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Characterization of beer-spoiling *Lactobacillus brevis* and their associated bacteriophages

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By

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I hereby declare that this thesis is my own work and has not been submitted for another degree, either at University College Cork or elsewhere.

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Abbreviations

ΔpH = Transmembrane proton gradient

Aa = Amino acid

ABC transporter = ATP-binding cassette transporter

Abi = Abortive infection

BLAST = Basic local alignment search tool

BS = Beer-spoiling

CDS = Coding sequence

Cm5 = Chloramphenicol 5 µg/mL

CO_2 = Carbon dioxide

COG = Cluster of orthologous group

COI = Centers of infection

CPS = Capsular polysaccharide

CRISPR = Clustered regularly interspaced short palindromic repeats

CWPS = Cell wall polysaccharide

Dit = Distal tail protein

DMG = Diagnostic marker genes

ECOI = Efficiency of the center of infection

EOP = Efficiency of plaquing

EPS = Exopolysaccharide

ESI-MS/MS = Electrospray ionization tandem mass spectrometry

EU = European Union

GIT = Gastrointestinal tract

GlcNAc = N-Acetylglucosamine

GRAS = Generally regarded as safe

IS = Insertion sequence

ITOL = Interactive tree of life

KIS = LL-K insertion sequence

LAB = Lactic acid bacteria

LTA = Lipoteichoic acid

MCL = Markov clustering algorithm

MFS = Major facilitator superfamily

MitC = Mitomycin C

MOI = Multiplicity of infection

MTP = Major tail protein

MurNAc = N-acetylmuramic acid

NBS = Non-beer spoiling

NCBI = National centre for biotechnology information

NSLAB = Non-starter lactic acid bacteria

nt = nucleotide

ORF = Open reading frame

PAGE = Polyacrylamide gel electrophoresis

PG = Peptidoglycan

PGH = Peptidoglycan hydrolase

PHASTER = Phage search tool enhanced release

PMF = Proton motive force

PS = Polysaccharide

QPS = Qualified presumption of safety

R/M = Restriction/modification system

RBP = Receptor binding protein

ROS = Reactive oxygen species

SDR = Short-chain dehydrogenase/reductase

SDS = Sodium dodecyl sulfate

Sie = Superinfection exclusion

SMRT = Single-molecule-real-time sequencing

SNP = Single-nucleotide polymorphism

TA = Teichoic acid

Tal = Tail fiber protein

TMP = Tape measure protein

WT = Wild-type

WTA = Wall teichoic acid

Abstract

Certain bacteria have acquired the ability to survive and even grow in beer, despite the antimicrobial nature of this harsh liquid environment. Bacterial growth in beer is often attributed to lactic acid bacteria (LAB) that are found in many environments, yet are commonly associated with plant material. While most LAB species bestow positive attributes to foods and food fermentations, others have the potential to survive and even thrive in inhospitable environments, including beer. *Lactobacillus brevis* strains are among the most frequently encountered LAB isolates in spoiled beer. In finished beer products, the presence of *Lb. brevis* is considered undesirable as they typically impart undesirable organoleptic properties on the product including off-flavors, odors and changes in the physical appearance and viscosity. The goal of the current study was to identify and characterize the molecular players that allow certain *Lb. brevis* to grow in and spoil in beer. It was also aimed to isolate phages that infect strains of *Lb. brevis* in order to assess their potential application in the brewing industry for the purpose of preventing or diminishing bacterial spoilage.

The findings described in this thesis have improved our understanding of *Lb. brevis* as a diverse species and as a beer-spoiling microorganism. The genomic features of 19 *Lb. brevis* strains (encompassing the genomes of six *Lb. brevis* strains sequenced in this study and thirteen *Lb. brevis* strains available in public databases) were evaluated in a comparative genome analysis of the species, paying particular attention to evolutionary aspects and adaptation to beer. Moreover, novel molecular players were identified revealing the importance of chromosomal genes for general stress response (pH tolerance), and the relevance of plasmid-encoded genes for beer-specific stress response (such as tolerance to hop compounds). The response of beer-spoiling *Lb. brevis* strains to stress factors associated with beer was assessed using a transcriptomic approach comparing growth in nutritive media with or without the

imposition of various beer-associated stressors including ethanol, low pH and hops. This analysis allowed the identification of a chromosomal gene encoding a presumed manganese transporter involved in low pH tolerance in *Lb. brevis*. Furthermore, the importance of plasmids for beer-spoiling *Lb. brevis* strains was investigated resulting in the identification of a plasmid-encoded putative glycosyltransferase involved in hop tolerance and thus growth in beer.

Furthermore, to alleviate the problem of beer spoilage associated with *Lb.* brevis, the possibility of employing lytic phages capable of infecting such strains can be envisaged as a bio-sanitation approach. This thesis reports on the isolation of five *Lb. brevis*-infecting virulent phages, which were shown to exhibit a high level of genetic and morphological diversity. Interestingly, certain phage isolates displayed activity against *Lb. brevis* beer-spoiling strains preventing them from growing optimally, thus providing a phage-mediated approach to control bacterial spoilage of beer. Moreover, the incidence of prophages among *Lb. brevis* strains was studied in order to understand their potential benefit for their bacterial carrier. Prophage-encoded phage resistance systems such as abortive infection system (Abi), confer to the host resistance to phage infection, thus increasing its overall fitness. Two adjacent genes encoding an Abi system were identified on the prophage sequence of a beer-spoiling *Lb. brevis* strain. This Abi system exhibits activity against phages infecting *Lb. brevis* and *Lactococcus lactis* strains. The presence of such prophage-encoded systems highlights the importance of temperate phages for *Lb. brevis* strains and, in some cases, may explain their resistance to phage infection. With the study and characterization of virulent and temperate *Lb. brevis* phages, we have significantly extended our knowledge on *Lb. brevis* phages. Furthermore, it has provided novel insights into the diversity of *Lb. brevis* phages and their potential application as a bio-sanitation tool in the beer brewing industry.

Chapter I

Introduction

Sections of this chapter have been published as part of a review: Viral genomics and evolution: the fascinating story of dairy phages. *Food Science*. 2020.

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1. Lactic acid bacteria

1.1. General introduction

The lactic acid bacteria (LAB) represent Gram-positive, catalase negative, non-sporulating, rod or coccus-shaped bacteria that produce lactic acid as a major end-product of carbohydrate fermentation [1]. This group includes several genera such as *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus* and *Streptococcus* [1]. LAB are found in many environments including milk, plants and grains, and are of particular interest for the food industry in the production of fermented foods [1,2]. They are predominantly exploited for their acidifying activity and the organoleptic properties that they confer on the final product [3,4]. LAB species have been employed by humans in food production for centuries and many LAB members have been granted the generally regarded as safe (GRAS) status which is required for the regulatory approval of these strains to be used in modern food fermentations [1].

1.2. *Lactobacillus*

Lactobacillus is a very large genus in terms of the number of species (> 200 currently recognized species) it comprises relative to other constituent genera of the LAB group [5]. Lactobacilli are widely used in the food industry for the production of fermented vegetables (e.g. sauerkraut, kimchi) and meats (sausages), and dairy products (cheese, yoghurt) [6]. Fermentation of vegetables often relies on the autochthonous LAB community that is typically present on the raw plant material including members of the *Lactobacillus* genus [7]. Conditions favorable for the growth of *Lactobacillus* species, including anaerobic conditions, high salt and sugar content, allow for their proliferation and consequently drive the associated fermentation

process [8]. Traditional Korean fermented vegetables called kimchi is a good example where acid-tolerant lactobacilli, such as *Lactobacillus plantarum* and *Lactobacillus sakei* become dominant as the fermentation progresses. *Lb. plantarum* is considered to be one of the most acid-tolerant species among the lactobacilli, and this species will dominate the advanced stages of the fermentation process when the environment becomes more acidic and anaerobic [8].

In the case of fermented meats, *Lb. sakei*, *Lb. plantarum* and *Lactobacillus curvatus* are commonly used as starter cultures and for flavor development. Moreover, many strains of these species have been demonstrated to exhibit antimicrobial activity against pathogenic organisms such as *Listeria monocytogenes* and *Staphylococcus aureus* [9].

Lactobacilli that are typically used as starter cultures for the production of fermented milk products, in particular yoghurt and cheeses where they contribute to acidification, include *Lactobacillus delbrueckii* subsp. *bulgaricus* (*Lb. bulgaricus*), *Lactobacillus delbrueckii* subsp. *lactis* (*Lb. lactis*) and *Lactobacillus helveticus* (*Lb. helveticus*) [10,11]. Moreover, *Lb. helveticus* strains commonly participate in flavor formation of the final product due to their proteolytic activities [3]. LAB can also be present in fermented milk products as naturally occurring non-starter LAB (NSLAB) that survived pasteurization and proliferate during the ripening step of the process, consequently contributing to flavor formation and ripening of the final product [12]. The most frequently isolated *Lactobacillus* NSLAB species are *Lb. plantarum, Lactobacillus casei, Lactobacillus paracasei, Lactobacillus rhamnosus* and *Lb. curvatus* [13].

2. *Lactobacillus brevis*

2.1. General characteristics

Lb. brevis is an obligate heterofermentative species, similar to *Lactobacillus reuteri* and *Lactobacillus fermentum*, and when such LAB metabolize carbohydrates this fermentation generates lactic acid, ethanol, acetic acid and carbon dioxide as metabolic end products. *Lb. brevis* strains are capable of fermenting galactose, maltose, lactose, raffinose, sucrose, and xylose [14]. They have been isolated from silage, fermented cabbage or other fermented foods [1,15]. Lactobacilli including *Lb. brevis* strains have also been found as part of the vaginal microbiota of a healthy female [16,17].

2.2. Genomics of *Lb. brevis*

Sixty-seven *Lb. brevis* genome assemblies are currently available on the NCBI (National Center for Biotechnology Information) database; twenty of these represent completely sequenced and fully assembled genomes (Table 1). Of these twenty completed genomes, six were sequenced and assembled as part of this thesis (see Chapter II). The size of a *Lb. brevis* chromosome ranges between 2.29 and 2.79 Mbp with a GC content of 45.8 to 46.6 % (Table 1). *Lb. brevis* strains have been isolated from silage, fermented vegetables (e.g. kimchi) and intestinal microbiota (e.g. in pig or *Drosophila*). Interestingly, almost half of them (nine out of twenty) were isolated from the brewery environment (Table 1). To date, studies of *Lb. brevis* have primarily focused on its role during food fermentation such as in kefir or in sourdough fermentation in the context of flavor development [14,18]. Research has also been conducted to assess the role of *Lb. brevis* in food and beverage spoilage where in some cases it is used to control food spoilage organisms, i.e. certain *Lb. brevis* strains isolated from fermented food

products are known to produce bacteriocins with a broad spectrum of inhibition against pathogenic and food spoilage organisms [19]. *Lb. brevis* strains have also been shown to elicit antifungal activity promoting their use in bread starter cultures to prevent mold spoilage [20]. On the other hand, *Lb. brevis* is itself a spoilage microorganism, in particular in beer where it can compromise the quality of beer [21].

Properties that provide *Lb. brevis* probiotic potential have also been studied revealing strain-dependent low pH and bile salt tolerance, conditions that would be compatible with those present in (parts of) the human gastrointestinal tract (GIT) [22,23]. The antifungal properties coupled with the probiotic and antioxidant characteristics of *Lb. brevis* strain P68 suggest a potential application of this strain in reducing the proliferation of food spoilage fungi as well as its potential application as a functional food supplement [24].

Based on their antibacterial properties against opportunistic pathogens, *Lb. brevis* strains have also been proposed as an alternative to antibiotics for the treatment of human vaginal infections, and for re-balancing the overall vaginal microbiota [17].

Table 1. *Lactobacillus brevis* strains whose genomes are fully sequenced.

Lb. brevis strain	GenBank accession	Ecological niche	Chromosome length (Mbp)	GC %	Plasmids	Reference
100D8	CP015338	Rye silage	2.35	46.1	3	
ATCC 367	CP000416	Silage	2.29	46.2	2	[1]
BDGP6	CP024635	*Drosophila melanogaster* gut	2.79	46.6	-	
KB290	AP012167	Suguki (fermented vegetable)	2.40	46.1	9	[15]
LMT1-73	CP033885	Kimchi	2.49	46.0	2	
NCTC13768	CP015398	Kimchi	2.49	46.0	-	
NPS-QW-145	LS483405	Unknown	2.55	45.8	-	[25]
SA-C12	CP031185	Silage	2.44	45.9	2	[26]
SRCM101106	CP021674	Food	2.44	45.9	4	
SRCM101174	CP021479	Food	2.41	46.1	5	
TMW 1.2108	CP019734	Wheat beer	2.57	45.8	8	[27]
TMW 1.2111	CP019743	Wheat beer	2.57	45.8	6	[27]
TMW 1.2112	CP016797	Wheat beer	2.49	46.0	5	[27]
TMW 1.2113	CP019750	Brewery-associated surface	2.54	45.9	4	[27]
UCCLB521	CP031208	Brewery environment	2.27	46.3	5	[26]
UCCLB556	CP031174	Brewery environment	2.38	46.1	7	[26]
UCCLB95	CP031182	Beer	2.51	45.9	2	[26]
UCCLBBS124	CP031169	Beer keg	2.61	45.8	4	[26]
UCCLBBS449	CP031198	Unpasteurized beer	2.58	45.8	9	[26]
ZLB004	CP021456	Pig's feces	2.66	46.0	5	

2.3. Physiology of *Lb. brevis*

The cell wall of Gram-positive bacteria plays an important role in the maintenance of the cell integrity, as well as interactions with its environment (e.g. surfaces, bacteriophages), and has been well described in LAB including lactobacilli. The cell wall is composed of a peptidoglycan (PG) layer surrounding the cytoplasmic membrane and can be decorated with other glycopolymers such as teichoic acids (TA) or polysaccharides (PS), and proteins (Figure 1) [28]. The PG is made up of glycan chains of alternating N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) that are linked *via* β-1,4 bonds. MurNAc can then covalently bind peptide chains, which form cross-linkage bridges with peptide chains from other PG glycan chains, thereby generating a three-dimensional, mesh-like structure surrounding the cell

and ensuring its structural integrity. PG formation starts in the cytoplasm with the synthesis of lipid II which is then transferred to the extracellular side of the membrane. These externalized lipid II subunits are then polymerized into a macromolecule. TAs are anionic polymers composed of alditol-phosphate repeating units and are classified into two groups: (i) wall teichoic acids (WTA) which are covalently linked to the PG, and (ii) lipoteichoic acids (LTA) which are anchored in the cytoplasmic membrane with a glycolipid moiety. Teichoic acids can be modified where the free hydroxyl groups of the alditol-phosphate chains are replaced by sugars or D-Ala, which modulate the net negative charge of the TA. WTA and LTA are involved in cell wall functionality. For example, they provide a reservoir of ions that may be required for enzymes but they also control autolysins; act as bacteriophage receptors; while they also mediate interactions with the host immune system [28,29]. In addition to PG and TA, Gram-positive cell walls are decorated with PS which can be classified into three groups: exopolysaccharides (EPS) associated with the cell surface and released into the environment, capsular polysaccharides (CPS) attached to the cell surface, and cell wall polysaccharides (CWPS) which are typically covalently bound to the cell wall. The genes responsible for PS biosynthesis are usually organized in clusters containing eight to 25 genes encoding, among other functions, glycosyltransferases and transport proteins. PS have been identified as bacteriophage receptors, and may also be involved in adhesion to abiotic surfaces and biofilm formation. Extracellular proteins are synthesized in the cytoplasm prior to their translocation across the cytoplasmic membrane *via* a signal peptide which is then cleaved off. The translocated protein may be released into the environment or retained in the cell envelope. The latter cell wall-associated proteins are believed to be involved in bacteria-host interactions by facilitating colonization and persistence in the gastrointestinal tract [28].

The structural and compositional diversity of the cell wall among bacterial species explains their differences in environmental stress response, probiotic properties and bacteriophage sensitivity.

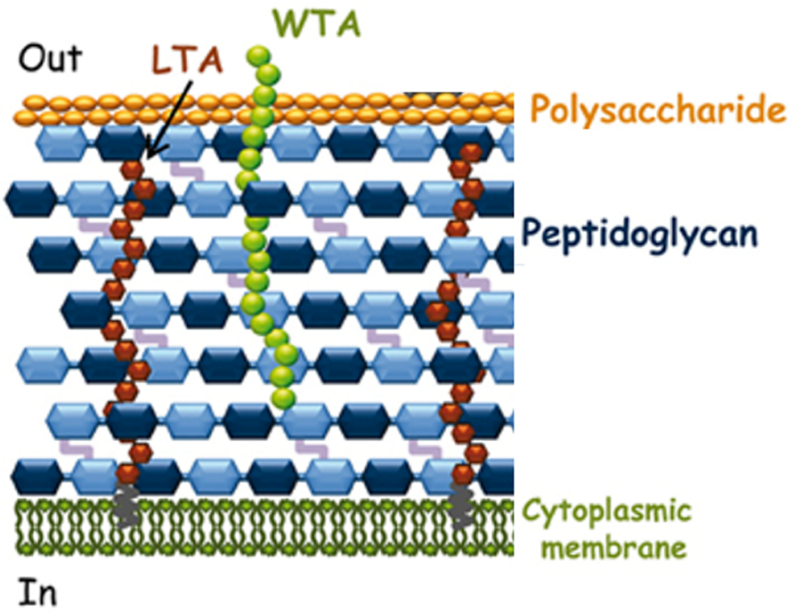

Figure 1. Schematic cross-sectional image of the lactic acid bacterial cell wall structure (LTA: lipoteichoic acids, WTA: wall teichoic acids). Adapted from Chapot-Chartier [29].

2.4. Plasmids of *Lb. brevis*

Lb. brevis strains naturally harbor a varying number of plasmids with (thus far) a maximum of nine plasmids observed in *Lb. brevis* KB290 and UCCLBBS449 (Table 1). *Lb. brevis* KB290 is a strain isolated from Japanese fermented vegetables, containing plasmid pKB290-1 required for GIT tolerance [15]. The presence of glycosyltransferases similar to members of the glycosyltransferase family 2 involved in the production of cell wall polysaccharide (CWPS) such as β-glucan was also identified in several *Lb. brevis* strains such as KB290 and TMW1.2112 [15,27]. Plasmids of *Lb. brevis* have also been associated with hop resistance and

plasmid-associated genes responsible for hop tolerance have been identified and characterized (see section 3.4.) [30,31]. Strains of *Lb. brevis* may encode antibacterial properties such as the production of bacteriocins which can be employed to inhibit growth and biofilm formation of pathogenic or spoilage bacteria [32,33]. For example, *Lb. brevis* 925A produces a bacteriocin, called brevicin 925A, which elicits antagonistic activity against *Ls. monocytogenes* (a food pathogen) and *Streptococcus mutans* (a cariogenic microbe). Detailed characterization found that both the structural gene *breB* and the immunity gene *breE* of the brevicin cluster were present on plasmid pLB925A04 of *Lb. brevis* 925A [34].

2.5. Positive roles of *Lb. brevis* in the fermentation industry

Lb. brevis strains have been widely used in the food fermentation industry primarily for flavor development. *Lb. brevis* strains are employed in dairy fermentations such as in the production of kefir where they contribute to the formation of lactic acid, carbon dioxide (CO_2) and sensory compounds in association with *Lactobacillus kefiri* and yeast [14]. They are also employed in combination with yeast during bread making for the purpose of aroma development (in particular for sourdough fermentation) [18]. As mentioned above, some strains of *Lb. brevis* possess potential probiotic activity [2,14]. In a study from Rönkä et al., *Lb. brevis*, added as a potential probiotic supplement, did not interfere with milk acidification nor did it negatively affect the taste or preservation characteristics of the final product (i.e. yoghurt) [23].

2.6. Undesirable activities of *Lb. brevis* in the fermentation industry

While in some cases LAB are deliberately added as a starter in food fermentation processes such as cheese-making in order to impart desirable traits to the final product, in other cases LAB are considered undesirable [21,35]. *Lb. brevis* strains have been associated with the

production of excessive CO_2 leading to undesirable gas pockets in cheese (a phenomenon referred to as 'blowing', which may cause cracks in cheese). Catabolism of amino acids during cheese ripening by heterofermentative LAB such as *Lb. brevis* may lead to the production of undesirable flavors and odors. Moreover, *Lb. brevis* have been identified as responsible for the formation of biogenic amines, which are formed by decarboxylation of certain amino acids and may cause intoxication with symptoms such as headache or nausea. Therefore, the control of available amino acids and the abundance of biogenic amine-producing bacteria during fermentation is crucial in order to prevent toxic levels of such compounds [14].

Furthermore, *Lb. brevis* strains have also been reported as a beverage spoiler, in particular during the production of wine [36] and beer [21]. They are among the most frequently encountered LAB isolates in spoiled beer, since more than half of the described incidents of beer spoilage have been associated with the presence of this bacterium representing a significant threat to the brewing industry [21]. Strains of this species may be present on raw materials used in breweries and represent a major microbial contaminant during the production and storage of beer. Spoilage by *Lb. brevis* can lead to off-flavor development, turbidity and super-attenuation of beer due to the ability of the species to ferment dextrins and starch [35]. Moreover, beer-spoiling bacteria may produce slime increasing the viscosity and turbidity of beer. This slime is due to the formation of exopolysaccharide (EPS) which, as described above, is believed to confer several advantages to the cells such as adherence or resistance to the environment [37].

3. *Lactobacillus brevis* and beer

3.1. Beer and the brewing process

In 2018, the global annual production of beer exceeded 1.90 billion hectoliters. More than 500 million hectoliters of beer were produced in Europe, representing almost a quarter of the world's production and rendering it a major export product. Heineken is the second biggest brewing company globally, behind AB InBev (Belgium), and accounted for 12.3 % of the world production beer in 2018 [38]. In 2018, the brewing industry was reported to employ more than 2 million people throughout the value chain in the European Union (EU), thus representing a significant contributor to the world economy accounting for around € 50 billion in revenue. These statistics reveal that the brewing industry is an economically significant and successful beverage sector. However, breweries are under constant pressure to improve, innovate, meet consumers' demands and maintain competitiveness in the market owing to new and emerging regulations, safety and quality control requirements, as well as changing consumer demands and preferences.

The principal ingredients of beer are water, malted barley, yeast and hops. Different steps are involved in the brewing process starting from malting, milling, wort boiling, fermentation, biological stabilization and packaging (Figure 2), and are further detailed below.

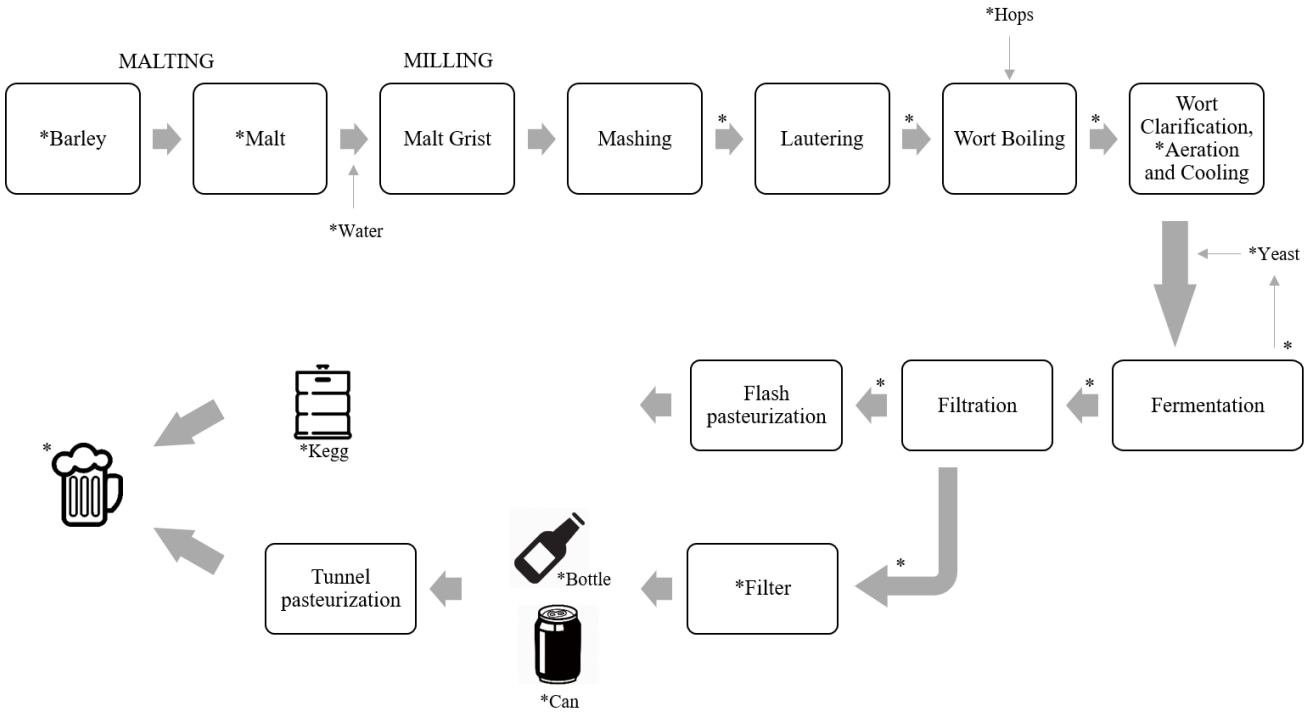

Figure 2. Schematic representation of the brewing process. Potential sources of microbiological contamination are indicated by *. Adapted from Vaughan et al. [35].

3.1.1. Malting

One of the primary activities in brewing is the malting of barley, which consists of three steps: (i) steeping to allow the barley to absorb water; (ii) barley germination during which enzymes are activated to depolymerize starch and proteins, and (iii) drying (kilning) to terminate germination and allow storage of the germinated barley called malt [39] (Figure 2).

3.1.2. Milling and mashing

After the malting step, milling is used to increase the contact surface between the malt and the brewing liquor. Once the grains have been milled, warm water is added, initiating the mashing step. The mixture is gradually warmed up throughout the process increasing enzyme activity to allow conversion of starch into fermentable sugars, mostly by α-amylase and β-amylase enzymes. The final mash mixture consists of the dissolved solution (wort) and an insoluble fraction, which is filtered out during the clarification (or lautering) step [35,39] (Figure 2).

3.1.3. Wort boiling

Following filtration the resulting wort is boiled for 90 to 120 minutes leading to water evaporation, sterilization, enzyme inactivation, as well as flavor and color intensification. During this step, hops are added, which are essential in beer production as they contribute to the flavor of the end-product. Hop compounds are derived from the flowers (cones) of the plant *Humulus lupulus* and are responsible for the bitterness and aromas in beer, while they also possess potent antibacterial properties [39] (Figure 2). Wort boiling also causes precipitation of proteins, β-acids and polyphenols which are removed as hot trub (sediment) in the whirlpool, thereby clarifying the hopped wort.

3.1.4. Fermentation

Once the wort is cooled and aerated, yeast is added to initiate the fermentation process, during which yeasts produce a variety of compounds including ethanol and CO_2. The brewing yeast *Saccharomyces* is the microorganism responsible for ethanol formation and can be categorized

into ale yeasts (*Saccharomyces cerevisiae*) and lager yeasts (*Saccharomyces pastorianus*) [40]. Yeasts assimilate fermentable sugars, amino acids and other nutrients to produce ethanol, CO_2, higher alcohols and esters prior to their removal from the mixture [39]. During the maturation, beer is clarified through yeast sedimentation while flavor formation also occurs at this stage. The beer is then stored, packaged and/or pasteurized [35,39,40] (Figure 2).

3.2. Beer spoilage

The typical composition of beer renders it a hostile environment for microorganisms to survive and grow in, and beer is therefore considered to be microbiologically stable. Indeed, the presence of ethanol (0.5 - 10 % w/w), carbon dioxide (~0.5 % w/w) and hop compounds (~14 - 55 ppm *iso*-α-acids), combined with a low oxygen content (<0.1 ppm), depleted nutritive substances (trace levels) and a low pH (3.8 - 4.7) generally do not support microbial growth. However, some bacteria appear to have undergone adaptations to survive and even grow in this environment [41]. Primary contaminants originate from the brewery environment, equipment and raw materials which each harbor their own microbiota. Secondary contaminants by airborne microorganisms are introduced to the beer during packaging (e.g. canning, bottling or kegging) [35] (Figure 2).

3.2.1. Beer-spoiling microorganisms

Only a small number of species of Gram-positive and Gram-negative bacteria, as well as wild yeasts are able to survive and grow in beer [21,42]. Strictly anaerobic Gram-negative genera such as *Pectinatus* spp. and *Megasphaera* spp. have also been described to grow and spoil beer. In Europe, more than 90 % of beer spoilage incidents are reportedly caused by specific bacteria

that belong to four genera: *Lactobacillus*, *Pediococcus*, *Pectinatus* and *Megasphaera* [21,42]. Growth of these microorganisms in beer is associated with the development of undesirable characteristics including turbidity, acidity and the production of off-flavors such as hydrogen sulfide [42].

Gram-positive beer-spoiling (BS) bacteria predominantly belong to LAB and are responsible for most of the beer spoilage incidents (60 to 90 %). The spoilage ability of lactobacilli is species-specific with a domination of beer spoilage by *Lb. brevis* (more than half of bacterial beer spoilage episodes) and *Lactobacillus lindneri* (15 to 25 % of beer spoilage incidents) (Figure 3). Other *Lactobacillus* species such as *Lb. casei* and *Lb. curvatus* have been encountered during beer spoilage but much less frequently when compared to *Lb. brevis*-related spoilage incidents [21]. Beer spoilage pediococci are predominantly *P. damnosus* and *Pediococcus claussenii* [21]. Among LAB, *Lb. brevis* and *Pediococcus damnosus* are the most problematic spoilage organisms for the brewery industry due to their resistance to hop compounds [21] (Figure 3).

Only a very small number of Gram-negative bacteria have been associated with beer spoilage, among them the obligate aerobic acetic acid bacteria including *Acetobacter* which are easily eliminated due to the low level of oxygen in finished beer. The most problematic organisms are *Pectinatus* and *Megasphaera*, which are strictly anaerobic. *Pectinatus* spp. have been reported in 20 to 30 % of bacterial incidents particularly in non-pasteurized beer [21,35,43] (Figure 3). Beer spoilage by *Pectinatus* spp. leads to extensive turbidity and the development of a “rotten-egg” smell as a result of hydrogen sulfide and methyl mercaptan production as mentioned earlier [43]. Up to 7 % of bacterial beer spoilage has been linked to *Megasphaera* (Figure 3) and more specifically to *Megasphaera cerevisiae*, although this species is sensitive to pH values below 4.1 and ethanol concentrations higher than 2.8 % (w/v) [44].

Yeasts that are present in the brewery environment, but that are not deliberately added to perform wort fermentation, are termed wild yeasts. They are divided into two categories: the *Saccharomyces* wild yeasts and the non-*Saccharomyces* (e.g. *Candida*, *Brettanomyces*). Wild yeasts are less problematic than bacteria in beer spoilage, but are difficult to discriminate from brewing yeasts and therefore their prevalence may be underestimated. They can lead to beer turbidity, off-flavor formation, fermentation failure or super-attenuation of the final product [21,35,45].

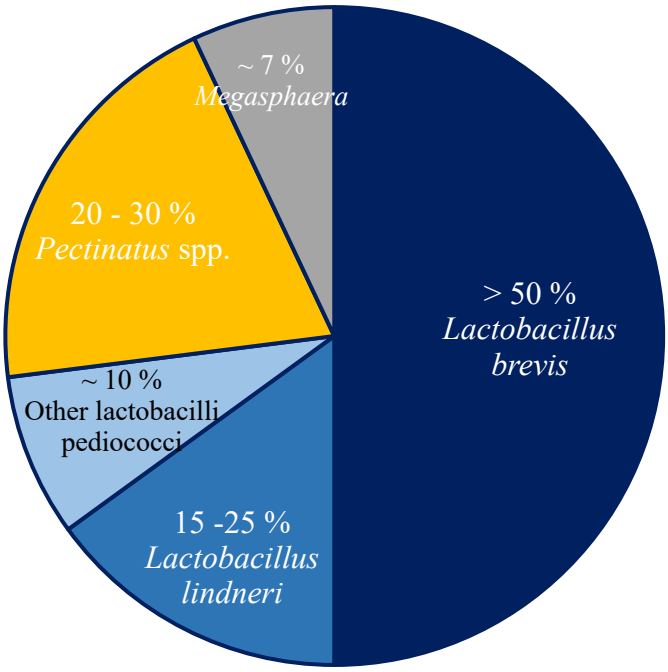

Figure 3. Beer-spoiling bacteria and their incidence in beer spoilage [21].

3.2.2. Beer-spoiling *Lactobacillus brevis*

Lb. brevis is the most frequently reported organism associated with beer spoilage and is, therefore, the greatest threat for breweries [42]. Isolates of this species can be found throughout the brewing process and on raw materials. However, contamination most often occurs in the

finished product, similar to contamination by *Pediococcus* which renders beer ropy and texturally undesirable [35,40]. Beer-spoiling isolates are generally resistant to hop compounds [21,35], while strains isolated outside of the brewery environment exhibit poor or no hop tolerance [42]. *Lb. brevis* strains have also been reported to lose their BS phenotype following repeated sub-cultivation in rich nutritive media and in the absence of hop compounds [41]. Due to the strain-specific nature of this beer spoilage ability, detection of *Lb. brevis* at strain level is required and represents a significant challenge for breweries.

3.3. Antibacterial mechanism of hop compounds

Hop compounds have been shown to exert antibacterial activity against Gram-positive bacteria including LAB strains. These antiseptic properties are due to soluble soft resins coming from the hop cones. These soft resins are then fractionated into α-acids (humulone) and β-acids (lupulone) (Figure 4). During the wort boiling stage of the brewing process α-acids are converted to more bitter and soluble compounds, which are referred to as *iso*-α-acids. Antibacterial activities of α-acids and β-acids are higher than that of *iso*-α-acids, however they are less efficient as they are less soluble in beer. Hop compounds are weak acids and the undissociated forms are responsible for inhibition of bacterial growth [21,35]. The hop constituents (lupulone and humulone) are believed to cause cytoplasmic membrane leakage leading to restriction in sugar and amino acid transport followed by the cessation of DNA, RNA and protein synthesis [46]. In 1993, Simpson showed that the *iso*-α-acid, *trans*-isohumulone, acts as a mobile-carrier ionophore and inhibits bacterial growth by dissipating the transmembrane pH gradient of the proton motive force. This activity is pH dependent and a low pH favors the antibacterial property of the derivative hop compounds [47]. Monovalent (e.g. K^+ or Na^+) and divalent cations (e.g. Mn^{2+}, Mg^{2+} or Ni^{2+}) seem to play a role in the optimal

antibacterial activity of *trans*-isohumulone, although their precise activity remains a matter of speculation [48].

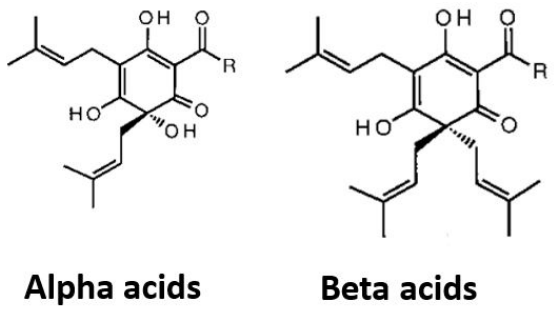

Figure 4. Chemical structures of hop compounds: α-acids (humulone) and β-acids (lupulone). Adapted from Sakamoto and Konings [21].

3.4. Hop resistance mechanisms

While beer is not an environment that is conducive to microbial growth, some bacteria have evolved to survive and grow in beer. *Lb. brevis* appears to be one of the most resistant species to hop compounds, but the degree of hop resistance varies depending on the *Lb. brevis* strain. The study and the understanding of hop resistance mechanisms in bacteria is therefore important for the microbiological control of BS isolates in breweries [21]. Hop resistance mechanisms have been associated with different bacterial properties such as the composition of the cytoplasmic membrane or the cell envelope as described below.

3.4.1. Hop resistance associated with the cytoplasmic membrane

The main mechanism of hop resistance is the active extrusion of the anti-microbial hop compounds from the cell. This function has been associated with the products of *horA* and *horC*, which were first identified in beer-spoling lactobacilli strains [41]. HorA functions as an

ATP-binding cassette multidrug transporter (ABC transporter) to extrude hop compounds from the bacterial cell [49] (Figure 5). HorA is a structural homologue of the multidrug transporter LmrA in *Lactococcus lactis* and was shown to transport similar substrates to LmrA such as ethidium bromide. Unlike *lmrA, horA* is a plasmid-associated gene that was identified in *Lb. brevis* ABBC45 [49]. Subsequently, it was found to be present on plasmids from various BS strains such as *Lb. brevis* BSO 464 [30] and *P. damnosus* ABBC478 [50] suggesting intra- and inter-species horizontal transfer of this gene or plasmid [42]. HorC is a proton motive force (PMF)-dependent multidrug efflux pump and its encoding gene was originally identified in *Lb. lindneri* DSM 20692, which harbors two genes, *horB* and *horC*, that were shown to be absent in a hop-sensitive variant of the wild-type strain [51]. HorB was identified as an AcrR family transcriptional regulator and is believed to control the expression of HorC, the transporter responsible for hop compounds extrusion [51] (Figure 5). The *horC* gene was shown to confer hop resistance and beer spoilage ability after its introduction into the hop-sensitive *Lb. brevis* strain ABBC45^{cc} [52].

Hop compounds can cross the cytoplasmic membrane where they dissociate internally. The release of protons from the hop compounds decreases the intracellular pH and results in the dissipation of the transmembrane proton gradient (ΔpH). This protonophoric action leads to a decrease in bacterial cell viability [21,42]. LAB strains are proposed to counteract the protonophoric activity of hops by increasing the rate of proton expulsion from the cells. To support this hypothesis it has been shown that hop-resistant strains maintain a larger ΔpH than hop-sensitive strains [47]. Moreover, *Lb. brevis* ABBC45 was demonstrated to increase its H^+-ATPase activity after its adaptation to hop compounds [53] suggesting the extrusion of protons by a proton-translocating ATPase. The ATP pool in hop-resistant strains was observed to be larger compared to hop-sensitive strains [54], which can be explained by the high amount of ATP required for the increased activity of the H^+-ATPase as well as the ATP-dependent

transporter HorA. When cultured in beer, *Lb. brevis* ABBC45, *Lb. lindneri* DSM 20690^T and *Lactobacillus paracollinoides* JCM 11969^T metabolize citrate, pyruvate, malate and arginine to support their growth, yielding significant levels of ATP [55] (Figure 5).

Passive defence mechanisms are also important and do not require energy sources such as ATP. In BS *Lb. brevis* the membrane composition is modified with the incorporation of saturated fatty acids (e.g. C16:0) decreasing the membrane fluidity and the intrusion of hop compounds [56] (Figure 5). This high fatty-acid composition has also been found in other spoilage bacteria such as in *Oenococcus oeni* increasing its resistance to ethanol during wine spoilage [57].

3.4.2. Hop resistance associated with the cell envelope

Beer-spoiling strains of *Lb. brevis* have been shown to increase higher molecular weight lipoteichoic acids (LTA) in their cell wall, in the presence of hop compounds [56,58]. This increase is believed to confer resistance to the bacteria by enhancing its extra-cytoplasmic barrier functions. It has also been hypothesized that LTA are reservoirs of divalent cations (e.g. Mn^{2+}), thus competing with hop compounds for access to these ions [56]. A transmembrane protein, HitA, was also identified in relation to hop tolerance and is thought to play a role in the uptake of divalent cations such as Mn^{2+}, while hop compounds have been claimed to exchange protons for cellular divalent cations such as Mn^{2+} [59] (Figure 5). Many proteins involved in energy generation and redox homeostasis are dependent on Mn^{2+}, suggesting the important role of these divalent cations in LAB metabolism and growth [60-62] (Figure 5).

Furthermore *gtf-2*, which encodes a glycosyltransferase-2, was demonstrated to be responsible for the production of excess β-glucan leading to the formation of exopolysaccharide (EPS) [27], thus forming a slimy capsule which is believed to provide BS strains robustness against environmental stress factors (e.g. ethanol, hop compounds). This gene was also identified in

other beverage spoiling strains such as the wine-associated *O. oeni* in which it confers ethanol tolerance [63].

3.4.3. Other hop resistance mechanisms

It has been suggested that LAB strains face oxidative stress due to the presence of hop compounds. The upregulation of Mn^{2+}-dependent enzymes responsible for redox homeostasis represents an adaptive response to oxidative stress [61,64]. Moreover, *Lb. brevis* and *Lb. lindneri* BS strains were observed as smaller rods presumably decreasing its cell size to reduce the surface area when in contact with beer [65].

Hop resistance mechanisms (Figure 5) are complex and multiple defence strategies have been acquired and employed by BS bacteria to survive and grow in beer. This variety of resistance mechanisms illustrates the difficulty for breweries to develop effective ways to identify, control and limit bacterial beer spoilage.

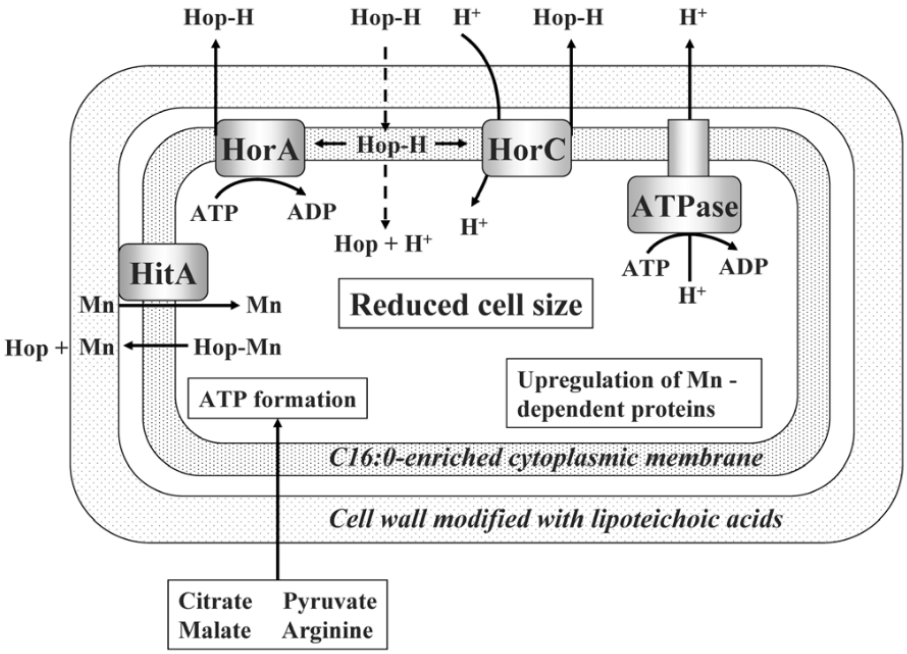

Figure 5. Hop resistance mechanisms in beer spoilage *Lactobacillus brevis* is exerted through a variety of defence strategies [42].

3.5. Bacterial spoilage prevention and control

The consequences of beer spoilage due to growth of unwanted microorganisms are significant to the brewing industry. Financial loss includes the value of the beverage contaminated, the logistical cost of product recall, the cost for replacing the contaminated product as well as eventual compensation for consumers. Further costs may also be incurred when factors such as production time lost due to investigations in identifying the origin of the contamination are considered. Moreover, it would negatively impact on the brand image, which is critical and may also have an economic impact. Therefore, a number of measures have been implemented to minimize the risk of contamination throughout the brewing process [35]. These measures involve working with raw materials that carry a low number of (harmless) microorganisms, producing a sterile wort as well as employing a pure yeast strain during the fermentation (Figure 2). The brewery should be rigorously cleaned and disinfected both internally and externally at all stages from brewing to packaging. Moreover, a suitable hygienic design of equipment should be adopted to minimize contamination. Finally pasteurization or membrane filtration of the final product is used to remove any microbial contamination, and extensive microbiological quality control from brewhouse to packaging is implemented to ensure the quality and safety of the final product [35].

Despite all of these preventive and control measures, contaminations leading to microbial spoilage and consequent costly product recalls still occur. Breweries therefore seek methods to reduce the risks associated with unwanted microorganisms. Currently, many consumers are concerned about food preservation using chemicals and demand for more natural and environmentally-friendly approaches. This has led to the investigation of alternative strategies such as bacteriophage-mediated bio-sanitation to control food and beverage spoilage [66].

4. Bacteriophages

Bacteriophages or phages are viruses that can infect bacterial cells; they are obligate parasites infecting and replicating within the cells leading to cell death and the release of multiple phages which will infect neighboring cells. They were first described in 1915 by Frederick Twort who observed ultramicroscopic viruses inhibiting bacterial growth [67] and a couple of years later by Felix d'Herelle [68]. The major structural components of a phage include a genome-containing capsid, a neck/collar and tail structures.

4.1. Classification of LAB-infecting phages

Classification of phages infecting LAB is based on genome composition/type and morphology. All LAB phages described to date belong to the *Caudovirales* order, characterized as tailed bacteriophages possessing doubled-stranded DNA genomes. The *Caudovirales* order comprises three families: the *Siphoviridae* with long non-contractile tails, the *Myoviridae* with long contractile tails and the *Podoviridae* with short non-contractile tails [69,70].

4.2. Lytic and lysogenic bacteriophage life cycle

Two different life cycles may be employed by a phage following cell infection: lytic infection which results in cell lysis after multiplication and release of phage particles, or lysogenic infection where the phage genome becomes incorporated into the host chromosome. In order to infect, a tailed phage typically recognizes and binds to a specific host-encoded receptor (e.g. carbohydrate or proteinaceous moiety) located on the cell surface of this host, after which the phage genome is injected into the cytoplasm. The injection process may be facilitated by

bacteriophage-mediated peptidoglycan degradation [71,72]. In the lytic life cycle, following phage adsorption and DNA injection, the internalized phage genome is replicated, transcribed and translated using the host cell machinery. Newly replicated phage DNA is packaged into assembled phage particles or virions. The combined action of the phage-encoded lysin and holin causes pore formation in the membrane, lysin-mediated peptidoglycan degradation and cell lysis, and consequently release of mature progeny phage particles [73]. Meanwhile, in the lysogenic life cycle, the phage genome is incorporated into that of the host cell in which state it is termed a prophage. Integration of the phage genome into the host genome is mediated by site-specific recombination between *attP* sites on the phage genome and *attB* sites on the bacterial chromosome. The phage genome is then replicated *in situ* along with the bacterial chromosome. Under certain (typically environmental stress) conditions, the prophage may excise from the bacterial chromosome and enter the lytic life cycle [73].

5. *Lactobacillus* phages

Phages infecting *Lactobacillus* species are genetically diverse and present different interactions with their respective host [72,74]. Currently, genome sequences of 36 *Lactobacillus* phages are available in public databases (https://www.ncbi.nlm.nih.gov, https://www.ebi.ac.uk/genomes/phage.html). Here, we provide an update on the genomic diversity and evolution within *Lactobacillus* phages highlighting their relevance to a variety of food and beverage fermentation industries.

5.1. *Lactobacillus* phages associated with the brewery industry

Strains belonging to the LAB species *Lb. brevis* are the most commonly encountered beer-spoilage microorganisms, yet surprisingly few studies have focused on phages of this species. In fact, only a single temperate phage induced from *Lb. brevis* C30 using mitomycin C has been described to date. The temperate phage LBR48 is 48 Kb in length, it encodes 90 putative Open Reading Frames (ORFs) and was classified as a member of the *Myoviridae* family (Table 2) [75]. In 2011, a virulent *Myoviridae Lb. brevis* phage SA-C12, isolated from silage, showed stability in beer and capable of controlling the growth of its host 56 (Table 2) [76]. Furthermore, in 2011 four *Lb. brevis* siphophages were isolated from sewage and farm slurries that demonstrated the ability to lyse *Lb. brevis* responsible for beer spoilage [77]. In relation to beer spoilage control, five virulent phages of *Lb. brevis* were recently isolated and characterized (Table 2) [78] (see Chapter V). These results indicate the potential of bacteriophage-based treatments for the control of bacterial contamination in beer.

Table 2. General characteristics of the genomes of *Lactobacillus brevis* phages.

Phage	Host	Lifestyle	Isolation source	Genome size (bp)	No. of ORFs	GC content (%)	Reference
Siphoviridae							
ATCCB	*Lb. brevis*	Lytic	Wastewater	80,538	96	30.8	[78]
Myoviridae							
LBR48	*Lb. brevis*	Lysogenic	Unknown	48,211	90	45.9	[75]
SA-C12	*Lb. brevis*	Lytic	Silage	79,099	121	37.5	[76]
3-521	*Lb. brevis*	Lytic	Wastewater	140,816	155	36.9	[78]
521B	*Lb. brevis*	Lytic	Wastewater	136,442	188	32.3	[78]
SAC12B	*Lb. brevis*	Lytic	Wastewater	136,608	191	32.4	[78]
3-SAC12	*Lb. brevis*	Lytic	Wastewater	41,292	61	40.0	[78]

5.2. *Lactobacillus* phages associated with the dairy industry

5.2.1. *Lactobacillus delbrueckii* phages

To date, the genomes of more than 20 strains of *Lb. delbrueckii* and the genomes of ten *Lb. delbrueckii* infecting phages have been fully sequenced, seven of which infect *Lb. delbrueckii* subsp. *bulgaricus* [79-82], and three of which infect *Lb. delbrueckii* subsp. *lactis* [79,83,84] (Table 3). *Lb. delbrueckii* phages are classified among the *Siphoviridae* family with five groups (**a-e**) identified based on their morphology and DNA homology [74]. Several genomic studies have been undertaken to explore the biodiversity of these phages including a study in 2011 by Riipinen et al. in which the genomes of *Lb. delbrueckii* phages belonging to groups **a**, **b** and **c** was undertaken [79]. Subsequently, six *Lb. delbrueckii* phages have been isolated, sequenced and characterized [80-83], adding a new group, group **e**, to the *Lb. delbrueckii* phage family [83].

Table 3. General characteristics of dairy *Lactobacillus* phages whose genomes have been completely sequenced.

Phage	Host	Lifestyle	Isolation source	Genome size (bp)	No. of ORFs	GC content (%)	Head shape	Notes	Reference
Siphoviridae									
c5	*Lb. delbrueckii* subsp. *bulgaricus*	Lytic	Yogurt	31,841	50	41.9	Icosahedral	Group b	[79]
Ld3	*Lb. delbrueckii* subsp. *bulgaricus*	Lytic	Whey	29,616	49	42.2	Icosahedral	Group b	[80]
Ld17	*Lb. delbrueckii* subsp. *bulgaricus*	Lytic	Whey	32,975	50	42.0	Icosahedral	Group b	[80]
Ld25A	*Lb. delbrueckii* subsp. *bulgaricus*	Lytic	Whey	32,799	51	42.2	Icosahedral	Group b	[80]
LLKu	*Lb. delbrueckii* subsp. *bulgaricus*	Lytic	Cheese plant	31,080	51	41.5	Icosahedral	Group b	[79]
phiJB	*Lb. delbrueckii* subsp. *bulgaricus*	Lysogenic	Induced	36,969	46	47.7	Icosahedral	Group a	[81]
phiLdb	*Lb. delbrueckii* subsp. *bulgaricus*	Lytic	Yogurt	33,996	59	41.9	Icosahedral	Group b	[82]
JCL1032	*Lb. delbrueckii* subsp. *lactis*	Lysogenic	Dairy plant	49,433	77	44.2	Prolate	Group c	[79]
Ldl1	*Lb. delbrueckii* subsp. *lactis*	Lytic	Cheese plant	74,806	79	37.76	Icosahedral	Group e	[83]
LL-H	*Lb. delbrueckii* subsp. *lactis*	Lytic	Whey	34,659	51	47.8	Icosahedral	Group a	[84]
Myoviridae									
AQ113	*Lb. helveticus*	Lytic	Grana Padano	36,566	56	37.0	Icosahedral		[85]

5.2.1.1. Classification and general features

Lb. delbrueckii group **a** phages have been studied in considerable detail and are represented by the prototype phage *Lb. delbrueckii* subsp. *lactis* LL-H [86] which exhibits high sequence similarity to the *Lb. delbrueckii* subsp. *lactis* phages LL-K and LL-S [87]. LL-K, LL-H and LL-S are almost identical with the exception of a 1.5 Kb region in LL-K, called the KIS element (LL-K insertion sequence) [87]. Phages in group **a** have isometric heads and *pac*-sites where packaging of phage DNA starts at the *pac* site and continues until the phage procapsid is filled (headful mechanism) [87].

Group **b** *Lb. delbrueckii* phages are the most prevalent in dairy fermentations and are represented by the *Lb. delbrueckii* subsp. *bulgaricus* phage c5 and the *Lb. delbrueckii* subsp. *lactis* phage LL-Ku, which are genetically and morphologically closely related [79,88]. Unlike group **a** phages, group **b** is represented by phages with an isometric head and a *cos*-site [79] where DNA packaging begins at the *cos* site and continues to the following *cos* site where the DNA is cut by a nuclease called the terminase [87].

Group **c** is represented by the temperate phage JCL1032 possessing a prolate head [89] and is the only sequenced dairy *Lactobacillus* phage harboring a prolate head. However, its overall genomic organization is similar to the *Siphoviridae* phage family possessing an isometric head [90]. JCL1032 possesses a larger genome (49.4 Kb) compared to the majority of *Lb. delbrueckii* phages (approximately 33 Kb).

Group **d** consists of the single temperate *Lb. lactis* phage 0252 that may be induced from the *Lb. delbrueckii* strain CNRZ252, and is notable for its long tail. It was placed in a separate group because of its unique hybridization and immunoblotting profiles, but has not yet received further scientific characterization [74].

The most recently identified group, i.e. group **e**, is represented by phage Ldl1, which shares little sequence relatedness to the other described *Lb. delbrueckii* phages highlighting the potential novelty and genetic diversity that may exist among phages of this species [83].

5.2.1.2. Lysogeny/Lysis modules

Group **a** *Lb. delbrueckii* phages may be lysogenic; for example phage mv4 [91] and phiJB [81]. In the case of *Lb. delbrueckii* phages belonging to group **a** (LL-H and phiJB), group **c** (JCL1032) and group **e** (Ldl1), the two-component lysis cassette is located downstream of the tail-associated genes consistent with other LAB phages [79,81,83]. Unlike most LAB phages [90], in *Lb. delbrueckii* phages of the group **b,** the two-component lysis cassette including a holin- and a lysin-encoding gene were identified within the late-transcribed gene module, i.e. after the DNA replication module [80] (Figure 6).

Temperate phages JCL1032 and phiJB harbor lysogeny-related genes with an integrase-encoded gene located downstream of the gene encoding the lysin [81,89] (Figure 6). Similar to other temperate LAB phages [92], the *attP* site is located downstream of the integrase gene in both lysogenic phage genomes, while *attB* sites were identified adjacent to tRNA or tmRNA genes [93]. In contrast, phages from groups **b** and **e,** as well as the group **a** phage LL-H, do not possess lysogeny-related genes indicating obligate lytic phages multiplying only via a lytic cycle.

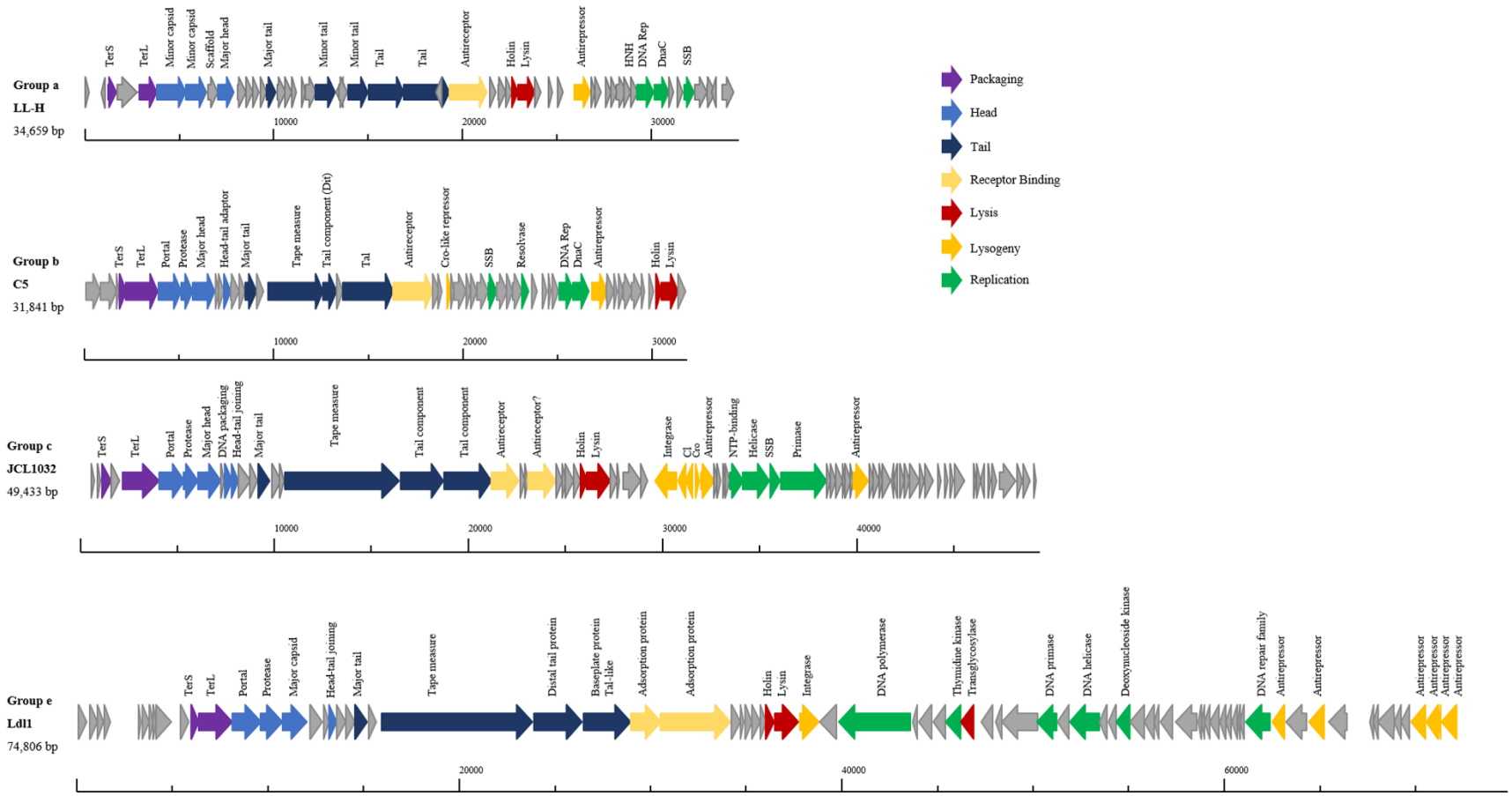

Figure 1. Genome organization of *Lactobacillus delbrueckii* phages. LL-H, c5, JCL1032 and Ldl1 were chosen as representative for each *Lb. delbrueckii* phage groups. The scale at the base of the genomes is in base pairs. Each arrow represents an ORF, with the color representing the putative function of the encoded protein indicated on the right.

5.2.1.3. DNA replication module of dairy *Lactobacillus* phages

Diversity is observed in gene organization of the DNA replication module of *Lactobacillus* phages [94]. *Lb. delbrueckii* phages belonging to group **b** and *Lb. delbrueckii* phage LL-H (group **a**) possess a similar DNA replication module with the gene encoding the putative DNA replication initiator (DnaA) directly upstream of the helicase loader (DnaC) [94] (Figure 6). However, *Lb. delbrueckii* temperate phages JCL1032 (group **c**) and phiJB (group **a**) contain a different replication module consisting of genes that are predicted to encode an NTP-binding protein, a helicase, a single strand binding protein and a primase [79,94]. The replication module of *Lb. delbrueckii* phage Ldl1, which represents group **e**, shares little homology to those of other phages. Ldl1 contains a gene encoding a DNA polymerase as well as a gene coding for a putative DNA repair protein that is absent in the genomes of other *Lb. delbrueckii* phages. Sequence similarity was observed with an equivalent gene encoded by the *Lb. plantarum* phage ATCC 8014-B2 (B2) [95].

5.2.1.4. Morphogenesis

The *pac*-type group **a** *Lb. delbrueckii* phage LL-H has been classified among the Sfi11-like *Siphoviridae* phages due to the gene organization within the head morphogenesis region, i.e. the genome encodes two major head proteins as well as a scaffolding protein [90]. The *cos*-type groups **b** and **c** *Lb. delbrueckii* phages appear to group with the Sf121-like *Siphoviridae* phages [90]. The organization of the structural module bears similarity to *Lb. delbrueckii* phages belonging to group **b** and **c** with a major head protein, a major tail protein, a tail tape measure protein and a tail component protein (two proteins in the case of JCL1032 belonging to group **c**) (Figure 6). Two genes encoding collagen repeat-containing regions and a putative

adsorption protein are present in *Lb. delbrueckii* phages Ld17 and Ld25A, yet are absent from the other *Lb. delbrueckii* group **b** phages suggestive of a deletion or an insertion event [80]. This genome sequence revealed similarity to a region found in the *Lb. delbrueckii* phage LL-K (group **a**) called the KIS element (LL-K insertion sequence) [96]. It is suggested that this "extra" DNA sequence present in Ld17 and Ld25A is responsible for the presence of the collar on these phages [80]. It was also linked to the *bppA* gene in lactococcal phages where the resulting protein interacts with the baseplate structure to increase phage-host affinity [97]. This insertion element may be tied to phage evolution with an increase and diversification in host infectivity [80].

Group **e** *Lb. delbrueckii* phage Ldl1 also exhibits similarity to *Lb. plantarum* phage ATCC 8014-B2, particularly in the structural module from the gene encoding the portal protein to that encoding the major tail protein. The predicted tail tape measure protein (TMP) is 2,627 amino acid long and is responsible for its particularly long tail [83]. The TMP was shown to harbor a peptidoglycan-binding domain as well as a lytic transglycosylase domain implicating this protein in the host infection process [98].

5.2.1.5. Phage receptor-binding proteins

Genes encoding receptor binding proteins (RBPs) (also called anti-receptors) have been identified for various *Lb. delbrueckii* phages. The RBP is responsible for recognition of, and binding to, the bacterial host receptor molecule, thus identified as the host recognition protein. The RBP is well conserved among *Lb. delbrueckii* phages leading to overlapping host ranges between different *Lb. delbrueckii* phage groups. However, divergence is observed in RBPs of phages belonging to the same group contributing to the difference in host specificity.

The group **a** *Lb. lactis* phage LL-H binds its host *Lb. lactis* ATCC 15808 *via* the RBP encoded by *gp71*$_{LL\text{-}H}$. This protein is predicted to protrude from the tail tip as a tail fiber. Adsorption of LL-H to the host cell surface has been shown to involve the C-terminal end of Gp71$_{LL\text{-}H}$ [99]. The Gp20$_{JCL1032}$ protein, which within its C-terminal region exhibits a high degree of similarity to the equivalent region of protein Gp71, was also linked to phage-host recognition in *Lb. delbrueckii* subsp. *lactis* phage JCL1032 [99]. Even though JCL1032 (group **c**) shares little DNA homology with phages from group **a** or **b**, it exhibits an overlapping host range (i.e. *Lb. lactis* ATCC 15808) with group **a** *Lb. delbrueckii* phages that may be explained by the sequence similarity observed between their predicted RBPs [100]. RBPs commonly possess a conserved N-terminal region and a variable C-terminal region believed to be involved in host recognition and specificity [101].

The putative RBPs of *Lb. delbrueckii* group **b** phages are very similar harboring more than 85 % sequence similarity [80]. Group **b** *Lb. delbrueckii* phages show a high degree of similarity within the N-terminal region of their predicted receptor binding proteins, diversity occurs within the C-terminal region that may explain the variation seen in their host range [79,80].

Group **e** *Lb. delbrueckii* phage Ldl1 encodes two potential RBPs downstream of the gene encoding the predicted tail fiber protein (Tal), forming the fiber protruding from the distal end of the tail tip [102]. The first of these predicted RBPs is similar to the N-terminal region of Gp71$_{LL\text{-}H}$ (RBP) [99] while the second protein bears sequence similarity to the C-terminus of the protein Gp71$_{LL\text{-}H}$ and Gp20$_{JCL1032}$ [83]. The role of the C-terminal region of these proteins in host recognition has previously been studied in LL-H and JCL1032 by Ravin et al. [99]. These phages harbor a broad baseplate reminiscent of that of lactococcal subgroup II P335 phages, implying similarity in host attachment mechanisms between these phages [72].

5.2.1.6. Host-encoded receptors

Among Gram-positive bacteria, peptidoglycans, wall teichoic acids (WTA) and lipoteichoic acids (LTA) have been implicated as phage receptors [72]. Adsorption of the phage *Lb. delbrueckii* LL-H to its host *Lb. delbrueckii* ATCC 15808 has been studied in considerable detail suggesting the interaction of the phage receptor protein with the LTA of the *Lb. delbrueckii* strain [103]. In a study conducted by Ravin et al. the results suggest the existence of at least three different types of LTA phage receptors in *Lb. delbrueckii* strains, two of these being specific for isometric-headed phages and one specific for prolate-headed phages [99]. Such differences in host receptors associated with different phage morphologies has previously been observed in phages of lactococci [104]. Meanwhile, Quiberoni et al. suggested the use of an accessory polysaccharide–peptidoglycan complex as phage receptor for *Lb. bulgaricus* phages YAB, BYM and the group **a** phage lb539 [105].

Recent studies on *Lb. delbrueckii* group **b** phages possessing isometric heads reveal that they do not seem to employ lipoteichoic acids as host receptors [106]. The RBP of phage Ld17 is believed to recognize a saccharidic cell surface receptor on the host [106]. The fact that phages infecting the same species, *Lb. delbrueckii,* use different receptors on the host cell, i.e. at least two LTA receptors [99] as well as CWPS [105] for group **a** phages, one LTA receptor for group **c** phage JCL1032 [99], and CWPS for group **b** phages [105,106], highlights the complexity of phage-host interactions (Table 4) and the ability of phages to adapt and increase their host-range infectivity in the dairy processing environment.

Table 4. *Lactobacillus* phage receptors.

Host	Phage	Predicted receptor	Reference
Lb. delbrueckii subsp. *lactis*	LL-H	Lipoteichoic Acid – Glucose moiety	[103]
	JCL1032	Lipoteichoic Acid	[103]
Lb. delbrueckii subsp. *bulgaricus*	Ldl17	Cell Wall Polysaccharide	[106]
	YAB	Cell Wall Polysaccharide – Glucose	[105]
	Lb539	Cell Wall Polysaccharide – Mannose	[105]
	BYM	Cell Wall Polysaccharide – Rhamnose/N-acetylglucosamine	[105]
Lb. helveticus	CNRZ832B1	S-Layer Protein	[107]
Lb. casei	J-1	Cell Wall Polysaccharide – Rhamnose/Galactosamine	[108]
	PL-1	Cell Wall Polysaccharide – Rhamnose	[108]
Lb. plantarum	ATCC8014-B1	Cell Wall Polysaccharide – Galactose	[109]
	ATCC8014-B2	Lipoteichoic Acid – Glucose moiety	[109]

5.2.2. *Lactobacillus helveticus* phages

To date, only one phage infecting *Lb. helveticus* has been sequenced and characterized, i.e. phage AQ113, which was isolated from a natural whey starter culture [110]. The phage belongs to the *Myoviridae* family and was suggested to employ a *pac*-site for DNA packaging [85] (Table 3). Its genome is organized as follows: DNA packaging, morphogenesis, lysis and DNA replication modules (Figure 7). Most of the phage genes shows similarity to phage genes carried by bacterial sequences suggesting a lysogenic origin to this phage. Although no similarity was observed with *Lb. delbrueckii* phages, AQ113 shows similarity with *Lactobacillus gasseri* myophage kc5a and *Lactobacillus johnsonii* Lj771 [85]. The similarity between *Lb. helveticus* phage AQ113 and phages belonging to species typically associated with the human gastrointestinal tract indicates a common ancestor.

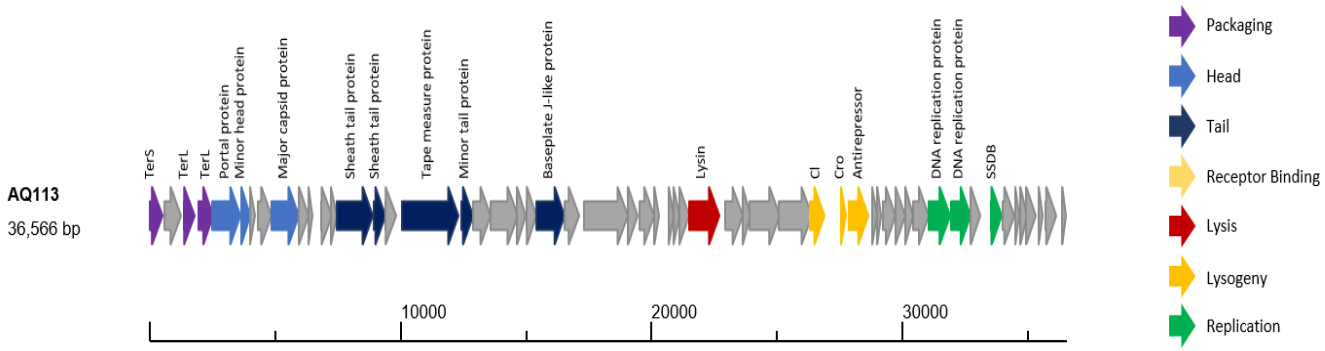

Figure 7. Genome organization of *Lactobacillus helveticus* phage AQ113. The scale at the base of the genome is in base pairs. Each arrow represents an ORF, with the color representing the putative function of the encoded protein indicated on the right.

5.3. Other *Lactobacillus* phages

Non-starter LAB (NSLAB) are naturally occurring LAB strains contributing to the ripening and flavor development during milk fermentation. The most commonly found are *Lactobacillus casei, Lactobacillus rhamnosus,* and *Lactobacillus plantarum*, among others [13]. For these bacterial strains, phages have been isolated, sequenced and characterized [13], and phage-host interactions have been studied as well in certain cases [111].

A2-like phages, such as *Lb. casei* phages phiAT3, J-1 and PL-1, and *Lactobacillus rhamnosus* phages Lc-Nu and Lrm1, are phages that show similarity to the *Lactobacillus casei* phage A2 [108]. *Lb. casei* phages A2 and phiAT3 bear high similarity to the *Lb. rhamnosus* phage Lc-Nu. Their predicted encoded anti-receptors diverge within their C-terminal regions (26 % similarity with A2 and 25 % similarity with phiAT3) compared to their N-terminal region (50 % similarity with A2 and 87 % similarity with phiAT3) [112]. As mentioned above, the C-terminal region of the RBP has been linked to host specificity explaining the ability of phage Lc-Nu and phages A2 and phiAT3 to infect different *Lactobacillus* species. Phages from this

group appear to have evolved over time from a common ancestor leading to closely related phages able to infect distinct *Lactobacillus* species. Phage J-1 and PL-1 host receptors have been identified as a saccharidic cell surface receptor containing galactosamine and/or rhamnose [108] (Table 4).

To date, the genomes of six *Lactobacillus plantarum* phages are available exhibiting similarities to other *Lactobacillus* phages such as the A2-like phages. *Lb. plantarum* phages ATCC8014-B1 and ATCC8014-B2 are genetically and morphologically distinct with ATCC8014-B2 displaying a much larger capsid and the so-far longest observed *Lactobacillus* phage tail (500 nm) [113]. They display divergence in terms of the host recognition component as ATCC8014-B1 employs a cell wall-associated polysaccharide as its receptor, while ATCC8014-B2 uses teichoic acid on the host cell surface to allow adsorption [109] (Table 4). The *Lb. plantarum* phage group is diverse and includes five phages belonging to the *Siphoviridae* family and the phage LP65 belonging to the *Myoviridae* family [114]. *Myoviridae* phages infecting *Lactobacillus* species are less common including, among others, *Lb. helveticus* phage AQ113, *Lb. plantarum* phage LP65 and the lysogenic *Lb. casei* phage Lb338-1.

5.4. Phage dynamics and evolution

A proteomic tree of all currently available *Lactobacillus* phages sequences was constructed to study their evolutionary relationship (Figure 8). The tree highlights the diversity of *Lactobacillus* phages with distinguishable clades observed grouping phages according to their bacterial host. Moreover, *Lb. delbrueckii* phage groups are clearly displayed on the tree reinforcing their division into distinct groups [83] (Figure 8). However, it is noteworthy that some *Lactobacillus* phages show similarity despite infecting distinct *Lactobacillus* species suggesting a common ancestor from which they have evolved and specialized through genetic mutations. Coevolution of phages and strains leads to genetic mutations, which can be beneficial for the strains where modifications within the receptors will prevent phage adsorption and infection, thus increasing the phage resistance of the host. The CWPS of three *Lb. helveticus* [115], *Lb. delbrueckii* Ld17 [116] and *Lb. casei* BL23 [117] strains have been analyzed showing diversity and complexity thus rendering it more difficult for a phage to extend its host range infectivity. *Lb. delbrueckii* strains have also developed defense mechanisms against phage infection using restriction/modification (R/M): mutants of the host strain ATCC 15808 adsorbed phage LL-H, yet did not allow phage infection and development, suggesting an alteration in the DNA specificity of an R/M system [99]. Prophages may also be beneficial to the host by carrying genes that will help the strain survive in its environment. These genes may encode phage-resistance systems such as abortive infection (Abi) systems. Abi systems were shown to block phage multiplication leading to the release of a very low number of phage particles and the death of the infected cells allowing survival of the overall bacterial population [118].

However, mutations may also benefit phages where alterations in the RBP will broaden the host range thus increasing the infective potential and range of the phage. This may explain why

phages of dairy *Lactobacillus* starter strains are genetically and morphologically diverse (Table 3 and Figure 8), being able to use different receptors on the surface of their host(s) (Table 4). Phages have evolved such that ORFs of the DNA replication module show similarity to proteins of bacterial origin highlighting the ancestral co-evolution of two entities (phage and bacterium) into a chimeric temperate phage, such as observed in phage phiJB [81]. Chimeric phages have also been observed among *Streptococcus* phages of the 987 group which exhibit DNA sequence similarity to the morphogenesis modules of certain P335 group *L. lactis* phages and to the replication modules of *Streptococcus thermophilus* phages [119]. This recombination-based system was suggested to be used by *S. thermophilus* phages to evade CRISPR-Cas-mediated immunity [120]. The chimeric phage phiJB may have been assembled after pressure of a CRISPR-Cas system from *Lb. delbrueckii* strains [121] presenting a novel strategy for *Lb. delbrueckii* phages to bypass host defense mechanisms and increase their overall dynamic adaptive ability [81]. Another mechanism of phage adaptation is their ability to lysogenize the host chromosome. Prophages are widely distributed among LAB genomes and may become a threat to a fermentation process if they are induced, thus eliminating their carrying host and targeting sensitive strains present in the starter cultures [122].

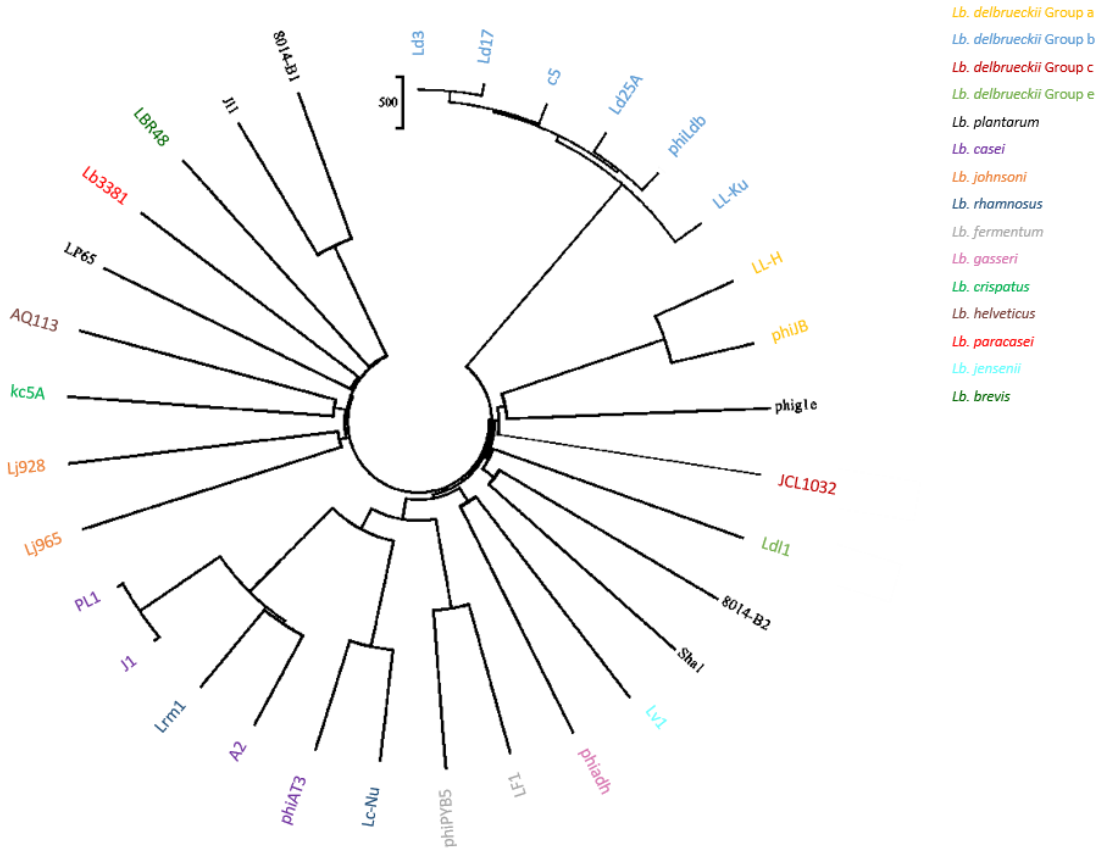

Figure 8. Proteomic tree of all currently available *Lactobacillus* phages constructed using the neighbor-joining method according to the number-of-differences model [83]. The bacterial species that each phage on the tree is known to infect are indicated with different colors. Figure adapted from Casey et al. [83].

5.5. Phages and their potential application in bioremediation

Despite the nature of beer and the array of antimicrobial compounds present in it, beer-tolerant and -resistant bacteria have emerged that negatively impact on the appearance and organoleptic properties of the final product [42]. Biological stabilization and safety approaches are used to increase the safety of beer, employing pasteurization, filtration, suitable packaging, strict cleaning and sanitation practices (Figure 2) [35]. However, the increased demand for non-

pasteurized or alcohol-free beer enhances the risk of microbial spoilage, particularly by LAB [123]. The overuse of antibiotics and chemical solutions has caused resistance development of these spoilage bacteria, moreover corrosive and/or toxic solutions are not allowed in food or beverage industry for consumer safety reasons [124]. The rising consumer demand for more natural and environmentally-friendly approaches has led to the development of a number of alternative strategies to control food and beverage spoilage using antimicrobials such as bacteriocins [125]. Bacteriophages are ubiquitous, specific to their bacterial host and do not represent a danger for humans and have reemerged as a potential bioremediation agent to limit growth of spoilage bacteria [66,76,126]. The impact of bacteriophages to prevent spoilage has mostly been studied for food fermentation applications [66]. Regarding beer fermentation spoilage, the virulent phage SA-C12 has demonstrated activity against *Lb. brevis* BS strains, extending the shelf-life of beer [76]. Moreover, *Lactobacillus* phage-derived endolysins exhibit lytic properties against contaminating lactobacilli found in bio-ethanol fermentations [127]. The potential of phages and phage-derived elements as antimicrobial agents in food and beverage fermentations is certainly becoming prevalent and a promising alternative to currently employed processes. Phages or phage-derived elements may be used during raw material growing (i.e. pre-harvest intervention), during the fermentation processing (i.e. sanitizer) or on the final product (i.e. natural preservative, incorporated into packaging). However, this approach presents challenges and technological hurdles (i.e. safety, phage concentration used, phage resistance potential, scale-up for industrial settings, cleaning) that need to be addressed before the approval by regulatory bodies for phage uses in fermentation control [128].

6. Conclusions and thesis objective

Lb. brevis is widely used in industry for fermentation purposes such as in dairy fermentations for the production of kefir or during sourdough fermentation of bread-making. However, it is also associated with the spoilage of foods and beverages, in particular beer. This thesis is aimed at increasing our current understanding of the mechanisms associated with beer spoilage by *Lb. brevis* as well as studying its associated bacteriophages and to explore their potential for application in bioremediation during the brewing process. Chapter II focuses on a comparative genome analysis of nineteen *Lb. brevis* strains to understand the genetic complexity of this species. Chapter III describes a transcriptomic analysis of BS strains of *Lb. brevis* cultured under different stress conditions encountered in the beer environment. This analysis highlights the role of a chromosomal gene for pH tolerance and manganese homeostasis. Chapter IV focuses on the importance of plasmids carried by *Lb. brevis* strains for hop tolerance and beer spoilage, and more specifically the relevance of a gene encoding a predicted cell surface protein widely present among BS strains of *Lb. brevis*. Chapter V describes the isolation of virulent *Lb. brevis* phages which were further characterized by genome sequencing, host-range analysis, morphology and structural proteome analysis. The activity of these phages against BS of *Lb. brevis* was also investigated with the aim of evaluating the potential of bacteriophage-based treatments in industry to prevent bacterial spoilage of beer. Chapter VI focuses on temperate phages of *Lb. brevis* strains studying their incidence, inducibility and relatedness. Gathering all *Lb. brevis* phages, virulent and temperate, a classification of these phages was proposed based on morphology, genome sequence analyses and phylogeny. Finally, Chapter VII highlights the identification of an Abi system, a phage resistance system encoded by a temperate phage and benefiting the host *Lb. brevis* against phage infection.

7. References

1. Makarova, K.; Slesarev, A.; Wolf, Y.; Sorokin, A.; Mirkin, B.; Koonin, E.; Pavlov, A.; Pavlova, N.; Karamychev, V.; Polouchine, N.; Shakhova, V.; Grigoriev, I.; Lou, Y.; Rohksar, D.; Lucas, S.; Huang, K.; Goodstein, D.M.; Hawkins, T.; Plengvidhya, V.; Welker, D.; Hughes, J.; Goh, Y.; Benson, A.; Baldwin, K.; Lee, J.; Díaz-Muñiz, I.; Dosti, B.; Smeianov, V.; Wechter, W.; Barabote, R.; Lorca, G.; Altermann, E.; Barrangou, R.; Ganesan, B.; Xie, Y.; Rawsthorne, H.; Tamir, D.; Parker, C.; Breidt, F.; Broadbent, J.; Hutkins, R.; O'Sullivan, D.; Steele, J.; Unlu, G.; Saier, M.; Klaenhammer, T.; Richardson, P.; Kozyavkin, S.; Weimer, B.; Mills, D. Comparative genomics of the lactic acid bacteria. *Proceedings of the National Academy of Sciences of the United States of America* **2006**, *103*, 15611-15616, doi:10.1073/pnas.0607117103.
2. Salvetti, E.; Torriani, S.; Felis, G.E. The genus *Lactobacillus*: a taxonomic update. *Probiotics and Antimicrobial Proteins* **2012**, *4*, 217-226, doi:10.1007/s12602-012-9117-8.
3. Wang, H.; Cui, L.; Chen, W.; Zhang, H. An application in gouda cheese manufacture for a strain of *Lactobacillus helveticus* ND01. *International Journal of Dairy Technology* **2011**, *64*, 386-393, doi:doi:10.1111/j.1471-0307.2011.00678.x.
4. Fox, P.F.; McSweeney, P.L.H. Cheese: an overview. In *Cheese (Fourth Edition)*, McSweeney, P.L.H., Fox, P.F., Cotter, P.D., Everett, D.W., Eds. Academic Press: San Diego, **2017**; https://doi.org/10.1016/B978-0-12-417012-4.00001-6pp. 5-21.
5. Sun, Z.; Harris, H.M.; McCann, A.; Guo, C.; Argimón, S.; Zhang, W.; Yang, X.; Jeffery, I.B.; Cooney, J.C.; Kagawa, T.F. Expanding the biotechnology potential of

lactobacilli through comparative genomics of 213 strains and associated genera. *Nature Communications* **2015**, *6*, 8322.

6. Claesson, M.J.; van Sinderen, D.; O'Toole, P.W. The genus *Lactobacillus*--a genomic basis for understanding its diversity. *FEMS Microbiology Letters* **2007**, *269*, 22-28, doi:10.1111/j.1574-6968.2006.00596.x.

7. Tamminen, M.; Joutsjoki, T.; Sjöblom, M.; Joutsen, M.; Palva, A.; Ryhänen, E.L.; Joutsjoki, V. Screening of lactic acid bacteria from fermented vegetables by carbohydrate profiling and PCR–ELISA. *Letters in Applied Microbiology* **2004**, *39*, 439-444.

8. Jung, J.Y.; Lee, S.H.; Jeon, C.O. Kimchi microflora: history, current status, and perspectives for industrial kimchi production. *Applied Microbiology and Biotechnology* **2014**, *98*, 2385-2393, doi:10.1007/s00253-014-5513-1.

9. Papamanoli, E.; Tzanetakis, N.; Litopoulou-Tzanetaki, E.; Kotzekidou, P. Characterization of lactic acid bacteria isolated from a Greek dry-fermented sausage in respect of their technological and probiotic properties. *Meat Science* **2003**, *65*, 859-867, doi:10.1016/s0309-1740(02)00292-9.

10. Sieuwerts, S.; de Bok, F.A.M.; Hugenholtz, J.; van Hylckama, V.; Johan, E.T. Unraveling microbial interactions in food fermentations: from classical to genomics approaches. *Applied and Environmental Microbiology* **2008**, *74*, 4997-5007, doi:10.1128/aem.00113-08.

11. Zago, M.; Suarez, V.; Reinheimer, J.A.; Carminati, D.; Giraffa, G. Spread and variability of the integrase gene in *Lactobacillus delbrueckii* ssp. *lactis* strains and phages isolated from whey starter cultures. *Journal of Applied Microbiology* **2007**, *102*, 344-351, doi:doi:10.1111/j.1365-2672.2006.03101.x.

12. Casey, M., G.; Häni, J., Pierre; Gruskovnjak, J.; Schaeren, W.; Wechsler, D. Characterisation of the non-starter lactic acid bacteria (NSLAB) of Gruyère PDO cheese. *Lait* **2006**, *86*, 407-414.

13. Beresford, T.P.; Fitzsimons, N.A.; Brennan, N.L.; Cogan, T.M. Recent advances in cheese microbiology. *International Dairy Journal* **2001**, *11*, 259-274, doi:https://doi.org/10.1016/S0958-6946(01)00056-5.

14. Ibrahim, S.A. Lactic acid bacteria: *Lactobacillus* spp.: other species. In *Reference Module in Food Science*, Elsevier: **2016**; https://doi.org/10.1016/B978-0-08-100596-5.00857-X.

15. Fukao, M.; Oshima, K.; Morita, H.; Toh, H.; Suda, W.; Kim, S.W.; Suzuki, S.; Yakabe, T.; Hattori, M.; Yajima, N. Genomic analysis by deep sequencing of the probiotic *Lactobacillus brevis* KB290 harboring nine plasmids reveals genomic stability. *PLoS One* **2013**, *8*, doi:10.1371/journal.pone.0060521.

16. Vasquez, A.; Jakobsson, T.; Ahrne, S.; Forsum, U.; Molin, G. Vaginal *Lactobacillus* flora of healthy Swedish women. *Journal of Clinical Microbiology* **2002**, *40*, 2746-2749, doi:10.1128/jcm.40.8.2746-2749.2002.

17. Mastromarino, P.; Brigidi, P.; Macchia, S.; Maggi, L.; Pirovano, F.; Trinchieri, V.; Conte, U.; Matteuzzi, D. Characterization and selection of vaginal *Lactobacillus* strains for the preparation of vaginal tablets. *Journal of Applied Microbiology* **2002**, *93*, 884-893, doi:10.1046/j.1365-2672.2002.01759.x.

18. Meignen, B.; Onno, B.; Gélinas, P.; Infantes, M.; Guilois, S.; Cahagnier, B. Optimization of sourdough fermentation with *Lactobacillus brevis* and baker's yeast. *Food Microbiology* **2001**, *18*, 239-245, doi:https://doi.org/10.1006/fmic.2000.0395.

19. Ogunbanwo, S.; Sanni, A.; Onilude, A. Characterization of bacteriocin produced by *Lactobacillus plantarum* F1 and *Lactobacillus brevis* OG1. *African Journal of Biotechnology* **2003**, *2*, 219-227.

20. Gerez, C.L.; Torino, M.I.; Rollán, G.; de Valdez, G.F. Prevention of bread mould spoilage by using lactic acid bacteria with antifungal properties. *Food Control* **2009**, *20*, 144-148.

21. Sakamoto, K.; Konings, W.N. Beer spoilage bacteria and hop resistance. *International Journal of Food Microbiology* **2003**, *89*, 105-124.

22. Ramos, C.L.; Thorsen, L.; Schwan, R.F.; Jespersen, L. Strain-specific probiotics properties of *Lactobacillus fermentum, Lactobacillus plantarum* and *Lactobacillus brevis* isolates from Brazilian food products. *Food Microbiology* **2013**, *36*, 22-29, doi:https://doi.org/10.1016/j.fm.2013.03.010.

23. Rönkä, E.; Malinen, E.; Saarela, M.; Rinta-Koski, M.; Aarnikunnas, J.; Palva, A. Probiotic and milk technological properties of *Lactobacillus brevis*. *International Journal of Food Microbiology* **2003**, *83*, 63-74, doi:https://doi.org/10.1016/S0168-1605(02)00315-X.

24. Arasu, M.V.; Al-Dhabi, N.A.; Rejiniemon, T.S.; Lee, K.D.; Huxley, V.A.J.; Kim, D.H.; Duraipandiyan, V.; Karuppiah, P.; Choi, K.C. Identification and characterization of *Lactobacillus brevis* P68 with antifungal, antioxidant and probiotic functional properties. *Indian Journal of Microbiology* **2015**, *55*, 19-28, doi:10.1007/s12088-014-0495-3.

25. Wu, Q.; Law, Y.S.; Shah, N.P. Dairy *Streptococcus thermophilus* improves cell viability of *Lactobacillus brevis* NPS-QW-145 and its γ-aminobutyric acid biosynthesis ability in milk. *Scientific Reports* **2015**, *5*, doi:10.1038/srep12885.

26. Feyereisen, M.; Mahony, J.; Kelleher, P.; Roberts, R.J.; O'Sullivan, T.; Geertman, J.-M.A.; van Sinderen, D. Comparative genome analysis of the *Lactobacillus brevis* species. *BMC Genomics* **2019**, *20*, 416, doi:10.1186/s12864-019-5783-1.

27. Fraunhofer, M.E.; Geissler, A.J.; Wefers, D.; Bunzel, M.; Jakob, F.; Vogel, R.F. Characterization of beta-glucan formation by *Lactobacillus brevis* TMW 1.2112 isolated from slimy spoiled beer. *International Journal of Biological Macromolecules* **2018**, *107*, 874-881, doi:10.1016/j.ijbiomac.2017.09.063.

28. Chapot-Chartier, M.-P.; Kulakauskas, S. Cell wall structure and function in lactic acid bacteria. *Microbial Cell Factories* **2014**, *13*, S9, doi:10.1186/1475-2859-13-S1-S9.

29. Chapot-Chartier, M.-P. Interactions of the cell-wall glycopolymers of lactic acid bacteria with their bacteriophages. *Frontiers in Microbiology* **2014**, *5*, 236.

30. Bergsveinson, J.; Baecker, N.; Pittet, V.; Ziola, B. Role of plasmids in *Lactobacillus brevis* BSO 464 hop tolerance and beer spoilage. *Applied and Environnmental Microbiology* **2015**, *81*, 1234-1241, doi:10.1128/aem.02870-14.

31. Sami, M.; Suzuki, K.; Sakamoto, K.; Kadokura, H.; Kitamoto, K.; Yoda, K. A plasmid pRH45 of *Lactobacillus brevis* confers hop resistance. *The Journal of General and Applied Microbiology* **1998**, *44*, 361-363.

32. Sari, N.P.; Sari, R.; Untari, E.K. Antibacterial activity test of bacteriocin from *Lactobacillus brevis, Lactobacillus casei* and *Lactobacillus plantarum* against Gram positive pathogenic bacteria. *Journal of Tropical Biodiversity and Biotechnology* **2018**, *3*, 85-91.

33. Kim, N.-N.; Kim, W.J.; Kang, S.-S. Anti-biofilm effect of crude bacteriocin derived from *Lactobacillus brevis* DF01 on *Escherichia coli* and *Salmonella Typhimurium*. *Food Control* **2019**, *98*, 274-280.

34. Wada, T.; Noda, M.; Kashiwabara, F.; Jeon, H.J.; Shirakawa, A.; Yabu, H.; Matoba, Y.; Kumagai, T.; Sugiyama, M. Characterization of four plasmids harboured in a *Lactobacillus brevis* strain encoding a novel bacteriocin, brevicin 925A, and construction of a shuttle vector for lactic acid bacteria and *Escherichia coli*. *Microbiology* **2009**, *155*, 1726-1737, doi:10.1099/mic.0.022871-0.

35. Vaughan, A.; O'Sullivan, T.; van Sinderen, D. Enhancing the microbiological stability of malt and beer — a review. *Journal of the Institute of Brewing* **2005**, *111*, 355-371, doi:10.1002/j.2050-0416.2005.tb00221.x.

36. Petri, A.; Pfannebecker, J.; Fröhlich, J.; König, H. Fast identification of wine related lactic acid bacteria by multiplex PCR. *Food Microbiology* **2013**, *33*, 48-54, doi:https://doi.org/10.1016/j.fm.2012.08.011.

37. Fraunhofer, M.E.; Geissler, A.J.; Wefers, D.; Bunzel, M.; Jakob, F.; Vogel, R.F. Characterization of β-glucan formation by *Lactobacillus brevis* TMW 1.2112 isolated from slimy spoiled beer. *International Journal of Biological Macromolecules* **2018**, *107*, 874-881, doi:https://doi.org/10.1016/j.ijbiomac.2017.09.063.

38. Barth, S.; Meier, H. The Barth report: hops 2018-2019. Barth-Haas group website. **2019**.

39. Pires, E.; Brányik, T. An overview of the brewing process. In *Biochemistry of Beer Fermentation*, Springer: **2015**; pp. 1-9.

40. Bokulich, N.A.; Bamforth, C.W. The microbiology of malting and brewing. *Microbiology and Molecular Biology Reviews* **2013**, *77*, 157-172, doi:10.1128/MMBR.00060-12.

41. Suzuki, K.; Iijima, K.; Sakamoto, K.; Sami, M.; Yamashita, H. A review of hop resistance in beer spoilage lactic acid bacteria. *Journal of the Institute of Brewing* **2006**, *112*, 173-191.

42. Suzuki, K. 125th anniversary review: microbiological instability of beer caused by spoilage bacteria. *Journal of the Institute of Brewing* **2011**, *117*, 131-155, doi:10.1002/j.2050-0416.2011.tb00454.x.

43. Lee, S.; Madee, M.; Jangaard, N.; Horiuchi, E. *Pectinatus*, a new genus of bacteria capable of growth in hopped beer. *Journal of the Institute of Brewing* **1980**, *86*, 28-30.

44. Haikara, A.; Lounatmaa, K. Characterization of *Megasphaera* sp, a new anaerobic beer spoilage coccus. In Proceedings of Journal of the Institute of Brewing: **1987**; pp. 161-161.

45. van der Aa Kuhle, A.; Jespersen, L. Detection and identification of wild yeasts in lager breweries. *International Journal of Food Microbiology* **1998**, *43*, 205-213.

46. Teuber, M.; Schmalreck, A.F. Membrane leakage in *Bacillus subtilis* 168 induced by the hop constituents lupulone, humulone, isohumulone and humulinic acid. *Archiv für Mikrobiologie* **1973**, *94*, 159-171, doi:10.1007/bf00416690.

47. Simpson, W.J. Studies on the sensitivity of lactic acid bacteria to hop bitter acids. *Journal of the Institute of Brewing* **1993**, *99*, 405-411, doi:10.1002/j.2050-0416.1993.tb01180.x.

48. Simpson, W.J.; Hughes, P.S. Cooperative binding of potassium ions to *trans*-isohumulone in the presence of divalent and trivalent cations. *Bioorganic and Medicinal Chemistry Letters* **1993**, *3*, 769-772.

49. Sakamoto, K.; Margolles, A.; van Veen, H.W.; Konings, W.N. Hop resistance in the beer spoilage bacterium *Lactobacillus brevis* is mediated by the ATP-Binding Cassette multidrug transporter HorA. *Journal of Bacteriology* **2001**, *183*, 5371-5375, doi:10.1128/JB.183.18.5371-5375.2001.

50. Suzuki, K.; Sami, M.; Iijima, K.; Ozaki, K.; Yamashita, H. Characterization of *horA* and its flanking regions of *Pediococcus damnosus* ABBC478 and development of more

specific and sensitive *horA* PCR method. *Letters in Applied Microbiology* **2006**, *42*, 392-399.

51. Suzuki, K.; Iijima, K.; Ozaki, K.; Yamashita, H. Isolation of a hop-sensitive variant of *Lactobacillus lindneri* and identification of genetic markers for beer spoilage ability of lactic acid bacteria. *Applied and Environmental Microbiology* **2005**, *71*, 5089-5097.

52. Iijima, K.; Suzuki, K.; Ozaki, K.; Yamashita, H. HorC confers beer-spoilage ability on hop-sensitive *Lactobacillus brevis* ABBC45cc. *Journal of Applied Microbiology* **2006**, *100*, 1282-1288, doi:10.1111/j.1365-2672.2006.02869.x.

53. Sakamoto, K.; Van Veen, H.; Saito, H.; Kobayashi, H.; Konings, W.N. Membrane-bound ATPase contributes to hop resistance of *Lactobacillus brevis*. *Applied and Environmental Microbiology* **2002**, *68*, 5374-5378.

54. Simpson, W.; Fernandez, J.L. Mechanism of resistance of lactic acid bacteria to *trans*-isohumulone. *Journal of the American Society of Brewing Chemists* **1994**, *52*, 9-11.

55. Suzuki, K.; Iijima, K.; Ozaki, K.; Yamashita, H. Study on ATP production of lactic acid bacteria in beer and development of a rapid pre-screening method for beer-spoilage bacteria. *Journal of the Institute of Brewing* **2005**, *111*, 328-335.

56. Behr, J.; Gänzle, M.G.; Vogel, R.F. Characterization of a highly hop-resistant *Lactobacillus brevis* strain lacking hop transport. *Applied and Environmental Microbiology* **2006**, *72*, 6483-6492.

57. Grandvalet, C.; Assad-García, J.S.; Chu-Ky, S.; Tollot, M.; Guzzo, J.; Gresti, J.; Tourdot-Marechal, R. Changes in membrane lipid composition in ethanol-and acid-adapted *Oenococcus oeni* cells: characterization of the *cfa* gene by heterologous complementation. *Microbiology* **2008**, *154*, 2611-2619.

58. Yasui, T.; Yoda, K. Purification and partial characterization of an antigen specific to *Lactobacillus brevis* strains with beer spoilage activity. *FEMS Microbiology Letters* **1997**, *151*, 169-176.

59. Hayashi, N.; Ito, M.; Horiike, S.; Taguchi, H. Molecular cloning of a putative divalent-cation transporter gene as a new genetic marker for the identification of *Lactobacillus brevis* strains capable of growing in beer. *Applied Microbiology and Biotechnology* **2001**, *55*, 596-603.

60. Watanabe, M.; van der Veen, S.; Nakajima, H.; Abee, T. Effect of respiration and manganese on oxidative stress resistance of *Lactobacillus plantarum* WCFS1. *Microbiology* **2012**, *158*, 293-300, doi:10.1099/mic.0.051250-0.

61. Zhao, Y.; Knøchel, S.; Siegumfeldt, H. Heterogeneity between and within strains of *Lactobacillus brevis e*xposed to beer compounds. *Frontiers in Microbiology* **2017**, *8*, 239-239, doi:10.3389/fmicb.2017.00239.

62. Archibald, F. Manganese: its acquisition by and function in the Lactic Acid Bacteria. *Critical Reviews in Microbiology* **1986**, *13*, 63-109, doi:10.3109/10408418609108735.

63. Dols-Lafargue, M.; Lee, H.Y.; Le Marrec, C.; Heyraud, A.; Chambat, G.; Lonvaud-Funel, A. Characterization of *gtf*, a glucosyltransferase gene in the genomes of *Pediococcus parvulus* and *Oenococcus oeni*, two bacterial species commonly found in wine. *Applied and Environmental Microbiology* **2008**, *74*, 4079-4090.

64. Behr, J.; Vogel, R.F. Mechanisms of hop inhibition include the transmembrane redox reaction. *Applied and Environmental Microbiology* **2010**, *76*, 142-149.

65. Asano, S.; Suzuki, K.; Iijima, K.; Motoyama, Y.; Kuriyama, H.; Kitagawa, Y. Effects of morphological changes in beer-spoilage lactic acid bacteria on membrane filtration in breweries. *Journal of Bioscience and Bioengineering* **2007**, *104*, 334-338.

66. Endersen, L.; O'Mahony, J.; Hill, C.; Ross, R.P.; McAuliffe, O.; Coffey, A. Phage therapy in the food industry. *Annual Review of Food Science and Technology* **2014**, *5*, 327-349, doi:10.1146/annurev-food-030713-092415.

67. Twort, F.W. An investigation on the nature of ultra-microscopic viruses. *The Lancet* **1915**, *186*, 1241-1243.

68. d'Herelle, F. Sur un microbe invisible antagoniste des bacilles dysentériques. *CR Académie des Sciences de Paris* **1917**, *165*, 373-375.

69. Deveau, H.; Labrie, S.J.; Chopin, M.-C.; Moineau, S. Biodiversity and classification of lactococcal phages. *Applied and Environmental Microbiology* **2006**, *72*, 4338-4346.

70. Mahony, J.; van Sinderen, D. Current taxonomy of phages infecting lactic acid bacteria. *Frontiers in Microbiology* **2014**, *5*, 7.

71. Mahony, J.; van Sinderen, D. Structural aspects of the interaction of dairy phages with their host bacteria. *Viruses* **2012**, *4*, 1410-1424, doi:10.3390/v4091410.

72. Mahony, J.; Cambillau, C.; van Sinderen, D. Host recognition by lactic acid bacterial phages. *FEMS Microbiology Reviews* **2017**, *41*, S16-S26, doi:10.1093/femsre/fux019.

73. Sturino, J.M.; Klaenhammer, T.R. Bacteriophage defense systems and strategies for lactic acid bacteria. *Advances in Applied Microbiology* **2004**, *56*, 332-378.

74. Villion, M.; Moineau, S. Bacteriophages of *Lactobacillus*. *Frontiers in Bioscience* **2009**, *14*, 1661-1683.

75. Jang, S.H.; Yoon, B.H.; Chang, H.I. Complete nucleotide sequence of the temperate bacteriophage LBR48, a new member of the family *Myoviridae*. *Archives of Virology* **2011**, *156*, 319-322, doi:10.1007/s00705-010-0841-7.

76. Deasy, T.; Mahony, J.; Neve, H.; Heller, K.J.; van Sinderen, D. Isolation of a virulent *Lactobacillus brevis* phage and its application in the control of beer spoilage. *Journal of Food Protection* **2011**, ***74***, 2157-2161, doi:10.4315/0362-028x.jfp-11-262.

77. Kelly, D.; Neve, H.; McAuliffe, O.; Ross, R.P.; Arendt, E.K.; Coffey, A. Isolation and characterization of bacteriophages that inhibit strains of *Pediococcus damnosus, Lactobacillus brevis,* and *Lactobacillus paraplantarum* that cause beer spoilage. *Journal of the American Society of Brewing Chemists* **2011**, *69*, 8-12.

78. Feyereisen, M.; Mahony, J.; Lugli, G.A.; Ventura, M.; Neve, H.; Franz, C.M.A.P.; Noben, J.-P.; O'Sullivan, T.; van Sinderen, D. Isolation and characterization of *Lactobacillus brevis* phages. *Viruses* **2019**, *11*, 393.

79. Riipinen, K.-A.; Forsman, P.; Alatossava, T. The genomes and comparative genomics of *Lactobacillus delbrueckii* phages. *Archives of Virology* **2011**, *156*, 1217-1233, doi:10.1007/s00705-011-0980-5.

80. Casey, E.; Mahony, J.; O'Connell-Motherway, M.; Bottacini, F.; Cornelissen, A.; Neve, H.; Heller, K.J.; Noben, J.-P.; Dal Bello, F.; van Sinderen, D. Molecular characterization of three *Lactobacillus delbrueckii* subsp. *bulgaricus* phages. *Applied and Environmental Microbiology* **2014**, 10.1128/aem.01268-14, doi:10.1128/aem.01268-14.

81. Guo, T.; Zhang, C.; Xin, Y.; Xin, M.; Kong, J. A novel chimeric prophage vB_LdeS-phiJB from commercial *Lactobacillus delbrueckii* subsp. *bulgaricus*. *Journal of Industrial Microbiology and Biotechnology* **2016**, *43*, 681-689, doi:10.1007/s10295-016-1739-5.

82. Wang, S.; Kong, J.; Gao, C.; Guo, T.; Liu, X. Isolation and characterization of a novel virulent phage (phiLdb) of *Lactobacillus delbrueckii*. *International Journal of Food Microbiology* **2010**, *137*, 22-27, doi:https://doi.org/10.1016/j.ijfoodmicro.2009.10.024.

83. Casey, E.; Mahony, J.; Neve, H.; Noben, J.-P.; Dal Bello, F.; van Sinderen, D. Novel phage group infecting *Lactobacillus delbrueckii* subsp. *lactis*, as revealed by genomic

and proteomic analysis of bacteriophage Ldl1. *Applied and Environmental Microbiology* **2015**, *81*, 1319-1326, doi:10.1128/aem.03413-14.

84. Mikkonen, M.; Räisänen, L.; Alatossava, T. The early gene region completes the nucleotide sequence of *Lactobacillus delbrueckii* subsp. *lactis* phage LL-H. *Gene* **1996**, *175*, 49-57, doi:https://doi.org/10.1016/0378-1119(96)00119-9.

85. Zago, M.; Scaltriti, E.; Rossetti, L.; Guffanti, A.; Armiento, A.; Fornasari, M.E.; Grolli, S.; Carminati, D.; Brini, E.; Pavan, P.; Felsani, A.; D'Urzo, A.; Moles, A.; Claude, J.-B.; Grandori, R.; Ramoni, R.; Giraffa, G. Characterization of the genome of the dairy *Lactobacillus helveticus* bacteriophage ΦAQ113. *Applied and Environmental Microbiology* **2013**, *79*, 4712-4718, doi:10.1128/aem.00620-13.

86. Alatossava, T.; Pyhtila, M. Characterization of a new *Lactobacillus lactis* bacteriophage. *IRCS Medical Science, Library Compendium* **1980**, *8*, 297-298.

87. Forsman, P.; Alatossava, T. Genetic variation of *Lactobacillus delbrueckii* subsp. *lactis* bacteriophages isolated from cheese processing plants in Finland. *Applied and Environmental Microbiology* **1991**, *57*, 1805-1812.

88. Mata, M.; Trautwetter, A.; Luthaud, G.; Ritzenthaler, P. Thirteen virulent and temperate bacteriophages of *Lactobacillus bulgaricus* and *Lactobacillus lactis* belong to a single DNA homology group. *Applied and Environmental Microbiology* **1986**, *52*, 812-818.

89. Riipinen, K.A.; Raisanen, L.; Alatossava, T. Integration of the group c phage JCL1032 of *Lactobacillus delbrueckii* subsp. *lactis* and complex phage resistance of the host. *Journal of Applied Microbiology* **2007**, *103*, 2465-2475, doi:10.1111/j.1365-2672.2007.03479.x.

90. Desiere, F.; Lucchini, S.; Canchaya, C.; Ventura, M.; Brüssow, H. Comparative genomics of phages and prophages in lactic acid bacteria. *Antonie van Leeuwenhoek* **2002**, *82*, 73-91, doi:10.1023/a:1020676825358.

91. Dupont, L.; Boizet-Bonhoure, B.; Coddeville, M.; Auvray, F.; Ritzenthaler, P. Characterization of genetic elements required for site-specific integration of *Lactobacillus delbrueckii* subsp. *bulgaricus* bacteriophage mv4 and construction of an integration-proficient vector for *Lactobacillus plantarum*. *Journal of Bacteriology* **1995**, *177*, 586-595.

92. Brüssow, H. Phages of dairy bacteria. *Annual Review of Microbiology* **2001**, *55*, 283-303, doi:10.1146/annurev.micro.55.1.283.

93. Williams, K.P. Integration sites for genetic elements in prokaryotic tRNA and tmRNA genes: sublocation preference of integrase subfamilies. *Nucleic Acids Research* **2002**, *30*, 866-875.

94. Weigel, C.; Seitz, H. Bacteriophage replication modules. *FEMS Microbiology Reviews* **2006**, *30*, 321-381, doi:10.1111/j.1574-6976.2006.00015.x.

95. Briggiler Marcó, M.; Garneau, J.E.; Tremblay, D.; Quiberoni, A.; Moineau, S. Characterization of two virulent phages of *Lactobacillus plantarum*. *Applied and Environmental Microbiology* **2012**, *78*, 8719-8734, doi:10.1128/aem.02565-12.

96. Forsman, P.; Alatossava, T. Repeated sequences and the sites of genome rearrangements in bacteriophages of *Lactobacillus delbrueckii* subsp. *lactis*. *Archives of Virology* **1994**, *137*, 43-54.

97. Collins, B.; Bebeacua, C.; Mahony, J.; Blangy, S.; Douillard, F.P.; Veesler, D.; Cambillau, C.; van Sinderen, D. Structure and functional analysis of the host recognition device of lactococcal phage Tuc2009. *Journal of Virology* **2013**, *87*, 8429-8440, doi:10.1128/jvi.00907-13.

98. Boulanger, P.; Jacquot, P.; Plançon, L.; Chami, M.; Engel, A.; Parquet, C.; Herbeuval, C.; Letellier, L. Phage T5 straight tail fiber is a multifunctional protein acting as a tape measure and carrying fusogenic and muralytic activities. *Journal of Biological Chemistry* **2008**.

99. Ravin, V.; Räisänen, L.; Alatossava, T. A conserved C-terminal region in Gp71 of the small isometric-head phage LL-H and ORF474 of the prolate-head phage JCL1032 is implicated in specificity of adsorption of phage to its host, *Lactobacillus delbrueckii*. *Journal of Bacteriology* **2002**, *184*, 2455-2459, doi:10.1128/jb.184.9.2455-2459.2002.

100. Forsman, P. Characterization of a prolate-headed bacteriophage of *Lactobacillus delbrueckii* subsp. *lactis*, and its DNA homology with isometric-headed phages. *Archives of Virology* **1993**, *132*, 321-330.

101. Dupont, K.; Vogensen, F.K.; Neve, H.; Bresciani, J.; Josephsen, J. Identification of the receptor-binding protein in 936-species lactococcal bacteriophages. *Applied and Environmental Microbiology* **2004**, *70*, 5818-5824.

102. Veesler, D.; Spinelli, S.; Mahony, J.; Lichière, J.; Blangy, S.; Bricogne, G.; Legrand, P.; Ortiz-Lombardia, M.; Campanacci, V.; van Sinderen, D. Structure of the phage TP901-1 1.8 MDa baseplate suggests an alternative host adhesion mechanism. *Proceedings of the National Academy of Sciences* **2012**, *109*, 8954-8958.

103. Räisänen, L.; Schubert, K.; Jaakonsaari, T.; Alatossava, T. Characterization of lipoteichoic acids as *Lactobacillus delbrueckii* phage receptor components. *Journal of Bacteriology* **2004**, *186*, 5529-5532, doi:10.1128/jb.186.16.5529-5532.2004.

104. Kraus, J.; Geller, B. Membrane receptor for prolate phages is not required for infection of *Lactococcus lactis* by small or large isometric phages. *Journal of Dairy Science* **1998**, *81*, 2329-2335.

105. Quiberoni, A.; Guglielmotti, D.; Binetti, A.; Reinheimer, J. Characterization of three *Lactobacillus delbrueckii* subsp. *bulgaricus* phages and the physicochemical analysis of phage adsorption. *Journal of Applied Microbiology* **2004**, *96*, 340-351, doi:doi:10.1046/j.1365-2672.2003.02147.x.

106. Cornelissen, A.; Sadovskaya, I.; Vinogradov, E.; Blangy, S.; Spinelli, S.; Casey, E.; Mahony, J.; Noben, J.-P.; Dal Bello, F.; Cambillau, C.; van Sinderen, D. The baseplate of *Lactobacillus delbrueckii* bacteriophage Ld17 harbours a glycerophosphodiesterase. *Journal of Biological Chemistry* **2016**, jbc. M116. 728279.

107. Ventura, M.; Callegari, M.; Morelli, L. Surface layer variations affecting phage adsorption on seven *Lactobacillus helveticus* strains. *Annali di Microbiologia ed Enzimologia* **1999**, *49*, 45-54.

108. Dieterle, M.E.; Bowman, C.; Batthyany, C.; Lanzarotti, E.; Turjanski, A.; Hatfull, G.; Piuri, M. Exposing the secrets of two well known *Lactobacillus casei* phages: Genomic and structural analysis of J-1 and PL-1. *Applied and Environmental Microbiology* **2014**, AEM. 02771-02714.

109. Wolin, M.; Douglas, L.J. Cell wall polymers and phage lysis of *Lactobacillus plantarum*. *Biochemistry* **1971**, *10*, 1551-1555.

110. Zago, M.; Bonvini, B.; Rossetti, L.; Meucci, A.; Giraffa, G.; Carminati, D. Biodiversity of *Lactobacillus helveticus* bacteriophages isolated from cheese whey starters. *The Journal of Dairy Research* **2015**, *82*, 242-247, doi:10.1017/s0022029915000151.

111. Mahony, J.; McDonnell, B.; Casey, E.; van Sinderen, D. Phage-host interactions of cheese-making lactic acid bacteria. *Annual Review of Food Science and Technology* **2016**, *7*, 267-285, doi:10.1146/annurev-food-041715-033322.

112. Tuohimaa, A.; Riipinen, K.-A.; Brandt, K.; Alatossava, T. The genome of the virulent phage Lc-Nu of probiotic *Lactobacillus rhamnosus*, and comparative genomics with *Lactobacillus casei* phages. *Archives of Virology* **2006**, *151*, 947.

113. Marcó, M.B.; Garneau, J.E.; Tremblay, D.; Quiberoni, A.; Moineau, S. Characterization of two virulent phages of *Lactobacillus plantarum*. *Applied and Environmental Microbiology* **2012**, AEM. 02565-02512.

114. Chibani-Chennoufi, S.; Dillmann, M.-L.; Marvin-Guy, L.; Rami-Shojaei, S.; Brüssow, H. *Lactobacillus plantarum* bacteriophage LP65: a new member of the SPO1-like genus of the family *Myoviridae*. *Journal of Bacteriology* **2004**, *186*, 7069-7083, doi:10.1128/jb.186.21.7069-7083.2004.

115. Vinogradov, E.; Valence, F.; Maes, E.; Jebava, I.; Chuat, V.; Lortal, S.; Grard, T.; Guerardel, Y.; Sadovskaya, I. Structural studies of the cell wall polysaccharides from three strains of *Lactobacillus helveticus* with different autolytic properties: DPC4571, BROI, and LH1. *Carbohydrate Research* **2013**, *379*, 7-12.

116. Vinogradov, E.; Sadovskaya, I.; Cornelissen, A.; van Sinderen, D. Structural investigation of cell wall polysaccharides of *Lactobacillus delbrueckii* subsp. *bulgaricus* 17. *Carbohydrate Research* **2015**, *413*, 93-99.

117. Vinogradov, E.; Sadovskaya, I.; Grard, T.; Chapot-Chartier, M.-P. Structural studies of the rhamnose-rich cell wall polysaccharide of *Lactobacillus casei* BL23. *Carbohydrate Research* **2016**, *435*, 156-161.

118. McGrath, S.; Fitzgerald, G.F.; van Sinderen, D. Identification and characterization of phage-resistance genes in temperate lactococcal bacteriophages. *Molecular Microbiology* **2002**, *43*, 509-520.

119. McDonnell, B.; Mahony, J.; Neve, H.; Hanemaaijer, L.; Noben, J.-P.; Kouwen, T.; van Sinderen, D. Identification and analysis of a novel group of bacteriophages infecting

the lactic acid bacterium *Streptococcus thermophilus*. *Applied and Environmental Microbiology* **2016**, *82*, 5153-5165.

120. Paez-Espino, D.; Sharon, I.; Morovic, W.; Stahl, B.; Thomas, B.C.; Barrangou, R.; Banfield, J.F. CRISPR immunity drives rapid phage genome evolution in *Streptococcus thermophilus*. *mBio* **2015**, *6*, doi:10.1128/mBio.00262-15.

121. Urshev, Z.; Ishlimova, D. Distribution of clustered regularly interspaced palindrome repeats CRISPR2 and CRISPR3 in *Lactobacillus delbrueckii* ssp. *bulgaricus* strains. *Biotechnology and Biotechnological Equipment* **2015**, *29*, 541-546, doi:10.1080/13102818.2015.1013351.

122. Marcó, M.B.; Moineau, S.; Quiberoni, A. Bacteriophages and dairy fermentations. *Bacteriophage* **2012**, *2*, 149-158, doi:10.4161/bact.21868.

123. Back, W.; Leibhard, M.; Bohak, I. Flash pasteurisation-membrane filtration. Biological security in comparison. Ins and outs of the both methods. *Brauwelt* **1990**.

124. Davidson, P.M.; Harrison, M.A. Resistance and adaptation to food antimicrobials, sanitizers, and other process controls. *Food Technology* **2002**, *56*, 69-78.

125. Vaughan, A.; Rouse, S.; van Sinderen, D. Investigating the antimicrobial efficacy of a lactococcal bacteriocin for the development of microbiologically stable beer. *Journal of the Institute of Brewing* **2004**, *110*, 181-188, doi:doi:10.1002/j.2050-0416.2004.tb00200.x.

126. Mahony, J.; McAuliffe, O.; Ross, R.P.; van Sinderen, D. Bacteriophages as biocontrol agents of food pathogens. *Current Opinion in Biotechnology* **2011**, *22*, 157-163, doi:10.1016/j.copbio.2010.10.008.

127. Roach, D.R.; Khatibi, P.A.; Bischoff, K.M.; Hughes, S.R.; Donovan, D.M. Bacteriophage-encoded lytic enzymes control growth of contaminating *Lactobacillus*

found in fuel ethanol fermentations. *Biotechnology for Biofuels* **2013**, *6*, 20, doi:10.1186/1754-6834-6-20.

128. Mahony, J.; van Sinderen, D. Novel strategies to prevent or exploit phages in fermentations, insights from phage–host interactions. *Current Opinion in Biotechnology* **2015**, *32*, 8-13, doi:https://doi.org/10.1016/j.copbio.2014.09.006.

Chapter II

Comparative genome analysis of the *Lactobacillus brevis* species

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Philip Kelleher helped with sequence assembly, annotation and data analysis, Richard John Roberts (New England BioLabs, USA) performed the methylome analysis.

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1. Abstract

Lactobacillus brevis is a member of the lactic acid bacteria (LAB), and strains of *Lb. brevis* have been isolated from silage, as well as from fermented cabbage and other fermented foods. However, this bacterium is also commonly associated with bacterial spoilage of beer.

In the current study, complete genome sequences of six isolated *Lb. brevis* strains were determined. Five of these *Lb. brevis* strains were isolated from beer (three isolates) or the brewing environment (two isolates), and were characterized as beer-spoilers or non-beer spoilers, respectively, while the sixth isolate had previously been isolated from silage. The genomic features of 19 *Lb. brevis* strains, encompassing the six *Lb. brevis* strains described in this study and thirteen *Lb. brevis* strains for which complete genome sequences were available in public databases, were analyzed with particular attention to evolutionary aspects and adaptation to beer.

Comparative genomic analysis highlighted evolution of the taxon allowing niche colonization, notably adaptation to the beer environment, with approximately 50 chromosomal genes acquired by *Lb. brevis* beer-spoiler strains representing approximately 2 % of their total chromosomal genetic content. These genes primarily encode proteins that are putatively involved in oxidation-reduction reactions, transcription regulation or membrane transport, functions that may be crucial to survive the harsh conditions associated with beer. The study emphasized the role of plasmids in beer spoilage with a number of unique genes identified among *Lb. brevis* beer-spoiler strains.

2. Introduction

Lactobacillus brevis is a member of the lactic acid bacteria (LAB), which are catalase-negative, non-sporulating, non-motile, rod or coccus-shaped Gram-positive bacteria. *Lb. brevis* grows optimally at 30 ˚C and within a pH range of 4 to 6 [1-3]. It is an obligatory hetero-fermentative bacterium producing lactic acid, carbon dioxide and ethanol and/or acetic acid [1-3]. Using phylogenomic and comparative genomic analysis, Duar et al. studied the relatedness within the *Lactobacillus* genus in light of their natural habitat in order to understand their evolutionary history [4]. They assigned lactobacilli species into three main lifestyle categories: free living (environmental and plant isolates), host adapted or as "nomadic" [4]. Sequenced genomes of the *Lactobacillus* genus range in size from 1.27 (*Lactobacillus iners*) to 4.91 (*Lactobacillus parakefiri*) Mb [4].

Lb. brevis has been isolated from silage, as well as from fermented cabbage and other fermented foods [5,6], and is assigned to the free-living lifestyle group of lactobacilli [4]. *Lb. brevis* strains, among other lactobacilli, are of particular interest as they have been granted Qualified Presumption of Safety (QPS) status and consequently have been widely used in the production of fermented foods [1,7]. In addition to their application in food fermentations they are purported to have potential as health-promoting or probiotic bacteria [1,7]. In contrast to these positive attributes, *Lb. brevis* strains have also been reported as the causative agent of food or beverage spoilage, in particular of beer [8,9]. LAB species are reported to cause approximately 70 % of microbial beer-spoilage incidents, and among this group *Lb. brevis* isolates are particularly problematic [10-12]. They are associated with the production of malodorous compounds, acidity and/or turbidity with negative impacts on the organoleptic properties of the final product. Bacterial spoilage of beer may result in product withdrawal or recall with concomitant economic losses for the brewing industry [10-12]. Beer spoilage by

Gram-positive bacteria has been studied previously and the main mechanism of hop resistance known so far involves an active extrusion of the toxic compound using transporters identified as: (a) HorA which functions as an ABC-type multidrug transporter to expel hop compounds, in particular *iso*-α-acids, from the bacterial cytoplasm, (b) HorC a proton motive force-dependent hop excretion transporter, and (c) the H^+-ATPase which increases the pumping of protons released from the hop compounds [13-15]. The transmembrane protein HitA is also thought to play a role in the transport of divalent cations, where *iso*-α-acids exchange protons for cellular divalent cations such as Mn^{2+} [16].

To date a number of comparative genome studies of the *Lactobacillus* genus have been described [1,17-19], some of which have provided insights into the taxonomy of the *Lactobacillus* genus [3,7], or its fermentation capabilities [3]. Carbohydrate metabolism has been assessed in several *Lactobacillus* species LAB such as *Lactobacillus casei* or *Lactobacillus plantarum* [2]. However, a broad comparative genome analysis of the *Lb. brevis* species has yet not been undertaken. Recent advances in next generation sequencing technologies has facilitated a rapid surge in the number of bacterial genomes now available for comparative analysis within a genus or a species.

In the current study, Single-Molecule-Real-Time (SMRT) sequencing technology [20,21] was employed to generate the complete genome sequence of an additional six *Lb. brevis* strains isolated from silage and the brewery environment. Using the dataset of 19 complete chromosomal sequences, a comparative genome analysis of the *Lb. brevis* taxon was undertaken through an assessment of the phylogeny, pan- and core-genome, and niche adaptation with particular emphasis on adaptation to the brewing environment. The importance of plasmids was also investigated in relation to beer spoilage ability.

3. Materials and Methods

3.1. Isolation of *Lb. brevis* strains

Five distinct *Lb. brevis* strains (UCCLB521, UCCLB556, UCCLB95, UCCLBBS124 and UCCLBBS449) were isolated from the brewing environment, while SA-C12 had previously been isolated from silage [22]. The strains were characterized by evaluating their plasmid content as well as growth curve profiles in MRS broth or in beer at 30 ˚C. Plasmids were isolated after overnight growth of the *Lb. brevis* strains in MRS broth at 30 ˚C, cells were harvested by centrifugation for 10 min at 5,000 rpm followed by lysozyme treatment (30 mg/mL lysozyme in TE + 25 % sucrose) at 37 ˚C for 30 minutes. Plasmid DNA was extracted using the GeneJET Plasmid Miniprep Kit (Thermo Scientific™). Plasmid profiles of the different *Lb. brevis* isolates were analyzed using a 1 % agarose gel. Growth profiles in MRS broth or in beer were performed at 30 ˚C by acquiring hourly OD_{600nm} measurements for a period of 55 hours. Moreover, colony morphology was recorded following growth on MRS agar plates at 30 ˚C.

3.2. Sequencing and annotation

Lb. brevis strains were streaked on MRS agar plates and grown at 30 ˚C for 24 hours. For each *Lb. brevis* strain, a single colony was inoculated into MRS broth and grown overnight at 30 ˚C. Cells were harvested by centrifugation at 5,000 rpm for 10 minutes. The supernatant was removed and the pelleted cells were frozen at -20 ˚C prior sending for sequencing. Sequencing was performed using the PacBio SMRT next generation sequencing technology (performed by GATC Biotech, Germany). *De novo* genome assemblies were performed using the Pacific Biosciences SMRT Portal analysis platform. Open Reading Frame (ORF) or coding sequence

(CDS) prediction was performed using Prodigal prediction software [23] and confirmed using BLASTX alignments (Basic Local Alignment Search Tool) [24]. Automatic annotations were refined using Artemis v16.0.0 where ORF predictions were manually checked, start codons adjusted and pseudogenes identified. Transfer RNA (tRNA) genes were predicted using tRNA-scan-SE v2.0 [25], while ribosomal RNA (rRNA) genes were identified using RNAmmer v1.2 [26]. The thirteen *Lb. brevis* genomes obtained from NCBI (National Center for Biotechnology Information) were re-annotated as described above in order to treat all sequenced genomes used in this study identically.

3.3. Methylome analysis

Following *de novo* genome assembly, the RS_Modification_and_Motif_Analysis.1 protocol of the SMRT Analysis portal was employed for base modification and methylated motif detection. This analysis was performed on *Lb. brevis* strains that had been sequenced, assembled and annotated as part of this study. Methylation motifs with a score equal or higher than 40 (corresponding to a P-value of <0.0001) were considered specific and were selected for further analysis. ORFs of genomes were investigated for the presence of restriction/modification systems using the BLASTP alignment function of the REBASE database [27] (cut-off E-value of 0.0001; with at least 30 % similarity over at least 80 % of the sequence length). A comparative genome analysis was employed to associate the presence of R/M (Restriction/Modification) system-encoding genes with the presence of methylation motif(s).

3.4. Comparative genomics

All protein sequence comparisons were performed using all-against-all, bi-directional BLAST alignments [24]. An alignment cut-off value of E-value 0.0001, and a similarity cut-off level of at least 30 % amino acid identity across 80 % of the sequence length was used. Results were analyzed with the Markov Clustering Algorithm (MCL) [28] and proteins encoded were categorized in predicted functional groups based on COG (Clusters of Orthologous Groups) assignments [29].

3.5. Phylogenetic analysis

The supertree was prepared using the BLAST-based comparative approach described above in order to identify chromosomal orthologous proteins. The set of chromosomal orthologous proteins was concatenated for each strain and an ungapped alignment was performed using MUSCLE v3.8.31 [30]. The phylogenetic tree was computed using the maximum-likelihood method in PhyML v3.0 and bootstrapped employing 1,000 replicates [31]. The final tree was visualized using MEGA7. A tree based on 16 S rRNA genes was constructed using ClustalW and visualized via ITOL (Interactive Tree Of Life) [32]. The chromosome sequence of *Enterococcus faecalis* V583 (Accession: AE016830) was included as an outgroup.

3.6. Pan/core-genome analysis

The pan-core genome analysis of the above-mentioned 19 *Lb. brevis* chromosomal sequences, was performed using PGAP v1.0 [33]. ORF content for each chromosome is classified in

functional gene clusters using the Gene Family method. From this analysis a pan/core genome profile was generated.

3.7. Genome accession numbers

Lb. brevis 100D8: CP015338, *Lb. brevis* ATCC 367: CP000416, *Lb. brevis* BDGP6: CP024635, *Lb. brevis* KB290: AP012167, *Lb. brevis* NCTC13768: LS483405, *Lb. brevis* NPS-QW-145: CP015398, *Lb. brevis* SA-C12: CP031185, *Lb. brevis* SA-C12_pA: CP031186, *Lb. brevis* SA-C12_pB: CP031187, *Lb. brevis* SRCM101106: CP021674, *Lb. brevis* SRCM101174: CP021479, *Lb. brevis* TMW 1.2108: CP019734, *Lb. brevis* TMW 1.2111: CP019743, *Lb. brevis* TMW 1.2112: CP016797, *Lb. brevis* TMW 1.2113: CP019750, *Lb. brevis* UCCLB521: CP031208, *Lb. brevis* UCCLB521_pA: CP031209, *Lb. brevis* UCCLB521_pB: CP031210, *Lb. brevis* UCCLB521_pC: CP031211, *Lb. brevis* UCCLB521_pD: CP031212, *Lb. brevis* UCCLB521_pE: CP031213, *Lb. brevis* UCCLB556: CP031174, *Lb. brevis* UCCLB556_pA: CP031175, *Lb. brevis* UCCLB556_pB: CP031176, *Lb. brevis* UCCLB556_pC: CP031177, *Lb. brevis* UCCLB556_pD: CP031178, *Lb. brevis* UCCLB556_pE: CP031179, *Lb. brevis* UCCLB556_pF: CP031180, *Lb. brevis* UCCLB556_pG: CP031181, *Lb. brevis* UCCLB95: CP031182, *Lb. brevis* UCCLB95_pA: CP031183, *Lb. brevis* UCCLB95_pB: CP031184, *Lb. brevis* UCCLBBS124: CP031169, *Lb. brevis* UCCLBBS124_pA: CP031170, *Lb. brevis* UCCLBBS124_pB: CP031171, *Lb. brevis* UCCLBBS124_pC: CP031172, *Lb. brevis* UCCLBBS124_pD: CP031173, *Lb. brevis* UCCLBBS449: CP031198, *Lb. brevis* UCCLBBS449_pA: CP031199, *Lb. brevis* UCCLBBS449_pB: CP031200, *Lb. brevis* UCCLBBS449_pC: CP031201, *Lb. brevis* UCCLBBS449_pD: CP031202, *Lb. brevis* UCCLBBS449_pE: CP031203, *Lb. brevis* UCCLBBS449_pF: CP031204, *Lb. brevis* UCCLBBS449_pG: CP031205, *Lb. brevis*

UCCLBBS449_pH: CP031206, *Lb. brevis* UCCLBBS449_pI: CP031207, *Lb. brevis* ZLB004: CP021456 and *Enterococcus faecalis* V583: AE016830.

4. Results and Discussion

4.1. Isolation of *Lb. brevis* strains

Six *Lb. brevis* strains were isolated and included as part of the study, with the aim of expanding the collection of *Lb. brevis* genome sequences currently available, as well as studying their ability to grow in and colonize harsh environments such as beer. Three *Lb. brevis* strains were isolated from beer and characterized as beer-spoilers based on their ability to grow in beer. Two additional *Lb. brevis* strains were isolated from the brewing environment, yet lacking the ability to grow in beer, were not classified as beer-spoilers (Table 1 and Figure 1). In addition, the sixth *Lb. brevis* strain sequenced as part of this study originates from silage [22] and was included as a non-brewing environmental isolate (Table 1). The different *Lb. brevis* isolates exhibited different colony morphologies ranging from a dry irregular colony type for *Lb. brevis* UCCLBBS449 and UCCLB95 to a slimy and ropy colony type for UCCLBBS124 (Table 1). Plasmid profiling of the different isolates revealed a distinct plasmid content for each isolate which was then corroborated with sequencing data. Growth curves in MRS broth demonstrated the unique growth profiles of the individual isolates confirming that the isolates were distinct from each other. Furthermore, only *Lb. brevis* UCCLB95, UCCLBBS124 and UCCLBBS449 were characterized as beer-spoilers having the ability to survive and grow in beer, while *Lb. brevis* SA-C12, UCCLB521 and UCCLB556 were defined as non-beer spoilers (Table 1 and Figure 1).

Table 1. Isolation of *Lb. brevis* strains.

Isolation source	*Lb. brevis*	Colony morphology	Plasmids	Ability to grow in beer	Spoiled beer characteristics
Silage	SA-C12	Rounded	2	No	N/A
Brewery	UCCLB521	Rounded	5	No	N/A
Brewery	UCCLB556	Rounded	7	No	N/A
Beer	UCCLB95	Dry, irregular edges	2	Yes	Turbid
Beer	UCCLBBS124	Slimy, ropy, rounded	4	Yes	Slimy, ropy, turbid
Beer	UCCLBBS449	Dry, irregular edges	9	Yes	Turbid

N/A not applicable

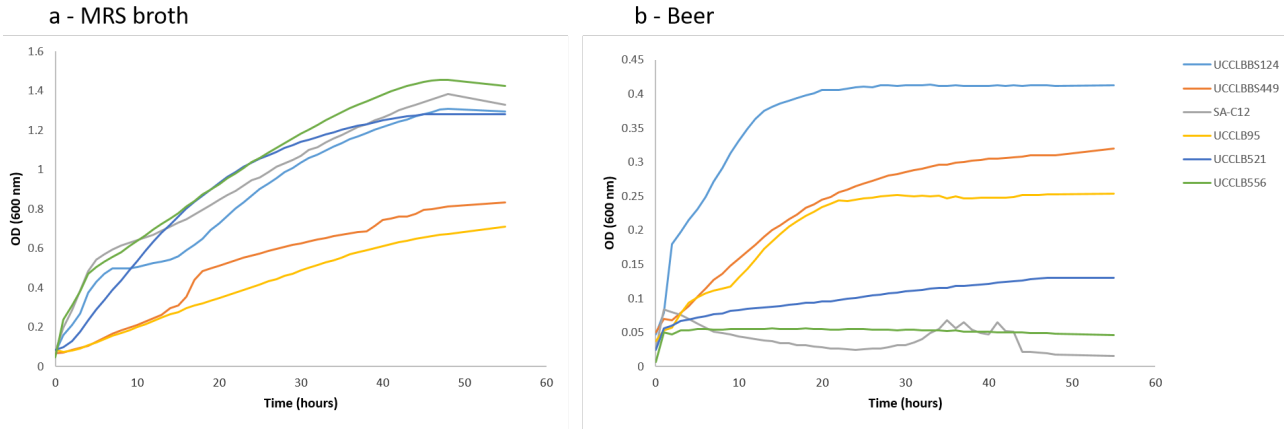

Figure 1. Growth profile of *Lb. brevis* strains sequenced in this study.

Growth profile of *Lb. brevis* strains UCCLBBS124, UCCLBBS449, UCCLB95, UCCLB521, UCCLB556 and SA-C12 in **(a)** MRS broth or **(b)** beer. Growth curves were performed in triplicate and the average of those measurements is displayed in the graph above.

4.2. General genome features

The complete chromosomal sequences of nineteen *Lb. brevis* strains were selected for analysis, thirteen of which were available at that time and were obtained from the NCBI database, while the remaining six were sequenced as part of this study using SMRT sequencing technology (Table 2). These 19 selected *Lb. brevis* strains had been isolated from different ecological niches: silage, fermented food, animal's gut and the brewery environment (Table 2). The general features of the 19 *Lb. brevis* genomes are indicated in Table 3 and include an average chromosome length of 2.49 Mbp (ranging from 2.27 to 2.79 Mbp) and a G+C content of 46 %. An average of 2,338 predicted CDSs per chromosome were identified to which approximately 78.3 % could be assigned a function based on *in silico* predictions using BLAST, while the remaining 21.7 % were annotated as hypothetical proteins (Table 3). A type II CRISPR-Cas (Clustered Regularly Interspaced Short Palindromic Repeats) locus was found in the chromosome of *Lb. brevis* BDGP6, *Lb. brevis* NPS-QW145 and *Lb. brevis* SRCM101106 where variability was observed in the spacer region, distinct spacers were observed in each of these three *Lb. brevis* strains suggesting an active system acquiring unique and various spacers for protection against invading DNA over time. Conversely, in the chromosome of *Lb. brevis* TMW1.2112 and *Lb. brevis* TMW1.2113 ten identical spacers were detected suggesting that these two strains are clonal or that this CRISPR-Cas system is inactive, and that these common spacers originate from a common ancestor that acquired genetic material from viruses/plasmids that it had encountered in the past [34]. The *Lb. brevis* strain ZLB004 chromosome revealed the presence of four CRISPR loci, one was associated to a type I-E CRISPR-Cas system, a second one was associated to a type II CRISPR-Cas system both potentially active systems. The two other CRISPR loci were not associated to any CRISPR-Cas proteins suggesting that they are likely inactive systems.

PacBio SMRT sequencing was used to determine the diversity and frequency of methylated motifs recognized by R/M systems within the six newly sequenced and annotated *Lb. brevis* strains as part of this study. R/M systems constitute one of the biological barriers exerted by a strain against foreign DNA [35]. This analysis revealed the presence of various m6A motifs and allowed the identification of three motifs assignable to Type I R/M system and six motifs assignable to Type II R/M system (Table 4). The presence of specific methylated motifs was linked to the presence of specific R/M systems in the corresponding *Lb. brevis* strains (Table 4). *Lb. brevis* UCCLB95 does not appear to encode any R/M systems.

Table 2. *Lactobacillus brevis* strains and/or genomes used in this study.

Strain name	Genbank accession	Ecological niche	Year	Citation
100D8	CP015338	Rye silage (South Korea)	2016	
ATCC 367	CP000416	Sourdough/Silage starter culture	2006	[5]
BDGP6	CP024635	*Drosophila melanogaster* female gut	2015	
KB290	AP012167	Suguki (fermented vegetable)	2013	[36]
NPS-QW-145	CP015398	Traditional Korean kimchi (Hong-Kong)	2016	[37]
NCTC13768	LS483405	Unknown		
SA-C12	CP031185	Silage (Ireland)	2008	[22]
SRCM101106	CP021674	Food (South Korea)	2017	
SRCM101174	CP021479	Food (South Korea)	2017	
TMW 1.2108	CP019734	Wheat beer (Germany)	2016	
TMW 1.2111	CP019743	Wheat beer (Germany)	2016	
TMW 1.2112	CP016797	Wheat beer (Germany)	2016	
TMW 1.2113	CP019750	Brewery-associated surface (Germany)	2016	
UCCLB521	CP031208	Brewery environment (The Netherlands)	2013	This study
UCCLB556	CP031174	Brewery environment (The Netherlands)	2014	This study
UCCLB95	CP031182	Beer (The Netherlands)	2001	This study
UCCLBBS124	CP031169	Beer keg (Singapore)	2003	This study
UCCLBBS449	CP031198	Unpasteurized beer (The Netherlands)	1994	This study
ZLB004	CP021456	Pig’s feces	2010	

Table 3. General chromosomal features and plasmid content among *Lb. brevis* strains.

Lb. brevis strain	Chromosome length (Mbp)	CDS	tRNA features	rRNA features	Hypothetical proteins %	Assigned function %	IS elements/ transposases	Prophage	CRISPR	GC %	Plasmids (Ranging size Kb)
100D8	2.35	2228	66	15	21.2	78.8	25	1 In[a] 3 Pa[b]	-	46.1	3 (39.9-45.1)
ATCC 367	2.29	2133	65	15	20.8	79.2	34	1 In	-	46.2	2 (13.4-35.6)
BDGP6	2.79	2674	71	15	23.1	76.9	24	4 In 3 Pa	1	46.6	-
KB290	2.40	2308	64	15	21.4	78.6	50	2 In 2 Pa	-	46.1	9 (5.9-42.4)
NCTC13768	2.49	2413	65	15	15.0	85.0	3	1 Pa	-	46.0	-
NPS-QW-145	2.55	2406	62	13	21.5	78.5	5	3 Pa	1	45.8	-
SA-C12	2.44	2344	66	15	23.2	76.7	42	2 In 3 Pa	-	45.9	2 (24.8-43.6)
SRCM101106	2.44	2379	67	15	23.0	77.0	46	3 In 4 Pa	1	45.9	4 (16.0-36.2)
SRCM101174	2.41	2353	68	15	24.0	76.0	37	3 In 2 Pa	-	46.1	5 (9.4-50.4)
TMW 1.2108	2.57	2448	66	15	22.8	77.2	17	2 In	-	45.8	8 (5.1-107.0)
TMW 1.2111	2.57	2458	66	15	21.8	78.2	22	2 In	-	45.8	6 (8.2-107.0)
TMW 1.2112	2.49	2283	65	15	19.6	80.4	29	1 In 1 Pa	1	46.0	5 (8.5-59.7)
TMW 1.2113	2.54	2376	69	15	22.5	77.5	30	2 In	1	45.9	4 (8.5-46.6)
UCCLB521	2.27	2088	62	15	20.0	80.0	32	2 Pa	-	46.3	5 (11.3-43.8)
UCCLB556	2.38	2201	66	18	22.8	77.2	32	1 Pa	-	46.1	7 (4.3-68.4)
UCCLB95	2.51	2283	65	15	22.7	77.3	132	1 In 1 Pa	-	45.9	2 (3.5-14.0)
UCCLBBS124	2.61	2442	66	15	21.8	78.2	60	1 In 2 Pa	-	45.8	4 (21.0-49.6)
UCCLBBS449	2.58	2404	66	15	21.1	78.9	114	1 In 3 Pa	-	45.8	9 (2.8-66.8)
ZLB004	2.66	2207	64	15	24.0	76.0	29	1 In	2	46.0	5 (16.7-78.1)
Average	2.49	2338	66	15	21.7	78.3	40	1.4 In 1.6 Pa	-	46.0	5

[a]In: Complete intact prophage [b]Pa: Partial/incomplete prophage

Table 4. *Lb. brevis* methyltransferases with their assigned recognition sequence.

Lb. brevis strain	Enzyme	Recognition sequence/motif	R/M type
UCCLBBS124	Lbr124II	CATCN**A**C	II
	M.Lbr124I	YTC**A**(N7)**T**TRG	I
UCCLB521	M.Lbr521I	**A**GG(N6)**T**TC	I
	Not assigned	G**A**TC	II
UCCLB556	M.Lbr556I	RTC**A**(N9)**T**CC	I
UCCLBBS449	Lbr449I	AGCC**A**G	II
	Not assigned	CTTGC**A**	II
UCCLB95	None detected		
SA-C12	M1.LbrSAC12IP	G**A**GGC	II
	M2.LbrSAC12I	G**A**GGC	II

Bold: m6A

4.3. The predicted mobilome of *Lb. brevis*

All complete chromosome sequences were analyzed for the presence of mobile elements such as IS (Insertion Sequence) elements and genes specified as encoding transposases. This analysis indicates that *Lb. brevis* strains UCCLBBS449 and UCCLB95 contain the highest number of IS elements/transposases, 114 and 132, respectively (Table 3). The genome sequences were also investigated for prophages, revealing various predicted intact or partial prophage regions (Table 3), displaying in most cases similarity to the published *Lb. brevis* temperate bacteriophage LBR48 [38]. The plasmid content of the *Lb. brevis* strains is detailed below.

4.4. Phylogenetic analysis

The phylogenetic relationship between the genomes of the nineteen *Lb. brevis* strains were investigated by a comparative analysis of their 16S rRNA sequences (Figure 2a). The resulting phylogenetic tree distinguishes five clades (clades A through to E). Clade A represents two *Lb. brevis* beer-spoiling strains UCCLB95 and UCCLBBS449 both isolated from spoiled beer (Table 1 and Figure 1). Clade B encompasses three *Lb. brevis* strains: *Lb. brevis* SRCM101106 isolated from food, *Lb. brevis* BDGP6 isolated from the gut of a *Drosophila* and *Lb. brevis* NCTC13768 from an unknown isolation source. Clade C is represented by six *Lb. brevis* strains, of which one was isolated from food (*Lb. brevis* SRCM101174), three from silage (*Lb. brevis* SA-C12, ATCC 367 and 100D8) and two strains (*Lb. brevis* UCCLB521 and UCCLB556), both isolated from the brewing environment, yet unable to survive and grow in beer (Table 1). These latter two strains may have been introduced into the brewery through raw materials such as cereal grains thus explaining the observed phylogenetic relationship to the silage *Lb. brevis* isolate SA-C12. Clade D includes five *Lb. brevis* strains, all retrieved as beer-spoiler strains from the brewing environment, and all exhibiting a slimy, ropy phenotype (Table 1) [9]. Clade E gathers three *Lb. brevis* strains, two isolated from fermented food (*Lb. brevis* KB290 and NPS-QW-145) as well as *Lb. brevis* ZLB004 isolated from pig's feces.

In order to obtain a more refined view of the phylogeny of the 19 analyzed strains, a so-called phylogenetic supertree was constructed based on 631 conserved orthologous proteins that had been identified as single-copy genes conserved across all investigated chromosomal sequences (19 *Lb. brevis* strains and *Enterococcus faecalis* V583 as an outgroup) [39,40]. This supertree does not display distinct clades separating the *Lb. brevis* strains in different groups as was observed with the 16S rRNA phylogenetic tree, suggesting a close relatedness within the species (Figure 2b). However, upon close inspection of this phylogenetic tree, it appears that

Lb. brevis strains isolated from food and silage cluster on one branch of the tree, while *Lb. brevis* brewery isolates cluster on another. The *Lb. brevis* strains isolated from the gut microbiota, BDGP6 and ZLB004 as well as *Lb. brevis* strains NCTC13768 and 100D8 gather in a smaller clade.

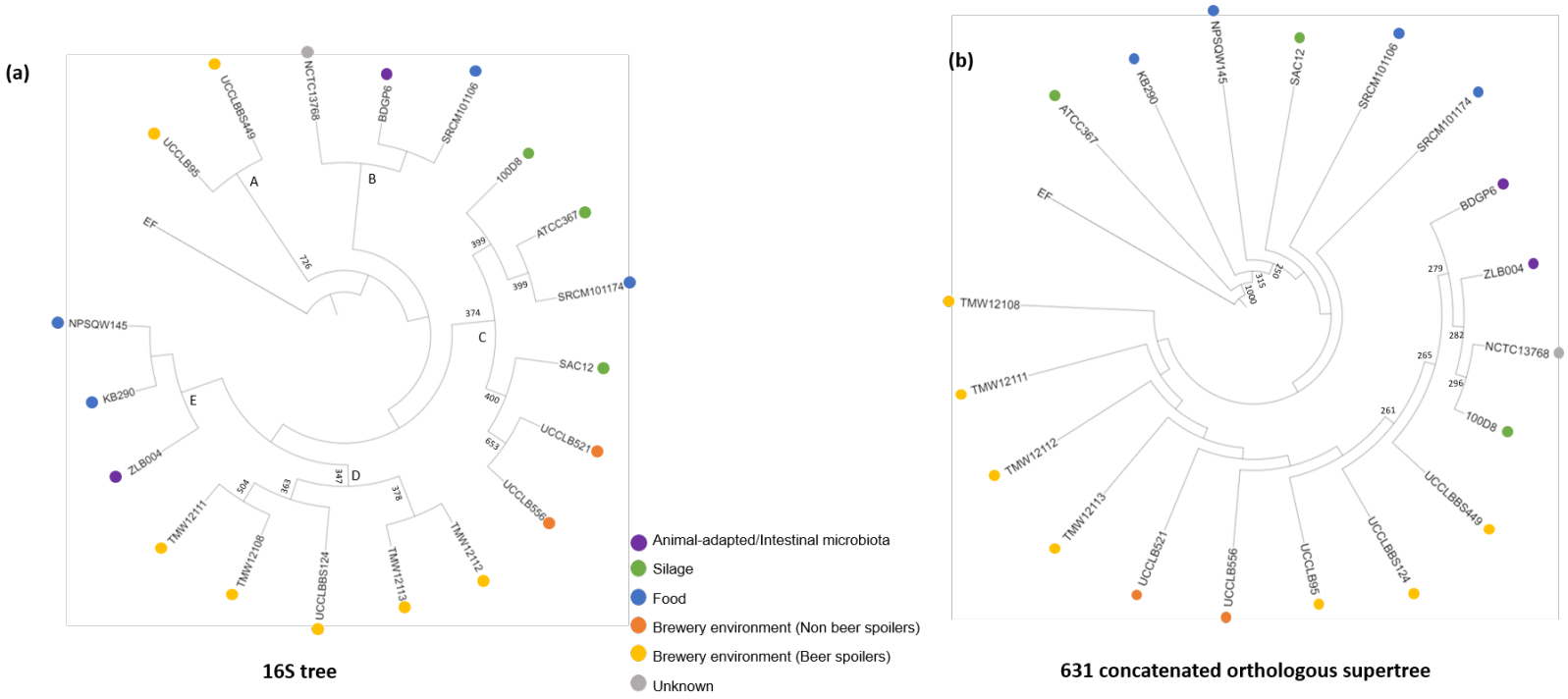

Figure 2. Phylogenetic analysis of *Lb. brevis* species.

(a) 16S ribosomal tree obtained from the alignment of the 16S rRNA-encoding genes of 19 *Lb. brevis* strains, bootstrapped x 1000 replicates, values > 250 are indicated. The 16S rRNA sequence of *Enterococcus faecalis* V583 (noted EF on the figure) was used as an outgroup. **(b)** Phylogenetic supertree obtained from the alignment of 631 orthologous genes among the 19 *Lb. brevis* strains used in this study as well as in *Enterococcus faecalis* V583 (noted EF on the figure) which was used as an outgroup, bootstrapped x 1000 replicates, values > 250 are indicated. Source of isolation for the different *Lb. brevis* strains are also indicated.

4.5. Pan/core-genome analysis

A pan-genome analysis was performed in order to determine the total number of distinct genes present on the combined chromosomal sequences of the analyzed *Lb. brevis* strains. The pan-genome curve displays an asymptotic trend, growing with an average rate of 136 genes per genome in the first nine iterations, then the number of new genes decreased leading to a total pan-genome content of 3,968 genes (Figure 3). The mathematical function displayed on the graph reveals an exponential value lower than 0.5 indicating that the pan-genome is in a closed state. The core genome was determined to encompass 1,428 genes (Figure 3).

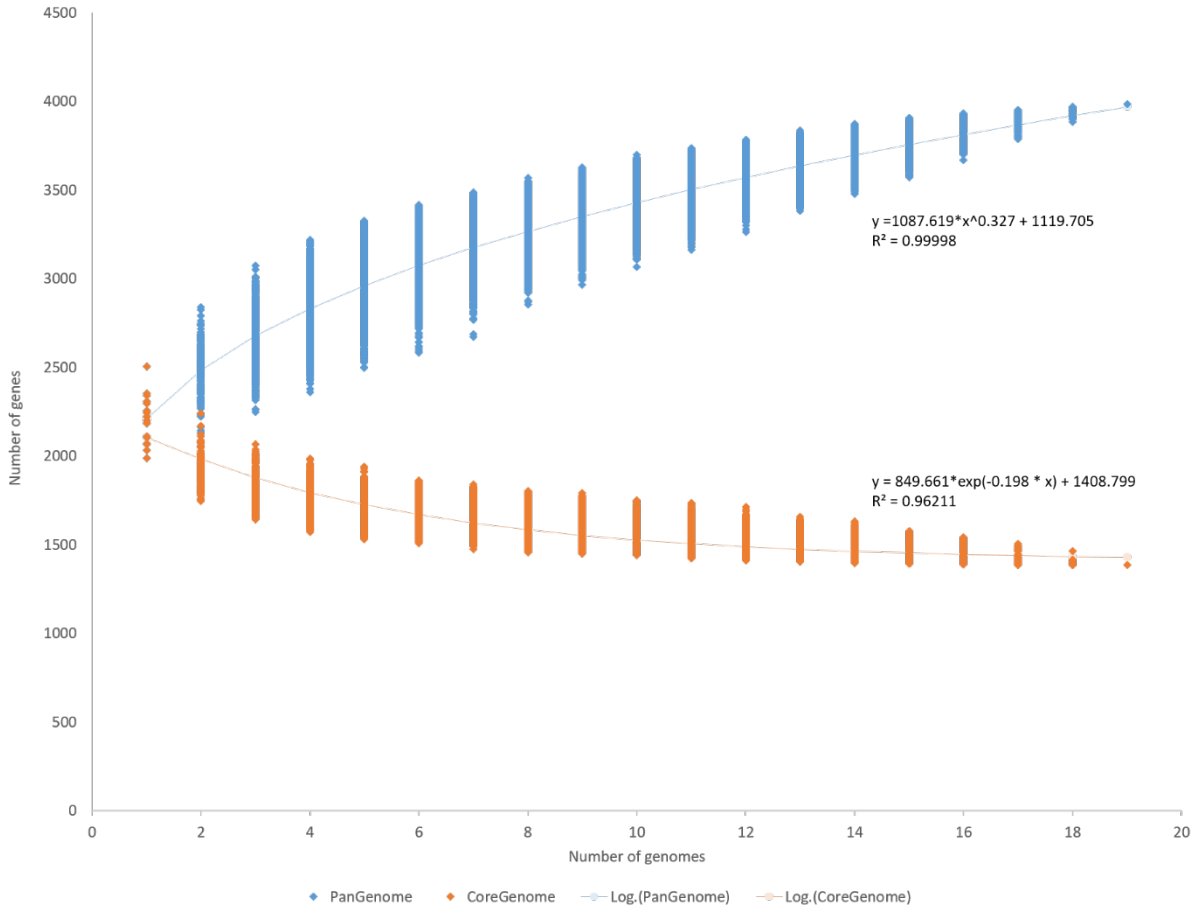

Figure 3. Pan- and core-genome of *Lb. brevis*.

Accumulated number of new genes in the *Lb. brevis* pan-genome plotted against the number of new genomes added as well as accumulated number of genes attributed to the core-genome plotted against the number of genomes added. Deduced mathematical functions are also displayed on the graph.

4.6. Comparative analysis of orthologous genes

The comparative analysis used in this study was based on chromosomal sequences. The core genome of 1,428 genes is divided in 1,170 orthologous gene families (single copy) and 258 paralogous gene families (multi-copy). Unique gene families to each chromosome were also recorded and 246 unique gene families were identified across the genomes of the nineteen *Lb. brevis* strains (Figure 4a). Functional assignment efforts revealed that 75.2 % of the unique gene families encoded proteins of unknown function (hypothetical proteins), while 4.5 % encoded (pro)phage-related proteins. The remaining unique gene families encode proteins that could benefit the fitness of the strain such as CRISPR-Cas system (e.g. Type I-E CRISPR Cas system in *Lb. brevis* ZLB004), restriction-modification systems (e.g. Type I R/M system in *Lb. brevis* strain UCCLBBS124), or cell wall polysaccharide synthesis (e.g. genes predicted to encode glycosyltransferases and a polysaccharide polymerase only found in the *Lb. brevis* strain NPS-QW-145).

In order to further investigate the functionality and diversity encoded by the core and dispensable genomes, a Cluster of Orthologous Group analysis was employed. The genome content of the 19 selected *Lb. brevis* strains was classified into different groups depending on their function. More than 75 % were predicted to be involved in housekeeping functions, vital for the strain to grow, such as those participating in transcription or translation. Approximately 16 % of the genes were assigned to COG groups with only a general function predicted or of unknown function (Figure 4b).

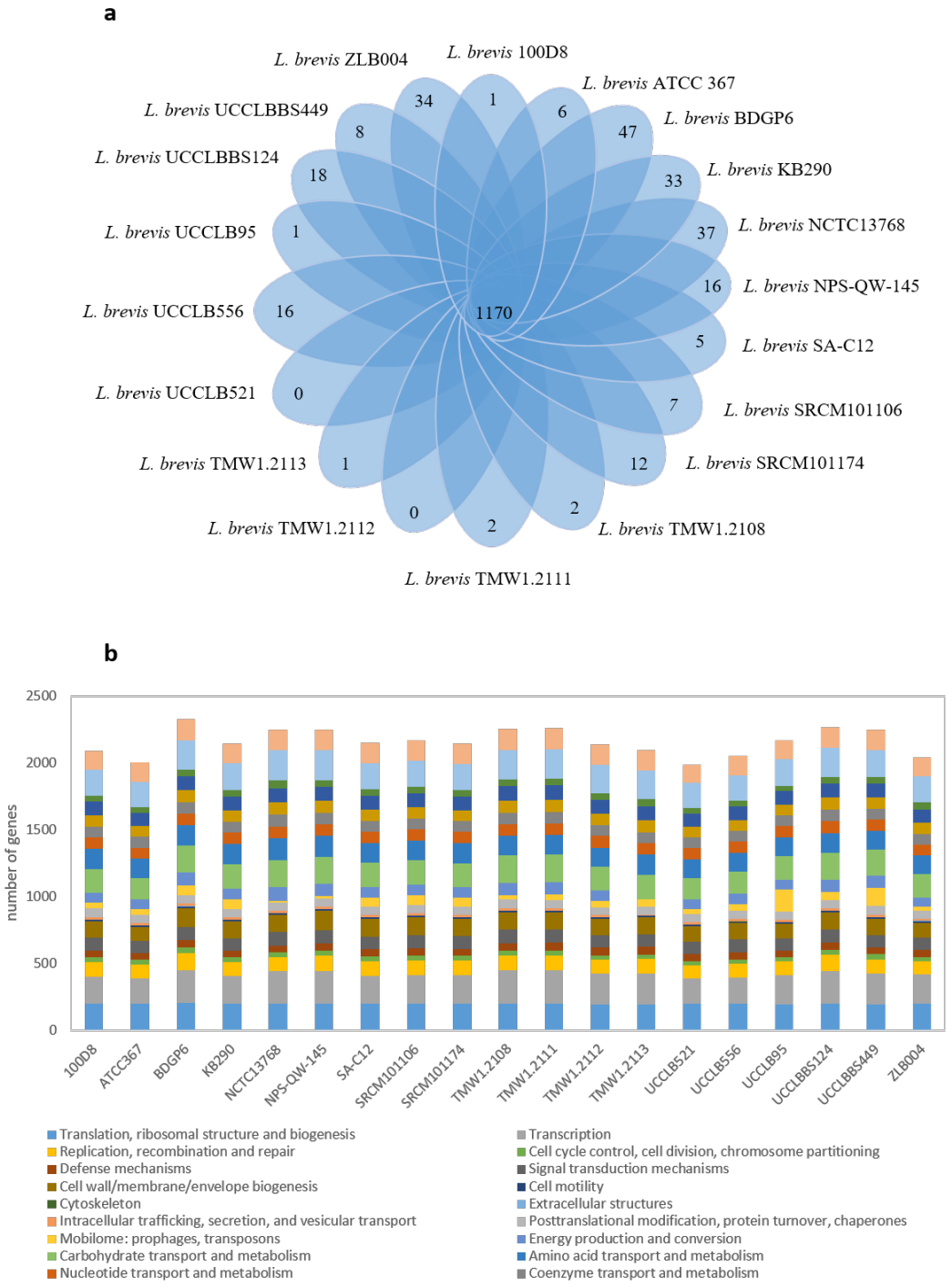

Figure 4. Comparative genomics of chromosomal orthologous proteins in *Lb. brevis.*

Panel a: Venn diagram representing the orthologous and unique gene families of 19 *Lb. brevis* strains obtained by MCL clustering. **Panel b:** Cluster of Orthologous Groups (COG) classification of *Lb. brevis*. Histograms represent COG predictions for each of the following 16 *Lb. brevis* isolates: *Lb. brevis* 100D8, *Lb. brevis* ATCC 367, *Lb. brevis* BDGP6, *Lb. brevis* KB290, *Lb. brevis* NCTC13768, *Lb. brevis* NPS-QW-145, *Lb. brevis* SA-C12, *Lb. brevis* SRCM101106, *Lb. brevis* SRCM101174, *Lb. brevis* TMW 1.2108, *Lb. brevis* TMW 1.2111, *Lb. brevis* TMW 1.2112, *Lb. brevis* TMW 1.2113, *Lb. brevis* UCCLB521, *Lb. brevis* UCCLB556, *Lb. brevis* UCCLB95, *Lb. brevis* UCCLBBS124, *Lb. brevis* UCCLBBS449, *Lb. brevis* ZLB004.

4.7. Evolution and adaptation to beer environment

When plotting the number of CDSs as a function of genome size for the different *Lb. brevis* strains, the beer spoilers were within those exhibiting the largest genome size as well as the highest number of CDS with *Lb. brevis* strain BDGP6 displaying the largest CDS number. *Lb. brevis* strains known to be beer-spoilers possess an average of 2,385 CDS, while those isolated from food, silage, animal's gut and non-beer spoiling brewery isolates display an average of 2,311 CDSs (Figure 5). This observation suggests a link to adaptation to a new environment, i.e. the beer or brewery environment, which may have necessitated the acquisition of novel genes and corresponding functions in order to survive in this harsh environment. To understand if the beer spoiling strains had acquired a specific set of genes or associated functions, genes that may putatively be associated to beer adaptation were first predicted to be those that would be present in the genomes of at least four beer spoiling strains (Table 5). From this analysis, 58 genes of interest were highlighted as well as 26 genes encoding hypothetical proteins. Of these 58 genes, approximately 21 % encode proteins related to oxido-reduction reactions (Flavodoxin, oxidoreductases and short-chain dehydrogenases), 22 % are linked to transcription (transcriptional regulators, RNA polymerase sigma-24 subunit ECF subfamily), 21 % encode membrane and cell surface proteins and 14 % are related to membrane transport (MFS transporter, permease, ABC transporters) (Table 5).

When exposed to beer, bacteria are subjected to various stresses, among them a low pH (3.8-4.7) and hop compounds [10]. When *iso*-α-acids enter the cell cytoplasm, they dissociate into hop anions and protons decreasing the intracellular pH [10]. Therefore, bacteria would have to adapt in order to regulate their internal pH in order to survive [41-43]. Furthermore, in beer the presence of ethanol (0.5-10 % w/w) causes oxidative stress in bacteria, this results in the production of Reactive Oxygen Species (ROS) such as hydrogen peroxide and free radicals

leading to cell damage [44,45]. Despite the stress and harsh environment imposed by the beer environment, some bacteria have evolved and acclimatized to this new medium. It may thus be possible that some of the *Lb. brevis* strains acquired additional functions which allow them to grow and survive in beer and which has led to an increased genome size. The fact that 21 % of these chromosomal genes encode proteins related to redox reactions is of interest and suggests a link between *Lb. brevis* beer-spoiler strains and oxidative stress response. Six of the 12 genes that encode functions relating to oxido-reduction reactions present in at least four beer-spoiler *Lb. brevis* strains are predicted to encode NADH oxidoreductases and short-chain dehydrogenases/reductases (SDRs). These proteins are part of the large family of NAD(P)(H)-dependent oxidoreductases and are believed to behave as scaffold proteins for an NAD(P)(H) redox sensor system [46]. In previous studies, the role of SDRs during oxidative stress was highlighted in species such as *Bacillus subtilis* where they are required for survival in severe ethanol stress [47], or in *Burkholderia pseudomallei* during salt stress [48].

Furthermore, 22 % of the chromosomal genes that seem to be specifically associated with beer-spoiling *Lb. brevis* strains are linked to transcriptional regulation, suggesting that these regulators act on specific genes to control their expression and confer an advantage when present in beer. It would be interesting to study which genes are affected by these transcriptional regulators to assess the mechanisms employed to survive in this harsh environment. Of the *Lb. brevis* beer-spoiler specific chromosomal genes 21 % encode membrane and cell surface proteins suggesting an adaptation to survive in the harsh beer environment. 14 % encode proteins associated with membrane transport such as permeases and ABC transporters suggesting exchange between the strain and its environment and possibly a role in extrusion where the *Lb. brevis* isolate would expel protons or *iso*-α-acids in order to survive and thrive in beer, as has been described previously [10,13,15].

Interestingly some of the chromosomal genes identified among *Lb. brevis* beer-spoiler strains in this analysis had also been highlighted in a previous study as beer-spoilage diagnostic marker genes (DMG) [49]. These genes are predicted to code for an oligogalacturonide transporter, a short chain dehydrogenase and a RNA polymerase sigma factor ECF subfamily, which reinforces the hypothesis for their involvement in beer spoilage adaptation.

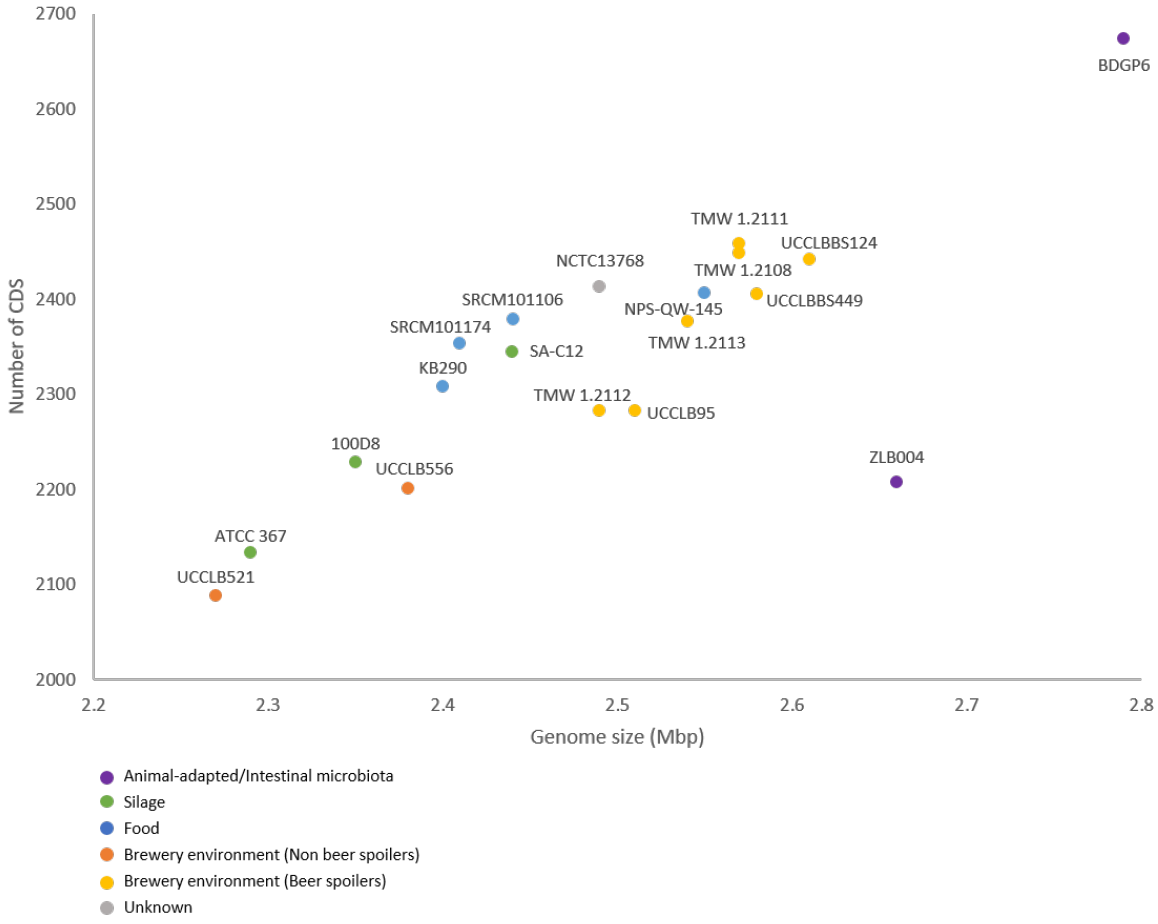

Figure 5. Association between chromosome size and CDS number in nineteen *Lb. brevis* complete chromosomal sequences.

Table 5. List of genes identified in the chromosome sequence of at least four *Lb. brevis* beer-spoiler strains. 26 genes coding for hypothetical proteins were also identified.

COG category and protein function	*Lb. brevis* beer-spoiler strains						
	TMW1.2108	**TMW1.2111**	**TMW1.2112**	**TMW1.2113**	**UCCLB95**	**UCCLBBS124**	**UCCLBBS449**
	Energy production and conversion						
Flavodoxin	+	+	+	+	+	+	+
NADH-Flavin reductase	+	+	-	-	+	+	+
Oxidoreductase	+	+	+	+	+	+	+
NADPH:quinone reductase	+	+	-	-	+	+	+
FMN-dependent NADH-azoreductase	+	+	-	-	+	+	+
Nitrobenzoate reductase	+	+	-	-	+	+	+
	Amino acid transport and metabolism						
Shikimate dehydrogenase	+	+	+	+	+	+	+
Acetyltransferase GNAT family	+	+	-	-	+	+	+
Serine O-acetyltransferase EC	+	+	-	-	+	+	+
	Carbohydrate transport and metabolism						
MFS transporter	+	+	-	-	+	+	+
Alpha-glucosidase	+	+	+	+	+	+	+
Glycoside hydrolase	+	+	-	-	-	+	+
Hydrolase	+	+	+	+	+	+	+
Transketolase	+	+	+	+	-	-	-
MFS transporter	+	+	+	-	+	+	-
PTS system2C IIA component 1	+	+	-	-	+	+	+
Putative integral membrane protein 1	+	+	-	-	+	+	+
PTS2C EIIB 1	+	+	-	-	+	+	+
PTS mannitol transporter subunit IIA	+	+	-	-	+	+	+
Putative oligogalacturonide transporter	+	+	+	+	+	-	+
	Coenzyme transport and metabolism						
6-pyruvoyl tetrahydropterin synthase	+	+	+	+	-	+	-
	Lipid transport and metabolism						
NADH peroxidase	+	+	+	+	+	+	+
Peroxidase	+	+	-	-	+	+	+
Citrate lyase	+	+	-	-	+	+	+
	Transcription						
Transcriptional regulator2C TetR family	+	+	-	-	+	+	+
Transcriptional regulator	+	+	+	+	+	+	+
Transcriptional regulator TetR family	+	+	-	-	+	+	+
Transcriptional regulator	+	+	+	+	+	+	+
Internalin-J	-	+	+	+	+	+	+
RNA polymerase sigma-24 subunit ECF subfamily	+	+	+	+	+	+	+
ECF-type sigma factor negative effector	+	+	+	+	+	+	+
Transcriptional regulator	+	+	+	+	+	+	+
Transcriptional regulator MarR family	+	+	-	-	+	+	+
Transcriptional regulator	+	+	-	-	+	+	+
Transcriptional regulator MarR family	+	+	-	-	+	+	+
Transcriptional regulator TetR	+	+	-	-	-	+	+
Transcriptional regulator ArsR family	+	+	-	-	+	+	+
	Cell wall/membrane/envelope biogenesis						
Membrane protein	+	+	-	+	-	+	-
Cell surface protein	+	+	+	+	-	-	-
Cell surface protein	+	+	-	-	-	+	+
Endo polygalacturonase	+	+	+	+	-	-	+
Glutamyl endopeptidase precursor	+	+	+	+	+	+	+
NLP-P60 protein	+	+	+	+	+	+	+
Short-chain dehydrogenase-oxidoreductase	+	+	-	-	+	+	+
	Inorganic ion transport and metabolism						
Permease	+	+	+	+	+	+	+
Permease	+	+	+	+	+	-	+
Na+-H+ antiporter	+	+	-	-	+	+	-
	General function prediction only						
NADPH-quinone reductase	+	+	+	+	+	+	+
Short-chain dehydrogenase-oxidoreductase	+	+	+	+	+	+	+
Short-chain dehydrogenase	+	+	+	+	+	+	-
Cell surface adherence protein	-	-	+	+	+	+	+
Mucus-binding protein LPXTG-motif cell wall anchor	+	+	+	+	+	-	+
	Function unknown						
Cell surface hydrolase	+	+	+	+	+	+	+
Membrane protein	+	+	+	+	-	+	+
Cell surface protein	+	+	-	-	+	+	+
	Defence mechanisms						
ABC transporter ATP-binding protein	+	+	+	+	+	+	+
ABC transporter permease	+	+	+	+	+	+	+
Prophage protein	+	+	+	+	-	-	+

+: gene present, -: gene absent

4.8. The role of plasmids in adaptation to beer environment

Different proteins involved in beer spoilage have been identified on plasmids indicating the importance of plasmids for bacterial strains in beer spoilage. This might suggest a role for plasmid mobilization and transfer between bacterial strains throughout evolution to adapt to a new environment such as beer.

The nineteen analyzed *Lb. brevis* strains were predicted to harbor up to nine plasmids with strains *Lb. brevis* KB290 and *Lb. brevis* UCCLBBS449 exhibiting the largest plasmid complements of the assessed strains. The plasmid size ranges from 2.8 Kb to 107.0 Kb (Table 3). The number of plasmids and their size do not appear to be linked to the isolation source of the *Lb. brevis* strains (e.g. four plasmids for *Lb. brevis* SRCM101106 *versus* nine plasmids for *Lb. brevis* KB290, both isolated from fermented food) or to the beer spoilage ability of the isolate (two plasmids for *Lb. brevis* UCCLB95 *versus* nine plasmids for *Lb. brevis* UCCLBBS449 both characterized as beer-spoilers). Investigating analogies between plasmids among *Lb. brevis* beer-spoiler strains revealed that the plasmid content of *Lb. brevis* TMW1.2108 and *Lb. brevis* TMW1.2111 were very similar. Indeed, the six plasmids of *Lb. brevis* TMW1.2111 show at least 90 % identity to seven of the eight plasmids contained by strain *Lb. brevis* TMW1.2108, with the exception of plasmid TMW1.2108-5. Similarly, *Lb. brevis* strains TMW1.2112 and TMW1.2113 present a close plasmid composition as the four plasmids of *Lb. brevis* TMW1.2113 are at least 90 % identical to four out of five plasmids of *Lb. brevis* TMW1.2112 with the exception of plasmid TMW1.2112-1.

Of the 38 plasmids shared between *Lb. brevis* beer-spoiler strains, only three plasmids seem to be unique, sharing less than 10 % similarity with any other plasmid. These three plasmids were found in *Lb. brevis* UCCLBBS449 (UCCLBBS449_pF, UCCLBBS449_pH and UCCLBBS449_pI) and contain mostly genes coding for hypothetical proteins, replication

proteins as well as genes coding for proteins involved in conjugation such as mobilization proteins and a relaxase.

Refined analysis of specific genes shared only between at least three *Lb. brevis* beer-spoiler strains, identified only twenty-five genes (Table 6). In this list of unique genes shared only between *Lb. brevis* beer-spoiler strains, the gene coding for the membrane protein HorC is noteworthy, as it is known to be involved in beer spoilage [14] and is present in all *Lb. brevis* beer-spoiler strains with the exception of *Lb. brevis* TMW1.2113.

Interestingly, the gene encoding the ABC transporter HorA [13] and present in *Lb. brevis* beer-spoiler strains TMW1.2108, TMW1.2111, TMW1.2113, UCCLBBS124 and UCCLBBS449 does not figure in this list as a similar protein can be found in plasmid sequences of the *Lb. brevis* strains KB290, SRCM101106 isolated from fermented food and *Lb. brevis* UCCLB556 isolated from the brewery and characterized as a non-beer spoiler. Moreover, the transmembrane protein HitA [16] has been identified only in two of the *Lb. brevis* beer-spoiler strains UCCLBBS449 and TMW1.2112. These observations reinforce the statement that involvement of these genes in beer survival and spoilage is not always verified as they are not consistently present in beer-spoiler organisms nor are they always corresponding to beer spoilage ability if present in a strain [15]. The list of genes present only in *Lb. brevis* beer-spoiler strains shows that strains *Lb. brevis* TMW1.2108 and TMW1.2111 possess more than 90 % of these genes whereas *Lb. brevis* UCCLB95 only possesses one gene coding for the membrane transporter HorC (Table 6). The remainder of the *Lb. brevis* beer-spoiler strains carry approximately 50 % of these particular genes (Table 6).

Of these 25 unique genes shared among *Lb. brevis* beer-spoiler strains approximately 25 % code for hypothetical proteins of unknown function. Meanwhile, ~ 30 % of these genes appear to encode cell wall-associated proteins either as membrane transporters (MFS transporter, HorC) or as cell wall biosynthesis (lipopolysaccharide glycosyltransferases, acyltransferases).

As mentioned above, a beer-spoiling strain would need to extrude toxic compounds using transporters and adapt its cell wall composition to survive the harsh beer environment. A smaller portion of these unique genes are linked to transcription regulation, replication or mobilome.

Interestingly, some of the plasmid-associated genes identified among *Lb. brevis* beer-spoiler strains in this analysis have also been highlighted previously as unique attributes of beer-spoiling strain plasmids [49]. The gene coding for the CrcB like-protein involved in ion transport was found on plasmid BSO 464-2 of the *Lb. brevis* beer-spoiler strain BSO 464 as well as a gene coding for enolase involved in glucose metabolism. A gene coding for cytosine deaminase is present in five out of the seven *Lb. brevis* beer-spoiler strains used in this analysis (Table 6) and was identified as a unique attribute on the plasmid pPECL-8 of the beer-spoiler *Pediococcus claussenii* ATCC BAA-344 [49]. Moreover, a plasmid-associated gene coding for a glycosyltransferase family 2 protein was highlighted in the analysis (Table 6), this protein was associated with excess β-glucan formation leading to a slimy ropy phenotype in the *Lb. brevis* beer-spoiler TMW1.2112 [9]. This slimy phenotype was observed in the *Lb. brevis* strain UCCLBBS124 first described in this study (Table 1), and the gene coding for the glycosyltransferase family 2 was identified on one of its plasmids UCCLBBS124_pB.

This overall examination of plasmid-associated genes shows the importance of extrachromosomal DNA in beer spoilage adaptation and opens new possibilities for understanding the beer spoilage process with an updated list of potential proteins of interest only present in *Lb. brevis* beer-spoiler strains.

Table 6. List of genes only present in plasmid sequences of at least three *Lb. brevis* strains characterized as beer-spoilers.

COG category and protein function	*Lb. brevis* beer-spoiler strains						
	TMW1.2108	**TMW1.2111**	**TMW1.2112**	**TMW1.2113**	**UCCLB95**	**UCCLBBS124**	**UCCLBBS449**
	Defence mechanisms						
Membrane protein HorC	+	+	+	-	+	+	+
	Cell wall biogenesis						
Lipopolysaccharide biosynthesis glycosyltransferase	+	+	+	+	-	+	+
Lipopolysaccharide biosynthesis glycosyltransferase	+	+	+	+	-	+	+
	Lipid transport and metabolism						
Phospholipid-glycerol acyltransferase	+	+	+	+	-	+	+
1-acyl-sn-glycerol-3-phosphate acyltransferase	+	+	+	+	-	+	+
Fatty acid-binding protein DegV	-	-	+	+	-	+	+
	Carbohydrate transport and metabolism						
Glycosyl transferase family 2	+	+	+	-	-	+	-
Enolase	+	+	-	-	-	-	+
MFS transporter	+	+	+	+	-	-	-
	Transcription						
Sigma-70 region 4 family protein	+	+	-	-	-	-	+
Transcriptional regulator TetR family	+	+	+	-	-	+	+
	Nucleotide transport and metabolism						
Cytosine deaminase	+	+	-	+	-	+	+
	Inorganic ion transport and metabolism						
CrcB-like protein	+	+	-	-	-	-	+
	Replication, recombination and repair						
Cytosine-specific methyltransferase	+	+	-	-	-	+	-
Initiator RepB protein	+	+	-	-	-	-	+
	Function unknown						
Hypothetical protein	+	+	-	-	-	-	+
Hypothetical protein	+	+	+	+	-	-	+
Hypothetical protein	+	+	+	+	-	-	-
Hypothetical protein	+	+	-	-	-	-	+
Hypothetical protein	+	+	-	-	-	+	-
Hypothetical protein	+	-	+	-	-	+	-
PemK family protein	+	+	-	-	-	+	-
	Mobilome						
Transposase	+	+	-	-	-	+	
Mobilization protein	+	+	-	-	-	-	+
Mobilization protein	+	+	-	-	-	-	+

+: gene present, -: gene absent

5. Conclusions

The isolation and genome sequencing of six *Lb. brevis* strains combined with thirteen additional, publicly available *Lb. brevis* genomes allowed a comparative genome analysis of the *Lb. brevis* species. Throughout evolution, it appears that *Lb. brevis* strains specified and differentiated one from another by acquiring plasmids and prophages, despite the presence of CRISPR-Cas and R/M systems which may have limited such foreign DNA invasion events. These latter systems are of relevance for future functional investigations that may necessitate the development of DNA transfer and/or mutagenesis tools. *Lb. brevis* strains represent a significant threat for the brewing industry being the most common cause of beer spoilage; however, this spoiling ability is strain specific. The comparative genome analysis performed here highlights that most of the *Lb. brevis* strains with the ability to grow in beer are within the strains with the highest number of CDSs in their overall chromosomal sequences. This observation suggests a link to evolution and adaptation to beer in which the strain would have acquired novel genes and functions in order to adapt and survive in the harsh environment that beer represents. The role(s) of the "acquired" or beer-specific CDSs revealed that almost a quarter of these are linked to oxidation-reduction reactions, possibly playing a role in the response to oxidative stress. Another 22 % are linked to transcription regulation, 21 % encode cell surface proteins while 14 % are encoding membrane transport related proteins and possibly associated to harmful compound extrusion encountered by the *Lb. brevis* strains when surviving and growing in beer. Additional genetic diversification of these *Lb. brevis* strains is expected to have occurred through plasmid acquisition that also likely contributes to beer adaptation. The plasmid content analysis of the different *Lb. brevis* beer-spoiler strains highlighted the presence of unique proteins shared among these strains. These proteins are mostly hypothetical proteins while approximately 30 % are linked to membrane transport, and

cell-wall synthesis. These observations demonstrate the complexity of microorganisms' beer spoilage ability and suggest that adaptation of the *Lb. brevis* strain to beer is a complex process, not due to the action of only one specific gene product, but more likely the intervention of a complex, multi-factorial response.

6. References

1. Vos, P.; Garrity, G.; Jones, D.; Krieg, N.R.; Ludwig, W.; Rainey, F.A.; Schleifer, K.-H.; Whitman, W. Bergey's manual of systematic bacteriology: volume 3: the Firmicutes. **2011**, *3*, 464-511.
2. Kandler, O. Carbohydrate metabolism in lactic acid bacteria. *Antonie van Leeuwenhoek* **1983**, *49*, 209-224, doi:10.1007/bf00399499.
3. Felis, G.E.; Dellaglio, F. Taxonomy of Lactobacilli and Bifidobacteria. *Current Issues in Intestinal Microbiology* **2007**, *8*, 44-61.
4. Duar, R.M.; Lin, X.B.; Zheng, J.; Martino, M.E.; Grenier, T.; Pérez-Muñoz, M.E.; Leulier, F.; Gänzle, M.; Walter, J. Lifestyles in transition: evolution and natural history of the genus *Lactobacillus*. *FEMS Microbiology Reviews* **2017**, *41*, S27-S48, doi:10.1093/femsre/fux030.
5. Makarova, K.; Slesarev, A.; Wolf, Y.; Sorokin, A.; Mirkin, B.; Koonin, E.; Pavlov, A.; Pavlova, N.; Karamychev, V.; Polouchine, N.; Shakhova, V.; Grigoriev, I.; Lou, Y.; Rohksar, D.; Lucas, S.; Huang, K.; Goodstein, D.M.; Hawkins, T.; Plengvidhya, V.; Welker, D.; Hughes, J.; Goh, Y.; Benson, A.; Baldwin, K.; Lee, J.; Díaz-Muñiz, I.; Dosti, B.; Smeianov, V.; Wechter, W.; Barabote, R.; Lorca, G.; Altermann, E.; Barrangou, R.; Ganesan, B.; Xie, Y.; Rawsthorne, H.; Tamir, D.; Parker, C.; Breidt, F.; Broadbent, J.; Hutkins, R.; O'Sullivan, D.; Steele, J.; Unlu, G.; Saier, M.; Klaenhammer, T.; Richardson, P.; Kozyavkin, S.; Weimer, B.; Mills, D. Comparative genomics of the lactic acid bacteria. *Proceedings of the National Academy of Sciences of the United States of America* **2006**, *103*, 15611-15616, doi:10.1073/pnas.0607117103.

6. Fukao, M.; Oshima, K.; Morita, H.; Toh, H.; Suda, W.; Kim, S.W.; Suzuki, S.; Yakabe, T.; Hattori, M.; Yajima, N. Genomic analysis by deep sequencing of the probiotic *Lactobacillus brevis* KB290 harboring nine plasmids reveals genomic stability. *PLoS One* **2013**, *8*, doi:10.1371/journal.pone.0060521.

7. Salvetti, E.; Torriani, S.; Felis, G.E. The genus *Lactobacillus*: a taxonomic update. *Probiotics and Antimicrobial Proteins* **2012**, *4*, 217-226, doi:10.1007/s12602-012-9117-8.

8. Bergsveinson, J.; Pittet, V.; Ewen, E.; Baecker, N.; Ziola, B. Genome sequence of rapid beer-spoiling isolate *Lactobacillus brevis* BSO 464. *Genome Announcements* **2015**, *3*, doi:10.1128/genomeA.01411-15.

9. Fraunhofer, M.E.; Geissler, A.J.; Wefers, D.; Bunzel, M.; Jakob, F.; Vogel, R.F. Characterization of β-glucan formation by *Lactobacillus brevis* TMW 1.2112 isolated from slimy spoiled beer. *International Journal of Biological Macromolecules* **2018**, *107*, 874-881, doi:https://doi.org/10.1016/j.ijbiomac.2017.09.063.

10. Sakamoto, K.; Konings, W.N. Beer spoilage bacteria and hop resistance. *International Journal of Food Microbiology* **2003**, *89*, 105-124.

11. Vaughan, A.; O'Sullivan, T.; van Sinderen, D. Enhancing the microbiological stability of malt and beer — a review. *Journal of the Institute of Brewing* **2005**, *111*, 355-371, doi:10.1002/j.2050-0416.2005.tb00221.x.

12. Bokulich, N.A.; Bamforth, C.W. The microbiology of malting and brewing. *Microbiology and Molecular Biology Reviews* **2013**, *77*, 157-172, doi:10.1128/MMBR.00060-12.

13. Sakamoto, K.; Margolles, A.; van Veen, H.W.; Konings, W.N. Hop resistance in the beer spoilage bacterium *Lactobacillus brevis* is mediated by the ATP-Binding Cassette

multidrug transporter HorA. *Journal of Bacteriology* **2001**, *183*, 5371-5375, doi:10.1128/JB.183.18.5371-5375.2001.

14. Iijima, K.; Suzuki, K.; Ozaki, K.; Yamashita, H. HorC confers beer-spoilage ability on hop-sensitive *Lactobacillus brevis* ABBC45cc. *Journal of Applied Microbiology* **2006**, *100*, 1282-1288, doi:10.1111/j.1365-2672.2006.02869.x.
15. Suzuki, K.; Iijima, K.; Sakamoto, K.; Sami, M.; Yamashita, H. A review of hop resistance in beer spoilage lactic acid bacteria. *Journal of the Institute of Brewing* **2006**, *112*, 173-191.
16. Hayashi, N.; Ito, M.; Horiike, S.; Taguchi, H. Molecular cloning of a putative divalent-cation transporter gene as a new genetic marker for the identification of *Lactobacillus brevis* strains capable of growing in beer. *Applied Microbiology and Biotechnology* **2001**, *55*, 596-603.
17. Kant, R.; Blom, J.; Palva, A.; Siezen, R.J.; de Vos, W.M. Comparative genomics of *Lactobacillus*. *Microbial Biotechnology* **2011**, *4*, 323-332, doi:10.1111/j.1751-7915.2010.00215.x.
18. Claesson, M.J.; van Sinderen, D.; O'Toole, P.W. *Lactobacillus* phylogenomics--towards a reclassification of the genus. *International Journal of Systematic and Evolutionary Microbiology* **2008**, *58*, 2945-2954, doi:10.1099/ijs.0.65848-0.
19. Goh, Y.J.; Klaenhammer, T.R. Genomic features of *Lactobacillus* species. *Frontiers in Bioscience* **2009**, *14*, 1362-1386.
20. Gupta, P.K. Single-molecule DNA sequencing technologies for future genomics research. *Trends in Biotechnology* **2008**, *26*, 602-611, doi:https://doi.org/10.1016/j.tibtech.2008.07.003.

21. McCarthy, A. Third generation DNA sequencing: Pacific Biosciences' single molecule real time technology. *Chemistry and Biology* **2010**, *17*, 675-676, doi:https://doi.org/10.1016/j.chembiol.2010.07.004.

22. Deasy, T.; Mahony, J.; Neve, H.; Heller, K.J.; van Sinderen, D. Isolation of a virulent *Lactobacillus brevis* phage and its application in the control of beer spoilage. *Journal of Food Protection* **2011**, ***74***, 2157-2161, doi:10.4315/0362-028x.jfp-11-262.

23. Hyatt, D.; Chen, G.-L.; LoCascio, P.F.; Land, M.L.; Larimer, F.W.; Hauser, L.J. Prodigal: prokaryotic gene recognition and translation initiation site identification. *BMC Bioinformatics* **2010**, *11*, 119, doi:10.1186/1471-2105-11-119.

24. Altschul, S.; Gish, W.; Miller, W.; Myers, E.; Lipman, D. Basic local alignment search tool. *Journal of Molecular Biology* **1990**, *215*, doi:10.1016/s0022-2836(05)80360-2.

25. Schattner, P.; Brooks, A.N.; Lowe, T.M. The tRNAscan-SE, snoscan and snoGPS web servers for the detection of tRNAs and snoRNAs. *Nucleic Acids Research* **2005**, *33*, W686-W689, doi:10.1093/nar/gki366.

26. Lagesen, K.; Hallin, P.; Rødland, E.A.; Stærfeldt, H.-H.; Rognes, T.; Ussery, D.W. RNAmmer: consistent and rapid annotation of ribosomal RNA genes. *Nucleic Acids Research* **2007**, *35*, 3100-3108, doi:10.1093/nar/gkm160.

27. Roberts, R.J.; Vincze, T.; Posfai, J.; Macelis, D. REBASE—a database for DNA restriction and modification: enzymes, genes and genomes. *Nucleic Acids Research* **2015**, *43*, D298-299, doi:10.1093/nar/gku1046.

28. Enright, A.J.; Van Dongen, S.; Ouzounis, C.A. An efficient algorithm for large-scale detection of protein families. *Nucleic Acids Research* **2002**, *30*, 1575-1584, doi:10.1093/nar/30.7.1575.

29. Tatusov, R.L.; Galperin, M.Y.; Natale, D.A.; Koonin, E.V. The COG database: a tool for genome-scale analysis of protein functions and evolution. *Nucleic Acids Research* **2000**, *28*, 33-36, doi:10.1093/nar/28.1.33.

30. Edgar, R.C. MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research* **2004**, *32*, 1792-1797, doi:10.1093/nar/gkh340.

31. Guindon, S.; Gascuel, O.; Rannala, B. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Systematic Biology* **2003**, *52*, 696-704, doi:10.1080/10635150390235520.

32. Letunic, I.; Bork, P. Interactive tree of life (iTOL) v3: an online tool for the display and annotation of phylogenetic and other trees. *Nucleic Acids Research* **2016**, *44*, W242-W245, doi:10.1093/nar/gkw290.

33. Zhao, Y.; Wu, J.; Yang, J.; Sun, S.; Xiao, J.; Yu, J. PGAP: pan-genomes analysis pipeline. *Bioinformatics* **2012**, *28*, doi:10.1093/bioinformatics/btr655.

34. Horvath, P.; Barrangou, R. CRISPR/Cas, the immune system of bacteria and archaea. *Science* **2010**, *327*, 167-170, doi:10.1126/science.1179555.

35. Modrich, P. Structures and mechanisms of DNA restriction and modification enzymes. *Quarterly Reviews of Biophysics* **2009**, *12*, 315-369, doi:10.1017/S0033583500005461.

36. Fukao, M.; Oshima, K.; Morita, H.; Toh, H.; Suda, W.; Kim, S.-W.; Suzuki, S.; Yakabe, T.; Hattori, M.; Yajima, N. Genomic analysis by deep sequencing of the probiotic *Lactobacillus brevis* KB290 harboring nine plasmids reveals genomic stability. *PLoS ONE* **2013**, *8*, e60521, doi:10.1371/journal.pone.0060521.

37. Wu, Q.; Law, Y.S.; Shah, N.P. Dairy *Streptococcus thermophilus* improves cell viability of *Lactobacillus brevis* NPS-QW-145 and its γ-aminobutyric acid biosynthesis ability in milk. *Scientific Reports* **2015**, *5*, doi:10.1038/srep12885.

38. Jang, S.H.; Yoon, B.H.; Chang, H.I. Complete nucleotide sequence of the temperate bacteriophage LBR48, a new member of the family *Myoviridae*. *Archives of Virology* **2011**, *156*, 319-322, doi:10.1007/s00705-010-0841-7.

39. Bottacini, F.; O'Connell-Motherway, M.; Kuczynski, J.; O'Connell, K.J.; Serafini, F.; Duranti, S.; Milani, C.; Turroni, F.; Lugli, G.A.; Zomer, A.; Zhurina, D.; Riedel, C.; Ventura, M.; van Sinderen, D. Comparative genomics of the *Bifidobacterium breve* taxon. *BMC Genomics* **2014**, *15*, doi:10.1186/1471-2164-15-170.

40. Kelleher, P.; Bottacini, F.; Mahony, J.; Kilcawley, K.N.; van Sinderen, D. Comparative and functional genomics of the *Lactococcus lactis* taxon; insights into evolution and niche adaptation. *BMC Genomics* **2017**, *18*, 267, doi:10.1186/s12864-017-3650-5.

41. Booth, I.R. Regulation of cytoplasmic pH in bacteria. *Microbiological Reviews* **1985**, *49*, 359-378.

42. Cook, G.M.; Russell, J.B. The effect of extracellular pH and lactic acid on pH homeostasis in *Lactococcus lactis* and *Streptococcus bovis*. *Current Microbiology* **1994**, *28*, 165-168, doi:10.1007/bf01571059.

43. Siegumfeldt, H.; Rechinger, K.B.; Jakobsen, M. Dynamic changes of intracellular pH in individual lactic acid bacterium cells in response to a rapid drop in extracellular pH. *Applied and Environmental Microbiology* **2000**, *66*, 2330-2335.

44. Betteridge, D.J. What is oxidative stress? *Metabolism: Clinical and Experimental* **2000**, *49*, 3-8.

45. van de Guchte, M.; Serror, P.; Chervaux, C.; Smokvina, T.; Ehrlich, S.D.; Maguin, E. Stress responses in lactic acid bacteria. *Antonie van Leeuwenhoek* **2002**, *82*, 187-216, doi:10.1023/a:1020631532202.

46. Kavanagh, K.L.; Jörnvall, H.; Persson, B.; Oppermann, U. Medium- and short-chain dehydrogenase/reductase gene and protein families: the SDR superfamily: functional

and structural diversity within a family of metabolic and regulatory enzymes. *Cellular and Molecular Life Sciences* **2008**, *65*, 3895-3906, doi:10.1007/s00018-008-8588-y.

47. Höper, D.; Völker, U.; Hecker, M. Comprehensive characterization of the contribution of individual sigB-dependent general stress genes to stress resistance of *Bacillus subtilis*. *Journal of Bacteriology* **2005**, *187*, 2810-2826, doi:10.1128/jb.187.8.2810-2826.2005.

48. Pumirat, P.; Boonyuen, U.; Vanaporn, M.; Pinweha, P.; Tandhavanant, S.; Korbsrisate, S.; Chantratita, N. The role of short-chain dehydrogenase/oxidoreductase, induced by salt stress, on host interaction of *B. pseudomallei*. *BMC Microbiology* **2014**, *14*, 1, doi:10.1186/1471-2180-14-1.

49. Bergsveinson, J.; Ziola, B. Comparative genomic and plasmid analysis of beer-spoiling and non-beer-spoiling *Lactobacillus brevis* isolates. *Canadian Journal of Microbiology* **2017**, *63*, 970-983, doi:10.1139/cjm-2017-0405.

Chapter III

Beer spoilage and low pH tolerance is linked to manganese homeostasis in *Lactobacillus brevis*

This chapter has been submitted as a research article: Beer spoilage and low pH tolerance is linked to manganese homeostasis in *Lactobacillus brevis*. *International Journal of Food Microbiology*. 2019.

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1. Abstract

Beer is a harsh medium for bacteria to survive in owing to the presence of ethanol, hop compounds, a low pH, and limiting nutrients. Despite this, some members of the lactic acid bacteria (LAB) including strains of *Lactobacillus brevis* have evolved the ability to grow in beer. Particular plasmid-encoded genes, such as *horA, horC* and *hitA*, are associated with hop tolerance; however, the presence of these genes among LAB is not always correlated with the ability to survive and grow in beer. In the current study a transcriptomic analysis of two *Lb. brevis* beer-spoiling strains was performed comparing growth in a nutritive medium with or without the imposition of a stressor related to the beer environment such as ethanol, low pH and hops. This allowed the identification of a gene predicted to encode a manganese transporter as being responsible for low pH tolerance, thereby facilitating growth in beer. Moreover, the importance of manganese for *Lb. brevis* growth and survival in a low pH environment (e.g. beer) was highlighted.

2. Introduction

The typical composition of beer makes it a rather hostile environment for microorganisms to grow and thrive. Indeed, the presence of ethanol (0.5-10 % w/w), carbon dioxide (approximately 0.5 % w/w) and hop compounds (approximately 14-55 ppm *iso*-α-acids) combined with a low oxygen content (<0.1 ppm), depleted nutritive substances (only traces) and a low pH (3.8-4.7) generally do not support microbial growth [1]. However, certain bacteria have evolved the ability to survive and even grow in this environment. In many cases, microbial beer spoilage incidents are reported to be caused by members of the lactic acid bacteria (LAB) particularly *Lactobacillus brevis* [1-3]. Although LAB have a so-called generally regarded as safe (GRAS) status, certain *Lb. brevis* isolates are known to be responsible for the production of off-flavor, acidity and/or turbidity in beer, thus negatively impacting on its organoleptic properties. Bacterial spoilage of beer may furthermore result in product withdrawal or recall with concomitant economic losses for the brewing industry [1-3]. Despite the problems caused by some members of this species, relatively little data exist regarding the mechanisms by which such *Lb. brevis* strains are able to grow in beer. In 2016, a transcriptional analysis of the beer-spoiling strain *Lb. brevis* BSO 464 grown in gassed beer suggested that the ability of the strain to grow and spoil beer is achieved through a multitude of genetic adaptations such as cell wall and membrane modifications, and/or nutrient scavenging [4]. Therefore, it is clear that adaptation to grow in beer is a complex process, and likely due to a complicated, multi-factorial adaptive response [4]. *Lb. brevis* is among the most resistant bacterial species to hop compounds, but the degree of hop resistance appears to be strain specific as indeed is beer spoilage capability. The main mechanism of hop resistance is the active extrusion of the hop compound *iso*-α-acid. The proteins that are known to be responsible for *iso*-α-acid export are HorA, an ABC-type multidrug transporter, and HorC, a proton motive force-dependent transporter [5-7]. Furthermore, the transmembrane protein,

HitA, is believed to contribute to *iso*-α-acid resistance since it acts to expel protons (generated due to cytoplasmic *iso*-α-acid dissociation) in conjunction with divalent cations such as Mn^{2+} [8].

However, the involvement of these genes and their encoded products in beer survival and spoilage is not always verified as they are not consistently present in beer-spoiling organisms and their presence in a given strain is not always synonymous with beer spoilage [6], demonstrating the complexity and multifactorial nature of bacterial beer spoilage. In the current study, two beer-spoiling *Lb. brevis* strains, UCCLBBS124 and UCCLBBS449, isolated from spoiled beer originating from Singapore and from an unpasteurized beer produced in the Netherlands, respectively, were employed as model strains in a transcriptomic study. The influence of environmental factors such as low pH, ethanol or hop content was assessed in this study. This resulted in the identification of a gene predicted to encode a manganese transporter protein involved in low pH tolerance, thereby facilitating the survival and dominance of these strains in beer. The relevance of divalent cations such as manganese cations (Mn^{2+}) and ferrous ion (Fe^{2+}) in low pH and beer survival was also investigated.

3. Materials and Methods

3.1. Bacterial strains and cultivation media

Bacterial strains used in this study are listed in Table 1. *Lb. brevis* strains were grown in MRS broth (Oxoid Ltd., England) at 30 ˚C, while *Lactococcus lactis* NZ9000 was grown in M17 broth (Oxoid Ltd., England) supplemented with 0.5 % glucose. 5 µg/mL chloramphenicol (Cm5) was added to the culture where indicated.

Table 1. Bacterial strains and plasmids used in this study.

Strain / Plasmid	Description	Reference
***Lb. brevis* strains**		
UCCLBBS124	Beer-spoiling strain isolated from spoiled beer keg (Singapore)	[9]
UCCLBBS449	Beer-spoiling strain isolated from unpasteurized spoiled beer (The Netherlands)	[9]
UCCLB521	Non-beer spoiler strain isolated from brewery environment (The Netherlands)	[9]
UCCLB556	Non-beer spoiler strain isolated from brewery environment (The Netherlands)	[9]
SA-C12	Non-beer spoiler strain isolated from silage (Ireland)	[9]
UCCLBBS124 pNZ44	UCCLBBS124 carrying pNZ44	This study
UCCLBBS124 pNZ44::*0274i*	UCCLBBS124 carrying a pNZ44 derivative for *0274* gene silencing	This study
***L. lactis* strain**		
NZ9000	Transformation host	[10]
Plasmids		
pNZ44	Transformation vector, chloramphenicol resistance gene	
pNZ44*::0274i*	*0274* gene silencing vector	This study

3.2. Transcriptomic growth conditions

The two isolates *Lb. brevis* UCCLBBS124 and UCCLBBS449 were grown in MRS broth to assess their differential transcription under nutritive growth conditions versus transcription during growth under acidic, ethanol or hop stress. They were grown directly from a -80 ˚C stock in MRS broth pH 6.2 at 30 ˚C for 24 h (UCCLBBS124) or 48 h (UCCLBBS449). A 2 % inoculum from the overnight culture was transferred into MRS broth pH 6.2 and incubated at

30 ˚C for 24 h (UCCLBBS124) or 48 h (UCCLBBS449) to achieve late log growth (OD600nm ~ 1.0). From this culture, MRS broth with the following modifications was inoculated with a 2 % inoculum in: (a) MRS broth pH 4.0, (b) MRS broth containing 5 % ethanol and (c) MRS broth containing 30 ppm tetra-iso-α-acids compounds (Hopsteiner, Mainburg, Germany). For each condition, three independent replicates were prepared. All cultures were grown in a final volume of 100 mL at 30 ˚C for 20 min. Cells were harvested after 20 min incubation in the medium by centrifugation at 5,000 × g for 10 min.

3.3. RNA isolation and mRNA processing

The resulting cell pellets were resuspended in 400 µL TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and flash-frozen in a -80 ˚C ethanol (EtOH) bath. Samples were then maintained at -80 ˚C until required for further processing and analysis. Cells were disrupted and total RNA was extracted using a High Pure RNA Isolation kit (Roche, Germany). RNA quality and yield were assessed by observation of band integrity on a 1 % agarose gel and determination of the OD_{260nm}. For all RNA preparations, OD_{260nm} / OD_{280nm} ratios of > 1.9 and OD_{260nm} / OD_{230nm} ratios of > 2.1 were deemed acceptable. For cDNA synthesis, 10 µg of total RNA was used in an annealing reaction with 1.6 $ng.\mu l^{-1}$ of random nonamers (MWG Biotech, Germany). The mixture was heated to 70 ˚C for 5 min followed by 10 min at room temperature. SuperScript III ΔReverse transcriptase (Invitrogen, USA) was added to the mixture for 16 h at 42 ˚C allowing reverse transcription. cDNA was purified using the Kreatech DSK-001 kit according to the manufacturer's instructions (Kreatech, Amsterdam, The Netherlands). cDNA was labelled, with Cy3 or Cy5 using the Kreatech DSK-001 labelling kit (Kreatech, Amsterdam, The Netherlands). Labelled cDNA was hybridized using the Agilent Gene Transcription hybridization kit as previously described [11].

3.4. DNA microarray analysis

Following hybridization, microarrays were washed and DNA microarrays were scanned and analyzed as previously described [11]. DNA microarrays containing oligonucleotide primers representing each of the 2,648 annotated genes on the genome of *Lb. brevis* UCCLBBS124 and the 2,719 annotated genes of the genome of *Lb. brevis* UCCLBBS449 were designed by eArray (Agilent, USA) and were obtained from Agilent Technologies (Palo Alto, CA, USA). The microarray data obtained in this study have been deposited in NCBI's Gene Expression Omnibus database and are accessible through GEO Series accession number GSE133065.

3.5. qRT-PCR microarray validation

cDNA was generated as described above. Primers for quantitative reverse transcription-PCR were designed using Primer3Plus [12]. qRT-PCR was performed using SYBR green I Master Mix (Roche, USA) according to the manufacturer's instructions in triplicate using a LightCycler 480 II detection system (Roche, USA). Cycling conditions consisted of an initial activation step of 95 ˚C for 10 min, followed by 30 cycles of 95 ˚C for 15 seconds, 53 ˚C for 5 seconds, and 72 ˚C for 15 seconds. The housekeeping gene *gyrB* was used to normalize results and transcription levels were determined using the $2^{-\Delta\Delta C_T}$ method [13] as follows:

$\Delta C_T = (\text{Average } C_{T,\text{target}} - \text{Average } C_{T,gyrB})$

$\Delta\Delta C_T = ((\text{Average } \Delta C_T)_{\text{stress condition}} - (\text{Average } \Delta C_T)_{\text{MRS broth}})$

Where C_T represents the threshold cycle indicating the number of cycle at which the amount of amplified target reaches a fixed threshold; target represents the target gene and *gyrB* the housekeeping gene, stress condition represents pH 4.0, 5 % ethanol or 30 ppm tetra-*iso*-α-acids

and MRS broth represents the nutritive medium MRS broth as the non-stressor reference growth condition.

$2^{-\Delta\Delta C_T}$ represents the final fold change in transcription level of the targeted gene when cultured in a given stress condition compared to culture in nutritive media (MRS broth).

3.6. Construction of antisense plasmid vectors

Genes of interest identified after microarray analysis were amplified by PCR (Table 2) and cloned into the pNZ44 plasmid in the reverse orientation relative to the P44 promoter [14]. PCR products and pNZ44 plasmid vector DNA were digested with the appropriate enzymes (Thermo Scientific, USA) at 37 ˚C for at least 4 h, following the manufacturer's instructions. A ratio of (3:1) was applied for the ligation of the PCR product with the pNZ44 vector using T4 DNA ligase (Promega, USA). The mixture was incubated at room temperature for at least 4 hours prior to electrotransformation into *L. lactis* NZ9000 competent cells [15].

Table 2. PCR primers used in this study. Incorporated restriction sites are indicated in capital letters.

Primer name	Sequence (5' - 3')	Target
pNZ44F	aacaattgtaacccatac	pNZ44 promoter
pNZ44R	gaacgtttcaagccttgg	pNZ44 MCS
2104F	aaaaaaTCTAGAcggatggagtttgatgat	Gene *UCCLBBS124_2104* in UCCLBBS124
2104R	aaaaaaCCATGGttagtcatgctgttgcccc	Gene *UCCLBBS124_2104* in UCCLBBS124
0274F	aaaaaaTCTAGAgctgcctaagtccttgata	Gene *UCCLBBS124_0274* in UCCLBBS124
0274R	aaaaaaCCATGGcgacagtccttttgccttaa	Gene *UCCLBBS124_0274* in UCCLBBS124
2102F	aaaaaaTCTAGAgcgtcagtcatgactagtt	Gene *UCCLBBS124_2102* in UCCLBBS124
2102R	aaaaaaCCATGGttagttggcgatagtttcg	Gene *UCCLBBS124_2102* in UCCLBBS124
0367F	aaaaaaTCTAGAgagtttatcgcaatgacccat	Gene *UCCLBBS124_0367* in UCCLBBS124
0367R	aaaaaaCCATGGctagtgcgcgtgagcaac	Gene *UCCLBBS124_0367* in UCCLBBS124
2396F	aaaaaaTCTAGAgtgcgatgatgaacgactt	Gene *UCCLBBS124_2396* in UCCLBBS124
2396R	aaaaaaCCATGGctaaaaaacggggtacg	Gene *UCCLBBS124_2396* in UCCLBBS124
2055F	aaaaaaTCTAGActatgagaaatgaagccg	Gene *UCCLBBS124_2055* in UCCLBBS124
2055R	aaaaaaCCATGGttacttcttaaggttttc	Gene *UCCLBBS124_2055* in UCCLBBS124
0227F	aaaaaaTCTAGAccacatggcagaaaatt	Gene *UCCLBBS124_0227* in UCCLBBS124
0227R	aaaaaaCCATGGctagttaattgatccttg	Gene *UCCLBBS124_0227* in UCCLBBS124
0167F	aaaaaaTCTAGAgattatggctaatgatg	Gene *UCCLBBS124_0167* in UCCLBBS124
0167R	aaaaaaCCATGGttagtcgatgctaatctcg	Gene *UCCLBBS124_0167* in UCCLBBS124
0909F	aaaaaaTCTAGAggggcgattaagatgaa	Gene *UCCLBBS124_0909* in UCCLBBS124
0909R	aaaaaaCCATGGttagtttgaagttttatcagtttgcg	Gene *UCCLBBS124_0909* in UCCLBBS124
2104F	aaaaaaTCTAGAcggatggagtttgatgat	Genes *UCCLBBS124_2102-2104* in UCCLBBS124
2102'R	aaaaaaCTGCAGcttttagttggcgatagtttc	Genes *UCCLBBS124_2102-2104* in UCCLBBS124

3.7. Preparation of competent cells and electrotransformation

Competent cells of *L. lactis* NZ9000 were prepared as previously described [15]. Competent cells of *Lb. brevis* UCCLBBS124 were prepared after adaptation from a previously described protocol [16]: an overnight culture was transferred (1 % inoculum) to 10 mL MRS broth containing 1 % glycine and incubated overnight at 30 ˚C. 5 mL of the overnight culture was transferred to fresh MRS broth containing 1 % glycine (50 mL final volume) and cells were grown to an OD_{600nm} of 0.6. Cells were harvested by centrifugation at 4,000 × *g* for 15 min at 4 ˚C and washed in ice-cold wash buffer (0.5 M sucrose, 10 % glycerol). The wash step was repeated twice and the cells were finally resuspended in 200 µL wash buffer prior to storage at -80 ˚C. All constructs were generated in *L. lactis* NZ9000, checked by sequencing after PCR amplification using the primers pNZ44F and pNZ44R (Table 2) prior to their transfer into *Lb. brevis* UCCLBBS124. Electrotransformation was performed using freshly prepared competent

cells as described above, where 45 µL of cells and 5 µL of plasmid construct were mixed into a pre-chilled 2 mm electroporation cuvette (Cell Projects, Kent, England) and subjected to electroporation at 1.5 kV (*Lb. brevis*) or 2.0 kV (*L. lactis*), 200 Ω, 25 µF. Following electroporation, 950 µL recovery broth was added (MRS broth supplemented with 0.5 M sucrose and 0.1 M $MgCl_2$ (*Lb. brevis*) or GM17 broth supplemented with 20 mM $MgCl_2$ and 2 mM $CaCl_2$ (*L. lactis*)). Cells were recovered at 30 ˚C for 3 h (*Lb. brevis*) or 2 h (*L. lactis*) prior to spread plating on MRS (*Lb. brevis*) or GM17 (*L. lactis*) agar supplemented with Cm5. Presumed transformants were purified on MRS agar supplemented with Cm5 and the integrity of single colony isolates was checked by sequencing after PCR amplification using the primers pNZ44F and pNZ44R (Table 2) and subjected to growth assays described below.

3.8. Growth assays

Growth profiles of the wild-type strain and mutant derivatives were performed by transferring an overnight culture (1 % inoculum) to MRS broth, MRS broth adjusted to pH 4.0 or beer supplemented with $MnCl_2$ or $FeSO_4$ as required. Cultures were incubated at 30 ˚C for 72 hours. One mL of culture was retrieved after 7, 24, 48 and 72 hours, diluted in ¼ strength Ringer's solution and plated on MRS agar plates. Plates were incubated at 30 ˚C anaerobically for 48 hours prior to colony counting. The growth profile of each bacterial strain was assessed after CFU/mL calculation. Similarly, growth profiles were also performed with non-beer spoiling *Lb. brevis* strains UCCLB521, UCCLB556 and SA-C12 (Table 1). Non-beer spoiling strains were grown in beer supplemented with 10 and 20 mM $MnCl_2$ and incubated at 30 ˚C for 72 hours. One mL of culture was retrieved after 24, 48 and 72 hours growth in beer, diluted in Ringer's solution and plated on MRS agar plates. Plates were incubated at 30 ˚C anaerobically for 48 hours prior to colony counting.

3.9. Gene expression in MRS broth at pH 4.0 +/- Mn^{2+}

Specific genes encoding proteins involved in oxidative stress response previously highlighted [9] were selected and their expression levels were evaluated by qRT-PCR analysis. The *Lb. brevis* beer-spoiling strain UCCLBBS124 and its derivative UCCLBBS124 pNZ44::*0274i* were grown as described above in MRS broth until late-log growth (OD_{600nm} ~ 1.0) prior to inoculation into experimental conditions: (a) MRS broth, (b) MRS broth at pH 4.0 and (c) MRS broth at pH 4.0 supplemented with 10 mM $MnCl_2$. All cultures were grown in a final volume of 100 mL at 30 ˚C for 20 min. Cells were harvested after 20 min of incubation, RNA was isolated and converted into cDNA as described above. qRT-PCR analysis was performed as described above.

4. Results and Discussion

This study was aimed at evaluating how certain *Lb. brevis* isolates are adapted so as to allow them to survive and grow in beer. A similar approach has been described previously where a transcriptomic analysis was performed on a beer-spoiling *Lb. brevis* strain during growth in both gassed and degassed beer [4]. Here, the strains were cultivated under specific stress conditions that they would typically encounter in beer: low pH, and the presence of *iso*-*α*-acids or ethanol, in order to study the specific and immediate transcriptomic response of a particular *Lb. brevis* strain to each distinct stress-inducing condition.

4.1. Transcriptome analysis

To determine the effect of ethanol, hop or low pH on the beer-spoiling *Lb. brevis* strains, UCCLBBS124 and UCCLBBS449 were exposed to these different stresses, after which total RNA was isolated. Transcriptomic profiles of both strains were obtained and compared to their respective control (growth in MRS broth). Analysis of microarray data demonstrated 92 and 41 chromosomal genes being differentially expressed (at least 4-fold difference between the control and the tested condition, with an associated *P* value of ≤0.001) when UCCLBBS124 or UCCLBBS449 were exposed to the different stresses, respectively (Table 3). Among the different stress conditions assessed, exposure of the strains to a low pH resulted in the highest level of adaptive gene transcription, accounting for more than 50 % of the total number of differentially expressed genes (Table 3).

Table 3. Number of genes differentially expressed in *Lb. brevis* strains UCCLBBS124 and UCCLBBS449 when exposed to different stress conditions: 30 ppm *iso*-α-acids, 5 % ethanol and pH 4.0.

	UCCLBBS124				**UCCLBBS449**			
	30 ppm *iso*-α-acids	**5 % ethanol**	**pH 4.0**	**Total**	**30 ppm *iso*-α-acids**	**5 % ethanol**	**pH 4.0**	**Total**
Number of up-regulated genes	4	15	33	**52**	1	1	18	**20**
Number of down-regulated genes	18	3	19	**40**	7	0	14	**21**

*At least 4 fold difference; $P \leq 0.001$

4.2. qRT-PCR validation

The ten most highly expressed genes in *Lb. brevis* UCCLBBS124 and UCCLBBS449, when cultured under a particular stress condition, were selected and their (induced) transcriptome levels were validated using qRT-PCR. Results obtained by qRT-PCR confirmed the microarray data where genes showing significant differential transcription by microarray analysis, also were shown to exhibit increased transcription when assessed with qRT-PCR (Table 4 and Table 5).

Table 4. qRT-PCR validation of the ten most over-expressed genes in *Lb. brevis* beer-spoiling UCCLBBS124 when exposed to different stress.

Condition	Gene	Putative function	Fold change by:	
			qRT-PCR	Microarray
pH 4.0	*UCCLBBS124_0274*	Manganese transport protein MntH	12.2	24.8
pH 4.0	*UCCLBBS124_2396*	Uncharacterized aminotransferase SSO0104	13.6	18.0
pH 4.0	*UCCLBBS124_2055*	Conserved hypothetical protein	15.3	17.9
pH 4.0	*UCCLBBS124_2104*	Transcriptional regulator GntR family	12.8	13.1
pH 4.0	*UCCLBBS124_2103*	Uncharacterized ABC transporter ATPase component	12.1	12.8
pH 4.0	*UCCLBBS124_0227*	ABC-type dipeptide-oligopeptide-nickel transport	16.4	12.8
pH 4.0	*UCCLBBS124_2102*	ABC Transporter Permease	17.2	12.5
30 ppm *iso*-*α*-acids	*UCCLBBS124_0367*	Nicotinamide mononucleotide transporter	9.47	11.9
5% ethanol	*UCCLBBS124_0167*	Heat shock protein HSP.16.4	26.4	11.7
pH 4.0	*UCCLBBS124_0909*	Uncharacterized transporter YdgF	18.4	11.2

Table 5. qRT-PCR validation of the ten most over-expressed genes in *Lb. brevis* beer-spoiling UCCLBBS449 when exposed to different stress.

Condition	Gene	Predicted function	Fold change by:	
			qRT-PCR	Microarray
pH 4.0	*UCCLBBS449_0258*	Manganese transport protein MntH	12.1	7.4
pH 4.0	*UCCLBBS449_2136*	Transcriptional regulator GntR family	5.2	7.0
pH 4.0	*UCCLBBS449_2359*	Malolactic enzyme	10.5	6.9
pH 4.0	*UCCLBBS449_0596*	Cellobiose-specific PTS system IIC component	17.3	5.9
pH 4.0	*UCCLBBS449_0782*	Conserved hypothetical protein	1.6	5.7
pH 4.0	*UCCLBBS449_1648*	Hypothetical protein	1.9	5.6
pH 4.0	*UCCLBBS449_2259*	Amino acid transporter	9.1	5.4
pH 4.0	*UCCLBBS449_0138*	Conserved hypothetical protein	8.4	5.1
pH 4.0	*UCCLBBS449_2135*	ABC transporter ATPase component	5.1	4.3
pH 4.0	*UCCLBBS449_2134*	ABC Transporter Permease	3.7	4.1

4.3. Gene silencing

The differential transcription observed for certain genes between growth in MRS broth and growth under a particular stress indicated that these genes are important for adaptation of *Lb. brevis* strains to the particular stress applied. Using a gene silencing approach, in which a target gene was cloned on a plasmid in reverse orientation behind a strong promoter so as to generate anti-sense mRNA of that gene, it was aimed to assess the importance of these genes for adaptation of the bacterial strain when grown under a given stress condition. Due to the very poor transformability of *Lb. brevis* UCCLBBS449, the gene silencing approach was exclusively applied to *Lb. brevis* UCCLBBS124. Derivatives of UCCLBBS124 harboring a gene silencing vector for each of the ten most highly overexpressed genes as identified in the transcriptome analysis described above (Table 4) were prepared. Interestingly, silencing of *UCCLBBS124_0274*, predicted to encode a manganese transporter protein ($MntH_{0274}$), imposed a growth phenotype on the beer-spoiling strain. Indeed, growth of the corresponding strain *Lb. brevis* UCCLBBS124 pNZ44::*0274i* was significantly ($P<0.05$) negatively impacted when cultured at a low pH (Figure 1) after 24 h. No significant impact was observed when a control carrying an empty plasmid pNZ44 was used or when the remaining nine candidate genes (Table 4) were subjected to the same transcriptional silencing strategy and then grown in MRS broth adjusted to pH 4.0 (data not shown), highlighting the importance of *UCCLBBS124_0274* (renamed here as $mntH_{0274}$) and its encoded protein in acid tolerance.

Interestingly, expression of genes involved in pH regulation and encoding manganese transporter proteins (showing low level of similarity with $MntH_{0274}$) was also upregulated during the transcriptomic analysis of *Lb. brevis* BSO 464 in beer and degassed beer corroborating results obtained in this study [4].

Clear homologues of *mntH*$_{0274}$ are present in all *Lb. brevis* complete genomes available on the NCBI website (https://www.ncbi.nlm.nih.gov/) with their deduced protein products exhibiting more than 98 % amino acid sequence similarity. MntH$_{0274}$ corresponds to the MntH protein encoded by the gene *UCCLBBS449_0258* in UCCLBBS449 which was also shown to elicit increased transcription when cultured at low pH (Table 5). MntH$_{0274}$ is predicted to possess twelve transmembrane domains, while exhibiting 60 % amino acid similarity with another MntH protein encoded by locus tag *UCCLBBS124_0489* located on the chromosome of the same strain (although the transcription level of *UCCLBBS124_0489* is not altered when cultured at low pH). Furthermore, MntH$_{0274}$ and HitA, a divalent cation transporter thought to be involved in hop resistance by extruding *iso*-*α*-acids in exchange for divalent cations such as Mn^{2+} [8], share approximately 50 % amino acid similarity, although *hitA* was not shown to be transcriptionally induced at low pH.

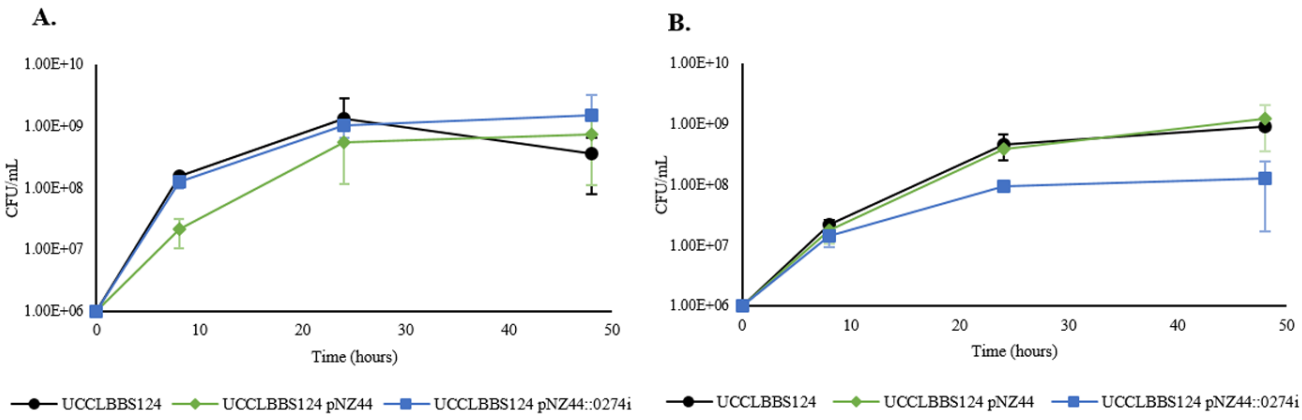


Figure 1. Effect of silencing the gene *0274* predicted to encode a manganese transporter protein in the *Lb. brevis* beer-spoiling UCCLBBS124 (UCCLBBS124 pNZ44::*0274i*) when cultured in **A.** MRS broth and **B.** MRS broth at pH 4.0. UCCLBBS124 wild-type strain and UCCLBBS124 carrying an empty plasmid (UCCLBBS124 pNZ44) were used as control.

4.4. Link between *mntH*$_{0274}$ and divalent cations

MntH$_{0274}$ and the role of divalent cations, particularly Mn^{2+}, were investigated in relation to low pH. The derivative strain UCCLBBS124 pNZ44::*0274i* (i.e. strain UCCLBBS124 in which the *mntH*$_{0274}$ gene is transcriptionally silenced by the introduction of plasmid pNZ44::*0274i*) was cultured in MRS broth at pH 4.0 supplemented with different concentrations of Mn^{2+} (1 and 10 mM $MnCl_2$) (Figure 2). Interestingly, the addition of Mn^{2+} under acidic conditions (MRS broth pH 4.0) allowed growth restoration of strain UCCLBBS124 pNZ44::*0274i*, which exhibited similar growth as the WT strain UCCLBBS124 as well as the strain carrying an empty plasmid UCCLBBS124 pNZ44 (data not shown), when 10 mM $MnCl_2$ was added to the low pH medium. Addition of Mn^{2+} to the medium is beneficial for the mutated strain at a concentration as low as 1 mM $MnCl_2$ (Figure 2) highlighting the importance of Mn^{2+} for the beer-spoiling strain UCCLBBS124 under acidic growth conditions. A link was established between *mntH*$_{0274}$ and Mn^{2+}, and for this reason the importance of other cations including Co^{2+}, Fe^{2+}, Cu^{2+}, Mg^{2+} on growth of *Lb. brevis* UCCLBBS124 pNZ44::*0274i* was investigated. An impact on growth was only observed when ferrous cations (Fe^{2+}) were added to the acidic environment (MRS broth pH 4.0). Interestingly, the addition of Fe^{2+} had a deleterious impact on growth of the *mntH*$_{0274}$-silenced strain when cultured in MRS broth and MRS broth adjusted to pH 4.0 (Figure 3). Furthermore, MRS broth pH 4.0 supplemented with Fe^{2+} affected growth of both the WT strain and its *mntH*$_{0274}$-silenced derivative (Figure 3). These results reveal the apparent toxicity of Fe^{2+} on the WT strain with an even higher toxicity of Fe^{2+} on UCCLBBS124 pNZ44::*0274i,* an impact that is even further exacerbated under acidic conditions (P<0.05). The link between iron metabolism and stress had previously been highlighted by the study of Bergsveinson et al. where they implicate the level of environmental ferrous ions in resistance to oxidative stress [4].

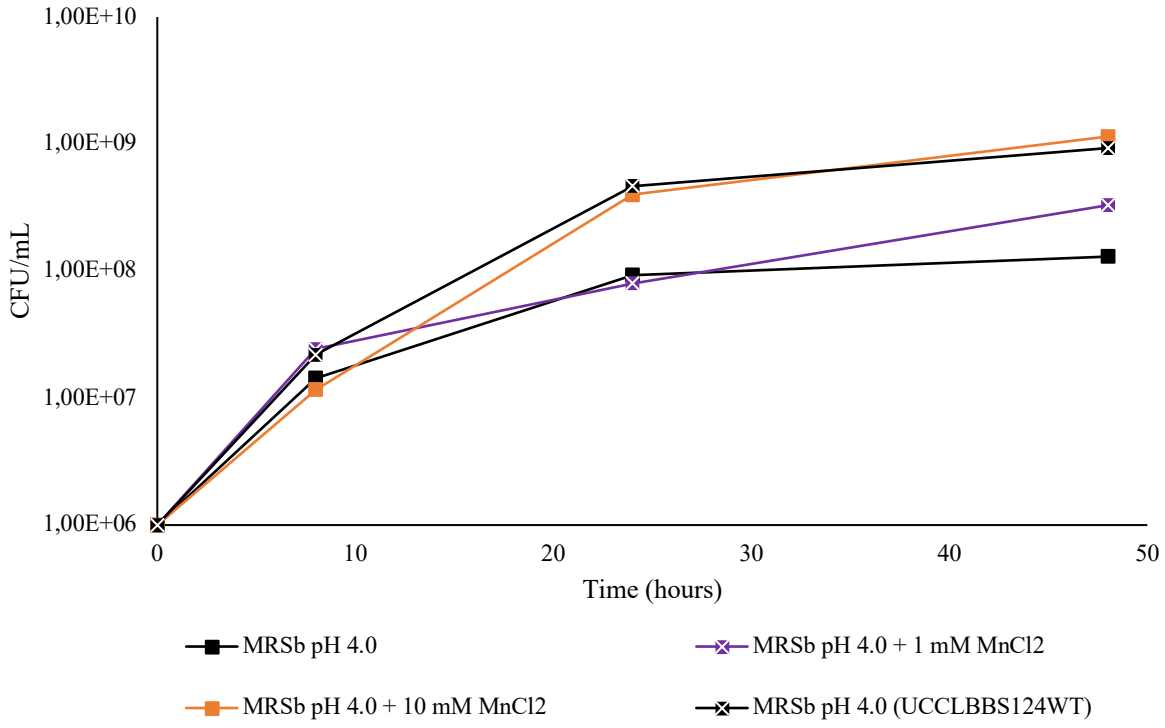
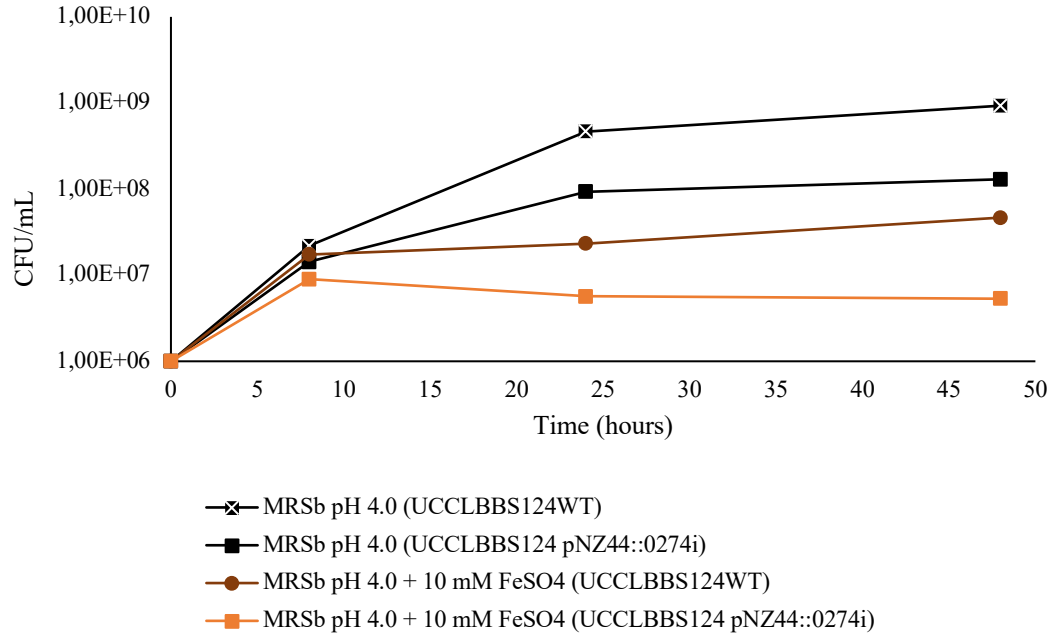


Figure 2. Growth curves of *Lb. brevis* UCCLBBS124 pNZ44::*0274i* when cultured in MRS broth at pH 4.0 supplemented with different concentrations of $MnCl_2$ (1 mM and 10 mM). The growth curve of the WT strain in MRS broth at pH 4.0 is also displayed on the graph (UCCLBBS124 WT). Growth curves were performed in triplicate and the average of those measurements is displayed in the graph above.

A.



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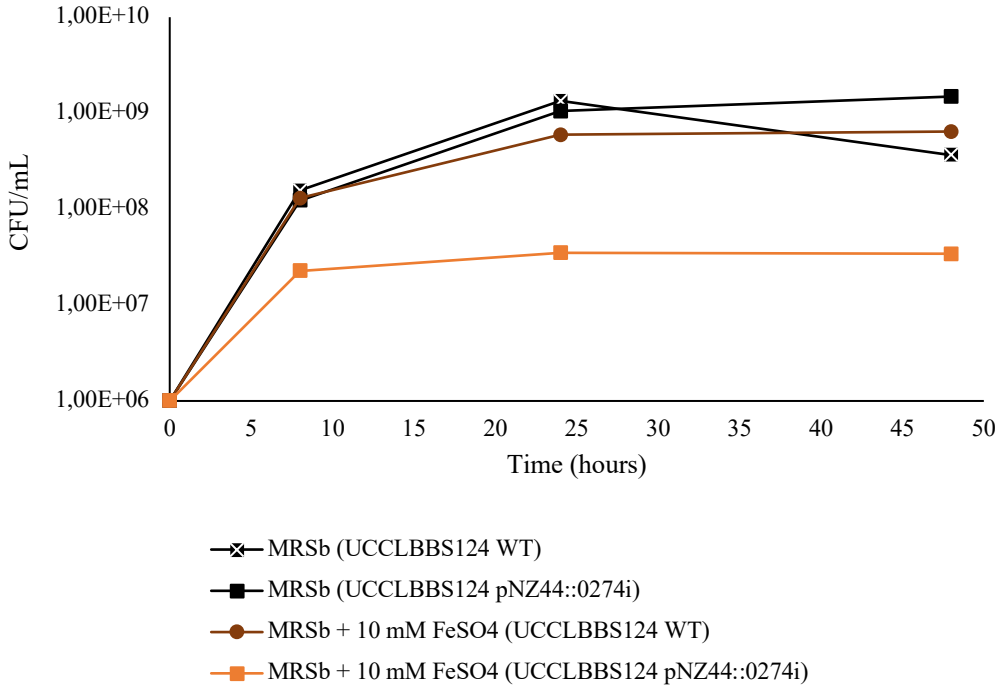

Figure 3. Growth curves of *Lb. brevis* UCCLBBS124 WT and UCCLBBS124 pNZ44::*0274i* when cultured in **A.** MRS broth at pH 4.0 supplemented or not with 10 mM $FeSO_4$ and **B.** MRS broth supplemented or not with 10 mM $FeSO_4$. Growth curves were performed in triplicate and the average of those measurements is displayed in the graph above.

4.5. Growth in beer

As shown above, inhibition of *mntH*$_{0274}$ transcription by the silencing approach negatively affects growth of the corresponding *Lb. brevis* strain in acidic environment (MRS broth pH 4.0). A low pH is one of the characteristics of beer that microorganisms are required to adapt to in order to survive and thrive in beer. The derivative strain UCCLBBS124 pNZ44::*0274i* was cultured in beer (pH 4.0) highlighting the inability of the strain to grow in beer when *mntH*$_{0274}$ is silenced (Figure 4A). The WT strain reaches 10^5 CFU/mL when grown in beer for 24 h, while UCCLBBS124 pNZ44::*0274i* exhibits a significantly lowered viable count compared to the WT (P<0.05) after 24 hours of incubation (Figure 4A). To ensure that low pH was the limiting growth factor for the derivative strain, UCCLBBS124 WT and UCCLBBS124 pNZ44::*0274i* were grown in beer with the pH adjusted to 6.5 (Figure 4A). Increasing the pH of beer, enhances growth of both strains, with *Lb. brevis* UCCLBBS124 (WT) and its derivative UCCLBBS124 pNZ44::*0274i* reaching a viable count of approximately 10^9 and 10^8 CFU/mL after 24 h of incubation, respectively (Figure 4A). These results indicate that *mntH*$_{0274}$ is required for growth *Lb. brevis* UCCLBBS124 at low pH (MRS broth or beer).

The addition of Mn^{2+} to MRS broth at pH 4.0 aided growth restoration of *Lb. brevis* UCCLBBS124 pNZ44::*0274i* to reach a comparable growth ability to that of the WT strain (Figure 2). *Lb. brevis* UCCLBBS124 pNZ44::*0274i* was furthermore grown in beer with varying concentrations of $MnCl_2$ to evaluate the impact of Mn^{2+} on its growth profile (Figure 4B). Addition of manganese to beer was shown to significantly (P<0.05) increase the viable count (CFU/mL) of UCCLBBS124 pNZ44::*0274i* by at least one hundred-fold even following $MnCl_2$ addition to a final concentration as low as 1 mM after 24 h of incubation (Figure 4B). Interestingly, adding Mn^{2+} to the medium also enhances the growth of the WT strain. Addition of 10 mM $MnCl_2$ to beer allowed both strains (WT and UCCLBBS124 pNZ44::*0274i*) to grow

with highly similar growth profiles and reaching viable counts of up to 10^7 CFU/mL after 24 h of growth (Figure 4B). These results corroborate the relevance of the *mntH*$_{0274}$ as well as the importance of Mn^{2+} for *Lb. brevis* growth in beer.

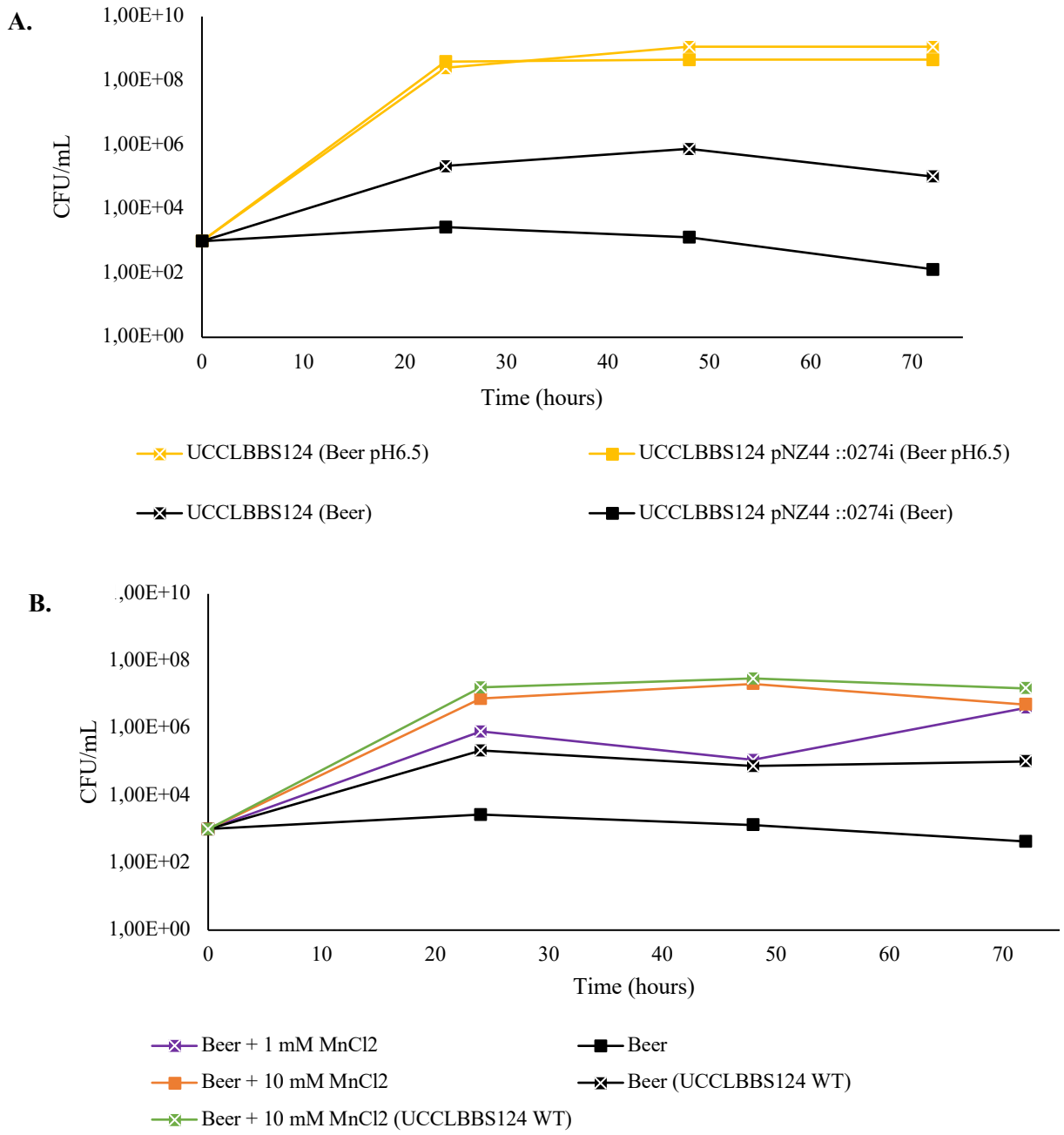

Figure 4. A. Growth curves of *Lb. brevis* UCCLBBS124 WT and UCCLBBS124 pNZ44::*0274i* when cultured in beer (black) and beer at pH6.5 (yellow). **B.** Growth curves of *Lb. brevis* UCCLBBS124 pNZ44::*0274i* in beer supplemented with different concentrations of $MnCl_2$ (1 mM and 10 mM). The growth curve of the WT strain in beer (supplemented or not with $MnCl_2$) is also displayed on the graph. Growth curves were performed in triplicate and the average of those measurements is displayed in the graph above.

4.6. Non-beer spoiling strains and Mn^{2+}

The importance of Mn^{2+} for survival and growth in beer for *Lb. brevis* beer-spoiling strains was clearly demonstrated from the data presented above. Therefore, we wanted to investigate the role of Mn^{2+} when non-beer spoiling strains are exposed to beer medium. *Lb. brevis* strains UCCLB521, SA-C12 and UCCLB556 are under normal circumstances unable to grow in beer and are thus characterized as non-beer spoiling strains [9]. When these strains are exposed to beer they lose viability after 24 to 48 hours of incubation in this medium (Figure 5), thus highlighting their inability to survive in beer. However, when beer is supplemented with 10 mM $MnCl_2$, which does not affect the pH of the medium, the strains can survive (strains UCCLB521 and UCCLB556) and even multiply (strain SA-C12) to 10^6 CFU/mL (Figure 5). In the presence of 20 mM $MnCl_2$, all tested strains were shown to significantly ($P<0.05$) grow in beer with SA-C12 achieving a viable count of up to 10^8 CFU/mL, which is comparable to the *Lb. brevis* beer-spoiling strain UCCLBBS124 (Figure 4). These results demonstrate the essential role of Mn^{2+} in *Lb. brevis* beer spoilage ability as non-beer spoiling strains were shown to be able to grow in beer upon Mn^{2+} addition to the medium. Similar results were observed in a previous study where a short term protection against hop compounds was obtained for hop tolerant and sensitive strains, after Mn^{2+} addition [17].

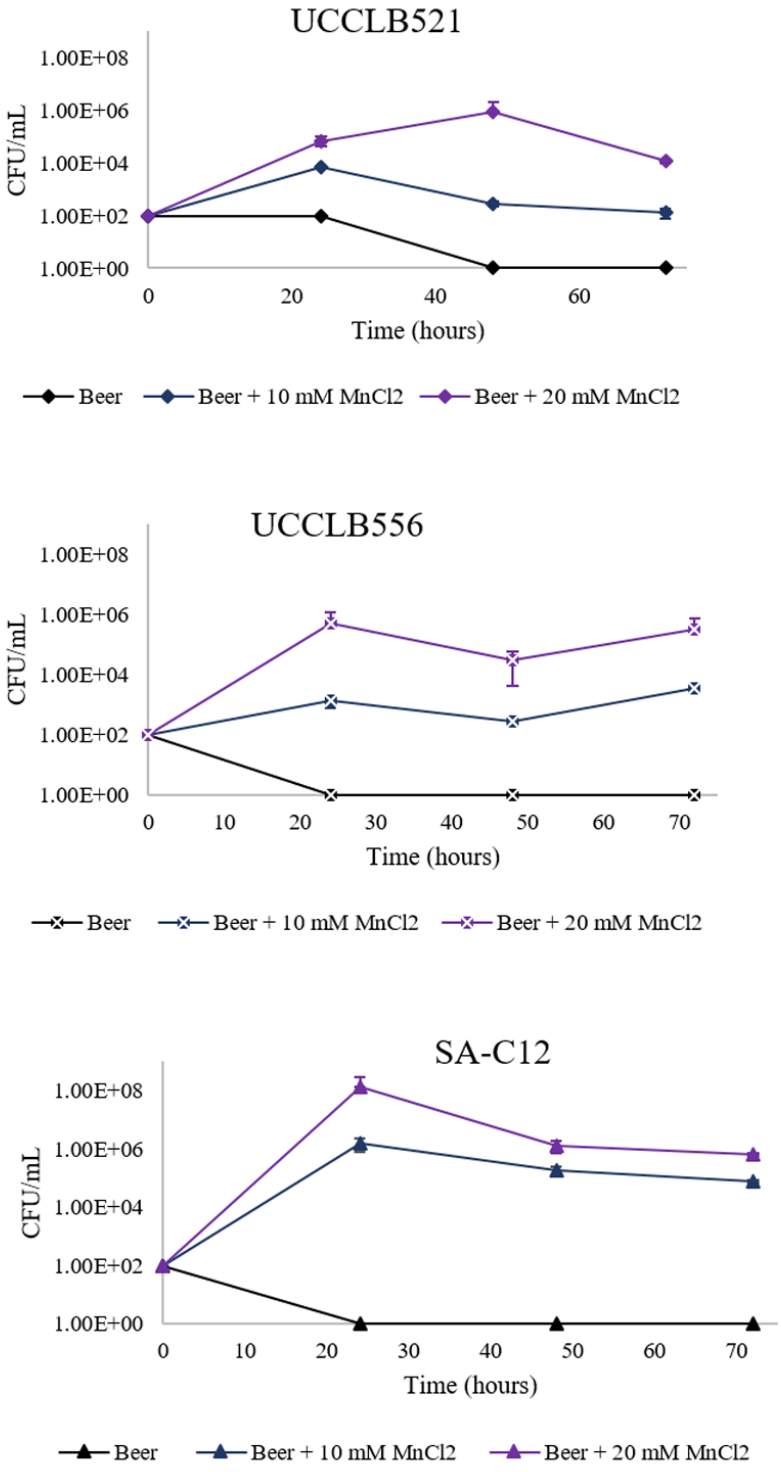

Figure 5. Growth curves of *Lb. brevis* non-beer spoiling strains UCCLB521, UCCLB556, and SA-C12 in beer (black) and beer supplemented with 10 mM $MnCl_2$ (dark blue) or 20 mM $MnCl_2$ (purple). Growth curves were performed in triplicate and the average of those measurements is displayed in the graph above.

4.7. Gene expression in MRS broth at pH 4.0 +/- Mn^{2+}

Results of the experiments presented above highlight the importance of Mn^{2+} in beer spoilage by *Lb. brevis* strains and this may be due to its role as an enzyme cofactor. It has previously been shown that Mn^{2+} can act as a cofactor in activating specific enzymes [18]. These enzymes include those involved in oxidative stress response and virulence [18-20]. Recently, *Lb. brevis* beer-spoiling strains were shown to more commonly contain (compared to non-beer-spoiling strains) genes encoding proteins involved in oxidative stress response such as dehydrogenases and peroxidase [9]. These particular genes were selected and their transcription level was evaluated by qRT-PCR when the *Lb. brevis* beer-spoiling strain UCCLBBS124 WT and its derivative UCCLBBS124 pNZ44::*0274i* were grown in MRS broth, MRS broth at pH 4.0 and MRS broth at pH 4.0 supplemented with 10 mM $MnCl_2$. When the WT strain is cultivated in MRS broth at pH 4.0 in the presence or absence of Mn^{2+}, transcription of $mntH_{0274}$ is, as expected, increased approximately 5-fold when compared to its transcription level when cells are grown in MRS broth (Table 6). When the WT strain is grown in MRS broth at pH 4.0 other genes were shown to exhibit increased transcription compared to growth in MRS broth: *UCCLBBS124_1100* encoding a peroxidase, and *UCCLBBS124_2021* and *UCCLBBS124_2022*, both of which are predicted to encode dehydrogenases (Table 6). These genes are therefore presumed to encode proteins that are involved in oxidative stress response and in environmental stress response in general [9]. However, when Mn^{2+} is added to MRS broth at pH 4.0 these genes are not overexpressed compared to the MRS broth control. In contrast, when UCCLBBS124 pNZ44::*0274i* is cultured in MRS broth at pH 4.0 in the presence or absence of Mn^{2+}, transcription of $mntH_{0274}$ is not induced as expected due to the production of the antisense mRNA targeting the gene (Table 6). Transcription of just a single tested gene was upregulated when cultured in MRS broth at pH 4.0 as compared to unmodified MRS broth.

This gene, associated with locus tag *UCCLBBS124_2394*, is predicted to encode a major facilitator superfamily (MFS) transporter. The increased transcription of this gene was not observed when cultured in MRS broth at pH 4.0 supplemented with Mn^{2+} (Table 6). Compounds transported by MFS transporters include cations [21] and it is tempting to speculate that this transporter may import Mn^{2+} as a way of compensating for the reduction in $mntH_{0274}$ transcription.

Table 6. Fold change of the gene expression level in *Lb. brevis* UCCLBBS124 and its derivative UCCLBBS124 pNZ44::*0274i* when cultured in MRS broth at pH 4.0 in the presence or absence of 10 mM $MnCl_2$ compared to growth in MRS broth.

		UCCLBBS124 WT		**UCCLBBS124 pNZ44 ::*0274i***	
		MRS pH 4.0	**MRS pH 4.0 + Mn2+**	**MRS pH 4.0**	**MRS pH 4.0 + Mn2+**
Gene	**Predicted function**	**compared to MRS broth**			
UCCLBBS124_0274	MntH	5.48	5.99	0.56	0.57
UCCLBBS124_0092	Transmembrane efflux protein	0.40	1.20	1.41	0.84
UCCLBBS124_0122	Short-chain dehydrogenase-reductase	1.80	1.64	1.13	0.78
UCCLBBS124_0279	Short-chain dehydrogenase-oxidoreductase	0.45	0.83	0.79	0.34
UCCLBBS124_0281	Short-chain dehydrogenase-oxidoreductase	0.33	0.46	1.20	0.31
UCCLBBS124_0479	Na+-H+ antiporter	0.79	1.27	1.57	1.65
UCCLBBS124_0922	Short-chain dehydrogenase	1.32	1.14	1.85	0.46
UCCLBBS124_1082	Short-chain dehydrogenase-reductase	0.59	0.56	0.41	0.44
UCCLBBS124_1095	Nitrobenzoate reductase	0.57	0.48	0.53	0.28
UCCLBBS124_1100	NADH peroxidase	5.19	1.80	0.78	0.51
UCCLBBS124_1186	Hypothetical protein/Oxidoreductase	0.43	0.72	1.39	0.49
UCCLBBS124_2021	Shikimate dehydrogenase	2.04	0.88	0.81	0.35
UCCLBBS124_2022	2-deoxy-D-gluconate 3-dehydrogenase	2.22	0.87	0.60	0.38
UCCLBBS124_2307	MFS transporter	0.88	0.55	0.69	0.29
UCCLBBS124_2334	NADPH-quinone reductase	0.40	0.60	1.42	0.53
UCCLBBS124_2335	Serine O-acetyltransferase	0.45	0.97	2.70	2.07
UCCLBBS124_2376	NADPH-quinone reductase	1.41	0.52	0.56	0.51
UCCLBBS124_2378	FMN-dependent NADH-azoreductase	1.27	0.57	0.98	0.73
UCCLBBS124_2394	MFS transporter	0.25	0.83	2.93	1.33
UCCLBBS124_2446	Flavodoxin	0.76	0.45	0.79	0.27

5. Conclusions

In the current study, we identified *mntH*$_{0274}$ encoding a putative manganese transporter MntH$_{0274}$, whose expression is essential for growth of *Lb. brevis* UCCLBBS124 at low pH, thus supporting its role in adapting to acid stress. When transcription of the gene is silenced *via* an anti-sense mRNA production strategy, growth of the derivative strain at low pH is negatively impacted. This may be explained by decreased import of manganese, which is known to be a cofactor of enzymes involved in stress response (Figure 6A and B). When manganese is added to the acidic environment, growth of the derivative strain is restored indicating the importance of Mn^{2+} for *Lb. brevis* and growth at low pH. We hypothesize that MntH$_{0274}$ is a high affinity manganese transporter and is important under limiting Mn^{2+} concentration (such as in beer and MRS). Reducing MntH$_{0274}$ expression was shown to negatively affect growth of the strain in beer, though this growth reduction can be reversed by the addition of Mn^{2+} which can be taken up by low affinity and/or general bivalent cation uptake systems (Figure 6C). The positive effect of Mn^{2+} addition on growth of *Lb. brevis* UCCLBBS124 and its derivative was also observed when both strains were cultivated in beer. The derivative strain was not able to grow in beer compared to the WT strain indicating the role of MntH$_{0274}$ in beer spoilage by *Lb. brevis* UCCLBBS124, while Mn^{2+} addition was shown to allow both strains to survive and grow in beer. A similar observation was made with the non-beer spoiling *Lb. brevis* strains UCCLB521, UCCLB556 and SA-C12 which were shown to be able to survive and in some cases grow in beer upon addition of Mn^{2+} to the medium. Here, we identified a novel beer spoilage-related chromosomal gene *mntH*$_{0274}$ encoding a manganese transporter protein MntH$_{0274}$ responsible for low pH tolerance. Moreover, the role of Mn^{2+} in acidic tolerance and beer spoilage was highlighted, possibly due to its role as an enzyme cofactor activating enzymes involved in environmental stress response.

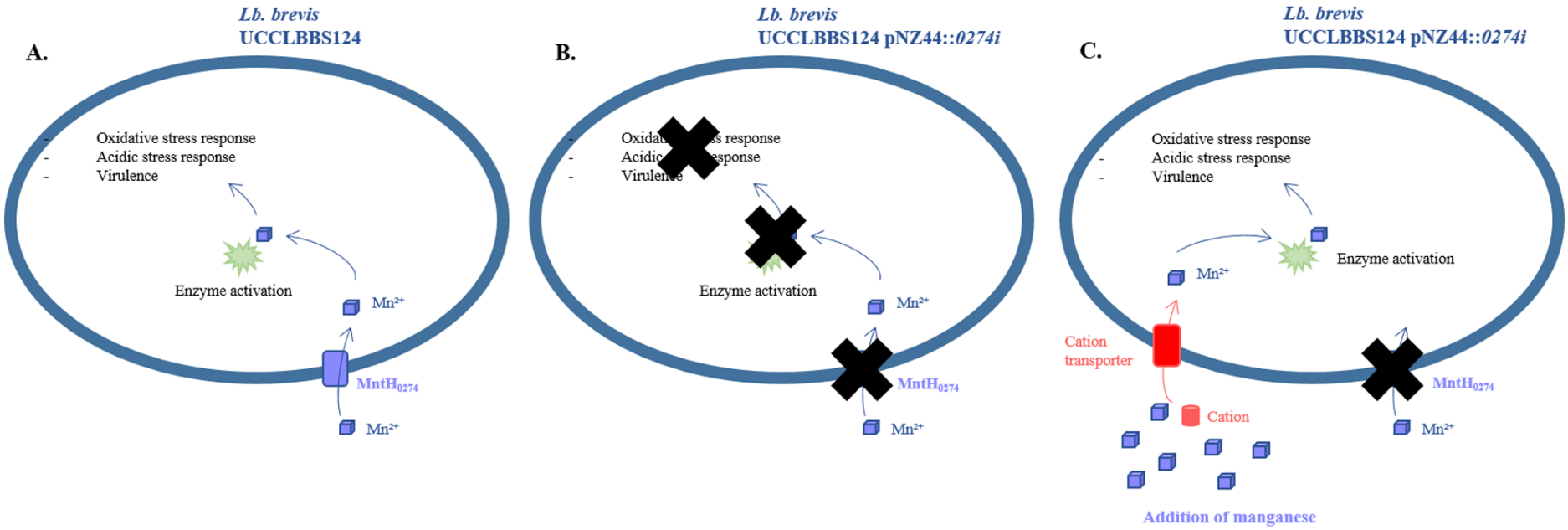

Figure 6. Importance of the manganese transporter protein $MntH_{0274}$ and manganese for acidic tolerance of the *Lb. brevis* strain UCCLBBS124. **A.** When the gene *mntH*$_{0274}$ is present in UCCLBBS124, it allows for manganese transport into the cells which will act as an enzyme cofactor allowing stress response, especially when cultured at low pH. **B.** When the gene *mntH*$_{0274}$ is silenced, the derivative UCCLBBS124 pNZ44::*0274i* can no longer transport manganese efficiently negatively affecting the stress response when cultured in an acidic environment. **C.** When the gene *mntH*$_{0274}$ is silenced but manganese is added in high concentration, manganese is competing with other cations for binding onto other cation transporters. The derivative strain UCCLBBS124 pNZ44::*0274i* is now able to transport manganese into the cells for enzyme activation allowing stress response to the acidic environment and growth of the strain.

6. References

1. Sakamoto, K.; Konings, W.N. Beer spoilage bacteria and hop resistance. *International Journal of Food Microbiology* **2003**, *89*, 105-124.
2. Vaughan, A.; O'Sullivan, T.; van Sinderen, D. Enhancing the microbiological stability of malt and beer — a review. *Journal of the Institute of Brewing* **2005**, *111*, 355-371, doi:10.1002/j.2050-0416.2005.tb00221.x.
3. Bokulich, N.A.; Bamforth, C.W. The microbiology of malting and brewing. *Microbiology and Molecular Biology Reviews : MMBR* **2013**, *77*, 157-172, doi:10.1128/MMBR.00060-12.
4. Bergsveinson, J.; Friesen, V.; Ziola, B. Transcriptome analysis of beer-spoiling *Lactobacillus brevis* BSO 464 during growth in degassed and gassed beer. *International Journal of Food Microbiology* **2016**, *235*, 28-35.
5. Sakamoto, K.; Margolles, A.; van Veen, H.W.; Konings, W.N. Hop resistance in the beer spoilage bacterium *Lactobacillus brevis* is mediated by the ATP-Binding Cassette multidrug transporter HorA. *Journal of Bacteriology* **2001**, *183*, 5371-5375, doi:10.1128/JB.183.18.5371-5375.2001.
6. Suzuki, K.; Iijima, K.; Sakamoto, K.; Sami, M.; Yamashita, H. A review of hop resistance in beer spoilage lactic acid bacteria. *Journal of the Institute of Brewing* **2006**, *112*, 173-191.
7. Iijima, K.; Suzuki, K.; Ozaki, K.; Yamashita, H. HorC confers beer-spoilage ability on hop-sensitive *Lactobacillus brevis* ABBC45cc. *Journal of Applied Microbiology* **2006**, *100*, 1282-1288, doi:10.1111/j.1365-2672.2006.02869.x.
8. Hayashi, N.; Ito, M.; Horiike, S.; Taguchi, H. Molecular cloning of a putative divalent-cation transporter gene as a new genetic marker for the identification of *Lactobacillus*

brevis strains capable of growing in beer. *Applied Microbiology and Biotechnology* **2001**, *55*, 596-603.

9. Feyereisen, M.; Mahony, J.; Kelleher, P.; Roberts, R.J.; O'Sullivan, T.; Geertman, J.-M.A.; van Sinderen, D. Comparative genome analysis of the *Lactobacillus brevis* species. *BMC Genomics* **2019**, *20*, 416, doi:10.1186/s12864-019-5783-1.
10. Kuipers, O.P.; de Ruyter, P.G.G.A.; Kleerebezem, M.; de Vos, W.M. Quorum sensing-controlled gene expression in lactic acid bacteria. *Journal of Biotechnology* **1998**, *64*, 15-21, doi:https://doi.org/10.1016/S0168-1656(98)00100-X.
11. Ainsworth, S.; Zomer, A.; Mahony, J.; van Sinderen, D. Lytic infection of *Lactococcus lactis* by bacteriophages Tuc2009 and c2 triggers alternative transcriptional host responses. *Applied and Environmental Microbiology* **2013**, *79*, 4786-4798, doi:10.1128/aem.01197-13.
12. Untergasser, A.; Nijveen, H.; Rao, X.; Bisseling, T.; Geurts, R.; Leunissen, J.A.M. Primer3Plus, an enhanced web interface to Primer3. *Nucleic Acids Research* **2007**, *35*, W71-74, doi:10.1093/nar/gkm306.
13. Livak, K.J.; Schmittgen, T.D. Analysis of relative gene expression data Using Real-Time Quantitative PCR and the 2−ΔΔCT method. *Methods* **2001**, *25*, 402-408, doi:https://doi.org/10.1006/meth.2001.1262.
14. McGrath, S.; Fitzgerald, G.F.; van Sinderen, D. Improvement and optimization of two engineered phage resistance mechanisms in *Lactococcus lactis*. *Applied and Environnemental Microbiology* **2001**, *67*, 608-616.
15. van Pijkeren, J.-P.; Britton, R.A. High efficiency recombineering in lactic acid bacteria. *Nucleic Acids Research* **2012**, *40*, e76-e76.

16. Ahrné, S.; Molin, G.; Axelsson, L. Transformation of *Lactobacillus reuteri* with electroporation: studies on the erythromycin resistance plasmid pLUL631. *Current Microbiology* **1992**, *24*, 199-205, doi:10.1007/bf01579282.

17. Zhao, Y.; Knøchel, S.; Siegumfeldt, H. Heterogeneity between and within strains of *Lactobacillus brevis e*xposed to beer compounds. *Frontiers in Microbiology* **2017**, *8*, 239-239, doi:10.3389/fmicb.2017.00239.

18. Archibald, F. Manganese: its acquisition by and function in the Lactic Acid Bacteria. *Critical Reviews in Microbiology* **1986**, *13*, 63-109, doi:10.3109/10408418609108735.

19. Juttukonda, L.J.; Skaar, E.P. Manganese homeostasis and utilization in pathogenic bacteria. *Molecular Microbiology* **2015**, *97*, 216-228, doi:10.1111/mmi.13034.

20. Watanabe, M.; van der Veen, S.; Nakajima, H.; Abee, T. Effect of respiration and manganese on oxidative stress resistance of *Lactobacillus plantarum* WCFS1. *Microbiology* **2012**, *158*, 293-300, doi:10.1099/mic.0.051250-0.

21. Pao, S.S.; Paulsen, I.T.; Saier, M.H. Major Facilitator Superfamily. *Microbiology and Molecular Biology Reviews* **1998**, *62*, 1-34.

Chapter IV

A plasmid-encoded putative glycosyltransferase is involved in hop tolerance and beer spoilage in *Lactobacillus brevis*

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1. Abstract

Lactobacillus brevis beer-spoiling strains harbor plasmids that contain genes such as *horA*, *horC* and *hitA*, which are known to confer hop tolerance. The *Lb. brevis* beer-spoiling strain UCCLBBS124, which possesses four plasmids, was treated with novobiocin resulting in the isolation of UCCLBBS124 derivatives exhibiting hop-sensitivity and an inability to grow in beer. One selected derivative was shown to have lost a single plasmid, designated here as UCCLLBS124_D, which harbors the *UCCLBBS124_pD0015* gene, predicted to encode a glycosyltransferase. Hop tolerance and growth in beer were restored when *UCCLBBS124_pD0015* was introduced in the hop-sensitive derivative on a plasmid. We hypothesize that this gene modifies the surface composition of the polysaccharide cell wall conferring protection against hop compounds. Furthermore, introduction of this gene *in trans* in *Lb. brevis* UCCLB521, a strain that cannot grow in and spoil beer, was shown to furnish the resulting strain with the ability to grow in beer while its expression also conferred phage-resistance. This study underscores how the acquisition of certain mobile genetic elements play a role in hop tolerance and beer spoilage for strains of this bacterial species.

2. Introduction

Lactobacillus brevis is a major threat for commercial and amateur brewers as strains of this species are the predominant bacterial contaminants associated with beer spoilage [1]. Such *Lb. brevis* strains can grow in beer despite the presence of ethanol, low pH and the depletion of oxygen and nutrients [2]. Moreover, hop compounds added to beer for bitter flavor development during the fermentation process also exert antibacterial activity through the presence of *iso*-α-acids [1,2]. *Lb. brevis* beer-spoiling (BS) strains appear to have acquired chromosomally- or plasmid-derived genetic content to survive and grow in beer [2]. *Lb. brevis* resistance to ethanol and low pH seems to be associated with chromosomal genes, possibly due to the general stressors they represent [3,4]. However, *Lb. brevis* BS strains are also known to harbor plasmids that are associated with their beer-spoilage phenotype and more specifically with hop tolerance [5-8]. Plasmid-derived genes that underpin hop-resistance in *Lb. brevis* include *horA, horC, hitA* and $orf5_{ABBC45}$ [1,2]. The genes *horA* and *horC* encode multidrug transporter proteins driven by ATP and proton motive force (PMF), respectively, and were identified as being involved in *iso*-α-acid extrusion from the bacterial cell [5,7]. The *hitA* gene encodes a transmembrane protein involved in the transport of divalent cations such as Mn^{2+} in exchange of protons released from hop compounds [8]. The $orf5_{ABBC45}$ gene was identified in *Lb. brevis* BS strain ABBC45 which was unable to grow in beer after it had lost a plasmid carrying this gene. The $orf5_{ABBC45}$ gene encodes a predicted transmembrane protein resembling a PMF-dependent multidrug transporter, which is presumed to be responsible for *iso*-α-acid export [9].

However, these genes are not always indicative of BS ability as the presence of such genes can be found among *Lb. brevis* strains that are unable to grow and consequently spoil beer (designated here as NBS strains) [10]. Indeed, *horA* is present in the *Lb. brevis* NBS strain

UCCLB556 [10]. Moreover, genes identified as conferring hop-resistance are not always simultaneously present in BS strains, e.g. the BS strain UCCLBBS124 carries plasmids harboring *horA* and *horC*, though it does not possess *hitA* [10]. Analysis of BS strain *Lb. brevis* BSO 464 has highlighted the importance of plasmids and genes on mobile genetic elements for bacterial growth in beer and beer spoilage ability [6]. Recently, a gene predicted to encode a glycosyltransferase was identified among BS strains responsible for excess β-glucan formation [11]. This gene is also present on the genome of *Lb. brevis* BS strain UCCLBBS124, while it is absent in that of BS strain UCCLBBS449 [10]. This indicates that beer spoilage is not uniquely governed by the presence of a few genes, but rather a combination of genes acting in concert to confer beer resistance to the strain. It also suggests that other plasmid-encoded genes involved in beer spoilage are yet to be discovered.

In the present study we generated plasmid-cured derivatives of *Lb. brevis* BS strain UCCLBBS124 using novobiocin. This approach has been successfully employed previously to cure plasmids from lactic acid bacteria (LAB) isolates [6,12]. Plasmid-cured derivatives were assessed for their ability to grow in the presence of hop and in beer. A derivative that showed inability to grow in beer was selected and analyzed to ascertain which plasmids were responsible for this phenotype. Bioinformatic analysis of the genetic content of such plasmids revealed candidate genes required for growth in beer. These genes were used in transformation experiments to revert the NBS phenotype.

3. Materials and Methods

3.1. Bacterial strains and cultivation media

Bacterial strains used in this study are listed in Table 1. *Lb. brevis* strains were grown in MRS broth (Oxoid Ltd., UK) at 30 ˚C, while *Lactococcus lactis* NZ9000 was grown in M17 broth (Oxoid Ltd., UK) supplemented with 0.5 % glucose. 5 µg/mL chloramphenicol (Cm5) was added to the medium when indicated.

Table 1. Bacterial strains and plasmids used in this study.

Strain / Plasmid	Description	References
***Lb. brevis* strains**		
UCCLBBS124	Beer-spoiling strain isolated from spoiled beer keg (Singapore)	[10]
UCCLBBS449	Beer-spoiling strain isolated from unpasteurized spoiled beer (The Netherlands)	[10]
MB569	Non-beer spoiling strain derivative of UCCLBBS124	This study
UCCLB521	Non-beer spoiling strain isolated from brewery environment (The Netherlands)	[10]
MB569 pNZ44	MB569 carrying pNZ44	This study
MB569 pNZ44:*gtf*$_{D15}$	MB569 carrying pNZ44 with *gtf*$_{D15}$	This study
UCCLB521 pNZ44:*gtf*$_{D15}$	UCCLB521 carrying pNZ44 with *gtf*$_{D15}$	This study
***L. lactis* strains**		
NZ9000	Transformation host	[13]
Plasmids		
pNZ44	Transformation vector, chloramphenicol resistance gene	
pNZ44:*gtf*$_{D15}$	pNZ44 harboring *gtf*$_{D15}$	This study

3.2. Plasmid curing and plasmid content analysis

Plasmid curing of the BS strain UCCLBBS124 was achieved using novobiocin treatment [14]. A 1 % inoculum of a WT strain overnight culture was used to inoculate 10 mL MRS broth containing 0.25 µg/mL novobiocin. Cultures were incubated at 26 °C for 72 h. After incubation, cells were diluted and plated on MRS agar. After 3 days of incubation at 26 °C, isolated colonies were randomly selected and derivatives with impaired growth in beer (no

growth observed after 72 h) were checked for the presence or loss of hop-resistance genes *horA*, *horC* and *orf5*$_{ABBC45}$ (Table 2). A derivative showing loss of hop-resistance genes was selected and sequenced using Illumina sequencing technology. Paired-end sequence reads were generated using an Illumina HiSeq2500 system. FASTQ sequence files were generated using the Illumina Casava pipeline version 1.8.3. After Illumina sequencing the obtained sequences were mapped back against the WT reference sequence to detect mutations by single nucleotide polymorphism (SNP) or plasmid content loss.

Table 2. PCR primers used in this study. Incorporated restriction sites are indicated in capital letters.

Primer name	Sequence (5' - 3')	Target
horAF	cgcaactgaggctaacttct	*horA* gene in UCCLBBS124
horAR	ggcttgctatgctaggata	*horA* gene in UCCLBBS124
horCF	gtatgcctaagtgacgt	*horC* gene in UCCLBBS124
horCR	cattctctgcctctatac	*horC* gene in UCCLBBS124
orf5F	Ctggattgaggtgaggg	*orf5* gene in UCCLBBS124
orf5R	Gctgtaaagggtagtgattg	*orf5* gene in UCCLBBS124
pNZ44F	Aacaattgtaacccatac	pNZ44 promoter
pNZ44R	Gaacgtttcaagccttgg	pNZ44 MCS
pD14F	aaaaaaCTGCAGgtccgaacagcgttcggatt	Gene *UCCLBBS124_pD0014* in UCCLBBS124_D
pD14R	aaaaaaTCTAGAttaatcttcgaaatagtt	Gene *UCCLBBS124_ pD0014* in UCCLBBS124_D
pD15F	aaaaaaCCATGGgcggtttggatattttatact	Gene *UCCLBBS124_ pD0015* in UCCLBBS124_D
pD15R	aaaaaaTCTAGAtcactcagttttcaattccc	Gene *UCCLBBS124_ pD0015* in UCCLBBS124_D
pD16F	aaaaaaCTGCAGaggcttgctatgctagg	Gene *UCCLBBS124_ pD0016* in UCCLBBS124_D
pD16R	aaaaaaTCTAGAtcacccgttgctcgt	Gene *UCCLBBS124_ pD0016* in UCCLBBS124_D
pD17-19F	aaaaaaCCATGGggggtagaatggttctgtt	Gene *UCCLBBS124_ pD0017-19* in UCCLBBS124_D
pD17-19R	aaaaaaTCTAGAttattgataatgaccagcaa	Gene *UCCLBBS124_ pD0017-19* in UCCLBBS124_D

3.3. Construction of plasmid vectors

Genes of interest were amplified by PCR (Table 2) and cloned into the expression vector pNZ44 [15]. PCR products and pNZ44 plasmid DNA were digested with the appropriate enzymes (Roche, USA) at 37 ˚C for at least 4 h, following the manufacturer's instructions (Table 2). A ratio of (3:1) was applied for the ligation of the PCR product with pNZ44 using T4 DNA ligase (Promega, USA). The mixture was incubated at room temperature for at least 4 hours prior to electrotransformation into *L. lactis* NZ9000 competent cells.

3.4. Preparation of competent cells and electrotransformation

Competent cells of *L. lactis* NZ9000 were prepared as previously described [16]. Competent cells of *Lb. brevis* UCCLBBS124 were prepared using an adapted version of a previously described protocol [17]: An overnight culture was transferred (1 % inoculum) to 10 mL MRS broth containing 1 % glycine and incubated overnight at 30 ˚C. 5 mL of the overnight culture was transferred to fresh MRS broth containing 1 % glycine (50 mL final volume) and cells were grown to an OD_{600nm} of 0.6. Cells were harvested by centrifugation at 4,000 × *g* for 15 min at 4 ˚C and washed in ice-cold wash buffer (0.5 M sucrose, 10 % glycerol). The wash step was repeated twice and the cells were finally resuspended in 200 µL wash buffer prior to storage at -80 ˚C and/or electroporation (see below). All constructs were generated using *L. lactis* NZ9000 as the cloning host, verified by sequencing after PCR amplification using the primers pNZ44F and pNZ44R (Table 2) prior to their transfer into *Lb. brevis* strains. Electrotransformation was performed using freshly prepared competent cells as described above, where 45 µL of cells and 5 µL of plasmid construct were mixed into a pre-chilled 2 mm electroporation cuvette (Cell Projects, Kent, England) and subjected to electroporation at 1.5

kV (*Lb. brevis*) or 2.0 kV (*L. lactis*), 200 Ω, 25 µF. Following electroporation, 950 µL recovery broth was added (MRS broth supplemented with 0.5 M sucrose and 0.1 M $MgCl_2$ (*Lb. brevis*) or GM17 broth supplemented with 20 mM $MgCl_2$ and 2 mM $CaCl_2$ (*L. lactis*)). Cells were recovered at 30 ˚C for 3 h (*Lb. brevis*) or 2 h (*L. lactis*) prior to spread plating on MRS (*Lb. brevis*) or GM17 (*L. lactis*) agar supplemented with Cm5. Presumed transformants were purified on MRS agar + Cm5 and colonies were checked by sequencing after PCR amplification using the primers pNZ44F and pNZ44R (Table 2) and applied to growth assays as described below.

3.5. Growth assays

Growth profiles of the wild-type strain and its derivative were obtained by transferring an overnight culture (1 % inoculum) to MRS broth, MRS broth supplemented with 30 ppm *iso*-α-acids or beer. Cultures were incubated at 30 ˚C for 72 hours. One mL of culture was retrieved after 24, 48, 72 and 96 hours, diluted in Ringer's solution and plated on MRS agar plates. Plates were incubated at 30 ˚C anaerobically for 48 hours prior to colony counting. The number of viable bacteria of each strain was assessed after CFU/mL calculation.

3.6. Phage activity against *Lb. brevis* strains and transformants

To assess phage sensitivity of *Lb. brevis* strains, transformants carrying genes of interest were compared to the wild-type (WT) strain using plaque assays, as previously described [18]. A 10 µL volume of the appropriate phage dilution and 200 µL of *Lb. brevis* culture were added to 4 mL of soft agar supplemented with 10 mM $CaCl_2$, mixed and poured onto an MRS agar plate supplemented with 10 mM $CaCl_2$ and 0.5% glycine. Plates were incubated at 30 °C overnight

and the resulting plaques were enumerated. Phage titer was determined as plaque-forming units per mL (PFU/mL). The ability of phages to propagate and multiply within the host cell was also tested. *Lb. brevis* strains were grown to early exponential phase (OD_{600nm} ~ 0.25), at which point phages were added to the culture (T0) at a MOI (multiplicity of infection) of 1, along with 10 mM $CaCl_2$. The mix was further incubated at 30 °C overnight (T1). The number of phages present in the medium (i.e. following removal of bacterial cells by centrifugation) at T1 was then determined by plaque assay. Phage propagation efficiency on a given host was then determined by dividing the phage titer (PFU/mL) at T1 by the phage titer (PFU/mL) at T0.

3.7. GenBank accession numbers

Lb. brevis UCCLBBS124: CP031169, *Lb. brevis* UCCLBBS124_A: CP031170, *Lb. brevis* UCCLBBS124_B: CP031171, *Lb. brevis* UCCLBBS124_C: CP031172 and *Lb. brevis* UCCLBBS124_D: CP031173.

4. Results and Discussion

4.1. Derivatives with impaired growth in beer reveal loss of plasmid UCCLBBS124_D

The beer-spoiling *Lb. brevis* strain UCCLBBS124 possesses four plasmids carrying genes of interest for bacterial beer spoilage (Table 3) [10]. Following exposure to novobiocin, surviving cells were plated and fifty isolated colonies of the BS strain *Lb. brevis* UCCLBBS124 were randomly selected. Thirty four of these fifty colonies displayed impaired growth in beer. PCR-based identification of the hop-resistance gene *horA* revealed the loss of this gene (located on plasmid UCCLBBS124_D) in 33 out of the 34 isolates. Of the 33 isolates, one derivative, designated here as MB569, was selected for genome sequencing, after which its sequence was compared to that of the WT, confirming that plasmid UCCLBBS124_D had been lost from strain MB569.

Table 3. *Lb. brevis* UCCLBBS124 plasmids and genes of interest for beer spoilage.

Plasmid	Size (bp)	Accession no.	Gene(s) of interest	Reference
UCCLBBS124_A	49,560	CP031170		
UCCLBBS124_B	23,078	CP031171	*gtf family 2*	[11]
UCCLBBS124_C	22,370	CP031172	*horB, horC, orf5*	[7,9]
UCCLBBS124_D	20,971	CP031173	*horA*	[5]

4.2. Tolerance of MB569 to *iso*-α-acids, ethanol and pH

The inability of strain MB569 to grow in beer highlights the apparent importance of plasmid UCCLBBS124_D in conferring a beer spoilage phenotype on strain UCCLBBS124 (Figure 1). Beer is a harsh environment incorporating a number of stresses such as low pH, lack of nutrients, and the presence of ethanol and hop compounds. In order to understand which of these stresses imposed a negative impact on growth of MB569, the WT strain and MB569 were grown in MRS broth and mimicking conditions encountered in beer, e.g. pH 4.0, 5.4 % ethanol, and 30 ppm *iso*-α-acids. Strain MB569 was shown to be capable of growth in MRS broth both at neutral pH and at pH 4.0, while it can also grow in the presence of ethanol comparable to the WT strain (Figure 1). However, MB569 is incapable of growth in the presence of *iso*-α-acids unlike the WT strain UCCLBBS124 (Figure 1). This indicates that plasmid-cured derivative MB569 has lost the ability to spoil beer due to its sensitivity to the antimicrobial compounds present in hops. Therefore, based on this phenotype and the finding that MB569 lacks plasmid UCCLBBS124_D (when compared to its parental strain), it indicates that this plasmid is linked to hop tolerance and thus contributes to the ability of strain UCCLBBS124 to cause beer spoilage.

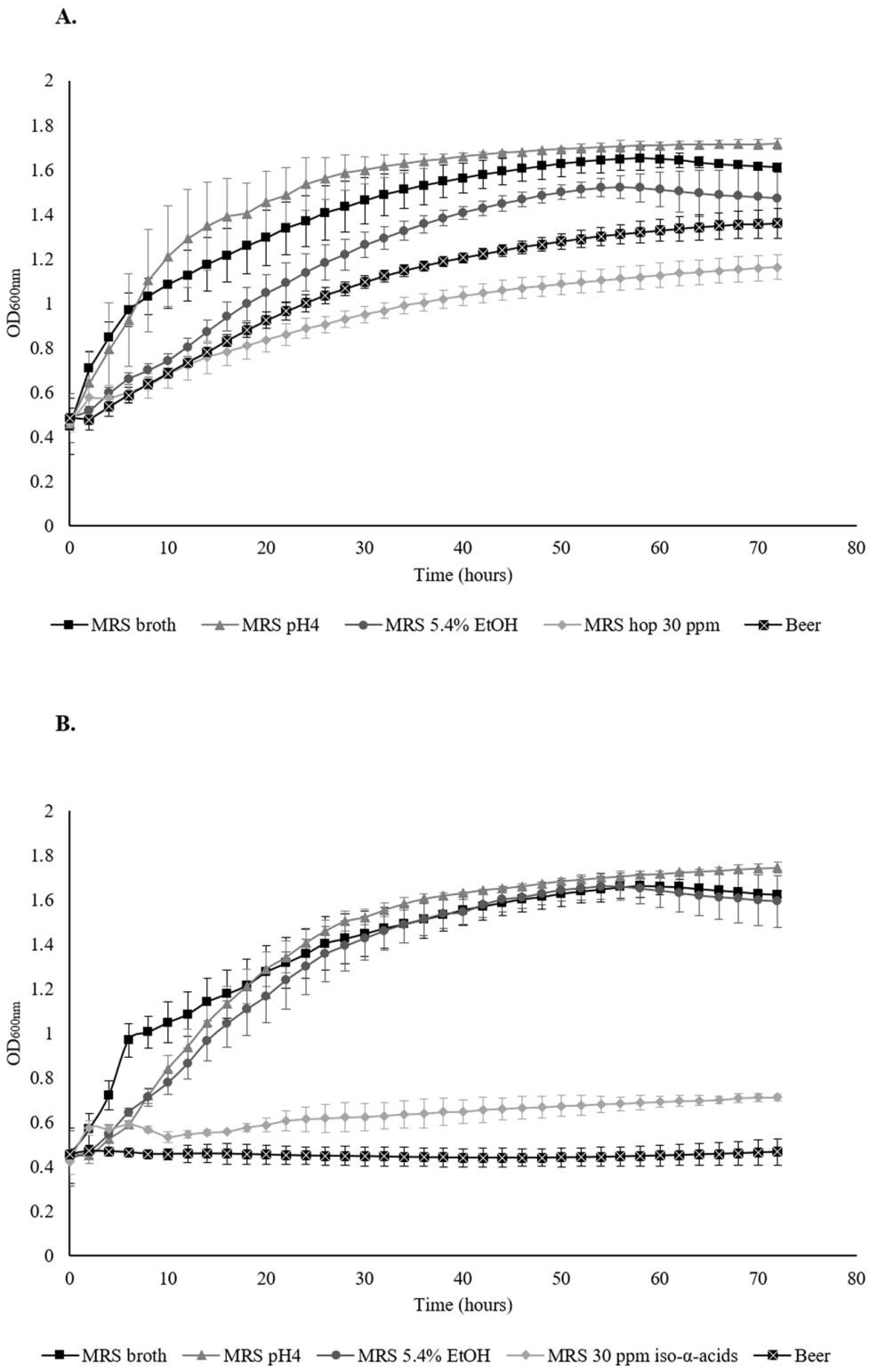


Figure 1. Growth of the WT beer-spoiling strain *Lb. brevis* UCCLBBS124 **(A)** and its plasmid-cured derivative MB569 **(B)** in beer, MRS broth, MRS broth at pH 4.0 and MRS broth supplemented with 5.4 % ethanol or 30 ppm *iso*-α-acids.

4.3. Identification and functional annotation of genes present on plasmid UCCLBBS124_D

Plasmid UCCLBBS124_D is 21 kb in size and is predicted to encompass 16 genes. Interestingly, a 7 kb region of this plasmid, contains six genes that are uniquely present among the plasmids of almost all (except UCCLB95) *Lb. brevis* BS strains (Table 4) [10]. In order to assess the possible role of these genes in beer spoilage, the BS plasmid-specific genes *UCCLBBS124_pD0014*, encoding a predicted cytosine deaminase*, UCCLBBS124_pD0015*, encoding a predicted glycosyltransferase*, UCCLBBS124_pD0016* encoding HorA (Table 4) were individually cloned into plasmid pNZ44 prior their transformation into NZ9000. The resulting plasmids were then introduced into strain MB569 to determine the ability of the obtained recombinant strains to grow in beer (where MB569 itself is unable to do so). Genes with locus tags *UCCLBBS124_pD0017, UCCLBBS124_pD0018* and *UCCLBBS124_pD0019* and encoding acyl-sn-glycerol-3-phosphate acyltransferases and a glycosyltransferase (Table 4) were cloned together as a cluster (as in plasmid UCCLBBS124_D) in pNZ44 prior their transformation into NZ9000 and MB569.

Introduction of the genes *UCCLBBS124_pD0014*, *UCCLBBS124_pD0016*, *UCCLBBS124_pD0017, UCCLBBS124_pD0018* and *UCCLBBS124_pD0019* in MB569 did not enable any obvious or significant improvement of growth in the presence of *iso*-α-acid (30 ppm) or beer (when compared to strain MB569) (data not shown).

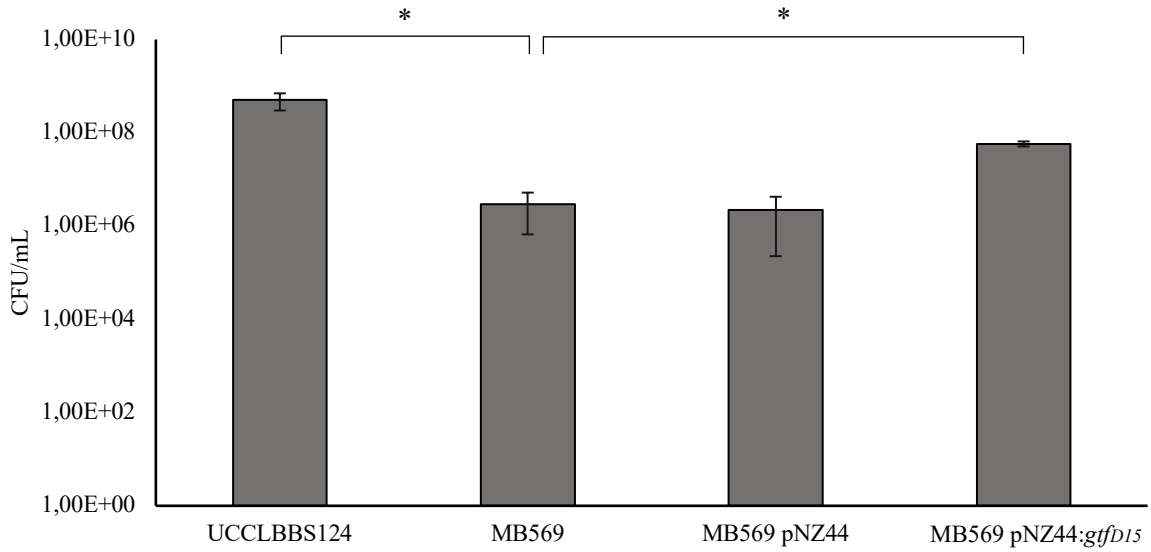
Interestingly, expression of the gene associated with locus tag *UCCLBBS124_pD0015* (and here referred to as gtf_{D15}) in MB569 was shown to confer a positive effect on its ability to grow in MRS broth containing 30 ppm *iso*-α-acids, with a significant (P value <0.05) growth increase after 72 h compared to the non-complemented strain or MB569 carrying the control plasmid pNZ44 (Figure 2A). When *Lb. brevis* MB569 pNZ44:gtf_{D15} was cultivated in beer, it also exhibited an ability to grow in beer that was significantly better than that of MB569 itself (P

value <0.05) (Figure 2B). Provision of *gtf$_{D15}$ in trans* in MB569 did not restore its growth in beer to the same level as the WT strain (i.e. the strain from which MB569 was derived), but nonetheless allowed survival and growth in beer for this recombinant strain across 96 h. MB569 and MB569 pNZ44 are still able to survive in the presence of *iso*-α-acids or beer after culture for 72 h (Figure 2A and 2B) which might be due to the presence of plasmid UCCLBBS124_C carrying *horC* (Table 3). The *gtf$_{D15}$* gene is predicted to encode a glycosyltransferase based on BLAST analysis and a HHPred analysis [19] predicted the protein to belong to the glycosyl-transferase family 8 associated with cell wall glycosylation. Further sequence scrutiny suggests that the Gtf$_{D15}$ protein is a surface protein (TMHMM Server 2.0 [20]) with a predicted signal peptide in its N-terminus that may act as a membrane anchor for the protein (http://phobius.sbc.su.se/ [21]). These predictions suggest that GtfD15 is a cell envelope-associated protein that confers protection against certain environmental stressors such as hop compounds.

Table 4. Presence and absence of genes of UCCLBBS124_D among *Lb. brevis* BS strains.

Gene	**Predicted function**	***Lb. brevis* BS strains**						
		UCCLBBS124	UCCLBBS449	UCCLB95	TMW1.2108	TMW1.2111	TMW1.2112	TMW1.2113
UCCLBBS124_pD0014	Cytosine deaminase	+	+	-	+	+	-	+
UCCLBBS124_pD0015 = gtf$_{D15}$	Glycosyltransferase family 8	+	+	-	+	+	+	+
UCCLBBS124_pD0016	HorA	+	+	-	+	+	-	+
UCCLBBS124_pD0017	Acyl-sn-glycerol-3-phosphate acyltransferase	+	+	-	+	+	+	+
UCCLBBS124_pD0018	Glycosyltransferase family 8	+	+	-	+	+	+	+
UCCLBBS124_pD0019	Acyl-sn-glycerol-3-phosphate acyltransferase	+	+	-	+	+	+	+

A.



B.

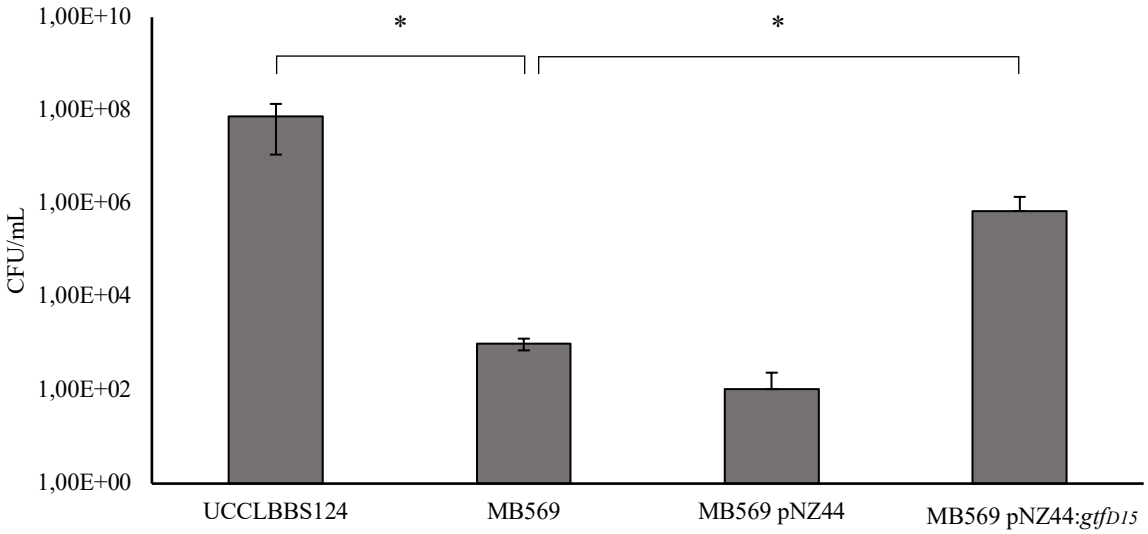
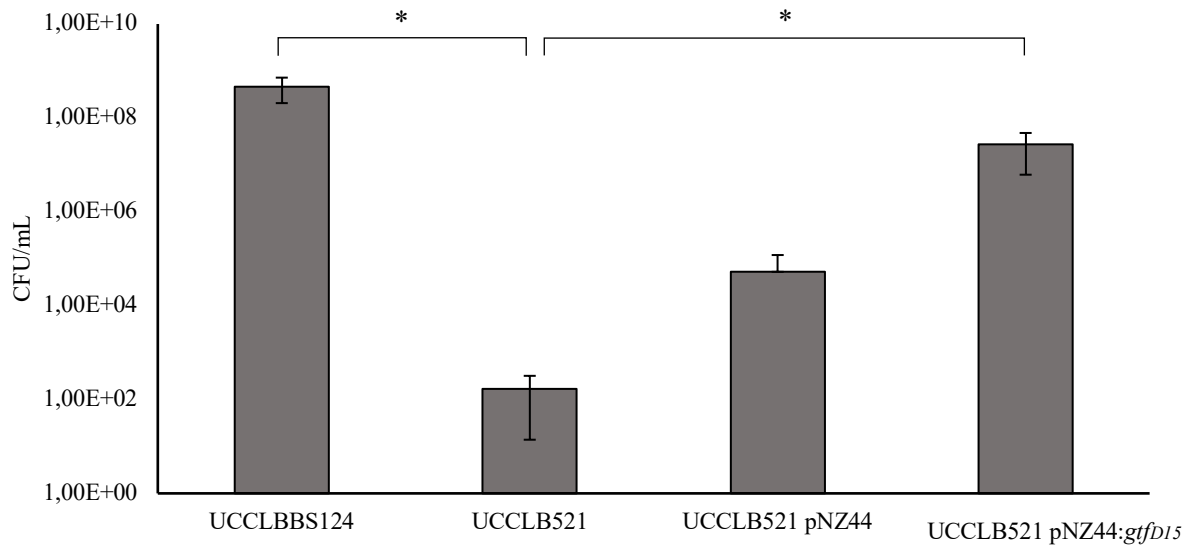


Figure 2. Number of viable bacteria (CFU/mL) of the WT BS strain *Lb. brevis* UCCLBBS124, the derivative MB569 +/- the empty plasmid pNZ44 and MB569 carrying the gene gtf_{D15} after growth in **(A)** MRS broth containing 30 ppm *iso*-α-acids for 72 h and **(B)** beer for 96 h (* indicates a $P<0.05$).

4.4. Introduction of *gtf*$_{D15}$ in NBS *Lb. brevis* strains allows growth in beer

The introduction of *gtf*$_{D15}$ in MB569 was shown to significantly improve growth of the strain in MRS broth containing hop compounds (30 ppm *iso*-α-acids) and in beer, indicating the importance of this gene for beer spoilage by *Lb. brevis* strain UCCLBBS124. In order to assess the potential growth-promoting effect of this gene for an NBS strain when inoculated in beer, *gtf*$_{D15}$ when cloned into pNZ44 (pNZ44:*gtf*$_{D15}$) was introduced into the NBS *Lb. brevis* strain UCCLB521 (Table 1). Remarkably, the presence of pNZ44:*gtf*$_{D15}$ in the NBS strain *Lb. brevis* UCCLB521 permitted the strain to grow significantly better (P value <0.05) in MRS broth containing 30 ppm *iso*-α-acids and in beer compared to the strain carrying an empty plasmid which is incapable of survival or growth in these environments (Figure 3). These observations reinforce our results above and highlight the significance of the *gtf*$_{D15}$ gene in hop tolerance and beer spoilage. However, introduction of pNZ44:*gtf*$_{D15}$ into ATCC 367, another NBS strain, did not allow improved survival or growth in the presence of hop compounds or in beer (data not shown). This suggests that a strain specific mechanism and possible involvement of other genes that are absent in ATCC 367 are responsible for increased hop tolerance.

A.



B.

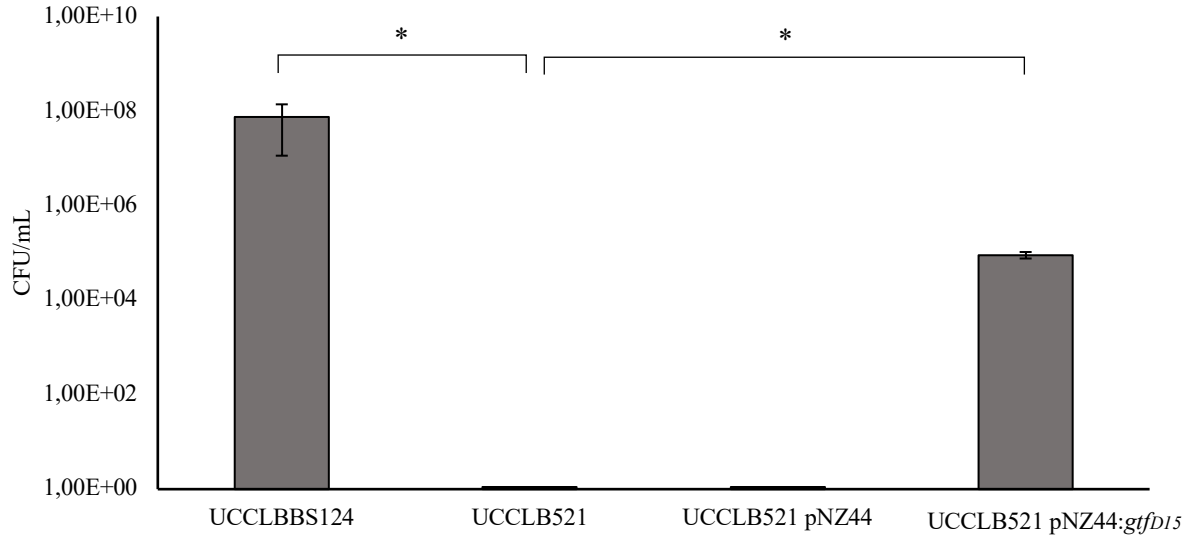


Figure 3. Number of viable bacteria (CFU/mL) of the WT BS strain *Lb. brevis* UCCLBBS124, the NBS UCCLB521 +/- the empty plasmid pNZ44 and the NBS UCCLB521 carrying the gene gtf_{D15} after growth in **(A)** MRS broth containing 30 ppm *iso*-α-acids and **(B)** beer for 96 h (* indicates a $P<0.05$).

4.5. Effect on phage sensitivity

As demonstrated above *gtf*$_{D15}$ was observed to play a role in hop and beer tolerance and is predicted to encode a glycosyltransferase. Since the protein is predicted to be involved in biosynthesis or modification of a cell surface-associated saccharidic polymer, the possible role of this protein in bacteriophage infection was investigated. *Lb. brevis* strain UCCLB521 is sensitive to *Lb. brevis* phages 3-521 and 521B [22]. Plaque assays employing these phages and *Lb. brevis* UCCLB521 harboring the empty vector pNZ44, or strain UCCLB521 containing pNZ44:*gtf*$_{D15}$ displayed similar EOP (Efficiency Of Plaquing) values with no significant difference to the WT (Table 5). However, notable differences in plaque morphology were observed, where plaques were faint and hard to distinguish on the bacterial lawn of UCCLB521 pNZ44:*gtf*$_{D15}$. Moreover, overnight incubation of the different strains with the two phages led to complete lysis-in-broth of UCCLB521 and UCCLB521 containing pNZ44 with an approximately 1000-fold increase of phage titer after overnight propagation (Table 5). In contrast, UCCLB521 pNZ44:*gtf*$_{D15}$ did not show visible lysis and was able to grow after overnight incubation with just a ten-fold increase in phage numbers after overnight propagation (Table 5). These results reinforce the role of the protein Gtf$_{D15}$ in bacterial protection against diverse environmental hazards such as hop compounds or bacteriophages.

Table 5. Effect of phages 3-521 and 521B on *Lb. brevis* strain UCCLB521 and derivatives.

		***Lb. brevis* strains**		
		UCCLB521	**UCCLB521 pNZ44**	**UCCLB521 pNZ44:*gtf*$_{D15}$**
	EOP	1.00	0.58 ± 0.29	0.64 ± 0.21
Phage 521B	**Plaque morphology**	Small clear plaques	Small clear plaques	Faint plaques
	Phage titer after O/N propagation* (PFU/mL)	2.90E+09	2.30E+09	3.00E+07
	EOP	1.00	1.52 ± 0.20	1.19 ± 0.19
Phage 3-521	**Plaque morphology**	Small clear plaques	Small clear plaques	Faint plaques
	Phage titer after O/N propagation* (PFU/mL)	4.30E+09	1.80E+09	4.80E+07

*Overnight propagation (O/N) was realized with a starting phage titer of 10^6 PFU/mL (results are average of triplicate assays).

5. Conclusions

In this study, we identified a novel genetic component required for beer spoilage and more specifically for hop tolerance. This gene is located on plasmid UCCLBBS124_D of *Lb. brevis* BS strain UCCLBBS124, validating the importance of plasmids to confer a beer spoilage phenotype. Moreover, this gene had been highlighted previously as common among BS strains [10]. Genes required for hop tolerance have all been identified on plasmids [5,7,8], reinforcing the importance of such mobile genetic elements in adaptation to the specific hurdles imposed by the beer environment. A derivative of UCCLBBS124, MB569 showed impaired growth in beer after the loss of plasmid UCCLBBS124_D and despite the presence of plasmids UCCLBBS124_B and UCCLBBS124_C which carry several genes of interest in beer spoilage. Transformation of MB569 with gtf_{D15} restored the hop tolerance phenotype of the strain which ultimately allowed it to grow in beer. Similar results were observed when the gene was introduced into a NBS strain confirming the notion that gtf_{D15} is required for the development of hop tolerance and beer spoilage. Furthermore, this gene impacts on phage sensitivity of its host. The gene is predicted to encode a glycosyltransferase and analysis of its topology suggests that it is a membrane-anchored protein involved in the biosynthesis or modification of a cell surface-associated saccharidic polymer. BS strains of *Lb. brevis* have been shown to increase higher molecular weight lipoteichoic acids (LTA) in their cell wall, in the presence of hop compounds, thus believed to confer resistance to the bacteria by enhancing the barrier functions of the cell wall and preventing intrusion of hop compounds [23,24]. Moreover, lipoteichoic acids have been described as phage receptors among phages infecting lactobacilli as seen for *Lactobacillus delbrueckii* phages LL-H and JCL1032 [25], as well as for *Lactobacillus plantarum* ATCC8014-B2 [26]. Therefore, we speculate that this glycosyltransferase is involved in replacing alanine residues with sugar residues on teichoic acids thereby changing

their charge and preventing *iso*-α-acids to penetrate the membrane as well as affecting phage adsorption and/or DNA injection. This predicted glycosyltransferase shows only limited similarity (36 % amino acid similarity in 20 % query cover) with the glycosyltransferase identified in a previous study as responsible for β-glucan formation [11], and is thus believed to play a different role in beer spoilage. Future studies will focus on defining the mechanism that underpins hop tolerance and on determining how the genes identified to date [5,7,8] are linked to each other. Moreover, located on the same plasmid as gtf_{D15} are genes predicted to encode a glycosyltransferase and acyltransferases (Table 2) suggesting a common action on teichoic acids with the acyltransferases involved in the acylation of alanine residues or the lipid moiety of the lipoteichoic acids [27]. Follow-up work may therefore focus on determining the precise function of the glycosyltransferase (and other associated genes) in the modification of the cell wall and/or cell surface. Another question to be addressed is if and how hop tolerance is enhanced when these genes are present in a certain combination, and how such tolerance is influenced by their expression level.

6. References

1. Sakamoto, K.; Konings, W.N. Beer spoilage bacteria and hop resistance. *International Journal of Food Microbiology* **2003**, *89*, 105-124.
2. Suzuki, K.; Iijima, K.; Sakamoto, K.; Sami, M.; Yamashita, H. A review of hop resistance in beer spoilage lactic acid bacteria. *Journal of the Institute of Brewing* **2006**, *112*, 173-191.
3. Wang, C.; Cui, Y.; Qu, X. Mechanisms and improvement of acid resistance in lactic acid bacteria. *Archives of Microbiology* **2018**, *200*, 195-201, doi:10.1007/s00203-017-1446-2.
4. Makarova, K.; Slesarev, A.; Wolf, Y.; Sorokin, A.; Mirkin, B.; Koonin, E.; Pavlov, A.; Pavlova, N.; Karamychev, V.; Polouchine, N.; Shakhova, V.; Grigoriev, I.; Lou, Y.; Rohksar, D.; Lucas, S.; Huang, K.; Goodstein, D.M.; Hawkins, T.; Plengvidhya, V.; Welker, D.; Hughes, J.; Goh, Y.; Benson, A.; Baldwin, K.; Lee, J.; Díaz-Muñiz, I.; Dosti, B.; Smeianov, V.; Wechter, W.; Barabote, R.; Lorca, G.; Altermann, E.; Barrangou, R.; Ganesan, B.; Xie, Y.; Rawsthorne, H.; Tamir, D.; Parker, C.; Breidt, F.; Broadbent, J.; Hutkins, R.; O'Sullivan, D.; Steele, J.; Unlu, G.; Saier, M.; Klaenhammer, T.; Richardson, P.; Kozyavkin, S.; Weimer, B.; Mills, D. Comparative genomics of the lactic acid bacteria. *Proceedings of the National Academy of Sciences of the United States of America* **2006**, *103*, 15611-15616, doi:10.1073/pnas.0607117103.
5. Sakamoto, K.; Margolles, A.; van Veen, H.W.; Konings, W.N. Hop resistance in the beer spoilage bacterium *Lactobacillus brevis* is mediated by the ATP-Binding Cassette multidrug transporter HorA. *Journal of Bacteriology* **2001**, *183*, 5371-5375, doi:10.1128/JB.183.18.5371-5375.2001.

6. Bergsveinson, J.; Baecker, N.; Pittet, V.; Ziola, B. Role of plasmids in *Lactobacillus brevis* BSO 464 hop tolerance and beer spoilage. *Applied and Environmental Microbiology* **2015**, *81*, 1234-1241, doi:10.1128/aem.02870-14.

7. Iijima, K.; Suzuki, K.; Ozaki, K.; Yamashita, H. HorC confers beer-spoilage ability on hop-sensitive *Lactobacillus brevis* ABBC45cc. *Journal of Applied Microbiology* **2006**, *100*, 1282-1288, doi:10.1111/j.1365-2672.2006.02869.x.

8. Hayashi, N.; Ito, M.; Horiike, S.; Taguchi, H. Molecular cloning of a putative divalent-cation transporter gene as a new genetic marker for the identification of *Lactobacillus brevis* strains capable of growing in beer. *Applied Microbiology and Biotechnology* **2001**, *55*, 596-603.

9. Suzuki, K.; Sami, M.; Kadokura, H.; Nakajima, H.; Kitamoto, K. Biochemical characterization of horA-independent hop resistance mechanism in *Lactobacillus brevis*. *International Journal of Food Microbiology* **2002**, *76*, 223-230.

10. Feyereisen, M.; Mahony, J.; Kelleher, P.; Roberts, R.J.; O'Sullivan, T.; Geertman, J.-M.A.; van Sinderen, D. Comparative genome analysis of the *Lactobacillus brevis* species. *BMC Genomics* **2019**, *20*, 416, doi:10.1186/s12864-019-5783-1.

11. Fraunhofer, M.E.; Geissler, A.J.; Wefers, D.; Bunzel, M.; Jakob, F.; Vogel, R.F. Characterization of beta-glucan formation by *Lactobacillus brevis* TMW 1.2112 isolated from slimy spoiled beer. *International Journal of Biological Macromolecules* **2018**, *107*, 874-881, doi:10.1016/j.ijbiomac.2017.09.063.

12. Karthikeyan, V.; Santosh, S.W. Comparing the efficacy of plasmid curing agents in *Lactobacillus acidophilus*. *Beneficial Microbes* **2010**, *1*, 155-158, doi:10.3920/bm2009.0038.

13. Kuipers, O.P.; de Ruyter, P.G.G.A.; Kleerebezem, M.; de Vos, W.M. Quorum sensing-controlled gene expression in lactic acid bacteria. *Journal of Biotechnology* **1998**, *64*, 15-21, doi:https://doi.org/10.1016/S0168-1656(98)00100-X.

14. Ruiz-Barba, J.L.; Piard, J.C.; Jiménez-Díaz, R. Plasmid profiles and curing of plasmids in *Lactobacillus plantarum* strains isolated from green olive fermentations. *Journal of Applied Bacteriology* **1991**, *71*, 417-421, doi:10.1111/j.1365-2672.1991.tb03810.x.

15. McGrath, S.; Fitzgerald, G.F.; van Sinderen, D. Improvement and optimization of two engineered phage resistance mechanisms in *Lactococcus lactis*. *Applied and Environmental Microbiology* **2001**, *67*, 608-616.

16. van Pijkeren, J.-P.; Britton, R.A. High efficiency recombineering in lactic acid bacteria. *Nucleic Acids Research* **2012**, *40*, e76-e76.

17. Ahrné, S.; Molin, G.; Axelsson, L. Transformation of *Lactobacillus reuteri* with electroporation: studies on the erythromycin resistance plasmid pLUL631. *Current Microbiology* **1992**, *24*, 199-205, doi:10.1007/bf01579282.

18. Svensson, U.; Christiansson, A. Methods for phage monitoring. *Bulletin of the International Dairy Federation* **1991**, *263*, 29-39.

19. Söding, J.; Biegert, A.; Lupas, A.N. The HHpred interactive server for protein homology detection and structure prediction. *Nucleic Acids Research* **2005**, *33*, doi:10.1093/nar/gki408.

20. Krogh, A.; Larsson, B.; von Heijne, G.; Sonnhammer, E.L. Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. *Journal of Molecular Biology* **2001**, *305*, 567-580, doi:10.1006/jmbi.2000.4315.

21. Käll, L.; Krogh, A.; Sonnhammer, E.L.L. Advantages of combined transmembrane topology and signal peptide prediction--the Phobius web server. *Nucleic Acids Research* **2007**, *35*, W429-W432, doi:10.1093/nar/gkm256.

22. Feyereisen, M.; Mahony, J.; Lugli, G.A.; Ventura, M.; Neve, H.; Franz, C.M.A.P.; Noben, J.-P.; O'Sullivan, T.; van Sinderen, D. Isolation and characterization of *Lactobacillus brevis* phages. *Viruses* **2019**, *11*, 393.

23. Behr, J.; Gänzle, M.G.; Vogel, R.F. Characterization of a highly hop-resistant *Lactobacillus brevis* strain lacking hop transport. *Applied and Environmental Microbiology* **2006**, *72*, 6483-6492.

24. Yasui, T.; Yoda, K. Purification and partial characterization of an antigen specific to *Lactobacillus brevis* strains with beer spoilage activity. *FEMS Microbiology Letters* **1997**, *151*, 169-176.

25. Räisänen, L.; Schubert, K.; Jaakonsaari, T.; Alatossava, T. Characterization of lipoteichoic acids as *Lactobacillus delbrueckii* phage receptor components. *Journal of Bacteriology* **2004**, *186*, 5529-5532, doi:10.1128/jb.186.16.5529-5532.2004.

26. Wolin, M.; Douglas, L.J. Cell wall polymers and phage lysis of *Lactobacillus plantarum*. *Biochemistry* **1971**, *10*, 1551-1555.

27. Kiriukhin, M.Y.; Neuhaus, F.C. d-alanylation of lipoteichoic acid: role of the d-alanyl carrier protein in acylation. *Journal of Bacteriology* **2001**, *183*, 2051-2058, doi:10.1128/jb.183.6.2051-2058.2001.

Chapter V

Isolation and characterization of *Lactobacillus brevis* phages

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Gabriele A. Lugli and Marco Ventura (GenProbio, Italy) carried out sequence assembly, Horst Neve and Charles M. Franz (Max Rubner Institut, Germany) carried out electron microscopy analysis, Jean-Paul Noben (Hasselt University, Belgium) performed mass-spectrometry analysis.

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1. Abstract

Lactobacillus brevis has been widely used in industry for fermentation purposes. However, it is also associated with the spoilage of foods and beverages, in particular, beer. There is an increasing demand for natural food preservation methods, and in this context, bacteriophages possess the potential to control such spoilage bacteria. Just a few studies on phages infecting *Lactobacillus brevis* have been performed to date and in the present study, we report the isolation and characterization of five virulent phages capable of infecting *Lb. brevis* strains. The analysis reveals a high diversity among the isolates, with members belonging to both, the *Myoviridae* and *Siphoviridae* families. One isolate, designated phage 3-521, possesses a genome of 140.8 kb, thus representing the largest *Lb. brevis* phage genome sequenced to date. While the isolated phages do not propagate on *Lb. brevis* beer-spoiling strains, phages showed activity against these strains, impairing the growth of some *Lb. brevis* strains. The results highlight the potential of bacteriophage-based treatments as an effective approach to prevent bacterial spoilage of beer.

2. Introduction

Lactobacillus brevis is a Gram-positive, heterofermentative lactic acid bacterium (LAB) that grows optimally at 30 °C and pH 4.0–6.0 [1]. *Lb. brevis* is used in the production of fermented foods [1,2]. Recently strains of this species have also been characterized as "probiotic" potentially promoting gut microbiota fitness and consumer health [2,3]. Conversely, *Lb. brevis* strains are also associated with food and beverage spoilage, particularly that of beer [4,5]. Beer is generally regarded as a harsh environment for microorganisms [5,6]. Indeed, the reduced availability of oxygen and nutrients coupled with the presence of an acidic environment, ethanol, carbon dioxide and hop compounds represent considerable challenges to microbial growth [5,6]. Despite the nature of beer and the array of antimicrobial compounds it contains, bacterial strains have emerged that can tolerate and grow in the presence of these hurdles [6,7]. This bacterial growth is mostly attributed to certain LAB, especially *Lb. brevis* and may result in the production of malodorous compounds, acidity and/or turbidity, thereby negatively impacting on the organoleptic properties of the final product [5,7,8]. Current approaches to increase the safety of beer include pasteurization, filtration, suitable materials and process packaging, strict cleaning and sanitation practices [7]. However, non-pasteurized beer products are in high demand, thus increasing the risk of microbial spoilage, for example by LAB, in particular when filtration cannot be applied [9]. The overuse of chemical sanitizers has led to an increase in biocidal-resistance of these food-spoilage bacteria [10]. Moreover, chemical sanitizers may be corrosive and/or toxic, thereby limiting the range of sanitizers that may be employed safely in industry. Various alternative strategies have been implemented to control bacterial spoilage using antimicrobials such as bacteriocins [11–13], and bioremediation using bacteriophages has re-appeared as a potential procedure for limiting spoilage bacteria in food and beverages [14–17]. Bacteriophages present an interesting bio-remediation approach,

because they are naturally ubiquitous and specific to their bacterial host [18]. The impact of bacteriophages in preventing/limiting spoilage has been thoroughly explored in the case of food fermentation applications [16], although at the same time the prevention or limitation of bacterial spoilage of fermented beverages such as beer using bacteriophages is poorly studied [17]. To date, the genome sequences of approximately 50 *Lb. brevis* strains (and their associated prophages) are available on the NCBI database while only one lytic phage (SA-C12) [17] and one temperate phage (LBR48) have been described [19]. Here, we report the isolation and characterization of phages active against *Lb. brevis* strains in order to increase our understanding of the diversity and therapeutic potential of *Lb. brevis* phages.

3. Materials and Methods

3.1. Bacterial strains and cultivation media

The *Lactobacillus brevis* strains used in this study are listed in Table 1. Bacterial stock cultures were stored in 20 % glycerol at −80 °C. Bacteria and phages were cultured and/or propagated in MRS broth (Oxoid Ltd., Hampshire, UK) at 30 °C without agitation. MRS broth was supplemented with 10 mM $CaCl_2$ (Sigma-Aldrich, St. Louis, MO, USA) where appropriate. Soft agar was prepared with MRS broth supplemented with 0.4 % agar and 0.5 % glycine [20].

3.2. Phage isolation and enrichment

Environmental samples were clarified by centrifugation at 4000 × g for 10 min followed by filtration through a 0.45 μm filter (Sarsted, Nümbrecht, Germany) and stored at 4 °C until required. The filtrate was added to equal amounts of MRS broth supplemented with 10 mM $CaCl_2$ and inoculated with an early log-phase host culture (Table 1). After incubation at 30 °C

overnight, the culture was centrifuged at 4000 × g for 10 min. This enrichment procedure was repeated twice. The filtered sample was then evaluated for the presence of phages active against a panel of *Lb. brevis* strains (Table 1). Each of the environmental samples were enriched and tested separately on each of the *Lb. brevis* strains listed in Table 1.

Table 1. *Lactobacillus brevis* strains used for phage isolation and characterization.

***Lactobacillus brevis* strains**	**Isolation source**	**Beer-Spoilers or Not**
ATCC367	Silage	Non-beer spoiler
UCCLBBS124	Beer	Beer-spoiler
UCCLB521	Brewery	Non-beer spoiler
UCCLB556	Brewery	Non-beer spoiler
SA-C12	Silage	Non-beer spoiler
UCCLBBS449	Beer	Beer-spoiler
UCCLB94	Beer	Beer-spoiler
UCCLB95	Beer	Beer-spoiler
RIBM 2-56	Beer	Beer-spoiler

3.3. Phage detection, purification and host range analysis

The spot test method was applied in first instance to detect the presence of phages [20]. Soft agar (4 mL) was seeded with 200 µL of fresh overnight culture and poured onto an MRS agar plate supplemented with 10 mM $CaCl_2$ and 0.5 % glycine. On the lawn of the series of *Lb. brevis* strains, 10 µL of the enriched samples was spotted and incubated at 30 °C overnight. The presence of phages was demonstrated by the presence of a clear zone on the plate. Presumptive positive samples were confirmed by plaque assay using the double-layer agar plate method [20]. A 10 µL volume of the appropriate phage dilution and 200 µL of *Lb. brevis* culture were added to 4 mL of soft agar supplemented with 10 mM $CaCl_2$, mixed and poured onto an MRS agar plate supplemented with 10 mM $CaCl_2$ and 0.5 % glycine. The plate was incubated at 30 °C overnight and resulting plaques were enumerated. Phages were purified by

single-plaque isolation using an appropriate *Lb. brevis* host strain. A single plaque was picked from the bacterial lawn, transferred into a tube containing 10 mL MRS broth, 10 mM $CaCl_2$ and 1 % inoculum of the propagating *Lb. brevis* culture. The tube was incubated at 30 °C overnight. The phage lysate was centrifuged at 4000 × g for 10 min at 4 °C. The supernatant was filtered (0.45 µm) and stored at 4 °C until required. Host range studies were performed using the spot and plaque assay techniques as described above where phage lysates were tested against available *Lb. brevis* strains (Table 1). The presence or absence of plaque formation was recorded indicating the susceptibility of *Lb. brevis* strains to isolated phages. Plaques were enumerated and phage titer determined as plaque-forming units (PFU/mL).

3.4. Phage concentration and purification

A 2 L phage lysate was centrifuged at 5000 × g for 10 min, 0.5 M NaCl was added to the supernatant and incubated for 1 h at 4 °C. The preparation was centrifuged at 5000 × g for 10 min and phages were precipitated by adding 10 % (w/v) polyethylene glycol 8000 (Sigma-Aldrich) and incubated overnight at 4 °C. Phages were harvested by centrifugation (as described above) and resuspended in 4 mL SM buffer (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 10 mM $MgSO_4$). Phages were extracted with chloroform (1:1 phage suspension:chloroform) applying multiple extraction steps where necessary (typically two or three times). The phage lysate was purified on a discontinuous CsCl (Sigma-Aldrich) gradient [21] and dialyzed against phage buffer (50 mM Tris-HCl, 100 mM NaCl, 8 mM $MgSO_4$) overnight at 4 °C.

3.5. Transmission electron microscopy

Purified bacteriophage lysates were analyzed by electron microscopy, as previously described [22]. Negative staining was performed using 2 % (w/v) uranyl acetate on freshly prepared ultrathin carbon films. Grids were analyzed in a Tecnai 10 transmission electron microscope (FEI Thermo Fisher Scientific, Eindhoven, The Netherlands) at an acceleration voltage of 80 kV. Micrographs were taken with a MegaView G2 charge-coupled device camera (Emsis, Muenster, Germany).

3.6. Phage DNA extraction and sequencing

Phage DNA was extracted using the Norgen Biotek Corp phage DNA isolation kit as per the manufacturer's instructions (Norgen Biotek Corp., Thorold, Ontario, Canada). Phage genome sequencing was performed by GenProbio at the University of Parma, Italy. Genomes were sequenced with Illumina MiSeq Sequencing System and assembled with MIRA v4.0.2. De novo sequence assemblies and automated gene calling was performed using the MEGAnnotator pipeline [23] and assessed for predicted transfer RNA genes via tRNAscan-SE v1.2.1 [24]. Predicted open reading frames (ORFs) were determined via Prodigal v2.6 [25]. A BLASTP [26] analysis was performed to assign functional annotations to the predicted ORFs (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The proposed functional annotations were further investigated by performing structural homology searches via HHpred [27] and querying the NCBI Conserved Domain Database (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). The annotated genomes were manually inspected, edited and finalized using the Artemis visualization tool [28].

3.7. Phage structural proteome and mass-spectrometry

An aliquot (30 µL) of CsCl-purified phage sample was mixed with 10 µL of SDS loading buffer containing 50 mM β-mercaptoethanol. The structural protein profile was generated by standard Tris-glycine sodium dodecyl sulfate (SDS)–12 % polyacrylamide gel electrophoresis (PAGE). Gel slices were then excized, trypsinized, and analyzed using electrospray ionization tandem mass spectrometry (ESI-MS/MS), as previously described [29].

3.8. Proteomic tree

To study the relationship between *Lactobacillus* phages a proteomic tree was constructed. The genomes of the five *Lb. brevis* phages isolated as part of this study as well as all *Lactobacillus* phage genomes available on the NCBI database were downloaded. All predicted protein-encoding sequences were extracted and concatenated beginning with the ORF encoding for the small terminase subunit (TerS) [22]. The concatenated sequences were aligned using ClustalW [30]. The phylogenetic tree was constructed using the neighbour-joining method and bootstrapped employing 1000 replicates. The final tree was visualized using MEGA7 [31].

3.9. Phage activity against *Lb. brevis* beer-spoiling strains

To assess if the isolated phages could affect *Lb. brevis* beer-spoiling strains' ability to grow, the strains were grown in MRS broth until an OD_{600nm} of 0.2 was reached, at which point phages were added at a MOI (Multiplicity Of Infection) of 1, along with 10 mM $CaCl_2$. The optical density at 600 nm (OD_{600nm}) was recorded at 30-min intervals for 48 h to monitor the impact of the addition of phages on the growth of *Lb. brevis* beer-spoiling strains. A control culture was also employed where the strain was grown in the absence of phage lysate but

treated identically in all other aspects, i.e., filtered MRS broth was added in place of phage lysate. Addition of calcium chloride, incubation time and temperature were identical for both scenarios.

Adsorption assays were adapted from a previously outlined protocol [32]. Briefly, strains were grown to mid-late exponential phase (OD_{600nm} ~ 0.5), at which point they were harvested by centrifugation at 4000 × g for 10 min and resuspended in 1/4-strength Ringer's solution. Phages were added to the cells at a final titer of 10^6 PFU/mL followed by incubation at 30 °C for 15 min. The supernatant was retained after centrifugation and tested for the residual phage concentration by plaque assay as described above. Adsorption efficiency was calculated using the formula: ((Ci – Cr) / Ci) × 100

where Ci represents the total phage concentration used in the adsorption assay and Cr represents the residual phage concentration after the adsorption step.

The ability of phages to propagate and multiply within the host cell was also tested. *Lb. brevis* strains were grown to mid-late exponential phase (OD_{600nm} ~ 0.5), at which point phages were added to the culture (T0), the mix was further incubated at 30 °C overnight (T1). The phage titer was enumerated at T0 and T1 to assess phage propagation efficiency.

3.10. Nucleotide sequence accession numbers

The genome sequences of the phages isolated in this study were deposited in the GenBank database under accession numbers: 3-521: MK504444; 521B: MK504443; 3-SAC12: MK504442; SAC12B: MK504446; ATCCB: MK504445. The GenBank accession numbers of phage genome sequences applied in the proteomic tree preparation are as follows: *Lactobacillus plantarum* phage ATCC8014-B1: JX486087; *Lactobacillus plantarum* phage ATCC8014-B2: JX486088; *Lactobacillus casei* prophage A2: AJ251789; *Lactobacillus helveticus* phage AQ113: HE956704; *Lactobacillus delbrueckii* phage c5: EU340421;

Lactobacillus casei phage J-1: KC171646; *Lactobacillus delbrueckii* phage JCL1032: EU409559; *Lactobacillus gasseri* phage kc5a: DQ320509; *Lactobacillus paracasei* phage Lb3381: FJ822135; *Lactobacillus brevis* phage LBR48: GU967410; *Lactobacillus rhamnosus* phage Lc-Nu: AY131267; *Lactobacillus delbrueckii* phage Ld3: KJ564038; *Lactobacillus delbrueckii* phage Ld17: KJ654037; *Lactobacillus delbrueckii* phage Ld25A: KJ654036; *Lactobacillus delbrueckii* phage Ldl1: KM514685; *Lactobacillus fermentum* phage LF1: HQ141410; *Lactobacillus delbrueckii* phage LL-H: EF455602; *Lactobacillus delbrueckii* phage LL-Ku: AY739900; *Lactobacillus johnsonii* phage Lj965: AY459535; *Lactobacillus johnsonii* phage Lj928: AY459533; *Lactobacillus plantarum* phage LP65: AY682195; *Lactobacillus rhamnosus* phage Lrm1: EU246945; *Lactobacillus jensenii* phage Lv1: EU871039; *Lactobacillus gasseri* phage phiadh: AJ131519; *Lactobacillus casei* phage phiAT3: AY605066; *Lactobacillus plantarum* phage phig1e: X98106; *Lactobacillus delbrueckii* phage phiJB: KF188409; *Lactobacillus delbrueckii* phage phiLdb: KF188410; *Lactobacillus fermentum* phage phiPYB5: GU323708; *Lactobacillus casei* phage PL1: KC171647; *Lactobacillus brevis* phage SA-C12: KU052488 and *Lactobacillus plantarum* phage Sha1: HQ141411.

4. Results and Discussion

4.1. Phage isolation and host range profile

Lactobacillus brevis is a persistent problem in the brewing industry due to its ability to grow in, and spoil, beer. Therefore, a screen for phages capable of infecting *Lb. brevis* strains with potential industrial relevance was undertaken. In excess of 200 environmental samples were screened for the presence of phages active against *Lb. brevis*. These environmental samples included silage, fermented foods and wastewater samples, collected at different locations (Ireland, Belgium and The Netherlands) over a period of three years. Five distinct virulent phages capable of infecting one or more *Lb. brevis* strain(s) within our collection were isolated from two different Irish wastewater samples (collected in 2017 and 2018), purified and characterized. Two phages were isolated that infected each of two strains, namely *Lb. brevis* UCCLB521 and *Lb. brevis* SA-C12, while a further isolate was identified that targeted *Lb. brevis* ATCC 367. These isolates were propagated to a titer of 10^9 PFU/mL (except for ATCCB, where only a titer of 10^7 PFU/mL could be reached) and applied to a host range analysis (Table 2) against the collection of *Lb. brevis* strains available. This analysis highlighted the narrow host range of the isolated phages, while also highlighting the relative sensitivity of two strains. The *Lb. brevis* strains UCCLB521 and SA-C12 exhibited sensitivity to three and two phages, respectively. On *Lb. brevis* strains UCCLBBS449, UCCLB95 and RIBM 2-56, a clearing zone was observed on bacterial lawns used in the spot assay technique. However, propagation of the phages using these *Lb. brevis* strains as hosts was not possible (see results below).

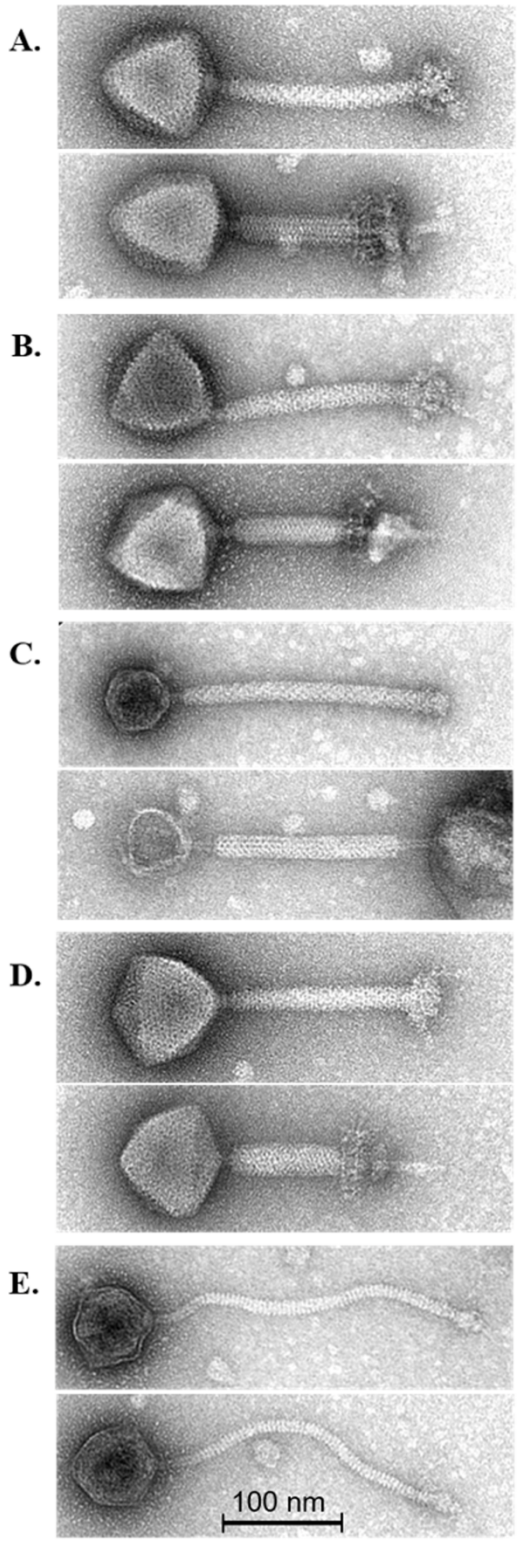
Table 2. *Lb. brevis* phage host range analysis.

		***Lb. brevis* non-beer spoiling strains**				***Lb. brevis* beer-spoiling strains**				
		ATCC367	UCCLB521	UCCLB556	SA-C12	UCCLBBS124	UCCLBBS449	UCCLB94	UCCLB95	RIBM 2-56
Phage	3-521	-	+ *	-	-	-	-	-	~	-
	521B	-	+ *	-	-	-	~	-	-	-
	3-SAC12	-	-	-	+ *	-	-	-	-	-
	SAC12B	-	+	-	+ *	-	-	-	-	~
	ATCCB	+ *	-	-	-	-	-	-	-	-

+: strain susceptible to phage infection; -: strain resistant to phage infection; ~: clearing zone was observed; *: host strain.

4.2. Phage morphology

The morphological diversity of the phage isolates was assessed by transmission electron microscopy (Figure 1). *Lb. brevis* phages 3-521, SAC12B and 521B possessed relatively short yet wide contractile tails and a large icosahedral head with a large complex baseplate structure at the distal end of the tail (Figure 1). These structural features are consistent with the typical attributes of *Myoviridae* phages [33] and revealed morphological similarity to the only virulent *Lb. brevis* phage identified to date, SA-C12 [17]. *Lb. brevis* phage 3-SAC12 possessed an icosahedral head, a defined baseplate structure and a long decorated contractile tail (Figure 1) and, therefore, also belongs to the *Myoviridae* family [33] and resembles the *Lb. brevis* temperate phage LBR48 [19]. *Lb. brevis* phage ATCCB was classified as a *Siphoviridae* phage due to the presence of a long non-contractile tail, a large icosahedral head and a discrete baseplate at the tip of the tail (Figure 1).



Head diameter	Tail length*	Tail width
95.3 ± 4.2 nm (n = 8)	201.9 ± 5.0 nm (n = 9)	21.8 ± 0.9 nm (n = 9)

* Incl. baseplate structures, when present

Head diameter	Tail length*	Tail width
87.1 ± 2.8 nm (n = 2)	201.0 ± 0.3 nm (n = 2)	20.5 ± 0.5 nm (n = 2)

* Incl. baseplate structures, when present

Head diameter	Tail length*	Tail width
58.3 ± 2.0 nm (n = 12)	237.8 ± 6.6 nm (n = 10)	17.9 ± 0.8 nm (n = 12)

Head diameter	Tail length*	Tail width
98.5 ± 1.2 nm (n = 3)	187.9 ± 3.0 nm (n = 4)	23.2 ± 0.7 nm (n = 4)

* Incl. baseplate structures, when present

Head diameter	Tail length*	Tail width
70.8 ± 2.7 nm (n = 11)	288.4 ± 8.4 nm (n = 11)	12.3 ± 0.4 nm (n = 11)

Figure 1. Electron micrographs of lytic *Lb. brevis* phages 3-521 (**A**), 521B (**B**), 3-SAC12 (**C**), SAC12B (**D**) and ATCCB (**E**). Head diameter, tail length and width are also indicated, where "n" represents the number of phage particles measured. For phage 521B and SAC212B, only few particles were detected with original extended tail sheaths (i.e., 2–4 particles). Tail lengths of phages 3-521, 521-B and SAC12B are also including the complex baseplate structures.

4.3. *Lb. brevis* phage comparative analysis and grouping

In order to evaluate the diversity of *Lb. brevis* phages and their phylogenetic links to phages of other lactobacilli, a proteomic tree was created gathering the five *Lb. brevis* phages characterized in this study as well as all previously sequenced *Lactobacillus* phages (Figure 2). The phylogenetic tree shows an interesting organization based seemingly on morphology rather than phage infecting-species. The right side of the tree displays exclusively phages belonging to the *Siphoviridae* family, while the left side predominantly gathered phages belonging to the *Myoviridae* family. It is noteworthy that *Lb. brevis* phages are quite diverse as they do not form a single cluster and are, in fact, spread across the phylogenetic tree with the exception of phages SAC12B and 521B, which form a clade next to the *Lb. helveticus* phage AQ113, a *Myoviridae* phage which shows similarity to phages of human gut-inhabiting species [34]. *Lb. brevis* phages 3-521, 3-SAC12, SA-C12, ATCCB and LBR48 all gathered closely on the tree in between *Lb. plantarum* phages 8014-B1, 8014-B2 and *Lb. delbrueckii* phage JCL1032, highlighting once again the interrelationships of the *Lactobacillus* phages. The relationship between these phages infecting similar host species might be explained by evolution over time from a common ancestor [22,35].

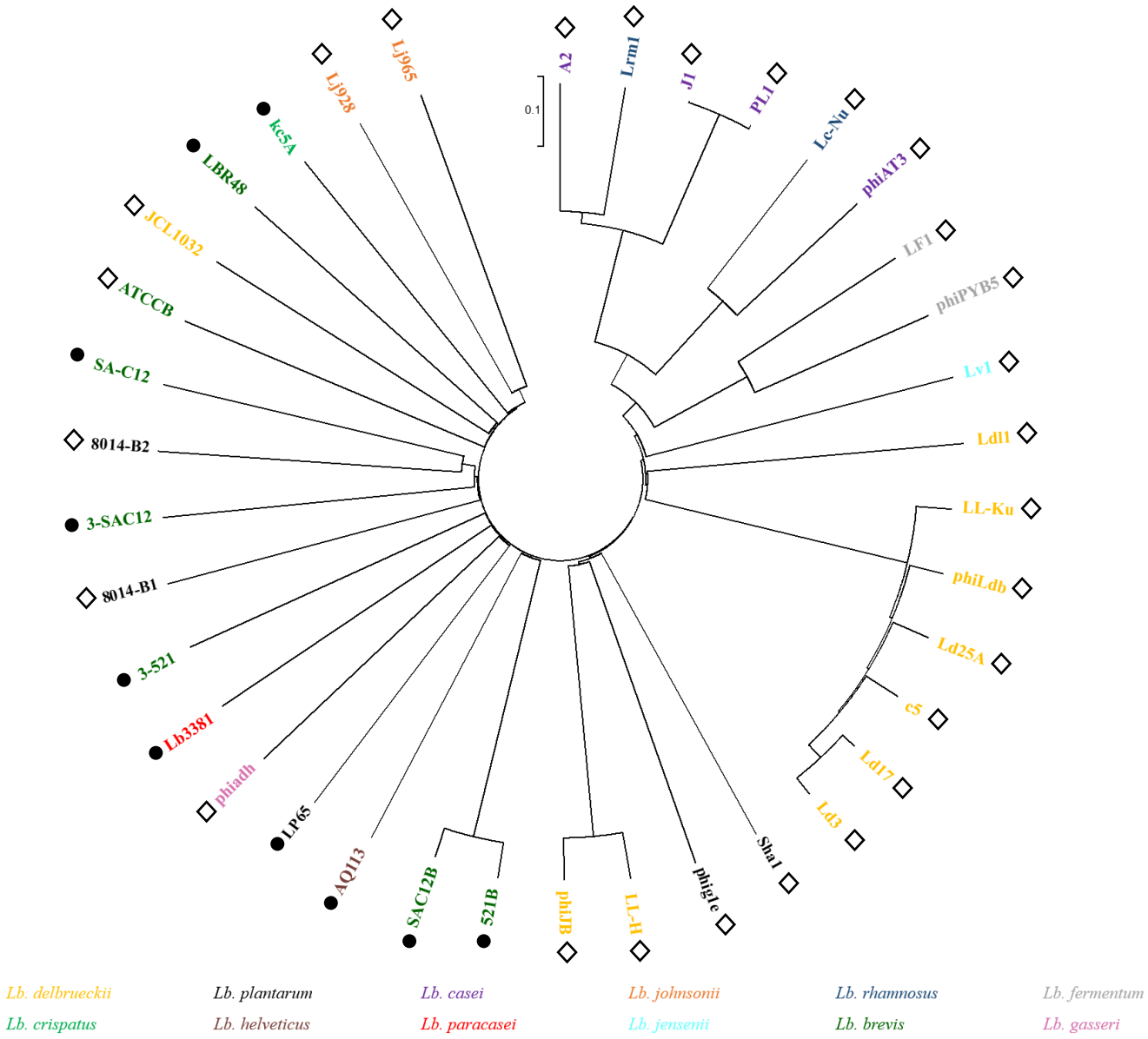


Figure 2. Proteomic tree of all *Lactobacillus* phages sequenced to date. Color coding indicates the host species for each phage. Black circles indicate *Myoviridae* phages, while white diamonds indicate *Siphoviridae* phages.

4.4. Genome analysis

Genomic DNA of the five lytic phages was isolated and sequenced revealing significant genetic disparity between these phages. General genome characteristics of the phage isolates are summarized in Table 3. The *Siphoviridae* phage ATCCB possesses a genome of 80.5 kb while the *Myoviridae* phage genomes vary in size from ~ 41–141 kb (Table 3). The largest phage genome among the isolates is that of 3-521 with a genome of 140.8 kb, which now represents the largest known *Lb.* brevis phage genome sequenced to date. Interestingly, the *Lb. brevis* myophages SAC12B, 521B and 3-521 are more closely related to myophages of other *Lactobacillus* spp. harboring a large genome size, such as *Lb. casei* Lb338-1 (142 kb) [36] and *Lb. plantarum* LP65 (131 kb) [37], than the previously characterized *Lb. brevis* phages (LBR48 and SA-C12) (Figure 2).

The genome of the phages investigated here display limited/no similarity to each other or to the genomes of other *Lb. brevis* phages, with the exception of phages 521B and SAC12B. These two phages share 97 % nucleotide sequence identity (88 % coverage) and their close relationship may be the result of their cohabitation within the same environment, as they were both isolated from the same wastewater sample in 2018 (Table 3). The absence of similarity with previously described *Lb. brevis* phages highlights the limited knowledge, and the apparent genetic diversity of these phages. The GC content of the phages is relatively low (Table 3) compared to that of the host (~ 46 %), implying that they may have evolved recently to infect *Lb. brevis* strains. The genomes of phages 3-521, 521B and SAC12B appear complex due to their size and their high number of predicted ORFs but present a similar genome organization. The genomes were organized into four functional modules: DNA packaging, morphogenesis, DNA replication and lysis modules (Figure 3A). For phages 3-SAC12 and ATCCB, the genome organization is similar but with a lysis module preceding the replication module

(Figure 3B and C). While these phage isolates are predicted to be obligatorily virulent, there are traces of temperate ancestry in some of their genomes. For example, phage 3-SAC12 possesses a predicted antirepressor-encoding gene (typically associated with lytic/lysogenic switch genomic regions) while ATCCB possesses a predicted recombinase/integrase-encoding gene [22].

Table 3. General characteristics of *Lb. brevis* phages.

	Phage	**Sample (Date)**	**Isolation source**	**Genome size (bp)**	**ORFs**	**GC content (%)**	**% nt identity (% coverage)**
Myoviridae	3-521	S1 (2017)	Wastewater (Ireland)	140,816	155	36.93	
	521B	S2 (2018)	Wastewater (Ireland)	136,442	188	32.27	97 (88) with SAC12B
	SAC12B	S2 (2018)	Wastewater (Ireland)	136,608	191	32.41	97 (88) with 521B
Siphoviridae	3-SAC12	S1 (2017)	Wastewater (Ireland)	41,292	61	40.01	
	ATCCB	S2 (2018)	Wastewater (Ireland)	80,538	96	30.80	

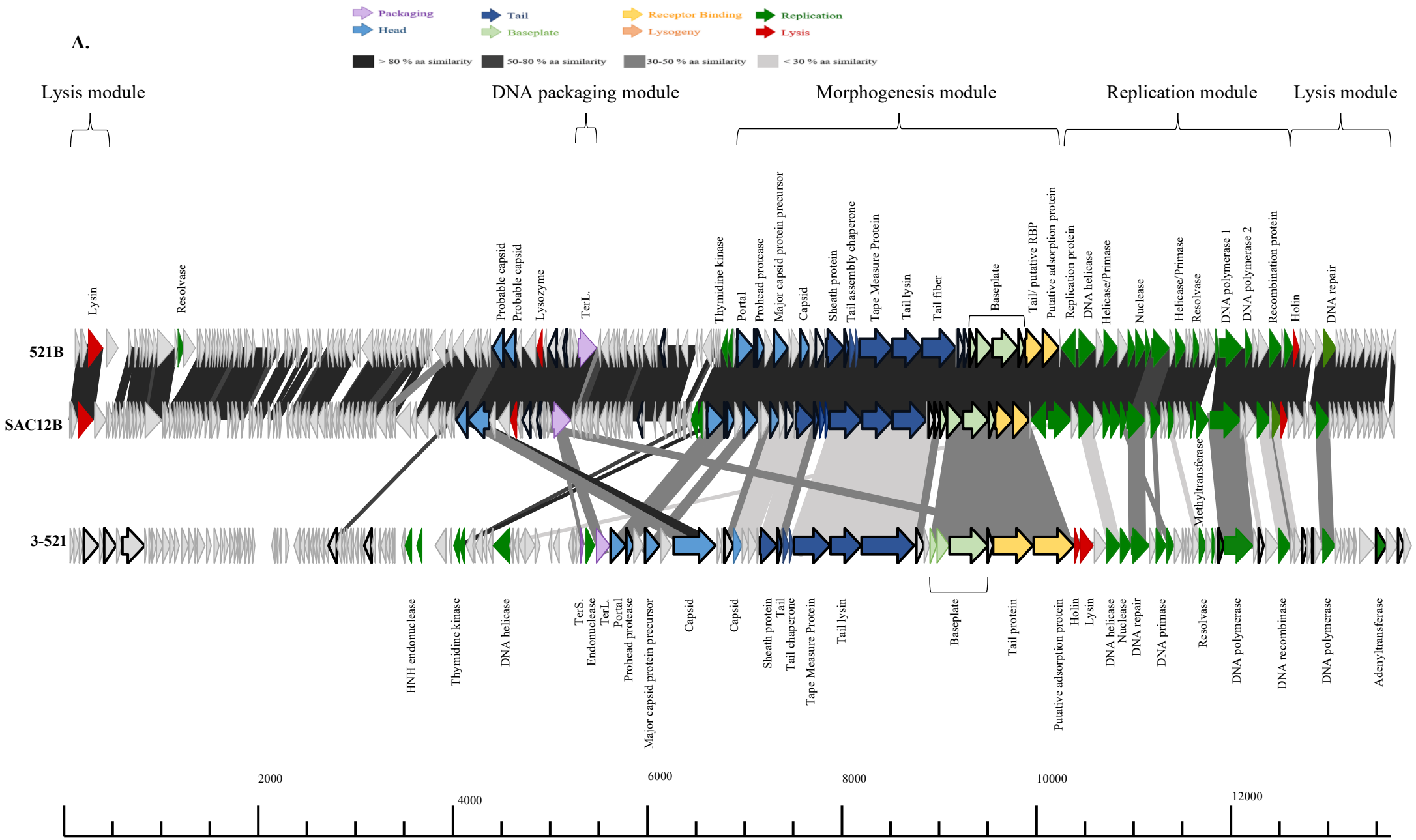


Figure 3 (continuing). Genomic organization of lytic *Lb. brevis* phages 3-521, 521B and SAC12B (**A**), 3-SAC12 (**B**) and ATCCB (**C**). The scale at the bottom of genomes is in base pairs. Each arrow represents an ORF, with the color representing the putative function of the encoded protein. Confirmed structural protein-encoding genes from mass spectrometry analysis are also highlighted (bold outline). TerS. Small terminase subunit, TerL. Large terminase subunit.

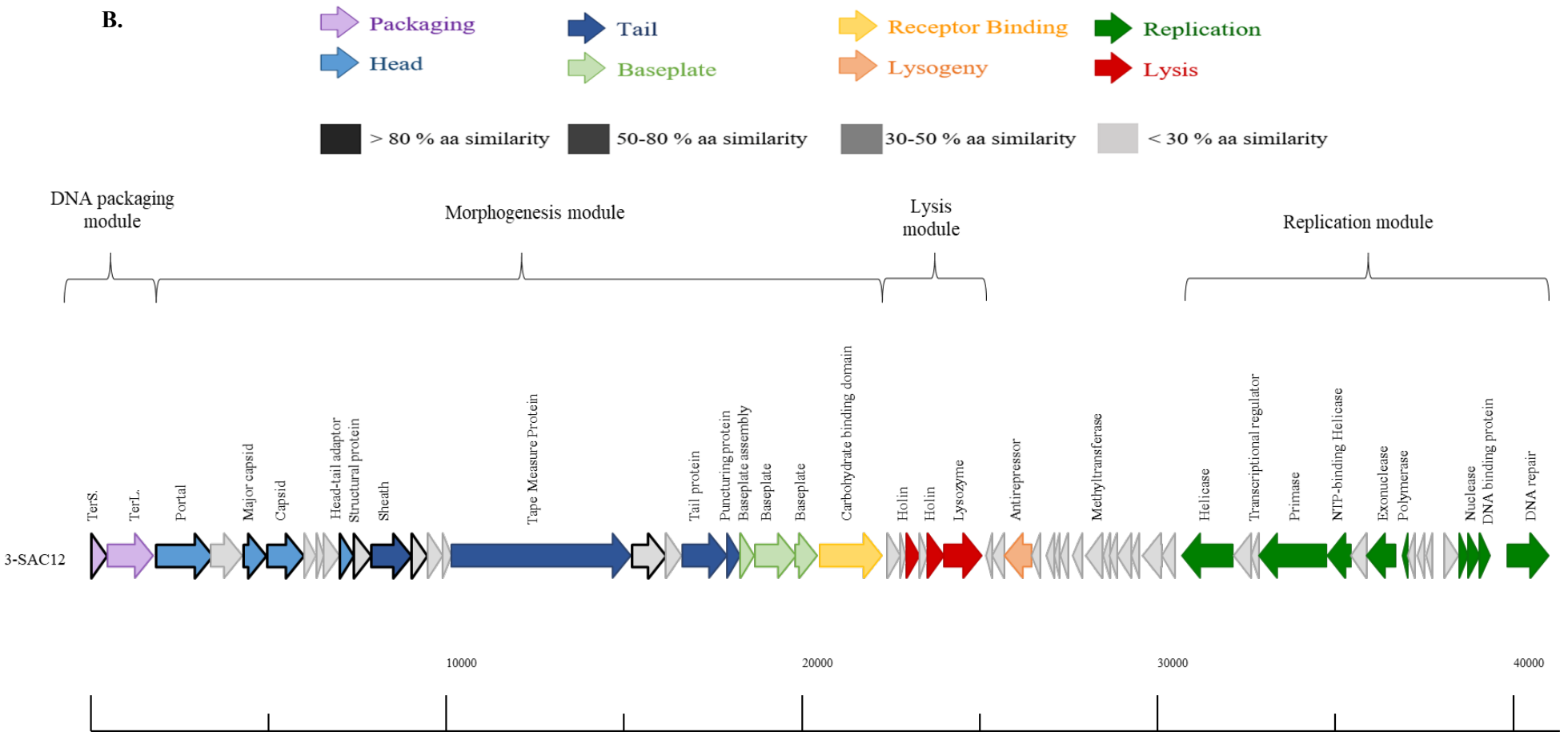


Figure 3 (continuing). Genomic organization of lytic *Lb. brevis* phages 3-521, 521B and SAC12B (**A**), 3-SAC12 (**B**) and ATCCB (**C**). The scale at the bottom of genomes is in base pairs. Each arrow represents an ORF, with the color representing the putative function of the encoded protein. Confirmed structural protein-encoding genes from mass spectrometry analysis are also highlighted (bold outline). TerS. Small terminase subunit, TerL. Large terminase subunit.

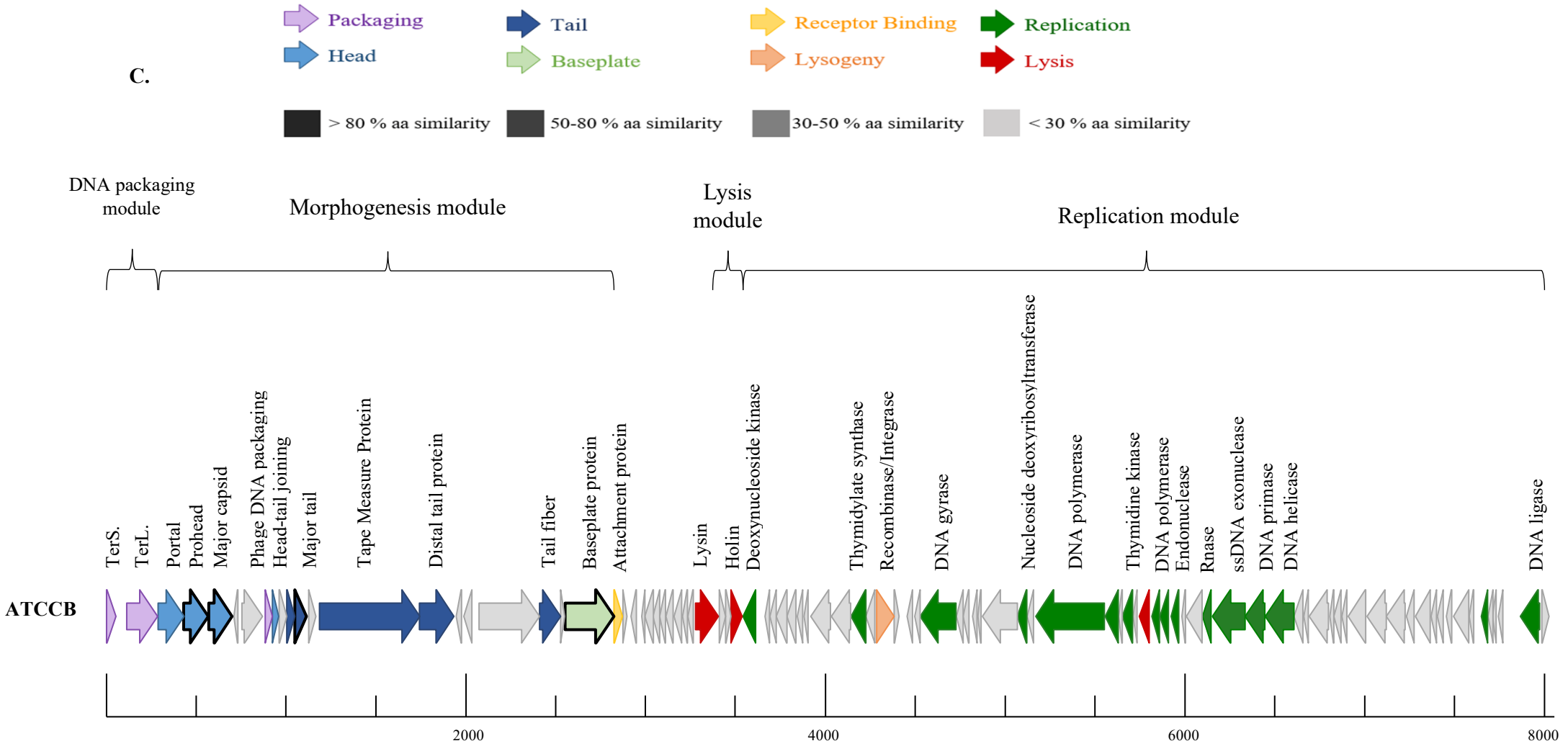


Figure 3 (end). Genomic organization of lytic *Lb. brevis* phages 3-521, 521B and SAC12B (**A**), 3-SAC12 (**B**) and ATCCB (**C**). The scale at the bottom of genomes is in base pairs. Each arrow represents an ORF, with the color representing the putative function of the encoded protein. Confirmed structural protein-encoding genes from mass spectrometry analysis are also highlighted (bold outline). TerS. Small terminase subunit, TerL. Large terminase subunit.

4.5. Morphogenesis module

The majority of the morphogenesis modules of *Lb. brevis* phages 3-521, 521B and SAC12B exhibited a high degree of synteny in the region encoding the portal protein through to the putative adsorption protein (Figure 3A). Phages 521B and SAC12B share more than 90 % amino acid (aa) sequence identity, while 3-521 shares less than 50 % aa sequence similarity with 521B and SAC12B across the morphogenesis module (Figure 3A). The most notable difference is the apparent insertion of an additional capsid-encoding protein in 3-521. The encoded predicted capsid protein, $ORF57_{3\text{-}521}$, is divided in two different proteins in 521B ($ORF80_{521B}$-$ORF81_{521B}$, 90 % and 45 % aa similarity, respectively) and in SAC12B ($ORF79_{SAC12B}$-$ORF80_{SAC12B}$, 26 % and 83 % aa similarity). In 521B and SAC12B, these protein-encoding genes are located upstream of the DNA packaging module in a divergently oriented cluster of genes of unknown function. Interestingly, it suggests the fusion of two ancestral phages into these two unique phages: 521B and SAC12B (Figure 3A).

The morphogenesis module of the *Myoviridae Lb. brevis* phage 3-SAC12 harbors genes encoding the phage capsid and tail structural components including a portal protein, two capsid proteins and a head-tail adaptor protein, the tail sheath protein, a tail tape measure protein (TMP), the major tail protein (MTP) and a "puncturing device" protein. This puncturing device comprises the tip of the central spike and is proposed to facilitate DNA ejection into the host cell [38]. Furthermore, at the distal tail region, there is a large organelle described as a baseplate complex that comprises three structural proteins ($ORF21_{3\text{-}SAC12}$, $ORF22_{3\text{-}SAC12}$ and $ORF23_{3\text{-}SAC12}$) and a protein that harbors a predicted carbohydrate binding domain ($ORF24_{3\text{-}SAC12}$) that we predict to bind to the host cell acting as the receptor binding protein (RBP) (Figure 3B).

The *Siphoviridae* phage ATCCB appears less complex in its morphogenesis module compared to the *Myoviridae* phages and genes encoding a portal protein, a prohead protease, a major

capsid protein, a head-tail joining protein, four predicted tail proteins, a distal tail (Dit) protein, a tail fiber protein, a baseplate protein and a predicted attachment protein, assumed to be involved in host recognition and binding, were identified (Figure 3C).

4.6. Structural proteome

The lytic *Lb. brevis* phages were analyzed by mass spectrometry to identify their structural proteomes (Table 4). Most of the predicted proteins encoded within the morphogenesis module of the genomes of 521B, SAC12B and 3-521 were confirmed as structural proteins with the predicted portal protein, prohead protease, major capsid precursor protein, capsid protein, tail sheath protein, tail proteins, tape measure protein, tail lysin, tail fiber, baseplate proteins, putative receptor binding protein and adsorption protein; all identified as structural proteins using this approach (Table 4 and Figure 3A). The majority of the predicted structural proteins forming the capsid and the tail components were identified in 3-SAC12 and ATCCB (Table 4, Figure 3B and C). Some (presumed) structural proteins were not identified in the experimentally determined proteome, which was likely due to their small size or their low relative abundance.

Table 3. Structural proteins extracted from purified phage particles by ESI-MS/MS. A minimum of two independent unique peptides or 5 % coverage were used as threshold values.

Phage	ORF	Putative function	No. of peptides	Sequence coverage (%)
521B	80	Probable capsid protein	8	29.4
	81	Probable capsid protein	9	28.4
	86	Structural protein	3	16.1
	88	Lipoprotein	5	50.8
	106	Structural protein	4	37.9
	121	Portal protein	12	28.3
	122	Structural protein	2	17.4
	123	Caudovirus prohead protease	4	20.8
	125	Major capsid protein precursor	19	59.7
	128	Capsid protein	3	16.8
	130	Gp91	8	35
	132	Major tail sheath protein	16	40.4
	133	Tail protein	5	59.9
	136	Tape measure protein	28	31.2
	137	Tail lysin	12	15.1
	138	Structural component of the tail fiber	8	10
	140	Structural protein	2	15.1
	141	Structural protein	5	42.2
	142	Baseplate protein	3	23.1
	143	Baseplate J-like protein	6	15.1
	144	Baseplate protein	7	9.4
	146	Tail protein	15	34.1
	147	Putative adsorption protein	9	22.2
	156	DNA starvation/stationary phase protein	6	48
	185	Structural protein	3	38.5
3-521	10	dUTP diphosphatase	2	9.9
	19	Zn-dependent protease	5	23.7
	52	Portal protein	3	7.9
	53	Prohead protease	1	8.3
	55	Major capsid protein	19	51.1
	57	Phage capsid and scaffold	19	18.6
	60	Structural protein	4	20.1
	65	Tail sheath protein	14	25
	66	Putative tail protein	6	53.3
	69	Tape measure protein	14	17.4
	70	Tail lysin protein	16	21.1
	71	gp673	2	1.7
	72	Structural protein	3	15.3
	76	Baseplate protein	4	3
	77	Structural protein	2	18
	78	Tail protein	22	24.5
	79	Tail associated protein	23	20.1
	98	Nucleoside 2-deoxyribosyltransferase	2	12.4
	101	Structural protein	1	6.1

	106	Structural protein	7	62
	108	Tail protein	1	18
	117	Adenyltransferase	6	16.9
	119	ADP-ribose pyrophosphatase	1	5.3
	124	Structural protein	6	15.7
	126	AAA superfamily ATPase	9	26.2
	128	Phosphatase	4	5.4
3-SAC12	1	Terminase small subunit	1	6.6
	3	Portal protein	13	31.4
	5	Major capsid protein	4	26.3
	6	Capsid protein	9	26.7
	10	Putative head-tail adaptor	1	9.8
	11	Structural protein	1	9
	12	Sheath protein	3	11.8
	13	Structural protein	2	22.4
	17	Structural protein	4	13.6
ATCCB	70	Baseplate protein	6	9.2
	79	Major tail protein	6	50.2
	86	Major capsid protein	8	25.9
	87	Prohead protease	2	7.5

4.7. Phage activity against *Lb. brevis* beer-spoiling strains

Phage adsorption experiments were performed in order to test the ability of the phages to recognize and bind to *Lb. brevis* beer-spoiling strains. Here, an adsorption efficiency higher than 50 % was considered as significantly effective adsorption of the phage to the strain. Phages were tested against all *Lb. brevis* beer-spoiling strains and efficient phage adsorption was only observed in the cases described below (Figure 4D, E and F). Adsorption of the lytic phages 521B and 3-521 to their *Lb. brevis* host UCCLB521 showed more than 90 % adsorption efficiency. Phages 521B and 3-521 were capable of high adsorption efficiencies to the *Lb. brevis* strains UCCLBBS449 and UCCLB95 (86.6 ± 4.7 % and 98.9 ± 0.5 %, respectively). Similarly, SAC12B adsorbed to its host strain SA-C12 and the beer-spoiling strain RIBM 2-56, with similar efficiencies (90.9 ± 0.9 % versus 87.7 ± 0.0 %). *Lb. brevis* strain UCCLBBS124 was not adsorbed efficiently by phages 3-521 and SAC12B (Figure 4E and F) and even if 521B showed an adsorption efficiency of 66.6 ± 7.0 % on UCCLBBS124 (Figure 4D), no infection or effect of the phage was observed against this strain (data not shown). Since

most of the phages were capable of adsorbing to *Lb. brevis* beer-spoiling strains, experiments were performed to study their ability to affect growth of *Lb. brevis* strains in nutritive media (MRS broth). *Lb. brevis* beer-spoiling strains were grown in nutritive media until they reached an OD_{600nm} of 0.2, at which point the relevant lytic phage showing adsorption capability (Figure 4D, E and F) were added to reach an MOI of 1. In some cases, the addition of the bacteriophage to the culture had a negative effect on growth of the *Lb. brevis* beer-spoiling strain, as the strains were not able to grow after addition of the phages even after 32 h of exposure (Figure 4A, B and C). Lytic phages 521B, 3-521 and SAC12B were shown to affect growth of *Lb. brevis* strains UCCLBBS449, UCCLB95 and RIBM 2-56, respectively (Figure 4A, B and C). Lytic phages isolated as part of this study adsorb onto *Lb. brevis* beer-spoiling strains and in most cases negatively affect their growth. However, they failed to form visible plaques on the beer-spoiling strains, thus we aimed to evaluate the potential of these phages to propagate within the host cell. Plaque assays after the enrichment did not reveal phage propagation and multiplication within the host (data not shown) as the phage titer did not increase after the incubation period. However, while they did not infect the beer-spoiling strains, they did appear to affect the growth rate of *Lb. brevis* beer-spoiling strains. It did not seem that the phages propagate lytically on these strains, but the negative impact of phages on certain *Lb. brevis* beer-spoiling strains might be caused by a high-multiplicity phage adsorption and/or by the action of exogenous phage-encoded lysin on the bacteria [39].

The negative impact of phages on *Lb. brevis* strains growth presents potential for the application of such entities to control bacterial spoilage of beer. In the experiment presented above, the number of cells to which phages were added is high and most certainly exceeds levels encountered during the beer fermentation process.

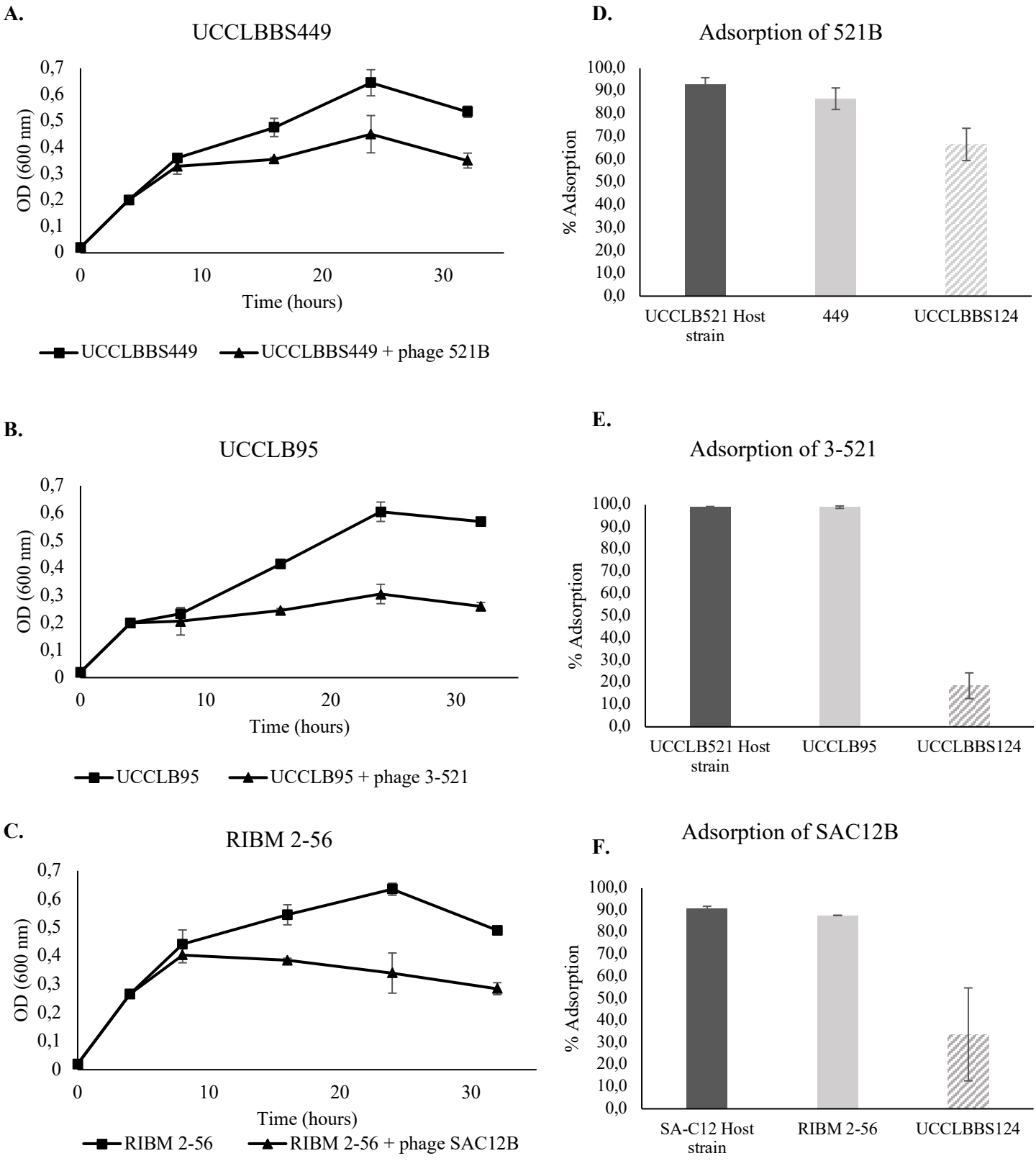


Figure 2. (**A**–**C**) Growth of *Lb. brevis* beer-spoiling strains when challenged with lytic phages (MOI = 1 when the culture reached an OD_{600nm} of 0.2). A culture of the bacterial strain where no phage was added was used as a control. (**D**–**F**) Adsorption assays of lytic phages 521B, 3-521 and SAC12B onto *Lb. brevis* strains. Respective *Lb. brevis* host strains were used as positive control while *Lb. brevis* UCCLBBS124 was used as a negative control.

5. Conclusions

In this study, the isolation and characterization of five *Lb. brevis*-infecting phages considerably increases knowledge of the genetic and morphological diversity of *Lb. brevis* phages, as only one lytic *Lb. brevis* phage had been isolated to date. Despite their shared host species, they show a high level of genetic diversity. Their morphology and genome size vary considerably with the largest phage isolated against *Lb. brevis* being that of 3-521 with a genome size of 141 kb. Some of the phages isolated as part of this study showed activity against *Lb. brevis* beer-spoiling strains preventing them from growing optimally, thus providing new approaches to control bacterial spoilage of beer. Indeed, such phages may be used in the future during beer fermentation to control and restrain growth of spoilage bacteria by bioremediation.

Interestingly, *Lb. brevis* bacteria are widely present in fermented foods, silage or microbiota; however, phages against this microorganism were not ubiquitously and easily isolated. Out of 200 environmental samples screened, only five lytic *Lb. brevis* phages were retrieved and only from Irish wastewater samples, indicating the rarity and the hurdle of isolating such entities. The study of *Lb. brevis* phages is in its infancy and many questions remain to be answered regarding their mode of action and their evolutionary strategies. For this reason, screening of phage populations from different sources (such as dairy fermentations, especially cheeses) is necessary to provide sufficient knowledge for their potential use in bioremediation applications.

6. References

1. Vos, P.; Garrity, G.; Jones, D.; Krieg, N.R.; Ludwig, W.; Rainey, F.A.; Schleifer, K.-H.; Whitman, W. Bergey's manual of systematic bacteriology: volume 3: The *Firmicutes.* **2011**, *3*, 464–511.
2. Salvetti, E.; Torriani, S.; Felis, G.E. The genus *Lactobacillus*: a taxonomic update. *Probiotics and Antimicrobial Proteins* **2012**, *4*, 217–226, doi:10.1007/s12602-012-9117-8.
3. Felis, G.E.; Dellaglio, F. Taxonomy of *Lactobacilli* and *Bifidobacteria*. *Current Issues in Intestinal Microbiology* **2007**, *8*, 44–61.
4. Bergsveinson, J.; Pittet, V.; Ewen, E.; Baecker, N.; Ziola, B. Genome sequence of rapid beer-spoiling isolate *Lactobacillus brevis* BSO 464. *Genome Announcements* **2015**, *3*, e01411–e01415, doi:10.1128/genomeA.01411-15.
5. Sakamoto, K.; Konings, W.N. Beer spoilage bacteria and hop resistance. *International Journal of Food Microbiology.* **2003**, *89*, 105–124.
6. Suzuki, K.; Iijima, K.; Sakamoto, K.; Sami, M.; Yamashita, H. A review of hop resistance in beer spoilage lactic acid bacteria. *Journal of the Institute of Brewing* **2006**, *112*, 173–191.
7. Vaughan, A.; O'Sullivan, T.; van Sinderen, D. Enhancing the microbiological stability of malt and beer—A review. *Journal of the Institute of Brewing* **2005**, *111*, 355–371, doi:10.1002/j.2050-0416.2005.tb00221.x.
8. Bokulich, N.A.; Bamforth, C.W. The microbiology of malting and brewing. *Microbiology Molecular Biology Reviews: MMBR* **2013**, *77*, 157–172, doi:10.1128/MMBR.00060-12.

9. Back, W.; Leibhard, M.; Bohak, I. Flash pasteurisation-membrane filtration. Biological security in comparison. *Brauwelt International* **1990**, 12, 42-49.

10. Davidson, P.M.; Harrison, M.A. Resistance and adaptation to food antimicrobials, sanitizers, and other process controls. *Food Technology* **2002**, *56*, 69–78.

11. Basanta, A.; Sánchez, J.; Gómez-Sala, B.; Herranz, C.; Hernández, P.E.; Cintas, L.M. Antimicrobial activity of *Enterococcus faecium* L50, a strain producing enterocins L50 (L50A and L50B), P and Q, against beer-spoilage lactic acid bacteria in broth, wort (hopped and unhopped), and alcoholic and non-alcoholic lager beers. *International Journal of Food Microbiology* **2008**, *125*, 293–307.

12. Gil, G.; del Monaco, S.; Cerrutti, P.; Galvagno, M. Selective antimicrobial activity of chitosan on beer spoilage bacteria and brewing yeasts. *Biotechnology Letters* **2004**, *26*, 569–574.

13. Vaughan, A.; Rouse, S.; van Sinderen, D. Investigating the antimicrobial efficacy of a Lactococcal bacteriocin for the development of microbiologically stable beer. *Journal of the Institute of Brewing* **2004**, *110*, 181–188, doi:10.1002/j.2050–0416.2004.tb00200.x.

14. Mahony, J.; McAuliffe, O.; Ross, R.P.; van Sinderen, D. Bacteriophages as biocontrol agents of food pathogens. *Current Opinion in Biotechnology* **2011**, *22*, 157–163.

15. Coffey, B.; Mills, S.; Coffey, A.; McAuliffe, O.; Ross, R.P. Phage and their lysins as biocontrol agents for food safety applications. *Annual Review of Food Science and Technology* **2010**, *1*, 449–468.

16. Endersen, L.; O'Mahony, J.; Hill, C.; Ross, R.P.; McAuliffe, O.; Coffey, A. phage therapy in the food industry. *Annual Review of Food Science and Technology* **2014**, *5*, 327–349, doi:10.1146/annurev-food-030713–092415.

17. Deasy, T.; Mahony, J.; Neve, H.; Heller, K.J.; van Sinderen, D. Isolation of a virulent *Lactobacillus brevis* phage and its application in the control of beer spoilage. *Journal of Food Protection* **2011**, *74*, 2157–2161.

18. Brüssow, H.; Desiere, F. Comparative phage genomics and the evolution of *Siphoviridae*: insights from dairy phages. *Molecular Microbiology.* **2001**, *39*, 213–222.

19. Jang, S.H.; Yoon, B.H.; Chang, H.I. Complete nucleotide sequence of the temperate bacteriophage LBR48, a new member of the family *Myoviridae*. *Archives of Virology* **2011**, *156*, 319–322.

20. Svensson, U.; Christiansson, A. Methods for phage monitoring. *Bulletin of the International Dairy Federation* **1991**, *263*, 29–39.

21. Sambrook, J.; Russell, D.W. Purification of bacteriophage λ particles by isopycnic centrifugation through CsCl gradients. *Cold Spring Harbor Protocols* **2006**, *1*, pdb-prot3968.

22. Casey, E.; Mahony, J.; Neve, H.; Noben, J.-P.; Dal Bello, F.; van Sinderen, D. Novel phage group infecting *Lactobacillus delbrueckii* subsp. *lactis*, as revealed by genomic and proteomic analysis of bacteriophage Ldl1. *Applied and Environmental Microbiology* **2015**, *81*, 1319–1326, doi:10.1128/aem.03413–14.

23. Lugli, G.A.; Milani, C.; Mancabelli, L.; van Sinderen, D.; Ventura, M. MEGAnnotator: a user-friendly pipeline for microbial genomes assembly and annotation. *FEMS Microbiology Letters* **2016**, *363*, doi:10.1093/femsle/fnw049.

24. Lowe, T.M.; Eddy, S.R. tRNAscan-SE: a program for improved detection of transfer RNA genes in genomic sequence. *Nucleic Acids Research* **1997**, *25*, 955–964.

25. Hyatt, D.; Chen, G.-L.; LoCascio, P.F.; Land, M.L.; Larimer, F.W.; Hauser, L.J. Prodigal: prokaryotic gene recognition and translation initiation site identification. *BMC Bioinformatics* **2010**, *11*, 119, doi:10.1186/1471–2105–11–119.

26. Altschul, S.F.; Madden, T.L.; Schaffer, A.A.; Zhang, J.; Zhang, Z.; Miller, W.; Lipman, D.J. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* **1997**, *25*, 3389–3402.

27. Soding, J.; Biegert, A.; Lupas, A.N. The HHpred interactive server for protein homology detection and structure prediction. *Nucleic Acids Research* **2005**, *33*, W244–W248, doi:10.1093/nar/gki408.

28. Rutherford, K.; Parkhill, J.; Crook, J.; Horsnell, T.; Rice, P.; Rajandream, M.A.; Barrell, B. Artemis: sequence visualization and annotation. *Bioinformatics* **2000**, *16*, 944–945.

29. Holtappels M., Vrancken K., Schoofs H., Deckers T., Remans T., Noben J.-P., Valcke R. A comparative proteome analysis reveals flagellin, chemotaxis regulated proteins and amylovoran to be involved in virulence differences between *Erwinia amylovora* strains. *Journal of Proteomics* **2015**, *123*, 54–69.

30. Thompson, J.D.; Gibson, T.J.; Higgins, D.G. Multiple sequence alignment using ClustalW and ClustalX. *Current Protocols in Bioinformatics* **2003**, *1*, 2–3.

31. Kumar, S.; Nei, M.; Dudley, J.; Tamura, K. MEGA: a biologist-centric software for evolutionary analysis of DNA and protein sequences. *Briefings in Bioinformatics* **2008**, *9*, 299–306, doi:10.1093/bib/bbn017.

32. Collins, B.; Bebeacua, C.; Mahony, J.; Blangy, S.; Douillard, F.P.; Veesler, D.; Cambillau, C.; van Sinderen, D. Structure and functional analysis of the host recognition device of lactococcal phage Tuc2009. *Journal of Virology* **2013**, *87*, 8429–8440, doi:10.1128/jvi.00907–13.

33. Matthews, R.E.F.; Maurin, J. Classification and nomenclature of viruses. Fourth report of the international committee on taxonomy of viruses. *Intervirology.* **1982**, 17, 199pp.

34. Zago, M.; Scaltriti, E.; Rossetti, L.; Guffanti, A.; Armiento, A.; Fornasari, M.E.; Grolli, S.; Carminati, D.; Brini, E.; Pavan, P.; et al. Characterization of the genome of the dairy *Lactobacillus helveticus* bacteriophage ΦAQ113. *Applied and Environmental Microbiology* **2013**, *79*, 4712–4718, doi:10.1128/aem.00620–13.

35. Tuohimaa, A.; Riipinen, K.-A.; Brandt, K.; Alatossava, T. The genome of the virulent phage Lc-Nu of probiotic *Lactobacillus rhamnosus*, and comparative genomics with *Lactobacillus casei* phages. *Archives of Virology* **2006**, *151*, 947.

36. Alemayehu, D.; Ross, R.P.; O'Sullivan, O.; Coffey, A.; Stanton, C.; Fitzgerald, G.F.; McAuliffe, O. Genome of a virulent bacteriophage Lb338–1 that lyses the probiotic *Lactobacillus paracasei* cheese strain. *Gene* **2009**, *448*, 29–39.

37. Chibani-Chennoufi, S.; Dillmann, M.-L.; Marvin-Guy, L.; Rami-Shojaei, S.; Brüssow, H. *Lactobacillus plantarum* bacteriophage LP65: a new member of the SPO1-like genus of the family *Myoviridae*. *Journal of Bacteriology* **2004**, *186*, 7069–7083, doi:10.1128/jb.186.21.7069–7083.2004.

38. Rossmann, M.G.; Mesyanzhinov, V.V.; Arisaka, F.; Leiman, P.G. The bacteriophage T4 DNA injection machine. *Current Opinion in Structural Biology* **2004**, *14*, 171–180, doi:https://doi.org/10.1016/j.sbi.2004.02.001.

39. Abedon, S.T. Lysis from without. *Bacteriophage* **2011**, *1*, 46–49, doi:10.4161/bact.1.1.13980.

Chapter VI

Biodiversity and classification of phages infecting *Lactobacillus brevis*

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Horst Neve and Charles M. Franz (Max Rubner Institut, Germany) carried out electron microscopy analysis, Jean-Paul Noben (Hasselt University, Belgium) performed mass-spectrometry analysis.

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1. Abstract

Lactobacillus brevis is a lactic acid bacterium that is known as a food and beverage spoilage organism, and more specifically as a beer-spoiler. Phages of *Lb. brevis* have been described, but very limited data is available regarding temperate phages of *Lb. brevis*. Temperate phages may exert benefits to the host, while they may also be employed to combat beer spoilage. The current study reports on the incidence of prophage sequences present in nineteen distinct *Lb. brevis* genomes. Prophage induction was evaluated using mitomycin C exposure followed by genome targeted-PCR, electron microscopy and structural proteome analysis. The morphological and genome sequence analyses revealed significant diversity among *Lb. brevis* (pro)phages, which appear to be dominated by members of the *Myoviridae* phage family. Based on this analysis, we propose a classification of *Lactobacillus brevis* phages into five groups.

2. Introduction

The most prevalent spoilage bacteria associated with beer fermentations are members of the lactic acid bacteria (LAB), which account for approximately 70 % of all microbial spoilage incidents [1-3]. *Lactobacillus brevis* strains are frequently reported to be the cause of such spoilage events as they have developed mechanisms to survive and grow in beer [4]. Strains of this species can be found on raw materials used in breweries and represent a major microbial contaminant during the production and storage of beer. Bacterial strains have acquired features throughout evolution allowing them to become more robust including resistance to virulent bacteriophages [5-7]. Moreover, the chromosomes of the majority of LAB are known to harbor one or more prophage regions and their presence may benefit the host by providing resistance attributes to its environment [8]. Upon induction, these phages enter the lytic cycle leading to bacterial cell death and formation of intact phage particles [9], and therefore it is relevant to examine their presence and functionality [10]. Prophages of LAB have been widely studied and particularly so in *Lactococcus lactis* [10] and *Streptococcus thermophilus* [11]. Temperate phages of the genus *Lactobacillus* have been sequenced and are primarily classified as members of the *Siphoviridae* family [12], being characterized by a non-contractile tail [13]. Successful induction of such prophages has been reported using UV, thermal exposure or treatment with DNA damaging/antimicrobial compounds such as mitomycin C and bacteriocins [14-17].

While the presence of prophages in LAB genomes is widely described, only a small number of studies have investigated their integrity and inducibility within these strains [18,19]. In the case of beer spoilage by *Lb. brevis,* prophage induction represents a beneficial attribute that could be harnessed as an alternative to chemical compounds in the eradication of spoilage bacteria. Indeed, induction of prophages is expected to cause bacterial cell lysis thus avoiding the

development of spoilage organisms in the brewing industry. Although prophages represent a reservoir for adaptation, prophages of *Lb. brevis* are currently poorly described. To date, the genome of a single temperate phage of *Lb. brevis*, namely LBR48, has been sequenced and characterized. The prophage was induced from *Lb. brevis* strain C30 using mitomycin C. LBR48 is 48 Kb long containing 90 putative Open Reading Frames (ORFs) and was classified as a member of the *Myoviridae* family. Interestingly, the LBR48 genome does not show protein sequence similarity with any other *Lactobacillus* phages [20].

Advances in genome sequencing technologies have considerably increased the number of available bacterial genomes sequences, improving also the detection capability of prophage-encoded regions within these genomes. In the current study, nineteen publically available *Lb. brevis* genome sequences were used to identify prophage-encoding regions. Five of these strains were available for prophage induction trials, allowing the assessment of the ability of these temperate phages to form intact phage particles and cause host cell lysis. The diversity of *Lb. brevis* temperate phages was studied to establish relatedness between *Lb. brevis* phages (temperate and virulent), resulting in a proposed classification scheme of *Lb. brevis* phages based on morphology and genome sequence data.

3. Materials and Methods

3.1. Bacterial strains and growth conditions

Lb. brevis strains used in this study are detailed in Table 1. Bacteria were cultured in MRS broth (Oxoid Ltd., Hampshire, UK) at 30 ˚C.

Table 1. *Lactobacillus brevis* strains used in this study and prophage regions predicted by PHASTER.

Strain name (Isolation source)	**Genbank accession**	**No. prophage regions detected by PHASTER**			**Reference**
		Intact	Questionable	Incomplete	
100D8* (Silage)	CP015338	1	1	1	
ATCC 367 (Silage)	CP000416	1	0	0	[21]
BDGP6* (*Drosophila's* gut)	CP024635	4	1	2	
KB290* (Fermented vegetable)	AP012167	2	0	1	[22]
NPS-QW-145* (Kimchi)	CP015398	0	1	2	[23]
NCTC13768* (Unknown)	LS483405	0	1	0	
SA-C12 (Silage)	CP031185	2	1	1	[24]
SRCM101106* (Food)	CP021674	3	0	1	
SRCM101174* (Food)	CP021479	3	0	1	
TMW 1.2108* (Beer)	CP019734	2	0	0	[25]
TMW 1.2111* (Beer)	CP019743	2	0	0	[25]
TMW 1.2112* (Beer)	CP016797	1	0	0	[25]
TMW 1.2113* (Brewery surface)	CP019750	2	0	0	[25]
UCCLB521 (Brewery surface)	CP031208	0	0	2	[24]
UCCLB556 (Brewery surface)	CP031174	0	1	0	[24]
UCCLB95 (Beer)	CP031182	1	0	2	[24]
UCCLBBS124 (Beer)	CP031169	1	0	1	[24]
UCCLBBS449 (Beer)	CP031198	1	0	3	[24]
ZLB004* (Pig feces)	CP021456	1	0	0	

Note: *These strains were not tested for prophage induction.

3.2. Prophage identification and genome annotation

PHASTER (PHAge Search Tool Enhanced Release) [26,27] was used to screen for prophage-specifying DNA regions within the genome of available *Lb. brevis* strains. Intact prophages were manually annotated to confirm the presence of all expected genes required to produce a fully functional phage particle including genes encoding proteins associated with replication functions (e.g. replisome, DNA-binding proteins) packaging (small and large terminases), morphogenesis (e.g. capsid and tail) and lysis (holin and lysin). Genes required for lysogeny maintenance (e.g. integrase and repressor) were also investigated [10]. Integration sites of prophages (attL and attR) were recorded and are presented in Supplementary Table S1. Prophage genome sequences were retrieved and annotated as previously described [10]. Briefly, ORF prediction was performed using the Prodigal prediction software [28] and confirmed using BLASTX alignments [29]. The automatic annotations were refined using Artemis v16.0.0 to allow visual inspection of ORF predictions [30]. Moreover, BLASTP [31] and HHPred [32] analyses were performed to assign functional annotations to the predicted ORFs. Transfer RNA (tRNA) genes were predicted using tRNA-scan-SE v2.0 [33] and added manually using Artemis.

3.3. Phylogenetic analysis

A proteomic tree was constructed using a concatenated amino acid sequence of all encoded proteins for each of the *Lb. brevis* phages sequenced to date. The concatenated amino acid sequence begins with the ORF encoding the small terminase subunit (TerS) [34]. The concatenated sequences were aligned using ClustalW [35]. The phylogenetic tree was

constructed using the neighbour-joining method and bootstrapped employing 1,000 replicates. The final tree was visualized using MEGA7 [36].

3.4. Genome characterization and organization

Following phylogenetic analysis, genomes of representative temperate phages were selected for further analysis where overall genome content and organization were studied. The genome content and architecture were analyzed based on the observation of the modular organization of the genomes into the following modules: packaging, morphogenesis, lysis, lysogeny and replication. Protein sequences of representative phages were compared using all-against-all, bi-directional BLAST alignments [29]. An alignment cut-off E-value of 0.0001, and a similarity cut-off level of at least 30 % amino acid identity across 80 % of the sequence length was applied. This analysis allowed the amino acid similarity assignment between temperate phage genomes and the study of the overall genome similarity/diversity among *Lb. brevis* phages.

3.5. Prophage induction trials

To assess the functionality of the identified prophage-encoding regions, prophage induction trials were performed using the DNA crosslinking agent mitomycin C (MitC). For this assay, five *Lb. brevis* strains were available for testing: ATCC367, SA-C12, UCCLB95, UCCLBBS124 and UCCLBBS449. 10 mL MRS broth was inoculated with 2 % of a fresh overnight culture of the relevant bacterial strain. Cultures were incubated at 30 ˚C until an OD_{600nm} of 0.1, 0.2 or 0.3 was reached at which point 0.1, 0.2 or 0.3 µg/mL MitC (final concentration) was added. A high concentration of 2 µg/mL MitC (final concentration) was

applied when cells reached an OD_{600nm} of 0.2. This was performed to ascertain if cell lysis occurred due to prophage induction or lethal MitC toxicity. Indeed, MitC levels between 0.1 and 0.3 µg/mL are relatively low and when induction occurs it would be considered genuine prophage-induction mediated cell lysis. Conversely, higher concentrations of MitC (e.g. 2 µg/mL MitC) are expected to cause growth arrest and cell death due to acute toxicity. Cultures were maintained at room temperature for 30 h during which the OD_{600nm} was recorded at 60 min time intervals for the first 8 h and then at 15, 20, 25 and 30 h.

Using the same protocol as MitC prophage induction, potential induction regimes using other stress-inducing chemicals (2 % v/v NaOH, 2 % v/v formic acid or 2 % v/v acetic acid) or physical treatment (direct UV light exposure (254 nm) of 30 min at a distance of 5 cm on a 1 cm culture suspension) were also assessed [37,38].

3.6. Validation of prophage induction by DNA sequencing and electron microscopy

To validate prophage induction from *Lb. brevis* strains cited above, the DNA derived from cell-free supernatants was extracted and sequenced. Phage DNA was isolated using a previously described phage DNA extraction protocol [39]. Primers were designed based on prophage sequences in order to confirm the presence of induced prophages in the cell-free supernatants of MitC-treated cultures (Table 2).

To validate prophage induction using electron microscopy, MitC-treated cultures were harvested by centrifugation at 5000 × *g* for 10 min, after which the supernatant was filtered twice through a 0.45 µm filter prior to electron microscopy analysis. Transmission electron microscopy of the samples was performed as previously described [34]. Negative staining was performed using 2 % (w/v) uranyl acetate on freshly prepared ultrathin carbon films. Grids were analyzed in a Tecnai 10 transmission electron microscope (FEI Thermo Fisher Scientific,

Eindhoven, The Netherlands) at an acceleration voltage of 80 kV. Micrographs were taken with a MegaView G2 charge-coupled device camera (Emsis, Muenster, Germany).

Table 2. Primer sequences used to amplify specific regions of induced prophages.

Primer name	Sequence (5' - 3')	Target
TPMB095F	gaatcctggcgataactag	TMP region of TPMB095 prophage
TPMB095R	gtggcaccagcgtatcgaa	
TPMB449F	cttcaatcaccatctaag	TMP region of TPMB449 prophage
TPMB449R	gactatcagcaatcgcatt	
TPMB124F	ggttgccttctgcaagg	TMP region of TPMB124 prophage
TPMB124R	gttaaggaggtgtgactaa	
TPSAC12-1F	gtatggcaatcaagcacac	TMP region of TPSAC12-1 prophage
TPSAC12-1R	tgccatctcattggtgac	
TPSAC12-2F	gacttcataacagcaat	TMP region of TPSAC12-2 prophage
TPSAC12-2R	ggtccactaatggcgac	
TPATCC367F	ggaaccttgtcgttcata	TMP region of TPATCC367 prophage
TPATCC367R	gcagcttctctagcaccac	

3.7. Phage structural proteome and mass-spectrometry

An aliquot (30 μL) of CsCl-purified phage sample was mixed with 10 μL of SDS loading buffer containing 50 mM β-mercaptoethanol. The structural protein profile was generated by standard Tris-glycine sodium dodecyl sulfate (SDS)–12 % polyacrylamide gel electrophoresis (PAGE). Gel slices were then excised, trypsinized, and analyzed using electrospray ionization tandem mass spectrometry (ESI-MS/MS), as previously described [40,41].

3.8. Genome accession numbers

Lb. brevis 100D8: CP015338, *Lb. brevis* ATCC 367: CP000416, *Lb. brevis* BDGP6: CP024635, *Lb. brevis* KB290: AP012167, *Lb. brevis* NCTC13768: LS483405, *Lb. brevis* NPS-QW-145: CP015398, *Lb. brevis* SA-C12: CP031185, *Lb. brevis* SRCM101106: CP021674, *Lb. brevis* SRCM101174: CP021479, *Lb. brevis* TMW 1.2108: CP019734, *Lb. brevis* TMW

1.2111: CP019743, *Lb. brevis* TMW 1.2112: CP016797, *Lb. brevis* TMW 1.2113: CP019750, *Lb. brevis* UCCLB521: CP031208, *Lb. brevis* UCCLB556: CP031174, *Lb. brevis* UCCLB95: CP031182, *Lb. brevis* UCCLBBS124: CP031169, *Lb. brevis* UCCLBBS449: CP031198, *Lb. brevis* ZLB004: CP021456, *Lb. brevis* phage 3-521: MK504444, *Lb. brevis* phage 521B: MK504443, *Lb. brevis* phage 3-SAC12: MK504442, *Lb. brevis* phage SAC12B: MK504446, *Lb. brevis* phage ATCCB: MK504445, *Lb. brevis* phage SA-C12: KU052488 and *Lb. brevis* phage LBR48: GU967410.

4. Results and Discussion

4.1. Prophage identification and characterization

Lb. brevis contamination of beer is a consistent threat for breweries as its survival and growth in beer cause spoilage thus leading to product withdrawals and associated negative financial consequences. The food and beverage industries aim to apply more natural, environmentally friendly and safer food preservation methods. Phage bioremediation or sanitation may represent a potential method to prevent bacterial growth and spoilage. LAB strains are known to carry prophage regions which, upon induction, may cause phage particle release and bacterial cell death.

The genomes of nineteen completely sequenced *Lb. brevis* strains were screened for the presence of prophage-encoding regions using PHASTER (Table 1). Of the nineteen bacterial strains of *Lb. brevis*, twenty-seven intact prophage sequences were predicted ranging from one to four prophage regions per strain. Twenty-three partial (marked as questionable and incomplete according to PHASTER analysis [26,27]) prophage regions were also identified among these strains. Four *Lb. brevis* strains do not appear to harbor intact prophage regions in their sequences, yet are predicted to carry remnant prophage sequences. The high number of prophage regions (intact and partial) identified shows that prophages are a very common occurrence in *Lb. brevis* genomes. Predicted intact prophage regions were manually examined and extracted for further analysis (general genome features are detailed in Table 3). Among the fifteen *Lb. brevis* strains whose genomes contain predicted intact prophage regions, *Lb. brevis* BDGP6 presented the highest number with four such prophage regions ranging in size from 42 to 74 Kb (Table 3). Interestingly, *Lb. brevis* strains SRCM101106 and SRCM101174 harbor an identical prophage, designated TPSRCM101106-3 and TPSRCM101174-3, respectively (100 % nucleotide similarity across the full length of their genomes), this can be explained by

the similarity between the two *Lb. brevis* host strains which share 99.8 % nt sequence identity across 94 % of their genomes. Similarly, *Lb. brevis* TMW1.2108 harbors two prophage regions that are nearly identical to those present in the genome of TMW1.2111 (TPTMW1-4 and TPTMW1-6 and; TPTMW1-5 and TPTMW1-7 bearing 99.99 % and 100 % nucleotide similarity, respectively) and in *Lb. brevis* TMW1.2112 and TMW1.2113 sharing a 99.99 % nucleotide identical prophage region (TPTMW1-1 and TPTMW1-2, respectively). These results are perhaps unsurprising as these *Lb. brevis* strains are more than 99 % identical in their genome sequences but mostly differ in their plasmid content [24]. The similarities observed in prophage content for some *Lb. brevis* strains might also be due to the common environment from which these strains have been isolated, i.e. food (South Korea) for *Lb. brevis* SRCM101106 and SRCM101174, and beer (Germany) for *Lb. brevis* TMW1.2108, TMW1.2111, TMW1.2112 and TMW1.2113.

Integration sites (*attL* and *attR* sites) of prophages were identified and are presented in Supplementary Table S1. Diversity is observed among these integration sites; however, closely related prophages share the same sites, such as TPSRCM101106-3 and TPSRCM101174-3 or TPBDGP6-1 and TPSAC12-2. Several integration site sequences seem to be shared by certain prophages such as 5'-aaatcctgtactctcctt-3' which was identified in five genomes. The presence of such sites acts as indicators of potential phage integration and movement, which is important in the context of phage and host evolution, fitness and adaptability in its ecological niche.

Temperate phages can be detrimental for the host if, for example, they switch to a lytic state but they can also be beneficial to the host by carrying genes that will help the strain survive in its environment. These genes may encode phage-resistance systems such as Abortive infection (Abi) system or Superinfection exclusion (Sie) proteins as previously identified [42]. Abi systems were shown to block phage multiplication leading to the release of few particles and the death of the infected cells allowing the survival of the bacterial overall population [43]. Sie

proteins were identified in *Lactococcus lactis* strains where they were shown to prevent DNA injection of certain phages without affecting phage adsorption [42]. Of the 19 *Lb. brevis* strains studied, nine strains were predicted to carry potential prophage-encoded Sie systems (Table 3). Meanwhile six were predicted to carry potential prophage-encoded Abi system (Table 3) such as observed in the prophages TPMB124 and TPSRCM101174-3 based on BlastN analysis. These two prophage regions encode the same potential Abi system which in turn exhibits similarity to the AbiL system identified in *Lactococcus lactis* [44]. The presence of these potential resistance systems is predicted to confer resistance to the host against phage infection thus increasing the overall host fitness. The absence of such systems in *Lb. brevis* UCCLB521 or SA-C12 may explain their higher sensitivity to lytic phage infection. Conversely, the presence of potential phage-resistance systems (i.e. Abi) in the prophage of the beer-spoiling *Lb. brevis* strain UCCLBBS124 could explain its resistance against lytic phage infection [45] (Chapter V, Table 2).

Table 3. General genome features of *Lactobacillus brevis* intact prophage regions and virulent phages.

Strain name	Phages	Genome size (bp)	ORFs No	GC (%)	Potential Abi system	Potential Sie system
Lysogen	**Prophages**					
100D8*	TP100D8	41,993	62	44.6	/	/
ATCC 367	TPATCC367	56,030	78	43.4	/	/
BDGP6*	TPBDGP6-1	41,938	63	42.1	/	/
	TPBDGP6-2	74,412	100	43.6	/	1
	TPBDGP6-3	46,262	71	44.9	1	/
	TPBDGP6-4	44,732	59	40.8	/	1
KB290*	TPKB290-1	43,639	64	44.5	/	/
	TPKB290-2	47,873	70	44.3	/	/
SA-C12	TPSAC12-1	57,452	83	43.5	/	/
	TPSAC12-2	38,492	50	43.4	/	/
SRCM101106*	TPSRCM101106-1	48,237	71	43.8	/	1
	TPSRCM101106-2	69,528	92	44.2	/	1
	TPSRCM101106-3	49,505	74	43.7	1	/
SRCM101174*	TPSRCM101174-1	39,697	47	42.2	/	/
	TPSRCM101174-2	70,358	97	44.3	/	/
	TPSRCM101174-3	49,505	74	43.7	1	/
TMW 1.2108*	TPTMW1-4	49,253	74	43.8	1	1
	TPTMW1-5	40,616	60	41.5	/	1
TMW 1.2111*	TPTMW1-6	49,251	77	43.8	1	1
	TPTMW1-7	40,616	61	41.5	/	1
TMW 1.2112*	TPTMW1-1	51,644	72	43.6	/	1
TMW 1.2113*	TPTMW1-2	51,643	74	43.6	/	1
	TPTMW1-3	51,532	76	43.5	/	1
UCCLB95	TPMB095	66,077	80	44.1	/	/
UCCLBBS124	TPMB124	46,131	73	43.7	1	1
UCCLBBS449	TPMB449	50,224	72	43.8	/	1
ZLB004*	TPZLB004	68,360	92	43.3	/	/
Host	**Virulent phages**					
UCCLB521	3-521	140,816	155	36.9	/	/
UCCLB521	521B	136,442	188	32.3	/	/
SA-C12	SAC12B	136,608	191	32.4	/	/
SA-C12	3-SAC12	41,292	61	40.0	/	/
ATTC 367	ATCCB	80,538	96	30.8	/	/

Note: *These strains were not tested for prophage induction.

4.2. Prophage inductions

Small scale prophage induction trials were performed for *Lb. brevis* strain UCCLBBS124. These trials were furthermore applied to ascertain the accuracy of the bioinformatic predictions of likely intact prophages within these genomes. MitC exposure was employed at sub-lethal (0.1-0.3 µg/mL) or lethal concentrations (2 µg/mL) to distinguish between genuine prophage induction-mediated cell lysis and cell death due to acute MitC toxicity. Prophage induction trials with *Lb. brevis* UCCLBBS124 generated different induction profiles (Figure 1), where both sub-lethal and lethal doses of MitC caused cell lysis, indicating that prophage induction may have occurred. Using the lowest concentration of MitC required for prophage induction in UCCLBBS124, phage inductions in other *Lb. brevis* strains were performed as described: cultures were grown and MitC was added at a sub-lethal concentration of 0.1 µg/mL MitC when the culture reached an OD_{600nm} of 0.1. *Lb. brevis* strains ATCC 367, SA-C12, UCCLB95, UCCLBBS124 and UCCLBBS449 exhibited lysis upon addition of 0.1 µg/mL MitC indicating prophage induction, cell lysis and phage particle release.

Prophage inductions using 2 % v/v formic acid or UV light exposure for 30 min were successful as indicated by PCR validation after phage DNA isolation. However, no apparent prophage induction was observed when acetic acid (2 % v/v) or sodium hydroxide (2 % v/v) was used as a potential inducing agent (data not shown). While in some cases formic acid and UV treatment appeared to cause cell death, phage particles were not observed by electron microscopy despite positive PCR assay results. This may be due to the detection limit of the microscopy approach limiting the ability to visualize the particles or to the poor growth characteristics of certain cultures (which appear to lyse) thus representing bacteriostasis rather than lysis.

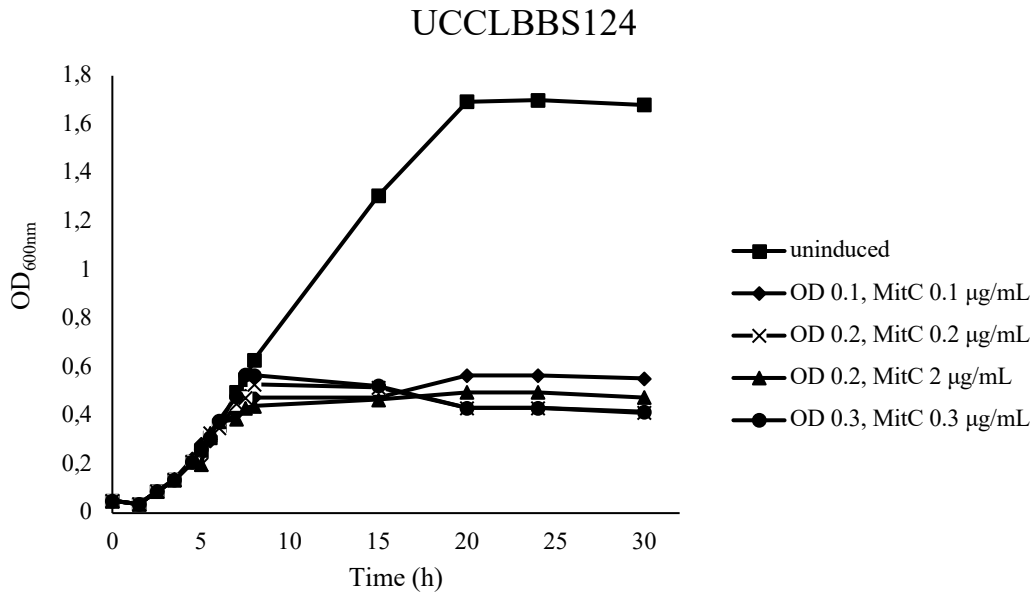


Figure 1. MitC induction profiles of *Lb. brevis* UCCLBBS124. Different concentrations of the inducing agent MitC: 0.1, 0.2, 0.3 and 2 µg/mL were added to the culture after growth of the bacterial strain at an OD_{600nm} of 0.1, 0.2, and 0.3. An uninduced culture was included as a control (indicated as 'uninduced'). Growth curves were performed in triplicate and the average of those measurements is displayed in the graph above.

4.3. Validation of prophage induction

The five *Lb. brevis* strains that were available for testing showed lysis following induction using 0.1 µg/mL MitC. To further validate that the observed lysis corresponds to phage particle release, filtered cell free supernatants of the induced cultures were analyzed by (i) electron microscopy, (ii) DNA extraction to confirm the prophage sequence using a PCR-based technique, and (iii) structural proteome analysis using mass spectrometry. This was also performed to match a specific prophage sequence to virion morphology in cases where more than one intact prophage region was identified in a bacterial strain such as *Lb. brevis* SA-C12, which harbors two predicted prophage regions (Table 3).

Prophage induction attempts for five *Lb. brevis* strains, i.e. UCCLBBS124, ATCC 367, SA-C12, UCCLBBS449 and UCCLB95, resulted in the identification of intact virions which in one case was shown to bear morphological characteristics of *Siphoviridae* phages: TPSAC12-2 (induced from SA-C12) which is characterized by a long thin, non-contractile tail (Figure 2 and Table 3), while in three cases *Myoviridae* phages were obtained: TPMB124 (induced from UCCLBBS124), TPATCC367 (induced from ATCC 367), TPMB449 (induced from UCCLBBS449) characterized by a decorated contractile-tailed phage (Figure 2 and Table 3). No phage particles were visible following induction of strain UCCLB95, which indicates that the prophage may not be inducible (to produce detectable virions) under the tested conditions despite causing cell lysis highlighting the limitations of in silico analysis. Such predictions have previously been shown to require manual evaluation and assessment in lactococcal prophages thus necessitating induction trials employing various chemical agents and/or UV treatment [10].

In parallel, PCRs targeting the gene encoding the Tape Measure Protein (TMP) of the temperate phages (Table 2) as well as phage structural proteome analysis using mass

spectrometry (Table 4 and Figure 4) further validated the induction and prophage identification findings. Predicted proteins encoded within the morphogenesis module of the phage genomes of TPSAC12-2, TPMB124, TPATCC367 and TPMB449 were confirmed as structural proteins. Tail proteins, the major capsid protein and the portal protein were identified as structural proteins of the temperate phage TPSAC12-2 confirming the induction of this prophage from strain SA-C12 (no structural proteins matching those encoded by the prophage region TPSAC12-1 were identified, suggesting that this prophage was not induced upon MitC treatment) (Table 4). The minor capsid protein and three hypothetical proteins were confirmed as structural proteins of the prophage region TPMB124 (Table 4). More than ten proteins of TPATCC367 were identified as structural proteins among which the tape measure protein, the head protein and the major capsid protein (Table 4). Structural proteins of the temperate phage TPMB449 were identified by mass spectrometry including the capsid, head and portal proteins. Some predicted structural proteins were not identified in the experimentally determined proteome, most likely due to their small size or their low relative abundance.

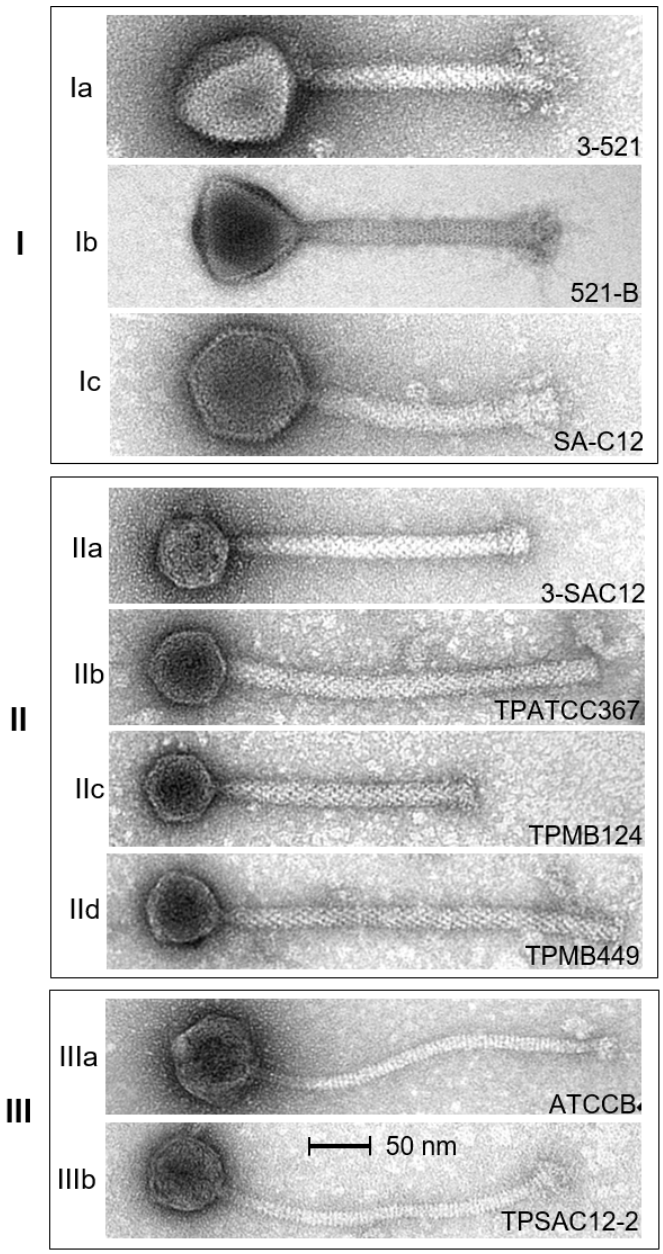


Figure 2. Electron micrographs of *Lb. brevis* phages representing morphotypes I to III. Ia: virulent phage 3-521 and Ib: virulent phage 521B [45], Ic: virulent phage SA-C12 [46]. IIa: virulent phage 3-SAC12 [45], IIb: temperate phage TPATCC367 induced from the *Lb. brevis* strain ATCC 367, IIc: temperate phage TPMB124 induced from the *Lb. brevis* strain UCCLBBS124, IId: temperate phage TPMB449 induced from the *Lb. brevis* strain UCCLBBS449. IIIa: virulent phage ATCCB [45], IIIb: temperate phage TPSAC12-2 induced from the *Lb. brevis* strain SA-C12. Temperate phages were induced from *Lb. brevis* strains using 0.1 µg/mL MitC.

Table 4. Structural proteins extracted from purified phage particles by ESI-MS/MS. A minimum of two independent unique peptides or 5 % coverage were used as threshold values.

Phage	ORF	Putative function	No. of peptides	Sequence coverage (%)
TPSAC12-2	SAC12_1335	Tail fiber protein	2	7.7
	SAC12_1361	Tail protein	5	30.2
	SAC12_1366	Major capsid protein	14	39.8
	SAC12_1368	Portal protein	4	19.8
TPMB124	UCCLBBS124_1395	Hypothetical protein	3	22.8
	UCCLBBS124_1396	Hypothetical protein	2	5.7
	UCCLBBS124_1402	Minor capsid protein	3	10.1
	UCCLBBS124_1457	Hypothetical protein	2	12.6
TPATCC367	LVIS_1073	Hypothetical protein	7	66.3
	LVIS_1080	Hypothetical protein	17	42.1
	LVIS_1081	Hypothetical protein	2	9.9
	LVIS_1084	Hypothetical protein	2	16.9
	LVIS_1085	Hypothetical protein	2	6.0
	LVIS_1087	Lysozyme	8	19.8
	LVIS_1088	Tape measure protein	24	13.2
	LVIS_1090	Hypothetical protein	6	38.9
	LVIS_1091	Hypothetical protein	7	49.4
	LVIS_1092	Hypothetical protein	12	56.4
	LVIS_1098	Major capsid protein	16	59.8
	LVIS_1099	Head protein	3	23.3
	LVIS_1102	Hypothetical protein	6	18.1
	LVIS_1128	Helicase	4	9.9
TPMB449	UCCLBBS449_1616	Hypothetical protein	5	10.6
	UCCLBBS449_1629	Hypothetical protein	3	33.3
	UCCLBBS449_1630	Structural protein	7	24.6
	UCCLBBS449_1636	Capsid protein	12	62.7
	UCCLBBS449_1638	Head protein	2	6.5
	UCCLBBS449_1641	Portal protein	3	6.2

4.4. Morphology of *Lb. brevis* phages

Electron microscopic analysis of *Lb. brevis* phages available to date were gathered, providing insights into the morphological diversity among *Lb. brevis* phages. From these analyses, three distinct morphologies were observed (Figure 2). Firstly, *Myoviridae* phages which exhibit imposing head structures and contractile tails ranging from 166 to 201.9 nm incorporating an organelle at the tail tip called a baseplate were termed *Myoviridae* Morphotype I phages (Figure 2). They are represented by the virulent phages 3-521, 521B, SAC12B [45] and SA-C12 [46]. Secondly, *Lb. brevis* phages such as 3-SAC12, TPATCC367, TPMB124 and TPMB449 also belong to the *Myoviridae* family, although their common morphology differs from that of the virulent *Myoviridae* Morphotype I phages mentioned above [45]. In this case, their morphology is represented by a small head structure and a decorated tail with a discrete baseplate, termed here as *Myoviridae* Morphotype II phages (Figure 2). This morphology is similar to the one observed for the prophage LBR48 [20] and interestingly, varying tail lengths were observed for phages of this morphotype (Figure 2).

Finally, some *Lb. brevis* phages were classified as members of the *Siphoviridae* family. Phages from this group, ATCCB and TPSAC12-2, are characterized as possessing a long non-contractile tail and an icosahedral head and were termed Morphotype III phages (Figure 2).

4.5. *Lb. brevis* phage phylogeny

In order to gain insight into the phylogeny of *Lb. brevis* (pro)phages, a proteomic tree was constructed with all available sequences of *Lb. brevis* phages (virulent and temperate). Phylogenetic analysis resulted in the identification of five different groups (Figure 3) highlighting the apparent uniqueness of *Lb. brevis* phages. The virulent *Myoviridae* phages previously studied [45] were gathered in group I. Group II comprises twenty-one (one virulent and twenty temperate phages) *Lb. brevis* phages and phages of this group for which the family is known are all part of the *Myoviridae* family. Meanwhile group III gathers phages of the *Siphoviridae* family (when phage family is known). Groups IV and V each comprise of a single prophage sequence with distinct genetic composition and as yet unknown morphology (Figure 3). Interestingly, the majority of phages belong to the *Myoviridae* family, which is unusual among phages of LAB where *Siphoviridae* phages are more typically reported [10,34,47].

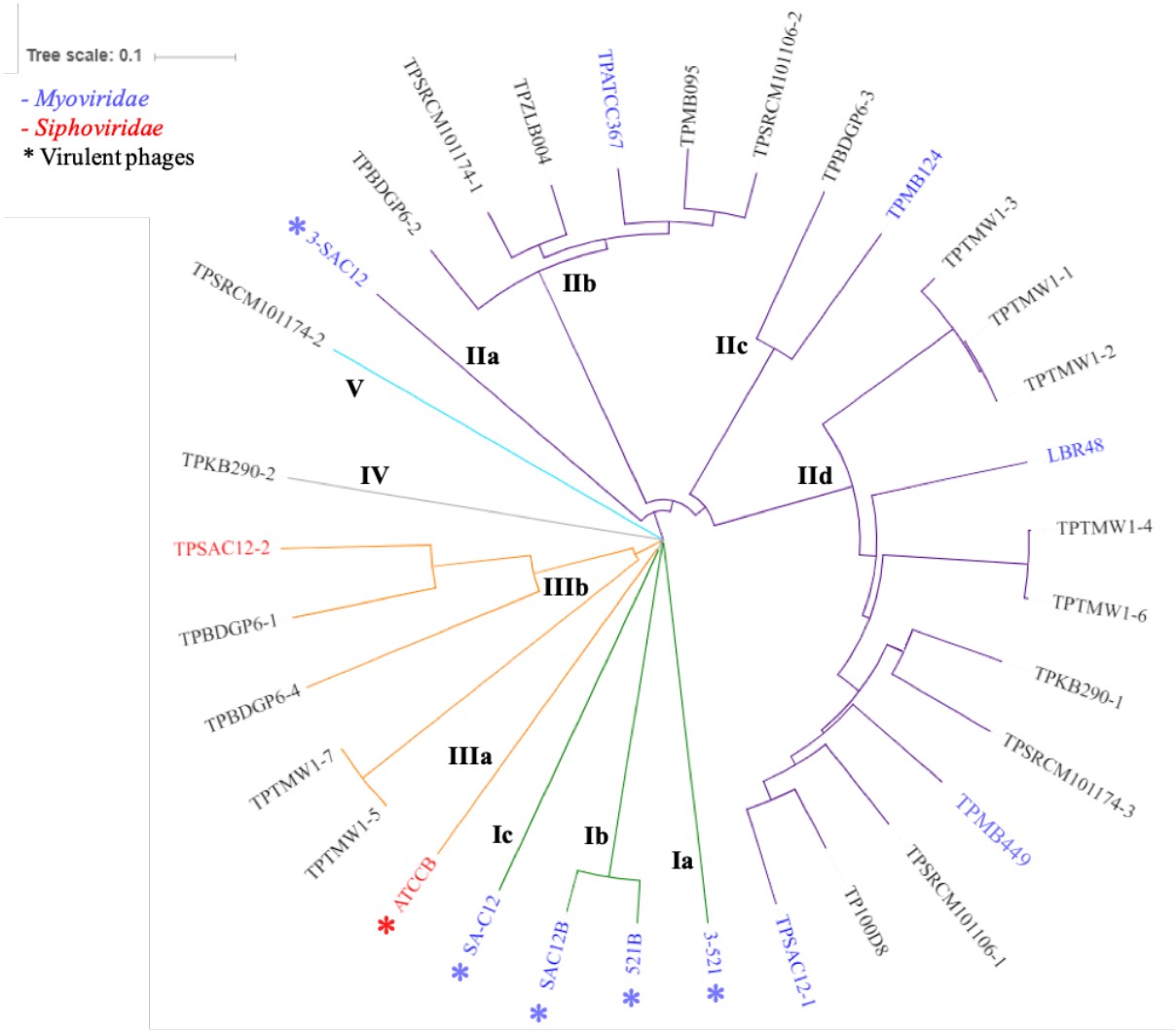


Figure 3. Proteomic tree of *Lactobacillus brevis* phages available so far: virulent (annotated with *) and temperate phages. The phylogenetic analysis revealed five distinct groups (I to V) highlighted by different colors on the tree (green I, purple II, orange III, grey IV and light blue V). *Myoviridae* phages are labelled in blue, while *Siphoviridae* phages are labelled in red.

4.6. Classification of *Lb. brevis* phages

To date, limited studies of *Lb. brevis* phages have been undertaken despite the commercial relevance of this bacterial species and its associated bacteriophages [20,46]. Here, we propose a classification of *Lb. brevis* phages similar to what has previously been undertaken for *Leuconostoc* phages [48] based on morphology, phylogeny and genomic diversity. The classification suggested here, divides *Lb. brevis* phages into five groups, I to V, linking the phylogeny and morphology analyses as described above.

The group I observed on the proteomic tree (Figure 3), gathers virulent *Myoviridae* Morphotype I phages (Figure 2). They are further divided into three subgroups based on their genetic diversity level, phage 3-521 (Ia), phages 521B and SAC12B (Ib) sharing 97 % nucleotide similarity (88 % coverage) and finally SA-C12 (Ic). The previously described [45] phages 3-521, 521B and SAC12B are characterized by a large genome size (> 136 Kb), probably in line with their imposing head structure, and a high degree of synteny throughout their genomes. Phage SA-C12 presents a similar morphology, yet harbors a smaller genome (79 Kb) [46]. The genome of SA-C12 is quite divergent and seems to be missing a certain number of genes encoding hypothetical proteins and proteins involved in the replication process compared to other phages of this group.

Group II (Figure 3) is represented by *Myoviridae* Morphotype II phages (Figure 2), thereby encompassing over half of the *Lb. brevis* phages. Genomic and morphological analysis of representative isolates suggest that all members of this group belong to the *Myoviridae* family. Based on their genetic diversity, group II phages are divided here into four subgroups (Figure 3). The subgroup IIa is comprised of the virulent phage 3-SAC12 (40 Kb) which has previously been described [45]. Subgroup IIb is composed of six temperate phages, including TPATCC367 (Figure 2), which share around 90 % nucleotide similarity (78 % sequence

coverage) and harbor a larger genome (average of 62 Kb) compared to other group II members. Subgroup IIc gathers temperate phages TPBDGP6-3 and TPMB124 with an average genome size of 46 Kb and sharing 92 % nucleotide similarity (56 % sequence coverage). The biggest subgroup, subgroup IId, is comprised of twelve temperate phages sharing at least 90 % nucleotide similarity (55 % sequence coverage) and harboring a genome with an average size of 49 Kb.

In depth comparative analysis highlighted the degree of amino acid similarity between these *Myoviridae* Morphotype II phages. Although divergence occurs within these phages, genomic synteny is observed and the genomes are organized into modules corresponding to DNA packaging, structure, lysis/lysogeny and replication (Figure 4). Different tail lengths were observed for phages belonging to the group II (Figure 2) and, as previously described, the length of the tail seems to be linked to the size of the TMP gene [49]. Indeed, phages belonging to the group IIc and presenting the smallest tail (Figure 2), have on average a TMP-encoding gene of 4.82 Kb, while phages belonging to the group IIb and IId which present the longest tailed-phages (Figure 2) possess a TMP-encoding gene with an average size of 5.67 Kb and 5.50 Kb, respectively (Figure 4). Proteins encoding holin and lysozyme (lysin) are highly conserved across the representative phages (more than 70 % amino acid (aa) similarity). The virulent phage 3-SAC12 (IIa) and the temperate phages TPMB095 (IIb) and TPMB124 (IIc) revealed low levels of similarity (between 30 and 50 % aa similarity) across their structural modules. Surprisingly, only temperate phage TPMB095 lacks sequence homology with other phages of this group in the region encoding the baseplate structure. The absence of such genes may be partly responsible for the finding that TPMB095 could not be induced by MitC treatment. Furthermore, the genome appears quite decayed with several transposase elements interjecting the genome and the overall genome appears to lack architectural conservation compared to many other phages of lactic acid bacteria. *Lb. brevis* prophages LBR48, TPMB449

and TPSAC12-1 (IId) share a high level of similarity (more than 70 % aa similarity) across the entire packaging, structural and lysis module (Figure 4). They also share between 30 and 50 % aa similarity between their predicted integrase proteins.

Group III (Figure 3) is represented by temperate and virulent phages, and gathers members of the *Siphoviridae* family (e.g. ATCCB and TPSAC12-2) characterized as Morphotype III (Figure 2). Other lysogenic bacteria carrying prophages of this group were not available for prophage induction, therefore their morphotype remains unknown. It is likely that they belong to the *Siphoviridae* family as they are most closely related to the siphophage TPSAC12-2. The group III is divided into two subgroups separating the virulent phage ATCCB (IIIa) with a genome size of approximately 80 Kb and five temperate phages (IIIb) harboring a smaller genome (size of around 40 Kb) and among which the siphophage TPSAC12-2 can be found (Figure 3). Representatives of group III for which electron microscopy images (where available) were chosen for further comparative analysis (Figure 4). Virulent phage ATCC-B and temperate phage TPSAC12-2 share synteny in terms of genome organization with the DNA packaging module followed by the structural module, the lysis/lysogeny module and the replication module. The two phages share a low level of similarity, yet synteny and amino acid (aa) similarity of around 30 % across the DNA packaging and structural modules with genes encoding terminase, capsid and tail morphogenesis-associated functions. Temperate phage TPSAC12-2, unlike virulent phage ATCC-B, harbors ORFs encoding predicted lysogeny functions, such as a Cro/Cl repressor and an antirepressor (Figure 4).

Groups IV and V contain single members, i.e. prophages TPKB290-2 and TPSRCM101174-2, respectively, and do not share any significant sequence similarity to the other groups. The morphology of these phages is unknown and based on their genome analysis it is difficult to derive assumptions on the morphology/classification of TPSRCM101174-2. However, based

on TPKB290-2 sequence data, a gene encoding a sheath protein is predicted suggesting that this phage is a *Myoviridae* member.

Induced temperate phages were tested for their potential ability to infect the seven *Lb. brevis* strains that were available in our collection (Table 1). However, these phages did not show any activity against the tested strains. Interestingly, the repressors encoded by (pro)phages of *Lb. brevis* do not share widespread sequence homology indicating that homo-immunity based on the activity of repressors is not the basis of the observed phage-insensitivity. It is likely that the observed phage-insensitivity is due to the use of alternative receptors on the cell surface by different phage groups or through the activity of phage-resistance mechanisms such as abortive infection systems.

In order to evaluate the diversity of *Lb. brevis* phages in relation to other *Lactobacillus* phages, a proteomic tree was created gathering *Lb. brevis* phages described in this study as well as previously sequenced *Lactobacillus* phages (Supplementary Figure S1). The phylogenetic tree clearly shows the distinct grouping of *Lb. brevis* phages when compared to other *Lactobacillus* phages. However, some *Lb. brevis* phages showed similarity with phages infecting other species such as observed for the *Lb. brevis* phage SA-C12 and the *Lb. plantarum* phage 8014-B2.

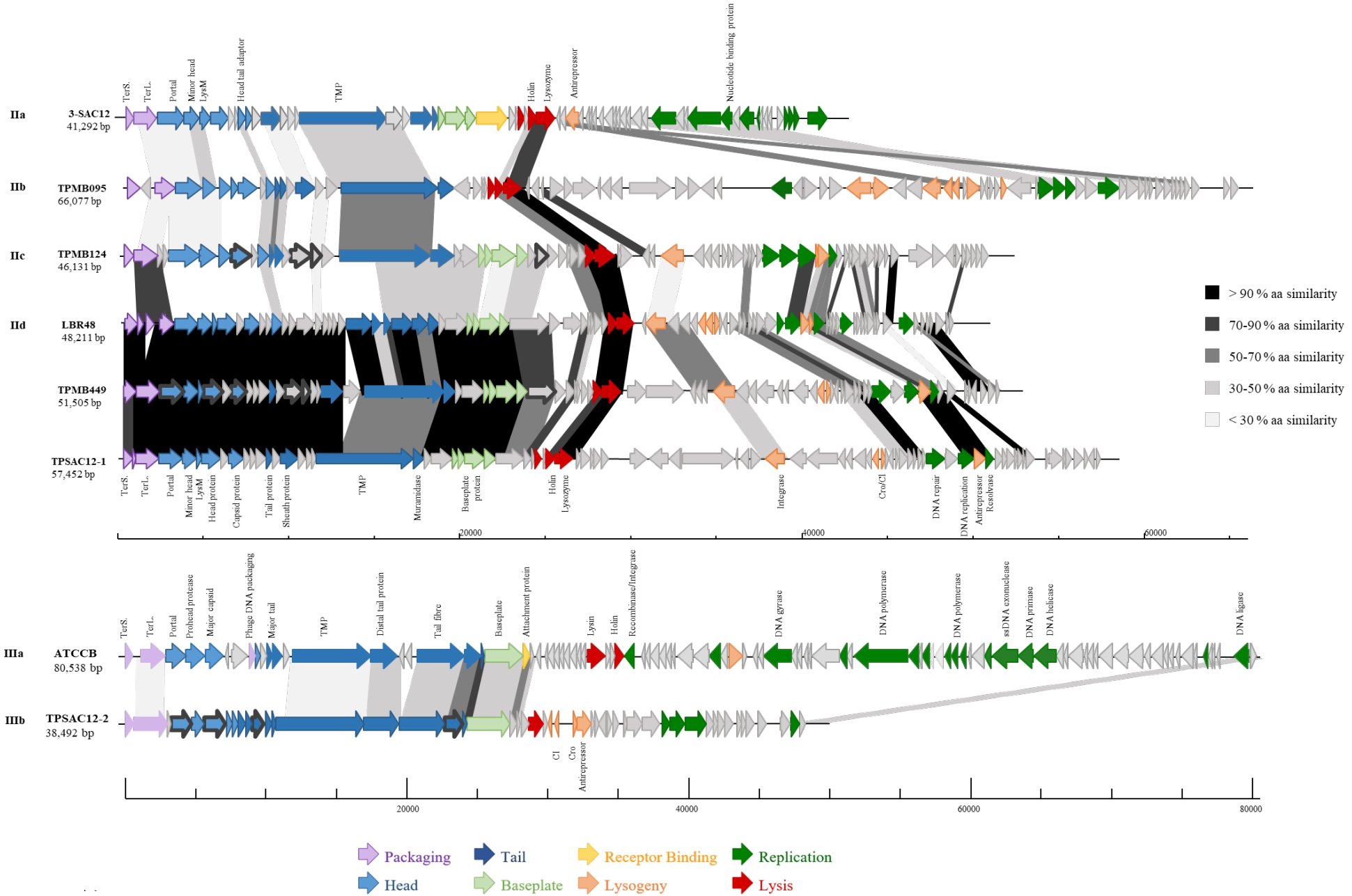


Figure 4. Genomic organization of representatives of *Lb. brevis* phages belonging to group II (*Myoviridae* Morphotype II phages): (IIa) 3-SAC12, (IIb) TPMB095, (IIc) TPMB124, (IId) LBR48, TPMB449 and TPSAC12-1 and group III: (IIIa) ATCCB and (IIIb) TPSAC12-2. The scale at the bottom of genomes is in base pairs. Each arrow represents an ORF, with the color representing the putative function of the encoded protein. Shaded boxes match the percentage of amino acid similarity between ORFs. (TerS: Small terminase; TerL: Large terminase; TMP: Tape measure protein). Confirmed structural protein-encoding genes from mass spectrometry analysis are also highlighted (bold outline).

5. Conclusions

The genomes of nineteen bacterial strains of *Lactobacillus brevis* analyzed in this study all harbor predicted prophage regions and twenty-seven intact prophage regions were identified. Only four *Lb. brevis* strains do not appear to contain intact prophage regions in their genomes. These numbers reveal the high incidence of prophages among *Lb. brevis* genomes with an average of 1.4 prophage region per strain. Of the five *Lb. brevis* strains available for prophage induction trials, four prophages were successfully induced and morphologically characterized by electron microscopy indicating a significant incidence of inducibility of these temperate phages. Electron microscopy observations, genome sequence analyses and phylogeny allowed the classification of *Lb. brevis* phages into five groups, I to V. The results show substantial diversity among *Lb. brevis* phages and interestingly these entities are mostly represented by members of the *Myoviridae* family, unlike the majority of LAB phages.

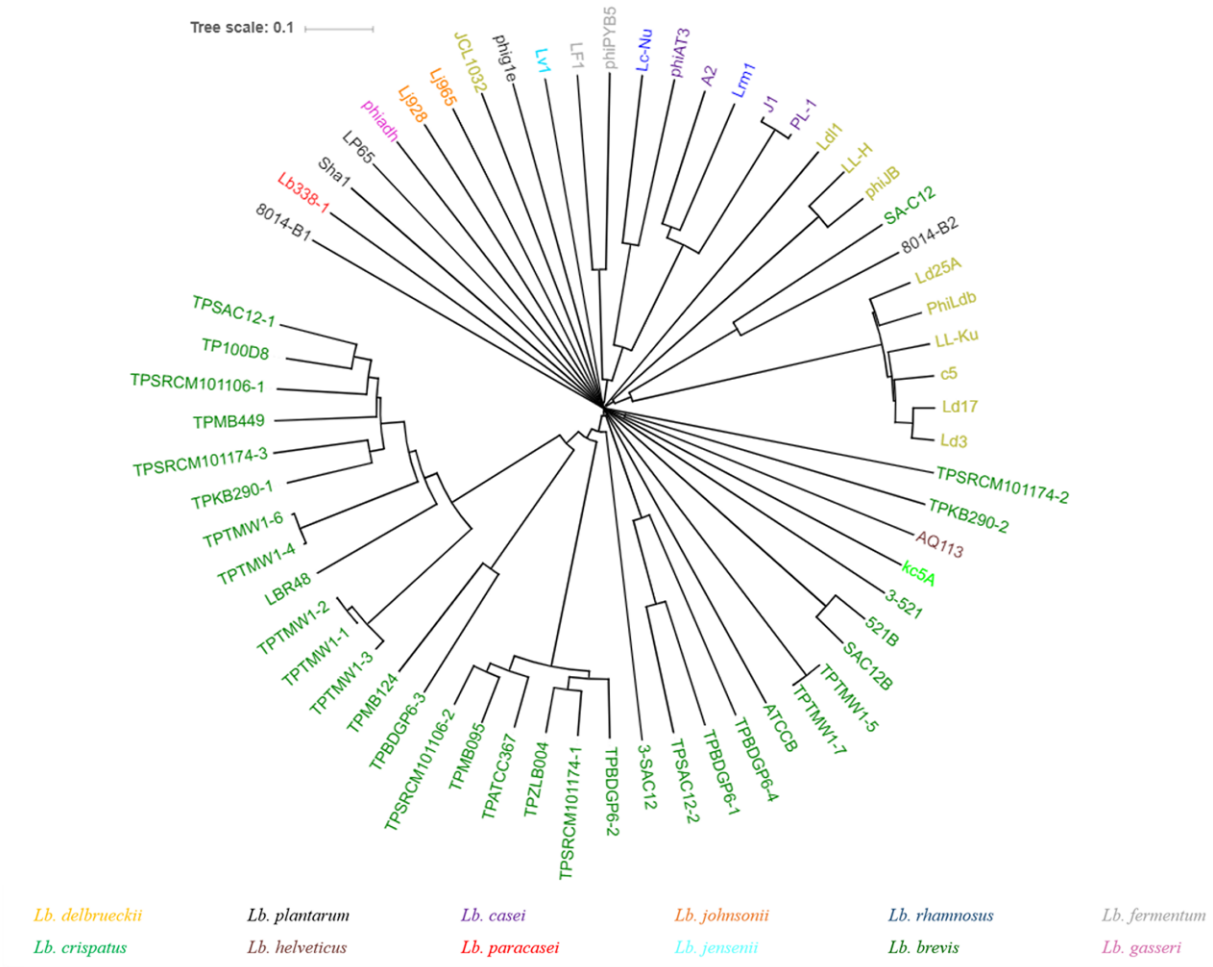
The potential of (pro)phages as antimicrobial agents in beer fermentation is a promising alternative to currently employed processes. Prophage induction in beer-spoiling *Lb. brevis* strain could be used during the cleaning process (such as coupled with sanitizers and/or UV treatment). However this approach presents challenges and hurdles (i.e. scale-up for industrial settings, phage-encoded resistance mechanisms, how to induce prophages during cleaning?, at which production stage?) that need to be addressed before their use in industry as bacterial spoilage control.

To date, very few *Lb. brevis* phages have been characterized and the identification and characterization of additional phages will provide greater insights into *Lb. brevis* phage biodiversity and their potential application and role in food spoilage prevention.

6. Supplementary Table S1. Prophage integration sites (attL and attR) and strain genome coordination.

***Lb. brevis* strains**	**Prophages**	**attL**	**attR**
100D8	TP100D8	atgccccgagcagg (2306504-2306517)	atgccccgagcagg (15706-15719)
ATCC 367	TPATCC367	ccgcattatatc (1091650-1091661)	ccgcattatatc (1142323-1142334)
BDGP6	TPBDGP6-1	aaatcctgtactctcctt (164125-164142)	aaatcctgtactctcctt (206062-206079)
	TPBDGP6-2	tccaaacagtgg (666293-666304)	tccaaacagtgg (740704-740715)
	TPBDGP6-3	ttcgactattgagtgggaata (2446977-2446997)	ttcgactattgagtgggaata (2493238-2493258)
	TPBDGP6-4	atgtatcaagcca (2740380-2740392)	atgtatcaagcca (2784356-2784368)
KB290	TPKB290-1	tttctttttga (1726892-1726903)	tttctttttga (1761666-1761677)
	TPKB290-2	attaaatgcgccccccgag (2131640-2131658)	aaatggttacatt (2179407-2179419)
SA-C12	TPSAC12-1	taacgggaattaaaat (795928-795943)	taacgggaattaaaat (853379-853394)
	TPSAC12-2	aaatcctgtactctcctt (1389949-1389966)	aaatcctgtactctcctt (1432997-1433014)
SRCM101106	TPSRCM101106-1	tatcacccgcacgg (697788-697801)	tatcacccgcacgg (746024-746037)
	TPSRCM101106-2	ccactgtttgga (894015-894026)	ccactgtttgga (963542-963553)
	TPSRCM101106-3	atgccccgagcagg (1534550-1534563)	atgccccgagcagg (1583259-1583272)
SRCM101174	TPSRCM101174-1	cccagtatacccaat (794755-794769)	cccagtatacccaat (809221-809235)
	TPSRCM101174-2	ccactgtttgga (944745-944756)	ccactgtttgga (1015102-1015113)
	TPSRCM101174-3	atgccccgagcagg (1534550-1534563)	atgccccgagcagg (1583259-1583272)
TMW 1.2108	TPTMW1-4	tatcacccgcacgg (875219-875232)	tatcacccgcacgg (924471-924484)
	TPTMW1-5	atgccccgagcagg (1646465-1646178)	atgccccgagcagg (1690290-1690303)
TMW 1.2111	TPTMW1-6	tatcacccgcacgg (877246-877259)	tatcacccgcacgg (926496-926509)
	TPTMW1-7	atgccccgagcagg (1650297-1650310)	atgccccgagcagg (1694423-1694436)
TMW 1.2112	TPTMW1-1	accccacctccggtata (872885-872901)	accccacctccggtata (924528-924544)
TMW 1.2113	TPTMW1-2	ttttttgttcttataaaatcatattcatacatacttatacaagttagtgctacagaagtgctatttttttagagtgcaat (870833-870912)	ttttttgttcttataaaatcatattcatacatacttatacaagttagtgctacagaagtgctatttttttagagtgcaat (922475-922554)
	TPTMW1-3	acagaattcctactacggctagggtgaaatgccggtttatcagtattttaaaagagtttccagaccccacctccggtata (922333-922412)	acagaattcctactacggctagggtgaaatgccggtttatcagtattttaaaagagtttccagaccccacctccggtata (973864-973943)
UCCLB95	TPMB095	ccactgtttgga (854234-854245)	ccactgtttgga (920310-920321)
UCCLBBS124	TPMB124	aaggagagtacagg (1461342-1461355)	aaggagagtacagg (1524547-1524560)
UCCLBBS449	TPMB449	agggctttatttagaactcaaaaagaacatacgtttgaaaatatcaatagttgcaatttccaaatcaaggaggaatcaa (1663018-1663097)	agggctttatttagaactcaaaaagaacatacgtttgaaaatatcaatagttgcaatttccaaatcaaggaggaatcaa (1713242-1713321)
ZLB004	TPZLB004	tggttagagcagac (1032476-1032489)	tggttagagcagac (1100835-1100848)

7. Supplementary Figure S1



Supplementary Figure S1. Proteomic tree of *Lb. brevis* phages characterized in this study and all *Lactobacillus* phages sequenced to date. Color coding indicates the host species for each phage.

8. References

1. Garofalo, C.; Osimani, A.; Milanovic, V.; Taccari, M.; Aquilanti, L.; Clementi, F. The occurrence of beer spoilage lactic acid bacteria in craft beer production. *Journal of Food Science* **2015**, *80*, 2845-2852, doi:10.1111/1750-3841.13112.
2. Bokulich, N.A.; Bamforth, C.W. The microbiology of malting and brewing. *Microbiology and Molecular Biology Reviews* **2013**, *77*, 157-172, doi:10.1128/MMBR.00060-12.
3. Vaughan, A.; O'Sullivan, T.; van Sinderen, D. Enhancing the microbiological stability of malt and beer — a review. *Journal of the Institute of Brewing* **2005**, *111*, 355-371, doi:10.1002/j.2050-0416.2005.tb00221.x.
4. Suzuki, K.; Iijima, K.; Sakamoto, K.; Sami, M.; Yamashita, H. A review of hop resistance in beer spoilage lactic acid bacteria. *Journal of the Institute of Brewing* **2006**, *112*, 173-191.
5. Brüssow, H. Phages of dairy bacteria. *Annual Review of Microbiology* **2001**, *55*, 283-303, doi:10.1146/annurev.micro.55.1.283.
6. Mahony, J.; McDonnell, B.; Casey, E.; van Sinderen, D. Phage-host interactions of cheese-making lactic acid bacteria. *Annual Review of Food Science and Technology* **2016**, *7*, 267-285, doi:10.1146/annurev-food-041715-033322.
7. Forde, A.; Fitzgerald, G.F. Bacteriophage defence systems in lactic acid bacteria. *Antonie van Leeuwenhoek* **1999**, *76*, 89-113, doi:10.1023/a:1002027321171.
8. Canchaya, C.; Proux, C.; Fournous, G.; Bruttin, A.; Brüssow, H. Prophage genomics. *Microbiology and Molecular Biology Reviews* **2003**, *67*, 238-276.
9. Davidson, B.E.; Powell, I.B.; Hillier, A.J. Temperate bacteriophages and lysogeny in lactic acid bacteria. *FEMS Microbiology Letters* **1990**, *87*, 79-90.

10. Kelleher, P.; Mahony, J.; Schweinlin, K.; Neve, H.; Franz, C.; van Sinderen, D. Assessing the functionality and genetic diversity of lactococcal prophages. *International Journal of Food Microbiology* **2018**, *272*, doi:10.1016/j.ijfoodmicro.2018.02.024.

11. Husson-Kao, C.; Mengaud, J.; Cesselin, B.; van Sinderen, D.; Benbadis, L.; Chapot-Chartier, M.-P. The *Streptococcus thermophilus* autolytic phenotype results from a leaky prophage. *Applied and Environmental Microbiology* **2000**, *66*, 558-565.

12. Villion, M.; Moineau, S. Bacteriophages of *Lactobacillus*. *Frontiers in Bioscience* **2009**, *14*, 1661-1683.

13. Forsman, P. Characterization of a prolate-headed bacteriophage of *Lactobacillus delbrueckii* subsp. *lactis*, and its DNA homology with isometric-headed phages. *Archives of Virology* **1993**, *132*, 321-330.

14. Chopin, M.C.; Chopin, A.; Rouault, A.; Galleron, N. Insertion and amplification of foreign genes in the *Lactococcus lactis* subsp. *lactis* chromosome. *Applied and Environmental Microbiology* **1989**, *55*, 1769-1774.

15. Chopin, A.; Bolotin, A.; Sorokin, A.; Ehrlich, S.D.; Chopin, M.C. Analysis of six prophages in *Lactococcus lactis* IL1403: different genetic structure of temperate and virulent phage populations. *Nucleic Acids Research* **2001**, *29*, doi:10.1093/nar/29.3.644.

16. Meijer, W.; Dobbelaar, C.; Hugenholtz, J. Thermoinducible lysis in *Lactococcus lactis* subsp. *cremoris* SK110: implications for cheese ripening. *International Dairy Journal* **1998**, *8*, 275-280, doi:https://doi.org/10.1016/S0958-6946(98)00052-1.

17. Madera, C.; García, P.; Rodríguez, A.; Suárez, J.E.; Martínez, B. Prophage induction in *Lactococcus lactis* by the bacteriocin Lactococcin 972. *International Journal of Food Microbiology* **2009**, *129*, 99-102.

18. Mittler, J.E. Evolution of the genetic switch in temperate bacteriophage. I. Basic theory. *Journal of Theoretical Biology* **1996**, *179*, 161-172, doi:10.1006/jtbi.1996.0056.

19. Lunde, M.; Blatny, J.M.; Lillehaug, D.; Aastveit, A.H.; Nes, I.F. Use of real-time quantitative PCR for the analysis of φLC3 prophage stability in lactococci. *Applied and Environmental Microbiology* **2003**, *69*, 41-48, doi:10.1128/aem.69.1.41-48.2003.

20. Jang, S.H.; Yoon, B.H.; Chang, H.I. Complete nucleotide sequence of the temperate bacteriophage LBR48, a new member of the family *Myoviridae*. *Archives of Virology* **2011**, *156*, 319-322, doi:10.1007/s00705-010-0841-7.

21. Makarova, K.; Slesarev, A.; Wolf, Y.; Sorokin, A.; Mirkin, B.; Koonin, E.; Pavlov, A.; Pavlova, N.; Karamychev, V.; Polouchine, N.; Shakhova, V.; Grigoriev, I.; Lou, Y.; Rohksar, D.; Lucas, S.; Huang, K.; Goodstein, D.M.; Hawkins, T.; Plengvidhya, V.; Welker, D.; Hughes, J.; Goh, Y.; Benson, A.; Baldwin, K.; Lee, J.; Díaz-Muñiz, I.; Dosti, B.; Smeianov, V.; Wechter, W.; Barabote, R.; Lorca, G.; Altermann, E.; Barrangou, R.; Ganesan, B.; Xie, Y.; Rawsthorne, H.; Tamir, D.; Parker, C.; Breidt, F.; Broadbent, J.; Hutkins, R.; O'Sullivan, D.; Steele, J.; Unlu, G.; Saier, M.; Klaenhammer, T.; Richardson, P.; Kozyavkin, S.; Weimer, B.; Mills, D. Comparative genomics of the lactic acid bacteria. *Proceedings of the National Academy of Sciences of the United States of America* **2006**, *103*, 15611-15616, doi:10.1073/pnas.0607117103.

22. Fukao, M.; Oshima, K.; Morita, H.; Toh, H.; Suda, W.; Kim, S.W.; Suzuki, S.; Yakabe, T.; Hattori, M.; Yajima, N. Genomic analysis by deep sequencing of the probiotic *Lactobacillus brevis* KB290 harboring nine plasmids reveals genomic stability. *PLoS One* **2013**, *8*, doi:10.1371/journal.pone.0060521.

23. Wu, Q.; Law, Y.S.; Shah, N.P. Dairy *Streptococcus thermophilus* improves cell viability of *Lactobacillus brevis* NPS-QW-145 and its γ-aminobutyric acid biosynthesis ability in milk. *Scientific Reports* **2015**, *5*, doi:10.1038/srep12885.

24. Feyereisen, M.; Mahony, J.; Kelleher, P.; Roberts, R.J.; O'Sullivan, T.; Geertman, J.-M.A.; van Sinderen, D. Comparative genome analysis of the *Lactobacillus brevis* species. *BMC Genomics* **2019**, *20*, 416, doi:10.1186/s12864-019-5783-1.

25. Fraunhofer, M.E.; Geissler, A.J.; Wefers, D.; Bunzel, M.; Jakob, F.; Vogel, R.F. Characterization of β-glucan formation by *Lactobacillus brevis* TMW 1.2112 isolated from slimy spoiled beer. *International Journal of Biological Macromolecules* **2018**, *107*, 874-881, doi:https://doi.org/10.1016/j.ijbiomac.2017.09.063.

26. Zhou, Y.; Liang, Y.; Lynch, K.H.; Dennis, J.J.; Wishart, D.S. PHAST: a fast phage search tool. *Nucleic Acids Research* **2011**, *39*, 347-352, doi:10.1093/nar/gkr485.

27. Arndt, D.; Grant, J.R.; Marcu, A.; Sajed, T.; Pon, A.; Liang, Y.; Wishart, D.S. PHASTER: a better, faster version of the PHAST phage search tool. *Nucleic Acids Research* **2016**, *44*, W16-21, doi:10.1093/nar/gkw387.

28. Hyatt, D.; Chen, G.-L.; LoCascio, P.F.; Land, M.L.; Larimer, F.W.; Hauser, L.J. Prodigal: prokaryotic gene recognition and translation initiation site identification. *BMC Bioinformatics* **2010**, *11*, 119, doi:10.1186/1471-2105-11-119.

29. Altschul, S.; Gish, W.; Miller, W.; Myers, E.; Lipman, D. Basic local alignment search tool. *Journal of Molecular Biology* **1990**, *215*, doi:10.1016/s0022-2836(05)80360-2.

30. Rutherford, K.; Parkhill, J.; Crook, J.; Horsnell, T.; Rice, P.; Rajandream, M.; Barrell, B. Artemis: sequence visualization and annotation. *Bioinformatics* **2000**, *16*, doi:10.1093/bioinformatics/16.10.944.

31. Altschul, S.F.; Madden, T.L.; Schaffer, A.A.; Zhang, J.; Zhang, Z.; Miller, W.; Lipman, D.J. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* **1997**, *25*, 3389-3402.

32. Söding, J.; Biegert, A.; Lupas, A.N. The HHpred interactive server for protein homology detection and structure prediction. *Nucleic Acids Research* **2005**, *33*, doi:10.1093/nar/gki408.

33. Schattner, P.; Brooks, A.N.; Lowe, T.M. The tRNAscan-SE, snoscan and snoGPS web servers for the detection of tRNAs and snoRNAs. *Nucleic Acids Research* **2005**, *33*, W686-W689, doi:10.1093/nar/gki366.

34. Casey, E.; Mahony, J.; Neve, H.; Noben, J.-P.; Dal Bello, F.; van Sinderen, D. Novel phage group infecting *Lactobacillus delbrueckii* subsp. *lactis*, as revealed by genomic and proteomic analysis of bacteriophage Ldl1. *Applied and Environmental Microbiology* **2015**, *81*, 1319-1326, doi:10.1128/aem.03413-14.

35. Thompson, J.D.; Gibson, T.J.; Higgins, D.G. Multiple sequence alignment using ClustalW and ClustalX. *Current Protocols in Bioinformatics* **2002**.

36. Kumar, S.; Nei, M.; Dudley, J.; Tamura, K. MEGA: a biologist-centric software for evolutionary analysis of DNA and protein sequences. *Briefings in Bioinformatics* **2008**, *9*, 299-306, doi:10.1093/bib/bbn017.

37. Loeffler, D. Modern brewery sanitation. In *Brewing*, Woodhead Publishing: Elsevier, **2006**; pp. 308-334.

38. Schmidt, R.H. *Basic elements of equipment cleaning and sanitizing in food processing and handling operations*; University of Florida cooperative extension service, institute of food and agricultural sciences, EDIS: **1997**.

39. Lavelle, K.; Murphy, J.; Fitzgerald, B.; Lugli, G.A.; Zomer, A.; Neve, H.; Ventura, M.; Franz, C.M.; Cambillau, C.; van Sinderen, D.; Mahony, J. A decade of *Streptococcus*

thermophilus phage evolution in an Irish dairy plant. *Applied and Environmental Microbiology* **2018**, *84*, e02855-02817, doi:10.1128/aem.02855-17.

40. Ceyssens, P.-J.; Mesyanzhinov, V.; Sykilinda, N.; Briers, Y.; Roucourt, B.; Lavigne, R.; Robben, J.; Domashin, A.; Miroshnikov, K.; Volckaert, G.; Hertveldt, K. The genome and structural proteome of YuA, a new *Pseudomonas aeruginosa* phage resembling M6. *Journal of Bacteriology* **2008**, *190*, 1429-1435, doi:10.1128/jb.01441-07.

41. Holtappels, M.; Vrancken, K.; Schoofs, H.; Deckers, T.; Remans, T.; Noben, J.P.; Valcke, R. A comparative proteome analysis reveals flagellin, chemotaxis regulated proteins and amylovoran to be involved in virulence differences between *Erwinia amylovora* strains. *Journal of Proteomics* **2015**, *123*, 54-69, doi:10.1016/j.jprot.2015.03.036.

42. Mahony, J.; McGrath, S.; Fitzgerald, G.F.; van Sinderen, D. Identification and characterization of lactococcal-prophage-carried superinfection exclusion genes. *Applied and Environmental Microbiology* **2008**, *74*, 6206-6215, doi:10.1128/aem.01053-08.

43. Chopin, M.-C.; Chopin, A.; Bidnenko, E. Phage abortive infection in lactococci: variations on a theme. *Current Opinion in Microbiology* **2005**, *8*, 473-479.

44. Deng, Y.-M.; Liu, C.-Q.; W. Dunn, N. Genetic organization and functional analysis of a novel phage abortive infection system, AbiL, from *Lactococcus lactis*. *Journal of Biotechnology* **1999**, *67*, 135-149, doi:https://doi.org/10.1016/S0168-1656(98)00175-8.

45. Feyereisen, M.; Mahony, J.; Lugli, G.A.; Ventura, M.; Neve, H.; Franz, C.M.A.P.; Noben, J.-P.; O'Sullivan, T.; van Sinderen, D. Isolation and characterization of *Lactobacillus brevis* phages. *Viruses* **2019**, *11*, 393.

46. Deasy, T.; Mahony, J.; Neve, H.; Heller, K.J.; van Sinderen, D. Isolation of a virulent *Lactobacillus brevis* phage and its application in the control of beer spoilage. *Journal of Food Protection* **2011**, ***74***, 2157-2161, doi:10.4315/0362-028x.jfp-11-262.

47. Brüssow, H.; Desiere, F. Comparative phage genomics and the evolution of *Siphoviridae*: insights from dairy phages. *Molecular Microbiology* **2001**, *39*, 213-222.

48. Kot, W.; Neve, H.; Heller, K.J.; Vogensen, F.K. Bacteriophages of *Leuconostoc, Oenococcus*, and *Weissella*. *Frontiers in Microbiology* **2014**, *5*, 186-186, doi:10.3389/fmicb.2014.00186.

49. Mahony, J.; Alqarni, M.; Stockdale, S.; Spinelli, S.; Feyereisen, M.; Cambillau, C.; van Sinderen, D. Functional and structural dissection of the tape measure protein of lactococcal phage TP901-1. *Scientific Reports* **2016**, *6*, 36667.

Chapter VII

Identification of a prophage-encoded abortive infection system in *Lactobacillus brevis*

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1. Abstract

Abortive infection systems (Abi) are phage resistance systems that can be chromosomally-, plasmid- or prophage-encoded. In this study, two genes encoding an Abi system were identified on the prophage sequence contained by the chromosome of the beer-spoiling strain *Lactobacillus brevis* UCCLBBS124. This Abi system is similar to the two-component AbiL system encoded by *Lactococcus lactis* biovar. *diacetylactis* LD10-1. The UCCLBBS124 prophage-derived Abi system (designated here as $AbiL_{124}$) was shown to exhibit specific activity against phages infecting *Lb. brevis* strains as well as *L. lactis* strains. Expression of the $AbiL_{124}$ system was shown for certain phages to cause a reduction in the efficiency of plaquing and cell lysis delay. Phage escape mutants capable of bypassing $AbiL_{124}$ exhibit a mutation in the tail fiber protein thereby identifying its possible role as the trigger for $AbiL_{124}$ activation following host infection.

2. Introduction

Lactobacillus brevis is a bacterial species which belongs to the lactic acid bacteria (LAB), and which has been widely used in industry for fermentation purposes such as in dairy fermentations for the production of kefir or during sourdough fermentation [1]. However, it is also associated with the spoilage of foods and beverages, in particular beer. There is an ever-increasing consumer demand for natural food preservation methods, and in this context, bacteriophages possess the potential to control such spoilage bacteria [2,3]. Virulent phages active against *Lb. brevis* have been isolated and characterized, revealing a narrow host range [4,5] and suggesting the presence of resistance mechanisms against these bacteriophages. Various naturally occurring, phage-derived defence systems against LAB phages have been identified, including abortive infection (Abi), superinfection exclusion (Sie) or restriction/modification systems [6-9]. Abi systems block phage multiplication leading to the release of few (if any) infective virions and cause death of infected cells, thereby protecting the overall bacterial population [10]. More specifically, Abi systems interfere with phage development following phage adsorption and DNA injection into the host's cytoplasm, resulting in an absence of plaques or a reduction in plaque size coupled with significant cell death [11]. Several Abi systems have been described in LAB, most notably in *Lactococcus lactis,* and their different modes of action have been studied. For example, AbiA [12] and AbiF [13] inhibit DNA replication, while AbiB is responsible for RNA degradation following infection [14]. AbiC has been shown to decrease the synthesis of a phage capsid protein [12], whereas AbiL is likely to act at the post-transcriptional level interfering with the synthesis of phage proteins or the assembly of phage particles [8]. Many Abi systems are encoded by a single gene, though two-component Abi systems have also been identified [8]. Interestingly, little homology is observed between protein sequences of different Abi systems, for example

AbiF shares just 26 to 47 % sequence homology with AbiD and AbiD1. However, a notable feature among Abi systems is the high A+T content (usually ~70 %) of the genes encoding these systems [15].

The majority of currently known Abi systems are plasmid-encoded [10] such as AbiL encoded by plasmid pND861 of *Lactococcus lactis* biovar. *diacetylactis* LD10-1 [8]. However, prophage-encoded systems have also been identified including AbiN encoded by a prophage of *L. lactis* subsp. *cremoris* S114. Publications on phage-resistance systems in lactobacilli is very limited and just a single, plasmid-encoded phage-resistance system has been described for *Lactobacillus plantarum* NGRI0101 plasmid pLKS [16].

Lb. brevis strains with the ability to spoil beer appear to be rather resistant to phage infection and/or propagation [5], suggesting the presence of phage-resistance systems. Analysis of *Lb. brevis* prophages revealed the presence of a potential prophage-encoded Abi system located within the chromosome of a beer-spoiling *Lb. brevis* strain (Chapter VI, Table 3). This Abi system was shown to be functional and is encoded by two genes, showing similarity to the previously characterized lactococcal AbiL system [8].

3. Materials and Methods

3.1. Bacterial strains and cultivation media

Bacterial strains used in this study are listed in Table 1. *Lb. brevis* strains were grown in MRS broth (Oxoid Ltd., England) at 30 ˚C, while *L. lactis* strains were grown in M17 broth (Oxoid Ltd., England) supplemented with 0.5 % glucose. 5 µg/mL chloramphenicol (Cm5) was added to the culture where indicated.

Table 1. Bacterial strains, phages and plasmids used in this study.

Strain / Plasmid / Phage	Description	Reference
***Lb. brevis* strains**		
UCCLBBS124	Beer-spoiling strain isolated from a spoiled beer keg (Singapore)	[17]
UCCLB521	Non-beer spoiler strain isolated from brewery environment (The Netherlands)	[17]
SA-C12	Non-beer spoiler strain isolated from silage (Ireland)	[17]
UCCLB521 pNZ44	UCCLB521 carrying pNZ44	This study
UCCLBB521 pNZ44:*abiL*$_{124}$	UCCLB521 carrying pNZ44 + *abiL*$_{124}$	This study
SA-C12 pNZ44	SA-C12 carrying pNZ44	This study
SA-C12 pNZ44:*abiL*$_{124}$	SA-C12 carrying pNZ44 + *abiL*$_{124}$	This study
***L. lactis* strain**		
NZ9000	Transformation host and phage sensitive strain	[18]
3107	Phage sensitive strain	[19]
NZ9000 pNZ44	NZ9000 carrying pNZ44	This study
NZ9000 pNZ44:*abiL*$_{124}$	NZ9000 carrying pNZ44 + *abiL*$_{124}$	This study
3107 pNZ44	3107 carrying pNZ44	This study
3107 pNZ44:*abiL*$_{124}$	3107 carrying pNZ44 + *abiL*$_{124}$	This study
Plasmids		
pNZ44	Transformation vector, chloramphenicol resistance gene	
pNZ44*:abiL*$_{124}$	Plasmid carrying *abiL*$_{124}$	This study
***Lb. brevis* phages**		
3-521	Virulent phage active against UCCLB521	[5]
521B	Virulent phage active against UCCLB521	[5]
3-SAC12	Virulent phage active against SA-C12	[5]
SAC12B	Virulent phage active against SA-C12	[5]
***L. lactis* phages**		
TP901-1	P335-type phage active against 3107	[20]
LC3	P335-type phage active against 3107	[21]
Dub35A	P335-type phage active against 3107	[22]
62601	936-type phage active against 3107	[23]
66901	936-type phage active against 3107	[24]
jj50	936-type phage active against NZ9000	[25]
p2	936-type phage active against NZ9000	[26]
sk1	936-type phage active against NZ9000	[27]
712	936-type phage active against NZ9000	[25]
949	949-type phage active against 3107	[28]
WRP3	949-type phage active against 3107	[29]
P087	P087-type phage active against 3107	[30]

3.2. Phage propagation and plaque assays

Phages used in this study were propagated on their respective host strain (*Lb. brevis* or *L. lactis*) as previously described [5,6]. Phage lysates were then stored at 4 ˚C. Plaque assays were performed as described previously [5,31]. These assays were used to determine the efficiency of plaquing (EOP) of the corresponding phages on the strain expressing the potential Abi system relative to that of the sensitive host strain.

3.3. Identification of potential abortive infection systems in *Lb. brevis* prophages

All complete chromosome sequences of *Lb. brevis* strains currently available in the NCBI (National Center for Biotechnology Information) database were analyzed for the presence of potential prophage-specifying DNA regions using PHASTER (PHAge Search Tool Enhanced Release) [32,33] (See Chapter VI). Potential Abi systems were detected by performing an all-against-all reciprocal blast of the prophage-encoded proteins against a database gathering all currently known proteins encoding Abi systems. An alignment cut-off value was used that employed an E-value of 0.0001, > 30 % amino acid identity across 50 % of the sequence length [7].

3.4. Construction of plasmid vectors

Putative *abi* genes were amplified by PCR and cloned into the expression vector pNZ44 [34]. PCR products and pNZ44 plasmid DNA were digested with the appropriate enzymes (Roche, USA) at 37 ˚C for at least 4 h, following the manufacturer's instructions. An insert:vector ratio of 3:1 was applied for the ligation of the PCR product with pNZ44 using T4 DNA ligase

(Promega, USA). The mixture was incubated at room temperature for at least 4 hours prior to electrotransformation into *L. lactis* NZ9000.

3.5. Preparation of competent cells and electrotransformation

Competent cells of *L. lactis* strains were prepared as previously described [35]. Competent cells of *Lb. brevis* strains were prepared using an adapted version of a previously described protocol [36]: An overnight culture was transferred (1 % inoculum) to 10 mL MRS broth containing 1 % glycine and incubated overnight at 30 ˚C. 5 mL of the overnight culture was transferred to fresh MRS broth containing 1 % glycine (50 mL final volume) and cells were grown to an OD_{600nm} of ~0.6. Cells were harvested by centrifugation at 4,000 × *g* for 15 min at 4 ˚C and washed in ice-cold wash buffer (0.5 M sucrose, 10 % glycerol). The wash step was repeated twice and cells were then resuspended in 200 µL wash buffer prior to storage at -80 ˚C. All constructs were generated in *L. lactis* NZ9000, verified by sequencing after PCR amplification prior to their transfer into *Lb. brevis* strains and *L. lactis* 3107. Electrotransformation was performed using freshly prepared competent cells as described above, where 45 µL of cells and 5 µL of plasmid construct were mixed into a pre-chilled 2 mm electroporation cuvette (Cell Projects, Kent, England) and subjected to electroporation at 1.5 kV (*Lb. brevis*) or 2.0 kV (*L. lactis*), 200 Ω, 25 µF. Following electroporation, 950 µL recovery broth was added (MRS broth supplemented with 0.5 M sucrose and 0.1 M $MgCl_2$ (*Lb. brevis*) or GM17 broth supplemented with 20 mM $MgCl_2$ and 2 mM $CaCl_2$ (*L. lactis*)). Cells were recovered at 30 ˚C for 3 h (*Lb. brevis*) or 2 h (*L. lactis*) prior to spread plating on MRS (*Lb. brevis*) or GM17 (*L. lactis*) agar supplemented with Cm5. Presumed transformants were purified on MRS agar + Cm5 and the presence of the appropriate construct was verified by Sanger sequencing.

3.6. Characterization of potential Abi systems

Lysis-in-broth assays were performed by infecting the *Lb. brevis* or *L. lactis* strain with the corresponding phage at a multiplicity of infection (MOI) of 1. The OD_{600nm} was measured every 2 h for the first 10 h and then at 15, 24 and 30 h. Cell survival assays were performed as previously described by Garvey et al. [37] where following phage infection of the *Lb. brevis* strain in broth for 30 h, cells were plated to determine the viable count as expressed in CFU/mL and to calculate the level of cell death. Adsorption assays were adapted from a previously outlined protocol [38]. Briefly, strains were grown to mid-late exponential phase (OD_{600nm} ~0.5), at which point they were harvested by centrifugation at 4000 × *g* for 10 min and resuspended in 1/4-strength Ringer's solution. Phages were added to the cells at a final titer of 10^6 PFU/mL followed by incubation at 30 °C for 15 min. The supernatant was retained after centrifugation and tested for the residual phage concentration by plaque assay as described above. Adsorption efficiency was calculated using the formula:

((Ci – Cr) / Ci) × 100; where Ci represents the total phage number used in the adsorption assay and Cr represents the residual phage number after the adsorption step.

To determine whether phage-infected strains released viable progeny, phage-host complexes were plated on a sensitive host and evaluated for infective centers detected as plaques. *Lb. brevis* strains were infected with phage at a MOI of 0.1 in the presence of 25 mM $CaCl_2$, and incubated for 30 min at 30 °C. Cultures were centrifuged at 4000 × *g* for 10 min, washed twice in MRS broth containing 25 mM $CaCl_2$, diluted, and assayed for infective centres. Efficiency of the center of infection (ECOI) was determined as the number of centers of infection (COI) from the test strain divided by the number of COI from the sensitive host as described by Sing and Klaenhammer [39].

3.7. Phage escape mutant isolation, DNA extraction and genome sequencing

Phage 3-521 was purified and concentrated as previously described [5] resulting in a concentrated lysate of approximately 10^{11} PFU/mL. 10 ml of *Lb. brevis* UCCLB521 was grown to an OD_{600nm} of 0.3-0.4 at which point the culture was centrifuged and re-suspended in 1 ml of ¼ strength Ringer's solution (Merck, Darmstadt, Germany) supplemented with 10 mM $CaCl_2$ (Sigma-Aldrich, Missouri, USA). The concentrated 3-521 phage lysate was added to the culture and incubated for 15 min at 30 ˚C. The mixture was then centrifuged and washed three times with an equal volume of ¼ strength Ringer's solution. Finally, 250 µl of the washed cell culture was incubated with 250 µl overnight culture of *Lb. brevis* UCCLB521 pNZ44:*abiL*$_{124}$ carrying the potential Abi system in a MRS semi-solid agar overlay. Following overnight incubation, visible plaques of escape mutant were picked and propagated on *Lb. brevis* UCCLB521 pNZ44:*abiL*$_{124}$ in order to purify and increase the phage titer. Genomic DNA of 3-521 phage escape mutant was extracted and sequenced using Illumina MiSeq sequencing technology (GenProbio, Parma, Italy) as previously described [5].

4. Results and Discussion

4.1. Identification of a potential prophage-encoded Abi system

Temperate phages may be beneficial to the host by carrying genes that provide a competitive advantage in surviving in its environment, for example by providing resistance against bacteriophage infection [6,9] thus increasing host fitness.

Predicted prophage regions were identified on the chromosome of nineteen *Lb. brevis* strains using PHASTER, resulting in the identification of twenty-seven intact prophage sequences as well as twenty-three partial prophage regions (see Chapter VI). Among these predicted prophage regions a BlastP analysis revealed the presence of an identical putative *abi* system carried by three prophage regions (approximately 56.5 % A+T content for the three prophage regions) located on the chromosome of the *Lb. brevis* strains UCCLBBS124, NPS-QW-145 and SRCM101174 (approximately 54 % A+T content for the three *Lb. brevis* strains). This *abi* system is composed of two genes with a relatively high A+T content of 68 % similar to that observed for other abortive infection genes [15]. The proteins encoded by this putative *abi* system display similarity (approximately 35 % at amino acid level; data not shown) to the two proteins of the AbiL system previously identified in *L. lactis* [8]. Of these three *Lb. brevis* strains, only *Lb. brevis* strain UCCLBBS124 was available in our collection for further characterization of this putative Abi system encoded on its temperate phage TPMB124. The putative *abi* system carried by prophage TPMB124, designated here as $abiL_{124}$, is composed of two genes, corresponding to locus tags *UCCLBBS124_1417* and *UCCLBBS124_1418*, and encodes proteins of 202 and 409 amino acids, respectively. The two genes that make up $abiL_{124}$ appear to be organized within a single operon as they appear to be translationally coupled as had previously been observed for the lactococcal AbiL system [8].

4.2. Phage resistance conferred by AbiL$_{124}$

To assess the effect of AbiL$_{124}$ on the phage sensitivity profile of different host bacteria, the genes encoding AbiL$_{124}$ were cloned into pNZ44 to generate plasmid pNZ44:*abiL$_{124}$*, which was then introduced into *Lb. brevis* strains UCCLB521 and SA-C12. These two strains are susceptible to *Lb. brevis* phages 3-521, 521B and SAC12B (in case of strain UCCLB521) and 3-SAC12 and SAC12B (for strain SA-C12) [5]. In the case of UCCLB521 pNZ44:*abiL$_{124}$* significant phage resistance was observed against phages 3-521, 521B and SAC12B with an EOP lower than 10^{-8} (Table 2), demonstrating the functionality of this prophage-encoded phage resistance system. Conversely, no such resistance was observed for SA-C12 and its derivative expressing the putative Abi system, SA-C12 pNZ44:*abiL$_{124}$* against phages 3-SAC12 and SAC12B (Table 2). The role of the individual genes constituting *abiL$_{124}$* in conferring phage resistance to UCCLB521 was also investigated. The genes were cloned individually in pNZ44, generating plasmids pNZ44:*abiLi$_{124}$* and pNZ44:*abiLii$_{124}$*, and were introduced into the phage sensitive strain UCCLB521. After introduction of the individual genes separately in UCCLB521, no resistance to phages 3-521, 521B and SAC12B was observed (data not shown) confirming the requirement for both genes for an active AbiL$_{124}$ phage resistance system. The presence of this Abi system in *Lb. brevis* UCCLBBS124 may therefore explain, at least in part, its high resistance to phage infection [5].

The phages that were shown to be sensitive to AbiL$_{124}$ (i.e. phages 3-521, 521B and SAC12B) are closely related to each other and represent virulent *Myoviridae* phages characterized by a large genome size (> 136 kb). The genomes of phages 521B and SAC12B share 97 % nucleotide (nt) similarity (88 % coverage) across their genome and similarity is observed with 3-521 notably within the morphogenesis module where a high degree of synteny is observed [5]. The AbiL$_{124}$ system was shown to be inactive against phage 3-SAC12 which is also a

Myoviridae yet differs from 521B, SAC12B and 3-521 by its size (41 kb) and its genome sequence [5]. Based on phylogeny analysis, the three phages targeted by AbiL$_{124}$ all belong to the *Myoviridae* Morphotype I phages (see Chapter VI), while 3-SAC12 is a member of the *Myoviridae* Morphotype II phage group (see Chapter VI). Remarkably, AbiL$_{124}$ was shown not to be active against phage SAC12B when *abiL$_{124}$* was introduced into (the SAC12B-sensitive host) *Lb. brevis* SA-C12, suggesting that the activity of the Abi system is dependent on particular host factors.

Table 2. EOP of tested bacteriophages against the putative *Lb. brevis* AbiL$_{124}$ system.

		***Lb. brevis* strains**					
		UCCLB521			**SA-C12**		
		WT	**pNZ44**	**pNZ44:*abiL$_{124}$***	**WT**	**pNZ44**	**pNZ44:*abiL$_{124}$***
Phage	**3-521 (Group I)**	1	0.58	$< 10^{-8}$	N/A	N/A	N/A
	521B (Group I)	1	1.52	$< 10^{-8}$	N/A	N/A	N/A
	SAC12B (Group I)	1	0.98	$< 10^{-8}$	1	0.97	0.90
	3-SAC12 (Group II)	N/A	N/A	N/A	1	0.96	0.94

N/A: not applicable

4.3. AbiL$_{124}$ characterization

Lysis-in-broth and cell death experiments were conducted to study the effect of the AbiL$_{124}$ system on actively growing cells. AbiL$_{124}$ system expressed in *Lb. brevis* UCCLB521 allowed survival after phage infection significantly better than the wild-type and the strain carrying an empty plasmid pNZ44 as shown by lysis-in-broth experiments (Figure 1). The strain expressing the AbiL$_{124}$ system exhibits growth in the presence of phages 3-521 or 521B, yet the level of growth is significantly lower than that observed for the strain in the absence of either of these phages (Figure 1). Cell death was calculated following phage infection of the *Lb. brevis* strain in broth for 30 h, which indicated that approximately 64 and 60 % of the cells of UCCLB521

expressing the AbiL$_{124}$ system lost viability following infection with phages 3-521 and 521B, respectively (Table 3). Adsorption assays revealed a significant lower adsorption efficiency of phages 3-521 and 521B on *Lb. brevis* strain UCCLB521 expressing the AbiL$_{124}$ system with an adsorption efficiency 2.5 (521B) to 4 (3-521) times lower than what observed for the WT sensitive strain (Table 3). These observations are unusual for an Abi system as Abi systems usually act intracellularly to interfere with phage development following phage adsorption and DNA injection [8]. To determine if phage-infected strains released viable phages, ECOI experiments were performed which demonstrated the significant inability of the strain expressing the AbiL$_{124}$ system (UCCLB521 pNZ44:*abiL$_{124}$*) to produce viable phages (Table 3). The results are consistent with what is observed for Abi systems where host strains use these phage resistance mechanisms to block phage multiplication by killing infected cells thus allowing the survival of the remaining uninfected bacterial population [10].

Table 3. Phenotypic characteristics of the potential AbiL$_{124}$ system.

***Lb. brevis* strains**	**Phage**	**Cell death (%)**	**Adsorption (%)**	**ECOI**
UCCLB521 WT	**3-521**	100	95.9 ± 2.6	1
	521B	100	96.1 ± 2.5	1
UCCLB521 pNZ44	**3-521**	99.2 ± 2.3	86.7 ± 1.7	0.92 ± 0.27
	521B	98.7 ± 1.3	94.4 ± 1.2	0.97 ± 0.03
UCCLB521 pNZ44:*abiL$_{124}$*	**3-521**	64.1 ± 0.9	22.7 ± 4.7	0
	521B	60.2 ± 0.7	37.1 ± 5.6	0.04 ± 0.03

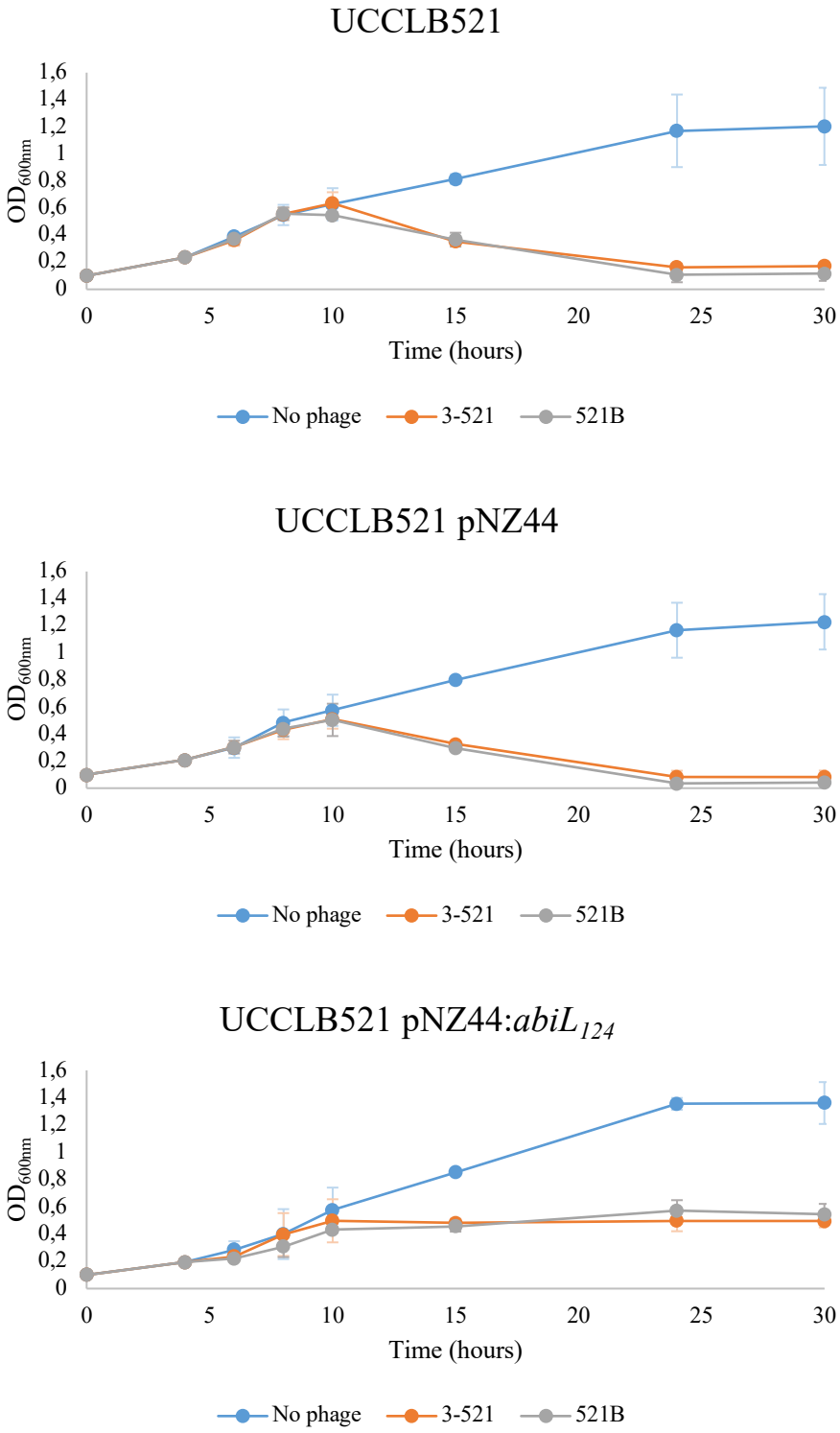

Figure 1. Lysis-in-broth experiments comparing the lysis profiles of UCCLB521 WT, harboring the empty vector pNZ44 or expressing the AbiL$_{124}$ system when infected with phages 3-521 (orange) or 521B (grey) (MOI = 1). Absence of phage in the culture was used as a negative control (blue).

4.4. Phage escape mutants of AbiL$_{124}$

Escape mutants that were able to overcome the AbiL$_{124}$ system were obtained by challenging strain UCCLB521 expressing the *abiL$_{124}$* genes with a high titer (around 10^{11} PFU/mL) of phage 3-521. Despite several attempts, a single stable escape phage mutant EM$_{3\text{-}521}$ was obtained and DNA sequencing revealed a single mutation located on the gene encoding the predicted tail fiber protein (Tal). This mutation led to the nucleotide substitution G65520A causing the amino acid substitution G1667E in the C-terminal region of this large (1857 aa) protein. The Tal protein of LAB phages has been shown to be involved in phage-host attachment [40]. The C-terminal part of the Tal has been associated to host adsorption but also in some cases to DNA passage facilitation due to a peptidoglycan hydrolase (PGH) activity [41]. HHpred analysis of the Tal$_{3\text{-}521}$ identifies a lytic domain within the protein sequence, however the C-terminal region of the protein did not provide any hits with known domains. The mutation in the C-terminal region of the Tal protein observed in escape mutant EM$_{3\text{-}521}$ allowed the phage to overcome the expressed AbiL$_{124}$ system in the host strain. Here, we hypothesize that activation of the AbiL$_{124}$ system is triggered by the Tal protein and that mutation within this protein allowed the phage mutant EM$_{3\text{-}521}$ to bypass the Abi system. The Tal protein in phage 3-521, even though at a low level, shows similarity to the Tal protein of 521B and SAC12B (~ 26 % aa similarity) explaining their sensitivity to the AbiL$_{124}$ system. Meanwhile no similarity was observed with any protein of phage 3-SAC12 which remained unaffected by the expression of this system.

4.5. AbiL$_{124}$ activity against *Lactococcus lactis* phages

AbiL$_{124}$ was shown to provide complete phage resistance for one strain of *Lb. brevis* species and its activity was also tested on *L. lactis* strains. The *abiL$_{124}$* genes were expressed in *L. lactis* NZ9000 and 3107 and tested for their effectiveness against a range of phages (Table 4). AbiL$_{124}$ was shown to confer almost complete resistance to *L. lactis* 3107 pNZ44:*abiL$_{124}$* against all tested phages with EOPs lower than 10^{-9}. The AbiL$_{124}$ system was shown to be active against phages belonging to different phage groups (i.e. the 936, 949, P335 and P087 groups), thus showing a broad activity-range against lactococcal phages. Phage resistance was also observed in *L. lactis* NZ9000 carrying the *abiL$_{124}$* genes but not to the same extent (Table 4) as in 3107. Significant ($P < 0.05$) resistance against phages jj50, p2 and sk1 were observed for NZ9000 expressing the AbiL$_{124}$ system, however no significant difference was observed against phage 712 which remained active in infection of the derivative strain (Table 4).

Obtaining phage mutants escaping the AbiL$_{124}$ system was attempted by challenging *L. lactis* 3107 pNZ44:*abiL$_{124}$* with high titer lysates of TP901-1 phages (> 10^{11} PFU/mL), however our attempts were unsuccessful supporting the notion that the AbiL$_{124}$ system is a robust phage resistance system.

Table 4. EOP of tested lactococcal bacteriophages against the putative *Lb. brevis* AbiL$_{124}$ system (results are average of triplicate assays).

		***L. lactis* strains**					
		3107			**NZ9000**		
		WT	**pNZ44**	**pNZ44:*abiL*$_{124}$**	**WT**	**pNZ44**	**pNZ44:*abiL*$_{124}$**
Phage (phage group)	**TP901-1 (P335)**	1	0.66	$< 10^{-11}$	N/A	N/A	N/A
	LC3 (P335)	1	0.75	$< 10^{-11}$	N/A	N/A	N/A
	Dub35A (P335)	1	1	$< 10^{-12}$	N/A	N/A	N/A
	62601 (936)	1	1	$< 10^{-12}$	N/A	N/A	N/A
	66901 (936)	1	1.57	$< 10^{-10}$	N/A	N/A	N/A
	949 (949)	1	0.96	$< 10^{-10}$	N/A	N/A	N/A
	WRP3 (949)	1	0.83	6.1×10^{-7}	N/A	N/A	N/A
	P087 (P087)	1	0.86	$< 10^{-9}$	N/A	N/A	N/A
	jj50 (936)	N/A	N/A	N/A	1	1	0.70
	p2 (936)	N/A	N/A	N/A	1	1.09	0.71
	sk1 (936)	N/A	N/A	N/A	1	0.96	0.71
	712 (936)	N/A	N/A	N/A	1	1.12	1.02

N/A: not applicable

5. Conclusions

In this study, a novel Abi system was identified on prophage regions of three *Lb. brevis* strains which could explain the high resistance of UCCLBBS124 against phage infection. This Abi system comprises two translationally coupled ORFs, both required for conferring phage resistance to the host. The AbiL$_{124}$ system was shown to be active against certain *Lb. brevis* phages and more specifically to the ones grouped as *Myoviridae* Morphotype I (see Chapter VI). Consequences of this AbiL$_{124}$ system on targeted phages are elimination of their efficiency of plaquing, a reduction in their adsorption efficiency and a significant decline in the number of progeny phage released. A phage mutant escaping this AbiL$_{124}$ system was isolated and revealed mutation in the gene encoding the Tal protein which suggests that it functions as the trigger for the activation of the AbiL$_{124}$ system after infection of the host in order to cause cell death.

Interestingly, the Abi system identified in this study on *Lb. brevis* prophages showed to be active against phages infecting *L. lactis* strains revealing a broad activity range. The phage resistance system described here shows similarities and impacts on phage/host interaction that are consistent with other Abi systems, though further experiments are needed to elucidate if this phage resistance mechanism is due to the inability of the phage to penetrate the host (low adsorption efficiency) or to exit the host once inside. The present work presented here reveals new insights into phage resistance mechanisms in *Lb. brevis* strains. Considering the negative impact of *Lb. brevis* strains on beer spoilage and the increased demand in bioremediation process during the fermentation process, it is important to understand natural phage defence systems in order to develop effective phage-based treatments to eliminate bacterial beer spoilage.

6. References

1. Meignen, B.; Onno, B.; Gélinas, P.; Infantes, M.; Guilois, S.; Cahagnier, B. Optimization of sourdough fermentation with *Lactobacillus brevis* and baker's yeast. *Food Microbiology* **2001**, *18*, 239-245, doi:https://doi.org/10.1006/fmic.2000.0395.
2. Endersen, L.; O'Mahony, J.; Hill, C.; Ross, R.P.; McAuliffe, O.; Coffey, A. Phage therapy in the food industry. *Annual Review of Food Science and Technology* **2014**, *5*, 327-349, doi:10.1146/annurev-food-030713-092415.
3. Mahony, J.; McAuliffe, O.; Ross, R.P.; van Sinderen, D. Bacteriophages as biocontrol agents of food pathogens. *Current Opinion in Biotechnology* **2011**, *22*, 157-163, doi:10.1016/j.copbio.2010.10.008.
4. Deasy, T.; Mahony, J.; Neve, H.; Heller, K.J.; van Sinderen, D. Isolation of a virulent *Lactobacillus brevis* phage and its application in the control of beer spoilage. *Journal of Food Protection* **2011**, ***74***, 2157-2161, doi:10.4315/0362-028x.jfp-11-262.
5. Feyereisen, M.; Mahony, J.; Lugli, G.A.; Ventura, M.; Neve, H.; Franz, C.M.A.P.; Noben, J.-P.; O'Sullivan, T.; van Sinderen, D. Isolation and characterization of *Lactobacillus brevis* phages. *Viruses* **2019**, *11*, 393.
6. Mahony, J.; McGrath, S.; Fitzgerald, G.F.; van Sinderen, D. Identification and characterization of lactococcal-prophage-carried superinfection exclusion genes. *Applied and Environmental Microbiology* **2008**, *74*, 6206-6215, doi:10.1128/aem.01053-08.
7. Kelleher, P.; Mahony, J.; Schweinlin, K.; Neve, H.; Franz, C.; van Sinderen, D. Assessing the functionality and genetic diversity of lactococcal prophages. *International Journal of Food Microbiology* **2018**, *272*, doi:10.1016/j.ijfoodmicro.2018.02.024.

8. Deng, Y.-M.; Liu, C.-Q.; W. Dunn, N. Genetic organization and functional analysis of a novel phage abortive infection system, AbiL, from *Lactococcus lactis*. *Journal of Biotechnology* **1999**, *67*, 135-149, doi:https://doi.org/10.1016/S0168-1656(98)00175-8.

9. McGrath, S.; Fitzgerald, G.F.; van Sinderen, D. Identification and characterization of phage-resistance genes in temperate lactococcal bacteriophages. *Molecular Microbiology* **2002**, *43*, 509-520.

10. Chopin, M.-C.; Chopin, A.; Bidnenko, E. Phage abortive infection in lactococci: variations on a theme. *Current Opinion in Microbiology* **2005**, *8*, 473-479.

11. Hill, C. Bacteriophage and bacteriophage resistance in lactic acid bacteria. *FEMS Microbiology Reviews* **1993**, *12*, 87-108, doi:10.1111/j.1574-6976.1993.tb00013.x.

12. Moineau, S.; Durmaz, E.; Pandian, S.; Klaenhammer, T.R. Differentiation of two abortive mechanisms by using monoclonal antibodies directed toward lactococcal bacteriophage capsid proteins. *Applied and Environmental Microbiology* **1993**, *59*, 208-212.

13. Garvey, P.; Fitzgerald, G.F.; Hill, C. Cloning and DNA sequence analysis of two abortive infection phage resistance determinants from the lactococcal plasmid pNP40. *Applied and Environmental Microbiology* **1995**, *61*, 4321-4328.

14. Parreira, R.; Ehrlich, S.D.; Chopin, M.C. Dramatic decay of phage transcripts in lactococcal cells carrying the abortive infection determinant AbiB. *Molecular Microbiology* **1996**, *19*, 221-230.

15. O'Connor, L.; Coffey, A.; Daly, C.; Fitzgerald, G.F. AbiG, a genotypically novel abortive infection mechanism encoded by plasmid pCI750 of *Lactococcus lactis* subsp. *cremoris* UC653. *Applied and Environmental Microbiology* **1996**, *62*, 3075-3082.

16. Eguchi, T.; Doi, K.; Nishiyama, K.; Ohmomo, S.; Ogata, S. Characterization of a phage resistance plasmid, pLKS, of silage-making *Lactobacillus plantarum* NGRI0101. *Bioscience, Biotechnology, and Biochemistry* **2000**, *64*, 751-756, doi:10.1271/bbb.64.751.

17. Feyereisen, M.; Mahony, J.; Kelleher, P.; Roberts, R.J.; O'Sullivan, T.; Geertman, J.-M.A.; van Sinderen, D. Comparative genome analysis of the *Lactobacillus brevis* species. *BMC Genomics* **2019**, *20*, 416, doi:10.1186/s12864-019-5783-1.

18. Kuipers, O.P.; de Ruyter, P.G.G.A.; Kleerebezem, M.; de Vos, W.M. Quorum sensing-controlled gene expression in lactic acid bacteria. *Journal of Biotechnology* **1998**, *64*, 15-21, doi:https://doi.org/10.1016/S0168-1656(98)00100-X.

19. Braun Jr, V.; Hertwig, S.; Neve, H.; Geis, A.; Teuber, M. Taxonomic differentiation of bacteriophages of *Lactococcus lactis* by electron microscopy, DNA-DNA hybridization, and protein profiles. *Microbiology* **1989**, *135*, 2551-2560.

20. Christiansen, B.; Johnsen, M.; Stenby, E.; Vogensen, F.; Hammer, K. Characterization of the lactococcal temperate phage TP901-1 and its site-specific integration. *Journal of Bacteriology* **1994**, *176*, 1069-1076.

21. Blatny, J.M.; Godager, L.; Lunde, M.; Nes, I.F. Complete genome sequence of the *Lactococcus lactis* temperate phage φLC3: comparative analysis of φLC3 and its relatives in lactococci and streptococci. *Virology* **2004**, *318*, 231-244, doi:https://doi.org/10.1016/j.virol.2003.09.019.

22. Mahony, J.; Oliveira, J.; Collins, B.; Hanemaaijer, L.; Lugli, G.A.; Neve, H.; Ventura, M.; Kouwen, T.R.; Cambillau, C.; van Sinderen, D. Genetic and functional characterisation of the lactococcal P335 phage-host interactions. *BMC Genomics* **2017**, *18*, 146, doi:10.1186/s12864-017-3537-5.

23. Oliveira, J.; Mahony, J.; Hanemaaijer, L.; Kouwen, T.R.H.M.; van Sinderen, D. Biodiversity of bacteriophages infecting *Lactococcus lactis* starter cultures. *Journal of Dairy Science* **2018**, *101*, 96-105, doi:https://doi.org/10.3168/jds.2017-13403.

24. Theodorou, I. Investigation of glycopolymer assembly systems in *Lactococcus lactis*. University College Cork, Ireland, cora.ucc.ie, **2019**.

25. Mahony, J.; Deveau, H.; Mc Grath, S.; Ventura, M.; Canchaya, C.; Moineau, S.; Fitzgerald, G.F.; van Sinderen, D. Sequence and comparative genomic analysis of lactococcal bacteriophages jj50, 712 and P008: evolutionary insights into the 936 phage species. *FEMS Microbiology Letters* **2006**, *261*, 253-261.

26. Bebeacua, C.; Tremblay, D.; Farenc, C.; Chapot-Chartier, M.-P.; Sadovskaya, I.; Van Heel, M.; Veesler, D.; Moineau, S.; Cambillau, C. Structure, adsorption to host, and infection mechanism of virulent lactococcal phage p2. *Journal of Virology* **2013**, *87*, 12302-12312.

27. Chandry, P.S.; Moore, S.C.; Boyce, J.D.; Davidson, B.E.; Hillier, A.J. Analysis of the DNA sequence, gene expression, origin of replication and modular structure of the Lactococcus lactis lytic bacteriophage sk1. *Molecular Microbiology* **1997**, *26*, 49-64.

28. Samson, J.E.; Moineau, S. Characterization of *Lactococcus lactis* phage 949 and comparison with other lactococcal phages. *Applied and Environmental Microbiology* **2010**, *76*, 6843-6852.

29. Mahony, J.; Randazzo, W.; Neve, H.; Settanni, L.; van Sinderen, D. Lactococcal 949 group phages recognize a carbohydrate receptor on the host cell surface. *Applied and Environmental Microbiology* **2015**, *81*, 3299-3305.

30. Villion, M.; Chopin, M.-C.; Deveau, H.; Ehrlich, S.D.; Moineau, S.; Chopin, A. P087, a lactococcal phage with a morphogenesis module similar to an *Enterococcus faecalis* prophage. *Virology* **2009**, *388*, 49-56.

31. Svensson, U.; Christiansson, A. Methods for phage monitoring. *Bulletin of the International Dairy Federation* **1991**, *263*, 29-39.

32. Zhou, Y.; Liang, Y.; Lynch, K.H.; Dennis, J.J.; Wishart, D.S. PHAST: a fast phage search tool. *Nucleic Acids Research* **2011**, *39*, 347-352, doi:10.1093/nar/gkr485.

33. Arndt, D.; Grant, J.R.; Marcu, A.; Sajed, T.; Pon, A.; Liang, Y.; Wishart, D.S. PHASTER: a better, faster version of the PHAST phage search tool. *Nucleic Acids Research* **2016**, *44*, W16-21, doi:10.1093/nar/gkw387.

34. McGrath, S.; Fitzgerald, G.F.; van Sinderen, D. Improvement and optimization of two engineered phage resistance mechanisms in *Lactococcus lactis*. *Applied and Environmental Microbiology* **2001**, *67*, 608-616.

35. van Pijkeren, J.-P.; Britton, R.A. High efficiency recombineering in lactic acid bacteria. *Nucleic Acids Research* **2012**, *40*, e76-e76.

36. Ahrné, S.; Molin, G.; Axelsson, L. Transformation of *Lactobacillus reuteri* with electroporation: studies on the erythromycin resistance plasmid pLUL631. *Current Microbiology* **1992**, *24*, 199-205, doi:10.1007/bf01579282.

37. Garvey, P.; Hill, C.; Fitzgerald, G. The lactococcal plasmid pNP40 encodes a third bacteriophage resistance mechanism, one which affects phage DNA penetration. *Applied and Environmental Microbiology* **1996**, *62*, 676-679.

38. Collins, B.; Bebeacua, C.; Mahony, J.; Blangy, S.; Douillard, F.P.; Veesler, D.; Cambillau, C.; van Sinderen, D. Structure and functional analysis of the host recognition device of lactococcal phage Tuc2009. *Journal of Virology* **2013**, *87*, 8429-8440, doi:10.1128/jvi.00907-13.

39. Sing, W.D.; Klaenhammer, T.R. Characteristics of phage abortion conferred in lactococci by the conjugal plasmid pTR2030. *Microbiology* **1990**, *136*, 1807-1815, doi:https://doi.org/10.1099/00221287-136-9-1807.

40. Ravin, V.; Räisänen, L.; Alatossava, T. A conserved C-terminal region in Gp71 of the small isometric-head phage LL-H and ORF474 of the prolate-head phage JCL1032 is implicated in specificity of adsorption of phage to its host, *Lactobacillus delbrueckii*. *Journal of Bacteriology* **2002**, *184*, 2455-2459, doi:10.1128/jb.184.9.2455-2459.2002.

41. Stockdale, S.R.; Mahony, J.; Courtin, P.; Chapot-Chartier, M.-P.; Van Pijkeren, J.-P.; Britton, R.A.; Neve, H.; Heller, K.J.; Aideh, B.; Vogensen, F.K.; van Sinderen, D. The lactococcal phages Tuc2009 and TP901-1 incorporate two alternate forms of their tail fiber into their virions for infection specialization. *Journal of Biological Chemistry* **2013**, *288*, 5581-5590.

Chapter VIII

Discussion and future perspectives

Despite the significant problem of beer spoilage caused by *Lactobacillus brevis*, the mechanisms by which they can survive and grow in this harsh environment remain only partially characterized. Just a small number of genes involved in beer spoilage have been identified, of which most represent genetic entities associated with hop resistance, such as *horA, hitA,* and *horC*. However, since none of these genes is universally present in *Lb. brevis* beer-spoiling strains, it is likely that there are (perhaps many) other molecular players involved in beer spoilage. The genes involved in beer spoilage are typically present on mobile genetic elements such as plasmids. In addition to being located on such extrachromosomal elements, it is possible that chromosomally-encoded genes are involved in this process, although data regarding such molecular elements is very limited to date. The aim of this thesis work was therefore to identify and characterize novel molecular players involved in beer spoilage by *Lb. brevis*.

In Chapter II, the genome sequences of six isolated *Lb. brevis* strains were determined and added to thirteen complete genomes of *Lb. brevis* already publicly accessible, thus considerably increasing the overall genomic data available for this species. The accompanied comparative genome analysis of *Lb. brevis* species generated novel insights into the species itself as well as on *Lb. brevis* as a beer-spoiling microorganism. It highlighted the intricacies of bacterial beer spoilage and allowed the identification of potential chromosomally- and plasmid-encoded genes involved in beer spoilage. These data were shown to be important for the identification of novel genes and/or plasmids involved in beer adaptation (see subsequent chapters of the thesis). Currently available PCR-based rapid detection kits for beer-spoiling bacteria rely on either the detection of 16S RNA genes or the hop resistance genes *horA* and *horC*. Using the genomic dataset obtained as part of this thesis it would be feasible to develop a multiplex PCR-typing approach to target a combination of genes highlighted in this study, as being prevalent among beer-spoiling strains of *Lb. brevis*. This approach could then be implemented as a complement to PCR-based approach already used in breweries during the quality control step to detect potential bacterial contamination of the beer. Furthermore, it is expected that advances

in sequencing technologies and the availability of newly isolated strains of *Lb. brevis* will in the coming years facilitate further expansion of the number of available genome sequences of *Lb. brevis*, thus increasing and refining the dataset presented here.

Chapters III and IV describe the identification of two genes that are involved in beer survival and spoilage. The first of these encodes a presumed manganese transporter that plays a role in pH tolerance, and thus beer spoilage. The second identified gene encodes a putative glycosyltransferase and reinforced the involvement of plasmids in hop tolerance by *Lb. brevis*. These results considerably enhance our understanding of the strain-specific nature of *Lb. brevis* as a beer spoiler, providing insights into the beer-imposed hurdles that need to be overcome. Further in depth investigations are needed in order to dissect the precise molecular mechanisms of action whereby these two identified genes enable growth in beer, while other resistance parameters will also be worth investigating, in particular ethanol resistance, which is currently poorly explored. Furthermore, it is now clear that adaptation to beer will require several resistance mechanisms (such as resistance to low pH, ethanol and hop compounds) involving distinct chromosomally- and/or plasmid-encoded genes (i.e. $mntH_{0274}$, *horA*, *hitA* or gtf_{D15}). However, a significant amount of research is still required in order to fully understand the complexity of beer spoilage and the interactions between the different beer spoilage-related genes that facilitate this. Study of functional complementarity and redundancy among these genes will very much help in the understanding of the adaptation process to the beer environment by *Lb. brevis*.

Current approaches to maintain the quality and safety of beer includes strict cleaning and sanitation practices, however, the overuse of chemical sanitizers has led to biocidal resistance of certain food/beverage spoilage bacteria. Moreover, an increased consumer awareness and demand for safe, natural and eco-friendly preservatives are leading the food and beverage industry to look for and implement novel approaches to control bacterial spoilage. Among these alternatives, bacteriophage

therapy is becoming prevalent as an alternative to chemical solutions to prevent bacterial food and/or beverage spoilage. Bacteriophages infecting *Lb. brevis* strains are understudied compared to phages infecting other lactic acid bacteria such as *Lactococcus lactis* or *Streptococcus thermophilus.* Only reports on the isolation of a single temperate (LBR48) and a single lytic phage (SA-C12) of *Lb. brevis* have been described, the latter exhibiting lytic activity against a beer-spoiling *Lb. brevis* strain. The work executed as part of this thesis reports on the isolation and characterization of novel phages that infect strains of *Lb. brevis* as well as studying the biodiversity among virulent and temperate phages of *Lb. brevis*.

With the study and characterization of virulent and temperate *Lb. brevis* phages in Chapters V and VI, significant data have been generated that considerably extend our knowledge of the genetic and morphological diversity of *Lb. brevis* phages. Furthermore, their potential for industrial application was explored as a means to reduce the microbial load of beer using natural methods. Surprisingly, the difficulties encountered when trying to isolate virulent phages active against *Lb. brevis* strains raises a number of questions: (i) are these entities rare, which is not typical for LAB phages (such as *Lactococcus lactis* or *Lactobacillus delbrueckii* phages); (ii) should the screening for *Lb. brevis* phages be performed using samples obtained from different environments, closer to its natural ecological niche; (iii) do *Lb. brevis* carry phage resistance mechanisms to prevent phage infection? In this case adopting a high-throughput screening methodology (increasing the number of environmental samples and *Lb. brevis* tested in a single experiment) may be beneficial to more successfully and efficiently isolate phages against *Lb. brevis* strains. Some phages were shown to exhibit a negative impact on the growth of beer-spoiling strains of *Lb. brevis* and may represent potential approaches to control bacterial spoilage of beer. This negative impact may be caused by the action of exogenous phage-encoded lysins on the bacteria providing a perspective for phage therapy in which overexpressing and purifying proteins with lysin activity could be used as an

antimicrobial during the brewing process to limit and prevent bacterial spoilage of beer (i.e. used as a surface decontaminant for BS eradication). However, this technique presents a potential drawback with the high cost of producing lysins as well as costs related to regulatory issues (e.g. immunogenicity). Phage lysins could eventually be used, together with other systems and in compliance with rigorous Good Manufacturing Practices, in the context of a hurdle strategy.

Strains of *Lb. brevis* are typically robust to bacteriophage infection rendering their removal difficult in an industrial context. The work described in Chapters VI and VII was aimed at assessing the role of temperate phages in the development of such resistance in *Lb. brevis*. In this thesis, a prophage-encoded phage resistance mechanism was identified benefiting the host and increasing its overall fitness. The success of using virulent phages in a bioremediation process in that case, is compromised by the presence of such prophage-encoded phage-resistance systems carried by beer-spoiling strains of *Lb. brevis.* An alternative approach to eliminate BS *Lb. brevis* strains could be the induction of resident prophages leading to the death of the bacterial spoiler. Indeed, temperate phages are widely present among *Lb. brevis* including beer-spoiling strains and these prophages are inducible using UV light for example. Therefore, prophage induction using UV irradiation of surfaces could be added as an additional step of the brewing process, thus avoiding the addition of virulent phages and the approval of regulatory authorities.

While the scientific findings presented in this thesis significantly add to the state of the art of *Lb. brevis* genomics, bacteriophages and phage bioremediation to control beer spoilage, knowledge gaps remain to be filled to fully understand the mechanisms by which *Lb. brevis* is able to grow in beer, and regarding the efficacy and safety of using phages in food and/or beverage production. Consumers should also be informed of these developments so they can fully appreciate the efforts made by the producer in making safe and high quality products,

while they may also contribute to provide guidance with regards to the changes they want (i.e. natural, eco-friendly, safe products).

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