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Characterization and translocation of lactic acid bacteria from the intestine of mothers to their milk and progeny

Rima ALBESHARAT

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Characterization and translocation of lactic acid bacteria from the intestine of mothers to their milk and progeny

BY

Rima Albesharat

B.S., Food Science (University of Damascus) 2002

Pg Diploma., Food Science (University of Damascus) 2003

M.S., Food Biotechnology (University of Damascus) 2007

DEDICATION

Dear God, today I want to spend a moment not to ask for anything from you, but to say sincere thank you for all what I have and don't have, thank you for giving me what I need instead of what I want, forgive me no thanks is enough for you

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These theses belonging to the dissertation of Rima Albesharat

I. وَإِنَّ لَكُمْ فِي الْأَنْعَامِ لَعِبْرَةً نُسْقِيكُمْ مِمَّا فِي بُطُونِهِ مِنْ بَيْنِ فَرْثٍ وَدَمٍ لَبَناً خَالِصاً سَائِغاً لِلشَّارِبِينَ
(النحل : 66)
And verily, in the cattle, there is a lesson for you. We give you to drink of that which is in their bellies, from between excretions and blood, pure milk; palatable to the drinkers. (the bees: 66)

II. Microbiota in human, from where it comes … how it survives and interacts in this special ecosystem (the gut)

III. The intestinal microbiota is special fingerprint for every individual and for every society or community

IV. Although every individual has its own special microbiota the most similar microbiota to it is its mothers microbiota as she was the source of the first colonization

V. Leadership criteria, when this concept is applied in bacteria community … the traits of the dominance species and how it can influence the other community

VI. (Beneficial) bacteria translocation depends on the bacteria and the body conditions

VII. Is newborn really sterile? … the first invasion or colonization starts before birth

VIII. Brest feeding is right for every infant … deprivation from it will influence all coming life.

IX. My story is about a woman who saw a butterfly struggling to get out of its chrysalis. She watched it struggle for a while and then stop. The women thought that it was so difficult for the butterfly to get out, that she would make it a little easier. She cut the cocoon just a little, just enough that the butterfly wouldn't have to struggle so much. It worked and the butterfly crawled out easily. She had a big fat body and withered wings. The women stuck around to watch the wings spread, but they never did. Without the struggle of getting out of the chrysalis, the butterfly had not been able to pump enough the fluid from its body into its wings. The struggle was what made the butterfly so beautiful. The women understand, that new life should come with straggle and pain to be beautiful … through birth pain the best mother microbiota pump to her baby …

Zusammenfassung

In dieser Arbeit wurden aus 70 verschiedenen Proben (Fäzes von je 15 Säuglingen bzw. Erwachsenen, 15 Frauenmilch Proben und 25 fermentierte Lebensmitteln) unter Verwendung von 9 verschiedenen Selektiv-Medien insgesamt 700 Milchsäurebakterien isoliert. Mittels Random Amplified Polymorphic DNA (RAPD) Analyse waren 171 unterschiedliche Genotypen differenzierbar. Je ein Vertreter eines Genotyps wurde durch 16S rDNA Sequenzierung bis zur Spezies identifiziert. Aus den Fäzes-und Milchproben wurden 36 verschiedene Arten aus den Gattungen *Lactobacillus, Enterococcus, Weissella, Streptococcus, Staphylococcus* und *Pediococcus* gefunden. Aus acht fermentierten Lebensmitteln konnten elf verschiedene Spezies aus den Gattungen *Lactobacillus, Enterococcus, Streptococcus, Staphylococcus, Pediococcus* und *Leuconostoc*. isoliert werden. Identifizierte Spezies waren *Lactobacillus plantarum, Lactobacillus fermentum, Lactobacillus casei, Lactobacillus brevis, Lactobacillus salivarius, Lactobacillus rhamnosus, Lactobacillus mucosae, Lactobacillus oris, Streptococcus gallolyticus, Streptococcus lutetiensis, Pediococcus pentosaceus, Enterococcus faecium, Enterococcus faecalis, Enterococcus durans, Enterococcus hirae, Enterococcus mundtii*.

Die meisten der Genotypen bzw. Stämme wurden in mehr als einer der Quellen gefunden. Nur ein Genotyp wurde innerhalb folgender Arten erkannt: *Lactobacillus reuteri*, *Lactobacillus sobrius, Lactobacillus animalis*, *Lactobacillus gasseri, Lactobacillus coleohominis, Lactobacillus vaginalis, Lactobacillus murinus, Lactobacillus helveticus, Streptococcus australis, Streptococcus vestibularis, Streptococcus equines, Streptococcus infantarius, Weissella confusa, Enterococcus gallinarum, Enterococcus avium, Enterococcus saccharolyticus,* und *Enterococcus casseliflavus.* Während einzelne Arten jeweils nur in einer der vier Quellen auftraten, konnte *L. plantarum, L. fermentum, L. brevis* und *P. pentosaceus* jeweils in allen Proben gefunden werden. In neun Fällen wurden identische Genotypen von *L. plantarum, L. fermentum, L. brevis, E. faecium* und *E. faecalis* sowohl in Faezes-Proben der Mütter, ihrer Milch und den entsprechenden Babys gefunden (Mutter/Milch/Kind-Paare).

Mittels 16S rDNA Amplicon sequencing wurde die Zusammensetzung der bakteriellen Microbiota der humanen Fäzesproben (Mütter und Babies) bestimmt. Es wurden signifikante Unterschiede zwischen Müttern und Babies, als auch zwischen Probanden unterschiedlicher Regionen gefunden. Diese Ergebnisse verdeutlichen den Einfluss regional unterschiedlicher Ernährungsgewohnheiten auf die Zusammensetzung der Darmflora.

Für ein besseres Verständnis der Eigenschaften potentiell translokierender Bakterien wurden in Fäzes und Milch Proben dominierende Isolate physiologisch näher charakterisiert. Die Isolate zeigten Unterschiede in ihrer Überlebensfähigkeit unter Stressbedingungen (Oxidativer – und Säurestress, Resistenz gegenüber Gallensalzen), der Bildung antagonistisch wirkender Substanzen, Enzymen und Sekundärmetaboliten.

Einzelne Stämme von *L. plantarum, L. brevis* und *E. faecium* waren sehr tolerant gegenüber den Bedingungen des Intestinaltraktes. Stämme von *L. plantarum, E. faecium, E. faecalis, E. hirae, E. mundtii* und *P. pentosaceus* zeigten die höchste Widerstandsfähigkeit gegen oxidativen Stress (Paraquat und H_2O_2). Insgesamt zeigte *L. plantarum* unter allen getesteten Bedingungen die beste Überlebensfähigkeit.
Interessanter weise zeigten genotypisch identische Isolate aus Mutter und Kind Proben untereinander ähnliche und deutlicher ausgeprägte Resistenzen als andere Isolate. Dies stützt die Hypothese einer möglichen vertikalen Übertragung bestimmter Stämme vom mütterlichen Intestinaltrakt über die Milch zum Baby.

In Ussing-Kammer Experimenten zeigten diese Isolate eine Translokation durch humanes Darm-Mucosa Gewebe. Allerdings induzierten im Gegensatz zu abgetöteten Zellen nur lebende *L. plantarum* Zellen auch eine verringerte Leitfähikgeit des Epithels. Diese Tatsache führt zu der Hypothese, dass bestimmte Isolate eventuell modulierend auf die Barriere-Eigenschaften von Epithelien Einfluss nehmen könnten beziehungsweise Epithelien nur unter bestimmten Bedingungen (z.B. Gestationsalter und/oder Lactationsphase) eine Passage von Bakterien erlauben.

Im Tierexperiment mit Mäusen konnte ein oral verabreichter *L. plantarum* Stamm TMW1.1623 (Dosis 10^7/d) in normalen Mäusen in keinem der getesteten Organe nachgewiesen werden. In trächtigen Tieren wurde eine Anreicherung in placentalem Gewebe gezeigt, nicht jedoch im Fetus. Bei säugenden Tieren wurde *L. plantarum* in den Milchdrüsen und im Darm der Welpen nachgewiesen.

Zusammenfassend lässt sich sagen dass in dieser Arbeit Hinweise dafür gefunden wurden, dass bestimmte, nicht pathogene Bakterienstämme in der Lage sind, das Darmepithel zu überwinden und in der Folge in lymphatischem bzw. placentalem Gewebe sowie in Milchdrüsen nachweisbar sind. Dies stützt sie Hypothese einer möglichen Übertragung von Bakterien von der Mutter zum Kind über die Placenta sowie eine Übertragung in der initialen Stillphase.

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General introduction and objectives

The small and large intestine

The intestinal tract is basically a tube, which its wall is composed of three tissue layers graded in a centric structure. The outer layer consists of several lamina of smooth muscle that, together with the collective enteric nervous system, proceeds the consistent peristaltic movements of the intestine. The space between the outer muscle and the inner epithelial layer is filled by connective tissue called submucosal, that contains numerous blood and lymph vessels, nerve fibers, and various cells of the immune system. From inside, the luminal surface consists of a simple epithelium, a single cell layer known as the mucosa. The mucosa is responsible for the transference and absorption of nutrients and also for the compaction of the stool (Sancho *et al.*, 2003).

Two well-defined parts can be anatomically distinguished in the intestinal tract, which are the small intestine and the large intestine or the colon. The first part or the small intestine is subdivided into three proximal-distal parts which are respectively, the duodenum, jejunum, and ileum. The mucosa (absorptive surface area) of the small intestine is spectacularly enlarged by plenty papilliform or finger-like bulges that point toward the lumen, the single is called villi, and intussusception into the submucosa layer called the crypts. However, in the large intestine the mucosal layer lacks villi and the crypts are deeper invaginated into the submucosa layer (Barker *et al.*, 2008).

There are four differentiated cell types proceeds the different functions in the intestinal epithelium (Sancho *et al.*, 2003) which are absorptive cells, enteroendocrine calls, muco-secreting cells and Paneth cells. The proportional abundance of each of these different cell types varies obviously within different parts of the intestine. Absorptive cells are also called enterocytes, they are the most plentiful cell type in the small intestine and are mainly responsible for the absorption of the simple food nutrients from the chyme and also for the secretion of a mixture of hydrolytic enzymes into the lumen. Markedly the numbers of the mucosecreting cells which are also named the goblet cells are increasing from the approaching part (small intestine) to distal part (large intestine and rectum) as the stool becomes increasingly compressed. The enteroendocrine cells are presented in small quantity comparing to other epithelium cells (<1%). Their function is controling gut physiology by secreting a variety of hormones like substance P, secretin, and serotonine. Varied subtypes cells can be defined by this specific produced intestinal hormones (Schonhoff *et al.*, 2004). The forth kind of cells called Paneth cells which dwell at the deep bottom of the crypts in the intestine. Their function is secreting antimicrobial compounds such as cryptidins (expressional defensins in humans) and lysozyme, they play a fundamental role in the controling of the microbial environment in the intestine (Porter *et al.*, 2002). There are more intestinal cell types which are lesser-known can be mentioned, such as the M cells that cover

the lymphoid Peyer's (Gebert *et al.*, 1996), brush/bunch/boracic cells (Nabeyama & Leblond, 1974), and cup cells (Madara, 1982). However, a little is known about their descent relationships (Barker *et al.*, 2008).

The gastrointestinal tract is a combination ecosystem consists of three main components that are constantly in contact and interact with each other, which are the intestinal host cells, food nutrients and microbiota. Intestinal ecology outcome from a movable equilibrium, reconstructed by food intakes, between the coexistence inhabitants of the gut lumen (Dogi & Perdigon, 2006), where between 1,000 and 1,150 prevalent bacterial species coexist (Qin *et al.*, 2010). Trillions of bacteria live in the intestinal tracts of healthy humans as well as those of many animals. This natural intestinal microbiota contributes to digestion and the metabolism of different food nutrients and vitamins which is a crucial important for the host organism. It has been recently proved the important role which intestinal microbiota also plays in the modulation of the immune system in the intestines (Ganal *et al.*, 2012). There are a continues challenge of taking in a foreign antigen in microbiota, result components of food digestion or drugs through the exposed surface of the intestinal mucosa. That explain why the largest collection of lymphoid tissues in the body is contained in the intestines (Brandtzaeg, 1985), in the form of lymphoid combinations in Peyer's patches and in the lamina propria (solitary lymphoid nodules) and as the dispersed lymphocyte inhabitants located in the epithelium and in the lamina propria. Although nascent Peyer's patches are obvious in the newborn, the epithelium and lamina propria are free of mononuclear cells. T lymphocytes migrating from the thymus rapidly inhabit the thymus-dependent areas of Peyer's patches and the epithelium. However exposure to microbiota in the normal environment is important to develop the B cell inhabitants and their germinal follicles as the experiments conducted in germ free animals shown (Crabbe *et al.*, 1970), (Doe, 1989).

Diversity of the human intestinal microbiota

The intestinal microbiota can be divided in two main groups: the autochthonous or mutualistic (C) microbiota, this microorganisms propagate in the intestine from the moment of birth or maybe before birth, becoming more steady after weaning; and the second group, called allochthone or non-mutualistic (NC) microbiota, this microorganisms existence are temporary and consist of various microorganisms inserted by ingestion together with the food. The C intestinal microbiota plays an influential role in maintaining the normal physiology and health of the coexistent host (Moreau & Gaboriau-Routhiau, 2001). The combination and performance of C microorganisms is responsible for three requisite functions: metabolic (food digestion and vitamin synthesis), barrier (protection against external and pervade pathogens) (Raibaud, 1992) and interactions with the host (in particular through the immune system). Moreover, C bacteria are essential for the maturation of the immune system because they function critical roles in promoting B cell development in Peyer's patches (Crabbé *et al.*,

1968). However, Dogi and Perdigon proved that the C microbiota would be contributed in the regulation of intestinal homeostasis rather than in the immune activation as the NC lactobacilli. (Dogi & Perdigon, 2006). Although, endogenous gastrointestinal microbiota plays an essential important role in health and disease, yet this complex ecosystem remains incompletely characterized and its diversity understanding is still developing.

Eckburg reported that of the 395 bacterial phylotypes, 244 (62%) were novel, and 80% represented sequences from species strictly anaerobic that have not been cultivated. Most of the appeared bacteria were members of the two phylums Firmicutes and Bacteroidetes (Hayashi *et al.*, 2002), (Wang *et al.*, 2003), (Eckburg *et al.*, 2005). The Firmicutes phylum consisted of 301 phylotypes, 191 of it are novel and most (95%) of the Firmicutes sequences were members of the Clostridia class. There are huge variety among the 65 Bacteroidetes phylotypes found between the different subjects (Hayashi *et al.*, 2002). Comparatively some of the sequences were related with the Proteobacteria**,** Actino bacteria, Fusobacteria, and Verrucomicrobia phyla. The low redundancy of the Proteobacteria sequences (including Escherichia coli) was normal considering that facultative species may represent a low amount ~0.1% of the bacteria in the strict anaerobic environment of the intestine (Hayashi *et al.*, 2002), (Wang *et al.*, 2003), (Eckburg *et al.*, 2005). In the newborn the compositions of the microbiota is strongly influenced and depends on the maternal input and the environment during delivery and during breast feeding. In the uterus, the growing fetus is expected to be completely free from bacterial flora. However, the same cannot be said of bacterial influence on the growing infants. During birth, the intestinal tract of the infant starts to be colonized, eventually creating a rich and various microenvironment. It has been demonstrated that human family members have very related similar microbiota as compared to those of unrelated individuals (Turnbaugh *et al.*, 2009). Furthermore, this trait was host genotype independent as there was no significant difference revealed in the rank of similarity between the mono- and dizygotic twins collected samples. This probably suggests that the maternal flora and environment, more than genetic factors alone, have greater influence on initiating intestinal bacterial communities (microbiota) in the new individual. This finding supports the statement that the largest degree of diversity between the intestinal microbiota could be explained by intersubject differences (Eckburg *et al.*, 2005). During the period of early childhood, microbial composition is influenced and changes with age and diet (Wu *et al.*, 2011). From the evolutionary point of view, the feature of intestinal microbiota substantially depends on the main nutritional source and consumption habits, can distinct bacterial profiles of omnivores, carnivores, and herbivores (Ley *et al.*, 2008), (Korecka & Arulampalam, 2012) which also probable consequences due to geographical circumstances.

Lactic Acid Bacteria (LAB) “are gram-positive, non-sporeforming cocci, coccobacilli or rods with a DNA base composition of less than 53mol% G+C. They generally are non-respiratory and lack catalytically active catalase. They ferment glucose primarily to lactic acid, or to lactic acid, CO_2 and ethanol” (Holzapfel *et al.*, 2001). All LAB are growing anaerobically, but unlike most anaerobes, they can also grow in the presence of O_2 as "aerotolerant anaerobes", even if some of them can express a functional respiratory chain in the presence of heme and menquinone. Although they lack catalase, they possess superoxide dismutase and have alternative tactics to detoxify peroxide radicals, usually through peroxidase enzymes. Although different genera of bacteria can produce lactic acid as a primary or secondary end-product of fermentation, the term Lactic Acid Bacteria is idiomatically confined for genera in the order *Lactobacillales*, which includes *Lactobacillus, Leuconostoc, Pediococcus, Lactococcus* and *Streptococcus,* in addition to *Enterococcus, Oenococcus, Carnobacterium, Vagococcus, Tetragenococcus,* and *Weisella* (Savadogo *et al.*, 2006). Lactic acid bacteria are mostly enteric microbiota, and are believed to play a beneficial role in the ecosystem of the human gastrointestinal (GI) tract. The beneficial scope of activity can be divided into nutritional, physiological and antimicrobial effects. LAB are also potential beneficial food supplements, and their oral administration stimulate both mucosal and systemic immune responses (Tuomola *et al.*, 2001). Multiple nutritional and therapeutic effects related to LAB can be summarized as: improvement of nutritional quality of food and feed, metabolic triggering of vitamin synthesis and enzyme production, constancy of gut microbiota and competitive exclusion of enteric pathogens, enhancement innate host defences by production of antimicrobial materials, reduction of serum cholesterol by absorption mechanism, decreased the risk of colon cancer by detoxification of carcinogens and tumor extinction by modulation of cell-mediated immunity (Naidu *et al.*, 1999). One of the effecting mechanisms to enhance host defence include the production of acid (Salminen *et al.*, 1996), and other by secondary products of bacterial metabolism (Ross *et al.*, 2002), (Tamime, 2002). The functional consequences of LAB in the human intestine highlight the need for detailed studies of bacterial properties, which shape this huge community cohabit synergies and competitions.

Bacterial translocation

Bacterial translocation (BT) is defined as “the passage of viable bacteria or endotoxins across the intestinal epithelium to mesenteric lymph nodes (MLN) and beyond, possibly contributing to sepsis at distant extra-intestinal sites” (Berg & Garlington, 1979). The mechanism by which these bacteria translocate remains unclear. Some researchers have suggested that these bacteria must have specific properties that predispose to translocation or belong to an invasive pathotype (O'Boyle *et al.*, 1998). Others however, have demonstrated that non-pathogenic mutualis bacteria were also able to translocate through

an intact intestinal epithelium (Nazli *et al.*, 2004), (Clark *et al.*, 2005). Bacterial translocation is important recommended indicator of administered bacteria safety, because it is the initial phase in the pathogenesis process for many opportunistic indigenous strains in the intestinal lumen (Steffen & Berg, 1983), (Ford *et al.*, 1996). However, the presence of some bacteria in the liver and spleen was previously described in healthy hosts also (Hale & Hill, 1973), (Ma *et al.*, 1990), (Gordon *et al.*, 1955)**,** (Martin *et al.*, 2004)**.** Actually bacterial translocation is a protective mechanism that gives the immune system continual contact with external antigens, although it can also contribute to the spreading of infection in an organism. It has been demonstrated in healthy animals that small amounts of bacteria penetrate systemically from the intestine tract into the tissues of the spleen and liver (Bengmark & Jeppsson, 1995), (Nikitenko *et al.*, 2011). However, in healthy body it is important to distinguish between two types of translocation, 1. Controlled translocation in normal host as the one described above, which aims to stimulate the immune system, and 2. Induced translocation which occurs due to specific body conditions (e.g. pregnancy and lactation (Perez *et al.*, 2007), (DiGiulio *et al.*, 2008)**,** (Jimenez *et al.*, 2005), (Martin *et al.*, 2004)).

Initial bacterial colonization in newborn and role of *L. plantarum* among the intestinal microbiota

Human and animal studies suggest that the intestinal microbiota during the neonatal period has a fundamental effect on nutritional status, gastrointestinal tract development, and maintenance of mucosal surface integrity (Dethlefsen *et al.*, 2006), (Hooper & Gordon, 2001), (Caicedo *et al.*, 2005). Also it has an important effect on health and specific inhabitant in this incident may influence his health and predispose to disease later in life (Kalliomaki *et al.*, 2001). Our understanding of the initiation and evolution of the intestinal microbiota during early infancy in humans has been limited. It could be demonstrated that the first colonizers might come from the mother microbiota (Favier *et al.*, 2003), (Matsumiya *et al.*, 2002), (Martin *et al.*, 2003). There is accumulating evidence that bacteria from mother are transmitted to the infant before birth (Jimenez *et al.*, 2005), (Perez *et al.*, 2007), (Jimenez *et al.*, 2008b). One important finding is represented by the fact that despite the maternal legacy of species diversity, each of the babies can develop its own microbiota pattern (Favier *et al.*, 2003). In the earliest stool samples, diversity varied depending on gestational age at delivery, use of maternal antibiotics, prolonged rupture of membranes, and feeding type (breast versus formula)(Mshvildadze *et al.*, 2010). During the early months of life for the neonate, breast milk is the best food for the speedy growing infant since it is a reach source with all the necessary nutritional requirements (Scientific Committee on Food, 2003). Moreover, several recent studies have shown that breastfeeding offer a continuous source of mutualistic microorganisms, which enhance the immunity and metabolism to support the infants health and protects against infectious diseases (Claud & Walker, 2001), (Martin *et al.*,

2005), (Caicedo *et al.*, 2005), (Lara-Villoslada *et al.*, 2007), (Heikkila & Saris, 2003), (Diaz-Ropero *et al.*, 2007), (Jimenez *et al.*, 2008a), (Lara-Villoslada *et al.*, 2009), (Maldonado *et al.*, 2010). It is estimated that an infant consuming approximately 800 mL of breast milk will receive about $1 \cdot 10^4 - 1 \cdot 10^6$ milk bacteria in each breast feeding, therefore, it is been expected that the bacterial composition of the infant fecal flora represents that one from the daily source breast milk and not the vaginal source (Heikkila & Saris, 2003). It has been proven that this microbiota is from maternal origin. However, milk had a less complex microbiota than maternal feces (Perez *et al.*, 2007), (Solis *et al.*, 2010), which leads to conclude that there is specific selection for this transmitted microbiota by milk. Among the lactic acid bacteria isolated from breast milk few species clearly predominated: *L. plantarum, L. fermentum, L. gasseri* and *E. faecium* (Martin *et al.*, 2004). While in human gut *L. plantarum*, *L. acidophilus* and *L. paracasei* are among the most predominant *Lactobacillus* species ((Ahrne *et al.*, 1998); (Reuter, 2001), (Vesa *et al.*, 2000), (de Vos *et al.*, 2004)). These species are considered among the probiotic bacteria (Holzapfel *et al.*, 1998) (Collins *et al.*, 1998). *L. plantarum* have effective survival after stomach passage (Ahrne *et al.*, 1998), (Vesa *et al.*, 2000) and high survival capacity in the ileum and faeces comparing to other lactobacillus species (Vesa *et al.*, 2000). *L. plantarum* species appear to be a dominant milk translocating strain.

Although different new infant formula milks are made to mimic human breast milk, they remain different from the natural biological fluid (Schwab & Gänzle, 2011). As a result, differences in the gut microbiota between breast-fed and formula-fed infants have been frequently reported (Conway, 1997), (Stark & Lee, 1982), (Harmsen *et al.*, 2000), (Claud & Walker, 2001), (Caicedo *et al.*, 2005) , (Penders *et al.*, 2006), (Mshvildadze *et al.*, 2010). “Human milk contains 1% of human milk oligosaccharides (HMOs) consisting of lactose with linked fucose, N-acetylglucosamine and sialic acid” (Gosling *et al.*, 2010). HMOs can activate growth of intestinal *Lactobacillus plantarum* and *bifidobacteria*, inhibiting the adhesion of infectious bacterial pathogens and provide prospective immunomodulatory properties (Kunz *et al.*, 2000; Schwab & Gänzle, 2011), (Claud & Walker, 2001). While in infant formula milks, galactooligosaccharides (GOSs) are added instead of HMOs. The different LAB which were isolated from the faeces of neonates were not able to digest complex HMOs but metabolized HMO simple components. Therefore general LAB depends on the presence of other gut microorganisms which is capable of dispersing simple monosaccharide components from the complex HMOs. *L. plantarum* is one of the LAB species, which is highly versatile in hydrolyzing HMOs (Schwab & Gänzle, 2011). Therefore *L. plantarum* play a major role in providing simple nutrient sources which might contribute to the selective persistence of the other LAB in the gut of infants fed with breast milk. It is not surprising that *L. plantarum* was the first *Lactobacillus* species isolated from breast milk (West *et al.*, 1979) while the

occurrence of other LAB seems not to have been explored until 2003 (Heikkila & Saris, 2003). Additional reason is that, between different LAB species, strains belonging to *L. plantarum* showed essential immunomodulatory properties due to its influence on innate cytokines (Vissers *et al.*, 2010), (Pavan *et al.*, 2003), (Maassen *et al.*, 2000), and this variation in the immunomodulatory effects between different species is generally larger than that between the strain of the same species (Vissers *et al.*, 2010).

One mechanism of bacteria used to adhere to epithelial cells is based on the binding to mannose-containing receptor sites. Several pathogenic species like enterotoxigenic *Escherichia coli, Pseudomonas aeruginosa ,* and *Salmonella enteritidis* are known to specifically adhere to mannose (Eshdat & Sharon, 1984), (Imberty *et al.*, 2004), and also *L. plantarum,* which is member of the endogenous microbiota, has been shown to bind to mannose-residues (Adlerberth *et al.*, 1996), (Molenaar *et al.*, 2005), (Gross, 2008), (Gross *et al.*, 2010). This ability to recognise the same receptor sites has been expected to enable beneficial microorganisms to prevent the colonisation of pathogens by competitive exclusion, which might prevent infections in the gastrointestinal tract (Reid & Burton, 2002). Keeping in mind that *E. coli* and other gram negative species are the first colonizer of the newborn gut , (Mackie *et al.*, 1999), (Harmsen *et al.*, 2000), (Penders *et al.*, 2006), (Bourlioux *et al.*, 2003), (Favier *et al.*, 2003), (Jimenez *et al.*, 2008b) and that *L. plantarum* had adherence competition rule with pathogenic bacteria, including *Escherichia coli* (de Vries *et al.*, 2006). For that it play important role in maintaining the integrity of gut barrier, stimulation the intestinal mucosal proliferation and improvement of barrier function (Adawi *et al.*, 1998). Different studies have reported the preventive effect of administrating *L. plantarum* on bacteria translocation of the global intestinal microbiota performed with healthy and injured rodents (Mao *et al.*, 1996), (Adawi *et al.*, 1997), (Adawi *et al.*, 1998), (Mangell *et al.*, 2002), (Pavan *et al.*, 2003), (Nguyen *et al.*, 2007), this special property will play major alteration role in case of pregnancy and lactation with the nitric oxide as will be described below. (Lara-Villoslada *et al.*, 2007) had presented the main probiotic properties which had been detected in different milk strains as intestinal colonisation, production of antimicrobial compounds, anti-microbial effect, immunomodulatory effect, anti-inflammatory effect, which all have been described in the species *L. plantarum*. For all this properties it is expect that the species *L. plantarum* may plays a key role in the initial colonization, modulation of intestinal function and influence intestinal microecology, and this makes it excellent candidate for a translocation study.

Translocation hypothesis during pregnancy (A ward of caution)

It has been suggested that the source of the live bacteria which was isolated from breast milk could be the mother mutualistic microbiota and “this bacteria would transfer to the mammary gland through the entero-mammary pathway, involving maternal dendritic cells

(DC) and macrophages" (Martin *et al.*, 2004), has been confirmed recently theoretically not practically (Martin *et al.*, 2004), (Perez *et al.*, 2007), (Jimenez *et al.*, 2008a). As in the tissue macrophages are nonmigrating resident cells, while DC are the migratory cells however, bacteria loaded DC are always restricted in the MLN and can't leave it to any farther niche (Rescigno *et al.*, 2001), (Macpherson & Uhr, 2004). It is also inconvenience that the free bacteria cells were released from the DC after they restricted them, as recent studies indicate that DC express osteoprotegerin (OPG), a secreted tumor necrosis factor receptor homolog, which regulates DC survival and monocyte chemotaxis (Schoppet *et al.*, 2007). Interestingly, this osteoprotegerin, a DC survival factor demonstrates elevated levels in the body during pregnancy and lactation (Uemura *et al.*, 2002) and is present in significant quantities in human breast milk (Vidal *et al.*, 2004).

An additional pregnancy condition, which inhibits DC action are glucocorticoids. When we live or have stressful circumstances our brain secretes corticotropin-releasing hormone (CRH). This master stress hormone stimulates the release of glucocorticoid stress hormones. During the second half of pregnancy period, circulating levels of corticotropin-releasing hormone (CRH) increase exponentially (Mastorakos & Ilias, 2003). Usually, this would have stimulated an overproduction of glucocorticoids in the mother. But orders are ineffective if nobody receives them, and hormonal orders are no exception. To be function, CRH must attach to special receptors in the brain (Dieterich *et al.*, 1997). Pregnant women body produce large amount of a CRH-binding protein ("CRH-BP") that prevent CRH from being recognized and used by its corresponding receptors. As an effect, most of the extra CRH is actually biologically inactive (McLean & Smith, 2001). But this situation shift in the last period of pregnancy. In the last three weeks of gestation, CRH levels escalate even higher. At the same time, CRH-binding proteins decrease. All at once, large quantities of CRH become available and biologically active (McLean & Smith, 2001). This rise in biologically-active CRH directs to a lift in glucocorticoids levels. Actually, glucocorticoid levels begin to climb during the second half of pregnancy period (Carr *et al.*, 1981), but they don't reach their peak until the late pregnancy. In the last weeks before birth, glucocorticoids levels are two to three times higher than normal (Dorr *et al.*, 1989). Interestingly, it is been proved that dendritic cells exist are decreased by glucocorticoids (de Baey *et al.*, 2003). This means that presence of DC will decrease during the last stage of pregnancy, which is the starting translocation point. Additional study showed that the frequencies of differentiated DC phenotypes tended to be lower in the circulation of lactating women during the first month after delivery than in that of virgin women (Perez *et al.*, 2007). Still it is not proven yet if DC is the real translocation transporter, where live bacteria can pass through from the intestine to the mammary gland of the mother. And if not, why the immune system didn't react and inhibit it? And why this only occurs during the last stage of pregnancy and lactation?

It is important to mention that human immune system could have tolerance to specific antigens (including bacterial antigens), defined strictly as the suppression of cellular and/or humoral immune responses or anti-inflammatory to that antigen (Weiner, 2000), (Sartor, 2002). Oral tolerance lead to treat exogenous bacterial agents that get access to the body via a natural way as internal components without danger signals, which then become part of self mutualistic microbiota (Weiner, 2000). This oral tolerance is an active immunologic process that is mediated by more than one mechanism and is dose-dependent (Friedman & Weiner, 1994). In addition to antigen dose, the nature of the antigen has impact on this tolerance (Weiner, 2000). However this only occurs during pregnancy and lactation. As during these periods body condition is very special and different factors could lead to bacteria translocation. The main reasonable and predictable mechanism which might control this special translocation occurring during this special period is organised by nitric oxide synthesis because nitric oxide appears to play a contradictory role in intestinal physiology (Nadler & Ford, 2000). Nitric oxide is synthesised in two different origins with two different mechanisms and rules between pregnancy and lactation. During pregnancy nitric oxide synthesis in the uterine artery endothelium, umbilical cord vessels and syncytiotrophoblast of the human placenta which is calcium sensitive activity (Sladek *et al.*, 1997). While during lactation nitric oxide is produced in the smooth muscle of human internal mammary artery (Qi *et al.*, 1993), (Lacasse *et al.*, 1996) which is calcium independent, and is different from that in endothelium (Qi *et al.*, 1993). The uterus produces nitric oxide during the pregnancy and this activity decrease between the late gestation period (16-18 days in rodent and 32-34 wk in human) and spontaneous labour at term time (Natuzzi *et al.*, 1993), (Sladek *et al.*, 1993), (Yallampalli *et al.*, 1993), (Sladek & Roberts, 1996). this decrease of nitric oxide production is capable of increasing intestinal permeability and bacteria translocation (Kubes, 1992), (Kanwar *et al.*, 1994), (Adawi *et al.*, 1998). Interestingly *L. plantarum* is the main species which influence with this decrease, it is proved the ability of *L. plantarum* to inhibit other bacteria translocation (Mao *et al.*, 1996), (Adawi *et al.*, 1997), (Adawi *et al.*, 1998), (Mangell *et al.*, 2002), (Pavan *et al.*, 2003), (Nguyen *et al.*, 2007). However, this ability of *L. plantarum* decreased with the decreases of nitric oxide production (Adawi *et al.*, 1998), and that is the circumstance during the late pregnancy

In another mechanism, nitric oxide generating system in the mammary gland is activated at the starting of lactation (Iizuka *et al.*, 1997), (Pero *et al.*, 2006). This nitric oxide which is synthesized in the breast or given as dietary supplementation during lactation play a fundamental influence in the development morphological and functional features of mammary gland as enhancing the secretion of breast milk due to increased nutrient and blood flow across the mammary gland (Iizuka *et al.*, 1997), (Pero *et al.*, 2006), (SW & G, 2008). This might increase the possibility for bacterial translocation by releasing intercellular

tight junction between intestinal epithelial cells (Nadler & Ford, 2000). This lead to wonder about the specific alteration rule *L. plantarum* and such ability having species, that it plays during pregnancy and lactation leading to bacteria translocation from mother to her infants. Although animal models such as mice are ideal systems for formulating hypotheses on bacteria translocation and its conditions, still the real answers come from experiments in human systems. However, it is worth mentioning that investigating differential bacterial gene expression in the intestine were different, as well as the hosts, mouse versus human, and for those genes which mainly involved in nutrient profits and synthesis, stress, and extracellular functions, they indicate that intestinal conditions of mouse and human may have similar effects on overall gene expression of *L. plantarum* in the intestine (de Vries *et al.*, 2006). However, it seems that the frequency of translocation in humans is much lower than that observed in murine model (Balzan *et al.*, 2007).

Motivations and objectives of the study

The aim of this work was to investigate the presence and identity of LAB in breast milk of healthy women, in mother and infant faeces, and in fermented food samples consumed by these people to study the distribution of different species in different sources. The predominant LAB species of all sources should be further investigated with respect to their survival, resistance, competition and stress tolerance properties to enable a better understanding of bacterial interaction and the rules of dominance. Strains should be identified, which are found in mothers as well as in babies versus strains present in only one of the sources to enable comparative studies on their properties and behavior. A model strain should be selected to probe translocation *in vitro* systems and in an *in vivo* murine model.

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CHAPTER I

Genotypic analysis and MALDI-TOF fingerprinting of lactic acid bacteria in local fermented food, breast milk and faeces of mothers and their babies

Abstract

Lactic acid bacteria (LAB) are generally accepted as beneficial to the host and their presence is directly influenced by ingestion of fermented food or probiotics. While the intestinal lactic microbiota is well-described knowledge on its routes of inoculation and competitiveness towards selective pressure shaping the intestinal microbiota is limited. In this study, LAB were isolated from faecal samples of breast feeding mothers living in Syria, from faeces of their infants, from breast milk as well as from fermented food, typically consumed in Syria. A total of 700 isolates were characterized by genetic fingerprinting with random amplified polymorphic DNA (RAPD) and identified by comparative 16S rDNA sequencing and Matrix Assisted Laser Desorption Ionization- Time-of-flight mass spectrometry (MALDI-TOF-MS) analyses. Thirty six different species of *Lactobacillus*, *Enterococcus*, *Streptococcus*, *Weissella* and *Pediococcus* were identified. RAPD and MALDI-TOF-MS patterns allowed comparison of the lactic microbiota on species and strain level. Whereas some species were unique for one source, *L. plantarum*, *L. fermentum*, *P. pentosaceus* and *L. brevis* were found in all sources. Interestingly, identical RAPD genotypes of *L. plantarum*, *L. fermentum, L. brevis, E. faecium*, *E. faecalis* and *P. pentosaceus* were found in the faeces of mothers, her milk and in faeces of her babies. Diversity of RAPD types found in food versus human samples suggest the importance of host factors in colonization and individual host specificity, and support the hypothesis that there is a vertical transfer of intestinal LAB from the mother's gut to her milk and through the milk to the infant´s gut.

Keywords Intestinal LAB; RAPD-PCR; 16S rDNA; MALDI-TOF; gut microbiota; breast milk; infant; translocation; *Lactobacillus plantarum*; fermented food.

Introduction

The human gastrointestinal (GI) tract is colonized by microorganisms from the oral cavity to the rectum, comprising nearly 800 species. Bacteria in the (human) gut achieve the highest cell densities recorded for any ecosystem (Bäckhed *et al.*, 2005), (Mshvildadze *et al.*, 2010) which makes it one of the most densely populated microbial ecosystems on earth (O'Hara & Shanahan, 2006). This population evolves over time. The faecal microbiota are highly stable within one individual over time, and a unique population is found in each individual (Stewart *et al.*, 2005). Whereas in adults the microbial community is rather stable, it appears unstable and shapeable in infants from birth until the age of 2–4 years (Egert *et al.*, 2006), (Hattori & Taylor, 2009). Beyond age, additional host factors e.g. secretory products, peristalsis, food transit time, host genotype and environmental factors e. g. type of diets or direct ingestion of alive microorganisms influence the microbial community structure (Bartosch *et al.*, 2005), (Dethlefsen *et al.*, 2006), (Guarner & Malagelada, 2003), (Penders *et al.*, 2006).

The large intestine is sterile at birth, but becomes rapidly colonized with bacteria derived from the maternal microbiota, the diet and environment. Numerous studies are available in the literature demonstrating prevalence, diversity, stability and source of the human lactic microbiota (Bäckhed *et al.*, 2005; Dethlefsen *et al.*, 2006), (Eckburg *et al.*, 2005), (Egert *et al.*, 2006). Only recently it became accepted that breast milk constitutes a source of microorganisms that may colonize the infant gut and modulate its function (Lara-Villoslada *et al.*, 2007a), (Lara-Villoslada *et al.*, 2007b), (Martín *et al.*, 2004), (Martin *et al.*, 2003). Human breast milk includes several predominant bacterial species, such as *staphylococci, streptococci, micrococci, lactobacilli, enterococci, lactococci* and *bifidobacteria* (Gueimonde *et al.*, 2007), (Martin *et al.*, 2009b), (Martin *et al.*, 2004), (Martin *et al.*, 2003), (Perez-Cano *et al.*, 2010), (Solis *et al.*, 2010). The origin of bacteria found in human milk is debated and it is suggested that, at least, some species may be endogenously delivered from the maternal gut to the mammary gland upon translocation through the intestinal epithelium. Translocation of viable (and dead) bacteria in low numbers from gut to extra-gut tissues, especially the gut-associated lymphoid tissues (GALT), is a normal and a beneficial physiological process associated with immune stimulation and the effects of alerting the local immune defense (Guarner & Malagelada, 2003), (MacFie, 2002). More recently, it has been shown that such bacteria are transferred from the maternal gut to the mammary gland during late pregnancy and lactation via the enteromammary pathway, an internal route that involves dendritic cells and macrophages (Martin *et al.*, 2004), (Perez *et al.*, 2007). This hypothesis is controversially discussed and requires further investigation as it opens new perspectives for bacteriotherapy, definition of new probiotic properties and formulation of infant foods.

In the current study, we investigated the presence and identity of LAB in breast milk of healthy women, in mother and infant faeces, and in fermented food samples at genotype level.

Materials and methods

Sampling and isolation of LAB

Strains were isolated from 15 fresh faecal samples of infants (age between 1 month to 2 years, vaginally delivered breast-fed full-term), 15 samples from their mother's milk (breast-milk samples were obtained in sterile tubes by manual expression using sterile gloves after cleaning the nipples and areola by wiping with a swab soaked in sterile water and discarding the first drops.), and another 15 faecal samples from the mothers and healthy adults (13-35 years) living in different villages in Syria. None of the adults, mothers or babies received antibiotic therapy during the sampling period. For the adults ten gram of faeces sample was added with 90 ml of saline water (8.5 g/l) to make an initial dilution (10^{-1}) and for the infants 5 gram of faeces in 45 ml of saline water. Serial dilutions were plated on nine specific culture media used for cultivation of bacteria: (a) for the isolation of bifidobacteria, modified Wilkins–Chalgren (WC) agar with the addition of glacial acetic acid (1 ml/l) and mupirocin (100 mg/l) as described by Rada and Koc (Rada & Koc, 2000), modified Trypticase Phytone Yeast extract medium (MTPY) agar with glacial acetic acid (1 ml/l) and mupirocin (100 mg/l) as described by Rada and Petr (Rada & Petr, 2000), Columbia Blood Agar (CBA) supplemented with propionic acid (5 ml), dicloxacillin (2 mg/l) and L-cysteine (0.5 g/l) as described by (Bonaparte, 1997), Bifidobacteria-Fermented Milk medium (BFM) medium with lactulose as carbon source (Nebra & Blanch, 1999), Raffinose-*Bifidobacterium* medium (RB) with raffinose as carbon source (Hattori & Taylor, 2009), (RF) (Hartemink *et al.*, 1996) and Beerens Agar supplemented with propionic acid (5 ml) (Beerens, 1991)**.** (b) MRS, Rogosa and M17 media supplemented with a vitamin mix containing biotin, folic acid, nicotinic acid, pyridoxal phosphate, thiamine, riboflavin, cobalamin and panthothenic acid (0.2 g/l each) for *Lactobacillus*. The agar plates were incubated at 37^{o}C under anaerobic conditions (85% nitrogen, 15% carbon dioxide) for 48h (MRS, Rogosa, M17, MTPY, WC, CBA, BFM, RF) and for 96h for Beerens Agar. Colonies with different morphology were purified by streaking on the appropriate media and tested for catalase reaction, Gram staining and microscopic examination. All isolates, which were catalase-negative and Gram-positive were selected and maintained as frozen cultures in MRS broth and 50% glycerol at -80 ^{o}C. Bacteria were also isolated by the described method from 25 samples of eight different types of fermented food (homemade and offered in markets) collected from different places in Syria (fermented vegetables, fermented olive, Kishk, Shanklish, Kashkawan, Bastirma, Qamer Eldeen and makdous) . All of the adults from whom faecal samples were collected had access to these different types of fermented food and consumed it daily. The food samples were cultured in the same culture media as above.

DNA extraction: Genomic DNA was directly isolated from overnight liquid culture using E.Z.N.A. bacterial DNA Kit (OMEGA BIOTEK, Inc.).

Typing of LAB isolates by RAPD-PCR

Randomly amplified polymorphic DNA (RAPD) polymerase chain reaction (PCR) analysis was performed using primer M13V (5'-GTTTTCCCAGTCACGAC-'3). The reaction mixture (final volume 25 µl) consisted of 16 µl H_2O, 2.5 µl 10x reaction buffer, 5.0 µl $MgCl_2$ (25 mM), 1.0 µl dNTP mixture, 0.15 µl Taq-polymerase, 0.25 µl M13V primer, 0.5 µl of the DNA. RAPD reactions were performed in a thermocycler by using the following temperature profile: initial denaturation at 94 ^oC for 3min followed by 40 ^oC, 5min; 72 ^oC, 5min; repeat it 3 time then 94 ^oC, 1min; 60 ^oC, 2min; 72 ^oC, 3min for 33 cycles. Amplification products were analysed by electrophoresis in 1.5% agarose gel with TBE buffer and stained with ethidium bromide 0.1%. RAPD-PCR pattern analysis and clustering was performed by the BioNumerics software (Applied Maths, Belgium).

Sequence analysis of the 16S rRNA gene

From each cluster one isolate was choosen for the 16S rDNA sequencing. Partial 16S rRNA genes were amplified with primer 616 forward (5'-AGAGTTTGATYMTGGCTCAG -'3) and 609 revers (5'-ACTACCAGGGTATCTAAAC -'3) the amplified DNA products were purified using the QIAquick PCR purification KIT (Qiagen) and sequenced by a commercial provider (GATC, Germany). The sequence was analysed, and the identification of isolates was achieved by comparison with sequences provided in public databases.

MALDI-TOF-MS

Matrix Assisted Laser Desorption Ionization-Time of flight mass analyzer (MALDI-TOF-MS) was performed for 280 strains from different species. 1 mL of bacterial cultures was centrifuged at 15.000 rpm for 5 min. Cells were washed in 300 mL of water and additionally in 900 mL of absolute ethanol. Cells were dissolved by vortexing for 2 min in 50 µl of 70% formic acid and additionally 50 µl of acetonitrile and vortexing for 2 min. The mixture was centrifuged at 15.000 rpm for 5 min to pellet the cell debris and transfer the cell extract in the supernatant to a new tube. 1 mL of the culture extract was spotted on microscout target plates and allowed to air dry, then overlaid with 1 mL of matrix solution containing 1.5 mg of α-cyano-4-hydroxynamic acid in 50% acetonitrile with 2.5% TFA and dried under ambient conditions. Each sample preparation was spotted in duplicate onto the MALDI target plate. Ion mass spectra in the linear mode were obtained on a Microflex LT mass spectrometer (Bruker Daltonics) analyzed by clinPro Tools software (version 2.0.365, Bruker Daltonics) for species identification. Cluster analyses were performed with Bionumerics.

Production of hydrogen peroxide

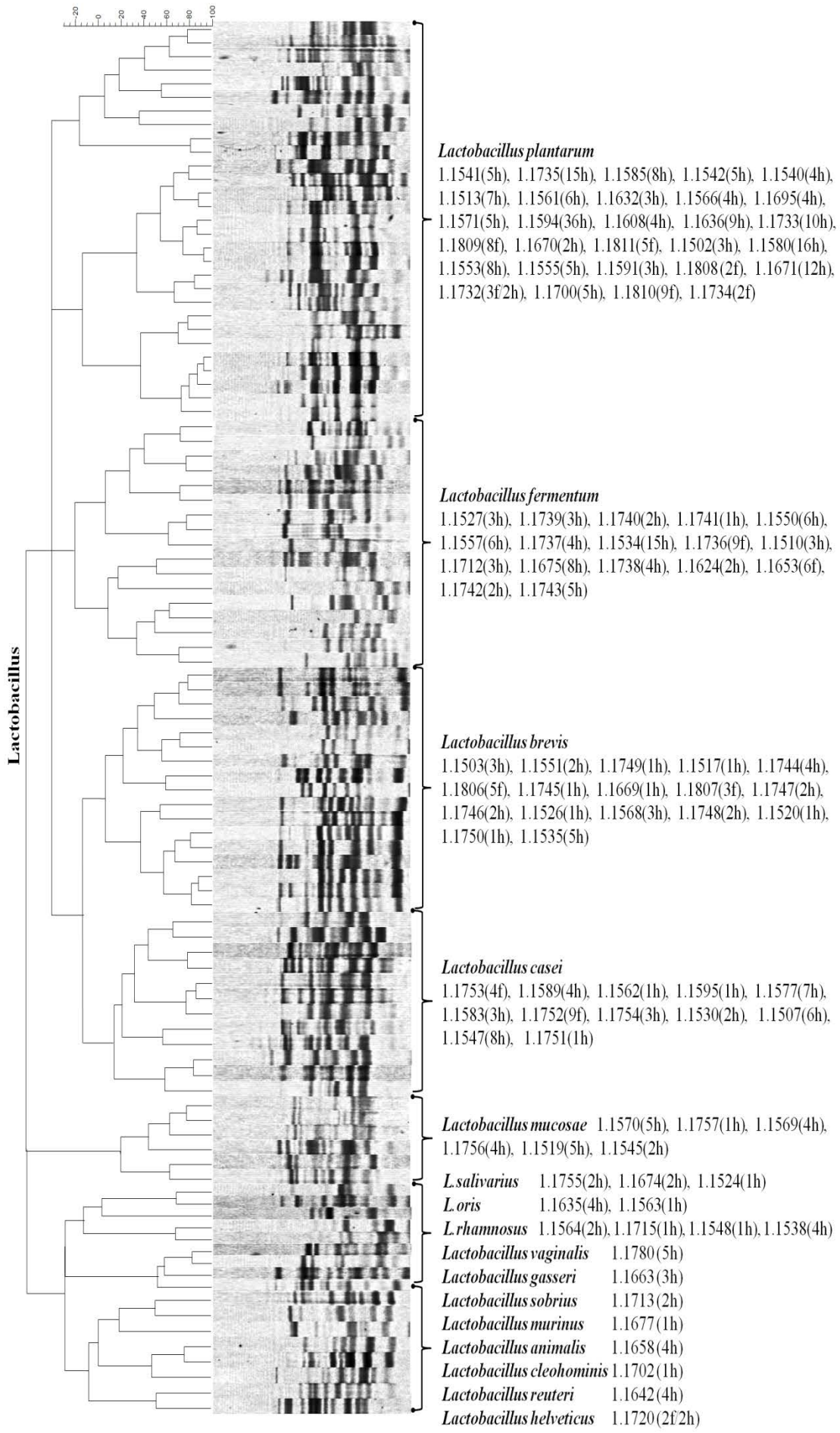
Overnight cultures were spotted either on TMB-plus agar plates as described by Rabe and Hillier (Rabe & Hillier, 2003). The plates were incubated anaerobically at 37 ^oC for 72h and then exposed to the atmosphere. H_20_2 production was considered positive when violet halos surrounding the colonies appeared. Results were counted after 1h, 3h and 24h.

Results

Bacteria were isolated from 70 different samples (15 infants and 15 adults and 15 breast milks and 25 fermented foods). Numbers of bacterial isolates were > 10^6 and varied different types of media, individuals and foods. As proven by the subsequent molecular identification, MRS, M17 and Rogosa recovered highest numbers of lactobacilli while MTPY, Beerens and WC proved effective in the isolation of *enterococci*. Strains of all species could be isolated on CBA even though some specific strains were only isolated from specific media (data not shown). Apart from the expectedly high cell numbers in faecal samples, bacteria were isolated from milk samples in CFU numbers of 2 x 10^1 – 2 x 10^3 (MRS), 5 x 10^2 – 3 x 10^3 (M17), to 2 x 10^1 (Rogosa), 1 x 10^1 (Beerens), 8 x 10^1 – 1 x 10^2 (Colombia) and 3 x 10^1 (RB). No milk isolates were obtained on MTPY, WC and BFM media. Interestingly, RB medium with raffinose as carbon source, which described as *Bifidobacterium* specific, could also encourage the growth of *Lactobacillus* species with even higher CFU counts than MRS and M17. From the 9 different media a total of 700 isolates of LAB were isolated and subjected to randomly amplified polymorphic DNA (RAPD) polymerase chain reaction analysis (Fig 1).

TABLE 1
Different bacterial species isolated in this study from fermented food in Syria as compared to the species found in these food types by (Campbell-Platt, 2000).

Food name	Composition	Isolated species	Campbell-Platt
Kishk	Dried mixture of fermented milk with crushed wheat (Triticum durum)	*L. plantarum* *L. brevis* *L. casei* *L. bulgaricus* *Str. thermophilus* *L. fermentum*	*Str. thermophilus, L. bulgaricus, Leuc. cremoris*
Shanklish	Hard, dry grating cheese from raw partly skimmed cow's or sheep's milk	*L. fermentum* *L. casei* *L. helveticus*	*L. lactis, L. helveticus*
Baldi cheese	Hard smooth spun-curd cheese, made from raw or pasteurized caw milk	*L. casei* *L. paraplantarum* *L. fermentum* *Str.thermophilus* *L.bulgaricus*	*Str. thermophilus, L. bulgaricus, Str. lactis*
Bastirma	Fermented beef meat	*P. pentosaceus* *E. hirae* *Staph. epidermidis*	*Lactobacillus Micrococcus*
Mekhalal	Fermented vegetables (cucumber, cabbage, turnip, carrot, capsicum, eggplant, cauliflower, lemon, onion, red beet, tomato, garlic, gage.. etc)	*L. plantarum* *L. brevis* *Staph. epidermidis*	*Pediococcus, Leuconostoc, Streptococcus*
Fermented Olives	Fermented green and black olives	*L. plantaru* *E. hirae* *Leuc.*	*Enterobacter aerogenes, Leuconostoc, Streptococcus, Pediococcus*
Makdous	Salted eggplants stuffed with the walnut, chili and garlic and covered with oil	*L. plantarum* *L. brevis* *L. paraplantarum* *L. fermentum*	
Qamer Eldeen	Dried concentrated apricot juice	*L. plantarum* *L. fermentum*	


-20
0
20
40
60
80
100
Lactobacillus
Lactobacillus plantarum
1.1541(5h), 1.1735(15h), 1.1585(8h), 1.1542(5h), 1.1540(4h), 1.1513(7h), 1.1561(6h), 1.1632(3h), 1.1566(4h), 1.1695(4h), 1.1571(5h), 1.1594(36h), 1.1608(4h), 1.1636(9h), 1.1733(10h), 1.1809(8f), 1.1670(2h), 1.1811(5f), 1.1502(3h), 1.1580(16h), 1.1553(8h), 1.1555(5h), 1.1591(3h), 1.1808(2f), 1.1671(12h), 1.1732(3f/2h), 1.1700(5h), 1.1810(9f), 1.1734(2f)
Lactobacillus fermentum
1.1527(3h), 1.1739(3h), 1.1740(2h), 1.1741(1h), 1.1550(6h), 1.1557(6h), 1.1737(4h), 1.1534(15h), 1.1736(9f), 1.1510(3h), 1.1712(3h), 1.1675(8h), 1.1738(4h), 1.1624(2h), 1.1653(6f), 1.1742(2h), 1.1743(5h)
Lactobacillus brevis
1.1503(3h), 1.1551(2h), 1.1749(1h), 1.1517(1h), 1.1744(4h), 1.1806(5f), 1.1745(1h), 1.1669(1h), 1.1807(3f), 1.1747(2h), 1.1746(2h), 1.1526(1h), 1.1568(3h), 1.1748(2h), 1.1520(1h), 1.1750(1h), 1.1535(5h)
Lactobacillus casei
1.1753(4f), 1.1589(4h), 1.1562(1h), 1.1595(1h), 1.1577(7h), 1.1583(3h), 1.1752(9f), 1.1754(3h), 1.1530(2h), 1.1507(6h), 1.1547(8h), 1.1751(1h)
Lactobacillus mucosae 1.1570(5h), 1.1757(1h), 1.1569(4h), 1.1756(4h), 1.1519(5h), 1.1545(2h)
L.salivarius 1.1755(2h), 1.1674(2h), 1.1524(1h)
L.oris 1.1635(4h), 1.1563(1h)
L.rhamnosus 1.1564(2h), 1.1715(1h), 1.1548(1h), 1.1538(4h)
Lactobacillus vaginalis 1.1780(5h)
Lactobacillus gasseri 1.1663(3h)
Lactobacillus sobrius 1.1713(2h)
Lactobacillus murinus 1.1677(1h)
Lactobacillus animalis 1.1658(4h)
Lactobacillus cleohominis 1.1702(1h)
Lactobacillus reuteri 1.1642(4h)
Lactobacillus helveticus 1.1720(2f/2h)

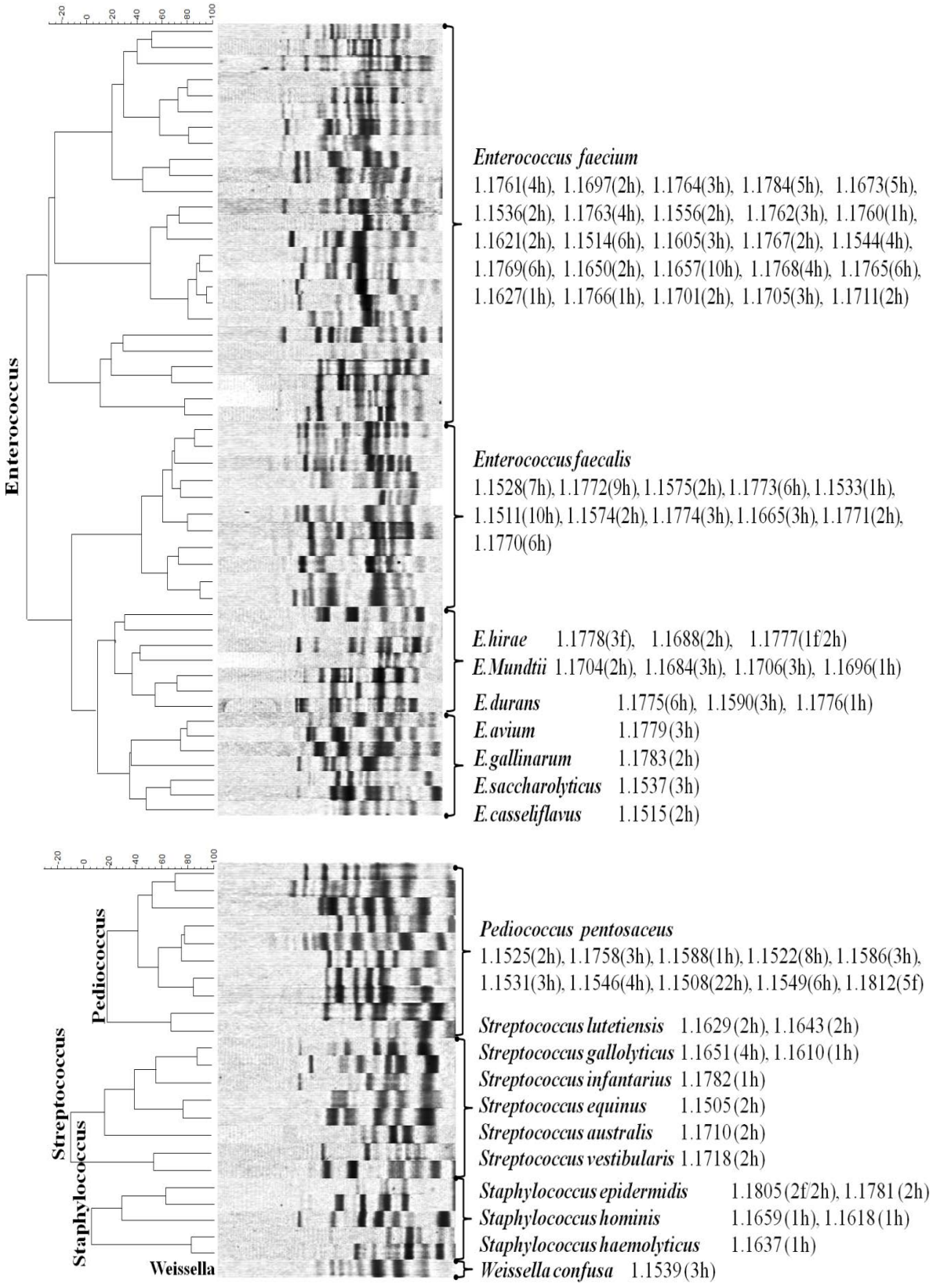


Figure 1. UPGMA cluster analysis of RAPD fingerprints pattern of different LAB strains isolated from mother milk, faeces samples of different age people living in Syria and local fermented food. Only patterns of representative strains showing different genotypes were presented. The numbering displays TMW numbers of the strain collection of "Technische Mikrobiologie Weihenstephan". Numbers in brackets indicate the number of isolates with an identical patterns; h = human isolate; f = food isolate.

All strains displaying different RAPD patterns were identified by 16S rDNA sequencing. From the faecal and milk samples we identified 36 different species of *Lactobacillus*, *Enterococcus, Weissella, Streptococcus, Staphylococcus* and *Pediococcus.* Eleven different LAB species of *Lactobacillus*, *Enterococcus, Streptococcus, Staphylococcus, Pediococcus* and *Leuconostoc* were isolated from different samples of eight types of fermented food typically consumed in Syria as shown in Table 1. The occurrence of the species with respect to the samples´ source is summarized in Fig 2.

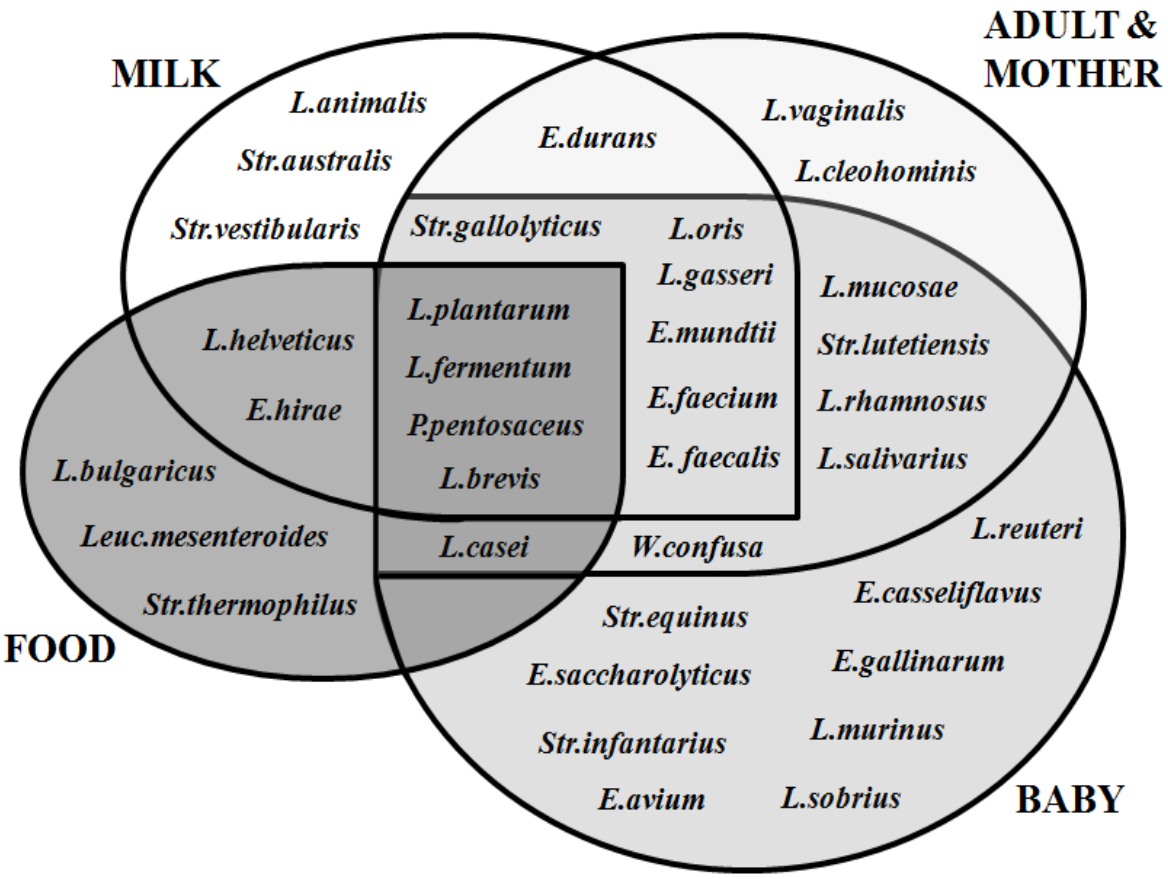


Figure 2. Lactic acid bacteria species distribution between the different sources, adults and mothers, babies, milk and food.

Most of the species were present in different RAPD genotypes. Within the 700 isolates 171 different strains (based on different RAPD genotypes) were differentiated, which were identified as (species (x total isolates / y different strains / z number of different RAPD genotypes obtained from human samples/*same RAPD genotype occurring in human and food)): *L. plantarum* (210/29/24/*1), *L. fermentum* (82/17/15), *L. casei* (49/12/10), *L. brevis* (38/17/15), *L. salivarius* (5/3/3), *L. rhamnosus* (8/4/4), *L. mucosae* (21/6/6), *L. oris* (5/2/2), *Str. gallolyticus* (5/2/2), *Str. lutetiensis* (4/2/2), *P. pentosaceus* (57/10/9), *E. faecium* (85/25/25), *E. faecalis* (51/11/11), *E. durans* (10/3/3), *E. hirae* (8/3/2/*2), *E. mundtii* (9/4/4). Most of these strains, were found in more than one of the sources, i. e. same RAPD

genotypes were found in mother and baby faeces as well as in milk and rarely also in food samples.

Only one RAPD genotype was detected for each (species (x total isolates/*same RAPD genotype exist in human and food)): *L. reuteri* (4), *L. sobrius* (2), *L. animalis* (4), *L. gasseri* (3), *L. coleohominis* (1), *L. vaginalis* (5), *L. murinus* (1), *L. helveticus* (4/*1), *Str. australis* (2), *Str. vestibularis* (2), *Str. equines* (2), *Str. infantarius* (1), *W. confusa* (3), *E. gallinarum* (2), *E. avium* (3), *E. saccharolyticus* (3) and *E. casseliflavus* (2). Also species of non-LAB were found, *Staph. hominis* (2/2/2), *Staph. haemolyticus* (1/1/1), *Staph. epidermidis* (6/2/1/*1) and one yeast *Kluyveromyces marxianus* (TMW 3.215)

As listed in Fig. 1, some strains were frequently isolated with identical RAPD genotypes, not necessarily recovered from the same sample. Very few identical RAPD genotypes were found in both, human and food samples. This is peculiar for *L. plantarum*, which was the most frequently found LAB species in these human and food samples. As a prominent example the RAPD genotype of *L. plantarum* TMW *1.1594* was found 36 times, in different individuals, 11 x in infants, 12 x in adults and 3 x in milk samples.

From the 16S identified 700 isolates a subset of 280 isolates was chosen for further characterization by MALDI-TOF–MS analyses to compare the accuracy of discrimination between the two methods of identification. This subset contained all strains exhibiting different RAPD patterns (171) plus those displaying identical RAPD genotypes but originating from different sources. In most cases MALDI-TOF–MS species identification confirmed 16S rDNA results. An insufficient identification was detected for strains of *P. pentosaceus* and *L. casei* probably due to a limit of MALDI-TOF-MS data base entries (data not shown). UPGMA clustering of MALDI-TOF-MS spectra discriminates at species level at a cutoff between 10-20% of similarity and for strain level at a cutoff value of 30% similarity, whereby these clusters were different from RAPD subgroupings (data not shown). 16S rDNA sequences do not differentiate between *L. plantarum* and *L. pentosus.* However both MALDI-TOF-MS and RAPD analysis clearly separated these two species.

The isolate TMW 1.1773 was identified by 16S rDNA sequencing as *E. faecalis* whereas MALDI-TOF identification software identifies it as *E. faecium.* However, the UPGMA cluster analysis of MALDI-TOF employing many strains of *E. faecium* and *E. faecalis* shows that TMW 1.1773 does not belong to any of the two species clusters and it can be differentiated from both of them at cutoff level of 10% (Fig 3).

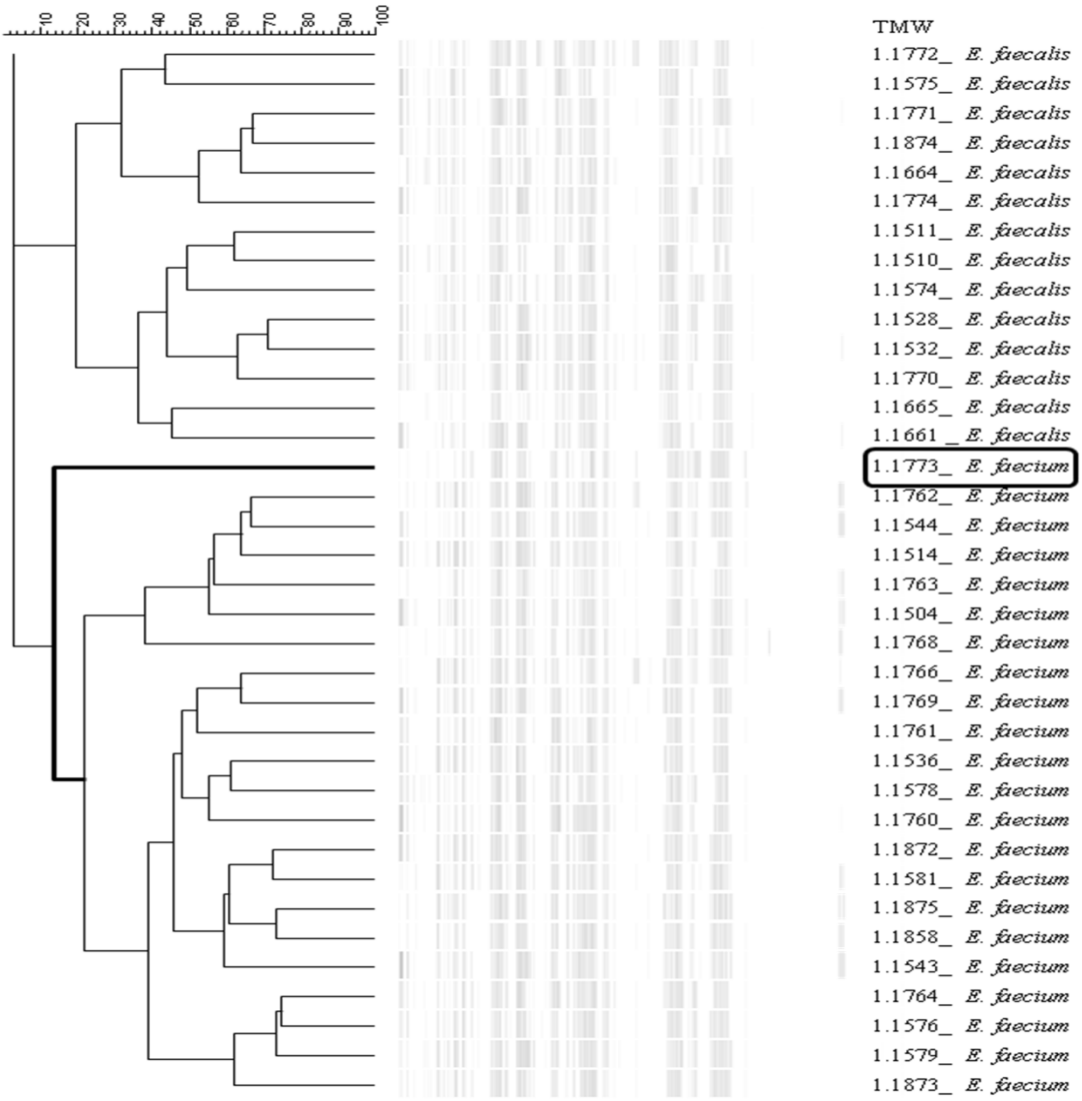


Figure 3. UPGMA analysis of MALDI-TOF patterns of the two species *Enterococcus faecium* and *E. faecalis.*

The distribution of LAB species found in the different sources are summarized in Fig 2. Whereas some species were unique for one of the four sources, *L. plantarum, L. fermentum, L. brevis* and *P. pentosaceus* were present in all different samples. *L. casei* was isolated from different fermented dairy products and the intestinal flora but not from the breast milk. As most of the species appear different in their RAPD genotypes, the genetic diversity and their occurrence and distribution in the different sources can be followed. For example, RAPD patterns within one species were generally more diverse in faecal than in food samples. Identical RAPD genotypes of *E. faecium* and *E. faecalis* were found in breast milk and faecal samples of mothers and their babies, but they were absent in foods. On the other hand, we did not find *L. bulgaricus* and *Str. thermophilus* used as starter cultures for fermented dairy products and *Leuc. mesenteroides* frequently found in fermented olives, in human samples.

Figure 4 demonstrates that in nine cases identical genotypes of *L. plantarum*, *L. fermentum, L. brevis, E. faecium* and *E. faecalis* were found in the mothers' faeces, their milk and the corresponding babies' faeces. These species were found in the three sources in some groups. While others were found in two of them, like in the same mother faeces and milk samples (*E. mundtii*) or in the mother milk and her baby faeces samples (*L. oris*) or in the mother and her baby faeces samples (*L. casei, L. salivarius, L. rhamnosus* and *Str. lutetiensis*) (Fig 4). In contrast, we found that identical RAPD genotypes in food and human samples were rare and restricted to one strain each of *L. plantarum*, *L. helveticus* and *Staph. epidermidis* (compare Fig. 1).

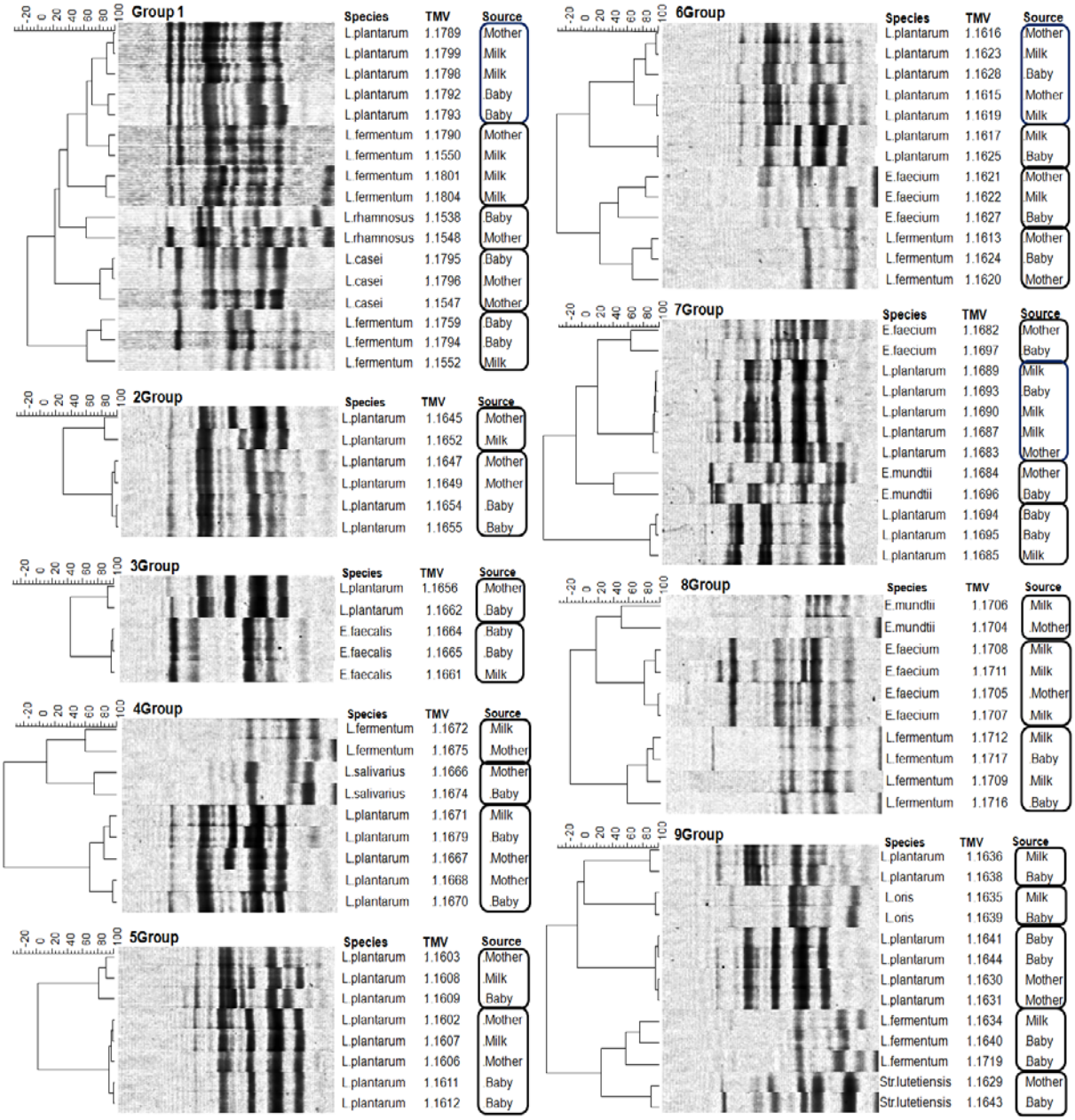


Figure 4. RAPD-PCR patterns of different species from mother faeces, her milk and her baby faeces in different mother–child pair groups (the rectangles represent common species existence in two or three sources of one group).

Additionally, comparative analysis by RAPD-PCR and MALDI-TOF analysis was performed for the 23 *L. plantarum* strains of 9 different mother/baby-pairs groups.

Among different strains of mother/baby-pairs groups *L. plantarum* was the dominant isolated species, which was found in the different groups as trio from the three sources (the mother faeces, her baby faeces and her own milk) of each group while other species groups were found in two sources of the three sources of each group. The nine different trio groups of *L. plantarum* had been distributed under three different RAPD-PCR genotypes patterns with high similarity as shown in Fig 5

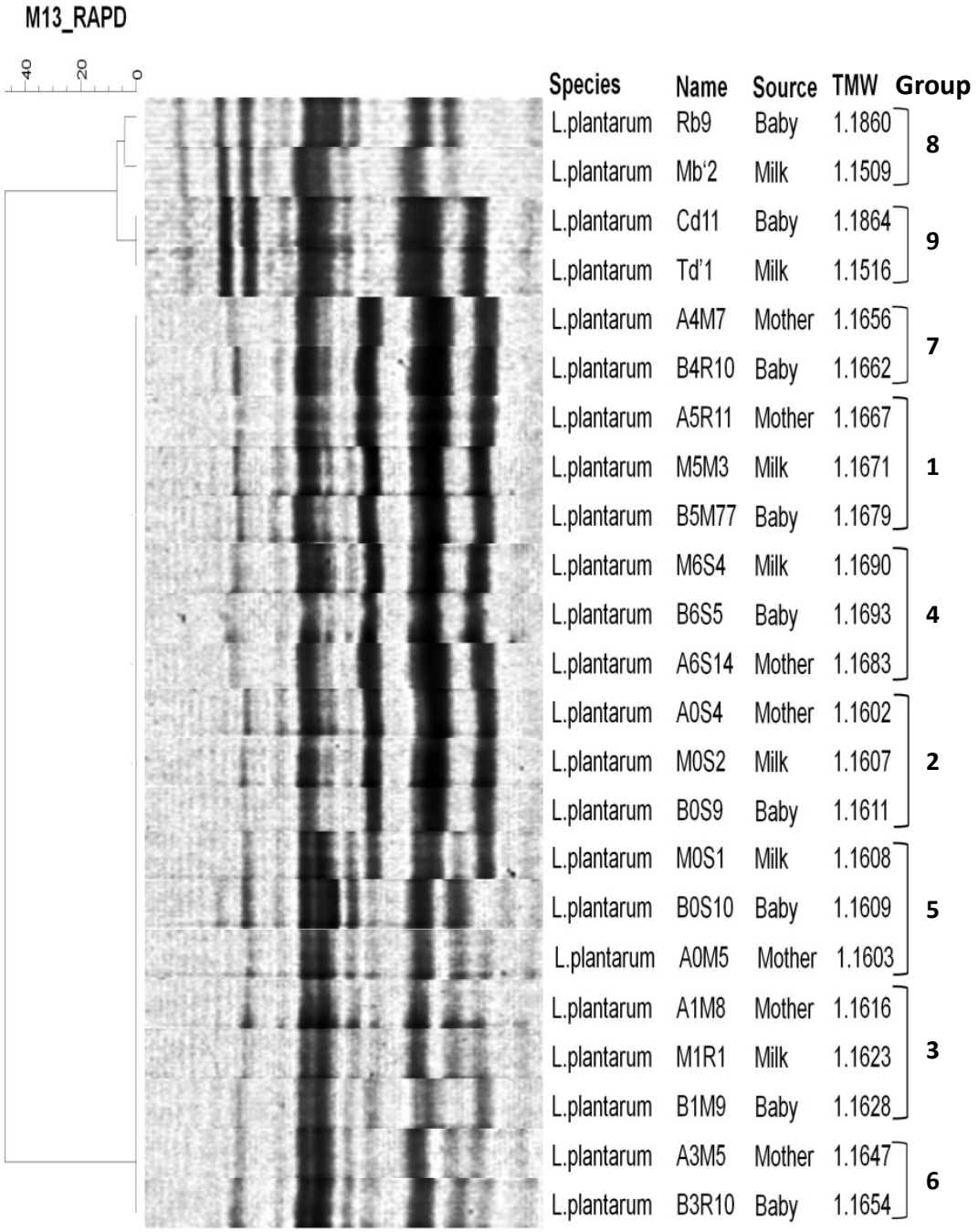


Figure 5. RAPD-PCR genotypes of *L. plantarum* strains isolated from three or two sources of different 9 mother/baby-pairs groups

The same *L. plantarum* strains were analysed also by MALDI-TOF test to see the possibility to be discriminated depending on the groups or the sources, however the result showed almost identical MALDI-TOF spectra for all strains and the analysis couldn't distinguish between the strains depending on their sources but it was able to distinguish only 5 group's strains from the 9 groups and cluster them together Fig (6).

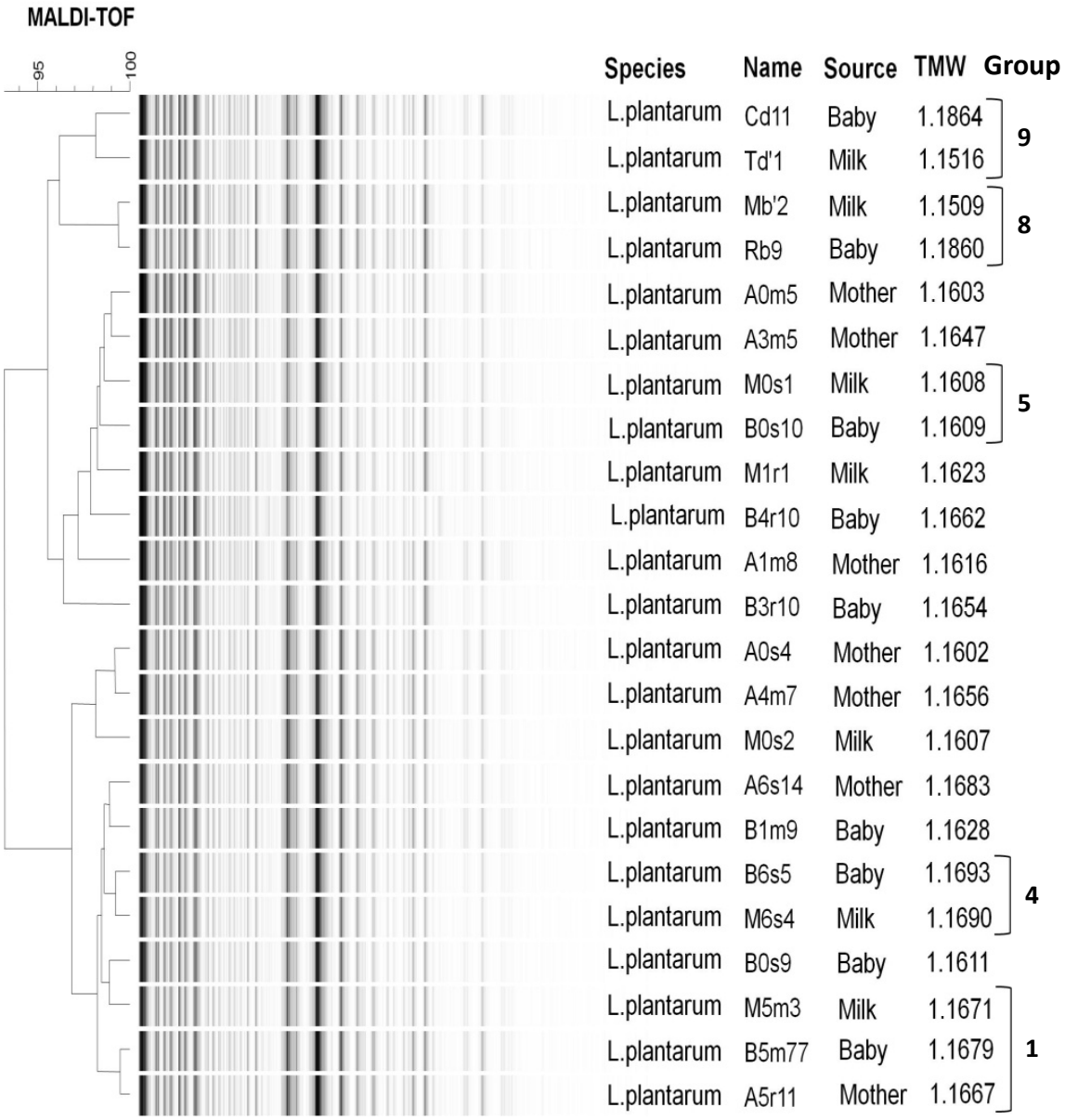


Figure 6. clustering of MALDI-TOF patterns of *L. plantarum* strains isolated from three or two sources of different 9 mother/baby-pairs groups

Discussion

Lactic Acid Bacteria diversity of healthy adults

The bacterial flora from the fecal samples showed a high bacterial diversity but low similarity between the individuals, indicating high variability of bacterial microorganisms among the different hosts. Such relations have been attributed to host selection for specific bacteria (Dethlefsen *et al.*, 2006). Also differences between individuals appeared greater than the differences between different samplings taken from one individual (Eckburg *et al.*, 2005). Our study suggests *Lactobacillus plantarum* as a key lactic acid bacterium (LAB) in the intestinal microbiota of the Syrian community as it was the dominant species almost found in all samples, even in the infants samples fed only by their mother's milk. As *L. plantarum* often is the dominating *Lactobacillus* species in traditional fermented food based on plant material the intestinal isolates were likely to originate from fermented vegetables frequently consumed in Syria. In addition to tannase activity promoting their prevalence in these polyphenol rich fermentations (Smith & Mackie, 2004) we found a high resistance to acid and bile salts supporting their persistence in the gastrointestinal tract (data not shown). Other prevalent species included *L. fermentum, L. brevis, L. casei, E. faecium, E. faecalis* and *P. pentosaceus.* Except for *E. faecium* and *E. faecalis*, they were also dominant species in local fermented food. Thus, an impact of local nutrition on the diversity of the individual intestinal microbiota is likely fitting the suggestion of Ley et al. (Ley *et al.*, 2008a), (Ley *et al.*, 2008b), Penders et al. (Penders *et al.*, 2006) and Smith and Mackie (Smith & Mackie, 2004) that diet is a potent parameter forming the faecal microbiota composition. On the other hand, the host genotype and resource competition can also influence the intestinal microbiota via the availability of attachment sites and host-derived nutrients, Dethlefsen et al. (Dethlefsen *et al.*, 2006). Many studies verify a significant positive relationship between the genetic relatedness of the hosts and the similarity between their bacterial communities (Caicedo *et al.*, 2005), (Hattori & Taylor, 2009), (Zoetendal *et al.*, 2002). As identical RAPD or MALDI-TOF-MS types in food and human samples were rare and restricted to one strain each of *L. plantarum*, *L. helveticus* and *Staph. epidermidis*, this study supports the impact of host factors, which may select for specifically equipped strains present in (fermented) food. In fermented food these would be hardly detectable if they were in low numbers besides the abundant fermentative flora.

Despite our expectations, no *bifidobacteria* were found in any of the samples matching the results of Hall et al. (Hall *et al.*, 1990). This could be due to the strict anaerobicity of *bifidobacteria*, which are very sensitive to superoxide and hydrogen peroxide (Kheadr *et al.*, 2007). On the other hand, the absence of *bifidobacteria* was also observed by other groups, who found presence or absence of *bifidobacteria* in some of their samples, despite appropriate isolation conditions or the use of molecular methods. For example, no

bifidobacteria were found in 1 out of 10 subjects by using both multiplex sequencing of 16S rRNA tags and specific qRT-PCR (Martínez *et al.*, 2010). Therefore, absence of *bifidobacteria* may also be caused by host determinants.

Fermented food isolates

The dominant species *L. plantarum* frequently occurs (spontaneously) in high numbers in most lactic acid fermented foods, especially when the food is based on plant material, for example, in brined olives, makdous, and fermented vegetables. Thus, individuals consuming these products also consume a large numbers of *L. plantarum* together with *L. casei, L. fermentum, L. brevis* and pediococci, which were found in these. As a result of their daily consumption some strains become the dominant species in the gut microbiota of the consumers. The high cell count of LAB on raffinose medium (RB) as compared to other media further suggests their plant origin. In contrast, we found that the RAPD genotypes of the LAB strains isolated from Syrian traditional fermented food were similar or identical between different samples, e. g. fermented olive but did not fit with those of the intestinal strains. Thus, even if it suggests itself as a LAB source for humans, the major fermentative flora of the food consumed must not comprise the strains adapted to the human GI tract, as the latter may also originate from sub-populations present in food at low numbers and exhibiting special adaptive properties.

Breast milk

Human milk is an important factor in the initiation, development, and composition of the neonatal gut microbiota. The interesting observation of West et al. (West *et al.*, 1979) that breast milk is not sterile, even when collected aseptically, raises the possibility that breast milk harbors a natural bacterial inoculum, which influences neonatal colonization. Breast milk not only provides a range of substrates for bacterial growth (Ward *et al.*, 2006). It has been found to be a significant source of lactic acid bacteria that appear to be of endogenous origin and not contaminants from the breast skin (Martin *et al.*, 2006), (Heikkila & Saris, 2003), , (Martin *et al.*, 2003), (Martin *et al.*, 2005). In previous studies the commonly found bacteria isolated from human milk included *staphylococci, streptococci, micrococci, lactobacilli, peptostreptococci, enterococci* and *bifidobacteria* with occasional *Escherichia* spp. (Martin *et al.*, 2009b), (Martin *et al.*, 2004), (Perez *et al.*, 2007). The breast milk collected in this study contained strains of *L. brevis, L. oris, L. animalis, E. durans, E. hirae, E. mundtii*, *Str. gallolyticus, Str. vestibularis, Str. australis and Staph. haemolyticus* and also pediococci, which were found in such samples for the first time. For a long time it has been considered that bacteria in breast milk were acquired by skin or faecal contamination. Obviously, sampling of milk for microbiological analysis must take into account that skin contamination is

almost unavoidable and that doubts about the original location (internal mammary gland (MG), skin) of the isolated bacteria may arise. However, it has been reported that lactobacilli present in human milk are genotypically different from those isolated from the skin within the same host (Martin *et al.*, 2009a), (Martin *et al.*, 2003). Therefore, it has been hypothesized that the origin of the live bacteria found in milk could be the maternal gut and the bacteria would arrive to the mammary gland (MG) through an endogenous route (the so-called entero-mammary pathway), involving maternal transporter cells (Man *et al.*, 2008), dendritic cells and macrophages (Martin *et al.*, 2004), (Perez *et al.*, 2007). The result from Mother/baby-pairs group's *L. plantarum* strains comparison by RAPD-PCR and MALDI-TOF analyses was able to cluster groups strains together which indirectly could lead us to conclude that each group strains which were isolated from different sources (mother faeces, baby faeces and mother milk) of the same mother and her own breast feed baby are identical strain which came from the same source (the mother intestinal strain) and transported in her body to her milk which were suckled by her own baby who receive her strain in his intestinal microbiota.

In summary our data demonstrate individual host specificity, but also support the hypothesis that there is a vertical transfer of intestinal LAB from the mother's gut to her milk and through the milk to the infant´s gut.

Discriminatory power of RAPD versus MALDI typing

To check for a more detailed typing technique as a prerequisite for studies on microbial diversity and transfer theories, a total of 280 isolates displaying different RAPD patterns or originating from different sources were further characterized by MALDI-TOF-MS analyses. Species clustering by MALDI-TOF-MS as compared to RAPD-PCR showed better clustering of the same species at the approximate cutoff level of 20%. However the species patterns were clustered in different unmatched ways by MALDI-TOF-MS and RAPD-PCR as the discrimination principles are different between the two techniques (analysing the whole cell proteins contents by MALDI versus genomic DNA by RAPD). These different principles gave different identification results in some cases like the *E. faecalis* TMW 1.1773, which was identified as *E. faecium* by MALDI-TOF-MS and has a matching pattern, but the cluster analysis could not delineate it as belonging to the *E. faecium* cluster. However, in contrast to RAPD typing with MALDI-TOF differentiation at the strain level was possible. As the pattern may be affected by the growth phase, medium composition and extracting protocol the experimental conditions should be restrictively controlled for all samples preparations. Upon evolution of the software, which currently is focussed on species differentiation MALDI-TOF-MS suggests itself as powerful strain typing technique, which may contribute to characterize breast milk microbiota and their putative intestinal origin.

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CHAPTER II

Determination of the phylobiome of faecal samples of adults, children and babies

Introduction

The human gastrointestinal tract is a habitat of diverse community of microorganisms which include a large number of mainly anaerobic bacteria. These have broadly been studied by plate count analysis of human faecal samples, which usually contain 10^{10} to 10^{11} CFU per g (Holdeman *et al.*, 1976), (Finegold *et al.*, 1983). One of the limitations in using conventional microbiological methods is that only aerotolerant cultivable microorganisms are counted. Bacteria which have obligate symbiotic with the host or other microbiota, or which require unknown specific growth conditions, will not be cultivated by this way. Assessment of culture ability of the gastrointestinal tract bacteria vary from 10 to 50% of the microbiota community (Langendijk *et al.*, 1995), (Wilson & Blitchington, 1996). Other limitations of cultivation include the specified and selectivity of the used medium, the stress mandatory by cultivation procedures, and the requirement of strictly anaerobic conditions. As an outcome, insight into the interaction between the host and the microbial community, and into the influence of environmental factors on microbial composition, is still lacking.

This decade has shown an explosive development in the application of molecular techniques based on 16S and 23S rRNA to the study of microbial diversity in different ecosystems (reviewed in references (Amann *et al.*, 1995) and (von Wintzingerode *et al.*, 1997)). So far, the rRNA approach has been used only incidentally to study human intestinal microbial ecology, and only specific groups of bacteria, such as *Bifidobacterium* and *Lactobacillus*, have been studied (Langendijk *et al.*, 1995), (McCartney *et al.*, 1996). In addition, PCR has been used to quantify specific groups of bacteria in human faeces (Wang *et al.*, 1996), (Zoetendal *et al.*, 1998b).

One initial observation was that the human gut microbiota belongs mainly to only two phyla – Firmicutes (mostly represented by *Clostridia*) and Bacteroidetes, with a smaller representation of bacteria belonging to Proteobacteria and Actinobacteria (Eckburg *et al.*, 2005), (Korecka & Arulampalam, 2012).

In this study we used RDP classifier as additional comparative method for detection general intestinal microbiota of the Syrian people. We did this study for faecal samples from Adults, children and babies.

Materials and methods

Collection of faecal samples and analysis by selective culture

Fecal samples were collected from 9 healthy Syrian Adults never been treated with antibiotics during the last two years (A1, A2, A3, A4, A5, A6, A8, A9 and A10), 5 healthy babies never been treated with antibiotics (B1, B2, B3, B4 and B5) and from 12 year age healthy child (C1). Fresh fecal samples were processed within an hour of defecation. A ten-fold dilution of each sample in sterile phosphate buffered saline (PBS) (pH 7.0) was immediately frozen at −80°C for later DNA extraction.

DNA extraction from fecal samples

Fecal homogenates (180-220mg) were thawed and transferred to sterile bead beating tubes (Biospec products, Bartlesville, OK, USA) containing 300 mg of zirconium beads. Cells were recovered by centrifugation (8,000×g for 5 min at room temperature) and suspended in ice-cold PBS to wash the cells. This step was repeated twice before cell pellets were suspended in 100 µl of lysis buffer (200 mM NaCl, 100 mM Tris, 20 mM EDTA, 20 mg/ml Lysozyme, pH 8.0) containing 20 mg/ml of Lysozyme (Sigma-Aldrich) and incubated at 37°C for half an hour. Buffer ASL (1.6 ml) from QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) was added to each sample, after which the samples were homogenized in a MiniBeadbeater-8 (BioSpec Products, OK, USA) for 2 min at maximum speed. 1.2 ml of supernatant was used to purify DNA with the QIAamp DNA Stool Mini Kit following the manufacturer's instructions.

Pyrosequencing of 16S rRNA tags

The V1-V3 region of the 16S rRNA gene was amplified by PCR from fecal DNA. The 16S primers were modified to work with the Roche-454 Titanium adapter sequences and contain the A or B Titanium sequencing adapter (shown in italics), followed immediately by a unique 8-base barcode sequence (BBBBBBBB) and finally the 5′ end of primer. A mixture (4:1) of the primers B-8FM (5′- *CCTATCCCCTGTGTGCCTTGGCAGTCTCAG*AGAGTTTGATCMTG GCTCAG—3′) and B-8FMBifido (5′-*CCTATCCCCTGTGTGCCTTGGCAGTCTCAGAGGGTT CGATTCTGGCTCAG—3′*) were used as the forward primer during PCR. As the reverse primer, the primer A-518R (5′- *CCATCTCATCCCTGCGTGTCTCCGACTCAG*BBBBBBBBAT TACCGCGGCTGCTGG —3′) was used. Individual samples were amplified with primers containing unique barcodes, which allowed the mixing of PCR products from multiple samples in a single run, followed by bioinformatic assignation of the sequences to their respective samples via the barcode. Primer 8FMBifido was used in combination with primer 8FM to detect bifidobacteria, as species within this genus do not match the latter primer (Martinez *et al.*, 2009). The PCR mixture contained 1 µl of forward primer mix, 1 µl of reverse primer, 0.25 µl of Ex-Taq polymerase (TaKaRa Bio, USA), 1.5 µl of the sample, 6.25 µl of Ex-

Taq buffer, 5 µl of deoxynucleotides and 37 µl of sterile dH_2O were used for the reaction. The PCR program consisted of an initial denaturing step for 5 min at 95°C, followed by 30 cycles of denaturation at 95°C for 45 sec, annealing at 57°C for 45 sec and extension at 72°C for 2 min, with a final step at 72°C for 10 min. The PCR products were quantified based on their staining intensity using the image acquisition software Genesnap (Syngene USA). PCR products were combined in equal amounts and gel purified using the QIAquick Gel Extraction Kit (Qiagen, USA).

Pyrosequencing was performed by the Core for Applied Genomics and Ecology (CAGE, University of Nebraska) from the A end with the 454/Roche A sequencing primer kit using a Roche Genome Sequencer GS-FLX following manufacturer's protocol for the Roche 454 GS FLX Titanium. Sequences were binned according to barcode using the 'Initial Process' tool of the Ribosomal Database Project (RDP) Pyrosequencing Pipeline (http://pyro.cme.msu.edu/) (Cole *et al.*, 2009) with default parameters (which included the removal of sequences containing at least one ambiguous nucleotide), except for the minimum sequence length, which was set to 300 bp. The quality approved sequences were trimmed to 495 bp before their submission to the sequence analyses.

The sequences were used for the Classifier have undergone the following quality checks (using QIIME):

(1) Length: 300<x<550 nt, (2) No ambiguous nucleotides (N=0), (3) No mismatches to primers, (4) No homopolymers > 6 nt, (5) Average quality score > 25, (6) No mismatches to barcodes and (7) Chimeras were removed.

Sequence analyses to characterize microbial populations

Two independent approaches were used to analyze the sequences obtained with pyrosequencing. First, the Classifier tool (with a minimum bootstrap value of 80%) of the RDP was applied to obtain a taxonomic assignment of all sequences. This approach allowed a fast determination of the proportions of bacterial groups at different taxonomic levels (phylum, family, genus). Second, sequences were assigned to Operational Taxonomic Units (OTUs) that were quantified in individual subjects. As the entire data from the ten subjects contained too many sequences for a quality alignment, sequences were aligned by subject using the Aligner web tool of the RDP, and then clustered using the Complete Linkage Clustering tool (with a maximum distance cutoff of 97%). OTUs that contained less than three sequences were excluded from the analyses. ANOVA was used to identify OTUs that were significantly affected by the dietary treatments in each of the ten subjects. These OTUs were subjected to a taxonomic classification and grouped according to phylum (Firmicutes, Bacteroidetes, and Actinobacteria). Within these phyla, five random sequences of each OTU identified above were aligned with the most closely related type strains and entry in the NCBI

database using Muscle 3.6 (Edgar, 2004). Phylogenetic trees were built with MEGA 4.0 Software (Tamura *et al.*, 2007) by neighbor-joining with 1,000 bootstrap replicates. These trees allowed us to visually assign OTUs as sequence clusters which, in most cases, encompassed sequences from several subjects, and consensus sequences were generated for each OTU. A local nucleotide database was established for each subject in BioEdit (Hall, 1999) containing all sequences detected by pyrosequencing, and the BLASTn algorithm was used with a 97% cutoff (min. length 300 bp) to quantify each OTU in the faecal bacterial populations in each sample. We verified that this approach did not result individual sequences being assigned to different OTUs. In two occasions, two OTUs that were initially identified as distinct had very high sequence similarities, and were thus combined.

Diversity of the fecal microbiota was determined using 16S rRNA sequence data with two different methods, Shannon's index and the generation of rarefraction curves. The DNA sequences of each sample were individually aligned and clustered using Aligner and Complete Linkage Cluster tools of the RDP. Individual cluster files corresponding to each fecal sample were used to determine the Shannon's Index and construct Rarefraction curves (Martínez *et al.*, 2010).

Results

16S rDNA amplicon sequencing revealed differences between adults and baby microbiota diversity:

Pyrosequencing of 16S rDNA amplicons from 15 fecal samples resulted in an average of 86.284 sequences per sample after quality control (1,344,601 sequences in total) with a mean sequence length of approximately 490 bp. The average number of operational taxonomic units (OTUs) identified per subject was (1.081).

The bacterial composition in the fifteen subjects during the baseline period was, dominated by the phyla Bacteroidetes (62%) and Firmicutes (28%). Other phyla detected were Proteobacteria (8%) and Actinobacteria (1%), and 1% of the sequences remained unclassified (Figure 1). Among the well characterized culturable genera were *Prevotella* (36.2%), *Bacteroides* (19.3%), *Veillonella* (6.3%), *Lachnospiraceae* (6.3%), *Succinivibrio* (3.9%), and *Dialister* (3.1%). Although every individual has his own special types of intestinal microbiota, there was much common similarity between babies' bacterial composition which can be differentiated from the common compositions found in adults. In adults the phyla Bacteroidetes (74%) was the dominant phyla, followed by the Firmicutes (19%) and Proteobacteria (7%) (Fig 2). While in babies the phyla Bacteroidetes (44%) and Firmicutes (42%) are both dominant followed by Proteobacteria (11%) and at last Actinobacteria (3%) which were absent in adults (Fig 3). The Lactobacillus genera of lactic acid bacteria which have been the dominant facultative aerobe isolated from different nine specific culture media represented a very small percentage (0.13%) of the complete human intestinal microbiom which include mostly different strict anaerobes that are difficult to cultivate and to study outside their host.

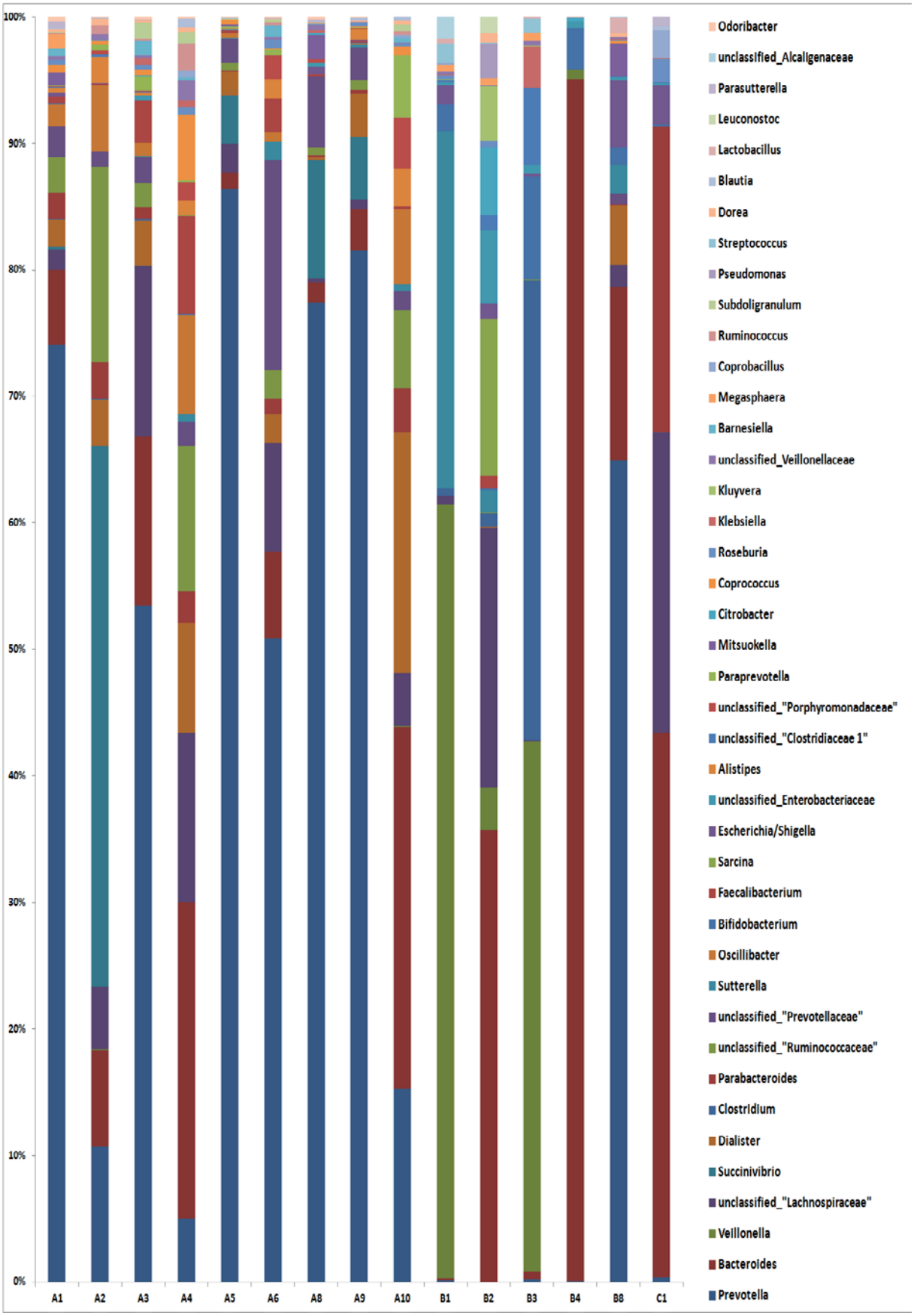

Figure1. Diversity and genus richness of the faecal microbiota in 15 Syrian human subjects, (An) refers to adult sample, (Bn) refers to baby sample, (Cn) refers to child sample and (n) refer to the sample number.

Phylum	Genus	A1	A2	A3	A4	A5	A6	A8	A9	A10	sum	%
Bacteroidetes	Prevotella	68,225	8,825	48,506	3,101	82,484	42,357	76,184	79,436	13,799	422,917	55.95
Bacteroidetes	Bacteroides	5,467	6,208	12,205	15,391	1,207	5,688	1,564	3,236	25,924	76,890	10.17
Firmicutes	Veillonella	0,000	0,043	0,000	0,000	0,000	0,000	0,022	0,000	0,058	0	0.00
Firmicutes	unclassified_"Lachnospiraceae"	1,470	4,110	12,158	8,203	2,146	7,147	0,308	0,670	3,811	39,045	5.17
Proteobacteria	Succinivibrio	0,206	35,172	0,016	0,000	3,675	0,000	9,253	4,862	0,000	52,962	7.01
Firmicutes	Dialister	2,028	3,007	3,239	5,391	1,770	1,867	0,242	3,316	17,206	37,824	5.00
Firmicutes	Clostridium	0,059	0,043	0,141	0,000	0,000	0,000	0,000	0,000	0,000	0	0.00
Bacteroidetes	Parabacteroides	1,881	2,379	0,861	1,536	0,080	1,021	0,132	0,255	3,176	9,993	1.32
Firmicutes	unclassified_"Ruminococcaceae"	2,587	12,719	1,706	7,072	0,563	1,838	0,595	0,829	5,543	31,465	4.16
Bacteroidetes	unclassified_"Prevotellaceae"	2,263	1,017	1,878	1,159	1,824	13,856	5,486	2,503	1,386	31,372	4.15
Proteobacteria	Sutterella	0,000	0,000	0,078	0,377	0,107	1,225	0,000	0,191	0,462	1,225	0.16
Firmicutes	Oscillibacter	1,587	4,261	0,908	4,812	0,322	0,583	0,022	0,128	5,427	16,087	2.13
Actinobacteria	Bifidobacterium	0,059	0,000	0,000	0,029	0,000	0,000	0,000	0,016	0,000	0	0.00
Firmicutes	Faecalibacterium	0,470	0,108	3,020	4,783	0,241	2,217	0,176	0,159	0,173	10,020	1.33
Firmicutes	Sarcina	0,000	0,000	0,000	0,029	0,000	0,000	0,000	0,000	0,000	0	0.00
Proteobacteria	Escherichia/Shigella	0,265	0,043	0,078	0,000	0,000	0,000	0,551	0,032	0,000	0	0.00
Proteobacteria	unclassified_Enterobacteriaceae	0,000	0,000	0,329	0,000	0,054	0,029	0,330	0,000	0,000	0	0.00
Bacteroidetes	Alistipes	0,353	1,709	0,219	0,725	0,080	1,254	0,000	0,845	2,656	5,619	0.74
Firmicutes	unclassified_"Clostridiaceae 1"	0,059	0,151	0,063	0,000	0,000	0,000	0,022	0,000	0,000	0	0.00
Bacteroidetes	unclass"Porphyromonadaceae"	0,088	0,260	0,016	0,928	0,000	1,546	0,220	0,128	3,695	5,241	0.69
Bacteroidetes	Paraprevotella	0,059	0,368	1,064	0,058	0,134	0,379	0,022	0,064	4,446	5,510	0.73
Firmicutes	Mitsuokella	0,970	0,000	0,000	0,000	0,161	0,029	1,917	0,000	0,000	1,917	0.25
Proteobacteria	Citrobacter	0,000	0,000	0,063	0,000	0,000	0,000	0,110	0,000	0,000	0	0.00
Firmicutes	Coprococcus	0,500	0,238	0,376	3,188	0,322	0,117	0,000	0,016	0,635	3,188	0.42
Firmicutes	Roseburia	0,353	0,065	0,360	0,377	0,027	0,496	0,066	0,287	0,231	0	0.00
Proteobacteria	Klebsiella	0,029	0,000	0,548	0,348	0,000	0,029	0,198	0,000	0,000	0	0.00
Proteobacteria	Kluyvera	0,000	0,000	0,016	0,000	0,000	0,000	0,000	0,000	0,000	0	0.00
Firmicutes	unclassified_Veillonellaceae	0,265	0,368	0,156	0,957	0,000	0,204	0,441	0,016	0,058	0	0.00
Bacteroidetes	Barnesiella	0,529	0,000	1,017	0,145	0,000	0,729	0,022	0,048	0,346	1,017	0.13
Firmicutes	Megasphaera	1,088	0,000	0,000	0,000	0,000	0,000	0,154	0,000	0,000	1,088	0.14
Firmicutes	Coprobacillus	0,000	0,022	0,031	0,319	0,000	0,029	0,000	0,000	0,173	0	0.00
Firmicutes	Ruminococcus	0,088	0,541	0,125	1,362	0,000	0,204	0,000	0,032	0,289	1,362	0.18
Firmicutes	Subdoligranulum	0,088	0,043	1,174	0,551	0,027	0,175	0,000	0,032	0,462	1,174	0.16
Proteobacteria	Pseudomonas	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0	0.00
Firmicutes	Streptococcus	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0	0.00
Firmicutes	Dorea	0,176	0,411	0,141	0,232	0,000	0,029	0,000	0,048	0,289	0	0.00
Firmicutes	Blautia	0,147	0,065	0,031	0,377	0,134	0,058	0,088	0,159	0,289	0	0.00
Firmicutes	Lactobacillus	0,029	0,110	0,016	0,029	0,054	0,240	0,019	0,026	0,020	0	0.00
Firmicutes	Leuconostoc	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0	0.00
Proteobacteria	Parasutterella	0,412	0,022	0,000	0,000	0,027	0,000	0,088	0,000	0,000	0	0.00
Proteobacteria	unclassified_Alcaligenaceae	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0	0.00
Bacteroidetes	Odoribacter	0,323	0,043	0,235	0,087	0,027	0,088	0,242	0,128	0,000	0	0.00

Figure 2. Collective faecal microbial composition including the major taxonomic groups at the phylum and genus levels averaged for 9 human subjects (9 adults), (An) refers to adult sample, and (n) refer to the sample number.

Phylum	Genus	B1	B2	B3	B4	B5	C1	sum	%
Bacteroidetes	Prevotella	0,077	0,000	0,186	0,082	63,335	0,310	63335	11.76
Bacteroidetes	Bacteroides	0,115	34,035	0,604	94,203	13,367	41,495	183100	34.01
Firmicutes	Veillonella	40,785	3,170	40,966	0,750	0,016	0,000	84921	15.77
Firmicutes	unclassified_"Lachnospiraceae"	0,423	19,549	0,093	0,000	1,718	22,906	44173	8.21
Proteobacteria	Succinivibrio	0,000	0,000	0,000	0,000	0,000	0,000	0	0.00
Firmicutes	Dialister	0,000	0,064	0,000	0,000	4,544	0,000	4544	0.84
Firmicutes	Clostridium	0,423	1,064	35,578	0,010	0,000	0,085	36642	6.81
Bacteroidetes	Parabacteroides	0,000	0,021	0,000	0,000	0,031	23,272	23272	4.32
Firmicutes	unclassified_"Ruminococcaceae"	0,000	0,043	0,046	0,000	0,000	0,000	0	0.00
Bacteroidetes	unclassified_"Prevotellaceae"	0,000	0,000	0,000	0,010	0,890	0,000	0	0.00
Proteobacteria	Sutterella	18,853	1,638	0,000	0,000	2,217	0,000	22708	4.22
Firmicutes	Oscillibacter	0,000	0,000	0,000	0,000	0,000	0,000	0	0.00
Actinobacteria	Bifidobacterium	1,385	0,191	7,989	3,217	1,343	0,197	13934	2.59
Firmicutes	Faecalibacterium	0,000	0,936	0,000	0,010	0,047	0,000	0	0.00
Firmicutes	Sarcina	0,000	11,763	0,000	0,000	0,000	0,000	11763	2.19
Proteobacteria	Escherichia/Shigella	1,000	1,149	0,186	0,051	5,106	2,962	10217	1.90
Proteobacteria	unclassified_Enterobacteriaceae	0,154	5,573	0,650	0,473	0,250	0,169	5573	1.04
Bacteroidetes	Alistipes	0,000	0,000	0,000	0,000	0,000	0,000	0	0.00
Firmicutes	unclassified_"Clostridiaceae 1"	0,154	1,127	5,945	0,000	0,031	0,056	7072	1.31
Bacteroidetes	unclass"Porphyromonadaceae"	0,000	0,000	0,000	0,010	0,000	0,028	0	0.00
Bacteroidetes	Paraprevotella	0,038	0,000	0,000	0,000	0,000	0,000	0	0.00
Firmicutes	Mitsuokella	0,000	0,000	0,000	0,000	2,592	0,000	2592	0.48
Proteobacteria	Citrobacter	0,000	5,105	0,000	0,319	0,031	0,000	5105	0.93
Firmicutes	Coprococcus	0,000	0,021	0,000	0,000	0,156	0,000	0	0.00
Firmicutes	Roseburia	0,154	0,468	0,000	0,000	0,078	1,749	1749	0.32
Proteobacteria	Klebsiella	0,038	0,021	3,251	0,041	0,047	0,056	3251	0.60
Proteobacteria	Kluyvera	0,000	4,063	0,046	0,000	0,031	0,000	4063	0.74
Firmicutes	unclassified_Veillonellaceae	0,192	0,085	0,372	0,000	0,172	0,000	0	0.00
Bacteroidetes	Barnesiella	0,000	0,000	0,000	0,000	0,000	0,056	0	0.00
Firmicutes	Megasphaera	0,346	0,574	0,650	0,000	0,000	0,000	0	0.00
Firmicutes	Coprobacillus	0,115	0,000	0,000	0,000	0,016	2,059	2059	0.38
Firmicutes	Ruminococcus	0,000	0,000	0,000	0,000	0,000	0,000	0	0.00
Firmicutes	Subdoligranulum	0,000	0,000	0,000	0,000	0,000	0,000	0	0.00
Proteobacteria	Pseudomonas	0,000	2,531	0,000	0,000	0,000	0,000	2531	0.47
Firmicutes	Streptococcus	1,000	0,149	1,068	0,000	0,000	0,028	2068	0.38
Firmicutes	Dorea	0,000	0,638	0,000	0,000	0,203	0,000	0	0.00
Firmicutes	Blautia	0,038	0,000	0,000	0,000	0,016	0,310	0	0.00
Firmicutes	Lactobacillus	0,231	0,000	0,139	0,010	1,249	0,021	1249	0.28
Firmicutes	Leuconostoc	0,000	1,276	0,000	0,000	0,000	0,000	1276	0.22
Proteobacteria	Parasutterella	0,000	0,000	0,000	0,000	0,000	0,705	0	0.00
Proteobacteria	unclassified_Alcaligenaceae	1,154	0,000	0,000	0,000	0,047	0,000	1154	0.21
Bacteroidetes	Odoribacter	0,000	0,000	0,000	0,000	0,000	0,000	0	0.00

Figure 3. Collective faecal microbial composition including the major taxonomic groups at the phylum and genus levels averaged for 6 human subjects (5 babies and 1 child), (Bn) refers to baby sample, (Cn) refers to child sample and (n) refer to the sample number.

Conclusions

The human intestine harbours a great comunity of microbes (microbiota) that provide important metabolic capabilities, including the ability to extract energy from otherwise indigestible dietary polysaccharides. Studies of some unrelated healthy humans have discovered abundant diversity in their intestinal communities, as measured by sequencing 16S rRNA genes. But, how this diversity relates to function and to the rest of the genes in the collective genomes of the microbiota (the gut microbiome) remains ambiguous. The initial infant intestinal microbiota is a simple structure, and through a series of progression and replacements, it evolves to a more complex adult pattern (Palmer *et al.*, 2007). The adult intestinal microbiota has been shown to be comparatively stable over time (Zoetendal *et al.*, 1998a) and is comparable between individuals (Claesson *et al.*, 2011). From our results the dominant phyla in Syrian people are Bacteroidetes (62%), Gram negative, nonsporeforming, anaerobic bacteria (Garrity, 2010). Bacteroides are prominent starch degraders and many strains are also capable of degrading some types of structural polysaccharides. The ability of *Bacteroides* spp. to import oligosaccharides into their periplasmic space for further hydrolysis (thus monopolizing the hydrolysis products) might contribute to their abundance (Dethlefsen *et al.*, 2006). While Firmicutes (28%) scientists once classified it to include all Gram-positive bacteria, but have recently defined them include both Gram-positive and Gram-negative and to be of a core group of related forms called the low-G+C group, in contrast to the Actinobacteria. They can be found in various environments, and the group includes some notable pathogens (Ludwig & Klenk, 2000). Species of Firmicutes given the genus names *Peptostreptococcus* and *Clostridium* are the predominant proteolytic and amino acid-fermenting organisms in the gut; some also ferment sugars (Cummings & Macfarlane, 1991). The other phyla detected were Proteobacteria (8%). Our results are partly in agreement with the data of Claesson et al, who found in a similar study on adults microbiota (n=161) that Firmicutes accounted for 8% to 80%, and Bacteroidetes varied from 14% to 92%. The phylum Actinobacteria includes *Bifidobacterium* spp., which are among the best characterized of the mutualistic organisms with beneficial or probiotic properties (Ventura *et al.*, 2009). In the latter study this phylum ranged from 0% to 8% in the total dataset (Claesson *et al.*, 2011). Zwielehner and coworkers reported that the proportion of Bacteroidetes in the fecal microbiota increase with the age (Zwielehner *et al.*, 2009) as our result showed obvious differences in the microbiota composition between infants and adults, Infants microbiota predominantly almost equally by both Firmicutes and Bacteroidetes (Marchesi, 2011). However, comparing the predominance and diversity of intestinal microbiota population in samples collected from Middle East people with Americans people revealed some differences (Martínez *et al.*, 2010), (Turnbaugh *et al.*, 2009). As the phyla Firmicutes (78%) is

much dominant over Bacteroidetes (13%) in western individual. Jeffrey Gordon and his colleagues found that obese humans and mice harbour intestinal microbiota with a lower percentage of Bacteroidetes and relatively more Firmicutes (Ley *et al.*, 2005). However, they are unsure if Bacteroidetes prevent obesity or if these intestinal microbiota are merely preferentially selected by intestinal conditions in those who are not obese (Ley *et al.*, 2006),(Turnbaugh *et al.*, 2006). Whereas it is taxonomically well established that the microbiota is mainly composed of members of the Bacteroidetes and Firmicutes, scientists are still struggling to determine the key functions that are important to the microbes and the host (Marchesi, 2011). Diet appears to be of the most important determinants shaping the pattern of intestinal bacteria (Dethlefsen *et al.*, 2006), (Ley *et al.*, 2008). Middle East people have their special general form of common diets in that area, mainly combining wheat bulgur with seasonal vegetables and some lamb meats tend to be staples in many of the main courses (fermented olives, olive oils, dates, black tea and no alcoholic beverages seems to be the most consumption habits shaping the people intestinal microbiota of that area).Thus, the main food consumption depends on plants and starch material that could explain the general predominance of Bacteroidetes (starch and polysaccharides degraders) among this people. These indicate the impact of age and diet ingredients on the general individuals' microbiota diversity in common community. Consequently the special genera deletion in adulthood (e.g. *Bifidobacteria*) could be due to the changes or additions in diet habits. For example, the influence of the high consumption of tea polyphenols among adults.

Trying to understand intestinal bacteria interaction, mutualism, dominancy and diversity which shape intestinal microbiota community, different properties and polyphenols influence were investigated for different bacteria species isolated from different people in the coming chapters.

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Chapter III

Comparative analysis of bacterial isolates along their physiology, biochemical capabilities and stress tolerance

Introduction

Intestinal Lactic acid bacteria

In the intestinal indigenous microbiota two bacterial species profiles can be distinct, first the autochthonous species (e.g. species, which are able to colonize the mucosal surface of the gastrointestinal tract due to special adhesion factors including compatibility with the immunological system of the host). These kind of bacteria should be differentiate from allochthonous species, which may be existing in the intestinal tract also, but which may only have a transient function. The existence of these bacteria in the intestinal tract will last for a limited passing time, likely only a few days (Tannock, 1999).

Lactic acid bacteria especially the genus Lactobacillus (the largest group of LAB containing 106 different species), contains the both types of indigenous microbiota, the autochthonous and allochthonous. Considering that they are almost ubiquitous bacteria: they can be found in different environments where carbohydrates are available, such as fermented food (dairy products, fermented meat, sour doughs, vegetables, fruits, beverages), GI and genital tracts of humans and animals, and in sewage and plant material (Holzapfel *et al.*, 2001), . The largest group of lactic acid bacteria belong to the genus of Lactobacillus which includes 106 different species (Felis & Dellaglio, 2007). As they exist in different fermented foods which will be transferred day by day through the stomach and small intestine into the colon and his supplementation depends on individual consumption habits (Reuter, 2001). Some of this species can't resist the low pH, bile salts and will not survive stomach and duodenal passage easily, while other species will resist very well and have ability to adhere and colonise the mucosal epithelium and became a part of the indigenous microbiota like LAB In many cases, these the fermentative conversion of sugars present in the raw materials into lactic acid is their main function, production of antimicrobial peptides, exopolysaccharides and a variety of other secondary metabolites are important properties in their competition and mutualism with other intestinal microbiota to persist in the gut (Ross *et al.*, 2002), (Tamime, 2002). Depending on this ability different species including *L. plantarum, L. fermentum, L. gasseri,* and *L. brevis* is mentioned as an autochthonous Lactobacillus species in the intestine (Ahrne *et al.*, 1998), (Cataloluk & Gogebakan, 2004). Lactobacillus species can be found in the intestinal tract of humans and other animals, while they vary depending on the species, the age of the host, and the location within the intestine. The human gastrointestinal tract contains food in different stages of digestion, digestive ferments, liquids and nondigestable dietary residues. Within the intestinal tract are also wide diversity of microbiota that may be either harmful or beneficial (Savadogo *et al.*, 2006). Our relationship with our microbiota is often described as "commensal" which mean that one partner get benefit from the other while the other is uninfluenced, this is not the real case, actually it can be described as "mutualistic" as both partner change benefits between each other (Bäckhed *et al.*, 2005). The

beneficial microbiota contribute in the digestion of food while they synthesize enzymes and also vitamins essential to the body, breaking down and extinguish some toxic compounds, which might have been ingested with the food or result from food bad fermentation. Under different body conditions, healthy and sick, in the intestinal microbiota community different species of bacteria fight or compete with each other to establish dominance in the moist and warm environment of the intestinal tract that serves as special ecosystem for their survival, propagation and mutualism. According to the definition of the World Health Organization, "probiotics are live microorganisms, which when administered in adequate amounts confer a health benefit on the host" (Gilliland *et al.*, 2001). Postulated properties for a probiotic LAB include human origin, survival of the gut passage (resistance to acid and bile), persistence in the host, production of antimicrobial substances and beneficial metabolites which influence metabolic activities such as vitamin production and proven safety for human consumption (Charteris *et al.*, 1998), (Tuomola *et al.*, 2001), (Salminen *et al.*, 1996), (Deshpande *et al.*, 2011). To understand the interaction, mutualism, roles whish shapes the gut inhabitants and determines the possibility for LAB species to coexist and dominance in this complex diverse ecosystem, we need close insight on different physiological and biochemical properties of human mutualistic microbiota. Taking in consideration also, the host selection ability for specific bacteria whose emergent collective behaviour is beneficial to the host (Bäckhed *et al.*, 2005). Moreover, to understand the previous interesting finding of the different "strains of mother/baby-pairs groups", which was found in the different groups and presented identical RAPD genotype as trio from the three sources (the mother faeces, her baby faeces and her own milk) of each group, we need to focus on the special properties of these strains comparing to normal adults strains and explore the possibility to be identical strains from one source in each group depending on the shared different physiological and biochemical properties between them.

Lactobacillus plantarum

L. plantarum is a versatile lactic acid bacterium, that is coexisting in varied environmental niches including dairy, meat and many vegetable fermentations. Moreover, it is commonly found in the human gastro-intestinal-tract (GIT). Furthermore, *L. plantarum* can be isolated from human milk, in our previous work *L. plantarum* species was the predominant milk isolated species (Albesharat *et al.*, 2011). Considering the special major role which *L. plantarum* plays in neonate gut colonization and shaping through mother milk metabolism abilities. Although different new infant formula milks are made to mimic human breast milk, they remain different from the natural biological fluid (Schwab & Gänzle, 2011). As a result, differences in the gut microbiota between breast-fed and formula-fed infants have been frequently reported (Conway, 1997), (Stark & Lee, 1982), (Harmsen *et al.*, 2000), (Claud & Walker, 2001), (Caicedo *et al.*, 2005) , (Penders *et al.*, 2006), (Mshvildadze *et al.*, 2010).

"Human milk contains 1% of human milk oligosaccharides (HMOs) consisting of lactose with linked fucose, N-acetylglucosamine and sialic acid" (Gosling *et al.*, 2010). HMOs can activate growth of intestinal *Lactobacillus plantarum* and bifidobacteria, inhibiting the adhesion of infectious bacterial pathogens and provide prospective immunomodulatory properties (Kunz *et al.*, 2000; Schwab & Gänzle, 2011), (Claud & Walker, 2001). While in infant formula milks, galactooligosaccharides (GOSs) are added instead of HMOs. The different LAB which were isolated from the faeces of neonates were not able to digest complex HMOs but metabolized HMO simple components. Therefore general LAB depends on the presence of other gut microorganisms which is capable of dispersing simple monosaccharide components from the complex HMOs. *L. plantarum* is one of the LAB species, which is highly versatile in hydrolyzing HMOs (Schwab & Gänzle, 2011). Therefore *L. plantarum* play a major role in providing simple nutrient sources which might contribute to the selective persistence of the other LAB in the gut of infants fed with breast milk. It is not surprising that *L. plantarum* was the first *Lactobacillus* species isolated from breast milk (West *et al.*, 1979) while the occurrence of other LAB seems not to have been explored until 2003 (Heikkila & Saris, 2003). To understand the real role of *L. plantarum* species in milk microbiota and to explain its dominance and diversity in all different isolation sources in this study we need to focus investigation on its properties and compare it with other coexistence species.

LAB influence by different stress conditions

There are different factors which shape the microbial community structure in the human intestine, that can be gathered under two main groups; the host factors include, secretory components (gastric acid and bile salts), peristalsis and food transit time and host genotype, and the environmental factors include, microbial ingestion, dietary components, oxidative metabolites, antagonistic metabolites, antibiotics and drugs, food structure and viscosity (Egert *et al.*, 2006).

Survival in the human GI-tract

After oral ingestion, bacteria experience a number of human defence systems that are linked with secretions. These include high concentrations of mucins that cover the inner surface of the intestine, gastric acid inducing a low pH in the stomach, and bile salts secreted into the luminal content in the proximal small intestine (Ouwehand *et al.*, 2005). Bile salts are produced from the liver by conjugation of a heterocyclic steroid derived from cholesterol (Hofmann & Mysels, 1992). The resulting conjugated bile salts are stored and concentrated in the gall bladder during the fasting state, and after ingestion of a food containing fats the bile salts are released from gall bladder into the duodenum, where they play a fundamental role in the emulsifying and absorption of fats, including bacterial phospholipids and cell membranes (Tannock *et al.*, 1994). A deconjugation process occurs to the bile salts due to

the intestinal microbiota followed by reabsorption in the distal small intestine and colon to be reintroduced in the liver in process known as enterohepatic circulation
(Hofmann *et al.*, 1983). This deconjugation reaction is performed by bacterial bile salt hydrolases, this enzymes can be produced by several intestinal bacteria, including Bifidobacterium and Lactobacillus species (Tanaka *et al.*, 1999), (Dussurget *et al.*, 2002), (Kleerebezem *et al.*, 2003). However both conjugated and deconjugated bile acids exhibit antibacterial activity (Lewis & Gorbach, 1972). Though the deconjugated forms are much inhibitory (Percy-Robb & Collee, 1972). Tolerance to digestive stress have focused mainly on physiological aspects, such as determination of the levels of gastric acid and bile salt tolerance (Hyronimus *et al.*, 2000), thus, intestinal stress condition resistance ability are important criteria for LAB which could shape the intestinal microbiota (Bron *et al.*, 2004a).

Bile salt hydrolases activity (BSH)

Initially bile acids are synthesized from cholesterol and conjugated to one of the two amino acid glycine or taurine in the liver to form conjugated bile acids (Savage, 1987). They then pass into the intestine, where the amino acid may be hydrolyzed from the conjugated bile acid by bacterial enzyme function to form the secondary bile acids (deoxycholic acid and lithocholic acid). These enzymes frame a class common known as conjugated bile salt hydrolases (BSHs). They are produced by gastrointestinal bacteria of several genera, including Bacteroides, Clostridium, Enterococcus, Bifidobacterium, and Lactobacillus (Hylemon, 1985). BSHs appear to enhance the bacterial colonization of the lower gastrointestinal tract of higher mammals. It has been hypothesized that deconjugation may be a mechanism of the detoxification of bile salts (Ridlon *et al.*, 2006). Desmet et al observed significantly higher ability of deconjugation of glycodeoxycholate (GDCA) over taurodeoxycholate (TDCA) bile salts in *L. plantarum* species (Desmet *et al.*, 1995), (Dashkevicz & Feighner, 1989). Investigating the (BSHs) activity in different intestinal species contributes in explaining its ability to resist bile salt existence stress condition.

Black tea influence on the intestinal bacteria

Additional to the acidic and bile salts stress condition which intestinal microbiota have to tolerate to survive in this ecosystem, there is the special dietary component (e.g. polyphenols) which reach the intestinal ecosystem from different food sources (e.g. beverages like tea and licorice). These components in general have no acute adverse influence on the intestinal microbiota but when consuming regularly it could shape and influence the microbiota diversity in this multiple external supplied ecosystem (Dethlefsen *et al.*, 2006), (Ley *et al.*, 2008).

After water, black tea is the most commonly consumed beverage in the Middle East. Thus, while tea may have many health benefits, though it is not panacea. And this high exposing to tea polyphenols may play a cretin role in shaping intestinal microbiota of those regularly

consumers, where the highest amounts of black tea are consumed. Oregano and rosemary can delay the growth and acid production of lactic acid bacteria (Zaika & Kissinger, 1981), (Zaika *et al.*, 1983). Other authors have reported that tannins, tannic acid and related compounds had slight influence on the growth of lactic acid bacteria at high concentrations (Chung *et al.*, 1998). Existing reports are conflicting regarding the influence of tea components on some species of lactic acid bacteria.

For green tea preparation, the leaves are steamed, rolled, and dried. This avoid them oxidization by inactivating certain chemicals in the leaves. While red and black teas are partially dried, smashed, incupated under controlled temperature and humidity environment to produce fermentation, and then totally dried. To "ferment" in this situation means "oxidize," or blacken; this occur as a result of chemicals heat reaction in the leaves. Black teas are kept to be fermented longer than red teas. The tea components that make tea a potential beneficial for health are called polyphenols; these may have antioxidant as well as anti-cancer effects. It is been thought once that green tea have the most polyphenols, however it turns out that black tea has a similar amount. The polyphenols in both green and black teas have antioxidant activity, that is, they help prevent cell-damaging cased by free radicals (Letter, 2000). Polyphenol compounds differs depending on the type of tea. The main polyphenols found in fresh green tea leaves are epigallocatechin, epicatechin gallate, epicatechin and epigallocatechin gallate, also known as EGCG, these compounds, collectively referred to as catechins. Teas that are more oxidized and dimerized have higher levels of different groups of polyphenols, which includes theaflavin and thearubigin. Tea also contains flavonoid polyphenols, including quercetin (Yang *et al.*, 2009).

Dietary polyphenols are not completely absorbed from the gastrointestinal tract and are metabolized by the intestinal microbiota so that they and their metabolites may accumulate to cause physiological effects. Intestinal bacteria varies in their ability to metabolize this complex compounds, and some bacterial species contains strains can significantly catalyzed tea phenolics. The microbial metabolism of the main tea polyphenols catechin, epicatechin, epicatechin gallate and epigallocatechin gallate, results in 4-hydroxybenzoic acid and vanillic acid, which are both transformed upon absorption to hippuric acid derivatives (Feng, 2006).

The antimicrobial effects of tea polyphenols have been well documented (Betts *et al.*, 2011). Tea polyphenols not only exhibit independent antibacterial effects but also show significant antibacterial synergy with more common antibiotics against gram positive pathogen, e.g. tetracycline (Sudano Roccaro *et al.*, 2004) and oxacillin (Zhao *et al.*, 2001) and with other antioxidants such as ascorbic acid (Hatano *et al.*, 2008). Black tea mixtures show synergy and hostile properties when accompanied with different clinical antibiotics against gram positive bacteria (Neyestani *et al.*, 2007). However, different strains of intestinal bacteria showed different levels of growth sensitivity to tea phenolics and metabolites (Lee *et al.*,

2006). There are noticeably few studies investigating the influence of polyphenols on the composition and activity of the intestinal microbial community. For this we need more investigating for more understanding of the polyphenol-microbe interactions. Nevertheless, most of this previous studies explained the inhibition role and mechanism of polyphenol on bacteria (table.1).

Table 1. Proposed mechanisms of antimicrobial activity of polyphenols (Kemperman *et al.*, 2010)

Mechanism	Compound	References
Membrane interaction	Green tea polyphenols, epicatechin gallate	Kumazawa et al. (2004), Sirk et al. (2009), Stapleton et al. (2007)
DNA gyrase inhibition	Quercetin	Cushnie & Lamb (2005)
Metal sequestering	Tannins	Smith et al. (2005)
Enzyme inhibition	Epigallocatechin gallate	Navarro-Martı´nez et al. (2005)
Reactive oxygen generation	Epigallocatechin gallate	Arakawa et al. (2004)
Inhibition of virulence factors	Tea polyphenols, resveratrol	Evensen & Braun (2009), Wang et al. (2006)

(Kumazawa *et al.*, 2004), (Sirk *et al.*, 2009), (Stapleton *et al.*, 2007), (Cushnie & Lamb, 2005), (Smith *et al.*, 2005), (Navarro-Martinez *et al.*, 2005), (Arakawa *et al.*, 2004), (Evensen & Braun, 2009), (Wang *et al.*, 2006).

Licorice influence on the intestinal bacteria

Other common consuming brevelage also in Syrian society is licorice. For centuries plants extracts have been used everywhere in the world as drugs and treatments for different diseases. Licorice (or liquorice) is a plant of ancient origin and rooted in history. It grows in subtropical climates in the Middle East and Western Asia. Licorice extracts and its principle component, glycyrrhizin, have spacious use in Middle East foods, and in traditional and herbal medicine. licorice has also been shown to help inhibit the growth of potentially harmful intestinal bacteria (Li *et al.*, 1998), such as Helicobacter pylori, which is associated with ulcers (Beil *et al.*, 1995). It has a significant antibacterial activities against two gram positive (*Bacillus subtilis* and *Stapphylococcus aureus*) and two gram negative (*Escherichia coli* and *Pseudomonas aeruginosa*) bacteria (Nitalikar *et al.*, 2010). As licorice is being used widely as traditional drink in Syria, it is expected to have influence on the intestinal microbiota of the consumer.

Oxidative stress and resistance mechanisms

Another challenge the intestinal bacteria have to face in the intestinal niche is the oxidative stress. Oxidative stress and antioxidative ability have been characterized as key processes in the molecular regulation of cellular stress responses. Oxidative stress occurs when abnormally high levels of reactive oxygen species (ROS) are generated, resulting in DNA, protein, enzyme, and lipid damage (Lee *et al.*, 2005) leading to growth arrest or mortality in bacteria. Cellular-derived reactive oxygen species (ROS) are enzymatically produced by macrophages is a part of an inflammatory-immune response to face a pathogen or exacerbate (Kirkham, 2007). Aerobes and facultative anaerobes have developed an effective mechanism for protection against the reactive oxygen species damage during

aerobic respiration or during defiance with oxidative stress. Although physiological adaptation to oxidative stress is typically complex, some bacteria can produce enzymatic defenses against oxidative stress that scavenge superoxide (O_2^-) and hydrogen peroxide (H_2O_2) and hydroxyl radical ($HO^{\cdot}$). These include two hemoprotein catalases, three types of superoxide dismutases (SOD), glutathione reductase (GR) (Cha *et al.*, 1995), and bacterioferritin comigratory protein (BCP) (Jeong *et al.*, 2000). Superoxide dismutase (SOD) are a class of enzymes that catalyze the dismutation of superoxide into oxygen and hydrogen peroxide. Thus, they are an important antioxidant defense in nearly all cells exposed to oxygen. One of the closely rare exceptions is *Lactobacillus plantarum* and Lactobacillus fermentum, which use a different mechanism. Facultative anaerobes such as lactic acid bacteria are defective in their capacity to synthesize heme (an essential cofactor of cytochrome oxidases) and quinone in some LAB, and does not require them for growth, but they expressed ability to undergo a respiration chain metabolism (catalase) when heme (*Enterococcus faecalis* and *Lactococcus lactis*) or heme and menaquinone (*Lactobacillus plantarum* and *Streptococcus agalactiae*) is available in the surrounding environment (Cesselin *et al.*, 2011) like the different food (meat and vitamin K) component in the intestinal tract. Moreover, lactic acid bacteria show a higher metabolic potential when the reduced cofactors (NADH is the most important), are regenerated by exogenous electron acceptors, and the easiest way to oxidize NADH is by the reduction of molecular oxygen by the activity of NADH oxidase (Higuchi *et al.*, 2000). Actually most aerotolerant strains in many lactic acid bacteria have developed protective mechanisms that employ antioxidant enzymes, such as superoxide dismutases and hydroperoxidases (e.g. catalases and peroxidases), which scavenge superoxide radicals and hydrogen peroxide, respectively, and thus, it can survive oxidative stress conditions in the intestinal tract (Higuchi, 1984), (Higuchi *et al.*, 2000).

Superoxide dismutase reaction:

- $2O^{2-} + 2H^+ \rightarrow O_2 + H_2O_2$

Catalase reaction: (Boon E M *et al.*, 2007)

1. H_2O_2 + Fe(catalase(red))-E → H_2O + Fe(catalase(ox))-E
2. H_2O_2 + Fe(catalase(ox))-E → H_2O + Fe(catalase(red))-E + O_2
3. 2 H_2O_2 → 2 H_2O + O_2

NADH oxidase reaction: (Higuchi *et al.*, 2000)

1. NADH + O_2 + H^+ → NAD^+ + H_2O_2
2. 2 NADH + O_2 + $2H^+$ → $2NAD^+$ + $2H_2O$

Table 2. presenting anti oxidative stress mechanisms or genes collected from different sources (De Angelis & Gobbetti, 2004), (Archibald & Fridovich, 1981b), (Hung *et al.*, 2005), (Lin & Yen, 1999), (Cha *et al.*, 1995)

Protein	Glutathione reductase	Thioredoxin	Thioredoxin reductase (NADPH)	NADH oxidase	NADH peroxidase	Catalase	pseudocatalase Mn catalase	Superoxide dismutase	FLP	RecA	Phosphate ABC transporter	L-cystine transporter
Gene	gor	trxA	trxB	nox	npr	kat	Mn-Kat	SodA	flpA, flpB	recA	pstS	
L. plantarum	+	+	+	+	+	+	+	-	-	+	+	-
L. fermentum	+	+	+	-	+	-	-	-	-	+	+	+
L. brevis	+	-	+	+	+	+	-	-	-	+	+	+
L. casie	-	+	+	+	+	-	-	-	+	+	+	+
L. rhmnosus	+	+	+	+	+	-	-	+	+	+	+	+
L. salivarius	-	+	+	-	+	-	-	-	-	+	+	+
L. gasseri	+	+	+	-	+	-	-	-	-	+	+	+
L.reuteri	+	+	+	+	-	-	-	-	-	+	+	+
E.faecalis	+	+	+	+	+	+	-	+	-	+	+	-
E.faecium	+	+	+	+	+	-	+	+	-	+	+	+
P.pentosaceus	-	+	-	-	-	-	+	-	-	+	+	-

Relating with our finding of (expected intestinal) bacteria in the mother milk, it is supposed from the bacteria which have ability to translocate during pregnancy or lactation to have any ability to resist oxidative stresses, considering macrophages oxidative stress (Kirkham, 2007). As periplasmic SOD evidently plays an additional role in several bacteria and helps resist the oxidative attack of the host macrophages (Imlay, 2008), bacteria possessing this scavenger enzyme might survive translocation. For more understanding of oxidative stress role and bacteria survival we tested strains different abilities as; survive in media containing hydrogen peroxide or paraquat and possessing some oxidative stress resistance mechanisms (catalase - NADH oxidase – SOD - GR)**.**

Producing of secondary metabolites and enzymes

The microbial community and diversity in the intestine are not limited to ecological exclusion (acid, bile, diet, antibiotics and oxygen) in the pre-existing niches, there are also the competitive exclusion factors (antagonistic metabolities) and microbial interaction and exchanges (secondary metabolites and enzymes). These include microbial-microbial and microbial-host exchange and interaction. For that we investigated the role of metabolic activity products in shaping intestinal microbiota, and focusing on those metabolites properties of the milk strains in the mother/baby-pairs groups to understand the special ability which enable these strains to coexist in this special complex biological fluid (breast milk), and to detect the possibility if these (identical genotype mother/baby-pairs groups) strains have the same properties to be from identical original source (the mother intestinal microbiota). Active metabolites study includes, antagonistic metabolites, secondary metabolites and enzymes (exopolysaccharides, caseinase, α-Galactosidases and β-Galactosidase (lactase)).

Production of antagonistic metabolites (antagonistic metabolities)

The average human intestine harbors between 1,000 and 1,150 prevalent bacterial species coexist (Qin *et al.*, 2010) with a total population far outnumbering even the number of human cells in the body. Under normal conditions of health and diet, the different strains of bacteria on microbiota compete or fight with each other to establish dominance. Intestinal microbiota

may prevent also infection by interfering with pathogens (Savadogo *et al.*, 2006). Several metabolic compounds produced by lactic acid bacteria (including organic acids, fatty acids, hydrogen peroxide, and diacetyl) have antimicrobial effects (Arthur & Satu, 2004). While production of lactic acid has been quite considered to be the main factor which enables Lactobacillus to dominate the intestinal and vaginal ecosystem, more recent data reported that H_2O_2 production by lactobacilli may be more efficient than lactic acid production. Lactic acid bacteria suppress the growth of competing microbes by using pyruvate and lactate oxidases to excrete large doses of H_2O_2 (Imlay, 2008). The antimicrobial role of H_2O_2 may come from the oxidation of sulfhydryl groups resulting in denaturing of substantial enzymes, and from the peroxidation of membrane phospholipids causing increasing membrane permeability (Kong & Davison, 1980). H_2O_2 may also be as a source for the production of bactericidal free radicals such as superoxide (O_2^-) and hydroxyl ($OH^·$) radicals which can disrupt the DNA (Byczkowski & Gessner, 1988). However, bacteriocins or proteinaceous bacteria product with specific inhibitory activity against closely related species are perhaps the most widely antimicrobial compound studied (Arthur & Satu, 2004), (McAuliffe *et al.*, 1998). Bacteriocins are usually ribosomally synthesized, extracellularly released bioactive peptides or peptide complexes (usually 30–60 amino acids), which have a bactericidal or bacteriostatic impact against other (usually closely related) species (Garneau *et al.*, 2002). However, the producer bacteria exhibit specific immunity to the action of its own bacteriocin. Generally, their main action is at the cytoplasmic membrane, as they disperse the proton motive force through the formation of pores in the phospholipids double layer (Montville *et al.*, 1995). The mostly known and described bacteriocins so far are produced by *Lactobacillus spp*., followed by *Enterococcus, Pediococcus* and *Leuconostoc spp*. (Luc de Vuyst & Vandamme, 1994). According to the definition of Klaenhammer (Klaenhammer, 1988), bacteriocins of lactic acid bacteria are active against closely related bacteria. However, a few exceptions to the principle have been reported, e.g. activity against Gram-negative bacteria. Bacteriocins or phages could cause rapid community changes by extermination established strains; the outcome of such dynamics might be unpredictable in practice because it might involve evolutionary changes in intestinal populations (Dethlefsen *et al.*, 2006).

Production of exopolysaccharides

Many strains of lactic acid bacteria are able to produce exopolysaccharides (EPS) (Ricciardi & Clementi, 2000). Different researches had reported that EPS produced by some lactic acid bacteria could support beneficial effects on human health, and have the possibility of acting as prebiotic substrates (Korakli *et al.*, 2002). It has been reported also that EPSs of LAB have anti-tumoral (Oda *et al.*, 1983), anti-ulcer (Nagaoka *et al.*, 1994), immunomodulating (Makino *et al.*, 2006) or cholesterol-lowering activity (Korakli *et al.*, 2002). Additionally,

metabolic products produced from one bacterial species may provide substrates to support the growth of other populations. Such a mutualistic mechanism, may lead to changes in bacterial communities within the colon as result of releasing of fermentation and other metabolic products formed by the bacterial metabolic activity (Flint *et al.*, 2008). So, species, which have this ability could play important role in shaping the intestinal diversity and inhabitation.

Production of caseinase

Casein is the main protein in mammalian milk making up 80% of the proteins in cow milk and between 20% and 45% of the proteins in human milk. When milk sours, the curd that is left is coagulated casein (due to the acid produced by the microbes in the milk). Casein also contains all 20 naturally occurring amino acids (making hydrolyzed casein an excellent nutritional source in microbiological environment) (Kunz & Lonnerdal, 1990). Casein is also the cause of many dairy allergies. The enzyme that is responsible for the hydrolysis of casein is caseinase. Some intestinal bacteria can secrete the caseinase, which breaks down the large casein molecules into smaller polypeptides which can be absorbed through the epithelial membrane. This hydrolytic reaction, called proteolytic action, will clear the area surrounding the colony as it attacks the casein molecules in the caseinase detecting test. For strains which isolated from breast milk it is important to investigate this property and compare with normal intestinal strains.

Production of α-galactosidase and β-galactosidase

Lactose is the most abundant carbohydrate in milk and milk-based products; thus, the ability to metabolize it is essential for *L. plantarum* and other LAB to be coexisting in milk or intestine of milk consumers. (Mayo *et al.*, 1989)**.** α-galactosidases (EC 3.2.1.22) and β-galactosidases (EC 3.2.1.23) are enzymes which are commonly found in nature and which are able to release α- or β-linked d-galactose from a wide range of compounds. β-galactosidase (lactase) is able to cleave β-linked galactose residues from various compounds and is commonly used to cleave lactose into galactose and glucose (Wigley, 1996). Induction of α-galactosidases has been observed on arabinoxylan, galactose, galactomannan, and wheat and rice bran (de Vries *et al.*, 1999).

Virulence Genes in Enterococcus species

Enterococci are ubiquitous microorganisms that inhabit the gastrointestinal tract of mammals and other animals. Additionally, strains belonging to such genera can be isolated from fresh breast milk of healthy women from different regions, confirming that they are a part of the natural microbiota of this biological fluid (Favier *et al.*, 2002), (Mackie *et al.*, 1999). Once they reach the infant gut through the milk, they contribute to the protective influence that breastfeeding support against infectious diseases (LopezAlarcon *et al.*, 1997), (Wright *et al.*, 1998) through competition and production of antimicrobials (enterocin A) (Nes *et al.*, 2007).

In the previous work we isolated a variety of lactic acid bacteria from human milk and found that *E. faecium* and *E. faecalis* was one of the predominant species since it was isolated from the samples provided by 6 of the 9 healthy women included in the study at a level (>10^2 cfu ml $^{-1}$). The randomly selected *Enterococcal* isolates could be grouped into 7 different genotypes by RAPD-PCR profiling. *Enterococci* considered an essential part of the original microbiota of humans, with *E. faecium* and *E. faecalis* being the most common species in the human gastrointestinal tract (Klein, 2003), (Murray, 1990), (Albesharat *et al.*, 2011). Moreover, and similarly to other lactic acid bacteria, some *Enterococcal* strains have found commercial applications as starter or protective cultures as well as probiotics in food and food supplements (Reviriego *et al.*, 2005). Otherwise, *Enterococci* are potential pathogens that cause clinical infections especially in patients (including neonates) whom have underlying diseases (Moellering, 2000). Despite this apparently mysterious nature of *Enterococci* (Franz *et al.*, 2003), the possibility of virulence behavior and/or any other actions of clinical significance, such as the antibiotic resistance or the virulence gene potential, appears to be strain specific among isolates studied so far involves both core and variable genetic traits and is not fully understood (Palmer *et al.*, 2010). In a recent study, Vancanneyt et al (Vancanneyt *et al.*, 2002) compared the genotypes of *E. faecium* strains from humans, animals, and foods and found that all human isolates involved in clinical infection fell into a well defined subgroup, which suggests that there may be a genetic basis for strains associated with human disease. Pillai et al (Pillai *et al.*, 2002) have also suggested that virulent subpopulations of *E. faecalis* may exist. Therefore, the safety of any *Enterococcal* strain of clinical or industrial interest should be carefully and individually evaluated. Since breastfeeding can be a significant source of *Enterococci* to the infant gut, (Heikkila & Saris, 2003), (Martin *et al.*, 2003), (Reviriego *et al.*, 2005) which may affect the overall composition of the neonate gut microbiota and exert biological functions that are unknown at present, the objective of this study was to evaluate the presence or absence of several genetic and phenotypic traits of clinical significance among *E. faecium* and *E. faecalis* strains previously isolated from human milk of healthy mothers.

Table 3. *Enterococcus* virulence factors identification. Adapted from (Eaton & Gasson, 2001)

Gene(s)	Role of product in virulence Reference(s)
Ace	adhesion to collagen. (Botes *et al.*, 2008)
Agg	Aggregation protein involved in adherence to eukaryotic cells; cell aggregation and conjugation. (Galli *et al.*, 1990)
gelE	Toxin; extracellular metalloendopeptidase that hydrolyzes and targets gelatin, collagen, hemoglobin and other bioactive compounds. (Su *et al.*, 1991)
cylM	Post translational modification of cytolysin. (Gilmore *et al.*, 1994)
cylB	Transport of cytolysin. (Gilmore *et al.*, 1994)
cylA	Activation of cytolysin. (Gilmore *et al.*, 1994)

esp	Cell wall-associated enterocooal protein involved adhesion and in immune evasion; may be associated with *cyl* genes on a pathogenicity island. (Shankar *et al.*, 1999)
efaAfs;	Cell wall adhesins expressed in serum by *E. faecalis* and *E. faecium*, respectively. (Lowe *et al.*, 1995)
cob	Sex pheromones, chemotactic for human leukocytes; facilitate conjugation. (Clewell *et al.*, 2000)
fsrB	described as important for biofilms (BopD), and genes predicted to encode the surface proteins EF0750 to -0757 and EF1097. (Bourgogne *et al.*, 2006)
sprE	*responsible for producing* serine protease that shares homology with *Staphylococcus aureus* V8 protease. (Engelbert *et al.*, 2004) .

Collectively, in this work we investigated intensively different physiological and biochemical prosperities of different LAB species isolated from different human sources, for more understanding for the rules which shape this community, the reason behind species dominance in different environments and the synergy or inhibitory rule which it can play influencing other intestinal LAB species. These investigations includes; ecological stresses resistance abilities (digestive stress (gastric acid and bile salt) - oxidative stress, dietary polyphenolics consumption - antibiotics), resistance mechanisms possessing (bile salt hydrolyse ability - oxidative resistance mechanisms (catalase (Cata), NADH oxidase, superoxide dismutase (SOD) and glutathione reductase (GR))), production of antagonistic metabolites (hydrogen peroxide - bacteriocin) and production of mutual secondary metabolites and enzymes (α-galactosidase & β-galactosidase - caseinase - exopolysaccharide (GTF – FTF)). Focusing on the common strains founded in the mothers milks and their own babies faeces and their faeces to confirm the possibility of being from on regional source and their special ability comparing to other intestinal strains, and finally investigating the safety of possible virulence strains (*Enterococcus*) which have been isolated from the mothers' milks.

Methods

Bacteria species and strains:

For the testing of physiological and biochemical criteria putatively contributing to the performance of a bacterial strain in the intestinal passage or putative passage to the mammary gland 150 strains were randomly selected from a total of 700 original isolates, which differed by their isolation of different individuals (adults, mothers and babies), species and RAPD patterns. They belonged to the species (species name (TMW number)): *Lactobacillus plantarum* (1.1671, 1.1679, 1.1667, 1.1607, 1.1611, 1.1602, 1.1623, 1.1628, 1.1616, 1.1690, 1.1693, 1.1683, 1.1608, 1.1609, 1.1603, 1.1647, 1.1654, 1.1656, 1.1662, 1.1861, 1.1862, 1.1516, 1.1864, 1.1793, 1.1798, 1.1860, 1.1509, 1.1617, 1.1625, 1.1867, 1.1868, 1.1869, 1.1541, 1.1571, 1.1748, 1.1619, 1.1636, 1.1633, 1.1631, 1.1553, 1.1555, 1.1585, 1.1735, 1.1542, 1.1567), *Lactobacillus fermentum* (1.1709, 1.1716, 1.1634, 1.1640, 1.1552, 1.1794, 1.1743, 1.1527, 1.1534, 1.1624, 1.1740, 1.1737, 1.1742, 1.1550), *Lactobacillus casei* (1.1870, 1.1871, 1.1751, 1.1507, 1.1583, 1.1562, 1.1547, 1.1795, 1.1841), *Lactobacillus brevis* (1.1517, 1.1863, 1.1503, 1.1750, 1.1568, 1.1744, 1.1526, 1.1859, 1.1669, 1.1748, 1.1747, 1.1746), *Lactobacillus salivarius* (1.1524, 1.1666, 1.1674, 1.1755), *Lactobacillus rhamnosus* (1.1548, 1.1538, 1.1715, 1.1564), *Lactobacillus mucosae* (1.1519, 1.1545, 1.1570, 1.1741), *Lactobacillus oris* (1.1635, 1.1639, 1.1563), *Lactobacillus helveticus* (1.1840), *Lactobacillus reuteri* (1.1642), *Lactobacillus vaginalis* (1.1780), *Lactobacillus gasseri* (1.1663), *Pediococcus pentosaceus* (1.1522, 1.1865, 1.1531, 1.1586, 1.1549, 1.1588), *Enterococcus faecium* (1.1872, 1.1873, 1.1556, 1.1536, 1.1858, 1.1650, 1.1708, 1.1784, 1.1514, 1.1768, 1.1762, 1.1673, 1.1660, 1.1707, 1.1763, 1.1760, 1.1697, 1.1764), *Enterococcus faecalis* (1.1661, 1.1665, 1.1510, 1.1773, 1.1528, 1.1575, 1.1533, 1.1774, 1.1770, 1.1574, 1.1772, 1.1771, 1.1511), *Enterococcus hirae* (1.1685, 1.1695, 1.1688), *Enterococcus durans* (1.1590, 1.1775), *Enterococcus mundtii* (1.1696, 1.1706), *Enterococcus gallinarum* (1.1783, 1.1537), *Enterococcus avium* (1.1779), *Enterococcus casseliflavus* (1.1515), *Streptococcus gallolyticus* (1.1610), *Streptococcus lutetiensis* (1.1629) and *Streptococcus infantarius* (1.1505). 54 strains of the 150 selected strains belong to the mother/baby-pairs groups (nine cases (groups) had identical RAPD genotypes of *L. plantarum, L. fermentum, L. brevis, E. faecium* and *E. faecalis* were found in the mothers' faeces, their milk and the corresponding babies' faeces. These species were found in all the three sources in some groups (e.g. five *L. plantarum* groups). While others were found in two of them, like in the same mother faeces and milk samples (*E. mundtii*) or in the mother milk and her baby faeces samples (*L. fermentum, L. oris*) or in the mother and her baby faeces samples (*L. casei, L. salivarius, L. rhamnosus* and *Str. lutetiensis*) (compare previous chapter). The 150 strains were tested for their growth behaviour in different acidic, bile salts presence, paraquat and hydrogen peroxide presence conditions.

A part of the 150 strains, i.e. 90 strains including the 54 mother/baby-pairs groups were tested for their ability to grow in tea and licorice medium, some oxidative stress resistance mechanisms (catalase - NADH oxidase – SOD - GR), production of antagonistic metabolites (hydrogen peroxide – bacteriocine), Produce metabolites and enzymes (α-galactosidase & β-galactosidase - exoenzyme caseinase production - exopolysaccharide production (GTF – FTF)). While only the 54 strains of the mother/baby-pairs groups were tested for their ability to grow in the presence of set of antibiotics.

Comparative study (physiological & biochemical criteria on different stresses and media for different species):

Microtiter assay method:

In the microtiter plate assay, 200 µL of mMRS broth or any tested broths were distributed in a 96-well microtiter plate, respectively. A volume of 5µL of overnight culture of the indicator strains appropriately diluted (to get final OD_{590} =2) was added to each well or the same quantity of water to the control. We used as control of cell growth the appropriate broth (mMRS) with sterile, distilled water instead of the tested condition solution, and it was inoculated with the test bacteria, which showed the growth of LAB species when nothing influenced their growth (Control 1). Thus we obtained the effect of different broths on tested bacteria strains and in this way we could predict the possibility of inhibitory effect of broths (contents or concentration) (Control 2). To ensure that the reaction conditions were (semi) anaerobic and to prevent evaporation during longer incubations, the wells were overlaid with 50 ml of light paraffin oil (0.85 g/ml; Merck, Darmstadt, Germany). The microtiter plates were incubated at 37 °C for 13 h and the absorbance, beginning the 0 hour of incubation, was measured at OD_{590} nm using a microtiter plate reader Tecan (Spectrafluor and Sunrise, Germany). The absorbance was measured every 20 minute for 40 cycles. The absorbance of wells containing only broth and acid or bile salt suspension without the indicator strain was subtracted from the absorbance of the wells that contained the indicator strain. Thus we obtained the absorbance of the cells. This absorbance is in direct fittings with the amount of bacteria cells, within certain limits. Growth on acidic and bile condition was scored as less than, equivalent to, or greater than that observed for the same growth on the mMRS broth. All different LAB species were able to grow well in mMRS media pH 7 and this was considered the standard medium. The mMRS contain the following components per liter: peptone from casein, 10 g; yeast extract, 5 g; meat extract, 5 g; $K_2HPO_4 \cdot 3H_2O$, 2.6 g; KH_2PO_4, 4 g; cysteine-HCl, 0.5 g; NH_4Cl, 3 g; Tween 80, 1 ml. The components were dissolved in 800 ml H_2O_{dest} resulting in a pH of 7.0 (if necessary, pH was adjusted by adding HCl). Stock sterile filtrated (pore size = 0.2 µm) solutions containing $MgSO_4 \cdot 3H_2O$ (100 g liter^{-1}) and $MnSO_4 \cdot 4H_2O$ (50 g liter^{-1}), and a stock solution of vitamin mix containing folic

acid, pyridoxal phosphate, thiamine, riboflavin, cobalamin, and pantothenic acid (0.2 g liter^{-1} each) was sterilized by filtration (stored at –20 °C). One milliliter of each stock solution liter^{-1} was added to the sterilized medium. Three types of sugars, 10 g of maltose liter$^{-1,}$ 5 g of glucose liter^{-1} and 5 g of fructose liter^{-1} were used unless otherwise indicated. Sugars were dissolved separately in 200 ml $H_2O_{dest.}$ To avoid the formation of Maillard products, basic medium and sugar solutions were autoclaved (121 °C, 20 min) separately and mixed after cooling.

Growth behaviour on acidic conditions

In the different mMRS media the pH was adjusted to 5, 4, 3, 2, 1 respectively by adding 10N HCl. The medium were adjusted with filter-sterlized 2N NaOH. All pH adjustments were done after autoclaving. 200 µL of the different pH mMRS broths were distributed in a 96-well microtiter plate and inoculated with different LAB species.

Growth behaviour in the presence of bile salts

Broth mMRS media were supplemented with bovine bile (Sigma Chemical Co Ltd, Poole, United Kingdom) and porcine bile (Sigma), to final concentrations of 0.4%, 0.8% and 1% (pH 7.0). These plates were then incubated at 37 °C under anaerobic conditions and growth was recorded after 13 h. 200 µL of the mMRS broths containing the same different concentrations of porcine and bovine respectively were distributed in a 96-well microtiter plate and inoculated with different LAB species for each concentration.

Bile salt hydrolases activity (BSH)

Qualitative BSH activity of the cultures was evaluated using the modified procedure described by (du Toit *et al.*, 1998). Sterile toothpicks were impregnated in an overnight culture of the test strain and placed on MRS agar plates supplemented with 0.5% (wt/vol) taurodeoxycholic acid sodium salt (TDCA; Sigma) and 0.37 g of CaCl2 (Merck)/liter. The plates were incubated anaerobically at 37°C for 72 h, and then the diameters of the precipitation zones were measured. mMRS agar plates without supplementation were used as controls. Each strain was tested in triplicate. BSH activity was detected when a halo of deoxycholic acid precipitated around colonies in the medium. The strain *L. acidophilus* TMW (1.18) or DSM (20079) were used as a positive control.

Growth behaviour in the presence of hydrogen peroxide and paraquat

Each strain was tested for its ability to survive ROS by exposure to hydroxyl radicals that were generated via the hydrogen peroxide (H_2O_2) and paraquat (a superoxide anion generator). Cultures were grown to stationary phase at 37°C in mMRS medium and washed twice (for 10 min each at 5,000 g) with assay medium without oxidants. Growth challenges with different oxidants were done in mMRS medium with additions of various concentrations of oxidants: H_2O_2 (maximum concentration tested [Cmax]= 3.5 mM, 2.1, 1.8 and 1.0) and paraquat (Cmax = 0.17 M, 0.14 and 0.03). Experiments with controls were performed without

the addition of oxidants. Growth challenges were carried out in microtiter plates sealed with parafilm. The inoculation density was set to an OD_{590} nm of (2). The survival tests were analyzed after one day incubation at 37°C. This test as described above was applied for different 150 milk and intestinal strains isolated in this study. Additionally, it was applied for two *Bifidobacteria* strains from our strain collection for comparison as no *Bifidobacteria* strains have been isolated from any sample in this study. The two additional strains are *Bifidobacterium breve* (TMW 2.477) and *Bifidobacterium lactis* (TMW 2.530).

Growth behaviour on black tea and licoric media

The influence was investigated of black tea, licoric and the phenolic components of a tea extract upon bacterial growth, for that mMRS medium components were solved in black tea solution (one black tea envelope in 250ml boiled water and left for 10 min) instead of deionised water (tea 100% medium), mMRS medium solved in 20% tea solution, mMRS components were solved in licoric solution (one licoric envelope in 250ml cold water and left for 10 min) instead of deionised water (licoric 100% medium), mMRS medium solved in 20% licoric solution and the last medium is MRS with all component except Tween 80.

5 different types of tea polyphenols mediums where prepared by adding Quercetin, (+)Catechin hydrate, (±)Catechin hydrate, (-)-Epicatechin and Epigallocatechin gallate in this concentrations : (0.02 – 0.05 – 0.1 – 0.2 – 0.5 – 1 – 2 – 2.25 – 2.50 – 2.27 – 3 – 3.25 – 3.50 – 3.75 – 4.0) mg/ml in MRS medium. 200 µL of this different concentrations and broths were distributed in a 96-well microtiter plate, respectively. A volume of 5µL of overnight culture of the different species appropriately diluted (to get final OD_{590} =2) was added to each well or the same quantity of water to the control. We used as control of cell growth the appropriate broth (mMRS) with sterile, distilled water instead of the tested condition solution, and it was inoculated with the test organisms, which showed the growth of LAB species when nothing influenced their growth. Thus we obtained the effect of different broths on test organisms and in this way we could predict the possibility of activation or inhibitory effect of broths (mainly the concentrated broths). The microtiter plates were sealed with parafilm and were incubated at 37 °C for 16 h and the absorbance, beginning the 0 hour of incubation, was measured at 590 nm using a microtiter plate reader. The absorbance was measured every 20 minute for 40 cycles. The absorbance of wells containing only broth and tea or polyphenols suspension without the indicator strain was subtracted from the absorbance of the wells that contained the indicator strain. Thus we obtained the absorbance of the cells. This absorbance is in direct proportion with the amount of cells, within certain limits.

Growth on tea and polyphenols suspension condition was scored as less than, equivalent to, or greater than that observed for the same growth on the mMRS broth. All this tests were performed for the 90 different LAB strains; additionally these tests were performed for two

Bifidobacteria strains *Bifidobacterium lactis* (TMW 2.530) and *Bifidobacterium longum* (TMW 2.448).

The presence of different amino acids in tea and licoric solutions (without the rest of the MRS components) where tested by High performance liquid chromatography HPLC using AAS18 standard from sigma containing all this 18 aminoacids (the following components are included in the amino acid standard: alanine, ammonium chloride, arginine, aspartic acid, cystine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tyrosine, valine) with additional gamma amino buytric acid (Gaba) and ornithine as described by (Benjamin Schurr *et al.*, 2013).

Investigating some oxidative stress resistance mechanisms:

Investigating oxidative stress resistance mechanisms (catalase - NADH oxidase)

We did systematically screen a large number of LAB isolates from human feaces and milk as to their capacity to resist oxidative stress. By screening Kat and Nox gens, using primers already described in literatures (table 4). For presence investigation, DNA was amplified with a Thermo Cycler (PRIMUS 96 plus, MWG-Biotech AG, Ebersberg, Germany) or Eppendorf Gradient Cycler (Eppendorf, Hamburg, Germany). The Taq Core Kit was used (2.5 µl of reaction buffer (with 1.5 mM $MgCl_2$), 0.5 µl dNTPs, 0.15 µl primer (for and rev 100um) and 0.15ul Taq and completed with PCR-H_2O to 21.55 ul were used for each reaction) with 1 µl DNA of different tested LAB strains. Standard cycling conditions for amplification with Taq polymerase were as follows; in a first step, genomic DNA was denatured (94 °C/2 min). Then an amplification cycle consisting of a denaturation step (94 °C/50 s), primer annealing step 50 °C/50 s) and an elongation step (72 °C, depending on fragment length – extension rate of Taq: 1 kb/min, 25 s for Kat and 90 s for Nox) was repeated for 32 times. Eventually a final elongation step (72 °C/5 min) finished PCR reactions. Visualization of DNA was performed by agarose gel electrophoresis. 1 - 1.3 % agarose gels were prepared with 0.5 x TBE buffer (stored as 10 x TBE buffer: 150 g/l Tris, 26.2 g/l boric acid, 9.0 g/l EDTA; pH 8.9) for analytical gels and with 1 x TAE buffer (stored as 50 x TAE buffer: 0.1 M EDTA, 1 M acetic acid (100 %), 2 M Tris, pH 8.2) for preparative gels. DNA samples were mixed with loading dye in ratio 6:1 (sample : loading dye). Separation was routinely performed in electrophoresis chambers (12 x 13.8 cm) at 90 – 120 V for 1 - 1.5 h. ready-to-use DNA size standards were applied (10 µl in small cavities (GeneRuler™ 100 bp DNA Ladder)) to determine DNA fragment sizes by comparison (table 7). Gels were stained with ethidium bromide/dimidium bromide and the banding profiles were visualized under UV light (wavelength 320 nm) and digitalized by gel documentation system from INTAS-science imaging instruments GmbH. Strain *L. sakei* (TMW: 1.1322) were used as a positive control for both genes Kat and Nox.

Table 4. Overview of the different primer pairs used to screen for Kat and Nox genes involved in catalase and NADH oxidase production (F: forward, R: reverse).

References	Sequence (5′-3′ orientation)	Target
(Waldschmitt *et al.*, 2009)	F: CGRGTBGTTCAYGCYAARGG	Catalase genes
	R: TTVACCCATTTRTTDGTRTG	
(Waldschmitt *et al.*, 2009)	F: GGTTGTACHCAYGCNGGMAC	NADH oxidase genes
	R: GGYTGGAADARCATATCHAC	

Investigating oxidative stress resistance mechanisms (superoxide dismutase - glutathione reductase)

Cell-free extracts obtention

Cells of the different strains were grown over night at 37°C in 15ml tubes and harvested by centrifugation at 5000 g for 10 minutes and discard the supernatant. The cell pellets were then quickly washed twice with deionized water and resuspended in TE buffer and transferred to 1.5 ml tubes. Then centrifugation at 5000 g for 5 minutes and resuspended in 1ml TE buffer contain 100mg/ml lysozyme, well mixed and incubated for 1 hour at room temperature followed by ultrasonic disruption (Ultra sonification, UP 200S, Dr. Hielscher GmbH, Teltow, Germany). Sonication was performed for 7 cycle each cycle 30 second (90% power) intervals in an ice bath. Cell debris was removed by centrifugation at 6.000 g for 10 minutes, and the resulting supernatant was the cell-free extract. The enzymes tests were performed directly after extraction.

Measurement of Superoxide dismutase activity

The activity of SOD was measured by using a commercially available reagent kit (Epiquik Superoxide Dismutase Activity/inhibition assay kit (Colorimatric), Epigentek Group Inc). This method employs xanthine, xanthine oxidase, and 2-(4-iodophenyl)-3-(4-nitrophenol)- 5-phenyltetrazolium chloride to form a red formazan dye. Inhibition of this reaction by samples indicates their SOD activity.

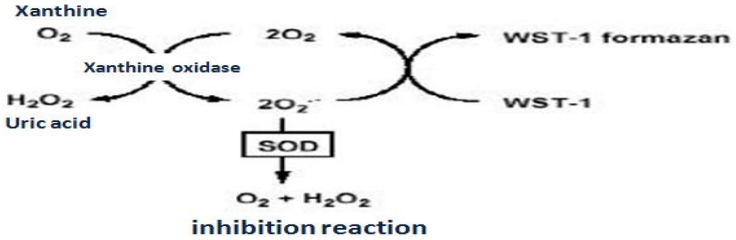


Figure 1. Principle of SOD Assay

Directly after cell-free extracts were obtained, 10µl of cell-free extract of lactobacilli was added to 80µl of mixed substrate in wells of a 96-well plate (dilution buffer instead of cell

extract was used as a positive control and dilution buffer instead of cell extract and reactive enzyme for blank), the mixture was incubated at 37°C. 10 µl of xanthine oxidase solution was added to all the wells and mixed to initiate the reaction, and the absorbance was read after 45-60 minutes at 470NM. (strain *L. sakei* TMW(1.1322) was used as positive control)

Measurement of Glutathione Reductase activity

The activity of GR was measured by using a commercially available reagent kit (glutathione reductase activity assay kit, Biomol). This method employs glutathione reductase together with its co-factor, NADPH, catalyzes the reduction of oxidized glutathione (glutathione disulfide, GSSG) to glutathione and $NADP^+$.

$$GSSG + NADPH + H^+ \xrightarrow{\text{Glutathione reductase}} 2GSH + NADP^+$$

(Absorbs at 340 nm)

(Reduction of glutathione disulfide (GSSG) by glutathione reductase and NADPH)

GSH is also a reactant for glutathione peroxidase, which converts hydrogen peroxide (H_2O_2) into water. In this assay the oxidation of NADPH to NADP+ is monitored by the decrease in absorbance at 340 nm. Directly after cell-free extracts were obtained, 50µl of cell-free extract of lactobacilli was added to 150µl of mixed substrate in wells of a 96-well plate (1XGR Buffer instead of cell extract and reactive enzyme for blank). 50µL of NADPH solution was added to all the wells to initiate the reaction, and the absorbance was read every minute for 10 minutes at room temperature at OD $_{340}$nm.

Production of antagonistic metabolites:

Production of antagonistic metabolites (Hydrogen peroxide)

To monitor hydrogen peroxide production in selected LAB strains, the qualitative method of **(Rabe & Hillier, 2003)** was used. Briefly, LAB species were subcultured on TMB-plus formulation, which contained Brucella agar 43g, distilled water 1 liter, soluble starch 20g, hemin solution 10ml, vitamin K solution 0.2ml, magnesium sulphate anhydrous 0.57g, manganese sulfate monohydrate 0.12g, peroxidase solution (1 mg/ml)10ml, horse serum 50ml. Plates were incubated for 48 h under anaerobic conditions at 37°C. After incubation, the plates were exposed to ambient air for 30 min to allow for color production. The peroxidase oxidizes TMB in presence of hydrogen peroxide produced by the bacteria and forms a blue pigment in presence of oxygen. As a consequence, hydrogen peroxide producing cells form dark blue colonies while non-producers are colourless. Growth and color production were compared by direct side-by-side observation.

Production of antagonistic metabolites (Bacteriocin)

Antimicrobial activity of LAB species was assessed with a colony overlay assay to screen a different set of microorganisms. This was assessed using the mMRS medium described

before, with slight modification to the sugar concentration to avoid high lactic acid production which might interfere or overlap the bacteriocin influence. The only sugar was added is glucose 3 g/L. Additionally 0.1% catalase was added to the medium to prevent H_2O_2 production which might also influence the detection of bacteriocin influence.

Overnight cultures of different LAB strains grown in mMRS medium were inoculated as a 3 ul spot on the special mMRS agar plates (described above) and incubated at 37°C for 24 h. A 1% v/v suspension of log-phase cells of every indicator strain culture at a concentration of about 2 x 10^7 colony forming units (CFU)/ml was inoculated in its appropriate soft agar (0.7% agar) (LB or Difco brain heart infusion agar (according to the requirements of the indicator strains) supplemented with 0.1% catalase) and overlaid on the surface. Plates were examined for zone of inhibition after incubation for 24 h at 37°C, including an initial pre-incubation of 3 h at 6°C. Zones of inhibition around the spots were scored as positive. The positive control strains were the plantaricin producer *L .plantarum* TMW (1.25) and the nisin producer *L. lactis* (2.25). The indicator bacterial cultures used in this study are *L. lactis* (TMW 2.26), *Listeria monocytogenes* (TMW 2.594) and *Staph. aureus* (TMW 1.1845).

Production of metabolites and enzymes (α-galactosidase & β-galactosidase – exoenzyme caseinase production - exopolysaccharide production (GTF – FTF):

α-Galactosidase Screening

The strains were grown on suitable plates (solid medium). X-α-gal (5-Bromo-4-Chloro-3-indolyl α-D-galactopyranoside) was added to the sterilized mMRS medium (121°C, 15 min) to prevent a chemical hydrolysis to final concentration 40µg/ml. Plates were incubated at 37°C for 48 hours. Colonies producing α-galactosidase will release substituted indol by α-galactosidase, oxidatively polymerises to give a blue colour (diazo) which does not diffuse in agar. The positive control strain was *E. coli* TMW (2.436).

β-Galactosidase Screening

The strains were grown on suitable plates (solid medium) mMRS containing X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) to final concentration 40µg/ml and 0.1 mM IPTG (iso-propyl-thio-β-D-galactopyranoside) as an inducer. Plates were incubated at 37°C for 48 hours. Colonies producing β-galactosidase were green to blue. The positive control strain was *E. coli* TMW (2.435).

Caseinase production

The strains were grown on suitable plates (solid medium) ready medium called calcium caseinate agar (Fluka) with additional sugars like the concentration in mMRS medium and 5g/L milk powder (121°C, 15 min) sugars solution and agar protein media were prepared and sterilized separately and mixed before plating at 50°C. After bacteria swapping on the

surface the plates were incubated at 37°C for 48 hours. Colonies producing caseinase will clear the area surrounding the colony as it attacks the casein molecules. The positive control strains were the food strains *L. plantarum* TMW (1.1723 and 1.701).

Exopolysaccharide (EPS) production

Screening for eps genes (GTFs – FTFs)

LAB are able to produce a great variety of exopolysaccharide (both homopolysaccharides (HoPS) and heteropolysaccharides (HePS)). We did systematically screen a large number of LAB isolates from human feaces and milk as to their capacity to produce EPS. By screening for eps genes (Glucosyltransferase, GTF and Fructosyltransferase, FTF genes), using primers already described in literatures targeting GTFs and FTFs (Table 5). Therefore, DNA from the tested LAB strains was extracted as described before. In total, five different PCR amplifications with the five pairs of primers were performed, using the same conditions as described by the corresponding authors (Table 5). Different strains were used as controls: *L. sanfranciscensis* and *L. reuteri* (TMW: 1.392 , 1.106) respectively. The PCR amplicons were visualized through gel electrophoresis on 1% (wt/vol) agarose gels in 1×TAE buffer (40 mM Tris acetate, 1 mM EDTA, pH 8.0), A 100-bp ladder (Invitrogen, Carlsbad, CA) was used to estimate the size of the bands (table7).

Table 5. Overview of the different primer pairs used to screen for eps genes (GTFs and FTFs) involved in HoPS and HePS production (F: forward, R: reverse).

References	Sequence (5′-3′ orientat	Target
(Kralj *et al.*, 2003)	F: GAYAAYWSIAAYCCIRYIGTIC	Glucansucrase genes
	R: ADRTCICCRTARTAIAVIYKIG	660bp
(Tieking *et al.*, 2003)	F: GAYGTITGGGAYWSITGGC	Fructansucrase genes
	R: TCITYYTCRTCISWIRMCAT	800bp

Production of EPS (extracellular polysaccharide)

Normal mMRS agar and mMRS with 80% sucrose as carbon source instead of glucose exopolysaccharide formation of positive colonies was observed by their slimy appearance. *L. sanfranciscensis* and *L. reuteri* (TMW: 1.392, 1.106) strains were used as controls.

Investigating virulence activity in Enterococcus species:

Generation of primers and probes for virulence genes investigation in Enterococcus species

PCR primers for the *efa, afs, cyl*, *esp* reverse, and *agg* forward virulence genes described in Table 6 were designed on the basis of published sequences and EMBL Nucleotide Sequence Database. PCR primers for *ace, fsrB, gelE, sprE, agg* reverse and *esp* forward were originally developed on the basis of primer sequences supplied by A. Lindenstrauss (TUM), and those for sex pheromone determinants *cop* were based on sequences in the *E.*

faecalis genome database at The Institute for Genomic Research. The primer sequences used were synthesized using the MWG online PCR primer design service (Eurofins MWG Operon).

A screening for *Enterococcus* virulence genes was performed using different primers targeting (*efaAfs, cyl*, *esp, agg, ace, fsrB, gelE, sprE, cob)* genes (Table 6). Therefore, DNA from the tested LAB strains was extracted as described before. Different PCR amplifications with the different pairs of primers were performed, using the same conditions as described by the corresponding authors (Table 6). Different *E. faecalis* strains were used as controls: (TMW: 2.9290, 2.6290, 2.852, 2.622 and 2.6300). The PCR amplicons were visualized through gel electrophoresis on 1% (wt/vol) agarose gels in 1×TAE buffer (40 mM Tris acetate, 1 mM EDTA, pH 8.0), A 100-bp ladder (Invitrogen, Carlsbad, CA) was used to estimate the size of the bands (table7).

Table 6. PCR primers and products for detection virulence determinants

Gene and primer	Sequence (5´ to 3´)	Product size(bp)	Reference
ace			
ace for	CCGAATTGAGCAAAAGTTC	746	Angela Lindenstrauss
ace rev	AGTGTAACGGACGATAAG		Angela Lindenstrauss
agg			
TE3	AAGAAAAAGAAGTAGACCAAC	1,553	Eaton & Gasson
agg rev	ACCTACAGCGTCCCAATCAC		Angela Lindenstrauss
gelE			
gelE for new	AATTGCTTTACACGGAACGG	419	Angela Lindenstrauss
gelE rev new	AGCCATGGTTTCTGGTTGTC		Angela Lindenstrauss
cob			
TE49	AACATTCAGCAAACAAAGC	1,405	Eaton & Gasson
TE50	TTGTCATAAAGAGTGGTCAT		Eaton & Gasson
cylM			
TE13	CTGATGGAAAGAAGATAGTAT	742	Eaton & Gasson
TE14	TGAGTTGGTCTGATTACATTT		Eaton & Gasson
cylB			
TE15	ATTCCTACCTATGTTCTGTTA	843	Eaton & Gasson
TE16	AATAAACTCTTCTTTTCCAAC		Eaton & Gasson
cylA			
TE17	TGGATGATAGTGATAGGAAGT	517	Eaton & Gasson
TE18	TCTACAGTAAATCTTTCGTCA		Eaton & Gasson
esp			
esp for multi	GAGTTAGCGGAACAGGTCA	933	Angela Lindenstrauss
TE36	GCGTCAACACTTGCATTGCCGAA		Eaton & Gasson
efaAfs			
TE5	GACAGACCCTCACGAATA	705	Eaton & Gasson
TE6	AGTTCATCATGCTGTAGTA		Eaton & Gasson
fsrB			
fsrB for multi	GTTTGTCCCATCCATTGTCC	346	Angela Lindenstrauss
fsrB rev multi	TTTATTGGTATGCGCCACAA		Angela Lindenstrauss
sprE			
sprE for	CCTGTCTGCAAATGCAGAAG	660	Angela Lindenstrauss
sprE rev	CGCCATTGGAATGAACACCA		Angela Lindenstrauss

(Lindenstrauss, 2012) and (Eaton & Gasson, 2001).

Gelatinase production

Production of gelatinase was determined for all *E. faecalis* and *E. faecium* strains which have the virulence genes (fsrB, gelE, sprE, the responsible about gelatinase production) by streaking single colonies of all thirteen *Enterococcus* strains (TMW 1.1528, TMW 1.1772,

TMW 1.1575, TMW 1.1773, TMW 1.1511, TMW 1.1574, TMW 1.1774, TMW 1.1665, TMW 1.1771, TMW 1.1770, TMW 1.1661, TMW 1.1650, TMW 1.1760) onto LB agar containing 3% gelatin. Plates were incubated at 37°C for 24 h. Gelatinase production, indicated by zones of opacity around colonies, was visualized after plates were incubated at 4°C for 5 h, as described by (Eaton & Gasson, 2001) and (Hew *et al.*, 2007).

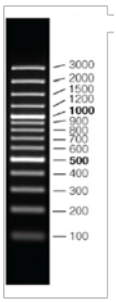
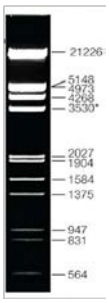
Antimicrobial susceptibility testing:

Antibiotic resistance patterns of the LAB strains were studied by disk diffusion and microdilution methods. mMRS agar was used to test the growth of the *bacteria of the mother baby pair group* strains tested. A total of 20 antibiotic substances: Ampicillin (0.0005g/ml), Aureomycin (0.0015g/ml), Chloramphenicol (0.0015g/ml), Tetracyclin (0.0015g/ml), Valinomycin (0.0015g/ml), Paromomycin (0.004g/ml), Kanamycin (0.0015g/ml) , Streptomycin (0.00125g/ml) and Erythromycin (0.0015g/ml) were tested by the disk diffusion assay. The agar plates were incubated aerobically for 24 h at 37°C. The degree of sensitivity was determined as the width of the growth inhibition area.

DNA and protein markers

DNA and protein markers used for size comparison were purchased from Fermentas GmbH, St. Leon-Rot and are listed in table 7.

Table 7. DNA and protein markers

GeneRuler™ 100 bp DNA Ladder	Lambda DNA/*EcoRI+HindIII* Marker, 3
	

Results

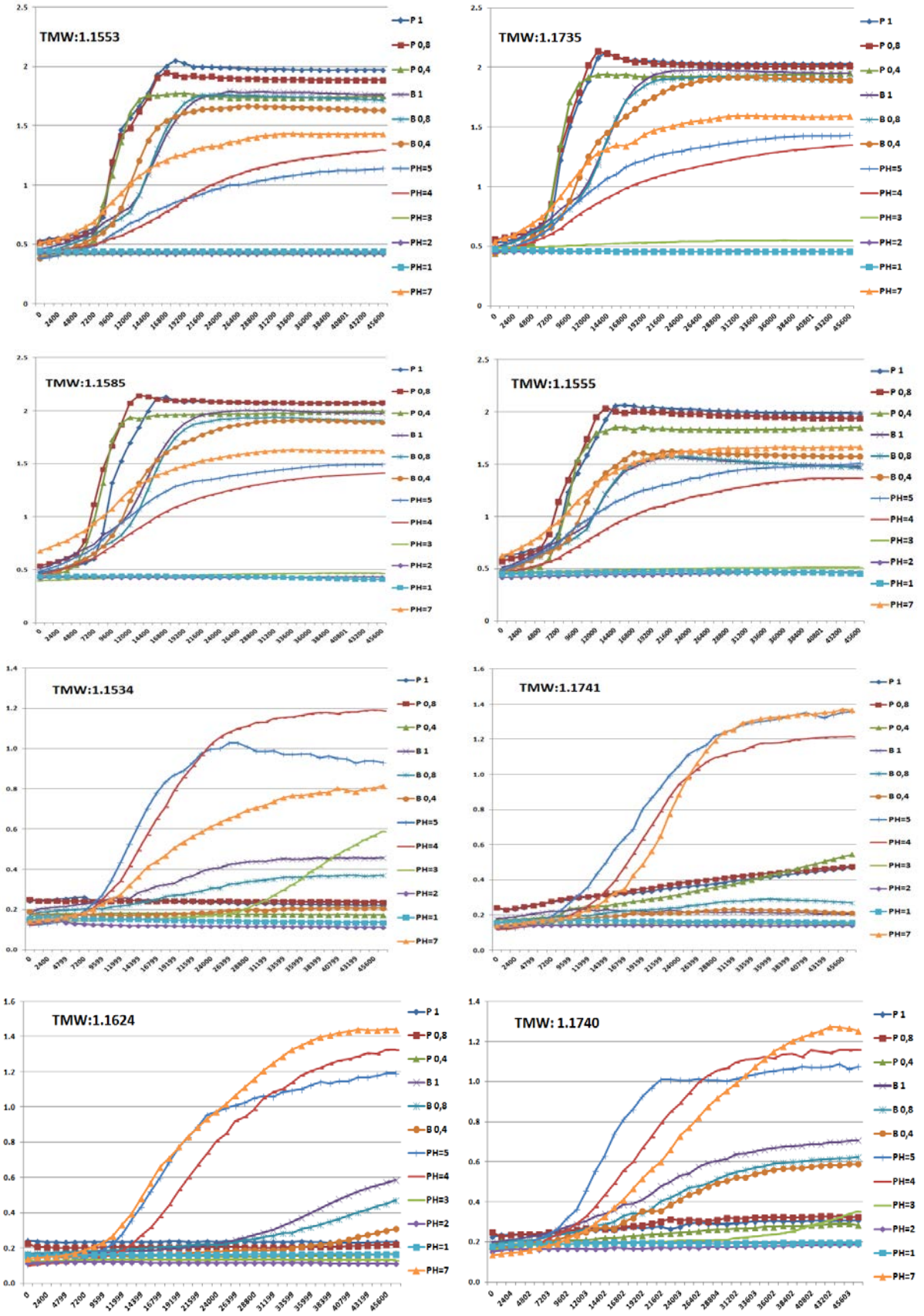
Growth behaviour in different stress conditions:

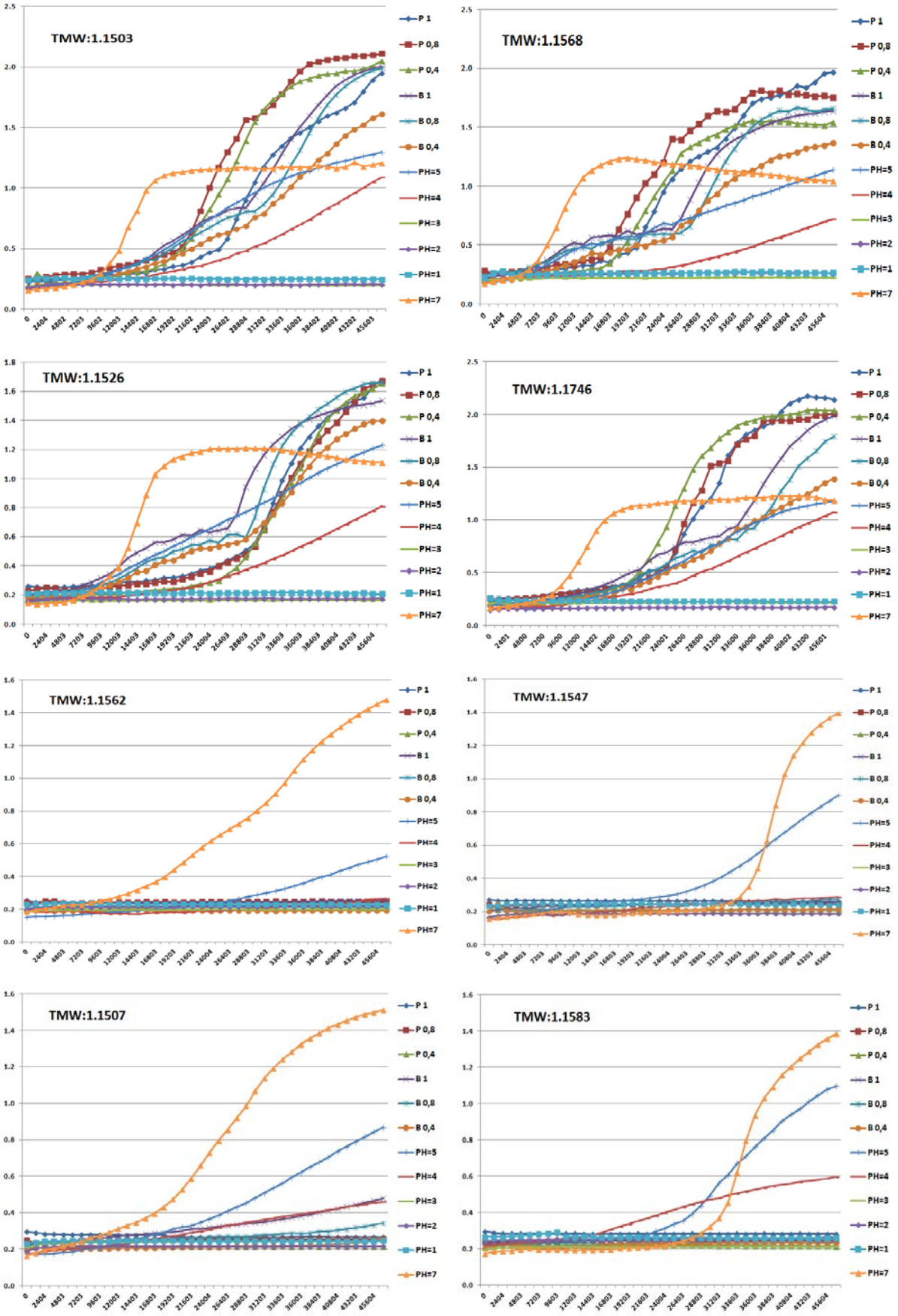
- **Growth behaviour on acidic conditions and in the presence of bile salts**

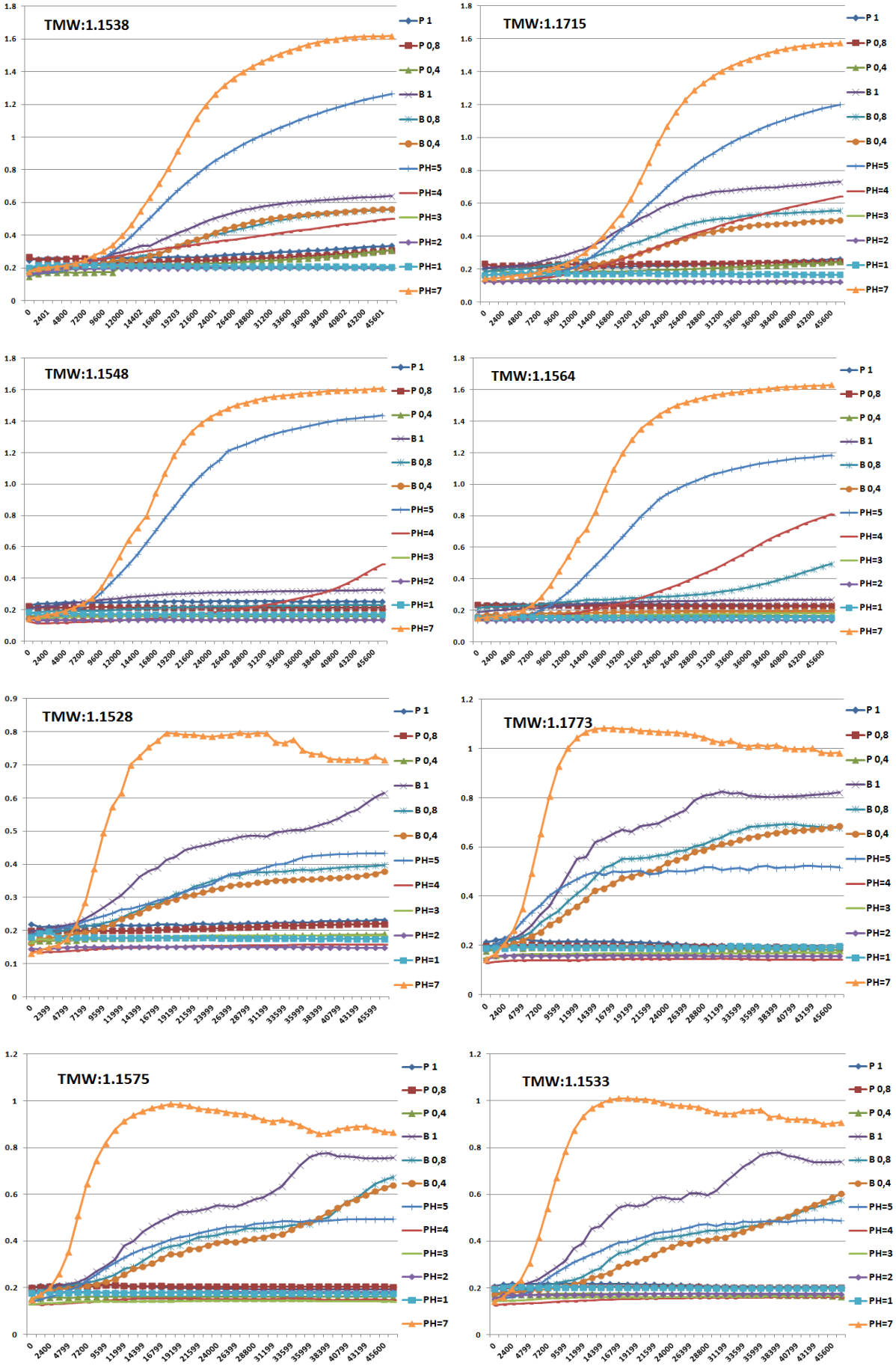
Our results for growth behaviour in different stress conditions couldn't be criterion (wide variation in growth behaviour, specific for each strain while general behaviour was similar for each species as coming described), it is a general conclusions from observation of growth curves of 150 different strains isolated from 30 different people (including the 54 strains of mother/baby-pairs groups) when they have been grown in different conditions. Different lactic acid bacteria species express different growth behaviour under different pH medium condition. Some strains showed high tolerance and resistance to low pH environment while other species could only grow in moderate acidity medium. Different strains in one species are variable in their resistance and growth tolerance but in general they express similar tendency differentiate them from the growth ability of strains belong to other species, as if every LAB species has its own special pattern of growth behaviour in different acidic stress medium. High acidic (pH=4 or less) resistance strains mostly belongs to the species *L. plantarum, L. fermentum* and *L. brevis*.

Similar behaviour was detected in bile salts mediums as general special common observation for each species and slight difference between strains in each species. Strains and species expressed different growth respond behaviour for the two different types of bile salts (the bovine and porcine bile salts). Although the tested Lactobacillus and Enterococcus strains exhibited resistance to the bovine bile used, the porcine bile used in these assays proved to be significantly more inhibitory to both of the bacterial groups. Even though bile salts are used as stress condition for specific species it acts as growth motivation factor (especially porcine bile) for other species as for *L. pantarum, L. brevis* and *E. faecium*. Figure (4) shows the slight differences in the strains´ growth patterns and the general common growth patterns specifics of each species, which differentiates it from other species patterns under different pH and different bile salt concentrations. Different strains of *L. plantarum* were found to show a high tolerance to the consecutive exposure to hydrochloric acid (pH 4.0) and bile salts. This was observed both for strains isolated from intestinal samples and for those isolated from fermented foods. In some strains of *L. plantarum* TMW(1.1541)*, L. brevis* TMW (1.1503, 1.1526, 1.1744,1.1568, 1.1748,1.1747, 1.1750), *L. fermentum* TMW(1.1740, 1.1737, 1.1742, 1.1624, 1.1741, 1.1510, 1.1527, 1.1534), *P. pentosaceus* TMW (1.1522, 1.1531, 1.1586), *E. avium* TMW (1.1779) and *L. casei* TMW (1.1507,1.1583, 1.1751) bile resistance appeared to be mediated by bile salt hydrolysis activity (as will be explained later). A general observation for acid resistant species like *L. plantarum* cells was that when grown under standard conditions they exhibited the characteristic rod-shaped,

smooth-surface morphology of *L. plantarum*. When acid or 0.4% bile was present in the medium, the cells had a slight tendency to clump together.




TMW:1.1503
TMW:1.1568
TMW:1.1526
TMW:1.1746
TMW:1.1562
TMW:1.1547
TMW:1.1507
TMW:1.1583
P 1
P 0,8
P 0,4
B 1
B 0,8
B 0,4
PH=5
PH=4
PH=3
PH=2
PH=1
PH=7


TMW:1.1538
TMW:1.1715
TMW:1.1548
TMW:1.1564
TMW:1.1528
TMW:1.1773
TMW:1.1575
TMW:1.1533
P 1
P 0,8
P 0,4
B 1
B 0,8
B 0,4
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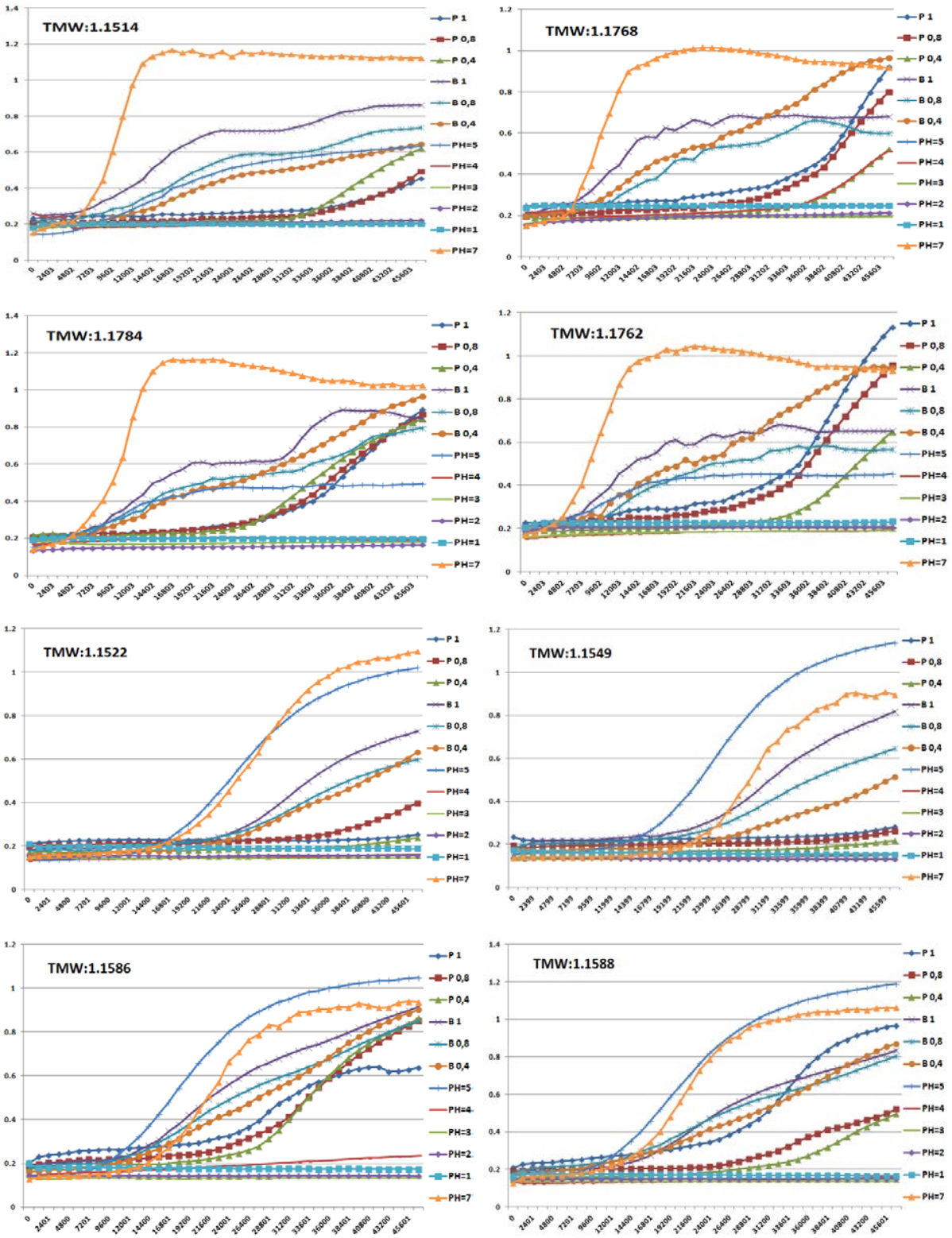


Figure 4. Growth curves of different LAB species, the vertical axis is the OD_{590} and the horizontal axis is the $TIME_{sec.}$ The parameters are (Px) mMRS with porcine bile salt concentration x%, (Bx) mMRS with bovine bile salt concentration x% and (pH=x) mMRS adjusted to the pH value x. Strains (1.1553,1.1735, 1.1585 and 1.1555) belong to *L.plantarum* species, Strains (1.1534,1.1741, 1.1624 and 1.1740) belong to *L.fermentum* species, Strains (1.1503,1.1568, 1.1526 and 1.1746) belong to *L.brevis* species, Strains (1.1562,1.1547, 1.1507 and 1.1583) belong to *L.cassei* species, Strains (1.1538,1.1715, 1.1548 and 1.1564) belong to *L. rhamnosus* species, Strains (1.1528,1.1773, 1.1575 and 1.1533) belong to *E. faecalis* species, Strains (1.1514,1.1768, 1.1784 and 1.1762) belong to *E. faecium* species and Strains (1.1522,1.1549, 1.1586 and 1.1588) belong to *P. pentosaceus* species.

In each species the strains, which were isolated as mother/baby-pairs groups (expressing similar RAPD-PCR genotypes), showed greater resistance to the stress condition (acidic and bile salt) than the strains only isolated from adult individuals of the same species. Figure 5 shows growth curves in different acidic and bile salt conditions for two identical strains patterns (one from adults and one from milk of mother/baby-pairs groups) of three different specious of intestinal LAB.

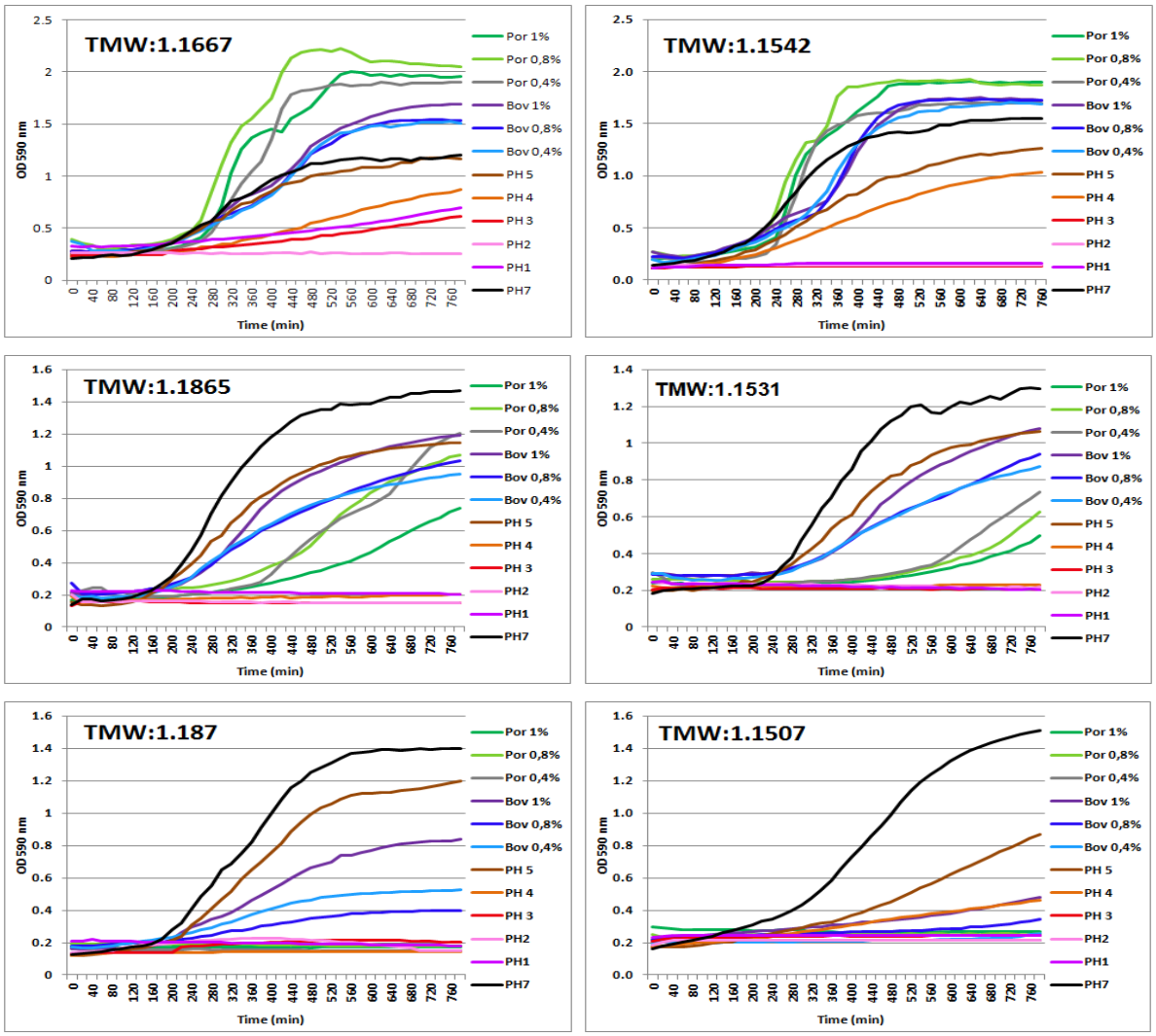


Figure 5. Growth curves in different acidic and bile salt condition of different LAB species of mother/baby-pairs groups strain (in the left) comparing to adults strain (in the right), The parameters are (Px) mMRS with porcine bile salt concentration x%, (Bx) mMRS with bovine bile salt concentration x% and (pH=x) mMRS adjusted to the pH value x. Strains TMW: (1.1667,1.1542) belong to *L. plantarum*, Strains TMW: (1.1870,1.1507) belong to *L. cassei* species, and Strains TMW: (1.1865,1.1531) belong to *P. pentosaceus* species.

Interestingly, mother/baby-pairs group's strains which include identical RAPD-PCR genotypes and MALDI-TOF spectra expressed identical growth behaviour in different acidic and bile salts condition. Figure 6 shows different conditions growth behaviour of triple *L. plantarum* strains isolated from mother's milk, mother's faeces and her baby faeces respectively belongs to different mother/baby-pairs groups.

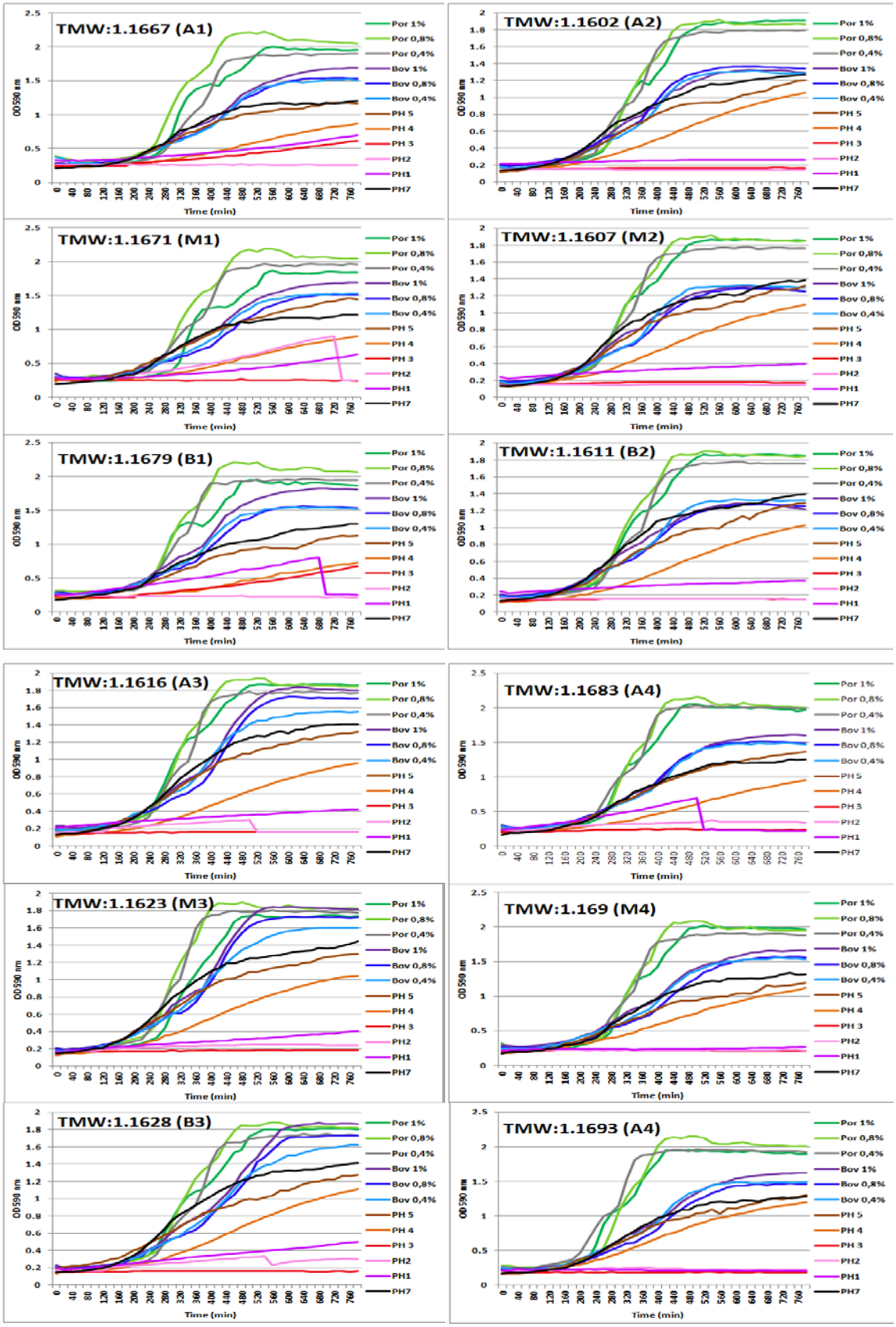


Figure 6. Growth curves in different acidic and bile salt condition of *L. plantarum* of mother/baby-pairs groups strain, The parameters are (Px) mMRS with porcine bile salt concentration x%, (Bx) mMRS with bovine bile salt concentration x% and (pH=x) mMRS adjusted to the pH value x. (Mn) refer to milk strain, (Bn) refer to baby strain, (An) refer to mother strain and (n) refer to the group number.

- **Growth behaviour in the presence of oxidative stress condition (hydrogen peroxide and paraquat)**

Using paraquat and hydrogen peroxide to induce oxidative stress in mMRS medium showed different ability to resist and grow between different LAB species and also different behaviour and resistance between these two kinds of oxidative stress inducer for every species. Strains of *L. plantarum, E. faecium, E. faecalis, E. mundtii, E. hirae* and *P. pentosaceus* showed well adaptation and growth at all tested concentration of paraquat and hydrogen peroxide than other LAB species. *L. fermentum, L. gasseri, L. oris, L. rhamnosus* and *E. avium* survived those conditions compared to no survival and very limited survival (0 or 0.7% for paraquat – 0.006% for H_2O_2) for *L. brevis, L. casei, L. reuteri* and *L. salivarius*. In general all intestinal species have higher resistance to hydrogen peroxide than paraquat.

General growth behaviour of different LAB species under different stress condition (acidic conditions, presence of bile salts, presence of hydrogen peroxide and presence of paraquat) is summarized in table (8). The *Bifidobacterium* strains from the strain collection *Bifidobacterium breve* (TMW 2.447) and *Bifidobacterium lactis* (TMW 2.530) had shown very low ability to survive in both oxidative stress condition paraquat and hydrogen peroxide, strain TMW 2.447 couldn't grow at all in any of the paraquat and H_2O_2 concentrations while strain TMW 2.530 had a weak grow at H_2O_2 concentration less than 3.5mM only.

Stress condition	Acid condition		Bile salts		Oxidative stresses	
Species	High acidity	Mild acidity	Bovine bile	Porcine bile	Paraquat	H2O2
L. plantarum	+++	+++	++	+++	++	+++
L. fermentum	+++	++	+	-	+	++
L. brevis	+++	++	++	+++	±	+
L. casei	+	+++	+	-	±	+
L. gasseri	++	++	-	-	++	++
L. oris	+	++	+	-	±	+++
L. mucosae	++	++	+	+	-	++
L. rhamnosus	++	+++	+	-	±	++
L. reuteri	+	++	++	++	-	+
L. salivarius	+	++	+	-	-	+
L. faecalis	±	++	++	-	+++	++
L. faecium	+	++	++	++	+++	+++
E. mundtii	±	++	+	++	+++	++
E. hirae	++	++	++	+	+++	++
E. avium	±	++	+	+	+	+
P. pentosaceus	±	+++	+	+	++	+++
Streptococcus	-	++	+	+	-	++

Table 8. General growth ability of different species of LAB when exposed to different stress condition; the used symbols are respectively in acid (high pH:4 and mild pH:5), bile (bovine and porcine) and oxidative conditions (paraquat and H_2O_2) refers to as: (+++) very well growth, enhance the growth more than standard condition or grow well at all concentrations, (++) well growth, like in standard condition or can grow at all concentrations, (+) can grow, less than standard condition or grow only at less than the first concentration, (±) survive, very weak growth or grow only at less than the second concentration and (-) inhibited growth, can't grow and can't grow at any concentration. Standard condition is mMRS at pH=7 without any addition.

Mother/baby-pairs group's strains, exhibit identical RAPD-PCR genotypes and MALDI-TOF spectra expressed identical growth behaviour in different paraquat and hydrogen peroxide oxidative stress condition. Interestingly, mother/baby-pairs group 2 strains (1.1602, 1.1607 and 1.1611) expressed similar ability of enhanced growth in the presence of H_2O_2 in the medium more than normal mMRS medium. Figure 7 shows examples for different oxidative stress conditions growth behaviour of *L. plantarum* strain triples isolated from Mother/baby-pairs group strains (1.1602, 1.1607 and 1.1611), other intestinal strains of different LAB species (1.1567, 1.1688, 1.1716 and 1.1784) and *Bifidobacterium* strain from strain collection (2.447).

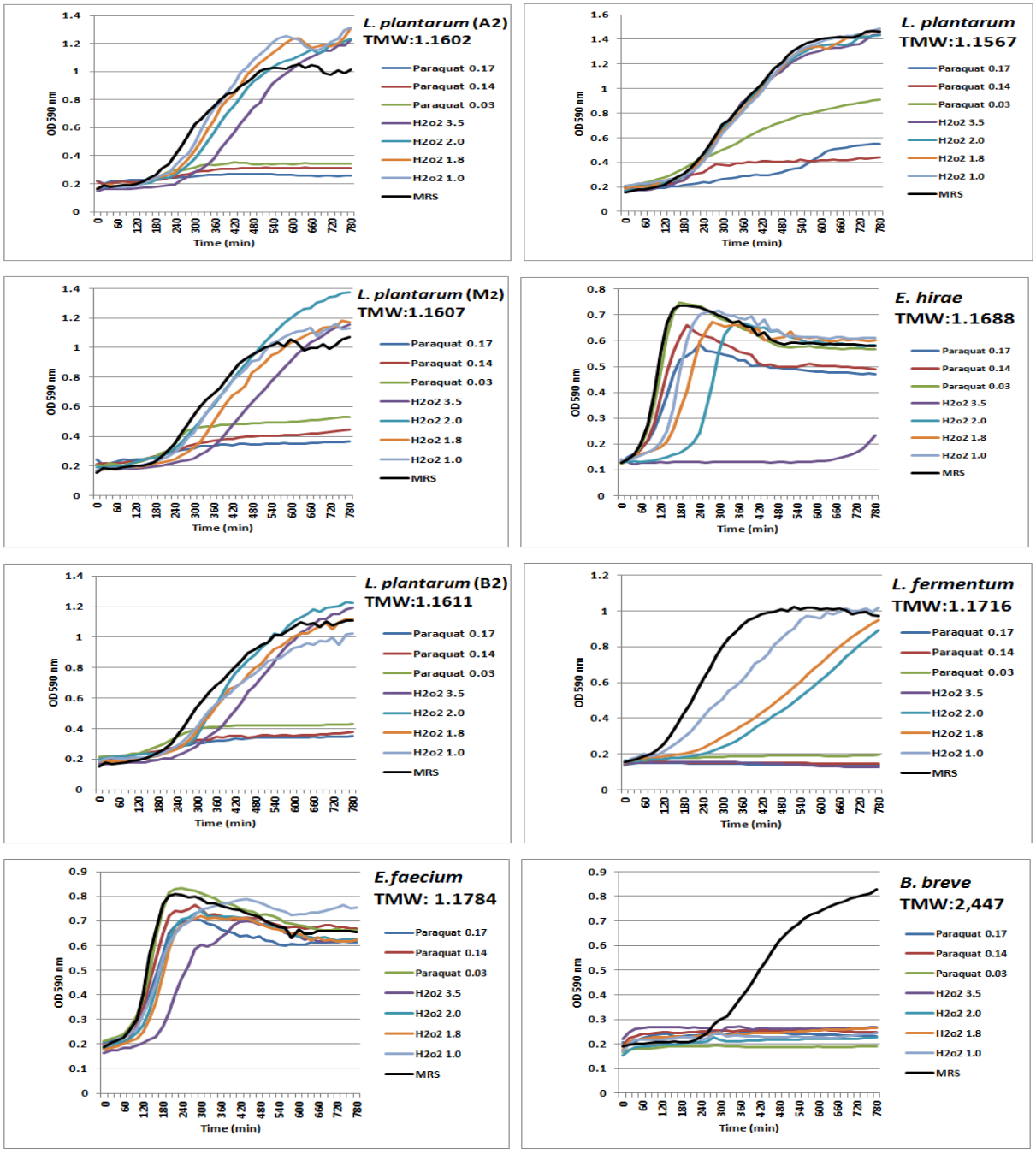


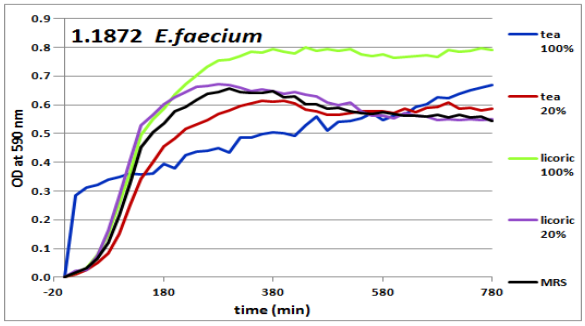
Figure 7. Growth curves in different LAB species at different oxidative stress conditions. For *L. plantarum* strains of mother/baby-pairs groups (1.1602, 1.1607, 1.1611) (Mn) refers to milk strain, (Bn) refers to baby strain, (An) refers to mother strain and (n) refer to the group number.

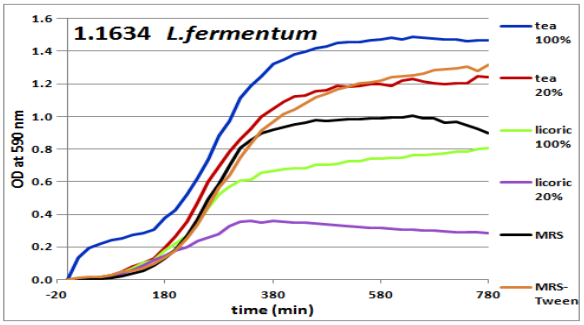
	Group	Name	Species	TMW	Source	Mild acidity	High acidity	Porcin Bile	Bovin Bile	H2O2	Paraquat
1	1	M5m3	L.plantarum	1.1671	milk	+ + +	+ + +	+ + +	+ + +	+	±
2	1	B5m77	L.plantarum	1.1679	baby	+ +	+ + +	+ + +	+ + +	+	±
3	1	A5r11	L.plantarum	1.1667	mother	+ +	+ + +	+ + +	+ + +	+	±
4	2	M0s2*	L.plantarum	1.1607	milk	+	+	+ + +	+ +	+ + +	+
5	2	B0s9	L.plantarum	1.1611	baby	+	+	+ + +	+ +	+ +	+
6	2	A0s4	L.plantarum	1.1602	mother	+	+	+ + +	+ +	+ + +	+
7	3	M1r1*	L.plantarum	1.1623	milk	+	+ +	+ + +	+ + +	+ +	+
8	3	B1m9	L.plantarum	1.1628	baby	+	+ +	+ + +	+ + +	+ +	±
9	3	A1m8	L.plantarum	1.1616	mother	+	+ +	+ + +	+ + +	+ +	±
10	4	M6s4	L.plantarum	1.1690	milk	+	±	+ + +	+ +	+	+
11	4	B6s5*	L.plantarum	1.1693	baby	+	±	+ + +	+ +	+	+ + +
12	4	A6s14	L.plantarum	1.1683	mother	+ +	+	+ + +	+ +	+	+
13	5	M0s1*	L.plantarum	1.1608	milk	+ +	+	+ + +	+ + +	+	±
14	5	B0s10	L.plantarum	1.1609	baby	+ +	+	+ + +	+ + +	±	±
15	5	A0m5	L.plantarum	1.1603	mother	+ +	+ +	+ + +	+ + +	+	±
16	6	A3m5	L.plantarum	1.1647	mother	+ +	±	+ + +	+ +	+	+
17	6	B3r10	L.plantarum	1.1654	baby	+	±	+ + +	+ +	+	+
18	7	A4m7	L.plantarum	1.1656	mother	+	±	+ +	±	+	±
19	7	B4r10	L.plantarum	1.1662	baby	+	±	+	±	+	+
20	8	Rb9	L.plantarum	1.1860	baby	+	±	+ + +	+ + +	+	+
21	8	Mb'2	L.plantarum	1.1509	milk	+ +	+	+ + +	+ + +	+	+
22	9	Td'1	L.plantarum	1.1516	milk	+ +	±	+ + +	+ +	+	+
23	9	Cd11	L.plantarum	1.1864	baby	+	+	+ + +	+ +	+	+
24	10	M7m4	L.fermentum	1.1709	milk	+ +	±	-	±	±	±
25	10	B7s3	L.fermentum	1.1716	baby	+ +	±	-	±	±	±
26	11	M2r5	L.fermentum	1.1634	milk	+ + +	+ +	-	±	±	±
27	11	B2s8	L.fermentum	1.1640	baby	+ + +	+ +	-	±	+	±
28	12	So'9	L.fermentum	1.1552	milk	+ +	+ +	-	±	+	-
29	12	Pk9*	L.fermentum	1.0794	baby	+ +	+ + +	±	±	+	±
30	13	So2	L.rhamnosus	1.1548	mother	+ +	±	-	±	+ +	±
31	13	Pk10	L.rhamnosus	1.1538	baby	+ +	+	-	±	+	+ +
32	14	M2s2	L. oris	1.1635	milk	+ +	+	-	±	+ +	+ +
33	14	B2s13*	L. oris	1.1639	baby	+ +	+	-	±	+ +	+ +
34	15	Rz12	L.casei	1.1870	mother	+	-	-	±	±	+ +
35	15	Rp20	L.casei	1.1871	child	+	+	-	±	±	+
36	16	Md'2	L.brevis	1.1517	milk	+ +	+ +	+ +	+	±	+ +
37	16	Cd9	L.brevis	1.1863	baby	+ +	+ +	+ +	+	±	+ +
38	17	M4m1	E.faecalis	1.1661	milk	+	-	+ +	+ + +	±	+ + +
39	17	B4m8	E.faecalis	1.1665	baby	+	-	+ +	+ + +	±	+ + +
40	18	Bp7*	E.faecium	1.1872	child	+	±	+ + +	+ + +	+	+ + +
41	18	Mz11	E.faecium	1.1873	mother	+	-	+ +	+ + +	±	+ + +
42	19	M6m10	E. hirae	1.1685	milk	+	-	+ +	+ + +	±	+ +
43	19	B6m11	E. hirae	1.1695	baby	+	-	+ +	+ + +	±	+ +
44	20	Ce'1	P.pentosaceus	1.1522	milk	+	+	±	±	+	+
45	20	Re10*	P.pentosaceus	1.1865	baby	+	-	±	±	+ +	+ + +
46	21	Sk16	L.plantarum	1.1793	baby	+	+ +	+ + +	+ +	+ +	+ + +
47	21	(Mo'1)	L.plantarum	1.1798	milk	+ +	+ + +	+ + +	+ +	+ +	+ +
48	22	Sb'3	L.plantarum	1.1861	milk	+ +	±	+ + +	+ +	+	+
49	22	Mb4	L.plantarum	1.1862	baby	+ +	±	+ + +	+ +	+	+
50	23	M1s4	L.plantarum	1.1617	milk	+ +	+	+ + +	+ +	+	-
51	23	B1m10	L.plantarum	1.1625	baby	+	±	+ + +	+ +	+	-
52	24	Mz10	L.plantarum	1.1867	mother	+ +	+ +	+ + +	+ + +	+	+
53	24	Sq19	L.plantarum	1.1868	child	+	+	+ + +	+ + +	+	+
54	24	Sp13	L.plantarum	1.1869	child	+	+	+ + +	+ + +	+	+

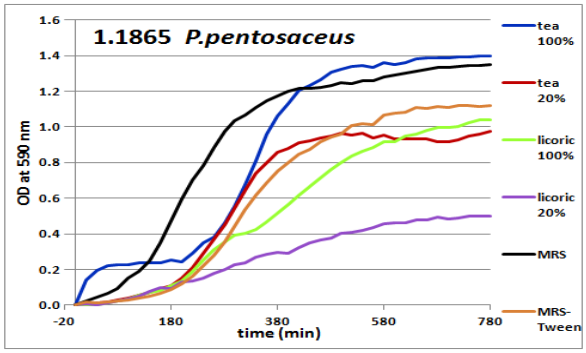
Table 9. General growth ability of mother/baby-pairs groups strains when exposed to different stress condition; the used symbols are respectively in, acid (pH=5 is mild acidic and pH=4-3-2-1 are high acidic), bile (porcine and bovine) salts and oxidative stress (H_2O_2 and paraquat) conditions refers to (Standard condition is mMRS at pH=7 without any addition):
(+++) for acidic: very well growth at pH 5 – can grow at pH 4-3-2-1, for bile: enhance the growth more than standard condition, for oxidative condition: grow well at all concentrations
(++) for acidic: well growth at pH 5 – can grow at pH 4-3-2 only, for bile: grow like in standard condition, for oxidative condition: can grow at all concentrations
(+) for acidic: can grow at pH 5 – can grow at pH 4-3 only, for bile: grow less than standard condition, for oxidative condition: can grow only at less than the first concentration,
(±) for acidic: weak grow at pH 5 – can grow at pH 4 only, for bile and oxidative condition: very weak growth or grow only at less than the second concentration
(-) inhibited growth, can't grow and can't grow at any concentration.

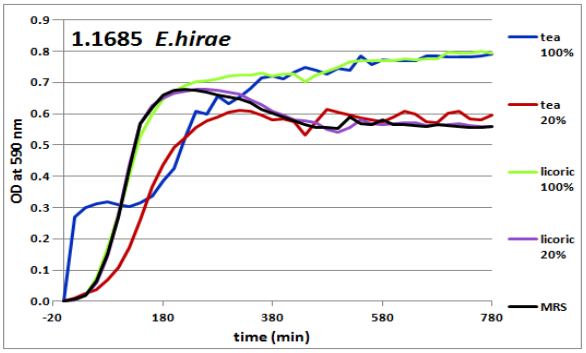
– **Growth behaviour in black tea and licoric media and their polyphenols**

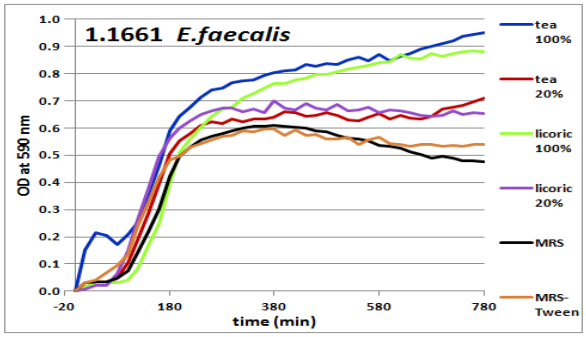
Different lactic acid bacteria strains express different growth behaviour under tea and licoric mMRS media comparing to the growth in normal mMRS medium. Unexpectedly, tea and licoric media enhanced the growth of some LAB species like *L. plantarum, L. fermentum, E. faecium, E. faecalis* and *E. hirae* while they have a weakening effect on growth of other species like *L. brevis, L. casei, P. pentosaceus* and *Bifidobacteria*. *Bifidobacteria* strains also couldn't grow in mMRS media, without tween 80 while other species did. Different strains in one species are variable in their resistance and growth tolerance but in general they express similar tendency differentiate them from the growth ability of strains belong to other species. Interestingly 100% tea media showed unusual influence on the growth behaviour of strains belong to all species, all strains which grow in tea 100% media had induce sudden jump or increase in the cell growth directly after inoculation which lead to jump directly to the log phase and escape the lag phase in the normal bacteria growth behaviour. Actually the strains didn't escape the lag phase of growth completely, it postponed the lag phase and started the growth with semi log phase for the first 40 to 60 min then inter in a short lag phase, then continue the log phase to reach the stationary phase with higher cell density or mass than in normal condition for the above mentioned species.

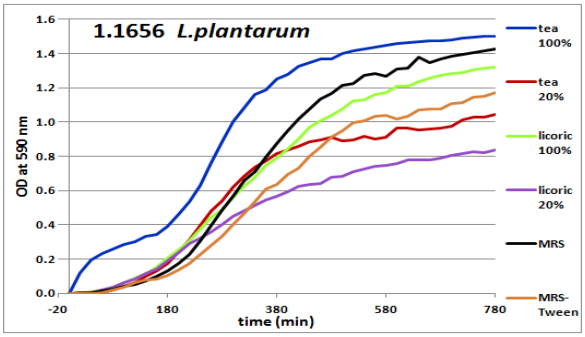












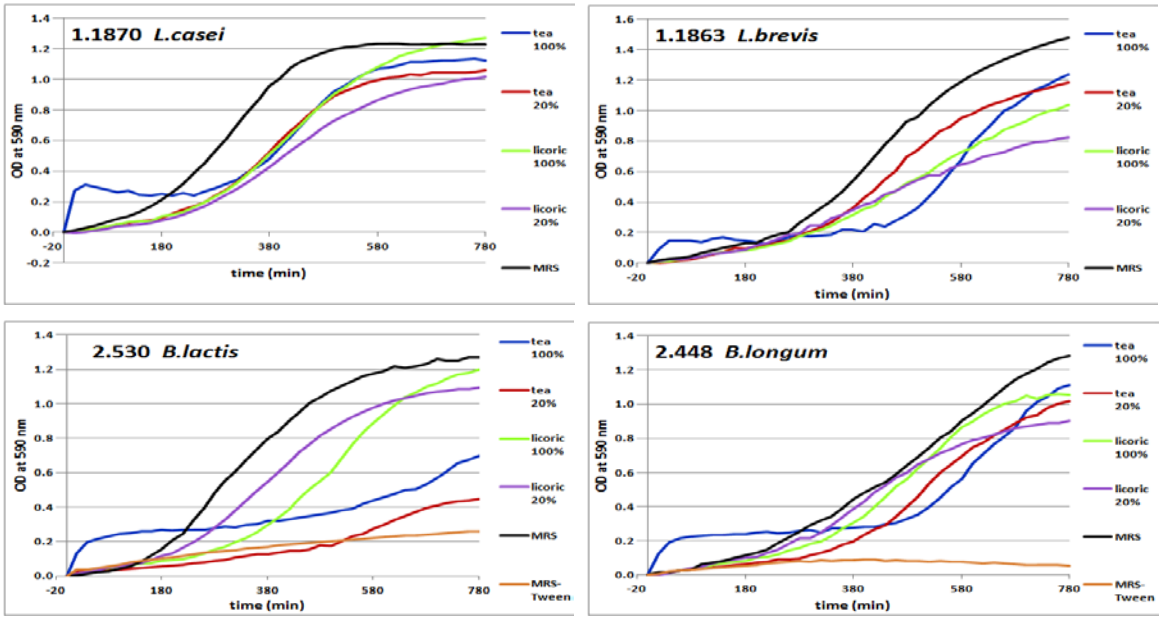


Figure 8. Growth curves in different LAB species at different tea and licoric media.

HPLC analysis of tea and licoric solutions amino acids compounds showed very poor content of different amino acids except gamma amino buytric acid (Gaba) in tea in (Fig 9) and alanine (Ala) in licoric (Fig 10) which showed exceptional high content than the other amino acids.

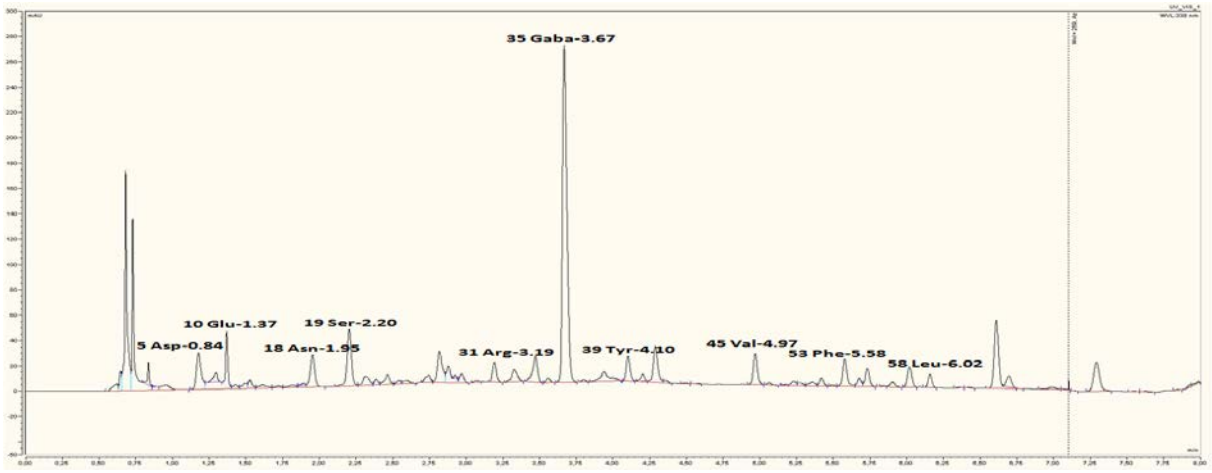


Figure 9. HPLC chromatogram and retention times of the amino acid standard AAS18 in tea

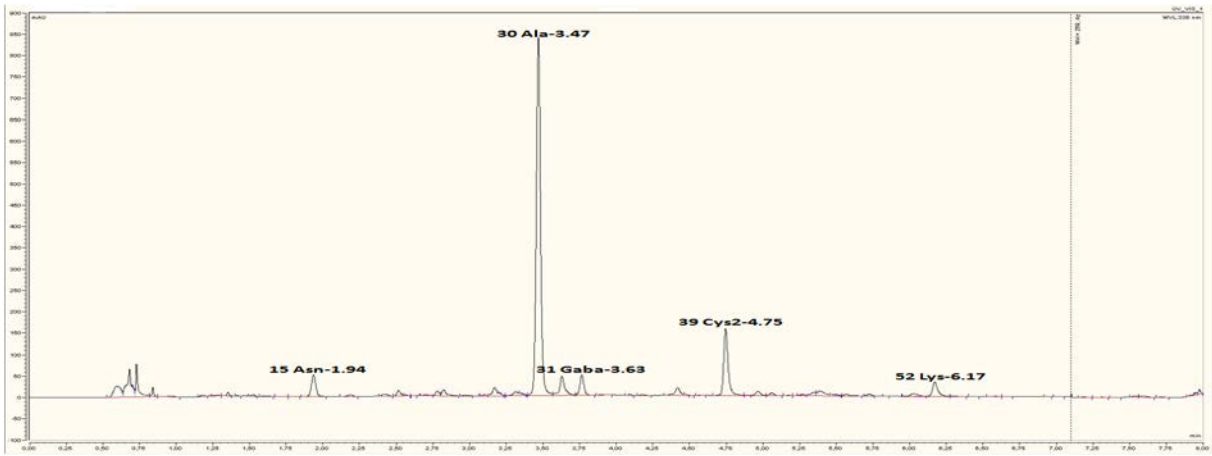


Figure 10. HPLC chromatogram and retention times of the amino acid standard AAS18 in licoric

To understand which of the tea components give this special interesting effect on bacteria growth initiation, the growth behavior was tested in different concentration of different tea polyphenols and the result showed no influence for quercetin and epigallocatechin gallate while (+)catechin hydreat and (±)catechin hydreat had showed slight influence at the concentrations: (2.25 – 2.50 – 2.27 – 3 – 3.25) mg/ml. (-)-epicatechin showed highest initial growth inducing at all concentration (2.25 – 2.50 – 2.27 – 3 – 3.25 – 3.50 – 3.75 – 4.0) mg/ml for all LAB species, very similarly to the tea medium influence.

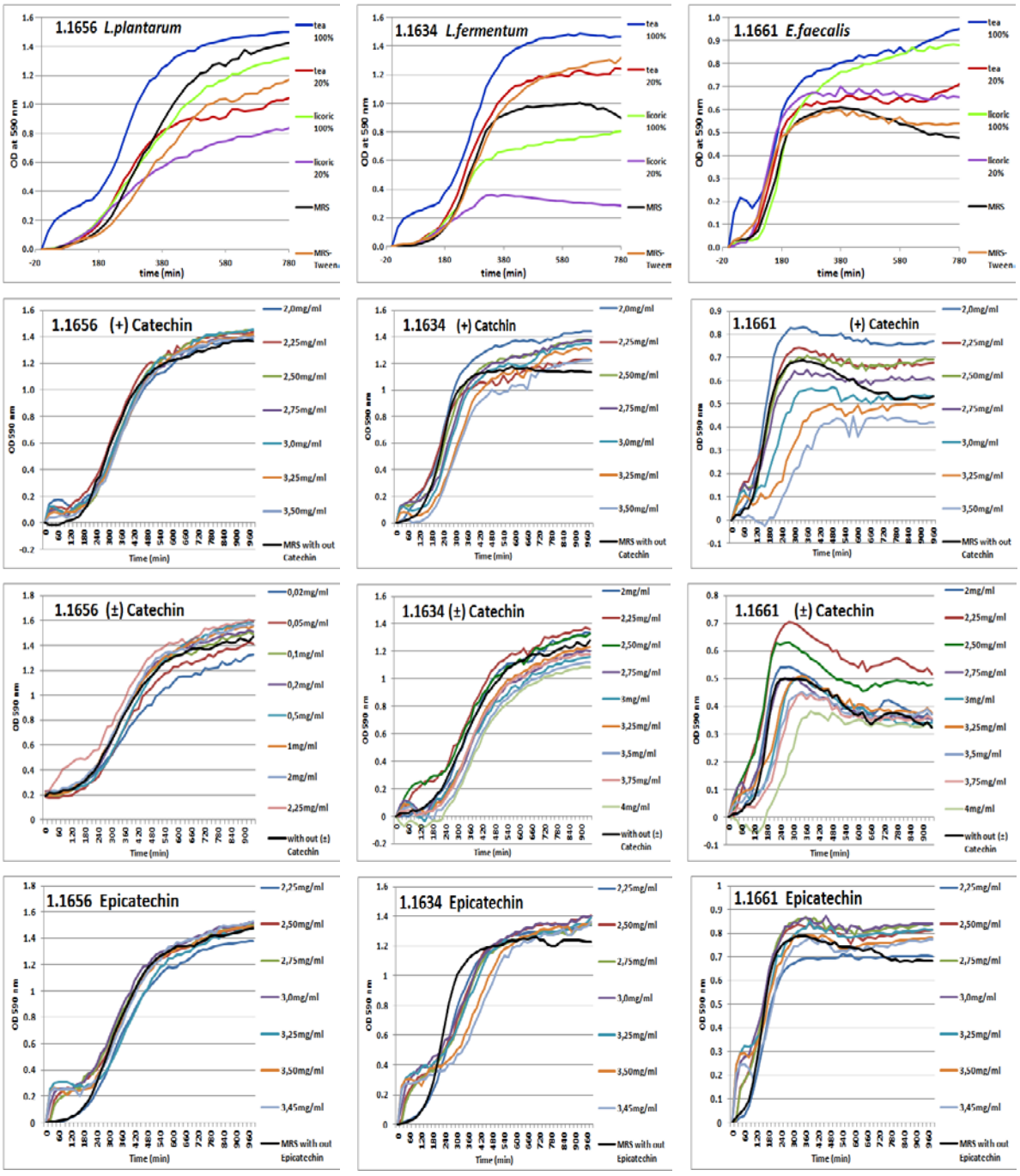


Figure 11. Growth curve diagrams of three LAB strains (*L. plantarum, L. fermantum* and *E. faecalis*) when they grow in different media containing different concentrations of different tea polyphenols. The diagrams of different polyphenols mediums are ordered from top to bottom as (in the top the growth curves in complete tea medium, (+)catechin, (±)catechin and at last in epicatechin medium)

Comparative study for some oxidative stress resistance mechanisms, production of antagonistic metabolites, produce of secondary metabolites and enzymes:

Table (10) and (11) summarize the results of different tests about oxidative stress resistance mechanisms (catalase - NADH oxidase – SOD - GR), production of antagonistic metabolites (hydrogen peroxide – bacteriocine), produce metabolites and enzymes (α-galactosidase & β-galactosidase - exoenzyme caseinase production) on 90 different LAB strains including the 54 mother/baby-pairs group's strains.

	Group	Name	Species	TMW	Source	Cata	NADH	SOD	GR	H2O2	Bacteriocine	α galacto	β galacto	Casienase
1	1	M5m3	L.plantarum	1.1671	milk	-	+	-	-	+	+	-	+	+
2	1	B5m77	L.plantarum	1.1679	baby	-	+	-	-	+	+	-	+	++
3	1	A5r11	L.plantarum	1.1667	mother	-	+	-	-	+	+	-	+	-
4	2	M0s2	L.plantarum	1.1607	milk	+	+	-	-	+	+	-	+	+
5	2	B0s9	L.plantarum	1.1611	baby	+	+	-	-	+	+	-	+	+
6	2	A0s4	L.plantarum	1.1602	mother	+	+	-	-	+	+	-	+	-
7	3	M1r1	L.plantarum	1.1623	milk	-	+	-	-	+	++	-	-	+
8	3	B1m9	L.plantarum	1.1628	baby	-	+	-	-	+	++	-	-	+
9	3	A1m8	L.plantarum	1.1616	mother	-	+	-	-	+	++	-	-	-
10	4	M6s4	L.plantarum	1.1690	milk	+	+	-	-	+	+	-	-	+
11	4	B6s5	L.plantarum	1.1693	baby	+	+	-	-	+	+	-	-	++
12	4	A6s14	L.plantarum	1.1683	mother	+	+	-	-	+	+	-	-	-
13	5	M0s1	L.plantarum	1.1608	milk	-	+	-	-	++	++	-	-	++
14	5	B0s10	L.plantarum	1.1609	baby	-	+	-	-	++	++	-	-	+
15	5	A0m5	L.plantarum	1.1603	mother	-	+	-	-	++	++	-	-	+
16	6	A3m5	L.plantarum	1.1647	mother	+	+	+	-	++	++	-	-	+
17	6	B3r10	L.plantarum	1.1654	baby	+	+	+	-	++	++	-	-	+
18	7	A4m7	L.plantarum	1.1656	mother	+	+	+	-	+	+	-	-	-
19	7	B4r10	L.plantarum	1.1662	baby	+	+	+	-	+	+	-	-	-
20	8	Rb9	L.plantarum	1.1860	baby	+	+	+	-	+	++	-	-	+
21	8	Mb'2	L.plantarum	1.1509	milk	+	+	+	-	+	++	-	-	+
22	9	Td'1	L.plantarum	1.1516	milk	+	+	+	-	-	++	-	-	+
23	9	Cd11	L.plantarum	1.1864	baby	+	+	+	-	-	++	-	-	+
24	10	M7m4	L.fermentum	1.1709	milk	-	-	-	+	-	-	+	+	+
25	10	B7s3	L.fermentum	1.1716	baby	-	-	-	+	-	-	+	+	+
26	11	M2r5	L.fermentum	1.1634	milk	-	-	-	+	-	-	+	+	-
27	11	B2s8	L.fermentum	1.1640	baby	-	-	-	+	-	-	+	+	+
28	12	So'9	L.fermentum	1.1552	milk	-	-	-	+	-	+	+	+	-
29	12	Pk9	L.fermentum	1.0794	baby	-	-	-	+	-	+	+	+	-
30	13	So2	L.rhamnosus	1.1548	mother	-	-	+	-	+	+	+	+	+
31	13	Pk10	L.rhamnosus	1.1538	baby	-	-	+	-	+	+	+	+	++
32	14	M2s2	L. oris	1.1635	milk	+	+	-	-	++	-	+	+	-
33	14	B2s13	L. oris	1.1639	baby	+	+	-	-	++	-	+	+	-
34	15	Rz12	L.casei	1.1870	mother	-	+	+	-	-	+	+	+	+
35	15	Rp20	L.casei	1.1871	child	-	+	+	-	-	+	+	+	+
36	16	Md'2	L.brevis	1.1517	milk	+	+	-	+	++	+++	+	+	-
37	16	Cd9	L.brevis	1.1863	baby	+	+	-	+	++	++	+	+	+
38	17	M4m1	E.faecalis	1.1661	milk	+	-	-	++	+++	-	-	-	-
39	17	B4m8	E.faecalis	1.1665	baby	+	-	-	++	+++	-	-	-	-
40	18	Bp7	E.faecium	1.1872	child	-	+	+	++	++++	-	-	-	-
41	18	Mz11	E.faecium	1.1873	mother	-	+	+	++	++++	-	-	-	-
42	19	M6m10	E. hirae	1.1685	milk	-	-	-	++	-	-	-	-	-
43	19	B6m11	E. hirae	1.1695	baby	-	-	-	+++	-	-	-	-	-
44	20	Ce'1	P.pentosaceus	1.1522	milk	-	+	-	+	-	-	+	+	-
45	20	Re10	P.pentosaceus	1.1865	baby	-	+	-	+	-	-	+	+	-
46	21	Sk16	L.plantarum	1.1793	baby	+	+	+	+	+	++	-	-	+
47	21	(Mo'1)	L.plantarum	1.1798	milk	+	+	+	+	+	++	-	-	+
48	22	Sb'3	L.plantarum	1.1861	milk	-	+	+	-	+	++	+	+	+
49	22	Mb4	L.plantarum	1.1862	baby	-	+	+	-	+	++	+	+	+
50	23	M1s4	L.plantarum	1.1617	milk	+	+	-	-	+	+	-	-	-
51	23	B1m10	L.plantarum	1.1625	baby	+	+	-	-	+	+	-	-	-
52	24	Mz10	L.plantarum	1.1867	mother	-	+	+	-	+	++	-	-	+
53	24	Sq19	L.plantarum	1.1868	child	-	+	+	-	+	++	-	-	+
54	24	Sp13	L.plantarum	1.1869	child	-	+	+	-	+	++	-	-	+

Table 10. Presence of different oxidative stress resistance mechanisms in mother/baby-pairs groups strains (catalase - NADH oxidase – superoxide dismutase SOD - glutathione reductase GR), ability to produce different antagonistic metabolites (hydrogen peroxide – bacteriocins), and ability to produce metabolites and enzymes (α-galactosidase & β-galactosidase - exoenzyme caseinase production)

	Name	Species	TMW	Source	Cata	NADH	SOD	GR	H2O2	Bacteriocine	α galacto	β galacto	Casienase
55	Ma4	L.brevis	1.1503	baby	+	+	-	+	-	-	+	+	+
56	Tv7	L.mucosae	1.1741	adult	-	+	+	+	+	-	+	+	-
57	Ph12	L.fermentum	1.1743	baby	-	-	-	+	-	-	+	+	-
58	Cc8	E.faecalis	1.1510	baby	-	-	-	+	-	-	+	+	+
59	Pg5	L.fermentum	1.1527	baby	-	-	-	+	-	-	+	+	-
60	TL9	L.plantarum	1.1541	baby	-	+	+	-	+	++	+	+	+
61	Sw10	L.plantarum	1.1571	adult	-	+	+	-	+	+	-	+	-
62	Md1	L.plantarum	1.1748	baby	+	+	-	-	-	+	+	+	+
63	Fe14	L.brevis	1.1750	baby	+	+	-	++	++	-	+	+	-
64	Mu11	L.brevis	1.1568	adult	+	+	-	+	+	+	+	+	-
65	Cn9	L.brevis	1.1744	adult	+	+	-	+	+	++	+	+	+
66	Ff3	L.brevis	1.1526	baby	+	+	-	+	+	-	+	+	-
67	Pj11	L.casei	1.1751	baby	-	+	+	-	-	+	+	+	-
68	Mj18	L.fermentum	1.1534	baby	-	-	-	++	-	-	+	+	-
69	Th6	P.pentosaceus	1.1531	baby	-	+	-	+	+	-	+	+	+
70	Tr5	E.faecium	1.1556	adult	-	+	+	++	++	-	-	-	-
71	Tj2	E.faecium	1.1536	baby	-	+	+	+	+++	-	-	+	-
72	Mo'2	L.brevis	1.1859	milk	+	+	-	-	+	++	+	-	+
73	Ca'22	E.faecium	1.1858	milk	-	+	+	+++	+++	-	-	-	-
74	Sf13	L.salivarius	1.1524	baby	-	-	+	+++	+	-	+	+	-
75	A5r9	L.salivarius	1.1666	adult	-	-	+	+++	-	-	-	-	+
76	B1s1	L.fermentum	1.1624	baby	-	-	-	+	-	+	+	+	+++
77	M6m8	E. hirae	1.1688	milk	+	+	-	-	+	+	-	-	+
78	A5s12	L.brevis	1.1669	adult	+	+	-	+	++	-	+	+	+
79	B4m9	L.gasseri	1.1663	baby	-	-	-	+	-	-	+	+	-
80	B6s66	E.mundtii	1.1696	baby	-	+	-	+++	-	-	-	-	-
81	B7s1	L.rhamnosus	1.1715	baby	-	-	+	-	+	+	+	-	+
82	M1s3	L.plantarum	1.1619	milk	+	+	-	-	+	++	-	+	+
83	M3m22	E.faecium	1.1650	milk	-	-	+	+	+	-	-	-	-
84	M2m3	L.plantarum	1.1636	milk	+	+	-	-	+	+++	-	-	+
85	M7s2	E.faecium	1.1708	milk	-	-	+	++	+++	-	+	-	++++
86	B2s7	L.reuteri	1.1642	baby	-	+	-	-	++	++	-	+	-
87	M2s11	L.plantarum	1.1633	milk	-	+	+	-	-	+	-	-	+
88	A2s15	L.plantarum	1.1631	adult	+	+	+	-	++	+	-	-	+
89	Ca17	E.faecalis	1.1773	baby	+	-	-	++	-	-	+	-	-
90	Fs6	E.faecium	1.1784	Adult	+	-	+	++	++	-	+	-	-

Table 11. Different LAB strains possession of different oxidative stress resistance mechanisms (catalase (Cata) - NADH oxidase – superoxide dismutase (SOD) and glutathione reductase (GR)), ability to produce different antagonistic metabolites (hydrogen peroxide – bacteriocine), ability to produce metabolites and enzymes (α-galactosidase & β-galactosidase - exoenzyme caseinase production)

PCR screening for eps genes:

All control strains displayed amplicons of the expected band size after PCR amplification with the corresponding primer pairs. Screening for eps genes revealed negative results for glucansucrase genes for all strains, while the screening for putative fructansucrase genes revealed some positive strains, belonging to the species *L. rhamnosus* (2/1.1548, 1.1538 strains), *E. faecalis* (2/1.1661, 1.1665 strains), *E. hirae* (2/1.1685, 1.1695 strains), *P. pentosaceus* (2/1.1522, 1.1865 strains) and *L. reuteri* (1/1.1642 strain). However these strains have no ability to produce exopolysaccharide on high glucose concentration medium.

Caseinase production

Caseinase production test revealed an exceptional behaviour for mother/baby-pair groups strains as compared with all the other tested properties, for all previous tested prosperities strains (from the three sources) of each group always possessed similar behaviour while caseinase production trait appear to be expressed in the milk and baby strains and absent in

the mother strain (for the first four mother/baby-pair groups). All *L. plantarum* strains, which were identified by MALDI-TOF as *L. pentosus* had caseinase production in all sources (adults baby milk and fermented food) while *L. plantarum* identified by MALDI-TOF showed caseinase production activity in all baby and milk strain and absence in adults strains. Also, *L. casei* identified as *L. paracasei* displayed caseinase production in all sources (Adults baby milk and fermented food) while *L. casei* identified by MALDI-TOF showed caseinase production activity in all baby and milk strain and absence in adults strains. *L. fermentum, L. rhamnosus, L. brevis, E. faecalis* and *E. faecium* had activity in some strains (mostly milk and baby strains) and did not have it in another. The milk strain *E. faecium* TMW 1.1708 expressed the highest production, even higher than the controls.

Figure 12 shows special unusual behaviour of faecal and milk bacteria cultures which can be explained with our recent date.

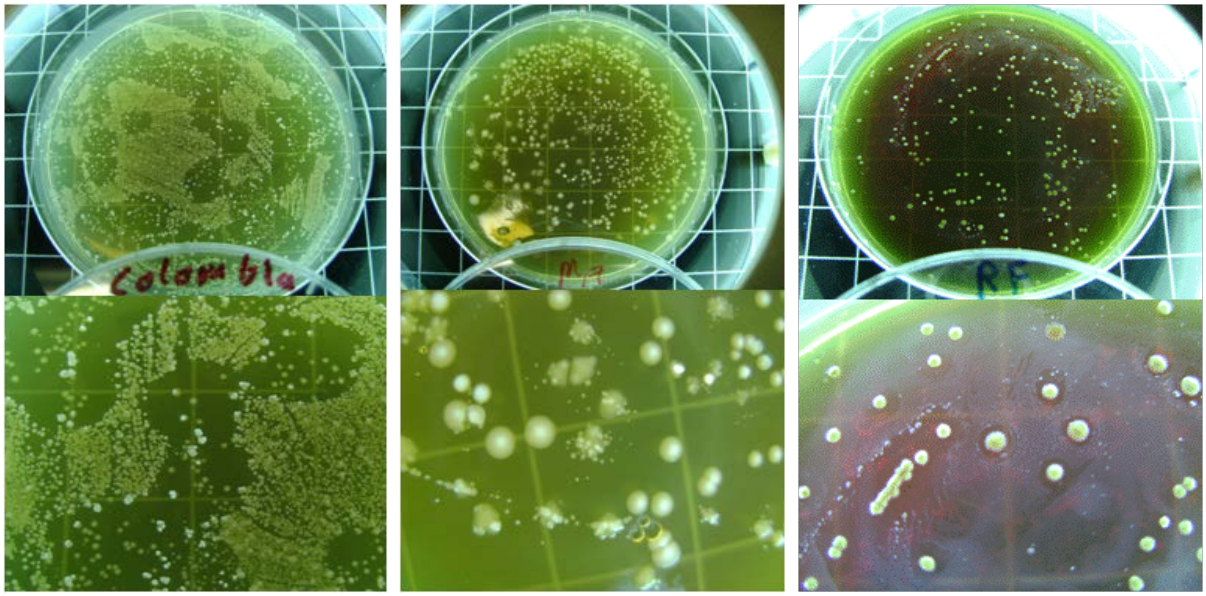


Figure 12. Photos took for the primer (first isolation work which mentioned in the first chapter) faecal and milk samples cultured on different specific media, expressing different interesting bacteria (competition or mutualistic) behaviours which can be explained with our recent results. The photo in the left presenting culture of different intestinal bacteria species with special inhibition behaviour of strains able to produce antagonistic metabolites, the photo in the middle presenting culture of different faecal bacteria species with special synergy activity (producing secondary metabolites) which enhance the growth of other species around the producing colony. The photo in the right presenting culture of different milk bacteria species with special milk protein hydrolysing ability (caseinase) formed clear zone around the colony.

Antimicrobial susceptibility test:

Antimicrobial susceptibility test for a set of different antibiotics ampicillin, aureomycin, chloramphenicol, tetracyclin , valinomycin , paromomycin , kanamycin , streptomycin and erythromycin were proceeded for mother/baby-pairs groups strains only. Table 12 summarizes all the results and present identical sensitivity behaviour to different antibiotics in every trio or pair of mother/baby-pairs group's strains. The child strain *E. faecium* 1.1872

showed high resistance to most antibiotics except tetracyclin, erythromycin and chloramphenicol.

	Group	Name	Species	TMW	Source	Ampiciline	Aureo*	Cloramphenicol	Tetracyclin	Erythro*	Valino*	Paromo*	Strepto*	Kana*
1	1	M5m3	L.plantarum	1.1671	milk	***	*	**	*	***	-	-	-	-
2	1	B5m77	L.plantarum	1.1679	baby	***	*	**	*	***	-	-	-	-
3	1	A5r11	L.plantarum	1.1667	mother	***	*	**	*	***	-	-	-	-
4	2	M0s2	L.plantarum	1.1607	milk	***	**	***	**	***	-	*	-	-
5	2	B0s9	L.plantarum	1.1611	baby	***	**	***	**	***	-	*	-	-
6	2	A0s4	L.plantarum	1.1602	mother	***	**	***	**	***	-	*	-	-
7	3	M1r1	L.plantarum	1.1623	milk	***	**	**	**	***	-	-	-	-
8	3	B1m9	L.plantarum	1.1628	baby	***	**	**	**	***	-	-	-	-
9	3	A1m8	L.plantarum	1.1616	mother	***	**	**	**	***	-	-	-	-
10	4	M6s4	L.plantarum	1.1690	milk	***	**	***	**	***	-	*	-	-
11	4	B6s5	L.plantarum	1.1693	baby	***	**	***	**	***	-	*	-	-
12	4	A6s14	L.plantarum	1.1683	mother	***	**	***	**	***	-	*	-	-
13	5	M0s1	L.plantarum	1.1608	milk	***	**	***	**	***	-	-	-	-
14	5	B0s10	L.plantarum	1.1609	baby	***	**	***	**	***	-	-	-	-
15	5	A0m5	L.plantarum	1.1603	mother	***	**	***	**	***	-	*	-	-
16	6	A3m5	L.plantarum	1.1647	mother	***	**	**	**	**	-	-	-	-
17	6	B3r10	L.plantarum	1.1654	baby	***	**	**	**	**	-	-	-	-
18	7	A4m7	L.plantarum	1.1656	mother	***	**	***	**	***	-	*	-	-
19	7	B4r10	L.plantarum	1.1662	baby	***	**	***	**	***	-	*	-	-
20	8	Rb9	L.plantarum	1.1860	baby	***	**	**	**	**	-	-	-	-
21	8	Mb'2	L.plantarum	1.1509	milk	***	**	**	**	**	-	-	-	-
22	9	Td'1	L.plantarum	1.1516	milk	***	*	**	*	**	-	-	-	-
23	9	Cd11	L.plantarum	1.1864	baby	***	*	**	*	**	-	-	-	-
24	10	M7m4	L.fermentum	1.1709	milk	****	***	***	**	***	-	**	-	-
25	10	B7s3	L.fermentum	1.1716	baby	****	***	***	**	***	-	-	-	-
26	11	M2r5	L.fermentum	1.1634	milk	***	**	***	**	***	*	*	-	-
27	11	B2s8	L.fermentum	1.1640	baby	***	**	***	**	**	-	*	-	-
28	12	So'9	L.fermentum	1.1552	milk	***	**	**	**	***	-	*	-	-
29	12	Pk9	L.fermentum	1.0794	baby	***	**	***	**	***	-	-	-	-
30	13	So2	L.rhamnosus	1.1548	mother	***	*	**	*	**	-	-	-	-
31	13	Pk10	L.rhamnosus	1.1538	baby	***	***	***	***	**	-	-	-	-
32	14	M2s2	L. oris	1.1635	milk	***	***	***	**	***	-	*	-	-
33	14	B2s13	L. oris	1.1639	baby	***	***	***	**	***	-	*	-	-
34	15	Rz12	L.casei	1.1870	mother	***	***	***	***	***	-	-	-	-
35	15	Rp20	L.casei	1.1871	child	***	***	***	***	***	-	-	-	-
36	16	Md'2	L.brevis	1.1517	milk	***	**	***	**	***	-	*	-	-
37	16	Cd9	L.brevis	1.1863	baby	***	**	***	**	***	-	*	-	-
38	17	M4m1	E.faecalis	1.1661	milk	***	**	***	*	**	-	-	-	-
39	17	B4m8	E.faecalis	1.1665	baby	***	**	***	*	**	-	-	-	-
40	18	Bp7*	E.faecium	1.1872	child	-	*	**	*	-	-	-	-	-
41	18	Mz11	E.faecium	1.1873	mother	**	***	***	***	*	-	-	-	-
42	19	M6m10	E. hirae	1.1685	milk	**	***	***	***	**	-	-	-	-
43	19	B6m11	E. hirae	1.1695	baby	**	***	***	***	**	-	-	-	-
44	20	Ce'1	P.pentosaceus	1.1522	milk	**	**	***	**	***	-	-	-	-
45	20	Re10	P.pentosaceus	1.1865	baby	**	**	***	**	***	-	-	-	-
46	21	Sk16	L.plantarum	1.1793	baby	***	**	**	**	**	-	-	-	-
47	21	(Mo'1)	L.plantarum	1.1798	milk	***	**	**	**	**	-	-	-	-
48	22	Sb'3	L.plantarum	1.1861	milk	**	**	***	**	***	-	-	-	-
49	22	Mb4	L.plantarum	1.1862	baby	***	**	**	**	***	-	-	-	-
50	23	M1s4	L.plantarum	1.1617	milk	**	**	***	**	**	-	-	-	-
51	23	B1m10	L.plantarum	1.1625	baby	***	**	***	**	**	-	-	-	-
52	24	Mz10	L.plantarum	1.1867	mother	***	**	**	**	**	-	-	-	-
53	24	Sq19	L.plantarum	1.1868	child	***	**	**	**	**	-	-	-	-
54	24	Sp13	L.plantarum	1.1869	child	***	**	**	**	**	-	-	-	-

Table 12. Sensitivity test for different mother/baby-pairs group's strains to different antibiotics, (antibiotic name abbreviation*) means the name of the antibiotic should be followed with –mycin, (*) refer to the degree of sensitivity as more (*) as more sensitive, (-) refer to insensitive.

Comparison of virulence gene profiles for Enterococcus isolates

All strains from milk and fecal origin possessed up to 10 virulence genes (Table 13), with the most common genes including *ace, agg, efaAfs, esp, fsrB gelE, sprE, cyl* and the pheromones *cob*. Interestingly, most of the strains which have the virulence genes *gelE* and *sprE* don't have the regulatory gene *fsrB* for the two previous genes. For those strains that possessed genes for both *gelE* and *fsrB* the gelatinase test shows no zones of clearing around colonies after 18 h incubation at 37 ^{o}C. Of the *E. faecalis* strains of interest, only two strains TMW 1.1574, 1.1511 possessed both *fsrB* and *gelE* genes. The milk strain *E. faecium* TMW 1.1760 only possessed the gelE gene but no gelatinase activity.

Name	Speciese	TMW-Nu	Source	ace	agg	cob	efaAfs/m	esp	fsr B	gel E	spr E	cyl M	cyl B	cyl A	Gelatinase prodaction
Bb6	E. faecalis	1.1774	feaces	+	-	+	+	+	-	+	+	-	-	-	-
Tc6	E. faecalis	1.1770	feaces	+	+	+	+	+	-	+	+	-	-	-	-
Ca17	E. faecalis	1.1773	feaces	+	-	+	+	+	-	+	+	-	-	-	-
Cg8	E. faecalis	1.1528	feaces	+	+	+	+	+	-	+	+	-	-	-	-
Bx8	E. faecalis	1.1574	feaces	+	-	+	+	-	+	+	+	-	-	-	-
Pr14	E. faecalis	1.1772	feaces	+	+	+	+	+	-	+	+	-	-	-	-
Wy15	E. faecalis	1.1575	feaces	+	+	+	+	+	-	+	+	+	+	+	-
Ci5	E. faecalis	1.1533	feaces	+	+	+	+	+	-	-	-	+	+	+	-
Ba8	E. faecalis	1.1771	feaces	+	-	+	+	+	-	+	+	-	-	-	-
B4m8	E. faecalis	1.1665	feaces	+	+	+	+	+	-	+	+	-	-	-	-
Bc15	E. faecalis	1.1511	feaces	+	-	+	+	-	+	+	+	-	-	-	-
M4m1	E. faecalis	1.1661	milk	+	+	+	+	+	-	+	+	-	-	-	-
M5o2	E. faecium	1.1673	milk	-	-	-	-	-	-	-	-	-	-	-	-
M4r4	E. faecium	1,1660	milk	-	-	-	-	-	-	-	-	-	-	-	-
M3m22	E. faecium	1.1650	milk	+	-	-	+	-	+	-	+	-	-	-	-
M7m3	E. faecium	1.1707	milk	-	-	-	-	-	-	-	-	-	-	-	-
Ce'2	E. faecium	1.1763	milk	-	-	-	+	-	-	-	-	-	-	-	-
(Td'1)	E. faecium	1.1760	milk	+	-	-	+	-	-	-	+	-	-	-	-
B6s7	E. faecium	1.1697	feaces	-	-	-	-	-	-	-	-	-	-	-	-
C$7	E. faecium	1.1764	feaces	+	-	-	+	+	-	+	+	-	-	-	-
Td22	E. gallinarum	1.1783	feaces	-	-	-	-	-	-	-	-	-	-	-	-
Cß13	E. duran	1.1590	feaces	-	-	-	-	-	-	-	-	-	-	-	-
Cb24	E. avium	1.1779	feaces	+	-	-	+	+	-	-	+	-	-	-	-
Cj15	E. saccharolyticus	1.1537	feaces	-	-	-	-	-	-	-	-	-	-	-	-
Wd13	E. casseliflavus	1.1515	feaces	-	-	-	-	-	-	-	-	-	-	-	-
B6s66	E. mundtii	1.1696	feaces	-	-	-	-	-	-	-	-	-	-	-	-
M7s55	E. mundtii	1.1706	milk	-	-	-	-	-	-	-	-	-	-	-	-
M6m8	E. hirae	1.1688	milk	-	-	-	-	-	-	-	-	-	-	-	-
control	E. faecalis	2.9290	clinical	+	+	+	+		+	+	+	+	+	+	+
control	E. faecalis	2.6290	cheese	+	+			+							-
control	E. faecalis	2.8520	clinical			+				+					-
control	E. faecalis	2.6220	clinical						+		+				-
control	E. faecalis	2.6300	beer									+	+	+	-

Table 13. Percent incidence of virulence determinants ace, agg, cob, efaAfs, esp, fsrB, gelE, sprE, cylM, cylB and cylA, among milk and faeces isolates strains of Enterococcus species (+): presence of the gene or activity, (-); negative result.

Discussion

Growth behaviour on stress condition (acidic conditions, presence of bile salts, presence of hydrogen peroxide and presence of Paraquat):

Growth behaviour on acidic conditions and in the presence of bile salts

After ingestion bacteria meet several biological barriers, the first of which is the gastric acidity encountered in the stomach of the host. Bacteria able to survive these harsh conditions transit to the intestine, where they encounter stresses associated with low oxygen availability, bile salts, and competition with the microbiota. Species of LAB, which have resistance mechanisms and high tolerance for this different stress condition, will have higher chance for survival and dominancy among the different microbiota species in the GIT. Our results present different abilities and growth tolerance between the different strains of intestinal LAB isolated from different people (Egert *et al.*, 2006). In spite of the slight difference and tolerance between different strains of the same species it was obvious that there is general fingerprint or pattern of each species, which can differentiate it from other intestinal LAB species. For example, *L. plantarum, L. brevis* and *E. faecium* species has a proven higher ability to survive gastric acid and different bile condition mimics the intestine comparing to other LAB species. This could be the reason why these three species were the dominant and the most various (PAPD genotype) isolated species as *L. plantarum* had expressed 29 different genotypes from 24 different individuals, 25 genotypes for *E. faecium* from 25 different individuals and 17 genotypes for *L. brevis* from 15 different individuals (from total 30 individuals) (Albesharat *et al.*, 2011). These results suggest that these human gut isolates could successfully transit the human stomach and may be capable of reaching the intestinal environment and functioning effectively there. Most LAB were susceptible to bovine and porcine bile in vitro. However, they were resistant to human bile, which correlated with the survival in the human GIT (Dunne *et al.*, 2001). Bron found that when high bile was present, the surfaces of the bacteria appeared to be less smooth and membrane present vesicle structures (Bron *et al.*, 2004b). This could explain that bacteria cells had a slight tendency to clump together when grow in high bile salt concentration medium.

Growth behaviour in the presence of oxidative stress condition (hydrogen peroxide and paraquat)

Survival and resistance to low hydrochloric pH and bile acid presence can be the general criterion to the majority of intestinal specious as they all live, grow and are often exposed to this condition in the GIT. Even if different strains are variable with respect to their influence and inhibition for this condition, which is reflected by their dominancy in this specific ecological niche, in general they all have ability to survive in it. However, survival and resistance to oxidative stress conditions beside acidic and bile salts might be the main criterion, which selected the most dominant species between all isolated LAB species in the

intestine. To evaluate the resistance of different LAB species, we tested the survival in 1 to 3.5mM hydrogen peroxide, 0.4 – 1.4 mM hydroxyl radicals, and superoxide anions induced by 30 to 170 mM paraquat. In fact, hydrogen peroxide is a relatively weak oxidant, but it is highly diffusive and has a long lifetime (Jaroni & Brashears, 2000). Because of these two basic characteristics, hydrogen peroxide contributes to oxidative damage either directly or as a precursor of hydroxyl radicals. Although its half-life is very short compared with hydrogen peroxide, hydroxyl radical generated by the Fenton reaction is more reactive and highly toxic, causing it to be especially potent in causing local oxidative damage to DNA (Achanta & Huang, 2004). Superoxide anion is more selective than hydroxyl radical since it has been shown to react quickly with Fe-S cluster containing proteins, while hydroxyl radical reacts with hemoproteins, lipids, and DNA (De Freitas & Meneghini, 2001). Because of this, superoxide anion may diffuse a greater distance to react with Fe-S cluster structures, resulting in an increase in hydrogen peroxide and free iron levels. Thus, the final product of the specific attack of superoxide radicals to Fe-S clusters is a hydroxyl radical that rapidly attacks nearby molecules (Lee *et al.*, 2005). In general LAB strains showed higher risistance to H_2O_2 comparing with paraquat oxidative radicals. These results indicate that survival of lactic acid bacteria from intestine was dependent upon the type of ROS they expose to. However, strains from different species of *L. plantarum, E. faecium, E. faecalis, E. hirae, E. mundtii* and *P. pentosaceus* exhibited oxidative stress resistance ability to reactive oxygen species (ROS) produced by paraquat and H_2O_2. Additionally to gastric acid and bile salts resistance ability the species *L. plantarum* seems to be the only species, which can survive in all different stress conditions and this could explain its dominancy between all different human isolated LAB in this study (Albesharat *et al.*, 2011). This explain the finding that, *L. plantarum* showed the highest isolates number (210 from 700) from different sources and the highest strains diversity (29 different strains (RAPD-PCR)) as compared with the other 36 different isolated species. Although many different parameters may coexist within the cell, the resistance to ROS may be, at least in part, due to the antioxidative activities of these different species. Many lactic acid bacteria possess enzymatic and non-enzymatic antioxidative mechanisms and minimize generation of ROS to levels that are not harmful to the cells. Antioxidant enzymes play a critical role in defence against ROS (Driscoll *et al.*, 2002). The screening results of different antioxidative enzymes in different species will help to understand the resistance mechanism.

Growth behaviour in black tea and licoric media

The aim of growth investigation of different intestinal LAB species in tea and licoric medium was to see the influence of regular tea and licoric consumption on the different intestinal species. While it was expected to find an inhibitory effect for tea on bacteria growth (Friedman, 2007) it was surprising to observe a special influence on growth of the tea

medium. Tea medium could increase the bacteria yield for all tested LAB species beside the interesting influence of inducing bacteria growth directly after culturing. Old research suggested that tannins inhibited pathogens but had a very slight inhibitory effect or no effect on intestinal microbiota (Ahn *et al.*, 1998). Chung et al found no inhibition of lactic acid bacteria when tannic acid was used (Chung *et al.*, 1998). They further indicated that this might be because of the absence of heme enzymes and the replacement of the crucial iron-containing ribonucleotide reductase with adenosylcobalamine in some lactic acid bacteria. Thus, although metal-chelating compounds would inhibit the growth of other bacteria by sequestering essential metal ions, it would have no effect on lactic acid bacteria (Ankolekar *et al.*, 2011). If tea medium showed no inhibitory effect on LAB growth, but additional growth enhancement influence, then which substance of the tea components is the responsible for the growth enhancement effect on LAB. Tea solution did not contain any sugars, and analysing the tea amino acid components showed high concentration for Gamma Amino Buytric Acid (Gaba) in tea solution, this is in agreement with (Omori *et al.*, 1987) who found it in tea also. However, it is inconclusive that (Gaba) which has been found in high concentration in the tea solution could be the reason for this exceptional growth behaviour, which occurred upon growth in tea medium. Gaba is not scarce compound in the intestinal bacteria environment and can be produced by different species of LAB like *L. plantarum, L. brevis, L. paracasei* .. etc (Li & Cao, 2010) where Gaba production in microbes is contribution to pH tolerance and ATP production for bacteria (Higuchi *et al.*, 1997), (Small & Waterman, 1998). So, tea is not special source for this component (Gaba) and its plenty in tea might not be the reason for this special bacteria growth enhancement influence.

High-performance liquid chromatography indicated the presence of gallic acid, quercetin, caffeine, and tea catechins (including catechin, epicatechin, and epigallocatechin) in tea samples (Ankolekar *et al.*, 2011), and the more expected influencing compounds are the tea polyphenols. Although, the antimicrobial effects of tea polyphenols have been well documented (Betts *et al.*, 2011), different strains of intestinal bacteria had varying degrees of growth sensitivity to tea phenolics and metabolites (Lee *et al.*, 2006). Tea can be potentially used as a low-cost dietary support to combat *H. pylori*-linked gastric diseases without affecting the beneficial intestinal bacteria (Ankolekar *et al.*, 2011). Lee proved that tea growth of certain pathogenic bacteria such as *Clostridium perfringens*, *Clostridium difficile* and *Bacteroides spp*. was significantly repressed by tea phenolics and their derivatives, while intestinal anaerobes like LAB bacteria were less severely affected (Lee *et al.*, 2006). We are in agreement with that as for different LAB bacteria species growth in different mediums supplemented with different tea polyphenols, no significant influence or difference on growth comparing with standard unsupplemented medium for quercetin, (+) catechin, (±) catechin and epigallocatechin. Only (-)-epicatechin showed the same special initial growth inducing

influence similar to the influence expressed in the tea medium with all LAB tested species. An investigation has shown that epicatechin has an antibacterial effect (Hirao *et al.*, 2010), which according to another study (Toda *et al.*, 1992) can be attributed to membrane disruption properties. However, different strains of intestinal bacteria had varying degrees of growth sensitivity to tea phenolics and metabolites (Lee *et al.*, 2006). Meselhy Found that human faecal bacteria extensively metabolise (-)-epicatechin after incubation for 24 h, whereas the (-)-epicatechin resisted any degradation by a rat fecal bacteria even after a prolonged incubation time (48 h) (Meselhy *et al.*, 1997). In this context our result on intestinal LAB species isolated from human consuming this polyphenols regularly shows direct growth enhancement lead to escape the lag growth phase of these bacteria in the presence of this polyphenols in the growth medium. This indirectly led to conclude special growth selective synergic influence of tea (-)-epicatechin polyphenol and beneficial intestinal microbiota. This indicates that tea phenolics exert significant effects on the intestinal environment by modulation of the intestinal bacterial population. This could be the reason for the *Bifidobacteria* absence in the adults intestinal LAB isolates of regular tea consuming people as this polyphenols showed inhibition growth effects on the two additional tested *Bifedobacteria* strains, this is in agreement with (Horiba *et al.*, 1991) who reported that green tea inhibited the growth of *Bifidobacterium bifidum*. More investigation to understand this influence is required.

Comparative study for some oxidative stress resistance mechanisms:

Macrophages are known to efficiently inhibit bacteria by causing oxidative stress (Kirkham, 2007), in which oxidizing molecules are generated at a higher rate than they are detoxified. Oxidative stress is one of the harsh conditions that intestinal microbes might have managed to endure during their translocation. To counteract oxidative stress (Miyoshi *et al.*, 2003), bacteria developed several defense strategies, namely, reduced generation of oxidizing molecules during metabolism, enzymatic or nonenzymatic detoxification of the latter, and repair of damaged cell components (Lushchak, 2001).

A number of LAB can use molecular oxygen or hydrogen peroxide to regenerate NAD^+, by the action of NADH oxidase and NADH peroxidase (Condon, 1987). Different tested LAB species expressed NADH oxidase presence except *L. fermentum, L. rhamnosus, L. salivarius, E. faecalis* and few strains of *E. faecium*. With the stepwise reduction of O_2 to H_2O the toxic intermediates O_2^- and H_2O_2 are generated. Hydrogen peroxide was found to inhibit growth of some LAB (Anders *et al.*, 1970). However, exposure of LAB to a sublethal dose of H_2O_2 induced an oxidative stress response (Condon, 1987), characterized by an increased survival after exposure to a lethal level of H_2O_2 compared with cells that were not pretreated. As most of our intestinal isolated species showed high ability to produce H_2O_2 in vitro, it is expected that it expressed this activity also in the intestine zone before, which make general

intestinal habitat participator microbiota have been exposed to H_2O_2 previously and this explain the general resistance ability for all intestinal LAB isolates to H_2O_2 even those species which have no ability to produce it.

Most LAB can deal with oxygen radicals by either a superoxide dismutase (Sod) or a high internal Mn^{2+} concentration (Archibald & Fridovich, 1981a). Sod dismutes oxygen radicals by catalyzing the reaction $2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$. Although most organisms use catalase for the breakdown of H_2O_2, some of them lack this activity; instead, they have NADH-peroxidase activity to decompose this compound (Anders *et al.*, 1970), (Sanders *et al.*, 1995). Gotz et al. (Gotz *et al.*, 1980a), (Gotz *et al.*, 1980b) found an NADH oxidase and peroxidase in *L. plantarum* and reported the presence of low levels of SOD. This can explained the few *L. plantarum* strains (in groups 6,7,8,9,21,22,24), which showed SOD presence although all *L. plantarum* strains showed NADH oxidase presence. In this study, activity of SOD was very low in *L. plantarum* and absent in *L. fermentum, L. brevis* and *E. faecalis*. It has been well documented that *L. rhamnosus* expresses SOD activity under either aerobic or anaerobic conditions (Lee *et al.*, 2005), and this is in agreement with our results. SOD activity of lactic acid bacteria in this study may not be sufficiently induced to remove ROS because these strains have been cultured under anaerobic conditions.

Regardless that many *L. plantarum* strains lack the SOD activity, all *L. plantarum* showed very well resistance and survival ability in oxidative stress condition and high H_2O_2 production ability. This result indicates that the antioxidative capability of *L. plantarum* may be dependent upon non-enzymatic rather than enzymatic antioxidative mechanisms. Thus, the antioxidative activity of *L. plantarum* species could result from its ability to chelate metal ions instead of increased expression of antioxidative enzymes such as SOD (Lee *et al.*, 2005). Archibald & Fridovich concluded that *L. plantarum* has an unusually large demand for $Mn2^+$ ions and accumulate more than 30 mM $Mn2^+$ (Archibald & Fridovich, 1981b). This bacterium does not contain a defense enzyme superoxide dismutase. A defense against oxygen toxicity in L. plantarum is provided using $Mn2^+$ ions, which perform the same function as the enzyme (Groot *et al.*, 2005).

Generally Lactic acid bacteria are considered to be devoid of catalase activity, since they are not able to synthesize heme compounds and does not require heme for growth (Kandler & Weiss, 1986). Nevertheless, certain species of lactic acid bacteria exhibit catalase activity. In our case some strains of *L. plantarum*, while all *L. brevis* and *E. faecalis* strains had catalase ability. Two types of catalase are described (Wolf & Hammes, 1988): (1) a heme-dependent catalase, induced by the addition of heme compounds and produced by some *Lactobacillus, Pediococcus, Enterococcus* and *Leuconostoc* strains; (2) a heme-independent catalase or pseudocatalase, produced by some *Leuconostoc, Pediococcus, Enterococcus* and *Lactobacillus plantarum* strains (Mares *et al.*, 1994). Although *L.*

fermentum strains lacks catalase, NADH oxides and SOD it all expressed Glutathione reductase (GR) presence which can explain it is ability to survive the oxidative stress condition (Kullisaar *et al.*, 2010).

Comparative study for production of antagonistic metabolites, and production of metabolites and enzymes:

Interestingly all *L. plantarum* and *L. brevis* milk strains (TMW 1.1623) showed high bacteriocin production ability which never been reported about lactobacillus from milk source before (Lara-Villoslada *et al.*, 2007). While some *L. fermentum, L. casei, L. reuteri, L. rhamnosus* and *E. hirae* have this ability and other don't have it. While almost all L. plantarum mother/baby-pairs group's strains are Hydrogen peroxide producers. *L. brevis, L. oris L. rhamnosus, E. faecalis* and *E. faecium* also have this ability corresponding with hydrogen peroxide resistance ability.

Intestinal bacteria, as a source of digestive exoenzymes, may depend upon the diet preference of the host (Park *et al.*, 2007). Production of caseinase (the proteolytic exo-enzyme), was classified as conditional criteria which can be expressed or activated in the bacteria depending on the growth environment components. As mother milk bacteria and baby intestinal bacteria live in environment rich with casein the bacteria expressed high casienase production ability than adult's strains whom consume less milk products. Interestingly, all different tested properties or activities of mother/baby-pairs group's strains showed similar activity or absence in all strains of different sources in each group (except caseinase production activity which appears that it is environment dependent trait) which indirectly lead to conclude that all these identical different properties expression (RAPD-PCR, MALDI-TOF, genes presence, production ability and resistance ability) is due to the identical source of this strains. This suggests that each mother/baby-pairs group's strains are identical strain from one source which is the mother intestinal microbiota transferred through her milk to her baby.

Virulence-related genes of Enterococcus:

The ability of *Enterococcus* to promote both health and illness at the same time is contradictory and currently not well understood. Studies have identified numerous virulence factors, although there is no clear association of a single gene or origin of isolation with pathogenicity, (Eaton & Gasson, 2001), (Martin *et al.*, 2005). In this study all the 6 *E. faecium* strains isolated from the human milk of healthy women were free of virulence genes (esp, agg, cob, gelE cylMBA) without a clear role in enterococcal pathogenesis. On the other hand, half of them contained the efaAfs gene, but the role of efaAfs adhesion in *E. faecium* for it's virulence has not yet been proved, in contrast to efaAfs of *E. faecalis* (Singh *et al.*, 1998). Presumably, the presence of efaAfs in the absence of other virulence factors have no value as a risk indicator since this gene was also found in 100% of the starter *E. faecium* strains

screened by Eaton and Gasson (Eaton & Gasson, 2001), which have a long record of safe use in food. Another enterococcal adhesion gene (esp) could not be detected in any *E. faecium* strain. The expression of cylA, cylB, and cylM are required for the biosynthesis of cytolysin, however the absence of this genes result in lack of hemolytic activity among the *Enterococci* (Gilmore *et al.*, 1994). Similarly, absence of gelE refers for the lack of gelatinase activity as shown by our results. Some *Enterococci* have highly effective gene transfer mechanisms, and both virulence and antibiotic resistance genes are accounts to be associated with specific highly transmissible plasmids (Reviriego *et al.*, 2005). However, in this study, neither the pheromone determinant cob nor the agg genes were present in any *E. faecium* strain. This finding is in agreement with previous report that examined milk *E. faecium* virulence activity (Reviriego *et al.*, 2005). The only *E. faecalis* strain isolated from milk showed presence of different virulence genes except fsrB and cylMBA. Despite the existence virulence genes pattern for *gelE* and *sprE* observed in some *E. faecalis* isolates examined for gelatinase production are unrelated. Although *gelE* and *sprE* genes have been proposed for the ability of bacterial migration and spread, this strain carrying these genes did not produce gelatinase on gelatine medium. However, these strains might have another non virulence mechanisms enabling them to translocate out of the intestinal niche as they were isolated from the breast milk of healthy women.

In this study, a disc diffusion method was used to determine the antibiotic resistance pattern of the *E. faecium* and *E. faecalis* strains. All the milk strains were sensitive to ampicillin, aureomycin, chloramphenicol, tetracyclin and erythromycin. The sensitivity against antibiotics is an important factor for the evaluation of the safety of *Enterococci*. In conclusion, no virulence factors could be detected in the milk *E. faecium* strains, which is the dominant *Enterococci* species isolated from the milk.

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Chapter IV

In vitro translocation of *Lactobacillus plantarum* in human and guinea pig resectates

Introduction

The Ussing chamber

Hans Ussing had invented a various applications device that was accordingly named after its inventor “Ussing - chamber“ (Ussing & Zerahn, 1951). The Ussing chamber consists of two functional parts. The main part is the chamber itself which consist of two halves and the other is the electrical circuitry. Chambers can be in different sizes and shapes depending on the special applications, while the electronic circuitry enable numerous measurement of resistance, current and voltage and other different parameters including impedance and capacitance. Recently, it is been widely applied two types of Ussing chambers: the circulating chamber and the continuously perfused chamber. Because of its simplicity the circulating chamber has been dependable by most labs, while the latter offers different featured advantages (Hug, 2002). In this study we used the circulating chamber as showed in fig.1

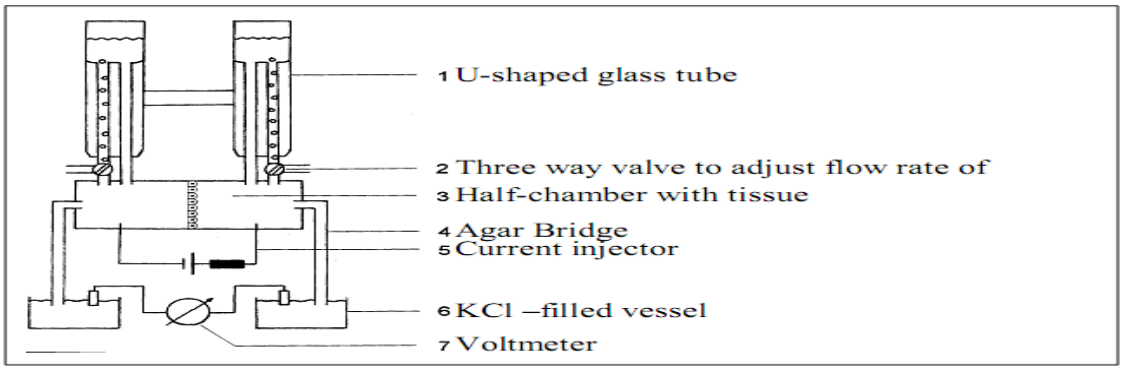

Figure 1. Schematic drawing illustration of the Ussing Chamber set up (Hug, 2002)

The circulating chamber consists of a U-shaped tubing system usually made of glass that is filled with the experimental solution (Krebs solution). The tubing temperature can be adjusted if needed and can be pumped either with air or different gases such as CO_2, O_2 or N_2. The gassing offers two necessary purposes: first, to oxygenate the experimental solution contents and second, to stir the liquid to ensure identical temperature distribution (termed “bubble lift”). The lumen of the tubes is connected to the chambers rebore. The U - shaped tube offer an equal hydrostatic pressure on both halves of the chamber and thus, avoids damage caused by curvature of the tissue. During the procession of an experiment, tested materials are usually added to one or both halves or sides of the chamber in a consecutive method. It is evidently that once applied the tested material, it hold in the solution until the end of the experiment. However, some investigators have overcome this (Hug, 2002). The two halves of the Ussing chamber bound together retaining the epithelia (sheet of mucosa or monolayer of epithelial cells grown on permeable supports) in between. The test subject, the epithelia are polar in nature, i.e., they have an apical or mucosal side and a basolateral or serosal side. The main interesting application possibility of the Ussing chamber is to isolate

the apical side from the basolateral side. The two half chambers should be first filled with equal amount of symmetrical Ringer solution in order to remove any chemical, mechanical and electrical driving forces. In normal life epithelium there is ion transport taking place through it from apical side to basolateral side or vice-versa. This ions movement generates a potential difference (voltage difference) across the epithelium. This generated voltage difference can be measured using two voltage electrodes that are placed on both sides of the live tissue (epithelium). This voltage can be rule out by inserting the current using another two current electrodes that are placed away from the epithelium. This value of the inserted current is called Short-circuit current (Isc) presenting accurate measure of net ion transport taking place across the epithlium. Epithelia present special two traits that differentiate them from all other tissues, which are polarity and permeability. Polarity results from the asymmetric distribution of proteins to either the apical or the basolateral membranes (Brown & Stow, 1996). While permeability results from intercellular junctions (an assembly of proteins called “tight junctions” separates epithelial monolayer and both membranes). The states and permeability of tight junctions determines the resistance and integrity of the tissue. Tightness can be accounted by the electrical resistance (R). Considering a tissue, R can be broken down in an arrangement of resistors. In application, R (Ω) can be calculated using Ohm’s law: $R_t = \frac{\Delta U}{\Delta I}$ (*U*: potential (mVolt) - *I*: current (µAmper)). The simplest approach is to apply a voltage and measure the resulting change in current (Hug, 2002).

The small intestine:

The small intestine is the primary locus of digestion. It is composed of three parts. The first part is called the duodenum, and connects to the stomach. The middle part is the jejunum. The final part, called the ileum, attaches to the first part of the large intestine (shown in fig 2 below). Unlike the large intestine (the colon), the small intestine plays a major role in absorption of foods and nutrients.

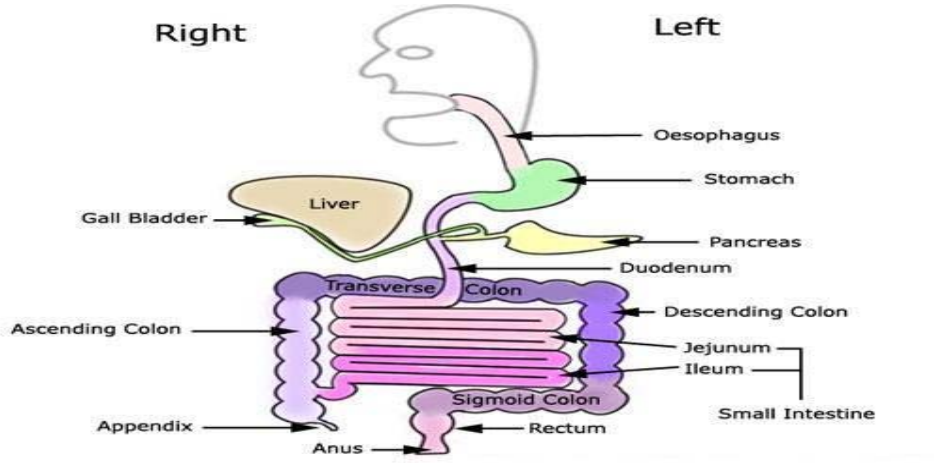

Figure 2: the digestive tract parts, and three sections of the small intestine, http://www.virtualmedicalcentre.com

The three sections of the small intestine look similar to each other at a macroscopic level, but there are some anatomically distinguished differences.

Table 1. The structure of the small intestine sections, duodenum, jejunum and ileum, (Brunner's glands are confined to the duodenum which is not in our interest in this study whoever Peyer's patch is abundant in the ileum which is our interest and will be explained in the text)

Layer	Duodenum	Jejunum	Ileum
serosa	normal	normal	normal
muscularis externa	longitudinal and circular layers, with Auerbach's (myenteric) plexus in between	same as duodenum	same as duodenum
submucosa	Brunner's glands and Meissner's (submucosal) plexus	no Brunner's glands	no Brunner's glands
mucosa: muscularis mucosae	normal	normal	normal
mucosa: lamina propria	no Peyer's patches	no Peyer's patches	Peyer's patches
mucosa: intestinal epithelium	simple columnar. Contains goblet cells, Paneth cells	Similar to duodenum. Villi very long.	Similar to duodenum. Villi very short.

The small intestine consists of many tissue layers, which can be seen in the cross section below (Solomon *et al.*, 2002)

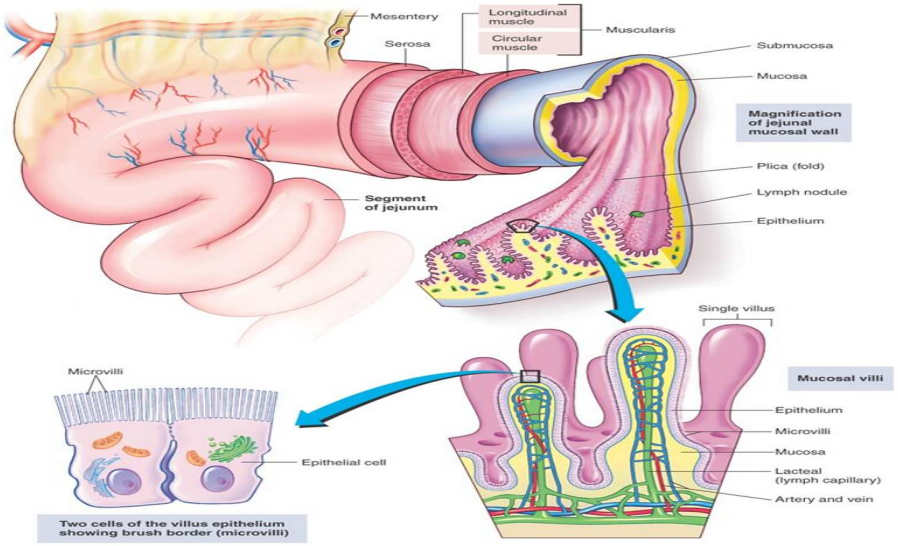

Figure 3. Anatomically the walls of the intestinal tract of human consist of four layers of intestinal tissue. These layers from inside to outside are: Mucose membrane, submucose membrane, muscular layer (circular and longitudinal) and serose membrane. http://www.baileybio.com

The microscopical anatomy of the intestinal mucous membrane reveals the following: The entire inner surface (that point toward the lumen) of the small intestine is composed of villi, certain papilliform bulges processes, and glands; an epithelial layer containing perpendicular epithelial cells with a striated border, and some goblet cells cover the entire surface. Each villus possesses a centrally located space for chyle which is covered with endothelial cells and connected with the lymphatics circulation of the intestinal mucosa. Every villus contains a thorough arrangement of blood-vessels and muscular fibres which arise in the muscularis

mucosa. This intestinal special structure enables to perform the function of suction and pumping. Villi are the main intestinal organs as they perform a fundamental rule of absorption in the small intestine. Solitary follicles or glands are found scattered throughout the mucous membrane of the small intestine. They are closely numerous in the lower part of the ileum. Each follicle has a diameter of between 3 to 6 mm. They have similar structure like the one of the lymph nodes which consists of a bushy retiform tissue assembled with lymph corpuscles and permeated by fine capillaries, having no ducts. The interspaces of the retiform structure are continued with wider lymph area at the base of the gland, by which they exchange with the lacteal system. The origin of the nodules is in the submucous tissue layer, where it penetrates through the muscularis mucosae and enters the mucous membrane forming a slight projection of its epithelial layer. The solitary follicles are the developing and differentiation site of the lymph cells. They are localized in two distinguished positions, either scattered singly, in which case they are called glandulae solitariae (Fig. 4, the first three images), or assembled in groups distance from one to three inches in length and about one-half inch in width. The surface of the solitary follicles is free from villi. Especially of an oval form, their long center is parallel with that of the intestine. In this situation they are termed glandulae agminatae or Peyer's patches (Fig. 4, the forth image). Generaly position are almost placed opposite the connection location of the mesentery (Toldt *et al.*, 1919).

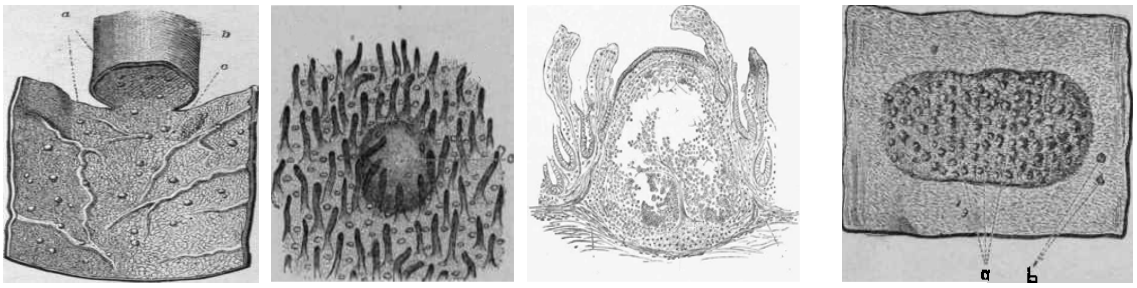

Figure 4. Three images in the left are presenting glandulae solitariae, the first image is for Ileum partly opened (a. Solitary lymph nodules; b, serosa; c, mucosa), the second image is for single solitary lymph nodule and the third image is Section of Single Lymph follicle of the Small Intestine. The forth image in the right is presenting Peyer's Patch (Noduli Lymphatici Aggregati) in the Ileum (a, Peyer's patch; b, solitary lymph nodules). (All images are from (Toldt *et al.*, 1919), except the third image from (Yeo, 1889))

Types of granulocytes

Normally, the bone marrow are the origin of the blood stem cells (immature cells) that develop and differentiate into mature blood cells over time. A blood stem cell may become a myeloid stem cell or a lymphoid stem cell. The lymphoid stem cell develops into a white blood cell. While the myeloid stem cell develops into one of three types of mature blood cells:

- Red blood cells that carry oxygen to all tissues of the body.
- Platelets that contribute in prevent bleeding by causing blood clots to form.
- Granulocytes (white blood cells) that fight microbes and infection

In the intestine different blood stem cells develop into a type of white blood cell called granulocytes. There are three types of granulocytes: eosinophils**,** basophils and neutrophils (see fig. 4)**.** There are also three types of agranulocytes: lymphocytes**,** monocytes and macrophages**.** Eosinophils deal with parasitic infections, while basophils are in charge for allergic response (releasing histamine), and finally the neutrophils are mainly responsible of defending us from bacteria and fungus (Alberts, 2005). Neutrophils are a principal component of the innate immune system and provide a first line of defence against bacteria and other invading pathogens. They recruited rapidly to sites of inflammation or infection, where they possess a different essential antimicrobial functions, including phagocytosis of bacteria, release of antimicrobial peptides, and cytolysis via ROS generation (Zhang *et al.*, 2009).

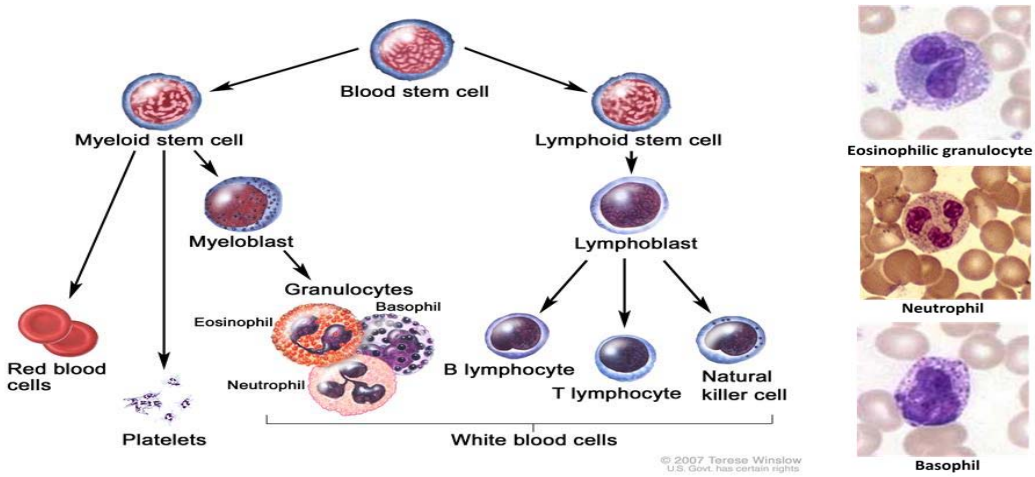

Figure 4. Blood cell development. A blood stem cell goes through several steps to become a red blood cell, platelet, or white blood cell, in the right the three types of granulcytes (white blood cells). (http://www.uchospitals.edu/online-library/content)

Human and Guinea pig intestine

Guinea pig is a widely applied model for human intestinal research. Guinea pigs have considerably been used as model organisms in medical research in the 19th and 20th centuries, resulting in the designation “guinea pig” for a test subject, but have recently been more replaced by other rodents such as mice and rats. However, there are certain human medical conditions for which guinea pigs appoint better models than other rodents. For example, unlike the case of mice and rats, the E-cadherin on the intestinal surface of guinea pigs is homologous to that of humans. This makes these animals one of the most suitable models in human like intestinal researches (Hildebrand *et al.*, 2012). The bacterial richness obtained for human samples was lower than for guinea pig samples. The intestinal microbiotas of both species were dominated by the two phyla Bacteroidetes and Firmicutes, but at genus level, the majority of distinguished genera (320 of 376) were differently numerous in the two hosts. Most microbiome functional groups were less numerous in guinea pigs than in humans (Hildebrand *et al.*, 2012). The permeability of the human

intestine to applied probe molecules is considerably different from that of three common laboratory rodents (rats, guinea pigs and hamsters), but is closest to that of guinea pigs (Delahunty & Hollander, 1987).

Bacterial translocation

Bacterial translocation (BT), "defined as the passage of viable and nonviable microbes, or their by-products, across an intact intestinal epithelial barrier" (Alexander *et al.*, 1990) may be a normal physiologic process that is important for mucosal antigen sampling in the intestine. Preconditions for transcellular passage assure bacterial attachment and internalization. The latter may performed either via pinocytosis or phagocytosis. The cell wall of Gram-positive bacteria is consisting of a thick, multilayered peptidoglycan sacculus (also called murein) containing teichoic acids, proteins and polysaccharides. In LAB, an additional outer structure of proteins, the S-layer, has been detected so far only in the genus Lactobacillus, in different species including *L. plantarum*, but the contribution of this protein layer to the functionality of the cell wall remains not fully understood (Delcour *et al.*, 1999), however it is been suggested that it contribute to some functions (i.e.: aggregation, adhesion) (Mobili *et al.*, 2007). Many components of Gram-positive bacteria cell walls have been shown to be involved in immune activation and cytokine induction (Miettinen *et al.*, 1996). In the case of lactobacilli, differences between strains concerning their ability to activate immune potentiating effects have been related to differences in cell wall structure (Sato *et al.*, 1988). Macrophages and lymphocytes play a fundamental role in humoral immunity response through the release of different cytokines such as the proinflammatory cytokines TNFa and IL-6 (Perdigón *et al.*, 1999) and of Interleukin-10 (IL-10), which is known to inhibit the synthesis of the above two cytokines (Miettinen *et al.*, 1996). Between different LAB species, strains belonging to *L. plantarum* showed intrinsic immunomodulatory properties due to its influence on innate cytokines. It has been reported that *L. plantarum* leads to interleukin IL-10 production, an antiinflammatory cytokine. While other LAB species like *Lactococcus lactis* stimulates TNF-α, IF-gamma and IL-12 production, proinflammatory cytokines, by human peripheral blood mononuclear cells (Müller-Alouf *et al.*, 1999). Neutrophils and inflammatory monocytes are forefront principal components of the innate immune respond and are essential for defence against a variety of microbial pathogens. However, it is proved that IL-10 can inhibit neutrophil apoptosis (Keel *et al.*, 1997). Additionally, it can inhibit neutrophil phagocytic and bactericidal activity (Laichalk *et al.*, 1996). This rise the question, how could that influence *L. plantarum* translocation ability. In this study, we examined whether milk isolate (translocation candidate) *L. plantarum* strain can passage across an intact intestinal mucosal membrane mounted in normal human like body conditions in the Ussing chamber. We investigated the passage possibility of the life and the dead bacteria and their respective influence on the tissue resistance.

Materials and Methods

Bacteria preparation

Milk isolate *L. plantarum* (TMW 1.1623) and faecal isolate (RAPD genotype never detected among milk isolates) *L. plantarum* (TMW 1.1566) were grown in MRS broth at 37°C for 16–18 h, and then the number of viable cells was determined by agar plate counting and/or OD_{590} measurements. For *in vitro* experiments, fresh cultures were used, whereas 1.1623 and 1.1566 bacterial stocks were made in MRS with 10% glycerol and kept frozen at −80°C until used for *in vivo* experiments. From frozen stocks (80°C) bacteria were suspended in Man-Rogosa-Sharpe liquid medium (MRS broth; Difco Laboratories, Detroit, MI) plated in MRS agar cultured anaerobically at 37°C for 24 hours, then daily inoculated in fresh MRS broth and grown at 37°C under anaerobic conditions for 24 hours in 15-ml tubes. After 1 day, tubes were centrifuged at 2,000 rpm for 15 minutes at 20°C and washed twice with sterile phosphate-buffered saline (PBS), and divided to two parts, one part kept in ice and second part exposed to 90 °C for 10 min (to kill all bacteria cells). The two separate bacterial suspensions (live and dead) were centrifuged in 15 ml tubes at 2,000 rpm for 15 minutes at 20°C, supernatants discarded, then bacteria were labelled with Fluorescein isothiocyanate (FITC;sigma) 10ug/ml incubated at room temperature for 2 hours in dark. Then extensively washed to remove unincorporated FITC, and resuspended in 15 ml Krebs solution (to a concentration of 6 $x10^7$ bacteria/ml). Then, 1 ml of each bacterial suspension was added over (mixed 3 times by pipetting and releasing the suspension with the solution) the 4ml of Krebs solution in the mucosal side of two separate Ussing chambers.

Tissue Samples

Tissue samples were obtained from male guinea pigs (Dunkin Hartley 300–550 g, Harlan-Winkelmann, Borchen, Germany; Harlan, Bichester, UK) (for primary experiment)). For the Ussing chamber studies colonic segments were removed after killing the animals by cervical dislocation and exsanguination in accordance with the German ethical guidelines for animal experiments. Segments were dissected in ice cold Krebs solution to obtain mucosa/submucosa preparations. Jejunal segments were removed from guinea pigs killed by cervical dislocation. The segments were immediately placed into oxygenated (95% O_2+5% CO_2, Carbogen) Krebs solution at room temperature.

Human tissue samples (not included in the primary experiment) of the ileum and jejunum were taken from 4 patients (ages, 57–70 y; mean age, 66 ± 6 y; 2 men, 2 women; 1 ileum and 3 jejunum) undergoing surgery at the Departments of Surgery at the University Hospital of the Technical University Munich and the Clinical Centre in Freising for the following diseases: carcinoma of the large bowel (1 patients), carcinoma of the stomach (3 patients). Specimens from non afflicted ileum and jejunum regions were used for our experiments. After removal of the specimen during surgery the tissue was placed in cold,

oxygenated, sterile Krebs solution and immediately transferred to the laboratory. The dissecting microscope was then used to separate the seromuscular layer from the underlying mucosa and submucosa. The remaining mucosa and submucosa were divided in two pieces of 1 cm^2 segments and were directly mounted in two different Ussing chambers and immediately perfused. All procedures were approved by the ethics committee of the Technical University Munich (project approval 1512/06).

Perform Ussing Chamber Experiment

To test the life and dead bacteria translocation ability through an in intact submucosa/mucosa preparations of distal guinea-pig and human intestine we used Ussing chamber techniques (Easy Mount chambers, Physiologic Instruments, San Diego, CA). The procedure how to set up an Ussing - chamber experiment can be described briefly: First the chambers and solutions should be prepared then all parts were put together without any tissue. The current and voltage electrodes are inserted into the half-cells and fill the system with the Kreps solution. When the electrodes are connected to the current/voltage pulse injectors and the volt-/amperemeter respectively the system were checked for noise and offset voltages. If the system proves to be watertight, the temperature were adjusted to the body temperature 37°C. The tissue exposing and recording chamber area is 0.5 cm^2. Mucosal and serosal sides were bathed separately in 5 mL Krebs solution. The bath was maintained at 37°C and continuously bubbled with 95% O_2 and 5% CO_2 (Carbogen). The transepithelial potential difference was measured by a pair of Ag/AgCl electrodes, connected to a voltage clamp device (VCC 600; Physiologic Instruments) that compensated for the solution resistance between the electrodes. The current electrodes were placed on each side of the tissue. Each electrode was inserted in a pipette tip, connected to the Krebs solution in the chamber via a 3-mol/L KCl-filled agar bridge (Easy Mount Electrode Set P2020, Physiologic Instruments). Ions secretion was measured as short-circuit current (Isc) and expressed in μAcm^{-2}. The Software Chart (Version 5, Chart Software: AD Instruments, Spechbach, Germany) detected the short-circuit-currents during the experiment. Positive Isc indicated a net anion current from the serosa to the lumen. Live or dead *L. plantarum* was added to the mucosal side of the preparation. Before starting the actual measurements, the tissues were allowed to equilibrate for at least 30 minutes. Change of the pH (7.4, ± 0.09) in the Krebs solution was observed with all of the bacterial addition used in this study. After equilibration of the system one millilitres of the bacterial suspension can be inoculated at time 0 in the mucosal side, approximately 1 x 10^8 colony-forming units (cfu) were the final bacteria concentration into the mucosal reservoir, and recording pH, voltage and current can begin every 5 min during the experiment. After 60 to 70 minutes of exposing the mucosal side to the bacteria the experiment were stopped by taking solution sample of both mucosal and serosal side of each chamber, transfer it in eppendorf tubes and keep it on ice till

measuring bacteria counts. Then the rest of the solution in each were bumped out with special pipette for every side and the tissue carrier were pulled out and rinsed carefully with Krebs solution from the mucosal side to remove all free bacteria from the surface of the tissue. The tissues were carefully separated from the carrier and stretched or fixed (the mucosal side to the top) on agar plate.

For histological studies, guinea-pig and human tissues were fixed overnight at room temperature in a solution containing 4% paraformaldehyde and 0.2% picric acid in 0.1 mol/L phosphate buffer, followed by several washes (3 x10 min) in phosphate buffer. Then the tissue were cut carefully to remove the surrounding part and get the exact part which were exposed to the bacteria in the using chamber (0.5 cm^2) and cut it in two halves, Then soaked it overnight at 4 C° in a solution containing sucrose 40% in 0.1 mol/L phosphate buffer. Without washing the samples were embedded in (Bright cryo-M-BED, Embedding compound for frozen tissue specimens) for frozen section. Sectioning was performed by exposing the serosal side to the knife and cutting from the serosal side to the mucosal side, additional wiping the knife with alcohol (Ethanol 70%) after every sectioning to avoid smearing the bacteria on the section. Tissue sections were cut at 75-100 µm thickness and stained with WGA Alexa Fluor 633 conjugate or Alexa Fluor 635 Phalloidin for the (Cytoskeleton) and with SYTOX Orange for the (DNA) (all from Invitrogen)—stained mammary and placenta sections. Staining solution (HBSS solution containing concentration of 0.5 µg/ml WGA Alexa Fluor 633 conjugate or 0.06 µM Alexa Fluor 635 Phalloidin and 0.01µM Sytox orange) were placed on the sections' coverslips for 10 minutes at room temperature followed by 3 times rinse with HBSS buffer. Section were mounted with antifade reagent (Molecular probes prolong Gold, Invitrogen), examined and inspection for florescent bacteria presence using Ti-U inverted research microscope with an e-C1plus confocal system (Nikon, Düsseldorf; Germany) and a 20 x (N.A. = 0.75) objective. Bacteria were monitored as green fluorescence images at (λ_{exc} = 488 nm, λ_{em} = 515/30 nm) and cell cytoskeleton as red at (λ_{exc} = 632 nm, λ_{em} = 650/LP nm) with 512 x 512 pixel resolution (636 x 636 µm) in a constant z-position, and representative scanning pictures of the sections were taken. Additional controls from the same tissue were performed by leaving out the Ussing chamber then apply similar procedure of fixation and staining of the tissue, a Zeiss microscope with appropriate filter blocks was used also. It is important to note, however, that samples containing bacteria prelabelled with FITC should be microscopically analysed as soon as possible, because the FITC label decays significantly when storage (even at 4 °C in the dark) exceeds more than one week.

Translocation calculation

The bacteria count in the solution samples of the mucosal and serosal side of the Ussing chamber was done immediately after experiment. The different cells suspensions were applied in hemocytometer (or haemocytometer or counting chamber) (Thoma: 0.02mm

depth, 0.0025mm^2), examined using florescent light microscopy (ZEISS, Germany) with appropriate excitation and emission filters and imaged with (Axio zeiss cam) camera (magnification 40x). Images were digitally processed with ImageJ plugin (ITCN: Image based Tool for Counting Nuclei). Results are expressed as the mean number of positive (florescent) cells per 32.5 fields or square; each square is 5x10^{8-} cm^3. The bacterial number calculation was done in a blind manner. The percentage of bacteria translocated through the intestinal tissue was calculated from the bacteria cell numbers of both sides of the Ussing chamber.

Primary experiments

Growth *L. plantarum* (TMW: 1.1623) on MRS and Krebs solution

MRS broth and Krebs solution were distributed in a 6 wells (triplicate) of 96-well microtiter plate, respectively. A volume of 5µL of overnight culture of *L. plantarum* (TMW: 1.1623) strain appropriately diluted (to get final OD_{590} =2) was added to each well or the same quantity of water to the control. Thus we obtained the effect of Krebs solution on the tested strain (inhibit or enable growth) and in this way we could predict the possibility of increase acidity effect by bacterial metabolites. To ensure that the reaction conditions were anaerobic and to prevent evaporation during longer incubations, the wells were overlaid with 50 ml of light paraffin oil (0.85 g/ml; Merck, Darmstadt, Germany). The microtiter plates were incubated at 37 °C for 13 h and the absorbance beginning from 0 hour of incubation was measured at 590 nm using a microtiter plate reader Tecan (Sunrise, Germany). The absorbance was measured every 20 minute for 40 cycles. The absorbance of wells containing only broths without the indicator strain was subtracted from the absorbance of the wells that contained the indicator strain. Thus we obtained the absorbance of the cells. This absorbance is in direct proportion with the amount of cells, within certain limits. Growth on Krebs solution was scored as less than, equivalent to, or greater than that observed for the same growth on the MRS broth

Influence of bacteria disintegration and pH on FITC staining stability

Cells of *L. plantarum* strain (TMW:1.1623) were grown over night at 37°C in 15 ml tubes and harvested by centrifugation at 5000 g for 10 minutes and discard the supernatant. Then bacteria were labelled with Fluorescein isothiocyanate (FITC;Sigma) 10 µg/ml incubated at room temperature for 2 hours in dark. Then extensively washed by PBS solution to remove unincorporated FITC, and resuspended in 1ml TE buffer contain 100 mg/ml lysozyme, well mixed and incubated for 1 hour at room temperature followed by dividing to 5 preparations each preparation adjusted at different pH =7, 6, 5, 4 and 3 by adding 10N HCl, followed by ultrasonic disruption (Ultra sonification, UP 200S, Dr. Hielscher GmbH, Teltow, Germany). Sonication was performed for 7 cycle each cycle 30 second (90% power) intervals in an ice bath. The different suspensions were examined using florescent light microscopy (ZEISS,

Germany). Images were digitally processed with ImageJ plugin (ITCN: Image based Tool for Counting Nuclei)

Primary Human and guinea pig experiments

The primary experiment includes all the guinea pigs experiments and two separate experiments on human tissues were taken from the ileum and large bowel of additional 2 patients (2 women; ages, 61–67 y). The tissues were prepared and treated as explained before with different bacteria concentration. The tissues were inoculated in the mucosal side of the Ussing chamber with live FITC labelled *L. plantarum* (TMW: 1.1623 and 1.1566) strains to final concentration $1x10^{10}$ CFU/ml in the mucosal reservoir as described in (Nadler *et al.*, 2000). Also it was tested for $1x10^{9}$ CFU/ml.

Results

Results from primary experiments

Growth *L. plantarum* (TMW: 1.1623) on MRS and Krebs solution

The milk and the faecal *L. plantarum* (TMW: 1.1623 and 1.1566) were able to grow in both media MRS broth and Krebs solution, which means that they can consume the sugars in this medium and produce acids which lower the pH of the medium and might have influence on the Ussing chamber experiment. However, the ability to grow in Krebs solution was much weaker and need longer lag phase (Fig 5).

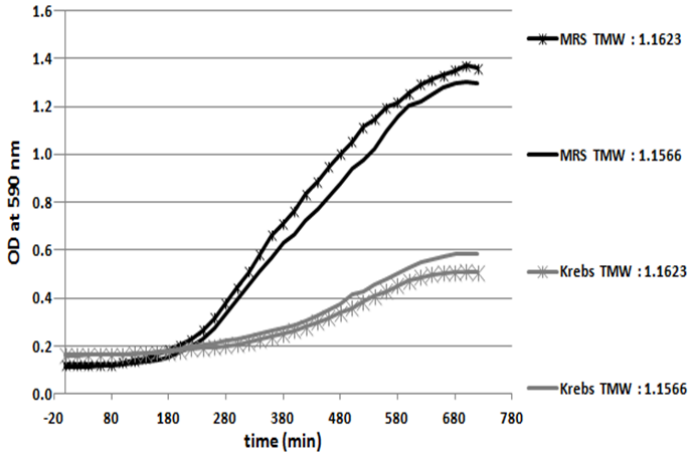

Figure 5. The different growth behavior of *L. plantarum* strain 1.1623 and 1,1566 when cultured in MRS medium and in Krebs solution

Influence of bacteria disintegration and pH on FITC staining stability

L. plantarum (TMW: 1.1623) were able to catch FITC staining and presented very well fluorescent cell signal under the florescent microscope. Even after disintegration the cells by sonication the florescent signal were still detected clearly (as this stain is able to react with

the surface proteins and peptides which still in the suspension after disintegration). However FITC staining can be influenced with the pH degree and the disintegrated florescent cells were giving less florescent signal as the solution pH value decrease from 7 to 3 and almost disappear at pH =3 (Fig. 6).

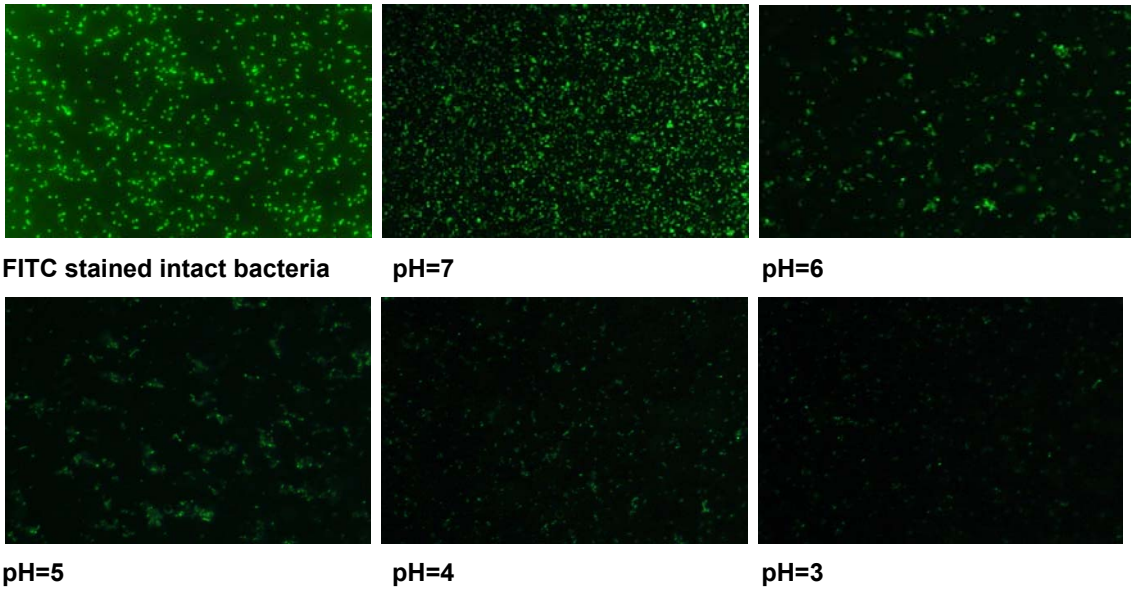

Figure 6. Microscope images of the same FITC-labeled bacteria suspension after sonication and different pH treatments.

Primary Human and guinea pig experiments

In primary experiments on human and guinea pigs tissues in the Ussing chamber the live *L. plantarum* (TMW: 1.1623 and 1.1566) strains were able to translocate through all human and guinea pigs tissues with translocation percentage about 0.07% ± 0.03 for human and about 0.1% ± 0.05 for guinea pigs when administrated at $1x10^{10}$ CFU. However, at this high bacteria dose the acidity in the mucosal reservoir increased from pH =7.4 to pH =6.5 after 60 minutes administration. The microscopic images for the tissue histological slices showed a very high active (suspected as) immune cell response as presented in figure 7, the shiny big spots are much bigger (10 time) than the bacteria cells size and was very florescent which never been seen in the control tissue which did not expose to the florescent labelled bacterial cells. Also it can be seen a very tiny shiny florescent pieces which is less than bacteria size and looks very much similar to the bacteria fragments after degradation (presented in the primary experiment results)

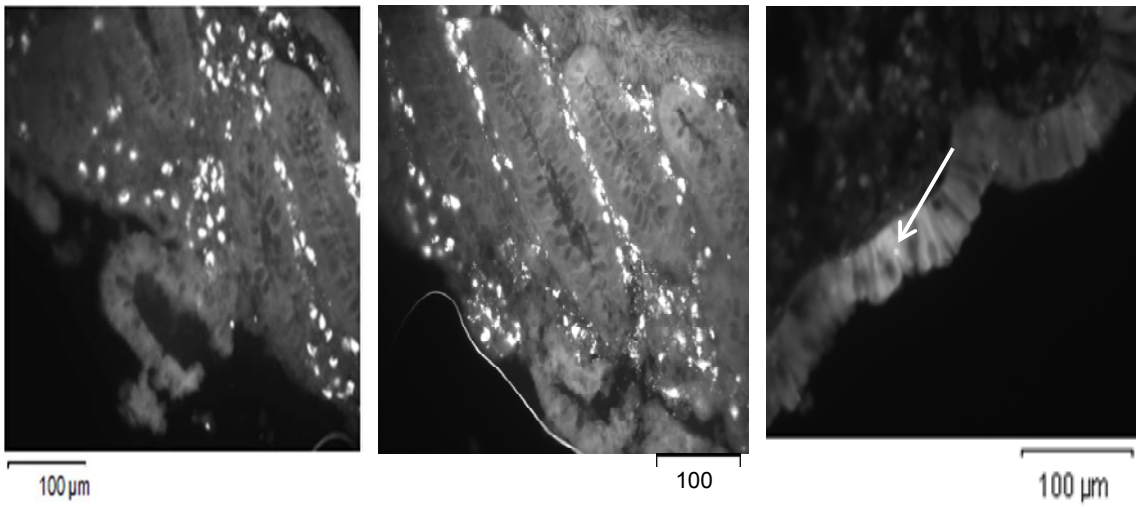

Figure 7. Histological slices of the human intestine. In the left and middle, photos from the primary experiments showing the active immune response for *L. plantarum* application (the shiny spots are immune cells which phagocytose the florescent bacteria; in the right single florescent FITC-labeled *L. plantarum* cell translocating in between epithelial cells (narrow arrows show the bacteria).

Under magnification 100x the florescent immune cells looks similar to neutrophils cells as shown in figure 8.

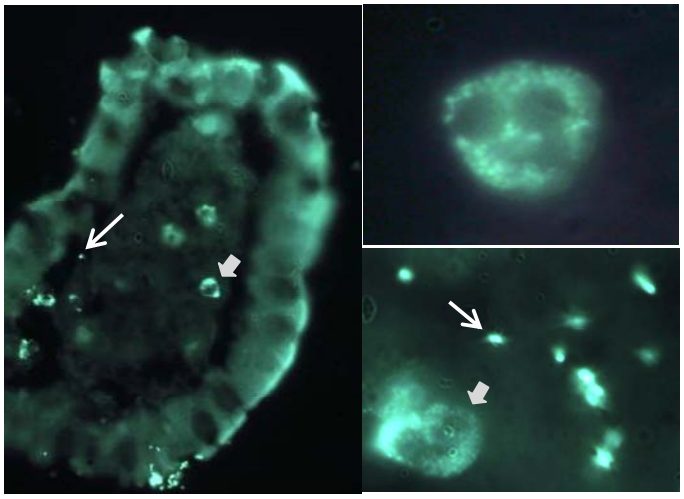

Figure 8. Histological slice of human small intestinal villi, the left photo shows the whole villus with flourescent immune cells (thick arrows) and florescent FITC-labeled bacteria (narrow arrow) (magnification 40x); in the right photos in the top single immune cells looks like neutrophils and in the bottom another neutrophils (narrow arrows) surrounded with FITC-labeled bacteria (thick arrow) (magnification 100x).

When we reduced the bacterial concentration in the mucosal reservoir to $1x10^9$ CFU the acidity increasing was reduced also from pH =7.4 to pH =7.2 after 60 minutes of the bacteria administration. The translocation percentage was reduced also to 0.005% ± 0.002 in human tissue. In the histological study by confocal microscope scanning of the 75-100µm tissue sections presented single intact florescent bacterial cells scattered in few parts of the sections. The scanning through the Z axis showed the single cells in the middle of the section which mean that it was translocating freely through the tissue (Fig. 9). In few sections some florescent immune cells (singles or groups) could be detected also. Many sections were free of bacteria (about 20% of the sections were not able to find translocating bacteria in it).

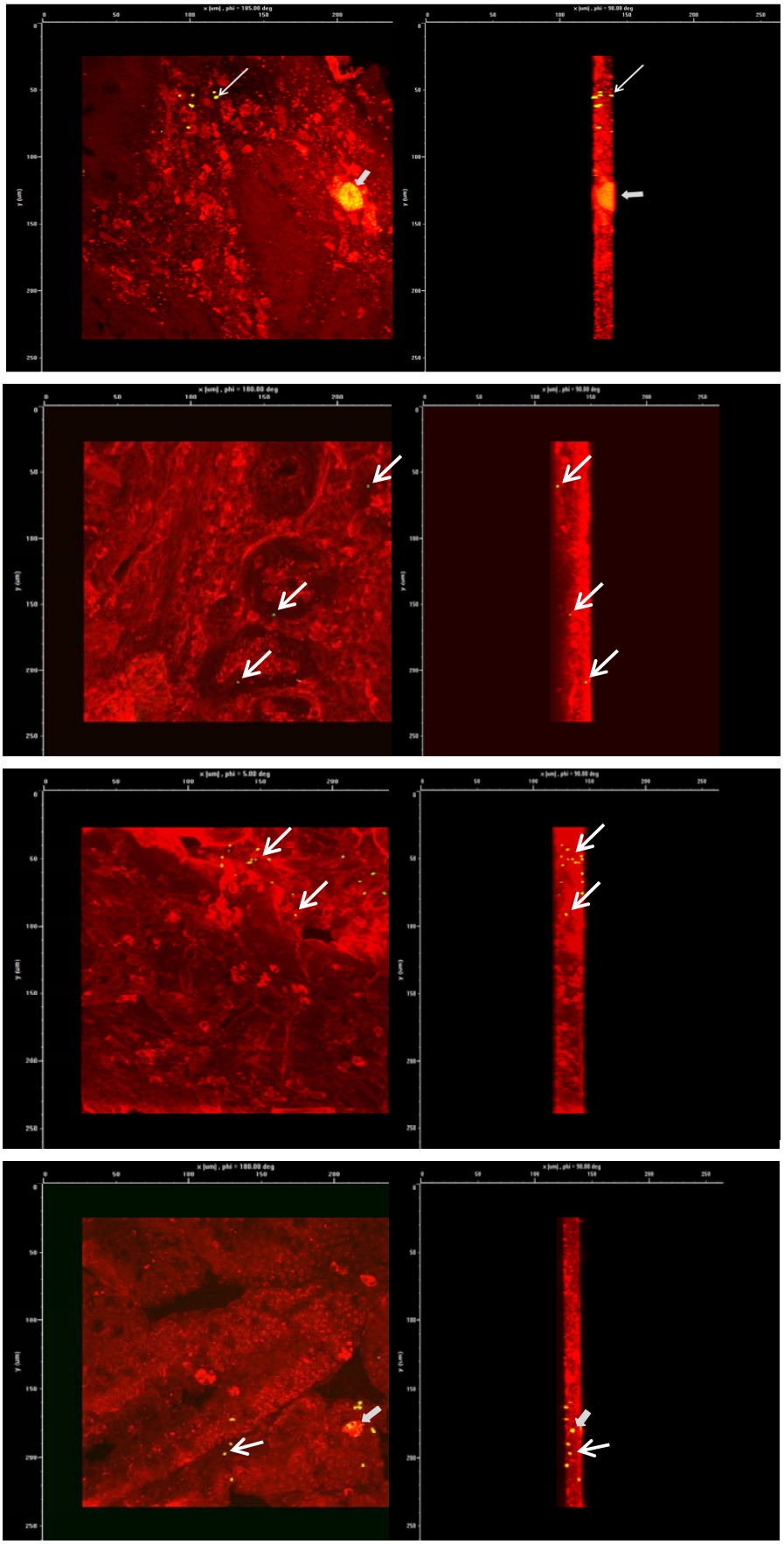

Figure 9. Histological slices of the small intestine (mucosa / submucosa) of human showing the interaction with live FITC-labelled *L. plantarum* (TMW: 1.1623) after 70 minutes of adminestration in the mucosal side of the intestine. The images in the right are on the Z axis and bacteria are visible as green spot in the middle of the 100µm thickness section, Magnification 60X. (narrow arrows refer to cluster of FITC-labeled bacteria cells While thick arrows refer to immmune cells).

In all primary experiments there were no significant or remarkable differences between the results of the milk *L. plantarum* strain TMW:1.1623 and faecal *L. plantarum* strain TMW:1.1566 in translocation through the different tissues.

Bacteria translocation through human tissue (experiment in the Ussing chamber)

In the main experiments of the 4 patient human tissues in the Ussing chamber, the live *L. plantarum* (TMW: 1.1623 and 1.1566) strains were not able to translocate through all tested human tissues with translocation percentage about 0.0% when administrated at $1x10^8$ CFU. The pH didn't significantly change pH (7.4, ± 0.09) after 70 minutes of $1x10^8$ CFU life bacteria administration in the mucosal reservoir. The dead *L. plantarum* (TMW: 1.1623 and 1.1566) strains were able to translocate through all tested human tissues with very low translocation percentage about 0.001% when administrated at $1x10^8$ CFU. There were no significant or remarkable different between the results of the live and dead milk *L. plantarum* strain TMW:1.1623 and live and dead faecal *L. plantarum* strain TMW:1.1566 in translocation through the different tissues (faecal *L. plantarum* strain TMW:1.1566 were tested only with tissues from two patients). All histological studies for different tissues sections couldn't present live translocating bacteria in any section, and very rarely florescent immune cell could be detected (Fig. 10). In the histological sections the bacteria were located outside the tissue in the mucosal side while the tissue section is clean, even the immune lymph follicle were free of bacteria.

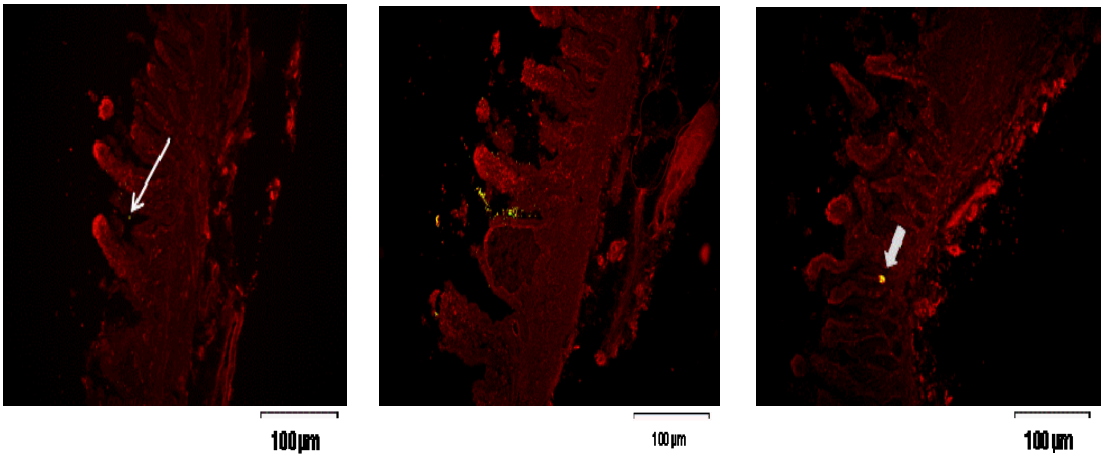

Figure 10. Histological slices of the human small intestine (the intestinal tissue stained with Alexa Fluor 635 Phalloidin red (Indodicarbocyanine, Cy5 filter) while live *L. plantarum* (TMW: 1.1623) strain is labeled with FITC green (Cyanine, Cy2 filter). the photo in the left showing single bacteria cell trying to passage through the intact intestinal tissue in between two villus, the photo in the middle present ileum section with single lymph follicle (solitary lymph nodule) surrounded with many bacterial cells in the mucosal side while the section was clean of any bacteria the, the photo in the right showing active immune respond to *L. plantarum* (the shiny pig spot is immune cell which engulfed the florescent bacteria (narrow arrows show the bacteria and the thick arrow show the immune cell).

Interestingly, the tissue resistance results presented significant deference between the tissue permeability change when tested with live and dead bacteria. When live *L. plantarum* (TMW: 1.1623) were adminestrated the tissue resistance increased significantly, which led to a decrease of the tissue permeability. On the contrary, when dead *L. plantarum* (TMW: 1.1623) were adminestrated the tissue resistance didn't change which lead to no change in the permeability of the tissue (Fig. 11).

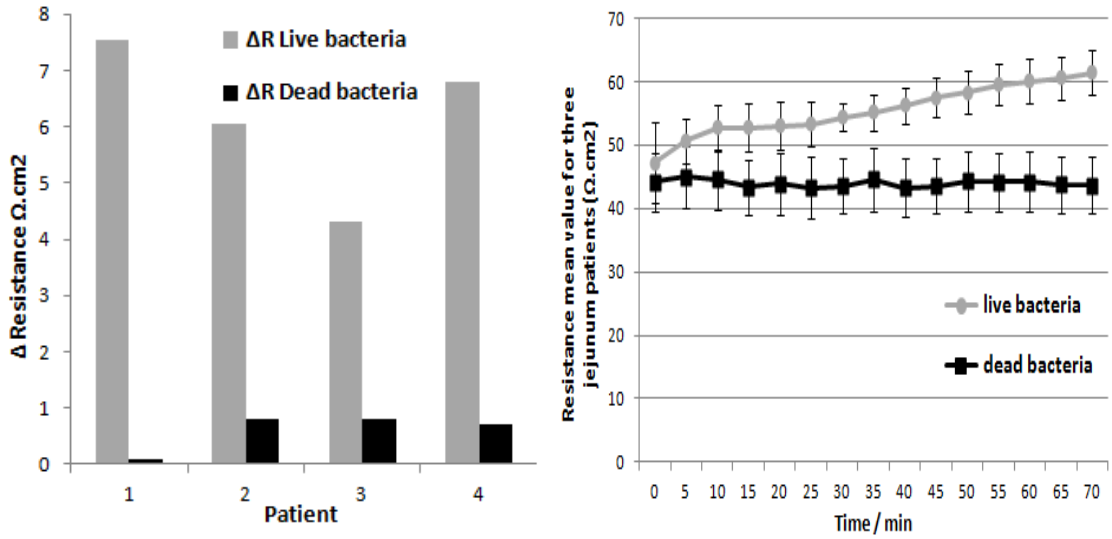

Figure 11. Segments of mucosa/submucosa intestinal of different 4 patients were mounted in Ussing chambers to measure changes in tissue resistance (an index of epithelial permeability) after 70 min of administration to live and dead bacteria of FITC-labelled *L. plantarum* (TMW: 1.1623) 1x10^8 CFU in the mucosal side of the intestine segment. (Normal jejunum resistance is 50.6 Ω ± 11.3 (personal information, Krueger D., unpublished)), the values indicated are mean±SE.

Discussion

Among the lactic acid bacteria isolated from breast milk few species clearly predominated: *Lactobacillus plantarum, L. fermentum, L. gasseri* and *E. faecium* (Albesharat *et al.*, 2011), (Martin *et al.*, 2004). While in human gut *L. plantarum*, *L. acidophilus* and *L. paracasei* are among the most predominant Lactobacillus species ((Albesharat *et al.*, 2011), (Ahrne *et al.*, 1998); (Reuter, 2001), (Vesa *et al.*, 2000), (de Vos *et al.*, 2004)). *L. plantarum* showed effective survival after stomach passage (Ahrne *et al.*, 1998), (Vesa *et al.*, 2000) and high survival capacity in the ileum and faeces comparing to other lactobacillus species (Vesa *et al.*, 2000). In the previous chapter results on *L. plantarum* species showed high survival ability in all different stress conditions with additional good H_2O_2, bacteriocin and beneficial metabolites producing ability. All this privileges of *L. plantarum* make it an excellent candidate for a translocation study. However, it has been well documented also that *L. plantarum* plays important role in maintaining the integrity of gut barrier, stimulation the intestinal mucosal proliferation and improvement of barrier function (Adawi *et al.*, 1998). Examining *L. plantarum* strain passage across a human and guinea pigs intestinal mucosal/submucosal membrane mounted in the Ussing chamber showed that barrier function and translocation ability is influenced by the change in the mucosal side pH, as a decrease in pH value lead to increase the permeability of the membrane (Emmanuel *et al.*, 2007). However, in spite of permeability change the immune respond was highly activated to prevent bacteria passage presenting in phagocytosis and engulfment of bacteria cells and degradation it in the neutrophils immune cells. Although the bacteria cell is disintegrated in the immune cells the florescent signals was still been able to be detected as the normal pH inside the phagosomes seems to be 4.7-5 (Henry *et al.*, 2004) were florescent signal can still be detected, whereas generation of reactive oxygen species (ROS) induces phagosomal pH increase which subsequently activates neutral proteases that digest and kill microorganisms (Narni-Mancinelli *et al.*, 2011) and from our previous results we found *L. plantarum* (TMW:1.1623) is highly H_2O_2 producer which can release ROS which increase the neutrophils phagosomes pH and cause higher florescent signal from the immune cells (neutrophils). The translocation percentage was higher in guinea pigs tissue comparing to human tissue and this is consistency with Balzan results who reported that frequency of translocation in humans is much lower than that observed in animal models (Balzan *et al.*, 2007).

In isolation from the pH impact on the intestinal membrane the live *L. plantarum* cells presented initiated a significantly increasing resistance leading to block membrane permeability, this influence was not observed in the case of dead bacteria which lead to confirm the results of variety of studies which exploit the capacity of *L. plantarum* to secrete bioactive molecules that evoke an immune response (Reveneau *et al.*, 2002), (de Vries *et*

al., 2006), (Vissers *et al.*, 2010) and evoke the membrane resistance. The data reported by Maassen et al. and Pavan et al. shows that *L. plantarum* is a weaker cytokine inducer comparing to other *Lactobacillus* species (Pavan *et al.*, 2003), (Maassen *et al.*, 2000). While Müller et al. demonstrated that heat inactivated *L. plantarum* and *L. lactis* differ substantially in their capacities to modulate cytokine production by human peripheral blood mononuclear cells. While the former leads to strong IL-10 (an anti-inflammatory cytokines) production, the latter is characterized by the stimulation of TNF-α, IFN-γ, and IL-12 (proinflammatory cytokines)(Müller-Alouf *et al.*, 1999), and this IL-10 can inhibit neutrophil phagocytic and bactericidal activity (Laichalk *et al.*, 1996). This can explain the slight translocation percentage observed in the case of dead bacteria with the absence of immune respond function. Our finding is in agreement with the different studies which have reported the preventive effect of administrating *Lactobacillus plantarum* on bacterial translocation of the global intestinal microflora performed with healthy and injured rodents (Mao *et al.*, 1996), (Adawi *et al.*, 1998), (Mangell *et al.*, 2002), (Pavan *et al.*, 2003), (Nguyen *et al.*, 2007). Adawi (Adawi *et al.*, 1997) had investigated the effect of administration five different *Lactobacillus* strains (*L. reuteri* R2LC, *L. rhamnosus* DSM 6594 (= strain 271), *L. plantarum* DSM 9843 (= strain 299v), *L. fermentum* 8704:3 (= strain 245), and *L. reuteri* (= strain 108) on bacterial translocation in rats, and he found that the incidence of bacterial translocation and the number of the translocated bacteria decreased significantly when supplement with *L. plantarum* DSM 9843 (= strain 299v) and this strain seemed superior to the other *Lactobacillus* strains, and he explained that this bacteria may execute their action via bacterial antagonism and/or enhancement of systemic and intestinal mucosal immunity. However, this special property of *Lactobacillus plantarum* will play major alteration rule in case of pregnancy and lactation with the nitric oxide synthesis. Nitric oxide appears to play a paradoxical role in intestinal physiology (Nadler & Ford, 2000). The uterus produces nitric oxide during the pregnancy and this activity decrease between late gestation (16-18 days in rodent and 32-34 wk in human) and spontaneous labour at term (Natuzzi *et al.*, 1993), (Sladek *et al.*, 1993), (Yallampalli *et al.*, 1993), (Sladek & Roberts, 1996). this decrease of nitric oxide production is capable of increasing intestinal permeability and bacteria translocation (Kubes, 1992), (Kanwar *et al.*, 1994), (Adawi *et al.*, 1998). Interestingly *L. plantarum* is the main species which influence with this decrease, the ability of *L. plantarum* to inhibit other bacteria translocation decreased with the decreases of nitric oxide production (Adawi *et al.*, 1998). This indirectly leads to conclude that *L. plantarum* translocation ability could be depending on certain body conditions, which induce it like the changes, which happen during lactation and pregnancy. To proof this theory further studies employing a murine module with pregnant and lactating animals was performed as described in the following chapter.

Acknowledgements

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CHAPTER V

In vivo translocation of *Lactobacillus plantarum* in pregnant and lactating mice

Abstract:

The characterization of bacterial isolates from mothers' milk, mothers' faeces and their babies has revealed identical, i.e. indistinguishable strains in all three sources of one such trio. This suggested that strains of several lactic acid bacteria including *Lactobacillus (L.) plantarum* from the gut microbiota of mothers can pass to their baby´s gut through her milk. Therefore, the ability of translocation of such bacteria during pregnancy and lactation from mother to her progeny was investigated in a murine model. Female mice were divided in groups, Three groups of pregnant mice were orally administered with the human milk isolate *L. plantarum* (TMW 1.1623), at 10^7 cfu/mouse per d for three days before birth and for one and two weeks after birth. Three pregnant groups receiving PBS, and three virgin groups receiving bacteria were used as controls. Food intake, body weight, bacterial translocation to the fetus, puppies and different organs of the mother were analyzed. The live tested strain could be isolated from fetus' gut, in the uterus, and after birth from puppies' gut before and after receiving milk from their inoculated mothers. Tested bacteria were recovered also from some mother's tissue samples (mesenteric lymph nodes, placenta, liver and spleen). No viable bacteria were detected in the samples obtained from virgin and PBS control groups. Based on these findings we suggest the possibility of bacterial translocation from the mothers' intestine being effective during pregnancy and lactation.

Introduction:

Human and animal studies suggest that the intestinal microbiota during the neonatal period has a profound effect on nutritional status, gastrointestinal (GI) tract development, and maintenance of mucosal surface integrity (Hooper & Gordon, 2001), (Caicedo *et al.*, 2005). Also it has an important effect on health and specific inhabitant in this process may predispose to disease later in life (Kalliomaki *et al.*, 2001). Our understanding of the establishment and evolution of the intestinal microbiota during early infancy in humans is limited. It could be confirmed that the first colonizers might come from the mother´s microbiota (Martin *et al.*, 2003), (Albesharat *et al.*, 2011). Apart from any obvious faecal, vaginal and skin contamination occurring during birth there is accumulating evidence that bacteria from mother are transmitted to the infant before birth (Jimenez *et al.*, 2005), (Perez *et al.*, 2007). Mutualistic bacteria have been recently isolated from human meconium (Kukkonen *et al.*, 2007), (Mshvildadze *et al.*, 2010), and the presence of some lactic acid bacteria species in chorioamnion samples of healthy mothers who underwent caesarean surgery has been described by (Bearfield *et al.*, 2002). In the earliest stool samples, diversity differed depending on gestational age at delivery, use of maternal antibiotics, and intended feeding type (breast versus formula)(Mshvildadze *et al.*, 2010). One remarkable finding is

represented by the fact that in spite of the maternal legacy of species diversity, each of the babies can develop its own species microbiota pattern (Favier *et al.*, 2003).

During the early months of life for the neonate, breast milk is the best food for the speedy growing infant since it is a rich source fulfilling all his nutritional requirements (Scientific Committee on Food, 2003). Additionally, several studies have shown that breastfeeding presents a continuous source of unharmful beneficial mutualistic microorganisms, which enhance the immunity and metabolism to support the infants´ health and protects against infectious diseases (Caicedo *et al.*, 2005), (Lara-Villoslada *et al.*, 2007), (Maldonado *et al.*, 2010), It is been demonstrated that this microbiota is from maternal origin. However, milk had a less variable microbiota than maternal feces (Perez *et al.*, 2007), (Albesharat *et al.*, 2011). This suggests that there is specific selection for this microbiota transmitted to milk. Among the lactic acid bacteria isolated from breast milk few species clearly predominated: *L. plantarum, L. fermentum, L. brevis, E. faecium* and *E. faecalis* (Albesharat *et al.*, 2011), (Martin *et al.*, 2004).

The real answer for formulating bacteria translocation hypotheses come from human system. However, animal model such as mice give alternative choices to study the hypotheses and its conditions. As every model or system has its restrictions, the mice module can provide special ability to study the delivery influence isolated from the vaginal contamination which human model can't provide it (the pups make their way down the birth canal with their placenta in a birth sac without contacting the vaginal environment and after delivery the mother release the puppies from the birth sac).

It is worth mentioning that investigating variable bacterial actions in the intestine were different, as well as the hosts, mouse *versus* human, and for those actions which fundamentally contribute in nutrient profit and synthesis, stress, and extracellular functions, they indicates that intestinal conditions of mouse and human may have similar effects on global actions of *L. plantarum* in the intestine (de Vries *et al.*, 2006).

The aim of the present work was to demonstrate the proof of concept of bacterial translocation from the mothers' intestine to their progeny during pregnancy and lactation. We selected the human milk isolate *L. plantarum* (TMW 1.1623) as a promising translocating candidate strain based on its presence in mothers, their milk and babies of one coherent such trio of indistinguishable isolates and employed it in a murine model.

Materials and Methods:

Bacterial strains:

L. plantarum strain (TMW 1.1623)**,** a human breast milk isolate **(Albesharat *et al.*, 2011)**, was used in this study. To permit detection of the strains within the endogenous intestinal and milk flora of the subjects, spontaneous mutants resistant to rifampicin (100mg/ml) were selected from the wildtype strain and designated (TMW 1.1623^{RF}). In order to monitor the bacteria after their administration to the mice and for their translocation to the mammary glands in the histological study, the strains were labelled with fluorescein isothiocyanate (FITC).

Bacterial culture preparation for feeding:

L. plantarum TMW 1.1623^{RF} were grown in MRS broth supplemented with 100 µg/mL rifampicin at 37°C for 16–18 h, and then number of viable cells was determined by agar plate counting and/or OD_{600} measurements. For *in vitro* experiments, fresh cultures were used, whereas 1.1623^{RF} bacterial stocks were made in MRS supplemented with 100 µg/mL rifampicin with 10% glycerol and kept frozen at −80°C until used for *in vivo* experiments. From frozen stocks (80°C) bacteria were suspended in de Man-Rogosa-Sharpe liquid medium (MRS broth; Difco Laboratories, Detroit, MI) plated in MRS agar supplemented with 100 µg/mL rifampicin, cultured anaerobically at 37°C for 24 hours, then daily inoculated in fresh MRS broth supplemented with 100 µg/mL rifampicin and grown at 37°C under anaerobic conditions for 24 hours in 15-ml tubes. After 1 day, tubes were centrifuged at 2,000 rpm for 15 minutes at 20°C and washed twice with sterile phosphate-buffered saline (PBS). Bacterial suspensions were centrifuged in 15 ml tubes at 2,000 rpm for 15 minutes at 20°C, supernatants discarded, then bacteria were labelled with Fluorescein isothiocyanate (FITC;Sigma) 10µg/ml incubated at room temperature for 2 hours in dark. Then extensively washed to remove unincorporated FITC, and resuspended in PBS before (to a concentration of 6 $x10^7$ bacteria/ml) administration by gavage to each animal.

Animals:

Fifty five females and 10 males of Balb/C SPF/VAF (specific pathogen-free (SPF), viral antibody-free (VAF)) mice aged 6–8 weeks, bred at the Charles River Laboratories (Mainland, China). Mice were maintained in specific pathogen free conditions at Nanchang University, China. were housed in the exclusion facility are held in ventilated racks in static micro-isolator cages under sterile conditions with a 12 h light/dark cycle (8 am–8 pm) in a controlled atmosphere (temperature 22°C±2°C, humidity 55% ± 2%). The animals were fed a standard purified diet (Charles River Laboratories, China) and had free access to water and mouse chow during the experimental protocol. The protocol was carried out according to the guidelines of Charles River, China and was approved by the Ethic procedure of the department of animal science laboratory at Nanchang University, China.

Animals' treatment and different samples processing:

After 7 days of acclimation, pregnant female mice were used around 8 weeks of age and 1.1623^{RF} or PBS-fed for the next 2 weeks (starting 2-3 days pre-birth) and age-matched, nongravid, nonlactating mice (virgin) were treated similarly. 1.1623^{RF}-fed animals received 10^7 CFU of live bacteria daily in 200 µL PBS, whereas control received only PBS. Mice were fed daily with new preparation of bacteria from the beginning until the end of the study.

Intragastric feeding was performed by means of metal feeding tube (animal feeding needle (AFN)) for rodent oral gavage. The intestinal microbiota of the mice did not contain any rifampicin resistant bacteria growing on the selective plates used in this study.

Collecting milk was performed under light anesthesia with trichloroacetaldehyde hydrate. Intraperitoneal (i.p.) injections of 10 µg of oxytocin were performed 10 min before milk collecting. Breast-milk samples were obtained by manual expression after cleaning the nipples and areola by wiping with a swab soaked in ethanol 70% and discarding the first drops. During milking process to the puppies a few drops were collected over microscope slide and tested.

Collecting fetus gut samples was performed before birth by sacrificing the pregnant mothers after three days of bacteria administration and collecting the fetus and placenta from the uterus aseptically.

Collecting puppies gut was performed in two ways (empty and full gut), when birth is impending there will be a bloody vaginal discharge. This occurs a day before, or the day of birth. Mouse delivered puppies range between 5 – 13. For empty gut puppies samples the puppies (one puppy from each mother) were collected on time directly after birth before the mother licks it or remove the birth sac. For full gut puppies samples the puppies where collected after 3 hours of the daily bacteria administration to the mother and after suckling (one puppy from each mother), that the full stomach with milk can be seen through the skin in the body of the puppies (see fig 10)**.** Full guts were collected from puppies every second day after suckling during the experimental period (one or two weeks after birth). For empty and full gut puppies the surface viscera was swiped with a sterile swab. The complete digestive tract were excised aseptically and weighed then collected into a set of 2 ml Eppendorf tubes containing 1 ml Ringer's solution. Then were treated them for bacterial count determination in the same way as described below.

Experimental design:

Fifty five female mice were divided in 11 groups (n = 5). Two groups of pregnant mice (A and B were treated orally with FITC labeled *L. plantarum* strain (TMW 1.1623^{RF}) dose of 10^7 cfu/mouse per d for three days before birth and during one week after birth then been sacrificed (A) and during two weeks after birth then been sacrificed (B) to collect its' blood and organs (liver, spleen and MLN) while its' puppies gut were collected one time before

suckling and every second day during the tested lactation period. Group (D) were treated similarly like group (A) but with unlabeled *L. plantarum* (TMW 1.1623^{RF}) to check for any adverse effect of the FITC labeling. One pregnant group (E) was treated with PBS before birth and with labeled *L. plantarum* after birth for one week and with PBS again for the second week after birth and its puppies gut was collected during the different treatments. Another pregnant group (C) was treated with labeled bacteria for three days and their fetus and organs were collected before parturition. Three additional pregnant groups (G, F and J) were administered the PBS alone for period similar to groups (A, B and C) respectively and was used as negative controls. The last three groups were virgins (H, I and K), which received the same treatment like the previous pregnant groups (A, B and C) respectively, and also were used as controls for comparison. Pregnant mice were sacrificed on day 1 to 3 before parturition, and lactation mice were sacrificed on day 7 and 15 after birth. Samples of blood, puppies' intestinal contents, mesenteric lymph nodes (MLNs), spleen, liver, and mammary gland were collected aseptically for microbiologic and histological analysis for all groups.

Translocating bacteria detection in blood, milk and different organs:

The translocation of fluorescent bacteria to the milk was detected under a florescent light microscope using Cy2 filter images at (λ_{exc} = 492 nm, λ_{em} = 520 nm). The number of rifampicin resistant bacteria was detected by plating the content of the puppies' full gut. On day 7 and 15 of lactation, after 3 hours of the last gavage and puppies milking, the blood was collected from the retro-orbital sinus in sterile conditions and animals were killed by cervical dislocation. Before excising tissue samples, the surface viscera of the mice was shaved from fur and swiped with a sterile swab. The mammary glands of the mothers, mesenteric lymph nodes (MLN), spleen and liver were excised aseptically and weighed then collected into a set of 2 ml Eppendorf tubes containing 1 ml Ringer's solution. Blood, MLN, spleen and liver were collected also from 10 age-matched, nongravid, nonlactating mice. Bacterial translocation was analysed in milk (full puppies gut), blood, MLN, liver and spleen. 500 microlitres of blood were collected, 100 microlitres were cultured in MRS agar supplemented with 100 µg/mL rifampicin and incubated at 37°C during 48 h in anaerobiosis. The puppies gut, whole MLN, spleen, and a portion of liver (after weighting) were immediately placed in one-third-strength Ringer's diluents (1 g/ml). The organ samples were homogenized with tissue grinder and serially diluted 3-fold in sterile saline. Selective enumeration of *L. plantarum* (TMW 1.1623) was performed by plating 100 µl of the resulting homogenates on plates of freshly prepared media. The medium used was MRS agar supplemented with 100 ug/mL rifampicin. After 48 h of anaerobic incubation at 37 °C, CFUs were counted and results were expressed as incidence of translocation. Positive growth on agar plates was defined by the presence of any micro-organisms (even a single colony). Matrix-assisted laser desorption/ionization-time

of flight mass spectrometer (MALDI-TOFms) analysis was used to identify the organisms detected in MLN, spleen and liver tissues. Following the described analysis procedure and protocol of 'direct transfer' in (Freiwald & Sauer, 2009) with one modified step to the protocol of additional 1µl of formic acid 70% over the cell smear before overlying with matrix. The MALDI-TOF spectrum patterns of suspected strains were compared with that of the test strains. The mammary glands were histologically scored as later described, the colony counting and histological scoring is performed in a blind manner.

Concomitant investigations:

Throughout the experiment, the animals' activity, behavior and general health status were observed twice daily. Whenever the situation warranted, the mice were weighed daily and their feed intake were weighed daily for each mouse. All animal displaying signs of pain or >10% weight loss have to be prematurely killed. Organs weight, spleen and liver were taken and considered as indicator for bacteria over passage. Also spleen weight index (SWI) was expressed as the actual spleen weight (mg) divided by the last measure of live body weight (g). Animal food intake (FI) and body live weight gain (LWG) were also measured daily.

Histological analysis:

The whole mammary glands, placenta, spleen and portion of liver were excised (from different groups except group D) and fixed by immersion in 4% paraformaldehyde in 0.1 M phosphate-buffered saline at room temperature for 8±12 hr then washed with PBS and transferred to be soaked in sucrose 43% solution for 8±12 hr. The samples were embedded in (Bright cryo-M-BED, Embedding compound for frozen tissue specimens) for frozen section. Tissue sections were cut at 75-100 µm thickness and stained with Alexa Fluor 635 Phalloidin (Cytoskeleton) and SYTOX Orange/DNA —stained mammary and placenta sections. The tissues was examined using Ti-U inverted research microscope with an e-C1plus confocal system (Nikon, Düsseldorf; Germany) and a 20 x (N.A. = 0.75) objective was used. Bacteria were monitored as green fluorescence images at (λ_{exc} = 488 nm, λ_{em} = 515/30 nm) and cell Cytoskeleton as red at (λ_{exc} = 632 nm, λ_{em} = 650/LP nm) and cell DNA as blue at (λ_{exc} = 543 nm, λ_{em} = 590/50 nm) with 512 x 512 pixel resolution (636 x 636 µm) in a constant z-position, and representative scanning pictures of the sections were taken.

Statistical Analysis:

Microbiological data, recorded as colony forming units (CFU) per gram of organ, or milliliter of milk or blood. All the data presented herein are expressed as mean ± SD. Comparison in the group and between groups was statistically analyzed by variance (ANOVA), performed by using Co-stat-ANOVA software. Treatment means were compared using Student-Newman-Keuls test at the level 5% of scientific. Bars indicate standard errors, Differences were considered statistically significant for P value ≤ 0.01, and n represents the number of experiments performed.

Results

Health status after repeated oral administration of *L. plantarum* in healthy mice:

Oral administrations of 10^7 CFU of *L. plantarum* were performed daily for 7 and 15 days to virgin, pregnant or lactation mice, and different parameters were monitored to evaluate the potential adverse effect of repeated bacterial ingestion. No variation in mouse activity and weight was observed in comparison to the activity and weight of the control group receiving PBS. Concerning tissue weights, there were no significant differences in liver and spleen weights between control mice and those receiving oral administration of *L. plantarum* for the three cases virgin, pregnant and lactation. As expected, there was a significant increase in liver weight during pregnancy and lactation as compared to virgin mice (P=0.0000) (Fig.3). The MLN and spleen cultures were negative for *L. plantarum* in virgin mice, indicating that repeated ingestion of 10^7 doses of this strain did not induce abnormal Bacteria translocation (BT) in healthy normal mice. From animal observation throughout the experiment period, no noticeable change in behaviour or activity was observed in the mice, and no treatment-related illness or death occurred. There was no observable difference in the animals' hair lustre between treatment and control groups.

Feed intake and live weight gain:

Oral administration of *L. plantarum* had no adverse effect on the animals' food intake and live weight gain (LWG). Figure 1 illustrates the animals' daily food intake. Statistical analysis revealed that there was no apparently significant difference in food intake and LWG among bacteria administrated groups and the control PBS groups ($P > 0.01$). There was a significant increase in food consumption after 5 days as compared with the first 5 days due to the increase of nutrient need for lactation.

Figure 2 illustrates animals' changes in weight gain between different groups. Statistical analysis revealed that there was no apparently significant difference in food intake and LWG among bacteria administrated groups and the control PBS groups (P=0.0000). There was a significant decrease in live weight gain after the day of birth as compared with the first 3 days, there was no significant difference or weight change between the 4^{th} and 15^{th} day.

In spite of bacterial detection in the liver and spleen and the increase in these tissue weights during pregnancy, the spleen index showed significant decrease during pregnancy and lactation comparing to virgin case (p=0.0000) (Fig.3). There was no statistically significant difference in translocation incidence between test and control groups (p=ns).

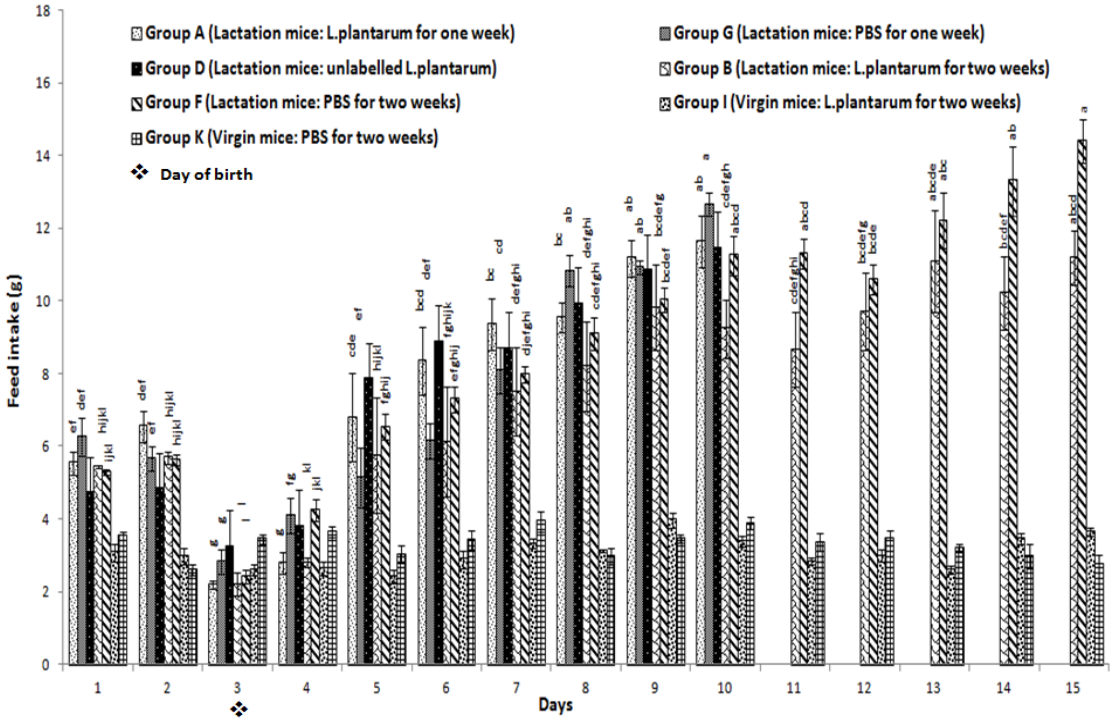

Fig. 1. Daily feed intake (g) of mice treated with *L.plantarum* strain or PBS for 15 days. Significant differences between group A&G, B&F are denoted by letters as determined by ANOVA with Co-stat multiple comparison using Student- Newman-Keuls test and this data was analyzed in two ways completely randomize (p<0.01). The values indicated are mean±SE.

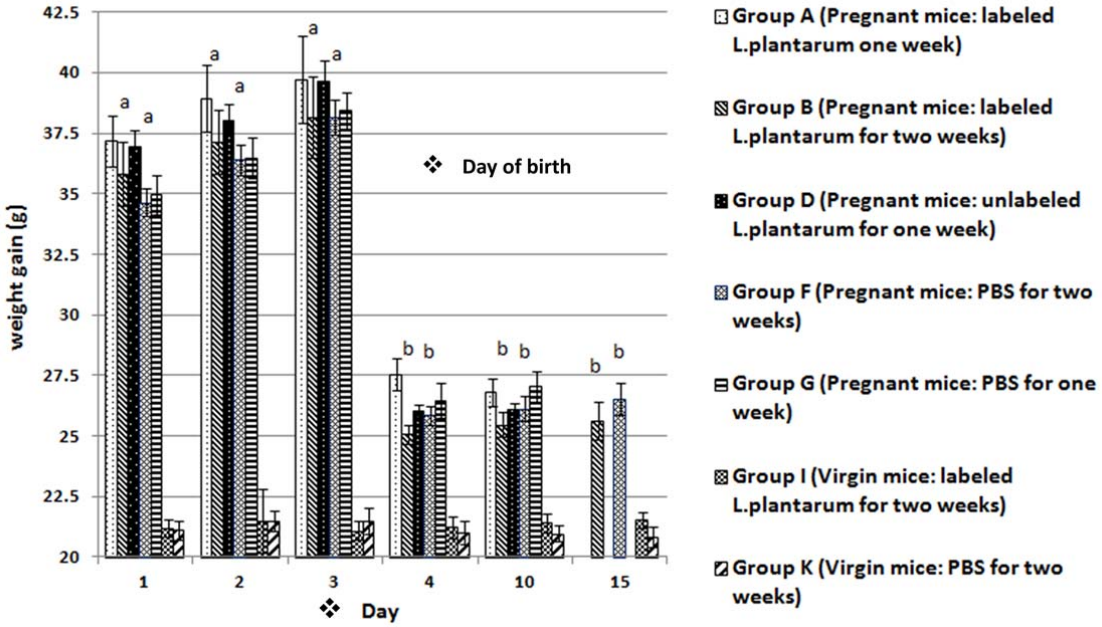

Fig. 2. Daily live weight gain (LWG) (g) of mice treated with *L.plantarum* strain or PBS for 15 days. Significant differences between group B&F are denoted by letters as determined by ANOVA with Co-stat multiple comparison using Student- Newman-Keuls test and this data was analyzed in two ways completely randomize (p<0.01). The values indicated are mean±SE.

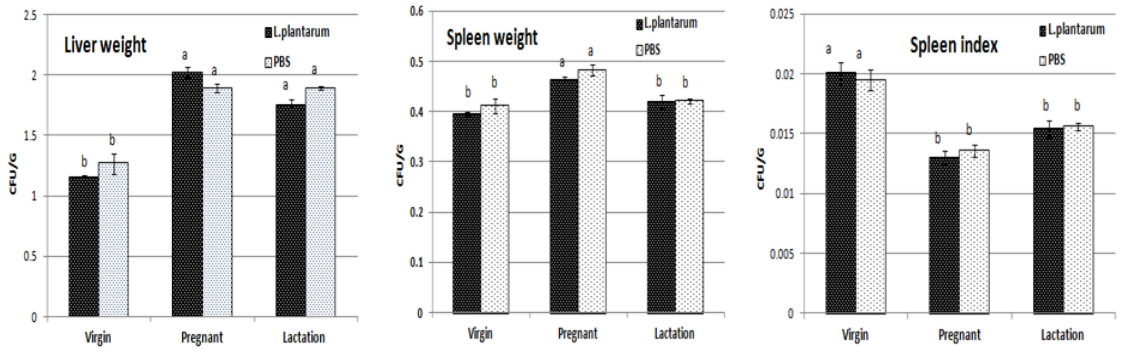

Fig. 3. From left to right, liver weight, spleen weight and Spleen index values after treatment with either PBS or *L.plantarum* of 10 days for virgin, 3 days for pregnant and 10 days for lactation mice. Significant differences are denoted by letters as determined by ANOVA with Co-stat multiple comparison using Student- Newman-Keuls test this data was analyzed in two ways completely randomize (p<0.01). The values indicated are mean±SE.

Detection of bacteria in organs of pregnant and lactating mice:

The presence of bacteria after oral administration was also determined in several organs of pregnant, lactation and virgin mice. The incidence of bacterial presence in different tissues is shown in (Fig 4). No bacteraemia was detected in any of the groups of animals. There was also a statistically significant difference in detection incidence between test and control groups.

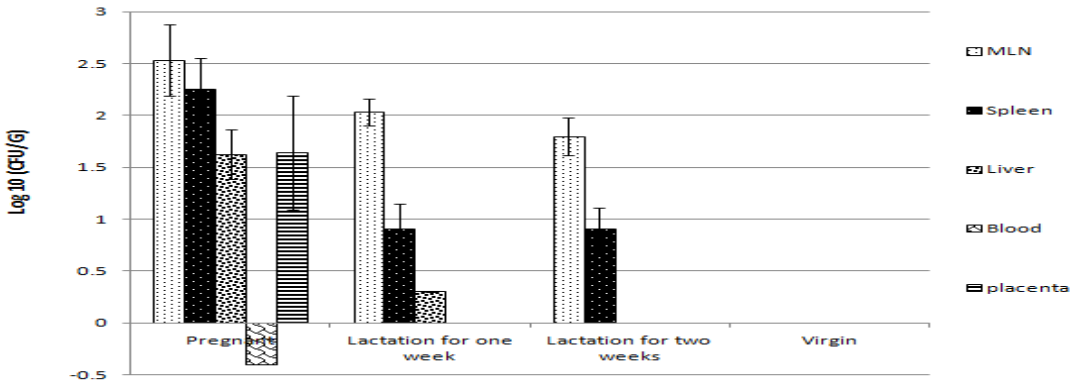

Fig. 4. Administrated bacteria presence in different organs of pregnant, lactation and virgin mice. Bacteria can be detected in the different organs of the pregnant mice. While it only exist in the MLN and spleen after two weeks of lactation. No viable bacteria were recovered from blood and tissue samples (mesenteric lymph nodes, liver and spleen) of virgin mice, this data was analyzed in two ways completely randomize (p<0.01).

In addition, colonies found on selective agar plates were checked by MALDI-TOF MS, and all MALDI-TOF MS spectra from suspected strains matched the pattern of the *L.plantarum* (TMW 1.1623^{RF}), which indicates that all the viable bacterial cells recovered from tissues were derived from the oral administration of this strain. Bacterial administration during pregnancy resulted in the detection of increased bacterial numbers during the last stage of

pregnancy (one or two day before parturition) in the MLN, spleen and liver in the pregnant mice, In these mice only blood remained free of bacteria, whereas blood, MLN, spleen and liver remained sterile in healthy virgin animals. Bacteria were then enumerated in each positive organ (Fig. 4). Oral administration caused a significant detection of *L. plantarum* in the MLN, spleen liver and placenta during pregnancy (P=0.0008). However, during the two weeks of lactation there was no significant difference except the liver bacteria count, whose value became 0 in the second week of lactation.

Occurrence of LAB in the puppies' intestine:

Comparisons of bacteria detection between animals in one group may sometimes be difficult because of slight differences in the animal milking efforts, which may be partly influenced by the different numbers of puppies per mouse. However, *L. plantarum* (TMW 1.1623^{RF}) was evaluated for its ability to persist in the GIT of mouse fetus after 3 days of oral administration to their pregnant mothers and in puppies after 1 day of oral administration to their lactating mother mouse, with bacterial levels ranging from 10^1 to 10^2 CFU/ml/g of the GIT during 1-2 weeks of daily oral administrations to the mother. All fetus samples were free of bacteria even the ones which collected at the parturition before birth. Interestingly in all empty guts from puppies analysed directly after birth and before milking the bacteria administered to the mother were detected. As mentioned before these bacteria were present in the placenta also before parturition. As shown in Figure 5, oral administration of *L. plantarum* to the pregnant mothers caused a significant increase (P= 0.0001) in bacterial detection in the fetus upon birth (sampled directly from the birth sac) as compared with the ones before birth (from the uterus).

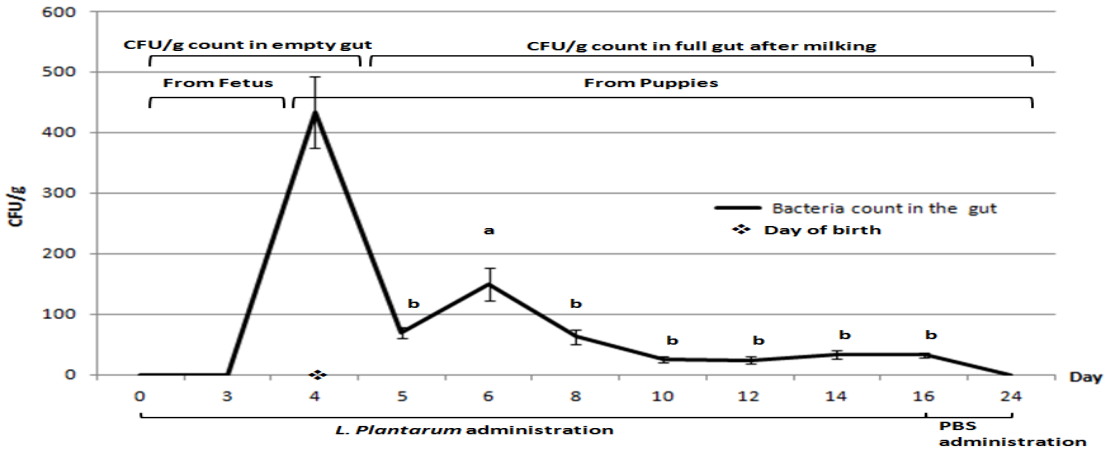

Fig. 5. The presence of the daily mother administrated strain in the gut of the fetus and puppies directly after birth and during two weeks of lactation and after one week from stopping administration. Day 0 is the first day of administration during pregnancy, which continue for two weeks during lactation and stop at day 16 where the last sample were taken in day 24. The values indicated are mean±SE.

After birth we distinguish between two kinds of puppies gut samples, empty gut (before milking) and full gut (after first suckling). The comparison between the empty gut and full gut bacterial count showed significant difference between them (P=0.0003), were the mean value of the empty gut was 433.8 CFU/g while the full gut was 68.8 CFU/g and this means that the gut count was decreased about 16 times after the first suckling. The bacterial count was refreshed and increased slightly after the second suckling, as the comparison between the puppies full gut samples during two weeks of lactation from mothers administrated daily to *L. plantarum* strain (TMW 1.1623^{RF}) showed that only in the 6th day (after second suckling) presents a significant increase in the bacteria count(mean value 149 CFU/g) while there was no significant difference between the other days samples until the day 16 (mean value 68, 62, 26, 24, 33 and 33 CFU/g respectively). Strain (TMW 1.1623^{RF}) was detected in the GIT of mouse puppies after 7 to 8 days of the last dose (P= 0.000). The tested stain was never detected in the PBS animals.

As the aim of the study was to detect the ability of the strain (TMW 1.1623^{RF}) to translocate through mother milk, Group E mice were treated differently with PBS starting before birth and for one day after birth and first suckling (sample of empty gut and full gut were taken), then started from the second day after birth to be treated with strain (TMW 1.1623^{RF}) for one week and collecting full gut samples. In this group oral administration of *L. plantarum* to the mother in the second day of lactation caused an increase (P < 0.01) in the tested bacterial count in the full gut of the puppies after suckling, and then it remained in stable during milking for one week (Fig.6).

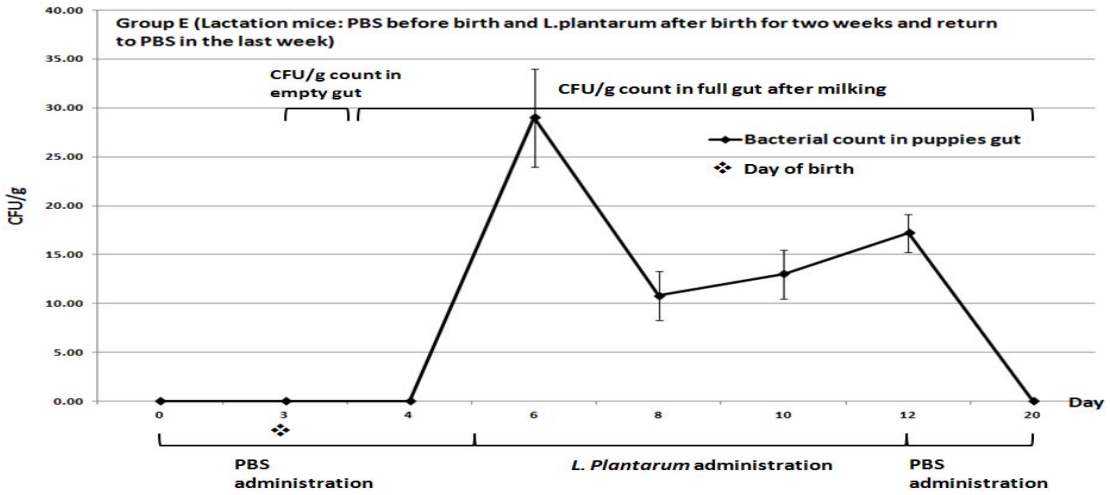

Fig. 6. Show Group E, administrated strain presence in the gut of the puppies as their pregnant mothers were administrated for PBS for three days before parturition and for one day after birth then started treatment with *L.plantarum* in the second day after birth for one week of lactation then return back for PBS administration for the second week . Day 0 is the first day of PBS administration during pregnancy, day 5 is the first day for *L.plantarum* treatment during

lactation for one week and day 12 return to PBS treatment and the last sample were taken in day 20. The values indicated are mean±SE.

This increase was dependent on the *L. plantarum* delivered through the milk, because there was no significant difference between the continued full gut sampling during one week of receiving the same doses of the potentially translocating bacteria. Additionally, the bacteria were lost after one week of the stop bacterial administration to the mothers.

Histological examinations:

Due to the small number of bacteria detected in microbiological analyses a statistical evaluation of the microscopic analysis cannot be expected. Therefore, the aim of this analysis was determination of a specific localization in the tissue. During lactation, bacteria were observed histologically in the glandular tissue between the alveolar spaces of the mammary gland in all mammary tissue samples of all *L. plantarum* groups except group D. Passage through the acidic stomach has slight reduction influence on the fluorescent signal of the bacteria. However, the fluorescent bacteria can still be clearly detected in the mammary tissue sections (Fig 7). The confocal scanning on Z axes for the tissue section, shows the presence of the bacteria in the middle of the section between the alveoli cells and capillary ducts (Fig.8). Microscopic examination revealed that single bacteria where distributed in the gland lobules, the bacteria look clear and free of connection with any other cells.

The treated strain were observed also in the placenta sections (Fig.9)

Counting the bacteria in the mammary gland was inaccurate as many bacteria might be washed with the staining sand washing steps, however we considered only the bacteria fluorescent signal which can be seen or detected deep inside the 100 µm section.

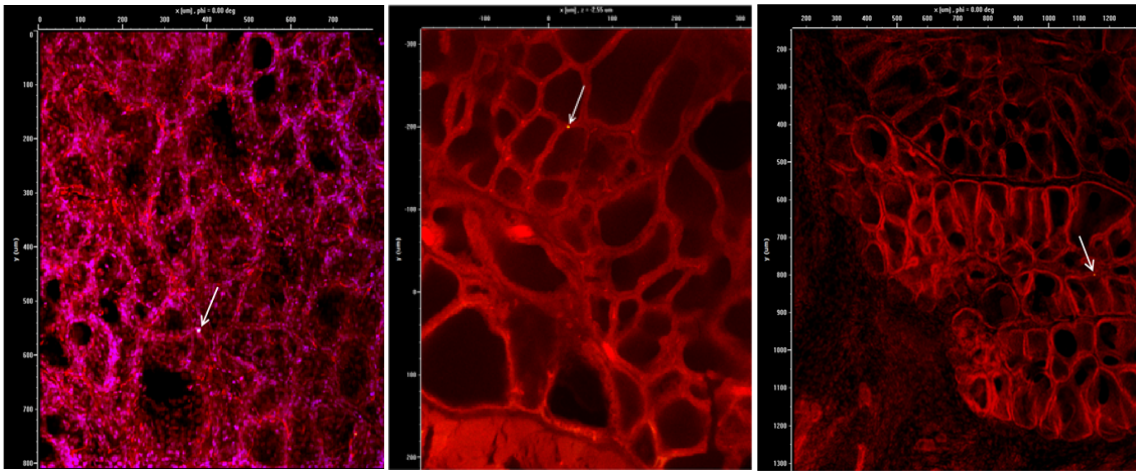

Fig. 7. Histological slices of mammary gland of lactation mice that received FITC-labelled *L.plantarum*. The bacteria are visible as green spot in the canals between the alveolar spaces, All images are representative of the entire sample and the histological results were very similar for each gland in a given mouse and between mice. Magnification 20X to 40X

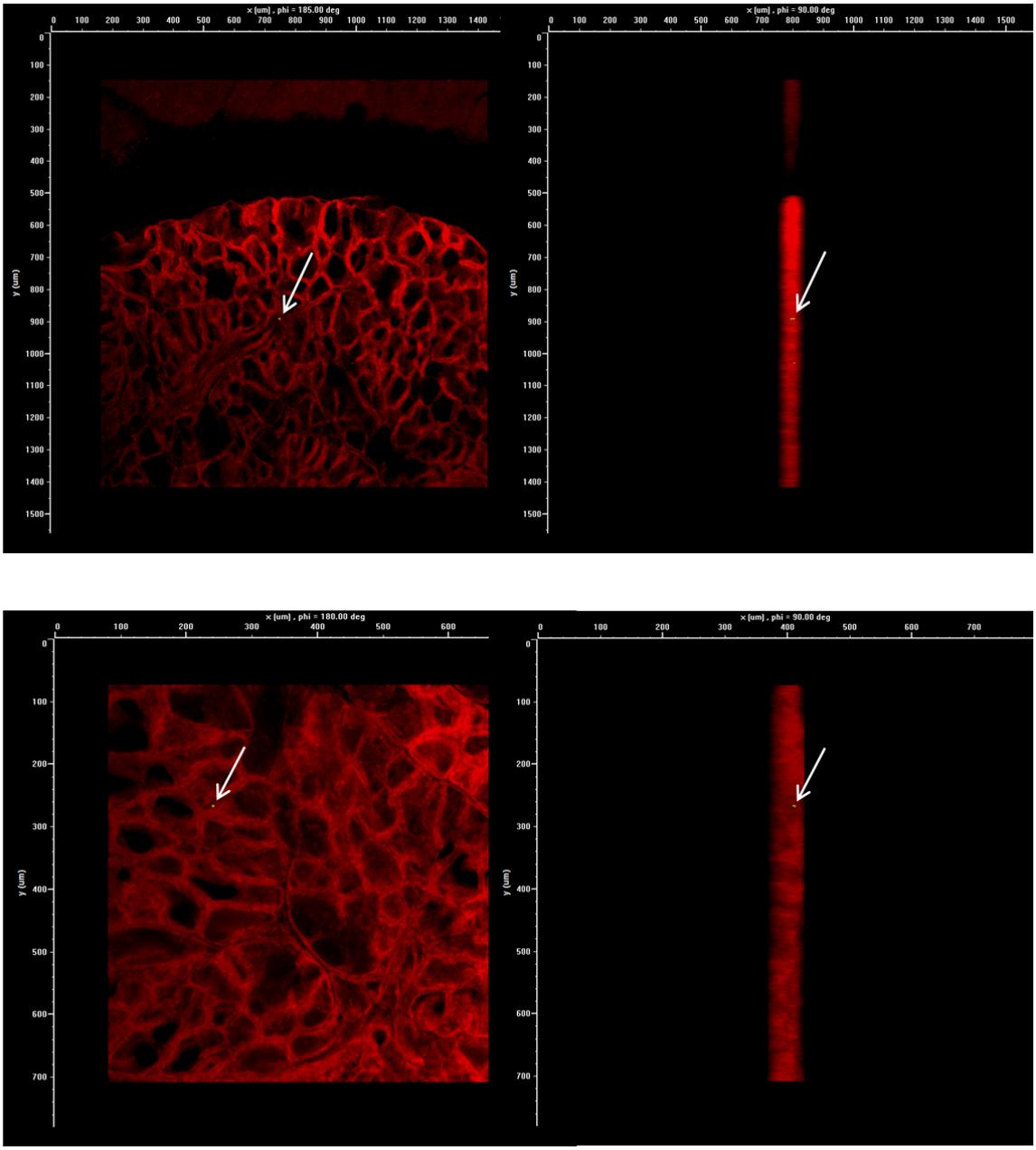

Fig. 8. Histological slices of mammary gland of lactation mice that received FITC-labelled *L.plantarum*. The image is on the Z axis and bacteria are visible as green spot in the middle of the 100 µm thickness section, Magnification 20X to 40X

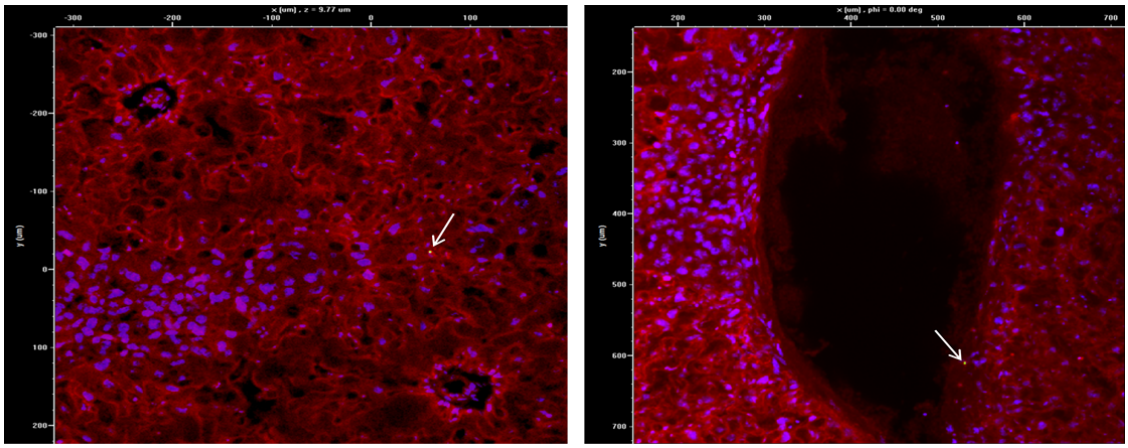

Fig. 9. Histological slices of placenta from pregnant mice that received FITC-labelled *L.plantarum*. The bacteria are visible as green spot between the chorionic villi, all images are representative of the entire sample and the histological results were very similar for each gland in a given mouse and between mice. Magnification 20X to 40X

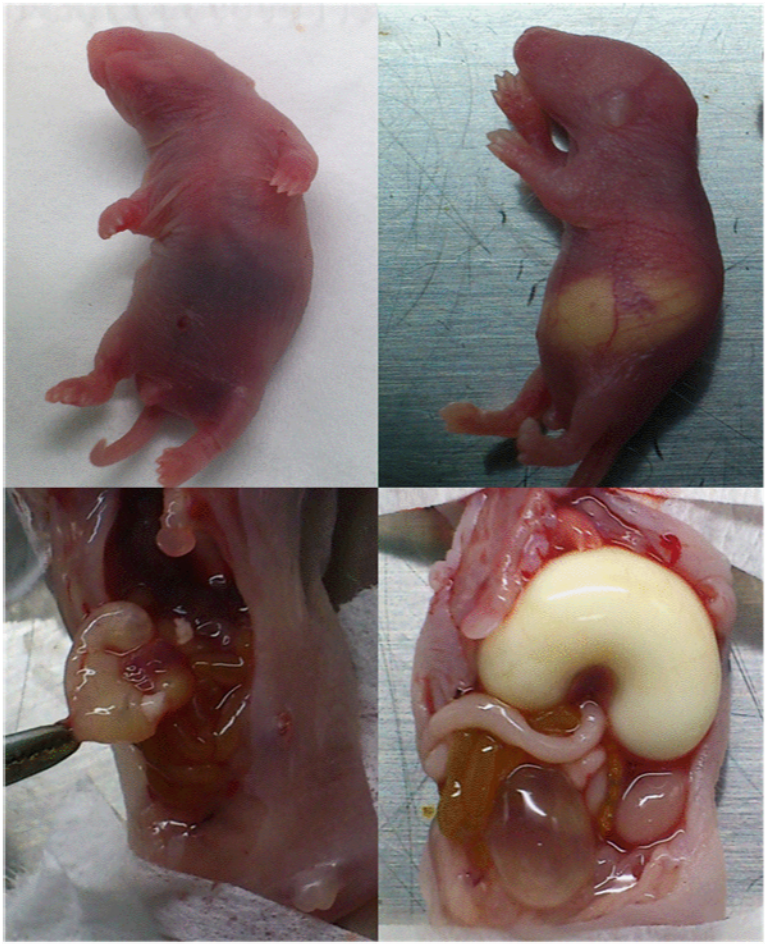

Fig. 10. Photos showing two puppies, before milking (empty gut) in the left and after suckling (full gut) in the right.

Discussion:

In previous work we explored the diversity of lactic acid bacteria (LAB) in human milk microbiota of healthy lactating women and compared it with the fecal lactic microbiota of the same women and their babies (Albesharat *et al.*, 2011). Among 700 different isolates from the mothers, their milk and babies we chose the milk isolate *L. plantarum* (TMW 1.1623), which is a member of a trio of bacterial strains isolated from these three sources, which could not be discriminated along RAPD patterns, tolerance to environmental stresses, growth behaviour under different conditions and other physiological and biochemical tests (chapter 3).

In the present study, this strain was used as a probe in a murine model to investigate the ability and the pathway of mutualistic bacteria to translocate from the maternal intestinal zone (or domain) to the intestinal tract of the progeny during pregnancy or lactation. As *L. plantarum* was the dominantly isolated species from mother milks (Albesharat *et al.*, 2011) we suspected that *L. plantarum* plays a key role in the initial colonization, modulation of intestinal function and influence intestinal microecology.

Setup of the experiment:

Different mice groups were chosen to enlighten the possibilities of mother mutualistic bacteria translocation in the three cases pregnancy, lactation and virgin. Pregnant group (C) present the occurrence of the mother-administrated strain in its fetus´ gut and its organs before birth. While the groups for one week of lactation (A) and two weeks (B) present the occurrence of bacteria in the puppies gut directly after birth (before suckling), after the first suckling and during lactation period. Moreover they present the occurrence of bacteria in the mother's organs after one week and two week of lactation. Group (E) was chosen as a supportive group to illustrate if the only chance of translocation is occurring during pregnancy or after it through the mother milk during lactation, and to show if the treated strain can be still detected in the puppies gut after stopping administration to their mothers. Groups H, I and K provide a comparison with A, B and C in the case of virgin animals, with respect to the behaviour of the strain in the mothers.

Preliminary studies in our laboratory showed that upon administration of a higher dose of the tested strain (10^8 and 10^9 cfu/d) translocation to several (all tested) organs occurred in virgin mice, rendering any observation of a specific translocation (phase) impossible (data not shown). Based on this observation a dose of 10^7 cfu/d was chosen for this test as translocation of the test strain from the gut to the other organs of the body in the treated virgin mice was below any detection limit.

General findings on the effect of strain administration to mice:

Appetite, activity and LWG are the most general and sensitive indicators of health status for animals (Zhou *et al.*, 2000). In accordance with this, we used feed intake and live weight gain

(LWG), as well as the behavior and condition of the mice, to evaluate the acute toxicity of test strains.

Feeding virgin, pregnant and lactating mice with the *L. plantarum* TMW 1.1623^{RF},at dose of 10^7 cfu/d had no adverse effects on food intake, body weight and general health status during 15 days as compared with the PBS groups. No bacterial translocation either to blood or to spleen or liver or MLN was observed in the virgin mice. The evidence of no change in spleen and liver weights (Fig. 3) in the virgin treatment animals also indicates that the *L. plantarum* strain (TMW 1.1623^{RF}), fed to mice did not cause or induce systemic infections or exhibit general acute oral toxicity effects on the experimental animals' general health status, growth and development. This suggests that, the tested human milk strain were non-invasive and is likely to be safe for food supplement or probiotic use in this respect.

Translocation to mothers' organs:

In the healthy body it is important to distinguish between two types of translocation, 1. Controlled translocation in normal healthy host (Martin *et al.*, 2004), (Nikitenko *et al.*, 2011) which aims to stimulate the immune system, and 2. Induced translocation, which occurs due to specific body conditions (e.g. pregnancy and lactation). Bacterial translocation from the gut to mesenteric lymph nodes and mammary gland occurring during late pregnancy and lactation in mice has been reported before (Perez *et al.*, 2007). The fact that *L. plantarum* could reach the placenta of pregnant mice after its oral administration (Fig.9) is not unexpected since it has been reported that bacteria from the digestive tract can reach amniotic fluid through the blood stream (Kornman & Loesche, 1980). Also oral bacteria can enter the uterine environment through the bloodstream and may influence the delivery process (Jimenez *et al.*, 2005), (Dasanayake *et al.*, 2005). Jimenez et al. (Jimenez *et al.*, 2005) had detected a very limited number of bacteria in amniotic fluid or chorioamnion and attributed it to the difficulty of bacteria to survive such stressing environments or being cultured from these. This explanation is supported as diversity of microbes can be detected in the amniotic fluid by using (culture independent) molecular methods (DiGiulio *et al.*, 2008). This diversity and number of initial newborn intestinal microbiota colonization decrease as the gestational phase ceases (full term versus premature neonates) (Mshvildadze *et al.*, 2010). Supportive explanation comes with our finding as pregnant mother organs were free of the tested strain in the early stages of pregnancy and increased translocation to MLNs, spleen and liver was observed in the early perinatal period followed by colonization of the placenta in the latest stage before birth and finally passage through mammary gland in the immediate postpartum period. These are in agreement with the recent finding that bacterial count present in the MLNs of pregnant and nonpregnant mice was higher in the former (Perez *et al.*, 2007). Nevertheless, in our study the tested strain accumulation in the placenta coincided with an increased number of occasional translocations to the spleen and liver in

the early perinatal period, which is rarely detected in the normal or lactation period (Fig.4). Spleen is the largest secondary lymphoid organ containing about one-fourth of the body's lymphocytes and initiates immune responses to blood-borne antigens (Wanda M. Haschek *et al.*, 2002). Some research reported a significant relation between spleen indices and pathogens resistance where larger spleen indices were related with higher inflammation resistance (Hadidi *et al.*, 2008), (OG Arinola *et al.*, 2005). Our data show decreases in the spleen index during pregnancy. However, the spleen weight could not be related to the weight of the mouse alone but refers to her weight including the fetus. As this significant decrease still is retained after birth and during lactation as compared to the virgin mice, this could be due to inhibited immune response to this special translocation activity during this period (Fig.3).

Translocation to the fetus during pregnancy:

Oral administration of *L. plantarum* TMW 1.1623^{RF} to pregnant mice, interestingly showed that the fetus guts were free of the treated bacteria during the whole pregnancy stages. Instead the placenta accumulated the tested strain and no transfer could be observed to the connected fetus during the whole pregnancy period until the fetus passed the complete gestational stage and was ready for birth. This could be a mechanism to delay bacterial translocation before fetal gut maturity is reached and it is ready for initiation of a natural, controlled and beneficial colonization. As microorganisms adhering and translocating across the intestine to a greater extent occurs with a higher rate in immature comparing to mature animals (Claud & Walker, 2001), and GI environment and susceptibility to disease are different between premature infants with that in term infants (Claud & Walker, 2001), (Mshvildadze *et al.*, 2010). A recent study showed that initial infant gut colonization was not affected by birth weight, mode of delivery, antibiotics given to the mother and infants, or type of feeding, but gestational age at birth was a significant condition for colonization (Butel *et al.*, 2007). This is compatible with our finding of the accumulation of the bacteria in the placenta until the last stage of pregnancy when the fetus gut was mature enough to receive the initial colonizers.

Translocation to the puppies during birth:

Matsumiya et al. suggest that approximately one-fourth of infants acquire vaginal lactobacilli from their mothers at birth, and that the acquired lactobacilli do not last in the intestine of the infant long-term, but rather, are replaced by ones from milk or unknown sources after birth (Matsumiya *et al.*, 2002). As we detected the tested strain in the puppies gut directly after birth and never in the fetus before birth it might be argued that sources of bacteria in the puppies are also mother vaginal as its been demonstrated that probiotic lactobacilli can be isolated from the vagina following oral intake (Reid *et al.*, 2001). However, the vaginal microbiota is not the source of our detected bacteria in the empty puppies gut as these

puppies were collected directly after birth from the birth sac before they contact with any other environment else than the mother-enclosed uterus. So the fetus in this study has been protected from the vaginal bacteria inside the birth sac. Moreover, the whole fetus gut samples before parturition (even from the one which had been collected when birth is impending as we saw the bloody vaginal discharge) were free of the tested bacteria and the whole empty gut puppies samples directly after birth were full with the tested bacteria while the only closest source (directly connected with the fetus) where the tested bacteria were detected is the placenta. So, we strongly suggest that the placenta is an important source for the initial gut microbiota colonization in the newborn, which accumulates (beneficial) bacteria from the mothers' mutualistic microbiota during the last stage of gestation. As the same bacteria can be delivered via milk over the lactation period their persistence is supported until the weaning phase.

Translocation to the puppies after suckling:

Colostrum, the milk produced by mothers in the first few days after birth, has antimicrobial effects it is especially rich in IgA (Kelly & Coutts, 2000), white blood cells, immunoglobulin, colostral cells, lysosyme, glycoproteins such as lactoferrin and bioactive peptides (Ho & Lawton, 1978), (van Hooijdonk *et al.*, 2000), (Georgiev, 2008). Certainly, breast milk also has a high proportion of phagocytes, however they are ineffective at killing ingested microbes (Ho & Lawton, 1978). It has been suggested that the ingestion of lipid results in disruption of lysosomes and depletion of lysosomal enzymes resulted in the decreasing killing power of leukocytes (Golde *et al.*, 1976), (Ho & Lawton, 1978). Considering that colostrum is higher in protein, with less sugar and significantly less fat (almost half concentration of the normal milk) (Kretchmer & Zimmermann, 1997). This could be the reason for the decline of bacterial counts in the puppies gut after the first suckling, while it increases again after the second suckling, which comes with a higher concentration of lipids and gives a higher chance to the translocated bacteria to survive the passage through the milk by reducing immune cells activity. Moreover, the fat content of the milk increases in relation to breast emptying, while the other macronutritients of milk show only slight changes (Saarela *et al.*, 2005). This might play the same role in bacteria quantity and passage ability during suckling. The reason for the bacterial count drop after the first milking might be also due to the few, bacterial cells are diluted in the full gut and lost in feces. After birth and suckling, from day 2 to day 3 of life, bacterial counts from puppies' full gut, raised from about 68 to 149 cfu/g then decrease to 26 in the fifth day and remained stable during the rest of the study (Fig. 5).

Translocation to the milk

The previous results rise doubt that all the bacteria detected during the experimental period were derived from the first colonization, which occurred before milking (puppies' empty gut). Group E mice results remove that doubt as they received oral administration of the tested

strain from the second day after birth where all first samples of their puppies we examined (empty gut and full gut) before administration were free of bacteria. The first sample we collected (full gut) after three hours of bacteria oral administration and milking contained about 30 CFU/g of the tested strain (TMW 1.1623^{RF}) and the count of the bacteria in the gut stays constant during the whole period of administration (one week) and lost after stopping administration (Fig.6). To provide conclusive evidence for bacterial translocation through the mammary gland as it was not achievable to collect representative amount of milk from the mice, the histological analysis of the mammary glands of showed the presence of the administrated florescent bacteria (TMW 1.1623^{RF}) in the middle of the mammary glands tissue sections of all administrated lactation mothers (Fig.7 & 8). This suggests that milk is an additional continuous source of mother intestinal bacteria and that translocation of bacteria to mammary gland indeed occurs during lactation. These results are in agreement with recent reports (Martin *et al.*, 2003), (Perez *et al.*, 2007), (Solis *et al.*, 2010).

L. plantarum TMW 1.1623^{RF} numbers observed in histological analysis of the mammary gland tissue and microscopically counted in the milk samples were higher than that ones recovered from the milk by plating (data not shown), indirectly suggesting that dead and live bacteria were translocating to the infants through the milk. This fits the findings of chapter 4 and may further be supported with the finding of Perez et al. that diversity of bacteria signals from milk mononuclear cells is 2 or 3 genera greater than that one of live bacteria isolated from milk (Perez *et al.*, 2007).

Taken together it can be concluded that organisms detected in mammary glands, placenta, puppies empty and full gut and other lymph systemic tissues are derived from dietary sources of the mother. In other words, the orally administered strain had the ability to translocate from gut to the placenta and the mothers' milk in pregnant and lactating mice.

Persistence in the weaned puppies

It is clear that neonates are exposed to diverse microbiota. However, not all components of these populations will colonize the digestive tract (Mackie *et al.*, 1999). Also, our experiment shows that puppies lost the tested strain after stopping administration. That could be due to the fact that the original habitat of *L. plantarum* TMW 1.1623 was human, while we used a murine model for obvious reasons. It has been suggested that bacteria have better chance of out-competing residents and establishing themselves at numerically significant levels if they belong to the natural intestinal flora of the species to be targeted (Morelli, 2000). Loss of the strain upon long term without administration may also be caused by impeded detection as a result of the occurrence of revertants of the rifampicin tolerance upon growth of many generations. It is also well known from animal and human (both adults and children) studies that shedding of probiotic organisms in the stool commonly stops about 2 to 3 weeks after the probiotic supplement is stopped (Crittenden *et al.*, 2005), (Gardiner *et al.*, 2004). So,

Lactobacillus plantarum strain (TMW 1.1623^{RF}) did not colonize the gut, but persisted throughout the 2-weeks period of administration.

The translocation incidence of bacteria in our study was similar between the same dose administrated groups and absence in the control group. This indicates that translocation was not associated with the feeding of LAB in this study but with the special condition of the body.

In summary, feeding mice with the mother milk LAB strain *L. plantarum* TMW 1.1623^{RF} at dose of 10^7 for 15 d had no deleterious effects on mice. Even in the case of translocation during pregnancy and lactation, toxicity of the bacteria seemed to be negligible. Our results suggest that placenta before birth and digestive tract of term infants after birth are not a sterile environment and therefore gut colonization may start before or during birth and the most likely source is the placenta not the vaginal. In addition, the bacterial composition of the maternal gut could affect (both qualitatively and quantitatively) the bacterial content of infant gut through its milk flora during the whole lactation period.

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CHAPTER VI

Dissertation concluding remarks

Concluding remarks:

Motivation and setup of the thesis

This thesis is based on the working hypothesis that inoculation of a human child to develop its own intestinal microbiota starts before birth and goes on during breast feeding by the mother. In that sense (selected) bacteria should be able to pass the intestinal barrier, go to placenta and fetus and also go to the mammary gland in a putative enteromammary pathway. To test this hypothesis this work follows a targeted approach described in several chapters, starting with the isolation and typing of bacteria, followed by determination of their molecular and biochemical properties and selection of appropriate strains for transfer studies. The transfer as such was probed with human biopsies in the Ussing chamber and culminated in a mouse model study employing pregnant mice with thorough investigation of bacterial distribution in the mother mice and their progeny, using labeled strains. The highlights of these chapters are summarized in the following to form a coherent view supporting our initial working hyopothesis.

Chapter I: Genotypic analysis and MALDI-TOF fingerprinting of lactic acid bacteria in local fermented food, breast milk and faeces of mothers and their babies

In this chapter, we investigated and identified lactic acid bacteria (LAB) at genotype level in the breast milk of healthy women, faeces of adults, mothers and infants and in the Syrian fermented food samples consumed regularly by the adult people. The microbiota from the faecal samples showed a high bacterial diversity but low similarity between the individuals. It indicates the high variability of bacterial microorganisms among the different hosts and the differences between the individuals appeared higher than the different samples from one individual. Among the all isolated LAB species, *Lactobacillus plantarum* is suggested as a key LAB in the intestinal microbiota of the Syrian community as it was the dominant species almost found in all samples, even in the infant's samples fed only by their mother's milk. As *L. plantarum* is often dominant *Lactobacillus* species in traditional fermented food based on plant material the intestinal isolates are likely to originate from fermented vegetables frequently consumed in Syria. As the biotypes found in humans were different from the major fermentative microbiota of foods they are only be contaminants in that food, which cannot be detected as a minority amongst the high numbers of fermentative microbiota. Other prevalent

species include, *L. fermentum, L. brevis, L. casei, E. faecium, E. faecalis* and *P. pentosaceus.* They were also dominant species in local fermented food except *E. faecium* and *E. faecalis*. These results show the impact of local nutrition on the diversity of the individual intestinal microbiota, namely the consumption of black tea appears to promote selection of tolerant strains, among which are specialized strains of *L. plantarum*. Typically, the diversity within one species were generally higher in faeces than in food samples.
The breast milk collected for this study contained strains of *L. brevis, L. oris, L. animalis, E. durans, E. hirae, E. mundtii*, *Str. gallolyticus, Str. vestibularis, Str. australis and Staph. haemolyticus* and also pediococci, which were observed in milk samples for the first time.

LAB strains comparison results by RAPD-PCR and MALDI-TOF analyses from the mother/baby-pair group's were used to combine cluster group strains. These findings indirectly suggest that each group strains which were isolated from different sources (mother and baby faeces and mother milk) of the same mother and her breast feed baby are identical strains which originated from the same source (the mother intestinal strain). These identical strains transported to baby by mother milk which were suckled by the baby who receive her strain in his intestinal microbiota.

This experimental data demonstrate that the faecal microbiota composition of the individual host specifically influenced by the diet. These results also hypothesize that there might be a vertical transfer of intestinal LAB from the mother's gut to her milk and then to the infant´s gut.

Chapter II: Determination of the phylobiome of faecal samples of adults, children and babies

In this chapter we used RDP classifier as an additional comparative method for the detection of general intestinal microbiota of the Syrian community. We conducted this study on faecal samples from adults, children and breast feed babies. These results showed significant differences in the microbiota composition between infants and adults. The initial infant intestinal microbiota is a simple structure, and through a series of progression and replacements, it evolves to a more complex adult pattern. Diet appears to be of the most important determinant shaping the pattern of intestinal micobiota. Middle East community have their special general form of common diet in that areas, mainly combining wheat bulgur with seasonal vegetables and some lamb meats tend to be staples in many of the main courses (fermented olives, olive oil, dates, black tea and no alcoholic beverages seems to be the most consumption habits shaping the local people intestinal microbiota). Thus, the main

food consumption depends on plants and starch material that could explain the general predominance of *Bacteroidetes* (starch and polysaccharides degraders) among the people.

These indicate the impact of age and diet ingredients on the general individuals' microbiota diversity in common community. Consequently the special genera deletion in adulthood (e.g. *Bifidobacteria*) can be due to the changes or additions in diet habits. For an example, the influence of the high consumption of tea polyphenols among the adults.
Trying to understand the intestinal bacterial interaction, mutualism, dominancy and diversity which shape the intestinal microbiota community, different properties and polyphenols influence were investigated for different bacterial species isolated from different people in the next chapters.

Chapter III: Comparative analysis of bacterial isolates along their physiology, biochemical capabilities and stress tolerance

In this chapter we mainly investigated the different physiological and biochemical properties of different LAB species isolated from the different human sources. These studies include; ecological stress resistance abilities (digestive stress (gastric acid and bile salt) - oxidative stress, dietary (black tea) polyphenolics consumption - antibiotics), resistance mechanisms possessing (bile salt hydrolyse ability - oxidative resistance mechanisms (catalase (Cata), NADH oxidase, superoxide dismutase (SOD) and glutathione reductase (GR))), production of antagonistic metabolites (hydrogen peroxide - bacteriocin) and production of mutual secondary metabolites and enzymes (α-galactosidase & β-galactosidase - caseinase - exopolysaccharide (GTF – FTF)). We focused on the common strains found in the mother's milk and faeces and their babies faeces to confirm the possibility of being from on regional source and their special ability comparing to other intestinal strains.

The results present different abilities and growth tolerance between the different strains of intestinal LAB isolated from the different people. Despite of the slight differences and tolerance between different strains of the same species, there is clear general fingerprint or pattern of each species, which can differentiate it from the other's intestinal LAB species. For an example, *L. plantarum* and *E. faecium* species have been shown high ability to survive in the gastric acid, different bile and oxidative condition mimics the intestine comparing to other LAB species. This may be the reason why these two species were the dominant and the most abundant (PAPD genotype) isolated species from the different individuals. Rich polyphenols and tannins black tea medium could increase the bacteria yield for all tested LAB species beside the interesting influence of inducing bacteria growth directly after

culturing while it give inhibition growth effects on the additional (external) tested *Bifidobacteria* strains.

Interestingly, all the different tested properties or activities of mother/baby-pair group's strains showed similar behaviour or pattern in all the strains of different sources in each group except caseinase production activity, which show that it is environmental dependent trait. This indirectly lead to conclude that all these identical different properties expression (RAPD-PCR, MALDI-TOF, genes presence, production ability and resistance ability) are due to the identical source of these strains. This suggests that each mother/baby-pair group's strains are identical strain from one source which is the mother intestinal microbiota transferred through her milk to baby. This hypothesis was further examined for one milk strain selected as a promising translocating candidate strain based on its presence in the mothers, their milk and babies of one coherent such trio of indistinguishable isolates and employed in two different body situations, first in normal body conditions (on intestinal mucosal membrane mounted in the Ussing chamber) and second in case of pregnant and lactation (murine model) as summarized in the next two chapters.

Chapter IV: *In vitro* translocation of *Lactobacillus plantarum* in human and guinea pig biopsies

In this chapter, we examined whether milk isolate (translocation candidate) *L. plantarum* strain (which showed from the previous results high production ability for different antagonistic metabolites and enzymes) can passage across an intact intestinal mucosal membrane mounted in normal human like body conditions in the Ussing chamber. We investigated the passage possibility of the live and the dead bacteria and their respective influence on the tissue resistance.

The results showed that, in isolation from the pH impact on the intestinal membrane the live *L. plantarum* cells significantly increased resistance leads to blocking of membrane permeability, this influence was not observed in the case of dead bacteria which could be due to the capacity of *L. plantarum* to secrete bioactive molecules that evoke an immune response and induce the membrane resistance in normal body conditions case.

Chapter V: *In vivo* translocation of *Lactobacillus plantarum* in pregnant and lactating mice

In this chapter we used the same previous milk isolate *L. plantarum* strain as a probe in a murine model to investigate the ability and the pathway of mutualistic bacteria to translocate from the maternal intestinal zone (or domain) to the intestinal tract of the progeny in three conditions pregnancy, lactation and virgin animals.

The results showed that feeding mice with the mother milk LAB strain *L. plantarum* TMW 1.1623^{RF} at dose of 10^7 for 15 days had no deleterious effects on mice. Even in the case of translocation during pregnancy and lactation, toxicity of the bacteria seemed to be negligible. No bacterial translocation was observed in the virgin mice, which is in agreement with the previous findings in case of normal body condition as shown in chapter IV. Results from mother's animals suggested that placenta before birth and digestive tract of term infants after birth are not a sterile environment and therefore gut colonization may start before or during birth and the most likely source is the placenta not the vagina. In addition, the bacterial composition of the maternal gut could affect (both qualitatively and quantitatively) the bacterial contents of infant gut through its milk flora during the whole lactation period.

Final Conclusion:

- The intestinal microbiota is special fingerprint for every individual of society or community which, apart from the hosts own determinants, can be influenced by food consumption habits of that people
- Survival and resistance to oxidative stress conditions beside acidic and bile salts might be the main criteria, which selected the most dominant species between all inhabitant LAB species in the intestine
- Bacterial (beneficial) translocation depends on the bacteria and the body conditions
- Although every individual has its own special microbiota the most similar microbiota to it is its mothers microbiota as she was the source of the first colonization
- A newborn is not really sterile. However, the first invasion or colonization is suggested to start before birth
- Human milk is a complex symbiotic food
- Brest feeding is right for every infant and deprivation from it will influence all coming life.
- New life come with straggle and pain to be beautiful; through birth pain the best mother microbiota pump to her baby

Summary

In this study, from 70 different samples (15 infants and 15 adults and 15 breast milks and 25 fermented foods) using 9 different selective media a total of 700 isolates of LAB were isolated and subjected to randomly amplified polymorphic DNA (RAPD) polymerase chain reaction analysis. All strains displaying different RAPD patterns were identified by 16S rDNA sequencing. From the faecal and milk samples we identified 36 different species of *Lactobacillus, Enterococcus, Weissella, Streptococcus, Staphylococcus* and *Pediococcus.* Eleven different LAB species of *Lactobacillus, Enterococcus, Streptococcus, Staphylococcus, Pediococcus* and *Leuconostoc* were isolated from different samples of eight types of fermented food typically consumed in Syria. Isolates varied dependent on different types of media, individuals and foods. Within the 700 isolates 171 different strains (based on different RAPD genotypes) were differentiated, which were identified as *Lactobacillus plantarum, Lactobacillus fermentum, Lactobacillus casei, Lactobacillus brevis, Lactobacillus salivarius, Lactobacillus rhamnosus, Lactobacillus mucosae, Lactobacillus oris, Lactobacillus reuteri*, *Lactobacillus sobrius, Lactobacillus animalis*, *Lactobacillus gasseri, Lactobacillus coleohominis, Lactobacillus vaginalis, Lactobacillus murinus, Lactobacillus helveticus, Streptococcus gallolyticus, Streptococcus lutetiensis, Streptococcus australis, Streptococcus vestibularis, Streptococcus equines, Streptococcus infantarius, Pediococcus pentosaceus, Enterococcus faecium, Enterococcus faecalis, Enterococcus durans, Enterococcus hirae, Enterococcus mundtii, Weissella confuse, Enterococcus gallinarum, Enterococcus avium, Enterococcus saccharolyticus,* and *Enterococcus casseliflavus.* Also species of non-LAB were found, *Staphylococcus hominis*, *Staphylococcus haemolyticus, Staphylococcus epidermidis* and one yeast *Kluyveromyces marxianus*. Whereas some species were unique for one of the four sources, *L. plantarum, L. fermentum, L. brevis* and *P. pentosaceus* were present in all different samples. Interestingly, we found in nine cases of mother/baby-pairs groups identical genotypes of *L. plantarum, L. fermentum, L. brevis, E. faecium* and *E. faecalis* were found in the mothers' faeces, their milk and the corresponding babies' faeces. These species were found in the three sources in some groups, while others were found in two of them. Studding the whole genome sequences of fecal samples from the previous people and their babies showed clear difference in phylum and genera distribution among the two different age groups and presented special microbiota diversity of people from this same area differentiate them from general microbiota diversity of inhabitance of different region which confirm the main role of type of diet and environment in shaping intestinal microbiota. For more understanding of bacteria interaction, mutualism, the rules of dominance and the selective criteria for the milk translocating bacteria, we selected the predominant species from human sources (milk and feces) for further investigation on its survival (in oxidative stress condition), resistance (for acidic and bile salt environment),

competition (ability to produce different antagonistic compounds) and production properties (of different beneficial enzymes and secondary metabolites). The greater survivability in different gastric acid and different bile condition mimics the gut were for the species of *L. plantarum, L. brevis* and *E. faecium* having higher ability to survive comparing to other LAB species, which could be the reason why this three species are the dominant isolated species as *L. plantarum* had expressed 29 different genotypes, 25 for *E. faecium* and 17 for *L. brevis.* The strains from different species of *L. plantarum, E. faecium, E. faecalis, E. hirae, E. mundtii* and *P. pentosaceus* have oxidative stress resistance ability to reactive oxygen species (ROS). Bringing together to gastric acid and bile salts resistance ability the species *L. pantarum* seems to be the only resistance species surviving all different stress condition and this could explain its dominancy between all different human isolated LAB in this study. Confirming the diet influence on shaping the intestinal microbiota diversity, the growth of the intestinal bacteria species in media containing black tea and licoric (regularly consumed in Middle East community) showed a good enhancement growth influence although the polyphenolic compounds in this beverage is known for it is antimicrobial effect on bacteria. The mother/baby-pairs strains which presented identical genotype (RAPD-PCR and MALDI-TOF) showed very similar behaviour in different stress condition and higher resistance than normal Intestinal strains, also they presented similar enzymes and metabolites production which confirmed that this strains belong to the same source which is the mother mutualistic microbiota and that support the hypothesis that there is a vertical transfer of intestinal LAB from the mother's gut to her milk and through the milk to the infant's gut. Still this translocation hypothesis needs to be tested in vivo, and for that we selected on milk *L. plantarum* strain depending on its different resistance and beneficial properties as translocating candidate for examining it is ability to passage across a human intestinal mucosal/submucosal membrane mounted in the Ussing chamber in normal intestinal condition. The life *L. plantarum* bacteria presented special increasing membrane resistance influence leading to block membrane permeability, this influence was not observed in the case of dead bacteria which lead to confirm the results of variety of studies which exploit the capacity of life *L. plantarum* to secrete bioactive molecules that evoke an immune response. However this result didn't refutes translocation hypothesis, but indirectly leads to conclude that *L. plantarum* translocation ability could be depending on certain body conditions which induce it like the changes which happen during lactation and pregnancy. To proof this theory further study of the same translocating candidate strain on pregnant and lactation animal model (mice) were performed in different mice gropes (virgin, pregnant and lactation) and puppies gut samples were collected as representative for the translocating bacteria in the milk. Our result shows that feeding mice with the life mother milk LAB strain *L. plantarum* at dose of 10^7 for 15 d had no deleterious effects on mice and there was no case of *L.*

plantarum translocation to any of the body organs at all in the virgin animals, and this was expected as *L. plantarum* had shown ability to increasing membrane resistance in normal case (Ussing chamber). Whoever oral administration of *L. plantarum* strain to pregnant mice, interestingly showed that the fetus gut were free of the treated bacteria during the whole pregnancy stages while the placenta accumulated the tested strain in it and didn't transferred it to the connected fetus during the whole pregnancy period until the fetus became completely mature and ready to birth. It was clear that the bacteria were accumulated in the placenta to be transferred later to the fetus (during birth). In case of lactation mice the *L. plantarum* was observed histologically in the mammary gland tissue and detected in the gut of puppies after milking from their bacteria treated mothers. From this we concluded that the same bacteria strain detected in mammary glands, placenta, puppies empty and full gut and other lymph systemic tissues are derived from dietary sources of the mother. In other words, the oral tested strain had ability to translocate from gut to the placenta and mother milk in pregnant and lactation case, and as translocation incidence of bacteria was similar between the same dose administrated groups and absence in the control group. This indicates that translocation was not associated with the feeding of LAB in this study but with the special condition of the body.

In conclusion, these data provide an important clue for future studies. These include evaluations of the very early microbiota sources, the rule of mother lactation on the early intestinal microbial colonisation and stability during lactation, and the possibility for the donated milk species to remain on the unstable intestinal microbial environment in the new host after weaning. More research efforts should be focused on this aspect of perinatal microbiology.

Abbreviations table

°C	degree Celcius
µg	micorgram
µl	microlitre
µm	micrometer
Ω	ohm
∆	change
%	percent
16S rRNA	the prokaryotic ribosomal subunit
ANOVA	analysis of variance
BCP	bacterioferritin comigratory protein
BD	Bacteroides Bile Esculin Agar
bp	base pair(s)
BT	Bacteria translocation
CFU	colony-forming units
DNA	desoxyribo nucleic acid
dNTP	desosy nucleotide triphosphate
EDTA	ethylendiaminetetraacetic acid
EMBL	*European Molecular Biology Laboratory*
EGCG	Epigallocatechin Gallate
EPS	exopolysaccharides
FITC	Fluorescein isothiocyanate
FTF	Fructosyltransferase
g	gram
GIT	gastro-intestinal-tract
GDCA	glycodeoxycholate
GOSs	galactooligosaccharides
GR	glutathione reductase
GRAS	generally regarded as safe
GSSG	Glutathione disulfide
GTF	Glucosyltransferase
H_2O_2	hydrogen peroxide
h	hours
HePS	heteropolysaccharides
HMOs	human milk oligosaccharides
HoPS	homopolysaccharides
HPLC	*High-performance liquid chromatography*
I	current
IFN-γ	*Interferon-gamma*
IL-10	Interleukin-10
k	kilo
ITCN	Image based Tool for Counting Nuclei
L	litre
LAB	lactic acid bacteria
M	molar, mol per litre

mA	milliampere
mg	milligram
min	minutes
ml	milliliter
mM	millimolar, millimol per litre
MRS	`de Man, Rogosa and Sharpe
mMRS	modified MRS medium
NADH	Nicotinamide adenine dinucleotide
NCBI	National Center for Biotechnology Information
NO	nitric oxide
OD	optical density
OD_{590}	optical density at 590 nm wavelength
OTUs	Operational Taxonomic Units
PBS	phosphate buffered saline
PCR	polymerase chain reaction
R	resistance
RAPD	random amplified polymorphic DNA
RDP Classifier	Ribosomal Database Project Classifier
rpm	rounds per minute
rRNA	Ribosomal ribonucleic acid
ROS	*Reactive oxygen species*
S-layer	surface layer
sec	second
Isc	Short-circuit current
SDS	sodium dodecyl sulfate
SOD	Superoxide dismutase
SPF	specific pathogen-free
TDCA	taurodeoxycholate
TAE	Tris-Acetat-EDTA
TBE	Tris-Borat-EDTA
TMW	Technische Mikrobiologie Weihenstephan
TNF-α	Tumor necrosis factor-alpha
Tris	Tris-(hydroxymethyl-) aminomethane
TUM	*Technische Universität München*
U	units
U	potential
UV	ultra violet
VAF	Viral antibody free
V	Volt
v	volume
X-Gal	5-Bromo-4-chloro-3-indolyl-ß-D-galactopyranoside