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Modulation of host innate immunity by health-promoting bacteria and dietary compounds

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ABSTRACT

Modulation of host innate immunity by health-promoting bacteria and dietary compounds

In its widest meaning, the probiotic approach consists in exogenous administration of microbial cells (or cell components) aimed at benefiting the host's health, both in terms of maintenance of homeostasis and also as alternative strategy for the prevention and/or treatment of infectious diseases. More recently, it has been demonstrated that an important way through which probiotics can exert their beneficial effects is the ability to interact with host's immune system, both at local and systemic level, thus having efficacy also in body niches different from the gut. Starting from these observations, in the first part of the PhD research activity we screened several bacterial strains for their potential use as probiotics for the pharyngeal mucosa. We tested the ability of bacteria employed in food industry and newly isolated from the pharynx of healthy volunteers to adhere to the human pharyngeal epithelium, and to antagonize the oro-pharyngeal pathogen *S. pyogenes* on FaDu cells and HaCat keratinocytes. Two bacterial strains, *Streptococcus salivarius* ST3, and the dairy starter *Lactobacillus helveticus* MIMLh5 were selected and compared with the oral commercial probiotic *S. salivarius* strain K12. These strains resulted sensitive to a variety of antibiotics routinely used for the control of upper respiratory tract infections. The *in vitro* immunological characterization performed on FaDu cells revealed that ST3 and MIMLh5 were both able to significantly reduce the activation of the nuclear-factor (NF)-κB in presence of the pro-inflammatory stimulus interleukin (IL)-1β, whereas presenting different modulatory abilities at baseline. Moreover these strain showed different cytokine profile under the above mentioned conditions. We subsequently decided to characterize the effects of the combined use of strain ST3 and MIMLh5. We found that strains MIMLh5 and ST3 activated innate immunity by inducing in U937 human macrophages the expression of cyclooxygenase (COX)-2, a balanced IL10/Tumor-Necrosis Factor (TNF)-α ratio, and we demonstrated that Toll-like receptor 2 (TLR-2) participates in the recognition of both strains. We also observed that these microorganisms grow efficiently when co-coltured in milk, suggesting that the preparation of a milk-based fermented product containing both strains can be a practical solution for the administration of these bacteria.

Considered the ability of *L. helveticus* MIMLh5 to trigger immune responses also on murine bone marrow-derived dendritic cells, in the second part of the research we focused our attention on the possible molecular determinants involved in the immunostimulating activity of this strain. We studied MIMLh5 surface layer protein (SlpA) and we found that the bacterium and its SlpA exerted anti-inflammatory effects on the intestinal epithelial Caco-2 cell line by reducing the activation of NF-κB. On the contrary, MIMLh5 and SlpA acted as stimulators of the innate immune system by triggering the expression of the pro-inflammatory factors TNF-α and COX-2 in the human macrophage cell line U937 via recognition through TLR-2, whereas having slighter effect on the anti-inflammatory IL10, particularly for SlpA. When we tested MIMLh5 bacterial cells depleted from the protein, we observed a reduced pro-inflammatory activity, suggesting that SlpA plays a major role in mediating the immunostimulatory attitude of the bacterium, which could help to induce host's defenses against and responses towards infections. Most of these results were confirmed when we tested the bacterium and the protein on murine macrophages isolated from bone marrow and from peritoneal cavity.

In the third part of the research we analyzed the effects on immune system of food compounds from vegetal origin. To this aim, we evaluated the immunomodulatory potential of different

fractions extracted from wild blueberries (WB) powder. We observed that only the anthocyanin (ACN) fraction was effective in reducing the activation of NF-κB on Caco-2 cells, whereas both the soluble and the phenolic fractions had no significant effects. Consequently, we used only the anthocyanin fraction for the subsequent characterization on U937 macrophages. We found that the presence of ACNs decreased the induction of TNF-α triggered by lipopolysaccharide (LPS) from *Escherichia coli* on U937, particularly when the cells were pretretated with ACNs and afterwards treated with LPS. These data suggest that ACNs from WB might have a protective role towards inflammation and that, probably, the described anti-oxidant features of these compound might be partially mediated by direct effects on immune system.

In conclusion, this PhD work evidenced the noticeable abilities of bacteria and dietary compounds to modulate host immune system responses. Particularly, this study suggests that the use of selected food-grade bacteria, bacterial components or dietary compounds has a promising potential for the maintenance of host health and the prevention of diseases.

RIASSUNTO

Modulazione dell'immunità innata dell'ospite da parte di batteri probiotici e di componenti della dieta.

Nel suo più ampio significato, l'approccio probiotico consiste nella somministrazione di batteri (o di componenti batteriche) finalizzate a un beneficio per la salute dell'ospite, sia in termini di mantenimento dell'omeostasi sia come strategia alternativa per la prevenzione di malattie infettive. Recentemente, è stato dimostrato che un'importante via di azione dei probiotici è la capacità di interagire con il sistema immunitario dell'ospite, sia a livello locale sia sistemico, agendo così anche in distretti corporei diversi dall'intestino. Partendo da queste considerazioni, nella prima parte del progetto di dottorato sono stati selezionati ceppi batterici come potenziali probiotici per la mucosa faringea. Sono stati utilizzati ceppi batterici alimentari e ceppi isolati dalla faringe di soggetti sani per valutare sulle linee cellulari FaDu e su cheratinociti HaCat la loro capacità di aderire all'epitelio della mucosa faringea umana, e di inibire *Streptococcus pyogenes*, il principale patogeno batterico della cavità oro-faringea. Un batterio di origine orale, *Sterptocossus salivarius* ST3, e lo starter caseario *Lactobacillus helveticus* MIMLh5 sono stati selezionati e confrontati con il probiotico orale commerciale *S. salivarius* K12. Tali ceppi sono risultati sensibili a diversi antibiotici usati comunemente per il controllo delle infezioni delle alte vie respiratorie. La caratterizzazione immunologica *in vitro* effettuata su cellule FaDu ha dimostrato che ST3 e MIMLh5 erano in grado di ridurre l'attivazione del nuclear-factor (NF)-κB in modo significativo in presenza dello stimolo pro-infiammatorio interleuchina (IL)-1β; presentando un'attività modulatoria diversa a livello basale. Inoltre, tali ceppi hanno mostrato un profilo di espressione di citochine diverso nelle medesime condizioni sopra indicate. Conseguentemente, abbiamo caratterizzato gli effetti dell'uso combinato dei 2 ceppi. È stato dimostrato che ST3 e MIMLh5 attivavano l'immunità innata inducendo nella linea cellulare U937 di macrofagi di origine umana l'espressione di ciclooxigenase (COX)-2 e un rapporto bilanciato IL10/*Tumor-Necrosis Factor* (TNF)-α. È stato inoltre dimostrato che il recettore Toll-like 2 (TLR-2) partecipa al riconoscimento di entrambi i ceppi. Abbiamo infine osservato che tali microorganismi crescono efficientemente in co-coltura in latte, suggerendo come possibile via di somministrazione per questi batteri un prodotto a base di latte fermentato.

Considerata la capacità di *L. helveticus* MIMLh5 di indurre risposte immunitarie anche in cellule dendritiche di origine murina estratte da midollo osseo, nella seconda parte della ricerca abbiamo focalizzato l'attenzione sui possibili determinanti molecolari coinvolti nell'attività immunostimolatoria di questo ceppo. È stata studiata la proteina di superficie *S-layer* (SlpA) di MIMLh5 ed è stato evidenziato che il batterio e la sua SlpA esercitavano effetti anti-infiammatori sulla linea cellulare epiteliale intestinale Caco-2 mediante la riduzione dell'attivazione del NF-κB. Al contrario, MIMLh5 e SlpA agivano da stimolatori del sistema immunitario innato attivando l'espressione dei fattori pro-infiammatori TNF-α e COX-2 in macrofagi U937, attraverso il riconoscimento da parte del TLR-2, e, particolarmente per SlpA, una più bassa espressione della citochina anti-infiammatoria IL-10,. Quando le cellule batteriche di MIMLh5 sono state private della proteina, è stata osservata una ridotta attività pro-infiammatoria.. Questo dato suggerisce come SlpA abbia un ruolo importante nel mediare l'attitudine immunostimolatoria del batterio, che può risultare positiva nell'aiutare l'ospite ad attivare meccanismi di difesa e di risposta contro le infezioni. Molti di questi dati si sono riconfermati su macrofagi di origine murina estratti da midollo osseo e da cavità peritoneale.

Nella terza parte del progetto di ricerca sono stati valutati gli effetti di componenti alimentari di origine vegetale sul sistema immunitario. A tale scopo, abbiamo valutato il potenziale immunomodulatorio di diverse frazioni estratte da polvere di mirtillo selvatico (WB). La sola frazione contenente antociani (ACN) è risultata efficace nel ridurre l'attivazione del NF-κB nelle cellule Caco-2, mentre la frazione solubile e quella fenolica non hanno dato variazioni significative. Di conseguenza, abbiamo utilizzato unicamente la frazione antocianica per la caratterizzazione immunologica sui macrofagi U937. Tali esperimenti hanno evidenziato come l'induzione di TNF-α indotta dal lipopolisaccaride (LPS) di *Escherichia coli* nelle cellule U937 diminuisse in presenza degli ACN. Questo effetto è stato particolarmente evidente quando le cellule sono state pretrattate con ACN e in seguito con LPS. Questi dati indicano come gli ACN derivati da WB possano avere un ruolo protettivo contro i processi infiammatori. Pertanto le proprietà anti-ossidanti descritte in letteratura per questi composti possono probabilmente in parte derivare da effetti diretti sul sistema immunitario.

Concludendo, il presente lavoro di dottorato di ricerca ha evidenziato le notevoli capacità dei batteri e di componenti bioattivi della dieta di modulare le risposte del sistema immunitario dell'ospite. In particolare, questo studio suggerisce l'impiego di batteri "food-grade", di componenti batteriche e di componenti alimentari come potenziale e promettente strategia per il mantenimento della salute dell'ospite e per la prevenzione di malattie.

PREFACE

There are increasing evidences of the importance that a proper and well-balanced functioning of the immune system plays in host's health maintenance (Wichers, 2009). Different endogenous and exogenous factors can take part in the modulation of the type and the magnitude of immune responses. Diet and nutrition have been demonstrated to affect diverse immune parameters (López-Varela et al., 2002; Veldhoen & Brucklacher-Waldert, 2012), and several studies revealed the pivotal role that microbiota has in shaping host's immune system (Macpherson & Harris, 2004; Round & Mazmanian, 2009). Moreover, the strong interrelationship between these factors has been shown both in terms of influence that diet can have on gut microbiota composition (Turnbaugh et al., 2009; Maslowski & Mackay, 2011) and impact that commensal and/or ingested bacteria have on host's nutritional status, depending on their metabolic activities (Sekirov et al., 2010; Tremaroli & Bäckhed, 2012). All these observations opened the way to an expanding field of studies targeted to the evaluation of the immunomodulatory potential of food-derived compounds, and food-associated and commensal/probiotic microorganisms. According to FAO/WHO, probiotics are defined as 'live microorganisms which when administered in adequate amounts confer a health benefit on the host' (FAO/WHO 2002). Increasing evidences support the idea that one of the main mechanisms through which beneficial microbes can positively affect the host's health involves their ability to interact with the host's immune system by eliciting responses at both local and systemic level (Borchers et al., 2009; Lebeer et al., 2010; Taverniti & Guglielmetti, 2011). Thus far probiotics have been most predominantly investigated for and applied to the intestinal tract; however, more recently it has been demonstrated that commensals and probiotic bacteria can act on immune functions beyond the gut (Noverr & Huffnagle, 2004; Smith et al., 2007), and that they can influence both innate and adaptive immunity (Chervonsky, 2010). But, even though commensal/beneficial microorganisms are important components of host's defence in many body sites, only a few applications beyond the gut suggested the potential positive role of probiotics, for instance for the stomach (Johnson-Henry et al., 2004), vaginal mucosa (Reid et al., 2009; Rose et al., 2012), urinary tract (Borchert et al., 2008), skin (Krutmann, 2009) and oral cavity (Tagg & Dierksen, 2003; Guglielmetti et al., 2010a,b). Moreover, elucidations of the mechanisms involved in the cross-talk between microorganisms and host's cells, in terms of identification of bacterial molecules involved and immune signaling pathways activated, are still needed. Several studies demonstrate that even non-viable bacterial cells or single cell components are able to drive immune responses, supporting the potentiality of a "paraprobiotic" approach (Taverniti & Guglielmetti, 2011). Identifying and characterizing unique bacterial components that act as effectors of the immune system is crucial for the elucidation of host-microbial interplay. In addition, a deeper understanding of the molecular mechanisms underlying the dialogue between bacteria and the host organism's system is of great importance for several reasons: to better define both the benefits and the potential risks associated with the administration of probiotic therapies (Besselink et al., 2008), and to open to new perspectives and alternative uses and applications of probiotics.

Considered all the previous observations concerning diet and microbes beneficial potential, the subsequent topics have been investigated during the PhD research activity:

1. Identification and characterization of bacterial strains isolated from the oro-pharyngeal cavity; evaluation of the probiotic abilities of food-derived bacteria and oral isolates for a potential application in the prevention and/or treatment of upper-respiratory tract infections; characterization of the immumodulatory properties derived from the combined use of a dairy strain of *Lactobacillus helveticus* with a selected strain of the

oral commensal *Streptococcus salivarius*, targeted to the improvement of host's surveillance at level of innate immunity.

2. Immunological evaluation of the previous characterized *L. helveticus* strain and study of its surface layer protein involvement in mediating the bacterial immunostimulating activity. Comparison with the immunological behavior of a commercial probiotic strain of *L. acidophilus*, and evaluation of the kind of immune responses and signaling pathways activated by our selected bacterium and its purified protein.

3. Evaluation of the immunomodulatory potential of food-associated compounds by characterizing the anthocyanin (ACN) fraction isolated from wild blueberries (*Vaccinium angustifolium*) powder, by analyzing the potential anti-inflammatory activities of ACNs in presence of pro-inflammatory stimuli.

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1 COMMENSALS AND FOOD-DERIVED BACTERIA AS POTENTIAL PROBIOTICS FOR THE ORO-PHARYNGEAL MUCOSA

1.1 STATE OF THE ART

1.1.1 History of probiotics

The term "probiotic" (derived from the ancient Greek meaning "for life") dates back to 1956, when it was probably used for the first time by the food scientist Werner Kollath to indicate microbial cells or molecules able to promote or improve the survival of live organisms (in contrast to the terms "antibiotics"). Although a relatively new word, the beneficial effects of certain fermented foods containing live bacteria have been recognized for centuries. The Russian physiologist Elie Metchnikoff (1845-1916) proposed, in fact, that shortening of life is a consequence of chronic poisoning due to intestinal putrefactions, and that the modification of microbiota composition through the consumption of viable microbes might help to improve health and longevity. Following Metchnikoff, in the early 20th century other investigators started to suggest that gut flora could be altered with beneficial bacteria replacing harmful microbes, leading to the concept of probiotics (Williams, 2010). Interestingly, the use of this word in scientific literature was negligible until the 1990s, but it reached 100 papers/y at the turn of the millennium, and is present today in about 900 papers/y. In 2001, the year of the Food and Agriculture Organization and World Health Oganization (FAO/WHO) Expert Consultation on "Health and Nutritional Properties on Probiotics in Food", there were 274 papers listed in PubMed under the keyword "probiotics," whereas in 2011 this number was as high as 975 (data from http://www.gopubmed.org; Morelli & Capurso, 2012).

For what it concerns the market of probiotics, the first probiotic product, including a bacterium belonging to the species *Lactobacillus paracasei*, has been isolated in 1930 by Prof. Shirota from human intestine, and later sold in yogurt drink "Yakult" in Japan in the 50's. In Europe, the first probiotic has been introduced in 1980, and nowadays the continuously growing interest in the field of probiotic microorganisms and products supports a global market expected to be worth $19.6 billion in 2013 (BCC Research 2008).

Table 1.1.1 *Commercial Strains Sold As Probiotics. Selected probiotic strains and products available in the US and Europe. This table does not constitute an endorsement of any of these products, nor does it include all strains/mixtures currently available (California Dairy Research Foundation, 2013).*

Strain[1]	Product containing strain[2]	Sold by
L. acidophilus NCFM *B. lactis* Bi-07 *B. lactis* HN019 (DR10) *L. rhamnosus* HN001 (DR20)	Sold as ingredient	DuPont Nutrition Biosciences ApS (Madison WI)
Saccharomyces cerevisiae boulardii	Florastor	Biocodex (Creswell OR)
B. infantis 35624	Align	Procter & Gamble (Mason OH)
L. rhamnosus R0011 *L. acidophilus* R0052	Sold as ingredient	Lallemand (Montreal, Canada)
B. lactis Bb-12 *L. acidophilus* LA5 *L. paracasei* CRL 431 *L. fermentum* VRI003 (PCC) *L. reuteri* RC-14 *L. rhamnosus* GR-1 *L. paracasei* F19	Sold as ingredient	Chr. Hansen (Milwaukee WI)
L. casei Shirota *B. breve* Yakult	Yakult	Yakult (Tokyo, Japan)
L. casei DN-114 001 ("L. casei Immunitas") *B. animalis* DN-173 010 ("Bifidis regularis")	DanActive fermented milk Activia yogurt	Danone (Paris, France) Dannon (Tarrytown, NY)
L. johnsonii Lj-1 (NCC533; *L. acidophilus* La-1)		Nestlé (Lausanne, Switzerland)
*L. plantarum*299V *L. rhamnosus* 271	Sold as ingredient Good Belly	Probi AB (Lund, Sweden)NextFoods (Boulder, Colorado)
L. reuteri ATCC 55730 ("Protectis")	BioGaia Probiotic chewable tablets or drops	Biogaia (Stockholm, Sweden)
L. rhamnosus GG ("LGG")	Culturelle	Valio Dairy (Helsinki, Finland)
L. rhamnosus LB21 *Lactococcus lactis* L1A	Sold as ingredient	Essum AB (Umeå, Sweden)
L. salivarius UCC118		University College (Cork, Ireland)
B. longum BB536	Sold as ingredient	Morinaga Milk Industry Co., Ltd. (Zama-City, Japan)
L. acidophilus LB	Sold as ingredient	Lacteol Laboratory (Houdan, France)
Bacillus coagulans BC30	Sustenex, Digestive Advantage;Sold as ingredient	Ganeden Biotech Inc. (Cleveland, OH)

[1]*Parenthetic entries indicate alternative strain designations;* B. lactis *is a shorthand designation for* Bifidobacterium animalis *subsp* lactis.
[2]*Strains sold as ingredients are available in numerous consumer products; contact responsible company for product list. Products listed are examples and do not reflect a comprehensive list of available products containing the indicated strain.*

1.1.2 Probiotic definition and regulation

According to the definition proposed by the FAO/WHO workshop of 2002, probiotics are 'live microorganisms which when administered in adequate amounts confer a health benefit on the host' ("Guidelines for the Evaluation of Probiotics in Food", FAO/WHO, 2002). The FAO/WHO consultation had as major outcomes the providing of an official definition for probiotics, together with guidelines regarding safety evaluation of the microorganisms possibly employed as probiotics, indications about the strategies for the assessment of nutritional and beneficial properties, and an overview of the rules needed for the elaboration of the health claims. An aspect of particular importance stated in these recommendations is that the probiotic properties must be strictly correlated to a single bacterial strain, meaning that benefits obtained from the ingestion of a specific bacterial strain cannot be extended to other strains, even belonging to the same species.

Nowadays, the situation of probiotics products regulation, especially in European Union (EU), is still a controversial issue. Probiotic products which have been introduced on market after 1997 must follow the indications regarding the Novel Foods reported in the Regulation (EC) of the European Parliament and of the Council No 258/1997, as well as the general guidelines reported in the Regulation (EC) No 1924/2006 on nutrition and health claims made on food, which thus encompass also the health claims allowed for probiotic products. Moreover, subsequent regulations on labeling, presentation and advertising of foodstuffs were introduced starting from 2007, aimed to provide a common regulation in all members states of EU, and to guarantee consumers safety and protection against misleading labels. To these specific aims, in 2002 the European Food Safety Authority (EFSA) was established by EU politicians in order to ensure an high level of consumer protection and to restore confidence in food, in particular after several food scares. According to current rules, food industry before introduce a new probiotic product on market must receive an EU authorization based on EFSA's assessment of safety.

In 2007 EFSA introduced the concept of "Qualified Presumption of Safety" (QPS), an evaluation tool to assess the safety of a microorganism for a deliberate introduction into food and feed. These guidelines establish that, to obtain the QPS status (akin to the concept of "Generally recognized as safe", the American Food and Drug Administration designation that a chemical or substance added to food must be considered safe by experts) for a microorganism it is required the deposition of the strain and its characterization to molecular level, together with data on antibiotic resistances, genetic stability, toxins and virulence factors, and a body of knowledge regarding history of safe use and established uses of the bacterium, accompanied by scientific literature on absence of clinical cases and evidences of beneficial effects from *in vitro* and *in vivo* studies. Thus, industry must submit to EFSA each health claim for scientific evaluation prior to its approval and use in EU. In particular, the European Commission in 2008 produced guidance that indicated that the Authority would have rejected any health claim not including human clinical data (Vogel, 2010). Thus far EFSA has rejected every submitted dossiers regarding health claims for probiotics.

Currently, in Italy the only label allowed from the Health Ministry for a probiotic product is "it supports gut flora balance", even though in the Opinion reported in "EFSA Journal 2009; 7(9):1232", the NDA (nutrition, dietetic and allergy) panel of EFSA states: "Increasing the number of any group of bacteria is not considered in itself as beneficial".

Even in the United States market, which is showing a growing interest for these products, probiotics regulation is very complex. Probiotics sold as dietary supplements do not require

Food and Drug Administration (FDA) approval before introduction on market. Labels allowed for probiotics sold as dietary supplements without FDA consent may refer only to effects on structure/function of the body (like claims for the strengthening of body's defense, caring for the digestive system..), but they cannot refer to reduction of risk of diseases without FDA approval. Thus far, FDA has not approved any health claims for probiotics (Saldanha, 2008). Similarly, in Australia probiotics sold for specific health benefits require premarket review by the Therapeutic Goods Administration and are regulated as complementary medicines, and in Japan a premarket review by Health Ministry is required (Boyle et al., 2006). Nonetheless, research on probiotics is moving on, both towards laboratory studies and clinical trials (Table 1.1.2), in order to evaluate the effects on several medical conditions.

Table 1.1.2 *The beneficial effects of probiotic bacteria documented in human intervention studies (adapted from Saxelin et al., 2005). The strains used are indicated, as are specific comments on the trials. META, indicates meta-analyses and LGG, 299v and BB12 refer to* L. rhamnosus *GG,* L. plantarum *299v and* B. lactis *BB1, respectively.*

Effect	Strain(s)	Comments
Treatment of acute diarrhoea in children, especially caused by rotavirus	META	Positive correlation
Reduction of the risk of antibiotic-associated symptoms in children and in adults	META	Positive correlation
Reduction of the recurrence of *Clostridium difficile* enterocolitis	299v	Preliminary, more research needed
Reduction of the risk of acute diarrhoea in children	LGG; BB12	Documented for certain strains
Relief of milk allergy/atopic dermatitis in infants	LGG; BB12; combination of *L. rhamnosus* 19070-2 and *L. reuteri* DSM 122460	Documented for certain strain(s), more research needed
Relief of allergic rhinitis	*L. paracasei* 33	Preliminary, more research needed
Reduction in the risk of atopic diseases in infants	LGG	4 years single study — repetition needed
Reduction in the risk of respiratory infections	LGG	Preliminary, more research needed
Amelioration of the immune response	Various	Documented for certain strains, more research needed
Reduction in the risk of dental caries	LGG	Preliminary, repetition needed
Suppression of *Helicobacter pylori*	Various	Preliminary, more research needed
Reducing the recurrence of pouchitis	VSL#3; LGG	Documented for certain combination(s), more research needed
Relief of IBS symptoms	299v	Preliminary, more research needed
Relief of rheumatoid arthritis symptoms	LGG	Preliminary, more research needed

1.1.3 Safety of probiotics

The scientific evaluation of probiotic beneficial properties is becoming increasingly rigorous since the last years. When performing a literature search, combining the key words "probiotics", "clinical trials" and "humans", more than 750 are listed, the major of which in top ranking nutritional and clinical journal. But, even though there exists a lot of strong evidences of probiotics administration efficacy in the treatment and prevention of several disorders, from acute gastroenteritis to intestinal neoplasia (Marteau, 2002), actually there are several risks due to a misuse of probiotics. In some conditions, in fact, these products might turn into detrimental factors, since their administration is often wider than the recommended and specific indications (Boyle et al., 2006), and the beneficial use of specific strains in healthy subjects might lead to detrimental consequences in patients (Besselink et al., 2008; Capurso & Morelli, 2010).

Safety evaluation of probiotics is controversial. Probiotics have been used for years in food industry, and there are also several studies confirming that the wider use of probiotic products has not caused any increase of bacteremia cases (Saxelin et al., 1996; Salminen et al., 2002). Moreover, some studies reported the safe use of probiotics even in high risk populations (Stansbridge et al., 1993; Wolf et al., 1998; Salminen et al., 2004). However, cases of sepsis are reported in literature (Ha et al., 1999; Kunz et al., 2004; Land et al., 2005). Along with translocation potential, other possible risks which can derive from the introduction of microorganisms include antibiotic resistance-transfers, gastrointestinal toxicity (caused by deleterious metabolic activities and production of dangerous metabolites), excessive immune stimulation (Snydman, 2008). The major obstacle in a safe definition of the possible applications of probiotic therapy is the deficiency in the knowledge of probiotic mechanism of action, in particular at immunological level. In general, the main aspect that still creates concern and confusion about probiotics use is the target consumer. FAO/WHO guidelines are in fact only related to food and the use of beneficial bacteria in healthy subject, thus excluding the use in pathologic conditions, which is properly a "therapeutic matter". Probiotics can be considered safe for the use in healthy people, but the observed beneficial properties cannot be extended to other populations, and administration should be evaluated with caution in medical conditions and in specific categories of person, like immunocompromised subjects, preterm infants or with underlying diseases, elderly patients". So each probiotic strain characterization activity should have a study-by-study and population-based approach (Snydman, 2008).

1.1.4 The probiotic approach

The probiotic approach could be an alternative and mild strategy for the prevention and treatment of either inflammatory or allergic diseases. It is estimated that the human microbiota contains as many as 10^{14} bacterial cells, a number that is 10 times greater than the number of human cells present in our bodies, starting from the skin surface to the genitourinary tract, oral cavity, respiratory tract, ear, and the gastrointestinal tract (Ley et al., 2006). Metagenomics and functional molecular immunology substantiate the interpretation of humans as holobionts, in the sense of functional superorganisms, combining self and microbes acting in concert to produce phenomena governed by the collective (Kelly, 1994; Zilber-Rosenberg & Rosenberg, 2008). The association between host and symbionts affects the fitness of the holobiont within its environment and it often governs the physiological homeostasis on the narrow balance between host wellbeing and dysfunction (Eberl & Lochner, 2009; Proal et al., 2009). The mechanisms underlying the cross-talk between a human host and microbes are only marginally understood. However, several studies have shown that different bacterial strains can exert their probiotic abilities by influencing the host's immune system, thereby modulating immune responses, at both the local level and the systemic level (Borchers et al., 2009; Lebeer et al., 2010; Taverniti & Guglielmetti, 2011). Thus, the elucidation of probiotic effects at molecular level could supply the theoretical bases to develop strategies for preventing or treating several human dysfunctions, through the reconstitution of a proper human-microbe mutualism. The probiotic approach, in its widest sense, falls into this context, since it consists of the modification of a human microbiota by exogenous administration of microbial cells or cell components, aimed at benefiting the host's health. The majority of probiotic bacteria belong to the genera *Lactobacillus* and *Bifidobacterium* (Table 1.1.1), which are Gram-positive bacteria that constitute a major part of the normal intestinal microflora in animals and humans (Kotzampassi & Giamarellos-Bourboulis, 2012). However, there are also evidences of probiotic roles of other microbes such as yeasts (*Saccharomyces boulardii* and *Saccharomyces cerevisiae*) and

some non-pathogenic strains of *Escherichia coli* and *Bacillus* spp. not normally found in the gastrointestinal tract (de Vrese & Schrezenmeir, 2008).

1.1.5 Probiotics in infectious diseases

Infectious diseases represent a perpetual issue to deal with. According to the World Health Organization (WHO)'s 2004 World Health Report, infectious diseases are the second cause of death (following cardiovascular diseases) responsible for about the 26 % of the deaths occurred worldwide in 2002. One of the major concerns is represented by the acquiring of antimicrobial resistance by pathogens, and thus to the spread of resistant microorganisms. This event is mainly caused by the misuse of antibiotics that often are prescribed unnecessarily (Wise et al., 1998). In this context, the use of probiotic microorganisms able to exert an antagonistic activity towards pathogens can represent an alternative intervention to prevent infections, and might also reduce excessive antibiotics administration. Regarding their role for the prevention and treatment of infectious diseases, there is an increasing evidence coming from randomized clinical trials (RCTs) for what it concerns antibiotic-associated diarrhea (AAD), *Clostridium difficile* infection (CDI), acute gastroenteritis and infectious complications following admission to the Intensive Care Unit (ICU).

Traditionally, probiotics have been associated with gastrointestinal tract (GIT) and used for the prevention and treatment of gastrointestinal disorders. However, more recently, probiotics effectiveness has been proposed also for a broader use. In fact, several probiotics have been designed for the vaginal mucosa (Reid et al., 2009), the urinary tract (Borchert et al., 2008), the skin (Krutmann, 2009), and the oro-pharyngeal cavity (Tagg & Dierksen, 2003). Particularly for the latter, related studies report that the mechanisms of action of probiotics at oro-pharyngeal level is analogous of that described for the gut (Rao et al., 2012).

1.1.6 Upper respiratory tract infections and probiotics for the oro-pharyngeal mucosa

The use of probiotics for the oro-pharyngeal tract (OPT) is particularly promising. OPT dysfunctions are often related to the presence of microbial pathogens (for instance, *Streptococcus mutans*, group A streptococci, or *Porphyromonas gingivalis*) or to microbial dysbiosis. Furthermore, compared with the distal GIT, the OPT is a more accessible site for microorganisms of exogenous origin. For these reasons, the OPT is a potential target for new, specifically designed probiotic products. Upper respiratory tract infections (URTIs) represent the most common acute illness in the patient outsetting, and they account for 9% of all consultations in general practice (Bourke, 2007). URTIs include rhinitis, rhinosinusitis, rhinopharyngitis, also called the common cold, pharyngitis, epiglottitis and laryngitis (Popova et al., 2012). There are reported different studies demonstrating probiotics beneficial effects in several pediatric infectious diseases (Weichert et al, 2012). In studies performed thus far on probiotic effects for URTIs, half of the species employed belong to *Lactobacillus* genus (*L. rhamnosus*, *L. acidophilus*, *L. debrueckii*, *L. paracasei*, *L. plantarum*) and *Bifidobacterium* genus (*B. animalis*, *B. bifidum*, *B. longum*); the rest are represented by *Streptococcus* genus (*S. salivarius*, *S. mitis*, *S. oralis*, *S. sanguinis*). Among these studies, an interesting example demonstrating the potential of the probiotic approach for the OPT and URTIs is represented by the research activity of Prof. J. R. Tagg and coworkers, who isolated the *Streptococcus*

salivarius strain K12 (Wescombe et al., 2006). Strain K12 has a marked ability to inhibit pathogenic bacteria, mainly due to the production of three different bacteriocins, salivaricin A2, B and 9 (Power et al., 2008). Strain K12 was also demonstrated to be able to colonize the upper respiratory tract (Horz et al., 2007) and to down-regulate the innate immune responses of human epithelial cells (Cosseau et al., 2008). The scientific results from the Tagg lab studies supported the creation of a set of probiotic pharmaceutical products (lozenges, powders and chewing gum), commercialized under the name of BLIS, that were specifically designed for the prevention or treatment of dysfunctions such as cavities, periodontitis, halitosis and pharyngitis (http://blis.co.nz/international/). In July 2011, the oral probiotic products BLIS K12 were granted GRAS status by FDA, enabling this probiotic to be included as an ingredient in food products within the United States.

1.1.7 The oro-pharyngeal environment

In any potential probiotic application it is important consider the peculiarity of the target body site. In the oro-pharyngeal cavity is present an organized secondary lymphoid tissue, namely the naso-pharynx associated lymphoid tissue (NALT), a specialized component of the family of mucosa-associated lymphoid tissue (MALTs), that all together constitute the mucosal immune system (Fukuyama et al., 2009). In humans NALT is mainly represented by the Waldeyer's ring, that comprise palatine tonsils, nasopharyngeal tonsil (adenoid) and lingual tonsil as major constituents (Hellings et al., 2000). Defense responses at level of mucous membranes are very critical, since these sites are continuously exposed to a large amount of different antigens, meaning that their role is not only to provide an efficient barrier to potentially harmful agents, but also to induce protective immune reactions (Debertin et al., 2003). In particular, the oro-pharyngeal tract is a way of entrance of both airborne and alimentary antigens. Characteristics and functions of the immune system at this level are organized in order to act either with local mucosal response or systemic immunological effects (Brandtzaeg, 2011). Considering the enormous number of microorganisms with which the mucosal immune system has constantly to interface, the ability to discriminate between “friends and foe”, and thus to recognize and eliminate pathogens while tolerating harmless commensals, represents the basis for the maintenance of homeostasis (Cutler & Jotwani, 2006).

1.1.8 Probiotics mechanisms of action: the modulation of the immune response

The modes of action by which probiotics are suggested to affect human health fall include three main categories (Lebeer et al., 2008). First, some probiotics can inhibit pathogens though various antagonism mechanisms. This is the most studied probiotic mechanism and has been exhaustively reviewed (Servin, 2004). A second way is the enhancement of the functions of mucosal barriers, particularly at intestinal level, and the modulation of various signalling pathways that lead to, for instance, the induction of mucus, and defensin production, enhancement of tight junction functioning, and prevention of apoptosis (Lebeer et al., 2010). The third mechanism is the modulation of host’s immune responses, both at local and systemic level (Smits et al., 2005). Increasing evidence supports this hypothesis (Borchers et al., 2009; Lebeer et al., 2010; Adams, 2010; Taverniti & Guglielmetti, 2011). Clinical applications of the immunomodulatory properties of bacteria have been performed particularly for lactobacilli and bifidobacteria. These studies refer to prevention and treatment of allergic diseases, in particular

atopic dermatitis in children, inflammatory bowel diseases, and prevention of virus infection and use as an adjuvant in vaccination (Borchers et al., 2009). In spite of this, the mechanisms determining the microbial ability to interact with the immune system of mammals are largely unexplored. Thus, studies targeted to elucidate the mechanisms involved in bacteria-host's cells cross-talk are required.

1.1.9 The first line of defense of the immune system: the innate immunity

The role of the immune system is to defend against diseases and to maintain homeostasis. Host's cells are continuously exposed to both endogenous and exogenous bacteria, which encompass harmless and potential detrimental microorganisms. Thus, one of the most important function of a balanced immune system is a proper recognition of self and non-self, that results in the mounting of non-inflammatory or sub-inflammatory responses against commensals, and activation of immune responses against pathogens. The immune system consists of two components, innate and acquired immunity, that induce both the systemic and the mucosal immune responses. The innate immunity is the first line of defense and is preposed to provide an early and non-specific response to antigens, targeted to a rapid elimination of the harmful agents. Innate immune responses can be initiated at level of epithelial cells and, more specifically, by phagocytes like neutrophils, dendritic cells, monocytes and macrophages, that play a crucial role in host defense (Galdeano & Perdigón, 2006).

Macrophages, once activated by microbial products, acquire microbicidal competence (Mackaness, 1964). Polarized macrophages have been classified into two main groups, M1 and M2, differing for their phenotype and effector functions: M1 subset, activated by bacterial products and inflammatory cytokines, produces cytokines like IL6, IL12, COX-2, IL1β and TNFα, and possesses microbicidal and inflammatory properties, while M2 are poorly microbicidal and with immunomodulating characteristics (Benoit et al., 2008). Macrophages are able to produce cytokines recruiting other inflammatory cells such as neutrophils. Phagocytic cells are attracted to the infection site by chemotaxis.

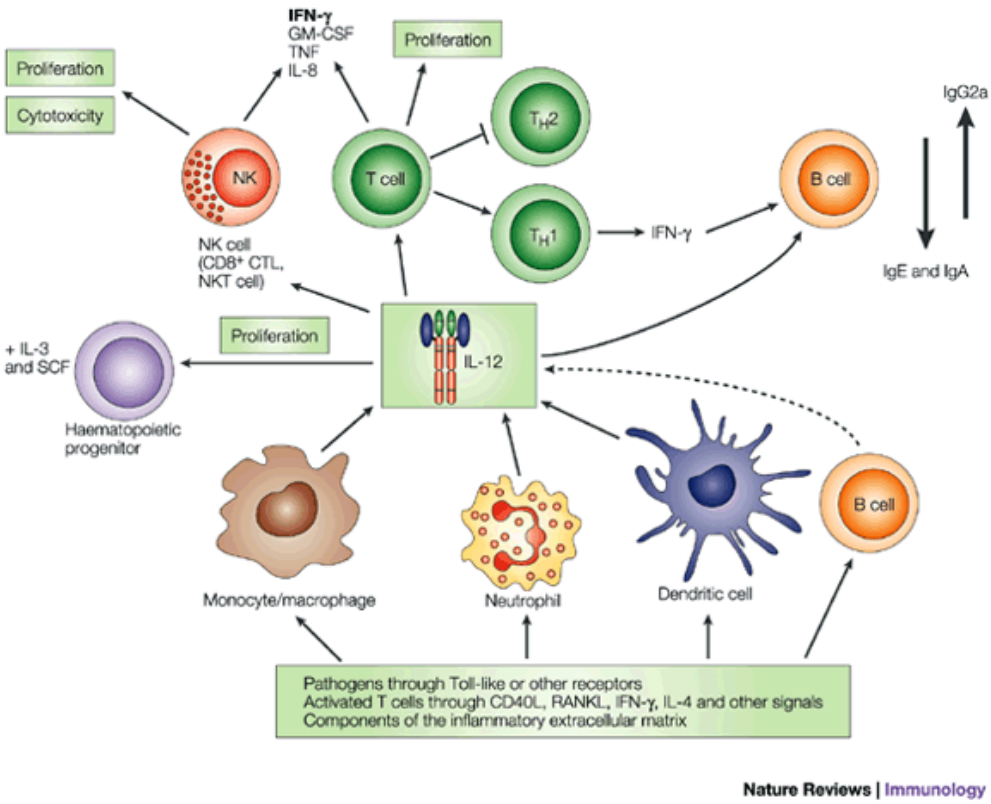
Natural Killer (NK) cells also participate in the innate immune response. NK cells rapidly react to the presence of virus infected cells in the early stages of infection by killing the infected target cells (Delcenserie et al., 2008).

Lymphocytes (B and T) are the main actors in the adaptive immune response. The adaptive immune system can provide a more effective protection against pathogens through the ability to recognize and remember an impressive number of antigens. (Delcenserie et al., 2008). Helper T cells are mainly found in two distinct cell types, Th1 and Th2, distinguished by the cytokines they produce and respond to, and the immune responses they are involved in. Th1 cells produce pro-inflammatory cytokines which stimulate the phagocytosis and destruction of microbial pathogens, while Th2 cytokines such as IL-4 generally stimulate the production of antibodies directed toward large extracellular parasites. Even though a true balance between Th1 and Th2 profiles is difficult to maintain, there are categories of immune cells, *i. e.* regulatory T cells (Treg), which are preposed to this crucial function. The balance between Th1 and Th2 responses determines, in fact, the direction and outcome of an immune response; moreover, prolonged or exaggerated responses in each direction can lead to the development of Th1-driven (chronic-inflammatory diseases) or Th2-driven (allergic diseases) pathologies (Delcenserie et al., 2008).

Dendritic cells (DCs), as well as macrophages and monocytes, are the link between the innate and adaptive immune systems, since they act as professional "antigen-presenting cells" (APCs). This function is crucial in initiating the adaptive immune response, as T cells do not respond to free-antigen but only to antigen that is presented by APCs. Cells of the innate immune system also influence the adaptive immune response through the production of chemical mediators called "cytokines": these are proteins which are involved in the communication among cells, and are essential in regulating cell priming, acquiring of a specific phenotype, and thus determining the outcomes of an immune response at both innate and adaptive levels (Fig. 1.1.1).

Epithelial cells and phagocytes interact with and respond to microorganisms by means of their pattern recognition receptors (PRRs). PRRs detect microorganism-associated molecular patterns (MAMPs), which are widespread and conserved among microorganisms (Lebeer et al., 2010). The best studied PRRs are Toll-like receptors (TLRs), which are transmembrane proteins present at the cell surface or on the membrane of endocytic vesicles or other intracellular organelles (Medzhitov, 2007). TLRs play a central role in alerting antigen-presenting cells to the presence of pathogenic material (Dunzendorfer et al., 2004).

Figure 1.1.1 *Cells and selected cytokines taking part in innate and adaptive immunity (adapted from Trinchieri, 2003).*



The modality through which bacteria can elicit a specific immune response, both in terms of cells signaling pathways activated and mechanisms involved in the host-microbes interplays, is still poorly understood. Thus, in order to identify the ways of action of health-promoting bacteria, and to find new applications in the prevention and treatment of several disorders, as well as for the evaluation of potential risks, studies at mechanistic level are required. Moreover, it is important to stress that bacteria interact with host cells in a strain-specific way. That leads to the need to identify which component in each bacterial strain is responsible for an immunomodulatory activity.

1.2 AIMS OF THE STUDY

Starting from the above-mentioned promising studies regarding the beneficial properties of food-grade bacteria in infectious diseases and on host innate immunity, we screened bacterial strains both from oral and food origin for their potential use as probiotics for the oro-pharyngeal mucosa. We tested the ability of bacteria, newly isolated from the pharynges of healthy volunteers or from dairy origin, to adhere to a human pharyngeal cell layer and to antagonize *S. pyogenes* on two different epithelial cell lines. As a result of these studies, we selected the oral isolate *Streptococcus salivarius* ST3 and the dairy strain *Lactobacillus helveticus* MIMLh5 as new probiotic candidates for the pharyngeal mucosa. These strains were further investigated for their *in vitro* probiotic properties when employed in combination, and also analyzed from an immunological point of view. Specifically, we examined their ability to adhere to FaDu human pharyngeal cells, to antagonize *Streptococcus pyogenes* and to modulate the immune response on U937 macrophages. Moreover, to explore the potential of combining strains ST3 and MIMLh5 in a fermented food product, we also studied the growth of *S. salivarius* ST3 in the presence of different sugars and in milk in co-culture with strain MIMLh5.

Our results support the possibility of producing a novel milk-based fermented food product to be used as probiotic for the oro-pharyngeal mucosa.

1.3 MATERIALS AND METHODS

1.3.1 Isolation of bacteria from pharyngeal mucosa and culture conditions

To isolate bacteria from pharynx, specimens were collected using polyester fiber-tipped applicator swabs (VWR, Milano, Italy) from 4 healthy donors (3 females, 58, 32 and 29 years old, and a 32 years old male). After serial dilutions in 0.1 % peptonated saline, speciments were plated on MRS agar (Fluka Feinchemikalien GmbH, Neu-Ulm, Germany) supplemented with 0,05 % cysteine-HCl (cMRS), M17 (Fluka Feinchemikalien GmbH) containing 2 % lactose (L-M17) and 2 % glucose TSA (Difco, Detroit, MI, USA). Around 50 colonies were randomly picked up and spread on plate with a loop. This procedure was repeated at least four time, in order to obtain pure cultures. Tables 1.3.1 and 1.3.2 list the bacterial strains used in this study. If not differently specified, oral streptococci, *Streptococcus thermophilus* and lactococci were routinely grown overnight at 37°C in L-M17. Lactobacilli were cultivated overnight in cMRS at 37°C. *Bifidobacterium animalis* subsp. *lactis* Bb12 was grown anaerobically at 37°C in pre-reduced cMRS, while *Streptococcus pyogenes* was grown overnight at 37°C in BHI (Difco) supplemented with 0.3 % yeast extract.

1.3.2 Identification and molecular characterization of bacterial isolates

The isolates from each subject have been clustered by means of BOX-PCR assay (Table 1.3.1), which was performed with primer BoxA1 (5′-CTACGGCAAGGCGACGCTGACG- 3′) in a PCR reaction mix consisting of of 20 mM Tris–HCl, 50 mM KCl, 200 μM of each deoxynucleoside triphosphate, 1 μM of the primer, 1.5 mM MgCl2 and 1.5 U of Taq polymerase (Fermentas, Vilnius, Lithuania). The 16S rRNA gene was amplified from at least one representative isolate from each BOX-genotypic group (Table 1.3.1) by PCR using the primers P0 (5′-GAAGAGTTTGATCCTGGCTCAG-3′) and P6 (5′-CTACGGCTACCTTGTTACGA-3′). Each PCR mixture (50 μl) was as for BOX-PCR analysis. Each PCR cycling profile consisted of an initial denaturation time of 2 min at 95°C followed by an amplification for 35 cycles of denaturation (30 s at 95°C), annealing (45 s at 55°C) and extension steps (2 min at 72°C). The PCR was completed with an elongation period (7 min at 72°C). The resulting amplicons were then sequenced by using an ABI Prism BigDye™ terminator technology in an ABI Prism™ 310 DNA sequencer (Applied Biosystems, Foster City, CA, USA). *Streptococcus salivarius* isolates were further characterized by RAPD analysis, performed with primers OPI17 (5'-CGAGGGTGGTGATC -3'), OPI02-mod (5'-GCTCGGAGGAGAGG-3'), M13 (5'-GTAAAACGACGGCCAGT-3') and PedAF (5'-ATACTACGGTAATGGGGT-3'). Reaction mix was as for BOX analysis, and the temperature profile was as follows: primary DNA denaturation step at 94°C for 2 min followed by 5 cycles of 45 s at 94°C, 45 s at 31°C and 2 min at 72°C; 35 additional cycles were carried out increasing the annealing temperature to 40°C. For all the amplification cycles the final extension was continued for 7 min at 72°C.

Similarity dendrogram was built using NTSYSpc v. 2.01 (Applied Biostatistic Inc., New York, NY).

Table 1.3.1 *Bacteria isolated from pharyngeal mucosa.*

Human source's designation	BOX-PCR genotype[1]	Denomination of the isolates[2]	Isolation medium	Taxonomic identification[3]	Additional identification method[4]
IS	IS-A1	**IS1**	cMRS	*S. salivarius*	
		IS8, IS10, IS12	L-M17		
	IS-A2	**IS5**, IS6,	L-M17	*S. salivarius*	
		IS7	gTSA		
	IS-B	**IS3**, IS4	cMRS	*S. sanguinis*	
	IS-C	**IS9**	L-M17	*Staphylococcus aureus*	
		IS15	gTSA		
	IS-D	**IS11**, IS13	L-M17	*Rothia mucillaginosa*	
		IS14	gTSA		
RS	RS-A1	**RS1**, RS8, RS10	L-M17	*S. salivarius*	
		RS3, RS6	cMRS		
		RS13, RS14	gTSA		
	RS-A2	**RS9**	L-M17	*S. salivarius*	
	RS-B	**RS4**	cMRS	*Alloscardovia omnicolens*	
	RS-C	**RS5**	cMRS	*Lactococcus lactis subsp. Lactis*	*his*
	RS-D	**RS11**	L-M17	*Micrococcus sp.* (*luteus*)	
	RS-E	**RS12**	gTSA	*Rothia mucillaginosa*	
	RS-F	**RS15**	gTSA	*Bacillus subtilis*	
SM	SM-A1	**SM1, SM2**, **SM4**, SM5	cMRS	*S. salivarius*	
		SM6, SM8, SM9	L-M17	*S. salivarius*	
		SM11, SM14, SM15	gTSA	*S. salivarius*	
	SM-A2	**SM12**	gTSA	*S. salivarius*	
	SM-B	**SM3**	cMRS	*S. oralis*	*gdh*
	SM-C	**SM7**	L-M17	*Rothia mucillaginosa*	
	SM-D	**SM13**	gTSA	*Neisseria* sp. (*subflava*)	
ST	ST-A1	**ST3**	cMRS	*S. salivarius*	
	ST-A2	**ST12**	L-M17	*S. salivarius*	
	ST-B	**ST2**, **ST5p**	cMRS	*S. oralis*	*gdh*
	ST-C	**ST5g**	cMRS	*S. sanguinis*	
	ST-D	**ST4**	cMRS	*S. oralis*	*gdh*
		ST15	gTSA		
	ST-E	**ST7**, **ST11**	L-M17	*S. infantis*	*gdh*
	ST-F	ST1, **ST8**	L-M17	*Bacillus* sp. (*cereus*)	
	ST-G	**ST9**	L-M17	*S. oralis*	*gdh*
	ST-H	**ST6**	cMRS	*Staphylococcus epidermidis*	
	ST-I	**ST13**, ST14	gTSA	*Rothia mucillaginosa*	

[1] *Similar (but not identical) BOX-PCR electrophoretic patters are indicated with the same letter and a different number.*

[2] *Representative strains chosen for 16S rDNA sequence analyses are in bold.*

[3] *S. salivarius stands for* Streptococcus salivarius. *Species designation between brackets refers to 16S rDNA sequence similarity below 95%.*

[4] *gdh: taxonomic identification based on the sequence of an approximately 500-bp internal fragment of the glucose-6-phosphate dehydrogenase gene (gdh) (Bek-Thomsen et al., 2008); his: taxonomic identification based on the length polymorphism of a PCR amplified fragment from histidine biosynthesis operon (Beimfohor et al., 1997).*

Table 1.3.2 Streptococcus salivarius *K12 and non-oral bacteria included in the study*.

Species	Strain	Source	Reference
Bifidobacterium animalis subsp. *lactis*	Bb12	Probiotic commercial strain	Juntunen et al., 2001
Lactobacillus acidophilus	LA5	Probiotic commercial strain	Juntunen et al., 2001
Lactobacillus paracasei	Shirota	Probiotic commercial strain	Juntunen et al., 2001
Lactobacillus helveticus	MIMLh5	Dairy natural starter	Guglielmetti et al., 2008
Lactobacillus rhamnosus	GG	Probiotic commercial strain	Juntunen et al., 2001
Lactococcus lactis subsp. *cremoris*	Viili	Finnish fermented milk	Kahala et al., 2008
Streptococcus salivarius	K12	BLIS throat guard	Burton et al., 2006
Streptococcus thermophilus	DSM20617^T	DSMZ type strain, yogurt	/

1.3.3 Antibacterial activity against *Streptococcus pyogenes* and PCR detection of bacteriocin encoding genes of pharyngeal isolates

In a first set of experiments, tester bacterial strains were spread with a loop on agar plate containing LM17 medium and incubated overnight at 37°C. Then, 15 ml of soft yeBHI agar containing about 10^6 cells of the indicator strain (*S. pyogenes* C11) were poured over the plates. The plates were checked for inhibition zones after incubation at 37°C for 24 and 48 h. The production of antimicrobial substances was also tested through disk-diffusion. Briefly, tester strains were grown until stationary growth phase in LM17 medium. Culture supernatants were neutralized to pH 7, filter-sterilized and spotted (0.1 ml) on a filter paper disk, which was previously placed on yeBHI softagar plates inoculated with about 10^5 *S. pyogenes* cells. The presence of an inhibition halo was checked after 24 and 48 h.The PCR reactions to detect previously characterized bacteriocin structural genes (salivaricin A, salivaricin B, streptin, and peptide SA-FF22) were performed as described by Wescombe et al. (2006).

1.3.4 Preparation of bioluminescent *Streptococcus pyogenes*

Reporter vector pCSS810, carrying a phage T5 promoter–lac operator upstream of the insect luciferase gene *lucFF* (Lampinen et al., 1992), was used to obtain the luminescent phenotype in *Streptococcus pyogenes* C11. In brief, electrocompetent *S. pyogenes* cells were prepared as follows. Fresh BHI broth (25 ml) supplemented with 0.3% yeast extract (yeBHI) was inoculated with 0.25 ml of an overnight culture of the bacterium and incubated at 37°C until an optical density (at 600 nm) of about 0.2 was reached. The cells were chilled on ice and concentrated in further washing steps of 25 and 10 ml (washing buffer EB: 0.3 M sucrose and 1 mM $MgCl_2$ in 5 mM phosphate buffer, pH 6.9). After washing, the pellet was resuspended in 2 ml of the same buffer, dispensed in 80 µl-aliquotes and maintained at -80°C until used for electroporation with an Eppendorf Multiporator 2510 (Eppendorf, Milano, Italy), at 12.5 kV/cm, in 2-mm cuvettes. The yeBHI supplemented with sucrose (final concentration 0.3 mM) was used as outgrowth medium. After 2 h of incubation at 37°C in the outgrowth medium, transformants were selected

on yeBHI agar plates with 5 μg ml^{-1} of chloramphenicol. The selected luminescent *S. pyogenes* clone was named $C11^{LucFF}$.

1.3.5 Antagonistic activity against *Streptococcus pyogenes*

FaDu (human pharynx carcinoma cell line; ATCC HTB-43) and HaCat cells (human keratinocytes from a spontaneous immortalized, non-tumorigenic cell line) were routinely grown in 24-well tissue culture plates in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10 % (v/v) heat-inactivated (30 min at 56°C) fetal calf serum, 100 U ml^{-1} penicillin, 100 mg ml^{-1} streptomycin, 0.1 mM non-essential amino acids, 2 mM L-glutamine, and incubated at 37°C in a water-jacketed incubator in an atmosphere of 95 % air and 5 % carbon dioxide, until a confluent monolayer was formed. The bacterial cell concentration of an overnight culture was determined microscopically with Neubauer Improved counting chamber (Marienfeld GmbH, Lauda-Königshofen, Germany). The antagonism was studied through exclusion and competition assays. Exclusion consisted of a pre-incubation of the FaDu layer with 1 ml of a tester strain suspension (5 x 10^8 cells ml^{-1}), followed by a washing step with phosphate-buffered saline (PBS) and the incubation with 1 ml of the indicator strain (*S. pyogenes* $C11^{LucFF}$) suspension (2 x 10^8 cells ml^{-1}). The concentration of 5 x 10^8 tester cells ml^{-1} was chosen because corresponded to the plateau of a dose-response curve which was prepared during the set-up of the experiment by measuring the antagonistic activity as a function of tester cell concentration (Data not shown).

Competition consisted of co-incubation of the same number of tester and indicator strains (2 x 10^8 cells ml^{-1}). After incubation, FaDu layers were quickly washed twice with 1 ml PBS (pH 7.3) and D-luciferin (Sigma-Aldrich, Steinheim, Germany) was added at the concentration of 12.5 μM in citrate buffer, pH 5. Immediately, the luminescence signal was measured with a Victor 3 luminometer (PerkinElmer, Monza, Italy). Each tester strain was analyzed in triplicate in at least two independent experiments. Unpaired Student's *t*-test was run for statistically significant differences.

1.3.6 Bacterial adhesion to FaDu cell layer

FaDu cells were grown in 3-cm Petri plates on microscopy cover glasses as described above. Cell monolayers were carefully washed with PBS pH 7.3 before bacterial cells were added. The bacterial cell concentration of an overnight culture was determined microscopically with Neubauer Improved counting chamber (Marienfeld GmbH). Approximately 2×10^8 cells of each strain resuspended in PBS (pH 7.3) were incubated with a monolayer of FaDu cells. After 1 h at 37°C, all monolayers were washed 3 times with PBS to release unbound bacteria. Cells were then fixed with 3 ml of methanol and incubated for 8 min at room temperature. After methanol was removed, cells were stained with 3 ml of Giemsa stain solution (1:20) (Carlo Erba, Milan, Italy) and left for 30 min at room temperature. Wells were then washed until no color was observed in the washing solution and dried in an incubator for 1 h. Microscopy cover glasses were then removed from the Petri plate and examined microscopically (magnification × 100) immersed in oil. Adherent bacteria in 20 randomly selected microscopic fields were counted and averaged.

1.3.7 Antibiotic susceptibility of selected bacteria

The inhibitory concentrations of the antimicrobial agents were determined by broth microdilution in commercial 96-well microtiter plates, for the following concentration ranges: ampicillin, chloramphenicol, erythromycin, oxytetracyclin and vancomycin 1-16 μg ml^{-1}; gentamicin 8-64 μg ml^{-1}; kanamycin and streptomycin 16-128 μg ml^{-1}. Antibiotics (Sigma-Aldrich) were serially 1:2 diluted in culture medium starting from a concentrated stock solution conserved at –20°C. In brief, bacterial cells from an overnight culture were washed once with saline and resuspended in fresh medium at a concentration corresponding to an optical density of 1 at 600 nm. Afterward, bacterial suspensions were 1:100 diluted in the liquid medium containing the appropriated antibiotic concentration, and loaded in duplicate in microtiter wells (200 μl $well^{-1}$). Three different liquid media were used in this experiment: L-M17, MRS and BHI (Table 3). The inoculated plates were subsequently incubated aerobically at 37°C for 48 h, after which the inhibitory concentrations of antibiotics were read as the lowest concentration.

1.3.8 Determination of urease activity and PCR detection of ureC gene

Urease activity was tested by evaluating the release of ammonia by means of the phenol red assay (Lanyi, 1987). Specifically, cells from 0.5 ml of an overnight culture were resuspended in a solution containing one volume of solution A (urea, 2 g dissolved in 2 ml of ethanol and 4 ml of sterilized water) and 19 volumes of solution B (KH_2PO_4, 1 g l^{-1}; K_2HPO_4, 1 g l^{-1}; NaCl, 5 g l^{-1}; phenol red, 20 μg ml^{-1}). The suspension was incubated at 37°C for 1-4 h and the development of a red-violet colour indicated positive urease activity. The amplification of the gene coding for the main subunit of the urease complex (*ureC*) was carried out as previously described (Mora et al., 2004), using primers ureC-F (5'-CTGTTCATGATCCTATTCAG-3') and ureC-R (5'-CGAATACCGAATTCATTCCCATG-3').

1.3.9 Stimulation of FaDu monolayers and enzyme-linked immunosorbent assay (ELISA) measurement of cytokine production

Human pharyngeal carcinoma cells (FaDu) were seeded into 24-well plates and grown as previously described. Bacterial cells were added to monolayers of FaDu cells in 0.5 ml of fresh antibiotic-free Eagle's Minimum Essential Medium (EMEM) containing 25 mM HEPES (pH 7.4) and incubated overnight at 37°C. Each bacterial strain was used at a multiplicity of infection (MOI) of about 1000, while EMEM/HEPES medium without bacterial cells was used as control. After overnight incubation, the supernatants were collected by pipetting, centrifuged to remove cells, and kept at -80°C. Finally, 17 different cytokines in the supernatants were determined on a Bio-plex Array Reader (LUMINEX 100, Bio-RadLaboratories, Hercules, CA) using Bio-Plex Human Cytokine 17-plex panel (Bio-Rad), according to the human cytokine Bio-Plex panel assay Bio-protocol (Bio-Rad). The list of tested cytokines and the corresponding detection limits were as follows: interleukin (IL)-1β, 0,3 pg ml^{-1}; IL-2, 0.2 pg ml^{-1}; IL-4, 0.1 pg ml^{-1}; IL-5, 0.3 pg ml^{-1}; IL-6, 0.2 pg ml^{-1}; IL-7, 0.3 pg ml^{-1}; IL-8, 0.3 pg ml^{-1}; IL-10, 0.2 pg ml^{-1}; IL-12 (p70), 0.4 pg ml^{-1}; IL-13, 0.3 pg ml^{-1}; IL-17, 0.5 pg ml^{-1}; granulocyte colony-stimulating factor (G-CSF), 0.2 pg ml^{-1}; granulocyte macrophage colony-stimulating factor (GM-CSF), 1.1 pg ml^{-1}; interferon (IFN)-γ, 2.6 pg ml^{-1}; monocyte chemotactic protein (MCP)-1, 0.8 pg ml^{-1};

macrophage inflammatory protein (MIP)-1β, 0.6 pg ml^{-1}; tumor necrosis factor (TNF)-α, 0.6 pg ml^{-1}.

1.3.10 Construction of stable NF-κB reporting FaDu cells

Stable transfectants of FaDu cell line were obtained after transfection with the plasmid pNiFty2 (InvivoGen, LaboGen, Rho, Italy; Fig. 1.3.1). This plasmid combines five NF-κB sites with the insect luciferase reporter gene *luc*. The presence of active NF-κB molecules in the cell activates the promoter, resulting in the expression of the luciferase gene. Transfection was performed by means of StoS Transfection Kit (GeneSpin, Milano, Italy) in accordance with the manufacturer's protocol. Afterward, cells were resuspended in fresh DMEM medium and seeded in 24-well plates for 48 h, in order to obtain the expression of the antibiotic resistance. Finally, stable recombinant clones were selected by adding into the culture medium 50 μg ml^{-1} of zeocin.

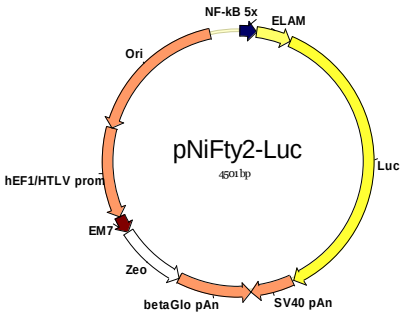


Figure 1.3.1. *Genetic map of a plasmid pNiFty2-Luc. This plasmid contains promoter combining five NF-κB-binding sites and the firefly luciferase reporter gene luc. The presence of NF-κB activating molecules in the cell activates this transcription factor, which binds to the promoter, resulting in the expression of the luciferase gene.*

1.3.11 Study of NF-κB activation

Recombinant FaDu cells were cultured using the same protocol as for non-transfected FaDu, in presence of 50 μg ml^{-1} of zeocin. After growth, FaDu layer was resuspended in EMEM at a concentration of 250.000 cells ml^{-1} in presence of 100 mM HEPES (pH 7.4). After that, 50 μl of tester bacterial suspension, containing 2 x 10^9, 2 x 10^8 or 2 x 10^7 cells ml^{-1}, were added to 450 μl of FaDu suspension, resulting in a MOI of about 1000, 100 or 10, respectively. After incubation at 37°C for 4 h, samples were kept in ice and sonicated at maximum speed for 5 s (Bandelin sonicator, Bandelin electronic GmbH & Co., Berlin, Germany). Bacterial cells and insoluble particles were removed by centrifugation and the supernatants were moved into a new tube. At this point, 200 μl of supernatants were aliquoted in duplicate into the wells of a 96-wells white microtiter plate (PerkinElmer); then 12.5 μl of a 10 mM ATP solution and 12.5 μl of 0.1 mM D-luciferin were added and the emitted bioluminescence was immediately recorded every 90 s with a Victor 3 luminometer (PerkinElmer). The maximum of the light-production curve was considered for comparing the results. In a different set of experiments, recombinant FaDu cells were simultaneously stimulated with IL-1β (2 ng ml^{-1}). All strains were analyzed in duplicate in at least three independent experiments for each MOI considered. Unpaired Student's *t*-test was run for statistically significant differences.

1.3.12 Study of the activation of the U937 human macrophage cell line. Cell culture, growth conditions, and stimulation protocol.

The cell line U937 (ATCC CRL-1593.2™) was derived from a human histiocytic lymphoma (Sundstrom et al., 1976). These cells are maintained as replicative, non-adherent cells and have many of the biochemical and morphological characteristics of blood monocytes (Harris and Ralph, 1985). When treated with phorbol myristate acetate (PMA), U937 cells differentiate to become adherent, non-replicative cells with characteristics similar to tissue macrophages, including similar isoenzyme patterns (Radzun et al., 1983) and other phenotypic markers (Harris and Ralph, 1985). The normal growth medium for the U937 cells consisted of RPMI 1640 medium (Lonza, Basel, Swiss) supplemented with 10 % (v/v) fetal bovine serum (FBS) (Gibco-BRL, Life Technologies, Milan. Italy), 2 mM L-glutamine, 100 units ml^{-1} penicillin and 100 μg ml^{-1} streptomycin (Sigma-Aldrich). U937 cells were seeded at a density of 5×10^5 cells $well^{-1}$ in 12-well plates and incubated at 37°C in a humidified atmosphere of 95 % air and 5 % CO_2. Differentiation was induced by the addition of PMA (Sigma-Aldrich) into the cellular medium at a final concentration of 100 nM and was allowed to proceed for 48 h. Afterwards, cells were washed once with sterile PBS buffer to remove all non-adherent cells. One hour before the bacteria were added to the cells, the culture media was replaced with RPMI 1640 medium supplemented with 1 % (v/v) FBS to allow the cells to adapt. Bacteria were used at MOIs of 100 and 1000, and lipopolysaccharide (LPS, final concentration of 1 μg ml^{-1}) from *Escherichia coli* 0127:B8 (Sigma-Aldrich) was used as positive control for pro-inflammatory stimulus in U937 cells. An untreated sample, *i.e.*, only RPMI 1640 medium with 1 % (v/v) FBS, was used as control.

1.3.13 Inhibition assay with Toll-like Receptor neutralizing antibodies on U937 cells.

Human anti-Toll-like receptor (TLR) 2 antibody (Invivogen) was added to U937 cells 1 hour before the stimulation with bacterial cells. A human immunoglobulin-A (IgA) 2 isotype control (Invivogen) was used as control to exclude non-specific binding and blocking activity of the antibody. Both the anti-TLR2 and IgA2 isotype control were used at 5 μg ml^{-1}. This concentration was determined by examining the neutralizing efficacy of anti-TLR2 with zymosan from *Saccharomyces cerevisiae* (Invivogen) as ligand; we observed that 5 μg ml^{-1} resulted in a 5-fold reduction in the expression level of tumor necrosis factor (TNF)-α, a cytokine known to be induced in the downstream pathway activated by TLR-2.

1.3.14 Preparation of RNA and reverse transcription.

After incubating U937 cells at 37 °C four 4 hours, the supernatant was carefully removed from each well and the total cellular RNA was isolated from the adhered U937 cells with an RNeasy Mini Kit (Qiagen Inc., Valencia, CA). Afterwards, RNA concentration and purity was determined with a Nanodrop Spectrophotometer (ND-1000, Thermo Fischer Scientific) and reverse transcription to cDNA was performed with the iScript™ Select cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA) using the following thermal cycle: 5 min at 25°C for, 30 min at 42°C, and 5 min at 85°C. *Real-time Quantitative PCR.* The mRNA expression levels of cytokines were analyzed with SYBR Green technology in real-time quantitative PCR using SsoFast EvaGreen Supermix (Bio-Rad) on a Bio-Rad CFX96 system according to the

manufacturer's instructions. The primers used are as follow (5'→3'): 18srRNA forward ATCCCTGAAAAGTTCCAGCA; 18srRNA reverse CCCTCTTGGTGAGGTCAATG; IL10 forward AGCAGAGTGAAGACTTTCTTTC; IL10 reverse CATCTCAGACAAGGCTTGG; TNF-α forward TCAGCTCCACGCCATT; TNF-α reverse CCCAGGCAGTCAGATCAT; COX2 forward CCCTTGGGTGTCAAAGGTAA; COX2 reverse TGAAAAGGCGCAGTTTACG. All primers were selected using the Primer3Plus software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi), and the specificity of the primers was tested with melting curves during amplification and by 1 % agarose gels. Quantitative PCR was carried out according to the following cycle: initial hold at 96°C for 30 s and then 40 cycles at 96°C for 2 s and 60°C for 5 s. Gene expression was normalized to the housekeeping gene 18s rDNA. The amount of template cDNA used for each sample was 12.5 ng. All results regarding cytokine mRNA expression levels are reported as the fold of induction (FOI) respective to the control (namely unstimulated U937), to which we attributed an FOI of 1.

1.3.15 Growth experiments.

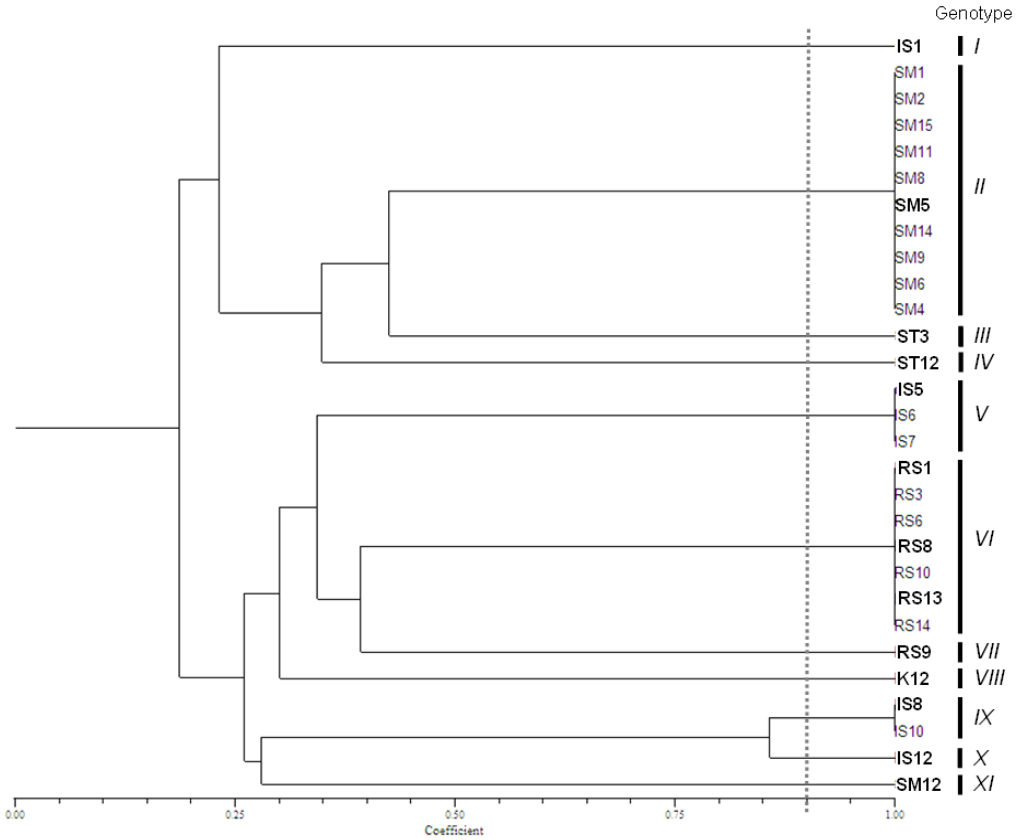
The growth curves of strain ST3 were determined in 384-well microtiter plates using a BioTek Synergy HT Multi-Mode Microplate Reader (AHSI S.p.A., Bernareggio Italy) to measure growth in M17 broth supplemented with 2 %, 1 %, 5 ‰, 2.5 ‰, 1.25 ‰ or 0.625 ‰ of one of the following sugars: fructose, galactose, glucose, inulin, lactose, lactulose, sucrose or fructooligosaccharides (FOS). All sugars were purchased from Sigma-Aldrich (Steinheim, Germany) with the exception of FOS, which were from Actilight® (Giulio Gross S.p.A., Trezzano sul Naviglio, Italy). The preparation of 384-well microtiter plates was conducted with an ep*Motion* Automated Pipetting System (Eppendorf, Milan, Italy). Acidification curves were determined by inoculating skim milk (Difco) (1 % inoculum) with an overnight culture of MIMLh5 and ST3 alone or in co-culture and recording the pH every hour for 24 hours with a pH-meter recorder (XS instruments pH 2100, Opto-Lab, Concordia, Italy).

1.4 RESULTS AND DISCUSSION

1.4.1 Identification and molecular characterization of bacterial isolates

In this study we aimed to select oral bacteria with potential probiotic features for the pharyngeal mucosa. We included bacteria newly isolated from the pharynges of healthy donors. Potentially, pharyngeal isolates could, in fact, display better performances in the colonization of the oral ecosystem than traditional dairy or intestinal probiotic bacteria. A total of 56 isolates were obtained from pharyngeal swab samples taken from four healthy donors by using three different culture media (Table 1.3.1). After a preliminary grouping of all isolated bacteria through BOX-PCR fingerprinting (Data not shown) 16S rRNA genes sequence analysis revealed that 39 of the isolates belonged to the *Streptococcus* genus. *Streptococcus salivarius* was the most represented species (28 isolates). We also isolated *Streptococcus oralis* (6 isolates) and *Streptococcus infantis* (2 isolates), which were distinguished by glucose-6-phosphate dehydrogenase gene (*gdh*) sequence analysis (Bek-Thomsen et al., 2008) and 1 strain of *Lactoccus lactis* sub. *Lactis*. The only species isolated from all four pharyngeal samples were *S. salivarius* and *Rothia mucilaginosa* (7 isolates). All the other bacteria are listed in Table 1.3.1. *Streptococcus salivarius* isolates were further characterized at the intraspecies level by means of BOX-PCR and RAPD-PCR analyses (Data not shown). A computer evaluation of similarities and clustering resulted in a total of 11 unique *S. salivarius* genotypes out of 28 pharyngeal isolates and the K12 commercial probiotic strain (Fig. 1.4.1). The isolates that were included in a single genotypic group originated from the same pharyngeal sample, suggesting that they were probably multiple isolates. In this research, half of the pharyngeal isolates were ascribed to the species *Streptococcus salivarius* (28 out of 56 isolates), in accordance with previous studies showing these bacteria to be the dominant cultivable species in the oro-pharyngeal tract (Kang et al., 2006). Nonpathogenic streptococci are the bacteria most largely present at the oro-pharyngeal level, and they have been proposed to exert a key role in the protection against pathogenic agents, which cause inflammation and infections (Tagg & Dierksen, 2003). In particular, *Streptococcus salivarius* already becomes a stable colonizer of the oral microbiota a few days after birth and represents, in adults, the major species at the levels of the pharyngeal mucosa and dorsal tongue.

Figure 1.4.1 *Unweighted-pair group method using average linkages (UPGMA) dendrogram derived from similarity coefficients calculated by the Jaccard method (simple Jaccard [Sj] coefficients; shown on the scale at the bottom), showing the relationship among* Streptococcus salivarius *pharyngeal isolates, analyzed by BOX-PCR and RAPD analysis using primers M13, OPI02mod, OPI17mod, and PedAF. Samples with a similarity coefficient higher than 0.9 have been included in the same genotype. Selected bacterial isolates included in antagonism experiments are indicated in boldface.*



1.4.2 Oral, dairy and probiotic strains differently adhered to FaDu human pharyngeal cell line

Thirty bacterial strains were studied for their ability to adhere to FaDu epithelial cell layer. Along with bacteria isolated from human pharyngeal mucosa we tested bacterial strains of dairy and probiotic origin already present on the market. In detail, strains MIMLh5, Viili and DSM 20617^{T} are used as starters in the production of Grana Padano cheese, Finnish fermented milk and yogurt, respectively. The strains *Bifidobacterium animalis* subsp. *lactis* Bb12, *Lactobacillus acidophilus* La-5, *Lactobacillus rhamnosus* GG and *Lactobacillus paracasei* Shirota are among the most widespread probiotic strains in the Western Countries. Certain properties of these strains have been compared to *Streptococcus salivarius* K12, the only probiotic bacterium specifically commercialized as probiotic for the pharyngeal mucosa up to date (*BLIS K12 Throat Guard* ™, Tagg et al., 2006). The first probiotic property that we considered was the adhesion to the host's target epithelium. In fact, the ability of commensals and probiotics to bind human mucosa has a pivotal importance because it can promote the colonization and sustain host epithelium-bacteria cross-talk (Lee et al., 1999). After extensive washings with PBS, a significant proportion of cells of most bacterial strains remained attached to the FaDu monolayer, providing evidence that the adhesion was not only non-specific physical entrapment. In particular, 5 strains displayed a strong adhesive phenotype, coinciding with an adhesion index (bacterial cells per 100 FaDu cells) of more than 2500 (Fig. 1.4.2). The 5 most adhesive strains included *L. helveticus* MIMLh5, *S. salivarius* ST3, *S. salivarius* K12 and two *Lactococcus lactis* strains (RS5 and Viili). A good adhesion ability was also displayed by 9 other strains, among which *L. rhamnosus* GG and 8 *S. salivarius* strains (Fig.1.4.2). Two strains in particular, *L. helveticus* MIMLh5 and *S. salivarius* ST3, showed to adhere to FaDu pharyngeal epithelial cell line comparably to the oral probiotic bacterium *S. salivarius* K12 that we used as reference (Horz et al., 2007).

Figure 1.4.2 *Adhesion of bacterial strains to FaDu epithelial cell layer according to their adhesion index (AdI, number of bacteria / 100 FaDu cells). (a) Adh = adhesion. +++, strong adhesion (AdI > 2500); ++, good adhesion (AdI between 2500 and 500); +, weak adhesion (AdI between 500 and 100); -, no adhesion (AdI < 100). (b) Adhesion to FaDu cell monolayers as observed with Giemsa staining under a light microscope. Bars, 8 µm. One FaDu nucleus for each layer is indicated with the letter N.*

Species	Strain	Adh
Lactobacillus helveticus	MIMLh5	+++
Lactococcus lactis subsp. cremoris	Vili	+++
Lactococcus lactis subsp. lactis	RS5	+++
Streptococcus salivarius	K12	+++
Streptococcus salivarius	ST3	+++
Lactobacillus rhamnosus	GG	++
Streptococcus salivarius	IS1	++
Streptococcus salivarius	IS12	++
Streptococcus salivarius	RS1	++
Streptococcus salivarius	RS9	++
Streptococcus salivarius	RS13	++
Streptococcus salivarius	SM5	++
Streptococcus salivarius	SM12	++
Streptococcus salivarius	ST12	++
Bacillus sp.	RS15	+
Lactobacillus acidophilus	LA6	+
Streptococcus oralis	SM3	+
Streptococcus oralis	ST4	+
Streptococcus oralis	ST5p	+
Streptococcus salivarius	IS5	+
Alloscardovia omnicolens	RS4	-
Bacillus sp.	ST8	-
Bifidobacterium animalis subsp. lactis	Bb12	-
Lactobacullus casei	Shirota	-
Rothia mucillaginosa	SM7	-
Streptococcus infantis	ST11	-
Streptococcus salivarius	IS8	-
Streptococcus sanguinis	ST5g	-
Streptococcus sanguinis	IS3	-
Streptococcus thermophilus	DSM20617	-

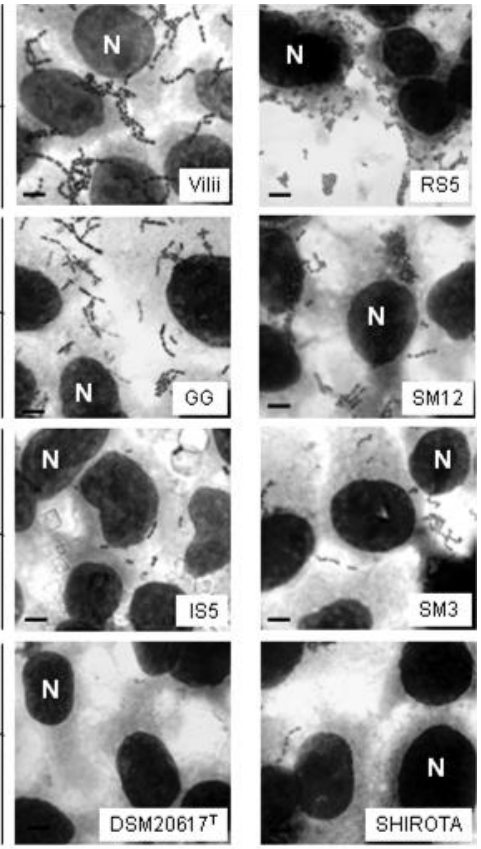
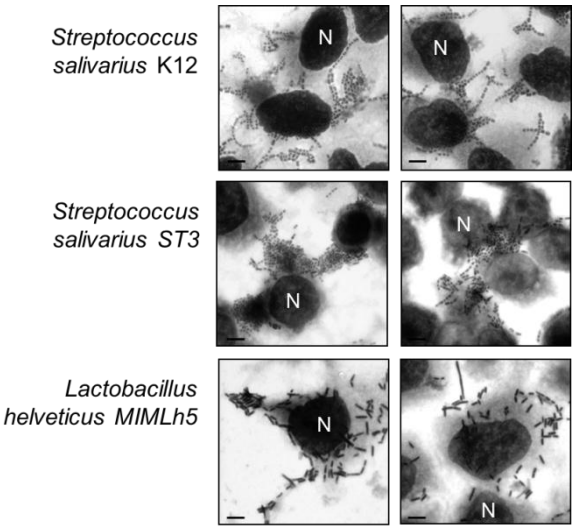


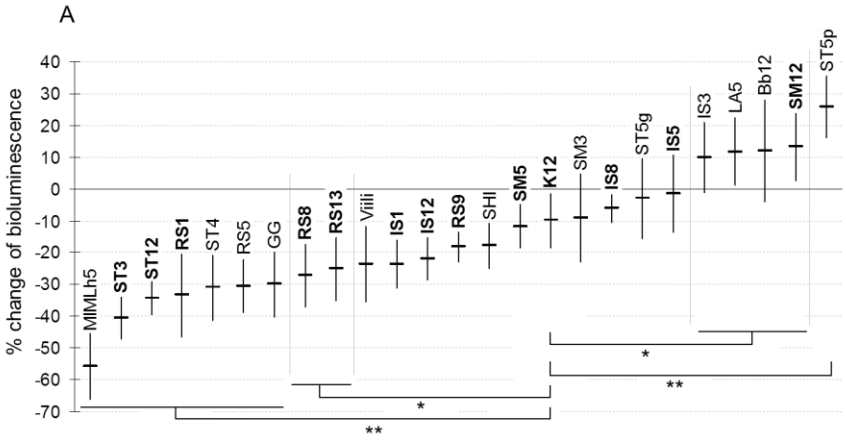
Figure 1.4.3 *Adhesion to FaDu epithelial cells of selected bacterial strains as observed with Giemsa staining under a light microscope. Bars, 8 µm. A FaDu nucleus for each layer is indicated with the letter N.*

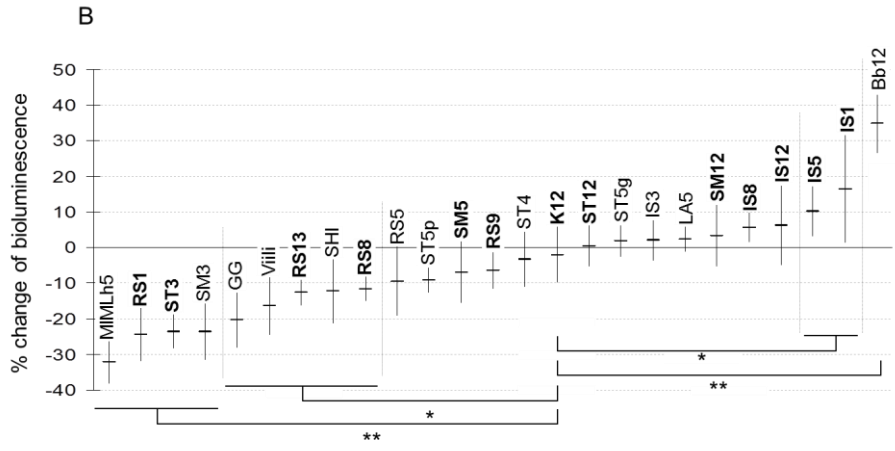


1.4.3 The antagonistic activity against *Streptococcus pyogenes* on human epithelial cell lines is strain-dependent

Streptococcus pyogenes (group A streptococci, GAS) causes approximately 15 to 30 % of pediatric sore throat (Bisno, 1996) and it is the etiological agent of skin and soft tissue infections, glomerulonephritis and acute rheumatic fever. The oral administration of probiotic bacteria could be a prophylactic strategy effective to reduce the transmission of GAS in the community (Wagenvoort et al., 2005). For this reason, we tested the ability of probiotic bacteria to antagonize GAS on *in vitro* epithelial layers. Since the oral cavity contains several types of surface, including keratinized and non-keratinized epithelia, besides FaDu, we also included in the experimentation the HaCat cell line, which resembles many characteristics of human keratinocytes (Boukamp et al., 1998).Twenty-five bacterial strains were tested for their ability to adhere to the FaDu epithelial cell layer. We studied 13 *S. salivarius* strains, including at least one representative isolate from each genotypic cluster, and the commercial probiotic strain K12. We also tested 5 intestinal probiotic and dairy lactic acid bacteria (Table 1.3.2) and 6 other pharyngeal isolates that have been arbitrarily selected. In this experiment we used a three-component system consisting of the epithelial cell layer, the *S. pyogenes* $C11^{LucFF}$ indicator bioluminescent strain, and the tester bacterium. We measured the reduction of bioluminescence produced by *S. pyogenes* $C11^{LucFF}$ as an indication of the antagonistic activity exerted by the tester strains. Antagonism through exclusion was tested on the layers of two different human epithelial cell lines, FaDu (Fig. 1.4.4A). and HaCaT (Fig. 1.4.4B). The results showed that antagonistic exclusion against *S. pyogenes* $C11^{LucFF}$ was generally stronger on FaDu hypopharyngeal carcinoma cells than HaCaT keratinocytes.

Figure 1.4.4 *Antagonistic exclusion activity of bacterial pharyngeal isolates and probiotic/food strains against bioluminescent* Streptococcus pyogenes *C11LucFF on FaDu hypopharyngeal carcinoma cells (A) and HaCaT keratinocytes (B). Data reported as percent variation of light emission, which referred to the cell layer treated with only PBS buffer before incubation with* S. pyogenes*. Numerical results are given as arithmetic means standard deviations. Each sample was processed in triplicate in at least two independent experiments. Strains belonging to species* S. salivarius *are indicated in boldface. Statistically significant differences compared to strain K12 were calculated according to an unpaired Student's t test (**, P< 0.001; *, P<0.05).*



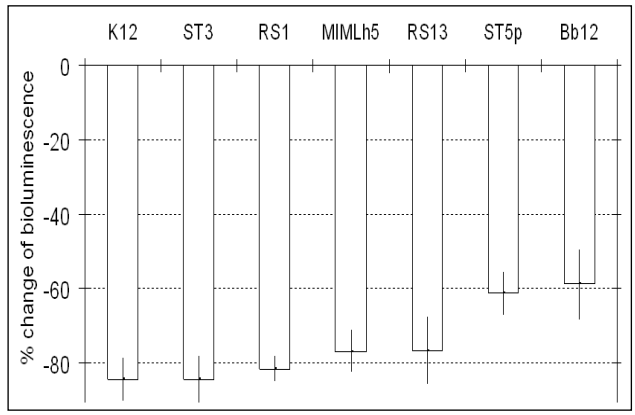


Adhesion experiments performed on the FaDu cell layer can give a partial explanation of the strong antagonizing activity displayed by these strains. (Fig. 1.4.3). In fact, it can be hypothesized that the competition for adhesion sites is a major mechanism through which these bacteria antagonize *S. pyogenes* on FaDu cells.

The results also demonstrated that the exclusion activity is strain specific, since significant differences were observed also among strains of the same species. We found a correlation between the results obtained with the two cell lines. In fact, with only a few exceptions, those strains that significantly reduced the light production in HaCaT cells also did so in the FaDu cell layer. Particularly, the following two strains were the most active in both cell lines: *L. helveticus* MIMLh5 (with an average reduction of bioluminescence of -55 % on FaDu and -32 % on HaCaT) and *S. salivarius* ST3 (with average reductions of bioluminescence of 40% in FaDu and 24% in HaCaT). In addition to MIMLh5 and ST3, the probiotic strains *L. rhamonosus* GG and *L. paracasei* Shirota and the dairy strain *L. lactis* subsp. *cremoris* Viili also displayed an antagonistic exclusion significantly stronger than the reference oral probiotic strain *S. salivarius* K12.

The antagonism by competition was tested only on the FaDu cell layer for the two selected strains *S. salivarius* ST3 and *L. helveticus* MIMLh5, the reference oral probiotic K12, three oral strains (*S. salivarius* RS13 and RS1, *S. oralis* ST5p) and the commercial probiotic *B. animalis lactis* Bb12, which were selected in order to have at least one representative strain for each of the four typologies of bacterial adhesion on the FaDu layer (Fig. 1.4.2). In this experiment, all tested strains markedly inhibited *S. pyogenes* bioluminescence (> 50 % reduction) and particularly *L. helveticus* MIMLh5 (78 %) and *S. salivarius* strains (ca. 80 % reduction) We also noticed a partial dependence of both exclusion and competition on adhesion ability, since the antagonism efficacy was slightly weaker for poorly adhesive strains like ST5p than strongly adhesive bacteria (e.g., K12 and ST3; *P* values of 0.001, according to an unpaired Student's *t* test) (Fig. 1.4.2 and Fig. 1.4.5).

Fig. 1.4.5 *Antagonistic competition activity of bacterial pharyngeal isolates against bioluminescent* Streptococcus pyogenes *C11lucFF on FaDu hypopharyngeal carcinoma cells. Data reported as percentage variation of light emission as referred to the cell layer treated with only* S. pyogenes *cells. Numerical results are given in the arithmetic means ± standard deviations. All samples resulted significantly different compared to control (P < 0.001, according to unpaired Student's t-test).*



1.4.4 Inhibition of *S. pyogenes* and PCR detection of bacteriocin encoding genes

The potential oral probiotic activity of pharyngeal streptococcal isolates were characterized by PCR for known lantibiotic structural genes coding for salivaricin A, salivaricin B, streptin and SA-FF22, which are known to be active against *S. pyogenes*. According to literature, strain K12 gave an amplicon of the expected length for salivaricin A and B. Among the other isolates, all SM *S. salivarius* isolates and *S. salivarius* IS7 resulted positive for the only salivaricin A. Sequence analyses revelaed that all salivaricin A amplicons, including K12 amplicon, belonged to the variant A2 (Wescombe et al., 2006). None of the streptococcal isolates resulted positive for streptin and SA-FF22 (Data not shown). When we performed inhibition test of *S. pyogenes* after 24 h incubation at 37°C we observed clear inhibition zones corresponding to the colonies of the strains K12 and ST3 (Data not shown). On the contrary, when cell-free neutralized broths were used in disk-diffusion tests, inhibition zones were observed only for the reference strain *S. salivarius* K12. Accordingly, PCR experiments suggested that ST3 could not possess bacteriocin genes which are known to be–encoded in strain K12 on a 190-kilobase transmissible plasmid (Hyink et al., 2007). In contrast, ST3 is a plasmid-free strain. Therefore, it appears plausible that the pharyngeal isolate ST3 inhibited the growth of *S. pyogenes* in plate probably partially due to its acid production. For what it concerns *L. helveticus* MIMLh5, this strain was not able to prevent *S. pyogenes* growth.

1.4.5 Immunomoduatory properties of selected LAB strains

One mechanism of action of probiotics is suggested to be their modulation of host immune responses. In a recent study, Cosseau and collaborators showed that the oral probiotic *S. salivarius* K12 can induce *in vitro* anti-inflammatory responses in epithelial cells, indicating a potential promotion of cellular health and homeostasis (Cosseau et al., 2008). In that study, after co-culture of human bronchial epithelial cells (16HBE14O-cells) with strain K12, they observed an inhibition of baseline secretion of the chemokine interleukin (IL)-8, in coincidence with the inhibition of activation of the NF-κB pathway (Cosseau et al., 2008). In our study, the immunomodulatory properties of *S. salivarius* K12, ST3 and *L. helveticus* MIMLh5 were tested on a FaDu layer by means of ELISA quantification of 17 secreted cytokines. Subsequently, in order to elucidate the possible mechanisms involved in the effects on cytokine production, we studied the modulation of NF-κB activation. Both experiments were performed in absence and presence of IL-1β, a prototypical proinflammatory cytokine that plays a central role in the inflammation amplification cascade.

1.4.6 Cytokine induction profile elicited by selected bacterial strains on FaDu cells.

Overnight incubation of a FaDu monolayer with selected bacteria resulted in a modulation of the cytokine production profile, as determined by the Bio-Plex human cytokine 17-plex array system. We observed different responses of FaDu cell layer to the three bacterial strains included in the experiment (Fig. 1.4.6). *Streptococcus salivarius* strain ST3 abolished the secretion of IL-1β and TNF-α and enhanced the production of IL-8 (from 1721 to 2331 pg ml^{-1}) and GM-CSF (from 0.2 to 1.9 pg ml^{-1}). The commercial oral probiotic *Streptococcus salivarius* K12 decreased IL-6 and IL-8 (respectively, from 78.1 to 24.2 pg ml^{-1} and from 1721 to 579 pg ml^{-1}). Furthermore, strain K12 induced a reduction of the secretion of TNF-α (from 1.9 to 0.9 pg ml^{-1}) and G-CSF (from 3.5 to 1.7 pg ml^{-1}) and increased GM-CSF (from 0.2 to 1.6 pg ml^{-1}). Finally, *Lactobacillus helveticus* MIMLh5 displayed the strongest ability to inhibit the secretion

of IL-6 (from 78.1 to 5.5 pg ml^{-1}), IL-8 (from 1721 to 111 pg ml^{-1}) and G-CSF (from 3.5 to 0.2 pg ml^{-1}). Moreover, strain MIMLh5 decreased TNF-α (from 1.9 to 0.8 pg ml^{-1}) and enhanced GM-CSF (from 0.2 to 2.4 pg ml^{-1}).

When FaDu layer was stimulated with 2 ng ml^{-1} of the pro-inflammatory cytokine IL-1β (Fig. 1.4.6), strains K12 and ST3 reduced IL-6 from 121.5 to 73.7, and 86.7 pg ml^{-1}, respectively, and IL-8 from 3353 to 2043 and 2261 pg ml^{-1}, respectively. For strain MIMLh5, with respect to baseline, the presence of IL-1β induced a drastic change of cytokine profile. Several cytokines, which were considerably reduced by *L. helveticus* MIMLh5 at baseline, in fact, resulted in an increased secretion when FaDu cells were incubated with MIMLh5 in the presence of IL-1β. The modification of MIMLh5's effect in the presence of IL-1β was particularly evident for cytokines IL-6, IL-8, G-CSF, INF-γ, and TNFα.

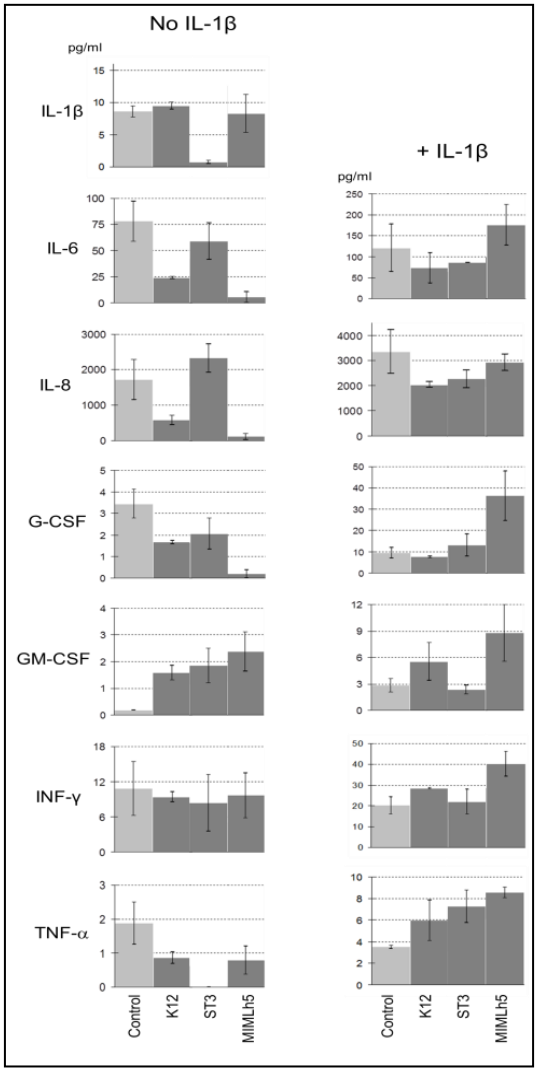
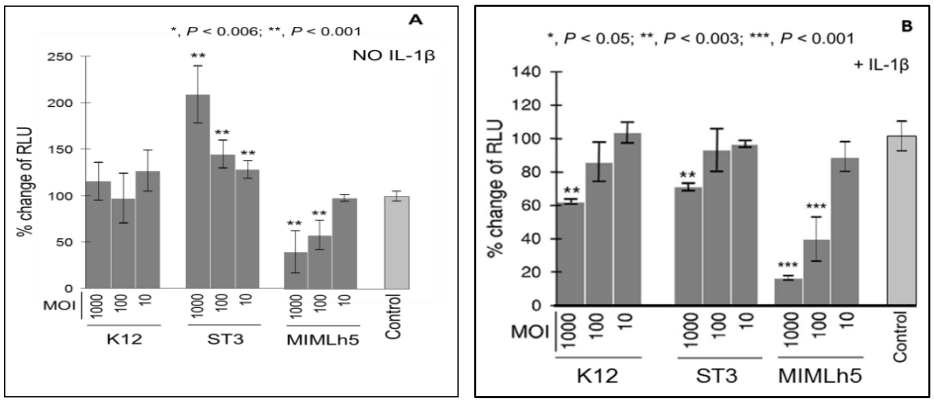


Figure 1.4.6 *Cytokine secretions that changed significantly after treatment of the FaDu layer with bacterial cells, as determined by using the Bio-Plex assay. FaDu layers were incubated overnight with bacterial cells (2 X 10^8 cells ml^{-1}) without and in the presence of 2 ng ml^{-1} of IL-1β. The values are the means from two experiments conducted in duplicate. The vertical bars indicate standard deviations.*

1.4.7 Modulation of NF-κB activation by tested bacteria in transfected FaDu cells.

To further explore the immunomodulatory properties of selected lactic acid bacteria, we tested the effect of the microorganisms on NF-κB activation using a reporter cell line, obtained by transfecting FaDu cells with a luciferase reporter vector induced by NF-κB. In FaDu cells, *L. helveticus* MIMLh5 was the only strain that decreased the NF-κB-dependent production of bioluminescence (Fig. 1.4.7A). On the contrary, strain ST3 showed a stimulatory effect. NF-κB activation was particularly evident for strain ST3, which doubled the bioluminescence produced by FaDu cells, when we employed a MOI of about 1000. *S. salivarius* K12 did not affect significantly NF-κB (Fig. 1.4.7A). In a following series of experiments, the effect of bacterial strains on NF-κB was assessed during stimulation of FaDu cells with the pro-inflammatory cytokine IL-1β (Fig. 1.4.7B). The addition of 2 ng ml^{-1} of IL-1β to FaDu culture broth caused nearly a two-fold increase of NF-κB activity after 4 h incubation. In presence of IL-1β, we observed that, at a MOI of 1000, strains K12 and ST3 reduced the NF-κB activation in a statistically significant manner, while the other conditions tested had no significant effect. In contrast, strain *L. helveticus* MIMLh5 strongly inhibited NF-κB activation by IL-1β in a concentration dependent manner (–84 % at MOI 1000 and –61 %; Fig. 1.4.7B).

Figure 1.4.7 *Effects of selected bacterial strains on FaDu cells stably transfected with an NF-κB/luciferase reporter vector, without (A) or with (B) stimulation with IL-1β (2 ng ml-1). Luciferase activity is expressed as percent change in relative luminescence units (RLU), assuming the control as 100 %. Control, FaDu layers incubated without bacterial cells. The values are the means (± standard deviations) of at least three independent experiments conducted in duplicate. Asterisks (*) indicate statistically significant differences compared to the control. MOI stands for multiplicity of infection (bacterial cells per FaDu cell).*



1.4.8 The selected LAB strains drive different immune responses *in vitro*.

In these experiments, none of the tested strains exhibited a potential pro-inflammatory effect, suggesting that they could be well tolerated by human epithelial cells *in vivo*. This statement is consistent with the induced reduction of baseline TNF-α secretion, which was observed with all

tested strains. In details, we found different behaviors among selected bacteria tested. While *S. salivarius* strain K12 reduced baseline IL-8 and IL-6 secretion, while ST3 inhibited drastically IL-1β . These results have been partially explained by the experiments on NF-κB activation. The reduced secretion of IL-8 and IL-6 provoked by strain K12 can be attributed to the inhibition of NF-κB activation, as already proposed by Cosseau et al. (2008). In contrast, *S. salivarius* ST3 promoted the baseline activation of NF-κB. Greten with collaborators have recently demonstrated that NF-κB activates the secretion of a selective inhibitor of caspase-1, a peptidase required for pro-IL-1β maturation (Greten et al., 2007). Therefore, we can reasonably speculate that the inhibition of IL-1β secretion by strain ST3 could also be mediated by a mechanism involving inhibition of the enzyme caspase-1. It is important to stress that the stimulatory activity of ST3 bacterial cells on NF-κB activation was eliminated in presence of the inflammatory stimulus due to IL-1β. Similarly, it is noteworthy that in IL-1β-treated FaDu *S. salivarius* strains can considerably reduce IL-6 and IL-8 secretion, suggesting their potential anti-inflammatory activity.

Finally, *Lactobacillus helveticus* MIMLh5 at baseline enhanced GM-CSF, decreased TNF-α and particularly, displayed the strongest ability to inhibit the secretion of IL-6 (from 78.1 to 5.5 pg ml^{-1}), IL-8 (from 1721 to 111 pg ml^{-1}) and G-CSF (from 3.5 to 0.2 pg ml^{-1}; Fig. 1.4.6). The reduced secretion of these cytokines promoted by strain MIMLh5 in FaDu cells can be potentially explained by the concomitant inhibition of NF-κB activation found in our experiments (Fig. 1.4.7). In fact, the transcriptional regulator NF-κB is an inducer of IL-8 and several other cytokines, and is a therapeutic target in a wide range of human (auto)-inflammatory diseases (Yamamoto & Gaynor, 2005).

It is well known that the mechanism of action of many anti-inflammatory compounds, including salicylate and corticosteroids, involves NF-κB inhibition (Auphan et al., 1995; Kopp & Ghosh, 1994). Furthermore, intestinal and/or probiotic microorganisms have also been proposed to exert their immunomodulatory activity through the modulation of the NF-κB signaling and the subsequent reduction of IL-8 production (Sokol et al., 2008).

Interestingly, the cell concentration-dependent ability of *L. helveticus* MIMLh5 to reduce NF-κB activation was particularly significant after stimulation by the pro-inflammatory cytokine IL-1β. Nevertheless, in this condition, we observed a simultaneous increase of the secretion of several cytokines, such as IL-6, G-CSF, INF-γ, and TNF-α. In particular, we found that MIMLh5 at baseline can inhibit G-CSF secretion, concomitantly with the stimulation of GM-CSF. Myeloid dendritic cells (DCs), which drive the T-cell response into a Th1 type, require the presence of GM-CSF for survival, in contrast to lymphoid DCs, which induce Th2 response and are mobilized by G-CSF (Arpinati et al., 2000; Moser et al., 2005; Rissoan et al., 1999). A systemic and local increase of GM-CSF/G-CSF ratio, for instance, has been correlated with a Th1-dominating response and good clinical status in cystic fibrosis patients (Moser et al., 2005).

1.4.9 Safety assessment of selected bacteria by antibiotic susceptibility test

We studied the antibiotic resistance of *Lactobacillus helveticus* MIMLh5 and *Streptococcus salivarius* K12 and ST3 with reference to the EFSA breakpoints for *Lactobacillus helveticus* and *Streptococcus thermophilus* (since *S. salivarius* is phylogenetically close to *S. thermophilus*) respectively (EFSA, 2008). Bacterial strains were found to be sensitive to ampicillin, chloramphenicol, erythromycin, oxytetracyclin and vancomycin. A variable resistance was observed with the aminoglycoside antibiotics (gentamicin, kanamycin and streptomycin)

depending on the strain and the growth medium. All strains were assessed to be resistant to kanamycin with the only exception of *S. salivarius* ST3, when it was grown in LM17 medium (Table 1.4.1). Strain K12 was resistant to gentamicin in LM17 medium and to streptomycin in BHI medium. Finally, *S. salivarius* ST3 was resistant to gentamicin in MRS medium and to streptomycin in BHI and MRS media (Table 1.4.1). For what it concerns *L. helveticus* MIMLh5, it was found to be sensitive to all tested antibiotics except kanamycin (Table 1.4.1). However, it appears reasonable to consider the resistance to kanamycin as intrinsic and not related to a specific gene. It is known, in fact, that several lactobacilli have a high natural resistance to kanamycin and other aminoglycosidic antibiotics (Bernardeu et al., 2008).

Table.1.4.1 *Antibiotic sensitivities of selected bacterial strains. Minimal inhibitory concentrations (MICs).*

	MIC (µg ml^{-1})						EFSA MIC S.t. [2]	EFSA MIC L.h. [2]
	K12		ST3			MIMLh5		
Antibiotic	BHI	M17[3]	BHI	M17[3]	MRS	MRS		
Ampicillin	< 1	< 1	< 1	< 1	< 1	< 1	2	1
Chloramphenicol	< 1	< 4 > 1	< 1	< 4 > 1	< 4 > 1	< 4 > 1	4	4
Erythromycin	< 1	< 1	< 1	< 1	< 1	< 1	2	1
Gentamicin[1]	< 32 > 16	> 64	< 16 > 8	< 8	> 64	< 16 > 8	32	16
Kanamycin[1]	> 128	> 128	> 128	< 16	> 128	< 128 > 64	64	16
Oxytetracyclin	< 1	< 1	< 1	< 1	< 1	< 4 > 1	4	4
Streptomycin[1]	< 128 > 64	< 16	< 128 > 64	< 32 > 16	< 128 > 64	< 16	64	16
Vancomycin	< 1	< 4 > 1	< 4 > 1	< 4 > 1	< 4 > 1	< 1	4	2

1 Possible interference of the growth medium is reported (EFSA, 2008).
2 Microbiological breakpoints categorizing bacteria as resistant (µg ml^{-1}) according to EFSA guidelines (EFSA, 2008); S.t. *and* L.h. *stands for* Streptococcus thermophilus *and* Lactobacillus helveticus, *respectively.*
3 M17 containing 2 % lactose.

Recently, the European Food Safety Authority (EFSA) assigned a "Qualified Presumption of Safety" (QPS) status (EFSA, 2004) to several lactic acid bacterial species, including *Lactobacillus helveticus*, *Streptococcus thermophilus*, but not *Streptococcus salivarius*. In Europe, *S. salivarius* belongs to the risk group 2 (such as *S. pyogenes* or *S. pneumoniae*), while the very closely related species *S. thermophilus*, *S. uberis* and *S. vestibularis* (Mora et al., 2003) belong to risk group 1. Presumably, *S. salivarius* is considered an opportunistic pathogen because, as with many food grade lactobacilli, there have been sporadic reports of infections, generally in subjects under adverse medical conditions (Ahmed et al., 2003; Afek et al., 2004; Corredoira et al., 2005). Quite the opposite, in other parts of the world, *S. salivarius* has already acquired the status of safe microorganism and has been commercialized for several years as

probiotic without any reported adverse consequences (Burton et al., 2006). In the light of the above mentioned facts, the optimal strategy to assess the safety of *S. salivarius*, would be considering every specific strain independently, in accordance with FAO/WHO guidelines on probiotics (FAO/WHO, 2002), as has been done for the strain *S. salivarius* K12 (Burton et al., 2006; Cosseau et al., 2008). From this perspective, the absence of transmissible antibiotic resistances is considered a key safety prerequisite for the selection of a probiotic microorganism (EFSA, 2004; FAO/WHO, 2002). In this study, *S. salivarius* strain ST3, according to the EFSA breakpoints suggested for *S. thermophilus* (2008), resulted sensitive to a variety of antibiotics that are routinely used for the control of URTIs. Differently, they showed resistance exclusively to the aminoglycosidic antibiotics gentamicin, kanamycin and streptomycin, for which an intrinsic resistance is known for several lactic acid bacteria (Corredoira et al., 2005; Hummel et al., 2007; EFSA, 2008; Maragkoudakis et al., 2009).

1.4.10 ST3 is a natural urease negative *S. salivarius* strain

All *Streptococcus salivarius* isolates obtained from pharyngeal mucosa and the probiotic strain K12 were tested for their ability to hydrolyze urea. All tested strains resulted urealytic when grown in a medium containing nickel cations, with the only exception of strain ST3. According to the phenotypic assay, we failed to amplify by PCR from strain ST3 *ureC* gene, which encodes the alpha subunit protein containing the active site and conserved nickel binding ligands of the urease complex. On the contrary, a PCR fragment of the expected length was obtained using primers targeting *ureC* from all the other *S. salivarius* strains (Data not shown). The urease activity is a bacterial feature that exerts an important role in the interaction with the human host is. Ammonia production from ureolysis in saliva and crevicular fluids is, in fact, a primary source of amino nitrogen and is thought to inhibit the initiation and progression of dental caries by reducing acidity (Kleinberg, 1967; Peterson et al., 1985). At the same time, a high ammonia concentration can have deleterious effects on host cells (Helgeland, 1985), such as fibroblasts and polymorphonuclear leukocytes, and may therefore contribute to tissue damage (Helgeland, 1985). Among the species of oral bacteria that have been identified as ureolytic, *S. salivarius* is known to produce high levels of urease (Sissons & Cutress, 1987). Unexpectedly, strain *S. salivarius* ST3, selected in this work for its potential probiotic properties, was unable to hydrolyze urea. In the consideration of its use as pharynx's probiotic, this bacterium would interact directly with oro-pharyngeal mucosa and tonsil crypts. Therefore, the inability to hydrolyze urea could be of potential benefit in preserving the host's mucosal cells from ammonia injury.

1.4.11 ST3/MIMLh5 co-suspension did not affect adhesion and antagonistic properties of the individual strains on the FaDu cell layer.

In the following series of experiments, we evaluated *in vitro* the potential probiotic properties of strains ST3 and MIMLh5 used in combination. We first studied the interaction with FaDu human pharyngeal cell line in terms of adhesion and ability to antagonize *Streptococcus pyogenes*. We found that the adhesion on pharyngeal epithelium of each strain was not affected when the other one was also present, indicating that there is no competition for adhesion sites between MIMLh5 and ST3 in our test conditions (MOI of 1000 for each bacterium; Fig. 1.4.8).

Afterwards, we measured the bioluminescence produced by *S. pyogenes* $C11^{LucFF}$ as an indication of the exclusion antagonism exerted by strains ST3, MIMLh5 and their mixed suspension. *L. helveticus* MIMLh5 showed a better antagonism against *S. pyogenes* (72 % reduction of luminescence), compared to *S. salivarius* strain ST3 (53 % luminescence reduction). When the tester bacteria were used in combination (MOI 1000 each), the antagonistic activity was maintained at the highest level (72 % reduction), indicating that the two strains can coexist without reducing their *in vitro* exclusion ability (Fig. 1.4.9).

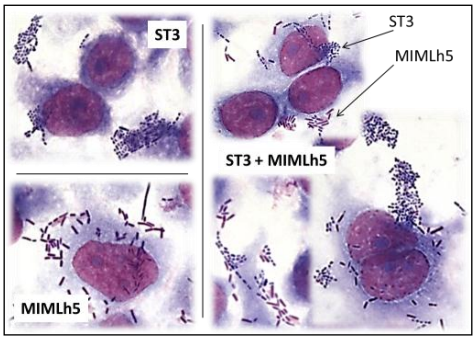


Figure 1.4.8 *Bacterial adhesion as observed with Giemsa staining under a light microscope. FaDu nuclei appear in red.*

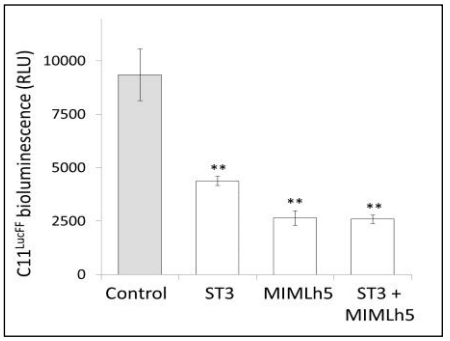


Figure 1.4.9 *Antagonistic exclusion activity against bioluminescent* S. pyogenes *C11lucFF; control is a cell layer treated with only PBS before incubation with* S. pyogenes.

1.4.12 The effect of strain MIMLh5 on NF-κB activation in FaDu epithelial cells is predominant over that of strain ST3.

In this study, we explored the effect of strains ST3 and MIMLh5 in combination on NF-κB activation in FaDu cells using the same luciferase reporter system previously described. As observed in previous investigations (Guglielmetti et al. 2010a;b), *L. helveticus* MIMLh5 decreased the NF-κB-dependent production of bioluminescence, while ST3 showed a stimulatory effect (Fig. 1.4.10). When the two bacterial strains were used in combination at the same multiplicity of infection (MOI 1000 each), activation of NF-κB was reduced to an extent not significantly dissimilar to that determined by MIMLh5 alone (Fig. 1.4.10), indicating that the inhibitory effect of this bacterium on NF-κB-mediated responses is predominant over the outcome induced by strain ST3.

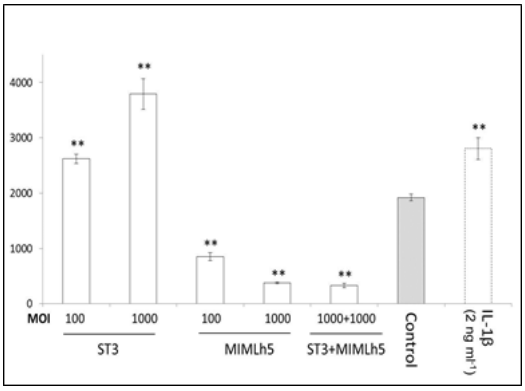


Figure 1.4.10. *Effect of bacteria on FaDu cells stably transfected with an NF-κB/luciferase reporter vector. Control is a FaDu layer incubated without bacterial cells; MOI stands for multiplicity of infection (bacterial cells per FaDu cell); interleukin IL-1β was used as positive control for NF-κB activation. Data in histograms are the means (± standard deviations) of at least three independent experiments conducted in triplicate. Bacterial and FaDu luciferase activity is expressed as relative luminescence units (RLU). Asterisks (**) indicate statistically significant differences compared to the control (P < 0.001).*

1.4.13 Effect of the strains MIMLh5 and ST3 on activation of U937 human macrophages.

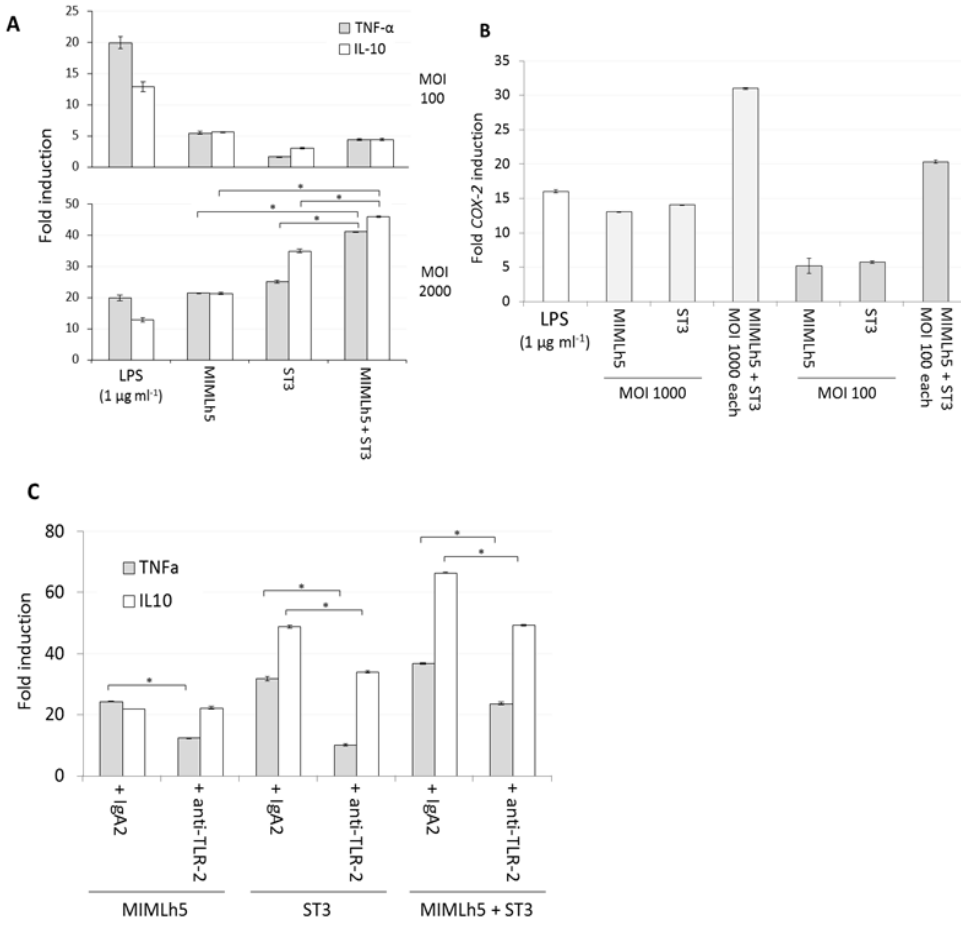
Studies on the immunomodulatory effects of probiotics for the oropharyngeal mucosa have thus far mainly concentrated on immune responses at the epithelial cell level (Cosseau et al., 2008). An evaluation of the responses by cells involved in the immune system is thus required. We used PMA-differentiated U937 human macrophages to study the innate immune responses induced by *L. helveticus* MIMLh5 and *S. salivarius* ST3. In this study, we first treated U937 cells with PMA for 48 hours to differentiate the cells into macrophages. Subsequently, we stimulated the differentiated cells with MIMLh5 and ST3 bacteria. After 4 h of incubation, we used RT-qPCR to quantify the gene expression of TNF-α (a cytokine involved in inflammatory responses; Belardelli, 1995) and IL-10 (a potent anti-inflammatory interleukin that inhibits the production of pro-inflammatory cytokines in several cell types; Fiorentino et al., 1991; D'Andrea, 1993). We tested the strains MIMLh5 and ST3 alone and in combination (co-incubation) at MOIs of 100 and 1000. In all tested conditions, strains MIMLh5 and ST3 produced a higher IL-10/TNF-α ratio than lipopolysaccharide (LPS) from *Escherichia coli*, a potent pro-inflammatory stimulus. Unlike LPS, the induction of IL-10 by MIMLh5 or ST3 alone was never lower than that of TNF-α (Fig. 1.4.11A). Strain ST3, in particular, triggered profound IL-10 expression. In co-incubation experiments, we used a total MOI (*i.e.*, the sum of the bacterial cell numbers of both strains) corresponding to the MOI of the bacterial strains employed individually to see possible synergistic effects. At the lower MOI tested, the mixed suspension of MIMLh5 and ST3 resulted in an intermediate cytokine induction compared to single strains (Fig. 1.4.11A). On the contrary, synergism was observed at the MOI of 1000; the combination of MIMLh5 and ST3 caused a significant increase in the expression of both TNF-α and IL-10 (Fig. 1.4.11A). These data show that the bacterial cell concentration affects the immune response of U937 cells; a dose-dependent approach should be therefore considered when defining a specific immunological effect of a bacterial strain, as demonstrated in previous works (Evrard et al., 2011). In this study, the strains MIMLh5 and ST3 significantly induced the expression of the pro-inflammatory cytokine TNF-α. Previous studies have shown that different

Lactobacillus species can trigger pronounced pro-inflammatory activity in DCs (Christensen et al., 2002; Weiss et al., 2010) and in macrophages (Morita et al., 2002). Macrophages are professional phagocytes that reside in the secondary lymphoid organs as well as in almost all tissues, including the tonsils (Yamamoto et al., 1988), serving as sentinels to detect microbial invaders (Adherem & Underhill, 1999). Upon the recognition of pathogen-associated molecular patterns (PAMPs; Janeway & Medzhitov, 2002), macrophages produce inflammatory mediators, such as cytokines and chemokines, that alert the immune system to the infection of injury. A pro-inflammatory behavior that results in the activation of cell-mediated (Th1 type) immunity, like that observed for MIMLh5 and ST3, could thus be crucial in combating intracellular pathogen attacks and viral infections and alerting the host's immune system. Other studies have reported that most lactic acid bacterial strains that have been tested could induce a Th1 response, even though this capability is strictly dependent on the specific strain and dose used (Perdigòn et al., 2002; Ongol et al., 2008). Notably, in the same studies, some strains of *S. thermophilus*, a species phylogenetically close to *S. salivarius*, induced a high level of IL-10 (Ongol et al., 2008). In our experiments, *S. salivarius* ST3 induced a higher level of IL-10 than TNF-α. Furthermore, the increased production of the anti-inflammatory cytokine was more pronounced in ST3 than in MIMLh5 at the highest MOI tested (Fig. 1.4.11A). Therefore, we speculate that the presence of both bacteria, even at high doses (MOI 1000), might not result in detrimental effects. The regulatory effect of the significant IL-10 expression could protect against secondary outcomes that can occur after streptococcal infections, namely, reactive inflammatory conditions such as rheumatic disease (Puliti et al., 2002).

1.4.14 The strains MIMLh5 and ST3 induce cyclooxygenase (COX)-2 expression in U937 cells and in BMDCs.

Cyclooxygenase (COX), or prostaglandin synthase H (PGH), is a homodimer enzyme involved in the synthesis of prostaglandins (PGs) from arachidonic acid (Funk, 2001). PGs are involved in several physiological processes and contribute to the protection of the gastrointestinal mucosa (Williams et al., 1999; Morteau et al., 2000). Two isoforms of the COX enzyme have been identified and described (Kujubu et al., 1991). COX-1 is constitutively expressed in a wide range of tissues, whereas COX-2 is constitutively expressed in very few tissues but is induced by several stimuli, including bacterial components (Herschmann, 1996). We investigated the effect of the strains MIMLh5 and ST3 on *COX-2* activation in the U937 cell model. The association of the two bacteria resulted in an enhanced transcription of the gene (Fig. 1.4.11B). In similar experiments, we also observed a strong increase in *COX-2* gene expression in murine bone marrow-derived dendritic cells (Data not shown). Although other studies have suggested that lactic acid bacteria could affect COX-2 secretion, there is no clear consensus in the literature. For instance, several probiotic preparations were found to reduce the amount of prostaglandins and levels *COX-2* expression at the intestinal level in murine models (Urbanska et al., 2010; Amdekar et al., 2011;) and *in vitro* (Lee et al., 2008). In contrast, other papers reported a significant up-regulation of *COX-2* expression levels by probiotic bacteria *in vitro* (Otte et al., 2009; Khailova et al., 2010) and *in vivo* (Khailova et al., 2010; Kwon et al., 2010). *Lactobacillus acidophilus*, a species phylogenetically related to *L. helveticus*, was reported to significantly increase *COX-2* expression and PGE_2 secretion in the human colon cancer cell line Colo320 (Otte et al, 2009). Differences in tested strains and model systems likely explain the lack of consensus found in the literature. The ability of the strains MIMLh5 and ST3 to induce *COX-2* expression suggests that these bacteria could directly affect inflammatory processes not only by modulating cytokine secretion but also inducing prostaglandin production.

Figure 1.4.11 *Transcription analysis of cytokine genes in U937 cells stimulated with* Lactobacillus helveticus *MIMLh5 and* Streptococcus salivarius *ST3 after 4 h of incubation with bacterial strains used alone or in association at two different MOIs. Lipopolysaccharide (LPS) was used as positive control at a concentration of 1 µg ml^{-1}. The values are the means (± standard deviations) for a result representative of three independent experiments, expressed as the fold change in induction respective to the control (U937 unstimulated cells) set at a value of 1. Asterisks indicate statistically significant differences compared to the corresponding control (*, P < 0.05). A, Expression levels of IL-10 and TNF-α. B, Expression levels of COX-2. C, Expression levels of IL10 and TNF-α in the presence of a neutralizing antibody against TLR-2 (anti-TLR2). Anti-TLR2 was added to U937 cells 1 h before stimulation with bacteria. Immunoglobulin-A2 isotype (IgA2) was used as control for non-specific blocking activity.*



COX-2 is involved in the induction of oral tolerance through the action of PGs in guiding T cells towards an immunosuppressive phenotype (Newberry et al., 1999) and in resolving inflammation (Wallace, 2006). The activity observed for MIMLh5 and ST3 on *COX-2* could thus be of immediate benefit for host's mucosa, and in light of the fact that, rapid *COX-2* up-regulation in response to injury or inflammation has been reported to restore mucosal integrity (Tan et al., 2000).

1.4.15 TLR-2 participates in the recognition of the strains MIMLh5 and ST3 by U937 cells.

To gain information on possible signaling pathways involved in the immune responses induced by *L. helveticus* MIMLh5 and *S. salivarius* ST3, we investigated which Toll-like receptor (TLR) recognizes these bacteria. TLRs are a class of transmembrane proteins involved in innate immunity (Akira and Hemmi, 2003); in mammals, they work as pattern recognition receptors (PRRs) and participate in detecting various conserved microbial molecules, such as lipopolysaccharide (LPS), lipoteichoic acid (LTA), peptidoglycan (PGN), bacterial lipoproteins, lipoarabinomannan, and zymosan (Means et al., 2000). More specifically, TLR-2 forms heterodimers with TLR-1 or TLR-6 and primarily interacts with Gram-positive bacteria by recognizing PGN, LTA, and lipoproteins (Takeda et al., 2003).

In this study, we performed experiments in U937 cells with a neutralizing antibody against TLR-2 (antiTLR2-Ab) to partially block its ability to bind ligands. An immunoglobulin-A (IgA) isotype control was also used to exclude non-specific binding to U937 cell receptors. After 1 hour pre-incubation with an anti-TLR2 neutralizing Ab (5 μg ml^{-1}), we stimulated the U937 cells for 4 hours with bacteria. We then evaluated the expression of the genes that encode TNF-α and IL-10 using RT-qPCR. When TLR-2 was blocked with anti-TLR2, we observed significantly less TNF-α mRNA, both when the strains MIMLh5 and ST3 were used individually and in combination (Fig. 1.4.11C). These results indicate that TLR-2 is involved in mediating the immunostimulatory activity of both strains. These data are consistent with previous studies that showed that the immunomodulatory activity elicited by lactobacilli involves TLR-2 in macrophages (Shida et al., 2009) and in dendritic cells (Zeuthen et al., 2008; Weiss et al., 2010). However, pre-incubation with anti-TLR2 resulted in a reduction of IL-10 expression only with strain ST3, whereas MIMLh5-mediated expression of IL-10 was not affected (Fig. 1.4.11C), indicating that receptors independent from TLR-2 may be involved in the induction of this cytokine. The interference of anti-TLR2-Ab with IL-10 induction that we observed in our study with strain ST3 is in agreement with the literature. Molecules able to induce IL-10 in a TLR2-dependent manner have been already described (Re & Strominger, 2004; Yamazaki et al., 2011). It is plausible that a similar response mechanism is triggered by *S. salivarius* ST3 in U937 cells.

When the strains were combined, the reduction of the anti-inflammatory cytokine TNF-α was similar to that of ST3 alone (1.43- and 1.34-fold reduction, respectively). Even though anti-TLR2 generally reduced TNF-α induction, the relative increase of TNF-α gene expression caused by the bacteria in combination compared to bacteria employed singularly was markedly higher when TLR-2 was blocked with anti-TLR2 than in control conditions (*i.e.*, in presence of IgA2, Fig. 1.4.11C). In other words, the synergistic effect of using both strains was intensified by the presence of the anti-TLR2 antibody. As a consequence, when TLR-2 was blocked, the IL-10/TNF-α ratio increased only slightly (approximately 18 %) with bacteria in combination,

whereas the ratio was nearly doubled by the individual strains. We can therefore speculate that PRRs other than TLR-2 are involved in the recognition of these bacteria. It might also be hypothesized that blocking TLR-2 can impact the formation of heterodimers with other TLRs and/or the cooperation of TLR-2 with other types of receptors (such as scavenger receptors), therefore causing different cytokine responses.

1.4.16 Strains ST3 and MIMLh5 can be used in combination to ferment bovine milk.

In light of the *in vitro* functional properties of *L. helveticus* MIMLh5 and *S. salivarius* ST3 in combination, we examined the possibility of co-cultivating these bacteria in bovine milk and potentially employing MIMLh5 and ST3 in dairy products. *L. helveticus* MIMLh5 is a natural whey starter culture that is well adapted to grow in bovine milk. In contrast, *S. salivarius* is a human oral commensal isolate that cannot proliferate when cultivated in milk due to its limited ability to use lactose as the sole carbon and energy source. We determined the growth curve of ST3 with eight different carbohydrates at six concentrations in M17 medium (Data not shown). Strain ST3 displayed optimal growth in the presence of glucose, sucrose, fructose and fructooligosaccharides (FOS); its growth was significantly reduced with inulin and lactose but was not detectable with galactose and lactitol (Fig. 1.4.12A). Interestingly, strain ST3 metabolized Actilight® (Fig. 1.4.12B), a commercial product consisting of a mixture of short-chain FOS (1-kestose, nystose, and fructosylnystose) that is frequently used as a prebiotic supplement. When we added one of the four sugars that are efficiently metabolized by ST3 to skim milk, ST3 growth became similar to that in M17 supplemented with the same sugar. Subsequently, we performed acidification curves by monitoring the pH in skim milk supplemented with 2 % glucose after 1 % inoculum of the overnight cultures of strains ST3 and MIMLh5 alone and in co-culture. The association of the two strains resulted in a faster acidification of the medium (Fig. 1.4.13). In fact, after 6 hours of incubation at 42 °C, ST3 and MIMLh5 monocultures reached pH 5.2 and 5.5, respectively, whereas their co-culture reduced the pH to 4.9 in skim milk supplemented with 2 % glucose or FOS (Fig. 1.4.13). After 6 hours of co-incubation in skim milk supplemented with glucose at 42°C, ST3 and MIMLh5 reached a number of viable cells of 9.5×10^8 and 8.9×10^7 CFU ml^{-1}, respectively (Fig. 1.4.13), indicating that both strains contributed to the milk acidification. These data support the potential of using a combination of strains ST3 and MIMLh5 to ferment milk for the production of a novel food product.

Figure 1.4.12. *Growth curves of* Streptococcus salivarius *ST3. A, Growth in M17 supplemented with 2 % of 8 different carbon sources. B, Growth in M17 supplemented with 6 different concentrations of Actilight® fructooligosaccharides (FOS). The curves are representatives of two experiments carried out in sextuplicate.*

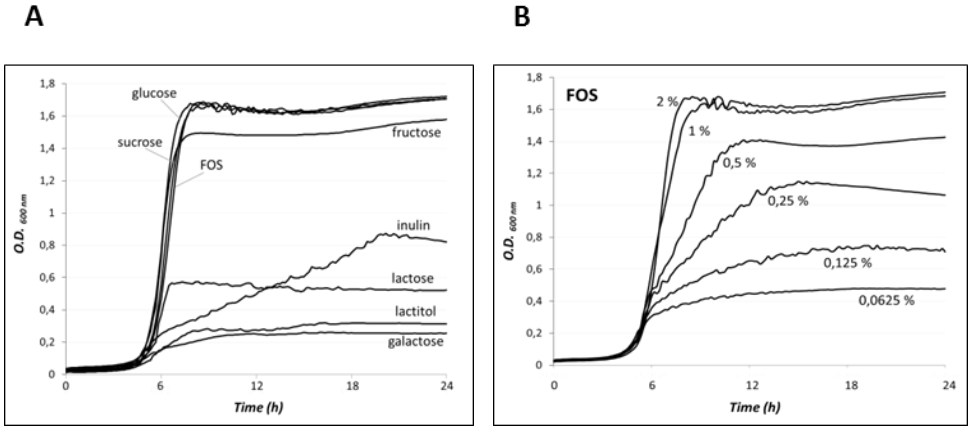
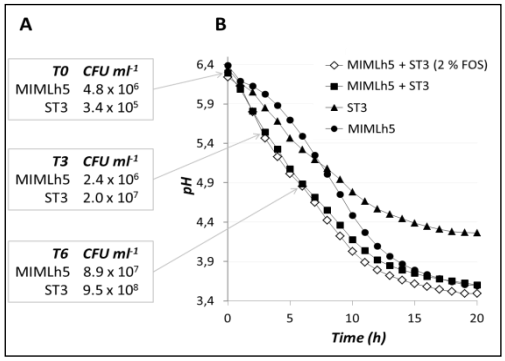


Figure 1.4.13 *Growth of* Lactobacillus helveticus *MIMLh5 and* Streprococcus salivarius *ST3 (single culture and co-culture) at 42°C in skim milk supplemented with 2 % glucose or FOS. A, bacterial plate counts of co-culture; T0, immediately after 1 % inoculum; T3 and T6, after 3 and 6 h, respectively. B, acidification curves.*



1.5 CONCLUSIONS

There is an increasing interest in prophylactic strategies effective for upper respiratory tract infections, that represent a major cause of medical prescription for antibiotics, especially in children. The identification of bacterial strains that could be used as probiotics for the pharyngeal mucosa is one potential strategy.

During this first part of the research, an *in vitro* rational process led us to select two potential probiotic bacterial strains for the pharyngeal mucosa, the commensal *S. salivarius* ST3 and the dairy bacterium *L. helveticus* MIMLh5. These bacteria efficiently adhered to human epithelial pharyngeal cell lines, antagonizing the adhesion of *Streptococcus pyogenes*. Furthermore, immunological tests suggested a good degree of adaptation of these selected strains to the host and potential immunomodulatory and anti-inflammatory abilities.

Therefore we further characterized the two selected strains, and as first we demonstrated that strains MIMLh5 and ST3, when used together, maintain their *in vitro* probiotic abilities. Subsequently, we gave particular attention to bacterial immunomodulatory properties because modulation of the host's immunity is one of the most commonly purported benefits of the consumption of probiotics and is supported by an increasing number of *in vitro* and *in vivo* studies (Corthesy et al., 2007). We found that the combination of MIMLh5 and ST3 resulted in a synergistic effect, according to cytokine induction, that might help the host immune system to react to potential pathogens while maintaining a balance between pro- and anti-inflammatory cytokines, thus preventing possible exaggerated responses. Finally, we observed that these microorganisms grow efficiently when co-cultured in milk, suggesting that the preparation of a milk-based fermented product containing both MIMLh5 and ST3 can be a practicable solution for the administration of these bacteria.

In conclusion, we propose the combined use of *Lactobacillus helveticus* MIMLh5 and *Streptococcus salivarius* ST3 for the preparation of novel products that can display probiotic properties for the pharyngeal mucosa (Taverniti et al., 2012).

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2 THE ROLE OF BACTERIAL CELL COMPONENTS IN THE INTERACTION WITH HOST IMMUNE SYSTEM: S-LAYER PROTEIN FROM *LACTOBACILLUS HELVETICUS* MIMLh5 AS MEDIATIOR OF THE STIMULATING ACTIVITY ON INNATE IMMUNITY

2.1 STATE OF THE ART

2.1.1 Beneficial effects reported for *L. helveticus* strains

Lactobacillus helveticus is a non-spore forming Gram positive thermophilic homofermentative lactic acid bacterium, which has a long history of use in the production of cheese types where high cooking temperatures are required. Besides its well-known technological importance in cheese making, a growing number of studies is demonstrating that *L. helveticus* strains can also exhibit significant health-promoting properties. There are, in fact, more than 50 scientific publications on the probiotic properties of *L. helveticus*, including *in vitro* characterizations, *in vivo* animal studies and interventional/clinical trials. These studies demonstrate that members of the species *L. helveticus* can potentially affect human health through direct or indirect mechanisms, such as the inhibition of pathogens, modification of gut microbiota and modulation of the host immune system. It has been shown the ability of strains belonging to the *L. helveticus* species to exert immunostimulatory effects, both when used alone or in combination with other bacterial strains, and at local and systemic level (Taverniti & Guglielmetti, 2012).

It is important to note that within the *L. delbrueckii* group, the species *L. acidophilus* and *L. helveticus* are phylogenetically very closely related (the 16S rDNA sequences of these bacteria differ by just 1.6%; Callanan et al., 2008); however, while *L. helveticus* is a specialist dairy culture, *L. acidophilus* is a natural inhabitant of the human gut. *L. acidophilus* strains, moreover, are commonly used as probiotics. If we compare these two species, more than 250 scientific studies have been published concerning the probiotic potential of *L. acidophilus* in less than 3 years (from January 2010 to August 2012, according to a PubMed search), whereas *L. helveticus* has been included in less than 20 studies on probiotics in the same period. Although limited respect to *L. acidophilus*, the literature on the potential beneficial effects of *L. helveticus* includes significant scientific facts that highlight the ability of *L. helveticus* strains to interact with the host and influence health. Thus, all these studies reporting that *L. helveticus* cells and cell components, or the enzymatic activities of this bacterium, are linked to specific effects on host physiology, support that the species *L. helveticus* includes strains that can be properly considered to be probiotic.

2.1.2 The impact of bacterial cell viability and the role of bacterial cell molecules: the paraprobiotic approach

The mechanisms underlying probiotic effects are generally attributed to the interaction of probiotics with other microorganisms (members of the microbiota or pathogens) or to the cross-talk of probiotics with host cells. The former type of interaction is typically (though not exclusively) dependent on the viability of probiotic cells, since it is exerted by competitive exclusion (competition for nutrients or adhesion sites), direct inhibition of certain microorganisms (production of antimicrobial molecules) or increased growth of healthy components of the microbiota (nutritional or environmental proto-cooperation). In contrast, direct interaction with the host can be mediated by bacterial cells independently from their viability and is based on the capacity of human cells to recognize specific bacterial components or products, giving rise to responses that commonly involve the mucosa associated lymphoid tissue (MALT) and, therefore, the immune system (Adams, 2010).

The FAO/WHO official definition of probiotics specifies that probiotic microorganisms must be “live”, and this stipulation is supported by an extensive number of studies suggesting that to

provide health benefits, probiotic microorganisms must be viable (Gobbetti et al. 2010). However, the administration of viable bacterial cells to certain categories, like individuals with weaker immune systems, enhanced inflammatory responses and/or compromised mucosal barrier functions, could turn "generally recognised as safe" harmless probiotic bacteria into detrimental microorganisms (Besselink et al., 2008). The emerging concern regarding safety problems arising from the extensive use of live microbial cells is enhancing the interest in non-viable microorganisms or microbial cell extracts and single bacterial components, as they could eliminate shelf-life problems and reduce the risks of microbial translocation and infection. There exist and expanding scientific literature concerning studies in which non-viable microbial cells or crude microbial cell fractions have been investigated as health-promoting agents (Kataria et al. 2009; Taverniti & Guglielmetti, 2011). The scientific literature suggests that loss of viability of probiotic microorganisms can induce complex effects in terms of immunomodulation.

Products intentionally containing non-viable microbial cells are already present on the market (*e.g., Lactéol Fort* from PUMC Pharmaceutical Co., Ltd and *Fermenti Lattici Tindalizzati* from Frau, AF United S.p.a.). Therefore, the use of killed/inactivated bacteria and/or bacterial components, as long as the beneficial effects are retained, would represent an advantage because it is possible to make these bacteria potentially harmless through a tailored inactivation treatment, or through the employment of a specific and characterized compound extracted from bacterial cell.

In the last fifteen years several definitions of probiotic have been proposed, some of them even comprising non-viable microbial cells. For instance, Prof. S. Salminen (1999) spoke about probiotics as "microbial cell preparations or components of microbial cells that have a beneficial effect on the health and well-being of the host". Therefore, since the FAO/WHO official definition of probiotics might results, in some cases, restrictive, a new terminology which could unambiguously define the use of nonviable microorganisms or microbial fractions to positively affect health would be required. To this aim, has also been recently proposed the term "paraprobiotic" (or "ghost probiotics"), to be defined as "nonviable microbial cells (intact or broken) or crude cell extracts (*i.e.*, with complex chemical composition), which, when administered (orally or topically) in adequate amounts, confer a benefit on the human or animal consumer" (Taverniti & Guglielmetti, 2011). Purified molecules of microbial origin or pure microbial cell products are omitted from the concept of paraprobiotic, since their use should be included in conventional pharmaceutical methodologies. However, this new approach and all the emerging studies regarding the bioactive properties of bacterial components open the way to new perspectives and possible use of probiotic and their single molecules.

2.1.3 Immunomodulatory properties of bacterial cell components

Several mechanistic studies have demonstrated that specific chemical compounds isolated from bacteria can induce specific immune responses. These investigations provide the scientific basis for a molecular explanation of the immunological effects observed *in vivo* after administration of inactivated probiotic bacteria or probiotic cell extracts. Excluding extracellular bacterial products, a major role in immunomodulatory activity should be mediated by the structural components of the cell, particularly the cell envelope, the outermost structure that immune system cells come into contact with first, which includes cell wall constituents or, if they are present, S-layer proteins, capsule and pellicle (Chapot-Chartier et al., 2009).

The immunological effects of bacterial cell envelope components are not surprising, considering data present in literature and the immunomodulatory properties attributed to specific molecular cell wall components.

Peptidoglycan (PGN) and lipopolysaccharide (LPS, also known as bacterial endotoxin) for instance, are well-known potent activators of immune responses. PGN is the main constituent of Gram-positive bacterial cell walls, accounting for up to 90% of their weight, whereas it constitutes only 15-20% of the cell wall in Gram-negative bacteria (Warshakoon, 2009). A thick PGN layer is generally the outermost structure covering Gram-positive cells, whereas in Gram-negative bacteria, there is an outer biological membrane that contains around 13% LPS and exposes the LPS-core polysaccharides and LPS-O-antigens to the external environment. PGN and LPS have traditionally been considered molecules that promote adverse events, such as fever, arthritis and auto-immune diseases (Marteau &Shanahan, 2003; Nahra &Dellinger, 2008; Opal, 2010). Nonetheless, the composition of PGN and LPS differs largely from one bacterium to another, and even small chemical modifications can yield significantly different immunological consequences (Lebeer et al., 2010).

In Gram-positive bacterial cell walls, there are molecules protruding from the external surface of the PGN layer known as teichoic acids (TAs). TAs are phosphodiester polymers of glycerol or ribitol, and they can be covalently linked to either peptidoglycan (wall teichoic acids, WTAs) or the cytoplasmic membrane (lipoteichoic acids, LTAs). Several studies have demonstrated the involvement of TAs in immune responses. With respect to microorganisms of probiotic interest, TAs from lactobacilli have been proposed to elicit proinflammatory responses through Toll-like receptor (TLR)-2 (Matsuguchi et al. 2003; Vidal et al., 2003).

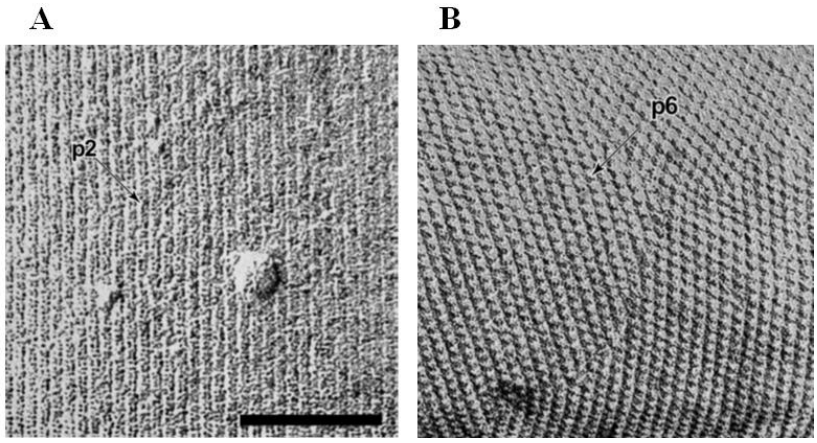
Proteins associated with probiotic bacteria have been shown to elicit immune responses. For example, the heat shock protein GroEL (Hsp60 class), of *L. johnsonii* La1 (NCC 533), which is also present at the cell surface, was expressed in *E. coli*, and its purified recombinant form (rGroEL) was shown to bind to mucins and epithelial cells, stimulating IL-8 secretion in macrophages and HT29 cells in a CD14-dependent manner (Bergonzelli et al. 2006). Another example is BopA, a cell surface-associated lipoprotein of *Bifidobacterium bifidum* MIMBb75 that mediates adhesion to human Caco-2 intestinal epithelial cells. Upon purification from strain MIMBb75, BopA has been demonstrated to induce the production of IL-8 by Caco-2 cells in a dose-dependent manner (Guglielmetti et al., 2008).

2.1.4 Surface layer proteins

Some microorganisms may also engage in cross-talk with the host immune system by means of their surface layer (S-layer) proteins. Surface (S-) layers are crystalline arrays of proteinaceous subunits present as the outermost component of cell wall in several species of the genus *Lactobacillus*, as well as in many other bacteria and Archaea (Sára & Sleytr, 2000). The presence of S-layer has been described in many species of lactobacilli such as *Lactobacillus acidophilus*, *L. helveticus*, *L. casei*, *L. brevis*, *L. buchneri*, *L. fermentum*, *L. bulgaricus*, *L. plantarum* , *L. crispatus*, and more recently in *L. kefir* and *L. parakefir* isolated from kefir grains (Mobili et al., 2010). S-layer is composed from one single protein or glycoprotein, forming crystalline structure of 5–15 nm with identical pores, which have diameter of 2–8 nm (Sleytr, 1997). The pores comprise up to 70 % of the lattice surface area (Sleytr & Beveridge, 1999). S-layer proteins have the ability to self-assemble into monomolecular crystalline arrays. The S-layer lattice can exhibit oblique, square, or hexagonal symmetry. From the *Lactobacillus* S-layer proteins, only oblique and hexagonal lattice types have been characterised (Figure 2.1.1), but in most cases, only a

periodic structure, without further characterisation, has been observed using electron microscopy analysis. Problems related to electron microscopy of *Lactobacillus* S-layers have been reported, and these problems may be one of the reasons why the lattice type and lattice constants have not been determined for most of the S-layer proteins of these bacteria (Åvall-Jääskeläinen & Palva, 2005).

Figure 2.1.1. *Scanning electron micrograph of a freeze-etched preparation of a bacterial cell with an Slayer with oblique (p2; A) and hexagonal (p6; B) lattice symmetry. Scale bar = 100 nm. Adapted from Sleytr & Beveridge (1999).*



S-layer proteins are normally the most abundant bacterial proteins, constituting even up to 15 % of total bacterial protein content (Åvall-Jääskeläinen & Palva, 2005). In the case of *L. helveticus*, it forms around 45 % of cell wall dry weight (Messner et al., 1997). Most of the S-layer proteins of the genus *Lactobacillus* appear to be non-glycosylated, and up to date all known *L. helveticus* S-layer proteins are known not to have this modification. The Slayer monomers are non-covalently linked to each other as well as to the supporting cell wall. The overall lack of identity between the S-layer proteins of different species is a common characteristic of all bacterial S-layer proteins. Lactobacilli make no exception in this respect (Åvall-Jääskeläinen & Palva, 2005; Gatti et al., 2005). The highest similarity can be found in the C-terminal region. The N-terminal regions are more variable except the signal peptides (Nterminal secretion signals) that share a high level of sequence similarity. Typical characteristics of *Lactobacillus* spp. S-layer proteins, distinguishing them from other Slayer proteins, is small size, ranging from 25 to 71 kDa and thus being among the smallest known for the S-layer proteins, which can be up to 200 kDa (Åvall-Jääskeläinen & Palva, 2005). S-layer proteins of lactobacilli have high content of positively charged residues, which makes them highly basic proteins: predicted isoelectric point (pI) values range from 9.35 to 10.4 (Mobili et al., 2010). A characteristic feature of *Lactobacillus* spp. S-layer proteins is a high percentage (23–33 %) of amino acid residues with hydroxyl groups and a high content of hydrophobic amino acid residues, ranging from 31.9 % to 38.7 %, which is a typical feature for all S-layer proteins (Åvall-Jääskeläinen & Palva, 2005).

2.1.5 Functional roles of Surface layer proteins

Even though S-layer protein is so abundant in carrying bacteria, its general role still remains elusive. Several specific functions have been assigned to the S-layer from different microorganisms (protective coats, molecular sieves, molecule and ion traps, cell adhesion and surface recognition, virulence factors). A protection role against hostile factors has been proposed for these superficial structures, considering that purified S-layers are stable toward non-physiological pH, radiation, temperature, some kind of proteolysis, high pressures and detergent treatments (Mobili et al., 2010). Moreover, when the S-layer is extracted with chaotropic agents such as 5M lithium chloride, microorganisms become more sensitive toward aggressive environments such as the gastro intestinal juices (Frece et al., 2005).

Several studies demonstrated participation of lactobacilli S-layer protein in mechanisms of aggregation (Kos et al., 2003; Chen et al., 2007), adhesion to epithelial cells (Åvall-Jääskeläinen et al., 2003; Tallon et al., 2007), activity against pathogens (Horie et al., 2002; Golowczyc et al., 2007), adhesion to mucus and extracellular matrix (ECM) protein (Antikainen et al., 2002; De Leeuw et al., 2006), development of complex microbial communities (Angulo et al., 1993; Garrote et al., 2004).

S-layer protein has also been proposed to play a role in the interaction with host immune system.

The commercial probiotic *Lactobacillus acidophilus* NCFM strain possesses an S-layer primarily composed of a 45 kDa protein named SlpA. Konstantinov and collaborators (2008) demonstrated that NCFM SlpA was recognized and bound by DC-SIGN (Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Nonintegrin, also known as CD209), a C-type lectin receptor present on both macrophages and dendritic cells. Notably, they found that NCFM-expressing SlpA was captured by DC-SIGN on DCs, resulting in an interaction that appeared to be crucial for the activation of IL-4-producing T cells; in contrast, a knockout mutant of *L. acidophilus* NCFM lacking SlpA demonstrated significantly impaired binding to DC-SIGN (Konstantinov et al., 2008). These data were confirmed by experiments performed with purified SlpA protein, which ligated to DC-SIGN and induced IL-10 expression by DCs in the presence of LPS (Konstantinov et al., 2008).

In particular for lactobacilli, different approaches have been used to study and comprehend the role of S-layer protein. Indirect approaches were targeted to test the impact of S-layer protein on specific properties of bacteria (like aggregation, adhesion, haemagglutination) before and after S-layer removal. Direct approaches mainly focused on the study of the isolated protein in its interaction with other bacteria, eukaryotic cells or different kind of matrices and inert surfaces. Both approaches seem to suggest that the role of S-layer is not only species-specific, but also strain-dependent. Thus, a general prediction of its role in carrying lactobacilli is not possible, and should be evaluated case by case for each specific strain (Mobili et al., 2010). Even small differences in the aminoacidic sequences and, consequentially, in the structural characteristic of S-layer proteins can result in diverse surface and/or probiotic properties of whole bacteria (Mobili et al., 2009). In this perspective we wanted to characterize S-layer protein of our strain MIMLh5.

2.2 AIMS OF THE STUDY

Considered the *L. helveticus* ability to support the cross-talk with host's immune system, and on the basis of our results obtained for strain MIMLh5 as potential probiotic for the oro-pharyngeal mucosa, we decided to deeper characterize its immunomodulatory potential. To this aim we tested MIMLh5 effects on different cell lines, including intestinal epithelial cells and different type of macrophages, by means of both *in vitro* and *ex vivo* experiments. Moreover, we tried to identify the bacterial cell components that could mediate the bacterial interaction with host's cells. Thus, using the same models above mentioned, we compared the immunomodulatory properties of the whole bacterium with the ones of its purified Surface-layer protein, which has been already demonstrated to play a role in the interaction with host immune system in other bacterial strains belonging, or closely related to *L. helveticus* species. We, moreover, tried to identify which host cell receptors can be involved in the recognition of both the whole bacterium and the protein, in order to possibly define which kind of signaling pathways could be activated.

2.3 MATERIALS AND METHODS

2.3.1 Bacterial strains, isolation, and growth conditions

L. helveticus MIMLh5 was grown in de Man-Rogosa-Sharpe (MRS) broth (Difco Laboratories Inc., Detroit, MI, USA) at 42°C, whereas *L. acidophilus* NCFM was grown in the same medium at 37°C. *Lactobacillus* strains were inoculated from frozen glycerol stocks and sub-cultured twice in MRS using 1:100 inoculum. For immunological experiments, bacterial cells from an overnight culture were collected, washed twice with sterile phosphate-buffered saline (PBS) and then resuspended in the same medium used to culture human or murine cells. Bacteria were tested at a multiplicity of infection (MOI) of 100 and 1000.

2.3.2 Extraction, purification and chemical characterization of the S-layer protein from *L. helveticus* MIMLh5

Extraction of the S-layer protein from *L. helveticus* MIMLh5 was performed with LiCl as described previously (Lortal et al., 1992; Smit et al., 2001; Johnson-Henry et al., 2007; Agave Biosystems, 2012). Briefly, 500 ml of an overnight culture of MIMLh5 was harvested by centrifugation at 10000 g for 20 min at 4°C and washed with 1 volume of cold sterile MilliQ water. The cell pellet was extracted with 0.1 volume of 1 M LiCl solution for 30 min at room temperature in the presence of Protease Inhibitor Cocktail (Sigma-Aldrich, St. Louis, MO, USA) with slight agitation and centrifuged. The cell pellet was then extracted with 0.1 volumes of 5 M LiCl solution for 1 h at room temperature in the presence of Protease Inhibitor Cocktail and centrifuged. The supernatant was filtered through a 0.2 µm filter and exhaustively dialyzed for 36 h at 4°C against distilled water using 12000 kDa cut-off membranes (Sigma-Aldrich), which were prepared for dialysis by boiling in 2% of $NaHCO_3$ and 1 mM EDTA solution. At each water change, 0.001% of Protease Inhibitor Cocktail was added. The dialysate was collected and centrifuged at 20000 g for 20 min at 4°C. The supernatant was removed, and the pellet resuspended in sterile MilliQ water and freeze dried. We calculated an approximately 4-fold underestimation in the quantification of S-layer protein with Bradford microassay method using bovine serum albumin (BSA) as a standard (probably due to the difference in aminoacidic residue composition between S-layer and BSA proteins). For this reason, for all the experiments in this study, the amount of S-layer was determined by measuring the weight of freeze dried dialyzed protein on an electronic analytical balance. Protein purity was determined by sodium dodecylsulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and RP–HPLC analysis. *SDS–PAGE*. S-layer protein and total bacterial lysates were resuspended in SDS–PAGE (Laemmli) sample buffer, boiled for 5 min, and separated on 10% polyacrylamide gel in TRIS–glycine–SDS buffer on Mini-PROTEAN 3 system (Bio-Rad Italia, Milan, Italy). Gels were stained with Coomassie brilliant blue G-250 (Sigma-Aldrich) or silver staining. *RP–HPLC/ESI–MS analysis of the S-layer protein.* RP–HPLC/ESI–MS analysis was performed on Waters Alliance 2695 instrument (Waters, Vimodrone, Italy) connected to a Q-Tof *micro* mass spectrometer (Micromass/Waters) equipped with an orthogonal electrospray source (Z-spray). S-layer protein was dissolved in 8 M urea and separated at 40 °C on PLRP-S column (2.1 mm i.d., 5 µm, 300 Å, Polymer Laboratories Ltd, Church Stretton, UK). The eluents used for the separation were: solvent A, 0.1 % TFA in MilliQ-treated water, and solvent B, 0.1 % TFA in acetonitrile. The linear elution gradient expressed as the solvent B proportion was as follows: 0 min, 25 %; 0 to 5 min, 25 %; 5 to 35 min, 25 to 55 %; 35 to 36 min, 55 to 95 %; 36 to 38 min, 95 %; 38 to 39 min, 95 to 25 % (run–to–run time, 40 min). Protein was eluted at a flow rate of 0.2 ml min^{-1} and monitored at 210 nm with Waters 2996 Photodiode Array Detector (Waters). Mass

spectra acquisition was performed in positive ion mode. The optimized mass spectrometry conditions were: capillary voltage 3200 V, source temperature 100 °C, and cone voltage 45 V. The electrospray ionization (ESI) gas was nitrogen. The Tof analyzer collected data between m/z 650 and 2000. The acquired MS spectrum was analyzed with the MassLynx 4.1 software (Waters). MS data were processed using *MaxEnt 1* algorithm.

2.3.3 Experiments with Caco-2 cell layers

In vitro *cultivation*. Caco-2 cells (human epithelial colorectal adenocarcinoma cell line; ATCC HTB-37) were routinely grown in Eagle's minimum essential medium (EMEM) supplemented with 10% (v/v) heat-inactivated (30 min at 56°C) fetal calf serum (FCS), 100 U/ml penicillin, 100 mg ml^{-1} streptomycin, 0.1 mM nonessential amino acids (NEAA), and 2 mM L-glutamine and were incubated at 37°C in a water-jacketed incubator in an atmosphere of 95% air and 5% carbon dioxide. *Study of NF-κB activation*. Stable recombinant Caco-2 cell line was generated by transfecting cells with the plasmid pNiFty2-Luc (Invivogen, Labogen, Rho, Italy) as described by Guglielmetti et al. (2010). This plasmid contains a promoter with five NF-κB-binding sites followed by the firefly luciferase reporter gene *luc*. Stimuli that activate NF-κB promote its binding to the vector promoter, resulting in the expression of the luciferase gene. After growth in the presence of 50 μg ml^{-1} zeocin, cell monolayers (approximately 3×10^5 cells $well^{-1}$) were carefully washed with 0.1 M Tris-HCl buffer (pH 8.0). Subsequently, 50 μl of a tester bacterial suspension containing 2.5×10^8 cells (or the purified S-layer protein) were added to 0.45 ml of fresh EMEM medium, containing 100 mM HEPES (pH 7.4). The resulting 0.5 ml were finally pipetted in the microtiter well containing Caco-2 layer, resulting in a MOI of approximately 1000. Stimulation was conducted both in presence and absence of 2 ng ml^{-1} of interleukin (IL)-1β. After incubation at 37°C for 4 h, the samples were treated and the bioluminescence was measured as described by Stuknyte et al. (2011). All conditions were analyzed in triplicate in at least two independent experiments.

2.3.4 Study of the activation of U937 human macrophage cell line

Cell culture, growth conditions, and stimulation protocol. The cell line U937 (ATCC CRL-1593.2) was derived from a human histiocytic lymphoma (Sundstrom & Nilsson, 1976). These cells are maintained as replicative, non-adherent cells and have many of the biochemical and morphological characteristics of blood monocytes (Harris & Ralph, 1985). When treated with phorbol myristate acetate (PMA), U937 cells differentiate to become adherent, non-replicative cells with characteristics similar to tissue macrophages, including similar isoenzyme patterns (Radzun et al., 1983) and other phenotypic markers (Harris & Ralph, 1985). The normal growth medium for the U937 cells consisted of RPMI 1640 medium (Lonza, Basel, Lonza, Basel, Switzerland) supplemented with 10% (v/v) fetal bovine serum (FBS) (Gibco-BRL, Life Technologies, Milan. Italy), 2 mM L-glutamine, 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin (Sigma-Aldrich). U937 cells were seeded at a density of 5×10^5 cells $well^{-1}$ in 12-well plates and incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Differentiation was induced by the addition of PMA (Sigma-Aldrich) into the cellular medium at a final concentration of 100 nM and was allowed to proceed for 48 h. Afterwards, cells were washed once with sterile PBS buffer to remove all non-adherent cells. One h before the bacteria were added to the cells, the culture medium was replaced with RPMI 1640 supplemented with 1% (v/v) FBS to allow the cells to adapt. Bacteria were used at MOIs of 100 and 1000.

Lipopolysaccharide (LPS, final concentration of 1 μg ml^{-1}) from *Escherichia coli* 0127:B8 (Sigma-Aldrich) was used as a positive control for pro-inflammatory stimulus in U937 cells. An untreated sample, *i.e.* only RPMI 1640 medium with 1% (v/v) FBS, was used as control. *Inhibition assay with TLR neutralizing antibodies*. Human anti-TLR4 and anti-TLR2 antibodies (Invivogen) were added to U937 cells 1 h before the stimulation with bacterial cells. A human immunoglobulin-A (IgA) 2 isotype (Invivogen) was used as control to exclude non-specific binding and blocking activity of the antibody. Anti-TLR4, anti-TLR2 and IgA2 isotype were all used at 5 μg ml^{-1}. This concentration was determined by examining the neutralizing efficacy with zymosan from *Saccharomyces cerevisiae* (Invivogen) as ligand for anti-TLR2, and LPS from *E. coli* 0127:B8 (Sigma-Aldrich); we observed that 5 μg ml^{-1} resulted in a 5-fold and 2-fold reduction when, respectively, anti-TLR2 and anti-TLR4 were used to partially block the action of their corresponding ligand in triggering the expression level of TNF-α, a cytokine known to be induced in the downstream pathway activated by TLR2 and TLR4 (Jones et al., 2001; Flo et al., 2002).

2.3.5 Isolation and differentiation of mouse bone marrow-derived macrophages (BMDMs)

Bone marrow cells from C57BL/6 mice were collected by flushing the femurs and tibias from 6- to 8-week-old mice with sterile PBS. Erythrocyte-depleted bone marrow cells were resuspended, counted and seeded in six-well plates at a density of 5×10^6 cells ml^{-1} in RPMI medium (2 ml $well^{-1}$) supplemented with 10% (v/v) heat-inactivated FBS (Gibco), penicillin (100 U ml^{-1}), streptomycin (100 μg ml^{-1}), glutamine (2 mM), 50 μM 2-mercaptoethanol, 15 mM HEPES (Sigma–Aldrich) and 30 ng ml^{-1} murine macrophage colony-stimulating factor (M-CSF) (Peprotech, Rocky Hill, NJ, USA). Cells were incubated at 37°C in 5% CO_2 humidified atmosphere. After 24 h, non-adherent cells were transferred to new six-well plates with complete RPMI medium supplemented with 30 ng ml^{-1} murine M-CSF. On days 3 and 5, the cultures were fed by adding 1 ml $well^{-1}$ of RPMI complete medium supplemented with 30 ng ml^{-1} murine M-CSF, and the cells were allowed to grow and differentiate for 7–8 days. Purity of BMDM cell populations (>90%) was acquired by flow cytometry (BD FACSCanto II, San Jose, CA, USA) and analyzed by FlowJo software. APC-labeled anti-CD11c (N418) (eBioscience, San Diego, CA, USA) and PerCP-Cy5.5-labeled anti-F4/80 (BM8) (eBioscience) were used as antibody mixtures. Prior to stimulation, non-adherent cells were discarded by aspirating the medium and washing once with sterile PBS. Fresh RPMI media supplemented with 1% FBS was added to adherent cells and was kept at 37°C in a 5% CO_2 humidified atmosphere for one h. Subsequently, macrophages were stimulated for 4 h. After stimulation, the cells were scraped and collected for RNA extraction.

2.3.6 Isolation of mouse peritoneal cavity macrophages (PCMs)

C57BL/6 mice were euthanized by CO_2 inhalation. Cells were elicited from the peritoneal cavity by injecting 5 ml of cold, sterile PBS supplemented with 3% FBS (Gibco) and 2 mM EDTA. A gentle massage of the peritoneum was performed to dislodge any attached cells into the PBS solution. The fluid was then aspirated through a syringe and immediately transferred to 50 ml polypropylene tubes kept on ice. This procedure was repeated twice. Peritoneal cells were then centrifuged and resuspended in RPMI supplemented with 10% (v/v) heat-inactivated FBS, penicillin (100 U ml^{-1}), streptomycin (100 μg ml^{-1}), glutamine (2 mM), 50 μM 2-mercaptoethanol, and 10 mM HEPES (Sigma-Aldrich). The cells were counted, seeded on 6

well-plates at a density of 1×10^6 cells ml^{-1}, and cultured for 24 h at 37°C in a 5% CO_2 humidified atmosphere. After 24 h, cells were washed three times with PBS to remove non-adherent cells, and fresh RPMI media supplemented with 1% FBS was added to adherent cells. After 1 h incubation at 37°C in a 5% CO_2 humidified atmosphere, the macrophages were stimulated for 4 h. Subsequently, the cells were scraped and collected for RNA extraction.

2.3.7 Ethics statement

All experimental methods have been accepted by the National Animal Experiment Board (Finland).

2.3.8 Preparation of RNA and reverse transcription

For both *in vitro* and *ex vivo* experiments, after incubating macrophages at 37°C for 4 h, the supernatant was carefully removed from each well and the total cellular RNA was isolated from the adhered cells with an RNeasy Mini Kit (Qiagen Inc., Valencia, CA, USA). Afterwards, RNA concentration and purity was determined with a Nanodrop Spectrophotometer (ND-1000, Thermo Fischer Scientific, Middletown, VA, USA) and reverse transcription to cDNA was performed with the iScript™ Select cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA) using the following thermal cycle: 5 min at 25°C, 30 min at 42°C, and 5 min at 85°C. *Reverse transcription quantitative PCR (RT-qPCR).* The mRNA expression levels of cytokines were analyzed with SYBR Green technology in RT-qPCR using SsoFast EvaGreen Supermix (Bio-Rad Italia, Segrate, Italy) on a Bio-Rad CFX96 system according to the manufacturer's instructions. The primers used are as follow (5'→3'): for human macrophages, 18S rRNA forward ATCCCTGAAAAGTTCCAGCA; 18S rRNA reverse CCCTCTTGGTGAGGTCAATG; IL10 forward AGCAGAGTGAAGACTTTCTTTC; IL10 reverse CATCTCAGACAAGGCTTGG; TNF-α forward TCAGCTCCACGCCATT; TNF-α reverse CCCAGGCAGTCAGATCAT; COX2 forward CCCTTGGGTGTCAAAGGTAA; COX2 reverse TGAAAAGGCGCAGTTTACG. For murine macrophages, 18S rRNA forward GTGATCCCTGAGAAGTTCCAG; 18S rRNA reverse TCGATGTCTGCTTTCCTCAAC; IL10 forward GCCCAGAAATCAAGGAGCAT; IL10 reverse TGTAGACACCTTGGTCTTGGAG; TNF-α forward CTTCTGTCTACTGAACTTCGGG; TNF-α reverse CAGGCTTGTCACTCGAATTTTG; COX-2 forward TGTGCTGACATCCAGATCAT; COX-2 reverse GGCAAAGAATGCAAACATCA. All primers were designed using the Primer3Plus software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi), and the specificity of the primers was tested with melting curves during amplification and by 1% TAE agarose gel electrophoresis. Quantitative PCR was carried out according to the following cycle: initial hold at 96°C for 30 s and then 40 cycles at 96°C for 2 s and 60°C for 5 s. Gene expression was normalized to the housekeeping gene coding for the18S rRNA. The amount of template cDNA used for each sample was 12.5 ng. All results regarding cytokine mRNA expression levels are reported as the fold of induction (FOI) in comparison to the control (namely unstimulated macrophages), to which we attributed a FOI of 1.

2.3.9 Statistical analysis

The significance of the results was analyzed by unpaired heteroscedastic Student's t test with two-tailed distribution. Differences of $P < 0.05$ were considered to be significant.

2.4 RESULTS AND DISCUSSION

2.4.1 Extraction, purification and analysis of MIMLh5 S-layer protein

The S-layer protein (SlpA) of *L. helveticus* MIMLh5 was extracted with LiCl and purified as described in the methods section. We verified that the protocol based on LiCl-washes efficiently removed most of the S-layer protein from the surface of *L. helveticus* cells. In fact, when we loaded the LiCl-treated and LiCl-untreated bacterial cells resuspended in SDS-PAGE loading buffer on a polyacrylamide gel, the electrophoretic profiles displayed a nearly complete disappearance of the band corresponding to SlpA (Fig.2.4.1A). SDS-PAGE and RP-HPLC analyses revealed that the protein was purified to apparent homogeneity (Fig. 2.4.1B, C and D). Furthermore, we did not consider as possible the contamination of the S-layer preparation with lipoteichoic acids (LTA), because it was previously demonstrate that LiCl treatments do not remove LTA from *L. helveticus* cells (Mozes & Lortal, 1995). Mass spectrometry indicated a molecular mass of 43853 Da, which is consistent with the average molecular mass calculated from the amino acid sequence deduced from the *slpA* gene sequence (Fig. 2.4.1E).

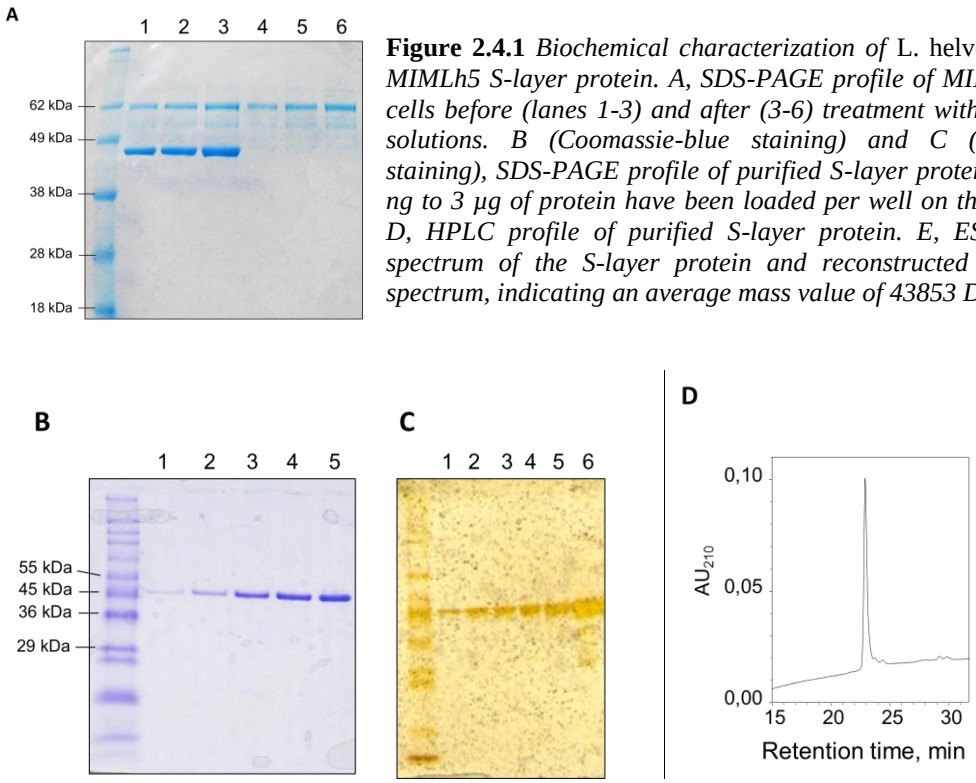
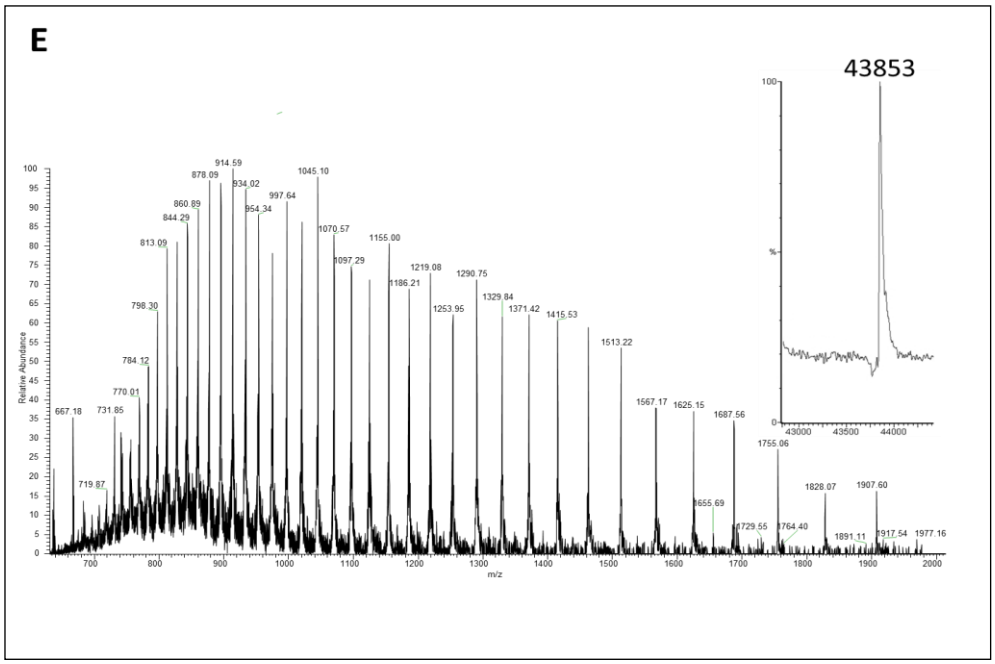


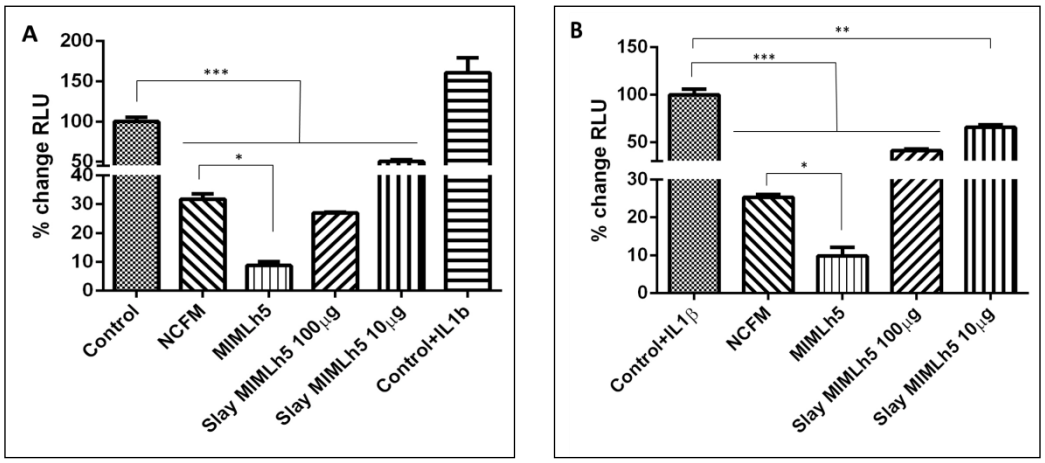
Figure 2.4.1 *Biochemical characterization of* L. helveticus *MIMLh5 S-layer protein. A, SDS-PAGE profile of MIMLh5 cells before (lanes 1-3) and after (3-6) treatment with LiCl solutions. B (Coomassie-blue staining) and C (silver staining), SDS-PAGE profile of purified S-layer protein; 50 ng to 3 µg of protein have been loaded per well on the gel. D, HPLC profile of purified S-layer protein. E, ESI-MS spectrum of the S-layer protein and reconstructed mass spectrum, indicating an average mass value of 43853 Da.*



2.4.2 *L. helveticus* MIMLh5 reduces NF-κB activation in transfected Caco-2 cells

In this study, we investigated *L. helveticus*, a bacterium specialized for colonizing dairy environments and traditionally used in the manufacture of Swiss-type and long-ripened Italian cheeses. Phylogenetically, *L. helveticus* is closely related to *L. acidophilus* (the 16S rDNA sequences of these bacteria differ by just 1.6%; Callanan et al., 2008), an intestinal bacterium regularly used as a probiotic and thoroughly investigated for its ability to modulate host immune responses (Konstantinov et al., 2008; Weiss et al., 2010; Maroof et al., 2012; Taverniti & Guglielmetti, 2012). Studying the interaction of the dairy bacterium *L. helveticus* with the host immune system could provide important insights into the health-modulating potential of a bacterium that, in contrast to the intestinal/probiotic *L. acidophilus*, had its adaptive evolution in dairy environments and, therefore, outside of a human/animal host. We performed a preliminary immunological characterization of *L. helveticus* MIMLh5 using a reporter cell line obtained by transfecting Caco-2 cells with a luciferase reporter vector induced by active NF-κB. Therefore, we compared MIMLh5 with the well-described commercial probiotic strain, *L. acidophilus* NCFM, which we included as a reference. In these experiments, *L. helveticus* MIMLh5 and *L. acidophilus* NCFM cells from an overnight culture were incubated with the transfected Caco-2 layer for 4 h at 37°C at an MOI of 1000. Similar to what we observed in our previous studies on the pharyngeal epithelial FaDu cell-line, *L. helveticus* MIMLh5 significantly reduced NF-κB activation both at baseline and in the presence of IL-1β (Fig. 2.4.2); bioluminescence was, in fact, always significantly lower when bacterial cells were added to the Caco-2 layer compared to the unstimulated control. In both tested conditions, strain MIMLh5 was significantly more effective in the immunomodulatory activity than *L. acidophilus* NCFM (Fig. 2.4.2).

Figure 2.4.2 *Effect of* Lactobacillus acidophilus *NCFM,* Lactobacillus helveticus *MIMLh5 and its purified S-layer protein on human epithelial colorectal Caco-2 cells stably transfected with an NF-κB/luciferase reporter vector at baseline (A) or stimulated with 2 ng ml^{-1} of IL-1β (B). A recombinant Caco-2 layer was incubated with EMEM medium only (A) or with the addition of IL-1β (B). Bacterial strains were used at a multiplicity of infection (MOI) of 1000 (bacterial cells per Caco-2 cell). S-layer protein was tested at two different concentrations (100 and 10 μg ml^{-1}). Data in histograms are the means (+ standard deviations) from at least three independent experiments conducted in triplicate. Data are reported as percentage variation of light emission (relative luminescence units, RLU), assuming that the corresponding control was 100%. Asterisks indicate statistically significant differences: ***, P < 0.001; **, P < 0.01; *, P < 0.05.*



2.4.3 S-layer protein from *L. helveticus* MIMLh5 reduces the activation of NF-κB in recombinant Caco-2 cells

In the following part of the study we proceeded with the characterization of strain MIMLh5 by studying the possible involvement of the S-layer protein (SlpA) in mediating bacterial effects on the host immune system. SlpA protein is an abundant molecule present on the outer surface of all *L. helveticus* strains, constituting up to 15% of total Lactobacillus protein content (Åvall-Jääskeläinen & Palva, 2005). For *L. helveticus*, SlpA forms approximately 45% of the cell wall dry weight (Messner et al., 1997). Even if many functions have been suggested/hypothesized (Beveridge et al., 1997; Horie et al., 2002; Smit et al., 2002), the actual role of the S-layer protein remains elusive. We isolated the S-layer protein from strain MIMLh5 to employ in experiments on recombinant Caco-2 cells. We found that similar to whole bacterial cells, purified S-layer protein was able to significantly reduce the activation of NF-κB at both concentrations tested (Fig. 2.4.2). Studies concerning the effects of *Lactobacillus* S-layer proteins on intestinal epithelial cells have already been performed (Frece et al., 2005; Johnson-Henry et al., 2007). Specifically, the properties described to date refer mainly to the role of S-layer proteins in mediating the bacterium's ability to antagonize pathogens due to the capability of the protein to efficiently adhere to the intestinal epithelium. This effect has been demonstrated for the *L. helveticus* (Kos et al., 2003; Frece et al., 2005) and *L. acidophilus* strains (Smit et al., 2002; Buck et al., 2005). Nonetheless, the S-layer protein was also demonstrated to exert direct effects

on the immune responses of intestinal epithelial cells. For instance, it was shown that the S-layer protein from *L. acidophilus* ATCC 4356^T reduced *Salmonella* Typhimurium FP1-induced apoptosis in Caco-2 cells, and this effect was dependent on the inhibition of caspase-3 activation (Li et al., 2011). In another study, the oral immunization of mice with the purified SlpA from *L. helveticus* M92 induced an increase in total levels of serum IgG, IgM and IgA (Beganović et al., 2011).

2.4.4 MIMLh5 strain and its S-layer protein elicit pro-inflammatory responses in human U937 macrophages

After a preliminary immunological investigation performed on epithelial cells, we employed cells belonging to the innate immune system to study the host's immune responses triggered by *L. helveticus* MIMLh5 and its S-layer protein. We used macrophages, which are professional phagocytes serving as sentinels to detect microbial host invaders (Benoit et al., 2008). Macrophages undergo activation upon environmental signals, including microbial products and cytokines (Aderem & Underhill, 1999). We quantified via RT-qPCR the gene expression of TNF-α (a cytokine involved in inflammatory responses; Belardelli, 1995), IL-10 (a multifunctional cytokine whose principal routine function appears to be to limit and ultimately terminate inflammatory responses; Fiorentino et al., 1991; D'Andrea, 1993) and COX-2. COX-2 is a homodimeric enzyme involved in the synthesis of prostaglandins (PGs), which are hormones that participate in physiological processes such as inflammation, as well as in protecting the gastrointestinal mucosa (Williams et al., 1999; Morteau et al., 2000). After 4 h of stimulation of the U937 cells, MIMLh5 induced a pronounced pro-inflammatory profile at MOI 100 and 1000, as evidenced by an enhanced induction of COX-2 and TNF-α compared to IL-10 (Fig. 2.4.5A). The cytokine-expression profile of the purified S-layer protein was similar to that induced by the MIMLh5 strain itself. The involvement of the S-layer protein in the immunostimulating effects of MIMLh5 was also confirmed in experiments involving bacterial cells without the S-layer (*i.e.*, after LiCl-extraction of the protein). In fact, we observed that at MOI 1000, the removal of the S-layer resulted in a decrease in COX-2 and TNF-α induction levels, whereas IL-10 levels did not change (Fig. 2.4.5A). A similar trend was observed at MOI 100, even if the data are not statistically significant (Fig. 2.4.5A). Furthermore, the addition of the purified S-layer protein to MIMLh5 cells without S-layer proteins resulted in a significant increase of COX-2 and TNF-α gene expression, while IL-10 remained unaffected (Fig. 2.4.5A). These results indicate that the S-layer protein from strain MIMLh5 could be primarily involved in inducing the expression of the pro-inflammatory factors COX-2 and TNF-α in human U937 macrophages.

2.4.5 Bone marrow-derived macrophages (BMDMs) display a pro-inflammatory profile upon stimulation with MIMLh5 and its S-layer protein

After the *in vitro* evaluation of the immunological activity of *L. helveticus* MIMLh5 and the purified S-layer protein on human U937 macrophages, we used the same approach to study their effects in *ex vivo* experiments by isolating and differentiating macrophages from mouse bone marrow. Similar to our previous results with human macrophages, we found that *L. helveticus* MIMLh5 induced, at both MOIs tested, a clear pro-inflammatory profile, as evidenced by a strong induction of COX-2 and TNF-α but not IL-10 (Fig. 2.4.3). The results obtained by incubating BMDMs with S-layer protein confirmed the pro-inflammatory properties of the protein on murine macrophages (Fig. 2.4.3). Furthermore, when we exposed BMDMs to the bacterium after the elimination of the S-layer, we observed a trend of reduction (although this

result was not statistically significant) of TNF-α levels for MIMLh5 (particularly at the highest MOI tested), whereas the absence of the protein led to a slight increase of IL-10 levels (Fig. 2.4.3).

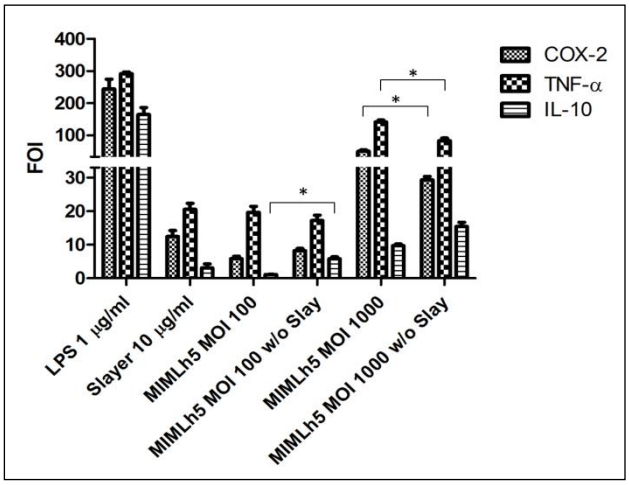
Thus, both in experiments on human U937 macrophages and on murine BMDMs, MIMLh5 exerted pro-inflammatory effects by inducing high levels of TNF-α and COX-2 and lower levels of IL-10. Interestingly, the purified SlpA extracted from *L. helveticus* MIMLh5, according to the expression levels of TNF-a and COX2, seems to be responsible for the pro-inflammatory behavior of the whole bacterium, as also confirmed by the experiments performed with SlpA-depleted MIMLh5 cells (Fig. 2.4.3 and Fig. 2.4.5A).

The ability to induce pro-inflammatory cytokines has already been shown in lactic acid bacteria (LAB) (Christensen et al., 2002; Morita et al., 2002; Peridigón et al., 2002; Ongol et al., 2008). Nonetheless, such ability of LAB should not be considered detrimental. In fact, even though cytokines belonging to the TNF-α superfamily are connected to the occurrence of inflammatory diseases (Kwon et al., 2010), they have also been shown to participate in the rejection of tumors and infections (Yasutake et al., 1999; Dinarello, 2003; Wajant et al., 2003). Furthermore, the induction of TNF-α could be important for the initiation of cross-talk among immune cells without causing any inflammation or detrimental effects (Galdeano et al., 2007). In addition, a transient inflammatory state could aid host defense. As recently suggested, differences among pathogenic, probiotic and commensal microorganisms lie in the magnitude of the immune response evoked, which can be defined as strong, intermediate or homeostatic (Lebeer et al., 2010).

Similarly, the up-regulation of COX-2 induced by LAB has been demonstrated both *in vitro* (Otte et al., 2009; Khailova et al., 2010) and *in vivo* (Khailova et al., 2010; Kwon et al., 2010). The pro-inflammatory role of COX-2, however, has recently been questioned. In fact, it has been proposed that COX-2 can induce the resolution of inflammation (Wallace, 2006) and an immunosuppressive phenotype in T-cells (Newberry et al., 1999) through the activity of PGs. In addition, it has also been shown that a rapid COX-2 up-regulation in response to injury or inflammation helps to restore mucosal integrity (Tan et al., 2000). In this context, the effects of MIMLh5 and SlpA on COX-2 expression could potentially be beneficial for the host's mucosa.

The ability of specific cell wall components to drive the immune response elicited by LAB has been rarely described (de Ambrosini et al., 1996; Tejada-Simon & Pestka, 1999). Concerning S-layer proteins, Konstantinov and colleagues (2008) demonstrated that SlpA from *L. acidophilus* NCFM mediates the interaction of the bacterium with human monocyte-derived dendritic cells (DCs) by specifically binding to the ligand of the dendritic cell-specific intercellular adhesion molecule (ICAM)-3-grabbing non-integrin (DC-SIGN). Even if the purified SlpA protein of NCFM did not affect DCs maturation, it was responsible for the anti-inflammatory cytokine profile observed for *L. acidophilus* NCFM because the protein induced higher levels of IL-10 in the presence of LPS when compared to SlpA or LPS alone.

Figure 2.4.3 *Quantitative analysis of cytokine gene expression in murine bone marrow-derived macrophages (BMDMs) after 4 h stimulation with* L. helveticus *MIMLh5 and its S-layer protein. Expression levels of TNF-α, IL-10 and COX-2 are indicated as the fold change of induction (FOI) relative to the control (unstimulated BMDMs), which was set at a value of 1. LPS was used as a positive control at a concentration of 1 μg ml^{-1}. S-layer protein was tested at a concentration of 10 μg ml^{-1}. MIMLh5 was used at MOIs 1000 and 100. MIMLh5 w/o Slay: MIMLh5 cells after removal of the S-layer protein by LiCl-extraction. Presented data are the means of measurements (+ standard deviations) for a result representative of three independent experiments. Asterisks indicate statistically significant differences compared to results for the corresponding control (*: P < 0.05).*



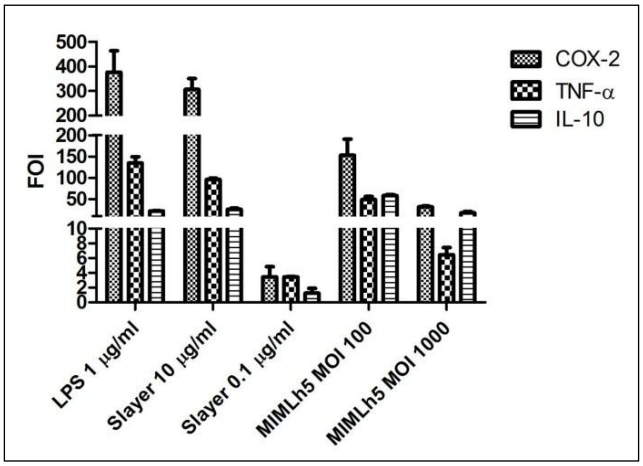
In comparison to the SlpA of *L. acidophilus* NCFM, our data suggest that the S-layer protein isolated from *L. helveticus* MIMLh5 exhibits a more pronounced pro-inflammatory behavior. Although the S-layer proteins of *L. acidophilus* NCFM and *L. helveticus* MIMLh5 are highly similar (73% identity, 83% positivity; Data not shown), it has been suggested that even small differences in surface proteins could alter the type of immune responses elicited by LAB (Vinderola et al., 2004; Lebeer et al., 2010). Nonetheless, the most plausible explanation for the different immunological behavior observed between MIMLh5 and NCFM SlpAs (Konstantinov et al., 2008) could reside in the different cell models utilized.

When we tested strain *L. helveticus* MIMLh5 and its SlpA on macrophages, we observed an immunological effect (stimulation of the pro-inflammatory factors) that was substantially different from what was noted for epithelial cells (*i.e.*, inhibition of NF-κB activation). These results are not surprising, considering that macrophages are immune cells that can express class and number of receptors on their cell surface considerably different from epithelial cells. This fact might result in recognition through diverse mechanisms and activation of alternative signaling pathways. Moreover, it is plausible (and desirable) that a potential probiotic candidate and its cell components do not trigger any inflammatory response at the host epithelium. This type of non-inflammatory interaction can be assimilated to a mechanism of oral tolerance, which, *in vivo*, is due to the natural cohabitation of epithelial cells with commensal and/or food-associated bacteria. In contrast, when bacteria come in direct contact with immune cells, for instance, when they cross the epithelial barrier following loss of epithelial integrity, the immune system is typically alerted through pro-inflammatory signaling.

2.4.6 The S-layer protein from the strain MIMLh5 induces pro-inflammatory activity in murine peritoneal cavity macrophages (PCMs)

Once we defined the immune profile induced by MIMLh5 and its S-layer protein *in vitro* on human U937 cells and *ex vivo* on BMDMs, we verified whether the same pro-inflammatory response could be confirmed in tissue-specialized macrophages. To this aim, we employed macrophages isolated from the mouse peritoneal cavity. Unlike the previous results, when we used the whole bacterium, we observed a balanced profile between pro- and anti-inflammatory cytokines in PCMs because the induction of IL-10 was not lower than the induction of TNF-α (Fig. 2.4.4). However, consistent with our findings in U937 cells and BMDMs, the S-layer protein triggered higher levels of COX-2 and TNF-α compared to IL-10 in peritoneal macrophages (Fig. 2.4.4). Furthermore, in contrast to U937 cells and BMDMs, the pro-inflammatory factor levels induced in PCMs by the S-layer protein were almost equal to the levels induced by LPS. This pronounced pro-inflammatory activity was shown to be dose-dependent because when we tested a lower concentration of the protein, 0.1 μg ml^{-1}, TNF-α and COX-2 levels were lower compared to LPS, although the pro-inflammatory factor profile was qualitatively the same. The results obtained by employing PCMs, therefore, confirm the involvement of S-layer protein principally in promoting pro-inflammatory immune responses.

Fig. 2.4.4 *Quantitative analysis of cytokine gene expression in murine macrophages isolated from peritoneal cavity after 4 h stimulation with* L. helveticus *MIMLh5 and its S-layer protein. Expression levels of TNF-α, IL-10 and COX-2 are shown as the fold change of induction (FOI) relative to the control (unstimulated peritoneal macrophages), which was set at a value of 1. LPS was used as a positive control at a concentration of 1 μg ml^{-1}. S-layer protein was tested at concentrations of 10 and 0.1 μg ml^{-1}. MIMLh5 was used at MOIs 1000 and 100. Presented data are the means of measurements (+ standard deviations) for a result representative of three independent experiments.*



This finding suggests that MIMLh5 bacterial cells could be more prone to induce an anti-inflammatory response in this cellular system compared to U937 cells and BMDMs. Such different behavior can be explained by considering that different immune responses can be induced by the same stimulus depending on the cell type, origin and polarization (Habil et al.,

2011). Moreover, because BMDMs and U937 cells have been maturated *in vitro*, it is plausible that they are less physiological than PCMs, and that they could not possess all the features of matured macrophages. It could also be speculated that macrophages from the peritoneal cavity can present different phenotypes compared to other macrophage populations due to the activity exerted on PCMs by the intestinal microbiota, which can promote a more tolerogenic activity based on differential expression of pattern-recognition receptors (PRRs). Accordingly, the induction of an anti-inflammatory cytokine profile by lactobacilli has been observed in murine PCMs (Marcinkiewicz et al., 2007). Nonetheless, in comparison to whole bacterial cells, the pro-inflammatory effect of S-layer protein was particularly evident in PCMs. In fact, for MIMLh5 cells, the TNF-α/IL-10 ratios were 0.84 at MOI 100, 0.35 at MOI 1000, and approximately 3.7 when purified SlpA protein was used as a stimulus.

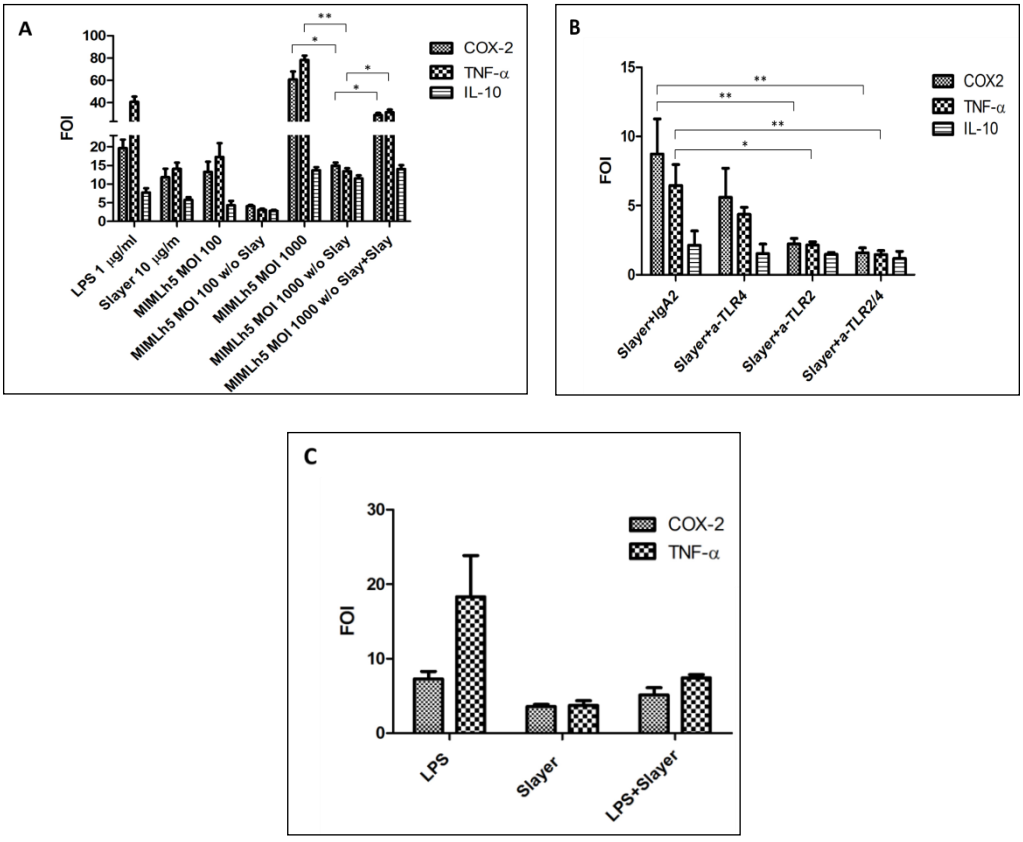
2.4.7 Toll-like receptor (TLR)-2 is involved in the recognition of the S-layer protein from strain MIMLh5 in human U937 cells

To gain information about the possible signaling pathways underlying the immune response activated by the S-layer protein isolated from *L. helveticus* MIMLh5, we evaluated whether TLR2 and TLR4 could be involved in the recognition of this protein. To this aim, we used neutralizing antibodies (Abs) on U937 cells to partially block the ability of TLR2 and TLR4 to bind ligands prior to the addition of the S-layer protein. We found that TLR2 and, to a lesser extent, TLR4 are involved in mediating the pro-inflammatory response elicited by S-layer protein because the treatments with Abs significantly reduced the S-layer-induced activation of TNF-α and COX-2, but not IL-10 (Fig. 2.4.5B). TLR2 is a receptor that was previously shown to be involved in mediating the immunological effects of LAB (Konstantivov et al., 2008; Zeuthen et al., 2008; Vizoso et al., 2009; Weiss et al., 2010). The observed differences when anti-TLR2 was used, either alone or in combination with anti-TLR4, were statistically significant compared to U937 cells stimulated with S-layer protein in the presence of Immunoglobulin-A2 isotype (IgA2), which was used as a control for nonspecific blocking activity. From these data, we can hypothesize that the S-layer, which is the outermost part of the bacterial cell wall, might come in contact first with host cells, mediate bacterial recognition, and therefore play a key role in triggering the immune response.

2.4.8 S-layer protein modulates the pro-inflammatory response triggered by LPS in human U937 macrophages

In a final set of experiments, we tested the effects of the S-layer protein on U937 cells in presence of LPS. Interestingly, we observed that the presence of the S-layer protein induced a clear reduction in the pro-inflammatory cytokine TNF-α, compared with the levels induced by LPS alone (Fig. 2.4.5C). These results suggest that although the S-layer protein and LPS presented similar pro-inflammatory effects, the simultaneous presence of both stimuli did not result in an additive or synergistic effect on the pro-inflammatory response. Rather, the addition of the S-layer protein induced a reduction of the stimulating activity of LPS. In our experiments on macrophages, the SlpA of MIMLh5 induced a cytokine profile qualitatively similar to that evoked by LPS; nonetheless, the cytokine induction levels on human U937 cells and murine BMDMs were lower for the S-layer protein than for LPS. In peritoneal macrophages, the S-layer protein triggered a cytokine induction similar to LPS only when ten-times more S-layer protein (10 μg) was present compared to LPS (1 μg).

Figure 2.4.5. *Quantitative analysis of cytokine gene expression in U937 human macrophages after 4 h of stimulation. Expression profiles of TNF-α, IL-10 and COX-2 are indicated as the fold change of induction (FOI) relative to the control, which was set at a value of 1. Presented data are the means (+ standard deviations) for a result representative of three independent experiments. Asterisks indicate statistically significant differences: **, P < 0.01; *, P < 0.05. A, U937 cells stimulated with* L. helveticus *MIMLh5 (MOIs 1000 and 100) and its S-layer protein (10 μg ml^{-1}); control: unstimulated U937 cells; MIMLh5 w/o Slay: MIMLh5 cells after LiCl-extraction of the S-layer protein; MIMLh5 w/o Slay + Slay: S-layer-depleted MIMLh5 cells supplemented with 10 μg ml^{-1} of the purified S-layer protein; lipopolysaccharide (LPS) was used as a positive control at a concentration of 1 μg ml^{-1}. B, Cytokine expression profiles in the presence of neutralizing antibodies against TLR2 (a-TLR2) and TLR4 (a-TLR4); immunoglobulin-A2 isotype (IgA2) was used as a control for nonspecific blocking activity; IgA2 and anti-TLRs were added at a concentration of 5 μg ml^{-1} on U937 cells 1 h before stimulation with the S-layer protein; anti-TLR2/4: anti-TLR2 and anti-TLR4 simultaneously added at a concentration of 2.5 μg ml^{-1} each; control: U937 cells incubated with IgA2). C, Cytokine expression profiles in U937 cells stimulated with* L. helveticus *MIMLh5 S-layer protein and LPS; LPS and S-layer were added at a concentration of 1 μg ml^{-1} and 10 μg ml^{-1}, respectively, both when used alone and in association; control: unstimulated U937 cells.*



These data indicate that the SlpA of MIMLh5 is not a pro-inflammatory stimulus as potent as LPS. We could hypothesize that the S-layer alone, in basal conditions, may exert a mild stimulatory effect on the immune system. However, in the presence of an inflammatory stimulus, such as LPS, the MIMLh5 S-layer could act as an immune modulator, representing a potential protective element against the septic shock caused by LPS, as already observed in murine models for certain probiotic bacteria (Arribas et al., 2009a;b).

Since S-layer protein and LPS interact with different TLRs, the observed modulatory effect of the S-layer protein should not be attributed to a competition for the same host cells' receptors but, more likely, to an activation of different immune response pathways.

2.5 CONCLUSIONS

The cross-talk between host and intestinal/probiotic bacteria principally relies on the capacity of host cells to recognize specific bacterial components or products, thus giving rise to responses that most frequently involve the mucosa-associated lymphoid tissue (MALT) and, therefore, the immune system (Adams, 2010; Taverniti & Guglielmetti, 2011). Specifically, cell surface components of commensal or food/probiotic bacteria, known as microbe-associated molecular patterns (MAMPs), can be identified by pattern recognition receptors (PRRs) on host's cells constituting the innate immune system, resulting in the activation of immune responses. The molecular mechanisms of such immune modulations are largely unknown. Therefore, the immunological characterization of single bacterial components, according to the strategy undertaken during this study, represents a reductionist approach of key importance for the elucidation of host–microbial interplays and bacterial modes of action that result in the immune modulation. In this study, we characterized the dairy strain *Lactobacillus helveticus* MIMLh5 and its surface layer protein (SlpA) using *in vitro* and *ex vivo* analyses. We found that MIMLh5 and SlpA exert anti-inflammatory effects by reducing the activation of NF-κB on the intestinal epithelial Caco-2 cell line. On the contrary, MIMLh5 and SlpA act as stimulators of the innate immune system by triggering the expression of pro-inflammatory factors (Taverniti et al., 2012). These results must not be surprising, since several studies reveal that probiotics exert stimulatory effects rather than suppressory effects on innate immunity. This mechanism is similar to a "physiologic inflammation", like the one induced by the commensal flora, that can be useful for immune system development and for combating pathogens (Pagnini et al., 2010).

Furthermore, our study supports the concept that the viability of bacterial cells is not always essential to exert immunomodulatory effects (Adams, 2010). In fact, dead or inactivated bacterial cells, or even their single molecular components, might also be effective in exerting beneficial immunostimulating properties, thus permitting the development of safer therapies for the treatment of specific diseases. This approach has recently been defined as paraprobiotic intervention (Taverniti & Guglielmetti, 2011). In addition, a deeper understanding of the molecular mechanisms of cross-talk between bacteria and the host organism's system is of great importance to better define the benefits and potential risks associated with the administration of probiotic therapies (Besselink et al., 2008).

Moreover, the possible medical and clinical uses of the S-layer protein are particularly intriguing, especially in light of the physicochemical properties of this molecule. The S-layer proteins are crystalline arrays of subunits forming a highly regular crystalline structure that has the capacity to reassemble spontaneously in suspension at the liquid-air interface, at solid surfaces, at floating lipid monolayers, on liposomes, and on nanocapsules (Sleytr et al., 2003; Sleytr et al., 2007). These features could support the use of the S-layer protein as a bioactive coating material, as a matrix for the immobilization and delivery of different molecules, and as a template for the formation of regularly arranged bioactive nanoparticles (Pum & Sleytr, 1999). Future efforts will explore the feasibility of such applications.

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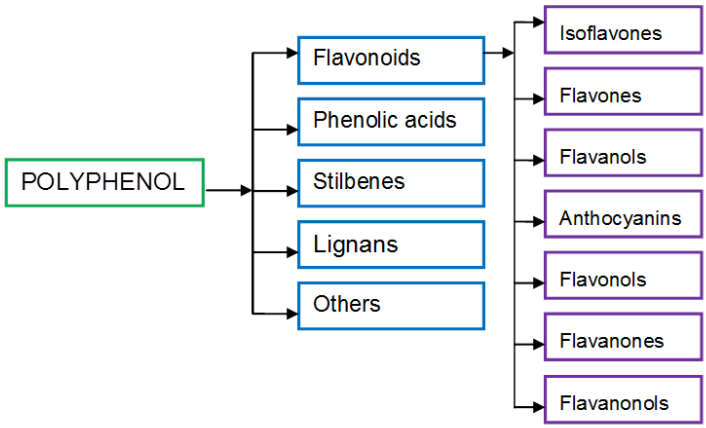
3 THE IMMUNOMODULATORY ACTIVITY OF ANTHOCYANINS FROM WILD BLUEBERRY ORIGIN

3.1 STATE OF THE ART

3.1.1 Anthocyanins: chemical structure, sources and bioavailability

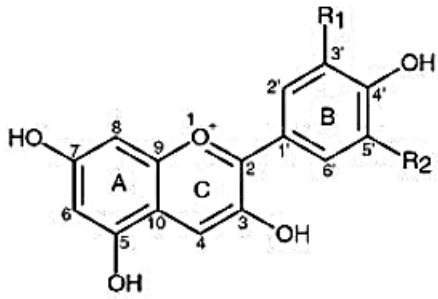
Berries such as cranberries, blueberries, strawberries, blackcurrant and raspberries contain significant amounts of non-nutritive phytochemicals including polyphenols and other antioxidants. Polyphenols are defined chemically as substances that have an aromatic ring with an hydroxyl substituents, including esters and glycosides. Polyphenols are grouped in different classes (Fig. 3.1.1) on the basis of the number of phenolic rings and of the type and number of elements bound to the rings (Manach et al., 2004). The classes include: simple phenolic acids; stilbenes; more complex chalcones; flavonoids, divided into seven subclasses that include flavonols, flavanols, flavones, flavanones, flavanonols, isoflavones and anthocyanins (Nicholson et al., 2008).

Figure 3.1.1. *Polyphenol classes in fruit and vegetables.*



Anthocyanins (ACNs) compounds, of the flavonoid class, are natural pigments that provide colours ranging from dark blue to purple to fruits and vegetables, including edible berries. They can be found in nature in different structural arrangements (Fig. 3.1.2) depending on the number of glycosilating sugars, their position in the aglycone (anthocyanidin) and the degree and nature of esterification of the sugar with aliphatic or aromatic acids (Giordano et al., 2007).
Flavonoids in general exist in plant predominantly as glycoside conjugates. The absorption of some (but not all) components into the circulatory system occurs in the small intestine (Silberberg et al., 2006). Flavonoids and their metabolites which have not been absorbed in the small intestine can be absorbed in the large intestine, upon colonic microflora action, which will lead to the production of phenolic acids and hydroxycinnamates. These molecules can also be absorbed and ultimately excreted in urine system (Manach et al., 2005).

Fig. 3.1.2 *Chemical structures of selected anthocyanidins (Adapeted from Nicholson et al., 2008.)*



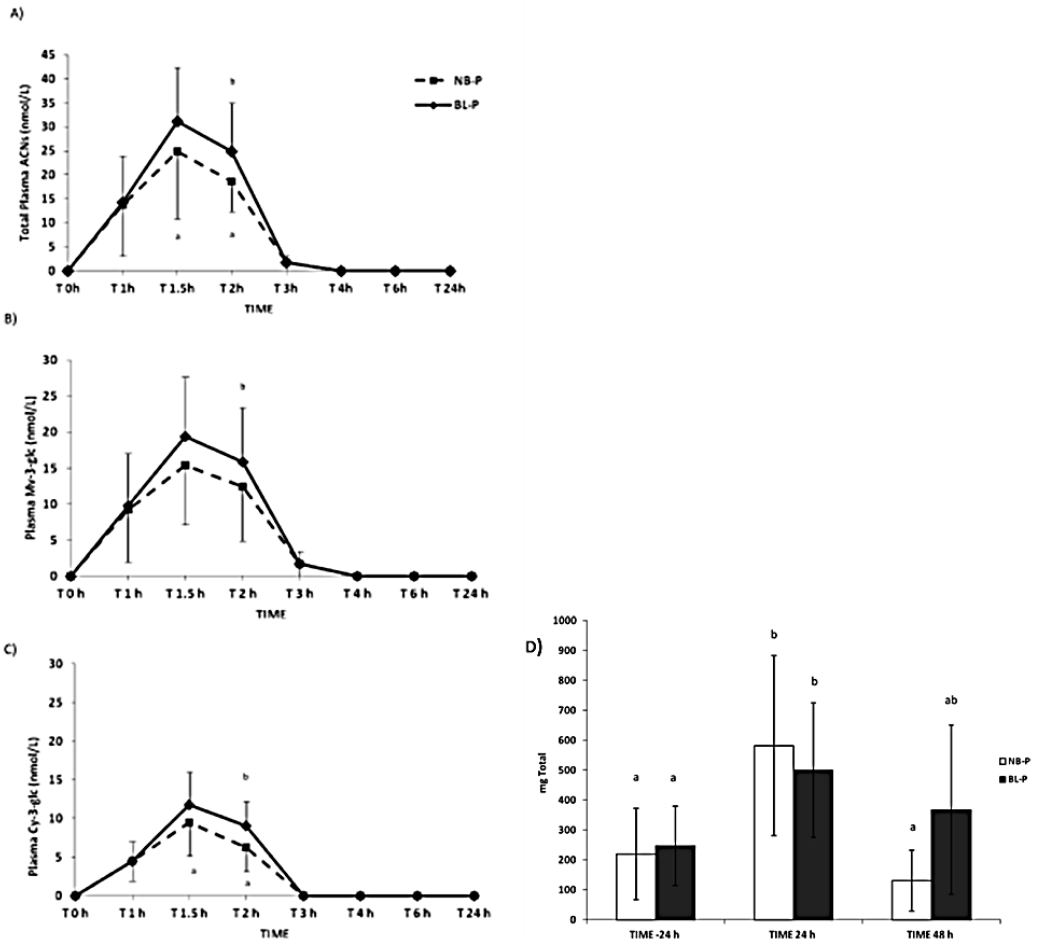
Cyanidin	R_1 = OH.	R_2 = H
Delphinidin	R_1 = OH.	R_2 = OH
Petunidin	R_1 = OCH_3	R_2 = OH
Peonidin	R_1 = OCH_3	R_2 = H
Malvidin	R_1 = OCH_3	R_2 = OCH_3
Pelargonidin	R_1 = H	R_2 = H

Wild blueberry (WB, *Vaccinium augustifolium*) is one of the richest sources in ACNs and is composed of different anthocyanidins such as delphinidin, malvidin, petunidin, cyanidin and peonidin. (Bushway et al., 1983).

In a recent study it was found that following the intake of a blueberry product, ACNs were rapidly absorbed, and their plasma concentration increased 1 h after consumption, achieving their maximum concentration at 1.5 h. In particular, the main compounds found at plasma levels were Cyanidin-3-glc, Malvidin-3-glc, and Delphinidin-3-glc (Del Bo' et al., 2012).
Moreover the excretion of hippuric acid increased within 24−48 h demonstrating that ACNs and other phenolic compounds were absorbed and metabolized.

Interestingly metabolites of ACNs in the plasma, urine, feces, and tissues were also found in an animal model, the Sprague-Dawley (SD) rats fed with a wild blueberry-enriched diet (8%) for 4 and 8 weeks (Del Bo' et al., 2010). However it was not possible to detect their native forms in liver, brain tissues or in the plasma since ACN metabolites are rapidly metabolized and the animals were sacrificed 3-4 h after the last meal, as also confirmed in previous studies (Felgines et al., 2002). ACNs content in the urine significantly increased at 8 weeks compared to that at 4 weeks. The percentage of intake that is absorbed can varys with structure and the food matrix. The exact yields and proportions of metabolites from any substrate can be also dependent on the individual's genetic profile and on the composition and competence of the individual's intestinal microbiota. In fact since most of the ACNs introduced with foods reach the colon, they can be extensively metabolized by intestinal microbiota, which contribute to the bioavailability and bioefficacy of ACNs and phenolic compounds in the systemic circulation. Moreover, it has been recently documented that ACNs and phenolic compounds from wild blueberry can positively modulate the composition of intestinal microbiota and promote the colonization of the gastrointestinal tract by beneficial bacteria *(i.e., Bifidobacterium* spp. and *Lactobacillus* spp.) at the expense of unhealthy bacteria (Vendrame et al., 2011).

Figure 3.1.3 *Absortption and excretion of ACNs and metabolites. (A) Total plasma ACN, (B) Mv-3-glc, and (C) Cy-3-glc concentrations after the consumption of 300 g of unblanched (NB-P) and blanched (BL-P) blueberry purées. Data are mean ± SD. (A) BL-P was significantly different with respect to NB-P at 1.5 and 2 h; $p \leq 0.05$. (B) BL-P at 2 h was significantly different with respect to BL-P at 1 h; $p \leq 0.05$. D) Total hippuric acid concentration in urine before and 24 and 48 h after the consumption of the unblanched (NB-P) and blanched (BL-P) blueberry purées. Data are mean ± SD. Data with different letters are significantly different at $p \leq 0.05$.*



3.1.2 Biological activities of ACNs

Different health-promoting properties have been attributed to anthocyanins.
The most documented and important property is the antioxidant activity (capacity to scavenge free-radicals) since it is involved in the development of several chronic degenerative diseases (Espin et al., 2007). Several studies, moreover, have shown that ACNs can play a critical role in promoting host health through diverse mechanisms: amelioration of lipid profile and vasomotor tone, modulation of detoxifying enzyme, reduction of blood pressure (Mazza et al., 2002; Norton

et al., 2005; Del Bo' et al., 2010), and by exerting anti-inflammatory and antitumoral properties (Bagchi et al., 2004).
Particularly, anti-inflammatory abilities have been demonstrated to be partially due to a direct action on host immune system (Karlsen et al., 2007). Many phenolic compounds have been shown to exert their effects through the modulation of pro-inflammatory cytokines, which are the chemical mediators of the immune system, involved in the cross-talk and priming.of the immune cells. Phenolic compounds in fact can be recognized by specific receptors of the intestinal mucosa triggering specific immune responses. Consequently, it has been proposed that they may decrease the risk of individuals to develop pathologies related to chronic exposures to pro-inflammatory cytokines (Clarke & Mullin, 2008). As an example, in fact, some pure polyphenols, such as quercetin, have been demonstrated to inhibit nuclear factor κB (NF-κB) activation in intestinal models of inflammation (Romier et al., 2008). The transcriptional regulator NF-κB is a transcriptional inducer of several inflammatory cytokines and is a therapeutic target in a wide range of human (auto)inflammatory diseases (Yamamoto & Gaynor, 2001).
In particular the effects described for ACNs seem to be dependent on the inhibition of NF-kB activation and down-stream signals, and possibly on modulating effects on other pro-inflammatory pathways (Calixto et al., 2004). Nevertheless, no single or definitive mechanism can explain all the effects of polyphenol compounds that appear to be signal specific and cell type dependent, with no general rule governing structure/activity relationships.
Further investigations are therefore necessary to validate a potential beneficial role of singles or mixtures of polyphenols contained in foods.

3.2 AIMS OF THE STUDY

Wild blueberry has been hypothesized to exert immunomodulatory potential due to its high content of ACNs and other bioactives. Considered the abilities, already reported in literature, of bioactive molecules of food-origins to exert anti-inflammatory effects on the host (like ACNs and other polyphenols), we wanted to better investigate the single contribution of different fractions isolated from wild blueberries. To this aim we extracted and characterized three fractions from WB and tested their immunomodulatory potential both on intestinal epithelial cells and on cells properly belonging to the innate immune system.

3.3 MATERIALS AND METHODS

3.3.1 Extraction and characterization of different fractions from Wild Blueberry (*Vaccinium angustifolium*) Powder

Extraction of bioactives from WB. Soluble (sugars and organic acids), phenolic (Phe) and anthocyanin (ACN) fractions were obtained from a freeze-dried Wild Blueberry (WB) powder provided by Future-Ceuticals Company (Momence, IL, USA). Extraction was performed following a method described by Wrolstad (2005) with few modifications. Briefly, the powder was suspended in a 1% TFA aqueous solution, sonicated for 10 min, and centrifuged at 3000 × *g* for 10 min. Fractions separation from the supernatant was obtained through solid-phase extraction (SPE)-cartridge (Strata-X 3 ml, Phenomenex, Torrence, CA) preactivated with methanol (5 ml) and then washed with water (5 ml). The elution of soluble, Phe and ACN fractions were carried out respectively with HCl 0.01 N (5 ml), ethyl acetate (10 ml) and methanol (5 ml) containing 0.1% HCl,. The fractions were dried under vacuum with rotavapor (RC Jouan 10, Jouan, Winchester, VA) at 20°C for ACN, 40°C for Phe and up to 60°C for the soluble fraction. The residues were dissolved in methanol acidified with HCl (0.05 mM) for the ACNs, methanol for the Phe, and water for the soluble fractions. The solutions were analyzed for the content of ACNs, Phe, sugars and organic acids, and stored at -20°C until use.

Analysis of Anthocyanin and phenol fractions. The analysis was performed with a liquid chromatographic system, which consisted of an Alliance mod. 2695 (Water, Milford, MA) equipped with a mod. 2998 photodiode array detector (Waters). The separation was carried out through a C_{18} Kinetex column (150 x 4.6 mm, 2.6 µm, Phenomenex, Torrence, CA) at 45°C and 1.7 ml/min as flow rate. The eluents were (A) 1% H_3PO_4 and (B) acetonitrile/water (35:65, v/v). The elution gradient was linear as indicated: 0-15 min 14% B; 15-25 min from 14 to 20% B; 25-35 min from 20 to 32% B; 35-45 min from 32 to 50% B; 45-48 min from 50 to 90% B; 90 % for 3 minutes. Chromatographic data were acquired from 200 to 700 nm and integrated at 520 (anthocyanins) and 320 nm (phenolic acids). Calibration curves ranged from 2 to 50 mg l^{-1} were obtained for cyanidin (Cy-), delphinidin (Dp-), petunidin (Pt-), peonidin (Pe-) and malvidin (Mv-) 3-*O*-glc, Cy- and Pt-3-*O*-gal and Pt-3-*O*-ara and chlorogenic acid. For the anthocyanins, the working solution was diluted from the stock solution with methanol acidified with 0.1% TFA. Each analysis, was carried out in duplicate. The identification of single ACNs was confirmed by LC coupled to ESI-MS (electro spray ionization - mass spectrometry) as already described by Del Bo' et al. (2010). Briefly, the mass spectrometer operated in positive full-scan mode in the range 200-800 Da. The capillary voltage was set to 3.5 kV, the cone voltage to 20 V, the source temperature to 130 °C, and the desolvating temperature to 350°C. Data were acquired by Masslinx 4.0 software (Micromass, Beverly, MA).

Analysis of sugars. Glucose and fructose were quantified in WB powder and fractions by UPLC (Acquity, Waters) coupled with a triple quadrupole mass spectrometer mod. Quattro micro (Micromass, Beverly, MA).
The separation was carried out on BEH (Ethylene Bridged Hybrid) Amide column (150 x 2.1 mm, 1.7 µm, Waters) at 35°C. Solvents were (A) triethylamine 0.2% and (B) triethylamine 0.2% in acetonitrile. Flow-rate was 0.40 ml min^{-1}. The calibration curve was obtained from 1 to 50 mg l^{-1} for both sugars. The mass spectrometer operated in ESI negative mode monitoring the ions with *m/z* 179 (glucose, fructose). The capillary voltage was set to 3.0 kV, the cone voltage to 20 V, the source temperature to 120 °C, and the desolvating temperature to 250°C.

Analysis of organic acids. Malic and citric acid were analyzed by UPLC (Acquity, Waters) coupled to a triple quadrupole mass spectrometer mod. Quattro micro (Micromass, Beverly, MA). The separation was performed on C_{18} Atlantis T3 column (150 x 2.1 mm, 1.7 µm, Waters)

at 45°C and the flow rate was 0.5 ml min^{-1}. The eluent was formic acid 0.05%. The calibration curves were obtained at concentration ranged from 2 to 25 mg l^{-1} for both malic and citric acid. The capillary voltage was set to 3.5 kV, the cone voltage and the collision energy was specific for each compound. The source temperature was 120°C, the desolvating temperature was 300°C and argon was used at $1.3x10^{-3}$ mbar to improve fragmentation in the collision cell. Masslinx 4.0 acquired data with Quan-Optimize option for fragmentation study. The fragmentation transitions for the multiple reaction monitoring (MRM) were $(m/z)^-$ 133→115, 133→71 for malic and $(m/z)^-$ 191→111, 191→87 for citric acid, with a dwell time of 0.2 s per transition.

3.3.2 Experiments with Caco-2 cell layers

In vitro *cultivation*. Caco-2 cells (human epithelial colorectal adenocarcinoma cell line; ATCC HTB-37) were routinely grown in Eagle's minimum essential medium (EMEM) supplemented with 10% (v/v) heat-inactivated (30 min at 56°c) fetal calf serum (FCS), 100 U/ml penicillin, 100 mg/ml streptomycin, 0.1 mM nonessential amino acids (NEAA), and 2 mM L-glutamine and were incubated at 37°c in a water-jacketed incubator in an atmosphere of 95% air and 5% carbon dioxide. *Study of NF-κB activation*. Stable recombinant Caco-2 cell line was generated by transfecting cells with the plasmid pNiFty2-Luc (Invivogen, Labogen, Rho, Italy) as described by Guglielmetti *et al.* (2010). This plasmid contains a promoter with five NF-κB-binding sites followed by the firefly luciferase reporter gene *luc*. Stimuli that activate NF-κB promote its binding to the vector promoter, resulting in the expression of the luciferase gene. Briefly, Caco-2 were transfected by means of the StoS transfection kit (GeneSpin, Milan, Italy), in accordance with the manufacturer's protocol. Afterwards, cells were resuspended in fresh EMEM, seeded in 24-well plates, and incubated for 48 h, in order to obtain the expression of the antibiotic resistance. Finally, stable recombinant clones were selected by adding into the culture medium 50 μg ml^{-1} of zeocin (InvivoGen).

3.3.3 Study of the immunomodulatory activity of WB ACNs

After growth in the presence of 50 μg/ml zeocin, cell monolayers (approximately 3×10^5 cells $well^{-1}$) were carefully washed with 0.1 M Tris-HCl buffer (pH 8.0). Subsequently, fresh EMEM medium, containing 100 mM HEPES (pH 7.4) was added to Caco-2 cells. The soluble, Phe and ACNs fractions obtained by WB powder have been tested. Concentrations used were 25, 50 and 100 μg ml^{-1} for the water soluble fraction (calculated considering the sugars and organic acid concentration), and 5, 25, 50 and 100 μg ml^{-1} for the phenolic (calculated considering the chlorogenic acid concentration) and the anthocyanin fractions (calculated considering the total ACNs concentration). Recombinant Caco-2 cells were simultaneously stimulated with IL-1β (2 ng ml^{-1}), used as pro-inflammatory stimulus. After incubation at 37°C for 4 h, 24-well plates were put on ice for 15 min; recombinant Caco-2 cells were detached mechanically from the bottom of a well, samples were transferred into an eppendorf tube and subjected to sonication at maximum power for 5 s using a Bandelin SONOPLUS Ultrasonic Homogenizer (Bandelin electronic GmbH & Co., Berlin, Germany). Insoluble particles were removed by centrifugation and the supernatants were transferred into a new tube. One hundred μl of supernatants were aliquoted in duplicate into the wells of a 96-well white microtiter plate (PerkinElmer, Monza, Italy) by means of ep*Motion* Automated Pipetting System (Eppendorf, Milan, Italy). Then 12.5 μl of a 10 mM ATP solution (i.e. up to the final concentration of 1 mM) and 12.5 μl of 0.1 mM D-luciferin were added and the emitted bioluminescence was immediately measured every 120 s

with a VICTOR3 1420 Multilabel Counter (PerkinElmer). The maximum of light production curve was considered for comparison of results. All conditions were analyzed in duplicate in eight independent experiments.

3.3.4 Study of the WB ACNs effect on U937 human macrophage cell line

Cell culture, growth conditions, and stimulation protocol. The cell line U937 (ATCC CRL-1593.2) was derived from a human histiocytic lymphoma (Sundstrom and Nilsson, 1976). These cells are maintained as replicative, non-adherent cells and have many of the biochemical and morphological characteristics of blood monocytes (Harris and Ralph, 1985). When treated with phorbol myristate acetate (PMA), U937 cells differentiate to become adherent, non-replicative cells with characteristics similar to tissue macrophages, including similar isoenzyme patterns (Radzun et al., 1983) and other phenotypic markers (Harris and Ralph, 1985). The normal growth medium for the U937 cells consisted of RPMI 1640 medium (Lonza, Basel, Lonza, Basel, Switzerland) supplemented with 10% (v/v) fetal bovine serum (FBS) (Gibco-BRL, Life Technologies, Milan. Italy), 2 mM L-glutamine, 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin (Sigma-Aldrich). U937 cells were seeded at a density of 5×10^5 cells $well^{-1}$ in 12-well plates and incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Differentiation was induced by the addition of PMA (Sigma-Aldrich) into the cellular medium at a final concentration of 100 nM and was allowed to proceed for 48 h. Afterwards, cells were washed once with sterile PBS buffer to remove all non-adherent cells. One h before the bacteria were added to the cells, the culture medium was replaced with RPMI 1640 supplemented with 1% (v/v) FBS to allow the cells to adapt. After adaptation U937 cells were stimulated for 4h at 37°C with three different concentration of the WB ACNs fraction. ACNs fraction were tested at final concentrations of 25, 10 and 1 μg ml^{-1}. Lipopolysaccharide (LPS, final concentration of 1 μg ml^{-1}) from *Escherichia coli* 0127:B8 (Sigma-Aldrich) was used as pro-inflammatory stimulus in U937 cells. We also added MetOH + 0.05 mM HCl to RPMI 1640 medium 1% (v/v) FBS (Data not shown) and to LPS in contact with U937 cells to evaluate any possible interference on macrophages responses from the solvent. In all these experiments, however, the final concentration of MetOH + 0.05 mM HCl in contact with U937 cells was always lower than the 0.1% of the final volume in the well. We performed two type of stimulations: 1 h of preincubation with LPS which followed the addition of ANCs for 3 h, and 1 h of preincubation with ACNs and subsequent addition of LPS for 3 h.

3.3.5 Preparation of RNA and reverse transcription

For both *in vitro* and *ex vivo* experiments, after incubating macrophages at 37°c for 4 h, the supernatant was carefully removed from each well and the total cellular RNA was isolated from the adhered cells with an RNeasy Mini Kit (Qiagen Inc., Valencia, CA, USA). Afterwards, RNA concentration and purity was determined with a Nanodrop Spectrophotometer (ND-1000, Thermo Fischer Scientific, Middletown, VA, USA) and reverse transcription to cDNA was performed with the iScript™ Select cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA) using the following thermal cycle: 5 min at 25°C, 30 min at 42°C, and 5 min at 85°C. *Reverse transcription quantitative PCR (RT-qPCR).* The mRNA expression levels of cytokines were analyzed with SYBR Green technology in RT-qPCR using SsoFast EvaGreen Supermix (Bio-Rad Italia, Segrate, Italy) on a Bio-Rad CFX96 system according to the manufacturer's instructions. The primers used are as follow (5'→3'): 18S rRNA forward ATCCCTGAAAAGTTCCAGCA; 18S rRNA reverse CCCTCTTGGTGAGGTCAATG; TNF-α

forward TCAGCTCCACGCCATT; TNF-α reverse CCCAGGCAGTCAGATCAT. Primers were designed using the Primer3Plus software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi), and the specificity of the primers was tested with melting curves during amplification and by 1% TAE agarose gel electrophoresis. Quantitative PCR was carried out according to the following cycle: initial hold at 96°C for 30 s and then 40 cycles at 96°C for 2 s and 70°C for 5 s. Gene expression was normalized to the housekeeping gene coding for the18S rRNA. The amount of template cDNA used for each sample was 50 ng. All results regarding cytokine mRNA expression levels are reported as the fold of induction (FOI) in comparison to the control (namely unstimulated U937 macrophages), to which we attributed a FOI of 1.

3.3.6 Statistical analysis

The statistical analysis was performed by means of STATISTICA software (Statsoft Inc., Tulsa, OK, US). Analysis of variance (ANOVA) with type of treatment as the dependent factor was used to evaluate the immunomodulatory activity. Post-hoc analysis of differences between treatments was assessed by the Least Significant Difference (LSD) test with $p \leq 0.05$ as level of statistical significance.

3.4 RESULTS AND DISCUSSION

3.4.1 Modulation of NF-κB activation by WB soluble, phenolic and anthocyanin fractions in Caco-2 cells

To evaluate the presence of potential bioactive molecules in our WB powder preparation, as preliminary immunological investigation, we tested the effects of the three different fractions isolated from WB on NF-κB, a transcriptional factor responsible for the induction of pro-inflammatory cytokines in immune responses. To this aim we used a reporter cell line obtained by transfecting Caco-2 cells with a luciferase reporter vector induced by NF-κB. In Table 3.4.1 the chemical composition of the different WB fractions is reported.

Table 3.4.1 *Different WB fractions characterization and concentration tested in* in vitro *immunological assay on NF-κB-transfected Caco-2 cells. A: water soluble fraction; B: phenolic fraction; C: anthocyanin (ACN) fraction.*

A

Compound	Extracted	CaCo cell addition		
	μg/ml	25 μg/ml	50 μg/ml	100 μg/ml
Glucose	2284,4	13,97	27,9	55,9
Fructose	1836,4	11,3	22,6	45,2
Malic acid	19,9	0,12	0,24	0,48
Citric acid	47,2	0,29	0,58	1,16

B

Compound	Extracted	CaCo cell addition			
	μg/ml	μg/ml	μg/ml	μg/ml	μg/ml
Chlorogenic acid	719,2	5	25	50	100

C

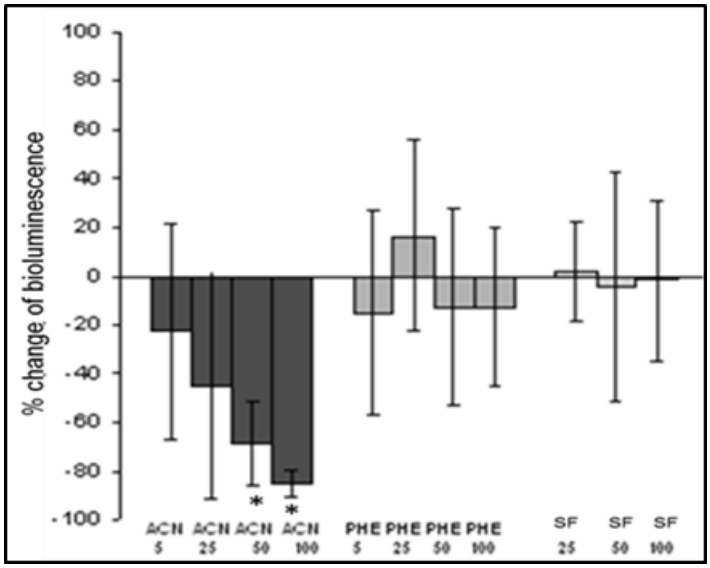
Compound	Extracted		CaCo cell addition			
	µg/ml	%	µg/ml	µg/ml	µg/ml	µg/ml
Delphinidin 3-gal	166	10,6	0,53	2,7	5,4	10,8
Delphinidin 3-glc	224	14,3	0,72	3,6	7,2	14,4
Cyanidin 3-gal	69	4,4	0,22	1,1	2,2	4,4
Delphinidin 3-ara	102	6,5	0,33	1,7	3,4	6,8
Cyanidin 3-glc	99	6,3	0,32	1,6	3,2	6,4
Petunidin 3-gal	107	6,8	0,35	1,8	3,6	7,2
Cyanidin 3-ara + Petunidin 3-glc	167	10,7	0,54	2,7	5,4	10,8
Peonidin 3-gal	38	2,4	0,12	0,6	1,2	2,4
Petunidin 3-ara + Malvidin 3-gal	247	15,8	0,96	3,8	7,6	15,2
Peonidin 3-glc + Malvidin 3-glc	279	17,9	0,89	4,4	8,8	17,6
Malvidin 3-ara	65	4,2	0,20	1,0	2,0	4,0
Total	1563	100	5	25	50	100

These experiments were performed in the presence of the pro-inflammatory stimulus IL-1β. All WB fractions, namely ACNs, Phe and water soluble, were tested at different concentrations (Fig 3.4.1). We found no effects for the Phe and the water soluble fractions on Caco-2 cells, while the ACNs fraction had a significant effect in reducing the activation of NF-κB, and in a dose-dependent manner. In fact, the addition of ACNs at the concentration of 50 and 100 µg/ml reduced the bioluminescence, and therefore the NF-κB activation, up to 70% and 85% ($p \leq 0.05$), respectively.

Roles of ACNs in modulating the activation of NF-κB, and the down-stream signals, have been already reported in literature (Epinat & Gilmore, 1999; Karlsen et al., 2007). Interestingly, ACNs isolated from bilberries and blackcurrant have been demonstrated to modulate LPS-

induced activation of NF-κB in monocytes, and supplementation with ACNs in a parallel-designed, controlled clinical trial reduced the levels of circulating NF-κB-dependent inflammatory mediators (Karlsen et al., 2007).

Figure 3.4.1 *Modulation of light emission expressed by Caco-2 cells stably transfected with a NF-κB/luciferase reporter vector and incubated in presence of Interleukin-1β with different fractions extracted from wild blueberry powder. Data are reported as percent variation of light emission, referred to the control. Control: Caco-2 cell layers incubated only with media supplemented with MetOH + 0.05 mM HCl at a concentration correspondent to the one present in highest concentration of fractions tested. ACN: anthocyanin fraction; PHE: phenolic fraction; S.F.: soluble fraction. Fraction concentrations reported (5-25-50-100) are referred to μg ml^{-1}. The values are the means (± standard deviations) for eight independent experiments conducted in duplicate. Asterisks indicate statistically significant differences compared to results for the control (*, P<0.05.)*



The transcription factor NF-kB is essential in orchestrating the inflammatory responses to a wide range of insults, by leading to the induction of genes involved in innate immune response and in the secretion of inflammatory responses mediators, like chemokines and cytokines (Pahl, 1999). High levels and/or prolonged presence of pro-inflammatory mediators is connected to increased risks of chronic-degenerative diseases onset (Libby, 2002; Boos and Lip, 2006). Therefore, the use of natural bioactive molecules, like WB ACNs which possess the ability to modulate NF-κB activation as we also tested and described in our study, might represent a mild an effective strategy in dampening inflammatory processes.

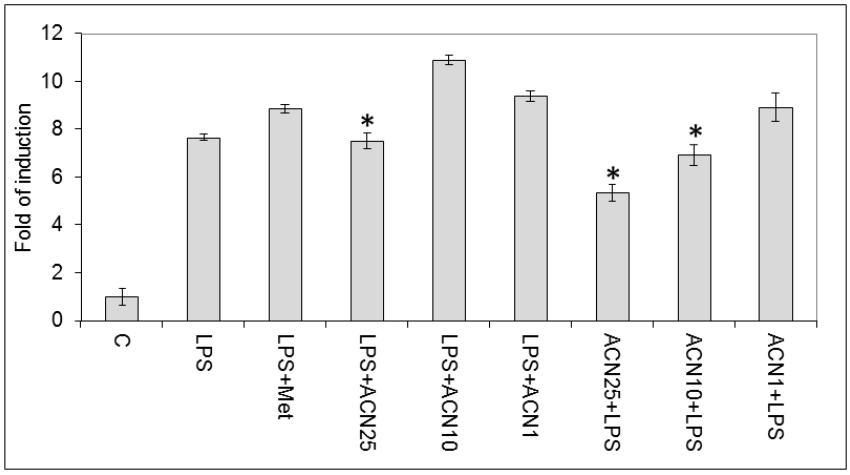
However, when the same ACNs fraction was tested on bone marrow-derived dendritic cells (BMDCs; Data not shown) we found no effect in inducing Th1-related nor Treg cytokines. Our results suggest that food molecules, like ACNs extracted from WB, may not have an effect on immune system *per se*, but rather they modulate the immune responses in presence of other stimuli, as demonstrated in our experiments on epithelial Caco-2 cells in presence of IL-1β, and as also confirmed in the experiments on U937 macrophages in presence of LPS.

3.4.2 WB ACNs fraction displays *in vitro* protective effects towards LPS-dependent inflammatory response on human U937 macrophages

In the subsequent immunological experiments we moved from epithelial cells to cells properly belonging to the immune system, and we decided to focus on ACNs, since it was the only fraction which showed immunomodulatory properties in our first characterization on Caco-2 cells. To study the host's responses elicited by ACN at level of innate immunity, we employed macrophages cells, which are professional phagocytes which undergo activation upon environmental signals (Benoit et al., 2008). We used PMA-differentiated U937 human macrophages, and we first treated U937 cells with PMA for 48 hours to differentiate the cells into macrophages. Subsequently, we stimulated the differentiated cells with WB ACNs at three different concentrations, namely 25, 10 and 1 μg ml^{-1}. We decided to use concentrations lower than the ones tested on Caco-2 cells, since it is plausible that *in vivo* epithelial cells are exposed to higher amounts of dietary molecules with respect to immune cells. After 4 h of incubation, we used RT-qPCR to quantify the gene expression of TNF-α, a cytokine involved in inflammatory responses (Belardelli, 1995). In these experiments we used LPS to induce a pro-inflammatory response on U937 macrophages.
Since ACNs fraction has been resuspended in MetOH + 0.05 mM HCl, as first we wanted to verify any possible interference of the solvent on cell permeability and/or on cell responses. When we incubated U937 cells with RPMI 1% (v/v) FBS media supplemented with MetOH + 0.05 mM HCl (at a concentration correspondent to that present in the highest concentration of ACNs used), we found that TNF-α expression levels were similar to the control (U937 cells incubated only with RPMI media 1% (v/v) FBS; Data not shown); moreover, the presence of MetOH + 0.05 mM HCl had only a slight impact on TNF-α expression level induced by LPS (Fig. 3.4.2).
When we stimulated U937 cells only with the ACNs extracts we found no difference in the induction of TNF-α compared to control, thus confirming the lack of immune response towards these molecules, as previously observed on BMDCs (Data not shown). Consequently, we tested the ACNs fraction on U937 cells together with LPS, by using two different experiment settings: 1 h of preincubation of U937 cells with LPS and then addition of ACNs, and 1h of preincubation of macrophages with ACNs and subsequent addition of LPS for 3 h. The aims were to mimic and evaluate, respectively, a possible anti-inflammatory activity of the ACNs in presence of an ongoing inflammatory status, or a protective role towards an incoming inflammatory agent. For the first hypothesis we found that only the highest concentration tested of ACNs had a significant effect in decreasing the induction of TNF-α with respect to U937 cells stimulated only with LPS. In the other experiment we observed, instead, a clear dose-response in the anti-inflammatory activity of ACNs. Moreover, two ACNs concentrations (25 and 10 μg ml^{-1}) resulted significantly effective in reducing the inflammatory response induced by LPS on U937 cells.

Summarizing our immunological experiments, we observed that ACNs fraction extracted from WB powder displayed anti-inflammatory properties, both on epithelial cells by decreasing the activation NF-κB in presence of IL-1β, and by exerting a protective role towards LPS-induced TNF-α in immune cells. These abilities might be particularly useful in several inflammatory disorders. Obesity, for instance, is a low-grade chronic inflammatory disease, characterized by macrophages infiltration in the white adipose tissue (WAT) (Trayhurn & Wood, 2004). The production of pro-inflammatory factors by activated macrophages can result in further inflammation of the WAT, contributing to the pathogenesis of obesity-related diseases, like hypertension, type 2 diabetes and atherosclerosis (Laine et al., 2007). It has been reported that obese subjects present high serum levels of pro-inflammatory factors, like TNF-α (Fantuzzi, 2004; Wellen and Hotamisligil, 2005). Diet-derived obesity has been also shown to lead to high level of serum LPS; moreover, endotoxemia and the presence of activated macrophages have been demonstrated to be connected with high plasma levels of LPS (Cani et al., 2008).

Fig. 3.4.2 *Quantitative analysis of TNF-α gene expression in U937 macrophages after total 4 h stimulation. Expression levels of TNF-α is indicated as the fold change of induction (FOI) relative to the control (unstimulated U937 cells), which was set at a value of 1. LPS was used at a concentration of 1 μg ml^{-1}. LPS+Met: U937 cells stimulated with LPS supplemented with MetOH + 0.05 mM HCl at a concentration correspondent to the one present in highest concentration of fractions tested. ACNs fractions were tested at concentrations of 25, 10 and 1 μg ml^{-1}. MetOH + 0.05 mM HCl was added in all samples in order to reach the same concentration present in the highest quantity of ACNs used. LPS+ACNs: U937 prestimulated for 1 h with LPS and afterwards for 3h also with ACNs; ACNs+LPS: U937prestimulated with ACNs for 1 h and then also with LPS for 3h. The values are the means (± standard deviations) for at least three independent experiments. Asterisks indicate statistically significant differences compared to results for U937 stimulated with LPS+MetOH + 0.05 mM HCl (*, P<0.05).*



As a matter of fact LPS may induce in macrophages, through the activation of NF-κB, the production of pro-inflammatory cytokines like TNF-α, which are detrimental for WTA by causing inflammation and insulin-resistance (Overman et al., 2010).

The use of compounds like WB ACNs, that have been shown to possess anti-inflammatory properties by acting on NF-κB and LPS responses, could be of help in the treatment or better in the prevention of pathological consequences associated to specific inflammatory disorders. Anti-inflammatory features have been already demonstrated for different categories of polyphenols, and isolated from diverse origins. For instance, a grape-powder extract used in *in vitro* experiments on U937 macrophages reduced the LPS-induced production of inflammatory cytokines (Overman et al., 2010). A proanthocyanidin-rich cranberry fraction has been reported to decrease the LPS-induced cytoxicity in macrophages and oral epithelial cells (La et at., 2009). In a recent study, protocatechuic acid (PCA), which is a major metabolite of anthocyanins, determined an attenuation of LPS-induced lung injury; moreover, the pretreatment of mice with PCA protected by LPS-caused histiologic alterations in lungs and inhibited the induction of pro-inflammatory cytokines like TNF-α (Wei et al., 2012).

Therefore, the intake of fruit and vegetables containing bioactive molecules like ACNs, might represent a natural and safe strategy in the prevention and management of chronic-degenerative diseases caused by inflammatory status.

3.5 CONCLUSIONS

Inflammation consists of a complex series of reactions and processes activated by the host in order to prevent ongoing tissue damage, and to repair processes and defense mechanisms against infectious diseases. However, if prolonged, inflammation may contribute to the pathogenesis of chronic diseases such as diabetes, neurodegenerative diseases, cancers, and cardiovascular disease (Nathan, 2002; Blomhoff , 2005; Karlsen et al., 2007). Thus, an attenuation of such processes might potentially help in retarding or preventing detrimental consequences.

Several studies report that anthocyanins display anti-inflammatory features. The anti-inflammatory activity of anthocyanins have mainly been attributed to a direct antioxidant action, since ACNs are well-known anti-oxidant factors able to control lipid peroxidation and to dampen detrimental effects of reactive-oxygen species.

However, more recently it has been proposed, and also demonstrated, that the anti-inflammatory attitude of such molecules is also mediated by a direct regulatory effects on the expression of genes involved in the inflammatory and immune responses. Several studied have shown ACNs immunomodulatory activity in cell cultures and animal models (Wang & Mazza, 2002; Herath et al., 2003; Cimino et al., 2006).

Accordingly, in our experiments on ACNs isolated from WB we demonstrated that these molecules can have immunomodulatory properties, both on epithelial cells and on immune cells, in particular in presence of pro-inflammatory stimuli.

In conclusion, exploring the role of food and, more specifically, the effect of bioactive compounds such as ACNs on the metabolic processes involved in diseases is crucial, also for designing public health directives for population approaches to prevention, and the results obtained in the present study add important knowledge and insights on the properties of ACN-rich foods and their exploitation for the prevention and treatment of chronic inflammatory diseases.

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APPENDIX 1. COPIES OF ABSTRACTS OF PAPERS, ORAL COMMUNICATIONS AND POSTERS

- Guglielmetti S, **Taverniti V**, Minuzzo M, Arioli S, Stuknyte M, Karp M, Mora D, 2010, Oral bacteria as potential probiotics for the pharyngeal mucosa. Appl Environ Microbiol 76:3948-3958.

- Guglielmetti S, **Taverniti V**, Minuzzo M, Arioli S, Zanoni I, Stuknyte M, Granucci F, Karp M, Mora D, 2010, A dairy bacterium displays *in vitro* probiotic properties for the pharyngeal mucosa by antagonizing group A streptococci and modulating the immune response. Infect Immun 78: 4734-4743.

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- **Taverniti V**, The role of probiotic microorganisms and dietary compounds in the modulation of immune response: a mechanistic approach. In Proceedings of the 15th Workshop on the Development in the Italian PhD Research on Food Science Technology and Biotechnology, Portici, Italy, 15-17 September, 2010.

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- **Taverniti V**, The role of probiotic microorganisms and dietary compounds in the modulation of immune response: a mechanistic approach. In Proceedings of the 16th Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology, Lodi, Italy, 21-23 September, 2011.

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- Fracasetti D, Del Bo'C, Guglielmetti S, Riso P, **Taverniti V**, Klimis-Zacas D, Porrini M. Immunomodulatory properties of Wild Blueberries (*Vaccinium Angustifolium*) in human intestinal epithelial Caco-2 Cells: preliminary data. In Abstracts of The Oxygen Club Of California - Oxidants And Antioxidants In Biology - Cell Signaling And Nutrient-Gene Interaction, 20-23 june, 2012 (Alba, Italy).

- **Taverniti V**, The role of food and probiotic microorganisms, and dietary compounds in the modulation of the immune response. In Proceedings of the 17th Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology, Cesena, Italy, 19-21 September, 2012.

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Oral Bacteria as Potential Probiotics for the Pharyngeal Mucosa[▽]

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The research described here was aimed at the selection of oral bacteria that displayed properties compatible with their potential use as probiotics for the pharyngeal mucosa. We included in the study 56 bacteria newly isolated from the pharynges of healthy donors, which were identified at the intraspecies level and characterized *in vitro* for their probiotic potential. The experiments led us to select two potential probiotic bacterial strains (*Streptococcus salivarius* RS1 and ST3) and to compare them with the prototype oral probiotic *S. salivarius* strain K12. All three strains efficiently bound to FaDu human epithelial pharyngeal cells and thereby antagonized *Streptococcus pyogenes* adhesion and growth. All were sensitive to a variety of antibiotics routinely used for the control of upper respiratory tract infections. Immunological *in vitro* testing on a FaDu layer revealed different responses to RS1, ST3, and K12. RS1 and ST3 modulated NF-κB activation and biased proinflammatory cytokines at baseline and after interleukin-1β (IL-1β) induction. In conclusion, we suggest that the selected commensal streptococci represent potential pharyngeal probiotic candidates. They could display a good degree of adaptation to the host and possess potential immunomodulatory and anti-inflammatory properties.

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A Dairy Bacterium Displays *In Vitro* Probiotic Properties for the Pharyngeal Mucosa by Antagonizing Group A Streptococci and Modulating the Immune Response▽

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The probiotic approach represents an alternative strategy in the prevention and treatment of infectious diseases, not only at the intestinal level but also at other sites of the body where the microbiota plays a role in the maintenance of physiological homeostasis. In this context, we evaluated *in vitro* the potential abilities of probiotic and dairy bacteria in controlling *Streptococcus pyogenes* infections at the pharyngeal level. Initially, we analyzed bacterial adhesion to FaDu hypopharyngeal carcinoma cells and the ability to antagonize *S. pyogenes* on FaDu cell layers and HaCat keratinocytes. Due to its promising adhesive and antagonistic features, we studied the dairy strain *Lactobacillus helveticus* MIMLh5, also through *in vitro* immunological experiments. First, we performed quantification of several cytokines and measurement of NF-κB activation in FaDu cells. MIMLh5 efficiently reduced the induction of interleukin-6 (IL-6), IL-8, and tumor necrosis factor alpha (TNF-α), in a dose-dependent manner. After stimulation of cells with IL-1β, active NF-κB was still markedly lowered. Nevertheless, we observed an increased secretion of IL-6, gamma interferon (IFN-γ), and granulocyte-macrophage colony-stimulating factor (GM-CSF) under these conditions. These effects were associated with the ability of MIMLh5 to enhance the expression of the heat shock protein coding gene *hsp70*. In addition, MIMLh5 increased the GM-CSF/G-CSF ratio. This is compatible with a switch of the immune response toward a TH1 pathway, as supported by our observation that MIMLh5, once in contact with bone marrow-derived dendritic cells, triggered the secretion of TNF-α and IL-2. In conclusion, we propose MIMLh5 as a potential probiotic bacterium for the human pharynx, with promising antagonistic and immunomodulatory properties.

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REVIEW

The immunomodulatory properties of probiotic microorganisms beyond their viability (ghost probiotics: proposal of paraprobiotic concept)

Valentina Taverniti · Simone Guglielmetti

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Abstract The probiotic approach represents a potentially effective and mild alternative strategy for the prevention and treatment of either inflammatory or allergic diseases. Several studies have shown that different bacterial strains can exert their probiotic abilities by influencing the host's immune system, thereby modulating immune responses. However, the emerging concern regarding safety problems arising from the extensive use of live microbial cells is enhancing the interest in non-viable microorganisms or microbial cell extracts, as they could eliminate shelf-life problems and reduce the risks of microbial translocation and infection. The purpose of this review is to provide an overview of the scientific literature concerning studies in which dead microbial cells or crude microbial cell fractions have been used as health-promoting agents. Particular attention will be given to the modulation of host immune responses. Possible mechanisms determining the effect on the immune system will also be discussed. Finally, in the light of the FAO/WHO definition of probiotics, indicating that the word 'probiotic' should be restricted to products that contain live microorganisms, and considering the scientific evidence indicating that inactivated microbes can positively affect human health, we propose the new term 'paraprobiotic' to indicate the use of inactivated microbial cells or cell fractions to confer a health benefit to the consumer.

Keywords Paraprobiotic · Probiotic · Immunomodulation · *Lactobacillus* · *Bifidobacterium*

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Introduction

A basic Google Internet search (executed on 21 January 2011) yielded approximately 6 million results for the word 'probiotic'. About 7500 scientific references were listed for the same word by PubMed, more than 20% of which appeared in 2010. These simple data indicate the growing interest in the field of probiotic microorganisms and products, which supports a global market that generated $15.9 billion in 2008 and is expected to be worth $19.6 billion in 2013 (BCC Research 2008).

The majority of the scientific reports define probiotics according to the definition recommended by an FAO/WHO workshop conducted in 2002, which describes probiotics as 'live microorganisms which when administered in adequate amounts confer a health benefit on the host' (FAO/WHO 2002). This definition specifies that probiotic microorganisms must be 'live', and this stipulation is supported by an extensive number of studies suggesting that to provide health benefits, probiotic microorganisms must be viable (Gobbetti et al. 2010). Nevertheless, scientific evidence indicating that inactivated microbes positively affect human health can also be found in the literature (Kataria et al. 2009). Accordingly, products intentionally containing non-viable microbial cells are already present in the market (e.g. *Lactéol Fort* from PUMC Pharmaceutical Co., Ltd and *Fermenti Lattici Tindalizzati* from Frau, AF United S.p.a.).

The mechanisms underlying probiotic effects are generally attributed to the interaction of probiotics with other microorganisms (members of the microbiota or pathogens) or to the cross-talk of probiotics with host cells. The former type of interaction is typically (though not exclusively) dependent on the viability of probiotic cells, since it is exerted by competitive exclusion (competition for nutrients

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In Vitro Functional and Immunomodulatory Properties of the *Lactobacillus helveticus* MIMLh5-*Streptococcus salivarius* ST3 Association That Are Relevant to the Development of a Pharyngeal Probiotic Product

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The use of proper bacterial strains as probiotics for the pharyngeal mucosa is a potential prophylactic strategy for upper respiratory tract infections. In this context, we characterized *in vitro* the functional and immunomodulatory properties of the strains *Lactobacillus helveticus* MIMLh5 and *Streptococcus salivarius* ST3 that were selected during previous investigations as promising pharyngeal probiotics. In this study, we demonstrated *in vitro* that strains MIMLh5 and ST3, alone and in combination, can efficiently adhere to pharyngeal epithelial cells, antagonize *Streptococcus pyogenes*, and modulate host innate immunity by inducing potentially protective effects. In particular, we found that the strains MIMLh5 and ST3 activate U937 human macrophages by significantly inducing the expression of the proinflammatory cytokine tumor necrosis factor alpha (TNF-α). Nonetheless, the induction of the anti-inflammatory interleukin-10 (IL-10) by MIMLh5 or ST3 was never lower than that of TNF-α, suggesting that these bacteria can potentially exert a regulatory rather than a proinflammatory effect. We also found that the strains MIMLh5 and ST3 induce cyclooxygenase 2 (COX-2) expression and demonstrated that toll-like receptor 2 (TLR-2) participates in the recognition of the strains MIMLh5 and ST3 by U937 cells. Finally, we observed that these microorganisms grow efficiently when cocultured in milk, suggesting that the preparation of a milk-based fermented product containing both MIMLh5 and ST3 can be a practical solution for the administration of these bacteria. In conclusion, we propose the combined use of *L. helveticus* MIMLh5 and *S. salivarius* ST3 for the preparation of novel products that display probiotic properties for the pharyngeal mucosa.

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Health-promoting properties of *Lactobacillus helveticus*

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Lactobacillus helveticus is an important industrial thermophilic starter that is predominantly employed in the fermentation of milk for the manufacture of several cheeses. In addition to its technological importance, a growing body of scientific evidence shows that strains belonging to the *L. helveticus* species have health-promoting properties. In this review, we synthesize the results of numerous primary literature papers concerning the ability of *L. helveticus* strains to positively influence human health. Several *in vitro* studies showed that *L. helveticus* possesses many common probiotic properties, such as the ability to survive gastrointestinal transit, adhere to epithelial cells, and antagonize pathogens. *In vivo* studies in murine models showed that *L. helveticus* could prevent gastrointestinal infections, enhance protection against pathogens, modulate host immune responses, and affect the composition of the intestinal microbiota. Interventional studies and clinical trials have also demonstrated a number of health-promoting properties of *L. helveticus*. Finally, several studies suggested that specific enzymatic activities of *L. helveticus* could indirectly benefit the human host by enhancing the bioavailability of nutrients, removing allergens and other undesired molecules from food, and producing bioactive peptides through the digestion of food proteins. In conclusion, this review demonstrates that in light of the scientific literature presented, *L. helveticus* can be included among the bacterial species that are generally considered to be probiotic.

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S-Layer Protein Mediates the Stimulatory Effect of *Lactobacillus helveticus* MIMLh5 on Innate Immunity

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The ability to positively affect host health through the modulation of the immune response is a feature of increasing importance in measuring the probiotic potential of a bacterial strain. However, the identities of the bacterial cell components involved in cross talk with immune cells remain elusive. In this study, we characterized the dairy strain *Lactobacillus helveticus* MIMLh5 and its surface-layer protein (SlpA) using *in vitro* and *ex vivo* analyses. We found that MIMLh5 and SlpA exert anti-inflammatory effects by reducing the activation of NF-κB on the intestinal epithelial Caco-2 cell line. On the contrary, MIMLh5 and SlpA act as stimulators of the innate immune system by triggering the expression of proinflammatory factors tumor necrosis factor alpha and COX-2 in the human macrophage cell line U937 via recognition through Toll-like receptor 2. In the same experiments, SlpA protein did not affect the expression of the anti-inflammatory cytokine interleukin-10. A similar response was observed following stimulation of macrophages isolated from mouse bone marrow or the peritoneal cavity. These results suggest that SlpA plays a major role in mediating bacterial immune-stimulating activity, which could help to induce the host's defenses against and responses toward infections. This study supports the concept that the viability of bacterial cells is not always essential to exert immunomodulatory effects, thus permitting the development of safer therapies for the treatment of specific diseases according to a paraprobiotic intervention.

15th Workshop on the Development in the Italian PhD Research on Food Science Technology and Biotechnology, 15-17 September, 2010 (Portici, Italy)

The role of probiotic microorganisms and dietary compounds in the modulation of immune response: a mechanistic approach

Valentina Taverniti

The research project of this PhD thesis deals with probiotic bacteria and dietary components and their role in immune responses, especially at intestinal level. The main goal of the project is the identification of bacterial and dietary molecules determining the immunomodulating activity. To this aim, we will employ *in vitro* experimental approaches such as luciferase reporter gene systems, real time quantitative PCR and ELISA tests, to evaluate the expression of immunologic key mediators on different cell lines.

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10th Symposium on Lactic Acid Bacteria, 28 August – 1 September, 2011 (Egmond aan Zee, The Netherlands)

Lactic acid bacteria as potential probiotics for the pharyngeal mucosa

Guglielmetti S, **Taverniti V**, Minuzzo M, Arioli S, Stuknyte M, Zanoni I, Granucci F, Karp M, Mora D

The probiotic approach represents an alternative strategy in the prevention and treatment of infections in those body sites where the microbiota plays a role in the maintenance of physiological homeostasis. In this context, we evaluated in vitro the potential abilities of oral, probiotic and dairy bacteria in controlling *Streptococcus pyogenes* infections at the pharyngeal level. Initially, we analysed the bacterial adhesion on FaDu hypopharyngeal carcinoma cells and the ability to antagonize *S. pyogenes* on FaDu layer and HaCat keratinocytes. Due to their promising adhesive and antagonistic features, we studied the oral isolate *Streptococcus salivarius* ST3 and the dairy strain *Lactobacillus helveticus* MIMLh5 also through immunological in vitro experiments. We performed the quantification of several cytokines and the measurement of NF-κB activation in FaDu cells. Strain ST3 significantly increased NF-κB activation at baseline, while it had an opposite effect when FaDu cells were co-stimulated with IL-1β. In addition, ST3 biased cytokines production at baseline (reduction of IL-1β and TNFα, increase of MIP-1β and GM-CSF) and after IL-1β-induction (reduction on IL-6 and IL-8). MIMLh5 efficiently reduced the induction of IL-6, IL-8 and TNFα in a dose-dependent manner. After stimulating with IL-1β, active NF-κB was still markedly lowered. Nevertheless, we observed an increased secretion of IL-6, IFN-γ and GM-CSF under these conditions. In addition, MIMLh5 increased the GM-CSF/G-CSF ratio. This is compatible with a switch of the immune response towards a TH1 pathway, as supported by our observation that MIMLh5, once in contact with bone marrow derived dendritic cells, triggered the secretion of TNFα and IL-2. In conclusion, we propose ST3 and MIMLh5 as a potential probiotic bacteria for the human pharynx, with promising antagonistic and immunomodulatory properties.

16th Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology, 21-23 September, 2011 (Lodi, Italy)

The role of probiotic microorganisms and dietary compounds in the modulation of immune response: a mechanistic approach

Valentina Taverniti

To investigate the immunomodulatory properties of probiotic bacteria, two strains of *Lactobacillus helveticus* have been selected through a screening assay based on a NF-κB/luciferase reporter gene system in Caco-2 human intestinal epithelial cell line. The setup of a purification protocol of their Surface (S)-layer proteins allowed testing the role of these molecules in the immune response. In vitro and ex vivo experiments, respectively performed on U937cell line and murine bone marrow-derived macrophages, demonstrated the involvement of S-layer protein in a transient activation of the immune system.

The 4th Interdepartmental Twinning Symposium, 28 September- 2 October, 2011 (Tammela, Finland)

Immunomodulatory characterization of S-layer protein from the dairy probiotic strain *Lactobacillus helveticus* MIMLh5

Taverniti V, Stuknyte M, Minuzzo M, Arioli S, Mora D, Karp M, Pesu M, Guglielmetti S

This work is targeted to a mechanistic investigation on the immunomodulatory properties of the dairy bacterium *Lactobacillus helveticus* MIMLh5, a strain isolated from Grana Padano cheese natural whey starter culture, that already showed to possess probiotic abilities and to modulate the immune response at pharyngeal level. Thus we decided to test the activity of this strain at gut level, using as proper model the intestinal epithelial Caco-2 cell line, and we observed that MIMLh5 was able to efficiently decrease the activation of the transcriptional factor NF-κB. To individuate which bacterial components could be responsible for the immunological activity, attention was given to Surface (S)-layer protein, which represents up to 14% of total protein content in *L. helveticus*. We improved an extraction protocol that allowed us to obtain the protein with a high level of purity, and thus suitable for immunological experiments. When we tested in Caco-2 model the purified protein isolated from *L. helveticus* MIMLh5, we saw that it was able to decrease the activation of NF-κB, similarly to the whole bacterium, both at baseline and in presence of IL-1β. We decided to study the effects on cells of the immune system; in *in vitro* experiments on U937 cell line, we observed by quantitative Real Time-PCR (RT-qPCR) that both the bacterium and the purified protein were more prone to induce the activation of pro-inflammatory cytokines such as COX2 and TNF-α, respect to the anti-inflammatory/regulatory IL10. These results were even confirmed when we performed *ex vivo* experiments by isolating macrophages from mouse peritoneal cavity and bone marrow (BMDMs), and moreover in bone marrow-derived dendritic cells. We found confirmation of the involvement of S-layer protein in the activation of the immune system elicited by the bacterium since, when we tested the bacterial cells without S-layer (after LiCl treatment for the removal of the protein), the propensity to induce pro-inflammatory cytokines decreased, while the level of IL10 did not change significantly. Moreover, when the protein was added to LiCl-washed bacterial cells the pro-inflammatory attitude raised again. We performed also preliminary *in vivo* experiments by injecting S-layer protein in Zebrafish embryos; even in this case we observed an increased level of some pro-inflammatory cytokines (like IFN-γ) respect to control embryos. As first step to individuate the signal pathways involved in the immune response elicited by MIMLh5 and its protein, we found that Toll-like Receptor (TLR)2 is involved in the recognition of both the whole bacterium and the purified protein. Its block resulted in a less production of TNF-α and COX2. Indeed the blocking effect was more efficient in LiCl-treated cells, probably due to the absence of the protein. In conclusion from all these data it seems that S-layer protein is a key molecular determinant for the immunomodulatory properties observed in *L. helveticus* MIMLh5. The pro-inflammatory activity elicited by the whole bacterium and the purified protein, as already demonstrated for other probiotics , could be transient and thus resulting in a "positive" activation of immune system that can support human-host defence and homeostasis.

The Oxygen Club Of California - Oxidants And Antioxidants In Biology - Cell Signaling And Nutrient-Gene Interaction, 20-23 june, 2012 (Alba, Italy)

Immunomodulatory properties of Wild Blueberries (*Vaccinium Angustifolium*) in human intestinal epithelial Caco-2 Cells: preliminary data

Fracasetti D, Del Bo'C, Guglielmetti S, Riso P, **Taverniti V**, Klimis-Zacas D, Porrini M

Wild blueberries (WB) are a rich source of polyphenols, particularly anthocyanins, which may be beneficial for human health because of their antioxidant, antiinflammatory and immunomodulatory potential. This study aims to investigate the immunomodulatory potential of three WB fractions (anthocyanin, phenolic and soluble fraction) in an inflammatory state model, based on human intestinal Caco-2 cells. A reporter gene system was prepared by transfecting Caco-2 cells with the pNiFty-SEAP reporter- construct containing an engineered ELAM promoter with 5 NF-kB binding sites and an insect luciferase reporter gene. The transfected Caco-2 cells were stimulated with interleukin (IL)-1β to mimic an inflammatory state and then supplemented with 5 mg/L, 25 mg/L, 50 mg/L and 100 mg/L of the anthocyanin, phenolic and soluble fractions. Immunomodulatory activity for each fraction was monitored in real time by quantification of bioluminescence with a luminometer. The addition of anthocyanins at the concentration of 50 and 100 mg/L reduced the bioluminescence, and therefore NF-κB activation, up to 70% and 85% ($p \leq 0.05$), respectively. No dose-response was observed after supplementing Caco-2 cells with both phenolic and soluble fractions. These data suggest that only the anthocyanin fraction can modulate Caco-2 cells inflammatory state in the experimental conditions adopted. Future experiments will address supplementation of Caco-2 cells with anthocyanin standards to elucidate the compound(s) responsible for the observed immunomodulatory effect.

17th Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology, 19-21 September, 2012 (Cesena, Italy)

The role of food and probiotic microorganisms, and dietary compounds in the modulation of the immune response

Valentina Taverniti

The aim of this PhD research project was to investigate the interaction between the immune system and (i) bacterial cells and (ii) their components, and (iii) food molecules. Specifically, our experiments were designed to study by means of *in vitro* and *ex vivo* analyses the possible signalling pathways involved in the cross-talk between epithelial and immune cells, and a dairy bacterium, its purified Surface (S)-layer protein and an anthocyanin fraction extracted from wild blueberry (*Vaccinium Angustifolium*).

APPENDIX 2. INDEX OF TABLES

APPENDIX 3. INDEX OF FIGURES

Itaca

Sempre devi avere in mente Itaca -
raggiungerla sia il pensiero costante.
Soprattutto, non affrettare il viaggio;
fa che duri a lungo, per anni, e che da vecchio
metta piede sull'isola, tu, ricco
dei tesori accumulati per strada
senza aspettarti ricchezze da Itaca.
Itaca ti ha dato il bel viaggio,
senza di lei mai ti saresti messo
sulla strada: che cos'altro ti aspetti?

E se la trovi povera, non per questo Itaca ti avrà deluso.
Fatto ormai savio, con tutta la tua esperienza addosso
già tu avrai capito ciò che Itaca vuole significare.

Ithaka

Keep Ithaka always in your mind.
Arriving there is what you are destined for.
But do not hurry the journey at all.
Better if it lasts for years,
so you are old by the time you reach the island,
wealthy with all you have gained on the way,
not expecting Ithaka to make you rich.
Ithaka gave you the marvelous journey.
Without her you would not have set out.
She has nothing left to give you now.

And if you find her poor, Ithaka won't have deceived you.
Wise as you will have become, so full of experience,
you will have understood by then what Ithaka means.

(1911 - Konstantinos P. Kavafis)

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