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IMPACT AND INDUCTION OF PROPHAGES IN *Lactococcus lactis*

MASTER IN BIOTECNOLOGY OF ENVIRONMENT AND HEALTH

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ABSTRACT

Lactic acid bacteria (LAB) are very important microorganisms in food industry, since they are used for the production of dairy products such as cheese or yogurt. Among LAB, *Lactococcus lactis* (gram-positive bacteria) is the main component of the starter cultures in cheese production. The technology used to manufacturing dairy products at large scale is standardized, simple and highly efficient, however, an unwanted lysis of *L. lactis* may have a negative impact in the final product, even causing the loss of the product. Phages are one of the causes of the lysis when they infect the cells. Temperate phages also known as prophages, can "switch" from the lysogenic state to the lytic cycle under specific environmental stress. Such induction has been widely studied and is mediated by the SOS response, although recently the cell envelope stress response (CESR) has been suggested to participate in prophage induction as well. The aim of this study was to assess the impact of two specific prophages, TP712 and CAP in growth and autolytic capacity. Additionally to elucidate the role of CESR in prophage induction, the *L. lactis* laboratory strains UKLc10 TP712, UKLc10 TP712/CAP and UKLc10 CAP were treated with MitC and two cell-wall antimicrobials, Lcn 972 and bacitracin, using qPCR as a quantification method to detect viral DNA. The results suggest that there is indeed, an impact of prophage content since strain UKLc10 CAP shows an adaptive advantage with a higher growth rate over the two other strains. Moreover, the lysogen carrying the two prophages is more autolytic. Interestingly, only the strain UKLc10 TP712/CAP lyses after treatment with MitC and prophage induction was observed by qPCR. In contrast, when both UKLc10 TP712 and UKLc10 TP712/CAP are treated with Lcn 972 or bacitracin, inhibition of spontaneous induction happens. The fact that we were not able to construct a Δ*cesSR* mutant, implies that we cannot evaluate at which extent CESR is involved in prophage induction. Further research is needed in order to find out the role of CAP and TP712 in the lysis of cells and how they behave in their coexistence as well as how CESR interferes in their lysis-lysogeny decision.

ABBREVIATIONS

- **CES**: Cell Envelope Stress
- **CESR**: Cell Envelope Stress Response
- **C_t**: Cycle threshold
- **DNA**: Deoxyribonucleic acid
- **DNase**: Deoxyribonuclease
- **dsDNA**: Double-stranded deoxyribonucleic acid
- **ECF**: Extracytoplasmatic function factor
- **Em**: Erythromycin
- **Em5**: Erythromycin at 5µg/ml
- **EmR**: Erythromycin Resistance
- **GM17**: M17 medium supplemented with 0.05% glucose
- **HK**: Histidine kinase
- **IC_{50}**: Specific concentration at which the 50% of the cells are dead
- **KPi**: Potassium phosphate
- **LAB**: Lactic acid bacteria
- **Lcn 972**: Lactococcocin 972
- **Lys-CAP**: Endolysin of prophage CAP
- **Min**: Minutes
- **MitC**: Mitomycin C
- **OD_{600}**: Optical density measure at a wavelength of 600 nm
- **P_L**: Promoter of the gene *cI*
- **P_R**: Promoter of the gene *cro*
- **qPCR**: Quantitative Polymerase Chain Reaction
- **RNA**: Ribonucleic acid
- **ROS**: Reactive oxygen species
- **RR**: Response regulator
- **ssDNA**: Single-stranded deoxyribonucleic acid
- **TCS**: Two-component system
- **UKLc10 CAP**: *Lactoccus lactis* strain containing prophage CAP
- **UKLc10 TP712**: *Lactococcus lactis* strain containing prophage TP712
- **UKLc10 TP712/CAP**: *Lactococcus lactis* strain containing prophages TP712 and CAP
- **UV**: Ultraviolet

1. INTRODUCTION

In industrial terms, lactic acid bacteria (LAB) are very important microorganisms, since they are used for the production of dairy products such as yogurt, cheese, butter and kefir. The species that are normally used with this application, are bacteria belonging to the group of gram-positive, such as *Lactobacillus, Lactococcus, Leuconostoc*, *Pediococcus* and the species *Streptococcus*. These bacteria are known for their fermentative ability that also improve food safety and organoleptic properties, enrich the product and increase health benefits (Gemechu, 2015).

Due to the fact that milk is a perishable food, the purpose of fermenting milk using LAB is to prolong the shelf life of milk and its derivatives, in addition to preserving all the nutritional characteristics of the product. On the other hand, in recent years interest has grown around the development of a variety of dairy products fermented for other purposes, especially those related to health and to prevent the production of toxins by foodborne pathogens (Gemechu, 2015).

For the fermentation of milk, starter cultures are used, being *Lactococcus lactis* one of the most used bacteria in this field since it is one of the most economical of its kind. In general, the technology used for the production of standardized large-scale dairy products is affordable, simple and highly efficient. (Gemechu, 2015; Sadiq et al., 2019). Additionally, it is necessary to use robust starter cultures for a correct and efficient production because an unwanted or premature lysis of the LAB can cause problems in the fermentation, which may incur in the production of low quality products or the loss thereof. However, one of the biggest problems in the production of dairy products, during the fermentation of milk is the presence of phages (Garneau & Moineau, 2011). These are found almost everywhere in the dairy processing environment and can negatively affect production processes. Besides, it is known that the genomes of many strains of *L. lactis* contain prophages and elements similar to prophages, which can be released in the form of virions, causing a complete failure of the fermentation and generating large economic losses (Sadiq et al., 2019).

1.1. Bacteriophages and prophages

Bacteriophages, also known as "phages" are viruses that infect bacteria and are widely distributed in the environment, being the most prevalent and ubiquitous entities on the planet (even above bacteria by an estimated factor of 10) (Emond and Moineau, 2007).

For decades, phages have been recognized as "enemies" of diverse industrial environments. Problems with phages are not only related to the food industry, but also to the pharmaceutical, chemical and pesticide factories. However, the dairy industry has been described as the industry most affected by these viruses (Garneau & Moineau, 2011).

Bacteriophages classification is based on the type of nucleic acid they possess and the morphology of the viral particles (Ackermann, 2006). The phages with double-stranded DNA (dsDNA) packed in the head (capsid) and with tail are grouped in the order *Caudovirale*, being all the phages belonging to this order responsible of infecting LAB (Ackermann, 1998; Pujato et al., 2019) and depending on the structure of the tail, three families are differentiated: *Myoviridae*, *Siphoviridae* and *Podoviridae* (Ackermann, 2006). However, there are other groups of phages which can have non-tailed capsids with dsDNA genome, non-tailed capsids with single-stranded DNA (ssDNA) or RNA genomes (**Figure 1**), but these do not infect of LAB (Ackermann, 2006).

In regards with the viral development, the recognition of the cells that will be susceptible to infection is specific. This implies that the viral recognition proteins are exposed on the surface of the virion, and in the case of the tailed bacteriophages those proteins can be located at tail, at its distal end or in the fibres that protrude in some cases from that end (Martín et al., 2019). Once its genome is injected into the host cell, the phage can proceed to the replication process using the bacterial biosynthetic machinery.

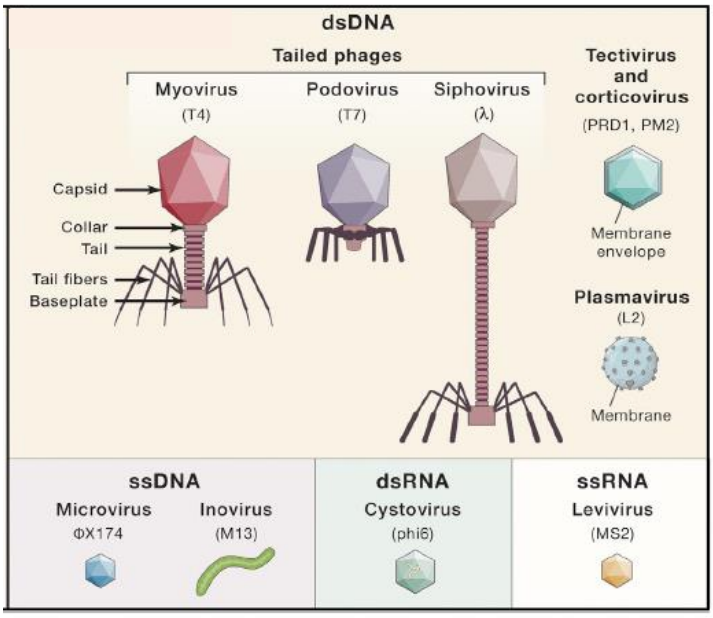

Figure 1: Phage taxonomy based on morphology and genome composition. A representative type phage for each taxonomical group is in parenthesis (Ofir & Sorek, 2018).

1.1.1. Lytic and lisogenic cylces

In general terms, phages can be classified into two categories based on their life cycle: i) the strictly lytic (or virulent) phages and ii) temperate phages (or prophages) (**Figure 2**). Phages can replicate by infecting host cells, stopping their metabolism to replicate and assemble the phage particles and finally release the virions after cell lysis (Pujato et al., 2019). Normally, the lytic cycle of the DNA phages is characterized by the phage adsorption on the surface of a bacterial receptor, followed by the injection of the DNA into the cytoplasm of the bacterium, replication of the genome of the virus and the transcription of the viral genes. Once the viral proteins that make up the capsid and tail are synthesized, they are assembled to form these structures to package the viral genome in the capsid to form the virions. At the end of the lytic cycle, the holin (a small protein that forms oligomers), creates pores in the cytoplasmic membrane allowing the endolysin to have access to the cell wall and hydrolyze the peptidoglycan causing the cell's lysis. Finally, hundreds of particles are released that are capable of re-infecting adjacent bacteria (Fortier & Sekulovic, 2013).

These virulent phages only present this type of replication strategy, however, the temperate ones can be integrated into the bacterial chromosome and multiply in a state of dormancy, also known as prophage, through the multiple divisions of the host cell until the external conditions trigger the lytic cycle (Pujato et al., 2019). Within a few minutes of the injection of the phage DNA into the bacterial cytoplasm, and depending on the metabolic state of the bacterial cell, the phage can "choose" to initiate a lytic cycle or integrate its DNA into the bacterial chromosome of its host to become a prophage (Fortier & Sekulovic, 2013)

Therefore, the temperate phages can choose between following a lytic cycle of development (similar to that of virulent phages) and a lysogenic one depending on the environmental conditions, and in this way, ensure their transmission to the descendants of the host (Martín et al., 2019).

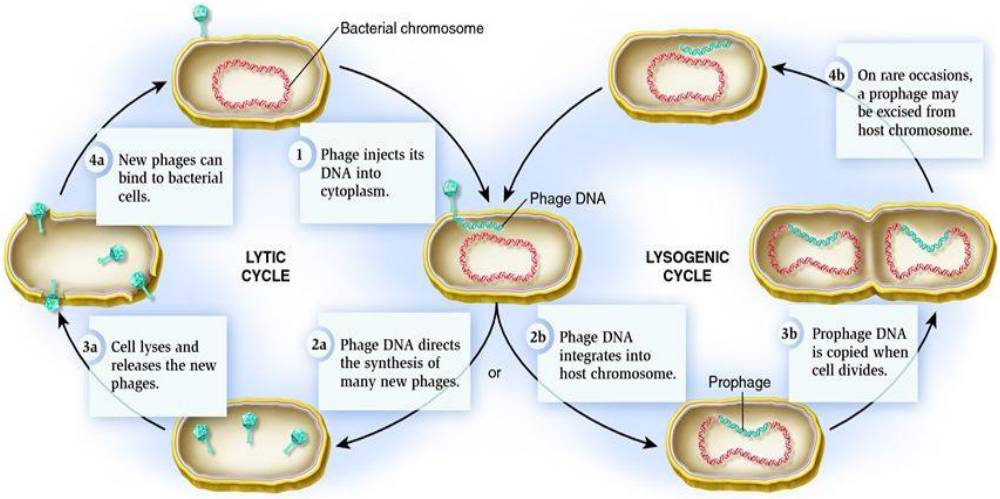

Figure 2: Different lifestyles adopted by phages (Goyal, 2017).

1.1.2. Lysis-lisogeny decision

Temperate phages can choose to infect either through the lytic or the lysogenic cycle. Whereas the lytic cycle leads to lysis of the bacterial cell, in the lysogenic cycle the phage genome integrates into bacterial genome, and the host becomes immune to further infection by the same phage (**Figure 2**), (Ofir

& Sorek, 2018). Whether a phage enters in lytic or lysogenic cycle it's named as lysis-lysogenic decision, and there are many factors that have been studied in order to understand better how the phages make this decision (Ofir & Sorek, 2018).

As it has been mentioned before, a temperate phage can "choose" between lytic cycle or integrate its DNA into the bacterial genome, becoming a prophage. This is mediated by the "molecular switch" and two main proteins are involved in the decision process (reviewed by Martín et al., 2019), CI and Cro, products of the expression of two genes named *cI* and *cro* respectively. These proteins are immediately synthesized after the injection of viral DNA in the bacterial host, and the fate of the phage depends on the relative concentration of CI and Cro proteins in the cytoplasm.

If the phage choose to integrate its DNA in the host genome, the CI repressor inhibits transcription of most of the phage genes, including those that are needed for the lytic cycle, so the prophage becomes quiescent (**Figure 2**). As is demonstrated for the phage A2 that infects *Lactobacillus casei*, CI has high affinity for the DNA sequence (operator) which overlaps with the promoter (P_R) of gene *cro* (O_1) and if it binds, it prevents from the recognition of RNA polymerase (Ladero et al., 2002). Thus, it inhibits Cro and viral proteins for replication synthesis which are forming an operon with *cro* (**Figure 3**). Under this situation, when the bacteria replicates its DNA, so does the prophage's DNA at each cell division process and this cycle can last for an infinite period of time (Fortier & Sekulovic, 2013; Martín et al., 2019).

On the contrary, the protein Cro blocks the beginning of the lysogenic cycle, due to the fact that it has a high affinity for the operator O_2, which overlaps with the promoter of the gene *cI* (P_L), preventing from the synthesis of CI and the integrase (**Figure 3**) (Ladero et al., 2002). This last protein, functions as a recombinase which catalyzes the integration of viral DNA in the host genome, ensuring its heritability. However, in some cases the viral DNA remains independent from host genome, like a plasmid (Martín et al. 2019).

Therefore, during the lysogenic cycle, CI is synthesized indefinitely, being the only active gene in many prophages. Such event is necessary because the host keeps growing and an accidental increase in its volume would provoke the displacement of the equilibrium between the two repressors. This would lead to the transcription of the *cro* and further genes under P_R control, inducing the lytic cycle. Finally, CI also recognizes and binds to O_2, with lesser affinity than to O_1, though. This is how it regulates its own synthesis, avoiding its accumulation but maintaining the lysogenic cycle of its host (Martín et al., 2019)

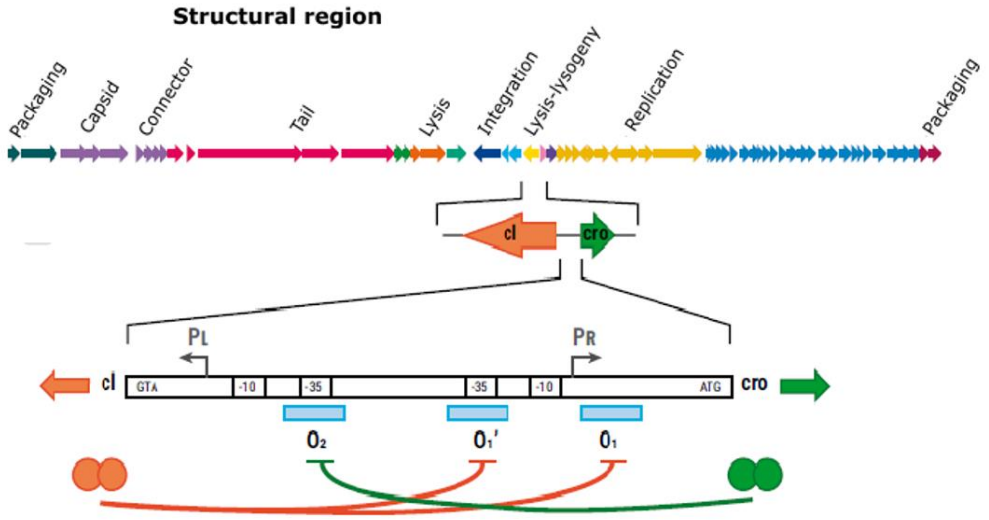

Figure 3: Structural organization of phage A2 genome. The 61 open reading frames that appear grouped in functional modules are shown; the lysis-lysogeny, integration and replication modules are of early transcription; the structural, packaging and lysis are expressed late as well as the genes *cI* and *cro*. Additionally, intergenic region between *cI-cro* is shown: regions 10 and 35 of the P_L and P_R promoters, transcription start points (broken arrows) and translation (ATG) and the two operating regions are represented (in this phage the operator O_1 has two parts to which can join CI, designated as O_1 and O_1 ') (Modification of Martín et al., 2019).

This "molecular switch", has been studied since the early days of molecular biology, specifically in the *Escherichia coli* phage lambda, where it was found to be a complex process which involves an intricate network, participating transcriptional repressors and activators, as well as RNA degradation, transcription antitermination and proteolysis (Ofir & Sorek, 2018). This complex network, integrates information of the metabolic state of the cell and the phage multiplicity of infection in order to make a decision depending on this factors (Oppenheim et al., 2005).

New studies with lambda phages infecting cells of larger volumes showed that they have lower chances of choosing lysogeny, presumably because the dilution of phage lysogeny-promoting proteins in a larger cell (St-Pierre & Endy, 2008). Additionally, it has been observed that one single cell of *E. coli* can be infected by multiple lambda phages. When this happens, each phage choose between lysis and lysogeny, however, that choice is dependent on the total viral concentration inside the cell. Then, the decision of all phages is integrated within the cell and a unanimous vote by all phages leads to establish a lysogenic state (Zeng et al., 2010).

Although there is enough information of lysis-lysogeny decision in lambda phage, the decision-making process in other phages remained almost completely unknown (Ofir & Sorek, 2018). However, a recent study performed by Erez et al., (2017) with phage phi3T in *Bacillus subtilis*, revealed that phages of the SPbeta group use a small-molecule (6 amino acid peptide, called arbitrium) communication system to coordinate lysis-lysogeny decisions. The peptide is released into the medium during the infection, and if the progeny phages detect a high concentration of this peptide, then they choose the lysogenic cycle. Moreover, they found that different phages encode different versions of the peptide, demonstrating a phage-specific peptide communication code for lysogeny decisions (Erez et al., 2017). Therefore, it is not unlikely to find that other phages and even non-phage viruses use such communication systems to coordinate group decisions (Ofir & Sorek, 2017).

1.1.3. Prophage's impact in bacterial host cell

The ability of temperate phages to establish a stable relationship with their host (lysogen), has a significant impact on the lifestyle, viability and virulence of the lysogen. Through the use of techniques such as next-generation sequencing, a large number of complete sequences of bacterial genomes are available in public databases. As a result, mining and comparative genomics have revealed the presence of prophages in most bacterial genomes (Fortier & Sekulovic, 2013).

The prophages are one of the sources of genetic diversity and variability within bacterial strains, associated with the virulence of many pathogenic bacteria such as *Escherichia coli*, *Streptococcus pyogenes*, *Salmonella enterica* and *Staphylococcus aureus*. In fact, numerous phages associated with virulent strains code for potent extracellular toxins, effector proteins that participate in the invasion and several enzymes (superoxide dismutase, staphylokinase, phospholipase, DNase, etc.) (Fortier & Sekulovic, 2013).

The phenomenon of lysogenic conversion, by which a phage converts a non-virulent bacterial strain into a virulent one, has been linked with the emergence of new virulent and epidemic clones such as the *E. coli* O157:H7 strain, which acquired two Shiga toxin prophages (Hayashi et al., 2001; Ohnishi et al., 2001). Moreover, prophage induction and mobility, which is often increased by the presence of antibiotics, can shape bacterial communities so antibiotic resistance genes can spread out and other mobile genetic elements as pathogenicity islands in *S. aureus* (Úbeda et al., 2005) promoting by this way their evolution (Fortier & Sekulovic, 2013). As it has been shown, the impact of prophages in bacteria has mainly been investigated in *E. coli* and in some pathogens. Although containing quiescent prophages can cause the destruction of a cell, there is a growing interest for the beneficial effects they offer to the host and how they can interfere in host's phenotypic plasticity related to growth, survival rate or susceptibility to antibiotics (Aucouturier et al., 2018).

1.2. Prophage induction

It has been shown that lysogeny represents beneficial and stable relationship between a phage and its bacterial host. However, sporadically this equilibrium can be broken as consequence, for example, of an accidental decrease on CI concentration in cytoplasm. Although seldom happens, cultures of lysogenic bacteria always present free phages, as a result of the process called "spontaneous induction" (Martín et al., 2019).

The main mechanism of prophage induction, widely described in the bibliography, is the SOS response. Nonetheless, recently it has been proposed another mechanism which may have a role in the prophage induction, known as cell

envelope stress response (CESR). As it will be explained below, both responses have been studied due to their importance in prophage's life cycle.

1.2.1. SOS response

The prophage is able to detect situations that put in danger the host's life and the response is the lytic cycle, so the main purpose is to produce a viral offspring before the bacteria dies. This emergency mechanism is the SOS response, and it's activated when DNA synthesis is blocked. It was first described in *E. coli* and there are two key proteins that govern the SOS response: LexA (repressor) and RecA (inducer). When the DNA remains undamaged, a LexA dimer binds to SOS boxes, a 20 base pair palindromic DNA sequence, repressing transcription of a regulon encompassing more than 50 genes (including *lexA* and *recA*). However, when the DNA is damaged, RecA is activated by binding to ssDNA to form a nucleoprotein filament. Thus, activated RecA stimulates self-cleavage of LexA, leading to depression of SOS genes (**Figure 4**), (Žgur-Bertok, 2013).

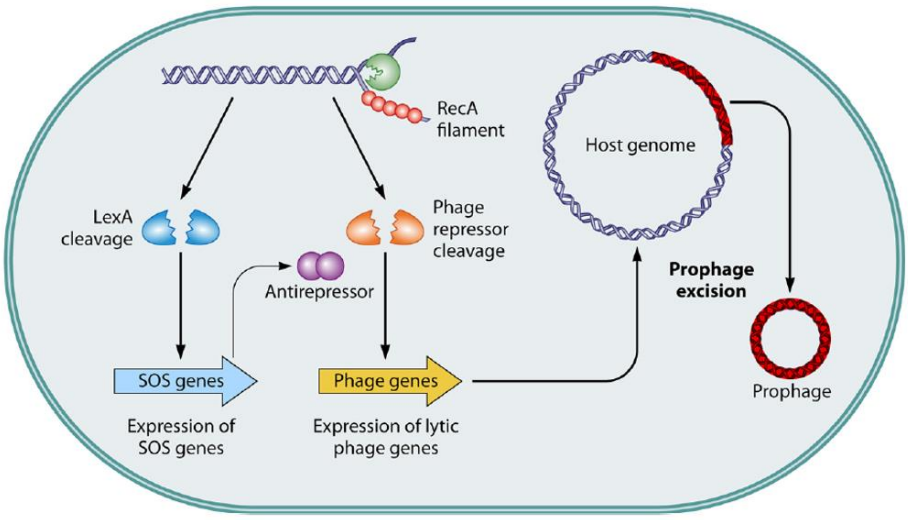

Figure 4: SOS dependent prophage induction (Nanda et al., 2015).

There are several factors that can trigger the SOS response and they are differentiated in two groups: intrinsic and extrinsic factors. Intrinsic factors are those related to the internal environment of the cell, for instance, during

growth, ongoing (multifork) replication has been shown to cause sporadic DNA damage resulting in the derepression of the SOS genes and even reactive oxygen species (ROS) provoke damages in DNA leading to prophage induction (reviewed by Nanda et al., 2015). Furthermore, it has been demonstrated that the intrinsic stability depends on the cell culture conditions in the same way as the occurrence of mutations which render the lysogen unstable. Therefore, prophages have evolved to optimize the switching frequency over a wide range of naturally occurring inducing conditions (Nanda et al., 2015)

However, as it has been highlighted before, besides intrinsic factors there are extrinsic factors that could affect the genomic DNA or RecA and induce the SOS response. For example, ROS, UV radiation, pH, heat or antibiotics such as mitomycin C (MitC). These all agents, can disrupt DNA, arrest synthesis and cell division leading to the accumulation of ssDNA, triggering and emergency response (Janion, 2008). Nevertheless, RecA-independent inductions also occur in *E. coli* that do not involve a SOS response (Rozanov et al., 1998), but the signal(s) participating in RecA-independent induction are being studied (Ghosh et al., 2009).

In the case of Gram-positive bacteria as *L. lactis*, also possess the SOS response as emergency mechanism to face, for example, environmental stress. Although the regulation of the response is different because *L. lactis* has LexA-like regulator HdiR instead of LexA repressor, the elemental components participating in SOS response are similar (Savijoki et al., 2003).

1.2.2. CESR mediated induction

Bacteria have developed adaptive responses to their environment and such adaptation requires the sensitive monitoring of numerous environmental parameters. This monitoring process is needed to coordinate the activity of intricate and complex regulatory systems that help the bacteria to readjust adequate cellular response (Jordan et al., 2008).

Due to the presence of agents and/or conditions which can threat the essential cellular structures, the cell envelope integrity is closely monitored by the own

bacterium. The stress that can affect to the cell envelope integrity is referred as cell envelope stress (CES). On the other hand, the regulatory systems of cell envelope stress response (CESR), respond to envelope altering conditions. (Jordan et al., 2008).

Hence, the CESR of a Gram-positive bacterium, consists of those signal-transducing regulatory systems (and their regulons) that are involved in sensing and responding to the presence of cell wall antibiotics or other perturbing conditions (Jordan et al., 2008).

The CESR is regulated by a two component system (TCS) which is constituted by a sensor and a transcriptional or response regulator (RR) (**Figure 5**). The sensor is the histidine kinase (HK) which is usually anchored to the membrane and it traduces a signal via phosphorylation of the RR. The nature of trigger activating the sensor varies between TCS and the activity of the protein encoded by genes that are under control of the regulator of TCS. In the case of LAB, the relative importance of TCS could be amplified since extracytoplasmic function (ECF) factors, an alternative regulatory mechanism that sometimes shares overlapping regulatory networks with TCS, seems almost virtually absent in LAB (Pinto, 2015).

CesSR (TCS) was originally described as LlkinD/LlrD in a systematic analysis of six TCS from *L. lactis* strain MG1363. Nevertheless, O'Connell-Motherway et al., (2000) were unable to generate an insertion mutant of *llknD*, encoding the HK, but the RR mutant MGRrD showed an increased salt-/osmosensitivity. Several years later, Martínez et al., (2007), showed that it responds to the extracellular presence of the lactococcal bacteriocin Lcn 972 and renamed it CesSR. Transcriptome analysis revealed that the expression of 26 genes was significantly up regulated in the presence of Lcn972, of which 23 responded in a CesSR dependent manner. Additionally, many of these genes encode putative membrane or stress-related proteins.

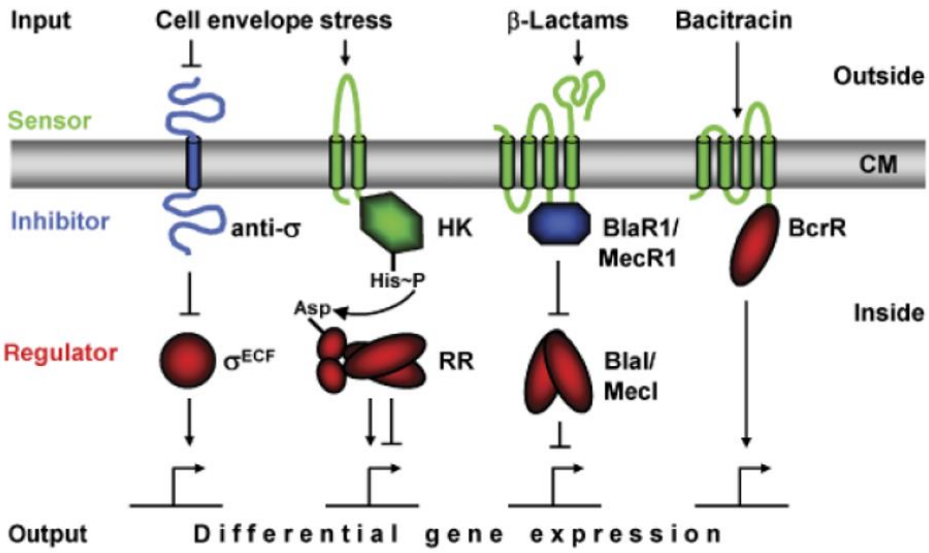

Figure 5: Regulatory mechanism of CESR in Gram-positive bacteria. From left to right: ECF s factors, two-component systems (HK, histidine kinase; RR, response regulator), BlaR1/MecR1 system, BcrR. Sensor proteins are shown in green, inhibitor in blue, transcriptional regulators in red (Jordan et al., 2008).

Besides Lcn 972, the CesSR is also induced by other cell wall antibiotics, such as bacitracin, plantaricin C and nisin which interfere with the Lipid II cycle, i.e. with cell wall biosynthesis (Martínez et al., 2007; 2008).

For instance, bacitracin (a cyclic nonribosomally synthesized dodecylpeptide) has been shown to inhibit bacterial cell wall biosynthesis through sequestration of undecaprenyl phosphate (C_{55}-PP), which is a key lipid involved in the biosynthesis of peptidoglycan and other cell-wall polysaccharide components. Therefore, the sequestration of that lipid leads to the loss of the cell wall integrity and lysis of the cell (Bouhss et al., 2008). However, bacitracin is not the only substance that disrupts the incorporation of key elements to the cell wall, in fact the bacteriocin Lcn 972 also blocks the incorporation of such elements.

Bacteriocins are ribosomally synthesized peptides that are used as a strategy for competition of space and resources. In the case of Lcn 972, which is a non-modified 66-aa hydrophilic bacteriocin synthesized by *L. lactis* IPLA 972 is bactericidal to lactococci, disrupting the incorporation of cell wall precursors,

such as lipid II in actively growing cells. This event causes a shift in cells growth, changing from an exponential to a linear model and subsequently becomes arrested (Martínez et al., 2000; 2008).

Interestingly, according to the study published by Madera et al., (2009), both Lcn 972 and bacitracin act as prophage inducers in early exponentially growing of *L. lactis*, indicating that CESR might be involved in the induction of prophages.

1.3. Lactococcal prophages in *L. lactis* laboratory strains

To date, most of studies relating to prophage induction have focused on one or a limited number of lactococcal strains, encumbering our ability to generally appreciate the risk presented by such prophages (Kelleher, 2017).

As it has been mentioned above, the interest in lactococcal phages originally arose from the economic impact of their attacks on *L. lactis* cultures that are used for the production of fermented dairy products. Due to this interest, several genomic studies are being made to study lactococcal phages' genetic organization, population genetics and mode of evolution (Ventura et al., 2007)

L. lactis is classified into two subspecies: i) *Lactococcus lactis* subsp. *lactis* (e.g., *L lactis* subsp. *lactis* IL-1403) and *Lactcoccus lactis* subsp. *cremoris* (e.g., *L. lactis* subsp. *cremoris* MG1363 and *L. lactis* subsp. *cremoris* SK11) (Ventura et al., 2007). These strains have been used to study prophages role and evolution within the bacteria and their prophage content have been even modified for such studies (Aucouturier et al., 2018). In fact, the strain MG1363 has been optimized for its use as a cell factory and the entire sequencing of its genome by (Wegmann et al., 2007), allowed the characterization of the prophages that it contains.

L. lactis MG1363 is a plasmid-free, and originally thought to be also prophage free, derivative of the dairy starter *L. lactis* NCDO712 (Gasson, 1983). However, according to Ventura et al., (2007) *L. lactis* MG1363 contains two intact prophages t712 (TP712 in this manuscript) (42,058 bp) and MG-3 (44,200 bp) as well as prophages MG-1 (19,053 bp), as MG-2 (6,019 bp), MG-4 (18,029 bp), and MG-5 (10,598 bp) which are presumed to be incomplete phage elements.

Intriguingly, there is no apparent lytic response of *L. lactis* MG1363 (or derivatives thereof) to treatment with both MitC and UV light (Ventura et al., 2007). Moreover, despite there is no apparent lytic response to induction with both mytomicin C and UV light (Ventura et al., 2007). Moreover, despite the presence of TP712 in the *L. lactis* MG1363 genome, this prophage does not provide immunity because lytic plaques could be observed when plating the original TP712 phage propagated on the mother strain *L. lactis* NCDO712 (Ventura et al., 2007). Later on, using a phage lysate from *L. lactis* NCDO712, putative TP712 lysogens able to lyse after MitC treatment were engineered in different *L. lactis* backgrounds such as MG1363 (*L. lactis* FI7274) and the *L. lactis* MG1363 derivative strains optimized for nisin inducible gene expression NZ9000 and UKLc10 (NZ9000/TP712 and UKLc10/TP712, respectively) (Roces et al., 2016, 2013; Wegmann et al., 2012). Although in these previous reports, these lysogens were regarded as "lytic or MitC- inducible TP712 lysogens", it was recently discovered by genome sequencing that all of them carry a new prophage named CAP (35,600 bp), in addition to the already described TP712 and MG3 (S. Escobedo, personal communication). In order to avoid confusion around the interpretation of the results of the current study, the nomenclature for these strains has been changed to reflect their actual prophage content. Thus, the *L. lactis* UKLc10 derivatives having prophages TP712 and CAP will be named as UKLc10 TP712/CAP whilst the strain without CAP will be named as UKLc10 TP712. In addition, a derivative of UKLc10 TP712/CAP was generated by our collaborator PhD. U. Wegmann (University of East Anglia, UK) in which the prophage TP712 was deleted by genetic engineering and, hence, this strains will be cited as UKLc10 CAP. The availability of several *L. lactis* lysogens carrying different prophages is seen as an excellent platform to: i) study the impact of prophages on the physiology of their bacterial host, ii) unravel conditions leading to prophage induction and even, iii) define putative prophage cross-talking.

2. OBJECTIVES

Taking into account all this information, the hypothesis of the current study is that there is a cascade of alternative or complementary signals to the SOS response that induce the lytic cycle in prophages and that would be caused by the damage of the cell wall, mediated

in this case by antimicrobials targeting the bacterial cell wall. Hence the main objectives of this study are:

1- To assess the impact of prophages in the phenotype of *Lactococcus lactis* regarding to their growth rate, autolytic capacity and susceptibility to cell wall antibiotics (Lcn 972 and bacitracin).
2- To determine and quantify by qPCR the induction of prophages after treatment with the cell wall antimicrobials, Lcn 972 and bacitracin using MitC as a positive induction control, in presence/absence of SOS response and/ or CES response.

3. MATERIALS AND METHODS

3.1. Bacterial strains and culture conditions

Three different strains derived from *L. lactis* MG1363 were used in order to perform the experiments: UKLc10 TP712 (Ventura et al., 2007) and UKLc10 TP712/CAP (Roces et al., 2013) and UKLc10 CAP (Laboratory collection). The strains (which were kept at -80ºC in the presence of 10% glycerol), were isolated by striations on a plate with solid M17 medium (2% agar) (supplemented with 0.5% of glucose, GM17). Subsequently they were incubated for 48h at 30ºC. For both experiments, microtiter based prophage induction and prophage induction for quantification by qPCR, the day before performing the inductions, pre-inocula were prepared in 4 ml of GM17 by taking one isolated colony and the culture was incubated at 30ºC during 16-18h. Next, for both experiments, strains were grown in GM17 and incubated until they reach the early exponential phase (Optical density, OD_{600}=0.2).

3.2. Microtiter based prophage induction: Growth curves

MitC, Lcn 972 and bacitracin were used to perform the inductions. Stocks of 40 µg/ml, 1600 UA/ml and 100 µg/ml were prepared respectively in GM17, and serial dilutions of factor 2 were made before adding 180 µl of culture (OD_{600}= 0.2) at each well. After this, the final concentration in the first well was: 2 µg/ml of MitC, 80 UA/ml of Lcn 972 and 5 µg/ml of bacitracin in a final volume of 200µl. Cells' growth was monitored each 10 min, at 30ºC in the microtiter plate reader

(BioRad), measuring the optical density (OD_{600}) during 6-8h. Three replicates were done with each antimicrobial for each strain.

3.3. Autolytic assay

L. lactis strains UKLc10 TP712, UKLc10 TP712/CAP and UKLc10 CAP were grown in GM17 to and OD_{600} of 0.3-0.5. Cells were harvested by centrifugation at 5000 g for 10 min at 4ºC, washed once with sterile 50 mM potassium phosphate (KPi) buffer (pH=7.0), and centrifuged again at the same conditions. Subsequently, cells were resuspended in KPi 50 mM buffer to OD_{600} 1.0. Two aliquots of 500µl were taken for each strain and third centrifugation step was done at 13000 rpm for 5 min. Finally, the cells were resuspended in 400µl of KPi 50 mM buffer and separately in KPi 50mM/Triton 0.05%. Cells suspensions were transferred to 96-well microplate and incubated at 30ºC. Autolysis was monitored by measuring OD_{600} at 10 min-intervals for 6h with a microtiter plate reader (BioRad). This experiment included three independent cultures of each strain and the extent of the autolysis was expressed as the percentage decrease in OD_{600}.

3.4. Prophage induction and quantification by qPCR

3.4.1. Bacterial strains

L. lactis strains UKLc10 TP712 and UKLc10 TP712/CAP and were induced with 1 µg/ml MitC, when cell cultures reached OD_{600}= 0.2. Samples were taken (200µl) before adding the MitC (0 min) and after the induction at 60 min, 75 min and 90 min. In order to stop the induction, these samples were rapidly frozen at -80ºC. The same procedure was done in the experiments with the cell wall antimicrobials Lcn 972 and bacitracin, using a concentration of 80 UA/ml and 0.5 µg/ml, respectively. In these cases, samples were taken at 60 min, 75 min, 90 min and 120 min after the inductions. Additionally, samples of cultures without any induction agent were taken in order to analyse the spontaneous induction. For each experiment, two biological replicates were used.

3.4.2. qPCR assays and primers

Quantitative PCR (qPCR) assays were carried out to follow the amplification of TP712 and CAP DNA (primers showed in **Table 1**). The single copy genome reference *tuf* gene was used as an internal control in order to normalize afterwards the copy number of the target genes.

First, calibration curves were made to test the efficiency of the primers. A calibration curve is built by making dilution series (e.g. from 10^0 to 10^{-4}) of known template concentration for either determining the initial amount of the target template in samples or for assessing the reaction efficiency. As it is explained in Real time PCR Handbook (Thermo Fisher Scientific, 2014), the log of each known concentration in the dilution series (x-axis) is plotted against the C_t value for that concentration (y-axis). This curve provide information about the performance of the reaction in addition to various reaction parameters such as slope, y-intercept and correlation coefficient (R^2). The slope value of the log-linear phase of the amplification plot is a measure of reaction efficiency, which value should be around to -3.32 when the efficiency is close to 100%. In the case of R^2, it measures how well the data fit the calibration curve, reflecting the linearity of the curve. Although the ideal value is 1, usually the obtained maximum value is 0.999. Finally, the y-intercept value gives the theoretical limit of the detection of the reaction and it is use as a direct measure sensitivity (recommended value is around 33.33). The obtained values for each pair of primers (Tuf-R and Tuf-F; TP03 and TP04; CAP-F and CAP-R) are shown in **Table 2** as well as the calibration curves are shown in **Figure 6**.

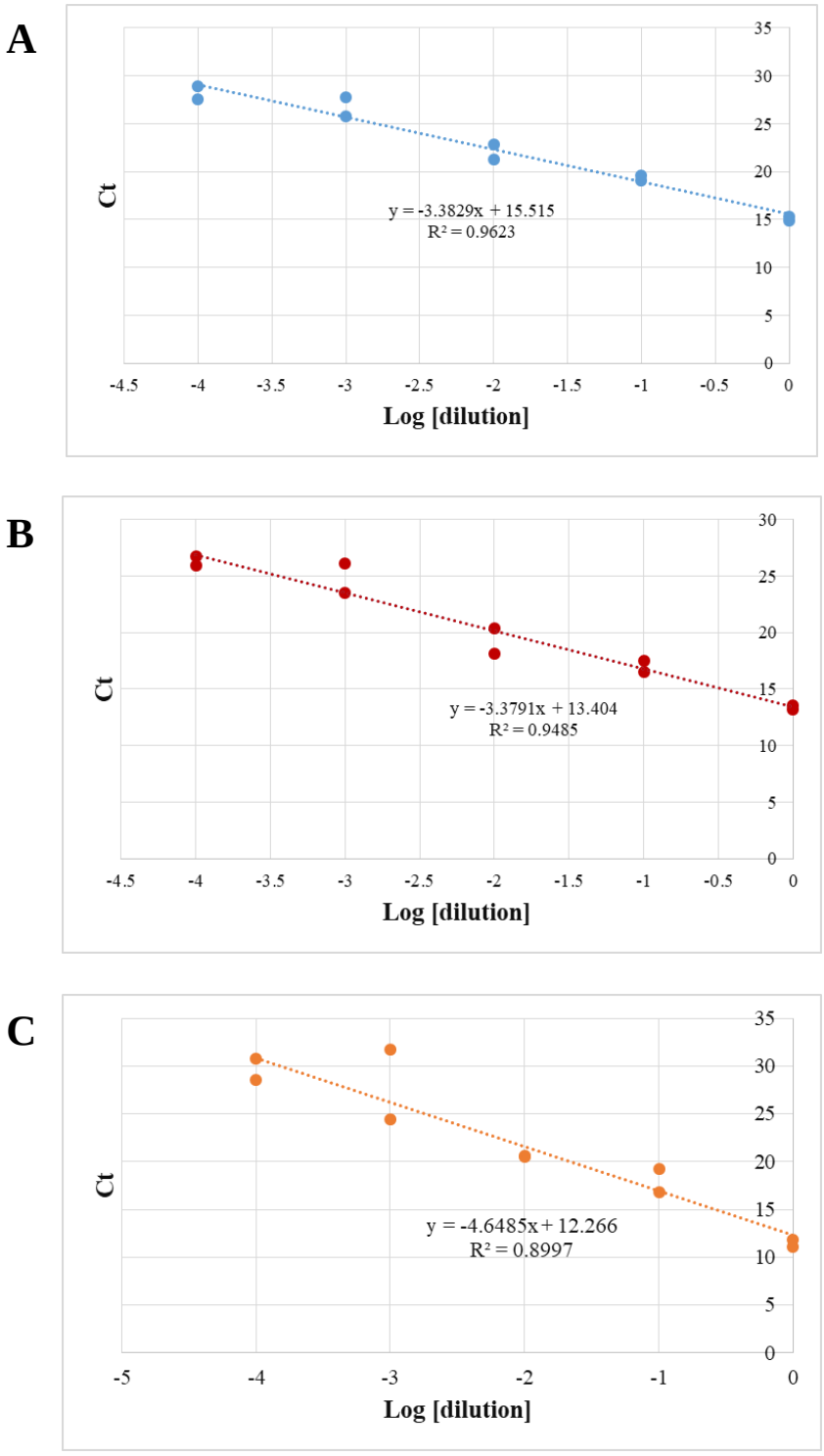

Figure 6: Calibration curves for pairs of primers: A) TP03 and TP04, B) CAP-F and CAP-R, and C) Tuf-F and Tuf-R.

Once they were tested, quantification experiments were made. The quantification method was the same followed by Ho et al., (2016) but without purification of the DNA and with slight changes in qPCR cycling parameters. Prior to qPCR, 50 µl of each culture samples were boiled for 15 minutes and diluted afterwards 1/10 (v/v) in distilled water. qPCR was performed by using SYBR Green PCR Master Mix Kit (Applied Biosystems, UK) and primers at final concentration of 10µM (**Table 1**). The qPCR conditions were the following ones: 50ºC 10 min; 95ºC 5 min; 45 cycles 95ºC 10s, 58ºC 30s; 95ºC 1 min, 55ºC 1 min, 55ºC 10s, in a final volume of 15 µl.

To determine the relative changes in copy number of the target genes, at 60 min, 75 min and 120 min several calculations were carried out using the $2^{-\Delta\Delta Ct}$ method as it is described in Livak & Schmittgen, (2001) and Ho et al., (2016), comparing the ΔCt value from time 0 corresponding samples. First step for the calculation is $\Delta Ct_{\text{target gene}} - \Delta Ct_{\textit{tuf}\text{ gene}}$ so as to normalize the copy number of genes of interest. Second step is to calculate the ΔΔCt value in order to relativize to time and the equation for that is $\Delta Ct_{\text{time } n} - \Delta Ct_{\text{time } 0}$. Finally, $2^{-\Delta\Delta Ct}$ is calculated to see the increment of induction over the time. Additionally, averages of two technical replicates for each biological replicates were calculated as well as standard deviations.

Table 1: Primers used for qPCR assay.

Name	Seq 5'-3'	gene	T °C
Tuf-F	GGTAGTTGTCGAAGAATGGAGTGTGA	Elongation factor Tuf	**68.1**
Tuf-R	TAAACCAGGTTCAATCACTCCACACA	Elongation factor Tuf	**69.1**
CAP-F	GCTGGTTCGGATGGACACAC	LysCAP	**68**
CAP-R	TGAACCTGAGCCAACGATTCG	LysCAP	**68**
TP03	CGCTGACAGTTTGACTGATG	3-4: convergent in head major prot TP712	**60**
TP04	GCCAACGACTTCGTTTAGAC		**62**

Table 2: Calibration curves values for each pair of primers.

Primers pairs	Slope	Y-inter	R^2	Efficiency (%)
TP03 and TP04	-3.383	32.43	0.962	97.521
CAP-F and CAP-R	-3.379	30.30	0.948	97.672
Tuf-F and Tuf-R	-4.648	35.508	0.900	64.106

3.5. *L. lactis* mutant by "gene knock-out" for cesSR genes

3.5.1. Bacterial strain, plasmids and primers

The *L. lactis* strain UKLc10 TP712/CAP, was used for the generation of new mutant in Δ*cesSR*. The plasmid used for the gene knock-out was pCS1996::cesSR (Pinto et al., 2011). The pCS1996::cesSR was designed for deletion of chromosomal *cesSR* gene and it harbours erithromycin resistance gene (EmR) and the *oroP* gene, which encodes for the orotate transporter expressed from the synthetic promoter obtained by selection (SP-oroP). This promoter confers *L. lactis* the ability to utilize orotate and in addition, the expressed transporter renders the cell sensitive to 5-fluoroorotate. The sensitivity is used to select for loss of the plasmid (Solem et al., 2008). On the other hand, selection for integration is performed primarily by resistance to erythromycin. As the plasmid does not replicate in *L. lactis*, it was previously amplified in *E. coli* background, grown in 2xYT broth with erythromycin (Em) at 120 μg/ml before performing the transformation.

3.5.2. *L. lactis* transformation and gene knock-out

For the transformation, cells were made electrocompetent. Firstly, o/n cultures of UKLc10 TP712/CAP were prepared in GM17 medium at 30ºC. Afterwards, 100 ml of GM17 supplemented with glycine at 1% were inoculated with 4 ml of o/n culture and cells were grown at 30ºC for 2.5h until they reached an OD_{600} between 0.2 and 0.3. Several centrifugation and resuspension steps were done as it is shown in the **Table 2**.

Electroporation was done using a Gene Pulser® from Bio-Rad, fitted with 200 Ohm, 2500V and 25μF on a 0.2 cm cuvette and after it 960 μl of SGM17 (20 mM $MgCl_2$, 2 mM $CaCl_2$) were added after electroporation. Cells were regenerated at 30ºC for 2 h in the incubator and plated on GM17 Erithromycin (Em) 5 μg/ml. Putative positives, which would have the plasmid integrated into the region of interest, were replicated on a GM17 Em and into 0.9 ml GM17 Em5. Overnight cultures were grown at 30ºC and in order to obtain more colonies. Subsequently,

to confirm which recombination happened lower frequency, samples of each positive colony were taken and grown in GM17 Em5 at 30ºC for PCR confirmation.

Table 2: Centrifugation and resuspension steps of electroporation protocol.

Steps
1. Centrifuge for 10 min at 500 rpm (250 ml bottles)
2. Resuspend pellet in 8 ml of H_2O mQ (sterile)
3. Centrifuge at 4ºC, 13000 rpm for 4 min
4. Resuspend pellet in 4 ml of H_2O mQ (sterile)
5. Centrifuge at 4ºC, 13000 rpm for 4 min
6. Resuspend pellet in 4 ml of EDTA 50mM buffer
7. Incubate in ice for 5 min
8. Centrifuge at 4ºC, 13000 rpm for 4 min
9. Resuspend pellet in in 4 ml of H_2O mQ (sterile)
10. Centrifuge at 4ºC, 13000 rpm for 4 min
11. Resuspend pellet in 4 ml of saccharose 0.3M
12. Centrifuge at 4ºC, 13000 rpm for 4 min
13. Resuspend pellet in 4 ml of saccharose 0.3M and make aliquots of 200µl (stock at -70ºC)

3.6. Statistics

To see if there were significant differences between *L. lactis* UKLc10 TP712, UKLc10 TP712/CAP and UKLc10 CAP, several statistical analyses were carried out. On the one hand, growth rates resulting from microtiter-based prophages induction and results from autolytic assays were analysed comparing strains by pairs using t-Student ($p<0.05$). In contrast, to compare values of IC_{50} obtained for each cell-wall antimicrobials (Lcn 972 and bacitracin) among strains one-way ANOVA ($p<0.05$) was applied.

4. RESULTS

4.1. Microtiter-based prophage induction: Growth curves

To elucidate the impact of prophages in *L. lactis* and how they are induced, three induction experiments were conducted in the micro-well plate for three strains (UKLc10 TP712, UKLc10 TP712/CAP and UKLc10 CAP), using MitC, Lcn 972 and bacitracin as induction agents. The results are shown in the **Figure 7**. Only the results of one replicate are shown for each induction experiment (in total three replicates were made with similar results).

For the experiment with MitC, the highest concentration (2 µg/ml) was lethal for the three strains whilst 1 µg/ml and 0.5 µg/ml were sub-inhibitory for UKLc10 TP712 and UKLc10 CAP (**Figure 7A** and **C**). In contrast it can be observed that the strain UKLc10 TP12/CAP was the only one which lysed at concentrations between 0.126 µg/ml and 0.0075 µg/ml after almost 2 hours (**Figure 7B**). Additionally, the lysis observed with 0.0156 µg/ml it was not as dramatic as at higher concentrations and cells seemed to recover after 3 hours reaching an OD_{600} of around 0.5 (**Figure 7B**). On the other hand, as it has been mentioned above, the strains UKLc10 TP712 and UKLc10 CAP, did not show lysis at any concentration but UKLc10 CAP showed a different growth curve at such high concentrations comparing to UKLc10 TP712 (**Figure 7B** and **C**). Despite the growth was slowed down at 1 µg/ml, the UKLc10 CAP culture reached an OD_{600} above 0.4 after 3 hours and at 1 µg/ml the culture reached an OD_{600} notoriously superior to that reached by UKLc10 TP712 after 5 hours.

Regarding to the experiments with cell wall antimicrobials Lcn 972 and bacitracin, no lysis was observed at any concentration for any strain (**Figure 7D, E,F,G,H** and **I**). For the induction with Lcn 972, the concentrations 80 UA/ml and 40 UA/ml were inhibitory for the three strains. The results with bacitracin show that the concentrations 5 µg/ml, 2.5 µg/ml, 1.25 µg/ml and 0.625 µg/ml were lethal for the three strains, nonetheless, at 0.315 µg/ml cells were able to grow reaching an OD_{600} above of 0.5-0.6 after 5 hours. However, as the results of IC_{50} (the concentration at which growth is inhibited by 50%) show, there were no

significant differences (One-way ANOVA, p>0.05) between strains regarding their susceptibility to Lcn 972 and bacitracin (**Table 3**). However, the results of IC_{50} with strains UKLc10 TP712 and UKLc10 TP712/CAP treated with Lcn972 were very variable while for the strain UKLc10 CAP the deviation is smaller, indicating that UKLc10 CAP might be more susceptible to Lcn 972.

Table 3: IC_{50} of the antibiotics Lcn 972 and Bacitracin for strains *L. lactis* UKLc10 TP712, UKLc10 TP712/CAP and UKLc10 CAP. There are no significant differences between strains (one-way ANOVA, p> 0.05).

Strains	IC50 Lcn972 (UA/ml)	IC50 Bacitracin (µg/ml)
UKLc10 TP712	13.46±10.89	0.21±0.14
UKLc10 TP712/CAP	7.68±4.71	0.24±0.07
UKLc10 CAP	1.74±1.77	0.15±0.05

Despite this fact, the growth rate of negative controls cultures of each induction experiment, including those from MitC experiments, show that there were significant differences between strains (**Table 4**). The strain UKLc10 CAP had a higher growth rate comparing to UKLc10 TP712 and UKLc10 TP712/CAP (comparison in pairs, t-Student p<0.05), however, there were no significant differences on the growth rate of these between last two strains (comparison in pairs, t-Student p>0.05) (**Table 4**).

Table 4: Growth rate for strains *L. lactis* UKLc10 TP712, UKLc10 TP712 / CAP and UKLc10 CAP. * Strain UKLc10 CAP shows significant differences with respect to the other two strains (Student t-

Strains	Growth rate (µ)
UKLc10 TP712	0.86±0.03
UKLc10 TP712/CAP	0.86±0.03
UKLc10 CAP	1.05±0.01*

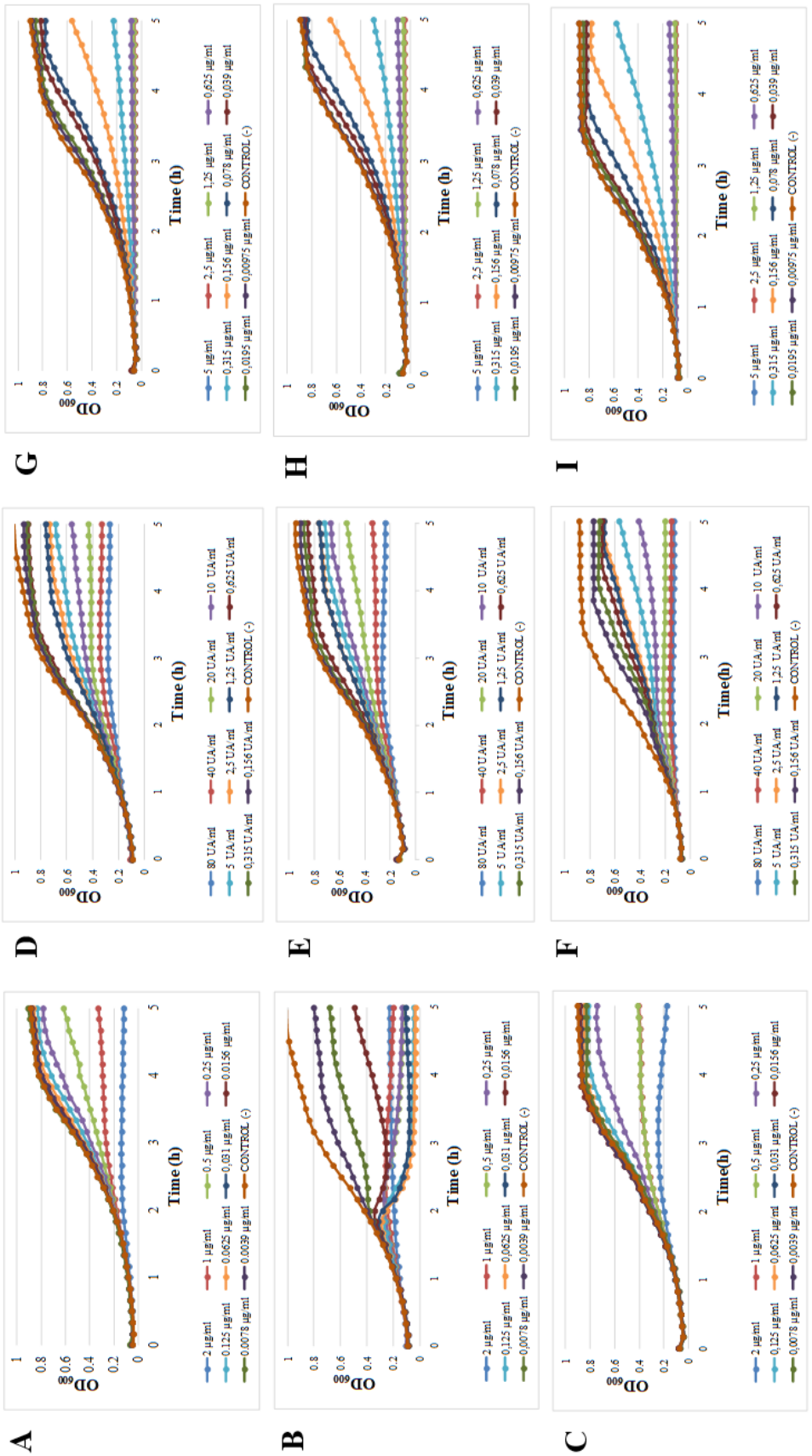

Figure 7: MitC inductions from A to C, Lcn 972 inductions from D to F and bacitracin inductions from G to I. *L. lactis* strain UKLc10 TP712 (A, D and G), UKLc10 TP712/CAP (B, E and H) and UKLc10 CAP (C, F and I). Only lysis is observed to strain UKLc10 TP712 under MitC treatment.

4.2. Autolysis capacity assay

To shed more light on the impact of the prophage in *L. lactis*, an autolysis capacity assay was performed with the strains UKLc10 TP712, UKLc10 TP712/CAP and UKLc10 CAP. The results are shown in the **Figure 8A** and **B**, the lysis in buffer KPi 50 mM serves as a control and the lytic activity of each strain was examined using KPi 50 mM/Triton X-100 (0.05%), respectively. The data shown is represented as the percentage of initial OD_{600} value against the time, and those values belong to the average of three replicates.

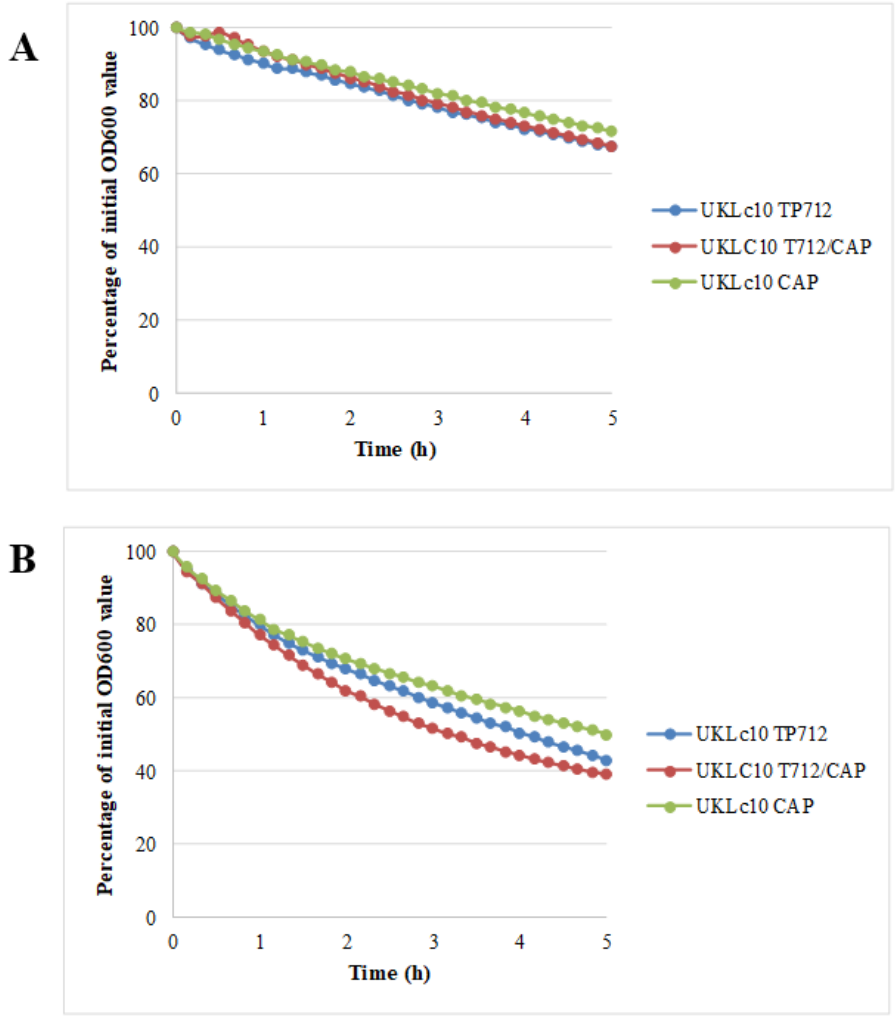

Figure 8: Autolysis in *L. lactis* lysogenic strains**.** A) Strains UKL10 TP712, UKLc10 TP712/CAP and UKLc10 CAP in KPi 50mM suspension. B) Strains UKL10 TP712, UKLc10 TP712/CAP and UKLc10 CAP in KPi 50mM/Triton (0.05%) suspension.

As it can be observed, in presence of KPi 50 mM/Triton X-100 (0.05%), the three strains lysed after 5 hours, however, UKLc10 CAP lysed slower than the other two strains, being the strain UKLc10 TP712/CAP which lysed quicker. Whereas, UKLc10 TP712 showed an intermediate lysis curve (**Figure 8B**). In fact, there were significant differences between stains when comparing the time at which 50% of the cells lysed (comparison in pairs, t-Student, $p<0.05$) (**Table 5**). The strains UKLc10 TP712 and UKLc10 CAP lysed significantly less than UKLc10 TP712/CAP. This results indicates that, indeed, there is an impact of prophage content on bacterial phenotype.

Table 5: Time (hours) at which the 50% of the *L. lactis* cells lysed (comparison in pairs, t-Student <0.05). *UKLc10 TP712/CAP shows significant differences in comparison with UKLc10 TP712 and UKLc10 CAP.

Strains	Time (h)
UKLc10 TP712	4.056±0.344
UKLc10 TP712/CAP	3.276±0.4225*
UKLc10 CAP	4.996±0.763

4.3. Quantification of prophage induction by qPCR assay

After knowing the results of microtiter-plate based prophage induction, quantification assays were conducted, using qPCR in order to estimate the increase or fold ($2^{-\Delta\Delta Ct}$) on the number of prophages' DNA copies. The results are shown in the **Figures 9** and **10** where the relative DNA increase is represented versus the induction time.

An experiment was conducted to observe the spontaneous induction of prophages TP712 and CAP in UKLc10 TP712^{+}/CAP^{+}. In this case, the results show that there was, indeed, a spontaneous induction of both prophages. However, the induction was very small and as it can be observed, CAP exhibited a higher increment over time in comparison to TP712, reaching a value around 4 and 2- fold using as reference time of induction, respectively (values of fold <2 it is not considered as induction) (**Figure 10C**). For the strain UKLc10 TP712, the experiment with MitC (1 µg/ml) although there was no obvious lysis of the cells (i.e. a decrease of

OD_{600}), there was some increase of the prophage TP712 DNA over time (**Figure 9A**), albeit just above the levels with are observed in untreated cultures (see **Figure 10C**). Therefore, in presence of MitC the SOS response is triggered, inducing the replication of the prophage. In contrast, the strain UKLc10 TP712/CAP lysed under the same conditions than UKLc10 TP712 did. In addition, the level of induction of the prophage was much higher at 90 min after the induction, having an increase of 25 whereas for UKLc10 TP712 was only of 4. On the other hand, the induction of the prophage CAP was minimal even after 90 min (**Figure 9A** and **B**). However, it seems that the presence of CAP is necessary for the release of the virions out the bacteria. In order to study the role of CESR in the induction of prophages, quantification assays were done with UKLc10 TP712/CAP using Lcn 972 (80 UA/ml) and bacitracin (0.5 µg/ml) as induction agents. Unexpectedly, with both antimicrobials, the results show a dramatic decrease on the number of DNA copies of both prophages over time (**Figure 10A** and **B**). These data show that an inhibition happened after the addition of the antimicrobials, repressing in some way the induction of the lytic cycle, and, thereby, spontaneous induction.

4.4. *L. lactis* mutant by "gene knock-out" for *cesSR* genes

To see how the CESR is involved and interferes with the life cycle of prophages, many attempts were done to build a *L. lactis* Δ*cesSR* lysogenic strain by gene knock-out. The experiment was performed with the strain UKLc10 TP712/CAP, transforming the cells with the plasmid pCS1966::cesSR for the gene knock-out. However, after plating and incubating the transformants, no growth was observed. In view of these results, the *L. lactis* strain NZ9000 was used to see if the problem was the background of our strain UKLc10 TP712/CAP which may be not suitable for the integration of the plasmid pCS1966::cesSR. We also tested transformation efficiency with the replicative plasmid pIL252 to determine if our cells were competent enough. Both strains were transformed with pIL252 and additionally, the strain NZ9000 was transformed with pCS1966::cesSR in order to compare the transformation efficiency. The values for NZ9000 transformation efficiency with pCS::cesSR was 0 as for UKLc10 TP712/CAP, whilst NZ9000 transformation efficiency with pIL252 was $1.1x10^4$ transformants/µg DNA. However, for

UKLc10 TP712/CAP the obtained value was 0. Therefore, the results indicate that the cells were not competent enough to incorporate successfully.

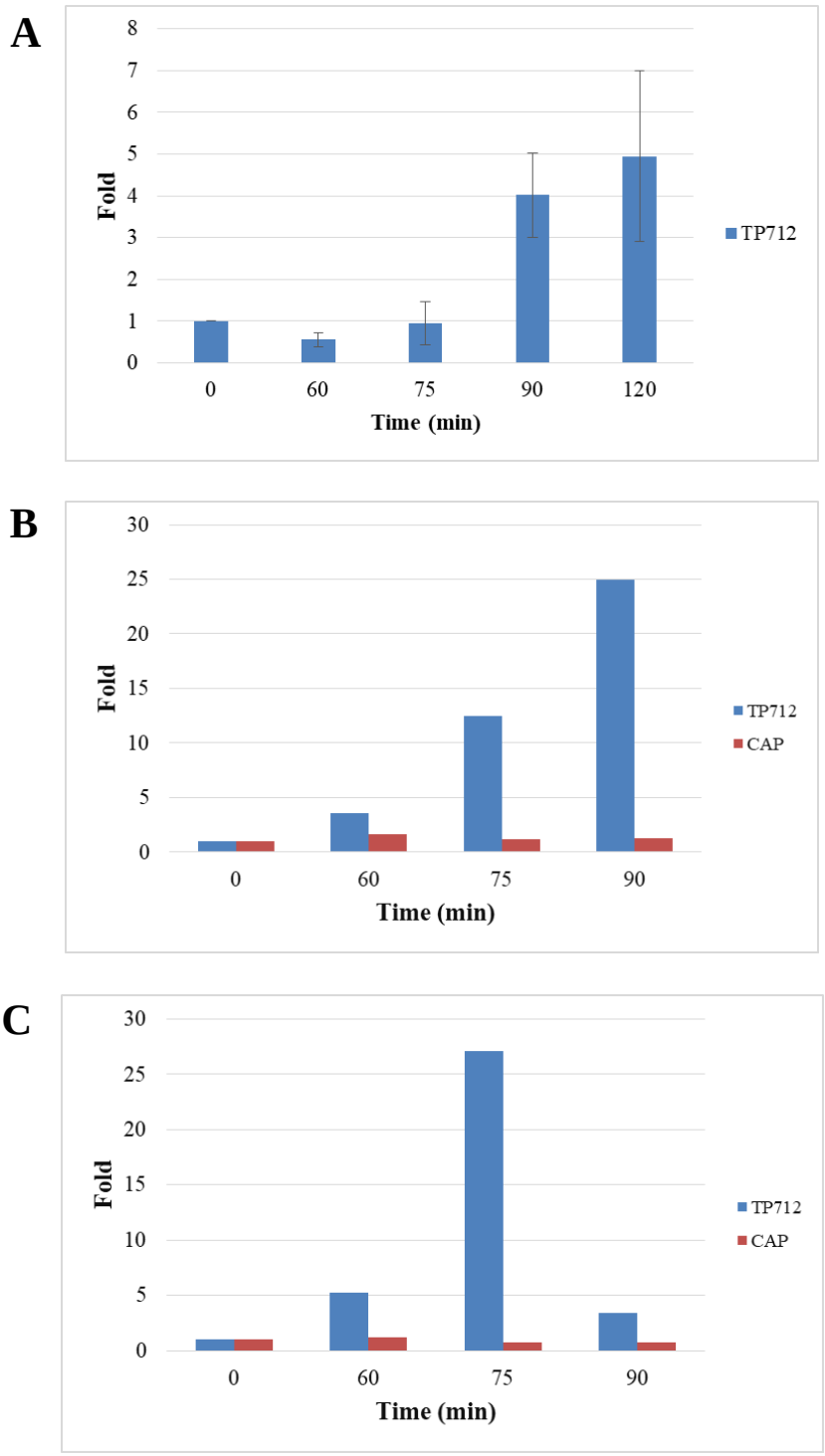

Figure 9: Fold induction of prophage DNA relative to time 0 min as determined by qPCR after induction of *L. lactis* with MitC. A) Strain UKL10 TP712, B) biological replicate 1 UKLc10 TP712/CAP and C) biological replicate 2 UKLc10 TP712/CAP.

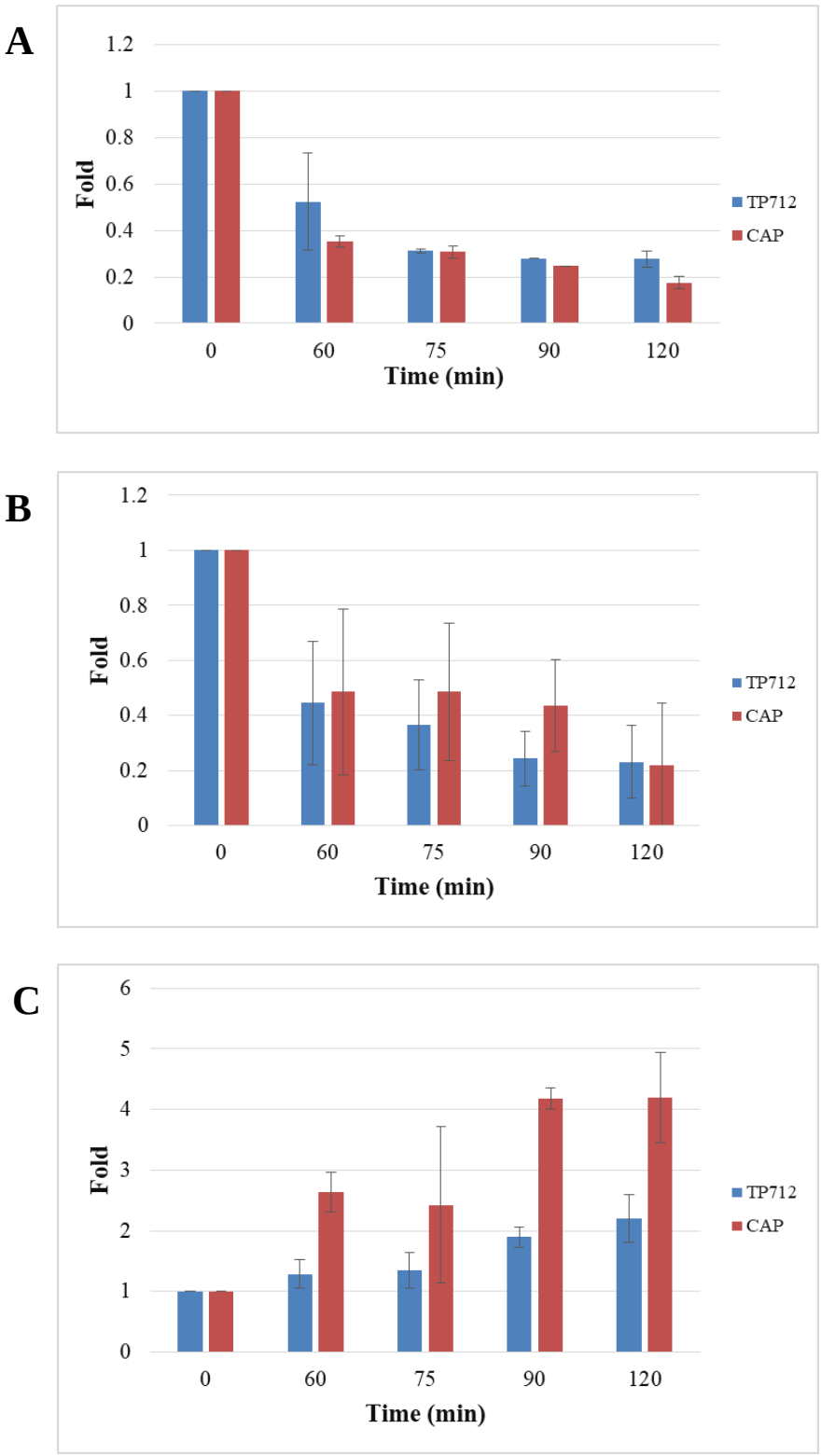

Figure 10: Fold induction of prophages TP712 and CAP DNA relative to time 0 min as determined by qPCR after induction of *L .lactis* with A) Lcn 972, B) bacitracin and C) no-treated culture (spontaneous induction).

5. DISCUSSION

In the current study, the phenotypic impact of prophage burden in *L. lactis* strains and prophage behaviour was investigated in presence of three different antimicrobials, so as to have an insight about the molecular pathways mediating their induction. According to the growth rate and autolytic capacity of UKLc10 TP712, UKLc10 TP712/CAP and UKLc10 CAP, there is indeed an negative impact on the growth of the bacteria when the bacteria carries more number of prophages in its genome. In fact, as it has been described by Aucouturier et al., (2018), some prophage-free strains show a better fitness compared to parental strains under conditions that trigger the induction of prophages. Additionally, as it is reviewed by (Ramisetty & Sudhakari, 2019), harbouring large prophages, as it is the TP712 (42,073 bp), and its genes expression is a metabolic burden so eliminating or minimizing such genetic elements could be beneficial to the host genome. This may explain why it was observed a faster growth rate for the strain UKLc10 CAP since prophage CAP (35,600 bp) is smaller than TP712, whilst for the autolytic capacity the differences may be matter of the number of prophages instead of which prophage is absent. However, in spite of the differences between strain UKLc10 CAP and UKLc10 TP712 are not significant, it can be observed that UKLc10 CAP lyses slower than UKLc10 TP712. Hence one possibility could be that both number and length of genomic elements as prophages have a metabolic impact on its host phenotype. Nevertheless, we detected that there was a spontaneous induction of prophages and this could affect negatively cells' growth rate, since their die after induction. This may explain as well the data from prophage impact assessment in *L. lactis*.

On the other hand, the inductions experiments gave us an insight about prophage behaviour in presence of different types of antimicrobials. It has been widely demonstrated that MitC triggers the SOS response, inducing the lytic cycle of prophages. This was observed in the UKLc10 TP712/CAP strain, but not in UKLc10 TP712 and TP712/CAP when tested under microtiter-based induction conditions. In contrast, quantifications by qPCR confirmed that induction of TP712 also occurred in UKLc10 TP712, although was not as dramatic as for UKLc10 TP712/CAP, having been detected more TP712 DNA copies with this last strain. However, although the cells lysed, an induction of the CAP phage was not observed, since there was no increase in its DNA

concentration. This event leads to think that the virions of TP712 are unable to get out of the cell in the absence of prophage CAP in the host genome.

In view of these results, the question which arises is what is blocking the virions release once they replicate within the bacterial host. As far as it is known, the genes that code for TP712 endolysin and holin system are complete and the proteins are functional in presence of MitC (Roces et al., 2016), hence the problem does not seem to lay down on its activity. Furthermore, in the article of Roces et al., (2016) it was described the presence of another endolysin after induction with MitC, presumably codified and expressed by CAP prophage (Lys-CAP), nevertheless, this has not been confirmed yet. Therefore, due to the complex nature of this phenomenon, in the current manuscript one hypothesis is proposed to explain the underlying molecular mechanisms of the lytic cycle of these prophages.

One possibility could be that the endolysins of both prophages have a synergistic effect, so as the cell only lyses when both proteins are expressed. In this sense two pathways are proposed: i) the signal may come directly from the MitC itself, promoting the transcription of only the Lys-CAP cassette or ii) during the induction of TP712 after treatment with MitC, a signal would be produced promoting the expression of Lys-CAP cassette, for the cooperative release of virions. Therefore, MitC may induce a signal that only activates the transcription the Lys-CAP cassette, without activating the transcription of the other viral genes. Doubts around the role of CAP on the lysis of the cells would be cleared off by performing an induction on the strain UKLc10 CAP with MitC to see whether CAP in absence of prophage TP712 is induced, despite the fact that in the current study it has been shown that this strain does not lyse under such conditions.

In addition, another question has arisen from the results of quantification by qPCR. After performing the induction experiments with Lcn 972 and bacitracin so as to elucidate the contribution of CESR to the induction of prophages, the results were surprisingly different to what it was expected. As it has been mentioned above, during both experiments a decrease of viral DNA was observed. Moreover, after the spontaneous induction experiment both TP712 and CAP DNA copies increased over time, being CAP at certain extent more induced than TP712. Consequently, under treatment of Lcn 972 and bacitracin an inhibition of spontaneous induction of both prophages happened. This phenomenon it is opposed to what was obtained by Madera et al., (2009), since in their

study both Lcn 972 and bacitracin induced the lytic cycle of temperate prophages in lactococcal strain IPLA 513.

Nevertheless, effect of environmental stressors on spontaneous induction have attracted the attention of scientific community. It has been reported that low concentrations of nutrients can stabilize the lysogenic situation of prophages (Alexeeva et al., 2018). Additionally, other researchers as Lunde et al., (2005) observed discrepancies regarding to prophage phILC3 prophage in *L. lactis* under different environmental stresses. They found out as Alexeeva et al., (2018), that the prophage was induced under high temperatures (34.5ºC). On the other hand, although under nutrient depletion conditions they also observed an increase on its induction, when they treated the bacterial cultures with a high concentration of NaCl (1.5%) the effect was the opposite. Ho et al., (2016) also study TP712 in industrial *L. lactis* MG1363, as well as other active prophages in two *L. lactis* strains, in the presence of heat, acid, osmotic, oxidative and antibiotic stressors and they were not able to detect any viral DNA increase either with bacitracin and other environmental stressors after qPCR quantifications. Therefore, our results could reinforce what Alexeeva et al., (2018) suggested in their study, that discrepancies regarding to how bacteria respond to environmental stresses are strain specific.

Finally, lysogenic stabilization mediated by environmental stressors as antimicrobials used in this study (Lcn 972 and bacitracin) and the role of CESR in prophage induction remains unclear because we were unable to generate a lysogen with *ces*SR knock-out mutation. What it is proposed here is that the CesSR system sends a signal in presence of these stressors, displacing the CI-Cro equilibrium towards CI lytic cycle repressor, favouring in this way the lysogeny state of both prophages TP712 and CAP. This could happen in similar way that happens with lamda phage. When the lysogenic pathway is activated, the CII protein is synthesized and stimulates the CI production, antagonizing Cro production. Once the lysogeny is established, CII is no longer needed and is degraded by the protease FtsH (Vohradsky, 2017). Hence, CII serves as key factor in the regulation of lysis-lysogeny decision together with FstH. In fact the gene that codifies for FtsH protein is one of the members of the CesSR regulon in *L. lactis* and it is induced upon CES (Matínez et al., 2007). However, in the study of Roces et al., (2016), it was demonstrated that in absence of *ftsH* gene in UKLc10 TP712/CAP, although, the release of viral progeny is inhibited after MitC treatment, the lytic cycle was activated, as judged

by the increase of phage DNA determined by qPCR. Nevertheless, under Lcn 972 and bacitracin treatment, the expression of *ftsH* gene could be affected at some extent, reinforcing lysogeny and repressing the lytic cycle. However, there is no tangible evidence that demonstrates this event. Moreover, the construction of Δ*cesSR* mutant would have given us more information about the involvement of CesSR system in prophage induction or prophage stabilization and would have help us to elucidate how these complex molecular pathways works.

6. CONCLUSION AND FUTURE PERSPECTIVES

The results obtained in this study demonstrate that prophage burden in *L. lactis* does have, indeed, an effect on its phenotypic features. The strain UKLc10 CAP seems to have an adaptive advantage over the other two strains UKLc10 TP712 and UKLc10 TP712/CAP since the presence of a large prophage could have at some extent a negative impact on host's metabolism.

Regarding to prophage inductions, it has been demonstrated that qPCR is a reliable method for quantification of viral DNA, but the obtained results lead to ask more questions about how CESR contributes to prophage induction in presence and absence of SOS response. Additionally, it remains unclear the role of CAP in the lysis of *L. lactis* after MitC induction as well as how the inhibition of both TP712 and CAP happens under Lcn 972 and bacitracin treatment. The results suggest that there is in fact, a cooperation between the two prophages when the lytic cycle is activated and that CESR has an important role in the inhibition of the spontaneous induction under laboratory conditions. This is the reason why further researches are needed and, in the future, quantification of CAP prophage in UKLc10 CAP by qPCR under the same treatment will help us to understand better its behaviour. Finally, a successful construction of a *L. lactis* Δ*cesSR* mutant will also provide a more accurate insight about CESR in the complex network of both prophage inhibition and induction.

7. BIBLIOGRAPHY

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