UNIVERSITÀ DEGLI STUDI DI MILANO

FACOLTÀ DI SCIENZE AGRARIE E ALIMENTARI
Department of Food, Environmental and Nutritional Sciences

Graduate School in Molecular Sciences and Plant, Food and Environmental Biotechnology

PhD programme in Food Science, Technology and Biotechnology

XXVIII cycle

Exploring *Lactobacillus paracasei* probiosis and metabolic potential

Scientific field AGR/16

SILVIA BALZARETTI

Tutor: Prof. Simone Guglielmetti
Co-tutor: Prof. Stefania Iametti

PhD Coordinator: Prof. Maria Grazia Fortina

2014/2015

Alla mia famiglia e a William

ABSTRACT

Exploring *Lactobacillus paracasei* probiosis and metabolic potential

Probiotics have been used so far for the prevention and treatment of various medical conditions and to support general wellness: for this reason, they are currently the subject of significant microbiological and clinical research. In fact, a body of literature suggests that including probiotics in the diet can be a strategy to reduce host-related immune diseases and, in general, modulate the intestinal microbiota composition. Although the mechanisms of action of probiotics are still largely unknown, particularly at molecular level, it is well understood that they can act in different ways, through interaction with the other bacteria residing the same niche, as well as the host, at both local and systemic levels. The genus *Lactobacillus*, which has important industrial applications as fermented food starter and probiotic adjunct, is a taxonomically broad and heterogeneous group and includes the species *Lactobacillus paracasei*, which is generally associated with habitats rich in nutrients, such as dairy food and human ecosystems, like gut and vagina.

The main purpose of my PhD activity, concerned the study of two *L. paracasei* strains, named DG and LPC-S01, isolated from two different ecological niches (human gut and vagina, respectively). They are already available on the market, included in two products as food supplements, but their characterization is still incomplete, particularly for LPC-S01. In order to deepen the knowledge about these strains, the first part of the work focused on exploring *L. paracasei* DG and LPC-S01 essential characteristics to define potential probiotics, by using *L. paracasei* Shirota as reference strain. The comparative genomic analyses evidenced that strain LPC-S01, a bacterium isolated from human vagina, but plausibly having its origin in the gut, resulted having the genetic features of a niche-generalist member of its species. Similarly, strain DG exhibited the potential ability to adapt to a wide range of environmental conditions if compared with other strains of dairy origin. *In vitro* tests conventionally used to evidence probiotic properties revealed that strains LPC-S01 and DG possessed comparable ability to resist to gastro-intestinal transit, as evidenced by tolerance to bile, and to decrease NF-κB activation in Caco-2 cells, with respect to strain Shirota. Moreover, LPC-S01 displayed higher tolerance to gastric juice and higher capacity to adhere to Caco-2 epithelial cells (whereas Shirota showed inability to adhere on Caco-2-cells). The *in vitro* observations were confirmed by setting up a pilot intervention trial on healthy adult volunteers, that demonstrated that LPC-S01 and DG can transiently colonize the gastrointestinal tract of the host, persisting for at least 5 days after the end of a 7-days oral consumption (corresponding to an average of 7 evacuations).

Thanks to the comparative genomic analysis on *L. paracasei* strains DG and LPC-S01, we identified two gene clusters putatively coding for exopolysaccharides biosynthesis related enzymes. Exopolysaccharides (EPSs), apart from their industrial applications, are found to be associated with many physiological functions, although their mechanism of action has not been fully clarified yet. In collaboration with Prof. Andy Laws, University of Huddersfield (United Kingdom), the second part of the work focused on the identification of the potential EPSs matrix from both strains DG and LPC-S01, and their structural characterization. Nonetheless, in the experimental conditions tested so far, only DG resulted able to synthetize EPSs. After performing its purification, we characterized DG derived EPS repeating unit by NMR spectroscopy based approaches. DG EPS structure resulted peculiar and unique compared to those identified in other lactic acid bacteria, prompting us to investigate its immunomodulatory potential, by using it as stimulus on phagocytes. Interestingly, THP-1 macrophages were highly responsive to the EPS stimulus, particularly through the activation of COX-2 expression. Moreover, COX-2 expression was also subjected to an additive effect due to

the combination of EPS with the pro-inflammatory stimulus of lipopolysaccharides (LPS). This results strongly suggest a role of DG and its secreted polysaccharidic molecule in triggering stimulatory immune responses together with the activation of protective mechanisms of the intestinal mucosa.

Starting by previous observations on other lactic acid bacteria, particularly on *Lactococcus lactis*, which demonstrated the ability to switch from fermentation to respiratory metabolism, and thanks to the identification of the operon *cydABCD* in *L. paracasei*, the third part of the work focused on evaluating if respiration was activated also in the strains under study. The respiratory metabolism, activated by the addition of heme and menaquinone, two essential co-factors not synthetized by the cell, typically results in two main advantages for the cell: increased biomass and long-term survival. Our preliminary data, however, indicated that only one of the two phenotypes occurred in *L. paracasei*, namely a very strong robustness achieved only upon addition of both heme and menaquinone, that resulted in the maintenance of viability for long periods of storage at 4° C (>200 days). The robustness phenotype was present also when the two co-factors were added during the storage time, and not during growth. This fact, along with (i) the detection of the added heme only outside the cells, (ii) the under regulation of *cydA* evaluated by gene expression analyses, and (iii) the decrease of ATP reservoir, suggested that the phenotype observed was not a consequence of a respiratory metabolism. Our data are instead more consistent with the presence of an external electron transfer, as already described for other intestinal bacteria. A deeper investigation is required to clarify the molecular basis of *L. paracasei* acquired resistance. However, our collected evidences, i.e. the robustness phenotype, can potentially be exploited in industrial applications to ameliorate the technological performances, but also in the niche colonized by the bacterium, where it can counteract environmental stresses and exert its probiotic potential by reducing and neutralizing damaging chemicals in the surrounding environment.

In conclusion, this PhD work evidenced the multiple properties of two strains belonging to *L. paracasei* species to colonize the human gut (and potentially the vagina) upon oral administration, and to interact with the host immune system. Moreover, *L. paracasei* possesses very interesting metabolic abilities that may be exploited both in *in vivo* conditions and in the industrial processes.

RIASSUNTO

Valutazione del potenziale probiotico e metabolico di *Lactobacillus paracasei*

I probiotici sono utilizzati attualmente per la prevenzione e il trattamento di varie condizioni mediche e per mantenere uno stato di salute generale: per questa ragione, sono tutt'ora il soggetto di molte ricerche in ambito clinico e microbiologico. Infatti, numerose pubblicazioni suggeriscono che includere probiotici nella dieta può essere una strategia per ridurre patologie legate al sistema immunitario dell'ospite e, in generale, modulare la composizione del microbiota intestinale. Sebbene i meccanismi di azione dei probiotici siano ancora largamente sconosciuti, soprattutto a livello molecolare, è assodato che essi possano agire in diversi modi, attraverso l'interazione con gli altri batteri residenti nella stessa nicchia ecologica ma anche con l'ospite stesso, sia a livello locale sia sistemico. Il genere *Lactobacillus*, il quale possiede importanti applicazioni industriali come starter alimentare e integratore probiotico, è un ampio ed eterogeneo gruppo dal punto di vista tassonomico ed include la specie *Lactobacillus paracasei*, la quale è generalmente associata ad habitat ricchi in nutrienti, come alimenti caseari ed ecosistemi umani, come l'intestino e la vagina.
Lo scopo principale della mia attività di dottorato ha riguardato lo studio di due ceppi di *L. paracasei*, denominati DG e LPC-S01, isolati da due nicchie ecologiche diverse (intestino e vagina umani, rispettivamente). Essi sono già disponibili sul mercato, inclusi in due prodotti come integratori alimentari, ma la loro caratterizzazione risulta ancora incompleta, particolarmente nel caso di LPC-S01. Al fine di approfondirne la conoscenza, la prima parte del lavoro si è focalizzata su esplorare le caratteristiche di *L. paracasei* DG e LPC-S01 essenziali per poterli definire come potenziali probiotici, utilizzando *L. paracasei* Shirota come ceppo di riferimento. Le analisi di comparazione genomica hanno evidenziato che il ceppo LPC-S01, un batterio isolato da vagina umana, ma plausibilmente avente origine intestinale, risulta avere le caratteristiche genetiche di un membro nicchia-generalista della sua specie. In modo simile, anche DG ha dimostrato la potenziale capacità di adattarsi ad un ampio range di condizioni ambientali se comparato ad altri ceppi di origine casearia. I test *in vitro* convenzionalmente usati per evidenziare proprietà probiotiche ha rivelato che i ceppi LPC-S01 e DG possiedono capacità di resistere al tratto gastrointestinale, come evidenziato dalla tolleranza alla bile, e di diminuire l'attivazione di NF-κB in cellule Caco-2, paragonabili al ceppo di riferimento Shirota. Inoltre, LPC-S01 mostrava più alta tolleranza al succo gastrico e più elevata capacità adesiva sul modello epiteliale intestinale Caco-2 (mentre Shirota ha dimostrato di non possedere quest'ultima capacità). Le osservazioni *in vitro* sono poi state confermate dalla messa a punto di uno studio di intervento pilota su volontari adulti sani, il quale ha dimostrato che LPC-S01 e DG sono in grado di colonizzare in modo transiente l'intestino dell'ospite, persistendo per almeno 5 giorni dopo la fine dell'assunzione orale del prodotto per 7 giorni (e corrispondente ad una media di evacuazioni pari a 7).
Grazie alle analisi comparative sui genomi di *L. paracasei* DG e LPC-S01, abbiamo identificato due cluster genetici che putativamente codificano enzimi correlati alla biosintesi degli esopolisaccaridi. Gli esopolisaccaridi (EPS), oltre all'applicabilità industriale, sono stati associati a molte funzioni fisiologiche, anche se non è stato ancora chiarito il loro meccanismo di azione. In collaborazione con il Prof. Andy Laws, University of Huddersfield (Regno Unito), la seconda parte del lavoro è stata incentrata sull'identificazione della potenziale matrice esopolisaccaridica prodotta da DG e LPC-S01 e sulla loro caratterizzazione strutturale. Tuttavia, nelle condizioni sperimentali testate ad oggi, solo il ceppo DG è risultato capace di sintetizzare EPS. In seguito alla purificazione, abbiamo caratterizzato l'unità ripetuta dell'EPS

di DG mediante approcci di spettroscopia NMR. La struttura dell'EPS di DG è risultata peculiare e unica quando comparata con quelle identificate in altri batteri lattici, inducendoci ad investigare le sue proprietà immunomodulatorie, utilizzandolo come stimolo su modelli di fagociti. È stato interessante verificare che i macrofagi THP-1 erano molto responsivi allo stimolo di EPS, particolarmente attraverso l'attivazione dell'espressione di COX-2. Inoltre, l'espressione di COX-2 era anche soggetta ad un effetto additivo dovuto alla combinazione di EPS con lo stimolo pro-infiammatorio del lipopolisaccaride (LPS). Questi risultati suggeriscono fortemente un ruolo di DG e della sua molecola secreta nell'innescare risposte immunitarie di tipo stimolatorio oltre all'attivazione di meccanismi di protezione della mucosa intestinale.
Partendo da osservazioni precedenti su altri batteri lattici, particolarmente su *Lactococcus lactis*, che dimostravano la capacità di passare da un metabolismo fermentativo ad uno respiratorio, e grazie all'identificazione dell'operone *cydABCD* in *L. paracasei*, la terza parte del lavoro si è focalizzata su valutare se la respirazione venisse attivata anche nei ceppi allo studio. Il metabolismo di tipo respiratorio, attivato dall'aggiunta di eme e menachinone, due cofattori essenziali non sintetizzati dalla cellula, risulta tipicamente in due principali fenotipi: formazione di biomassa e sopravvivenza a lungo termine aumentati. Dati preliminari, tuttavia, hanno indicato che uno solo dei due fenotipi si verificava in *L. paracasei*, ossia una elevata resistenza ottenuta solamente in seguito ad aggiunta di entrambi eme e menachinone, la quale risultava nel mantenimento di vitalità per lunghi periodi di conservazione a 4° C (>200 giorni). Il fenotipo di resistenza era presente anche quando i due cofattori venivano aggiunti durante il periodo di conservazione, e non durante la crescita. Questo fatto, oltre a (i) la rilevazione del gruppo eme aggiunto solo all'esterno delle cellule, (ii) l'inibizione dell'espressione di *cydA*, (iii) la diminuzione del reservoir di ATP, suggerivano che il fenotipo osservato non era conseguenza di un metabolismo respiratorio. I nostri dati sono invece più coerenti con la presenza di un trasporto di elettroni esterno, come già descritto per altri batteri intestinali. È richiesta un'investigazione più approfondita per chiarire le basi molecolari dell'acquisita resistenza di *L. paracasei*. Tuttavia, le prove raccolte, i.e. il fenotipo di resistenza, può essere potenzialmente sfruttato in applicazioni industriali per migliorare le prestazioni tecnologiche, ma anche nella nicchia colonizzata dalla batterio, dove può essere in grado di controbilanciare gli stress ambientali e eseguire il suo potenziale probiotico mediante la riduzione e neutralizzazione di molecole dannose nell'ambiente circostante.
In conclusione, questo lavoro di dottorato ha evidenziato le molteplici proprietà di due ceppi di *L. paracasei* di colonizzare l'intestino umano (e potenzialmente la vagina) in seguito a somministrazione orale, e ad interagire con il sistema immunitario dell'ospite. Inoltre, *L. paracasei* possiede abilità metaboliche molto interessanti che si posso sfruttare sia in condizioni *in vivo* sia in processi industriali.

PREFACE

The activity of many microorganisms is necessarily connected to the survival of the multicellular eukaryotic organism in which they reside. During a common evolution, the host-microbe relationship has, indeed, far exceeded the limits of simple commensalism, evolving into deep symbiosis (Rosenberg and Zilber-Rosenberg, 2011). For instance, a constantly increasing number of scientific reports is demonstrating that microbes associated to the human body carry out activities such as vitamin production, competitive exclusion of pathogens, regulation of intestinal motility, energy harvest from indigestible components of host diet, and modulation of host immune system (Frick and Autenrieth, 2013). Most of these activities are exerted by the trillions of microorganisms colonizing the intestinal tract, collectively known as the gut microbiota. Skin, oral, and vaginal microbiota are of key importance as well in maintaining the functionality of the corresponding host's body site (Ma, 2012; Hanski, 2012; Guglielmetti, 2010; Borges, 2014).

In particular, the modulation of host immune responses is the primary means by which commensal, food, or probiotic microorganisms are believed to exert their bioactivities (Adams, 2010; Collier-Hyams and Neish, 2005). In spite of this, the mechanisms determining the microbial ability to interact with the immune system of mammals are largely unexplored, particularly in the case of non-pathogen microorganisms such as commensal, food-associated and probiotic microbes. In this context, the study of host-microbe interactions can supply an essential fund of knowledge in order (i) to understand pathologies and dis-functional syndromes (for example ulcerative colitis, irritable bowel syndrome, atopic eczema, recurrent vaginal candidiasis) and (ii) to develop targeted treatment and prevention strategies, such as the probiotic intervention, which is intended - in its broadest sense - as the use of selected live microbial cells to benefit the host's health.

The interest on probiotic microorganisms has greatly increased during the past decade, because of the widespread belief in benefits of the probiotic approach towards disease prevention or management. Accordingly, sales of probiotic dietary supplements and foods are growing. The microorganisms most commonly employed as probiotics belong to the genus *Lactobacillus*. More specifically, species like *L. acidophilus*, *L. rhamnosus* and *L. paracasei* include the most important probiotics worldwide. For instance, the first probiotic product in history (the Yakult fermented milk) contains *L. paracasei* strain Shirota. The most studied probiotic nowadays is *L. rhamnosus* strain GG, whereas the most popular probiotic sold in Italy is *L. paracasei* DG, a strain included in the food supplement Enterolactis (Sofar S.p.A.). Nonetheless, at present, the Italian Ministry of Health accepts only one use indication concerning probiotics ("*Favorisce l'equilibrio della flora intestinale*" i.e. "*Helps balance of the intestinal flora*"), whereas the European Community, through the Food Safety Authority (EFSA) and the regulation (EC) N° 1924/2006, has not yet approved any health claim related to probiotic products. It is unanimously accepted that the use of microorganisms as probiotics is drastically limited by the inadequate knowledge concerning the molecular mechanisms that support the potential effects of probiotic use (Rijkers, 2010). In this context, the immunologic characterization of single microbial cell components is a reductionistic approach based on scientific research, which can significantly contribute to unveil the molecular mechanisms governing the cross-talk between microorganisms and host.

Another important factor to consider when characterizing a probiotic strain, it is how its metabolism can influence all the industrial processes to which the bacterium will be subjected, as well as the quality and shelf-life of the final product to be released on the market. Indeed, the food ingredient companies are constantly putting efforts in order to improve the quality and produce new innovative products, as well as increase the efficiency in their factories (Pedersen, 2005; Gregoret, 2013). Many data are now available on *Lactococcus lactis* and its improvement

of growth efficiency and resistance due to the establishment of a second type of metabolism (apart from the classic fermentation) that is respiratory-like (Pedersen, 2012; Duwat, 2001). This capacity can be exploited at laboratory level, but the industrial scale production has already been confirmed for the above mentioned *L. lactis*, and this evidence leads to interesting perspectives also for other genera of bacteria used as probiotics, such as lactobacilli.

Considered all the previous observations concerning probiotic bacteria and their components, the following topics have been investigated during the PhD research activity:

1. Characterization of two *L. paracasei* strains, namely DG and LPC-S01 of gut and vaginal origin, respectively, for their use as probiotics by means of a comparative approach using *L. paracasei* Shirota as reference strain. In order to unravel their properties, the selected bacteria have been analysed through *in silico*, *in vitro* and *in vivo* techniques. Particularly, a draft genome of DG and LPC-S01 was generated and a comparative analysis was performed against other *L. paracasei* reference strains. *In vitro* methods conventionally employed for the assessment of the probiotic potential of microbial cells were included (resistance to acidity and bile salts, adhesion to Caco-2 human epithelial cell line, inhibition of several microbial pathogens, production of bacteriocins, antibiotic resistances; Guglielmetti, 2008). Finally, a double-blind cross-over pilot intervention trial was carried out in order to assess the ability of LPC-S01 and DG to transiently persist in the gastrointestinal tract of healthy adults.

2. Investigation of *L. paracasei* DG and LPC-S01 potential capacity to produce exopolysaccharides. The extraction and purification protocol allowed to obtain a clean exopolysaccharides fraction, which was further employed to identify the repeating unit composing the polymer by means of NMR spectrometry and other analytical chromatographic methods. The purified molecule was then used to assess its immunomodulatory potential on a transfected Caco-2 cell line for the measurement of NF-κB activation by means of bioluminescence quantification, and on two models of human macrophages (U937 and THP-1).

3. Evaluation of the metabolic potential of *L. paracasei* as respiratory species, as hypothesized by Pedersen (2012) after identification of the *cydABCD* operon (coding for a terminal cytochrome oxidase), and on the basis of previous works conducted on *L. lactis* and *Lactobacillus plantarum* (Arioli, 2012; Brooijmans, 2009a; Brooijmans 2009b; Pedersen, 2008). To assess the respiratory potential, classic microbiology methods (biomass formation, final pH, and long-term resistance evaluation) along with several molecular approaches were performed in presence of the essential co-factors, i.e. heme and menaquinone.

REFERENCES

- Adams CA, 2010, The probiotic paradox: live and dead cells are biological response modifiers. Nutr Res Rev. 23:37-46.
- Arioli S et al., 2012, Increasing the heme-dependent respiratory efficiency of *Lactococcus lactis* by inhibition of lactate dehydrogenase. Appl Environ Microbiol. 79:376-80.
- Borges S et al., 2014, The role of lactobacilli and probiotics in maintaining vaginal health. Arch Gynecol Obstet. 289:479–489.
- Brooijmans RJ et al., 2009a, Heme and menaquinone induced electron transport in lactic acid bacteria. Microb Cell Fact. 8:28.
- Brooijmans RJ et al., 2009b, *Lactobacillus plantarum* WCFS1 electron transport chains. Appl Environ Microbiol. 75:3580-3585.
- Collier-Hyams LS and Neish AS, 2005, Innate immune relationship between commensal flora and the mammalian intestinal epithelium. Cell Mol Life Sci. 62:1339-48.
- Duwat P et al., 2001, Respiration capacity of the fermenting bacterium *Lactococcus lactis* and its positive effects on growth and survival. J Bacteriol. 183:4509-16.
- Frick JS and Autenrieth IB, 2013, The gut microflora and its variety of roles in health and disease. Curr Top Microbiol Immunol. 358:273-89.
- Gregoret V et al., 2013, A comprehensive approach to determine the probiotic potential of human-derived *Lactobacillus* for industrial use. Food Microbiol. 34:19-28.
- Guglielmetti S et al., 2008, Implication of an outer surface lipoprotein in adhesion of *Bifidobacterium bifidum* to Caco-2 cells. Appl Environ Microb. 74: 4695–4702.
- Guglielmetti S et al., 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. 78:4734-43.
- Hanski I et al., 2012, Environmental biodiversity, human microbiota, and allergy are interrelated. Proc Natl Acad Sci USA. 109:8334-9.
- Ma B, 2012, Vaginal microbiome: rethinking health and disease. Annu Rev Microbiol. 66:371-89.
- Pedersen MB et al., 2005, The long and winding road from the research laboratory to industrial applications of lactic acid bacteria. FEMS Microbiol Rev. 29:611-24.
- Pedersen MB et al., 2012, Aerobic respiration metabolism in lactic acid bacteria and uses in biotechnology. Annu Rev Food Sci Technol. 3:37-58.
- Rijkers GT et al., 2010, Guidance for substantiating the evidence for beneficial effects of probiotics: current status and recommendations for future research. J Nutr. 140:671S-6S.
- Rosenberg E and Zilber-Rosenberg I, 2011, Symbiosis and development: the hologenome concept. Birth Defects Res C Embryo Today. 93:56-66.

1. CHARACTERIZATION OF TWO STRAINS OF *LACTOBACILLUS PARACASEI* FOR THEIR USE AS PROBIOTICS.

1.1 STATE OF THE ART

1.1.1. The gastrointestinal tract and the gut microbiota

The gastrointestinal tract (GIT) represents an ecosystem of the highest complexity. The mucosal surface provides a large area for the microbial adherence and colonization in the small intestine, which corresponds to 150–200 m^2 area (Waldeck, 1990). A three-fold increase in the surface area is accomplished by circular folds, 7–10-fold by folding of the epithelium (intestinal villi) and 15–40-fold by the formation of microvilli in the enterocyte absorptive luminal membrane. Thereby, the necessary space for interactions between host and microorganisms during the digestive process and for microbial adhesion to the mucosal wall and concomitant colonisation is provided (Holzapfel, 2002).
The GIT is a sterile environment at birth, and bacterial colonization begins during the delivery process (from the maternal faecal or vaginal flora and/or the environment). The bacteria that colonize the large gut initially are facultative anaerobic strains such as *Escherichia coli* and *Streptococcus* spp. These first colonizers metabolize any trace of oxygen in the gut, thereby reducing the environment into one with strong anaerobic conditions. The subsequent colonizing bacteria are largely determined by the feeding profile of the infant during the nursing and the weaning, when a complex microflora develops (Wallace, 2011).
The human intestinal ecosystem acts as an "organ" synergistically with the host, which houses over 10^{14} microbial cells (which comprise bacteria, archaea, some protozoa, and anaerobic fungi) and different bacteriophages and viruses. The presence of a great number of microbial species (more than 1000) represents an immense metabolic potential and suggests strong regulatory effects on the human host. The diversity of the gut microbiota varies from segment to segment and, in addition, is determined by factors such as the diet, the genetic background and the physiological state of the host. A beneficial stable microbiota, found in the healthy host, is instead subject to dramatic changes as a consequence of pathological mechanisms: for example, patients with inflammatory bowel disease (IBD), either Crohn's disease (CD) or ulcerative colitis (UC), exhibit reduced microbial diversity and disproportionate quantities of gram-negative bacteria when compared to healthy subjects (Qin, 2010).
The composition and function of human microbial populations associated with various body sites have been studied with the help of metagenomic tools as part of two recent initiatives – the NIH Human Microbiome Project (HMP) and the European Metagenomics of the Human Intestine (metaHIT) project (Peterson, 2009; Dusko Ehrlich and MetaHIT Consortium, 2011). These massive molecular approaches have already revealed the presence of three different clusters, or enterotypes, which correspond to one of three most abundant genera of human intestine: *Bacteroides*, *Prevotella*, and *Ruminococcus* (Arumugam, 2013; Varankovich, 2015).
The following may be considered as the major physiological functions of the gut and its microbiota including the health-promoting effects.

Barrier function and restoration. The gut mucosa provides the first physical line of defense to prevent the passage of harmful intraluminal entities including foreign antigens, microorganisms and their toxins (Groschwitz and Hogan, 2009). The single cell layer that forms the physical epithelial barrier includes absorptive and secretory cells, goblet cells and Paneth cells. Goblet cells contribute to the formation of a protective mucus layer, whereas Paneth cells are located near the base of small intestinal crypts and secrete potent antimicrobial peptides, known as defensins, and antibodies (Figure 1.1; Cho, 2008). The regulation of barrier integrity and function is mediated by endogenous and exogenous factors, such as cytokines, chemicals and drugs. Pathogens and their toxins also have the ability to directly or indirectly modulate the intestinal barrier, as well as commensal bacteria. These latter have been proposed as key

modulators of intestinal barrier function (Natividad and Verdù, 2013). For instance, a recent study has indicated that the probiotic bacterium *Lactobacillus rhamnosus* GG promotes cell renewal and augments mucosal repair following dextran sodium sulfate (DSS) induced colitis via reactive oxygen species generation in epithelial cells (Swanson, 2011).

Immune system stimulation. The immune system is immature at birth and develops, inducing the gut-associated lymphoid system (GALT) in the lamina propria and beyond, in the mesenteric lymph nodes, establishing immune tolerance, upon exposure to the intestinal lumen microbiota. Gut microbiota indeed stimulates the innate and the acquired immunity but, on the other hand, the key challenge of the host intestinal immune system is balancing the need to respond to pathogens (through humoral and cell-mediated immune mechanisms) while coexisting with commensal bacteria and food antigens (through a mechanism of immune tolerance/hypo-responsiveness) (Hardy, 2013). In normal conditions, the stimulation of the mucosal immune system by gut microbiota determines a state of "low-grade physiological inflammation", a status of continuous activation in response to commensals and, if needed, to pathogens. Mucosal homeostasis (tolerance/suppression versus activation) requires a continuous balance between pro- and anti-inflammatory components (Rakoff-Nahoum, 2004; Scaldaferri, 2013).
Being considered as the largest "immune organ" in the human body, the gut architecture comprises three different types of antigen-presenting cells (APCs): epithelial cells, specialized epithelial cells (microfold M cells) and dendritic cells (DCs) (Fig.1.1). Epithelial cells or enterocytes, at the apical surface, linked by tight junctions, prevent the penetration of microbial pathogens but can also facilitate vesicular bacterial/antigenic transfer across the barrier by receptor-mediated pinocytosis. The antigenic material is then processed and presented in form of a major histocompatibility complex molecule (MHC) expressed on the basolateral membrane, with both chemokines and cytokines that modulate underlying dendritic cells and macrophage responses (Collier-Hyams and Neish, 2005).
Microfold (M) cells are inserted in the epithelial monolayer above areas of follicular lymphoid tissue, namely Peyer's patches, where they form a gateway, trans-cytosing microorganisms and allowing controlled access to a range of immune cell types. They can induce the expansion and activation of follicular lymphocytes but, unlike enterocytes, they do not secrete anti-microbial components or present antigens but, instead, shuttle macromolecules and microorganisms to other effector cells such as DCs and macrophages present in the M cell pocket (Jang, 2004).
Pathogenic bacteria or disruptions of the epithelial barrier activate DCs through interaction with their arm-like extensions (dendrites) present between the tight junctions that allow the independent sampling of the luminal contents (Chieppa, 2006; Rescigno, 2001). Upon sampling, DCs are primed to activate T cells towards a Th1/Th2 polarization. In particular, the cytokine milieu skews the differentiation of naive CD4+ T cells into T helper Th1-, Th2-, Th17- or regulatory T-cell subsets (Cho, 2008).

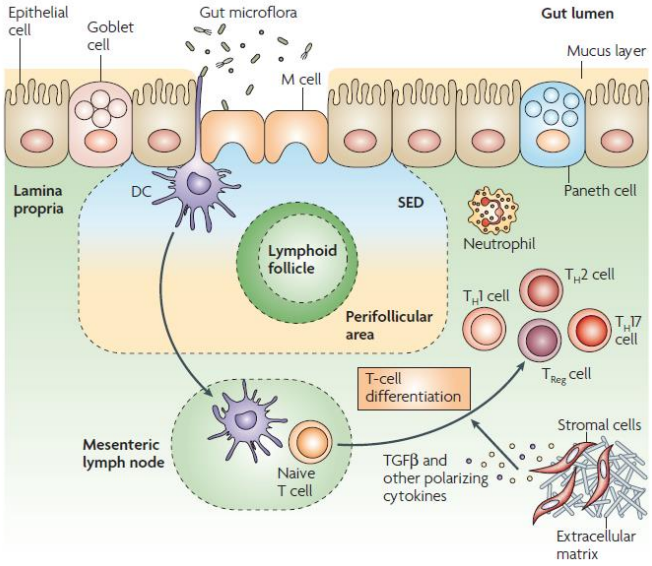
Production of nutrients and improvement of their bioavailability. Several members of the intestinal microbiota produce vitamins and minerals and provide them to the host. For instance, it has been observed that germ-free animals require 30% more energy in their diet, and supplementation with vitamins K and B is mandatory to maintain their body weight (Lee and Hase, 2014). Moreover, the gut microbiota possesses glycosidases able to hydrolyse the linkages of oligo- and polysaccharides non-digested or incompletely digested by the intestinal enzymes. In this way, the resulting monosaccharides are available to be metabolized (Perez Chaia and Oliver, 2003).

Impact on both weight and mood. Gut microbiota regulates several host metabolic functions, and microbial dysbiosis is associated with altered energy homeostasis, as observed in humans and animal models of obesity. Particularly, a reduction in bacterial diversity, coupled with overall compositional shifts, such as reduced abundance of Bacteroidetes and a proportional increase in Firmicutes phylum, have been verified. Moreover, the microbiome of the obesity model was significantly better equipped with genes involved in the transport and metabolism of undigested carbohydrates (Turnbaugh, 2006; Duca, 2014).
Depression is a multi-factorial disease being caused by biological, psychological, and social factors. There has been recent major interest in the gut–brain axis in relation to depression. Three general mechanisms have been suggested to describe how the gut microbiota influences depression, namely through inflammation, the Hypothalamic–Pituitary–Adrenal axis (HPA), or interference with neurotransmitter signaling. (Dantzer, 2008; Naseribafrouei, 2014).

Production of signalling molecules. Carbohydrate fermentation leads to short-chain fatty acids (SCFAs) production, such as butyrate, which has been known to inhibit DNA synthesis and stimulate apoptosis, and to significantly improve the absorption of calcium, magnesium, and phosphorus (Perez Chaia and Oliver, 2003). Moreover, several studies demonstrated that butyrate and butyrate-producer bacteria levels were strongly decreased in colon affected by ulcerative colitis and cancer (Machiels, 2014; Singh, 2014).

It can be concluded that the establishment and maintenance of a stable gut microbiota, through the appropriate exposure to commensal organisms and their prebiotic substrates, has a paramount impact on the colonization dynamics and the well-being of the host.

Fig. 1.1. Key features of the intestinal immune system (Cho, 2008).



1.1.2. The vaginal tract and its microbiota

The vaginal mucosa of reproductive aged women is comprised of a multilayered stratified squamous epithelium that rests on a lamina propria and reaches a large surface area up to 360 cm^2. It is kept moist by a fluid that is partly secreted as a plasma transudate through the vaginal wall with contributions from the cervical and vestibular glands. Like the epidermis, the human vaginal epithelium contains basal and suprabasal layers and undergoes terminal differentiation to form a cornified envelope comprised of a flattened layer of specialized cells. However, vaginal *stratum corneum* does not have intercellular tight junctions, and does not usually keratinize or form a complete lipid envelope, so it is penetrable by microorganisms and cellular and molecular mediators of the immune system (Anderson, 2014; Merk, 2015). Immune cells residing in the reproductive tract have two roles: maintain immunity against vaginal pathogens in the lower tract and establish immune tolerance for sperm and the embryo/fetus in the upper tract. The leukocytes are distributed in either an aggregated or a dispersed form in the epithelial layer, lamina propria, and stroma. Antimicrobial molecules are another important factor in mucosal immunity and are produced in the mucosa of the female reproductive tract. Their production is significantly influenced by estrogen, which acts differently in the upper and lower reproductive tracts. High levels of estrogen, a characteristic of the preovulatory period, increase the production of some antimicrobial peptides, such as secretory leukocyte peptidase inhibitor (SLPI), β-defensin 1-2 (HBD 1-2), and elafin from the endometrial epithelium (Lee, 2015). On the other hand, estrogen suppresses the LPS- and poly (I:C)-induced secretion of proinflammatory cytokines, including TNF-α, macrophage inflammatory protein 3α (MIP3α, CCL20), IL-1β, IL-6, and IL-8 from uterine epithelial cells. IgG levels in the uterine cavity peak in the periovulatory period. In contrast, the lower reproductive tract, including the cervix and the vagina, maintains a low level of IgA, IgG, and lactoferrin and decreased secretions of β-defensin 2, elafin, SLPI, and α-defensin1-3 (HNP1-3) (Lee, 2015; Pudney, 2005; Wira, 2014).

Despite the proximity of the vagina to the anus, the variety of microorganisms present in this district is much lower than in the gut. The reduced receptivity of the vagina, different nutrient availability and competition with indigenous microorganisms can be the reasons for this lower diversity in the vaginal tract. Some microbes found in the gut can also be found in the vagina, demonstrating that the proper receptors, nutrients, and oxygen tension are present for these organisms to grow (Cribby, 2012). The microbial species that inhabit the vaginal tract play an important role in preventing illnesses of the host, including bacterial vaginosis (BV), urinary tract infections (UTI), yeast vaginitis, and sexually transmitted diseases including human immunodeficiency virus (HIV) (Reid and Bocking, 2003). Historically, it was demonstrated that the vaginal microbiota was composed only from "gram positive bacilli" (Döderlein, 1892) but, nowadays, knowledge has significantly broadened and we know that it is characterized by a high number of lactobacilli (10^7–10^8 cfu/g of vaginal fluid in healthy premenopausal women). Among them, those belonging to the *Lactobacillus acidophilus* group and *Lactobacillus fermentum* are most frequently isolated, although others, such as *L. plantarum*, *L. brevis*, *L. jonsonii*, *L. casei*, *L. delbrueckii*, and *L. salivarius*, are isolated as well (Redondo-Lopez, 1990). However, other lactic acid bacteria genera have been found in the vaginal tract, such as *Pediococcus* spp., *Weisella* spp., *Streptococcus* spp. and *Leuconostoc* spp. The vaginal vault is colonized within 24 h of a female child's birth and remains colonized until death. Lactobacilli become the predominant inhabitants of the vagina at the time of puberty, while menopause is marked by a dramatic reduction in estrogen production, resulting in drying and atrophy of the vaginal epithelium. When estrogen levels drop, glycogen content in the vaginal epithelium decreases, leading to depletion of lactobacilli (Farage, 2010). The balance between a healthy and diseased state can change depending on a number of factors such as environmental

exposures, microbial interspecies competition or commensalism, hormone levels and host defenses. In general, the presence of high numbers of lactic acid bacteria (LAB) in the vagina is equated with “healthy”, whereas low numbers or absence as being “abnormal”.
The mechanisms by which LABs stabilize the vaginal microbiota are the production of antimicrobial compounds, i.e. hydrogen peroxide, lactic acid, and bacteriocin-like substances. Vaginal pH varies from 6.6 (±0.3) to 4.2 (±0.2) between day 2 and day 14 of the menstrual cycle and the maintenance of these values is achieved by means of the production of lactic acid by LABs as well as the secretion of organic acids by the vaginal epithelial cells (Charlier, 2009). Another mechanism exploited by endogenous LABs in the healthy urogenital tract, is the capability to adhere and compete for adhesion sites in the vagina thus excluding the colonization of pathogenic bacteria by occupying or masking (by steric hindrance) their potential binding sites to the mucosa (Borges, 2014).
When LABs, and particularly lactobacilli, are disrupted, they are subsequently replaced by predominatly anaerobic bacteria including *Gardnerella vaginalis*, *Mycoplasma hominis*, *Prevotella* and *Peptostreptococcus*, causing bacterial vaginosis (BV). This urogenital disease is the most common in women, affecting about 19-24% of them in reproductive age (Allsworth and Peipert, 2007; Bastani, 2012). It is associated with high pH, a decrease in antimicrobial activity of the vaginal fluid compared to healthy women, and local impairment of the innate immune pathways, but it does not exhibits inflammation, except for a slight increase in IL-1 and IL-8, which prevent the attraction of inflammatory cells (macrophages and neutrophils) (Donati, 2010). A second major abnormality of the vaginal microbiota is instead caused by the replacement of the normally present *Lactobacillus* spp. with aerobic microorganisms, and for this reason it is denominated aerobic vaginitis (AV). These bacteria are predominantly enteric commensals or pathogens: group B streptococci (GBS), *Escherichia coli*, and *Staphylococcus aureus* are the organisms most frequently associated with AV (Rampersaud, 2012).

In this context, probiotics are currently the subject of significant clinical research. A growing body of work now exists describing the role of various probiotic strains in ameliorating chronic intestinal inflammation, diarrhea, constipation, vaginitis, irritable bowel syndrome, atopic dermatitis, sepsis, food allergies, and liver disease (Saez-Lara, 2015; Parma, 2014; Didari, 2015; Baquerizo Nole, 2014; Marchesi, 2015).

1.1.3. History and use of probiotics

Throughout the history of microbiology, most human studies have focused on the disease-causing organisms found on or in people; in the last 100 years (and mainly in the last 20 years), however, many reports have centred on the benefits of the resident bacteria and, as a consequence, on probiotics (Gregoret, 2013). The term “probiotic” was initially used in the 1960s and comes from a Greek word meaning "for life". Although a relatively new word, the use of probiotics stretches back in the 1800s, when a beneficial association of “lactic acid producing” microorganisms with the human host has been suggested by Döderlein (1892) for vaginal bacteria and, more particularly, for lactic acid bacteria (LAB) in gut ecology studies conducted by Moro (1900), Beijerinck (1901), and Cahn (1901). However, scientific literature about this topic did not appear until 1905, when Elie Metchnikoff, who had worked with Pasteur in the 1860s, was credited with making the association of longevity among Caucasians, not to the yogurt they consumed, but rather to the lactobacilli used to ferment the yogurt and the presence of these lactobacilli in the colon (Metchnikoff, 1908). It was probably Vergio (1954) who first introduced the term “probiotic” and compared the detrimental effects of antibiotics and other antimicrobial substances on the gut microbial population with factors (“Probiotika”)

favourable to the gut microflora in his manuscript “Anti- und Probiotika” (Holzapfel, 2002). In the 1950s–1980s, research focused on screening potential probiotic strains from isolates in nature or from human hosts, and on defining their mechanisms of action, in order to understand the complex interactions of normal microbiota and the ability to resist pathogenic invasions (McFarland, 2015; Williams, 2010). So far, probiotics have been used for the prevention and treatment of various medical conditions and to support general wellness; among them, *Lactobacillus* and *Bifidobacterium* constitute the most frequently used genera. Some of their beneficial health effects have been validated, whereas other uses are supported by limited evidences. Illnesses associated with the gastrointestinal tract have been a common target of probiotics, mainly due to their ability to restore gut microbiota. The strongest evidence for the use of probiotics lies in the treatment of certain diarrheal diseases, especially rotaviral diarrhea in children (Pham, 2008; Vanderhoof and Young, 2008). Although clinical trial results are still conflicting, probiotic therapy may also be beneficial in the treatment of Crohn's disease, ulcerative colitis (UC), irritable bowel syndrome (IBS), and *Helicobacter pylori* infections (Santosa, 2006; Pham, 2008; Scarpellini, 2008). Other illnesses not associated with the gastrointestinal tract, including various urogenital problems, upper respiratory infections, allergic diseases, atopy, may also respond to probiotics (Pham, 2008; Scarpellini, 2008; Senok, 2005; Reid, 2003; Goldin and Gorbach, 2008; Williams, 2010).

1.1.4. Regulation and definition of probiotics

The Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) define probiotics as “Live microorganisms which when administered in adequate amounts confer a health benefit on the host” (FAO/WHO, 2002). This definition, formulated during the FAO/WHO Expert Consultation in 2002, origins from the need to clarify the role of probiotics. Indeed, recognizing the inequality of many products on the market and the lack of any regulatory guidelines, the Experts panel had the task to examine whether or not probiotics had proven benefits and if so, to establish a set of guidelines that would ensure product safety and reliability. The resultant report not only established that there were indeed excellent data on probiotics, but also developed a set of Guidelines or Operating Standards. These guidelines, which have been received favourably by Codex Alimentarius, constitute a set of parameters required in order to term a product or strain as “probiotic” (Reid, 2005). In Europe, the European Food Safety Authority (EFSA) has proposed a frame-work for the safety evaluation of micro-organisms in the food chain, similar in purpose to the generally recognized as safe (GRAS) approach, and taking into account the experience of use (Qualified Presumption of Safety, QPS, concept) (EFSA, 2012; Vankerckhoven, 2008).
According to the Guidelines proposed by FAO/WHO and EFSA mentioned above, the criteria of selection can be summarized in evaluation steps. The first step in the selection of an organism or product being referred to as a “probiotic” is to identify and characterize the organism to genus and species level using internationally accepted methodologies (Vankerckhoven, 2008). Moreover, each organism must be designated with its strain name. This is important in order to differentiate reliable and proven strains and products from those of dubious quality and safety. This clear identification will ensure that consumers know exactly what strains are in the food products (Reid, 2005).
Secondly, an extensive characterization performed under *in vitro* conditions is necessary in order to gain knowledge about the selected strains and their mechanisms of action (FAO/WHO, 2002). A list of the main *in vitro* tests for the study of probiosis is shown in Table 1.1.

Tab. 1.1. Main currently used *in vitro* tests for the study of probiotic strains ("Guideline for the Evaluation of Probiotics in Food", FAO/WHO, 2002).

Resistance to gastric acidity
Bile acid resistance
Adherence to mucus and/or human epithelial cells and cell lines
Antimicrobial activity against potentially pathogenic bacteria
Ability to reduce pathogen adhesion to surfaces
Bile salt hydrolase activity
Ability to modulate immune responses

All of these data are valuable and can provide scientific insight in the characteristics of probiotic organisms, but validation with *in vivo* trials (at least using an animal model) are strongly suggested by the FAO/WHO, to prove that the probiotic confers a significant improvement in health. These assays generally include evaluations of the capacity to stimulate the host immune system, as well as to prevent enteric infections or other gut-associated pathologies (Gregoret, 2013).
Given their long history of consumption in traditional fermented foods and their residency in the gastrointestinal and genitourinary tracts of vertebrates, the most common microorganisms used as probiotic (i.e. lactobacilli and bifidobacteria) have been awarded the status of GRAS by the American Food and Drug Agency. However, a probiotic strain can only be utilized if it fulfills certain criteria related to safety aspects (Adams and Marteau, 1995; Ammor, 2007) and, specifically, it should be tested using laboratory and human studies to confirm that the product under study is not responsible for a number of side-effects: (i) systemic infections, (ii) antibiotic resistances, (iii) deleterious metabolic activities (biogenic amines or toxins production, haemolytic activity, bile salt deconjugation), (iv) excessive immune stimulation in susceptible individuals, and (v) genetic transfer (Marteau, 2002).
Among technological criteria, strain viability and maintenance of desirable characteristics during product manufacture and storage is a requirement for probiotic strains to assure a beneficial effect on the consumer (Champagne, 2011).
European Union (EU) consumers have used probiotic foods for decades, but with the implementation of EU legislation on health claims starting in 2009, no specific health claims for probiotic foods have been approved by EFSA, which is responsible for reviewing health claim substantiation in the EU. As a result, probiotic food labels cannot communicate any health benefits to consumers in the EU (Glanville, 2015).

1.1.5. Mechanism of action of probiotics

Many rigorous studies have suggested that emerging nutritional strategies including probiotics diets may be able to reduce these host-related immune diseases and modulate the intestinal microbiota. Although the mechanisms of action of probiotics are largely unknown at molecular level, a probiotic can act in a number of ways, that can be summarized as follows (Fig. 1.2).
A first level of interaction is direct and takes place within the gut lumen between the probiotic strain and the microbiota. The mucosal epithelia provide an enormous surface area for invading pathogens, but its integrity can be enhanced by probiotics through competition for adhesive access to epithelial cells. Moreover, this transient intimate association allows exchanging mediators between the bacterium and the host immune system. It has been considered that multiple factors (either unspecific or specific) of the bacterial cell surface are responsible for the adhesion capacity: electrostatic forces (such as divalent cations, probably Ca^{2+}), biosurfactants

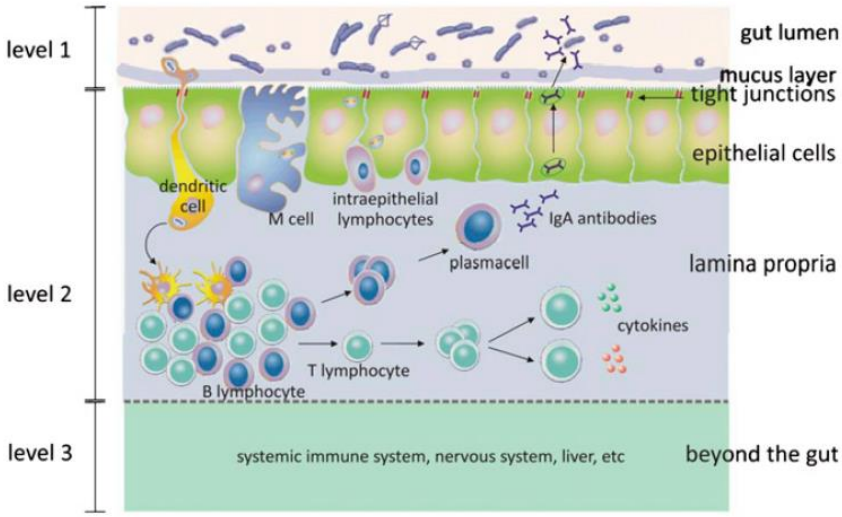
(such as glycolipids, lipopeptides, protein-like substances, phospholipids, substituted fatty acids, and lipopolysaccharides), glycoproteins, carbohydrates (Boris, 1998; Ren, 2014; Merk, 2005).
There are also many other mechanisms underlying effects of probiotics and they are likely to be multifactorial: reduction of luminal pH, competition for nutritional sources, and the production of organic acids, hydrogen peroxide, bacteriocin or bacteriocin-like substances (de LeBlanc Ade, 2010; Chenoll, 2011; Collado, 2007; Hardy, 2013). Bacteriocins are antimicrobial peptides, with a variety of killing mechanisms, including cytoplasmic membrane pore formation, interference with cellular enzymatic reactions (such as cell wall synthesis) and nuclease activity. Nisin (a lantibiotic produced by certain strains of *Lactococcus lactis*) is the only bacteriocin that has been approved by the World Health Organization for use as a food preservative (Gillor, 2005; Borges, 2014). A few bacteriocins from vaginal isolates of *Lactobacillus* spp. have also been identified, such as salivaricin produced by the vaginal bacterium *L. salivarius* CRL 1328, that exhibited activity against *E. faecalis* by dissipating membrane potential and transmembrane proton gradient (Vera Pingitore, 2009). Acid production has long been known to be detrimental to some microorganisms in the gut as well as the vagina, not only inhibiting viruses such as HIV, rotavirus and even influenza virus, but also displacing some pathogens from surfaces (Reid, 2006).

A second level concerns the interaction with the gut mucus and the epithelium, and includes barrier effects, digestive processes, mucosal immune system, and enteric nervous system.
The mucus, composed of mucins secreted by goblet cells and polymerized, grants protection from pathogens, enzymes, toxins, dehydration and abrasion: it has been demonstrated that *Lactobacillus plantarum* 299v and *Lactobacillus rhamnosus* GG are able to up-regulate the production of intestinal mucins (MUC2 and MUC3) which overturn the adherence of the enteropathogenic bacterium *Escherichia coli* O157:H7 to intestinal epithelial cells (Mack, 1999; Caballero-Franco, 2007). Other studies have shown that *S. thermophilus* and *L. acidophilus* enhance barrier integrity by phosphorylation of actinin and occludin in the tight junctions region of epithelial cells, thereby inhibiting the invasion of pathogens into human intestinal epithelial cell lines (Resta-Lenert, 2003). Similarly, probiotic bacterial cell wall products such as peptidoglycan and lipoteichoic acids have been shown to augment apical tightening and sealing of tight junctions and the Th2-type cytokine release by activation of the pattern recognition receptor TLR-2 (Cario, 2004). Other TLRs involved in recognition of bacterial components are TLR-4 and TLR-9, which are activated by lipopolysaccharides of Gram-negative bacteria and CpG DNA sequences, respectively (Gomez-Llorente, 2010; Zhong, 2012). TLR-4 activation by LPS activates Th1-type cytokine secretion (Cario, 2007), which, in IBD, is upregulated and contributes to sensitizing the mucosa to the proinflammatory effects of bacterial endotoxins. Through TLRs and via the release of soluble mediators, probiotics can also modulate the nuclear factor-kappa B (NF-κB) signal transduction pathway in epithelial cells, resulting in inhibition of NF-κB translocation to the nucleus and reduction in the degradation of IκB kinase through modulation of proteasome function (Petrof, 2004).
Since TLRs contribute to establish the level of mucosal responsiveness to intestinal microbiota-derived products and to regulate the amplitudes of the inflammatory responses, it is likely that probiotic immunomodulatory effects depend, at least partially, on TLR modulation. This has been evidenced by D'Incà (2011), who observed reduced TLR-4 levels in patients with ulcerative colitis (UC) after rectal administration of *L. paracasei* DG. Simultaneously, in the pro-inflammatory cytokines TNF-α and IL-1β were reduced and the anti-inflammatory cytokine IL-10 was increased. This action, orchestrated by a probiotic strain, may make the mucosa more sensitive to commensal non-pathogenic Gram-positive bacteria, recognized by TLR-2 receptors, which are then able to induce protective responses.

Substantial evidence has shown that probiotics can modulate mucosal immune function, in a strain-dependent fashion, also by the control of antibodies pool, as seen for the probiotic strains *L. rhamnosus* GG, *Bifidobacterium animalis* subsp. *lactis* Bb12 and *Saccharomyces boulardii*, which can enhance production and secretion of protease-resistant IgA through alteration of the cytokine milieu in the gut mucosa, after induction of B-cells maturation (Shang, 2008). Other mechanisms include the increase of phagocyte and natural killer cell activity, and the induction of regulatory dendritic cells and CD4+Foxp3+T cells responses (Paineau, 2008; de LeBlanc Ade, 2010; Kwon, 2010).

Signalling to the host beyond the gut to the liver, systemic immune system, and other potential organs such as the brain, is considered the third level of interaction. Physiological studies on the role of the intestinal microbiota in maintaining normal GI function, are now supported by molecular work, indicating that indeed, colonization with common commensals can modify the expression of a variety of genes involved not only in immunity and barrier function, but in also in motility and neurotransmission. Verdù et al. (2009) observed that, in gnotobiotic models and in animal models of post-infective gut dysfunction, oral probiotic therapy may influence the neuromotor apparatus. To investigate the effect of different probiotic strains on this type of dysfunction, mice were treated post-*Trichinella spiralis* infection with different probiotic strains. Among these, *Lactobacillus paracasei* NCC2461 significantly attenuated post-infective muscle hypercontractility, and this was accompanied by decreased expression of inflammatory mediators in the muscle layer, such as COX-2 (Verdù, 2004).

Fig. 1.2. The three levels of action of probiotics (Rijkers, 2010).



It is important to underline, however, that it is not possible to generalize these individual effects for every probiotic, and each individual strain must be tested for each property. The key to the effective use of probiotics in treating human disease is to match the correct probiotic strain with the desired clinical outcome (Wallace, 2011).

1.1.6. Lactic Acid Bacteria (LAB)

Lactic Acid Bacteria (LAB) consist of several genera but we can consider a core group consisting of four: *Lactobacillus, Leuconostoc, Pediococcus* and *Streptococcus*. Recent taxonomic revisions have proposed several new genera and the remaining group now comprises the following: *Aerococcus, Alloiococcus, Carnobacterium, Dolosigranulum, Enterococcus, Globicatella, Lactococcus, Oenococcus, Tetragenococcus, Vagococcus, and Weissella*. *Lactobacilli, Carnobacteria* and some *Weissella* are rods while the remaining genera are cocci.
LAB are gram-positive, usually non-motile, non-spore-forming, and catalase-negative. The bacteria within this group produce lactic acid as their major end product through fermentation of carbohydrates. Lacking the ability to synthesize cytochromes and porphyrins (components of respiratory chains), they are not able to generate ATP by creation of a proton gradient. Therefore, LAB can only obtain ATP by fermentation (Khalid, 2011).

1.1.6.1. The genus *Lactobacillus*

The genus *Lactobacillus* is a taxonomically broad and heterogeneous group, which has important industrial applications as fermented food starter and probiotic adjunct. It comprises more than 130 species, and is generally associated with habitats rich in nutrients. It has been reported as the main autochthonous microbiota in cheeses made from different milk types (Martín-Platero, 2009; Abriouel, 2008), with *Lactobacillus plantarum* and *Lactobacillus paracasei* species being dominant. Moreover, lactobacilli are found in the gastrointestinal tract soon after birth. In healthy humans, they are normally present in the oral cavity (10^3-10^4 cfu/g), the ileum (10^3-10^7 cfu/g), and the colon (10^4-10^8 cfu/g), and they are the dominant microorganisms in the vagina (Laville-Larma, 2012).
Owing to their non-pathogenic profile lactobacilli (together with bifidobacteria) are the most commonly used species and significantly influence human health through a range of effects which include: detoxification of xenobiotics, biosynthesis of vitamin K, metabolic effects of fermentation of indigestible dietary fibre, positive influence on transit of luminal contents by peristalsis, competition with pathogenic microbes for nutrients and binding sites on mucosal epithelial cells and modulation of the host's immune response (Hardy, 2013; Maurice, 2013; Nilsson, 2008). Regarding the latter mentioned function, lactobacilli have shown a role in modulating the immune response, both innate and adaptive, through induction of dendritic cell maturation and further stimulation of lymphocytes to release pro-inflammatory cytokines, such as tumour necrosis factor alpha (TNF-α), interferon-gamma (IFN- γ) and interleukin-12 (IL-12) (Chiang, 2012).
Inside the *Lactobacillus* genus, the *L. casei* group of species (i.e., *L. casei, L. paracasei*, and *L. rhamnosus*) includes some of the most conventional and well-characterized probiotic strains, such as *L. paracasei* Shirota (considered the first probiotic ever; Nanno, 2011), and *L. rhamnosus* GG (Szajewska and Chmielewska, 2013). Our research group developed a method according to a technique known as high resolution melting analysis (HRMa), which was applied to a 150 bp groEL gene fragment. The HRMa clustered *L. casei* group strains in three groups that exactly corresponded to the three species, providing a simple, labor-saving, and rapid strategy to obtain the genotyping of a bacterial isolate and simultaneously potentially confirm its affiliation to the *L. casei* group of species (Koirala, 2015).

1.1.6.2. *Lactobacillus paracasei*

Strains of the *L. paracasei* species have been isolated from several diverse ecological niches such as raw milk, plants, fermented artisanal products (fermented milk, cheese, sourdough bread starter, and fermented vegetables), and the intestinal tracts and reproductive systems of humans and animals. The remarkable ecological adaptability of *L. paracasei* to diverse habitats can be plausibly explained by very frequent accumulation of indels and genome rearrangements, which originated a high level of genotypic and phenotypic diversity in the species (Cai, 2007; Smokvina, 2013).
L. paracasei Shirota is one of the most intensively studied probiotics that has been used in the production of the fermented milk Yakult since 1935. Its benefits include improvement in the balance of intestinal microbes and volatile fatty acids in the gastrointestinal environment, antitumor activity, activation of the immune system, antimicrobial activity and improvement in the frequency of defecation and stool quality (van den Nieuwboer, 2015; Wang, 2015; Matsumoto, 2010). Sgouras (2004) studied the potential inhibitory effect of strain Shirota on *Helicobacter pylori* and found out that it possessed activity, but it was not dependent on a secreted molecule. On the contrary, the entire bacterium showed inhibition suggesting that the lactic acid produced was involved in inhibition of *H. pylori* urease system and its reduced ability to survive at a low pH in the absence of urea.
Chiang (2012) reviewed the role of *L. paracasei* strain NTU 101 in modulation of numerous immune responses and prevention of several diseases. This strain (and its fermented products), isolated from a newborn infant faeces, proved to be effective for the management of blood cholesterol and pressure, prevention of gastric mucosal lesion development, immunomodulation and alleviation of allergies, anti-osteoporosis and inhibition of fat tissue accumulation. Regarding immunomodulation, Tsai (2008) studied the effect of a daily consumption of *L. paracasei* NTU 101 (10^8 cfu/day) for 3, 6 and 9 weeks on BALB/c mice. The antigen-presenting ability of DCs and the expression of natural killer group-2 D molecules, capable of triggering natural killer cell-mediated cytotoxicity, were found to be upregulated. Lymphocyte proliferation and antibody production were also significantly increased in mice after the feeding. Interestingly, innate and adaptive immunity remained constant even after the most protracted feeding time, thus indicating the time dependence of the bacterial-mediated enhanced immunity (Tsai, 2008). Furthermore, feeding mice with strain NTU 101, significantly increased the percentages of CD4+ T cells in both Peyer's patches and the spleen. The data also showed that Peyer's patch-derived immunomodulation induced intestinal IgA+-producing cells in the lamina propria and increased CD4+ T cell - dendritic cell interactions, and higher mRNA expression levels of IL-1β, IL-10, IL-12, IFN-γ and TNF-α (Tsai, 2010).

Given that most effects of probiotics are strain-specific (Azaïs-Braesco, 2010), the intrinsic high heterogeneity existing among *L. paracasei* strains makes this species an optimal source for the selection of novel candidate probiotic strains possessing unique technological and health-promoting traits.

1.2 AIMS OF THE STUDY

The regular intake of beneficial microorganisms, or probiotics, is an extensively-studied approach for tapping into the health benefits bestowed by commensal microorganisms colonizing the gastrointestinal tract (GIT) of the healthy human host. During my PhD activities, I was involved in the study of two *Lactobacillus paracasei* strains, named DG and LPC-S01, isolated from two different ecological niches (human gut and vagina, respectively). These strains are already available on the market, since they are included in two products as food supplements. However, a deep characterization is still missing, particularly for LPC-S01. On the contrary, *L. paracasei* DG, which is included in a commercial product named Enterolactis®, has recently demonstrated probiotic properties thanks to *in vivo* studies that showed its ability to interact with the host (D'Incà, 2011; Tursi, 2013; Ferrario, 2014). Moreover, it is one of the most popular probiotic supplements in Italy.
Firstly, we focused our attention on the evaluation of their probiotic potential by means of several techniques using a comparative approach. Indeed, we added a third strain to utilize as reference, that is *L. paracasei* Shirota, the first probiotic product sold in the fermented milk drink "Yakult". In order to unravel their properties, the selected bacteria had been analysed through *in silico*, *in vitro* and *in vivo* analyses. Particularly, we generated a draft genome of DG and LPC-S01 and performed a comparative genomic analysis against other *L. paracasei* reference strains. In addition, they were included in several *in vitro* tests conventionally employed to establish the intestinal probiotic potential of microbial strains. Finally, we carried out a double-blind cross-over pilot intervention trial in order to assess the ability of LPC-S01 and DG to transiently persist in the gastrointestinal tract of healthy adults.

1.3 MATERIALS AND METHODS

1.3.1 Bacterial strains and culture conditions

Unless differently specified, lactobacilli were grown at 37° C in De Man-Rogosa-Sharpe (MRS) broth (Difco Laboratories Inc., Detroit, MI) for 24 h. *L. paracasei* LPC-S01 and DG were isolated from the commercial products PreGyn® and Enterolactis® Plus (Sofar S.p.A.), respectively; *L. paracasei* strain Shirota was isolated from Yakult fermented milk; strain FMBr3 was isolated from Raschera artisanal Italian cheese.

1.3.2. Genome sequencing, sequence annotation, and comparative analysis

The draft genome sequences of *L. paracasei* LPC-S01, DG, and FMBr3 were obtained through Ion Torrent PGM (Life Technologies, Germany) according to a previously described protocol (Milani, 2013) by GenProbio Ltd. The raw sequence data were assembled using MIRA v.3.9 (http://www.chevreux.org/projects_mira.html), applying default parameters recommended for Ion Torrent data processing. Initial automated annotation of the L. paracasei genomes was performed using RAST, combined with BLASTX. Results of the gene-finder program were combined manually with data from BLASTP analysis against a non-redundant protein database provided by the National Center for Biotechnology Information (NCBI). LPC-S01 draft genome sequence was compared with other *L. paracasei* genome sequences by means of BLAST Ring Image Generator (BRIG; Alikhan, 2011).

1.3.3. Bacterial resistance to simulated gastric juices and bile

One hundred microliters of *L. paracasei* culture containing 10^8–10^9 CFU/ml were transferred to 6 ml of simulated gastric juice at pH 2 (1 g l^{-1} DIFCO peptone, 100 mM KCl, 500 U ml^{-1} pepsin) or simulated gastric juice at pH 3 (125 mM NaCl, 45 mM $NaHCO_3$, 7 mM KCl, 500 U ml^{-1} pepsin). Cells in 0.1 M phosphate buffer pH 7 were used as control. After 90 min of incubation at 37° C, viability was monitored by plating on MRS agar. Afterwards, cell suspension was neutralized by adding 1 M phosphate buffer pH 8 and centrifuged at 10,000 × g for 12 min. Bacterial cell pellet was then suspended in 5 ml bile solution (1 g l^{-1} peptone, 0.3% Difco Oxgall in 0.1 M phosphate buffer at pH 6.5). Cells in 0.1 M phosphate buffer pH 6.5 in the presence of 1 g l^{-1} peptone were used as control. The number of viable cells was determined after 3 h of incubation at 37°C by plating on MRS agar. Plates were incubated under anaerobic conditions and colonies were counted after 48 h. The ability to grow in presence of bile was also investigated by inoculating bacteria in MRS broth supplemented with increasing concentrations of Oxgall (from 0.0625 to 9.6%). Growth was assessed by measuring the optical density at 600 nm.

1.3.4. Bacterial adhesion to Caco-2 cell line

The adhesion of *L. paracasei* strains to Caco-2 (ATCC HTB-37) cell layer was assessed as previously described (Guglielmetti, 2008). In brief, Caco-2 cells were grown in Dulbecco's Modified Eagle's Medium (MEM) supplemented with 10% (v/v) heat-inactivated 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 an atmosphere of 95% air and 5% carbon dioxide. For adhesion experiments, fully differentiated Caco-2 were used (*i.e.*, 15 days after confluence). Approximately 2×10^8 cells for each bacterial strain (determined microscopically with Neubauer Improved counting chamber; Marienfeld GmbH, Lauda-Königshofen, Germany) were incubated with a monolayer of Caco-2 cells for 1 h at 37° C. Monolayers were washed three times with phosphate-buffered saline pH 7.3 (PBS) to release unbound bacteria and incubated with 3 ml of methanol for 8 min at room temperature to fix cells. Afterwards, cells were stained with 3 ml of Giemsa stain solution (1:20; Carlo Erba, Milano, Italy) and left 30 min at room temperature in the dark. Finally, monolayers were washed three times with PBS, dried in an incubator for 1 h, and examined microscopically (magnification, 400×) under oil immersion. All experiments were performed in duplicate.

1.3.5. Antagonistic activity against pathogens

We investigated the ability of *L. paracasei* strains to secret inhibitor molecules against pathogens by disc diffusion assay. *Lactobacillus plantarum* strain WHE 92 was used as a positive control for bacteriocin production (Ennahar, 1996). We used seven indicator microorganisms: *Salmonella enterica* MIMms, *Listeria monocytogenes* FMB4b, *Escherichia coli* VE7108, *Streptococcus pyogenes* emm TYPE 77, *Pseudomonas aeruginosa* FMBpa18, *Staphylococcus aureus* FMBDR, and *Candida albicans* ATCC MYA2876. Indicator bacteria were cultivated in Brain Heart Infusion (BHI) agar medium (Oxoid S.p.A., Milan, Italy), supplemented with 0.3% yeast extract and 1% glucose (ygBHI), whereas *C. albicans* was cultivated on Sabouraud dextrose agar (Oxoid S.p.A., Basingstoke, UK). Three different fractions for each *Lactobacillus* strain (tester bacteria) were used: (i) broth culture at stationary growth phase, (ii) cell-free broth (obtained by centrifugation at 10,000 × g for 10 min and sterilization with a 0.22 μm syringe filter), and (iii) cell-free broth neutralized with NaOH to pH 7.4. Sterile MRS was used as negative control. The assay was carried out as follows: 0.1 ml from an over-night culture of the indicator strains were plated on ygBHI (or Sabouraud) agar in order to obtain a confluent growth after incubation. Sterile paper discs (Whatman ™ Grade AA 9 mm disc, Maidstone, UK) were dipped into three different preparations from tester bacteria and subsequently positioned on the Petri dishes inoculated with the indicator strain. Plates were kept at 4° C for 2 h and then incubated at 37° C. The presence of an inhibition halo was checked after 24 or 48 h.

1.3.6. Fermentation profile

Carbon source fermentation was determined in a 96-well microtiter plate in a final volume of 200 μl with a basal CHL medium at pH 6.3 (Bio-Merieux, Montelieu-Vercieu, France) containing bromocresol purple as pH indicator, and the desired filter-sterilized carbohydrate at a final concentration of 0.5% (w/v). The 45 different substrates tested (Tab. 1.2) were from Sigma-Aldrich (St. Louis, MO, USA), with the exception of fructooligosaccharides (FOS) which were from Actilight® (Giulio Gross S.p.A., Trezzano sul Naviglio, Italy), and blastose, which was purified in our laboratory. Cells from an over-night culture of *L. paracasei* were collected by centrifugation, washed with PBS, and used to inoculate the liquid medium in microtiter wells (1/100 inoculation). Plates were examined for colour change (from purple to yellow) after 24 and 48 h incubation at 37° C.

1.3.7. NF-κB activation assay

The activation nuclear factor κB (NF-κB) was studied by means of a recombinant Caco-2 cell line stably transfected with vector pNiFty2-Luc (InvivoGen, Labogen, Rho, Italy) as in Taverniti (2013). In brief, recombinant Caco-2 monolayers (approximately 3×10^5 cells/well), cultivated in the presence of 50 μg ml^{-1} zeocin, were washed with 0.1 M Tris-HCl buffer (pH 8.0) and then incubated with 3.5×10^8 cells of *L. paracasei* suspended in fresh DMEM containing 100 mM HEPES (pH 7.4), resulting in a MOI of approximately 1000. In a different set of experiments, Caco-2 monolayers were incubated with 0.1 ml of cell-free broth obtained by centrifugation and filter-sterilization from a stationary-phase culture of *L. paracasei*. Stimulation was conducted by adding 2 ng ml^{-1} of IL-1β. After incubation at 37° C for 4 h, the samples were treated and the bioluminescence was measured as described by Stuknyte (2011). Two independent experiments were conducted in triplicate for each condition.

1.3.8. Susceptibility to antibiotics and production of biogenic amines

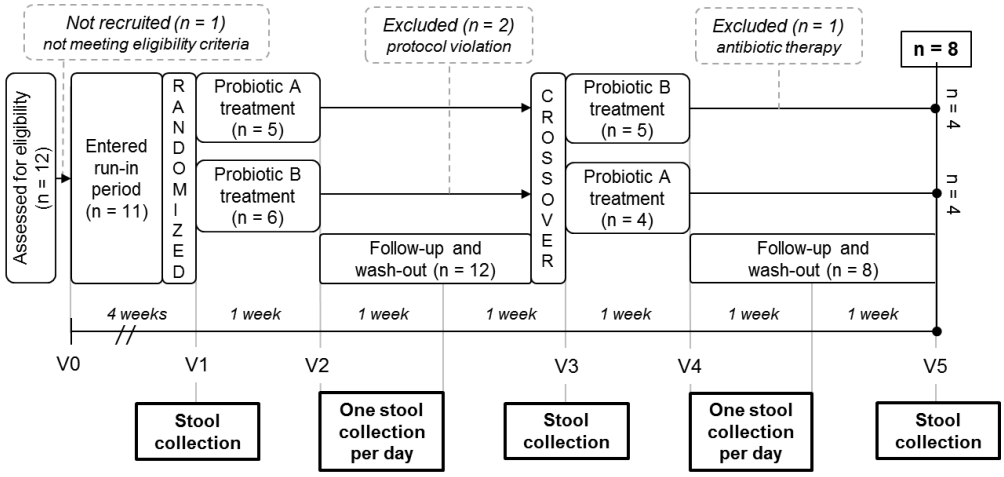
The minimal inhibitory concentrations (MICs) of *L. paracasei* have been determined using a micro-dilution method in LSM broth (ISO-Sensitest broth, Oxoid supplemented with 10% v/v MRS Difco) as described in the ISO10932 IDF 223 document and recommended by EFSA (2012). The data are reported as average of two independent assays. The ability to produce cadaverine, tyramine, histamine and putrescine respectively from L-lysine, tyrosine disodium salt, L-histidine monohydrochloride and L-ornithine monohydrochloride was investigated by a qualitative test, according to the method proposed by Bover-Cid and Holzapfel (1999). All amino acids were purchased from Sigma. Strains were inoculated (1%) in MRS broth containing 0.1% of each amino acid precursor and incubated at 37° C for 18 h. The qualitative test to evaluate decarboxylase activity was performed in an agar medium specially formulated (0.5% tryptone, 0.5% yeast extract, 0.5% meat extract, 0.25% NaCl, 0.05% glucose, 0.1% Tween 80, 0.02% $MgSO_4$, 0.005% $MnSO_4$, 0.004% $FeSO_4$, 0.2% ammonium citrate, 0.001% thiamine, 0.2% K_2HPO4, 0.01% $CaCO_3$, 0.005% pyridoxal phosphate, 1% amino acid precursor, 0.006% bromocresol purple, 2% agar; pH 5.3). 10 μl of each culture were placed on the medium containing the same amino acid precursor. The plates were incubated at 37°C for 4 days. Positive test is indicated by the colour change and by amino acid precipitation around the corresponding spot (for tyramine only).

1.3.9. Human intervention trial

The study protocol was approved by the Research Ethics Committee of the Università degli Studi di Milano (opinion no. 37/12, December 2012). Written informed consent was obtained from all subjects before recruitment. The intervention study consisted of a randomized, double blind, cross-over pilot trial with two parallel groups (Fig. 1.3). Each volunteer was asked to participate to 6 visits: before run-in period (visit V0); before the first treatment (V1); after the first treatment (V2); before the second treatment (V3); after the second treatment (V4), and at the end of the trial (V5) (Fig. 1.3). After a 4-week pre-recruitment phase, volunteers have been randomized to receive 1 capsule daily of *L. paracasei* LPC-S01 (product A, 5 subjects) or *L. paracasei* DG (product B, 6 subjects) every day for 1 week, in addition to their habitual diet. After a 2 week wash-out period, the volunteers received a daily capsule of the other product for 1 week. Volunteers received directions to keep the products at room temperature and to avoid exposure to heat sources. Volunteers received also oral and written instructions to consume the

capsule during the morning, while drinking natural water, at least 15 min before breakfast; alternatively, volunteers were allowed to consume capsule in the evening at least 3 h after the last meal of the day. The two probiotic preparations (provided by Sofar S.p.A., Trezzano Rosa, Italy) consisted of a gelatine capsule containing about 24 billion viable cells of the bacterial strain *L. paracasei* DG (CNCM I-1572) and LPC-S01 (DSM 26760), respectively; silicon dioxide and magnesium stearate were also added inside capsules as antiagglomerants. Capsules were delivered to participants in metal boxes sealed with a plastic cap containing desiccant salts. From the last day of consumption of the probiotic capsules, for the following 7 days, the volunteers provided one stool sample per day. Furthermore, participants provided a faecal sample at visits V1, V3, and V5 (Fig. 1.3). The sample was collected in a sterile plastic pot no more than 24 h before visit. Volunteers were asked to preserve the faecal sample at room temperature until delivery to the laboratory, according to the recommendations on storage conditions of intestinal microbiota matter in metagenomic analysis provided by Cardona (2012). During the trial period, the participants compiled a weekly diary (including a Bristol stool chart) of their bowel habits.

Fig. 1.3. Schematic of study design and flow. V0–V5, visits before the run-in period, before the first treatment, after the first treatment, before the second treatment, after the second treatment, and after final follow-up, respectively.



1.3.10. Faecal DNA extraction and qPCR

Stools were stored at −80° C until DNA extraction, performed by means of QIAamp DNA Stool Mini Kit (Qiagen, Valencia, CA). Before the extraction, the sample was homogenized in a Bead Beater Precellys 24 (Bertin Technologies, Montigny le Bretonneux, France) after addition of 0.45 g 0.5 mm glass beads. Subsequently, the extraction proceeded consistently with manufacturer's recommendations. Real-Time quantitative (qPCR) protocols were adopted for the quantification of *L. paracasei* DG in faecal metagenomic DNA (targeting the glycosyltransferase gene welF, with primers rtWELFf, 5′-TACTAAAGAAATTAGCTTTTGT-3′ and rtWELFr, 5′- AGTAATGTCTGCATCCTCCA -3′ ; Ferrario, 2014) and LPC-S01 (targeting an hypothetical protein coding sequence with primers qS01a-F, 5 ′ - TGGAAGAGACCCTGCGAA-3 ′ and qS01a-R, 5′-GAGGTTGATTCACAAACCGTGC-3′).

These two genes were selected because they resulted unique to the respective strain as revealed by a search in the GenBank nucleotide database. A gradient PCR was initially performed to standardize the qPCR conditions. qPCR amplifications were carried out in a final volume of 15 µl containing 7.5 µl of EvaGreen® Supermix and 0.5 µM of each primer. We used 100 ng of faecal DNA template in each reaction. Samples were amplified with the following programs: for rtWELF primers, initial hold at 95° C for 3 min, and 44 cycles at 95° C for 30 s, 58° C for 30 s and 72° C for 30 s; for qS01a primers, initial hold at 95° C for 3 min, and 40 cycles at 95° C for 30 s, 62° C for 30 s and 72° C for 30 s. Melting curves were analysed to confirm the specificity of the amplification products. To generate the standard curves with spiked faeces for DG and LPC-S01 detection, a 200 mg matrix of faecal sample was added with 1×10^9 bacterial cells and DNA was extracted as reported above. Six-fold dilution series of isolated DNA were prepared and used in qPCR reactions. The equation of the derived standard curves was used to calculate the correlation between Ct values and the concentration of bacterial cells per g of faeces.

1.4 RESULTS AND DISCUSSION

1.4.1. Comparative genomics to reveal indels of chromosomal regions in *L. paracasei* LPC-S01 and DG

We generated a draft genome sequence of *Lactobacillus paracasei* LPC-S01 and DG, consisting of 17 and 20 contigs, respectively. Comparative genomic analysis was performed using strain LPC-S01 or DG as reference against the other following five *L. paracasei* strains: the type strain of the species (*L. paracasei* JCM 8130T), the probiotic strain Shirota, a strain isolated from Raschera Italian artisanal cheese (FMBr3), and two other *L. paracasei* strains whose complete genome is available in GenBank (strains 8700:2 and N1115) (Fig. 1.4). The draft genome sequences of strains *L. paracasei* LPC-S01, DG and FMBr3 were generated by our group. When we used *L. paracasei* LPC-S01 as reference genome in the comparative analysis, we identified 14 genomic islands with putatively known biological functions, which have not been found in the genomes of type strain JCM 8130T or other *L. paracasei* strains. These chromosomal islands, which discriminate LPC-S01 genome from the others, include 7 mobile genetic elements (e.g. prophages, phage remnants, and integrated plasmids; Fig. 1.4), a transposon containing a phospho-β-galactosidase operon (region a in Fig. 1.4; Fig. 1.5A), a putative taurine transport and metabolization operon (b; Fig. 1.5B), a large sugars transport/metabolization island (c; Fig. 1.5C), an ABC-type Fe^{3+} transport system (d; Fig. 1.5D), and a putative nucleotide transport and metabolism operon (e; Fig. 1.5E). We also found in the genome of strain LPC-S01 two regions putatively involved in exopolysaccharide (EPS) synthesis. Specifically, one of these EPS regions (namely, EPS2; Fig. 2.4A in Chapter 2) was found exclusively in the genome of *L. paracasei* LPC-S01 and is constituted of 14 putative genes, including 6 genes potentially coding for glycosyl transferases. Notably, BLASTN search revealed inside EPS2 a region of about 8 kb encompassing 8 putative genes, which did not find any significant match in the GenBank database (genes from A to H in Fig. 2.4A).

In addition, strain DG possessed three genetic regions that are absent in LPC-S01: a maltose/maltodextrin utilization gene cluster (referred as a in Fig. 1.6; in Fig. 1.7A) an arsenical resistance operon (b, in Fig. 1.7B), and a fumarate reductase gene (c, in Fig. 1.7C). As in LPC-S01, we could identify other genomic islands including 6 mobile genetic elements, two of them corresponding to prophages and phage remnants. Interestingly, we could identify an EPS region also in DG genome, which resulted intriguing because, as for the one belonging to LPC-S01, did not find any match on NCBI database for a long extent of its length (Fig. 2.4B). For this reason we focused further attention on this kind of macromolecule production, as described in Chapter 2.

In subsequent comparative analyses, we used the genome of the other *L. paracasei* strains as reference to identify missing chromosomal islands in the genome of strains LPC-S01 and DG. Largely most of the genomic regions that are absent in our strains genome refer to mobile genetic elements and DNA restriction-modification systems. Furthermore, the genome of strain *L. paracasei* 8700:2 had a tetracycline efflux region, a galactitol/sorbitol phosphotransferase transport and utilization system, and a α-mannosidase operon. Finally, the genetic regions putatively coding for β-glucosidase and chitinase operons were found in the genome of strain FMBr3 but not in DG and LPC-S01 (data not shown).

Fig. 1.4 Comparative genomic analysis of *Lactobacillus paracasei* LPC-S01(reference genome) with the genome sequences of other six *L. paracasei* strains. Grey rectangles indicate mobile genetic elements. pφ, prophage-related regions. mge, mobile genetic element; mge1, putative integrated plasmid; mge2, putative mobile genetic element containing a restriction-modification system. EPS, putative exopolysaccharide coding operon. Letters flanking red rectangles refers to panels of Figure 1.5.

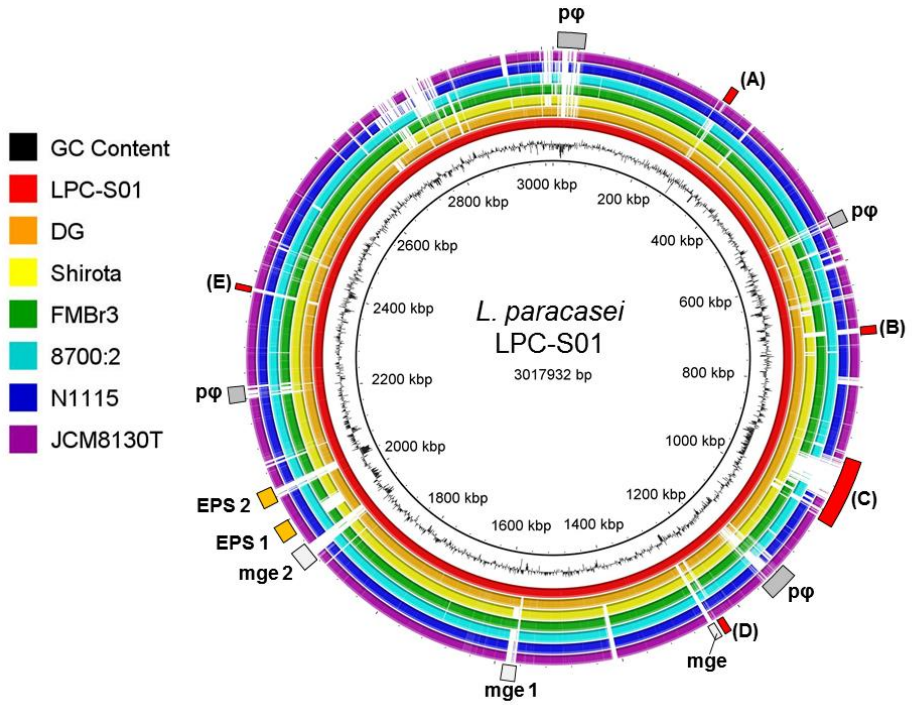
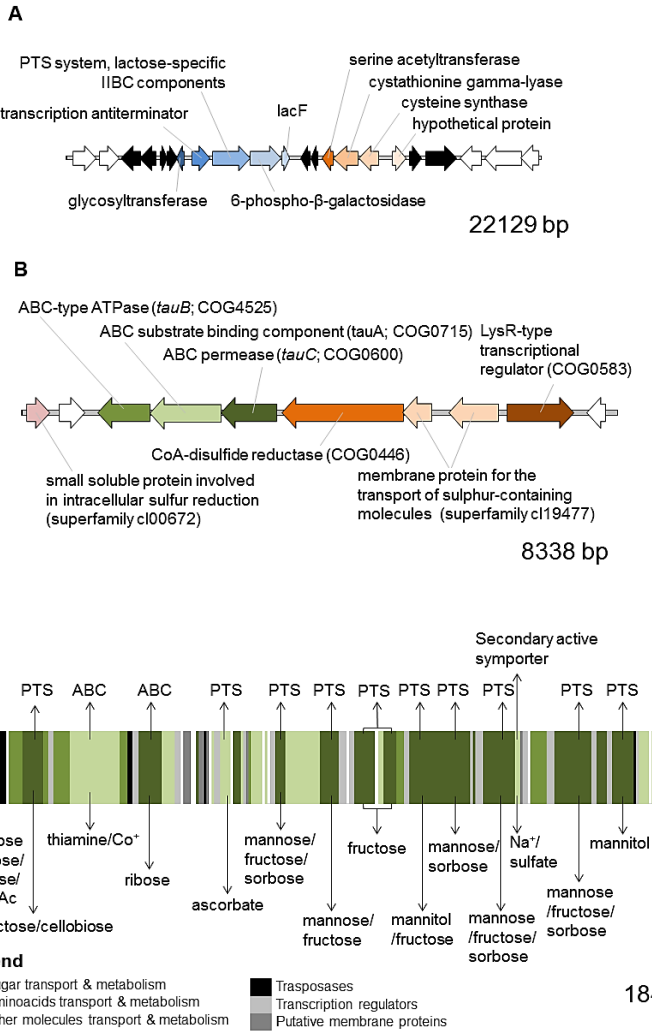


Fig.1.5 (next pages). *In silico* predicted functional organization of the chromosomal regions of *Lactobacillus paracasei* LPC-S01 that discriminates it from the other strains included in the comparative genomic analysis depicted in Figure 1.4. Letters in the panels of this figure refer to the letters flanking red rectangles in Figure 1.4. **(A)** Putative phospho-β-galactosidase operon. In black are indicated transposase-associated putative genes. In white are indicated putative genes that are also present in the genome of the other *L. paracasei* strains considered for comparative genomic analysis. **(B)** Putative taurine ABC-type transport and metabolization operon. **(C)** Multi-transport region for the uptake of sugars and other small molecules. The membrane transport mechanism is indicated above the picture, whereas the transported molecule is indicated below. ABC, ATP binding cassette transport system, PTS, phosphotransferase transport system. Gal-NAc, N-acetylgalactosamine; Glu-NAc, N-acetylglucosamine; Co^{+}, cobalt ions. **(D)** Putative ABC-type Fe^{3+} transport system. Gene "a", putative acetyltransferase (COG0456) coding gene; gene "b", putative gene coding for an uncharacterized protein (DegV family, COG1307). **(E)** Putative nucleotide transport and metabolism operon. MFS, major facilitator superfamily. In white are indicated putative genes that are also present in the genome of all the other *L. paracasei* strains considered.


A
PTS system, lactose-specific IIBC components
transcription antiterminator
lacF
serine acetyltransferase
cystathionine gamma-lyase
cysteine synthase
hypothetical protein
glycosyltransferase
6-phospho-β-galactosidase
22129 bp
B
ABC-type ATPase (tauB; COG4525)
ABC substrate binding component (tauA; COG0715)
ABC permease (tauC; COG0600)
LysR-type transcriptional regulator (COG0583)
CoA-disulfide reductase (COG0446)
small soluble protein involved in intracellular sulfur reduction (superfamily cl00672)
membrane protein for the transport of sulphur-containing molecules (superfamily cl19477)
8338 bp
C
Secondary active symporter
PTS PTS ABC ABC PTS PTS PTS PTS PTS PTS PTS PTS PTS PTS
mannose /fructose/ sorbose/ Gal-NAc
lactose/cellobiose
thiamine/Co+
ribose
ascorbate
mannose/ fructose/ sorbose
mannose/ fructose
fructose
mannitol /fructose
mannose/ sorbose
mannose /fructose/ sorbose
Na+/ sulfate
mannitol
mannose /fructose/ sorbose
mannose /fructose/ sorbose/ Gal-NAc/ Glu-NAc
Legend
Sugar transport & metabolism
Aminoacids transport & metabolism
Other molecules transport & metabolism
Trasposases
Transcription regulators
Putative membrane proteins
184938 bp

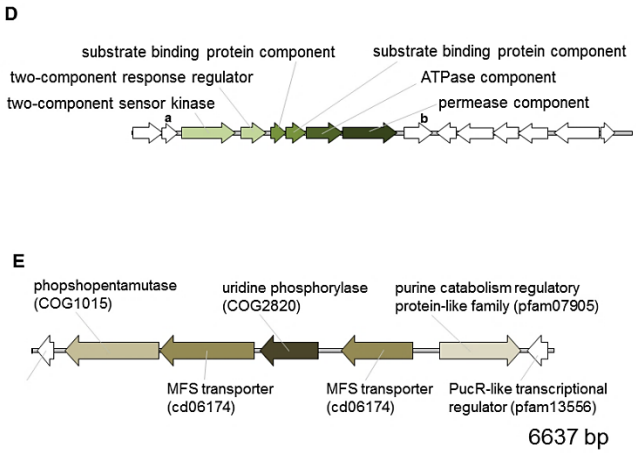


Fig.1.6. Comparative genomic analysis of *Lactobacillus paracasei* DG (reference genome) with the genome sequences of other six *L. paracasei* strains. Grey rectangles indicate mobile genetic elements. pφ, prophage-related regions. mge, mobile genetic element. EPS, putative exopolysaccharide coding operon. Letters flanking red rectangles refers to panels of Figure 1.7.

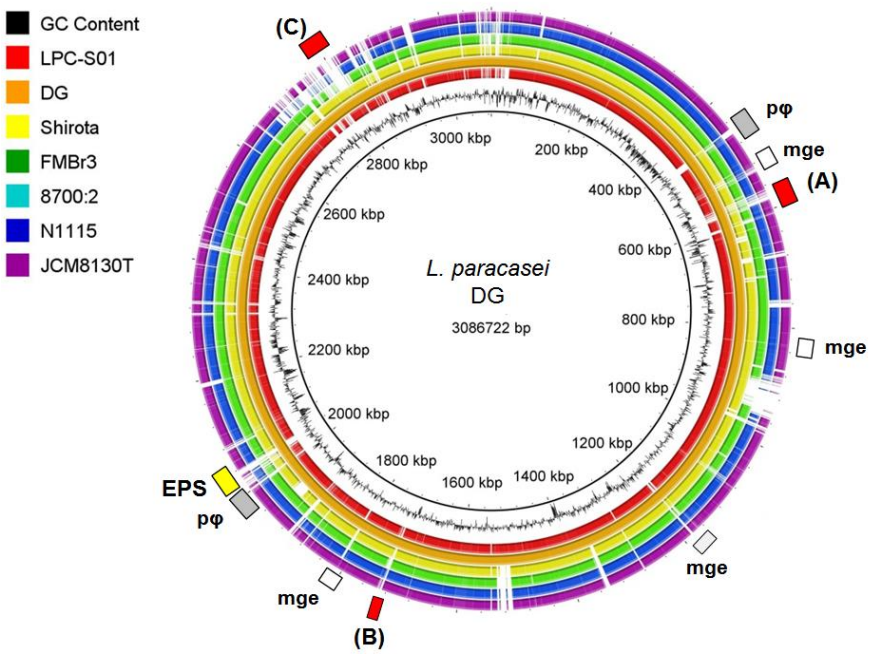
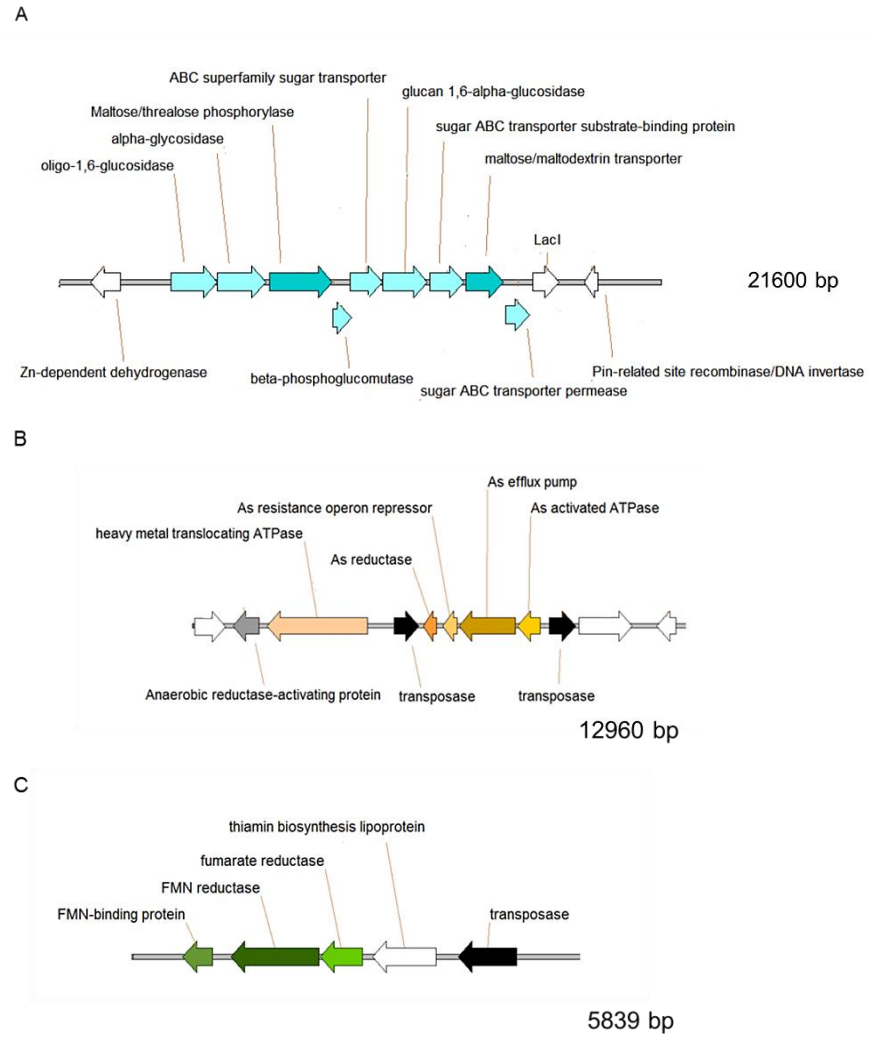


Fig.1.7. *In silico* predicted functional organization of the chromosomal regions of *Lactobacillus paracasei* DG that discriminates it from the other strains included in the comparative genomic analysis depicted in Figure 1.6. Letters in the panels of this figure refer to the letters flanking red rectangles in Figure 1.6. **(A)** Putative maltose/maltodextrin utilization gene cluster. **(B)** Putative arsenic resistance operon. **(C)** FMN and fumarate reductase coding sequences. In black are indicated transposase-associated putative genes. In white are indicated putative genes that are also present in the genome of all the other *L. paracasei* strains considered.



Summarizing, we found the presence of numerous accessory genes (i.e., unique to a particular strain) that suggests the niche-generalist nature of DG and, more evident, of LPC-S01; such potential ability to adapt to a wide range of environmental conditions is evidenced, for instance, by the presence of large chromosomal regions including genes for the utilization of numerous sugars (Fig. 1.5C). Similar regions are also present in the genome of DG and Shirota, which are of human intestinal origin. On the contrary, it was proposed that the adaptation of *L. paracasei*

to the nutrient-rich milk environment had been gone along with extensive decay of genes involved in carbohydrate utilization (Broadbent, 2012). Accordingly, we found the lack of genes for sugar transport and metabolism in the chromosome of the dairy strains *L. paracasei* FMBr3 and N1115 compared to LPC-S01, DG, and Shirota (Fig. 1.4 and Fig. 1.6).

1.4.2. *In vitro* assays to evaluate acid tolerance, adhesion ability and pathogen inhibition capacity

To meet the main criteria for the selection of potential probiotic strains and crucial factors for beneficial health effects, probiotic bacteria have to withstand the harsh environment of digestive enzymes and gastric juice in the stomach as well as bile secreted in the duodenum (Lim and Ahn, 2012). The probiotic potential of strains *L. paracasei* LPC-S01 and DG was assessed through *in vitro* experiments including *L. paracasei* Shirota as reference probiotic strain. The potential ability to survive the gastro-intestinal transit was assessed in simulated gastric juices at pH 3 or 2 (90 min incubation), followed by 3 h incubation in bile (Oxgall) solution. All strains showed high tolerance to pH 3; cell viability, in fact, was not significantly reduced after 4.5 h (Fig. 1.8A). Conversely, pH 2 determined a drastic reduction of the viability of all tested strains. However, *L. paracasei* LPC-S01 displayed the highest tolerance, which was significantly higher than that of the reference strain *L. paracasei* Shirota. This latter is an acid-tolerant bacterial strain with demonstrated survivability to human gastrointestinal transit (Tuohy, 2007; Wang, 2015).
Growing concentrations of Oxgall were added to the MRS broth to assess the ability to grow in presence of bile. The adaptation to bile salts is strain-specific and is related to changes in carbohydrate fermentation, glycosidase activity (Burns, 2010), exopolysaccharide production (Ruas-Madiedo, 2009; Ruiz, 2007), composition of membrane proteins and fatty acids (Gueimonde, 2007), increased adhesion to human mucus and inhibition of pathogen adhesion (Muñoz-Quezada, 2013). In the present study, all of the tested strains exhibited similar bile tolerance at concentrations between 0.3% and 0.5% (corresponding to the physiological concentrations of human bile (Ren, 2014)) with a dose-dependent inhibition of the bacterial growth. On the contrary, growth was completely arrested at Oxgall concentrations higher than 1%. In specific, Shirota displayed the best tolerance to bile, even though not statistically significant (Fig. 1.8B).

The Caco-2 cell line is commonly used as a model for assessing the ability of probiotics to adhere to human intestinal epithelial cells. Bacterial adhesion properties mainly depend on the molecules exposed at the external cell surface (Guglielmetti, 2008; Polak-Berecka, 2014) and there exists a great variability among strains; however, many probiotics are known to inhibit adhesion and displace pathogens such as *Salmonella enterica*, *Escherichia coli*, *Listeria monocytogenes*, *Staphylococcus aureus*, and *Clostridium difficile* (Collado, 2007). Furthermore, the adhesion capacity is a very important parameter for a probiotic candidate because it avoids the bacterial elimination by peristaltic movements and allows the interaction with the immune system (Muñoz-Quezada, 2013). When the ability of the bacterial strains under study to adhere to the Caco-2 epithelial cell layer was tested, we found that only LPC-S01 displayed a marked adhesive phenotype, corresponding to an adhesion index (i.e., bacterial cells per 100 Caco-2 cells) of more than 2000 (Fig. 1.9). Also strain DG displayed adhesion ability, whereas strain Shirota was unable to adhere on Caco-2 cells, as observed in previous studies (Botes, 2008; Guglielmetti, 2008). As mentioned above, genome analysis revealed the presence in LPC-S01 of putative exopolysaccharide coding region (EPS2) containing a cluster of five genes which are not present in the other known *L. paracasei* genomes. Therefore, EPS2 could potentially

support the synthesis of a peculiar exopolysaccharide molecule, which could plausibly contribute to the adhesion ability of LPC-S01. Exopolysaccharides have been proposed to promote *Lactobacillus* adhesion (Ren, 2014), although, more frequently, the presence of a polysaccharide capsule has been shown to reduce adhesion by masking cell surface *Lactobacillus* adhesive determinants (Lebeer, 2012; Horn, 2013; Dertli, 2015), which are most commonly proteins (Lebeer, 2012; Polak-Berecka, 2014), (lipo)teichoicacids (Granato, 1999), and/or fatty acids (Polak-Berecka, 2014).

Fig. 1.8. **(A)** Tolerance of *Lactobacillus paracasei* strains to simulated gastric juice at pH 2 (90 min incubation) and bile (180 min incubation). Dashed lines refer to controls (i.e., bacterial cells incubated in phosphate buffer, pH 6.5). Data are reported as the number of viable bacterial cells (CFU) plotted on a semi-logarithmic diagram. Vertical bars at each point refer to standard deviation calculated on three independent experiments conducted in duplicate. Asterisk between strain LPC-S01 and Shirota at the end of the experiment indicates statistically significant difference according to two-tailed unpaired Student's t test (*P < 0.05). **(B)** Growth of *Lactobacillus paracasei* strains in MRS broth supplemented with increasing concentrations of bile (Oxgall).

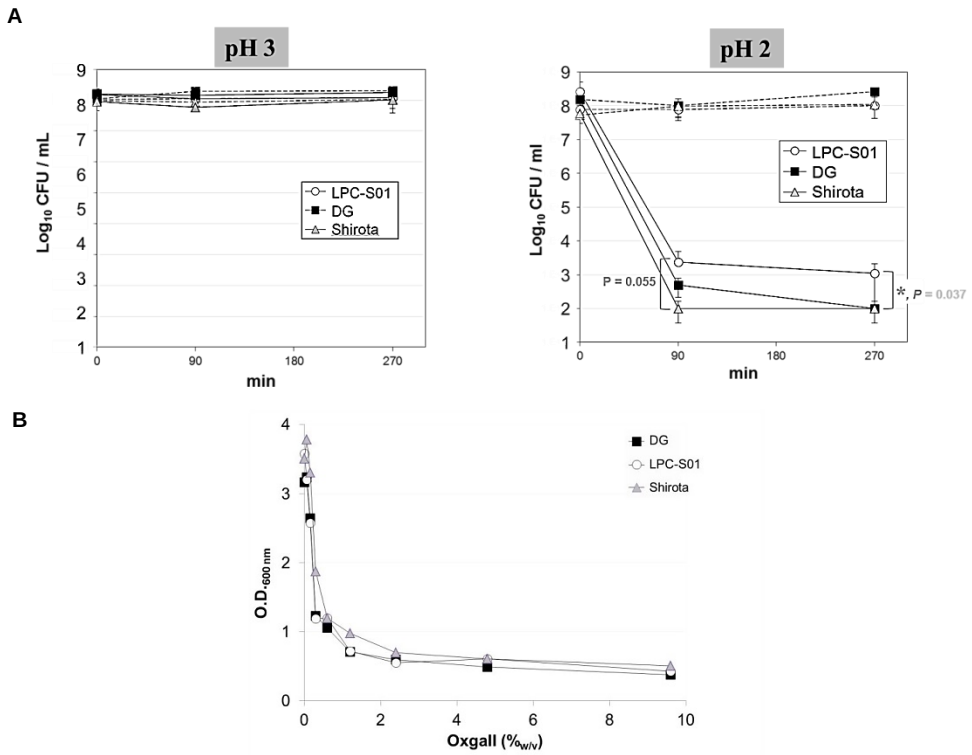
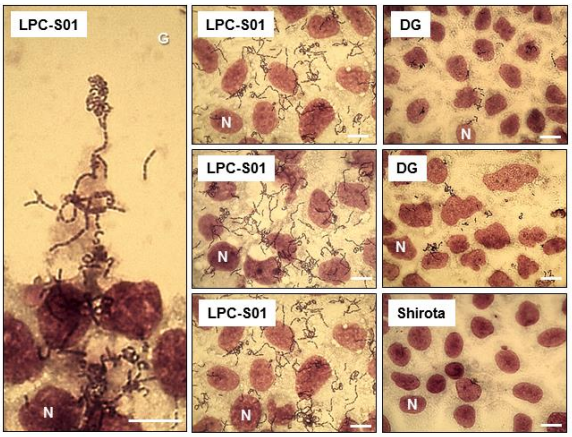


Fig. 1.9. Adhesion of *Lactobacillus paracasei* strains to the Caco-2 epithelial cell layer as observed with Giemsa staining under a light microscope. Bars, 25 µm. One Caco-2 nucleus for each layer is indicated with the letter N. LPC-S01 adhesion was specific to Caco-2 cells: no adhesion was detected on the cover glass underlying Caco-2 cells (G; top part of the panel on the left).



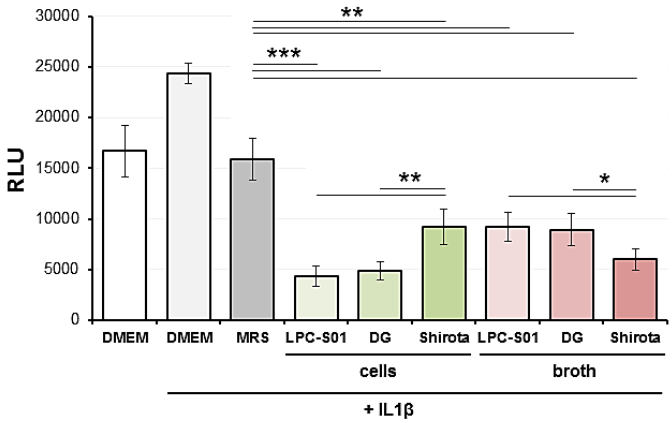
We also tested the ability of the bacteria under investigation to produce inhibitor molecules against pathogens by disc diffusion assay carried out by using cell-free MRS broths cultures. With the only exception of *L. plantarum* WHE 92 against *L. monocytogenes*, used as positive control, none of the neutralized broths was able to prevent pathogens growth, suggesting that, plausibly, the only inhibitory molecules produced by tested *L. paracasei* strains were organic acids. Regarding the lack of activity against *C. albicans*, it is known that many yeasts can tolerate acids and resist hydrogen peroxide action, probably because of their cell wall structure and the ability to form biofilm, so it is very difficult to identify probiotic strains that are effective against this pathogenic microorganism in the vagina (Reid, 2006).

1.4.3. Activation of NF-κB in transfected pNiFty2-Luc Caco-2 cells

We employed a reporter cell line obtained by transfecting Caco-2 cells with a luciferase reporter vector induced by active NF-κB (Guglielmetti, 2010a). The NF-κB reporter system was chosen to study the immunomodulatory properties of the strains under study, as this transcription factor regulates many physiological processes, including the innate and adaptive immune responses, cell death and inflammation (Gilmore, 2006; Perkins, 2007). NF-κB indeed owns a critical function since it can enhance the transcription of many cytokines (including tumour necrosis factor (TNF)-α, IL-1, IL-6, and IL-8, which are thought to be important in the generation of acute inflammatory responses), growth factors, adhesion molecules, immunoreceptors, and acute-phase proteins. Thus, activated NF-κB initiates both extracellular and intracellular regulatory events that result in priming the inflammatory cascade (Stuknyte, 2011). In our experiments, we tested the effect of the microorganisms on NF-κB activation under the presence of the pro-inflammatory cytokine IL-1β. For all three *L. paracasei* strains, both bacterial cells and cell-free neutralized broths were able to significantly decrease the NF-κB-dependent production of bioluminescence. Particularly, we found that the highest ability to

reduce NF-κB activation was exerted by DG and LPC-S01 cells, and by broth from Shirota culture (Fig. 1.10).

Fig.1.10. Effect of *L. paracasei* strains on Caco-2 cells stably transfected with an NF-κB/luciferase reporter vector, in the presence of 5 ng ml^{-1} of IL-1β. Data in the histograms are the means (± standard deviations) from two independent experiments conducted in triplicate. RLU, relative luminescence units. Asterisks indicate statistically significant differences according to two-tailed unpaired Student's t-test: ***P < 0.001; **P < 0.01; *P < 0.05.



1.4.4. Utilization of sugars as carbon sources

Afterwards, the *in vitro* probiotic properties of LPC-S01 and DG were assessed by testing their ability to produce acid from 45 different carbohydrate sources. Gut microorganisms are readily able to degrade available substrates, derived from either the diet or endogenous secretions. Major substrates available are starches and soluble dietary fibres, but also oligosaccharides and portions of non-absorbable sugars and sugar alcohols (in lower concentrations) (Wallace, 2011). Among them, the fructans (inulin, fructo-oligosacharides (FOS)) and galacto-oligosaccharides (GOS) are the most extensively studied prebiotics which, owing to their chemical structure, are indigestible in the small intestine and are anaerobically fermented by bacteria in the colon (Hardy, 2013). These carbohydrates fermentation results in the production of short chain fatty acids (SCFAs), especially acetate, propionate and butyrate, that have significant positive impacts on intestinal epithelial cell function, including maintenance of metabolism, proliferation, differentiation and promotion of low pH levels in the gut environment, favouring beneficial microbes with a concomitant reduction in pathogens viability (Ohigashi, 2013). In addition, histamine, carbon dioxide, and other neutral, acidic, and basic end products derive from these reactions.

The fermentation patterns of *L. paracasei* strains were very similar. In detail, the three tested strains fermented cellobiose, FOS, fructose, galactose, glucose, inulin, maltulose, mannitol, mannose, ribose, salicine, sorbose, sucrose, trehalose, and turanose. In addition, LPC-S01 was the only strain that fermented (although weakly) esculin and L-arabinose, and the only not fermenting sorbitol. Moreover, only strain DG was able to rapidly ferment maltose and maltodextrins, plausibly due to the presence of the putative genetic cluster described above (Fig. 1.7A), and unable to use lactose and lactulose. Finally, Shirota was the only strain unable to ferment tagatose (Tab. 1.2). Interestingly, all the three *L. paracasei* strains were able to

utilize the non-conventional disaccharide blastose (6-O-β-D-fructofuranosyl-α, β-D-glucopyranoside) produced in a FOS mixture by *Cladosporium cladosporioides* MUT 5506 and recently identified and fully characterized (Zambelli, 2014; Zambelli, 2016).

Tab. 1.2. Phenotypic characterization of *Lactobacillus paracasei* strains based on acid production from 45 carbon sources. +++, positive; –, negative; +, weak activity.

	LPC-S01	**DG**	**Shirota**
D-Glucose	+++	+++	+++
Lactose	+++	-	+++
D-Fructose	+++	+++	+++
D(+)-Xylose	-	-	-
D-Galactose	+++	+++	+++
Sucrose	+++	+++	+++
Maltose	+	+++	-
L(+)-Arabinose	+	-	-
D(-)-Arabinose	-	-	-
D(+)-Trehalose	+++	+++	+++
D(+)-Mannose	+++	+++	+++
L-Rhamnose	-	-	-
Cellobiose	+++	+++	+++
L(+)-Sorbose	+++	+++	+++
D-Sorbitol	-	+++	+++
Inulina	+++	+++	+++
Lactulose	+++	-	+++
D-Mannitol	+++	+++	+++
FOS (Actilight)	+++	+++	+++
Salicine	+++	+++	+++
Maltodextrin	-	+++	-
α-ciclodextrin	-	-	-
β-ciclodextrin	-	-	-
Arabinogalactan	-	-	-
Maltulose	+++	+++	+++
D(+)-Turanose	+++	+++	+++
Isomaltulose (palatinose hydrate)	-	-	-
GOS	+++	+	+++
D(-)-Ribose	+++	+++	+++
Xylan from beechwood	-	-	-
Red Arabinian from sugar-beet	-	-	-
Arbutin	-	-	-
D(-)-Tagatose	+++	+++	-
D(+)-Fucose	-	-	-
D(+)-Raffinose	-	-	-
Glycerol	-	-	-
D-Gluconic acid	+	+	-
Meso-Erythritol	-	-	-
Melibiose	-	-	-
Myo-Inositol	-	-	-
Inositol	-	-	-
D-Glucoron-gamma-Lactone	-	-	-
Glycogen	-	-	-
Esculin	+	-	-
Blastose	+++	+++	++

1.4.5. Safety evaluation of *L. paracasei* DG and LPC-S01

In order for probiotics to be considered as safe, they should be free of transmissible antibiotic-resistance genes (FAO/WHO, 2002; EFSA, 2008), since antibiotic-resistant foodborne species can act as "reservoirs" of resistance genes and persist in the human host environment in the absence of selective pressure (Ammor, 2007; Comunian, 2010). On the basis of this precautionary principle and to prevent potential risks to human health, the European Food Safety Authority (EFSA) has proposed a framework for the safety evaluation of microorganisms in the food chain, similar in purpose to the Generally Recognised As Safe (GRAS) approach, taking into account experience of use (Qualified Presumption of Safety, QPS) (Gregoret, 2013).
We studied the antibiotic resistance of *L. paracasei* DG and LPC-S01 by the microdilution assay recommended by International Organization for Standardization (ISO, 2010) with reference to the EFSA breakpoints for *L. casei*/*paracasei* (EFSA, 2012). The minimal inhibitory concentrations for *L. paracasei* LPC-S01 and Shirota were below the breakpoints for all antibiotics (Tab. 1.3). Only strain DG displayed resistances to gentamycin, kanamycin and chloramphenicol slightly higher than EFSA breakpoints. In general, MICs were higher for *L. paracasei* DG than the other strains for most antibiotics. However, we did not find any acquired antibiotic resistance gene in the genome (chromosome and plasmids) of *L. paracasei* DG; moreover, subsequent experiments (described in Chapter 2) demonstrated that the modestly increased ability of strain DG to tolerate antibiotics is probably due to the presence of an abundant and peculiar exopolysaccharide, which partially hinders antibiotic penetration into the cell. In addition, it was reported that often lactobacilli have a high natural resistance to aminoglycosides, such as gentamicin and kanamycin (Bernardeau, 2008; Guglielmetti, 2010b). Therefore, the observed increased resistance of strain DG to certain antibiotics may be plausibly considered intrinsic and not associated to horizontally transmissible genetic elements.

Tab. 1.3. Antibiotic sensitivities of *Lactobacillus paracasei* strains determined according to the microdilution assay recommended by EFSA (2012). Data are reported in mg l^{-1}. MIC, minimal inhibitory concentration (MIC). *, MIC higher than EFSA breakpoint.

	EFSA breakpoint	DG	LPC-S01	Shirota
Ampicillin	2	2	2	0.25
Vancomycin	not required	>256	>256	>256
Gentamicin	32	64*	32	8
Kanamycin	64	<256*	32	64
Streptomycin	not required	32	32	32
Erythromycin	1	1	0.5	1
Clindamycin	1	0.25	0.062	0.062
Tetracycline	4	1	2	1
Chloramphenicol	4	8*	4	4
Amoxicillin	not indicated	8	1	0.5

Finally, the safety of *L. paracasei* strains was also estimated by assessing the bacterial decarboxylation of amino acids, which generates biogenic amines in food (Deepika Priyadarshani and Rakshit, 2014). According to our experiments, none of the tested *L. paracasei* strains produced histamine, tyramine, putrescine and cadaverine (data not shown), which are among the most common biogenic amines found in food products (Naila, 2010).

1.4.6. *L. paracasei* LPC-S01 and DG persistence in the gut of healthy adults

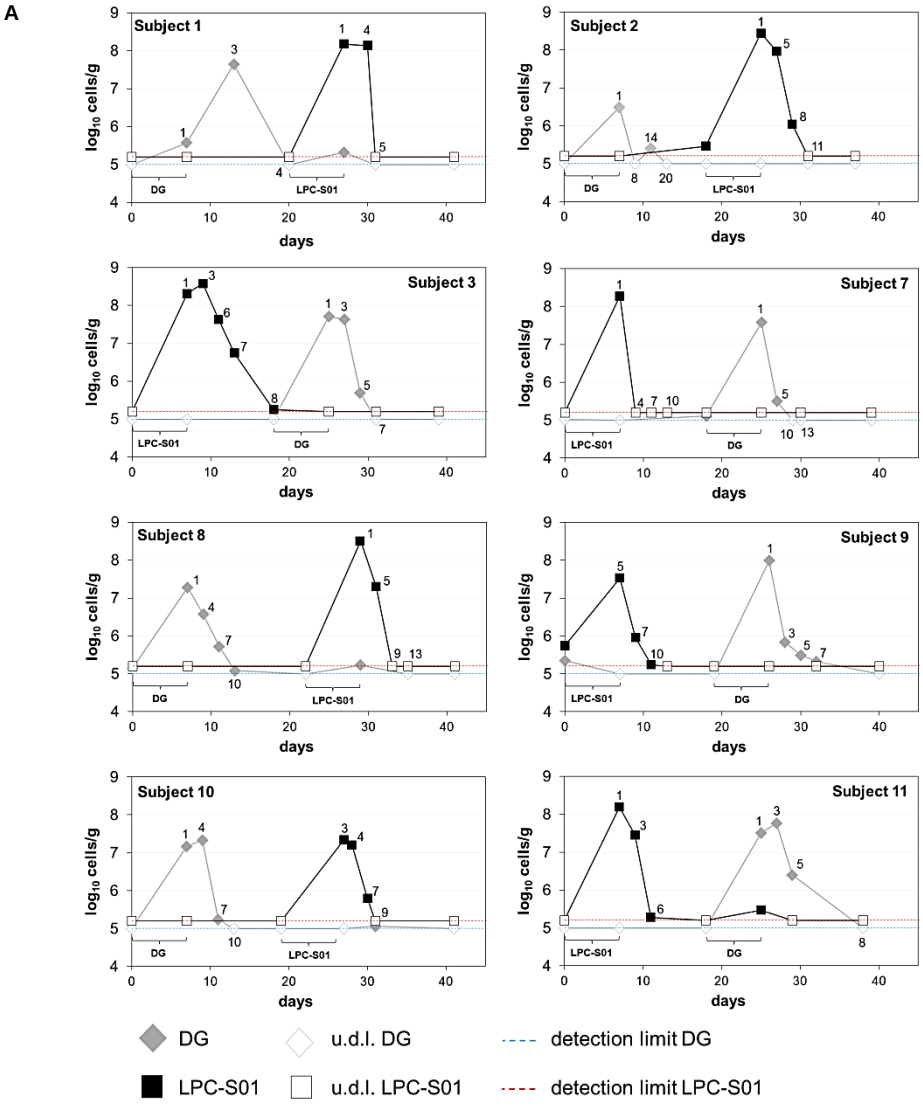
Ingested bacteria are exposed to the harsh environmental conditions in the human stomach (gastric acid) and small intestine (especially the proximal sections, which contain hydrolytic enzymes and bile salts), along with the highly diverse and competitive environment presented by the human gut microflora, which limit the ability of probiotic strains to persist in the GIT. As a consequence, distinction should be made between microorganisms capable of colonizing the human gut and those able to persist or remain metabolically active during passage through the gut (Tuohy, 2007). The acid tolerance and adhesion properties of LPC-S01 suggested that this bacterium might tolerate and adapt to the gastrointestinal environment. Effectively, probiotics can be administered vaginally or orally because lactobacilli can migrate passively from the rectum to the vagina via perineum (Vásquez, 2005). Consistently, bacteria colonizing the vaginal mucosa (both commensals and vaginosis-associated microbes) have been isolated from the rectum and the mouth, suggesting that gut and oral cavity act as extra-vaginal reservoirs of vaginal microbiota bacteria (van de Wijgert, 2014). Therefore, it appears plausible that the oral administration of probiotic bacteria may potentially influence the vaginal microbiota through two possible mechanisms: (i) modification of the intestinal microbiota (e.g., by reducing potentially harmful bacteria and increasing endogenous lactobacilli); (ii) direct migration to the vaginal mucosa via the gastrointestinal route. The time for this intervention to affect the vaginal tract is evidently longer than direct vaginal instillation, however an advantage of the oral approach is the possibility to deliver probiotics in foods and dietary supplements thus favouring the accomplishment of long-term treatments.

To test this hypothesis, we performed a pilot intervention trial, in which we compared the gut persistence of *L. paracasei* LPC-S01 with that of strain DG. We enrolled 11 healthy adult volunteers, who were asked to consume for 1 week a capsule per day containing LPC-S01 or DG cells. Out of 11 enrolled volunteers, 8 concluded the study. Three volunteers dropped out of the study due to non-conformity (n = 1) and protocol violation (missed probiotic consumption; n = 2) (Fig. 1.3). Capsules, which contained about 24 billion CFU of *L. paracasei*, were well tolerated by all participants and no adverse events were reported. In order to quantify *L. paracasei* cells in the faecal samples all over the trial, we used qPCR with primers targeting gene *welF* (specific for strain DG; Ferrario, 2014) and a gene coding for a hypothetical protein (specific for strain LPC-S01). We estimated that strain LPC-S01 reached a concentration of about 10^8 cells per g of faeces, persisting above the detection limit (10^5 cells per g of faeces) for about 5 days, which was not significantly dissimilar to the persistence of the reference *L. paracasei* DG, a strain already demonstrated to colonize the human gut of healthy adults and induce specific modifications in the microbiota composition (DeVecchi, 2008; Ferrario, 2014). In effect, according to repeated measure ANOVA, we did not find significant differences in the persistence of the two *L. paracasei* strains (Fig. 1.11). Our data are in agreement with other studies, which observed that the persistence of *L. paracasei* Shirota in the gastrointestinal tract of healthy adults is lower than 1 week after cessation of the probiotic ingestion (Tuohy, 2007; Wang, 2015).

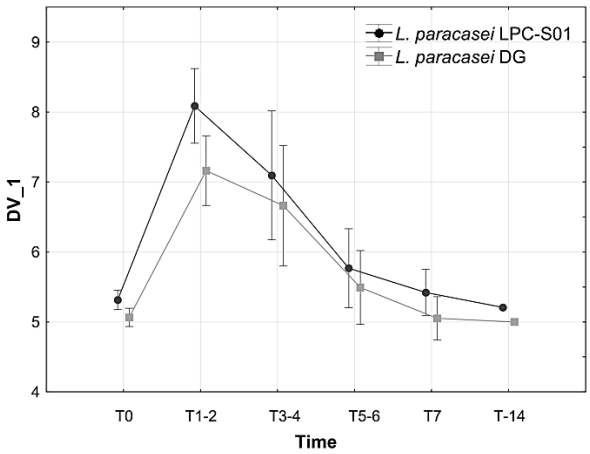
The persistence of a certain probiotic strain in the gut plausibly depends on the evacuation frequency. In our study, we found persistence of strains LPC-S01 and DG as average for 7

evacuations (Fig. 1.11A). No data correlating the day of persistence with the number of evacuations were found in literature.

Fig. 1.11. (A) Bacterial concentration of *L. paracasei* DG and LPC-S01 in the faecal samples collected during the study per single participant. **(B)** Average change in the faecal concentration of *L. paracasei* cells after the probiotic-intake period. Vertical bars denote 0.95 confidence intervals. Current effect according to repeated measures ANOVA performed to determine the statistical significance of the treatment × time interaction: F (5,65) = 0.794; p = 0.558.



B


L. paracasei LPC-S01
L. paracasei DG
DV_1
9
8
7
6
5
4
T0
T1-2
T3-4
T5-6
T7
T-14
Time

1.5 CONCLUSIONS

We explored the essential characteristics of *L. paracasei* DG and LPC-S01 (two strains already present on the market as food supplements), which can help to define them as potential probiotics. According to comparative genomic analyses, *L. paracasei* LPC-S01, a bacterium isolated from human vagina but plausibly having origin from the gut, possesses the genetic features of a niche-generalist member of its species. Similarly, also strain DG exhibits the potential ability to adapt to a wide range of environmental conditions if compared with strains of dairy origin. *In vitro* tests evidenced that the probiotic properties of strain LPC-S01 and DG, with respect to the reference probiotic strain *L. paracasei* Shirota, are equal for the bile tolerance and the reduction of NF-κB activation in Caco-2 cells. Strain LPC-S01, however, displayed a higher tolerance to gastric juice and higher capacity to adhere to Caco-2 epithelial cells (Shirota is unable to adhere on Caco-2-cells). We could confirm that both strains under study are safe for human consumption, since they do not contain any acquired antibiotic resistance and do not produce biogenic amines, and they can be administered in high number (24 billion CFU) to healthy people without adverse events. The pilot intervention trial results demonstrated that LPC-S01 and DG transiently colonize the gastrointestinal tract of the host, persisting for at least 5 days after the end of a 7-days oral consumption (corresponding in average to 7 evacuations).
Finally, we consider of particular interest the remarkable ecological adaptability demonstrated by the vaginal isolate *L. paracasei* LPC-S01: indeed, its safety and functional properties may support its use as probiotic to be administered *per os* for potential intestinal as well as vaginal applications. In this perspective, our future perspectives include the design of a clinical trial consisting on the oral administration of LPC-S01 to healthy adult women, in order to verify the potential ability of this probiotic bacterium to (i) modulate the intestinal and vaginal microbiota, and (ii) colonize the human vagina via gastrointestinal route.

1.6 REFERENCES

- Abriouel H et al., 2008, Biodiversity of the microbial community in a Spanish farmhouse cheese as revealed by culture-dependent and culture-independent methods. Int J Food Microbiol. 127:200-8.
- Adams MR and Marteau P, 1995, On the safety of lactic acid bacteria from food. Int J Food Microbiol. 27:263-4.
- Alikhan NF et al., 2011, BLAST Ring Image Generator (BRIG): simple prokaryote genome comparisons. BMC Genomics. 12:402.
- Allsworth JE and Peipert JF, 2007, Prevalence of bacterial vaginosis: 2001-2004 National Health and Nutrition Examination Survey data. Obstet Gynecol. 109:114-20.
- Ammor MS et al., 2007, Antibiotic resistance in non-enterococcal lactic acid bacteria and bifidobacteria. Food Microbiol. 24:559e570.
- Anderson DJ et al., 2014, The structure of the human vaginal stratum corneum and its role in immune defense. Am J Reprod Immunol. 71:618-23.
- Arumugam M, 2011, Enterotypes of the human gut microbiome. Nature. 473:174-80.
- Azaïs-Braesco V et al, 2010, Not all lactic acid bacteria are probiotics,...but some are. Br J Nutr. 103:1079–1081.
- Baquerizo Nole KL et al., 2014, Probiotics and prebiotics in dermatology. J Am Acad Dermatol. 71:814-21.
- Bastani P et al., 2012, Dairy Probiotic Foods and Bacterial Vaginosis: A Review on Mechanism of Action. In: Probiotics (Prof. Everlon Rigobelo Ed.), ISBN: 978-953-51-0776-7, InTech.
- Bernardeau M et al., 2008, Safety assessment of dairy microorganisms: the *Lactobacillus* genus. Int J Food Microbiol. 126:278–285.
- Borges S et al., 2014, The role of lactobacilli and probiotics in maintaining vaginal health. Arch Gynecol Obstet. 289:479–489.
- Boris S et al., 1998, Adherence of human vaginal lactobacilli to vaginal epithelial cells and interaction with uropathogens. Infect Immun. 66:1985–1989.
- Botes M et al., 2008, Adhesion of the probiotic strains *Enterococcus mundtii* ST4SA and *Lactobacillus plantarum* 423 to Caco-2 cells under conditions simulating the intestinal tract, and in the presence of antibiotics and anti-inflammatory medicaments. Arch. Microbiol. 190:573–584.
- Bover-Cid S and Holzapfel WH, 1999, Improved screening procedure for biogenic amine production by lactic acid bacteria. Int. J Food Microbiol. 53:33–41.
- Broadbent JR et al., 2012, Analysis of the *Lactobacillus casei* supragenome and its influence in species evolution and lifestyle adaptation. BMC Genomics. 13:533.
- Burns P et al, 2010, Inside the adaptation process of *Lactobacillus delbrueckii* subsp. *lactis* to bile. Int J Food Microbiol. 142:132–141.
- Caballero-Franco C et al., 2007, The VSL#3 probiotic formula induces mucin gene expression and secretion in colonic epithelial cells. Am J Physiol Gastrointest Liver Physiol. 292:315–322.

- Cai H et al., 2007, Genotypic and phenotypic characterization of *Lactobacillus casei* strains isolated from different ecological niches suggests frequent recombination and niche specificity. Microbiology 153:2655–2665.
- Cardona S et al., 2012, Storage conditions of intestinal microbiota matter in metagenomic analysis. BMC Microbiol. 12:158.
- Cario E et al., 2004, Toll-like receptor 2 enhances ZO-1-associated intestinal epithelial barrier integrity via protein kinase C. Gastroenterology. 127: 224–238.
- Cario E et al., 2007, Toll-like receptor 2 controls mucosal inflammation by regulating epithelial barrier function. Gastroenterology. 132:1359–1374.
- Champagne CP et al., 2011, Recommendations for the viability assessment of probiotics as concentrated cultures and in food matrices. Int J Food Microbiol. 149:185-93.
- Charlier C et al., 2009, Interactions between *Staphylococcus aureus* and lactic acid bacteria: an old
- Chenoll E et al., 2011, Novel probiotic *Bifidobacterium bifidum* CECT 7366 strain active against the pathogenic bacterium *Helicobacter pylori*. Appl Environ Microbiol. 77:1335-43.
- Chiang SS et al., 2012, Beneficial effects of *Lactobacillus paracasei* subsp. *paracasei* NTU 101 and its fermented products. Appl Microbiol Biotechnol. 93:903-16.
- Chieppa M et al., 2006, Dynamic imaging of dendritic cell extension into the small bowel lumen in response to epithelial cell TLR engagement. J Exp Med. 203: 2841–2852.
- Cho JH et al., 2008, The genetics and immunopathogenesis of inflammatory bowel disease. Nat Rev Immunol. 8:458-66.
- Collado MC, 2007, Role of commercial probiotic strains against human pathogen adhesion to intestinal mucus. Lett Appl Microbiol. 45:454-60.
- Collier-Hyams LS and Neish AS, 2005, Innate immune relationship between commensal flora and the mammalian intestinal epithelium. Cell Mol Life Sci. 62:1339-48.
- Comunian R et al., 2010, Susceptibility to tetracycline and erythromycin of *Lactobacillus paracasei* strains isolated from traditional Italian fermented foods. Int J Food Microbiol. 138:151e156.
- Cribby S et al., 2008, Vaginal microbiota and the use of probiotics. Interdiscip Perspect Infect Dis. 2008:256490
- D'Incà R et al., 2011, Rectal administration of *Lactobacillus casei* DG modifies flora composition and Toll-like receptor expression in colonic mucosa of patients with mild ulcerative colitis. Dig Dis Sci. 56:1178–1187.
- Dantzer R et al., 2008, From inflammation to sickness and depression: when the immune system subjugates the brain. Nat Rev Neurosci. 9:46-56.
- de LeBlanc Ade M et al., 2010, Anti-infective mechanisms induced by a probiotic *Lactobacillus* strain against *Salmonella enterica* serovar *Typhimurium* infection. Int J Food Microbiol. 138:223-31.
- De Vecchi E et al., 2008, *In vitro* screening of probiotic characteristics of some Italian products. J Chemother. 20:341–347.

- Deepika Priyadarshani WM and Rakshit SK, 2014, Growth and biogenic amine (histamine and tyramine) potential of probiotic *Lactobacillus casei* in skim milk. Am J Food Technol. 9:69–79.
- Dertli E et al., 2015, Impact of the exopolysaccharide layer on biofilms, adhesion and resistance to stress in *Lactobacillus johnsonii* FI9785. BMC Microbiol. 15:18.
- Didari T et al., 2015, Effectiveness of probiotics in irritable bowel syndrome: Updated systematic review with meta-analysis. World J Gastroenterol. 21:3072-84.
- D'Incà R et al., 2011, Rectal administration of *Lactobacillus casei* DG modifies flora composition and Toll-like receptor expression in colonic mucosa of patients with mild ulcerative colitis. Dig Dis Sci. 56:1178-87.
- Döderlein A, 1892, Das scheidensekret und seine bedeutung fur puerperalfieber. Zbl Bakt., 11:699.
- Donati L et al., 2010, Vaginal microbial flora and outcome of pregnancy. Arch Gynecol Obstet. 281:589–600.
- Duca FA et al., 2014, Replication of obesity and associated signaling pathways through transfer of microbiota from obese-prone rats. Diabetes. 63:1624-36.
- Dusko Ehrlich S and MetaHIT Consortium, 2011, "MetaHIT: the European Union Project on metagenomics of the human intestinal tract" in Metagenomics of the Human Body, Chap.15. ed. K. Nelson (Berlin:Springer).
- EFSA, 2012, Guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance. EFSA J. 10:2740.
- Ennahar S et al., 1996, Production of pediocin AcH by *Lactobacillus plantarum* WHE 92 isolated from cheese. Appl Environ Microbiol. 62:4381–4387.
- European Food Safety Authority (EFSA), 2008, Technical guidance prepared by the Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) on the update of the criteria used in the assessment of bacterial resistance to antibiotics of human or veterinary importance. EFSA Journal 732:1e15.
- FAO/WHO, 2002, Report of a joint FAO/WHO expert consultation on guidelines for the evaluation of probiotics in food. World Health Organization and Food and Agriculture Organization of the United Nations, London, Ontario, Canada.
- Farage MA et al., 2010, The possible relevance of sex hormones on irritant and allergic responses: their importance for skin testing. Contact Dermatitis. 62:67-74.
- Ferrario C et al., 2014, Modulation of fecal Clostridiales bacteria and butyrate by probiotic intervention with *Lactobacillus paracasei* DG varies among healthy adults. J Nutr. 144:1787–1796.
- Gillor O et al., 2005, Genetically engineered bacteriocins and their potential as the next generation of antimicrobials. Curr Pharm Des. 11:1067–1075.
- Gilmore TD, 2006, Introduction to NF-kappa B: players, pathways, perspectives. Oncogene. 25:6680e6684.
- Glanville J, 2015, A review of the systematic review process and its applicability for use in evaluating evidence for health claims on probiotic foods in the European Union. Nutr J. 14:16.

- Goldin BR and Gorbach SL, 2008, Clinical indications for probiotics: an overview. Clin Infect Dis. 46:S96–100.
- Gomez-Llorente C et al., 2010, Role of toll-like receptors in the development of immunotolerance mediated by probiotics. Proc Nutr Soc. 69:381e9.
- Granato D et al., 1999, Cell surface-associated lipoteichoic acid acts as an adhesion factor for attachment of *Lactobacillus johnsonii* La1 to human enterocyte-like Caco-2 cells. Appl Environ Microbiol. 65:1071–1077.
- Gregoret V et al., 2013, A comprehensive approach to determine the probiotic potential of human-derived *Lactobacillus* for industrial use. Food Microbiol. 34:19-28.
- Groschwitz KR and Hogan SP, 2009, Intestinal barrier function: molecular regulation and disease pathogenesis. J Allergy Clin Immunol. 124:3-20.
- Gueimonde M et al., 2007, Competitive exclusion of enteropathogens from human intestinal mucus by *Bifidobacterium* strains with acquired resistance to bile: a preliminary study. Int J Food Microbiol. 113:228–232.
- Guglielmetti S et al., 2008, Implication of an outer surface lipoprotein in adhesion of *Bifidobacterium bifidum* to Caco-2 cells. Appl Environ Microb. 74: 4695–4702.
- Guglielmetti S et al., 2010a, Oral bacteria as potential probiotics for the pharyngeal mucosa. Appl Environ Microbiol. 76:3948-58.
- Guglielmetti S et al., 2010b, 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.
- Hardy H et al., 2013, Probiotics, Prebiotics and Immunomodulation of Gut Mucosal Defences: Homeostasis and Immunopathology. Nutrients. 5:1869-1912.
- Holzapfel WH and Schillinger U, 2002, Introduction to pre- and probiotics. Food Res Int. 35:109-116 .
- Horn N et al., 2013, Spontaneous mutation reveals influence of exopolysaccharide on *Lactobacillus johnsonii* surface characteristics. PLoS ONE 8:e59957.
- ISO ,2010, Milk and Milk Products - Determination of the Minimal Inhibitory Concentration (MIC) of Antibiotics Applicable to Bifidobacteria and Non-enterococcal Lactic acid Bacteria (LAB). Geneva: International Organization for Standardization 10932:2010 (IDF 223:2010).
- Jang MH et al., 2004, Intestinal villous M cells: An antigen entry site in the mucosal epithelium. Proc Nat Acad Sci USA. 101:6110–6115.
- Khalid K, 2011, An overview of lactic acid bacteria. Int J Biosci 3:1-13
- Koirala R et al., 2015, Melting curve analysis of a groEL PCR fragment for the rapid genotyping of strains belonging to the Lactobacillus casei group of species. Microbiol Res. 173:50-8.
- Kwon HK et al., 2010, Generation of regulatory dendritic cells and CD4+Foxp3+ T cells by probiotics administration suppresses immune disorders. Proc Natl Acad Sci USA. 107:2159-64.
- Lavilla-Lerma L et al., 2013, Characterization of functional, safety, and gut survival related characteristics of *Lactobacillus* strains isolated from farmhouse goat's milk cheeses. Int J Food Microbiol. 163:136-45.

- Lebeer S et al., 2012, Functional analysis of *Lactobacillus rhamnosus* GG pili in relation to adhesion and immunomodulatory interactions with intestinal epithelial cells. Appl Environ Microbiol. 78:185–193.
- Lee SK et al., 2015, Immune cells in the female reproductive tract. Immune Netw. 15:16-26.
- Lee WJ and Hase K, 2014, Gut microbiota-generated metabolites in animal health and disease. Nat Chem Biol. 10:416-24.
- Lim SM and Ahn DH, 2012, Factors affecting adhesion of lactic acid bacteria to Caco-2 cells and inhibitory effect on infection of *Salmonella typhimurium*. J Microbiol Biotechnol. 22:1731-9.
- Machiels K et al., 2014, A decrease of the butyrate-producing species *Roseburia hominis* and *Faecalibacterium prausnitzii* defines dysbiosis in patients with ulcerative colitis. Gut. 63:1275-83.
- Mack DR et al., 1999, Probiotics inhibit enteropathogenic *E. coli* adherence *in vitro* by inducing intestinal mucin gene expression. Am J Physiol. 276:G941–G950.
- Marchesi JR et al., 2015, The gut microbiota and host health: a new clinical frontier. Gut. pii: gutjnl-2015-309990. (Epub ahead of print)
- Marteau PR, 2002, Probiotics in clinical conditions. Clin Rev Allergy Immunol. 22:255-73.
- Martín-Platero AM et al., 2009, Polyphasic study of microbial communities of two Spanish farmhouse goats' milk cheeses from Sierra de Aracena. Food Microbiol. 26:294-304.
- Matsumoto K et al., 2010, Effects of a probiotic fermented milk beverage containing *Lactobacillus casei* strain Shirota on defecation frequency, intestinal microbiota, and the intestinal environment of healthy individuals with soft stools. J Biosci Bioeng. 110:547–52.
- Maurice CF et al., 2013, Xenobiotics shape the physiology and gene expression of the active human gut microbiome. Cell. 152: 39–50.
- McFarland LV, 2015, From yaks to yogurt: the history, development, and current use of probiotics. Clin Infect Dis. 2:S85-90.
- Merk K et al., 2005, Lactobacilli-bacteria-host interactions with special regard to the urogenital tract. Int J Med Microbiol. 295:9-18.
- Metchnikoff E, 1908, Chapter V. Lactic acid as inhibiting intestinal putrefaction. In: The prolongation of life. Optimistic studies. New York, London: G.P. Putnam's Sons, 161–83.
- Milani C et al., 2013, Assessing the fecal microbiota: an optimized ion torrent 16S rRNA gene-based analysis protocol. PLoS ONE 8:e68739.
- Muñoz-Quezada S et al., 2013, Competitive inhibition of three novel bacteria isolated from faeces of breast milk-fed infants against selected enteropathogens. Br J Nutr. 2:S63-9.
- Naila A et al., 2010, Control of biogenic amines in food-existing and emerging approaches. J Food Sci. 75:R139–R150.
- Nanno M et al., 2011, Biological effects of probiotics: what impact does *Lactobacillus casei* shirota have on us? Int J Immunopathol Pharmacol. 24:45S-50S.
- Naseribafrouei A et al., 2014, Correlation between the human fecal microbiota and depression. Neurogastroenterol Motil. 26:1155-62.

- Natividad JM and Verdù EF, 2013, Modulation of intestinal barrier by intestinal microbiota: pathological and therapeutic implications. Pharmacol Res. 69:42-51.
- Nilsson, AC et al., 2008, Including indigestible carbohydrates in the evening meal of healthy subjects improves glucose tolerance, lowers inflammatory marks, and increases satiety after subsequent standardized breakfast. J Nutr. 138: 732–739.
- Ohigashi S et al., 2013, Changes of the intestinal microbiota, short chain fatty acids, and fecal pH in patients with colorectal cancer. Dig Dis Sci. 58:1717-26.
- Paineau D et al., 2008, Effects of seven potential probiotic strains on specific immune responses in healthy adults: a double-blind, randomized, controlled trial. FEMS Immunol Med Microbiol. 53:107-13.
- Parma M et al., 2014, Probiotics in the prevention of recurrences of bacterial vaginosis. Altern Ther Health Med. S1:52-7.
- Perez Chaia A and Oliver G, 2003, Intestinal microflora and metabolic activity. In: Gut Flora, Nutrition, Immunity and Health (eds R. Fuller and G. Perdigón), Blackwell Publishing Ltd, Oxford, UK.
- Perkins ND, 2007, Integrating cell-signalling pathways with NF-kappa B and IKK function. Nat Rev Mol Cell Biol. 8:49e62.
- Peterson J et al., 2009, The NIH Human Microbiome Project. Genome Res. 19:2317-23.
- Petrof EO et al., 2004, Probiotics inhibit nuclear factor-kappaB and induce heat shock proteins in colonic epithelial cells through proteasome inhibition. Gastroenterology 127:1474-87.
- Pham M et al., 2008, Probiotics: sorting the evidence from the myths. Med J Aust. 188:304–8.
- Polak-Berecka M et al., 2014, The effect of cell surface components on adhesion ability of *Lactobacillus rhamnosus*. Antonie Van Leeuwenhoek 106:751–762.
- Pudney J et al., 2005, Immunological microenvironments in the human vagina and cervix: mediators of cellular immunity are concentrated in the cervical transformation zone. Biol Reprod. 73:1253-63.
- Qin J et al., 2010, A human gut microbial gene catalogue established by metagenomic sequencing. Nature. 464:59–65.
- Rakoff-Nahoum S et al., 2004, Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis. Cell. 118:229-41.
- Rampersaud R et al., 2012, Microbiota of the upper and lower genital tract. Semin Fetal Neonatal Med. 17:51–57.
- Redondo-Lopez V et al., 1990, Emerging role of lactobacilli in the control and maintenance of the vaginal bacterial microflora. Rev Infect Dis. 12:856–872.
- Reid G and Bocking A, 2003, The potential for probiotics to prevent bacterial vaginosis and preterm labor. Am J Obstet Gynecol. 189:1202–1208.
- Reid G et al., 2003, Potential uses of probiotics in clinical practice. Clin Microbiol Rev. 16:658–72.
- Reid G et al., 2006, Selecting, testing and understanding probiotic microorganisms. FEMS Immunol Med Microbiol. 46:149–157.

- Reid G., 2005, The importance of guidelines in the development and application of probiotics. Curr Pharm Des. 11:11-6.
- Ren D et al., 2014, *In vitro* evaluation of the probiotic and functional potential of *Lactobacillus* strains isolated from fermented food and human intestine. Anaerobe. 30:1-10.
- Rescigno M et al., 2001, Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria. Nat Immunol. 2:361–367.
- Resta-Lenert S et al., 2003, Live probiotics protect intestinal epithelial cells from the effects of infection with enteroinvasive *Escherichia coli* (EIEC). Gut. 52:988-97.
- Rijkers GT et al., 2010, Guidance for substantiating the evidence for beneficial effects of probiotics: current status and recommendations for future research. J Nutr. 140:671S-6S.
- Ruas-Madiedo P et al., 2009, Bile affects the synthesis of exopolysaccharides by *Bifidobacterium animalis*. Appl Environ Microbiol. 75:1204–1207.
- Ruiz L et al., 2007, Cell envelope changes in *Bifidobacterium animalis* ssp. l*actis* as a response to bile. FEMS Microbiol Lett. 274:316–322.
- Saez-Lara MJ et al., 2015, The role of probiotic lactic acid bacteria and bifidobacteria in the prevention and treatment of inflammatory bowel disease and other related diseases: a systematic review of randomized human clinical trials. Biomed Res Int. 2015:505878.
- Santosa S et al., 2006, Probiotics and their potential health claims. Nutr Rev. 64:265–74.
- Scaldaferri F et al., 2013, Gut microbial flora, prebiotics, and probiotics in IBD: their current usage and utility. Biomed Res Int. 2013:435268.
- Scarpellini E et al., 2008, Probiotics: which and when? Dig Dis. 26:175–82.
- Senok AC et al., 2005, Probiotics: facts and myths. Clin Microbiol Infect. 11:958–66.
- Sgouras D et al., 2004, *In vitro* and *in vivo* inhibition of *Helicobacter pylori* by *Lactobacillus casei* strain Shirota. Appl Environ Microbiol. 70:518-26.
- Shang L et al., 2008, Toll-like receptor signaling in small intestinal epithelium promotes B-cell recruitment and IgA production in lamina propria. Gastroenterology, 135:529–538.
- Singh N et al., 2014, Activation of Gpr109a, receptor for niacin and the commensal metabolite butyrate, suppresses colonic inflammation and carcinogenesis. Immunity. 40:128-39.
- Smokvina T et al., 2013, *Lactobacillus paracasei* comparative genomics: towards species pan-genome definition and exploitation of diversity. PLoS ONE 8:e68731.
- Stuknyte M et al., 2011, Potential immunomodulatory activity of bovine casein hydrolysates produced after digestion with proteinases of lactic acid bacteria. Int Dairy J. 21:763–769.
- Swanson PA 2nd et al., 2011, Enteric commensal bacteria potentiate epithelial restitution via reactive oxygen species-mediated inactivation of focal adhesion kinase phosphatases. Proc Natl Acad Sci USA. 108:8803-8.
- Szajewska H and Chmielewska A, 2013, Growth of infants fed formula supplemented with *Bifidobacterium lactis* Bb12 or *Lactobacillus* GG: a systematic review of randomized controlled trials. BMC Pediatr. 13:185.

- Taverniti V et al., 2013, S-layer protein mediates the stimulatory effect of *Lactobacillus helveticus* MIMLh5 on innate immunity. Appl Environ Microbiol. 79:1221–1231.
- Tsai YT et al., 2008, Time-dependent persistence of enhanced immune response by a potential probiotic strain *Lactobacillus paracasei* subsp. *paracasei* NTU 101. Int J Food Microbiol. 128:219-25.
- Tsai YT et al., 2010, Effect of the administration of *Lactobacillus paracasei* subsp. *paracasei* NTU 101 on Peyer's patch-mediated mucosal immunity. Int Immunopharmacol. 10:791-8.
- Tuohy KM et al., 2007, Survivability of a probiotic *Lactobacillus casei* in the gastrointestinal tract of healthy human volunteers and its impact on the faecal microflora. J Appl Microbiol. 102:1026–1032.
- Turnbaugh PJ et al., 2006, An obesity-associated gut microbiome with increased capacity for energy harvest. Nature. 444:1027-31.
- Tursi A et al., 2013, Randomised clinical trial: mesalazine and/or probiotics in maintaining remission of symptomatic uncomplicated diverticular disease—a double-blind, randomised, placebo-controlled study. Aliment Pharmacol Ther. 38:741–751.
- van de Wijgert JH et al., 2014, The vaginal microbiota: what have we learned after a decade of molecular characterization? PLoS ONE. 9:e105998.
- van den Nieuwboer M et al., 2015, Improving the bowel habits of elderly residents in a nursing home using probiotic fermented milk. Benef. Microbes 17:1–8.
- Vanderhoof JA and Young R, 2008, Probiotics in the United States. Clin Infect Dis. 46:S67–72.
- Vankerckhoven V et al., 2008, Biosafety assessment of probiotics used for human consumption: recommendations from the EU-PROSAFE project. Trends Food Sci Tech. 19:102-114.
- Varankovich NV et al., 2015, Probiotic-based strategies for therapeutic and prophylactic use against multiple gastrointestinal diseases. Front Microbiol 6:685.
- Vásquez A et al., 2005, Oral administration of *Lactobacillus* and *Bifidobacterium* strains of intestinal and vaginal origin to healthy human females: re-isolation from faeces and vagina. Microb Ecol Health Dis. 17:15–20.
- Vera Pingitore E et al., 2009, Characterization of salivaricin CRL 1328, a two-peptide bacteriocin produced by *Lactobacillus salivarius* CRL 1328 isolated from the human vagina. Res Microbiol. 160:401–408.
- Verdù EF et al., 2004, *Lactobacillus paracasei* normalizes muscle hypercontractility in a murine model of post-infective gut dysfunction. Gastroenterology 127: 826-837.
- Verdú EF et al., 2009, Effect of probiotics on gastrointestinal function: evidence from animal models. Therap Adv Gastroenterol. 2:31-5.
- Waldeck F, 1990, Funktionen des Magen-Darm-Kanals. In: Schmidt RF, Thews G, Physiologie des Menschen (24. Aufl.) Springer-Verlag, Berlin
- Wallace TC et al., 2011, Human gut microbiota and its relationship to health and disease. Nutr Rev. 69:392-403.
- Wang R et al., 2015, Survival of *Lactobacillus casei* strain Shirota in the intestines of healthy Chinese adults. Microbiol Immunol. 59:268–276.

- Williams NT, 2010, Probiotics. Am J Health Syst Pharm. 67:449-58.
- Wira CR, 2014, Regulation of mucosal immunity in the female reproductive tract: the role of sex hormones in immune protection against sexually transmitted pathogens. Am J Reprod Immunol. 72:236-258.
- Zambelli P et al., 2014, Production of fructooligosaccharides by myceliumbound transfructosylation activity present in *Cladosporium cladosporioides* and *Penicilium sizovae*. Process Biochem. 49:2174–2180.
- Zambelli P et al., 2016, An efficient continuous flow process for the synthesis of a non-conventional mixture of fructooligosaccharides. Food Chem. 190:607-13.
- Zhong Y et al., 2012, Effects of probiotics, probiotic DNA and the CpG oligodeoxynucleotides on ovalbumin-sensitized brown-Norway rats via TLR9/NF- k B pathway. FEMS Immunol Med Microbiol. 66:71e82.

2 CHARACTERIZATION OF *LACTOBACILLUS PARACASEI* EXOPOLYSACCHARIDES AND EVALUATION OF THEIR ABILITY TO MODULATE IMMUNE RESPONSES *IN VITRO*

2.1 STATE OF THE ART

2.1.1. Overview on exopolysaccharides

Exopolysaccharides are long-chain carbohydrate polymers released by a wide range of microorganisms, including yeasts, moulds, microalgae and bacteria (Donot, 2012). In general, the exocellular polymers comprise the capsular polysaccharides, which form a cohesive layer or capsule covalently linked to the cell surface, and the exopolysaccharides (EPS), which form a slime layer loosely attached to the cell surface or secreted into the environment. Some bacteria produce only one type of polysaccharide, whereas, in some cases, they can produce both forms (Nwodo, 2012; Degeest, 2001; Ruas-Madiedo and de los Reyes-Gavilán, 2005). The history of bacterial exopolysaccharides began during the mid-20th century with the discovery of an exopolysaccharide in wine, which would later be known as dextran and the prokaryote responsible for the production was identified as *Leuconostoc mesenteroides* (Linker and Jones, 1966). Over the course of time, other exopolysaccharides have been discovered, including cellulose, alginate and xanthan. Most of the LAB producing EPS belong to the genera *Streptococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, and *Pediococcus*. Production of EPS is also reported for bifidobacteria (Patel and Prajapati, 2013; Hidalgo-Cantabrana, 2014).

2.1.2. Classification of exopolysaccharides on the basis of the chemical structure

The overwhelming diversity of bacterial polysaccharides allows categorizing them based on chemical structure, functionality, molecular weight and linkage bonds. Following the chemical composition, exopolysaccharides can be further classified as homopolysaccharides (HoPS) and heteropolysaccharides (HePS). HoPS are the polymeric form of one type of monosaccharide unit, and can be clustered into three groups according to the linkage bonds and the monomeric unit: α-D-glucans, β-D-glucans, and fructans. They exhibit molecular weights ranging from 4×10^4 to 2×10^7 Da, and the most common glycosidic bonds are 1-6, 1-3 and 2-6 linkages. Glucans vary in their degrees of branching and, consequently, result in the formation of different polymer types: for instance, dextran, mutan, and alternan are α-glucans, whereas levan and inulin-type HoPS are fructans (Ruas-Madiedo and de los Reyes-Gavilán, 2005).
On the other hand, HePS are composed of repeating units, varying in size from disaccharides to octasaccharides, and demonstrate little structural similarity to one another (Tab. 2.1). The monomers forming the repeating unit include D-glucose, D-galactose, L-rhamnose and, in some cases, N-acetylglucosamine (GlcNAc), N-acetylgalactosamine (GalNAc), or glucuronic acid (GlcA). Non-carbohydrate substituent such as phosphate, acetyl and glycerol are sometimes present (Ruas-Madiedo, 2002; Hidalgo-Cantabrana, 2012). Bonds between monomeric units at the backbone of the polymers are 1,4-β- or 1,3-β- linkages and 1,2-α- or 1,6-α- linkages. The former are characterized by strong rigidity whereas the latter are more flexible. In spite of their heterogenic composition, HePS exhibit a smaller variability in size, ranging from 1×10^4 to 6×10^6 Da (Badel, 2011).
It is easily deducible that the great variety in bacterial EPSs is conferred by the presence of different isomers, linkage types, and organic or inorganic monosaccharide substituents that could be combined in repeating units which have, as well, different degrees of polymerization and branching patterns. Moreover, depending on the substituents present in the repeating units of HePSs, we can distinguish among non-charged, charged or neutral polymers. Within the latter, a special mention goes to the "zwitterionic" EPSs, characterized by having both positively (e.g. free amine) and negatively (e.g. phosphate or carboxylate) charged moieties within their repeating units. These molecules have been reported to be able to modulate the immune system,

both innate and adaptive response, although they are very rare among bacteria (Hidalgo-Cantabrana, 2012; McLoughlin and Kasper, 2009).

Tab. 2.1. Monomer composition of heteropolysaccharides produced by a number of representative *Lactobacillus* strains (Patten and Laws, 2015).

Strain	**Monosaccharide composition**
L. acidophilus 5e2	glucose, galactose, N-acetyl-D-glucosamine (3:3:1)
L. acidophilus LMG9433	glucose, galactose (4:1)
L. brevis	glucose, galactose (3:3)
L. casei CG11	glucose, rhamnose (variable)
L. delbrueckii subsp. *bulgaricus* 291	glucose, galactose (3:3)
L. delbrueckii subsp. *bulgaricus* EU23	rhamnose, glucose (3:3)
L. delbrueckii subsp. *bulgaricus* LY03, 24, 25	galactose, glucose, rhamnose (5:1:1)
L. delbrueckii subsp. *bulgaricus* NCFB2074	glucose, galactose (3:3)
L. delbrueckii subsp. *bulgaricus* NCDC 285	rhamnose, galactose (1:7)
L. fermentum V10	glucose, rhamnose, galactose (1:13: 2)
L. helveticus MB2-1	EPS 1: galactose, glucose and mannose (1:3:1) EPS 2: galactose, glucose and mannose (1:1:9) EPS 3: galactose, glucose and mannose (1:1:3)
L. helveticus subsp. Rosyjski	glucose, galactose, N-acetyl-D-mannosamine (2:2:1)
L. johnsonii 151	galactose, glucose (3:2)
L. johnsonii FI9785	EPS-2: glucose, galactose (4:2)
L. kefiranofaciens WT-2B	glucose, galactose (3:3)
L. mucosae DPC 6426	mannose, glucose, galactose (3:2:2)
L. plantarum EP56	glucose, galactose and rhamnose (3:1:1)
L. plantarum MTCC 9510	glucose, mannose (2:1)
L. rhamnosus C83	glucose, galactose (2:3)
L. rhamnosus GG	galactose, rhamnose, N-acetyl-D-glucosamine (4:1:1)
L. rhamnosus KF5 S1	S1: glucose, arabinose, glucosamine, galactosamine, galactose (2:1:1:1:1) S2: rhamnose, glucose, galactose (2:1:1)
L. rhamnosus R	glucose, galactose, rhamnose (2:1:4)
L. rhamnosus RW-9595M	rhamnose, glucose, galactose, pyruvate (4:2:1:1)
L. sakei O-1	glucose, rhamnose (3:2)

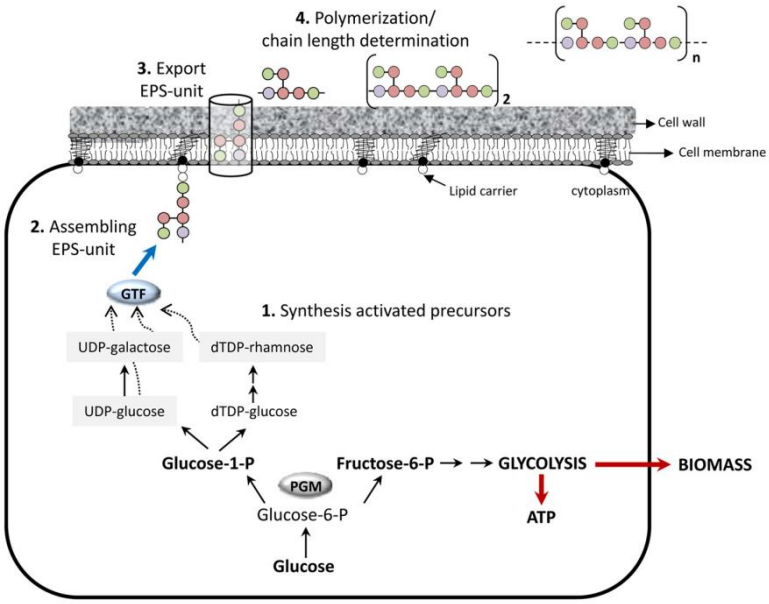
2.1.3. Biosynthetic pathways leading to the production of EPSs in lactic acid bacteria

The differences between homopolysaccharides and heteropolysaccharides do not reside exclusively in the chemical structure but also in enzymes and site of their synthesis.
Concerning HoPS, their synthesis requires the presence of sucrose as substrate, which donates the corresponding glycosyl moiety in a reaction catalysed by the glycansucrases, members of the glycoside hydrolase (GH) family and referred to as glucansucrases and fructansucrases (family GH68 and GH70, according to CAZy classification), involved in the polymerization of α-glucans and β-fructans, respectively. This reaction occurs extracellularly, where the sucrase catalyses the transfer of the released monosaccharides to an acceptor molecule in creating a glycosidic bond. The energy liberated by degradation of sucrose present in the culture medium is employed to catalyse the transfer of a glycosyl residue on forming the polysaccharide (van Hijum, 2006; Badel, 2011). On the other hand, β-glucans are less frequent in LAB, and to date, the few that have been described have the same structure: 1,3-β-D-glucans with side ramifications of a single

1,2-linked β-D-glucose (Zeković, 2005; Kearney, 2011). These β-glucans are synthetized through a different mechanism in which another single enzyme type, a glucosyltransferase, which does not use sucrose as substrate, is involved (Dols-Lafarge, 2008). This enzyme belongs to the COG1215 membrane-bound glycosyltransferase family; topology predictions show that a conserved cytosolic domain is flanked by two and four transmembrane segments (Werning, 2006; Hidalgo-Cantabrana, 2014). The formation of HoPS can occur separately from the bacterial cell or within its cell wall (Donot, 2012).

In contrast, HePS biosynthesis occurs mainly within the bacterial cell and is altogether a more complex process (summarized in Fig. 2.1), which can be separated into four distinct stages:
1) Active transport of the sugar into the bacterial cytoplasm through protein complexes, such as the phosphoenolpyruvate (PEP)–sugar phosphotransferase system (PTS) (Postma, 1993). During the transmembrane PEP-PTS transport, free-state sugar molecules are phosphorylated to their sugar-6-phosphate form.
2) Synthesis of sugar-1-phosphates via phosphomutase enzymes. Phosphoglucomutase (PGM), the enzyme involved in the conversion of glucose-6-phosphate to glucose-1-phosphate, potentially has an important role in the bifurcation of the flux towards the catabolic (e.g. formation of fructose-6-phosphate and, consequently, glycolysis with biomass and ATP formation) or the anabolic pathways (biosynthesis of sugar nucleotides, the precursors of EPSs). Alternatively, sugar molecules may also enter the bacterial cell in a pre-phosphorylated state (Welman and Maddox, 2003; Torino, 2005).
3) Activation and coupling of sugars with nucleotides. As an example, UDP–glucose and dTDP–glucose are formed via the action of UDP–glucose pyrophosphorylase and dTDP–glucose pyrophosphorylase, respectively. These sugar nucleotides are probably bound to a phosphorylated lipid carrier on the cytoplasmic face of the bacterial cell wall via the enzymatic action of a membrane-associated priming glycosyltransferase (GTF). Following the attachment, a series of additional EPS-specific GTF enzymes catalyses the synthesis of the repeated unit (Lebeer, 2009).
4) Exportation of the repeated unit from the cytoplasmic face to the periplasmic face of the membrane after the attachment on a C55-isoprenoid–lipid carrier linked to the cytoplasmic membrane and the intervention of a flippase enzyme. The subsequent polymerization occurs extracellularly (Lebeer, 2009; Welman and Maddox, 2003).
As the biosynthesis of homopolysaccharides only requires one enzyme, a single gene encoding the appropriate enzyme is present (Korakli and Vogel, 2006; van Hijum, 2006). Conversely, those strains which synthesize heteropolysaccharides require the combined action of a series of gene products, in order to reach the complexity of the chemical and structural composition of the repeating units. This complexity is also reflected in the organization of the genes encoding the proteins involved in their synthesis, which are present in *eps* clusters, located within the chromosomal DNA or on plasmids (Suresh Kumar, 2007). Specifically, heteropolysaccharides *eps* gene clusters are typically arranged in operons with all the genes orientated in the same direction, having a high coding density and a highly conserved structural–functional organization. (Jolly and Stingele, 2001; Lebeer, 2008). This pattern is, in some cases, characterized by the subsequent categories: (i) regulation, (ii) chain length determination, (iii) biosynthesis of the repeating unit, and (iv) polymerization and export (Lebeer, 2008).
Mobile elements are often located bordering the *eps* clusters, which could explain the instability of HePS production phenotype in some LAB (De Vuyst, 2011; Ruas-Madiedo, 2009).

Fig. 2.1. Hypothetical heteropolysaccharide biosynthesis pathway. GTF, glycosyltransferase; PMG, phosphoglucomutase (Hidalgo-Cantabrana, 2014).



2.1.4. Importance of the exopolysaccharides produced by LAB

The polysaccharides derived from different LAB show large variation in composition, charge, spatial arrangement, rigidity and ability to interact with proteins: for this reason, several functions have been attributed to these macromolecules.

2.1.4.1. Functions of EPSs in bacteria

EPSs play a major role in bacterial biofilm structure allowing the stabilization of the 3D structure, helping to withstand shear forces to minimize the intercellular repulsions among the bacteria by shielding the electrostatic charges on their surfaces (Watnick and Kolter, 1999; Sutherland, 2001). In addition to a structural role in biofilms, EPSs are thought to be responsible for the enhancement of nutrient and water entrapping and have also been shown to protect bacteria towards environmental threats, such as bacteriophages, antibiotics, lysozyme, metal ions and acidic conditions (Patten and Laws, 2015; Badel, 2011). Another indirect function of EPSs is that they facilitate the communication between cells because they aggregate bacteria and decrease cell-to-cell distances. Moreover, in various pathogens, EPS molecules (specifically, the capsular fraction) act as shield of other cell surface effector molecules recognized by the host pattern recognition receptors (PRRs), thus impeding macrophage and neutrophil-mediated phagocytosis (Lebeer, 2010).

2.1.4.2. Technological applications

Polysaccharides may function in foods as viscosifying agents, stabilizers, emulsifiers, gelling agents, or water-binding agents. Concerning EPSs from LAB, they have found their most valuable application in the improvement of the rheology, texture and "mouthfeel" of fermented milk products, such as yoghurts, as well as the reduction of syneresis. Indeed, although having no taste on their own, EPSs increase the time the milk product spends in the mouth, and hence impart an enhanced perception of taste (Duboc and Mollet, 2001). Moreover, there is a high consumer demand for smooth and creamy yoghurt products, which is typically met by increasing the content of fat, sugars, proteins or stabilizers (e.g. pectin, starch, alginate or gelatine). Afterwards, the consumer demand for products with low fat or sugar content and low levels of additives, as well as cost factors, makes EPSs a valuable alternative (Jolly, 2002). Among others, they have long been used in traditional fermented drinks, such as Kefir, or in the Scandinavian fermented milk drinks Viili (Vinderola, 2006; Ruas-Madiedo, 2006).
Even if there are no defined chemical characteristics that allow to predict the viscosity of an EPS molecule, some generalized trends have, however, become apparent: high concentrations, high specific volumes, chemical composition, chain length, structure of the subunits, molar mass and radius of gyration of the EPS molecule determine the physical characteristics and thereby their viscosity-intensifying properties (Ruas-Madiedo and de los Reyes-Gavilán, 2005; Tuinier, 1999). Low yields of EPS (corresponding normally to about 0.8-2.5 g/l) can be optimized by modulating the fermentation conditions, such as the carbon source and its quantities, pH, temperature, and salt concentrations (Patten and Laws, 2015; Årsköld, 2007; Mende, 2012). In general, long chains of subunits (high molecular mass) and/or stiff chains are required to improve viscosity. As mentioned above, backbone linkages of the 1,4-β type, as found in *Lactococcus lactis* subsp. *cremoris* B40, for example, result in stiffer chains than 1,4-α or 1,3-β linkages (Laws and Marshall, 2001).

2.1.4.3. Physiological benefits in the host

2.1.4.3.1. Prebiotic effects

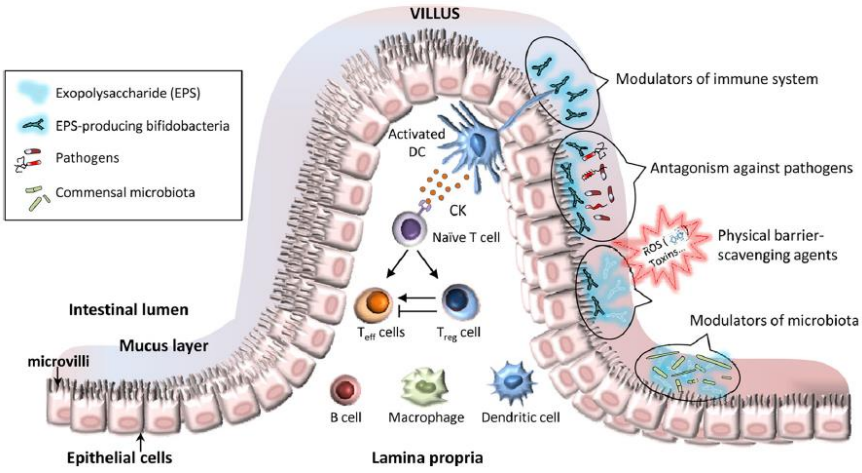
EPSs are not hydrolysed by host digestive enzymes, and therefore allow probiotic bacteria to overcome the environmental challenges and to persist for longer periods in the gut, thus enhancing their colonization (Fanning, 2012). An early study by Ruijssenaars (2000) demonstrated the resistance to biodegradation of EPSs from strains *L. sakei* O-1 and *L. helveticus* Lh59, thus suggesting that EPSs could survive the enzymatic stresses of the colonic milieu. Additionally, Hongpattarakere (2012) documented the ability of EPSs isolated from *L. plantarum* A3 to highly resist *in vitro* gastric and intestinal digestion. However, once the EPSs arrive in the colon lumen, they should then be able to selectively stimulate the growth of those bacterial species capable of eliciting a beneficial effect on the host. Bifidogenic activities have been observed for EPSs isolated from *L. rhamnosus* E/N and *L. plantarum* DM5 (Polak-Berecka, 2013; Das, 2014). It is clear that *Lactobacillus*-produced EPSs show some promises as prebiotic biopolymers *in vitro*, nevertheless, these effects have yet to be observed *in vivo*.

2.1.4.3.2. Immunomodulatory activity

An increasing number of studies on the biological effects of LAB EPSs is demonstrating the ability of these polymers to interact with the host immune system. Figure 2.2 summarizes the pathways and immune cells involved in the effects EPSs might exert at intestinal level.

Most investigations of EPSs immunoregulatory effects have focused on macrophages: initial studies in this field described their mitogenic activities, as observed for EPSs isolated from *L. rhamnosus* RW-9595M and *L. delbrueckii* subsp. *bulgaricus* 1073R-1, which significantly promoted the proliferation of human and murine macrophages (Chabot, 2001; Kitazawa, 1998). The capacity of *L. delbrueckii* subsp. *bulgaricus* 1073R-1 to trigger immune responses and mitogenic effects has been attributed to the acidic portion of its HePS, specifically to the phosphate group attached to one monomer of the repeated unit, as confirmed by the reduction of the stimulatory effect determined by chemical dephosphorylation of the EPS molecule (Nishimura-Uemura, 2003; Kitazawa, 1998).
Regarding the high-molecular weight (HMW) EPS isolated from *L. rhamnosus* RW-9595M, it has also been demonstrated the inhibition of TNF-α, IL-6 and IL-12 expression and the stimulation of IL-10 secretion in murine peritoneal macrophages, thus suggesting an anti-inflammatory role for this molecule (Bleau, 2010). In addition, *L. paracasei* Shirota harbours an *eps* cluster of 10 genes which is involved in the synthesis of a HMW cell-wall polysaccharide capable of suppressing inflammatory responses; Yasuda (2008) showed that knockout-mutants of *eps* genes were able to induce the production of TNFα, IL-12, IL-10 and IL-6 in mouse macrophages (RAW-264.7) and mouse spleen cells, to a higher extent than the wild-type bacterium. It has been proposed that this kind of inhibitory activity towards the activation of pro-inflammatory responses can be attributed to the HMW nature of the EPS (Hidalgo-Cantabrana, 2012). On the contrary, EPSs isolated from *L. paracasei* subsp. *paracasei* NTU101 and *L. plantarum* NTU102 exhibited only pro-inflammatory effects on macrophages, stimulating the expression of IL-6 and TNF-α (Liu, 2011). Finally, Vinderola (2006) demonstrated that the oral administration of kefiran, the EPS isolated from *Lactobacillus kefiranofaciens*, increased the secretion of luminal IgA in the small and large intestine in BALB/C mice, thus suggesting a potential role in the stimulation of the adaptive immunity and, specifically, of B-cell-dependent humoral (antibody-mediated) immunity.

Fig. 2.2. Beneficial activities potentially attributed to exopolysaccharides. CK, cytokines; DC, dendritic cell; T_{eff} cells, lymphocyte T effector cells; T_{reg} cell, lymphocyte T regulatory cell; B cell, lymphocyte B cell; ROS, reactive oxygen species (Hidalgo-Cantabrana, 2014).



2.2 AIMS OF THE STUDY

In light of (i) the importance of exopolysaccharides in technological applications and, more interestingly, in the cross-talk between bacteria and host, and (ii) the presence of putative *eps* operons in the genome, we investigated the potential EPS producing properties of *L. paracasei* DG and LPC-S01. To this aim, we performed an extraction and purification protocol in order to obtain a clean exopolysaccharides fraction, which was further employed to identify the repeating unit composing the polymer by means of NMR spectrometry and other analytical chromatographic methods. The purified molecule was then used to assess its immunomodulatory potential on the transfected pNiFty2-Luc Caco-2 cell line and on two models of human macrophages, namely U937 and THP-1.

2.3 MATERIALS AND METHODS

2.3.1. Bacterial strains and culture conditions

Lactobacillus paracasei strains DG and LPC-S01 were grown at 37° C in de Man-Rogosa-Sharpe (MRS) broth (Difco Laboratories Inc., Detroit, MI) for 24 h. This culture was used to inoculate 500 ml-1.5 l of chemically defined medium (CDM, Tab. 2.2).

Tab. 2.2 Chemically defined medium (CDM) used to cultivate *L. paracasei.*

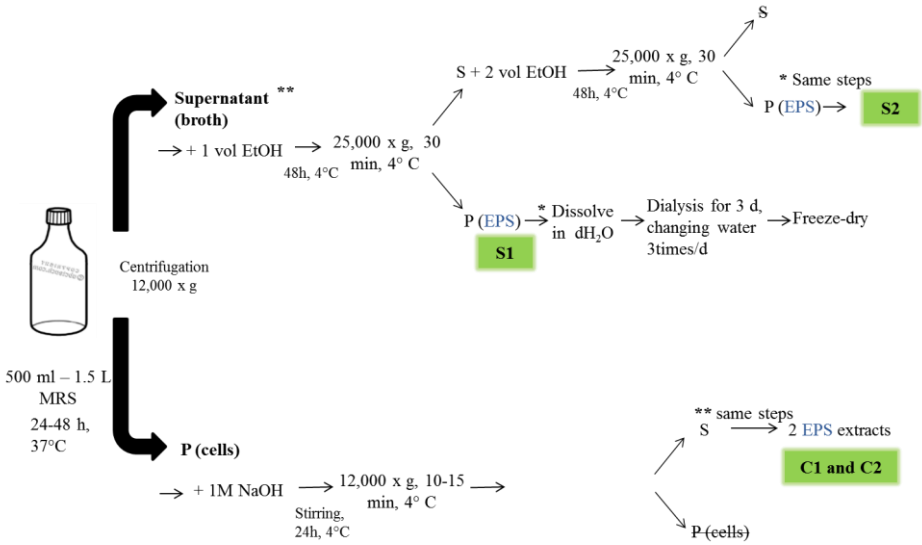
	Medium ($g\ l^{-1}$)
Sol. 1	
$(NH_4)_2SO_4$	2
$MgSO_4$ x 7 H_2O	0.15
$MnSO_4$ x 4 H_2O	0.02
Sol. 2	
Adenine	0.005
Pyridoxal	0.002
Nicotinic acid	0.001
Ca^{2+}-D-pantothenate	0.001
Riboflavin	0.001
Thiamine	0.001
Vitamin B12	0.000001
Biotin	0.00001
p-aminobenzoic acid	0.000005
Folic acid	0.00001
Sol. 4 *	
Guanine	0.005
Xanthine	0.005
Uracil	0.005
Sol. 5	
K_2HPO_4	4.56
Sol. 6	
Sodium acetate	0.05
Sodium citrate	0.02
KH_2PO_4	0.01
NaCl	0.002
$CaCl_2$	0.002
Sol. 7	
Tween 80	1
Tween 20	1
Glycerol	1
Glucose	20
Casaminoacids	1

* Dissolved in 1M NaOH. An equal volume of 1M HCl was added to the medium to neutralize the pH.

2.3.2. EPS isolation and purification

The multistep extraction and purification of EPS was performed from 0.5-1.5 l CDM supplemented with 2% glucose. After growth at 37° C for 48 h, cells were collected by centrifugation at 12,000 x g for 15 min at 4°C (Avant J-26 XPI, Beckman Coulter Ltd, High Wycombe, UK) and separated from the exhausted medium. The two fractions were then treated separately. The exhausted medium was added with an equal volume of absolute ethanol and stored at 4°C for 48 h. After storage, it was centrifuged at 25,000 x g for 35 min at 4°C. The pellet obtained (fraction S1, Fig. 2.3) was dissolved in deionized water (about 20-50 ml), whereas the supernatant was added with a second volume of ethanol and stored again at 4°C for 48 h. Subsequently, the centrifugation step was repeated and the pellet (fraction S2, Fig. 2.3) was dissolved in deionized water as above. Concerning cell fractions, the pellet was dissolved in 100 ml 1 M NaOH and stirred overnight at 4°C. Afterwards, it was centrifuged again at 12,000 x g 4°C for 15 min in order to remove sodium hydroxide. After resuspension of the pellet in PBS buffer, cells were sonicated at a power of 45% for 10 min, in order to detach the EPS from the cell wall. The resulting slurry was centrifuged at 10,000 x g 4°C for 10 min and the pellet (containing bacterial cells) was discarded. Crude EPS was precipitated by the addition of an equal volume of chilled absolute ethanol; this was stored 48 h at 4°C and then centrifuged at 25,000 x g 4°C for 35 min. The recovered pellet (fraction C1, Fig. 2.3) was re-dissolved in deionized water (about 20 ml). The resulting supernatant was instead added with a second volume of absolute ethanol and again incubated 48 h at 4°C. Another centrifugation, as described above, let us recover the second precipitated fraction (C2, Fig. 2.3), which was dissolved in deionized water.
Small neutral sugars and proteins were then removed by dialysis (with 100 kDa cut-off cellulose acetate membranes) of the extracted fractions for 72 h at 4°C, against three changes of deionized water per day. After three days, the contents of the dialysis membrane were collected and lyophilized in a freeze-dryer (Northern Scientific, York, UK). The extraction protocol is outlined in Fig. 2.3. The dry mass of EPS was then determined.

Fig. 2.3 Outline of the extraction protocol used to purify EPS from *L. paracasei.*



2.3.3. Monomer composition and linkages of the EPS

For sugar composition determinations, 1 mg ml^{-1} EPS was hydrolysed prior to analysis by treatment with 2 ml of 2 M TFA (120°C for 2 h); the solution was then let evaporate under a constant stream of nitrogen atmosphere at 60°C to give monomers, whose identity was determined using high performance anion exchange chromatography (HPAEC) on a Dionex ICS-5000 system (Dionex Corporation, Sunnyvale, USA) equipped with a CarboPac PA20 anion exchange analytical column (150 mm x 3 mm). Monomers were eluted using a 10 mM sodium hydroxide mobile phase at a flow rate of 0.5 ml min^{-1} and detected using a pulsed amperometric detector (PAD) ED40 (Dionex) operating with a gold working electrode and an Ag/AgCl reference electrode. The identity was determined by comparison of the detector response to pure standards of the individual monomers (100 ppm).
The hydrolysed monomers were also analysed by gas-chromatography mass-spectrometry (GC/MS). To derivatize the sample, the monomers mix resuspended in 1 ml Milli-Q water was added with 10 mg $NaBH_4$ and the reduction step ran at 40°C for 2 h. After evaporation of the solution as described above, 1 ml glacial acetic acid was added to the residue, and again evaporated to dryness. Subsequently, 3 ml methanol were added and then evaporated in order to remove the borate complex and to give the methylated sugar alditols. They were then added with 2 ml pyridine and 2 ml acetic anhydride; acetylation reaction ran at 100°C for 2 h. At the end of the reaction the solution was evaporated and the acetylated monomers resuspended in water. Extraction with chloroform was performed to collect the organic phase, containing the alditol acetate sugars. Any trace of water was removed by adding anhydrous sodium sulphate and storing the sample 30 min at 4°C. Sodium sulphate was removed by filtration on filter paper and chloroform by evaporation. The resulting residue was resuspended in 2 ml acetone for the GC/MS analysis, performed on an Agilent 7890A GC system (Santa Clara, CA, USA) coupled to an Agilent 5675c quadrupole MS. The samples were eluted from a SGE column (BPX5, 25 m 0.32 mm-id, 0.5 lm film; HP5, 15 m 0.32 mm-id) with helium (9 psi, flow rate 1 ml min^{-1}) and using a temperature program (start temperature 150°C, hold time 4 min and a final column temperature of 250 °C reached via a rising gradient of 4°C/min).
For linkage analysis, 3 mg EPS were permethylated using 0.7 ml anhydrous dimethylsulphoxide (DMSO) and stirring at room temperature for 20 min (until formation of a slurry). Afterwards, 70 mg sodium hydroxide along with 60 µl methyl iodide were added and, after 20 min, the resulting solution (methylated polysaccharide) was separated and isolated. Specifically, after addition of 3 ml of Milli-Q water, extraction with dichloromethane was performed and the organic phase was collected. After evaporation, the protocol followed was the same described above for the monomer analysis (e.g. hydrolysis, reduction and acetylation steps). The identity of the various methylated alditol acetates was determined by GC/MS and by analysis of the individual fragmentation patterns observed in the MS. The samples were eluted from a HP-5 column (30 m 0.25 mm-id, 0.25 lm film) using helium as carrier (9 psi, flow rate 1 ml min^{-1}) and using the following temperature programme: start temperature of 155 °C, hold time of 1 min and a final column temperature of 195 °C reached via a rising gradient of 0.75 °C min^{-1}.

2.3.4. NMR analyses of exopolysaccharides

For NMR analyses, EPS freeze-dried samples were dissolved (10 mg ml^{-1}) directly in D_2O (Goss Scientific Instruments Ltd, Essex, UK). NMR spectra were recorded at an elevated probe temperature of 70°C. The elevated temperature was initially chosen as it shifted the HOD signal to a higher field, into a clear region of the spectrum. The higher temperature also increased spectral resolution by reducing the sample viscosity. All of the NMR spectra were recorded on a Bruker Avance 500.13 MHz ^{1}H (125.75 MHz ^{13}C) spectrometer (Bruker-Biospin, Coventry, UK) operating with Z-field gradients where appropriate, and using Bruker's pulse programs. Chemical

shifts are expressed in ppm relative to either internal or external acetone; δ 2.225 for ^{1}H and δ 31.55 for ^{13}C.
The 2D gs-DQF-COSY spectrum was recorded in magnitude mode at 70°C. TOCSY experiments were recorded with variable mixing times (60, 90 and 120 ms). The 2D-heteronuclear ^{1}H–^{13}C HSQC, and phase sensitive HSQC-TOCSY spectra were recorded using Bruker pulse sequences and 256 experiments of 1024 data points. The NOESY spectrum was recorded using a Bruker pulse sequence and 256 experiments of 1024 data points using a mixing time of 200 ms. For the majority of spectra, time-domain data were multiplied by phase-shifted (squared-) sine-bell functions. After applying zero-filling and Fourier transformation, data sets of 1024–1024 points were obtained.

2.3.5. NF-κB activation by exopolysaccharides

The experiment was conducted on the transfected Caco-2 cell line described in Chapter 1, Paragraph 1.3.7. In this set of experiments, Caco-2 pNiFty2-Luc monolayers were incubated with 0.1 ml of *L. paracasei* DG EPS corresponding to a final concentration of 100 μg ml^{-1}. The stimulation was conducted by adding 10 ng ml^{-1} of IL-1β. After incubation at 37° C for 4 h, the samples were treated and the bioluminescence was measured as described by Stuknyte (2011). Two independent experiments were conducted in triplicate for each condition.

2.3.6. Activation of U937 and THP-1 human macrophage cell lines: 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, 1983) and other phenotypic markers (Harris and Ralph, 1985). The monocytic THP-1 cell line was purchased from American Type Culture Collection (Manassas, VA, USA). THP-1 cells were originally cultured from the peripheral blood of 1 year child with acute monocytic leukemia (Tsuchiya, 1980). They are non-adherent cells, which can be differentiated into macrophage-like cells using PMA through a protein kinase C-mediated ROS-dependent signaling pathway (Traore, 2005). Treatment of THP-1 with PMA (10 ng ml^{-1} overnight) leads to a decrease in the expression of LDL receptor and an increase in the expression of scavenger receptors (Johnson, 2003). These alterations allow the cells to take up modified lipoproteins and to become foam cells with excessive accumulation of lipid, primarily cholesterol ester, that make THP-1 a reasonable model to mimic monocyte–macrophage behavior during the atherogenesis process (Del Bò, 2015).
The normal growth medium for U937 and THP-1 cells consisted of RPMI 1640 medium (Lonza, Basel, Switzerland) 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). 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 24 and 48 h, for THP-1 and U937, respectively. 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 *Salmonella enterica* (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.

2.3.7. Preparation of RNA and qRT-PCR

After incubating U937 or THP-1 cells at 37 °C four 4 h, the supernatant was carefully removed from each well and the total cellular RNA was isolated from the adhered cells with the Total RNA Blood and Cultured cells Kit (GeneAid, New Taipei City, Taiwan) . Afterwards, DNA traces were removed by treatment with DNAse enzyme (Sigma-Aldrich), following the manufacturer's instructions. RNA concentration and purity was determined with a Take3 Multivolume Plate Reader (Biotek, Luzern, Switzerland) 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 (qRT-PCR) was carried out in order to measure the mRNA expression levels of cytokines by means of the SYBR Green technology using the SsoFast EvaGreen Supermix (Bio-Rad) on a Bio-Rad CFX96 system according to the manufacturer's instructions. The primers used are as follows (5'→3'): GAPDH forward GGGAAGGTGAAGGTCGGAGT; GAPDH reverse TCAGCCTTGACGGTGCCATG; IL10 forward AGCAGAGTGAAGACTTTCTTTC; IL10 reverse CATCTCAGACAAGGCTTGG; TNF-α forward TCAGCTCCACGCCATT; TNF-α reverse CCCAGGCAGTCAGATCAT; COX2 forward CCCTTGGGTGTCAAAGGTAA; COX2 reverse TGAAAAGGCGCAGTTTACG. All primers were designed previously and their specificity was assessed with melting curves during amplification and by 1 % agarose gels (Taverniti, 2012). Quantitative PCR was carried out according to the following cycle: initial hold at 95° C for 30 s and then 39 cycles at 95° C for 2 s and 60° C for 5 s. Gene expression was normalized to the reference gene *gapdh*. The amount of template cDNA used for each sample was 15 ng. All results regarding cytokine mRNA expression levels are reported as the fold of induction (FOI) respective to the control (namely unstimulated U937 or THP-1), to which we attributed a FOI of 1.

2.4 RESULTS AND DISCUSSION

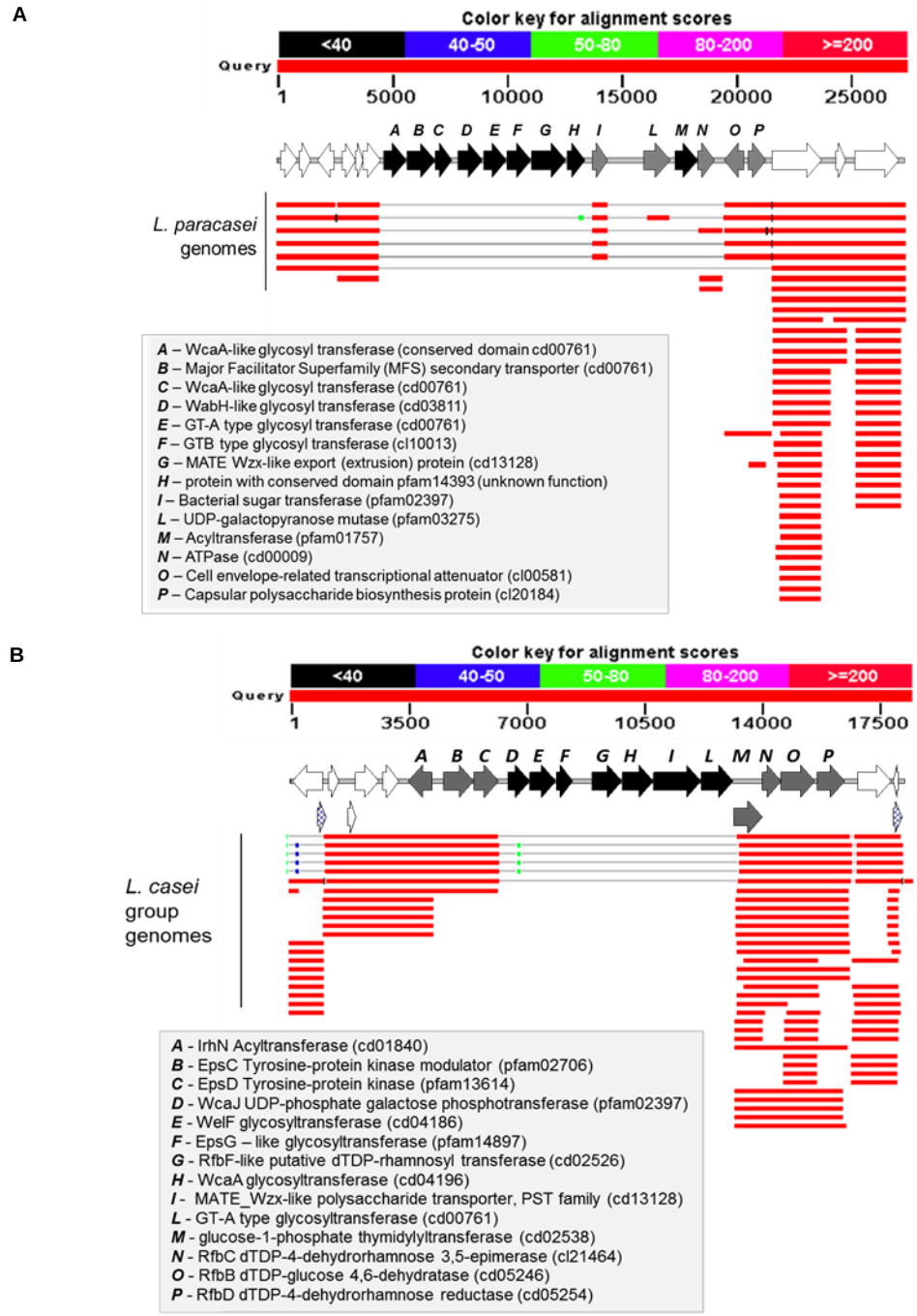
2.4.1. Identification of putative genetic clusters for exopolysaccharide biosynthesis

As mentioned in Chapter 1, we generated a draft genome sequence of *L. paracasei* DG and LPC-S01 and, by comparative analysis with other genomes of the same species, we identified several genomic islands with putatively known biological functions. Among them, we found two different unique genomic regions in DG and LPC-S01 genomes containing a cluster putatively encoding genes involved in the biosynthesis of exopolysaccharide (EPS) molecules. Interestingly, these two regions were different between each other and, more importantly, did not find any match with other sequences in GenBank database, as observed by performing a BLASTN search. Specifically, search on strain LPC-S01 revealed a region of about 8 kb encompassing eight putative genes (genes from A to H in Fig. 2.4A). Similarly, we identified in DG genome a 13 kb region coding for several putative glycosyltransferases involved in EPS synthesis and, even in this case, a region of about 7 kb in the centre of the cluster found no match in the database (genes from D to L in Fig. 2.4B).

2.4.2. Purification of EPS from *L. paracasei* DG and LPC-S01

The quantity of exopolysaccharides produced varies with bacterial species and, in laboratory conditions, several physicochemical factors play a crucial role in EPS production yield, including pH, temperature, incubation time and, above all, medium composition (carbon, nitrogen and cation sources) (Nwodo, 2012). During my experience at the University of Huddersfield (United Kingdom), under the guide of Prof. Andy Laws, I attempted to extract the EPS from the strains under study by using different media, specifically MRS, Huddersfield Bifidobacteria Medium (HBM) and, finally, the chemically defined medium (CDM) indicated in Tab. 2.2. HBM has been developed by Alhudhud (2014) and its composition is based on Reinforced Clostridial Medium (RCM). Specifically, RCM has been modified in order to remove all those molecules that are EPS equivalents (EPS-E), in particular casein and yeast extracts components, which both interfere with the quantification and characterization of the EPS. In our experiments, HBM did not prove to be effective since *L. paracasei* strains showed a very slow growth rate and a low biomass formation, probably due to the absence of various salts and amino acids in the modified medium. On the other hand, MRS allowed a very fast and abundant biomass formation but, nonetheless, the EPS fraction was partially contaminated by the EPS-E molecules present in the extracts included in the medium. For this reason, the best results were obtained by developing the chemically defined medium (CDM, Tab. 2.2), which contains all the micro- and macro-elements needed by *L. paracasei* without adding complex components such as beef and yeast extracts. Under the conditions tested, and by following the extraction protocol indicated in Fig. 2.3, we obtained a final product ascribable to a crude EPS isolate only from *L. paracasei* strain DG batch. We concluded that strain *L. paracasei* LPC-S01 did not produce EPS under the tested conditions; however, there are several factors, as mentioned above, which can influence the EPS synthesis. For this reason, further investigation is needed in order to test the hypothesis that strain LPC-S01 express the putative *eps* operon we identified in its genome. For instance, a screening of different carbon sources, such as disaccharides (e.g. lactose instead of glucose) or oligosaccharides, could be useful to investigate more extensively LPC-S01 capacity to secrete EPSs (Laws, 2008; Polak-Berecka, 2013). In addition, bacterial growth phase may be also crucial since, reportedly, EPS production may be limited to the exponential rather than stationary growth phase (Pham, 2000; Yang, 2010; Arsköld, 2007).

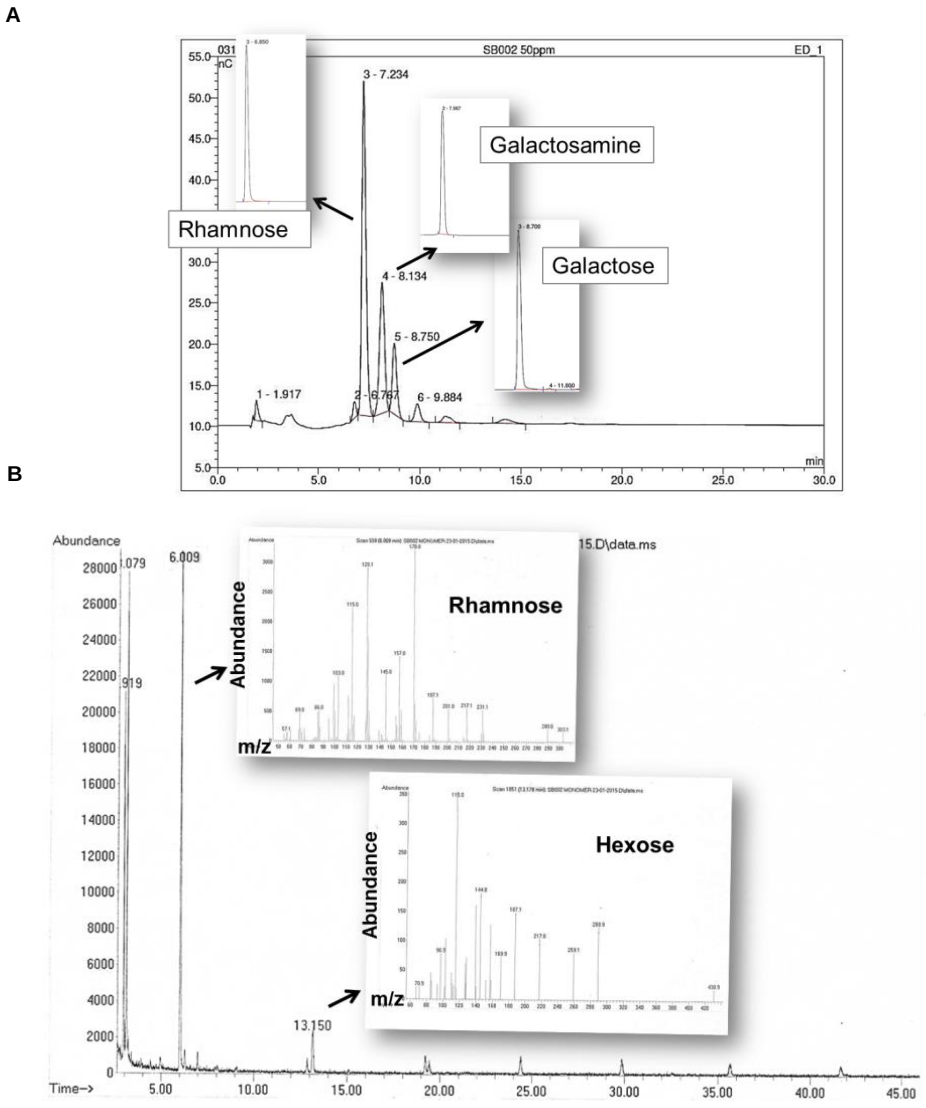
Fig. 2.4. BLASTN search results for the regions from *Lactobacillus paracasei* LPC-S01 **(A)** and DG **(B)**, which putatively code for the enzymes involved in the synthesis of an exopolysaccharide (EPS). The figure has been obtained adding a picture of the putative EPS operon over the graphic representation of the BLASTN output. Genes outside the putative EPS operon are indicated in white, genes belonging to the EPS cluster are indicated in grey or black if they did find or not a match on BLASTN, respectively.



2.4.3. Identification of monomers composing *L. paracasei* DG EPS

The analysis of the hydrolysed macromolecule extracted by strain DG was performed by means of HPAEC and GC/MS, in order to discover the identity of the monomers composing the polymer. In Fig. 2.5A, the chromatogram indicates three main signals, with retention times corresponding to the standards of rhamnose, galactosamine and galactose, respectively. Thanks to the gas-chromatography mass spectrometry analysis, we could confirm these data: in Fig. 2.5B the chromatogram corresponding to DG EPS indicates two peaks with retention times of 6.009 and 13.150 min. By analysing their fragmentation profile we could identify that they corresponded to those of rhamnose and of an hexose, respectively. The galactosamine signal was missing, probably due to the loss of the amino group during the derivation steps.

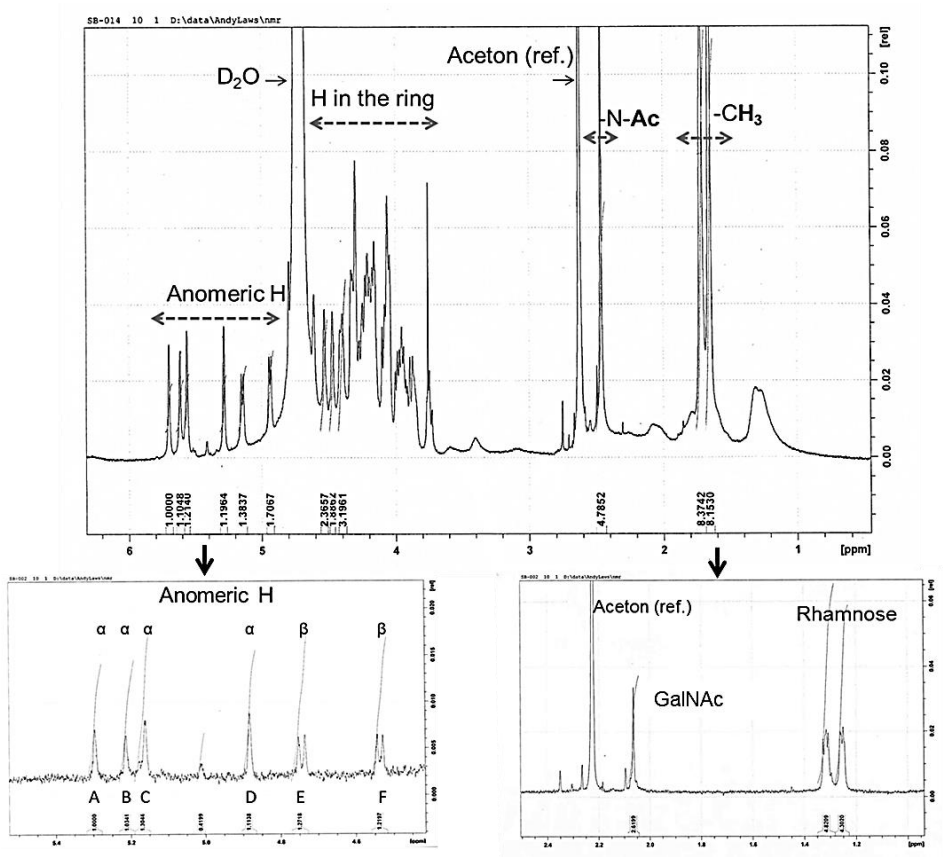
Fig. 2.5. HPAEC **(A)** and GC/MS spectrum **(B)** indicating the monomer composition of DG EPS.



2.4.4. Structural identification of *L. paracasei* DG EPS repeating unit

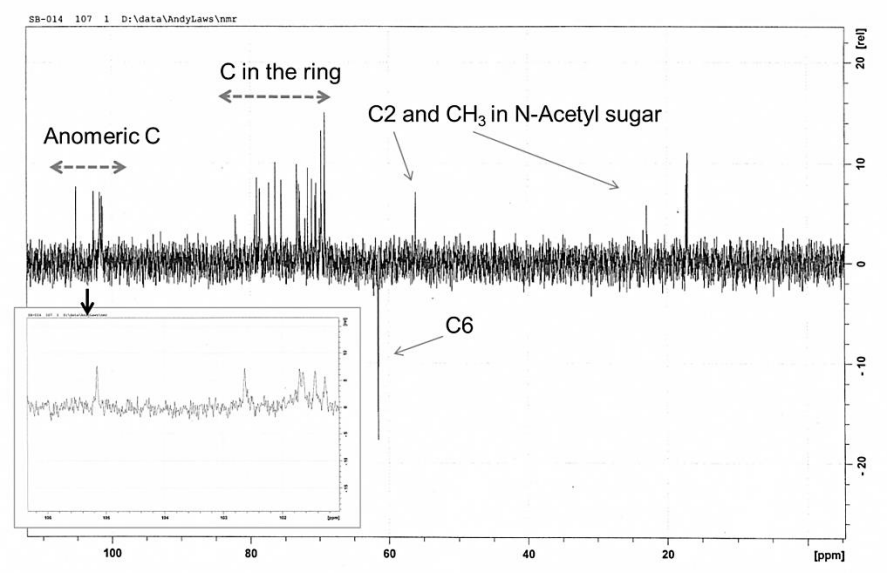
The previous analyses helped us to identify the components of DG's EPS repeating unit. In order to gain information about the quantity and the sequence of monomers, a detailed analysis of a number of 1D and 2D-NMR spectra was then necessary. The analysis of the anomeric region of the ^{1}H NMR (Fig. 2.6, δ= 4.4–6 ppm) identified six proton resonances: four corresponded to anomeric α sugars signals since they displayed a single peak (labelled A to D with decreasing chemical shift), whereas the two remaining signals belonged to β sugars (E and F), because having doublet peaks. The presence of six major anomeric protons thus suggested that the repeating unit is a exasaccharide. The ^{1}H NMR also gave us additional information: the presence of methylated sugars, as deduced by the appearance of two signals in the low region of the spectrum (between 1.2 and 1.4 ppm), ascribable to the rhamnose identified by the chromatographic analyses, and the presence of one signal with chemical shift δ= 2.08, typical of the proton bounded to the acetyl group of N-acetylated sugars, such as GlcNAc and GalNAc.

Fig. 2.6. ^{1}H NMR spectrum for *L. paracasei* DG EPS recorded at 70 °C in D_2O. Magnified region showing anomeric H1's is inserted in the bottom left, magnified region showing the low ppm are of the spectrum is in the bottom right.



The ^{13}C 135 DEPT spectrum, shown in Fig. 2.7, contains six signals in the anomeric region confirming that the EPS has a exasaccharidic repeated unit. A high field ring carbon at 53.86 ppm and an acetyl–methyl group at 21 ppm confirmed the presence of an N-acetyl amino sugar. We could also detect a negative peak in the DEPT 135 spectrum corresponding to C6, thus suggesting that at least one of the six sugars is a hexose.

Fig. 2.7. DEPT 135 ^{13}C NMR spectrum of *L. paracasei* DG EPS recorded at 70 °C in D_2O; magnified region showing anomeric C1's is inserted in the bottom left.



The position of the remaining ring protons was established through analysis of the scalar coupling observed in the corresponding $^{1}H–^{1}H$ COSY and $^{1}H–^{1}H$ TOCSY spectrum, as shown in Fig. 2.8. The COSY spectrum (blue in Fig. 2.8) allowed us to assign the protons on the spectrum based on the vicinal coupling, that is to identify the protons belonging to ring carbons linked together. On the other hand, TOCSY spectrum (performed with mixing times 30–250 ms, red in Fig. 2.8), gave us information about the correlations among all the protons in the sugar ring. By assigning all the cross peaks in the 2D spectra, we could identify four residues in the repeating unit, corresponding to rhamnose (sugars from A to D), as observed in the panel on the right of Fig. 2.8 which highlights the low ppm area of the spectrum. The ^{1}H-^{13}C HSQC spectrum helped us to confirm the identity of the remaining two residues: N-acetyl galactosamine (GalNAc, sugar E), and galactose (Gal, sugar F) (data not shown). Finally, in order to uncover how these residues are linked together in the repeating unit, we performed a NOESY NMR (red, Fig. 2.9) , which reveals the spatially proximal proton pairs and helps to determine a sequence of molecules. As shown in Fig. 2.9, we could identify six cross peaks in the 2D spectrum corresponding to the interaction between the protons close in the space and belonging to the carbons involved in the glycosidic bond. The linkages of the different monomers in the oligosaccharide repeat unit were confirmed using a combination of these latter chemical shift data with the linkage analysis of the methylated alditol acetates performed with GC/MS. The GC/MS profiles shown in Fig. 2.10 indicate the presence of a terminal hexose (galactose, 6.993 min), two 1,2-linked rhamnoses (4.046 min), a 1,3-linked rhamnose (4.218 min), a 1,2,3-linked rhamnose (5.207 min), and a 1,3-linked N-acetylated residue (GalNAc, 6.277).

Fig. 2.8. COSY (blue) and TOCSY (red) 2D NMR spectrum of EPS from *Lactobacillus paracasei* DG recorded at 70 °C in D_2O. On the right a panel shows a selected region of the spectrum, corresponding to the low ppm area (1-2.5 ppm). The identity of cross peaks is noted by the sugar residue, as A–F, and by identifying the location of hydrogens within the ring as 1–6.

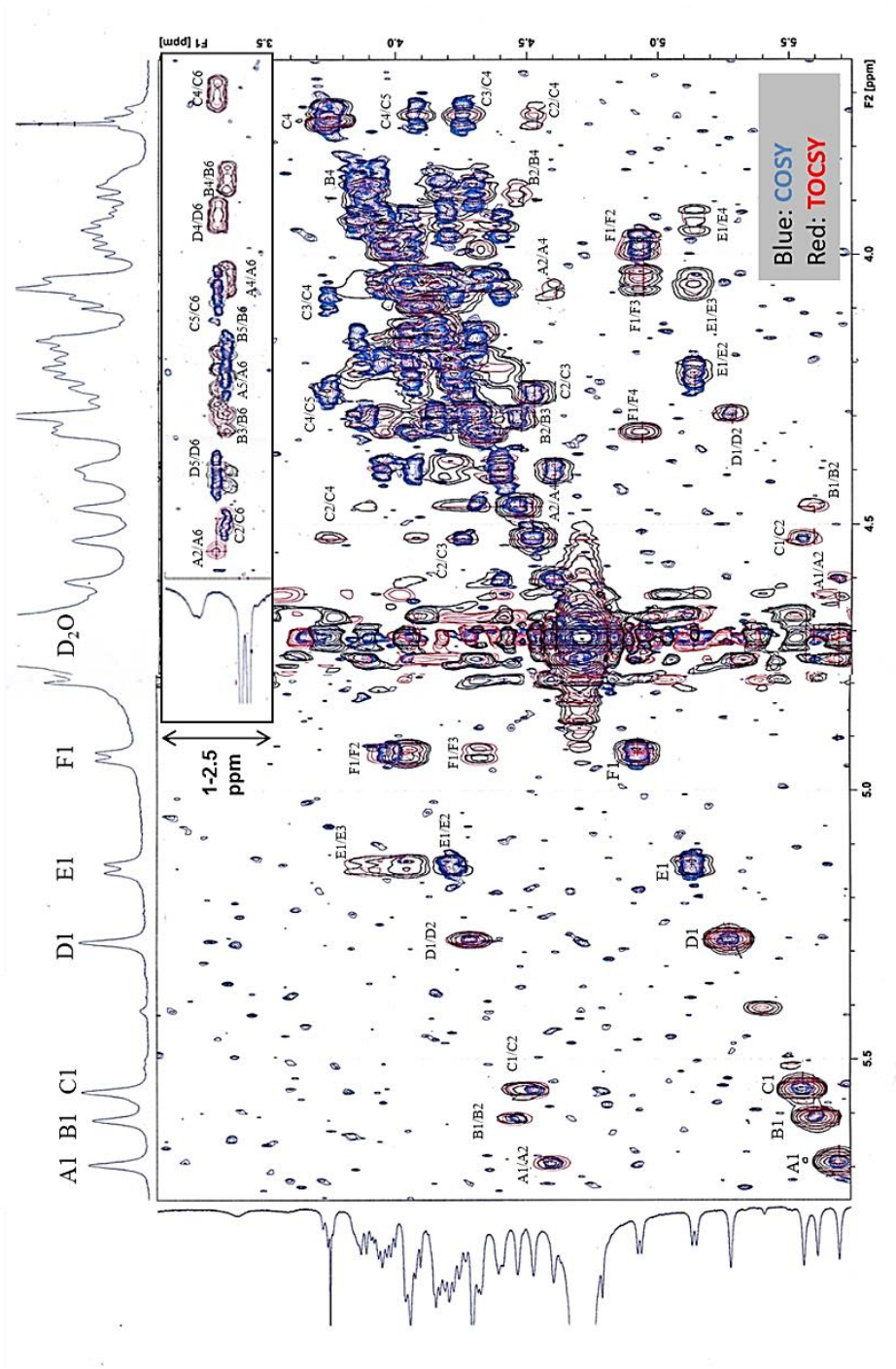


Fig. 2.9. COSY (green) and NOESY (red) 2D NMR spectrum of EPS from *Lactobacillus paracasei* DG recorded at 70 °C in D_2O. The identity of cross peaks is noted by the sugar residue, as A–F, and by identifying the location of hydrogens within the ring as 1–6.

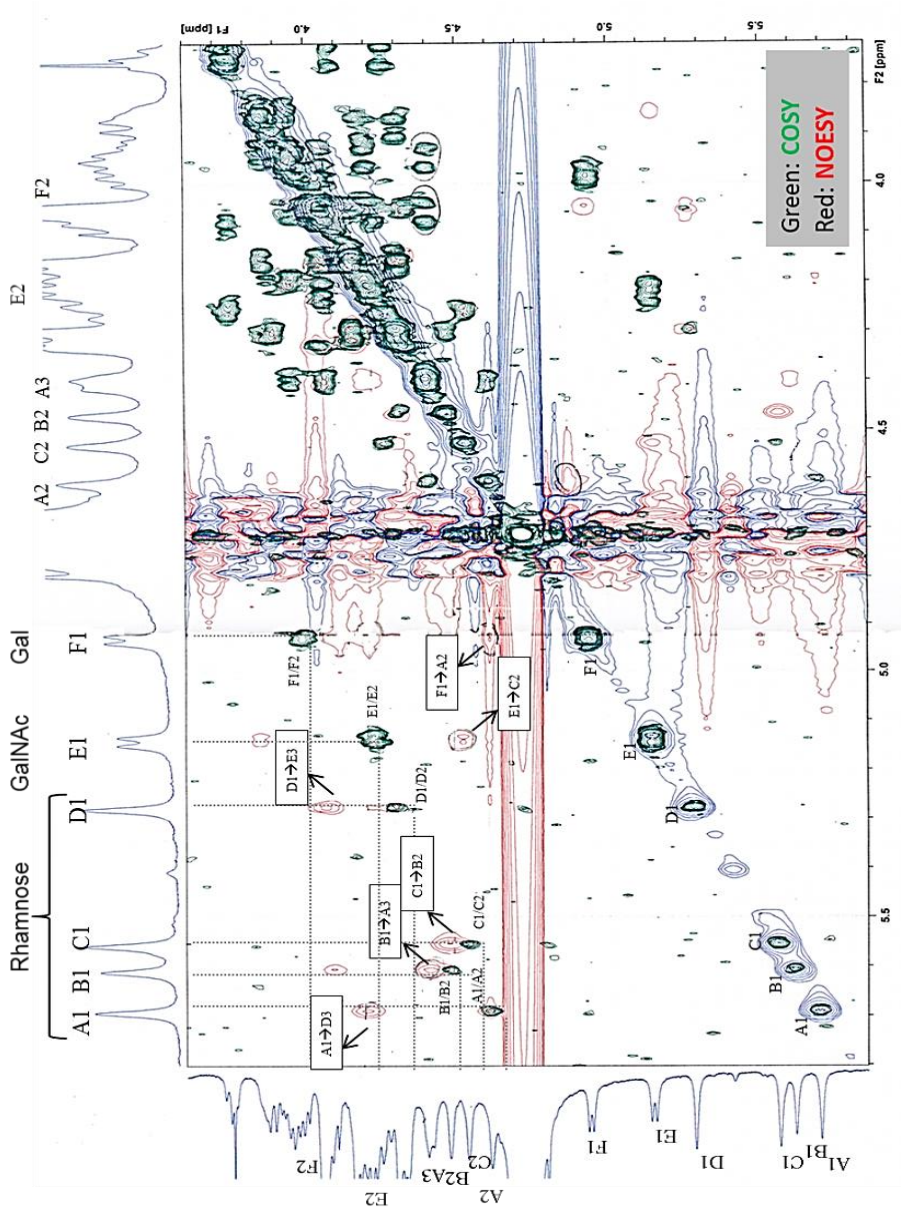
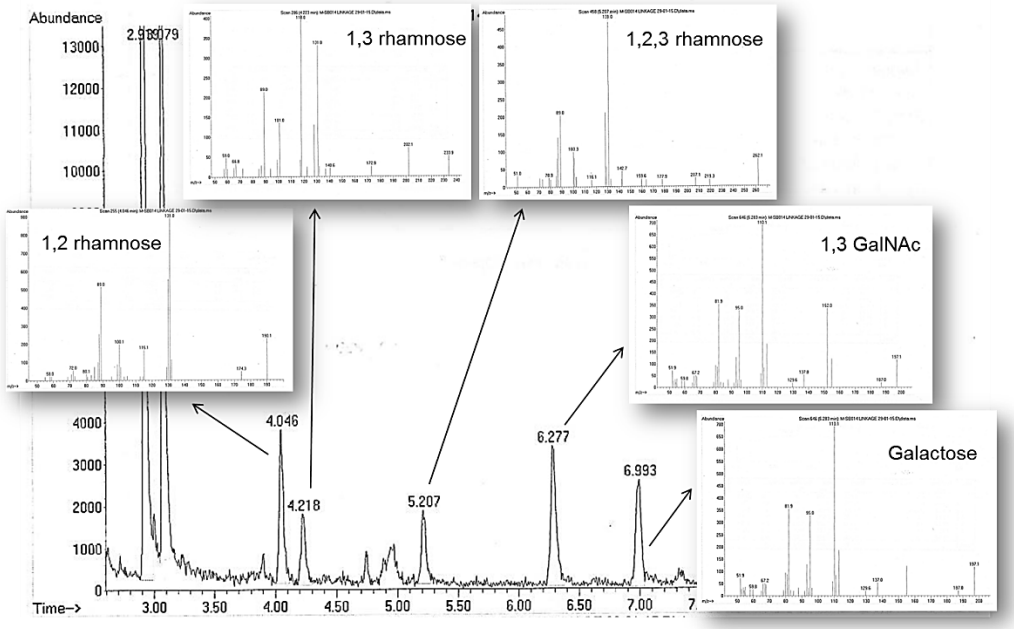


Fig. 2.10. GC/MS profiles indicate the different type methylated alditol acetates obtained by derivation of DG EPS for the linkage analysis.



In conclusion, the combined results of the chemical and spectroscopic analyses let us build the repeating unit of *L. paracasei* DG EPS, which results being a branched exasaccharide with the novel following structure:

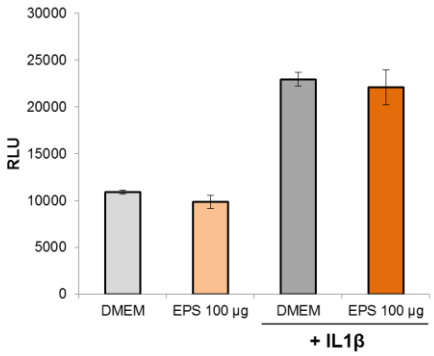
→3)-a-L-Rha*p*-(1→3)-a-L-Rha*p*-(1→3)-b-D-Gal*p*NAc-(1→2)-a-D-Rha*p*-(1→2)-a-D-Rha*(1→*
2
↑
1
b-D-Gal*p*

2.4.5. Activation of NF-κB in recombinant Caco-2 cells by *L. paracasei* DG EPS

After the structure characterization, the EPS isolated from *L. paracasei* DG, we studied its possible involvement in mediating the bacterial effects on the host immune system. Indeed, there are many evidences about the ability of EPSs isolated from lactobacilli to interact with the host immune cells and trigger immunomodulatory responses (Liu, 2011; Vinderola, 2006; Gao, 2015; Ciszek-Lenda, 2011a; Ciszek-Lenda, 2011b; Patten, 2014); however, the molecular mechanisms by which they promote these effects, and their overall role in immune homeostasis, are yet to be elucidated. NF-κB is an important intracellular signalling molecule that mediates the activation of various cytokine genes in response to microbial products. In the cytosol, the nuclear

localization sequence of NF-κB is hidden by IκB but, upon stimulation, IκB is degraded, allowing NF-κB to translocate into the nucleus where it may bind the promoters of cytokine genes (Hayden and Ghosh, 2004). This transcription factor was proved to be activated in mouse peritoneal macrophages upon stimulation with the EPS producing strain *L. paracasei* KB28, causing induction of TNF-α, IL-6, and IL-12 via its modulation (Kang, 2011). Similar effects have been reported for the EPS produced by *Lactobacillus acidophilus* NCFM on Caco-2 cells (Li, 2014).
We utilized the isolated DG's EPS macromolecule as stimulus in the experiments on the recombinant pNiFty2-Luc Caco-2 cell line. We found that, differently from the whole bacterial cells and their exhausted broth (Fig. 1.10), the purified EPS molecule was unable to either decrease or increase the activation of NF-κB, with or without addition of the pro-inflammatory stimulus of IL-1β (Fig. 2.11).

Fig. 2.11. Effect of the purified *Lactobacillus paracasei* DG EPS on the human transfected pNiFty2-Luc Caco-2 cell line, at baseline or stimulated with 10 ng ml^{-1} of IL-1β. EPS was tested at a concentration of 100 μg ml^{-1}. Data in histograms are the means (+ standard deviations) from at least three independent experiments conducted in triplicate. Data are reported as relative luminescence units (RLU).



2.4.6. Immunomodulatory activity of *L. paracasei* DG and its isolated exopolysaccharide

After the 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. paracasei* DG and its isolated EPS. We utilized two models of macrophages, U937 and THP-1, both professional phagocytes that undergo activation upon environmental signals such as microbial products and cytokines (Benoit, 2008; Italiani and Boraschi, 2014). We quantified through qRT-PCR the gene expression of TNF-α (a cytokine involved in inflammatory responses; Belardelli, 1995), IL-10 (a potent anti-inflammatory interleukin inhibiting the production of proinflammatory cytokines in several cell types; Taverniti, 2012) and COX-2. Cyclooxygenase (COX), or prostaglandin synthase H (PGH), is a homodimer enzyme involved in the synthesis of prostaglandins (PGs) starting from arachidonic acid (Funk, 2001). PGs are involved in several physiological processes and contribute to the protection of the gastrointestinal mucosa (Williams, 1999; Morteau, 2000). Two isoforms of the COX enzyme have been identified and described (Kujubu, 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 (Herschman, 1996; Taverniti, 2012). At first, we investigated the effect of strains DG, Shirota (both at a MOI= 50), and the isolated EPS molecule (30 μg ml^{-1}) on the genes activation in the U937 cell model upon presence or absence of an inflammatory

stimulus, that is LPS from *S. enterica*. After 4 h of stimulation, U937 cells proved not to be responsive to LPS; consequently we limited our evaluation on the bacterial stimuli at a basal level. DG EPS induced a pronounced pro-inflammatory profile, as evidenced by an enhanced induction of COX-2 and TNF-α, as compared to IL-10 (Fig. 2.12A). We also found that the cells of strain DG triggered COX-2 expression more than strain Shirota (Fig. 2.12A).

Afterwards, we utilized another macrophages cell line model, named THP-1. The involvement of the EPS in the immunostimulating effects of DG was confirmed by adding DG bacterial cells without the EPS (naked DG = nDG, *i.e.,* strain DG after detachment of its EPS by washes with PBS and mild sonication) as stimulus. In this set of experiments, we could observe that all the whole cells showed very weak effects on the modulation of the cytokines expression. However, interestingly, we observed that the removal of the EPS resulted in a decrease in COX-2 and TNF-α induction levels (Fig. 2.12B). The use of the isolated EPS molecule to immunostimulate the THP-1 macrophages led to an increase of TNF-α and, to even higher values, of COX-2, with a similar trend observed in U937 macrophages experiments (Fig. 2.12C). Finally, when co-stimulating the THP-1 with LPS from *S. enterica*, we could observe that an additive effect had occurred between LPS and EPS on COX-2 expression, which nearly doubled (from 80 and 85 FOI with the single stimuli LPS or EPS, respectively, to about 140 FOI when combined together; Fig. 2.12D). The same effect occurred when THP-1 cells were co-stimulated with LPS and strain DG but not with nDG. Taken together, these data suggest that EPS could play a dominant role in the ability of strain DG to trigger COX-2 expression in macrophages.

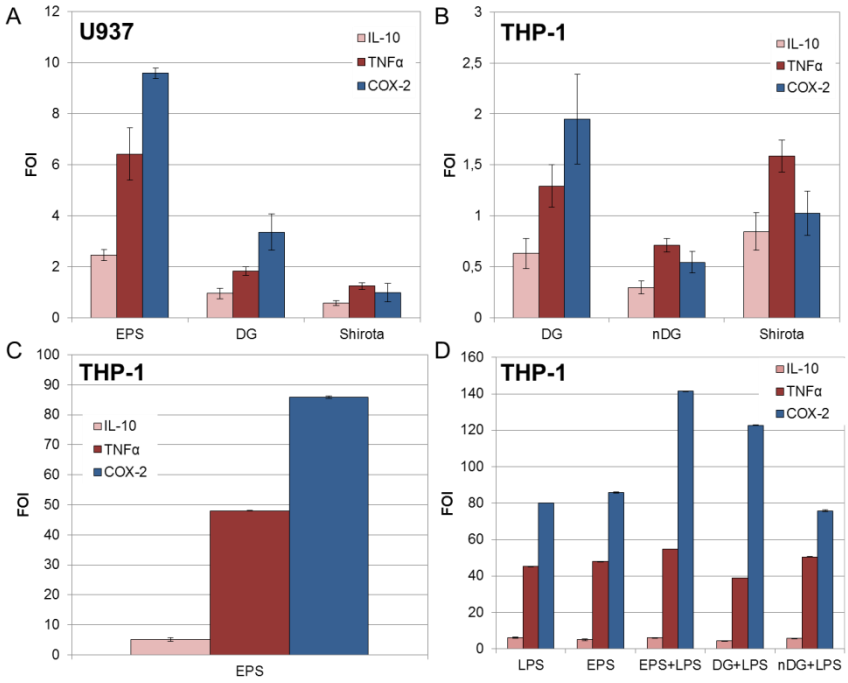
Although other studies have suggested that lactic acid bacteria could affect COX-2 secretion, there is no clear consensus in the literature. For instance, several probiotic preparations were found to reduce the amount of prostaglandins and levels of COX-2 gene expression at the intestinal level in murine models and *in vitro* (Lee, 2008; Urbanska, 2010; Amdekar, 2011). In contrast, other papers reported a significant up-regulation of COX-2 expression levels by probiotic bacteria *in vitro* and *in vivo* (Khailova, 2010; Otte, 2009; Kwon, 2010). Differences in tested strains and model systems likely explain the lack of consensus found in the literature.

The ability of the strain DG and its EPS to induce COX-2 expression suggests that this bacterium could directly affect inflammatory processes not only by modulating cytokine secretion but also by inducing prostaglandin production. The observed effect on the activity of the COX-2 gene could thus be of immediate benefit for the host's mucosa; furthermore, rapid COX-2 upregulation in response to injury or inflammation has been reported to restore mucosal integrity (Tan, 2000).

Our results appear also in accordance with the study conducted by D'Incà (2011), who reported a beneficial role of *L. paracasei* DG in patients with ulcerative colitis after rectal administration of the probiotic. Accordingly, the authors propose that DG's possible mechanisms of action are related to the alterations in the microbiota (as observed also in healthy volunteers by Ferrario, 2014) and the contribution to the modulation of the mucosal immune response through signalling molecules, ultimately modifying the overall cytokine balance.

A number of *Lactobacillus*-derived EPSs have demonstrated beneficial effects on the innate and adaptive immunity of the intestine (Amdekar, 2011; Bleau, 2010); therefore, these biopolymers, and particularly DG's EPS, could be potentially exploited to reduce intestinal inflammation associated with chronic diseases. Further efforts need to be undertaken to identify the EPS receptor and, consequently, elucidate the immunomodulatory mechanism of action of DG's EPS.

Fig. 2.12. Quantitative analysis of cytokine gene expression in human macrophages U937 **(A)** and THP-1 **(B,C** and **D)** after 4 h stimulation with *L. paracasei* DG, *L. paracasei* Shirota, *L. paracasei* nDG and the isolated DG EPS molecule. Expression levels of TNF-α, IL-10 and COX-2 are shown as the fold change of induction (FOI) relative to the control (unstimulated macrophages), which was set at a value of 1. LPS was used as a co-stimulator at a concentration of 1 μg ml^{-1}. EPS was tested at concentrations of 30 μg ml^{-1}. Bacterial cells were used at a MOI of 50. Presented data are the means of measurements (+ standard deviations) for a result representative of three independent experiments.



2.5 CONCLUSIONS

Among the modes of action by which probiotics are thought to contribute to human health, the modulation of the immune responses, resulting in both local and systemic effects, is probably the most important but, at the moment, also the more obscure (Goldin and Gorbach, 2008). For this reason, a better understanding of how probiotic bacteria interact with host cells is needed for their optimal application.

Bacterial cell wall and secreted molecules are key probiotic ligands that can interact with the host receptors and activate various signalling pathways, thus triggering the final probiotic effect. Among the most studied probiotics, lactic-acid bacteria possess a cell wall that is typically composed of a thick peptidoglycan layer decorated with proteins, teichoic acids and polysaccharides (Lebeer, 2010). Exopolysaccharides, apart from their industrial applications, are found to be associated with many physiological functions, but their mechanism of action has not been clearly established, and is probably diverse and complex (Patel and Prajapati, 2013). Thanks to the comparative genomic analysis on *L. paracasei* strains DG and LPC-S01, we could identify two gene clusters putatively coding for exopolysaccharides biosynthesis. Interestingly, GenBank search revealed that such regions are unprecedented. For this reason, during my period as visiting scholar at the University of Huddersfield, United Kingdom, I attempted to extract the EPSs molecules produced by both strains under study: however, only strain DG synthetized this kind of macromolecules in the tested conditions. After purification, we were able to characterize DG EPS repeating unit by means of chromatographic and NMR spectroscopy, and we could uncover that it possessed a novel structure, never identified before in any other lactic acid bacterium. Afterwards, the purified molecule was utilized as stimulus to assess its immunomodulatory potential on Caco-2 cells and two macrophages cell lines, namely U937 and THP-1. These latter macrophages were highly responsive to the EPS stimulus. Particularly, the purified molecule showed a great ability to enhance the expression of TNF-α and COX-2, leading to the activation of an innate immune response with a pro-inflammatory direction. Nonetheless, the activation of COX-2, also subjected to an additive effect due to the combination of EPS with the pro-inflammatory stimulus of LPS, strongly suggests a role of DG and its secreted polysaccharidic molecule in the protection of intestinal mucosa, as observed in previous studies (Tan, 2000; D'Incà, 2011). In conclusion, these results provide us additional information about strain DG probiotic potential and its mechanisms of action in the cross-talk with the host.

2.6 REFERENCES

- Amdekar S et al., 2011, *Lactobacillus casei* reduces the inflammatory joint damage associated with collagen-induced arthritis (CIA) by reducing the pro-inflammatory cytokines: *Lactobacillus casei*: COX-2 inhibitor. J Clin Immunol. 31:147-154.
- Arsköld E et al., 2007, Environmental influences on exopolysaccharide formation in *Lactobacillus reuteri* ATCC 55730. Int J Food Microbiol. 116:159-67.
- Badel S et al., 2011, New perspectives for lactobacilli exopolysaccharides. Biotechnol Adv. 29:54-66.
- Belardelli F, 1995, Role of interferons and other cytokines in the regulation of the immune response. APMIS. 103:161-179.
- Benoit M et al., 2008, Macrophage polarization in bacterial infections. J Immunol. 181:3733-3739.
- Bleau et al., 2010, Intermediate chains of exopolysaccharides from *Lactobacillus rhamnosus* RW-9595M increase IL-10 production by macrophages. J Appl Microbiol. 108:666-675.
- Chabot S et al., 2001, Exopolysaccharides from *Lactobacillus rhamnosus* RW-9595M stimulate TNF, IL-6 and IL-12 in human and mouse cultured immunocompetent cells, and IFN-γ in mouse splenocytes. Lait. 81:683-697.
- Ciszek-Lenda M et al., 2011a, Immunoregulatory potential of exopolysaccharide from *Lactobacillus rhamnosus* KL37. Effects on the production of inflammatory mediators by mouse macrophages. Int J Exp Pathol. 92:382-391.
- Ciszek-Lenda M et al., 2011b, Strain specific immunostimulatory potential of lactobacilli-derived exopolysaccharides. Cent Eur J Immunol. 36:121-129.
- D'Incà R et al., 2011, Rectal administration of *Lactobacillus casei* DG modifies flora composition and Toll-like receptor expression in colonic mucosa of patients with mild ulcerative colitis. Dig Dis Sci. 56:1178–1187.
- Das D et al., 2014, A food additive with prebiotic properties of an α-D-glucan from *Lactobacillus plantarum* DM5. Int J Biol Macromol. 69:20-26.
- De Vuyst L et al., 2011, New insights into the exopolysaccharide production of *Streptococcus thermophilus*. Int Dairy J. 21:586–591.
- Degeest B et al., 2001, Microbial physiology, fermentation kinetics, and process engineering of heteropolysaccharide production by lactic acid bacteria. Int Dairy J. 11:747–757.
- Del Bò C et al., 2015, Anthocyanins and phenolic acids from a wild blueberry (*Vaccinium angustifolium*) powder counteract lipid accumulation in THP-1-derived macrophages. Eur J Nutr. [Epub ahead of print].
- Dols-Lafarge M et al, 2008, Characterization of *gtf*, a glucosyltransferase gene in the genomes of *Pediococcus parvulus* and *Oenococcus oeni*, two bacterial species commonly found in wine. Appl Environ Microbiol. 74:4079–4090.
- Donot F et al., 2012, Microbial exopolysaccharides: Main examples of synthesis, excretion, genetics and extraction. Carbohydr Polym. 87: 951-962.
- Duboc P and Mollet B, 2001, Applications of exopolysaccharides in the dairy industry. Int Dairy J. 11:759–768.
- Fanning S et al., 2012, Bifidobacterial surface-exopolysaccharide facilitates commensal-host interaction through immune modulation and pathogen protection. Proc Nat Acad Sci USA. 109:2108–2113.

- Ferrario C et al., 2014, Modulation of fecal Clostridiales bacteria and butyrate by probiotic intervention with *Lactobacillus paracasei* DG varies among healthy adults. J Nutr. 144:1787–1796.
- Funk CD, 2001, Prostaglandins and leukotrienes: advances in eicosanoid biology. Science. 294:1871-1875.
- Gao K et al., 2015, Immunomodulation and signaling mechanism of *Lactobacillus rhamnosus* GG and its components on porcine intestinal epithelial cells stimulated by lipopolysaccharide. J Microbiol Immunol Infect. S1684-1182(15)00748-3. [Epub ahead of print].
- Goldin BR and Gorbach SL, 2008, Clinical indications for probiotics: an overview. Clin Infect Dis. 46:S96–S100.
- Harris P and Ralph P, 1985, Human leukemic models of myelomonocytic development: a review of the HL-60 and U937 cell lines. J Leukoc Biol. 37:407-422.
- Hayden MS and Ghosh S, 2004, Signaling to NF-kappaB. Genes Dev. 18:2195-2224.
- Herschman HR, 1996, Prostaglandin synthase 2. Biochim Biophys Acta. 1299:125-140.
- Hidalgo-Cantabrana C et al., 2012, Immune Modulation Capability of Exopolysaccharides Synthesised by Lactic Acid Bacteria and Bifidobacteria. Probiotics & Antimicro. Prot. 4:227–237.
- Hidalgo-Cantabrana C et al., 2014, Genomic overview and biological functions of exopolysaccharide biosynthesis in *Bifidobacterium* spp. Appl Environ Microbiol. 80:9-18.
- Hongpattarakere T et al., 2012, *In vitro* prebiotic evaluation of exopolysaccharides produced by marine isolated lactic acid bacteria. Carbohyd Polym. 87: 846-852.
- Italiani P and Boraschi D, 2014, From Monocytes to M1/M2 Macrophages: Phenotypical vs. Functional Differentiation. Front Immunol. 5:514.
- Johnson AC et al., 2003, Experimental glomerulopathy alters renal cortical cholesterol, SRB1, ABCA1, and HMG CoA, reductase expression. Am J Pathol. 162:283–291.
- Jolly L and Stingele F, 2001, Molecular organization and functionality of exopolysaccharide gene clusters in lactic acid bacteria. Int Dairy J. 11:733-745.
- Jolly L et al. ,2002, Exploiting exopolysaccharides from lactic acid bacteria. Antonie Van Leeuwenhoek 82:367–374.
- Kang H et al., 2011, Exopolysaccharide-overproducing *Lactobacillus paracasei* KB28 induces cytokines in mouse peritoneal macrophages via modulation of NF-κβ and MAPKs. J Microbiol Biotechnol. 21:1174-8.
- Kearney N et al., 2011, *Lactobacillus paracasei* NFBC 338 producing recombinant beta-glucan positively influences the functional properties of yoghurt. Int Dairy J. 21:561–567.
- Khailova L et al., 2010, *Bifidobacterium bifidum* reduces apoptosis in the intestinal epithelium in necrotizing enterocolitis. Am J Physiol Gastrointest Liver Physiol. 299:G1118-G1127.
- Kitazawa H et al, 1998, Phosphate group requirement for mitogenic activation of lymphocytes by an extracellular phosphopolysaccharide from *Lactobacillus delbrueckii* ssp. *bulgaricus*. Int J Food Microbiol. 40:169–175.
- Korakli M and Vogel RF, 2006, Structure/function relationship of homopolysaccharide producing glycansucrases and therapeutic potential of their synthesised glycans. Appl Microbiol Biotechnol. 71: 790-803.

- Kujubu DA et al., 1991, TIS10, a phorbol ester tumor promoter-inducible mRNA from Swiss 3T3 cells, encodes a novel prostaglandin synthase/cyclooxygenase homologue. J Biol Chem. 266:12866-12872.
- Kwon HK et al., 2010, Generation of regulatory dendritic cells and $CD4^{+}Foxp3^{+}$ T cells by probiotics administration suppresses immune disorders. Proc Natl Acad Sci USA. 107:2159 -2164.
- Laws AP and Marshall VM, 2001, The relevance of exopolysaccharides to the rheological properties in milk fermented with ropy strains of lactic acid bacteria. Int Dairy J. 11:709–721
- Laws AP et al., 2008, Determination of the structure and molecular weights of the exopolysaccharide produced by *Lactobacillus acidophilus* 5e2 when grown on different carbon feeds. Carbohydr Res. 343:301-7.
- Lebeer S et al., 2008, Genes and molecules of lactobacilli supporting probiotic action. Microbiol Mol Biol R. 72:728-764.
- Lebeer S et al., 2009, Identification of a gene cluster for the biosynthesis of a long, galactose-rich exopolysaccharide in *Lactobacillus rhamnosus* GG and functional analysis of the priming glycosyltransferase. Appl. Environ. Microbiol. 75:3554-3563.
- Lebeer S et al., 2010, Host interactions of probiotic bacterial surface molecules: comparison with commensals and pathogens. Nat Rev Microbiol. 8:171-84.
- Lee JM et al., 2008, Antiinflammatory effect of lactic acid bacteria: inhibition of cyclooxygenase-2 by suppressing nuclear factor-kappaB in Raw264.7 macrophage cells. J Microbiol Biotechnol. 18:1683-1688.
- Li L et al., 2014, Immunoregulatory effects on Caco-2 cells and mice of exopolysaccharides isolated from *Lactobacillus acidophilus* NCFM. Food Funct. 5:3261-8.
- Linker A and Jones RS, 1966, A new polysaccharide resembling alginic acid isolated from *Pseudomonas*. J Biol Chem. 241:3845–3851.
- Liu CF et al., 2011, Immunomodulatory and antioxidant potential of *Lactobacillus* exopolysaccharides. J Sci Food Agric. 91:2284-2291.
- Looijesteijn PJ et al., 2000, Influence of different substrate limitations on the yield, composition, and molecular mass of exopolysaccharides produced by *Lactococcus lactis* subsp. c*remoris* in continuous cultures. J Appl Microbiol. 89:116–122
- McLoughlin RM and Kasper DL, 2009, Immunomodulation by zwitterionic polysaccharides. In: Moran A, Holst O, Brennan PJ, von Itzstein M (eds) Microbial glycobiology. Structures, relevance and applications. Academic Press, Elsevier, London, pp 957–980
- Mende S et al., 2012, Growth and exopolysaccharide yield of *Lactobacillus delbrueckii* ssp. *bulgaricus* DSM 20081 in batch and continuous bioreactor experiments at constant pH. J Biosci Bioeng. 113:185-191.
- Morteau O et al., 2000, Impaired mucosal defense to acute colonic injury in mice lacking cyclooxygenase-1 or cyclooxygenase-2. J Clin Invest. 105:469-478.
- Nishimura-Uemura J et al., 2003, Functional alteration of mucrine macrophages stimulated with extracellular polysaccharides from *Lactobacillus delbrueckii* ssp. *bulgaricus* OLL1073R-1. Food Microbiol. 20:267–273.
- Nwodo UU et al., 2012, Bacterial exopolysaccharides: functionality and prospects. Int J Mol Sci. 13:14002-15.

- Otte JM et al., 2009, Probiotics regulate the expression of COX-2 in intestinal epithelial cells. Nutr Cancer. 61:103-113.
- Patel A and Prajapati JB, 2013, Food and health applications of exopolysaccharides produced by lactic acid bacteria. J Adv Dairy Res 1:2.
- Patten DA and Laws AP, 2015, *Lactobacillus*-produced exopolysaccharides and their potential health benefits: a review. Benef Microbes. 6:457-71.
- Patten DA et al., 2014, The structure and immunomodulatory activity on intestinal epithelial cells of the EPSs isolated from *Lactobacillus helveticus* sp. Rosyjski and *Lactobacillus acidophilus* sp. 5e2. Carbohydr Res. 384:119-27.
- Pham PL et al., 2000, Production of exopolysaccharide by *Lactobacillus rhamnosus* R and analysis of its enzymatic degradation during prolonged fermentation. Appl Environ Microbiol. 66:2302-10.
- Polak-Berecka M et al., 2013, Bifidogenic and antioxidant activity of exopolysaccharides produced by *Lactobacillus rhamnosus* E/N cultivated on different carbon sources. Pol J Microbiol. 62:181-189.
- Postma PW et al., 1993, Phosphoenolpyruvate:carbohydrate phosphotransferase systems of bacteria. Microbiol Rev. 57:543-94.
- Radzun HJ et al., 1983, Monocytic origin of the human hematopoietic cell line U-937 and its convertibility to macrophages evidenced by isoenzyme mapping. Int J Cancer. 31:181-186.
- Ruas-Madiedo P and de los Reyes-Gavilán CG, 2005, Invited review: methods for the screening, isolation, and characterization of exopolysaccharides produced by lactic acid bacteria. J Dairy Sci. 88:843-56.
- Ruas-Madiedo P et al., 2002, An overview of the functionality of exopolysaccharides produced by lactic acid bacteria. Int Dairy J. 12:163–171.
- Ruas-Madiedo P et al., 2006, Short communication: Effect of exopolysaccharide isolated from "viili" on the adhesion of probiotics and pathogens to intestinal mucus. J Dairy Sci. 89:2355-2358.
- Ruas-Madiedo P et al., 2009, Biosynthesis and chemical composition of exopolysaccharides produced by lactic acid bacteria. In: Ullrich M (ed) Bacterial polysaccharides. Current innovations and futures trends. Caister Academic Press, Norfolk, pp 279–310.
- Ruijssenaars HJ et al., 2000, Biodegradibility of food-associated extracellular polysaccharides. Curr Microbiol. 40:194-199.
- Stuknyte M et al., 2011, Potential immunomodulatory activity of bovine casein hydrolysates produced after digestion with proteinases of lactic acid bacteria. Int Dairy J. 21:763–769.
- Sundstrom C and Nilsson K, 1976, Establishment and characterization of a human histiocytic lymphoma cell line (U-937). Int J Cancer. 17:565-577.
- Suresh Kumar A et al., 2007, Bacterial exopolysaccharides–a perception. J Basic Microbiol. 47:103-117.
- Sutherland IW, 2001, Biofilm exopolysaccharides: a strong and sticky framework. Microbiology. 147:3-9.
- Tan XD et al., 2000, Prostanoids mediate the protective effect of trefoil factor 3 in oxidant-induced intestinal epithelial cell injury: role of cyclooxygenase-2. J Cell Sci. 113:2149-2155.

- Taverniti V et al., 2012, *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. Appl Environ Microbiol. 78:4209-16.
- Torino MI et al., 2005, Exopolysaccharide biosynthesis by *Lactobacillus helveticus* ATCC 15807. Appl Microbiol Biotechnol. 68: 259-265.
- Traore K et al., 2005, Signal transduction of phorbol 12-myristate 13-acetate (PMA)-induced growth inhibition of human monocytic leukemia THP-1 cells is reactive oxygen dependent. Leuk Res. 29:863–879.
- Tsuchiya S et al., 1980, Establishment and characterization of a human acute monocytic leukemia cell line (THP-1). Int J Cancer. 26:171–176.
- Tuinier R et al., 1999, Isolation and physical characterization of an exocellular polysaccharide. Biopolymers 49:1–9
- Urbanska AM et al., 2010, Suppression of tumorigenesis: modulation of inflammatory cytokines by oral administration of microencapsulated probiotic yogurt formulation. Int J Inflam. 2010:894972.
- van Hijum SAFT et al., 2006, Structure-function relationships of glucansucrase and fructansucrase enzymes from lactic acid bacteria. Microbiol Mol Biol Rev. 70:157-176.
- Vinderola G et al., 2006, Effects of the oral administration of the exopolysaccharide produced by *Lactobacillus kefiranofaciens* on the gut mucosal immunity. Cytokine. 36: 254-260.
- Wachi S et al., 2014, *Lactobacillus delbruecki* TUA4408L and its extracellular polysaccharides attenuate enterotoxigenic *Escherichia coli* - induced inflammatory response in porcine intestinal epitheliocytes via Toll-like receptor-2 and -4. Mol Nutr Food Res. 58:2080-93.
- Watnick PI and Kolter R, 1999, Steps in the development of a *Vibrio cholerae* El Tor biofilm. Mol Microbiol. 34: 586-595.
- Welman AD and Maddox IS, 2003, Exopolysaccharides from lactic acid bacteria: perspectives and challenges. Trends Biotechnol. 21:269-74.
- Werning ML et al., 2006, *Pediococcus parvulus gtf* gene encoding the GTF glycosyltransferase and its application for specific PCR detection of β-D-glucan-producing bacteria in foods and beverages. J Food Prot. 69:161–169.
- Williams CS et al., 1999, The role of cyclooxygenases in inflammation, cancer, and development. Oncogene. 18:7908 -7916.
- Yang Z et al., 2010, Capsular and slime-polysaccharide production by *Lactobacillus rhamnosus* JAAS8 isolated from Chinese sauerkraut: potential application in fermented milk products. J Biosci Bioeng. 110:53-7.
- Yasuda E et al., 2008, Suppressive effect on activation of macrophages by *Lactobacillus casei* strain Shirota genes determining the synthesis of cell wall-associated polysaccharide. Appl Environ Microbiol. 74:4746–4755.
- Zeković DB et al.,2005, Natural and modified (1→3)-beta-D-glucans in health promotion and disease alleviation. Crit Rev Biotechnol. 25:205-30.

3 EXPLOITING THE ENERGETIC METABOLISM POTENTIAL OF *LACTOBACILLUS PARACASEI*

3.1 STATE OF THE ART

3.1.1. Overview on lactic acid bacteria metabolism

Lactic acid bacteria (LAB) have been traditionally used for the production of a diverse range of fermented foods with improved shelf-life, taste and nutritional properties. For this reason, their metabolic and genetic properties have been studied by means of several approaches oriented to metabolic and genetic manipulation (Brooijmans, 2009a). LAB have been classified as non-respiring, obligate anaerobes, producing lactic acid as a major end product from glucose. Specifically, they can be divided in obligate homofermentative, facultative heterofermentative, and obligate heterofermentative (Axelsson, 2003). The homofermentative species metabolize hexoses via glycolysis generating pyruvate, which is reduced to lactic acid as the sole end product. They are not able to metabolize pentoses. Obligate heterofermentative LAB do not utilize hexoses through glycolysis since they do not harbour the enzyme fructose 1,6-biphosphate aldolase, which catalyses fructose 1,6-biphosphate scission in glyceraldehyde 3-phosphate and dihydroxyacetone phosphate. For this reason, they ferment glucose through the pentose phosphate pathway, generating three final products in equimolar ratio: lactic acid, ethanol and CO_2 (and small quantities of acetic acid). They can utilize also pentoses, but without production of CO_2. Facultative heterofermentative bacteria, which comprise *Lactobacillus paracasei*, can utilize both hexoses and pentoses. Hexoses are fermented via glycolysis with production of lactic acid as sole final product (as in homofermentative LAB); pentoses are oxidized through the pentose phosphate pathway, without production of CO_2 (Kandler, 1983; Gänzle, 2015).

3.1.2. Respiratory metabolism in LAB: state of the art

Several LAB species have been reported to have a double metabolic life: they can switch from fermentation to aerobic respiration metabolism when provided with heme, and for some bacteria, heme and menaquinone (Pedersen, 2012). This observation dates back to the early seventies, when some studies demonstrated that exogenous heme could induce a behavior that resembled respiration in various LAB, including *Lactococcus lactis*, *Enterococcus faecalis*, *Streptococcus dysgalactiae* species and *Leuconostoc mesenteroides*. The first evidences related to the respiratory behavior concerned two main phenotypes: growth improvement and/or induction of cytochrome formation (Sijpesteijn , 1970).
The first requisite for a lactic acid bacterium to have a respiratory metabolism is the presence of three main membrane components:

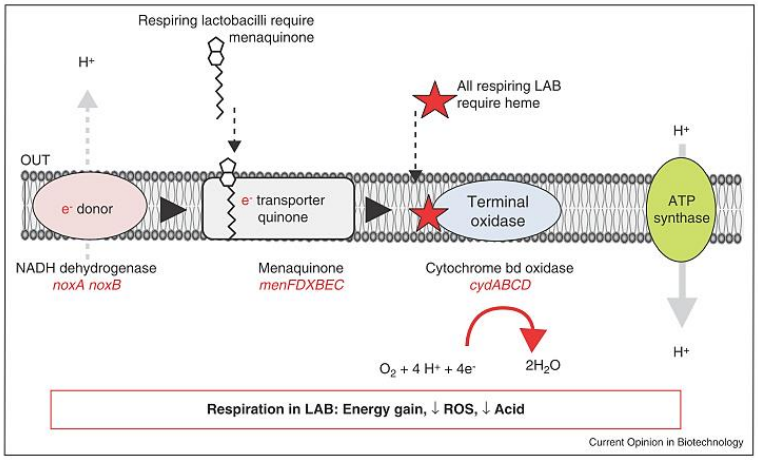
(i) an NADH dehydrogenase which acts as electron donor;

(ii) a quinone (in Gram-positive bacteria menaquinones), which is a lipophilic non-protein molecule that delivers electrons from the dehydrogenase to a final acceptor enzyme complex and concomitantly contributes to maintaining an electrochemical gradient (proton-motive force). Numerous LAB lack menaquinones biosynthesis genes, but can assimilate them from the environment (for instance ad vitamin K2).

(iii) an acceptor protein complex, namely a cytochrome oxidase, that reduces oxygen to water in the presence of H^+. This enzyme is encoded by the operon *cydABCD* , where *cydA* and *cydB* encode for the structural subunits, whereas *cydC* and *cydD* are required for the assembly (Puri-Taneja, 2007). Its activity depends on heme cofactor, which has to be administered exogenously. Various LAB encode genes for heme biosynthesis pathway; for instance, *Lactobacillus reuteri* harbors genes which may lead to vitamin B12 synthesis; others, like *L. lactis*, encode genes that allow iron insertion in the protoporphyrin. Nonetheless, to date no LAB encode the full complement of genes (Lechardeur, 2010).

Subsequently, protons extruded by the respiratory chain can be utilized by the FoF1 ATP synthase to generate ATP (Fig. 3.1). Unlike fermentation, energy from the respiration chain is generated by the activity of the ATP synthase, which recovers H^+ and generates ATP. In LAB, and particularly in *L. lactis*, the ATP synthase enzyme complex was initially identified for having the reverse role in fermentation, *i.e.* the elimination of intracellular H^+ that accumulates with lactic acid production. However, more recent studies suggest that H^+ flux may be bidirectional (Blank, 2001; Koebmann, 2008).

Fig. 3.1 Schematic figure of electron transport chain components in respiration-competent LAB. Benefits of respiration as compared to fermentation growth are indicated. ROS, reactive oxygen species. (Lechardeur, 2010).



In his review Pedersen (2012) classified LAB in the context of respiratory potential according to genetic characteristics, particularly for the presence of *cyd* genes (coding for the terminal cytochrome oxidase), and genes involved in the synthesis of menaquinones. Such classification is shown in Tab. 3.1.

The most extensively investigated LAB for the respiratory metabolism is *L. lactis*, an important species in the dairy industry, that conventionally converts 1 mole of glucose to 2 moles of lactate with the generation of 2 moles of ATP during glycolysis. Nonetheless, *L. lactis* is very tolerant to oxygen and satisfies the above cited requisites for respiration. In fact, its putative respiratory chain contains a heme-dependent bd-type cytochrome, encoded by the *cydABCD* operon that is capable of generating a proton motive force (Brooijmans, 2007).

Furthermore, *L. lactis* chromosome harbors genes encoding menaquinone biosynthesis enzymes; menaquinone production was in fact demonstrated for this bacterium (Tab. 3.1; Morishita, 1999; Wegmann, 2007). For these reasons, a well-documented literature provides data about *L. lactis* respiratory metabolism upon addition of heme in aerobic conditions, which leads to intense alteration of its phenotype, consisting in improved growth efficiency, increased bacterial biomass, resistance to oxygen, and robustness (long-term survival)(Duwat, 2001; Gaudu, 2002; Arioli, 2012).

Tab 3.1. Lactic acid bacteria classification of predicted or demonstrated respiration proficiency (Pedersen, 2012).

	Streptococcaceae	Lactobacillaceae
Respire with exogenous heme	*Enterococcus casseliflavus* ***Enterococcus faecalis*** *Enterococcus gallinarum* *Enterococcus italicus* *Eremococcus coleocola* ***Lactococcus lactis*** *Lactococcus garviae* *Leuconostoc argentinum* *Leuconostoc citreum* *Leuconostoc fallax* *Leuconostoc gasicomitatum* *Leuconostoc kimchii* ***Leuconostoc mesenteroides*** *Weissella cibaria* *Weissella paramesenteroides*	
Respire with exogenous heme + menaquinone	*Oenococcus oenii* ***Streptococcus agalactiae*** *Streptococcus dysgalactiae* *Streptococcus parauberis* *Streptococcus pseudoporcinus* *Streptococcus uberis*	*Lactobacillus antri* *Lactobacillus brevis* *Lactobacillus buchneri* *Lactobacillus casei* *Lactobacillus coryniformis* *Lactobacillus crispatus* *Lactobacillus fermentum* *Lactobacillus gasseri* *Lactobacillus hilgardii* *Lactobacillus johnsonnii* *Lactobacillus oris* *Lactobacillus paracasei* ***Lactobacillus plantarum*** *Lactobacillus reuteri* *Lactobacillus rhamnosus* *Lactobacillus salivarius* *Lactobacillus ultunensis* *Lactobacillus vaginalis*
Never respire[b]	*Enterococcus faecium* *Streptococcus equi* *Streptococcus gallolyticus* *Streptococcus gordonii* *Streptococcus infantis* *Streptococcus mitis* *Streptococcus mutans* *Streptococcus parasanguinis* *Streptococcus pneumoniae* *Streptococcus pyogenes* *Streptococcus sanguinis* *Streptococcus suis* *Streptococcus thermophilus*	*Lactobacillus acidophilus* *Lactobacillus delbrueckii* *Lactobacillus iners* *Lactobacillus sakei*

[a]Lactic acid bacteria (LAB) are classified for respiration proficiency using as criteria presence or absence of *cydABCD* genes. No LAB as known to date are genetically equipped for heme synthesis, although some encode an incomplete heme biosynthesis pathway. Only some LAB synthesize menaquinones. Here LAB are classified according to their capacity to respire aerobically in the presence of exogenous heme, or heme and menaquinone. [b]Nonrespiring LAB lack *cyd* genes. Closely related species are not necessarily in the same category with respect to respiration potential or required cofactors. Species in bold were tested experimentally, whereas the others carry the *cyd* genes. Species in each category are used in food fermentations.

These phenotypes are attributed to 4 main features intrinsic to the respiration process:

i) it is energetically more efficient than fermentation;

ii) it generates less acid stress: NADH is regenerated to NAD^+ by respiration chain NADH activity, to the detriment of lactate dehydrogenase, which uses NADH as cofactor for the reduction of pyruvate to lactate;

iii) it generates less oxidative stress: respiration reduces oxygen to water, decreasing intracellular oxygen;

iv) it acts as a "sink" for free electrons generated by the membrane NADH dehydrogenase (Lechardeur, 2010).

The extensive characterization of respiration in *L. lactis* has led to the examination of other LAB; studies on some *Leuconostoc mesenteroides* isolates revealed similar effects of respiration on growth and long-term survival. Among lactobacilli, which are deficient for both heme and menaquinone synthesis, *Lactobacillus plantarum* is probably the most studied, especially strain WCFS1 (Kleerebezem, 2003). Results indicate that conventional (static) fermentation conditions give better growth yields than aerobiosis; nevertheless, in aerobiosis, at the presence of heme and menaquinone, yields and long term survival are greatly improved (Brooijmans, 2009a). Interestingly, the same authors suggested that heme is also able to activate an anaerobic electron transport chain in *L. plantarum* WCFS1, in which nitrate is the terminal electron acceptor (Brooijmans, 2009b).

Regarding *L. casei* group species, a recent work by Zotta (2014) investigated the capability of more than a hundred strains belonging to *L. casei*, *L. paracasei* and *L. rhamnosus* species to cope with the presence of oxygen, ROS (reactive oxygen species) generating compounds, heme and menaquinone. They found that *L. casei* and *L. rhamnosus* exhibited the best adaptation to the aerobic conditions and most strains were also capable to consume oxygen. On the other hand, in the tested *L. paracasei* strains, heme supplementation impaired the growth of aerobic cultures, suggesting a reduced ability to use this compound and/or toxic accumulation into the cells.

3.1.3. External electron acceptors

Besides the extensive work on respiration, a smaller number of papers have demonstrated that the metabolic shift described above for *L. lactis* can occur also in the absence of heme, with the final electron transfer step being carried out mostly by quinones to the external environment. In fact, quinones in their reduced form (hydroquinones or quinols) can also directly reduce O_2 to some extent and thus enable the bacterium to endure microaerophilic conditions (Newman and Kolter, 2000). The same phenomenon can also occur in anaerobic environments, such as the human colon or the soil sediments, where redox potentials are substantially lower (-500 to -200mV), and where microbial growth and metabolism can rely (apart from fermentation) on the presence of alternative electron acceptors through an extracellular electron transfer (EET) to recycle electron carriers (such as NADH to NAD^+). These acceptors can be exogenous or endogenous, as for *L. lactis*, in which the production of a variety of soluble and insoluble quinones leads to the establishment of this transfer (Freguia, 2009). Specifically, *L. lactis* along with *L. plantarum* can exploit an exogenous soluble quinone, namely 2-amino-3-dicarboxy-1,4-naphthoquinone (ACNQ), the amino-functionalised precursor of menaquinone biosynthesis, for the reduction of hexacyanoferrate. This reduction was concomitant with a metabolic shift that generate pyruvate and acetate (in *L. plantarum*) and also acetoin (in *L. lactis*) (Yamazaki, 2002).

In other notable works, Khan (2012a and 2012b) studied the intestinal bacterium *Faecalibacterium prausnitzii*, a well-recognized butyrate-producing health-producing species inhabiting the human gut. They employed a microbial fuel cell system to investigate how the microorganism exploits the vitamin riboflavin, a soluble redox-active component commonly

present in the gut lumen, as an electronophore for extracellular electron transfer (EET). They showed that *F. prausnitzii* can electrochemically reduce riboflavin with the concomitant oxidation of NADH to NAD^+. The utilization of riboflavin as electron acceptor was observed also in *L. lactis* by Masuda (2010).

The fact that exogenous extracellular molecules can participate in the electron transfer to inorganic or organic compounds indicates that they may give a significant contribution to the dissipation of oxidative stress and redox-active substances in many environments, such as soil and human gut (Watanabe, 2009; Newman and Kolter, 2000). In fact, the human gut bears many redox-active substances derived from host, diet, and gut microbiota (van Niel, 2012; Khan, 2012a; Khan, 2012b). For this reason, the possibility that endogenous or exogenous extracellular molecules can be exploited in such environments remains an important open question.

.

3.2 AIMS OF THE STUDY

Lactobacillus paracasei is an obligate anaerobic lactic acid bacterium, with a facultative heterofermentative metabolism. From the genomic analysis conducted by Pedersen (2012), it has been classified as a potential respiratory species since it harbors the *cydABCD* operon, coding for a terminal cytochrome oxidase. Starting by this genetic prediction, and basing on previous several works conducted on *Lactococcus lactis* and *Lactobacillus plantarum* (Arioli, 2012, Brooijmans, 2009a; Brooijmans 2009b; Pedersen, 2008), we decided to evaluate if *L. paracasei* is able to establish this kind of alternative metabolism and, eventually, to gain technological advantages spendable at industrial level, as well as in the colonization of the human host.
To assess the respiratory potential, we performed experiments in the presence of the co-factors responsible for respiration, i.e. heme and menaquinone, in order to measure biomass formation and long-term resistance. Along with these methods, we performed cytofluorimetric analyses to measure membrane damage and oxidative stress, gene expression analyses on the main genes involved in the fermentation pathways and the cytochrome oxidase, and chromatographic analyses to measure the production of organic acids. Moreover, we constructed a bioluminescent reporter system by transforming *L. paracasei* with the pNZ-T5-LucGR vector, which allows the real-time measurement of ATP production rate during bacterial growth in different cultural conditions. The experiments were conducted on DG and LPC-S01 strains; however, having obtained the same results on both strains, we decided to show only the data regarding strain LPC-S01.

3.3 MATERIALS AND METHODS

3.3.1. Strains and cultural conditions

L. paracasei strains LPC-S01 and DG were refreshed from a glycerol stock suspension in de Man-Rogosa-Sharpe (MRS, Difco Laboratories Inc., Detroit, MI, USA) through an overnight incubation at 37°C. This pre-inoculum was then used to inoculate a chemically defined medium (CDM), whose composition is indicated in Tab. 2.2. *L. paracasei* was cultivated in anaerobiosis (An, allowed by the use of sealed vials) and aerobiosis (Ae, using Erlenmeyer flasks filled to less than 1/5-volume capacity in a shaking incubator at 180 rpm) at 37 °C. Co-factors were differentially added to the broth culture. Heme was supplied by adding 10 µg ml^{-1} hemin porcine (Sigma-Aldrich), corresponding to a molarity of 15 µM. Menaquinone was added to a final concentration of 50 µg ml^{-1} (corresponding to 11 µM) in the form of vitamin K2 (Sigma-Aldrich). Samples were then stored at 4°C for further analyses.

3.3.2. Long-term resistance evaluation

Concerning the viable count, an aliquot from samples stored at 4° was used to make serial decimal dilutions in saline solution pH 7.5, which were then plated on MRS agar medium. Colonies were then counted after 48-72 h of incubation at 37° C.
Another aliquot was collected from the samples stored at 4°C and used for the cytofluorimetric analyses, performed with a BD ACCURY TM C6 cytometer (BD Bioscience, San José, USA). To prepare unstained samples (to use as control of the background of fluorescence), serial decimal dilutions were made by using filter sterilized saline solution. The right dilution to perform the analysis had to give a number of event $µl^{-1}$ comprised between 1000 and 2000.
The dyes we utilized were propidium iodide (PI, dissolved in dH_2O) and dihydroethidium (DHE, dissolved in DMSO), both purchased by Sigma-Aldrich, that were added to 200 µl of broth culture at a final concentration of 5 µg ml^{-1} and incubated at 37° C for 10 min. Afterwards, serial dilutions were made as described above, and the analysis was performed by using a channel with filter of 610/20 (FL-3).

3.3.3. Detection of heme by bathophenantroline sulfonate (BPS) assay

For the BPS assay, 0.9 ml of broth culture with or without addition of heme were collected before the inoculum (T0). After 24 and 48 h growth, 1 ml of broth culture was collected and cells and medium were separated by centrifugation at 10,000 x g for 10 min. Cells were then washed with 1 ml of 50 mM Tris buffer pH 7.4 and centrifuged again, in order to obtain the washed fraction. Cells were then resuspended in 1 ml Tris buffer and added with about 200 mg 0.1 mm glass beads to obtain a clarified lysate fraction after treatment with a Bead Beater Precellys 24 (Bertin Technologies, Montigny le Bretonneux, France). All the fractions were added with 100 µl of 6 M TCA in order to detach complexed iron. Afterwards, the sample was neutralized by addition of Tris-base salt until reaching pH 7.4. The colorimetric assay was performed by addition of 0.2 mM bathophenanthrolinedisulfonic acid (BPS) and 10 mM sodium dithionite. The complex Fe(II)-bathophenantroline was detected at a wavelength of 535 nm, as described elsewhere (Ljones and Burris, 1978; Bonomi, 1998; Miriani, 2014). The calculus of iron concentration was performed by considering the molar extinction coefficient for the complex Fe(II)-bathophenantroline, which is 25000 M^{-1} cm^{-1}.

3.3.4. Construction of the bioluminescent reporter system *L. paracasei* LPC-S01-LucGR

L. paracasei strains were transformed, after the induction of competence, with the expression vector pNZ-T5-LucGR, by following a protocol suggested by Palomino (2010). Briefly, pre-cultures of *L. paracasei* were obtained in MRS medium after incubation without aeration at 37°C for 18 h. Competent cultures were obtained after inoculating 1 ml of the pre-culture to 100 ml MRS medium containing 0.9 M NaCl, and then further incubated. When OD_{600nm} reached 2–2.5, growth was stopped by cooling cultures on ice for 10 min. The cells were harvested by cool centrifugation and washed three times with ice-cold ultrapure water. Cells were resuspended in 1 ml of water (100-fold concentration of cell suspension), resulting in a total count of about 10^{12} CFU/ml and fractions of 50 µl were either used directly for electroporation or stored at −80 °C. Electroporation was carried out using a MicroPulser Electroporator (Biorad, Milano, Italy). 50 µl of competent cells were mixed
with 5 µl plasmid DNA (50 to 100 ng/experiment) in 0.2-cm electrode gap cuvettes and subjected to a voltage of 2.5 KV. Following electroporation, cells were mixed with 0.95 ml MRS medium and incubated at 37 °C for 3 h. About 100 µl of a 1/5 dilution was plated on MRS agar medium with chloramphenicol (12 µg ml^{-1}). After 48–72 h of anaerobic incubation, colonies were screened to find positivity by checking the level of production of light.
The obtained recombinant strains were able to express constitutively the gene *lucGR*, coding for *P. plagiophtalamus* luciferase enzyme. *L. paracasei* LPC-S01-LucGR, grown overnight in 10 ml CDM added with 12 µg ml^{-1} chloramphenicol, was used to inoculate 20 ml CDM in aerobiosis and anaerobiosis at 37°C, with addition of heme and/or menaquinone to the final concentration indicated above. Every h one aliquot of broth culture was collected and used to monitor the light production: specifically, to 200 µl of bacteria were added 50 µl d-luciferin dissolved in 0.1 M citrate buffer pH 5.5 (Sigma-Aldrich), corresponding to 125 µM of final concentration. The bioluminescence emitted by the recombinant cells was measured by means of a Victor3 luminometer (Perkin-Elmer, USA), maintaining a constant temperature of 37°C inside the instrument.

3.3.5. Gene expression analysis

RNA from exponential and stationary phase cells was extracted as described by Arioli (2010). Briefly, about 2 x 10^9 cells were collected and centrifuged to remove the medium. The pellet was dissolved in PureZol® buffer (provided by the Aurum™ Total RNA Fatty and Fibrous Tissue Kit, Bio-rad) and about 100 mg of 0.1 mm glass beads were added, to lyse properly the cells by means of a Bead Beater Precellys 24 (Bertin Technologies, Montigny le Bretonneux, France). After the removal of the beads by mild centrifugation, we followed the instructions provided by the Biorad kit. Afterwards, we treated 15 µl of extracted RNA by adding 1 unit of DNAse (Sigma-Aldrich) and by incubating at RT for 30 min. Stop solution provided by the manufacterer was added to inhibit the enzyme at 70°C for 10 min. After that, we quantified and checked the integrity of the RNA obtained by reading the absorbance at 260/280 nm and by running 1 µl of sample on a 1% agarose gel. 1 µg of RNA was then used for the reverse transcriptase reaction (iScript™ cDNA Synthesis Kit, Bio-rad) following the kit procedure.
In the Real Time qPCR 50 ng of cDNA were used for each reaction. Real-time Quantitative PCR (RT-qPCR) was carried out in order to measure the mRNA expression levels of metabolic genes by means of the SYBR Green technology using the SsoFast EvaGreen Supermix (Bio-Rad) on a Bio-Rad CFX96 system according to the manufacturer's instructions. The primers used are as follows (5'→3'): Tuf forward: CCGTGGTTCTGCTCTGAAGG; Tuf reverse: TGTTTCACGAACAGGTGTTGGG; GAPDH forward: GGCTATCGGTTTGGTTATCCCA; GAPDH reverse: GTTGTTTTCGGTGTGCTTCTTG; Pol forward:

GATTCAGCAAGGGCTCGGTAAG; Pol reverse: GATTGGGCTGCTTGTTCCTCAT; Rpo forward: CGGGCTTTCTCGGATGGAAC; Rpo reverse: AACTCACCAAGCGGATTCGTCT; Cydq forward: CAGTCAAAGGCATGGAGACAGC; Cydq reverse: CCGAGCAGGAACAGAAGAACG; Ldh1 forward: GCGACAAGCCCCAGTGTTGATT; Ldh1reverse: GCAAGACGGTCAAGGGACTCCT; Ldh2 forward: CCTGTATGGTCCCACGCTAACA; Ldh2 reverse: CGGATAGTGGCAGAACCGCATT; Ldh3 forward: GTTGCTGACGTGCGTGGTTAC; Ldh3 reverse: GACCGCCTGTGTCATTTCAGTC; Ldh4 forward: CCAGGTCAGACACGCCTACAAC; Ldh4 reverse: AACGCAGACCGATCTCAGACCG; Pfl forward: GTATTGCTTGCTGTGTCTCGG; Pfl reverse: GTTGATCGCATACAGAACCGTC; Pdh forward: CAGAGGGCACGTACACGGTA; Pdh reverse: CAGCAGCTTTCAACGCTTCGC; Ack1 forward: AGCCGACTGTATCGCCATCT; Ack1 reverse: CGCATTTGTGCCAATGGTTTGC; Ack2 forward: GTGTTTGATACGGCGTTCCACC; Ack2 reverse: CCTTGGACTGCGGTAACACTG. All primers were designed previously and their specificity was assessed with melting curves during amplification and by 1 % agarose gels. Quantitative PCR was carried out according to the following cycle: initial hold at 95 °C for 30 s and then 39 cycles at 95°C for 10 s and 60.2°C for 30 s. Gene expression was normalized to the reference genes *gapdh*, *pol*, *rpoB*, and *tuf*. The amount of template cDNA used for each sample was 20 ng. All results regarding target mRNA expression levels are reported as the fold of induction (FOI) respective to the control (namely sample without co-factors), to which we attributed a FOI of 1.

3.3.6. NAD pool and organic acids quantification

NAD pool was measured by means of the Abcam® NAD/NADH Colorimetric Assay Kit (Abcam, Cambridge, UK) following the manufacturer instructions. To quantify glucose, lactic acid and acetic acid, 1 ml of broth culture was centrifuged at 9,000 x g for 10 min, and the obtained exhausted broth was used for the chromatographic analyses. For the quantification of glucose, the chromatographic system consisted of an Alliance model 2695 (Waters) equipped with a model 2420 (Waters, Milford, MA, USA) evaporative light scattering detector (ELSD). A 5 µm Prevail Amino column (250x4.6 mm, Alltech) was used for the separation in isocratic mode at a flow-rate of 1.5 ml min^{-1} and the eluent was acetonitrile:water (80:20, v/v). Acetonitrile was purchased from Merck (Darmstadt, Germany). The column and the sample were maintained at 35 and 20° C, respectively. The ELSD conditions were the following: gain 100, pressure 40 psi and drift tube temperature 40° C. All data were acquired by Empower 2 software (Waters). Calibration curves were obtained from glucose stock solutions prepared by dissolving 200 mg of standard powder in 20 ml of water. The working solutions in water:acetonitrile (1:1, v/v) were prepared in the range of 0.2-1 mg ml^{-1}. For the quantification of lactic and acetic acid, an UPLC-HR-MS analysis was carried out on an Acquity UPLC separation module (Waters,) coupled with an Exactive Orbitrap MS through an HESI-II probe for electrospray ionization (Thermo Scientific, San Jose, CA, USA). The ion source and interface conditions were: spray voltage -3.0 kV, sheath gas flow-rate 35, auxiliary gas-flow rate 10 and temperature 120° C, capillary temperature 320° C. A 1.8 µm HSS T3 column (150x2.1 mm, Waters) was used for separation at a flow-rate of 0.2 ml min^{-1}. The eluents were 0.001% HCOOH in MilliQ-treated water (solvent A) and CH3OH:CH3CN (1:1, v/v, solvent B, Darmstadt). Five µl of the sample were separated by the UPLC using the following elution gradient: 0% B for 4 min, 0-15% B in 6 min, 15-20% B in 5 min, 20% for 13 min and then return to initial conditions in 1 min. The column and samples were kept at 30 and 15°C, respectively. The UPLC eluate was analyzed in full scan MS in the range 50-130 m/z. The resolution was set at 50 K, the AGC target was 1E6 and the maximum ion injection time was 100 ms. The ion with m/z 91.0038, corresponding to the formic acid dimer [2M-H]-, has been used as lock mass. The mass tolerance was 2 ppm. The MS data were processed using Xcalibur software (Thermo Scientific). Lactic and acetic standard mother

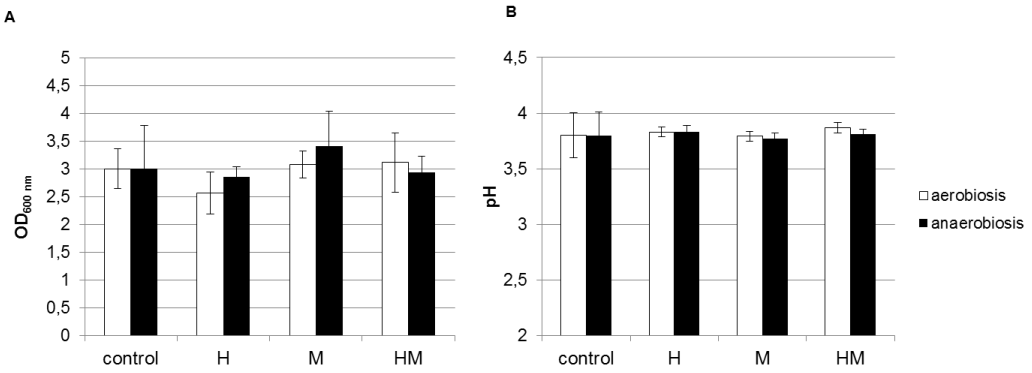
solutions (1 mg ml^{-1}, Sigma-Aldrich) were prepared by dissolving 0.1 ml of standard in 100 ml of water. The working solutions in 0.001% formic acid were prepared in the range of 0.2-20 µg ml^{-1}. Six-point external calibration curves were adopted to quantify lactic and acetic acid in fecal samples. Acid concentrations were expressed in mg per ml of exhausted broth.

3.4 RESULTS AND DISCUSSION

3.4.1. Effect of heme and menaquinone on *L. paracasei* LPC-S01 growth

The first phenotype observed in lactic acid bacterial species with demonstrated respiratory metabolism (i.e. *L. lactis*) was an increased biomass formation when the microorganism was inoculated in a medium containing heme. Moreover, it has been reported that higher biomass formation is associated with higher final pH (Gaudu, 2002; Arioli, 2012). The same observations have been made by other authors when studying *L. plantarum* metabolism, even if, in this case, also a source of menaquinones was added to the medium due to the inability of this species to synthesize them (Brooijmans, 2009a). Similarly, in our experiments on *L. paracasei* LPC-S01, we added 10 μg ml^{-1} hemin and 50 μg ml^{-1} vitamin K2 to the chemically defined medium (CDM) we developed (Tab. 2.2). However, after 24 h growth, when cells achieved stationary phase, we found that the addition of the two co-factors did not improve either *L. paracasei* growth or pH if compared to controls (Fig. 3.2). The same result was found in both aerobiosis (Ae-HM) and anaerobiosis (An-HM).

Fig. 3.2. Growth rate expressed in OD_{600nm} **(A)** and pH **(B)** of *L. paracasei* LPC-S01 cultures in CDM after 24 h in anaerobiosis (An, black bar) and aerobiosis (Ae, white bar), without cofactors or with addition of heme (H) and/or menaquinone (M). Data have been normalized against the respective control and indicate average geometric means of three replicates ± standard deviation, calculated from at least three independent assays.



3.4.2. Effect of heme and menaquinone on *L. paracasei* long-term survival

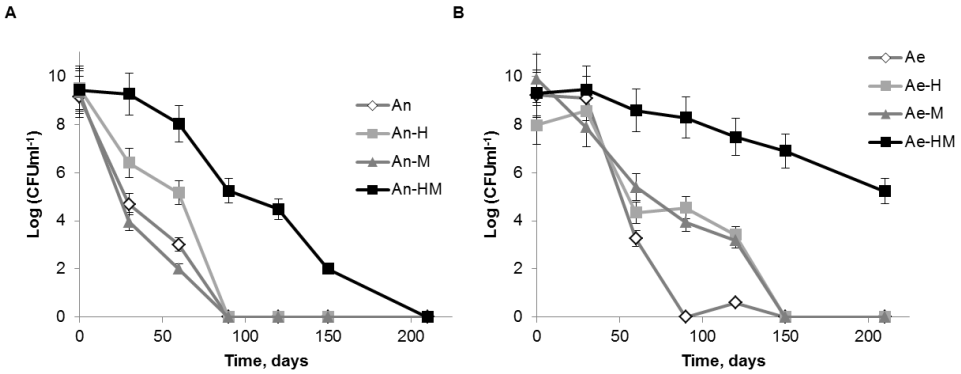
The second clear phenotype observed in respiring *L. lactis* cells was prolonged survival, or robustness, when stored at 4°C for a long period (Arioli, 2012). Consequently, the effects of the different growth conditions on the long-term storage survival of *L. paracasei* were examined by monitoring the cell viability during a period of storage at 4°C. The viable cell population of the anaerobic cultures after 24 h growth reached approximately 10^9-10^{10} CFU ml^{-1}, whereas the viability of aerobic culture was slightly lower (between 10^8-10^9 CFU ml^{-1}), particularly in culture supplemented with heme, even if not statistically significant. Interestingly, when LPC-S01 was cultured in presence of heme and menaquinone, it was able to survive for a much longer period if compared to the cultures supplemented with none or just one of the molecules. Specifically, concerning anaerobic cultures, we observed a drastic reduction of viability after 30 days of storage and the complete abatement of the survival after 90 days for all samples with the

exception of An-HM (Fig. 3.3A). Similarly, aerobic cultures showed a strong decrease and complete loss of viability after 60 and 150 days, respectively, with the only exception of sample Ae-HM) (Fig. 3.3B). These data suggest that:

(i) both heme and menaquinone are necessary to confer to *L. paracasei* peculiar robustness, characterized by a viability decrease of only 3 logs after 210 days of storage;
(ii) oxygen plays an important role during growth in the establishment of robustness.

These results are in accordance with previous studies performed on *L. lactis* IL-1403, in which survivability increase was observed during 90 days of storage (from about 10^{10} to 10^{5} CFU ml^{-1}; Arioli, 2012). An even stronger phenotype was observed in a study on *L. lactis* strain MG1363, in which a viability of 10^{8} CFU ml^{-1} was not affected during 70 days (Duwat, 2001).

Fig. 3.3. Viability of bacterial cells grown for 24 h in anaerobiosis **(A)** and aerobiosis **(B)**, and then stored at 4°C. Time 0 corresponds to sampling performed after 24 h of growth. Cell survival is expressed as log CFU ml^{-1} in CDM medium. Cells were grown under four conditions at 37°C: negative control (no co-factors added; white diamonds), addition of heme (grey squares), addition of menaquinone (grey triangles), and addition of both heme and menaquinone (black squares). Data points indicate average geometric means of three replicates ± standard deviation, calculated from three independent biological assays for each time point.



To gain more information about the reason of this acquired robustness, we investigated membrane integrity and level of radical formation by means of flow cytofluorimetric assays, using propidium iodide (PI) and dihydroethidium (DHE) to stain bacterial cells. Specifically PI has been used to estimate dead bacterial according to severe membrane damage. PI, in fact, does not enter cells with intact membranes, i.e. viable cells. On the contrary, cells with damaged membrane allow PI entrance into the cytoplasm, where it binds nucleic acids, causing the rise of emitted fluorescence (Watanabe, 2012). On the contrary, DHE provides a measure of the oxidative stress levels inside the cell. In detail, this dye can freely cross cell membrane and, once in the cytoplasm it can be oxidized by intracellular oxidizing molecules, thus being converted into ethidium. Ethidium can intercalate DNA, thus increasing fluorescence. Particularly, DHE oxidation is relatively specific for superoxide anion, but it can also be induced by hydrogen peroxide, peroxynitrate, and hypochlorous acid (Herrera, 2003).

We performed the analyses on samples Ae-HM, An, and Ae immediately after growth (day 0) and after 30, 50, 100 and 150 days of storage. We expressed the data as ratio between

fluorescence emitted in An or Ae (controls) and fluorescence of sample Ae-HM (Fig. 3.4). Fig. 3.4A summarizes the analyses performed with PI; after 24 h growth the An/AeHM and, to a lesser extent, the Ae/AeHM ratios indicated that membrane damage was lower in cells grown in presence of heme and menaquinone. This can be observed also after several days of storage, reflecting the higher viability of sample Ae-HM for longer periods. After 150 days, the ratio was equal to 1 so, at that stage, the damage was similar in all samples; however, at that time point exclusively sample Ae-HM still contained viable cells. This difference could be due to the fact that two populations of cells normally exist: viable but non cultivable (VBNC) and viable-cultivable (VC) cells (Khan, 2010). For this reason, we can hypothesize that Ae-HM cells exhibit a limited membrane damage that did not compromise their survival.
Regarding oxidative stress, we found a similar trend regarding the ratio between Ae or An, and Ae-HM sample, which was always above 1 (Fig. 3.4B). Interestingly, the ratio An/Ae-HM showed a nearly linear growth, indicating that increasing formation of reactive oxygen species (ROSs) was occurring in the sample cultivated in absence of oxygen. This is plausibly explained by the hypothesis that growing in aerobiosis triggers the activation of defensive mechanisms which help bacterial cells to counteract the oxidative stress due to the presence of oxygen (Imlay, 2013).

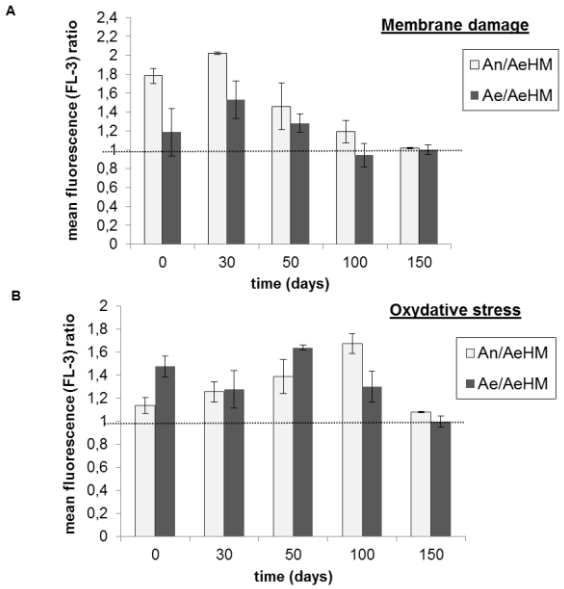
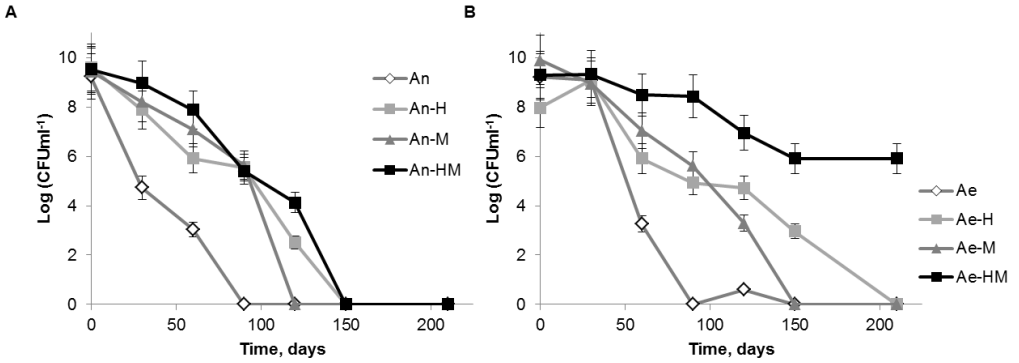


FIG 3.4. Membrane integrity **(A)** and radical formation **(B)** expressed as mean fluorescence (FL-3) ratio between samples Ae/Ae-HM (dark grey bar) and An/Ae-HM (white bar). Samples were analyzed at time 0 (corresponding to 24 h of growth at 37° C) and after 30, 50 100 and 150 days of incubation. Data indicate average geometric means of three replicates ± 1 standard deviation, calculated from three independent biological assays for each time point.

Since we just observed one of the two main phenotypes related to the respiratory metabolism, we wondered if the contribute of heme and menaquinone to the observed phenotype occurred during the bacterium growth, as stated in the "respiration theory", or only during storage. To assess this hypothesis, we added none, only one or both molecules to four different aliquots of a unique broth culture, grown 24 h in aerobiosis or anaerobiosis, and we stored them at 4°C. We observed that the addition of only one or both co-factors conferred robustness to *L. paracasei* in an independent way with respect to the condition of growth (Fig. 3.5). We found a big difference again between anaerobiosis and aerobiosis growth conditions, since the latter allowed a higher gain in survival, particularly when both heme and menaquinone were added. This phenotype has been never described before and strongly suggests that, in our strain, the observed robustness is

plausibly independent from either a respiratory metabolism or any pathway related to the growth phase of the bacterium.

Fig. 3.5. Bacterial survival for cells grown for 24 h in anaerobiosis **(A)** and aerobiosis **(B)**, and then stored at 4°C. Time 0 corresponds to sampling performed after 24 h of growth. Cell survival is expressed as log CFU ml^{-1} in CDM medium. After growth, cells were splitted into four aliquotes: negative control (no co-factors added; white diamond), addition of heme (grey squares), addition of menaquinone (grey triangles), and addition of both heme and menaquinone (black squares). Data points indicate average geometric means of three replicates ± standard deviation, calculated from three independent biological assays for each time point.



3.4.3. Determination of heme absorption by bacterial cells

The results indicated above instilled us the doubt that hemin and vitamin K2 added to the medium could act just externally, since their final effect (i.e. the increasing survival) occurred also when they were added after the bacterial growth was completed, i.e. while samples were stored at 4°C, a temperature that plausibly should not allow an efficient transport and metabolism of the co-factor inside the cell. To confirm this hypothesis, we quantified heme in broth and cells fractions after bacterial growth in presence of the heme cofactor; in specific, we used a colorimetric quantitative assay based on the use of bathophenantroline sulfonate (BPS), which specifically allows the measurement of total iron in the sample (Landers JW and Zak, 1958). Since chemically defined medium does not contain any source of iron apart from the one deriving from the hemin prosthetic group, we could detect the presence of hemin outside or inside the bacterial cells. We collected one aliquot of broth with and without addition of hemin at the beginning of the experiment (i.e., before inoculating *L. paracasei* LPC-S01), and then an aliquot at 24 and 48 h of growth. Specifically, we analyzed broth, Tris buffer used to wash once bacterial cells (in order to remove hemin loosely attached to the cell wall or membrane), and cell lysate fractions. As presented in Tab. 3.2, heme was detectable only in the broth fraction, indicating that it is not imported inside the cells at a significant amount. It can be also hypothesized that heme is internalized but rapidly excreted by efflux pumps due to its intracellular toxicity. In *L. lactis* and *Streptococcus agalactiae* heme-specific efflux pumps were identified, having the function to maintain heme homeostasis inside the cells, coded by operons *ygfCBA* and *pefAB*/*pefRCD*. This latter manages heme and iron-free protoporphyrin IX homeostasis at low heme concentrations (below 1 µM) (Fernandez, 2010; Stauff, 2008; Lechardeur, 2011).

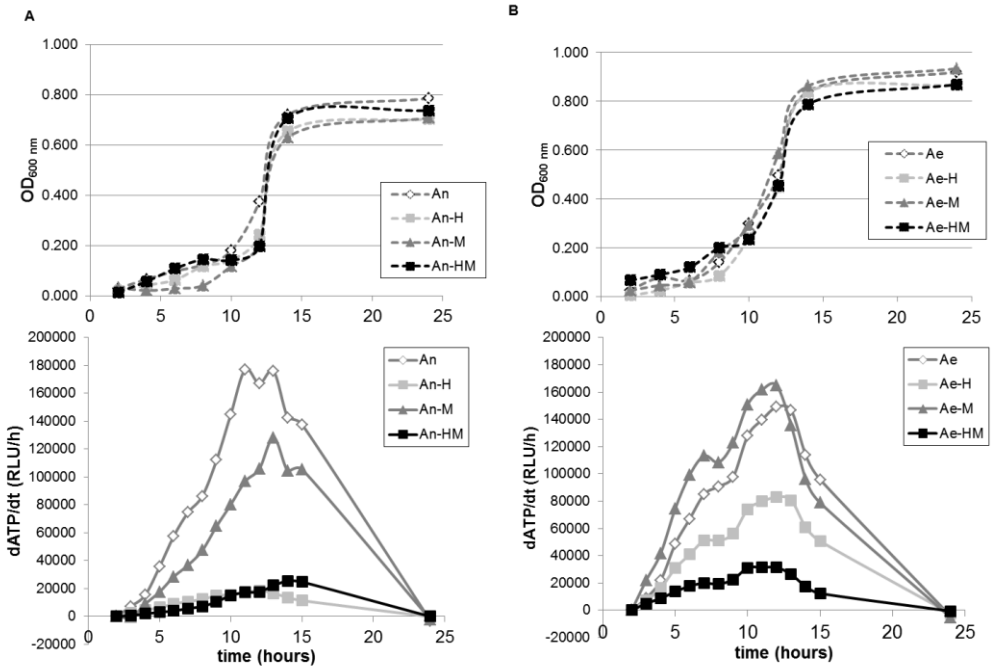
Tab.3.2. Heme quantification by means of bathophenantroline sulfonate (BPS) colorimetric assay. Data are average geometric means of two replicates ± standard deviation, calculated from two independent biological assays for each time point. nd, heme not detected.

	time (h)		
	0	24	48
	heme (µM)		
broth	14.11 ±1.19	16.18 ±1.38	14.76 ±1.16
washed		nd	nd
cells		nd	nd

3.4.4. Effect of heme and menaquinone on ATP production rate

We constructed a bioluminescent biosensor that can be used for a quick analysis of the metabolic state of cells under different conditions. To construct luminescent *L. paracasei*, we electroporated LPC-S01 with vector pNZ-T5-LucGR, which contains the luciferase gene (*lucGR*) from *P. plagiophtalamus* under the control of a T5 phage promoter. The insect luciferase is a reporter gene, which catalyzes a reaction in which ATP, D-luciferin and O_2 are converted to AMP, CO_2, inorganic pyrophosphate and oxyluciferin, together with yellowish light production at 560 nm. Therefore, firefly luciferase catalyzes a bioluminescent reaction that depends stoichiometrically on ATP. ATP is involved in many essential metabolic processes, and its intracellular level provides a measure of the metabolic state of a cell. Quantification of light emission, i.e. bioluminescence, is one of the most sensitive real-time means of assessing metabolic state of a cell (Guglielmetti, 2008). For this reason, we used the recombinant strain LPC-S01-LucGR to monitor intracellular ATP in anaerobiosis and aerobiosis with the addition of heme and/or menaquinone (Fig. 3.6). Light emission measured as a function of growth time showed a similar overall trend and revealed that bioluminescence drops drastically when cells reach the mid-log phase (i.e., after about 12 h from the inoculum). Light emission by recombinant cells was highest at the early-logarithmic stage of growth; particularly, the highest dATP/dt value was observed for cultures growing without co-factors in both conditions and with menaquinone, particularly in aerobiosis. Conversely, we observed that heme alone or combined with menaquinone, brings to lower intracellular ATP during the entire growth curve. This observation is in accordance with the hypothesis of the existence of a transporter involved in an ATP-dependent import/export of heme (Lechardeur, 2011).
Our results are in contrast to the experiments conducted with bioluminescent *L. lactis* 1403-945 (a derivative of IL1403). In fact, Arioli (2012) demonstrated that the maximum dATP/dt value was measured in the early exponential phase of growth during fermentation and in the late exponential phase of growth during respiratory metabolism. Therefore, our results do not support the hypothesis of respiration, which implies a higher yield of ATP production (the number of units of ATP generated per unit of sugar molecule consumed) due the presence of the FoF1-ATPase, that exploits the proton gradient force generated by the electron transport chain (Pfeiffer, 2001). In addition, respiration should be characterized by low growth rate and high biomass production, whereas fermentation is characterized by high rate of glucose consumption and low biomass production.

Fig. 3.6. ATP production rate (dATP/dt) expressed as light emission during growth (panel above) of *Lactobacillus paracasei* LPC-S01-LucGR in CDM in anaerobiosis **(A)** and aerobiosis **(B)** in four conditions: negative control (no co-factors added; white rombi), addition of heme (grey squares), addition of menaquinone (grey triangles), and addition of both heme and menaquinone (black squares). Broken lines represent cell densities. Solid lines represent light emission values.



3.4.4. Gene expression analysis, quantification of organic acids, and NAD^+/NADH ratio evaluation in *L. paracasei* LPC-S01 grown in aerobiosis

Considering the pivotal role of oxygen in the observed phenotype, we evaluated the gene expression levels, the production of lactic and acetic acid, and the ratio between NAD^+ and NADH in *L. paracasei* LPC-S01 grown in aerobic condition. Specifically, we determined by qRT-PCR the relative expression levels of *ldh* (lactate dehydrogenase), *cydA* (cytochrome bd oxidase subunit I), *pfl* (pyruvate-formate lyase), *pdhB* (pyruvate dehydrogenase E1 component β subunit), and *ack* (acetate kinase). Gene expression and the NAD^+/NADH analyses were performed at two different stages of growth: mid-exponential phase (exp) and beginning of the stationary phase (stat, Fig. 3.7A). In addition, we collected a sample after 24 h of growth to measure by UPLC/MS residual glucose, lactate and acetate.

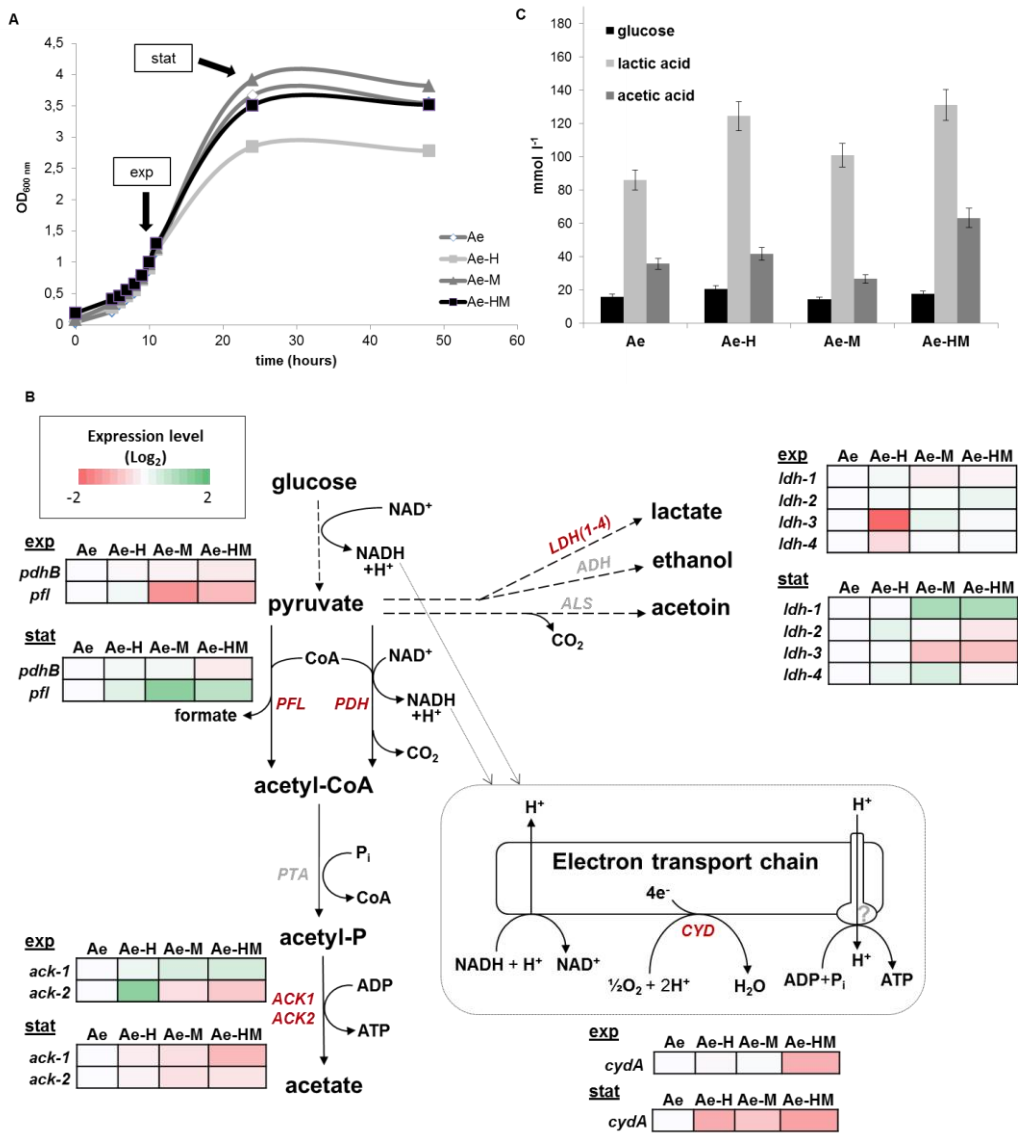
Regarding gene expression levels, we found only few significant differences among the four conditions tested. In specific, among the four *ldh* genes identified in *L. paracasei* LPC-S01 genome, the isoform *ldh-1* was constitutively expressed in all conditions, as also observed in previous studies (Rico et al., 2008; Fig. 3.7B). Particularly, we could detect an induction of its expression in stationary phase in presence of menaquinone with and without heme. Isoforms *ldh-2* and *ldh-4* were constitutively expressed as well; on the contrary, *ldh-3* showed a strong inhibition during exponential phase in the Ae-H sample. Interestingly, NAD^+/NADH ratio in AE-H at the exponential phase was 0.97 (Tab. 3.3), i.e. significantly lower than other conditions,

suggesting that the inhibition of *ldh-3* by heme may imply a slower rate of pyruvate fermentation to lactic acid, thus reducing also glycolysis process efficiency due to the lack of the oxidized form of NAD (Garrigues, 1997). However, after 24 h of incubation, lactic acid was accumulated in Ae-H broth (Ae; Fig. 3.7C).
Regarding pyruvate-formate lyase (*pfl*) expression, an enzyme that evolves pyruvate to acetyl-CoA towards production of acetate, the presence of menaquinone (combined or not with heme) led to its inhibition during exponential phase and to activation during the stationary phase (Fig 3.7B). Although having found no significant differences in acetate kinase (*ack-1* and *ack-2*) expression, we observed higher production of acetate in Ae-HM condition (Fig. 3.7C). Addressing pyruvate to acetate instead of lactate, can give an energetic advantage to the cell, since it involves the gain of one molecule of ATP for each molecule of acid produced. Differently inform previous observation in respiring *L. lactis* cells (Arioli, 2012), acetoin and diacetyl were not detectable in our samples. The reason why the combination of aerobiosis condition with the two exogenous molecules heme and menaquinone led to the increase of acetic acid is still under investigation, but we can speculate that relies on the NAD pool status (Tab.3.3): NADH generated by glycolysis can take other ways to be re-oxidized that differ from the lactic fermentation, possibly the electron transfer chain, as in respiration metabolism in *L. lactis*, or an extracellular electron transfer (EET) (Freguia, 2009; Yamazaki, 2002). The fact that, in our experiments, the terminal cytochrome oxidase subunit A (*cydA*) expression resulted inhibited in presence of heme and/or menaquinone (Fig. 3.7B), further support the hypothesis of the absence of an actual respiratory metabolism in the *L. paracasei* strains under study. On the contrary, data collected so far suggest that the strong robustness we observed in presence of both heme and menaquinone (which is boosted by the presence of oxygen) is due to an external or periplasmic phenomenon. At this point, it would be of interest to carry out a more comprehensive gene expression analysis by means of transcriptomic approaches, in order to have more detailed information about the metabolic scenery and the involvement of specific enzymes in the predicted extracellular electron transfer (Pedersen, 2008; Stevens, 2008).

Tab. 3.3. NAD^+/NADH ratio measured in exponential and stationary collections as indicated in Fig. 3.7A.

	NAD^+/NADH ratio	
sample	***Exponential***	***Stationary***
Ae	15.44	0
Ae-H	0.97	0
Ae-M	11.94	0
Ae-HM	7.4	0.02

Fig. 3.7. (A) *Lactobacillus paracasei* LPC-S01 growth curve under four conditions: aerobiosis (Ae), aerobiosis with addition of heme (Ae-H), aerobiosis with addition of menaquinone (Ae-HM) and aerobiosis with addition of both molecules (Ae-HM). Aliquots for the extraction of mRNA and quantification of NAD pool were collected as indicated: during exponential (exp) and at the beginning of stationary (stat) phase. **(B)** Quantitative analysis of gene expression. Expression levels of *ldh-1, ldh-2, ldh-3, ldh-4, pdh, pfl, ack-1, ack-2* and *cydA* are shown as the Log_2 of the fold change of induction (FOI) relative to the control (Ae sample), which was set at a value of 1. Presented data are the means of measurements for a result representative of three independent experiments. **(C)** Quantification by means of LC/MS of glucose, lactic acid and acetic acid in the broth of *L. paracasei* LPC-S01 at the beginning of the stationary phase (24 h growth).



3.5 CONCLUSIONS

Lactic acid bacteria are widely used in food industry for the production of a diverse range of fermented foods with improved shelf-life, taste and nutritional properties. A growing body of literature demonstrates how some species belonging to this group of bacteria have a double metabolic life: they can switch from fermentation to a respiratory-like metabolism (Pedersen, 2012). However, such metabolic switch is achieved only upon exogenous exposure to a source of heme and, in some cases, menaquinones, which together with a terminal cytochrome oxidase help to establish a functional electron transfer chain (Koebmann, 2008; Gaudu, 2002; Brooijmans, 2009b). This kind of metabolism results in two main advantages for the cell: increased biomass and long-term survival. Starting by these previous observations, and thanks to the identification of the operon *cydABCD* in the *L. paracasei* strains under investigation, we carried out experiments aimed to evaluate if the respiratory-like metabolism was activated also in the strains DG and LPC-S01. All presented data refer to strain LPC-S01 since we did not find any significant difference between them, apart for the better capacity of LPC-S01 to internalize dyes used in cytofluorimetric analyses.

We verified that only one of the two phenotypes indicative of the respiratory metabolism occurred in *L. paracasei*. However, the addition of heme and menaquinone led to a very strong robustness acquisition, particularly if bacterial cells were grown in aerobiosis; particularly, LPC-S01 cells grown in aerobiosis in presence of heme and vitamin K2 survived storage at 4°C for more than 200 days, along with a decreased membrane damage and a lower oxidative stress level, as measured by cytofluorimetric analyses. Subsequently, we tried to understand why the contribution of heme and menaquinone was apparent only during the storage time, and not during growth; to this aim, we added the co-factors only after *L. paracasei* had reached the stationary phase, i.e. just before storage. This experiment allowed us to observed that bacterial cells stored in presence of heme and menaquinone acquired the robustness phenotype independently from the condition in which they were cultivated. This fact, along with the detection of heme exclusively outside the bacterial cells during growth, and the under regulation of gene *cydA*, suggested that the observed phenotype was not a consequence of a respiratory metabolism but, potentially, was due to an export of electrons outside cell, as proposed by other works (Khan (2012a and 2012b, Yamazaki 2002 and Freguia 2009). Other evidences supporting our hypothesis were obtained by measuring ATP production rate by means of a bioluminescent reporter system inserted in strain LPC-S01, which demonstrated that the presence of heme (alone or with menaquinone) led to the decrease of energy reservoir if compared to controls. Also this evidence is not in accordance with the establishment of the respiratory electron transport chain, which, contrarily, should be coupled to a slower growth rate but an increased ATP production by the FoF1 ATPase (Pfeiffer, 2001). Gene expression analysis, NAD^+/NADH ratio assay, and organic acids quantification gave us some insights about the possible mechanisms at the basis of the acquired robustness. In specific, we could speculate that *L. paracasei*, converting pyruvate to acetate instead of lactate, can give an energetic advantage to the cell, by gaining an extra ATP one molecule. This metabolic switch towards acetate production can be a consequence of the decreased need of the bacterial cell to re-oxidate NADH to NAD^+ because of the reduction of exogenous (and potentially extracellular) molecules. Our speculation has still several unclear points that may be clarified by two possible approaches: (i) a comprehensive transcriptomic approach in order to identify the regulation of other genes we have not considered yet and that could be involved in establishment of the acquired resistance; (ii) the use of microbial fuel cells, as described in previous works (Khan, 2012a; Freguia, 2009), in order to understand if an extracellular electron transfer (EET) occurs upon addition of heme and menaquinone (or other similar molecules, such as riboflavin) and if oxygen plays a role in the process.

Even if we do not have conclusive data about the molecular mechanisms at the basis of *L. paracasei* acquired resistance, we consider this phenotype of particular interest that can be exploited at industrial level, since it can confer to the bacterium improved viability, as observed

for *L. lactis*, and a potential higher capacity to resist to environmental stresses (Pedersen, 2005). Moreover, *L. paracasei* is used as probiotic and can be delivered to the human gut, an environment that is a natural source of external molecules that can be electron acceptors. This fact could be an advantage for the bacterium itself, because it improves its resistance to the complex habitat in which it resides. Furthermore, potential electron acceptors, especially ketones and aldehydes provided with diet (e.g. acrylamide) or produced in the colon by the digestion process of proteins (e.g. acetaldehyde, acrolein, diacetyl, crotonaldehyde, formaldehyde, furan, furfural and hydroxymethylfurfural) have been connected to several human neurodegenerative disorders, diabetic complications, hypertension and inflammation (Ellis, 2007; Guillén and Goicoechea, 2008; van Niel, 2012). For this reason, the microbiota, as well as the probiotic intervention, may be crucial for the *in vivo* biodetoxification of these detrimental molecules; in this context, the potential capability of *L. paracasei* to reduce external molecule could be an advantage also for the host.

3.6 REFERENCES

- Arioli S et al., 2010, Alkalizing reactions streamline cellular metabolism in acidogenic microorganisms. PLoS One 5:e15520.
- Arioli S et al., 2012, Increasing the heme-dependent respiratory efficiency of *Lactococcus lactis* by inhibition of lactate dehydrogenase. Appl Environ Microbiol. 79:376-80.
- Axelsson L, 2003, Lactic acid bacteria: classification and physiology. In: Lactic Acid Bacteria: Microbiological and Functional Aspects, ed 3. Edited by Salminen S, von Wright A, Ouwehand A, Marcel Dekker; 2003:1-66.
- Blank LM et al., 2001, Hemin reconstitutes proton extrusion in an $H(^{+})$-ATPase-negative mutant of *Lactococcus lactis*. J Bacteriol. 183:6707-6709.
- Bonomi F et al., 1998, Direct metal ion substitution at the $[M(SCys)_4]_2$- site of rubredoxin. J Biol Inorg Chem. 3:595–605.
- Brooijmans RJ et al., 2007, Generation of a membrane potential by *Lactococcus lactis* through aerobic electron transport. J Bacteriol. 189:5203-5209.
- Brooijmans RJ et al., 2009a, Heme and menaquinone induced electron transport in lactic acid bacteria. Microb Cell Fact. 8:28.
- Brooijmans RJ et al., 2009b, *Lactobacillus plantarum* WCFS1 electron transport chains. Appl Environ Microbiol. 75:3580-3585.
- Duwat P et al., 2001, Respiration capacity of the fermenting bacterium *Lactococcus lactis* and its positive effects on growth and survival. J Bacteriol. 183:4509-16.
- Ellis EM et al., 2007, Reactive carbonyls and oxidative stress: potential for therapeutic intervention. Pharmacol Ther. 115:13-24.
- Fernandez A et al., 2010, Two coregulated efflux transporters modulate intracellular heme and protoporphyrin IX availability in *Streptococcus agalactiae*. PLoS Pathog. 6:e1000860.
- Freguia S et al., 2009, *Lactococcus lactis* catalyses electricity generation at microbial fuel cell anodes via excretion of a soluble quinone. Bioelectrochemistry. 76:14-8.
- Gänzle MG, 2015, Lactic metabolism revisited: metabolism of lactic acid bacteria in food fermentations and food spoilage. Curr Opin Food Microbiol. 2:106-117
- Garrigues C et al., 1997, Control of the shift from homolactic acid to mixed-acid fermentation in *Lactococcus lactis*: predominant role of the $NADH/NAD^{+}$ ratio. J Bacteriol. 179:5282-7.
- Gaudu P et al., 2002, Respiration capacity and consequences in *Lactococcus lactis*. Antonie Van Leeuwenhoek 82:263–69.
- Guglielmetti S et al., 2008, Construction, characterization and exemplificative application of bioluminescent *Bifidobacterium longum* biovar *longum*. Int J Food Microbiol. 124:285-90.
- Guillén MD and Goicoechea E, 2008, Toxic oxygenated alpha,beta-unsaturated aldehydes and their study in foods: a review. Crit Rev Food Sci Nutr. 48:119-36.
- Herrera G et al., 2003, Functional assays of oxidative stress using genetically engineered *Escherichia coli* strains. Curr Protoc Cytom. 2003:11.16.1–11.16.9.
- Imlay JA, 2013, The molecular mechanisms and physiological consequences of oxidative stress: lessons from a model bacterium. Nat Rev Microbiol. 11:443-54.
- Kandler O, 1983, Carbohydrate metabolism in lactic acid bacteria. Antonie Van Leeuwenhoek. 49:209-224.

- Khan MM et al., 2010, Specific and rapid enumeration of viable but nonculturable and viable-culturable gram-negative bacteria by using flow cytometry. Appl Environ Microbiol. 76:5088-96.
- Khan MT et al., 2012a, How can *Faecalibacterium prausnitzii* employ riboflavin for extracellular electron transfer? Antioxid Redox Signal. 17:1433-40.
- Khan MT et al., 2012b, The gut anaerobe *Faecalibacterium prausnitzii* uses an extracellular electron shuttle to grow at oxic-anoxic interphases. ISME J. 6:1578-85.
- Kleerebezem M et al., 2003, Complete genome sequence of *Lactobacillus plantarum* WCFS1. Proc Natl Acad Sci USA. 100:1990-5.
- Koebmann B et al., 2008, Increased biomass yield of *Lactococcus lactis* during energetically limited growth and respiratory conditions. Biotechnol Appl Biochem. 50:25-33.
- Landers JW and Zak B, 1958, Determination of serum copper and iron in a single small sample. Am J Clin Pathol. 29:590-2.
- Lechardeur D et al, 2011, Using heme as an energy boost for lactic acid bacteria. Curr. Opin. Biotechnol. 22:143-49.
- Ljones T and Burris RH, 1978, Nitrogenase: the reaction between iron protein and bathophenanthrolinedisulfonate as a probe for interactions with MgATP. Biochem. 17:1866–1872.
- Masuda M et al., 2010, Flavins contained in yeast extract are exploited for anodic electron transfer by *Lactococcus lactis*. Bioelectrochemistry. 78:173-5.
- Miriani M et al., 2014, Rubredoxin refolding on nanostructured hydrophobic surfaces: Evidence for a new type of biomimetic chaperones. Proteins. 82:3154–3162.
- Morishita T et al., 1999, Production of menaquinones by lactic acid bacteria. J Dairy Sci. 82:1897-1903.
- Newman DK and Kolter R, 2000, A role for excreted quinones in extracellular electron transfer. Nature. 405:94-7.
- Palomino MM et al., 2010, New method for electroporation of *Lactobacillus* species grown in high salt. J Microbiol Methods. 83(2):164-7.
- Pedersen MB et al., 2005, The long and winding road from the research laboratory to industrial applications of lactic acid bacteria. FEMS Microbiol Rev. 29:611-24.
- Pedersen MB et al., 2012, Aerobic respiration metabolism in lactic acid bacteria and uses in biotechnology. Annu Rev Food Sci Technol. 3:37-58.
- Pedersen MB, 2008, Impact of aeration and heme-activated respiration on *Lactococcus lactis* gene expression: identification of a heme-responsive operon. J Bacteriol. 190:4903-11.
- Pfeiffer T et al., 2001, Cooperation and competition in the evolution of ATP-producing pathways. Science. 292:504–507.
- Puri-Taneja A et al, 2007, Regulators of the *Bacillus subtilis cydABCD* operon: identification of a negative regulator, CcpA, and a positive regulator, ResD. J Bacteriol. 189:3348-3358.
- Rezaïki L et al., 2008, *Lactococcus lactis* produces short-chain quinones that cross-feed Group B *Streptococcus* to activate respiration growth. Mol Microbiol. 67:947-57.
- Rico J et al., 2008, Analysis of *ldh* genes in *Lactobacillus casei* BL23: role on lactic acid production. J Ind Microbiol Biotechnol. 35:579-86.

- Sijpesteijn AK, 1970, Induction of cytochrome formation and stimulation of oxidative dissimilation by hemin in *Streptococcus lactis* and *Leuconostoc mesenteroides*. Antonie Van Leeuwenhoek 36:335-348.
- Stauff DL et al., 2008, *Staphylococcus aureus* HrtA is an ATPase required for protection against heme toxicity and prevention of a transcriptional heme stress response. J Bacteriol. 190:3588-3596.
- Stevens MJ et al., 2008, Improvement of *Lactobacillus plantarum* aerobic growth as directed by comprehensive transcriptome analysis. Appl Environ Microbiol. 74:4776-8.
- van Niel EW et al., 2012, The potential of biodetoxification activity as a probiotic property of *Lactobacillus reuteri.* Int J Food Microbiol. 152:206-10.
- Watanabe K et al., 2009, Electron shuttles in biotechnology. Curr Opin Biotechnol. 20:633-41.
- Watanabe M et al., 2012, Impact of respiration on resistance of *Lactobacillus plantarum* WCFS1 to acid stress. Appl Environ Microbiol. 78:4062-4.
- Wegmann U et al, 2007, Complete genome sequence of the prototype lactic acid bacterium *Lactococcus lactis* subsp. *cremoris* MG1363. J Bacteriol. 189:3256-3270.
- Yamazaki S et al., 2002, Glucose metabolism of lactic acid bacteria changed by quinone-mediated extracellular electron transfer, Biosci Biotechnol Biochem. 66:2100–2106.
- Zotta T et al., 2014, Assessment of aerobic and respiratory growth in the *Lactobacillus casei* group. PLoS One. 9:e99189.

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

- Guglielmetti S, **Balzaretti S**, Taverniti V, Miriani M, Milani C, Scarafoni A, Corona S, Ciranna A, Arioli S, Santala V, Iametti S, Bonomi F, Ventura M, Mora D, Karp M, 2014, TgaA, a VirB1-like component belonging to a putative type IV secretion system of *Bifidobacterium bifidum* MIMBb75. Appl Environ Microbiol. 80:5161-9.

- Guglielmetti S, Zanoni I, **Balzaretti S**, Miriani M, Taverniti V, De Noni I, Presti I, Stuknyte M, Scarafoni A, Arioli S, Iametti S, Bonomi F, Mora D, Karp M, Granucci F, 2014, Murein lytic enzyme TgaA of *Bifidobacterium bifidum* MIMBb75 modulates dendritic cell maturation through its cysteine- and histidine-dependent amidohydrolase/peptidase (CHAP) amidase domain. Appl Environ Microbiol. 80:5170-7.

- Koirala R, Taverniti V, **Balzaretti S**, Ricci G, Fortina MG, Guglielmetti S, 2015, Melting curve analysis of a groEL PCR fragment for the rapid genotyping of strains belonging to the *Lactobacillus casei* group of species. Microbiol Res. 173:50-8.

- **Balzaretti S**, Taverniti V, Rondini G, Marcolegio G, Minuzzo M, Remagni MC, Fiore W, Arioli S, Guglielmetti S, 2015, The vaginal isolate *Lactobacillus paracasei* LPC-S01 (DSM 26760) is suitable for oral administration. Front Microbiol. http://dx.doi.org/10.3389/fmicb.2015.00952

- Zambelli P, Tamborini L, Cazzamalli S, Pinto A, Arioli S, **Balzaretti S**, Plou FJ, Fernandez-Arrojo L, Molinari F, Conti P, Romano D, 2016, An efficient continuous flow process for the synthesis of a non-conventional mixture of fructooligosaccharides. Food Chem. 190:607-13. Epub 2015 Jun 3.

- **Balzaretti S,** 2013, Functional genome analysis, metabolism study, and probiotic characterization of *Lactobacillus paracasei* strains. 18th Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology, Universities of Padova and Udine, Conegliano, 25-27 Sept, 2013.

- **Balzaretti S,** Arioli S, Taverniti V, Mora D, Guglielmetti SD, 2014, Respiratory condition induces a catabolic shift that promotes long-term survival in *Lactobacillus paracasei*.11th International Symposium on Lactic Acid Bacteria, Egmond aan Zee, 31 Aug-4 Sept, 2014.

- **Balzaretti S,** 2014, Probiotic characterization and metabolism study of *Lactobacillus paracasei*. 19th Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology, University of Bari, Bari, 24-26 Sept, 2014.

- Taverniti V, Ferrario C, **Balzaretti S**, Colombo S, Mugetti S, Gargari G, Guglielmetti S, 2015, Probiotics Can Exert Profoundly Different Effects On The Intestinal Microbial Ecosystem Of Healthy Subjects Depending On The Product Formulation: Comparison Of

Two Randomized, Double-Blind, Crossover, Placebo-controlled Intervention Studies. International Yakult Symposium, Berlin, 23-24 Apr, 2015.

- **Balzaretti S,** 2015, Probiotic and energetic metabolism characterization of *Lactobacillus paracasei*. 20th Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology, University of Perugia, Perugia, 23-25 Sept, 2015**.**
- **Balzaretti S**, Taverniti V, Guglielmetti S, Fiore W, Ngehnyuiy H, Humphreys P, Laws AP, 2015, A novel hetero-exopolysaccharide mediates the recognition of *Lactobacillus paracasei* DG by the immune system. Pharmabiotics Conference, Paris, 29-30 Oct, 2015.

AEM
Journals.ASM.org

TgaA, a VirB1-Like Component Belonging to a Putative Type IV Secretion System of *Bifidobacterium bifidum* MIMBb75

Simone Guglielmetti,[a] Silvia Balzaretti,[a] Valentina Taverniti,[a] Matteo Miriani,[a] Christian Milani,[b] Alessio Scarafoni,[a] Silvia Corona,[c] Alessandro Ciranna,[d] Stefania Arioli,[a] Ville Santala,[d] Stefania Iametti,[a] Francesco Bonomi,[a] Marco Ventura,[b] Diego Mora,[a] Matti Karp[d]

Department of Food Environmental and Nutritional Sciences, Università degli Studi di Milano, Milan, Italy[a]; Laboratory of Probiogenomics, Department of Life Sciences, University of Parma, Parma, Italy[b]; IRCCS Policlinico San Matteo, Pavia, Italy[c]; Department of Chemistry and Bioengineering, Tampere University of Technology, Tampere, Finland[d]

Bifidobacterium bifidum* MIMBb75 is a human intestinal isolate demonstrated to be interactive with the host and efficacious as a probiotic. However, the molecular biology of this microorganism is yet largely unknown. For this reason, we undertook whole-genome sequencing of *B. bifidum* MIMBb75 to identify potential genetic factors that would explain the metabolic and probiotic attributes of this bacterium. Comparative genomic analysis revealed a 45-kb chromosomal region that comprises 19 putative genes coding for a potential type IV secretion system (T4SS). Thus, we undertook the initial characterization of this genetic region by studying the putative *virB1*-like gene, named *tgaA*. Gene *tgaA* encodes a peptidoglycan lytic enzyme containing two active domains: lytic murein transglycosylase (LT, cd00254.3) and cysteine- and histidine-dependent amidohydrolase/peptidase (CHAP, pfam05257.4). By means of several *in vitro* assays, we experimentally confirmed that protein TgaA, consistent with its computationally assigned role, has peptidoglycan lytic activity, which is principally associated to the LT domain. Furthermore, immunofluorescence and immunogold labeling showed that the protein TgaA is abundantly expressed on the cell surface of *B. bifidum* MIMBb75. According to the literature, the T4SSs, which have not been characterized before in bifidobacteria, can have important implications for bacterial cell-to-cell communication as well as cross talk with host cells, justifying the interest for further studies aimed at the investigation of this genetic region.

AEM
Journals.ASM.org

Murein Lytic Enzyme TgaA of *Bifidobacterium bifidum* MIMBb75 Modulates Dendritic Cell Maturation through Its Cysteine- and Histidine-Dependent Amidohydrolase/Peptidase (CHAP) Amidase Domain

Simone Guglielmetti,[a] Ivan Zanoni,[b] Silvia Balzaretti,[a] Matteo Miriani,[a] Valentina Taverniti,[a] Ivano De Noni,[a] Ilaria Presti,[a] Milda Stuknyte,[a] Alessio Scarafoni,[a] Stefania Arioli,[a] Stefania Iametti,[a] Francesco Bonomi,[a] Diego Mora,[a] Matti Karp,[c] Francesca Granucci[b]

Department of Food Environmental and Nutritional Sciences, Università degli Studi di Milano, Milan, Italy[a]; Department of Biotechnology and Biosciences, University of Milano-Bicocca, Milan, Italy[b]; Department of Chemistry and Bioengineering, Tampere University of Technology, Tampere, Finland[c]

Bifidobacteria are Gram-positive inhabitants of the human gastrointestinal tract that have evolved close interaction with their host and especially with the host's immune system. The molecular mechanisms underlying such interactions, however, are largely unidentified. In this study, we investigated the immunomodulatory potential of *Bifidobacterium bifidum* MIMBb75, a bacterium of human intestinal origin commercially used as a probiotic. Particularly, we focused our attention on TgaA, a protein expressed on the outer surface of MIMBb75's cells and homologous to other known bacterial immunoactive proteins. TgaA is a peptidoglycan lytic enzyme containing two active domains: lytic murein transglycosylase (LT) and cysteine- and histidine-dependent amidohydrolase/peptidase (CHAP). We ran immunological experiments stimulating dendritic cells (DCs) with the *B. bifidum* MIMBb75 and TgaA, with the result that both the bacterium and the protein activated DCs and triggered interleukin-2 (IL-2) production. In addition, we observed that the heterologous expression of TgaA in *Bifidobacterium longum* transferred to the bacterium the ability to induce IL-2. Subsequently, immunological experiments performed using two purified recombinant proteins corresponding to the single domains LT and CHAP demonstrated that the CHAP domain is the immune-reactive region of TgaA. Finally, we also showed that TgaA-dependent activation of DCs requires the protein CD14, marginally involves TRIF, and is independent of Toll-like receptor 4 (TLR4) and MyD88. In conclusion, our study suggests that the bacterial CHAP domain is a novel microbe-associated molecular pattern actively participating in the cross talk mechanisms between bifidobacteria and the host's immune system.

Microbiological Research 173 (2015) 50–58

Contents lists available at ScienceDirect

Microbiological Research

journal homepage: www.elsevier.com/locate/micres

Melting curve analysis of a *groEL* PCR fragment for the rapid genotyping of strains belonging to the *Lactobacillus casei* group of species

Ranjan Koirala[a,b], Valentina Taverniti[c], Silvia Balzaretti[c], Giovanni Ricci[c], Maria Grazia Fortina[c], Simone Guglielmetti[c,*]

[a] *Nepal Academy of Science and Technology (NAST), Khumaltar, Lalitpur, Nepal*
[b] *Central Department of Biotechnology, Tribhuvan University, Kirtipur, Kathmandu, Nepal*
[c] *Department of Food, Environmental and Nutritional Sciences (DeFENS), Division of Food Microbiology and Bioprocessing, Università degli Studi di Milano, Italy*

ARTICLE INFO

Article history:
Received 12 May 2014
Received in revised form
13 December 2014
Accepted 2 January 2015
Available online 12 February 2015

Keywords:
Probiotic
Lactobacillus
HRM analysis
Molecular typing

ABSTRACT

Lactobacillus casei group (*Lcs*) consists of three phylogenetically closely related species (*L. casei*, *L. paracasei*, and *L. rhamnosus*), which are widely used in the dairy and probiotic industrial sectors. Strategies to easily and rapidly characterize *Lcs* are therefore of interest. To this aim, we developed a method according to a technique known as high resolution melting analysis (HRMa), which was applied to a 150 bp *groEL* gene fragment. The analysis was performed on 53 *Lcs* strains and 29 strains representatives of species that are commonly present in dairy and probiotic products and can be most probably co-isolated with *Lcs* strains. DNA amplification was obtained only from *Lcs* strains, demonstrating the specificity of the *groEL* primers designed in this study. The HRMa clustered *Lcs* strains in three groups that exactly corresponded to the species of the *L. casei* group. A following HRMa separated the 39 *L. paracasei* strains in two well distinct intraspecific groups, indicating the possible existence of at least two distinct genotypes inside the species. Nonetheless, the phenotypic characterization demonstrated that the genotypes do not correspond to the two *L. paracasei* subspecies, namely *paracasei* and *tolerans*. In conclusion, the melting curve analysis developed in this study is demonstrably a simple, labor-saving, and rapid strategy obtain the genotyping of a bacterial isolate and simultaneously potentially confirm its affiliation to the *L. casei* group of species. The application of this method to a larger collection of strains may validate the possibility to use the proposed HRMa protocol for the taxonomic discrimination of *L. casei* group of species. In general, this study suggests that HRMa can be a suitable technique for the genetic typization of *Lactobacillus* strains.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 15 September 2015
doi: 10.3389/fmicb.2015.00952

The vaginal isolate *Lactobacillus paracasei* LPC-S01 (DSM 26760) is suitable for oral administration

Silvia Balzaretti[1], Valentina Taverniti[1], Greta Rondini[1], Giorgio Marcolegio[1], Mario Minuzzo[2], Maria C. Remagni[1†], Walter Fiore[3], Stefania Arioli[1] and Simone Guglielmetti[1]*

[1] Division of Food Microbiology and Bioprocessing, Department of Food Environmental and Nutritional Sciences, Università degli Studi di Milano, Milano, Italy, [2] Department of Biosciences, Università degli Studi di Milano, Milano, Italy, [3] Sofar S.p.A., Trezzano Rosa, Italy

OPEN ACCESS

Edited by:
Fabio Minervini, Università degli Studi di Bari Aldo Moro, Italy

Reviewed by:
Marcela Carina Audisio, Instituto de Investigaciones para la Industria Química-CONICET, Argentina
Amit Kumar Tyagi, The University of Texas MD Anderson Cancer Center, USA

***Correspondence:**
Simone Guglielmetti, Division of Food Microbiology and Bioprocessing, Department of Food, Environmental and Nutritional Sciences, Università degli Studi di Milano, Via Celoria 2, Milano 20133, Italy
simone.guglielmetti@unimi.it

†Present Address:
Maria C. Remagni, Sacco S.r.l., Cadorago, Italy

Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: *30 April 2015*
Accepted: *28 August 2015*
Published: *15 September 2015*

Citation:
Balzaretti S, Taverniti V, Rondini G, Marcolegio G, Minuzzo M, Remagni MC, Fiore W, Arioli S and Guglielmetti S (2015) The vaginal isolate Lactobacillus paracasei LPC-S01 (DSM 26760) is suitable for oral administration. Front. Microbiol. 6:952. doi: 10.3389/fmicb.2015.00952

Bacterial vaginosis is one of the most common urogenital diseases affecting women in reproductive age. The administration of probiotics as vaginal suppository has been proposed as a strategy to cure this condition and reduce its recurrence. Nonetheless, also oral consumption of probiotics, which is a more practical route of administration, proved to be an efficient strategy. In this perspective, we studied *Lactobacillus paracasei* LPC-S01 (DSM 26760), a human vaginal isolate included in commercial probiotic preparations for topical use, in order to assess if this bacterium can also perform as gastrointestinal probiotic. Comparative genomics revealed the presence of several accessory genes suggesting that LPC-S01 is a niche-generalist member of its species. According to a procedure conventionally used to predict the probiotic potential, we demonstrated that the probiotic properties of strain LPC-S01, with respect to those of the well-known probiotic references *L. paracasei* Shirota and DG, are equal for the bile tolerance and the reduction of NF-κB activation in Caco-2 cells, or superior for the tolerance to gastric juice and the adhesion to Caco-2 epithelial cells. We then demonstrated that LPC-S01 is susceptible to antibiotics indicated by EFSA and does not produce biogenic amines. Finally, a double-blind cross-over pilot intervention trial on healthy human volunteers showed that, after a 7-days oral consumption of capsules containing about 24 billion live cells, the fecal cell concentrations of strains LPC-S01 and DG (evaluated by qPCR) were not dissimilar. Specifically, both probiotics' cell concentrations were above the detection limit for an average of 5 days from the end of the treatment, corresponding to a mean number of evacuations of 7 ± 2. Taken together, these data demonstrate that the vaginal isolate *L. paracasei* LPC-S01 possesses safety and functional properties that may support its use as probiotic to be administered *per os* for potential intestinal as well as vaginal applications.

Keywords: lactobacilli, probiotic, Caco-2 adhesion, NF-κB, *in vivo* trial, gastrointestinal persistence

Food Chemistry 190 (2016) 607–613

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

An efficient continuous flow process for the synthesis of a non-conventional mixture of fructooligosaccharides

Paolo Zambelli [a], Lucia Tamborini [b,*], Samuele Cazzamalli [b], Andrea Pinto [b], Stefania Arioli [a], Silvia Balzaretti [a], Francisco J. Plou [c], Lucia Fernandez-Arrojo [c], Francesco Molinari [a], Paola Conti [b], Diego Romano [a,*]

[a] Department of Food Environmental and Nutritional Science (DeFENS), University of Milan, Via Mangiagalli, 20133 Milan, Italy
[b] Department of Pharmaceutical Sciences (DISFARM), University of Milan, Via Mangiagalli 25, 20133 Milan, Italy
[c] Instituto de Catálisis y Petroleoquímica, CSIC, 28049 Madrid, Spain

ARTICLE INFO

Article history:
Received 15 February 2015
Received in revised form 12 May 2015
Accepted 1 June 2015
Available online 3 June 2015

Keywords:
Fructooligosaccharides
Flow chemistry
Cladosporium cladosporioides
Dried alginate entrapped enzymes (DALGEE)
Prebiotics

ABSTRACT

A sustainable and scalable process for the production of a new mixture of fructooligosaccharides (FOS) was developed using a continuous-flow approach based on an immobilized whole cells-packed bed reactor. The technological transfer from a classical batch system to an innovative flow environment allowed a significant improvement of the productivity. Moreover, the stability of this production system was ascertained by up to 7 days of continuous working. These results suggest the suitability of the proposed method for a large-scale production of the desired FOS mixture, in view of a foreseeable use as a novel prebiotic preparation.

18th Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology, Universities of Padova and Udine, Conegliano 25-27 September, 2013

Functional genome analysis, metabolism study, and probiotic characterization of *Lactobacillus paracasei* strains.

Balzaretti S

This research project is focused on the evaluation of the probiotic potential of different strains of *Lactobacillus paracasei*. Particular attention will be spent on the characterization of three strains isolated from different environments. To this aim, *in vitro* methods conventionally employed for the assessment of the probiotic potential of microbial cells will be used (resistance to acidity and bile salts, adhesion to Caco-2 human epithelial cell line, inhibition of several microbial pathogens). Furthermore, we will employ functional genome analysis and some molecular tools to characterize the catabolic properties and the host-microbe relationship of *L. paracasei.*

11th Symposium on Lactic Acid Bacteria, 31 August – 4 September, 2014 (Egmond aan Zee, The Netherlands)

Respiratory condition induces a catabolic shift that promotes long-term survival in *Lactobacillus paracasei*

Balzaretti S, Arioli S, Taverniti V, Mora D, Guglielmetti SD

Lactic acid bacteria are conventionally classified as obligate anaerobic microorganisms, however several studies support the hypothesis that certain species, such as *Lactococcus lactis* and *Lactobacillus plantarum*, may shift to a respiratory metabolism when exogenous heme and, if necessary, menaquinone sources are added to the cultural medium. Since this capacity has never been investigated in *Lactobacillus paracasei*, the aim of this study was to understand if the same metabolic change may occur in *L.paracasei* LPC-S01 and DG, two strains used industrially as probiotics. The addition of heme and vitamin K2 (source of menaquinone) to an aerobic culture (Ae-HM) in MRS did not improve *L. paracasei* growth compared to anaerobic (An) and aerobic (Ae) cultures without co-factors. Nonetheless, Ae-HM condition strongly increased long-term cell survival at 4°C in exhausted MRS broth. Interestingly, drastically enhanced cell viability was also observed when *L. paracasei* Ae-HM cultures were prepared in an industrial culture medium. To understand the molecular basis of this acquired resistance capacity, we performed cytofluorimetric analyses, which evidenced that Ae-HM cells had reduced membrane damage and oxidative stress levels compared to An and Ae cultures. Moreover, we observed by HPLC the production of a higher amount of acetate by the Ae-HM compared to Ae and An cells. This result was also supported by RT-qPCR analysis, which showed higher expression levels of acetate kinase, an enzyme that converts acetyl-phosphate to acetate with production of ATP. In conclusion, we propose that *L. paracasei* can be able to activate the electron transport chain upon exogenous administration of heme and menaquinone, shifting the metabolism towards the production of acetic acid; consequently, bacterial cells may benefit of a reduced oxidative stress and an enhanced energy yielding, which may preserve cell viability. The respiratory-like metabolism of *L. paracasei* can represent a method to improve the resistance performances of *L. paracasei* probiotic strains at industrial level.

19th Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology, University of Bari, Bari, September 24th-26th, 2014

Probiotic characterization and metabolism study of *Lactobacillus paracasei*

Balzaretti S

The first two activities of the PhD thesis project are described. Firstly, we evaluated the probiotic potential of several strains of *Lactobacillus paracasei*, according to the *in vitro* methods suggested by EFSA. Secondly, we studied the shift into the respiratory metabolism in *L. paracasei* induced by exogenous heme and menaquinone sources in the cultural medium by means of microbiology methods, metabolite pool and gene expression analysis.

International Yakult Symposium, Berlin, 23-24 Apr, 2015.

Probiotics can exert profoundly different effects on the intestinal microbial ecosystem of healthy subjects depending on the product formulation: comparison of two randomized, double-blind, crossover, placebo-controlled intervention trials

Taverniti V, Ferrario C, **Balzaretti S**, Colombo S, Mugetti S, Gargari G, Guglielmetti S

Introduction. Because of a lack of consensually accepted and validated biomarkers, it is difficult to assess the benefits of probiotics in a healthy population. The impact of a probiotic product on the gut microbiota composition is thus the initial target for any evaluation of its efficacy. We report here the results of two intervention studies called PROBIOTA-DG and PROBIOTA-Bb, aimed to assess the modifications induced in the fecal microbiota of healthy adults by the consumption of commercial probiotic products.

Methods. We used Ion Torrent PGM high-throughput DNA sequencing technology according to a randomized, double-blind, crossover, placebo-controlled study design to characterize the fecal microbiota composition of healthy adults after four weeks of daily consumption of one capsule of the probiotic supplement (per capsule: 24 billion viable cells of Lactobacillus paracasei in PROBIOTA-DG, 1 billion of viable cells of a Bifidobacterium bifidum strain in PROBIOTA-Bb). In post-hoc analysis, we also evaluated the fecal concentrations of short chain fatty acids (SCFAs) by HPLC.

Results and discussion. No significant effect was observed upon the consumption of capsules containing Bifidobacterium bifidum on the fecal bacterial-community structure and SCFAs levels. On the contrary, the Lactobacillus paracasei-based product significantly affected the relative abundance of specific members of the bacterial order Clostridiales, resulting in subject-dependent modulation of fecal SCFAs. Specifically, the intake of L. paracasei increased the Blautia/Coprococcus ratio, which, according to literature, can confer a health benefit on the host. Moreover, the probiotic intervention showed a rebalancing effect on SCFA concentrations (particularly butyrate), highly dependent on the initial characteristics of the intestinal microbiota (J. Nutr. 2014;jn.114.197723). Interestingly, the strain L. paracasei DG employed in this trial was demonstrated to be beneficial in several intestinal diseases when administered in the same formulation used in PROBIOTA-DG (Curr. Clin. Pharmacol. 2013;8:169-72; Aliment. Pharmacol. Ther.2013;38:741-51).

Conclusion. This work demonstrates that both bacterial strain and dose may be crucial for the efficacy of a probiotic product. In addition, this work indicates that a probiotic preparation that is beneficial in a pathological or dysfunctional context (such as that including L. paracasei DG) may also exert significant potential benefit when consumed by healthy people.

20th Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology, University of Perugia, Perugia, September 23rd-25th, 2015

Probiotic and energetic metabolism characterization of *Lactobacillus paracasei*.

Balzaretti S

This research activity aimed to highlight the probiotic properties of two *Lactobacillus paracasei* strains, by means of *in vitro* and *in vivo* techniques, and by the identification of a unique genetic cluster for the synthesis of exopolysaccharides. The peculiar ability of this species to counteract oxidative stress and overcome redox imbalance by exploiting external electron acceptors, also naturally localized in the gut, was further investigated.

Pharmabiotics Conference 2015, Paris, 29-30 Oct, 2015.

A novel hetero-exopolysaccharide mediates the recognition of *Lactobacillus paracasei* DG by the immune system

Balzaretti S, Taverniti V, Guglielmetti S, Fiore W, Ngehnyuiy H, Humphreys P, Laws AP.

Lactobacillus paracasei DG is a bacterial strain that has been commercialized as a probiotic for more than 15 years under the commercial name of Enterolactis®. Reportedly, *L. paracasei* DG exerts health-promoting properties including maintaining remission of symptomatic uncomplicated diverticulosis, reducing inflammation in mild ulcerative colitis, and reducing side effects during therapy for *Helicobacter pylori* eradication and after antibiotic treatment of small intestinal bacterial overgrowth. Furthermore, *L. paracasei* DG was demonstrated to modulate fecal Clostridiales bacteria and butyrate levels in healthy adults. The mechanisms underlying the probiotic properties of strain DG, however, are still largely unknown. For this reason, we undertook the genome sequencing of this strain.

Comparative analysis of DG's draft genome allowed the identification of a gene cluster putatively involved in exopolysaccharide (EPS) synthesis. Specifically, we found a 16 kb region composed of 19 ORFs, which includes several putative glycosyltransferase genes and contains a region of about 8 kb that was found to have no significant similarity with other DNA sequences deposited in GenBank.

We isolated and purified the capsular fraction of the EPS produced by DG cells. NMR analyses revealed that DG's EPS is a hetero-exopolysaccharide composed of a branched repeating unit with a chemical structure that has never been described before in lactobacilli and probiotic bacteria.

The purified EPS was then used, in vitro, to stimulate macrophages obtained from the human cell line U937. Immunological experiments showed that the EPS had the ability to activate macrophages by triggering the expression of TNF-α and COX-2. The same ability is provided by EPS to whole DG cells, which, in fact, displayed an increased immunostimulatory activity compared to other *L. paracasei* strains, including the reference probiotic strain Shirota.

This study demonstrates that *L. paracasei* DG expresses an exopolysaccharide with a novel structure conferring distinctive immunomodulatory properties to this probiotic bacterium.

APPENDIX 2. INDEX OF TABLES

APPENDIX 3. INDEX OF FIGURES.

ACKNOWLEDGEMENTS

I would like to thank my tutor Simone Guglielmetti for this great experience, and for teaching me a lot of things, especially that " banal mistakes don't exist, but they are always around the corner ";

Thanks to Valentina Taverniti, a unique colleague and friend;

Thanks to all my colleagues and students I met through all these years;

Thanks to Prof. Andy Laws from the University of Huddersfield for his help with the EPS troubles, and thank you to my friends Hansel and Jim;

Thanks to all my family, my friends , and especially thank you Wiwi