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"Engineered strains of *Lactococcus lactis* envisioning to deliver exogenous β-galactosidases for relieving lactose intolerance symptoms"

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Abstract

Lactose intolerance (LI) is a condition affecting many adults. This condition is triggered by the physiological decline of lactase expression in adulthood, which results in a range of gastrointestinal symptoms following the ingestion of lactose-containing foods. Despite the beneficial effect of probiotic and prebiotic preparations, dairy avoidance remains the most widely adopted strategy for treating lactose intolerance. However, intolerant individuals often report a poor life quality associated with dairy avoidance and the occurrence of occasional gastrointestinal symptoms.

Lactase replacement strategies based on enzyme supplemented through tablets also exist, but gastric inactivation limits their efficacy.

The development of preparations effective in alleviating intolerance symptoms upon dairy products ingestion could result in a significant life quality improvement in intolerant individuals. In recent years, studies highlighted the potential of *Lactococcus lactis* subspecies *lactis* for delivering therapeutic molecules in humans.

In this work, we constructed a vector for the expression of heterologous β-galactosidases in the NZ9000 *L. lactis* to employ for treating lactose intolerance. We selected three galactosidases from different microorganisms based on their reported pH and temperature profiles of activity. After this first selection step, we cloned the galactosidases encoding genes under a nisin-inducible promoter and evaluated their activity in different environmental conditions.

Among the selected galactosidases, the one encoded by the *LacZ* gene of *L. bulgaricus* showed the most promising results. The *L. bulgaricus* enzyme retained a good activity at 37°C and within a pH range typical of the small intestine. Although affected by intestinal fluids concentrations, this enzyme showed significant activity in this latter. Moreover, the enzyme remained stable for up to 90 minutes in simulated intestinal fluids. These observations allowed us speculating that the galactosidase from *L. bulgaricus* could perform well during the entire transit throughout the small intestine.

Additionally, intestinal fluids seem to trigger the lysis of the transiting *L. lactis*, promoting the release of the intracellular proteins, including the galactosidase of interest.

While these data were promising, some critical aspects need to be evaluated. Firstly, the expression system was designed to allow the extracellular exportation of the galactosidase. However, the activity detectable in the extracellular compartment was limited. The employment of the 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) instead of lactose as a substrate to assess the enzyme activity has several advantages, but the findings (enzyme activity and activity in different environmental conditions) need to be confirmed in presence of lactose as substrate of the reaction

catalyzed by beta-galactosidases. Lastly, the evidence presented here come entirely from *in vitro* experiments. To corroborate these results, we will carry out additional experiments using lactose as a substrate. We will also optimize the secretion efficiency by employing a modified *L. lactis* lacking the HtrA protease. Assuming positive results, we will test the effect of this modified *L. lactis* on a mice model of lactose intolerance and eventually implement a food-grade system for a human application.

1. Introduction

In humans, the ability to digest lactose is strictly dependant on the activity of an enzyme named "lactase-phlorizin hydrolase" (LPH) encoded by the lactase gene (*LCT*; Chr. 2q21).

The most striking and fascinating characteristic of LPH is probably its pattern of expression.

The LPH activity starts to increase in the proximal part of the duodenum, peaks in the mid/distal jejunum, and drops in the ileum. Overall, the expression and activity of LPH are limited to the small intestinal enterocytes. Even more importantly, in the majority of humans (~ 70%), and mammals in general, the LCT expression declines after weaning, defining the so-called "lactase non-persistence" phenotype (LNP). This limited window of expression is the main reason behind the inability to digest lactose that characterizes many adults and can trigger the onset of lactose intolerance symptoms.

Among humans, however, a 30% retain the expression of lactase and the ability to digest lactose even during adulthood showing a "lactase persistence" phenotype (LP).

During the last decades, many researchers, fascinated by the mechanism that drove the evolution of LNP and LP worldwide, analysed thousands of genetic sequences from individuals all around the world. The aim of these studies? To identify the actors orchestrating this pattern of expression and the forces behind the selection of LP phenotype.

Today we know that the LP evolved multiple times independently in different populations (convergent evolution), with LP in Europe and Asia associated with genetic variants different from those found in Africa or the Middle East. We also know that at least two genetic regions regulate the spatiotemporal expression of the *LCT* gene: a promoter located immediately upstream of the transcription starting site (TSS) and a distal enhancer located about 14kb upstream of the TSS that spans the intron 13 of the minichromosome maintenance 6 (*MCM6*) gene.

Before going into the details of its regulation, however, we should draw a picture of the lactase-phlorizin hydrolase.

1.1. Lactase-phlorizin hydrolase

Lactase-phlorizin hydrolase (LPH) is a membrane-bound glycoprotein responsible for the digestion of lactose. The nascent LPH is a 195 kDa peptide (pre-pro-LPH) characterized by four regions of internal repeats, which suggest that the enzyme derived from two events of partial gene duplication. Despite the similarities, these four regions show different functions. The first two regions serve as internal chaperones that promote the correct folding and have no catalytic activities. Conversely, regions III and IV constitute the catalytic domain of LPH, showing a phlorizin hydrolase (**EC 3.2.1.62**) and a lactase (**EC 3.2.1.108**) activity, respectively **(Figure 1)**. Overall, five functional domains

characterize the pre-pro-LPH: **(1)** a short signal peptide (19 amino acids) at the N-terminus that mediates the translocation over the endoplasmatic reticulum; **(2)** the internal chaperone and **(3)** the catalytic domains described above**; (4)** a transmembrane domain and **(5)** a cytoplasmic tail that seem to be both involved in the dimerization that occurs during the maturation process. The first maturation step is the translocation over the endoplasmic reticulum membrane and the subsequent cleavage of the signal peptide, which converts the "pre-pro-LPH" into the "pro-LPH". Within the endoplasmic reticulum, pro-LPH undergoes several glycosylation events that affect the catalytic domain. Subsequent O- and N-glycosylations also occur in the Golgi apparatus, increasing the molecular mass to 220 kDa. These co-translational modifications seem to affect the correct folding, the intracellular transport, and the catalytic activity of LPH. The last steps see the cleavage of the internal chaperon and an extracellular proteolytic trimming that leads to the mature form of LPH [1], [2].

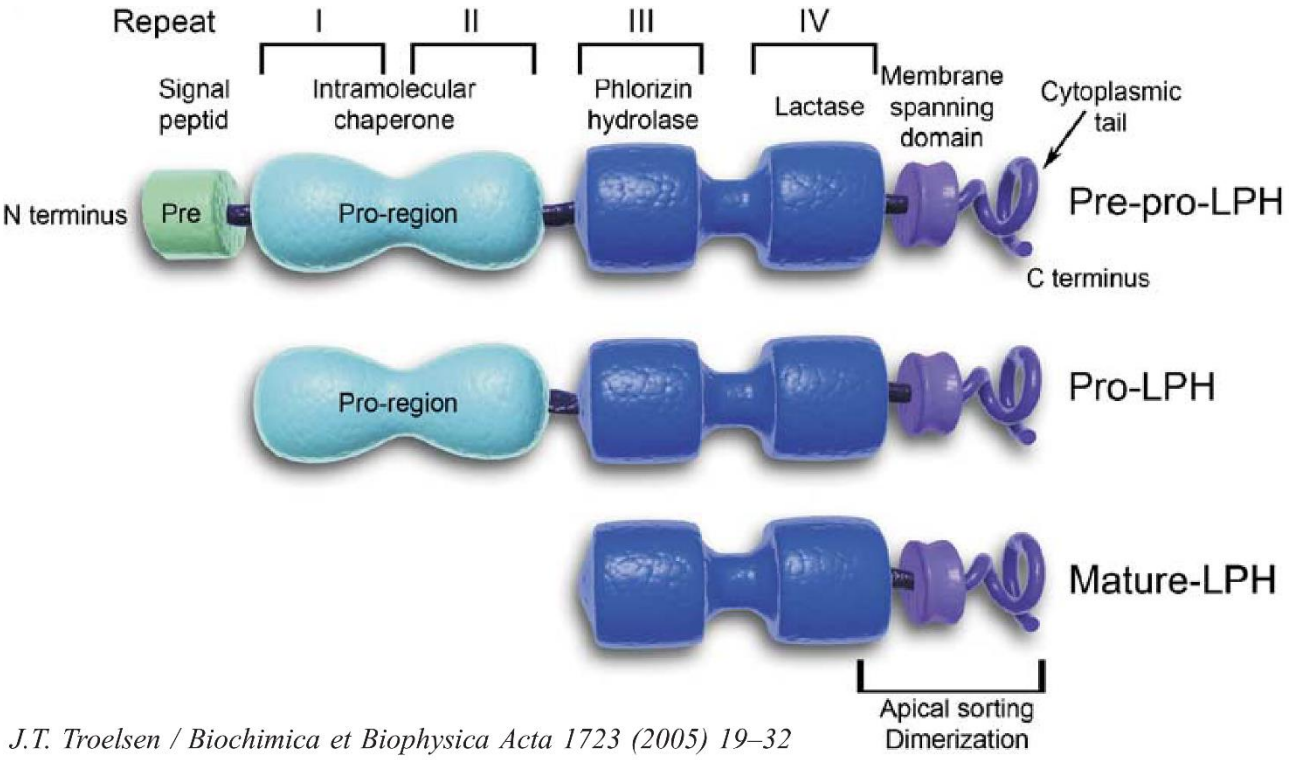

Figure 1: *schematic representation of the lactase phlorizin hydrolase (LPH) and its maturation. LPH originates as a pre-pro-LPH, which, following the proteolytic cleavage of signal peptide and intramolecular chaperone regions, is converted to the mature LPH. The enzyme in its mature form consists in two catalytic domains (Phlorizin hydrolase and lactase) and a C-terminal tail, which is critical for protein sorting and dimerization.*

1.2. The evolution of lactase persistence in humans

The functional significance of the post-weaning decline of LPH is still a bit mysterious. The majority of mammals stop ingesting milk after weaning, and they do not need the ability to digest lactose

during adulthood. But, what is the advantage in turning-off the *LCT* gene? While this question remains unanswered, studies are progressively unveiling the story behind the evolution and selection of LP.

This story began around 12000 BP in the Middle East, with the beginning of the Neolithic revolution [3]. During this era, humans started an unstoppable process that saw the transition from a culture of hunting and gathering, to one of agriculture, domestication, and settlement. By 6000 BP, farming had spread through all the Europe and hunting-based cultures had become very rare **(Figure 2)**.

Among the many innovations brought by this revolution, was the potential availability of a new food

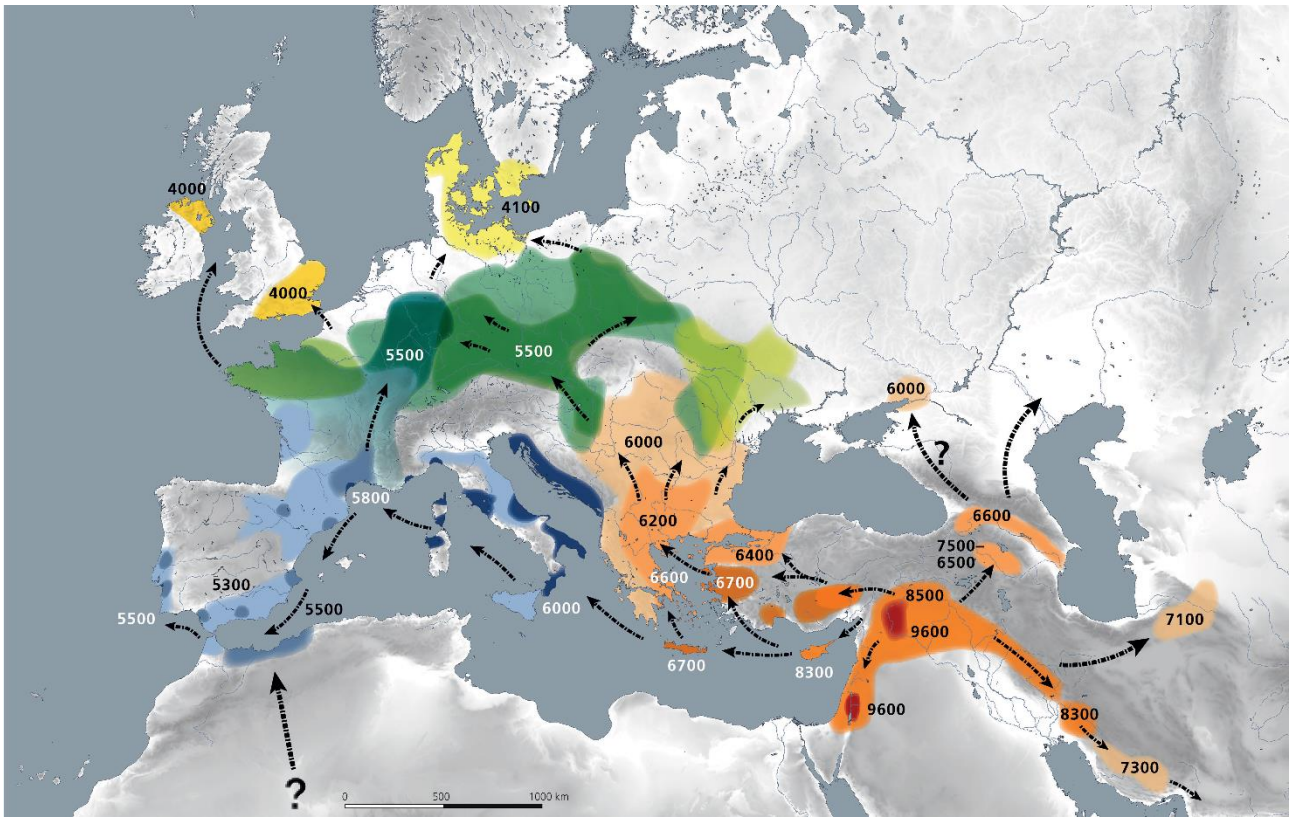

Figure 2: *spreading of agriculture, domestication, and settling through the Europe and Middle East during the Neolithic revolution.*

source: fresh milk. Given the physiological function of LPH, it was not surprising that studies dated the beginning of the selection of LP trait back to the same historical period that saw the domestication of dairy cattle [3], [4]. Accordingly, the frequency of LP paralleled the milk-drinking habits, with high frequency found in pastoralist populations [3]. However, to be astonishing was that the LP-associated alleles become so frequents in such a short amount of time (on the evolutionary scale) [5]. Researchers calculated a selection coefficient comprised between 0.014 and 0.15 for the allele associated with LP in Europeans. As a comparison, the selection coefficient for

the malaria resistance alleles is between 0.04 and 0.09 [4], making the selective pressure for LP trait one of the higher in human history. But why the ability to digest lactose in adulthood conferred such a selective advantage? To date, there are three main theories elaborated to answer this question [5].

The first theory, suggests that, by representing an additional source of fat and proteins, milk could confer an advantage to lactose tolerant individuals when other food sources were scarce (e.g. in case of crop failure, or between harvesting seasons).

According to the "calcium assimilation hypothesis", on the other hand, milk may have been fundamental for the populations living at latitudes where the limited solar irradiation could cause a deficit of vitamin D (which is required for calcium assimilation). Studies showed that lactose could partially prevent the detrimental effects of vitamin D deficit by promoting calcium absorption, supported this idea [6].

Lastly, milk may have represented a source of pathogen-free fluids for those populations that lived in arid and hot regions.

Overall, the most likely picture that emerges from the studies conducted so far is that the LP trait has been selected in various geographical areas for different reasons. These reasons range from the advantage of having a clean source of fluids in desert regions to the importance of lactose for the absorption of calcium at high latitudes. In every case, the selective pressure exerted was extremely strong and led to the allele frequencies observable today.

1.3. The spatiotemporal regulation of the lactase gene

Before examining the genetic variants that drive the development of the LP phenotype, we should clarify which elements participate in the *LCT* regulation.

The lactase expression is restricted to the brush border of the small intestine. In particular, the mRNA levels and enzymatic activity of LPH start increasing in the proximal duodenum, peaks in the jejunum, and drops in the ileum. Moreover, for the majority of humans, its expression declines after weaning. To date, we know two regulatory regions that contribute to this pattern: one surrounding the gene itself and containing the proximal and distal promoter, and another one, located about 14kb upstream, which spans the sequence of the *MCM6* gene and serves as an enhancer.

1.3.1. Molecular characterization of the LCT promoter

The first studies conducted on the 5'-upstream region of *LCT* allowed identifying a short sequence of 150 bp conserved between human, pig, rat, and mouse. This region harbours binding sited for the hepatocyte nuclear factor 1 (HNF-1α), caudal type homeobox 2 (Cdx-2), GATA transcription factors, and (in rat and mouse only) for the transcriptional repressor pancreatic and duodenal homeobox 1 (Pdx-1) [2] **(Figure 3A)**.

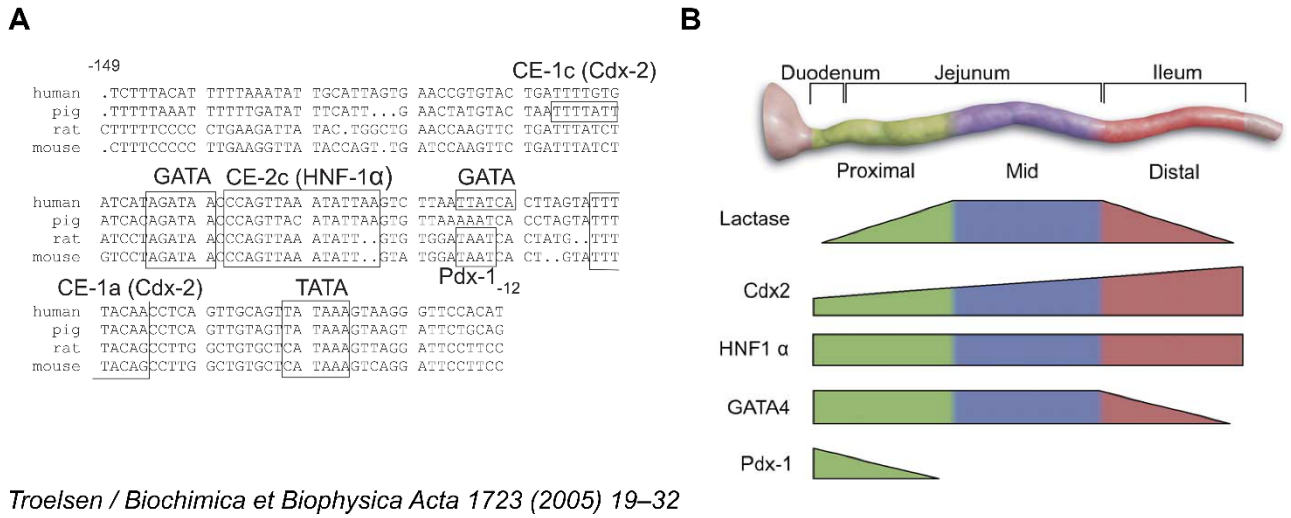

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Figure 3: (A) *Sequence alignment of the proximal LCT promoter of human, pig, rat, and mouse along with the putative transcription factors binding sites (boxed).* ***(B)*** *Expression pattern of the LCT gene along with its putative transcription factors through the small intestine.*

To better understand how these factors interact to orchestrate the complex regulation of *LCT*, studies analysed their expression throughout the small intestine and in other organs. Cdx-2 was the only intestinal-specific factor, with its level that progressively increased following a proximal to distal axis and peaked in the colon [7]. Conversely, the expression of HNF-1α and GATA-4 (the main LCT-interacting GATA factor) was not restricted to intestine but could be detected in several other tissues and organs. In respect to the small intestine, HNF-1α showed a constant level, while the expression of GATA-4 remained stable in the duodenum and jejunum and dropped in the ileum [8]. In vitro analyses showed that HNF-1α could interact with both Cdx-2 and GATA-4, suggesting that they could synergistically cooperate in activating the *LCT* promoter [9]. Interestingly, the expression of the repressor Pdx-1 was high in the proximal duodenum and dropped before the jejunum, showing an inverse correlation with the lactase expression [10]. While not present in the proximal promoter of the human *LCT* gene, the observation that Pdx-1 repressed the endogenous expression of lactase in the human cell line Caco2, suggested the presence of some distal binding sites for this repressor. Overall, it is conceivable to hypothesize that these four transcription factors cooperate

in the spatial regulation of *LCT*, with Pdx-1 and GATA-4 defining the proximal and distal border of lactase expression **(Figure 3B)**.

Further studies conducted on pig [11] and rat [12] *LCT* promoters allowed identifying additional regulatory elements within a region of 1 kb and 2 kb respectively. Experiments using transgenic mice and the human Caco2 cell line showed that the sequences from pig and rat promoters drove a small intestine specific expression and a post-weaning decline that mimicked those observed for the endogenous lactase.

However, the porcine sequence also promoted a high level of expression in the proximal duodenum that was not consistent with the low lactase activity observed in this region, suggesting that additional, more distal, regulatory elements might play an important role.

Additional analyses led to the identification of some of the transcription factors that could bind these regions. In particular, the hepatocyte nuclear factors (HNF) and the fork head factors (FREAC) seemed to act as transcriptional activators and repressors respectively, with several binding sites for these regulators being present in the promoter of both pig and rat [11] **(Figure 4)**.

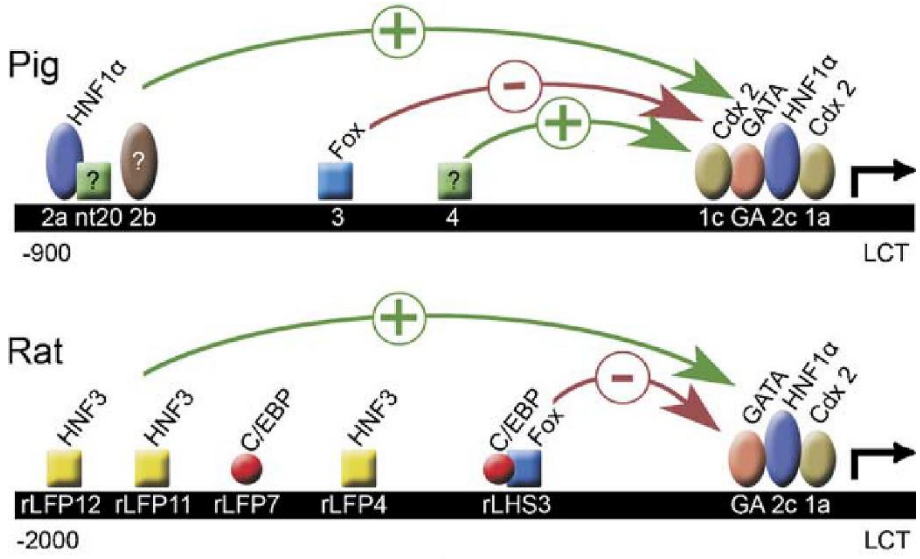

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Figure 4: *image showing the proximal promoter and the upstream distal elements that regulate the expression of LCT gene in pig and rat. Green arrows (+) indicate a transcriptional activator; red arrows (-) indicate a transcriptional repressor.*

1.3.2. A distal enhancer mediates the high levels of expression of LCT and the acquisition of the LP phenotype

Despite a long story of efforts to identify the genetic variants associated with lactase persistence, the first positive result arrived only in 2002, when Enattah et al. reported an association between

adult-type hypolactasia and a polymorphic site located almost 14 kb upstream of the *LCT* transcriptional starting site (TSS) [13]. The study of Enattah et al. was a breakthrough in the search for LP-associated alleles as it suggested for the first time the importance of this distant locus (within the *MCM6* gene) for the *LCT* regulation. The next few years saw the identification of several other single nucleotide polymorphisms (SNPs) associated with LP in Europeans, Asians, Africans, and the Middle East populations. Interestingly, all of these SNPs resided within the intron 13 of *MCM6* and evolved independently from each other. To date, we know a total of twenty-three LP-associated SNPs, for five of which *in vitro* and *in vivo* studies highlighted a functional role [14] **(Table 1)**.

Table 1: *functional single nucleotide polymorphisms (SNP) affecting lactase persistence trait. Table reports the SNP identifier, the SNP position relative to the transcriptional starting site, and the geographical areas with the higher incidence of the alternative variant.*

Identifier	Relative position	Geographical Area
rs4988235 C>T	-13910:T	Eurasia, North Africa, Central Africa
rs41380347 A>G	-13915:G	Middle East
rs41525747 C>G	-13907:G	East Africa (Ethiopia and Sudan)
rs869051967 T>G	-14009:G	East Africa (Ethiopia and Sudan)
rs145946881 G>C	-14010:C	East Africa (Kenya and Tanzania) South Africa

The first LP-associated SNP described by Enattah et al. was the -13910 C>T (rs4988235). The authors found an association between the LP-trait and the alternative variant of this SNP in a cohort of nine extended Finnish families [13]. Moreover, the same group highlighted an association between this SNP and the *LCT* mRNA levels in intestinal mucosa biopsies [15]. Following studies showed that the region of 200 bp surrounding the -13910 SNP increased the *LCT* promoter activity, explaining the higher level of *LCT* mRNA observed by Enattah et al. and suggesting the enhancing activity of this region [16], [17]. Moreover, Olds and Sibley demonstrated that the two allelic variants of the -13910 C>T interacted differentially with intestinal cell nuclear proteins on EMSAs [16]. This result provided the first evidence that the rs4988235 could participate in the development of LP trait by affecting the binding of a not yet identified transcription factor. A few years later, Lewinsky et al. found a putative binding sequence for the octamer transcription factor 1 (Oct-1), encompassing the polymorphic site. Interestingly, in silico analyses predicted a higher Oct-1 binding affinity for the variant T-13910 than the ancestral C-13910 [18]. Gel shift assays confirmed this prediction, paving to the possibility that the Oct-1 binding to the 13910:T could prevent the lactase post-weaning decline *in vivo.* To evaluate the presence of other regulatory elements, the author carried out DNase

footprint and supershift assays. These experiments allowed identifying additional binding sites for GATA-6, HNF4/Fox/HNF3, and Cdx-2 surrounding the -13910 SNP [18] **(Figure 5)**.

The ultimate evidence that the interaction between Oct-1 and the -13910:T could promote the occurrence of lactase persistence in adulthood come from an *in vivo* study conducted by Sibley's group. With their work, the authors showed that the -13910:T abolished the post-weaning decline of LPH in mouse [19]. This works represented an important milestone as they allowed the molecular characterization of the distal enhancer of *LCT*, offered a mechanistic explanation for the lactase persistence in adulthood, and pave the way for the studies that led to the identification of the other LP-associated SPNs.

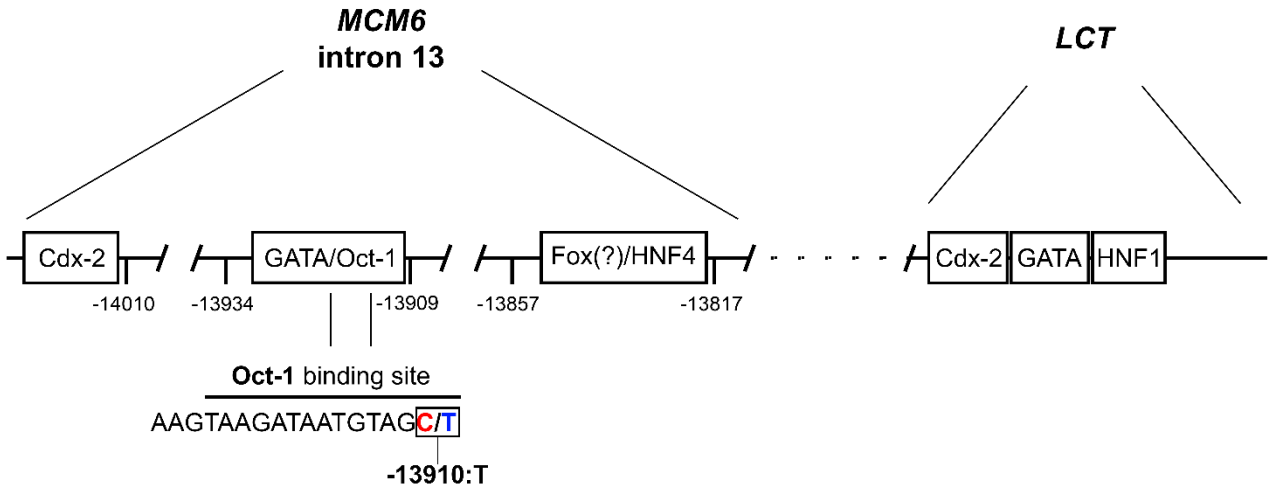

Figure 5: *transcription factor binding sites within the MCM6 intron 13 encompassing the European SNP -13910, along with the binding sites lying within the LCT proximal promoter.*

The -13910:T accounts for the LP-trait in Eurasia, and North and Central Africa. However, its frequency in other geographical regions, such as East and South Africa, or the Middle East, is too low to explain the LP-trait found among the inhabitants of these areas. Through the years, studies have identified several other SNPs associated with the persistence of LPH in the Middle East and Africa. Among these, the most widespread and strongly associated LP variants were the -14010:C (rs145946881 G>C), -13907:G (rs41525747 C>G), -14009:G (rs869051967 T>G), and -13915:G (rs41380347 T>G) **(Table 1)**. For these SNPs, functional *in vitro* analyses supported their role in affecting the expression of *LCT* (see references from Anguita-Ruiz et al.) [4]. Moreover, for some of these SNPs an interaction with Oct-1 has been reported, even if the difference in the bonding strength between the alternative and ancestral alleles was not comparable to those observed for the -13910 [20]–[23]. Thus, it is possible that other, not yet identified, transcription factors

participate in the *LCT* regulation through binding these regions. For example, Liebert et al. [22] observed that the -14009:G interacted differentially with nuclear extracts compared to the sequence carrying the ancestral variant (-14009:T), but, in this case, Oct-1 was not responsible for this difference. Conversely, the authors showed that the allele affected the binding of some member of the Ets transcription factor family, most likely c-Ets-1 [22], adding another element to explain the occurrence of lactose persistence **(Figure 6)**. Based on these observations we could conclude that the interaction between the distal enhancer located within the intron 13 of *MCM6* and the proximal promoter determines the spatiotemporal expression of *LCT*. However, genetic variants that create binding sites for additional transcription factors (in most cases for Oct-1) within the enhancer region abolish the typical post-weaning decline determining the persistence of LPH in adulthood.

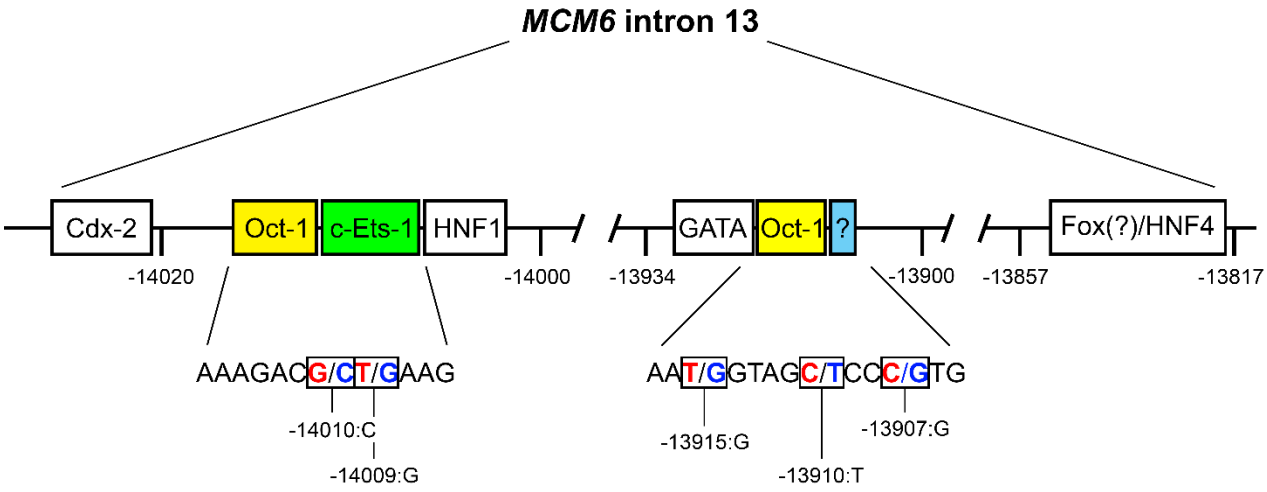

Figure 6: *transcription factor binding sites located within the MCM6 intron 13 region and encompassing the single nucleotide polymorphisms associated with lactase persistence in several human ethnicity.*

1.3.3. Epigenetic modifications play a critical role in the regulation of LCT expression

The genetic factors described above are not the only elements that drive the pattern of *LCT* expression. Indeed, a recent study showed that epigenetic modifications, and in particular DNA methylation at CpG sites, play a critical role in establishing the tissue specificity, post-weaning decline, and inter-individual variations of *LCT* expression.

The authors found a significantly greater density of modified cytosines in jejunum samples of adult mice, which clustered into three LCT's regions (intron2-exon3, intron8-exon9, and exon10).

Additionally, the jejunum samples from 115 unrelated adult people showed a significant inter-individual difference between DNA modifications in 11 CpGs sites. These sites clustered into two regions of *MCM6* (intron 13-exon 13 and exon 16) and the density of cytosines modifications was inversely proportional to the *LCT* mRNA levels. Moreover, 35 CpG sites showed a significantly

different modification density based on cell type, with greater densities found in enterocytes-deficient jejunum samples compared with jejunum enterocytes. These sites clustered into seven regions comprehending the *LCT* intron 5, intron 3, intron 2, exon 1, the *MCM6* exon 17, exon 16, and the *MCM6* intron 13.

The most fascinating finding of this study was that carriers 13910:C showed a greater density of DNA modification at *MCM6* intron 13- exon 13 than carries of 13910:T. The authors speculated a competition for binding this region between transcription factors and the DNA methylation machinery. By creating a strong-affinity binding site for Oct-1, the T allele at -13910 could prevent the accumulation of age-dependent DNA modification, thus abolishing the post-weaning decline of *LCT*. These findings suggest that the interaction between genetics and epigenetics drives the *LCT* expression in childhood and determines its post-weaning decline or its persistence through adulthood.

1.4. Lactose intolerance

The lactase activity allows the hydrolysis of lactose to D-Glucose and D-Galactose **(Figure 7)**, which are readily absorbed by the small intestinal enterocytes. The decline of lactase activity prevents this process, leading to "lactose malabsorption" (LM) and representing the triggering cause of lactose intolerance (LI).

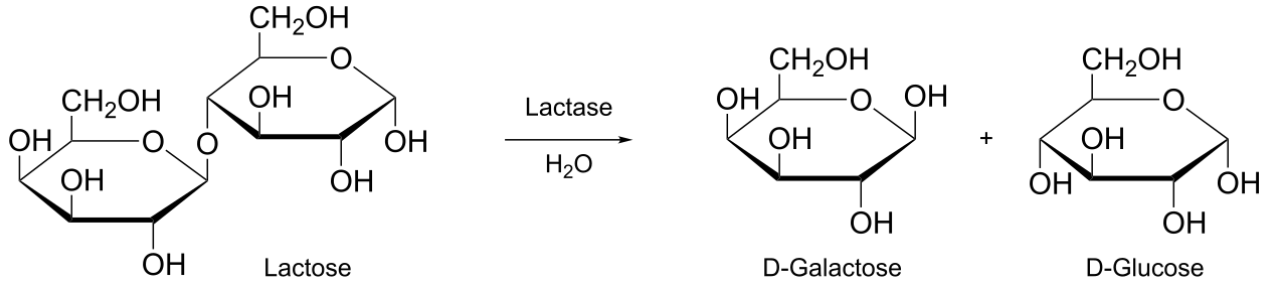

Figure 7: *hydrolysis of lactose into D-galactose and D-glucose mediated by lactase phlorizin hydrolase.*

There are three possible causes behind a loss of lactase activity: the first one is the genetically programmed post-weaning decline described in the previous chapters, also defined as "adult-type hypolactasia" or "primary lactase deficiency". Conversely, a "secondary lactase deficiency" can also occur in lactase-persistent individuals, following inflammatory bowel disease, gastrointestinal infections, or, in general, health conditions that compromise the lactase activity by damaging the brush border of the small intestine [5], [24].

Another, yet extremely rare, cause of lactase deficiency is a set of missense/nonsense mutations on the *LCT* gene that compromise the correct lactase functions resulting in a "congenital lactase deficiency" [25].

Whatever the cause, malabsorption often leads to LI symptoms such as diarrhea, nausea, vomiting, cramps, bloating, and flatulence. However, the onset of these symptoms depends on several factors other than the lack of a lactase activity and not all the malabsorbers eventually become lactose intolerant.

1.4.1. Factors affecting the onset of lactose intolerance symptoms

Lactose malabsorption is not synonymous of lactose intolerance. Some malabsorber individuals can tolerate small amounts of lactose without developing any gastrointestinal symptoms. The heterogeneity of the response to lactose among malabsorbers prompted the search for the individual factors affecting the occurrence of gastrointestinal symptoms upon lactose ingestion.

Lactose is an osmotic-active element, meaning that, if not absorbed, it increases the retention of water in the intestine. Thus, when transiting through the intestine, unabsorbed lactose causes an osmotic load that can result in intestinal distention and diarrhea. This osmotic shock is the principal cause of LI-related symptoms **(Figure 8)**.

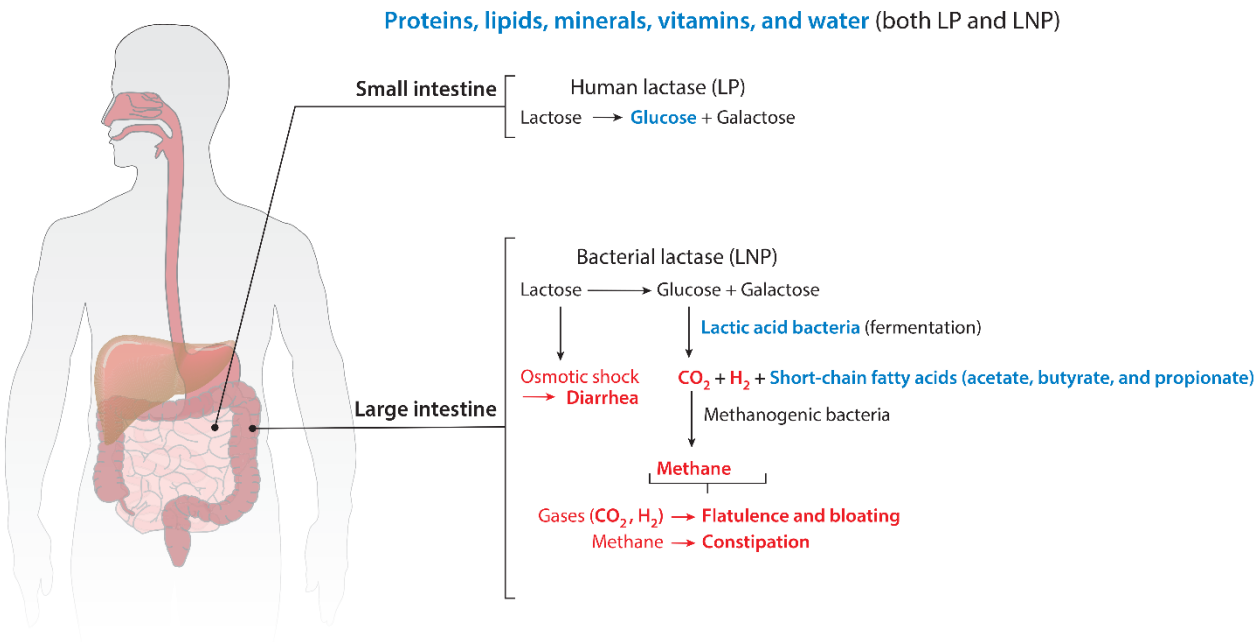

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Figure 8: *role of the bacterial lactases in lactase persistent and non-persistent individuals. Red indicates the products exerting a negative effect, which promote the occurrence of lactose intolerance (LI) symptoms, while blue indicates the products with a potential positive effect, which could alleviate the symptoms of LI.*

The individual susceptibility to the intestinal distention, with symptoms that can range from painful abdominal cramps to mild discomfort, is the first element that can explain the mentioned heterogeneity [26]. Once in the large intestine, unabsorbed lactose became the substrate of bacterial fermentative processes, resulting in the production of gasses (CO_2/NH_4/H_2) and short-chain fatty acids (SCFA; acetate, butyrate, and propionate) [27].

These processes can either improve or worsen gastrointestinal symptoms. Generally speaking, these fermentative reactions remove lactose from the intestine producing molecules that can be readily absorbed by the colonic mucosa. Thus, the expected result would be a decrease in the osmotic load and a consequent improvement of the symptoms.

However, if not removed, gasses can result in flatulence, bloating, and constipation. Similarly, when not efficiently absorbed, SCFAs can significantly increase the osmotic load in the intestine. Both events would result in a significant worsening of the symptoms **(Figure 8)**.

This scenario can occur under several circumstances in malabsorber individuals: **(i)** upon the ingestion of an excessive quantity of lactose; **(ii)** when the individual colonic absorption capabilities are limited for some reason; **(iii)** when the intestinal bacteria produces SCFA in excessive quantities or at a too high rate.

Interestingly, studies found that the metabolic activity of faecal bacteria differed between intolerant and tolerant malabsorbers. In particular, when incubated with lactose, faecal bacteria from intolerant individuals showed a higher rate of production of SCFA than bacteria from tolerant maldigesters. The bacteria from the two groups did not show any difference in lactose hydrolysis rate. Overall, these considerations highlight the importance of the intestinal microbiota metabolic activity in determining the balance between the production and removal of SCFAs and, thus, the improvement or worsening of gastrointestinal symptoms among lactose malabsorbers [27].

Interestingly, studies showed that, while the ingestion of lactose has no consequences on the expression of the *LCT* gene, nor on lactase activity, conversely it can shape the composition and the metabolism of the gut microflora [28], [29]. As we will see, this phenomenon, knowns as “colonic adaptation”, may have potential implications in the treatment of LI.

1.4.2. The challenges behind the diagnosis of lactose intolerance

A correct diagnosis of lactose intolerance would require assessing the relationship between the ingestion of lactose and the occurrence of intolerance-related symptoms in a lactose malabsorber individual.

Malabsorption assessment is quite simple, as many objective tests exist. These include the hydrogen breath test (HBT), the lactose tolerance test, the genetic test for the -13910 polymorphism, the serum gaxilose or urine galactose test, and the duodenal lactase activity evaluation.

On the other hand, establishing a correlation between the ingestion of lactose and the development of gastrointestinal symptoms is not an easy task. Indeed, self-reported symptoms are usually poorly reliable for several reasons. Firstly, symptoms that are typical of lactose intolerance are quite common and can have many causes including irritable bowel syndrome (IBS), inflammatory bowel disease (IBD), celiac disease, and others [30]. Additionally, psychological factors can influence the onset of these symptoms. Anxiety increases the risk of symptoms after lactose ingestion, and the fear of symptoms increases the anxiety itself in a loop that complicates the objective evaluation of lactose tolerance [31].

Consistently with these considerations, several studies showed that patients subjected to lactose tolerance test or HBT often reported gastrointestinal symptoms even in the absence of an objective lactose malabsorbance [30], [32]–[37]. Moreover, in double-blind tests, a significant proportion of patients often report the occurrence of symptoms even after the administration of placebo in place of lactose [30], [38], [39].

Consequently, in February 2010, the NIH consensus conference defined LI as “symptoms developing after ingestion of lactose which not develop after placebo challenge in a person with lactose maldigestion”, implying the need for placebo-controlled tests in the diagnosis of LI [40].

Indeed, double-blind, multi-dose, placebo-controlled tests would result in higher specificity. Moreover, this kind of tests would allow determining the maximum amount of lactose tolerated by each patient. Ten years later, however, the idea of a placebo-controlled test is still far from finding an application in clinical practice. To date, the diagnosis of LI relies solely on the symptomatic evaluation in a single-dose lactose tolerance test performed during the HBT. This approach has several limits, especially in terms of specificity, as it cannot exclude that the occurrence of symptoms depends on factors other than lactose ingestion.

The assessment of a dose-response effect in a blind test would remove any doubts on the relationship between lactose ingestion and symptoms occurrence, allow better dietary management, and prevent an unnecessary exclusion of lactose from diet.

Moreover, following the confirmation of a lactase deficiency, this test could be carried out by the patients themselves in a home-environment, overcoming the practical difficulties of implementing this kind of test in the clinical practice.

However, to date, these suggestions remain unheard.

1.4.3. The poor management of lactose intolerance and its consequences

When experiencing painful symptoms after the ingestion of a specific food, the first reaction is usually the effort of avoiding that and any other foods, which could trigger those symptoms again. Thus, driven by a condition of fear and anxiety, patients often adopt restrictive diets that could negatively affect their health condition [31]. Moreover, it is objectively hard to understand which of the diet's constituents is responsible for the experienced symptoms (if any). Consequently, the tendency is to attribute these symptoms to foods known to cause similar problems, such as lactose, gluten, or fat. [33]. Concerning lactose intolerance, the observation that self-reported symptoms of LI poorly correlate with the actual lactose absorption capacity, suggests that many of the patients with self-diagnosed LI are often lactose tolerant or a least capable of tolerating moderate lactose amounts [30], [32]–[37].

The first problem with a wrong self-diagnosis of LI is that symptoms of LI sometimes overlap those of other gastrointestinal diseases. As a result, the misbelief of being lactose intolerant could delay the correct diagnosis and treatment of other conditions.

Another reason for concern is that self-prescribed avoidance of dairy products may negatively affect health and life quality.

Indeed, studies showed an association between the CC-13910 genotype, a lower bone mineral density (BMD), a higher bone fracture incidence, and, as expected, a lower calcium intake from dairy food in postmenopausal women and elderly people [41], [42].

Other research groups found that self-perceived LI was associated with a lower calcium intake from dairy foods [37], [43]–[46]. In these subjects, the self-perception of lactose intolerance was associated with a lower perceived quality of life [37], [46], and dairy avoidance was associated with lower BMD and higher incidence of bone fractures [43]–[45].

Several other studies seemed to confirm the negative effect of dairy avoidance on bone health [47]–[49]. Dairy avoidance has been associated with many other health issues such as a higher incidence of diabetes [50]–[53], hypertension [54]–[58], and increased risk of developing colorectal cancer [59]–[61]. Additionally, while the diet cannot affect lactase expression or activity, conversely it can influence the composition and the metabolism of the colonic microbiota [29], [62], which, in turn, influences the development of intolerance symptoms [27]. Moreover, the microbiota participates in many human diseases and conditions, including obesity, hypertension, diabetes, cancer, inflammatory bowel disease, and many others [62], [63].

As concluded by Nicklas et al., self-perception of lactose intolerance may have a detrimental effect on nutrient intake and health outcome due to unnecessary avoidance of dairy consumption [45]. These observations reinforce the need for an accurate diagnostic process, which should allow a precise determination of the source of symptoms and, for those resulting as LI, the assessment of a maximum amount of lactose tolerated.

1.4.4. Treatment of lactose intolerance

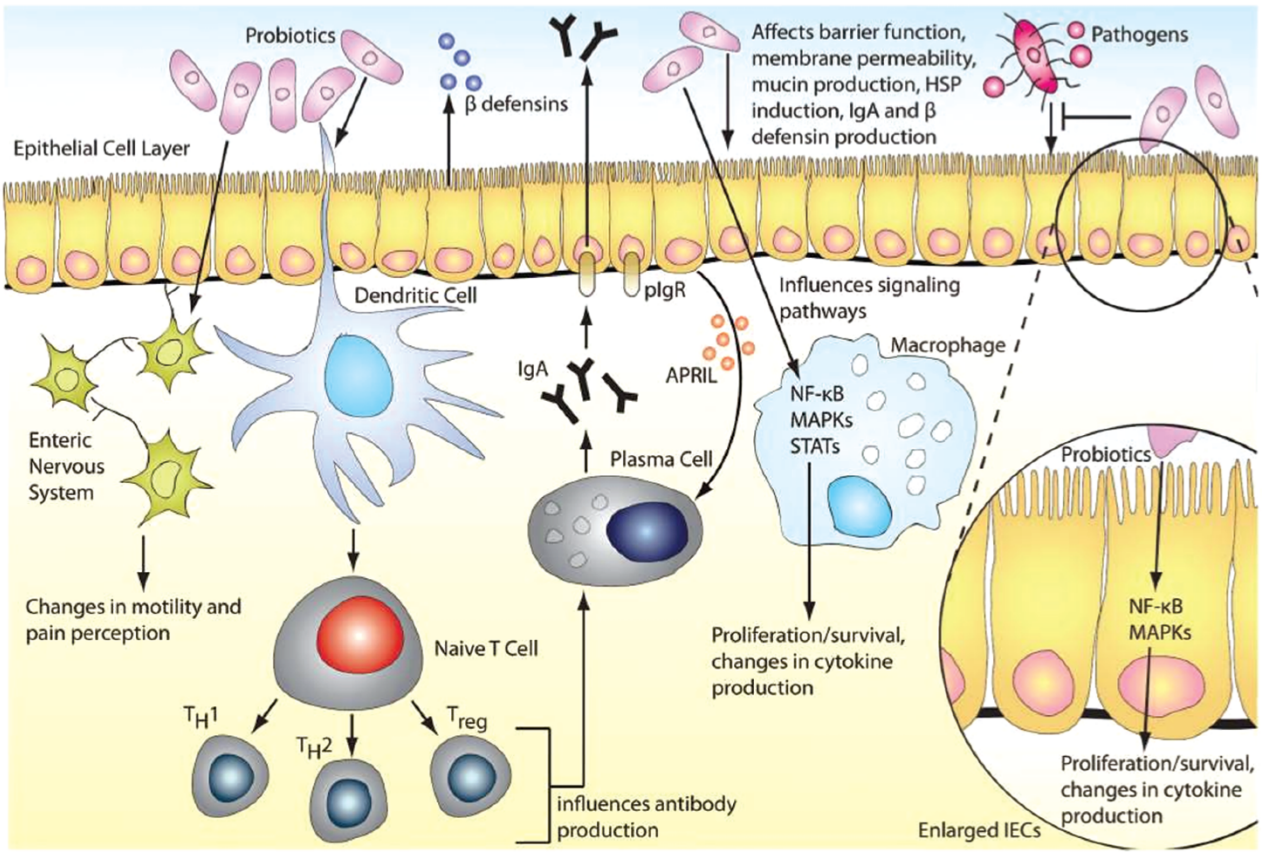
To date, the treatment for LI relies essentially on a reduction/avoidance of lactose intake sometimes accompanied by lactase supplements and the employment of pre-/pro-biotics to stimulate the colonic adaptation. For the reasons stated in the previous chapter, and considering that LI individuals can often tolerate small/moderate amount of lactose without experiencing any symptoms (usually between 5 to 20g in a single meal), an absolute exclusion of dairy products from the diet is not recommended. Thus, a correct dietetic approach, aimed to avoid the occurrence of gastrointestinal symptoms, and the health risk related to dairy avoidance, is the first critical step in the management of LI [64].

In addition to a controlled diet, patients affected by LI may undergo a "lactase replacement" therapy, which consists of adding exogenous β-galactosidases to milk some hours of just before the consumption. This addition results in a pre-hydrolysis of the contained lactose and a significant reduction of symptoms. Alternatively, β-galactosidases containing supplements (tablets or capsules) can be ingested just before consuming lactose-containing foods. Studies have confirmed the efficacy of both methods [24], [65], [66]. However, lactase supplements seem to be less effective than pre-hydrolyzed milk, probably due to the gastric enzyme inactivation caused by the low pH found in the stomach [65]. Another approach to improve the symptoms of LI is the employment of prebiotics and probiotics. As discussed in the previous chapters, diet can influence the composition and the metabolism of the intestinal microflora, inducing the so-called "colonic adaptation" [29], [62].

The elements of the diet that exert this effect are known as "prebiotics", defined as "non-digestible substances that promote the growth or bioactivity of beneficial microorganisms in the intestinal tract" [67]. Moreover, the observation that the microbiota participates in many human health issues, including LI, prompts exploring the employment of prebiotics for treating this condition. Among the possible prebiotic-based strategies, the use of galactooligosaccharides (GOSs) is showing probably the most promising results. GOSs are lactose-like molecules, which contain 2-4 galactose units per molecule. As such, GOS are not digestible nor absorbable and can travel through all the large intestine where they seem to exert a prebiotic effect potentially similar to those of undigested

lactose [68]. The prebiotic properties of GOSs and their use as the treatment for LI have been studied since the early ’90 [69]–[72].

Recently, two randomized, double-blind, placebo-controlled, clinical trials conducted on LI individuals showed that the administration of the GOS RP-G28 resulted in significantly reduced symptoms of LI. Moreover, the administration of RP-G28 increased the relative intestinal abundance of *Lactobacilli* and *Bifidobacteria* and the consumption of milk and dairy products in general [73], [74]. Another strategy to shape the intestinal microbiota is the employment of probiotics, defined as “live microorganisms (bacteria or yeasts) that, when ingested, provide equilibration of the intestinal microflora with a positive effect on consumer’s health” [67]. Among the most relevant characteristics of a probiotic are the tolerance to the gastrointestinal conditions, the ability to adhere to the gastrointestinal mucosa, and the competitive exclusion of pathogens [67], [75].



Hemarajata and Versalovic / Therapeutic Advances in Gastroenterology (2013) 39-51.

Figure 9: *ways and pathways through which probiotics can affect the functionality of the human gastrointestinal tract.*

Probiotics could alter the intestinal microflora composition by several mechanisms, such as by producing antimicrobial agents or metabolites that inhibit microbial growth, competing for the

adhesion to the intestinal mucosa with other bacteria, and modulating the immune responses of the host [62], [75]. Additionally, as for the microbiota, probiotics can interact with the host by modulating the expression of intestinal genes involved in immune response, nutrient absorption and genetic metabolism, affecting the gastrointestinal tract functionality [62] **(Figure 9)**. In respect to LI, probiotics can promote lactose digestion by increasing the hydrolytic capacity of the small intestine and the colonic fermentation, and by affecting the composition and the metabolic activities of the microflora [75].

As reviewed by Leis et al., many probiotic preparations for the treatment of LI contain some species of *Lactobacilli*, *Bifidobacteria*, and, in some cases, *Streptococcus thermophiles* [76]. The first evidence of the beneficial effects of probiotic-containing formulations dated back to 1990-2000 [77]–[79]. The following 20 years saw many other studies conducted to assess the efficacy of probiotics in LI treatments. The most widely employed strains belong to the genus of *Lactobacillus* (*L. bulgaricus, L. reuteri, L. acidophilus*, and *L. rhamnosus*) and *Bifidobacterium* (*B. longum*, and *B. animalis*). Additionally, some studies assessed the beneficial properties of *Streptococcus thermophiles* and the yeast *Saccharomyces boulardii.* While for some of these strains further analyses are needed to evaluate their effectiveness in reducing the intolerance symptoms, overall, the findings summarized by Oak and Jha [75] and by Leis et al. [76] strongly support the potential of probiotics in the treatment of LI.

1.5. The role of Lactococcus lactis in human health

Lactococcus lactis is a Gram-positive, nonmotile, noncolonizing, and nonsporulating lactic acid bacterium (LAB) belonging to the *Streptococcaceae* family [80]. Generally recognized as safe (GRAS) by the American food and drug administration (FDA), *L. lactis* has a long history of employment in the dairy industry, mainly due to its lactose fermentations capabilities, its role in the formation of the aroma and texture, and the secretion of antimicrobial peptides that contribute to food preservation [81]–[83].

Thus, a large number of researches focused on characterizing the industrial properties of *L. lactis*, from its sugar carbohydrate metabolism to its sensitivity to a wide range of stressors. In the last decades, these studies unveiled so many details about the genome, transcriptome, metabolic pathways, and extra-chromosomal elements, to make *L. lactis* the most deeply characterized organism among LAB [84].

Alongside with the molecular characterization of *lactococci*, came progresses in the development of genetic engineering tools such as new transformation protocols, the availability of a growing number of different vectors, and the evolution of recent genome editing techniques.

All of these factors contributed making *L. lactis* the first microorganism employed as a carrier, deliverer, and displayer of biologically active molecules including oral vaccines, antimicrobial peptides, and therapeutic proteins.

In August 2000, Steidler et al. used a recombinant strain of *L. lactis* for the delivery of interleukin-10 (IL-10) in the gastrointestinal tract of two mouse models of inflammatory bowel disease [85]. The authors showed that the intragastric administration of IL-10-secreting *L. lactis* significantly reduced chemically induced colitis and prevented the onset of colitis in IL-$10^{-/-}$ mouse models.

Few years later, the same research group assessed the feasibility of using the MG1363 strain of *L. lactis* as a vehicle for delivering murine Trefoil Factors (TFF) [86]. To do so, they fused the coding sequence of the TFF with the secretion signal peptide sequence Usp45 (Unidentified secreted 45-kDa protein) [87] under the control of the lactococcal P1 promoter [88]. This construct allowed the efficient production and secretion of the TFF. Moreover, results indicated that the intragastric administration of TFF-secreting *L. lactis* was effective in preventing and healing chemically induced colitis in mice. Importantly, the authors highlighted that this *Lactococcus*-based system of delivery worked significantly better that the administration of purified TFF as *Lactococcus,* probably, protected the TFF from gastric inactivation, allowing its effective delivery to the colon [86]. Similarly, the authors showed that the administration of an IL-27 secreting *L. lactis* reduced the symptoms of chemically induced acute colitis in mice [89]. Steidler's group was a pioneer in the employment of *L. lactis* as a delivery system for therapeutic molecules and conducted other similar works obtaining promising results [90], [91]. However, the most important breakthrough was probably the assessment of the safety of this strategy in a phase I clinical trial [92]. In this work, the authors replaced the thymidylate synthase gene of *L. lactis* with a synthetic sequence encoding for the human IL-10 fused with the Usp45 signal peptide for an efficient secretion. The resulting genetically modified strain (*LL-Thy12*) was orally administered to 10 patients affected by Chron's disease to assess the safety of the treatment. Results showed that treatment with *LL-Thy12* was well tolerated as no severe nor moderate adverse effects was reported. Clinical benefits were also observed in the majority of the patients but the study design did not allow any conclusion about the effectiveness of this strain in the treatment of Chron's disease [92]. This study was an important milestone as it showed for the first time the feasibility and safety of employing *L. lactis* as a vehicle for the delivery of therapeutic

proteins in humans. Following this evidence, another clinical trial assessed the safety of a *L. lactis*-containing preparation (AG013), with similar results. Once again, authors showed that the treatment was well tolerated resulting only in modest side effects. Importantly, none of the participants developed sepsis from AG013, and bacteria were not detectable in the blood of any patient [93].
While reliable studies assessing the clinical benefits of these strategies are still lacking, these data are quite promising and encourages the exploration of this route for the treatment of many conditions, including LI.

1.5.1. Modified microorganism for the treatment of human diseases: safety concerns

Following his long history of employment in dairy production, *L. lactis* received the "generally recognized as safe" status by the FDA, meaning that it is safe for humans under the conditions of its intended use. Despite its GRAS status, however, the modification of *L. lactis* requires cautions, as special restrictions exist for genetically modified organisms (GMO) especially when employed for human-related applications. The main reasons of concern are the safety of the host strains, the biological containment of the transgene and/or the modified microorganism, and the horizontal transfer of foreign genetic material to other bacteria. Additionally, the manipulation procedures should follow the "self-cloning" principles, meaning that the introduced coding and regulatory elements should come from organisms of the same genus or, at least, other GRAS organisms. When adherent to the self-cloning principles, a manipulated microorganism usually do not follow under the definition of GMO. However, things change if the microorganism is released in the environment in its viable form, a scenario that may include the excretion of modified *lactococci* through the faeces. In this case, even a self-cloning based organism is subjected to the same rules as GMOs [94]. Thus, strategies to avoid an uncontrolled spread of the organism in the environment are needed.
A good example of biological containment comes from the Staidler's group work, in which the authors replaced the thymidylate synthase gene (*thyA*) of *L. lactis* MG1363 with the human *IL-10* coding sequence [95]. The result was a modified auxotrophic strain (*LL-Thy12*) that was strictly dependent on thymidine or thymine for growth and survival. After an initial validation in pigs, this strain underwent a phase I clinical trial, which highlighted is safety and confirmed the efficacy of the containment strategy [92]. Interestingly, the substitution of the *thyA* gene with the *IL-10* transgene also implied the spontaneous removal of the transgene itself in the unlikely event of an acquisition of an intact *thyA* gene from a donor strain. The aforementioned works provide an example of a "passive containment" strategy. This kind of approach relies on the complementation of an

auxothrophy, with the modified bacteria that can survive only when supplemented with an essential metabolite that is not present in the environment.

Alternatively, constructs that enable killing the host strain through an environmentally controlled activation or repression of a specific gene can be employed. However, these “active containment” strategies often required the introduction of large foreign DNA, thus impeding their use in humans [95], [96].

An even greater concern derives from the risk of transferring antibiotic resistances (AR) from harmless bacteria to other, pathogenic, microorganisms.

In the laboratory practice, plasmids carrying the transgene of interest often harbor also a AR encoding gene that serves for the selection of the successfully transformed bacteria.

However, for the aforementioned reason, selection methods based on AR-harboring plasmids, which can be easily transferred to other bacteria by transduction, conjugation, or transformation, cannot be employed for the manipulation of microorganisms intended for human usage (or “food-grade”) [96]. To overcome this problem, researchers have developed alternative selection strategies based on dominant and complementation markers. For their mechanism of actions, dominant markers are comparable to AR, as they allow the direct selection of positive transformants by conferring resistance to an otherwise toxic substance. It is worth noting that, for the system to be considered “food-grade”, the selective substance has to be “food-grade” themselves, thus being recognized as harmless for humans. Example of strategies employing these markers are plasmids carrying the *nsr* gene, which encodes for a hydrophobic protein that prevent the bactericidal action of nisin, or the *bsh* gene, which, by encoding for a bile salt hydrolase, confers resistance to high bile salt concentrations. In goes without saying that both, nisin and bile salts, are harmless substances, which perfectly adhere to the definition of “food-grade” compounds.

On the other hand, the selection by compensation requires a first step that consists in the knockout of a specific gene, followed by the construction of an expression vector carrying the relative complementation. An example of food-grade selection marker based on complementation is the *alr* gene. *Alr* encodes for an alaline racemase that catalyzed the conversion of L-alanine into D-alanine. Since the reaction catalyzed by the racemase is essential for the synthesis of the cell wall in *L. lactis*, $alr^{-/-}$ strains require either supplemented D-alanine or an *alr* carrying vector for growth and survival. Thus, positive transformants can be selected for their ability to growth on L-alanine containing medium.

Another targeted gene for the construction of food-grade plasmids is *lacF*, which is essential for using lactose as an alternative carbon source. Thus, when lactose is added to the medium as the only source of carbon, *lacF*$^{-/-}$ strains are not able to growth unless they carry an expression plasmid bearing the *lacF* gene. Two recent reviews elucidated the advantages and drawbacks of these strategies [94], [96].

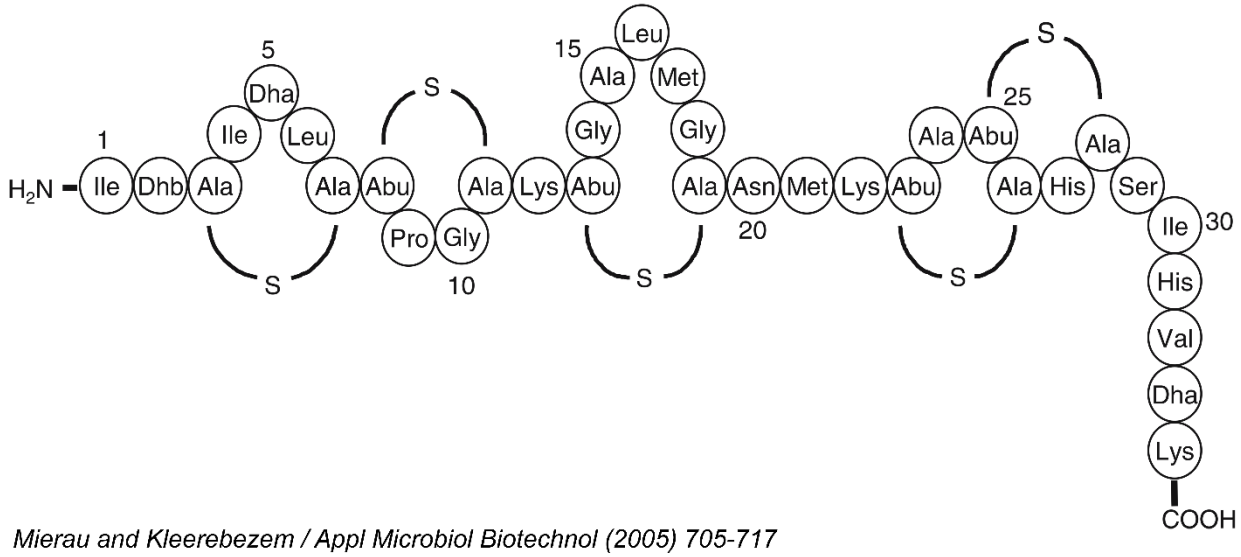
1.5.2. The Nisin Controlled gene Expression system: NICE

Details about the regulatory region of *L. lactis* started emerging from the early '90s studies.

These works showed that the promoters driving lactococci gene expression were similar to the classical promoter of *E. coli*, harbouring a -35 and a -10 element, constituted by a TTGACA and a TATAAT consensus sequence respectively, spaced by 17-18 base pairs (bp) from each other [97]–[100] (with some exceptions [88]). The gene structure showed the classical Shine Dalgarno consensus, containing the AGGAGG sequence and, usually, an ATG as a translation starting site [97]–[100]. Other studies allowed characterizing several constitutive and inducible promoters, along with signal sequences required for protein secretion and anchorage.

These pieces of information were fundamental for the first bioengineering attempts on *L. lactis.* However, the real breakthrough was the discovery of the nisin induction mechanism described for the first time by Kuipers et al. back in 1995 [101], and reviewed by Mierau & Kleerebezem [83].

Nisin is a short antimicrobial peptide of 34 a.a. that form five rings structures **(Figure 10)**, produced and secreted by some strains of *L. lactis* through a quorum-sensitive auto-inducible mechanism.



Mierau and Kleerebezem / Appl Microbiol Biotechnol (2005) 705-717

Figure 10: *structure of the antimicrobial peptide nisin.*

To work properly, the entire system requires 11 clustered genes [102], with *nisA*, *nisB*, *nisT*, *nisC*, and *nisP* directing the synthesis of the precursor as well as its translocation and processing, and *nisI*, *nisF*, *nisE*, and *nisG* involved in the immunity against nisin itself. The expression of these genes is strictly dependent on the activity of NisK and NisR proteins (encoded by the *nisK* and *nisR* genes), which constitute the two-component signal transduction system **(Figure 11)**. Upon binding with nisin, the membrane-located NisK kinase phosphorylates NisR. In turns, phosphorylated NisR activates P_{nisA} and P_{nisF} promoters, which drive the expression of the aforementioned genes.

This knowledge led to developing the Nisin Controlled gene Expression system (NICE), which, to date, is one of the most widely used for the expression of homologous and heterologous genes in *L. lactis.* To exploit this induction system, Kuipers et al. [102] cloned the two signal transduction genes

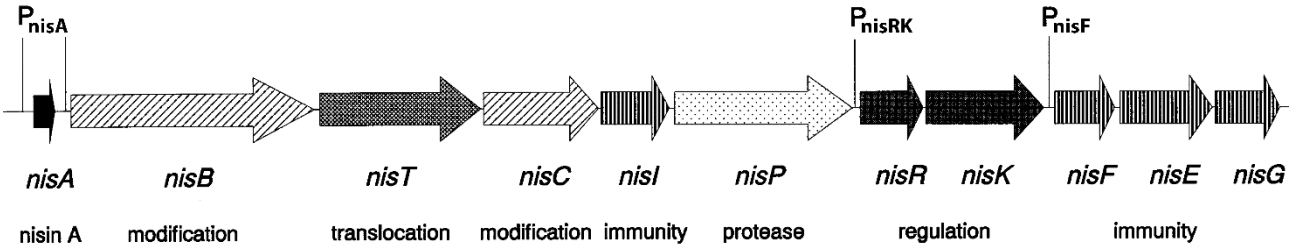


Figure 11*: genes and regulatory elements involved in the expression and translocation of, and immunity to, the antimicrobial peptide nisin.*

(nisK and nisR) into the chromosome of the nisin-negative strain MG1363 of *L. lactis*, generating the NZ9000. Upon transformation with a plasmid harbouring a gene under the control of a P_{nisA} or P_{nisF} promoter, the NZ9000 allows inducing gene expression by adding a sub-inhibitory amount of nisin (0.1-5 ng/ml) to the medium. Importantly, studies showed a linear dose-response curve for nisin induction. These observations implied the possibility to exploit NICE for both switching on and off gene expression and dosing the target protein levels.

Through the years, the MoBiTech developed several NICE-based strains and plasmids to employ in a wide range of applications. The NZ9000 strain remains the most used in the laboratory practice as it represents the easiest tool for a controlled expression of homologous and heterologous proteins in *L. lactis*. However, two other strains are worth mentioning for their potential as *in vivo* delivery systems for human health-related applications: the NZ3900, and the NZ9130.

The first strain derives from a two-steps process: the chromosomal integration of the lactose operon and the following deletion of the *lacF* gene. The resulting NZ3900 cannot grow on media that contain lactose as the only source of carbon unless provided with a *lacF*-bearing plasmid. Similarly,

the deletion of the alanine racemase gene (*alr*) results in a strain, the NZ9130, which cannot grow in the absence of D-alanine or an alr-producing plasmid. Both strains are suitable for the food-grade systems, as they do not require employing AR markers for selecting positive transformants. Additionally, the NZ9130 could be useful for biological containment strategies by exploiting its auxotrophy for D-alanine.

The MoBiTech also developed many types of plasmids suitable for the NICE system.

Among these, the most widely used harbour a chloramphenicol resistance gene (*Cm*^{r}) as a selectable marker (i.e. pNZ8148 and pNZ8123). However, plasmids exist that allow a food-grade selection of positive transformants using the NZ3900 or the NZ9130 as host strains (i.e. pNZ8149 and pNZ8152), by carrying the *lacF* or the *alr* genes in place of the *Cm*^{r} gene. Based on the presence of specific signal peptides, some constructs allow the secretion of the protein of interest into the extracellular environment (i.e. pNZ8121, pNZ8122, pNZ8123).

As reviewed by Mierau and Kleerebezem, the NICE system offered advantages for many applications, from metabolic engineering to industrial-scale processes, in addition to the already mentioned production of cytoplasmic, secreted, and membrane-anchored proteins [83].

It is worth noting that the NICE system would represent a perfect platform for developing a probiotic-based therapeutic strategy to employ in the treatment of lactose intolerance. Indeed, by cloning bacterial galactosidases under the control of a nisin-inducible promoter, it would be possible to achieve their delivery to the small intestine using an appropriate strain of *L. lactis*. The nisin-induction would lead to a high but switchable expression of the transgene, thus diminishing safety concerns. Additionally, the food-grade strains NZ3900, and NZ9130 could allow an antibiotic-free selection and the planning of an efficient containment strategy.

2. Aims

The aim of this study was the construction of an *L. lactis*-based expression system with potential application in the treatment of lactose intolerance. To this end, the first critical step was the selection of galactosidases retaining a good enzyme activity at pH and temperature that characterized the human small intestine. These galactosidases were then cloned into vectors allowing their nisin-inducible expression and secretion. Lastly, we aimed to evaluate whether *L. lactis* could survive the transit through the stomach and release the heterologous galactosidases into the small intestine.

3. Materials and Methods

3.1. Selection and cloning of β-galactosidases into pLB141 vector

3.1.1. β-galactosidases selection

The effectiveness of a therapeutic strategy for LI employing a *Lactococcus*-based delivery of exogenous galactosidases depends on several key aspects. Firstly, the selected β-galactosidases must retain a good activity when exposed to environmental conditions that mimic those found in the small intestine. As such, the first step was the selection of exogenous β-galactosidases based on enzyme activity and stability at 37°C and a pH comprised between 6.5 and 7.5. Through literature researches, we found three β-galactosidases (EC:3.2.1.23) corresponding to these criteria encoded by the following genes: **(i)** *GanA* of *Bacillus licheniformis* (ATCC 14580), **(ii)** *LacZ* of *Lactobacillus delbrueckii* subsp. *bulgaricus* (ATCC 11842) and **(iii)** *LAC4* of *Kluyveromyces lactis* (ATCC 8585). Details on the kinetic properties of these galactosidases are reported in the "results" sections.

3.1.2. Vector pLB141

For *in vivo* applications, food-grade vectors and hosts are the ideal choices. However, the lack of an antibiotic selection marker complicates the initial manipulation steps. For this reason, and considering the explorative nature of this study, we decided to employ the pLB141 vector (kindly donated by Prof. Luis G. Bermudez-Humaran, Institut National de la Recherche Agronomique, Paris), which harboured the following sites of interest:

- Chloramphenicol resistance gene Cm^R
- P_{nisA} nisin-inducible promoter
- Exp4 signal peptide
- Multiple cloning site containing restriction sites for *NsiI* and *NotI*
- Micrococcal nuclease coding sequence (*nuc*) located between *NsiI* and *NotI*
- TrpA terminator

The vector also harboured all the sequences needed for its replication within a *L. lactis* host. This vector was originally employed for the nisin-inducible expression and Exp4-mediated secretion of the micrococcal nuclease nuc [103]. For our purposes, replacing the *nuc* sequence with that of the selected galactosidases should allow their expression and translocation to the extracellular environment.

3.1.3. Cloning procedures

For galactosidases cloning, we employed a ligase-based strategy, as illustrated in **Figure 12A**. The procedure consisted of PCR amplification of the galactosidase sequences followed by *NsiI-NotI* double digestion of PCR products and pLB141 vector. Upon purification, digested product and vector were ligated using a T4 DNA ligase and transformed into *E.coli* for subsequent selection and amplification. The following paragraphs illustrate the detailed cloning steps.

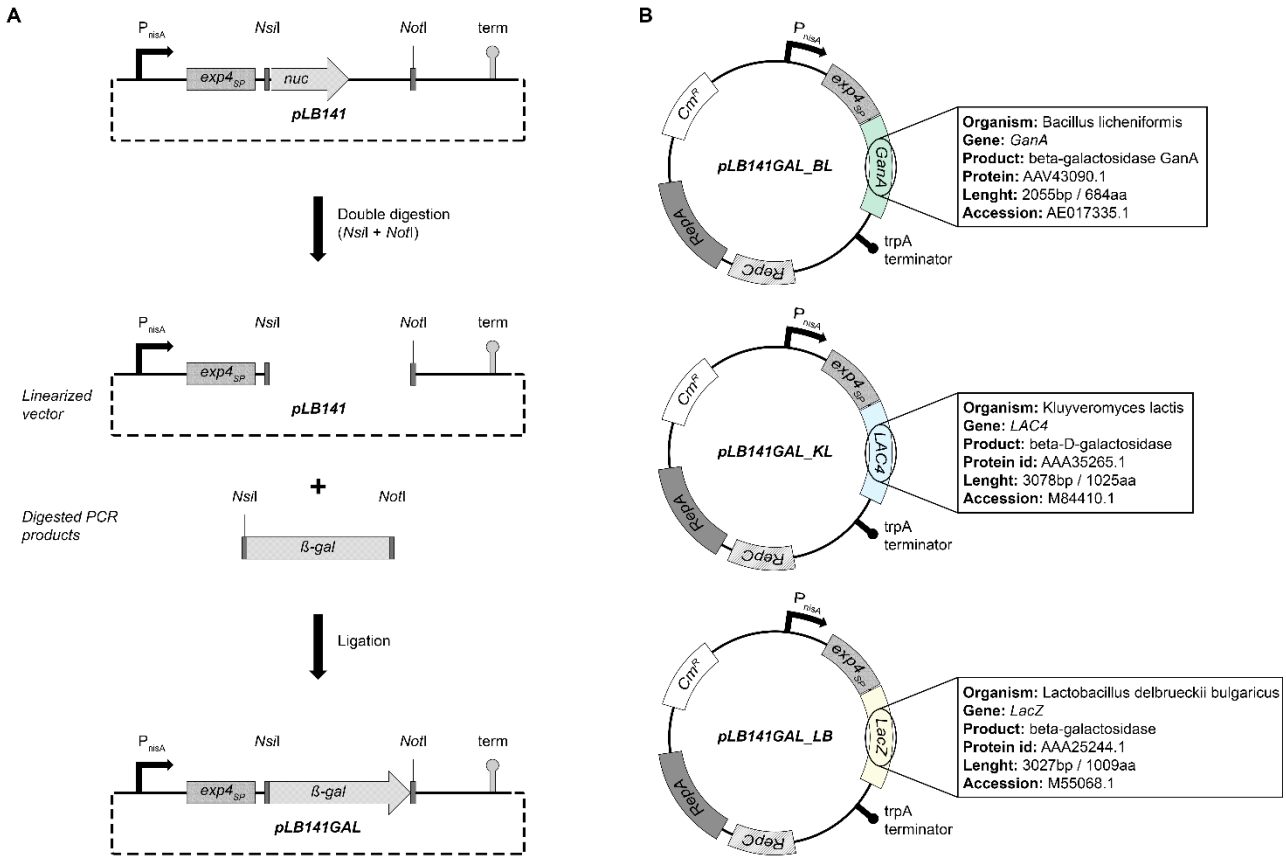


Figure 12: (A) *Schematic representation of the cloning strategy employed to assemble the vectors harbouring a ß-galactosidase coding sequence (ß-gal) fused with the signal peptide exp4$_{SP}$, to allow protein secretion, and under the control of a nisin-inducible promoter (P$_{nisA}$).* ***(B)*** *The three vectors employed for the production of exogenous ß-galactosidases from Bacillus licheniformis, Kluyveromices lactis, and Lactobacillus delbrueckii bulgaricus respectively.*

3.1.3.1 β-galactosidases amplification by PCR

For cloning purposes, the sequences encoding the selected β-galactosidases were PCR-amplified using a Q5® High-Fidelity DNA Polymerase (M0491L; New England Biolabs), according to manufacturer's instructions. Genomic DNA of *B. licheniformis* (ATCC 14580D-5) and *K. lactis* (ATCC 8585D-5) was purchased from American Type Culture Collection (ATCC) and 1ng used as a template to PCR amplifying *GanA* and *LAC4* gene respectively. Conversely, the *LacZ* gene was PCR-amplified

using the diluted live *L. bulgaricus* directly as PCR template. Specifically, *L. bulgaricus* (kindly donated by Prof. Roberto Di Marco, Università degli Studi del Molise) from the stab was streaked on MRS plates and incubated at 42°C for 48h. After the incubation time, a single colony was peaked and dissolved in 5ul of sterile water and 1ul of this solution used as PCR template. Upon verification on a 1% agarose gel, PCR products were purified using the Expin combo GP mini kit (112-102; GeneAll). **Table 2** reports the sequences of the primers employed for the amplification of the mentioned genes.

Table 2: *Sequences of the primers employed for cloning, colony PCR and sanger sequencing.*

Primer name	Primer sequence (5'-3')	Primer usage
Lig_KL_144_F	*ATGATGCATCTTCTTGCCTTATTCCTGAG*	Cloning of *LAC4* gene in pLB141
Lig_KL_177_R	*CTGCGGCCGCTTATTCAAAAGCGAGATCAAAC*	
Lig_LB_144_F	*ATGATGCATCTAGCAATAAGTTAGTAAAAG*	Cloning of *LacZ* gene in pLB141
Lig_LB_177_R	*CTGCGGCCGCTTATTTTAGTAAAAGGGG*	
Lig_BL_144_F	*ATGATGCATTGCCAAAAATTTATACGACC*	Cloning of *GanA* gene in pLB141
Lib_BL_177_R	*CTGCGGCCGCCTATTCTTTTGCTTTTACCGCTA*	
pLB141_F2	*GCATCACCACGCATTACAAGT*	Colony PCR and junction regions sequencing
pLB141_R2	*TCATTGAGAAGATTGCCGAA*	
K.lactis_2_F	*TCAGATAATCCAGAGTACGAGG*	Sequencing of the internal regions of *LAC4*
K.lactis_3_F	*ATAAGCACGACTTCATTACGAC*	
B.licheniformis_1_F	*TTCCGTTTCAAGGGCTGGACTAC*	Sequencing of the internal region of *GanA*
L.bulgaricus_1_F	*AAATCCGCTCGCTTCTTATGTCAG*	Sequencing of the internal regions of *LacZ*
L.bulgaricus_2_F	*CGGGCTATCACAGAAGAGGA*	

3.1.3.2 Double digestion of PCR products and pLB141

To remove the *nuc* sequence and create compatible sticky ends for the directional cloning of the β-galactosidases, PCR products and pLB141 vector were double-digested with *NsiI* (Anza, IVGN0376) and *NotI* (Anza, IVGN0014) restriction enzymes, as illustrated in **Table 3**. The reaction mix was incubated overnight at 37°C. After the overnight incubation, 1µl of Anza™ Alkaline Phosphatase was added to the vector-containing mix and incubated at 37°C for 15 min. The overnight incubation and the phosphatase treatment allowed complete digestion and prevented vector self-ligation. The enzyme was then heat-inactivated at 80°C for 20 min. Digested PCR products and vector were loaded on a 0.8 agarose gel and gel-purified using the Expin combo GP mini kit (112-102; GeneAll).

Table 3: *reaction mix for vector and PCR products digestion.*

Reagent	Vector digestion	PCR products digestion
Nuclease-free water	To reach a final volume of 40 µL	
Anza 10X RED Buffer	4 µL	4 µL
pLB141	2 µg	-
PCR products	-	2 µg
NsiI	2 µL	2 µL
NotI	2 µL	2 µL

3.1.3.3 Ligation

Ligation was achieved using T4 DNA ligase (M0202; New England Biolabs) and a 1:1 vector-insert molar ratio. Upon an overnight incubation at 16°C, 5µl of the reaction mix was then used to transform the ultra-competent *E.coli* cells NZY5α (MB00401; Nzytech) following the heat-shock protocol suggested by the manufacturer. After the heat-shock treatment, cells were incubated for 30 min at 37°C in antibiotic-free Luria-Bertani (LB) medium and then plated on Agar-LB plates containing 40µg/mL of chloramphenicol and maintained at 37°C for 48h.

3.1.3.4 Colony PCR, digestion, purification and sequencing

The cloning outcome was evaluated by colony PCR, enzyme digestion and Sanger sequencing as follow. After a two-day incubation at 37°C on Agar-LB, colonies were picked and dissolved in 5µl of sterile water. A PCR reaction was carried out, according to the manufacturer's instructions, using the HotFirePol DNA polymerase (01-02-00500; Solis BioDyne) and 1µl of the dissolved colonies solution as a template. Primers specific for the pLB141 vector and flanking the cloning site **(Table 2)** were used to discriminate between positive (i.e. carrying the vector with the correct insert) and negative (carrying the empty vector) colonies. The results of the PCR were verified on a 0.8% agarose gel. Positive colonies were inoculated in LB medium containing 40µg/mL of chloramphenicol and incubated overnight at 37°C. After the overnight incubation, plasmids were purified using the PureYield™ Plasmid Miniprep System (A1222; Promega). Purified plasmids were digested with *NotI* and *NsiI* as previously described, and the digestion outcome gel-verified to corroborate the colony PCR results. Sanger sequencing was carried out by Eurofins Genomics and employed to confirm the cloning outcome and the fidelity of the cloned sequences. **Table 2** reports the sequencing primers. The pLB141GAL-BL/KL/LB vectors, harbouring the *GanA*, *LAC4* and *LacZ* genes, respectively **(Figure**

12B), were purified from *E.coli* after the cloning and verification steps reported above, using the NucleoBond Xtra Midi Plus EF purification kit (740422.50; Macherey-Nagel).

3.2. Electroporation of β-gal plasmids in L. lactis

While cloning and plasmid expansion was carried out in *E.coli*, studying the nisin-induction and characterizing the β-galactosidases expression and activity required the employment of *L. lactis* as a bacterial host. To this end, the pLB141GAL plasmids described above were electroporated in the NZ9000 strain of *L. lactis* (kindly donated by Prof. Luis G. Bermudez-Humaran, Institut National de la Recherche Agronomique, Paris), following the protocol reported by MoBiTec for the NICE expression system (https://www.mobitec.com/media/datasheets/mobitecgmbh/NICE_Expression_System-Handbook.pdf) and detailed below. Before the electroporation, *L. lactis* cells were grown for two days at 30°C in a modified ESTY Broth (1254.00; Condalab), named G-SGM17B. Specifically, in addition to a 0.5% glucose as carbon source, the medium contained 2.5% of glycine as a cell wall weakening agent and sucrose at a final concentration of 0.5M as an osmotic stabilizer. On the third day, 50mL of the full growth culture were inoculated into 450mL of G-SGM17B and incubated 30°C until an OD_{595} of 0.25 (about 4h). Following three centrifugations and washing steps with a solution containing 0.5M sucrose and 10% glycerol (detailed in NICE expression system protocol), cells were resuspended in a final volume of 4mL of this solution and stored at -80°C. The electroporation was carried out using a Biorad Genepulser and applying the following parameters: 2000 V, 25 µF, 200 Ω and a pulse of 4.5-5 msec. For each sample, 40µl of the cells described above and 250ng of plasmid DNA were used. Immediately after the electroporation, cells were transferred in 960µl of a recovery medium (ESTY Broth + 0.5% glucose + 20 mM $MgCl_2$ + 2 mM $CaCl_2$) and incubated 1h at 30°C. After this incubation step, cells were plated in Agar-ESTY plates with +0.5% glucose and 10µg/mL chloramphenicol and incubated for 48h at 30°C. As for the cloning procedure, colony PCR and plasmid digestion were carried out to verify the presence of the correct plasmids and inserts. These verification steps were performed as described in the *"colony PCR, digestion, purification and sequencing"* paragraph.

3.3. Evaluation of the optimal nisin concentration

The system employed for the expression of the exogenous β-galactosidases relied on a nisin-inducible promoter P_{nisA}. Upon the induction with a sub-inhibitory concentration of nisin, the promoter drives the transcription of downstream genes, leading to the production of the exogenous

β-galactosidase. To maximize the induction efficiency, nisin concentration should be evaluated based on its effect on cell growth and enzyme production.

3.3.1. Effect of nisin concentrations on L.lactis growth

According to MoBiTec documentations and literature [83], a nisin concentration as high as 5ng/mL should not impair *L. lactis* growth. To verify this aspect, we evaluated the growth curve of *L. lactis* in ESTY-broth containing increasing concentrations of nisin. Specifically, *L. lactis* harbouring the pLB141GAL_LB vector was grown overnight at 30°C in ESTY broth containing 0.5% glucose and 10µg/mL chloramphenicol. The day after, 100µl of full-grown cells were inoculated in 10mL of fresh medium and incubated at 30°C until an OD_{595} of about 0.2. Increasing concentrations of nisin, ranging from 0 to 5ng/mL, were then added to the medium and the growth monitored by measuring the OD_{595} every 15 min for 12 hours in a 96-well plate, using the microplate reader Fluostar OPTIMA (BMG Labtech) and a controlled temperature of 30°C. Each condition was assessed in triplicate, and three independent experiments were conducted.

3.3.2. Effect of nisin concentration on enzyme activity

Once establishing the sub-inhibitor range of nisin concentrations, we evaluated the relationship between nisin concentration and intracellular enzyme activity.

3.3.2.1. *L. lactis* permeabilization and X-Gal assay

Before assessing the relationship between nisin concentration and enzyme activity, we evaluated whether a mild protein extraction reagent (78248; B-PER™ Bacterial Protein Extraction Reagent) could promote the internalization of the 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) used as substrate at a final concentration of 80 µg/mL. To this end, *L. lactis* harbouring the pLB141GAL_LB vector was growth as described in the previous chapter. Once the OD_{595} reached 0.2, 0.5ng/mL of nisin were added to the medium and cells incubated for two additional hours at 30°C. Following the incubation, cells were centrifuged. The pellets were weighed and treated with B-PER reagent, 100µg/mL of lysozyme, and EDTA-free protease inhibitors. After a 15min incubation at room temperature, cells were centrifuged and pellets resuspended in an adequate volume of Phosphate Buffered Saline (PBS). The activity of the *L. bulgaricus* β-galactosidase was evaluated in the supernatant and pellet fraction and compared with the control (not treated with B-PER). X-Gal at a final concentration of 80µg/mL was used as a substrate, and enzyme activity measured by monitoring the absorbance at 595nm over time (1h).

3.3.2.2. Determination of the optimal nisin concentration

The optimal concentration of nisin to induce the expression of the exogenous β-galactosidases was determined by exposing the L. lactis harbouring the pLB141GAL_LB vector to increasing nisin concentrations ranging from 0 to 5ng/mL. After a two-hour incubation at 30°C, the enzyme activity was measured in the B-PER treated pellet using the X-Gal as substrate and following the same procedures reported in the previous chapter.

3.4. Kinetics of nisin-induction

To understand the kinetics of nisin-induction, we measured mRNA expression and intra- and extra-cellular enzyme activity at different time points. The induction was performed using the same host and procedures described above with a single dose of 5ng/mL of nisin.

3.4.1. Evaluation of mRNA expression after nisin-induction

To evaluate how nisin-induction could affect the expression of the L. bulgaricus galactosidase over time, we employed quantitative real-time PCR. Since DNA contamination, primers efficiency, and reference genes stability are all factors that could affect the outcome of this analysis, we performed the following preliminary evaluations.

3.4.1.1. RNA extraction and DNA-contamination assessment

RNA was extracted from the induced *L. lactis* harbouring the pLB141GAL_LB vector using the Ribospin II RNA extraction kit (314-103; GeneAll) following the manufacturer's instructions. To promote the lysis of the cell wall, bacteria were incubated at 37°C in a solution of TE-Buffer + 10mg/mL lysozyme for 30min before the extraction. The digestion of DNA contaminants was carried out using the TURBO DNA-free kit (AM1907; Invitrogen) following the "rigorous, two steps, DNA treatment" reported in the user manual. To this extent, 5µg of RNA were incubated with 0.5µl of TURBO DNase™ Enzyme in the appropriate buffer and a final reaction volume of 50µL.

After a 30min incubation at 37°C, an additional 0.5µL of DNAse were added and the mix incubated for a further 30min. RNA was quantified using the NanoDrop™ Lite Spectrophotometer (ThermoFisher Scientific) and the RNA integrity assessed on an agarose gel.

Table 4: *real-time PCR cycling conditions.*

Temperature	Time (min)	Cycles
95 °C	15:00	X 40
95 °C	00:15	
60 °C	00:30	

Plate read		
65 °C	00:30	
65 °C	00:31	
65 °C +0.3 °C/cycle Ramp. 0.3°C/sec	00:10	X 100
Plate read		

To assess the degree of DNA contamination, 500ng of RNA were reverse-transcribed using the iScript™ cDNA Synthesis Kit (170-8891; BIO-RAD) with or without (negative control) the addition of the iScript Reverse Transcriptase to the reaction mix. The cDNA obtained as described was used as a template for quantitative real-time PCR. Real-time PCR was carried out using the HOT FIREPol® EvaGreen ® qPCR Mix Plus (08-25-00020; Solis BioDyne) and 1ng of cDNA as a template. For this step, primer pairs specific for the *L. lactis* GTPase *Era* gene and the *L. bulgaricus LacZ* were used to evaluate the degree of genomic and plasmid DNA contamination. Primer sequences and cycling conditions are reported in **Table 5 and 4**.

3.4.1.2. Determination of primer efficiency

To evaluate the primer efficiency, real-time PCRs were performed using each of the selected primer pairs **(Table 5)** and increasing, ten-fold, dilutions of the cDNA (ranging from 1:10 to 1:100000) obtained from the nisin-induced *L. lactis* harbouring the pLB141GAL_LB vector. Nisin-induction, RNA extraction and retro-transcription were carried out as reported in the previous chapters. Primer efficiency was calculated from the slope of the log-linear portion of the calibration curve as follow:

$$Efficiency = 10^{(-1/slope)-1)*100}$$

The specificity of each primer pair was also estimated from the melting profile of the reaction.

Table 5: *primers employed in for the real-time PCR experiments. Table shows the target gene along with the sequence of the forward and reverse primer and the expected amplicon length for each reaction.*

Target	Forward primer	Reverse primer	Amplicon length (bp)
GyrA	*TTGCTGACTTGACGGATA*	*GTAAGAACCTCACCAACTAAC*	130
GyrB	*ACAACTACGCCAAACAAAAT*	*TCAATCCTTCACGCACAT*	78
Adk	*ATGCTCTTGATACAATGCT*	*TTTTGTGATAAGTTGCTCC*	138
DnaG	*TTATGGCTAACTCAGGCTT*	*CGTATTCATTGGTAATTGGA*	93
Era	*TACTTTGCGTGAATGTGAT*	*TGACTGGAACTTCTGCTTT*	108
FtsZ	*CGATCTACTCACCACTTCTT*	*ATATCCATTCCACCTGTCA*	79

RecA	*GATGCTCTTGCTCTGATT*	*AACCTGAACTTACAACTGAT*	97
RpoA	*ACTGATAGCGACATTGAA*	*GAACATAGCCGTATCCTT*	109
RpoB	*TGAGTATGTTGACGGTAATGA*	*TCCTGAGTTAGAACGACGAT*	94
RpoC	*CCAGTTATTCCACCAGATT*	*CAAGTCGTTCAAGTCAGA*	78
RpoD	*TGCTCAAGAACCAGTATCA*	*GTGTCCATCACTTCATCTAAC*	150
Gmk	*CAGACATTGGATGAAGGA*	*CTGGTGGAGTCAAGAAGA*	109
SecA	*TCAACAAGGAGCAGTAACG*	*CCAATAACAGCAAGTCCAC*	117
LacZ	*GGCTGAAGCAGAAGAAGTA*	*TGACCTTGGAGAAGAGAAT*	129

3.4.1.3. Reference genes selection

The last critical step for setting up the optimal conditions for evaluating the nisin-induced mRNA expression was the reference genes selection. To obtain an accurate normalization, we aimed to select at least three reference genes. We started from an initial list of 12 potential targets from which 4 (*Ftsz*, *DnaG*, *RecA*, *RpoB*) were excluded as the primer efficiency was outside the optimal range. Among the remaining genes, 6 (*Adk*, *GmK*, *GyrB*, *Era*, *SecA*, *RpoD*) were evaluated for their stability regardless of the experimental conditions (cell culturing time and nisin-induction). The remaining two genes, *GyrA* and *RpoC*, were excluded as they shared the same cellular pathway with *GyrB* and *RpoD,* respectively. The selection of the three most stable genes was carried out with geNorm following the guidelines reported by [104].

Briefly, *L. lactis* harbouring the pLB141GAL_LB vector induced with 5ng/mL of nisin, or without induction, was growth for 4 hours at 30°C. At time 0 and after each hour (T0-T4), RNA was extracted and retro-transcribed as described in the previous chapters. Real-time PCR was performed for each sample using the 1ng of cDNA and the same conditions previously reported. For each gene, quantitation cycles (Cq) were used to determine the pairwise variation with all the other reference genes. The stepwise exclusion of the gene with the lowest stability led to selecting *Era*, *SecA* and *RpoD* as the most stable reference genes.

3.4.1.4. Effect of nisin-induction on mRNA expression

To evaluate how nisin-induction affect the expression of P_{nisA}-regulated genes, we induced the *L. lactis* carrying the pLB141GAL_LB with 5ng/mL of nisin, as previously described. RNA was extracted at time 0 post-induction and every hour for 4 hours (T0-T4). Real-time PCR was carried out to evaluate the relative expression of *LacZ* as previously described, using 1ng of cDNA and *Era*, *SecA*, and *RpoD* as reference genes. The relative expression was calculated by comparing each time point with T0 and using the $2^{\Delta\Delta Ct}$ method.

3.4.2. Evaluation of β-galactosidase intra- and extracellular activity following nisin-induction

To evaluate the effect of nisin on the intracellular and extracellular enzyme activity over time, we induced the *L. lactis* as described above. Immediately after the induction and each hour for 7 hours, the OD_{595} was measured for cellular density estimation and samples collected as follow: an adequate volume of cell culture (ranging from 10 mL at T0 to 3 mL at T7) was centrifuged for 10min at 600g and supernatant and pellet fractions recovered. The pellet was treated with B-PER reagent as described in the *"L. lactis permeabilization and X-Gal assay"* and resuspended in an adequate volume of culture medium (equal to the centrifuged volume). The supernatant was filtered with a 0.22µm PES filter to remove any residue of bacterial cells. The permeabilized pellets and the filtered supernatants were used in the X-Gal assay or store at -80°C. The activity was estimated from the OD_{595} measurement as previously described. The relative activity was calculated as the ratio between the OD_{595} at T1 (intracellular fraction) or T5 (supernatant fraction) and all the other time points normalized by the cellular density.

3.5. Secretion efficiency evaluation

To estimate the percentage of $Exp4_{SP}$-mediated enzyme secretion, we compare the enzyme activity detectable in the supernatant (intracellular) with that in the intracellular compartment or the whole cell culture (supernatant + intracellular; total fraction). To this end, we employed the *L. lactis* carrying either pLB141GAL_BL, pLB141GAL_KL or pLB141GAL_LB, induced with 5ng/mL on nisin. Five hours post induction, supernatants and pellets were collected and treated as described in the previous chapter and enzyme activity estimated using the X-Gal assay. The relative activity was expressed as a ratio between the OD_{595} of the intracellular or extracellular compartment and the OD_{595} of the total fraction. While this method gave us an estimation of the extracellular enzyme activity, it did not allow a precise assessment of the secretion efficiency. Indeed, several factors could affect the activity in the extracellular environment.

3.6. Effect of pH and temperature on enzyme activity

To be employed as a treatment for LI, the selected β-galactosidases must retain good activity within the human gastrointestinal tract. Among the factor that can potentially affect enzyme activity are pH and temperature. As such, we decided to evaluate the enzyme activity at pH between 2 and 8 and temperatures comprised between 27 and 45°C. To assess the pH effects on enzyme activity, we employed *L. lactis* carrying either one of the three β-galactosidase expressing vectors. The activity was measured as described in the previous chapters from the intracellular or the supernatant fraction two or five hours post-induction, respectively. Before measuring the activity, the pH was

adjusted to the desired level (0.5 points increment, ranging from 2 to 8). The relative enzyme activity was then measured using the X-Gal as substrate and a controlled temperature of 37°C. A similar procedure was followed to evaluate the effect of temperature. In this case, the activity was measured at different temperatures (27, 32, 37, 42 and 45°C) while keeping a constant pH7.

To evaluate whether the *L. bulgaricus* galactosidase could resist the transit through the stomach, we measure the enzyme stability at pH3. To this end, the enzyme was incubated for 0, 30, 60 and 90 minutes at pH3 and 37°C. After the incubation, the pH was raised to 7 and the activity measured and compared with that of the control (enzyme kept at pH7 and 37°C).

3.6.1. Combination of L. bulgaricus and K. lactis galactosidases

To evaluate whether combining the galactosidases of *L. bulgaricus* and *K. lactis* could improve the overall activity, we combined the two enzymes in a 1:1 ratio (based on cellular density). We then measured the activity of the intracellular fraction over a wide range of pH. To this end, the cellular density of *L. lactis* cell cultures, carrying either the pLB141GAL_KL or pLB141GAL_LB vector, was measured two hours post-induction. Cell cultures were mixed in a 1:1 ratio, and the OD_{595} measured again. An aliquot of the mixed and individual cultures was centrifuged, and the pellets recovered, treated with the B-PER reagent, and resuspended in an adequate volume of PBS. The pH was adjusted to the desired level, and the activity evaluated using X-Gal as substrate, normalized by cellular density and expressed as relative activity.

3.7. L. lactis in simulated gastrointestinal fluids

3.7.1. Survival of L. lactis in simulated gastrointestinal fluids

The aim of this study was the construction of an expression system to employ for the *L. lactis*-mediated delivery of exogenous β-galactosidases into the human small intestine. As such, a percentage of *L. lactis* has to survive the transit through the gastric compartment and reach the small intestine, where the bacteria have to release or secrete the β-galactosidases. A study conducted by Drouault et al. suggested that about 90-98% of *L. lactis* could survive the transit through the stomach and reach the small intestine when force-fed to mice [105]. Conversely, only 10-30% survived in the duodenum. Interestingly, the same study suggested that dead bacteria underwent cell lysis, thus releasing their intracellular components in the intestinal lumen. To verify this aspect, we incubated *L. lactis* in simulated gastric (SGF) and intestinal (SIF) fluids. For the preparation of SGF and SIF, we followed the protocol reported by Brodkorb et al. [106]. Electrolytes solutions were prepared as illustrated in **Table 6**.

Table 6: *composition of the electrolyte solutions for the preparation of simulated gastric (SGF) and intestinal (SIF) fluids.*

Salt Solution	Final concentration in SGF (pH3) (mM)	Final concentration in SIF (pH7) (mM)
KCl	6.9	6.8
KH_2PO_4	0.9	0.8
$NaHCO_3$	25	85
NaCl	47.2	38.4
$MgCl_2(H_2O)_6$	0.12	0.33
$(NH_4)_2CO_3$	0.5	-
HCl	15.6	8.4

Fresh SGF and SIF were prepared the day of the experiment as illustrated in **Table 7**.

Table 7: *composition of simulated gastric (SGF) and intestinal (SIF) fluids for 5g/mL of food.*

	SGF (pH3)	SIF (pH7)
PBS (mL)	10	20
Electrolyte solution	8	8
CaCl2(H2O)2	0.005	0.04
Enzymes	Pepsine - Gastric lipase	Trypsin in pancreatine - Bile salts
Enzyme activity/concentration	2000 U/mL – 60 U/mL	100 U/mL – 10mM
H2O	0.448	3.16

To assess the effect of SGF and SIF on bacteria survival and growth we proceeded as it follows: *L. lactis* carrying pLB141GAL_LB was growth at 30°C overnight in ESTY broth + 0.5% glucose + 10µg/mL chloramphenicol. The following day, the full-grown cultures were diluted in the same medium and incubated at 30°C until they reached an OD_{595} of 0.2. *L. lactis* cultures were then induced with 5ng/mL nisin and incubated for 2 additional hours at 30°C. After the incubation, four different samples were prepared as illustrated in **Table 8**. The pH was adjusted to 3 or 7, and the solutions incubated at 37°C for 2 hours.

Table 8: *samples employed to assess the effect of simulated gastric (SGF) and intestinal (SIF) fluids on L. lactis survival and growth.*

	SGF-control (pH3)	SGF	SIF-control (pH7)	SIF
PBS	5.5 mL	0.5 mL	15.5 mL	5.5 mL
Milk	2.5 mL	2.5 mL	2.5 mL	2.5 mL
Bacterial culture	2 mL	2 mL	2 mL	2 mL
SGF	-	5 mL	-	-

SIF	-	-	-	10 mL

After the incubation, the bacteria were plated at different concentrations on Agar-ESTY plates containing 0.5% glucose and 10µg/mL chloramphenicol to evaluate the survival rate. The plates were incubated two days at 30°C before colony counting.

3.7.2. Release and activity of L. bulgaricus β-galactosidase in simulated intestinal fluids.

To evaluate the hypothesis that the SIF-mediated lysis of *L. lactis* could promote the extracellular release of the heterologous β-galactosidase, we proceeded as follow.

L. lactis carrying the pLB141GAL_LB vector was inoculated in 10 mL of ESTY broth + 0.5% glucose + 10 µg/mL chloramphenicol and grown overnight at 30°C. Full-grown cells were then diluted 1:20 in the same medium and incubated at 30°C until an OD_{595} of 0.2-0.3 (about one hour). Once the optimal OD has been reached, cells were incubated for two additional hours at 30°C with 5 ng/mL of nisin. Induced cells were diluted 1:1 in either SIF or PBS, and the β-galactosidase activity measured at time zero and every 30 minutes for two hours, as previously described.

3.7.3. Effect of simulated intestinal fluids on enzyme activity and stability

Since the results of the experiments described in the previous chapter could be affected by the release rate of the β-galactosidase within the extracellular compartment and did not directly reflect the effect of SIF on enzyme activity and stability, we carried out additional tests to evaluate this aspect. The *LacZ* expression was induced with 5ng/mL of nisin, and the intracellular fraction recovered as previously described. The enzyme was then incubated at 37°C and pH7 for one hour in a 1:1 ratio with either SIF or PBS. To assess the effect of SIF on enzyme stability, an aliquot of each sample was taken at time zero and every 30 minutes for two hours. The aliquots were diluted in a 1:100 ratio with PBS, then X-Gas at a final concentration of 80 µg/mL was added and the activity of the treated sample (enzyme + SIF) measured, and compared with those of the control (enzyme + PBS). To evaluate the effect of SIF on enzyme activity, enzyme was diluted in reaction mixes containing PBS and increasing concentration of SIF (ranging from 0% to 20%). X-Gal at a final concentration of 80 µg/mL was then added on each sample, and enzyme activity measured as previously described.

3.8. Statistical analyses

All the experiments were carried out in triplicates. When needed, data distribution was assessed using the Kolmogorov-Smirnov test before carrying out the statistical analyses.

The effect of the B-PER reagent on cell permeabilization and the effect of gastric and intestinal fluids on cell survival was assessed with a t-test analysis. For the real-time experiments, the relative expression of each gene was calculated using the $2^{\Delta\Delta Ct}$ method. For the remaining experiments, statistical differences were assessed with the analysis of variance (ANOVA) and Tukey's multiple comparison test.

4. Results

4.1. Literature researches leaded to the selection of three suitable β-galactosidases

For constructing expression plasmids that adhered to the self-cloning principles, we select only galactosidases from GRAS microorganisms. Additionally, we carried out literature researches to identify those galactosidases reported to perform well at 37°C and within the pH range typical of the human intestine (6.5-7.5) [107], [108]. These searches resulted in the selection of the three β-galactosidases reported below and illustrated in **Table 9**.

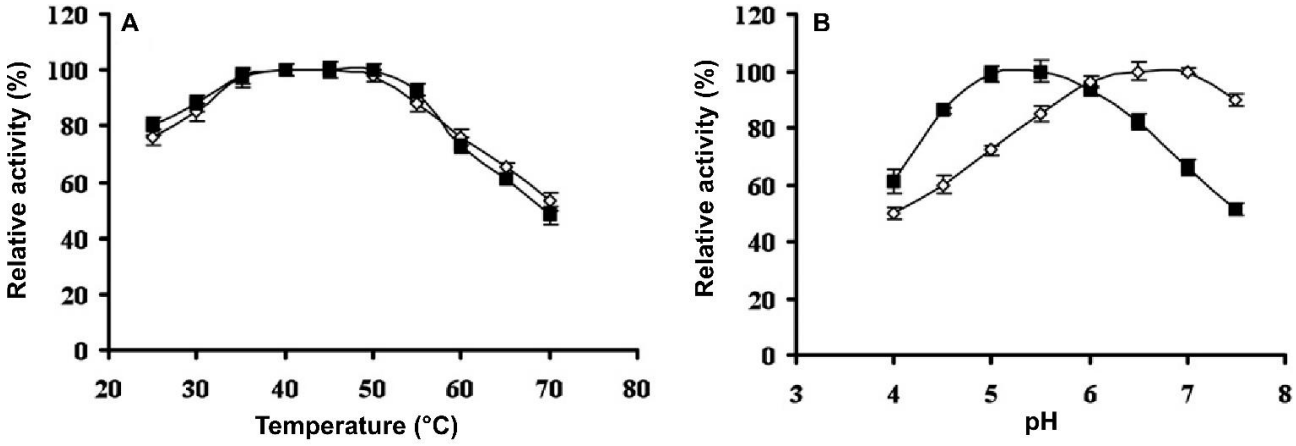
Table 9. *Table summarizing the characteristics of the main galactosidases examined during the selection process.*

Source	Optimal pH	Optimal temperature	Substrate	Study
Bacillus circulans	5-6	60°C	Galβ-pNP	[109]
	6	44°C	oNPG	[110]
	4	74°C		
	4	60°C		
	6	60/45°C	oNPG/Lactose	[111]
	6	60°C		
Bacillus licheniformis	6.5	50°C	oNPG/Lactose	[112]
Bacillus stearothermophilus	7	70°C	oNPG	[113]
Bacillus subtilis	8	35°C	oNPG	[114]
	7	55°C	oNPG	[115]
Kluyveromyces lactis	7	40°C	Lactose	[116]
	6.5	50°C	oNPG	[117]
	7	37/40°C	oNPG/Lactose	[118]
Lactobacillus delbrueckii bulgaricus	5-5.5	35-50°C	oNPG	[119]

4.1.1. Lactobacillus delbrueckii bulgaricus

Lactobacillus delbrueckii bulgaricus is a homofermentative lactic acid bacterium employed for yoghurt and cheese production. *L. bulgaricus* is a safe microorganism and, on the basis of its use as ingredient in yoghurt and cheese, it is considered a GRAS organism by FDA (GRAS Notice GRN No. 378, Food and Drug Administration, 2012). The *lacZ gene* of *L. bulgaricus* encodes for an enzyme showing a β-galactosidase activity, which properties make it ideal for our purposes.

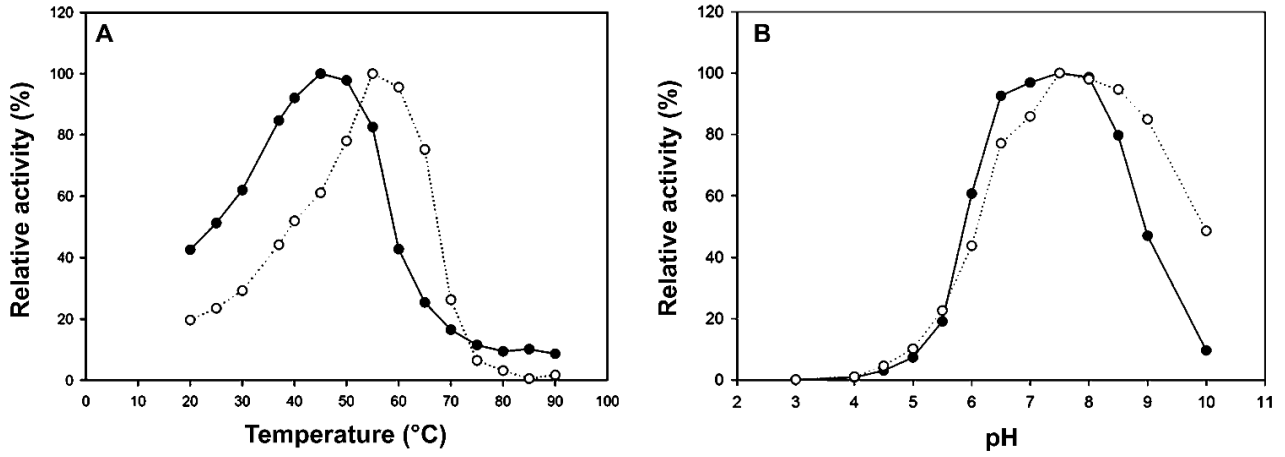
A study conducted by Rhimi et al. suggested that the enzyme had a full activity at pH between 5 and 5.5 and at a temperature between 35°C and 50°C. Interestingly, the enzyme performed well within a wide range of pHs (from 4 to 7.5) and temperatures (from 25°C to 60°C) [119] (**Figure 13A**-**13B**). Similar results were obtained by Nyguen et al. in respect to the activity measured at different



Rhimi et al. / Research in Microbiology (2009) 775-784

Figure 13: *Temperature* ***(A)*** *and pH* ***(B)*** *activity profiles of the purified wild-type (■) and mutated (◊) β-galactosidases of L. delbrueckii bulgaricus. Activities at optimal pH and temperature were defined as 100%. Error bars represent the standard deviation from three separate experiments* ***[Rhimi et al., 2009]****.*

temperatures, with the activity peaking between 45°C and 60°C, depending on the substrate [120]. Conversely, the authors reported an optimal pH of 7.5, with the activity dropping down at pHs below 6 **(Figure 14A-14B)**. Despite the differences between the two studies, since the enzyme showed



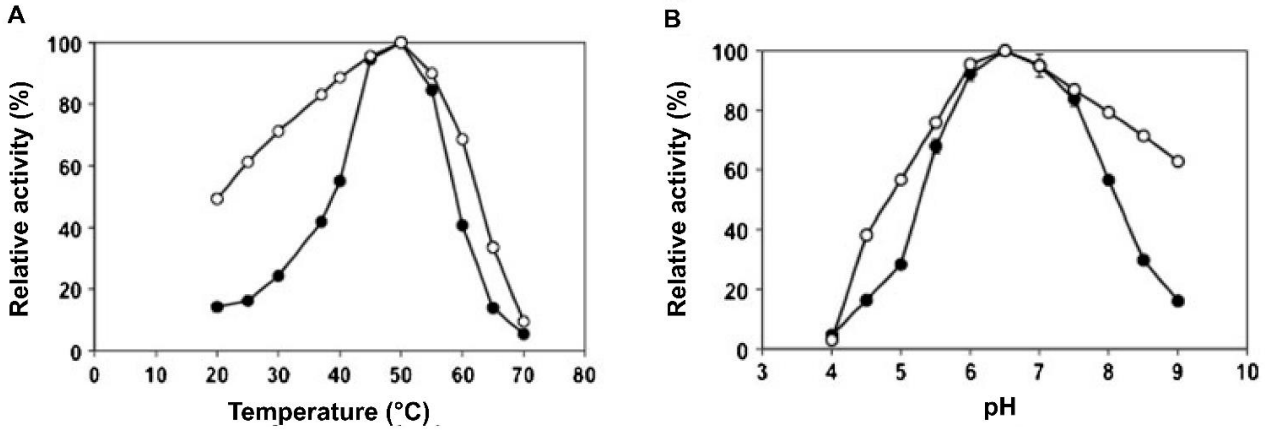
Adapted from Nguyen et al. / J. Agric. Food Chem. (2012) 1713-1721

Figure 14: *Temperature* ***(A)*** *and pH* ***(B)*** *optima of the activity of recombinant β-galactosidase from L. bulgaricus: (○) lactose as substrate; (●) oNPG as substrate. Relative activities are given in comparison with the maximum activities measured under optimal conditions (100)* ***[Nyguen et al., 2012]****.*

good performances at 37°C and between the range of pHs typical of the small intestine, we considered it a promising candidate for our study.

4.1.2. Bacillus licheniformis

Bacillus licheniformis is another GRAS organism widely employed for industrial processes such as proteases, amylases, and antibiotics production [121]. Additionally, recent studies analyzed its potential for probiotic-based applications. The *lacA* (or *GanA*) gene of *B. licheniformis* DSM13 encodes for a homodimeric β-galactosidase. This enzyme is stable and works well at temperatures between 45°C and 55°C, making it suitable for the industrial processes that require lactose



Adapted from Juajun et al. / Appl Microbiol Biotechnol (2011)645–654

Figure 15: *Effect of the temperature* ***(A)*** *and pH* ***(B)*** *on the activity of β-galactosidase (lacA) from B. licheniformis using either oNPG (filled circle) or lactose (empty circle) as substrates* ***[Juajun et al., 2011]****.*

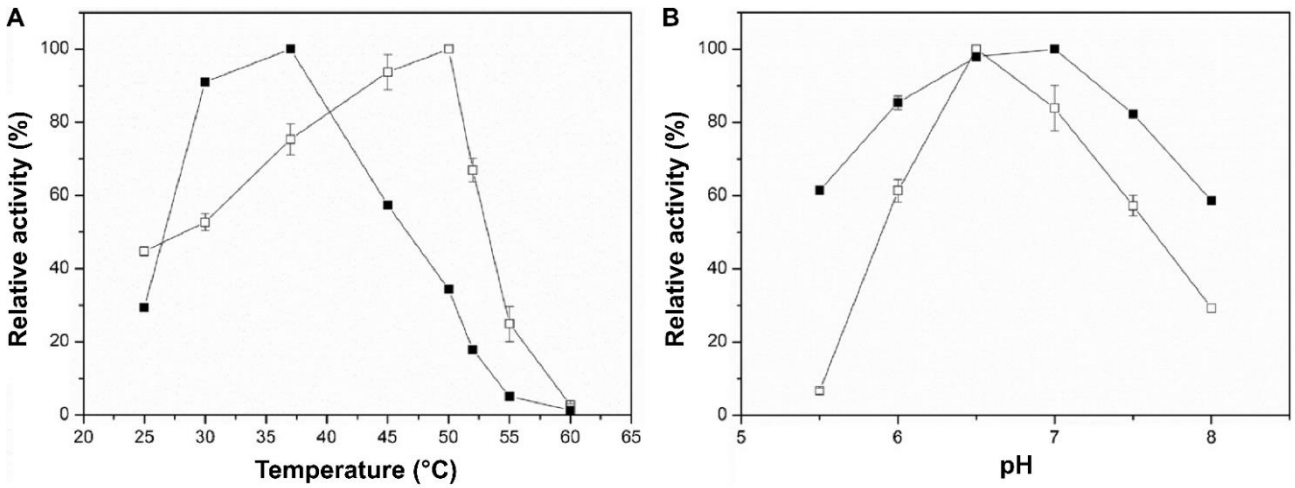
hydrolysis. While the galactosidase activity peak at 50°C, the enzyme retains a good activity (about 80%) also at 37°C [112] **(Figure 15A)**. Moreover, Juajun et al. reported an optimal pH value of 6.5 and a good activity between pH5 and pH9 [112] **(Figure 15B)**, making this galactosidase potentially interesting for our purposes.

4.1.3. Kluyveromyces lactis

The last enzyme we chose for our project came from the yeast *Kluyveromyces lactis.* While phylogenetically distant from *L. lactis*, *K. lactis* hold the GRAS status. Moreover, the β-galactosidase encoded by the *LAC4* gene is safe and often added to milk and other dairy products without any safety concerns. Thus, the expression of *LAC4* in *L. lactis* should adhere to the "self-cloning" principles. The employment of this enzyme in the production of galactooligosaccharides [122], prompted many studies aimed to elucidate its biochemical properties. Among these, some evaluated the enzymatic performances at different pHs and temperatures.

Lima et al. reported an optimal pH of 6.5, and a good activity between 6 and 7.5, a range overlapping those typical of the gastrointestinal tract **(Figure 16B)**. Conversely, the optimal reported temperature was 50°C, with the activity drastically dropping down at 52.5-55°C.

While these temperatures were significantly higher than the 37°C of the human gastrointestinal tract, the enzyme showed good performances even at 37°C, retaining about 75% of the maximum activity [117] **(Figure 16A)**.



Adapted from Lima et al. / Process Biochemistry 48 (2013) 443–452

Figure 16: *Temperature effects on the hydrolytic activity of soluble (□) and immobilized (■) β-gal from K. lactis (LAC4) when hydrolysing a 1.25 mM ONPG solution in 50 mM of potassium phosphate buffer supplemented with 0.1 mM MnCl2 at pH 6.6* ***(A)****. pH effects on the on hydrolytic activity of soluble (□) and immobilized (■) β-gal at 37 °C when hydrolysing a 1.25 mM ONPG solution in 50 mM of potassium phosphate buffer supplemented with 0.1 mM MnCl2 at pH 6.6* ***(B)*** ***[Lima et al., 2013]****.*

These results were consistent with those obtained by other groups [116], [118], making this enzyme another promising candidate for our work. In conclusion, all these three selected enzymes could perform potentially well throughout the human gastrointestinal tract as, overall, they maintain their activity within a wide range of pHs (pH 4 - pH 9).

4.2. Cloning of the three galactosidases into the pLB141

For the expression and secretion of the selected β-galactosidases, we employed the pLB141 vector described in the "material and methods" section. We cloned *LacZ*, *GanA*, or *LAC4* gene downstream and in-frame with the signal peptide Exp4, under the control of the nisin-inducible promoter P_{nisA}. To verify the cloning outcome, we perform a colony PCR using primers flanking the region of interest. Each of the screened colony carryed the insert of interest as expected. **Figure 17** shows the outcome of the colony PCR.

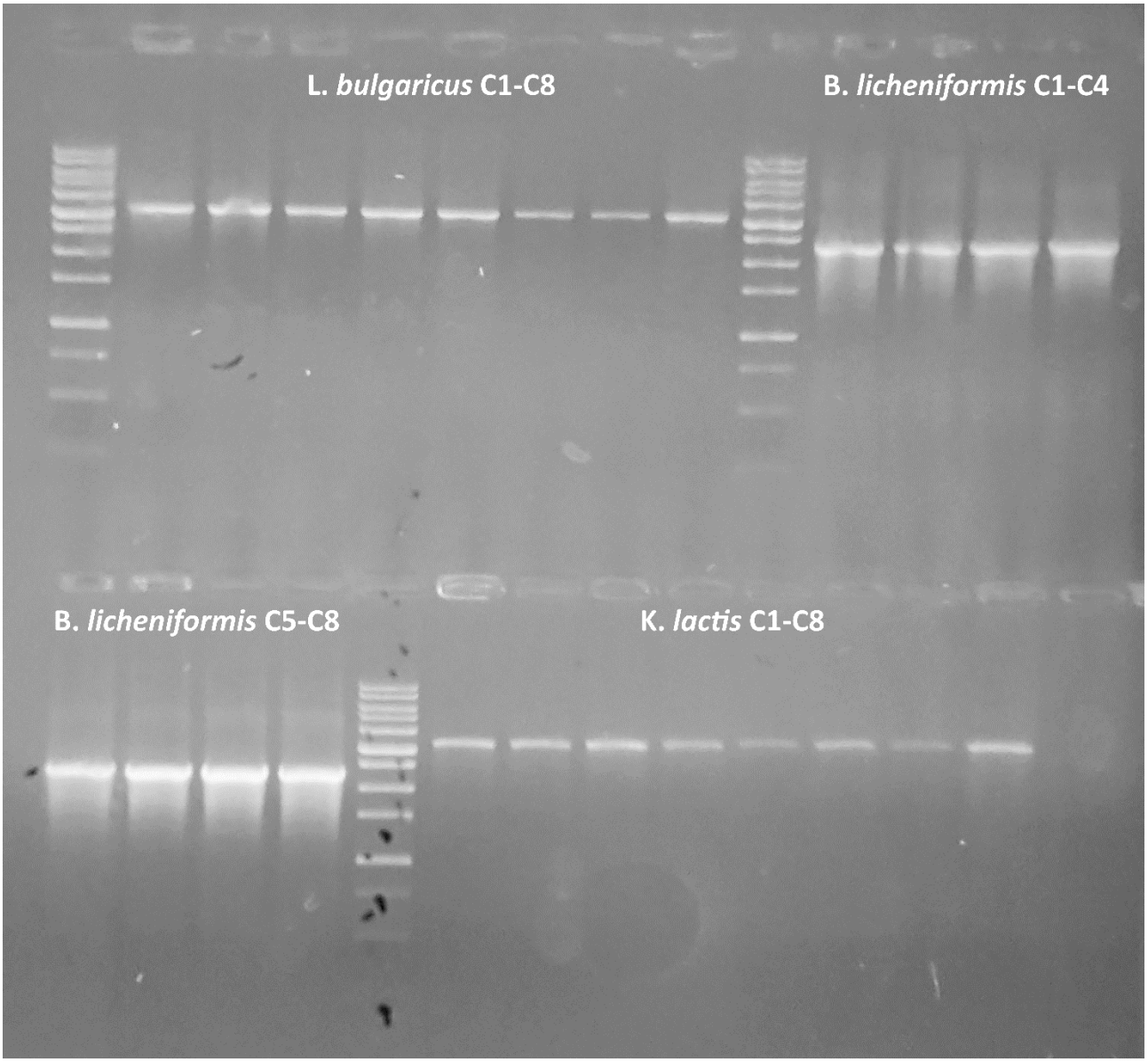


Figure 17: *Verification of the cloning outcome through colony PCR. The image shows the results of the agarose-gel (0.8%) run of the PCR products. The expected amplicon length for L. bulgaricus and K. lactis genes was about 3kB. The expected amplicon length for B. licheniformis was about 2kB. All the screened colonies were positive for the desired insert.*

Additionally, we further corroborated these observations and verified the nucleotide sequence of the cloning target region of the plasmids by Sanger sequencing. **Figure 18** shows the resulting chromatograms limited to the vector-insert junctions, including vector backbone (grey), restriction sites (dark grey) and the 3' and 5' ends of each insert, validating the cloning results. Moreover, by analyzing the whole cloned region, we confirmed the fidelity of the three inserted genes.

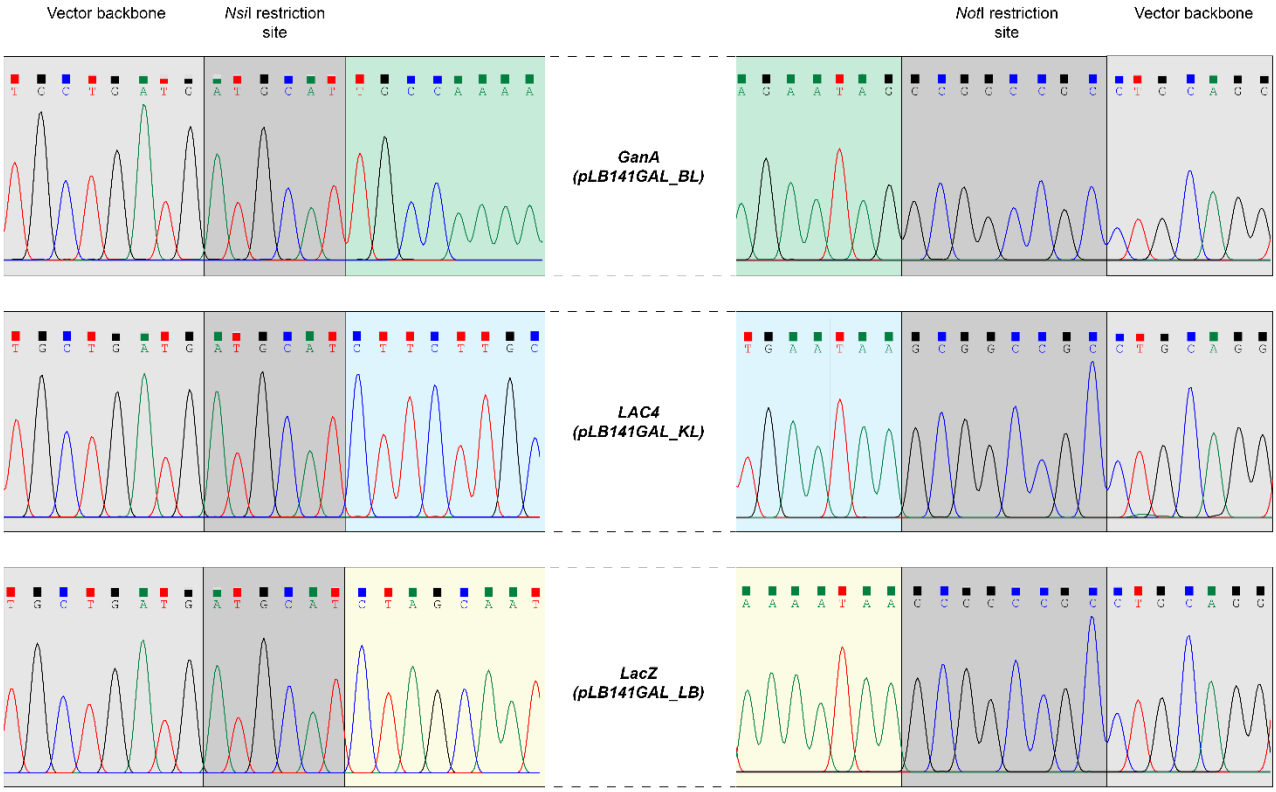


Figure 18: *Image showing the results of the vector-insert junctions sequencing of the plasmids bearing the coding sequence of the β-galactosidases from Bacillus licheniformis (pLB141GAL_BL), Kluyveromyces lactis (pLB141GAL_KL), and Lactobacillus delbrueckii bulgaricus (pLB141GAL_LB).*

4.3. Increasing nisin concentrations enhanced LacZ transcription and enzyme activity without affecting cell growth

Previous studies reported that 0.1 to 5 ng/mL of nisin could efficiently activate the P_{nisA} promoter without inhibiting bacterial growth [83]. To evaluate this aspect, we used either 2, 3.5, or 5ng/mL to induce the *LacZ* expression in *L. lactis* transformed with pLB141GAL_LB. Two hours post-induction, we measured the enzyme activity as described in the material and method section. Additionally, we evaluated whether the employment of a mild extraction reagent could promote cell permeabilization, thus allowing the assessment of the intracellular β-galactosidase activity. Compared with cells treated with the B-PER reagent, untreated cells showed only a 9.92% ± 0.32 of enzyme activity (p-value < 0.0001) **(Figure 19A)**. These findings suggested the importance of B-PER

in allowing the inflow of X-Gal into the intracellular compartment. As expected, the incubation in nisin up to 5ng/mL did not significantly impair cell growth, as we did not observe any significant difference in the OD_{595} between the control and any of the treatments **(Figure 19B)**. Concerning the effect of nisin on enzyme activity, we observed the highest activity using 5ng/mL (100% ± 2.68), which significantly decreased with 3.5ng/mL (72.42% ± 0.996, p-value=0.0008). The activity further dropped to 51.3% ± 1.92 (p-value=0.013) when we employed 2ng/mL and was almost undetectable without nisin induction (4.02% ± 1.01) **(Figure 19C)**. Following these results, we chose a final concentration of 5ng/mL as this dosage did not hamper the cell growth and resulted in the highest enzyme activity.

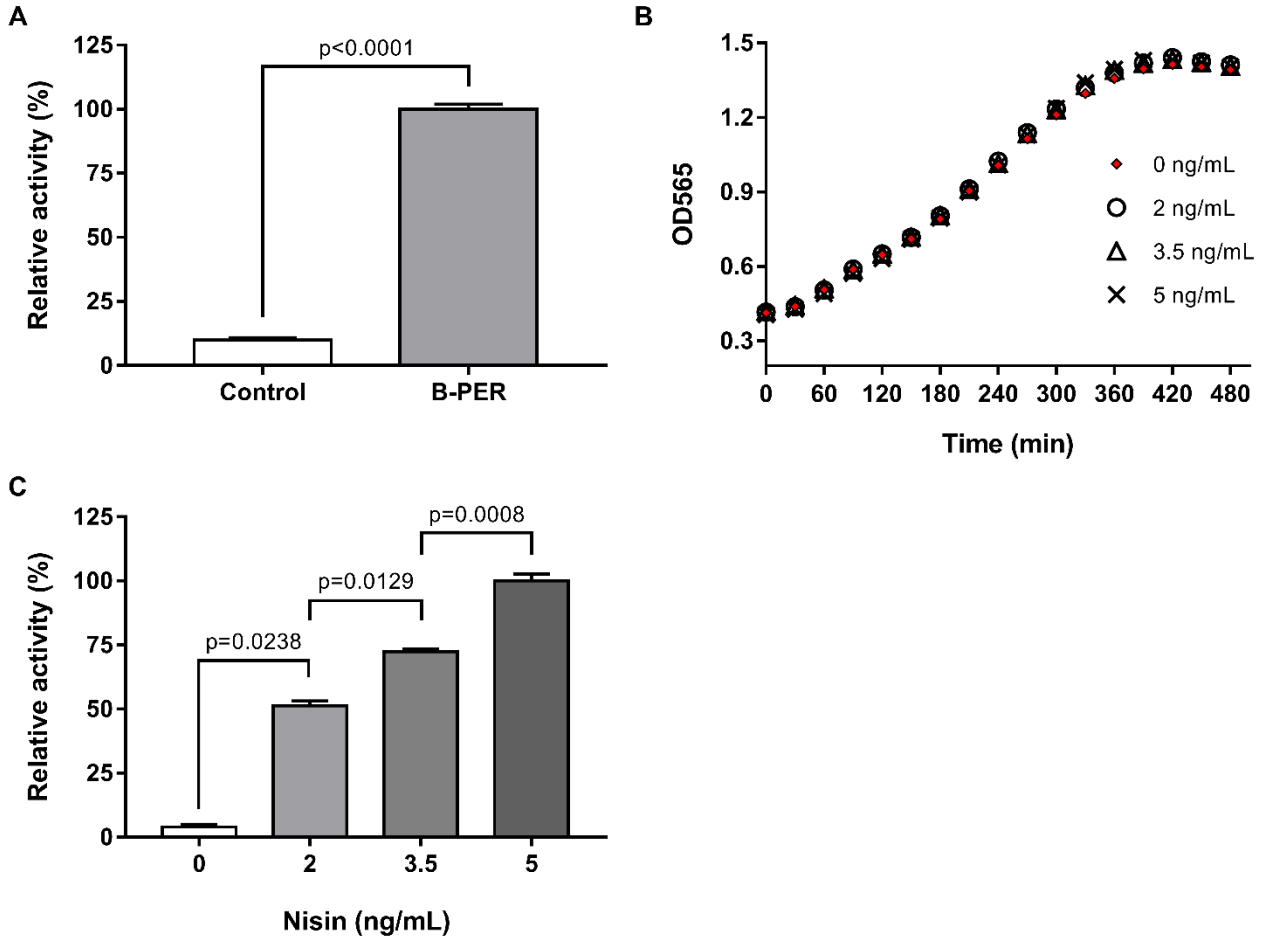


***Figure 19**: **(A)** Bar chart showing the effect of B-PER reagent on enzyme activity detectable in the permeabilized bacteria. Control represent the untreated bacteria. Columns report the enzyme activity relative to the B-PER treated cells. Error bars represent the standard error of the three experimental replicates. **(B)** Graph showing the effect of different nisin concentrations on cell growth. Different symbols represent different nisin concentration. The OD_{595} was monitored over time until bacteria reached the stationary phase. No significant difference was observed between the treatments and the control (0 ng/mL nisin). **(C)** Bar chart reporting the effect of different concentration of nisin on enzyme activity, along with the p-values of the difference and the standard errors. The activity was expressed as relative to the activity of the optimal condition.*

4.4. Kinetics of nisin induction

Once determined the optimal nisin concentration, we evaluated the induction kinetics by measuring the mRNA expression, and the intracellular and extracellular enzyme activity.

4.4.1. Preliminary tests for qPCR

The evaluation of mRNA expression required some preliminary steps: **(i)** the assessment of the degree of DNA contamination, **(ii)** the determination of primer efficiency and **(iii)** the choice of the appropriate reference genes. The "materials and methods" section reports the detailed procedures followed to evaluate these aspects.

To estimate the degree of DNA contamination, we carried out a qPCR comparing the Cq values of a positive (cDNA, reverse transcription) and a negative (RNA, no-reverse transcription) control. To this extent, we used either primers specific for the reference gene *Era* or the *LacZ* gene of *L. delbrueckii bulgaricus*. For the reference gene *Era,* the average Cq was 31,07 ± 0.24 and >40 for the positive and negative control respectively **(Figure 20A)**. Similarly, the average Cq value for *LacZ* was 17.32 ± 0.13 (positive control) and 36.78 ± 1.29 (negative control) **(Figure 20B)**.

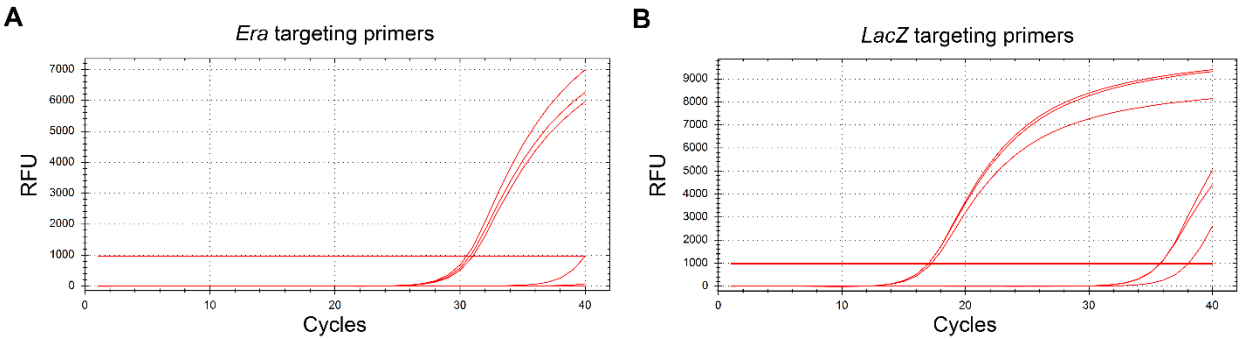


Figure 20: *real-time amplification curves using either Era-specific* ***(A)*** *or LacZ-specific* ***(B)*** *primers and cDNA of RNA (contamination control) as a template. The average Cq >40 and = 36.78 for Era and LacZ controls suggest only a minimal degree of DNA contamination in the RNA samples.*

Once established the degree of DNA contamination, we evaluated the efficiency and specificity of our primers. We calculated the efficiency of each primer-pairs from the slope of the log-linear portion of the calibration curve. As shown in **Table 10**, except for the primer-pairs targeting *Ftsz, DnaG, RecA and RpoB,* all the other primers worked well, with an efficiency ranging from about 95% to 110%. Moreover, the melting profiles suggested a high degree of specificity for each primer-pairs (data not shown). **Figure 21** reported the melting curves for the primers employed in the kinetics experiments: **(A)** *SecA*, **(B)** *Era*, **(C)** *RpoD* and **(D)** *LacZ.*

Table 10: *Table showing the primer efficiency for each target genes, along with R^2, slope and standard error of the slope for the calibration curve.*

Target	R^2	Slope	SE of the Slope	Efficiency %
GyrB	0.996	-3.259	0.119	102.694
GyrA	0.998	-3.237	0.007	103.670
Era	0.999	-3.257	0.061	102.783
Ftsz	0.997	-2.910	0.067	120.619
SecA	0.988	-3.129	0.198	108.733
AdK	0.990	-3.430	0.194	95.679
DnaG	0.851	-2.801	0.676	127.518
RecA	0.998	-3.042	0.066	113.172
GmK	0.997	-3.239	0.107	103.581
RpoB	0.998	-3.076	0.074	111.396
RpoC	0.992	-3.131	0.162	108.635
RpoD	0.990	-3.439	0.197	95.336
LacZ	0.999	-3.424	0.054	95.910

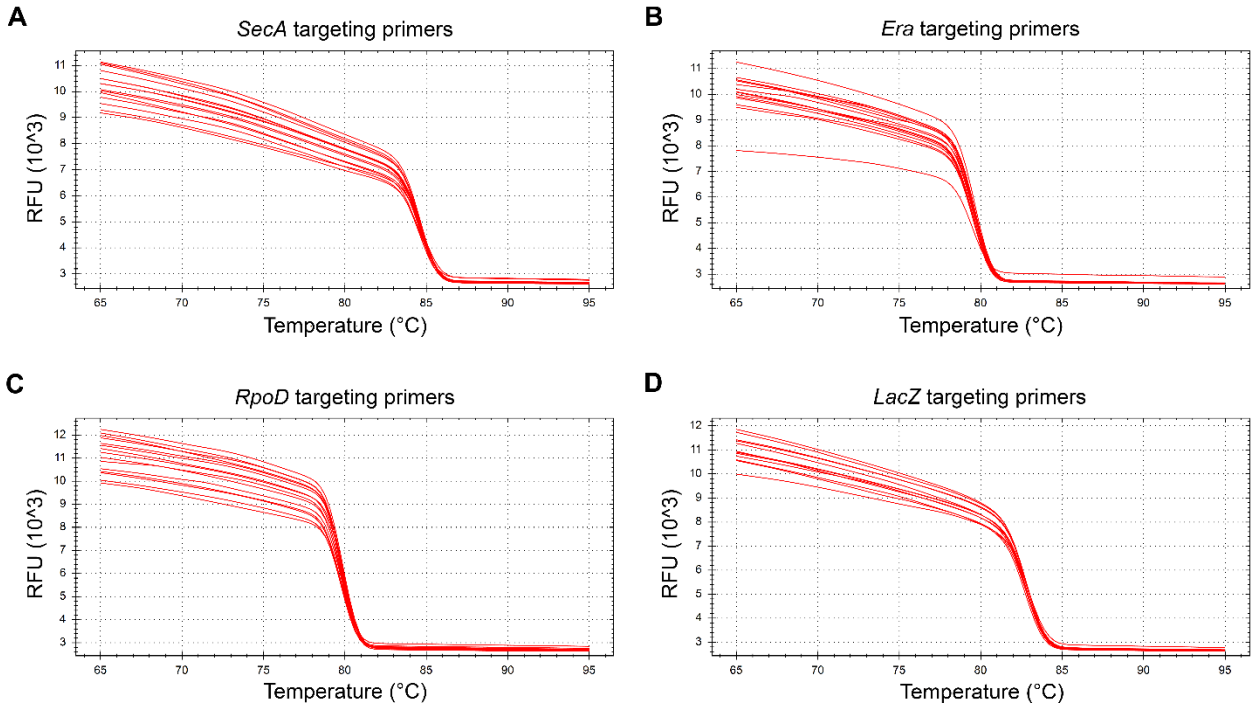


Figure 21: *Melting curves showing the specificity of the primer pairs targeting SecA* ***(A)****, Era* ***(B)****, RpoD* ***(C)****, and LacZ* ***(D)****.*

Another aspect to consider was the stability of the reference genes. To achieve a reliable relative quantification of mRNA expression, we aimed to select three genes to be used as a reference. Starting from an initial pool of 12 genes, we employed geNorm to test their stability under the

experimental conditions. As shown in **Figure 22**, the geNorm analyses led to the selection of *Era, SecA* and *RpoD* as the most stable among the candidate reference genes, showing relative stability (M) of 0.277, 0.264 and 0.249 respectively.

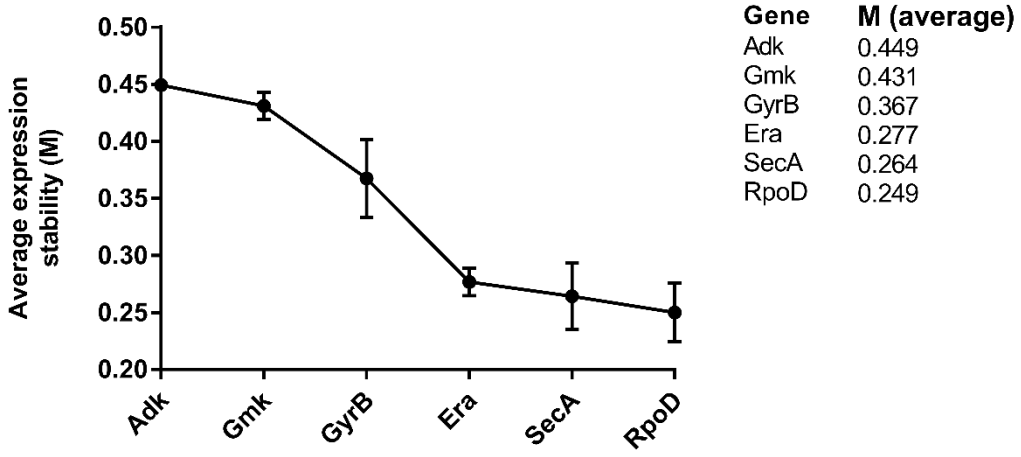


Figure 22: *Graph showing the average stability values (M) of the six housekeeping genes during the stepwise exclusion of the less stable gene.*

4.4.2. Nisin induces a fast transcriptional activation followed by a slower protein secretion

Once selected the optimal reference genes, we proceeded with the evaluation of the induction kinetics. As shown in **Figure 23A**, the mRNA reached a full expression 1 hour post-induction (100% ± 4.88), slightly decreased after 2 hours (90.76 ± 11.55, p-value=0.4589), and dropped after 3-4 (57.07 ± 6.37, p-value=0.0006; 36.68 ± 6.91, p-value=0.0501). The intracellular galactosidase activity followed a similar trend, reaching almost 70% after 1 hour (68.38 ± 8.79), and peaking after 2 hours (100% ± 2.15). The activity significantly dropped after 3 hours (67.48% ± 3.43, p-value<0.0001) and reached a minimum of 41.57% (± 2.607) 6 hours post-induction. No activity was detectable just after the induction (T0) **(Figure 23B)**. The kinetics of the soluble fraction was significantly slower, probably as a result of the translocation processes. Within the first 3 hours from the induction, the activity remained below 50% (T0 and T1 undetectable; T2: 27.26% ± 8.56; T3: 42.36% ± 3.50). The activity reached its maximum between 4 and 5 hours post-induction (75.18% ± 6.94 and 100% ± 0.97) and dropped again at T6 and T7 (45.88% ± 12.15 and 22.63% ± 1.83) **(Figure 23C)**. Overall, these results suggest a fast induction at a transcriptional level followed by a rapid enzyme production, consistent with the co-transcriptional translation mechanism typical of the bacteria. Conversely, protein processing and translocation seems to require more time as it takes 5 hours after the induction for the enzyme activity to reach its maximum.

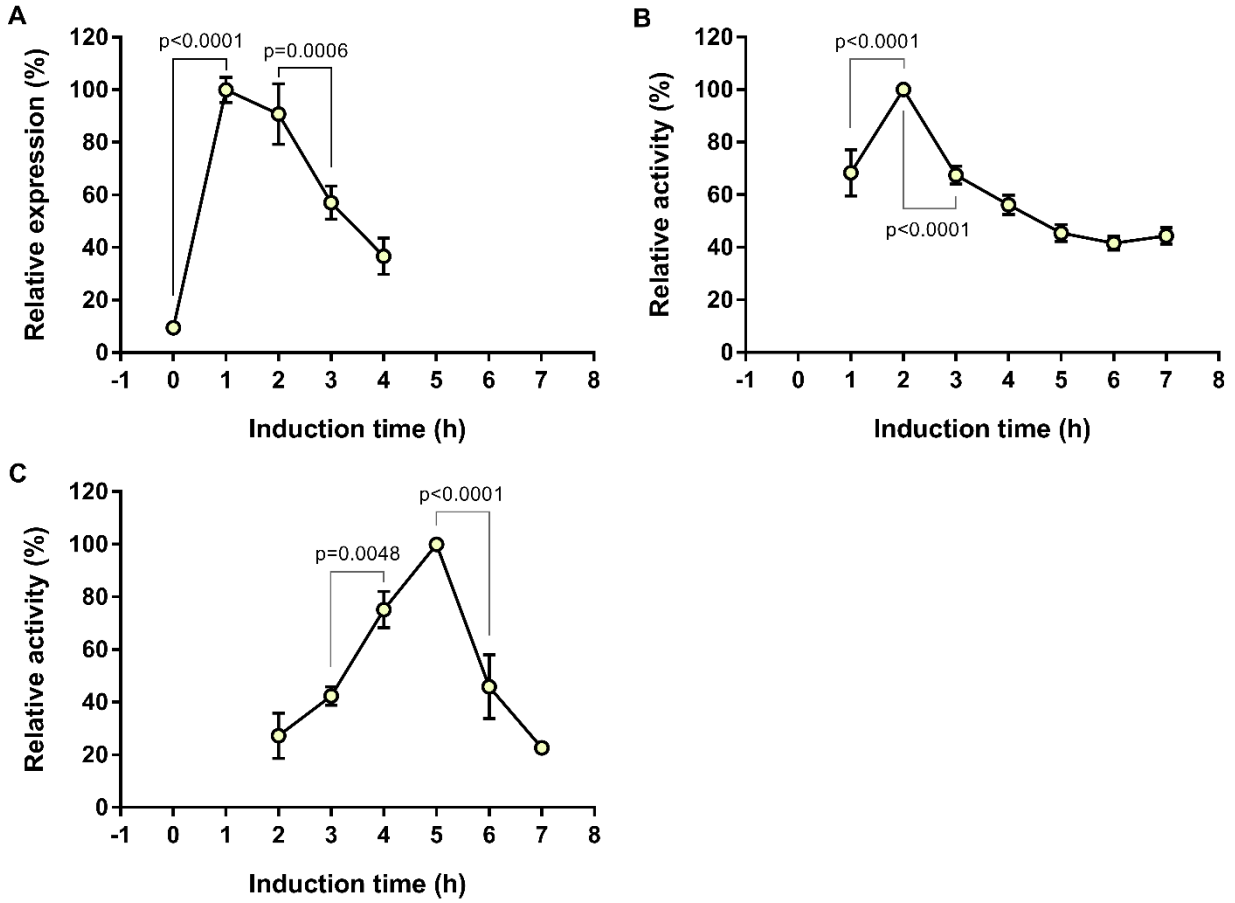


Figure 23: *Graphs showing the effect of nisin induction on mRNA expression* ***(A)****, intracellular enzyme activity* ***(B)****, and extracellular enzyme activity* ***(C)****. Relative expression and activity are reported in comparison with the maximum expression/activity measured. Circles represent the average relative expression/activity; bars represent the standard errors. P-values are reported only for the time points showing a significant difference between each other.*

4.5. Activity of the secreted enzyme

The evaluation of the induction kinetics allowed identifying the optimal time points for the following analyses. To estimate the secretion efficiency, we compared the enzyme activity in the medium and intracellular compartment. To maximize the proportion of secreted protein, we carried out the X-Gal assay 5 hours post-induction. However, the enzyme activity was significantly lower in the medium than in the intracellular compartment. When we transformed the bacterial cells with the pLB141GAL_LB, the activity of the secreted enzyme was only 0.36% ± 0.002 of the total (p-value<0.0001) **(Figure 24A)**. Similarly, the activity of the pLB141GAL_KL-derived enzyme detectable in the medium was only 0.2% ± 0.033 of the total (p-value<0.0001) **(Figure 24B)**. Conversely, we did not observe any significant difference between the total and the intracellular activity for any of the two enzymes. No activity was detectable in any compartment when we employed cells transformed with the pLB141GAL_BL harbouring the *GanA* gene.

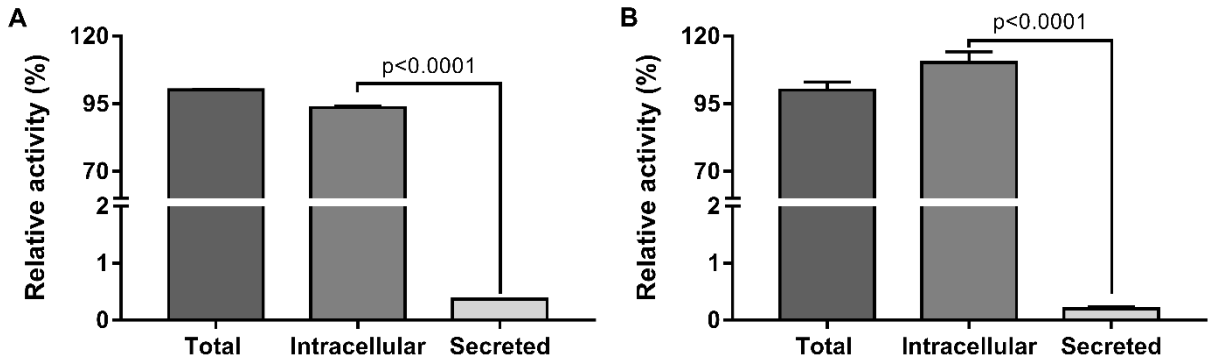


Figure 25: *Graphs reporting the activity of the L. bulgaricus* ***(A)*** *and K. lactis* ***(B)*** *β-galactosidases detectable in the total, intracellular, and secreted fraction. Columns show the average activity relative to the total fraction; bars represent the standard error. P-value is reported for the comparison between the activity detectable in the intracellular and secreted fractions.*

4.6. Effect of pH on enzyme activity

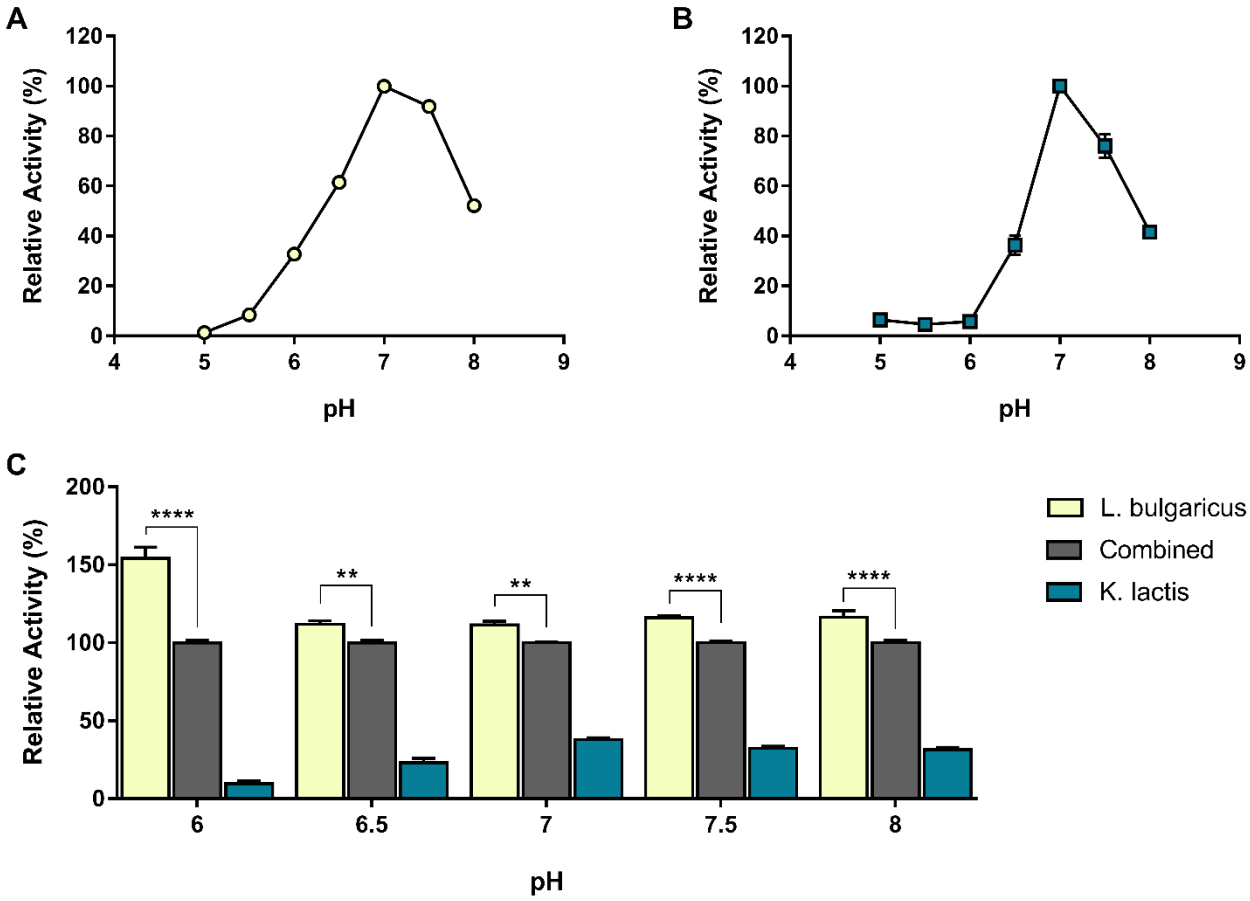


Figure 24: *Graphs showing the activity of β-galactosidases from L. bulgaricus* ***(A)*** *and K. lactis* ***(B)*** *at different pH and a constant temperature of 37°C. Dots represent the average activity relative to the maximum activity measured. Bars represent the standard errors.* ***(C)*** *Bar-chart showing the comparison between the activity of β-galactosidases form L. bulgaricus, K. lactis, and the combination of the two enzyme at different pH. The activity is reported as relative to the activity measured when the enzymes were combined.*

To evaluate whether the selected enzymes could perform well through the gastrointestinal tract, we measured the relative activity over a wide range of pHs. The β-galactosidase encoded by the *LacZ* of *L. bulgaricus* worked better at pH 7 and retained more than 50% of its activity between pH 6.5 and 8 **(Figure 25A)**. Similarly, the optimal pH for the β-galactosidase encoded by *LAC4* (*K. lactis*) was 7, but the activity was as low as 36% ± 3.8 at pH 6.5 and 42% ± 1.6 at pH 8 **(Figure 25B)**. As for the previous evaluations, we did not detect any activity in the intracellular fraction of cells transformed with the pLB141GAL_BL (data not shown). Despite the similar activity profile, we decided to evaluate whether the combination of the two enzymes could represent an effective strategy for obtaining a higher global activity within the pH range typical of the small intestine. To this end, we compared the relative activity of the combined enzymes (1:1 ratio) with those of the individual enzymes at pHs comprised between 6 and 8. As expected, the combination of the two enzymes did not result in any performance improvement. Moreover, data showed that the β-galactosidase from *L. bulgaricus* outperformed both, the β-galactosidase from *K. lactis,* and the combination of the two enzymes despite the pH **(Figure 25C)**.

4.7. Effect of temperature on enzyme activity

To test whether the selected enzymes worked well at the physiological temperature of about 37°C, we assessed their activity between 27 and 45°C. As shown by **Figure 26A-26B**, both enzymes performed well within this range of temperature, with the activity that never dropped below 55%.

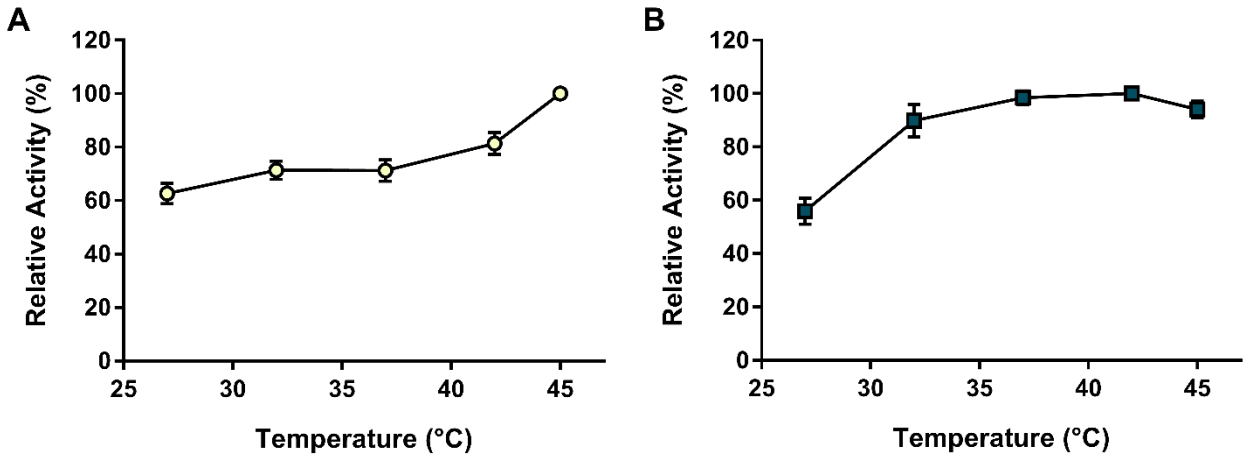


Figure 26: *Graphs showing the activity of β-galactosidases from L. bulgaricus* ***(A)*** *and K. lactis* ***(B)*** *at different temperature and a constant pH 7. Dots represent the average activity relative to the maximum activity measured. Bars represent the standard errors.*

The β-galactosidase derived from *L. bulgaricus* reached its maximum activity at 45°C (100% ± 1.74)

and a minimum of 62.6% ± 3.80 at 27°C. The activity at 37°C was 71.25% ± 3.99 **Figure 26A**. The activity of the β-galactosidase derived from *K. lactis* peaked at 42°C, but remained above 90% from 32 to 45°C, showing a minimum of 56% ± 4.87 at 27°C and a value of 98.47 % ± 2.63 at 37°C **(Figure 26B)**.

4.8. Survival of L. lactis and enzyme activity and stability in gastrointestinal environment

4.8.1. Incubation in simulated gastrointestinal fluids significantly reduced L. lactis survival rate

With the perspective of an *in vivo* application, data regarding the survival of *L. lactis* in the gastrointestinal transit are of paramount importance. To evaluate this aspect, we employed the INFOGEST protocol described in the "material and methods" section. As shown by **Figure 27A**, a two hours incubation in simulated gastric fluids (SGF) significantly reduced bacteria viability. Indeed, compared to the control (PBS at pH 7), only the 0.003% of the cells survived after the incubation at pH 3 in SGF, with an average of 144.4 ± 53 CFU/mL compared with $4.4x10^6$ ± 3.74 x 10^5 CFU/mL of

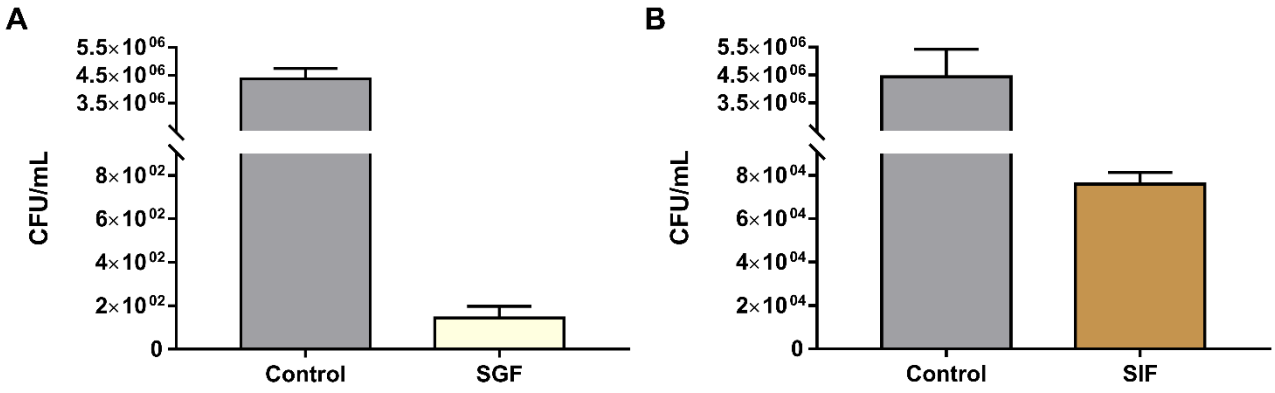


Figure 27: *Bar charts illustrating the colony counting results for L. lactis after a two hours incubation in PBS + milk (control) or Milk + simulated gastric **(A)** or intestinal **(B)** fluids. Graphs report the average colony forming units per millilitre along with the standard errors.*

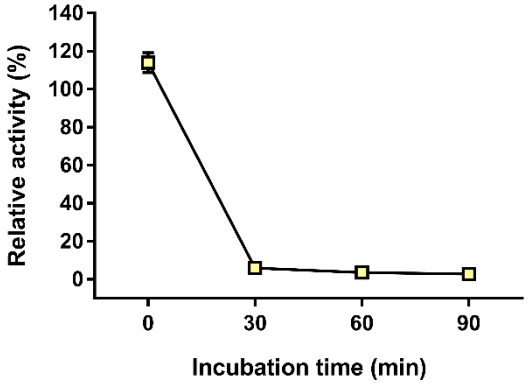
the control. Similarly, a two hours incubation in simulated intestinal fluids (pH 7) significantly reduced bacteria viability. In this case, the survability rate was 4.07%, with an avarage of 7.59 x 10^4 ± 5.359 x 10^3 CFU/mL in the treated sample, compared with an average of 4.38 x 10^6 ± 9.87049 x 10^5 CFU/mL in the control **Figure 27B**.

4.8.2. L. bulgaricus β-galactosidase activity was abolished after incubation at gastric pH

Given the low survival rate of *L. lactis* in SGF, we decided to verify if the β-galactosidase encoded by *L. bulgaricus* could retain its activity after transiting the stomach. To this end, we exposed the enzyme to pH 3 for 0, 30, 60, and 90 minutes and then measured its residual activity at pH7. As

showed in **Figure 28**, 30 minutes of incubation at pH3 significantly decreased the enzyme activity. Indeed, the residual average activity was only 5.91% ± 0.80 compared with that of the control

Figure 28: *Graph showing the effect of an incubation at pH 3 on the activity of L. bulgaricus galactosidase. The enzyme was incubated for 0, 30, 60 and 90 minutes before measuring its activity relative to that of the control (incubated at pH 7 for the same amount of time). Graph report the average relative activity along with the stander errors for each time point. No significant difference was observed between 30, 60 and 90 minutes of incubation.*



(enzyme incubated in PBS at pH 7 for the same amount of time). The activity further dropped to 3.59 ± 0.55 and 2.86 ± 0.46 after 60 and 90 minutes, however this additional decreses was not statistically significant.

4.8.3. Incubation of L. lactis in simulated intestinal fluids promoted the enzyme-substrate interaction

Since the incubation of SIF determined a significant reduction of *L. lactis* viability and the observation from previous studies suggested lysis of dead bacteria within the small intestine [105], we decided to evaluate whether the exposure of *L. lactis* to SIF could promote an enzyme-substrate interaction. As shown in **Figure 29**, incubation in SIF of nisin-induced *L. lactis* strain carrying the

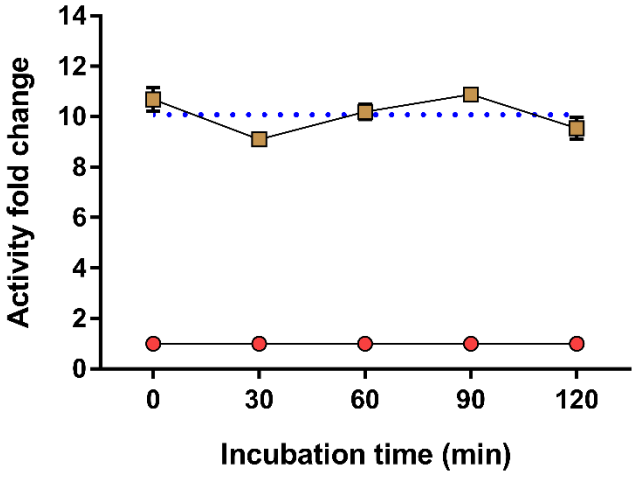


Figure 29: *Effects of SIF on detectable enzyme activity from L. lactis strain carrying the pLB141GAL_LB plasmid. The activity was reported as fold-change relative to the control (L. lactis incubated in PBS). Squares and circles represent the average activity fold-change for the treated and the control at each time point. Blue dashed line represent the average activity fold-change over time.*

pLB141GAL_LB plasmid resulted a significant increased in the detectable enzyme activity (p-value < 0.0001). Indeed, the incubation in SIF resulted in an average 10.08 ± 0.18 fold-change over time

compared with control (incubation of the same strain in PBS for an equal amount of time). Interestingly, the only statistically significant difference among the activity fold-change at different time points was observed between 0 and 30 min (p-value 0.0068). This observation suggests that SIf exerts most of its effect as soon as the incubation starts, probably by inducing lysis or permeabilization of *L. lactis.*

4.8.4. Simulated intestinal fluids affected enzyme activity but not stability

To evaluate whether the *L. bulgaricus* galactosidase could perform well in the intestinal environment, we assessed enzyme stability and activity in SIF. When the enzyme was incubated in SIF, it retained its activity for as long as 90 minutes. After 120 minutes a statistically significant decrease was observed as compared with time zero (T0: 92.9% ± 4.41; T120: 79.68 ± 2.32; p-value = 0.027) **(Figure 30A)**. While SIF seemed to exert only a moderate effect on enzyme stability, increasing concentrations of SIF added to the reaction mix significantly decreased enzyme activity. Specifically, the activity dropped to 63.29 ± 0.46 in reaction mix containing 2.5% SIF compared with the control (0% SIF; p-value < 0.0001). Each concentration increased corresponded to a significant decrease in enzyme activity. The lowest level of activity was reached with 20% SIF (17.22 ± 0.49) **(Figure 30B)**.

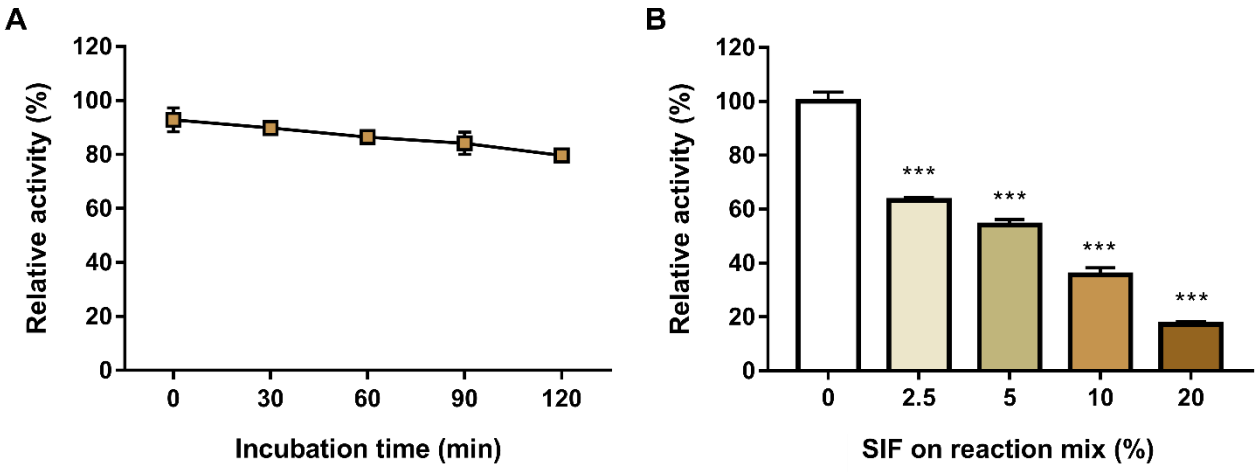


Figure 30: *Graphs showing the stability* ***(A)*** *and activity* ***(B)*** *of L. bulgaricus galactosidase on SIF. Bars represent the standard errors; asterisks indicate a statistically significant difference (*** p<0001).*

5. Discussion

Lactose malabsorption (LM) is a condition affecting almost 2/3 of the adult population worldwide [123], which can result in the development of gastrointestinal symptoms leading to lactose intolerance (LI). To date, no effective therapies exist, and the management of LI relies mainly on dairy avoidance. In this work, we evaluated whether an *L. lactis* NZ9000 strain harbouring a β-galactosidase-expressing plasmid could represent a potential therapeutic approach for treating LI.

To this end, we first selected three galactosidases reported to have a good activity at temperature and pH typical of the human intestine [107], [108]. When we verified this aspect, our results partially confirmed data from previous studies. Indeed, the β-galactosidases encoded by the *LacZ* and *LAC4* genes of *Lactobacillus delbrueckii bulgaricus* (*L. bulgaricus*) and *Kluyveromyces lactis* (*K. lactis*), respectively, retained a high activity between pH 6.5 and 8. In particular, the enzyme from *L. bulgaricus* showed a peak of activity at pH 7.5 that was consistent with the results reported by Nguyen et al. [120]. Concerning the activity of the galactosidase from *K. lactis*, it also peaked at pH 7.5 but dropped at pH 6 and lower. This result was slightly different from the data reported by Lima et al. [117]. Indeed, the authors observed an activity peak at pH 6.5 and a sharp drop at pH equal and below 5.5. The differences between our results and the previous findings may be explained by the different substrates employed, which can slightly affect the enzyme activity at different pH. Concerning the impact of temperature, both enzymes worked well at 37°C and between 32°C and 45°C. Specifically, we observed a relative activity at 37°C of 71.25% and 98.47% for the galactosidases derived from *L. bulgaricus* and *K. lactis*. On notice, we were not able to detect any activity when we evaluated the effect of pH and temperature on the β-galactosidase from *Bacillus licheniformis*. This observation conflicted with other authors results reporting a good activity under the conditions employed in our study [112]. The reasons behind this discrepancy remains elusive. Sanger sequencing excluded any possible sequence alteration in the pLB141GAL_BL plasmid. Culturing the strain in a chloramphenicol-containing medium should prevent a spontaneous plasmid loss. Colony PCR and double digestion with *NsiI/NotI* confirmed the plasmid presence in the employed *L. lactis*. Lastly, differences in the enzyme activity may arise when using X-Gal instead of lactose/oNPG. However, it is unlikely that the employment of X-Gal could result in a complete loss of activity. Other possible explanations remain to be explored. The mRNA translation into a mature protein may be hampered by differences in the codon usage between the host strain *L. lactis* and the original strain *B. licheniformis*, resulting in a low protein expression level. Unfortunately, the lack of a short N-terminus peptide tag in our galactosidases prevented us from verifying this aspect.

However, since we aimed to evaluate the enzyme activity under different conditions, we preferred avoiding any unneeded sequence modification and, thus, reducing variables potentially influencing the effectiveness of our system. Another possible explanation behind this lack of enzyme activity is that the protein itself could be unstable and undergo degradation for reasons yet to be clarified.

Since we aimed to employ *L. lactis* for delivering exogenous galactosidases to the small intestine, an important aspect to evaluate was the secretion efficiency of our system. To this extent, we compared the enzyme activity in the intracellular and extracellular fraction five hours after nisin induction. The results showed most of the enzyme activity within the intracellular compartment, with the extracellular enzyme activity limited to 0.2-0.36% of the total, depending on the plasmid carried by the cells. Since the *Exp4*$_{SP}$ harboured by both plasmids should allow an efficient translocation in the extracellular space, this result was unexpected.

As recently reviewed by Morello et al., [103] host factors can affect the production and secretion of heterologous proteins in *L. lactis*. A way to overcome this problem could be the employment of modified *L. lactis* strains carrying inactivating mutations in the *HtrA* gene. Indeed, HtrA is the only known L. lactis protease participating in protein quality control at the extracellular surface level. Thus, the inactivation of HtrA decreases the degradation of exogenous secreted proteins. As reviewed by Morello et al. [103], inactivating the *HtrA* gene could result in a 4-fold increased yield of heterologous proteins [82]. Moreover, overexpressing proteins such as SecD and SecF from *B. subtilis*, or the isomerase PmpA from *L. lactis*, could further improve the secretion efficiency. Modifying the expression plasmid sequence could also increase the secretion efficiency. Indeed, several authors reported that inserting a short synthetic spacer between the signal peptide and the protein sequence could improve the secretion and the overall protein yield [82], [103]. Lastly, studies showed a similar efficiency for the SPExp4 and the canonical SPUsp45 of *L. lactis*, suggesting that the type of signal peptide employed only plays a minor role in the actual secretion efficiency.

The secretion efficiency was not the only meter of concern. The observation that a pH as low as three hugely reduced the enzyme activity implies the need to protect the enzyme from the gastric environment during its transit through the GI tract. Drouault et al. [105] reported that *L. lactis* ingested with solid food could survive the transit through the rats' stomach. Specifically, the authors showed that between 90% and 98% of the bacteria ingested with powder diet survived the gastric environment. Conversely, only 10-30% survived the duodenum. Interestingly, the authors found that most of the dead cells underwent rapid lysis in the duodenum. This observation suggested the possibility to exploit this event for the delivery of intracellular proteins. Concerning the present

work, the intestinal lysis observed by Drouault et al. could allow a massive release of the intracellular β-galactosidases, overcoming the problems related to the low secretion efficiency. Moreover, *L. lactis* could protect the exogenous β-galactosidases from gastric inactivation while transiting through the stomach. To evaluate this aspect, we firstly incubated *L. lactis* in simulated gastric (SGF) and intestinal fluids (SIF) and subsequently assessed its viability on agar plates.

Results showed that both fluids significantly reduced *L. lactis* viability. When incubated two hours in SGF, only 0.003% of bacterial cells survived. Similarly, the two hours incubation in SIF determined a viability reduction to 4.07%. The low survival rate we observed in the SGF was not consistent with Drouault observation reporting a high survival of *L. lactis* in the rats' gastric environment. However, results from a recent study conducted by the Rottiers group showed reduced viability of *L. lactis* NZ9000 incubated in gastric juice [124]. Indeed, a 30 minutes incubation in 50% gastric juice caused a 96.2% decrease in cell viability. These data support our results, highlighting the sensitivity of this strain to gastric fluids. To promote *L. lactis* survival, freeze-dried bacteria could be filled within enteric-coated capsules, as reported by Huyghebaert et al. [125]. While the encapsulation did not fully protect *L. lactis* from gastric juice, it resulted in a significant survival increase during the gastric transit. Additionally, Termont et al. showed that expressing the trehalose synthesizing genes in *L. lactis* conferred enhanced resistance to bile and gastric juice [124]. Moreover, this system did not interfere with the production and secretion of heterologous proteins and could be considered a valid alternative for protecting *L. lactis* from gastric lysis. Once in the small intestine, *L. lactis* should release the galactosidase of interest. Given the low survival rate of *L. lactis* in intestinal fluids, we hypothesized that the incubation in SIF could promote a lysis-mediated release of the intracellular proteins, including the galactosidase of interest. To verify this aspect, we incubated *L. lactis* in SIF and measured the enzyme activity at different time points. Results showed an average ten-fold increase in the activity detectable from SIF exposed cells compared with the control. Moreover, the enzyme activity remained almost unchanged for 90 minutes, suggesting high enzyme stability in SIF. These results were promising since they allowed us speculating that the galactosidase from *L. bulgaricus* would retain a stable activity for the whole transit throughout the small intestine. However, intestinal fluids seems to affect the galactosidase activity. Indeed, adding 2.5, 5, 10, and 20% of SIF to the X-Gal assay reaction mix reduced the enzyme activity to 63.29, 54.13, 35.67, and 17.22%. Whether the residual activity could be enough to allow efficient lactose digestion in an *in vivo* setting remains to be unveiled.

6. Limitations and future perspectives

In our opinion, this work represents a solid proof of concept that the *L. lactis*-mediated intestinal delivery of galactosidases could become a valuable option for treating lactose intolerance. By showing the stability and activity of *L. bulgaricus* galactosidase in simulated intestinal fluids, we highlighted its potential for an *in vivo* application. However, our work also presents some limitations.

X-gal is a versatile substrate for the colorimetric assessment of galactosidase activity. By using X-gal as substrate, we were able to monitor the galactosidase activity over time. This method allowed us to evaluate the characteristics of the selected enzymes with limited previous knowledge. On the other hand, the affinity of the studied galactosidases could differ between lactose and X-gal. This difference could slightly change some of the results reported here. While it is unlikely that this could significantly affect enzyme performance in the gastrointestinal environment, lactose will be the natural *in vivo* substrate. Thus, future experiments employing lactose as a substrate will be needed to corroborate our results.

Although bacterial exposure to intestinal fluids enhanced the enzyme-substrate interaction through a cell-lysis mediated release or a cell membrane permeabilization, a secretion efficiency improvement is needed. As discussed in the previous section, several strategies exist to increase heterologous protein secretion. In this respect, the first attempt will be the use of a *HtrA*$^{-}$ strain. If the results obtained so far will be confirmed on lactose and assuming an improvement in the secretion efficiency, the entire system should be tested in relevant *in vitro* and pre-clinical *in vivo* models. Those in vitro systems mimicking the intestinal barriers, based on organoids technology, could give us additional insights into the potential of this method. The MIVO system consists of a 3D reconstructed intestinal epithelium integrated into a fluid-dynamic bioreactor, which allows simulating the *in vivo* small intestinal absorptions of molecules, including sugars [126]. Reconstructing an *in vitro* model of lactose malabsorption using MIVO would allow a more accurate evaluation of the potential therapeutic properties of the *L. lactis* strain developed here.

On the other hands, animal models such as post-weaning mice represent an *in vivo* experimental model that could be informative as well on the reliability and effectiveness of our approach.

By orally administering the modified *L. lactis* strain to post-weaning mice, we should be able to evaluate lactase activity *in vivo* and *in vitro,* as reported by Heijning et al. [127]. These data will give us additional information about the *in vivo* behaviour of our strain. Moreover, we could evaluate

the effect of *L. lactis* in alleviating the symptoms of lactose intolerance by exposing the same animals to lactose challenge.

Since the long term aim of this work is to employ the modified *L. lactis* strain for treating lactose intolerance in humans, one of the final steps will be the preparation of a food-grade system. To this extent, we could adopt some of the strategies described in the "introduction" section. By removing the chloramphenicol resistance from the pLB141GAL_LB plasmid, we could create "food-grade" plasmids, which will allow a lactose-based selection of positive bacterial colonies. As previous evidence suggested that *L. lactis* has limited colonizing capabilities [80], removing the antibiotic resistance is a mandatory step to avoid or, at least, reduce at minimum risks for human health.

However, the possible release of the modified strain into the environment poses additional safety concerns. *L. lactis* could be modified to create an auxotrophic strain and meet all safety requirements. To this extend, we could employ the CRISPR-based strategy illustrated by Vo et al. to substitute the thymidylate synthase gene (*thyA*) in the genome of *L. lactis* with the P_{nisA}-LacZ gene expression cassette carried by the pLB141GAL_LB vector [128]. The modified strain would be dependent on thymidine or thymine for growth and survival. Staidler et al. highlighted the efficiency of this containment method [95]. Moreover, a phase I clinical trial showed the safety of this modified *L. lactis* strain [92], paving the way for future human applications.

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