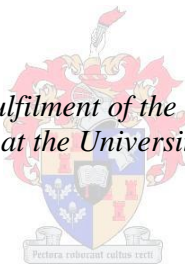


**Fluorescence and bioluminescence imaging of the
intestinal colonization of *Enterococcus mundtii* ST4SA and
Lactobacillus plantarum 423 in mice infected with *Listeria
monocytogenes* EGde**

by

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*Thesis presented in partial fulfilment of the requirements for the degree
Master of Science at the University of Stellenbosch*



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March 2015

Declaration

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

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Summary

Lactic acid bacteria (LAB) are common inhabitants of the human gastro-intestinal tract (GIT). Some LAB, especially lactobacilli, are well known for their application in fermented foods and probiotic properties. These microorganisms exert many beneficial effects on human health, such as digestion and assimilation of food and preventing pathogens colonising the GIT. Furthermore, some selected probiotic strains are believed to perform a critical role in the treatment of gastro-intestinal disorders, lactose intolerance and in the stimulation of the immune system.

Despite the ever increasing consumer interest in probiotic LAB, the mechanisms by which they exert their beneficial effects and the activities of probiotics in the GIT often remain poorly understood. Understanding survival mechanisms of LAB in the GIT, especially the interaction between LAB and pathogens, would be facilitated by the direct *in vivo* monitoring of these processes.

Using the *mCherry* fluorescence gene, we successfully constructed *Lactobacillus plantarum* 423 and *Enterococcus mundtii* ST4SA reporter strains. With this study we showed that fluorescence imaging can be used to detect *Lb. plantarum* 423 and *Ent. mundtii* ST4SA in the GIT of mice. The two species colonized the cecum and colon for at least 24 h after one oral administration. To our knowledge, this is the first report on fluorescence imaging of LAB expressing mCherry in a mouse model.

Using a bioluminescence marker system, we evaluated the impact of *Lb. plantarum* 423 and *Ent. mundtii* ST4SA on orally acquired *Listeria monocytogenes* infection and the ability of the probiotics to compete with the pathogen in the GIT of mice. Challenging *Lb. plantarum* 423 and *Ent. mundtii* ST4SA that were already established in the GIT of mice with *L. monocytogenes* EGDe had no effect on the survival and persistence of the probiotic strains.

We demonstrated that the colonization of mice with *Lb. plantarum* 423 and *Ent. mundtii* ST4SA, or a combination of the strains, protected the animals against colonization of the GIT by *L. monocytogenes* EGDe. *Enterococcus mundtii* proved more effective than *Lb. plantarum* 423 in reducing the number of *L. monocytogenes* EGDe in the mouse model.

Opsomming

Melksuurbakterieë (MSB) kom algemeen in die mens se spysverteringskanaal (SVK) voor. Verskeie MSB, veral lactobacilli, is bekend vir hul gebruik in gefermenteerde voedsel en as probiotika. Die bakterieë het baie eienskappe wat die mens se gesondheid kan bevoordeel, insluitend vertering en assimilasië van voedsel en voorkoming van kolonisering van die SVK deur patogeeniese bakterieë. Sekere probiotiese rasse speel ook 'n belangrike rol in die behandeling van SVK verstoringe, laktose intoleransië en die stimulering van die immuun stelsel.

Alhoewel die belangstelling in probiotiese bakterieë toeneem, is daar min inligting bekend oor die meganismes wat MSB gebruik om hulle voordelige eienskappe in die SVK uit te voer. Die oorlewing van MSB in die SVK, veral die interaksies tussen MSB en patogene, kan met behulp van 'n *in vivo* moniteringsstelsel bestudeer word.

Deur die *mCherry* fluoresensie geen in *Lactobacillus plantarum* 423 en *Enterococcus mundtii* ST4SA te kloner, het ons daarin geslaag om 'n effektiewe verklikker stelsel te ontwikkel en kon die voorkoms en migrasie van die twee spesies in die SVK van muis bestudeer word. *Lactobacillus plantarum* 423 en *Ent. mundtii* ST4SA het veral die blindederm en kolon gekoloniseer. Beide rasse het na 'n enkele mondelinge toediening vir ten minste 24 h in die SVK oorleef. Sover ons kennis strek, is hierdie die eerste verslag van fluoreserende MSB wat met behulp van die *mCherry* geenprodukt in die SVK bestudeer is.

Deur gebruik te maak van 'n bioliggewende verklikker stelsel, het ons die vermoë van *Lb. plantarum* 423 en *Ent. mundtii* ST4SA om met *Listeria monocytogenes* in die SVK te kompeteer, bestudeer. *Listeria monocytogenes* het geen invloed gehad op die kolonisering van *Lb. plantarum* 423 en *Ent. mundtii* ST4SA nie. Deur die muis vooraf met *Lb. plantarum* 423 en *Ent. mundtii* ST4SA te koloniseer (in kombinasie of met net een van die twee rasse), kon

ons daarin slaag om koloniserings van die SVK met *L. monocytogenes* te voorkom. In die muis model wat in hierdie studie gebruik is, was *Ent. mundtii* ST4SA meer effektief as *Lb. plantarum* 423 in die verlaging van *Listeria* selgetalle.

This thesis is dedicated to my best friend and fiancé Lauren Jansen

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.

Preface

All chapters have been written according to the instructions for the Journal of Applied and Environmental Microbiology.

Acknowledgements

I sincerely want to thank:

My family and friends for their constant motivation and support.

Prof. L.M.T. Dicks (Department of Microbiology, University of Stellenbosch) for granting me this opportunity and all his support and guidance.

Dr. S.D. Deane for her valuable insight and assistance with some of the experiments.

All my co-workers in the lab and Department of Microbiology for their insight and moral support.

The National Research Foundation (NRF) of South Africa for financial support.

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Chapter 1

Introduction

Introduction

Reporter genes are invaluable when studying gene expression, the labelling of bacteria for molecular biology studies and biomedical tests, and in research on cellular processes and microbial interactions. Reporter proteins used as molecular markers are essential to the better understanding of all cellular processes and microbial functioning. Microorganisms are easily identified *in vivo* and over a prolonged period, when the expression of reporter genes are detected with sensitive imaging (1, 2), such as the Caliper *in vivo* imaging system (IVIS). These proteins have to be expressed constitutively under physiological conditions (3). Research using reporter systems is thus important in understanding the competition between antimicrobial-producing probiotic bacteria and disease causing pathogens.

The common functional limitations and drawbacks of most reporter proteins are low photo stability, photo bleaching and slow maturation of the expressed proteins (4). One of the new variants of a reporter gene is the mCherry red fluorescent protein (rfp), derived by mutagenesis from the more functionally limited tetrameric rfp from *Discosoma*, also known as DsRed (5). The excitation and emission spectra of rfp's occur in the region of the spectrum where auto-fluorescence is minimal, rendering them the preferred choice as molecular markers for *in vivo* experiments (6). In addition, the protein expressed by the *mCherry* gene is stable and resistant to photo-bleaching (4).

One of the best examples in which reporter systems are used, is the study conducted by Mortin et al. (2007) in the evaluation of daptomycin against infection by *Staphylococcus aureus* (7). A luciferase-labelled strain of *S. aureus* was used in a murine model. The drug was later approved by the Food and Drug Administration (FDA). The same technology was used to study the spread of an infection in mice caused by *Listeria monocytogenes* (8).

Rat and mouse models are the predominant choice for *in vivo* evaluation of probiotic properties and have been used to study the persistence and localization of lactic acid bacteria (LAB) that show potential as probiotics (9, 10). Probiotic bacteria are evaluated by the ability of the strains to colonise the intestinal tract or the ability to compete against pathogens for adhesion sites (11, 12). Most studies performed on probiotic LAB are based on *in vitro* tests and models simulating the human gastro-intestinal tract (GIT) (9, 11, 13). However, data generated from these studies can only be used to predict the survival of LAB and serve only as an indication as to where the strain may potentially colonize the GIT. Conventional *in vivo* approaches are frequently limited by the need to sacrifice large numbers of animals to establish the precise localization of these bacteria. Thus, a better understanding of the persistence and colonization of LAB would be facilitated by direct *in vivo* monitoring of these biological processes in animals.

Lactobacillus plantarum 423 and *Enterococcus mundtii* ST4SA are excellent probiotic strains (14, 15). They are safe to use and survive conditions that simulate the GIT, as determined by a computerised GIT model (9, 11, 14). Both strains inhibited the growth of *L. monocytogenes* ScottA and adhered to Caco-2 cells (9, 15). The strains also reduced symptoms associated with *Salmonella enterica* serovar Typhimurium infection in Wistar rats (11).

The present study evaluated fluorescent imaging (FI) in the real time monitoring of *Lb. plantarum* 423 and *Ent. mundtii* ST4SA stably expressing the *mCherry* fluorescent gene. The *mCherry* gene has been codon optimized to enhance expression in *Lb. plantarum* 423 and *Ent. mundtii* ST4SA. Due to a growing interest in LAB in the biological sciences and industry, there is a clear need for a more extensive range of genetic tools to facilitate their study. The mCherry fluorescence marker was used to establish whether *Lb. plantarum* 423 and *Ent. mundtii* ST4SA persists and colonises the GIT of mice. In addition, the viability and duration of colonization was determined. The effect of *Lb. plantarum* 423 and *Ent. mundtii* ST4SA on

the colonization of *L. monocytogenes* in the GIT of mice were also investigated. Mice colonized with labelled *Ent. mundtii* ST4SA and *Lb. plantarum* 423 were used to determine whether these probiotic strains could inhibit intestinal infection by a bioluminescent *L. monocytogenes* strain. Using bioluminescence, the progression of infection could be monitored in real time without sacrificing the animals.

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Chapter 2

Literature Review

1. Introduction

Bioluminescence (BLI) and fluorescence imaging (FI) are probably the most effective methods to detect viable microorganisms in living tissue. Optical imaging is highly sensitive, non-toxic and detects a signal from the excitation of fluorescent proteins, or through an enzyme (luciferase)-catalysed oxidation reaction (1, 2). Moreover, optical imaging allows for the non-invasive detection of microorganisms from within living tissue (3). Thus, optical imaging technologies are at the forefront of shedding light on our understanding of numerous host-microorganism interactions, including the beneficial health effects probiotic microorganisms may have (4-6). *In vivo* application of BLI and FI has the added advantage of reducing the number of animals required, and enables researchers to obtain more information in less time compared to traditional pre-clinical animal models (7-8). The development of recombinant bioluminescent/fluorescent microorganisms allows for the real-time monitoring of their spatial and temporal persistence in living hosts.

This review discusses several advances in *in vivo* bioluminescence and fluorescence imaging and the limitations encountered. The potential of applying optical imaging to lactic acid bacteria (LAB) to improve our understanding of their survival in the gastro-intestinal tract (GIT) is discussed. The idea of using BLI to monitor *Listeria monocytogenes* infection in mice colonized with LAB is also reviewed.

2. Reporter Systems

Genes are selected as reporters when the characteristics of the expressed protein(s) allow easy detection of the cells carrying the reporter in a complex microbial environment (9). A number of reporter molecules with improved activity have been developed over the past two decades (10-13). Light emitted by these molecules is detected using advanced photon detectors in charge-coupled device (CCD) cameras mounted within light-tight specimen chambers (14).

Examples of useful imaging systems that are commercially available, including the various features of each, are listed in Table 1. Over the past decade, advancements in detection systems have significantly improved sensitivity and allowed for the detection of weak *in vivo* light sources emitted by reporter proteins (15).

Before developing a bioluminescent or fluorescent microorganism, the suitability of the reporter gene for a given experiment must be assessed, based on several factors. When a bioluminescent phenotype is desired, the choice of the luciferase system generally depends on the microorganism of interest. An inherent advantage of the bacterial luciferase system is the ability to produce the substrate required for light production, thus it does not require the substrate to be added exogenously. However, it is known that the *lux* genes are generally not well-expressed in Gram-positive bacteria (6, 16). Some versions of the *lux* genes have been developed in which the operon has been modified with the insertion of Gram-positive ribosomal binding sites (4, 17). When a fluorescent phenotype is required, numerous factors have to be taken into consideration including the excitation and emission wavelengths, photostability and maturation speed (7).

A vast array of reporter systems is available (Tables 2 and 3). The most popular include bioluminescence imaging with bacterial *lux* genes (*lux*), firefly and click beetle luciferase (*luc*) and fluorescence imaging, i.e. green and red fluorescence proteins (34-36). Other well established imaging systems include magnetic resonance imaging (MRI) and positron emission tomography (1, 12). Bioluminescent and fluorescent reporters are the most popular choices when tagging bacteria for *in vitro* and *in vivo* studies and will be focussed on in this review.

2.1 *In vivo* Fluorescence

Just over 150 years has passed since John Herschel first described fluorescence in 1845. He observed a blue glow shining from a solution of fluorescent quinine sulfate (37). Today, fluorescent proteins (FPs) and probes are used universally in biological and molecular research. Fluorescence is simply defined as the emission of light from a chemical substrate upon light excitation. Green and red fluorescent proteins are discussed.

2.1.1 Fluorescent proteins

Fluorescent proteins are versatile, genetically encoded *in vivo* reporters, that are easily imaged and they have become invaluable tools in biological and biotechnological sciences (18, 38). These proteins are widely used to tag other proteins, eukaryotic cells and microorganisms (39, 40). Vectors expressing modified variants of red and green fluorescent proteins are used extensively as reporters in bacteria and will be discussed in detail. A comparison of three of the most common and widely used reporter systems that confer identifiable characteristics on the organisms expressing them are listed in Table 4. For a fluorescent protein to be effectively used as a reporter molecule for *in vivo* optical imaging, it must not lose FP emission when constantly illuminated and has to reach peak intensity within a specific time period, which is referred to as maturation time of the full chromophore (7).

2.1.1.1 Green fluorescent protein

Green fluorescent protein (GFP) is encoded by a gene that was originally isolated from the bioluminescent jellyfish, *Aequoria victoria* (41). Osamu Shimomura, Roger Tsien and Martin Chalfie were awarded the Nobel Prize in Chemistry for the remarkable impact GFP technology has on research in life sciences. The first GFP gene was cloned and expressed in other organisms, including *E. coli*, during the early 1990's (42). Since then, numerous

derivatives of GFP with enhanced fluorescence and the potential to be expressed in a wide variety of organisms have been constructed.

Cells expressing GFP, and derivatives thereof, emit light in the blue, green and yellow range of the spectrum. The GFP protein emits light when excited by long-wavelength UV-light, without the requirement for exogenous substrate or complex media (34). When the GFP gene is cloned and transformed into the genome of a living organism, the resultant fluorescent protein functions as a reporter molecule for detection and visualization of cells and even whole organisms under UV-light.

Green fluorescent proteins have been expressed in numerous LAB species, such as *Lb. sakei*, *Lb. fructosus*, *Lb. delbrueckii* subsp. *lactis* and *Ent. faecalis* (43-45). Most notably, Yu and co-workers (2007), illustrated that GFP could be used to detect *Lactobacillus* spp. in the gastro-intestinal tract (GIT) of chickens (43).

2.1.1.2 Red fluorescent proteins

A wide variety of FPs have been discovered and developed since the first application of GFP as a marker of gene expression in the nematode *Caenorhabditis elegans* (46). This includes the discovery of the DsRed FP from the coral *Discosoma* sp. and the development of a range of far-red FPs termed the 'mFruits' (18). The use of the DsRed fluorescent protein in live-cell imaging was hampered due to its slow maturation time (caused by its tetrameric form) and low photo stability (47). The 'mFruit' FPs, developed in the laboratory of Roger Tsien are based on molecular-directed evolution of the DsRed FP (18). The characteristics of FPs that render them most suitable for optical imaging are listed in Table 2.

To improve maturation time, brightness and photo stability, optimized variants of RFP had been developed, of which mCherry is one of the best known derivatives (18, 48). Additionally, the *mCherry* gene was used successfully as a reporter in several *in vitro* studies

(49, 50). The mCherry protein has a monomeric structure which matures from a nascent polypeptide into a folded fluorescent form within 15 min. In addition, the mCherry molecule reaches 100% fluorescence in less than 2 h in *E. coli*, and matures four times faster than the DsRed protein it was derived from (18).

The monomeric structure of the mCherry fluorescent protein makes it ideal for protein fusions, as monomeric proteins tend to be the least disruptive to the function of the protein to which they are fused. The mCherry protein is non-toxic, and it can be expressed at high levels without adding any unwanted physiological stresses on host organisms (5). The mCherry fluorophore is also highly resistant to photo bleaching, which means that the fluorophore will not lose its ability to fluoresce during continuous illumination (51). The coding sequence of the *mCherry* gene has also been codon-optimized to be expressed in mammalian cells.

2.1.2 Alternatives to fluorescent proteins

2.1.2.1 Near-infrared molecular probes

Genetic engineering of microorganisms is often time consuming and requires a considerable level of expertise in molecular biology. Therefore, although genetically encoded markers have proven to be very useful in the biological sciences, they are not always easy to apply. A second strategy employs an injectable near-infrared molecular (NIR) probe consisting of a fluorescent reporter group (52). The authors used a synthetic zinc(II) dipicolylamine (Zn-DPA) coordination complex that binds to the anionic surfaces of bacterial cells. Carbocyanine dye, as NIR probe, attaches to an affinity group with two Zn-DPA units on the bacterial cell surface, which leads to the selective staining of the whole cell. The resulting bacterial imaging probe has an excitation wavelength of 794 nm and emission wavelength of 810 nm. By using *in vitro* fluorescence microscopy, the authors have shown that the probe can be used to stain

the periphery of *Staphylococcus aureus* cells. In addition, they also demonstrated that the probe could be used *in vivo* to target *S. aureus* in a mouse leg infection model (52).

2.1.3 Limitations and side effects of fluorescent proteins in marker applications

In recent years, bacterial imaging has become an emerging technology with many applications in biological science. Despite this, there are numerous limitations and challenges that affect the application of molecular markers (2). Optical imaging methods such as radio-imaging and MRI, are much more developed than the *in vivo* imaging of bacteria (52). A clear drawback of optical imaging within live animals is the penetration of light through tissue. Although higher wavelength bacterial imaging systems such as those using NIR probes can penetrate readily through animal tissue, the imaging of bacterial cells in deeper tissues with higher background fluorescence could be problematic (7). The deeper the tissue penetration, the more bacterial cells would be required to produce a detectable fluorescent signal.

In many applications, the use of fluorescent proteins such as DsRed is hampered by a slow maturation time, photobleaching, oligomerization, brightness of the chromophore and cell toxicity (18). According to Wiedemann et al. (2009), a critical characteristic of FPs suitable for *in vivo* applications is their brightness (2). Brightness is defined as the combination of the capabilities of the chromophores to absorb excitation light and to re-emit photons. Furthermore, the brightness of a chromophore is significantly influenced by factors such as the total number of functional molecules expressed, the strength by which it is transcribed or translated and how many of the proteins develop into mature chromophores.

One of the most adverse side effects FPs may have on living cells and tissues is phototoxicity. Light of short wavelengths induces a phototoxic effect in cells (53, 54). Many of the negative effects related to the exposure of living cells to UV and visible light can be avoided by the use of microscopic methods that employ the use of infrared light for imaging of FPs (55).

2.2 *In vivo* bioluminescence imaging

Bioluminescence is defined as the release of energy by a chemical reaction in the form of light emission. Bioluminescence is a naturally occurring process and is widespread in nature. Biological entities such as bacteria, eukaryotic cells or genes can be labelled with a reporter gene or gene complex that encodes light-producing enzymes known as luciferases (14, 32).

A diverse set of organisms can produce bioluminescent light, and it occurs most commonly in bacteria, marine crustaceans, fungi, fish and insects (7). Luciferase enzymes produced by these organisms generate visible light that arises from the oxidation of a substrate in the presence of ATP as an energy source (11). The most commonly used bioluminescent systems applied in *in vivo* optical imaging originate from bacteria (*Photobacterium luminescens*), the firefly (*Photinus pyralis*) and click beetle (*Pyrophorus plagiophthalmus*) and the marine copepod *Gaussia princeps* (7).

2.2.1 Bacterial lux bioluminescence system

Luminous bacteria are the most widely distributed light-emitting organisms, with the majority existing in seawater and the remainder living in terrestrial or freshwater environments. The bacteria are classified into three major genera; *Photobacterium*, *Vibrio* and *Photobacterium*. Species existing in the marine environment are mainly grouped into the genera *Photobacterium* and *Vibrio*, with the terrestrial species being classified in the genus *Photobacterium* (56). Species found in the seawater environment are generally free-living, but the majority live in symbiosis with host organisms that include fish, squid and nematodes (57).

For bacterial bioluminescence production, a molecular substrate reacts with oxygen to create light. The luciferase enzymes act as a catalyst to speed up this reaction. The expression of

genes related to bioluminescence is controlled by the lux operon (58). The lux operon used in most molecular studies (16, 32), was originally isolated from a *Photobacterium* sp (Fig. 1).

Bacterial luciferase is encoded by the structural genes *luxA* and *luxB*. Luciferase is thus a heterodimer composed of two different polypeptides known as alpha and beta, respectively. Substrates for the bacterial luciferase are reduced flavin mononucleotide (FMN_{H2}), oxygen (O₂) and long chain fatty aldehyde produced by the fatty acid reductase complex that is encoded by the *lux* genes *CDE* (Fig. 1). Two regulatory genes, *luxR* and *luxI*, encoding the regulatory protein and auto-inducer synthetase, respectively, are also required for light emission.

One of the most significant advantages of the bacterial luciferase system is the ability of the bacteria to synthesize the substrate (long chain aldehydes and FMN_{H2}) required for light production. When eukaryotic bioluminescence systems such as the click beetle luciferase are expressed by bacteria, the substrate (luciferin) has to be added exogenously (6). The optimum temperature of the *P. luminescence* lux operon lies within the same range as that of mammalian tissues, making it one of the most suitable bacterial luciferase systems for *in vivo* applications (59).

The bacterial luciferase system is predominantly used when bacterial infection models are studied (16, 60). Example of this are the cloning of the *P. luminescence* lux operon into infectious pathogens such as *E. coli*, *Citrobacter rodentium* and *Salmonella* spp. (26, 28, 36). Pathogenicity of the labeled bacteria remained unaffected and they were visualized throughout infection. Bacteria labelled with the *lux* genes can be detected in the tissue of mice, revealing the precise location of the infection. Furthermore, the integration of the *lux* genes into the chromosome of bacteria significantly increases the stability in terms of the light emitted and is generally preferred over the use of plasmid expression.

2.2.2 Firefly bioluminescence system

Other than the bacterial luciferase, the most commonly used luciferases for *in vivo* bioluminescence imaging are the luciferase (*luc*) genes from the North American firefly, *P. pyralis*, and the click beetle, *P. plagiopthalmus*. Expression in bacteria requires codon-optimization of the *luc* genes (6). The firefly luciferase enzyme is approximately 62 kDa in size and is encoded by a single gene, *luc* (61). The luminescence reaction involves the oxidation of the native D-luciferin (D-(-)-2-(6'-hydroxy-2'benzothiazolyl) thiazone-4-carboxylic acid) substrate in the presence of Mg-ATP, resulting in the production of oxyluciferin, CO₂ and the emission of light (14, 62).

The *P. pyralis* luciferase enzyme produces light with an emission peak at 560 nm (Hastings, 1996). However, the generation of light by firefly luciferase is affected by temperature, producing light at a peak of 610 nm at 37°C, rendering it suitable for *in vivo* applications (14). Researchers have developed a number of *luc* genes with improved expression in bacterial and eukaryotic cells, with shifted emission spectra by genetically modifying the wild type *luc* genes (58).

The amount of light emitted in relation to energy consumed by the firefly luciferase represents the most efficient bioluminescent reaction known and is therefore the most attractive choice for expression by mammalian cells (7, 63). Researchers have expressed the firefly luciferase successfully in the muscle tissue and nasal airways of mice (64, 65). Daniel and co-workers (6) tagged lactic acid bacteria with the *P. pyralis* luciferase using a plasmid expression system, revealing valuable information about the spatio-temporal persistence of the bacteria in mice.

2.2.3 Limitations and side-effects of luciferase systems in marker applications

One of the most significant potential drawbacks of the bacterial and firefly luciferases is the relation of bioluminescent light production to the metabolic activity of the microorganisms expressing the luciferase enzymes (7). Luciferases rely heavily on the availability of microbial metabolites in order for the light reactions to occur continuously. Bacterial luciferase relies on the bio-availability of FMNH₂, whereas firefly luciferase requires ATP. This is important to note during *in vivo* infection studies, as a decrease or an absence of bioluminescence production by marked pathogens could be due to the bacteria being in stationary growth phase and not due to eradication of the infection (15, 17, 26). Supporting this notion, an absence of bioluminescence production as a result of bacteria being in a phase of dormancy has been reported for *lux*-expressing *Leishmania amazonensis* (66) and *Mycobacterium smegmatis* (5).

The absorption of light by mammalian tissues is one of the main factors influencing the sensitivity and detection limits of optical imaging (Table 5). Haemoglobin serves as one of the most significant causes of light absorption in animals (67). While haemoglobin absorbs light in the visible spectrum (400-760 nm), longer wavelengths of light (above 600 nm) can propagate through mammalian tissues more readily (68). Furthermore, melanin influences the absorption of light in animals with dark fur. Nude animals or animals with white fur are thus more suited for *in vivo* optical imaging studies.

Since luciferases are oxygenases, molecular oxygen is a basic requirement for all luciferase enzymes to produce light. The lack of oxygen may thus limit the application of luciferases as reporters in anaerobic environments such as the gastro-intestinal tract (GIT) (14). However, oxygen enters the GIT via diffusion from oxygen-rich tissue surrounding the lumen. A low level of oxygen was sufficient for production of detectable levels of bioluminescence by *lux*-expressing *Citrobacter rodentium* cells that colonized the murine GIT as reported by Wiles and co-workers (26).

3. Probiotic lactic acid bacteria

Probiotics are defined as microorganisms that provide the consumer with health-promoting effects when ingested in certain numbers (69). Over 2000 years has passed since the ingestion of live bacteria was recorded for the first time (70). However, in the early 20th century Nobel prize recipient Elie Metchnikoff was the first to suggest that colonizing the gut with beneficial flora could exert beneficial effects on the host (71). Since then, a remarkable number of microorganisms have been described as probiotics.

Some of the most notable potential beneficial effects of probiotics are the alleviation of constipation, treatment and prevention of atopic dermatitis, cancer treatment, prevention of cardiovascular incidents and the treatment of irritable bowel syndrome, also known as IBS (70, 72, 73). However, for the maintenance of a healthy human gastro-intestinal microflora and treatment of GIT disorders, lactic acid bacteria are the most important species used as probiotics (74, 75). Moreover, species of the genera *Lactobacillus* and *Bifidobacterium*, dominate a large percentage of the probiotic market (76). The evaluation of two relatively new LAB strains as probiotics will be discussed. *Lactobacillus plantarum* 423 and *Ent. mundtii* ST4SA are commercially available as probiotics and are distributed by Cipla Medpro (Pty) Ltd., South Africa. Both strains have excellent adhesion properties and displace *Clostridium sporogenes* and *Ent. faecalis* in competitive exclusion experiments (77). Moreover, both strains constantly produced antimicrobial peptides active against a number of commonly occurring intestinal Gram-positive pathogens (78).

3.1 *Lactobacillus plantarum* 423

Lactobacillus plantarum is encountered in many habitats, including fermented milk, meat and vegetables. The species is a naturally occurring inhabitant of the human GIT (79). *Lactobacillus plantarum* 423 was originally isolated from sorghum beer (80). Probiotic

properties of strain 423 have been reported on in extensive *in vitro* and *in vivo* studies. *Lactobacillus plantarum* 423 is also regarded as safe to use as a probiotic as was determined in trials using Wistar rats (78). The bacterium was administered to animals via intragastric gavage over a period of 14 days and the rats showed no physical or behavioural abnormalities or changes in bodyweight, suggesting that *Lb. plantarum* 423 is not pathogenic. Using fluorescent *in situ* hybridization (FISH) with strain specific probes, it was indicated that *Lb. plantarum* 423 colonises the lower section of the small intestine. However, this method was insufficient in providing information on the amount of viable cells in the different sections of the GIT and feces (78).

Previous studies reported that *Lb. plantarum* 423 survived the harsh conditions of the GIT (78, 81, 82). Furthermore, an *in vitro* model simulating the human GIT and using infant milk as substrate was used to demonstrate the survival of the strain (82). The authors showed that the survival of strain 423 was significantly improved when used in combination with *Ent. mundtii* ST4SA. This suggests that the strains grew in symbiosis, thus degrading complex sugars to fermentable substrates more rapidly when both strains are present.

A variety of potential pathogens such as *L. monocytogenes*, *Bacillus cereus*, *Ent. faecalis* and *Clostridium sporogenes* are inhibited *in vitro* by *Lb. plantarum* 423 (78, 80, 83). Furthermore, symptoms of *Salmonella* infection were alleviated by *Lb. plantarum* 423 as determined in Wistar rats (84). When administered as a single culture *Lb. plantarum* 423 was the most effective at reducing the symptoms associated with *Salmonella* infection. According to these results, it can be speculated that *Lb. plantarum* 423 should be the probiotic of choice for treatment of *S. enterica* serovar Typhimurium infections compared to *Ent. mundtii* ST4SA.

3.2 *Enterococcus mundtii* ST4SA

Enterococcus mundtii ST4SA, isolated from soybeans, is regarded as a safe probiotic as shown by the absence of haematological and histological abnormalities in studies using the rat model (85). Enterococci are common inhabitants of the human GIT and produce antimicrobial peptides active against intestinal pathogens. However, it is known that in some cases *Enterococcus* spp., such as *Ent. faecalis* have been associated with immunosuppression, bacteraemia and urinary tract infections (86). Botes and co-workers (2008) reported on the presence of three virulence factors in *Ent. mundtii* ST4SA that are transcriptionally silent (81). Furthermore, the safety of the strain was confirmed in rat studies (78).

A previous study recorded high numbers of *Ent. mundtii* ST4SA in the ileum as determined in *in vitro* conditions simulating the GIT (82). This suggested that the strain survives the more anaerobic conditions of the colon. The ability of strain ST4SA to adhere to Caco-2 cells under conditions simulating those of the intestinal tract was also reported (81). The strain also relieved symptoms of *Salmonella* infection as determined in Wistar rats (84).

Enterococcus mundtii ST4SA and *Lb. plantarum* 423 produce antimicrobial peptides with activity against a number of Gram-positive and Gram-negative bacteria (85). These antimicrobial peptides, termed bacteriocins, may play a critical role in the competitive exclusion of pathogens in the human GIT.

4. Expression of reporter genes in LAB

Lactic acid bacteria are widely distributed in nature, representing one of the most important groups of microorganisms. They are very well known for being exploited in the commercial industry either in the production of fermented foods, or for their medicinal properties (87, 88). Hence, tools to genetically engineer LAB strains to improve their performance are constantly being developed (89).

A critical part of improving and studying the interaction of LAB strains with hosts is the introduction of foreign genes into bacterial cells. In fact, the idea of adding new and improved properties to microorganisms has underpinned biotechnology for decades (90). An example of this is the growing interest in using reporter genes to track labelled bacteria in a complex environment such as the human GIT. Using controlled *in vivo* conditions or *in vitro* simulations of the human GIT, the ability to track marked strains in real time can provide valuable information such as the location of bacterial colonization (6, 44).

4.1 Electro-transformation of LAB

Lactic acid bacteria are Gram-positive, and have a very thick and rigid cell wall. This affects the transformation of LAB, resulting in variable transformation efficiencies. Furthermore, the heterogeneity of LAB, means that electroporation protocols have to be optimized for each strain, including strains within the same species (91). To support this, Sieo et al. (2006) showed that by using the same method, strains of *Lb. crispatus* electro-transformed with plasmid DNA produced very different numbers of transformants per μg DNA (89, 92, 95).

Electroporation is generally regarded as the most efficient and reproducible method of transferring foreign genetic material to LAB (93, 94). Almost three decades ago, Chassy and Flickinger (1987) were one of the first to successfully use electroporation to transform LAB with plasmid DNA (96). Since then, researchers have developed methods to transform a number of LAB species (97, 98).

For some LAB species, failure to obtain transformants after electro-transformation may be due to the presence of restriction modification (R-M) systems (93). In bacteria, R-M enzymes are responsible for the cleavage of foreign genetic material that enters the cell by transformation, conjugation or by infectious prophages (99). Almost all bacteria have R-M systems that protect them from possibly harmful foreign DNA. Hence, the R-M status of any

LAB strain must be taken into consideration when attempting the transformation of the cells with recombinant DNA.

4.2 Integration and expression of reporter genes in LAB

Lactic acid bacteria can be marked with reporter genes by using either replicative plasmids or by integration of the gene into the host chromosome. The stable expression of genes by replicative plasmids in LAB is achieved when the plasmid DNA can be maintained stably in the host for many generations without antibiotic selection. The integration of genes into the host chromosome, on the other hand, provides genetic stability and eliminates selection requirements (100).

4.2.1 Plasmid expression

Maintenance of replicative plasmids inside LAB requires selective pressure, which is usually achieved by the presence of antibiotic marker genes. Clearly, this limits the utility of plasmids for *in vivo* applications (100). Yet, the use of multiple copy number plasmids may seem an attractive choice for the constitutive expression of a reporter gene (101). As a result, numerous studies have successfully used LAB marked with reporter plasmids for *in vivo* applications, providing that the plasmids are maintained by the host (6, 87).

4.2.2 Integration of reporter genes

Replicative plasmids are inherently unstable, while the integration of a reporter gene into the LAB host chromosome provides genetic stability. The integration of genes into the host bacterial chromosome eliminates selection requirements for maintenance inside the bacterial cell. Integration also provides the added advantage of stabilizing the exogenous DNA in the host chromosome by irreversible incorporation (90). However, very few chromosomal sites have been investigated that will allow high levels of reporter gene expression in LAB.

Furthermore, plasmid reporter gene expression levels are higher than those of some chromosomally integrated genes (102).

Plasmids that are unable to replicate in a specific host LAB, such as plasmids harbouring replication genes of Gram-negative bacteria (termed ‘suicide vectors’), but contain sequences that share homology with the selected strain’s DNA, may integrate into the host chromosome by homologous recombination (103). Consequently, the reporter gene will be incorporated at the chromosomal location corresponding to that of the homologous fragment. This type of integration has been described in numerous LAB species, including *Lb. plantarum*, *Lc. lactis* and *Streptococcus pneumonia* (104, 105).

Target sequences for the genomic integration of reporter genes should be genes or regions that are non-essential to the host. It is essential that the physiology of the host cell remains unchanged. The region or gene that is disrupted has to have no impact on the phenotype (106). Furthermore, recombinant strains must only differ from the wild type by the newly acquired characteristics (fluorescence or bioluminescence). Complex experiments to prove that the host cell physiology has not been negatively affected, can be avoided by disrupting a non-functional genetic locus on the host chromosome. Prophage genes that are generally considered to be transcriptionally silent in the lysogenic phase are a good example of genetic loci that are often used for homologous recombination of foreign genes in LAB. Several LAB species harbour phage genes on their chromosomes, and heterologous DNA can be inserted, for example into the *attB* sites present in many prophages (100, 102). A more refined integration strategy makes use of two recombination events for integration. Following the integration of the entire plasmid, a second recombination event occurs to remove the plasmid backbone, while the gene of interest is irreversibly left behind in the host chromosome (107). The event in which recombination occurs followed by looping out of the vector is determined by how the homologous sequences are arranged on the vector. Double recombination

integration has been used with many LAB species, e.g. *Lb. casei*, *Lb. plantarum* and *Lb. acidophilus* (108).

4.3 Codon optimization

This is a technique employed by many researchers with the aim of maximizing the translational efficiency of a gene in a particular host organism. To encode any particular amino acid, different organisms have their preferred choice of nucleotide usage (109). In other words, the codon usage of a plant will differ from that of a bacterium for the expression of the same gene product. Therefore, the translation efficiency of a protein in a heterologous host organism can be significantly increased by modifying the codon usage frequency.

To enhance the translational efficiency of a reporter gene in LAB, the gene can be chemically synthesized with an optimized codon usage for expression in the specific LAB host. Garcia-Cayuela et al. (2011) constructed fluorescent protein vectors for analysing the expression strength of different promoters in LAB and *E. coli* (38). Here, the researchers used a synthetic red fluorescent protein that had been codon-optimized for increased expression in *Lb. plantarum* and *Ent. faecalis*.

Luciferase reporter genes from eukaryotes such as the firefly (*P. pyralis*) or click beetle (*P. plagiophthalmus*) have a codon usage that is not suitable for expression in bacteria. Therefore, the nucleotide sequences have to be altered to the preferred ones to maximize the luciferase reporter protein expression in LAB. Firefly luciferase expression in *M. tuberculosis* increased 30-fold in signal intensity after codon-optimization (7).

5. Effect of probiotic LAB on *Listeria monocytogenes*

Listeria monocytogenes is an opportunistic food-borne pathogen, which can often cause a life-threatening systemic disease, known as listeriosis, in individuals with a weakened immune

system (110). Newborns, elderly people and those with HIV or any other disease compromising immunity are the most susceptible to listeriosis (111). *Listeria monocytogenes* has an innate ability to grow at low temperatures (15). Furthermore, *L. monocytogenes* is widely distributed in nature, thus rendering many food products vulnerable to contamination (112).

Listeriosis affects the central nervous system, and may lead to meningitis, brain abscess and bacteraemia (113, 114) Furthermore, previous studies reported that infections of the liver and placenta were due to *L. monocytogenes* (115, 116). Healthy individuals may develop gastroenteritis when high cell numbers of *L. monocytogenes* are ingested. The replication and consequent colonization of a bioluminescent strain of *L. monocytogenes* in bone marrow, the intestinal tract and the gall bladder have been investigated (15, 110, 113). The murine listeriosis model remains one of the best studied models of infection in which bacterial pathogens are studied. However, the understanding of colonization and the processes of infection by *L. monocytogenes* remain unclear, despite decades of research on host-pathogen interactions.

Treatment of infections caused by *Listeria*, generally includes administration of antibiotics to which the organism is highly susceptible, such as ampicillin, gentamicin, erythromycin and vancomycin (117). However, with an increase in antibiotic resistant pathogens primarily resulting from antibiotic over dosage, the need for alternative antimicrobial agents against resistant pathogens is obvious. Several studies reported that antimicrobial peptides, classified as bacteriocins and produced by some LAB, can be used to effectively treat multi-drug resistant *S. aureus* infections in the respiratory tract and peritoneal cavity as determined in Wistar rats (118, 119). These antimicrobial agents are ribosomally synthesized peptides which have a bactericidal or bacteriostatic effect on other species (111, 120). Nisin V, a bioengineered version of the nisin A bacteriocin that was first discovered in 1928, was found

to be an effective treatment for an infection by a lux-tagged *L. monocytogenes* strain in a murine model (119).

Bacteriocins have been applied as bio-preservative in the food industry for decades, particularly against contamination by *L. monocytogenes*. A bacteriocin produced by *Lb. casei* CRL 705, known as Lactocin 705 was reported to have a bacteriostatic effect on the growth of *L. monocytogenes* in ground beef (112).

Numerous studies have suggested that probiotic LAB, especially *Lactobacillus* spp., can inhibit and control the growth of *L. monocytogenes in vitro* (121-123). A recent study reported that an infection by *L. monocytogenes* in gnotobiotic mice could be modulated by treatment with *Lb. casei* BL23 and *Lb. paracasei* CNCM I-3689 (124). Treatment of the mice with the two *Lactobacilli* strains may have altered the regulation of several *L. monocytogenes* genes involved in metabolism. Additionally, treatment of the mice with *Lactobacillus* caused a decrease in *L. monocytogenes* dissemination in the gnotobiotic humanized mouse model (125).

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TABLE 1. Commercially available Imaging Systems (7)

Manufacturer	Instrument	Features
Caliper Life Sciences (http://www.caliperls.com)	IVIS (several models)	Bioluminescence Fluorescence Digital X-ray (Lumina XR) Image freely moving animals (kinetic)
Berthold Technologies (http://www.bertholdtech.com)	NightOwl (two camera options)	Bioluminescence Fluorescence
Biospace Lab (http://www.biospacelab.com)	PhotonImager	Bioluminescence Fluorescence Macrolens to convert to bioluminescence microscope Image freely moving animals (In Actio ^R)
Cambridge Research and Instrumentation (CRi) (http://www.cri-inc.com)	Maestro	Fluorescence Spectral scanning
Carestream Health (http://www.carestreamhealth.com)	Kodak <i>In Vivo</i> Imaging systems (several models)	Bioluminescence Fluorescence Digital X-ray (FX/FX-Pro)
Li-Cor Biosciences (http://www.licor.com)	Pearl (1 mouse) Odyssey ^R Imager+ Mousepod TM (3 mice)	Near infra-red fluorescence
VisEn (http://www.visenmedical.com)	FMT 2500 Imaging system	Near infra-red fluorescence Two modes: Reflectance Imaging and Quantitative Tomography Multimodality adaptors for CT/MR/PET

TABLE 2. Properties of selected fluorescent reporter proteins (2)

Fluorescent protein variant	Origin	Excitation max. (nm)	Emission max. (nm)
DsRed (18)	<i>Discosoma</i> sp.	558	583
mCherry (18)	DsRed	587	610
tdTomato (18)	DsRed	554	581
mRaspberry (19)	DsRed	598	610
mPlum (19)	DsRed	590	649
TurboRFP (20)	eqFP578	553	574
mKate (20)	eqFP578	588	635
Katushka (21)	eqFP578	588	635
TagRFP (20)	eqFP578	553	574
RFP693 (22)	eqFP611	588	639
tdRFP611 (22)	eqFP611	558	609
AQ143 (23)	aeCP597	595	655
IFP1.4 (24)	DrBphP	684	708

TABLE 3. Imaging systems utilizing bioluminescent bacteria (7)

Bacteria	Reporter system
<i>Bacillus anthracis</i> (25)	lux
<i>Citrobacter rodentium</i> (26)	lux
<i>Brucella melitensis</i> (27)	lux
<i>Bifidobacterium breve</i> (4)	lux
<i>Escherichia coli</i> (28)	lux, luc
<i>Listeria monocytogenes</i> (15)	lux
<i>Mycobacterium tuberculosis</i> (29)	lux, luc
<i>Pseudomonas aeruginosa</i> (30)	lux
<i>Pseudomonas fluorescence</i> (31)	lux
<i>Salmonella enterica</i> Typhimurium (32)	lux
<i>Staphylococcus aureus</i> (16, 33)	lux
<i>Streptococcus pneumonia</i> (17)	lux

TABLE 4. Comparison of Lux, GFP and mCherry (18)

Characteristic	Lux	GFP	mCherry
Color of light	Bluish-white	Green but variants are yellow, blue and cyan	Red, shade of purple in visible light
External requirements for light emission	None; except must view in dark room with dark adapted eyes	Requires excitation by UV light	Short wavelengths of light – excitation maximum of 587nm
Intrinsic requirements for light emission	5 additional genes plus <i>luxR</i> and <i>luxI</i>	None	None
Duration/stability of light emission	Lasts 5-9 days, stops after nutrient depletion	Last months as long as plates do not dry out	Shade of purple in visible light, resistant to photo bleaching
O ₂ dependency for light emission	Requires O ₂	None; some O ₂ needed for chromophore formation	Functions under hypoxic conditions

TABLE 5. Factors that can affect the sensitivity of imaging systems (7)

Reporter gene (fluorescent/bioluminescent) expression level
Emission light wavelength (referring to reporter gene chosen)
Excitation wavelength (fluorescence only)
Sensitivity of detection system
Background fluorescence/bioluminescence
Animal fur colour or pigmentation
Signal location within the animal (type and depth of tissue)

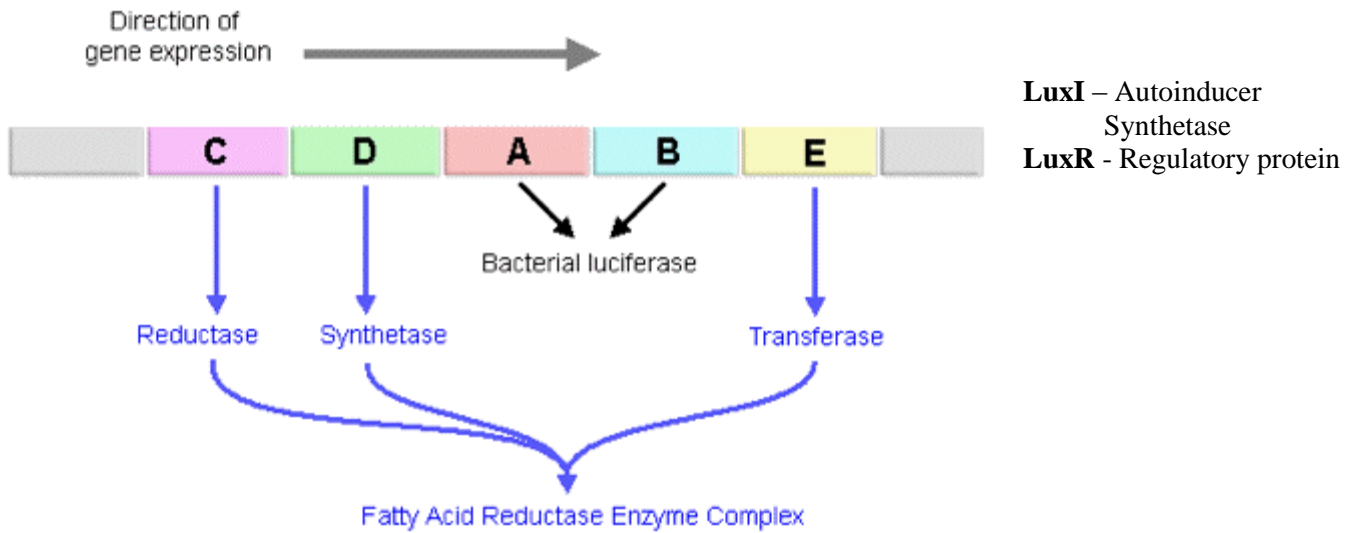


FIG. 1. The arrangement of the *luxCDABE* open reading frames found in *Photobacterium* spp, *Vibrio* spp. and *Photorhabdus* spp. (56). The *luxI* and *luxR* genes are adjacent to one another and *luxI* is the first of the seven genes in the operon. LuxI and LuxR are required for activation of the luminescence genes.

STELLENBOSCH UNIVERSITY

Chapter 3

Construction of a Fluorescent Reporter System for Lactic Acid Bacteria, using the *mCherry* Gene

Construction of a Fluorescent Reporter System for Lactic Acid Bacteria, using the *mCherry* Gene

Abstract

Fluorescent reporter genes are invaluable when studying gene expressions and following the migration of bacteria in the gastro-intestinal tract (GIT). The *mCherry* gene, encoding a red fluorescent protein (rfp), was cloned into the pGKV223D LAB/*E. coli* expression vector and two integration vectors were constructed. The integration vectors were constructed to allow stable integration of mCherry into non-functional regions on the chromosomes of the probiotics *Lactobacillus plantarum* 423 and *Enterococcus mundtii* ST4SA by homologous recombination. Insertion of the *mCherry* gene did not alter the growth rate of strains 423 and ST4SA and had no effect on bacteriocin production. Fluorescent cells were recorded using the Caliper *in vivo* imaging system (IVIS). The *mCherry* gene may be used as a marker to tag *Lb. plantarum* and *Ent. mundtii* in the GIT.

Introduction

Lactic acid bacteria (LAB) are used in many applications, such as probiotics, as starter cultures in food fermentations, biological control agents and in pharmaceutical preparations. They form part of the natural microbiota of the intestines, mouth, skin, urinary and genital organs of both humans and animals (1). The use of some strains as probiotics exert beneficial effects on human and animal health by improving the microbial balance of the gastrointestinal tract (GIT) and by producing antimicrobial peptides active against pathogens (2, 3). *Lactobacillus plantarum* is a lactic acid bacterium that is commonly applied to industrial and artisanal dairy, meat or plant fermentation and some strains are marketed as probiotics (4-6). Lactic acid bacteria strains belonging to the genus *Enterococcus*, such as *Enterococcus faecium* and *Enterococcus faecalis*, play a key role in environmental, food and clinical microbiology (7, 8). Enterococci are also known to produce antimicrobial peptides active against intestinal pathogens (9).

Due to the increasing consumer interest in probiotics as health supplements and natural alternatives to antibiotics, techniques to study the survival and metabolic activities of LAB are required. The *in vivo* monitoring of these processes is achieved by labelling LAB with reporter genes. Fluorescence, the emission of light by fluorophores at different wavelengths, is a versatile technique which may be applied *in vitro* and *in vivo* (10).

To develop a fluorescent microorganism, it is critical that the reporter gene is efficiently and stably expressed (11). The Expression of reporter genes in LAB using plasmids requires selective pressure for maintenance which complicates their use in *in vivo* applications. Integration of a selected reporter gene into the genome of LAB for expression eliminates selection requirements and provides genetic stability (12). Furthermore, the presence of the

fluorescent vector or gene in the recombinant organism should not alter the physiology of the host or its ability to survive.

The green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* is a versatile reporter that is widely used to label bacteria, study gene functionality or tag proteins (13, 14). In addition, GFP have been used to track labelled cells in tissues (15-17) and to provide valuable information on the localization of bacteria in simulated GIT studies (18).

Since the discovery of GFP in 1962, numerous red-emitting variants have been developed with optimized properties for *in vivo* applications (19). Of these, the mCherry red fluorescence protein (rfp) is considered the most appropriate for *in vivo* studies. Fluorescent reporters with emission wavelengths above 600 nm were required, as the absorption of light by mammalian tissues is lower in this range of the light spectrum. Excitation and emission of the mCherry rfp is at a wavelength where auto-fluorescence is minimal (20). Red fluorescent proteins with excitation and emission wavelengths in the far-red region of the spectrum are preferred for *in vivo* experiments.

The mCherry protein is highly photo-stable and was thus chosen as a reporter for *in vivo* application in the current study. In addition, mCherry has an improved brightness and maturation time compared to the *Discosoma* red (DsRed) protein it was derived from (21, 22). The *mCherry* gene was used with great success in several bacteria, including LAB (23-25). Furthermore, the mCherry protein is non-toxic and expression at high levels will not inflict additional physiological stress on the host organism (26). The mCherry fluorophore is also highly resistant to photo bleaching and the fluorophore will not lose its ability to fluoresce during continuous illumination (27).

This paper describes the construction of mCherry reporter plasmids. The constructs were specially designed for integration into the genomes of the probiotics, *Lb. plantarum* 423 and

Ent. mundtii ST4SA. We report on the introduction of the *mCherry* gene into the bacterial chromosomes by homologous integration into selected target genes. Expression of fluorescence in the two LAB was detected using the *in vivo* imaging system (IVIS). We also show that introduction of the *mCherry* fluorescence gene into *Lb. plantarum* 423 and *Ent. mundtii* ST4SA had no effect on their ability to produce antibacterial peptides (bacteriocins).

Materials and Methods

Bacterial strains, media and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. All subcloning experiments were carried out using *Escherichia coli* DH5 α (28) as host. *Escherichia coli* DH5 α was grown in Brain Heart Infusion (BHI) or Luria-Bertani (LB) broth (Biolab Diagnostics, Midrand, South Africa) at 37°C on an orbital shaker (200 rpm). All LAB were grown in MRS broth (Biolab Diagnostics) without shaking, or on MRS agar at 30°C. When necessary, antibiotics were added to growth media at the following final concentrations: 200 μ g/ml erythromycin (Em) for *E. coli* DH5 α , and 10 μ g/ml Em for *Lb. plantarum* 423 and *Ent. mundtii* ST4SA. Chloramphenicol (Cm) was incorporated at a final concentration of 5 μ g/ml for *Ent. mundtii* ST4SA, 10 μ g/ml for *Lb. plantarum* 423 and 20 μ g/ml for *E. coli* DH5 α . Ampicillin (Amp) was added to a final concentration of 100 μ g/ml for *E. coli* DH5 α .

General molecular techniques

Recombinant DNA methods and gel electrophoresis were carried out according to standard protocols, as described by Sambrook and Russel (2001). DNA restriction and modification enzymes were purchased from Roche Molecular Chemicals (Mannheim, Germany) or Fermentas (Vilnius, Lithuania) and were used according to the suppliers' instructions. Oligonucleotides (Table 2) were obtained from Inqaba Biotechnical Industries (Pretoria,

South Africa). PCR amplifications (Table 2) were performed on a Swift™ Minipro Thermal Cycler (Esco Healthcare, Malaysia), using Takara Ex Taq High-Fidelity PCR DNA polymerase (Clontech Laboratories, Inc., Randburg, South Africa). DNA sequencing was performed by the Central DNA Sequencing Facility of the University of Stellenbosch. DNA fragments were purified from agarose gels using the QIAquick PCR Purification Kit (Qiagen Inc., Valencia, CA). *Escherichia coli* plasmid DNA was purified using the standard Qiagen® Plasmid Mini and Midi Kits. Genomic DNA of LAB strains was isolated using the ZR Fungal/Bacterial DNA Miniprep Kit (Zymoresearch Corporation, Irvine, CA, U.S.A.). The presence of recombinant plasmid DNA in LAB transformants was confirmed by using the plasmid isolation method of Qiagen®, but modified by using 10 mg/ml lysozyme for cell lysis and STE buffer (6.7% saccharose; 50 mM Tris-Cl, pH 8.0; 1 mM EDTA) for wash steps.

Construction of pGKV223D expression and integration vectors containing the *mCherry* fluorescence gene

Expression and integration plasmids constructed during this study are based on the pGKV223D LAB/*E. coli* expression vector (University of Grönigen, Netherlands). The vector contains an *Em^R* gene for erythromycin resistance, the L23 LAB promoter, a multiple cloning site (MCS) and the *repA* gene for replication in LAB/*E. coli* hosts, which was derived from *Lactococcus* and *Enterococcus* spp. All primers are listed in Table 2. A schematic diagram summarizing the construction of pGKCherry is shown in Figure 1. The mCherry cassette was chemically synthesized by Invitrogen, Germany, with codon-optimization for enhanced expression in *Lb. plantarum* and *Ent. mundtii*. The mCherry gene cassette was amplified from plasmid pMK-RQCherry (Invitrogen, Germany) by using primers ForPldh and RevTT that contain the restriction sites *EcoRI* and *XbaI*, respectively. The 1.5 kb amplicon, containing the mCherry gene, flanked by the *Pldhl* promoter and a transcriptional terminator (TT), was

digested with *EcoRI* and *XbaI* and introduced into the MCS of pGKV223D after digestion of the vector with the same restriction enzymes (Fig. 1). Fragments were ligated overnight at 15°C, before being transformed into competent *E. coli* DH5 α for plasmid propagation. Transformants with an Em-resistant phenotype and a color change from white to pink were selected.

To construct the integration vectors PGKRm1CmRCherry and PGKEmuNCmRCherry, a gene for chloramphenicol resistance (*cat*) was fused to the *mCherry* gene cassette as a second selection marker (Fig. 2). Plasmid pKSCmR was digested with *EcoRI* and an 800 bp DNA fragment containing the *cat* gene was purified from a 0.8% (w/v) agarose gel. Plasmid pKSCCherry carrying the codon-optimized *mCherry* gene cassette was linearized following digestion with *EcoRI*, purified and then ligated with T₄ DNA ligase (Roche Molecular Chemicals, Mannheim, Germany) to the 800 bp *cat* gene fragment. *Escherichia coli* DH5 α electro-competent cells were then transformed for propagation of the resulting plasmid, named pKSCmRCherry. Transformants were screened by selecting for a Cm resistant and a red colony color phenotype. The integrity of the plasmid was verified by restriction digests and PCR with primers ForCmR and RevTT.

The targets used for homologous recombination were the *restriction modification type 1* (*Rm1*) and *DNA-entry* (*EmuN*) genes for *Lb. plantarum* 423 and *Ent. Mundtii* ST4SA, respectively. Unique sites were chosen within these genes for the insertion of the *CmRCherry* gene cassette. The integration vectors were constructed in such a way that, the *CmRCherry* gene cassette (Fig. 2) was flanked with the target genes' sequences including some of their upstream and downstream regions, to facilitate homologous recombination. The *Rm1* and *EmuN* genes were amplified by PCR from genomic DNA extracted from *Lb. plantarum* 423 and *Ent. mundtii* ST4SA, respectively. Amplification of the target genes *Rm1* and *EmuN* was performed using primers ForRm1/RevRm1 and ForEmuN/RevEmuN, respectively. Both

amplicons were digested with *EcoRI* and *XbaI* and then ligated to the LAB/*E. coli* shuttle vector pGKV223D that was cut with the same enzymes. The presence of target genes in the resulting recombinant plasmids pGKRm1 and pGKEmuN isolated from Em resistant transformants was confirmed by sequencing with primers ForRm1/RevRm1 and ForEmuN/RevEmuN for both constructs, respectively.

For insertion of the *CmRCherry* cassette into the target gene sequences of plasmids pGKRm1 and pGKEmuN, pKSCmRCherry was digested with *SalI* and *XbaI*. The resulting 2.2 kb DNA fragment containing the *cat* gene, *PldhI* promoter, *mCherry* gene and the TT transcriptional terminator, in that specific order (Fig. 2), was purified from a 0.8% agarose gel. The flushing of both the *SalI* and *XbaI* restriction site ends of the 2.2 kb DNA fragment was accomplished by filling in the 5' sticky overhang ends with *E. coli* T₄ DNA polymerase in the presence of all four dNTP's (31). Plasmids pGKRm1 and pGKEmuN were digested at unique restriction sites in the target gene sequences namely, *PvuI* and *EcoRV*, respectively. After digestion of the plasmids, the *PvuI* restriction site ends of the linearized pGKRm1 plasmid were flushed by filling in the 5' sticky ends as described before. Since, *EcoRV* is a blunt end restriction enzyme, flushing of the ends was not necessary for compatibility with the blunt ended *CmRCherry* gene cassette. The linearized blunt ended pGKRm1 and pGKEmuN fragments were purified and ligated with T₄ DNA ligase to the blunt ended *CmRCherry* gene cassette from pKSCmRCherry. The ligation mixture was introduced into competent *E. coli* DH5 α cells through electroporation. Transformants with both an Em and Cm resistant phenotype were selected. The presence of the *CmRCherry* gene cassette fused within upstream and downstream flanking regions of the target genes *Rm1* and *EmuN* in the resulting plasmids designated pGKRm1CmRCherry and pGKEmuNCmRCherry was confirmed by DNA sequencing. Sequence confirmation of pGKRm1CmRCherry and pGKEmuNCmRCherry was carried out using primers ForCmR/RevRm1 and ForCmR/RevEmuN, respectively. Following

sequence confirmation of the integration vectors, the plasmids were extracted for subsequent transformation of LAB. The Cm marker was associated with the *mCherry* gene to enable selection of integrants that have lost the Em^R-associated vector.

Targets for genomic integration

At the time of completion of this study, the whole genome sequences of *Lb. plantarum* 423 and *Ent. mundtii* ST4SA were not available. Therefore, published whole genome sequences of closely related *Lb. plantarum* strains, WCFS1, JDM1 and ZJ316 were used as reference strains in the search for non-essential target sequences for the genomic integration of the CmRCherry gene cassette into the *Lb. plantarum* 423 chromosome. The presence of the five selected targets genes was screened for by PCR using *Lb. plantarum* strains 423, ATCC8014 and ATCC14917 gDNA as templates. Three transcriptionally silent prophage and two restriction modification (RM) genes were identified as potential non-essential target genes for homologous recombination in the *Lb. plantarum* 423.

Similarly, two draft genome sequences of *Ent. mundtii* strains CRL1656 and ATCC882 were used as reference in the screening of potential target gene sequences for homologous recombination in strain ST4SA. Two RM genes and a DNA-entry nuclease gene (*EmuN*) were identified as potential target sequences for homologous recombination in strain ST4SA.

Transformation of bacterial cells

***Escherichia coli* DH5 α**

Electrocompetent *E. coli* DH5 α cells were prepared as previously described (32), except that the LB growth medium contained 50% less NaCl in this study. Cells were electroporated with 5 μ l plasmid DNA and transformants selected with Em, Amp or Cm.

***Lactobacillus plantarum* 423**

Electroporation of *Lb. plantarum* was carried out as previously described (33), with minor modifications. Overnight cultures of actively growing *Lb. plantarum* 423 cells were diluted 1/20 (5 ml) into filter-sterilized MRS growth medium (100 ml) supplemented with 8 % glycine and 0.3 M sucrose as cell wall weakening agents. Cultures were incubated at 30°C for approximately 4h until optical density at 600 nm of 0.4 was reached, representing the late exponential growth phase. After incubation, cultures were harvested at 1500 x g for 5 min, washed twice in ultra-pure water, kept on ice for 5 min in 50 mM EDTA, washed once more in milliQ water, once in 0.3 M sucrose and resuspended in 2 ml of the latter solution. Aliquots of 50 µl electro-competent bacteria were mixed with 1 µl plasmid DNA (500 ng) and transferred to 2 mm cuvettes for electroporation. In addition, electro-competent *Lb. plantarum* 423 cells were used immediately for electroporation and not frozen. The electroporation parameters used were 1.5 kV, 25 µF and 200Ω. Directly after electroporation, 1 ml pre-warmed MRS media was added, the cells incubated at 30°C for 2h for phenotypic expression before plating on MRS agar containing 10 µg/ml Em or Cm and subsequently incubated at 30°C for 48h.

***Enterococcus mundtii* ST4SA**

A modified version of a *Pediococcus* sp. protocol for the electro-transformation of *Ent. mundtii* ST4SA was used in this study (34). For the preparation of electro-competent *Ent. mundtii* ST4SA cells, 10 ml of an overnight culture was inoculated into 100 ml MRS broth, supplemented with 4.5 % glycine, 0.5 M sucrose and 40 mM threonine as cell wall weakening agents. Incubation was at 30°C for 4h, without aeration, to an optical density at 600 nm of 0.5. The cells were harvested (1500 x g, 10 min), resuspended in filter-sterilized buffer solution (0.5 M sucrose and 10 % glycerol), and kept on ice for 1h before being washed three times

with the same solution. Cells were resuspended in electroporation buffer, containing 1 mM K_2HPO_4/KH_2PO_4 (pH 7.0), 1 mM $MgCl_2$ and 0.5 M sucrose. Subsequently, 100 μ l aliquots was mixed with 5 μ l plasmid DNA (1 μ g), transferred to 2 mm cuvettes and electroporated at 2.2 kV, 25 μ F and 200 Ω . Following electroporation, cells were promptly transferred into MRS broth supplemented with 0.5 M sucrose, 20 mM $MgCl_2$ and 2 mM $CaCl_2$, before incubation at 30°C for 2h and plating onto MRS agar containing 10 μ g/ml Em.

Transformant selection

Escherichia coli DH5 α and LAB transformants harbouring recombinant vectors encoding the *mCherry* gene, were analyzed on agar plates for fluorescence emission using the IVIS[®] 100 In Vivo Imaging System (Caliper Life Sciences, Hopkinton, MA, U.S.A.). Colonies with the highest fluorescence emission were selected and transferred to 10 ml liquid broth containing an appropriate antibiotic. Following the transformation of LAB strains with the integration vectors pGKRm1CmRCherry and pGKEmuNCmRCherry, the electroporated culture was directly transferred into 10 ml MRS broth containing Cm for the enrichment of successful integrants. After a 48 hour incubation period, cultures were replica plated on MRS agar supplemented with Em and Cm, respectively. Erythromycin-sensitive and Cm-resistant colonies were then selected and scanned for fluorescence. Background auto fluorescence emitted by the agar plate was used to normalize the fluorescence signal of colonies.

Fluorescence detection

Fluorescence assays of bacterial strains expressing the *mCherry* gene were carried out using black non-autofluorescent 96-well microtiter trays. Four millilitres of 12 h-old cultures expressing the *mCherry* gene were harvested (1500 x g for 3 min), the cells washed twice with sterile saline solution (0.85% (w/v) NaCl) to remove trace elements of growth media and

then resuspended in 300 µl saline. Before fluorescent image capturing, the 300 µl cell suspensions were transferred to wells in a microtiter tray. Bacterial strains with plasmid constructs without the *mCherry* gene insert were used as controls.

Growth of *Lb. plantatum* 423 and *Ent. mundtii* ST4SA compared to the chromosomally marked strains

Wild-type and recombinant *Lb. plantarum* 423 and *Ent. mundtii* ST4SA transformed with the integration vectors were grown in MRS broth for 12 h. One millilitre of the 12-h old cultures was used to inoculate 100 ml of pre-warmed MRS broth. The 100 ml cultures were then aliquoted into nine 2 ml Eppendorf tubes (one Eppendorf tube for each time point) and incubated at 30°C for 9 h. Every 1 h the optical density reading at 600nm of each of the cultures in one Eppendorf tube was measured, serially diluted and plated on MRS agar.

Determination of antibacterial activities of fluorescing LAB strains

The ability of the recombinant LAB containing the *mCherry* gene to produce antimicrobial peptides (bacteriocins) was tested using *Listeria monocytogenes* EGDe in a lawn assay, as described by Van Reenen et al. (30). An actively growing overnight culture (12 h) of *L. monocytogenes* EGDe was overlaid onto a BHI agar plate by inoculating 100 µl of the bacterial suspension in 10 ml semi-solid BHI agar (0.8 %, w/v). The cell-free supernatants (adjusted to pH 7.0 with NaOH) of 2 ml actively growing fluorescent LAB was collected by centrifugation (8000 x g for 3 min). The supernatants were passed through a 0.22 µm pore size filter, 10 µl was spotted onto BHI agar overlaid with *L. monocytogenes* EGDe, and the plates incubated at 37°C for 24h. As positive controls, supernatants from LAB strains not harbouring control plasmids vectors were used.

Quantification of fluorescent signals and CFU determination

The quantification of fluorescence signals relative to the amount of bacterial cells required for a specific emission signal was determined. Overnight cultures (12 h) of *Lb. plantarum* 423::CmRCherry and *Ent. mundtii* ST4SA (pCmRCherry) were diluted as follows: 1:2, 1:4, 1:8, 1:16 and 1:32. Fluorescence of each dilution was measured using the IVIS imaging system. For the determination of the colony forming units (CFU), each dilution was further serially diluted in 0.85 % NaCl and plated out on MRS agar. For the estimation of CFU, 100 μ l of each serial dilution was plated out, followed by incubation at 30° for 48h.

The amount of fluorescence emitted in p/cm²/s/sr for each dilution was plotted against the corresponding CFUs of *Lb. plantarum* 423::CmRCherry and *Ent. mundtii* ST4SA (pCmRCherry), with each point on the graphs representing the amount of photons emitted correlating to the CFUs of each dilution.

Results

Cloning of pGKCherry and introduction into LAB

Escherichia coli colonies harbouring pGKCherry were easily detected on agar plates as a result of their bright purple appearance (Fig. 3a). Bright fluorescence was detected on agar plates using the IVIS (Fig. 3b). High fluorescence intensity was observed for cells harbouring pGKCherry, compared to control cells harbouring the pGKV223D vector (Fig. 3c).

Lactobacillus plantarum 423 and *Ent. mundtii* ST4SA colonies transformed with pGKCherry had a white appearance growing on agar plates, but showed varying intensities of fluorescence. Colonies with the strongest intensity were selected for further analysis. Fluorescent imaging of *Lb. plantarum* 423 (pGKCherry) and *Ent. mundtii* ST4SA (pGKCherry) strains resuspended in microtitre trays revealed bright red fluorescence

compared to the control strains as shown in Figure 4a and Figure 4b, respectively. *Lactobacillus plantarum* 423 pGKCherry was also imaged in microfuge tubes using the IVIS with a pGKV223D transformant as control (Fig. 4c).

The presence of the 5 kb pGKCherry plasmid was confirmed in *Lb. plantarum* 423 strains by isolation and visualization after electrophoresis. The presence of the pGKCherry vector in the recombinant *Lb. plantarum* 423 is indicated in Fig. 5a. The probiotic *Lb. plantarum* 423 contain five natural plasmids as shown in Fig. 5b. The presence of the pGKCherry vector in *Ent. mundtii* ST4SA was similarly confirmed, with *Ent. mundtii* ST4SA containing two large natural plasmids of 50 kb and 100 kb with the bacteriocin ST4SA operon situated on the 50 kb plasmid as indicated in Fig. 6.

Screening of targets for genomic integration

Of the five target genes tested for, PCR results confirmed that the 1 kb type 1 RM methyltransferase gene (*Rm1*) was the only gene present in *Lb. plantarum* 423 (Table 3). Therefore, the *Rm1* gene was used as target sequence for homologous recombination. *Lactobacillus plantarum* 423 was significantly different compared to *Lb. plantarum* strains that already have their genomes published, particularly with the regards to the genes we screened for. One of the target genes tested, the *Mrrp* type IV RM gene, was present in six sequenced *Lb. plantarum* strains and in three strains we have tested, but was not present in *Lb. plantarum* 423.

In the case of *Ent. mundtii* ST4SA, PCR results confirmed that the DNA-entry nuclease gene (*EmuN*), associated with the natural competence of the strain, was the only target sequence present in the strain out of the three potential targets that was screened for. The *EmuN* gene was used as target sequence for homologous recombination in *Ent. mundtii* ST4SA.

Introduction of integration vectors containing the *mCherry* gene into *Lb. plantarum* 423 and *Ent. mundtii* ST4SA

Escherichia coli DH5 α cells transformed with the integration vectors, pGKRm1CmRCherry and pGKEmuNCmRCherry, resulted in fluorescent colonies that were both Em and Cm resistant. The vectors are able to replicate in Gram-negative and Gram-positive bacteria, and contain a Cm marker that could be selected for in integrants that have lost the Em^R-associated vector. *Lactobacillus plantarum* 423 and *Ent. mundtii* ST4SA transformants could either contain the autonomous plasmids, integrated plasmids with Cm and Em resistance, or an integrated but recombined *CmRCherry* cassette in which the Em^R-associated plasmid was lost. *Lactobacillus plantarum* 423 transformants retained resistance to Cm, but not Em, indicating that the *CmRCherry* gene cassette successfully integrated into the host chromosome without the plasmid DNA encoding the *Em^R* gene. However, *Ent. mundtii* ST4SA transformants retained resistance to both Em and Cm, indicating that the Em^R-associated vector was not lost.

The presence of the *cat* and *mCherry* genes in *Lb. plantarum* 423 and *Ent. mundtii* ST4SA transformants was confirmed by PCR using primers ForCmR/RevCmR and ForCherry/RevTT (Fig. 7). No 1 kb *Rm1* target gene fragments were amplified from potential *Lb. plantarum* 423 integrants with primers ForRm1 and RevRm1. However, amplification with primers ForCmR and RevRm1 resulted in a 2.5 kb DNA fragment consisting of the *CmRCherry* gene cassette and a 270 bp downstream *Rm1* gene fragment (Fig. 8). These results suggested that the *Rm1* gene was successfully disrupted by the insertion of the *CmRCherry* gene cassette. Amplification of *Ent. mundtii* ST4SA transformant DNA with primers ForEmuN and RevEMuN resulted in a 720 bp *DNA-entry* gene fragment (data not shown), indicating that the *EmuN* gene was not knocked out. *Lactobacillus plantarum* 423 and *Ent. mundtii* ST4SA

transformed with the integration vectors were fluorescent when examined using the IVIS (Fig. 9).

Impact of the genomic disruption of target genes on LAB phenotypes

No significant difference in growth was observed between the wild-type and engineered strains after a nine-hour incubation period (Fig. 10). The potential genomic interruption of the target genes *RmI* and *EmuN* on the chromosomes of *Lb. plantarum* 423 and *Ent. mundtii* ST4SA had no detectable impact on the bacterial growth.

Bacteriocins isolated from *Lb. plantarum* 423::CmRCherry and *Ent. mundtii* ST4SA (pCmRCherry) resulted in the formation of clear inhibition zones of the sensitive *L. monocytogenes* EGDe strain (Fig. 11).

Quantification of fluorescence signals and CFU determination

The excellent correlation between the fluorescence emitted and CFU counts for all the dilutions made for *Lb. plantarum* 423 ($R^2=0.9889$) and *Ent. mundtii* ST4SA ($R^2=0.9788$) recombinant strains indicated that photon emission levels accurately reflect bacterial numbers in total cultures (Fig. 12).

Discussion

The *mCherry* gene was chemically synthesized and codon optimized for optimal expression in *Lb. plantarum* 423 and *Ent. mundtii* ST4SA. The expression and integration plasmids were derived from the pGKV223D shuttle vector which is based on a *Lactococcal* spp. origin of replication functional in *E. coli* and LAB.

Firstly, we developed the pGKCherry expression vector carrying the *mCherry* gene under constitutive expression from the strong *Lb. plantarum* *Pldhl* promoter to evaluate the expression of *mCherry* in LAB (36, 37). The expression of *mCherry* in *E. coli* DH5 α resulted in a change in colony colour on agar plates, indicating that the fluorescence protein was functionally expressed from the *Pldhl* promoter. Following the electroporation of the pGKCherry plasmid into *Lb. plantarum* 423 and *Ent. mundtii* ST4SA, fluorescence imaging revealed strong fluorescence emission of the bacteria, suggesting that there was functional expression of the mCherry protein in these Gram-positive hosts. Expression of the mCherry protein in the LAB hosts did not affect colony color. Similarly, in another study the expression of the *mCherry* gene in *Lb. sakei* was also reported not to affect colony color, with subsequent observation of the bacteria by fluorescence microscopy revealing bright red fluorescent cells (38). These results demonstrate the usefulness of the pGKCherry plasmid to fluorescently tag LAB host strains for *in vitro* applications.

Our interest is in using the *mCherry* gene to track labeled strains of LAB in the GIT of mice, thereby obtaining valuable information on the location and colonization by the bacteria. Therefore, we developed a system to integrate the *mCherry* fluorescent marker gene onto the chromosomes of *Lb. plantarum* 423 and *Ent. mundtii* ST4SA to circumvent the use of autonomous plasmids. This ensures that the marker gene will be maintained inside the host genomes without the need for antibiotic selection to ensure enhanced stability and continuous expression (12, 39-41). Integration vectors pGKRm1CmRCherry and pGKEmuNCmRCherry were constructed to enable the homologous recombination of the *CmRCherry* cassette containing a gene for Cm resistance and the *mCherry* fluorescence gene into the *Rm1* and *EmuN* gene sequences of *Lb. plantarum* 423 and *Ent. mundtii* ST4SA, respectively. Previous studies have described the integration of plasmids, unable to replicate in Gram-positive bacteria, in LAB by using randomly selected heterologous chromosomal fragments as regions

of homology (39, 41, 42). However, the disruption or inactivation of random genes without knowing their function may be detrimental to the relevant LAB strain.

We hypothesized that the disruption or inactivation of potentially inactive prophage and restriction modification (RM) genes would not affect the host strains' growth capacity or cell physiology. Since, the homologous recombination of the engineered construct can only be designed using known chromosomal DNA sequences, the screening for target sequences proved to be challenging. As the whole genome sequences of *Lb. plantarum* 423 and *Ent. mundtii* ST4SA had not yet been published, we had to turn to genome sequences of closely related bacteria. Our investigation showed that both strains are significantly different to the *Lb. plantarum* and *Ent. mundtii* strains that already had their genomes published. In support of this notion, the *Mrrp* RM gene was present in all of the *Lb. plantarum* strains whose genomic sequences were available and in the strains we tested for in our lab but was not in strain 423.

The *CmRCherry* gene cassette was successfully integrated into the chromosome of *Lb. plantarum* 423 by homologous recombination. Several other studies have reported on the successful use of homologous recombination for the stable integration of genes in bacterial chromosomes (18, 42, 43). Our results indicate that disruption or inactivation of the chromosomally located *Lb. plantarum* 423 *Rm1* gene is not lethal to the survival of the host. In addition, it was essential that the genomic interruption of the *Rm1* target gene by homologous recombination on the chromosome of *Lb. plantatum* 423 have no impact on the strain's growth capacity. Thus, in favour of this, the growth capacity of the chromosomally marked *Lb. plantatum* 423 remained unaffected following the disruption of the target gene sequence.

Fluorescent *Ent. mundtii* ST4SA (pCmRCherry) transformants retained resistance to both Em and Cm. This was an indication that the integration of the *CmRCherry* gene cassette without the plasmid backbone did not occur. PCR amplification confirmed that the target *EmuN* gene was not disrupted. Therefore, it can be speculated that disruption of the *EmuN* gene was not achieved as its inactivation could potentially be harmful to the host cell. However, despite this, *Ent. mundtii* ST4SA (pCmRCherry) transformants retained resistance to both Em and Cm following culturing for five days in the absence of the antibiotics, indicating the stability of the autonomous plasmid.

The recombinant fluorescent probiotic reporter strains, *Lb. plantarum* 423::CmRCherry and *Ent. mundtii* ST4SA (pCmRCherry), were shown to still be producing their antimicrobial peptides as evident from the formation of inhibition zones on overlaid MRS plates containing the sensitive *L. monocytogenes* EGDe strain. This result also confirmed that the metabolic load of carrying and expressing the fluorescence gene had no effect on the probiotic strains' ability to produce their antimicrobial peptides. The operons coding for the bacteriocins produced by *Lb. plantarum* 423 and *Ent. mundtii* ST4SA strains are situated on plasmids (34, 35). The replicons of the bacteriocin-containing plasmids and the plasmid vectors used to introduce the *mCherry* marker are compatible, as was confirmed by plasmid isolations.

Furthermore, for both *Ent. mundtii* ST4SA (pCmRCherry) and *Lb. plantarum* 423::CmRCherry a positive or linear correlation between different fluorescence intensities and CFUs exists. This suggests that in future experiments the amount of labeled bacteria (CFU) present in a sample, correlating to a given fluorescent signal, can be estimated using the linear graphs.

In this study, we describe the construction and application of a LAB/*E. coli* expression plasmid as well as two integration vectors carrying the synthetic *mCherry* fluorescence gene.

It was shown that the *mCherry* gene can be used as a useful marker to fluorescently tag the *Lb. plantarum* 423 and *Ent. mundtii* ST4SA probiotic LAB strains for *in vivo* application. The introduction of the *mCherry* fluorescence gene into the LAB strains has no effect on the bacteria's ability to produce their antibacterial peptides. Furthermore, we have demonstrated that target genes with known DNA sequences that are likely to be non-functional in the cells can be used for the homologous integration of foreign genes in *Lb. plantarum* 423 and *Ent. mundtii* ST4SA. The integration system described here may be tested and applied to other *Lb. plantarum* and *Ent. mundtii* strains using the target genes we have identified.

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TABLE 1. Description of bacterial strains and plasmids used in this study

Strain or plasmid	Description	Reference or source
Strains		
<i>E. coli</i>		
DH5 α	General purpose host strain for subcloning	(28)
<i>Lactobacillus plantarum</i>		
423	Probiotic (with adhesion genes) which lines the gut; constantly produces a powerful anti-microbial peptide that kills microbes; originally isolated from sorghum beer	Cipla Medpro (Pty) Ltd
423 pGKCherry	Contains pGKCherry plasmid; Em ^R	This study
423::CmRCherry	Contains CmR gene fused to <i>mCherry</i> gene cassette stably integrated to the Rm1 locus; Cm ^R	This study
423 bac ⁻	Derivative of probiotic 423, cured of plasmid harbouring plantaricin 423	(30)
ATCC ^R 8014 TM	Quality control strain used for molecular research on LAB; origin not specified	ATCC
ATCC ^R 14917 TM	Quality control strain; Originally isolated from pickled cabbage.	ATCC
<i>Enterococcus mundtii</i>		
ST4SA	Probiotic (with adhesion genes) which lines the gut and constitutively produces antimicrobial peptides; originally isolated from soybeans	Cipla Medpro (Pty) Ltd
ST4SA pGKCherry	Contains pGKCherry plasmid; Em ^R	
ST4SA pCmRCherry	Contains pGKEmuNCmRCherry plasmid; Cm ^R , Em ^R	
Plasmids		
pMK-RQmCherry	Cloning vector carrying <i>mCherry</i> gene cassette codon optimized for <i>Lb. plantarum</i> and <i>Ent. mundtii</i> fused to the <i>Lb. plantarum Pldhl</i> promoter (lactate dehydrogenase); Kan ^R	This study. Invitrogen, Germany
pGKV223D	<i>E. coli</i> /Lactic acid bacteria shuttle vector; Em ^R ; LAB expression vector with L23 promoter	University of Grönigen, The Netherlands
pGKCherry	pGKV223D plasmid carrying <i>mCherry</i> gene cassette codon optimized for <i>Lb. plantarum</i> and <i>Ent. mundtii</i> fused to the <i>Lb. plantarum Pldhl</i> promoter (lactate dehydrogenase); Em ^R	This study
pKSBluescript	PCR cloning vector	Promega, Madison, WI
pKSCherry	pKSBluescript plasmid carrying codon optimized <i>mCherry</i> gene cassette; Amp ^R	This study

pKSCmR	pKSBluescript plasmid carrying Chloramphenicol resistance gene (Cm ^R); Amp ^R	This study
pKSCmRCherry	pKSBluescript plasmid carrying Cm ^R gene fused to <i>mCherry</i> gene cassette; Cm ^R , Amp ^R	This study
pGKRm1	pGKV223D plasmid carrying <i>Lb. plantarum</i> 423 Type 1 restriction modification gene (<i>Rm1</i>); Em ^R	This study
pGKEmuN	Contains pGKV223D plasmid carrying <i>Ent. mundtii</i> ST4SA DNA entry nuclease gene (<i>EmuN</i>); Em ^R	This study
pGKRm1CmRCherry	pGKV223D plasmid carrying <i>Lb. plantarum</i> 423 <i>Rm1</i> gene with Cm ^R and <i>mCherry</i> cassette insert; Cm ^R , Em ^R	This study
pGKEmuNCmRCherry	pGKV223D plasmid carrying <i>Ent. mundtii</i> ST4SA <i>EmuN</i> gene with Cm ^R and <i>mCherry</i> cassette insert; Cm ^R , Em ^R	This study

^a Em^R, resistance to erythromycin

^b Amp^R, resistance to ampicillin

^c Kan^R, resistance to kanamycin

^d Cm^R, resistance to chloramphenicol

TABLE 2. Oligonucleotides used in this study

Target	Primer	Sequence 5' to 3'	Restriction sites	Product size (bp)
<i>Pldh</i>	ForPldh RevPldh	<u>GAATTC</u> AAATCTTCTCACCGTCTTG <u>GGATCCT</u> CATCCTCTCGTAGTG	<i>Eco</i> R1 <i>Bam</i> HI	500
TT	ForTT RevTT	<u>AAGCTT</u> CCTGCAGGCATG GCT <u>TCTAGA</u> ACCGTTTCTACTCAATGAAC	<i>Hind</i> III <i>Xba</i> I	274
<i>Lp2</i>	ForLp2 RevLp2	<u>GAATTC</u> TGGTACTTCTTGCAATTCATCTG <u>TCTAGA</u> TGCTAATTTACGATCATCAATATACTTC	<i>Eco</i> R1 <i>Xba</i> I	963
<i>Plys</i>	ForPlys RevPlys	<u>GAATTC</u> GTGATATATAATGATGGAATTTATTC <u>TCTAGA</u> GCCTTAGCGTACTTATCCCAGGC	<i>Eco</i> R1 <i>Xba</i> I	900
<i>Mrrp</i>	ForMrrp RevMrrp	<u>GAATTC</u> CATGAGTTATAAGCGTTGGAATG <u>TCTAGA</u> TCAATCTTGTTTCATAATAATATGC	<i>Eco</i> R1 <i>Xba</i> I	936
<i>Lp1</i>	ForLp1 RevLp1	<u>GAATTC</u> AAAGCATTGGCTATCATTACG <u>TCTAGA</u> TATCAAAGTCAACGTCATCTAATATTG	<i>Eco</i> R1 <i>Xba</i> I	2349
<i>Rm1</i>	ForRm1 RevRm1	<u>GAATTC</u> TAGCGAATAATATTCTGGTCGAC <u>TCTAGA</u> CTGGCACAAGCAGAAACCGAG	<i>Eco</i> R1 <i>Xba</i> I	1011
<i>Emu</i>	ForEmu RevEmu	<u>GAATTC</u> AGGGATTGGCGGTTTTTCG <u>TCTAGA</u> TTTAACTTCCACTACTTCATAATGAG	<i>Eco</i> R1 <i>Xba</i> I	2157
<i>Emu1</i>	ForEmu1 RevEmu1	<u>GAATTC</u> CATGCATTATTTTAGTATTGG <u>AAGCTT</u> CAATTGATTTTTTCAGTTC	<i>Eco</i> R1 <i>Hind</i> III	743
<i>EmuN</i>	ForEmuN RevEmuN	<u>GAATTC</u> TTAGCTTGCCCCGTTTGATAG <u>TCTAGA</u> ACAAAAAACAACCAAAACAATCATAG	<i>Eco</i> R1 <i>Xba</i> I	720
<i>cat</i>	ForCmR RevCmR	<u>GTTCGAA</u> GGATCCATTCTAATGAAG <u>ATTCGAA</u> GCTTAACGTCAATAAAGC	<i>Asu</i> II <i>Asu</i> II	800
<i>mCherry</i>	ForCherry	ATGGCAATCATCAAAGAATTTATGCG	-	-

TABLE 3. Presence or absence of target genes in *Lb. plantarum* strains tested

<i>Lb. plantarum</i>	Target genes				
	<i>Lp2</i>	<i>Plys</i>	<i>Lp1</i>	<i>Mrrp</i>	<i>Rml</i>
423	-	-	-	-	+
ATCC 8014	+	+	-	+	+
ATCC 14917	-	-	-	+	+

^a Presence of target genes indicated by -

^b Absence of target genes indicated by +

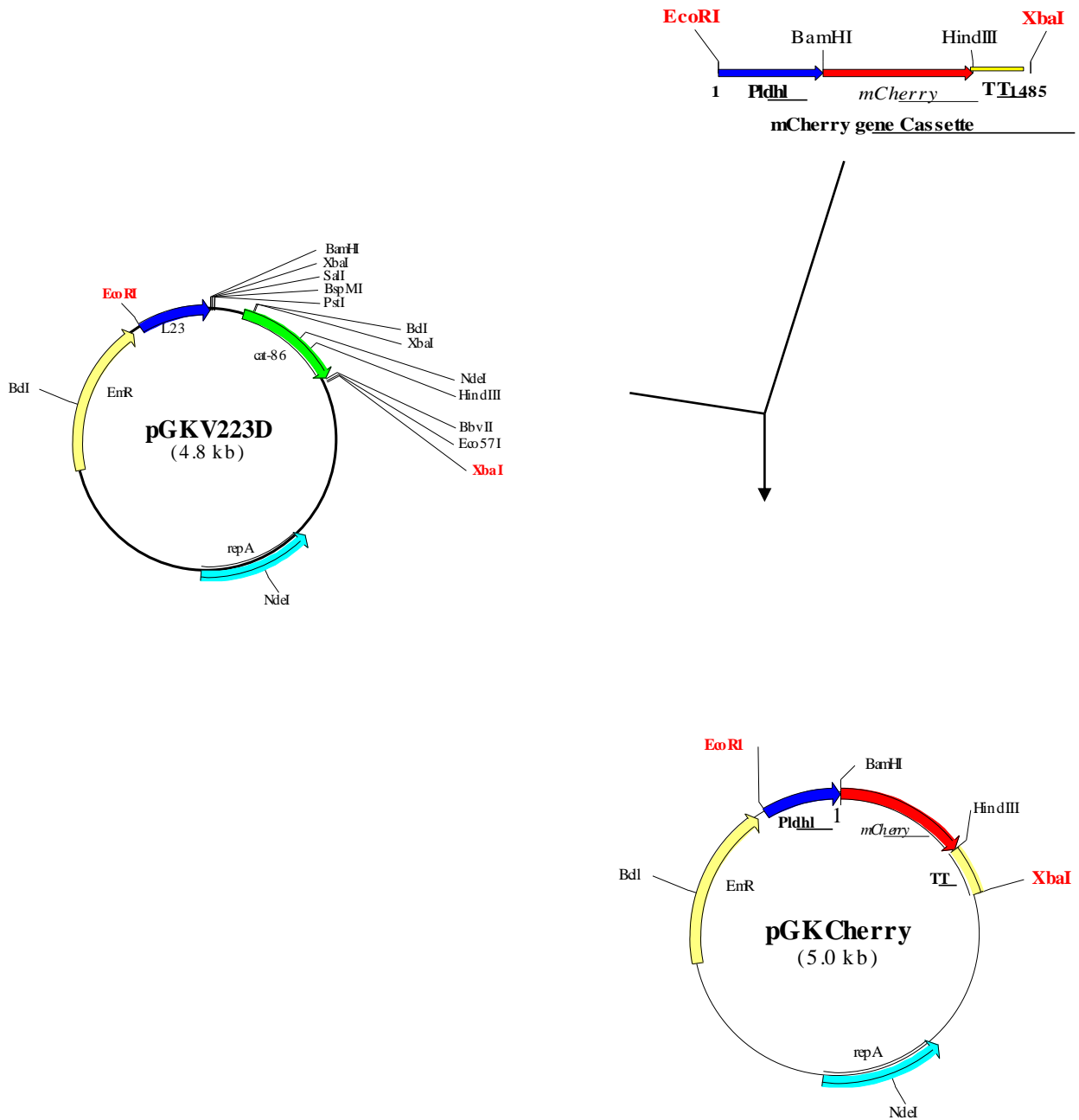


FIG. 1. Schematic representing the construction of pGKCherry. Relevant restriction sites are shown. For details, refer to “Materials and Methods”. Genes shown are *mCherry* and *Em^R* that encode the *mCherry* fluorescence gene and the protein for erythromycin resistance, respectively. Promoters: L23 originating from a *Lactococcal* spp.; *Pldhl* *Lb. plantarum* 423 lactate dehydrogenase promoter.

FIG. 2. Schematic representing the construction of pGKRm1CmRCherry and pGKEmuNCmRCherry. For details, refer to “Materials and Methods”.

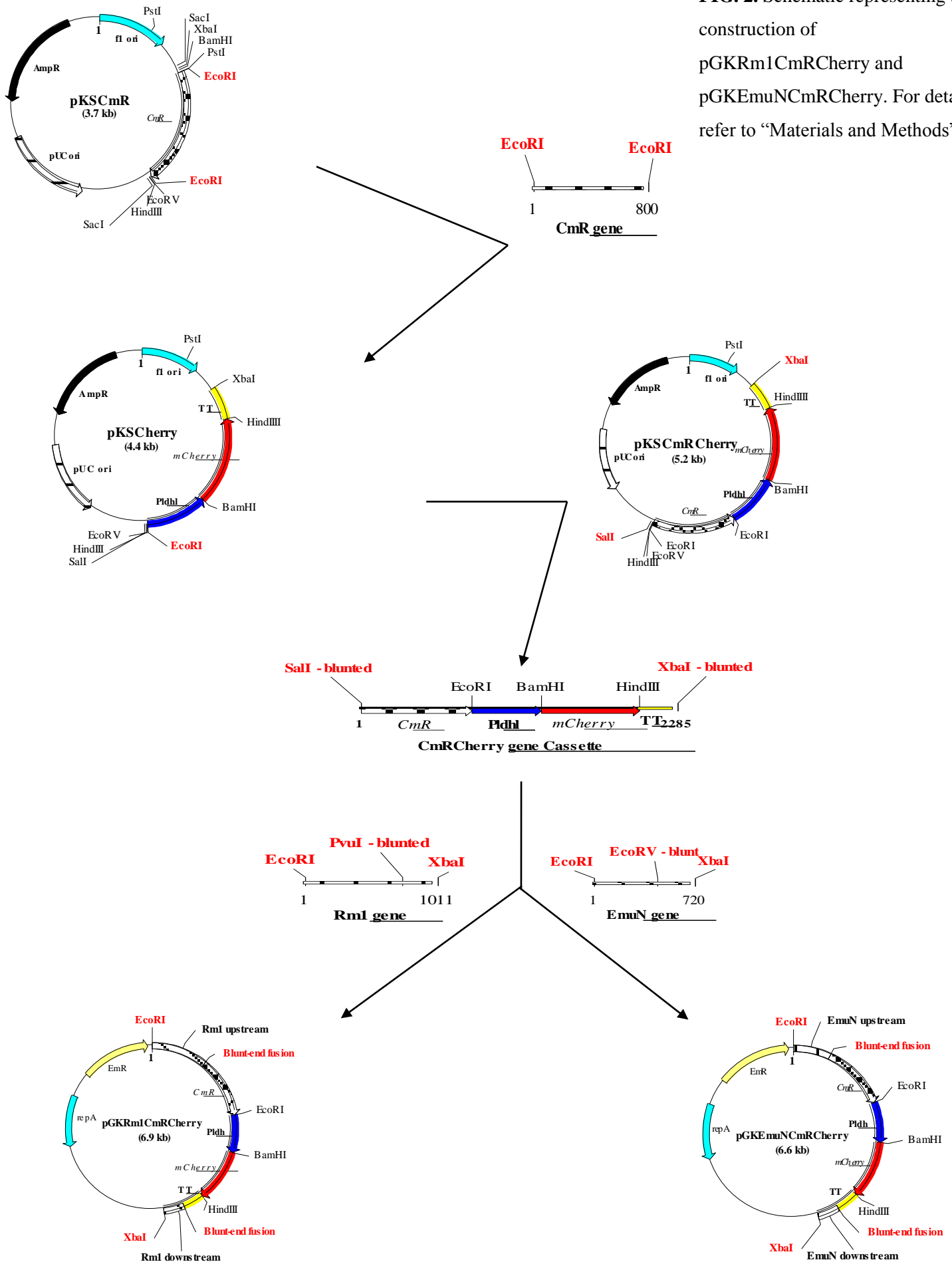




FIG. 3. Fluorescence detection of *E. coli* DH5 α harbouring pGKCherry. (a) Overnight culture of *E. coli* DH5 α containing pGKCherry inoculated onto surface of BHI agar using sterile swab and incubated for 24h at 37°C before and (b) after imaging for fluorescence using the IVIS spectrum imaging system, (c) Fluorescence emission of *E. coli* (pGKCherry) on the left compared to non-fluorescent *E. coli* (pGKV223D) on the right.

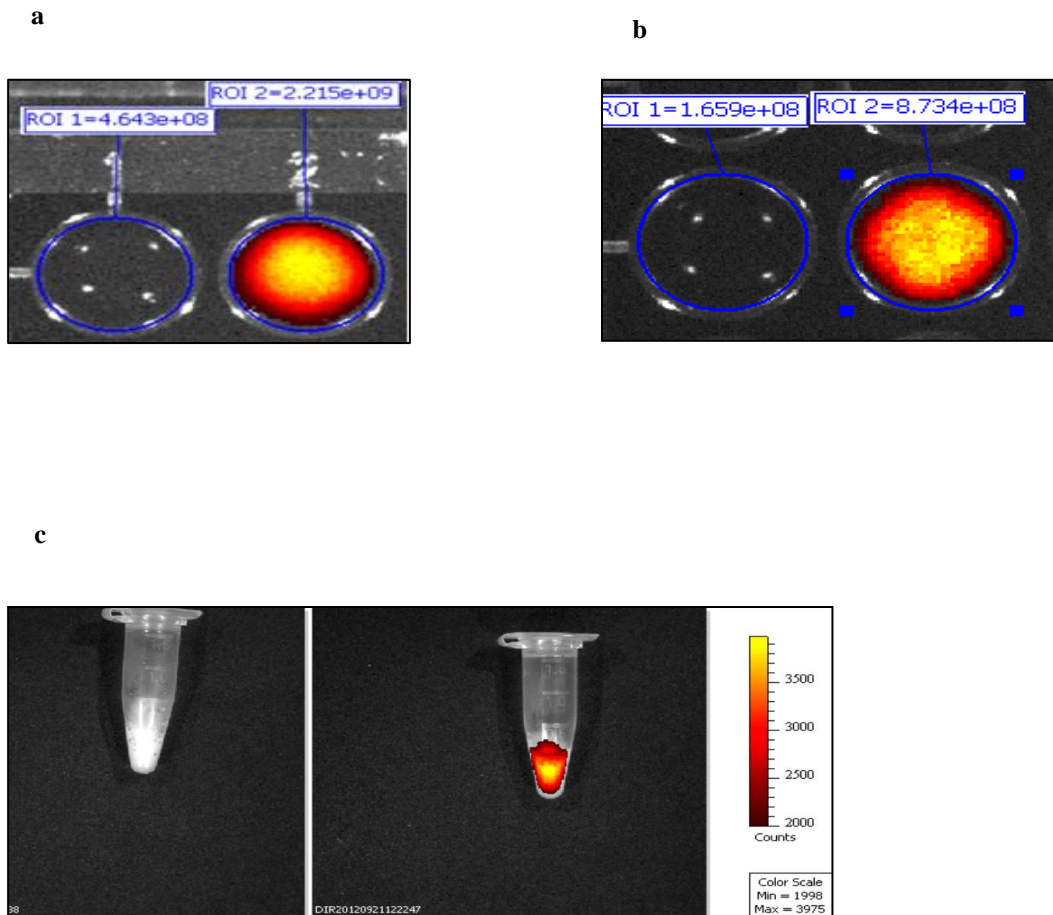


FIG. 4. Fluorescence detection of *Lb. plantarum* 423 and *Ent. mundtii* ST4SA harbouring pGKCherry (a) Left: control non-fluorescing *Lb. plantarum* 423 strain harbouring pGKV223D, right: fluorescing *Lb. plantarum* 423 strain harbouring pGKCherry; (b) left: control non-fluorescing *Ent.mundtii* ST4SA strain harbouring pGKV223D, right: fluorescing *Ent. mundtii* ST4SA strain harbouring pGKCherry; (c) microfuge tube containing (left) non-fluorescing *Lb. plantarum* 423 strain harbouring pGKV223D, and fluorescing (right) *Lb. plantarum* 423 strain harbouring pGKCherry. Regions of Interest (ROI) represent the average amount of fluorescence emitted at 572 nm in $\text{p/cm}^2/\text{s/sr}$.

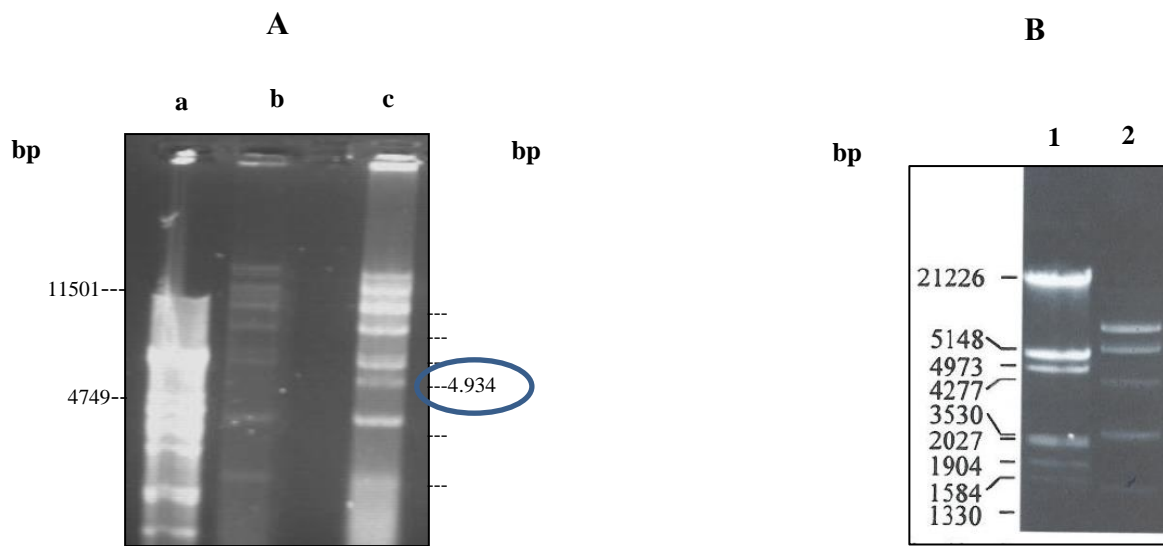


FIG. 5. Plasmid profiles of *Lb. plantarum* 423 transformed with pGKCherry and wild-type *Lb. plantarum* 423. **A.** Profile of plasmids in transformed *Lb. plantarum* 423 (this study): (a) Lambda DNA digested with *Pst*I (Promega, Madison, USA); (b) plasmid profile of *Lb. plantarum* 423; (c) plasmid profile of transformant *Lb. plantarum* 423 harbouring pGKCherry (position and molecular weight indicated). **B.** Profile of naturally occurring plasmids of *Lb. plantarum* 423 (30): (1) Lambda DNA digested with *Eco*RI and *Hind*III; (2) Five naturally occurring plasmids of *Lb. plantarum* 423.

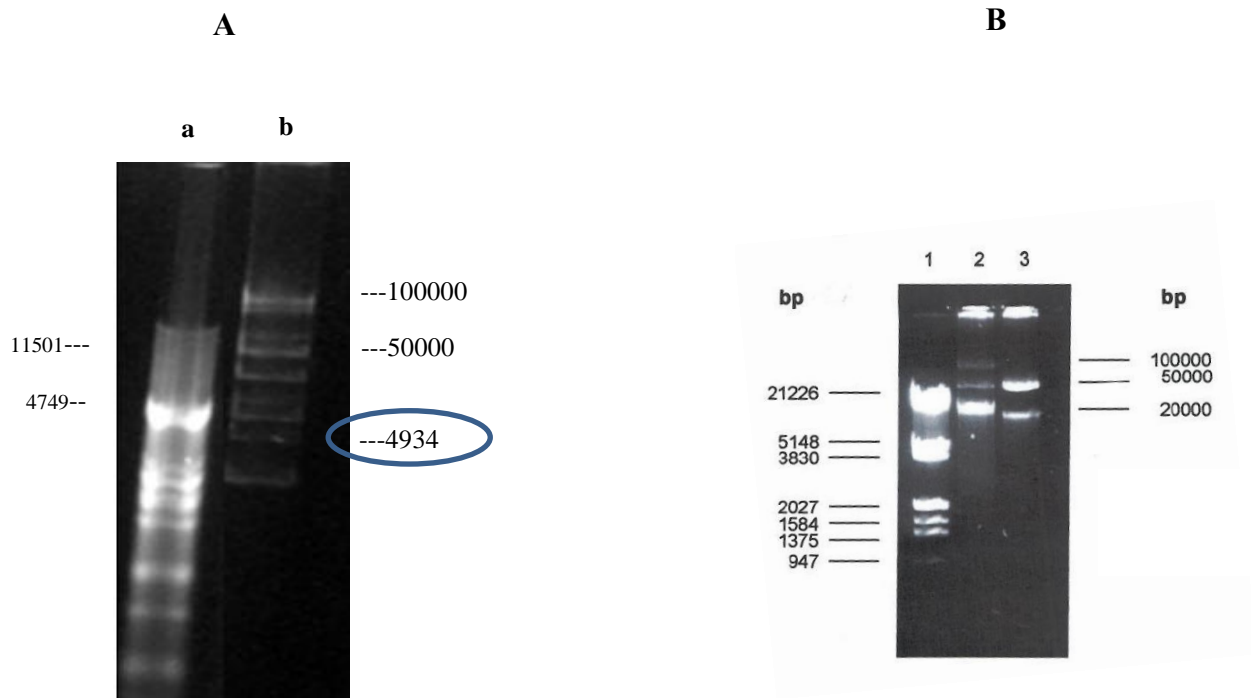


FIG. 6. Plasmid profiles of *Ent. mundtii* ST4SA transformed with pGKCherry and wild-type *Ent. mundtii* ST4SA. **A.** Profile of plasmids in transformed *Ent. mundtii* ST4SA (this study). (a) Lambda DNA digested with *Pst*I (Promega, Madison, USA); (b) 100 kb and 50 kb plasmids of transformant *Ent. mundtii* ST4SA with 4934 bp pGKCherry plasmid (indicated with dashed lines). **B.** Naturally occurring plasmid profile of *Ent. mundtii* ST4SA (35). (1) Lambda DNA digested with *Eco*RI and *Hind*III; (2 and 3) 50 kb and 100 kb natural plasmids of ST4SA.

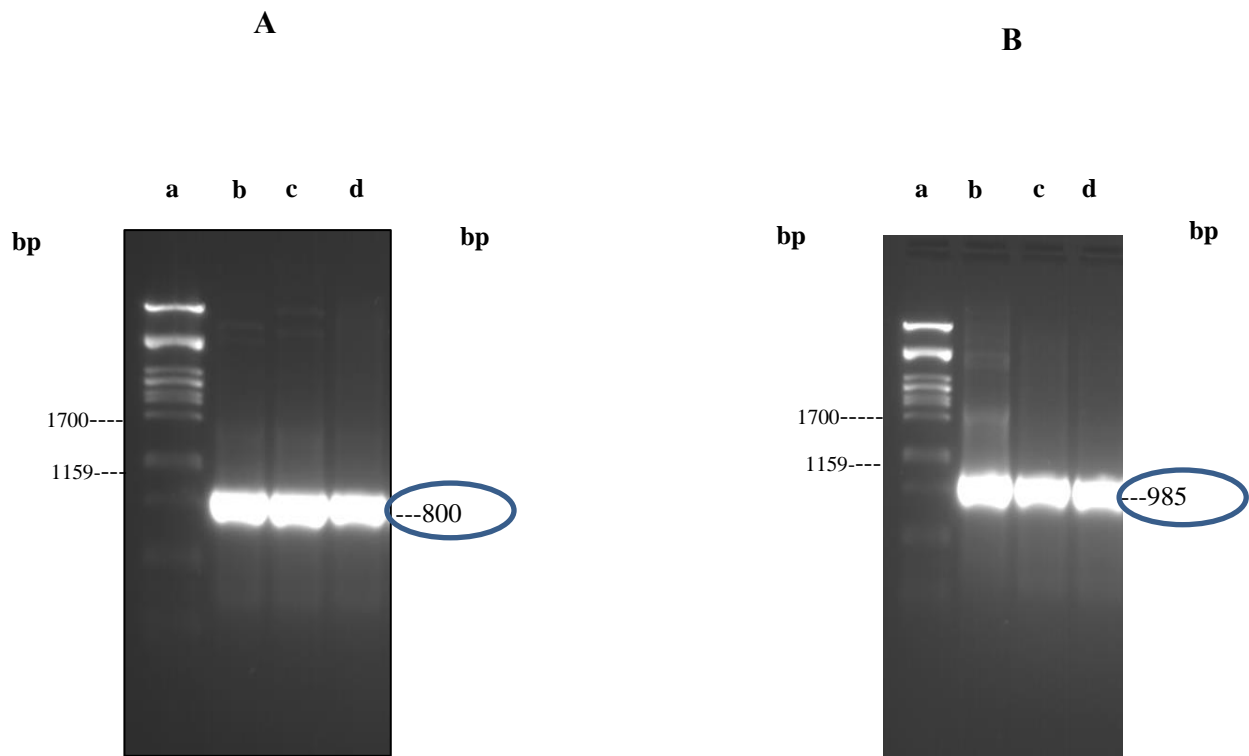


FIG. 7. PCR amplification of the *cat* and *mCherry* genes in *Lb. plantarum* 423 and *Ent. mundtii* ST4SA transformed with integration vectors. **A.** (a) Lambda DNA digested with *Pst*I (Promega, Madison, USA); 0.8 kb *cat* gene PCR amplicon amplified using (b) pKSCmR plasmid template DNA as positive control, (c) *Lb. plantarum* 423::CmRCherry and (c) *Ent. mundtii* ST4SA (pCmRCherry) gDNA as template DNA. **B.** (a) Lambda DNA digested with *Pst*I (Promega, Madison, USA); 0.98 kb *mCherry* gene plus TT transcriptional terminator PCR amplicon amplified using (b) pGKCherry plasmid template DNA as positive control, (b) *Lb. plantarum* 42::CmRCherry and (c) *Ent. mundtii* ST4SA (pCmRCherry) gDNA as template DNA.

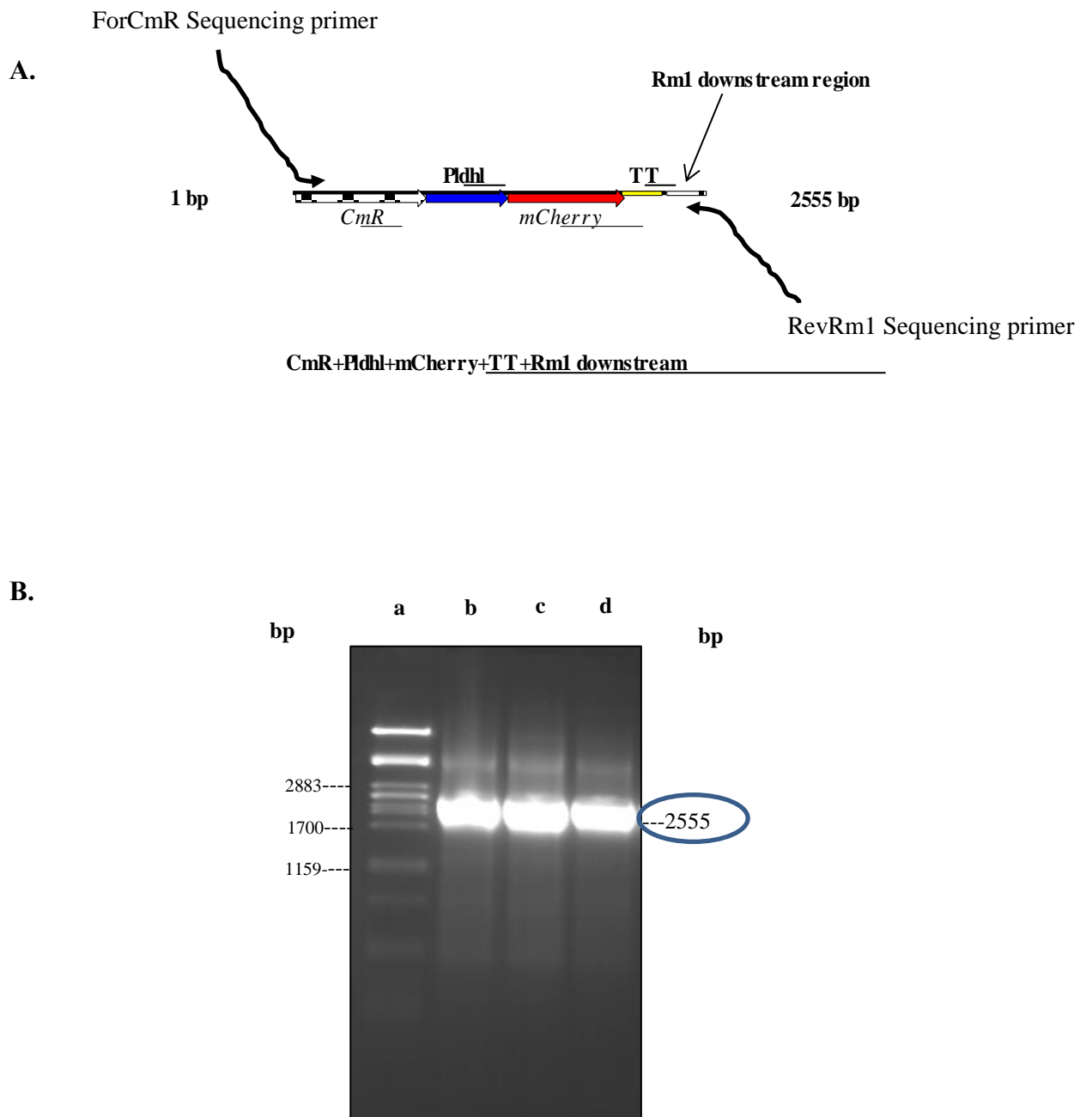


FIG. 8. A. Schematic diagram representing the 2.5 kb CmR+*Pldhl*+*mCherry*+TT+*Rm1* amplicon. The predicted fragment size and the binding regions of sequencing primers ForCmR and RevRm1 are indicated. **B.** PCR amplification confirming the integration of the CmRCherry cassette in the chromosome *Lb. plantarum* 423. (a) Lambda DNA digested with *Pst*I (Promega, Madison, USA); 2.6 kb PCR DNA fragment containing the *cat* gene, *Pldhl* promoter, *mCherry* gene, TT transcriptional terminator and the downstream *Rm1* gene fragment amplified using (b) pGKRm1CmRCherry plasmid template DNA as positive control and (b & c) gDNA of two transformants of *Lb. plantarum* 423::CmRCherry as template DNA.

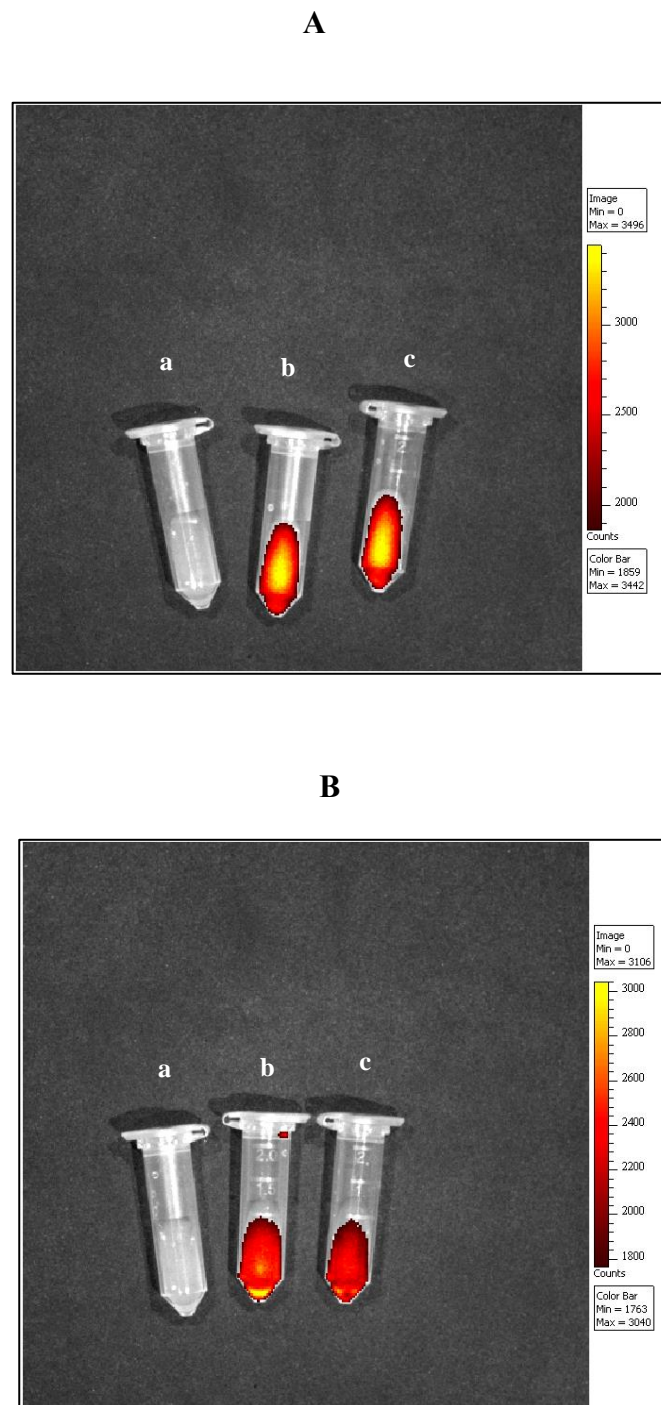


FIG. 9. Real-time fluorescence detection of *Lb. plantarum* 423 and *Ent. mundtii* ST4SA transformed with integration vectors. **A.** (a) Control non-fluorescing *Lb. plantarum* 423 strain harbouring pGKV223D and (b & c) fluorescing *Lb. plantarum* 423::CmRCherry; **B.** (a) Control non-fluorescing *Ent. mundtii* ST4SA strain harbouring pGKV223D and (b & c) fluorescing *Ent. mundtii* ST4SA (pCmRCherry).

FIG. 10. A.

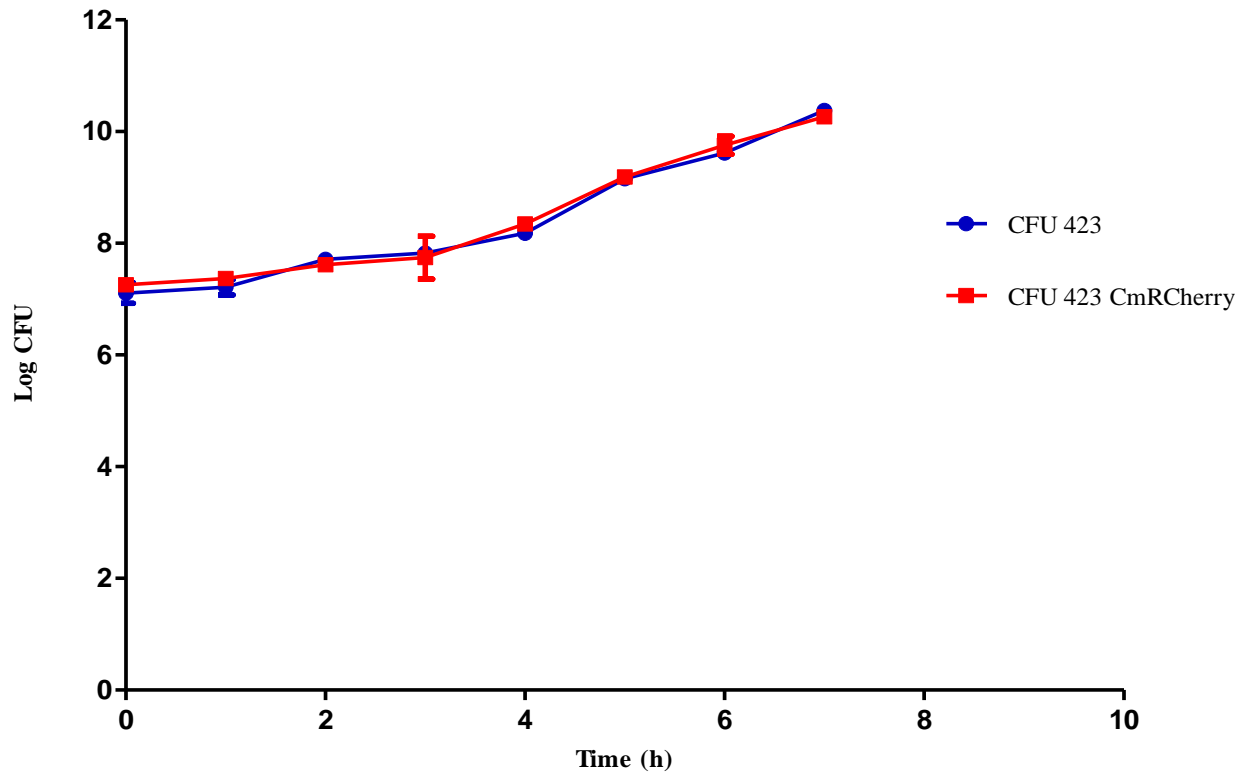


FIG. 10. B.

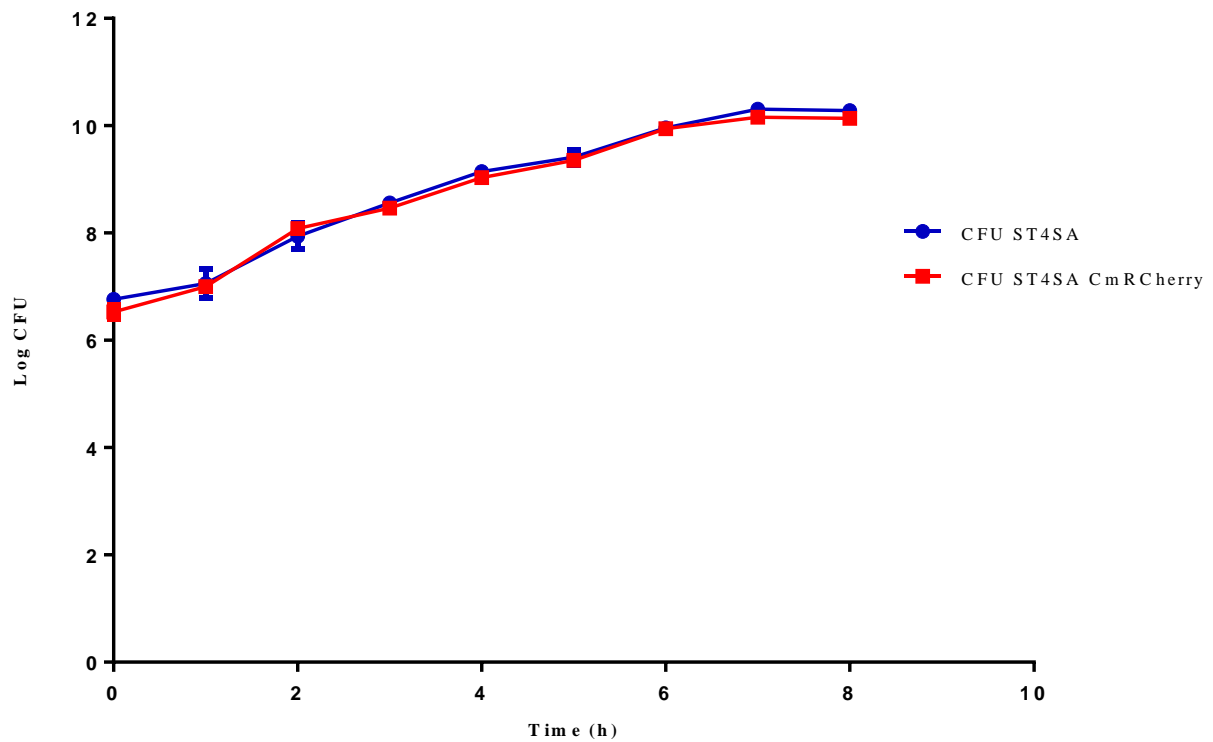


FIG. 10. Growth comparison of wild-type probiotic strains (in blue) and probiotic strains harbouring the Cherry gene (red): (A) *Lb. plantarum*::CmCherry and (B) *Ent. mundtii* ST4SA (pCmRCherry). Values are averages of triplicate plate counts.

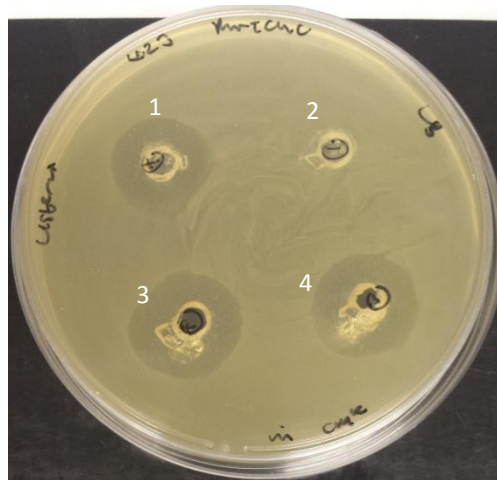
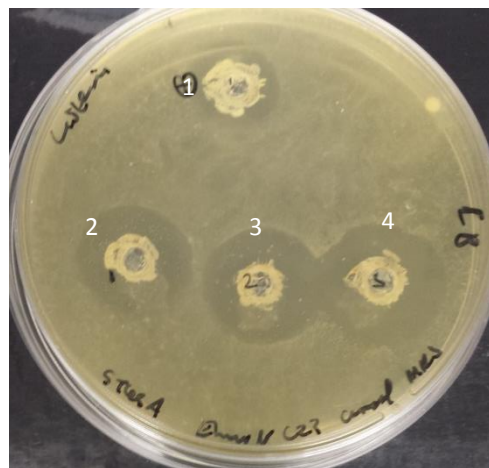
A**B**

FIG. 11. Inhibition zones on overlay plates with *L. monocytogenes* EGDe as indicator strain. **A.** (1) Bacteriocin 423 isolated from wild-type *Lb. plantarum* 423 as positive control, (2) sterile non-bacteriocin supernatant isolated from *Lb. plantarum* 423 bac⁻ as negative control and (3 & 4) bacteriocin 423 isolated from two *Lb. plantarum*::CmRCherry transformants; **B.** Bacteriocin ST4SA isolated from wild-type *Ent. mundtii* ST4SA as positive control and (2, 3 & 4) bacteriocin ST4SA isolated from three *Ent. mundtii* ST4SA (pCmRCherry) transformants.

FIG. 12. A

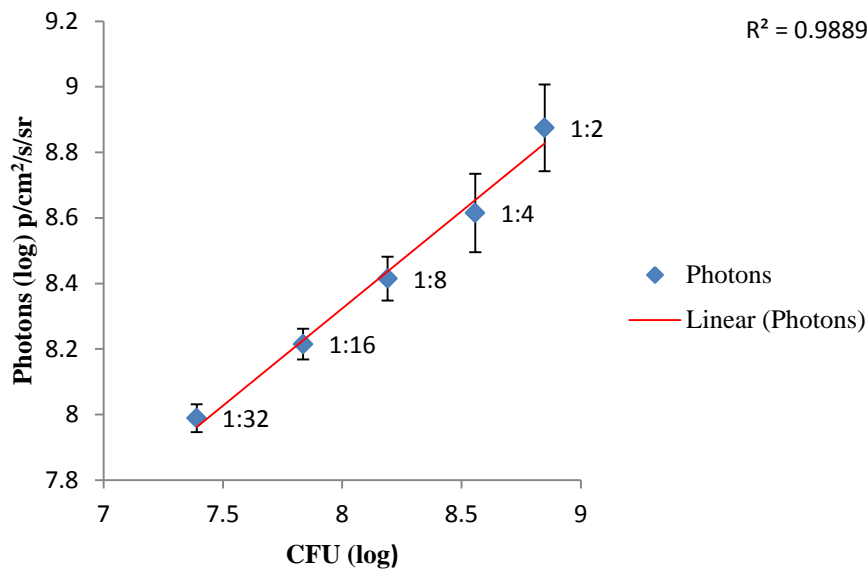


FIG. 12. B

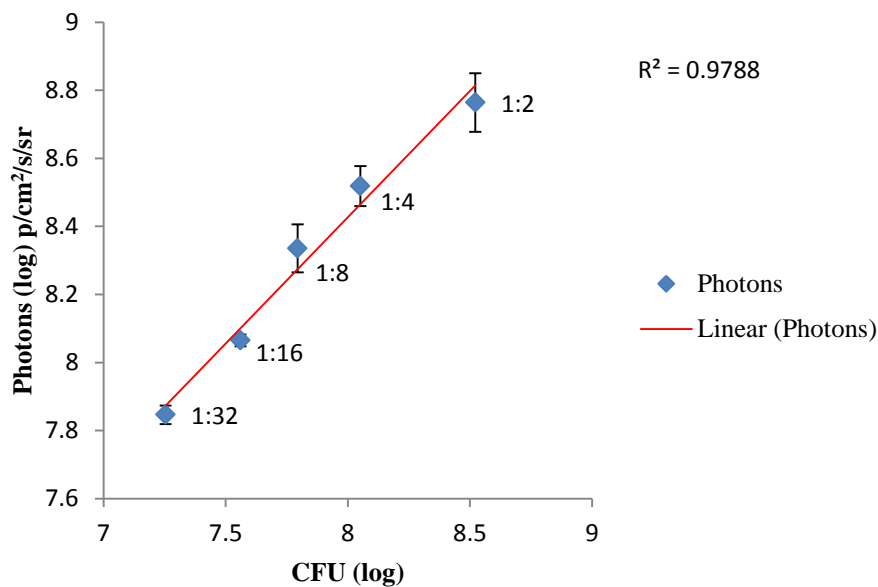


FIG. 12. Correlation between fluorescence signals and total CFUs of (A) *Lb. plantarum* 423::CmRCherry and (B) *Ent. mundtii* ST4SA (pCmRCherry). Positive correlations ($R^2=0.9889$ and $R^2=0.9788$) between fluorescence signals and CFUs were determined for all dilutions for both strains. Log₁₀ averages of three repeats are plotted, with standard deviations. The logarithmic trendline (red line) and the correlation coefficient of determination (R^2) between fluorescence signals and CFUs are shown.

Chapter 4

**Intestinal Colonization of *Enterococcus mundtii* ST4SA
and *Lactobacillus plantarum* 423 in Mice, as Reported with
Fluorescent Imaging**

Intestinal Colonization of *Enterococcus mundtii* ST4SA and *Lactobacillus plantarum* 423 in Mice, as Reported with Fluorescent Imaging

Abstract

Lactic acid bacteria (LAB) are frequently encountered as natural inhabitants of the gastrointestinal tract (GIT) of humans and animals. Some species received considerable attention due to the beneficial effects they exert on health. In this study we report on the application of the mCherry protein in the *in vivo* tagging of two probiotic strains, *Enterococcus mundtii* ST4SA and *Lactobacillus plantarum* 423. Using fluorescence imaging, we determined that both strains were predominantly colonized in the cecum and colon of mice. The location and duration of colonization of the two strains in each section of the GIT were also revealed. We report for the first time on the fluorescence imaging of LAB expressing mCherry in a mouse model.

Introduction

Lactic acid bacteria (LAB) are common inhabitants of the human gastro-intestinal tract (GIT). Some LAB, especially lactobacilli, are well known for their application in fermented foods and probiotic properties (1, 2). These microorganisms exert many beneficial effects on human health, such as the digestion and assimilation of food and the prevention of pathogens from colonizing the GIT. (3-6). Furthermore, some selected probiotic strains are believed to perform a critical role in the treatment of gastro-intestinal disorders, lactose intolerance and in the stimulation of the immune system (7, 8).

Despite the ever increasing consumer interest in probiotic LAB due to their health-promoting characteristics, the mechanisms by which they exert their beneficial effects and the activities of the probiotics in the GIT often remain uncertain (9). The understanding of the survival mechanisms of LAB in the GIT, especially the interaction between LAB and pathogens would be facilitated by the direct *in vivo* monitoring of these processes.

During the past three decades, a wide range of molecular biology tools have been developed to facilitate the study of LAB. The labelling of bacteria with marker genes with an easily detectable phenotype has proven to be invaluable for the tracking of various LAB strains in complex environments such as the mammalian GIT. Several reporter genes, such as the green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* (1, 10), the bacterial *lux* genes from *Photobacterium luminescens* (11, 12), the click beetle luciferase system from *Pyrophorus plagiophthalmus* (13, 14), chloramphenicol acetyl-transferase gene (15, 16), the *Escherichia coli* β -glucuronidase gene (17) and the *Vibrio fischeri* luciferase gene (18) have been used to label LAB for *in vitro* and *in vivo* studies. However, many of these systems require an exogenous substrate to detect the expression of the reporter gene. Furthermore, oxygen requirements needed for the expression of reporter genes is often a limiting factor for *in vivo* applications (19). To circumvent these drawbacks, red fluorescent proteins, such as mCherry,

have been developed (20), and have been used successfully to label *Mycobacterium tuberculosis* (21), *E. coli*, *Lactococcus lactis* and *Enterococcus faecalis* (23).

In this study we evaluate the potential of fluorescent imaging (FI) in real time monitoring of *Lb. plantarum* 423 and *Ent. mundtii* ST4SA *in vivo*. Survival and colonization of *Lb. plantarum* 423 and *Ent. mundtii* ST4SA in the GIT was studied using a simulated gastrointestinal model (32). However, simulated conditions of the GIT, are not always a true indication of what happens *in vivo*. Here, we demonstrate that *Lb. plantarum* 423 and *Ent. mundtii* ST4SA persists and colonises the GIT of mice. We also determined the location and duration of colonization of the two strains in each section of the GIT.

Materials and methods

Ethical approval for *in vivo* experiments was obtained from the Ethics Committee of the University of Stellenbosch (ethics reference number: SU-ACUM14-00015). Eight-week-old female BALB/c mice were used in all experiments. The animals were kept under controlled environmental conditions with water and feed provided *ad libitum* and changed daily.

Preparation of bacterial strains and administration to mice

Fluorescent strains of *Lb. plantarum* 423::CmRCherry and *Ent. mundtii* ST4SA (pCmRCherry) that express the synthetic *mCherry* red fluorescence gene were constructed as described previously (23). Both recombinant strains contain a gene for chloramphenicol resistance. The strains were grown for 12 h at 30°C in 10 ml MRS broth (Biolab Diagnostics, Midrand, South Africa), without aeration. One milliliter of each culture was inoculated into fresh 10 ml MRS broth and incubated to an optical density at 600 nm of 2.5 for *Lb. plantarum* 423::CmRCherry and 2.3 for *Ent. mundtii* ST4SA (pCmRCherry). The cells were harvested (3 min at 8000 x g) and washed twice with sterile phosphate-buffered saline (PBS).

The cells of each of the strains were diluted to 2×10^{10} CFU/ml and resuspended in 200 μ l gavage buffer (0.2 M NaHCO₃ buffer containing 1% glucose, pH 8) to a final concentration of 4×10^9 CFU. Viable bacterial cell suspensions were administered to mice by intragastric gavage.

***In vitro* fluorescence detection**

Fluorescence assays of bacterial strains expressing the *mCherry* gene were carried out using black non-autofluorescent 96-well microtiter trays. Bacterial suspensions (4 ml of 12 h old cultures) of strains with plasmids containing the *mCherry* gene were harvested (1500 x g for 3 min), the cell pellets were washed twice with sterile saline (0.85% (w/v) NaCl) to remove traces of medium, and resuspended in 300 μ l sterile saline. Before fluorescent image capturing, the 300 μ l cell suspensions were transferred to wells in a microtiter tray. Bacterial strains with plasmid constructs without the *mCherry* gene insert were used as controls for each fluorescing strain throughout.

Gastro-intestinal persistence of LAB

Two groups of nine mice each received one oral administration of fluorescent *Lb. plantarum* 423::CmRCherry and *Ent. mundtii* ST4SA (pCmRCherry), respectively. Control mice received non-fluorescent *Lb. plantarum* 423 and *Ent. mundtii* ST4SA containing a vector without the *mCherry* gene insert. Mice fecal material was collected at 30 min, 1, 2, 4, 6, 24, 48 and 72 h after administration of *Lb. plantarum* 423::CmRCherry and *Ent. mundtii* ST4SA (pCmRCherry). For the enumeration of viable bacteria in feces, samples (100 mg) were mechanically homogenized in 1 ml PBS and serially diluted in PBS and plated onto MRS media containing chloramphenicol (Cm). Mice were sacrificed by cervical dislocation at 24, 48 and 72 h after the administration of bacteria. The GITs of the mice were dissected and the small intestine (duodenum, jejunum and ileum) and large intestine (cecum and colon) were

studied for the presence of fluorescence signals. Background auto-fluorescence was removed by overlaying images recorded of intestines with fluorescent bacteria with images of the intestines of non-fluorescent bacteria. The small and large intestines were homogenized separately in 3 ml PBS, serially diluted and plated onto MRS agar containing 5 and 10 $\mu\text{g/ml}$ Cm for *Ent. mundtii* ST4SA (pCmRCherry) and *Lb. plantarum* 423::CmRCherry, respectively.

Evaluation of *In vivo* fluorescence

Fluorescence imaging (FI) was carried out using the IVIS[®] 100 In Vivo Imaging System (Caliper Life Sciences, Hopkinton, MA, U.S.A.) which consists of a cooled charge-coupled-device camera mounted on a light-tight specimen chamber. Photons emitted at 620 nm were expressed as photons/cm²/second/steradian (p/cm²/s/sr), using the Living Image[®] software, version 3.0 of Caliper Life Sciences. Regions of interest (ROI) were manually selected. Signals produced from mice administered with fluorescent strains were compared to signals produced from mice administered non-fluorescent strains. Before fluorescence imaging, mice were anesthetized with 2% (v/v) isoflurane and placed into the IVIS camera chamber. The anaesthetic (1.5% (v/v) isoflurane) was administered throughout examination through a nose cone. Mice were killed at endpoints and the skin tissue of the abdomen was removed before imaging in order to enhance fluorescence signal penetration. Reference images of mice administered with control non-fluorescent strains were used for the removal of background signals throughout the experiment.

Results

Monitoring of *Lb. plantarum* 423 and *Ent. mundtii* ST4SA in the GIT

At time zero (before administration of mice), no fluorescence signal was detected. During the first 24 h post inoculation, no significant difference could be detected transcutaneously between mice treated with fluorescent LAB and mice treated with control strains (data not shown). However, when mice were imaged after sacrifice with the removal of the abdomen skin tissue 24 h after administration of bacteria, a notable difference in fluorescence was detected (Fig. 1).

The intestines were also imaged *ex vivo*, to determine the localization of the fluorescent bacteria in the GIT (Fig. 2). At 24 h post inoculation of bacteria, both strains were observed to have colonized predominantly in the cecum and colon. However, no fluorescence was observed in the small intestines of mice administered *Lb. plantarum* 423::CmRCherry, whereas fluorescent cells could be detected in the small intestines of mice administered *Ent. mundtii* ST4SA (pCmRCherry). After 48 h, no fluorescent *Lb. plantarum* 423::CmRCherry and *Ent. mundtii* ST4SA (pCmRCherry) could be detected in either the small or large intestines.

Colonization of *Lb. plantarum* 423 and *Ent. mundtii* ST4SA in small and large intestine

After 24 h, mice administered with *Lb. plantarum* 423::CmRCherry harboured approximately 7.5×10^2 CFU in the small intestine (Fig. 3A) and more than 2×10^5 CFU in the large intestine (Fig. 3B). Mice administered *Ent. mundtii* ST4SA (pCmRCherry) harboured approximately 2×10^4 CFU in the small intestinal compartment (Fig. 3A) and 2×10^5 in the large intestinal compartment after 24 h (Fig. 3B). After 72 h, no *Lb. plantarum* 423::CmRCherry was detected in the intestines of mice. No viable *Ent. mundtii* ST4SA (pCmRCherry) cells were detected in the small intestine 72 h after administration. However,

the number of *Ent. mundtii* ST4SA (pCmRCherry) cells in the large intestinal compartment was still approximately 5×10^3 CFU.

Enumeration of viable *Lb. plantarum* 423 and *Ent. mundtii* ST4SA in mouse feces

Fluorescent cells of *Lb. plantarum* 423::CmRCherry and *Ent. mundtii* ST4SA (pCmRCherry) were observed in the feces collected after 24 h. High numbers of both strains were excreted in the feces (Fig. 4). For *Lb. plantarum* 423::CmRCherry, the maximum level of viable cells excreted after 4 h reached approximately 3×10^7 CFU/100 mg feces. *Enterococcus mundtii* ST4SA (pCmRCherry), on the other hand, reached a maximum level of 3×10^8 CFU/100 mg feces after 4 h and then slightly declined to 8×10^7 CFU/100 mg feces after 24 h. After 72 h, the number of *Lb. plantarum* 423::CmRCherry organisms declined to approximately 5×10^3 CFU/100 mg feces, while the *Ent. mundtii* ST4SA (pCmRCherry) declined to 4×10^4 CFU/100 mg feces.

Discussion

The emission of strong fluorescence by *Lb. plantarum* 423::CmRCherry and *Ent. mundtii* ST4SA (pCmRCherry) harbouring the codon-optimized synthetic *mCherry* has been reported (23). Similar results were recorded for the *in vitro* expression of the *mCherry* gene in *Lactococcus lactis*, *Enterococcus faecalis* and *Mycobacterium tuberculosis* (21, 22).

In the current study, *in vivo* results demonstrated that transcutaneous signals in mice administered fluorescent *Lb. plantarum* 423::CmRCherry and *Ent. mundtii* ST4SA (pCmRCherry), could not be distinguished from the background signals of mice administered non-fluorescent control strains. Factors that usually influence the application of a particular fluorescence protein for *in vivo* experimentation include the propagation of fluorescent signals through tissue and brightness of the fluorophore compared to the inherent background

auto-fluorescence of the sample or animal (21, 24, 25). However, the excitation and emission spectrum of the mCherry fluorophore is outside the region where endogenous chromophores, such as haemoglobin, absorb light within tissues (26).

In a similar study, abdominal auto-fluorescence effected the detection of intentional fluorescence signals in mice (27). However, the effects of auto-fluorescence can usually be avoided by the use of fluorophores with excitation and emission wavelengths in the far red and near infrared region (600 nm > 780 nm) of the visible spectrum (28). Furthermore, impairment of transcutaneous signals could also be explained by the inherent background signals generated by the auto-fluorescence of the animal diet intake (29). This merits further investigation. Since no other studies regarding the *in vivo* application of the *mCherry* gene are reported in literature, this is the first study reporting the *in vivo* detection of LAB in mice using mCherry.

By removal of the abdominal tissue and skin of euthanized animals at 24 h post-inoculation, fluorescence was detected in mice colonized with *Lb. plantarum* 423::CmRCherry and *Ent. mundtii* ST4SA (pCmRCherry). The fluorescent signal was weakened significantly by passage through the animal tissue and skin for transcutaneous detection compared to the signal obtained after tissue and skin removal.

The digestive tracts of mice colonized with fluorescent LAB were also imaged *ex vivo*. The compensation for background auto-fluorescence, possibly due to chromophores in mice feed, significantly increased detection of the signal. Both strains were predominantly detected in the anaerobic cecum and colon. Similarly, previous studies found *Lb. plantarum* to predominantly persist in the cecum and colon (5, 30). In another study, *Lb. plantarum* 423 was reported to adhere to mucus isolated from the ileum and cecum of piglets (31). The CFU counts confirmed that the cecum and colon were the major sites of *Lb. plantarum* 423 and *Ent. mundtii* ST4SA colonization (Fig. 3). However, a fluorescent signal and high numbers of

Ent. mundtii ST4SA cells were also found in the small intestine. This is in contrast to a previous investigation which reported that *Lb. plantarum* 423 colonized the ileum, cecum and colon, whereas *Ent. mundtii* ST4SA only colonized the cecum (8). The authors used fluorescent *in situ* hybridisation (FISH) with strain specific oligonucleotide probes to detect which surfaces in the GIT the strains adhered to. However, *Lb. plantarum* 423 was also detected in the intestines of rats not treated with *Lb. plantarum* 423. This suggests that the oligonucleotide probes that were used may have hybridized to DNA from other closely related strains in the rat GIT and may have affected the results. The CFU counts revealed that strain ST4SA colonises the gut more effectively as low levels of the bacteria were still present 72 h post-feeding (Fig 3).

The colonization ability of *Lb. plantarum* 423 and *Ent. mundtii* ST4SA were assessed by determining the number of cells excreted at different time points over 72 h. Both strains survived conditions in the GIT as clearly indicated by the attainment of viable fluorescing cells in the feces of mice (Fig. 4). Similar results were obtained in a previous study, with the prediction of the survival of *Lb. plantarum* 423 and *Ent. mundtii* ST4SA in the GIT of infants using a computerized gastro-intestinal model (32). High cell numbers were excreted for both strains during the first 24 h after administration of the bacteria to mice (Fig. 4). *Enterococcus mundtii* ST4SA was excreted in higher numbers during the first 24 h compared to *Lb. plantarum* 423, suggesting that *Lb. plantarum* 423 colonized the GIT more effectively. After 72 h, the number of cells of both bacteria excreted declined to low levels. These transit dynamics are similar to those reported for several LAB strains` including *Lb. casei*, *Lb. plantarum* WCFS1 and *Lc. lactis* (5, 11, 33, 34). The decline in the number of *Lb. plantarum* 423 and *Ent. mundtii* ST4SA cells excreted over the 72 h period suggests that it is a non-permanent colonization. However, Pavan and co-workers (2002), found that *Lb. plantarum* WCFS1 was still excreted in the feces of mice for up to 7 days after one oral administration (33).

In summary, we have shown that mCherry can be used to detect LAB *ex vivo*. Fluorescence imaging proved useful in demonstrating that *Lb. plantarum* 423 and *Ent. mundtii* ST4SA predominantly colonise the cecum and colon. Both strains persisted in the gut over 24 h. However, no *Lb. plantarum* 423 could be found in the digestive tract after 72 h, while low numbers of *Ent. mundtii* ST4SA were still present. The detection of fluorescence in whole animals was greatly facilitated by the removal of the mice abdominal region skin tissue after sacrifice. We could not detect any transcutaneous fluorescence. The propagation of mCherry fluorescence through tissue was restricted. As far as we know, this is the first report on fluorescence imaging of LAB expressing mCherry in a mouse model.

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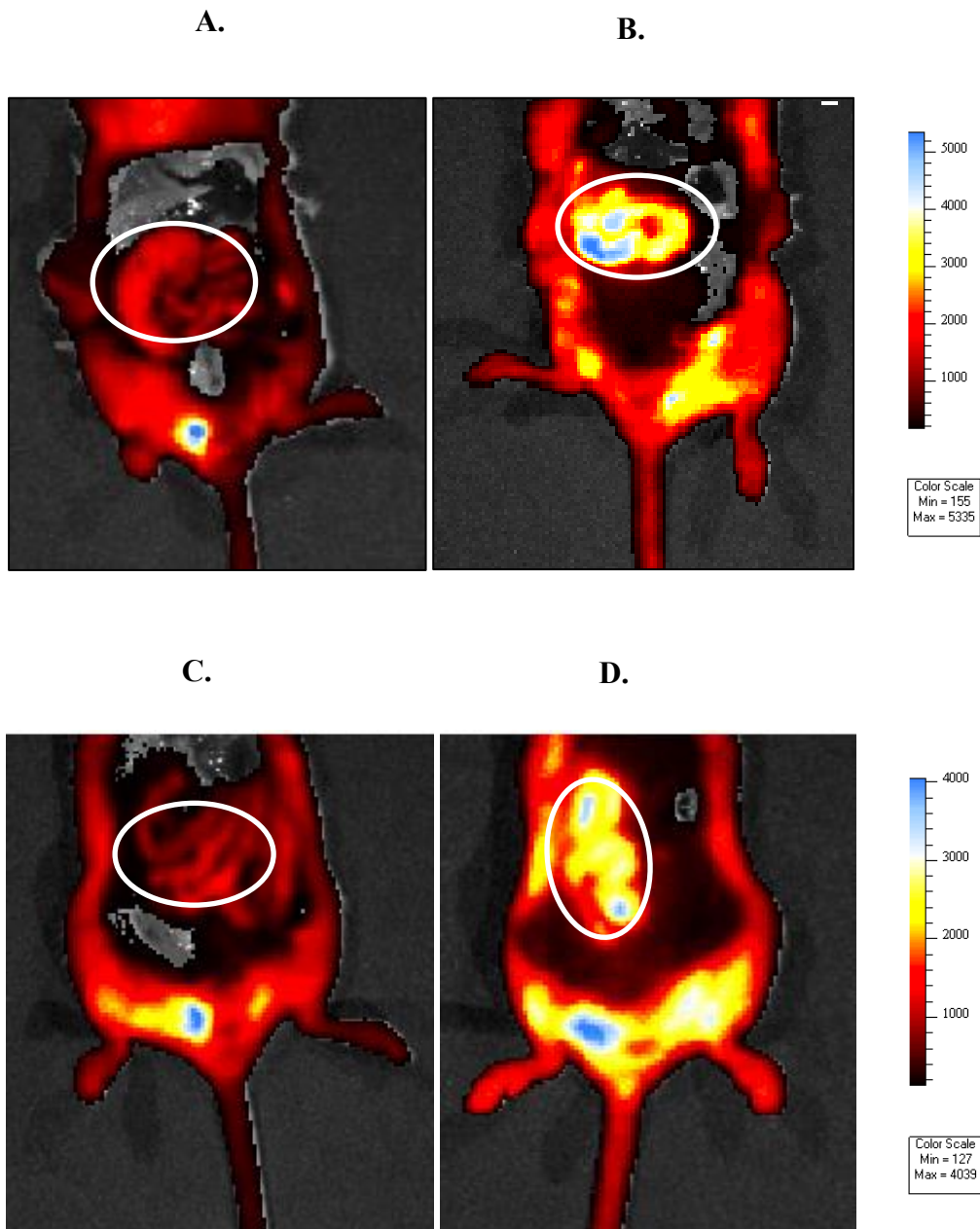


FIG. 1. Fluorescent imaging of *Lb. plantarum* 423 and *Ent. mundtii* ST4SA *in vivo*. *Lactobacillus plantarum* 423::CmRCherry, *Ent. mundtii* ST4SA (pCmRCherry) and control strains, *Lb. plantarum* 423 (pGKV) and *Ent. mundtii* ST4SA (pGKV) (4×10^9 CFU) were inoculated intragastrically into sets of three mice, and the fluorescent signal was detected after removal of the skin tissue on the ventral side after euthanasia. Shown is one representative image of one mouse, 24 h post-feeding. The mouse abdominal regions are circled. (A) Mouse fed with control non-fluorescent strain 423 (pGKV) and (B) mouse fed with fluorescent strain 423::CmRCherry. (C) Mouse fed with control non-fluorescent strain ST4SA (pGKV) and (D) mouse fed with fluorescent strain ST4SA (pCmRCherry).

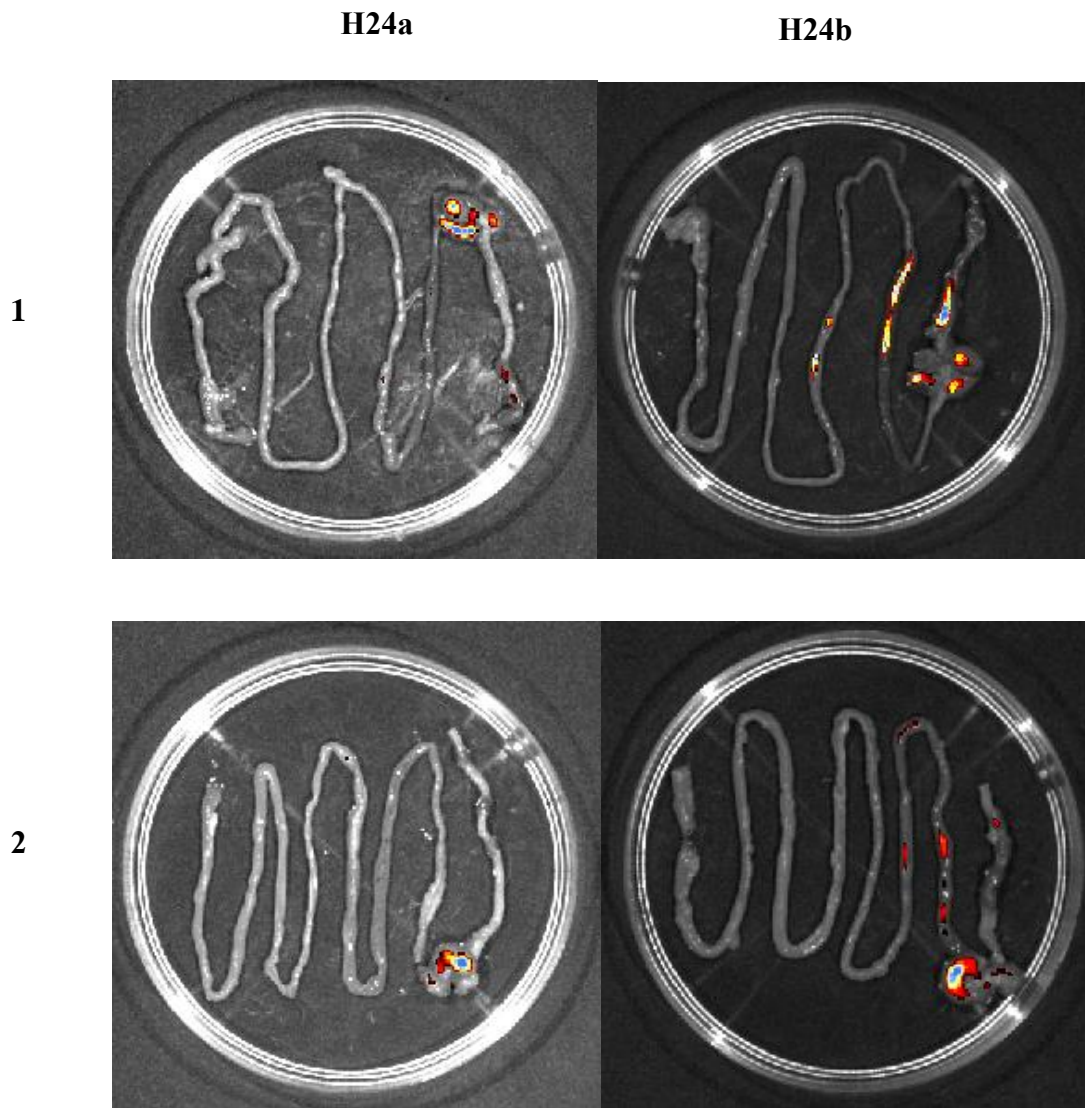


FIG. 2. Localization of fluorescent *Lb. plantarum* 423 and *Ent. mundtii* ST4SA in the digestive tracts of mice. Groups of mice were gavaged once with *Lb. plantarum* 423::CmRCherry and *Ent. mundtii* ST4SA (pCmRCherry) and the intestines resected 24 h post-feeding. Three mice were sacrificed, and shown are representative images of the digestive tracts of two mice (1 and 2) at 24 h (H24), in mouse fed with *Lb. plantarum* 423::CmRCherry (a) and mouse fed with *Ent. mundtii* ST4SA (pCmRCherry) (b). The background auto-fluorescence was compensated for by using images of the digestive tracts of mice fed with control strains, 423 (pGKV) and ST4SA (pGKV).

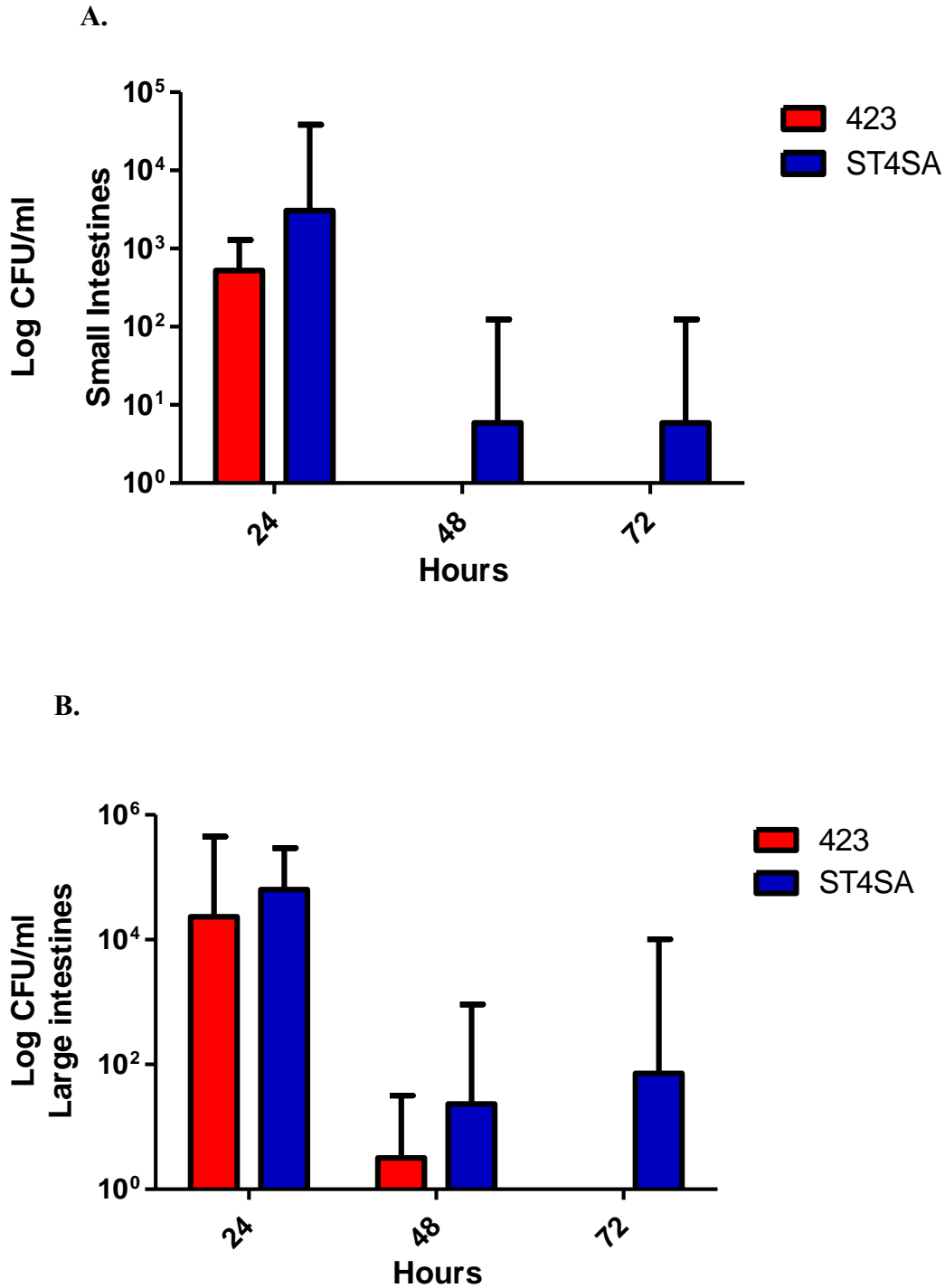


FIG. 3. Persistence of *Lb. plantarum* 423 and *Ent. mundtii* ST4SA in the (A) small and (B) large intestinal compartments of mice after one oral administration. Groups of mice were gavaged once with *Lb. plantarum* 423::CmRCherry and *Ent. mundtii* ST4SA (pCmRCherry) and the intestines resected 24, 48 and 72 h post-feeding. At the indicated times, log₁₀, averages of three mice are plotted with standard deviations.

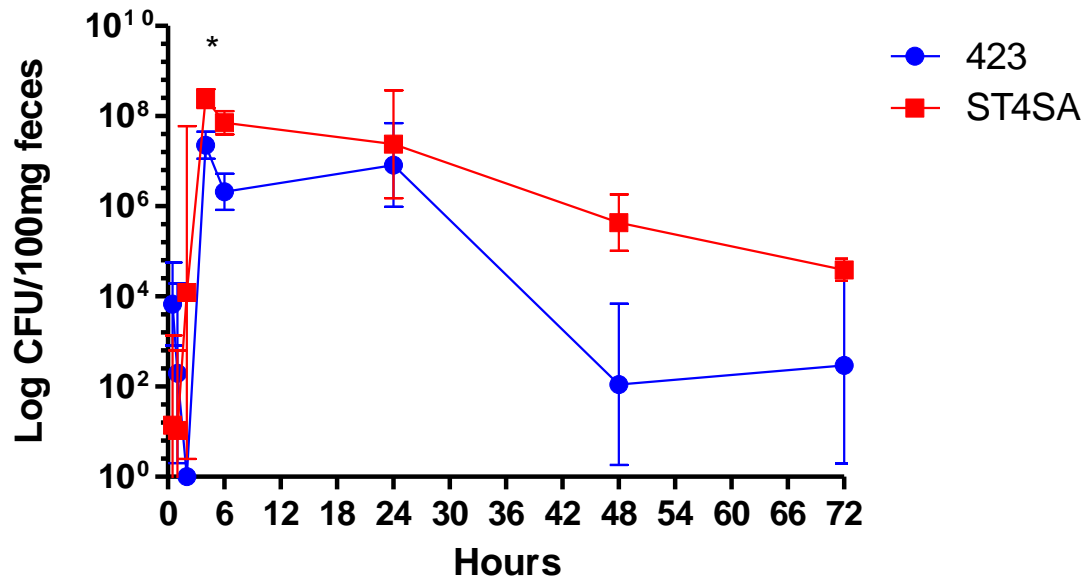


FIG. 4. Transit of *Lb. plantarum* 423 and *Ent. mundtii* ST4SA in the feces of mice. Sets of three mice were fed once with 4×10^9 CFU of *Lb. plantarum* 423::CmRCherry and *Ent. mundtii* ST4SA (pCmRCherry). At every time point, \log_{10} , means of the CFU per 100 mg feces for each set of three mice are plotted with standard deviations. At the times indicated, colonization was monitored by enumeration of bacteria in feces plated on MRS containing chloramphenicol. Overall differences between strains 423 and ST4SA, were assessed using the Man-Whitney nonparametric test and those found to be significant ($P < 0.05$) are shown with an asterisk at 4 h.

Chapter 5

**Bioluminescence Imaging of *Listeria monocytogenes* in
Mice Colonized with *Enterococcus mundtii* ST4SA and
Lactobacillus plantarum 423**

Bioluminescence Imaging of *Listeria monocytogenes* in Mice Colonized with *Enterococcus mundtii* ST4SA and *Lactobacillus plantarum* 423

Abstract

Listeria monocytogenes is an opportunistic food-borne pathogen, which can be life-threatening to individuals with a weakened immune system. The aim of this study was to evaluate whether *Lactobacillus plantarum* 423 and *Enterococcus mundtii* ST4SA could prevent the colonization of *L. monocytogenes* in the gastro-intestinal tract of mice. Mice pre-colonized with *Lb. plantarum* 423 and *Ent. mundtii* ST4SA were challenged with a bioluminescent strain of *L. monocytogenes*. Using *in vivo* bioluminescence, we determined that the colonization of mice with *Lb. plantarum* 423 and *Ent. mundtii* ST4SA, or a combination of the strains, protected the animals from systemic *L. monocytogenes* EGDe infection. Treatment of *Listeria* infections with probiotic lactic acid bacteria may offer an alternative to conventional antibiotics.

Introduction

Listeria monocytogenes is the causative agent of a serious food-borne illness. Immunocompromised individuals are the most susceptible to listeriosis and it results in a high mortality rate (1, 2). *Listeria monocytogenes* is a ubiquitous gram positive organism that can survive low pH, low water activity and high sodium chloride levels associated with meat drying and fermentation (3, 4). Its ability to proliferate at low temperatures renders many food products at risk to contamination.

Effective antibiotic treatment against infections caused by *Listeria* is essential. Although several antibiotics have activity against the organism, the frequent use of antibiotics may increase the chances of developing antibiotic resistant strains. Treatment with probiotic lactic acid bacteria (LAB) may offer an alternative to conventional antibiotics. Several studies have reported that LAB exert inhibitory activity against *Listeria monocytogenes in vitro* (5-9). The anti-microbial effects of LAB are exerted either directly via competition of live microbial cells with pathogens or indirectly via the production of organic acids, hydrogen peroxide or bacteriocins (10).

Several bacteriocins, including nisin, lactocin 705, enterocin 81 and lacticin 3147 have been described with *in vitro* activity against *L. monocytogenes* (4, 7, 11-13). They are small ribosomally synthesized antimicrobial peptides that inhibit the growth of other species (14). Bacteriocins have been applied as natural preservatives in the food industry for decades, particularly against contamination by *L. monocytogenes*.

One of the prerequisites of a good probiotic is the ability of the strain to adhere to epithelial cells or its ability to compete against pathogens for adhesion sites (13, 15). The effects of a healthy intestinal microbiota composition on *L. monocytogenes* induced infection have previously been assessed *in vivo* (16, 17). Treatment of infections caused by *Listeria* with live probiotic LAB enhances the host's resistance by stimulating the immune system (18-20).

In the current study, we evaluated the impact of *Lb. plantarum* 423 and *Ent. mundtii* ST4SA on orally acquired *L. monocytogenes* infection and their ability to compete with the pathogen in the gastro-intestinal tract (GIT) of mice. Mice were colonized with the probiotic LAB prior to being challenged with a bioluminescent strain of *L. monocytogenes*. Changes in infection were recorded by monitoring changes in bioluminescence and viable cell counts.

Materials and Methods

Approval for experiments on animals was obtained from the Ethics Committee of the University of Stellenbosch (ethics reference number: SU-ACUM14-00015). Eight-week-old female BALB/c mice were used in all experiments. The animals were kept under controlled environmental conditions and fed a standard rodent diet.

Preparation of bacterial strains and growth conditions

The bioluminescent strain, *L. monocytogenes* EGDe, harbouring the pPL2 lux plasmid containing the *luxABCDE* operon of *Photobacterium luminescence* was purchased from Bio-ware™ Microorganisms (Caliper Life Sciences, Hopkinton, MA, U.S.A.). *Listeria monocytogenes* EGDe was cultured in Brain Heart Infusion (BHI) broth (Biolab Diagnostics, Midrand, South Africa) or BHI agar at 37°C supplemented with 7.5 µg/ml chloramphenicol (Cm). *Enterococcus mundtii* ST4SA and *Lb. plantarum* 423 harbouring a gene for Cm resistance were grown in MRS broth (Biolab Diagnostics, Midrand, South Africa) or MRS agar at 30°C containing 5 µg/ml Cm for strain ST4SA and 10 µg/ml Cm for strain 423 (21). After 12 h, the cultures were used to inoculate fresh media and were grown to exponential phase, harvested (8000g, 3 min) and washed twice with phosphate buffered saline. A dilution series was prepared and the optical density of each culture recorded at 600 nm.

Dosage of mice

Sets of six mice received a daily dose of LAB for six consecutive days. Mice in group L received 4×10^9 CFU *Lb. plantarum* 423, mice in group E received 4×10^9 CFU *Ent. mundtii* ST4SA, and mice in group EL received a 1:1 combination of *Ent. mundtii* ST4SA and *Lb. plantarum* 423 in 200 μ l gavage buffer (0.2 M NaHCO₃ buffer containing 1% glucose, pH 8). Mice in group P received 200 μ l sterile physiological water (control group). On day 6, the mice were infected intragastrically with 9.6×10^8 CFU of *L. monocytogenes* EGDe, resuspended in 200 μ l gavage buffer.

Mouse colonization

Fresh fecal samples were collected at 30 min, 1, 2, 4, 6 and 24 h after the administration of *L. monocytogenes* EGDe. For the enumeration of viable bacteria, fecal samples were weighed (100 mg), mechanically homogenized in PBS, serially diluted and plated on BHI and MRS agar containing Cm at the concentrations described above. Mice were sacrificed at 4, 6 and 24 h by cervical dislocation and immediately thereafter the digestive tracts (stomach to rectum) were resected and positioned in a Petri dish for *ex vivo* bioluminescence imaging. The bioluminescence signal was enhanced by imaging longitudinally opened organs. To determine the number of viable bacteria per organ homogenate, the small and large intestines were homogenized in 3 ml PBS, respectively, diluted and plated on BHI and MRS agar containing Cm as described above.

***In vitro* and *in vivo* bioluminescence imaging**

Bioluminescent images were obtained using the IVIS[®] 100 In Vivo Imaging System (Caliper Life Sciences, Hopkinton, MA, U.S.A.), which consists of a cooled charge-coupled-device camera mounted on a light-tight specimen chamber. For the detection of bioluminescent *L.*

monocytogenes EGDe in fecal samples homogenized in PBS, 300 µl homogenate was added to black 96-well microtiter trays and imaged by IVIS. Prior to live animal bioluminescence imaging, isoflurane gas anesthetic was administered to mice at 2%, from which the mice recovered within 2 min of removal of gas. During imaging, anesthesia was maintained by a controlled air flow of 1.5% (v/v) isoflurane through a nose cone. Photon emission of bacteria in microtitre trays, or anesthetized mice placed in the ventral position, was measured with exposure times ranging from 1 to 5 min, depending on bioluminescence signal intensity. Bioluminescence was quantified using the Living Image[®] software, version 3.0 (Caliper Life Sciences, Massachusetts, U.S.A.) and measured as photons per second (p/s). Bioluminescence signals originating from the abdomen of each individual mouse were quantified using identical regions of interest (ROI) for all images and were determined manually.

Results

Bioluminescence monitoring of colonization by *Listeria monocytogenes* in mouse intestines

Before the administration of *L. monocytogenes* EGDe to mice, no bioluminescence signal was detected (7.24×10^4 p/s being the background signal). As early as 30 min after the administration of *L. monocytogenes* EGDe, a strong bioluminescence signal was detected in the abdominal region of all mice (Fig. 1A). After 24 h, a bioluminescent signal could be observed from mice in the control group (group P). Mice in the control group, showed symptoms of listeriosis, such as lethargy, hunched appearance and conjunctivitis of the eye throughout the infection. Mice that had received *Lb. plantarum* 423, *Ent. mundtii* ST4SA and a combination of the two strains showed no physical abnormalities. The bioluminescence signal from mice that had received *Lb. plantarum* 423 (group L) could not be observed after 4 h post-administration of *L. monocytogenes* EGDe, whereas the signal from mice that had

received *Ent. mundtii* ST4SA (group E) or the combination of both strains (group EL) could not be observed after 2 h. The highest signal measured in whole animals was recorded from mice in the control group (mean value of 2.66×10^6 p/s) compared to that from mice in group L (mean value of 1.46×10^6 p/s), group E (mean value of 1.26×10^6 p/s) and group EL (mean value of 9.72×10^5 p/s), respectively (Fig. 1B). After 6 h, the signal from mice in group L decreased to the background level, whereas the signals from both groups E and EL declined to approximately 1.30×10^5 p/s. The bioluminescent signals of mice in the control group declined to approximately 3×10^5 p/s after 6 h, and remained at a plateau until 24 h.

Dissected intestines were also imaged *ex vivo* for *L. monocytogenes* EGDe bioluminescence (Fig. 2). The highest bioluminescent signals were obtained when the small and large intestines were longitudinally opened. By 6 h after infection, *L. monocytogenes* EGDe was detected throughout the lower section of the small intestine (group P). After 24 h, a bioluminescence signal was detected from the small intestine of mice in the control group alone. After 6 h, no bioluminescent *L. monocytogenes* EGDe could be detected from the intestines of mice that had received *Lb. plantarum* 423, *Ent. mundtii* ST4SA or the combination of both strains, respectively. The CFU counts confirmed that the small and large intestines were colonized by *L. monocytogenes* EGDe (Fig. 3). After 6 h, the number of *L. monocytogenes* EGDe cells (mean value of 5×10^2 CFU) in the small intestines of mice that had received *Ent. mundtii* ST4SA was significantly lower compared to that of group P (mean value of 8.9×10^6 CFU) as indicated in Fig. 3A. The number of *L. monocytogenes* EGDe cells in the large intestines of mice that received *Ent. mundtii* ST4SA decreased to low levels (mean value of 1×10^3 CFU) after 24 h (Fig. 3B). While the number of *L. monocytogenes* EGDe cells in the GIT of mice that had received *Lb. plantarum* 423 also decreased compared to the control group. The amount of *L. monocytogenes* EGDe cells in the intestines of mice that had received *Ent. mundtii* ST4SA was always lower. Viable cell counts of the individual bacterial species in the intestines of mice in group EL could not be determined as *L.*

monocytogenes EGDe, *Lb. plantarum* 423 and *Ent. mundtii* ST4SA could not be differentiated from each other based on colony morphology. Twenty four hours post-administration of *L. monocytogenes* EGDe, the large intestine (cecum and colon) was the major site of colonization of *Lb. plantarum* 423, whereas *Ent. mundtii* ST4SA was colonizing both the small and large intestines (Fig. 4).

Enumeration of viable *L. monocytogenes* EGDe, *Lb. plantarum* 423 and *Ent. mundtii* ST4SA in mouse feces

The number of viable *L. monocytogenes* EGDe increased with time in the feces of all the mice, reaching a maximum level after 4 h in mice administered *Lb. plantarum* 423 (mean value of 3.54×10^8 CFU) and *Ent. mundtii* ST4SA (mean value of 2.61×10^8 CFU), respectively (Fig. 5A). After 4 h, the number of *L. monocytogenes* EGDe excreted in the feces of mice in groups E and L was significantly higher compared to that of group P (mean value of 2×10^2 CFU). There was excellent correlation between this peak and the maximum bioluminescence signal of 3.15×10^5 p/s/100mg of feces and 1.03×10^6 p/s/100mg of feces after 4 h for mice in groups E and L, respectively (Fig. 5B). By 6 h, a maximum of approximately 1.78×10^8 CFU *L. monocytogenes* EGDe with a bioluminescence signal of 5.87×10^5 p/s/100mg of feces were detected in the feces of mice in group P and declined to approximately 1.67×10^5 CFU after 24 h. The number of *Lb. plantarum* 423 and *Ent. mundtii* ST4SA, increased with time in the feces of mice in groups L and E, respectively, reaching maximums of approximately 4.27×10^7 and 1.53×10^8 CFU (Fig. 6).

Discussion

Bioluminescence has been used in a number of previous studies to follow *in vivo* infection processes (12, 22-26). In the current study, *Listeria monocytogenes* EGDe bioluminescence could be detected transcutaneously in whole animals after a single oral administration. No

physical symptoms of listeriosis were observed in any mice that received the probiotic LAB, suggesting that the LAB protected the animals from systemic infection. The highest bioluminescence signals were obtained with mice in the control group and could be observed transcutaneously throughout the 24 h period of infection (Fig. 1). After 4 h, the bioluminescent signal of mice that received *Lb. plantarum* 423, *Ent. mundtii* ST4SA and the combination of the strains declined below transcutaneous detection levels. This suggests that interactions between the probiotic LAB and *L. monocytogenes* EGDe in the mouse GIT results in the inhibition of the pathogen as observed with live animal imaging. The ability of *Lb. plantarum* 423 and *Ent. mundtii* ST4SA to inhibit the growth of *L. monocytogenes* EGDe and *L. monocytogenes* ScottA *in vitro* has been shown previously (21, 27). Our results show that the real-time bioluminescence imaging of live animals plays a key role in displaying the difference in effects of *L. monocytogenes* EGDe infection on mice already colonized with *Lb. plantarum* 423 and *Ent. mundtii* ST4SA compared to the control group.

Listeria monocytogenes EGDe lux bioluminescence emission is optimal in an aerobic environment (28). Transcutaneous lux bioluminescence signal detection may not be a true representation of the actual colonization due to limited oxygen availability in the intestinal cavity. Longitudinally opened mouse digestive tracts were imaged *ex vivo* with the aim of exposing *L. monocytogenes* EGDe to oxygen. A previous study has shown that a 10-fold-higher bioluminescent signal was observed by imaging opened digestive tracts (24). Bioluminescent *L. monocytogenes* EGDe was detected only in the small intestine of mice in the control group after 24 h. However, CFU counts confirmed that *L. monocytogenes* EGDe also colonized the large intestine but did not emit bioluminescence, suggesting that oxygen may have been the limiting factor in the more anaerobic cecum and colon (Fig. 3B). Microorganisms harbouring the bacterial *lux* genes can only produce bioluminescence when the cells are metabolically active (28). Although CFU counts confirmed the presence of high numbers of *L. monocytogenes* EGDe cells in the digestive tracts of mice that received *Lb.*

plantarum 423 and *Ent. mundtii* ST4SA, respectively, no bioluminescence was detected after 6 h. This suggests that the probiotic LAB may have had a bacteriostatic effect on *L. monocytogenes* EGDe which resulted in the inability of the strain to emit bioluminescence. Additionally, the lack of oxygen in the intestines having an effect on bioluminescence emission can also be ruled out, since bioluminescence was detected from the mice in the control group (no LAB administered). Bacteriocins produced by some LAB strains have previously been shown to have a bacteriostatic effect on other species (14). Ramiah et al. (2008) reported that *Lb. plantarum* 423 could compete with *Clostridium sporogenes* and *Ent. faecalis* for adhesion to Caco-2 cells (29). In another study, treatment of an orally acquired *L. monocytogenes* infection in a gnotobiotic humanized mouse model with live *Lb. casei* BL23 and *Lb. paracasei* CNCM limited *L. monocytogenes* systemic dissemination (30). Mice pre-colonized with *Ent. mundtii* ST4SA showed the most rapid decline in viable *L. monocytogenes* EGDe cell counts after infection. This suggests that *Ent. mundtii* ST4SA was the most effective in controlling the growth of *L. monocytogenes* EGDe in the GIT compared to *Lb. plantarum* 423. Using fluorescence imaging, *Ent. mundtii* ST4SA has been reported to colonize in the ileum and cecum, whereas *Lb. plantarum* 423 only colonized the large intestine (21). Challenging the two probiotic LAB strains colonized in mice with *L. monocytogenes* EGDe did not affect the colonization ability of either probiotic strain.

Colonization was further assessed by monitoring the number of CFU per 100 mg feces at different time points. Mice administered probiotic LAB excreted *L. monocytogenes* EGDe more rapidly in the feces after 4 h compared to mice in the control group (Fig. 5A). This indicates that the ability of *L. monocytogenes* EGDe to colonize the GIT in the presence of either LAB strain was greatly reduced. The good correlation between the maximum CFU counts and the maximum bioluminescence signals emitted indicates that photon emission accurately reflects bacterial numbers in the feces. Transit kinetics of the two LAB strains in

the feces of mice was not affected by challenging with *L. monocytogenes* EGDe and is comparable to a previous study that assessed the colonization of the strains *in vivo* (21).

In conclusion, real-time bioluminescence monitoring proved useful in comparing the inhibition of *L. monocytogenes* by *Lb. plantarum* 423, *Ent. mundtii* ST4SA and a combination of the strains, respectively. Challenging *Lb. plantarum* 423 and *Ent. mundtii* ST4SA colonized in the GIT of mice with *L. monocytogenes* EGDe had no effect on the survival of the LAB strains. The results reported here suggest that the colonization of mice with *Lb. plantarum* 423 and *Ent. mundtii* ST4SA or a combination of the strains protects the animals from systemic *L. monocytogenes* EGDe infection. The probiotic LAB may have inhibited *L. monocytogenes* EGDe, either directly by competing for adhesion sites in the GIT or indirectly through the antimicrobial effect of bacteriocins produced. In addition, the probiotic LAB may have stimulated the animals' immune response against the pathogen. *Enterococcus mundtii* proved more effective than *Lb. plantarum* 423 in reducing the number of *L. monocytogenes* EGDe *in vivo*.

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Figure 1. A.

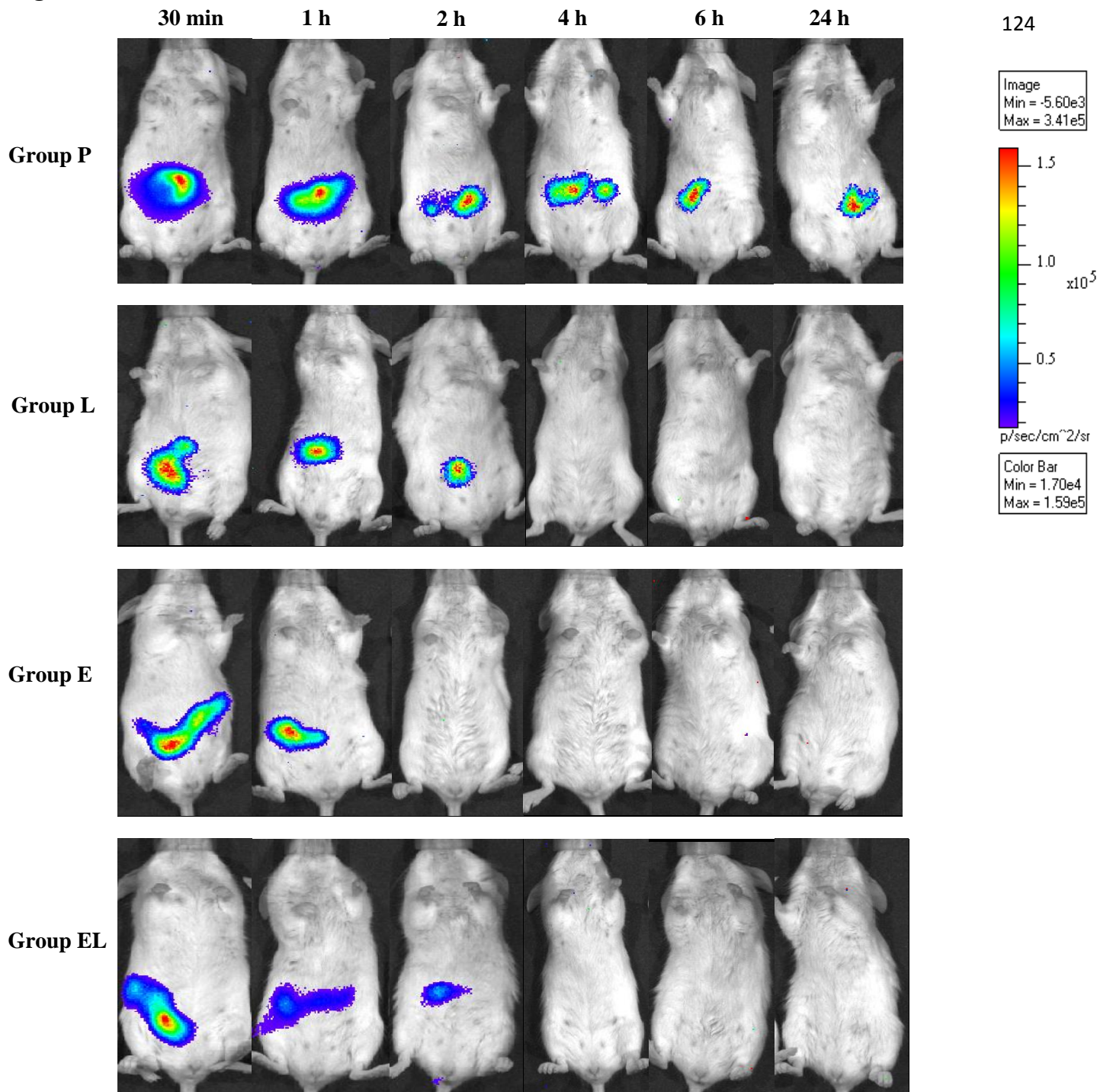


Figure 1. B.

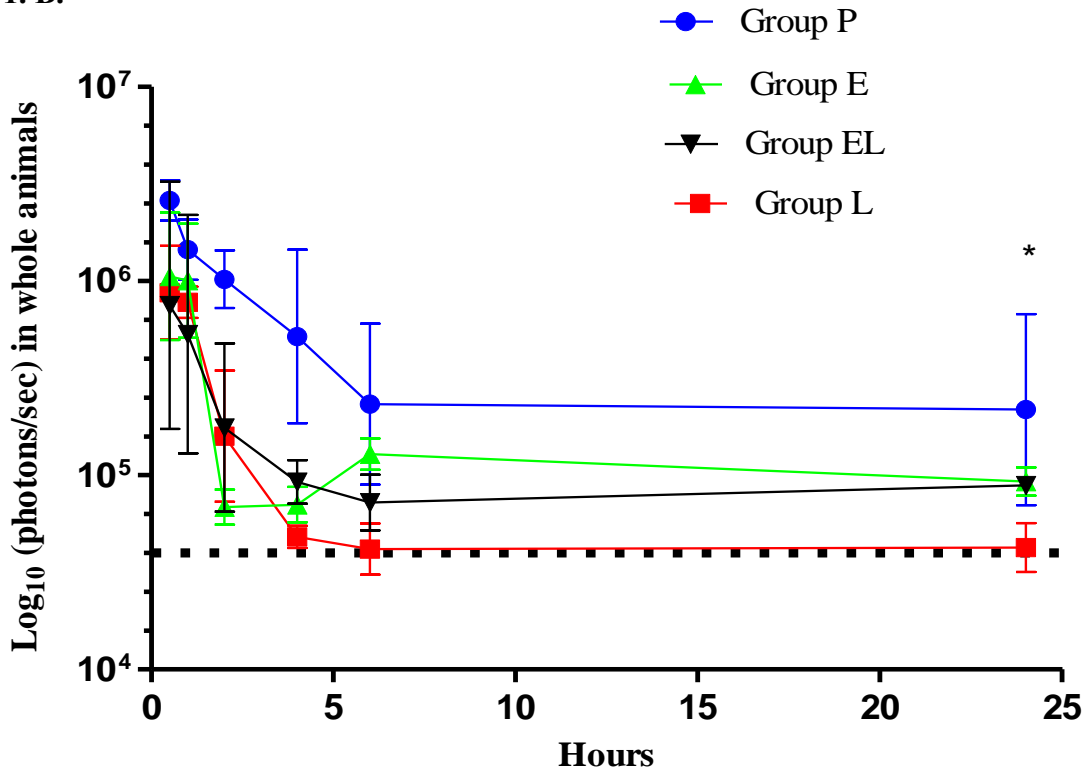


FIG. 1. Monitoring of intestinal colonization by *L. monocytogenes* EGDe using bioluminescence imaging in whole animals. *Listeria monocytogenes* EGDe (9.6×10^8 CFU) was intragastrically administered to groups of mice that had received sterile saline (Group P), 4×10^9 CFU of *Lb. plantarum* 423 (Group L), 4×10^9 CFU of *Ent. mundtii* ST4SA (Group E) and a 1:1 combination of the LAB strains (Group EL) for 6 days. (A) The bioluminescence signal was measured transcutaneously in whole animals at different time points. The intensity of the photon emission is represented as a pseudocolor image. One representative image of one mouse is shown. At every time point, the bioluminescence signal in p/s in whole animals (B) is plotted for sets of three mice, with standard deviations. Overall differences between the bioluminescence signals of the groups were assessed using the Kruskal-Wallis nonparametric test and those found to be significant ($P < 0.05$) are indicated with an asterisk for comparison between Group P and Group L (24 h). The background level of the bioluminescence signal is represented by a dashed line.

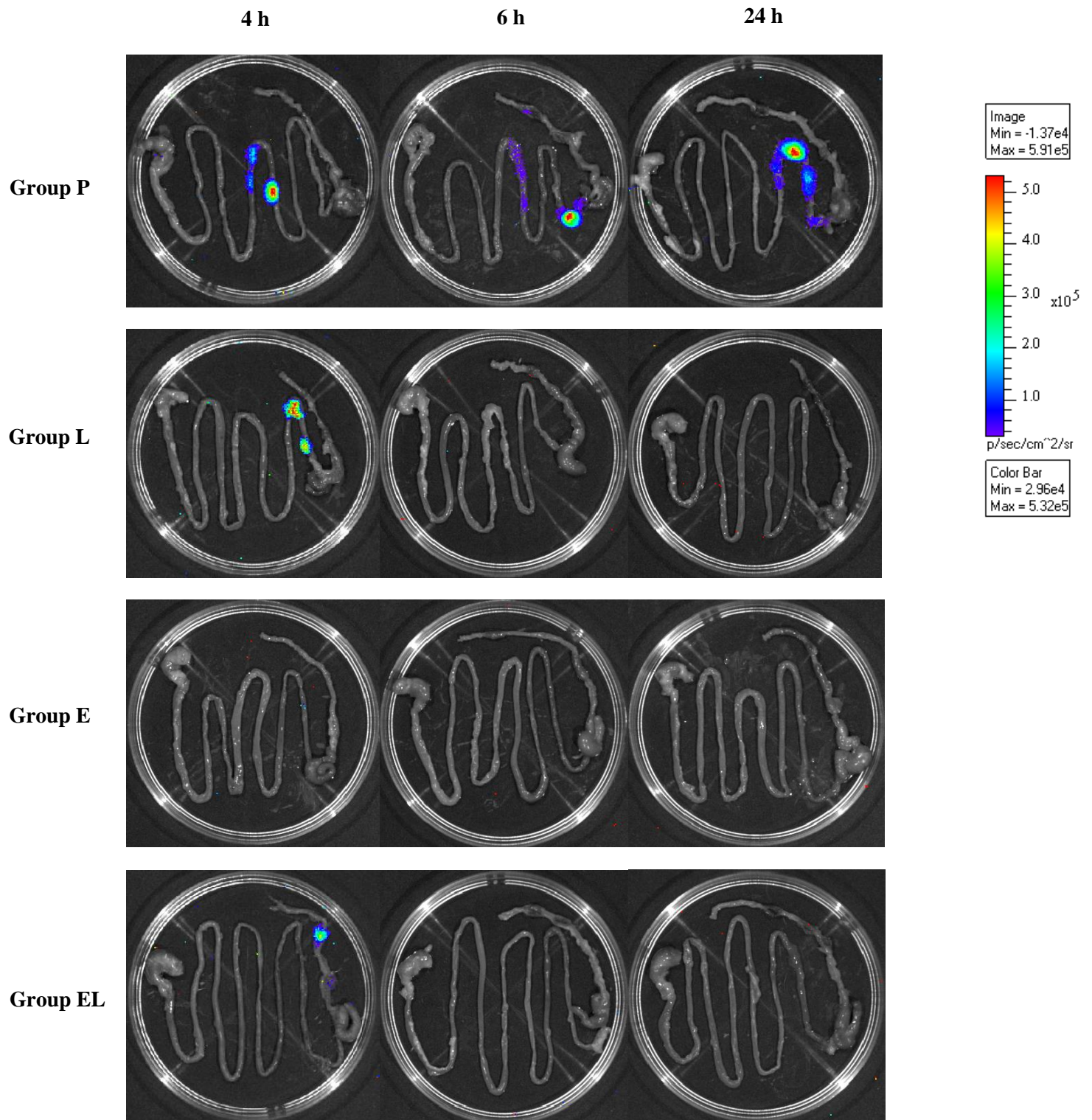
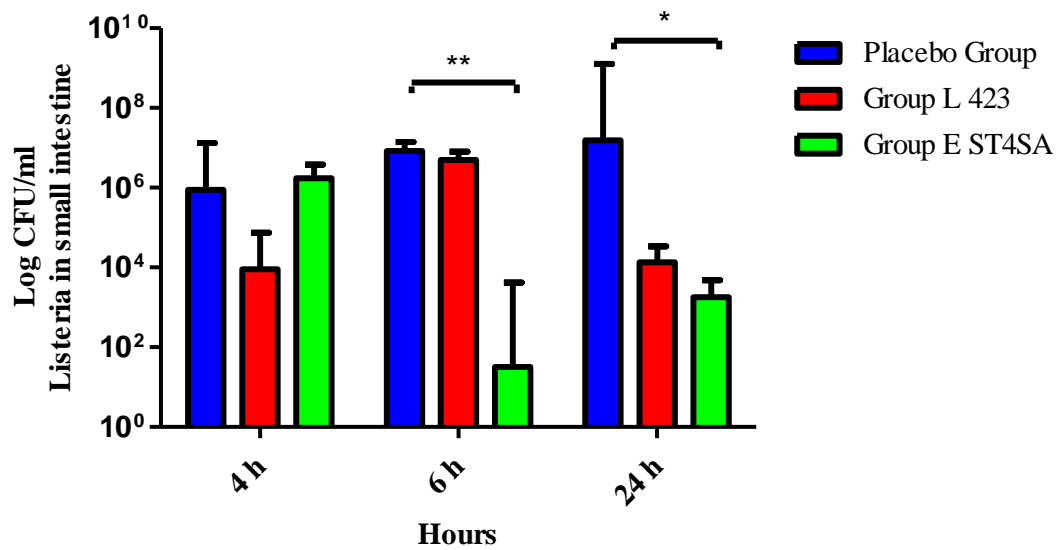


FIG. 2. Bioluminescence monitoring of *L. monocytogenes* EGDe in the digestive tract of mice. Two mice were sacrificed at each time point and one representative image of the digestive tract of one mouse is shown at 4, 6 and 24 h after administration of *L. monocytogenes* EGDe (9.6×10^8 CFU) on day 6. Mice that had received sterile saline (Group P), 4×10^9 CFU of *Lb. plantarum* 423 (Group L), 4×10^9 CFU of *Ent. mundtii* ST4SA (Group E) and a 1:1 combination of the LAB strains (Group EL) for 6 days.

A.



B.

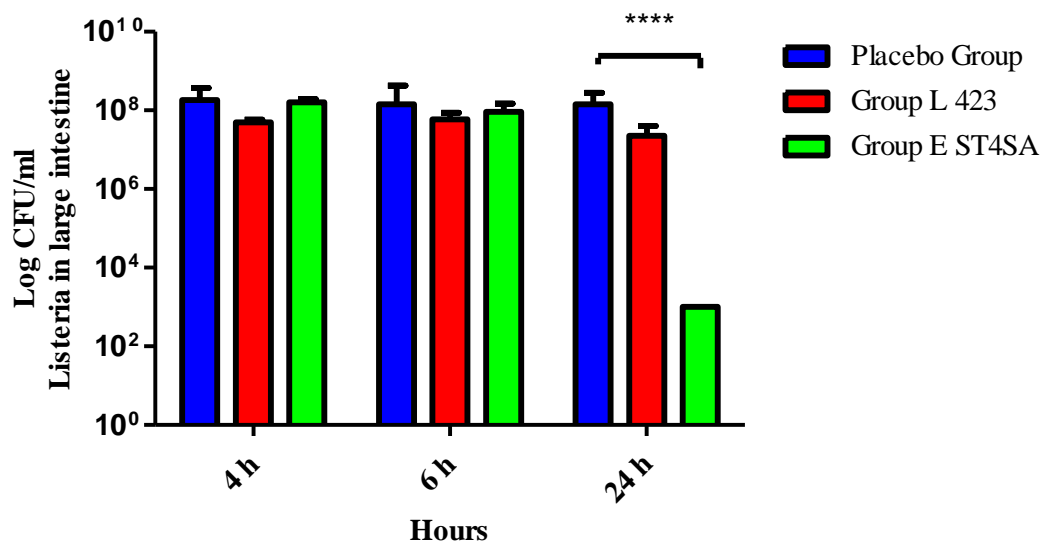
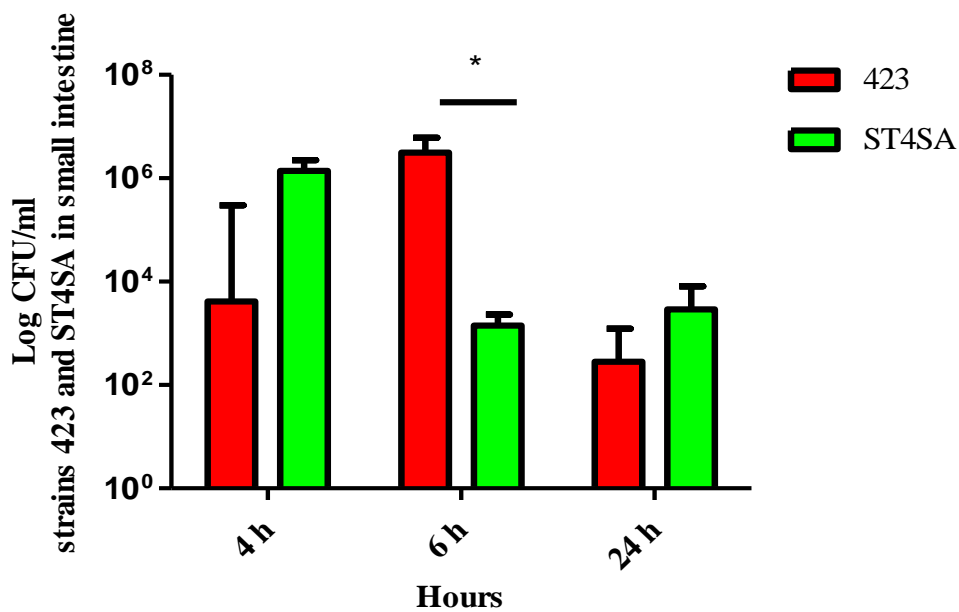


FIG. 3. Persistence of *L. monocytogenes* EGDe in the (A) small and (B) large intestinal compartments of mice that had been administered sterile saline (Control Group), 4×10^9 CFU of *Lb. plantarum* 423 (Group L 423) and 4×10^9 CFU of *Ent. mundtii* ST4SA (Group E ST4SA) for 6 days. Two mice were sacrificed at each time point and the digestive tracts resected at 4, 6 and 24 h after the administration of *L. monocytogenes* EGDe (9.6×10^8 CFU). Differences between the groups were assessed using the Kruskal-Wallis nonparametric test and those found to be significant are indicated with one ($P < 0.05$), two ($P < 0.01$) or four ($P < 0.0001$) asterisks for comparison between the Control Group and Group E ST4SA.

A.



B.

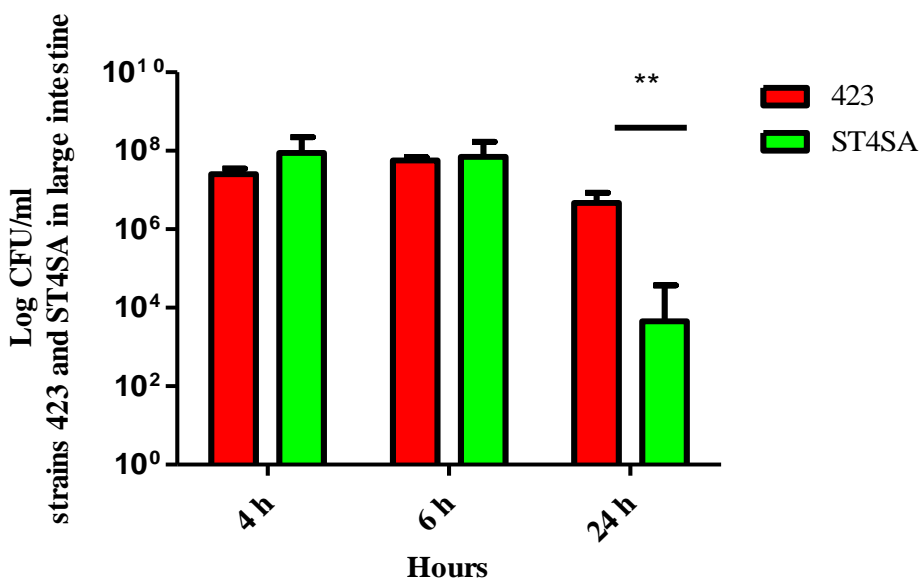
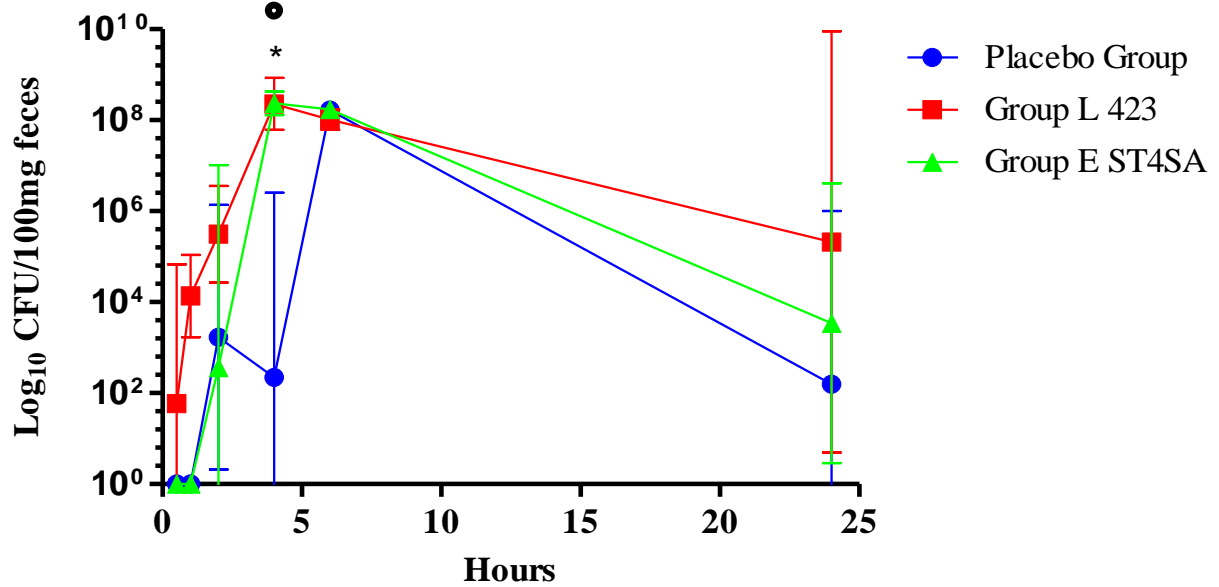


FIG. 4. Persistence of *Lb. plantarum* 423 and *Ent. mundtii* ST4SA in the (A) small and (B) large intestinal compartments of mice that had been administered 4×10^9 CFU of *Lb. plantarum* 423 (Group L) and 4×10^9 CFU of *Ent. mundtii* ST4SA (Group E) for 6 days. Two mice were sacrificed at each time point and the digestive tracts resected at 4, 6 and 24 h after the administration of *L. monocytogenes* EGDe (9.6×10^8 CFU). Differences between the groups were assessed using the Mann-Whitney nonparametric test and those found to be significant are indicated with one (P < 0.05) or two (P < 0.01) asterisks.

A.



B.

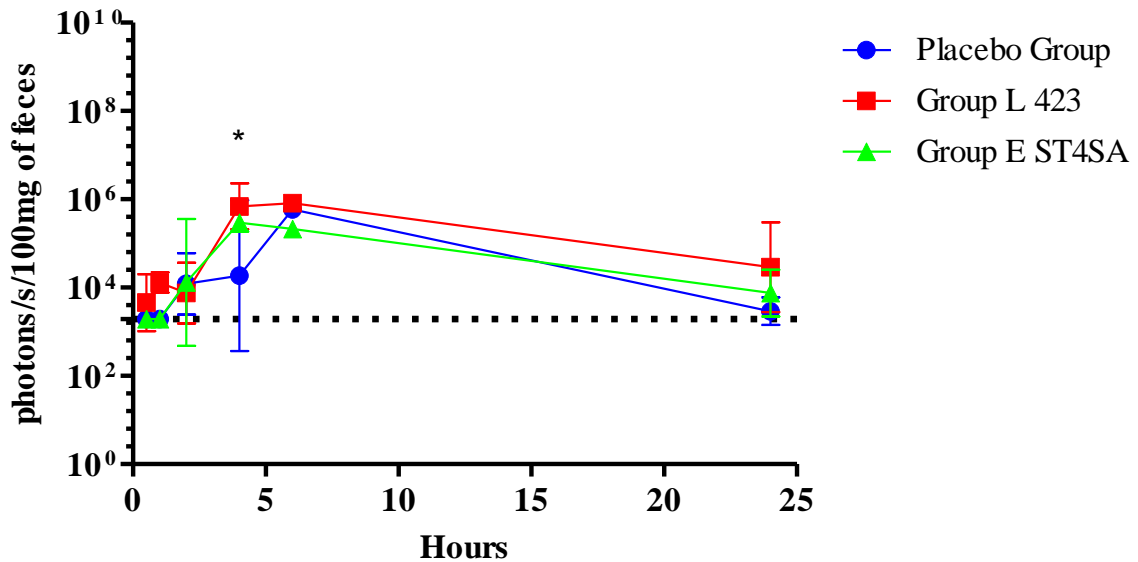


FIG. 5. Transit of *L. monocytogenes* EGDe in the GIT as assessed in the feces of mice. *Listeria monocytogenes* EGDe (9.6×10^8 CFU) was fed once to groups of mice that had received sterile saline (Control Group), 4×10^9 CFU of *Lb. plantarum* 423 (Group L 423) or 4×10^9 CFU of *Ent. mundtii* ST4SA (Group E ST4SA) for 6 days. At the indicated times, \log_{10} averages of the CFU per 100 mg feces (A) and p/s per 100 mg of feces (B) are plotted for each set of three mice, with standard deviations. Differences between the groups, were assessed using the Kruskal-Wallis nonparametric test and those found to be significant ($P < 0.05$) are indicated with an asterisk (for comparison of the Control Group and Group L 423) and a circle (for comparison of the Control Group and Group E ST4SA). The background level of the bioluminescence signal is represented by a dashed line.

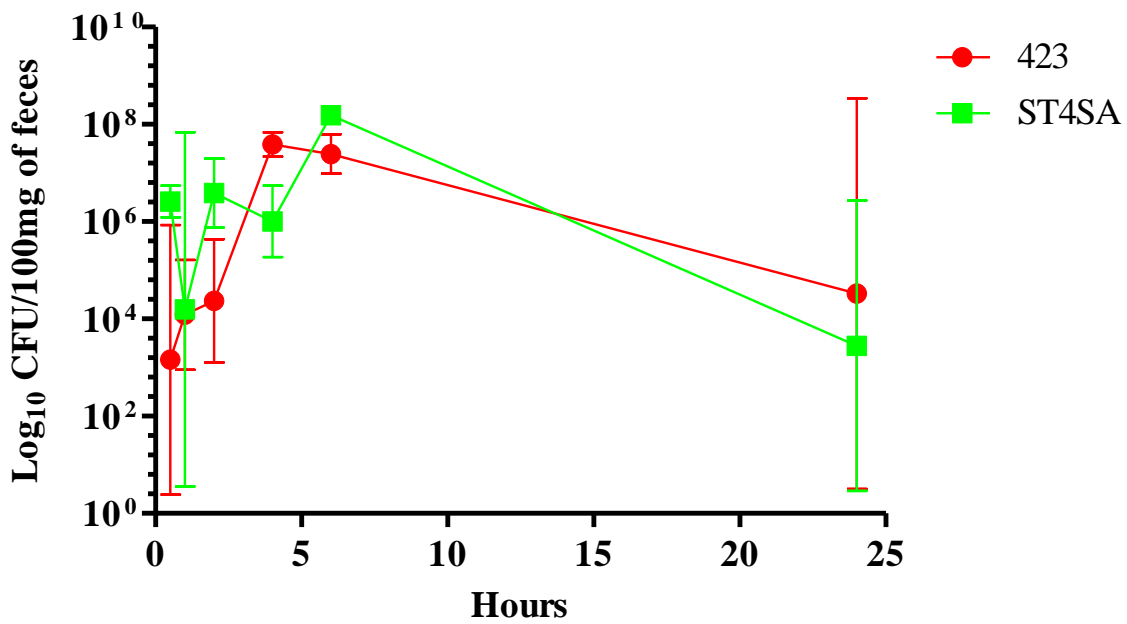


FIG. 6. Transit of *Lb. plantarum* 423 and *Ent. mundtii* ST4SA in the GIT as assessed in the feces of mice challenged with *L. monocytogenes* EGde. Groups of mice were administered daily (4×10^9 CFU) with *Lb. plantarum* 423 (Group L) and *Ent. mundtii* ST4SA (Group E) for 6 days before administration of *L. monocytogenes* EGde (9.6×10^8 CFU) on day 6. At selected time points, \log_{10} averages of the CFU per 100 mg feces for each set of three mice are plotted with standard deviations.

Chapter 6

General Discussion and Conclusions

General Discussion and Conclusions

Genomic integration and expression of mCherry in LAB

The *mCherry* gene was successfully expressed in *Lb. plantarum* 423 and *Ent. mundtii* ST4SA, resulting in the emission of bright fluorescence. The use of mCherry as a marker gene to label LAB, including *Lactobacillus sakei*, *Lactococcus lactis* and *Enterococcus faecalis* has been reported previously (1-3). Codon-optimization of the *mCherry* reporter gene for expression in *Lb. plantarum* 423 and *Ent. mundtii* ST4SA significantly improved fluorescence emission. A LAB/*E. coli* expression plasmid carrying the synthetic *mCherry* gene was constructed and proved useful for the *in vitro* detection of fluorescent *Lb. plantarum* 423 and *Ent. mundtii* ST4SA. However, our interest was in using the *mCherry* gene to track labeled strains of LAB in the GIT of mice. Therefore, we developed a system to integrate the *mCherry* fluorescent marker gene onto the chromosomes of *Lb. plantarum* 423 and *Ent. mundtii* ST4SA to circumvent the use of autonomous plasmids. This ensures that the marker gene will be maintained inside the host genomes without the need for antibiotic selection for enhanced stability and continuous expression (4-7).

The *mCherry* and a gene for chloramphenicol resistance (*cat*) were successfully integrated into the chromosome of *Lb. plantarum* 423 by homologous recombination. This was an indication that the disruption of the target gene for integration was not lethal to the survival of the host. Disruption of the target gene for integration of the marker genes into the chromosome of *Ent. mundtii* ST4SA proved unsuccessful. However, the integration plasmid was stably maintained in *Ent. mundtii* ST4SA without recombination. Therefore, it appears that disruption of the target gene was not achieved as its inactivation could potentially be harmful to the host cell.

The introduction of the *mCherry* fluorescence gene into the LAB strains had no effect on the bacteria's ability to produce their antibacterial peptides or on their growth compared to the

wild-type strains. Furthermore, we have demonstrated that target genes with known DNA sequences that are likely to be non-functional in the cells can be used for the homologous integration of foreign genes in *Lb. plantarum* 423 and *Ent. mundtii* ST4SA. The integration system we have described could potentially be applied to other *Lb. plantarum* and *Ent. mundtii* strains using the target genes we have identified.

Intestinal colonization by *Ent. mundtii* ST4SA and *Lb. plantarum* 423 in mice

The aim of this work was to evaluate the potential of fluorescent imaging (FI) for the real time monitoring of *Lb. plantarum* 423 and *Ent. mundtii* ST4SA *in vivo*, to reveal valuable information on the strains' ability to colonize the gastro-intestinal tract (GIT). The survival of *Lb. plantarum* 423 and *Ent. mundtii* ST4SA in the GIT of infants using a computerized gastro-intestinal model has been reported (Botes et al., 2008). However, simulated *in vitro* conditions are not always the same as what happens *in vivo*.

We demonstrated that transcutaneous signals in mice administered fluorescent *Lb. plantarum* 423::CmRCherry and *Ent. mundtii* ST4SA (pCmRCherry), could not be distinguished from the background signals of mice administered non-fluorescent control strains. This was unexpected as the excitation and emission spectrum of the mCherry fluorophore is outside the region where endogenous chromophores, such as haemoglobin, absorb light within tissues (8, 9). Factors that usually influence the application of a particular fluorescence protein for *in vivo* experimentation include the penetration of fluorescent signals through tissue and brightness of the fluorophore compared to the inherent background auto-fluorescence of the sample or animal (9-11). Our findings merit further investigation.

Detection of the fluorescent signals emitted by the LAB strains was achieved by imaging of the digestive tracts *ex vivo*. Therefore, there is no doubt that the signal was weakened by passage through the mouse tissue and skin. Both strains were detected predominantly in the

anaerobic cecum and colon and persisted in the gut over 24 h. Similarly, previous studies found *Lb. plantarum* to persist predominantly in the cecum and colon (12, 13). However, a fluorescent signal and high numbers of *Ent. mundtii* ST4SA cells were also found in the small intestine. The CFU counts from the intestines revealed that strain ST4SA colonises the gut more effectively than strain 423 as low levels of the bacteria were still present 72 h post-feeding. To our knowledge this is the first report on fluorescence imaging of LAB expressing mCherry in a mouse model.

Alleviation of *Listeria monocytogenes* infection by *Lb. plantarum* 423 and *Ent. mundtii* ST4SA

Effective treatment against *Listeria* infections is essential. Treatment of infections caused by *Listeria monocytogenes* generally includes administration of antibiotics to which the organism is highly susceptible (14). However, the frequent use of antibiotics may increase the chances of developing antibiotic resistant strains. Therefore, the need for alternative antimicrobial agents against resistant pathogens is obvious.

Treatment with probiotic lactic acid bacteria (LAB) may offer an alternative to conventional antibiotics. Several studies have reported that LAB possess inhibitory activity against *Listeria monocytogenes in vitro* (15-18). The anti-microbial effects of LAB are exerted either directly via competition of live microbial cells with pathogens or indirectly via the production of antimicrobial peptides, classified as bacteriocins (19).

The aim of this work was to evaluate the impact of *Lb. plantarum* 423 and *Ent. mundtii* ST4SA on orally acquired *L. monocytogenes* infection and their ability to compete with the pathogen in the GIT of mice. Mice pre-colonized with the probiotic LAB were challenged with a bioluminescent strain of *L. monocytogenes*. Infection rates could be recorded by monitoring bioluminescence emission which proved useful in comparing the inhibition of *L.*

monocytogenes by *Lb. plantarum* 423, *Ent. mundtii* ST4SA and a combination of the strains, respectively.

Challenging *Lb. plantarum* 423 and *Ent. mundtii* ST4SA colonized in the GIT of mice with *L. monocytogenes* EGDe had no effect on the survival of the strains. Our results demonstrated that the colonization of mice with *Lb. plantarum* 423 and *Ent. mundtii* ST4SA or a combination of the strains protected the animals from systemic *L. monocytogenes* EGDe infection. The ability of *Lb. plantarum* 423 and *Ent. mundtii* ST4SA to inhibit the growth of *L. monocytogenes* EGDe and *L. monocytogenes* ScottA *in vitro* has been shown previously (20, 21). In another study it was reported that an infection by *L. monocytogenes* in gnotobiotic mice could be modulated by treatment with live *Lb. casei* BL23 and *Lb. paracasei* CNCM I-3689 (22).

In conclusion, the probiotic LAB tested in this study inhibited *L. monocytogenes* EGDe, possibly directly, by competing for adhesion sites in the GIT, or indirectly through the antimicrobial effect of the bacteriocins produced. *Enterococcus mundtii* ST4SA produces a broad-spectrum antimicrobial peptide active against Gram-positive and Gram-negative bacteria, whereas *Lb. plantarum* 423 produces a bacteriocin with activity against a number of Gram-positive bacteria (23). Stimulation of the animals' immune response by the probiotic LAB may have played a key role in protection against pathogen colonization.

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