

**Gastrointestinal persistence of the probiotic bacteria
Lactobacillus plantarum 423 and *Enterococcus mundtii*
ST4SA, and their anti-listerial activity**

by

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Declaration

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December 2018

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Summary

Gastrointestinal diseases, and in particular those caused by bacterial infections, are a major cause of morbidity and mortality worldwide. Treatment is becoming increasingly difficult due to the increase in number of species developing resistance to antibiotics. Different treatment strategies need to be developed.

Probiotic lactic acid bacteria (LAB) have considerable potential as alternatives to antibiotics, both in prophylactic and therapeutic applications. Lactic acid bacteria have a long history of safe use in food and therapeutic products and is increasingly recognised for their beneficial effects. However, the underlying mechanisms by which probiotic LAB enhance the health of the consumer have not been fully elucidated. Demonstrating key antimicrobial and protective probiotic mechanisms *in vivo* will allow for industry and consumers to choose scientifically validated probiotics for the prevention or treatment of targeted gastrointestinal diseases. The present study aimed to contribute to this area of probiotic research.

The first part of this study focus on monitoring the survival, metabolic activities and persistence of *Lactobacillus plantarum* 423 and *Enterococcus mundtii* ST4SA in the gastrointestinal tract (GIT) of mice using a bioluminescence imaging (BLI) system. The route and destination of both probiotic strains in the GIT were determined after single and multiple doses. Both strains prominently colonized the cecum and colon. *Enterococcus mundtii* ST4SA persisted in the GIT and faeces the longest and in higher numbers while also actively colonizing the small intestine. This is the first report of *in vivo* and *ex vivo* BLI of *E. mundtii* ST4SA in a murine model.

The second part of the study encompassed the development of a novel system that facilitates the rapid and efficient isolation of double-crossover integration or deletion mutants of *L. plantarum* 423 and *E. mundtii* ST4SA. The system was useful in the construction of *L. plantarum* 423 and *E. mundtii* ST4SA bacteriocin and adhesion gene mutants. The newly described method expands the LAB molecular research genetic toolkit and has significant potential to allow genetic modification of most, if not all LAB species. This provides the unique opportunity to study the role of specific probiotic LAB genes in complex environments using reverse genetics analysis.

In the final part of the study, the ability of *L. plantarum* 423 and *E. mundtii* ST4SA to competitively exclude *L. monocytogenes* EGDe, an intestinal pathogen, from the GIT of mice

was proven. Valuable insight was gained on the molecular modes of action of the two probiotic strains. Plantaricin 423 and mundticin ST are bacteriocins produced by *L. plantarum* 423 and *E. mundtii* ST4SA, respectively. Bacteriocin-negative mutants of *L. plantarum* 423 and *E. mundtii* ST4SA failed to exclude *L. monocytogenes* EGDe from the GIT, confirming *in situ* bacteriocin production as an anti-infective mediator. Additional confirmation of *in situ* bacteriocin production as a mechanism of action was provided by using variant strains of *L. monocytogenes* EGDe expressing the immunity genes of plantaricin 423 and mundticin ST, respectively, which provided resistance to the respective bacteriocins. Furthermore, the exclusion of *L. monocytogenes* EGDe from the GIT was reduced when mice were administered with *L. plantarum* 423 and *E. mundtii* ST4SA adhesion gene knockout strains. These results substantiate our understanding of the functional attributes of probiotics currently available to consumers and the improvement of future probiotic products.

Opsomming

Spysverteringskanaal (SVK) siektes, en veral dié wat veroorsaak word deur bakteriële infeksies, is wêreldwyd 'n belangrike oorsaak van morbiditeit en mortaliteit. Behandeling van SVK siektes word toenemend moeiliker weens 'n toename in die getal spesies wat weerstand ontwikkel teen antibiotika. Verskillende behandel strategieë moet ontwikkel word.

Probiotiese melksuur bakterieë (MSB) het potensiaal as 'n alternatief vir antibiotika, in profilaktiese- sowel as terapeutiese-toepassings. Melksuur bakterieë het 'n lang geskiedenis van veilige gebruik in voedsel- en terapeutiese produkte en geniet toenemend erkenning vir hul voordelige uitwerking op die mens se gesondheid. Die onderliggende meganismes waarvolgens probiotiese MSB die gesondheid van die verbruiker verbeter, is egter nie ten volle toegelig nie. Demonstrasie van belangrike antimikrobiese en beskermende probiotiese meganismes *in vivo* sal die industrie en verbruikers toelaat om wetenskaplik gevalideerde probiotika te kies vir die voorkoming of behandeling van geteikende SVK siektes. Die huidige studie beoog om by te dra tot hierdie gebied van probiotiese navorsing.

Die eerste gedeelte van die studie fokus op die monitering van die oorlewing, metaboliese aktiwiteite en volharding van *Lactobacillus plantarum* 423 en *Enterococcus mundtii* ST4SA in die SVK van muise deur gebruik te maak van 'n bioluminisensie beeldvorming (BLB) stelsel. Die roete en ligging van beide probiotiese stamme in die SVK is suksesvol bepaal ná enkel- en veelvuldige dosisse. Beide stamme is prominent teenwoordig in die cecum en kolon. *Enterococcus mundtii* ST4SA het die langste, en teen hoër selgetalle, in die SVK oorleef, terwyl die dunderm ook aktief gekoloniseer is. Hierdie is die eerste bekendmaking van *in vivo* en *ex vivo* BLB van *E. mundtii* ST4SA in 'n muis model.

Die tweede deel van die studie het betrekking op die ontwikkeling van 'n nuwe sisteem wat die vinnige en doeltreffende isolasie van dubbel oorkruis-integrasie of delesie mutante van *L. plantarum* 423 en *E. mundtii* ST4SA insluit. Die sisteem is nuttig vir die konstruksie van *L. plantarum* 423 en *E. mundtii* ST4SA bakteriosien- en adhesie mutante. Die nuut beskryfde metode maak 'n belangrike bydrae tot die molekulêre tegnieke vir MSB en het die potensiaal om genetiese modifikasie van die meeste, indien nie alle, MSB-spesies, toe te laat. Dit bied die unieke geleentheid om die rol van spesifieke probiotiese MSB-gene in komplekse omgewings te bestudeer deur middel van omgekeerde genetiese analise.

In die finale deel van die studie is bewys dat *L. plantarum* 423 en *E. mundtii* ST4SA die vermoë het om *L. monocytogenes* EGDe, 'n patoëen, uit die SVK te verwyder. Waardevolle insig is verkry rakende die molekulêre werkswyse van die twee probiotiese stamme. Plantaricin 423 and mundticin ST is bakteriosiene wat onderskeidelik deur *L. plantarum* 423 en *E. mundtii* ST4SA geproduseer word. Bakteriosien-negatiewe mutante van *L. plantarum* 423 en *E. mundtii* ST4SA het nie daarin geslaag om *L. monocytogenes* EGDe in die SVK te onderdruk nie. Dit bevestig dat die *in situ* produksie van bakteriosiene 'n belangrike rol speel in die bekamping van infeksies in die SVK. Ter bevestiging hiervan het stamme van *L. monocytogenes* EGDe, waarin die immuniteitsgene van plantaricin 423 en mundticin ST gekloneer is, weerstand gebied teen die twee bakteriosiene. Verdermeer het pogings om *L. monocytogenes* EGDe van die SVK te verwyder afgeneem met die dosering van *L. plantarum* 423 en *E. mundtii* ST4SA waarvan adhesie gene verwyder is. Hierdie resultate ondersteun ons begrip van die funksionele eienskappe van probiotika wat tans beskikbaar is vir verbruikers en die verbetering van toekomstige probiotiese produkte.

Biographical sketch

Winschau Fayghan van Zyl was born in Cape Town, South Africa on the 11th of February, 1989. He matriculated at St. Andrews High School, South Africa, in 2006. In 2007 he enrolled as B.Sc. student in Molecular Biology and Biotechnology at the University of Stellenbosch and obtained the degree in 2011. In 2012 he obtained his B.Sc (Hons) in Microbiology, also at the University of Stellenbosch. In January 2013 he enrolled as M.Sc. student in Microbiology at the University of Stellenbosch, receiving his M. Sc. (Cum Laude) in 2015. In 2015 he enrolled as a Ph.D student in Microbiology at the University of Stellenbosch.

Preface

This dissertation is represented as a compilation of 6 chapters. Each chapter is introduced separately.

Chapter 1: Introduction

Chapter 2: Literature Review: Probiotic-Pathogen Interactions

Chapter 3: Spatial and Temporal Colonization of *Lactobacillus plantarum* 423 and *Enterococcus mundtii* ST4SA in the Intestinal Tract, as Revealed by *In vivo* Bioluminescence Imaging in a Murine Model

Chapter 4: Development of a Novel Selection/Counter-selection System for Efficient Isolation of Double-crossover Homologously Recombined Mutants in Lactic Acid Bacteria, Enabling Directed Chromosomal Gene Deletions and DNA Integrations

Chapter 5: Anti-infective Gastrointestinal Properties of *Lactobacillus plantarum* 423 and *Enterococcus mundtii* ST4SA, as Determined in Mice Infected with *Listeria monocytogenes* EGDe

Chapter 6: General Discussion and Conclusions

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Chapter 1

Introduction

Introduction

Motivation for the study

The term probiotics stems from the Greek word “pro-bios”, meaning- for life and is defined as viable microorganisms which, when administered in sufficient amounts, confer health-promoting effects on the host (1). Probiotics play a major role in maintaining a healthy and stable intestinal microbiota, primarily by preventing infection (2). Several studies have shown that probiotics, when administered orally, can be used for the prevention or treatment of infectious diseases, acute or antibiotic-associated diarrhoea, the alleviation of constipation, bloating and atopic dermatitis, colon cancer and irritable bowel syndrome, also known as IBS (3-8). As a result of these findings, there is a growing appreciation for the potential of probiotic microorganisms (9). In recent years, a remarkable number of probiotic-containing commercial products that confer beneficial health-effects on the host have been developed. However, the mechanisms employed by probiotic microorganisms responsible for these effects have not been fully elucidated.

Lactic acid bacteria (LAB) are the most important species used as probiotics for the maintenance of a healthy and stable human gastrointestinal microflora and the treatment of gastrointestinal tract (GIT) disorders (10, 11). The most predominant genera used in probiotics are *Lactococcus*, *Lactobacillus* and *Bifidobacterium* (9). Lactic acid bacteria strains are characterized by their production of lactic acid and comprise a large variety of microorganisms that have been used for thousands of years for the production and preservation of fermented foods (12). Moreover, LAB are indigenous colonists of the GIT of humans and animals, and are believed to be among the most prevailing inhabitants of the small and large intestine. The GIT is considered to be the most important field of activity where probiotic LAB exert their host-health promoting effects when orally administered (2, 9). Thus, it is important to understand the mechanisms whereby the administered bacteria exert their beneficial effects and the interaction between the bacteria and the host GIT. In the past, several *in vitro* studies simulating gastrointestinal models have been used to evaluate probiotics (13-17). Although *in vitro* models have produced valuable data on the survival of probiotic LAB in gastrointestinal conditions, these methods are not a true reflection of *in situ* conditions. A more complete understanding of the *in vivo* metabolic activities, adherence to epithelial cells, antimicrobial activity and interaction of probiotic LAB with their host GIT

system can be facilitated by direct *in vivo* tracking of these processes using cells labelled with reporter genes.

Bioluminescence imaging (BLI) represents one of the most remarkable uses of imaging technologies by allowing for *in vivo* non-invasive monitoring of cells expressing luciferase genes in real-time (18-20). Researchers have used bacterial and eukaryotic luciferase genes to track viral and bacterial infections, and as reporters of gene expression and tumour growth in small animals (18-23). The BLI technique is based on the detection and quantification of visible light produced by luciferase enzymes in the presence of ATP (23).

Numerous studies using rodent models of infection have demonstrated the role of probiotic LAB in the treatment or prevention of infectious diseases caused by *Salmonella typhimurium*, *Helicobacter pylori*, enteropathogenic *Escherichia coli*, *Citrobacter rodentium* and *Listeria monocytogenes* (24-28). Although many of these papers have provided incremental support to the health-benefits of probiotic LAB, few address the causal relationship between their effects and their mechanistic basis of action. Thus, *in vivo* identification of the precise mechanistic basis of the beneficial effects conferred by probiotic LAB to the host remains a significant goal.

In this study, BLI was implemented to allow a better understanding of the survival and metabolic activities of two relatively new LAB strains included in the probiotic product entiro™. Bioluminescence imaging allowed the direct *in vivo* monitoring of the spatial and temporal persistence of the strains *Lactobacillus plantarum* 423 and *Enterococcus mundtii* ST4SA. In the second part of the study, the mechanisms by which the two probiotic strains suppresses the growth of *Listeria monocytogenes* EGDe in the GIT was studied. Specifically, the role of bacteriocins and adhesion proteins as *in vivo* probiotic mechanisms of action were investigated. Mice were used as experimental animals. A novel system was developed for the efficient detection of single or multiple gene knockouts in *L. plantarum* 423 and *E. mundtii* ST4SA. In addition, bacteriocin immune variant strains of *L. monocytogenes* EGDe were constructed. Using BLI, the interaction of the probiotic strains with *L. monocytogenes* EGDe in the GIT could be monitored in real-time, at molecular level.

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Chapter 2

Literature Review:

Probiotic-Pathogen Interactions

Probiotic-Pathogen Interactions

1. Introduction

Lactic acid bacteria (LAB) play an important role in various applications, as starter cultures in fermented food products, food bio-preservation agents and as probiotic health supplements (1-4). The beneficial effects of probiotic LAB on human and animal health have been extensively researched. Some of the most important probiotic effects are stimulation of the host's immune system, the prevention of antibiotic-associated diarrhoea, treatment of inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS), the alleviation of lactose intolerance, lowering of cholesterol levels and prevention of life-threatening gastrointestinal infectious diseases such as *Clostridium difficile*-associated diarrhoea (5-10). It is thus not surprising that in recent years a profitable probiotic market has emerged together with an increasing number of probiotic-containing supplements conferring specific health benefits to the consumer (5, 11). Examples of successful probiotics and their effects are listed in Table 1.

The primary habitat for LAB species used as probiotic health-supplements is either the small intestine or colon (large intestine) where they are among the most dominant colonizing organisms (11, 15). It is therefore important to have an in-depth understanding of the specific metabolic and genetic interactions between the administered probiotic bacteria, the host intestinal mucosa and enteric pathogens in the gastrointestinal tract (GIT). Ultimately, the detailed characterization of these interactions will significantly improve the application of probiotics to support and enhance human health.

Probiotic LAB have shown remarkable potential for preventative and therapeutic applications in various gastrointestinal disorders, especially those caused by enteric pathogens (Table 2). Probiotic bacteria mediate their beneficial effects by interacting with the enteric pathogens to prevent the intestinal colonization of the pathogenic bacteria and the occurrence of disease. The mechanisms underlying these probiotics-pathogen interactions generally include the following categories: competitive exclusion, secretion of antimicrobial substances, strengthening of the gut mucosal barrier and modulation of immune responses (30-33). Figure 1 depicts a summarized overview of the different modes of action by which probiotics exclude pathogens from the GIT. Several probiotic strains, particularly strains from *Bifidobacterium* and *Lactobacillus* genera, improve resistance to intestinal infections by

inhibiting the growth of potentially harmful bacteria in the gut (11, 30). Nevertheless, for probiotics to confer their beneficial effects on the host, they must be able to resist stomach acid and bile and persist at high levels in the intestinal tract (11, 34).

In the first part of this chapter, the survival of probiotics in the GIT and their *in vivo* persistence are reviewed. Finally, probiotic mechanisms of action and their health-promoting effects through interaction with enteric pathogens are discussed.

TABLE 1. Examples of successful probiotics and reported effects

Strain	Reported effects
<i>Lactobacillus acidophilus</i> LA1	Immune enhancement, modulation of intestinal microflora, adherence to human intestinal cells, adjuvant in <i>Helicobacter pylori</i> treatment
<i>L. acidophilus</i> NFCO 1748	Treatment of constipation, used in prevention or alleviation of radiotherapy diarrhoea, lowering of faecal enzymes
<i>L. rhamnosus</i> GG (ATCC 53103)	Reduction of antibiotic-associated diarrhoea, treatment and prevention of rotavirus and <i>Clostridium difficile</i> associated diarrhoea, immune enhancement, treatment of Crohn's disease, used for alleviation of atopic dermatitis in children, stabilisation of intestinal permeability
<i>L. plantarum</i> 299v	Modulation of intestinal microflora, prevention of antibiotic-associated diarrhoea
<i>L. reuteri</i> SD2112	Significantly decreases diarrhoea symptoms in infants and children
<i>Bifidobacterium bifidum</i>	Prevention and treatment of rotavirus diarrhoea, modulates intestinal microflora
<i>Pediococcus pentasaceus</i> NB-17	Stimulation of immune cells and cytokines, stimulation of allergic inhibitory effects
<i>Oenococcus oeni</i> 9115	Immune enhancer, significantly decreases acid-induced colitis in mice
<i>Lactobacillus casei</i> Shirota	Immune enhancer, modulates intestinal microflora, treatment of rotavirus diarrhoea, lowering of faecal enzymes, positive effects in the treatment of superficial bladder and cervical cancer
<i>Lactobacillus reuteri</i> (BioGaia Biologicals)	Reduces rotavirus diarrhoea symptoms in children, treatment of acute diarrhoea, safe in HIV-positive adult patients
<i>Lactobacillus bulgaricus</i>	Treatment of diarrhoea, modulates intestinal flora
<i>Bifidobacterium infantis</i> UCC 3624	Modulates intestinal flora, reduces clostridia levels, immune enhancer, increased blood phagocytic levels
<i>Saccharomyces boulardii</i>	Prevention and treatment of antibiotics associated and acute diarrhoea in children, treatment of <i>C. difficile</i> colitis, prevention of diarrhoea in critically ill tube-fed patients

Adapted from (5, 12-14)

TABLE 2. Effects of different probiotics on selected gastrointestinal pathogens

Probiotic strains	Pathogen	Reported outcomes (<i>in vivo</i>)
<i>Lactobacillus rhamnosus</i> GG (16)	<i>Helicobacter pylori</i>	Reduced diarrhoea and nausea in a human trial
<i>L. johnsonii</i> La1 (17)	<i>H. pylori</i>	Regular ingestion modulated <i>H. pylori</i> colonization in children
<i>L. casei</i> DG (18)	<i>H. pylori</i>	Increased eradication rate of <i>H. pylori</i> infection when supplemented with first-line therapies
<i>L. casei</i> CRL431 (19)	<i>Salmonella enterica</i> serovar Typhimurium	Preventative administration protected mice against infection
<i>L. rhamnosus</i> HN001 (20)	<i>Salmonella enterica</i> serovar Typhimurium	Conferred immune enhancement and protection against <i>Salmonella</i> infection in mice
<i>Bifidobacterium longum</i> Bb46 (21)	<i>Salmonella enterica</i> serovar Typhimurium	Protective effect against <i>Salmonella</i> challenge in gnotobiotic mice
<i>L. plantarum</i> 423 and <i>Enterococcus mundtii</i> ST4SA (22)	<i>Salmonella enterica</i> serovar Typhimurium	Alleviated symptoms of <i>Salmonella</i> infections in challenge study using rats
<i>L. casei</i> BL23 and <i>L. paracasei</i> CNCM I-3689 (23)	<i>Listeria monocytogenes</i>	Decreased pathogen systemic dissemination in orally infected mice
<i>L. salivarius</i> UC118 (24)	<i>L. monocytogenes</i>	Protected mice from pathogenic infection in liver and spleen
<i>L. plantarum</i> 423 and <i>E. mundtii</i> ST4SA (25)	<i>L. monocytogenes</i>	Excluded the pathogen from the intestinal tract of mice after daily administrations of probiotic strains
<i>Lactococcus lactis</i> MM19 and <i>Pediocin acidilactici</i> MM33 (26)	Vancomycin resistant enterococci (VRE)	Modulated intestinal microbiota and reduced pathogen intestinal colonization in mice.
<i>L. rhamnosus</i> R0011 and <i>L. acidophilus</i> R0052 (27)	<i>Citrobacter rodentium</i>	Pre-treatment of the probiotic strains attenuated pathogen infection in mice
<i>L. reuteri</i> (28)	<i>C. rodentium</i>	Attenuated <i>C. rodentium</i> -induced colitis in mice
<i>B. breve</i> (29)	<i>Escherichia coli</i> O157:H7	Protected mice from Shiga toxic-producing <i>E. coli</i>

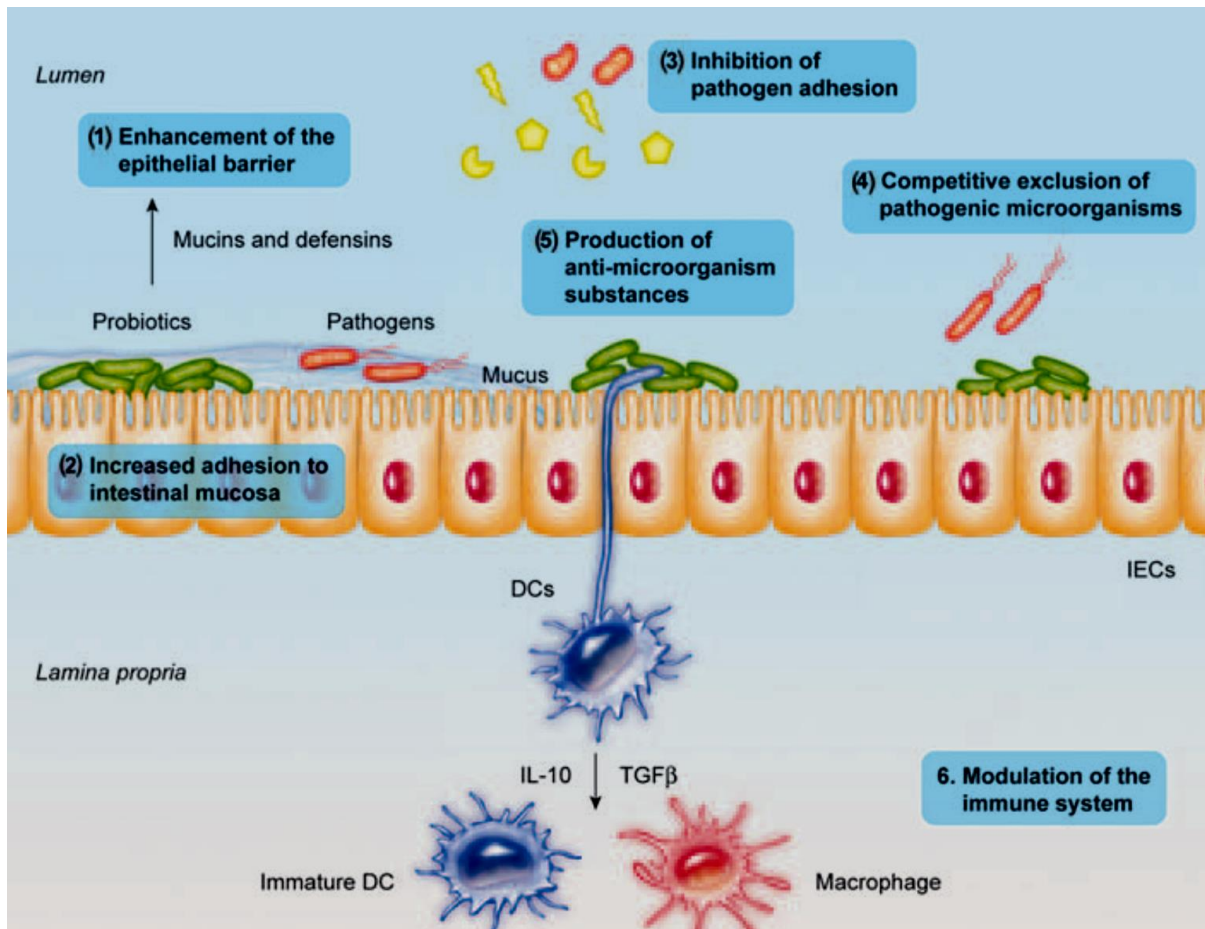


FIG. 1. Major modes of probiotic mechanisms of action against enteric pathogens in the GIT (32).

2. Survival and persistence of probiotic bacteria in the GIT

The human intestinal tract is colonized by a large number of microorganisms, representing a highly complex microbial ecosystem. This ecosystem is primarily inhabited by bacteria and is mainly confined to the ileum and colon. The large intestine is the most heavily populated area of the GIT, containing estimated bacterial concentrations ranging from 10^{11} - 10^{12} CFU/ml of contents (35). Moreover, according to a recent study, the ratio between bacterial cells and human cells in the human body is one to one (36). These bacteria form part of a microbiota that plays an essential role in the breakdown of food particles and the metabolism of faecal residue (5). In addition, the microbiota also acts as a protective barrier against pathogens by providing mucosal protection and stimulation of the immune system (5). Species of the genera *Lactobacillus* and *Bifidobacterium* are responsible for many of the beneficial effects of the microbiota (37) and are incorporated in most of the commercially available probiotics (11, 38) (Table 1).

Probiotic species are mainly isolated from the commensal gut microbiota (39). Thus, the potential modes of action employed by probiotic bacteria to defend against infectious enteric diseases may not differ much from those employed by the natural microbiota. A prerequisite of a good probiotic strain is its survival during transit of the acidic conditions of the stomach and bile salts in the small intestine in order to colonize the GIT in sufficient numbers (40, 41). Moreover, the most obvious mechanism of probiotic action against enteric pathogens in the GIT involves the production of antimicrobial agents, a task that can only be performed by viable and metabolically active cells. The number of viable cells that reach the intestine to gain therapeutic benefits may be strain specific and vary by the amount ingested and the duration of administration.

2.1 Factors affecting the survival of probiotic bacteria in the GIT

In vitro methods that mimic the conditions during gastrointestinal transit are routinely used to assess the effect of exposure to different pH values and the presence of bile on potential probiotics (42-44). The survival of probiotic cells in stomach acid and bile varies among strains and depends on degree of gastric juice acidity, bile concentration and length of exposure.

Once in the stomach, probiotic bacteria are confronted by gastric acid where the pH level can be as low as 1.5 (45). Low pH may decrease the survival of probiotic LAB. The best probiotics should survive at least a pH of 3.0 (46). Hydrochloric acid (HCl) present in the human stomach is a strong oxidizing agent that can destroy important biological compounds such as fatty acids, proteins, cholesterol and DNA (47, 48). In an *in vitro* study, two *Bifidobacterium* strains demonstrated different levels of growth inhibition after exposure to simulated gastric juice for 90 min (49). The growth of one strain was inhibited by only 0.5 log units, whereas the growth of the other strain declined by 4 log units. In another study, *in vitro* growth of six *Lactobacillus acidophilus* and nine *Bifidobacterium* strains was maintained between pH levels of 1.5-3.0 for a 3 h incubation period (45). The authors reported that the growth of *Bifidobacterium adolescentis* and *Bifidobacterium breve* was retarded at all pH levels (1.5, 2.0, 2.5 and 3.0), while the most robust strains were *L. acidophilus* (strains 2401, 2409 and 2415), *Bifidobacterium longum* 1941 and *B. pseudolongum* 1969 (45). A report by Hood and Zottola showed that *L. acidophilus* BG2FO4 cell numbers declined rapidly at pH 2.0 but that cell numbers did not decrease significantly at

pH 4 (50). Moreover, Conway and co-workers (51) reported that bacterial strains that originate from the gut have increased levels of gastric acid resistance. In addition to a rapid transit time of probiotics in the stomach, the consumption of food also protects probiotic LAB during transit through the GIT (52). The buffering capacity of yogurt and milk provides a protective effect for bacteria in the GIT (53). Furthermore, Gram-positive bacteria can employ several mechanisms that aid resistance to acid. These include, cell envelope structural changes, proton pumps, electrogenic transport systems, the decarboxylation of amino acids, and the repair or degradation of damaged proteins by chaperones (54).

Once probiotic cultures reach the upper section of the small intestine (duodenum), the secretion of bile greatly affects probiotic survival. Bile salts act as a biological detergent that alters the lipids and fatty acid composition in cell membranes and increases the permeability of cells (42, 55). In humans, bile and the associated bile acids are continuously produced by the liver and stored in the gallbladder (56). After food intake, this stored bile is secreted into the duodenum and aids the digestion of lipids and oils (57). The two primary bile acids synthesized by the liver are cholic acid and chenodeoxycholic acid which have strong inhibitory effects on intestinal bacteria (58). Secondary bile acids such as deoxycholic acids (DCA) are toxic to LAB (59). Exposure of *Lactobacillus reuteri* CRL 1098 to DCA revealed changes in the integrity of the cell wall, rendering cells completely permeable and preventing glucose uptake, resulting in cell death (59). The rate at which bile is secreted depends on the food composition. Food with a high fat content could affect the levels of faecal bile acids, and fatty acids may decrease the survival of *Lactobacillus* and *Bifidobacterium* spp. (60).

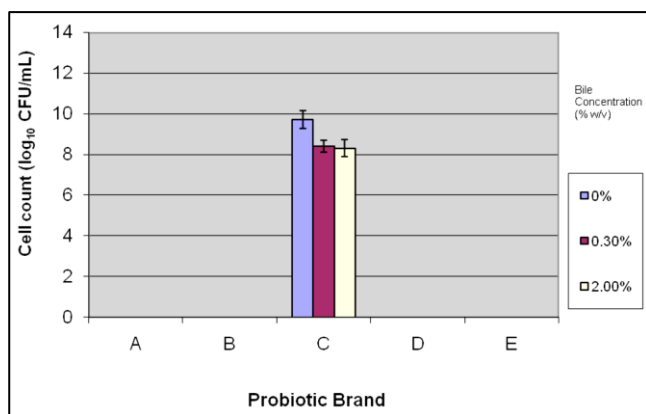
Probiotics contained in five different brands of commercial milk drinks showed increasing levels of inhibition as bile concentrations and pH acidity levels increased (61). The authors demonstrated that bile tolerance of the probiotic strains showed a similar trend after pre-exposure of cells at pH levels 1.5, 3.0 and 7.2 (Table 3 and Fig. 2). Bile concentrations in the small intestine range from 0.5 to 2.0 % during the first hour of food digestion (57). Clark and Martin reported that the survival rate of *B. longum* is best in 2% - 4% bile (62). In another study, Lankaputhra and Shah reported that the survival rates in bile varied (1-1.5%) significantly among strains of *Lactobacillus* and *Bifidobacterium* (45). The authors showed that while some strains were bile tolerant (*L. acidophilus* 2404 and *B. longum* 1941), other strains (*L. acidophilus* 2409 and *B. longum* 1963) survived poorly in the presence of bile. Moreover, yogurt starter cultures *Lactobacillus delbrueckii* subsp. *bulgaricus* and

Streptococcus thermophilus are not bile resistant and thus unable to survive gastrointestinal transit (63).

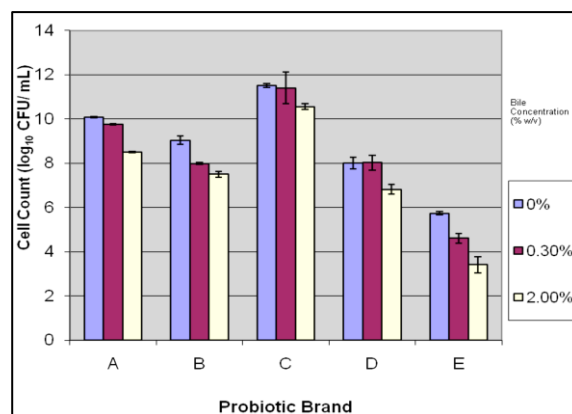
TABLE 3. List of probiotic species in commercial milk brands (61)

Brand	Species
A	<i>L. acidophilus</i>
B	<i>L. acidophilus</i>
C	<i>L. casei</i> Shirota
D	<i>L. acidophilus</i> , <i>Streptococcus thermophilus</i> , <i>Bifidobacterium</i> strains
E	<i>L. acidophilus</i> , <i>L. casei</i> , <i>Bifidobacterium</i>

A.



B.



C.

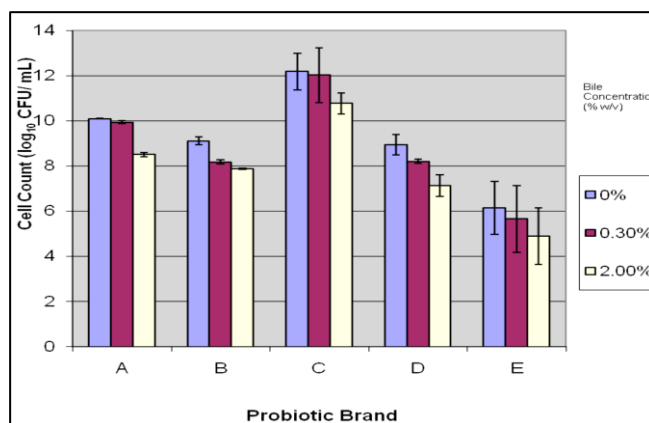


FIG. 2. Survival of probiotic bacteria in different probiotic brands (A-E, Table 3) at bile concentrations of 0% (control), 0.3% and 2% after 24 h incubation period at 37°C. Before bile resistance test, cells were pre-exposed to pH (A) 1.5, (B) 3 and (C) 7.2 for 3 h (61).

Changes in cell wall integrity can protect cells from harsh intestinal conditions (64). Using DNA micro-arrays it was demonstrated that genes of *Lactobacillus plantarum* WCFSI involved in membrane and cell wall-associated functions, stress response and redox reactions were all up-regulated in the presence of 0.1% porcine bile (64, 65). Survival in bile-rich environments also depends on the regulation of gene transcription, amino acid transport and fatty acid biosynthesis (66, 67). Bile salt hydrolase (BSH) activity is also important for the survival of probiotic LAB in bile. Bile salt hydrolase-encoding genes are present in several *Lactobacillus* spp., thus emphasizing the potential importance of these genes to the survival and persistence of strains in the GIT (68). Conjugated bile acids produced in the liver are hydrolysed by BSH, thus reducing their bactericidal effects (61, 69).

The survival of probiotic bacteria during gastrointestinal transit has also been studied *in vivo* by using several animal models (70, 71). In these assessments, the re-isolation of orally administered probiotic cells in faecal samples was used to assess the survival of the probiotic in the intestinal tract. In a murine model, *L. plantarum* 423 and *Enterococcus mundtii* ST4SA survived passage through the intestinal tract and persisted in faeces for at least 72 h after a single administration (72). *Lactobacillus casei* L26 survived within the GIT of mice for at least 48 h after a single oral administration (73). The number of probiotic cells that survive gastrointestinal transit is also important. In a human intubation study, the sampling of *Bifidobacterium* spp. from the cecum after administration in fermented milk showed that the average number recovered were $23.5 \pm 10.4\%$ of the ingested dose (74). In another study, the estimated survival of *L. bulgaricus* NCIMB 8826 in faeces was $25 \pm 29\%$ at day 7 (10^8 CFU/g) after 7 consecutive daily administrations of 10^8 CFU and could be detected in faeces for two weeks after the last administered dose (75).

2.2 Colonization and persistence of probiotic bacteria in the GIT

For a probiotic bacterium to successfully fulfil a prophylactic role it must be able to colonize and persist in the digestive tract to confer functional beneficial effects on the host (76, 77). While colonization is not an absolute requirement for probiotic activity, LAB strains that transit only passively through the GIT are usually not selected as probiotics for industrial purposes (78). The *in vivo* persistence of probiotic organisms is related not only to the physiological or genotypical properties of strains, but also the dosage, administration method and metabolic activity (79). The intestinal persistence of probiotic LAB is also limited by

interactions with the enteric microbiota. Commensal enteric microorganisms compete with exogenously administered probiotic bacteria for essential nutritional substrates and mucosal adhesion sites (80).

For probiotic LAB to persist and become permanently established in the host's intestine, they must be able to attach to intestinal mucosal cells (80). The persistence or at least temporary colonization of probiotics in the host's intestine is a phenotypical trait that can be related to the beneficial action of probiotics against enteric pathogens. Many enteric pathogens require attachment to intestinal mucosal cells to exert their deleterious effects and probiotics have been demonstrated to interfere with this adherence (81). This implies that probiotics compete with enteric pathogens for intestinal adhesion sites to decrease the activity of the detrimental bacteria and in turn proceed to colonize the gut themselves. Several *in vitro* studies have reported that probiotics interfere with the ability of gastrointestinal pathogens such as *Salmonella typhimurium*, *Clostridium sporogenes* and *Enterococcus faecalis*, to adhere to Caco-2 cells (82-84).

A review of the literature suggests that many of the earlier studies on the colonization of probiotics were based on the ability of the strains to adhere to *in vitro* cell lines such as Caco-2, HT-29 and HT29-MTX (85, 86). Although these studies using simulated GIT-models have provided valuable insights into the adherence of probiotic cells, they remain an *in vitro* approach (37). Moreover, colonization studies using cell lines is laborious and requires special facilities to maintain the cells. Hence, studies on the survival and colonization of administered probiotic bacteria are most frequently done by analysing faecal samples (70). Faecal recuperation enables microbiologists to compare the intestinal persistence of different probiotic strains provided that the same ingested doses and methods for enumeration are used. The *in vivo* pharmacokinetics of probiotics can be studied by comparing specific strains before and after ingestion and whether the ingested bacteria increase in number (in faecal material) following consumption (75, 87). The pharmacokinetics of different probiotic LAB are listed in Table 4. Species of the *Lactobacillus* and *Bifidobacterium* genera have been extensively explored as probiotics, since they form an integral part of the natural gut microbiome of both humans and animals (88). Strains that persist in high numbers for the longest time in faeces are usually considered to be the best probiotics. *Bifidobacterium lactis* LAFTI B94 and *B. longum* SB T2928 and *Lactobacillus rhamnosus* DR20 persisted in highest proportions or for the longest duration in faeces (Table 4). In comparison, faecal

recuperation of *Lactococcus lactis* MG 1363, *L. rhamnosus* GG and *Lactobacillus fermentum* KLD was much lower.

TABLE 4. Pharmacokinetics of probiotic strains

Strain	Dosage	Faecal recuperation	Persistence (day)
<i>B. lactis</i> LAFTI B94	1 x 10 ¹¹ CFU for 7 days	1.8 x 10 ⁹ CFU/g	28
<i>B. lactis</i> Bb12	1 x 10 ¹¹ CFU	8 x 10 ⁷ CFU/g	14
<i>B. longum</i> SB T2928	7 x 10 ¹¹ CFU for 7 days	1 x 10 ⁹ CFU/g	>30
<i>L. rhamnosus</i> GG	6 x 10 ¹⁰ CFU for 12 days	4 x 10 ⁴ CFU/g	14
<i>L. rhamnosus</i> DR20	1.6 x 10 ⁹ CFU for ±182 days	6.3 x 10 ⁵ CFU/g	60
<i>L. salivarius</i> UCC118	1 x 10 ¹⁰ CFU for 21 days	1 x 10 ³ -1 x 10 ⁷ CFU/g	>21
<i>L. plantarum</i> 299v	2 x 10 ¹⁰ CFU for 21 days	1 x 10 ⁷ CFU/g	>8
<i>L. plantarum</i> NCIMB 8826	5 x 10 ¹⁰ CFU for 7 days	1 x 10 ⁸ CFU/g	14
<i>L. fermentum</i> KLD	1.5 x 10 ⁹ CFU	3.2 x 10 ⁴ CFU/g	1
<i>L. gasseri</i> SBT2055	1 x 10 ¹¹ CFU for 7 days	1 x 10 ⁷ CFU/g	>31
<i>S. thermophilus</i>	1.2 x 10 ¹² CFU	5 x 10 ⁶ CFU/g	6
<i>Lc. lactis</i> MG 1363	1 x 10 ¹¹ CFU for 4 days	1 x 10 ⁴ CFU/g	3

Adapted from (78).

Some reports have suggested that non-viable and thus non-colonizing probiotics may also confer certain health benefits to the host (11, 89, 90). According to these studies the death of probiotic cells in the GIT is not related to the absence of a positive effect given that certain beneficial effects produced by live microorganisms may also be due to their metabolites, DNA or cell wall components (78). During gastrointestinal passage, non-colonizing or transiently colonizing probiotic bacteria continue to be metabolically active, thus conferring beneficial health effects to their host (40). In a study by Kullen and co-workers (91), human volunteers were administered a *Bifidobacterium* probiotic strain and the recovery of the strain in the faeces was monitored. It was observed that faecal recuperation of the strain increased during the days of administration, but that it was no longer recovered in faeces after administration was discontinued. The authors concluded that although the administered strain did not colonize the human GIT, colonization and prolonged persistence may not be required to achieve a significant probiotic effect. Similar results were reported by Fujiwara and co-workers (92). The authors found that bifidobacteria produce a 100 000-kDa protein, which

actively prevents the adherence of pathogenic *Escherichia coli* to intestinal mucosal cells. Therefore, the competitive exclusion of the pathogenic strain may not have been related to direct live cell-to-cell competition for intestinal adhesion sites.

A major drawback of using only faecal recuperation to analyse *in vivo* probiotic persistence is that it does not accurately reflect the amount of cells that remain colonized in the intestinal tract. Furthermore, the microbial inhabitants of faecal samples represent a part of the luminal environment that is more indicative of the lower intestine, such as the colon (5). Colonization studies in humans using intubation at specific intestinal sites is a better indicator of probiotic persistence in the upper sections of the GIT. Biopsies can be taken of the portions of the intestinal tract where probiotics are likely to colonize, proliferate and produce their metabolites (75, 78, 93, 94).

Lactobacillus rhamnosus GG is one of the most studied probiotic strains and reportedly plays a role in the prevention or treatment of antibiotic-associated diarrhoea, flatulence, rotavirus gastro-enteritis, stomach and abdominal pain (12-14, 95-97). However, when *L. rhamnosus* GG in fermented milk was administered to human volunteers, the strain showed only limited persistence in faeces after feeding was stopped (96). The strain was no longer recovered in 67% of the subjects within 7 days of the last dosage. The same results were obtained when the strain was fed in milk formulas to premature infants (98). However, Alander and co-workers (94) reported that *L. rhamnosus* GG was recoverable from colonic biopsies for lengthy periods after its administration was ceased. Human volunteers were administered with 6×10^{10} CFU of *L. rhamnosus* GG twice a day for 12 consecutive days. The authors observed that the amount of *L. rhamnosus* GG recuperated in the faeces decreased with time after the last bacterial dosage was administered and that the strain disappeared completely from faeces 14 days after the last dosage. However, *L. rhamnosus* GG persisted in biopsies taken from the colonic mucosa for up to 21 days at 7×10^4 CFU/biopsy sample after consumption ceased (94). Concluded from these results and others, faecal enumeration for analysis of probiotic persistence is not truly reflective of real *in situ* conditions in the human digestive tract.

2.3 Optical imaging techniques to study probiotic bacteria *in vivo*

The survival and persistence of probiotic or pathogenic bacteria in the GIT are mostly based on *in vitro* models and the analysis of faecal samples (37, 70). However, *in vitro* models are

not a true reflection of real-life conditions and faecal recuperation does not accurately reflect the amount of cells that remain colonized in the intestinal tract or the bacterial sites of colonization in the intestinal tract. Using bioluminescence imaging (BLI), the spatial and temporal course of colonization or infection can be detected without killing large numbers of animals (99). For a review on the application of optical imaging systems in the *in vivo* tracking of LAB in small animals, refer to the article by Van Zyl et al. (100).

Optical imaging techniques are used in *in vivo* studies to track probiotic or pathogenic bacteria in the GIT of small animals (101). Bioluminescence imaging is used for real-time, non-invasive, monitoring of bacterial cells that are genetically tagged with light-producing luciferase genes (101). The light produced by cells tagged with luciferase is detectable through the tissues of live small animals using an *in vivo* imaging system (IVIS). Luciferases produce bioluminescent light in the presence of ATP and oxygen (100). The BLI technique is a means to rapidly and accurately determine sites of bacterial replication because only growing or metabolically active cells emit detectable levels of light (100). Numerous studies have demonstrated the use of bioluminescent bacteria tagged with bacterial or eukaryotic luciferase genes to monitor real-time progression of infection or bacterial colonization *in vivo* (25, 100-103).

3. Interactions between probiotic and pathogenic bacteria

Although the viability and persistence of probiotic bacteria in the GIT contribute immensely to the ability of strains to exert their beneficial effects on the host, the ultimate goal of probiotic research is the characterization of specific probiotic-pathogenic bacteria interactions that result in health-promoting effects. However, the exact modes of probiotic action during these interactions remain largely uncharacterized (11). Only a few scientific papers provide evidence to support the inhibitory activities of probiotic LAB on gastrointestinal pathogens (104).

Elucidating the mechanisms of action of probiotic strains is a difficult task given the complex nature of the human GIT. A specific health benefit may also be attributed to a combination of mechanisms. The mechanism(s) of action of one specific probiotic strain against a particular disease or pathogen cannot be generalized since different strains evoke different responses in the host (31). This suggests that probiotic effects may vary from one probiotic strain to another. Thus, the health benefits conferred by one strain are not applicable to another strain,

even within the same species (32, 105). Furthermore, different probiotic strains are associated with different effects relating to their specific capacities to secrete antimicrobial substances or to express particular surface proteins interacting directly with host or pathogenic cells (32). The specific aspects of potential probiotic mechanisms of action are discussed below (Fig. 1).

3.1 Competitive exclusion of enteric pathogens

The term competitive exclusion was first used in a report published in 1969 by Greenberg, who used the term to describe the total exclusion of *S. typhimurium* from maggots of blowflies (106). This anti-pathogenic mechanism describes the scenario in which one species of bacteria more rigorously competes for intestinal adhesion sites in the GIT than another species. The mechanisms of action used by one species of bacteria to exclude another species from the GIT are varied and may include microbe-microbe interactions mediated by binding to the host mucosal interface at specific attachment sites, the secretion of antimicrobial substances and competition for available nutrients (32, 33).

For enteropathogens to initiate infection, they have to cross the intestinal mucosal barrier before colonization of the intestine can occur (107). Once pathogens have penetrated the mucus layer overlying the intestinal epithelium they can attach to binding sites on the epithelial cells (108). Subsequently, attachment is followed by intestinal colonization and infection. One of the mechanisms by which probiotics can protect the gut against enteric infections is by preventing this attachment (109). Results from *in vitro* studies using human or animal mucosal material have demonstrated the effect of probiotic LAB on the competitive exclusion of pathogens (84, 110-112). A *L. rhamnosus* strain with excellent adhesion properties was able to prevent the internalization of enterohemorrhagic *E. coli* (EHEC) in human intestinal cell lines (110). Enteric pathogens such as EHEC, utilize their mannose-sensitive type 1 fimbriae to attach to oligosaccharide receptor sites on intestinal epithelial cells (IECs) (113). Evidence suggests that probiotics, including lactobacilli and bifidobacteria, are capable of attaching to the same specific carbohydrate receptor sites on host gut cells so that enteric pathogens are in constant competition for binding and are thereby prevented from subsequent pathogenic elaboration of toxins or invasion (32, 114, 115).

The host-beneficial effects of different probiotic strains vary and therefore it is reasonable to suggest that different probiotics may vary in their effectiveness to block pathogen adhesion

sites in the gut. Specific adhesion proteins may contribute to the probiotic competitive exclusion mechanism(s) of action, although probiotic inhibition of pathogen attachment to enterocyte receptors in the gut based on steric hindrance is also possible (31-33, 116). Carbohydrate-binding adhesion proteins on the bacterial cell surface stereo-specifically bind to carbohydrate moieties on the intestinal surface (117). For an overview of studies that analysed the effect of lactobacilli surface proteins on adhesion using mutant analysis, see Table 5. One example of a specific adhesion protein involved in competitive exclusion adhesion-receptor interactions in the gut is the *L. plantarum* mannose-specific adhesion (Msa) protein (117). A spontaneously mutated strain of the probiotic *L. plantarum* 299v, thought to be affected in the *msa* gene, was unable to inhibit the attachment of EHEC to HT-29 epithelial cells compared to the wild-type (123). This suggests that Msa-containing probiotic strains could effectively exclude several other, if not all, type 1 fimbriated enteropathogens.

TABLE 5. *Lactobacillus* cell surface proteins putatively involved in adhesion

Strain	Gene	Predicted function	Mutant phenotype
<i>L. plantarum</i> WCFS1 (118)	<i>srtA</i>	Sortase	Reduced mannose-specific binding; competitive ability in murine GIT not affected
<i>L. plantarum</i> WCFS1 (118)	<i>msa</i>	Mannose-specific adhesin	Reduced mannose-specific binding
<i>L. plantarum</i> WCFS1 (119)	<i>lp_2940</i>	Sortase-dependent cell wall protein	Reduced persistence in murine GIT
<i>L. acidophilus</i> NCFM (120)	<i>mub</i>	Mucus-binding protein (MUB)	Reduced binding to human Caco-2 cells
<i>L. acidophilus</i> NCFM (120)	<i>slpA</i>	S-layer protein	Reduced binding to human Caco-2 cells
<i>L. salivarius</i> UCC18 (121)	<i>srtA</i>	Sortase	Reduced binding to human Caco-2 and HT-29 cells
<i>L. salivarius</i> UCC18 (121)	<i>lspA</i>	Large surface protein (LSP), putative MUB	Reduced binding to human Caco-2 and HT-29 cells
<i>L. salivarius</i> UCC18 (121)	<i>lspB</i>	LSP, putative MUB	Binding to human Caco-2 and HT-29 cells not affected
<i>L. reuteri</i> 100-23 (122)	<i>lsp</i>	LSP	Reduced persistence in murine GIT
<i>L. johnsonii</i> NCC533 (79)	LJ1476	Transpeptidase Sortase	Colonization dynamics similar to that of wild-type

Another example of a putative competitive exclusion factor is the collagen-binding protein of *L. fermentum*. Heinemann and co-workers (124) characterized and purified the collagen surface-binding protein of the *L. fermentum* RC-14 strain, which was shown to inhibit the adherence of *E. faecalis* 1131. Other studies have demonstrated the role of surface layer (S-layer) extracts in the prevention of pathogens from attaching to and thus colonizing IECs (125, 126). Chen and co-workers (125) showed that S-layer proteins of *Lactobacillus crispatus* ZJ001 were responsible for competitive exclusion against *S. typhimurium* and EHEC. Similar results were obtained by Johnson-Henry and co-workers (126), who showed that S-layer protein extracts from *Lactobacillus helveticus* R0052 inhibited the adhesion of *E. coli* O157:H7 to the Caco-2 intestinal cell line. However, due to the high hydrophobicity of S-layer proteins, the process of pathogen adherence inhibition by S-layers was suggested to be mediated by hydrophobic group interactions as opposed to adhesion-receptor interactions.

Previous studies suggest that sortase-dependent cell surface proteins (SDPs) play a crucial role in probiotic-host interactions, adherence and colonization (127). Several SDPs have been identified with a role in *in vitro* and *in vivo* adhesion to intestinal cells, including mucus-binding cell surface proteins (Table 5). In Gram-positive bacteria, sortase enzymes decorate the cell surface with a diverse array of proteins by covalently joining them to the cell wall (Sortase A) or by polymerizing proteins to construct complex multi-subunit pilin structures (Sortase C) on the cell surface (128). Sortase enzymes are characterized as cysteine transpeptidases that join SDPs containing a specific cell wall sorting signal (CWSS) to an amino group located on the cell surface (129). Sortase A enzymes anchor proteins that contain a CWSS with a LPXTG (where X donates any amino acid) C-terminal motif to the cell surface (128) (Fig. 3). The LPXTG motif is recognized by the SrtA enzyme, which breaks the threonine and glycine peptide bond and then covalently links the threonine residue to the amino group of the pentaglycine cell wall cross bridge of the bacterium (121, 130). Sortase C proteins catalyse a similar transpeptidation reaction, but recognize a QVPTGV sorting motif to construct pili that promote microbial adhesion (131).

Several strains of lactobacilli and bifidobacteria inhibit, displace and adhere to the same enterocyte layer previously colonized by enteropathogenic *Salmonella choleraesuis* serovar Typhimurium (132). This indicates that the probiotic strains have the ability to effectively displace the pathogen after pathogenic colonization of the gut has occurred instead of being administered only in a preventative setup. To gain a competitive advantage, the probiotics can thus modify the gut environment by producing inhibitory compounds, lowering pH levels

and competing for nutrients (32). *Lactobacillus* species such as *L. acidophilus* and *L. plantarum* have the ability to utilize complex carbohydrates such as fructans (133). Similarly, bifidobacteria are capable of metabolising various plant dietary fibres using several depolymerizing enzymes (134). Utilizing carbohydrate sources other than those used by enteropathogenic bacteria enable probiotic bacteria to widen their areas of colonization in the GIT and inhibit pathogens.

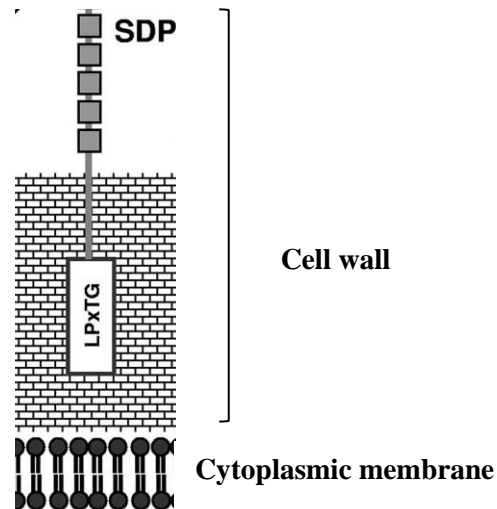


FIG. 3. Sortase-dependent cell surface proteins containing the LPXTG C-terminal motif are anchored to the bacterial cell wall by Sortase A enzymes. Image modified from (31).

3.2 Production of antimicrobial compounds

Besides competing for nutrients, competitive inhibition of the epithelial and mucosal adherence of pathogens and thus prevention of pathogenic colonization, probiotic bacteria also produce a variety of compounds that exert direct antimicrobial action toward competing enteropathogens (Fig. 4).

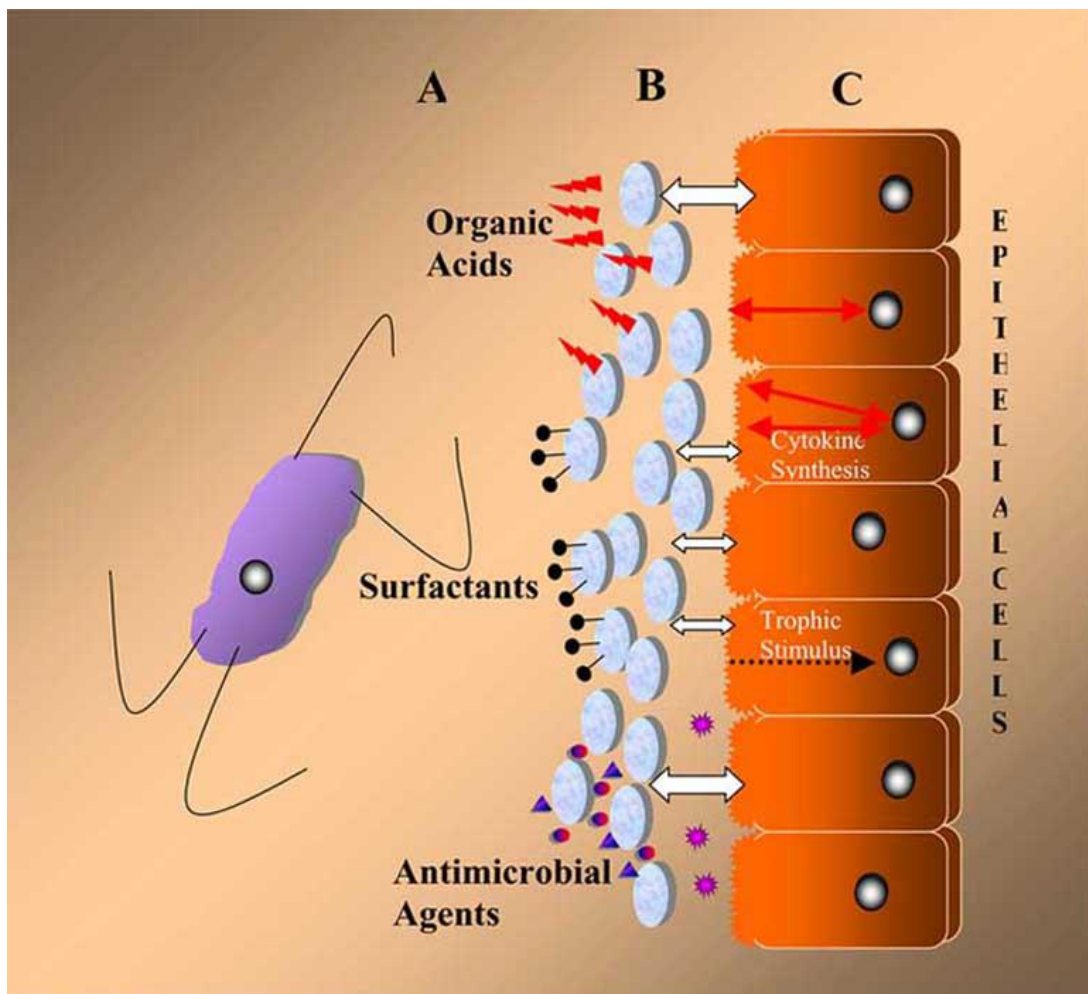









FIG. 4. Inhibition of pathogens by probiotic bacteria adhered to gut intestinal epithelial cells (5). Inhibitory activity of probiotic bacteria is achieved by (A) secretion of organic acids ; surfactants , and antimicrobial compounds including bacteriocins  and hydrogen peroxide . (B) Probiotic bacteria compete with pathogens by adherence and signal exchange through epithelial cell receptors . (C) Probiotics can stimulate a host immune response (via trophic stimuli and cytokine synthesis) against pathogens , including viruses .

3.2.1 Bacteriocins

The production of bacteriocins by probiotic bacteria (usually LAB) is a key mechanism by which the inhibition of pathogenic adhesion to IECs and invasion can occur (Fig. 5). Bacteriocins are ribosomally produced antimicrobial peptides that differ in terms of their size (2-6 kDa), mechanisms of action and immunity (for a review, see reference 135). Bacteriocins usually only inhibit specific species, often those closely related to the strain producing them (135, 136). Bacteriocins are produced by many LAB strains (for example, nisin from *Lc. lactis*, plantaricin from *L. plantarum* and lacticin B from *L. acidophilus*) that are active against food-borne enteropathogens such as *Listeria*, *Clostridium*, *Bacillus* and antibiotic-resistant pathogens such as methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant enterococci (VRE) (32, 137-141). Bacteriocins may have a bacteriostatic or direct bactericidal effect on pathogens, thus limiting their ability to colonize the gut. The associated antimicrobial activities of bacteriocins allow bacteriocin-producing probiotic strains to gain a competitive advantage within the complex gastrointestinal environment (142).

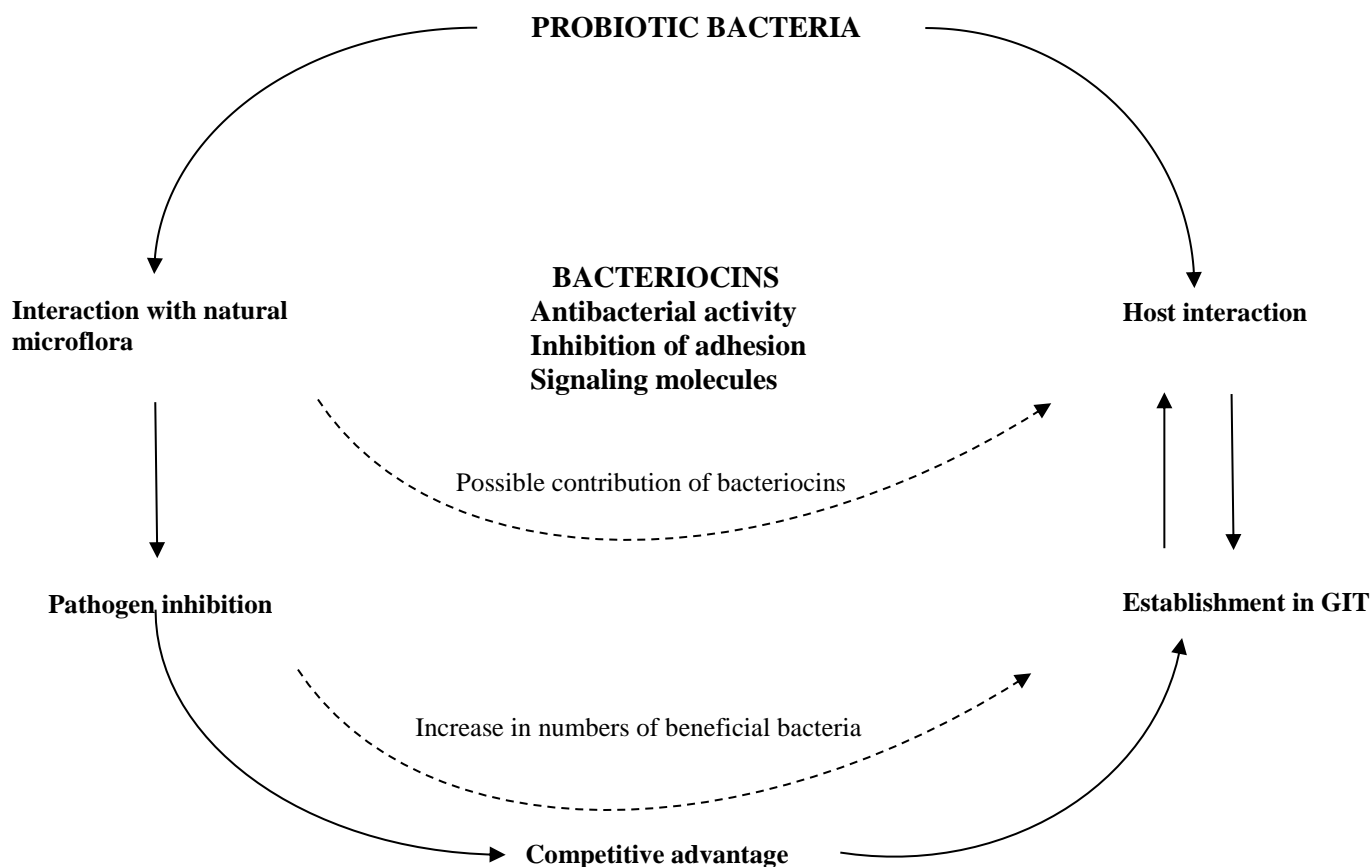


FIG. 5. Beneficial effects of probiotic bacteria and their bacteriocins against enteric pathogens.

The general mechanisms of bacteriocin-mediated pathogen killing include the induction of cytoplasmic membrane permeabilization of sensitive bacteria that leads to cell leakages and/or the inhibition of cell wall synthesis (143). For instance, nisin acts by forming a complex with the cell membrane lipid II precursor, which is followed by the aggregation and incorporation of peptides to form discrete pores in the bacterial cell membrane (144). A unique bacteriocin, bifidocin B, produced by *Bifidobacterium bifidum* NCFB, is active against several Gram-positive bacteria, including *Listeria*, *Enterococcus*, *Bacillus* and *Lactobacillus*, but shows no activity towards several other Gram-positive and Gram-negative bacteria (145). The difference in activity between strains is related to the ability of Gram-negative bacteria to resist adsorption of bifidocin B, due to their cell wall composition (146). However, several bacteriocins, such as mutacins (A-D), nisins (A and Z), lacticins (A164 and BH5), bacteriocins E 50-52 and OR7 have been reported with activity against medically important Gram-negative organisms such as *Campylobacter*, *Helicobacter*, *Haemophilus*, *Neisseria* and *Salmonella* spp., by disruption of their outer cell membrane (147-150).

In addition to *in vitro* studies, several *in vivo* studies have demonstrated the inhibitory effect of purified bacteriocins and probiotic bacteriocin-producing strains in infectious animal models. Simonova and co-workers (151) observed that feeding rabbits with bacteriocin-producing *Enterococcus faecium* CCM7420 and its partially purified bacteriocin significantly reduced *Staphylococci* spp. cell numbers in the cecum thus protecting the animals against infection. Other studies found that the *E. faecium* EK13, enterocin A producing strain reduced *Salmonella* cell numbers in gnotobiotic Japanese quails and also reduced the colonization of pathogenic *Staphylococcus* in the digestive tract of rabbits (152, 153). The capacity of human-isolated nisin- and pediocin-producing LAB to reduce the intestinal colonization of VRE in mice was demonstrated for the first time by Millette and co-workers (26). Svetoch and co-workers (154) reported a significant reduction of *Salmonella enteritidis* in broilers after oral administration of the *E. faecium* E 50-52 bacteriocin. Corr and co-workers (24) demonstrated that feeding mice with the *Lactobacillus salivarius* UCC11 bacteriocin Abp118- producing strain reduced *Listeria monocytogenes* cell numbers in the liver and spleen. A similar reduction in cell numbers of the same pathogen in the GIT of mice was observed when the animals were pre-treated with probiotic strains *L. plantarum* 423 and *E. mundtii* ST4SA, producing bacteriocins plantaricin 423 and mundticin ST, respectively (25).

It is also important to consider that not all potential or developed probiotic strains that show *in vitro* antimicrobial activity against enteropathogens will be active *in vivo* (30). For example, despite the fact that a *Lactobacillus* sp. strain adhered to the jejunum and ileum of gnotobiotic pigs after oral administration and that the strain showed *in vitro* activity against enteropathogenic *E. coli* (EPEC), the LAB strain failed to prevent EPEC colonization in the GIT of infected animals (155). Similar results were observed when *L. casei* subsp. *casei* failed to prevent the intestinal colonization of EPEC in the GIT of gnotobiotic or conventional piglets when the LAB strain was administered in a preventative setup (156). Nevertheless, the probiotics discussed in the above experiments use their bacteriocins to effectively interact with enteropathogens through either bacteriostatic or bactericidal activities. In doing so, they prevent pathogenic colonization of the host GIT and subsequent occurrence of disease.

3.2.2 Bacteriocin-like compounds

Other antimicrobial bacteriocin-like compounds that are incompletely defined or that do not share the typical characteristics of bacteriocins, are produced by probiotic bacteria (30). As opposed to bacteriocins, bacteriocin-like compounds are characterized by having a broader spectrum of antimicrobial activity. In addition, their antimicrobial activities are not related to the production of lactic or organic acids or hydrogen peroxide (30, 157). *Lactobacillus* sp. strain GG secretes an antimicrobial substance with inhibitory activity against *Clostridium* spp., *Staphylococcus* spp., *Enterobacteriaceae*, *Streptococcus* spp., *Bacteriodes* spp., and *Pseudomonas* spp. (158). This low molecular weight (LMW) substance was characterized as heat-stable, distinct from lactic and acetic acids and closely resembled a microcin that is normally produced by *Enterobacteriaceae* spp. These characteristics suggest that it could be a non-bacteriocin bacteriocin-like substance (30). Similar substances with molecular weights and broad activity spectrums uncharacteristic of bacteriocins have been shown to be produced by other lactobacilli strains, including *L. acidophilus*, *L. delbrueckii* and *L. rhamnosus* strains, whose bactericidal affects are related to neither lactic acid nor hydrogen peroxide (30, 159, 160). Other studies have identified bacteriocin-like antimicrobial substances produced by several *Bifidobacterium* strains with broad spectrums of activity against both Gram-positive and Gram-negative pathogens such as *L. monocytogenes*, *Salmonella* spp. and *E. coli* spp. (161-163).

3.2.3 Lactic and other organic acids

An additional mechanism of pathogen displacement in the gut employed by probiotic bacteria is their ability to make the intestinal environment less suitable for pathogen growth. Probiotic LAB and commensal microbiota ferment carbohydrates in the GIT that lead to the production of metabolites such as acetic, formic, succinic and lactic acids, rendering the intestinal environment acidic and inhibiting the growth of bacterial pathogens (164). Organic acids, in particular lactic and acetic acid repress the growth of many pathogenic bacteria in the GIT (30, 32, 37, 165). The undissociated form of lactic acid functions as a permeabilizer of the Gram-negative bacterial outer cell membrane, after which it dissociates inside the bacterial cytoplasm following entry. The bacterial killing activity is then exerted by lowering the intracellular pH level, through the accumulation of ionized forms of the organic acid and other antimicrobial compounds inside the cytoplasm (32, 166).

De Keersmaecker and co-workers (167) demonstrated that the strong inhibitory effects of *L. rhamnosus* GG against *S. typhimurium* was due to lactic acid production. The potential role of lactic acid in the ability of *Lactobacillus* strain GG to prevent the invasion of Caco-2 cells by *S. enterica* serovar Typhimurium was demonstrated by Lehto and Salminen (168). The authors suggested a pH-dependent mechanism after they observed that inhibition of the pathogen was eliminated when the LAB culture was set to pH 7. In another study, the growth and expression of virulence factors by *Salmonella* were shown to be affected by lactic acid (169).

The inhibition of *E. coli* O157:H7 by different *Lactococcus* and *Lactobacillus* strains was attributed to the production of lactic acid and low pH (170, 171). The growth of *Helicobacter pylori* was inhibited by different *Lactobacillus* and *Bifidobacterium* strains including *L. acidophilus*, *L. bulgaricus* and *Bifidobacterium bifidus* (172, 173). These effects were attributed to the production of lactic, acetic and hydrochloric acid. In another study, the growth of four species of known enteropathogens, *H. pylori*, *Campylobacter jejuni*, *Campylobacter coli* and *C. difficile* were inhibited by *Lactobacillus* strains isolated from the human GIT, probably due to the production of organic acids (174). Based on these studies, it is reasonable to suggest that the production of organic acids by probiotics in the GIT makes the intestinal environment less favourable for their competitors and decreases the risk of enteric infections by pathogens.

3.2.4 Hydrogen peroxide

In addition to lactic acid and bacteriocin production, hydrogen peroxide (H_2O_2) production by commensal or probiotic LAB may be an important antimicrobial mechanism against pathogens (175). Hydrogen peroxide may cause reduced pathogen virulence, reduced pathogen invasion of epithelial cells or death of intestinal pathogens after intracellular diffusion which alters gene transcription and signal transduction (176-179). Several H_2O_2 -producing bacterial species with probiotic properties have been isolated, such as *B. bifidum*, the *Lactobacillus johnsonii* NCC 533 gut isolate, a *L. delbrueckii* subsp. *bulgaricus* yogurt isolate and normal microflora vaginal isolates such as *L. crispatus* and *Lactobacillus gasseri* (178-184).

The ability of *L. johnsonii* NCC533 to generate up to millimolar quantities of H_2O_2 under aerobic conditions has been demonstrated (179). The authors demonstrated the antimicrobial role of *L. johnsonii* NCC533 produced- H_2O_2 against *S. enterica* serova Typhimurium *in vitro*, and proposed that *L. johnsonii* NCC533 H_2O_2 -production could contribute to protection against the pathogen *in vivo*. Other studies have shown that H_2O_2 -producing *L. crispatus* F117 and *Lactobacillus paracasei* strains (F2 and F28) inhibited the growth of *S. aureus* *in vitro* (184, 185). The beneficial role of H_2O_2 -producing probiotic LAB that form part of the vaginal microflora of healthy women has been studied extensively (183-186). Previous studies have reported that women carrying H_2O_2 -producing lactobacilli are less likely to develop bacterial vaginosis (183, 186).

3.2.5 Siderophores

Iron is an essential micronutrient that plays a central role in the metabolism and proliferation of most gut microbes, including commensal bacteria and gut pathogens (187). Siderophores are LMW organic, high-affinity iron-chelating compounds produced by microorganisms such as bacteria and fungi (188). These compounds inhibit the growth, proliferation and persistence of competing microbes by depriving them of iron. In doing so, siderophore-producing bacteria sequester free iron available in their environment that is essential to other microorganisms. For example, the growth of *Lc. lactis*, *C. difficile* and *Clostridium perfringens* was inhibited in the GIT by iron-binding *Bifidobacterium* strains that produce siderophores (189). In a recent study, the growth and adhesion to IECs of enteropathogenic *S.*

typhimurium N15 and EHEC were inhibited by *B. pseudolongum* PV8-2 and *Bifidobacterium kashiwanohense* PV20-2 with high iron sequestration properties (190).

3.2.6 Biosurfactants

The production of biosurfactants by some LAB is another mechanism that can interfere with pathogen growth in the GIT. Biosurfactants are a group of compounds with surface and emulsifying activities that can be used in many different biomedical applications (191, 192). Several LAB strains have been isolated that produce either cell-bound or excreted biosurfactants with antibacterial, antiviral and antifungal properties (191-196). Biosurfactants cause permeabilization of cells by effecting changes that disrupt or lyse the physical cell membrane structure (197). The use of biosurfactant-producing lactobacilli in the prevention of urogenital tract infections is of considerable interest (191). These organisms are believed to compete with urogenital bacterial pathogens and yeast for adhesion sites on epithelial cells and control their growth by the production of biosurfactants (198-200). In another study, *L. casei* MRTL3 that produces a bacteriocin and a biosurfactant was shown to inhibit several food-borne pathogens (201).

3.2.7 Compounds inhibiting pathogen adhesion to intestinal cells

Adhesion to intestinal cells and subsequent colonization by enteropathogens is regarded as a prerequisite for their virulence (202). Probiotic bacteria have been shown to produce compounds that do not have a direct bactericidal effect, but which inhibit the binding of pathogenic bacteria to intestinal cells. Fujiwara and co-workers purified and characterized a novel proteinaceous compound in culture supernatants of *B. longum* SBT2928, termed BIF, that inhibits the adhesion of enterotoxigenic *E. coli* Pb176 (ETEC) to human HCT-8 IECs (92, 203, 204). The authors demonstrated that BIF blocks the binding of the ETEC Pb176 colonization factor antigen (CFA) II adhesive factor to gangliotetraosylceramide (bacterial binding structure) receptors on the intestinal cell surface (92), thereby preventing ETEC Pb176 colonization. Two *Bifidobacterium* strains, CA1 and F9, isolated from the GIT of infants produce a LMW lipophilic antibacterial compound that inhibits the adhesion of several pathogenic bacteria, including *S. typhimurium* SL1344 and *E. coli* C1845 (205).

3.3 Stabilization of intestinal epithelial barrier

The intestinal epithelium consists of a uni-layer of cells covered by a mucus layer that is constantly exposed to the luminal contents and various enteric bacteria (107, 206). The intestinal epithelial barrier consists of the mucus layer, the intestinal cells and the gut innate immune system (206). This intestinal barrier functions as a key defence mechanism required to maintain epithelial integrity and to prevent infection by pathogens and excessive inflammation (206). Stabilization and maintenance of this barrier is thus of utmost importance to the host. Important defence mechanisms of the intestinal barrier against unwelcome intrusion of harmful antigens include the mucosal layer (mucin production), intercellular junctional complexes (tight and adherence junctions) and the secretion of antimicrobial peptides (such as defensins) and immunoglobulin A (IgA) (206-209). Disruptions of this barrier function can lead to inappropriate inflammatory responses due to invasion of the submucosa by bacteria or food antigens, which may result in intestinal disorders such as inflammatory bowel disease (IBD) and ulcerative colitis (207, 210, 211). Consumption of colonizing or non-colonizing probiotics can enhance barrier integrity which helps to protect the intestinal epithelium against enteric pathogens and chronic inflammation by direct effects on the epithelium (e.g. increasing mucin expression), modulation of the immune system and by direct effects on commensal and pathogenic bacteria (e.g. antimicrobial peptides and competition for adherence) (Fig. 6).

Intestinal epithelium cells are overlaid with a protective inner and outer mucus layer that limits bacterial movement and acts as a dynamic defence barrier against enteropathogens and other potentially harmful antigens (208). For enteropathogens to colonize the intestine, they have to penetrate the mucus layer before they reach the intestinal epithelium (212). Mucins are the major macromolecular constituents of the epithelial mucus layer and are produced by specialized goblet cells in the intestinal tract (213) (Fig. 6). Probiotics are able to inhibit pathogen adherence to IECs by promoting the secretion of intestinal mucins. Several *Lactobacillus* species have been shown to increase the expression of specific mucin genes in human intestinal Caco-2 and HT29 cells, thus preventing the adherence and internalization of pathogenic *E. coli* (123, 214, 215). The adherence of EPEC was inhibited by *L. plantarum* 299v-mediated increase in expression of the MUC2 and MUC3 mucins (123, 215). Rats administered with VSL3 (pre- and probiotic mixture) for 7 consecutive days showed a 60-fold increase in MUC2 expression and an associated increase in mucin production (216). Therefore, increased mucus production mediated by probiotic bacteria *in vivo* may be a key

mechanism in their interactions with enteropathogens to prevent infections and to improve intestinal barrier function.

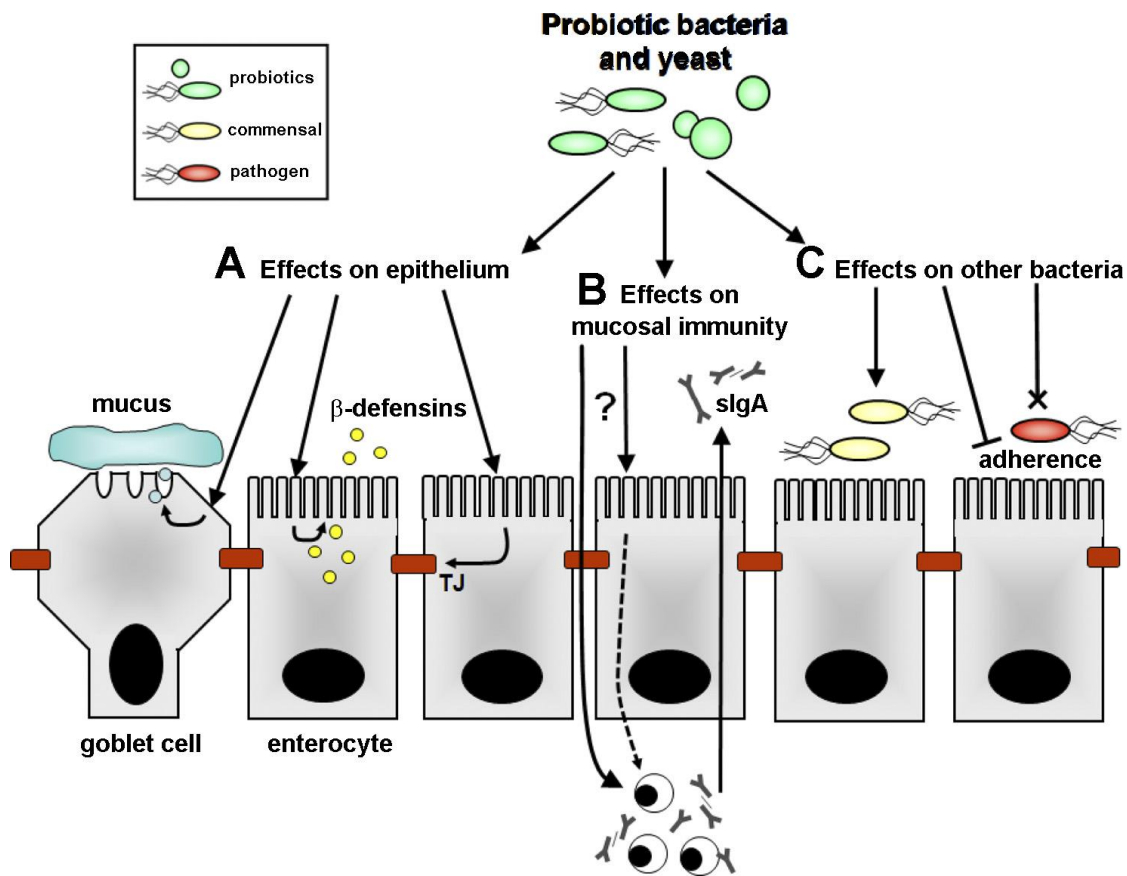


FIG. 6. Beneficial effects of probiotics on intestinal epithelial barrier function (206). Probiotics can affect epithelial barrier integrity by numerous mechanisms. These include: **A.** direct effects on the intestinal epithelial cells (IECs). Probiotics can increase the secretion of mucin glycoproteins by goblet cells that assemble into a thick mucus layer. Probiotics can augment the secretion of antimicrobial proteins (defensins) by IECs that help to eliminate commensals or pathogens that penetrate the mucus layer. Probiotics can enhance the stability of intercellular junctional complexes (tight junctions (TJ)), which decreases the intercellular permeability of IECs to pathogens and other antigens. **B.** Mucosal immunomodulation. Probiotics can augment the levels of IgA-secreting plasma cells in the lamina propria and promote the transcytosis of secretory IgA (sIgA) across the epithelial cell layer and secretion into the luminal mucus layer, preventing and limiting bacterial penetration of host tissues. **C.** Effects on commensal and infectious bacteria. Probiotics can alter the natural gut microbiota composition and/or gene expression, enhancing barrier integrity through the commensal microbiota. Most probiotics can inhibit enteric pathogens via the production of antimicrobial substances such as bacteriocins. Finally, probiotics can compete with commensals and enteric pathogens for adherence sites in the mucus layer or IECs, thereby preventing harmful colonization and enhancing barrier function.

3.4 Co-aggregation

Probiotic bacteria can prevent enteropathogenic adherence and intestinal colonization by co-aggregating with pathogens (217-219). This process allows probiotic bacteria to interact closely with pathogens, allowing them the opportunity to release their anti-pathogenic substances in very close proximity. *Lactobacillus plantarum* strains (S1, A and B) co-aggregate with selected food-borne pathogens including *S. typhimurium* and *L. monocytogenes* (220). The adherence of ETEC to porcine enterocytes was affected by co-aggregation of the pathogen with selected *Lactobacillus* spp. (221). Schachtsiek and co-workers (222) described the role of a *Lactobacillus coryniformis* DSM 20001 surface protein encoded by a *cpf* gene (co/aggregation-promoting factor) in the ability of the LAB strain to co-aggregate with *E. coli* K-88, *C. coli* and *C. jejuni*.

3.5 Inhibition of flagella motility

Foodborne pathogens such as *S. enterica* serovar Typhimurium require actively rotating flagella to rapidly contact and to efficiently penetrate gastrointestinal epithelial cells (223, 224). A recent study by Lieveen-Le Moal and co-workers (225) demonstrated that antidiarrhoeic *L. acidophilus* LB inhibited the entry of *S. enterica* serovar Typhimurium into human intestinal Caco-2 cells by disrupting the swimming motility of the diarrhoea-associated enteropathogen. Therefore, impairment of the flagella motility of enteropathogens by probiotic bacteria in the intestinal tract could prevent pathogenic colonization of the gut.

3.6 Immune system modulation

It is well known that probiotic bacteria can exert regulatory effects on host innate and adaptive immune responses (226). These bacteria have the ability to modulate the functions of dendritic cells (DC), monocytes/macrophages, and T and B lymphocytes, which enhances phagocytosis of invading gut pathogens (227, 228). By stimulation of the host immune responses (specific and non-specific), probiotic bacteria can displace pathogens in the GIT and prevent intestinal diseases (228-231).

Probiotic bacteria can interact with pathogens in the gut by antagonizing inflammatory responses induced by the gut pathogens, which can lead to intestinal diseases such as gastroenteritis and irritable bowel syndrome (226). Inflammation allows pathogens to flourish

at the expense of the natural microbiota and host intestinal health. Probiotic bacteria are able to trigger an anti-inflammatory response from the innate immune system by signaling DCs to secrete anti-inflammatory cytokines such as interleukin 10 (IL-10) (232, 233) (Fig. 1). They can also elicit a decrease in pro-inflammatory cytokines during inflammation (227). Down-regulation of pro-inflammatory cytokine secretion from immune cells occurs as a result of probiotic bacterial interference with inflammatory signaling pathways such as nuclear factor-kappa B (NF- κ B) and mitogen activated protein kinases (MAPK) (234, 235) (Fig. 7). The activation of these pathways leads to the secretion of pro-inflammatory cytokines that can severely damage the intestinal epithelial barrier. NF- κ B and MAPK signaling pathways are activated by enteric pathogens to stimulate the secretion of pro-inflammatory cytokines (e.g. IL-8), that lead to the recruitment of inflammatory immune cells (e.g. neutrophils) to the infected area resulting in severe inflammation, tissue damage and disease (234, 235). Several studies have identified probiotic strains with the ability to suppress pro-inflammatory cytokine production to avoid pathogen-induced inflammation at infection sites (226, 235, 237, 238). A recent study by Finamore and co-workers (237) reported that *Lactobacillus amylovorus* DSM 16698 protected IECs against the pro-inflammatory response induced by ETEC K88 through the suppression of pro-inflammatory cytokines IL-8 and IL-1 β . Another study demonstrated the ability of *L. casei* OLL2768 to suppress the ETEC-induced pro-inflammatory response by inhibition of NF- κ B and MAPK pathways which reduced pro-inflammatory cytokine levels (238).

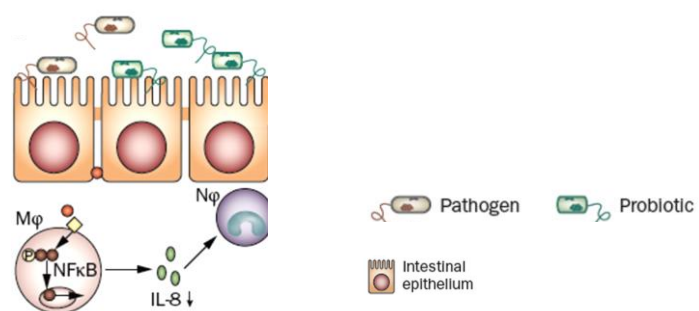


FIG. 7. Down-regulation of pro-inflammatory cytokine (IL-8) secretion by probiotic bacteria in the GIT (236). Probiotic bacteria (or their products) may dampen an innate immune response by inhibiting the NF- κ B inflammatory signaling pathway and influencing the production of IL-8 and subsequent recruitment of inflammatory immune cells to sites of intestinal injury. Abbreviations: IL-8, interleukin 8; M Φ , macrophage; N Φ , neutrophil; NF- κ B, nuclear factor-kappa B.

Probiotics also play a role in the stimulation and production of antibodies in the gut, particularly immunoglobulin A (IgA) (226, 227) (Fig. 6). Antibodies released in the intestinal lumen can inhibit pathogen adherence to IECs by interfering with adhesive cell receptors on the pathogen's cell membrane (33). Previous studies have indicated that *Saccharomyces boulardii* and *L. rhamnosus* GG increased secretory IgA levels or immunoglobulin-secreting cell levels in the GIT (239, 240). Other studies have reported that oral administration of probiotic lactobacilli increased IgA levels in children suffering from diarrhoea, thereby shortening the duration of symptoms (33, 227, 241-243). Several probiotic strains can also modulate the host immune mechanisms by influencing phagocytosis of enteric pathogens by host immune phagocytic cells such as macrophages (37, 239, 242, 244, 245). The inhibition of enteropathogenic *Pseudomonas aeruginosa* and *L. monocytogenes* in mice by a strain of *L. casei* was correlated to an increase in abundance of macrophages (246). Furthermore, probiotic bacteria can affect phagocytotic cell activities not only in clinical situations but also in healthy subjects (247-249).

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Chapter 3

Spatial and Temporal Colonization of *Lactobacillus plantarum* 423 and *Enterococcus mundtii* ST4SA in the Intestinal Tract, as Revealed by *In vivo* Bioluminescence Imaging in a Murine Model

Spatial and Temporal Colonization of *Lactobacillus plantarum* 423 and *Enterococcus mundtii* ST4SA in the Intestinal Tract, as Revealed by *In vivo* Bioluminescence Imaging in a Murine Model

Abstract

Lactic acid bacteria (LAB) are major inhabitants and part of the normal microflora of the gastrointestinal tract (GIT) of humans and animals. Despite substantial evidence supporting the beneficial properties of LAB, only a few studies addressed the migration and colonization of probiotic bacteria in the GIT. The reason for this is mostly due to the limitations, or lack of, efficient reporter systems. This study reports on the application of the firefly luciferase gene (*ffluc*) from *Photinus pyralis* to develop luciferase-expressing *Lactobacillus plantarum* 423 and *Enterococcus mundtii* ST4SA using a *Lactococcus lactis* NICE system high copy number plasmid (pNZ8048) and strong constitutive lactate dehydrogenase gene promoters (*Pldh* and *STldh*). The reporter system was used for the *in vivo* and *ex vivo* monitoring of both probiotic LAB strains in the GIT of mice after single and multiple oral administrations. *Enterococcus mundtii* ST4SA reached the large intestine 45 min after gavage, while *L. plantarum* 423 reached the cecum/colon after 90 min. Both strains predominantly colonized the cecum and colon after five consecutive daily administrations. *Enterococcus mundtii* ST4SA persisted in faeces at higher numbers and for more days compared to *L. plantarum* 423. This is the first report of bioluminescence imaging of a luciferase-expressing *E. mundtii* strain to study colonization dynamics in the murine model. The bioluminescence system developed in this study has the potential to show the *in vivo* colonization dynamics of other important probiotic LAB species.

Introduction

Lactic acid bacteria (LAB) are common inhabitants of a healthy human and animal gastrointestinal tract (GIT) and they play a major role in keeping the gut microbiota in a balanced state (1-4). Beneficial properties include the inhibition of enteric pathogens (5), alleviation of constipation (6) and diarrhoea (7), stimulation of the immune system (8), repression of cancer cell and tumor growth (9, 10), and synthesis of essential metabolites such as vitamins (11). Key to the function of probiotic bacteria is their ability to survive stomach acids and bile salts (12). Some strains have adapted to these harsh conditions by over-expressing specific genes when exposed to acids and bile salts (13-14). Changes on a genetic level will undoubtedly alter the way probiotic bacteria compete against pathogens and colonize the GIT.

The survival and colonization of LAB in the GIT is usually studied *in vitro* and *ex vivo* by using models simulating the GIT (16-19). Although these studies are valuable in understanding the survival of LAB in the GIT, the findings seldom reflect real-life conditions. More *in vivo* studies are needed to understand the interactions between probiotic bacteria, pathogens, commensal bacteria and gut epithelial cells. The best approach to study real-time interactions between probiotic bacteria and their mammalian host in the GIT is by labelling the cells with fluorescent or bioluminescent markers (20, 21). A selection of genes, encoding proteins that emit light at specific wavelengths, are available for cloning into plasmids or insertion into the genomes of recipient cells (21, 22). The most commonly used luciferase labelling systems used in *in vivo* and *ex vivo* tracking of bacteria are bacterial *luxABCDE* from *Photobacterium luminescence* (23), click beetle luciferase (CBluc) from *Pyrophorus plagiophthalmus* (24) and firefly luciferase (Ffluc) from *Photinus pyralis* (25). The CBluc and Ffluc luciferases require the exogenous addition of D-Luciferin, whereas the bacterial *lux* substrate is naturally present within the bacterial cells (20). The half-life of luciferase is only several seconds and does not represent bioluminescence accumulated over a period (26). Another advantage is that only low levels of background luminescence are emitted by mammalian tissue.

To date, very few studies have used whole-body imaging to monitor the persistence of LAB in the GIT, and with variable degrees of success. This is mostly due to the weak penetration of photons through muscles and tissue. Furthermore, labelled cells orally administered are often dispersed throughout the GIT or become metabolically inactive and emit

bioluminescent signals too weak to detect. Cronin and co-workers (27) used a bacterial *lux* system to study the persistence of *Bifidobacterium breve* in mice. The bioluminescent signal was, however, not emitted from the GIT of live mice and all imaging had to be done *ex vivo* after dissecting the GIT. In our own studies (28), fluorescence encoded by the *mCherry* gene, transformed into *Lactobacillus plantarum* 423 and *Enterococcus mundtii* ST4SA, was also only detected after surgical removal of the GIT. This is not unusual, as also reported by Oozeer et al. (23) and Corthier et al. (29) with studies done on mice. Lee and Moon (30) were one of the first to detect *Lactococcus lactis* in the GIT of live mice by using a pMG36e Ffluc plasmid vector, although the strain could only be detected for up to 2 h. In another study, Daniel and co-workers (31) successfully monitored the colonization and persistence of *Lc. lactis* and *L. plantarum* in live mice using the CBluc luciferase system.

Lactobacillus plantarum 423, isolated from sorghum beer and *E. mundtii* ST4SA, isolated from soybeans both have probiotic properties (32-34). The strains survive conditions in the human GIT, as shown with studies performed on a computerized model simulating the intestinal conditions of infants (16). Both strains adhere to human intestinal epithelial cells (35) and produce antimicrobial peptides (36, 37), active against *Listeria monocytogenes*, *Enterococcus faecalis*, *Clostridium sporogenes* and *Salmonella typhimurium* (32, 33, 38-40). In a previous report, using the *mCherry* fluorescence gene, we have shown that *L. plantarum* 423 and *E. mundtii* ST4SA were localized in the cecum and colon of mice after a single oral dosage (28).

This study reports on the application of a red-shifted thermostable firefly luciferase-system (Ffluc) to study the spatial and temporal persistence of *L. plantarum* 423 and *E. mundtii* ST4SA in the GIT of mice after single and multiple dosages. The use of a red-emitting luciferase with a longer wavelength (620 nm) enabled optimal light penetration through intestinal and skin tissue. The *in vitro* and *in vivo* expression of the Ffluc system was optimized using a combination of a high-copy number plasmid vector and strong constitutive promoters. Differences between the two strains in viability and persistence in the GIT of mice were demonstrated by monitoring *in vivo* and *ex vivo* bioluminescence, using the Caliper *in vivo* imaging system (IVIS; Caliper Life Sciences, Hopkinton, MA). The bioluminescence system also allowed tracking each of the strains in different sections of the GIT.

Materials and methods

Animals used

Ethical approval for *in vivo* experiments was granted by the Ethics Committee of Stellenbosch University (reference number SU-ACU-2017-0206-454). Eight-week-old female BALB/c mice were used in all experiments. Animals were housed separately under controlled environmental conditions and fed a standard rodent diet. Animal procedures were performed according to the Stellenbosch University ethical guidelines.

Bacterial strains, plasmid construction and culture conditions

Lactobacillus plantarum 423 and *E. mundtii* ST4SA were labelled by transformation with plasmids encoding the red-shifted thermostable firefly luciferase gene from *P. pyralis* (*ffluc*) (41). The bioluminescence expression vectors are based on the pNZ8048 *Lc. lactis* NICE system high copy number plasmid (Mobitech, Goettingen, Germany). The vector contains the *cat* gene for chloramphenicol (Cm) resistance, the *nisA* gene promoter region (*PnisA*), a multiple cloning site (MCS), replication genes (*repC* and *repA*) for replication in LAB/*E. coli* and the termination (T) sequence of the *Lc. lactis pepN* gene (42). Primers used for PCR amplification are listed in Table S1 and were from Inqaba Biotechnical Industries (Pretoria, South Africa). DNA restriction enzymes and PCR polymerase were from New England Biolabs (NEB, Ipswich, MA, USA). The construction of pNZPldhFfluc and pNZSTldhFfluc luciferase expression vectors is shown in Fig. S1. Plasmid pNZPldhFfluc carried the *ffluc* gene under control of the strong constitutive *L. plantarum* 423 lactate dehydrogenase gene promoter (*Pldh*). In the pNZSTldhFfluc construct, the *ffluc* gene was cloned under control of the strong constitutive *E. mundtii* ST4SA lactate dehydrogenase gene promoter (*STldh*). The *Pldh* and *STldh* promoters were amplified from *L. plantarum* 423 and *E. mundtii* ST4SA genomic DNA, using primer pairs Pldh1/Pldh2 and ldhS1/ldhS2, respectively. The *ffluc* bioluminescence gene was amplified from plasmid pMV306G13+FflucRT using primers FlucFor and FlucRev. Briefly, the *Pldh* (520 bp), *STldh* (166 bp) and *ffluc* (1.6 kb) PCR fragments were cloned into pNZ8048 after digestion of *Pldh* and *STldh* with *BglIII/NcoI*, digestion of *ffluc* with *NcoI/XbaI* and digestion of pNZ8048 with *BglIII/XbaI*, yielding plasmids pNZPldhFfluc and pNZSTldhFfluc, respectively.

The two bioluminescence expression vectors were introduced into *L. plantarum* 423 and *E. mundtii* ST4SA by electro-transformation as described by Van Zyl and co-workers (28) and

were named *L. plantarum* 423 Fluc and *E. mundtii* ST4SA Fluc. *Lactobacillus plantarum* 423 and *E. mundtii* ST4SA containing the empty pNZ8048 vector were used as controls and were labelled *L. plantarum* 423 (pNZ8048) and *E. mundtii* ST4SA (pNZ8048). Plasmid stability in *L. plantarum* 423 Fluc and *E. mundtii* ST4SA Fluc, and growth comparison between wild-type (WT) and recombinant strains was tested by standard methodology as described previously (28).

Escherichia coli MC1061 (Mobitech) was used as a cloning host for construction of pNZ8048-derived bioluminescence expression vectors and was cultured aerobically at 37°C in Luria-Bertani (LB) broth, or brain heart infusion (BHI) and streaked onto the same media, supplemented with 1.5% (w/v) agar (all from Biolab Diagnostics, Midrand, South Africa). *Lactobacillus plantarum* 423 and *E. mundtii* ST4SA were grown without shaking at 30°C in MRS broth and streaked onto MRS agar (both from Biolab Diagnostics). Where appropriate, Cm was added at 10 µg/ml to growth media of *E. coli* MC1061 and *L. plantarum* 423 and 5 µg/ml to media of *E. mundtii* ST4SA.

Correlation between *in vitro* bioluminescence measurements and viable cell numbers

Lactobacillus plantarum 423 Fluc and *E. mundtii* ST4SA Fluc were grown for 12 h at 30°C in MRS broth, supplemented with Cm as mentioned elsewhere. From these cultures, 1 ml was inoculated into freshly prepared MRS broth and incubated at 30°C to an optical density (OD_{550nm}) of 2.5 (for *L. plantarum* 423) and 2.3 (for *E. mundtii* ST4SA). Viable cell numbers were determined by plating onto MRS agar containing Cm. The bacterial suspensions were harvested (3 min at 8000 x g), washed twice with sterile phosphate buffered saline (PBS), resuspended in gavage buffer (0.2 M NaHCO₃ with 1%, w/v, glucose, pH 8.0) and serially diluted to 1/128 in the same buffer. Two-hundred microliters of each dilution was added in triplicate to black 96-well microtitre plates and bioluminescence measured after the addition of 5 µl of D-Luciferin potassium salt (Anatech Instruments, Bellville, South Africa) at 470 µM. Bioluminescent readings were recorded using the Caliper *in vivo* imaging system (IVIS; Caliper Life Sciences, Hopkinton, MA, USA) and the photons emitted from regions of interest (ROI) calculated using the Living Image[®] software, version 3.0 (Caliper Life Sciences). The ROI of each well were manually selected. Exposure times ranged from 30 s to 2 min, depending on the intensity of the signal. Bacterial cell numbers were plotted against bioluminescence emitted, recorded as photons per second (p/s). Non-bioluminescent *L.*

plantarum 423 (pNZ8048) and *E. mundtii* ST4SA (pNZ8048) were used to set the background bioluminescence.

Preparation of bacterial strains and dosing of mice

Lactobacillus plantarum 423 Fluc and *E. mundtii* ST4SA Fluc were grown at 30°C for 12 h, whereafter 1 ml of each culture was inoculated into freshly prepared 10 ml MRS broth. *Lactobacillus plantarum* 423 Fluc was grown to an OD₅₅₀ of 2.5 and *E. mundtii* ST4SA Fluc to an OD₅₅₀ of 2.3. The cells were harvested (3 min at 8000 x g), washed twice with sterile PBS and resuspended in gavage buffer at a final concentration of 4 x 10⁹ CFU. Mice in each group were then gavaged with 200 µl (4 x 10⁹ CFU) of each strain.

***In vivo* gastrointestinal persistence of LAB in the murine model**

Groups of mice each received a daily dose of 200 µl (4 x 10⁹ CFU) of live *L. plantarum* 423 Fluc or *E. mundtii* ST4SA Fluc for one or five consecutive days by intragastric gavage. Control mice received 200 µl (4 x 10⁹ CFU) of non-bioluminescent *L. plantarum* 423 (pNZ8048) or *E. mundtii* ST4SA (pNZ8048) in all experiments. Faeces (100 mg) were collected at different time points and vortexed in 1 ml sterile PBS for 5 min, followed by serial dilution in sterile PBS, and plating onto MRS agar supplemented with Cm and incubated, as described elsewhere. Viable cell numbers were expressed as CFU per 100 mg faeces. Two mice per strain were sacrificed by cervical dislocation at predetermined time points, the intestines surgically removed and immediately separated in a sterile Petri dish (refer to Fig. S2 for different sections of mouse GIT). The lumen of all intestinal sections was injected with air using a 27-gauge needle and syringe, as described by Rhee and co-workers (43), and bioluminescence recorded using the IVIS. The complete duodenum, jejunum, ileum and large intestine (cecum plus colon) were homogenized, separately, in 3 ml sterile PBS, serially diluted and plated (in duplicate) onto MRS agar. The plates were incubated and cell numbers determined as described elsewhere.

***In vivo* bioluminescence measurements**

In vivo bioluminescence imaging (BLI) was recorded using the IVIS, equipped with a cooled-charged-device camera mounted on a light-tight specimen chamber (dark box) and a Windows computer system. Mice were gavaged with 200 µl of a D-Luciferin potassium salt

suspension (30 mg/ml) 30 min before gavage with *L. plantarum* 423 Fluc and *E. mundtii* ST4SA Fluc. Mice were anesthetized with 2% (vol/vol) isoflurane in an oxygen-rich induction chamber before administering the D-Luciferin and bacteria. Mice were kept subdued during bioluminescent readings with a mixture of isoflurane (1.5%, vol/vol) and oxygen. Mice in ventral position were imaged for quantification of bioluminescent photon emission with exposure times ranging from 1 to 5 min, depending on the signal intensity. Pseudo-color images superimposed over grayscale reference images representing light intensity (red, most intense and purple being the least intense) were generated using the Living Image[®] software program. ROIs were manually selected and bioluminescence expressed as photons emitted per second (p/s).

Results

***In vitro* functionality and stability of bioluminescent *L. plantarum* 423 and *E. mundtii* ST4SA**

Bacterial cultures resuspended in PBS were used to image the intensity of bioluminescent signals produced by *L. plantarum* 423 Fluc and *E. mundtii* ST4SA Fluc (Fig. 1A). No significant difference was observed in the maximum intensities of bioluminescent signals produced by *L. plantarum* 423 Fluc or *E. mundtii* ST4SA Fluc. Maximum bioluminescence was recorded for *E. mundtii* ST4SA Fluc with mean values of 2.49×10^8 p/s, while slightly lower mean values of 1.94×10^8 p/s was recorded for *L. plantarum* 423 Fluc. None of the control strains (*L. plantarum* 423 (pNZ8048) or *E. mundtii* ST4SA (pNZ8048)), emitted bioluminescent signals. No bioluminescence was detected in culture supernatants of *L. plantarum* 423 Fluc or *E. mundtii* ST4SA Fluc, indicating that bioluminescent light production was strictly intracellular.

Bioluminescence emitted by cells that expressed firefly luciferase correlated with serial dilutions of total CFUs of cultures of *L. plantarum* 423 Fluc ($R^2=0.9830$) and *E. mundtii* ST4SA Fluc ($R^2=0.9870$), indicating that photon emission accurately reflects bacterial cell numbers (Figs 1B and 1C). The bioluminescence signal corresponded to the detection of bacterial CFU over a broad range, from approximately 6×10^6 CFU to 8.5×10^8 CFU for *L. plantarum* 423 Fluc (Fig. 1B) and from approximately 1×10^7 CFU to 2×10^9 CFU for *E. mundtii* ST4SA Fluc (Fig. 1C).

Fig 1A.

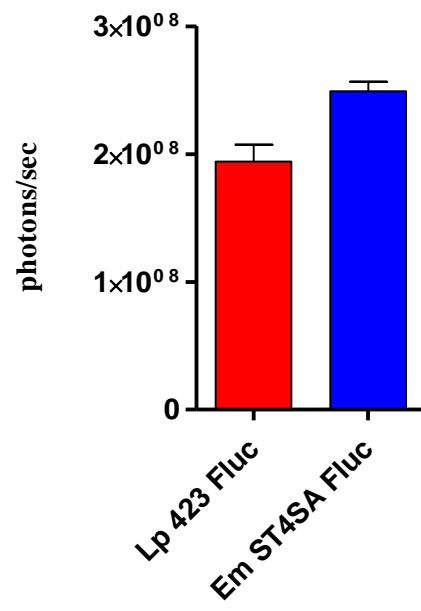


Fig 1B.

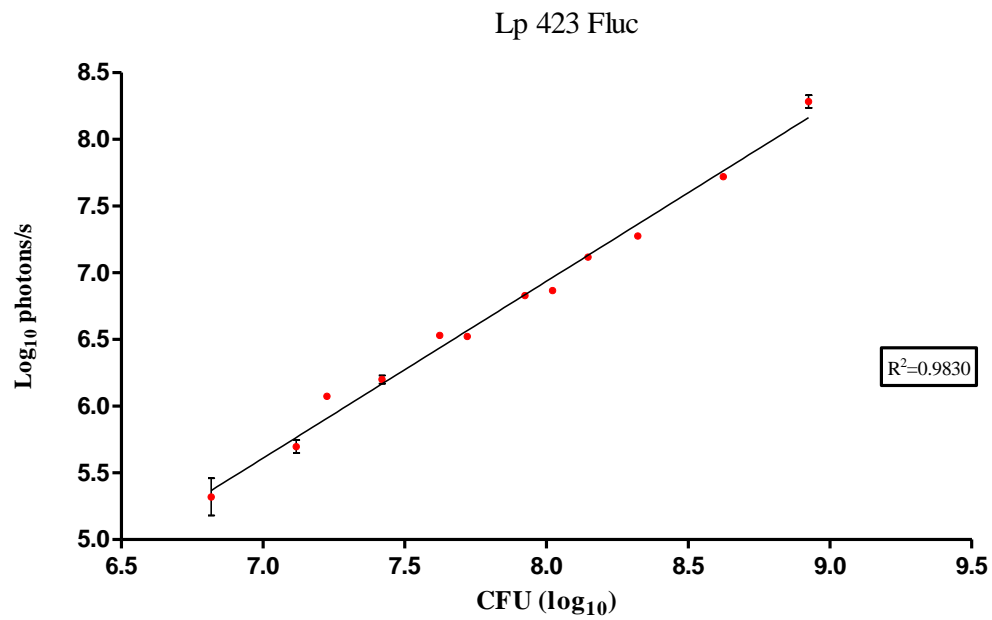


Fig 1C.

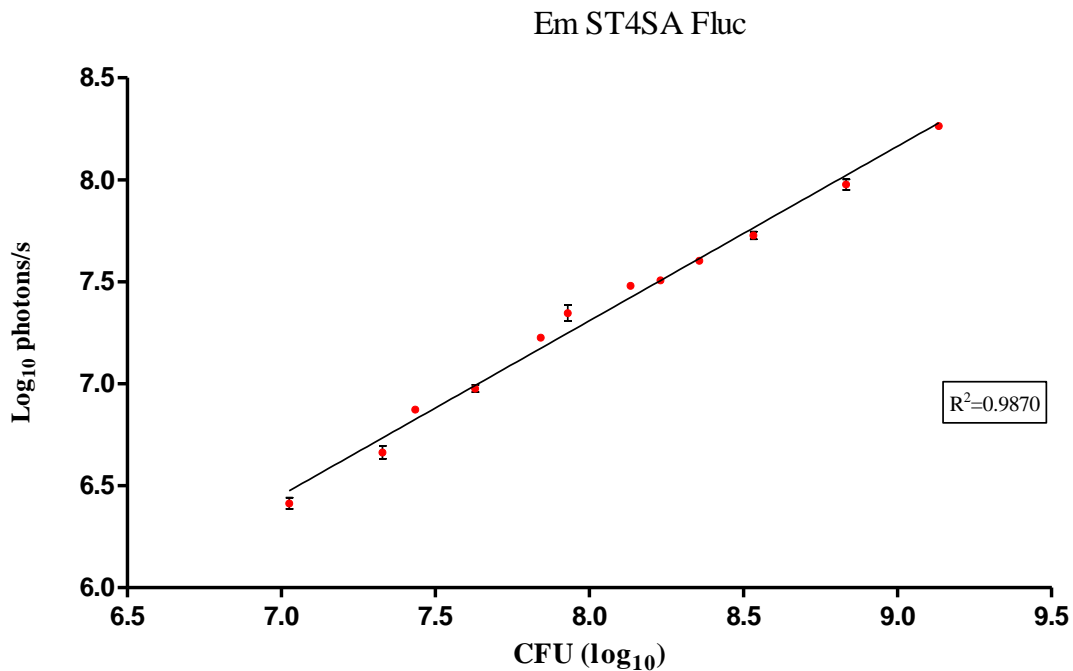


FIG. 1. Quantification of bioluminescent *L. plantarum* 423 Fluc and *E. mundtii* ST4SA Fluc. (A) Bioluminescence measured in cultures of *L. plantarum* 423 Fluc and *E. mundtii* ST4SA Fluc distributed in black 96-well microtitre plates. Means from six independent cultures are shown with standard deviations, and the background from each strain has been subtracted from each respective measurement. Correlation between bioluminescent signals and bacterial cell numbers of (B) *L. plantarum* 423 Fluc and (C) *E. mundtii* ST4SA Fluc. Cultures of each strain were serially diluted in black microplates and the bioluminescent signals quantified using the IVIS and then correlated with CFUs. Log₁₀ averages of three cultures are plotted, with error bars indicating standard deviations. The logarithmic trendline and the correlation of determination (R^2) between bioluminescence measurements and bacterial numbers of each strain are shown.

No significant difference in growth was observed between wild-types *L. plantarum* 423 and *E. mundtii* ST4SA and recombinants *L. plantarum* 423 Fluc and *E. mundtii* ST4SA Fluc, respectively, after 9 h of growth (not shown). Bioluminescent light production and the presence of the luciferase expressing plasmids in recombinant *L. plantarum* 423 Fluc and *E. mundtii* ST4SA Fluc strains had no detectable effect on bacterial growth. The stability of the luciferase-expressing plasmids pNZPldhFfluc in *L. plantarum* 423 Fluc and pNZSTldhFfluc in *E. mundtii* ST4SA Fluc was tested *in vitro* by subculturing for up to 7 days with replica-plating on non-selective and selective media (Fig 2A). The stability of the autonomous plasmids in *L. plantarum* 423 Fluc and *E. mundtii* ST4SA Fluc transformants was indicated by 100% plasmid retention and retained resistance to Cm following culturing for 7 days in

the absence of the antibiotic. The bioluminescent signals of the recombinant strains were also imaged with the IVIS in parallel to replica plating (Figs 2B and 2C).

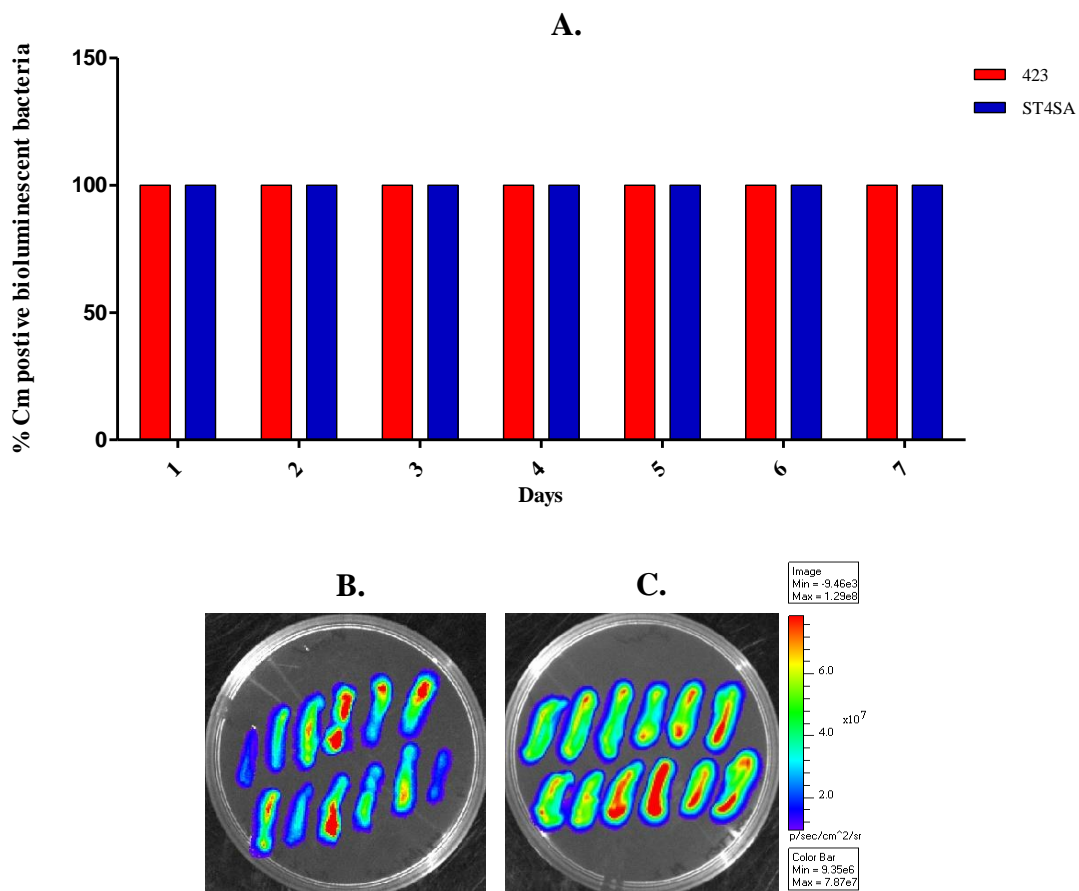


FIG. 2. *In vitro* stability of bioluminescence. (A) Stability of plasmid pNZPldhFluc in *L. plantarum* 423 Fluc and plasmid pNZSTldhFluc in *E. mundtii* ST4SA Fluc after subculturing for 7 days with replica plating on non-selective (antibiotic-free) and selective (Cm) media. The percentages of Cm-resistant colonies of three independent cultures of each respective strain are shown. Bioluminescent colonies of (B) *L. plantarum* 423 Fluc and (C) *E. mundtii* ST4SA Fluc after 7 days of subculture in antibiotic-free MRS media.

Colonization dynamics of bioluminescent *L. plantarum* 423 and *E. mundtii* ST4SA in the GIT of mice after a single dosage

To determine the spatial and temporal colonization of *L. plantarum* 423 Fluc and *E. mundtii* ST4SA Fluc after a single oral administration, groups of mice (n=13, per strain) were monitored over a 24 h period by transcutaneous *in vivo* BLI and *ex vivo* BLI of GITs and faeces. The viable bacteria numbers in the GIT and faecal samples were also recorded. Three anaesthetised mice (n=3, per strain) were imaged at 15 and 30 min and, 1, 1.5, 2, 3, 4, 6 and 24

h after the administration of *L. plantarum* 423 Fluc and *E. mundtii* ST4SA Fluc, respectively (Fig. 3). The same three mice were used throughout the 24 h trial period. At time zero (before administration) no bioluminescence was recorded (background signal corresponded to approximately 3×10^4 p/s). A maximum bioluminescent signal of approximately 2×10^8 p/s was detected for *L. plantarum* 423 Fluc and *E. mundtii* ST4SA Fluc at 1 h and 30 min, respectively. The bioluminescent signal of *L. plantarum* 423 Fluc remained at high levels until 2 h, but declined to lower levels 1 h later (mean value of approximately 9×10^6 p/s). The bioluminescent signal of *L. plantarum* 423 Fluc remained at a plateau (mean value of approximately 4×10^5 p/s) until 6 h. The bioluminescent signal of *E. mundtii* ST4SA Fluc steadily declined between 1 and 6 h and was significantly higher than that observed for *L. plantarum* 423 Fluc between 3 and 4 h. After 24 h, no bioluminescent signal could be detected for *L. plantarum* 423 Fluc (background level), whereas the signal of *E. mundtii* ST4SA Fluc declined to approximately 2×10^5 p/s.

Next, the localization of bioluminescent *L. plantarum* 423 Fluc and *E. mundtii* ST4SA Fluc in the GITs of mice after oral administration was determined by *ex vivo* imaging and recording viable cell numbers in the small and large intestinal tracts (Fig. 4). Results showed that 15 min after administration of bacterial strains, both *L. plantarum* 423 Fluc and *E. mundtii* ST4SA Fluc survived passage through the stomach by the observation of high cell numbers and bioluminescent cells throughout the small intestine (Fig. 4A and 4B). After approximately 45 min, bioluminescent cells of *E. mundtii* ST4SA Fluc reached the cecum and colon. From 90 to 240 min after oral administration of bacteria to mice, the majority of bioluminescent *L. plantarum* 423 Fluc and *E. mundtii* ST4SA Fluc had travelled through the small intestine and were located exclusively in the cecum and colon. Some of the viable cells of both strains remained in the small intestine after 90 min, but emitted weak or no bioluminescence signals (Fig. 4B). No bioluminescence of either strain was detected in the large intestine 15 min (Fig. 4A) after intragastric administration, suggesting that the viable cells' bioluminescence emission was below the detection limit of the IVIS through the intestinal tissue or the cells were metabolically inactive (Fig. 4C). After 24 h, a significantly higher number of viable *E. mundtii* ST4SA Fluc was detected in the cecum/colon compared to *L. plantarum* 423 Fluc.

Fig. 3A.

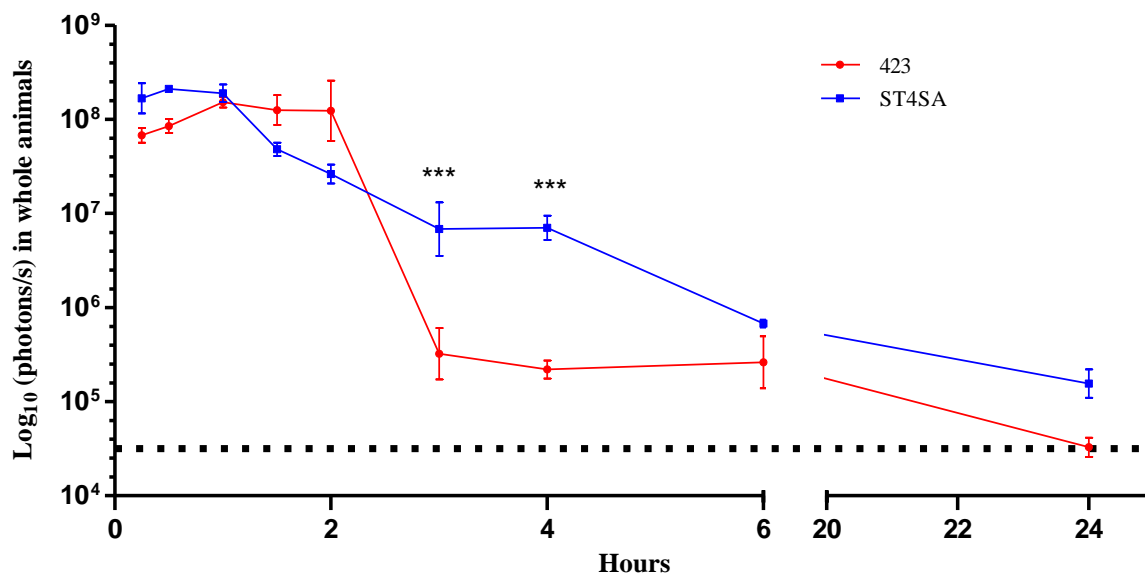


Fig. 3B.

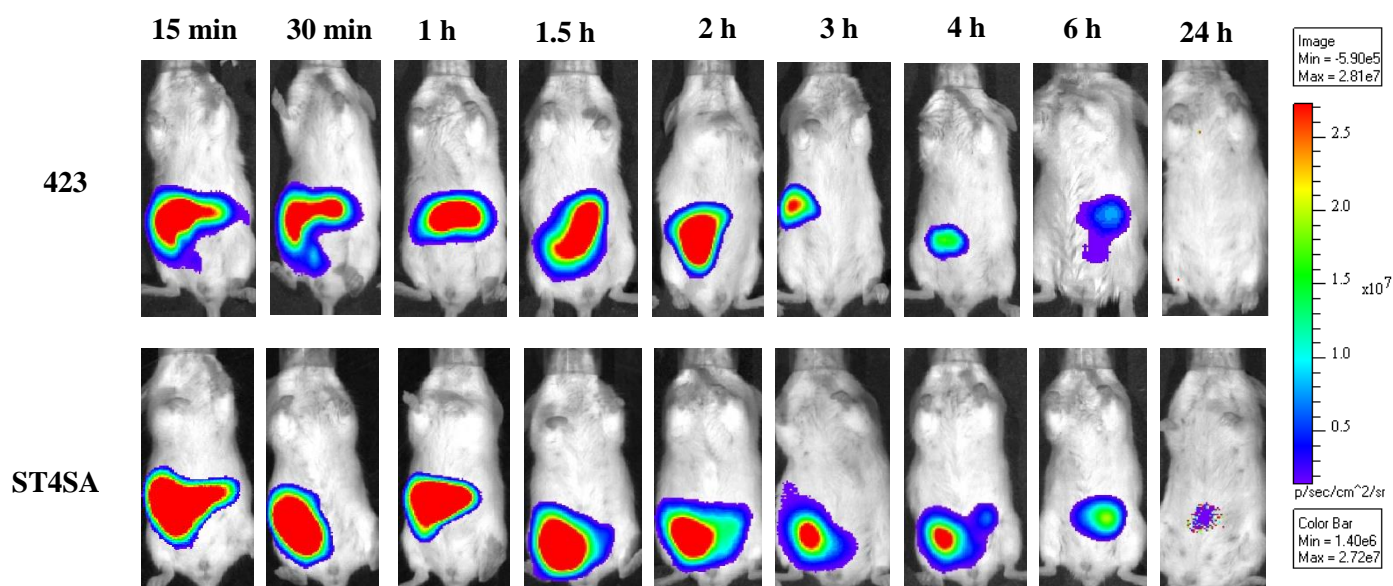


FIG. 3. Monitoring of *L. plantarum* 423 and *E. mundtii* ST4SA colonization in the digestive tract of mice by bioluminescence imaging in whole animals after one oral administration. *Lactobacillus plantarum* 423 Fluc and *E. mundtii* ST4SA Fluc were fed intragastrically (4×10^9 CFU) to two sets of three mice. The bioluminescent signals in \log_{10} photons/s measured from whole animals at different time-points over a 24 h period (A) are plotted, with standard deviations. Significant statistical differences between the bioluminescence signals of the two groups of mice are indicated with three asterisks ($P < 0.001$); Mann-Whitney nonparametric test). The background bioluminescence signal emitted is represented by a dashed line. (B) Visual images of bioluminescence emission in whole animals by mice fed once with *L. plantarum* 423 Fluc or *E. mundtii* ST4SA Fluc. In each case, one representative image of one mouse is shown. The intensity of the photon emission is represented as a pseudo-color image. One representative scale bar is shown (p/s).

Fig. 4A.

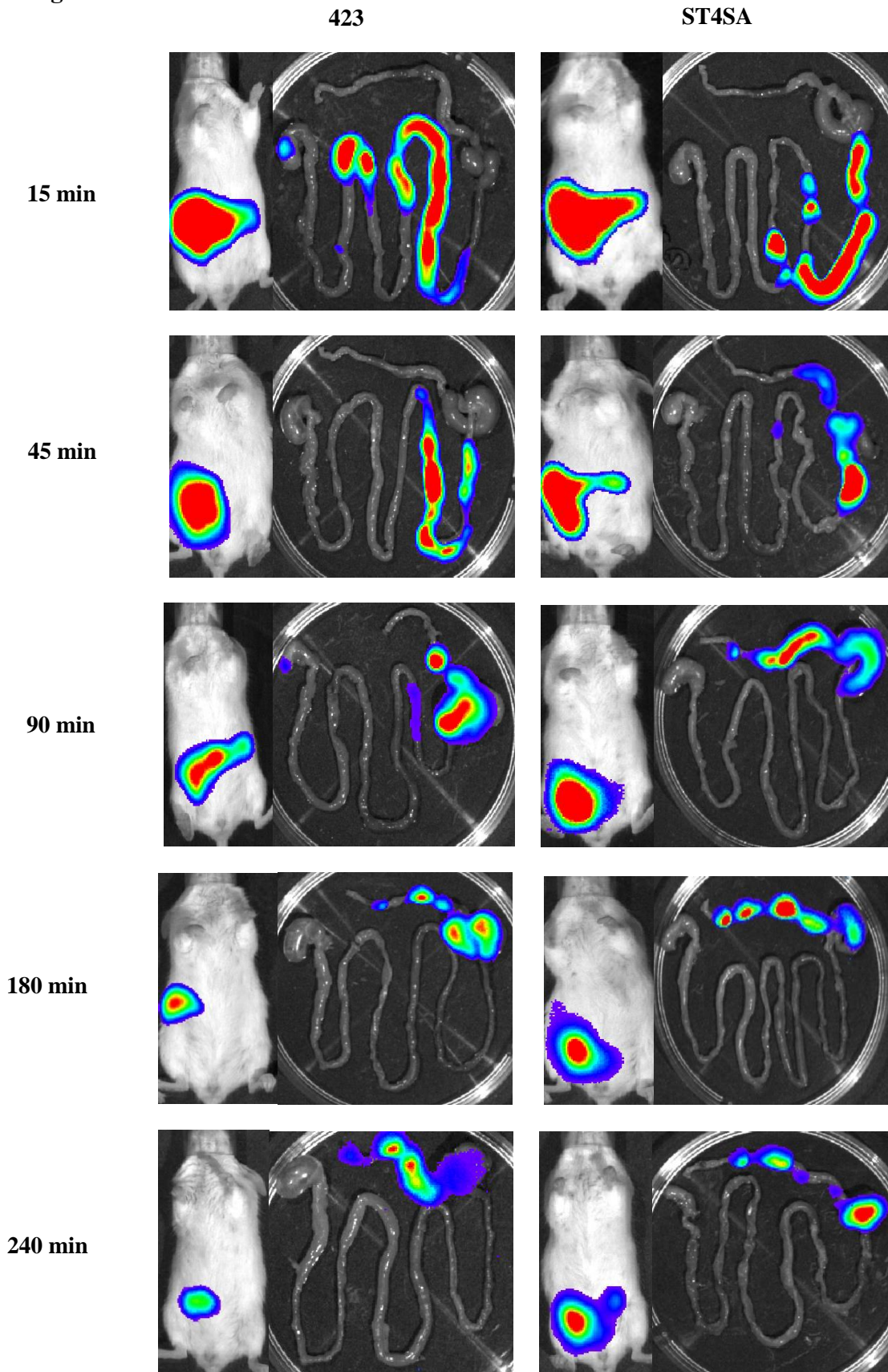


Fig. 4B.

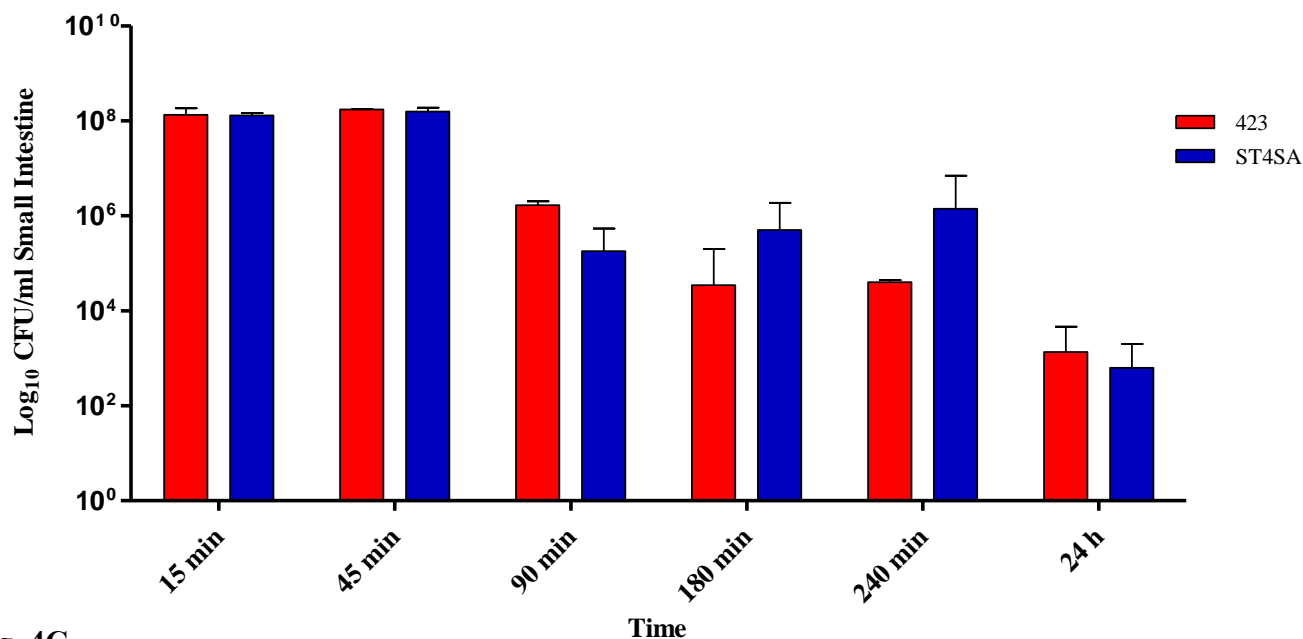


Fig. 4C.

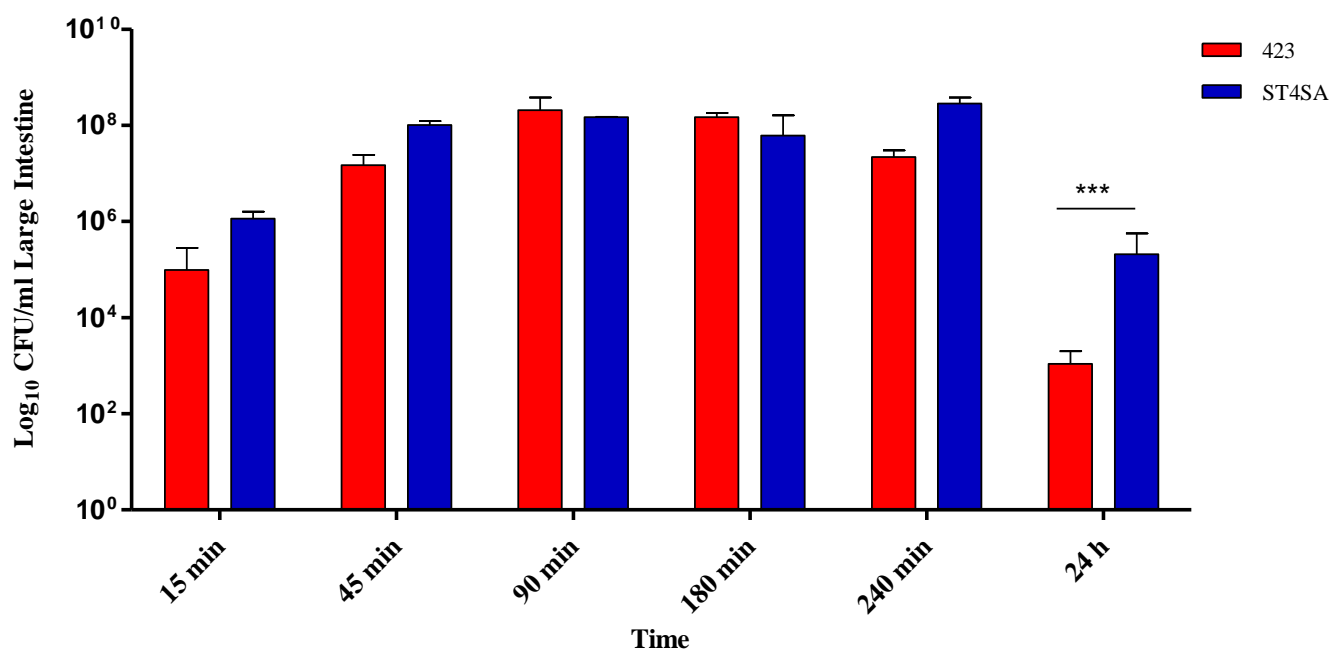


Fig. 4. Transit of *L. plantarum* 423 and *E. mundtii* ST4SA through the digestive tract of mice after one oral administration. Groups of mice were gavaged once with 4×10^9 CFU of *L. plantarum* 423 Fluc or *E. mundtii* ST4SA Fluc and the intestines resected at 15, 45, 90, 180, 240 min and 24 h. At each time point two mice were sacrificed, and (A) one representative image of one mouse and its GIT are shown. Persistence of viable *L. plantarum* 423 Fluc or *E. mundtii* ST4SA Fluc cells in (B) the small and (C) large intestinal tract of mice sacrificed at time points indicated in A. Significant differences between the two groups were assessed using the Mann-Whitney nonparametric test are indicated with asterisks ($P < 0.001$).

Colonization of *L. plantarum* 423 Fluc and *E. mundtii* ST4SA Fluc in the GIT of mice was also determined by monitoring the number of viable bacterial cells in faeces at different time points after intragastric administration (Fig. 5A). The respective bioluminescent signals of *L. plantarum* 423 Fluc and *E. mundtii* ST4SA Fluc in faeces were also monitored with the IVIS (Fig. 5 B). High cell numbers of both strains were excreted in the faeces and were proportionate to the respective bioluminescence signals emitted. The bacterial populations of both strains in faeces increased with time. Both strains reached a maximum number of approximately 2×10^8 CFU/100 mg faeces after 4 h and remained at this level for the following 2 h. These peaks correlated excellently with maximum bioluminescent signals of approximately 4×10^6 p/s/100 mg of faeces for *L. plantarum* 423 Fluc and 2×10^7 p/s/100 mg of faeces for *E. mundtii* ST4SA Fluc, from 4 to 6 h. The maximum level of viable *E. mundtii* ST4SA Fluc cells shed in the faeces was significantly higher in the first 2 h, reaching approximately 8×10^7 p/s/100 mg of faeces. Bioluminescence signals emitted by *E. mundtii* ST4SA Fluc cells in the faeces were higher compared to those of *L. plantarum* 423 Fluc throughout the 24 h study period. After 24 h, the number of *L. plantarum* 423 Fluc declined to approximately 4×10^4 CFU/100 mg faeces with no bioluminescent signal (background), while *E. mundtii* ST4SA Fluc cells declined to approximately 1×10^5 CFU/100 mg faeces with a weak bioluminescent signal of 1.5×10^4 p/s/100 mg of faeces.

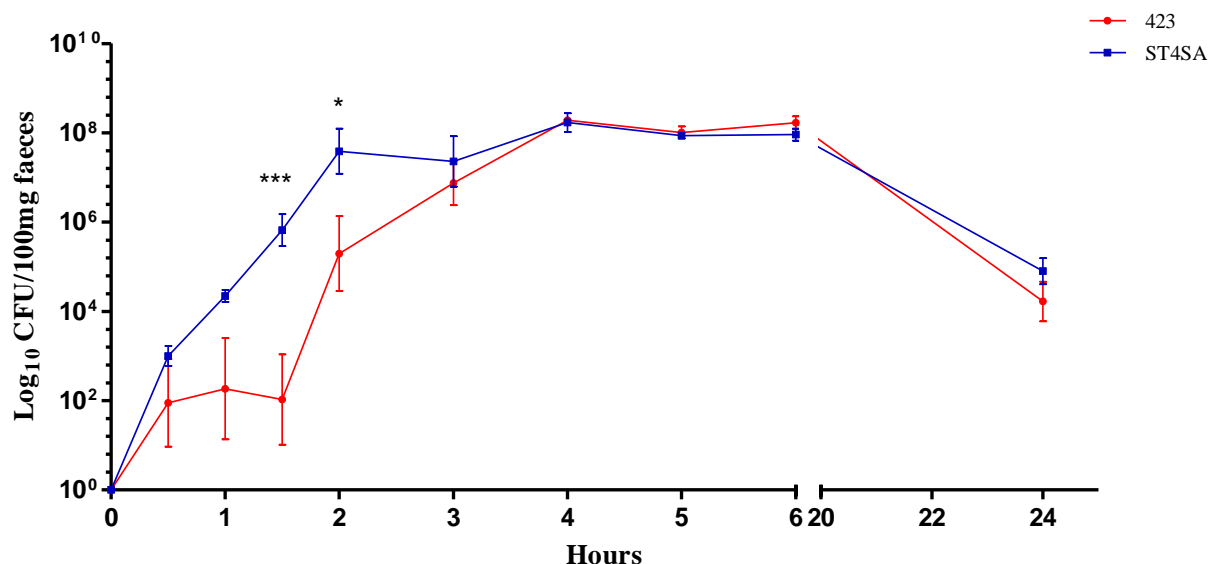
Fig. 5A.

Fig. 5B.

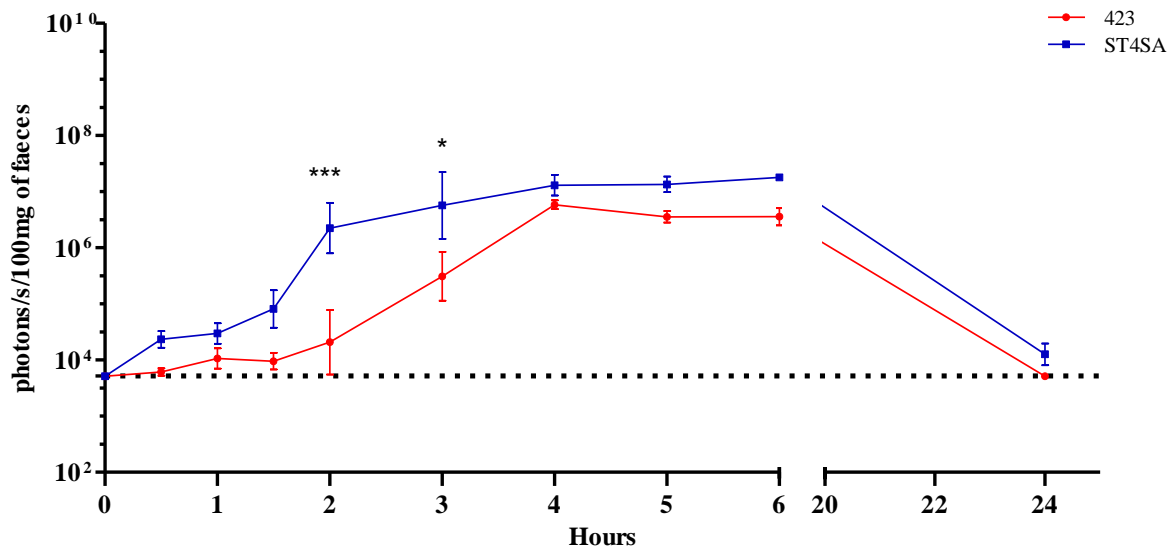


FIG. 5. Transit of *L. plantarum* 423 and *E. mundtii* ST4SA in the faeces of mice after a single oral administration. Groups of three mice each were administered once with 4×10^9 CFU *L. plantarum* 423 Fluc or *E. mundtii* ST4SA Fluc. At each time point, \log_{10} averages of the (A) cell counts per 100 mg faeces and the corresponding (B) bioluminescence in \log_{10} p/s per 100 mg faeces for each group of three mice are plotted with standard deviations. Significant differences between the two groups were assessed using the Mann-Whitney nonparametric test and are indicated with one ($P < 0.05$) or three ($P < 0.001$) asterisks. The background bioluminescence signal emitted is represented by a dashed line.

Persistence of bioluminescent *L. plantarum* 423 and *E. mundtii* ST4SA in the GIT of mice after five oral dosages

In vivo bioluminescent signals were measured everyday (1 h after administration of bacteria to mice on days 1 to 5) for nine days after two groups of mice ($n=22$, per strain) each received daily doses of *L. plantarum* 423 Fluc and *E. mundtii* ST4SA Fluc for five consecutive days (Fig. 6). The experimental design is described in Fig. S3. High intensity bioluminescence was emitted by mice in both groups from days 1 to 5. Highest bioluminescent signals were recorded for *E. mundtii* ST4SA Fluc (mean = 4.27×10^8 p/s) compared to *L. plantarum* 423 Fluc (mean = 7.78×10^7 p/s) during the first 5 days. After 6 days the signal for *L. plantarum* 423 Fluc rapidly declined to approximately 1×10^5 p/s, while the signal for *E. mundtii* ST4SA Fluc declined to approximately 5×10^5 p/s. From day 6, the *L. plantarum* 423 Fluc bioluminescent signal remained at a plateau until day 9 (approximately 1×10^5 p/s). The *E. mundtii* ST4SA Fluc bioluminescent signal declined to approximately 2×10^5 p/s on day 7 and to approximately 8×10^4 p/s after 9 days.

Fig. 6A.

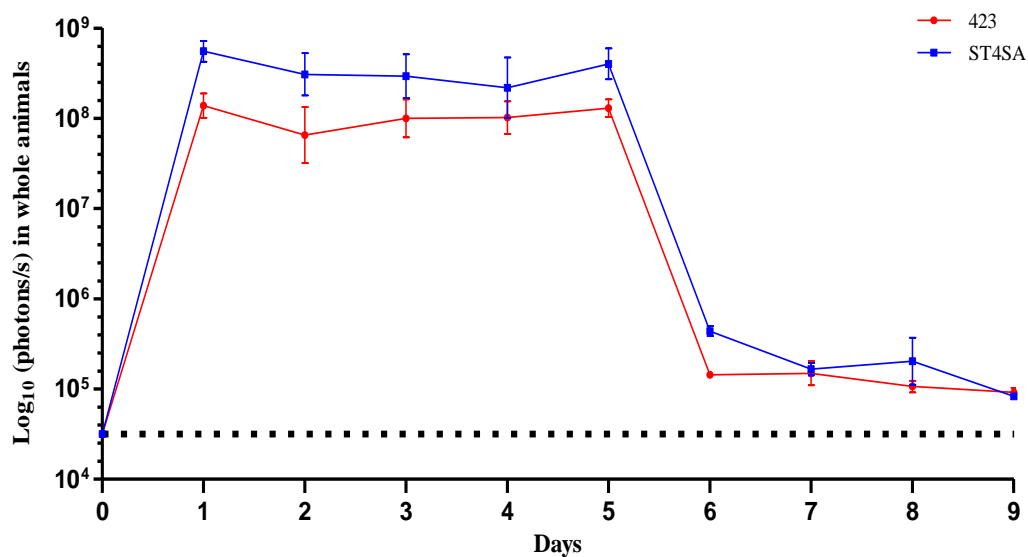


Fig. 6B.

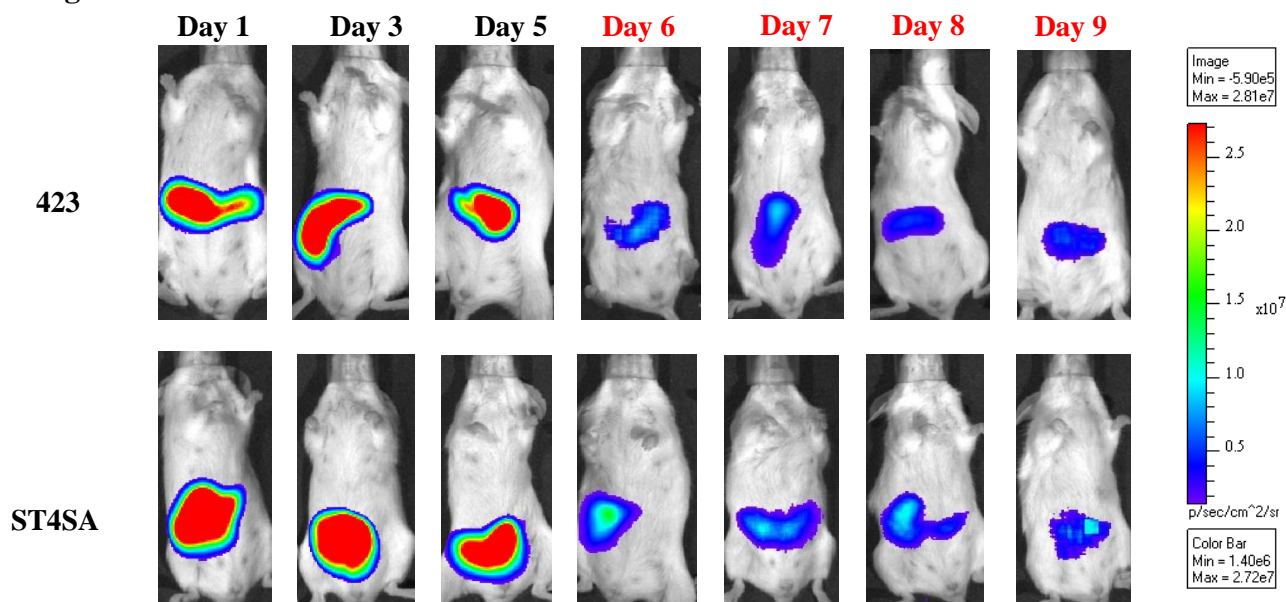


FIG. 6. Monitoring of colonization and persistence of *L. plantarum* 423 and *E. mundtii* ST4SA in the GIT of mice by bioluminescence imaging in whole animals after five daily administrations. *Lactobacillus plantarum* 423 Fluc and *E. mundtii* ST4SA Fluc were fed once daily by intragastric gavage (4×10^9 CFU) to two groups of mice for five consecutive days (days 1 to 5). The bioluminescent signals in \log_{10} photons/s measured in whole animals from day 1 to 9 for each set of three mice (A) are plotted, with standard deviations. No statistical differences between the bioluminescence signals of the two groups of mice were observed. The background bioluminescence signal emitted is represented by a dashed line. (B) Visual images of bioluminescence emission in whole animals by mice fed once daily for five consecutive days with *L. plantarum* 423 Fluc or *E. mundtii* ST4SA Fluc. One representative image of one mouse is shown. The intensity of the photon emission is represented as a pseudo-color image. One representative scale bar is shown (p/s).

The intestines of mice were imaged *ex vivo* to determine which sections of the GIT the bacterial strains colonized after 5 oral administrations (Fig. 7). On day 1, both strains were detected throughout the small intestine as of 30 min after oral administration of bacteria. From day 2 to day 5, both strains were detected in the jejunum and ileum sections of the small intestine (jejunum and ileum) and in both the cecum and colon sections of the large intestinal tract. At days 6 and 7 (1 and 2 days after last bacterial dose), *L. plantarum* 423 Fluc colonized the small intestine and cecum readily, but the bioluminescent signal detected from the small intestine progressively declined and by day 9 a very weak or no bioluminescent signal remained (Fig. 7A). On day 9, *L. plantarum* 423 Fluc was predominantly localized in the cecum and colon. From day 6 to day 9, *E. mundtii* ST4SA Fluc colonized the upper section of the small intestine and the cecum/colon most prominently (Fig. 7B).

Viable counts of the small and large intestine taken on days 1 to 5 revealed high numbers (approximately 10^8 CFU) of both *L. plantarum* 423 Fluc and *E. mundtii* ST4SA Fluc (Fig. 7C and D). At day 7 (2 days after last bacterial dose), the colonization of the large intestine (mean value of 3×10^5 CFU) by *L. plantarum* 423 Fluc was 10-fold superior to that of the small intestine (mean value of 3×10^6 CFU). Two days later, the difference between the amount of viable *L. plantarum* 423 Fluc cells in the small (mean value of 9×10^2 CFU) and large intestine (mean value of 8×10^4 CFU) increased to 100-fold. In the case of *E. mundtii* ST4SA Fluc, the number of viable cells in the small intestine increased from approximately of 1×10^3 CFU on day 6 to 2×10^4 CFU on day 9. On day 9, mice administered with *L. plantarum* 423 Fluc harboured approximately 9×10^4 CFU in the cecum/colon, while mice administered *E. mundtii* ST4SA Fluc, approximately to 7×10^4 CFU in the cecum/colon. After 20 days, no *L. plantarum* 423 Fluc were detected in the intestines of mice. However, the number of *E. mundtii* ST4SA Fluc cells in the small intestine was still approximately 1.4×10^3 CFU and 5×10^3 CFU in the large intestine.

Fig. 7A.

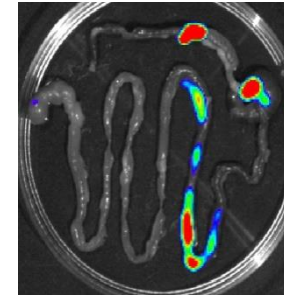
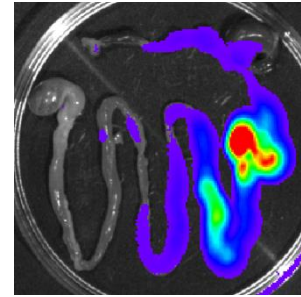
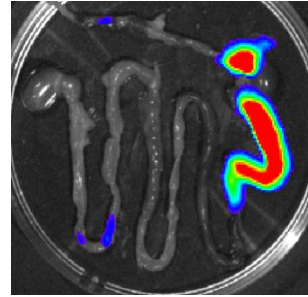
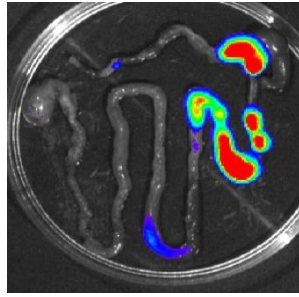
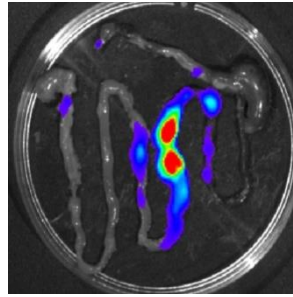
Day 1

Day 2

Day 3

Day 4

Day 5

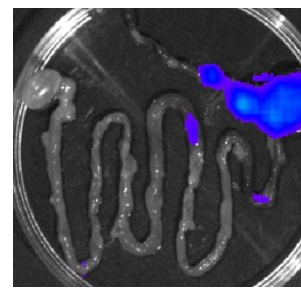
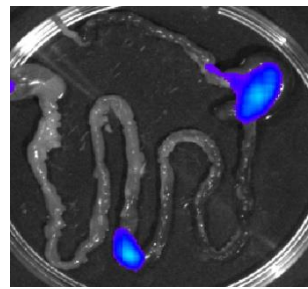
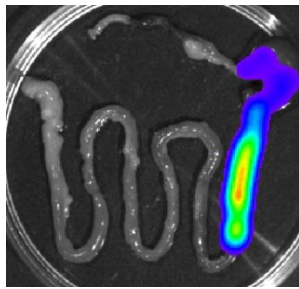
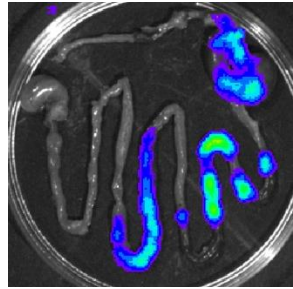


Day 6

Day 7

Day 8

Day 9



423

Fig. 7B.

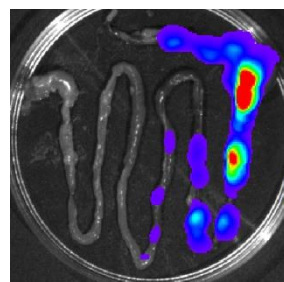
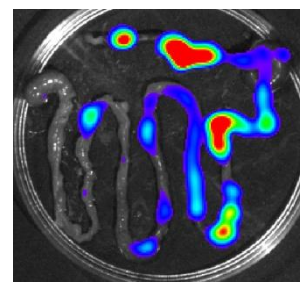
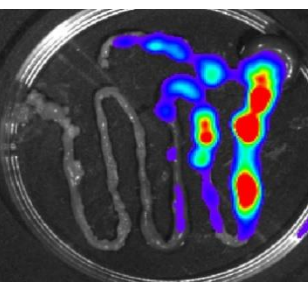
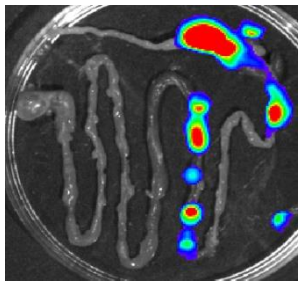
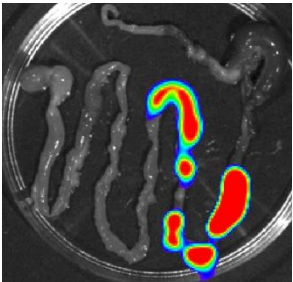
Day 1

Day 2

Day 3

Day 4

Day 5

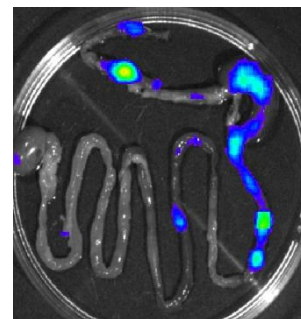
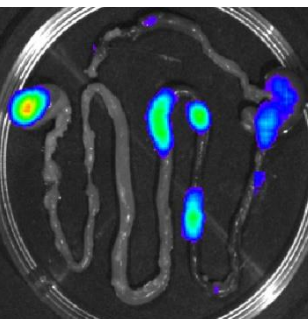
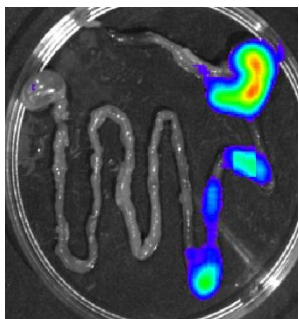
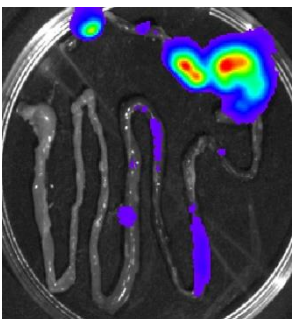


Day 6

Day 7

Day 8

Day 9



ST4SA

Fig. 7C.

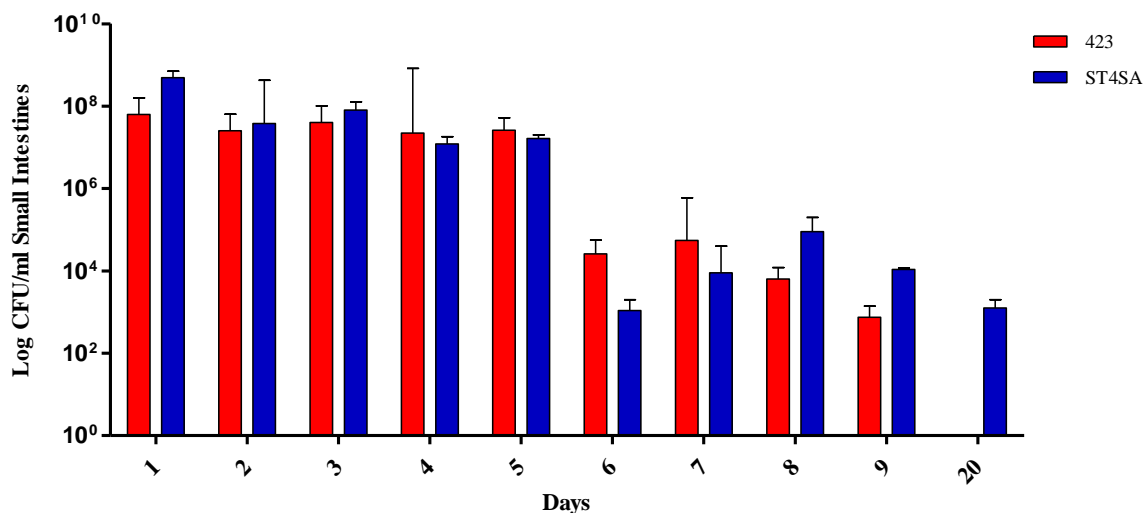


Fig. 7D.

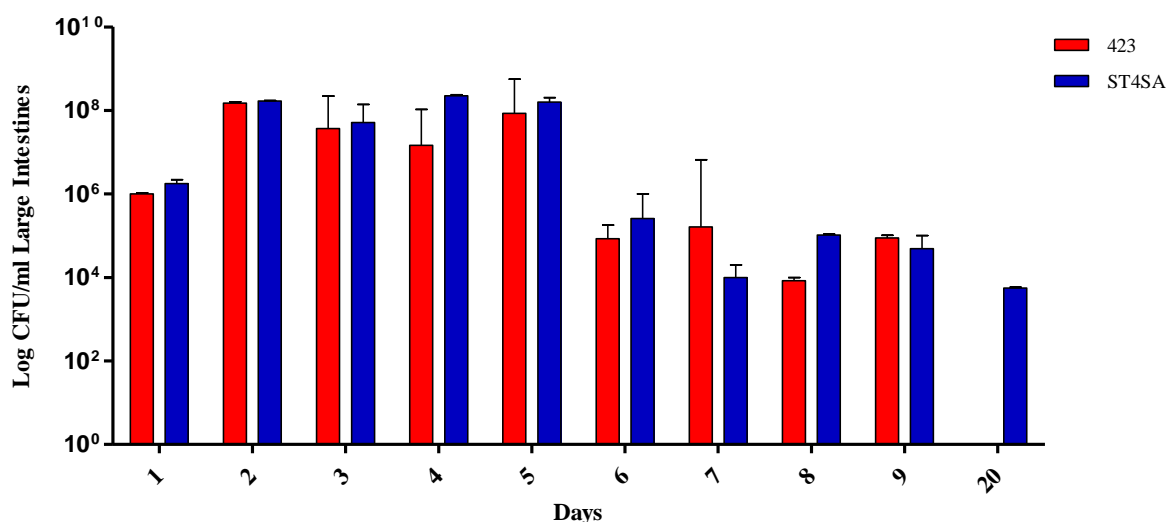


Fig. 7. Comparison of colonization abilities of *L. plantarum* 423 and *E. mundtii* ST4SA in the GIT of mice after five daily oral administrations. *Lactobacillus plantarum* 423 Fluc and *E. mundtii* ST4SA Fluc were fed once daily by oral gavage (4×10^9 CFU) to two groups of mice for five consecutive days (days 1 to 5). Four mice (two per group) were sacrificed from day 1 to 9, and a representative image of the GIT of one mouse is shown (days 1 to 9) in mice fed with (A) *L. plantarum* 423 Fluc or (B) *E. mundtii* ST4SA Fluc. Persistence of viable *L. plantarum* 423 Fluc or *E. mundtii* ST4SA Fluc cells in (C) the small and (D) large intestinal tract of mice sacrificed at time points indicated in A and B and day 20.

Persistence of *L. plantarum* 423 and *E. mundtii* ST4SA in faeces after five oral dosages

The persistence of bioluminescent *L. plantarum* 423 Fluc and *E. mundtii* ST4SA Fluc in faeces and their respective bioluminescent signals were monitored every day for 20 consecutive days (Fig. 8A and B). *Enterococcus mundtii* ST4SA Fluc persisted in the faeces throughout the trial, with significantly higher cell numbers recorded at days 4, 19 and 20 compared to *L. plantarum* 423 Fluc. The maximum amount of viable *E. mundtii* ST4SA Fluc cells in faeces was excreted at day 4 (approximately 2×10^8 CFU/100 mg faeces). From day 4 to 9 the level of *E. mundtii* ST4SA Fluc cells in faeces declined to approximately 1×10^4 CFU/100 mg faeces. After day 9 there is a slight increase in the number of *E. mundtii* ST4SA Fluc cells in faeces until day 11 after which levels of approximately 10^4 to 10^5 CFU/100 mg faeces were maintained from days 12 to 20. *Lactobacillus plantarum* 423 Fluc, on the other hand, reached a maximum level of approximately 2×10^8 CFU/100 mg faeces at day 1 and then steadily declined until day 19 (mean value of 1×10^4 CFU/100 mg faeces). *Lactobacillus plantarum* 423 Fluc persisted for only 13 days in faeces after the last day of intragastric administration of bacteria (day 5).

The bioluminescent signal of *E. mundtii* ST4SA Fluc cells in faeces was detected throughout the trial period, while the *L. plantarum* 423 Fluc bioluminescent signal declined to the background level at day 19. The peaks of the amount of viable cells per 100 mg faeces of both strains correlated perfectly with the amount of bioluminescent signals emitted at different time points. The bioluminescent signal of *L. plantarum* 423 Fluc was detected at lower levels between days 2 and 6, but displayed levels similar to those observed for *E. mundtii* ST4SA Fluc from day 7 until 18. The bioluminescent system allowed the detection of both *L. plantarum* 423 Fluc and *E. mundtii* ST4SA Fluc viable cells in faeces as low as 10^4 CFU/100 mg faeces.

Fig. 8A.

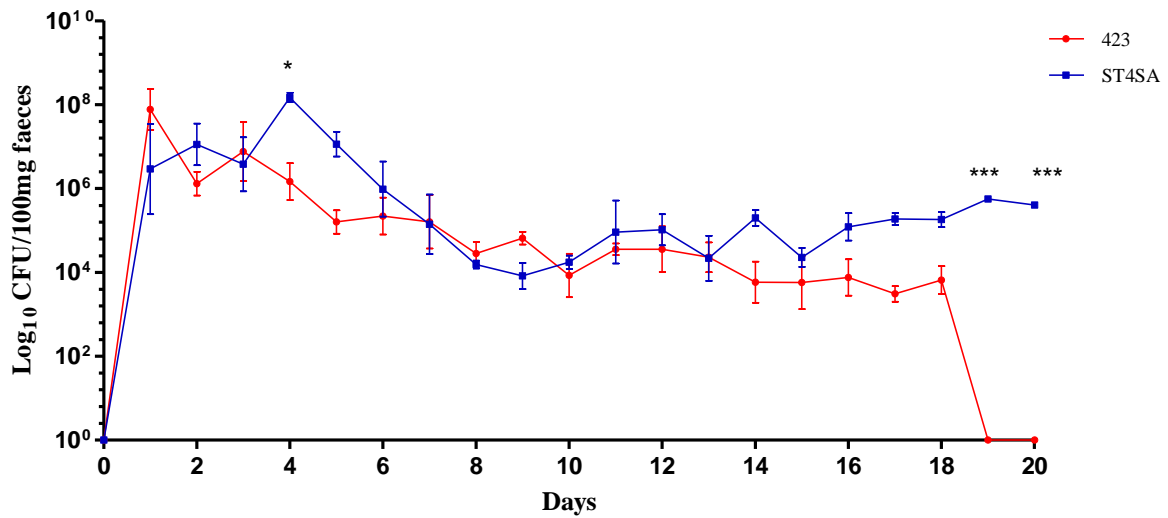


Fig. 8B.

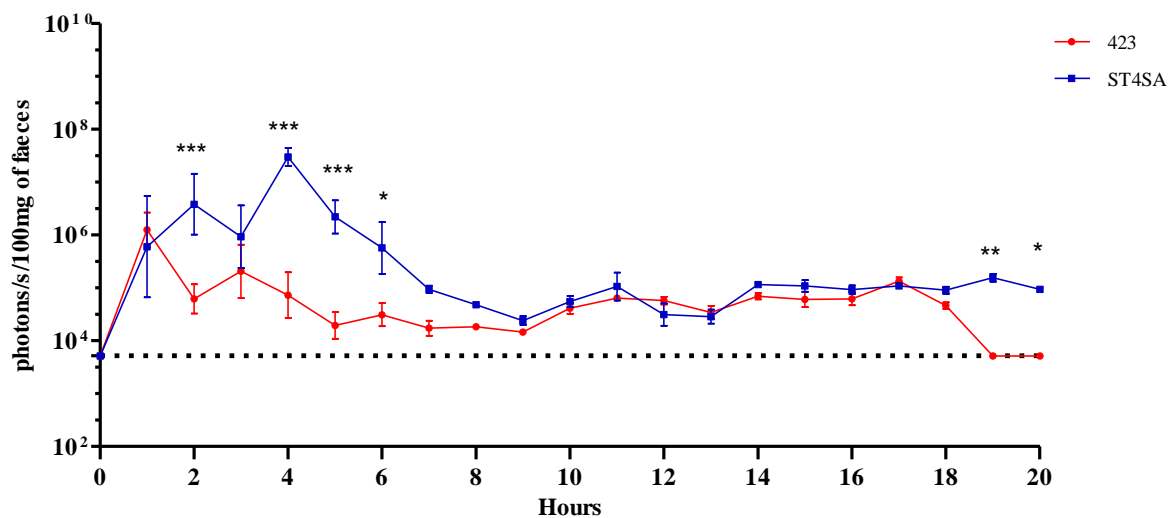


FIG. 8. Persistence of *L. plantarum* 423 and *E. mundtii* ST4SA in mouse faeces after five daily oral administrations. Groups of three mice each were administered once daily (days 1 to 5) with 4×10^9 CFU *L. plantarum* 423 Fluc or *E. mundtii* ST4SA Fluc for five consecutive days. Mouse faeces were collected daily from days 1 to 20. At each time point, log₁₀ averages of the (A) cell counts per 100 mg faeces and the corresponding (B) bioluminescence in log₁₀ p/s per 100 mg faeces for each group of three mice are plotted with standard deviations. Significant differences between the two groups were assessed using the Mann-Whitney nonparametric test and are indicated with one ($P < 0.05$), two ($P < 0.01$) or three ($P < 0.001$) asterisks. The background bioluminescence signal emitted is represented by a dashed line.

Discussion

A major advantage of using the BLI technique compared to conventional approaches is that it allows for drastic reductions in the number of animals to be sacrificed to establish the precise location of bacteria in mouse or rat models (20). Moreover, more information is gathered over a shorter period per experiment compared to traditional pre-clinical animal trials (21). In the current study, an IVIS system and the BLI technique were used to study the colonization dynamics of the probiotic LAB strains *L. plantarum* 423 and *E. mundtii* ST4SA in the GIT of mice.

The stable expression of reporter genes during *in vivo* imaging is an absolute prerequisite for the detection of luminescent light through animal tissues. Stable expression of reporter genes depends on the nature of the expression vector used, including the promoters used to drive expression and plasmid copy numbers. With this in mind, expression of the firefly luciferase gene from *P. pyralis* (*ffluc*) was optimized for use in *L. plantarum* 423 and *E. mundtii* ST4SA. Results in this study demonstrate that highest bioluminescence signals were achieved using the pNZ8048 high-copy number plasmid (42) for luciferase gene expression (Fig 1). Attempts to use a low-copy number plasmid and chromosomally integrated *ffluc* genes in *L. plantarum* 423 and *E. mundtii* ST4SA resulted only in the emission of weak and inconsistent bioluminescent signals (not shown). The former results are most likely linked to the presence of multiple copies of the pNZ8048 plasmid that in turn leads to higher expression levels of the *ffluc* gene within the respective LAB hosts. The expression of reporter genes in bacteria using plasmids is widely used. Bacterial plasmids play an important role in the ability of bacteria to adapt in diverse environments (44). However, when introducing a new recombinant plasmid harbouring a reporter gene, it is critical to evaluate its stability and persistence within the bacterial host with and without antibiotic pressure. This study demonstrates that the *ffluc* expression plasmids were remarkably stable in *L. plantarum* 423 Fluc and *E. mundtii* ST4SA Fluc and that bioluminescence did not affect the growth of the respective host strains compared to the wild-type derivatives (Fig. 2). In terms of promoter selection, the lactate dehydrogenase gene (*ldh*) promoters of *L. plantarum* 423 and *E. mundtii* ST4SA, respectively, drove highest bioluminescence for the *ffluc* gene. The *L. plantarum* *ldh* gene promoter has been used for the constitutive expression of genes in LAB in several studies (28, 31, 44, 46). These results demonstrated that both *L. plantarum* 423 Fluc and *E. mundtii* ST4SA Fluc could produce bioluminescence with no loss of the firefly luciferase-expressing plasmids.

The current study was designed to study the colonization (spatial and temporal) dynamics of *L. plantarum* 423 and *E. mundtii* ST4SA orally inoculated once or for five consecutive days over a period of 24 h and 20 days, respectively. Strong bioluminescence signals emitted by *L. plantarum* 423 Fluc and *E. mundtii* ST4SA Fluc could be detected as soon as 15 min after oral administration with a single bacterial dosage of the respective strains (Fig. 3). The bioluminescence signals of both strains as recorded with whole body imaging showed an overall decline after a single oral administration to mice. This is an indication that the probiotic strains only transiently colonized the GIT of mice after a single oral administration. In a previous report, using the *mCherry* fluorescence gene and a conventional culture-based method, we showed similar transit dynamics of viable *L. plantarum* 423 and *E. mundtii* ST4SA after a single dose of each bacterial strain (28). However, we could not obtain fluorescence signals from *in vivo* whole body imaging for real-time analysis and could only locate the bacterial strains in the GIT after killing the animals. Interestingly, in the current study bioluminescence signals were detected up to 6 h in whole animals fed with either *L. plantarum* 423 or *E. mundtii* ST4SA and up to 24 h in mice fed with *E. mundtii* ST4SA. Eom and co-workers (25) could not detect a bioluminescence signal after 3 h from mice administered bioluminescent *L. casei* CJNU 0588. Based on whole body bioluminescence imaging in real-time, these results clearly reflect the superior active replication and colonization abilities of *L. plantarum* 423 and *E. mundtii* ST4SA in the mouse GIT compared to *L. casei* CJNU 0588.

The bacterial transit time in the GIT for viable *L. plantarum* 423 and *E. mundtii* ST4SA varied. *Lactobacillus plantarum* 423 showed a slower transit time and was detected in the lower part of the jejunum and in the ileum 45 min post gavage, while *E. mundtii* ST4SA was detected in the ileum, cecum and colon (Fig. 4A). Similarly, Karimi and co-workers (47) demonstrated that the gastrointestinal transit time differs between strains of *L. reuteri*, using fluorescence and bioluminescence imaging techniques. However, the authors did not selectively monitor the bacterial cell numbers of the LAB strains present in gastrointestinal tissue or stool samples. Although it was shown in the current study that there is excellent correlation between bioluminescence and bacterial load, it is important to verify that a certain level of bioluminescence correlates to a certain number of cells. Enumeration of viable counts in dissected intestines revealed that both bioluminescent LAB strains were located predominantly in the large intestine (cecum/colon) 3 h post gavage (Fig. 4B). No bioluminescent cells of *L. plantarum* 423 or *E. mundtii* ST4SA were detected in the stomach

or duodenum compartments 45 min after intragastric administration of the respective bacteria (Fig. 4A). In a previous study, similar transit dynamics were observed with a *L. plantarum* and a *Lc. lactis* strain expressing click beetle luciferase (31). These results are indicative of the harsh conditions the LAB strains are exposed to in those compartments of the GIT and may be the reason for the rapid transit time in those sections of the gut. The CFU counts revealed the presence of viable cells of *L. plantarum* 423 and *E. mundtii* ST4SA in the small intestine at 4 and 24 h post gavage, but no bioluminescent signals could be detected. This might be caused by reduced cell activity or the inhibition of protein synthesis during passage in the stomach and duodenum compartments of the gut, which leads to inefficient bioluminescence emission. Bioluminescent light can only be produced in metabolically active cells (20). Both strains could be detected in the cecum and colon after 4 h, indicating the metabolically active state of the bacteria in the large intestine (Fig. 4A).

After 24 h, no bioluminescent signal could be detected from mice administered with *L. plantarum* 423, while a detectable bioluminescent signal was still observed from mice fed with *E. mundtii* ST4SA (Fig. 3). This indicates that *L. plantarum* 423 was eliminated from the GIT of mice more rapidly than *E. mundtii* ST4SA, and is associated with a significantly higher amount of viable *E. mundtii* ST4SA in the large intestine compared to *L. plantarum* 423 (Fig. 4C). Both strains were excreted in high numbers in the faeces of mice over the 24 h period, but it is clear that a small amount of each LAB strain persisted in the GIT after a single oral dose as demonstrated with intestinal tissue CFU counts (Figs 4 and 5). Overall, the gastrointestinal colonization dynamics of *L. plantarum* 423 and *E. mundtii* ST4SA after a single dosage are similar to those reported for several LAB strains, including *L. reuteri* 6475, *L. casei*, *L. plantarum* SS2, *L. plantarum* WCFSI, *L. plantarum* NCIMB8826 and *Lc. lactis* MG1363 (31, 47-49).

To study more thoroughly the persistence of the bioluminescent strains in the GIT, mice were administered orally with either *L. plantarum* 423 or *E. mundtii* ST4SA for five consecutive days. Results demonstrate that *L. plantarum* 423 and *E. mundtii* ST4SA had similar GIT transit dynamics during the first 5 days of administration of the respective strains to mice, despite the detection of higher bioluminescent signals for *E. mundtii* ST4SA compared to *L. plantarum* 423 (Fig. 6). At day 6 (one day after last bacterial dosage), the *in vivo* bioluminescent signals of both strains declined to low levels but were maintained at similar levels for the next 3 days. This suggests that while most of the administered bacteria transited the GIT of mice, small populations of both strains persisted until day 9 (4 days after last

bacterial dosage). These observations were confirmed by *ex vivo* imaging of dissected intestines (Fig. 7A and B). At day 9, bioluminescent cells of both strains were predominantly localized in the cecum and colon. It has been suggested that the murine cecum may be the site where microorganisms adapt to the gastrointestinal environment and where the activation of genes required for colonization of the colon occur (27). Interestingly, the cecum and colon have also been shown to be the major sites of colonization of several enteric pathogens including *E. coli* O157:H7, *Citrobacter rodentium*, *Yersinia enterocolitica*, *L. monocytogenes* and *S. typhimurium* (40, 50-53). Both *L. plantarum* 423 and *E. mundtii* ST4SA have been demonstrated to exclude pathogens such as *L. monocytogenes*, *S. typhimurium*, *C. sporogenes* and *E. faecalis* in *in vitro* or *in vivo* competitive exclusion experiments (38-40). The presence of persistent populations of *L. plantarum* 423 and *E. mundtii* ST4SA in the murine cecum/colon as demonstrated in this study suggest that their presence may have prevented the pathogenic bacteria from becoming established. It is also interesting to note that the population of *E. mundtii* ST4SA in the small intestine increased until day 9 compared to that of *L. plantarum* 423 (Fig. 7C). This could explain why mice pre-colonized with *E. mundtii* ST4SA showed a more rapid decline in *L. monocytogenes* EGDe cell numbers compared *L. plantarum* 423 in a competitive exclusion experiment (40). *Enterococcus mundtii* ST4SA cells were able to persist in the faeces of mice throughout the trial period (Fig. 8). In contrast, *L. plantarum* 423 could not be detected in faeces after 13 days after the last oral administration to mice and in lower numbers compared to *E. mundtii* ST4SA. Based on bioluminescence, the amount of bacteria administered to mice per day (4×10^9 CFU) and the amount of *E. mundtii* ST4SA cells shed in the faeces per day, there is a clear indication that *E. mundtii* ST4SA persists better than *L. plantarum* 423 in the murine GIT of mice.

The construction and optimization of LAB reporter strains is an important step towards a better understanding of the route and destination of orally administered probiotics in the GIT, and the interactions between probiotics and the host. This study demonstrates the application of the firefly luciferase system to compare the colonization dynamics of *L. plantarum* 423 and *E. mundtii* ST4SA in mice. The *in vivo* BLI system revealed the precise location of the bacterial strains within the murine GIT after single or multiple doses. Both strains prominently colonized the cecum and colon. *Enterococcus mundtii* ST4SA persisted in the GIT and faeces of mice throughout the trial period and also actively colonized the small intestine. This is the first report of bioluminescence *in vivo* imaging of *E. mundtii* ST4SA in a

mouse model. The bioluminescence system developed in this study has the potential to elucidate the *in vivo* colonization dynamics of other important probiotic LAB species.

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Chapter 3

Spatial and Temporal Colonization of *Lactobacillus plantarum* 423 and *Enterococcus mundtii* ST4SA in the Intestinal Tract, as Revealed by *In vivo* Bioluminescence Imaging in a Murine Model

Supplementary Material

TABLE S1. Primers used in this study

Target	Primer	Sequence 5' to 3'	Restriction sites	Product size (bp)
<i>Pldh</i>	Pldh1	GCGC <u>AGATCT</u> AATCTTCTCACCGTCTTG	<i>Bgl</i> III	520
	Pldh2	ATAT <u>CCATGG</u> TAAGTCATCCTCTCGTAGTG	<i>Nco</i> I	
<i>STldh</i>	ldhS1	GCGC <u>AGATCT</u> GAAGAACACAATCCGACT	<i>Bgl</i> III	166
	ldhS2	TCCT <u>CCATGG</u> TCTAACACACCTTTCA	<i>Nco</i> I	
<i>ffluc</i>	FlucFor	ATAT <u>CCATGG</u> AGGACGCTAAGAACATCA	<i>Nco</i> I	1669
	FlucRev	GCGC <u>TCTAGAC</u> CACAATTTGACTTGCCAC	<i>Xba</i> I	

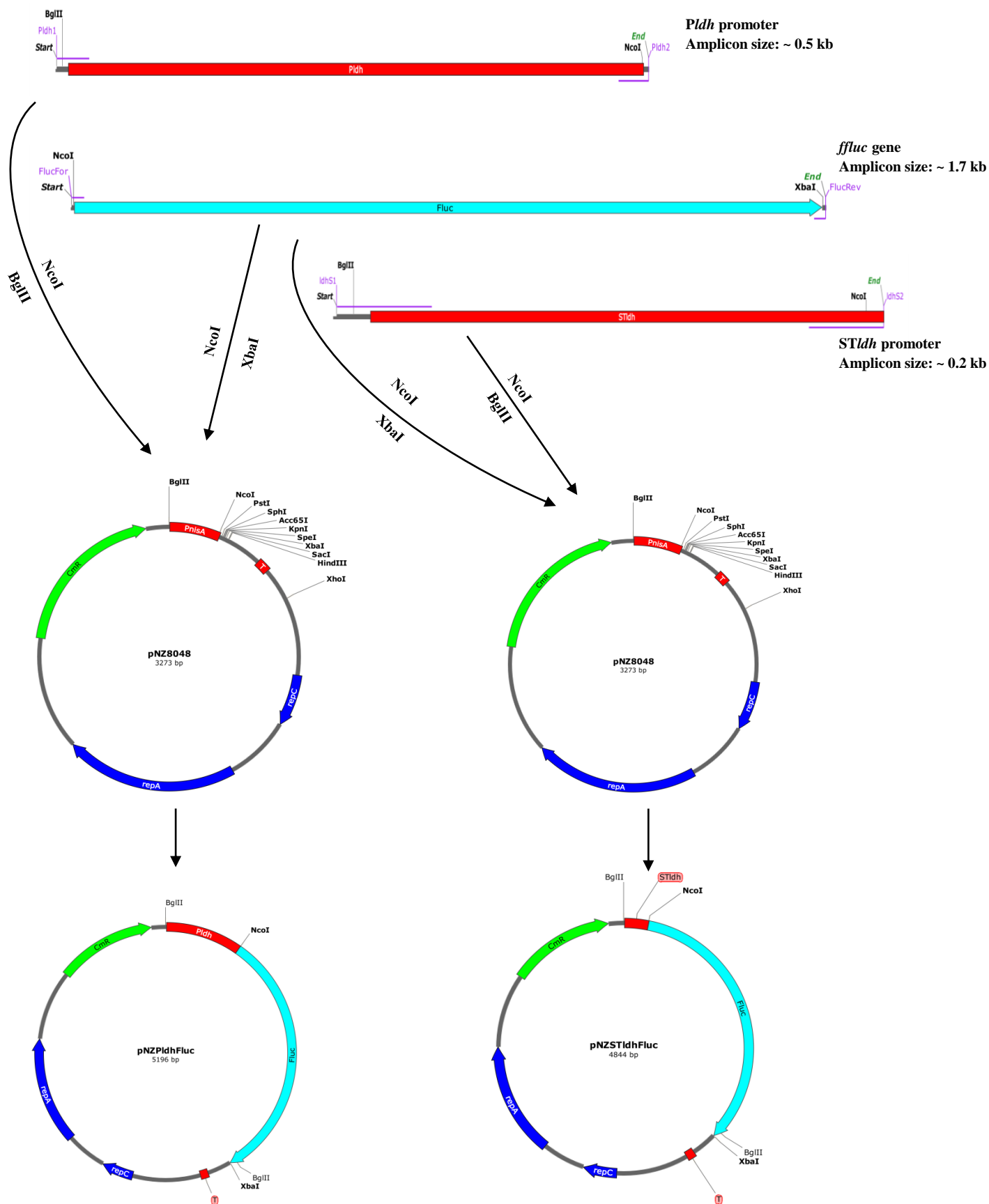


FIG. S1. Schematic representing the construction of the pNZPldhFluc and pNZSTldhFluc luciferase expression plasmids. Relevant features are indicated, including restriction sites and PCR primers used for

cloning; the *E. coli*/LAB *repA* and *repC* replication ori; the chloramphenicol acetyltransferase (*cat*) gene conferring resistance to chloramphenicol; the *Pldh* promoter from the *L. plantarum* 423 lactate dehydrogenase gene and the *STldh* promoter from the *E. mundtii* ST4SA lactate dehydrogenase gene.

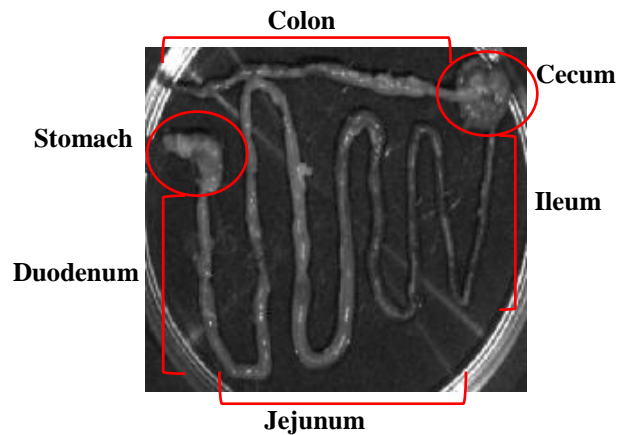


FIG. S2. Different sections of dissected mouse intestinal tract

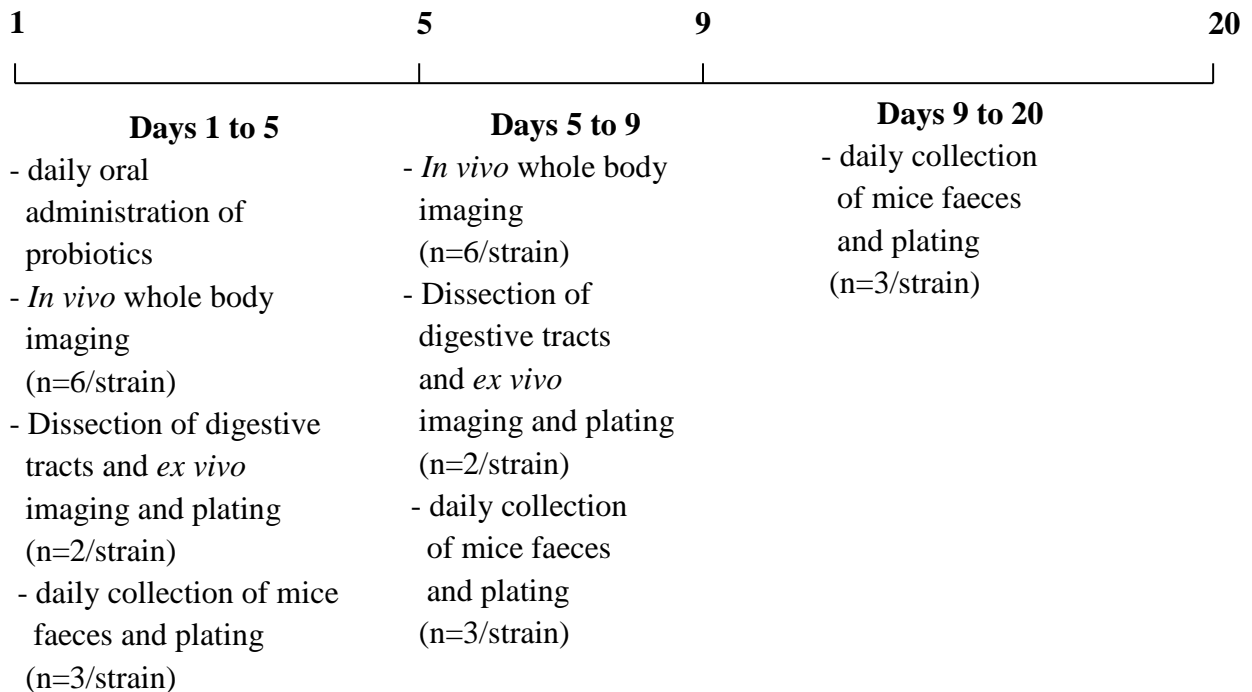


FIG. S3. Experimental design.

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Chapter 4

**Development of a Novel Selection/Counter-selection
System for Efficient Isolation of Double-crossover
Homologously Recombined Mutants in Lactic Acid
Bacteria, Enabling Directed Chromosomal Gene Deletions
and DNA Integrations**

Development of a Novel Selection/Counter-selection System for Efficient Isolation of Double-crossover Homologously Recombined Mutants in Lactic Acid Bacteria, Enabling Directed Chromosomal Gene Deletions and DNA Integrations

Abstract

Lactic acid bacteria (LAB) have significant potential as probiotics due to the beneficial effects they exert on consumer health. The isolation of stable recombinant DNA integration or deletion LAB mutants is desirable when studying probiotic modes of action. The functional effects of genes expressed by LAB *in vitro* or *in vivo* can only be truly assessed by the creation of knockout derivatives of strains expressing these genes, yet this remains a major limitation for this important bacterial group. In this report the construction of a novel selection/counter-selection system that facilitates the rapid and efficient isolation of stable double-crossover integration or deletion mutants is described. The study reports on the application of the *Lactococcus lactis* nisin A inducible promoter in LAB for the expression of a toxin gene counter-selection marker to generate stable DNA integrations or deletions at any genomic loci. This system is based on the *Escherichia coli mazF* toxin gene as counter-selectable marker under control of the inducible nisin promoter and simultaneous expression of NisR and NisK signalling proteins. Although this work focuses on two probiotic strains, *Lactobacillus plantarum* 423 and *Enterococcus mundtii* ST4SA, the system developed here could be adapted to most, if not all, LAB species. Large sequences can be inserted or deleted in a series of steps, as demonstrated by insertion of the firefly bioluminescence gene (*ffluc*) and erythromycin resistance marker into the bacteriocin operons or adhesion genes of *L. plantarum* 423 and *E. mundtii* ST4SA. Furthermore, this study reports on the use of the FLP/FRT recombination system and an antisense RNA transcript to create markerless chromosomal gene integrations/deletions in LAB. This work opens the way to the isolation of reliable integration/deletion mutants of LAB and other diverse microorganisms.

Introduction

In recent years a profitable probiotic market has emerged, with an increasing number of probiotic-containing supplements conferring specific health benefits to the consumer (1). Strains of lactic acid bacteria (LAB) and bifidobacteria are the most frequently used as probiotics and form part of many functional foods and dietary supplements (2-4). Probiotic LAB have a long history of safe use in food and therapeutic products and an increased recognition of their beneficial effects on human health (5). However, the underlying mechanisms responsible for these effects on the health of the consumer have yet to be fully elucidated and are likely to be multifactorial. If the growing consumer interest in probiotics is to continue, it is crucial to identify the precise mechanisms of action by which probiotics influence human health. One way to provide verification of probiotic modes of action is the use of single or multiple gene knockout analyses of bacterial mutants in *in vitro* or *in vivo* models (6).

The functional analysis of proteins expressed by genes that confer specific phenotypical properties has underpinned biotechnology for decades. The potential scope of such an approach has grown exponentially with the availability of whole-genome sequencing, commercial *de novo* DNA synthesis and synthetic biology (7). While many species of LAB are now transformable by electroporation and have thus become amenable to genetic manipulation using plasmid vectors, methods for the isolation of stable and irreversible genetic mutants are still underdeveloped (8-10). The construction of tailor-made LAB strains for functional genetic analyses is dependent on efficient genetic methods and is often reliant on chromosomal integrations or deletions of specific target genes (11). This calls for the development of techniques that will allow for the easy and efficient selection of chromosomally or plasmid located gene excisions/integrations.

For many years, researchers have used replicative plasmids to express foreign DNA in microorganisms, but these are inherently unstable when expressed *in vitro* or *in vivo* where antibiotic selection is not possible, thus limiting their applied utility (12). To circumvent antibiotic selection-related issues, exogenous DNA must be irreversibly incorporated into a DNA molecule inside the cell, usually on a chromosome or plasmid of the host microorganism. In this way, integration of recombinant DNA is achieved by positioning a selectable marker gene alongside a DNA sequence that is homologous to the target gene of interest within an allele exchange cassette. This has been achieved successfully in yeast and

naturally competent *Bacillus subtilis* that are easily transformable with linear DNA gene exchange cassettes (13). However, most bacteria, including LAB, cannot be transformed with linear DNA, thus integration plasmids bearing DNA homologous to the site of chromosomal integration are used instead. Desired recombinant cells are specifically selected and isolated using the selectable marker, typically an antibiotic resistance gene. Several sequences can be inserted at multiple loci by simply alternating between selectable markers, usually antibiotic resistance genes as described in the ‘domino’ method of Itaya and co-workers (14). This method can be effective, but is not without limitations. A major drawback is the availability of suitable antibiotic resistance markers for use as selection/counter-selection markers in the strain of interest. According to this strategy, different antibiotic resistance genes have to be used to introduce multiple chromosomal modifications. Moreover, multi-antibiotic selection pressure could potentially modify the physiology of the recombinant strain.

One method commonly used for the construction of stable integration mutants in LAB is the use of plasmid vectors containing the chromosomally located conjugative transposon Tn919, which is utilized as the region of homology required to mediate insertion into the host genome (15, 16). While this method has been successfully applied in *Enterococcus faecalis* and *Lactococcus lactis*, it cannot be used for specific targeted gene deletions. Another method often employed for targeted gene inactivation and DNA chromosomal integrations is based on the use of suicide vectors. Suicide vectors are plasmids that are unable to replicate in a specific host, but which express antibiotic resistance genes and carry sequences homologous to a target sequence on the chromosome of the strain to be modified. The site of DNA integration then corresponds to the chromosomal locus of the sequence carried by the plasmid. This type of insertion has been successfully used in Gram-negative and -positive bacteria, including *Escherichia coli* (17-18), *Lc. lactis* (16, 19, 20), *B. subtilis* (21-23), *L. plantarum* (24, 25) and *Streptococcus pneumonia* (26, 27) to map non-selectable genes. In most of these studies, the isolation of successful integrations was straightforward, but strains that have lost the plasmid backbone may be difficult to isolate. Moreover, as plasmids are circular, single homologous recombination events may be unstable and reversible, resulting in single- crossover mutants with the potential to revert back to the wild-type. The isolation of stable double-crossover mutants from single-crossover cells is further complicated as both contain the selectable marker present in the allele exchange cassette. This can be overcome by the use of a counter-selection marker located on the plasmid backbone, but the identification of a suitable counter-selection marker under specific conditions to use as a

genetic tool can be a challenging task (28, 29). The use of the *upp* gene, which codes for uracil phosphoribosyltransferase has been used as a counter-selectable marker in *Lc. lactis* (30-31). The *upp* gene is responsible for conferring toxicity to cells in the presence of 5-fluorouracil, whereas the loss thereof leads to resistance to 5-FU. The main limitation of the applied utility of the *upp* gene as counter-selectable marker is that it is present in the nucleotide metabolic pathway of almost every organism (32). Another disadvantage is that 5-FU may be toxic even in *upp* mutants, thus further hampering its use as a heterologous counter-selectable marker. Nonetheless, counter-selectable markers have been proven invaluable in the construction of clean and unmarked gene deletions or insertions in various unicellular microorganisms (28, 33-39).

This study reports on the development of a strategy generally applicable to all LAB species for the quick and efficient isolation of double-crossover homologously recombined mutants at any genomic loci. The use of a toxin gene as counter-selectable marker placed under the control of the *Lc. lactis* nisin inducible promoter (PnisA) that forms part of the well-characterized nisin-controlled expression (NICE) system (for a complete review on the NICE expression system, see reference 40) is described. The PnisA promoter is auto-inducible by nisin in *Lc. lactis*, but can be induced heterologously in other LAB strains using sub-inhibitory concentrations of nisin (41). For heterologous exploitation of the NICE system, the *nisK* and *nisR* nisin regulatory genes required for signal transduction have to be expressed in conjunction with the use of the PnisA promoter (42). The NICE system has been successfully adapted for gene expression or protein secretion in several LAB species (41, 43). The *E. coli* *mazF* gene was chosen as a toxin gene for plasmid-borne counter-selection that has been used successfully as a counter-selection marker in *B. subtilis* (38) and *Clostridium* spp. (39). The *mazF* gene is an mRNA interferase (sequence-specific endoribonuclease) that forms part of the MazE-MazF toxin-antitoxin (TA) system of *E. coli*, encoded by the *mazEF* operon (44). The *mazEF* TA system comprises a stable MazF toxic peptide that is neutralized by an unstable MazE antitoxin under normal conditions. Once the cells are subjected to cellular stress, the MazE protein is degraded by proteases, releasing the MazF protein from inhibition (45). Ectopic expression of the MazF toxin then promotes cell death by specifically targeting mRNAs at ACA sequences (46). In the counter-selection system described here, the expression of the MazF toxin under the control of PnisA is directly coupled to the formation of desired double-crossover mutants using an associated selectable phenotype. Using this method, DNA fragments can be irreversibly inserted or deleted at any genomic locus in *L.*

plantarum 423 and *E. mundtii* ST4SA in a step-by-step manner. The application of the FLP-FRT recombination system to generate markerless homologously recombined mutants is also demonstrated. This method may be applicable to many, if not all, transformable LAB species.

Materials and Methods

Bacterial strains, plasmids and culture media

Bacterial strains and general cloning plasmids are listed in Table 1. The integration vectors and their relevant characteristics are listed in Table 2. All subcloning experiments were done in *E. coli* DH5 α (47) and *E. coli* MC1061 (Mobictech, Goettingen, Germany). *Escherichia coli* strains were grown in Luria-Bertani (LB), brain heart infusion (BHI) broth or solid agar (1.5% w/v) (Biolab Diagnostics, Midrand, South Africa) and incubated at 37°C with rotary shaking at 200 rpm. Forty micrograms per milliliter of X-gal was added to *E. coli* LB growth media when required for blue-white colony screening. The probiotic LAB strains *L. plantarum* 423 and *E. mundtii* ST4SA were grown as static cultures at 30°C in MRS broth (Biolab Diagnostics) or on MRS agar plates. *Lactococcus lactis* pNZ9000 was grown at 30°C in M17 broth (Biolab Diagnostics) without shaking, or on agar plates supplemented with 0.5% (w/v) glucose. *Escherichia coli* strains containing plasmids (Table 1) were cultured in LB or BHI medium supplemented with either 200 μ g/ml erythromycin (Em), 10 μ g/ml chloramphenicol (Cm) or 100 μ g/ml ampicillin (Amp). Recombinant LAB strains containing plasmids, and integrative mutants (Table 2), were cultured in MRS or M17 medium supplemented with 10 μ g/ml Em or 10 μ g/ml Cm for *L. plantarum* 423, and 5 μ g/ml Em or 5 μ g/ml Cm for *E. mundtii* ST4SA and *Lc. lactis* pNZ9000. *Listeria monocytogenes* EGDe was grown in BHI media supplemented with 7.5 μ g/ml Cm and incubated at 37°C on an orbital shaker (200 rpm).

DNA manipulation procedures and transformation

Nucleic acid manipulations and general cloning procedures were carried out according to standard protocols, as described by Sambrook and Russel (48). DNA restriction and modification enzymes were purchased from New England Biolabs (NEB, Ipswich, MA, USA) and were used as recommended by the manufacturer. Oligonucleotides (Table 3) were purchased from Inqaba Biotechnical Industries (Pretoria, South Africa). PCR amplifications were performed using Q5 high-fidelity PCR DNA polymerase (NEB) in a SwiftMinipro

thermal cycler (Esco Healthcare, Malaysia). DNA fragments were purified from agarose gels using the Zymoclean™ gel DNA recovery kit (Zymo Research Corporation, Irvine, CA, USA). *Escherichia coli* plasmid DNA was purified using the PureYield™ plasmid miniprep system (Promega, Madison, WI, USA). Genomic DNA (gDNA) of *E. coli* and LAB strains was purified using the ZR Fungal/Bacterial DNA miniprep kit (Zymo Research Corporation) following the manufacturer's instructions. Electrotransformation of *E. coli*, *L. plantarum* 423 and *E. mundtii* ST4SA was achieved as described previously (25), using the Bio-Rad Gene Pulser electroporation system (Bio-Rad Laboratories, Hercules, CA, USA). *Lactococcus lactis* NZ9000 was electroporated as suggested by the supplier, using standard procedures (Mobitech).

TABLE 1. Plasmids and strains

Strain or plasmid	Description	Reference or source
Strains		
<i>E. coli</i>		
DH5α	Host strain used for general subcloning	(47)
MC1061	Host strain used for subcloning with <i>Lc. lactis</i> derived pNZ8048 vector; <i>recA</i> positive strain	Mobitec, Göttingen, Germany.
<i>Lactobacillus plantarum</i>		
423	Probiotic with multiple adhesion genes and a plantaricin bacteriocin producer; forms part of the entiro™ probiotic; originally isolated from sorghum beer	Cipla Medpro (Pty.) Ltd.
423 pNZmazFnisRK	Contains the pNZmazFnisRK plasmid; Cm ^R	This study
423 pNZ8048	Contains the pNZ8048 plasmid; Cm ^R	This study
423 pNZCherryinisRK	Contains the pNZCherryinisRK plasmid; Cm ^R	This study
423 bac ⁻	Derivative of <i>L. plantarum</i> 423, cured of the plasmid (pPLA4) harboring the <i>plaA</i> bacteriocin gene	(49)
<i>Enterococcus mundtii</i>		
ST4SA	Probiotic with multiple adhesion genes and a mundticin bacteriocin producer; forms part of the entiro™ probiotic; originally isolated from soybeans	Cipla Medpro (Pty.) Ltd.
ST4SA pNZmazFnisRK	Contains the pNZmazFnisRK plasmid; Cm ^R	This study
ST4SA pNZ8048	Contains the pNZ8048 plasmid; Cm ^R	This study
ST4SA pNZCherryinisRK	Contains the pNZCherryinisRK plasmid; Cm ^R	

<i>Lactococcus lactis</i>		
pNZ9000	Standard host strain for nisin regulated gene expression; harbors the <i>nisR</i> and <i>nisK</i> nisin regulatory genes integrated into the <i>pepN</i> gene locus	Mobitec, Göttingen, Germany.
pNZ9000 pNZmazF	Contains the pNZmazF plasmid; Cm ^R	This study
pNZ9000 pNZCherry	Contains the pNZCherry plasmid; Cm ^R	This study
<i>Listeria monocytogenes</i>		
EGDe	Food-borne clinical pathogen; harbors the pPL2 lux plasmid; Cm ^R	Caliper Life Sciences, Hopkinton, MA, U.S.A.
Plasmids		
pNZ8048	Broad-host range vector; <i>E. coli</i> shuttle vector; LAB expression vector containing nisin A inducible-promoter (PnisA); Cm ^R	Mobitec, Göttingen, Germany; (40)
pGKV223D	<i>E. coli</i> /LAB shuttle vector; LAB expression vector with L23 promoter; Em ^R	University of Gröningen, The Netherlands
pNZnisRK	pNZ8048 vector carrying the <i>nisR</i> and <i>nisK</i> regulatory genes for cloning in LAB strains that do not have the regulatory genes integrated onto the chromosome; Cm ^R	This study
pNZmazF	pNZ8048 vector carrying the <i>E. coli mazF</i> toxin gene under the control of the PnisA promoter; Cm ^R	This study
pNZmazFnisRK	pNZnisRK vector carrying the <i>E. coli mazF</i> toxin gene under the control of the PnisA promoter; Cm ^R	This study
pNZmazFnisRkerm	pNZ8048nisRK vector carrying the <i>E. coli mazF</i> toxin gene under the control of the PnisA promoter and the <i>erm</i> gene; Cm ^R , Em ^R	This study
pNZErmFluc423	pNZ8048 vector carrying the <i>erm</i> gene fused to the <i>Photinus pyralis</i> firefly luciferase gene (<i>ffluc</i>) under the control of the constitutive <i>L. plantarum</i> 423 lactate dehydrogenase (<i>ldh</i>) gene promoter (Pldh); Em ^R , Cm ^R	This study
pGKVCatFflucST4SA	pGKV223D vector carrying the <i>cat</i> gene fused to the <i>ffluc</i> gene under the control of the constitutive <i>E. mundtii</i> ST4SA lactate dehydrogenase (<i>ldh</i>) gene promoter (Pstldh); Em ^R , Cm ^R	This study
pBluescriptKS	PCR cloning vector; Amp ^R	Stratagene, California, USA.
pKSplaA::ErmFfluc	pBluescriptKS plasmid carrying the <i>erm-ffluc</i> gene cassette flanked by <i>L. plantarum</i> 423 <i>plaA</i> gene regions of homology ; Em ^R , Amp ^R	This study

pKSmunA::CatFfluc	pBluescriptKS plasmid carrying the <i>cat-ffluc</i> gene cassette flanked by <i>E. mundtii</i> ST4SA <i>munA</i> gene regions of homology; Cm ^R , Amp ^R	This study
pKСаap::FRTerm	pBluescriptKS plasmid carrying the FRT-flanked <i>erm</i> gene flanked by <i>L. plantarum</i> 423 <i>aap</i> gene regions of homology ; Em ^R , Amp ^R	This study
pKSsrtA::FRTerm	pBluescriptKS plasmid carrying the FRT-flanked <i>erm</i> gene flanked by <i>E. mundtii</i> ST4SA <i>srtA</i> gene regions of homology ; Em ^R , Amp ^R	This study
pKSsrtC::FRTerm	pBluescriptKS plasmid carrying the FRT-flanked <i>erm</i> gene flanked by <i>E. mundtii</i> ST4SA <i>srtC</i> gene regions of homology; Em ^R , Amp ^R	This study
pKSFRT	pBluescriptKS plasmid carrying two <i>flippase</i> (FLP) recombination target sequences (FRT) in a direct repeat orientation; Amp ^R	This study
pKSFRTerm	pBluescriptKS plasmid carrying the <i>erm</i> gene flanked by two FRT sequences in a direct repeat orientation; Em ^R , Amp ^R	This study
pGKVPldhFLP	pGKV223D vector carrying the <i>flippase</i> (FLP) gene downstream of the constitutive <i>L. plantarum</i> 423 <i>lactate dehydrogenase</i> gene <i>Pldh</i> promoter; Em ^R	This study
pNZasRNA_repAnisRK	pNZmazFnisRK vector carrying a 350 bp asRNA_repA transcript downstream of the nisin-inducible <i>PnisA</i> promoter; Cm ^R	This study
pNZasFLPasRNA_repA	pNZasRNA_repAnisRK vector carrying the <i>flippase</i> (FLP) gene downstream of the constitutive <i>L. plantarum</i> 423 <i>lactate dehydrogenase</i> gene <i>Pldh</i> promoter; Cm ^R	This study
pNZCherry	pNZ8048 vector carrying the <i>mCherry</i> fluorescence gene under the control of the <i>PnisA</i> promoter; Cm ^R	This study
pNZCherrynisRK	pNZnisRK vector carrying <i>mCherry</i> fluorescence gene under the control of the <i>PnisA</i> promoter; Cm ^R	This study

Cm^R, chloramphenicol resistance; Em^R, erythromycin resistance; Amp^R, ampicillin resistance

TABLE 2. List of integration vectors and recombinant LAB strains

Plasmid	Modified strain	Locus	Upstream region of homology	Downstream region of homology	Region deleted on chromosome/plasmid	Element (s) integrated onto chromosome/plasmid
pNZKOplaA::ErmFfluc	<i>L. plantarum</i> 423 <i>plaA::erm-ffluc</i>	<i>plaA</i>	902 bp upstream of <i>plaA</i>	69 bp internal fragment of <i>plaA</i> , 330 bp <i>plaB</i> bacteriocin immunity gene and 214 bp of <i>plaC</i> bacteriocin translocation protein	208 bp, including 102 bp of <i>plaA</i> ORF	3204 bp <i>erm-ffluc</i> gene cassette
pNZKOMunA::CatFfluc	<i>E. mundtii</i> ST4SA <i>munA::cat-ffluc</i>	<i>munA</i>	911 bp upstream of <i>munA</i> , including 135 bp internal fragment of <i>munA</i> ORF	633 bp internal fragment of <i>munB</i> translocation gene	260 bp, including 18 bp of <i>munA</i> ORF	2471 bp <i>cat-ffluc</i> gene cassette
pNZKIaap::FRTerm	<i>L. plantarum</i> 423 <i>aap::frt-erm</i>	<i>aap</i>	909 bp internal fragment of <i>aap</i> ORF	929 bp internal fragment of <i>aap</i> ORF	None	1458 bp FRT-flanked <i>erm</i> gene
pNZKIaap::FRTerm	<i>L. plantarum</i> 423 <i>aap::frt_um</i>		909 bp internal fragment of <i>aap</i> ORF	929 bp internal fragment of <i>aap</i> ORF	1063 bp FRT-flanked <i>erm</i> resistance gene excised	335 bp including a 96 bp FRT-scar
pNZKOsrtA::FRTerm	<i>E. mundtii</i> ST4SA <i>srtA::frt-erm</i>	<i>srtA</i>	1011 bp upstream of <i>srtA</i> , including 57 bp internal fragment of <i>srtA</i> ORF, 333 bp <i>mazG</i> nucleotide pyrophosphohydrolase gene and 270 bp fragment of <i>mfs</i> transporter ORF	1011 bp downstream of <i>srtA</i> , including 57 bp internal fragment of <i>srtA</i> ORF and 576 bp internal fragment of <i>bglG</i> transcriptional anti-terminator ORF	580 bp internal fragment of the <i>srtA</i> ORF	1458 bp FRT-flanked <i>erm</i> gene
pNZKOsrtCFRTerm	<i>E. mundtii</i> ST4SA <i>srtC::frt-erm</i>	<i>srtC</i>	1011 bp upstream of <i>srtC</i> , including 59 bp of <i>srtC</i> ORF and 892 bp of <i>EbpCfm</i> pilus subunit protein gene	376 bp downstream of <i>srtA</i> , including 60 bp of <i>srtC</i> ORF and 225 bp internal fragment of <i>hemolysin III</i> ORF	726 bp internal fragment of the <i>srtC</i> ORF	1458 bp FRT-flanked <i>erm</i> gene

TABLE 3. Oligonucleotides utilized in this study

Target	Primer	Sequence 5' to 3'	Restriction sites	Product size (bp)
<i>nisR-nisK</i>	nisRK1	GCGCA <u>AAGCTT</u> CCCCGGCTTTAGGTATAG	<i>Hind</i> III	2162
	nisRK2	ATCC <u>CTCGAG</u> TACTTTTTTATTTTAGGATA	<i>Xho</i> I	
<i>mazF</i>	mazF1	GCTTGGAT <u>CCATGG</u> TAAGCCGATACGTACC	<i>Nco</i> I	342
	mazF2	AGTCA <u>AAGCTT</u> CTACCCAATCAGTACGTTAAT T	<i>Hind</i> III	
<i>erm-ffluc</i> gene cassette	erm1	ATAC <u>GAATTC</u> CACGACCAAACTATAAAAACC	<i>Eco</i> RI	3204
	fluc2	GCGCA <u>AAGCTT</u> CACAATTTGACTTGCCA	<i>Hind</i> III	
<i>plaA</i> KO region	plaAKO1	<u>GAATTC</u> GTGATATATAATGATGGAATTTATT C	<i>Eco</i> RI	2012
	plaAKO2	<u>TCTAGAG</u> CCCTTAGCGTACTTATCCCAGGC	<i>Xba</i> I	
<i>munA</i> KO region	munAKO1	GCCG <u>GAATTC</u> GTTTATACGATTAGTGGAAATT A	<i>Eco</i> RI	1809
	munAKO2	ATACT <u>TCTAGAG</u> ACAATATCTCCAGTTTT	<i>Xba</i> I	
<i>cat-ffluc</i> gene cassette	cat1	<u>GAATTC</u> GGATCCATTCTAATGAAG	<i>Eco</i> RI	2349
	fluc2	GCGCA <u>AAGCTT</u> CACAATTTGACTTGCCA	<i>Hind</i> III	
<i>aap</i> KI region	aapKO1	GCCG <u>GAATTC</u> GAGTACTGTGACGACAGCTA	<i>Eco</i> RI	1848
	aapKO2	ATACT <u>TCTAGAG</u> TAATCGTCCGGGTAACCGTT	<i>Xba</i> I	
FRT- <i>erm</i> gene	M13for	GTAAAACGACGGCCAG	blunt	1458
	M13rev	CAGGAAACAGCTATGA	blunt	
<i>srtA</i> upstream region	srtAKO1for	CACCA <u>AAGCTT</u> GTAGTTACTTCATCAACGG	<i>Hind</i> III	1011
	srtAKO1rev	GATAG <u>GTAACT</u> AGTAGAAACAATAGGAAAT	<i>Hpa</i> I	
<i>srtA</i> down- stream region	srtAKO2for	TATAG <u>GTAACT</u> AAAGAAGCCACCCAAGAAAT	<i>Hpa</i> I	376
	srtAKO2rev	GATAT <u>TCTAGAG</u> CCGAAAAATTTACTGATGA	<i>Xba</i> I	
<i>srtC</i> upstream region	srtCKO1for	GAGGA <u>AAGCTT</u> ATTAGATGGGCAAGACGTAG	<i>Hind</i> III	1011
	strCKO1rev	GATAG <u>GTAACT</u> CCGATAAAAAATACCGAGAA	<i>Hpa</i> I	
<i>srtC</i> down- stream region	srtCKO2for	CACCG <u>GTAACT</u> TTTTTGTGCTATTTCTTAT	<i>Hpa</i> I	1035
	srtCKO2rev	CACCT <u>TCTAGAG</u> AAGATTATAATTTGAATTTTA	<i>Xba</i> I	

FRT target 1	FRTfor1	AGAT <u>GGATCC</u> CTCGTTTTTCGGAAACGCTTT	<i>Bam</i> HI	121
	FRTrev1	GATA <u>CTGCAG</u> TCAGAGCGCTTTTGGTTTT	<i>Pst</i> I	
FRT target 2	FRTfor2	AGATA <u>AAGCTT</u> CTCGTTTTTCGGAAACGCTTT	<i>Hind</i> III	121
	FRTrev2	GATA <u>GTCGAC</u> TCAGAGCGCTTTTGGTTTT	<i>Sal</i> I	
<i>erm</i> gene	<i>erm</i> 1	ATAC <u>GAATTC</u> CACGACCAAAACTATAAAACC	<i>Eco</i> RI	1063
	<i>erm</i> 2	GCTT <u>GAATTC</u> TACTTATTAATAATTTATAG		
FLP gene	FLPfor	GATA <u>CCATGG</u> ATGCCACAATTTGGTATATT	<i>Nco</i> I	1293
	FLPprev	AGTA <u>GTCGAC</u> TATATGCGTCTATTTATGT	<i>Sal</i> I	
asRNA repA	asRNAfor	ATAT <u>CCATGG</u> TCATATGAACCTTTGAT	<i>Nco</i> I	350
	asRNArev	CACGA <u>AAGCTT</u> GATAAGGTAATTATATCAT		
Pldh-FLP amplicon	Pldh1	<u>GAATTC</u> AATCTTCTCACCGTCT	<i>Eco</i> RI	1794
	FLPprev	AGTA <u>GTCGAC</u> TATATGCGTCTATTTATGT	<i>Sal</i> I	
<i>plaA</i> KO integration region	<i>plaA</i> KOc	AATATCTTCGTTGCTGTGAT	-	5118
	<i>plaA</i> KO2	<u>TCTAG</u> AGCCTTAGCGTACTTATCCCAGGC	<i>Xba</i> I	
<i>munA</i> KO intergration region	<i>munA</i> KOc	ATTCTTGAGAACATTCCACA	-	4190
	<i>munA</i> KO2	ATACT <u>TCTAG</u> AGACAATATCTCCAGTTTT	<i>Xba</i> I	
<i>aap</i> KI integration region	<i>aap</i> KOc	GGCGCCAGCAGCCAACTCAA	-	3696
	<i>aap</i> KOc2	CGTAGCCCTCATGGCTCAGA	-	
<i>aap</i> KI unmarked integration region	<i>aap</i> KOc	GGCGCCAGCAGCCAACTCAA	-	2573
	<i>aap</i> KOc2	CGTAGCCCTCATGGCTCAGA	-	
<i>srtA</i> KO integration region	<i>srtA</i> KOc	GATGGTTTTGTTTATTCGAA	-	3853
	<i>srtA</i> KO2rev	GATATCTAGAGCCGAAAAATTTACTGATGA	-	
<i>srtC</i> KO integration region	<i>srtC</i> KOc	GAAGGAACGCTGAAGGTCAA	-	3183
	<i>srtC</i> KOc2	AAACTAGTCCTGTCGTTTCCTT	-	

Construction of plasmids

The integration vectors for use in *L. plantarum* 423 and *E. mundtii* ST4SA were based on the pNZ8048 *Lc. lactis* NICE system plasmid (Mobitech), containing the PnisA *nisA* gene promoter region, a multiple cloning site (MCS), *E. coli/Lc. lactis repC* and *repA* replication genes for replication in LAB and *E. coli*, the *cat* gene for Cm resistance and the termination (T) sequence of the *Lc. lactis pepN* gene. DNA primers used are listed in Table 3. The nisin *nisK* and *nisR* regulatory genes were amplified from *Lc. lactis* pNZ9000 gDNA, using primers nisRK1 and nisRK2. The 2162 bp amplicon was digested with *HindIII* and *XhoI* and cloned into pNZ8048 after digestion of the vector with the same restriction enzymes, yielding plasmid pNZnisRK (Fig. 1). A schematic diagram summarizing the construction of pNZmazF and pNZmazFnisRK is shown in Fig. 2. Plasmids pNZmazF and pNZmazFnisRK were constructed to test the functionality of the nisin-inducible promoter in *Lc. lactis* pNZ9000, *L. plantarum* 423 and *E. mundtii* ST4SA. The *mazF* toxin gene was amplified from genomic DNA isolated from *E. coli* DH5 α , using primers mazF1 and mazF2. The generated 342 bp amplicon was cloned into the MCS's of pNZ8048 and pNZnisRK, using *NcoI* and *HindIII*, to yield pNZmazF and pNZmazFnisRK, respectively (Fig. 2). All fragments were ligated using T₄ DNA ligase (NEB) and incubated overnight at 15°C, before transformation into electrocompetent *E. coli* MC1061 for plasmid propagation.

The *L. plantarum* 423 *plaA* bacteriocin gene knockout (KO) plasmid was constructed by PCR, amplifying the complete 2012 bp region of homology that included the *plaA* open reading frame (ORF), using primers plaAKO1 and plaAKO2 (see Fig. S1 in the supplemental material). The generated amplicon was triple digested with *EcoRI*, *HpaI* and *XbaI*, resulting in the removal of 108 bp from the *plaA* ORF, ligated to a blunt-ended *erm-ffluc* gene cassette and pBluescriptKS digested with *EcoRI* and *XbaI*, yielding plasmid pKSplaA::ErmFfluc. The 3204 bp *erm-ffluc* gene cassette contained the *erm* gene for erythromycin resistance, the firefly luciferase gene (*ffluc*) from *Photinus pyralis* fused to the strong constitutive *L. plantarum ldh* gene (Pldh) promoter, generated via PCR using primer pair erm1/fluc2 and plasmid pNZErmFfluc423 (Table 1) as source DNA. The pKSplaA::ErmFfluc construct was used as template for PCR amplification of a 5013 bp amplicon containing the *plaA* upstream, *plaA* downstream and *erm-ffluc* gene cassette using primer pair plaAKO1/plaAKO2. Next, the full-length blunt-ended 5013 bp PCR fragment was cloned into the destination plasmid pNZmazFnisRK, before digestion of the plasmid with *BglIII* and filling in the 5' sticky ends, yielding plasmid pNZKOplaA::ErmFfluc (Fig. S1).

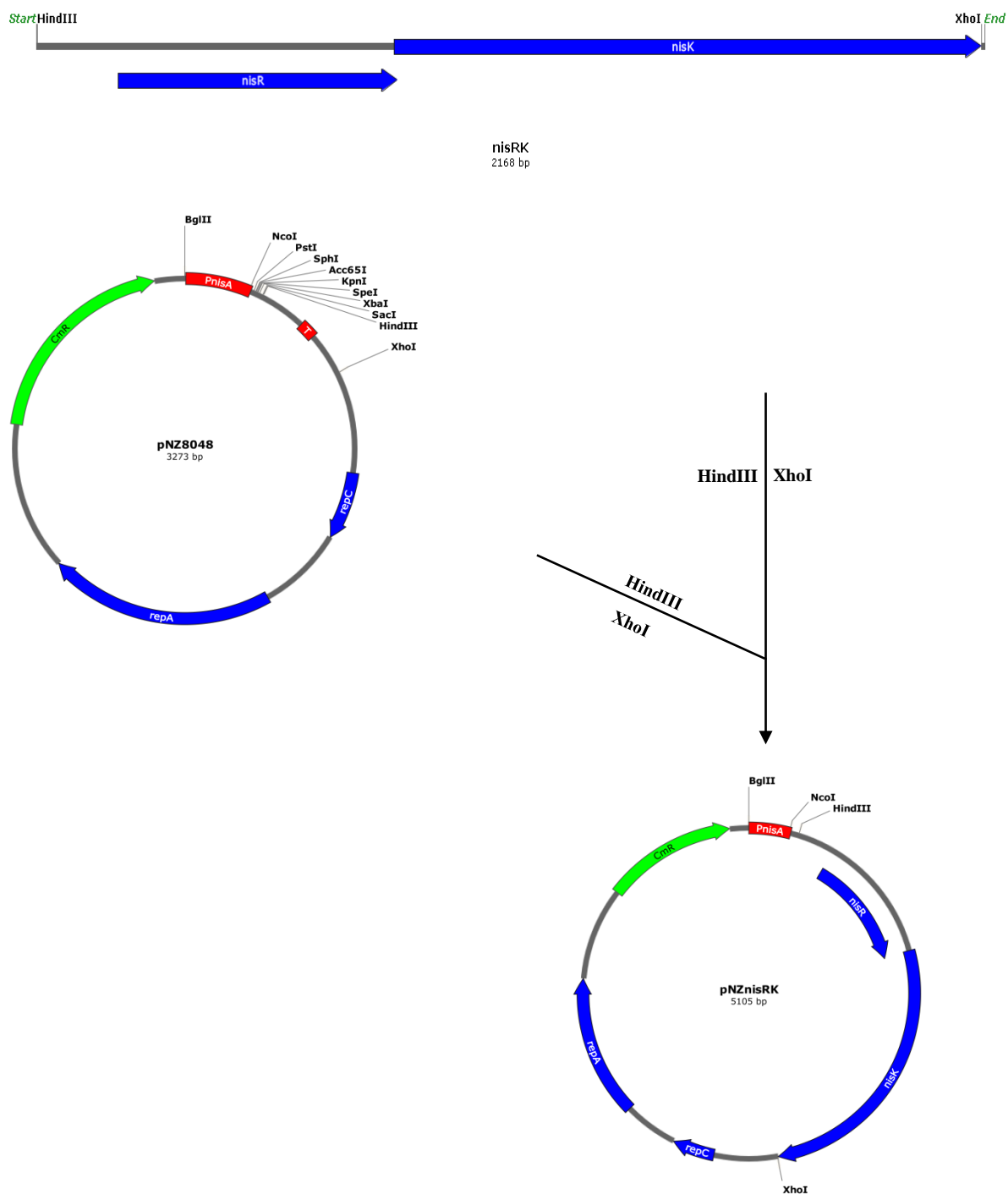


FIG. 1. Schematic representing the construction of pNZnisRK. Relevant features are indicated, including restriction sites used for cloning; the *E. coli*/LAB *repA* and *repC* replication ori; the chloramphenicol (*cat*) gene conferring resistance to chloramphenicol; the *nisR* and *nisK* nisin regulatory genes; and the nisin-inducible *PnisA* promoter from *Lc. lactis* pNZ9000.

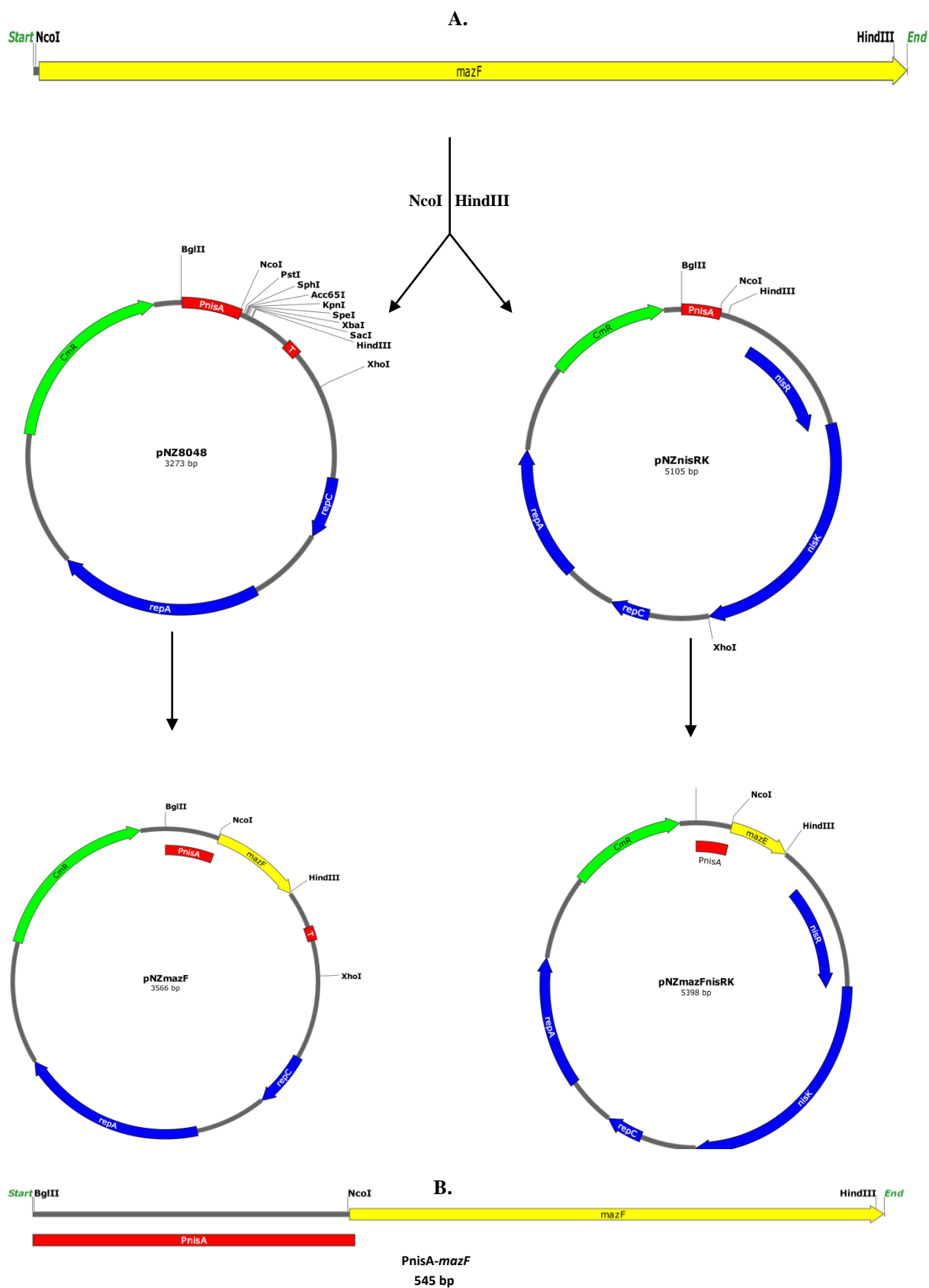


FIG. 2. (A) Schematic representing the construction of the MazF toxin expression plasmids pNZmazF, pNZmazFnisRK and (B) the arrangement of PnisA promoter placed upstream of the *mazF* gene. Relevant restriction sites are shown. For details refer to “Materials and Methods”.

The *E. mundtii* ST4SA *munA* bacteriocin gene KO plasmid was constructed as follows. First, a complete 1809 bp region of homology that included the *munA* ORF was obtained by PCR using the primer pair munAKO1/munAKO2, incorporating *EcoRI* and *XbaI* digestion sites (Fig. S2). The generated amplicon was digested with *EcoRI/PvuII* and *HpaI/XbaI*, resulting in a *munA* 911 bp upstream- and a 633 bp downstream-region of homology, respectively, and the removal of 18 bp from the *munA* ORF. Next, the two regions of homology were joined together with a blunt-ended 2480 bp *cat-ffluc* gene cassette generated via PCR amplification using primer pair cat1/fluc2 and plasmid pGKVCatFflucST4SA (Table 1) as source DNA. This *cat-ffluc*-interrupted *munA* region was then cloned into the *EcoRI/XbaI* double-digested pBluescriptKS cloning vector, yielding plasmid pKSmunA::CatFfluc. In this construct, the *ffluc* gene was fused to the strong *E. mundtii ldh* gene (*Pstldh*) promoter for constitutive expression of the bioluminescence gene. Finally, the *cat-ffluc* gene cassette flanked by the *munA* upstream/downstream regions of homology (4024 bp) was PCR amplified from pKSmunA::CatFfluc and cloned into the *StuI*-linearized pNZmazFnisRKerm plasmid, containing both *cat* and *erm* antibiotic resistance genes, yielding plasmid pNZKOMunA::CatFfluc (Fig. S2).

The *L. plantarum* 423 *aap* adhesion gene knock-in (KI) plasmid was constructed by PCR, amplifying a 1848 bp internal fragment of the *aap* ORF using primer pair aapKO1/aapKO2 (Fig. S3). The resulting amplicon was triple-digested with *EcoRI/HpaI/XbaI* (*HpaI* has a single cut site approximately in the middle of the *aap* fragment), and ligated to the blunt-ended *flippase* (FLP) recombination target (FRT) – flanked *erm* gene PCR fragment as well as the pBluescriptKS plasmid double-digested with *EcoRI/XbaI*, to yield plasmid pKSaap::FRTerm. The 1458 bp FRT-flanked *erm* gene was generated using primer pair M13for/M13rev and plasmid pKSFRTerm as source DNA (Fig. S6). The FRT-*erm* gene flanked by the *aap* upstream/downstream regions of homology (3305 bp) was PCR amplified from pKSaap::FRTerm using primer pair aapKO1/aapKO2 and cloned into the blunt-ended (*BglIII*, blunted) destination plasmid pNZmazFnisRK to yield plasmid pNZKIaap::FRTerm. (Fig. S3).

The *E. mundtii* ST4SA *sortase A* (*srtA*) deletion plasmid was constructed by amplifying two regions of homology flanking the *srtA* gene, using primer pairs, srtAKO1for/srtAKO1rev and srtAKO2for/srtAKO2rev. The generated amplicons (~1 kb each) were designed to include small internal (<60-bp) fragments of the *srtA* ORF in both the upstream and downstream regions of homology (Fig. S4). The upstream and downstream regions were then digested

with *HindIII/HpaI* and *HpaI/XbaI*, respectively, ligated to the blunt-ended FRT-*erm* gene PCR fragment and pBluescriptKS digested with *HindIII* and *XbaI*, yielding plasmid pKSsrtA::FRTerm. The complete *srtA* region of homology including the FRT-*erm* gene was then PCR amplified from pKSsrtA::FRTerm using primer pair srtAKO1for/srtAKO2rev, and cloned into the *HindIII*-linearized and blunt-ended plasmid pNZmazFnisRK, yielding plasmid pNZKOsrta::FRTerm (Fig. S4).

The pNZKOsrtaC::FRTerm plasmid, designed for disruption of the *E. mundtii* ST4SA sortase C (*srtC*) gene was constructed as follows. Two regions of homology (~1 kb upstream and ~0.4 kb downstream) flanking the *srtC* gene were PCR amplified using primer pairs srtCKO1for/srtCKO1rev and srtCKO2for/srtCKO2rev, respectively (Fig. S5). The generated fragments were digested with *HindIII/HpaI* and *HpaI/XbaI*, ligated to the blunt-ended FRT-*erm* gene and cloned to the *HindIII/XbaI* double-digested pBluescriptKS plasmid, yielding plasmid pKSsrtC::FRTerm. Finally, the complete FRT-*erm* –*srtC* and flanking region was amplified from pKSsrtC::FRTerm using primer pair srtCKO1for/srtCKO2rev and cloned into the pNZmazFnisRK plasmid as described above, to yield plasmid pNZKOsrtaC::FRTerm (Fig. S5).

Plasmids pKSFRTerm and pNZFLPasRNA_repA were constructed for utilization of the FLP/FRT recombination system of *Saccharomyces cerevisiae* for the generation of unmarked LAB knockout mutants and antibiotic marker recycling, as follows (see Figs S6 and S7). The pKSFRTerm plasmid was constructed by PCR amplification of two 48 bp FRT target sequences using primer pairs FRTfor1/FRTrev1, FRTfor2/FRTrev2 and the *S. cerevisiae* 2 μ m (~6.4 kb) plasmid as template DNA. The two generated amplicons containing the FRT target sequences were then double-digested with *HindIII/SalI* and *BamHI/PstI*, respectively, and cloned into the pBluescriptKS plasmid in a direct repeat orientation before digestion with the same restriction enzymes. The resulting plasmid (pKSFRT) was then digested with *EcoRI* (located in between the two FRT repeats) and ligated to an *erm* gene PCR fragment (erm1/erm2 primer pair) digested with the same enzyme to yield plasmid pKSFRTerm (Fig. S6).

The pNZFLPasRNA_repA plasmid was constructed as follows. The 1284 bp *flippase* (FLP) gene was amplified from the 2 μ m plasmid using primer pair FLPfor/FLPprev, incorporating the *NcoI/SalI* digestion sites, and ligated to the *EcoRI/NcoI* double-digested *Pldh* promoter and double-digested *EcoRI/SalI* pGKV223D plasmid, yielding plasmid pGKVPldhFLP (see

Fig. S7). Next, a 350 bp *repA* antisense RNA (asRNA) PCR fragment was generated, using primers asRNAfor and asRNArev, and was fused upstream of the nisin-inducible promoter by digestion of the amplicon with *NcoI* and *HindIII* and cloned to the pNZmazFnisRK plasmid after digestion with the same restriction enzymes, creating pNZasRNA_repAnisRK. The asRNA_repA fragment was designed for inactivation of the *repA* replication protein by binding to the first 350 bp of the *repA* transcript as a reverse and complement RNA strand thus inhibiting its translation. Finally, the 1794 bp Pldh-FLP PCR fragment was amplified from plasmid pGKVPldhFLP using primer pair Pldh1/FLPprev and subsequently cloned into the *BglIII* blunt-ended pNZasRNA_repAnisRK plasmid, yielding plasmid pNZFLPasRNA_repA. The integrity of all plasmids constructed was verified by restriction digests and by PCR using appropriate primer combinations (Table 3).

Optimization of MazF toxin expression using the nisin-inducible promoter in LAB

Control *L. plantarum* 423 and *E. mundtii* ST4SA strains transformed with empty pNZ8048 plasmid and recombinant *L. plantarum* 423 and *E. mundtii* ST4SA transformed with the pNZmazFnisRK plasmid were grown in MRS broth for 12 h. One millilitre of the 12-h old cultures was used to inoculate 50 ml of pre-warmed MRS broth. The 50 ml cultures were supplemented with 0 ng/ml, 100 ng/ml, 200 ng/ml, 300 ng/ml, 400 ng/ml and 600 ng/ml nisin (Sigma-Aldrich, St. Louis, MI, USA), respectively, and incubated at 30°C for 7 h. Every 30 min the optical density (OD_{550nm}) reading for each 50 ml culture was measured. All experiments were done with three repeats.

***mCherry* reporter gene expression in LAB using the nisin-inducible promoter**

To demonstrate the utility of the nisin-induction system using a reporter gene, *Lc. lactis* pNZ9000 transformed with plasmid pNZCherry, *L. plantarum* 423 and *E. mundtii* ST4SA each transformed with plasmid pNZCherrynisRK were grown in MRS or M17 broth for 12 h (Table 1). Each 12-h old culture was then serially diluted and plated onto MRS or M17 agar plates supplemented with or without nisin. Expression of the *mCherry* reporter gene was confirmed visually by a colony color change from white to pink or purple. Strains harboring the empty pNZ8048 vector were used as controls.

Confirmation of bacteriocin gene knockouts

To confirm bacteriocin gene KO integration mutants of strains *L. plantarum* 423 and *E. mundtii* ST4SA an overlay lawn assay was performed as described by Van Zyl and co-workers (25) with the following modifications. The *L. monocytogenes* EGDe bacteriocin sensitive strain was grown for 12 h and 100 μ l plated onto a BHI agar plate. The cell-free supernatants (adjusted to pH 7.0 with NaOH) of actively growing (12 h) bacteriocin KO strains were collected by centrifugation (8000 x g for 5 min). The supernatants were sterilized by passage through a 0.22- μ m-pore size filter using a 5 ml syringe and 30 μ l spotted into wells on BHI agar overlaid with *L. monocytogenes* EGDe. This was followed by incubation at 37°C for 24 h. As positive controls, supernatant from wild-type strains was used. Additionally, bacteriocin KO strains were sonicated for 3 min using an Omni-Ruptor 400 (Omni International, Kennesaw, GA, USA) for release of intracellular proteins. Briefly, bacteriocin KO cultures were grown for 12 h in 100 MRS broth. Cells were harvested at 13000 x g for 10 min, resuspended in 30 ml 10 mM Tris buffer supplemented with 500 mM NaCl and 10 mg/ml lysozyme, and incubated at 37°C for 2 h. After incubation, the cells were sonicated for 3 min followed by centrifugation (13000 x g for 5 min) and collection of the cell free supernatants. The supernatants containing intracellular proteins were then spotted onto BHI agar plates spread with *L. monocytogenes* EGDe as described before for visualization of inhibitions zones.

Clumping response protocol

The bacterial clumping protocol for confirmation of the *E. mundtii* ST4SA *srtA* gene KO strain was adapted from previous studies (51-52) with the following modifications. A pheromone-containing supernatant was collected by centrifugation (13000 x g for 5 min) of a 12-h old wild-type *E. mundtii* ST4SA strain, followed by filter sterilization through a 0.22- μ m-pore size cellulose nitrate filter using a 5 ml syringe. Wild-type and *srtA* KO mutant strains were grown for 12 h in MRS broth and diluted to an OD_{600nm} of ~0.06 in 5 ml MRS broth supplemented with 250 μ l sterile supernatant from wild-type *E. mundtii* ST4SA. The cultures were incubated for 12 h at 30°C without shaking before visualization of clumping.

Detection of *in vitro* bioluminescence

Lactobacillus plantarum 423 and *E. mundtii* ST4SA recombinant strains carrying the *ffluc* bioluminescence gene were analyzed on agar plates for bioluminescence emission using the Caliper *in vivo* imaging system (IVIS; Caliper Life Sciences, Hopkinton, MA, USA). Cultures were grown for 12 h in MRS broth, plated onto MRS agar plates using sterile swabs and incubated at 30°C for 24 h. Prior to imaging, one ml of beetle D-luciferin potassium salt substrate (Anatech Instruments, Bellville, South Africa) at 150 µg/ml dissolved in phosphate-buffered saline (PBS) was added directly to the colonies.

Confirmation of successful chromosomal or plasmid integrations

To confirm successful double-crossover recombination events in LAB gene deletion or integration mutants, gDNA was isolated for PCR amplification using the appropriate primer combinations listed in Table 3. All LAB KO or KI mutants were confirmed by DNA sequencing of PCR products performed by the Central DNA Sequencing Facility of the University of Stellenbosch.

Protocol for isolating double-crossover mutants

The complete step-by-step design and timeline for this method of quick and easy isolation of double-crossover KO or KI mutants is shown in Fig. S8. Upon completion of the construction and transformation of an appropriate integration plasmid in LAB strains, transformants were enriched for by either direct inoculation into MRS broth or by plating on MRS agar plates supplemented with both Cm and Em. After an incubation period of 2 – 3 days at 30°C, cells grown in broth or colonies observed on agar plates were streaked on fresh agar plates and re-incubated for 24 h at 30°C until single colonies were observed. Cells carrying the integration plasmid were then inoculated into fresh MRS broth supplemented with Cm and Em, followed by further incubation at 30°C for 12 h. Two-hundred microliters of the cell suspension was then inoculated into fresh MRS broth supplemented with the antibiotic present in the integrative gene cassette (either Cm or Em) and the appropriate concentration of nisin for MazF toxin expression, followed by incubation at 30°C for 6 h. After 6 h of incubation and growth recorded at OD_{600 nm}, the cell suspensions were serially diluted, and plated onto MRS agar plates supplemented with the appropriate antibiotic and nisin as described above. The plates were incubated for 24 to 48 h at 30°C or until colonies were observed. Double-

crossover recombined mutants appearing on the antibiotic-nisin plates were isolated and screened for the loss of the plasmid-backbone-located antibiotic marker and by PCR for confirmation of the desired recombination event.

Mutant cells carrying a FRT-flanked *erm* gene were transformed with the pNZFLPasRNA_repA plasmid for excision of the *erm* resistance gene. Upon transforming the plasmid in FRT-*erm*-containing LAB mutants and the appearance of transformants on MRS agar plates supplemented with Cm, single colonies were inoculated into MRS broth and incubated at 30°C for 6 h for constitutive expression of FLP recombinase. Cell suspensions were then serially diluted and plated onto MRS agar plates containing Cm for propagation. Unmarked mutants that have lost the *erm* gene via FRT-FLP excision were identified by replica plating on MRS agar plates containing Cm or Em. Colonies that did not grow on MRS plates supplemented with Em were screened by PCR to confirm *erm* gene excision.

To generate unmarked mutants that would allow for further genetic manipulation using the nisin-MazF counter-selection marker system, cells carrying the FLP recombinase pNZFLPasRNA_repA plasmid were induced with nisin to stimulate plasmid loss. Cells carrying the pNZFLPasRNA_repA plasmid were inoculated into MRS broth supplemented with nisin, followed by incubation at 30°C for 6 h for expression of the asRNA transcript. After incubation, the cell suspensions were serially diluted, and plated onto MRS agar plates not supplemented with Cm and incubated at 30°C for 24 h. Mutant cells that have lost the pNZFLPasRNA_repA plasmid were identified by replica plating on MRS agar plates supplemented with and without Cm. Colonies that showed no growth on MRS plates supplemented with Cm indicated plasmid loss.

Results

Construction and optimization of the nisin-inducible counter-selection marker

The nisin-inducible promoter (PnisA) originating from the *Lc. lactis* nisin A lantibiotic producer strain was developed and optimized for its heterologous use in the probiotics *L. plantarum* 423 and *E. mundtii* ST4SA. The *mazF* toxin gene was placed downstream of the pNZnisRK plasmid PnisA promoter region that also carries the *nisR* and *nisK* nisin signal regulatory genes on the plasmid backbone required for heterologous induction of gene expression by PnisA (Figs 1 and 2). The resulting plasmid pNZmazFnisRK was introduced

into the probiotic LAB strains, giving rise to strains *E. mundtii* ST4SA pNZmazFnisRK and *L. plantarum* 423 pNZmazFnisRK. Their MazF response to induction with varying concentrations of nisin was then evaluated (Figs 3 and 4). Previous studies (40, 41) reported that low sub-inhibitory concentrations of nisin, ranging from 0.1 to 5.0 ng/ml, were sufficient for Pnis- controlled gene expression in *Lc. lactis* pNZ9000. Results in this study showed that the PnisA promoter responded to much higher sub-inhibitory concentrations of nisin, ranging from 300 to 400 ng/ml in *E. mundtii* ST4SA pNZmazFnisRK (Fig. 3A) and 300 to 600 ng/ml nisin in *L. plantarum* 423 pNZmazFnisRK after 7 h of induction (Fig. 4A). Control strains transformed with pNZ8048 and recombinant strains transformed with pNZmazFnisRK without nisin induction showed no significant differences in growth after 7 h. These results suggested that no significant promoter leakiness was occurring that could potentially result in premature cell growth arrest without nisin induction of PnisA. No significant difference in growth rate was recorded for control strain *E. mundtii* ST4SA pNZ8048 with (300 ng/ml) or without nisin induction, whereas a significant growth difference was recorded for recombinant *E. mundtii* ST4SA pNZmazFnisRK when induced with nisin at the same concentration (Fig. 3A). No significant growth inhibition was observed for control strain *L. plantarum* 423 pNZ8048 when induced with nisin concentrations ranging from 100-600 ng/ml compared to un-induced. A significant inhibition of growth was recorded when strain *L. plantarum* 423 pNZmazFnisRK was induced with nisin at 600 ng/ml as compared to when no nisin was present (Fig. 4A). For all further nisin induction experiments, a nisin induction concentration of 300 ng/ml was used for PnisA-carrying *E. mundtii* ST4SA and 600 ng/ml for *L. plantarum* 423. Taken together, these results confirmed that the MazF protein toxicity would enable its application in LAB as an efficient counter-selection marker for the isolation of double-crossover mutants.

Lactococcus lactis pNZ9000 transformed with plasmid pNZmazF, recombinant strains *L. plantarum* 423 pNZmazFnisRK and *E. mundtii* ST4SA pNZmazFnisRK cultures were plated onto M17 or MRS agar plates supplemented with Cm in the absence and presence of nisin. After 24 h of incubation at 30°C, plates containing nisin showed very little or no growth, while those no nisin showed an abundance of growth (Figs 3B, 4B and 5A). To further demonstrate the application of the nisin-induction system in LAB, *Lc. lactis* pNZ9000 was transformed with plasmid pNZCherry, while *L. plantarum* 423 and *E. mundtii* ST4SA were transformed with plasmid pNZCherryFnisRK. In both vectors, the *mCherry* red fluorescence gene was placed downstream of the PnisA promoter (Table 1). The PnisA nisin-induced

expression of mCherry fluorescence protein was easily detected on agar plates containing nisin by the appearance of pink colonies, while those lacking nisin remained white or cream (Figs 5B, 5C and 5D).

Fig. 3A.

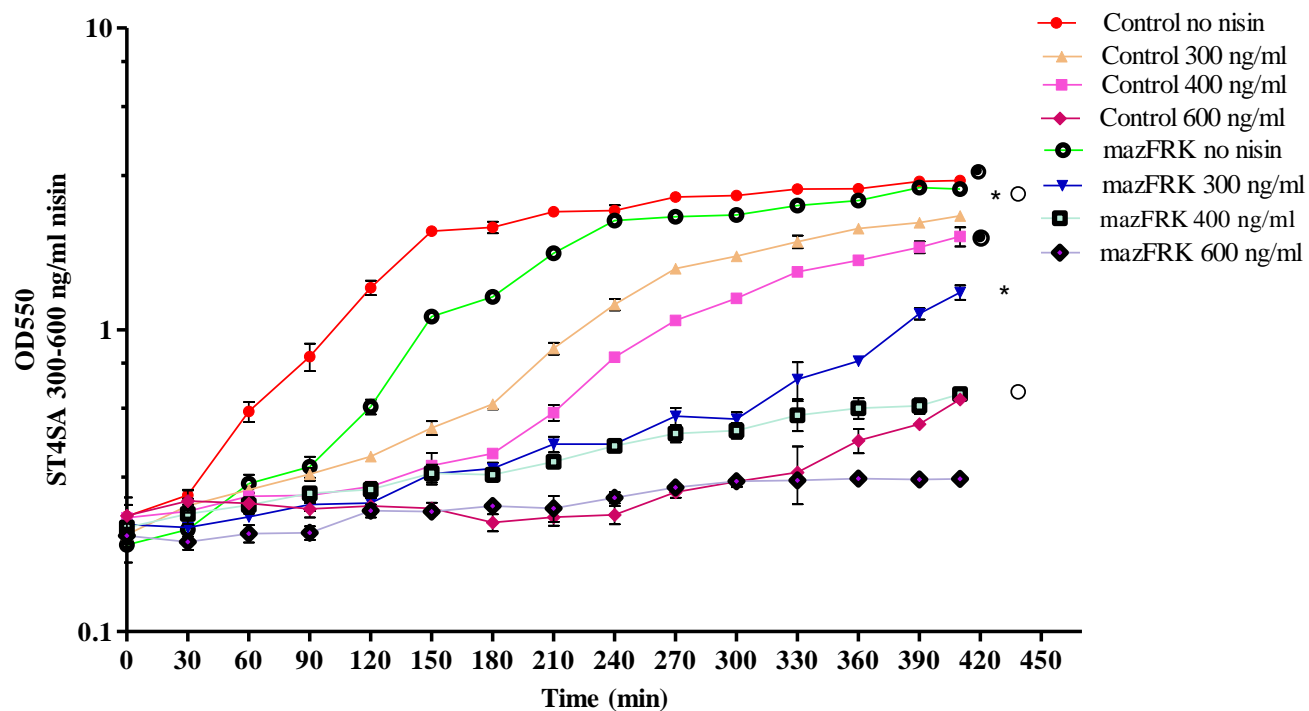


Fig. 3B.

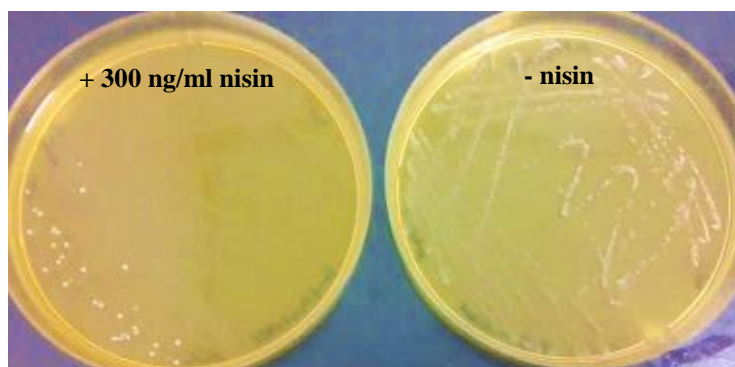


FIG. 3. Optimization of nisin-controlled *mazF* gene expression in *E. mundtii* ST4SA. **(A)** Growth comparison of *E. mundtii* ST4SA transformed with the empty pNZ8048 vector (control) and *E. mundtii* ST4SA transformed with the PnisA-controlled *mazF* gene pNZmazFnisRK plasmid in sub-inhibitory concentrations of nisin (0 - 600 ng/ml). Differences were assessed using the Kruskal-Wallis nonparametric test, ● ($P < 0.05$) for comparison between Control no nisin and Control 400 ng/ml, * ($P < 0.05$) for comparison between mazFRK no nisin and mazFRK 300 ng/ml, ○ ($P < 0.05$) for comparison between mazFRK no nisin and mazFRK 400 ng/ml. **(B)** MRS agar plates representative of the effect of MazF protein expression in *E. mundtii* ST4SA harboring the pNZmazFnisRK plasmid in the absence of nisin (-nisin) and in the presence of nisin (+300 ng/ml nisin).

Fig. 4A.

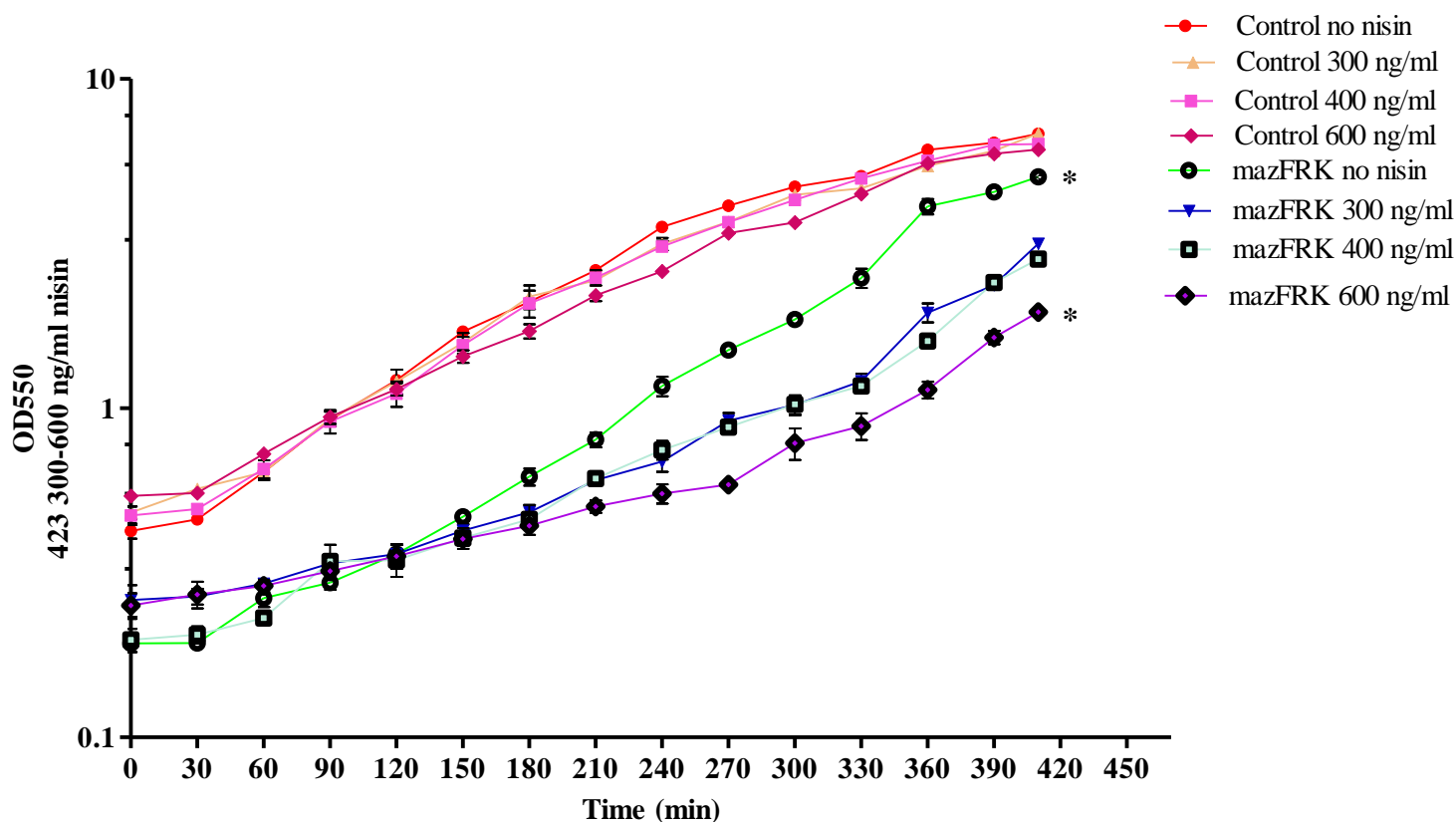


Fig. 4B.

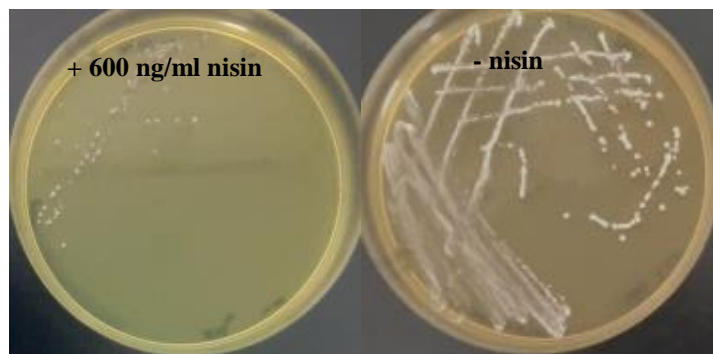


FIG. 4. Optimization of nisin-controlled *mazF* gene expression in *L. plantarum* 423. **(A)** Growth comparison of *L. plantarum* 423 transformed with the empty pNZ8048 vector (control) and *L. plantarum* 423 transformed with the PnisA controlled *mazF* gene pNZmazFnisRK plasmid in sub-inhibitory concentrations of nisin (0 - 600 ng/ml). Significant differences ($P < 0.05$; Kruskal-Wallis nonparametric test) between mazFRK no nisin and mazFRK 600 ng/ml are indicated with an asterisk. **(B)** MRS agar plates representative of the effect of MazF protein expression in *L. plantarum* 423 harboring the pNZmazFnisRK plasmid in the absence of nisin (-nisin) and in the presence of nisin (+600 ng/ml nisin).

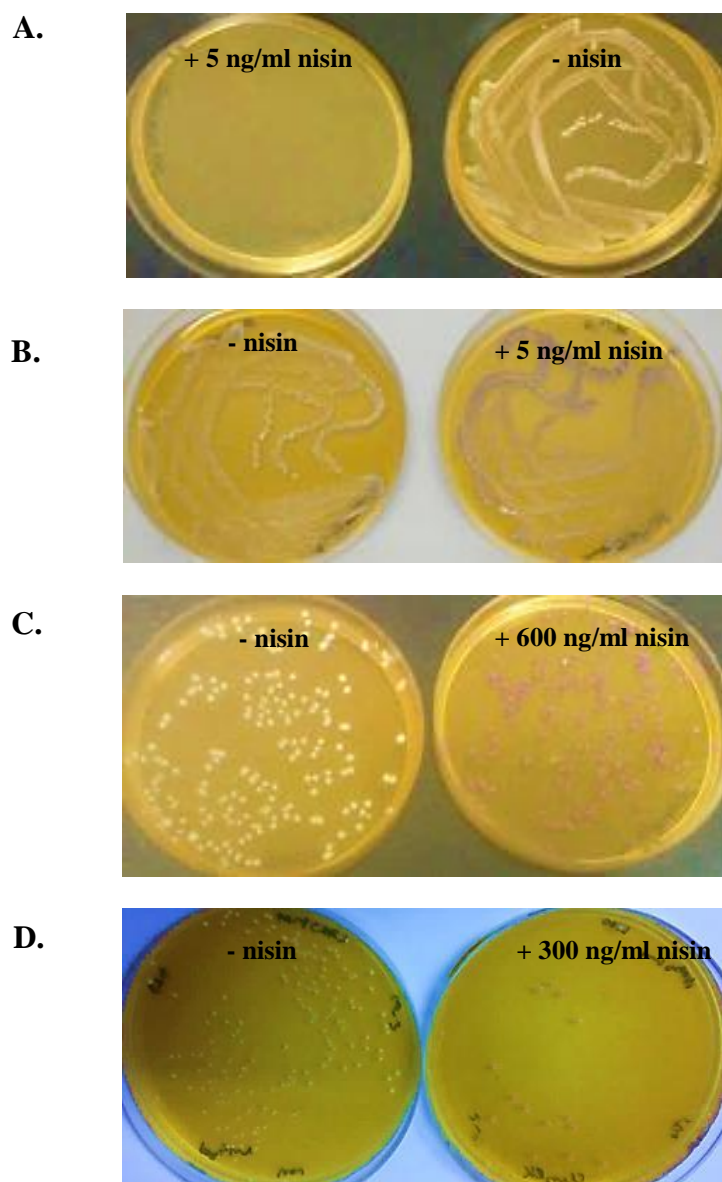


FIG. 5. Plates showing the effect of PnisA promoter-controlled MazF protein expression in *Lc. lactis* pNZ9000 and PnisA promoter-controlled mCherry fluorescence protein expression in *Lc. lactis* pNZ9000, *L. plantarum* 423 and *E. mundtii* ST4SA. **(A)** M17 agar plates representative of the effect of MazF protein expression in *Lc. lactis* pNZ9000 harboring the pNZmazF plasmid in the absence of nisin (-nisin) and in the presence of nisin (+5 ng/ml nisin). **(B)** M17 agar plates showing the expression of the *mCherry* fluorescence gene in *Lc. lactis* pNZ9000 harboring the pNZCherry plasmid in the absence of nisin (-nisin) and in the presence of nisin (+5 ng/ml nisin). **(C)** MRS agar plates showing the expression of the *mCherry* fluorescence gene in *L. plantarum* 423 harboring the pNZCherrynisRK plasmid in the absence of nisin (-nisin) and in the presence of nisin (+600 ng/ml nisin). **(D)** MRS agar plates showing the expression of the *mCherry* fluorescence gene in *E. mundtii* ST4SA harboring the pNZCherrynisRK plasmid in the absence of nisin (-nisin) and in the presence of nisin (+300 ng/ml nisin).

Integration of large DNA fragments and deletion of the *L. plantarum* 423 and *E. mundtii* ST4SA bacteriocin genes

To demonstrate the feasibility and efficiency of the nisin-MazF counter-selection marker system in LAB, the *plaA* plantaricin bacteriocin gene located on the *L. plantarum* 423 pPLA4 plasmid (49) and the *E. mundtii* ST4SA *munA* mundticin bacteriocin gene located on a megaplasmid (52) were targeted for inactivation. The *plaA* KO plasmid carried a 3204 bp *erm-ffluc* gene cassette (see Fig. S1), while the *munA* KO plasmid carried a 2471 bp *cat-ffluc* gene cassette (see Fig. S2), flanked by regions of homology required for homologous recombination. The pNZKO*plaA::ErmFfluc* plasmid included a 69 bp internal fragment of the *plaA* ORF in the downstream region of homology, resulting in the deletion of 102 bp of the 171 bp *plaA* gene in all *L. plantarum* 423 *plaA::erm-ffluc* mutants (Fig. 6A). Refer to Table 2 for a detailed description of the components of all regions of homology for targeted gene inactivation. Similarly, plasmid pNZKOMunA::CatFfluc included a 135 bp internal fragment of the *munA* ORF in the upstream region of homology, resulting in the deletion of 18 bp of the 153 bp *munA* gene in all *E. mundtii* ST4SA *munA::cat-ffluc* mutants (Fig 7A). Double crossover mutants were isolated by following the protocol described in ‘Materials and Methods’ (Also refer to Fig. S8). *Lactobacillus plantarum* 423 *plaA::erm-ffluc* mutants retained resistance to Em, but not to Cm, indicating that the *erm-ffluc* gene cassette was successfully integrated onto the pPLA4 plasmid without the plasmid backbone DNA encoding the *mazF* and *cat* genes. *Enterococcus mundtii* ST4SA *munA::cat-ffluc* double-crossover mutants retained resistance to Cm, but not Em, in turn indicating the loss of the plasmid backbone DNA encoding the *mazF* and *erm* genes. Knockout mutants that appeared on nisin plates were screened by PCR and DNA sequencing using primer combinations listed in Table 3. *Lactobacillus plantarum* 423 *plaA::erm-ffluc* mutants contained the 3204 bp *erm-ffluc* “cargo” DNA via allelic exchange, with the consequent deletion of a 208 bp DNA fragment on the pPLA4 native plasmid as confirmed by PCR as well as sequencing (Fig. 6B). Similarly, *E. mundtii* ST4SA *munA::cat-ffluc* mutants retained the *cat-ffluc* cassette via allelic exchange and the deletion of a 260 bp fragment from the megaplasmid harboring the *munA* gene as confirmed by PCR and sequencing (Fig. 7B).

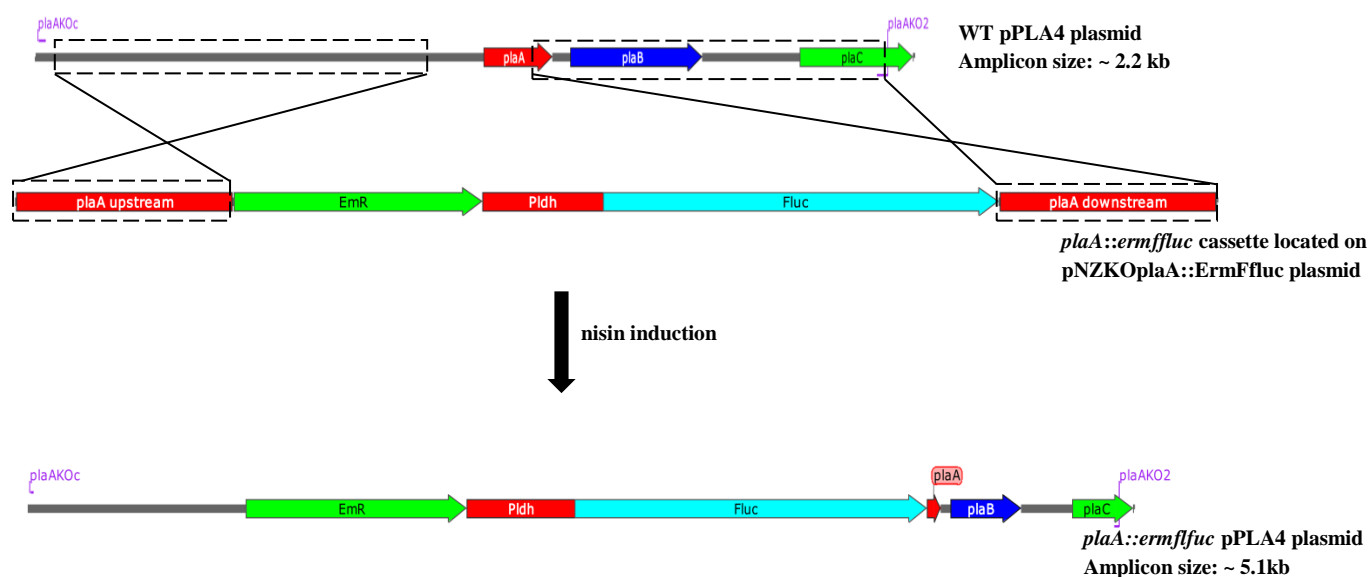
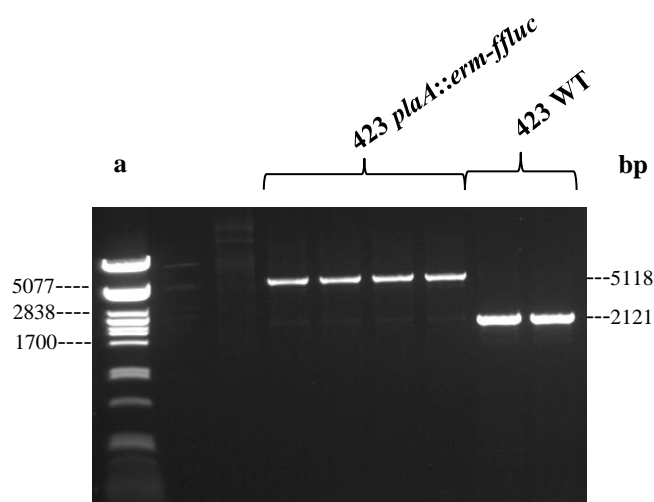
Fig. 6A.**Fig. 6B.**

FIG. 6. Gene deletion and integration via homologous recombination into the genome of *L. plantarum* 423 at the *plaA* bacteriocin gene locus to create *L. plantarum* 423 *plaA::erm-ffluc*. (A) Homologous recombination between the WT pPLA4 plasmid and the *plaA::ermffluc* cassette. Boxed regions show the upstream and downstream regions of homology (~ 0.9 kb) on plasmid pPLA4 and the pNZKOplaAErmFfluc KO vector. Cells harboring the *plaA* KO vector were selected on Cm and Em, followed by nisin induction for MazF toxin expression to select for mutants that have lost the plasmid backbone bearing *cat* and *mazF* genes. Double crossover mutants were selected and screened by PCR using the primer combinations indicated in purple. (B) PCR amplification of WT and *plaA* deletion and insertion mutants using the primer pair indicated in panel A. (a) Lambda DNA digested with *Pst*I (NEB). Amplicons from four *plaA* mutant and two WT colonies, respectively, are shown.

Fig. 7A.

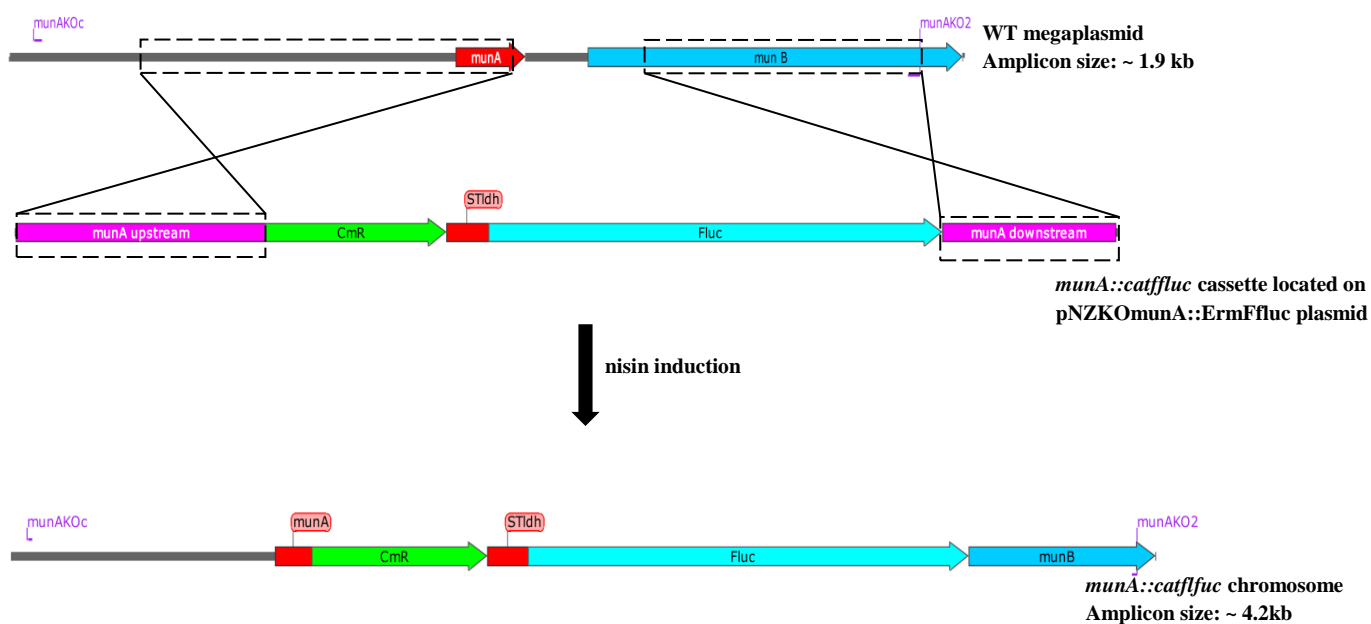


Fig. 7B.

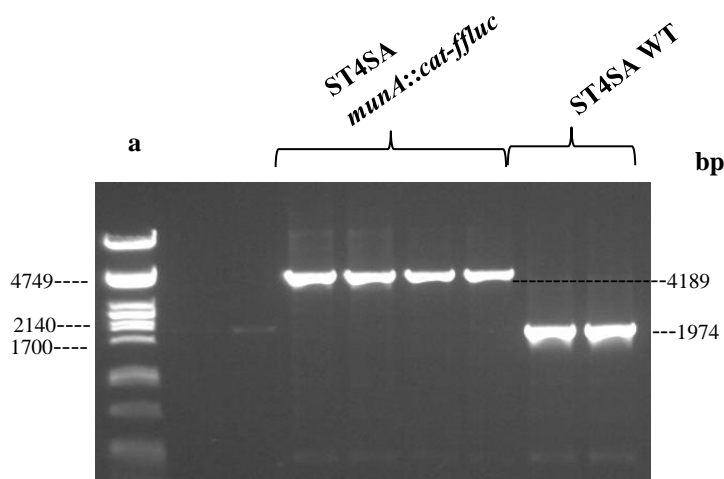


FIG. 7. Gene deletion and integration via homologous recombination into the genome of *E. mundtii* ST4SA at the *munA* bacteriocin gene locus to create *E. mundtii* ST4SA *munA::cat-ffluc*. (A) Homologous recombination between the WT *E. mundtii* ST4SA *munA*-carrying megaplasmid and the *munA::catffluc* cassette. Boxed regions show the upstream (~ 0.9 kb) and downstream regions (~ 0.6) of homology on the megaplasmid and the pNZKOmunaCatFfluc KO vector. Cells harboring the *munA* KO vector were selected on Cm and Em, followed by nisin induction for MazF toxin expression to select for mutants that have lost the plasmid backbone bearing *erm* and *mazF* genes. Double crossover mutants were selected and screened by PCR using the indicated primer combinations. (B) PCR amplification of WT and *munA* deletion and insertion mutants using the primer pair indicated in panel A. (a) Lambda DNA digested with *Pst*I (NEB). Amplicons from four *munA* mutant and two WT colonies, respectively, are shown.

Supernatants isolated from *L. plantarum* 423 *plaA::erm-ffluc* (Fig. 8A) and *E. mundtii* ST4SA *munA::cat-ffluc* (Fig. 8B) mutants did not result in the formation of clear inhibition zones on plates overlaid with the sensitive *L. monocytogenes* EGDe strain as compared to the wild-type derivatives of each strain. Similarly, supernatants isolated from sonicated *plaA*- and *munA*- mutant cell cultures did not result in the formation of inhibition zones on agar plates overlaid with *L. monocytogenes* EGDe (not shown). These results confirmed that the bacteriocin genes of the probiotic strains were successfully inactivated using the nisin-MazF counter-selection method. To test the active expression of the integrated *ffluc* bioluminescence gene, *L. plantarum* 423 *plaA::erm-ffluc* (Fig. 9A) and *E. mundtii* ST4SA *munA::cat-ffluc* (Fig. 9B) mutants were imaged using the IVIS. A strong bioluminescent signal was detected from colonies formed on agar plates for each of the mutant strains.

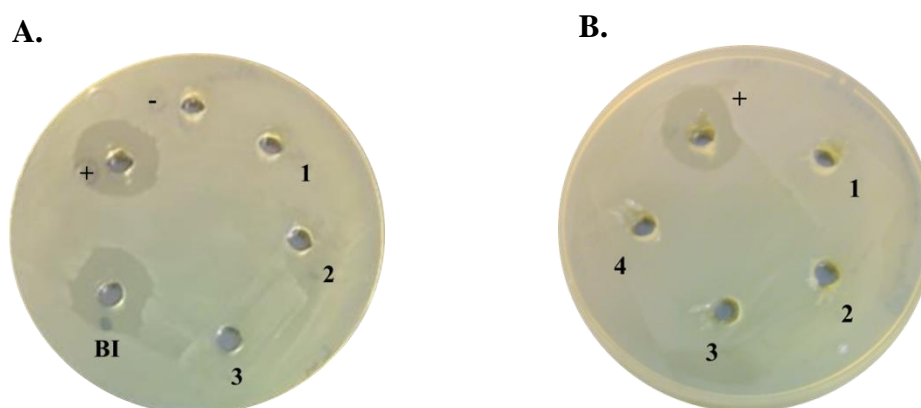
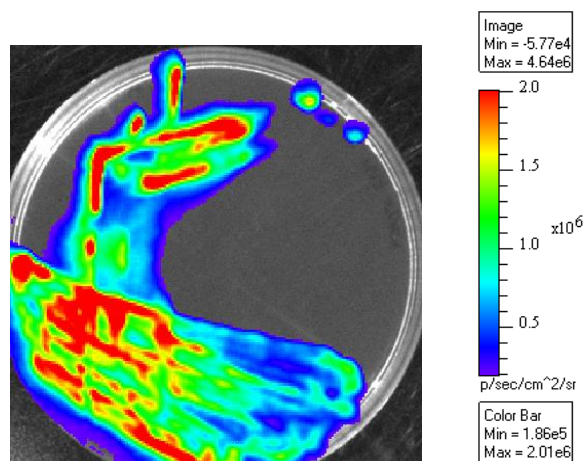


FIG. 8. Zones of inhibition on plates overlaid with *L. monocytogenes* EGDe. **A.** (BI) Supernatant containing bacteriocin plantaricin 423 isolated from *L. plantarum* 423 transformed with KO plasmid pNZKO $plaA::ErmFfluc$ before induction with nisin, (+) supernatant containing bacteriocin plantaricin 423 isolated from WT *L. plantarum* 423 as positive control, (-) supernatant lacking plantaricin 423 bacteriocin isolated from *L. plantarum* 423 bac^- as negative control and supernatants (1, 2 & 3) lacking bacteriocin plantaricin 423 isolated from three *L. plantarum* 423 *plaA::erm-ffluc plaA* gene deletion mutants. **B.** (+) Supernatant containing bacteriocin mundtacin ST4SA isolated from WT *E. mundtii* ST4SA as positive control and supernatants (1, 2, 3 & 4) lacking bacteriocin mundtacin ST4SA isolated from four *E. mundtii* ST4SA *munA::cat-ffluc munA* gene deletion mutants.

A.



B.

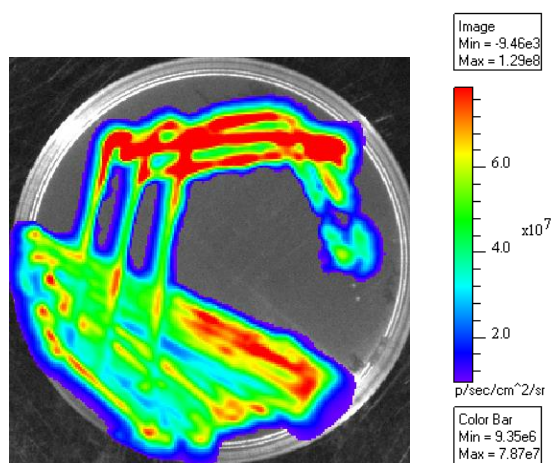


FIG. 9. MRS agar plates showing bioluminescence emission of *ffluc* luciferase gene integration mutants. **(A)** Bioluminescent *L. plantarum* 423 *plaA::erm-ffluc* double-crossover mutant colonies harboring the *ffluc* gene integrated at the *plaA* locus. **(B)** Bioluminescent *E. mundtii* ST4SA *munA::cat-ffluc* double-crossover mutant colonies harboring the *ffluc* gene integrated at the *munA* locus.

Integration and removal of the FRT-flanked *erm* gene in *L. plantarum* 423 for marker recycling

To overcome the limited availability of antibiotic resistance markers that are suitable for use in LAB as integrative selective markers, the *S. cerevisiae* FLP-FRT recombination system was adapted for marker recycling. A gene disruption mutant of the *L. plantarum* 423 *aap* adhesion-associated gene was generated by utilizing the *erm* gene flanked by two direct repeat FRT recombination targets for excision by FLP recombinase. The pNZKIaap::FRTerm plasmid carried the FRT-*erm* gene flanked by two internal regions (~0.9 kb each) of homology of the 4035 bp *aap* ORF that would result in the insertion of the FRT-*erm* gene and simultaneous disruption of the *aap* gene (see Fig. S3). The generated sizes of the wild-type *L. plantarum* 423 and integrant loci are shown in Fig. 10A. *Lactobacillus plantarum* 423 *aap::ftr-erm* mutants were successfully isolated via nisin induction of the *mazF* toxin gene as described elsewhere (see Materials and Methods; also refer to Fig. S8). Double-crossover mutants selected on nisin-supplemented MRS agar plates retained resistance to Em, but not Cm, confirming the loss of the *cat* and *mazF* gene encoded plasmid backbone. The recombination event, namely, the insertion of the FRT-*erm* gene onto the *aap* ORF locus, was confirmed by PCR and the resulting amplicon sizes are shown in Fig. 10B. PCR amplification of *aap* mutant gDNA resulted in a 3696 bp fragment compared to the 2238 bp fragment in the wild-type derivative.

Lactobacillus plantarum 423 *aap::ftr-erm* mutants were electro-transformed with the pNZFLPasRNA_repA plasmid for FRT-flanked *erm* antibiotic resistance marker excision. Once successful transformants were isolated and confirmed to be resistant to both Em and Cm, FLP recombinase activity was induced as described in Materials and Methods. This resulted in the isolation of unmarked *aap* mutant *L. plantarum* *aap::ftr_um* (um - unmarked) colonies that have lost the *erm* gene via FRT-FLP excision as confirmed by PCR and sequencing (Figs 10A and 10B).

Once excision of the *erm* antibiotic resistance marker in *L. plantarum* *aap::ftr_um* colonies was confirmed, loss of the FLP recombinase plasmid was stimulated by the expression of a 350 bp asRNA *repA* transcript by induction with nisin. *Lactobacillus plantarum* *aap::ftr_um* mutants isolated via replica plating did not show any colony growth on MRS agar plates supplemented with nisin, indicating plasmid loss, while colonies that were not induced by nisin showed an abundance of growth (indicating the presence of the plasmid) (Fig. 10C).

Fig. 10A.

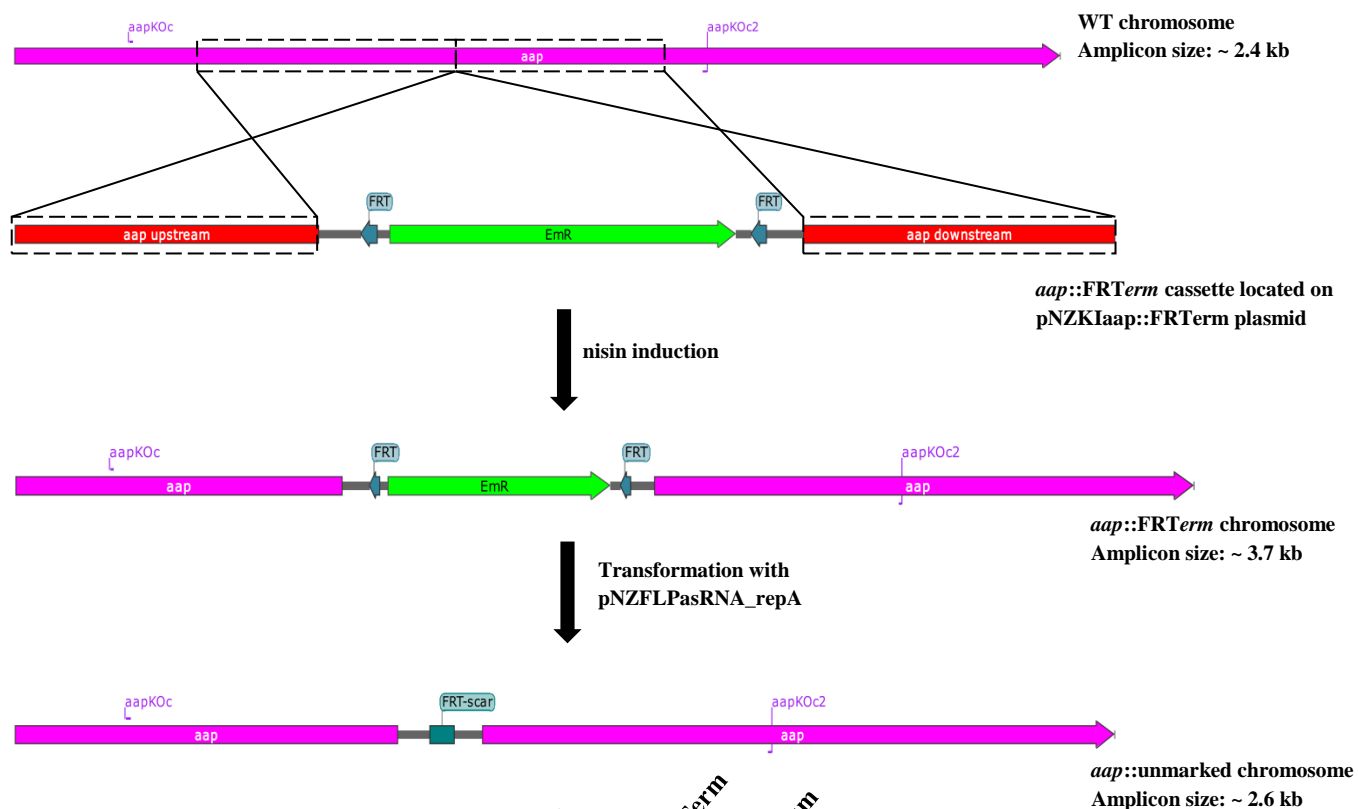


Fig. 10B.

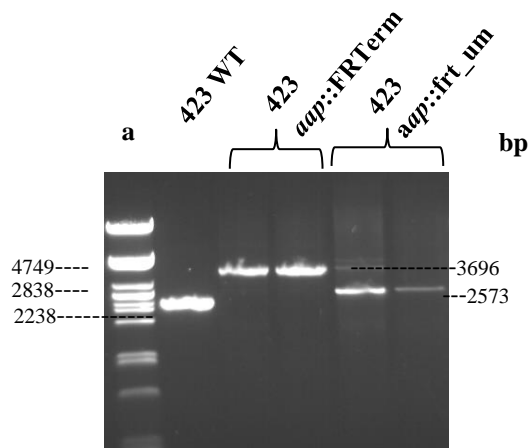


Fig. 10C.

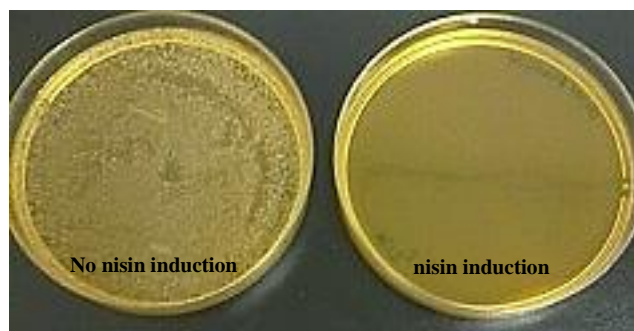


FIG. 10. Gene inactivation and integration via homologous recombination into the genome of *L. plantarum* 423 at the *aap* adhesion gene locus to create *L. plantarum* 423 *aap*::FRTerm and *L. plantarum* 423 *aap*::frit_{um} (A) Homologous recombination between the WT *L. plantarum* 423 chromosome and the *aap*::FRTerm cassette and selection of unmarked *aap* double-crossover mutants.

Boxed regions show the upstream and downstream regions of homology (~ 0.9 kb) on the WT *L. plantarum* 423 chromosome and plasmid pNZKIaap::FRTerm KI vector. Cells harboring the *aap* KI vector were selected on Cm and Em, followed by nisin induction for MazF toxin expression to select for mutants that have lost the plasmid backbone bearing *cat* and *mazF* genes. Double crossover mutants were selected and screened by PCR using the primer combinations indicated in purple. **(B)** PCR amplification of WT *L. plantarum* 423 and *aap* insertion mutants using the primer pair indicated in panel A. Additionally, the Em resistance marker was recycled via excision by FLP recombinase as described in Materials and Methods. **(a)** Lambda DNA digested with *Pst*I (NEB). Amplicons from one WT, two *aap*::FRTerm insertion mutant and two *aap*::unmarked colonies are shown. **(C)** MRS agar plates showing the effectiveness of the *repA* asRNA induction of FLP recombinase-bearing plasmid loss in the absence of nisin (no nisin induction) and in the presence of nisin (nisin induction). Colonies that have lost the *repA*-bearing plasmid were isolated via replica plating.

Isolation of *srtA* and *srtC* deletion mutants using regions of homology containing small (<60 bp) 5' and 3' end sequences

Although the PnisA-MazF counter-selection system proved to be functional, the *E. mundtii* ST4SA *srtA* and *srtC* genes did not contain sufficient restriction enzyme sites to allow cloning of integrative gene cassettes flanked by homologous arms. A strategy to introduce new restriction sites would facilitate the cloning of integrative genes inbetween homologous regions to enable the inactivation of target sequences with insufficient restriction enzyme digestion sites. To do this, *srtA* and *srtC* KO plasmids carrying upstream and downstream regions of homology with new restriction sites for integrative gene cassette insertion were constructed (see Figs S4 and S5). Both KO plasmids were designed to facilitate the deletion of the majority of the *srtA* and *srtC* genes sequences (with <60 bp of the 5' and 3' ends included in the upstream and downstream regions of homology). The FRT-flanked *erm* gene was utilized as integrative cassette and the anticipated sizes of the wild-type *E. mundtii* ST4SA and integrant loci are shown in Figs 11A and 11B. The deletion mutant isolation protocol was followed to enrich for recombinant cells that have undergone the desired double-crossover recombination event (refer to 'Materials and Methods' and Fig. S8). *Enterococcus mundtii* ST4SA *srtA*::*frt-erm* mutants were successfully isolated that resulted in the deletion of a 580 bp fragment of the 714 bp *srtA* ORF as confirmed by PCR and sequencing (Fig. 11C). Similarly, the expected band sizes of *E. mundtii* ST4SA *srtC*::*frt-erm* mutants were obtained as confirmed by PCR, sequencing and restriction digests, that resulted in the deletion of a 726 bp internal fragment of the 826 bp *srtC* ORF (Fig. 11D).

Fig. 11A.

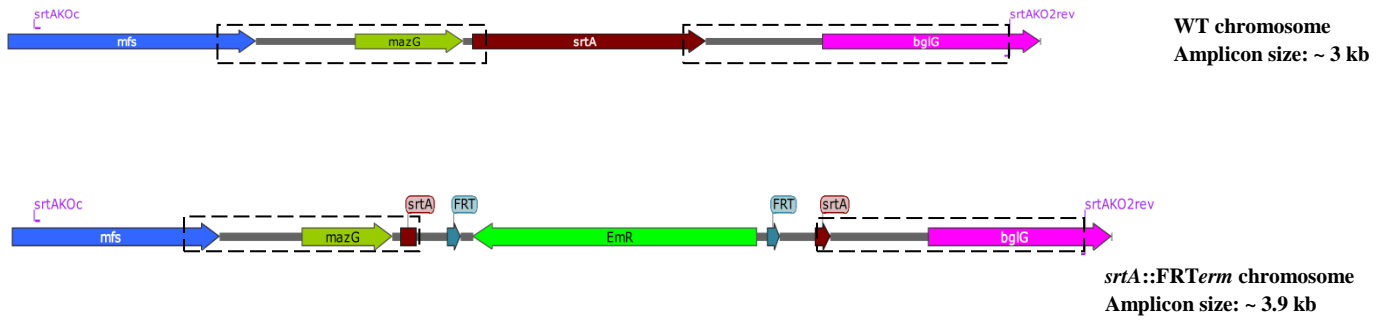


Fig. 11B.

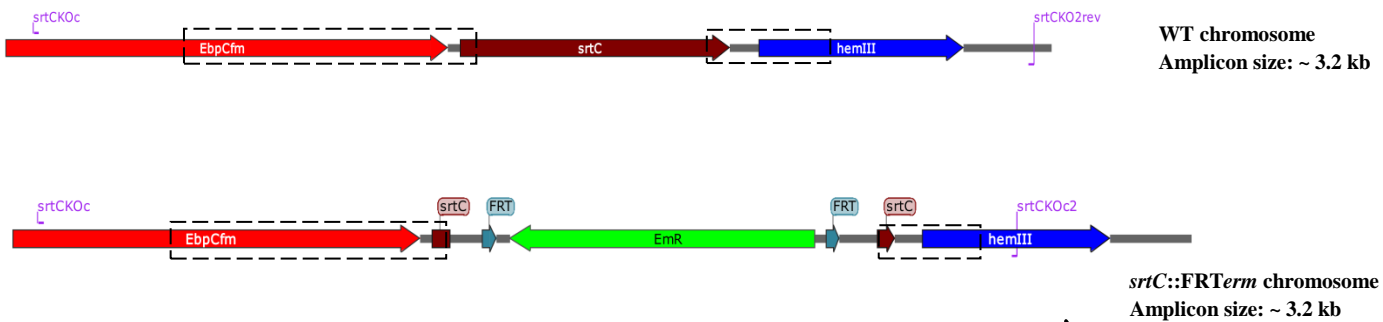
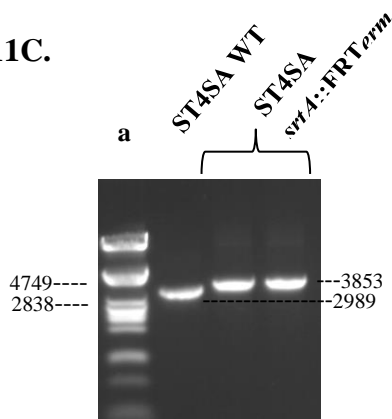


Fig. 11C.



D.

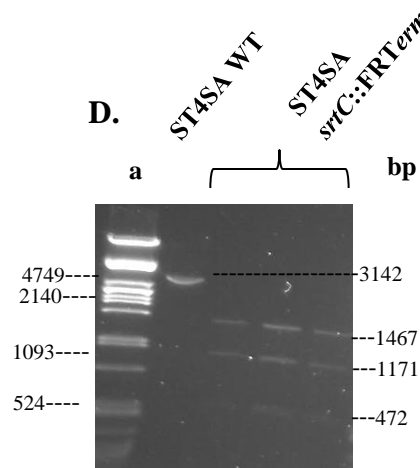


FIG. 11. Gene deletion and integration via homologous recombination into the genome of *E. mundtii* ST4SA at the *srtA* and *srtC* loci to create *E. mundtii* ST4SA *srtA::FRTerm* and *E. mundtii* ST4SA *srtC::FRTerm*. **(A)** Schematic representing the WT *E. mundtii* ST4SA *srtA* gene locus and the recombinant *srtA* deletion and FRT-*erm* integration site. Boxed regions show the upstream and downstream regions of homology (~ 1 kb) on the WT chromosome and the recombinant *srtA::FRTerm* locus. Cells harboring the *srtA* KO vector were selected on Cm and Em, followed by nisin induction for MazF toxin expression to select for mutants that have lost the plasmid backbone bearing *cat* and *mazF* genes. Double crossover mutants were selected and screened by PCR using the indicated primer combinations. **(B)** Schematic representing the WT *E. mundtii* ST4SA *srtC* gene locus

and the recombinant *srtC* deletion and FRT-*erm* integration site. Boxed regions show the upstream (~ 1 kb) and downstream (~ 0.4 kb) regions of homology on the WT chromosome and the recombinant *srtC::FRTerm* locus. *srtC* deletion and integration mutants were isolated as described in Fig. 11A. (C) PCR amplification of WT and *srtA* deletion and insertion mutants using the primer pair indicated in panel A. (a) Lambda DNA digested with *PstI* (NEB). Amplicons from one WT and two *srtA::FRTerm* insertion mutant colonies are shown. (D) PCR amplification of WT (3151 bp) and *srtC* deletion and insertion mutants (3183 bp) using the primer pairs indicated in panel B, followed by restriction digestion of the amplicons with *XbaI*. The WT amplicon contained one *XbaI* restriction site, while the *srtC* mutant amplicons contained three *XbaI* restriction sites. (a) Lambda DNA digested with *PstI* (NEB). Digested amplicons from one WT and three *srtC::FRTerm* insertion mutant colonies are shown.

To verify the loss of the *E. mundtii* ST4SA *sortaseA* aggregation substances' (AS) ability in *srtA* knockout mutants to attach secreted adhesion proteins to the bacterial cell surface, a cell clumping assay was performed. In wild-type *E. mundtii* ST4SA cells, AS expression lead to a marked clumping of bacterial cells to form large cell aggregates that settled at the bottom of the tube (Fig. 12A). In the *srtA* knockout strain lacking AS, the cell suspension remained turbid, thus indicating the loss of the cells' ability to form aggregates compared to the wild-type (Fig. 12B). In the tube containing *E. mundtii* ST4SA *sortaseC* knockout cells, the bacteria maintained the ability to form aggregates that settled at the bottom of the tube (Fig. 12C). These results confirmed that sorting of AS and subsequent cell clumping is dependent on *srtA* but not *srtC*.

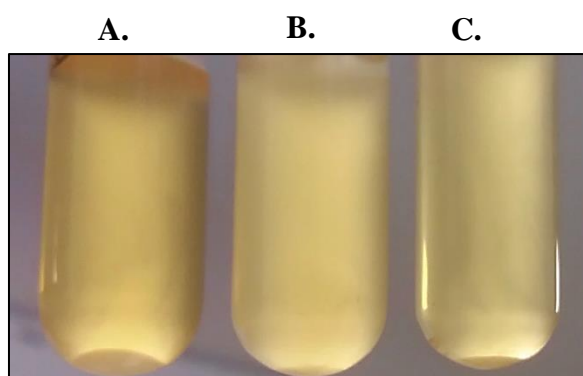


FIG. 12. *Enterococcus mundtii* ST4SA *sortase A* aggregation substance (AS) cell clumping assay. (A) MRS broth with the WT strain containing SrtA AS. (B) MRS broth containing the *E. mundtii* ST4SA *srtA::FRTerm* deletion mutant strain lacking SrtA AS. (C) MRS broth with *E. mundtii* ST4SA *srtC::FRTerm* deletion mutant strain containing SrtA AS.

Discussion

The functional genetic analysis of proteins that confer specific phenotypical properties in LAB is highly dependent on the application of an effective counter-selection marker system for the easy and efficient isolation of chromosomally- or plasmid-located allelic exchange mutants (11, 28-29). While the use of replicative plasmids to study protein functions is easy to implement, multi-copy plasmids are not suited for *in vitro* or *in vivo* models where antibiotic selection for plasmid maintenance is not possible (12). The integration or deletion of genes at any specified locus circumvents antibiotic selection related issues. To date, most of the techniques used for the isolation of homologous recombination mutants in LAB are limited by a lack of broad applicability amongst different LAB species, their inability to target specific gene loci, availability of suitable antibiotic resistance markers and the isolation of reversible single-crossover mutants (14, 15, 16, 30-32). Counter-selection markers are invaluable for the construction of stable double-crossover mutants, especially in probiotic LAB, where the mechanisms by which they exert their beneficial effects on the consumer can be studied by reverse genetic analysis. Reverse genetics is a powerful tool that can be used to study gene function, in which a gene of interest is inactivated to study the resulting effects on the microorganism in a particular environment (28, 53). Nevertheless, the identification and optimization of suitable counter-selection markers is a challenging and laborious task (28).

In this study, a method was developed to easily and efficiently isolate stable irreversible double-crossover mutants in *L. plantarum* 423 and *E. mundtii* ST4SA. This method is based on the use of the *E. coli mazF* toxin gene, an mRNA interferase, as a counter-selection marker that has been used successfully in *Bacillus subtilis* and *Clostridium acetobutylicum* (38-39). The *mazF* gene was placed under the control of the PnisA nisin-inducible promoter that is strictly associated with the *nisR* and *nisK* nisin signal regulatory genes to form the pNZmazFnisRK destination plasmid (40). A major advantage of the nisin-induction system (NICE) over other inducible gene expression systems, is the tightly controlled gene expression that has been used to produce large amounts of enzymes for food, medical or technical applications (54-55). Compared with the use of the lactose-inducible promoter and its divergent regulator as described by Al-Hinai and co-workers (39), results in this study have shown that MazF expression is tightly controlled by the nisin-inducible promoter. This rules out any potential MazF-induced premature cell growth arrest as a result of promoter leakiness. In this study, attempts to use the *L. plantarum* 423 lactose-inducible promoter and

its divergent regulator as an inducible expression system showed it to be poorly regulated (unpublished data).

Growth conditions were successfully optimized for the *mazF* counter-selection gene to promote the death of cells harbouring *mazF* upon induction of the PnisA promoter with nisin. The approach of this method ensures that transformants that have undergone only a single event of homologous recombination (thus retaining a copy of the plasmid-located counter-selection marker in the chromosome), are eliminated in the presence of nisin-induced MazF toxin. The method was used to construct deletions within the ORFs of specific genes, to introduce a gene of interest, to construct an unmarked mutation and to realize gene deletion mutants using small regions of homology. In the first case, bacteriocin gene deletion mutants of *L. plantarum* 423 and *E. mundtii* ST4SA were constructed, while simultaneously inserting the large 1.6 kb *ffluc* firefly luciferase reporter gene. The *L. plantarum* 423 *plaA* bacteriocin KO plasmid was designed to delete the majority of the *plaA* ORF while ensuring that none of the adjacent genes would be affected. In a previous study (56), the pPLA4 *plaA* gene-encoding plasmid was cured from the *L. plantarum* 423 wild-type strain to produce bacteriocin-deficient mutants. While their approach proved successful, the loss of the whole plasmid may be undesirable because of the consequent loss of all bacteriocin-adjacent genes. The *E. mundtii* ST4SA *munA* bacteriocin KO plasmid was designed to delete 18 bp of the *munA* ORF. The bacteriocin gene deletions of both *L. plantarum* 423 and *E. mundtii* ST4SA resulted in the loss of bacteriocin production, as was confirmed against *L. monocytogenes* EGDe. Supernatants isolated from the bacteriocin-deficient mutant strains lacked the ability to form clear inhibition zones as compared to the wild type derivatives. Bioluminescence imaging revealed that the integrated *ffluc* gene was actively expressed in each of the bacteriocin KO mutants. Strong bioluminescence emission was detected from colonies on MRS agar plates, indicating the functional expression of the Ffluc protein at the integrated loci. These results demonstrate the feasibility and applied utility of the newly developed counter-selectable marker system.

Desired double-crossover mutants are usually isolated using an antibiotic resistance gene as integrative selective marker (14). However, repeated manipulations of any bacterial chromosome can only be achieved by the generation of markerless integration mutants due to a limited availability of suitable antibiotic resistance markers. An easy-to-implement marker recycling system for LAB allows microbiologists to genetically engineer LAB strains for biotechnological production processes. In this study, the *S. cerevisiae* FLP-FRT

recombination system was modified for application in LAB. The FLP-FRT recombination system is present in most yeast strains and is encoded by the 2 μ m (6.4 kb) plasmid (57-58). Two 34 bp FLP recognition targets (FRT) are necessary for successful excision of an integrated fragment by FLP recombinase. The yeast FLP-FRT system has been successfully applied in several pathogenic and non-pathogenic Gram-negative and Gram-positive bacteria (38-39, 59-61). The *L. plantarum* 423 *aap* mucus adhesion gene was targeted for inactivation with the insertion of an FRT-flanked *erm* resistance gene (62). Once FRT-*erm* gene insertion was confirmed, a two-step process was followed for marker recycling. In the first step, FLP recombinase activity was induced by transformation with plasmid pNZFLPasRNA_repA to produce unmarked *aap* KO mutants. Secondly, once excision of the *erm* gene was confirmed, loss of the pNZFLPasRNA_repA plasmid was stimulated by a 350 bp *repA* antisense RNA (asRNA) transcript placed under the control of the PnisA nisin-inducible promoter. The *repA* asRNA is a single-stranded RNA molecule that is complementary to the *repA* mRNA molecule of pNZ8048 derived vectors. The *repA* asRNA inhibits translation of the *repA* origin of replication (*ori*) by binding to the first 350 bp of the complementary *repA* mRNA transcript (63). As a result, the strain was cured of the *repA* *ori*-containing plasmid via *repA* *ori* protein synthesis inhibition. Several studies have used asRNA successfully to modulate the expression of specific genes or to prevent the proliferation of bacteriophages in Gram-positive bacteria, including many LAB sp. (64-70). In a previous study (39), numerous days of sub-culturing and replica plating were required to isolate unmarked mutants via FLP-FRT excision of an antibiotic resistance gene. The system developed in this study requires less effort (days) for the isolation of unmarked mutants based on the strength and specificity of the NICE system. The resulting *aap* KO unmarked strain, free of antibiotic selection markers may be used for further genetic manipulations of the *L. plantarum* 423 chromosome using the newly developed method.

To further demonstrate the strength of the newly developed system, gene deletion mutants of the *E. mundtii* ST4SA *srtA* and *srtC* genes were generated using homologous arms containing <60 bp regions (5' and 3') homologous to the target genes. This approach ensured that none of the adjacent genes were affected while deleting the majority of each target. Emphasis is placed on the introduction of new restriction sites in homologous arm regions for the insertion of integration cassettes to ensure optimal cloning of integrative cassettes. This strategy ensures the inactivation of smaller gene targets (<200 bp), that may not contain sufficient restriction sites to facilitate the cloning of integrative genes, usually antibiotic

resistance genes, flanked by homologous regions. Previous studies reported that the rate of recovery of homologous recombinants significantly decreases when the size of homologous regions is less than 70 bp in *B. subtilis* (71), less than 100 bp in *Cryptococcus neoformans* (72) and less than 200 bp in mammalian cells (73). Notwithstanding this, the technique used in this study ensures the isolation of recombination mutants by using small regions (<60 bp) of homology included in homologous arms also containing sequences adjacent to the target genes.

In conclusion, the method described in this paper is easy to implement, highly efficient and can be used to manipulate the chromosomes of *Lactobacillus* and *Enterococcus* spp. Furthermore, the strategy is well adapted for use in other LAB sp. due to the broad applicability of the nisin expression system. This provides a unique opportunity to study the role of specific probiotic LAB genes in complex environments using reverse genetics analysis. In addition to the efficient deletion or integration of genes at any defined loci, the use of FLP/FRT recombination system provides marker recycling for further manipulations of LAB strain chromosomes.

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Chapter 4

**Development of a Novel Selection/Counter-selection
System for Efficient Isolation of Double-crossover
Homologously Recombined Mutants in Lactic Acid
Bacteria, Enabling Directed Chromosomal Gene Deletions
and DNA Integrations**

Supplementary Material

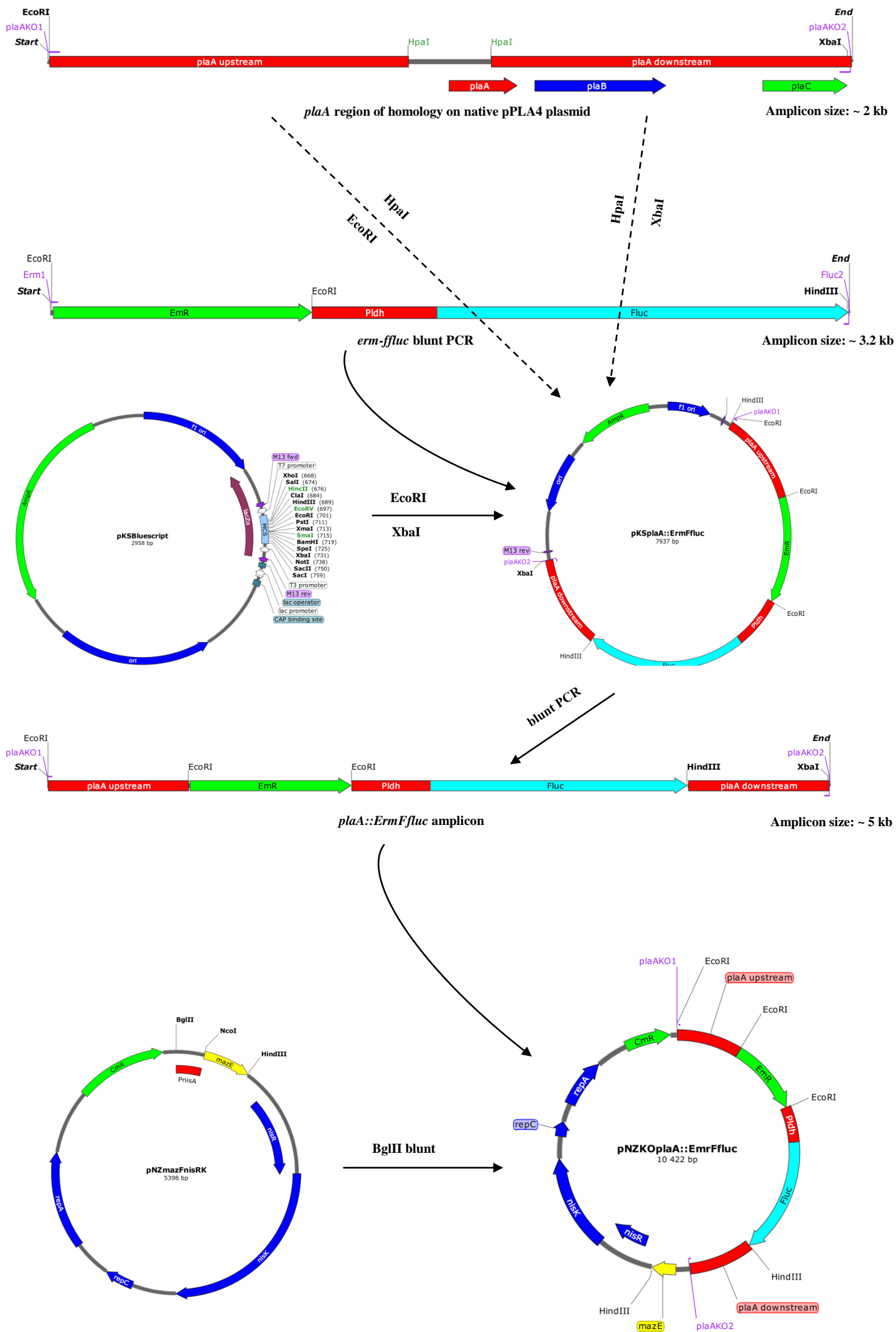


FIG. S1. Schematic representing the construction of the pNZKO $\text{plaA}::\text{ErmFfluc}$ integrative plasmid containing the *erm* gene and two flanking sequences of the *plaA* bacteriocin gene from *L. plantarum* 423. Relevant genes, restriction sites and PCR primers are shown. For details refer to “Materials and Methods”.

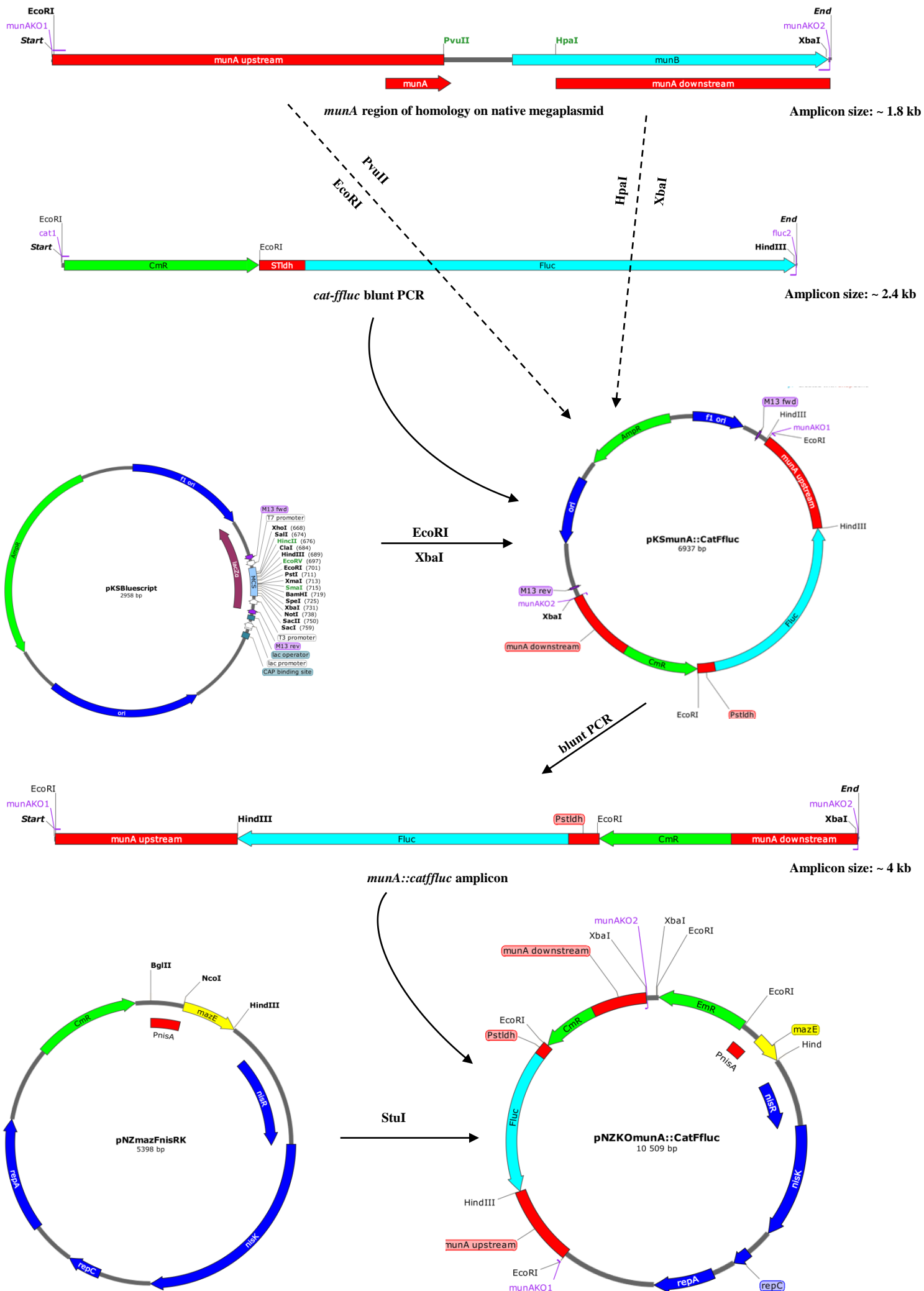
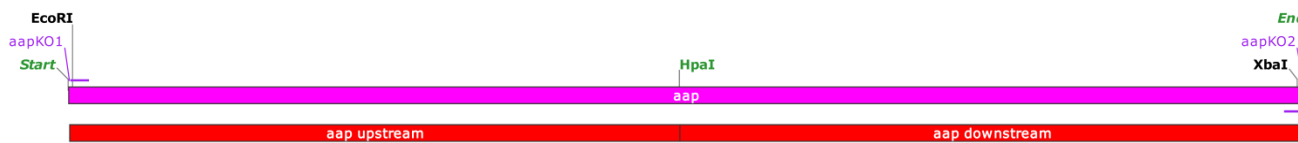
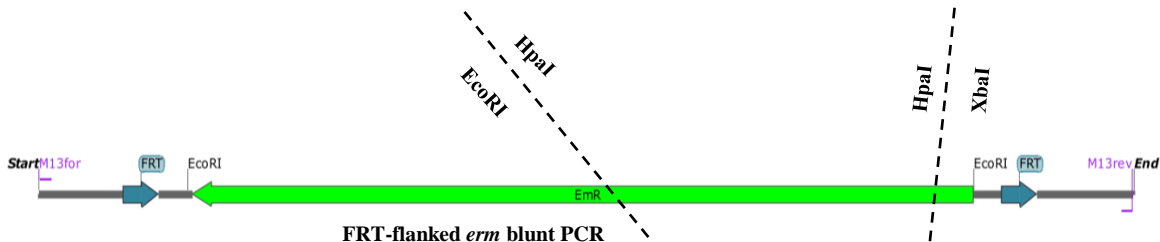


FIG. S2. Schematic representing the construction of the pNZKOMunA::CatFfluc integrative plasmid containing the *cat* gene and two flanking sequences of the *munA* bacteriocin gene from *E. mundtii* ST4SA. Relevant genes, restriction sites and PCR primers are shown. For details refer to “Materials and Methods”.

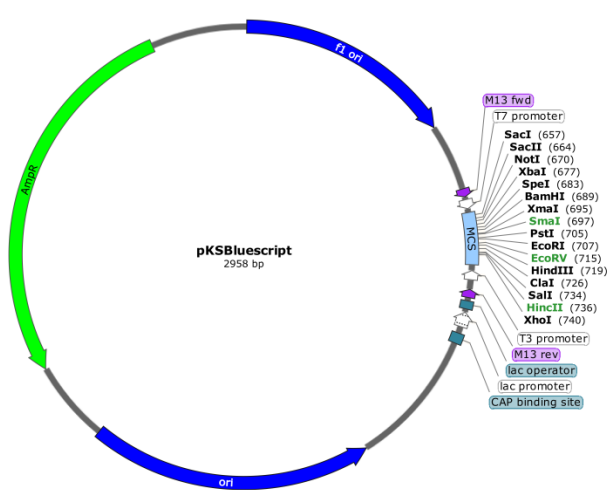


aap gene internal region of homology on WT chromosome

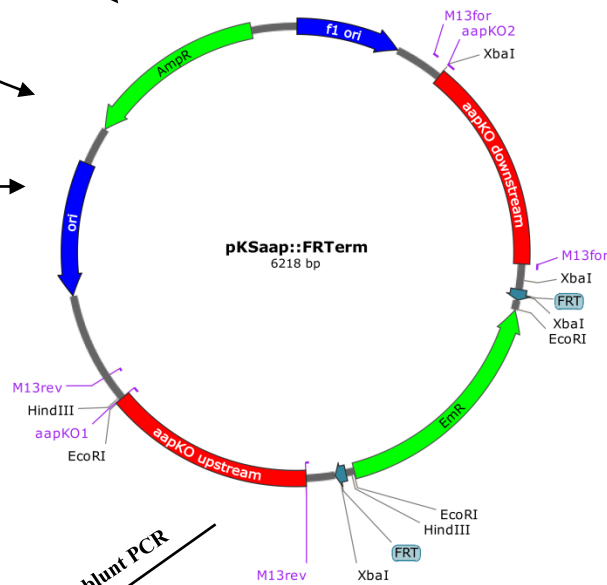
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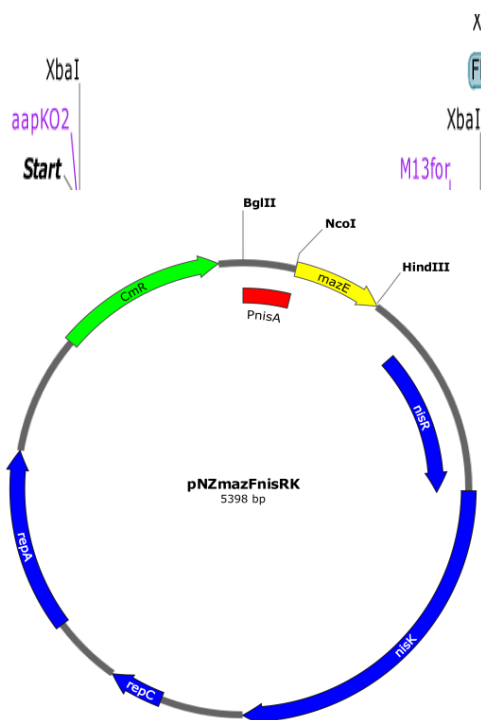
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EcoRI
XbaI

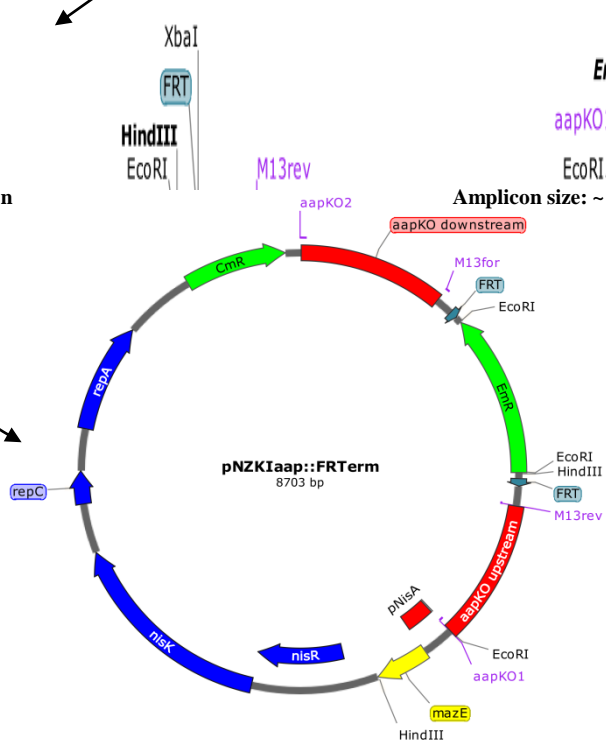


blunt PCR



aap::FRTerm amplicon

BglII, blunt



Amplicon size: ~ 3.3 kb

FIG. S3. Schematic representing the construction of the pNZKIaap::FRTerm integrative plasmid containing the *erm* gene and two flanking sequences of the *aap* mucus adhesion gene from *L. plantarum* 423. Relevant genes, restriction sites and PCR primers are shown. For details refer to “Materials and Methods.

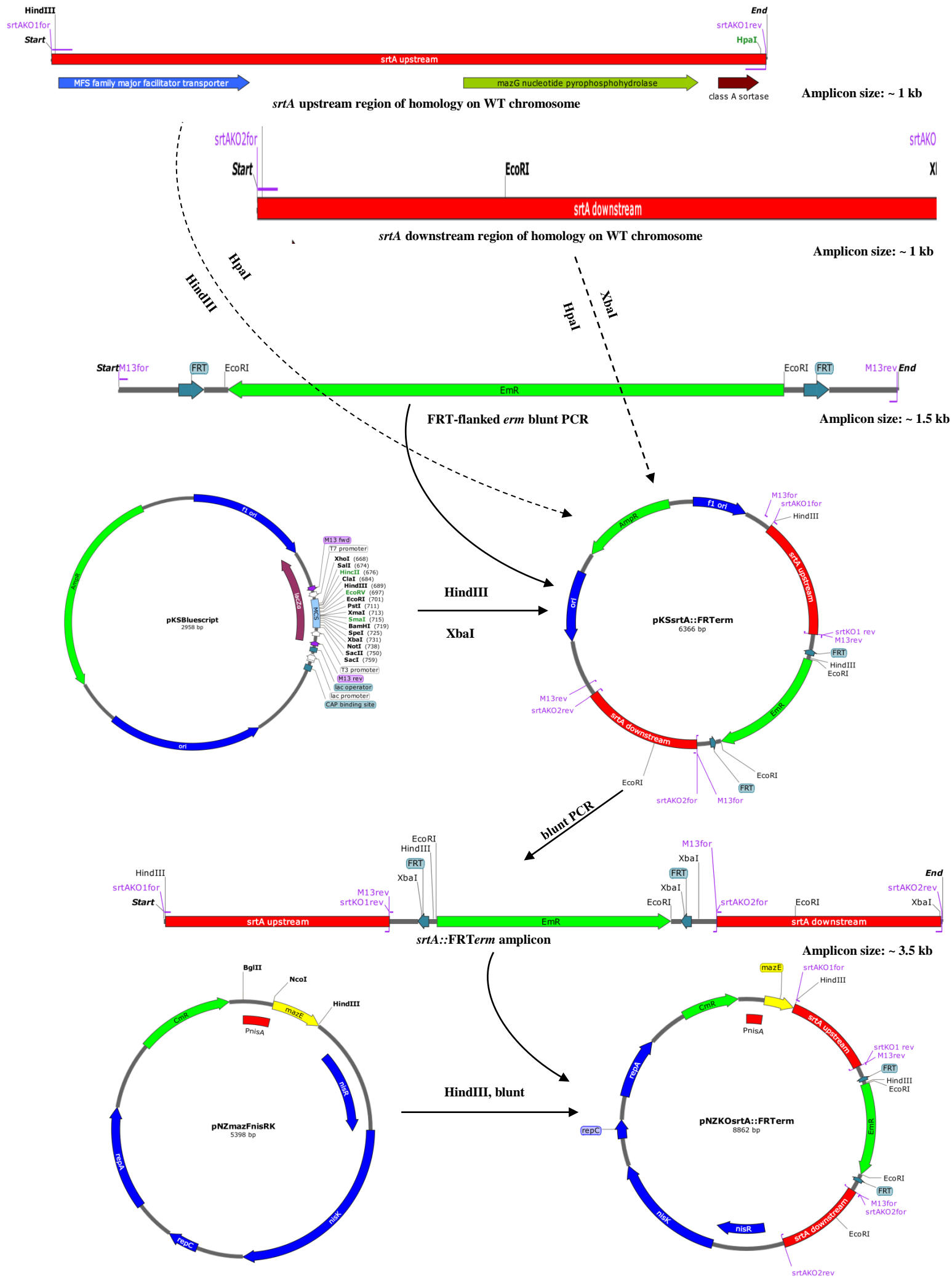


FIG. S4. Schematic representing the construction of the pNZKOsrA::FRTerm integrative plasmid containing the *erm* gene and two flanking sequences of the *srtA* cell wall adhesion associated gene from *E. mundtii* ST4SA. Relevant genes, restriction sites and PCR primers are shown. For details refer to “Materials and Methods”.

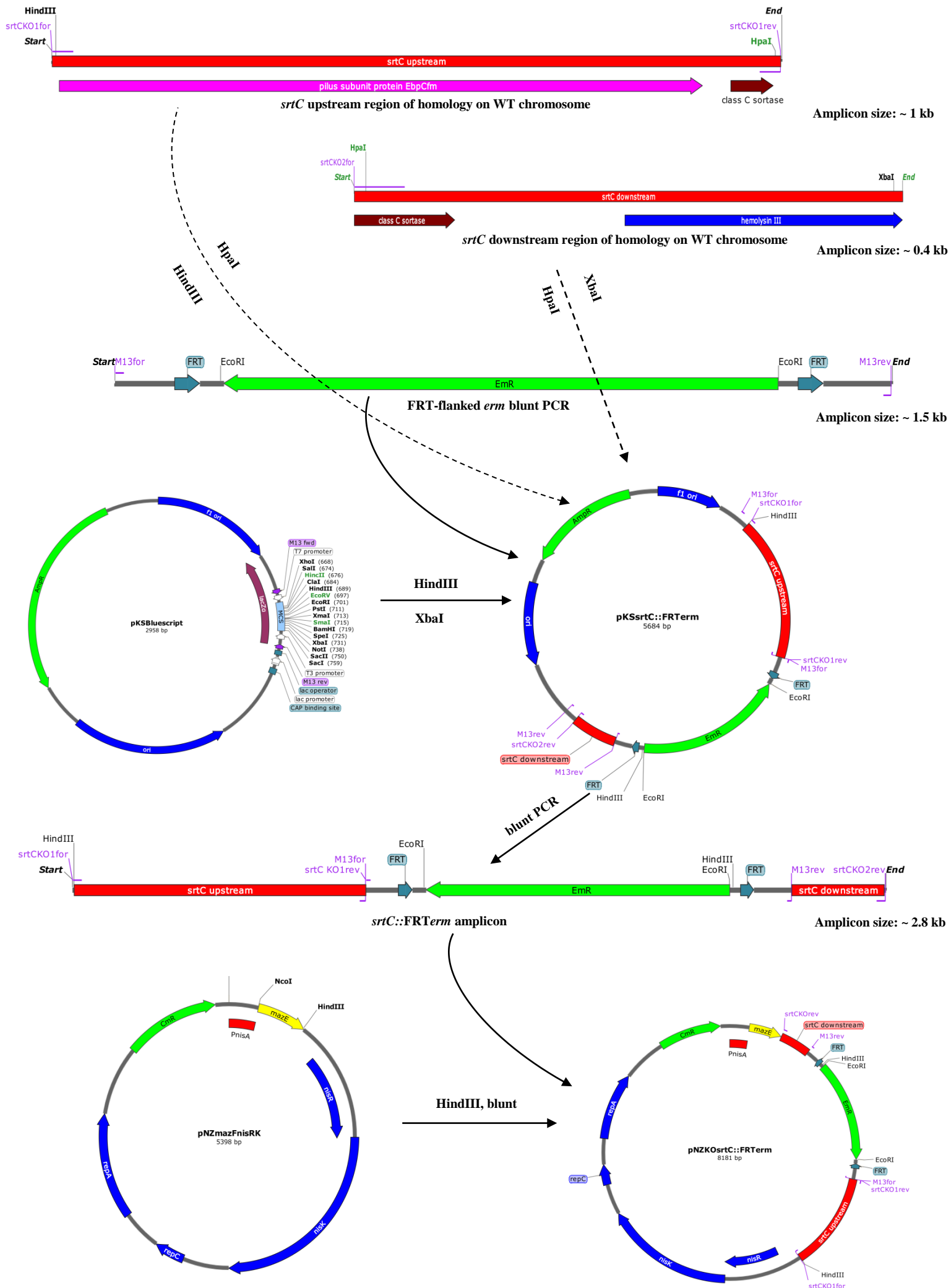


FIG. S5. Schematic representing the construction of the pNZKO_{srtC}::FRTerm integrative plasmid containing the *erm* gene and two flanking sequences of the *srtC* cell pilus associated gene from *E. mundtii* ST4SA. Relevant genes, restriction sites and PCR primers are shown. For details refer to “Materials and Methods”.

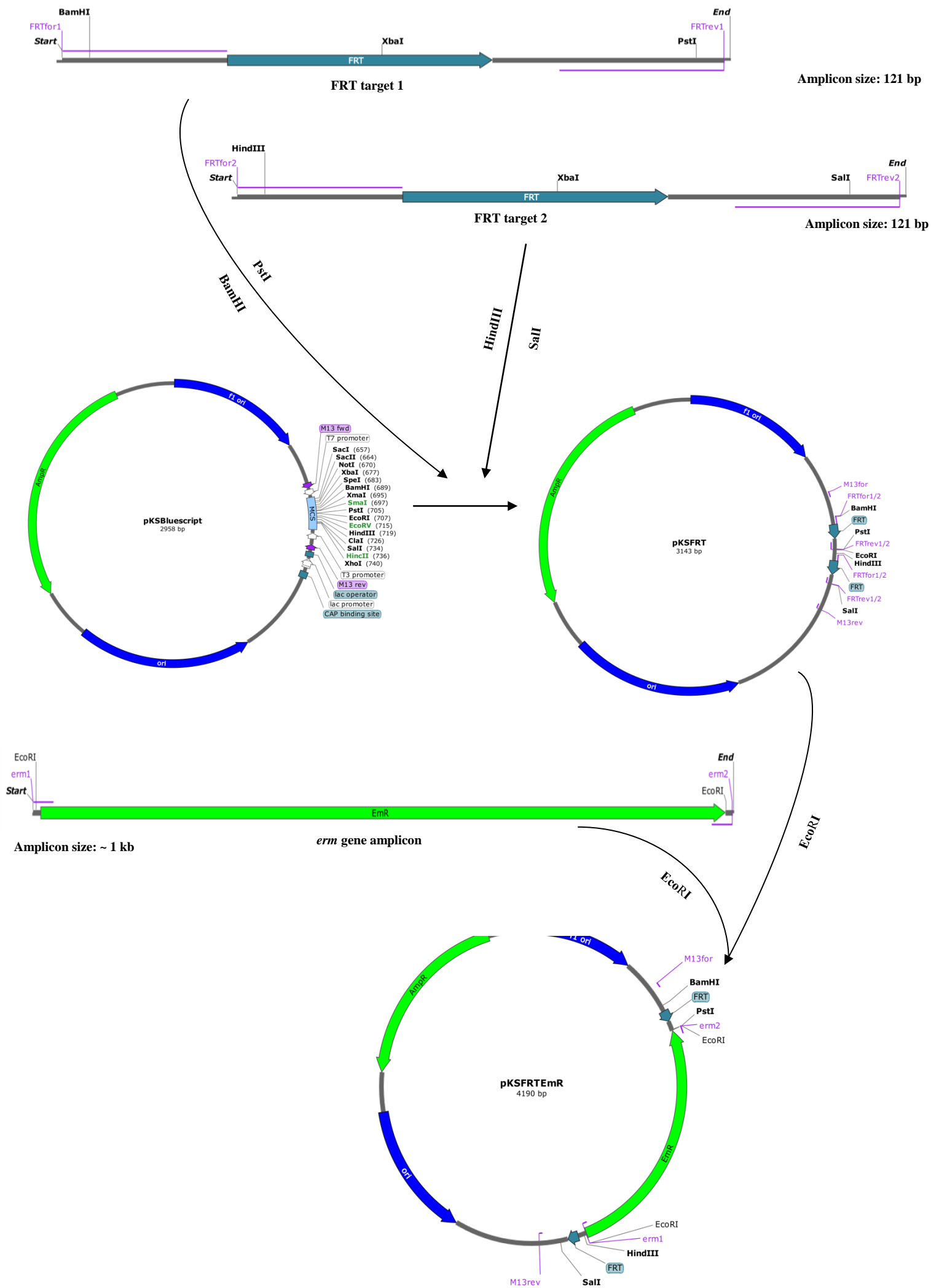


FIG. S6. Schematic representing the construction of plasmid pKSFRTerm containing the FRT-flanked *erm* gene. Relevant genes, restriction sites and PCR primers are shown. For details refer to “Materials and Methods”.

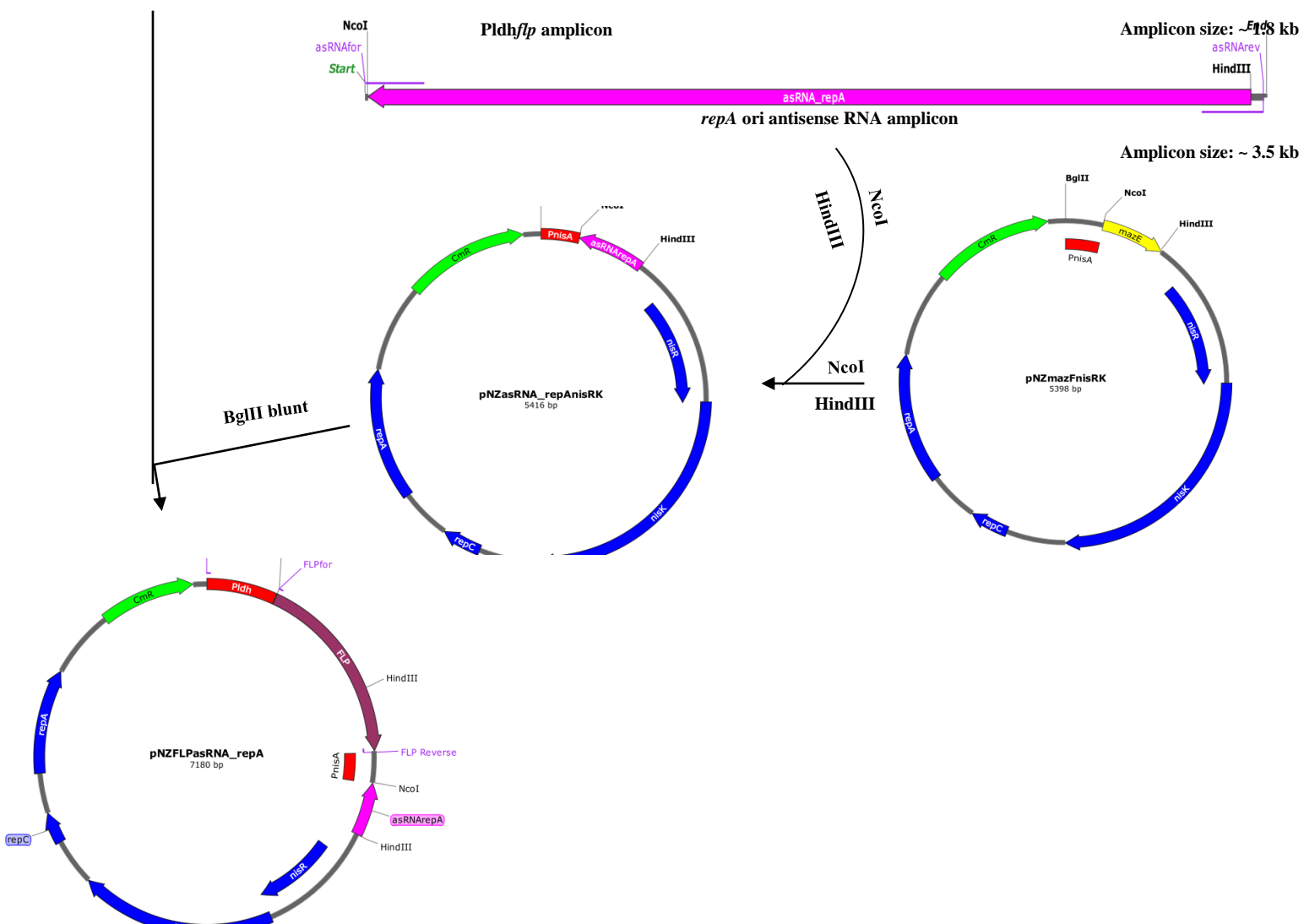
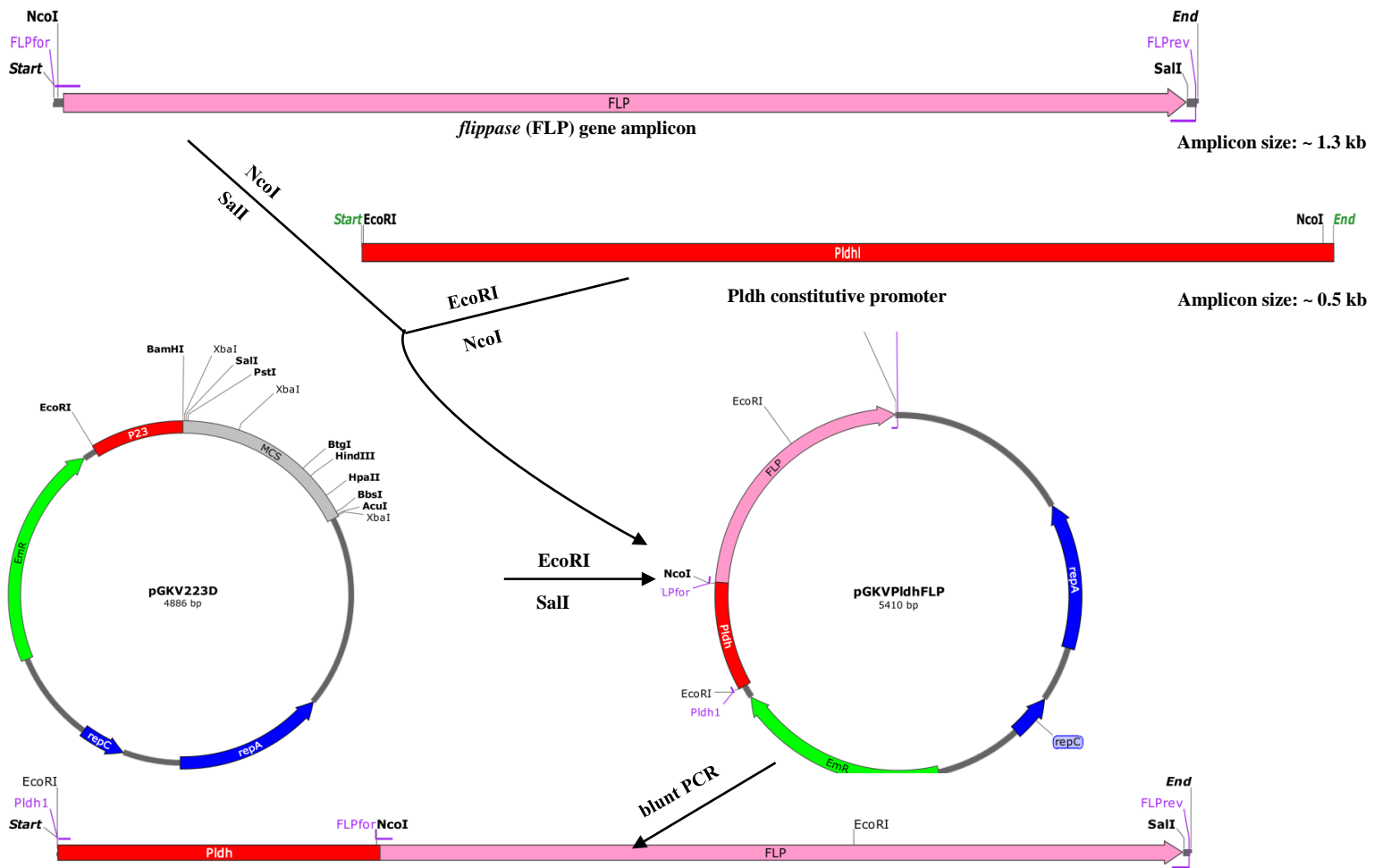


FIG. S7. Schematic representing the construction of plasmid pNZFLPasRNA_repA containing the *flp* recombinase gene and a *repA* asRNA fragment. Relevant genes, restriction sites and PCR primers are shown. For details refer to “Materials and Methods”.

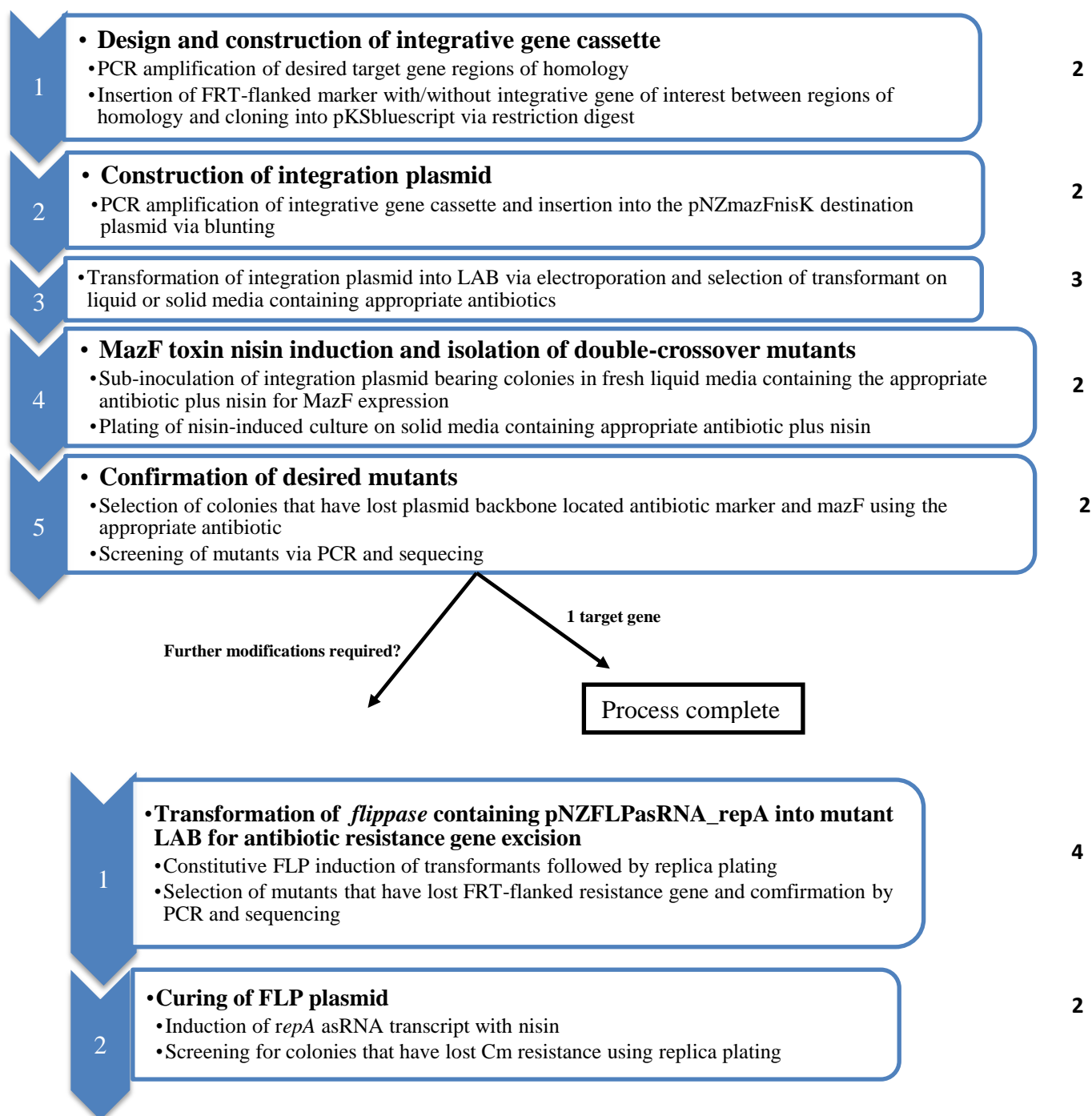


FIG. S8. A workflow diagram showing the step-by-step design and protocol of the newly developed counterselection method for the deletion and inactivation of LAB genes or to introduce genes of interest followed by resistance marker recycling.

STELLENBOSCH UNIVERSITY

Chapter 5

Anti-infective Gastrointestinal Properties of *Lactobacillus plantarum* 423 and *Enterococcus mundtii* ST4SA, as Determined in Mice Infected with *Listeria monocytogenes* EGDe

Anti-infective Gastrointestinal Properties of *Lactobacillus plantarum* 423 and *Enterococcus mundtii* ST4SA, as Determined in Mice Infected with *Listeria monocytogenes* EGDe

Abstract

Intestinal infectious diseases, and in particular those caused by enteric pathogenic bacteria, are a major cause of morbidity and mortality. Probiotics play an important role in maintaining a healthy and stable intestinal microbiota, primarily by preventing infection. Probiotic lactic acid bacteria (LAB) are known to be inhibitory to many bacterial enteric pathogens, including antibiotic-resistant strains. Whilst the positive role that probiotics have on human physiology, specifically in the treatment or prevention of specific infectious diseases of the gastrointestinal tract (GIT) is known, the precise mechanistic basis of these effects remains a major research goal. In this study, molecular evidence to underpin the protective and anti-listerial effect of *Lactobacillus plantarum* 423 and *Enterococcus mundtii* ST4SA against orally administered *Listeria monocytogenes* EGDe in the GIT of mice is provided. *Lactobacillus plantarum* 423 and *E. mundtii* ST4SA significantly reduced *L. monocytogenes* EGDe bioluminescence and viable bacterial cell numbers. Bacteriocin-negative mutants of *L. plantarum* 423 and *E. mundtii* ST4SA failed to exclude *L. monocytogenes* EGDe from the GIT, confirming bacteriocin production as an anti-infective mediator. *Lactobacillus plantarum* 423 and *E. mundtii* ST4SA failed to reduce *Listeria* bioluminescence or viable bacterial cell numbers in the GIT when mice were administered with *L. monocytogenes* EGDe strains expressing the bacteriocin immunity proteins. Furthermore, the exclusion of *L. monocytogenes* EGDe from the GIT was reduced when mice were administered with *L. plantarum* 423 and *E. mundtii* ST4SA adhesion gene knockout strains. These results substantiate our understanding of the functional attributes of probiotics currently available to consumers and the improvement of future probiotic products.

Introduction

The gastrointestinal tract (GIT) is inhabited by the largest microbial community in the human body and represents a highly complex ecosystem composed of mainly bacteria (1). The intestinal microbiota is essential in the breakdown of food and faecal residue, but also plays a major role in the protection of epithelial mucosa, stimulation of the immune system and formation of a protective barrier against pathogens (2). The intestinal microbiome is thus a ‘virtual organ’ that performs a number of significant biochemical and physiological functions (3).

Imbalances of the microbiota (for example following antibiotic treatment) have been associated with several disease states, including increased susceptibility to pathogens such as *Clostridium difficile*, irritable bowel syndrome, diabetes, allergies, acute gastro-enteritis and obesity (4-6). Significant differences in the microbiota composition of healthy individuals compared to patients with various clinical conditions were revealed in a study by Khor and co-workers (7). Intervention studies using live probiotic cultures aimed at restoring microbiota composition impact health in preventing or ameliorating gastrointestinal infections (3, 8, 9). Most probiotics are composed of lactic acid bacteria (LAB) that prevent enteric infections caused by *C. difficile*, *Helicobacter pylori*, vancomycin resistant enterococci (VRE), *Campylobacter*, *Salmonella enterica* serovar Typhimurium and *Listeria monocytogenes* (6, 10-15). Hence, in the last few decades, the use of probiotic LAB for health benefits by introducing transiently colonizing probiotic strains has grown significantly (3).

Listeria monocytogenes, the etiological agent of listeriosis, is a ubiquitous Gram-positive bacterium that can cause life-threatening systemic disease by mainly affecting immunocompromised individuals (16). The cells can grow at low temperatures, presenting a high risk for the contamination of refrigerated foods (17). *Listeria monocytogenes* can also proliferate in harsh environments, such as those with low acidity, low water activity and high NaCl levels (18). Upon oral infection, *L. monocytogenes* crosses the intestinal barrier and may infect the GIT, liver, gallbladder, brain and spinal cord (2, 19-22). *Listeria monocytogenes* may also affect the central nervous system and cause meningitis, brain abscess or bacteraemia (19). Treatment of listeriosis includes the administration of antibiotics such as erythromycin, ampicillin, gentamicin and vancomycin (23). Continuous use of these antibiotics increases the risk that infectious pathogenic strains may develop resistance. The

use of probiotics to treat or prevent gastric infections could potentially decrease the use of antibiotics and prevent the emergence of antibiotic-resistant strains.

Several *in vitro* and *in vivo* studies using the murine listeriosis model have shown that LAB have antagonistic activities against the growth of *L. monocytogenes* (2, 24-27). Our own studies have shown that the probiotics *Lactobacillus plantarum* 423 and *Enterococcus mundtii* ST4SA successfully excluded *L. monocytogenes in vivo* (15). However, the exact mode of action remains uncertain (28). Only a few scientific papers provide evidence to support the inhibitory activities of probiotic LAB on gastrointestinal pathogens (29). It is likely that the probiotic-pathogen inhibitory effects in the GIT are underpinned by diverse, poorly understood mechanisms. Probiotics may repress the growth of pathogens by producing anti-inflammatory molecules (30) or antimicrobial compounds such as organic acids, ammonia, hydrogen peroxide and antimicrobial peptides (bacteriocins) (29, 31-33). Probiotic bacteria compete with pathogens for nutrients and receptor sites on intestinal mucus and epithelial cells (34). In all of these cases, probiotic bacteria execute their beneficial effects by interacting with enteric pathogens to prevent intestinal colonization of the gut and subsequent epithelial invasion, infection and the occurrence of disease.

Bioluminescence imaging (BLI) is used for real-time, non-invasive, monitoring of bacterial cells that are genetically tagged with light-producing luciferase enzymes (35). The light produced by cells tagged with luciferase is detectable through the tissues of live small animals using an *in vivo* imaging system (IVIS). Numerous studies have demonstrated the use of bioluminescent bacteria genetically tagged with bacterial or eukaryotic luciferase genes to monitor real-time progression of infection *in vivo* (15, 36-39). The BLI technique is based on the detection and quantification of visible light produced by luciferases in the presence of ATP and oxygen (37). Bioluminescence can thus only be detected if cells are metabolically active. The application of the murine listeriosis model using *L. monocytogenes* tagged with the bacterial lux operon, revealed strong bioluminescence signals from within the intestinal tract (15). The BLI technique thus provides a way of monitoring the colonization of *L. monocytogenes* in the mouse GIT while at the same time reducing the amount of animals required to make significant experimental conclusions.

In this study, the role of bacteriocins and adhesion proteins in the protective effect of the probiotics *L. plantarum* 423 and *E. mundtii* ST4SA against mice orally administered with *L. monocytogenes* EGDe are investigated. Both strains constantly produce potent class IIa

bacteriocins active against several Gram-positive enteric pathogens, including *L. monocytogenes* (40, 41). Both strains line the gut by using adhesion proteins and displace *Enterococcus faecalis* and *Clostridium sporogenes* in competitive exclusion experiments (42). This study investigated involvement of bacteriocins in the competitive exclusion of *L. monocytogenes* EGDe from the GIT of mice using non-bacteriocin producing mutants of both probiotic strains. Plantaricin 423 and mundticin ST bacteriocin immune strains of *L. monocytogenes* EGDe were constructed to confirm the role of the respective bacteriocins in the *in vivo* anti-listerial effect. The involvement of adhesion proteins in the gastrointestinal probiotic anti-listerial effect was also investigated. The capacity of a *L. plantarum* 423 mutant lacking the *aap* adhesion associated gene and *E. mundtii* ST4SA lacking the Sortase A and Sortase C genes associated with the anchoring of cell surface proteins to the cell surface, to competitively exclude *L. monocytogenes* EGDe from the GIT was tested. The intestinal colonization and exclusion of *L. monocytogenes* from the GIT were recorded using bioluminescence imaging monitored with the IVIS.

Materials and Methods

Animals used

Approval to conduct research on animals was obtained from the ethics committee of Stellenbosch University (reference number SU-ACU-2017-0206-454). Eight-week-old non-specific-pathogen-free (non-SPF) female BALB/c mice were used for challenge studies. Animals were fed a standard rodent diet and kept under controlled environmental conditions (12 h light/dark cycles, 20-22°C). At least nine mice were used per bacterial strain. Animal procedures were performed according to the Stellenbosch University ethical guidelines.

Bacterial strains, media, growth conditions and preparation of dosages

Lactobacillus plantarum 423 and *E. mundtii* ST4SA strains were grown in MRS broth (Biolab Diagnostics, Midrand, South Africa) without shaking, or on MRS agar, at 30°C. When necessary, 10 µg/ml chloramphenicol (Cm), or erythromycin (Em) were added to MRS medium for recombinant *L. plantarum* 423 and 5 µg/ml Cm, or Em for recombinant *E. mundtii* ST4SA. Before probiotic dosing of mice, 12-h old cultures were inoculated into freshly prepared MRS broth, grown to optical densities (OD_{550nm}) of 2.5 (*L. plantarum* 423) and 2.3 (*E. mundtii* ST4SA), harvested (8000 x g for 3 min) and washed with sterile

phosphate buffered saline (PBS) to remove residual media. Cell numbers were determined by plating onto MRS agar, supplemented with the appropriate antibiotic, and diluted with sterile PBS to 2×10^{10} CFU/ml.

Listeria monocytogenes EGDe, genetically labelled with an integrated bacterial lux operon of *Photobacterium luminescence*, was obtained from Bio-ware™ Microorganisms (Caliper Life Sciences, Hopkinton, MA, USA). *Listeria monocytogenes* EGDe is a lineage type II, serotype 1/2a strain and strains of this serotype are major contributors to the microorganisms-related foodborne outbreaks (43, 44).

Listeria monocytogenes EGDe was grown in Brain Heart Infusion medium (BHI, Biolab Diagnostics) at 37°C (supplemented with 7.5 µg/ml Cm or 10 µg/ml Em where required) on an orbital shaker (200 rpm). Before administration to mice, cell counts were determined by plating onto BHI agar supplemented with the appropriate antibiotic, and diluted with sterile PBS to 2×10^{10} CFU/ml.

Construction of recombinant *L. plantarum* 423 and *E. mundtii* ST4SA mutant strains

Lactobacillus plantarum 423 and *E. mundtii* ST4SA bacteriocin- and adhesion-gene knockout (KO) mutants were constructed as described previously (chapter 4). Briefly, an *erm* gene for Em resistance was stably integrated into the plantaricin 423 bacteriocin operon (at *plaA* gene locus) of *L. plantarum* 423 and a *cat* gene for Cm resistance into the *E. mundtii* ST4SA mundticin ST bacteriocin operon (at *munA* gene locus) via homologous recombination, yielding *L. plantarum* 423 *plaA::erm* and *E. mundtii* ST4SA *munA::cat*, respectively. *Lactobacillus plantarum* 423 *aap* adhesion gene-negative, and *srtA* and *srtC* adhesion protein related-negative mutants of *E. mundtii* ST4SA were constructed using the same protocol, to yield strains *L. plantarum* 423 *aap::erm*, *E. mundtii* ST4SA *srtA::erm* and *E. mundtii* ST4SA *srtC::erm*, respectively.

Construction of *L. monocytogenes* EGDe bacteriocin-resistant strains

The bacteriocin immunity proteins of *L. plantarum* 423 (*plaB*) and *E. mundtii* ST4SA (*munC*) were expressed in *L. monocytogenes* EGDe using the *Lactococcus lactis* pNZ8048 plasmid vector containing an *erm* gene for Em resistance. (45). The *plaB* and *munC* bacteriocin immunity proteins were placed under control of the strong constitutive *E. mundtii* ST4SA lactate dehydrogenase (*ldh*) gene promoter. Preparation of electro-competent cells, and

electroporation of *L. monocytogenes* EGDe, was carried out using the protocol described by Monk and co-workers (46). *Listeria monocytogenes* EGDe was transformed with plasmids pNZSTldhplaB and pNZSTldhmunC, yielding recombinant strains *L. monocytogenes* EGDe (pNZSTldhplaB) and *L. monocytogenes* EGDe (pNZSTldhmunC), respectively.

***In vitro* assessment of growth of *L. monocytogenes* EGDe in the presence of plantaricin 423 and mundtacin ST bacteriocins**

Wild-type (WT) *L. monocytogenes* EGDe, recombinant *L. monocytogenes* EGDe (pNZSTldhplaB) and *L. monocytogenes* EGDe (pNZSTldhmunC) were grown in BHI broth for 12 h. One millilitre of 12-h old cultures was used to inoculate 40 ml BHI broth to a starting OD_{550nm} of 0.1. The 40 ml cultures were supplemented with either 10 ml MRS (control), sterile supernatant of the bacteriocin producer or bacteriocin-negative strains and incubated at 37°C for 9 h. Every hour the OD₅₅₀ reading for each 50 ml culture was measured. All experiments were done with three biological replicates. Bacteriocin activity on solid media was determined using the overlay lawn assay as described previously (47), but with the following modifications. The WT *L. monocytogenes* EGDe bacteriocin sensitive and recombinant bacteriocin-resistant strains were grown for 12 h and 100 µl liquid culture spread-plated onto a BHI agar plate. Cell-free supernatants (adjusted to pH 7.0 with NaOH) of actively growing (12 h) bacteriocin-producing, or bacteriocin-non-producing, strains were collected by centrifugation (8000 x g for 5 min). The supernatants were filter-sterilized by passage through a 0.22-µm-pore size cellulose acetate filter using a 5 ml syringe, 30 µl spotted into wells on BHI agar spread with WT, or bacteriocin-resistant *L. monocytogenes* EGDe, followed by incubation at 37°C for 24 h.

Dosing of mice

Ten groups of mice were administered a daily dose of LAB for six consecutive days. At least nine mice were used per LAB strain (4 x 10⁹ CFU per mouse). All cell suspensions were resuspended in 200 µl gavage buffer (0.2 M NaHCO₃ with 1%, w/v, glucose), pH 8.0. Mice in groups WT-423 and WT-ST4SA received WT *L. plantarum* 423 (pGKV) (containing pGKV223 empty vector) and *E. mundtii* ST4SA (pGKV) (containing pGKV223 empty vector), respectively. A placebo group (group PBS) of mice were administered 200 µl sterile PBS for six days. Mice in groups B-423 and B-ST4SA received bacteriocin-negative *L. plantarum* 423 *plaA::erm* and *E. mundtii* ST4SA *munA::cat*, respectively. Mice in group A-

423 received *L. plantarum* 423 *aap::erm*, mice in group A-ST4SA received *E. mundtii* ST4SA *srtA::erm* and mice in group C-ST4SA received *E. mundtii* ST4SA *srtC::erm*. On day 6, 4 h after administering LAB, the mice in each group were fed 4×10^9 CFU (per mouse) *L. monocytogenes* EGDe by oral gavage. Mice in groups IL-423 and IL-ST4SA were administered WT *L. plantarum* 423 and *E. mundtii* ST4SA, respectively, before oral administration with *L. monocytogenes* EGDe bacteriocin-resistant (4×10^9 CFU) variant strains on day 6.

Monitoring of *in vivo* and *ex vivo* bioluminescence

Bioluminescence imaging of *L. monocytogenes* EGDe in the GIT of infected mice was monitored using the Caliper *in vivo* imaging system (IVIS[®] 100, Caliper Life Sciences, Hopkinton, MA, USA), equipped with a cooled charge-coupled-device camera mounted on a light-tight specimen chamber. Bioluminescent *L. monocytogenes* EGDe in the GIT of mice was imaged transcutaneously in whole animals at 15 min, 30 min, 1 h, 1.5 h, 2 h, 3 h, 4 h, 5 h, 6 h and 24 h after oral administration. Before bioluminescence imaging, mice were anesthetized with 2% (vol/vol) isoflurane in O₂. A constant flow of isoflurane (1.5%, v/v) was administered throughout examination through a nose cone. Exposure in the IVIS was from 3 to 5 min, depending on the signal strength. Photons emitted from manually selected regions of interest (ROIs) were quantified using the Living Image software, version 3.0 (Caliper Life Sciences).

Recording of bacteria in faeces

Faecal samples were collected and weighed (100 mg) over a period of up to 48 h after oral administration with *L. monocytogenes* EGDe on day 6. The faecal pellets were homogenised after a few minutes in PBS for softening, vortexed for 5 min, serially diluted in the same buffer and plated onto MRS agar containing the appropriate antibiotic for enumeration of LAB strains. Cells of *L. monocytogenes* EGDe were selected by plating onto BHI agar supplemented with either Em or Cm. After a 24 h incubation period, the number of viable LAB and *L. monocytogenes* EGDe cells present per 100 mg faeces was determined. Bioluminescence of *L. monocytogenes* EGDe cells in faecal samples was recorded by transferring 300 µl of homogenised faeces to a 96-well microtiter tray for imaging using the IVIS.

Recording of bacteria in the GIT

Two mice from each group were euthanized by cervical dislocation at 4 h, 6 h, 24 h and 48 h after oral administration with *L. monocytogenes* EGDe for *ex-vivo* bioluminescence imaging of their digestive tracts. The digestive tracts were immediately resected, rearranged in a Petri-dish and examined for the presence of bioluminescent *L. monocytogenes* EGDe cells using the IVIS (refer to Fig. S1 for different sections of mouse GIT). Prior to imaging, each resected digestive tract was longitudinally opened with a sterile surgical blade for enhancement of the bioluminescent signal. For CFU counts in the digestive tracts, the small (duodenum, jejunum and ileum) and large (cecum and colon) intestinal tracts were homogenized separately in 3 ml sterile PBS, serially diluted and plated in duplicate onto BHI agar (for *L. monocytogenes* EGDe counts) or MRS agar (for LAB counts) supplemented with the appropriate antibiotics. Plates were incubated as described before for 24 h to 48 h and the number of viable cells determined in each section of the GIT.

Statistical Analysis

All data were analysed using GraphPad Prism (version 6.05) and statistical differences between groups were determined using the Kruskal-Wallis nonparametric test and two-way analysis of variance (ANOVA). Statistical differences are shown for each data set. Error was calculated as standard error of mean (SEM).

Results

In vitro effect of plantaricin and mundtacin on the growth of *L. monocytogenes* EGDe

Listeria monocytogenes EGDe is sensitive to the bacteriocins plantaricin 423 and mundtacin ST produced by *L. plantarum* 423 and *E. mundtii* ST4SA, respectively, as determined by well diffusion assay (Fig. 1A). Wild-type *L. monocytogenes* EGDe was inhibited by twofold dilutions of plantaricin 423 up to 1/64 (Fig. 1B), while *L. monocytogenes* EGDe, transformed with pNZSTldhplaB for bacteriocin immunity, showed a complete loss of inhibition at a dilution of 1/4 (Fig. 1C). *Listeria monocytogenes* EGDe transformed with pNZSTldhmunC, containing the gene coding for mundtacin ST immunity, was resistant to undiluted supernatants containing mundtacin ST (Fig. 1D). *Listeria monocytogenes* EGDe (pNZSTldhmunC) were inhibited by plantaricin 423, indicating that the immunity protein of mundtacin ST did not protect the cells against plantaricin 423 (Fig. 1D). Similarly, *L.*

monocytogenes EGDe (pNZSTldhplaB) were inhibited by mundtacin ST. Both *L. monocytogenes* EGDe (pNZSTldhplaB) and *L. monocytogenes* EGDe (pNZSTldhmunC) retained resistance to their respective bacteriocins after 7 days of culturing in non-selective media, indicating the stability of the autonomous plasmids in the host (not shown).

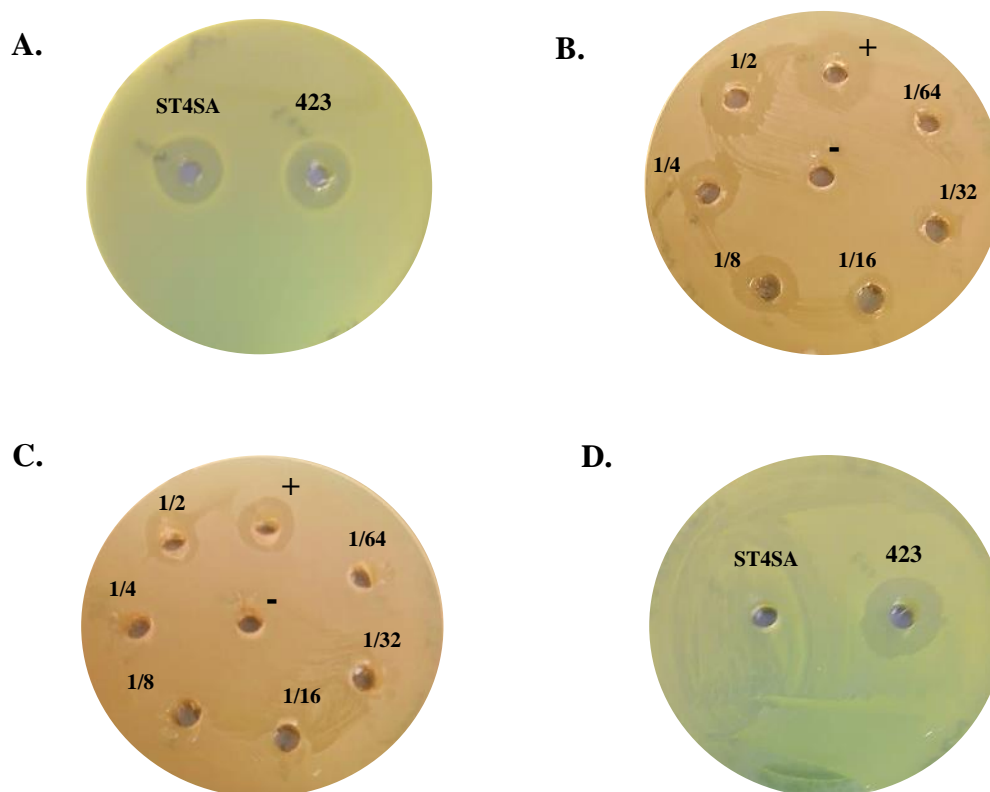


FIG. 1. Zones of inhibition on plates overlaid with *L. monocytogenes* EGDe. **A.** (ST4SA) Supernatant containing bacteriocin mundtacin ST isolated from WT *E. mundtii* ST4SA and (423) supernatant containing bacteriocin plantaricin 423 isolated from WT *L. plantarum* 423 spotted into wells on plate spread with WT *L. monocytogenes* EGDe. **B, C.** (+) Supernatant containing bacteriocin plantaricin 423 isolated from WT *L. plantarum* 423, (-) supernatant lacking plantaricin 423 bacteriocin isolated from *L. plantarum* 423 bac⁻ as negative control, and (1/2 to 1/64) supernatants containing bacteriocin plantaricin 423 diluted twofold spotted into wells on plates spread with **(B)** WT *L. monocytogenes* EGDe and **(C)** *L. monocytogenes* EGDe (pNZSTldhplaB). **D.** (ST4SA) Supernatant containing bacteriocin mundtacin ST isolated from WT *E. mundtii* ST4SA and (423) supernatant containing bacteriocin plantaricin 423 isolated from WT *L. plantarum* 423 spotted into wells on plate spread with *L. monocytogenes* EGDe (pNZSTldhmunC).

Loss of inhibition as determined by a cell growth assay in BHI broth for *L. monocytogenes* EGDe (pNZSTldhplaB) and *L. monocytogenes* EGDe (pNZSTldhmunC) expressing the plantaricin 423 and mundtacin ST immunity proteins, respectively, is depicted in Fig. 2. The growth of WT *L. monocytogenes* EGDe was completely inhibited in the presence of two-fold dilutions of plantaricin 423 up to 1/64, while the growth rate in non-bacteriocin containing MRS remained unaffected (Fig. 2A). A significant increase in growth was recorded for *L. monocytogenes* EGDe (pNZSTldhplaB) in the presence of plantaricin 423 diluted to 1/32 compared to the WT strain. The growth of *L. monocytogenes* EGDe (pNZSTldhmunC) was not affected in the presence of undiluted mundtacin ST compared to a lack of growth for the WT strain after the 9 h period (Fig. 2B). The growth of WT, PlaB and MunC immunity protein expressing *L. monocytogenes* EGDe remained unaffected in the presence of non-bacteriocin containing supernatants isolated from *L. plantarum* 423 and *E. mundtii* ST4SA bacteriocin-negative strains (Fig. 2A and 2B).

Fig. 2A.

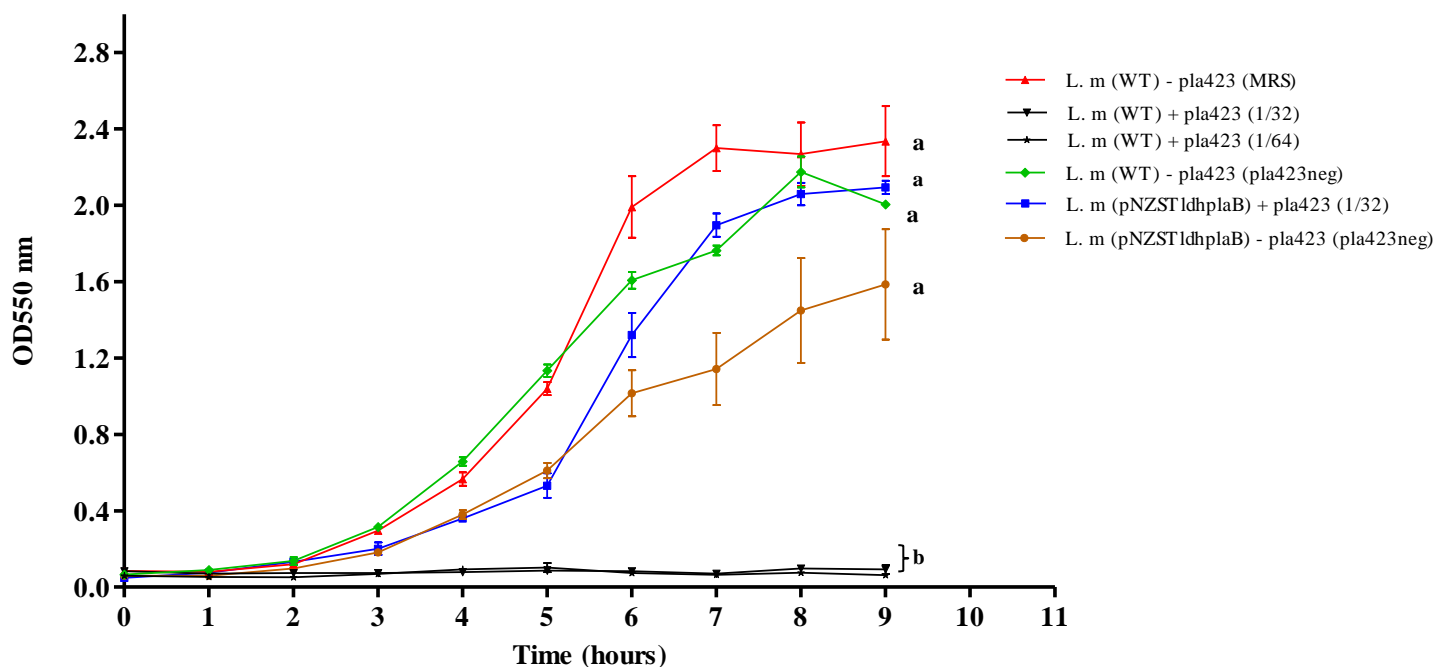


Fig. 2B.

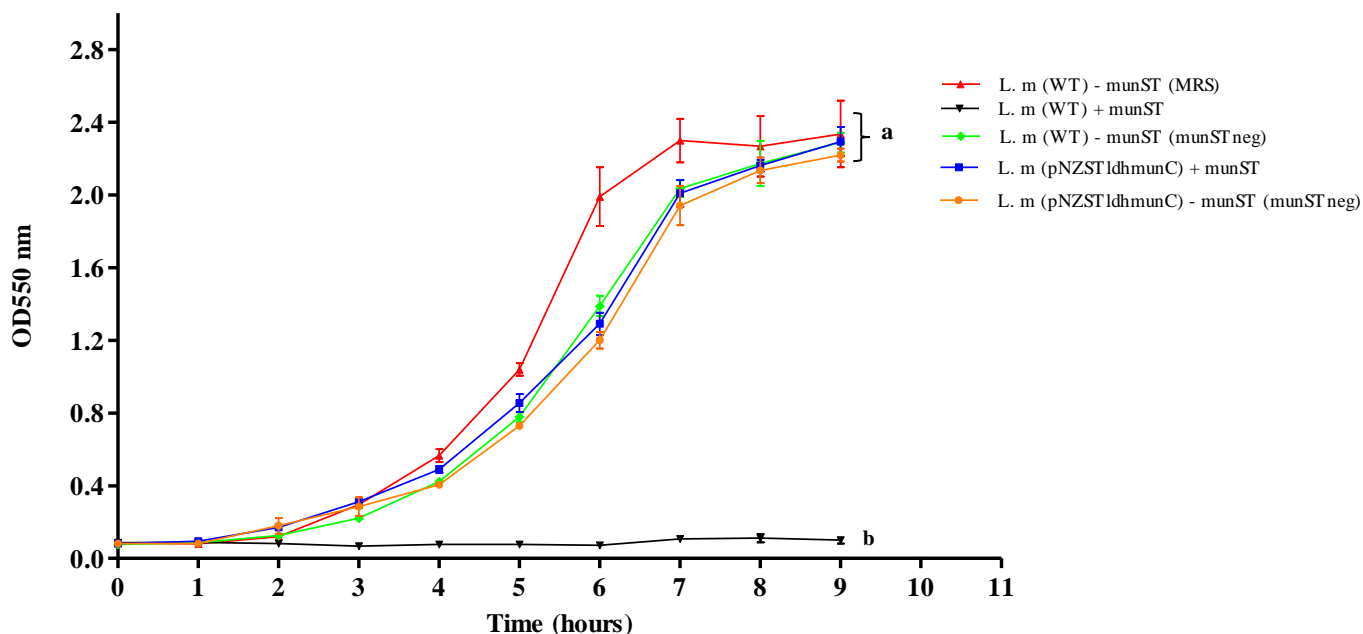


FIG. 2. Effect of (A) plantaricin 423 and (B) mundtacin ST on growth of WT *L. monocytogenes* EGDe, *L. monocytogenes* EGDe transformed with pNZSTldhplaB or *L. monocytogenes* EGDe transformed with pNZSTldhmunC, for 9 h, as determined by cell growth in BHI broth. Statistical differences were assessed using the Kruskal-Wallis nonparametric test ($P < 0.05$). Identical letters a and b denote no significant difference, and different letters indicate which groups have statistically significant differences with each other.

Probiotic administration excludes *L. monocytogenes* from the GIT

Luciferase expression by *L. monocytogenes* EGDe was used to follow the intestinal colonization and exclusion of cells from the intestinal tract after probiotic dosing. As early as 15 min after administering *L. monocytogenes* EGDe, a strong bioluminescence signal could be detected for mice in all groups (Fig. 3). A strong bioluminescence signal was detected from mice in the placebo control group (PBS) for 24 h. Mice administered with WT *L. plantarum* 423 or WT *E. mundtii* ST4SA for six consecutive days before challenge with luminescent *L. monocytogenes* EGDe elicited a significant reduction in the bioluminescent signal emitted from the abdominal region of all mice throughout the study period (Fig. 3A and 4A). From as early as 2 to 3 h post-infection the bioluminescence signal emitted from mice in groups WT-423 and WT-ST4SA was significantly reduced to the background signal compared to mice administered with the placebo control (Fig. 3B and 4B).

Fig. 3A.

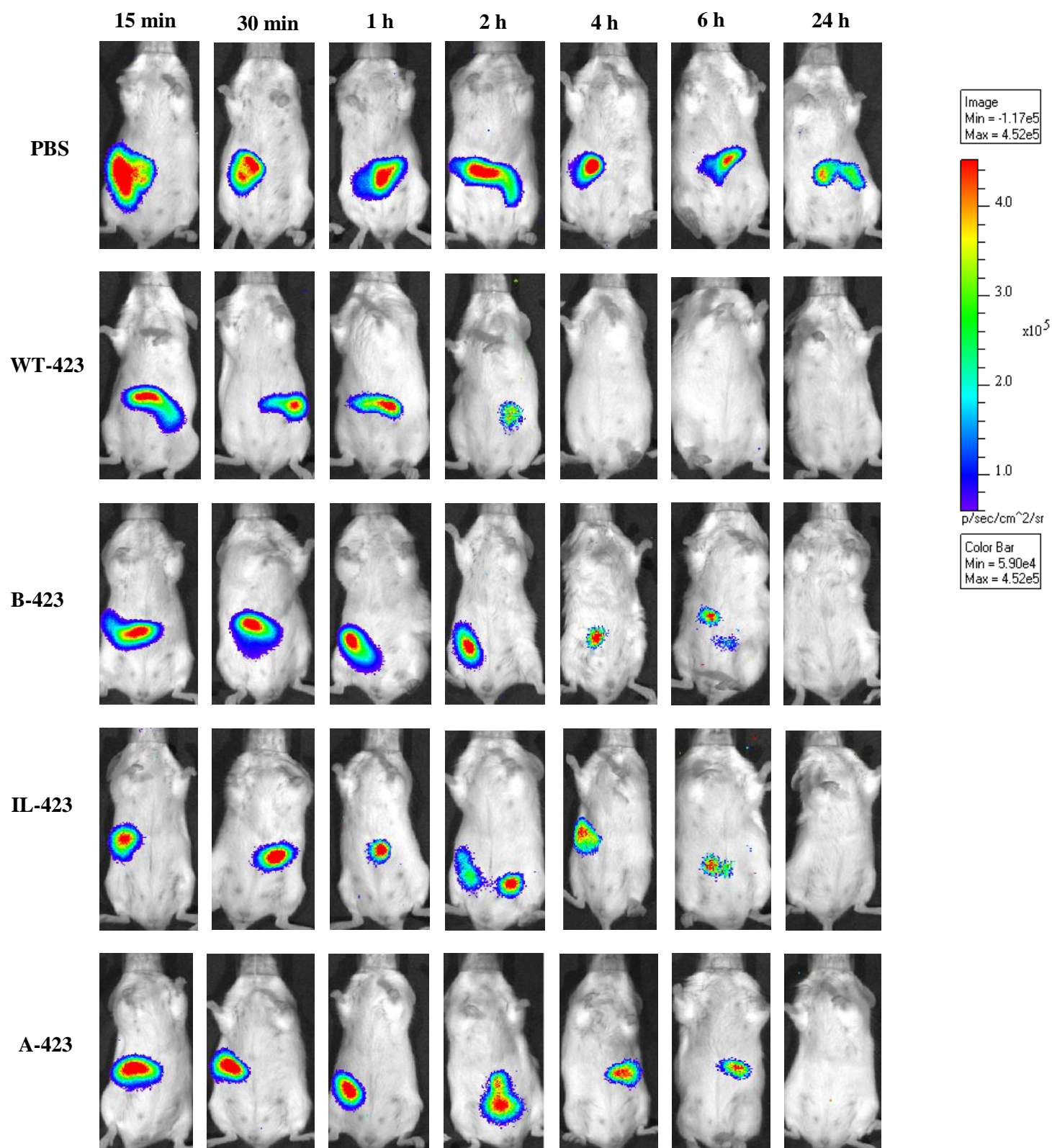


Fig. 3B.

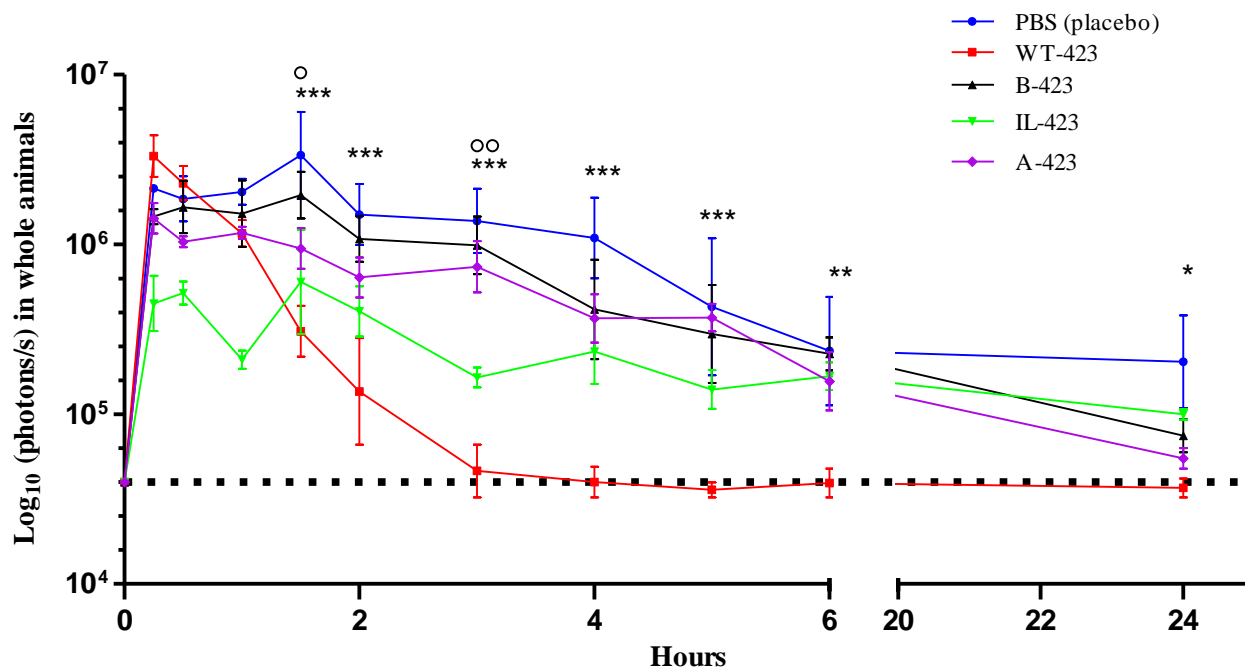


FIG. 3. (A) Visual images of bioluminescence and **(B)** the representative signals emitted used to track colonization and bacterial load of *L. monocytogenes* EGDe in the gastrointestinal tract of mice administered with PBS (placebo control), WT and recombinant *L. plantarum* 423 strains for 6 days before *Listeria* challenge. **PBS**: mice administered with sterile PBS, placebo control; **WT-423**: wild-type *L. plantarum* 423; **B-423**: plantaricin 423 bacteriocin-negative *L. plantarum* 423 *plaA::erm* and **A-423**: Aap-negative *L. plantarum* 423 *aap::erm* for 6 days before *Listeria* challenge. **IL-423**: mice administered with wild-type *L. plantarum* 423 for 6 days before challenge with plantaricin 423-resistant *L. monocytogenes* EGDe (pNZST1dhplaB). The intensity of the photon emission is represented as a pseudo-color image. One representative scale bar is shown ($\text{ps}^{-1}\text{cm}^{-2}\text{sr}^{-1}$) in A. Statistical analysis was determined by two-way ANOVA ($P < 0.05$, $P < 0.01$ or $P < 0.001$). Significant differences between groups PBS and WT-423 are indicated with asterisks and with empty circles between groups PBS and IL-423. The background level of the bioluminescence signal is represented by a dashed line.

Fig. 4A.

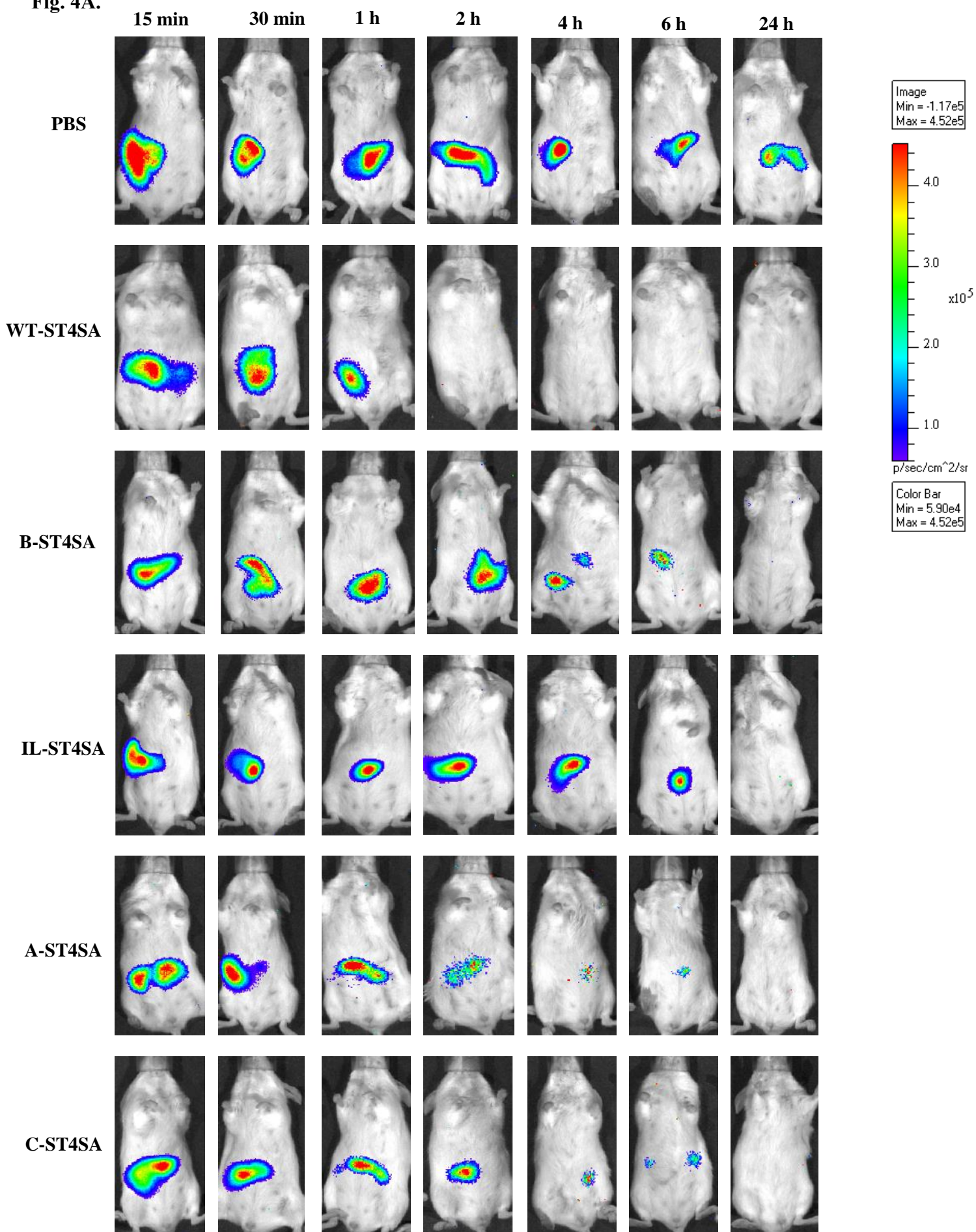


Fig. 4B.

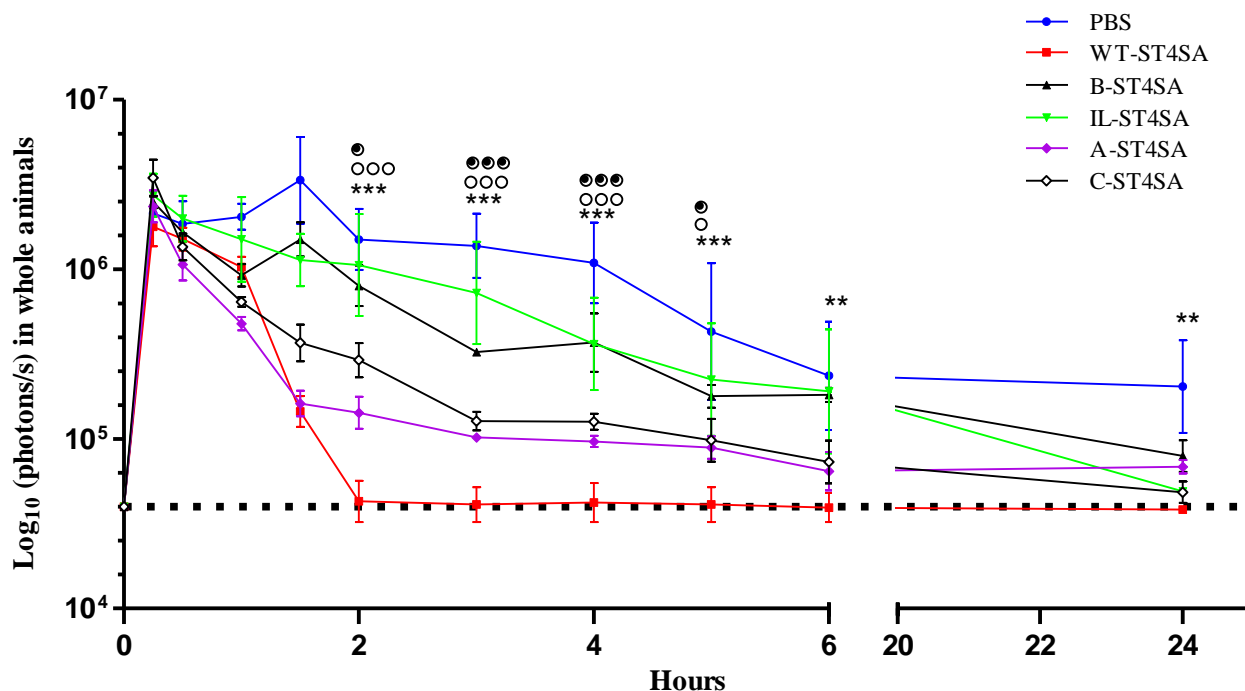


FIG. 4. (A) Visual images of bioluminescence and (B) the representative signals emitted used to track colonization and bacterial load of *L. monocytogenes* EGDe in the gastrointestinal tract of mice administered with PBS (placebo control), WT and recombinant *E. mundtii* ST4SA strains for 6 days before *Listeria* challenge. **PBS:** mice administered with sterile PBS, placebo control; **WT-ST4SA:** wild-type *E. mundtii* ST4SA; **B-ST4SA:** mundticin ST bacteriocin-negative *E. mundtii* ST4SA *munA::cat*; **A-ST4SA:** SrtA-negative *E. mundtii* ST4SA *srtA::erm* and **C-ST4SA:** SrtC-negative *E. mundtii* ST4SA *srtC::erm* for 6 days before *Listeria* challenge. **IL-ST4SA:** mice administered with wild-type *E. mundtii* ST4SA for 6 days before challenge with mundticin ST-resistant *L. monocytogenes* EGDe (pNZSTldhmunC). The intensity of the photon emission is represented as a pseudo-color image. One representative scale bar is shown ($\text{ps}^{-1}\text{cm}^{-2}\text{sr}^{-1}$) in A. Statistical analysis was determined by two-way ANOVA ($P < 0.05$, $P < 0.01$ or $P < 0.001$). Significant differences between groups PBS and WT-ST4SA are indicated with asterisks; empty circles between groups PBS and A-ST4SA and with filled circles between groups PBS and C-ST4SA.

Strong bioluminescence emission was detected in dissected intestines of mice in the PBS placebo control group (PBS, Fig. 5A). From 4 h post-infection, no bioluminescence was observed in the GIT of mice that received *L. plantarum* 423 and *E. mundtii* ST4SA for 6 days before oral administration with *L. monocytogenes* EGDe (WT-423 and WT-ST4SA, Fig. 5A). The small and large intestinal tracts of mice in all groups were homogenized separately to

enumerate *L. monocytogenes* EGDe viable cell numbers and these were expressed as the percentage reduction of the PBS (PR^P) placebo control group CFU throughout the trial period. Results shown in Figs 5 to 8 represent absolute CFU values, while values in the text refer to PR^P. Enumeration of *L. monocytogenes* EGDe cells in the small intestinal tracts of mice that were pre-treated with WT *L. plantarum* 423 revealed a highly significant percentage reduction of 99.7% after 4 h and stayed consistent until 48 h post-infection compared to the PBS placebo control (Fig. 5B). In the same group, cell numbers of *L. monocytogenes* EGDe in the large intestinal tracts were significantly reduced by 89.5% PR^P after 4 h and increased to 99.5% PR^P after 48 h (Fig. 5C). The number of *L. monocytogenes* EGDe cells in the small intestinal tracts of mice pre-treated with *E. mundtii* ST4SA was significantly reduced by 41.3% after 4 h but increased further to 98.9% PR^P after 48 h (Fig. 5B). Despite a higher number of *L. monocytogenes* EGDe cells in the small intestinal tract of mice in group WT-ST4SA (41.4% PR^P) after 4 h compared to mice in group WT-423 (99.5% PR^P), no bioluminescent cells were detected in the small intestines of mice in group WT-ST4SA (Fig. 5 B). Significant differences in the *L. monocytogenes* EGDe population between mice in group WT-ST4SA and the PBS placebo control group were also confirmed in the large intestinal tract by a percentage reduction of 95% to 98.5% during the study period (Fig. 5C).

Fig. 5A.

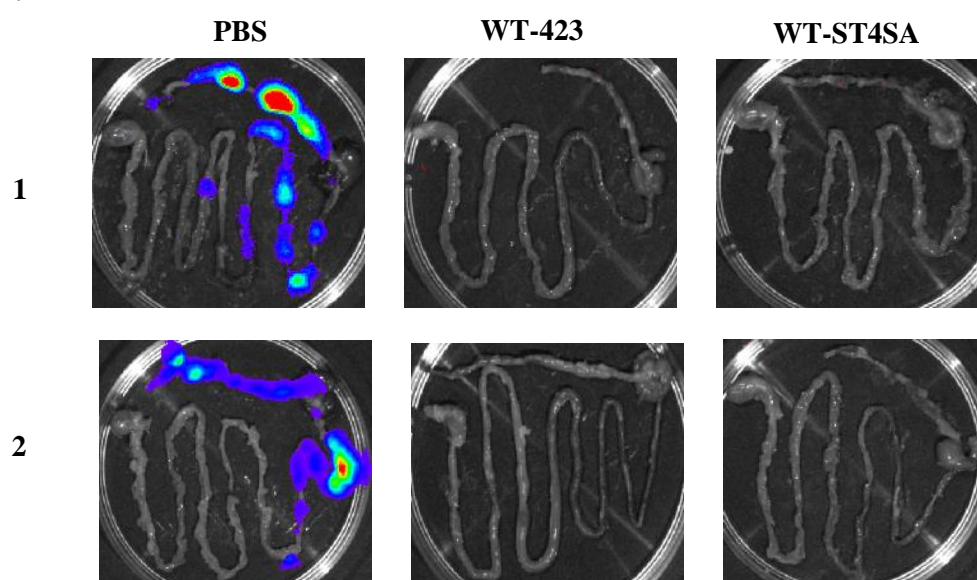


Fig. 5B.

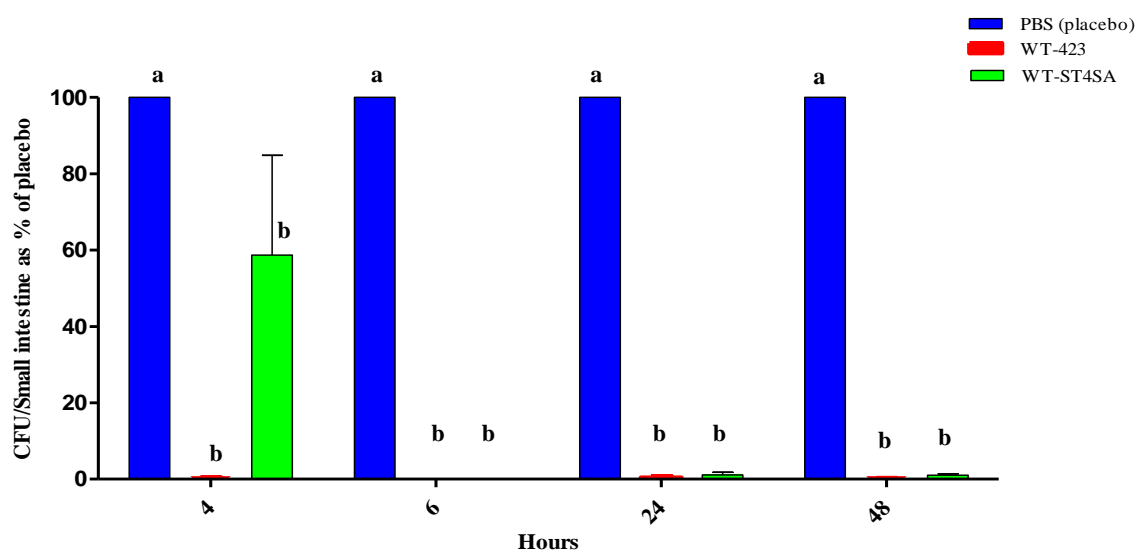


Fig. 5C.

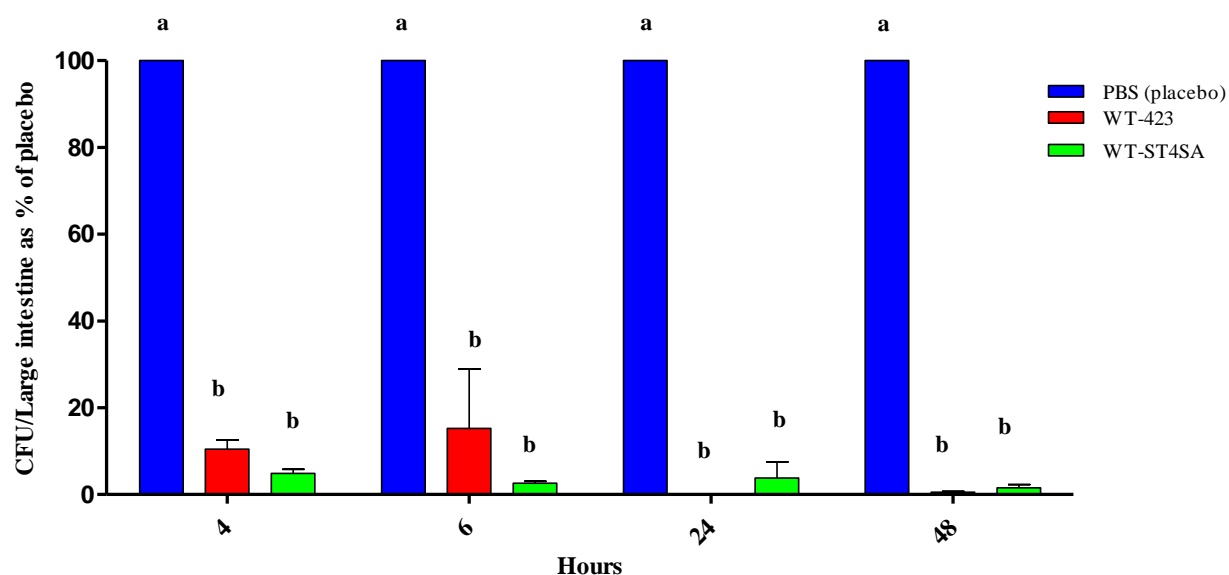


Fig. 5. A. Visual images to track bioluminescent cells of *L. monocytogenes* EGDe in the different sections of the digestive tract of mice in groups **PBS** (placebo control), **WT-423** and **WT-ST4SA** (1) 4 and (2) 6 h after *Listeria* challenge. **PBS**: mice administered with sterile PBS, placebo control; **WT-423**: wild-type *L. plantarum* 423 and **WT-ST4SA**: wild-type *E. mundtii* ST4SA for 6 days before *Listeria* challenge. Persistence of viable *L. monocytogenes* EGDe cells in the (B) small and (C) large intestinal tracts of mice in groups **PBS** (placebo control), **WT-423** and **WT-ST4SA**. Results are expressed as the percentage reduction of the PBS placebo control group *L. monocytogenes* EGDe CFU. Statistical differences were assessed using two-way ANOVA ($P < 0.01$ and $P < 0.001$). Identical letters a and b denote no significant difference, and different letters indicate which groups have significant statistical differences with each other.

Bacteriocin production as a protective mechanism against *L. monocytogenes* in the GIT

Mice administered with bacteriocin-negative *L. plantarum* 423 *plaA::erm* and *E. mundtii* ST4SA *munA::cat* for 6 days emitted a significantly higher bioluminescence signal compared to mice administered with WT *L. plantarum* 423 and WT *E. mundtii* ST4SA (Fig 3A and 4A). Throughout the trial period (24 h), mice in groups B-423 and B-ST4SA displayed levels of bioluminescence emission similar to mice fed the PBS placebo control compared to a significant reduction in bioluminescence in groups WT-423 and WT-ST4SA (Fig. 3B and 4B). Bioluminescent cells of *L. monocytogenes* EGDe were detected in the upper section of the small intestine in mice that received *L. plantarum* 423 *plaA::erm* 4 h post-infection (1, B-423, Fig. 6A). Two h later, bioluminescent cells of *L. monocytogenes* EGDe migrated further into the large intestinal tract (2, B-423, Fig. 6A). High-intensity bioluminescence was observed in the small intestine of mice that were administered *E. mundtii* ST4SA *munA::cat*, 4 and 6 h after challenge with *Listeria monocytogenes* EGDe (B-ST4SA, Fig. 6A). A significantly higher amount of viable *L. monocytogenes* EGDe cells were observed in the small intestinal tract of mice in group B-423 (38.9% PR^P) compared to mice in group WT-423 (99.7% PR^P) 4 h after challenge (Fig. 6B), while there were no significant differences between the *L. monocytogenes* EGDe CFU counts in the large intestinal tracts of mice in group B-423 and the PBS placebo control group from 6 to 48 h post-infection (Fig. 6C). There was no significant reduction in CFU counts of *L. monocytogenes* EGDe in the small intestinal tract of mice pre-treated with *E. mundtii* ST4SA *munA::cat* (33.7% PR^P) or mice treated with the PBS placebo control 4h after they were infected, but there was a significant increase to 94.5% PR^P 44 h later (Fig. 6B). High cell counts of *L. monocytogenes* EGDe were observed in the large intestinal tract of mice in group B-ST4SA with 61.5% PR^P at 6 h and 52.3% PR^P after 48 h (Fig. 6C).

Fig. 6A.

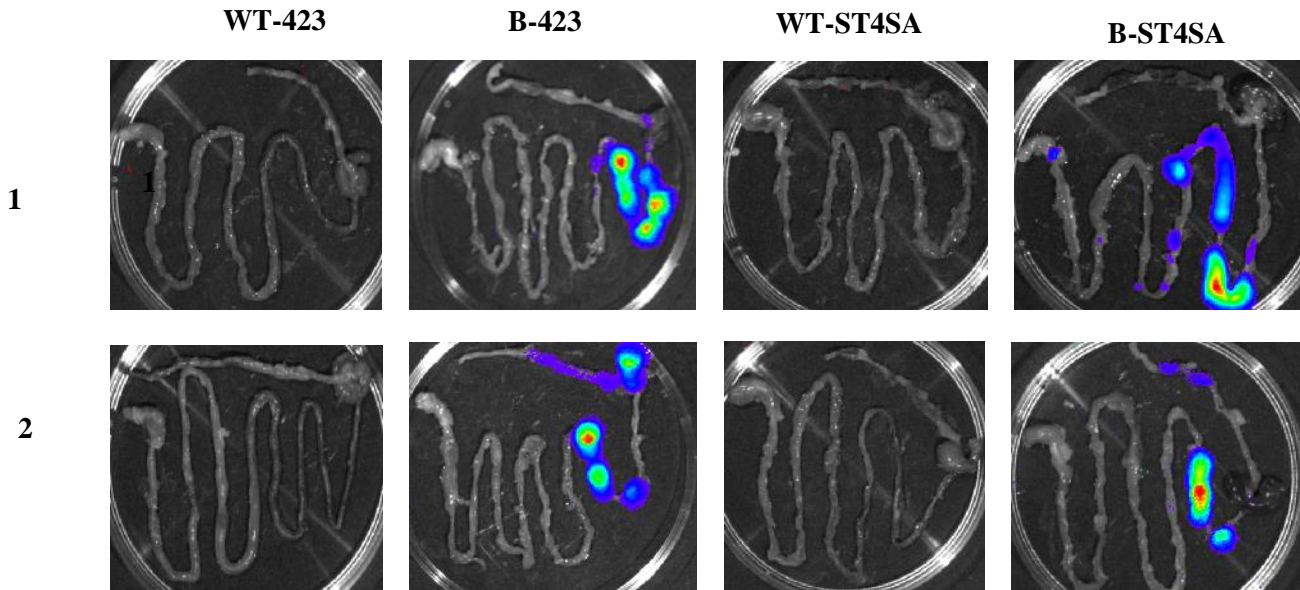


Fig. 6B.

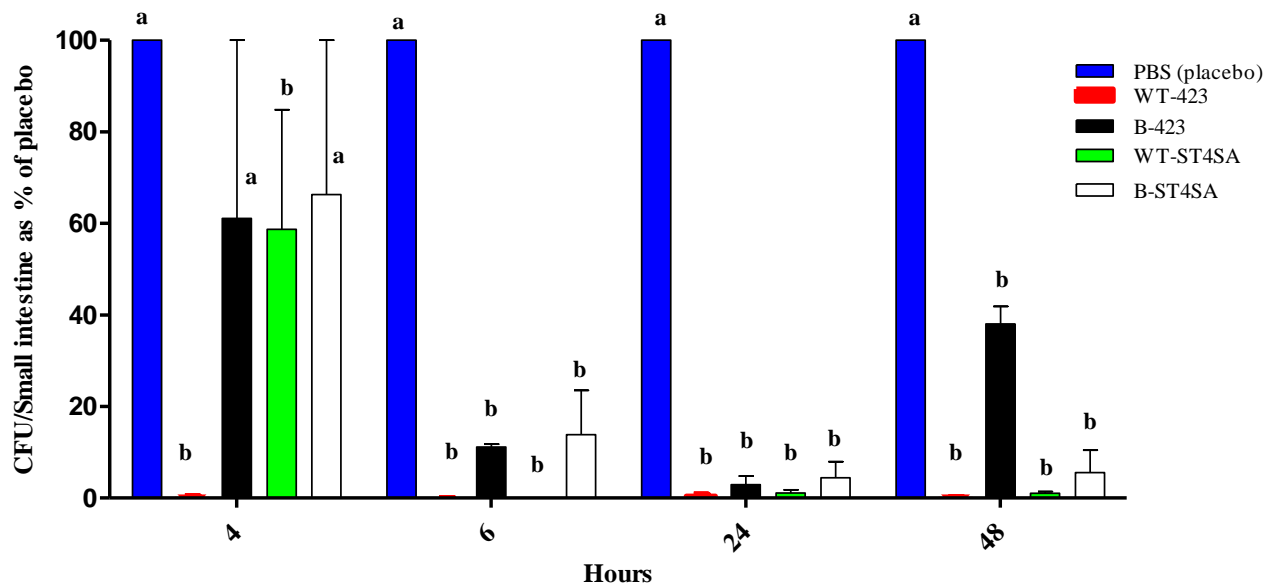


Fig. 6C.

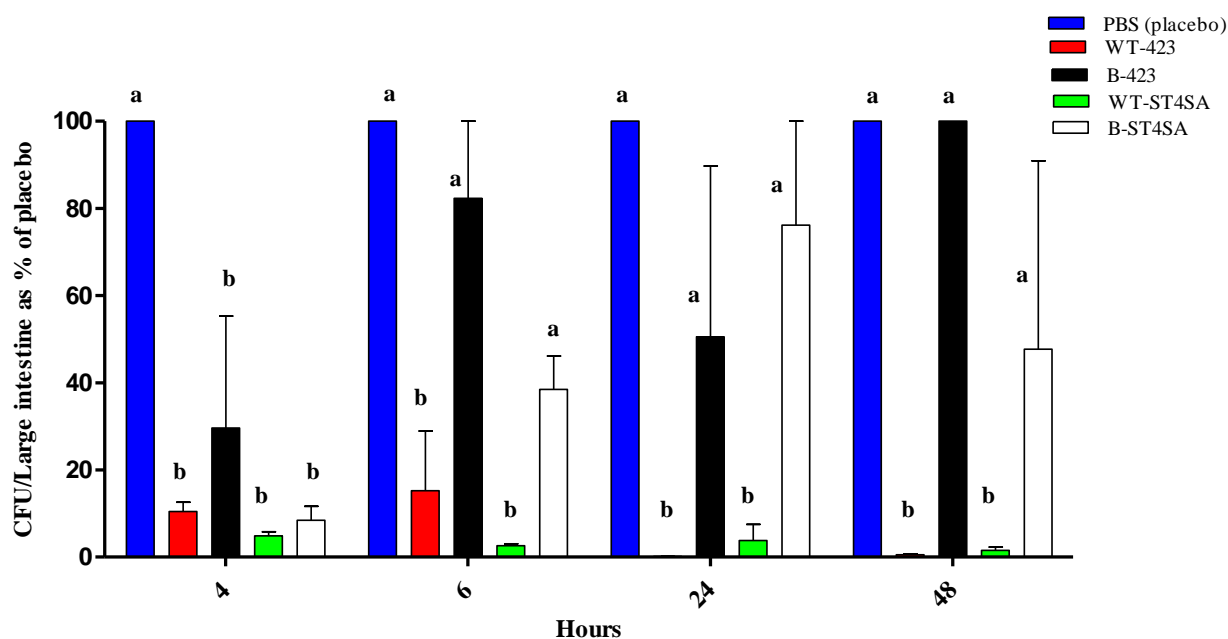


Fig. 6. A. Visual images to track bioluminescent cells of *L. monocytogenes* EGDe in the different sections of the digestive tract of mice in groups **WT-423**, **B-423**, **WT-ST4SA** and **B-ST4SA** (1) 4 and (2) 6 h after *Listeria* challenge. **WT-423**: mice administered with wild-type *L. plantarum* 423; **B-423**: plantaricin 423 bacteriocin-negative *L. plantarum* 423 *plaA::erm*; **WT-ST4SA**: wild-type *E. mundtii* ST4SA and **B-ST4SA**: mundticin ST bacteriocin-negative *E. mundtii* ST4SA *munA::cat* for 6 days before *Listeria* challenge. Persistence of viable *L. monocytogenes* EGDe cells in the (B) small and (C) large intestinal tracts of mice in groups **PBS** (placebo control), **WT-423**, **B-423**, **WT-ST4SA** and **B-ST4SA**. Results are expressed as the percentage reduction of the PBS placebo control group *L. monocytogenes* EGDe CFU. Statistical differences were assessed using two-way ANOVA ($P < 0.05$, $P < 0.01$ and $P < 0.001$). Identical letters a and b denote no significant difference, and different letters indicate which groups have significant statistical differences with each other.

To provide additional confirmation of bacteriocin involvement in the anti-infective activity of *L. plantarum* 423 and *E. mundtii* ST4SA against *L. monocytogenes* EGDe in the GIT, mice administered with the WT probiotic strains for 6 days were challenged with *L. monocytogenes* EGDe (pNZSTldhplaB) and *L. monocytogenes* EGDe (pNZSTldhmunc) expressing the *plaB* plantaricin 423 and *munC* mundticin ST bacteriocin immunity genes, respectively. Mice challenged with *L. monocytogenes* EGDe (pNZSTldhplaB) and *L. monocytogenes* EGDe (pNZSTldhmunc) in groups IL-423 (Fig. 3A) and IL-ST4SA (Fig. 4A), respectively, elicited an increase in bioluminescence emission compared to mice

challenged with the WT *L. monocytogenes* EGDe strains for 6 h. This result served as an early indication of an increase in *L. monocytogenes* EGDe bacterial load as a direct consequence of bacteriocin immunity gene expression. Bioluminescence readings of mice in group IL-423 significantly decreased 3 h after they were infected compared to readings recorded for mice in the PBS placebo control group (Fig. 3B). However, when examined 1 h later, the bioluminescence signal increased and remained at a similar level as observed for mice in group PBS for 20 h. There were no significant differences in bioluminescence recorded between mice in groups IL-ST4SA and mice in the B-ST4SA or PBS placebo control group (Fig. 4B).

Strong bioluminescence was emitted from *L. monocytogenes* EGDe (pNZSTldhplaB) in dissected intestines of mice in group IL-423 4 h after they were infected, and changed to a reduced signal 2 h later (IL-423, Fig. 7A). Similar results were observed for mice in group IL-ST4SA, with high bioluminescence emitted from the upper section of the small intestine (IL-ST4SA, Fig. 7A). Enumeration of viable cells of *L. monocytogenes* EGDe (pNZSTldhplaB) (IL-423) and *L. monocytogenes* EGDe (pNZSTldhmunC) (IL-ST4SA) in the small intestinal tract revealed levels similar to mice group PBS after 4 h infection, but significantly decreased 2 h later (Fig. 7B). The *L. monocytogenes* EGDe (pNZSTldhplaB) and *L. monocytogenes* EGDe (pNZSTldhmunC) populations in the large intestinal tract displayed levels similar to mice that received the placebo control (PBS) throughout the trial period (Fig. 7C).

Fig. 7A.

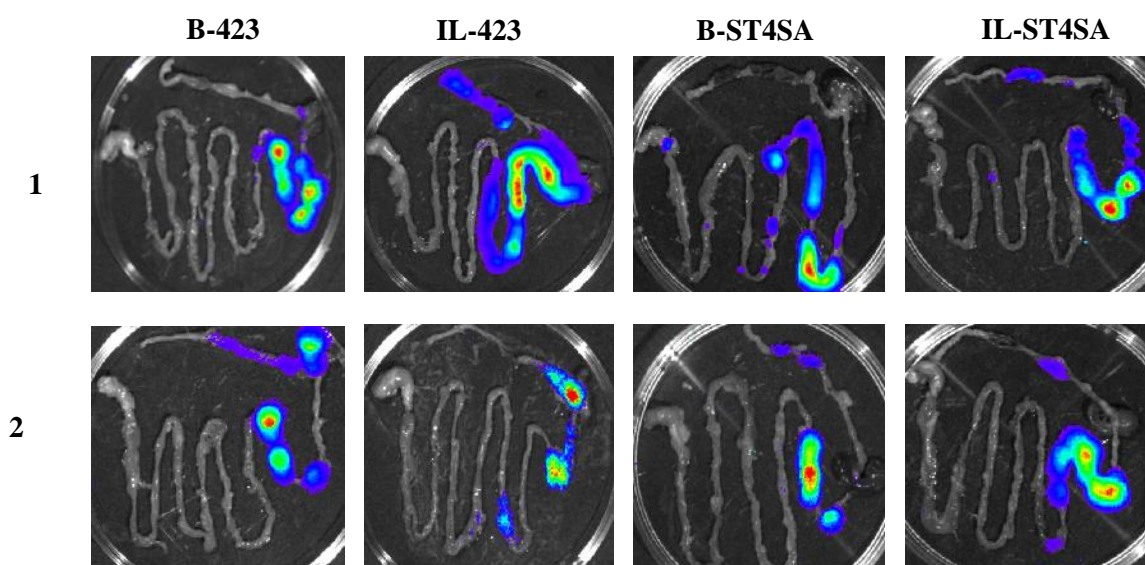


Fig. 7B.

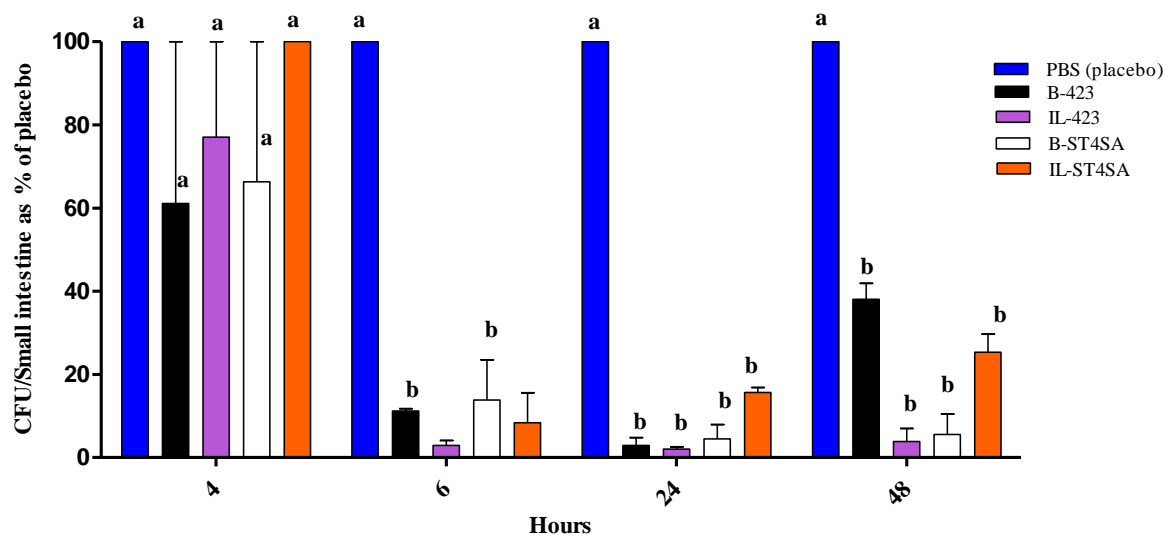


Fig. 7C.

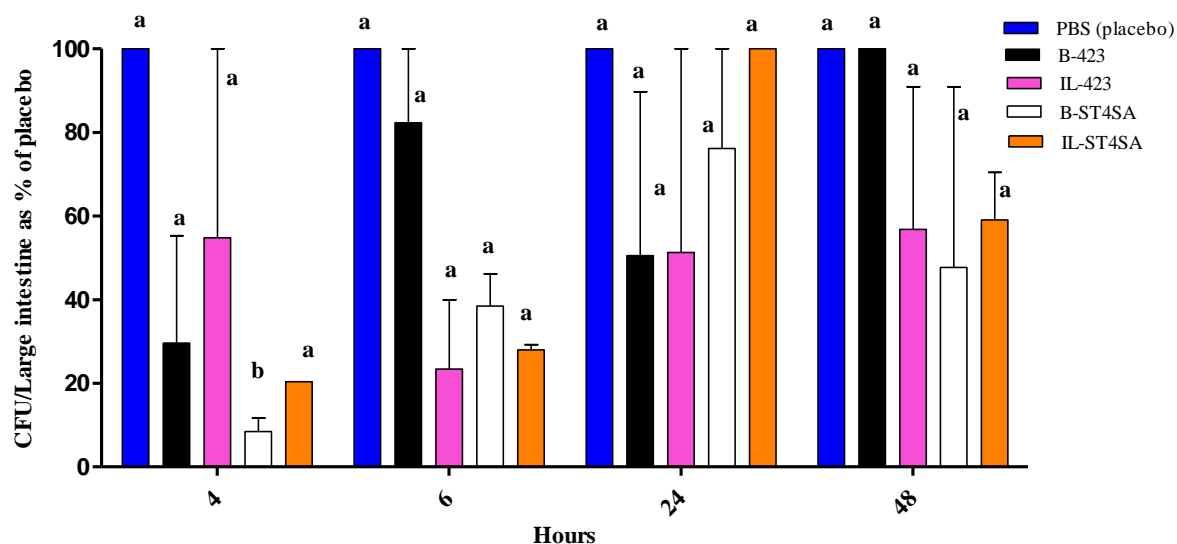


Fig. 7. A. Visual images to track bioluminescent cells of *L. monocytogenes* EGDe in the different sections of the digestive tract of mice in groups **B-423**, **IL-423**, **B-ST4SA** and **IL-ST4SA** (1) 4 and (2) 6 h after *Listeria* challenge. **B-423**: mice administered with plantaricin 423 bacteriocin-negative *L. plantarum* 423 *plaA::erm* for 6 days before *Listeria* challenge and **IL-423**: mice administered with wild-type *L. plantarum* 423 for 6 days before challenge with plantaricin 423-resistant *L. monocytogenes* EGDe (pNZST1dhplaB). **B-ST4SA**: mice administered with mundticin ST bacteriocin-negative *E. mundtii* ST4SA *munA::cat* for 6 days before *Listeria* challenge and **IL-ST4SA**: mice administered with wild-type *E. mundtii* ST4SA before challenge with mundticin ST-resistant *L. monocytogenes* EGDe (pNZST1dhmunC) on day 6. Persistence of viable *L. monocytogenes* EGDe cells in the (B) small and (C) large intestinal tracts of mice in groups **PBS**

(placebo control), **B-423**, **IL-423**, **B-ST4SA** and **IL-ST4SA**. Results are expressed as the percentage reduction of the PBS placebo control group *L. monocytogenes* EGDe CFU. Statistical differences were assessed using two-way ANOVA ($P < 0.05$, $P < 0.01$ and $P < 0.001$). Identical letters a and b denote no significant difference, and different letters indicate which groups have significant statistical differences with each other.

Probiotic adhesion properties as a protective mechanism against *L. monocytogenes* in the GIT

No significant differences in bioluminescence readings between mice administered with *L. plantarum* 423 *aap::erm* (lacking the *aap* adhesion gene) and the PBS placebo control were recorded. (Fig. 3A and B). Significantly lower bioluminescence readings, between 2 and 5 h, were recorded for mice in groups A-ST4SA and C-ST4SA compared to mice administered with the PBS placebo control (Fig. 4A and B). High-intensity bioluminescence was observed in the small intestine of dissected mice in group A-423 4 h after they were infected, but decreased to a lower intensity 2 h later (A-423, Fig. 8A). Bioluminescent cells of *L. monocytogenes* EGDe were detected in the GIT of mice in group A-ST4SA 4 h after infection and were detectable over a larger area of the small and large intestine over the next 2 h (A-ST4SA, Fig. 8A). No bioluminescence emission was detectable in the small intestine of mice administered with *E. mundtii* ST4SA *srtC::erm* (C-ST4SA, Fig. 8A). In the same group, low-intensity bioluminescence was observed in the cecum of the large intestinal tract 6 h after infection.

The *L. monocytogenes* EGDe PR^P in the small intestine of mice in group A-423 were significantly lower compared to those in the WT-423 group after 4 h but decreased to higher levels between 6 and 48 h with a percentage reduction of 83.1% to 87%, respectively (A-423, Fig. 8B). However, the *L. monocytogenes* EGDe PR^P in the large intestinal tract of mice in group A-423 were comparable to those observed in the WT-423 group after 6 h, but significantly decreased at 24 h (3.2% PR^P) and 48 h (39% PR^P) (A-423, Fig. 8C). At 4 and 6 h after infection, there was no difference between *L. monocytogenes* EGDe CFUs in the small intestinal tract of mice in group A-ST4SA and the PBS placebo control group (A-ST4SA, Fig. 8B), while significant differences in CFUs were observed between group C-ST4SA and the PBS placebo control group throughout the study period (C-ST4SA, Fig. 8B). Highest *L. monocytogenes* EGDe cell numbers in the large intestinal tract of mice in groups A-ST4SA

and C-ST4SA were recorded at 6 h (47.7% PR^P) and 24 h (45% PR^P), respectively, followed by a decrease to similar levels observed for mice in group WT-ST4SA after 48 h. (Fig. 8C).

Fig. 8A.

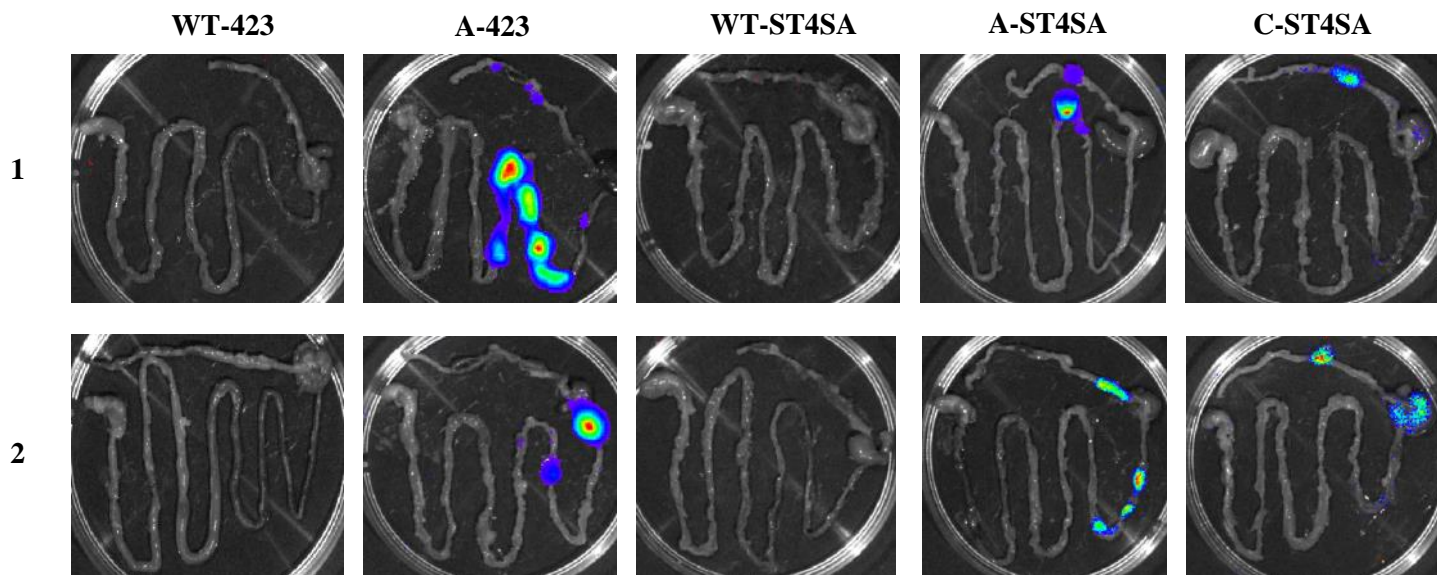


Fig. 8B.

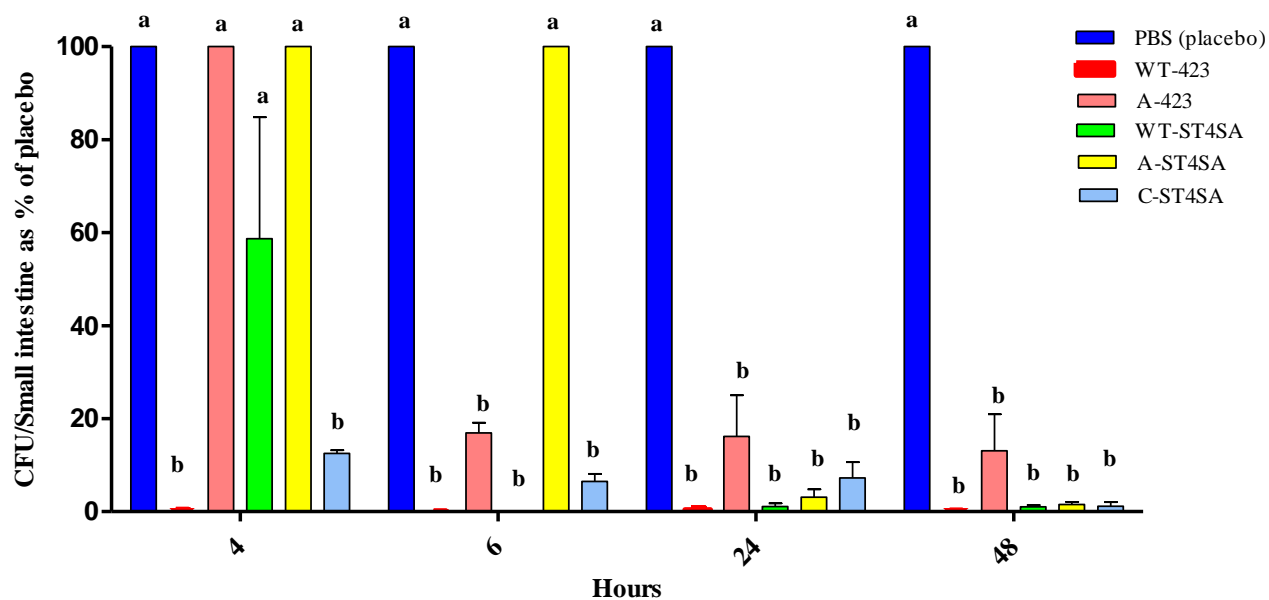


Fig. 8C.

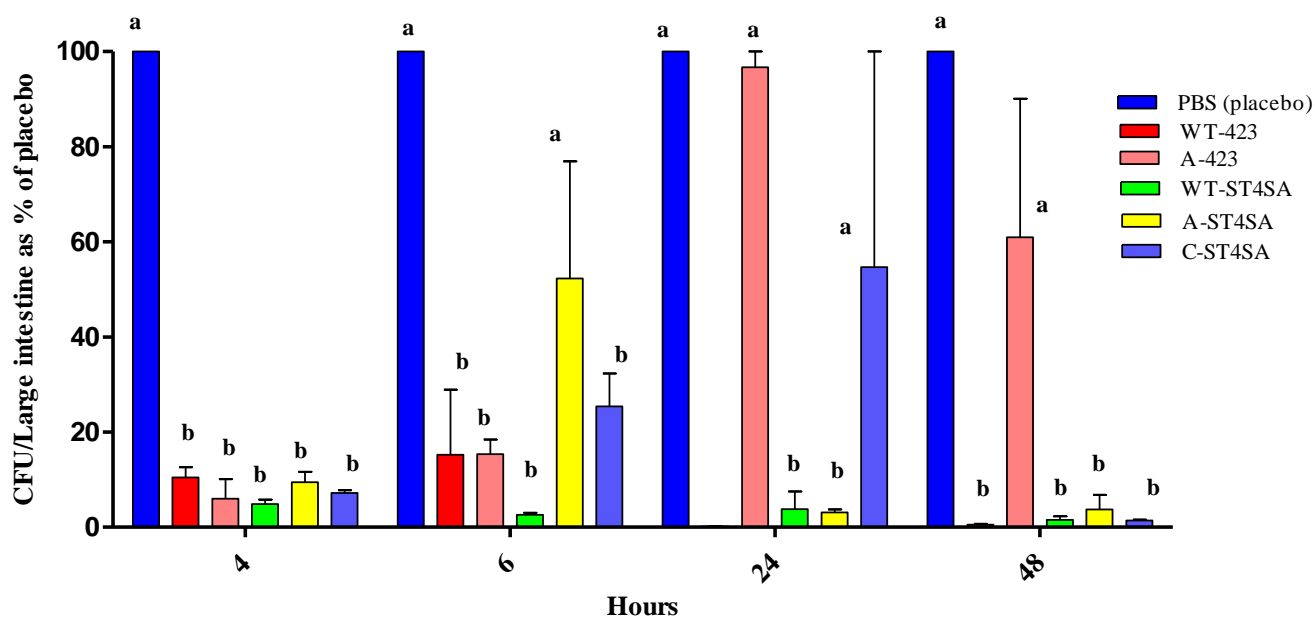


Fig. 8. A. Visual images to track bioluminescent cells of *L. monocytogenes* EGDe in the different sections of the digestive tract of mice in groups **WT-423**, **A-423**, **WT-ST4SA**, **A-ST4SA** and **C-ST4SA** (1) 4 and (2) 6 h after *Listeria* challenge. **WT-423**: mice administered with wild-type *L. plantarum* 423; **A-423**: Aap-negative *L. plantarum* 423 *aap::erm*; **WT-ST4SA**: wild-type *E. mundtii* ST4SA; **A-ST4SA**: SrtA-negative *E. mundtii* ST4SA *srtA::erm* and **C-ST4SA**: SrtC-negative *E. mundtii* ST4SA *srtC::erm* for 6 days before *Listeria* challenge. Persistence of viable *L. monocytogenes* EGDe cells in the (B) small and (C) large intestinal tracts of mice in groups **PBS** (placebo control), **WT-423**, **A-423**, **WT-ST4SA**, **A-ST4SA** and **C-ST4SA**. Results are expressed as the percentage reduction of the PBS placebo control group *L. monocytogenes* EGDe CFU. Statistical differences were assessed using two-way ANOVA ($P < 0.05$, $P < 0.01$ and $P < 0.001$). Identical letters a and b denote no significant difference, and different letters indicate which groups have significant statistical differences with each other.

Enumeration of viable *L. monocytogenes* in mouse faeces

No viable cells of *L. monocytogenes* EGDe or bioluminescent signals were detected in the faeces of mice administered with the PBS placebo control 3 h after infection (Fig. 9). One hour later (at 4 h), 9.54×10^7 CFU/100 mg faeces *L. monocytogenes* EGDe were recorded in the faeces of mice in the PBS placebo control group which then increased to 5.73×10^8 CFU/100 mg faeces after 6 h (Fig. 9A). In group PBS, the maximum bioluminescence signals were detected between 5 (1.15×10^6 p/s/100 mg of faeces) and 6 h (2.74×10^5 p/s/100 mg of

faeces) after infection, correlating perfectly with the amount of viable cells recorded during the same period (Fig. 9B). The number of viable *L. monocytogenes* EGDe cells increased with time in faeces of mice in groups WT-423, B-423 and IL-423, with the most significant differences observed in the first 3 h after infection compared to mice in the PBS placebo control group (Fig. 9A). During the same period, there were no significant differences between *L. monocytogenes* EGDe counts or bioluminescence emissions in faeces of mice in group A-423 and group PBS (Fig. 9A and B). Highest numbers of *L. monocytogenes* EGDe were excreted in the faeces of mice in groups B-423 and IL-423, reaching maximums of approximately 1.8×10^6 CFU/100 mg faeces after 1.5 h and 1.8×10^6 CFU/100 mg faeces after 48 h for each group (Fig. 9A) with corresponding bioluminescence signals of approximately 1.41×10^4 p/s/100 mg of faeces and 7.19×10^4 p/s/100 mg of faeces, respectively (Fig. 9B). Significantly higher cell numbers of *L. monocytogenes* EGDe were excreted in the faeces of mice in group WT-423 compared to group A-423 during the first 4 h, suggesting that the WT 423 strain more effectively excluded the pathogen from the GIT compared to the *L. plantarum* adhesion gene KO strain.

Fig. 9A.

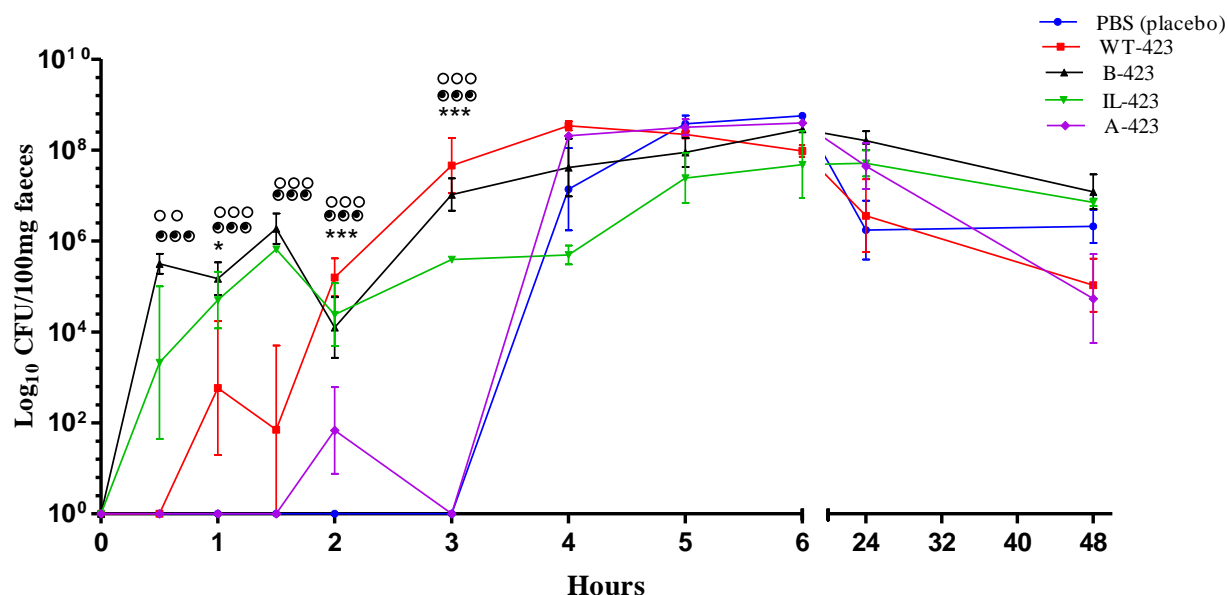


Fig. 9B.

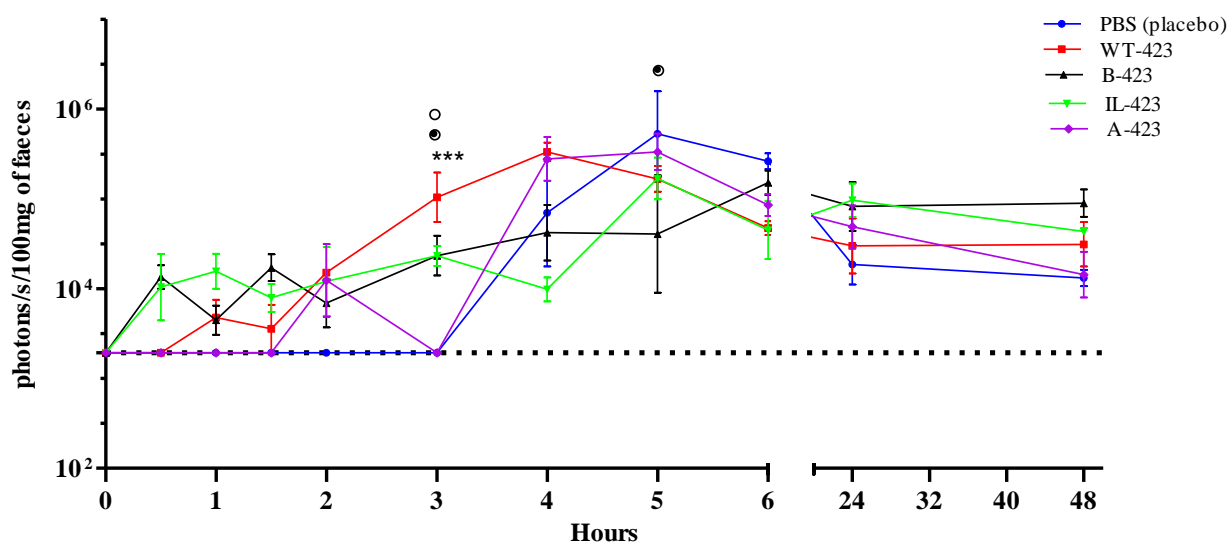


Fig. 9. Persistence of viable *L. monocytogenes* EGDe in the faeces of mice in groups **PBS** (placebo control), **WT-423**, **B-423**, **IL-423** and **A-423**, as recorded with (A) cell counts and (B) bioluminescence imaging. **PBS**: mice administered with sterile PBS, placebo control; **WT-423**: wild-type *L. plantarum* 423; **B-423**: plantaricin 423 bacteriocin-negative *L. plantarum* 423 *plaA::erm* and **A-423**: Aap-negative *L. plantarum* 423 *aap::erm* for 6 days before *Listeria* challenge. **IL-423**: mice administered with wild-type *L. plantarum* 423 for 6 days before challenge with plantaricin 423-resistant *L. monocytogenes* EGDe (pNZST1dhplaB). Statistical analysis was determined by two-way ANOVA ($P < 0.05$, $P < 0.01$ or $P < 0.001$). Significant differences between groups PBS and WT-423 are indicated with asterisks, empty circles between groups PBS and IL-423, and with filled circles between groups PBS and B-423. The background level of the bioluminescence signal is represented by a dashed line.

For mice in group WT-ST4SA, the *L. monocytogenes* EGDe counts in faeces reached approximately 6.23×10^7 CFU/100 mg faeces after 2 h with a bioluminescent signal of 2.58×10^5 p/s/100 mg of faeces and remained at a plateau for about 4 h (Fig. 10). After 2 h, no *L. monocytogenes* EGDe could be found in the faeces of mice in groups B-ST4SA, IL-ST4SA and A-ST4SA, while the number of *L. monocytogenes* EGDe recorded in the faeces of mice in group C-ST4SA reached 7.6×10^5 CFU/100 mg faeces with a bioluminescence signal of 3.86×10^4 p/s/100 mg of faeces (Fig. 10). After 3 h, the number of *L. monocytogenes* EGDe organisms in the faeces of all probiotic pre-treated mice was significantly higher compared to the PBS placebo control group (Fig. 10A). These peaks correlated well with the bioluminescent signals recorded at the same time, except for mice in group IL-ST4SA (Fig. 10B).

Fig. 10A.

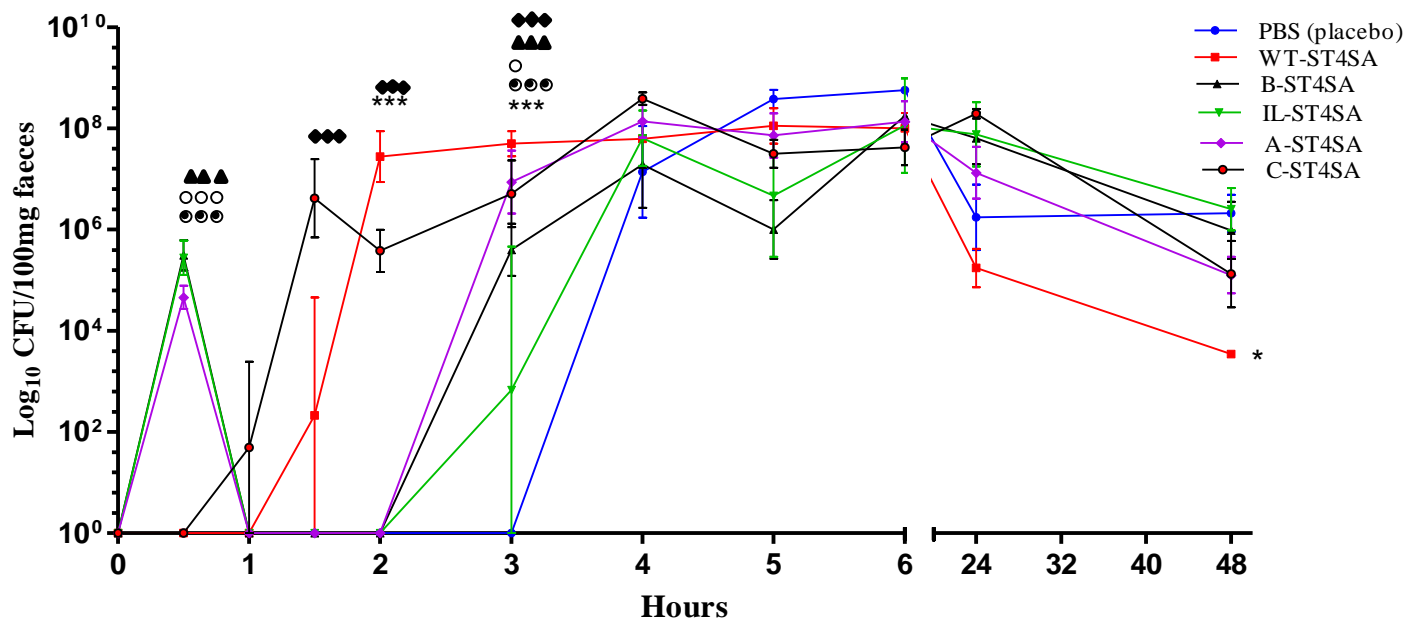


Fig. 10B.

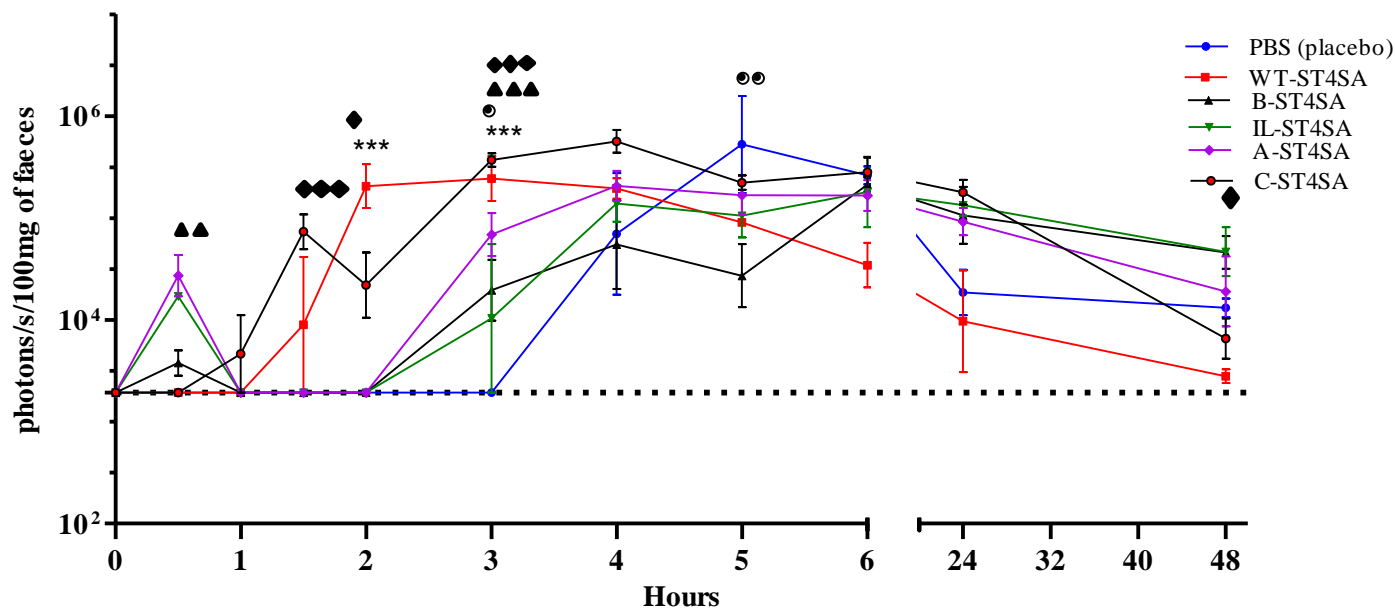


Fig. 10. Persistence of viable *L. monocytogenes* EGDe in the faeces of mice in groups **PBS** (placebo control), **WT-ST4SA**, **B-ST4SA**, **IL-ST4SA**, **A-ST4SA** and **C-ST4SA**, as recorded with (A) cell counts and (B) bioluminescence imaging. **PBS**: mice administered with sterile PBS, placebo control; **WT-ST4SA**: wild-type *E. mundtii* ST4SA; **B-ST4SA**: mundticin ST bacteriocin-negative *E. mundtii* ST4SA *muna::cat*; **A-ST4SA**: SrtA-negative *E. mundtii* ST4SA *srtA::erm* and **C-ST4SA**: SrtC-negative *E. mundtii* ST4SA *srtC::erm* for 6 days before *Listeria* challenge. **IL-ST4SA**: mice administered with wild-type *E. mundtii* ST4SA for 6 days before challenge with mundticin ST-

resistant *L. monocytogenes* EGDe (pNZSTldhmunC). Statistical analysis was determined by two-way ANOVA ($P < 0.05$, $P < 0.01$ or $P < 0.001$). Significant differences between groups PBS and WT-ST4SA are indicated with asterisks, empty circles between groups PBS and IL-ST4SA, filled circles between groups PBS and B-ST4SA, triangles between groups PBS and A-ST4SA and with diamonds between groups PBS and C-ST4SA. The background level of the bioluminescence signal is represented by a dashed line.

Cell numbers of *L. monocytogenes* EGDe isolated from the faeces of all groups displayed similar levels between 4 and 24 h (Fig. 10 A). After 48 h, the number of *L. monocytogenes* EGDe cells recorded in the faeces of mice in group WT-ST4SA declined to approximately 3.5×10^3 CFU/100 mg faeces compared to 3.5×10^6 CFU/100 mg faeces isolated from mice in the PBS placebo control group (Fig. 10A).

Probiotic CFU counts in the GIT and faeces of mice challenged with *L. monocytogenes*

Highest cell numbers of WT *L. plantarum* 423 and all recombinant *L. plantarum* 423 strains were detected in the large intestine when examined 4, 6, 25 and 48 h after *Listeria* infection compared to the small intestine (Fig. 11A and B). Compared to WT *L. plantarum* 423 (WT-423 group), cell numbers of *L. plantarum* 423 *aap::erm* (A-423 group) were approximately a 1000-fold higher in the small intestine 4 h after infection but decreased 2 h later (Fig. 11A). Cell counts of WT *L. plantarum* 423 in both the small and large intestinal tract were generally lower compared to that of recombinant *L. plantarum* 423 strains, with the most significant reduction after 24 h in the large intestinal tract (Fig. 11B). There were no significant differences between WT *L. plantarum* 423 and all recombinant strains' viable counts in the large intestinal tract between 4 and 6 h after they were infected. The number of viable WT *E. mundtii* ST4SA and recombinant *E. mundtii* ST4SA bacteria in the small intestinal tract displayed similar levels between 4 and 6 h after infection, ranging from approximately 3.44×10^5 CFU/ml to 2.40×10^7 CFU/ml (Fig. 12A). After 24 h, the number of viable *E. mundtii* ST4SA *srtA::erm* (A-ST4SA group) in the small intestine declined to approximately 100 cells per ml. Cell numbers similar to those of WT-ST4SA were recorded for all recombinant *E. mundtii* ST4SA strains in the large intestine between 4 and 6 h, where after viable WT *E. mundtii* ST4SA (WT-ST4SA group) and *E. mundtii* ST4SA *srtA::erm* (A-ST4SA group) cells declined to approximately 2.52×10^5 CFU/ml and 7×10^3 CFU/ml, respectively, at 24 h (Fig. 12B). After 48 h, no viable *E. mundtii* ST4SA *srtA::erm* cells were detected in the intestines of mice (Fig. 12A and B).

Fig. 11A.

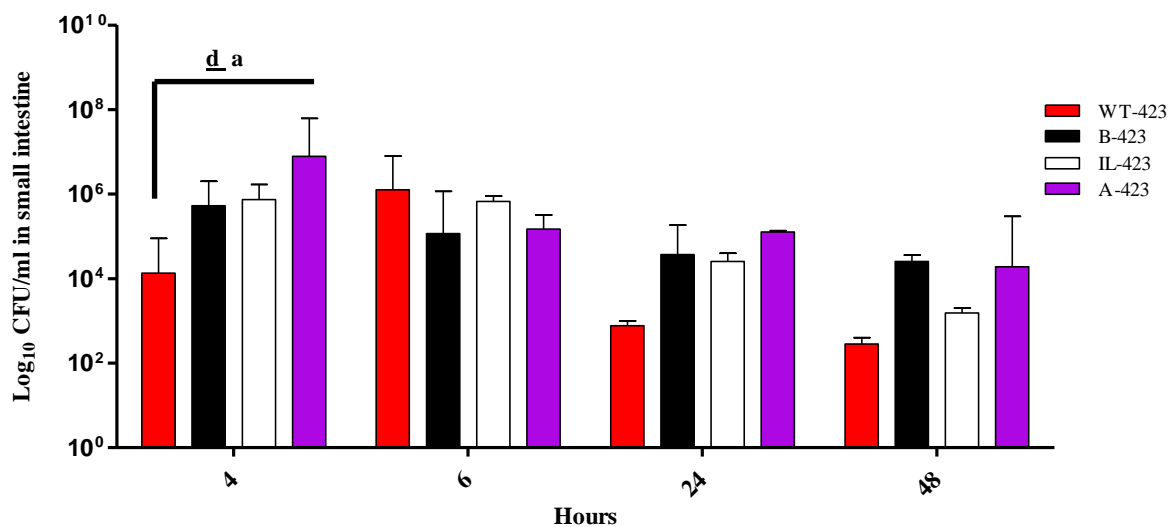


Fig. 11B.

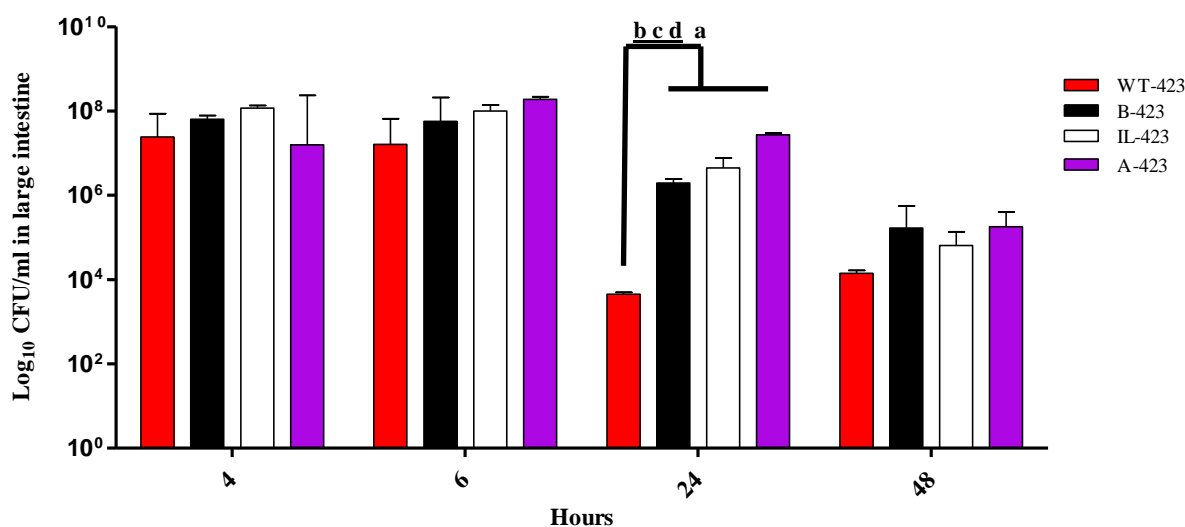


Fig. 11. Persistence of viable *L. plantarum* 423 strains in the (A) small and (B) large intestinal tracts of mice post *Listeria* challenge in groups **WT-423**, **B-423**, **IL-423** and **A-423**. **WT-423**: mice administered with wild-type *L. plantarum* 423; **B-423**: plantaricin 423 bacteriocin-negative *L. plantarum* 423 *plaA::erm* and **A-423**: Aap-negative *L. plantarum* 423 *aap::erm* for 6 days before *Listeria* challenge. **IL-423**: mice administered with wild-type *L. plantarum* 423 for 6 days before challenge with plantaricin 423-resistant *L. monocytogenes* EGDe (pNZST1dhplaB). Statistical analysis was determined by two-way ANOVA ($P < 0.05$, $P < 0.01$ or $P < 0.001$). Underlined letters and lines above bars indicate which groups have statistically significant differences.

Fig. 12A.

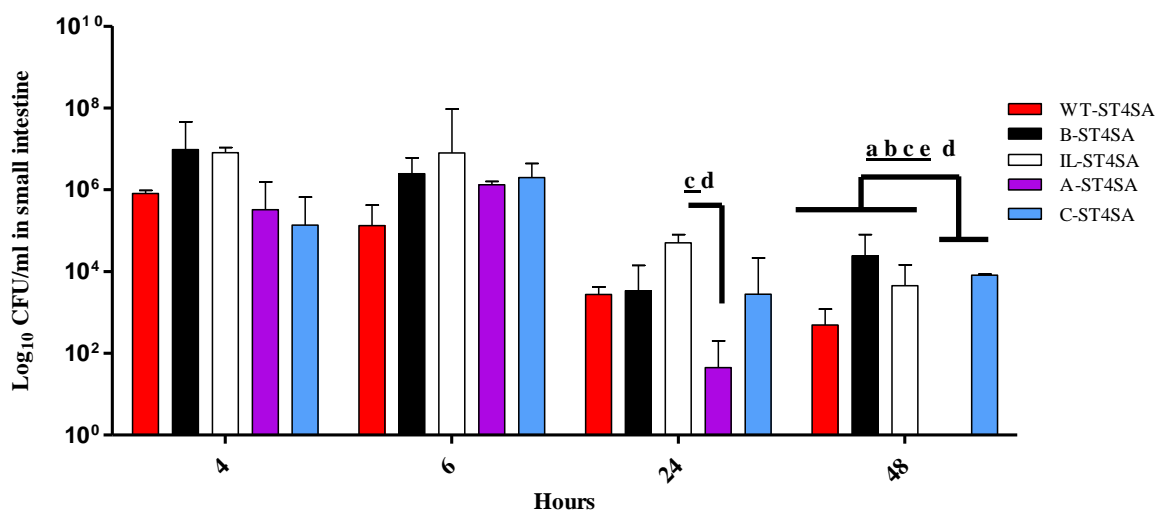


Fig. 12B.

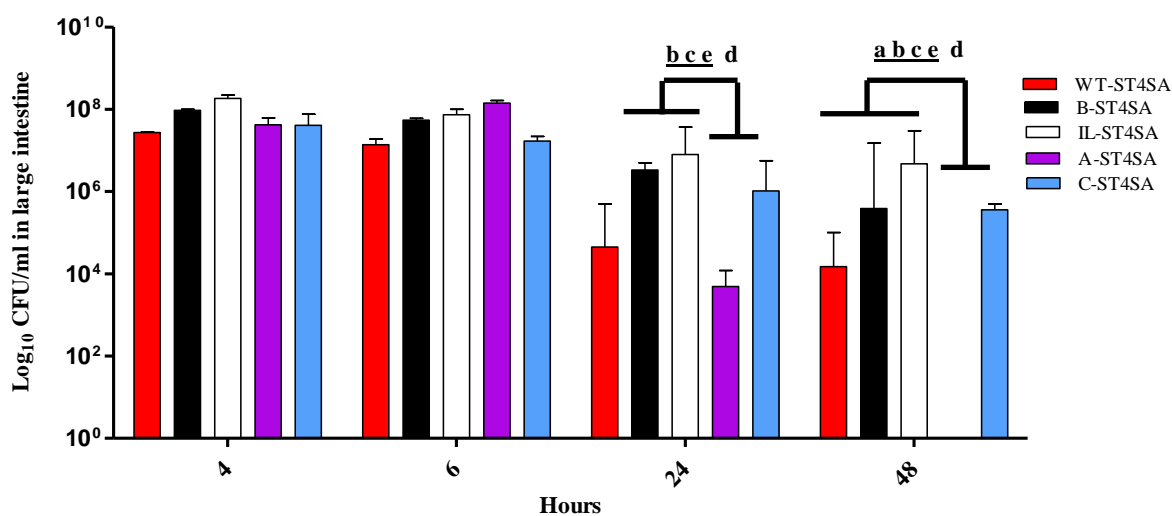


Fig. 12. Persistence of viable *E. mundtii* ST4SA strains in the (A) small and (B) large intestinal tracts of mice post *Listeria* challenge in groups **WT-ST4SA**, **B-ST4SA**, **IL-ST4SA**, **A-ST4SA** and **C-ST4SA**. **WT-ST4SA**: mice administered with wild-type *E. mundtii* ST4SA; **B-ST4SA**: mundtacin ST bacteriocin-negative *E. mundtii* ST4SA *munA::cat*; **A-ST4SA**: SrtA-negative *E. mundtii* ST4SA *srtA::erm* and **C-ST4SA**: SrtC-negative *E. mundtii* ST4SA *srtC::erm* for 6 days before *Listeria* challenge. **IL-ST4SA**: mice administered with wild-type *E. mundtii* ST4SA for 6 days before challenge with mundtacin ST-resistant *L. monocytogenes* EGDe (pNZST1dhmunC). Statistical analysis was determined by two-way ANOVA ($P < 0.05$, $P < 0.01$ or $P < 0.001$). Underlined letters and lines above bars indicate which groups have statistically significant differences.

On day 6 (last day of probiotic administration to mice), cell number in faeces of WT *L. plantarum* 423 (WT-423 group) increased with time, reaching its maximum level at 2 h after oral administration of *L. monocytogenes* EGDe of approximately 3×10^8 CFU/100 mg faeces, but declined to 6.9×10^5 CFU/100 mg faeces (Fig. 13A) at 48 h. Lowest cell numbers were recorded in the faeces of mice in groups IL-423 and A-423, declining to 9.1×10^4 CFU/100 mg faeces WT *L. plantarum* 423 (IL-423) after 1.5 h and 4.2×10^4 CFU/100 mg faeces *L. plantarum* 423 *aap::erm* (A-423) after 2 h, respectively (Fig. 13A). The number of WT *E. mundtii* ST4SA reached approximately 3×10^8 CFU/100 mg faeces 1 h after infection with *L. monocytogenes* EGDe, remained at a plateau for about 5 h and then declined (Fig. 13B). During the first 6 h after infection, the number WT *E. mundtii* ST4SA and recombinant *E. mundtii* ST4SA in the faeces of mice in all groups displayed similar levels, except for significant reductions in the first 2 h in the faeces of mice in groups B-ST4SA and IL-ST4SA compared to WT-ST4SA (Fig.13 B). After 48 h, no significant differences in cell counts were observed in the faeces of mice in all groups (Fig. 13A and B).

Fig. 13A.

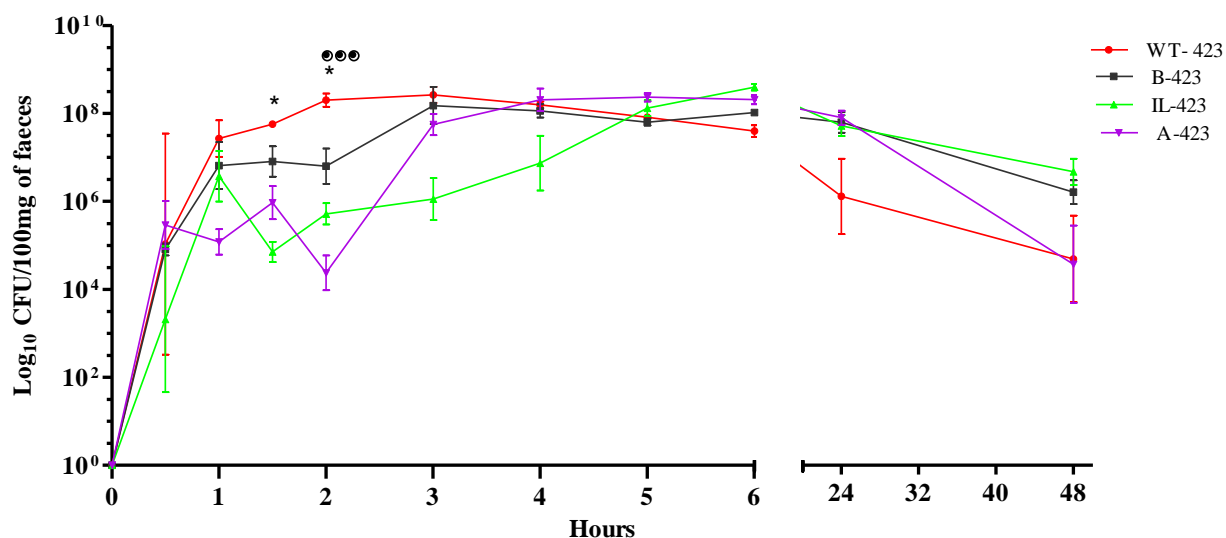


Fig. 13B.

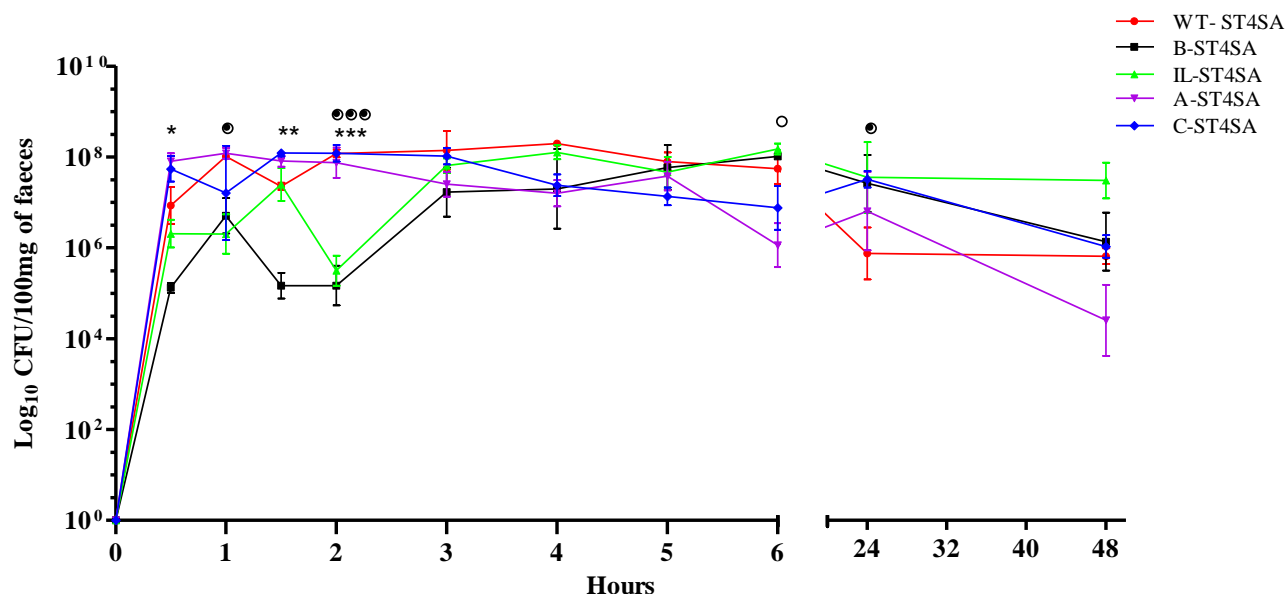


Fig. 13. A. Persistence of viable *L. plantarum* 423 strains in the faeces of mice post *Listeria* challenge in groups **WT-423**, **B-423**, **IL-423** and **A-423**. **WT-423**: mice administered with wild-type *L. plantarum* 423; **B-423**: plantaricin 423 bacteriocin-negative *L. plantarum* 423 *plaA::erm* and **A-423**: Aap-negative *L. plantarum* 423 *aap::erm* for 6 days before *Listeria* challenge. **IL-423**: mice administered with wild-type *L. plantarum* 423 for 6 days before challenge with plantaricin 423-resistant *L. monocytogenes* EGDe (pNZST1dhplaB). **B.** Persistence of viable *E. mundtii* ST4SA strains in the faeces of mice post *Listeria* challenge at t=0 in groups **WT-ST4SA**, **B-ST4SA**, **IL-ST4SA**, **A-ST4SA** and **C-ST4SA**. **WT-ST4SA**: mice administered with wild-type *E. mundtii* ST4SA; **B-ST4SA**: mundtacin ST bacteriocin-negative *E. mundtii* ST4SA *munA::cat*; **A-ST4SA**: SrtA-negative *E. mundtii* ST4SA *srtA::erm* and **C-ST4SA**: SrtC-negative *E. mundtii* ST4SA *srtC::erm* for 6 days before *Listeria* challenge. **IL-ST4SA**: mice administered with wild-type *E. mundtii* ST4SA for 6 days before challenge with mundtacin ST-resistant *L. monocytogenes* EGDe (pNZST1dhmunC). Statistical analysis was determined by two-way ANOVA ($P < 0.05$, $P < 0.01$ or $P < 0.001$). In A, significant differences between groups WT-423 and IL-423 are indicated by an asterisk and with filled circles between groups WT-423 and A-423. In B, significant differences between groups WT-ST4SA and B-ST4SA are indicated with asterisks, empty circles between WT-ST4SA and A-ST4SA and with filled circles between groups WT-ST4SA and IL-ST4SA.

Discussion

Numerous studies have demonstrated the ability of some LAB strains to control the growth of *L. monocytogenes* both *in vitro* and *in vivo* (2, 15, 24-27, 48-51). We have shown previously (and reconfirmed in this study), that the probiotics *L. plantarum* 423 and *E. mundtii* ST4SA successfully excluded *L. monocytogenes* from the GIT and protected mice from systemic infection (15). The functional effects of genes expressed by LAB *in vitro* or *in vivo* can only be truly assessed by the creation of knockout derivatives of strains expressing these genes. In the present study, two potential probiotic mechanisms of action responsible for a defined protective and anti-listerial effect in the GIT of mice using reverse genetic analysis are investigated.

First, the *in vitro* effect of bacteriocins plantaricin 423 and mundticin ST on the growth of wild-type (WT) *L. monocytogenes* EGDe and recombinant *L. monocytogenes* EGDe variant strains expressing the plantaricin 423 *plaB* and mundticin ST *munC* bacteriocin immunity genes, respectively, were evaluated. The growth of WT *L. monocytogenes* EGDe was completely inhibited in the presence of either plantaricin 423 or mundticin ST (Fig. 1). *In vitro* expression of the mundticin ST *munC* by *L. monocytogenes* (pNZSTldhmunC) resulted in a complete loss of sensitivity to mundticin ST, while expression of the plantaricin *plaB* immunity gene by *L. monocytogenes* EGDe (pNZSTldhplaB) resulted only in a partial loss of sensitivity to plantaricin 423 (Fig. 2). Usually, the expression of a bacteriocin immunity protein provides the bacteriocin-producing cell resistance to its own bacteriocin (52). While exact modes of action of immunity proteins remain largely uncharacterized (52-55), several studies have indicated that these proteins disrupt bacteriocin aggregation, pore formation or they interact with possible bacteriocin membrane-located receptors (47, 52, 54, 55). In this study it was observed that a bacteriocin negative *L. plantarum* 423 strain, cured of the bacteriocin and immunity gene containing pPLA4 plasmid (41), remained immune to plantaricin 423 (not shown). Therefore it is possible that the mechanism(s) involved in bacteriocin plantaricin 423 resistance in the WT strain include changes in the cell wall structure, such as fatty acid and phospholipid composition. These changes could prevent the interaction of the bacteriocin and its membrane located receptor, providing the WT *L. plantarum* 423 strain resistance even in the absence of *plaB* immunity gene expression (54). The absence of these changes in the cell membrane of *L. monocytogenes* EGDe could explain why the partial loss of *in vitro* sensitivity by *L. monocytogenes* EGDe (pNZSTldhplaB) to plantaricin 423.

The current study involves the transient colonization of bacterial strains resulting in the direct antagonism between an intestinal pathogen and two probiotic strains. Significantly higher bioluminescence signals were detected transcutaneously from mice in the PBS placebo control group for 24 h, while there was a significant reduction of detectable signals recorded from mice that received WT probiotic strains in groups WT-423 and ST4SA from as early as 2 h after infection with *L. monocytogenes* EGDe (Figs 3 and 4). This result served as the first line of evidence to support the anti-listerial effect of *L. plantarum* 423 and *E. mundtii* ST4SA. A reduction in bioluminescence correlates with a reduction of bacterial burden, but it is important to verify such a reduction in bioluminescence with viable cell counts. *Ex vivo* imaging of dissected intestines revealed strong bioluminescence emission in the small and large intestinal tract of mice in the PBS placebo control group, whereas no bioluminescent cells were detected in the intestines of mice in groups WT-423 or WT-ST4SA 4 h after they were infected (Fig. 5A). Enumeration of viable *L. monocytogenes* EGDe from homogenized intestines of mice in groups WT-423 and WT-ST4SA confirmed significant differences between probiotic treated mice and the PBS placebo control group. It is interesting to note that significant differences in viable *L. monocytogenes* EGDe were recorded in the small intestines between groups WT-423 and WT-ST4SA 4 h after infection. This result is supported by a previous study that suggested that particular probiotic strains differ greatly in their *in vivo* effectiveness (56). Despite a higher number of *L. monocytogenes* EGDe cells in the small intestinal tract of mice in group WT-ST4SA after 4 h compared to mice in group WT-423, no bioluminescent cells were detected in the small intestines of mice in group WT-ST4SA (Fig. 5A and B). This result is probably due to an initial bacteriostatic effect exerted by *E. mundtii* ST4SA on *L. monocytogenes* EGDe cells in the small intestine that was followed by a large reduction in *L. monocytogenes* EGDe after 6 h, since only metabolizing cells can emit bioluminescence.

The most obvious explanation of the anti-listerial effect of *L. plantarum* 423 and *E. mundtii* ST4SA suggests direct antagonism in the GIT between each of the probiotics and the pathogen as a result of bacteriocin production. *Lactobacillus plantarum* 423 bacteriocin production in an *in vitro* gastrointestinal model simulating the GIT of a pre-weaned piglet has been demonstrated previously (57). To provide *in vivo* experimental proof of the role of bacteriocin production as a mechanism of action against *L. monocytogenes* EGDe, stable mutants of *L. plantarum* 423 and *E. mundtii* ST4SA that do not produce the bacteriocins plantaricin 423 and mundticin ST, respectively, were constructed. Both bacteriocin-negative

probiotic strains failed to exclude *L. monocytogenes* from the mouse GIT in comparison with the WT strains as observed with whole body bioluminescence imaging (Figs 3 and 4) and by the presence of bioluminescent *Listeria* cells in the small and large intestinal tracts (Fig. 6A). This was most evident in the large intestinal tract and was confirmed with CFU counts (Fig. 6B and C). Bernbom and co-workers (27) have demonstrated previously that pediocin AcH-producing *L. plantarum* was responsible for the inhibition of *L. monocytogenes* compared to a less successful inhibition from its non-bacteriocin producing variant in an *in vitro* GIT model. Similarly, Corr and co-workers (58) demonstrated that bacteriocin production by *L. salivarius* UCC118 was responsible for the protective effect of the probiotic strain against *L. monocytogenes* in the liver and spleen of mice.

Viable cell counts also revealed that both bacteriocin-negative strains significantly excluded *L. monocytogenes* EGDe from the small intestinal tract from 6 h after infection (Fig. 6B). However, it was clear that the remaining viable *L. monocytogenes* EGDe cells were still able to emit strong bioluminescence, indicating active colonization as a result of a lack of the *in vivo* bacteriocin-related anti-listerial effect. The rapid drop of viable *L. monocytogenes* EGDe cells in the small intestines of mice in groups B-423 and B-ST4SA could also be due to anti-infective mechanisms other than bacteriocin production. Previous studies have shown that *L. plantarum* 423 and *E. mundtii* ST4SA adhere to different sections of the GIT and have excellent adhesion properties and displace *Clostridium sporogenes* and *E. faecalis* in *in vitro* competitive-exclusion experiments (42, 57, 59). Probiotic or commensal bacteria also produce antimicrobial factors (other than bacteriocins) such as organic acids, ammonia and hydrogen peroxide that are inhibitory to invading pathogens (29, 31, 32). In another study, *L. plantarum* 423 and *E. mundtii* ST4SA alleviated symptoms of *Salmonella* infection, as determined in Wistar rats challenged with *S. enterica* serovar Typhimurium, which is insensitive to both plantaricin 423 and mundticin ST bacteriocins (60). This implies that protection against *S. enterica* serovar Typhimurium was not linked to bacteriocin production and is likely to be more complex and might be pathogen-specific.

In addition to direct bactericidal or bacteriostatic effects on pathogenic microorganisms, it is important to note that antimicrobial peptides, including bacteriocins, could affect the established host microflora and have important roles as host immunomodulators (61). Several probiotic LAB are known to produce and excrete extracellular proteins involved in adhesion to intestinal surfaces and modulation of the function of epithelial and immune cells, and thus to actively participate in cross-talk between themselves and the host (62, 63). If this were the

case in the present study, the role of bacteriocins as a mechanism of probiotic action would be evident as less significant relative to the sensitivity of *L. monocytogenes* EGDe to the bacteriocins produced by *L. plantarum* 423 and *E. mundtii* ST4SA. Therefore, to provide additional confirmation of the *in situ* bacteriocin production in the GIT as a mechanism of action against *Listeria* infection, the bacteriocin immunity genes of plantaricin 423 and mundticin ST4SA were expressed in *L. monocytogenes* EGDe. Results in this study show that whole body bioluminescence readings from mice in groups IL-423 (4-24 h, Fig. 3) and IL-ST4SA (Fig. 4), challenged with *L. monocytogenes* EGDe strains expressing the respective bacteriocin immunity genes, display similar levels compared to that of the PBS placebo control group and the respective bacteriocin-negative groups (B-423 and B-ST4SA). The high bioluminescence readings were correlated directly with the bacterial load present, due to the heterologous expression of the bacteriocin immunity proteins by *L. monocytogenes* EGDe. Bioluminescent signals recorded from mice in group IL-423 were generally less intense than those recorded for IL-ST4SA. This was expected, as plantaricin *plaB* immunity gene expression by *L. monocytogenes* EGDe only provided significant protection from plantaricin 423 at a dilution of 1/32 in an *in vitro* environment. Further confirmation of the role of immunity gene expression by *L. monocytogenes* EGDe in groups IL-423 and IL-ST4SA is provided by the presence of actively metabolizing bioluminescent cells in dissected intestines (Fig. 7A). Viable cell counts of *L. monocytogenes* EGDe in the intestines of mice in groups IL-423 and IL-ST4SA revealed similar results for those obtained from the respective bacteriocin-negative groups (Fig. 7B and C). These results support the proposal that *in situ* bacteriocin production by the probiotic LAB acts as a direct mechanism of action against infectious *L. monocytogenes* EGDe in the GIT.

One of the major selection criteria of a good probiotic is its ability to adhere to intestinal mucus and epithelial cells (64). Probiotic LAB with excellent adhesion properties are able to successfully compete with intestinal pathogens for adhesion sites on epithelial cell surfaces and thus prevent pathogens from colonizing (42, 65, 66). However, despite extensive genetic reports on the adhesion capabilities of probiotic LAB, the mechanisms of attachment are not fully understood. The complex and kinetic nature of the human intestinal environment has made it difficult to study these processes. Several *in vitro* intestinal models have been used to study the adhesion of LAB to mucosal and epithelial surfaces using human colon adenocarcinoma cells such as the Caco-2 and HT-29 cell lines (5, 67, 68). *Lactobacillus* spp. have been the best studied of which a few strains produce specific adhesion proteins. The best

researched examples are the mucus adhesion-associated protein (*MapA*) and the mucus-binding proteins (*mub*) produced by *L. reuteri* (69). Proteins homologous to the MapA and Mub proteins of *L. reuteri* have been described for several other *Lactobacillus* spp. (69-71). Mub and MapA homologs have been reported for *L. plantarum* 423, of which MapA (referred to as Aap in this study) was shown to be extensively up-regulated under normal physiological concentrations of bile and pancreatin simulating GIT conditions (72). In this study a *L. plantarum* 423 mutant lacking the *aap* adhesion gene was constructed to investigate its role in the competitive exclusion of *L. monocytogenes* EGDe in the GIT. Using *in vivo* and *ex vivo* bioluminescence imaging, results showed that *L. plantarum* 423 *aap::erm* (lacking the *aap* adhesion gene) failed to exclude *L. monocytogenes* EGDe from the GIT of mice in group A-423 compared to WT *L. plantarum* 423 (WT-423 group) (Figs 3 and 8). The presence of high numbers of viable *L. monocytogenes* EGDe cells in the large intestinal tract of mice in group A-423 between 24 and 48 h after infection provided further evidence of the role of the *L. plantarum* Aap adhesion protein as a mechanism of action involved in the exclusion of the pathogen from the GIT (Fig. 8C). These results are in agreement with studies that suggest that probiotic adhesion is a requirement for the realization of specific probiotic effects such as immunomodulation and competitive exclusion of pathogens (65-67, 73).

To investigate the role of cell surface adhesion proteins produced by *E. mundtii* ST4SA in the competitive exclusion of *L. monocytogenes* EGDe from the GIT, groups of mice were orally administered with stable mutants of *E. mundtii* ST4SA lacking the Sortase A (*srtA*) and Sortase C (*srtC*) cell wall protein sorting enzymes for 6 days before *Listeria* infection. In Gram-positive bacteria, sortase enzymes decorate the cell surface with a diverse array of proteins by covalently joining them to the cell wall (Sortase A) or by polymerizing proteins to construct complex multi-subunit pilin structures (Sortase C) on the cell surface (74). Although sortase enzymes are not essential to bacterial viability, they play an important role by displaying or 'sorting' surface proteins on the cell wall that are essential for bacterial adhesion to host intestinal mucosal surfaces, colonization and the acquisition of essential nutrients (74-76). Furthermore, the surface proteins displayed by probiotic LAB play a key role in their interaction with intestinal epithelial cells and mucus by promoting adhesion (64). These specific interactions have been associated with the competitive exclusion of pathogens from the GIT by probiotic bacteria (64, 71, 77, 78). Using an *E. mundtii* ST4SA draft genome sequence and all available fully annotated reference *E. mundtii* full genome sequences, 18 putative Sortase A-dependent cell wall anchored proteins together with a fully dedicated

sortaseA gene were identified. In addition, a three-gene locus encoding structural pilus-subunit proteins consisting of two putative accessory pilus subunits (*EbpAfm* and *EbpCfm*) and a major pilus subunit protein (*EbpCfm*) located adjacent to the *E. mundtii* pilus-dedicated *sortaseC* gene was also identified.

In vivo whole body bioluminescence signals for mice in groups A-ST4SA and C-ST4SA were only slightly higher than those observed for mice in the WT-ST4SA group and significantly lower than those recorded for mice in the PBS placebo control group between 2 and 5 h after infection (Fig. 4). This result indicates that *E. mundtii* ST4SA mutants lacking the *srtA* or *srtC* genes are capable of competitively excluding the intestinal pathogen and thus lowering the bacterial burden. On the other hand, actively growing bioluminescent cells in dissected intestines of mice in groups A-ST4SA and C-ST4SA compared to no bioluminescent cells detected in the WT-ST4SA group were detected (Fig. 8A). The *Listeria monocytogenes* EGDe CFU counts in the intestinal tracts of mice in group A-ST4SA confirmed the presence of high numbers in the small intestinal tract between 4 and 6 h (Fig. 8B). Previous studies have demonstrated that Sortase A play a role in the *in vitro* adhesion of bacterial cells to human cell lines (89, 80), intestinal colonization (75, 81) and virulence (81, 82). Lalioui and co-workers (75) showed that the capacity of a *Streptococcus agalactiae* SrtA negative strain to colonize the murine intestine was significantly impaired compared to a *S. agalactiae* SrtA positive strain, in a competition assay. However, the current study investigates the role of Sortase A and C in the direct antagonism between a probiotic and an enteric pathogen, rather than intraspecies competitive exclusion.

Listeria monocytogenes EGDe cell counts recorded in the intestines of mice in group C-ST4SA confirmed that the *E. mundtii* ST4SA *srtC* mutant efficiently excluded *L. monocytogenes* from the GIT, except for a significant increase in *L. monocytogenes* EGDe cell numbers after 24 h in the large intestinal tract (Fig. 8B). In a recent study by Tytgat and co-workers (83), it was demonstrated that *L. rhamnosus* GG outcompeted vancomycin-resistant *E. faecium* via mucus-binding pili *in vitro*. The role of sortase C and sortase C-dependent pilus-encoding genes in bacterial adhesion to epithelial cells, host invasion and biofilm formation have been studied extensively in virulent *E. faecium* and *E. faecalis* strains (84, 85). Sillanpää and co-workers (85) identified four pilus-containing gene clusters in the genome of *E. faecium* TX16 each located with an adjacent *sortase C* gene that is predicted to encode four distinct types of pili. However, only one such gene cluster was identified in the genome of *E. mundtii* ST4SA. This could explain the less significant effect that *E. mundtii*

ST4SA Sortase C-negative strain had on the exclusion of *L. monocytogenes* from the GIT. If it were the case that *E. mundtii* SrtC-dependent pilus proteins played a major role in the competitive exclusion of *L. monocytogenes* EGDe from the GIT, one would expect much higher *L. monocytogenes* EGDe cell numbers in the intestines of mice in group C-ST4SA.

Listeria monocytogenes EGDe cells excreted in the faeces of mice were then enumerated in all groups and the respective bioluminescent signals emitted quantified as a measure of the colonization ability of the intestinal pathogen in the presence of the probiotic strains. However, it must be noted that enumerations of bacteria in faeces does not truly reflect the number of cells that remain colonized in the GIT (86). In general, there was excellent correlation between the number of *L. monocytogenes* EGDe cells excreted in the faeces of mice in all groups and the respective bioluminescence signals emitted at each time point (Figs 9 and 10). This is also an indication that *L. monocytogenes* EGDe survived conditions in the intestinal tract and is in agreement with previous work (15). *Listeria monocytogenes* EGDe was eliminated more rapidly in the faeces of mice in groups WT-423, B-423, IL-423 than in the PBS placebo control group and A-423 during the first 3 h of the trial (Fig. 9). This result coincided with highest numbers of *L. monocytogenes* EGDe observed in the intestinal tract of mice in groups PBS and A-423 during the same period. At 3 h after infection, high cell numbers of *L. monocytogenes* EGDe were excreted in the faeces of mice treated with either WT *E. mundtii* ST4SA or recombinant *E. mundtii* ST4SA (Fig. 10). The excretion of high cell numbers of *L. monocytogenes* EGDe in the faeces of mice during the first 2 h after infection in groups WT-ST4SA and C-ST4SA suggests that *E. mundtii* ST4SA and *E. mundtii* ST4SA *srtC::erm* initially outcompete *L. monocytogenes* EGDe for intestinal adhesion sites more effectively than the recombinant *E. mundtii* ST4SA strains in the other respective groups (Fig. 10). This also correlates with a significant decrease in *L. monocytogenes* EGDe cell numbers in the intestines of mice in groups WT-ST4SA and C-ST4SA, suggesting that the majority of cells are excreted from the GIT during the first few h after infection with *L. monocytogenes* EGDe.

Viable cell counts of mice treated with either WT *L. plantarum* 423 or recombinant *L. plantarum* 423 confirmed the presence of colonizing cells in the intestines of mice in all groups for the duration of the study period (Fig. 11). Highest cell numbers of *L. plantarum* 423 strains were observed in the large intestinal tract compared to the small intestinal tract. *Lactobacillus plantarum* 423 are reported to colonize the cecum and colon (large intestine) of mice more successfully compared to the small intestinal tract (59). The decline in the

numbers of WT or recombinant probiotic strains over the 48 h period suggests that it is a transient colonization, or at least when in the presence of a competing pathogen. It is interesting to note that, in comparison with *L. plantarum* 423 CFU counts in the intestines of mice in groups B-423, IL-423 and A-423, the cell numbers of WT *L. plantarum* 423 in group WT-423 were always lower despite being the most effective in excluding *L. monocytogenes* EGDe from the GIT (Fig. 11). This supports the theory that single gene deletions (or different probiotic strains) and the presence of pathogens during competitive exclusion experiments may affect probiotic behaviour *in vivo* (56). Another important observation was the detection of high cell numbers of the respective *L. plantarum* 423 recombinant strains in the small intestines of mice in groups B-423, IL-423 and A-423. The fact that *L. monocytogenes* EGDe failed to be excluded from the large intestines of mice in the same groups but was significantly excluded from the small intestinal tracts, implies that the ability to produce bacteriocins or to adhere to intestinal cells may not be central to all anti-infective mechanisms and is likely to be more complex (30, 34). Similar results were obtained for mice treated with WT *E. mundtii* ST4SA and recombinant *E. mundtii* ST4SA, except for mice in group A-ST4SA. After 24 h, the number of viable *E. mundtii* ST4SA *srtA::erm* in the intestines of mice in group A-ST4SA declined to low levels and it was completely excluded from the GIT a further 24 h later. This result supports the proposal that sortase A-dependant cell wall anchored proteins of *E. mundtii* ST4SA are directly involved in bacterial adhesion to intestinal epithelial cells (76, 82). The colonization abilities of the probiotic strains in mice challenged with *L. monocytogenes* EGDe were also assessed by determining the number of cells excreted in the faeces over the 48 h trial period. The probiotic cells in all groups survived passage through the intestinal tract, with high viable cell numbers recorded in the faeces (Fig. 13). This is in agreement with previous *in vitro* and *in vivo* studies that demonstrated the survival of *L. plantarum* 423 and *E. mundtii* ST4SA in the GIT (15, 59, 87). Based on the number of viable cells dosed on a daily basis (4×10^9 CFU), the steady excretion of high cell numbers (approximately 10^6 CFU) of all probiotic LAB strains in the faeces and the presence of high cell numbers in the GIT, it is safe to conclude that the probiotics colonized the GIT readily.

In conclusion, the current study performed a comprehensive evaluation on the impact of *L. plantarum* 423 and *E. mundtii* ST4SA on the exclusion of *L. monocytogenes* EGDe from the GIT of mice. Molecular evidence to underpin the protective and anti-listerial effect of the probiotic strains against orally administered *L. monocytogenes* EGDe, as monitored by

bioluminescence imaging is provided. Bacteriocin production by *L. plantarum* 423 and *E. mundtii* ST4SA represents a direct mechanism of probiotic action in the competitive exclusion of *L. monocytogenes* EGDe from the GIT. A significant increase in viable *L. monocytogenes* EGDe cells in the intestines of mice pre-treated with non-bacteriocin variants of *L. plantarum* 423 and *E. mundtii* ST4SA confirm this. Additional confirmation of *in situ* bacteriocin production as a mechanism of action was provided by using variant strains of *L. monocytogenes* EGDe expressing the immunity genes of plantaricin 423 and mundticin ST, respectively, which provide resistance to the respective bacteriocins. The relevance of probiotic adhesion proteins as *in vivo* probiotic mechanisms of action was demonstrated. Wild-type *L. plantarum* 423 excluded *L. monocytogenes* EGDe from the GIT more effectively than an *aap* adhesion gene knockout *L. plantarum* 423 variant strain, clearly indicating the direct effect of the adhesion protein. Sortase A- and sortase C-dependent adhesion genes of *E. mundtii* ST4SA had a slightly less effective role in the competitive exclusion of *L. monocytogenes* EGDe from the GIT. This is the first *in vivo* study using targeted gene knockout mutants to investigate the direct roles of bacteriocin and adhesion proteins in competitive exclusion experiments in the GIT. Gaining further insights into key antimicrobial and protective mechanisms of probiotics *in vivo* will not only help to improve the credibility of the probiotic concept but also contribute to the identification and selection of the most suitable ones for prophylactic or therapeutic treatments against specific gastrointestinal diseases (64).

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Chapter 5

Anti-infective Gastrointestinal Properties of *Lactobacillus plantarum* 423 and *Enterococcus mundtii* ST4SA, as Determined in Mice Infected with *Listeria monocytogenes* EGDe

Supplementary Material

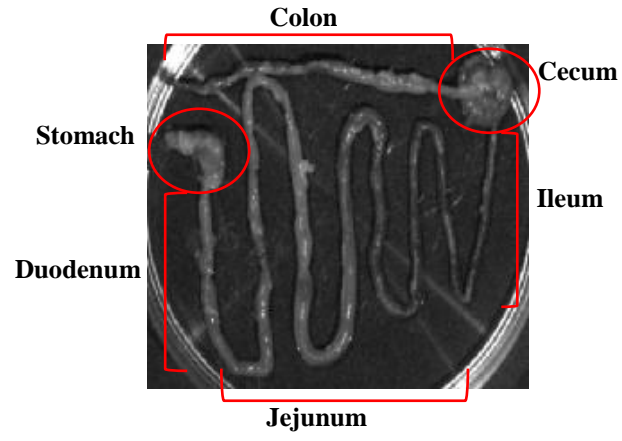


FIG. S1. Regions of dissected mouse intestinal tract

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Chapter 6

General Discussion and Conclusions

General Discussion

Probiotics have an important role in maintaining a healthy and stable intestinal microbiota, primarily by preventing infection (1). As a result, there is currently a growing appreciation for the beneficial properties of probiotic microorganisms (2, 3). The gastrointestinal tract (GIT) is believed to be the major region where orally administered probiotic lactic acid bacteria (LAB) exert their health modulating effects against enteric pathogens to prevent the occurrence of disease (1). Therefore, understanding the specific activities amongst administered probiotic bacteria, the host gastrointestinal system and enteric pathogens is crucial. This will permit the selection of the most suitable probiotic strains for the prevention and/or treatment of specific enteric diseases. Unfortunately, two aspects still poorly understood are the fate of probiotic bacteria in the GIT after oral administration and the underlying relationship between their beneficial effects on health and molecular mechanisms of action. Gaining insight into these interactions will significantly improve the application of probiotics to support and enhance human health. The present study aimed to contribute to this area of research and reports on the use of bioluminescence imaging (BLI) and novel molecular techniques to provide insight into the gastrointestinal persistence of the probiotic strains *Lactobacillus plantarum* 423 and *Enterococcus mundtii* ST4SA, and the mechanisms of their anti-listerial activity.

Persistence of *Lactobacillus plantarum* 423 and *Enterococcus mundtii* ST4SA in the gastrointestinal tract, as reported with bioluminescence imaging in mice

For probiotics to confer their beneficial effects on the host, they must be able to survive through gastrointestinal stresses and persist at high levels in the intestinal tract (3, 4). The analysis of faecal samples is often used to study the survival and persistence of orally administered probiotic bacteria in the GIT (5). The best probiotic strains generally persist in faeces in high numbers and for the longest periods. However, a major limitation of using only faecal recuperation to analyse *in vivo* probiotic persistence is that it does not accurately reflect the amount of cells that remain colonized in the GIT nor does it reveal the bacterial sites of colonization. This study reports on the application of the firefly luciferase gene (*ffluc*) from *Photinus pyralis* to monitor the colonization and persistence of *L. plantarum* 423 and *E. mundtii* ST4SA in the murine GIT. *In vivo* BLI is a non-invasive imaging technique that allows infection rates or colonization dynamics of viable microorganisms to be studied in

real-time (6). As opposed to conventional approaches, one of the key advantages of using the BLI technique is that it allows for drastic reductions in the number of animals to be sacrificed to establish the precise location of bacteria in mouse models (7).

The stable expression of reporter genes during *in vivo* imaging is critical for the detection of luminescent light through animal tissues (6). The expression of the *ffluc* luciferase gene in *L. plantarum* 423 and *E. mundtii* ST4SA was successfully optimized using a high-copy number plasmid and strong constitutive promoters. The presence of plasmids carrying reporter genes in the recombinant LAB strains should not alter the physiology of the host or its ability to survive (6). The *ffluc* expression plasmids were stable in recombinant *L. plantarum* 423 Fluc and *E. mundtii* ST4SA Fluc and bioluminescence did not affect the growth of the respective host strains compared to the wild-type (WT) derivatives. These results indicate that both recombinant LAB strains can produce bioluminescence while stably maintaining the firefly luciferase-expressing plasmids. The bioluminescent plasmids constructed may thus be functional in other species of LAB.

Transcutaneous LAB bioluminescence could be detected in live anesthetized mice after a single oral administration. In a previous study (8) with strains expressing the mCherry fluorescent protein, it was shown that both probiotic strains survived *in vivo* intestinal transit. The current study reconfirmed the survival of the strains using bioluminescence imaging. However, with the mCherry construct, no fluorescence was detected when live anesthetized mice were scanned. Fluorescence was only recorded when sections of the GIT were scanned after animals were sacrificed. Nonetheless, these results clearly demonstrated that both probiotic strains survived transit through the stomach, were resistant to high bile concentrations in the small intestine and were metabolically active in the large intestinal tract.

Different transit times were recorded for *L. plantarum* 423 and *E. mundtii* ST4SA through the GIT of mice after a single oral administration. *Lactobacillus plantarum* 423 had a slower transit time, and was detected in the lower part of the jejunum and in the ileum 45 min post gavage, while *E. mundtii* ST4SA migrated to the ileum, cecum and colon during the same period. After 3 h strong bioluminescent signals of both strains were detected in the cecum and colon. The rapid transit time through the stomach and small intestine may be indicative of the harsh conditions the LAB strains are exposed to in these sections of the GIT and is in agreement with previous studies (9, 10). After 24 h, bioluminescent signals could be detected from the GIT of mice administered with *E. mundtii* ST4SA, while no bioluminescent signals

could be detected from mice administered with *L. plantarum* 423. This indicated that *L. plantarum* 423 was eliminated from the GIT of mice more rapidly than *E. mundtii* ST4SA. It also suggests that *E. mundtii* ST4SA colonizes the GIT of mice at higher numbers compared to *L. plantarum* 423. These findings are in agreement with previous studies (10, 11) reporting on the gastrointestinal transit time of *Lactobacillus reuteri*, *L. plantarum* and *Lactococcus lactis*. The authors showed that the intestinal transit times differed between two strains of *L. reuteri*, and that a *Lc. lactis* strain had shorter survival times in the GIT compared to a *L. plantarum* strain.

In vivo and *ex vivo* bioluminescence imaging confirmed that both probiotic strains persisted in the GIT of mice for several days after five consecutive days of oral administration. The persistence of *L. plantarum* 423 and *E. mundtii* ST4SA compared well to several other commercial probiotic strains, including *Lactobacillus rhamnosus* GG, *Bifidobacterium lactis* LAFTI B94, *L. plantarum* 299v and *Lactobacillus gasseri* SBT2055 (12-15). At day 9 (4 days after the last bacterial dosage), bioluminescent cells of both strains were predominantly localized in the cecum and colon. However, bioluminescent cells of *E. mundtii* ST4SA were also detected in the ileum of mice and persisted in the faeces of mice in higher numbers and for a longer period compared to *L. plantarum* 423, as recorded with bioluminescence imaging and CFU counts. This could explain why *E. mundtii* ST4SA was more effective in reducing *Listeria monocytogenes* EGDe cell numbers compared to *L. plantarum* 423 in an *in vivo* competitive exclusion experiment (16). These results suggested that the persistence of *E. mundtii* ST4SA in the murine GIT is superior to that of *L. plantarum* 423. Since the same dosage and administration methods were used for both strains, the difference in the intestinal persistence between the two probiotic strains might be due to differences in the physiological and genotypic properties of the strains. This merits further investigation. The combination of bioluminescence and mutant analysis may provide a powerful tool to elucidate the differences in intestinal transit and persistence between the two probiotic strains.

Efficient isolation of double-crossover homologously recombined mutants in lactic acid bacteria

The isolation of stable LAB recombinant DNA integration or deletion mutants is often desirable when studying probiotic molecular modes of action (3). The functional effects of genes expressed by LAB *in vitro* or *in vivo* can only be truly assessed by the creation of

knockout (KO) derivatives of strains expressing those genes. However, broadly applicable and efficient methods for the isolation of rare and reliable genetic mutants at any genetic locus are still underdeveloped. The development of techniques that will allow for the easy and efficient selection of chromosomally or plasmid located gene excisions will undoubtedly contribute to this area of probiotic research. To date, most of the techniques used for the isolation of homologous recombination mutants in LAB are limited by a lack of broad applicability amongst species, their inability to target specific gene loci, availability of suitable antibiotic resistance markers and the isolation of reversible single-crossover mutants (17-20). The aim of this study was to develop a method to easily and efficiently isolate rare double-crossover mutants in *L. plantarum* 423 and *E. mundtii* ST4SA at any genomic locus.

The method described here is based on the use of the *Lc. lactis* nisin A inducible promoter (PnisA) and the *E. coli mazF* toxin gene, an mRNA interferase, as a counter-selection marker (21, 22). The PnisA promoter forms part of the well-characterized nisin-controlled expression (NICE) system that has been successfully adapted for gene expression or protein secretion in several LAB species (21, 23). Tightly controlled gene expression is a major advantage of the NICE system over other inducible gene expression systems and it has been used to produce large amounts of enzymes for food, medical or technical applications (24, 25). This advantage rules out any potential MazF toxin induced premature cell growth arrest as a result of promoter leakiness. The appropriate growth conditions required for the *mazF* counter-selection gene to promote cell death upon induction of the PnisA promoter with nisin was optimized successfully. Crucially, the developed method ensures that transformants are eliminated in the presence of nisin-induced MazF toxin when cells have only undergone a single event of homologous recombination (or no homologous crossover at all) and retain a copy of the plasmid-located counter-selection marker in the chromosome or on the episomal plasmid. This ensures the isolation of stable irreversible double-crossover LAB mutants.

The method was used to construct deletions of specific genes, to introduce a gene of interest, to construct an unmarked mutation and to isolate gene deletion mutants using small regions of homology. Large sequences can be inserted or deleted in a series of steps, as demonstrated by the insertion of the *ffluc* bioluminescence gene and erythromycin (Em) resistance marker into the bacteriocin operons or adhesion genes of *L. plantarum* 423 and *E. mundtii* ST4SA. All integration plasmids were designed to delete the majority of the targeted ORF, while ensuring that none of the adjacent genes would be affected. The successful construction of bacteriocin-negative *L. plantarum* 423 and *E. mundtii* ST4SA strains were confirmed by

overlaying MRS agar plates seeded with *L. monocytogenes* EGDe and by recording bioluminescence. Compared to WT derivatives, supernatants isolated from the bacteriocin-negative mutant strains lacked the ability to form clear inhibition zones. Bioluminescence imaging revealed that the integrated *ffluc* gene was actively expressed in each of the bacteriocin KO mutants.

Repeated manipulations of any bacterial chromosome can only be achieved by the generation of markerless integration mutants due to a limited availability of suitable antibiotic resistance markers. Lactic acid bacterial strains can be genetically engineered for biotechnological production processes using an easy to implement marker recycling system. The *Saccharomyces cerevisiae* FLP-FRT recombination system was applied successfully to recycle the Em antibiotic resistance marker in a *L. plantarum* 423 *aap* mucus adhesion gene mutant (26, 27). The yeast FLP-FRT system has been successfully applied in several pathogenic and non-pathogenic Gram-negative and Gram-positive bacteria (28-30). The resulting *aap* KO unmarked strain, free of antibiotic selection markers may be used for further genetic manipulations of the *L. plantarum* 423 chromosome using the newly developed method.

To further demonstrate the strength of this novel system, gene deletion mutants of the *E. mundtii* ST4SA *sortaseA* (*srtA*) and *sortaseC* (*srtC*) genes using homologous arms containing <60 bp regions (5' and 3') homologous to the target genes were generated. Previous studies reported that the rate of recovery of homologous recombinants significantly decreases when the size of homologous regions is less than 200 bp (31-33). Although the developed strategy may be adapted for use in other LAB spp. due to the broad applicability of the nisin expression system, further studies are needed to confirm this. Nonetheless, the easy to implement and highly efficient gene integration/deletion method described here provides the unique opportunity to study the role of specific probiotic LAB genes in complex environments using reverse genetics analysis.

Characterization of the anti-listerial mechanisms of *L. plantarum* 423 and *E. mundtii* ST4SA in the GIT

Intestinal infectious diseases, particularly those caused by enteric pathogenic bacteria, are a major cause of morbidity and mortality at all ages. Listeriosis has a relatively high mortality rate of 20-30% compared to most other food-borne pathogens (34, 35). In the United States, a

multistate outbreak of food-borne listeriosis occurred in 2011, with 147 confirmed cases, including 33 deaths (36). More recently, the largest ever *L. monocytogenes* outbreak occurred in South Africa. Between January 2017 and April 2018, 1011 laboratory-confirmed listeriosis cases were reported, resulting in 193 deaths in just over a year (37).

In most cases, prevention of infection caused by foodborne enteric pathogens relies heavily on good hygiene and sanitation during food preparation (38). Although listeriosis can be treated with several antibiotics, this is not without limitations. First, depending on the form or location of the disease, the cure rate of debilitated patients infected with *L. monocytogenes* can be low (35). Second, the emergence of antibiotic-resistant bacteria is one of the major problems faced by the medical industry today. Bacteria have the ability to acquire resistance against antibiotics via random mutation or the dissemination of specific resistance mechanisms via the exchange of genetic material (e.g. horizontal gene transfer) (39). Therefore, despite having a large arsenal of antibiotics available, it is inevitable that bacteria will undergo mutations or adapt. The inappropriate use of antibiotics and over-prescription further exacerbate this crisis (40, 41). The search for, and development of, alternative prophylactic and therapeutic strategies is therefore of the utmost importance.

Probiotic LAB have considerable potential as alternatives to antibiotics for prophylactic or therapeutic applications in various gastrointestinal disorders (42). Numerous studies have demonstrated the ability of some LAB strains to control the growth of *L. monocytogenes* *in vitro* and *in vivo* (16, 43-48). However, while many reports on probiotic health claims are substantiated by experimental evidence, few address the causal relationship between their health benefiting effects and the underlying mechanisms of probiotic action (3). Molecular elucidation of the mechanisms of probiotic action *in vivo* is crucial to identify and select the most suitable probiotics for the prevention and/or treatment of particular diseases (49, 50). In this study, molecular evidence to underpin the protective and anti-listerial effect of *L. plantarum* 423 and *E. mundtii* ST4SA against orally administered *L. monocytogenes* EGDe in the GIT is provided. A mouse model was used. The role of bacteriocins and adhesion proteins as *in vivo* probiotic mechanisms of action were investigated.

Lactobacillus plantarum 423 and *E. mundtii* ST4SA produce peptide antimicrobial bacteriocins known as plantaricin 423 and mundticin ST, respectively, and are active against numerous intestinal Gram-positive pathogens, including *L. monocytogenes* EGDe (51-53). Bacteriocins are small, heat stable peptides with potent antimicrobial activity and have

received considerable attention as bio-preservatives and as potential alternatives to antibiotics (54). Bacteriocin genes may be located on the chromosome or on plasmids and usually occur in operons encoding a structural protein, a bacteriocin immunity protein and genes for translocation (55). *In vitro* results in this study demonstrated that the growth of WT *L. monocytogenes* EGDe was completely inhibited in the presence of either plantaricin 423 or mundtacin ST. These results are consistent with previous studies that have shown that both plantaricin 423 and mundtacin ST inhibit the growth of *Listeria* spp. (16, 52, 56). *In vitro* expression of the mundtacin ST *munC* bacteriocin immunity gene in *L. monocytogenes* EGDe resulted in a complete loss of sensitivity to mundtacin ST. However, *in vitro* expression of the plantaricin 423 *plaB* in *L. monocytogenes* EGDe resulted only in a partial loss of sensitivity to plantaricin 423. It is possible that the mechanism(s) involved in bacteriocin plantaricin 423 resistance in the WT strain include changes in the cell wall structure, such as fatty acid and phospholipid composition, which could interfere with bacteriocin binding to membrane-located receptors. The absence of these changes in the cell membrane of *L. monocytogenes* EGDe could explain the only partial loss of *in vitro* sensitivity by *plaB*-expressing *L. monocytogenes* EGDe to plantaricin 423. This merits further investigation. Nonetheless, the *in vitro* activity of plantaricin 423 and mundtacin ST against *L. monocytogenes* EGDe suggests a possible mechanism of probiotic action *in vivo*.

Significantly reduced transcutaneous bioluminescence signals from *L. monocytogenes* EGDe were detected from mice that were orally administered with either *L. plantarum* 423 or *E. mundtii* ST4SA for six consecutive days compared to mice that received the PBS placebo control prior to infection. This result served as the first line of evidence to support the gastrointestinal anti-listerial effect of *L. plantarum* 423 and *E. mundtii* ST4SA. *Ex vivo* imaging of dissected intestines and enumeration of viable *L. monocytogenes* EGDe from homogenized intestines confirmed the anti-listerial effect of the probiotics. No bioluminescent *L. monocytogenes* EGDe cells were detected in the intestines of mice administered with either *L. plantarum* 423 or *E. mundtii* ST4SA as early as 4 h after infection compared to the PBS placebo control group. These results correlated with a previous study that demonstrated the ability of *L. plantarum* 423 and *E. mundtii* ST4SA to successfully exclude *L. monocytogenes* from the GIT when administered to mice either individually or in combination (16).

Cell-free bacteriocin-containing culture supernatants of *L. plantarum* 423 and *E. mundtii* ST4SA have been shown to inhibit the invasion of Caco-2 cells by *L. monocytogenes* Scott A

(57). To provide experimental proof of the role of bacteriocin production as an *in vivo* mechanism of action against *L. monocytogenes* EGDe, stable mutants of *L. plantarum* 423 and *E. mundtii* ST4SA that do not produce the bacteriocins plantaricin 423 and mundticin ST, respectively, were constructed. *Lactobacillus plantarum* 423 and *E. mundtii* ST4SA bacteriocin production in *in vitro* GIT models simulating human gastrointestinal conditions have been demonstrated previously (57, 58). Both bacteriocin-negative mutants of *L. plantarum* 423 and *E. mundtii* ST4SA failed to exclude *L. monocytogenes* EGDe from the GIT, in comparison with the WT strains as observed with whole body bioluminescence imaging and by the presence of bioluminescent *Listeria* cells in the small and large intestinal tracts. These results support the proposal that bacteriocin production is an anti-infective mediator against *L. monocytogenes* in the GIT. Previous studies (45, 46) have shown that other bacteriocin-producing LAB strains inhibit *L. monocytogenes* *in vitro* and *in vivo* compared to non-bacteriocin producing strains.

To provide additional confirmation of the *in situ* bacteriocin production in the GIT as a mechanism of action against *L. monocytogenes*, the bacteriocin immunity genes of plantaricin 423 and mundticin ST4SA were expressed in *L. monocytogenes* EGDe. *Lactobacillus plantarum* 423 and *E. mundtii* ST4SA failed to reduce *L. monocytogenes* EGDe bioluminescence or viable bacterial cell numbers in the GIT when mice were administered with *L. monocytogenes* EGDe strains expressing the bacteriocin immunity proteins. Mice challenged with bacteriocin-immune *L. monocytogenes* EGDe strains, displayed similar bioluminescence levels compared to that of the PBS placebo control group and the respective bacteriocin-negative groups. These results confirm the proposal that *in situ* bacteriocin production by the probiotic LAB acts as a direct mechanism of action against infectious *L. monocytogenes* EGDe in the GIT. Results in this study indicates that *in vivo* bacteriocin production as a probiotic mechanism of action is specific for *L. monocytogenes* or other bacteriocin-sensitive pathogens since the alleviation of infection by *L. plantarum* 423 and *E. mundtii* ST4SA in rats challenged with the naturally bacteriocin-resistant *Salmonella enterica* serovar Typhimurium foodborne pathogen is clearly not linked to bacteriocin production (59).

Probiotic LAB expressing adhesion genes are able to successfully compete with intestinal pathogens for adhesion sites on epithelial cell surfaces, which help to prevent pathogens from colonizing (53, 60, 61). The surface proteins displayed by probiotic LAB play a key role in their interaction with intestinal epithelial cells and mucus by promoting adhesion,

colonization and the acquisition of essential nutrients (50, 62). These specific interactions have also been associated with probiotic competitive exclusion of pathogens (50, 64-66). The role of several surface bound proteins of *L. plantarum* 423 in the adhesion of the strain to Caco-2 cells and in the competitive exclusion of pathogens *in vitro* was demonstrated previously (53). Chemical removal of selected surface-bound proteins of *L. plantarum* 423 reduced the adhesion of the strain to Caco-2 cells and altered the strain's competitive ability *in vitro* (53). To assess the involvement of adhesion proteins in the probiotics' gastrointestinal anti-listerial effect, groups of mice were administered (for 6 consecutive days) with either a *L. plantarum* 423 mutant lacking the *aap* adhesion associated gene, or *E. mundtii* ST4SA lacking the *srtA* or *srtC* genes associated with the anchoring of cell surface proteins to the cell surface, before challenge with *L. monocytogenes* EGDe.

Using *in vivo* and *ex vivo* bioluminescence imaging, it was demonstrated that *L. plantarum* 423 *aap::erm* (lacking the *aap* adhesion gene) failed to exclude *L. monocytogenes* EGDe from the GIT of mice compared to WT *L. plantarum* 423. These results were confirmed by the presence of high numbers of viable *L. monocytogenes* EGDe cells in the large intestinal tract of mice administered with *L. plantarum* 423 *aap::erm* between 24 and 48 h after infection. In a previous study, expression of the *L. plantarum* 423 Aap protein was shown to be extensively up-regulated under normal physiological concentrations of bile and pancreatin, simulating GIT conditions (67). Furthermore, the role of several *Lactobacillus* spp. cell wall proteins in adhesion to enterocytes *in vitro* and in *in vivo* intestinal persistence is well documented (68-71). The results presented here indicate that the *L. plantarum* Aap adhesion protein is actively involved in the exclusion of the *L. monocytogenes* EGDe from the GIT.

In Gram-positive bacteria, sortase enzymes covalently join cell surface proteins to the cell wall (Sortase A) or polymerize proteins to construct complex multi-subunit pilin structures (Sortase C) on the cell surface (72). *In silico* analysis of an *E. mundtii* ST4SA draft genome sequence and all available fully annotated reference *E. mundtii* full genome sequences, revealed 18 putative Sortase A-dependent cell wall anchored proteins, two putative accessory pilus subunits (EbpAfm and EbpCfm) and a major pilus subunit protein (*EbpCfm*). *In vivo* whole body bioluminescence signals for mice administered with stable mutants of *E. mundtii* ST4SA lacking the *srtA* (group A-ST4SA) and *srtC* (C-ST4SA) cell wall protein sorting enzymes were only slightly higher than those observed for mice administered with WT *E. mundtii* ST4SA and significantly lower than those recorded for mice in the PBS placebo control group between 2 and 5 h after infection. However, actively growing bioluminescent

cells in dissected intestines of mice in groups A-ST4SA and C-ST4SA were detected compared to no bioluminescent cells detected in the WT-ST4SA group. Taken together, these results indicate that the Sortase A- and Sortase C-dependent adhesion genes of *E. mundtii* ST4SA have a slightly less effective role in the competitive exclusion of *L. monocytogenes* EGDe from the GIT compared to the WT strain. It has been reported previously, that Sortase A plays a role in the *in vitro* adhesion of bacterial cells to human cell lines, intestinal colonization and virulence (73-75). Recently, it was demonstrated that *L. rhamnosus* GG outcompeted vancomycin-resistant *E. faecium* via mucus-binding pili *in vitro* (76). The role of sortase C and sortase C-dependent pilus-encoding genes in bacterial adhesion to epithelial cells, host invasion and biofilm formation have been studied extensively in virulent *E. faecium* and *E. faecalis* strains (77, 78).

Concluding remarks and future perspectives

Understanding the full potential of probiotics for therapeutic or prophylactic applications against gastrointestinal diseases requires thorough investigations of probiotic-host and probiotic-pathogen interactions. Enhanced understanding of these interactions will enable the identification of true probiotics to target specific enteric diseases. The BLI imaging technique and a novel molecular recombination system were successfully applied to gain crucial insights into the persistence of *L. plantarum* 423 and *E. mundtii* ST4SA in the GIT and the molecular mechanisms of action against *L. monocytogenes* EGDe.

The application of the BLI technique allowed demonstration of the precise location of the bacterial strains and their persistence within the murine GIT after single or multiple doses. To exploit all the beneficial effects of these two organisms to improve human health, understanding the fate of the individual strains in the GIT is an important step. Future recommendations will encompass the expression of different reporter genes in each probiotic strain to assess possible *in vivo* interactions between the two strains that may potentially affect their individual colonization dynamics in the GIT.

A novel selection/counter-selection system that facilitates the rapid and efficient isolation of double-crossover integration/deletion mutants of *L. plantarum* 423 and *E. mundtii* ST4SA was developed. The system was useful in the construction of *L. plantarum* 423 and *E. mundtii* ST4SA bacteriocin and adhesion gene mutants. The newly described method expands the LAB molecular research genetic toolkit and has significant potential to allow genetic

modification of most, if not all LAB species. Besides mutant analysis, the method may be used to expand the repertoire of beneficial probiotic strains by genetically engineering designer probiotics for the treatment or prevention of particular gastrointestinal diseases. The genetic modification of not only other LAB species but also other Gram-positive bacteria using the described method is underway. The system also has significant potential for application in Gram-negative bacteria in future research.

Finally, this study showed that the probiotics strains *L. plantarum* 423 and *E. mundtii* ST4SA can competitively exclude *L. monocytogenes* EGDe from the GIT. Additionally, valuable information is gained on the *in vivo* molecular mechanisms of the probiotic strains responsible for the protective and antimicrobial effect against *L. monocytogenes* EGDe. The characterization of other potential mechanisms of probiotic action against gastrointestinal pathogens such as the inactivation of novel adhesion genes should be considered for future research. Although the objectives of this study have been achieved, greater emphasis is required in this area of probiotic research. Unravelling the intricacies of probiotic-host and probiotic-pathogen interactions will not only improve the *in vitro* selection of the best probiotics based on key properties such as bacteriocin production and adhesion genes but will also increase the likelihood of *in vivo* probiotic efficacy. Demonstrating key antimicrobial and protective probiotic mechanisms *in vivo* will allow for industry and consumers to choose scientifically validated probiotics for the prevention or treatment of various health problems.

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