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Study of fluoroquinolone resistance in
Lactobacillus spp.

TESI DI DOTTORATO

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*Una vita senza ricerca
non vale la pena
di essere vissuta*

Platone

Abstract

Introduction. The genus *Lactobacillus* and, more generally, the commensal organisms, which colonize the human gastro-intestinal and genital tracts, can potentially serve as reservoirs of resistance genes(1). The main danger associated with such condition regards products made of viable *Lactobacillus*, such as probiotics and fermented foods, that can transfer resistance genes to commensal or pathogens bacteria; this set-up can potentially result in a spread of antibiotic resistance among microorganism.

Several studies have confirmed the presence of genes encoding antibiotic resistance in *Lactobacillus* (2)(3)(4)(5)(6)(7)(8)(9)(10)(11). Some of these genes are localized in plasmids and / or trasposonics that could be transferred horizontally between lactobacilli and other species of the intestinal microbiota, increasing significantly their pathogenic potential. Probiotics and fermented foods are a vehicle for massive amounts of living bacteria, which could represent human reservoirs of antibiotic resistance genes. However, many phenomena of resistance are not due to the presence of mobile genetic elements, but to the onset of mutated clones that give rise to resistant strains.

In particular the resistance to quinolones in lactobacilli has been described since 2003 by Fukao et al., which have demonstrated the absence of changes in the *Lactobacillus gyrA* and *parC* genes (12).

Aims of the study and results. 244 strains previously classified as *Lactobacillus* spp., isolated from women's vagina and belonging to the collection of Department of Bio-Medical Science section of Microbiology, University of Catania, have been characterized to the species level using a polyphasic approach. This approach provides both isolation on selective media, and the use of genotyping techniques: 16S-RFLP (13), two steps multiplex PCR (14) and *tuf* gene species-specific primer for *L. paracesei-L.rhamnusius* discrimination(15).

The susceptibility profiles for ciprofloxacin, levofloxacin, ofloxacin and ulifloxacin have been determined (16).

In particular, we have studied the mechanisms of genotypic resistance of four strains of *L. fermentum* that showed reduced *in vitro* susceptibility or resistance to the fluoroquinolone ciprofloxacin (assuming as resistant strains with MIC \geq 4 mg/mL).

The first hypothesized mechanism of resistance involves mutations in QRDR regions (Quinolone stance made Determining Regions) of the DNA gyrase and topoisomerase IV subunits genes. In order to identify these mutations, QRDR of the *parC* and *gyrA* genes were amplified (17). The sequencing results revealed the presence of nucleotide mutations, which, however, did not result in changes of the amino acid sequence. These results are consistent with those obtained by Fukao et al. in 2003 (12).

The quinolone resistance mechanisms mediated by efflux pumps MDR (Multi Drug Resistance) was also investigated. The trend of the intracellular concentrations of ciprofloxacin in an interval between zero and four hours has been measured; ciprofloxacin concentrations were analyzed by exploiting the values of maximum absorption at 275 nm which give rise to an emission peak at 447 nm (18)(19)(20).

Further studies, conducted with phenotypic uncouples (CCC carbonyl-cianil-chlorophenyl hydrazone) and with MDR channel blockers (Verapamil and reserpine) have revealed a reduction of ciprofloxacin MIC values (2 fold reduction) (21).

The comparative genomic analysis performed on GenBank showed that in *L. fermentum* ATCC 14931 there are two hypothetical proteins: one (GenBank ref.ZP_03944345.1) belonging to the MFS (Major Facilitator Superfamily) family which has a homology of 98% with Nora (GenBank ref.CCE58495.1), the protein responsible for quinolones efflux in *S. aureus*; the other one (GenBank ref.ZP_03944509.1) belonging to the ABC (ATP Binding Cassette) family, which has a sequence homology of 90% with LmrA (GenBank ref.YP_005868060.1) responsible for quinolones efflux in *L. lactis*.

Future outlooks Studies of characterization of this protein in collaboration with Professor Patrizia Brigidi and Dr. Beatrice Vitali University of Bologna are currently ongoing.

Introduzione. I lattobacilli e, più in generale, i microrganismi commensali che nell'uomo colonizzano il tratto gastro-intestinale e le vie genitali, possono potenzialmente servire da serbatoi di geni di resistenza (1). Il pericolo maggiore, associato ad una tale condizione, è quello che i prodotti in cui si fa uso di lattobacilli vitali, quali i probiotici ed i cibi fermentati, possano trasferire geni di resistenza a batteri commensali o potenzialmente patogeni con conseguente aumento del fenomeno della resistenza agli antibiotici.

Diversi studi hanno confermato la presenza, nei lattobacilli, di geni codificanti la resistenza antibiotica (2)(3)(4)(5)(6)(7)(8)(9)(10)(11). Alcuni di questi geni sono a localizzazione plasmidica e/o trasposonica per cui potrebbero essere trasferiti orizzontalmente fra lattobacilli e altre specie commensali del microbiota intestinale, incidendo notevolmente sul loro potenziale patogeno. Probiotici e alimenti fermentati sono veicolo di enormi quantità di batteri viventi i quali potrebbero rappresentare nell'uomo dei serbatoi di geni di resistenza agli antibiotici. Tuttavia molti fenomeni di resistenza non sono dovuti alla presenza di elementi mobili ma all'insorgenza di cloni mutati che danno a ceppi resistenti.

In particolare la resistenza ai chinoloni nei lattobacilli è stata descritta sin dal 2003 da Fukao et al i quali hanno dimostrato l'assenza di modificazioni a carico dei geni *gyrA* e *parC* in *Lactobacillus* (12).

Obiettivi della ricerca e risultati. 244 ceppi in precedenza classificati come *Lactobacillus* spp., di origine vaginale e appartenenti alla batterioteca del Dipartimento di Scienze Bio-Mediche sez. Microbiologia dell'Università degli studi di Catania, sono stati caratterizzati a livello di specie mediante un approccio di tipo polifasico. Tale approccio prevede sia l'isolamento su terreni selettivi, sia l'uso di tecniche genotipiche: 16S-RFLP (13), *two steps multiplex* PCR (14) e *tuf* gene PCR per la discriminazione di *L. paracasei*-*L. rhamnosus*.

Sono stati determinati i profili di sensibilità per quattro fluorochinoloni: ciprofloxacin, levofloxacin, ofloxacin e ulifloxacin (16).

In particolare, sono stati studiati i meccanismi di resistenza genotipica di quattro ceppi di *L. fermentum* che hanno mostrato ridotta sensibilità *in vitro* o

resistenza verso ciprofloxacina (assumendo come resistenti, i ceppi con MIC \geq 4 μ g/mL).

Il primo meccanismo di resistenza ipotizzato coinvolge le mutazioni presenti a livello delle regioni QRDR (*Quinolone Resistance Determining Regions*) dei geni delle subunità della DNA girasi e della topoisomerasi IV, per individuare tali mutazioni sono state amplificate le QRDR dei geni *parC* e *gyrA*, delle rispettive subunità della Topoisomerasi IV e della DNA girasi, bersagli farmacologici dei chinoloni. I risultati del sequenziamento hanno evidenziato la presenza di mutazioni nucleotidiche, che però non hanno determinato variazioni nella sequenza aminoacidica (17). Tale risultato è in linea con quanto descritto nel 2003. da Fukao et al. (12), i quali hanno dimostrato l'assenza di modificazioni a carico dei geni *gyrA* e *parC*, quindi lo studio si è orientato alla ricerca di meccanismi di efflusso mediate da pompe MDR (*Multi Drug Resistance*). A tal fine è stato misurato l'andamento delle concentrazioni intracellulari in un intervallo di tempo compreso tra zero e quattro ore; la variazione delle concentrazioni di ciprofloxacina è stata analizzata sfruttando i valori di assorbimento massimo a 275 nm da cui scaturisce un picco di emissione a 447 nm (18)(19)(20).

Lo studio fenotipico condotto sia con disaccoppianti (CCC carbonil-cianil-clorofenil idrazone), sia con bloccanti dei canali tipo MDR (Verapamil e reserpina), hanno rivelato una riduzione dei valori di MIC per ciprofloxacina (riduzione di due diluizioni) (21).

L'analisi genomica comparata condotta su GenBank ha mostrato che in *L. fermentum* ATCC 14931 sono presenti due proteine ipotetiche: una (GenBank ref.ZP_03944345.1) appartenente alla famiglia MFS (Major Facilitator Superfamily) che presenta un'omologia del 98% con NorA (GenBank ref.CCE58495.1) proteina responsabile dell'efflusso dei chinoloni in *S. aureus*; un'altra (GenBank ref.ZP_03944509.1), appartenente alla famiglia ABC, che presenta una omologia del 90% con LmrA (GenBank ref.YP_005868060.1) responsabile dell'efflusso dei chinoloni in *L. lactis*.

Prospettive future Sono attualmente in corso gli studi di caratterizzazione di questa proteina in collaborazione con la Prof. Patrizia Brigidi e la Dott.ssa Beatrice Vitali dell'Università di Bologna.

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1 The genus *Lactobacillus*

The genus *Lactobacillus* includes microorganisms Gram-positive, catalase negative, nonspore-forming, with a shape that can vary from long and thin (rod shaped) to short and curved (coccobacillary, coryneform); they are generally facultative anaerobic, or microaerophilic almost always motionless (22)

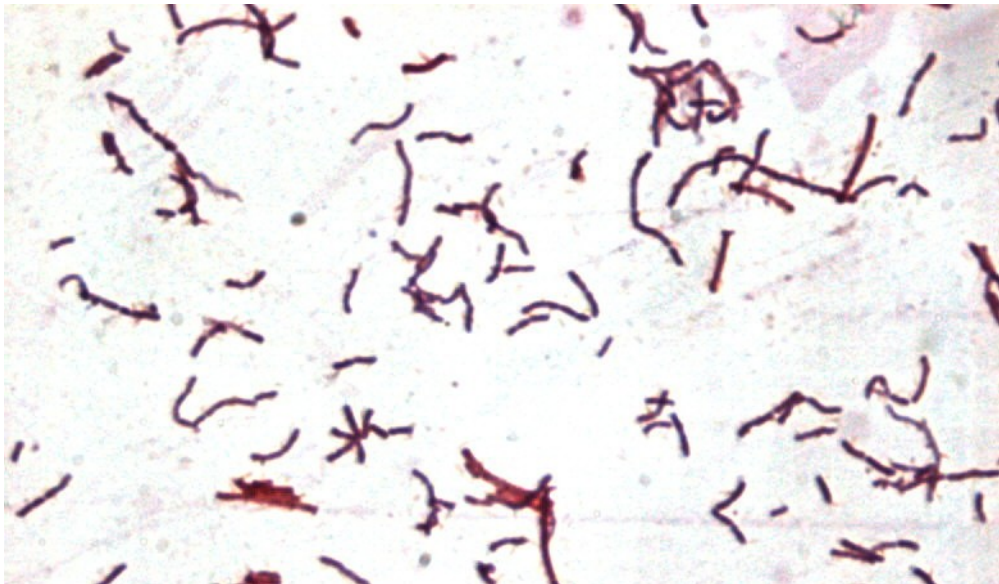


Figure 1-1 Gram stain

1.1 Cell morphology

The degree of curvature and the length of the rods depend on the age of the culture, the composition of the medium (availability of esters of oleic acid) and the oxygen pressure. Some species of gas-producing lactobacilli (*L. fermentum*, *L. brevis*) are represented as rods long and short together. The morphological differences between species are still evident and discriminating in the case of lactobacilli coconut-bacillary form, these may appear so short as to be incorrectly identified in the genus *Leuconostoc* (eg *L. confusus*, originally considered as a species of the genus *Leuconostoc*) or in the genus *Streptococcus* (eg *L. xylosus* and *L. hordniae* classified as lactobacilli and only recently reclassified as streptococci).

Lactobacilli tend to arrange themselves in chains: this characteristic is variable among species, sometimes from strain to strain of the same species. This variability depends on the growth phase and on the pH of the medium.

The asymmetric development of coryneform lactobacilli during cell division leads to the formation of corrugated chains or even rings. Forms wrapped in an irregular manner can be observed in the case of symbiotic growth (in kefir grains) or under the high

concentrations of glycine, amino acids or antibiotics active on the cell wall.

The motility in lactobacilli is very rare and, if present, it is due to the presence of peritrichous flagella, and it is dependent on cultivation parameters (medium components and age of the culture). It can be observed during isolation, and it is lost during subsequent transplantation in artificial medium.

Some strains show bipolar bodies with internal granulation that let them appear striped after Gram staining with methylene blue, especially the rods of homofermentative species. The large bipolar structures likely to contain polyphosphates and appear very electron dense to an electron microscope (22).

1.2 Metabolism

Lactobacilli are microorganisms which are obligately saccharo-
clastic and at least half of the final product of carbon is lactate;
additional products may be acetate, ethanol carbon dioxide, formate
and succinate. Volatile acids with more than two carbon atoms are
not produced.

The reduction of nitrate is unusual and present only when the pH is
increased up to 6.0. The gelatin is not liquefied and casein is not
digested, but small amounts of soluble nitrogen are made from most
of the strains. They are not indole or hydrogen sulfide producers.
Overall lactobacilli are catalase negative due to lack of cytochromes
(porphyrins are absent), but some strains can decompose the
peroxide through a pseudo-catalase; they give a negative reaction
with benzidine. The production of pigments is rare and if present it
becomes yellow orange-rust or red-brick.

Species belonging to the genus *Lactobacillus* are divided into three
metabolic groups according to the presence of enzymes responsible
for sugars omofermentation (fructose 1-6 diphosphate) or hetero-
fermentation (aldolase and phosphoketolase):

Obligated homofermentative lactobacilli.

Belong to this group species that ferment hexoses carbohydrates almost exclusively producing lactic acid through the glycolytic pathway of Embden-Meyerhof-Parnas (EMP) and which are not able to ferment the pentose and the gluconate. From a morphological point of view they are generally like isolated long cells or arranged in very long or coiled chains. The species of the group live in different habitats and are phylogenetically related. The group also includes the most acidifying species (2.7% of lactic acid) and can be subdivided into two subgroups: the homofermentative psychrophilic that grow at a low temperature ($\sim 15^{\circ}\text{C}$) and thermophilic homofermentative that grow at high temperature ($\sim 45^{\circ}\text{C}$).

Facoltative heterofermentative lactobacilli.

The species of the group ferment hexoses through the route of EMP and produce almost exclusively lactic acid, in the presence of small quantities of glucose, lactate acetate, ethanol and formic acid can also be produced; they are able to ferment pentoses to lactate and acetate through an inducible phosphoketolase due to the presence of pentoses.

Facultative heterofermentative lactobacilli are usually mesophilic, with the exception of some species; the cells morphology is variable from coccoid to stubby coccus most often arranged in very long chains. They also have vegetables and fermented meats as habitats.

Obligated heterofermentative lactobacilli.

Species belonging to this group ferment hexoses to lactate, acetate (or ethanol) and carbon dioxide through the phosphogluconate pathway, the pentoses are also fermented to lactate and acetate, again through the action of the enzyme phosphoketolase. These heterofermentative lactobacilli are characterized by their ability to produce volatile aromatic substances and by their poor acidifier power (0.5% lactic acid). Cells are very short and generally isolated; they tend to develop in association with other species of lactic acid bacteria or other microorganisms both in fermented foods and in the digestive tract (22) (23)

1.3 Nutritional requirements and cultural characteristics

The majority of lactobacilli species are very demanding from a nutritional point of view with specific characteristics for different species. Complex substrates are required for growth such as sources of carbon, nitrogen, phosphorus and sulphur compounds, also growth factors, vitamins, amino acids and trace elements. In order to supply their nutritional needs, culture media must contain fermentable carbohydrates, peptones, meat extract and yeast extract, in addition to substances and elements such as tomato juice, manganese, and acetate esters of oleic acid as growth promoters. Substances such as pantothenic acid and nicotinic acid are essential for all species while compounds such as thiamine are necessary for the growth of heterofermentative lactobacilli. Even riboflavin is a compound frequently required while the biotin and vitamin B12 are necessary to only some strains.

Lactobacilli grow well in slightly acid medium with an initial pH of 6.4-4.5, the growth stops when it reaches a pH of 4.0-3.6, this is a condition variable between species and between individual strains. Since they are acidophilous, the optimum pH is generally 5.5-6.2;

the speed of growth is often reduced when the initial pH is neutral or alkaline. Many strains are weakly aerotolerant so optimal growth occurs in anaerobic or microaerobic conditions; the increase of the concentration of carbon dioxide (5% approx.) can stimulate the growth itself. The growth temperature ranges from 2 to 53 °C and the optimum is generally between 30 °C-40 °C.

Colonies on agar are very small (2-5mm in diameter), with well-defined margins, convex, smooth, translucent or opaque, in rare cases, pigmented yellow or red. Some species form characteristically irregular colonies and thin distinct colonies are formed only by *L. confusus*.

When growth occurs on agar containing dispersed proteins or fat, there are no light areas generated by exoenzymes. However, many strains show a weak proteolytic activity, which is made by proteases and peptidases released from the cell wall and a weak lipolytic activity, carried out mostly by intracellular lipases.

Growth in liquid medium generally occurs in suspension and, once completed, the cells fall, with a smooth and even sediment, which is rarely granular or gelatinous. Lactobacilli do not develop characteristic odours as they grow in common culture media. When they represent as the predominant microbiota, MRS agar (DeMan

Rogosa Sharpe) can be used; while, if they represent only a part of a complex microbial population, selective media such as, acetate Rogosa SL2, commonly called Rogosa, are needed. Although this is not completely selective, other lactic acid bacteria, for example, *Leuconostoc*, *Pediococcus*, *Enterococcus*, and *Bifidobacterium* as well as yeasts can grow in this culture medium. In addition, some lactic acid bacteria, mostly from unusual environments, do not grow on Rogosa SL. Depending on the source from which they were isolated, minor changes in Rogosa SL, such as the addition of specific growth factors [meat extract, tomato juice, yeast extract, malt extract, ethanol, or even some of the mevalonate natural substrates (beer, juices)] may facilitate the isolation of lactobacilli that have adapted themselves to the growing conditions of their ecological niche (22).

1.4 Habitat

Lactobacilli colonize some specific parts of the human body: at these sites they play specific functions.

1.4.1 Gastro-Intestinal (GI) Tract

Lactobacilli are part of the normal microbiota of the mouth and intestines of healthy people and animals. Species composition and their amount depend on individuals, host age and GI area considered (24). It is difficult to distinguish from those indigenous (oral cavity) to allochthonous one (fermented foods)(25).

Lactobacilli form only a small part of the fecal microbiota of the adult (0.01% -0.6% of total bacteria) (26) (27). *L. gasseri*, *L. reuteri*, *L. crispatus*, *L. salivarius* and *L. ruminis* are considered more prevalent as indigenous, while *L. acidophilus*, *L. fermentum*, *L. casei*, *L. rhamnosus*, *L. johnsonii*, *L. plantarum*, *L. brevis*, *L. delbrueckii*, *L. curvatus* and *L. sakei* are present in variable amounts (25).

In comparison to the adult microbiota, the one present in children is highly unstable and contains lactobacilli in varying amounts. The number of lactobacilli in infants varies from 10^5 CFU/g of to 10^6

CFU/g feces, while in a newborn of one month old or more, this interval increases to 10^6 - 10^8 CFU/ of feces (28).

The presence of lactobacilli as indigenous microbiota is guaranteed by their ability to adhere to the mucous membranes of the districts concerned. In the case of *L. plantarum* is a mannose binding adesine to ensure colonization (29).

Lactobacilli appear in the mouth during the first year of neonatal life. Their presence depends on numerous factors including the existence of ecological niches, for example the natural cavities of the teeth. In many cases, lactobacilli may play a beneficial role by inhibiting the proliferation of cariogenic bacteria. Ahumada et al. (30) have shown that 36% of lactobacilli isolated from the tongue are able to prevent the growth of *S. mutans*. The homofermentative ones produce a greater number of inhibitory substances, compared to those belonging to the group of heterofermentative. Many authors have studied the role of lactobacilli as probiotics for oral health. In agreement with Busscher *et al.* (31), *L. acidophylus* and *L. casei*, present in yogurt, can colonize the oral cavity due to their ability to adhere to tooth enamel (32).

1.4.2 Vaginal microbiota

Unlike the G.I. tract, the presence of lactobacilli is very pronounced in the female urogenital tract. The role of lactobacilli in these districts is potentially important because of the protective role against pathogenic microorganisms.

Aerobic and anaerobic microorganisms, generally constitute the microbiota of a healthy woman. Lactobacilli are present in the absolute majority in the vaginal fluid of healthy not menopause women. Their metabolic products, such as hydrogen peroxide (H_2O_2), lactic acid, bacteriocins, play an important role in the maintenance of normal vaginal microbiota inhibiting the colonization of pathogenic bacteria. The predominant species detected by molecular biology studies are *L. crispatus*, *L. jensenii* and *L. gasseri* (33) (34). In recent studies, *L. iners* (*L. 1086V*) described by Anthony et al. (35) has been identified among species colonizing the human vagina (36) (34) (37)(38).

The high levels of estrogen present during the reproductive age cause the storage of large quantities of glycogen vaginal epithelium (39), which can be metabolized by vaginal microbiota in organic acids (40). Since the vaginal pH in women of reproductive age is

around 3.5/4.5, it is believed that these drastic conditions allow the proliferation of acidophilic species like lactobacilli. Lactic acid and other acids produced by lactobacilli inhibit the proliferation of pathogenic microorganisms such as *E. coli*, *C. albicans*, *G. vaginalis*. Also, the hydrogen peroxide is toxic to fungi, viruses, etc. (41).

Bacterial Vaginosis (BV) is a disorder of the vaginal microbiota, where the normal lactobacilli colonizers, are overwhelmed by an abnormal growth of different anaerobic bacteria (*Gardnerella vaginalis*, *Mycoplasma hominis*, *Mobiluncus spp.*, *Peptostreptococcus*, *Prevotella*, *Bacteroides*, etc.) (42). This condition is common in women of reproductive age (43)(44) and can cause foul-smelling vaginal secretions, although many women are asymptomatic (45). In pregnant women this may represent a risk factor for the occurrence of perinatal complications, including preterm labor and chorioamnionitis (43)(44)(45) (46)(47).

BV may be associated with different behaviours of women, including sexual ones (relations with multiple partners, use of intrauterine devices for contraception, etc.) (31). The incidence of BV varies markedly between different ethnic groups: about 6% in Asian women, 9% in the white, 16% in spanish and 23% in african

americans. The rationalization of this phenomenon lies in the socio-demographic characteristics, sexual behaviour, and personal hygiene (48).

1.5 The salutary effects of lactobacilli

It has been demonstrated that the lactobacilli carry healthy effects when applied under various conditions. The best results have been obtained in the treatment and prevention of enteric infections and in post-antibiotic syndromes. Some lactobacilli are able to reduce the diarrhea associated with *Clostridium difficile* (49) and preventing necrotizing enterocolitis in preterm infants (50).

The GI tract is certainly the district where it is believed that lactobacilli exert major effects on the health of host; nevertheless probiotic applications of some species, in other districts, seem to be promising, for example, in the prevention and treatment of urogenital infections and bacterial vaginosis (37), in the prevention of atopic disease, in food hypersusceptibility (51) and in the prevention of dental caries (52). The probiotic lactobacilli must possess the status of GRAS (Generally Regarded as Safe) and so must be well tolerated. There have been rare cases of infection, presumably caused by probiotics in immunocompromised individuals or in patients with severe disease (53) (54).

1.5.1 General mechanisms of the action of probiotic lactobacilli

Abilities of lactobacilli to carry salutary effects for the host, can be recognized in one or more of the following mechanisms (Figure 2) (55)(56):

- i. Bacterial pathogens inhibition and homeostasis restoration through microbe-microbe interaction;
- ii Enhancement of epithelial barrier function;
- iii Modulation of immune responses.

The ability of lactobacilli to inhibit pathogenic microorganisms is well-known, given that they have been used for centuries for the preservation of foods. Subsequently the immunostimulating and immunomodulating capacity of these microorganisms were investigated by molecular studies.

Different strains of probiotic lactobacilli, have been associated with different effects in relation to specific properties such as the ability to express particular surface molecules, to secrete specific proteins and metabolites that can interact directly with host cells (24).

Molecular characterization of probiotic strains is extremely important and has two main objectives:

- a. Define the best conditions that determine the "performance" of the best probiotic strains;
- b. Select well-defined molecular criteria for new probiotic strains.

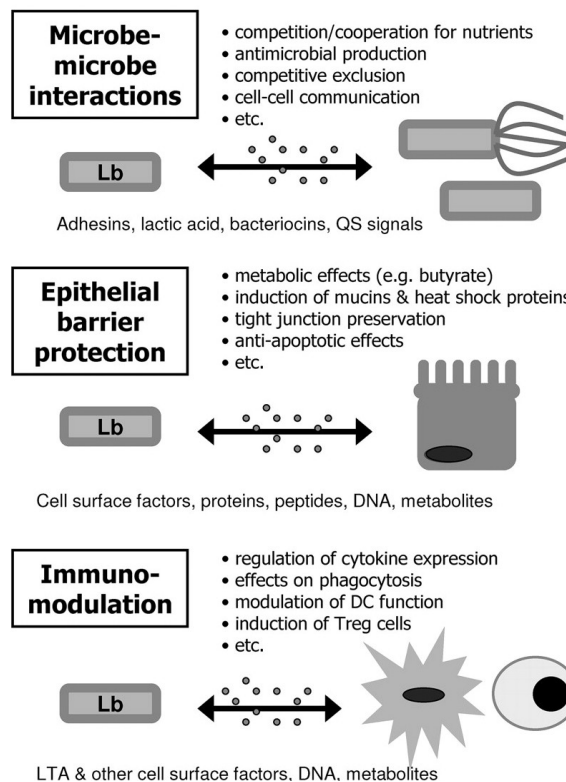


Figure 1-2 Probiotic lactobacilli mechanistic view. Molecular studies on probiotics lactobacilli in order to identify factors that promote survival, as a result of adaptation, and host colonization factors (adaptation) and factors that directly promote the health benefits (on probiotic factors)(24)

There are two main categories of factors that contribute to optimize the activity of probiotic lactobacilli: factors that promote optimum adaptation to ecological niches that the bacteria met temporarily in the host (adaptation factors) and factors that directly contribute to

the promotion of the beneficial effects (probiotic factors) (figure 1-2). Probiotic Factors include three main mechanisms: the maintenance of microbial balance, epithelial protection and immunomodulation. Adaptation Factors include resistance to stress, adaptation to host metabolism and adherence to intestinal mucosa (24).

Specific metabolic and physiological features of lactic acid bacteria play a key role to adaptation in host environment. In Gram-positive bacteria, the cell wall is made up of unique structures: a thin, multi-layer peptidoglycan (PG), surrounded with protein, teichoic acid and polysaccharides, in some species (*L. acidophilus*, *L. gasseri*, *L. johnsonii*, *L. brevis*, *L. crispatus*, *L. helveticus*) there is a shell protein packed in a crystalline layer (S layer) (57).

Wall macromolecules determine the specific properties of the strain including the ability to adapt to environmental changes and interaction with host epithelial cells and receptors of immune responses (58).

1.6 Probiotics side effects

Probiotics are not selected among pathogens, and the theoretical risk of infection is thus very low. The risk of their passage in blood, eventually by translocation, is, however, important to determine. Bacterial translocation is defined as the passage of micro-organisms from the gastrointestinal to extra-intestinal sites such as the mesenteric lymph nodes, liver, spleen and bloodstream. Indigenous bacteria are continuously translocating in low numbers but are rapidly killed in the lymphoid organs. Bacterial translocation is a major cause of severe infection in immunosuppressed, trauma and post-surgical patients. This may result from three mechanisms: intestinal bacterial overgrowth, increased permeability or damage of the intestinal mucosal barrier, and immunodeficiency (59).

Rare cases of infection, including septicaemia and endocarditis caused by lactobacilli, bifidobacteria or other lactic acid bacteria, have been reported (60). *Enterococcus faecium* and *E. faecalis* are more frequently involved in clinical infection, and there is concern over the emergence of vancomycin-resistant strains. In most cases of infection, the organism appeared to have come from the patient's own microbiota, but, in a few cases, the recent consumption of

probiotics was proposed as a potential cause. About 30 cases of fungaemia have been reported in patients treated with *Saccharomyces boulardii*(61)(62), and two cases of infection have been traced back to food-borne *L.rhamnosus* (63)(64). Nearly all subjects who had fungaemia involving *Saccharomyces boulardii* had an indwelling vascular catheter (61)(62).

Infection caused by *L. rhamnosus* similar to the probiotic GG strain was observed in a 74-year-old woman with non-insulin-dependent diabetes, who suffered from a liver abscess, which proved to contain *Lb. rhamnosus*, and pleuropulmonary infection(63). No cause for this infection was found, but the woman reported a regular consumption of dairy drinks containing *L. rhamnosus GG*. The clinical strain appeared to be indistinguishable from the GG strain. The other case of infection occurred in a 67-year-old man with mild mitral valve regurgitation who habitually chewed a probiotic mixture and had carious teeth to be removed and suffered after a few days from an endocarditis. *Lactobacillus rhamnosus* was isolated from his blood, further analysis showing that it was indistinguishable from one of the organisms present in the probiotic preparation. (64) Saxelin and colleagues (65)(66) studied the prevalence of bacteraemia caused by *Lactobacillus* species in

Southern Finland and compared the characteristics of the blood culture isolates and the probiotic dairy strains. In their first study, lactobacilli were identified in eight of 3317 blood culture isolates, none of the isolates corresponding to a dairy strain. In the second study, 5912 blood cultures were analysed, none of the 12 lactobacilli isolated being identical to any of the commercial *Lactobacillus* strains. To summarise, there is no evidence that ingested probiotic lactobacilli or bifidobacteria pose any greater risk of infection than do commensal strains, but there is insufficient knowledge on the risks or benefits of probiotics in immunodeficiency. Other risk factors for opportunist infection, such as extremes of age, pregnancy and digestive lesions, have not been identified as risk factors for probiotic infections.

According to EFSA (*European Food Saefety Authority*) since the 2010 only few reports have been published concerning lactobacilli and clinical infections (67)

One article from Turkey (68) detected a '*Lactobacillus acidophilus* or *Lactobacillus jensenii*' strain in clinical specimen amongst other species in an immunocompromised patient. The clinical relevance of this isolate was not clear. In addition, the taxonomic identification was done with the API system and therefore no clear

attribution to a taxonomic unit can be done. Several reports related to the well known association of lactobacilli with dental caries (69) (70)(71). Kneist et al. (2010) (69) found five species from carious dentine: *Lactobacillus paracasei subsp. paracasei*, *Lactobacillus paracasei subsp. tolerans*, *Lactobacillus rhamnosus*, *Lactobacillus gasseri*, and *Lactobacillus alimentarius*.

They concluded that *Lactobacillus rhamnosus* and *Lactobacillus paracasei subsp. paracasei* occurred in all caries progression stages, whilst the other species were found only sporadically. There is a connection to endocarditis via caries which has been reported on several occasions earlier. There is also a possibility of lactic acid bacteria (LAB) being involved in endocarditis after dental surgery(72).

Caries is a multifactorial disease, including bacteria from the oral cavity, eating and drinking habits (high sugar amounts) and insufficient oral and dental hygiene (73). Bacteria involved change through different stages of caries proliferation. In the primary phase mainly mutans streptococci are involved, whereas in secondary caries with lesions already present also lactobacilli, bifidobacteria and other LAB are involved(74) (73). The conclusions of these studies are that without bacteria caries development is not possible.

LAB, however, are present in caries stages with predisposing factors such as lesions and insufficient dental hygiene. The origin of those LAB (if deriving from food or as autochthonous oral microbiota) has not been studied so far. In conclusion, LAB are not the initial cause of these diseases and they are present in the human organism as commensal microbiota.

Doi et al. (2010) (75) found *Lactobacillus paracasei* involved in a splenic abscess. But again as in previous similar case reports, the patient had an underlying disease and was immunocompromised. In a similar case with an immunocompromised patient (stem cell transplantation in a child) *L. Rhamnosus* was found to be the causative agent for meningitis after recurrent episodes of bacteremia (76)) Russo et al. (2010) (77) isolated a presumptive *Lactobacillus casei* strain from a bacteraemia case, where heavy consumption of dairy products was involved in the case history. However, no strains from dairy products (mainly cheese) were isolated and compared to the clinical strain. The identification was most probably misinterpreted, as only a 16S rDNA sequence analysis was done, which matched equally to *Lactobacillus casei* and *Lactobacillus paracasei*. Therefore *Lactobacillus paracasei* seems to be the correct identification given the distribution of

species in humans and dairy products. *Lactobacillus casei* does not naturally occur in such environments. *Lactobacillus rhamnosus* can be associated with unexplained sustained bacteremia like in the TIPSS syndrome (tipsitis). This is a rare disease where *Lactobacillus rhamnosus* may be involved inter alia(78).

A research including 'urinary tract infection' (UTI) revealed one review(79) indicating a relatively higher risk for elderly women for urinary tract infections with *L. delbrueckii*.

2 Taxonomy

The taxonomy can be defined as a scientific study that is able to "classify" the biodiversity of microorganisms, or more generally of organisms (80). Advances in bacterial taxonomy have always been dependent on the technological ones: the modern taxonomy is built primarily on molecular data. The complete study on the identification of a microorganism is closely related to the methods of analysis used. For this reason, the correct taxonomic status can only be taken out from the comparative study of a wide range of techniques, molecular and otherwise: the polyphasic approach.

The phylogenetic approach has revolutionized systematic studies on bacteria. Phylogenetic relationships between microorganisms can be effectively estimated through the comparison of molecular sequences such as those of the genes coding for 16S rRNA. This target is chosen for several reasons: the 16S rRNA genes are highly conserved, as ribosomes play a key role in protein biosynthesis since the earliest stages of the development of micro-organisms; although phenomena of horizontal gene transfer between microorganisms have never occurred in these genes, they represent ancestral molecules and there are enough variables to differentiate

microorganisms, representing their variance genomics. These statements are not always true, or at least some experimental evidence has cast some doubt on this. In general the molecular analysis of the 16S rRNA has enabled us to draw an evolutionary scenario of bacteria although other molecular targets such as RecA and elongation factor Tu can be used in parallel taxonomy studies and bacterial phylogeny (81).

2.1 Classification

According to outline Taxonomy of Prokaryotes, the genus *Lactobacillus* belongs to the *Firmicutes* phylum, *Bacilli* class, *Lactobacillales* order, *Lactobacillaceae* family. NCBI taxonomy database today recognizes today 148 species belonging to the genus *Lactobacillus* that represents, therefore, the most numerous of the *Lactobacillales* order (82).

Recently, a new species has been described, *Lactobacillus tuceti* (83) and the last entry in the classification are the species *L. compounds*, *L. farraginis*, and *L. parafarraginis* (84)(85) and *L. secaliphilus* (86), in addition to the species described by Dellaglio and collaborators (81).

Only seven species of the genus *Lactobacillus* described by Dellaglio et al. comprise two or more subspecies: *Lactobacillus aviarius* (*L. aviarius* subsp. *aviarius* and *L. aviarius* subsp. *araffinosus*), *Lactobacillus coryniformis* (*L. coryniformis* subsp. *coryniformis* and *L. coryniformis* subsp. *torquens*), *Lactobacillus delbrueckii* (*L. delbrueckii* subsp. *delbrueckii*, *L. delbrueckii* subsp. *bulgaricus*, *L. delbrueckii* subsp. *indicus* and *L. delbrueckii* subsp. *lactis*), *Lactobacillus kefiranofaciens*

(*kefiranofaciens* *L. subsp. kefiranofaciens kefiranofaciens* and *L. subsp. kefirgranum*), *Lactobacillus paracasei* (*L. paracasei subsp. paracasei* and *L. paracasei subsp. Tolerans*), *Lactobacillus plantarum* (*L. plantarum subsp. plantarum* and *L. plantarum subsp. argentoratensis*), and *Lactobacillus sakei* (*L. sakei subsp. sakei* and *L. sakei subsp. carnosus*).

The phylogenetic structure of the *Lactobacillaceae* family considers *Lactococcus lactis* and *Streptococcus thermophilus* as group limits.

The first phylogenetic analysis of lactobacilli was carried out by Collins et al. in 1991, on a small number of species known at that time: they proposed to divide the genus *Lactobacillus* into 3 groups: *L. delbrueckii* group, *L. casei-Pediococcus* group and *Leuconostoc* group, which contained some lactobacillus.

In 1995, Schleifer and Ludwig confirmed Collins evidences and they changed *L. delbrueckii* group with group name *L. acidophilus*. In addition, these authors noted that the group *L. casei-Pediococcus* could be divided into four sub clusters. In the first group the percentage of homology of 16S rDNA varies from 90.8% to 99.3%. It includes *L. delbrueckii* (G+ C = 50%) with the three subspecies (*L. delbrueckii subsp. lactis*, which includes the two old species *L. lactis* and *L. leichmannii*, *L. delbrueckii subsp. delbrueckii* and *L.*

delbrueckii subsp. bulgaricus) and species in the group identified by Collins, such as *L. acidophilus* (G+ C = 34-37%), *L. amylovorus* (G+ C = 40-41%), *L. crispatus* (G+ C = 33-35%), *L. gallinarum* (G+ C = 36-37%), *L. gasseri* (G+ C = 33-35%) and *L. johnsonii* (G+ C = 35-38%).

The group *Lactobacillus casei-Pediococcus* is the largest and heterogeneous, where the percentage of homology of 16S rDNA varies from 90.3% to 99%. It includes 37 species of *Lactobacillus* and 5 species of *Pediococcus*. Finally, *Leuconostoc* group: includes species assigned to the new genus *Weissella*, *Oenococcus oeni* and heterofermentative lactobacilli.

The recent description of a large number of species and the consequent re-examination of the phylogenesis splits these groups into smaller and more flexible groups. This strategy of "grouping" has been adopted by Hammes and Hertel (2003) and by Dellaglio Felis in 2005 (81) (Table 2-1).

Table 2-1 Phylogenetic grouping

Group	<i>Hammes and Hertel (2003)</i>	<i>Dellaglio and Felis (2005)</i>	<i>Felis and Dellaglio</i>
<i>L. delbrueckii</i> group (delb)	<i>L. acetotolerans, L. acidophilus, L. amylolyticus, L. amylophilus, L. amylovorus, L. crispatus, L. delbrueckii, L. fornicalis, L. gallinarum, L. gasserii, L. hamsteri, L. helveticus, L. iners, L. intestinalis, L. jensenii, L. johnsonii, L. kefirano-faciens, L. kefirgranum, L. psittaci</i>	<i>L. acetotolerans, L. acidophilus, L. amylolyticus, L. amylophilus, L. amylovorus, L. crispatus, L. delbrueckii, L. fornicalis, L. gallinarum, L. gasserii, L. hamsteri, L. helveticus, L. iners, L. intestinalis, L. jensenii, L. johnsonii, L. kalixensis, L. kefirano-faciens, L. kefirgranum, L. kitasatonis, L. psittaci, L. suntoryeus, L. ultunensis</i>	<i>L. acetotolerans, L. acidophilus, L. amylolyticus, L. amylophilus, L. amylo-trophicus, L. amylovorus, L. crispatus, L. delbrueckii, L. fornicalis, L. gallinarum, L. gasserii, L. hamsteri, L. helveticus, L. iners, L. intestinalis, L. jensenii, L. johnsonii, L. kalixensis, L. kefirano-faciens, L. kitasatonis, L. psittaci, L. sobrius, L. ultunensis</i>
<i>L. salivarius</i> group	<i>L. acidipiscis, L. agilis, L. algidus, L. animalis, L. aviarius, L. cypricasei, L. equi, L. mali, L. murinus, L. nagelii, L. ruminis, L. salivarius</i>	<i>L. acidipiscis, L. agilis, L. algidus, L. animalis, L. aviarius, L. cypricasei, L. equi, L. mali, L. murinus, L. nagelii, L. ruminis, L. saerimneri, L. salivarius, L. satsumensis</i>	<i>L. acidipiscis, L. agilis, L. algidus*, L. animalis, L. apodemi, L. aviarius, L. equi, L. mali, L. murinus, L. nageli, L. ruminis, L. saerimneri, L. salivarius, L. satsumensis, L. vini</i>
<i>L. reuteri</i> group (reu)	<i>L. coleohominis, L. durianis, L. fermentum, L. frumenti, L. ingluviei, L. mucosae, L. oris, L. panis, L. pontis, L. reuteri, L. suebicus, L. thermotolerans, L. vaccinostercus, L. vaginalis</i>	<i>L. antri, L. coleohominis, L. fermentum, L. frumenti, L. gastricus, L. ingluviei, L. mucosae, L. oris, L. panis, L. pontis, L. reuteri, L. thermotolerans, L. vaginalis (L. reuteri group-a) associated with L. durianis, L. vaccinostercus, L. suebicus, L. rossii (L. reuteri group-b)</i>	<i>L. antri, L. coleohominis, L. fermentum, L. frumenti, L. gastricus, L. ingluviei, L. mucosae, L. oris, L. panis, L. pontis, L. reuteri, L. secaliphilus, L. vaginalis</i>
<i>L. buchneri</i> group (buch)	<i>L. buchneri, L. diolivorans, L. ferintoshensis, L. fructivorans, L. hilgardii, L. homohiochii, L. kefir, L. kunkeei, L. lindneri, L. parabuchneri, L. parakefiri, L. sanfranciscensis</i>	<i>L. buchneri, L. diolivorans, L. ferintoshensis, L. hilgardii, L. kefir, L. parabuchneri, L. parakefiri (L. buchneri group-a) associated with L. fructivorans, L. homohiochii, L. lindneri, L. sanfranciscensis (L. buchneri group-b)</i>	<i>L. buchneri, L. diolivorans, L. farraginis, L. hilgardii, L. kefir, L. parabuchneri, L. parafarraginis, L. parakefiri associated with L. acidifarinae, L. namurensis, L. spicheri, and L. zymae (which form a robust group)</i>
<i>L. alimentarius-L. farciminis</i> group (al-far)	/	/	<i>L. alimentarius, L. farciminis, L. kimchii, L. mindensis, L. nantensis, L. paralimentarius, L. tuccei, L. versmoldensis</i>
<i>L. casei</i> group (cas)	<i>L. casei, L. manihotivorans, L. pantheris, L. paracasei, L. rhamnosus, L. sharpeae, L. zeae</i>	<i>L. casei, L. paracasei, L. rhamnosus, L. zeae (L. casei group-a) L. manihotivorans, L. pantheris, L. sharpeae (L. casei group-b) appear as distinct clusters, not robustly associated with each other</i>	<i>L. casei, L. paracasei, L. rhamnosus, L. Zeae</i>
<i>L. sakei</i> group (sakei)	<i>L. curvatus, L. fuchuensis, L. graminis, L. sakei</i>	<i>L. curvatus, L. fuchuensis, L. graminis, L. sakei</i>	<i>L. curvatus, L. fuchuensis, L. graminis, L. Sakei</i>

<i>L. fructivorans</i> group (fru)	/	/	<i>L. fructivorans</i> , <i>L. homohiochii</i> , <i>L. lindneri</i> , <i>L. sanfranciscensis</i>
<i>L. coryniformis</i> group (cor)	/	/	<i>L. bifermentans</i> , <i>L. coryniformis</i> , <i>L. rennini</i> , not robustly associated with <i>L. composti</i>
<i>L. plantarum</i> group (plan)	<i>L. alimentarius</i> , <i>L. arizonensis</i> , <i>L. collinoides</i> , <i>L. farciminis</i> , <i>L. kimchii</i> , <i>L. malefermentans</i> , <i>L. mindensis</i> , <i>L. paralimentarius</i> , <i>L. paraplantarum</i> , <i>L. pentosus</i> , <i>L. plantarum</i> , <i>L. versmoldensis</i>	<i>L. arizonensis</i> , <i>L. collinoides</i> , <i>L. paraplantarum</i> , <i>L. pentosus</i> , <i>L. plantarum</i> (<i>L. plantarum</i> group-a) associated with <i>L. alimentarius</i> , <i>L. farciminis</i> , <i>L. kimchii</i> , <i>L. mindensis</i> , <i>L. paralimentarius</i> , <i>L. versmoldensis</i> (<i>L. plantarum</i> group-b) the affiliation of <i>L. collinoides</i> was poorly supported	<i>L. plantarum</i> , <i>L. paraplantarum</i> , <i>L. pentosus</i>
<i>L. perolens</i> group (per)	/	/	<i>L. perolens</i> , <i>L. harbinensis</i> , <i>L. paracollinoides</i>
<i>L. brevis</i> group (bre)	/	<i>L. acidifarinae</i> , <i>L. brevis</i> , <i>L. hammesii</i> , <i>L. spicheri</i> , <i>L. zymae</i>	<i>L. brevis</i> , <i>L. hammesii</i> , <i>L. parabrevis</i>
<i>Pediococcus dextrinicus</i> group (Pdex)			<i>P. dextrinicus</i> , <i>L. concavus</i> , <i>L. oligofermentans</i> (the latter sometimes poorly supported)
<i>Pediococcus</i>	Not reported	1 single cluster (not including <i>P. dextrinicus</i>)	2 clusters, not associated: the first comprises <i>P. cellicola</i> , <i>P. damnosus</i> <i>P. parvulus</i> , <i>P. inopinatus</i> , while the second includes <i>P. acidilactici</i> , <i>P. claussenii</i> , <i>P. pentosaceus</i> and <i>P. stilesii</i>
Couples (couple)			<i>L. rossiae</i> - <i>L. siliginis</i> (1) <i>L. vaccinostercus</i> - <i>L. suebicus</i> (2) <i>L. manihotivorans</i> - <i>L. collinoides</i> (3)
Single species (ss)	<i>L. bifermentans</i> , <i>L. brevis</i> , <i>L. coryniformis</i> and <i>L. perolens</i>	<i>L. algidus</i> , <i>L. kunkeei</i> , <i>L. malefermentans</i> , <i>L. paracollinoides</i> , <i>L. perolens</i> , <i>Paralactobacillus selangorensis</i>	<i>L. kunkeei</i> , <i>L. malefermentans</i> , <i>L. pantheris</i> , <i>L. sharpeae</i> , <i>Paralactobacillus selangorensis</i>

Table 2-1 Continue

The greatest discrepancy in the taxonomy of the genus *Lactobacillus* is due to the lack of ability to correlate between the phylogenetic position and metabolic properties of the species. The

historical division of the genus *Lactobacillus* based on the type fermentation was excellently revisited by Pot et al. (1994) (87), who pointed out that the terms "homofermentative", "heterofermentative", "obliged homofermentative", "facultative heterofermentative" and "obliged heterofermentative" can have different meanings according to the authors, creating misunderstandings. The most recently accepted definition is given by Hammes and Vogel (1995) as described in the paragraph on the metabolism of lactic acid bacteria (Table 2-2).

Table 2-2 Taxonomic characteristics of the species belonging to the genus *Lactobacillus*

Species	Metabolism	Phylogenetic group	GC content (%)
<i>L. fuchuensis</i> (Sakala et al. 2002)	B	sakei	41-42
<i>L. gallinarum</i> (Fujisawa et al. 1992)	A	delb	36-37
<i>L. gasseri</i> (Lauer and Kandler 1980)	A	delb	33-35
<i>L. gastricus</i> (Roos et al., 2005)	C	reu	41-42
<i>L. graminis</i> (Beck et al. 1989)	B	sakei	41-43
<i>L. hammesii</i> (Valcheva et al. 2005)	B	bre	nd
<i>L. hamsteri</i> (Mitsuoka and Fujisawa 1988)	B	delb	33-35
<i>L. harbinensis</i> (Miyamoto et al. 2006)	B	per	53-54
<i>L. helveticus</i> (Orla-Jensen 1919) (Bergey et al. 1925)	A	delb	38-40
<i>L. hilgardii</i> (Douglas and Cruess 1936)	C	buch	39-41
<i>L. homohiochii</i> (Kitahara et al. 1957)	B	fru	35-38
<i>L. iners</i> (Falsen et al. 1999)	A	delb	34-35
<i>L. ingluviei</i> (Baele et al. 2003)	C	reu	49-50
<i>L. intestinalis</i> (ex Hemme 1974) (Fujisawa et al. 1990)	B	delb	33-35
<i>L. jensenii</i> (Gasser et al. 1970)	B	delb	35-37
<i>L. johnsonii</i> (Fujisawa et al. 1992)	A	delb	33-35
<i>L. kalixensis</i> (Roos et al., 2005)	A	delb	35-36
<i>L. kefiranofaciens</i> subsp. <i>kefiranofaciens</i> (Fujisawa et al. 1988)	A	delb	34-38
<i>L. kefiranofaciens</i> subsp. <i>kefirgranum</i> (Takizawa et al. 1994) (Vancanneyt et al. 2004)	A	delb	34-38
<i>L. kefirii</i> (Kandler and Kunath 1983)	C	buch	41-42
<i>L. kimchii</i> (Yoon et al. 2000)	B	al-far	35
<i>L. kitasatonis</i> (Mukai et al. 2003)	B	delb	37-40
<i>L. kunkeei</i> (Edwards et al. 1998)	C	ss	nd
<i>L. lindneri</i> (Back et al. 1997)	C	fru	35
<i>L. malefermentans</i> (Farrow et al. 1989)	C	ss	41-42
<i>L. mali</i> (Carr and Davies 1970, emend. Kaneuchi et al., 1998)	A	sal	32-34
<i>L. manihotivorans</i> (Morlon-Guyot et al. 1998)	A	coppia3	48-49
<i>L. mindensis</i> (Ehrmann et al. 2003)	A	al-far	37-38
<i>L. mucosae</i> (Roos et al. 2000)	C	reu	46-47
<i>L. murinus</i> (Hemme et al. 1982)	B	sal	43-44
<i>L. nagelii</i> (Edwards et al. 2000)	A	sal	nd
<i>L. namurensis</i> (Scheirlinck et al. 2007)	C	buch	52
<i>L. nantensis</i> (Valcheva et al. 2006)	B	al-far	38,6
<i>L. oligofermentans</i> (Koort et al. 2005)	C	Pdex	35,3-39,9
<i>L. oris</i> (Farrow and Collins 1988)	C	reu	49-51
<i>L. panis</i> (Wiese et al. 1996)	C	reu	49-51
<i>L. pantheris</i> (Liu and Dong 2002)	A	ss	52-53
<i>L. parabrevis</i> (Vancanneyt et al. 2006)	C	bre	49
<i>L. parabuchneri</i> (Farrow et al. 1989)	C	buch	44
<i>L. paracasei</i> subsp. <i>paracasei</i> (Collins et al. 1989)	B	cas	45-47
<i>L. paracasei</i> subsp. <i>tolerans</i> (Collins et al. 1989)	B	cas	45-47
<i>L. paracollinoides</i> (Suzuki et al. 2004)	C	per	44-45
<i>L. parafarraginis</i> (Endo and Okada 2007)	B	buch	40
<i>L. parakefirii</i> (Takizawa et al. 1994)	C	buch	41-42
<i>L. paralimentarius</i> (Cai et al. 1999)	B	al-far	37-38

A: Obligated homofermentative; B: Facoltative heterofermentative; C: Facoltative heterofermentative; nd: not determinated; na: not classified

Species	Metabolism	Phylogenetic group	GC content (%)
<i>L. paraplantarum</i> (Curk et al. 1996)	B	plan	44-45
<i>L. pentosus</i> (Zanoni et al. 1987)	B	plan	46-47
<i>L. perolens</i> (Back et al. 2000)	B	per	49-53
<i>L. plantarum</i> (Orla-Jensen 1919) (Bergey et al. 1923)	B	plan	44-46
<i>L. plantarum</i> subsp. <i>argentoratensis</i> (Bringel et al. 2005)	B	plan	44-46
<i>L. pontis</i> (Vogel et al. 1994)	C	reu	53-56
<i>L. psittaci</i> (Lawson et al. 2001)	C	delb	nd
<i>L. rennini</i> (Chenoll et al. 2006)	B	cor	nd
<i>L. reuteri</i> (Kandler et al. 1982)	C	reu	40-42
<i>L. rhamnosus</i> (Hansen 1968) (Collins et al. 1989)	B	cas	45-47
<i>L. rogosae</i> (Holdeman and Moore 1974)	na	na	na
<i>L. rossiae</i> (Corsetti et al. 2005)	C	coppia1	44-45
<i>L. ruminis</i> (Sharpe et al. 1973)	A	sal	44-47
<i>L. saerimneri</i> (Pedersen and Roos, 2004)	A	sal	42-43
<i>L. sakei</i> subsp. <i>carosus</i> (Torriani et al. 1996)	B	sakei	42-44
<i>L. sakei</i> subsp. <i>sakei</i> (Katagiri et al. 1934 emend. Klein et al. 1996)	B	sakei	42-44
<i>L. salivarius</i> (Rogosa et al. 1953 emend. Li et al. 2006)	A	sal	34-36
<i>L. sanfranciscensis</i> (Weiss and Schillinger 1984)	C	fru	36-38
<i>L. satsumensis</i> (Endo and Okada, 2005)	A	sal	39-41
<i>L. secaliphilus</i> (Ehrmann et al. 2007)	B	reu	48
<i>L. sharpeae</i> (Weiss et al. 1982)	A	ss	53
<i>L. siliginis</i> (Aslam et al. 2006)	C	coppia1	44,5
<i>L. sobrius</i> (Konstantinov et al. 2006)	B	delb	35-36
<i>L. spicheri</i> (Meroth et al. 2004)	B	buch	55
<i>L. suebicus</i> (Kleynmans et al. 1989)	C	coppia2	40-41
<i>L. tuccei</i>	A	al-far	ND
<i>L. ultunensis</i> (Roos et al., 2005)	A	delb	35-36
<i>L. vaccinostercus</i> (Kozaki and Okada 1983)	C	coppia2	36-37
<i>L. vaginalis</i> (Embley et al. 1989)	C	reu	38-41
<i>L. versmoldensis</i> (Kröckel et al. 2003)	A	al-far	40-41
<i>L. vini</i> (Rodas et al. 2006)	B	sal	39,4
<i>L. vitulinus</i> (Sharpe et al. 1973)	A		34-37
<i>L. zaeae</i> (Dicks et al. 1996)	B	cas	48-49
<i>L. zymae</i> (Vancanneyt et al. 2005)	C	buch	53-54

Table 2-2 Continue

The phylogenetic structure of lactobacilli is extremely complicated and from the data recorded in the literature it is evident that the taxonomy of the genus is still ambiguous for certain species and for this reason subjected to periodic reinterpretations.

2.2 Comparative genomic analysis

Phylogenetic distance between species is now highlighted by different methods of comparative analysis, which can help to explore the characteristics of *Lactobacillus* species whose genome has been sequenced.

L. plantarum has a relatively large number of proteins involved in the sugars and lipids metabolism and amino acids transport, these became relevant when compared to *L.johnsonii*, which, however, can only use an extracellular proteinase to degrade peptides. This could explain and justify their different ecological niches; *L.johnsonii*, in fact is only found in the GI tract rich in proteins. The most "flexible" *L. plantarum* is able to colonize other environments such as plants, which require the ability to ferment a wider range of sugars.

There is only slight evidence of the correlation between the two genomes. Dot-plot comparisons and genomic alignments show little homology, have been found only 28 genes' clusters in common, with 6 genes in the same order. In addition, these clusters are also present in *Bacillus subtilis*, *Enterococcus faecalis* *Listeria monocytogenes*: this has led to affirm that the phylogenetic

correlation between *L. plantarum* and *L. johnsonii* is only minimally higher than that exhibited with other Gram-positive bacteria (88)

Although *L. gasseri* and *L. acidophilus* genomes group were compared. Many similarities have been identified between the members of the *L. acidophilus* group , which includes *L. acidophilus*, *L. gasseri* and *L. plantarum*. The genes' disposition is highly conserved and especially in the genomes of the two latter species mentioned and with the exception of a chromosomal reverse in the replication terminal site. Synteny studies have also revealed a gene region probably related with production of exopolysaccharides cell surface. This cluster is conserved among the three species and may have important properties in dairy products fermented by these microorganisms (89).

3 *Molecular methods of identification*

Traditionally, lactobacilli, and in general all the species belonging to the LAB (Lactic Acid Bacteria), have been identified on the basis of phenotypic characteristics, such as cell morphology, type of sugar fermentation, different growing temperatures, protein patterns of cell wall or the entire cell (90). Unfortunately, these phenotypic/biochemical typings are not completely accurate. Their limits are: lack of reproducibility, ambiguity of some techniques (often resulting from flexibility of bacterial growth), lack of reproducibility on a large scale and lack of discriminatory power. Another disadvantage of phenotypic analysis is represented by the fact that information carried by the entire genome is not always expressed, for example the expression of a gene may be related to environmental conditions (90).

Genotypic techniques are recognized as very important for identification purposes. Benefits arising from their use are high discriminatory power and their universal applicability. Strains strongly correlated with similar phenotypic characteristics, can be effectively identified by molecular techniques, mainly based on the PCR, such as RFLP (*Restriction Fragment Length Polymorphism*),

ARDRA (*Amplified Ribosomal Restriction Fragment Analysis*), AFLP (*Amplified Fragment Length Polymorphism*), RAPD (*Randomly Amplified Polymorphic DNA*), PFGE (*Pulse-Field Gel Electrophoresis*), ribotyping, DGGE (*Denaturing Gradient Gel Electrophoresis*) and TGGE (*Terminal Gradient Gel Electrophoresis*), etc..

RFLP was the first method of molecular typing used. Profiles in bands that result from enzymatic cuts. Subsequent separation into DNA fragments, obtained by electrophoretic run, is known as DNA fingerprinting. Due to the high specificity of endonuclease and the stability of the chromosomal DNA, the profiles obtained after complete digestion of the DNA, are reproducible. A general criticism of this method is the complexity of the profile of bands. However, many scholars believe that the proper choice of enzymes and the use of specific conditions make the RFLP technique relatively fast and easily accomplished (90).

ARDRA (90)(91) is essentially a restriction polymorphisms analysis of amplified gene rRNA encoding. The effectiveness of the method in distinguishing between species or between the subspecies depends on the choice of restriction enzymes and the length of the amplicon. Since some bacterial species have high

homology of the sequence of rDNA genes, it is difficult to select endonucleases capable of producing distinct patterns of restriction for species closely related. The method generates highly reproducible restriction patterns reproducible in different laboratories.

AFLP (92) combines the power of RFLP technique to the flexibility of PCR-based methods. The genomic DNA is digested with two restriction enzymes, one with a low cut-off frequency, the second with high-frequency cutting. Nucleotide adapters are ligated to the double stranded DNA fragments using the binding sites of primers for PCR amplification. The use of complementary primers to adapters and sequence of the cleavage sites allow amplification patterns strain-specific to be obtained (93). AFLP technique is mainly used in clinical trials also it had effective application for typing strains belonging to the *L. acidophilus* and *L. johnsonii* (94)(95).

In RAPD, also known as AP-PCR (arbitrarily Primed PCR), random sequences oligonucleotides with a length of 10bp are used in PCR at low stringency. The profile that emerges is of an array of gene amplicons "anonymous." Generally this method is used to discriminate the species and sometimes different strains of the same

species. It has been widely used for typing lactobacilli and bifidobacteria from various habitats (96)(97). For example, Tynkkynen et al. (98), have distinct 12 genotypes between 24 belonging to the *L. casei* group, many of which are of human origin.

The method is simple to perform and rapid, but the reproducibility of the results is poor, therefore optimization and standardization of the technique is required. The use of different thermal cyclers, different DNA polymerases in different concentrations, methods of preparation and DNA extraction, concentrations of the primers and magnesium chloride, can cause variations in the RAPD patterns and, they are not comparable if carried out in different laboratories.

PFGE technique allows the separation of large DNA fragments, in a continuous redirecting electrical field. Entire genome is digested with endonucleases (which rarely cut) and resulting macro fragments are separated by PFGE.

PFGE protocols have been designed for both lactobacilli and for bifidobacteria, and it shows a greater discriminating power if compared to other molecular typing methods, such as ribotyping. (90) (98). Due to the intense work involved, PFGE is not a feasible technique for large-scale typing of isolates.

Ribotyping is a RFLP variation, where some fragments are recognized by probes to obtain less complex profiles easier to interpret. Fragments obtained are derived from rDNA operons and their adjacent regions, by hybridization with specific probes. With this technique, the microorganisms are discriminated at the species level; it depends on probe length and restriction enzymes used (90) (99). In this context, Zhong et al. (100) evaluated the effectiveness of ribotyping of lactobacillus type strains and reference strains (*L. johnsonii*, *L. rhamnosus*, *L. casei*, *L. acidophilus*, *L. plantarum*, *L. fermentum*).

DGGE and TGGE techniques are used in screening of heterogeneity rRNA among bacterial species and in fingerprinting of bacterial communities (101). Nucleic acids are extracted from environmental samples and specific primers for bacteria or other phylogenetic groups are used. Then the amplicons are separated using specific electrophoretic techniques.

The PCR-T/DGGE of 16S rRNA is a culture-independent method widely used to determine the diversity and dynamics of microbial communities. These techniques can detect only the predominant members of the community, which must represent at least 1% of the microbial community (101). The limit of detection for the major

groups of intestinal bacteria is approximately 10^5 cells/mL of fecal sample; this value depends also on methods of DNA extraction used (102).

Recently, these methods have been developed for the selective monitoring of LAB (103)(104). The T/DGGE is a useful tool to analyse the complexity of the microbial community, while each member can be individually identified by subsequent cloning and sequencing of the fragments. Alternatively, to obtain information about the individual species T/DGGE profiles can be hybridized with phylogenetic probes (91).

Recently, these methods have been used for the study of microbial populations of the gastro-intestinal tract. The composition of the microbial community (bifidobacteria and lactobacilli) proved to be unique for each individual (microbiota host-specific), and, in general, the population of bifidobacteria seems to be more stable over time, both numerically and in composition, as compared to the lactobacilli (105)(106).

3.1 Macromolecules as “molecular clock” of microbial diversity

Macromolecules have represented "documents" in the history of microbial evolution and for decades have been used to explore the phylogenetic diversity and evolutionary relationships between organisms (90)(107).

Since the 80s, new methods have been developed for identification of bacterial species. Woese et al. demonstrated that phylogenetic relationships between bacteria, and in general among all forms of life, could be detected through the comparison of stable portions of the genetic code (108). Candidates of these genetic studies were represented by genes that coded for subunits 5S, 16S (also called small subunit), 23S rRNA and the interspacers regions between these genes. today the region of DNA mostly used for taxonomic purposes is 16S rRNA (109)(110).

3.2 The Choice of the 16S rRNA as Sequencing Gene

In the '60s, Dubnau et al. (111) observed the 16S rRNA conservation between phylogenetically close species belonging to the genus *Bacillus*. The widespread use of the 16S rRNA gene sequence for the taxonomy study and bacterial phylogeny occurred as a result of a study by Woese et al. (108), which defined important characteristics. The finding that 16S rRNA acts as a molecular clock (108) was of great importance. This assumption stems from the importance of the 16S rRNA as a critical component of cellular function. In fact, if we take into consideration the genes that code for certain enzymes, it is intuitive to assume that mutations in these genes can be more easily tolerated, since they do not affect the essential and unique structures as the rRNA (if a bacterium does not have a gene that encodes a specific enzyme necessary for the use of lactose, it may use an alternative sugar or a different protein to obtain energy). Furthermore, few other genes are highly conserved as those encoding the 16S rRNA.

The 16S rRNA is also the target of numerous antimicrobial agents. Consequently, mutations of its sequence may affect the

susceptibility of microorganisms; for this reason the sequence of the 16S rRNA gene can distinguish resistance phenotypes to antimicrobial agents (107). However, these characteristics do not negatively affect the choice of the 16S rRNA gene for bacterial identification or assignment of close phylogenetic relationships at the level of genus or species. They may have a greater impact in the allocation of relations between branches less strongly correlated by a phylogenetic point of view (112).

The 16S rRNA gene has a sequence of approximately 1,550 bp and it is composed of both variable and conserved regions. This gene is large enough, with sufficient polymorphisms to allow distinguishable detections between microorganisms (Figure 3-1)

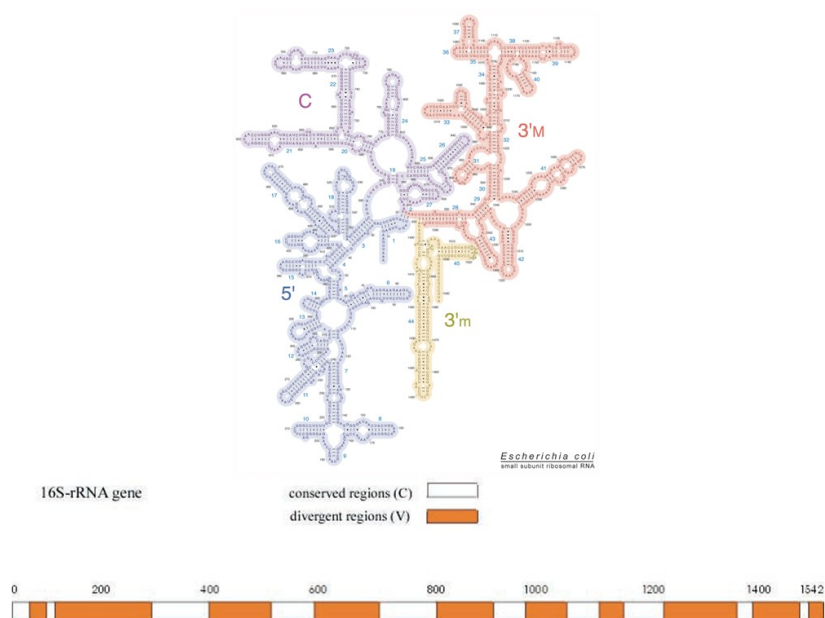


Figure 3-1 16S rDNA is the target of molecular analysis. The use of ribosomal RNA (rRNA) as molecular marker to identify microbial populations is now routinely used in microbial ecology.

Primers used are designed for conserved regions located at the beginning of the gene and region over 540bp or at the end of the entire sequence (approximately 1550bp).

Variable region sequence is used in taxonomic comparison studies (113). Fragments of about 500bp or approximately 1500bp lengths are commonly amplified, sequenced, and compared to the ones contained in the Gene Bank database.

It is the largest database of nucleotide sequences, that has more than 20 million sequences deposited, of which 90.000 are those of 16SrRNA genes.

Many other genomic regions have been used to study the phylogenetic relationships between bacteria. The analysis of the entire genome has been carried out, but it is particularly difficult since the genomes have different amplitude and duplication, deletion, fusion and splitting genes frequently occurs. There are less than 100 genomes for comparative analysis. However, it was observed that phylogenetic trees constructed on the entire genome or the ones based on the 16S rDNA are similar to each other (114).

Song et al. (14) have developed a protocol for rapid identification of 11 species of intestinal lactobacilli isolated from human feces, by a two steps multiplex PCR assay through the designation of

species-specific primers obtained from analysis of 16S-23S and ISR-flanking region of the 23S rRNA sequence. This target shows greater variability compared to the 16S rDNA, that makes it more advantageous to use (115).

3.3 Other phylogenetic markers

Many other genes other than 16S rDNA, are studied to explore the microbial diversity. Some of these genes are universal, possessed by all bacteria, where they perform similar functions. The advantage of using these genes for identification purposes is that they are found in multiple copies in different bacterial species. Some are taxa-specific, and reveal a wider genetic diversity among closely related species. Examples are: the *dsr* gene for sulfate-reducer bacteria, the gene *pmoA* for methanotrophic, *nifH* gene for cyanobacteria (100).

A highly conserved protein such as RecA (small protein involved in homologous recombination of DNA, in SOS response and in induced mutagenesis) has been proposed as an alternative phylogenetic marker in studies of comparative phylogenetic analysis of the genus *Bifidobacterium* and *L. plantarum* (116).

The highly conserved function and the ubiquitous distribution of gene encoding elongation factor Tu (EF-Tu) has made this gene another phylogenetic marker available to eubacteria, it also gave satisfactory results for the identification of enterococcal species (15).

Ventura et al. (15) studied short sequences of different LAB *tuf* genes to perform phylogenetic analysis of many species of lactobacilli and bifidobacteria. They have designed, on the basis of available genomic sequences, species-specific primers for the identification of members belonging to *L. casei* group by a multiplex PCR assay.

3.3.1 Elongation factor Tu and GTPs superfamily

Elongation factor Tu (EF-Tu) is the most abundant protein of the bacterial cell, it is a GTP-binding protein and it plays an important role in protein synthesis. It mediates recognition and transport of aminoacyl-tRNAs and their positioning at the site of the ribosome during translation (116)(15)(117)(118)(119).

EF-Tu belongs to GTPs superfamily, whose members regulate various cellular functions from protein synthesis, cell cycle and differentiation, to hormonal signalling in eukaryotes (120).

Although GTPs carry out a variety of functions, it is believed that all of them derive from a single common precursor. In fact, there are many similarities in all superfamily members: from conservation of action mechanism and core structure, to conserved regions found in the sequences (121).

Bacterial GTPs are numerically and functionally more limited than eukaryotic ones (122). First of all, unlike eukaryotic cells, there is a lack of GTPs signalling in bacterial membrane. The α subunits of heterotrimeric proteins, essential in the transmission of the signal to membrane receptors on eukaryotic cells, are absent in bacteria. In addition, small GTPases subfamilies (Ras, Rho, ARF, Rab and Ran) appear to be absent (123).

The bacteria with larger genomes possess 20-30GTPs, while those with smaller genomes only 11 (124). It is interesting to note that these 11 GTPases are universally and highly conserved among all bacteria phylogenetically related. These include three factors involved in the translation process (EF-G, EF-Tu and IF2) and two GTPases families: FtsY (FtsY and Ffh), Era, THDF/TrmE, Enga, YchF, OBG and LEPA. Phylogenetic analysis studies showed that EF-G, EF-Tu and IF2 belong to the same cluster, while FtsY and Ffh form another distinct one (120).

***tuf* gene**

The elongation factor Tu is encoded by the gene *tuf* in eubacteria and is present in more than one copy per bacterial genome (116) (15).

Many Gram-negative bacteria contain two *tuf* genes (116)(125). In *E. coli* there are two genes with the same sequence, located in different parts of the bacterial chromosome (126) (127). Recently, genome maps have revealed that there is a single copy of the *tuf* gene in *Helicobacter pylori*, in other bacteria (*Borrelia burgdorferi*, *Rickettsia prowazekii*, and *Treponema pallidum*) and in some cyanobacteria (127) (128)(128)(129). Many Gram-positive bacteria (low G+ C content) carry a single copy of *tuf* gene (125)(128)(130). For this reason, *tuf* gene is a good candidate for phylogeny studies (131).

However, Southern hybridization has shown that there are two genes in some clostridia, as *Streptomyces coelicolor* and *Streptomyces lividans* (125).

The *tuf* gene belongs to a large transcriptional unit, the *str* operon, that encodes ribosomal proteins and it is related to many regulatory proteins (132). *str* operon of *E. coli* is composed of 4 genes: *rpsL* (encoding ribosomal protein S12), *rpsG* (ribosomal protein S7), *fus* (elongation factor G) and *tufa* (EF-Tu). The order of these genes in the transcriptional unit is similar to that observed and described in many species, such as *Enterococcus spp.*, *Bacillus subtilis* and *Neisseria meningitidis* (117).

Ventura et al. (15) analysed the *tuf* gene sequences of different LABs to study lactobacilli and bifidobacteria phylogeny. They also have described the positions of *tuf* genes in some species genome, and their transcriptional patterns. They have also designed species-specific primers for *L. casei* group members' identification by a multiplex PCR protocol. The *tuf* gene sequences comparative analysis revealed that there are conserved regions in all strains of the same species, with a good sequence variability between different species. The *tuf* sequences identity between studied lactobacilli ranged from 78% to 98% (reaching a value of 100% for strains of the same species), and from 76% to 100% for the translated products. Many differences among species in nucleotide sequences, proved to be silent in terms of amino acid encoding . (15).

Lactobacilli and bifidobacteria *tuf* genes amino acid sequences alignments with other EF-Tu sequences available in databases have shown that products are highly conserved and their amino acid composition is similar to that found in prokaryotes (133).

Many species of *Lactobacillus* and *Bifidobacterium* have been identified by gene *tuf* comparative analysis and a phylogenetic tree shown in Figure 3 was obtained. The phylogenetic tree constructed

on 16S DNA known sequences (GenBank), have shown a phylogenetic arrangement very similar to that obtained from *tuf* genes sequences. An important feature of phylogenetic study based on *tuf* genes was that *tuf* sequences of *L. delbrueckii* are closely relate with those of *L. acidophilus* group A, while they are phylogenetically more distant from *L. acidophilus* group B. It is also interesting to note that strains strongly related, having almost identical 16S rRNA sequences, such as the *L. casei* group (*L. casei*, *L. paracasei subsp. paracasei*, and *L.rhamnosus*) and the *L. acidophilus* group B (*L. gasseri* and *L.johnsonii*) are clearly divided into separate branches in phylogenetic trees constructed on *tuf* genes (Figure 3-2) (15).

The location of the *tuf* gene, in *L. gasseri* ATCC 33323 and *L. johnsonii* NCC533, is downstream of a gene coding for a beta-lactamase and a *rpsO* gene [ribosomal protein (subunit 30S) S20]; while directly downstream of the *tuf* gene a *tig* (transcription regulator trigger factor) gene is located, followed by a gene encoding for a Clp protease (*clp*). However, only EF-Tu, trigger factor, Clp protease and the GTP-binding protein appear to be highly conserved, corroborating the hypothesis that these genes belong to the same operon. In fact, the trigger factor is a protein associated with ribosomes that interacts with EF-Tu and with a wide variety of nascent polypeptides(144). Clp ATP-dependent proteins are stress-induced and they act on refolding proteins degrading or denatured proteins (134).

Despite substantial gene transfer in prokaryotes world represents one of the main factors responsible for bacterial genome evolution, genes encoding components involved in protein synthesis are highly conserved and they are hardly horizontally transferred (146). However, recent studies have shown a horizontal transfer of the 16S rDNA and some aminoacyl-tRNA synthetases (135)(136)(137). Previous studies have indicated that duplicate copies of *tuf* gene in some bacteria genomes are the result of an ancestral gene

duplication (125). In addition, a study on the *tuf* gene in *R. prowazekii* suggested an intrachromosomal recombination in microorganism evolutionary scenario (127).

4 Antibiotic resistance in lactic acid bacteria (non-enterococcal)

4.1 LAB, Lactic Acid Bacteria

The Lactic Acid Bacteria (LAB) are a group of taxonomically different microorganisms, able to transform fermentable carbohydrates into lactic acid (138). Because of their microaerophilic nature, these bacteria are found in many natural environments; the most representative members are Gram-positive, catalase-negative, low G+C. The lactobacilli, along with the genera *Lactococcus*, *Leuconostoc* and *Pediococcus*, belong to this group (139).

Many species of LAB are involved in production and storage of fermented foods and raw foods (milk, cheese, vegetables and cereals) where they are present as contaminants or they are deliberately added as starters to control fermentation process. In addition, lactic acid bacteria determines organoleptic and rheological properties as well as nutrient requirements of fermented foods (138). By virtue of their historical use in traditional fermented foods, the status of GRAS," Generally Regarded As Safe" was

given to them by the American Food and Drug Agency. Many species of LAB are often found in the microbiota of the gastrointestinal tract and the genitourinary tracts of vertebrates (139), where it is believed that they play benevolent effects on health (140). As probiotics are defined all the non-pathogenic microorganisms that, when ingested in certain quantities, have positively affected the host physiology and health (140). A large number of species is now consumed for maintaining and restoring intestinal microbiota balance, or to counteract the action of dangerous microbial populations, etc. However it should be taken into great consideration potential transfer of antibiotic resistance to pathogenic microorganisms (140) (141).

4.2 Antibiotics resistance: acquisition and dissemination

The antibiotics use has extended to veterinary medicine, where they are used as therapeutic agents, in prophylaxis and as growth promoters, and even agriculture, for control of plant diseases. This extensive use, sometimes excessive, can lead to the selection of resistant strains. In fact correlation between indiscriminate use of antibiotics and emergence of antibiotic resistance, has been repeatedly observed (142).

The development of antibiotic resistance in bacteria is mainly based on two factors: presence of resistance genes and selective pressure generated by use of antibiotics (143).

Resistance to a given antibiotic can be intrinsic to a bacterial species or to a genus (natural resistance): is the ability of a microorganism to survive in the presence of the antimicrobial agent, thanks to the presence of its own peculiar characteristics. Intrinsic resistance is not transferable horizontally. On the contrary, acquired resistance is present in some strains of a species, which is generally susceptible to that particular antibiotic, and can be diffused for horizontal transfer among bacteria. Acquired resistance

can result from mutations in bacterial genome or for acquisition of genes encoding molecules involved in resistance. These genetic changes alter defensive functions of bacteria, resulting in different mechanisms, such as alteration of drug molecular target, membrane permeability alteration, antibiotic enzymatic inactivation (by β -lactamases, aminoglycoside acetyl transferase fosforiltrasferasi, etc.); antibiotic active transport (for example ATP-dependent efflux systems); target modification (for example mono-methylation of the 23S rRNA, mutation of topoisomerase amino acid sequence) (144).

A single gene can select resistance to a particular drug, and simultaneously to same class drugs; for example tetracycline resistance mediated by *tet* (M) includes resistance to oxytetracycline, chlortetracycline, doxycycline and minocycline (145). When there are different classes of antibiotics that act on the same target and one of them is modified by the product encoded by a resistance gene, then cross-resistance between structurally unrelated antibiotics is observed; for example, combined resistance to macrolides, lincosamides and streptogramin B mediated by *erm* genes (146).

Determinants of antibiotic resistance can be transferred both vertically and horizontally between natural microbial communities.

Vertical transmission is mediated by the clonal diffusion of a particular resistant strain. For horizontal transmission, three mechanisms have been identified (147): natural transformation, which involves free DNA uptake and incorporation from extracellular medium; conjugation, a DNA transfer mechanism that entails contact between two cells and transduction, mechanism mediated by bacteriophages. Relative contribution of each mechanism is not known, but conjugation is believed to be the main mechanism of the transfer of antibiotic resistance (1). The reasons that confirm this assertion are different. One of these resides in the fact that many of antibiotic resistance determinants are located on mobile genetic elements such as plasmids and conjugative transposons. A second reason is that conjugation can occur between different species and genera, while transformation and transduction are generally limited to strains of the same species.

Conjugative plasmids are common in lactococci, in *Leuconostoc*, *Pediococcus* genera and in some species of lactobacilli, bifidobacteria while they are quite rare in other species of lactobacilli (147).

R plasmids encoding resistance to tetracycline, erythromycin, chloramphenicol or macrolides-lincosamides-streptogramins, were

found in *L. reuteri* (148)(149) (150) (151), *L. fermentum* (152)(153), *L. acidophilus*(150) and in *L. plantarum* (154)(9), isolated from raw meat and feces. Many R plasmids are sizes less than 10Kb (5,7-18Kb) in size. A strain of *L. fermentum* isolated from pig feces carries a plasmid of 5.7 Kb with an *erm* gene which confers high resistance to erythromycin and has a homology of 98.2% with that found in conjugative enterococcus transposon Tn1545 (152).

R plasmids carrying *tet* (M) were found in lactobacilli isolated from fermented and raw foods (155). The two allelic forms of the gene *tet* (M) show a high sequence homology (> 99.6%) with the gene *tet* (M) previously described in *S. aureus* MRSA101 and in *Neisseria meningitides*.

Recently it has been found in *L. fermentum* a plasmid of 19.3 Kb, which brings a new resistance determinant for erythromycin, *erm* (LF). Genes *tet* (M) in 6 different strains of *Lactobacillus*, not plasmid placed, probably chromosomal were found in the same study. (11).

4.3 Intestinal bacteria as reservoirs of antibiotic resistance

Human intestinal bacteria play a variety of roles for health, many of which benefit the host. Today, thanks to microorganisms' genome characterization, their involvement as "traffickers" of antibiotic resistance genes is emerging. A lot of evidence supports the hypothesis of resistance determinants exchange, not only between same intestinal bacteria, but also between normal microbiota and opportunistic pathogens (1).

4.4 Lactobacilli susceptibility/resistance profiles

Susceptibility pattern of the genera belonging to LAB are species specific (141).

Lactobacilli are generally susceptible to cell wall synthesis inhibitors antibiotics as penicillins (ampicillin and piperacillin) and β -lactamases inhibitors, however, they are less susceptible to oxacillin and cephalosporins (cefoxitin and ceftriaxone) (156)(157).

Cell wall impermeability is the main mechanism of resistance (158). However, cooperation of non-specific mechanisms, such as multi-drug transporters (159) and autolytic cell wall defective systems (160), may explain differences between species. Many species of lactobacilli have high natural resistance to the glycopeptide vancomycin, a characteristic that can be used to distinguish them from other Gram-positive organisms (161)(162).

Such intrinsic resistance to vancomycin is due to the presence of enzymes related to D-alanine/D-alanine ligase (163). Resistant species have dimer D-alanine/D-lactate, in place of D-ala/D-ala dimer in their peptidoglycan (164). However, this glycopeptide resistance in lactobacilli is not comparable to the transmissible one,

plasmid placed, recovered in enterococcal species (165). Susceptibility to the antibiotic bacitracin varies greatly (157)(166). Lactobacilli are generally susceptible to antibiotics that inhibit protein synthesis, such as chloramphenicol, erythromycin, clindamycin and tetracycline and more resistant to aminoglycosides (neomycin, kanamycin, streptomycin and gentamicin) (157) (167) (168). However resistant strains to the above antibiotics (156)(167)(169)(170) were isolated and many resistance determinants have been studied: *cat* gene for chloramphenicol resistance, found in *L. reuteri* (2) and *L. plantarum* (154); different genes *erm* (erythromycin resistance methylase) (171)(152)(149) and *tet* genes (K, M, O, Q, S, W) for tetracycline resistance (145)(172) (173)(174). Lactobacilli are usually resistant to most of the nucleic acids synthesis inhibitors such as enoxacin, pefloxacin, norfloxacin, nalidixic acid, ciprofloxacin, sulfamethoxazole, trimethoprim and cotrimazole (156)(157)(167). Resistance to these antibiotics is, in most cases, intrinsic. High frequency of spontaneous mutations toward nitrofurazone kanamycin and streptomycin was observed for a number of lactobacilli (175). From these data it is clear that there are intergenus and interspecies differences; consequently, species level identification is necessary for data interpretation on

phenotypic susceptibility. In the study by Danielsen and Wind (156) it has been observed that susceptibility to different chemotherapeutic agents can be considered as species-dependent, especially to vancomycin, teicoplanin, tetracycline, norfloxacin, ciprofloxacin, fusidic acid and clindamycin.

In a study by Temmerman et al. (2002), antibiotic resistance of bacterial isolates taken from 55 European probiotic products have been studied. By disc diffusion test about 79% of the isolates (187 strains) were found resistant to kanamycin, 65% to vancomycin; less resistance was observed to tetracycline (26%), penicillin G (23%), erythromycin (16%) and chloramphenicol (11%) (176).

4.5 Antibiotic susceptibility/resistance profiles determination in LAB

Clear definitions of breakpoint values are necessary to discriminate between susceptible and resistant species, they have vital importance in antibiotics phenotypic susceptibility studies. It is also necessary to distinguish between apparently intrinsic resistance (non-transferable) and acquired resistance (177).

In addition to traditional clinical breakpoints, which help clinicians in the choice of antibiotic therapy, concept of "microbiological breakpoints" was introduced by studying the distribution of minimum inhibitory concentration (MIC) for a given antibiotic in a bacterial population (178).

Due to the variety of available methods, there is a lack of agreement about the breakpoints values that define LAB susceptible/resistant to many antibiotics. Different methods for *in vitro*, determination, such as E-test, agar dilution, disc diffusion test, broth microdilution, are the source of confusion, as the results produced can not be directly compared (141). In addition, some susceptibility testing are not feasible for certain antibiotics. For example, disk diffusion test cannot be used to detect enterococci with low levels of resistance to

vancomycin (MIC values from 8 to 32 η g/mL) (179). Furthermore, the culture medium can influence susceptibility testing results to certain antibiotics (180). Variations in content or in concentration of cationic compounds is critical, such as thiamine or folic acid, inoculation, temperature, incubation period, etc.. can change the results. Finally, many species of LAB grow poorly in the common medium used such as Mueller-Hinton or Isosensitest; the MRS medium can also inactivate certain antibiotics, such as imipenem (141).

5 Quinolones

5.1 Structure and classification

Quinolones are a family of chemotherapeutic agents in continuous and progressive evolution. Their basic chemical structure is that of 4-quinolones, ie azaquinolones, with a carboxyl group in position 3 (Figure 5-1).

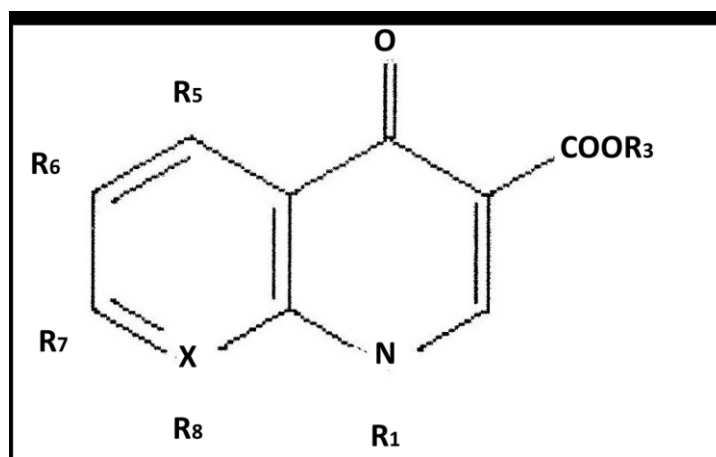


Figure 5-1 "4-quinolone" (4-oxo-1,4-dihydroquinolone)

They were discovered in the late fifties during synthesis process and purification of chloroquine (antimalarial agent). Repeated chemical modifications of this lead compound allowed the obtainment of structural analogues that were assayed as antibacterials, thus arriving to the discovery of nalidixic acid (Figure 5-2).

This compound, with naphthyridinic structure, was the founder of the quinolone family.

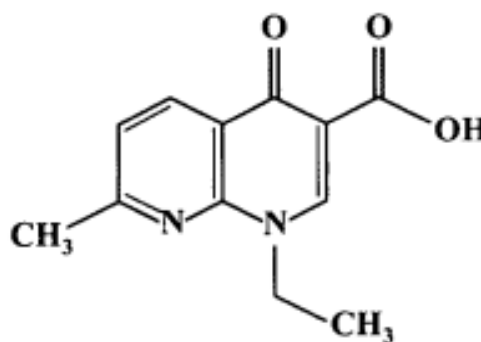


Figure 6-2 Nalidixic Acid

Introduced in therapy in 1962, the compound showed only a modest activity on Gram-negative and low oral absorption, with a peak plasma level of less than 0.5 mg/L. Their clinical use was limited to the treatment of urinary tract infections (UTIs) (181).

Structural modifications on first-generation quinolones have brought a target activity spectrum expansion: in the sixties and seventies there was the development of a series of similar second-generation quinolones. Marginal structural modifications have been carried out obtaining the development of the first and second-generation compounds, such as oxolinic acid (quinoline nucleus) and the cinoxacin (with cinnolonic nucleus). Better clinical results about activity expansion on Gram negative, including anti *Pseudomonas* activity, have been achieved with the replacement of a piperazine ring at R7, leading to the development of various compounds such as pipemidic acid (structure pyrido [2,3 -d] pyrimidine) and

pyromidic acid (structure pyrido [2,3-d] pyrimidine). Despite the spectrum of action enlargement, clinical use of second-generation quinolones has remained limited because of their poor systemic bioavailability and renal toxicity risk (182)(183).

Third generation Quinolones are characterized by the insertion of a fluorine atom in position R6, leading to the classification of the successive compounds such as fluoroquinolones (184).

Inclusion of a fluorine atom has caused an exceptional antibacterial activity enhancement, while the addition of a piperazine ring in position 7 has improved the capacity of penetration at tissue level. The clinical use of fluoroquinolones is expanded in ophthalmic field by reason of a good level of activity against Gram positive, achieved with the fluorination, together with good solubility observed in ophthalmic preparations.

Norfloxacin was the first quinolone used in management of ocular infections, for treatment of bacterial conjunctivitis (181). Norfloxacin shows anti *Pseudomonas* and Gram-negative bacilli activity, while limited activity towards susceptible Gram-positive bacteria (185). Replacement of a carbon atom in place of a nitrogen (linked to R8 chain in Figure 6) and changes in R1 and R8 have led to the development of several third generation compounds.

Addition of a cyclopropyl ring at R1 position, has led to the development of ciprofloxacin; addition of a ring with six atoms (piridobenzossazine) between positions R1 and R8 has led to ofloxacin (184). Both compounds show good activity on susceptible Gram-negative bacteria and extended activities towards Gram-positive microorganisms frequently associated with eye infections. Ofloxacin has activities also on anaerobic microorganism such as *Propionibacterium acnes* (186). Levofloxacin, ofloxacin active enantiomer, has better activity towards Gram-positive bacteria, including *S. pneumoniae* and *S. viridans* (182)(186).

Their extended spectrum of action has selected these compounds as first choice antibacterial agents in topical therapy and for the treatment of infections such as bacterial keratitis (181).

Development of resistance to third generation fluoroquinolones, among pathogens associated with eye infections, especially among Gram-positive microorganisms, has led to research and development of new compounds (186). Addition to a methoxy group in R8 side chain, has led to the development of fourth-generation fluoroquinolones, gatifloxacin and moxifloxacin. Moxifloxacin has also a bicyclic ring constructed on R7 position (182); gatifloxacin has a methyl group on piperazilidic ring. These

changes appear to be responsible for the dual mechanism of action (inhibitory activity on bacterial DNA topoisomerase gyrase and topoisomerase IV) in Gram-positive bacteria, in addition to a reduced efflux from bacterial cells. Activity spectrum includes *Streptococcus* strains and *Staphylococcus* third-generation quinolone-resistant (186). Methoxy group is responsible for the activity of these compounds towards anaerobic microorganisms (Figure 5-3) (182).

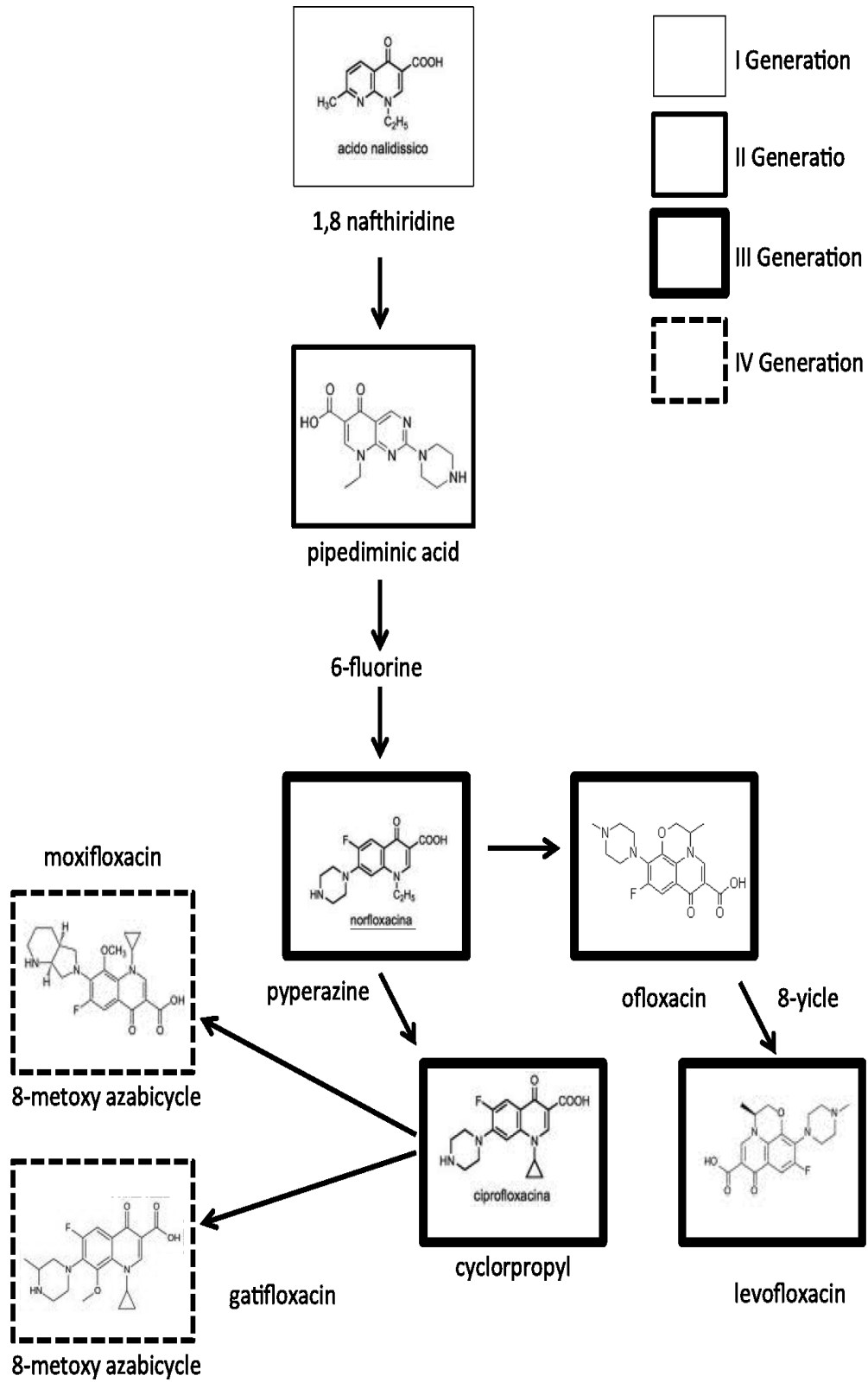


Figure 5-3 Structural evolution of quinolones

In Italy in 2004, a new fluoroquinolone, prulifloxacin was marketed. Rapidly absorbed after oral administration, prulifloxacin undergoes through first-pass hepatic metabolism becoming its active metabolite ulifloxacin. In urinary tract infections treatment, this pro drug is more effective than ciprofloxacin, enoxacin, norfloxacin and amoxicillin/clavulanic acid, also does not modify normal composition of vaginal microbiota in healthy women (187).

5.2 Ciprofloxacin

Ciprofloxacin [acid 1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinyl)-3-quinoline-carboxylic acid] was introduced in therapy at the end of the eighties. nalidixic acid structure changes, concerning pharmacophoric groups, improved both antibacterial activity and pharmacokinetic properties. They are:

- Change in naftiridinic structure (binding to plasma proteins is reduced);
- Introduction of a fluorine atom in position 6, (activity of ciprofloxacin towards DNA gyrase is increased and its entrance inside the bacterial cell is enhanced);
- Piperazine ring introduction in position 7 (half-life is longer);
- Ethyl group in N-1 replacement with a cyclopropyl group (potential power towards Gram-positive and Gram-negative is increased) (188).

Ciprofloxacin is active against many Gram-negative and Gram-positive cocci, and it has anti-*Pseudomonas* activity. It is also active against *Acinetobacter spp.*; Very active on *H. influenzae*, *Moraxella catarrhalis*, *Neisseria spp.* including strains of *N. gonorrhoeae* beta-lactamase producers. After oral administration, is widely

distributed in body fluids and tissue concentrations and phagocytes it is comparable to those in plasma. Adverse reactions are rare, and if they are represented, they are manifest by disorders of the gastrointestinal tract (approximately 3.4%), and rash (<1%). CNS disorders, typical of quinolones, have been reported in approximately 1% of patients treated (189).

It has proven effective in treating a wide range of infections, including UTIs, osteomyelitis caused by *Enterobacteriaceae*, gonococcal infections and chronic bacterial prostatitis (189).

5.3 Levofloxacin

Levofloxacin is the L-isomer of the fluoroquinolone ofloxacin. *In vitro* studies have demonstrated a spectrum of activity extended to Gram-positive, Gram-negative and anaerobic bacteria. This drug is more active against Gram-positive than ciprofloxacin, but less active when compared to fourth-generation fluoroquinolones gatifloxacin. Its activity against *S. pneumoniae* and is not influenced by the presence of penicillin-resistance exhibited by these microorganisms.

Levofloxacin inhibits both bacterial target of quinolones, ie DNA gyrase and topoisomerase IV. Depending on the type of bacterium, primary target may be one or the other enzyme.

Levofloxacin is generally well tolerated in clinical trials. Main adverse effects are usually transient and moderate in severity. The most common are: nausea (1.3%), diarrhea (1.1%), vaginitis (0.7%), pruritus (0.5%), flatulence (0.4%), rash (0.4%), etc. (190)(191).

5.4 Fluoroquinolones mechanism of action

Fluoroquinolones inhibit two enzymes involved in bacterial DNA synthesis; they are both type 2 topoisomerase that are absent in eukaryotic cells. They are essential in bacterial DNA replication (181)(192). DNA topoisomerases are responsible for double helix separation, insertion of a filament breakage and, subsequently, in reconstitution of filaments originally separated (193). Specifically, fluoroquinolones inhibit DNA gyrase and topoisomerase IV.

5.4.1 DNA gyrase and topoisomerase IV

Structure

DNA gyrase is a tetramer consisting of two A subunits (about 97KDa) and two B subunits (about 90KDa), encoded by *gyrA* and *gyrB* genes. In the genome of *E. coli*, *gyrA* and *gyrB* are located at 48 and 83 min, but in some bacteria are adjacent to each other and close to *OriA*; this configuration is known as QRDR (Quinolone Resistance Determining Region) (194)(195).

Topoisomerase IV is DNA gyrase homologous, it comprises four subunits, two C and two E, encoded by the genes *parC* and *parE*. Topoisomerase IV *locus* has been described for the first time in 1990. In *S. aureus*, FLQ locus, today reported as GRLA, is

equivalent to *parC* of other bacteria such as *S. pneumoniae*. In *E. coli*, the locus *nfxD* is now recognized *parE*.

In *E. coli*, *gyrA* gene has 36% identity and 60% similarity with *parC*, in amino acid sequence; genes *gyrB* and *parE* instead have 42% identity and 62% similarity (188).

Function

DNA gyrase and topoisomerase IV are important in cellular functions such as DNA replication and repair, recombination and transcription of some operons, but also are essential in maintaining bacterial DNA supercoiled form.

The DNA gyrase is a bacterial enzyme that facilitates exclusively the conformational changes of DNA during replication, in particular it removes supercoiling (superhelix positive) that accumulates on replication fork, or as a result of transcription of some gene (192). It Introduces negative supercoiling (superhelix negative, one every 15-20 turns of the DNA helix) so that DNA can clog in to the cell, facilitating initiation of replication (196)(197). Energy required to achieve supercoiling is made available by ATP hydrolysis (ATP-dependent reaction), a process that is involved in gyrase B subunit. ATP binding domain site is on N-terminal end of B subunit (188). On localized sites on the subunit A occur DNA binding, its

breakage and interaction between two subunits. The tyrosine residue in 122 At GyrA N-terminal participates in breakage-reunion DNA reaction and it is considered DNA gyrase active site (197).

The topoisomerase IV acts in terminal stages of replication, leading to daughter chromosomes separation in daughter cells. It removes chains created by replication and thereby breaks DNA strands before completing replication round, unlike gyrase that breaks DNA two strands only after replication round is completed.

Ternary complex formation

It is thought that fluoroquinolones forming bonds, both with DNA and enzymes, to form a ternary complex quinolone-gyrase/topoisomerase IV-DNA (Figure 5-4); formation of such a complex would be responsible for DNA replication inhibition by hindering duplicative apparatus movement along the replicative fork, forming a DNA-topoisomerase irreversible complex. Bactericidal effect itself is result of bacterial growth alteration and subsequent cell lysis. This could be linked to SOS response which represents a adaptation reaction to DNA molecular damage, followed by different exonucleases derepression that activate SOS genes, whose expression is responsible for the arrest of cell

division. At higher concentrations, cell death is also due to DNA release from quinolone-gyrase and/or topoisomerase IV complex. Lastly, molecule interaction with quinolone DNA-gyrase complex, would give rise to a series of lethal phenomena for bacterium, such as a rapid inhibition of DNA replication, growth arrest and errors in genome system recovery (188).

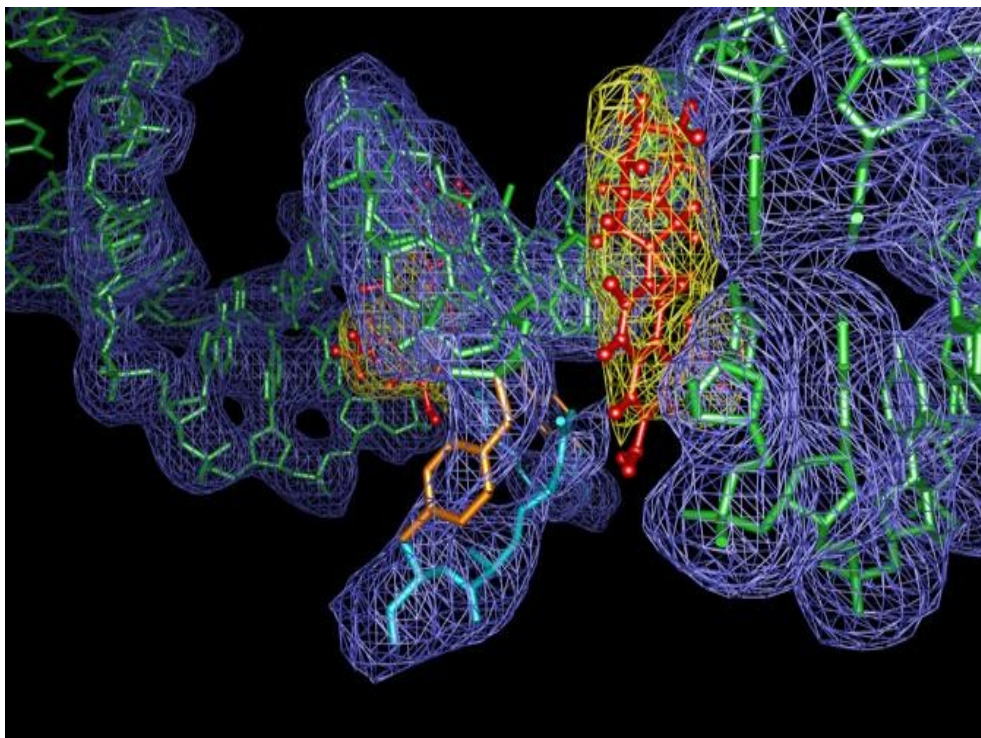


Figure 5-4 Ternary complex formation

6 Mechanisms of resistance

Antimicrobial agents must enter in cells to find their target, destroy cell function and eliminate infecting organisms to achieve therapeutic success. There are many mechanisms, chromosomal placed or plasmid-encoded, which help to define phenotypes of drug-resistance in certain infecting organisms. Potential mechanisms are:

- Reduced drug accumulation, for transport failure into the cell or for efflux pump systems activation, that expel it out of the cell;
- Pharmacological target alteration, for target mutation or overexpression
- Enzymatic drug inactivation that modifies it, inactivating it, or degrading it.

A micro-organism resistant to a given drug has at least one of the above-mentioned molecular mechanisms (188).

6.1 Fluoroquinolones resistance

Molecular target Alterations by DNA alteration of gyrase and/or topoisomerase IV, and reduced intracellular accumulation (for reduced permeability and/or increased activity of efflux), are the main mechanisms of resistance to fluoroquinolones (Figure 6-1) (181)(192)(194)(198).

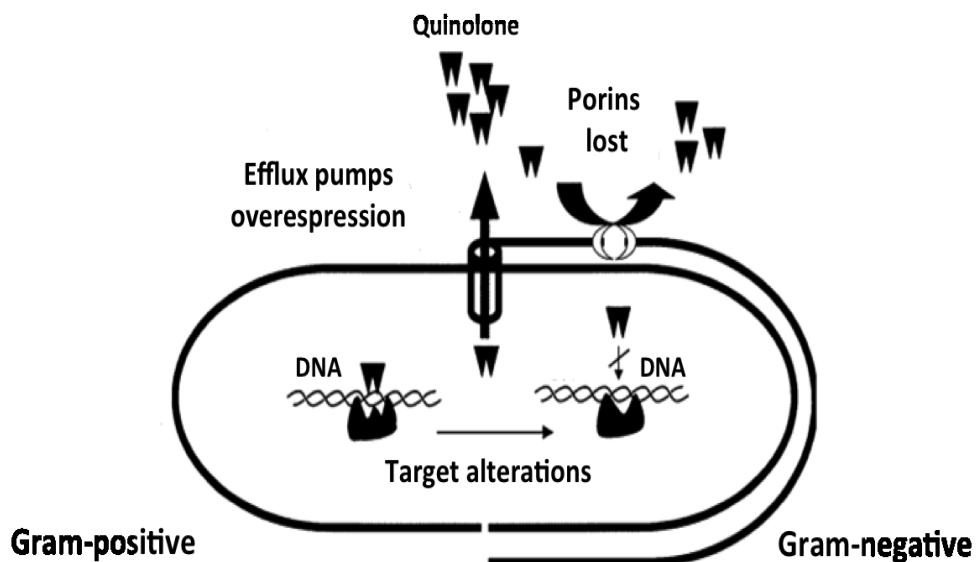


Figure 6-1 Representation of molecular resistance to quinolones mechanisms

Both mechanisms are mediated at chromosomal level. Nevertheless, movable elements carrying the *qnr* gene that confers resistance to quinolones have been described (181).

6.1.1 Target Alteration

Despite the fact that quinolones act against DNA gyrase and topoisomerase IV, they differ in activity against these enzymes. In recent studies on *E. coli*, DNA gyrase proved to be quinolone primary target (199); activity on topoisomerase IV seems to be limited, therefore represents a secondary target (199). Later, studies of *S. aureus* have revealed that the topoisomerase IV is the preferred target in such Gram-positive microorganisms (192)(200). In addition, studies performed in *E. coli* and *S. aureus* have shown that different levels of resistance to quinolones, depend on primary target or secondary target alterations. Mutations in the primary target precede those of the secondary, selecting resistance strains; mutations in both targets lead to high levels of resistance. Blanch et al. (201) studied several fluoroquinolones (ciprofloxacin, sparfloxacin, ofloxacin and norfloxacin) inhibitory activity against purified DNA gyrase and topoisomerase IV in strains of *E. coli* and *S. aureus*. Regarding *E. coli*, quinolone concentration required the inhibition of 50% of DNA gyrase activity (IC₅₀) was between 0.5 g/mL and 1.5 g/mL; on the contrary, that required to inhibit topoisomerase IV was higher (from 2 g/mL to 12 micrograms/mL).

In *S. aureus*, instead, topoisomerase IV was much more susceptible to fluoroquinolones compared to DNA gyrase: IC50 values ranged between 4-10 micrograms/mL and 12-100 g/mL, for topoisomerase IV and DNA gyrase, respectively. For both microorganisms, MIC values observed were closer to IC50 values for the most susceptible enzymes, suggesting that MIC is determined predominantly on inhibitory effects on the primary target.

In Gram-negative bacteria, such as *E. coli*, *gyrA* subunit is the primary target, in Gram-positive bacteria such as *S. aureus*, is *parC* subunit of topoisomerase IV.

DNA gyrase Alterations

Many mutations observed in *gyrA* are localized in a small terminal region of GyrA protein (residues 67-106 in *E. coli*), called QRDR (Quinolone Resistance Determining Region)(202), near the residue Tyr122 that binds cleaved DNA. A similar QRDR was found in *parC*.

Mutations in codons 67, 81, 82, 83, 84, 87 and 106 of *gyrA* were considered responsible for resistance in *E. coli* (Table 6-1) (203). Recently, codon 51, a region outside QRDR, has been proposed as a novel mutation that causes quinolones decreased susceptibility (194).

Presence of a single mutation in the above-mentioned positions of *gyrA* QRDR, generally, determines a high level of resistance to nalidixic acid; to obtain equally high levels of fluoroquinolones resistance, an additional mutation in *gyrA* and/or *parC*, is required.

Most frequently observed mutation in quinolone-resistant *E. coli* strains, is the one that affects *gyrA* codon 83. However, this mutation was also found in quinolone-resistant isolates belonging to *Enterobacteriaceae*, such as *Citrobacter freundii* and *Shigella spp.* or pathogens such as *Neisseria gonorrhoeae* or *Acinetobacter baumannii* (194).

The second most observed mutation in clinical isolates is at codon 87 of *gyrA*. Strains with a double mutation in codons 83 and 87, exhibit quinolones increased MICs. This is true for other Gram-negative microorganisms, such as *C. freundii*, *Pseudomonas aeruginosa* or *N. gonorrhoeae*. Substitutions at equivalent positions to those mentioned above, have been frequently observed also in Gram-positive microorganisms (194).

The mutations (Table 6-1) observed in *E. coli gyrB* are on positions 426 and 447. Substitution at position 426 appears to confer resistance to all quinolones, while that in the position 447 leads to an increased level of resistance to nalidixic acid, and at a higher

susceptibility to the fluoroquinolones. Mutations in equivalent positions have been described for other Gram-positive microorganisms (194).

Table 6-1 Mutations described in *gyrA* and *gyrB* subunit of quinolone-resistant *E. coli* strains (198)

	Codon	Original aa.	Mutation described
<i>GyrA</i>			
	51 ^b	Ala	Val
	67 ^b	Ala	Ser
	81	Gly	Cys, Asp
	82 ^b	Asp	Gly
	83	Ser	Leu, Trp, Ala, Val
	84	Ala	Pro, Val
	87	Asp	Asn, Gly, Val, Tyr, His
	106 ^b	Gln	Arg, His
<i>GyrB</i>			
	426	Asp	Asn
	447	Lys	Glu

In Table 6-2 mutations observed in *gyrA* of different Gram-positive and Gram-negative (198) are reported; in Table 6-3 mutations observed in *gyrB*.

Table 6-2 Mutations in *gyrA* observed in Gram-positive and Gram-negative(198)

Microorganism	aa. sostitution ^(*)
<i>Acinetobacter baumannii</i>	Gly81→Val Ser83→Leu Glu87→Gly
<i>Aeromonas salmonicida</i>	Ser83→Ile; Ala67→Gly
<i>Campilobacter fetus</i>	Asp91→Tyr
<i>C. jejuni</i>	Ala70→Thr Thr86→Phe, Lys Asp90→Asn
<i>C. lari</i>	Thr86→Ile Asp90→Ala, Asn Pro104→Ser
<i>Enterobacter cloacae</i>	Ser83→Leu, Tyr, Phe Asp87→His, Gly, Val, Ala, Asn
<i>E. faecalis</i>	Ser83→Ile, Ang, Asn Glu87→Gly
<i>Haemophilus influenza</i>	Ser84→Leu, Tyr Asp88→Asn, Tyr
<i>Helicobacter pylori</i>	Asn87→Lys Ala88→Val Asp91→Gly, Asn, Tyr, Asn, Val
<i>K. pneumonia</i>	Ser83→Tyr, Phe Asp87→ Gly, Asn, Ala
<i>Mycobacterium avium</i>	Ala90→Val
<i>M. smegmatis</i>	Ala90→Val Asp94→Gly
<i>M. tuberculosis</i>	Gly88→Cys Ala90→Pro, Val Ser91→Pro Asp→Asn, His, Gly, Tyr, Ala
<i>Neisseria gonorrhoeae</i>	Ser83→Phe; Asp87→asn Ser91→Phe, Tyr, Phe Asp95→Asn, Gly; Ala75→Ser
<i>Pseudomonas aeruginosa</i>	Thr83→Ile Asp87→Tyr, Asn, Gly, His
<i>Shigella dysenteriae</i>	Ser83→Leu
<i>Serratia marcescens</i>	Ser83→Arg Asp87→Tyr
<i>Streptococcus pneumonia</i>	Ser84→Tyr, Phe Glu88→Lys
<i>Salmonella enteritidis</i>	Ser83→Phe
<i>S. hadar</i>	Ser83→Phe
<i>S. typhi</i>	Ser83→Phe
<i>S. typhimurium</i>	Ala67→Pro Gly81→Ser Ser83→Phe, Tyr, Ala Asp87→Gly, Tyr, Asn Ala119→Glu
<i>Staphylococcus aureus</i>	Asp73→Gly Ser84→Leu, Ala, Phe, Val Ser85→Pro Glu88→Gly, Lys
<i>S. epidermidis</i>	Ser84→Phe
<i>S. haemolyticus</i>	Ser84→Leu

(*)The position of the codon is based on the sequence of *E. coli*

gyrA

Table 6-3 Mutations in *gyrB*(198)

Microorganism	aa. sostitution
<i>Staphylococcus aureus</i>	Asp437→Asn Arg458→Gln Pro456→Ser
<i>Streptococcus pneumponiae</i>	Glu474→Lys
<i>Salmonella typhimurium</i>	Ser464→Tyr

Topoisomerase IV Alterations

In *E. coli parC* gene, the most frequent substitutions occur at codons 80 and 84 (Table 6) (194).

Table 6-4 Described mutations in *parC* and *parE* in strains of *E. coli* quinolone-resistant

Codon	Original aa.	Mutation described
<i>ParC</i>		
78	Gly	Asp
80	Ser	Ile, Arg
84	Glu	Lys, Val, Gly
<i>parE</i>		
445	Leu	His

Another substitution has also been described in *E. coli* (Gly78 → Asp) both in clinical isolates that in mutant strains obtained *in vitro* (211). The substitution Asp79 → Ala in *parC* gene in mutant strains of *Shigella flexneri* has been described. Other substitutions in the same codon for other microorganisms both Gram-negative (such as *Haemophilus influenzae*: Asp79 → Asn) and Gram-positive bacteria (*Streptococcus pneumoniae*: Asp79 → Asn) were observed. However, other mutations in *gyrA* or *parC* were observed at the same time (194).

A mutation described in only GRLA strains of *S. aureus*, concerns codon 116 (Ala116 → Glu or Pro). Other mutations found in this organism affecting codons 23 (Lys23 → Asp), 69 (Asp69 → Tyr), 176 (Ala176 → Gly) or 451 (Pro451 → Gln). However, the real effect they have on susceptibility to quinolones has not yet been determined.

The role of substitutions that occur in *parE*, in quinolone-resistance diffusion of Gram-negative microorganisms, seems irrelevant. In fact, only the replacement Leu445 → His has been described in *parE* of a single *E. coli* mutant strain. Changes in this subunit have been described in both clinical isolates and in quinolone-resistant strains obtained in laboratory. For example, in *S. pneumoniae*, mutations are observed Asp435 → Asn and His102 → Tyr, in *S. aureus* Pro25 → His, Glu422 → Asp, Asp432 → Asn or Gly, Pro451 → Ser or Gln and Asn470 → Asp.

Tables 6-5 and 6-6 show the mutations observed in *parC/parE* GRLA and/*grlB*, respectively, for microorganisms other than *E. coli* (Table 7).

Table 6-5 *parE/grlB* mutations

Microorganism	aa sostituitioin
<i>Streptococcus pneumoniae</i>	Asp435→Asn Pro454→Ser
<i>Staphilococcus aureus</i>	Asp432→Val Asn470→Asp

Table 6-6 *parC/grlA* mutations

Microorganism	Aa sostituitioin
<i>Acinetobacter baumannii</i>	Ser80→Leu Glu84→Lys
<i>Enterobacter cloacae</i>	Ser80→Ile Glu84→Gly, Lys
<i>Enterococcus faecalis</i>	Ser80→ Ang, Ile Glu84→Ala
<i>Haemophiule influenza</i>	Ser84→Ile Glu88→Lys
<i>Klebsiella pneumonia</i>	Ser80→Ile, Ang Glu84→Gly, Lys
<i>Neisseria gonhorroaeae</i>	Asp86→Asn Ser87→Ile Ser88→Pro Gly85→Cys Glu91→Gly Ang116→Leu
<i>Pseudomonas aeruginosa</i>	Ser80→Leu Glu 84→Lys
<i>Staphilococcus aureus</i>	Ser80→Phe, Tyr Ser81→Pro Glu84→Lys Ala116→Pro, Glu
<i>Streptococcus pneumoniae</i>	Ser79→Tyr, Phe Asp83→Gly Asp84→His Ser80→Tyr

6.1.2 Are there alterations of target in quinolone-resistant lactobacilli?

To date, there are only few papers (181)(182)that characterized genetic resistance to ciprofloxacin in quinolone-resistant lactobacilli.

In Hummel et al. study (2007) (17), mutation was detected in the QRDR of *gyrA* and *parC*: substitution Glu87 → Leu in GyrA in a strain of *L. acidophilus* BFE 7429 and mutations in position 74, 84 and 88 in relative ParC. These substitutions have not, however, been associated with the increase of quinolone-resistance in other microorganisms.

In Fukao et al study (2009) (12), the QRDR of *gyrA* and *parC* of a probiotic strain, *L. brevis* KB290 used since 1993 in Japan in fermented foods and freeze-dried powders have been sequenced. No mutation was observed. These findings have led scholars to assert that moderately high levels of resistance to ciprofloxacin may result from intrinsic connotation, like cell wall structure, its permeability or efflux systems.

6.2 Decreased uptake

The decreased uptake of quinolone may be associated with two factors: a membrane increase impermeability or an efflux pumps overexpression.

To reach their target in cytoplasm, fluoroquinolones must cross cytoplasmic membrane and, in Gram-negative bacteria, also the outer membrane. They are however sufficiently small, such as to be able to cross these barriers in two different ways: through specific porins (proteins which form channels of diffusion) or by diffusion in phospholipid bilayer. A low drug uptake can be attributed to its hydrophobicity level: only quinolone with a high index of hydrophobicity can easily cross cell membrane, by passive diffusion, while the less hydrophobic ones must use porins. Therefore, alterations in porins content or altered lipopolysaccharide membrane composition, can alter quinolones susceptibility profile (194)(200)

6.2.1 Decreased expression of porins

The alteration of membrane permeability is mostly associated with porins decreased expression. This has been widely described in *E. coli* and other Gram-negative microorganisms (194).

E. coli outer membrane has three major porins (OmpA, OmpF OMPC). OmpF decreased expression is related to some quinolones increasing resistance, but does not affect others MIC values, like the one for tosufloxacin and sparfloxacin. However, its reduced expression leads to reduced susceptibility to other antibacterial agents such as beta-lactams, tetracyclines and chloramphenicol (194).

Some chromosomal *loci* as *marRAB* (consisting of three genes: *marR* that encodes a repressor protein, MarA which encodes a transcription activator and MarB which encodes a protein whose function is unknown) or SoxRS (this operon encodes two proteins, SoxR, a regulatory protein, and SOXS, transcription activator) regulate both OmpF expression levels in *E. coli* (194).

It was also shown that chloramphenicol, tetracycline and other substrates such as salicylate, can induce MarA expression, with a consequent increase in antisense regulator expression *micF*, which

induces a post-transcriptional repression in OmpF synthesis. *micF* expression can also be adjusted by SoxRS operon (194).

Outer membrane composition of some microorganisms like *A. baumannii* or *P. aeruginosa*, has been associated with their intrinsic resistance to quinolones. *A. baumannii* wild type strains have MIC for ciprofloxacin that fall in the range 0.125-1 $\mu\text{g/mL}$. In contrast, wild-type strains of *E. coli* have lower MIC range (0.007-0.25 $\mu\text{g/mL}$). This result is interpreted admitting that it is intrinsic resistance or it has an efflux pumps overexpression. *P. aeruginosa* outer membrane has a non-selective permeability to small hydrophobic molecules, thus justifying quinolones intrinsic resistance.

6.2.2 Efflux Pumps

In both Gram-positive and Gram negative microorganisms, the resistance caused by a reduced intracellular accumulation of antibiotic is due to an overexpression of efflux pumps that transport drug outside of bacterial cell. These outflow systems, may be drug-specific, caring only one type of antimicrobial agent, or non-specific, accommodating a wide range of antimicrobials. They can

be expressed in cell or constitutively or be controlled by regulatory systems or induced by mutations. Antimicrobials efflux systems have been grouped into five superfamilies on the basis of amino acids homologous sequences: Major Facilitor Superfamily (MFS), ATP-Binding Cassette (ABC), Resistance-Nodulation-Division (RND), Small Multidrug Resistance (SMR), drug/metabolite transporter (DMT), and Multidrug And Toxic Extrusion (MATE) superfamily (159). While ABC-type transporters are primary active transporters and use ATP as an energy source, transporters SMR, RND, MFS and probably also MATE are secondary active (204)(205).

In Gram-negative bacteria these pumps are formed by three structural components: a conveyor in the cytoplasmic membrane, a porin in the outer membrane protein level and a linker that connects the two. In Gram-positive bacteria, given the absence of an outer membrane, system outflow consists only of a cytoplasmic pump (206)(Figure 6-2).

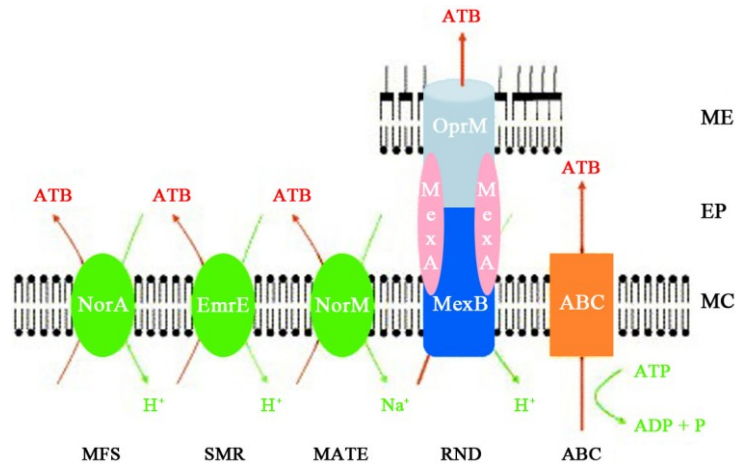


Figure 6-2 efflux pumps ATB = Substrate pump, ME = external membrane EP = periplasmic Space, MC = cytoplasmic membrane (207)

ABC (ATP-binding cassette)

All this superfamily members are derived from a single ancestral gene encoding a membrane transporter, therefore, all ABC transporter proteins have similar organization of domains. There are four distinct domains: two of these are Trans Membrane Domains (TMD), they consist of 5-10 alpha-helices that span membrane forming the channel through which substrate is pumped out and they also contain substrate binding site (208). The other two domains, Nucleotides Binders Domain (NBD) are localized in cytoplasm and they are closely associated with each other, as well as with trans membrane domains. TMD and NBD domains can be expressed as individual polypeptides, or may exist in a variety of fused domains. These systems are generally capable of causing

resistance to several structural types of compounds and therefore are referred to as multidrug resistance (MDR). MDR transporters may be formed by all four domains (two TMD and two NBD) or by a pair of TMD and NBD. MDR substrates are very varied and each pump can interact with hundreds of very different structure substrates. ATP binding and hydrolysis in NBD site induces conformational changes in coupled TMD allowing substrate transport (208). They seem to be present in many if not all bacteria and pathogens such as, *E.coli*, *P.aeruginosa*, *S.aureus*, and *S.pneumoniae*. In *E. coli*, AcrAB-TolC plays an important role in quinolones extrusion and it has multiple controls. Mutations in *acrRR* gene (repressor of *acrAB*) increase pump activity.

In contrast, mutations that inactivate *marR* (a repressor of *marA*) cause a drug influx reduction and increase in its outflow.

Table 6-7 Efflux sistem (208)

Microorganism	Efflux sistem	
Gram-negative		
<i>A. baumannii</i>	AdeABC	
<i>C. jejuni</i>	CmeABC	
<i>E. coli</i>	AcrAB	MdfA
	AcrEF	YdhE
	EmrAB	
<i>P. aeruginosa</i>	MexAB-OprM	MexEF-OprN
	MexCD-OprJ	MexxY-OprM
<i>S. maltophilia</i>	SmeDEF	
<i>Vibrio colera</i>	VceAB	
<i>Vibrio parahaemolyticus</i>	NorM	
Gram positivi		
<i>B. subtilis</i>	Blt	Bmr3
	BmrA	
<i>S. aureus</i>	NorA	
<i>S. pneumoniae</i>	PmrA	

MFS (Major Facilitator Superfamily)

In Gram-positive efflux pumps best characterized by the extrusion from the cell of fluoroquinolones belong to Major facilitator Superfamily, a large and diverse group of secondary transporters that includes uniport, symport and antiport. They are generally formed from 400-600 a.a., and the analysis of the profiles of hydrophobicity and the alignment of the conserved domains allowed to divide this superfamily into two sub-families: the pumps to 12 transmembrane segments (TMS) and pumps with 14 transmembrane segments (207) (Table. 6-8). Both ends (N and C) of these proteins present analogies, probably due to duplication or partial melting of the genes at the chromosomal level. The MFS proteins facilitate transport through the cytoplasmic membranes of a wide variety of substrates, including ions, phosphorylated sugars, antimicrobials, neurotransmitters, nucleosides, a.a. and peptides, using the electrochemical potential of the substrates transported (207).

Table 6-8 MFS efflux pumps involved in antibiotics resistance(207)

Pumps	MFP/OMF^a	Microorganisms	Antibiotics/substrate^b
12 transmembrane segments MFS (12 TMS)			
BcrA		<i>B. cepacia</i>	TC, Nal
Blt		<i>B. subtilis</i>	FQ
Bmr		<i>B. subtilis</i>	C, FQ
CmlA		<i>P. aeruginosa</i>	C
EmeA		<i>E. faecalis</i>	FQ, Ery, L, Nov
Lde		<i>L. monocytogenes</i>	FQ
LmrP		<i>L. lactis</i>	TC, M14, 15, L
MdfA		<i>E. coli</i>	C, TC, AG, Ery, Rif, FQ
MdrL		<i>L. monocytogenes</i>	Ctx, ML
MefE		<i>S. pneumoniae</i>	M14, 15
NorA		<i>S. aureus</i>	C, FQ
PmrA		<i>S. pneumoniae</i>	FQ
MefA		<i>S. pyogenes, S. agalactiae,</i> oral streptococci, C e G streptococci	M14, 15
Tap		<i>M. fortuitum, M.</i> <i>tuberculosis</i>	TC, AG
TetA-E		Enterobacteria, <i>Pseudomonas, Vibrio,</i> <i>Aeromonas...</i>	TC
TetH		<i>Pasteurella, Mannheimia</i>	TC
14 transmembrane segments MFS (14 TMS)			
EmrBc	EmrA/ TolC	<i>E. coli</i>	Nal
MdeA		<i>S. aureus</i>	QAC, Vir, Novo
QacA, B		<i>S. aureus</i>	QAC
TetK-L		<i>Staphylococcus, Streptococcus,</i> <i>Enterococcus, Listeria,</i> <i>Bacillus, Clostridium,</i> <i>Mycobacterim</i>	TC
VceB	VceA	<i>V. cholerae</i>	Nal

a MFP (membrane fusion protein) and OMF (outer membrane factor).

b Atibiotics : AG : aminoglycosides ; Amp : ampicillin ; Azi : azithromycin; β L : beta-lactam; β L# : beta-lactams except carbapenems; C : chloramphenicol ; C4G : cefepime, Cefpirome ; Carb : carbenicillin ; Cip : ciprofloxacin ; Ctx : cefotaxime ; Ery : erythromycin; FQ : fluoroquinolonese ; Fus : fusidic acid; Gm : gentamicin ; Ipm : imipenem ; L : lincosamides ; M14,15 : 14 or 15 atoms macrolides; ML : macrolide-lincosamide ; Nal : nalidixic acid; Nor : norfloxacin ; Nov : novobiocin; Pen : penicillin ; QAC : quaternary ammonium; QD : quinupristine-dalfopristine ; Rif : rifampicin ; SA: streptogramin A, SB: streptogramin B, Sm: streptomycin; Sul: sulfa, TC: tetracycline Tmp: trimethoprim; Vir: virginiamycin.

NorA

NorA is a proton motive force (PMF)-dependent multidrug (MDR) efflux pump in *Staphylococcus aureus*. It is a member of the major facilitator superfamily formed by 388 a.a. and it has 12 transmembrane-spanning segments. Hydrophilic fluoroquinolones and monocationic organic compounds such as acriflavine, ethidium, and tetraphenylphosphonium bromide (TPP) are substrates of this pump (209).

NorA is under promoter control that binds a not yet isolated protein. A single mutation in NorA promoter region, cause a pump overexpression that extrude from cell hydrophilic quinolones such as ciprofloxacin and norfloxacin but does not interfere with hydrophobic molecules accumulation such as sparfloxacin and gatifloxacin (210).

Two efflux pumps relate to NorA, Bmr and Blt, have been found in *Bacillus subtilis*. Other pumps similar to NorA, have been found in other Gram-positive microorganisms, such as *S. pneumoniae* (PmrA) and *Streptococcus viridans* group (194).

LmrA

LmrA is drug transport system in *L. Lacts*. It belongs to the ATP-Binding Cassette (ABC) Superfamily and is driven by ATP hydrolysis. LmrA is a 590-aa polypeptide that contains an N-terminal membrane domain with six membrane spanning segments followed by the ABC domain (211). LmrA is homologous to prokaryotic ABC transporters such as the hop-resistance protein HorA in *Lactobacillus brevis*(212) and ABC proteins in *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, *Helicobacter pylori*, *Haemophilus influenzae*, and *Mycoplasma genitalium* (213). LmrA is also homologous to eukaryotic ABC transporters, and is a half-molecule version of the human multidrug resistance P-glycoprotein which is a cause of multidrug resistance of tumor cells during chemotherapy (214). Recent studies of the expression of covalently linked dimers of LmrA in *L. lactis* suggest that, by analogy with P-glycoprotein, LmrA is transport-active as a homodimer (215)

Overexpression of LmrA resulted in increased resistance to 17 of 21 antibiotics, including broad-spectrum antibiotics belonging to the classes of aminoglycosides, lincosamides, macrolides, quinolones, streptogramins, and tetracyclines (216)(217)

MDR inhibitors

Inhibition of efflux is potentially one way to improve the clinical efficacy of an antibiotic, even in the presence of target based mutations, by increasing intracellular antibiotic concentrations.

Because of emerging resistance to all classes of antibiotics, in particular the fluoroquinolones, there has been a significant focus by the pharmaceutical industry on addressing this problem (218).

It is not known exactly how inhibitors of MDR transporters function but there are a few proposed mechanisms of action: direct binding of inhibitor to one or more binding sites on the protein therefore blocking transport as either competitive or non-competitive inhibitors, depletion of pump energy by inhibiting binding of ATP and modifying protein conformation by an inhibitor interaction with the cell membrane (219).

Since the efflux of fluoroquinolones can be mediated by a proton motive force process (like NorA in *S.aureus*), and by an ATP hydrolysis active mechanism (like LmrA in *L. lactis*), uncouplers like carbonyl-CCC cyanil-chlorophenyl hydrazone (CCCP) (220), that are able to dissipate the proton motive force of the cytoplasmic membrane and block the ATP synthesis, leads to an intracellular drug accumulation (221)(222).

The plant alkaloid Reserpine and the phenylalkylamine verapamil are a known inhibitor of both mammalian and gram-positive bacterial efflux. However, their clinical utility is limited by its neurotoxicity. They have activity against both the NorA pump and LmrP, a known contributors to fluoroquinolone resistance in clinical isolates(215)(218).

In 2003 Zloh at al conducted a series of molecular modelling experiments with fluoroquinolones inhibitor reserpine to study the affinity that inhibitors of MDR phenomena have with MDR substrates. They found out that inhibitors of MDR have affinity for substrates of efflux transporters, and that they may form complexes which could have a number of roles in the mechanism of MDR inhibition. These complexes may facilitate entry of drugs into the cell and secondly the drug in such a complex may be hidden from MDR transporters (219).

7 Materials and methods

Two hundred forty-four strains previously classified as *Lactobacillus* spp. and collected from women vaginas, belonging to Department of Bio-Medical Section of Microbiology University of Catania, were examined in this study

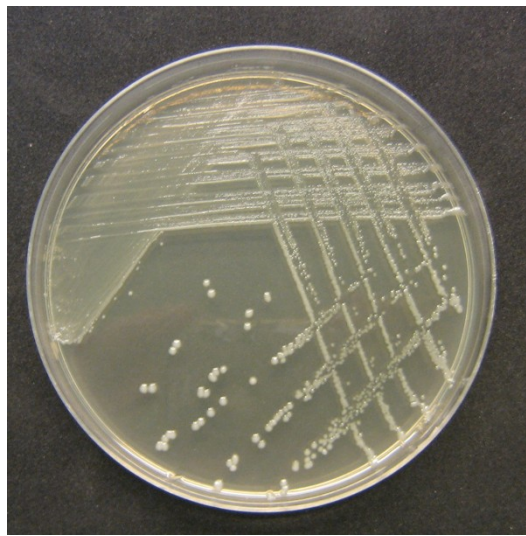


Photo 7-1 Growth of *Lactobacillus fermentum* in LSM agar

7.1 Cultivation

Isolated lactobacilli were previously cryopreserved in MRS with 20% glycerol, at -80 °C. For each sample subcultures in LSM agar (Isosensitest agar 90%, MRS agar 10%) (226) was prepared; then they were grown at 37 °C in a microaerobic atmosphere, for 24-48 hours

7.2 Susceptibility testing

Minimum inhibitory concentration (MIC) was done by microdilution method, using 96 wells polystyrene microplates (Bibby Sterilin), according to CLSI M45-A procedure(16). However, CAMHB (Cation Adjusted Mueller Hinton Broth, with 2.5-5% of lysed horse blood) was replaced with LSM broth (89) because most of the *Lactobacillus* strains grow poorly in CAMHB; our previous observations showed that susceptibility-testing results obtained with LSM are more reliable due to a better grow of microorganisms (Furneri unpublished data).

The following fluoroquinolones were investigated for their activity: ciprofloxacin (CIP), ofloxacin (OFL), ulifloxacin (ULI), levofloxacin (LEV).

7.3 Molecular identification

7.3.1 DNA Extraction

Each strain was inoculated into LSM broth at 37 °C overnight; broths were centrifuged at 3000-4000 rpm for 30 minutes, at room temperature. supernatant was discarded and cell pellet was suspended in 5 mL of PBS pH 7 (phosphate buffered saline). 3 washes in PBS buffer were carried out. In each tube was added a lysozyme solution (100mg/mL). samples were thus incubated for 1 hour at 37 °C (up to dissolve the pellet)., then, 200µl of Sodium Dodecyl Sulphate (SDS) were added, together with 100 uL of Proteinase K solution (10mg/mL). samples were incubated at 55 °C overnight. following morning, saturated NaCl (an amount equal to 1/3 of the total volume) was added, continuing with a new incubation for 20 minutes at 40°C. 3 volumes of TE buffer (Tris/EDTA) were added and samples were centrifuged at 3500 rpm at 4°C. obtained supernatant was centrifuged at 13000 rpm for 30 minutes at 4 °C. supernatant collection, was performed by adding 3 volumes of 95% EtOH. Flocculated DNA was washed in 70% EtOH and air-dried.

Extracted DNA was finally resuspended in 1 mL of TE buffer and stored at 4 °C (223).

Spectrophotometric analysis

DNA samples were subjected to spectrophotometric analysis (spectrophotometer Ultrospec 2000). absorbance reading of 100 uL of DNA in TE solution was performed at two different wavelength values (λ), 260nm and 280nm. DNA purity degree was deduced from sample absorbance ratio. Values were all near to 1.8.

DNA samples were visualized by electrophoresis on 1% agarose gel, stained with SYBR Safe 1X (Invitrogen) and observed on a transilluminator Safe Imager (Invitrogen).

7.3.2 PCR/RFLP analysis of the 16s rDNA (13)

PCR amplification was performed with *Taq* Dna polymerase Recombinant (Invitrogen-Life Technologies, UK) using primers 7f 5'-AGAGTTTGATC/TA/CTGGCTCAG-3' and 1510rev 5'-ACGG(C/T)TACCTTGTTACGACTT-3'. Reaction mixtures consisted of 20 mM Tris-HCl (pH 8,4), 50 mM KCl, 3 mM MgCl₂, 50 mM each dNTP, 1.25 U of *Taq* polymerase, 5 pmol of each primer and 1 μ l of appropriately diluted template DNA in a final

volume of 50 μ l. DNA from isolates and type strains, amplification program used was as follows: : 94 $^{\circ}$ C for 3 min; 30 cycles of 94 $^{\circ}$ C for 30 s, 52 $^{\circ}$ C for 30 s and 68 $^{\circ}$ C for 1.5 min; and finally, 68 $^{\circ}$ C for 7 min. Amplified obtained (about 1500bp) (Photo 72) were visualized by electrophoresis (70V) on 1, 5% agarose gel in 1X TAE buffer (Tris, acetic acid, EDTA), stained with SYBR Safe 1X (Invitrogen) and observed on transilluminator Safe Imager (Invitrogen). DNA ladder was used 1Kb (BioRad)

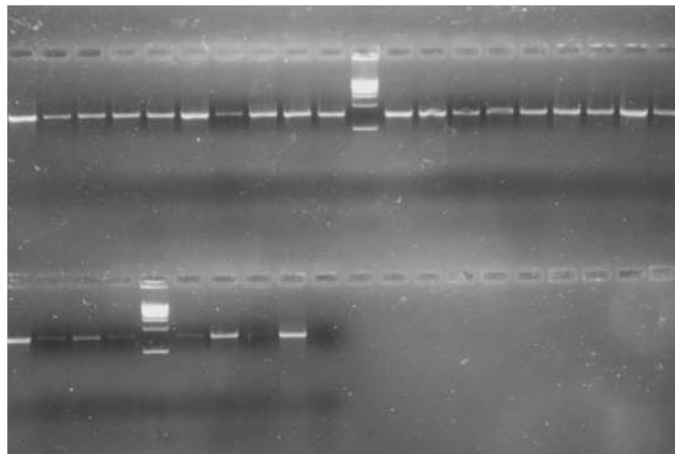


Photo 7-2 Electrophoretic amplified 16S rDNA: 1-10/11-20 wells (I row) samples; 1-4/6-10 wells (row II) samples, 11 wells (row I) / 5 (row II) 1Kb DNA ladder (BioRad).

Restriction fragments length polymorphism (RFLP) of 16S rDNA PCR products was performed by restriction enzyme digestion with *Hae* III, *Msp*I I and *Alu* I (MBI Fermentas), followed by electrophoresis of the products on a 2% (w/v) agarose gel in 1X TBE buffer (89 mM Tris–borate, 89 mM boric acid, 2 mM EDTA; pH 8.0) containing Sybr Safe 1X (Invitrogen). 100bp DNA Ladder (BioRad) was used as size standard. Gel was visualized after electrophoresis on a 1.2% agarose gel containing Sybr Safe 1X (Invitrogen).

Unidentified strains restriction profiles have been compared with those obtained from known *Lactobacillus* type strains belonging to DSMZ catalog (table 7-1). After rehydration through a special protocol provided by DSMZ, type strains were subjected to the same experimental procedures (DNA isolation, PCR / RFLP, etc.).

Cleavage sites of *Hae*III, *Alu*I and *Msp*I are respectively: 5'... GC ↑ CC ... 3', 5'... AG ↑ CT ... 3' and 5'... C ↑ CGG. ... 3'.

Table 7-1 DSMZ type strains

Descrizione ceppo	Numero DSMZ	Altri numeri di collezione
<i>Lactobacillus acidophilus</i>	20079	ATCC 4356
<i>Lactobacillus antri</i>	16041	CCUG 48456
<i>Lactobacillus brevis</i>	20054	ATCC 14869
<i>Lactobacillus casei</i>	20011	ATCC 393
<i>Lactobacillus colohemini</i>	14050	CCUG 44007
<i>Lactobacillus concavus</i>	17758	AS 1.5017
<i>Lactobacillus crispatus</i>	20584	ATCC 33820
<i>Lactobacillus delbrueckii subsp. bulgaricus</i>	20081	ATCC 11842
<i>Lactobacillus delbrueckii subsp. delbrueckii</i>	20074	ATCC 9649
<i>Lactobacillus delbrueckii subsp. indicus</i>	15996	LMG 22083
<i>Lactobacillus delbrueckii subsp. Lactis</i>	20072	ATCC 12315
<i>Lactobacillus fermentum</i>	20052	ATCC 14931
<i>Lactobacillus gasseri</i>	20243	ATCC 33323
<i>Lactobacillus gastricus</i>	16045	CCUG 48454
<i>Lactobacillus intestinali</i>	6629	ATCC 49335
<i>Lactobacillus jensenii</i>	20557	ATCC 25258
<i>Lactobacillus johnsonii</i>	10533	ATCC 33200
<i>Lactobacillus oris</i>	4854	ATCC 49062
<i>Lactobacillus parabuckneri</i>	5707	ATCC 49374
<i>Lactobacillus paracasei subsp. paracasei</i>	5622	ATCC 25302
<i>Lactobacillus paraplantarum</i>	10667	CIP 104668
<i>Lactobacillus plantarum</i>	20174	ATCC 14917
<i>Lactobacillus reuteri</i>	20016	ATCC 23272
<i>Lactobacillus rhamnosus</i>	20021	ATCC 7469
<i>Lactobacillus salivarius</i>	20555	ATCC 11741
<i>Lactobacillus vaginalis</i>	5837	ATCC 49540
<i>Lactobacillus zeae</i>	20178	ATCC 15820
<i>Weissella confusa</i>	20196	ATCC 10881

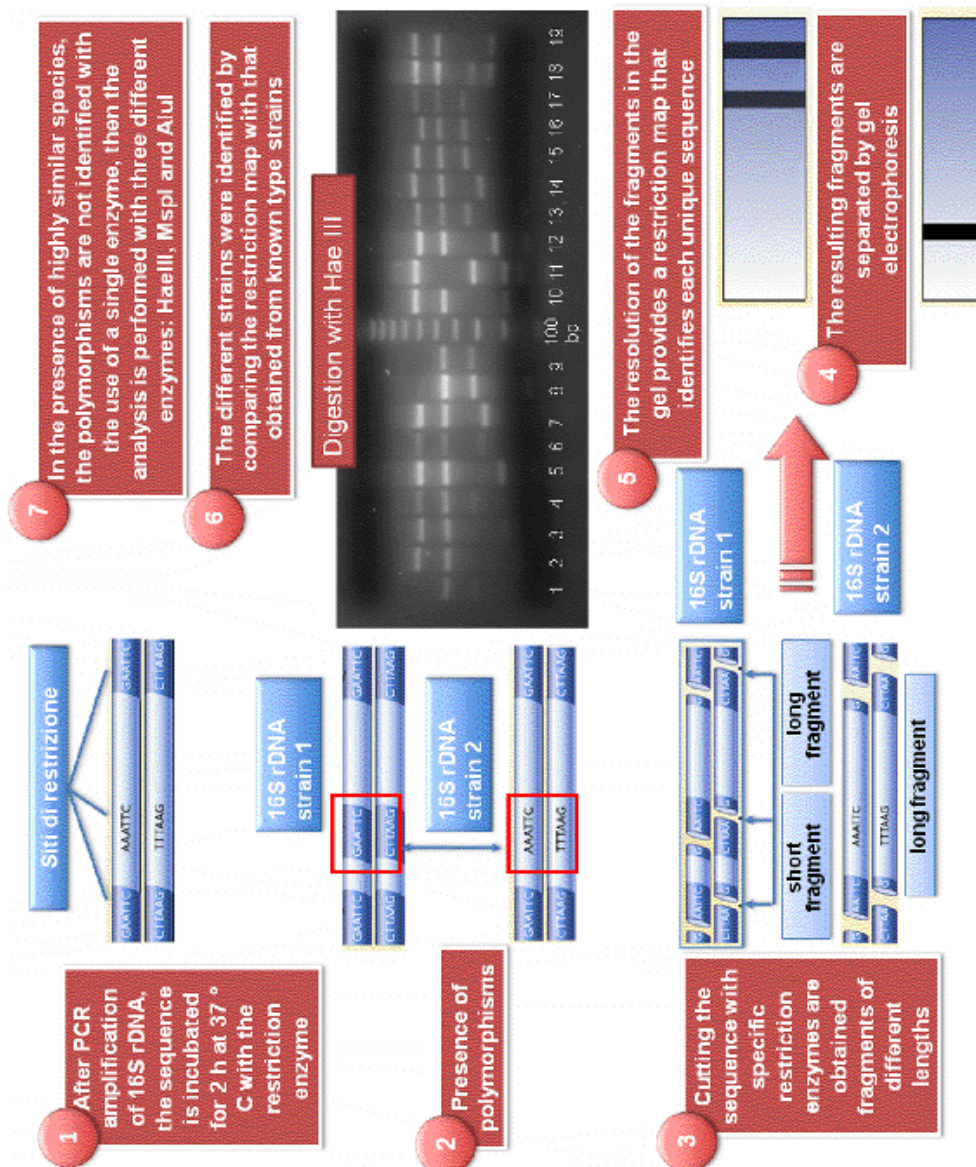


Figure 7-1 Schematic representation of 16S RFLP

Photos below depict the electrophoresis runs a digestion with HaeIII (photos 7-3 and 7-4) and one with MspI (photo 7-5).

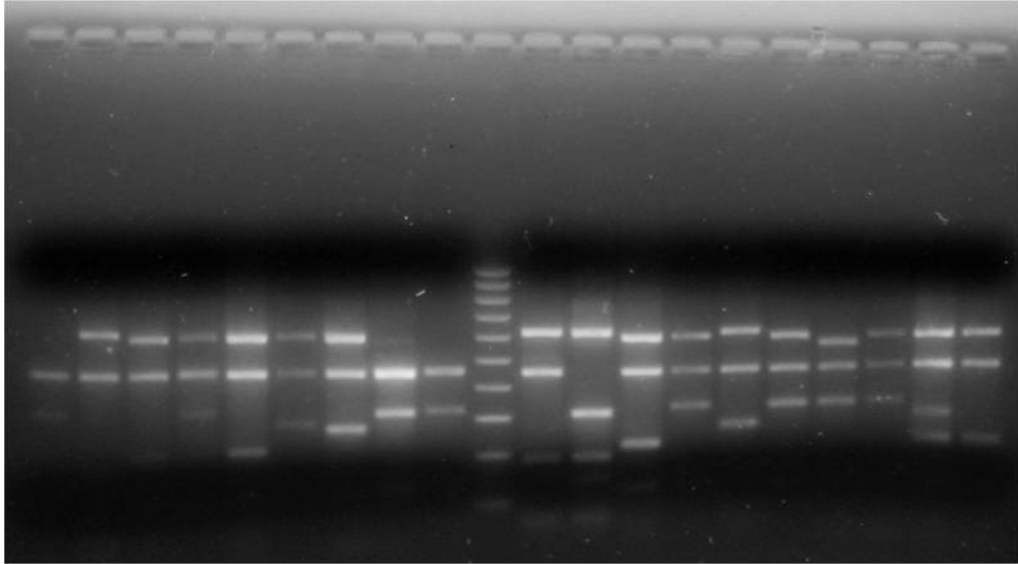


Photo 7-3 *HaeIII* digestion Electrophoresis: wells 1-5/18-20 samples; well 6 *L. zeae* DSM20178; well 7 *L. salivarius* DSM20555; well 8 *L. jensenii* DSM20557; well 9 *L. intestinalis* DSM6629; well 10 100bp DNA ladder (BioRad); well 11 *L. colehominis* DSM14050; well 12 *L. caves* DSM16041; well 13 *L. gastricus* DSM16045; well 14 *L. paracasei subsp. paracasei* DSM5622; well 15 *L. reuteri* DSM20016; well 16 *L. rhamnosus* DSM20021; well 17 *L. plantarum subsp. plantarum* DSM 20174

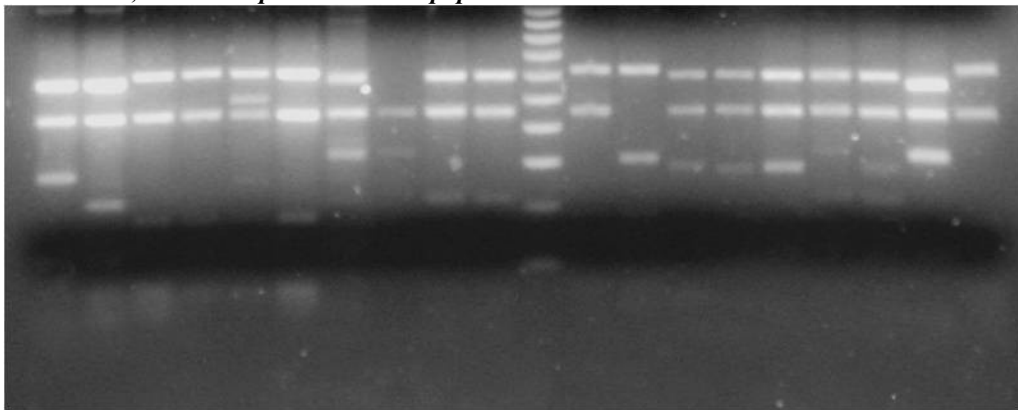


Photo 7-4 Electrophoresis digestion with *HaeIII*. wells 1-7 / 16-19 samples, 8 *L. gasseri* DSM20243; well 9 *L. fermentum* DSM20052; well 10 *L. gastricus* DSM16045; well 11 100bp DNA ladder (BioRad); well 12 *L. vaginalis* DSM5837; well 13 *L. caves* DSM16041; well 14 *L. parabuchneri* DSM5707; well 15 *L. brevis* DSM20054; well 20 *L. colehominis* DSM14060.

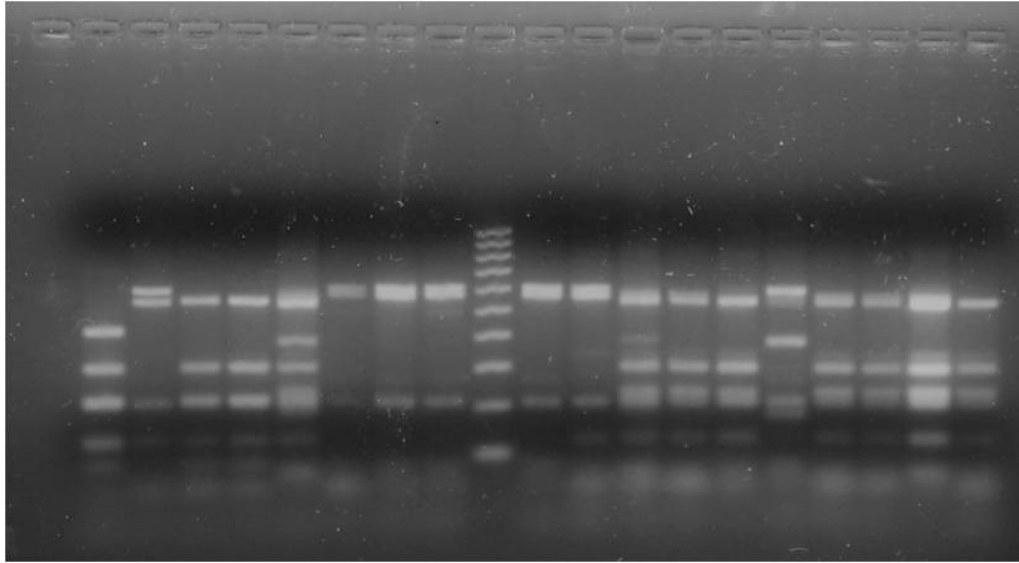


Photo 7-5 Electrophoresis digestion with *MspI*: wells 3-7 / 13-20 samples; well 2 *L. reuteri* DSM 20016; well 8 *L. rhamnosus* DSM 20021; well 9 *L. paracasei subsp. paracasei* DSM5622; well 10 100bp DNA ladder (BioRad); well 11 *L. casei* DSM20011; well 12 *L. plantarum subsp. plantarum*

Virtual restriction maps, obtained on the basis of 16S rDNA sequences found in the NCBI (220), are shown in table 7-2.

Table 7-2 Virtual restriction maps of 16S rRNA genes deposited in NCBI database.

Species	16S rRNA (bp)	<i>HaeIII</i> (cut number) fragment (bp)	<i>MspI</i> (cut number) fragment (bp)	<i>AluI</i> (cut number) fragment(bp)
<i>L. acidophilus</i>	1553	(8) 242-35-44-22-139-459-457-55-100	(6) 177-389-606-11-211-53-106	(9) 59-146-20-33-186-429-221-44-208
<i>L. antri</i>	1520	(7) 62-191-78-620-312-145-55-57	(8) 24-552-88-518-11-71-48-92-116	(7) 268-186-429-105-102-189-204-37
<i>L. brevis</i>	1430	(5) 227-44-22-618-457-82	(6) 516-68-538-11-211-53-33	(5) 823-105-102-207-193
<i>L. delbrueckii subsp. bulgaricus</i>	1561	(5) 280-44-22-598-457-160	(6) 180-389-289-317-11-211-164	(7) 64-164-33-186-636-221-44-213
<i>L. casei</i>	1517	(4) 320-22-598-457-120	(5) 565-606-11-211-53-71	(5) 224-33-615-207-207-231
<i>L. colehominis</i>	1564	(7) 65-191-78-23-596-457-55-99	(8) 27-551-68-234-304-11-211-158	(6) 271-494-120-105-102-207-265
<i>L. concavus</i>	1528	(5) 313-22-139-458-457-139	(5) 558-605-11-211-53-90	(7) 217-33-615-206-207-14-169-67
<i>L. confusus</i>	1525	(2) 347-22-1156	(7) 437-155-376-11-211-53-52	(6) 96-188-370-245-207-207-212
<i>L. crispatus</i>	1518	(7) 226-79-22-139-459-457-55-81	(6) 161-389-606-11-211-53-87	(8) 189-20-33-219-615-207-221-233
<i>L. fermentum</i>	1364	(5) 67-225-44-22-598-408	(6) 29-552-68-234-304-11-166	(5) 273-186-429-105-269
<i>L. gasseri</i>	1747	(5) 405-22-139-459-457-261	(7) 266-388-68-538-11-211-53-212	(8) 40-15-85-206-186-429-207-275-314
<i>L. gastricus</i>	1550	(6) 48-224-44-22-598-457-157	(6) 10-551-302-304-11-211-161	(5) 253-186-429-105-102-475
<i>L. iners</i>	1539	(5) 276-44-22-598-457-142	(8) 177-388-68-96-442-11-211-53-93	(6) 257-186-217-212-207-265-195
<i>L. intestinalis</i>	1513	(6) 306-22-139-459-457-55-75	(7) 162-389-59-547-11-211-53-81	(9) 44-134-32-33-186-429-207-221-44-183
<i>L. jensenii</i>	1496	(5) 288-22-139-459-457-131	(5) 144-389-606-11-211-135	(10) 26-40-88-38-32-187-429-207-221-44-184
<i>L. johnsonii</i>	1487	(5) 302-22-139-459-457-108	(7) 159-388-68-538-11-211-53-59	(6) 33-206-186-429-207-265-161
<i>L. oris</i>	1359	(7) 134-78-234-384-311-145-55-18	(9) 457-88-375-140-11-71-48-92-60-17	(4) 149-364-455-186-205
<i>L. parabuchneri</i>	1497	(5) 272-44-22-598-458-103	(6) 561-68-539-11-211-53-54	(4) 253-615-208-207-214
<i>L. paracasei</i>	1558	(4) 153-457-598-22-328	(5) 102-53-211-11-606-575	(5) 264-207-207-615-33-232
<i>L. paraplantarum</i>	1502	(5) 309-22-564-34-457-116	(4) 554-606-11-211-120	(5) 246-615-105-102-207-227
<i>L. plantarum</i>	1555	(5) 327-22-564-34-457-151	(4) 572-606-11-211-155	(6) 213-51-615-105-102-207-262
<i>L. reuteri</i>	1571	(5) 67-269-620-457-55-103	(8) 29-125-427-88-214-304-11-211-162	(7) 273-186-429-105-102-195-14-269
<i>L. rhamnosus</i>	1540	(4) 306-22-597-457-158	(5) 550-606-11-211-53-109	(5) 210-33-614-207-207-269
<i>L. salivarius</i>	1570	(4) 279-44-620-457-170	(7) 568-68-538-11-211-53-117-4	(4) 875-207-207-268-13
<i>L. vaginalis</i>	1541	(6) 42-190-78-621-457-55-98	(6) 555-89-214-304-11-211-157	(7) 247-186-430-105-102-207-231-33
<i>L. zeae</i>	1559	(4) 324-22-598-457-158	(5) 569-606-11-211-53-109	(5) 228-33-615-207-207-269

In red terminal fragments resulting from cuts

7.3.3 Two-steps multiplex PCRs: 16S-ITS-23S and 23S rDNA flanking region (Song et al., 2000)(14)

A multiplex PCR assays using group- and species-specific primers derived from the 16S-23S rRNA intergenic spacer region and its flanking 23S rRNA was used for lactobacilli identification.

Lactobacilli were first grouped by a multiplex PCR (designated multiplex PCR-G) and then identified to the species level by four multiplex PCR assays (named multiplex PCR II-1, multiplex PCR II-2, multiplex PCR III and multiplex PCR IV).

Multiplex PCR (II-1, II-2, III and IV) were carried out with primers designed on 16S-23S rDNA and ITS-flanking region of 23S rDNA sequences alignments, because, just exploring this region, Song et al. were able to design species specific primers, which would generate amplicons with significantly different amplitudes (Figure 7-2).

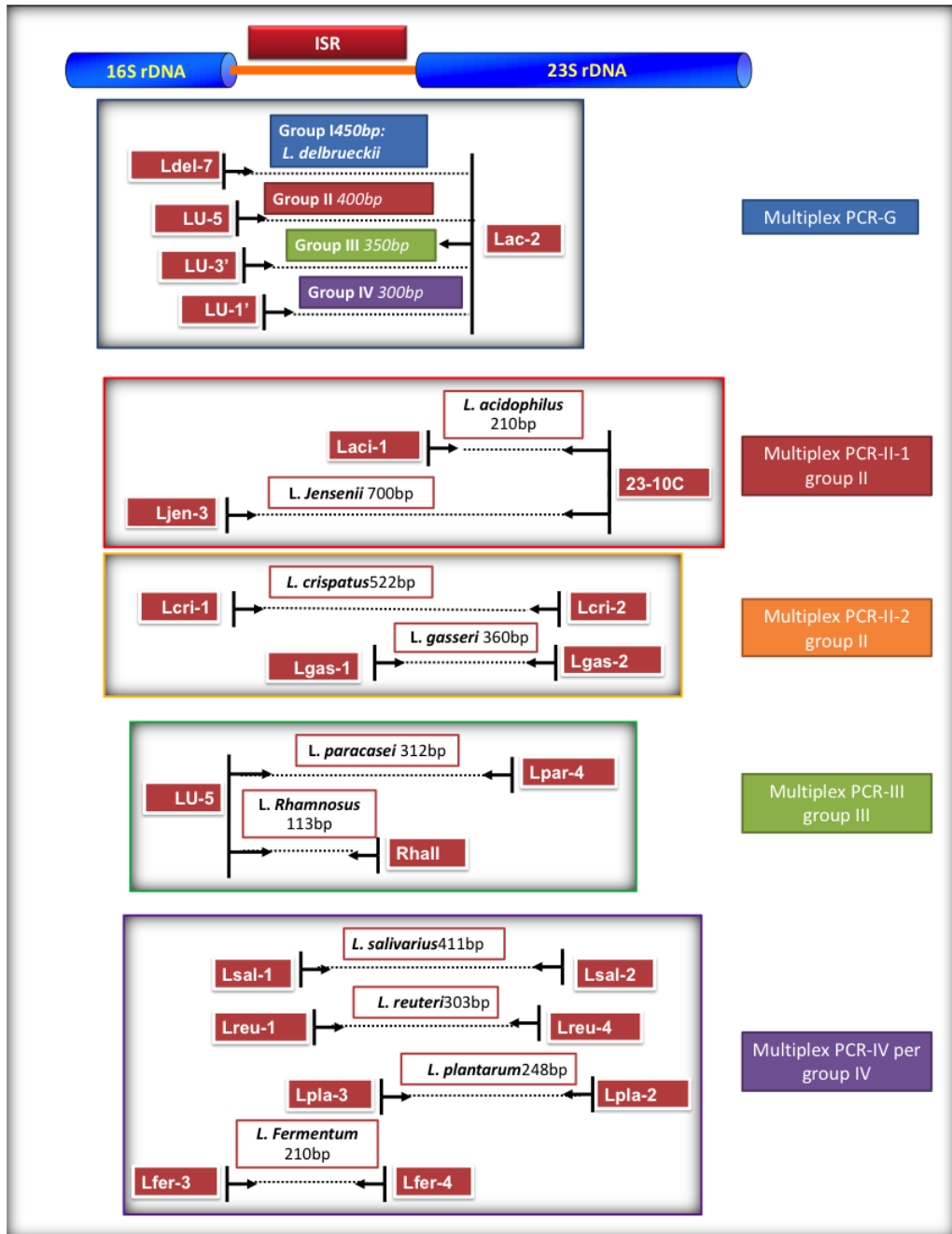


Figure. Schematic representation of two steps multiplex PCR assays by Song et al. (2002) (14)

Primers oligonucleotide sequences used in multiplex PCR assays are shown in Table 7-3:

Table 7-3 Multiplex PCR primer sequences (Song et al., 2000)

Primer	Sequence (5'-3')	Multiplex	
Ldel-7	ACAGATGGATGGAGAGCAGA	FW	G
LU-1'	ATTGTAGAGCGCGACCGAGAAG	FW	G
LU-3'	AAACCGGAGAACACCGCGTT	FW	G
LU-5	CTAGCGGGTGC GACTTTGTT	FW	G; III
Lac-2	CCTCTTCGCTCGCCGCTACT	REV	G
Laci-1	TGCAAAGTGGTAGCGTAAGC	FW	IIA
Ljens-3	AAGAAGGCACTGAGTACGGA	FW	IIA
2310-C	CCTTCCCTCACGGTACTG	REV	IIA
Lcri-1	AGGATATGGAGAGCAGGAT	FW	IIB
Lcri-2	CAACTATCTCTTTACTACTGCC	REV	IIB
L. gas-2	TGCTATCGCTTCAAGTGCTT	FW	IIB
Lgas-3	AGCGACCGAGAAGAGAGAGA	REV	IIB
Lpar-4	GGCCAGCTATGTATTCCTGA	REV	III
RhaII	GCGATGCGAATTTCTATTATT	REV	III
Lfer-3	ACTAACTTGACTGATCTACGA	FW	IV
Lfer-4	TTCACTGCTCAAGTAATCATC	REV	IV
L-pla-3	ATTCATAGTCTAGTTGGAGGT	FW	IV
Lpla-2	CCTGAACTGAGAGAATTTGA	REV	IV
Lreu-1	CAGACAATCTTTGATTGTTTAG	FW	IV
Lreu-4	GCTTGTTGGTTTGGGCTCTTC	REV	IV
Lsal-1	AATCGCTAAACTCATAACCT	FW	IV
Lsal-2	CACTCTCTTTGGCTAATCTT	REV	IV

Amplified obtained with multiplex PCR-G are 450bp, 300bp, 400BP, 350bp, for group I, II, III and IV, respectively. Multiplex-PCR, primers used were species-specific.

Group I, has not been set up no further multiplexes, because members belonging to this group have all been brought back to *delbrueckii* species; for this, it was not possible to discriminate the subspecies.

Group II, composed by two multiplex-PCR (II-1 and II-2) for discrimination of *L. acidophilus* (ca. 210bp) and *L. jensenii* (ca. 700bp) with multiplex II-1; *L. crispatus* (ca. 522bp) and *L. gasseri* (ca. 360bp) with multiplex II-2.

L. rhamnosus (ca. 113bp) and *L. paracasei* (ca. 312) were identified with the multiplex III; by multiplex IV has identified 4 species, *L. fermentum* (ca. 192bp), *L. salivarius* (ca. 411bp), *L. reuteri* (ca. 303bp) and *L. plantarum* (ca. 248bp).

Mixx composition (50µL each) prepared for each multiplexPCR are shown in table 7-4

Table 7-4

Components	Concentration	Multiplex
Buffer (Biotools) 1X	1X	G, IIA, IIB, III, IV
MgCl ₂ (Biotools)	2,5mM	G
	2mM	IIA, IIA, IIB, III, IV
dNTP (TaKaRa)	1mM	G, IIA, IIB, III, IV
Primers (Invitrogen)	0,4mM	G
	0,3mM	IIA, IIB, III, IV
Taq polymerase(Biotools)	1U	G, IIA, IIB, III, IV
H ₂ O sterile	Up to volume	G, IIA, IIB, III, IV
DNA	10-15ng	G, IIA, IIB, III, IV

PCR termocycler program (tabella 7-5):

Table 7-5

Step	Temperature (°C)	Time (minute)	Multiplex-PCR
Denaturation	94	5	G, IIa,IIb, III, IV
30 cicli	Denaturation	95	G, IIa,IIb, III, IV
	Annealing	55	G
		68	IIA
		65	IIB
		62	III
		60	IV
elongation	72	0.5	G, IIa,IIb, III, IV
Final elongation	72	10	G, IIa,IIb, III, IV

All amplified products were visualized by electrophoresis on a 2% agarose gel, stained with SYBR Safe 1X (Invitrogen) and observed on a transilluminator Safe Imager (Invitrogen).

Photos below illustrate electrophoresis runs of a multiplex PCR-G and multiplex PCR, specific group.

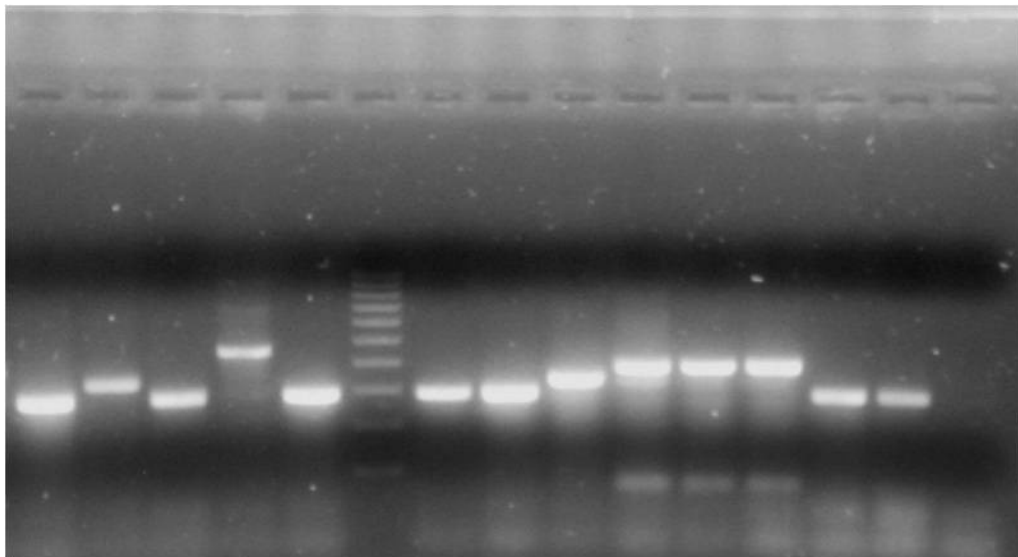


Photo 7-6 Electrophoresis PCR-G: wells 1-3, 5, 7, 11-14 samples; well 4 *L. delbrueckii subsp. delbrueckii* DSM20074; well 8 *L. rhamnosus* DSM20021; well 9 *L. gasseri* DSM20243; well 10 *L. salivarius* DSM20555; well 6 100bp DNA ladder (BioRad); negative control well 15.

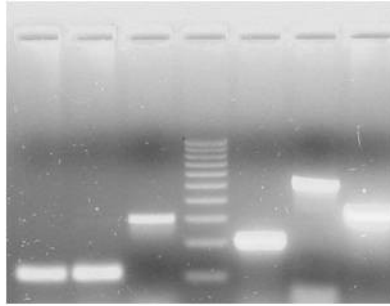


Photo 7-7 Electrophoresis after multiplex PCR grouping: wells 1 and 2 *L. rhamnosus* DSM 20021 (about 110bp amplified); well 3 *L. reuteri* DSM20016 (amplified by about 300bp); well 5 *L. acidophilus* DSM20079 (amplified by about 210bp); well 7 *L. crispatus* DSM20584 (amplified by about 520bp); wells 7 and 8 *L. gasseri* DSM20243 (amplified by about 360bp) DNA ladder 100bp well 4.

7.3.4 *tuf* gene amplification (15)

Amplification reactions were performed with a 50- μ l (total volume) solution containing buffer (Biotools) 1X [10mM Tris-HCl (pH 8.0), 50 mM KCl, 1 mM EDTA, 0.1% Triton X-100, 50% glicerolo (v/v)]; MgCl₂ 1,5 mM; dNTP mix 0,8 mM; 0,2 μ M for each forward primers PAR (5'-GACGGTTAAGATTGGTGAC-3'), CAS (5'-ACTGAAGGCGACAAGGA-3'), and RHA (5'-GCGTCAGGTTGGTGTGTTG-3'), 0,6 μ M of reverse primer CPR (5'-CAANTGGATNGAACCTGGCTTT-3'), 1.5 U Taq polymerase (Biotools); 10-15 ng of DNA. Amplification reactions were performed by the following temperature profiles: 1 cycle at 95°C for 5 min; 30 cycles at 95°C for 30 s, 60°C for 1 min, and 72°C for 1.5 min; and 1 cycle at 72°C for 10 min. PCR amplicons were analyzed by 2% (wt/vol) agarose gel electrophoresis in Tris-acetate-EDTA buffer at a constant voltage of 7 V/cm, visualized with Sybr

Safe 1X (Invitrogen™), and photographed under Safe Imager (Invitrogen™).

obtained amplified were the following: about 540 bp for *L. rhamnosus* and approximately 200 bp for *L. paracasei*, (photos 7-8 and 7-9).

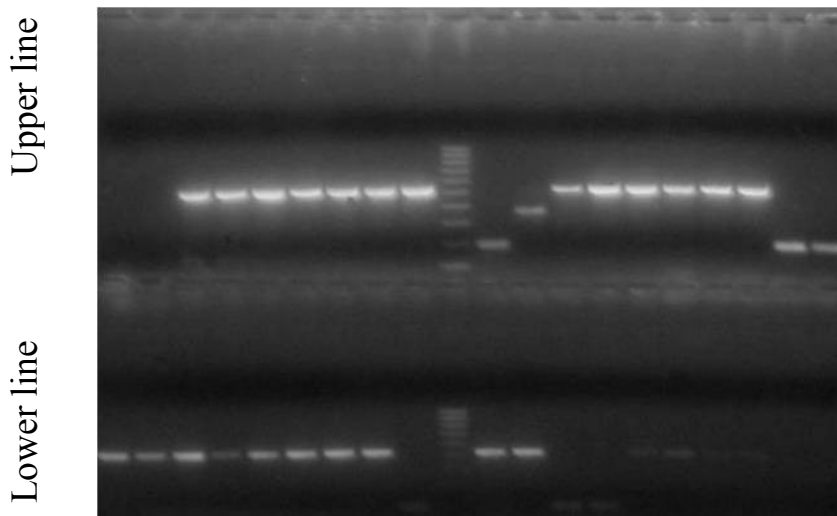


Photo 7-8(*) Upper line: (wells 1 to 2: negative controls (*L. plantarum* subsp. 20174 *plantarum*, *L. jensenii* DSM20557); wells 3 to 8-13 to 29-31 to 38 studied strains; well 9: *Lactobacillus rhamnosus* DSM2021 (ATCC7469); well 10: 100bp DNA ladder (BioRad); well 11: *Lactobacillus paracasei* subsp. *paracasei* DSM5622 (ATCC25302); well 12: *Lactobacillus casei* (ATCC 393); Lower line: well 10: 100bp DNA ladder (BioRad); wells 1 to 8 and 11 to12: studied strains; 9:negative control *L. reuteri* DSM20016; well 20: mix negative control.

(*) electrophoresis photo refers to human samples (in this study) and strains belonging to dipartiment of Bio-Medical Sciences Microbiology section, previously studied with other molecular techniques.

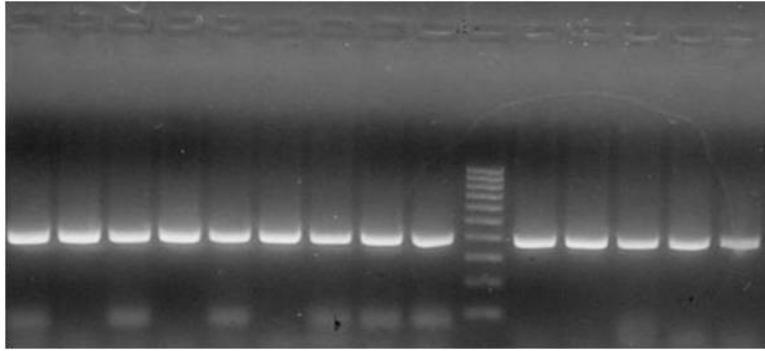


Photo 7-9(*) Electrophoresis: wells 1 to 8 studied strains; well 9: *Lactobacillus rhamnosus* DSM2021 (ATCC 7469), well 10: DNA ladder 100bp (BioRad); wells 11 to 15: strains studied; well 16: negative control

(*) electrophoresis photo refers to samples isolated from swabs (in this study) and strains belonging to dipartiment of Bio-Medical Sciences Microbiology section, previously studied with other molecular techniques.

7.4 Mechanisms of resistance to ciprofloxacin in *L. fermentum*

Four of sixteen *L. fermentum* strains that showed reduced *in vitro* susceptibility or resistance to quinolones ciprofloxacin (assuming as resistant strains with MIC \geq 4 mg/mL) were investigated for genotypic resistance mechanisms searching.

7.4.1 QRDR amplification in *gyrA* and *parC* (17)

To investigate whether observed fluoroquinolone resistances were due to mutations in the quinolone resistance-determining regions (QRDR) of the *gyrA* and *parC* genes. The QRDR encoding regions of *L. fermentum* strains were PCR amplified, using the following mix: buffer (Biotools) 1X [10mM Tris-HCl (pH 8.0), 50 mM KCl, 1 mM EDTA, 0.1% Triton X-100, 50 % glycerol (v/v)]; MgCl₂ (Biotools) 5 mM; dNTP mix (TaKaRa) 200 uM, 0.2 uM each primer (Invitrogen); 1U Taq polymerase (Biotools); 10-15 ng of DNA; water sterile injectable enough to make up the volume.

designed primers for the *gyrA* gene were GyrAfw (5'-CAM CGK CGK ATT CTT TAC GGA ATG-3') and GyrArev (5'-TTR TTG ATA TCR CGB AGC ATT TC-3'), and the primers for the *parC*

gene were ParCfw (5'-TAT TCY AAA TAY ATC ATT CAR GA-3') and ParCrev (5'-GCY TCN GTA TAA CGC ATM GCC G-3').

program used was as follows: initial denaturation at 94 °C for 5 minutes; 30 cycles (94 °C for 1 minute, 52 °C for 1 minute, 72 °C for 30 seconds); final elongation at 72 °C for 10 minutes.

The PCR products were loaded on agarose gel at 1, 5% stained with SYBR Safe 1X (Invitrogen TM) and displayed on transilluminator Safe Imager (Invitrogen TM). The amplified obtained were of 286bp, both for *gyrA* for both *parC*.

The amplification conditions for both the *gyrA* and the *parC* genes consisted of 35 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 30 s.

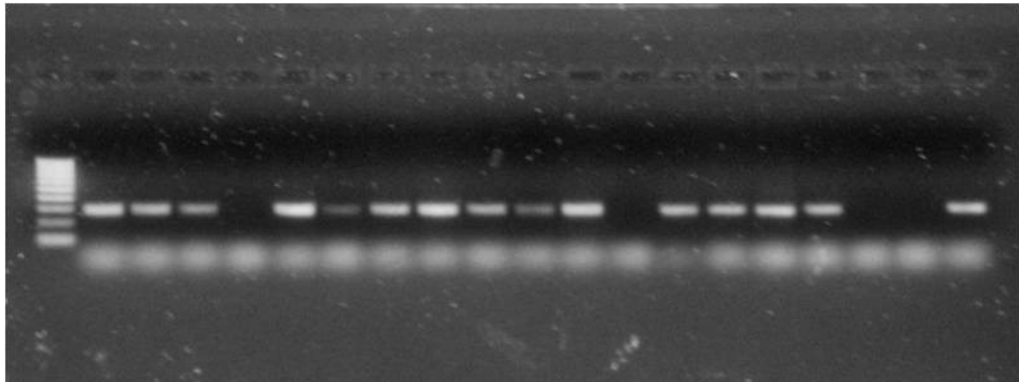


Photo 7-10 Electrophoresis *gyrA* PCR: well 1 DNA 100bp ladder (BioRad); wells 2-18, 20 samples; pozzetto19 negative control

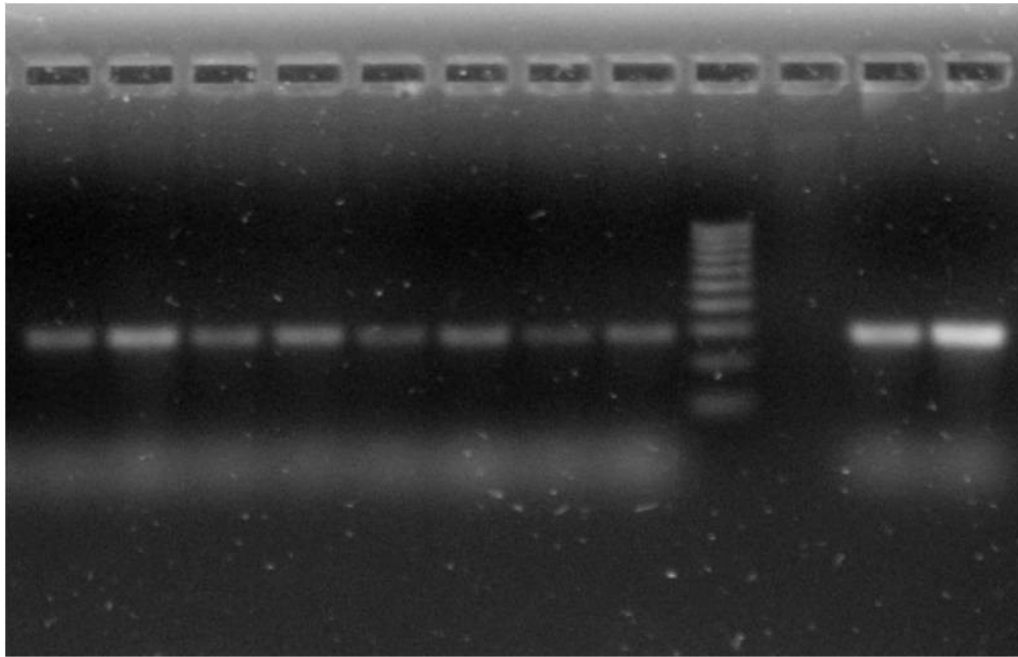


Photo 7-11 Electrophoresis parC PCR: 1-8/11 wells, 12 samples; well 9 DNA ladder 100bp (BioRad); well 10 negative control.

Sequencing protocol

The sequencing protocol was performed as follow:

1. Purification of amplified using QIAquick PCR Purification Kit ® column – Qiagen.
2. Quantitative analysis by spectrophotometric ULTROSPEC 200 nm. (determination of the concentration of DNA solution in TE, 260/280 absorbance).
3. ABI 3730 capillary sequencers (BIO-FAB RESEARCH©).

Sequencing of double-stranded, alignment of the sequences with their respective gyrA and parC of ATCC strains of Lactobacillus spp. and contig sequences with the program BioEdit, version 7.0.9.

7.4.2 Fluoroquinolones accumulation essay

(18)(19)(20)

Ciprofloxacin intracellular accumulation was performed as follows:

1. Strains were incubated in LSM broth with addition of inulin to 0.5% w/v, under stirring, at 37 °C until reaching OD660 between 0.7 and 0.8.
2. After reaching optimal optical density, we proceeded to collection by centrifugation (3000 g for 15 min)
3. Strains were resuspended in 50 mM PBS at pH 7.0 After washing and concentration to 20 times in a final volume of 50mL.
4. Suspensions were equilibrated at 37 °C for 10 minutes under stirring.
5. At time zero 0.5 mL of samples devoid of antibiotic were collected; that has been used as a blank.
6. A solution of antibiotic at a final concentration of 10 mg/L was added to the suspension
7. At regular time intervals 0.5 mL were collected and they were processed as follows:
 - a) Aliquots were transferred into centrifuge tubes with 1mL of PBS 50 mM pH 7.0 and centrifuged immediately at 4 °C for 10 minutes.

b) Recovered pellet was resuspended in 1 mL of 0.1 M glycine at pH 3.0 and incubated overnight at room temperature to lyse the cells.

c) The suspension was centrifuged to remove cell debris.

8. Supernatant was transferred to new 1.5 mL tubes and re-centrifuged to remove any debris left.

Same protocol was repeated in the presence of CCCP (Sigma-Aldrich) (m.-cCarbonyl cyanide m-chlorophenyl hydrazone) to a final concentration of 10 mM.

The experiment was repeated twice for each *L. fermentum* strain studied. Standard Deviation for each point is reported

Reading fluorescence spectrophotometer

Study on ciprofloxacin concentrations variation has been obtained by spectrophotometric analysis (Varian Cary Eclipse Fluorescence Spectrophotometer 5.1) exploiting maximum absorption at 275 nm values which give rise to a peak emission at 447 nm. (53)

Calibration curve

For antibiotics calibration curves, serial dilutions in 0.1 M glycine at pH 3.0 have been made using following concentrations: 4, 2, 1, 0.5, 0.1, 0.05, 0.01, 0.005 and 0.001 g/mL. In this way it was

possible to relate maximum absorption at 275 nm values with concentrations expressed as micrograms/mL.

7.4.3 Inhibitors influence on fluoroquinolone MICs(224)

Influence of efflux system inhibitors on fluoroquinolone MICs was studied using competitive ion channel blockers (Verapamil and reserpine).

For each strain of *L. fermentum*, MICs of fluoroquinolone and efflux inhibitor alone and in combinations were determined by broth microdilution according to the guidelines of the National Committee for Clinical Laboratory Standards(16). A starting inoculum of 10^5 to 10^6 CFU/mL was used, and combinations of fluoroquinolone and inhibitors were initially tested with doubling serial dilutions of the antibiotic and each inhibitor. Preliminary results from these MICs revealed a range of inhibitor concentrations that had either no effect or a maximal effect on fluoroquinolone MICs. A fixed concentration of 0.1 mg/mL was chosen as a low inhibitor concentration to evaluate any effects not detected by

changes in MICs. A high inhibitor concentration of 50 mg/mL was chosen to evaluate the effects of maximal MIC reductions.

8 Results

8.1 Molecular identification of *Lactobacillus* species

All *Lactobacillus* species object of the present study were identified using molecular techniques. In particular, the species distribution (Chart 8-1) was as follows: *L. gasseri*, 50 strains (20.5%), *L. acidophilus* 46 strains (18.8%), *L. crispatus*, 35 strains (14.3%), *L. vaginalis* 30 strains (12.3%), *L. rhamnosus* 25 strains (10.2%), *L. delbrueckii* 23 strains (9.4%), *L. paracasei* 19 strains (7,8%), and *L. fermentum*, 16 strains (6,5%).

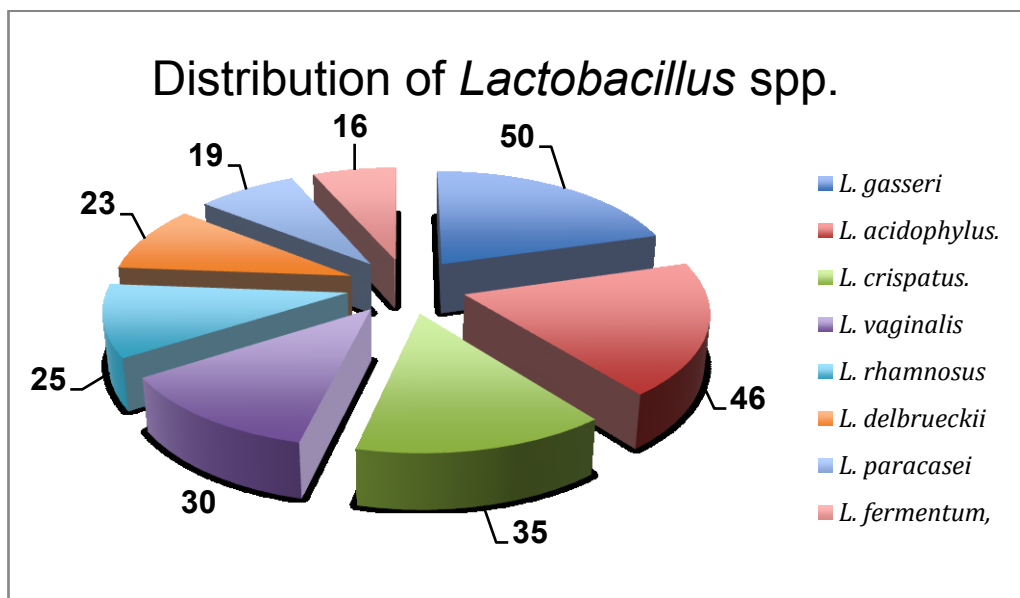


Chart 8-1 Distribution (no. of strains) of *Lactobacillus* spp. identified by genotypic methods.

Two hundred strains out of two hundred forty-four (81.9%) were identified by *16S rDNA PCR/RFLP*. The remaining 44 strains (18.1%) were all attributable to the *L. casei* group (*L. paracasei*, *L. rhamnosus*), since the restriction profiles with *HaeIII*, *MspI*, and *AluI*, obtained for the respective type strains (*L. paracasei subsp. paracasei DSM5622*, *L. rhamnosus DSM20021*), were indistinguishable.

According to the study of Song *et al.* (2000), a multiplex PCR that amplifies different *locus* of the gene region 16S-ITS-23S and the region flanking the gene 23S rDNA was carried out.

Then, all strains were divided into 4 groups by the first multiplex PCR (PCR-G). In particular, 23 strains belonged to group 1, and have thus been identified as *L. delbrueckii*.

One hundred thirty-one strains were awarded at Group II, 44 strains at Group III, and 46 strains at Group IV. It should be emphasized that the strains identified as *L. vaginalis* (30 strains) by *16S rDNA PCR-RFLP*, gave an amplicon of 350bp, and for this reason they have been classified in Group IV. Even the corresponding type strains (*L. vaginalis DSM5837*) gave the same result.

According to the results obtained by multiplex PCR-G were carried out subsequent multiplex-PCRs. For each multiplex, the 11 type

strains listed in Song *et al* study's were used as positive controls and not listed type strains as internal negative controls. No amplicon was obtained from not listed type strains.

two hundred fourteen strains out of two hundred forty-four were identified (87.7%), 30 strains have not identified. These are those strains that the RFLP technique has identified as *L. vaginalis*.

The *tuf* gene multiplex PCR was conducted in parallel to Song *et al*. two-step multiplex PCR

The 44 strains previously classified as *L. casei* group by 16S RFLP were analyzed with the *tuf* gene PCR (Ventura).

In contrast to the results obtained in the study of Ventura, we have displayed only one band for each identified species and in particular: about 540bp for *L. rhamnosus*, approximately 200 bp for *L. paracasei* and about 350bp for *L. casei*. However our results have been confirmed by Nucleotide Blast studies, performed with the primers sequences reported by authors.

The results obtained by *tuf* gene multiplex PCR were the same as those achieved by the two-steps multiplex PCR (Song et al., 2000): 25 *Lactobacillus* strains have been identified as *L. rhamnosus* strains and 19 as *L. paracasei*.

8.2 Determination of antibiotic susceptibility profiles

The *in vitro* determination of antibiotic susceptibility of lactobacilli is influenced by the choice of the medium used as well as by the breakpoint MIC. In fact, many authors use different media (Mueller Hinton with 5% lysed horse blood, MRS, LSM, isosensitest, etc) and different standards of interpretation (89).

In our study, we evaluated the *in vitro* activity of various antibiotics by microdilution broth method (16).

The medium used was not the CAMHB (Cation adjusted Mueller Hinton Broth, with 2.5-5% lysed horse blood), as reported in the CLSI M45-A, but LSM broth (89). Most of the *Lactobacillus* strains grow poorly in CAMHB; our previous observations showed that susceptibility-testing results obtained with LSM are more reliable due to a better grow of microorganism (Furneri unpublished data).

Table 8-1 shows for each species of identified *Lactobacillus* sp., the MIC 50/90 $\mu\text{g/mL}$ for the four fluoroquinolones tested.

Table 8-1 *Lactobacillus* species MIC 50/90 [manuscript in preparation]

Bacteria	MIC 50/90 (µg/mL)			
	CIP	OFL	LEV	ULI
<i>L. rhamnosus</i> (n.25)	2/≥32	2/≥32	2/32	2/16
<i>L. gasseri</i> (n.50)	2/≥32	2/≥32	2/32	2/16
<i>L. fermentum</i> (n.16)	2/≥32	2/≥32	2/32	2/16
<i>L. paracasei</i> (n.19)	1/≥32	2/≥32	2/32	2/16
<i>L. vaginalis</i> (n.30)	4/≥32	4/≥32	4/32	4/16
<i>L. delbrueckii</i> (n.23)	4/≥32	4/≥32	4/32	4/16
<i>L. crispatus</i> (n.35)	2/≥32	4/≥32	4/32	4/16
<i>L. acidophilus</i> (n.46)	1/≥32	1/≥32	1/16	1/16

CIP ciprofloxacin; OFL ofloxacin LEV levofloxacin; ULI ulifloxacin

In table 8-2 clinical (CLSI(16) and EUCAST(225)) and not clinical (SCAN(67) and Danielsen (156)) breakpoints values are reported.

Table 8-2 Breakpoints values for *Lactobacillus* spp.

Antibiotics	Breakpoints values (µg/mL)				
	EUCAST(225) Non-species related**		CLSI M45- A(16) <i>Lactobacillus</i> species	SCAN(226) proposed breakpoints	Danielsen et al. proposed breakpoints(156)
Fluoroquinolones	S≤	R>	S	I	R
ciprofloxacin	0.5	1	≤1* 2* ≥4*	4	≥32
ofloxacin	0.5	1	Levofloxacin breakpoints	-	-
levofloxacin	1	2	≤2* 4* ≥8*	-	-
ulifloxacin	-	-	-	-	-

* CLSI M45-A: interpretative criteria adapted from those for *Enterococcus* spp. As published in the current edition of CLSI document M100

**Non-species-related breakpoints. Those have been determined mainly on the basis of pharmacokinetic/pharmacodynamic data, and are independent of the MIC distributions for specific species. They are used in clinical breakpoint development and can be a guide to interpretation in situations where there is no species-specific clinical breakpoint.

According to clinical breakpoints (CLSI(16) and EUCAST(225)), susceptibilities and resistances profiles (percentages and strains numbers) were determined only for ciprofloxacin and levofloxacin.

Table 8-3 Susceptibilities/resistances percentages to fluoroquinolones [manuscript in preparation]

Strains	CIP			OFL*		LEV***			ULI	
	MIC 50/90	%R*	n. ceppi	MIC 50/90	%R	MIC 50/90	R**%	n. ceppi	MIC 50/90	% R**
<i>L. rhamnosus</i> (n.25)	2/≥32	20	5/25	2/≥32	NA	2/32	20	5/25	2/16	NA
<i>L. gasseri</i> (n.50)	4/≥32	76	38/50	4/≥32	NA	4/32	76	38/50	2/16	NA
<i>L. fermentum</i> (n.16)	4/≥32	100	16/16	4/≥32	NA	4/32	100	16/16	2/16	NA
<i>L. paracasei</i> (n.19)	1/≥32	47.4	9/19	2/≥32	NA	2/32	42.1	8/19	2/16	NA
<i>L. vaginalis</i> (n.30)	4/≥32	100	30/30	4/≥32	NA	4/32	100	30/30	4/16	NA
<i>L. delbrueckii</i> (n.23)	4/≥32	100	23/23	4/≥32	NA	4/32	100	23/23	4/16	NA
<i>L. crispatus</i> (n.35)	4/≥32	77.1	27/35	4/≥32	NA	4/32	51.4	18/35	4/16	NA
<i>L. acidophilus</i> (n.46)	2/≥32	45.6	21/46	2/≥32	NA	2/16	41.3	19/46	4/16	NA

CIP ciprofloxacin; OFL ofloxacin; LEV levofloxacin; ULI ulifloxacin

***Eucast Breakpoints: all strains are resistant.**

**** MIC breakpoint not available**

***** M100 S20 CLSI 2011: S= ≤2; I=4; R=≥8**

8.3 Mechanisms of resistance to ciprofloxacin in *L. fermentum*

Four of sixteen *L. fermentum* strains that showed reduced *in vitro* susceptibility or resistance to quinolones ciprofloxacin (assuming as resistant strains with MIC \geq 4 mg/mL) were investigated for their genotypic mechanisms of resistance.

8.3.1 Sequence analysis of *gyrA* and *parC*

QRDR regions of *gyrA* and *parC* genes of four strains of *L. fermentum* with MIC \geq 4 mg/mL for ciprofloxacin were amplified.

The results of the sequencing revealed nucleotide mutations for two strains in the *gyrA* sequence (Table), which have proved to be silent mutations, as they did not result in a change to the amino acid sequence of DNA gyrase and topoisomerase IV.

[manuscript in preparation]

Table 8-4 *gyrA* and *parC* mutation

Strains	MIC CIP	nucleotide mutations		AA mutations
		<i>gyrA</i>	<i>parC</i>	
<i>L. fermentum</i>	8	T237G; C270T; T339C; C396T	No mutation	/
<i>L. fermentum</i>	16	No mutation	No mutation	/
<i>L. fermentum</i>	8	No mutation	No mutation	/
<i>L. fermentum</i>	8	C270T; T339C; C396T	No mutation	/

8.3.2 Ciprofloxacin intracellular accumulation

The graph (Figure 8-1) shows the trend of the intracellular concentrations of ciprofloxacin in the 4 strains of *L. fermentum* investigated both in the absence and in the presence of CCCP (Carbonyl cyanide m-chlorophenyl hydrazone), in a time interval (minutes) between thirty seconds and four hours.

The progressive reduction of concentrations, in the absence of CCCP, suggests the existence of a system of efflux of the drug.

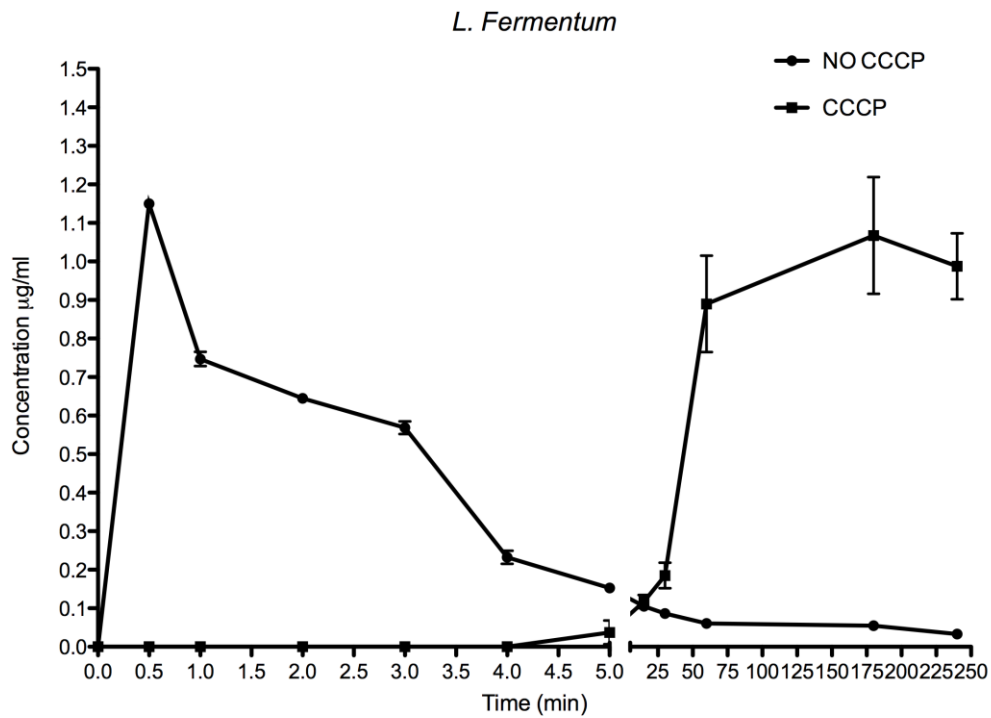


Figure 8-1 Ciprofloxacin accumulation. ● Accumulation without CCCP ■ Accumulation with CCCP. The experiment was repeated twice for each *L. fermentum* strain studied. Standard Deviation for each point is reported.

The accumulation of the drug in the presence of CCCP suggests that the type of pump involved requires an ATP consumption (ABC transporter) or a proton motive force (MFS Major Facilitator Superfamily).[manuscript in preparation]

8.3.3 Inhibitors influence on fluoroquinolones MICs

The MIC results are summarized in Table . The MICs of all of the potential efflux inhibitors against the four strains of *L. fermentum* when tested alone with ciprofloxacin were 8 µg/mL for 3 strains and 16 µg/mL for one strain. For the two channel blockers tested (verapamil 50 µg/mL and reserpine 50 µg/mL) reductions in the MICs were 2 twofold dilution for all isolates.

[manuscript in preparation]

Table 8-5 Inhibitors influence on fluoroquinolones MICs

Strains	Ciprofloxacin MIC (µg/mL)	Ciprofloxacin MIC (µg/mL) + reserpine (50 µg/mL)	Ciprofloxacin MIC (µg/mL) + verapamil (50 µg/mL)
<i>L. fermentum</i>	8	2	2
<i>L. fermentum</i>	8	2	2
<i>L. fermentum</i>	8	2	2
<i>L. fermentum</i>	16	4	4

8.3.4 *L. fermentum* ATCC 14931 genome Analysis

In order to find possible target proteins in the genomic sequence of *L. fermentum*, two “lead” proteins responsible for efflux of quinolones in Gram-positive were chosen: NorA (MFS) found in *S. aureus* and LmrA (ABC) found in *L. lactis*.

Our studies on *L. fermentum* ATCC 14931 genome allowed us to identify two proteins:

GeneBank Reference Sequence: ZP_03944345.1 belonging to MFS superfamily.

```
1  mtrvvkrtin imlvqcfllic lgmsslifpve paikqayhls afdmgvmaal falvqfvasp
61  vvgrvsdkwg rkqmlvwglg ifagaeflfa acnslaafnt sraidglaaa mfvptsmala
121 adittpaqra kvigwlsasf sggililgpgi ggilaadnfk lpfwvagvlv vistivaaiw
181 lpsdekvgtv hheehrqsks tmtalkeiws ptvsllflmi lvaafglagf eaiyslyvng
241 vhgfdlqgia lvtltnngiis lvlqvfcfea mvkwlgelrl vrwayllaav gtvfviydgj
301 awqitlatlv vfeafdllrp aittlltdlg ednqglingm nmsltsvgnv igplmagall
361 drnylypywv viafllvawv ltfavrrrrfr a
```

GeneBank Reference Sequence: ZP_03944067.1 belonging to ABC superfamily

```
1  midralfkklp garsmimglv gldvlqalli igqalflsqz itglwqghal ktvagpiayf
61  alcfigrqli nwnarrlidd fagsvakdmr kqllqkvfal gpeavakkgt gsmvtvtldg
121 isnvedylql tlskivtmmi tpmiliava flnwqsaaam lviypliilf miilgyaaqt
181 radrqyenfq rlsnnfidsl rgidtlkyfg lskrynsif kssesfrkst mdvlkvamls
241 tfaldffttl siaivavylg fglidaeipl fpalatlila pdyflpirnf andyhatldg
301 knsfrdvmei vgqkqapape fklhawqadd qleindlafr yheggkiapl svrlrgyqkv
361 giigmsgsgk ttlinllagf ltpeqgeikf qgqtsatmni adwqhqiyyi pqspyvfaas
421 lrdnvafytp gvsdeevkda ihvvgldd11 adlpagldtm igggkralsg gqaqrialar
481 afldhkrrvm ifdeptahld ieteldlker mlplmenrlv ffathrlhwm kkmdyilvmd
541 hgqlveqgty qellakngyf tkliqqtkge geqdvq
```

The alignments between the amino acid sequence of ZP_03944345.1 with NorA (GeneBank ref. CCE58495.1) sequence (Figure 8-2) and ZP_03944067.1 with LmrA (GeneBank ref.

YP_005868060.1) (Figure 8-3), conducted with the BLAST program (Basic Local Alignment Search Tool), have revealed similarities between the proteins.

The proteinblast alignments results are shown in Table 8-3.

Table 8-6 Proteinblast alignments results

Sequences	ZP_03944345.1 CCE58495.1		ZP_03944067.1 YP_005868060.1	
Query coverage	98%		90%	
Identities	131/387	34%	127/537	24%
Positives	222/387	57%	249/537	46%
Gaps	6/387	2%	36/537	7%

```

ZP_03944345.1 5 VKRTINIMLCVQFLICLGMSLIFVEPAIKQAYHLSAFDMGVMALFALVQFVASPVVCR 64
+ + I ++ FLI LG+ L+ PV P + L+ D+G++ A FAL Q + SP G
CCE58495.1 1 MNQIVLYFNIFLIFLGIGLVIPVLEDLGLTGSDLGLLVAFALSQMIISPFCGT 60

ZP_03944345.1 65 VSDKWRKQMLVGLGIFAGAEFLFACNSLAFNTSRAIDGLAAMFVPTSMALAADIT 124
++DK G+K ++ GL +F+ +EF+FA ++ + SR I G++A M +P L ADI+
CCE58495.1 61 LADKLGKKLICIGLILFSAEFMFAVGHNFSILMSRIIGMSAGMVMPGVTGLIADIS 120

ZP_03944345.1 125 TPAQRAKVIGWLSASFSGLILGPGIGILADNFKLPFWACVLGVISTIVAAIWLPSD 184
Q+AK G++SA + G ILGPGIGG +A + +LFF+ AG LG+++ I++ I +
CCE58495.1 121 PSHQKAKNFGMSAINSGFILGPGIGFMAEVSHRLPFFAGLGIVAFIMSVILLYDP 180

ZP_03944345.1 185 EKVGVTHEEHRQEKSTMTALKEIWSPTVSLLFLMILVARFLAGFEAISLYVNQVEGF 244
+K T ++ + T K +P + + LV AFGL+ FE ++SLY + +
CCE58495.1 181 KKSTTGFQKLEPQLLTKINWKVFITPAI-----LTLVLAFGLSAFETLFSLYTSYKVNY 235

ZP_03944345.1 245 DLGQIALVLTLNGIISLVLQVFCFEAMVKWLGELRLVRWALLAAVGTVFIYDGIAWQI 304
I++ +T GI + Q++ F+ +K+ EL + W+ L + + + +++ W I
CCE58495.1 236 TPKDISIAITGGIFGAIFQIYFFDKFMKYFSELTFIAWSLLYSVIVLLLVFANCYSI 295

ZP_03944345.1 305 TLATLVFEAFDLLRPAITLLTDLGEDNQGLINGMNSLTSVGNVIGPLMAGALLDRNY 364
+ + +VF FD++RPAIT +++ D QG G+N + TS+GN IGPL+AGAL D +
CCE58495.1 296 MIISFIVFIGFDMIRPAITNFSNIAGDRQGFAGLNSTFTSMGNFIGLIAGALFDVHI 355

ZP_03944345.1 365 LYPWVVIAFLLVANVLTFAVRRRFRA 391
P ++ IA L A ++ + ++ RA
CCE58495.1 356 EAPIYMAIAVSL-AGIIVLIEKQHRA 391

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Figure 8-2 Amino acid sequence alignments between ZP_03944345.1 with NorA (GeneBank ref. CCE58495.1)


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ZP_03944067      47  GHALKTVAGPIAFALCFIGRQLINWFNARRLDFAGSVAKDMRKQLLKVFALGPEAVA      106
GH + G +A +IG ++ A L F SV K++R ++ K+ L +
YP_005868060.1  67  GHGVN--GGVALVIALYIGSAAVSAIAIVLGIFGSVVKNLRTRVWDKMILPVKYFD      124
ZP_03944067      107 KKGFGSMVTVTLDGISNVEDYLQLTSKIVTMITPMILLIAVAFNWSAIMLVIYPL      166
+ TG M + + + V++ + ++ + T ++ V ++ + + W+ M++ P+
YP_005868060.1  125 EVKTGEMSRLANDITQVKNLIANSIPOAFTSILLVSIVFMLQMWRLTAMIAVFV      184
ZP_03944067      167 ILFMI-ILGYAQTRADRQ--YENFORLSNFIDSLRGIDTLKFGLSKRYS-----      216
++L M I+ + + RQ NFQ +++ +SL I +K K+ S
YP_005868060.1  185 VMLTMFPIMTFGKIGRTRQDSLANFOGIAS--ESLSEIRLVKSSNAEKQASKKAENDV      241
ZP_03944067      217 NSIFKSSEFRKSTMD-VLKVMLSTFALDFETLSIAIVAVLGFGLIDAEIPLFPALA      275
N+++K +++ D ++ M+ + L F L+ I + G + + + L
YP_005868060.1  242 NALYKIG--VKEAIFDGLMSPVMLSMMLIFGLLAYGILISTGVMSLGTLGMMYIM      299
ZP_03944067      276 TLLLAPDYFLPIRNEANDYHATLDGKNSFRDVEIVGQRQAPAPEFKLHAWQADD-----      331
LI A + A + S + E++ ++Q LH ++ D
YP_005868060.1  300 NLIGA-----VPTVAFFTELAKASTGRLTELDEEQEV-----LHQESLDLEGKT      348
ZP_03944067      332 LEINDLAFRVHEGGKI--APLSVRLGYQKVGIGMSGSGKTLINLLAGLTPEQEIKF      390
L + F Y + +I +S + + G SG GK+T+ +LL F P GEI
YP_005868060.1  349 LSARHVDFAYDDSEQILRDISFEAQPNSIIAFAGPSGGKSTIFSLLERFYQPTAGEITI      408
ZP_03944067      391 QGQTSATMNIADWHQITVIPQSPVVFASLRDNVAFYTPG--VSDEVKDAIHVVGLDL      449
GQ +++ ++Q I ++ Q + A ++R++ + G +DE++ + +
YP_005868060.1  409 DGQPDNISLENWRSQIGFVSQSAIMAGTIRENLTYGLEGDYTDELWVLDLAFARSF      468
ZP_03944067      450 LADLPAGLDMIGGKRALSGQQRIALARAFLDHKRRVMIFDEPTAHLDIELDLKE      509
+ ++P L+T +G +SGG QR+A+ARAFI + +++ DE TA LD E+E +++
YP_005868060.1  469 VENMPDQLNTEVGERGVKISGQRIAIARAFLRNP--KILMLDEATASLDSESESMVQR      527
ZP_03944067      510 RMLPLMENLVFFAHRLHMKRMDYLVMDHGOLVEQTYQELLAKNGYFTKLIQQ      566
+ LM+ R HRL + D I ++ GQ+ G + EL+A + + K + +
YP_005868060.1  528 ALDSLMKGRTTVIAHRLSTIVDADKIYFIEKQITSGKHNELVATHPLYAKYSE      584

```

Figure 8-3 Amino acid sequence alignments between ZP_03944067.1with LmrA (GeneBank ref. YP_005868060.1)

9 Discussion

9.1 Strains identification

Both prokaryotic and eukaryotic organisms are classified by their phenotypic similarities and differences. The exclusive use of phenotypic methods for the taxonomic classification may be insufficient because of the variability of their phenotypic characteristics. About three decades ago, Carl Woese et al. began to analyze the sequence of 16S rDNA of different bacteria, using the sequencing technique. The sequences obtained were used for the first time in phylogenetic studies (108)

In the last three decades, the invention of PCR and DNA sequencing have led to an extensive collection of genes sequences coding for rRNA of the small ribosomal subunits of many living organisms. By the comparison of these sequences, was easy to deduce that the rDNA genes are highly conserved between organisms of the same species. Using 16S rDNA sequences, numerous genera and species of bacteria have been renamed and reclassified, and has been possible to classify non-cultivable species and the discovery of new ones. Given the considerable advances in PCR and sequencing, the use of 16S rDNA sequence was not given

only to research, but has entered the routine clinical microbiology laboratory.

In the present study, we wanted to compare the use of different molecular techniques to identify species of lactobacilli of human origin. The molecular target used was the gene 16S rDNA associated with the RFLP technique. The combination of a highly sensitive method such as PCR with the use of specific endonuclease, allowed us to achieve a saving, simple and reproducible laboratory practice. Our results showed that the 16S rDNA-RFLP technique (13) has clearly identified all the species found, except for those belonging to the group *L. casei* (*L. casei*, *L. rhamnosus*, *L. paracasei*), which showed very similar restriction profiles. Virtual restriction maps carried out by digesting the 16S rDNA sequences of the respective type strains, deposited in GenBank, have highlighted this problem.

The study of Song et al. (14), have been effectively identified 11 species of lactobacilli, including *L. paracasei* and *L. rhamnosus* by multiplex PCR. The target region was the 16S-23S rDNA-ITS and the area flanking the 23S rDNA since the only region 16S rDNA-ITS-23S rDNA is not variable enough to allow the design of species-specific primers. The results obtained were all coincident

with those obtained by PCR-RFLP technique 16SrDNA. Since the multiplex PCR technique has the advantage of reducing working time, and it has been preferred in the preliminary identification study.

According to the study of Ventura et al, another molecular target used was the *tuf* gene (15). They demonstrated that the phylogenetic analysis of lactobacilli and bifidobacteria, established through the use of *tuf* genes sequences, it is valid to the same extent as the one obtained from the comparison of 16S rRNA sequences. In our study, the method was used to discriminate species belong to *L. casei* group (*L. paracasei* and *L. rhamnosus*), thereby overcoming the limit exhibited by the method 16S rDNA PCR-RFLP.

9.2 Lactobacilli resistance profiles distribution

The *in vitro* activity of quinolones against Lactobacilli is quite variable. While older quinolones are almost inactive, the *in vitro* activity of new quinolones is variable. Variability in susceptibility profile has been reported by numerous strains depending of source. Vaginal lactobacilli have been reported as resistant to ciprofloxacin (227), or intermediate to resistant by Herra et Al. 1995 (228), and resistant to ofloxacin by Choi et al(229). Lactobacilli from other apparatus, including those from bacteriema have shown a more variable MIC range from susceptibility to resistance (65) (179) (230) (231).

Lactobacilli used as probiotics or from starter culture appear to be more susceptible to ciprofloxacin than those of human origins(156). Although all the strains were resistant to norfloxacin, and to nalidixic acid (168). In a molecular orientated study ciprofloxacin resistance of lactobacilli used as starter or as probiotic was reported higher as than 70%, indicating that these may constitute intrinsic resistance. Unlikely, the genetic basis of a such resistance could not be demonstrated, since no mutations typical of quinilones resistance

were detected in the quinolone determining regions of the *parC* and *gyrA* genes(17).

Lactobacilli from gastrointestinal apparatus showed MICs to moxifloxacin randomly distributed from less than 0.12 to ≥ 16 $\mu\text{g/mL}$ (Table 9-1)(169).

Table 9-1 Distribution of MICs to the fluoroquinolone moxifloxacin for Lactobacillus species of the human gastrointestinal tract (modified from (169))

Antibiotic	Species	No. of strains	Number of isolates for which MIC ($\mu\text{g/mL}$) was as follows:							
			≤ 0.12	0.25	0.50	1	2	4	8	≥ 16
Moxifloxacin	<i>L.gasseri</i>	20				1	5	8		6
	<i>L. delbrueckii</i>	8		1	1			4		2
	<i>L. casei/</i>									
	<i>L. paracasei</i>	7		1	4		2			
	<i>L. rhamnosus</i>	5		3	2					
	<i>L. acidophilus</i>	2					2			
	<i>L. plantarum</i>	1								1
	<i>L. parabuchneri</i>	1					1			
	<i>L. brevis</i>	1								1
	<i>L. vaginalis</i>	1								1

A study of the effect of ciprofloxacin on the vaginal microbiota of healthy people and patients with bacterial vaginosis, showed no significant changes in the microbiota of both groups (232).

In order to assign susceptibilities/resistances percentages, only clinical breakpoints can be used (CLSI(16) and EUCAST(225)).

Charts 9-1 (ciprofloxacin) and 9-2 (levofloxacin) show resistance percentages for the *Lactobacillus* spp. investigated.

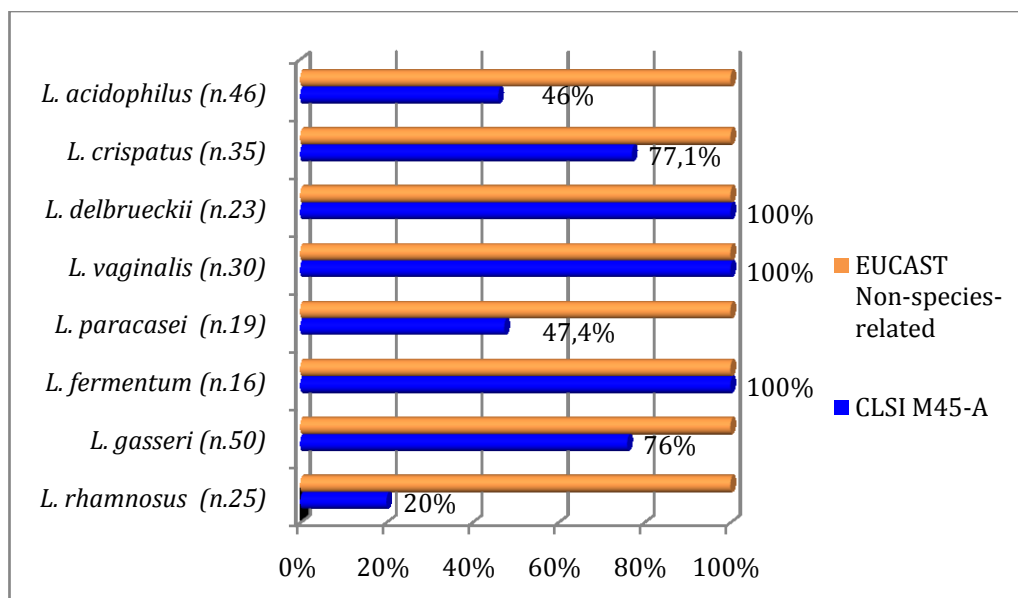


Chart 9-1 Susceptibilities/resistances percentages to ciprofloxacin

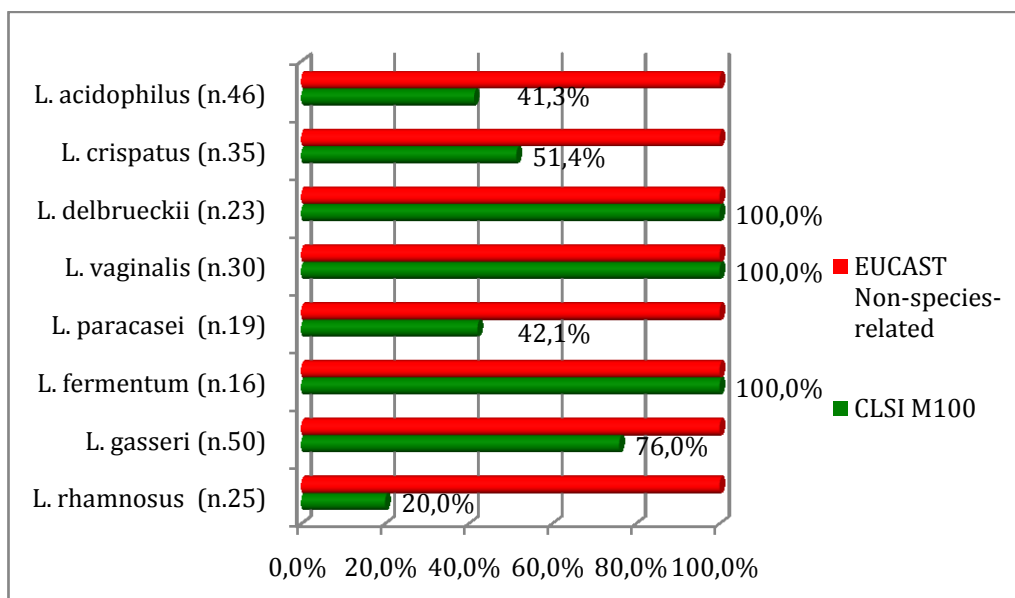


Chart 9-2 Susceptibilities/resistances percentages to levofloxacin

According to EUCAST breakpoints resistance values (ciprofloxacin 1 µg/mL and levofloxacin 2 µg/mL), all strains are resistant for both fluoroquinolones. The values used from EUCAST are “non-species-related breakpoints”. This have been determined mainly on the basis of pharmacokinetic/pharmacodynamic data, and are independent of the MIC distributions for specific species. They are used in clinical breakpoint development and can be a guide to interpretation in situations where there is no species-specific clinical breakpoint (225).

On the other hand CLSI breakpoints for ciprofloxacin increase the variability of the percentages of resistance among the *Lactobacillus* species studied (≥ 4 µg/mL).

The resistance value for levofloxacin ($\geq 8 \mu\text{g/mL}$) is not specific for the genus *Lactobacillus*, but it adapted from those for *Enterococcus spp.* As published in the current edition of CLSI document M100 (16).

[manuscript in preparation]

9.3 Possible mechanism of ciprofloxacin resistance

The resistance to fluoroquinolones has been studied also at genotypic level. In particular, amplification and subsequent sequencing of the QRDR of *gyrA* and *parC* genes of four *L. fermentum* considered as ciprofloxacin-resistant ($\text{MIC} \geq 4$), has confirmed the data obtained in a study of Fukao et al. (12), i.e. the absence of mutations in amino acid sequence of DNA gyrase and topoisomerase IV proteins which are associated with resistance. In the literature there is only one work (17) that contains amino acid substitutions in *gyrA* sequence (Glu87 to Leu) of an *L. acidophilus* “starter strain”, but this mutation was not associated with quinolones resistance.

Since the alteration of the pharmacological target is only one of the possible mechanisms of resistance to quinolones, the high MIC

values found for ciprofloxacin may be explained by admitting the existence of other mechanisms of resistance in lactobacilli, such as the alteration of the cell wall permeability or a multi-drug efflux systems.

In order to investigate the possible mechanism of ciprofloxacin resistance, the same strains of *L. fermentum* previously studied for *gyrA* and *parC* mutation with MIC values ≥ 8 $\mu\text{g/mL}$ were chosen for fluoroquinolone accumulation studies. These studies suggest that changes in intracellular concentrations of ciprofloxacin can be due to an efflux mechanism that explains the high MIC values. The block efflux in the presence of CCCP, a mitochondrial uncoupling, suggests that the type of pump is involved or active type (ATP consumption), or requires a proton motive force (221)(222).

In addition the influence of different pumps inhibitors (reserpine and verapamil) was also evaluated by microdilution broth. Unfortunately, the reductions of the MIC values for ciprofloxacin in the presence of the two blockers (2 fold) does not allow to discern the type of pump involved (MFS or ABC), for this reason we supposed the presence of more than one transporter involved in the efflux of ciprofloxacin. This hypothesis is supported by the study of

Lactobacillus fermentum ATCC 14931 genome, where the presence of about 40 transporters have been reported.

The analysis of *L. fermentum* ATCC14931 genome allowed us to identify two proteins (ZP_03944345.1 and ZP_03944067) that have significant homology (see Table 8-3) with NorA (*S. aureus*) and LmrA (*L. lactis*) involved in quinolones efflux. To confirm the hypothesis of a possible involvement of these two proteins in ciprofloxacin resistance in *L. fermentum*, CD (Conserved Domains) analysis was carried out. Domains can be thought of as distinct functional and/or structural units of a protein. These two classifications coincide rather often, as a matter of fact, and what is found as an independently folding unit of a polypeptide chain also carries specific function. Domains are often identified as recurring (sequence or structure) units, which may exist in various contexts.

The Conserved Domain Database from NCBI is a resource for the annotation of functional units in proteins. Its collection of domain models utilizes 3D structure to provide insights into sequence/structure/function relationships (233) (234)(235).

The analysis of the CD (Conserved Domains) of ZP_0394435.1 (Figure 9-1) shows conserved domain analogies to the EmrB protein of *E. coli*. This subfamily of drug efflux proteins, a part of

the major facilitator family, is predicted to have 14 potential membrane-spanning regions. Members with known activities include EmrB (multiple drug resistance efflux pump) in *E. coli*, FarB (antibacterial fatty acid resistance) in *Neisseria gonorrhoeae*, TcmA (tetracenomycin C resistance) in *Streptomyces glaucescens*, etc. In most cases, the efflux pump is described as having a second component encoded in the same operon, such as EmrA of *E. coli* involved in cellular processes, toxin production and resistance, transport and binding proteins, etc. (233) (234)(235).

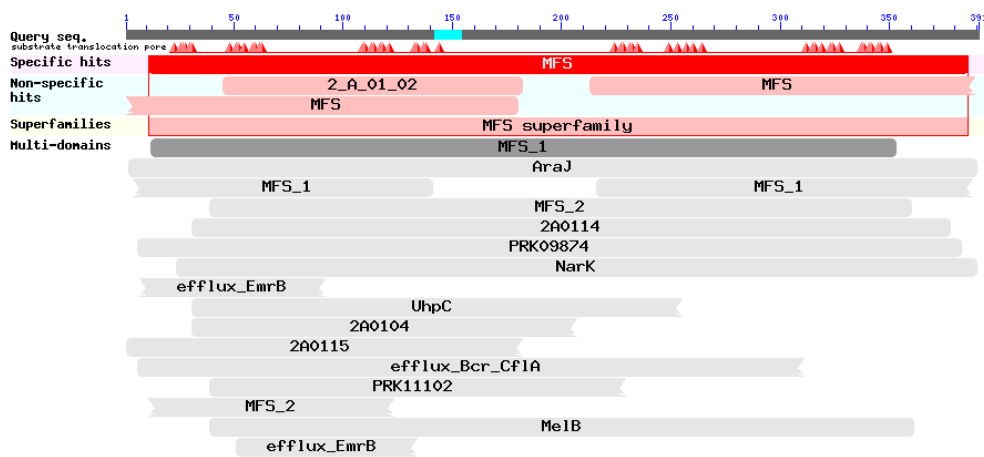


Figure 9-1 Conserved domains on ZP_03944345.1: MFS family major facilitator transporter [Lactobacillus fermentum ATCC 14931]

The analysis of the CD (Conserved Domains) of ZP_03944067.1 (Figure 9-2) shows conserved domain analogies to the ABCC_MRP subfamily. The MRP (Multidrug Resistance Protein)-like transporters are involved in drug, peptide, and lipid export. They

belong to the subfamily C of the ATP-binding cassette (ABC) superfamily of transport proteins. The ABCc subfamily contains transporters with a diverse functional spectrum that includes ion transport, cell surface receptor, and toxin secretion activities. The MRP-like family, similar to all ABC proteins, have a common four-domain core structure constituted by two membrane-spanning domains, each composed of six transmembrane (TM) helices, and two nucleotide-binding domains (NBD). ABC transporters are a subset of nucleotide hydrolases that contain a signature motif, Q-loop, and H-loop/switch region, in addition to, the Walker A motif/P-loop and Walker B motif commonly found in a number of ATP- and GTP-binding and hydrolyzing proteins (233) (234)(235).

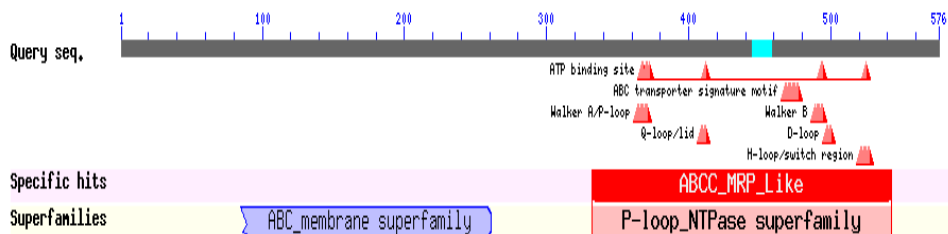


Figure 9-2 Conserved domains on ZP_03944067: ABC superfamily ATP binding cassette transporter, ABC/membrane protein [Lactobacillus fermentum ATCC 14931]

[manuscript in preparation]

10 Future outlooks

In order to discriminate the type of protein involved in the quinolones efflux mechanism, studies of characterization of this proteins are currently underway in collaboration with Professor Patrizia Brigidi and Dr. Beatrice Vitali University of Bologna.

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