes (30 mL/min at 37°C) with the same buffer containing 10 mmol/L EDTA. Then, the colons were gently squeezed and the luminal fluid collected and centrifuged (150x*g* for 3 minutes) to harvest the cell fraction. An additional 15 minutes incubation was performed in a shaking water bath (100 oscillations/min at 37°C) using the same buffer without EDTA and in the presence of hyaluronidase (4 g/L). The isolated cells were washed twice and resuspended in the same bicarbonate buffer medium (pH 7.4) without dithiothreitol and containing $CaC1_2$ (1.3 mmol/L), $MgCl_2$ (2 mmol/L), and 10 g/L albumin (incubation buffer). After two washes and centrifugations, the colonic cell pellet was immediately used for RNA extraction.

III-3) GENE EXPRESSION ANALYSIS

III-3-1) TARGET PREPARATION AND HYBRIDIZATION TO MICROARRAYS

Total RNA was extracted from epithelial cells by the guanidinium thiocyanate method (Chomczynski P *et al*, 1987). The RNA yield was quantified by Biophotometer (Eppendorf AG Hamburg, Germany) and the RNA purity was determined with the A260-A280 ratio. Further, the quality and integrity of the RNA were assessed with an RNA 6000 Nano LabChip Kit using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) . After the quality controls, the total RNA extracted, from controls (GF) and treated (LMD9) rats was amplified using the RNA ampULSe Amplification and Labeling Kit (KREATECH's ULS™ RNA ampULSe with cy5 for combimatrix arrays: Amplification and Labeling Kit; KREATECH BIOTECHNOLOGY, Amsterdam) to generate antisense RNA (aRNA) which was Labeled and prepared for Hybridization. Steps of amplification were performed according to the standard manufacturer's protocol (KREATECH BIOTECHNOLOGY). Briefly: 1000 ng of total RNA was transcribed to synthesize First Strand cDNA using T7 Oligo(dT) primer and incubation 10minutes at 70 °C using thermal cycler a second incubation for 2 hours at 42 was performed, using an air incubator (GRANT BOIKEL, MOD: KIR10M), after added the following reagents: 10X First Strand Buffer, dNTP Mix, RNase Inhibitor, and ArrayScript.

To synthesize the second strand cDNA, the following reagent were added directly to the first – strand reaction mixture: Nuclease-free Water, 10X Second Strand Buffer, dNTP Mix, DNA Polymerase, and RNase H. and all mixture was Incubated for 2 hr in a16°C thermal cycler.

After incubation the product was subjected to a direct purification step. This step is designed to prevent carryover of unincorporated dNTPs, Primers, and inactivated enzymes into the subsequent in vitro transcription reaction. The purified double stranded cDNA was subjected to a linear amplification by in vitro transcription to generate aRNA. At room temp, an in vitro transcription(

IVT) Master Mix was prepared by adding the following reagents to a nuclease-free microfuge tube: Double-stranded cDNA in Nuclease-free Water, T7 rNTP mix (75mM), T7 10X Reaction Buffer, and T7 Enzyme Mix. Once assembled, the samples were incubated using an in air incubator (GRANT BOIKEL, MOD: KIR10M) at 37°C for 14 hours. After Stop the reaction by adding Nuclease-free Water to each aRNA, a purification step was recommended to removes enzymes, salts and unincorporated nucleotides from the aRNA. At the end of the purification, the aRNA is eluted from the filter with Nuclease-free Water. Concentration of aRNA was determined by measuring the absorbance at 260nm. aRNA products were conserver at - 80°C or directly labelled using ULS aRNA Fluorescent Labelling kit KREATECH's ULS™ RNA ampULSe with cy5 for combimatrix arrays: Amplification and Labeling Kit; KREATECH BIOTECHNOLOGY, Amsterdam). 5 ugs of each purified aRNA were added to 5 ul of cy5, a 1/10 volume of 10x Labeling solution was added and the final volume was adjusted with RNase-free water for each sample. Samples were Labeled by incubation for 30 min at 85ºC and finally purified using the *KREApure* columns. The product were assessed by measuring A260 and A650 for determining the DOL of Cy5-ULS labeled aRNA and the degree of labeling (DOL) using the following formula:

- ng / µL =A260 *dilution factor* 40/cuvet length (in cm)

Degree of labeling (amount of dyes per 100 nucleotides)

- Labeling % = (340 * pmoldye /ng nucleic acid *1000) x 100%

Gene expression experiments were performed according to the standard CombiMatrix protocol described in detail at http://www.combimatrix.com/docs/CustomArray 90K Protocol.pdf. according to manufacturer's protocol. Briefly: before hybridization step the microarray was Filled with nuclease-free water and incubated at 65 ^{o}C for 10 minutes to hydrate it. A pre-hybridization solution was introduced into hybridization chamber and incubated at 42 °C for 30 min using Rotisserie oven (GRANT BOIKEL; KIR10M). 5X RNA fragmentation solution were prepared and a defined volume (see: CustomArray™ 90K Microarray: Hybridization and Imaging Protocol (PTL020)) was added to 4 ug of labeled aRNA of each sample. Samples were incubated for 20min at 95°C

thermo cycler and the entire Fragmentation Reaction volume was added to the Hybridization Solution. the product was Denatured at 95^{o}C for 3 minutes and Filled into the hybridization chamber. The microarrays were placed onto the rotisserie in the hybridization oven and incubate at 42 °C for 16 hours with gentle rotation. Figure 2 summarize the Hybridization and Imaging of CustomArray™ 90K Workflow Fluorochrome. After hybridization the arrays were then washed and conserved in PBS solution for imagining.

Slides were then scanned using a Gene Pix Professional 4200A scanner (Molecular Devices, Sunnyvale, CA, USA) ,that operate using sequential scanning, at variable photomultiplier tube to obtain optimized signal intensities, the PMT was optimized to obtain 2^{-6} level of saturation. The image was saved as a tiff image file. Spot identification, intensity quantification, and data filtered and extracted was performed using GenePix Pro software 6.0 (Molecular Devices, Sunnyvale, CA, USA) .

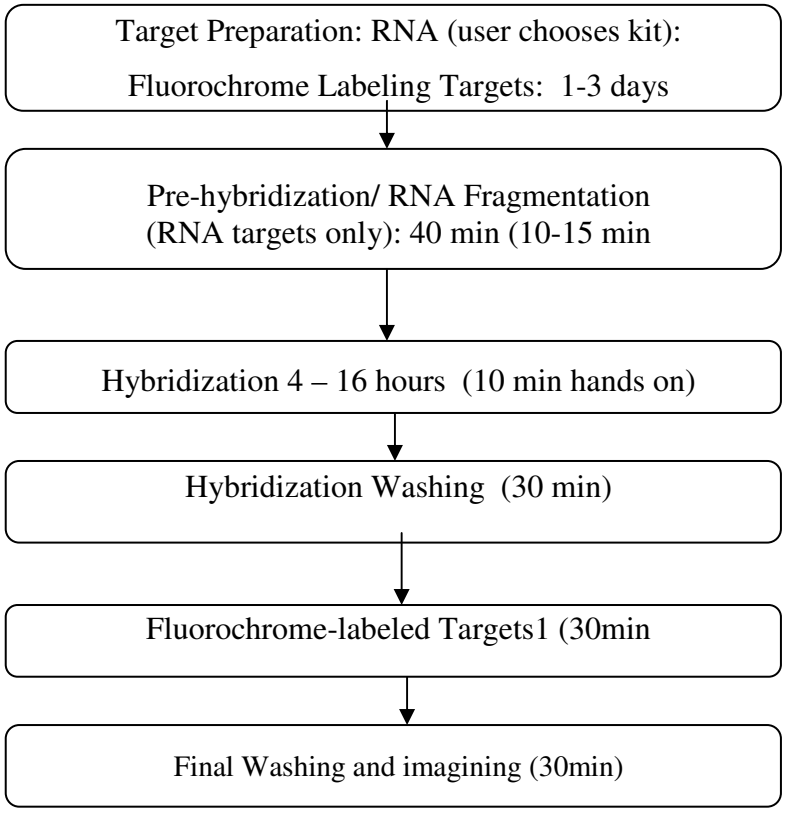


Figure 2: Hybridization and Imaging of CustomArray™ 90K Workflow Fluorochrome.

III-3-2) MICROARRAY RE-USE (MICROARRAY STRIPPING)

The microarray stripping procedure was performed using the CombiMatrix CustomArrayTM Stripping Kit according to the standard protocol: http://www.combimatrix.com/docs/ StrippingProtocol CustomArray90K.pdf (Stripping and Preparation of 90K Microarrays for Re-hybridization Protocol) . The stripped microarrays were stored then store wet in Imaging Solution or 1X PBS at 4 ^{o}C for a maximum of 2 weeks. The CombiMatrix platform is the first and only commercially available microarray that can be stripped from hybridized targets and re-used three times resulting in four uses for a single microarray. Figure 3 summarize the workflow of the stripping and re-hybridization of microarray.

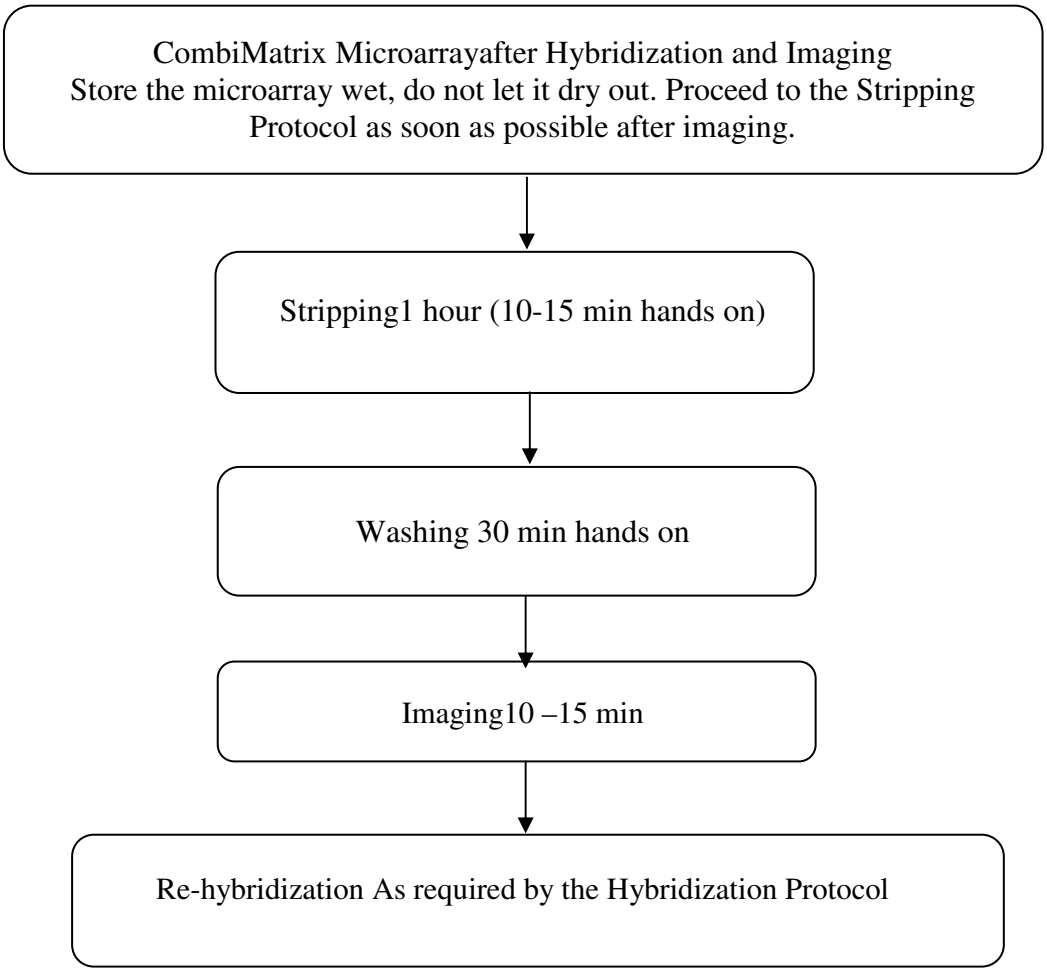


Figure 3: Stripping and Preparation of 90K Microarrays for Re-hybridization Protocol Workflow

III-3-3) MICROARRAY SYNTHESIS

Transcriptomic analysis was performed on a 90K Rattus 1.1 microarray synthesized using the CombiMatrix platform at the Plant Functional Genomics Center of the University of Verona http://ddlab.sci.univr.it/Functional_ Genomics/. The 90K Rattus 1.1 array contains 90,000 silicon electrodes including 30,082 unique 35-40 mer DNA oligonucleotide probes synthesized *in situ* with three replications. The target sequences for designing probes to be included on the microarray were selected from the RefSeq (http://www.ncbi.nlm.nih.gov/projects/RefSeq/) and UniGene (http://www.ncbi.nlm.nih.gov/unigene/) databases. The Reference Sequence (RefSeq) collection aims to provide a comprehensive, integrated, non-redundant, well-annotated set of sequences and the UniGene collection represents an organized view of the transciptome where each entry is a set of transcript sequences that appear to come from the same transcription locus. The full dataset of rat RefSeq transcript sequences (35033 entries on April, 8th 2009) where selected from the mammalian dataset downloaded from ftp://ftp.ncbi.nih.gov/refseq/release/vertebrate_mammalian/. The unique UniGene sequences (63440 entries on March, 29th 2009) where downloaded from ftp://ftp.ncbi.nih.gov/repository/UniGene/Rattus_norvegicus/Rn.seq.uniq.gz. In total 98473 rat sequences were available. Since part of the UniGene entries are represented in the RefSeq database the dataset was filtered in order to reduce redundancy. Identical or highly similar sequences (E-value > e^{-100}) where identified with a custom script employing Blast (Altschul et al. 1997; Gish 1996-2009) and the longest sequence among two or more similar sequences was selected giving priority to RefSeq entries. After the filtering 59815 unique sequences, including 55 sequences from SSH experiments, constituted the final dataset used to design the probes with the OligoArray 2.1. software (http://berry.engin.umich.edu/oligoarray2_1/utilization.html) . From the output of the software, about 30000 unique probes were selected, matching 25198 RefSeq, 4721 UniGene and 46 SSH targets. The selection was performed by giving priority to RefSeq targets, longer sequences and available annotation derivedretrieved from NCBI database or by similarity with known protein

in other species. The limited selection of 30000 probes was necessary in order to allow the representation of three replicates for each target on the chip. Only the sequences with the necessary thermodynamics allowing the design of specific probes were represented on the chip . These three replicates of each probe were distributed randomly across the array to control for internal variability. The Table 1 presents the number and descriptions hows the composition of the final layout of the microarray. Besides the probes for the target genes, the array carries 31 different housekeeping genes (replicated three time), selected from the commercial arrays for rat prepared by Combimatrix, and used as positive controls to evaluate labeling and sample hybridization. This is based on the assumption that these genes show a constant expression between samples. However, no set of housekeeping genes has proven to be universally stable. In addition, these housekeeping genes are often highly expressed and therefore possibly influenced by saturation effects. Moreover, probes targeting phage, bacterial and plant DNA used as negative control and probes for quality and factory controls were included in the layout. These last probes were used of quality control of data by indentified outlier samples, contamination samples and or decide whether a sample needs to be repeated or removed from data set.

Probe Number	Probe discriptions
25198	Refseq
4721	Unigene
46	Transcripts generated by supressive subtractive hybridization (SSH)
31	Housekeeping
32	negative controls (lambda phage, *Agrobacterium tumefaciens*, *Alteromonas* phage, *Arabidopsis thaliana*)
19	Quality Controls (controls of microarray hybridization quality)
35	factory negative (controls

Table1: number and description of probe 90K Rattus microarray synthesized using the CombiMatrix platform.

The Oligoarray 2.1. software (Rouillard et al, 2003). computes oligonucleotide specificity by searching for similar sequences in a database containing all the transcribed sequence derived from

the genome. When the genome sequence is not known, the Blast database that is used to compute oligo's specificity is represented by the set of transcribed sequences often derived from the expressed sequence tags (EST) available.

III-3-4) MICROARRAY DATA ANALYSIS

Before any kind of microarray data analyzed for differential expression we have taken several preprocessing steps to assesses raw data quality and to ensure its integrity. The most appropriate method that we have used to preprocess the raw data before differential expression analysis were linked at least.

III-3-4-1) Background Correction

The first step is to background correct the intensity reading for each spot. Background fluorescence can arise from many sources, such as non-specific binding of labeled sample to the array surface, processing effects such as deposits left after the wash stage or optical noise from the scanner. There is always some level of background noise, even if nothing but sterile water is labeled and hybridized to the array, some fluorescence will still be picked up by the scanner. For our analysis we have used "normexp" (Matthew E et al, 2007) a new method has been implemented from Bioconductor's limma package. This method offers a very important advantage over other background corrections: It dosen't produce negative values. This means that we don't loose any spot, even if very high background signal was measured.

III-3-4-2) Normalization between arrays

Oligonucleotide microarrays often suffer substantial scale differences because of technical variation, which could be down to any number of factors. Performing normalization between arrays will compensate for such effects and thus yield more reliable results. The purpose of this step is to

adjust data for technical variation, as opposed to biological differences between the samples. There will always be slight discrepancy between the hybridization processes for each array and these variations tend to lead to scaling differences between the overall fluorescence intensity levels of various arrays.The Quantile normalization method was implemented through the Affy module of the bioconductor microarray analysis. The goal of the Quantile method is to make the distribution of probe intensities for each array in a set of arrays the same.

III-3-4-3) Data Quality Assessment Methods

Quality assessment is an important phase that applies to analysis of all types of microarrays. Quality assessment of data ensures that the best use is made of the information available and ensures meaningful results at the end of an analysis. Boxplot is is a convenient means by which to compare the probe intensity levels between the arrays of a dataset. For microarray data, these graphs are always constructed using log2 transformed probe intensity values, as the graph would be virtually unreadable using raw values.

III-3-4-4) Identification of differentially expressed genes

To assess inter-animal variation, colonic epithelium samples were not pooled in this experiment. Therefore, each animal was analyzed as an individual experimental unit. Differential expression of the microarray data was evaluated using the limma (Smyth GK, 2004) a R library which is part of the bioconductor project . This procedure first fit a linear model to the expression data for each probe. Differences between groups were then extracted from the model as contrasts (LMD9 versus Germ Free). An empirical Bayes ‘‘shrinkage’’ method was employed on the standard errors to improve power for small sample sizes (Smyth GK, 2004). Lastly, multiple test correction of p-values was done using the false discovery rate (FDR) method (Benjamini Y et al, 1995). Gene transcripts having $\geq$1 log-fold change and FDR $\geq$ 0.1 were considered significantly different.

III-3-5) NETWORKING ANALYSIS

The analysis was performed using Gene Ontology (Ensembl; http:/ www.**ensembl**.org) to identify the functional categories of our list of genes differentially expressed, and Ingenuity Pathway Analysis (IPA; http://www.ingenuity.com , Redwood City, CA). IPA is a web –based application that enables the discovery, visualization , and exploration of functions, canonical pathway, and interaction networks, the software relies on currently known relationships (i.e., published manuscripts) among human, mouse, and rat genes/proteins. The IPA Functional Analysis identifies the biological functions, canonical pathway, and networks that are most significant to the data set. Genes from the dataset that meet the cutoff of p 0.05 and are associated with biological functions in the ingenuity knowledge base are considered for the analysis.

III-3-5-1) Network Generation

A data set of genes (differentially expressed in the mono-associated rats models relative to control) containing gene identifiers differentially expressed were uploaded into the application. Each gene identifier was mapped to its corresponding gene object in the Ingenuity Pathways Knowledge Base. These genes, called focus genes, were overlaid onto a global molecular network developed from information contained in the Ingenuity Pathways Knowledge Base. Networks of these focus genes were then algorithmically generated based on their connectivity.

III-3-5-2) Functional analysis

Functional Analysis identified the biological functions that were most significant to the data set. Genes from the dataset were associated with biological functions in the Ingenuity Pathways Knowledge Base were considered for the analysis. Fischer's exact test was used to calculate a p-value determining the probability that each biological function assigned to that data set is due to chance alone.

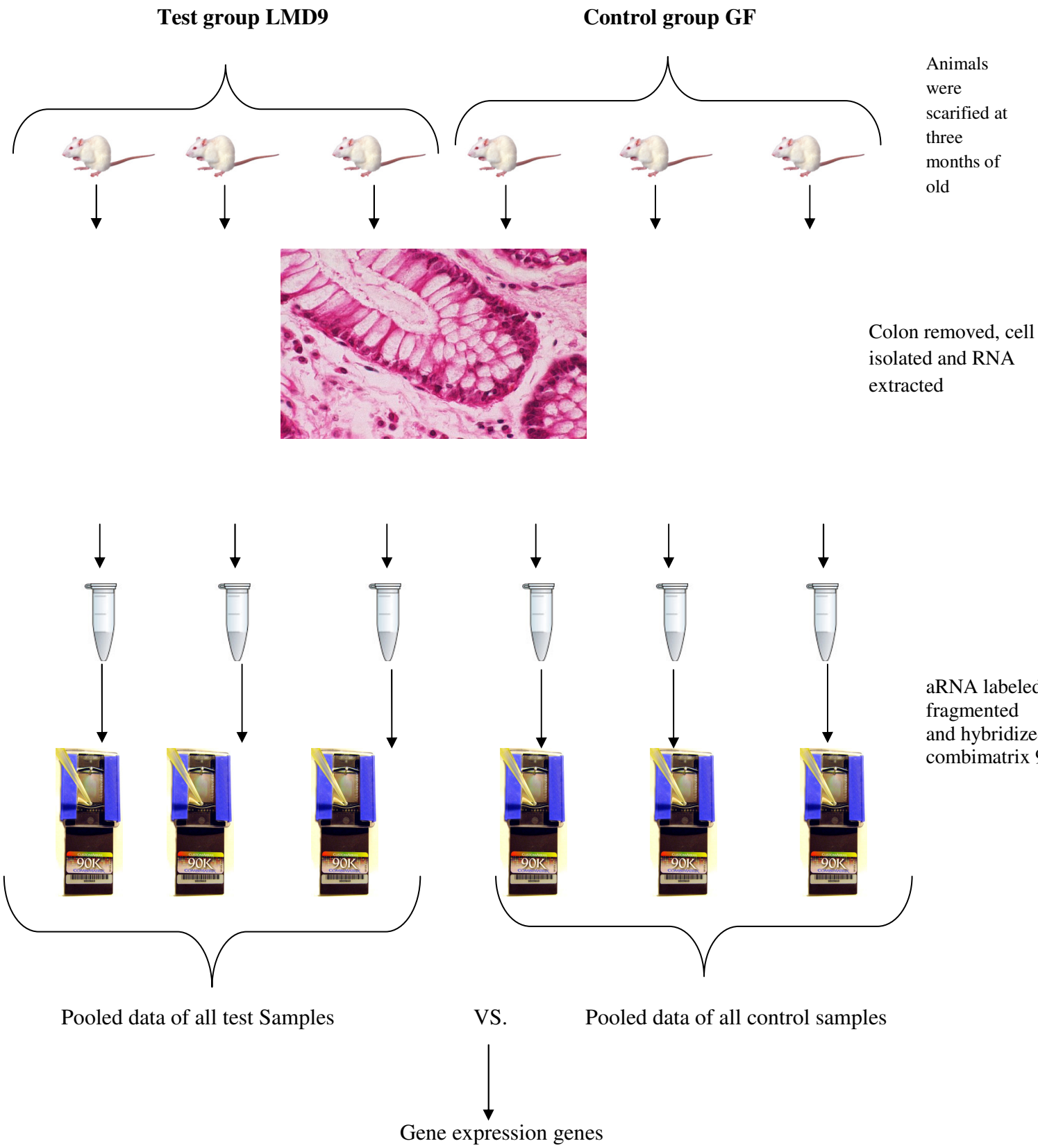


Figure 4: Schematic diagram illustrating the design of the pilot microarray experiment that investigated gene changes in the colonic epithelium of mono-associated rat versus Germ free rats .

IV) RESULTS

II-1) Association of Germ-Free rats with *Streptococcus thermophilus*

Two groups of three 3-weeks-old, germ-free male rats belonging to the Ficher inbred strain 344 and maintained on a standard diet,one of the two group was colonized with a single gavage of 10^9 colony forming units (CFU) of *Streptococcus thermophilus* in fermented milk (Rul F.et al, 2010 submetted) And the second group was maintained as a control. The rats were sacrificed 30 days later, a period of time sufficient for *S. thermophilus* to colonize the colonic epithelium. The number of viable bacteria was determined in the faeces of each rat during 4 week and at the time of their sacrifice (Figure1). Furthermore, the colonization experiment revealed that the *S.thermophilus* strain LMD9 can implanted after one time of adaptation.

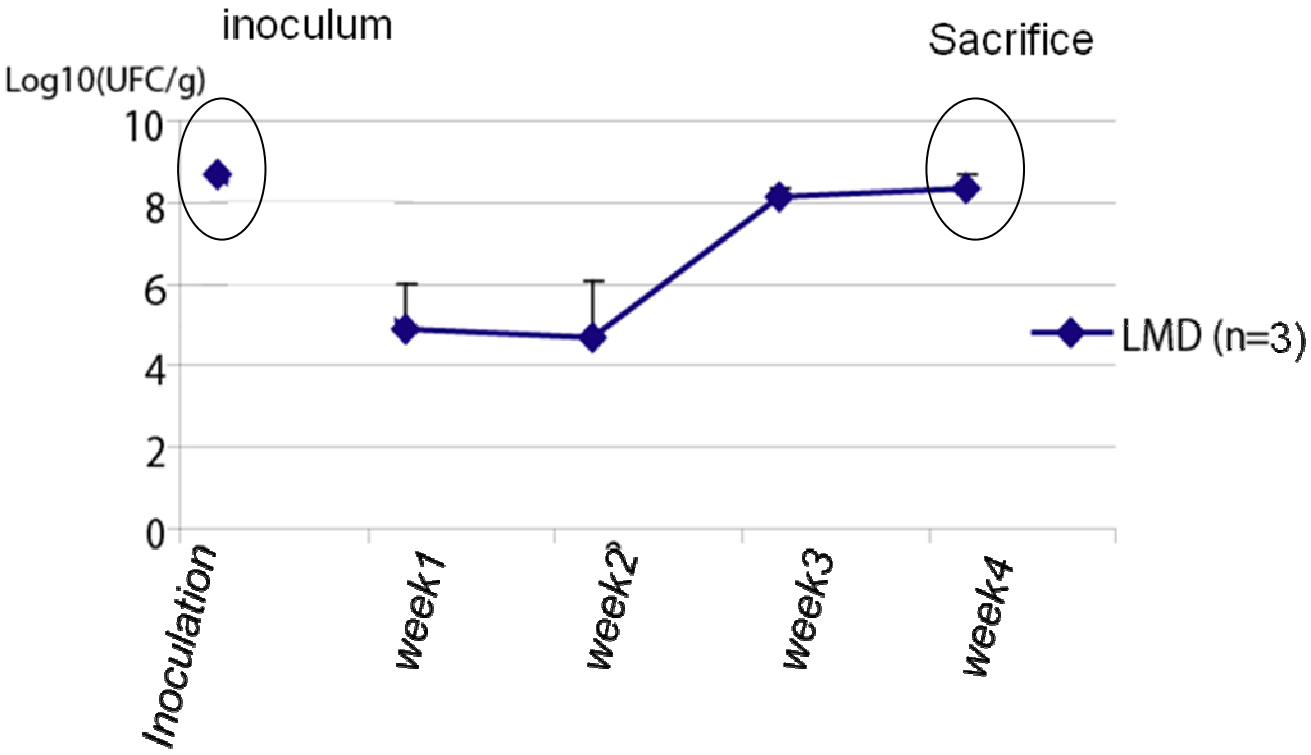


Figure 5: level of implantation during 4 weeks after inoculation of the Germ Free rats with *Streptococcus thermophilus* strain LMD9 by single gavage . Control of viable bacteria determined in the faeces during 4 weeks. At the moment of the animals sacrifice, *S.thermophilus* population was similar in feaces to the inoculum

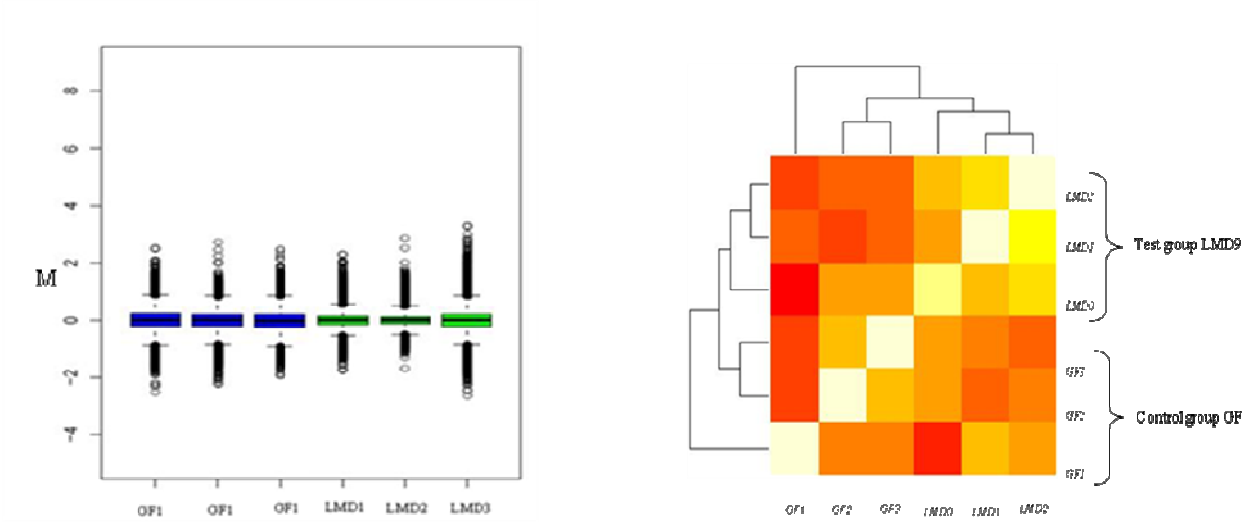
II-2) GLOBAL ALTERATIONS IN GENE EXPRESSION DUE TO THE PRESENCE OF *STREPTOCOCCUS THERMOPHILUS* IN HOST COLONIC EPITHELIUM.

We have used oligonucleotide microarray to analyse the host transcriptional responses caused by oral delivery of *Streptococcus thermophilus* bacteria (strain LMD9). Rats from the test group received LMD9 strain of *Streptococcus thermophilus* in milk, while those from the Germ Free (GF) control group received only milk. Experiments were performed using age-matched rats raised under Germ Free and mono-associated conditions. After the period of colonization (30 days), the presence of the corresponding *Streptococcus thermophilus* LMD9 strains in the intestinal content of the test group, and the lack of these bacteria in the control, were confirmed. Importantly, from the colon of the rats from test group, only the administrated strain could be re-isolated, while the corresponding control group remained Germ Free during the whole study period.

RNA was isolated from the colonocytes cells of each animal in each Treated and control group. The resulting RNAs were subjected to microarray analysis (combimatrix technology).

II-2-1) MICROARRAY QUALITY ASSESSMENT

After hybridization and scanning steps, we obtained spots of good quality, with high intensity, homogeneous size, and very low background. After we have performed the normalization step to eliminate variations between biological replicates, results were presented by box plot graph (Figure6a). The mean correlation coefficient for microarray values, obtained by scatter plot was 0.90 for biological replicates (Figure 6b)



a b

Figure 6: a – boxplot of the M-values from 6 arrays after Quantile normalization to equalize the median absolute deviation for each array. b- heatmap representig correlation between different biological samples after normalization

II-2-2) IDENTIFICATION OF DIFFERENTIALLY EXPRESSED GENES

Gene that were up- or down-regulated by log -1 ≤ fold-change ≤ 1 genes altered by administration of *Streptococcus thermophilus* are presented in table 2 ("Top table"). Top table is a function provided by limma package (described in Material and Method section). In the mono-associated rats LMD9 in comparaison to Germ Free rats , A total of 95 genes were designated as differentially expressed using selections criteria (*adj.P*≤ 0.1), and maintained just the unique genes (NB: we have eliminated all probe none and predicted genes). A number of summary statistics are presented by topTable for the top genes and the selected contrast (Table2).

ID	description	logFC	t	*P.Value*	*Adj.P. Value*
NM_175838.1	eukaryotic translation elongation factor 1 alpha 1 (Eef1a1), mRNA	2,63	5,54	0,0010	0,1042
NM_199498.1	keratin 19 (Krt19), mRNA	2,39	6,63	0,0003	0,0873
NM_138827.1	solute carrier family 2 (facilitated glucose transporter), member 1 (Slc2a1), mRNA	2,30	5,64	0,0009	0,1015
NM_001011918.1	annexin A11 (Anxa11), mRNA	2,17	7,54	0,0002	0,0736
NM_139257.1	lymphocyte antigen 6 complex, locus B (Ly6b), mRNA	2,06	6,23	0,0005	0,0958
NM_134409.2	zymogen granule protein 16 (Zg16), mRNA	1,97	7,85	0,0001	0,0736
NM_182738.1	calcineurin B homologous protein 2 (Chp2), mRNA	1,90	8,86	0,0001	0,0665
NM_053586.1	cytochrome c oxidase subunit Vb (Cox5b), nuclear gene encoding mitochondrial protein, mRNA	1,88	12,1	0,0000	0,0365
NM_001107660.1	carbonic anhydrase 1 (Car1), mRNA	1,86	6,27	0,0005	0,0958
NM_001034151.1	transmembrane protein 54 (Tmem54), mRNA	1,85	8,60	0,0001	0,0665

NM_021740.1	prothymosin alpha (Ptma), mRNA	1,77	13,1	0,0000	0,0365
NM_199207.1	family with sequence similarity 21, member C (Fam21c), mRNA	1,76	10,1	0,0000	0,0566
NM_153469.4	protein kinase inhibitor, gamma (Pkig), mRNA	1,66	5,68	0,0008	0,1015
NM_001007701.1	translocation associated membrane protein 1 (Tram1), mRNA	1,64	8,55	0,0001	0,0665
NM_001126120.1	alcohol dehydrogenase 5 (Adh5), mRNA	1,63	7,55	0,0002	0,0736
NM_001004209.1	hydroxysteroid (17-beta) dehydrogenase 11 (Hsd17b11), mRNA	1,58	6,47	0,0004	0,0898
NM_017231.1	phosphatidylinositol transfer protein, alpha (Pitpna), mRNA	1,56	7,69	0,0001	0,0736
NM_001025418.1	protein phosphatase 2 (formerly 2A), regulatory subunit A (PR 65), beta isoform (Ppp2r1b), mRNA	1,55	7,15	0,0002	0,0845
NM_053299.1	ubiquitin D (Ubd), mRNA	1,55	6,17	0,0005	0,0958
NM_053475.1	protein tyrosine phosphatase 4a2 (Ptp4a2), mRNA	1,52	5,50	0,0010	0,1060
NM_031614.2	thioredoxin reductase 1 (Txnrd1), mRNA	1,52	6,24	0,0005	0,0958
NM_199370.1	keratin 8 (Krt8), mRNA	1,51	6,66	0,0003	0,0873
NM_017320.1	cathepsin S (Ctss), mRNA	1,49	5,59	0,0009	0,1026
NM_001108826.1	TNFAIP3 interacting protein 1 (Tnip1), mRNA	1,48	5,39	0,0011	0,1097
NM_001106957.1	glyoxalase domain containing 5 (Glod5), mRNA	1,48	5,40	0,0011	0,1097
NM_001106557.1	endothelial differentiation-related factor 1 (Edf1), mRNA	1,47	7,36	0,0002	0,0781
NM_019299.1	clathrin, heavy chain (Hc) (Cltc), mRNA	1,45	6,60	0,0003	0,0873
NM_001034104.1	transmembrane channel-like 4 (Tmc4), mRNA	1,45	8,75	0,0001	0,0665
NM_001108624.2	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 2 (Ndufb2), mRNA	1,45	6,74	0,0003	0,0865
NM_001106029.2	cornichon homolog (Drosophila) (Cnih), mRNA	1,42	9,32	0,0000	0,0577
NM_031000.3	aldo-keto reductase family 1, member A1 (aldehyde reductase) (Akr1a1), mRNA	1,40	6,44	0,0004	0,0898
NM_001100995.1	similar to 4930438D12Rik protein (RGD1563224), mRNA	1,39	10,6	0,0000	0,0566
NM_031026.1	dynein, cytoplasmic 1 light intermediate chain 2 (Dync1li2), mRNA	1,37	12,5	0,0000	0,0365
NM_001013919.1	galactokinase 2 (Galk2), mRNA	1,35	6,04	0,0006	0,0975
NM_031603.1	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, (Ywhae), mRNA	1,35	6,46	0,0004	0,0898
NM_139099.1	ATP synthase, H+ transporting, mitochondrial F1 complex, epsilon subunit (Atp5e), mRNA	1,35	5,93	0,0006	0,1009
NM_001108442.1	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 7 (Ndufb7), mRNA	1,34	6,09	0,0006	0,0962
NM_012653.2	solute carrier family 9 (sodium/hydrogen exchanger), member 2 (Slc9a2), transcript variant 2, mRNA	1,33	13,4	0,0000	0,0365
NM_001106289.1	TMEM9 domain family, member B (Tmem9b), mRNA	1,32	5,67	0,0008	0,1015
NM_054002.1	zinc finger and BTB domain containing 7a (Zbtb7a), mRNA	1,32	9,01	0,0001	0,0653
NM_175757.2	crystallin, lambda 1 (Cryl1), mRNA	1,31	9,48	0,0000	0,0566
NM_001025419.1	Tax1 (human T-cell leukemia virus type I) binding protein 3 (Tax1bp3), mRNA	1,31	6,14	0,0005	0,0961
NM_053755.1	solute carrier family 26, member 3 (Slc26a3), mRNA	1,31	8,52	0,0001	0,0665
NM_001008319.1	macrophage erythroblast attacher (Maea), mRNA	1,29	7,50	0,0002	0,0736
NM_012975.1	lectin, galactoside-binding, soluble, 4 (Lgals4), mRNA	1,29	10,4	0,0000	0,0566
NM_031731.2	aldehyde dehydrogenase 3 family, member A2 (Aldh3a2), mRNA	1,28	8,55	0,0001	0,0665
NM_001100756.1	tetratricopeptide repeat domain 7 (Ttc7), mRNA	1,28	7,43	0,0002	0,0769
NM_031132.3	transforming growth factor, beta receptor II (Tgfbr2), mRNA	1,27	9,52	0,0000	0,0566
NM_001107449.1	chloride channel calcium activated 3 (Clca3), mRNA	1,26	6,50	0,0004	0,0898
NM_001014119.1	dehydrogenase/reductase (SDR family) member 11 (Dhrs11), mRNA	1,26	6,04	0,0006	0,0975
NM_001130559.1	NIPA-like domain containing 2 (Npal2), mRNA	1,26	5,71	0,0008	0,1015
NM_001007600.1	ribosomal protein S4, X-linked (Rps4x), mRNA	1,25	7,71	0,0001	0,0736
NM_017318.2	PTK2B protein tyrosine kinase 2 beta (Ptk2b), mRNA	1,25	5,94	0,0006	0,1009
NM_001134687.1	solute carrier family 35, member E3 (Slc35e3), mRNA	1,23	8,43	0,0001	0,0691
NM_001031654.1	annexin A8 (Anxa8), mRNA	1,22	5,99	0,0006	0,0996
NM_001007662.1	archain 1 (Arcn1), mRNA	1,19	6,94	0,0003	0,0859
NM_001134701.1	MHC class I RT1.Aa alpha-chain (Rt1.aa), mRNA	1,19	6,49	0,0004	0,0898
NM_198748.1	scinderin (Scin), mRNA	1,18	8,54	0,0001	0,0665
NM_001109678.1	NAD kinase (Nadk), mRNA	1,18	6,27	0,0005	0,0958

NM_001039031.1	dihydroxyacetone kinase 2 homolog (S. cerevisiae) (Dak), mRNA	1,18	5,45	0,0010	0,1075
NM_001108330.1	similar to CG14977-PA (RGD1309735), mRNA	1,17	6,97	0,0002	0,0859
NM_019376.2	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, (Ywhag), mRNA	1,16	6,56	0,0004	0,0888
NM_001106066.1	6-phosphogluconolactonase (Pgls), mRNA	1,14	6,61	0,0003	0,0873
NM_182671.2	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 10-like 1 (Ndufa10l1), mRNA	1,14	9,56	0,0000	0,0566
NM_012762.2	caspase 1 (Casp1), mRNA	1,12	5,64	0,0009	0,1015
NM_001008348.1	FERM domain containing 8 (Frmd8), mRNA	1,11	6,76	0,0003	0,0859
NM_001077670.1	eukaryotic translation initiation factor 3, subunit J (Eif3j), mRNA	1,10	6,19	0,0005	0,0958
NM_133526.1	tetraspanin 8 (Tspan8), mRNA	1,10	6,16	0,0005	0,0958
NM_001079887.1	inhibitor of growth family, member 4 (Ing4), mRNA	1,08	6,87	0,0003	0,0859
NM_001025773.1	tumor necrosis factor receptor superfamily, member 9 (Tnfrsf9), mRNA	1,07	5,90	0,0007	0,1015
NM_001013150.1	mitogen-activated protein kinase kinase kinase 11 (Map3k11), mRNA	1,07	5,75	0,0008	0,1015
NM_001014253.2	selenoprotein T (Selt), mRNA	1,06	5,54	0,0010	0,1042
NM_134365.1	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit B1 (Atp5f1), mRNA	1,06	5,83	0,0007	0,1015
NM_001033706.1	aldehyde dehydrogenase 16 family, member A1 (Aldh16a1), mRNA	1,05	6,43	0,0004	0,0898
NM_207595.1	ankyrin repeat, family A (RFXANK-like), 2 (Ankra2), mRNA	1,05	5,85	0,0007	0,1015
NM_017040.1	protein phosphatase 2 (formerly 2A), catalytic subunit, beta isoform (Ppp2cb), mRNA	1,05	5,66	0,0008	0,1015
NM_001012118.1	odd-skipped related 2 (Drosophila) (Osr2), mRNA	1,04	5,47	0,0010	0,1069
NM_001106063.1	hypothetical gene supported by AF152002 (LOC290595), mRNA	1,04	6,65	0,0003	0,0873
NM_001107586.1	cleavage stimulation factor, 3' pre-RNA subunit 2, tau (Cstf2t), mRNA	1,03	5,49	0,0010	0,1060
NM_139105.1	ribonuclease/angiogenin inhibitor 1 (Rnh1), mRNA	1,02	8,05	0,0001	0,0736
NM_001009622.1	SAR1 homolog B (S. cerevisiae) (Sar1b), mRNA	1,02	6,11	0,0005	0,0962
NM_001107111.1	interleukin 10 receptor, beta (Il10rb), mRNA	1,02	6,00	0,0006	0,0996
NM_001008298.1	STARD3 N-terminal like (Stard3nl), mRNA	1,02	7,78	0,0001	0,0736
NM_001006981.2	dihydrolipoamide S-succinyltransferase (E2 component of 2-oxo-glutarate complex) (Dlst), mRNA	1,02	6,72	0,0003	0,0867
NM_001012359.1	angiogenin, ribonuclease, RNase A family, 5 (Ang), mRNA	1,02	5,78	0,0007	0,1015
NM_133583.1	N-myc downstream regulated gene 2 (Ndrg2), mRNA	1,02	5,80	0,0007	0,1015
NM_001004220.1	electron-transfer-flavoprotein, beta polypeptide (Etfb), mRNA	1,01	5,85	0,0007	0,1015
NM_031682.1	hydroxysteroid (17-beta) dehydrogenase 10 (Hsd17b10), mRNA	1,00	6,41	0,0004	0,0900
NM_057196.1	BAI1-associated protein 2 (Baiap2), mRNA	-1,11	-6,85	0,0003	0,0859
NM_001014011.1	GRAM domain containing 3 (Gramd3), mRNA	-1,11	-7,84	0,0001	0,0736
NM_001047877.1	arylsulfatase G (Arsg), mRNA	-1,14	-7,60	0,0001	0,0736
NM_001108125.1	sestrin 3 (Sesn3), mRNA	-1,15	-7,04	0,0002	0,0859
NM_001033075.1	defensin alpha 7 (Defa7), mRNA	-1,17	-5,86	0,0007	0,1015
NM_013085.3	plasminogen activator, urokinase (Plau), mRNA	-1,26	-5,66	0,0008	0,1015
NM_133598.2	glycine cleavage system protein H (aminomethyl carrier) (Gcsh), mRNA	-1,29	-7,02	0,0002	0,0859

Table2: summary of the results of the linear model, after performing the hypothesis tests and adjusting the *p*-values for multiple testing. Results include (log) fold changes, *t*-statistics, and *p*-values. The basic statistics used for significance analysis is the moderated *t*-statistic, which is computed for each probe and for each contrast.95 Selected differentially expressed genes in mono-associated rats (LMD9) in comparison with Germ Free rats. ID represent the accession number in gene bank, (log)FC represent a level of expression en logarithmic function, *adj.p.val* represent the *p value* adjusted.

II-2) ANALYSIS OF THE HOST RESPONSE TO THE PRESENCE OF *STREPTOCOCCUS THERMOPHILUS*

Basic analyses computed with gene ontology using the "Biomart" (http://www.ensembl.org/biomart/martview) function available at Ensembl public database allowed to divide differentially expressed genes into different functional categories based on the biological process efficiently stimulated the gene expression involved in the cellular metabolism and proteins processing, Cell growth and development, signal transduction, ion homeostasis, transport activity, and unknown function were presented by "other" (Figure7).

Analysis computed by Ingenuity Pathway Analysis software(IPA; http://www.ingenuity.com), a Functional Analysis that identifies the biological functions, canonical pathway, and networks that are most significant to the data set, we have shown that IPA mapped and annotated 95 genes , among which 45 could be associated to 9 networks. Six of these are main networks interconnected by at least one common gene. The remaining 3 networks are deemed to be independent. Six of these are main networks interconnected by at least one common gene (Figure8). The remaining 3 networks are deemed to be independent. The composition of each network is given in Table 4, which are classified into major and minor networks according to their score and to the number of genes identified and linked to these networks. The first six networks were identified as major networks fairly high scores ranging from 35 for the best of them to 19 for the 6th. The other networks are comprised of few gene products and cannot be directly associated to major networks, which means that only 3 out of the total gene list products were not associated to major networks.. In Figure 9, we present in more detail, the most likely network (6 networks).

Network 1 contains 2 under-expressed gene, which are colored in green, and 18 over-expressed genes, which are colored in red. This principal network is interconnected on 3 most important complexes: NFκ-B which is known to play a central role in survival cell pathway, 38pMAPK which plays an important role in cell differentiation process , Akt which is important in the embryonic

development, cell growth and cell survival. NFκ-B in this network up-regulates the TGFRB2, gene involved in the development process of colonic epithelium by governing a cell differentiation and migration and down-regulates PLAU gene involved in the cell cycle arrest. Network 1 showed also that JNK complex, which is a member of the mitogen-activated protein kinase group of signaling proteins and that plays a key role in the differentiation of ES cells (Ping Xu et al, 2009), upregulates PTK2B and KRT8. PTK2B has a direct interaction with SLC2A1 and ANXA11. KRT8 has a direct interaction with KRT19. 38pMAPK complex upregulates PTK2B by a direct interaction.

Network 2 contain one gene down-regulated (green)and 16 genes upregulated (red). This second principal network is centered on TGFRB1 which is known to play a central role in developmental processes. We noticed that TGFB1 in this network interacts and upregulates target genes as TGFBR. TGFRB1 indirectly upregulates MUC5 that indirectly interacts and upregulates SLC2A1.

Network 3 contains 13 upregulated genes (red)and one downregulated gene (green). This network shows that HNF4A complex orchestrates the interconnection of most genes. In particularly, this gene complex interacts directly and upregulates DLST and CSTF2T.

Network 4 contain 12 upregulated genes (red) This network is orchestrated by APP complex that has a direct interaction with HSD17B10 and indirectly with CA1.

The two last Networks 5 and 6 contain 11 upregulated genes (red) each. The network 5 presents important interactions between products of target genes and has one gene downregulated relatively to network 6. It's also formed by two sub-clusters: the up cluster and the bottom cluster (Figure 5)The bottom cluster is constructed by 6 target genes NDUFB2, NDUFA10, NDUFB7, NADH2 deshydrogenase (ubiquitinone), NADH2 dehydrogenase and NADH dehydrogenase which are involved in the oxidative phosphorylation pathway. The network 6 is centered on TNF, a tumor necrosis factor which plays an important role in cell signaling and inflammatory response. The cellular response (e.g., proliferation versus apoptosis) to TNF is determined by the balance between the apoptotic signaling pathway and NF-κB survival pathway stimulated by TNF.

The minor networks, which were at first considered to be less pertinent, were emerged of the networks considered principals. The result revealed interconnection between network 3 and 9 (figure 10), and the interconnection is presented by a direct interaction between MIR 124 and STOM. In contrast network 8 was remained single.

II-3) GENES AND MOLECULAR PATHWAYS AFFECTED BY *STREPTOCOCCUS THERMOPHILUS* STIMULATION.

Among the 95 mono-associated LMD9 genes undergoing knowledge expression variation upon *Streptococcus thermophilus* stimulation, the pathway analysis knowledge database has revealed that 45 gene products could be defined as "Top Bio Functions". Most relevant functions were associated with:

- Molecular and Cellular Functions that included, Small Molecule Biochemistry (15 gene; *p value* :9,57E-04 - 4,09E-02) , Cellular Assembly and Organization (10 gene; *p value*: 2,22E-03 - 4,96E-02), Molecular Transport (10 gene; *p value*: 2,56E-03 - 4,09E-02), Energy Production (5 gene; *p value*: 2,56E-03 - 1,11E-02), and Lipid Metabolism (5 gene; *p value*: 9,57E-04 - 3,52E-02).
- Physiological System Development and Function associated with Embryonic Development (5 gene; *pvalue:*1,04E-04 - 1,19E-02), Tissue Morphology (11 gene; *pvalue:* 1,04E-04 - 4,67E-02), Reproductive System Development and Function (2 gene; pvalue: 3,46E-04 - 3,46E-04), and Organ Morphology (5 gene; *pvalue:* 4,24E-04 - 3,52E-02).
- Diseases and Disorders composed by Genetic Disorder (gene; *pvalue*: 3,50E-05 - 4,67E-02), Metabolic Disease(8 gene; *pvalue*: 3,50E-05 - 2,94E-02), Gastrointestinal Disease(19 gene products; *pvalue*: 1,93E-04 - 4,24E-02), and Hematological Disease (12 gene; *pvalue*: 1,18E-03 - 4,67E-02).

- Canonical pathway analysis of targets gene list showed metabolic pathway including oxidative phosphorylation associated with 5 of gene targets such as NADH deshydrogenase, NADH dehydrogenase (ubiquinone), hydroxysteroid (17-beta) dehydrogenase. Wntβ-catenin signaling pathway including 3 of gene targets such as TGFBR, PP2A and ub. And PI3KAkt signaling including 3 of gene targets such as 14-3-3 γ, 14-3-3ε and PP2A.

V) DISCUSSION

The primary role of the colon has been known for years to maintain water and electrolyte balance and to excrete undigested food materials. Currently, the colon is appreciated as a metabolically active organ and, therefore, colonic health is closely linked with overall health of humans and animals (O'Keefe SJ , 2008). Until recently, however, the physiology of the colon has received little attention in biological studies as compared to other body organs. Dietary composition may be the most important factor affecting colonic health because of its direct effects on microbial fermentation, morphology, and metabolism. Microbial populations, and their fermentation play a key role in colonic health and physiology, previous study reported by cherbuy et al have shown that microbiota have an impotant role in maturation of colonic epithelium (cherbuy et al, 2010). Gut bacteria play a critical role in the maintenance of colonic health, but the molecular mechanisms involved in the process are not well understood. A considerable focus has been given to the Lactic acid bacteria that have demonstrated in the last 10 years a most important benefice on the colonic health (Turpin et al, 2010).

In recent years, micro-array studies have been increasingly used to analyze tissue or cell response to a given stimuli (Childs RA. Et al, 2009; Febbo PG. et al, 2006; Mayburd AL. et al, 2009), and provide a lot of data for analysis. However, improved integration of this huge mass of data is needed to better understand the biological processes for which slight modifications in gene expression can have consequences. To this end, in the present study, we carried out pathway and

functional analysis on the *Streptococcus thermophilus* induced genes in colonic epithelium cells using Ingenuity Pathway Analysis tool.

In the present study we have investigated the gene expression profile of mono-associated rats with *Streptococcus thermophilus* bacteria to understand the behaviour of host colonic epithelium in the presence of lactic acid bacteria (*S.thermophilus* LMD9), here we presented *in vivo* the resulting host response after 30 days of inoculation. Implantation of *S. thermophilus* strain LMD9 in GF rats occurred progressively in accordance with study presented by Rull et al (Rul et al, 2010 submetted). Analysis of gene expression profil has shown that this adaptive response of *S.thermophilus* (LMD9) was accompanied by significant physiological and functional changes in the host colonic epithelium.

The gene expression analysis of the host colonic epithelium have revealed a up-regulation of most important genes involved in the cellular development and maintenance; cellular Growth and Proliferation; cell Cycle; energy Production, molecular transport, and metabolism process among the functional categories examined by Ingenuity Pathway Analysis.

Molecular transport:

Genes for the transport category in particularly genes coding of solutes carriers SLC2A1, SLC1A4, SLC9A2, SLC26A3. Transporters were identified as being more highly expressed in the colon than in the small intestine (Barrett KE, et al 2001). Many of these transporters have been described as highly expressed in the colon and involved in the bi-directional transport of electrolytes and fluids, which is the principal role of the colon (Geibel JP et al, 2004). However, the majority of known drug and nutrient transporters or carriers of bioactive compounds (Steffansen B et al, 2004) are not differently expressed between the small intestine and the colon. Recent study computed by Gail et al at 2009, on colonic gene expression in conventional and Germ Free mice has provided that expression of SLC26A3 is reduced in the Germ Free mice, but the level return to normal upon bacterial colonization (Gail A. et al, 2009). other study reported by Ingrid et al has shown down-regulation of SLC2A1, SLC1A4, and CA1 during DSS treatment in comparison to normal

conditions (Ingrid B. et al, 2002).The SLC2A1, SLC1A4, and CAI are the specifics products expressed by enterocytes and that are involved in colonic CO2 excretion, intracellular pH regulation, NA+ and Cl- absorption and, indirectly, in water transport (Charney AN et al, 1994). Combined our results of all observations that were listed in this contest suggest strongly that *Streptococcus thermophilus* has a positive impact on stimulation and maintenance of colonic absorption function by stimulation of membrane permeability.

Regulation of Cell cycle (proliferation and differentiation):

This study have showed also an up-regulation of genes involved in the regulation of cell cycle such as, transforming growth factor that is a gene that provides instructions for producing protein called transforming growth factor beta. The TGFβ-1 protein helps control the growth and division (proliferation) of cells, the process by which cells mature to carry out specific functions (differentiation), cell movement (motility), and the self-destruction of cells (apoptosis). The TGFβ-1 protein is found throughout the body and plays a role in development before birth the formation of blood vessels, the regulation of muscle tissue and body fat development, wound healing, and immune system function. TGFβ-1 is particularly abundant in tissues that make up the skeleton, where it helps regulate bone growth, and in the intricate lattice that forms in the spaces between cells (the extracellular matrix) (Gijs R. Van Den Brink,2003). Within cells, this protein is turned off (inactive) until it receives a chemical signal to become active. However, TGFB2 is involved in the development process of colonic epithelium by governing a cell differentiation and migration, their inactivation lead to a pathogenic role in the formation of human colon cancer (Muñoz NM et al, 2006). previous findings in colon cancers showed inactivation of TGF-β signaling (Biswas S. et al,2004; Biasi F. et al, 2008). In the present study we have found that this gene was regulated by NF-κB-complex. The NF-κB a complex pathway involved in the cell survival, growth and differentiation (valeriu B et al, 2009). Our results were Reinforced by direct Interaction with target genes KRT8 and K19. KRT8 is one of the major intermediate filament proteins expressed in single-layered epithelia of the gastrointestinal tract (Matthias T. et al, 2006), the function of KRT8 as a

regulator of cell division and cell differentiation suggest that altered KRT8 might contribute to tumor development (Matthias T. et al, 2006), loss of this gene in transgeneic mice lead to increased cell proliferation (Casanova ML. et al, 1999). KRT9 that is found expressed in the ducts of the human pancreas (Schussler MH et al, 1992), is responsible for the structural integrity of epithelial cells. Expression of KRT19 is often accompagned with expression of KRT8 differentiate cells of epithelial origin(Jeffrey Allard W. et al, 2004). In this study we have also shown an indirect interaction between genes target and p38Mitogen-activated Protein Kinase complex. Several recent finding have demonstrated that p38MAPK is involved in various vertebrate cell differentiation process, namely adipocytic (Engelman JA. et al, 1998) and myogenic differentiation (Wu Z. et al, 2000). Analysis computed by Mathieu H et al (Mathieu H. et al, 2001) have demonstrated that p38MAPK was found to be activated rapidly in intestinal cells induced to differentiate. Additionally specific inhibition of p38 significantly reduced the expression of several differentiation markers (Matheiu et al, 2001). In the seam context , this study has shown a direct interconnection between JNK complex, that was involved in the differentiation of ES cells (Ping Xu et al, 2009), and PTK2B gene, that is a cytosolic tyrosine kinases that are known to be important for processes such as cell proliferation, differentiation, and motility (Annemiek Beverdam et al, 2010), lead some evidence of the role of *S.thermophilus* to promote cell differentiation process.

All of these data were reinforced by down-regulation of Sesn3 named sestrin-3 involved in the arrest of cell cycle, and were in concordance with expression of Kruppel like factor 4 and 14-3-3σ, genes resulted differentially expressed using Supressive subtractive hybridization technology (chapter III), that were involved in the arrest of cell cycle and start of cell differentiation process.

These observations suggest that *Streptococcus thermophilus* has an anti proliferative impact on colonic epithelium cells and a positive effect on the cell differentiation.

Metabolism and energy production:

On the other hand, we identified multiple genes associated with metabolism and energy production processes, such as mitochondrial genes (NADH2 dehydrogenase, NADH2 dehydrogenasa ubiquinone, NDUFB). Genes that were distributed on the surface epithelial cells of the normal small intestine and colon (Andrew JS. Macpherson et al, 1992). Induction of the mitochondrial genes is commonly observed during disturbed ATP homeostatsis caused by increased energy demand or decreased mitochondrial energy suplly (Montalto M. et al, 2004; Schulzke JD. Et al, 2006). However, up-regulation of these genes lead to suggest that these genes were important to produce energy necessary to enhance intestinal permeability (Somasundaram S. et al, 1997) consequently, facilitate electrolytes and indirect water transport. This result was also in concordance with production of lactose by *Streptococcus thermophilus* (Rull et al, 2010 submetted).

Immune response:

Down-regulation of the Defaα7, Mammalian α-defensins constitute a family of cysteine-rich, cationic antimicrobial peptides produced by phagocytes and intestinal Paneth cells, playing an important role in innate host defense (Ganz T et al, 2002), and Plau, a urokinase gene that her over-expression was due to the mucosal inflammation(Blassi F. et al 1988; Elliott R. et al, 1987), indicated that *Streptococcus thermophilus* did not caused damage of the colonic epithelium of mono-associated rats.

In conclusion, the current experiment used microarray technology to identify global changes in colonic gene expression induced by *Streptococcus thermophilus* . In particular, mono-associated colonic epithelium had an up-regulation of genes associated with cell differentiation, metabolic and transport functions a down-regulation of genes associated with proliferation and immune response, highlighting potential genes and pathways that may be responsible for the signaling pathway. Up-regulation of genes related to transport activity in the mono-associated colonic epithelium may indicate an elevated absorption rate in the colonic epithelium in the presence of *Streptococcus thermophilus*. This report give another evidences for utility of microbita and use of probiotic.

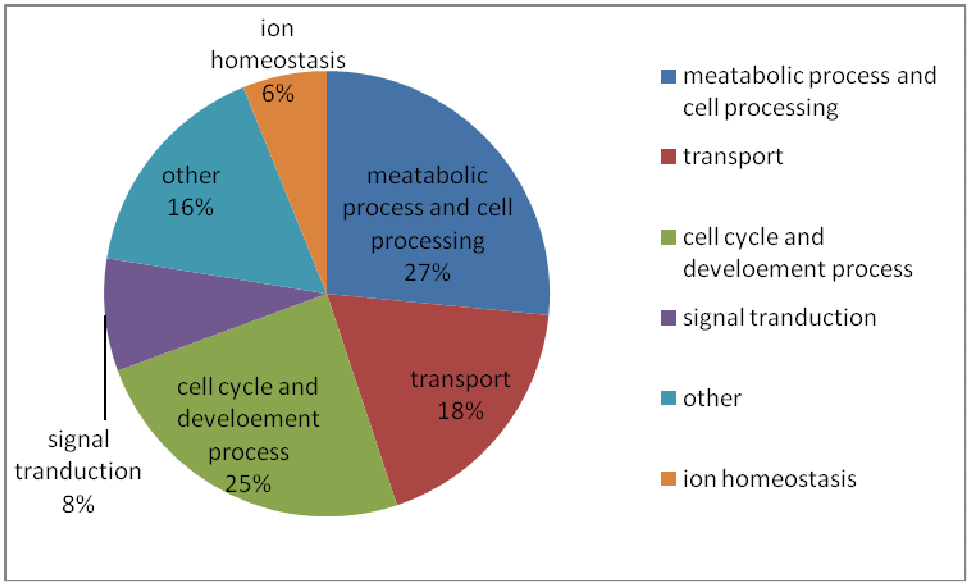


Figure 7: Functional categories of selected induced genes by *streptococcus thermophilus* in rat colonic epithelium. Analysis based on gene ontology using ensemble public database. The percentual represent a percentual of genes relative of total genes.

Network	Score	Focus Molecules	Top Functions
1	35	18	Embryonic Development, Tissue Morphology, Reproductive System Development and Function
2	31	16	Cellular Movement, Genetic Disorder, Immunological Disease
3	24	13	Cellular Development, Cellular Function and Maintenance, Cell Death
4	20	12	Energy Production, Molecular Transport, Nucleic Acid Metabolism
5	19	11	Carbohydrate Metabolism, Lipid Metabolism, Small Molecule Biochemistry
6	19	11	Antigen Presentation, Antimicrobial Response, Humoral Immune Response
8	2	1	Cancer, Reproductive System Disease, Carbohydrate Metabolism
9	2	1	Cellular Function and Maintenance, Small Molecule Biochemistry, Cellular Development

Table 3: the networks generated by genes differentially expressed in the assay using IPA. The score indicates the likelihood that the assembly of a set of focus genes in a network could be explained by random chance alone . the data base attributed general general functions of each network which are determined by interrogating the ingenuity pathway knowledge base for relationships between the genes in the network and the cellular functions they impact.

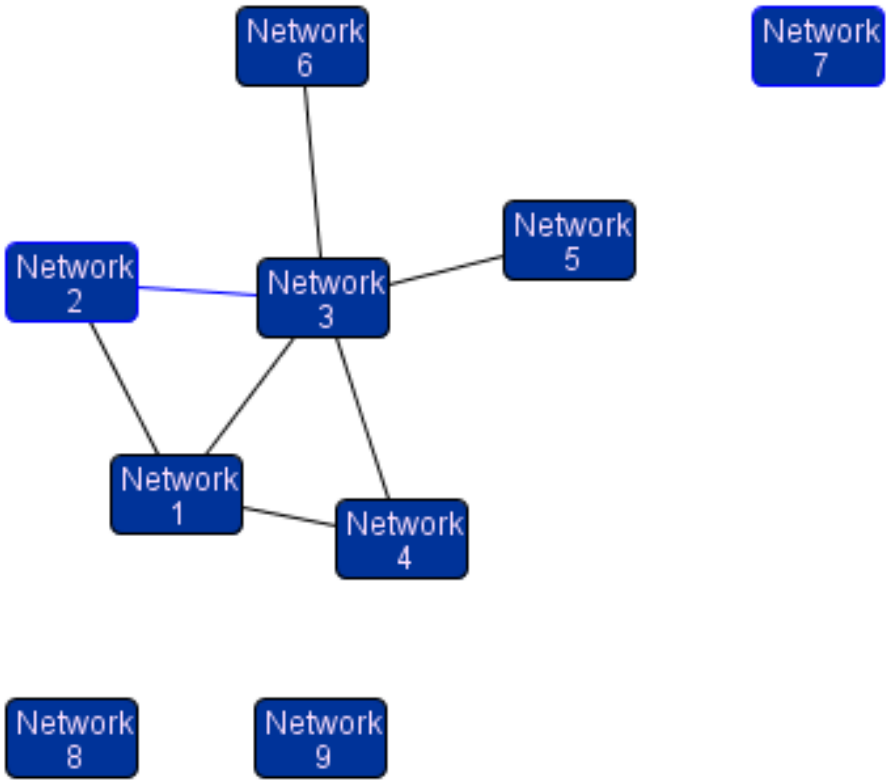
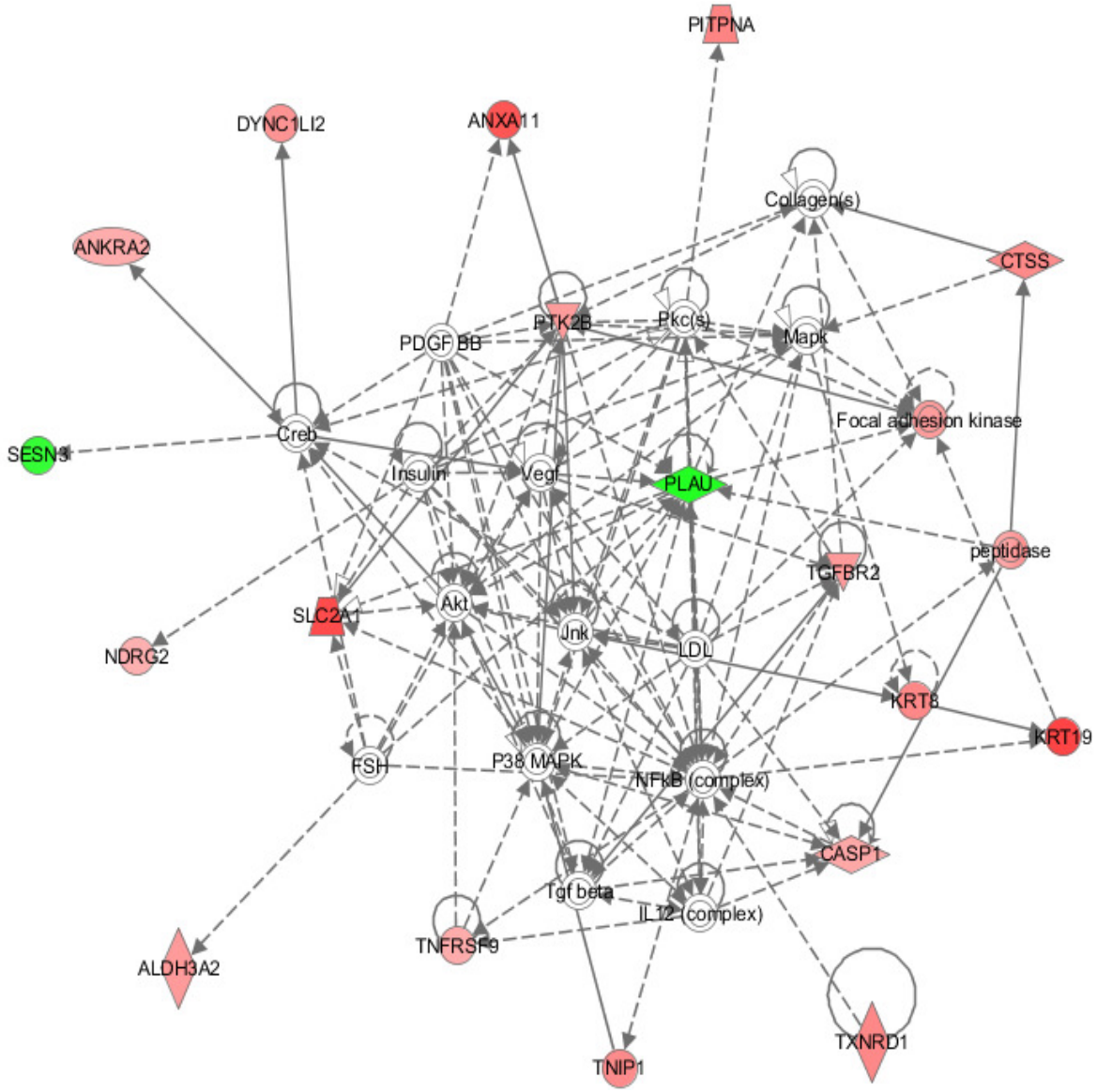
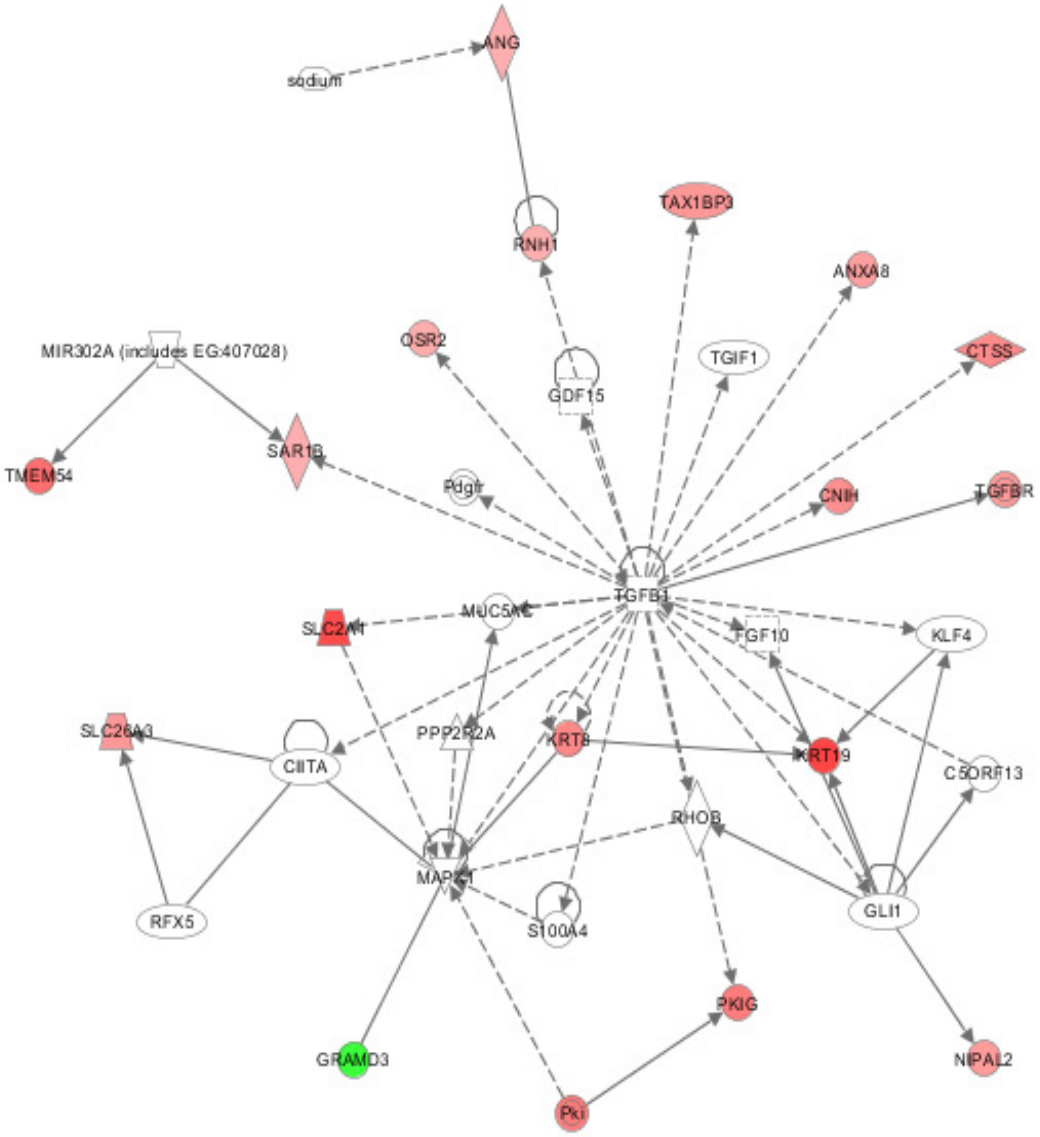


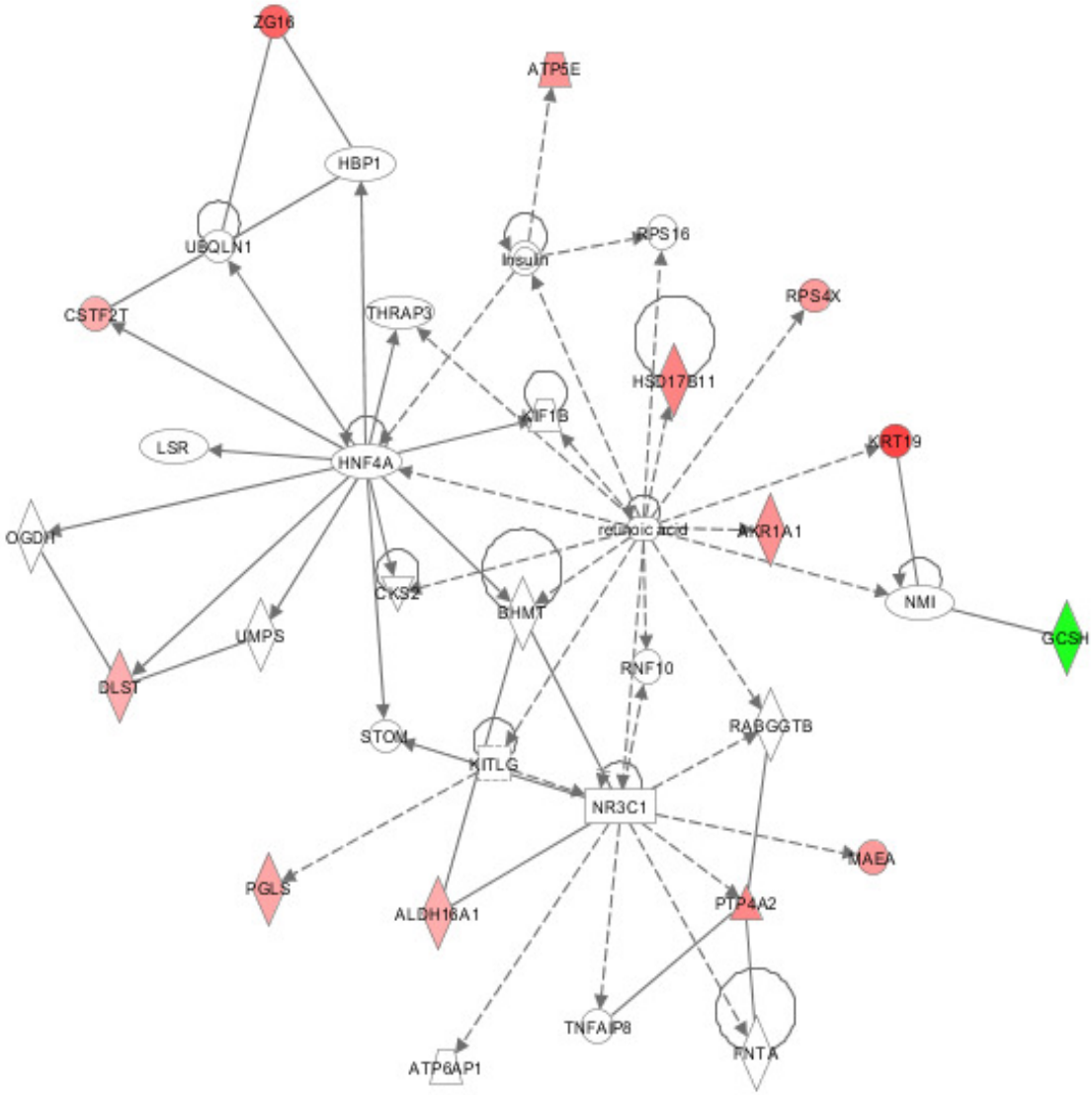
Figure 8: Interconnections between networks. From our 45 differentially expressed genes, the data base has identified 9 networks. The first six are heavily interconnected as shown by solid lines between the networks. Networks from 7 to 9 do not share common genes.


PITPNA
DYNC1LI2
ANXA11
Collagen(s)
ANKRA2
CTSS
PTK2B
Pkc(s)
Mapk
PDGF BB
Focal adhesion kinase
Creb
SESN3
Insulin
Vegf
PLAU
peptidase
TGFBR2
SLC2A1
Akt
Jnk
LDL
NDRG2
KRT8
KRT19
FSH
P38 MAPK
NFkB (complex)
CASP1
Tgf beta
IL12 (complex)
TNFRSF9
ALDH3A2
TXNRD1
TNIP1

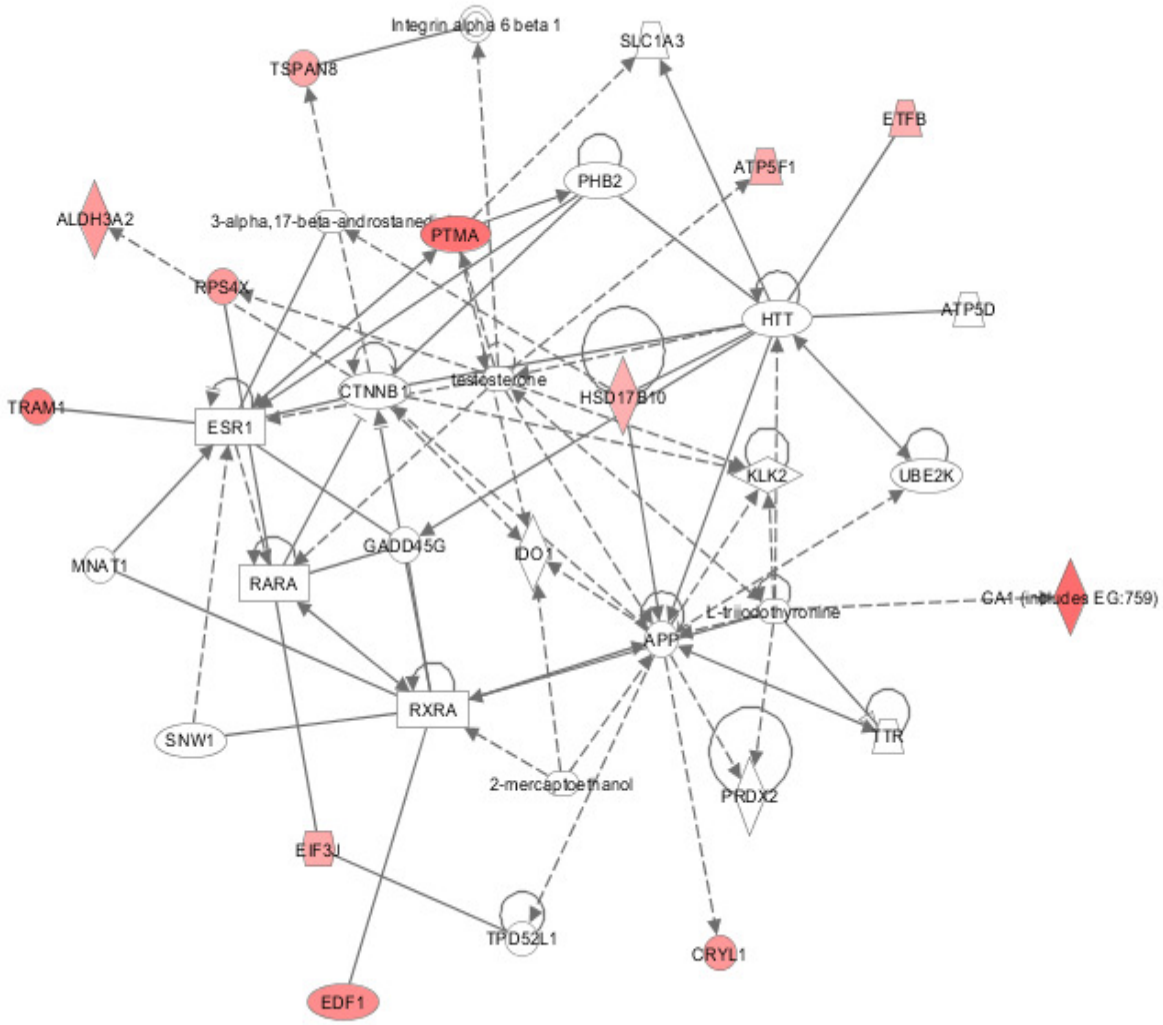
Network 1


ANG
sodium
RNH1
TAX1BP3
ANXA8
MIR302A (includes EG:407028)
OSR2
GDF15
TGIF1
CTSS
TMEM54
SAR1B
Pdgf
CNIH
TGFBR
TGFB1
SLC2A1
MUC5AC
FGF10
KLF4
SLC26A3
PPP2R2A
KRT8
KRT19
C5ORF13
CIITA
RHOB
MAPK1
GLI1
RFX5
S100A4
PKIG
GRAMD3
NIPAL2
Pki

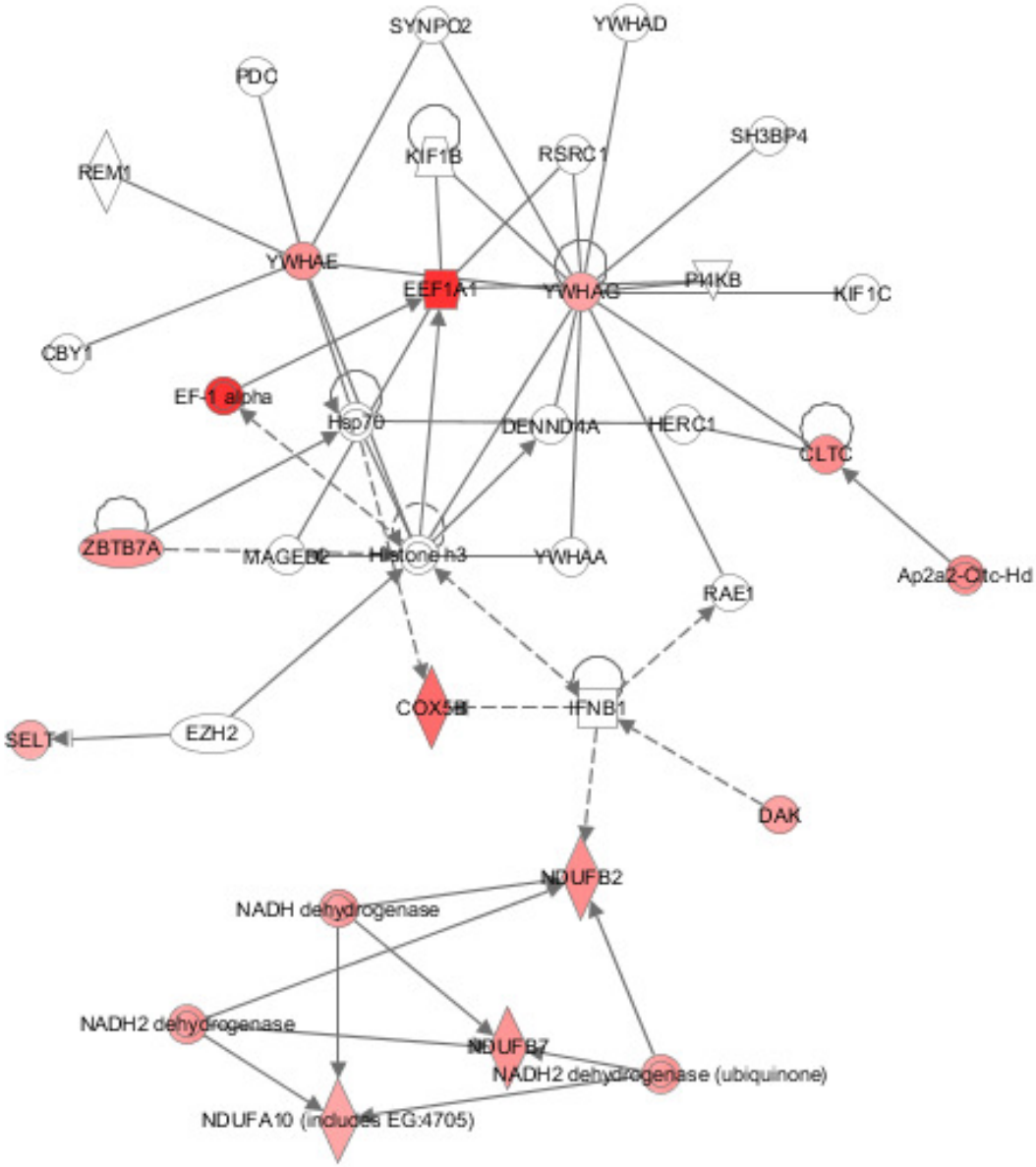
Network 2


ZG16
ATP5E
HBP1
UBQLN1
Insulin
RPS16
CSTF2T
THRAP3
RPS4X
HSD17B11
KIF1B
LSR
HNF4A
KRT19
retinoic acid
AKR1A1
OGDH
CKS2
BHMT
NMI
GCSH
UMPS
RNF10
DLST
STOM
RABGGTB
KITLG
NR3C1
MAEA
PGLS
ALDH16A1
PTP4A2
TNFAIP8
FNTA
ATP6AP1

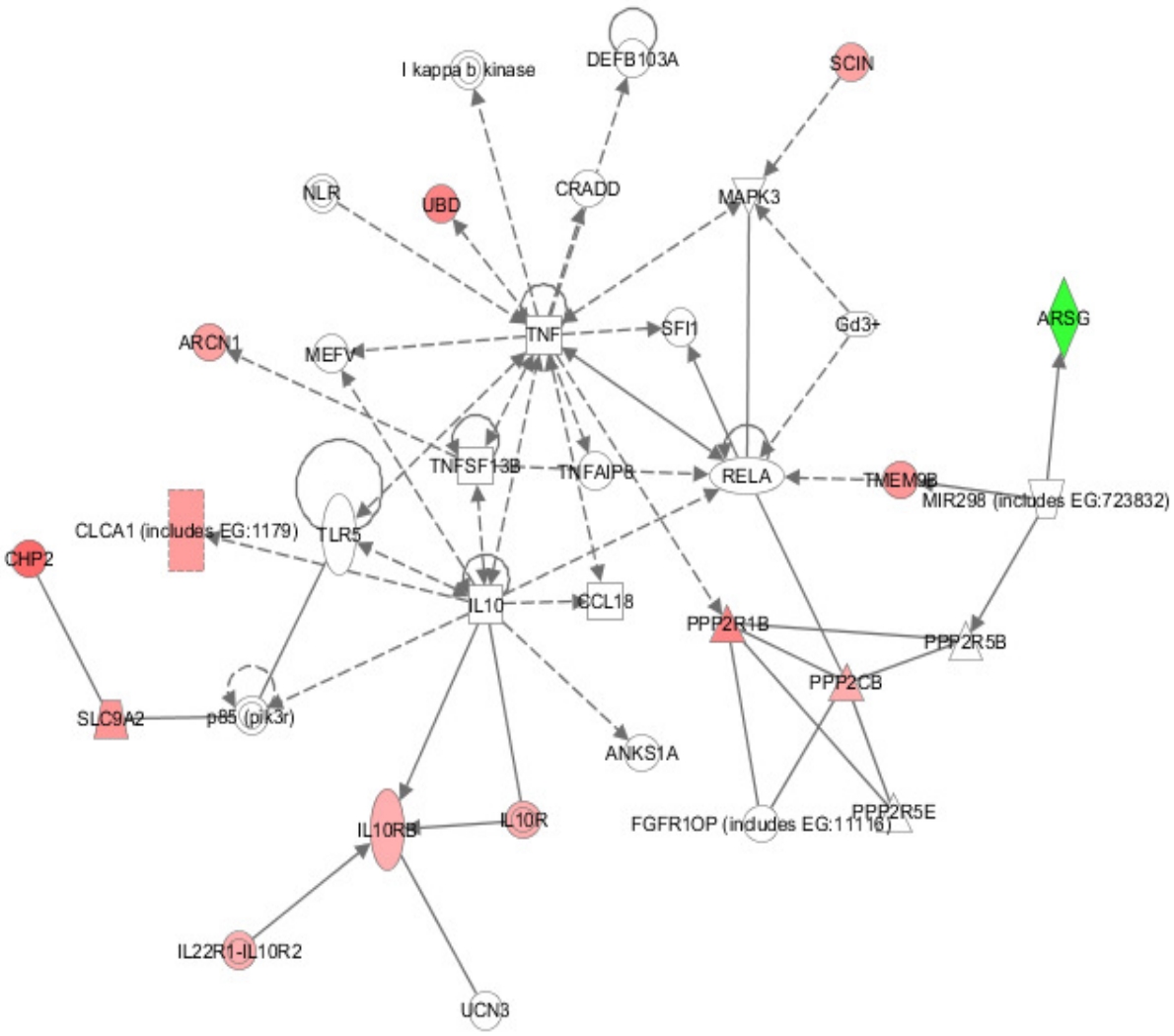
Network3


Integrin alpha 6 beta 1
SLC1A3
TSPAN8
ETFB
ATP5F1
PHB2
ALDH3A2
3-alpha,17-beta-androstanediol
PTMA
RPS4X
HTT
ATP5D
testosterone
CTNNB1
HSD17B10
TRAM1
ESR1
KLK2
UBE2K
GADD45G
IDO1
MNAT1
RARA
L-triiodothyronine
APP
RXRA
SNW1
TTR
2-mercaptoethanol
PRDX2
EIF3J
TPD52L1
CRYL1
EDF1

Network4

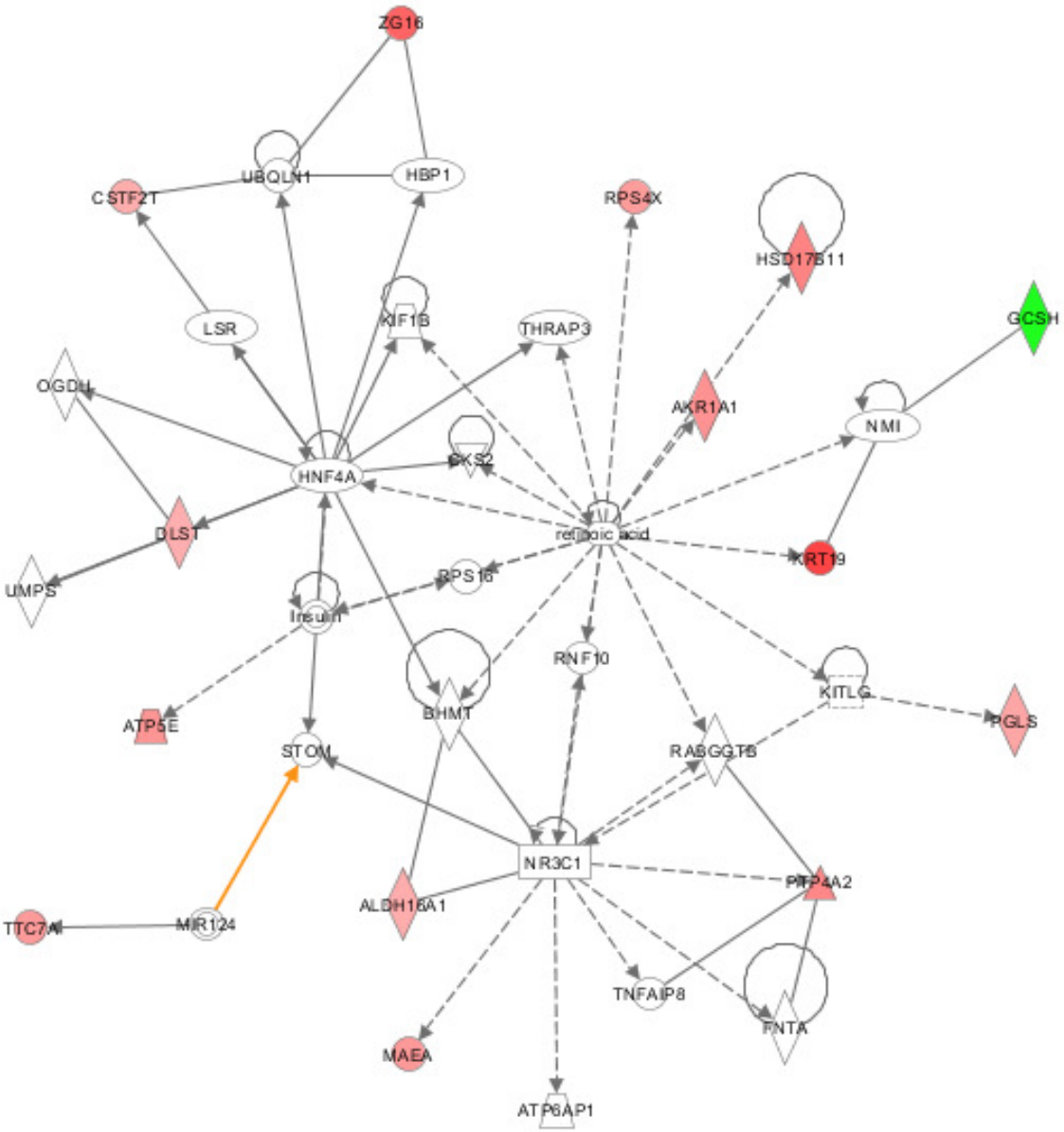

SYNPO2
YWHAD
PDC
KIF1B
RSRC1
SH3BP4
REM1
YWHAE
EEF1A1
YWHAG
PI4KB
KIF1C
CBY1
EF-1 alpha
Hsp70
DENND4A
HERC1
CLTC
ZBTB7A
MAGEB2
Histone h3
YWHAA
Ap2a2-Clc-Hd
RAE1
COX5B
IFNB1
SELT
EZH2
DAK
NDUFB2
NADH dehydrogenase
NADH2 dehydrogenase
NDUFB7
NADH2 dehydrogenase (ubiquinone)
NDUFA10 (includes EG:4705)

Network5



Network6

Figure 9: Close up of network (Network 1-6). A maximum autorized number of 35 genes were used to generate a network. Direct interactions between each gene within a network were represented. Genes highlighted in green were down-regulated whereas genes highlighted in red were up-regulated. Genes in white, which were not found in the assay, were added by the data base as they are relevant to the network. Solid lines represents a direct interaction whereas a dashed line represents an indirect interaction.



Network 3-9

Figure 10: Connection of network 3 with minor network 9. Network s are built as previously described in figure 4. Genes that are in green were down regulated whereas genes in red were up- regulated. Genes in white, which were not found in the assay, were added by the data base as they are relevant to the network. Solid lines represent direct interaction between gene products whereas dashed lines represents indirect interactions. Orange line display interconnection between minor network (network9) and major network 3.

ID	Molecules in Network	Score	Focus Molecules	Top Functions
1	Akt, ALDH3A2, ANKRA2, ANXA11, CASP1, Collagen(s), Creb, CTSS, DYNC1LI2, Focal adhesion kinase, FSH, IL12 (complex), Insulin, Jnk, KRT8, KRT19, LDL, Mapk, NDRG2, NFkB (complex), P38 MAPK, PDGF BB, peptidase, PITPNA, Pkc(s), PLAU, PTK2B, SESN3, SLC2A1, Tgf beta, TGFBR2, TNFRSF9, TNIP1, TXNRD1, Vegf	35	18	Embryonic Development, Tissue Morphology, Reproductive System Development and Function
2	ANG, ANXA8, C5ORF13, CIITA, CNIH, CTSS, FGF10, GDF15, GLI1, GRAMD3, KLF4, KRT8, KRT19, MAPK1, MIR302A (includes EG:407028), MUC5AC, NIPAL2, OSR2, Pdgfr, Pki, PKIG, PPP2R2A, RFX5, RHOB, RNH1, S100A4, SAR1B, SLC26A3, SLC2A1, sodium, TAX1BP3, TGFB1, TGFBR, TGIF1, TMEM54	31	16	Cellular Movement, Genetic Disorder, Immunological Disease
3	AKR1A1, ALDH16A1, ATP5E, ATP6AP1, BHMT, CKS2, CSTF2T, DLST, FNTA, GCSH, HBP1, HNF4A, HSD17B11, Insulin, KIF1B, KITLG, KRT19, LSR, MAEA, NMI, NR3C1, OGDH, PGLS, PTP4A2, RABGGTB, retinoic acid, RNF10, RPS16, RPS4X, STOM, THRAP3, TNFAIP8, UBQLN1, UMPS, ZG16	24	13	Cellular Development, Cellular Function and Maintenance, Cell Death
4	2-mercaptoethanol, 3-alpha,17-beta-androstanediol, ALDH3A2, APP, ATP5D, ATP5F1, CA1 (includes EG:759), CRYL1, CTNNB1, EDF1, EIF3J, ESR1, ETFB, GADD45G, HSD17B10, HTT, IDO1, Integrin alpha 6 beta 1, KLK2, L-triiodothyronine, MNAT1, PHB2, PRDX2, PTMA, RARA, RPS4X, RXRA, SLC1A3, SNW1, testosterone, TPD52L1, TRAM1, TSPAN8, TTR, UBE2K	20	12	Energy Production, Molecular Transport, Nucleic Acid Metabolism
5	Ap2a2-Cltc-Hd, CBY1, CLTC, COX5B, DAK, DENND4A, EEF1A1, EF-1 alpha, EZH2, HERC1, Histone h3, Hsp70, IFNB1, KIF1B, KIF1C, MAGED2, NADH dehydrogenase, NADH2 dehydrogenase, NADH2 dehydrogenase (ubiquinone), NDUFA10 (includes EG:4705), NDUFB2, NDUFB7, PDC, PI4KB, RAE1, REM1, RSRC1, SELT, SH3BP4, SYNPO2, YWHAA, YWHAD, YWHAE, YWHAG, ZBTB7A	19	11	Carbohydrate Metabolism, Lipid Metabolism, Small Molecule Biochemistry
6	ANKS1A, ARCN1, ARSG, CCL18, CHP2, CLCA1 (includes EG:1179), CRADD, DEFB103A, FGFR1OP (includes EG:11116), Gd3+, I kappa b kinase, IL10, IL10R, IL10RB, IL22R1-IL10R2, MAPK3, MEFV, MIR298 (includes EG:723832), NLR, p85 (pik3r), PPP2CB, PPP2R1B, PPP2R5B, PPP2R5E, RELA, SCIN, SFI1, SLC9A2, TLR5, TMEM9B, TNF, TNFAIP8, TNFSF13B, UBD, UCN3	19	11	Antigen Presentation, Antimicrobial Response, Humoral Immune Response
7	DHRS11, MIR198	2	1	-
8	galactokinase, GALK2	2	1	Cancer, Reproductive System Disease, Carbohydrate Metabolism
9	MIR124, TTC7A	2	1	Cellular Function and Maintenance, Small Molecule Biochemistry, Cellular Development

Table 4: the genes found to be differentially regulated in our experiments and the number of such genes displayed in the “focus molecules” . the score is generated using a pvalue calculation and is displayed as the negative log p-value. This score indicates the likelihood that the assembly of a set of focus genes in a network could be explained by random chance alone. A score of 2 indicates that there is a 1 in 100 chance that the focus genes are toghether in a network due to random chance. Therefore, network s with scores of 2 or higher have at least a 99% confidence of not being generated by random chance alone. The database attributed general cellular functions to each network which are determined by interrogating the Ingenuity Pathway Knowledge base for relationships between the genes in the network and the cellular functions they impact.

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GENERAL CONCLUSION

Vertebrates are essentially born germ-free but normally acquire a complex intestinal microbiota soon after birth. Most of these organisms are non-pathogenic to immunocompetent hosts; in fact, many are beneficial, supplying vitamins for host nutrition and filling the available microbiological niche to limit access and consequent pathology when pathogens are encountered. Thus, colonic epithelium health depends on interplay between host and microbiota. This is evident in inflammatory conditions such as inflammatory bowel disease, where aberrant responses to microbiota can result in host pathology. Studies with axenic (germ-free) or deliberately colonised animals have revealed that commensal organisms are required for the development of a fully functional immune system and affect many physiological processes within the host.

The prospects of this work are to understand the molecular mechanisms by which the microbiota influence the homeostasis of the intestinal epithelium. For this aim, we have colonized gnotobiotic rats with lactic acid bacteria such as *Streptococcus thermophilus*.

S.thermophilus has been used in this study for a number of reasons. It's among the first to colonize the intestinal tract in the birth (>10^8 /g faeces) (Moreau *et al*, 1986) (Palmer *et al*, 2007) and its presence in the faces and in breast milk has been recently described (Perez *et al*, 2007). Therefore, *S.thermophilus* can have an impact on the maturation and homeostasis of intestinal epithelium after birth. T*his bacterium* is important since it is used as probiotics in the manufacturing of some industrial products, particularly yoghurt and milk fermentation. Moreover, recent data indicate that the *S.thermophilus* LMD-9 strain used in this study is among the 57 bacteria species found in 90% of 124 European human individuals (Qin J. et al, 2010).

The research discussed in this thesis was carried out in three steps. The first one, was the generation of mono-associated rats with *Streptococcus thermophilus* by oral administration and the integrative study of the crosstalk established between the bacterium and the host. The results have

demonstrated that *S. thermophilus* is able to adapt its global metabolism to gut environment by emphasizing its carbohydrate metabolism in the digestive tract. This is in accord with the beneficial role of fermented milk consumption in improving lactose intolerance. Moreover *S.thermophilus* is able to induce the host colonic epithelium $p27^{kip1}$ protein which is involved in the cell cycle arrest, leading to a differentiation of colonic epithelium cells..

In the second step we have used the suppressive subtractive hybridization (SSH) technology to identify differentially expressed genes. The subtraction library was prepared by subtracting mRNA between epithelial cells isolated from colonic mono-associated rats (inoculated with LMD9 strain of *S. thermophilus*) and germ-free rats (without bacteria). Eighty transcripts were sequenced after SSH and among these, 32 corresponded to genes whose expression in colonic epithelial cells is potentially activated in the presence of *S.thermophilus.* Candidate genes can be grouped into four essential functional groups: genes that play a role in cell-cycle arrest and induction of differentiation, genes involved in the pathway of cell-communication, genes involved in binding and genes involved in regulation of biological processes.

The two most important candidate genes, Kruppel like factor4 and 14-3-3 σ, were tested by conventional RT-PCR, quantitative RT-PCR, and Western blot analysis in mono-associated animals versus germ-free animals. The results confirmed that selected candidate genes increase their expression in the presence of *S. thermophilus,* suggesting that the bacerium has an antiproliferative impact on colonic epithelium cells reinforced by a promotion of the differentiation of these cells. These results partly confirmed the role of *S. thermophilus* in the modulation of the expression of genes involved in essential intestinal functions such as the maturation of cells.

Finally, in the third step, we have used gene array technology to comparatively characterize differences in gene transcription in the colon of mono-associated (LMD) and germ-free (GF) rats evoked by oral administration of *Streptococcus thermophilus* LMD9 strains used in fermented dairy products .

We showed that *Streptococcus thermophilus* caused important changes on the transcriptional profile of the host. This bacteria, also considered a probiotic, evoked a complex response in the gut, reflected by differential regulation of a number of genes involved in essential biological functions such as metabolic activity, transport activity, development and cell growth, which are essential of a healthy colonic functionality.

Differential expression of an array of genes described in this report evokes novel hypothesis of possible interactions between the *Streptococcus thermophilus* bacteria and the host organism and warrants further studies to evaluate the functional significance of these transcriptional changes on the functionality of the host.

In summary, these results proved that the normal colonic host function of many biological functions is dependent on bacterial colonization as commensal or probiotic. The critical tools of strain combination experiments, axenic /gnotobiotic , and omics technologies will allow us to understand the mechanisms that underlie host-bacteria in health and improve the therapies of the defects that occur in the colonic diseases.