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ABSTRACT

In this study, we isolated probiotic yeasts and lactic acid bacteria (LAB) from different microbial sources. Eighty-one (81) yeasts and seventy (70) LAB isolates were randomly selected and identified from fecal samples of poultry feces and healthy Iraqi infants, respectively.

The yeast strains were obtained from a farm of broiler chickens located in the city of Lille. They were clustered into 22 groups by GTG_5-rep PCR technique, then identified as *Debaryomyces hansenii*, (teleomorph of *Candida famata*) species using the biochemical ID-32C system and molecular sequencing of 26S rDNA and ITS1-5.8-ITS2 rDNA region methods.

Only one yeast strain, designated as *Candida famata* Y.5 (*C. famata* Y.5), exhibited antimicrobial activity against *Listeria innocua*. For more accurate discrimination, the antagonistic strain *C. famata* Y.5 was identified by MALDI-TOF-MS technology. Further characterization of this anti-*Listeria* strain, permitted to unveil its probiotic potential. Thus, *C. famata* Y.5 appeared to be a non-hemolytic strain. *In vitro* tests of cytotoxicity and adhesion on human Caco-2 epithelial cells confirmed the safety traits of this strain. *C. famata* Y.5 displayed good surface properties, especially auto-aggregation, in addition to high survival ability under harsh conditions mimicking those of the gastrointestinal tract (GIT).

The LAB strains were isolated from fecal samples of a group of Iraqi children living in the north of France. LAB strains were obtained from six blind donors and then identified as 41 cocci and 29 bacilli. Two strains displayed antagonistic activities against Gram-positive bacteria (GPB) including: *Listeria monocytogenes*, *Staphylococcus aureus*, methicillin-resistant *Staphylococcus aureus* (MRSA), and *Clostridium perfringens* but not against fungi or Gram-negative bacteria (GNB), except for *Salmonella* Newport. The biochemical, MALDI-TOF-MS, and molecular (16S rDNA sequencing) methods identified these two strains as *Enterococcus faecalis* B3A-B3B and B20A-B20B . Bacteriocin produced by strain B3A-B3B, designed as

enterocin B3A-B3B, was purified by a simplified two-step procedure including a liquid-liquid phase extraction and reverse phase high-performance liquid chromatography (RP-HPLC). The predicted molecular mass of this enterocin consists of two peptides of 5,176.31 Da (B3A) and 5,182.21 Da (B3B). Notably, B3A-B3B hampered the biofilm installation of *L. monocytogenes* strain grown on AISI 304 stainless steel slides. The treatment of stainless steel with nisin (1 mg. ml^{-1} or 16 mg. ml^{-1}) diminished the cell numbers by about 2 logs CFU. ml^{-1}, preventing therefore the biofilm formation by *L. monocytogenes* 162 or by its nisin-resistant variant *L. monocytogenes* 162R. Further combination of nisin and B3A-B3B enterocin reduced the MIC value needed to inhibit this pathogen about 2 logs CFU. ml^{-1}.

To gain insights on the probiotic profile of the *E. faecalis* B3A-B3B strain, the whole genome was sequenced and *in silico* analysis was performed and compared with those of clinical strains as *E. faecalis* MMH594, *E. faecalis* V583, and *E. faecalis* OG1RF from humans, and also compared to that of the well-known probiotic *E. faecalis* Symbioflor1 strain. Even harboring *gelE*, *cpd*, *efaAfm*, *ccf*, *agg*, and *cob* coding for virulence factors, the B3A-B3B strain resulted to be sensitive to most antibiotics tested here, non-cytotoxic, non-hemolytic, and devoid of inflammatory effects. Moreover, B3A-B3B strain showed remarkable hydrophobicity, auto-aggregation, adhesion to human Caco-2 cells, viability in simulated GIT conditions, and cholesterol assimilation. These features together introduce the *E. faecalis* B3A-B3B strain as an interesting probiotic candidate.

RESUME

Dans cette étude, nous avons isolé des levures et des bactéries lactiques (BL) potentiellement probiotiques, à partir de différents écosystèmes microbiens. Quatre-vingt-une (81) levures, et soixante-dix (70) BL, ont été isolées et identifiées à partir de matières fécales animales (poulet) et humaines (enfants Irakiens en bonne santé).

Ainsi, les souches de levures ont été isolées à partir de matières fécales de poulets, dans une ferme située dans la région de Lille (France). Elles ont été regroupées en 22 groupes par la technique de Rep-PCR utilisant une amorce unique 5'-GTG5-3', puis identifiées comme appartenant à l'espèce *Debaryomyces hansenii* (téléomorphe de *Candida famata*) en utilisant des méthodes biochimique (système ID-32C) et moléculaire (séquençage de l'ADNr 26S et les régions ITS1-5.8-ITS2 de l'ADNr). Dans le criblage des activités antibactériennes, seule la souche nommée, *Candida famata* Y.5, a montré une activité contre *Listeria innocua*. L'identification de cette souche a été confirmée par la méthode robuste de MALDI-TOF-MS. Une ample caractérisation de cette souche, a permis de révéler son potentiel probiotique. Ainsi *C. famata* Y.5 est non-hémolytique, non-cytotoxique et présente une capacité d'adhésion remarquable sur les cellules Caco-2 épithéliales. Cette souche s'avère posséder des propriétés de surface intéressantes en particulier les capacités d'auto-agrégation, et de survie dans les conditions du tractus gastro-intestinal.

Comme précédemment indiqué, les bactéries lactiques, quant à elles, ont été isolées à partir d'échantillons fécaux, provenant d'un groupe d'enfants irakiens résidant dans le nord de la France. 70 souches lactiques ont été obtenues à partir de six donneurs, celles-ci ont été caractérisées comme étant 41 cocci et 29 bacilles avec une coloration différentielle de Gram positive et une absence de catalase. Le criblage des activités antagonistes, a permis de mettre en évidence une activité contre des bactéries à Gram-positif comprenant *Listeria monocytogenes*, *Staphylococcus aureus*, *Staphylococcus aureus* résistant à la méthicilline (SARM), et *Clostridium perfringens*, mais pas contre des champignons microscopiques ou les bactéries à Gram-négatif (GNB), à l'exception de *Salmonella* Newport. L'utilisation de plusieurs méthodes d'identification comme les méthodes biochimiques, et moléculaires (séquençage de l'ADNr 16S et MALDI-TOF-MS) ont permis d'identifier avec certitude ces deux

souches comme appartenant à l'espèce *Enterococcus faecalis*. Ces souches sont alors nommées *E. faecalis* B3A-B3B et B20A-B20B. L'activité antagoniste est attribuée à une activité bactériocinogénique. Ainsi, la bactériocine produite par la souche B3A-B3B est désignée entérocine B3A-B3B. Celle-ci a été purifiée par une procédure en deux étapes comportant une extraction en phase liquide-liquide, suivie par une chromatographie liquide à haute performance en phase inversée (HPLC-RP). Les masses moléculaires prédites pour les deux peptides composant cette entérocine seraient de 5 176,31 Da (B3A) et 5 182,21 Da (B3B). Par ailleurs, cette entérocine a montré une activité limitant l'installation de biofilms de *L. monocytogenes* cultivées sur des lames AISI 304 en acier inoxydable. Le prétraitement de ces lames avec de la nisine à 1 ou 16 mg. ml^{-1} permet de réduire le nombre de cellules bactériennes sur cette surface d'environ 2 logs UFC. ml^{-1}, perturbant ainsi la formation de biofilms par *L. monocytogenes* 162 ou son variant résistant à la nisine, appelé *L. monocytogenes* 162R. En plus la combinaison des deux bactériocines (nisine et entérocine B3A-B3B) permet de réduire la valeur de la concentration minimal inhibitrice (CMI) nécessaire pour inhiber ce pathogène d'environ 2 logs CFU. ml^{-1}. Pour déterminer le profil probiotique de la souche *E. faecalis* B3, son génome a été séquencé et comparé aux génomes des souches cliniques notamment *E. faecalis* MMH594, *E. faecalis* V583 et *E. faecalis* OG1RF d'origine humaine, et au génome de la souche probiotique *E. faecalis* Symbioflor1. Même si six gènes codant pour des facteurs de virulence, *gelE, cpd, efaAfm, ccf, agg,* et *cob*, ont été retrouvés dans l'ADN de notre souche, cell-ci s'est globalement avérée sensible aux antibiotiques utilisés dans cette étude, non-cytotoxique, non-hémolytique et dépourvue d'effets inflammatoires sur les lignées cellulaires de type Caco-2. En outre, *E. faecalis* B3A-B3B a montré des caractéristiques d'hydrophobicité, d'auto-agrégation, d'adhésion aux cellules Caco-2, un taux de survie aux conditions gastro-intestinales simulées, et une capacité d'assimilation du cholestérol remarquables. Les résultats obtenus laissent à penser et considérer la souche *E. faecalis* B3A-B3B comme une souche avec un potentiel probiotique intéressant.

Table of contents

Acknowledgements II
Scientific valorization IV
ABSTRACT V
List of figures XI
List of tables XII
List of frequently used abbreviations XIII
General Introduction 1
Chapter 1. Literature Review 6
1. Microbial community in gastrointestinal tract and feces 6
1.1. Development of gut microbiota 7
1.1.1. Neonate and infancy 8
1.1.2. Early 8
1.1.3. Adult 9
1.1.4. Elderly 9
1.2. Factors affecting microbiota development and composition 9
1.2.1 Maternal microbiota 10
1.2.2 Mode of delivery 11
1.2.3 Mode of feeding 11
1.2.4. Geographical location and environment 12
1.2.5 Antibiotics, probiotics and prebiotics 13
1.2.6. Other factors 14
2. Host microbe interactions 14
2.1. Host–microbiota cross-talk 17
2.2. Microbial adhesion and interaction with pathogens 17
3. Probiotics 18
3.1. What are probiotics? 18
3.2. Probiotic criteria and safety assessment 19
3.3. Probiotics and their health effects 23
4. Fecal origin microbes with probiotic potential 25
4.1. Yeasts as probiotics 26
4.1.1 General characteristics of yeast 26
4.1.2. *Saccharomyces cerevisiae* var. *boulardii* 28

4.1.3. *Debaryomyces hansenii* (*Candida famata*) 29
4.1.3.1. Taxonomy, morphology and physiology 29
4.1.3.2. Biochemical properties with industrial importance and applications 31
4.1.4. Probiotic and antagonistic potential of *Debaryomyces hansenii* 34
4.2. Lactic acid bacteria as probiotics 36
4.2.1. General characteristics of lactic acid bacteria 36
4.2.2. Taxonomy of the genus *Enterococcus* 36
4.2.3. Phenotypic characteristics of *Enterococcus* genus 38
4.2.4. Genotypic characterization of the genus *Enterococcus* 40
4.2.5. Physiological properties 44
4.2.6. Biochemical properties with industrial importance and applications 45
4.2.6.1. Lactic acid production 46
4.2.6.2. Proteolytic activity 47
4.2.6.3. Lipolytic activity 48
4.2.6.4. Citrate and pyruvate metabolism 48
4.2.6.5. Production of volatile compounds 50
4.2.6.6. Bacteriocin production 51
4.2.6.6.1 Class I bacteriocins (Lantibiotics) 52
4.2.6.6.2 Class II bacteriocins 54
4.2.6.6.3 Class IIa bacteriocins (pediocin-like peptides) 54
4.2.6.6.4 Class IIb bacteriocins (two-peptide bacteriocins) 56
4.2.6.6.5 Class IIc bacteriocins (circular bacteriocins) 60
4.2.6.6.6 Class IId bacteriocins (unmodified, linear, non-pediocin-like bacteriocins) 60
4.3 Enterococci as probiotics 61
Chapter 2. Yeasts from fecal samples of chicken displayed anti-*Listeria* activities and further probiotic properties 65
Chapter 3.Enterocin B3A-B3B produced by LAB collected from infant feces: potential utilization in the food industry for *Listeria monocytogenes* biofilm management 94
Chapter 4. Bacteriocinogenic LAB from the feces of Iraqi infants and their potential as probiotics 139
General Conclusions and Prospects 176
References 181

List of figures

Figure 1. Distribution and variations in microbial numbers and composition across the length of the GIT.

Figure 2. Factors influencing the development of infant, adult, and elderly gut microbiota.

Figure 3. Pattern recognition receptors (PRRs) signaling promotes immune homeostasis.

Figure 4. Some examples of commonly used prokaryotic and eukaryotic genera as probiotics.

Figure5. Guidelines for the Evaluation of Probiotics for Food Use.

Figure 6. Probiotics effect on animal health and production.

Figure 7. A commercial probiotic product for human use containing 32 probiotic strains of bacteria and yeasts including *D. hansenii*.

Figure 8. Phylogenetic position of the genus *Enterococcus* demonstrated by 16S rRNA-dendrogram of Gram-positive genera.

Figure 9. Schematic pathway showing the metabolic relationships between citrate and glucose.

Figure 10. Diagramatic representation of *Enterococcus faecalis* Fly1 type 1 lantibiotic.

Figure 11. The model of killing by Man-PTS targeting and immunity of Class IIa bacteriocins.

Figure 12. A structural model of lactococcin G and its orientation in target-cell membranes.

Figure 13. Some commercial products of *Enterococcus faecalis* validated as probiotics.

Figure 14. The chicken gut microbiome.

Figure 15. The rep-PCR DNA fingerprints generated in 1.0% agarose gel, requested by the use of the GTG5 primer 5'–GTGGTGGTGGTGGTG- 3'.

Figure 16. (a) RP-HPLC chromatogram of the bacteriocin purification with a zoom on the active peak corresponding to the enterocin B3A-B3B. (b) Mass spectrometry of enterocins B3A-B3B.

Figure 17. Alignments of the 3' ends of structural genes coding for enterocins.

Figure 18. Mode of action of Nisin.

Figure 19. Some mechanisms and genes involved in bacterial resistance to bacteriocin of Gram positive bacteria.

Figure20. *E. faecalis* cytolysin expression.

Figure 21. The main mechanisms of *Enterococcus* spp. antibiotic resistance.

List of tables

Table 1. Some beneficial effects of probiotics on human health.

Table 2. Assimilation of different substrates by *D. hansenii*.

Table 3. Phenotypic properties of recently identified *Enterococcus* species.

Table 4. Species of *Enterococcus* and genotyping methods used for their identification.

Table 5. Physiological properties of validly described *Enterococcus* species.

Table 6. Effect of enzymes on antilisterial activity of neutralized cell free supernatant (CFS) and semi-purified enterocin B3A-B3B (SP).

Table 7. Spectrum registered with the cell-free supernatant (CFS) before its neutralization (BN) and after its neutralization (AN) as well as that obtained with the semi-purified bacteriocin (SP).

Table 8. Determination of minimum inhibitory concentrations (MIC) of Enterocin B3A-B3B.

Table 9. Minimum inhibitory concentrations (MICs) of Nisin and Enterocin B3.

Table 10. Numbers of LAB isolates in the fecal samples of Iraqi infants.

Table 11. Virulence genes present in *E. faecalis* B3.

Table 12. Antibiotic susceptibility of *E. faecalis* B3A-B3B strain in comparison with a pathogenic strain *E. faecalis* ATCC29212.

List of frequently used abbreviations

AAD	Antibiotic-Associated Diarrhea
ABC	ATP-Binding Cassette
AEDS	Atopic Eczema/Dermatitis Syndrome
AFLP	Amplified Fragment Length Polymorphism
AID	Acute Infections Diarrhea
AMPs	Antimicrobial Peptides
AN	Antimicrobial Activity Before Neutralization Of Supernatant With 1M NaOH
ATP	Adenosine Triphosphate
ATCC	American Type Culture Collection
a_w	Water Activity
BN	Antimicrobial Activity Before Neutralization Of Supernatant
CDC	Centre For Disease Control And Prevention
CFS	Cell Free Supernatant
CFU	Colony Forming Unit
Cls	Cardiolipin Synthase
CM	Cytoplasmic Membrane
cpn60	60-Kilodalton Chaperonin
CW	Cell Wall
DBPC	Double-Blind, Randomized, And Placebo-Controlled
DC	Dendritic Cell
DMDS	Dimethylsulphide
DMTS	Dimethyltrisulphide
DNA	Deoxyribonucleic Acid
ECDC	European Centre For Disease Prevention And Control
ED	Entner-Doudoroff
EFSA	European Food Safety Authority
EMP	Embden-Meyerhof-Parnas
EPS	Extracellular Polymeric Substances
FAO	Food And Agriculture Organisation
FIC	Fractional Inhibitory Concentration
gad	Glutamate Decarboxylase
GdpD	Glycerophosphoryl Diester Phosphodiesterase
GF	Germfree
GIT	Gastro Intestinal Tract
GNB	Gram Negative Bacteria
GPB	Gram Positive Bacteria
GRAS	Generally Recognized As Safe
h	Hour
HIV	Human Immunodeficiency Virus
IBD	Inflammatory Bowel Disease
IBS	Irritable Bowel Syndrome
IgE	Immunoglobulin E

IGS	Intergenic Spacer
IGSAF	Intergenic Spacer rDNA Amplification and *AluI* Fingerprinting
IL	Interleukin
imm	Immunity
ITS	Intergenic Transcribed Spacer
kDa	Kilodalton
LAB	Lactic Acid Bacteria
LAPase	Leucine Aminopeptidase
LC	Laboratory Collection
LTA	Lipoteichoic Acid
M	Molar Concentration
MAI	Medium After Incubation
MALDI-TOF-MS	Matrix-Assisted Laser Desorption/Ionization-Time of Flight-Mass Spectrometry
Man-PTS	Mannose Phosphotransferase System
MBI	Medium Before Incubation
MIC	Minimum Inhibitory Concentration
MLN	Mesenteric Lymph Node
MLSA	Multilocus Sequence Analysis
MRS	de Man, Rogosa And Sharpe
MRSA	Methicillin Resistant Staphylococcus Aureus
MsrC	Macrolide–Streptogramin Resistance Protein
MTA	Methyl Thioacetate
MTL	Methanethiol
NEC	Necrotizing Enterocolitis
OVA	Ovalbumin
PBP	Penicillin-Binding Protein
PCR	Polymerase Chain Reaction
PFGE	Pulsed-Field Gel Electrophoresis
pI	Iso-Electric Point
PP	Pentose Phosphate
PRRs	Pattern Recognition Receptors
PYR	Pyrrolidonyl-ß- Naphtylamide
Q–D	Quinupristin-Dalfopristin
QPS	Qualified Presumption Of Safety Of Microorganisms In Food And Feed
RAPD	Randomly Amplified Polymorphic DNA
rDNA	Ribosomal DNA
RegIIIγ	Regenerating Islet-Derived 3 Gamma
rep-PCR	Repetitive Element Palindromic Polymerase Chain Reaction
RP-HPLC	Reversed-Phase High-Performance Liquid Chromatography
rRNA	Ribosomal Ribonucleic Acid
RTE	Ready To Eat Foods
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
sig	Sigma Factor
SOD	Superoxide Dismutase
SP	Semi-Purified Bacteriocin
SPF	Pathogen-Free Facility

TA	Teichoic Acid
tDNA	Transfer DNA
Th2	T Helper2
TIV	Trivalent Inactivated Influenza Vaccine
tRNA	Transfer RNA
Vat	Virginiamycin Acetyltransferase
Vgb	Virginiamycin B Lysase
VRE	Vancomycin Resistant *Enterococcus Faecalis*
WHO	World Health Organization
YPD	Yeast Extract Peptone Dextrose
2/3CS	Two/Three-Component Signal Transduction System
°C	Degree Celsius

General Introduction

Human and animal gastrointestinal tracts (GIT) contain trillions of microorganisms which play an important role in the host's physiology, metabolism, nutrition, and immune function. For these reasons, these microorganisms are considered to be a fully functional virtual organ within the body [Guinane and Cotter, 2013]. The microbial diversity in the GIT evolves with the host's age until reaching stability [Pan and Yu, 2014]. The estimated weight of total biomass of these microorganisms exceeds 1kg [Scarpellini et al. 2010]. They are mainly present in the large intestine, which contains about 10^{12} bacteria per gram of colonic tissue [Rial et al. 2016]. The GIT shows variations which depend on its length, oxygen levels, and the flow rates of digesta that move through the stomach to the large intestine [Karasov and Douglas, 2013]. These factors, in addition to plenty of substrates, make the gut microbiota one of the most complex ecosystems on the planet [Round and Mazmanian, 2009; Roeselers et al. 2013].

It has been reported that gut microbiome has a clear impact on a large number of diseases such as dementia, obesity, cancer, irritable bowel syndrome (IBS), inflammatory bowel disease (IBD), rheumatoid arthritis, and ankylosing spondylitis [Myers, 2004; Putignani et al. 2016; Cheema et al. 2016]. Thus, gut microbiota has been proven to be a determining factor in energy metabolism, lipids β-oxidation, bile acid, amino acids and glutathione metabolism, in addition to oxidative stress and immune response metabolites [Mardinoglu et al. 2015; Sommer et al. 2016; Rial et al. 2016]. The symbiotic relationship between the resident microbes and GIT is necessary in order to preserve the health and wellbeing of the host, whereas the alterations attributed to environmental changes such as infections or diets, could alter this stability and trigger disease [Gagnière et al. 2016].

Fecal samples are an easy and informative way to investigate and explore the gut microbiota, which contribute to 60% of the fecal mass [O'Hara and Shanahan, 2006]. However, it still necessary to study the fecal microbial contents and estimate to what degree they differ from the mucosal microbiota in composition and function [Eckburg et al. 2005]. Fecal materials harbour wide varieties of beneficial microbes such as yeasts and bacteria, which might fulfil criteria defined in the probiotic guideline [Psomas et al.

2003; Gareau et al. 2010; Ait Seddik et al. 2016]. Probiotics are “live microorganisms, which when administrated in adequate amounts, confer a health benefit on the host” [FAO/WHO, 2002]. Probiotics have been used for treating diarrhea, anti-pathogen colonization, reducing inflammation, and improving normal colonic flora. [McFarland, 2015; Cruchet et al. 2015].

With the discovery and production of antibiotics, the added-value of probiotics has been neglected. [Bengmark, 2001; Meier and Steuerwald, 2005]. Because of the antibiotic resistance concern around the world and the resurgence of some infectious diseases, probiotics are considered by the World Health Organization (WHO) and the medical community to be a sustainable approach to treating certain diseases such as acute infectious diarrhea (AID), nosocomial diarrhea, antibiotic-associated diarrhea (AAD), necrotizing enterocolitis (NEC), irritable bowel syndrome (IBS), and allergies, among others. [Gareau et al. 2010; Sanders et al. 2014; Cruchet et al, 2015, Riddle et al. 2016; Urbanska et al. 2016; Szajewska et al. 2016]. Studies undertaken during the recent years on probiotics have led to rapid commercial interest [Scarpellini et al. 2008; McFarland, 2015]. In order to be "candidates" for probiotics applications, microorganisms are expected to be safe, to survive in the digestive tract, to produce antimicrobial compounds, to possess good adhesive properties in the intestinal epithelial cells, to modulate immune response, and to tolerate technological processes [Heyman and Ménard, 2002; Wedajo, 2015]. Importantly, antagonism is considered a key criterion for the selection of probiotics; this function enables the killing or inhibition of pathogens [Georgieva et al. 2015].

Lactic acid bacteria (LAB) are an important part of the intestinal microbiota [Derrien and van Hylckama Vlieg, 2015]. This group is a good provider of antimicrobial agents such as the antimicrobial peptides (AMPs), which include bacteriocins, in addition to LAB’s role in the production of lactic acid and H_2O_2 [Berstad et al. 2016]. LAB group has been classified into many genera, and those with relevance to food and probiotics include *Lactobacillus, Lactococcus, Enterococcus, Pediococcus, Leuconostoc and Streptococcus* [Holzapfel, 2012].

Enterococcus species have several phenotypic properties. Indeed, they are able to grow under moderately restrictive conditions such as temperatures ranging from 10 to

45°C, NaCl up to 6.5%, and high pH (pH 9.6) [Sherman, 1937, Teixeira and Facklam, 2003]. Many species belonging to *Enterococcus* have been reported as commensal bacteria, and have also been proven to be associated with beneficial effects in the intestines of human and animals [Khan et al. 2010]. They also exist as natural microbiota in food such as milk, cheese, and fermented meat, as well as in vegetables and plant materials because of their potential to defy various environmental conditions [Gomes et al., 2010; Henning et al. 2015]. Moreover, *Enterococcus* spp are commonly found in manufactured food products where they contribute to the development of aroma and ripening of different cheeses and meat products [Gomes et al. 2010; Santos et al. 2015]. They also produce bacteriocins that inhibit the growth of some pathogens and spoilage microorganisms [Yang et al. 2014].

Bacteriocins are ribosomally synthesized antimicrobial peptides produced by both Gram negative and Gram positive bacteria [Drider and Rebuffat, 2011]. They are able to inhibit or kill other competing bacteria by pore-forming in the cell membranes. Bacteriocins can also act as anti-viral, anti-cancer, plant protective, and microbiota regulatory agents [Drider et al. 2016]. The bacteriocins produced by LAB include: (I) Lantibiotics, small (<5 kDa) peptides containing lanthionine and β-methyllanthionine, (II) Small (<10 kDa), heat-stable, non-lanthionine-containing peptides, and (III) Large molecules heat sensitive [Perez et al. 2014].

Enterocins, which are bacteriocins produced by enterococci, are remarkable for their spectra, mode of action, molecular weight, and chemical structures [Cintas et al. 2001; Foulquié- Moreno et al. 2003]. They are mainly included the class II bacteriocins. Interest in enterocins has recently increased because of their activity against important food pathogens, including *Staphylococcus spp., Clostridium spp., Bacillus spp., Escherichia coli, Campylobacter Pseudomonas spp.* and *Listeria monocytogenes* [Franz et al. 1996; Galvez et al. 1998; Giraffa, 1995; Jennes et al. 2000; Caly et al. 2015; Drider et al. 2016; Liu et al. 2016].

Listeria monocytogenes is a ubiquitous and facultative Gram-positive bacteria causing listeriosis in both humans and animals [Leong et al. 2016]. Listeriosis is a serious foodborne disease frequently incriminated in food poisoning outbreaks around the world [Miyamoto et al. 2015]. In Europe, about 1,642 cases (0.41 cases per 100,000 population)

of listeriosis were confirmed in 2012 with an average case-fatality rate of 17.8 % [EFSA and ECDC, 2014]. However, of over 600 cases of listeriosis confirmed per year in the United States, 100 deaths have been registered by the Centers for Disease Control and Prevention [CDC, 2011]. This infection is particularly hazardous to specific high-risk groups including neonates, older adults, pregnant women, and those with weak immune systems (e.g. cancer, leukemia, HIV, IBD, etc...) [Liu et al. 2010; Miranda-Bautista et al. 2014]. The abilities of *L. monocytogenes* to grow in up to 10% NaCl, pH 4.7, temperatures ranging from -1.5^{o} to 45^{o} C, and to colonize biotic devices leading to biofilm formation, make this bacterium one of the most important contaminants hitting the food processing industry [Liu et al. 2010; Leong et al. 2016].

A biofilm is an aggregate of microorganisms that adheres to a biotic or abiotic surface, and the adherent cells are embedded in a self-produced matrix of extracellular polymeric substances (EPS), encompassing nucleic acids, proteins, polysaccharides, and lipids [Jung et al. 2015]. The biofilm formation has been involved in many outbreaks recorded around the world, and therefore is considered a serious problem in food industries such as dairy, fish processing, poultry, meat, and ready-to-eat foods (RTE)[Srey et al., 2013]. The mechanism of biofilm formation on the contaminated surfaces includes (1) the microbial adsorption or accumulation on the surface; (2) the attachment and formation of polymer bridges between the microbes and the surface; (3) the colonization or microbial growth and division on the surface [Garrett et al. 2008]. Biofilm helps microbe persistence and resilience to physical and chemical stress by acting as a protective layer from the hostile environment in addition to its nutritional role by trapping the necessary elements [Poulsen, 1999]. *L. monocytogenes'* ability to form biofilms is strain-dependent [Milanov et al. 2009]. Even if many physical and chemical techniques (non-bacteriocin methods) such as irradiation, high pressure, ultrasound, ultraviolet light, acids, and other chemicals have been exploited to control *L. monocytogenes* in foods, they are generally associated with certain undesirable effects in the final product [Liu et al. 2010].

Bacteriocins produced by food-grade bacteria have received great attention, especially with the increasing requests for more natural foods [Chen and Hoover, 2003]. Currently, the lantibiotic nisin is the only bacteriocin that is used as a food additive in more than 50 countries under the designation E234 [Ramu et al. 2015]. Nonetheless, the

bactericidal efficiency of nisin could be affected by environmental conditions such as pH, temperature, food composition, structure, and food microbiota [Zhou et al. 2014]. Moreover, the efficacy of nisin could face the emergence of *L. monocytogenes* nisin-resistant strains [Crandall and Montville, 1998; Draper et al. 2015]. To overcome this problem, different studies suggested the use of bacteriocins combinations [Vignolo et al. 2000; Naghmouchi et al. 2007]. This study provides meaningful insights on this topic by showing that amount of nisin used to master *L. monocytogenes* could drastically be reduced in combination with enterocin B3A-B3B.

The main purpose of this project is the exploration of animal and human microbiota, targeting the isolation of yeasts (non-*Saccharomyces*) and LAB with anti-*Listeria* properties and overall probiotic applications. To this end, poultry feces were used for isolation of yeasts with probiotics properties, while the feces of Iraqi children were used as sources of novel LAB.

Chapter 1. Literature Review

1. Microbial community in gastrointestinal tract and feces

The GIT from human or animal origins consists of various anatomical sections extending from the mouth to the anus [Spainhour, 2007]. The microbiological studies of GIT are usually limited to the microbiota of the stomach, small intestine, large intestine, and fecal materials [Mackie and Gaskins, 1999]. As indicated, this organ is composed of trillions of microbes including bacteria, archaea, and eukaryotes, which play a key role in the physiology and health of the host [Roeselers et al. 2013]. Different factors including length, oxygen levels and flow rates of ingested materials, and substrates render the gut one of the most complex ecosystems [Roeselers et al. 2013; Donaldson and Toskes, 1989; He et al. 1999].

The exchanges among gut microorganisms, as well as their interaction with the host immune system, influence the development of health and disease [Clemente et al. 2014]. Humans and animals hold and maintain a diverse but host-specific gut microbial community [Tannock, 1995; Donaldson et al. 2016].The distribution of intestinal microorganisms is varied depending on the anatomic sites (**Figure 1**). Fecal sampling permits a preliminary insight into the cecal microbial content, but it does not reflect the real composition of microbiota or activities in the proximal large intestine [Eckburg et al. 2005; Mai et al. 2010]. Gut microbial populations have been described in humans, animals and in a wide range of zoological classes, where they contribute to the nutrition, physiology, immunology and protection of their hosts [Eckburg et al. 2005; Mackie and White, 1997; Mackie et al. 1997].

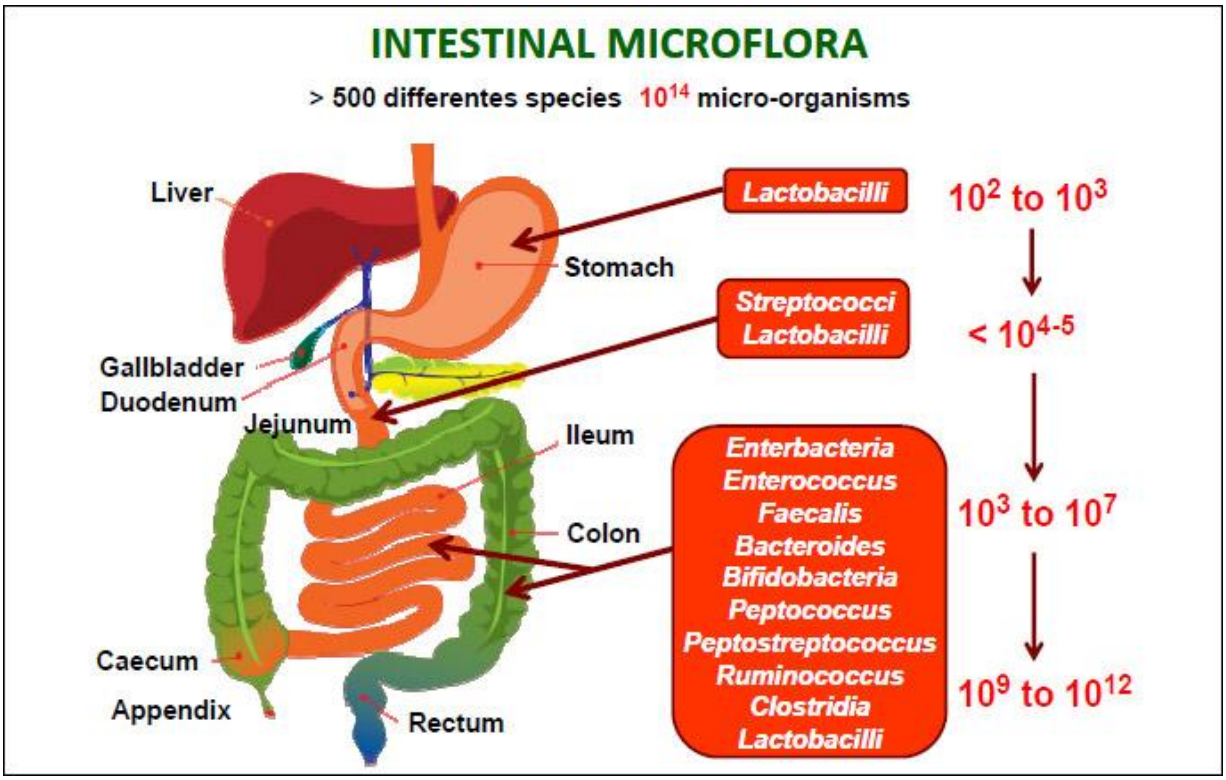

Fig. 1. Distribution and variations in microbial numbers and composition across the length of the GIT. The human microbiota includes about 10^{14} bacterial cells; this number is 10 folds higher than the number of human cells present in the body. The bacterial cell numbers goes from 10^{2} to 10^{3} bacteria per gram of contents in the stomach and duodenum, progressing to 10^{4} to 10^{7} bacteria per gram in the jejunum and ileum, then ends with 10^{9} to 10^{12}cells per gram in the colon [Konturek et al. 2015].

1.1. Development of gut microbiota

Immediately and rapidly after birth, microorganisms, from the mother and the surrounding environment get access to the GIT of neonates [Mead and Adams, 1975; Park et al. 2005]. Development of this microbiota is influenced by several factors during the different steps of life; however, the first three years of life are considered the most critical period to establish the intestinal microbiota of newborns [Rodríguez et al. 2015].

1.1.1. Neonate and infancy

In spite of serious studies considering the fetus' GIT as "microbiologically sterile," it has been established that the first bacterial exposure begins when the neonate is in contact with the intestinal, vaginal and surrounding environment microbiota [Jimenez et al. 2005; Onderdonk et al. 2008; Satokari et al. 2009]. The neonate's GIT is then gradually and constantly colonized by diverse microorganisms. In the GIT of a typical infant, facultative anaerobes such as streptococci, enterobacteria, coliforms and lactobacilli are the first microbes that colonize the host's intestine, normally in the second to third days of life, while anaerobes including *Bifidiobacteria*, *Clostridia*, *Bacteriodes* and *Eubacteria* become the dominant microorganisms in the infant's feces at 1 to 2 weeks of age [Mitsuoka, 1992; Cong et al. 2016].

Three phases of microbe acquisition have been described in infants. The first one occurs during the initial hours of life when the microbial content in feces is zero. The second one takes place between the 10^{th} and 12^{th} hours of life and does include variant microbiota, and the third one occurs when the maternal milk comes through the intestinal tract of the infant. At that point, the microbiota is predominated by *Bifidobacteria* [McCartney and Gibson, 2006]. Remarkably, a fourth phase related to the introduction of solid food (weaning) to the intestinal tract, which modulates its microbiota as adult type and makes it more complex and diverse, was also described [Benno and Mitsuoka, 1986; Edwards and Parrett, 2002; Bourlioux et al. 2003; Park et al. 2005; Rodríguez et al. 2015].

1.1.2. Early

During the weaning period and when solid foods are introduced to the baby's intestinal tract, the gut microbiota becomes more diverse and complex [Flint et al. 2012; Rodríguez et al. 2015]. The levels of *Bifidobacteria* are decreased by about 1 log and became more stable in this period of life. Meanwhile, a remarkable increase of *Bacteroides*, anaerobic cocci and *Clostridia* occurs. Additional colonization starts during this period until the adult profile microbiota is shaped [Morelli, 2008; Fallani et al. 2011].

1.1.3. Adult

The intestinal microbiota of adults is relatively stable over time, and the microbiota of the large intestine becomes more complex conversely to that of children [Zoetendal et al. 1998; Claesson et al. 2011]. The percentage of beneficial bacteria encountered in the gut microbiota such as *Bifidobacteria* decreases following the weaning and the introduction of solid foods [Salminen and Wright, 2004]. Nevertheless, in adults, *Bifidobacteria* represent about 1-5% of the total fecal bacterial content [Salminen and Wright, 2004]. Remarkably, the dominant species in the GIT changes with age. For example, *B. adolescentis*, *B. catenulatum/pseudocatenulatum*, *B. bifidum*, and *B. longum* are the most dominant species displaying considerable variation between individuals [Lahtinen et al. 2011].

1.1.4. Elderly

Limited studies report that structural changes occur in the GIT microbiota of the elderly. The number of beneficial species such as *Bifidobacteria* diminishes, while the number of species from *Clostridia* and *Enterobacteria* populations viewed as detrimental ones for health increase [Gorbach et al. 1967; Mitsuoka, 1982; Rodríguez et al. 2015]. Hopkins and Macfarlane [2002] revealed that *Bacteroides* species diversity was slightly increased in the feces of elderly subjects, whereas that of *Bifidobacteria* was decreased. On the other hand, Woodmansey et al. [2004] and Woodmansey et al. [2007] suggested that both *Bacteroides* numbers and species diversity decreased in the elderly. Overall, the shape of microbiota in the elderly displayed a variation from that established for adults, with the highest levels of *Bacteroides* spp. and evident abundance of *Clostridium* spp. [Claesson et al. 2011; Rodríguez et al. 2015].

1.2. Factors affecting microbiota development and composition

Prenatal factors such as the mother's microbiota, mode of delivery, diet, environment, use of antibiotics and others are associated with establishment and development of GIT microbiota (**Figure 2**) [Moschen et al. 2012; Rodríguez et al. 2015].

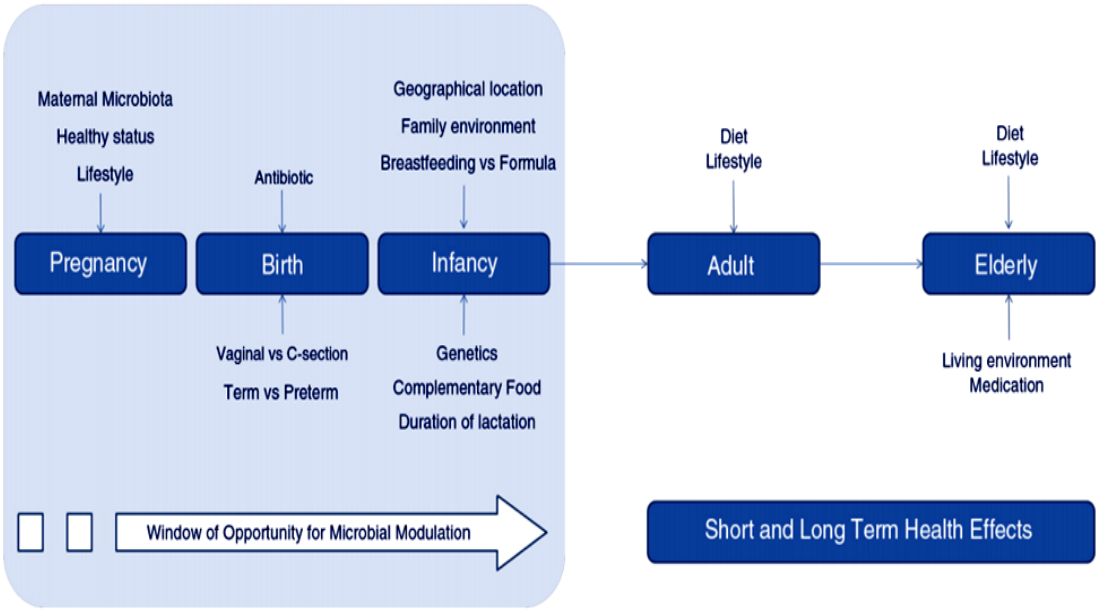

Fig. 2. Factors influencing the development of infant, adult, and elderly gut microbiota [Rodriguez et al. 2015]

1.2.1 Maternal microbiota

Uterine microbiota is always correlated with intrauterine infection, which is the main cause of infant mortality [Blencowe et al. 2013]. However, recent studies on uterine microbiota with healthy-term pregnancies underpinned a possible transfer of bacteria, or their DNA, from mothers to the placenta tissue [Satokari et al. 2009; Aagaard et al. 2014; Romano-Keeler and Weitkamp, 2014], fetal membranes [Steel et al. 2005; Rautava et al. 2012], amniotic fluid [Bearfield et al. 2002], and umbilical cord blood [Jiménez et al. 2005] of healthy neonates without any indicator of inflammation or infection. Moreover, significant changes in the establishment of neonates' gut microbiota are supposed as a result of probiotic consumption by mothers during pregnancy [Gueimonde et al. 2006].

1.2.2 Mode of delivery

It was reported that microbial colonization of the neonate gut is correlated with the mode of delivery, either vaginal delivery or cesarean section [Dominguez-Bello et al. 2016]. Studies point out a strong correlation between the first gut microbiota and the microbial communities of the mother's vagina (*Lactobacillus*, *Prevotella*, or *Sneathia*) upon vaginal delivery, or of the mother's skin (*Staphylococcus*, *Corynebacterium*, and *Propionibacterium*) upon cesarean section, when the meconium of newborns has been analyzed [Mueller et al. 2015]. Thus, the vaginal delivery of neonates exposes them to the mother's vaginal and fecal microbiota, which is a very important source of *Bifidobacteria*, *Bacteroides*, and *Escherichia coli*. Different studies showed that infants born by cesarean section (CS) contain more *Bifidobacteria* and *Bacteroides* than those vaginally born [Huurre et al. 2008; Biasucci et al. 2010; Dominguez-Bello et al. 2010].

1.2.3 Mode of feeding

The mode of feeding plays a major role in the development of the infant's intestinal microbiota. In human cases as a typical model *Bifidobacteria* and Lactobacilli are frequently detected in the plated samples of breast milk that make it an important source of probiotic bacteria [Fernández et al. 2013]. The comparative studies between breast-fed and formula-fed infants showed a significantly higher number of *Bifidobacteria* and Lactobacilli and lower counts of *Clostridium* sp., *Bacteroides*, *Enterobacteriaceae* and Staphylococci in the breast-fed infants' samples [Harmsen et al. 2000; Rinne et al. 2005; Fallani et al. 2010]. Identical *Bifidobacterium*, *Lactobacillus*, and *Staphylococcus* strains were detected in breast milk of mothers and fecal samples of their infants, thus confirming a strong relationship between the mother's milk and early colonization of the infant's digestive tract [Martín et al. 2012]. The predominant microbes in the intestinal tract of formula-fed infants were the facultative anaerobes *Bacteroides*, *Clostridium Enterobacteriaceae*, *Streptococcus*, and *Staphylococcus*, whereas the *Bifidobacteria* colonization was decreased, leading to a complex microbiota similar to that of adults in these cases [Harmsen et al. 2000; Marques et al. 2010; Fernández et al. 2013].

1.2.4. Geographical location and environment

Different studies have pointed out a relationship between the intestinal microbiota of the host and the geographical location. Related to this, Hill et al [1971] studied the geographical variations in the incidence of breast cancer and assumed that gut bacteria can produce oestrogens from the biliary steroids present in the colon which could play a direct role in the ætiology of breast cancer. Mueller et al. [2006] conducted a cross-sectional study on intestinal microbiota composition on 230 healthy subjects located in Germany, Italy, France, and Sweden. Significant country-age interactions were detected for the German and Italian groups. Notably, the variations between the European intestinal microbiota were only noticed for the *Bifidobacterium* group. The *Bifidobacteria* proportion was 2-3 fold higher in the Italian intestinal microbiota than in any other group, and the effect was independent of age.

Lee et al. [2011] conducted a comparative study of the composition of fecal microbiota of Korean and American adult twins. They concluded that every geographical area has its own unique microbial "fingerprint," or identity. Grzeskowiak et al. [2012] studied the gut microbiota of Malawian and Finish infants; they mentioned that *Bifidobacteria* were dominant in six month old infants, with a higher percentage in Malawian than in Finnish infants. According to these authors, *Bifidobacterium adolescentis*, *Clostridium perfringens,* and *Staphylococcus aureus* were absent in the Malawian infants but present in the Finnish ones.

Furthermore, variations in the fecal microbiota content of infants from five European countries with different lifestyle characteristics (Sweden, Scotland, Germany, Italy and Spain) were studied. *Bifidobacterium* was found to be the predominant species, with about a 40% average of total detectable bacteria, followed by *Bacteroides* with 11.4% and the *Enterobacteriaceae* with 7.5%. Infants from the northern European countries appeared to contain high proportions of *Bifidobacteria* in their feces, in contrast to infants from southern European countries, whose feces contained a diversified microbiota. A higher proportion of fecal microbiota of *Bifidobacteria* has been associated with breast-fed babies, while formula-fed infants have a significant concentration of *Lactobacilli*, *Bacteroides* and *Clostridium* [Fallani et al. 2010].

The composition of the colon microbiota was also studied for northern Europeans. To this end, fecal samples were collected from 91 healthy humans between age 7 and 52 in France, Germany, Denmark, The Netherlands, and the United Kingdom. The results revealed large inter-individual differences, with *Clostridium coccoides* and *Clostridium leptum* as the dominant group followed by *Bacteroides*. Nonetheless, no significant variation related to geographic origin, age, or gender was noticed [Lay et al. 2005].

1.2.5 Antibiotics, probiotics and prebiotics

The use of antibiotics for bacterial infections has led to the preservation and extension of both human and animal lives, but the development of antibiotic-resistant bacteria became worrisome worldwide. Clearly, resistant bacteria can be found in the intestinal tract [Jernberg et al. 2010; Andersson et al. 2012]. The antibiotic therapies target the pathogenic bacteria, but their effects can afflict the normal microbial communities of the host, mainly those in the gastrointestinal tract [Lode et al. 2001; Bartosch et al. 2004]. The content of the GIT-beneficial anaerobic *Bifidobacteria*, Lactobacilli, or *Bacteroides* species could be reduced or even eradicated upon antibiotic treatment, leading to the development of pathogenic species [Sullivan et al. 2001]. A recent study of the short-term effects of antibiotics on human gut microbiota showed that fluoroquinolones and β-lactams reduced about 25% of the intestinal microbial diversity and decreased the core phylogenetic microbiota from 29 to 12 taxa [Panda et al, 2014].

Hospitalized patients subjected to a broad spectrum of antibiotic treatment can acquire the symptoms of antibiotic-associated diarrhea resulting from the modification of the composition of their gut microbiota and the proliferation of pathogens such as *Clostridium difficile* [Vollaard, 1994; Sun et al. 2011; Vincent and Manges, 2015].

The composition of intestinal microbiota could be restored and modulated by probiotics (see page 17) and prebiotics, which play a beneficial role in the gut microbial communities, preventing gut inflammation and other intestinal disease [Hemarajata and Versalovic, 2012; Pourabedin and Zhao, 2015].

1.2.6. Other factors

Dietary lifestyle and environmental factors have an important impact on the modulation of the composition and metabolic activities of the intestinal microbiota [Gonzaga et al. 2016]. The influence of short-term and long-term dietary changes on the intestinal microbial profile may lead to life-long consequences for the host's health resulting from the microbial modulation of the immune system [Conlon and Bird, 2014].

Non-dietary lifestyle factors such as smoking, lack of physical exercise, stress, and air-borne toxic particles are tightly linked to intestinal microbiota and host health. Indeed, smoking can significantly affect the intestinal microbiota community and increase the genus *Prevotella*, mainly in subjects with Crohn's disease and in healthy subjects that have an increased risk of Crohn's disease [Benjamin et al. 2012]. Air pollutants can pass to the large intestine via mucociliary clearance from the lungs, in addition to food and water, which affect directly the epithelial cells, immune system and modulation of the intestinal microbiota that linked to increasing of inflammatory bowel diseases (IBD) cases [Beamish et al. 2011].

Stress is another lifestyle factor influencing the bowel activities through the gut-brain axis. Intestinal microbiota of the stressed hosts present a decreasing number of the beneficial intestinal microbiota such as lactobacilli, while pathogenic bacteria such as *Escherichia coli* and *Pseudomonas* show high growth ability and epithelial adhesion [Lutgendorff et al. 2008].

2. Host microbe interactions

The microbiota of GIT provides essential health benefits to its host, especially the regulation of immune homeostasis [Honda and Littman, 2016]. The host's innate immune system consists of the intestinal epithelium and immune system cells such as neutrophils, dendritic cells, monocytes/macrophages, and innate lymphoid cells [Haag and Siegmund, 2015]. The largest interface between host and environment is the luminal surface of the GIT, where the diverse microbiota are in close contact with the immune system of the intestinal mucosa and underlying tissue [Wu and Wu, 2012; Tomasello and Bedoui, 2013]. The intestinal homeostasis is maintained by different protective mechanisms.

Several mechanisms depend on the pattern recognition receptors (PRRs) signaling which are triggered by commensal microbes [Kamada et al. 2013]. In the small intestine, AMPs such as peptidoglycan recognition proteins (PGRPs), regenerating islet-derived 3 gamma (RegIIIγ) and defensins are induced as a result of PRR's stimulation by commensals. However, in the colon, microbes such as *Bacteroides fragilis* and *Bifidobacterium breve* induce Treg cells by TLR2 signal (**Figure 3**) [Chu and Mazmanian, 2013].

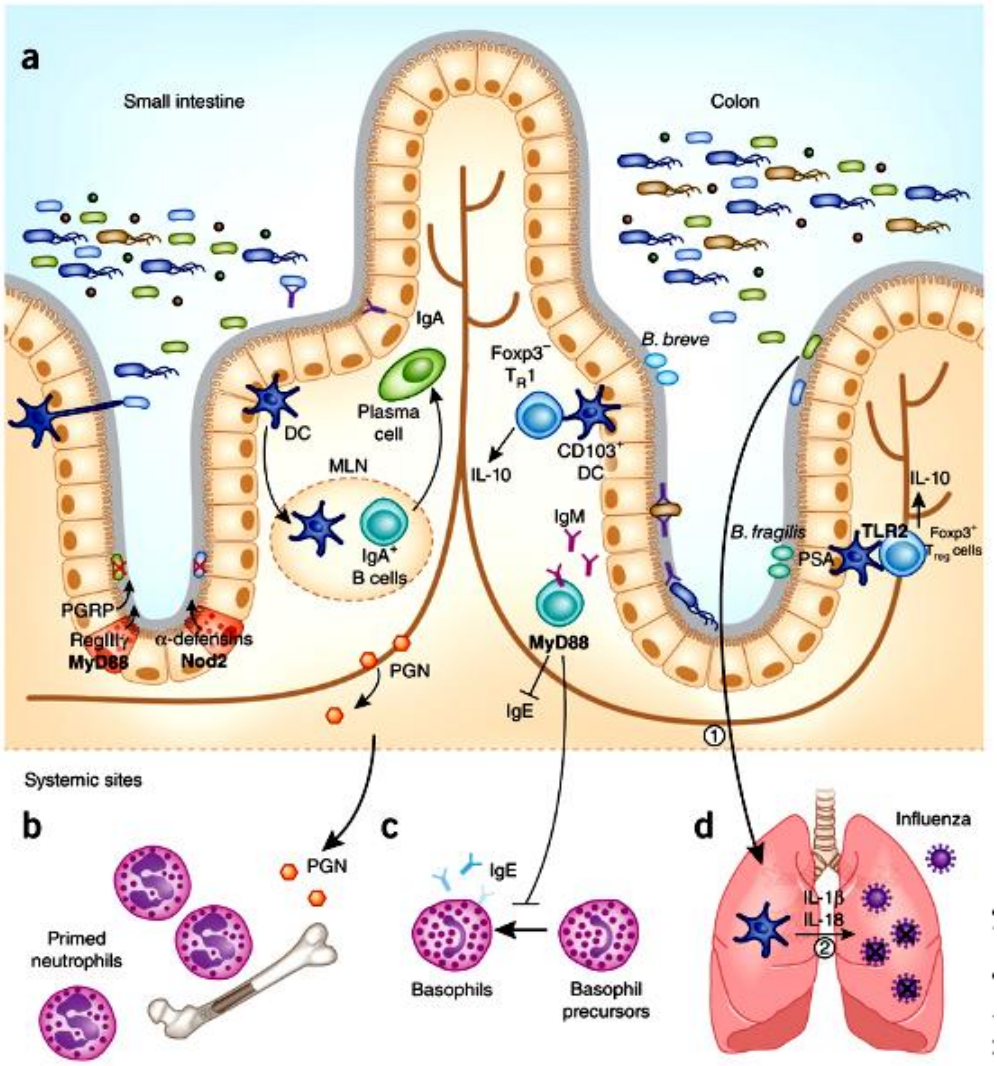

Fig. 3. Pattern recognition receptors (PRRs) signaling promotes immune homeostasis. (**a**) The small intestine and colon comprise a single layer of intestinal epithelial cells separating the abundant microbiota from host tissues. (**b**) Peptidoglycan derived from the gut microbiota is necessary to prime neutrophils in bone marrow stores in a Nod1-dependent manner. (**c**) MyD88 signaling in B cells suppresses serum IgE and inhibits the differentiation of basophils in systemic sites. (**d**) Commensal gut microbiota induces the production of pro-IL-1β and pro-IL-18 during steady state (signal (1)). During an influenza infection in the lungs, activation of IL-1β and IL-18 mediated by caspase-1 (signal (2)) is critical for clearance of influenza. DC, dendritic cell; MLN, mesenteric lymph node [Chu and Mazmanian, 2013].

2.1. Host–microbiota cross-talk

After birth, the development of the newborn intestinal microbiota is governed by the interaction between the gut microbial community and the host's immune system [Critz and Bhandari, 2015]. The neonatal immune system rapidly matures as a result of the influence of microbiota and other important factors [Maynard et al. 2012].

Germfree (GF) mice models showed a defect in intestinal barrier functions and a decrease in inflammatory responses as a consequence of the absence of early microbial stimuli [Sudo et al. 1997]. Oh et al. [2014] studied the responsibility of the gut microbiota to restore and promote the immunity to vaccination of orally inoculated germfree mice models, which previously showed no immune response to the trivalent inactivated influenza vaccine (TIV). Another study conducted on mice models, housed separately in two rooms of the same specific pathogen-free facility (SPF) with two different microbiota, resulted in different mucus barrier properties depending on the influence of intestinal bacteria and their community structure [Jakobsson et al. 2015]. Probiotics were capable of modulating the intestinal immune response by "talking" with the immune cells, implying recognition receptors sensitive to probiotic-derived products such as cell wall components, metabolic products, and DNA [Corthésy et al. 2007].

2.2. Microbial adhesion and interaction with pathogens

The ability of bacteria to adhere to the intestinal surface is a prerequisite for colonization of the squamous and epithelium in the host digestive tract by both beneficial and pathogenic microbes [Pedersen and Tannock, 1989]. The mechanism of adhesion is based on the interaction between the microbe and the targeted surfaces. Hydrophobicity plays a key role in this process owing to the strong correlation between the electrostatic balance, van der Waals interactions, surface hydrophobic character, and the cells' adhesion behavior [Polak-Berecka et al. 2014]. Various structures such as flagella, fimbriae, and cell wall components are associated with the targeted cell wall to set up the adhesion ability of microorganisms to the intestinal surfaces [Kline et al. 2009; Haiko and Westerlund-Wikström, 2013].

Different factors govern the bacterial attachment to the host intestinal surfaces. These include digestive enzymes, bile salts, presence of zinc, calcium, magnesium and mucin concentration as well as the pH of the intestine [Ouwehand and Salminen, 2001; Sanchez et al. 2010].

The adhesion ability of beneficial microbes seems to be necessary for the competitive exclusion and displacement of pathogenic microbes and immune system modulation [Castagliuolo et al. 2005; Haiko and Westerlund-Wikström, 2013]. Adhesion of pathogens to intestinal surfaces is the first step of the intestinal infection [Beachey, 1981; Finlay and Falkow, 1997]. Probiotics could develop different mechanisms aimed at inhibiting pathogens colonization of the host intestine, including competition with the pathogens for colonization of the host GIT and therefore occupy the binding sites on the mucus [Vesterlund et al. 2006; Collado et al. 2007a]. Probiotics' ability to inhibit the adhesion of pathogens showed a high specificity to pathogenic strains [Gueimonde et al. 2006; Collado et al. 2007b]. However, many studies reported that exclusion of pathogenic bacteria by probiotic strains is not related to the adhesion ability of these strains, but could result from different mechanisms involved in the inhibition of pathogens installation [Lee and Salminen, 2009].

3. Probiotics

3.1. What are probiotics?

The term "probiotic" is derived from a combination of the Latin preposition "*pro*" which means "for" and the Greek noun "*bios*" meaning "biotic" or "life". Therefore, this term represents the opposite of "antibiotic" which means "against life" [Guarner et al. 2005; Watson and Preedy, 2015; Nami et al. 2015]. According to a report of the Food and Agriculture Organization (FAO) and World Health Organization (WHO), a probiotic is defined as a "live microorganism which when administrated in adequate amounts confers a health benefit on the host" [FAO/WHO, 2002].

Microorganisms have to fulfil several characteristics to be a candidate for probiotic status. They have to be safe for health, be resistant to digestive tract conditions with as

much viability as possible, produce antagonistic molecules, adhere to the intestinal epithelial cells, modulate the host immune responses, and be tolerant to technological processes [Heyman and Ménard, 2002]. The bacteria most commonly used as probiotics are the lactic acid bacteria (LAB) group, especially *Lactobacillus* spp., as well as *Bifidobacteria,* which received Generally Recognized as Safe (GRAS) status. Nevertheless, different species from other bacteria groups were also tested for their probiotic properties (**Figure 4**) [Salminen and von Wright, 1998, Watson and Preedy, 2015]. At the same time, the fungal genera *Saccharomyces, Debaryomyces, Candida, Kluyveromyces, Pichia, Yarrowia, Metschnikowia, Isaatchenkia* and *Aspergillus* were also commonly proposed as eukaryotes for possible probiotic applications [Nayak, 2011].

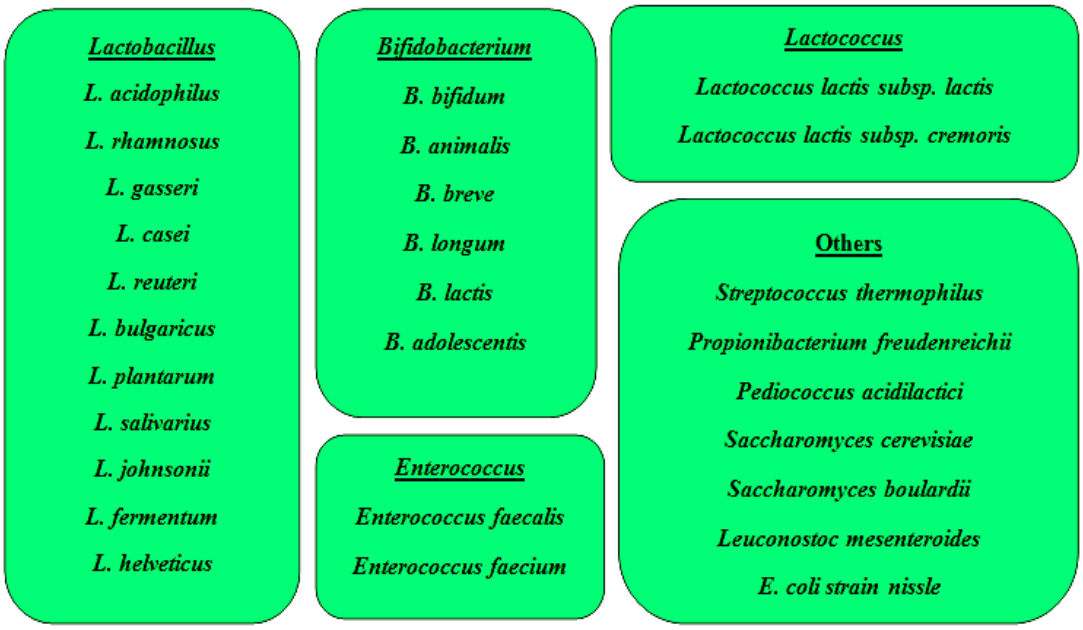

Fig. 4. Some examples of prokaryotic and eukaryotic genera commonly used as probiotics [Preedy, 2015].

3.2. Probiotic criteria and safety assessment

To be a candidate for probiotic use, a microorganism has to fulfil the criteria approved by the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) as outlined in the "Guidelines for the Evaluation and

Selection of Probiotics for Food Use" in 2002 (**Figure 5**) [FAO/WHO, 2002]. These criteria indicate that the identification of a probiotic strain has to be restricted to the strain level because the probiotic effects are strain-specific. Strain identification has to link it to a specific health effect and permit precise observation and epidemiological studies. It was also recommended that updated identification methods be used, and the probiotic strains have to be deposited in an internationally recognized culture collection [FAO/WHO, 2002].

The probiotic selection criteria can be summarized into five major categories [Dunne et al. 2001, Salminen et al. 2004, Holzapfel, 2006, Diez-Gonzalez and Shamberger, 2006, Kailasapathy, 2010].

- ecological, genetic and biochemical properties such as origin, identity, biochemical characteristics, and genetic and metabolic stability.

- safety properties; probiotics have to be GRAS (Generally Recognized as Safe), with no invasive potential; non-transferable antibiotic resistance traits, have to be devoid of virulence factors.

- physiological properties including (i) resistance to the harsh environmental conditions of the GIT like the low pH of gastric juice, concentration of bile salts and adverse effects of gastric enzymes, and (ii) adhesion ability and viability in the GIT as long as possible.

- functional properties related to host health claims such as (i) the ability to colonize the epithelial cells or intestinal mucus and exert competitive exclusion or displacement of target pathogens, (ii) specific antimicrobial activity against pathogens, (iii) stimulation of the immune responses, (iv) selective stimulation of beneficial indigenous bacteria, and (v) restoration of the normal population.

- technological properties and performance of the probiotics during manufacturing including growth characteristics *in vitro*, and survival during the shelf life of the product.

In vitro studies are widely used to gain information about the functional characteristics and safety of probiotics. However, it has been recognized that available tests are not always accurate in predicting the performance and efficacy of probiotics *in vivo* [Siró, 2011]. The selection of strains and their validation as probiotics should be

based on both *in vitro* and *in vivo* demonstrated activities [Siró, 2011]. Probiotics have to pass through various levels of clinical trials that are described by Charalampopoulos [2009]:

1. Phase I trials: Clinical pharmacology and toxicity, (safety).
2. Phase II trials: Initial clinical investigation effect, (efficacy).
3. Phase III trials: Evaluation of intervention, (effectiveness).
4. Phase IV trials: Post marketing surveillance, (surveillance).

Phase II human trials should be designed in the form of double-blind, randomized, and placebo-controlled (DBPC) [FAO/WHO, 2002]. The evaluation of probiotic efficacy in clinical trials is more challenging than the other potential functional foods because the probiotic effects are dependent on the microorganism status [Siró, 2011].

The use of probiotic has significantly increased around the world, and novel probiotic strains are continuously appearing in the markets. Thus, it is recommended that consumers of these probiotics be advised which strains have been used, the minimum concentration of viable cells, their shelf-life, storage conditions, and producer contact details [FAO/WHO, 2002].

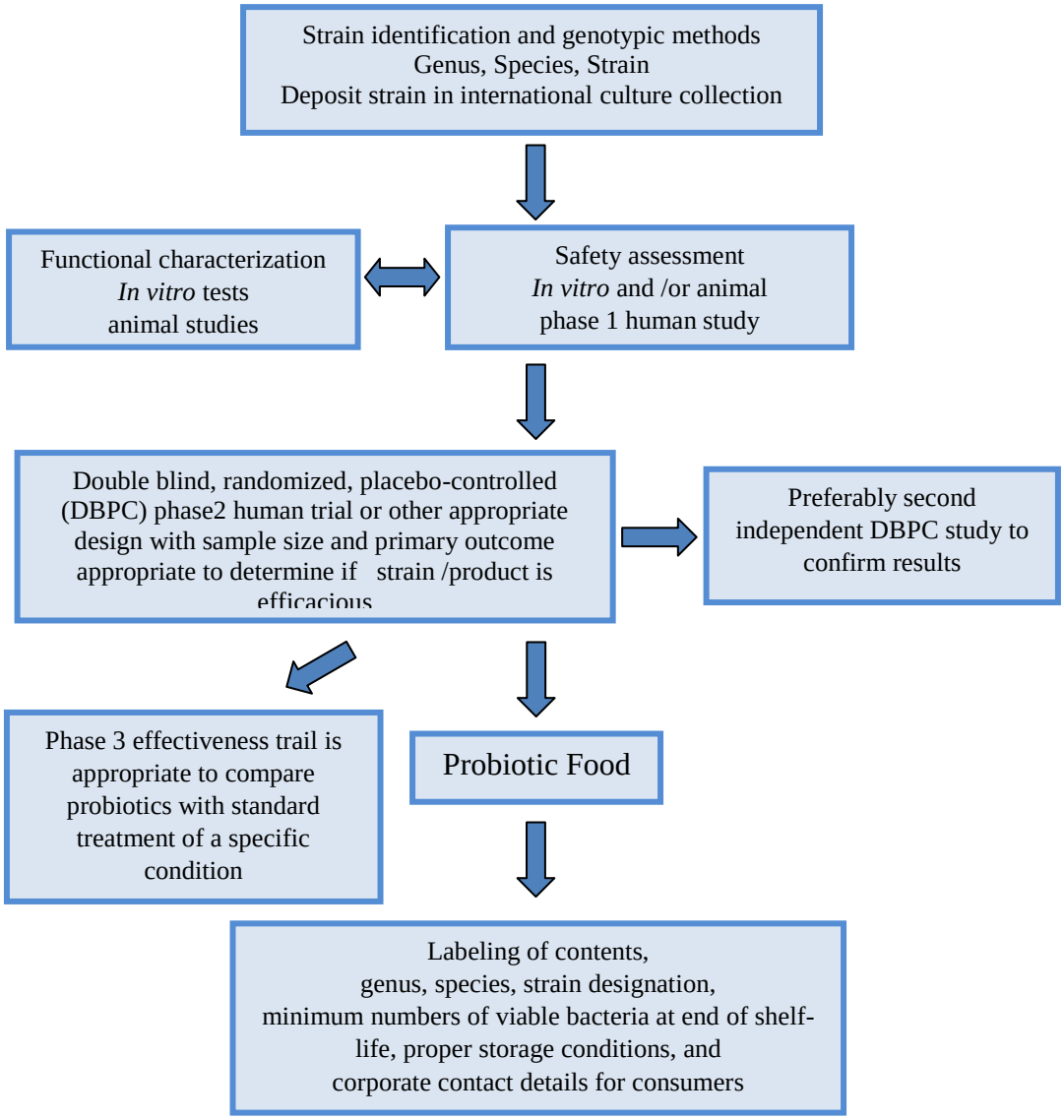

Fig. 5. Guidelines for the Evaluation of Probiotics for Food Use [FAO/WHO, 2002]

3.3. Probiotics and their health effects

Beneficial microorganisms can act on their hosts' health by modulating the composition of the intestinal microbiota or the innate immune system [Frei et al. 2015]. Many studies have highlighted the beneficial effects of probiotic strains by decreasing the risks and treatments of human diseases based on well conducted trials (**Table 1**).

Table 1. Some beneficial effects of probiotics on human health

Probiotic effect	References
stimulation of immune system, gut immune responses and intestinal homeostasis	[Savard et al. 2011]
prevention and treatment of diarrhea	[Guarino et al. 2015]
treatment of irritable bowel syndrome (IBS)	
enhancement of fecal properties and microbiota	[Moayyedi et al. 2010
treatment of inflammatory bowel disease and constipation	Saez-Lara et al. 2015]
protection and cure of *Clostridium difficile*-associated diarrhea	[Lau et al. 2016]
mitigation of lactose intolerance symptoms and food allergies	[Pakdaman et al. 2016]
prevention of necrotizing enterocolitis	[Lambæk et al. 2016]
cholesterol-lowering effect	Gilliland et al. 1985; Ooi and Liong, 2010]
therapeutic influence on human immunodeficiency virus (HIV) patients by supporting their immune function	[Hemsworth et al. 2011; Hemsworth et al., 2012]
reduction of pulmonary damage which resulted from viral infection by development of immune coagulative responses	[Zelaya et al. 2014]
alleviation of the atopic eczema/dermatitis syndrome (AEDS) in specific cases of food-allergic infants	[Viljanen et al. 2005].

Probiotics are used in animal production including poultry, pigs, and ruminants, as well as in aquaculture to improve health, and welfare [Bouchard et al. 2015]. Probiotics permit protection of these animals from severe pathogens including *E. coli*, *Salmonella*, *Campylobacter*, and *Clostridium* [Papadimitriou et al. 2015]. The relevant beneficial claims of probiotics on farm animals are listed in **figure 6** [Cooper et al. 2007].

Growth-promoting effects in farm animals are one of the most important benefits of probiotic strains [Bryan et al. 2015]. These microorganisms are expected to help hydrolysis of complex food elements into simple and easy to absorb units. Related to this, the use of *Lactobacillus* and Bifidobacteria in calves and young piglets can contribute to weight gain and decrease the rate of mortality [Abe et al. 1995; Mountzouris et al. 2007; Delia et al. 2012].

Host protective effects from intestinal infections were described for animals [Bouhafs et al. 2015]. Probiotics' adhesion to the intestinal cecum and epithelial cells prevents colonization of pathogens by competitive exclusion, secretion of antimicrobial compounds, competition for nutrients, and stimulation of the immune responses [Scanlan, 1997; Doyle, 2001; Patterson and Burkholder, 2003; Mazmanian et al. 2008]. Moreover**,** Mikulski et al. [2012] described significant effects of the dietary probiotic *Pediococcus acidilactici* MA18/5M on the performance, nutrient digestibility, egg traits, egg yolk cholesterol, and fatty acids profile of laying hens. In another study carried out on cow nutrition, Sretenović et al. [2008] investigated the efficiency of a commercial preparation of the live yeast *Saccharomyces cerevisiae* combined with *Lactobacillus casei*, *Streptococcus faecium*, *Aspergillus oryzae*, *Lactobacillus acidophilus*, and enzymes (1,3-b and 1,6 D-Glucan, hemicellulase, protease, cellulase, α- amylase. In this study, the proposed probiotic formulation showed a significant influence on the quantity and composition of the milk of the treated group compared to the control group.

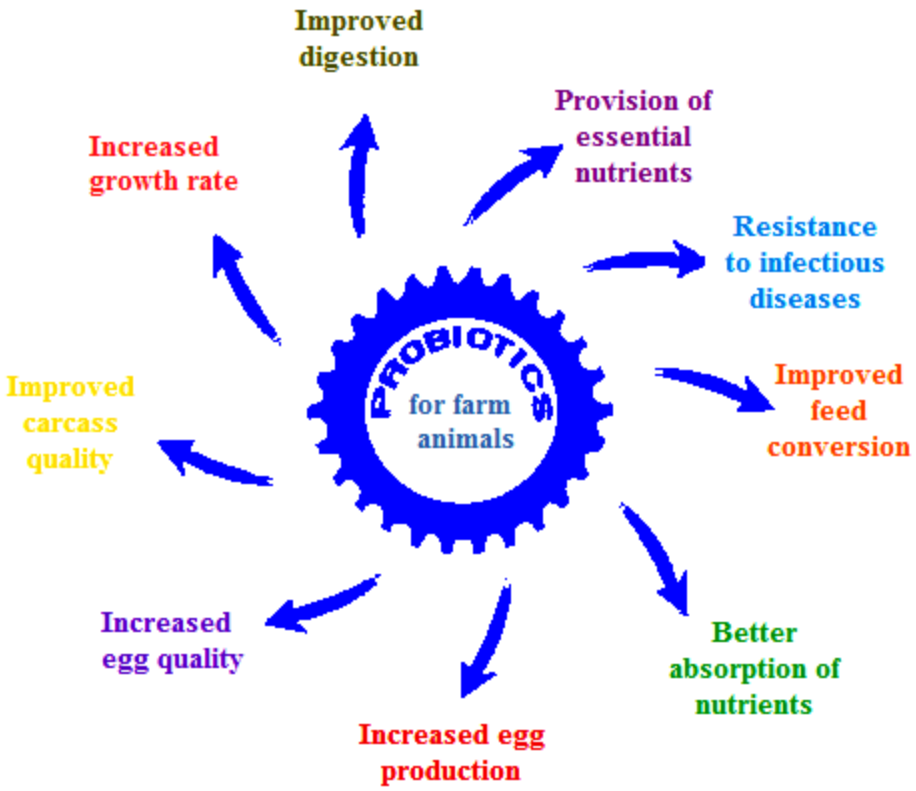

Fig. 6. Probiotics' effect on animal health and production [Cooper et al. 2007]

4. Fecal origin microbes with probiotic potential

The essential components of fecal materials include water, intestinal bacteria (free-living microorganisms and single cell), dead bacteria, and cells derived from the body, undigested carbohydrates, fiber, proteins, fat , minerals, and coloring pigments [Khurana, 2014; Rose et al. 2015]. Intestinal bacteria were reported as the largest component of feces, as more than 400 species, with at least 40 genera were isolated from human feces and constitute about 30 to 50% of total dry matter [Monastyrsky, 2005; Gropper and Smith, 2012]. Archaea and fungi, especially *Saccharomyces* sp. and *Candida* sp., were found to be normal inhabitants of the digestive tract in human and animal feces [Somas et al. 2001; Gupta and Ayyachamy, 2012; Hamilton et al. 2012; Kemoi et al. 2013; Hoffmann et al. 2013; Kantarcioglu et al. 2016]. Moreover, different studies showed the

beneficial effects of microorganisms of fecal origin and recommended their use as probiotics.

4.1. Yeasts as probiotics

Yeasts are a part of the intestinal microbial community with a content estimated to be less than 0.1% [Czerucka, and Rampal, 2007]. They have been subjected to intensive studies and have attracted significant attention for a long time as feeding agents in the animal health field as well as various human uses [Palma et al. 2015; Uyeno et al. 2015]. Yeasts were first used for cattle, pigs, and poultry diets because of their high nutritional content, including proteins, peptides, amino-acids, minerals, and vitamin B [Lyons et al. 1993]. Many species of yeasts were recognized and confirmed as safe strains for probiotic applications as they were never been involved in any outbreaks or food-borne illness-related cases [Foligné et al. 2010].

4.1.1 General characteristics of yeast

The word "yeast" originates from the Dutch word "*gist'* referring to foam in a brewing fermentation; whereas yeast in French corresponds to "*levure*" which means "bread dough rising" [Goldman and Green, 2008]. Yeasts can be identified as eukaryotic unicellular fungi, basically forming rounded, ovoid or cylindrical cells. They can reproduce asexually by forming vegetative buds known as blastospores or by cell fission. Some yeasts can also reproduce sexually by formation of spores [Barnett et al. 1983; Kurtzman and Fell, 1998]. Yeasts are characterized, classified, and identified according to their morphological and physiological features. However, the most important criteria for classification of yeasts are the composition of DNA bases, the homology of DNA, and the sequences of rDNA molecules [Kurtzman and Robnett, 1998]. More than 100 genera of yeasts have been identified through today, with approximately 1,500 species [Hutzler et al. 2015] that represent about 1% of yeasts on the planet [Tokuoka and Ishitani, 1991; Jindamorakot, 2000; Satyanarayana and Kunze, 2009; Vaz et al. 2011]. Yeasts are naturally present in different environments including plants, animals, and insects. They

are also isolated from different special extreme environments such as low water environments (e.g., high concentrations of sugar or salt), low oxygen environments (e.g., human and animal intestine), and low temperature environments (e.g., Antarctic origin yeasts) [Satyanarayana and Kunze, 2009; Vaz et al. 2011].

Yeasts have a unique status among microorganisms because they have to possess two valid names depending on their sexual state, which is called teleomorph, or imperfect, and their asexual state, which is known as anamorph, or perfect. Related to this, the teleomorph of *Hanseniaspora uvarum* is the anamorph of *Kloeckera apiculata* [Deak, 2008]. Yeasts also show a phenomenon called "dimorphism" when the anamorphic and telemorphic genera may found either as a "yeast–like", unicellular microorganism or as a "mold-like" filamentous microorganism depending on environmental conditions such as temperature and carbon dioxide levels [Goldman and Green, 2015]. Filamentous morphology was found in yeasts cultivated at room temperature (25^{o}C) while typical morphologies were encountered inside the host body at 37^{o}C , *Histoplasma capsulatum* is a typical example of this dual morphology [Deak, 2008]. Some species of yeasts can form a true mycelium, whereas other ones like *Candida* sp. are able to form a developed pseudo mycelium, or both "true'' and "pseudo" mycelium, as in *Candida tropicalis* [Deak, 2008; Kurtzman and Boekhout, 2011; Goldman and Green, 2015].

Yeasts are heterotrophic microorganisms using the organic elements to form their environment for growth [Kurtzman and Fell, 1998]. In order to degrade carbon sources and produce energy, yeasts use two different metabolic pathways known as respiration and alcoholic fermentation [Deak, 2006]. The metabolic requirements of yeasts are relatively modest. Carbon, nitrogen, phosphate, and sulphate, plus low concentrations of minerals such as potassium, magnesium, calcium, iron, zinc, and certain vitamins such as biotin, thiamine or pantothenic acid, together form a perfect growth medium [Deak, 2006; Conrad et al. 2014]. Carbohydrates, especially the hexose monosaccharides such as glucose, fructose, galactose, and mannose or the disaccharides such as maltose and sucrose form the main carbon sources [Deak, 2006; Buglass, 2011]. Yeasts are classified according to their ability to ferment glucides into three types: non-fermentative (e.g. *Cryptococcus* spp.), obligately fermentative (e.g. *Candida pintolopesii*), and facultatively

fermentative (e.g. *Saccharomyces cerevisiae*) [Jennings, 1995; Zaragoza et al, 2011; Schaechter, 2012].

In aerobic respiration, yeasts degrade glucides in the presence of oxygen in order to produce Carbon dioxide and water as primary products. However, anaerobic respiration occurs in the absence of oxygen, and the main products of this process are carbon dioxide with ethanol plus other metabolites such as fusel alcohols, polyols, esters, organic acids, vicinyl diketones and aldehydes [Schaechter, 2012]. Glucose and oxygen are the environmental factors regulating yeast cells' respiration, and their availability is linked to regulatory phenomena defined as the Pasteur effect (reduction in the rate of glycolysis under aerobic conditions), Crabtree effect (the short-term effect is the ability of stimulating alcoholic fermentation upon a sudden excess of glucose to inactivate the respiratory enzymes, while the long-term effect is the alcohol production in aerobic conditions because the excess of glucose acts to repress respiratory genes), Custers effect (stimulation of alcoholic fermentation by oxygen), and Kluyver effect (obligate aerobic utilization of disaccharides) [Briggs et al. 2004; Branett and Entian, 2005].

There are many species of non-pathogenic yeasts which reveal potential applications based on their probiotic claims such as production of antagonistic molecules, stimulation of the host immune system, and resistance to digestive tract conditions. [Hatoum et al. 2012, Romanin et al. 2016, Hudson et al. 2016].

4.1.2. *Saccharomyces cerevisiae* var. *boulardii*

Saccharomyces boulardii is closely related to the well-known *Saccharomyces cerevisiae*. The main difference between the two strains is the ability of *S. boulardii* to survive under low pH conditions and at 37°C [Edwards-Ingram, 2007]. The classification of yeasts on the basis of their biochemical and physiological properties remains the main approach to distinguishing between multiple species. This has led to a discussion about *S. boulardii,* which is considered a species or subspecies of *S. cerevisiae.* The developed molecular methods which were successfully used for yeast identification revealed that *S. boulardii* should be referred as *S. cerevisiae var. boulardii* [Rajkowska and Kunicka-Styczyńska, 2009].

S. boulardii was patented in 1947 by Henri Boulard as a eukaryotic probiotic microorganism when he sold it to Biocodex Company, created for probiotic production, and in 1953 it was registered as a drug for the first time [Łukaszewicz, 2012]. Extensive studies focused on investigating the efficacy and safety of *S. boulardii* in medical applications. *S. boulardii* was widely used for improving health by preventing antibiotic-associated diarrhea, viral diarrhea, and bacterial diarrhea [Szajewska et al. 2010; Surawicz, 2010; McFarland, 2010]. *S. boulardii* is active against pathogens through inactivation of bacterial toxins, nutritional competition, trophic effect, anti-inflammatory effects, cell restitution, epithelial barrier maintenance, immuno-modulatory effects and release of quorum-sensing molecules [Murzyn et al. 2010]. These applications were supported by a high number of publications and reports dedicated to this specific point [Castagliuolo et al. 1996; Murzyn et al. 2010].

4.1.3. *Debaryomyces hansenii* (*Candida famata*)

Debaryomyces hansenii, the teleomorph of *Candida famata*, is an ascomycetous yeast widespread in nature as it is regularly found in soil, air and water. It is also common in saline environments such as high salt foods, dairy products, and sea water [Vasdinyei and Deak, 2003; Aggarwal and Mondal, 2009]. Similarly, it has been detected in the human gut microbiota of individuals who regularly consume cheese, and it was also isolated in the GIT of animals [Raggi et al, 2014; Suhr et al. 2015].

4.1.3.1. Taxonomy, morphology and physiology

The exact taxonomic position of *D. hansenii* (*C. famata*) is Eukaryota (*Opisthokonta*); Fungi (*Dikarya*); Ascomycota (*Saccharomyceta*); *Saccharomycotina*; *Saccharomycetes*; *Saccharomycetales*; *Debaryomycetaceae*; *Debaryomyces*, *Debaryomyces hansenii* (http://www.ncbi.nlm.nih.gov/Taxonomy) and (http://www.uniprot.org/taxonomy) .

The species *D. hansenii* (anamorph: *C. famata*) forms white to cream-colored, smooth, glabrous colonies on rich yeast extract peptone dextrose (YPD) solid medium [Dmytruk and Sibirny, 2012]. Microscopic observation revels ovoid to broadly ellipsoidal

budding blastoconidia, 3.5-5 x 2-3.5 µm in size. No pseudohyphae are produced. Asci, when present, are spherical and persistent, containing 1-2 spherical ascospores with rough walls [Ellis et al. 2007]. According to Fitzpatrick et al. [2006], *D. hansenii* belongs to the monophyletic clade comprised of organisms that translate CTG as serine instead of leucine. The species *D. hansenii* (Zopf) Lodder and Kreger encompasses two varieties: *D. hansenii* (Zopf) Lodder and Kreger var. *hansenii* (anamorph: *C. famata* (Harrison) S.A. Meyer and Yarrow var. *famata*) and *D. hansenii* var. *fabryi* (Ota) Nakase & M. Suzuki (anamorph: *C. famata* (Harrison) S.A. Meyer and Yarrow var. *flareri* (Ciferri and Redaelli) Nakase and M. Suzuki). Recently, the intergenic spacer rDNA amplification and *AluI* fingerprinting (IGSAF) method revealed four distinct groups of *D. hansenii* strains. These are *D. hansenii* var. *hansenii*; *C. famata* var. *famata*; *D. hansenii* var. *fabryi*; and *C. famata* var. *flareri* [Nguyen et al. 2009]. The IGS sequence comparison of representative strains showed that *D. hansenii* var. *hansenii* and *C. famata var. famata* belong to one species, while *D. hansenii* var. *fabryi* and *C. famata* var. *flareri* belong to two different species that were finally approved as three new species of the genus *Debaryomyces* as the following: *Debaryomyces hansenii* (= *Candida famata*), *Debaryomyces fabryi* and *Debaryomyces subglobosus* (= *Candida flareri*) [Nguyen et al. 2009]. The species *D. hansenii* has the ability to grow up to 2.5M of NaCl and tolerate the highest concentrations up to 4M. [Prista et al. 1997; Lépingle et al. 2000]. They are able to assimilate a wide range of carbon sources such as glucose, galactose, sucrose, maltose, cellobiose, trehalose, raffinose, xylose, and arabinose (**Table2**) [Nakase et al. 1998; Davis et al. 2007].

Table 2: Assimilation of different substrates by *D. hansenii* [Nakase et al. 1998; Davis et al. 2007]

Glucose	+	N-Acetyl-D-glucosamine	V
Galactose	+	Methanol	-
L-Sorbose	V	Ethanol	+/w
Sucrose	+	Glycerol	+
Maltose	+	Erythritol	V
Cellobiose	+	Ribitol	+
Trehalose	+	Galacitol	V
Lactose	V	D-Mannitol	+
Melibiose	V	D-Glucitol	+/w
Raffinose	+	*A*-Methyl-D-glucoside	+
Melezitose	V	Salicin	+/w
Inulin	V	D-Gluconate	+/w
Soluble starch	V	DL-Lactate	V
D-Xylose	+	Succinate	-
L-Arabinose	+/w	Citrate	+/w
D-Arabinose	V	Inositol	-
D-Ribose	V	Hexadecan	V
L-Rhamnose	V	Nitrate	-
D-Glucosamine	V	Nitrite	V
2-Keto-D-gluconate	+	5-Keto-D-gluconate	V
Saccharate	-		

(+): positive; (W): weak; (V): variable; (-): negative.

D. hansenii is able to metabolize the n-alkanes and benzenoid compounds such as phenol, dihydroxybenzenes (catechol, resorcinol) and dihydroxybenzoic acids [Middelhoven, 1993; Yadav and Loper, 1999]. This species can grow under temperatures ranging from 5°C to 37°C, but the optimum temperature is between 25-28°C [Skinner and Davenport, 1980]. The fermentation of glucose, sucrose, maltose, trehalose, and raffinose by *D. hansenii* is weak mainly in the absence of oxygen [Nakase et al. 1998]. In iron-deficient media, *D. hansenii* appeared to overproduce riboflavin [Gadd and Edwards, 1986; Stahmann et al. 2000].

4.1.3.2. Biochemical properties with industrial importance and applications

D. hansenii was studied for possible industrial applications ascribed to its distinguished physiological and biochemical properties which include growth under high

saline concentrations up to 4 M of NaCl, conversely to *S. cerevisiae* whose growth is limited to less than 1.7 M of NaCl [Bansal et al. 2001]. This species also functioned as a cell factory as a result of its ability to produce a wide range of important biological molecules such as the following:

-riboflavin. Overproduction of riboflavin (vitamin B2) under iron limitation by *D. hansenii* has been known for more than 60 years [Tanner et al. 1945]. This species possesses the highest flavinogenic potential among riboflavin-producing yeasts [Dmytruk et al. 2014]. Wild-type strains of *D. hansenii* are capable of producing about 600 µg/ml of riboflavin [Levine et al. 1949], while other flavinogenic yeasts' productivity ranged between 5-300 µg/ml, and riboflavin production for non-flavinogenic yeasts is not more than 1-2 µg /ml [Sibirny et al. 2006].

- lignocellulose fermentation. *D. hansenii* is able to efficiently metabolize the lignocellulosic materials, which are the largest source of hexose and pentose with potential applications for chemical production. The ability to produce polyols from xylose and /or arabinose-containing media was also demonstrated [Parajó et al. 1997; Gírio et al. 2000].

- lytic enzymes production. Many lytic enzymes of *D. hansenii* are important for wine production, in particular, the β–glucosidases, which play a key role in increasing flavor compounds of the terpenol-containing juices by liberating monoterpenoles from D-glucopyranoside, β -D-xylopyranoside, α -L-arabinofuranoside and α -L-rhamnopyranoside [Yanai and Sato, 1999]. Esterase, one of the main enzymes involved with wine production, can be also produced by *D. hansenii* along with other non-*Saccharomyces* wine yeasts [Besancon et al. 1995; Esteve-Zarsoso et al. 1998]. *D. hansenii* can produce superoxide dismutase (SOD), a metalloenzyme which catalyses the dismutation of superoxide radicals into either ordinary molecular oxygen (O_2) or hydrogen peroxide (H_2O_2). Superoxide dismutase has important applications in the food industry. It also has medical relevance related to anti-inflammation, immune-response modulation, malignant tumor regression, radiation and chemotherapy protection, premenstrual syndrome, arthritis, and anti-aging treatments during the use of hyperbaric chambers, and uses against oxidative stress in general [Garcia-Gonzalez and Ochoa, 1999 ; Orozco et al. 1998; Zeinali et al. 2015].

-dairy production. *D. hansenii* is widely applied in the dairy industry. It is commonly found as a dominate yeast species in most cheese varieties including soft cheeses and the brines of semi-hard and hard cheeses [Fleet, 1990; Reps, 1993; Ceugniez et al. 2015]. It is also prevalent in yogurt and fruit preparations [Seiler, 1991; Viljoen and Greyling, 1995]. The desired features of *D. hansenii* such as the ability to adhere to solid devices, halo tolerance, low temperature demands, low water activity (a_w), metabolism of milk proteins and lipids, inhibitory effects against undesirable microbes in cheeses brines, together endow this species with the ability to play an important role in cheese manufacturing [Fatichenti et al. 1983; Breuer and Harms, 2006; Gori et al. 2007]. Moreover, *D. hansenii* is able to produce significant amounts of volatile Sulphur compounds such as methanethiol (MTL), dimethylsulphide (DMDS), dimethyltrisulphide (DMTS) and S-methyl thioacetate (MTA) in comparison to other cheese-ripening yeasts [Arfi et al. 2002].

- meat production. *D. hansenii* was shown to be involved in meat fermentation. This yeast is considered the most commonly one encountered in dry-cured meat products [Andrade et al. 2009]. In addition to its ability to produce volatile compounds, this species also influences the sensory properties of the meat [Andrade et al. 2009; Durá et al. 2004a, 2004b]. A few enzymes like prolyl amino peptidase, arginyl amino peptidase [Bolumar et al. 2003a, 2003b] and glutaminase [Durá et al. 2004c] produced by *D. hansenii* were isolated and characterized. However, their role and possible involvement in the ripening of a fermented sausage and in meat fermentation attracted wide attention [Durá et al. 2004a; Cano-García et al. 2014; Corral et al. 2015]. This also contributes to the stability of the color of dry-fermented sausages through oxygen consumption [Encinas et al. 2000]. Besides, *D. hansenii* showed antioxidant effects in ripened meat products contributing to the other microbiota by reducing the partial pressure of oxygen on the surface, degrading peroxides, and protecting them from pro-oxidant effects [Bai, 2014]. On other hand, *D. hansenii* was applied as an antagonistic agent in different food products because of its tolerance to high salt concentrations. This makes it very valuable for application as a bio preservative in such meat products [Chalutz and Wilson, 1990; Droby et al. 1989; Hernández-Montiel et al. 2010].

4.1.4. Probiotic and antagonistic potential of *Debaryomyces hansenii*

Probiotic microorganisms that are known to be beneficial to health can be obtained by using the viable cells (lyophilic preparations and tablets) or ingested through enrichment of various foods and fermented dairy products with these beneficial microbes [Yerlikaya, 2014]. The fulfillment of *D. hansenii* of QPS (Qualified Presumption of Safety) of microorganisms in food and feed status has qualified this species for permission by the European Union to be used in food production such as cheeses, fermented sausages and other food fermentations [Bourdichon et al. 2012]. Strain characteristics such as antimicrobial activity against food-borne pathogens and acid and bile stability may contribute to a fermented product with potential probiotic properties [De Smet et al. 1995]. Also, sensory characteristics such as unpleasant flavors or textures are important criteria to select probiotics [Crittenden and Tannock, 1999]. Interestingly, yeast existence in the GIT was found to be significantly correlated to recently consumed foods [Hoffmann et al. 2013]. *D. hansenii* is frequently detected in gut microbiota from individuals who consume cheese [Desnos-Ollivier et al. 2008]. The antimicrobial effect of yeast strains was first associated with yeast killer toxins (mycocins), which are proteinaceous compounds lethal to sensitive microorganisms such as yeasts, filamentous fungi, bacteria and parasites [Valzano et al. 2016]. Killer yeasts could have several applications in the food industry, including as bio-preservatives and starter strains in order to control the wild strains during production of products like beer [Muccilli and Restuccia, 2015], wine [Mehlomakulu et al. 2015], and bread [Kang–Heui et al. 2015]. The interest in *D. hansenii* as a probiotic has increased, especially in aquaculture. Related to this, *D. hansenii* and *S. cerevisiae* were used as probiotic strains for sea bass (*Dicentrarchus labrax*) larvae to investigate the effect of live cell incorporation in a compound diet on digestive enzyme activity [Tovar et al. 2002]. According to these authors, polyamines production was three-fold higher than that of *S. cerevisiae*. On the other hand, the synthesis of amylase and trypsin was increased in the presence of a *D. hansenii* diet group compared to that fed with *S. cerevisiae*. Reyes-Becerril et al. [2011] studied the effects of dietary supplementation with probiotic live yeast *D. hansenii* on the immune and antioxidant systems of leopard grouper fish (*Mycteroperca rosacea*) infected with *Aeromonas hydrophila*. They conducted their study in two trials, and they obtained

results showing a significant enhancement in fish growth as well as a significant increase in the levels of plasmatic immunoglobulin M in post-infected fish with *A. hydrophila* compared with the control. Mahdhi et al. [2011] investigated the probiotic properties of *Candida famata* and *Geobacillus thermoleovorans* isolated from pure oil waste. The two strains showed an antagonistic effect against some important pathogens such as *Salmonella* Typhimurium, *E. coli, Vibrio parahemolitycus* and *Staphylococcus aureus* in addition to their ability to express enzymes such as alkaline phosphatase, esterase lipase (C8), amylase, lipase, lecithenase and caseinase. This makes these strains possible candidates for probiotic applications. *D. hansenii* is marketed as a probiotic for human use in tablets form associated with other beneficial microorganisms (**Figure 7**).

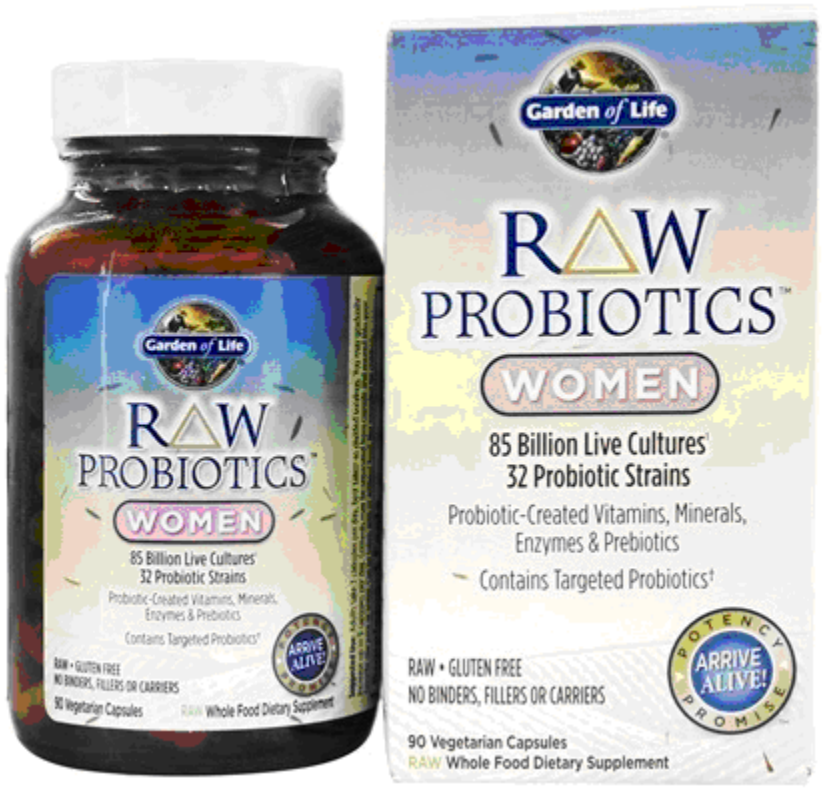

Fig. 7. A commercial probiotic product for human use containing 32 probiotic strains of bacteria and yeasts including *D. hansenii*. (http://www.powerofprobiotics.com/Raw-Probiotics-Women.html).

4.2. Lactic acid bacteria as probiotics

LAB is a group of useful bacteria which have been used unintentionally for thousands of years as starters in fermented dairy products (cheese, yogurt and butter), as documented in archaic texts from Uruk/Warka (Iraq) around 3,200 B.C. [Stiles and Holzapfel, 1997]. Lactobacilli (among LAB group), and bifidiobacteria are considered "generally recognized as safe" (GRAS) by the Food and Drug Administration in the USA and were allowed for food applications because they are devoid of harmful effects on human health - except one strain belonging to the *Lactobacillus rhamnosus* species [Otles, 2013].

4.2.1. General characteristics of lactic acid bacteria

A typical lactic acid bacterium grown under standard conditions (non-limiting glucose concentration, growth factors and oxygen limitation) is Gram-positive, nonsporing, catalase negative, areotolerant, acid tolerant, organotrophic and strictly fermentative rod or coccus, producing lactic acid as a major end product. It lacks cytochromes and is unable to synthesize porphyrins [König and Fröhlich. 2009]. LAB are classed into many genera, and those of relevance in food include the following genera: *Lactobacillus, Lactococcus, Enterococcus, Pediococcus, Leuconostoc and Streptococcus.* Notably, *Enterococcus* is found in the intestines of humans and animals, where they live as commensals and provide many beneficial effects [Khan et al., 2010].

4.2.2. Taxonomy of the genus *Enterococcus*

We cannot separate the early history of the enterococci from that of the genus *Streptococcus*. The term "entérocoque" was first coined by Thiercelin in 1899 to describe a new Gram-positive diplococcus in human feces [Thiercelin, 1899]. The publication of Thiercelin and Jouhaud [1903] was the first effort to designate the genus *Enterococcus*. However, enterococci were renamed by Andrewes and Horder [1906] as *Streptococcus faecalis (Strep. faecalis*) based on its ability to form short or long chains. Consequently, the enterococci were considered a subgroup of the genus *Streptococcus*. Orla-Jensen

[1919] described *Strep. faecium*, *Strep. glycerinaceus* and *Strep. liquefaciens* in a study of heat-resistant streptococci isolated from fecal origin. The properties and possible similarities between *Enterococcus* and fecal *Streptococcus* were studied by Dible [1921]. This author postulated that these bacteria may belong to the *Streptococcus* genus. A serological grouping of streptococci was proposed by Lancefield [1933]; who used the alphabet letters to nominate the microorganisms and installed the enterococci under the group D antigen. Afterwards, streptococci were divided into four groups, specifically pyogenic, viridians, lactic and enterococci, based on their physiological and biochemical characteristics [Sherman, 1937]. The word *Enterococcus* was used again by Kalina [1970], who proposed the separation of *Enterococcus* from *Streptococcus* as an independent genus and renamed *Strep. faecalis* as *E. faecalis.* The author also introduced the *E. faecium* as a second species of the genus *Enterococcus,* which previously was mentioned by Orla-Jensen. In 1978, Jones used the names "oral" and "fecal" streptococci instead of "viridians" and "enterococci" based on new biochemical and physiological features [Jones, 1978]. He suggested the groupings of pneumococci, anaerobic and other streptococci. The taxonomic debate was finally settled by Schleifer and Kilpper-Bälz [1984], who confirmed the separation of *Streptococcus faecalis and Streptococcus faecium* from the other streptococci and clearly differentiated these two species by using DNA and DNA-rRNA hybridization. They classified them under the genus *Enterococcus*. Moreover, Collins et al [1984] reclassified the species that named *Streptococcus avium* [Nowlan and Deibel, 1967], *Streptococcus casseliflavus* [Vaughn et al. 1979], *Streptococcus durans* [Sherman and Wing, 1937], *Streptococcus faecalis subsp. malodoratus* [Pette, 1955], and *Streptococcus gallinarum* [Bridge and Sneath, 1982], as members of the *Enterococcus* genus. They presented these taxa as *Enterococcus avium nom. rev., comb. nov., Enterococcus casselifavus nom. rev., comb. nov., Enterococcus durans nom. rev., comb. nov., Enterococcus malodoratus sp. nov.,* and *Enterococcus gallinarum comb. nov.,* respectively. In 1986, the genus *Enterococcus* was officially validated by the Bergey's Manual of Systematic Bacteriology, but it was mentioned as an editorial note in an *Enterococcus* chapter which was already completed [Mundt, 1986].

4.2.3. Phenotypic characteristics of *Enterococcus* genus

The members of *Enterococcus* genus are Gram-positive and catalase-negative spherical or ovoid cells 0.6-2.0 × 0.6-2.5 µm; they occur singly, in pairs, or in short chains in liquid media. They are facultative anaerobes, nonsporing, lake obvious capsule, chemoorganotrophs with fermentative metabolism of a wide range of carbohydrates with the production of mainly L(+)-lactic acid but no gas and final pH of 4.2-4.6 [Teixeira and Facklam, 2003]. They are tolerant of extreme conditions of temperatures, pH, and salinity [Sherman, 1937, Teixeira and Facklam, 2003]. They hydrolyze esculin in the presence of 40% bile-salts. Most of enterococci are able to hydrolyze L-pyrrolidonyl-ß- naphtylamide (PYR) and all of them hydrolyze leucine-ß-naphtylamide by producing leucine aminopeptidase (LAPase). They usually ferment lactose and seldom reduce nitrate. Some species, such as *E. gallinarum*, are motile [Graudal, 1957; Mundt, 1986; Holt et al. 1994]. The phenotypic traits of strains belonging to *Enterococcus* genus are very similar to those of other Gram-positive and catalase-negative enterococci. However, there are no phenotypic characteristics which can precisely differentiate between them [Klein, 2003; Fisher and Phillips, 2009] (**Figure 8**).

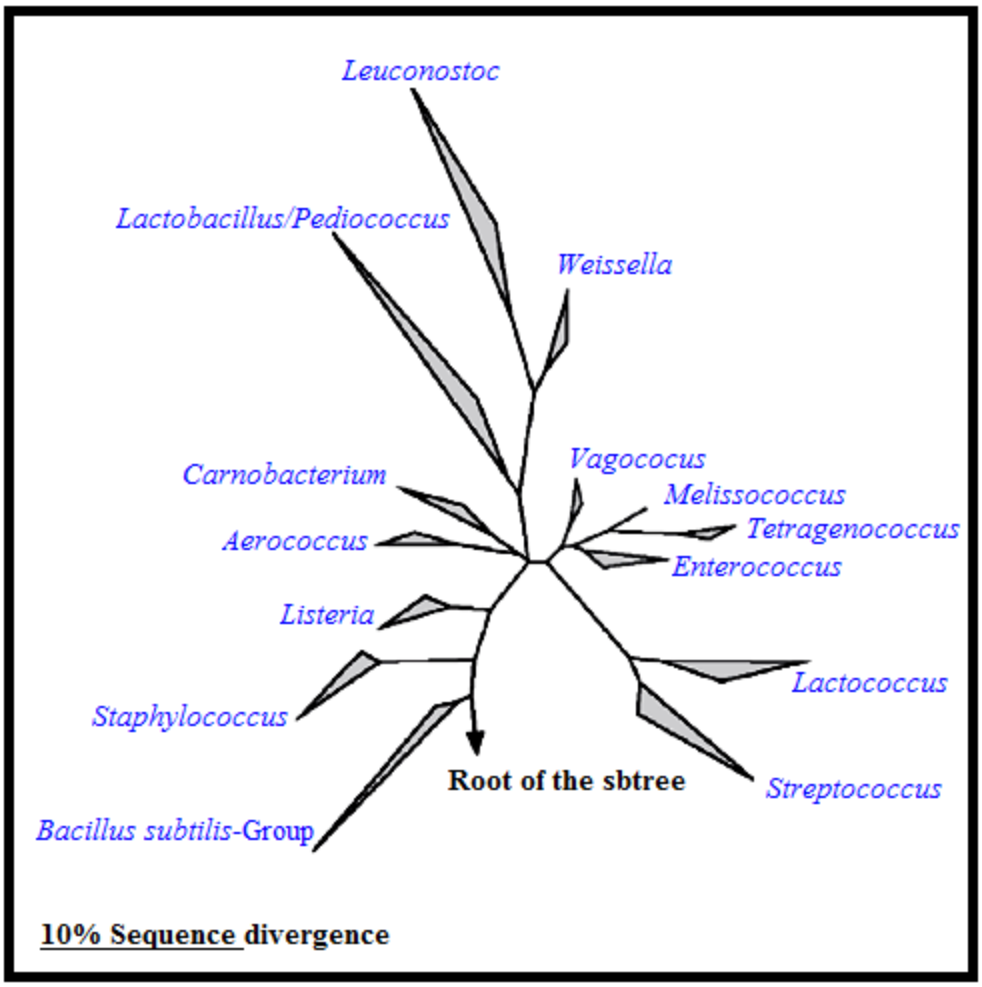

Fig. 8. Phylogenetic position of the genus *Enterococcus* demonstrated by 16S rRNA-dendrogram of Gram-positive genera, including *Streptococcus* and *Lactococcus*. The length of the branches indicates a 10% estimated sequence divergence [Klein, 2003].

Enterococci have been divided into five groups on the basis of their acid production in mannitol and sorbose broth plus their ability to hydrolyze arginine. This grouping included 22 *Enterococcus* species which were recently identified [Lebreton et al. 2014]. As depicted in **Table 3**, group I comprises the *Enterococcus* species that are unable to hydrolyze arginine but are able to form acid by fermenting both mannitol and sorbose broths. Group II consists of species that hydrolyze arginine and form acid in mannitol broth but are unable to ferment the sorbose. Species that belong to group III are able to hydrolyze arginine, but they are not able to form acid in mannitol and sorbitol broths.

Table 3: Phenotypic properties of recently identified *Enterococcus* species [Lebreton et al. 2014]

Species	Phenotypic Characteristic										Group
	MAN	SOR	ARG	ARA	SBL	RAF	MOT	PIG	SUC	MGP	
E. phoeniculicola	+	+	-	+	+	-	-	-	+	nd	I
E. devriesei	+	+	-	v	V	V	-	-	+	-	I
E. canis	+	+	-	+	+	-	-	-	v	+	I
E. canintestini	+	-	+	-	-	-	-	-	+	+	II
E. lactis	+	-	+	+	-	-	-	-	-	-	II
E. thailandicus	+	-	+	-	-	-	-	-	+	-	II
E. sanguinicola	+	-	+	-	-	-	-	-	+	-	II
E. silesiacus	-	-	+	-	-	-	-	-	-	-	III
E. rotai	-	-	+	-	-	-	-	+	+	-	III
E. ratti	-	-	+	-	-	-	-	-	-	-	III
E. aquimarinus	-	-	-	+	-	+	-	-	+	-	IV
E. caccae	-	-	-	-	-	-	-	-	+	-	IV
E. plantarum	-	-	-	-	-	-	-	w	+	-	IV
E. termitis	-	-	-	-	-	-	-	-	-	+	IV
E. rivorum	+	-	-	-	+	-	-	-	+	-	V
E. hermaniensis	+	-	-	-	-	-	-	-	-	nd	V
E. camelliae	+	-	-	-	-	-	-	-	+	-	V
E. viikkiensis	+	-	-	-	-	-	-	-	nd	-	V
E. ureilyticus	-	-	-	-	-	-	-	+	+	-	VI
E. quebecensis	+	nd	-	-	-	-	-	-	+	nd	NA
E. italicus	v	-	-	-	v	-	-	-	+	+	NA
E. ureasiticus	v	nd	-	-	-	-	-	-	+	nd	NA

Abbreviations and symbols: MAN, mannitol; SOR, sorbose; ARG, arginine; ARA, arabinose; SBL, sorbitol; RAF, raffinose; MOT, motility; PIG, pigment; SUC, sucrose; MGP, methyl- α-D-glucopyranoside; +, >80% positive; ⁻,<20% positive; v, variable.

Group IV contains the species that are unable to hydrolyze arginine and fail to form acid in mannitol and sorbose broths. Finally, the species of group V are unable to hydrolyze arginine, and they are able to form acid in mannitol broth but unable to ferment the sorbose [Facklam, et al. 2002; Lebreton et al. 2014].

4.2.4. Genotypic characterization of the genus *Enterococcus*

As mentioned previously, the *Enterococcus* genus was separated from the streptococci based on DNA-DNA and DNA-rRNA hybridization studies [Schleifer and Kilpper-Bälz, 1984; Schleifer et al. 1985; Schleifer and Kilpper-Bälz, 1987]. Ludwig et al. [1985] affirmed the separation of *Enterococcus* genus from those of *Streptococcus* and *Lactococcus* by 16S ribosomal RNA sequences (rRNA oligonucleotide cataloging). The rRNA sequence analysis revealed that the *Enterococcus* genus belongs to the Gram-

positive bacteria with low (≤50 mol %) guanine and cytosine (G+C) content in the DNA, as clostridia and bacilli. Different molecular techniques were developed to identify the enterococci to species and even sub-species levels using 16S rRNA gene sequencing, multilocus sequence analysis (MLSA), pulsed-field gel electrophoresis (PFGE), amplified fragment length polymorphism (AFLP) analysis, randomly amplified polymorphic DNA (RAPD) analysis, intergenic ribosomal PCR and *atpA* gene Sequence Analysis [Angeletti et al. 2001, Homan et al. 2002, Descheemaeker et al. 1997, Bruinsma et al. 2002, Domig et al. 2003, Baele et al. 2000, Ozawa et al. 2000, Baher et al. 2000, Naser et al.2005]. Up through today, fifty five *Enterococcus* species have been identified within the genus due to the genotyping methods as illustrated in **Table 4**.

Table 4: Species of *Enterococcus* and genotyping methods used for their identification

Species	Genotyping Methods	Author(s) references
E. alcedinis	(GTG)5-PCR fingerprinting, 16S rRNA gene sequences analysis, sequencing of the genes for (sodA), phenylalanyl-tRNA synthase (pheS) and the RNA polymerase alpha subunit (rpoA), and whole-cell protein fingerprinting.	Frolkov et al. 2013
E.. aquimarinus	16S rRNA gene sequence, (GTG)5-PCR fingerprinting, housekeeping gene phenylalanyl-tRNA synthase (pheS).	Švec et al. 2005a
E. asini	16S rRNA gene sequence analysis.	De Vaux et al. 1998
E. avium	DNA-DNA relatedness	Collins et al. 1984
E. caccae	16S rRNA gene sequence analysis, DNA–DNA relatedness experiments.	Carvalho et al. 2006
E. camelliae	16S rRNA sequence analysis and DNA-DNA hybridization.	Sukontasing et al. 2007
E. canintestini	16S rRNA sequence analysis, MLSA, DNA-DNA hybridization.	Naser et al. 2005
E. canis	16S rRNA sequence analysis, and DNA–DNA hybridization, tDNA interspacer gene PCR, SDS-PAGE of whole-cell proteins.	De Graef et al. 2003
E. casseliflavus	DNA-DNA relatedness	Collins et al. 1984
E. cecorum	Reverse transcriptase sequencing of 16S rRNA	Williams et al. 1989
E. columbae	16S rRNA sequence analysis and DNA-DNA hybridization.	Devriese et al. 1990
E. devriesei	16S rRNA sequence analysis, housekeeping gene sequence pheS (encoding the phenylalanyl-tRNA synthase a-subunit), (GTG)5-PCR fingerprinting, ribotyping and DNA–DNA hybridization.	Švec et al. 2005b
E. diestrammenae	16S rRNA gene sequences, comparative (pheS) and (rpoA) sequence analyses, DNA (G+C) content.	Kim et al. 2013
E. dispar	Partial 16S rRNA sequence analysis	Collins et al. 1991
E. durans	DNA-DNA relatedness	Collins et al. 1984
E. eurekensis	16S rRNA, RNA polymerase-subunit (rpoA), and the 60-kilodalton chaperonin (cpn60) gene sequence analyses.	Cotta et al. 2013
E. faecalis	DNA-DNA and DNA-rRNA hybridization.	Schleifer and Kilpper-Bälz 1984
E. faecium	DNA-DNA and DNA-rRNA hybridization.	Schleifer and Kilpper-Bälz 1984
E. flavescens	DNA-DNA hybridization and DNA base composition.	Pompei et al. 1992
E. gallinarum	DNA-DNA relatedness	Collins et al. 1984

E. gilvus	16S rRNA gene sequence analysis	Tyrrell et al. 2002
E. haemoperoxidus	tRNA intergenic length polymorphism analysis, 16S rRNA sequence analysis, DNA base composition and DNA-DNA hybridization.	Švec et al., 2001
E. hermanniensis	16S rRNA sequence analysis and DNA-DNA hybridization	Koort et al. 2004
E. hirae	DNA base composition and DNA-DNA hybridization	Farrow and Collins 1985
E. Italicus	tRNA intergenic length polymorphism analysis, 16S rRNA sequence analysis and DNA-DNA hybridization	Fortina et al. 2004
E. lactis	16S rRNA sequence analysis, phenylalanyl-tRNA synthase alpha subunit (pheS), RNA polymerase alpha subunit (rpoA), 16S–23S rRNA intergenic transcribed spacer (ITS) sequences, randomly amplified polymorphic DNA (RAPD) PCR.	Morandi et al. 2012
E. lemanii	16S rRNA, RNA polymerase-subunit (rpoA), and the 60-kilodalton chaperonin (cpn60) gene sequence analyses	Cotta et al. 2013
E. moraviensis	tRNA intergenic length polymorphism analysis, 16S rRNA sequence analysis, DNA base composition and DNA-DNA hybridization.	Švec et al. 2001
E. mundtii	DNA base composition and DNA-DNA hybridization.	Collins et al. 1986
E. olivae	16S rRNA sequence analysis, DNA–DNA relatedness and DNA G+C content, RNA polymerase alpha subunit (rpoA)	Lucena-Padrós et al. 2014
E..pallens	16S rRNA gene sequence analysis	Tyrrell et al. 2002
E. phoeniculicola	16S rRNA gene sequence analysis	Law-Brown and Meyers 2003
E. plantarum	(GTG)5-PCR fingerprinting, 16S rRNA gene sequence, phenylalanyl-tRNA synthase alpha subunit (pheS) and the RNA polymerase alpha subunit (rpoA) sequence analyses.	Švec et al. 2012
E. porcinus	DNA-DNA relatedness	Teixeira et al. 2001
E. pseudoavium	DNA base composition, DNA-DNA hybridization	Collins et al. 1989
E. quebecensis	16S rRNA gene sequence analysis, housekeeping genes rpoA (encoding RNA polymerase a subunit), pheS (phenylalanyl-tRNA synthase), tufA (elongation factor Tu) and atpD (ATP synthase b-subunit), amplified fragment length polymorphism (AFLP) DNA fingerprinting and DNA–DNA hybridization.	Sistek et al. 2012
E. raffinosus	DNA base composition, DNA-DNA hybridization	Collins et al. 1989
E. ratti	DNA-DNA relatedness	Teixeira et al. 2001
E. rivorum	16S rRNA gene sequence analysis, partial atpA and pheS gene sequence, (GTG)5-PCR fingerprints.	Niemi et al. 2012
E. rotai	16S rRNA gene sequence analysis, DNA base composition, rep-PCR fingerprinting and automated ribotyping, DNA–DNA hybridization, pheS and rpoA sequencing.	Sedláček et al. 2013
E. saccharolyticus	16S rRNA gene sequence analysis	Rodrigues and Collins, 1990.
E. saccharolyticus subsp. saccharolyticus	DNA–DNA hybridization, DNA G+C content, 16S rRNA, pheS and rpoA gene sequences, rep-PCR fingerprinting.	Chen et al. 2013
E. saccharolyticus Subsp. taiwanensis	DNA–DNA hybridization, DNA G+C content, 16S rRNA, pheS and rpoA gene sequences, rep-PCR fingerprinting	Chen et al. 2013

E. saccharominimus	16S rRNA gene sequence analysis, tRNA intergenic length polymorphism analysis, DNA–DNA hybridization, DNA G+C contents	Vancanneyt et al. 2004
E. seriolicida	DNA–DNA hybridization, DNA G+C contents	Kusuda et al. 1991
E. silesiacus	(GTG)5-PCR fingerprints, 16S rRNA gene sequence analysis, housekeeping gene pheS (encoding the phenylalanyl-tRNA synthase a-subunit), DNA–DNA hybridization.	Švec et al. 2006
E. solitarius	DNA base composition, DNA-DNA hybridization	Collins et al. 1989
E. sulfureus	16S rRNA sequences analysis	Martinez-Murcia, and Collins 1991
E. termitis	(GTG)5-PCR fingerprints, 16S rRNA gene sequence analysis, housekeeping gene pheS (encoding the phenylalanyl-tRNA synthase a-subunit), DNA–DNA hybridization.	Švec et al. 2006
E. thailandicus	DNA G+C content, 16S rRNA and RNA polymerase a-subunit (rpoA) gene sequence analysis.	Tanasupawat et al. 2008
E. ureasiticus	16S rRNA gene sequences, housekeeping genes rpoA (encoding RNA polymerase a subunit), pheS (phenylalanyl-tRNA synthase), tufA (elongation factor Tu) and atpD (ATP synthase b-subunit) amplified fragment length polymorphism (AFLP) DNA fingerprinting and DNA–DNA hybridization.	Sistek et al. 2012
E. ureilyticus	16S rRNA gene sequence analysis, DNA base composition, rep-PCR fingerprinting and automated ribotyping, DNA–DNA hybridization, pheS and rpoA sequencing.	Sedláček et al. 2013
E. viikkiensis	16S rRNA gene sequence analysis, DNA-directed RNA polymerase subunit A (rpoA) and phenylalanyl-tRNA synthetase α chain (pheS) housekeeping gene analyses, DNA-DNA hybridization.	Rahkila et al. 2011
E. villorum	16S rDNA sequence analysis, DNA–DNA hybridizations, and DNA base-ratio determinations.	Vancanneyt et al. 2001
E. xiangfangensis	16S rRNA gene sequence analysis, phenylalanyl-tRNA synthase (pheS) gene sequence analysis, RNA polymerase A subunit (rpoA) gene sequence analysis, DNA G+C content, DNA–DNA hybridization.	Li et al. 2014

4.2.5. Physiological properties

Enterococci are chemo-organotrophic organisms with the capability of converting sugars into L-lactic acid by homo-fermentative pathways [Franz et al. 2003]. These bacteria can grow in a wide range of temperatures and restrictive environments such as the presence of high salt concentrations and low pH. Nevertheless, the ability of the *Enterococcus* genus to grow at temperatures ranging from 10 to 45 °C, pH ranging from 4 to 9.6, NaCl concentration between 5 to 10%, and in the presence of bile and sodium azide facilitate the differentiation between this genus and other homo-fermentative cocci such as Streptococci and Lactococci, according to Franz et al. [2003]. The typical physiological properties of valid *Enterococcus* species are shown in Table 4 according to Domig et al. [2003]. However, recently-identified *Enterococcus* species vary in their physiological characteristics from those of typical enterococci. Related to this, there are discrepancies in different studies about the group D antigen in enterococcal species (**Table 5)**. Some enterococcal strains may not react with group D antiserum; they are considered unable to produce group D antigens [Franz et al. 2003]. Conversely, other reports suppose that all *Enterococcus* species definitely produce the group D antigen and attributed the problem to laboratory techniques used that failed to detect its presence in these isolates [Hartman et al. 2013].

Several species of *Streptococcus*, *Pediococcus* and *Leuconostoc* strains are capable of reacting with group D antiserum, which could lead to a misleading identification of *Enterococci*. In direct line, several *Enterococcus* strains are unable to grow at a 6.5% NaCl concentration (**Table 5**), while other strains belonging to *Lactococcus*, *Pediococcus*, *Aerococcus* and *Leuconostoc* are capable of growing under this concentration [Christensen and Ruoff, 2015]. Conversely to *Pediococcus* and some *Lactococcus* strains, some *Enterococcus* species fail to grow at 45°C (**Table 5**). The growth at 10 °C is not only limited to the *Enterococcus* species; most *Leuconostocs*, *Lactococci* and several species of *Streptococci* are also able to grow at this same temperature while some *Enterococcus* species do not (**Table 5**).

Table 5: Physiological properties of validly described enterococcal species [Domig et al. 2003].

Species	Growth at			Growth in the presence of			Aeusculin hydrolysis	Group D antigen
	10°C	45°C	pH 9.6	6.5% NaCl	40% bile	0.04% sodium Azide		
E. asini	(+)	(+)	n.d	-	+	n.d	+	+
E. avium	v	+	+	v	v/+	n.d	+	+
E casseliflavus	+	+	+	v/+	+	+	+	+
E. cecorum	-	+	(+)	-	(+)	-	+	-
E. columbae	-	n.d	n.d	-	(+)	-	+	-
E. dispar	+	-	n.d	+/-	+	-	+	-
E. durans	+	+	+	+	+	+	+	(+)
E. faecalis	+	+	+	+	+	+	+	+
E. faecium	+	+	+	+	+	+	+	v
E. flavescens	v/-	v/+	n.d	+	+	+	+	+
E. gallinarum	+	+	+	+	+	+	+	+
E. haemoperoxidus	+	-	n.d	+	+	+	+	+
E. hirae	+	+	+	+	+	+	+	v
E. malodoratus	+	-	+	+	+	n.d	+	+
E. moraviensis	+	-	n.d	+	+	+	+	+
E. mundtii	+	+	+	+	+	+	+	+
E. porcinus	+	+	n.d	+	n.d	n.d	+	+
E. pseudoavium	+	+	+	+/-	v/+	n.d	+	-
E. raffinosus	(+)	+	+	+	v/+	n.d	+	n.d
E. ratti	+	+	n.d	+	n.d	n.d	+	(+)
E. saccharolyticus	+	+	n.d	(+)	+	n.d	+	-
E. solitarius	+	+	n.d	+	+	n.d	+	+
E. sulfureus	+	-	n.d	+	+	n.d	+	-
E. villorum	n.d	n.d	n.d	+	+	+	+	n.d

n.d.: not determined; (+): weak positive; V: variable; +/-: differing reports in literature

4.2.6. Biochemical properties with industrial importance and applications

Enterococci are known as ubiquitous bacteria, which are commonly associated with GIT microbiota of human and animals in addition to a wide variety of foods [Singh and Nakayama, 2015]. Interest in this genus has increased during the last decade due to their beneficial effects and negative impact on human health [Giraffa, 2002; Franz et al. 2003]. Enterococci are one of the most common LAB commercially used as silage inoculants [Aragón, 2012]. The contribution of the *Enterococcus* species to the development of sensorial traits when used as adjunct starter cultures in dairy production, as well as their potential as probiotic organisms were reported early [Moreno et al. 2006]. In addition,

enterococci are essential constituents of the natural microbiota involved in the ripening of fermented sausages [Hugas et al. 2003].

4.2.6.1. Lactic acid production

Lactic acid is recognized as GRAS (Generally Regarded as Safe) by the US Food and Drug Administration due to its exclusive physicochemical properties and its wide application in food and chemical industries [Benthin and Villadsen, 1995; Subramanian et al. 2015]. Bacteria use glucides as a source of energy through different catabolic pathways in order to produce ATP and other reducing equivalents. The well-characterized pathways for glucides catabolism in bacteria are the Embden-Meyerhof-Parnas (EMP), the pentose phosphate (PP), and the Entner-Doudoroff (ED) pathways [Ray and Joshi, 2014]. LAB are classified into homofermentative and heterofermentative, depending on the end product of the fermentation process. The pathways of lactic acid production differ for these two groups. Indeed, the homofermentative group produces mainly lactic acid via the glycolytic (Embden-Meyerhof-Parnas) pathway, while heterofermentative group use the pentose-phosphate (6-phosphogluconate/phosphoketolase) pathway for carbohydrate fermentation and produce lactic acid plus noticeable amounts of ethanol, acetate and carbon dioxide [Axelsson, 2004].

The fermentation of carbon leads to stereospecific D(-)-lactic acid (which is known to be harmful to human metabolism) or L(+)-lactic acid depending on the strains used [Hamdan and Sonomoto, 2011]. Thus, D(-)-lactic acid is produced by *Leuconostoc* and *Lactobacillus bulgaricus* while *Lactobacillus, Bacillus, Rhizopus, Streptococcus* and *Enterococcus* produce L(+)-lactic acid which is the upmost one used in food and pharmaceutical technologies [Park et al. 2010; Subramanian et al. 2015]. Only few strains of lactic acid bacteria belonging to the genus *Lactobacillus, Lactococcus* and *Enterococcus* are capable of producing optically pure L(+) -lactic acid [Benthin and Villadsen, 1995; Yun et al. 2003]. A novel strain *E. faecalis* RKY1 was successfully applied to production of optically pure L(+)-lactic acid from various carbohydrates by batch fermentation in high yields ranging from 130 to 333 g/l through the

homofermentative pathway [Yun et al. 2003; Nandasana and Kumar, 2008; Reddy et al. 2016]. Abdel-Rahman et al. [2011] identified a novel strain named *E. mundtii* Q25, which efficiently metabolize xylose into L(+)-lactic acid in batch fermentation with optical purity of ≥ 99.9%, and without acetate production. Subramanian et al. [2015] described production of L(+)-lactic acid at high concentration, yield and volumetric productivity with *E. faecalis* CBRD10. The highest concentration of L(+)-lactic acid was 182 g/l after 38 h of glucose fermentation in fed-batch culture, and conversion efficiency of glucose to lactic acid was 87 to 98%. Moreover, Oltuszak-Walczak and Walczak [2015] isolated and characterized eight strains of *E. faecium* able to ferment the D-xylose to L-lactic acid. The concentration of lactic acid in the growth medium after 72 h of fermentation was between 16.8 and 29.1 g/l. Strains *E. faecium* KD31 and *E. faecium* K4 produced more than 28 g/l of L-lactic acid, whereas *E. faecium* 2B2 only produced 16.8 g/l, and the chiral purity of produced lactic acid varied between 93.8 and 100 %.

4.2.6.2. Proteolytic activity

Proteolytic systems of LAB, to which the *Enterococcus* genus belongs, are considered to have one of the most important traits essential to technological applications, enabling them to grow in protein-containing media like milk, ripened cheese and fermented meat products [Sarantinopoulos et al. 2002; Savijoki et al. 2006; Ruiz-Moyano et al. 2009]. *E. faecalis* and *E. faecium* are the most frequently detected species in certain types of cheeses and fermented sausages from several Mediterranean countries [Moreno et al. 2006; Aquilanti et al. 2015]. They are unintentionally used as starter cultures to enhance the product's quality by contributing to ripening and flavour development [Psoni et al. 2006; Franz et al. 2011]. Nevertheless, strains like *E. faecalis* ND3, *E. faecalis* HM3C, and *E. faecalis* G32 previously isolated from fermented wheat dough exhibited high proteolytic activity on wheat proteins [M'hir et al. 2008]. These strains showing proteolytic activity could be of high value in cereal-based fermented foods by helping to reduce gliadin involved in coeliac disease [M'hir et al. 2008]. Proteolytic activity of the enterococci varies depending on the species; however, *E. faecalis* scored the highest in proteolytic activity among them [Giraffa, 2003]. There is no obvious relationship linking

the acidification capability and proteolytic activity of enterococci [Durlu-Ozkaya et al. 2001]. Despite that, these two properties were fortuitously correlated [Hagrass et al. 1991; Giraffa et al. 1993; Suzzi et al. 2000].

4.2.6.3. Lipolytic activity

Esterases and lipases are found throughout all kingdoms of life. While lipases exhibited high activity in the aggregated state of its substrate, esterases demonstrated the highest activity in the soluble state of its substrate [Fojan et al. 2000]. The *Enterococcus* species showed lipolytic and esterolytic activities which contribute to the development of organoleptic properties during the ripening of fermented products such as cheeses and sausages [Centeno et al. 1999; Ogier and Serror, 2008]. The role of enterococci in sausage aromatization by glycolytic, proteolytic and lipolytic activities has been already reported [Hugas et al. 2003; Moreno et al. 2006; Tarantella et al. 2012; Aquilanti et al. 2015]. Nevertheless, esterases linked to flavour development and cheese texture through milk fat hydrolyses, and further conversion of free fatty acids produced methyl ketones and thioesters which function as cheese flavour compounds [Cárdenas et al., 2016]. Lipolysis is involved indirectly in cheese rheology. Its effect on cheese texture is related to partial glycerides which are tensio-active compounds influencing the molecular organisation [Giraffa, 2003]. Milk triglycerides hydrolysis by *Enterococcus* species was reported, and *E. faecalis*, *E. faecium*, and *E. durans* were described as the most lipolytic species [Dovat et al. 1970; Macedo and Malcata, 1997; Sarantinopoulos et al. 2001; Durlu-Ozkaya et al. 2001]. The esterolytic system of enterococci is more complex and efficient than their lipolytic system [Giraffa, 2003]. *E. faecium* is the most esterolytic species among the enterococci. Furthermore, many strains of enterococci exhibited higher esterolytic activity than other genera of LAB [Giraffa, 2003].

4.2.6.4. Citrate and pyruvate metabolism

Citrate and pyruvate metabolism by LAB plays an important role in the fermentation of many foods due to the production of several flavour compounds [Martino et al. 2016].

In milk, citrate is metabolized by different LAB species into flavour compounds such as acetate, acetaldehyde, and diacetyl [Hugenholtz, 1993; Giraffa, 2003]. Studies centered on citrate metabolism by *Enterococcus* species have increased to inspect their behavior in citrate transport and aroma generation [Cabral et al. 2007; Martino et al. 2016]. In LAB, citrate uptake is mediated via a citrate permease and then is broken down by a citrate lyase to oxaloacetate and acetate. Decarboxylation of oxaloacetate results in pyruvate which is then converted into acetaldehyde-thiamine pyrophosphate by α-acetolactate and decarboxyated under oxidizing conditions to produce diacetyl. However, acetoin is formed by decarboxylation of α- acetolactate by α- acetolactate decarboxylase or from the reduction of diacetyl by diacetyl reductase (**Figure 9**) [Hugenholtz, 1993; Drider et al. 2004].

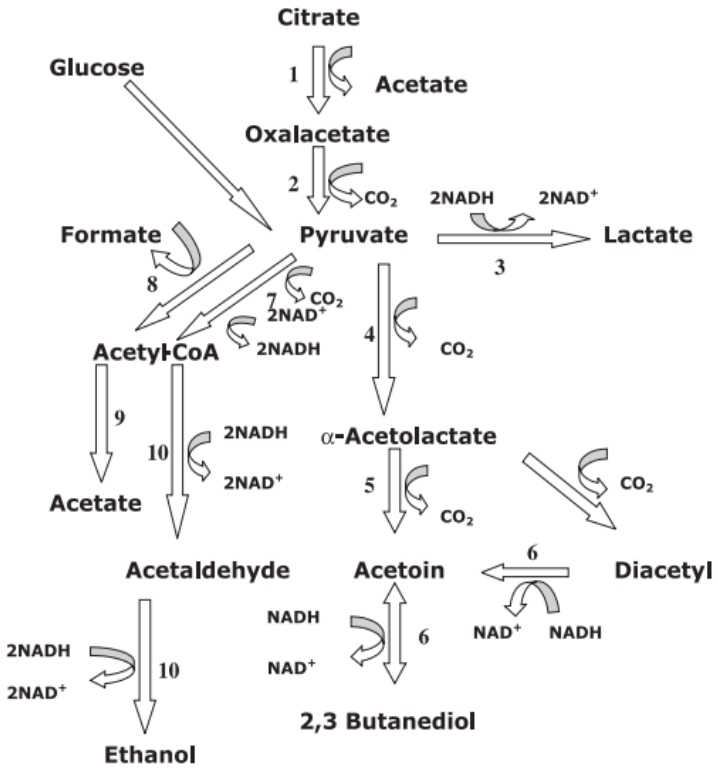

Fig. 9. Schematic pathway showing the metabolic relationships between citrate and glucose. 1, citrate lyase; 2, oxaloacetate decarboxylase; 3, lactate dehydrogenase; 4, α- aceto lactate synthase; 5, α-acetolactate decarboxylase; 6, diacetyl and/or acetoin reductase; 7, pyruvate dehydrogenase complex; 8, pyruvate formate lyase; 9, acetate kinase; and 10, alcohol dehydrogenase [adapted from Hugenholtz, 1993].

Freitas et al. [1999] considered citrate metabolism in milk as evidence of *E. faecalis* and, to a lesser extent, of *E. faecium*, isolated from Picante cheese. Citrate metabolism by *E. faecalis* FAIR-E229 in various growth media containing citrate, either in the presence of lactose or glucose as the sole carbon source, was investigated by Sarantinopoulos et al. [2001]. Their results revealed that *E. faecalis* FAIR-E229 could co-metabolize citrate and lactose in milk in the presence of yeast extract, whereas it was unable to metabolize citrate in presence of lactose or glucose in a more complex medium such as MRS broth.

4.2.6.5. Production of volatile compounds

As previously mentioned, important volatile compounds such as acetaldehyde, ethanol, diacetyl, and acetoin could be produced by citrate and lactose breakdown by the *Enterococcus* species, which may further contribute to flavour development during cheese ripening [Martino et al. 2016]. To this end, many *enterococcal* strains like *E. faecalis* and *E. faecium* were isolated from dairy products and shown to be good producers of acetaldehyde, ethanol, diacetyl, and acetoin when grown in milk, contributing therefore to the development of cheese flavour and aroma [Hagrass et al. 1991; Sarantinopoulos et al. 2001; Giraffa 2003]. Sarantinopoulos et al. [2001], isolated 129 *E. faecium, E. faecalis* and *E. durans* strains of food, animal, and human origin and assessed them for their biochemical properties and technological performances. Acetaldehyde, ethanol and acetoin were the main volatile compounds. None of the strains decarboxylated histidine, lysine and ornithine, but the majority produced tyramine from tyrosine, independently of origin or species. The results also showed that, regarding the origin of the isolates, *E. faecalis* isolates of food origin were the main acetaldehyde producers. Ethanol concentrations were also highest among *E. faecalis* isolates of food origin. Acetoin concentrations were found in the highest concentrations and more frequently among *E. faecium* strains of food origin. Generally, of all the three species, *E. faecalis*, and to a lesser degree *E. faecium* produced the highest concentrations of these compounds, and most of them were of the same origin.

On the other hand, Latorre-Moratalla et al. [2011] investigated the volatile profiles of slightly-fermented sausages inoculated with eight different *enterococcal* strains in comparison with a fermented sausage prepared without any inoculation. After 21 days of

fermentation, a total of 121 volatile and semi-volatile compounds were identified in the samples of the final products. Terpenes were the main group of compounds in the volatile profile that derived from spices added during the manufacture of dry-fermented sausages. Other groups including alcohols, aldehydes, alkanes and alkenes, and ketones resulted from degradation of lipids, proteins or carbohydrates and were formed during fermentation and ripening. In respect to total volatile composition, the batches inoculated with enterococci were similar to those of non-inoculated batches. Nevertheless, some quantitative variations were observed in the volatile profiles of different batches, mostly in the compounds derived from amino acids catabolism (i.e.: propanol and isoamyl), from free fatty acids oxidation (i.e.: hexanal, hexanol), or from carbohydrate fermentation (i.e.: 2-butanone, acetoin). However, some inoculated batches were more resistant to oxidation in comparison with non-inoculated sausages; they contained fewer volatile compounds arising from lipid oxidation [Latorre-Moratalla et al. 2011].

Moreover, Ono et al. [2015] studied the characteristic odor of the volatile oils obtained from liquid medium after incubation (MAI) and liquid medium before incubation (MBI) in the cultivation process of *E. faecalis* in order to estimate the role of liquid medium. Fifty-six and thirty-two compounds were found in the volatile oils from the MAI (MAI oil) and MBI (MBI oil), respectively. The main components of MAI oil were 2,5-dimethylepyrazine (19.3%), phenyl acetaldehyde (19.3%), and phenyl ethyl alcohol (9.3%). Thirteen aroma-active compounds were identified; particularly, 5-methyl-2-furanmethanol, phenyl acetaldehyde, and phenyl ethyl alcohol were the most aroma-active compounds in MAI oil. These results suggest that industrial cultivation medium after incubation (AMI) of *E. faecalis* offer novel applications such as in the perfume industry.

4.2.6.6. Bacteriocin production

Bacteriocins are ribosomally synthesized antimicrobial peptides produced by Gram positive and Gram negative bacteria. They exhibit an ability to kill species both related (narrow spectrum) and non-related (broad spectrum) to the bacteriocin-producing strain in nano-molar concentrations, contrary to traditional antibiotics that are used in much higher concentrations [Nes, 2011; de Freire Bastos et al. 2015]. According to

Klaenhammer [1993], there are four classes of bacteriocins. Class I are designed as lantibiotics, Class II are small heat-sable peptides (<10 kDa), class III is made up of large heat-labile (>30 kDa), and class IV contains complex bacteriocins composed of essential lipids or carbohydrate moieties in addition to protein. Because of the increasing number of bacteriocins and novel data accumulated, this classification is regularly revised [Drider et al. 2016]. Currently, bacteriocins encompasses three main classes: Class I (lantibiotics), Class II which consists of four subclasses (IIa, IIb, IIc, and IId), and Class III that contains the large molecule heat sensitive peptides [Perez et al. 2014].Even though bacteriocins classification is still a contradictory subject, they were divided into two major, well-defined classes of heat-stable, ribosomally synthesized antimicrobial peptides. Class I comprises the lantibiotics, whereas Class II encompasses the unmodified non-lantibiotics [Perez et al. 2014; Nes et al. 2014].

Within LAB groups, *Enterococcus* species portrayed the ability to produce bacteriocins belonging to different classes and subclasses. Most of enterococcal bacteriocins arose from strains isolated from human and animal digestive tracts and also from other environments [Foulquié Moreno et al. 2003; Hanchi et al. 2016]. The majority of the identified enterocins are from *E. faecalis* and *E. faecium* [Zendo et al. 2005], but also come from *E. durans* [Hanchi et al. 2016], *E. Mundtii* [Yusuf et al. 2015], *E. hirae* [Gupta et al. 2016], and *E. avium* [Audisio et al. 2005].

4.2.6.6.1 Class I bacteriocins (Lantibiotics)

Lantibiotics are gene-encoded peptides that include intramolecular ring structures by thioether bridge formation between dehydrated serine and threonine and cysteines which give lanthionine and methyllanthionine residues, respectively [Cotter et al. 2015]. Lantibiotics are rarely produced by *Enterococcus* species. Only two enterocins with two-peptides produced by *E. faecalis* have been characterized and genetically identified as lantibiotics [Sawa et al. 2012]. Cytolysin is the most characterized enterococcal lantibiotics. This bacteriocin showed antagonism towards a broad spectrum of Gram positive bacteria and some eukaryotic cells such as erythrocytes of various animals [Dong et al. 2015]. This peptide lyses polymorphonuclear leukocytes, retinal cells, and human

intestinal epithelial cells [Coburn and Gilmore, 2003]. Cytolysin activity is related to two unique peptides carrying modifications and physiochemical properties of lantibiotic bacteriocins [Van Tyne et al. 2013].

The expression of this bacteriocin is strongly controlled by a two-component regulatory system known as quorum-sensing regulation. In this regulatory system, the high-level expression of the cytolysin genes is induced by the smaller peptide of cytolysin through binding to a membrane-bound receptor histidine kinas ensued by phosphorylation relay resulting in a phosphorylated response activator that stimulates the genes implicated in cytolysin biosynthesis [Coburn et al. 2004; Van Tyne et al. 2013].

Enterocin W is the second two-peptide lantibiotic produced by *E. faecalis* to be reported by Sawa et al. [2012]. Enterocin W shares high similarities with plantaricin W, a bacteriocin produced by *Lb. plantarum*. Its amino-acid sequences showed 63.3% and 44.7% similarity to those of plantaricin two-peptides [Sawa et al. 2012]. Enterocin W demonstrates a strong antagonistic effect against several Gram-positive bacteria [Sawa et al. 2012]. The recent technologies of high through-put DNA sequencing have provided massive genomic information concerning the bioactive molecules. To this end, Marsh et al. [2010] described a gene cluster of one-peptide enterocin using *in silico* analysis, which potentially encodes the *Enterococcus*-associated lantibiotic, but this bioinformatic data still needs to be approved by laboratory work (**Figure 10**).

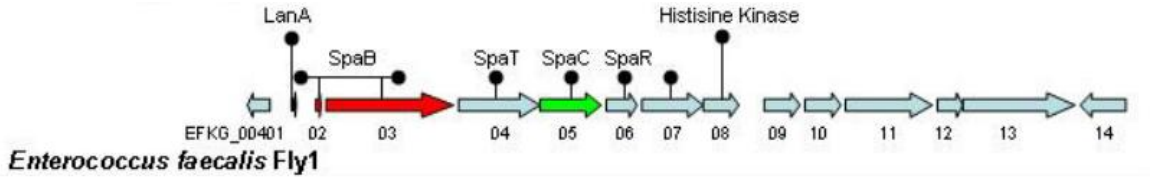

Fig. 10. Diagrammatic representation of *Enterococcus faecalis* Fly1 type 1 lantibiotic operon, found in the original NisC screen, which contain genes predicted to encode a structural peptide LanA, and the modification enzymes [adapted from Marsh et al. 2010].

4.2.6.6.2 Class II bacteriocins

Bacteriocins of class II contain diverse subclasses that are still under debate with respect to their classification. Bacteriocins that belong to this class are defined as non-modified and heat stable peptides [Perez et al. 2014]. In spite of some differences between the bacteriocins of class II, there is a general agreement that the class IIa and IIb are well defined classes.

4.2.6.6.3 Class IIa bacteriocins (pediocin-like peptides)

The class IIa represents the largest group of class II bacteriocins. They are frequently defined as listericidal, small (<10-kDa), heat stable, unmodified peptides consisting of 37 (e.g. leucocin A and mesentericin Y105) to 48 (eg. carnobacteriocin B2 and enterocin SE-K4) amino acids, with a net positive charge and (pI) values ranging from 8 to 10 [Drider et al. 2006]. Despite the differences in length among the bacteriocins class IIa, they share the following consensus sequences motifs in their N-terminal parts **YGNGV**(X)C(X)$_4$C(X)V(X)$_4$A. In addition to this signature, class IIa contains at least two cysteines, which are involved in the disulfide bridge involved in their antimicrobial effect [Eijsink et al. 1998; Drider et al. 2006; Perez et al. 2014]. This class of bacteriocins was encountered in *E. faecalis. E. faecium, E. durans, E. mundtii, E. avium* and *E. hirae*. They have been a focus of particular interest because of their strong antagonism toward *L. monocytogenes* and other pathogenic bacteria. Enterocin A is one of the most effective antimicrobial peptide [Aymerich et al. 1996; Eijsink et al. 1998; McClintock et al. 2016]. The antibacterial effect of bacteriocin was shown to result from two intramolecular disulfide bridges [Drider and Rebuffat, 2011]. The nuclear magnetic resonance spectroscopy revealed that the second bridge is placed in the C-terminal part, stabilizing therefore the α-helical structure in this region, and enhancing the bacteriocin antagonism [Johnsen et al. 2005]. Different studies pointed out that several class IIa bacteriocins lack the C-terminal disulfide bond including enterocin P, sakacin P, curvacin A, leucocin A, and carnobacteriocin B2 [Fimland, et al. 2000; Uteng et al. 2003]. These bacteriocins were less active by about 30 to 50 times at 37°C compared with their activity at 25°C,

While bacteriocins that have C-terminal disulfide bonds like Pediocin PA-11 do not show any variation in their antimicrobial activity at 37 °C and 25°C [Kaur et al. 2004].

Class II bacteriocins are usually synthesized as an inactive pre-peptide which holds a typical double-glycine site that plays an important role in peptide recognition and secretion [Perez et al. 2014]. The majority of class IIa bacteriocins are secreted by proteolytic cleaving and removal of double-glycine-type leader peptide through the ATP-binding cassette (ABC) transporters and their accessory proteins. Whereas few class IIa bacteriocins such as enterocin P, enterocin SE-K4, listeriocin743A, bacteriocin31, and hiracin JM79 are secreted by sec-dependent export pathway [Cui et al. 2012].

Class IIa bacteriocins target the membrane located part (Man-CD proteins) of mannose phosphotransferase system (Man-PTS), the main permease in the majority of Gram-positive and Gram-negative bacteria. The Man-PTS system includes four domains IIA, IIB, IIC, and IID arranged into two to four subunits. The cytoplasmic domains IIA and BII are responsible for phosphorylation, while the membrane domains IIC and IID are responsible for the transport [Belguesmia et al. 2011]. It was clearly shown that class IIa bacteriocins lead to permeabilization of targeted bacteria that encode a phylogenetically defined subgroup of Man-PTS. Specifically, a defined region of 40 amino acids that encloses an expected extracellular loop in the domain IIC seems to encompass the specific targeting site of class IIa bacteriocins. The specific interaction of class IIa bacteriocins with Man-C in the presence of Man-D forms an irretrievable opening of the transporter which allows a free diffusion of ions across the cell membrane that ultimately kills the target cell. Nevertheless, it has been conclusively shown that the class IIa bacteriocins and their immunity proteins form a strong complex with the Man-PTS system which prevents the permeabilization and death of the target cell (**Figure 11**) [Nes et al. 2014].

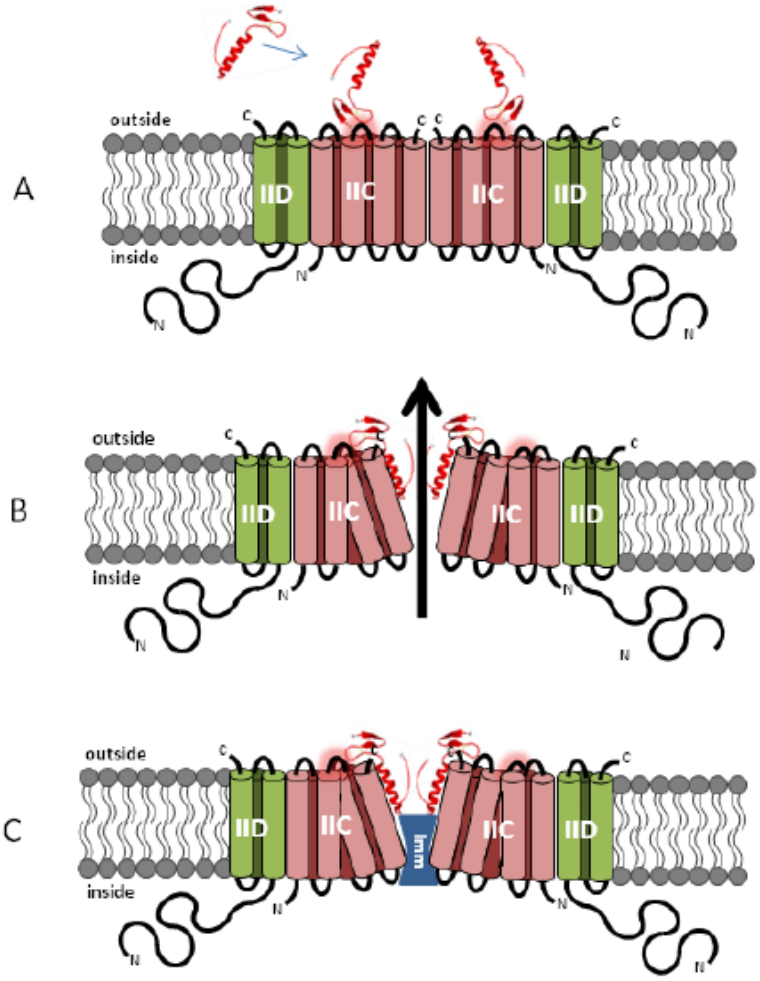

Fig. 11. The model of killing by Man-PTS targeting and immunity of Class IIa bacteriocins. A) a Class IIa bacteriocin specifically targets an extracellular loop of IIC, one of the two membrane-embedded components (IIC and IID, also called Man-CD) of man-PTS; and **B**) the initial interaction leads to further interactions with some membrane helices of man-PTS, somehow causing the channel of the sugar permease to remain open, leading to leakage of solutes, destruction of membrane integrity, and eventually cell death. **C**) In producer cells, the cognate immunity protein binds to IICD and locks the bacteriocin in a tight complex, thereby preventing the bacteriocin from opening the pore [adapted from Nes et al. 2014].

4.2.6.6.4 Class IIb bacteriocins (two-peptide bacteriocins)

This type of bacteriocins contains two different peptides; each of them is usually synthesized with a double-glycine-type N-terminal leader sequence consisting of 15 to 30 residues [Nissen-Meyer et al. 2010]. During the bacteriocin maturation, this leader is cleaved off at the C-terminal side of the two glycine residue by a dedicated ABC-type transporter that concurrently transfers the bacteriocin outside the cell membrane

[Havarstein et al. 1995]. The class IIb bacteriocins share several features that form their identity: (i) the bacteriocin consists of two different peptides that are encoded by two genes located next to each other on the same operon (ii) the two peptides act synergistically in one to one molar value against the target cells, but they show slight or no effect if they work individually, (iii) they share only one immunity protein to protect their producing cell [Oppegård et al. 2007].

For some class IIb bacteriocins such as enterocin 1071, and plantaricin (J/K and E/F), the dedicated ABC-transporter and so-called accessory protein are encoded by genes found on a separate operon close by the operon with the genes that encode to the preforms of the two- peptides and immunity protein, whereas for other class IIb bacteriocins, like lactococcin G, the genes encoded to the dedicated ABC-transporter, accessory protein, the preforms of two- peptides, and the immunity proteins are located on the same operon with each other [Nissen-Meyer et al. 2010].

Some class IIb bacteriocins are produced constitutively, while the production of others is transcriptionally regulated by a three-component regulatory system comprised of a peptide pheromone, a membrane-associated histidine protein kinase, and response regulators [Nissen-Meyer et al. 2010]. The secretion process of these types of bacteriocins can be explained by the interaction between the two-peptide pheromone and the membrane-associated histidine kinase, thus arousing the kinase to phosphorylate the intracellular response regulator, thereby allowing the response regulators to activate the operon responsible for bacteriocin synthesis and secretion [Kleerebezem and Quadri, 2001].

Similarly to other bacteriocins, class IIb bacteriocins permeabilize the target cell membrane causing depletion in the intracellular components. All the available studies about the class IIb mode of action propose that the two peptides of such bacteriocins may cause a membrane-penetrating helix-helix structure involving a helix-helix interacting **GxxxG**- motif which is present virtually in the two-peptide bacteriocins. Furthermore, it has been also stated that the helix–helix structure interacts with an integrated membrane protein, thus leading to a conformational modification in the protein which finally causes the membrane-leakage and eventually cell death [Nissen-Meyer et al. 2010]. Findings also suggested that the beta-peptide of lactococcin G (the first two-peptide bacteriocin isolated

from *Lactococcus lactis*) and enterocin 1071 (first characterized two-peptide enterocin of *Enterococcus faecalis* BFE1071, isolated from mini pig feces) is important for target specificity [Nissen-Meyer et al. 2010]. The individual peptides of lactococcin G attach separately to the target cell membrane, but their antimicrobial effect appears only when they are present concomitantly [Nissen-Meyer et al. 1992]. It has been suggested that peptides attach immediately and irreversibly to the target cell membrane or surface. Moreover, a selective conductivity of ions was noticed for plantaricin EF and JK. Both two-peptide plantaricins form pores in the membranes of the target cells and dispel the transmembrane electrical potential and pH gradient, but plantaricin EF pores efficiently conduct the small monovalent cations while the conductivity for the anions is low or absent. Conversely, plantaricin JK pores show high conductivity for specific anions but low conductivity for cations [Moll et al. 1999]. Nissen-Meyer et al. [2010] explained the mode of action of the class IIb bacteriocins on the target membrane, which seem to be dependent on structural information, genetic, mutagenic, and complementary studies (**Figure 12**). This model displays how the two-peptide bacteriocins initiate a helix-helix structure when they bind to the target membrane and form an open pore for free passage of ions outside the cell.

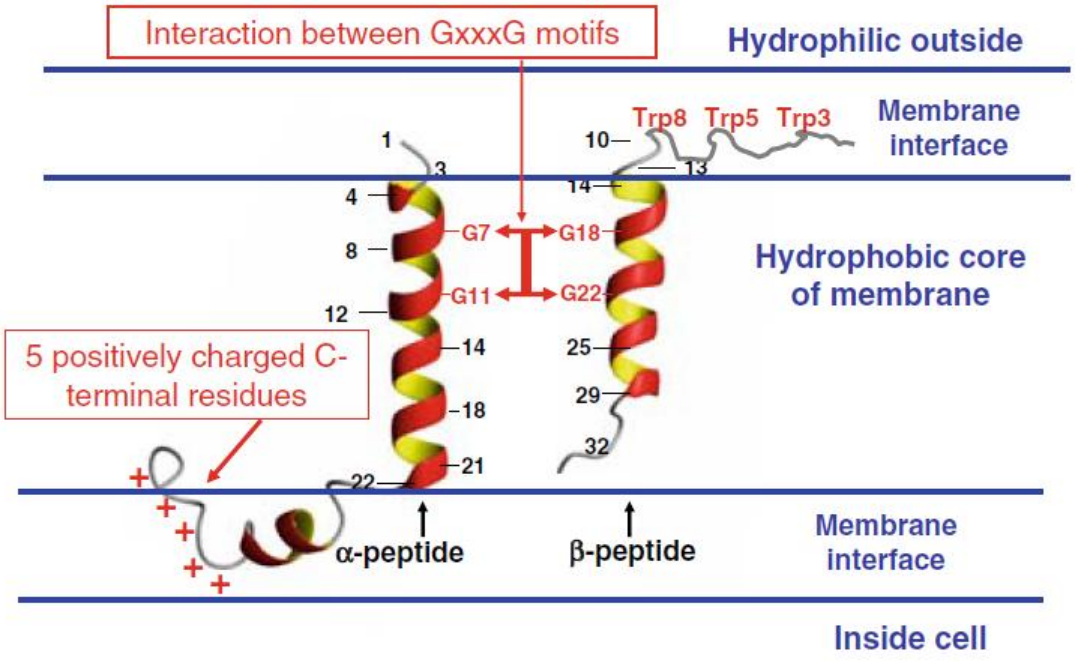

Fig. 12. A structural model of lactococcin G and its orientation in target-cell membranes. The two peptides interact through the G7xxxG11-motif in the a-peptide and the G18xxxG22-motif in the b-peptide and form a trans-membrane helix–helix structure. The highly positively charged and structurally flexible C-terminal end of the a-peptide is forced through the membrane by the trans-membrane potential (negative inside). The tryptophan residues in the structurally flexible N-terminal region of the b-peptide are in or near the outer membrane interface [adapted from Nissen-Meyer et al. 2010].

The well characterized bacteriocins produced by *Enterococcus* species including enterocin 1071 A and B, enterocin 31, enterocin CRL35, enterocin A, enterocin P, mundticin, mundticin KS, enterocin RC714, T8, and enterolysin A, which are synthesized with a leader peptide, can be classified under the group of class IIb according to Klaenhammer [1993] and Nes et al. [1996]. Other two-peptide enterocins are synthesized without leader peptide (leaderless bacteriocins) including L50 A/B and MR10 A/B. These enterocins show high identity between their two peptides and share a strong homology among them [Franz et al. 2007]. The genetic determinants for these enterocins can be located on the plasmid, as in the case of enterocin L50A/B or on the chromosome as in enterocin MR10 A/B [Martín-Platero et al. 2006; Franz et al. 2007]. It is worth mentioning that the classification of these leaderless bacteriocins is still a controversial subject [Cotter et al. 2005; Franz et al. 2007; Iwatani et al. 2011; Nes et al. 2014; Perez et al. 2014].

4.2.6.6.5 Class IIc bacteriocins (circular bacteriocins)

Bacteriocins of this subclass are circularized by an α-amino acid group of one residue coupled to the carboxyl group of the terminal residue of the linear peptide as an amide bond, therefore forming a four-or five- α-helix structure [Van Belkum et al. 2011]. These cyclic bacteriocins are ribosomally synthesized with a defined leader peptide which varies in length and sequence from one to another. However, they are divided into two groups: (i) cyclic bacteriocins with very short leader sequences (2-6 amino acids residues), and (ii) cyclic bacteriocins with long amino acid residues (>20 amino acid residues) [Van Belkum et al. 2011]. The antimicrobial activity of these cyclic bacteriocins is related to their ability to permeabilize the membrane of the target cell, causing ions depletion and dispersion of the membrane potential [Gong et al. 2009]. Few circular bacteriocins were found in LAB, of which enterocin AS-48, which was originally produced by *E. faecalis*, has been well studied and characterized [Franz et al. 2007]. About ten ORFs coding for production and immunity function are located in two operons identified for this enterocin [Martínez-Bueno et al. 1998; Diaz et al. 2003] The structural genes of enterocin AS-48 are located on the pheromone-responsive plasmid pMB2, while another circular enterocin called (AS-48 RJ) has been categorized from *E. faecium* which contains a gene cluster identical to the strain S-48 but it is located on the bacterial chromosome [Franz et al. 2007].

4.2.6.6.6 Class IId bacteriocins (unmodified, linear, non-pediocin-like bacteriocins)

This subclass of bacteriocins comprises the unmodified, linear, non-pediocin-like bacteriocins that do not fulfill any criteria of previous classification schemes [Iwatani et al. 2011]. Enterocin B and lactococcin A and B are members of class IId bacteriocins. Even though these bacteriocins do not share any sequence similarity to the pediocin-like bacteriocins, some of them (such as lactococcin A and B) have a similar mode of action and immunity systems [Diep et al. 2007]. For example, lactococcin A exploits the membrane-located components (ManCD) of the Man-PTS as a receptor, as in the case of class IIa (pediocin-like bacteriocins). Nevertheless, they differ totally from each other in their spectrum of antimicrobial activity. Lactococcin is generally active only in

Lactococcal cells, whereas pediocin-like bacteriocins targets include various bacterial genera [Kjos et al. 2010; Kjos et al. 2011].

4.3 Enterococci as probiotics

Enterococci are an important member of LAB, which are naturally colonized in human and animal GIT [Devriese et al. 2006]. These bacteria are also found in many food products as natural microbiota [Franz et al. 2003]. The use of enterococci as probiotics is subjected to controversial debates because of their safety [Franz et al. 2011]. Indeed, some *Enterococcus* strains were rejected as pathogens involved in nosocomial infections and displayed extended resistance to antibiotics [Miller et al. 2016]. They are considered as emerging pathogens, especially *Enterococcus faecalis* and *Enterococcus faecium*, and sometimes *Enterococcus gallinarum*, *Enterococcus hirae*, and *Enterococcus mundtii*, which were involved in endophtalmitis and native valve endocarditis [Bhardwaj et al. 2008]. The incidence of virulence factors and antibiotic resistance are mostly considered to be strain-specific among *Enterococcus* species isolated from food [Franz et al. 2001].

In Europe, laws consider the probiotic or starter strain to be under the responsibility of the producer; therefore, each strain has to be evaluated vigilantly [Franz et al. 2003]. Indeed, there are no obvious criteria to identify the *Enterococcus* species as probiotic or pathogen, so it is necessary to study each probiotic strain individually [Lauková, 2011]. It is worth noting that *Enterococcus faecalis*, *Enterococcus faecium* and *Enterococcus durans* were allowed to be used as starter cultures by the Australian authorities according to Quarantine (Cocos Islands) Proclamation 2004 (https://www.legislation.gov.au/details/f2006c00825).

Enterococcus faecalis has attracted more interest as a probiotic strain for humans and animals. Heat-treated and live cells of *Enterococcus faecalis* showed interesting beneficial probiotic effects on host's health. The autoclaved cells of *Enterococcus faecalis* FK-23, a commercial strain in Japan isolated from feces of a healthy human subject, showed a significant ability to bind to the amino acid pyrolysates as Trp-P1 and Trp-P2 in addition to aflatoxins B1, B2, G1, and G2 [Hosono and Hisamatsu 1995].

Heat-treated cells of *Enterococcus faecalis* FK-23 significantly reduced the obesity levels in a FK-23 mice diet group compared with a high fat-fed group in a study conducted by Motonaga et al. [2009]. Moreover, *Enterococcus faecalis* FK-23 heat-treated cells showed a significant reduction in the hepatic steatosis and the expression of fatty acid oxidation responsible genes in the lever tissues of treated mice in comparison with the control [Kondoh et al. 2014]. The ability of *Enterococcus faecalis* EC-12, a commercial strain in Japan, was evaluated to prevent the colonization of vancomycin-resistant *Enterococcus faecalis* (VRE) in the cecum of newly-hatched chicks. Accordingly; it seems that dietary EC-12 stimulated the gut immune system and increased the immune reaction against the VRE [Sakai et al. 2006]. Furthermore, the administration of *Enterococcus faecalis* LAB31, a feed additive certified by the Chinese ministry of agriculture, demonstrated significant potential to improve the growth performance of weaned piglets. It also reduced diarrhea incidence and positively altered the balance of intestinal communities by stimulating the growth of beneficial microbes, especially *Lactobacillus* group, and inhibiting a wide range of Gram-negative bacteria [Hu et al. 2015]. The same beneficial effect of *Enterococcus faecalis* on increasing the numbers of favorable microbiota has already been reported by Han et al. [2013] who studied the effect of dietary supplementation with microencapsulated *Enterococcus faecalis* CG1.0007 on growth performance, anti-oxidation activity and intestinal microbiota in broiler chickens. The authors noticed that microcapsule-treated groups showed considerable changes in terms of anti-oxidation in addition to a significant increase in the number of intestinal *Lactobacillus* and *Bifidobacterium.*

In direct line, Hayakawa et al. [2016] studied the probiotic effect of BIO-THREE, a commercial probiotic marketed by TOA Pharmaceutical Co. Ltd. in Tokyo, Japan, that contains three probiotic strains (*Bacillus mesentericus* strain TO-A, *Clostridium butyricum* strain TO-A, and *Enterococcus faecalis* strain T-110) on diets administered to sows and their neonates. They showed that the growth performance and the ratio of return to estrus (one of the most important reproduction factors) of sows were significantly improved by BIO-THREE administration. Moreover, the feed intake was superior in the probiotic-administrated group compared to the control during the late lactation period. Also, post-weaning diarrheal incidence and growth performance was improved by

administration of three probiotic species to the neonates, whereas the combined use of probiotics in sows and their neonates triggered the enlargement of villous height and prohibited muscle layer thinning in the small intestine of weaning piglets. Nevertheless, *Enterococcus faecalis* CECT7121, a probiotic strain recovered from natural corn silage, demonstrated a significant effect on the early development of nematode larvae *Toxocara canis* that completely disappeared in the feces of infected mice who had orally received the probiotic bacterial suspension. In addition, a significant decrease in the number of *Toxocara canis* larvae recovered from the livers and lungs of the probiotic group was noticed in comparison with untreated infected mice [Basualdo et al. 2007]. These results have been confirmed by Chiodo et al. [2010] who evaluated the larvicidal effect of *Enterococcus faecalis* CECT7121 on *Toxocara canis* cycles both *in vitro and in vivo*. For *in vitro* experiments, the viability of the larvae was significantly reduced after incubation for 48 h with the supernatant of CECT7121, pre-culture of *Enterococcus faecalis* CECT7121, and a fresh culture of *Enterococcus faecalis* CECT7121, while the inhibitory effect was decreased after incubation with the supernatant and fresh culture of mutant *Enterococcus faecalis* CECT7121. However, for *in vivo* experiments, the interference of CECT7121 was evaluated in mice challenged with *Toxocara canis*. It was significant when the mice were challenged with probiotic and *Toxocara canis* simultaneously, while no significant interference was observed when the challenge was applied 15 days after oral administration of probiotic sustention. Another study on the same probiotic strain *Enterococcus faecalis* CECT7121 was conducted by Castro et al. [2011] to evaluate anti-allergic effects of CECT7121 in mice. The results revealed that the administration of the probiotic CECT7121 decreased the specific immunoglobulin E (IgE) levels; the anti-ovalbumin (OVA) lgG2a levels increased while the levels of IgG and lgG1 remained unchanged, a reduction in proliferation rate of memory cells was observed, and a decrease in the levels of the T helper2 (Th2) cytokines IL-4, IL-5, and IL-13 was noticed, while the secretion of IL-10, IL-12, and IFN-γ remained unaltered. In addition, the incubation of human basophils (a type of white blood cells) with non-viable cells of CECT7121 together with an allergen preparation triggered the release of ß-hexosaminidase at lower levels than control reactions. Furthermore, *Enterococcus faecalis* MN1, a strain isolated in healthy women, significantly repressed the interleukin-

8 production of human vaginal epithelial cells in response to the vaginal pathogens *Candida albicans, Gardnerella vaginalis* and *Neisseria gonorrhoeae* as well as to toxic shock syndrome toxin-1[Brosnahan et al. 2013].

Nowadays, several strains of *Enterococcus faecalis* are commercialized in the market as probiotics for human use in addition to some enterocins that are commercially available for animal protection and treatment (**Figure 13**).

Fig. 13. Some commercial products of *Enterococcus faecalis* validated as probiotics. A:http://drohhiraprobiotics.com/; B: http://www.synergy-health.co.uk/shop/fivelac-60-sachets/, C: http://www.symbiopharm.de/de/produkte/symbioflor-1.html; D:http://www.nichinichi-phar.co.jp/en/houjin/fk23.html; E: http://old.aor.ca/products-page/baccilus-mesentericus/probiotic-3/ ; F: http://www.leedstone.com/enterocin-c-reg.html

Chapter 2. Yeasts from fecal samples of chicken displayed anti-Listeria activities and further probiotic properties

This chapter is aimed at exploring and then unveiling the yeast content of fecal samples derived from broiler chickens harvested on a farm located in the north of France. Isolated strains were identified and categorized by their antagonism towards pathogens including *L. monocytogenes*. As mentioned, the intestinal microbiota is a widely studied topic because of its complexity and involvement in host health and disease [Marchesi et al. 2016]. Broiler chicken intestine is colonized by a complex community including bacteria, methanogenic archaea, and fungi [Perumbakkam et al. 2014]. Similarly to other birds, chickens have smaller GIT and shorter digestion transition time than mammals, but they do not show any less efficiency in the digestive process, which could be attributed, in part, to the complex microbial community that inhabits their intestine (**Figure 14**) [Sergeant et al. 2014].

There are many factors governing the composition of intestinal microbiota. The most important are the surrounding environment, diet variation, pathological conditions, antibiotic therapy and others [Cisek and Binek, 2014]. Indeed; research focused on the gut microbiome is very bacteria-centric; therefore, only a handful papers dealing with viral components, micro-eukaryotes and protozoa have been reported [Marchesi et al. 2016]. As of today, there are approximately 1.500 species of yeasts distributed among 149 genera belonging to two phyla, Ascomycota and Basidiomycota [Fell, 2012]. Most of the current information on yeast diversity in the GIT of vertebrates, especially those of farm animals, is still based on findings from the 1950s and 1970s during the 20th Century [Urubschurov and Janczyk, 2011].

Notably, fecal samples are considered a rich reservoir to isolate the beneficial microbes with probiotic properties [Psomas et al. 2003; Gareau et al. 2010; Kizerwetter-Świda and Binek, 2016]. In 2002, the FAO/WHO working group established new guidelines for the use of microorganisms as probiotics. Safety and effectiveness were the most important issues that were recommended for assessment by both *in vitro* tests to

evaluate the probiotic potential and *in vivo* tests for clinical experiments on animals or humans [FAO/WHO, 2002].

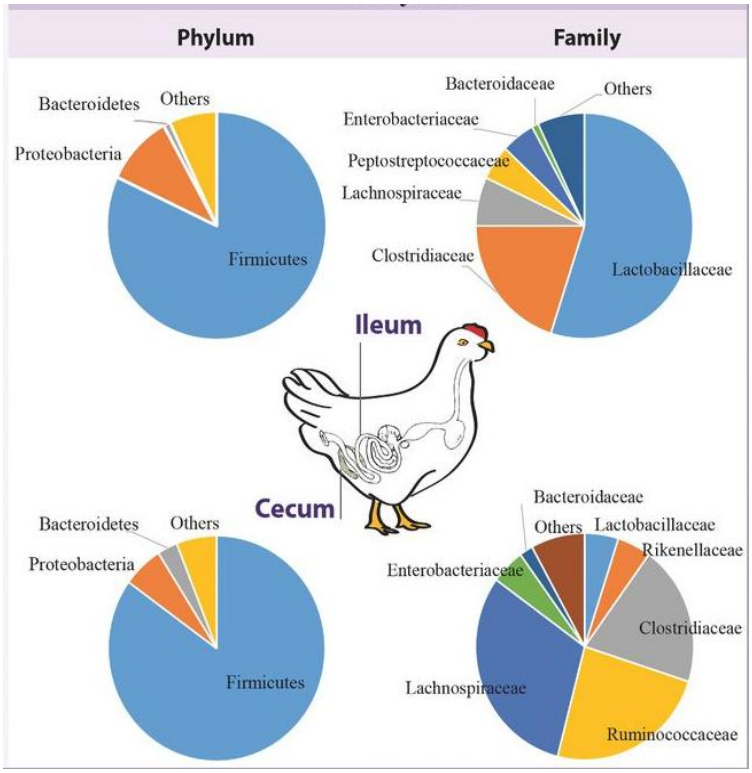

Fig. 14. **The chicken gut microbiome**. The graphs provide an overview of the relative abundance of dominant bacterial phyla and families of the broiler chicken ileal (top level) and cecal (bottom level) microbiota [adapted from Pourabedin and Zhao, 2015].

Herein, we studied the yeast diversity in the fecal samples of healthy broiler chickens collected randomly from an industrial farm located near the city of Lille in France. The fecal samples were serially diluted in a 0.9% (w/v) sterile saline solution from 10^{-1} to10^{-6}. One milliliter of each dilution was platted onto Sabouraud agar plates supplemented with chloramphenicol at 0.05 µg/ ml. Thus, 81 yeast colonies were isolated after incubation of plates at 30°C for 48-72 h.

First, isolated yeast strains were grouped by Rep-PCR method, which is based on the primer $(GTG)_5$-rep-PCR fingerprinting technique that encompasses the repetitive and palindromic DNA sequences with a length between 21 and 65 bases has been successfully used for yeast classification and identification [Tobes and Pareja, 2006; Ait Seddik et al. 2016]. The repetitive sequence-based PCR or rep-PCR DNA fingerprint technique employs primers targeting several repetitive elements, which exist in different

sites in the genome of bacteria, archaea and eukaryotes, to generate unique DNA profiles, or fingerprints, of individual microbial strains [Ishii and Sadowsky, 2009]. This technique enabled us to identify and separate 22 groups from a total of 81 isolated strains with a cut-off scale of 70%. The sequences presumably varied in number and position on the targeted yeast chromosomes, since different rep-PCR profiles were observed for the tested strains (**Figure 15**). The PCR amplicons generated with this primer showed highly discriminatory patterns by agarose gel electrophoresis.

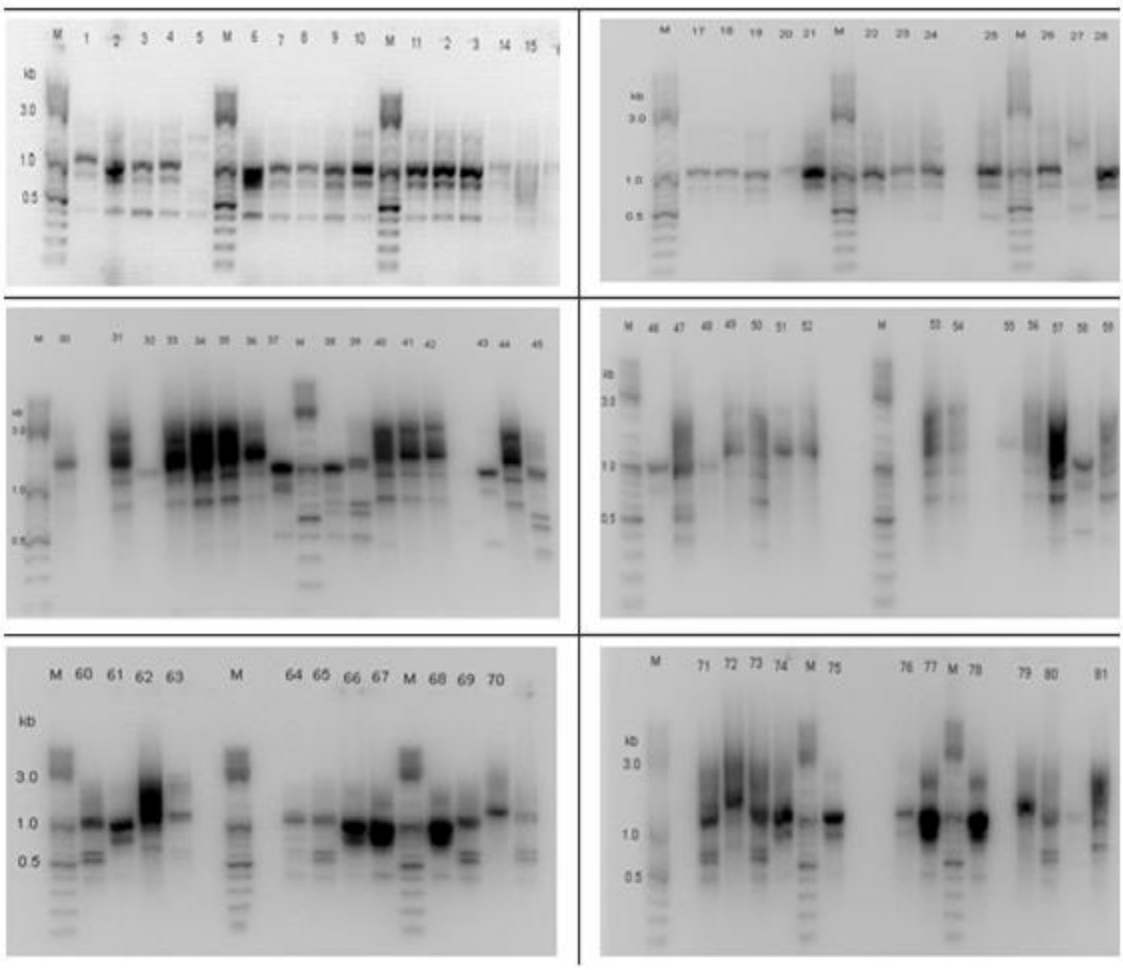

Fig. 15. The rep-PCR DNA fingerprints generated in 1.0% agarose gel, requested by the use of the GTG_5 primer 5'–GTGGTGGTGGTGGTG- 3'. The genetic relatedness of 81 strains permitted us to gather 22 groups. M: DNA markers (Gene Ruler TM DNA) 10 Kb (5µl); 1 to 81: yeast strains (GTG)5-rep-PCR DNA fragments (3 µl PCR Product +7 µl H2O +2 µl of loading dye).

The biochemical identification carried out with the ID 32C system showed that all these strains were identified as the *Candida famata* species, which is the telemorphic

form of *Debaryomyces hansenii*, with a confidence score of 99%. These results have been confirmed by the sequence analysis of the 26S rDNA gene and the ITS1-5.8-ITS2 region. Indeed, the biochemical and molecular methods converge to identify these strains as the *Candida famata* (*Debaryomyces hansenii*) species, with a percentage of identity ranging from 99 to 100%.

Remarkably, the sequencing of the ITS1-5.8-ITS2 region has been proposed as the most accurate for identification of fungal population in complex systems [Richard et al. 2015].

Antagonism assessments carried out on the 81 strains isolated in this study underpinned the capabilities of *Candida famata* Y.5 to inhibit the growth of *Listeria innocua* under defined conditions. To lend a broader context to our finding, we examined the probiotic properties of this strain. Thus, *Candia famata* Y.5 turned out to be safe, as no cytotoxic effect was observed towards human epithelial cells Caco-2, and no hemolytic activity was observed when the strain was cultivated on blood agar plates. Moreover, *Candida famata* Y5 exhibited a significant ability to survive under simulated gastric and intestinal environments. Taken together, these data delineate the potential of *Candida famata* Y.5 as a candidate for probiotic application.

The data obtained here sheds light on the potential of non-*Saccharomyces* yeasts to be used as probiotics. Further isolation and sampling are expected in the future, and hopefully they will allow identification of further species.

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Characterizations of *Candida famata* isolated from poultry feces for possible probiotic applications

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ABSTRACT

We studied here the yeast content of poultry feces, collected randomly from a French farm located in the north of the country. Thus, 81 yeast colonies were isolated and clustered into 22 distinct groups using the rep- PCR method. A single colony was taken from each group and identified using biochemical (ID 32C system) and molecular (sequencing of the D1 domain of 26S rDNA and ITS1-5.8-ITS2 rDNA region) methods. Both methods led to the identification of Candida famata species. One isolate of *C. famata* strains, named strain Y5, was further studied for its cytotoxicity, adhesion, and surface properties, hemolytic activity, and its survival in simulated gastric and intestine environments. The data obtained advocate the probiotic potential of this isolate.

Keywords: Yeasts, Poultry feces, *Candida famata*, Rep-PCR, Probiotics.

INTRODUCTION

The use of probiotics in agriculture has become an alternative to replace antibiotics that were misused as growth promoters and in some cases, to control specific enteric pathogens [1, 2]. Poultry remains a source of transmission of foodborne pathogens, particularly the thermophilic species [3]. Many enteropathogens are known to colonize the intestinal tract of broiler chickens as harmless commensals, often remaining undetectable before slaughtering. The gastrointestinal microbiota of broiler chickens could help to decipher the microbial interactions with the host. The first days of chicks'

lives are critical in establishing a normal stable microbiota [4], which advocates that young chicks are often well advanced in establishing a stable microbiota before they leave the hatchery. The established microbiota is highly diverse, with over 1000 bacterial species in chickens [5]. The composition of intestinal microbiome appeared to be dependent on several factors including the surrounding environment, diet variation, pathological conditions and antibiotic therapy [6]. The number of anaerobic bacteria, lactic acid bacteria and yeasts in cecal contents of birds fed with rye-based diets was higher than in birds fed with barley-based diets [7]. Thus, the most abundant bacterial groups found in the broiler caeca were *Clostridium*, *Eubacterium* followed by *Bacteroides* spp., *Lactobacillus* spp., *Enterococcus* spp., *Bifidobacterium* spp. and *Enterobacteriaceae* [7]. A recent analysis of fecal microbiome of low and high feed conversion ratio (FCR) broilers using shotgun sequencing technology has established the microbial diversity and metabolic potential in low and high FCR birds [8]. According to these authors, bacteria were highly present (>95 %), whereas eukaryotes (>2 %), archaea (>0.2 %) and viruses (>0.2 %) were poorly present. Moreover, probiotic supplementation, such as live yeasts or bacteria, enhanced broilers' performance rendering thereof chickens resistant to *Salmonella* spp., *Escherichia coli* or *Clostridium perfringens* infections [9–13]. Related to this, recent reports on poultry probiotics have demonstrated a set of beneficial effects, such as counteraction of dysbiosis, promotion of gut health and homeostasis, enhancement of immune defenses and neutralization of infectious agents [6].

This work aimed at studying the yeast content in chicken feces randomly sampled in a farm located near to Lille city (north of France). *C. famata* turned to be the only species

isolated from these samples, and one isolate designated as *C. famata* Y5 was studied for its safety for the eukaryotic host, bearing in mind its possible use as a probiotic microorganism, should any beneficial characteristics be revealed in the future studies.

MATERIALS AND METHODS

Isolation of Yeasts from Poultry Feces

Samples of feces from healthy birds were randomly collected from an industrial farm located near to Lille city (north of France). The samples were collected in sterile dry plastic containers, and were serial diluted in 0.9 % (w/v) sterile saline solution (TS) from 10^{-1} to 10^{-6}. One milliliter of each dilution was platted onto Sabouraud agar plates (Sigma-Aldrich, India) supplemented with 0.05 µg/ml of chloramphenicol. Plates were incubated at 30°C for 48-72 h. The colonies fitting yeasts characteristics were selected and stored at -80°C in Sabouraud broth supplemented with 30% of glycerol.

DNA Extraction and Yeasts Genotyping with Rep- PCR

The yeasts isolated from chicken feces were clustered by rep-PCR method. To this end, total DNA was extracted for each isolate using the Bust n' Grab method [14] and then quantification with a Nanodrop (Biowave II, Biochrom WPA, Cambridge, UK).

The rep-PCR technique utilized a universal GTG_5 primer, which sequence is 5'–GTGGTGGTGGTGGTG-3' [15-17]. The rep-PCR programme consisted of the following steps: initial amplification (4 min at 94°C), 30 cycles of denaturation (1 min at

94°C), annealing (1 min at 40°C), elongation (8 min at 72°C), and a final elongation (16 min at 72°C).

Biochemical Identification of Yeasts by ID32C System

Yeast isolates grouped according to the results obtained with rep-PCR were identified using

ID32C galleries (Biomérieux, Marcy-l'Etoile, France). Before inoculation of the ID 32C strip, strains were streaked on YEG agar plates and incubated for 24 h at 30°C. After inoculation, strips were incubated at 30°C for an additional 48 h and the identification was performed according to the manufacturer's instructions using an ApiWeb^{TM} online software (Biomérieux) (https://apiweb.biomerieux.com).

Molecular Identification of the Yeast Isolates

Yeasts were identified by sequencing about 250 nucleotides covering most of the expansion loop of the D1 domain, present at the 5'-end of the 26S rDNA [18-19], and by sequencing 500-800 bp within the ITS1-5.8S-ITS2 region [20-21]. Total DNA extracted from the appropriate isolate was amplified using the primers NL-1/NL-2 for26S rDNA and the primers ITS1/ITS4 for ITS1-5.8S-ITS2 region.

Sequencing of the D1 Domain of 26S rDNAs

Total DNA isolated as described above was amplified with the forward NL-1 (5'-CGCCCGCCGCGCGCGGCGGGCGGGGGGGGGGCCATATCAATAAGCGGAGGAAAAG-3') and reverse primers LS-2 (5'-ATTCCCAAACAACT CGACTC-3'). The PCR program consisted of initial denaturation (5 min at 95 °C), 30 cycles of

denaturation(1 min at 95 °C), annealing (45 s at 52 °C), elongation(1 min at 72 °C) and a final elongation (1 min at 72 °C). The PCR products were purified using the ‘‘Nucleospin® Gel and PCR Clean-Up kit of Macherey–Nagel’’ (Düren, Germany). PCR products were sequenced at Eurofins Genomics (Ebersberg, Germany). All partial 26S rDNA sequences obtained were compared to those available within the online at NCBI database using a megablast research and confirmed with Ribosomal Database project on sequence match with default parameters except size placed at ‘‘<1200’’ parameter [22].

Sequencing of the ITS1-5.8-ITS2 Region

Total DNA isolated as described above was amplified with the forward ITS1 (forward) 5’-TCC GTA GGT GAA CCT GCG G-3’ and the reverse ITS4 primers 5’-TCC TCC GCT TAT TGA TAT GC-3’. The PCR program consisted of initial denaturation (5 min at 94 °C), followed by 35 cycles of denaturation (30 s at 94 °C), annealing (30 s at 57 °C) and elongation (1 min at 72 °C). A final elongation step (5 min at 72 °C) ended this amplification.

Agarose Gel Electrophoresis and Gel Analysis

PCR products were separated on a 1.0 % agarose gel labeled with 0.5 % (v/v) GelRed for 2 h at 100 V. Gels were analyzed with a Gel-Doc 2000® (Bio-Rad, Hercules, CA, USA). In the case of rep-PCR electrophoresis, the gels were analyzed with GelCompar (Biosystematica, Ceredigion, Wales, UK) in order to generate the resulting dendrogram.

Matrix-Assisted Laser Desorption/Ionization (MALDI) Time-of-Flight (TOF) Mass Spectrometry (MS) of Antagonistic Yeasts

Cells of 5 colony-forming units (CFU) were classically picked up with pipette tips and separately smeared as a thin layer onto a ground steel MALDI-target. The on-target deposits were overlaid with 1 µl of 70 % formic acid solution, dried at room temperature then overlaid again with 1 µL of matrix solution (10 mg/ml of α-Cyano-4-hydroxycinnamic acid (HCCA) dissolved in acetonitrile/water/trifluoroacetic acid (50/47.5/2.5; v/v/v)) and dried again. MALDI-MS analyses were performed on an Autoflex speed^{TM} (Bruker Daltonics, Bremen, Germany) running Flexcontrol 3.3. The MALDI-TOF mass spectrometer calibration was performed using the bacterial test standard (BTS) according to the Bruker's recommendations. MALDI-MS profiles were acquired in positive linear mode across the m/z range of 2,000-20,000 using the "LP_12kDa.par" measurement automatic method. Each MALDI-MS profile was the sum of the ions obtained from 5000 laser shots performed randomly on different regions of the same spot. The determination of m/z ratios of detected ions in each MALDI-MS profile was performed under Flexanalysis 3.4.

Hydrophobicity of *C. famata* Y5

The *in vitro* method described by Rosenberg *et al.* [23] was used to detect the bacterial adhesion to hydrocarbons. The antagonistic isolate *C. famata* Y5 was grown overnight, at 30 °C, in Sabouraud containing 0.05 µg/ml of chloramphenicol. Cells harvested by centrifugation (5,000 x *g*, 4 °C, 15 min) and washed twice with phosphate buffer solution (PBS) (pH 7.0). The resulting sample's optical density (A600) was then measured. One milliliter of xylene (Fluka, Germany) was added to 3 ml of cell suspension and vortexed

for 2 min after 10 min of incubation at room temperature. The aqueous phase was removed after 2 h of incubation at room temperature and the A600 was determined. The percentage of hydrophobicity was calculated using the formula given below. All experiments were performed in triplicate.

% hydrophobicity = [(A600 reading 1 – A600 reading 2)/ A600 reading 1] x 100.

Evaluation of Hemolytic Activity of *C. famata* Y5

The hemolytic activity of *C. famata* Y5 was determined upon its inoculation on Muller Hinton agar supplemented with 5% (v/v) of horse blood. The plates were incubated at 30^{o}C for 48 h. The development of a clear zone around the colonies was considered as positive result. In this experiment, the probiotic *S. cerevisiae* var. *boulardii* and the human fungal pathogen *C. albicans* ATCC 10231, recognized as the most frequently agent of candidiasis, were used as positive and negative controls, respectively. This experiment was performed in triplicate.

Cytotoxicity Assay

Intestinal Caco-2 cell line obtained from Sigma-Aldrich (France). All chemicals for the cell culture were from PAN – Biotech GmbH. Cells were routinely grown in 75 cm^2 flasks at 37°C, 5% CO_2 atmosphere in Dulbecco's modified Eagle's Medium (DMEM) (PAN, Biotech) supplemented with 4.5 g/l glucose, 10% of fetal calf serum, 2 mM glutamine, 100 U/ml of penicillin and 100 µg/ml of streptomycin. Sub-confluence (60-80%) cultures were split (1:3 to 1:6) using trypsin (0.25%).

Adhesion Evaluation of *C. famata*

Effects of *C. famata* to intestinal cell proliferation were assayed using Cell Proliferation Kit II (Roche Applied Science, USA) based on the reduction of a tetrazolium salt (XTT) into yellow formazan salt by active mitochondria. When sub-confluence was reached, cells were seeded into 96-well plate at a density of 10,000 cells per well in 100 µl of supplemented DMEM. After 48-72h, at 37°C, 5% CO_2 atmosphere, sub-confluent cells were washed twice with PBS. In parallel, yeasts Sabouraud broth cultures (18 h, 10 ml) was harvested by centrifugation (8 000×*g*, 10 min, 4°C) and washed twice with 2 ml of minimum medium (DMEM without serum and antibiotics). After Caco-2 counting, yeast were re-suspended and diluted in minimum medium. 150 µl of yeast suspension were added into wells to obtained 1:10 and 1:100 Caco-2:yeast ratios. 150 µl of minimum medium were used for control. Plates were then incubated at 37°C, 5% CO_2 atmosphere for 3h.

For XTT assay, 50 µl of XTT was then added in each well and plates incubated for 3 h. Absorbance (490 nm, against 655 nm reference) was measured in a microplate reader spectrophotometer (Xenius, Safas, Monaco). Results were expressed as fold of control.

For adhesion assessment, Caco-2 monolayers were washed two times with PBS to remove non-adherent yeast. Caco-2 cells were then lysed by addition of 300 µl of 0.1% Triton X100 in PBS. Lysates were then collected and diluted before being plated onto Sabouraud agar. After culture, yeasts were counted and adhesion percentages calculated.

In vitro Survival in Gastric and Intestinal Environments

The cultures of *C. famata* Y5 and *S. cerevisiae* var. *boulardii* IS (control) were grown in YPD broth (1 % yeast extract, 2 % peptone and 2 % dextrose) for 18–24 h at30 °C. Cells were harvested by centrifugation (3000g, 10 min, 4 °C) and washed three times in PBS (pH 7) and inoculated at a concentration of 10^6 CFU/ml into a stimulated gastric aqueous solution containing 3 g/l pepsin (3260 U/mg) and 5 g/l NaCl, pH 2.0 which was prepared fresh daily and sterilized by filtration through 0.22 lm Millipore filter [24]. Human intestine conditions were further simulated by addition of 1 g/l pancreatin (903 U/ mg) and 5 g/l NaCl, pH 8.0. The viable cells of C. famata Y5 and S. cerevisiae var. boulardii IS were determined by the plate count method after 20, 40, 60, 120, 180 and240 min of incubation at 37 °C. The results were given as the mean value of three replicates and presented as percentage log survival according to Williamson and Johnson [25]. The log survival = (log N/log N0) 9 100, where N is count (CFU/ml) after incubation, N0 is count (CFU/ml) at time 0.

NCBI Sequence of *C. famata* Y5

The sequences resulting from 26S rDNA and 5.8S rDNA are available as BankIt1829967 rDNA_26S KT023570 and BankIt1829967 rDNA_5.8S KT023571, respectively.

RESULTS

Candida famata is the Unique Species Recovered from Chicken Caeca

The 81 yeast colonies isolated from poultry feces were clustered into 22 distinct groups using rep-PCR method (Fig. 1), as recently described [15-16]. Afterward, one colony was randomly taken from each group and then identified by biochemical (ID32C system) and methods. These methods ascertained the identification and thereof the presence of C. famata as unique species. Indeed, these independent methods resulted to be fully concordant regarding the identification of this species (Table 1). Moreover, the MALDI-TOF mass spectrometry (MALDI-TOF MS) profile of *C. famata* Y5 was established, constituting thereof the first MALDI-TOF MS of *C. famata* strain from poultry origin. We established in this study by the MALDI-TOF MS technique the MS profiles of *C. famata* (laboratory collection) (Fig. 2) that were used as the positive controls. Briefly, the MALDI-MS profiles (Fig. 2a, m/z range of 2000–14,000) obtained for *C. famata* isolates are clearly matching. However, they are very different from MALDI-MS of *S. cerevisiae* var. boulardii IS. The enlarged areas (Fig. 2b) of mass spectra emphasize the high degree of concordance of MALDI-MS profiles obtained from *C. albicans* ATCC10231 and *C. famata* Y5. Indeed, in the 5700–7600 m/z range, the mass signals are fully identical (Fig. 2b, center).

Candida famata Y5: A Safe Poultry Isolate with Possible Benefits to the Host

Candida famata Y5 appeared to possess no hemolytic activity, as no blood hemolysis was observed around the grown colonies (data not shown). The adhesion score of C. famata Y5 to human Caco-2 cells as studied here was less than 1 % (Fig. 3a), which

could explain the absence of cytotoxicity against these eukaryotic cells (Fig. 3b). The cell surface properties of *C. famata* Y5 were not very different from those obtained for *C. albicans* ATCC10231 and S. cerevisiae var. boulardii IS. Indeed, the hydrophobicity value obtained for *C. famata* Y5 was32.85 ± 1.38 and appeared slightly lower than the values obtained for C. albicans ATCC 10231 (41.85 ± 0.77) and *S. cerevisiae* var. *boulardii* IS (39.01 ± 1). The auto-aggregation value obtained from *C. famata* Y5 was 62.61 ± 0.85, and it was also slightly lower than those obtained for *C. albicans* ATCC 10231 (64.27 ± 0.70) and *S. cerevisiae* var. *boulardii* IS (71.31 ± 1.06). The cytotoxicity of *C. famata* Y5 on the Caco-2 cell lines was measured after 3 h of contact by determining the dehydrogenase activity of mitochondria. Results obtained with XTT assay showed that at 1:10 and 1:100 ratios (intestinal cell vs. yeast cell), the three different strains were not toxic for Caco-2 cells. Moreover, *C. albicans* (1:100 ratio) stimulated intestinal cell metabolic activity (Fig. 3). The adherence to intestinal epithelial cells of the three different strains was evaluated. Indeed, after 3 h of contact, the results showed for the three strains a negligible adhesion lower than0.01 % for both ratios assayed (results not shown). In simulated gastric and intestine environments, the survival of *C. famata* Y5 equaled 86.71 ± 0.11–89.31 ± 0.04, after 4 h of incubation at 37 °C, in media containing pepsin and pancreatin, respectively (Tables 2, 3). Remarkably, the survival of probiotic strain *S. cerevisiae* var. *boulardii* IS in similar conditions equaled 89.39 ± 0.17–90.87 ± 0.16(Tables 2, 3).

DISCUSSION

Methodological achievements of the last decade have shed light on the recognition of microbiome as complex communities with important influences on the health and disease

status of the host [26]. Indeed, the microbes play an important role in the growth and development of chickens [27] by providing useful function such as antagonism against human pathogens that are commonly found in this ecosystem [28]. Studies dedicated to chicken gastrointestinal microbiota indicate that bacteria are dominant and diverse [8], contrarily to yeasts, which are not seemingly abundant in chicken gastrointestinal tract, and whose influence on chicken health remains to be studied. Recently, Garcı´a-Herna´ndez et al. [29] reported the presence of *Trichosporon* sp., *Wickerhamomyces anomalus*, *Pichia kudriavzevii*, *Kodamaea ohmeri* and *Trichosporon asahii* from chicken origin. The excreta used were collected from healthy animals that have ad libitum access to water and a diet based mainly on soybean and corn [29]. The present study analyzed yeast content in chicken feces collected randomly in a farm located in the north of France. The culture-dependent approach used here permitted isolation of only *C. famata* species from different samples. Remarkably, *C. famata* (teleomorph *Debaryomyces hansenii*) is also called *C. flareri*. This species belongs to the group of ‘‘flavinogenic yeasts,’’ which overproduce riboflavin under iron limitation [30]. *C. famata* is usually recovered from food and environment and occasionally could be responsible for human infections [30, 31]. The biochemical and molecular methods permitted to identify successfully *C. famata* species. To be noted that contradictory diagnostics and misleading identifications were reported precisely for *C. famata* species [32]. According to Castanheira et al. [32], the accurate identification of this species was attributed to the newly introduced MALDI-TOF MS as performed in this study for *C. famata* Y5 isolated from chicken feces. Search of antagonistic isolates unveiled the potential of *C. famata* Y5 to inhibit L. innocua. Even this inhibitory activity was slight, the finding remains of

major importance because not much studies have so far been dedicated to yeasts and particularly to non-*Saccharomyces* with antagonism [33], contrary to bacterial antagonism that has been deeply investigated [34]. Further production of hemolysin is considered as an important virulence factor for yeast from the *Candida* genus. Rossoni et al. [35] reported that *Candida* species producing hemolysin were not necessarily hemolytic. The strain *C. famata* Y5 did not exhibit any hemolytic activity. This is also the case of *C. albicans* ATCC 10231, which was used in this study as the negative control, and known to be the member of candidiasis lesions in humans [36]. On the other hand, the research presented hereby showed that the cell surface hydrophobicity of *C. famata* Y5 was weaker than those of *C. albicans* ATCC 10231 and S. cerevisiae var. boulardii IS, explaining likely the very slight adhesion observed for these three strains. Recently, the *Candida* strains described with hydrophobicity values displayed weak even no adhesion to Caco-2 cells [37]. Besides, *C. famata* Y5, *C. albicans* ATCC 10231 and *S. cerevisiae* var. boulardii IS were not cytotoxic to the human Caco-2 intestinal epithelial cells. These results are in good agreement with those obtained previously on *C. famata* adhesion and its internalization by epithelial HeLa cells [38]. It can be easily concluded that chicken feces might constitute a reservoir of non-*Saccharomyces* yeasts with potential probiotics. This study permitted to isolate exclusively *C. famata* isolates from chicken feces. In the best of our knowledge, this report is the first one to establish the presence of antagonistic and safe *C. famata* in chicken feces. Details on inhibitory mechanisms of *C. famata* Y5 and its stability in vivo using animal model will be our next goal.

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Compliance with Ethical Standards

Conflict of interest Alaa Al-Seraih, Christophe Flahaut, François Krier, Benoit Cudennec and Djamel Drider declare that they have no conflict of interest.

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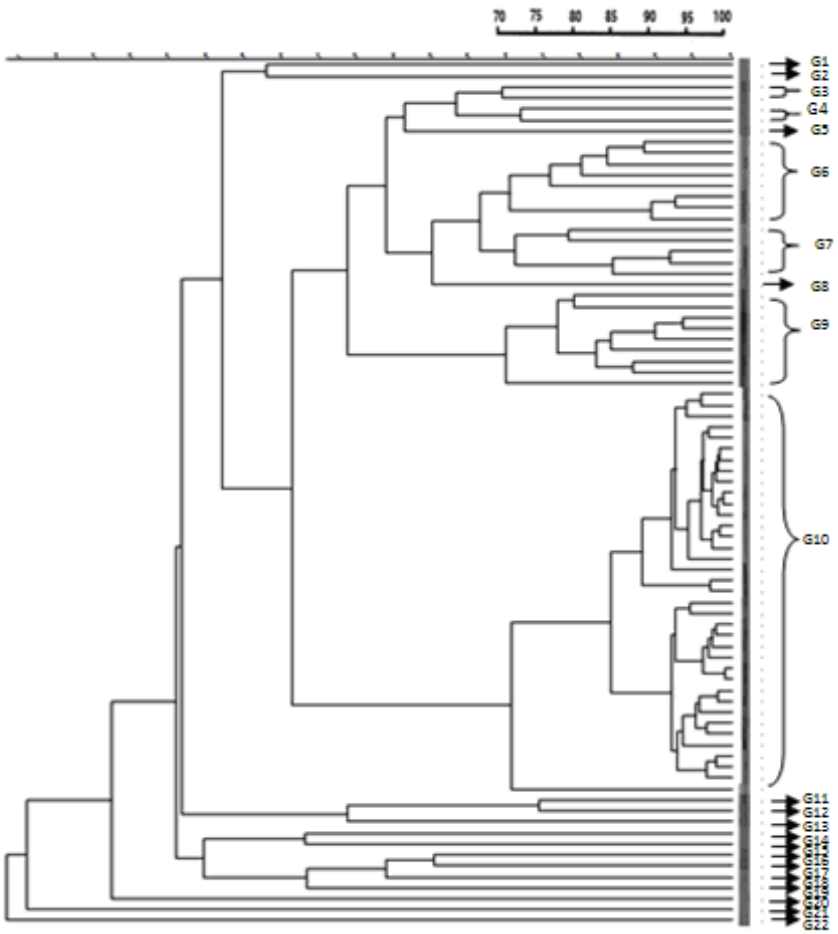

Fig. 1 Dendrogram resulting from the rep-PCR clustering of81 yeasts colonies isolated from the chicken feces. Isolates displaying less than 80 % of Pearson correlation were considered as different, while those displaying more than80 % of correlation were considered as similar. Thus, 22distinct groups were gathered from the 81 isolates obtained in this study.

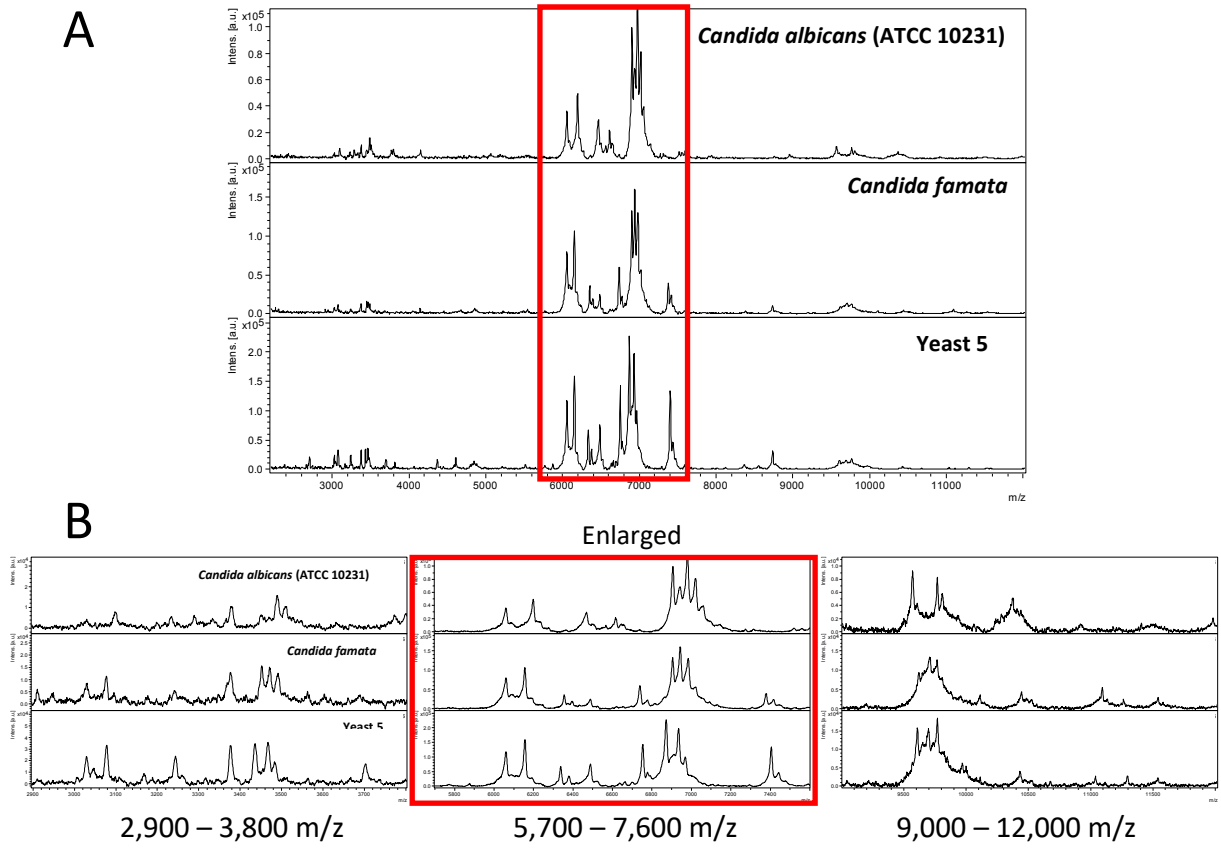

Fig. 2 MALDI-TOF MS profiles of *Candida famata* isolated in our laboratory, *Candida albicans* ATCC 10231 and *C. famata* Y5.

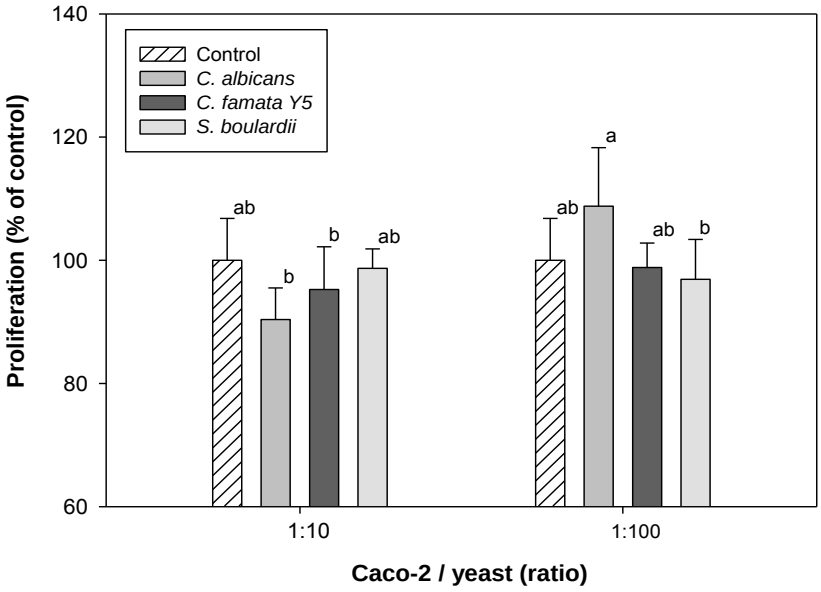

Fig. 3 Cytotoxicity of yeasts against Caco-2 cells. Reduction of a tetrazolium salt (XTT) after 3 h of contact with *C. famata* Y5 (dark gray), C. albicans ATCC 10231 (gray) and Saccharomyces cerevisiae var. boulardii IS, (black) assayed at 1:10 and 1:100 Caco-2/yeast ratios. Data are expressed as percentage of the control and represent the mean of six values. Means without a common letter are different (p\0.05) using one-way ANOVA with Tukey test.

Table 1 Identification of 22 yeasts colonies with ID32C system and molecular methods

Isolate Number	ID32C system (%ID)	Identity %	D1 domain of 26S rDNA	Identity %	ITS1-5.8S-ITS2 region	Identity %
1	*C. famata*	99.9	*D. hansenii*	99	*D. hansenii*	99
2	*C.famata*	99.9	*D. hansenii*	98	*D. hansenii*	99
3	*C. famata*	99.8	*D. hansenii*	99	*D. hansenii*	99
4	*C.famata*	99.8	*D. hansenii*	100	*D. hansenii*	99
5	*C.famata*	99.8	*D. hansenii*	99	*D. hansenii*	100
6	*C. famata*	99.7	*D. hansenii*	97	*D. hansenii*	100
7	*C. famata*	99.9	*D. hansenii*	100	*D. hansenii*	99
8	*C. famata*	99.8	*D. hansenii*	99	*D. hansenii*	100
9	*C. famata*	99.8	*D. hansenii*	99	*D. hansenii*	99
10	*C.famata*	99.8	*D. hansenii*	100	*D. hansenii*	100
11	*C. famata*	99.8	*D. hansenii*	96	*D. hansenii*	100
12	*C. famata*	99.8	*D. hansenii*	99	*D. hansenii*	ND
13	*C.famata*	99.7	*D. hansenii*	99	*D. hansenii*	99
14	*C. famata*	99.7	*D. hansenii*	96	*D. hansenii*	99
15	*C. famata*	99.7	*D. hansenii*	99	*D. hansenii*	100
16	*C. famata*	99.5	*D. hansenii*	99	*D. hansenii*	99
17	*C. famata*	99.0	*D. hansenii*	82	*D. hansenii*	99
18	*C. famata*	99.7	*D. hansenii*	99	*D. hansenii*	99
19	*C. famata*	99.8	*D. hansenii*	99	*D. hansenii*	100
20	*C. famata*	99.0	*D. hansenii*	97	*D. hansenii*	85
21	*C.famata*	99.5	*D. hansenii*	98	*D. hansenii*	99
22	*C. famata*	99.8	*D. hansenii*	99	*D. hansenii*	100

C. famata: *Candida famata, D. hansenii*: *Debaryomyces hansenii*, ND not determined

Table 2 Survival of *Candida famata* Y5 in simulated gastric juice

Strains	Time of incubation					
	20 min	40 min	60 min	120 min	180 min	240 min
C. famata Y5	99.10±0.05	97.30±0.06	96.76±0,05	92.63±0.12	89.94±0.11	86.71±0.11
S. boulardii	99.63±0.06	99.26±0.08	98.17±0.07	94.88±0.12	93.96±0.13	89.39±0.17

The simulated gastric juice contains pepsin (3 g/l), NaCl (5 g/l) and pH 2.0. The data (±SD) are the average of at least three independent experiments.

Table 3 Survival of *Candida famata* Y5 in simulated intestine environment

Strains	Time of incubation					
	20 min	40 min	60 min	120 min	180 min	240 min
C. famata Y5	99.81±0.05	98.91±0.07	96.55±0.03	94.38±0.11	90.94±0.10	89.31±0.04
S. boulardii	99.08±0.06	98.54±0.07	97.99±0.08	95.62±0.16	93.61±0.15	90.87±0.16

Simulated intestine environment contains pancreatin (1 g/l), NaCl (5 g/l) and pH 8.0. The data (±SD) are the average of at least three independent experiments.

Chapter 3.Enterocin B3A-B3B produced by LAB collected from infant feces: potential utilization in the food industry *for Listeria monocytogenes* biofilm management

Bacteriocin designed as enterocin B3A-B3B was isolated and characterized as class II b bacteriocin. B3A-B3B enterocin is produced by *Enterococcus faecalis* B3A-B3B; a strain isolated from the feces of Iraqi infants. Activity of the B3A-B3B enterocin was significantly reduced upon treatment with proteinase K and trypsin, suggesting a proteinaceous nature. On the other hand, B3A-B3B enterocin was insensitive to amylase and lysozyme treatments, confirming the proteinaceous nature and absence of carbohydrates moieties (**Table 6**). A significant reduction in the antimicrobial activity of B3A-B3B enterocin was observed after treatment with lipase. This could be attributed to the high hydrophobicity of class II bacteriocins, which helps with the interaction of some MRS components such as Tween80 ($C_{64}H_{124}O_{26}$) [Dezwaan et al. 2007].

Table 6. Effect of enzymes on antilisterial activity of neutralized cell free supernatant (CFS) and semi-purified enterocin B3A-B3B (SP).

Enzymes (1mg/ml)	***E. faecalis* B3A-B3B CFS**	***E. faecalis* B3A-B3B SP**
Proteinase K (sigma)	9.8±0.289	12.6±0.577
Trypsin(sigma)	10.3±0.764	14.1±0.289
Papain(sigma)	11.0±0.500	15.1±0.289
Lipase(sigma)	0	8.8 ±0.764
Catalase(sigma)	12.5±0.500	18.1 ±0.360
Amylase(sigma)	12.8±0.764	18±0.500
Lysozyme(sigma)	12.1±0.289	18±0.289
Control	13.1±0.289	18.0±0.500

The inhibitory effect was determined by measuring the inhibition zone diameter (mm) in the agar plates inoculated with *L. innocua* ATCC ATCC51742 after 24h of incubation at 37 °C. CFS: cell free supernatant after neutralization with 1M NaOH. SP: Semi-purified B3A-B3B enterocin. Control: CFS and SP without enzymatic treatments.

Further characterization assays revealed that antibacterial activity of B3A-B3B enterocin was stable after heat treatments, and also not affected by pH modification values, indicating that B3A-B3B enterocin is likely a class II bacteriocin.

B3A-B3B enterocin was partially purified by chloroform using liquid-liquid phase separation [Burianek and Yousef, 2000]. Therefore, semi-purified enterocin B3A-B3B was loaded onto a reverse-phase high-performance liquid chromatography (RP-HPLC) column. The HPLC fractions were collected and their antimicrobial activity was tested against *L. innocua* ATCC51742. The purified enterocin B3A-B3B was analyzed by mass spectrometry, which showed a precise molecular mass of 5203.927 Da. (**Figure 16)**.

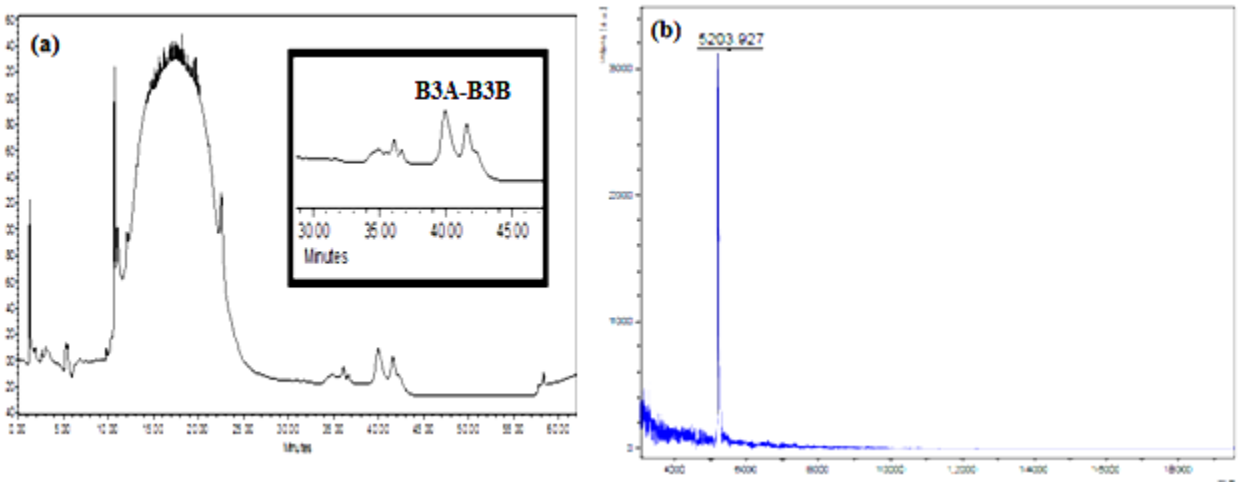

Fig. 16. (**a**) RP-HPLC chromatogram of the bacteriocin purification with a zoom on the active peak corresponding to the enterocin B3A-B3B. (**b**) Mass spectrometry of enterocins B3A-B3B

PCR amplification of total DNA extracted from strain *Enterococcus faecalis* B3A-B3B was performed with forward primer 5′-GATCATGTTGATGACTAGAATTCTTTA-3′ and reverse primer 5′-CAAGGATCCACTTATTATTTCACA-3′ previously designed to amplify DNA coding for enterocin MR10 A and MR10 B. The resulting amplicon was cloned into the pGEM-T Easy vector, transferred to *Escherichia coli* JM 109 and sequenced. Sequence alignment revealed only a two nucleotides difference between B3A-B3B enterocin and enterocin MR10A and MR10B. Nevertheless, this slight difference in DNA content does not modify the amino acid sequences, enabling us to conclude that both enterocins are identical (**Figure 17**).

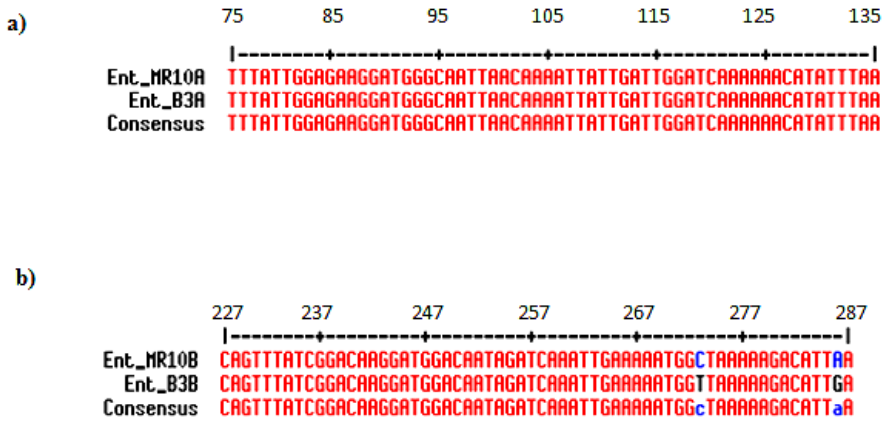

Fig. 17. Alignments of the 3' ends of structural genes coding for enterocins obtained with multalin software (http://multalin.toulouse.inra.fr/multalin/multalin.html) of (**a**) enterocins B3A with MR10A and (**b**) enterocin B3B with MR10B.

The antimicrobial activity of B3A-B3B enterocin was tested against a wide range of pathogens including *Listeria monocytogenes*, *Clostridium perfringens*, *Staphylococcus aureus*, and methicillin-resistant *Staphylococcus aureus* (MRSA-S1) **(Table 7).**

Table 7. Spectrum registered with the cell-free supernatant (CFS) before its neutralization (BN) and after its neutralization (AN) as well as that obtained with the semi-purified bacteriocin (SP).

Target strain	*E. faecalis* B3A-B3B (BN)	*E. faecalis* B3A-B3B (AN)	*E. faecalis* B3A-B3B (SP)
Listeria innocua F	13.3 ±0.667	11.5±0.255	17.8±0.570
L. innocua ATCC 51742	12 ±0.192	12±0.694	18±0.500
L. innocua CIP80.11	12.1 ±0.500	12.2 ±0.255	18.1±0.360
L. monocytogenes 162	11.8±0.577	11.6±0.577	15.00±1.00
L. monocytogenes 162R	11.6±0288	11.3±0.577	14.6±1.154
S. aureus ATCC33862	8.5±0.404	8.5±0.700	10.5±0.500
S. aureus MRSA	0	0	12.6±0.763
Kocuria rhizophila CIP 53.45	13.2±0.450	12.8±0.208	17.7±0.251
Bacillus subtilis ATCC6633	13±0.404	12.3±0.350	16.1±0.288
Salmonella Newport LC	0	0	11.1±1.258
C. perfringens DSM756	8±0.602	7.3±0.404	11.5±0.500
C. perfringens NCTC 6785	11.4±0.450	9.5±0.503	14.3±1.041
C. perfringens NCTC 8789	12.8±0.288	11.6±0577	17.6±0.764

The inhibitory effect was determined by measuring the inhibition zone diameter (mm) in the agar plates after 24h of incubation at 37 °C, **BN:** Antimicrobial activity before neutralization of supernatant. **AN:** Antimicrobial activity before neutralization of Supernatant with 1M NaOH. **SP:** Semi-purified bacteriocin. LC: Laboratory collection.

Notably, the combination of B3A-B3B enterocin and tobramycin as tested by the checkerboard assays exhibited a synergic effect against MRSA-S1, which reduced the minimum inhibitory concentration from 32 mg/l to 4 mg/l, and 400 mg/l to 100mg/l for tobramycin and B3A-B3B enterocin, respectively (**Table 8**).

Table 8. Determination of minimum inhibitory concentrations (MIC) of Enterocin B3A-B3B and tobramycin antibiotic and their combination against MRSA-S1.

Bacterial strain	B3A-B3B Enterocin (mg/l)	Tobramycin (mg/l)	B3A-B3B Enterocin /Tobramycin (mg/l)	Fractional Inhibitory Concentration (FIC)
S. aureus MRSA-S1	400	32	100/4	0.375

Fractional inhibitory concentration (FIC) index. The data (±SD) are the average of at least three independent experiments. Tobramycin was obtained from Sigma-Aldrich.

Taking advantage of the anti-*Listeria monocytogenes* activity of B3A-B3B enterocin, we examined the effect of this bacteriocin on this tremendous foodborne pathogen grown under planktonic and biofilm modes of life. For the simulation of this activity, we used both *L. monocytogenes* with sensitive and resistant-nisin phenotypes. *L. monocytogenes* used here was kindly provided by Dr. Françoise Leroi (IFREMER, Nantes) and Dr. Marie-France PILET (ONIRIS, Nantes). This strain was isolated from seafood products and designated as *L. monocytogenes* 162. Importantly, this strain was sensitive to nisin and the nisin-variant phenotypes were obtained according to Naghmouchi et al. 2007. The minimum inhibitory concentrations (MICs) of nisin were 1mg/ml and 16 mg/ml for *L. monocytogenes* 162 and *L. monocytogenes* 162 R respectively; whereas that of enterocin B3A-B3B was 0.064 mg/ml. These concentrations were reduced significantly by the combination of the two bacteriocins (**Table 9**).

Table 9. Minimum inhibitory concentrations (MICs) of nisin and enterocin B3A-B3B

Strains	Nisin (mg. ml^{-1})	Enterocin B3A-B3B (mg. ml^{-1})	Nisin+Enterocin B3A-B3B (mg. ml^{-1})
Listeria monocytogenes 162 R	16	0.064	4/0.008
Listeria monocytogenes 162	1	0.064	0.256/0.008

The data (±SD) are the average of at least three independent experiments.

The food additive Lantibiotic Nisin E234 is marketed under Nisaplin™ by Danisco [Alvarez-Sieiro et al. 2016]. Nisin was reported to act on sensitive strains through two killing mechanisms. The first one targets the cell-wall synthesis, while the second is a pore- forming mode of action (**Figure 18**). These mechanisms required a binding of nisin to the lipid II, which is the main transporter of peptidoglycan that is responsible for the synthesis of the bacterial cell wall building blocks. The cell wall synthesis is affected by dislocation of the lipid II from its assigned position at lower concentrations of nisin; whilst at the high concentrations nisin binds to lipid II causing membrane insertion and pore formation and then cell death [Miyamoto et al. 2015; Perez et al. 2015].

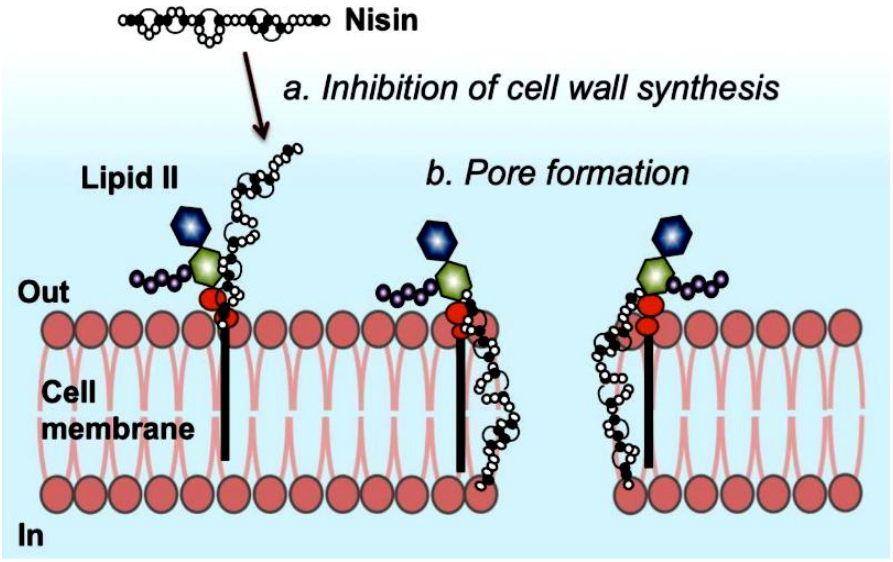

Fig. 18. Mode of action of Nisin. Nisin has a dual mechanism in killing the targeted cells through (**a**) inhibition of cell wall synthesis and (**b**) pore formation [Perez et al. 2015].

Nisin as an antimicrobial agent is active against a wide range of foodborne pathogens including *Listeria monocytogenes*, which is responsible for thousands of food poisoning outbreaks worldwide every year [Miyamoto et al. 2015]. The generation of nisin-resistant *Listeria monocytogenes* is easy to carry out in the laboratory by exposure to nisin at high concentrations [Crandall and Montville, 1998]. Enhanced nisin resistance in *Listeria monocytogenes* is generally defined as an increase in the minimum inhibitory concentration (MIC) by 10 fold or more; therefore, if the strain is capable of growing in

the presence of the highest concentration of tested bacteriocin, it is generally considered to be a high-level resistant mutant [Gravesen et al. 2004]. Different strategies for bacterial resistance to bacteriocins have been reported, including innate resistance, bacteriocin degradation, resistance associated with growth conditions, change of the bacterial cell envelope, and other genetic loci involved in innate resistance. These are summarized in **Figure 19** [de Freire Bastos et al. 2015].

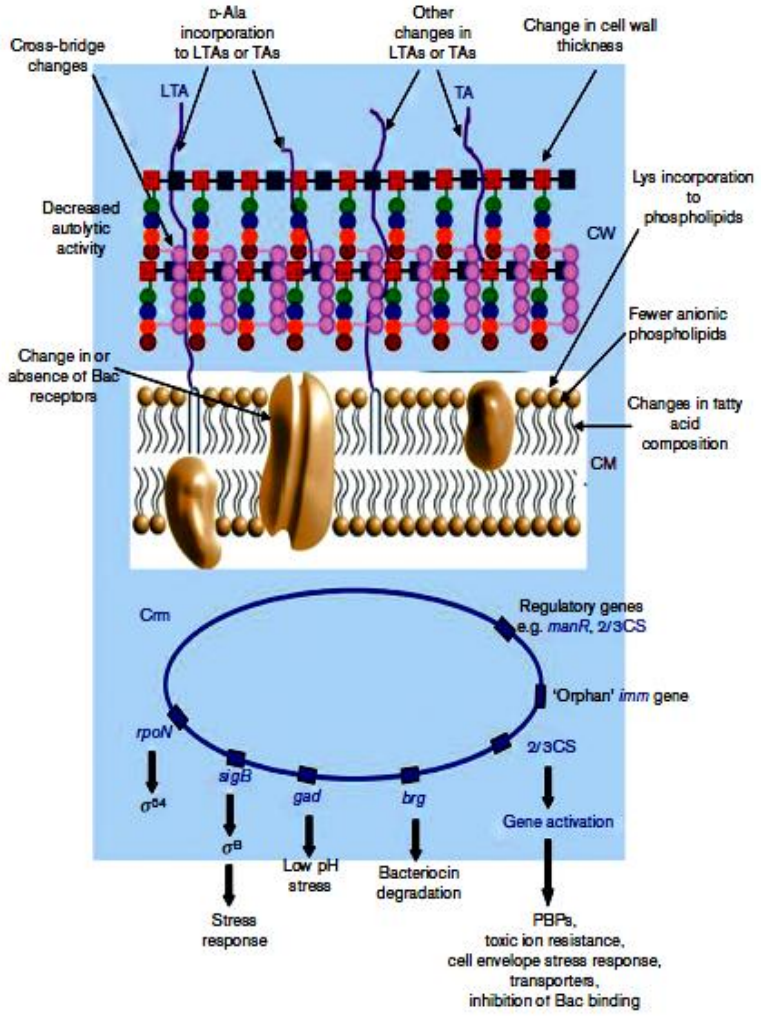

Fig. 19. Some mechanisms and genes involved in bacterial resistance to bacteriocin of Gram positive bacteria. Bac, bacteriocin; *brg*, bacteriocin resistance gene; CM, cytoplasmic membrane; Crm, chromosome; CW, cell wall; gad, glutamate decarboxylase; *imm*, immunity; LTA, lipoteichoic acid; PBP, penicillin-binding protein; *sig*, sigma factor; TA, teichoic acid; 2/3CS, two/three-component signal transduction system [adapted from de Freire Bastos et al. 2015].

As mentioned above, the enterocin B3A-B3B disrupted the biofilm formation of the *L. monocytogenes* 162 when it was cultivated on AISI 304L stainless steel slides. Interestingly, the pretreatment of stainless steel slides with nisin at 1 mg/ml or 16 mg/ml decreased the listerial cell numbers about two logs CFU/ml for both the wild-type *L. monocytogenes* 162 and the nisin-resistant *L. monocytogenes* 162R. However, the combination of nisin with enterocin B3A-B3B reduced the cell counts for 2 logs CFU/ml and minimized the required inhibitory concentration of nisin about 4 fold. The general aims of this chapter can be described as the following:

1. Biochemical characterization and purification of the bacteriocin produced by *Enterococcus faecalis* isolated from infants fecal samples
2. Characterization and molecular identification of the bacteriocin and its safety for human use
3. Inspection of its antimicrobial activity against foodborne pathogens
4. Study of the antimicrobial potential of characterized bacteriocin on biofilm formation by nisin-resistant *Listeria monocytogenes* in combination with nisin

To provide perspective, different studies can be developed on the enterocin B3A-B3B such as its efficacy to control other important antibiotic-resistant or foodborne pathogens individually or in combination with other antibiotics, bacteriocins, and nano-particles. In addition, studies can evaluate its anti-viral, anti-fungal and anti-spore activities and study the therapeutic potential of enterocin B3A-B3B by *in vitro* and *in vivo* experiments such as anti-cancer effects, as several bacteriocins were active against various types of cancer cell lines. Finally, enterocin B3A-B3B may be engineered to increase its stability, potency, and spectrum of activity.

Enterocin B3A-B3B produced by LAB collected from infant feces: potential utilization in the food industry for *Listeria monocytogenes* biofilm management

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Keywords: class IIb Enterocin B3A-B3B, nisin, *Listeria monocytogenes*, resistance, biofilm

Abstract

Enterococcus faecalis B3A-B3B produces a bacteriocin B3A-B3B with activity against *Listeria monocytogenes, Staphylococcus aureus,* methicillin-resistant *Staphylococcus aureus* (MRSA), and *Clostridium perfringens*; but not against fungi or Gram-negative bacteria, except for *Salmonella* Newport. B3A-B3B enterocin has two nucleotides different but similar amino-acids content to class IIb MR10A-MR10B enterocin. The predicted molecular mass of B3A-B3B consists of two peptides of 5,176.31 Da (B3A) and 5,182.21 Da (B3B). Importantly, B3A-B3B impeded biofilm formation of the foodborne pathogen *L. monocytogenes* 162 grown on stainless steel. The antimicrobial treatment of stainless steel with nisin (1 mg. ml^{-1} or 16 mg. ml^{-1}) decreased the cell numbers of about 2 logs CFU. ml^{-1}, impeding thereof the biofilm formation by *L. monocytogenes* 162 or its nisin-resistant derivative strain named *L. monocytogenes* 162R. Further the combination of nisin and B3A-B3B enterocin reduced the MIC value requested to inhibit this pathogen grown in planktonic or biofilm cultures.

Introduction

Listeria are ubiquitous microorganisms widely distributed in the environment. The main reservoirs of *Listeria* are soil, forage and water; however they can be found in other environments such as cattle, sheep and goats, sources for domestic infections. While infections transmitted directly from infected animals to humans or between infected humans are rare, human infections are the result of *Listeria* bacteria transmitted from food products (Allerberger and Wagner 2010; EFSA 2014).

Listeria are resistant to various harsh environmental conditions and can grow even at low temperatures, forming biofilms on different devices, resisting a range of environmental stresses, and consequently contaminate food products by cross-contamination (Gandhi and Chikindas 2007).

L. monocytogenes is recognized as the etiologic agent of listeriosis, a severe food-borne disease that particularly affects high-risk individuals including pregnant women, newborns, the elderly, and immunocompromised individuals. Contamination of foodstuffs by *L. monocytogenes* can occur at all stages of the food chain (Evans and Redmond 2014). Ready to eat (RTE) foods such as the uncooked sea food and non-pasteurized cheeses are the most sensitive products to contamination by *L. monocytogenes,* especially when hygiene and cold chain rules or preparation conditions are compromised (Evans and Redmond 2014).

Moreover, *L. monocytogenes* was shown to form biofilms on different surfaces used in the food industry such as stainless steel, and can therefore persist and survive for long periods of time (Orgaz et al. 2013). *L. monocytogenes* strains embedded in biofilm are resistant to disinfectants and heating (Garrett et al. 2008). The persistence of such

structure on surfaces albeit with routine cleaning and strict hygiene conditions was reported in several cases (Carpentier and Cerf 2011; Ibarreche et al. 2014; Camargo et al. 2013). To enhance food safety and setup novel practices, the use of lactic acid bacteria (LAB) or their metabolites as bacteriocins has been proposed. Related to that, the use of protective cultures, particularly LAB alone or in combination with natural antimicrobials might extend the shelf-life of the minimally processed products without any detrimental effect on their organoleptic qualities (Siroli et al. 2015). Bacteriocins are natural antimicrobials produced by Gram-negative and Gram-positive bacteria (Drider and Rebuffat 2011). Bacteriocins are ribosomally synthesized antimicrobial peptides that are able to kill or inhibit close (narrow spectrum) or distant (broad spectrum) phylogenetically bacteria (Cotter et al. 2005). Production of bacteriocins is strategically an important mean to control competing and surrounding bacteria in an environmental niche. According to Riley and Wertz (2002), many bacteria produce at least one bacteriocin, impacting thus on the global population dynamics.

Bacteriocins produced by LAB may be classified into two distinct classes based on their modification status: Modified (class I), and minimally modified or cyclic (class II) (Cotter et al. 2013). Bacteriocins produced by enterococci are referred as enterocins, which have specific spectrum of inhibitory activity, mode of action, molecular weight and chemical structures (Cintas et al. 2001; Foulquié Moreno et al. 2003; Franz et al. 2007). They consist of different subclasses of bacteriocins; the most common is class II. Some of them belong to class IIb, which are commonly known as two peptides bacteriocins (Nissen-Meyer et al. 2010). These bacteriocins form pores in the cytoplasmic membrane of bacterial cell and provoke loss of internal compounds

leading to cell death (Nissen-Meyer et al. 2009). Lately, interest to enterocins has significantly increased because of their activity against foodborne pathogens such as *Staphylococcus spp., Clostridium spp., Bacillus spp.* and *Listeria monocytogenes* (Franz et al. 1996; Gálvez et al. 1998; Giraffa 1995; Jennes et al. 2000). Further studies permitted to broaden this spectrum as inhibitory activities were reported against Gram-negative bacteria such as *Salmonella* spp. *Pseudomonas* spp., *Shigella* spp. and *Escherichia* coli (Maia et al. 2001; Lauková et al. 2002a,b; Abriouel et al. 2003; Levkut et al. 2009) Nisin (in the commercial forms Nisaplin™ and Chrisin™) is the only bacteriocin currently approved as a food preservative (E234). It should be pointed out that *L. monocytogenes* strains, exposed to sub-inhibitory concentration of nisin, are able to develop spontaneous nisin-tolerant and even nisin-resistant phenotypes depending on the concentration of exposure (Schillinger et al. 1998; Crandall and Montville, 1998).

This study aims at exploring additional reservoirs of bacteriocinogenic LAB and unveiling novel strategies to control the growth of the foodborne pathogens. *L. monocytogenes* 162 isolated from smoked salmon production factory as well as its nisin-resistant variant designed as *L. monocytogenes* 162R were afflicted by the combinations of the food additive and class IIb B3A-B3B enterocin.

Materials and Methods

Bacteria isolation and identification

Six feces samples were obtained from different Iraqi children of 1 to 10 years old. Then 1 gram of each sample was re-suspended in 9 ml of a saline solution (Trypton Salt Broth 0.09%). A series of dilution from 10^{-1} to 10^{-7} were performed and plated

onto de Man–Rogosa–Sharpe (MRS) agar Sigma Aldrich, Germany)(De Man et al. 1960). The plates were incubated in CO_2 jar at 37°C for 48 h. The obtained isolates were maintained in 20% glycerol at -80°C until use.

Presumptive LAB strains fulfilling the Gram-positive staining and absence of catalase activity were identified by mass-spectrometry using the VITEK MS v2.0 MALDI-TOF according to manufacturer's instructions.

Antimicrobial activity

Filter-sterilized cell free supernatant (CFS) was prepared from each LAB isolate by centrifugation (9,000 × *g*, 10 min, 4°C) of overnight cultures grown in MRS broth at 37°C. Then, wells in Mueller Hinton agar (MHA) previously seeded with 1% of overnight culture of target strain including Gram-positive bacteria and Gram-negative bacteria were filled with 50 µl of each CFS or neutralized cell-free supernatant (NCFS) (pH6.5). The Petri plates were left at 4°C for 1 h before incubation at 37°C for 24 h. After this period of incubation, the antibacterial activity was determined by measuring the inhibition zones diameters around the wells containing the CFS or NCFS.

Characterizations of antagonistic substances produced by LAB isolates

CFS gathered from each presumptive LAB strain was treated with proteinase K, trypsin, papain, lipase, catalase, and lysozyme at a final concentration of 1mg. ml^{-1}. The reaction mixture was incubated at 37°C for 1h. On the other hand, 1 ml of NCFS was sterilized by filtration (0.22 µm pore) size filter and incubated at 65, 80 and 100°C for 1h. Similarly, the pH sensitivity of the active substances was determined by

adjusting the CFS pH to values ranging from 2 to 10 using 1M NaOH or 1M HCl. After 2 h of incubation at 37°C, the pH was readjusted to 6.5 and the CFS was filter-sterilized. In all cases, the bacteriocin activity was tested against *L. innocua* ATCC51742 by the agar well diffusion assay (Tagg and McGiven 1971).

Molecular identification of bacteriocinogenic strains

Total DNA was extracted from each antagonistic strain with the Wizard® Genomic DNA Purification Kit (Promega, USA). The 16S rDNA gene was amplified using primers forward 5′-AGAGTTTGATCMTGGCTCAG-3′ and reverse 5′-GGMTACCTTGTTACGAYTTC-3′. The PCR programme and electrophoresis conditions were carried out as recently described (Al Kassaa et al. 2014; Al Atya et al. 2015), Prior to sequencing, the gathered PCR product was extracted from 0.6 % agarose gel using MinElute Gel Extraction Kit (Qiagen, Germany), cloned into the pGEM-T Easy vector (Promega, USA), and transferred to *Escherichia coli* JM 109 (Promega, USA) by heat shock treatment. The recombinant plasmids were selected for their resistance to ampicillin (10 mg. ml^{-1}) (Sigma- Aldrich, Germany). Each recombinant plasmid was extracted from the host strain using the Gene JET Plasmid Miniprep Kit (ThermoScientific, USA). The 16S rDNA was contained in each recombinant plasmid was sequenced at Eurofins MWG (Germany). The blast of each 16S rDNA sequence was performed in NCBI-Standard Nucleotide BLAST and the Ribosomal Database Project (RDP) databases (rdp.cme.msu.edu/seqmatch).

B3A-B3B enterocin production and purification

One liter of MRS broth was inoculated with 1% of an overnight culture of the bacteriocinogenic strain *E. faecalis* B3A-B3B and incubated for 24 h at 37^{o}C. The culture was centrifuged (7,100 x g, 12^{o}C, 15 min), and the CFS containing B3A-B3B enterocin was collected. Then 500 ml of chloroform (Sigma-Aldrich, Germany) were added to the CFS containing B3A-B3B enterocin, stirred vigorously using a magnetic stirrer for 20 min, distributed into 250 ml polypropylene bottles and centrifuged (10,400 g, 12^{o}C, 20 min). Chloroform was removed and the semi-purified B3A-B3B enterocin was obtained as previously reported (Burianek and Yousef. 2000). Then, semi-purified active B3A-B3B enterocin was filtrated on Millipore filter (0.2 µm) and loaded onto a reversed-phase high-performance liquid chromatography (RP-HPLC) column C-18 (5µm, 250 x 3mm, Vydac 218 TP53) equilibrated with solvent A (10mM trifluoroacetic acid, TFA; Sigma-Aldrich), at a flow rate of 5 ml. min^{-1}. Non-absorbed substances were eliminated by washing the column with solvent A until the UV absorbance of the effluent at 210 nm (A210 nm) reached baseline. The substances retained in the column were eluted with a gradient of 0 – 40% of solvent B [isopropyl alcohol/acetonitrile (2:1, v/v) in 40 mM TFA] over 5 min followed by 40 – 100% of solvent B over 60 min at a flow rate of 2.5 ml/min. Fractions of the column effluent were collected according to their UV light absorbance, lyophilized and redissolved in distilled water before being tested for bacteriocin activity as reported by Abriouel et al. (2003).

Bacteriocin activity was assayed by the agar well diffusion method against *L. innocua* ATCC 51742 as the reference organism (Tagg and McGiven 1971). The bacteriocin

titer was performed as previously described (Apolônio et al. 2008) and the arbitrary unit (AU) of antimicrobial activity per milliliter (AU/ml) was defined as: (AUml) = 2^n x 1000 µl x V $µl^{-1}$; where V is the volume of bacteriocin used in the test (Apolônio et al. 2008). The protein concentration of enterocin B3A-B3B was determined by BCA assay by using Sigma-Aldrich BCA kit (USA). The molecular mass of the purified B3A-B3B enterocin was determined by the MALDI-TOF/TOF mass spectrometer (Bruker, Bremen, Germany) equipped with a smart beam laser as recently reported (Al Atya et al. 2016).

Amplification and sequencing of structural gene coding for B3A-B3B enterocin

DNA coding for B3A-B3B enterocin was amplified with forward primer 5′-GATCATGTTGATGACTAGAATTCTTTA-3′ and reverse primer 5′-CAAGGATCCACTTATTATTTCACA-3′. Amplification of the gene coding for B3A-B3B enterocin was performed with following programme: pre-denaturation step of 5 min at 94°C, followed by 30 cycles of 1 min at 94°C, 30s at 55°C, 1 min at 72°C and a final extension step of 5 min at 72°C (Achemchem et al. 2005). The PCR product was extracted, cloned and sequenced as above-detailed. B3A-B3B enterocin gene was identified using the online database automated bacteriocin mining BAGEL3 (http://bagel.molgenrug.nl/).

Minimal inhibitory concentrations (MIC) and checkerboard assay

L. monocytogenes 162 was isolated from smoked salmon factory by Dr. Pilet and Dr. Leroi (Nantes, France). The nisin-resistant variant, *L. monocytogenes* 162 R, was obtained as described by Naghmouchi et al. (2007a). These strains were grown overnight on Tryptic Soy Broth (TSB) at 37°C. Afterwhich, 10µl of each culture were inoculated into the wells of 96- wells cell culture microplate (Cellstar) containing different concentrations of B3A-B3B enterocin ranging from 0.002 to 0.400 mg.ml^{-1}. The MIC was considered as the lowest concentration that inhibited the visible growth of the target strain after incubation at 37°C for 24h. Checkerboard assay was used to elucidate the nature of interaction between the tested antimicrobials. The concentrations used for B3A-B3B enterocin were ranging from 0.002 to 0.400 mg. ml^{-1}, whereas those used for nisin were ranging from 1 to 16 mg. ml^{-1}. Microplates were inoculated with both strains at about 10^6 CFU. ml^{-1}, in a final volume of 200 µl per well and incubated at 37 °C for 24h.

Cytotoxicity of the enterocin B3A- B3B

The cytotoxicity assay was performed using adapted protocol described by Belguesmia et al. (2010). Briefly, Caco-2 cells (Sigma Aldrich, Germany) were cultivated on 96-well tissue culture plates for 48-72h, at 37°C, in atmosphere containing 5% CO_2, until the formation of a continuous confluent cell culture on the bottom of each well. The B3A-B3B enterocin and nisin were tested at 0.064 mg. ml^{-1} and 1 mg. ml^{-1}, which correspond to their MIC values, respectively. The required concentrations were prepared in DMEM without antibiotics and serum and were added to the corresponding Caco-2 cells in the wells, after washing with the same medium

without enterocin B3A-B3B and nisin and then the plate containing Caco-2 cells and samples were incubated for 24h at 37°C, in atmosphere containing 5% CO_2. CCK-8 assay (Dojindo Molecular Technologies, Japan) based on the reduction of tetrazolium salt by active mitochondria was used to assess cell viability of the treated Caco-2 cells. 150 µl of DMEM containing 7.5 µl of CCK-8 reagent were added in each well and cells were incubated for 2h. Plates were then read at 450 nm in a microplate reader spectrophotometer (Xenius, Safas, Monaco). Results were expressed in % of basal growth observed with non-treated cells.

Inhibition of biofilm formation by *Listeria* strains on stainless steel AISI 304L

Preparation of stainless steel slides and bacterial adhesion assay protocol was adapted from Ait Ouali et al. (2014). After washing and cleaning, AISI 304L stainless steel slides were placed in sterile Petri plates, with 2 ml of antimicrobial agents at their MIC. The concentrations used for *L. monocytogenes* 162 were 1 mg. ml^{-1} for nisin, 0.064 $mg.ml^{-1}$ for B3A-B3B enterocin, and 0.256/0.008 $mg.ml^{-1}$ for nisin-enterocin B3A-B3B. The concentrations used for *L. monocytogenes* 162R were 16 mg. ml^{-1} (nisin), 0.064 mg. ml^{-1} (B3A-B3B enterocin) and 4/0.008 mg. ml^{-1} for their combination. AISI 304L slides treated with sterile TSB were used as controls. After 2h of incubation at 37°C, the antimicrobial agents and TSB were removed and replaced with 2 ml of target strains at about 10^7 CFU. ml^{-1}. After incubation for 1h at 37°C, the supernatant containing non-adhered bacterial cells was removed by pipetting and replaced with 2 ml of a sterile TSB medium, and the incubation was conducted for 0, 3, 6, and 24 h at 37°C. After each period of incubation, the slides were washed twice

with 30 ml of PBS (pH 7). Finally, the AISI 304L slides were immersed individually in 30ml of sterilized phosphate buffer and sonicated for 5min at 50 kHz. An additional scraping step, with sterile flexible brush, was applied to complete the removing of biofilms and bacterial cells. The detached *L. monocytogenes* 162 and *L. monocytogenes* 162R viable cells were counted by plating on Tryptic Soy Agar (TSA) after incubation at 37°C for 24h. Additional slides were prepared at the same conditions and used for epifluorescence observation. The biofilm-containing AISI 304L slides were stained with BacLight LIVE/DEAD bacterial viability kit according to the manufacturer's instruction (Molecular Probes, Invitrogen, France). Briefly, 1.5 µl of each reagent were diluted with 1 ml of physiological water (0.85% w/v NaCl), and the mixture was gently added onto the upper surface of the slides followed by incubation of the slides in dark for 15min. The staining solution was decanted and biofilms were observed by epifluorescence microscope (Nikon Optiphot-2 EFD3, Japan). Further, Scan Electron Microscopy (SEM) was performed on biofilms formed by *L. monocytogenes* 162R on glass slides devices. The biofilm formation was maintained for 5 days, with changing the growth medium (TSB) every day to keep the viability of bacterial cells, remove non adherent cells and excess biological materials. The glass slides devices were then treated for 2h at 37 ^{o}C with antimicrobial compounds, nisin and B3A-B3B enterocin alone or in combination, at the same concentrations used previously. At the end of the experiment, the glass slides were fixed with 1% of glutaraldehyde solution for 30 minutes then washed several times with demineralized water

Scanning Electron Microscopy (SEM)

Bacteria grown on surfaces were fixed with glutaraldehyde (1 %) in PBS 1X for 30 minutes. After washing, bacteria were dehydrated with increasing ethanol concentration baths. After two pure ethanol washes, cells were dried with a critical point drier (Quorum Technologies K850, Elexience, France). Finally, dry coverslips were mounted on stubs and coated with 5 nm platinum (Quorum Technologies Q150T, Elexience, France). Cells were observed with a secondary electron detector in a Zeiss SEM Merlin Compact VP (Zeiss, France) operating at 5 kV.

Statistical analysis

Data are expressed as mean ± standard error (SE) calculated over three independent experiments performed in triplicate.

Results

Screening of lactic acid bacteria with anti-*Listeria* activities

Out of 500 colonies selected on MRS agar from six different donors, 70 colonies were Gram-positive bacteria and devoid of catalase activity, 41 of the isolates were cocci and 29 bacilli. Thus, they were considered as presumptive LAB. The distribution of the colonies was as follows: six isolates from the donor 1, five isolates from the donor 2, nine isolates from the donor 3, five isolates from the donor 4, fifteen isolates from the donor 5, and thirty isolates from the sixth one. The CFS and NCFS (pH 6.5) gathered from these presumptive LAB strains were tested against *L. innocua* ATCC 51742 as indicator organism. These assays underline the abilities of strains B3A-B3B and B20A-B20B, identified as *E. faecalis*, to produce enterocin-like substances. The

NCFS (pH 6.5) treated with different proteinases diminished the inhibitory activities of B3A-B3B and B20A-B20B enterocins (data not shown). NCFS treated with lipase, were devoid of inhibitory activities. According to Dezwaan et al. (2007), the loss of this activity could be ascribed to the interactions between the high hydrophobic bacteriocins and some MRS medium contents such as Tween 80. Furthermore, the NCFS from *E. faecalis* B3A-B3B and *E. faecalis* B20A-B20B strains were active independently of the pH and temperature variations.

Overall, the CFS gathered from each of these strains displayed bacteriocin-like characteristics, as their antagonism was pH and temperature independent and abolished upon treatment with different proteases. The CFS gathered from the bacteriocinogenic strains *E. faecalis* B3A-B3B and B20A-B20B was tested before and after neutralization against a set of Gram-positive bacteria, Gram-negative bacteria and yeasts such as the well known pathogenic *Candida albicans* or the probiotic *Saccharomyces boulardii* (Table 1). The NCFS from *E. faecalis* B3A-B3B and B20A-B20B inhibited the growth of Gram-positive bacteria, mainly *Listeria*, *Bacilli* and *Clostridia* species except methicillin resistant *Staphylococcus aureus* (MRSA) or *Staphylococcus epidermidis*, advocating a narrow spectrum. Notably, the growth of MRSA was, in turn, affected by the semi-purified B3A-B3B and B20A-B20B enterocins. No antagonism was observed against *Salmonella*, *Escherichia coli* and *Pseudomonas* with the CFS of *E. faecalis* B3A-B3B and B20A-B20B before and after neutralization. The use of semi-purified enterocins B3A-B3B, and B20A-B20B inhibited the growth of *Salmonella* Newport (data not shown). Finally, enterocins B3A-B3B, B20A-B20B were not active against the phylogenetically closely related *E.*

faecalis ATCC 29212, a vancomycin sensitive strain which is used as a standard control in clinical and food safety tests (Kim et al. 2012). For this study enterocin B3A-B3B was purified and further characterized.

Enterocin B3A-B3B purification

Enterocin B3A-B3B was purified by liquid–liquid extraction (LLC) consisting of mixing the CFS with chloroform as the extraction solvent. Then, the semi-purified enterocin B3A-B3B was separated by RP-HPLC column, and the active fraction was identified as one peak at 40 min of retention time and 100% of elution solvent (isopropanol and acetonitrile) (Fig. 1A). During the purification process, the specific activity of enterocin B3A-B3B increased, as expected (Table 2). The purified B3A-B3B enterocin analyzed by mass spectrometry displayed a molecular mass of 5,203.927 Da. (Fig. 1B), which is slightly higher than the predicted masses. The DNA sequences coding for this peptide (these peptides) performed as described in the materials and methods sections unveiled similarities with enterocins MR10A and MR10B. B3A-B3B enterocin differs in terms of nucleotides content from MR10A-MR10B enterocin only in two nucleotides. Indeed, the 3'-region of the *entB3B* gene, the nucleotide C was replaced by T (position 273), while the nucleotide A was replaced by G (position 286), compared to *entMR10B* gene (Fig. 2B). The sequences analysis using the online GeneScript Software (https://www.genscript.com) predicted a molecular mass of 5,176.31 for peptide B3A and 5,182.21 Da for peptide B3B.

Minimal inhibitory concentrations (MIC) and cytotoxicity of bacteriocins on Caco-2

MICs values of nisin were 1 mg.ml^{-1} and 16 mg. ml^{-1}, for *L. monocytogenes* 162 and *L. monocytogenes* 162 R respectively, whilst that of B3A-B3B enterocin was 0.064 mg. ml^{-1} for both strains. The combination of nisin and enterocin B3A-B3B has diminished the MIC values for both bacteriocins. Indeed the checkerboard assay determined for *L. monocytogenes* 162 a MIC value of 0.008 mg. ml^{-1} for enterocin B3A-B3B and 0.256 mg. ml^{-1} for nisin, and for *L. monocytogenes* 162R a MIC of 0.008 mg. ml^{-1} for B3A-B3B enterocin and 4 mg. ml^{-1} for nisin (Table 3). Cytotoxicity assay realized on Caco-2 cells showed 94±4.0% survivability for nisin at 1 mg.ml^{-1}, and 75.1±6.0% for the semi-purified B3A-B3B enterocin at the MIC value of 0.064 mg. ml^{-1} (Fig. 3).

Effect of enterocin B3A-B3B on biofilm formation *L. monocytogenes* 162 and *L. monocytogenes* 162R on AISI 304L stainless steel and glass slides

Biofilm formation on the AISI 304L stainless steel slides by *L. monocytogenes* 162 and *L. monocytogenes* 162R was inspected after conditioning the devices by nisin, enterocin B3A-B3B and combination of nisin/ B3A-B3B enterocin. The data reveals that about 1 mg. ml^{-1} of nisin or 0.064 mg. ml^{-1} of B3A-B3B enterocin alone inhibit about 2 logs of biofilm formation of *L. monocytogenes* 162. The combination of nisin at its MIC value with B3A-B3B enterocin at 0.256 and 0.008 mg. ml^{-1} showed a significant drop in the numbers of viable cells. This drop was about 2 logs for *L. monocytogenes* 162 after 0, 3, 6 and 24 h of incubation at 37°C (Table 4; Fig. 4A).

Enterocin B3A-B3B demonstrated a clear effect on the biofilm formation by *L. monocytogenes* 162R. On the other hand, the concentrations of 16 mg. ml^{-1} of nisin or 0.064 of enterocin B3A-B3B were needed to inhibit the biofilm formation for *L. monocytogenes* 162R, with a reduction of cell count number by 2 logs. The combination of nisin and B3A-B3B enterocin, at a MIC value of 4 and 0.008 mg. ml^{-1} respectively, resulted in a decrease of about 2 logs in the numbers of viable cells and biofilm formation after incubation at 37°C for 0, 3, 6, and 24h for *L. monocytogenes* 162R. As shown in Table 5 and Fig. 4B, when nisin and enterocin B3A-B3B were combined, lower concentration of nisin was required to impede biofilm formation by *L. monocytogenes* 162 and its derivative strain resistant to nisin *L. monocytogenes* 162 R.

SEM analysis showed that *L. monocytogenes* 162R biofilms formation, on glass slide devices, were affected by treatment with nisin at 16 mg. ml^{-1} and enterocin B3A-B3B at 0.064 mg. ml^{-1}. Important reduction of biofilms and modification of the bacterial cells form and shape with decrease of the number of adherent cells were observed (Fig. 5B,C). The combination of nisin (4 $mg.ml^{-1}$) and B3A-B3B enterocin (0.008 $mg.ml^{-1}$), showed similar effects, with reduction of biofilms and number of adherent bacterial cells with lower concentrations of bacteriocins.

Discussion

The use of the *Enterococcus* species in food, conversely to other LAB species, remains to be clarified. Indeed, some species were incriminated in urinary tract infections, bacteremia and endocarditis (Franz et al. 1999; Kayser 2003), while Khan et al. (2010) and Franz et al. (2011) reported their use in fermented traditional foods

and also as bioprotective cultures to limit the development of foodborne pathogens and spoilage microorganisms. De Vuyst et al. (2003) suggested that *Enterococcus* species are safely usable in foodstuffs, only when genes coding for virulence factors are absent. Related to this, Jaouani et al. (2015) examined the presence of virulence and antibiotic resistance genes on different bacteriocinogenic enterococci and concluded that only 22/55 of the strains tested were safe for use in food based on these criteria. All these indications enable us to say that until this ambivalent situation is solved, we can consider *Enterococcus* species as great reservoirs of bacteriocins.

Here we studied and characterized B3A-B3B enterocin produced by *E. faecalis* B3A-B3B, a strain isolated from feces of Iraqi infants. Enterocin B3A-B3B was purified using three distinct steps. An unconventional initial step requiring chloroform solvent extraction instead of ammonium sulfate precipitation was introduced in the procedure to minimize the interaction of the active compound with Tween 80 and scale-up the production yield as suggested by Burianek and Yousef (2000). Genetic analysis of DNA coding for enterocin B3A-B3B displayed difference only in a couple of nucleotides with DNA sequences of class IIb MR10A-MR10B enterocin produced by *E. faecalis* MRR 10-3 (Martín-Platero et al. 2006). Notwithstanding that these minor genetic differences do not result in any difference in the amino-acids sequence. Albeit their distal ecological niches, the strains *E. faecalis* B3A-B3B (Iraqi infant) and *E. faecalis* MRR 10-3 (hoopoe *Upupa epops*) (Martín-Platero et al. 2006) produce similar bacteriocins. MR10A and MR10B peptides are cationic compounds rich in basic residues. The predicted isoelectric points for MR10A and MR10B are 10.02 and 10.23, respectively. The high proportion of hydrophobic polar-uncharged amino acids

(21 hydrophobic residues plus 4 polar-uncharged residues in MR10A and 19 hydrophobic residues, plus 4 polar uncharged residues in MR10B) indicates the hydrophobic nature of these peptides (Martín-Platero et al. 2006). As enterocin B3A-B3B has almost similar DNA sequence, and fully similar amino-acid sequence, we can conclude that enterocin B3A-B3B and enterocins MR10A and MR10B share similar biochemical characteristics. The purification procedure used here resulted in only one active peak collected after RP-HPLC step, but the molecular analysis of the coding genes led to the identification of two very similar peptides belonging to class IIb bacteriocins, designated hence as B3A-B3B.

Batdorj et al. (2006) faced similar challenge when they purified A5-11A and A5-11B produced by *E. durans* A511. To overcome this challenge, the authors have used two successive reversed phase HPLC to separate the two peptides which were very similar to the enterocin B3A-B3B identified in this study. Cytotoxicity assay on Caco-2, a human epithelial colorectal cells, showed 20 to 25% of cell death after treatment with the enterocin B3A-B3B, this mortality is likely ascribed to the presence of some toxic compounds in the semi-purified enterocin B3A-B3B and the data should be minimized regarding the toxicity of enterocins yet reported (Belguesmia et al. 2010).

L. monocytogenes has the capacity to adhere rapidly to the surface of stainless steel equipment leading to mature biofilms that are difficult to eradicate (de Oliveira et al. 2010). The initial and reversible adhesion step occurs during the few earlier minutes of the biofilm formation. At this stage, bacteria are easily removable (Garrett et al. 2008). Then an irreversible adhesion is initiated after 20 min to a maximum of 4 h of contact at temperature ranging from 4 to 20º C (Garrett et al. 2008). Once the biofilm process

is completed, the removal of irreversibly adhered cells becomes harsh and requires strong mechanical or chemical treatments (de Oliveira et al. 2010). Novel class of antibiofilms agents consisting of safe and sustainable natural compounds could be developed in order to secure food industry process. Bacteriocins endowed with broad spectrum activity are great candidates for the biofunctionalization of such devices. In this study, the functionalization of stainless steel slides with the food additive E234 (nisin) and enterocin B3A-B3B decreased the counts of the *L. monocytogenes* 162 and its nisin-resistant derivative by at least 2 log CFU/ml. In *L. monocytogenes*, spontaneous nisin-resistant derivative strains were generally attributed to the fatty acids cell membrane modification (Mazzotta and Montville 1997; Naghmouchi et al. 2007a,b). Moreover, interestingly these bacteriocins were found to be active against established *L. monocytogenes* 162R biofilms on glass slide devices, reducing significantly the biofilms and the number of visible adherent bacterial cells. Another finding of importance evidenced here is the absence of cross-resistance of *L. monocytogenes* to nisin and enterocin B3A-B3B. Indeed, *L. monocytogenes* 162R resistant to the food additive E234 was sensitive to enterocin B3A-B3B, arguing the absence of cross-resistance phenomenon and also different mode of actions of these bacteriocins. This finding is in good agreement with studies from Duffes et al. (2000) and Kaur et al. (2013) who revealed the absence of cross resistance between nisin and class IIa bacteriocins. On the other hand, other studies by Gravesen et al. (2004) then Naghmouchi et al. (2007a) portrayed a cross resistance phenomenon in resistant variants strains of *L. monocytogenes* strains for nisin and class IIa bacteriocins. According to Gravesen et al (2004) resistance to nisin in some *L. monocytogenes*

strains involved modification in the expression of *pbp2229*, *hpk1021* or *lmo2487*, which code for penicillin-binding protein, histidine kinase and putative protein of unknown function.

In this study, we show that the use of nisin and enterocin B3A-B3B combination towards *L. monocytogenes* 162 nisin-resistant derivative strains required four fold less of nisin and enterocin, delineating thereof a novel strategy to fight against resistance to bacteriocins. Related to this, Shillinger et al. (1998; 2001) proposed to use of protective culture including class II bacteriocins LAB producers, in combination with nisin to limit the emergence of nisin-tolerant and nisin-resistant strains of *L. monocytogenes.* Bacteriocins are anticipated to cause cell death through membrane permeabilization consecutively to pores forming. Nisin was reported to impair cell wall formation, thus compromising cell envelope strength (Bastos Mdo et al. 2015; Bierbaum and Sahl 2009) Indeed, nisin interacts with lipid II and inhibits cell wall synthesis by blocking the lipid II cycle (Breukink et al. 2003; Brotz et al. 1998; van Heusden et al. 2002) Class IIb bacteriocins act by membrane permeabilization to a variety of small molecules including ATP, ions and nutrients (Niseen-Meyer et al. 2009). Structure–function analysis, proposed that the two peptides, of two-peptide class IIb bacteriocins, form a membrane-penetrating helix–helix structure involving helix–helix-interacting GxxxG-motifs, which play a major role in the interaction with the cytoplasmic membrane of the cell target (Niseen-Meyer et al. 2010). The amino-acid sequences of enterocin B3A-B3B displayed GxG motif at the positions 28th and the 30th and this motif could likely have the same role as that debated by Nissen-Meyer et al. (2010). Some class IIb bacteriocins exhibited cross-immunity in a

dependent manner, suggesting a relationship with a specific cellular component, likely a bacteriocin receptor (Oppegård et al. 2010) This could explain the absence of cross-resistance between enterocin B3A-B3B and nisin.

We looked in addition at the effect of combination of bacteriocins from different classes and their advantages to fight the development of spontaneous nisin-resistant *L. monocytogenes* strains as *L. monocytogenes* 162R. This approach permitted us to show that resistance to nisin in *L. monocytogenes* is not a fatality and can be easily solved by addition of safe and active bacteriocin of another class such as enterocin B3A-B3B.

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Table 1. Spectrum registered with the inhibitions zones expressed in millimeters (mm) with cell-free supernatant (CFS) before its neutralization (BN) and after its neutralization (AN) as well as that obtained with the semi-purified bacteriocin (SP).

BN: Antimicrobial activity before neutralization of supernatant. **AN:** Antimicrobial activity after neutralization of supernatant with 1M NaOH. **SP:** Semi-purified bacteriocin

Target strain	*E. faecalis* B3A-B3B (BN)	*E. faecalis* B3A-B3B (AN)	*E. faecalis* B3A-B3B (SP)	*E. faecalis* B20A-B20B (BN)	*E. faecalis* B20A-B20B (AN)	*E. faecalis* B20A-B20B (SP)
Listeria innocua F	13.3 ±0.667	11.5±0.255	17.8±0.570	12.6 ±0.763	12.3±0.255	17.4±0.790
L. innocua ATCC 51742	12 ±0.192	12±0.694	18±0.500	12 ±0.500	11.5±0.500	16.1±0.289
L. innocua CIP80.11	12.1 ±0.500	12.2 ±0.255	18.1±0.360	12.7 ±0.251	12.1 ±0.404	17.7±0.680
L. monocytogenes 162	11.8±0.577	11.6±0.577	15.00±1.00	11.0±0.577	10.6±1.154	14.6±0.577
L. monocytogenes 162R	11.6±0288	11.3±0.577	14.6±1.154	10,8±0,500	10.6±0.577	14.3±0.577
S. aureus ATCC33862	8.5±0.404	8.5±0.700	10.5±0.500	8.1±0.360	8.2±0.680	10.5±0.500
S. aureus MRSA	0	0	12.6±0.763	0	0	12.3±0.577
Kocuria rhizophila CIP 53.45	13.2±0.450	12.8±0.208	17.7±0.251	13.4±0.404	12.53±0.472	17.1±0.230
Bacillus subtilis ATCC6633	13±0.404	12.3±0.350	16.1±0.288	12.5±0.500	11.3±0.754	15.8±0.288
Salmonella Newport LC	0	0	11.1±1.258	0	0	9.3 ±0.764
C. perfringens DSM756	8±0.602	7.3±0.404	11.5±0.500	7.4±0.450	7.2±0.450	11.3±0.577
C. perfringens NCTC 6785	11.4±0.450	9.5±0.503	14.3±1.041	10.4±0.404	9.3±0.288	14.6±0.764
C. perfringens NCTC 8789	12.8±0.288	11.6±0577	17.6±0.764	11.5±0.500	10.5±0.500	17.0±1.000

No activity was observed against *Candida albicans* ATCC 10231, *Saccharomyces cerevisiae*, *Escherichia coli* ATCC 8739, *Salmonella* Heidelberg, *Pseudomonas aeruginosa*, *Enterococcus faecalis* ATCC29212, *Staphylococcus epidermidis*

LC : Laboratory collection

Table 2. Assessment of enterocin B3A-B3B purification procedure

Purification Step	Volume (ml)	Activity AU. ml^{-1}	Protein (mg. ml^{-1})	Total protein (mg)*	Total activity (AU)	Specific activity** ($AU.mg^{-1}$)	Purification Factor
Supernatant	1,000	400	11.2	11,200	400,000	35.7	1
Chloroform	500	1,600	3.2	1,600	800,000	500	14
C18 RP-HPLC	1	6,400	0.2	0.2	6,400	32,000	896

*Total protein concentration refers to protein concentration per milliliter multiplied by the volume (ml). It was determined by BCA assay.

** Specific activity is arbitrary units (AU) divided by the total protein concentration.

Table 3. Minimum inhibitory concentrations (MICs) of Nisin and Enterocin B3A-B3B

Strains	Nisin (mg. ml^{-1})	Enterocin B3A-B3B (mg. ml^{-1})	Nisin+Enterocin B3A-B3B (mg. ml^{-1})
Listeria monocytogenes 162 R	16	0.064	4/0.008
Listeria monocytogenes 162	1	0.064	0.256/0.008

Table 4. Total count of biofilm formation on AISI 304L stainless steel slides by strain *Listeria monocytogenes* 162

Incubation time	Control Log CFU. ml^{-1}	Nisin at 1 mg. ml^{-1} Log CFU. ml^{-1}	Enterocin B3A-B3B at 0.064 mg. ml^{-1} Log CFU. ml^{-1}	Nisin+Enterocin B3A-B3I at 0.256+0.008 mg. ml^{-1} Log CFU. ml^{-1}
0h	4.7±0.352	3.9±0.316	4.1±0.229	3.3±0.114
3h	5.4±0.345	4.2±0.252	4.4±0.212	4.0±0.253
6h	6.1±0.270	4.6±0.160	4.7±0.401	4.2±0152
24h	7.7±0.0.655	5.6±0.222	5.7±0.162	5.4±0.212

Table 5. Total count of biofilm formation AISI 304L stainless steel slides by strain nisin-resistant *L. monocytogenes* 162R.

Incubation Time	Control Log CFU. ml^{-1}	Nisin at 16mg. ml^{-1} Log CFU. ml^{-1}	Enterocin B3A-B3B at 0.064 mg. ml^{-1} Log CFU. ml^{-1}	Nisin+Enterocin B3A-B3B at 4+0.008 mg. ml^{-1} Log CFU. ml^{-1}
0h	4.2±0.141	3.76±0.395	3.80±0.217	3.69±0.330
3h	4.9±0.144	4.1±0.115	4.2±0.117	4.0±0.193
6h	6.1±0.184	4.7±0.707	4.9±0.748	4.5±0.144
24h	7.9±0.269	5.7±0.337	5.8±0.539	5.5±0.307

Figures Captions

Figure 1. (a) RP-HPLC chromatogram of the bacteriocin purification with a zoom on the active peak corresponding to the enterocin B3A-B3B. (b) Mass spectrometry of enterocins B3A-B3B

Figure 2. Alignments of the 3' ends of structural genes coding for enterocins obtained with multalin software (http://multalin.toulouse.inra.fr/multalin/multalin.html) of (a) enterocins B3A with MR10A and (b) enterocin B3B with MR10B.

Figure 3. Survivability of Caco-2 cells after 24 h of contact with antimicrobials at their MIC; enterocin B3A-B3B (0.064 $mg.ml^{-1}$) and nisin (1 $mg.ml^{-1}$).

Figure 4. Epifluorescence microscopy imaging of biofilm formation by *L. monocytogenes* 162 (A) and *L. monocytogenes* 162R (B).

Figure 5. Scanning electron microscope (SEM) of biofilm formation by *L. monocytogenes* 162 R (A), upon treatment with nisin (16 mg. ml^{-1}) (B), with enterocin B3A-B3B (0.064 mg. ml^{-1}) (C) and with combination of nisin/enterocin B3A-B3B a(4/0.008 mg. ml^{-1} respectively) (D).

Fig. 1

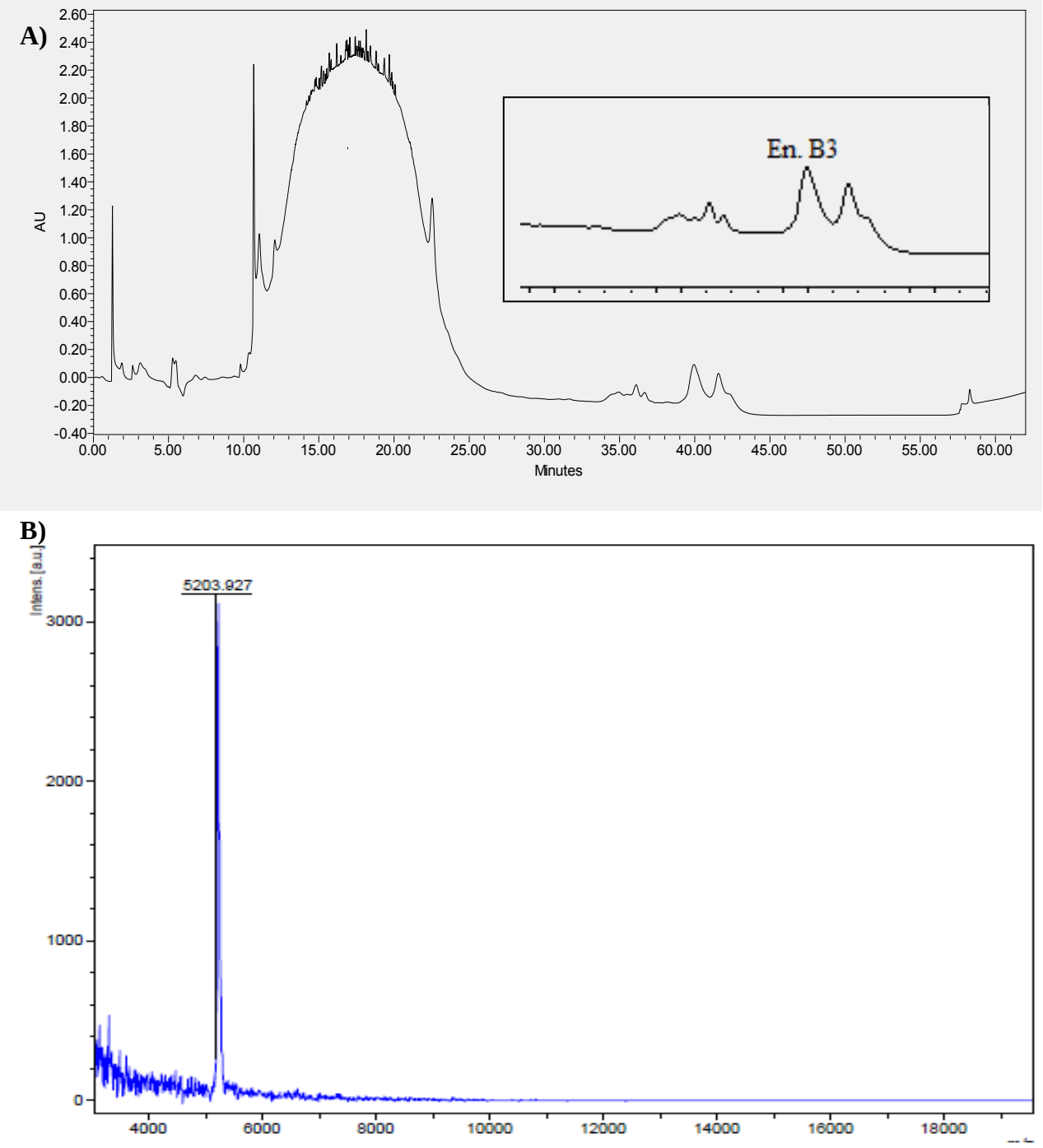
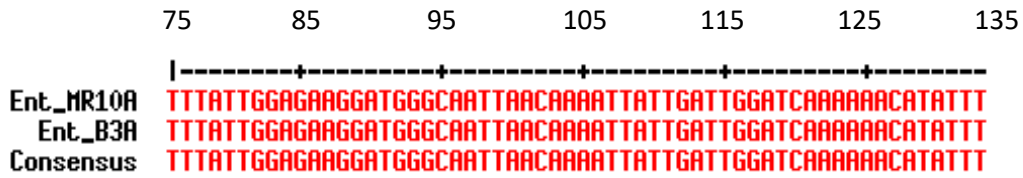


Fig. 2

A)



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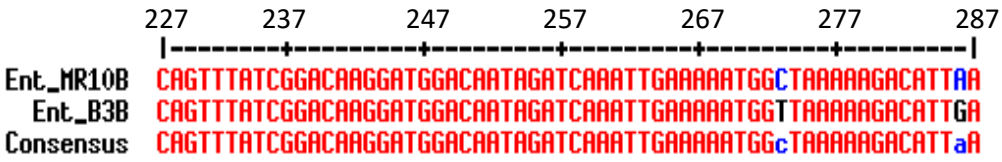


Fig 3

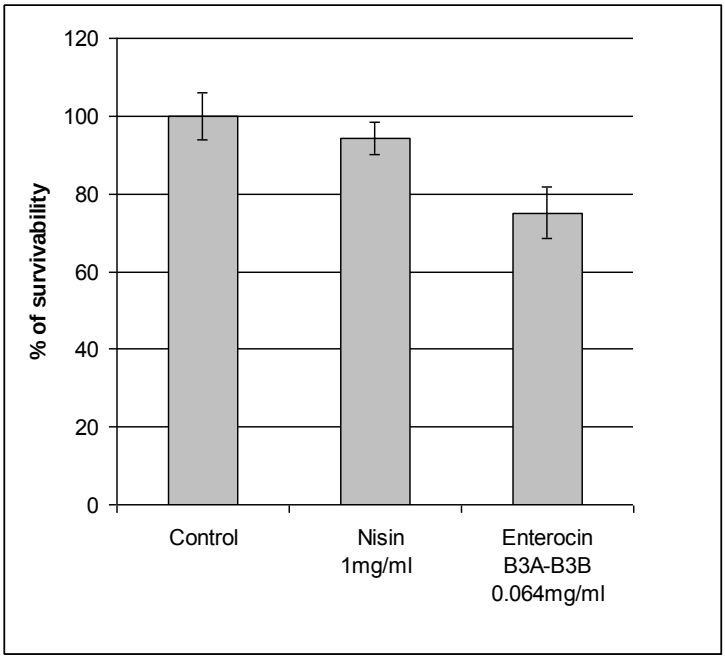
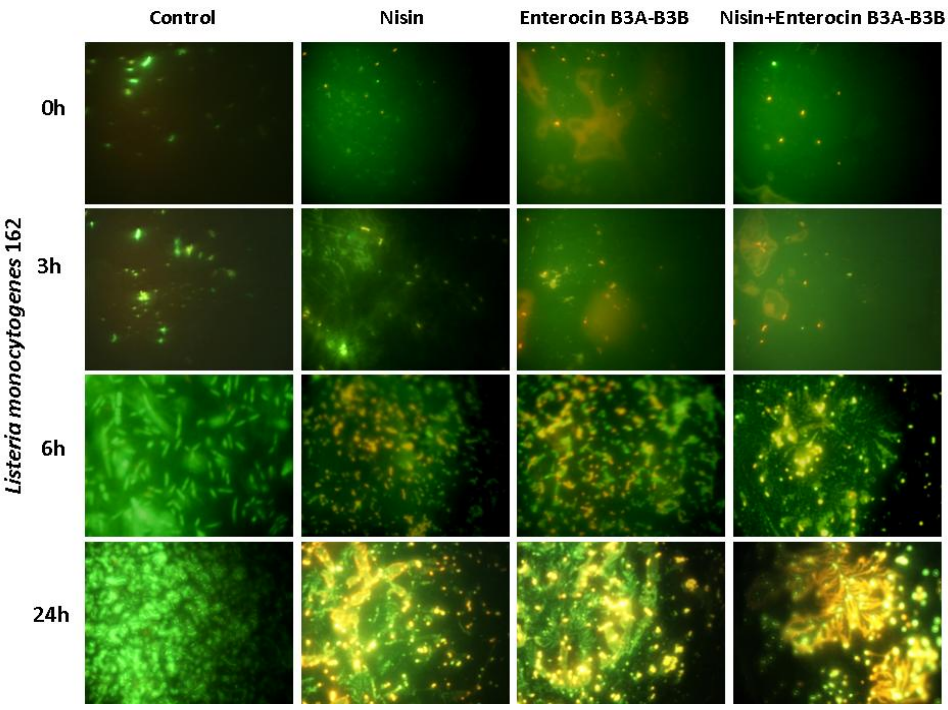


Fig. 4

(A)



(B)

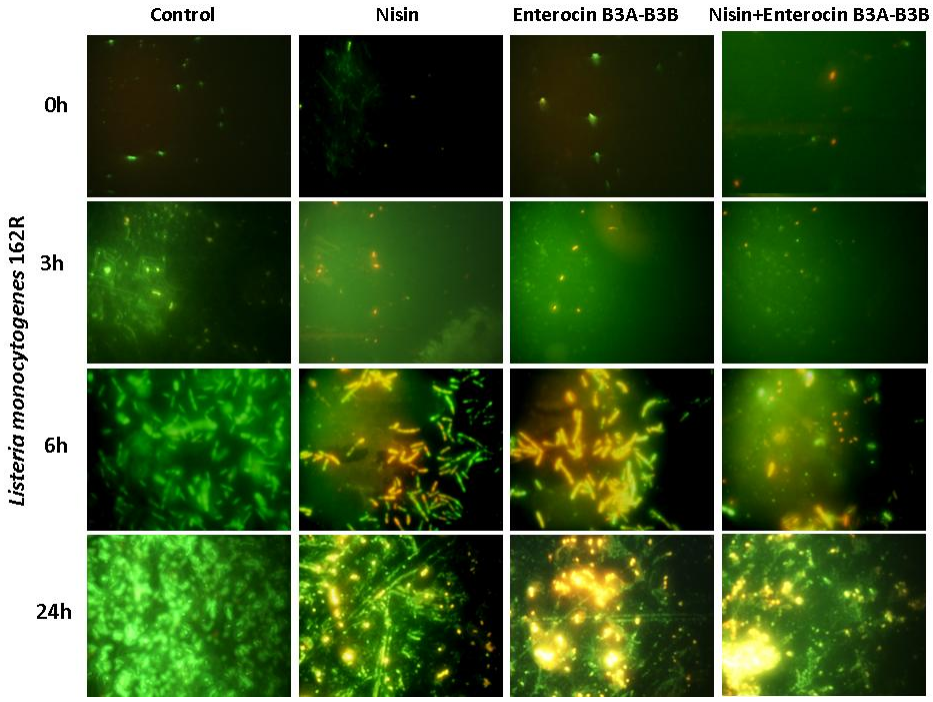
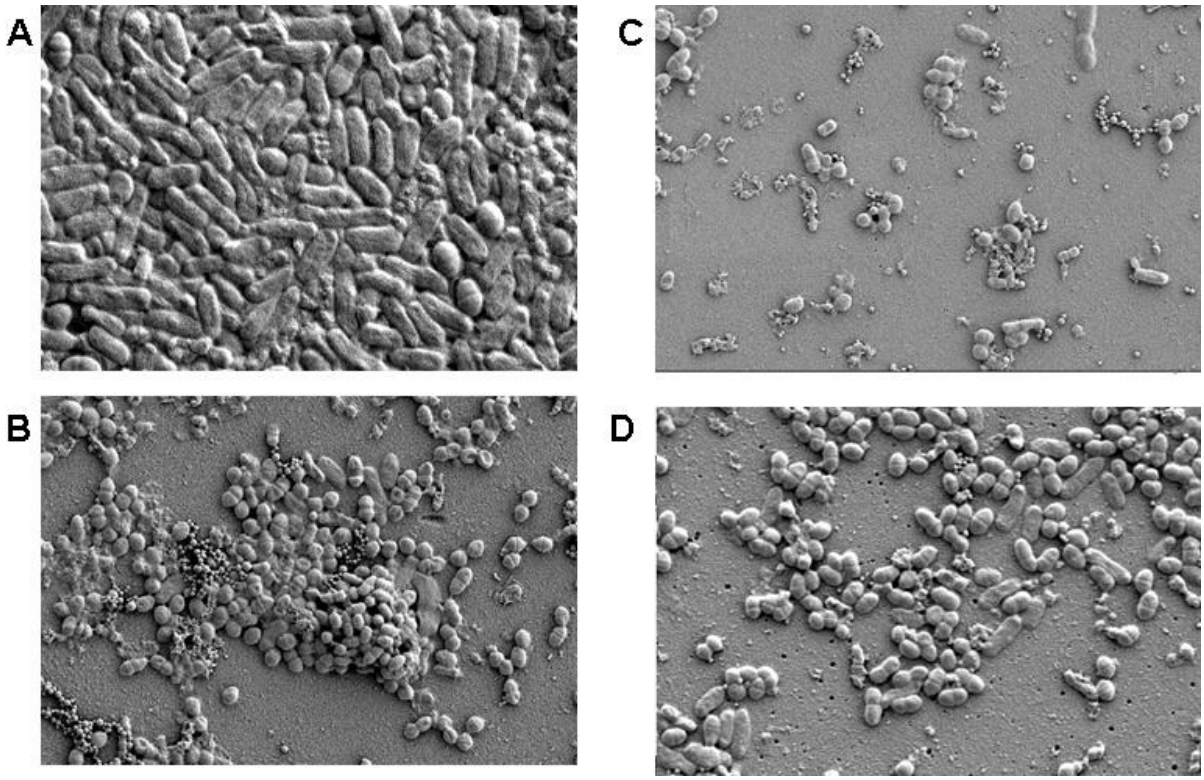


Fig. 5



Chapter 4. Bacteriocinogenic LAB from the feces of Iraqi infants and their potential as probiotics

This chapter is aimed at isolation of bacteriocinogenic LAB with probiotic properties from the fecal material of Iraqi infants living in France. Six feces samples were obtained from different children ranging from one to ten years old. Afterward, one gram of each sample was re-suspended in 9 ml of a saline solution (Trypton Salt Broth 0.09%). A series of dilutions from 10^{-1} to 10^{-7} were performed and plated onto de Man–Rogosa–Sharpe (MRS) agar Sigma Aldrich, (Germany). The plates were incubated in a CO_2 jar at 37°C for 48 hours. The obtained isolates were maintained in 20% glycerol at -80^{o}C until use. Among more than 500 selected colonies on MRS agar from the six different donors, 70 colonies were Gram- positive bacteria and catalase-negative activity, 41 of the isolates contained 41 cocci and 29 bacilli. Thus, they were considered as presumptive LAB. The distribution of the colonies was as set forth in **Table 10**.

Table 10. Numbers of LAB isolates in the fecal samples of Iraqi infants

Fecal samples	LAB isolates
1	6
2	5
3	9
4	5
5	15
6	30

Only two strains with antagonistic activities due to bacteriocin production were obtained and then identified as *E. faecalis* B3A-B3B and *E. faecalis* B20A-B20B by 16S rDNA sequencing. Their bacteriocins were designated as enterocin B3A-B3B and enterocin B20A-B20B (see **chapter 3**).

Despite the fact that enterococci are core members of the commensal intestinal microbiota, they were listed among the most prevalent multidrug-resistant hospital pathogens worldwide [Van Tyne et al. 2013]. About a dozen putative virulence factors have been reported for enterococci which contribute to a variety of infections, including endocarditis, sepsis, and urinary tract infections [Rathnayake et al. 2012; Anderson et al. 2016]. Cytolysin is criminated as the major risk factor involved in the pathogenicity of *E. faecalis* such as bacteremia [Hansen et al. 2015], endophthalmitis [Ozcimen et al. 2016], endocarditis [Dubé et al. 2012], and intraperitoneal infection [Muller et al. 2015]. Cytolysin production is a variable trait among *E. faecalis* isolates [McBride et al. 2007]. The operon of cytolysin is comprised of six genes responsible for toxin biosynthesis and two divergently-transcribed genes encoding regulatory proteins [Pham et al. 2014]. This operon is found either in pheromone-responsive plasmids or within the chromosomal pathogenicity island [Van Tyne et al. 2014]. A general scheme for cytolysin production, processing, secretion, and regulation is displayed in **Figure 20** [Van Tyne et al. 2013].

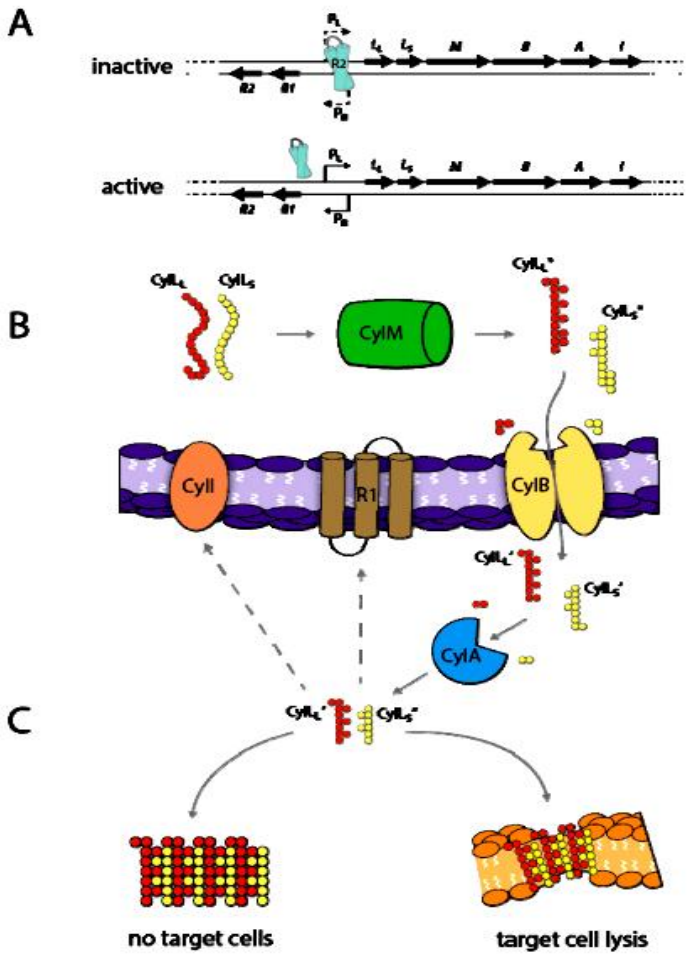


Fig.20. *E. faecalis* cytolysin expression. (**A**) Cytolysin operon in the inactive and active states. In the inactive state, CylR2 binds to the PLys (PL) promoter. Auto-induction via quorum sensing triggers an inferred change in the binding of the cytolysin promoter by the CylR2 protein, resulting in high-level expression of the cytolysin operon. (**B**) Cytolysin processing and secretion. Large and small subunits are post-translationally modified by CylM, secreted and trimmed by CylB, and further processed by CylA. (**C**) Cytolysin activity, in the absence and presence of target cells. In the absence of target cells the subunits form inactive and insoluble multimeric complexes. In the presence of target cells they coordinate to form a pore in the target cell membrane [Van Tyne et al. 2013].

Before investigating probiotic properties for the *Enterococcus faecalis* B3A-B3B strain, we studied its pathogenicity and revealed the presence of possible virulence genes, hemolytic activity, cytotoxicity towards human intestinal epithelial cell line Caco-2, pro-inflammatory activity, and antibiotic resistance. Further DNA-PCR analyses revealed that the *E. faecalis* B3A-B3B strain is missing gene-coding cytolysin, endocarditis antigen, and hemolysin, whilst other genes coding for *gelE, cpd, efaAfm, ccf, agg, and cob* were present (**Table 11)**.

Table 11. Virulence genes present in *E. faecalis* B3A-B3B strain

Virulence genes	Result of the PCR	Encoded virulence factor
cylA	-	Cytolysin activator
*cyl*B	-	Cytolysin
cylM	-	Cytolysin synthetase
gelE	+	Gelatinase
Esp	-	Enterococcal surface protein
Agg	+	Aggregation substance
efaAfs	-	Enterococcal endocarditis antigen
efaAfm	+	Enterococcal surface antigen
Cpd	+	Sex pheromone
Cob	+	Sex pheromone
Ccf	+	Sex pheromone
Ace	-	Collagen binding protein

Nevertheless, the presence of the *gelE, cpd, efaAfm, ccf, agg, and cob* putative virulence genes on the genome of *E. faecalis* B3A-B3B does not hinder its potential applications. In direct line, many *Enterococcus* species that are used as starters or naturally inhabited the traditional fermented foods and mammalian milk (even human milk), were also reported to harbor such types of virulence genes [Gelsomino et al. 2004; Drahovská et al. 2004; Moraes et al. 2012; Jiménez et al. 2013].

Enterococci are resistant to several commonly used antibiotics [Gilmore et al. 2014]. They exhibit either intrinsic resistance (naturally found within the genome of the species), or acquired resistance (via acquisition of exogenous genes or through sporadic mutations to intrinsic genes) [Hollenbeck and Rice, 2012]. The resistance of *Enterococcus* spp. to ampicillin is related to the expression of penicillin-binding proteins (PBPs) as (PBP4 in *E. faecalis*, and PBP5 in *E. faecium*) which show low affinity for β-

lactams [Hollenbeck and Rice, 2012]. They also exhibit low-level resistance to aminoglycosides such as gentamicin or streptomycin, throughout the low uptake of these highly polar molecules [Arias and Murray, 2012], whilst high-level resistance to these aminoglycosides results from the acquisition of aminoglycoside-modifying enzymes, or due to the ribosomal mutations that is caused by altered target binding [Hollenbeck and Rice, 2012]. Resistance to glycopeptides such as vancomycin belongs to the reduced vancomycin-binding affinity, involving modifications in the peptidoglycan synthesis pathway [Gilmore et al. 2014]. Moreover, enterococci show resistance to the streptogramin quinupristin–dalfopristin (Q–D) throughout several pathways including: (i) drug modification (through virginiamycin acetyltransferase (Vat)), (ii) drug inactivation (via virginiamycin B lysase (Vgb)), (iii) drug efflux (by the ATP-binding cassette protein macrolide–streptogramin resistance protein (MsrC)) [Arias and Murray, 2012]. The resistance of enterococci towards oxazolidinone linezolid is rare, but the most common mechanism is the modification of the 23S rRNA ribosome-binding site [Long and Vester, 2012; Gilmore et al. 2014]. *E. faecalis* exhibits resistance to the lipopeptide Daptomycin owing to the altered interactions with the cell membrane, which require the membrane protein LiaF and enzymes charged with phospholipid metabolism such as cardiolipin synthase (Cls) and glycerophosphoryl diester phosphodiesterase (GdpD) [Arias and Murray, 2012]. Nevertheless, the mechanisms discussed above are summarized in **Figure 21.**

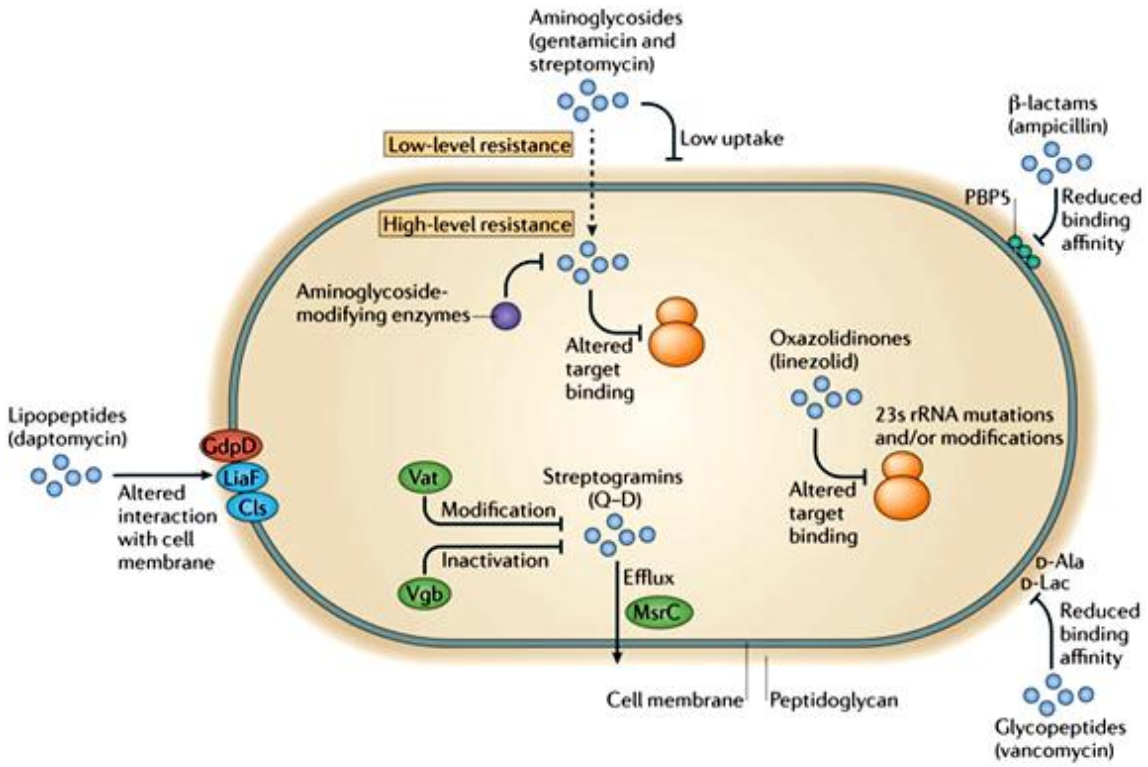


Fig. 21. The main mechanisms of *Enterococcus* spp. antibiotic resistance [Arias and Murray, 2012].

In direct line, we investigated the antibiotic susceptibility of the strain *E. faecalis* B3A-B3B. The test was achieved by three independent methods: disk diffusion method, minimal inhibitory concentrations (MICs) using E test (Bio-Mérieux, France), and VITEK 2 system (Bio-Mérieux, France). The antibiotic sensitivity tested towards the most important antibiotics including ampicillin, gentamicin, kanamycin, streptomycin, levofloxacin, moxifloxacin, erythromycin, clindamycin, linezolid, teicoplanin, vancomycin, tetracycline, nitrofurantoin chloramphenicol, and trimethoprim sulfamethoxazole. The strain *E. faecalis* B3A-B3B was sensitive to most of the antibiotics used, and no vancomycin resistance was noticed (**Table 12**).

Table 12. Antibiotic susceptibility of *E. faecalis* B3A-B3B in comparison with a pathogenic strain *E. faecalis*ATCC29212

Antibiotics MIC (mg/l)	***E. faecalis* B3A-B3B**	***E. faecalis* ATCC29212**
Ampicillin	S (<= 2)	S (<= 2)
Gentamicin (high level)	S	S
Kanamycin (high level)	S	S
Streptomycin (high level)	S	S
Levofloxacin	S(1)	S(0.5)
Moxifloxacin	S(<= 0.25)	S(<= 0.25)
Clindamycin	R(>=8)	R(>=8)
Linezolid	S(2)	S(2)
Teicoplanin	S(<= 0.5)	S(<= 0.5)
Vancomycin	S(1)	S(2)
Tetracycline	S(<= 1)	R(>=10)
Nitrofurantoin	S(<= 16)	S(<= 16)
Chloramphenicol	S(<= 4)	S(<= 4)
Trimethoprim/sulfamethoxazole	R(>=10)	R(>=10)
Erythromycin	I(4)	I(4)

R, Resistant; S, Sensitive; I, Intermediate; MIC, Minimal inhibitory concentration determined by Vitek 2 system.

Afterward, we shed light on the whole sequenced genome of *E. faecalis* B3A-B3B strain by *in silico* analyses tools to reveal the genetic similarities to probiotic *E. faecalis* Symbioflor 1 [Domman et al. 2007] or clinical strains MMH594 [Huycke et al. 1991] and V583 [Paulsen et al. 2003]. *In vitro* tests revealed that the *E. faecalis* B3A-B3B strain was safe for human intestinal epithelial cells, as no cytotoxic or pro-inflammatory effects were observed. This strain exhibited high surviving abilities under harsh conditions simulating the GIT. The *E. faecalis* B3A-B3B strain showed good hydrophobicity and auto aggregation rates as well as high adhesion capabilities with human epithelium intestine Caco-2 cells. Moreover, it demonstrated an encouraging capability of assimilating cholesterol.

In conclusion, the whole genome analysis of the *E. faecalis* B3A-B3B strain seems to be virtually similar in some aspects to the clinical strains MMH594 and V583 rather than the well-known probiotic *E. faecalis* Symbioflor 1. Nevertheless, the experimental data emphasized an absence of cytotoxicity to Caco-2 cells, a pro-inflammatory effect, and hemolytic activity. Besides the absence of these major depressing effects, the B3A-

B3B strain showed slight intrinsic resistance towards some antibiotics (Clindamycin and Trimethoprim/sulfamethoxazole) and an insignificant presence of gene coding for virulence factors like *gelE, cpd, efaAfm, ccf, agg, and cob* which naturally exist in most food-based enterococcal isolates. In addition to production of antibacterial compounds (shown in **chapter 3**), the B3A-B3B strain resisted the simulated harsh conditions of the GIT, displayed a good adhesion score, and showed a promising ability to reduce cholesterol. Considering this data, we can optimistically suggest the strain *E. faecalis* B3A-B3B for further probiotic applications.

Enterococcus faecalis B3A-B3B: Genome analysis and experimental evidences to claim its beneficial attributes

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ABSTRACT

In this study, we evaluated the probiotic potential of the bacteriocinogenic *Enterococcus faecalis* B3A-B3B recently isolated form healthy Iraqi infants and characterized for its antagonism potency. The whole genome was sequenced, analyzed and compared to those of clinical strains *E. faecalis* MMH594 (Huycke *et al.,* 1991), *E. faecalis* V583 (Paulsen *et al.,* 2003) and *E. faecalis* OG1RF from human origin (Bourgogne *et al.,* 2008) and probiotic *E. faecalis* Symbioflor1 strain (Domann *et al.,* 2007). Although carrying six genes coding for virulence factors (*gelE, cpd, efaAfm, ccf, agg, and cob*), B3A-B3B strain was nonetheless non-cytotoxic, non-hemolytic, devoid of inflammatory effects and sensitive to most antibiotics tested except for clindamycin and trimethoprim, which intrinsic resistance was also reported for the pathogenic strain *E. faecalis* ATCC29212. In turns, B3A-B3B strain displayed interesting hydrophobicity, auto-aggregation, adhesion to human Caco-2 cells, and survival in simulated gastrointestinal conditions, and cholesterol assimilation. The data gathered claim the beneficial effects for *E. faecalis* B3A-B3B, and the possibility of using this strain as probiotic.

1. Introduction

Lactic acid bacteria (LAB), which are considered as Generally Recognized As Safe organisms are widespread and ubiquitous frequently isolated from different sources such as plants, fermented foods, humans and animals (Klaenhammer *et al.*, 2005). They constitute a substantial part of human and animal microbiota, and are especially abundant in the gastrointestinal (GIT) and genitourinary tracts along with other bacteria species and microorganisms (Aureli *et al.*, 2011; Barinov *et al.*, 2011). Enterococci are doubtless part of LAB group with capabilities to grow under the presence bile salts up to 40%, temperatures ranging from 10 to 45°C, NaCl concentration up to 6.5% and pH 9.6 (Sherman, 1937; Teixeira and Facklam, 2003). Enterococci are Gram-positive bacteria that are found in plants, soil and as commensals of the gastrointestinal tract of humans, animals, and insects (Guzman *et al.*, 2016). The GIT of animals was reported as the largest reservoir of enterococci (Gilmore *et al.*, 2013). Enterococci have emerged during the last decades as the main cause of healthcare-associated infections around the world (Arias and Murray, 2012), constituting the third main cause of infective endocarditis (IE) worldwide (Pericás *et al.*, 2015). Vancomycin-resistant enterococci (VRE) are major opportunistic pathogens in immunocompromised populations. VRE can easily grow and colonize the gastro-intestinal tract of patients. Management of VRE by anti-biotherapy stands as a challenge because of a possible dysbiosis effect, which could allow persistence of VRE in the GIT (Crouzet *et al.*, 2015). Within 54 *Enterococcus* species (Parte, 2014), *E. faecalis* and *E. faecium* are clinically most relevant species with incrimination as endocartidis, urinary tract infections, meningitis, bacteremia and wound infections agents (Anderson *et al.*, 2016; Domann *et al.*, 2007).

Even LAB are considered as GRAS, the status of Enterococci remains unclear. Enterococci were isolated from different foodstuffs and used as artisanal starters (Franz *et al.*, 2011; Jamet *et al.*, 2012). Nonetheless, their food application was subjected to the absence of genes coding for virulence factors and antibiotic resistance (De Vuyst *et al.*, 2001). These criteria were refuted because of Enterococci ease abilities to acquire virulence and antibiotic resistance genes mainly through mobile genetic elements (Franz *et al.*, 2011). Thus the suitability of Enterococci in foodstuffs is a unsolved question.

On other hand, their use as probiotics may result in some advantages. Probiotics are defined as live microorganisms that, when administered in adequate amounts, confer a health benefit to the host (FAO/WHO, 2002). Indeed, they can be used to prevent intestinal overgrowth or colonization by bacterial pathogens (Buffie and Pamer, 2013). Enterococci strains with specific health claims including *E. faecalis* Symbioflor 1 (Domman *et al.*, 2007), *E. faecalis* DSM 16440 (Enck *et al.*, 2008), *E. faecalis* PC1.1 (Cuív *et al*,. 2013) and *E. faecalis* EC12 (Sakai *et al.* 2006) have been reported. The industrial use of enterococci as probiotics or starters appears clearly as controversial topic, which is complicated by the absence of official recommendations and specific guideline. In the absence of such obvious criteria, the discrimination of *Enterococcus* species for their positive and negative impacts has to be strain dependent (Lauková, 2011). In direct line, we established in this study the probiotic related features vs. pathogenic functions of *E. faecalis* B3A-B3B strain, recently isolated from feces of healthy Iraqi infants. This analysis was then strengthened by the whole genome of this strain to address its probiotic portrait.

2. Materials and Methods

2.1. Genomic analysis

Alignment of genomes of *E. faecalis* B3A-B3B, isolated during this study, with published whole genomes sequences of a probiotic strain *E. faecalis* Symbioflor1 (Domann *et al.*, 2007), clinical isolates *E. faecalis* MMH594 (Huycke *et al.*, 1991), *E. faecalis* V583 (Paulsen *et al.*, 2003) and *E. faecalis* OG1RF from human origin (Bourgogne *et al.*, 2008), was done. *E. faecium* NRRL B-2354 was included as out-groups for the phylogenetic tree construction using MEGA 5.0 software with appropriate definitions.

2.2. Bacteria used and their growth conditions

E. faecalis B3A-B3B and *E. faecalis* ATCC29212 were maintained as frozen stock cultures in de Man Rogosa Sharpe broth (MRS, Sigma-Aldrich) (de Man *et al.*, 1960) containing 20% (v/v) glycerol. They were grown on MRS agar plates at 37°C for 24 h to use for the further experiments.

2.3. Adverse effects of *E. faecalis* B3A-B3B

2.3.1. Antibiotic Susceptibility

Antibiotic susceptibility was achieved by three independent methods: disk diffusion method, minimal inhibitory concentrations (MICs) using E test (Bio-Mérieux, France), VITEK 2 system (Bio-Mérieux, France) equipped with ASTP606 card. The antibiotics tested included ampicillin, gentamicin, kanamycin, streptomycin, levofloxacin, moxifloxacin, erythromycin, clindamycin, linezolid, teicoplanin, vancomycin,

tetracycline, nitrofurantoin chloramphenicoland trimethoprimsulfamethoxazole. Antibiotic susceptibility interpretation was realized according to the recommendations of the French Committee on Antimicrobial Susceptibility Testing (CA-SFM 2013).

2.3.2. Genes coding for virulence factors

Total DNA of the strain *E. faecalis* B3A-B3B was used for PCR amplification of the genes responsible for cytolysin (*cylA, cylB and cylM*), gelatinase (*gelE*), enterococcal surface protein (*esp*), aggregation substance (*agg*), cell wall adhesins (*efaAfs and efaAfm*), sex-pheromones (*cpd, cob and ccf*),and the collagen adhesin (*ace*) according the procedures described by (Eaton and Gasson, 2001; Duprè *et al.*,2003; Hickey *et al.*,2003; Zoletti *et al.*, 2011). Samples were subjected to a cycle of denaturation (94°C for 1 min), annealing (at an appropriate temperature for 1 min) and elongation (72 °C for 1 min) for 35 cycles using primers and annealing temperatures listed in table 1. Electrophoresis was carried out at 100 V for 2h using 1× Tris–borate–EDTA to separate the PCR products on a 1.0 % agarose gel labeled with 0.5 % (v/v) GelRed. The gels were visualized by a Gel-Doc 2000® (Bio-Rad, Hercules, CA, USA).

2.3.3. Hemolytic activity

E. faecalis B3A-B3B (isolated from feces of Iraqi healthy child) and *E. faecalis* ATCC29212 strains were grown overnight in Brain heart infusion agar (BHIA) (Sigma-Aldrich) at 37°C were streaked on Columbia agar (Biokar Diagnostics, France), supplemented with 5% horse blood (BioMérieux; Marcy l'Etoile, France). Agar plates were incubated at 37°C for 48 h under aerobic conditions. The hemolytic activity was determined by observation of a clear zone around the colonies (ß-hemolysis), a partial

hydrolysis with green zone (α-haemolysis), or absence of clear zone (γ-hemolysis) (Semedo *et al.*, 2003).

2.4. Probiotic related features

2.4.1. *In vitro* adhesion assay on human intestinal epithelial cell line Caco-2

Adhesion assessment was performed according to Nueno-Palop and Narbad, (2011). Thus, the monolayers of the Caco2 cells were prepared in 24-wells tissue culture plates. Overnight bacterial culture was harvested by centrifugation, resuspended in DMEM cell culture medium without serum and antibiotics, and then applied on confluent Caco2 cells monolayers (10^7 CFU/well). After 2 h of incubation at 37°C, 5% CO_2, monolayers were washed twice with PBS to remove non adherent bacteria, and then lysed by incubation for 15 min with 0.1% Triton X100. Lysates were diluted and plated onto appropriate agar medium to determine the number of adherent bacteria.

2.4.2. Autoaggregation Assay

Autoaggregation test was performed according to Del Re et al. (2000). The *E. faecalis* B3A-B3B strain was grown on MRS for 18 h at 37°C, afterward cells were recovered by centrifugation (5,000 × g, 4°C, 15 min), washed three times with phosphate saline (PBS) buffer (pH7.0) and re-suspended in sterile PBS to get a viable cell count of 10^8 CFU/ml. Precisely 4 ml of the bacterial suspension were mixed by vortexing for 10 seconds and maintained at room temperature for 0, 1, 2, 3, 4, and 5 h. At each period of time, 0.1 ml of the upper suspension was transformed to another tube containing 3.9 ml of PBS and the absorbance (*A*) was measured at 600 nm. The percentage of autoaggregation was expressed as the following formula:

$$[(A_0 - A_t) / A_0] \text{ X } 100 = \% \text{ autoaggregation}$$

Where A_t represents the absorbance at time t=1, 2, 3, 4, 5h and A_0 is the absorbance at t= 0.

2.4.3. Hydrophobicity

Hydrophobicity was performed using the protocol previously reported by Rosenberg *et al.* (1980). *E. faecalis* B3A-B3B strain was cultured in MRS broth at 37°C for 18 h. Cells harvested by centrifugation (5,000 × g, 4°C, 15 min), were washed twice with PBS (pH 7), re-suspended in the same solution to approximately 10^8 CFU/ml and the absorbance (A_0) at 600 nm was measured. One milliliter of xylene (Fluka, Germany) was added to 3 ml of bacterial cell suspension and vortexed for 2 min after 10 min of incubation at room temperature. The aqueous phase was removed after 2 h of incubation at room temperature and the absorbance (A_t) at 600 nm was determined. The percentage of hydrophobicity was calculated depending on the formula below:

$$[(A_0 - A_t) / A_0] \text{ X } 100 = \% \text{ hydrophobicity}$$

Where A_t represents the absorbance at time t= 2 h and A_0 is the absorbance at t= 0.

2.4.4. *In vitro* survival in gastric and intestinal juice

The survival ability in gastric and intestinal juice was evaluated according to Charteris *et al.* (1998) with some modifications. Briefly, the cultures of *E. faecalis* B3A-B3B was grown in MRS broth for overnight at 37°C. Cells harvested by centrifugation (3,000×g, 10 min, 4 °C), were washed three times in PBS (pH7) and inoculated at a concentration of 10^6 CFU/ml into simulated human gastric aqueous solution containing 3 g/l pepsin (Sigma-Aldrich) and 5 g/l NaCl (Sigma-Aldrich) at pH of 2.0 or 3.4. These solutions were sterilized by filtration through 0.22 µm Millipore filter. Human intestinal simulated juice was prepared by mixing 1g/l pancreatin (Sigma-Aldrich) and 5 g/l NaCl (Sigma-

Aldrich) at pH 8.0, in addition of 0.3 or 3.0 % w/v of bile extract (Sigma-Aldrich) as described by Melgar-Lalanne *et al.* (2014). The viable cells of *E .faecalis* B3A-B3B were determined by plating on MRS agar after 1, 2, 3, and 4 h of incubation at 37°C. The surviving of bacterial cells was calculated according to the formula given below:

The log survival = (log N/log N0) X 100, where N is count (CFU/ml) after incubation, N0 is count (CFU/ml) at time 0.

2.4.5. Cholesterol assimilation assay

The measurement of cholesterol was performed as previously reported Lavanya *et al.* (2011). Briefly, *E. faecalis* B3A-B3B was grown in MRS broth supplemented with 0.3% bile salts. Then a solution of ethanol (10 mg of cholesterol dissolved in 500µl of ethanol) was added to 100 ml of MRS broth containing bile salts. After incubation of cultures at 37°C for 24h, cells were harvested by centrifugation (8,000× g, 10 min, 4°C). The supernatant was used to determine the amount of cholesterol. The non-inculcated broth was used as control. 3ml of ethyl alcohol (95%) was added to 1ml of supernatant followed by 2ml of potassium hydroxide and the contents were mixed after addition of each component. Afterwards, the tubes were heated for 10min at 60°C in water bath, after cooling; 5ml of hexane were added and tubes were vortexed for 5 min. Then, 3ml of water were added and thoroughly mixed. Tubes were incubated for 20min at 30°C to allow phases separation. After that 2.5ml of hexane layer was transferred onto a fresh tube and dried completely under chemical hood. Ferric chloride reagent (1.5 ml) was added and kept at room temperature for 10 min. 1 ml of sulphuric acid at 97% was added to each tube. After vortexing, the mixture was incubated for 45 min at 30°C. The optical

density (OD) was measured at 540nm in UV spectrometer (UVmini-1240, Shimadzu, Kyoto, Japan). A standard curve permitted to determine the cholesterol concentrations. The percentage of cholesterol assimilation was calculated by using the following formula:

$$\text{Assimilation}(\%) = \frac{\text{Conc. of cholesterol in control} - \text{Conc. of cholesterol in sample}}{\text{Con. of cholesterol in control}}$$

2.4.6. Inflammatory effect on human intestinal epithelial cell line Caco-2

Putative inflammatory effect of *E. faecalis* B3A-B3B was determined as described by Belguesmia *et al*, (2016). Caco-2 cells were cultivated in 24-well tissue culture plate for 48-72h. Then, obtained confluent monolayer was treated with IL-1β to induce inflammation. A second set of tissue culture plate was prepared in the same conditions but without inducing inflammation. After 24h of incubation, 10^8 CFU.mL^{-1} of *Enterococcus* strain were added on inflamed and non inflamed Caco2 cells and were left at 37°C with 5% CO_2 for further 24h. The levels of interleukins (IL-6, IL-8 and IL-10) produced by Caco-2 cells were measured in the culture supernatant using ELISA Quantikine kits (R&D Systems, USA).

2.5. Statistical analysis

Data are expressed as mean ± standard error (SE) calculated over three independent experiments performed in triplicate. SigmaPlot 11.0 Software (Germany) was used to carry out most statistical analyses on the data obtained after inflammation test. All statistical differences between groups were measured using one way-ANOVA, and

Tukey post-hoc Test. The differences between the means were considered significant when p value < 0.05 (n.s.: non significant).

3. Results

3.1. Genomic analysis displayed proximity to pathogenic Enterococci

The genome size of *E. faecalis* B3A-B3B was 2,887,406 bp with an average G+C content of 37.3%. As depicted on Figure 1, the phylogenetic tree was gathered upon genomes alignment with those of *E. faecalis* MMH584 and *E. faecalis* V583 strains.

3.2. Evidences on safety of *E. faecalis* B3A-B3B

E. faecalis B3A-B3B was sensitive to most of antibiotics except clindamycin and trimethoprim, which are defined as natural characteristics of Enterococci. Resistance to these antibiotics was also observed for pathogenic *E. faecalis* ATCC29212, which also was resistant to tetracycline (Table 2). Furthermore, genes coding for hemolysin and cytolysin were not amplified by PCR. Genome whole sequence analysis confirm lack of the genes coding these two compounds (data not shown). Nonetheless, genes *gelE, cpd, efaAfm, ccf, agg,* and *cob* were amplified (Table 3). As expected, no hemolytic activity on the blood agar plates, was observed conversely the pathogenic strain *E. faecalis* ATCC29212 which showed a clear zone around the colonies, advocating a □-hemolytic activity (data not shown).

Furthermore, *E. faecalis* B3A-B3B strain did not induce statistically significant secretion of IL-6 and IL-8 pro-inflammatory and IL-10 anti-inflammatory interleukins by human intestine Caco-2 cells, as the levels of the aforementioned interleukins measured along with B3A-B3B strain were similar to those measured for the control test (Fig. 3a,b).

3.3. *E. faecalis* B3A-B3B displayed quite similar cell-surface properties vs. the pathogenic *E. faecalis* ATCC29212

Adhesion of *E. faecalis* B3A-B3B on the human intestinal epithelial Caco2-cells was about 1.8×10^5 CFU/ml, whilst that of the pathogenic strain *E. faecalis* ATCC29212 was about 1.4×10^5 CFU/ml (Table 4). Furthermore, B3A-B3B strain autoaggregation percentage was 60.14±2.05%, which is slightly highest than that of the pathogenic strain *E. faecalis* ATCC29212, which was about 57.24±2.64%. The hydrophobicity scores of these strains were almost similar. The score of *E. faecalis* B3A-B3B was 47.62±1.00% while that of the pathogenic strain *E. faecalis* ATCC29212 was 44.85±2.33% (Table 4).

3.4. Surviving simulated gastric juice and intestinal juices and *in vitro* cholesterol assimilation

E. faecalis B3A-B3B strain showed low viability (5.06 %) after 3h of incubation in simulated gastric juice in fasting condition (pH 2), while no viable cells were observed after 4 h of incubation. Nevertheless, a high surviving ability was observed (90.81%) after 4 h of incubation in simulated gastric juice with higher pH value (3.4) simulating feeding state in the stomach. The surviving ability in simulated intestinal juice (pH 8) was also high with (92.67%) in supplemented one with 0.3% of bile extract and (91.51%) in supplemented one with 3.0% (Table 5). Moreover**,** *E. faecalis* B3A-B3B showed an ability to reduce the cholesterol of about 21 ± 0.033 % compared to the untreated control.

4. Discussion

E. faecalis B3A-B3B was recently isolated and characterized for production of class IIb bacteriocin with strong inhibition of tremendous pathogen *Listeria monocytogenes* grown either under planktonic and biofilm cultures (to be published elsewhere). Before claiming any further probiotic attribute for B3A-B3B strain, we investigated its probiotic related features and revealed its potential adverse effects such as its resistance to antibiotics, hemolytic activity, cytotoxicity towards mammalian cells, detection of genes coding for virulence factors and pro-inflammatory activity. Then, we looked at the sequenced whole genome of this strain and proceeded to *in silico* analysis to establish genetic relatedness with probiotic *E. faecalis* Symbioflor 1 (Domman *et al.,* 2007), or clinical strains MMH594 (Huycke et al., 1991) and V583 (Paulsen *et al.,* 2003). The resulting phylogenetic tree argues the human organism as ancestry of these distinct strains. Further B3A-B3B strain genome analyses indicated that B3A-B3B strain is missing genes coding hemolysin, cytolysin and endocarditis antigen, according to the genome analysis and the PCR assay. Nonetheless, other genes coding for *gelE, cpd, efaAfm, ccf, agg, and cob* were amplified by PCR. Nevertheless, the detection of these genes on B3A-B3B strain is not hampering its potential of application. Related to this, many Enterococci usable as starters or naturally present in traditional fermented cheeses and meats microbiota were also reported to carry these genes. Albeit, *E. faecalis* CP58 from GIT of human origin, was reported to harbor *efaAfs, gelE, agg, cpd, cob, ccf* and *cad* genes and display resistance to kanamycin and chloramphenicol, the authors claimed optimistically the use of this strain as probiotic (Nueno-Palop and Narbad, 2011). The genes coding for virulence factors is highly occurring in clinical strains. Drahovská *et al.* (2004)

underpinned the presence of genes *cylA* coding for hemolysin, *gelE* coding for gelatinase and *esp* coding for surface protein in Enterococci isolated from cheese. Importantly, 45 % of *E. faecalis* of clinical origin appeared to harbor the *cylA* gene, whilst it was only 22% in isolates of food origin (Drahovská *et al.*, 2004). Moreover the distribution of *gelE* and *esp* genes, was not significantly different in the clinical vs. food *E. faecalis* strains (Drahovská *et al.*, 2004). Gelsomino *et al.* (2004), showed that *Enterococcus* strains isolated from an Irish artisanal cheese were hosting at least two, as long is not more genes coding for virulence factors such as *agg*, *gel*, *cyl*, *esp*, *ace*, *efaAfs* and *efaAfm*. Moraes *et al.* (2012) portrayed the presence of *asa1* (100%), *gelE* (93%) and *efaA* (83.7%) in forty three *Enterococcus* strains isolated from raw milk and cheeses. Interestingly 53.5% of them were endowed with ß-hemolytic activity, conversely to B3A-B3B strain isolated and studied here. B3A-B3B strain is also devoid of pro-and anti-inflammatory properties, which are also added value regarding its safety. Ultimately, the resistance to antibiotics, there is not a major resistance which could breakdown the design of this strain as probiotic candidate.

In terms of probiotic related features, B3A-B3B strain exhibited good survival rates under conditions mimicking the GIT. This trait was reported for Enterococci, which are normal inhabitant of this ecosystem (Valenzuela *et al.*, 2008; Gu *et al.*, 2008).

Furthermore, auto-aggregation and cell surface hydrophobicity were depicted as key criteria for probiotic design (Del Re *et al.*, 2000; Collado *et al.*, 2007; Li *et al.*, 2015), as a relationship was mentioned between auto-aggregation and adhesion (Del Re *et al.*, 2000; Bujnakova *et al.*, 2004), and between adhesion and cell-surface hydrophobicity (Wadstrom *et al.*, 1987). B3A-B3B strain has overall good hydrophobicity and

autoaggregation scores, and also adhesion capabilities human epithelium intestine Caco2 cells through mechanisms, which need to be studied. The levels of adhesion to Caco-2 cells of B3A-B3B (1.8 10^5 CFU/ml) were quite similar to those of pathogenic *E. faecalis* ATCC29212 (1.4 × 10^5 CFU/ml), making a ratio of 1.8 and 1.4%, respectively. As the adhesion is strain dependant, highest or weakest values could be reported in the literatures.

Adhesion to the epithelial cells is a key parameter that is taken into account for probiotic. Probiotics adhesion onto intestinal cecum and epithelial cells prevent pathogens colonization by several mechanisms such as competitive exclusion, secretion of antimicrobial compounds (bacteriocins, organic acids etc..), competition for nutritive elements and stimulation of the immune responses (Scanlan, 1997; Doyle, 2001; Patterson and Burkholder, 2003; Mazmanian *et al*, 2008).

Inflammatory response is a fundamental immune reaction resulting after infections caused by "foreign" agents as viruses and bacteria (Reinoso Webb *et al.*, 2014). This mechanism is highly complex involving different signalization pathways and membrane receptors (Reinoso Webb *et al.*, 2014). The interactions between host cells and probiotic strains, should have a positive impact on the immunomodulation response, allowing to maintain homeostasis and integrity of the GIST (Perdigón *et al.*, 2001; Belkaid and Hand, 2014). As mentioned, B3A-B3B strain did not impact on the pro-inflammatory interleukins Il-6 and Il-8, nor on the anti-inflammatory Il-10, and its cytotoxicity on Caco-2 cells as conducted here confirmed the innocuity of this strain (data not shown). In our opinion, these criteria are seldom used to characterize Enterococci for their probiotic related features and should be here as meaningful insight. These aspects could be

considered as positive attributes in potential use of the *E. faecalis* B3A-B3B strain as probiotic. Ultimately, B3A-B3B strain provide another health claim, at least *in vitro*. Indeed, this strain was able to reduce, *in vitro*, cholesterol of about 20%. This interesting physiological property (Pereira *et al.*, 2002; Guo *et al.*, 2015) needs to be studied in vivo.

As a conclusion, the whole genome analysis of B3A-B3B strain permitted to shape a genetic proximity to clinical strains MMH594 and V583, rather than well known probiotic *E. faecalis* Symbioflor 1. Nonetheless, the experimental data pointed out the absence of both cytotoxicity to Caco-2 cells, and hemolytic activity. In addition to the absence these major adverse effects, B3A-B3B strain displayed negligible resistance to antibiotics and insignificant presence of genes coding for virulence factors. Besides production of antibacterial compounds (not shown in this study), B3A-B3B strain defied the simulated harsh conditions of the GIT and good adhesion level, health issue possibility as cholesterol lowering. Taken the data together, B3A-B3B strain, which could be proposed use to prevent pathobiont infections because they contribute to colonization resistance against specific intestinal pathogens.

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Table 1
PCR primers and the annealing temperatures used to detect the virulence genes in *E. faecalis* B3A-B3B.

Primer	Sequence(5'-3')	Product (bp)	MT (^{o}C)	Time (s)	Reference
cylA	f:TGGATGATAGTGATAGGAAGT r:TCTACAGTAAATCTTTCGTCA	517	53	30	Eaton and Gasson, 2001
*cyl*B	f:ATTCCTACCTATGTTCTGTTA r:AATAAACTCTTCTTTTCCAAC	843	51	30	Eaton and Gasson, 2001
cylM	f:CTGATGGAAAGAAGATAGTAT r:TGAGTTGGTCTGATTACATTT	742	52	30	Eaton and Gasson, 2001
gelE	f:ACCCCGTATCATTGGTTT r:ACGCATTGCTTTTCCATC	419	52	30	Eaton and Gasson, 2001
esp	f:TTGCTAATGCTAGTCCACGACC r:GCGTCAACACTTGCATTGCCGAA	933	62	30	Zoletti et al., 2011
agg	f:AAGAAAAAGAAGTAGACCAAC r:AAACGGCAAGACAAGTAAATA	1553	55	30	Hickey et al.,2003
efaAfs	f:GACAGACCCTCACGAATA r:AGTTCATCATGCTGTAGTA	705	55	30	Hickey et al.,2003
efaAfm	f:AACAGATCCGCATGA ATA r:CATTTCATCATCTGATAGTA	735	53	30	Eaton and Gasson, 2001
cpd	f:TGGTGGGTTATTTTTCAATTC r:TACGGCTCTGGCTTACTA	782	53	30	Hickey et al.,2003
cob	f:AACATTCAGCAAACAAAGC r:TTGTCATAAAGAGTGGTCAT	1405	51	30	Hickey et al.,2003
ccf	f:GGGAATTGAGTAGTGAAGAAG r:AGCCGCTAAAATCGGTAAAAT	543	57	30	Eaton and Gasson, 2001
ace	f:AAAGTAGAATTAGATCCACAC r:TCTATCACATTCGGTTGCG	320	56	30	Duprè *et al.*,2003

Table 2
Antibiotic susceptibility of antagonistic isolates

Antibiotics MIC (mg/l)	*E. faecalis* B3A-B3B	*E. faecalis* ATCC29212
Ampicillin	S (<= 2)	S (<= 2)
Gentamicin (high level)	S	S
Kanamycin (high level)	S	S
Streptomycin (high level)	S	S
Levofloxacin	S(1)	S(0.5)
Moxifloxacin	S(<= 0.25)	S(<= 0.25)
Clindamycin	R(>=8)	R(>=8)
Linezolid	S(2)	S(2)
Teicoplanin	S(<= 0.5)	S(<= 0.5)
Vancomycin	S(1)	S(2)
Tetracyclin	S(<= 1)	R(>=10)
Nitrofurantoin	S(<= 16)	S(<= 16)
Chloramphenicol	S(<= 4)	S(<= 4)
Trimethoprim/sulfamethoxazole	R(>=10)	R(>=10)
Erythromycin	I(4)	I(4)

R, Resistant; S, Sensitive; I, Intermediate; MIC, Minimal inhibitory concentration determined by Vitek 2 system.

Table 3
Virulence genes present in *E. faecalis* B3A-B3B strain

Virulence genes	Result of the PCR	Encoded virulence factor
cylA	-	Cytolysin activator
*cyl*B	-	Cytolysin
cylM	-	Cytolysin synthetase
gelE	+	Gelatinase
esp	-	Enterococcal surface protein
agg	+	Aggregation substance
efaAfs	-	Enterococcal endocarditis antigen
efaAfm	+	Enterococcal surface antigen
cpd	+	Sex pheromone
cob	+	Sex pheromone
ccf	+	Sex pheromone
ace	-	Collagen binding protein

Table 4
Adhesion of tested *E. faecalis* strains on Caco-2 cells

Strain	Autoaggregation%	Hydrophobicity %	Adhesion CFU/ml
E .faecalis B3A-B3B	60.14 ±2.05	47.62±1.00	1.8×10^5
E. faecalis ATCC29212	57.24±2.64	44.85±2.33	1.4×10^5

Table 5
Percentage of survival of *E. faecalis* B3A-B3B under simulated gut conditions

	Incubation Time (h)			
Gastric juice	1	2	3	4
Pepsine pH 2	17,95±0,208	11,40±0,157	5,06±0,213	0±0,000
Pepsine pH 3.4	95,82±0.260	94,98±0.074	91,96±0.260	90,81±0.137
Intestinal juice				
Pancreatine + Bile extract 0,3%	96,65±0.259	94,71±0.680	93,42±0.586	92,67±0.528
Pancreatine + Bile extract 3%	95,97±0.734	94,66±0.704	92,60±0.137	91,51±0.307

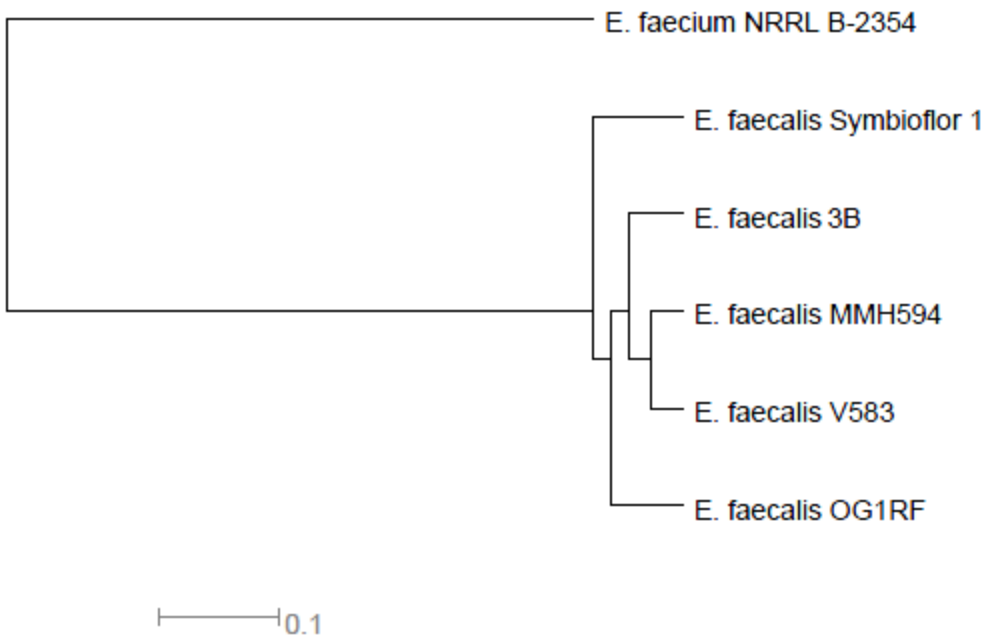


Fig. 1. Phylogenetic tree based on genomic comparison of *E. faecalis* B3A-B3B with other sequenced *E. faecalis* strains referenced in pubmed genome database

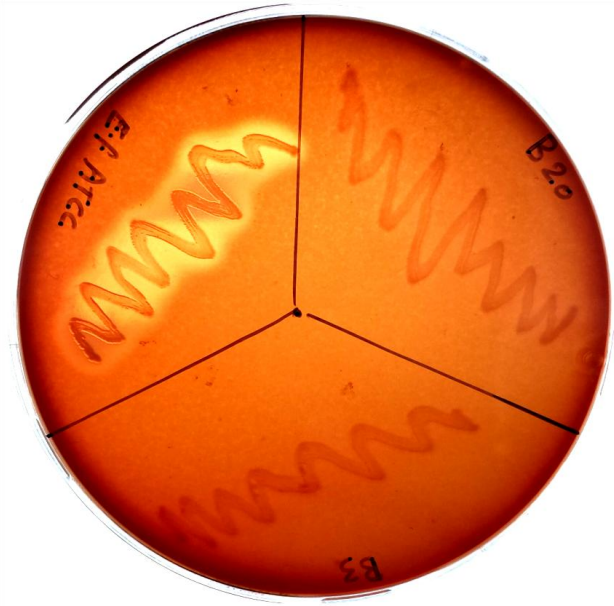
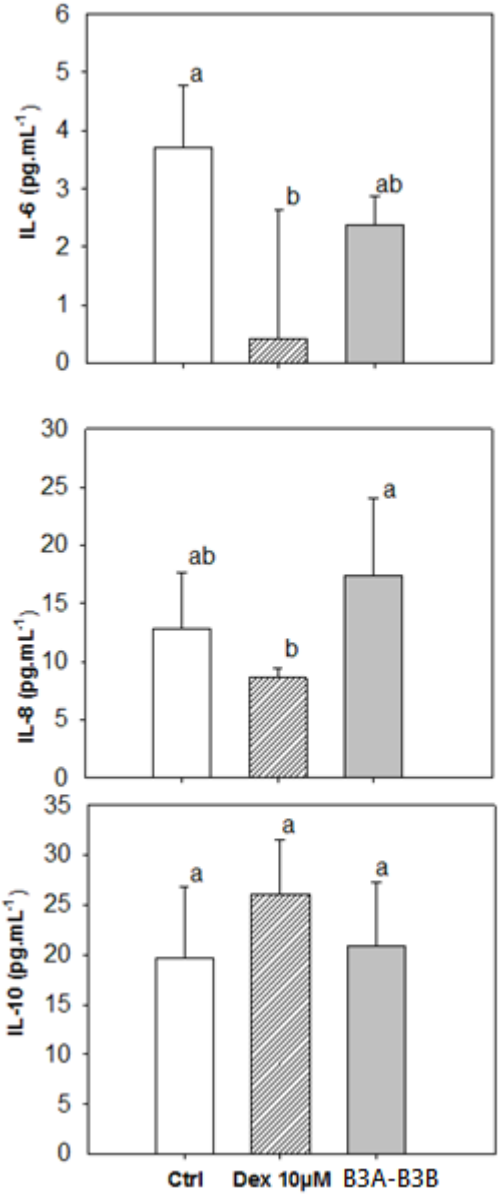


Fig. 2. Hemolytic activity of *E.faecalis* B3A-B3B, *E. faecalis* B20A-B20B and *E. faecalis* ATCC29212

A)



B)

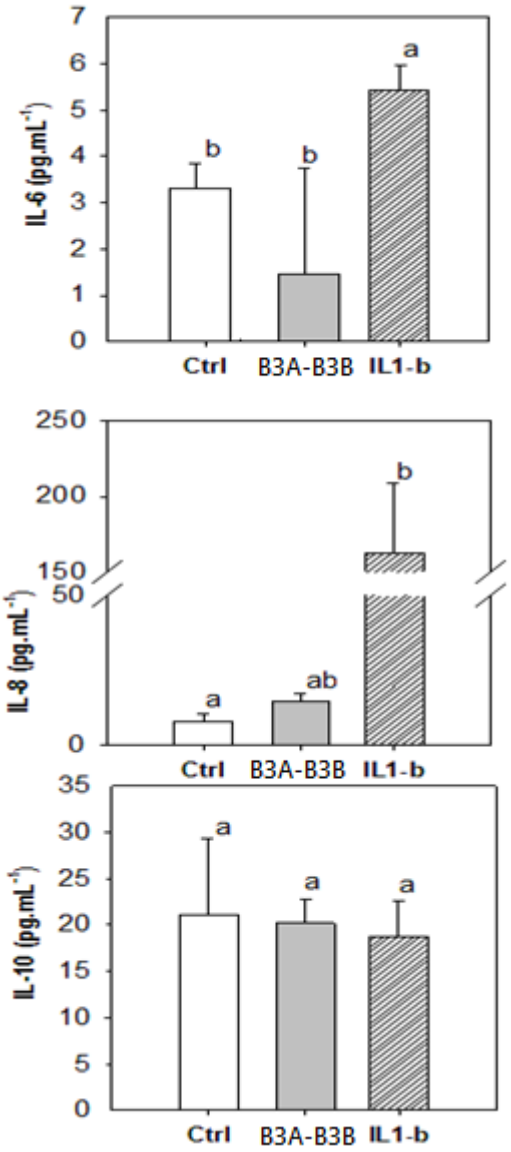


Fig. 3. Effect of *E. faecalis* B3A-B3B contact with Caco2 cells on secretion of pro-inflammatory interleukins IL-6, IL-8 and anti-inflammatory IL-10. **A**) Pre-inflammated Caco2 cells with IL-1β before contact with *E. faecalis* strains. Dexamethasone (Dex 10µM) was used here as positive control for anti-inflammatory effect **B**) Without pre-treatment of Caco2 cells. IL-1β was used as positive control for inflammation effect.. Values are means ± SD of three repeated measurements. Means without a common letter are different ($p < 0.05$) using one way ANOVA with Tukey Test for pairwise comparisons.

General Conclusions and Prospects

In this study, we focused on the isolation and characterization of antagonistic yeasts and LAB from different ecosystems in order to evaluate their probiotic potential and to characterize and purify the antimicrobial molecules produced by these strains - especially those with antilisterial effects. Due to the lack of information about the normal eukaryotic community of the human and animal gastrointestinal tract (such as fungi and protozoa) which are still not well investigated in contrast to the massive data concerning bacterial diversity, we started our project to get a better understanding of a particular part of this community by exploring the diversity of antagonistic yeasts in the fecal samples collected randomly from a farm located in the north of France. In this respect, our study could be the first look at the intestinal yeast diversity of broiler chicken in the Nord Pas de Calais, at least to the best of our knowledge.

Eighty one yeast strains have been identified by biochemical methods such as 32 ID systems in addition to identification by molecular methods 26S rDNA and ITS 5.8S rDNA. The phylogenetic analysis using the repetitive element palindromic PCR (rep-PCR) revealed twenty different groups that all belong to the genus *Candida* under the species of *Candida famata* which is the teleomorph of (*Debaryomyces hansenii*) which was also confirmed by MALDI-TOF identification technology for the strain of interest.

We concluded from these results that yeast belonging to the gastrointestinal tract of the broiler chicken loaded with their fecal material could be related to only one species, and it is not necessary to be more diversified as bacteria; that may also confirm the very low percentage of yeast populations in comparison with bacterial contents and diversity in the gastrointestinal tract, which is strictly governed by different determinants such as the dietary system, geographical location, antimicrobial uses and other factors as reported in the literature.

Moreover, only one strain among the identified fecal yeasts has been demonstrated to have an antilisterial effect. This motivated us to inspect the probiotic potential of this antagonistic strain. The safety aspects of this interesting antilisterial yeast, designated as *Candida famata* Y.5, were investigated by studying its hemolytic activity, adhesion, and cytotoxic effect on caco-2 intestinal epithelial cells. The results show that there is no observation recorded concerning hemolysin production and cytotoxic effect on Caco-2

cells. These results led to investigation of the surface properties, such as auto-aggregation and hydrophobicity, in addition to this yeast's ability to survive in the harsh environmental conditions of simulated gastrointestinal tract juices. The data obtained from these experiments demonstrated interesting capacities of auto aggregation and hydrophobicity as well as a high surviving capability under simulated gastric and intestinal conditions which enhance the probiotic potential of this fecal origin strain. This could be an encouraging basis for further perspectives to investigate its beneficial effects *in vitro* and *in vivo* experiments, including anti-viral and anti-fungal activity, anti-cancer potentials, and the ability to improve the immune system. In addition, an analysis of its whole genome to investigate antimicrobial structural genes may provide us with more knowledge about this strain for future studies. In direct line, antilisterial molecules were produced by microorganisms of fecal origin, and the second part of this study focused on the lactic acid bacteria isolated from fecal samples of healthy infants. The screening for antagonistic yeasts or fungal contents did not show any results after plating the samples on appropriate media. Out of seventy lactic acid bacteria obtained from six different donors, only two strains showed antilisterial activity after the neutralization of the cell-free supernatant (CFS). The biochemical tests, MALDI-TOF technology and 16S rDNA sequencing revealed that the two strains belong to the genus *Enterococcus* under the species *Enterococcus faecalis*. These results confirmed previous studies which reported the absence or deficiency of fungal contents in the infants' intestinal microbiota in comparison with bacteria, which, as mentioned previously, depend on different factors. Moreover, the data obtained by this study introduced the *Enterococcus faecalis* as a friendly intestinal inhabitant armed with antimicrobial defensive agents, and consequently confirmed the fecal materials of healthy individuals as a natural reservoir of antagonistic microbes.

Thus, we inspected the spectrum of the antimicrobial activity of these two strains, which were nominated as *Enterococcus faecalis* B3A-B3B and *Enterococcus faecalis* B20A-B20B. These two strains were active against important foodborne pathogens such as *Listeria monocytogenes*, *Clostridium perfringens*, *Staphylococcus aureus*, and methicillin resistant *Staphylococcus aureus* (MRSA).

Next, we purified and characterized the bacteriocin produced by the strain *E. faecalis* B3A-B3B. The biochemical and physical characterizations have been confirmed the proteinaceous nature, tolerance to the high and low pH values, and high thermo-stability of this bacteriocin that reinforce its favorable properties for industrial uses. To avoid the misidentification of the molecular mass of this bacteriocin caused by the interference between the polyethylene glycol, derived from Tween80 of MRS medium, and the analyte of interest, we proposed in this work an unfamiliar purification protocol by using the chloroform liquid–liquid separation instead of the classical precipitation of bacteriocins by ammonium sulfate. The mass spectrometry displayed a molecular mass of 5.203.927 Da that approximately agreed with the predicted mass 5.176.31 Da and 5.182.21 Da obtained from the sequencing of bacteriocin structural genes. This data revealed that this bacteriocin, designated as enterocin B3A-B3B, belongs to the class IIb (two peptide bacteriocins). The DNA sequence coding for these two peptides disclosed similarities with enterocins MR10A and MR10B. Enterocin B3A-B3B differ from enterocin MR10A and MR10B only in two nucleotides. Definitely, the 3'-region of the *entB3B* gene, the nucleotide C was replaced by T (position 273), while the nucleotide A was replaced by G (position 286), in comparison to the *entMR10B* gene. These differences did not change the final structure of amino acids.

In addition to the previous interesting technological features of enterocin B3A-B3B, we confirmed the non-cytotoxic effect on caco-2 intestinal epithelial cells which allowed us to study its potential as a bio preservative. The anti-biofilm effect of enterocin B3A-B3B has been evaluated alone and in combination with nisin against *Listeria monocytogenes* 162 and its derivative nisin-resistant variant. Biofilm formation on the AISI 304 stainless steel slides was significantly affected. The data revealed that about 1 mg/ml of nisin or 0.064 mg/ml of enterocin B3A-B3B alone inhibited the biofilm formation of *L. monocytogenes* 162 about 2 logs. The combination of nisin at its MIC value with enterocin B3A-B3B at 0.256 and 0.008 mg/ml showed a significant drop in the number of viable cells by about 2 logs also. The data gathered by this PhD work allowed us to present the enterocin B3A-B3B as an effective antimicrobial agent with a significant anti-biofilm activity against the nisin-resistant *Listeria monocytogenes* and other foodborne pathogens such as methicillin-resistant *Staphylococcus aureus.*

To configure the probiotic outline of the strain *E. faecalis* B3A-B3B, the whole genome was analyzed *in silico* and compared with other available genomes of clinical and probiotic *E. faecalis* strains. It appears that *E. faecalis* B3A-B3B shares some similarities with the clinical strains *E. faecalis* MMH594, *E. faecalis* V583, and *E. faecalis* OG1RF instead of the *E. faecalis* Symbioflor1 probiotic strain. Notwithstanding, the experimental studies of this bacteriocinogenic strain verified its probiotic properties. *E. faecalis* B3A-B3B was a non-hemolytic, non-cytotoxic, and non-inflammatory strain. The virulence factors of this strain (including *gelE, cpd, efaAfm, ccf, agg,* and *cob*), were commonly found in most dairy and food-origin *Enterococcus* isolates. No vancomycin resistance was shown by the *E. faecalis* B3A-B3B strain, and it was sensitive to most of the antibiotics used in this study. Moreover, the strain *E. faecalis* B3A-B3B scored promising values of hydrophobicity, auto-aggregation, adhesion to human Caco-2 cells, viability in simulated GIT conditions, and cholesterol assimilation. As a conclusion, this data led to submission of *E. faecalis* B3A-B3B as a remarkable probiotic strain. Nevertheless, as further continuation of this project, we propose the following targets:

i. Optimizing enterocin B3A-B3B production by studying the effect of carbon and nitrogen sources, necessary minerals, pH values, culture temperatures, and other necessary factors to increase the productivity of the strain *E. faecalis* B3A-B3B as an industrial producer strain in addition to the genetic regulation of the enterocin B3A-B3B production.
ii. Exploring possible applications of enterocin B3A-B3B against other foodborne pathogens alone or in combination with other antibiotics, bacteriocins, chemical food additives, and nano-particles, as well as evaluation of its anti-viral, anti-fungal and anti-spores activities.
iii. Studying the therapeutic potential of enterocin B3A-B3B by *in vitro* and *in vivo* experiments such as anti-cancer effects since several experimental studies have reported the therapeutic potential of bacteriocins against various types of cancer cell lines.
iv. Engineering of enterocin B3A-B3B is a possible subject to develop its efficacy to control other important antibiotic-resistant or foodborne pathogens.

v. Extending the investigation of probiotic properties of the *E. faecalis* strain by *in vivo* experiments.

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