Modification and development of probiotic strains *Saccharomyces boulardii* CNCM I-745 and *Lacticaseibacillus rhamnosus* GG

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Cover page picture: A microscopic view of probiotic strains *Saccharomyces boulardii* CNCM I-745 and *Lacticaseibacillus rhamnosus* GG (taken by Vy Anh Huynh, prepared by Ran Li)

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List of original publications

I. **Li, R.**, Wan, X., Takala, T. M., & Saris, P. E. J. (**2021**). Heterologous expression of the *Leuconostoc* bacteriocin leucocin C in probiotic yeast *Saccharomyces boulardii*. Probiotics and Antimicrobial Proteins, 13(1), 229-237.

II. **Li, R.**, Yassami, S., Kiviniemi, E. A., Qiao, W. J., Takala, T. M., & Saris, P. E. J. (**2021**). *Listeria* decontamination of chicken meat with beer brewed with bacteriocin producing *Saccharomyces boulardii*. LWT-Food Science and Technology, 112323.

III. Hussain, N., **Li, R.**, Takala, T. M., Tariq, M., Zaidi, A. H., & Saris, P. E. J. (**2021**). Generation of lactose- and protease-positive probiotic *Lacticaseibacillus rhamnosus* GG by conjugation with *Lactococcus lactis* NCDO 712. Applied and Environmental Microbiology, 87(6), e02957-20. **(Co-first author)**

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I. **Li, R.** participated in the design of the study, performed all the experiments, participated in data analysis and results interpretation, and wrote the manuscript together with the co-authors.

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III. **Li, R.** performed half of the experiments (except for the conjugation, transconjugants screening, plasmid stability, and casein degradation), participated in data analysis and results interpretation, and wrote the corresponding part of the manuscript together with the co-authors.

The publications are referred to in the following text by their roman numerals.

Abbreviations

AIDS	acquired immunodeficiency syndrome
AMPs	antimicrobial peptides
BBM	brush border membrane
BCP	bromocresol purple
BMI	body mass index
CFU	colony forming unit
CMPA	cow's milk protein allergy
CRISPR	clustered regularly interspaced short palindromic repeats
C-terminal	carboxyterminal
DNA	deoxyribonucleic acid
EDQM	European Directorate for the Quality of Medicines and healthcare
EFSA	European Food Safety Authority
FAO	Food and Agriculture Organization
FDA	Food and Drug Administration
GFP	green fluorescent protein
GI	gastrointestinal
GMO	genetically modified organisms
GRAS	generally regarded as safe
HIV	human immunodeficiency virus
HPLC	High-performance liquid chromatography
IBD	inflammatory bowel disease
IBS	irritable bowel syndrome
IL-10	interleukin 10
ISAPP	International Scientific Association for Probiotics and Prebiotics
kDa	kilodalton
LAB	lactic acid bacteria
LBP	live biotherapeutic product

Man-PTS	mannose phosphotransferase system
MRS	De Man, Rogosa and Sharpe, medium name
NGPs	next generation probiotics
OVA	ovalbumin
PCR	polymerase chain reaction
QPS	qualified presumption of safety
SCFAs	short chain fatty acids
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
TEF1	yeast translation elongation factor 1
UV	ultraviolet
WHO	World Health Organization

ABSTRACT

Probiotics are defined as “live microorganisms which when administered in adequate amounts confer a health benefit on the host”. In this doctoral dissertation, probiotic strains *Saccharomyces boulardii* CNCM I-745 and *Lacticaseibacillus rhamnosus* GG (LGG) were developed as next-generation probiotics.

The constructed *S. boulardii* strains, *i.e.*, SAC4 based on the wild-type *S. boulardii* CNCM I-745 and SAC12 based on the *URA3* auxotrophic derivative, secreted bacteriocin leucocin C, which showed inhibitory activity against the foodborne pathogen *Listeria monocytogenes*. Interestingly, the leucocin C secretion ability of *S. boulardii* SAC12 was stronger than that of SAC4. *S. boulardii* SAC4 killed *L. monocytogenes* effectively when cells of yeast and *Listeria* were incubated together without selection pressure, demonstrating the potential of cell mediated inhibition instead of using concentrated supernatant. Beer fermented with SAC12 was evaluated to be efficient in *Listeria* decontamination of chicken breast strips, with the maximum reduction of 2.2 log units from $(1.8\pm0.3) \times 10^5$ CFU/g.

LGG is one of the most studied probiotic strains, and it has been commercially used as a probiotic supplement in dairy products. The challenge of using LGG in dairy products is that it cannot metabolize the lactose and casein of milk, thus causing its poor growth in milk. We aimed to abolish this deficiency of LGG by bacterial conjugation, a non-GMO method. The dairy strain *Lactococcus lactis* NCDO 712 was used as donor, as it carries the plasmid pLP712 with the gene encoding the protease for casein degradation as well as the gene for lactose catabolism. In this study, a successful conjugation was done between *L. lactis* NCDO 712 and LGG. The plasmid pLP712 was conjugated into LGG, verified by plasmid-specific PCR and plasmid DNA isolation. The transconjugant *L. rhamnosus* LAB49 showed a clear ability of lactose utilization on indicator plate, in which lactose was the only carbon source. LAB49 was incubated in MRS, and all tested colonies (n= 80) lost their lactose-fermenting ability after 100 generations. The proteolytic activity of LAB49 was analyzed by SDS-PAGE and it

showed that β-casein was fully digested in 4 h by LAB49 and NCDO 712 but not at all by LGG. The growth curve indicated that LAB49 grew well in milk, reaching stationary phase in 11 to 12 h after inoculation. These results collectively suggested that, *L. rhamnosus* LAB49, an upgraded food-grade and non-GMO derivative of LGG had been generated.

TIIVISTELMÄ (abstract in Finnish)

Probiootit ovat eläviä mikrobeja, jotka riittävissä määrin syötyinä vaikuttavat terveyteen myönteisellä tavalla. Tässä väitöskirjatutkimuksessa kehitettiin terveyttä edistävistä *Saccharomyces boulardii* CNCM I-745 ja *Lacticaseibacillus rhamnosus* GG (LGG) mikrobikannoista ns. uuden sukupolven probiootteja.

S. boulardii CNCM I-745 on probioottinen hiivakanta, joka estää bakteerien haitallisia vaikutuksia suolistossa. Tässä työssä sekä *S. boulardii* CNCM I-745 villityyppi että auksotrofinen *URA3* –mutantti saatiin erittämään leukosiini C –nimistä bakteriosiinia, joka on tehokas estämään patogeenisen *Listeria monocytogenes* –bakteerin kasvua. *URA3*-mutantti-pohjaisen SAC12-kannan havaittiin olevan tehokkaampi leukosiinin erittäjä kuin villityyppiin perustuvan SAC4-kannan. *S. boulardii* SAC4 –kanta tappoi *Listeria*a tehokkaasti, kun *Listeria*- ja hiivasoluja inkuboitiin yhdessä. Tämä koejärjestely osoitti nimenomaan soluvälitteisen inhibition olevan tehokasta kasvatusliemen sijaan. *S. boulardii* SAC12 –kannalla valmistettiin olutta, jolla marinoitiin *Listeria*lla kontaminoituja kananrintasuikaleita. Olutmarinadi vähensi tehokkaasti *Listeria*-määriä kananrintasuikaleissa.

LGG on maailman tutkituimpia probioottisia maitohappobakteerikantoja, ja sitä käytetään probioottisena lisänä monissa kaupallisissa maitopohjaisissa tuotteissa. LGG ei kuitenkaan hajota maitosokeri laktoosia eikä maitoproteiini kaseiinia, eikä se siksi pysty kasvamaan maidossa. Tässä työssä nämä puutteet korjattiin bakteerien konjugaation avulla, koska konjugaatiota pidetään ns. non-GMO -geeninsiirtomenetelmänä. Laktoosin ja kaseiinin hajotukseen tarvittavat geenit saatiin juustohapatebakteeri *Lactococcus lactis* NCDO 712 –kannan pLP712-plasmidista. LGG ja *L. lactis* NCDO 712 saatiin konjugoitumaan, jolloin plasmidi pLP712 siirtyi laktokokista LGG:hen. DNA:n siirtyminen varmistettiin PCR:llä ja plasmidieristyksellä. Transkonjuganttikanta *L. rhamnosus* LAB49 hajotti laktoosia kasvatusmaljalla, jossa laktoosi oli ainoa hiilenlähde kasvulle. Kun LAB49-kantaa kasvatettiin 100 sukupolvea ilman laktoosin tuottamaa selektiopainetta, se menetti

laktoosinkäyttökykynsä, mikä johtui plasmidin pLP712 rakenteellisesta epävakaudesta. LAB49-kannan proteolyyttinen aktiivisuus varmistettiin SDS-PAGE-geelissä, mikä osoitti LAB49:n hajottavan kaseiinin täydellisesti neljässä tunnissa. Näiden LAB49-kannan uusien ominaisuuksien vaikutus kykyyn kasvaa maidossa testattiin vertaamalla kasvua LGG-kannan kanssa. Toisin kuin LGG, joka ei kasvanut maidossa, LAB49 kasvoi hyvin, ja saavutti stationäärivaiheen noin 12 tunnissa. LAB49-kantaa voidaan pitää ei-geneettisesti-muunneltuna elintarvikekelpoisena paranneltuna LGG-versiona, jota voitaisiin käyttää hapatteena maitotuotteissa.

1 INTRODUCTION

1.1 Probiotics, the “good bugs”

1.1.1 Definition, classification, and source of probiotics

Microorganisms, such as archaea, bacteria, fungi, protozoans, and even virus, are not always in good reputation. People tend to think of pathogens, disease, corruption, or spoilage when microorganisms are mentioned. However, the truth is that microorganisms are not all “bad bugs”, instead there is a certain portion of “good bugs” that are beneficial to human and animals, for example probiotics. According to the definition of FAO/WHO, probiotics are “live microorganisms which when administered in adequate amounts confer a health benefit on the host” (Food and Agriculture Organization and World Health Organization Expert Consultation, 2001).

The concept “probiotics” originates in 1954 from the Greek word meaning “for life” (Binns, 2013). Nevertheless, for centuries people have realized the benefits of fermented food that contains live microbes. In academia, the idea that consuming fermented foods may connect to an improved and prolonged life is attributed to the work of the Nobel Prize winner, Elie Metchnikoff (Metchnikoff, 1907). Within a century, thousands of studies about microbes having beneficial effects on humans have been emerging, and the amount of commercial products related to “probiotics” has rocketed sky-high. Such increasing in both academia and industry has led to misuse of the term “probiotics” and misleading claims. In 2013, an expert panel by the International Scientific Association for Probiotics and Prebiotics (ISAPP) suggested that under two scenarios the term “probiotics” cannot be used: **a)** fermented foods with undefined microbial content; **b)** undefined consortia, including fecal microbiota transplant (Hill et al., 2014). **Table 1** shows the acknowledged microorganisms that confer probiotic effects in human so far, bacteria like *Lactobacillus* spp., *Bifidobacterium* spp., and yeast (*Saccharomyces boulardii*) are included.

Table 1. Microorganisms used as probiotic in human. Modified according to the schemes of Senok et al., 2005, Williams, 2010, and Zommiti et al., 2020.

Lactobacillaceae [a]	***Bifidobacterium* spp.**	**Other bacteria**	**Yeast**
Lactobacillus acidophilus	*B. adolescentis*	*Bacillus clausii*	*Saccharomyces boulardii*
Lactobacillus amylovorus	*B. animalis*	*Bacillus coagulans*	
Lactobacillus bulgaricus [b]	*B. bifidum*	*Bacillus subtlis*	
Lacticaseibacillus casei	*B. breve*	*Lactococcus lactis*	
Lactobacillus crispatus	*B. infantis*	*Enterococcus faecalis*	
Limosilactobacillus fermentum	*B. lactis*	*Enterococcus faecium* [c]	
Lactobacillus gasseri	*B. longum*	*Escherichia coli Nissle* 1917	
Lactobacillus helveticus		*Streptococcus thermophilus* [b]	
Lactobacillus johnsonii			
Lactiplantibacillus pentosus			
Lactiplantibacillus plantarum			
Limosilactobacillus reuteri			
Lacticaseibacillus rhamnosus GG			

[a] The genus name under Lactobacillaceae family are according to the taxonomic reclassification of Zheng et al., 2020, in which the genus *Lactobacillus* was reclassified into 25 genera under the Lactobacillaceae family; in this thesis, the new taxonomic names will be adopted, and the abbreviation "*L.*" still refers to "*Lactobacillus*" when the species of *Lactobacillus* are mentioned.

[b] The probiotic activity is still debated (Uriot et al., 2017).

[c] Safety concerns remain because of potential pathogenicity and vancomycin resistance.

In daily life, for adults there are two main ways of obtaining probiotics, *i.e.*, from foods supplemented with probiotics, or directly from dietary supplements. People may also directly use probiotic pills or capsules, in which high dose of targeted microbes are supplied. For infants, the mother milk is an important source of acquiring potential probiotics like *Bifidobacterium*, and this is also a crucial process for forming the normal gut microbiota of infants' (Peirotén et al., 2018). Potential probiotics can be isolated from human microbiota (gut, skin, urogenital), fermented foods, and environment (soil, plants, animals) (Cunningham et al., 2021).

1.1.2 Properties and health benefits of probiotics

The presently acknowledged probiotics are well-selected and characterized strains, with scientific evidence of their capacity to confer health benefits on the host when

consumed in adequate amounts. The core feature of probiotics is that they should be safe to humans and not have pathogenic properties. Besides, probiotics aimed for the intestine must survive under the harsh condition, *e.g.*, acid in stomach, bile and enzymes of small intestine, in order to lively exert their functions in gastrointestinal (GI) tract. From the perspective of production and ingestion, probiotics should be easily cultured to a large scale and maintain its viability in food product or supplement (Binns, 2013).

In the recent decades, some probiotic strains have displayed promising effects in the topical application of skin (Blanchet-Réthoré et al., 2017; Lopes et al., 2017; Muizzuddin et al., 2012), nose (Michalickova et al., 2016; Van den Broek et al., 2018), and vagina (Coste et al., 2012; Gille et al., 2016). While this section will focus on the health benefits of "traditional probiotics" used by oral administration and exert their functions through human GI tract. The effects of many probiotic strains or their combinations have been extensively evaluated clinically. Clinical significance of probiotics is mainly indicated at their roles in metabolic disorders (*e.g.*, obesity, diabetes), dysbiosis, gastrointestinal disorders (*e.g.*, antibiotic-associated diarrhea, constipation), depression, anxiety, and mental disorders. Selected clinical studies evaluating the benefits of probiotics in human are shown in **Table 2.**

Table 2. Selected clinical studies evaluating the benefits of probiotics in human. Modified according to the schemes of Zommiti et al., 2020.

Disorders/Probiotics	Health benefits	References
Obesity		
Enterococcus faecium, Streptococcus thermophilus	Decreased body weight, increased fibrinogen levels	Agerholm-Larsen et al., 2000
Lactobacillus gasseri SBT2055	Decreased BMI and arterial blood pressure values	Kadooka et al., 2013
Bifidobacterium, Streptococcus thermophilus	Improved lipid profile, insulin sensitivity, and decreased C-reactive protein	Rajkumar et al., 2014
Lactobacillus acidophilus La5, *Bifidobacterium animalis* BB12	Decreased fasting glucose concentration, increased homeostasis model assessment of insulin resistance	Ivey et al., 2015
Type-2 diabetes		
Lactiplantibacillus plantarum A7	Decreased methylation process and superoxide dismutase	Hariri et al., 2015
Limosilactobacillus reuteri NCIMB 30242	Decreased low-density lipoprotein cholesterol by 11.64% and total cholesterol by 9.14% in hypercholesterolemic adults	Jones et al., 2012
Lactobacillus acidophilus	A significant reduction was found in low-density lipoprotein	Sun and Buys, 2015
Constipation		
Bifidobacterium animalis DN-173010, *Escherichia coli* Nissle 1917, *Lacticaseibacillus casei* Lcr35	Alleviated the symptoms of functional constipation in adults	Dimidi et al., 2014
Bifidobacterium lactis	Improve the whole gut *Bifidobacterium lactis* transit time, stool frequency, and stool consistency	Chmielewska and Szajewska, 2010
Antibiotic-associated diarrhea, diarrhea, inflammatory bowel disease		
Saccharomyces boulardii	Decreased diarrhea rates in children receiving probiotic yeast (7.5%) compared to those receiving placebo (23%)	Kotowska et al., 2005
Escherichia coli Nissle 1917	Alleviated the symptoms of inflammatory bowel disease	Behnsen et al., 2013
Bacillus clausii	Alleviated the symptoms of acute diarrhea in children	Ianiro et al., 2018
Saccharomyces boulardii	A protective role in preventing antibiotic-associated diarrhea after intake of 5×10^9 CFU/day	Johnston et al., 2011
Lacticaseibacillus rhamnosus GG	Decreased the duration of acute gastroenteritis in children	Szajewska et al., 2013

Cancer and side effects associated with cancer		
Lactobacillus acidophilus and *Bifidobacterium* spp.	Inhibited the growth of tumor cells, produced anti-carcinogens and reduces cancer risks	Vasiljevic and Shah, 2008
Lacticaseibacillus rhamnosus 573	Patients experienced less abdominal discomfort, less hospital care and lower chemo dose were needed	Österlund et al., 2007
Depression, anxiety, and mental disorders		
Lactobacillus helveticus R0052 *Bifidobacterium longum* R0175	Decreased aggressive and ruminative thoughts in response to sad mood	Messaoudi et al., 2011
Bifidobacterium bifidum W23, *Bifidobacterium lactis* W52, *Lactobacillus acidophilus* W37, *Levilactobacillus brevis* W63, *Lacticaseibacillus casei* W56, *Ligilactobacillus salivarius* W24, and *Lactococcus lactis (*W19 and W58*)*	Significantly reduced overall cognitive reactivity to sad mood	Steenbergen et al., 2015

1.1.3 Overview of the probiotic yeast *Saccharomyces boulardii*

Saccharomyces boulardii is the only yeast used as probiotics, and the type strain is *S. boulardii* CNCM I-745 (or *S. boulardii* Hansen CBS 5926). In 1923, a French scientist Henri Boulard isolated *S. boulardii* from tropical fruit lychee in Southeast Asia. Later in 1962, the lyophilized powder of *S. boulardii* was commercially available from Laboratoires Biocodex (Montrouge, France) and mainly used for antibiotic-associated diarrhea (McFarland and Bernasconi, 1993). Nowadays, *S. boulardii* is regulated as dietary supplement for human (Venugopalan et al., 2010) and can be easily found in pharmacy as capsules or pills.

S. boulardii was first identified as a distinct species in the genus *Saccharomyces* since it does not sporulate and metabolize galactose (McFarland, 1996; Mitterdorfer et al., 2001). However, molecular typing (Edwards-Ingram et al., 2004; Fietto et al., 2004; Mitterdorfer et al., 2002) and the comparative genomic analysis (Khatri et al., 2017) argued that *S. boulardii* should be considered as a strain of *Saccharomyces cerevisiae* instead of a different species. Mitterdorfer et al., 2002 found that *S. boulardii* clustered

to a separate group, but belonged to *S. cerevisiae* species. Khatri et al., 2017 compared the genomes of 5 *S. boulardii* strains with 145 *S. cerevisiae* strains. The genetic characteristic of *S. boulardii* was that it did not carry Ty elements Ty1, Ty3, Ty4 and associated long terminal repeat, while complete Ty2 and Ty5 were identified. More importantly, *S. boulardii* and *S. cerevisiae* shared more than 99% relatedness in genome, and strains of *S. boulardii* were closely related to the wine strains of *S. cerevisiae*.

Even though they are genetically similar, *S. boulardii* does own some unique physiological phenotypes that make it a probiotic yeast. Comparing with *S. cerevisiae*, the heat and acid resistance of *S. boulardii* is superior, and its optimal growth temperature is 37 °C (Fietto et al., 2004). *S. boulardii* also resists the proteolytic cleavage (Fietto et al., 2004). These features guarantee that *S. boulardii* could tolerate the harsh condition of human stomach and GI tract, thus reaching the target organ to exert its function. Since *S. boulardii* is not a natural colonizer in the human intestine, it can easily be cleared within 3-5 days (Klein et al., 1993). In a word, *S. boulardii* has a long history of applying as probiotics and it is similar to the well-known *S. cerevisiae*, but with unique properties.

In addition to the physiological phenotypes of *S. boulardii* that enable its better survival in GI tract, the safety and function also matter for probiotics. *S. boulardii* is granted as GRAS (generally regarded as safe) status by the Food and Drug Administration (FDA) of USA (Czerucka et al., 2007), and QPS (qualified presumption of safety) by European Food Safety Authority (EFSA) (EFSA Panel on Biological Hazards (BIOHAZ) et al., 2018). Although *S. boulardii* has been linked to fungemia in immunocompromised individuals (Santino et al., 2014), no fungemia cases caused by *S. boulardii* were reported in clinical trials (Kelesidis and Pothoulakis, 2012; McFarland, 2017). The benefits of *S. boulardii* in gastrointestinal diseases have been widely assessed in clinical trials. It has been summarized by McFarland, 2017 that 90 randomized controlled trials have been done within 40 years. These trials investigated the efficacy of *S. boulardii* in 15 types of disease, including diarrheas like antibiotic-associated diarrhea (D'souza et al., 2002; Surawicz et al., 1989; Szajewska and Mrukowicz, 2005), traveler's diarrhea

(Kollaritsch et al., 1993; McFarland, 2007), AIDS-associated diarrhea (Saint-Marc et al., 1995; Villar-García et al., 2015), acute diarrhea in children (Dinleyici et al., 2015; Feizizadeh et al., 2014); IBD (inflammatory bowel disease) like Crohn's disease (Guslandi et al., 2000), ulcerative colitis (Guslandi et al., 2003); IBS (irritable bowel syndrome) (Maupas et al., 1983); *Helicobacter pylori* infections (Cremonini et al., 2002; Duman et al., 2005) and so on.

The mechanisms of action of *S. boulardii* to display its probiotic benefits have been described as multiple ways. They mainly take place through pathogen binding, antitoxin effects, trophic actions on the intestinal mucosa, and immune regulations (**Figure 1**).

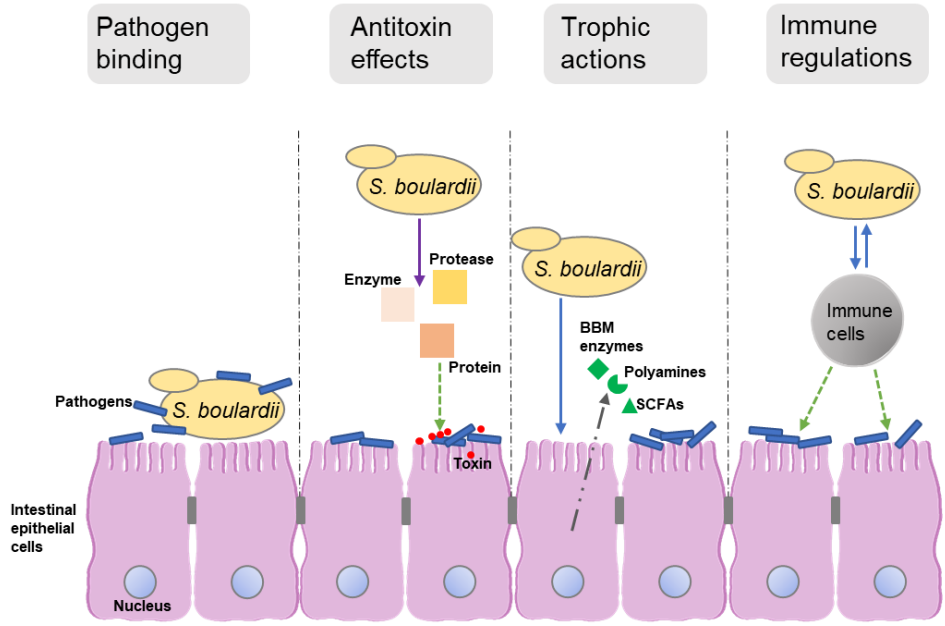

Figure 1. Mechanisms of action of *S. boulardii* to display its probiotic benefits. Modified from the figure of Pais et al., 2020 and the scheme of Kelesidis et al., 2012. Briefly, *S. boulardii* displays its benefits through: **a)** directly binding and excluding intestinal pathogens (Chen et al., 2006; Czerucka et al., 1994; Dalmasso et al., 2006; Geyik et al., 2006; Herek et al., 2004), the yeast cell wall components are responsible for the binding; **b)** deactivating the toxin produced by pathogens, mainly *S. boulardii* produces serine protease (Pothoulakis et al., 1993), phosphatase (Buts et al., 2006; Castagliuolo et al., 1996), or some unknown proteins (Czerucka et al., 1994) for toxin lysis; **c)** stimulating the trophic effects on intestinal epithelial cells, for example stimulating producing brush border membrane (BBM) enzymes

(Buts et al., 1986), polyamines (Buts et al., 2002; Jahn et al., 1996; Schneider et al., 2005) and restoring the normal level of colonic short chain fatty acids (SCFAs) (Breves et al., 2000; Sezer et al., 2009) to enhance the intestinal barrier; **d)** acting as an immune stimulant (Rodrigues et al., 2000) or reducing pro-inflammatory cytokines (Generoso et al., 2011).

1.1.4 Overview of the probiotic bacterium *Lacticaseibacillus rhamnosus* GG

As summarized in **Table 1**, majority of the probiotics, with the exception of the probiotic yeast *S. boulardii*, belong to the bacterial domain. Specifically, the genus *Lactobacillus* is the dominant group among the probiotics market and recently reclassified into several genera (Zheng et al., 2020). At present, many strains from *L. acidophilus*, *Lactiplantibacillus plantarum*, *Lacticaseibacillus casei*, *L. johnsonii* and *Lactiplantibacillus rhamnosus* are commercially available with certain trademarked names. For example, *Lacticaseibacillus rhamnosus* GG (ATCC 53103, LGG) is known under several commercial names, *e.g.*, Actifit$^{Plus®}$, Gefilus$^{®}$, LGG$^{®}$, Onaka He GG! $^{®}$, and Vifit$^{®}$ (Saxelin et al., 2005). The wide utilization of LGG in industry is a result of extensive studies that have demonstrated the safety and benefits of this famous strain. Unlike *S. boulardii*, LGG was isolated from healthy human intestine by Sherwood Gorbach and Barry Goldin in 1983. The patented name of LGG in 1989 (Gorbach and Goldin, 1989) was *L. acidophilus* GG, and it was later reclassified into a strain of *L. rhamnosus.* Notably, Zheng et al., 2020 proposed a new classification of genus *Lactobacillus* into 25 genera according to the whole genome sequences analysis, in which LGG should be called *Lacticaseibacillus rhamnosus* GG.

After its discovery, LGG has become one of the most broadly researched probiotic strains and thus the safety and benefits of LGG have been well documented. Studies have shown the safe usage of LGG covering varied-age or -status people: newborns (Arvola et al., 1999), preterm infants (Underwood et al., 2009), children (American Academy of Pediatrics, 2001; Hojsak et al., 2010; Vanderhoof et al., 1999), adults (Szajewska and Kołodziej, 2015) and in the elderly (Hatakka et al., 2007); pregnant women (Lahtinen et al., 2009), and HIV patients (Salminen et al., 2004). Generally, LGG is safe for human consumption and its QPS (EFSA Panel on Biological Hazards

et al., 2018) and GRAS (Food and Drug Administration, 2019) status have been acknowledged. Clinical trials have shown the efficacy of LGG, mainly in alleviating GI disorders like acute rotavirus gastroenteritis (Fang et al., 2009; Oberhelman et al., 1999; Szajewska et al., 2001), antibiotic-associated diarrhea (Arvola et al., 1999; Thomas et al., 2001; Vanderhoof et al., 1999), IBS (O'Sullivan and O'Morain, 2000; Pedersen et al., 2014), travelers' diarrhea (Hilton et al., 1997; Oksanen et al., 1990), abdominal pain-related functional gastrointestinal disorders in children (Bausserman and Michail, 2005; Francavilla et al., 2010; Gawrońska et al., 2007), acute diarrhea in children (Guandalini et al., 2000; Misra et al., 2009; Ritchie et al., 2010); enhancing microbiota balance (Apostolou et al., 2001; Gueimonde et al., 2006; Lahti et al., 2013); modulating immune response (Kekkonen et al., 2008; Schultz et al., 2003); preventing onset of allergic diseases (Isolauri et al., 1991; Kalliomäki et al., 2001; Majamaa and Isolauri, 1997).

Apart from the ability of surviving and proliferating in gastric acid and bile, a noteworthy feature of LGG is its strong adhesion ability to epithelial cells of intestine. As claimed in the patent of Sherwood Gorbach and Barry Goldin in 1989, LGG is characterized "in that an average of at least 50, more preferably at least 100, of the bacteria can adhere to one human small intestinal mucosal cell after five minutes incubation of the bacteria with the cells". The *in vivo* human intervention study of Kankainen et al., 2009 also revealed that LGG could stay longer and in higher density in human intestinal tract compared to *L. rhamnosus* LC705. This feature enables active interactions between LGG and intestinal epithelial cells or immune cells, and the so-called LGG-host interactions (Bron et al., 2012; Segers and Lebeer, 2014) are also important mechanism of action responsible for LGG's probiotic benefits. From the molecular insights, Segers and Lebeer, 2014 proposed several ways engaging in LGG-host interactions, *i.e.*, pili-mediated adhesive capacity, lipoteichoic acid as key immune effector, major secreted proteins as probiotic effectors, exopolysaccharides as modulating adaptation factors, and secreted antimicrobials.

1.2 Modification and development on the property of probiotics

1.2.1 Genetic engineering as a way to develop next-generation probiotics

Considering the increasing commercial and clinical relevance of probiotics, the modification and development of the properties of probiotics as a new frontier in probiotics research have attracted many scholars' interests. Modifying the properties of probiotics is mainly targeted to: **a)** enhancing the resistance of probiotics to the stresses encountered during preparation and storage of the delivery matrices; **b)** enhancing *in vivo* resistance of probiotics to the stresses in human GI tract; and **c)** enhancing the benefits of probiotics by engineering them for secretion of bioactive molecules, or targeting specific pathogens/toxins (Sleator and Hill, 2008). The third-type modified probiotics could be regarded a kind of next-generation probiotics (NGPs).

The term NGPs is a new concept proposed to describe live microorganisms that are not traditionally used as probiotics, but own potential health benefits to humans, even though many of these are still at an early stage of investigation (Langella et al., 2019; Martín and Langella, 2019; O'Toole et al., 2017). On the other hand, as summarized by O'Toole et al., 2017, NGPs are "more likely to be delivered under a drug regulatory framework" like live biotherapeutic product (LBP). The formally recognized concept by FDA (Food and Drug Administration, 2016) and European Directorate for the Quality of Medicines and healthcare (EDQM) (European Pharmacopoeia Commission, 2019), LBP, is defined by FDA as a biological product that "contains live organisms; is applicable to the prevention, treatment, or cure of a disease or condition of human beings; is not a vaccine". Even though NGPs conform to the definition of LBP, it is suggested that the term LBP should not be systematically applied to replace NGPs. For one thing, the term NGPs indicates that they are different from traditional probiotics; for another thing, NGPs include microorganisms that are being investigated and do not correspond to a defined product yet, *e.g.*, genetically modified microorganisms and potential beneficial commensal bacteria (Martín and Langella, 2019; O'Toole et al., 2017).

Two strategies are being employed to develop NGPs (O'Toole et al., 2017). Like current probiotics, the first strategy is to evaluate if a specific strain carries health phenotype when administered in sufficient quantities. For example, the benefits of *Bacteroides xylanisolvens* DSM 23694 (Ulsemer et al., 2016), *Bacteroides ovatus* D-6 (Ulsemer et al., 2013), *Clostridium butyricum* MIYAIRI 588 (Shimbo et al., 2005; Woo et al., 2011), and *Faecalibacterium prausnitzii* (Rossi et al., 2016; Song et al., 2016) have been evaluated. Another strategy is to apply well-characterized probiotic strains, GRAS microorganisms, or commensals as delivery vehicles for bioactive molecules like anti-inflammatory cytokines, transforming growth factor, or bacteriocins. The advantages of modifying and using aforementioned microbes as delivery vehicles are that they are usually identified as not producing virulence factors, and will be tolerated by the host, and if chosen carefully, may not even colonize the host.

In the second strategy, targeted modification of the properties of the microorganisms is needed, in such case, genetic engineering could serve as a useful tool. Taken the genetic engineering of probiotics as examples, the serine protease inhibitor Elafin was expressed in *L. lactis*, and the administration of the modified strain reduced inflammation in a mice model of colitis (Motta et al., 2012). In addition, interleukin 10 (IL-10) controlling allergen sensitivity (Frossard et al., 2007) and trefoil factor 1 controlling oral mucositis (Robert and Steidler, 2014), were produced by different *L. lactis* strains and proved to be useful in preclinical trials in mice. A plasmid expressing anti-HIV hybrid peptides was introduced into probiotic strain *E. coli* Nissle 1917, and mice trial showed that the modified probiotic inhibited HIV infection by blocking its entry into host cells (Rao et al., 2005). Plasmid-based techniques have also been applied in the genetic engineering of *Lactiplantibacillus plantarum* (Shi et al., 2014) and *Lacticaseibacillus casei* (Alvarez-Sieiro et al., 2014; Liu et al., 2014; Qiu et al., 2013). Besides, several bioactive molecules were engineered to be secreted in *S. thermophilus* (Selle et al., 2015), *Limosilactobacillus reuteri* (Oh and van Pijkeren, 2014; Van Pijkeren and Britton, 2014) using CRISPR-Cas9, in *L. lactis* and *Limosilactobacillus reuteri* (van Pijkeren and Britton, 2012; van Pijkeren et al., 2012), and

Lactiplantibacillus plantarum (van Pijkeren and Britton, 2012) using chromosome modification system.

1.2.2 Studies on the modification and development of *S. boulardii* and LGG

To secrete bioactive molecules, various laboratory strains of *S. cerevisiae* have been genetically engineered. While natural auxotrophic mutants of *S. boulardii* have not been reported, only *URA3* mutant has been generated by UV mutagenesis (Hamedi et al., 2013; Hudson et al., 2014) and CRISPR-cas9 (Bagherpour et al., 2018; Liu et al., 2016). Douradinha et al., 2014 investigated factors concerning the genetic manipulation of *S. boulardii*, such as plasmid transformation and screening of positive strains. In the newest study of Jin et al., 2021, four signal peptides, namely chicken lysozyme signal peptide (CL), α-mating factor signal peptide (α-MF) from *S. cerevisiae*, Sta1 signal peptide (STA1) from *Saccharomyces diastaticus*, and Sed1 signal peptide (SED1) from *S. cerevisiae* were tested for controlling the production of target substance. SED1 was shown to be the most efficient signal peptide in producing the endo-type β-agarase *Bp*GH16A in *S. boulardii* ATCC MYA-796.

Several studies have been performed aiming to modify or develop the properties of *S. boulardii* by genetic engineering. Glucoamylase (Latorre-García et al., 2008), mouse IL-10 (Michael et al., 2013; Pöhlmann et al., 2013), and *Eimeria tenella* microneme-2 antigen (Wang et al., 2014) were produced in wild-type *S. boulardii* using antibiotics selection. Using *URA3* auxotrophic mutant of *S. boulardii* as host, green fluorescent protein (GFP) (Hudson et al., 2014), human lysozyme (Liu et al., 2016), ovalbumin (OVA) (Bagherpour et al., 2018), and endo-type β-agarase (Jin et al., 2021) were produced. Three studies have evaluated the effects of engineered *S. boulardii* with mice model. Michael et al., 2013 tested the anti-inflammatory function of IL-10 producing *S. boulardii* in the colitis mice model. No significant differences were observed between the treated and untreated groups, suggesting that IL-10 was not sufficiently secreted into the intestine to decrease the inflammatory response. Hudson et al., 2014 revealed in their studies that the GFP-producing *S. boulardii* strain could be recovered from the Peyer's patches of mice fed with this strain, and the isolated yeasts still carried the GFP

production capacity. Via mice oral administration, Bagherpour et al., 2018 used the OVA-producing *S. boulardii* to deliver antigenic peptide ovalbumin into intestinal lumen and observed an increased antibody response in treated group compared with control group. It has been suggested that probiotic *S. boulardii* should be desirable vehicle for expressing and delivering functional molecules, as it carries native benefits and tolerance to human GI tract, and most importantly it will not exchange genetic material with other microorganisms (Palma et al., 2015; Pöhlmann et al., 2013).

As for LGG, very few studies aiming to modify or improve its properties have been implemented. Vélez et al., 2007 deleted *dltD* gene encoding DltD membrane protein in LGG, which resulted in modifications of the bacterial cell surface properties; later, the *dltD* LGG mutant was applied in a mice colitis model, and the mutant displayed an improved therapeutic efficacy compared to wild-type LGG (Claes et al., 2010). The HIV-inhibiting lectin, griffiths, was produced in LGG and the recombinant strain showed *in vitro* anti-HIV activity against M-tropic and T-tropic HIV-1 strains (Petrova et al., 2018). In addition, novel fluorescent derivative (Spacova et al., 2018) and highly mucus-adherent derivative (Rasinkangas et al., 2020) of LGG were generated, which could be used to further study the LGG-host interactions. On the other hand, one patent (Heidtman et al., 2015) described the generation of a LGG strain that its lactose utilization deficiency has been fixed, the details are elaborated in section **1.4.2**.

1.3 LAB bacteriocins and *Leuconostoc* bacteriocin leucocin C

1.3.1 LAB bacteriocins

Antimicrobial peptides (AMPs) are molecules that exist in several organisms like bacteria, plants, animals and human, and they play an important role in the defense system against pathogens. In bacteria, the gene-encoded AMPs are called bacteriocins, and they primarily kill closely related organisms of the producer. It has been proposed that bacteriocins may be viable alternatives to antibiotics due to the advantages of bacteriocins: *in vitro* and *in vivo* efficacy, the availability of both broad- and narrow-spectrum peptides and the possibility of *in situ* production by probiotics (Cotter et al.,

2013). Most importantly, unlike antibiotics that are secondary metabolites, the biosynthetic mechanisms of bacteriocins are comparatively simple (Perez et al., 2014), making bacteriocins easily bioengineered (Cotter et al., 2013; Perez et al., 2014).

Bacteriocins produced by LAB have received much attention because of their antimicrobial activity against foodborne pathogens like *Listeria monocytogenes* and *Staphylococcus aureus* (Arqués et al., 2011), *Bacillus cereus* (Kaya and Simsek, 2019), *Clostridium perfringens* (Voidarou et al., 2020); or food spoilage microbes like *Pseudomonas fluorescens* (Voidarou et al., 2020) and *Pediococcus damnosus* (Ahn et al., 2017). Studies have shown that a number of LAB including *Lactobacillus*, *Lactococcus*, and *Leuconostoc* secrete bacteriocins, of which many have been well characterized (Zacharof and Lovitt, 2012). The most well-known LAB bacteriocin is nisin, which has been accepted as a safe food additive and commercially used to facilitate the food preservation (Gálvez et al., 2007).

Presently, 230 characterized bacteriocins have been archived by the online database Bactibase (http://bactibase.hammamilab.org/main.php). The LAB (order Lactobacillales) are the predominant group of producers. In 2019, Kassaa et al., 2019 developed a dedicated database for LAB bacteriocins: LABiocin (www.labiocin.net), collecting 517 LAB bacteriocins from research articles up to August 2017. Notably, *Lactobacillus* (old classification) made up 30.95% of the producer genus, followed by *Enterococcus* (24.18%) and *Streptococcus* (16.83%). The antimicrobial spectrum showed that 62.08% of the archived LAB bacteriocins could inhibit Gram-positive bacteria, and 22.05% could inhibit both Gram-positive and Gram-negative bacteria.

Put aside the bacteriocins containing non-protein moieties, LAB bacteriocins are generally classified into three categories (Class I, II, and III). The proposed classification and typical bacteriocin are presented in **Figure 2**. Class I and II bacteriocins are small peptides (<10 kDa) and are heat stable, while class III bacteriocins are generally considered to be large (>10 kDa) and thermo-labile AMPs. Specifically, class I bacteriocins need post-translational modifications before they are biologically active. For class II bacteriocins modifications are not needed, and they

usually have a narrow spectrum. Class III are unmodified AMPs that consist of bacteriolysins and non-lytic bacteriocins.

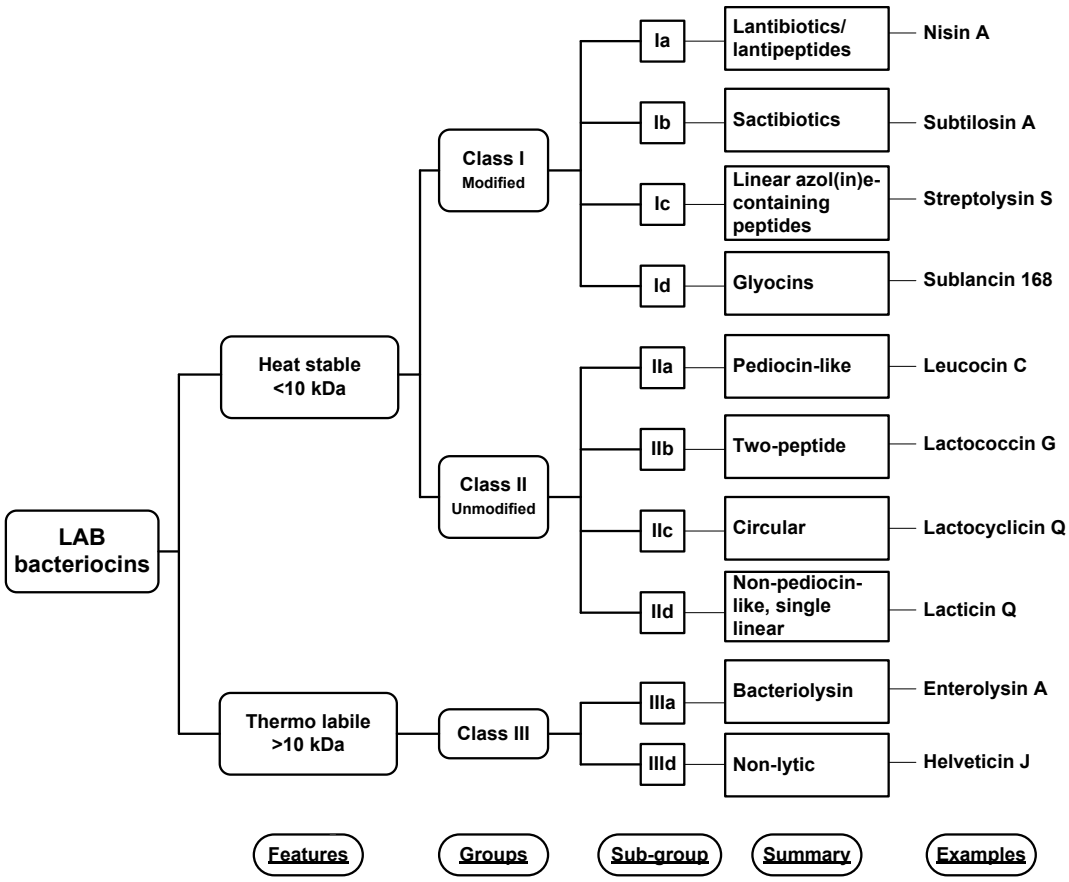

Figure 2. Proposed scheme of classification for LAB bacteriocins. Modified according to the schemes of Alvarez-Sieiro et al., 2016 and Wan, 2017.

1.3.2 *Leuconostoc* bacteriocin leucocin C

Leucocin C is a class IIa (also known as pediocin-like) bacteriocin secreted by *Leuconostoc* species, mainly *Ln. carnosum* and *Ln. mesenteroides*. Besides leucocin C, the bacteriocins produced by *Leuconostoc* species known as leucocins are leucocin A, leucocin B, and other leucocins like leucocin Q, N and so on. There are also some other

Leuconostoc bacteriocins named as mesentericins, which are produced by *Ln. mesenteroides* strains. So far, more than 40 *Leuconostoc* bacteriocins have been characterized (Wan, 2017).

Like other class IIa bacteriocins, leucocin C is best known for its antimicrobial activity against the foodborne pathogen *L. monocytogenes*. It has been well accepted that the class IIa bacteriocins exert their function through a fast-acting system: holing the cell membrane of target microbes and causing internals release (Eijsink et al., 2002; Tiwari et al., 2015). In detail, three steps are included in the model of action for class IIa bacteriocins killing: first, the bacteriocin attaches to the extracellular loop of Man-PTS (mannose phosphotransferase system) IIC domain; then, the C-terminal helix of the bacteriocin breaks into the cytoplasmic membrane after interacting with transmembrane helices of the IIC domain, and finally forms pores and leading to cell death (Diep et al., 2007; Kjos et al., 2011).

So far, the amino acid sequence of leucocin C from six different producers have been published (Budde et al., 2003; Fimland et al., 2002; Papathanasopoulos et al., 1998; Vaughan et al., 2001; Wan et al., 2013), among which only Wan et al., 2013 performed the genetic characterization of leucocin C from *Ln. carnosum* 4010. The new culture, *Ln. carnosum* 4010, was isolated by Budde et al., 2003 from vacuum-packed meat products; they found that the strain 4010 displayed strong anti-listerial activity. By N-terminal sequencing, Budde et al., 2003 identified the purified and characterized leucocin B-4010, which is actually closely similar to leucocin C TA33a (Papathanasopoulos et al., 1998). In 2013, the work from our group characterized the genes encoding leucocins A and C in *Ln. carnosum* 4010, and leucocin C cluster included two intact operons in the plasmid; the gene encoding the mature peptide of leucocin C was cloned and expressed in *L. lactis*, and heterologously secreted leucocin C was active in killing *L. monocytogenes* (Wan et al., 2013). With the availability of the genetic information, more molecular-biological work could be done to either increase the activity or expand the specificity of leucocin C against target microbes. For example, Liu et al., 2013 constructed a leucocin C-producing *E. coli* that was equipped with cell

wall binding domain of *Listeria* phage endolysin. In this way the *E. coli* bound to *Listeria* cells and exhibited enhanced ability to kill *L. monocytogenes*.

1.3.3 Application of LAB bacteriocins in foods

As mentioned in **1.3.1**, LAB bacteriocins are mainly applied in foods as bio-preservatives against foodborne pathogens or food spoilage microbes. For such application, there are three considerations: first, the LAB bacteriocins are produced by lactic acid bacteria that are generally considered to be food-grade; second, unlike antibiotics, bacteriocins do not show toxicity towards eukaryotic cells (Perez et al., 2014); third, most LAB bacteriocins are heat-stable and maintain their activity over a wide range of pH. Thus, considering that the external additives used in food have to be safe to humans and tolerate the "natural intrinsic hurdles" of foods, and the technologies used in food processing, the features of LAB bacteriocins make them good candidates for use in foods to improve the safety (And and Hoover, 2003; O'Sullivan et al., 2002), quality (O'Sullivan et al., 2002), or even flavor (Garde et al., 2002; O'Sullivan et al., 2003). The utilization of bacteriocins in food preservation may also reduce the needs for chemical or physical treatments, resulting in more naturally preserved foods.

Presently, only nisin and pediocin PA1/AcH, for example commercial products Nisaplin (DuPont Nutrition & Biosciences) and ALTA 2431 (Quest International), respectively, are widely used in food industry. There are three main ways of introducing LAB bacteriocins into foods: **a)** *in situ* secretion by the producer strain added in fermented food or beverage, thereby the purification of bacteriocins is not needed; **b)** if the dose of bacteriocins has to be controlled, then purified or semi-purified bacteriocins like Nisaplin can be added into food either directly, or by the incorporation to food packaging films (Franklin et al., 2004; Mauriello et al., 2005); **c)** using food ingredients like ALTA 2431 containing natural metabolites including organic acids and pediocin of LAB is also a method of introducing LAB bacteriocins into food. In addition, studies have shown that use of LAB bacteriocins could also be combined with traditional food processing techniques such as heating (Boziaris et al., 1998; Budu-Amoako et al., 1999), adding of chelating agents (Cutter and Siragusa, 1995) or chemical antimicrobials

(Mansour and Millière, 2001; Periago and Moezelaar, 2001), high hydrostatic pressure (Masschalck et al., 2001), and pulsed electric field (Pol et al., 2000).

Studies in recent years have clearly demonstrated the benefits of using LAB bacteriocins in food. For example, Arqués et al., 2011 found a strong synergistic effect against *L. monocytogenes* when nisin, lacticin 481 or enterocin AS-48 was used together with reuterin in milk. Besides, enterocin AS-48 could increase the thermal sensitivity of *Bacillus licheniformis* (Grande et al., 2006), a bacterium that forms slime and causes ropy appearance in cider (Larpin et al., 2002), and thus makes it more efficiently deactivated when combined with heat treatment. Nisin applied on fresh-cut fruits could reduce up to 3.2 log of *L. monocytogenes* spiked on honeydew melon slices, and up to 2.0 log on apple slices (Leverentz et al., 2003). In particular, using LAB bacteriocins to inhibit the highly pathogenic and foodborne *L. monocytogenes* has drawn a lot of attention of scientists, since *L. monocytogenes* is ubiquitously found in meat (Kurpas et al., 2018; Pesavento et al., 2010), raw vegetables (Beuchat, 1996; Farber et al., 1998), and dairy products (Hayes et al., 1986; Rudol and Scherer, 2001) and it can cause human listeriosis. Studies regarding using nisin (Leverentz et al., 2003), leucocin C (Fu et al., 2018), and bacteriocin PA-1 (Pucci et al., 1988) in *L. monocytogenes*-contaminated foods have indicated the effectiveness of bacteriocins. As for leucocin C, Budde et al., 2003 added the leucocin C-producing strain *Ln. carnosum* 4010 to vacuum-packaged meat sausage, and the viable cells of *L. monocytogenes* decreased below 10 CFU/g after 21-days storage at 5 ºC. Fu et al., 2018 co-expressed nisin Z and leucocin C in *L. lactis* N8. After that, they confirmed that the supernatant containing leucocin C and nisin Z reduced the viable cell count of *L. monocytogenes* inoculated into pasteurized milk by 2 log after 16 h at 4 ºC.

Traditionally, bacteriocins that are added into foods are mainly for the purpose of preservation or safety of foods. As bacteriocins could be introduced into foods through adding bacteriocin-producing probiotics, in such case foods in turn could act as vehicles for delivering bacteriocins into GI tract to inhibit pathogenic bacteria (Cotter et al., 2005; Cotter et al., 2013). Bacteriocins directly applied in food may be inactivated by

the interaction with food components like fat (Degnan et al., 1993) or protein, or by the proteolytic enzymes of the stomach. Bacteriocins-producing probiotics added in foods could reach the GI tract alive, allowing the *in vivo* production of bacteriocins.

1.4 Lactose and casein utilization deficiency of LGG

1.4.1 Lactose intolerance and cow's milk protein allergy (CMPA)

Milk is an important source of minerals, vitamins, carbohydrates, and proteins for humans, yet some disorders like lactose intolerance and cow's milk protein allergy (CMPA) caused by milk ingestion in certain people cannot be ignored. There is approximately 4.8% lactose in bovine milk, and up to 7% lactose in human milk (Jenness, 1979; Ohlsson et al., 2017). Lactose intolerance is caused by the lack of endogenous lactase in the human small intestine. The decrease in the amount of lactase usually happens after weaning and can extend into adulthood. The prevalence of lactose intolerance is estimated to be 68% globally, and 28% in Western, Southern, and Northern Europe (Storhaug et al., 2017). Moreover, around 3% of infants are affected by CMPA (Gupta et al., 2011; Sicherer and Sampson, 2014), a type of immune system malfunction that is mainly triggered by casein and whey β-lactoglobulin in milk.

So far, the commonly used preventive measure for lactose intolerance and CMPA is consuming milk alternatives with reduced lactose and casein content (EFSA Panel on Dietetic Products, 2010), for example, hydrolyzed formulas and soy-based formulas. The use of probiotic or lactose- and/or casein hydrolyzing bacteria in dairy products is another candidate for alleviating the symptoms. In lactose intolerance, probiotics improve the lactose absorption through enhancing the overall hydrolytic capacity in the small intestine and increasing the colonic fermentation (Dhama et al., 2016). In addition, bacteria in fermented dairy products can decrease the lactose contents and bring active lactase into the intestine (He et al., 2008). Several probiotics have been reported to play a role in lactose intolerance prevention, such as *B. animalis* (Roškar et al., 2017), *B. longum* (He et al., 2008; Jiang et al., 1996), *L. bulgaricus* (Parra and Martínez, 2007; Rizkalla et al., 2000), and *Lacticaseibacillus rhamnosus* (Agustina et al., 2007) (strain

LMG P-22799). In terms of CMPA, the main probiotic used was LGG, which was usually supplied in extensively hydrolyzed formula, either hydrolyzed casein (Berni Canani et al., 2017; Berni Canani et al., 2012; Scalabrin et al., 2017) or hydrolyzed whey (Kirjavainen et al., 2003), to ease the symptoms and accelerate the acquisition of CMPA tolerance. When used in hydrolyzed formula for CMPA, the main benefits of LGG are mediating the gut immune response and boosting the intestinal mucosal barrier (Scalabrin et al., 2017).

1.4.2 Lactose/casein utilization deficiency of LGG and ways to improve LGG

As aforementioned in **1.4.1**, LGG could be added as a probiotic supplement in hydrolyzed formulas. Besides, LGG has also been added to several dairy products, such as yogurt, cheese, and fermented milk. However, studies have indicated that some dairy products like yogurt, cannot maintain an adequate level of viable probiotics during their shelf-life. Thus, their health value might be compromised in this respect (Heenan et al., 2004; Schillinger, 1999; Shah et al., 2000; Shin et al., 2000). LGG does not metabolize lactose and casein, which limits cell growth and number in dairy products (Liptáková et al., 2008). It is known that LGG carries the *lacTEGF* gene cluster encoding the lactose-PTS operon (Kankainen et al., 2009), while the two mutations occurred in *lacG* (a phospho-β-galactosidase gene) and *lacT* (an anti-terminator gene) cause the lactose metabolization deficiency of LGG. The reason of casein metabolization deficiency of LGG has not been clarified. Using comparative genomic analysis, Kankainen et al., 2009 did not find evident differences in the predicted enzymatic system for casein digestion between LGG and *Lacticaseibacillus rhamnosus* LC705, while the strain LC705 is capable of using casein. LGG does not carry the gene *LC705_02680* encoding a subtilisin-like serine protease. However, this gene presents in the strain LC705, where it is predicted to be involved in casein degradation of LC705. Both strains carry intact genes encoding the cell envelope serine protease (PrtP) and maturation protein (PrtM), which are supposed to be responsible for casein degradation, for example in *L. lactis* (Wegmann et al., 2012). Nevertheless, it is not clear if *PrtP* or *PrtM* is expressed in LGG.

Efforts have been made to fix such deficiency of LGG. At genetic level, Heidtman et al., 2015 patented a LGG strain, which is capable of growing on lactose. This strain was generated by spontaneous mutagenesis using a *lacT* expression plasmid. Focusing on improving the growth of LGG in milk is another way. To realize such purpose, it is suggested that the milk matrices should be supplied carbohydrates that LGG could use such as glucose, or proteolytic enzymes, or partially hydrolyzed nitrogen sources such as yeast extract. Besides, Kort et al., 2015 used *S. thermophilus* C106 as adjuvant culture with LGG in fermenting milk in Africa, and LGG could reach a final density of 1×10^9 CFU/ml. Genome-scale metabolic model of LGG and *S. thermophilus* C106 revealed that the folic acid, succinate, glycerol, and galactose released by *S. thermophilus* C106 during fermentation might be the reason of improved growth of LGG. Therefore, it is also feasible to select suitable starter strains to facilitate the growth of LGG in milk due to the starter's native proteolytic activity, lactose degradation ability and other synergistic effects.

As summarized in **1.2**, the properties of probiotics can be modified and improved by genetic engineering. If the ability deficiency in lactose/casein metabolization could be fixed by genetic engineering, LGG could be applied in milk fermentation to exhibit its dual effects in probiotic benefits with enhanced proliferation, and in reducing the chance of lactose intolerance/CMPA. In addition to the method of Heidtman et al., 2015, non-GMO transformation methods are also ideal techniques, for example, transduction and conjugation of natural phages, plasmids, or transposons (Börner et al., 2019). Conjugation naturally exists in bacteria, and the most common conjugation is the antibiotic-resistance transferring. Unlike the vertical transferring of DNA from parent to offspring, bacterial conjugation is a type of horizontal gene transfer (Keeling and Palmer, 2008) between cells, even though they are not genetically related. Regarding the topic of lactose/casein metabolization deficiency, conjugation has been used to transfer a pLP712 plasmid from a dairy strain *L. lactis* NCDO 712 into lactose-deficient strains (Gasson, 1990). The pLP712 plasmid contains the gene that encodes the protease for casein degradation as well as the genes for lactose catabolism. Thus, the dairy strain

L. lactis NCDO 712 carrying pLP712 could be considered as a donor for LGG to solve its lactose/casein metabolization issue by conjugation.

1.5 Use of LGG and *S. boulardii* in food

The idea of introducing probiotics into food as a strategy to facilitate food processing or preservation has been well accepted in the food industry. The purpose is to develop products containing live probiotics or their metabolites to realize the concept of "health-promoting properties"; or utilize the antimicrobial characteristic of certain probiotics like bacteriocins-secreting LAB to improve the food safety and shelf life.

LGG has been widely used in food and many products have been developed, for example, in Finland there is a "Gefilus®" product series (Valio Oy, Helsinki, Finland), including juice, yogurt, buttermilk and kefir, in which LGG has been supplied. In academia, the effects of LGG in food processing and food preservation have been evaluated (**Table 3**).

Table 3. The effects of LGG used in food.

Purpose/product	Description	References
Food processing		
Mixed jussara and mango juice	LGG added presented a better survival rate under optimized condition obtained from this study	Prates et al., 2020
Hawthorn berry tea	A functional food containing LGG with enhanced storage stability was developed	Lai et al., 2020
Fermented juice with blueberry pomace	LGG-fermented juice showed improved active ingredients, antioxidant and cholesterol clearance capacity, and anti-fatigue effects in mice	Yan et al., 2019
Yoba probiotic yogurt containing LGG	*S. thermophilus* C106 as used as an adjuvant culture of LGG to ferment yogurt; children consuming the yogurt showed significant reduction in skin allergies and diarrhea	Kort et al., 2015; Westerik et al., 2018
Orange juice	LGG added showed excellent survival in orange juice; encapsulated LGG reduced the acidification and negative sensory properties of juice	Sohail et al., 2012
Yoghurt containing LGG	Combination of fiber-rich rye bread and LGG yoghurt improves bowel function in women suffering from constipation	Hongisto et al., 2006

Food preservation		
Fresh-cut bell pepper	The combination of biochemical additives and LGG reduced the total counts of bacteria (25.10%), *Salmonella* (38.32%), *Listeria* (23.75%), fungus (61.90%) in fresh-cut bell pepper	Saravanakumar et al., 2020
Fresh-cut pear	LGG reduced the *L. monocytogenes* population by 1.8 logs in fresh-cut pear without compromising its quality	Iglesias et al., 2018
Fresh-cut pear	LGG population added on fresh-cut pear remained constant in 7-day storage at 10 °C; LGG also reduced the resistance of the *L. monocytogenes* to GI simulation at the end of the storage	Iglesias et al., 2017
Others		
Edible film	Several edible films incorporated with LGG to introduce LGG into processed food were developed	Soukoulis et al., 2017
Oils (canola oil, flaxseed oil etc.)	The effects of moisture content and cell conditions on the survival of LGG with oils as matrices were evaluated	Endo et al., 2014

While *S. boulardii* is usually used by oral administration as capsules or pills, in human foods the use of *S. boulardii* has not been largely evaluated. Only few studies have investigated the function of *S. boulardii* in fruit juice and fermented beverage (Değirmencioğlu et al., 2016; Fratianni et al., 2014), dairy products (Karaolis et al., 2013; Lourens-Hattingh and Viljoen, 2001; Parrella et al., 2012), cereals (Heenan et al., 2004), legumes (Rekha and Vijayalakshmi, 2010) and products derived from them (Ryan et al., 2011). In these studies, *S. boulardii* was not just used as probiotic additive, but also acted as a core player to alter the food components and produce bioactive substances (Lazo-Vélez et al., 2018). When Rekha and Vijayalakshmi, 2010 used the combination of lactic acid bacteria and *S. boulardii* to ferment soymilk, they observed an increase in bioactive isoflavone and mineral bioavailability, and decrease in the antinutrient phytic acid. In the black carrot juice fermented by *S. boulardii*, the final concentration of total phenolic compounds was increased, which led to a high antioxidant activity (Değirmencioğlu et al., 2016). Ryan et al., 2011 fermented rice bran for human use by *S. boulardii* and an enhanced metabolite diversity after fermentation was found. Notably, the extracts from *S. boulardii*-fermented rice bran inhibited lymphoma viability, possibly due to the increased ferulic acid contents compared to

nonfermented extracts.

Yeast, due to its ability to metabolize sugars into carbon dioxide and alcohol, is crucial in manufacturing alcoholic beverages (Walker and Stewart, 2016). *S. cerevisiae* is the dominant species that is used for the production of beer, wine, whisky, gin and so on. Recent research has revealed the close phylogenetic relationship of *S. boulardii* with *S. cerevisiae* wine strains. Therefore, the use of *S. boulardii* directly to perform alcoholic beverages fermentation holds promise. Osnaya et al., 2017 had determined the growth kinetics of *S. boulardii* in the wort of barley malt. The yeast was found to be well adapted, and it was able to hydrolyze the carbohydrates in wort. During recent years, scientists have started to put effort on investigating the possibility of using *S. boulardii*, either solely or in combination with *S. cerevisiae*, to brew "probiotic beer" including craft beer (Capece et al., 2018; Mulero-Cerezo et al., 2019; Ramírez-Cota et al., 2021), alcohol-free beer (Senkarcinova et al., 2019), and wheat beer (de Paula et al., 2021). When applied in combination, *S. boulardii* did not have negative effects on the beer aroma as shown by the analysis of main volatile compounds, and the mixed starter increased the antioxidant activity and polyphenols content of the final product (Capece et al., 2018). The performance of *S. boulardii* when applied solely has also been evaluated. The viable cell count of *S. boulardii* in the final product, which matters for probiotics, has been found to be maintained at a similar level to *S. cerevisiae*, *i.e.*, 8.3×10^6 CFU/ml and ranged from 8.0×10^6 to 7.0×10^6 CFU/ml in the study of Mulero-Cerezo et al., 2019 and Capece et al., 2018, respectively. Interestingly, Mulero-Cerezo et al., 2019 also found a higher antioxidant activity, lower alcohol content, but similar sensory attributes in the *S. boulardii*-fermented beer compared to the *S. cerevisiae*-fermented beer. It seems that *S. boulardii*-fermented beer may have a better health value without compromised the flavor. Ramírez-Cota et al., 2021 investigated the ethanol tolerance of *S. boulardii* in beer, and they found *S. boulardii* could withstand 6%-8% (v/v) of ethanol. On the other hand, beer was often used in meat marinating, and studies have shown it could reduce the formation of carcinogens in meat during cooking (Melo et al., 2008; Viegas et al., 2015; Viegas et al., 2014; Wang et al., 2019).

Based on the aforementioned studies, it is feasible to produce “probiotic beer” by *S. boulardii*, in this way *S. boulardii* may display its dual effects as a probiotic and a starter. Yet, it still needs more investigations to elaborate the metabolisms of *S. boulardii* in beer and the optimized parameters to proceed beer fermentation.

2 AIMS OF THE STUDY

The main aims of this doctoral study were to modify and develop the properties of probiotic strains *Saccharomyces boulardii* CNCM I-745 (**I, II**) and *Lacticaseibacillus rhamnosus* GG (LGG) (**III**). Detailed aims were as follows:

a) study the possibility of cloning and expressing the gene encoding the *Leuconostoc* bacteriocin leucocin C, using wild-type *S. boulardii* CNCM I-745 and its CRISPR-Cas9 modified *URA3* auxotrophic derivative as host;

b) utilize leucocin C-secreting *S. boulardii* to ferment beer and evaluate its efficacy in *Listeria* decontamination in chicken meat;

c) fix the lactose and casein utilization deficiency of LGG, and generate its non-GMO derivative which could grow well in milk.

3 MATERIALS AND METHODS

All the strains and plasmids are listed in **Table 4** and **Table 5**. Experimental methods are summarized in **Table 6**, and details have been elaborated in publications I, II and III. Primers and sequences are listed in **Table 7**.

Table 4. Strains used in this thesis.

Strains	Descriptions	Source/reference	Used in
Escherichia coli DH5α	Library Efficiency™ DH5α™ Competent Cells; intermediate host for preserving plasmids	Invitrogen, Carlsbad, CA, USA	I, II
S. boulardii SAA655	*S. boulardii* CNCM I-745, wild-type probiotic strain	Capsule PRECOSA, Biocodex, Espoo, Finland	I, II
S. boulardii SAC2	SAA655 carrying pSF-TEF1-TPI1-Blast (OG539), vector control	This study	I (named as Sb-vector)
S. boulardii SAC4	SAA655 carrying pSF-TEF1-TPI1-Blast-*lecC* for leucocin C expression	This study	I (named as Sb-LecC), II
S. boulardii SAA940	*URA3* auxotrophic strain of *S. boulardii* CNCM I-745 generated by CRISPR-Cas9	Bagherpour et al., 2018; Prof. Vahid Khalaj, Pasteur Institute of Iran, Tehran, Iran	II
S. boulardii SAC10	SAA940 carrying pSF-TEF1-URA3, vector control	This study	II
S. boulardii SAC12	SAA940 carrying pSF-TEF1-URA3-*lecC*, used for leucocin C secretion based on uracil selection	This study	II
Listeria monocytogenes WSLC 1018	Indicator strain, sensitive to leucocin C, ATCC 19118	Prof. Martin Loessner, ETH Zurich, Switzerland	I, II
L. lactis LAC409	Recombinant *L. lactis* NZ9000 that produces leucocin C	Wan et al., 2013	I, II
L. lactis NCDO 712	*L. lactis* subsp. *cremoris* NCDO 712, plasmid donor strain, lactose/casein positive	Gasson, 1983	III
L. lactis MG1614	Plasmid-cured derivative of *L. lactis* NCDO712, streptomycin and rifampicin resistant, used as positive control in conjugation	Gasson, 1983	III
LGG	*Lacticaseibacillus rhamnosus* GG, ATCC 53103	Gorbach and Goldin, 1989	III

Lacticaseibacillus rhamnosus LAB49	LGG that has been conjugated with plasmid pLP712 from *L. lactis* NCDO 712, lactose/casein positive	This study	III

Table 5. Plasmids used in this thesis.

Plasmids	Descriptions	Source/reference	Used in
pSF-TEF1-TPI1-Blast (OG539)	*E. coli*-yeast shuttle vector, antibiotic blasticidin S selection	Oxford Genetics, Oxford, UK	I
pSF-TEF1-TPI1-Blast-*lecC*	pSF-TEF1-TPI1-Blast plasmid that has been inserted with leucocin C expression cassette	GenScript, Piscataway, NJ, USA	I
pSF-TEF1-URA3	*E. coli*-yeast shuttle vector, uracil selection	Oxford Genetics, Oxford, UK	II
pSF-TEF1-URA3-*lecC*	pSF-TEF1-URA3 plasmid that has been inserted with leucocin C expression cassette	GenScript, Piscataway, NJ, USA	II

Table 6. Methods used in this thesis.

Methods	Source/reference	Used in
Molecular cloning, nucleic acid techniques, *e.g.*, PCR, enzymatic modifications, electrophoresis	Sambrook et al., 1989; manuals of the enzyme supplier	I, II, III
Electroporation		
E. coli	Zabarovsky and Winberg, 1990	I, II
S. boulardii	Kawai et al., 2010	I, II
DNA isolation		
E. coli	GeneJET Plasmid Miniprep Kit, Thermo Fischer Scientific	I, II
L. lactis NCDO 712	Anderson and Mckay, 1983; Wan et al., 2013	III
LGG	Anderson and Mckay, 1983; Wan et al., 2013	III
Lacticaseibacillus rhamnosus LAB49	Anderson and Mckay, 1983; Wan et al., 2013	III
Ammonium sulfate precipitation	Budde et al., 2003; Wan et al., 2013	I, II
Agar well diffusion assay	Lehrer et al., 1991	I, II
Tricine-SDS-PAGE	Schägger, 2006	I
Glycine-SDS-PAGE	Laemmli, 1970	III

Gel overlay assay	Bhunia et al., 1987	I, II
Bacteria broth conjugation	McKay et al., 1980	III
Agar halo method for verifying protease activity	van den Berg et al., 1993	III
Casein degradation assay	Vukotic et al., 2015	III
Growth rate determination	Zierdt and Swan, 1981	III
Ethanol analysis by HPLC	Mattila et al., 2018	II
Inhibition of *L. monocytogenes* attached to meat	Nielsen et al., 1990	II

Table 7. PCR primers sequences and source.

Primers	Sequence 5' end to 3' end	Reference	Size	Use
pSF specific-F	CATATCACATAGGAAGCAACAG	This study	1400 bp	*lecC* insertion verification
pSF specific-R	CTACGATACCGATAGAGATGG			
***spaC*-FW**	CCAAATTGGCAACAGACCTT	de Vos et al., 2010	801 bp	LGG- specific
***spaC*-RV**	GCCATCTGGTGCTTTTGTTT			
GG-spec-A	CGCCCTTAACAGCAGTCTTC	Ahlroos and Tynkkynen, 2009	757 bp	LGG- specific
GG-spec-B	GCCCTCCGTATGCTTAAACC			
***prtP*-FW**	CTCGAGGCTAGCTCGTTTGATTTAATTGTG	This study	2550 bp	pLP712 specific
***prtP*-RV**	CCAGAATTCGGGCCCTATTCTTCACGTTGTTTCCG			
***repAC*-FW**	CGTTTCTGAGACGTTTTAGCG	This study	1680 bp	pSH71 specific
***repAC*-RV**	AAATAAAAGCCCCCTTCGACT			
pSH72-FW	GCTTTTTCGTTGGTTTGCTC	Tarazanova et al., 2016	466 bp	pSH72 specific
pSH72-RV	GCCCAAAATAGTGGGTTAGTG			
pSH73-FW	TTTCAGTAGAAGGCCAAACAAC	Tarazanova et al., 2016	803 bp	pSH73 specific
pSH73-RV	TGCAAATTTATCTACAAAGGCTTG			
pSH74-newFW	GTGGATGAACAAACAAAATACG	This study	195 bp	pSH74 specific
pSH74-newRV	GGATTGTGTCGATTTGCTTTACGC			
pNZ712-FW	CACTCTAGTTTCCTACCTTCGTTGCAAGC	Tarazanova et al., 2016	1120 bp	pNZ712 specific
pNZ712-RV	GCTATACTTATACGGAGGATTAGCACTGG			

4 RESULTS AND DISCUSSION

4.1 Heterologous expression of leucocin C in *S. boulardii* (I, II)

The DNA sequence of the complete leucocin C gene cluster from *Ln. carnosum* 4010 has been reported by Wan et al., 2013 (GenBank accession number: JQ061256.3). The leucocin C precursor gene has 204 bp nucleotides encoding 67 amino acids, in which there is a signal peptide with 24 aa in length. In this present study, the 132 bp gene fragment (*lecC*) encoding the mature peptide of leucocin C was selected to perform the molecular cloning in *S. boulardii*. To guide the extracellular secretion in yeast cells, a α-mating factor signal sequence from *S. cerevisiae* (Brake et al., 1984) was fused before *lecC* (**Figure 3**).

For heterologous expression of leucocin C in *S. boulardii* SAA655 (**I**) and SAA940 (**II**), the vector pSF-TEF1-TPI1-Blast (pSF-Blast) (**Figure 3**) and pSF-TEF1-URA3 (pSF-URA3) were used, respectively. These two vectors are both equipped with basic plasmid components, *e.g.*, kanamycin-resistant gene (KanR) for bacterial transformants selection, constitutive promoter TEF1 (yeast translation elongation factor 1), and 2Micron replicon (2μ). The difference between the plasmids is that the selection marker is dedicated to different yeast hosts. For the purpose of antibiotic selection, blasticidin S resistance cassette (Blast) was provided in pSF-Blast used in wild-type *S. boulardii* SAA655. While for uracil selection, *URA3* gene encoding orotidine-5'-phosphate decarboxylase was provided in pSF-URA3 to allow the growth of the auxotrophic strain *S. boulardii* SAA940 in medium without uracil. Taken pSF-Blast as the example, the cloning strategy is shown in **Figure 3**.

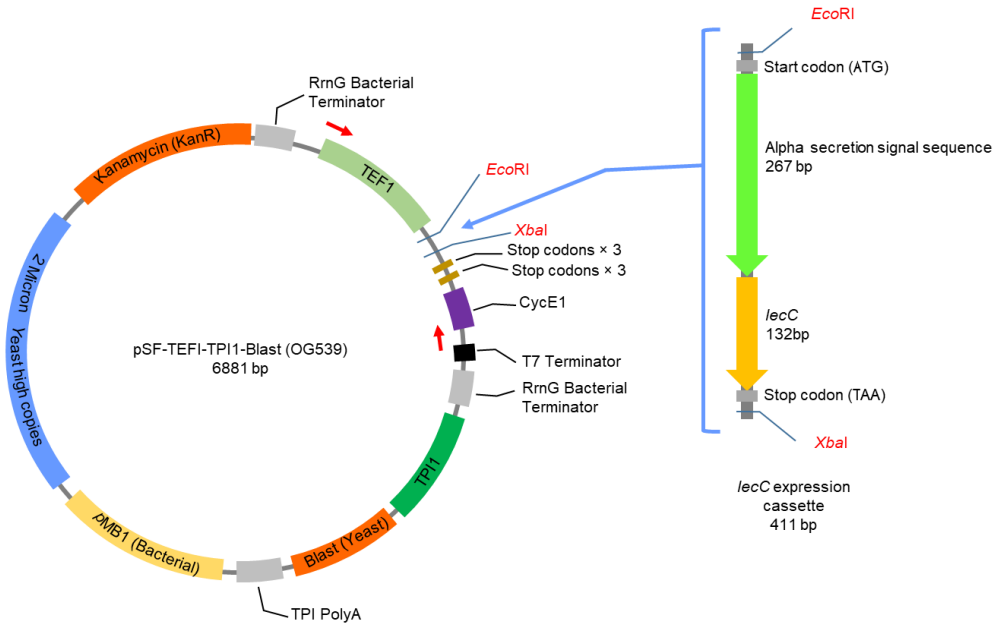

Figure 3. Cloning of *lecC* expression cassette into pSF-TEF1-TPI1-Blast. The expression cassette containing DNA fragment of *lecC* and α-mating factor was cloned into in the vector pSF-TEF1-TPI1-Blast (OG539) at the enzyme sites *Eco*RI and *Xba*I. Red arrows indicated the binding sites of plasmid specific primers pSF specific-F/R (Table 7).

The probiotic yeast *S. boulardii* is not typical host for heterologous gene expression, since only few genetic tools are developed or examined for *S. boulardii*. However, *S. boulardii* tolerates heat and acid better than *S. cerevisiae* (Edwards-Ingram et al., 2007), and it can be easily cured from intestine (Klein et al., 1993). These features make *S. boulardii* an ideal vehicle for bioactive molecules delivery. Therefore, the genetic manipulation of *S. boulardii* has become more and more attractive for scientists. We first used the antibiotic selection system, which is commonly used (Douradinha et al., 2014; Latorre-García et al., 2008; Michael et al., 2013; Pöhlmann et al., 2013; Wang et al., 2014) when the auxotrophic strains of *S. boulardii* CNCM I-745 are not yet available. The constructed strain *S. boulardii* SAC4 (named as Sb-LecC in publication I) secreted the *Leuconostoc* bacteriocin leucocin C, which actively inhibited the growth of *L. monocytogenes* (**Figure 4b**; also in publication I, Fig. S2 in supplementary materials). This was the first study presenting the secretion of antimicrobial peptides in

S. boulardii.

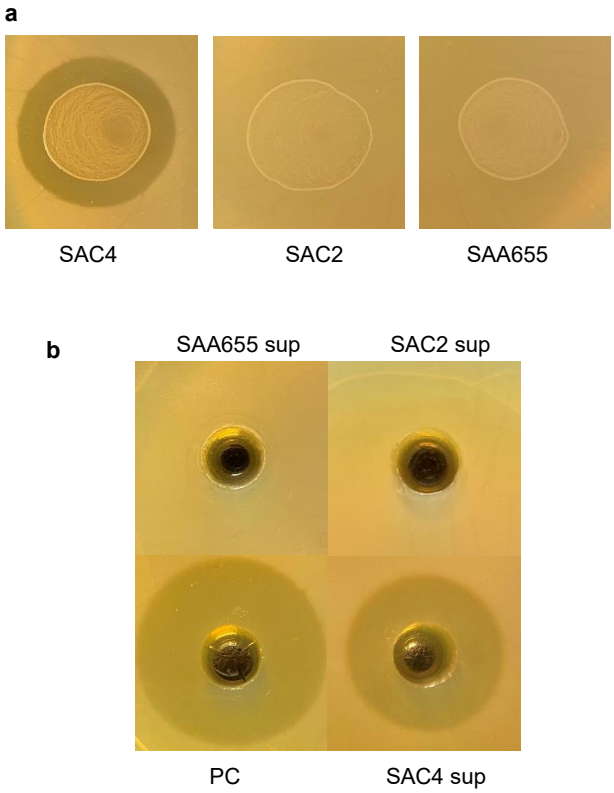

Figure 4. Anti-listerial test. (a) Agar inhibition assay. The overnight culture of yeast was added on *Listeria* indicator plate after washing to indicate the inhibition of *Listeria* by directly using *S. boulardii* SAC4 cells. The cell density of SAC4 culture was 5.1×10^7 CFU/ml, SAC2 was 5.4×10^7 CFU/ml, and SAA655 was 7.4×10^7 CFU/ml. An inhibition halo was observed only around SAC4 cell lawn. **(b)** Agar well diffusion assay. Supernatant from SAA655, SAC2, and SAC5 was collected and concentrated by ammonium sulfate precipitation. SAA655 sup: 90 µl of the supernatant from wild-type *S. boulardii* CNCM I-745; SAC2 sup: 90 µl of the supernatant from SAC2 strain; PC: positive control, 10 µl of the supernatant from the leucocin C producing *L. lactis* LAC409 strain; SAC4 sup: 90 µl of the supernatant from SAC4.

Later we obtained a *URA3* auxotrophic strain of *S. boulardii* CNCM I-745. This strain was named as *S. boulardii* SAA940, and the same cloning strategy was applied to yield a *S. boulardii* SAC12 strain (**II**). The auxotrophic host SAA940 originated from the wild-type strain *S. boulardii* CNCM I-745, which is the same wild-type strain we used previously in cloning with antibiotic selection. Therefore, the different behaviors of *S.*

boulardii under two different selection systems can be compared. As expected, anti-listerial peptide leucocin C could be secreted by the new constructed strain *S. boulardii* SAC12 (publication II, Fig. 2). Interestingly, we found that the anti-listerial effect of leucocin C from SAC12 was much stronger than leucocin C from SAC4 (publication II, Fig. 3A). Even though SAC4 and SAC12 grew in different selective media with different pattern, both strains could reach stationary phase within 36-h incubation (publication II, Fig. 3B). The final cell density of SAC4 and SAC12 was not decided, the reason was that we considered the ability to grow in each strain's selective medium also matters when compare the leucocin C-secreting ability. Since the initial cell density of SAC4 and SAC12 after inoculation was similar, their leucocin C-secreting ability was comparable within the same growth period. Besides the differences in leucocin C secretion level, antibiotic blasticidin S selection used for SAC4 strain was expensive and not environmentally friendly. The presence of the antibiotic also made the recovery of secreted product more complicated, since the antibiotic remained in the yeast supernatant. Considering the two important features of *URA3* selection system in this study: antibiotic free and higher production of leucocin C, it might potentially act as a superior gene expression method for *S. boulardii* CNCM I-745 and expand its usage. Based on this consideration, we decided to test the possibility of using SAC12 in beer fermentation and use the beer containing leucocin C in food matrix, as higher production of leucocin C might help killing more *L. monocytogenes* (**II**).

Strictly speaking, the word "probiotics" is not a term based on taxonomy, but refers more to functionality. Regarding this topic, O'Toole et al., 2017 proposed an interesting idea that "nothing in the definition of the term probiotics limits the species, genus or even kingdom from which probiotics can be selected, nor does it dictate whether they must be native strains or whether they can have been subjected to any form of genetic manipulation." Therefore, studies about commensals or genetically engineered probiotics that could be used as NGPs are emerging. Specifically, engineered probiotic microorganisms secreting bacteriocins, for example *L. lactis* secreting Hiracin JM79 (Sánchez et al., 2008) and lactococcin A (Chikindas et al., 1995), and

Lactiplantibacillus plantarum secreting ABP-118 (Flynn et al., 2002), could be applied as a dual therapy to control bacterial infections. Direct antimicrobial effects have not been reported for the probiotic yeast *S. boulardii*, only studies showing its binding to some enteric pathogens like *Salmonella* (Martins et al., 2013), *E. coli* (Dahan et al., 2003), and *Shigella* (Mumy et al., 2008) are available. In our case, we focused on improving the probiotic effects of *S. boulardii* by adding the ability to kill *Listeria*. Expression of leucocin C enabled *S. boulardii* to directly kill *Listeria* even without selection pressure (publication I, Fig. 3). The engineered leucocin C secreting *S. boulardii* could be developed as a type of NGPs for controlling human listeriosis.

4.2 Beer fermentation by leucocin C-secreting *S. boulardii* (II)

Our strategy of constructing leucocin C-secreting *S. boulardii* was successful, presenting the first anti-listerial probiotic *S. boulardii*. Especially, the *S. boulardii* SAC12 secreted more leucocin C than the *S. boulardii* SAC4, and antibiotic selection was not needed. Even though, usually *S.cerevisiae* is used in beer fermentation instead of *S. boulardii,* the beer fermented by *S. boulardii* SAC12 had an average ethanol contents of 4.3% (v/v). The typical content of ethanol produced by yeast during fermentation is 3.5%-5.0% (v/v) (Menz et al., 2009). Thus, using *S. boulardii* SAC12 to perform beer fermentation seems to be feasible. More importantly, the beer fermented by *S. boulardii* SAC12 carried anti-listerial effect due to the accumulation of the secreted leucocin C in beer (publication II, Fig. 4). When the beer was used in real food matrix, it displayed an inhibition of up to 2.2 log units, against the *L. monocytogenes* contaminated in chicken breast strips (publication II, Fig. 6). On the other hand, the beer might also be equipped with the probiotic substances secreted by *S. boulardii* as a kind of "probiotic anti-listerial beer".

4.3 Generation of lactose and protease positive LGG (III)

Genetic manipulation is one way for bringing new features to probiotics, but public acceptance of GMOs is low and the regulatory requirements make the process to the market slow and expensive (Shew et al., 2018). Therefore, it is preferable to add new

properties to probiotics using non-GMO techniques. Conjugation happens naturally in the world of microorganisms, and it could be used as an excellent tool if proper features are transferred. Based on this consideration, we made such attempt to make the wild-type LGG lactose and PrtP protease positive by conjugal transfer of the lactose-protease plasmid pLP712 from *Lactococcus lactis* NCDO 712. The key part of conjugation is the selection of the expected recipient, because the contact between the donor strain and recipient is essential for horizontal gene transfer. Here in our study, we used vancomycin to counter select the donor strain from the bacteria mixture and lactose to select the transconjugant LGG (publication III, Fig. 2). The presence of the pLP712 plasmid in the transconjugant was confirmed by PCR (publication III, Fig. 3).

Later we isolated the total DNA from the transconjugant LGG: *L. rhamnosus* LAB49. The transconjugant strain LAB49 also carried several plasmids, while from the wild-type LGG no plasmids were obtained, only chromosomal DNA (publication III, Fig. 4). The explanation was that other plasmids of NCDO 712 were mobilized along with the conjugation of pLP712. This phenomenon was also observed in the study of Barry et al., 2019, in which the mobilization of non-conjugative plasmids along with the conjugative plasmid occurred in all 143 conjugation tests. After confirmation of the successful conjugation, the phenotypes associated with pLP712 plasmid were characterized. The lactose utilization ability was easily identified by the growth of LAB49 on xMRS-lactose-BCP plate (MRS without glucose and meat extract, and supplemented with 1% lactose, 100 mg/ml vancomycin, and 50 mg/ml bromocresol purple) (publication III, Fig. 2), and the proteolytic activity of LAB49 was demonstrated by its degradation of β-casein on SDS-PAGE (publication III, Fig. 7). As LAB49 was equipped with these two new features, we tested if LAB49 could grow better than LGG in milk. LAB49 and LGG were inoculated separately into milk and incubated at 37 °C for 36 h. The final cell density is presented in **Figure 5.** The results showed that the transconjugant LAB49 reached 43-fold higher CFU/ml, than the parental strain LGG ($p<0.05$) after 36-h growth in milk. While for LGG, a slight growth was observed from 6.35×10^6 CFU/ml to 5.28×10^7 CFU/ml. The slight growth was also

found when LGG was cultured in xMRS-lactose medium, which means that LGG can grow, although poorly, in this medium with lactose as the main carbon source. In addition, only LAB49 coagulated the milk, whereas LGG-milk was still liquid after 36 h at 37 °C (unpublished).

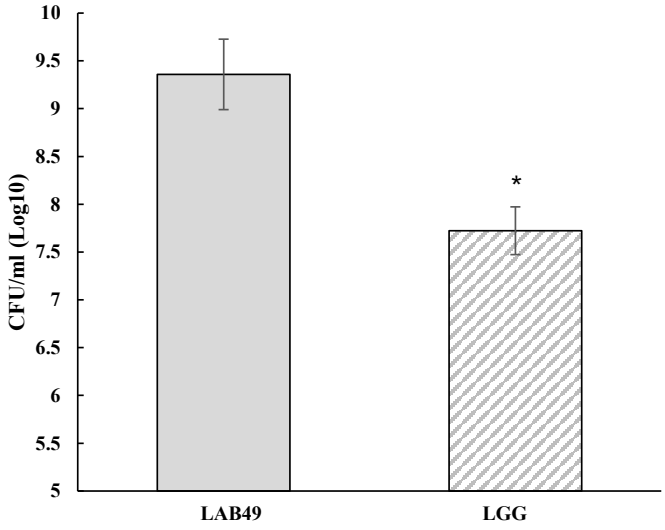

Figure 5. Growth of LAB49 and LGG in milk. The starting cell density inoculated in milk of LGG was 6.35×10^6 CFU/ml, and LAB49 was 7.4×10^6 CFU/ml. The final cell densities of LGG and LAB49 were 5.28×10^7 CFU/ml and 2.28×10^9 CFU/ml, respectively. Error bars show calculated standard errors of the means, * represents the significant difference between the CFU/ml of each group ($P<0.05$).

The growth efficiency of LAB49 in milk was also investigated (**III**). A growth curve of time versus CFU/ml of LAB49 was generated to demonstrate its better growth efficiency (publication III, Fig. 8) than wild-type LGG. However, before LAB49 could display such a "normal" growth profile, it actually needed to be activated for 20 h in milk. When LAB49 was directly inoculated from medium into milk, it still needed a long lag phase before entering a rapid growth (**Figure 6**). Such phenomenon might be caused by the time needed to activate the transcription of *lac* gene cluster or *prtMP* genes, which was introduced externally into LAB49. The induction of carbohydrate metabolism when the cells encounter different sugar sources is common in LAB (Afzal et al., 2014; Gosalbes et al., 2001; Tsai and Lin, 2006). Another reason might be the lack of free amino acids in milk, which are needed for the bacterial growth. After

LAB49 produced PrtP, it takes some time before the enzyme has digested casein, followed by intake of peptide fragments and activity of peptidases to release free amino acids for growth.

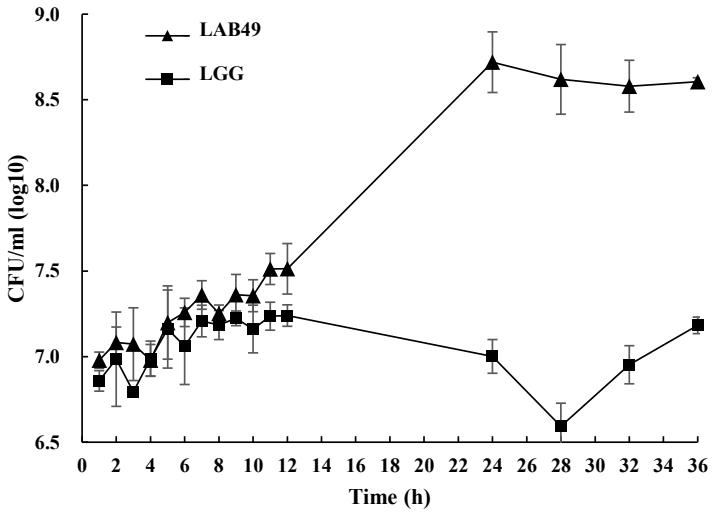

Figure 6. Growth of LAB49 and LGG that are directly inoculated from medium into milk. The starting cell densities of LGG and LAB49 were 5.0×10^6 CFU/ml and 6.31×10^6 CFU/ml, respectively**.** Both strains grew slightly during the first 12 hours, after which LAB49 started rapid growth until 24 h. LGG maintained low growth during the 36-h incubation. Error bars show calculated standard errors of the mean.

Compared to wild-type LGG, the lactose and casein positive *Lacticaseibacillus rhamnosus* LAB49 could exert new functions in dairy applications, such as, alleviating the symptoms of lactose intolerance or CMPA. Even though wild-type LGG has been reported to function in CMPA by mediating the gut immune response and thereby boosting the host tolerance (Scalabrin et al., 2017), it would still be interesting to test the efficacy of LAB49 as it directly hydrolyzes casein and might reduce more efficiently the risk of CMPA.

5 CONCLUSIONS AND FUTURE PROSPECTS

In this thesis, the main aim was to explore ways of modifying probiotic strains *S. boulardii* CNCM-I715 and LGG, and thereby develop their benefits or usage. Regarding *S. boulardii,* the genetic tools and selection systems we used were demonstrated to be valid in expressing heterogeneous genes in this probiotic yeast. The introduced *lecC* gene enabled the secretion of *Leuconostoc* bacteriocin leucocin C in *S. boulardii,* and the recombinant strain displayed sufficient capability to kill foodborne pathogen *L. monocytogenes* without selection pressure. One usage of the leucocin C secreting *S. boulardii* was to ferment "probiotic anti-listerial beer", which could help with excluding *L. monocytogenes* from contaminated meats upon marination. Here in this thesis, we demonstrated such possibility and thus the "probiotic anti-listerial beer" might be equipped with the dual effects of anti-listeria activity and probiotic benefits of the engineered *S. boulardii*. Another usage in the future is to test the *in vivo* efficacy of leucocin C secreting *S. boulardii* in mice model, and apply as a type of "next generation probiotic" or "live biotherapeutic product" aiming to deliver leucocin C into gastrointestinal tract and control *Listeria* infection.

As for LGG, a probiotic bacterium that is commercially used in food, a non-GMO derivative strain LAB49 was generated by bacterial conjugation. The strategy of applying *L. lactis* NCDO712 as donor strain made it possible to fix the deficiency of lactose and casein utilization of LGG. Even though the transfer frequency was low, with proper screening method it was possible to identify the transconjugants. The utilization of lactose and casein of LAB49 was verified at both gene and phenotype level and most importantly it grew better in milk than the wild-type LGG. Therefore, the *L. rhamnosus* LAB49 can be regarded as an upgraded food-grade and non-GMO derivative of LGG. Since the conjugated plasmid has provided new properties to LAB49 compared to LGG, it may raise a concern about the probiotic capacity of LAB49. In the further studies, it would be good to investigate whether LAB49 still holds the probiotic activity of LGG, *e.g.*, the adherence to epithelial cells. Moreover, the possibility of applying LAB49 in

fermenting “probiotic yogurt” and its benefits in reducing symptoms of lactose intolerance and CMPA would also be interesting to test *in vivo*.

6 Acknowledgements

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First, I deeply appreciate my supervisor Prof. Per Saris for his guidance, encouragement, and support during my four-year of pursuing PhD. His expertise in both academia and life enlightened me a lot when I was confused. Prof. Per Saris has offered me an insight of how to be a decent researcher in the future.

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