Determination and Expression Analysis of Functional Genes in *Lactobacillus plantarum*

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Ich versichere, dass ich meine Dissertation mit dem Titel "Determination and Expression Analysis of Functional Genes in *Lactobacillus plantarum*" selbständig, ohne unerlaubte Hilfe angefertigt und mich dabei keiner anderen als der von mir ausdrücklich bezeichneten Quellen und Hilfen bedient habe.

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Karlsruhe, 09.03.2010 Gyu-Sung Cho

Zusammenfassung

In dieser Studie wurde die Bacteriocinproduktion von zwei *Lactobacillus plantarum* Stämmen und ihre Wirksamkeit als Schutzkulturen zur Biokonservierung von Truthahnfleisch untersucht. Da Bakterien als Starterkulturen zur Lebensmittelherstellung, als Schutzkulturen oder als Probiotika eingesetzt werden, ist es zuerst notwendig die Sicherheit dieser Stämme bezüglich ihrer Antibiotikaresistenz zu untersuchen Daher wurde in dieser Studie das Vorhandensein von Antibiotikaresistenzen der *Lactobacillus plantarum* Stämme BFE 5092 und PCS20 bestimmt. Diese *Lactobacillus plantarum* Stämme zeigten eine intrinsische Resistenz gegen bestimmte Antibiotika, d.h. Vancomycin und Streptomycin. Genetisch übertragbare Resistenzen, wie z.B. Resistenzen gegen Tetracyclin, Erythromycin oder Chloramphenicol, wurden nicht nachgewiesen. Diese Stämme konnten bezüglich ihrer Antibiotikaresistenzen somit als sicher angesehen werden. Dies ist Vorraussetzung, um den Status der qualifizierten Sicherheitsannahme („Qualified Presumption of Safety“, QPS) der europäischen Behörde für Lebensmittelsicherheit („European Safety Authority“, EFSA) für mikrobielle Stämme als Lebensmittelzusatz zu erhalten.

Lactobacillus plantarum Stämme produzieren eine Vielfalt chromosomal kodierter Bacteriocine und oftmals werden mehrere Bacteriocine von einem einzigen Stamm kodiert. In dieser Studie wurden die genetischen Loci für Bacteriocinproduktion der *Lactobacillus plantarum* Stämme BFE 5092 und PCS20 vollständig analysiert. Ein PCR „Screening“ für verschiedene, bekannte Plantaricingene zeigte, das *Lactobacillus plantarum* BFE 5092 die Gene für *pln*EF, *pln*JK und *pln*N besaß, wohingegen der Stamm PCS20 nur positiv für das *pln*EF Gene war. Weitere Untersuchungen des Bacteriocin-Locus von BFE 5092 zeigten beträchtliche Ähnlichkeiten zu Plantaricin Loci vorher beschriebener *Lactobacillus plantarum* Stämme C11 und WCFS1. Jedoch war der Locus von *Lactobacillus plantarum* PCS20 ungewöhnlich, da er eine Mutation, als Resultat einer Deletion innerhalb des *pln*E Gens, zeigte. Diese Deletionen resultierten in einem hypothetisch produzierten Peptid, welche zwei Aminosäuren kürzer als Plantaricin E ist. Weiterhin unterschied es sich durch 24 Aminosäuren und besaß aber auch 30 identische Aminosäuren (15 am Aminoende und 15 am Carboxylende). Als Konsequenz hieraus ist die Aminosäuresequenz insofern verändert als dass kein funktionelles Signalpeptid vom „double-glycine“ Typ kodiert wird. Dies bedeutete wiederum, dass kein funktionelles Peptid produziert wird, obwohl RT-PCR Untersuchungen zeigten, dass das *pln*E Gen offensichtlich exprimiert wurde. Außerdem war ein Transposase Gen stromaufwärts im Plantaricin EFI Gencluster lokalisiert. Dieses war im Bacteriocin-Regulatorgen dem Histidin Protein Kinase Gen eingefügt. Zusammenfassend lässt sich sagen, dass diese Fakten auf einen Verlust der Plantaricingen-Funktion in *Lactobacillus plantarum* PCS20 als Resultat einer Transposition und Mutation hinwiesen.

Aufgrund des Vorhandenseins des mutierten Plantaricingens in *Lactobacillus plantarum*

PCS20 wurde nur der Stamm *Lactobacillus plantarum* BFE 5092 auf eine mögliche Anwendung als Schutzkultur zur Konservierung von Truthahnfleisch mit *Salmonella* Typhimurium oder *Listeria monocytogenes* als Zielpathogene näher untersucht. Der Stamm *Lactobacillus plantarum* BFE 5092 wuchs (wenn auch nicht sehr gut) und produzierte Bacteriocin bei 8 oder 10°C und auch während des sessilen Wachstums auf Truthahnfleisch. Jedoch war dieser Stamm nicht in der Lage das Wachstum der Zielpathogene auf Truthahnfleisch unter den in dieser Studie verwendeten Bedingungen zu unterdrücken. Dies resultierte höchstwahrscheinlich daraus, dass dieser Stamm nicht in der Lage war bei geringen Temperaturen zu wachsen und in dieser Umgebung nicht wettbewerbsfähig war. Obwohl dieser Stamm für diesen Produkttyp in dieser Studie nicht geeignet erschien, könnte er trotzdem zur Biokonservierung anderer Lebensmittel, die unter anderen Bedingungen produziert und gelagert werden und für das Wachstum und die Bacteriocinproduktion dieses Stammes (*Lactobacillus plantarum* BFE 5092) besser geeignet sind, erfolgreich eingesetzt werden. Dies könnte in weiteren Studien untersucht werden.

ABSTRACT

In this study, the bacteriocin production of two *Lactobacillus plantarum* strains was investigated and their effectiveness as protective cultures for the biopreservation of turkey meat was assessed. As bacteria used as starter cultures for the production of foods, as protective cultures or as probiotics, could possible contain antibiotic resistance genes, it was first necessary to determine the safety of these strains as pertaining to their antibiotic resistances. Therefore, in this study the incidence of antibiotic resistance was determined for the *Lactobacillus plantarum* strains BFE 5092 and PCS20. These *Lactobacillus plantarum* strains only showed an intrinsic resistance towards certain antibiotics, i.e., vancomycin and streptomycin, and no genetically transferable resistances, such as resistance towards tetrycycline, erythromycin or chloramphenicol, were detected. Thus, the strains could be regarded as safe from an antibiotic resistance point of view, which was considered as a prerequisite for obtaining 'Qualified Presumption of Safety' (QPS) status as proposed by the European Food Safety Authority (EFSA) for microbial strains added to foods.

Lactobacillus plantarum strains produce a variety of chromosomally encoded bacteriocins and often multiple bacteriocins are encoded by a single strain. In this study, the genetic loci for bacteriocin production of *Lactobacillus plantarum* strains BFE 5092 and PCS20 were completely analysed. PCR screening for various known plantaricin genes showed that *Lactobacillus plantarum* BFE strain 5092 contained the genes for *pln*EF, *pln*JK and *pln*N, while strain PCS20 was only positive for the *pln*EF genes. Further investigation of the bacteriocin locus of strain BFE 5092 showed remarkable similarity to the plantaricin loci previously described for *Lactobacillus plantarum* strains C11 and WCFS1. However, the locus of the *Lactobacillus plantarum* PCS20 strain was unusual in that it showed a mutation as a result of deletions within the *pln*E gene. These deletions led to a hypothetically produced peptide which is 2 amino acids shorter than plantaricin E. Furthermore, it differs by 24 amino acids, while it shares 30 identical amino acids, i.e., 15 at the amino end and 15 at the carboxyl end of the hypothetical peptide. As a consequence, the amino acid sequence is changed such that a double-glycine-type leader peptide would not be encoded. This implied that a functional peptide was not being produced, even though RT-PCR studies showed that the *pln*E gene was obviously expressed. Furthermore, a transposase gene was located upstream of the plantaricin EFI gene cluster. This was inserted into a bacteriocin regulatory gene, the histidine protein kinase gene. Taken together, these facts indicated a loss of plantaricin gene function in *Lactobacillus plantarum* PCS20 as a result of transposition and mutation.

Because of the presence of a mutated plantaricin gene in *Lactobacillus plantarum* PCS20, only the *Lactobacillus plantarum* BFE 5092 strain was further investigated for its possible application as protective cultures in preservation on turkey meat using *Salmonella* Typhimurium or *Listeria monocytogenes* as target pathogens. The protective *Lactobacillus*

plantarum BFE 5092 grew (although not well) and produced bacteriocin at 8 or 10°C as well as during sessile growth on turkey meat. This strain, however, was not capable of inhibiting the growth of the target pathogens on the turkey meat under the conditions of this study, most probably as a consequence of its low growth capability at low temperature, and its failure to compete in this environment. Although this strain was unsuitable for the type of product used in this study, it may nevertheless be successful for biopreservation of other food commodities, which are produced and stored under conditions and may be better suited for the growth and bacteriocin production of *Lactobacillus plantarum* BFE 5092, which could be further investigated in future studies.

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INTRODUCTION

1.1 Lactic Acid Bacteria

1.1.1 Taxonomy of the lactic acid bacteria

The *Firmicutes* and *Actinobacteria* are the only Gram-positive phyla among the major eubacterial lineages. The *Firmicutes* consist of the classes *Clostridia* (class I), *Mollicutes* (class II) and *Bacilli* (Class III). The lactic acid bacteria (LAB) occur in class III, order II i.e., the '*Lactobacillales*' (Garrity and Holt, 2001). Lactic acid bacteria can generally be described as Gram-positive, non-motile, non-sporeforming, rod- or coccus-shaped organisms that ferment carbohydrates to form mainly lactic acid. Early LAB taxonomy was based on morphological and physiological features such as determination of end products of metabolism, the enantiomer of lactic acid produced, composition of cell wall amino acids as well as sugar fermentation patterns (Stiles and Holzapfel, 1997). However, a modern approach for classification of LAB is based on the premise that an unequivocal definition does not exist for this group of bacteria (Stiles and Holzapfel, 1997; Axelsson, 2004). Therefore, it is more appropriate to describe the 'typical' lactic acid bacterium, which is Gram-positive, non-sporeforming, catalase-negative, devoid of cytochromes, non-aerobic but aerotolerant, fastidious, acid-tolerant and strictly fermentative, with lactic acid as the major end-product of sugar fermentation (Klein et al., 1998; Axelsson, 2004). Variations of this general description are common, and it is only the Gram-positive character really that cannot be argued with (Axelsson, 2004).

The common ancestor of the order *Lactobacillales* to which all LAB belong developed from a common ancestor of all *Bacilli* (Makarova et al., 2006). The evolution of the *Lactobacillales* involved extensive loss of ancestral genes. Thus, while the common ancestor of the *Bacilli* possessed about 2700 to 3700 genes, the common ancestor of the *Lactobacillales* possessed about 2100-2200 genes, losing 600-1200 genes during evolution and these gene reductions were most probably a result of adaptation to nutritionally rich habitats (Makarova et al., 2006). Genes for biosynthesis of cofactors such as heme, molybdenum coenzyme, and panthothenate were lost, while cofactor transporters were acquired e.g., nicotinamide mononucleotide transporter (Makarova and Koonin, 2007). Another notable acquisition was a group of diverse peptidases, which are important commodities in the protein rich environments inhabited by LAB. The loss of heme/copper type cytochromes/quinol oxidase-related genes and catalase are characteristic for aerobic bacteria, indicating that the ancestor of *Lactobacillales* was a microaerophile or an anaerobe (Makarova and Koonin, 2007). Beyond gene loss, the *Lactobacillales* exhibit clear ancestral adaptations for nutritionally rich and microaerophilic environments, which include acquisition of genes via horizontal gene transfer and duplication of

genes for various enzymes and transporters of sugar and amino acid metabolism (Makarova et al., 2006, Makarova and Koonin, 2007).

Before the advent of bacterial genomics, the taxonomy of the LAB was somewhat unclear. Based mostly on 16S rRNA gene sequences, three closely related lineages of the LAB were initially described by Woese (1987), i.e. the *Leuconostoc* group, the *Lactobacillus casei/Pediococcus* group and the *Lactobacillus delbrueckii* group. *Carnobacterium, Enterococcus, Vagococcus, Aerococcus* and *Tetragenococcus* were considered more closely related to each other than to any other LAB, while *Lactococcus* and *Streptococcus* appeared to be very closely related to each other and were described to form a separate branch (Schleifer und Ludwig, 1995). The genus *Lactobacillus* alone, which contains more than 145 species, is unusually diverse and its taxonomy has long been considered unsatisfactory because of the highly heterogenous nature of its members (Schleifer and Ludwig, 1995; Cachaya et al., 2006; Felis and Dellaglio, 2007).

The recent availability of complete genomes of representative LAB strains of all major families of the *Lactobacillales* enables a more definitive analysis of their evolutionary relationships (Makarova et al., 2006; Makarova and Koonin, 2007). Accordingly, the streptococci-lactococci branch is considered to be basal in the *Lactobacillales* tree, and the *Pediococcus* group is a sister to the *Leuconostoc* group within the *Lactobacillus* clade. Thus, the *Lactobacillus* genus appeared to be paraphyletic with respect to the *Pediococccus-Leuconostoc* group and *L. casei* was placed at the base of the *L. delbrueckii* group (Makarova et al., 2006; Makarova and Koonin, 2007). Makarova et al. (2006) and Makarova and Koonin (2007) showed on the basis of a phylogenetic tree, reconstructed from concatenated alignments of four subunits of the DNA-dependant RNA polymerase sequences, a division of *Lactobacillus* into three distant groups, the first being comprised of *L. brevis, L. plantarum* and *P. pentosaceus* to which *L. salivarius* was basal. The second group consisted of *L. gasseri, L. johnsonii*, *L. delbrueckii* and *L. acidophilus* (Makarova and Koonin, 2007). An additional branch with *Leuconostoc mesenteroides* and *Oenococcus oeni* is wedged between *L. brevis, P. pentosaceus* and the *L. plantarum* group and *L. salivarius,* and thus *L. salivarius* is also basal to this branch. The third group consisted of *Lactobacillus casei* and *L. sakei* and was basal to the *L. gasseri, L. johnsonii, L. delbrueckii* and *L. acidophilus* groups (Makarova and Koonin, 2007) (Figure 1.1).

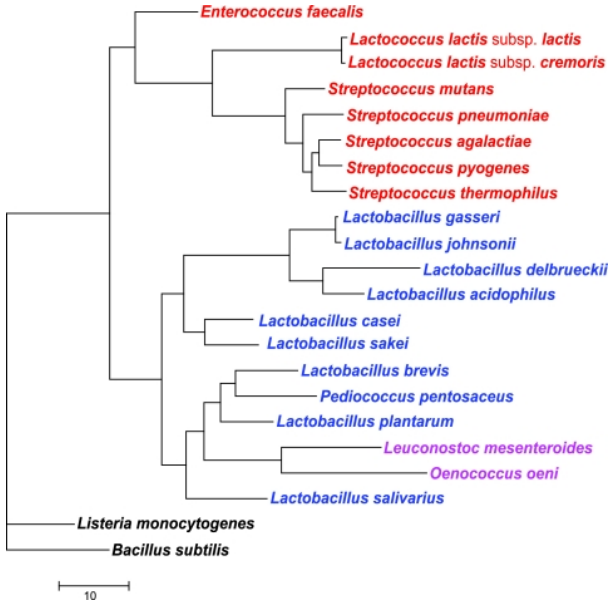

Figure 1.1 Phylogenetic tree of the *Lactobacillales* constructed on the basis of concatenated alignments of four (α,β,β',δ) of the DNA—dependant RNA polymerase subunit protein sequences (adapted from Makarova and Koonin, 2007).

Whole genome comparisons of five *Lactobacillus* species (*L. salivarius*, *L. plantarum*, *L. acidophilus*, *L. johnsonii* and *L. sakei*) that were completely sequenced showed that there is no extensive synteny of the genome sequences of these five species (Canchaya et al., 2006), and the observed extreme divergence of the *Lactobacillus* genomes supports the recognition of new subdivisions as proposed by Dellaglio and Felis (2005). Whole genome alignments showed that the sequences with the best alignments were *L. johnsonii* and *L. acidophilus*, but alignments of these two species with the other three species showed much lower degrees of synteny at the interspecies level, than observed in other species genome comparisons with high and low-G+C content Gram-positive bacteria (Canchaya et al., 2006). These stepwise-decreasing degrees of similarity observed after genome alignments of members of the *L. delbrueckii* / *L. acidophilus* group were considered as a 'hallmark of Darwinian evolution' by Berger et al. (2007). Claessen et al. (2008) used the genomic data from 12 *Lactobacillus* strains to investigate whether a single, congruent phylogeny could be inferred. By reconstructing phylogenetic tress from concatenated sequences of 141 core proteins, as well as concatenated RNA polymerase subunit sequences, considerable incongruence was noticed, but it was still possible to distinguish four subgeneric groups i.e., group A (*L. acidophilus, L. helveticus, L. delbrueckii* ssp. *bulgaricus, L. johnsonii* and *L. gasseri*), group B (*L. salivarius, L. plantarum, L. reuteri, L. brevis* and *P. pentosaceus*), group C (*L. sakei* and *L. casei*) and group D (*L. mesenteroides* and *O. oeni*) (Claesson et al., 2008). However, the authors concluded that based on significantly different branching patterns within some groups and the availability of genomic data for too few members of the groups, three of the four groups could not confidently be identified as candidate novel genera within the current genus (Claesson et al., 2008).

The genera of LAB associated with foods include *Lactococcus, Enterococcus, Streptococcus, Pediococcus, Tetragenococcus, Leuconostoc, Weissella* and *Oenococcus* which have coccus shaped cells, as well as the genera *Carnobacterium* and *Lactobacillus* which are rod-shaped. The genus *Weissella* is somewhat unusual in that it includes some species which are rod-shaped, while others are coccus-shaped. In addition, these genera are furthermore quite heterogenous in their nutrient requirements, growth niches, growth temperatures and other phenotypic properties, and also differ in the respective pathways they utilise for metabolism.

1.1.2 Lactic Acid Bacteria Metabolism

Metabolically, LAB are on the threshold of anaerobic-to-aerobic life. They possess efficient carbohydrate fermentation pathways coupled with substrate level phosphorylation (Hammes and Hertel, 2009). In addition to substrate level phosphorylation, energy is generated by secondary transport systems including uniporters, proton-solute symporters and antiporters, all contributing to the generation of a proton motive force (Konings, 2002). Lactobacilli contain no isoprenoid quinones and no cytochromes systems to perform oxidative phosphorylation. However, they do possess flavine-containing oxidases and peroxidases to carry out the oxidation of $NADH_2$ with O_2 as the final electron acceptor (Hammes and Hertel, 2009).

LAB are all chemo-organotrophs, i.e., they require carbohydrates for their metabolism and growth. They have rather complex nutritional requirements and also need vitamins and amino acids, as well as nucleotides for growth (Axelsson, 2004). There are two major pathways that are utilized for hexoses sugars. The first pathway (Emden Meyerhof pathway) is through the glycolytic pathway by splitting fructose-1,6-disphosphate in two triose sugar moieties, which are further converted to private and finally to lactate (Kandler et al,. 1983; Axelsson, 2004), with a net gain of 2 ATP/mol glucose. As the major end product here is lactate, this fermentation is also known as a homolactic fermentation (Axelsson, 2004). The second one (phosphogluconate/phosphoketolase pathway) is characterized by an initial dehydrogenation step with the formation of 6-phosphogluconate by oxidation of glucose-6-phosphate. This is followed by a decarboxylation reaction and the remaining pentose-5-phosphate is split by phosphoketolase into glyceraldehyde-3-phosphate and acetyl-phosphate, which are further metabolized to lactate and ethanol with a net gain of 1ATP/mol glucose (Kandler et al,. 1983). If fructose is present as an alternative electron acceptor, acetyl-phosphate may also be converted to acetic acid, with the concurrent reduction of fructose to manitol. The end products of this pathway thus include lactic acid, acetic acid, ethanol and CO_2 and, based on the diversity of end products obtained this type of fermentation, it is also known as the heterolactic fermentation. Facultatively heterofermentative LAB can utilize pentoses such as arabinose or ribose, as they possess an inducible phosphoketolase and are thus able to convert these pentose sugars to glyceraldehyde-3-phosphate and acetyl-phosphate. Thus, they produce lactic and acetic acid

(or ethanol) as end-products of fermentation, without the production of CO_2 (Cogan, 1995; Axelsson, 2004).

Many LAB can also metabolize arginine by a deiminase pathway forming ornithine, citrulline, NH_3 and CO_2 and generating 1 mol ATP from each mol of arginine used in the process (Poolman, 1993; Chaillou et al., 2005; Cogan et al., 2006). It is also well known that LAB may change their metabolism in response to various conditions, resulting in a different end-product pattern than seen with glucose fermentation under normal conditions. In most of these cases, the change can be attributed to an altered pyruvate metabolism, the use of external electron acceptors, or both. Depending on conditions and enzymatic capacity, LAB can use alternative ways of utilizing pyruvate. For example, one of the well known pathways among the LAB is leading to diacetyl (butter aroma) and acetoin/2.3-butanediol. This pathway is very significant technologically in the fermentation of milk (Axelsson, 2004).

1.1.3 Lactic Acid Bacteria as Starters for Food Fermentations

Historically, food fermentation developed by default rather than by design (Stiles, 1996). The term fermentation is often used imprecisely when referring to foods (Adams, 1999). Strictly, it describes the type of energy yielding anaerobic metabolism in which an organic substrate is incompletely oxidized and an organic compound acts as an electron acceptor. Examples are the production of ethanol by yeasts and production of organic acids by e.g. the LAB. However, in a more general use, the term fermentation is applied to any foods that have been subjected to the action of microorganisms or enzymes, so that desirable biochemical changes cause significant modification of the food (Cambell-Platt, 1987). LAB play an important role in food fermentations, causing characteristic flavor changes and exercising a preservative effect on the fermented product (Stiles, 1996). It is estimated that 25% of the European diet and 60% of the diet in developing countries consist of fermented foods (Holzapfel, 2005).

The main effect of LAB in food fermentations is illustrated by their contribution to rapid acidification of the raw material by production mainly of lactic acid. In addition, some strains also produce other important compounds such as acetic acid, ethanol, aroma compounds, bacteriocins, exopolysaccharides and other important enzymes (e.g., proteases) that contribute to enhancing the shelf life and microbial safety of the fermented product and to improving the texture of product (Leroy and De Vuyst, 2004).

LAB are associated with dairy, vegetable, meat and cereal fermentations (Stiles and Holzapfel, 1997; Nout and Sarkar, 1999; Holzapfel, 2002). Such foods can be fermented in three different ways, based on the source of the starter cultures, i.e. 1) natural fermentation (spontaneous fermentation), 2) back slopping and 3) controlled fermentation. Spontaneous fermentation results from the competitive activities of different microorganisms present naturally on the raw material. The strains with the highest growth rate will dominate the fermentation and

as the natural microbial flora of the raw material is not always the same, it is difficult to produce a product with consistent quality over a long time (Ray, 2001). Backslopping involves the use of a residue from a previous fermentation batch of acceptable quality (Holzapfel, 1997). The drawback with this practice is that retention of product characteristics over a long period may be difficult also due to changes in microbial types (Ray, 2001). Controlled fermentations which rely on the use of starter cultures lead to decreased production times and products with a more consistent quality. The starters are usually adapted to the substrate and are added to the raw materials at high numbers, which serves to accelerate the fermentation process. This enables stricter control of the fermentation and the outcome is more predictable. The first pure starter cultures were strains of *Lactococcus lactis* introduced simultaneously in Denmark and Germany in 1890 in the fermentation of milk for cheese and sourmilk production (Holzapfel, 1997).

Still today, traditional technologies for processing cheese and fermented meat without using starter cultures are being applied even in developed countries (Stiles and Holzapfel, 1997). Nevertheless, process technology and use of starter cultures have also allowed increasing sizes of fermentations to industrial scale and specific species, which affect product flavor, texture, and quality, have been further selected. Today, about 100 species from all LAB genera are used as starters for food fermentations (Leroy and De Vuyst, 2004).

1.1.4 Use of LAB as Protective Cultures

The most important aspect for the use of LAB in food fermentations is their contribution to food safety. A significant feature of a fermented food is the high titratable acidity. In cereal and vegetable products which are weakly buffered, an efficient lactic fermentation will produce a pH of 4.0 or less, at which the growth of bacterial pathogens is inhibited and many bacteria will die at a rate which increases with increasing ambient temperature (Nout and Motarjemi, 1997). However, organic acids such as lactic and acetic acids are not the only compounds produced by LAB that have a pronounced antimicrobial activity. Other fermentation end-products with antimicrobial activity include hydrogen peroxide (in the presence of oxygen), diacetyl, aldehydes (e.g., β-hydroxypropionaldehyde) and bacteriocins (Lindgren and Dobrogosz, 1990; Holzapfel et al., 1995).

Since the role of microorganisms in spontaneous food fermentations became clear, man has tried to apply controlled fermentations in order to preserve food products. Today, food safety is more than ever an important issue and the search for new preservation methods goes on. The consumer wants food products which are fresh, natural, healthy and convenient and which are less heavily preserved (Ohlsson, 1994; Gould 1996). These demands are addressed in the marketplace by the emergence of a new generation of chill stored, minimally processed foods (Stiles, 1996). However, many of the new ready-to-eat and novel food types bring along new health hazards and new spoilage associations. Against this background, and relying on an

improved understanding and knowledge of microbial interactions, milder preservation approaches such as biopreservation have been developed (Holzapfel, 1997; Hugas, 1998). Biopreservation can be defined as a preservation method to improve safety and stability of food products in a natural way by using desired microorganisms (cultures) and or their metabolites without necessarily changing the sensory quality (Holzapfel et al., 1995; Lücke et al., 2000).

Protective cultures can be defined as antagonistic microorganisms (cultures) that are added to a food product only to inhibit pathogens and/or to extend shelf life, while changing the sensory properties of the product as little as possible (Lücke, 2000). Protective cultures differ from starter cultures in their functional objectives. Starter cultures are, by definition, used in food fermentations in order to modify the raw material to give it new sensory properties. This relies on the metabolic activity (acid production) of the culture, while the preservation effect (antimicrobial effect) is of secondary importance. For a protective culture, the functional objectives are the inverse. Although distinguished by their definition, in reality a starter culture and protective culture may be the same culture applied for different purposes under different conditions (Holzapfel et al., 1995). Biopreservation by protective cultures can be applied to food products by 1) adding crude, semi purified or purified microbial metabolites with inhibitory activity or 2) by adding pure and viable microorganisms (Hugas et al., 1998). Bacteriocins or their producer cultures are most often applied for biopreservation to inhibit the growth of spoilage or pathogenic bacteria.

1.2 Bacteriocin Production by Lactic Acid Bacteria

Bacteriocins were first discovered in 1925 and have subsequently been studied with growing interest. Bacteriocins are a heterogenous group of anti-bacterial peptides that vary in spectrum of activity, mode of action, molecular weight, genetic origin and biochemical properties. Currently, artificial chemical preservatives are employed to limit the number of microorganisms capable of growing within foods, but increasing consumer awareness of potential health risks associated with some of these substances has led researchers to investigate preservative agents such as bacteriocin produced by LAB (Abee, 1995).

1.2.1 Classification of bacteriocins produced by lactic acid bacteria

Bacteriocins produced by LAB can be grouped into three different classes, i.e., class I: lantibiotics or small, heat-stable, lanthionine containing, single- and two-peptide bacteriocins whose inactive pre-peptides are subject to extensive post-translational modification; class II: non-lanthionine containing and unmodified bacteriocins which are subdivided into three subclasses, namely, class IIa (pediocin-like bacteriocins), class IIb (two-peptide bacteriocins), and IIc (non-pediocin like, one-peptide bacteriocins); and class III the bacteriolysins or large, heat-labile, lytic proteins, often murein hydrolases (Drider et al., 2006). The inhibitory spectrum

of common LAB bacteriocins is relatively narrow compared to that of the antimicrobial peptides produced by eukaryotic cells. The current understanding of only the class IIa and IIb bacteriocins and their varied structural features, production, regulation, biological activity, and potential applications will be discussed below as these are relevant to this study.

1.2.2 Class IIa bacteriocins

The past few years have seen the emergence of class IIa bacteriocins produced by LAB as one of the most interesting groups of antimicrobials for use in food preservation (Cleveland et al., 2001) and in medicine, as antibiotic complement in treating infectious diseases (Ingham et al., 2003) or as antiviral agents (Wachsman et al., 1999, 2003). Some of the peptides inhibit the growth of Gram-positive food spoilage and pathogenic bacteria such as *Bacillus cereus*, *Clostridium perfringens*, *Staphylococcus aureus* and *Listeria monocytogenes* and thus the interest in these compounds for use in foods to prevent foodborne illness has been a primary objective (Drider et al., 2006). Class IIa bacteriocins are described as small (<10kDa), active against *L. monocytogenes*, heat stable and produced as unmodified peptides of 37 to 48 amino acids in length, having a net positive charge with pI values ranging from 8 to 10 (Nes et al., 1996; Drider et al., 2006; Nissen Meyer et al., 2009). Sequence alignment of class IIa bacteriocins reveals that they consist of a highly conserved hydrophilic and charged N-terminal part (residues 1 to 16) containing a consensus amino acid sequence YGNGV(X)C(X)$_4$C(X)V(X)$_4$A (X denotes any amino acid) (Klaenhammer, 1993; Cleveland et al., 2001; Drider et al., 2006) and a more variable hydrophobic and/or amphiphilic C-terminal part (Fig. 1.2). Based on amino acid sequence alignments three subgroups (see Fig 1.2) were suggested. It appears that the different subgroups have somewhat different three dimensional (3D) structures which in turn reflect differences in their target cell specificities.

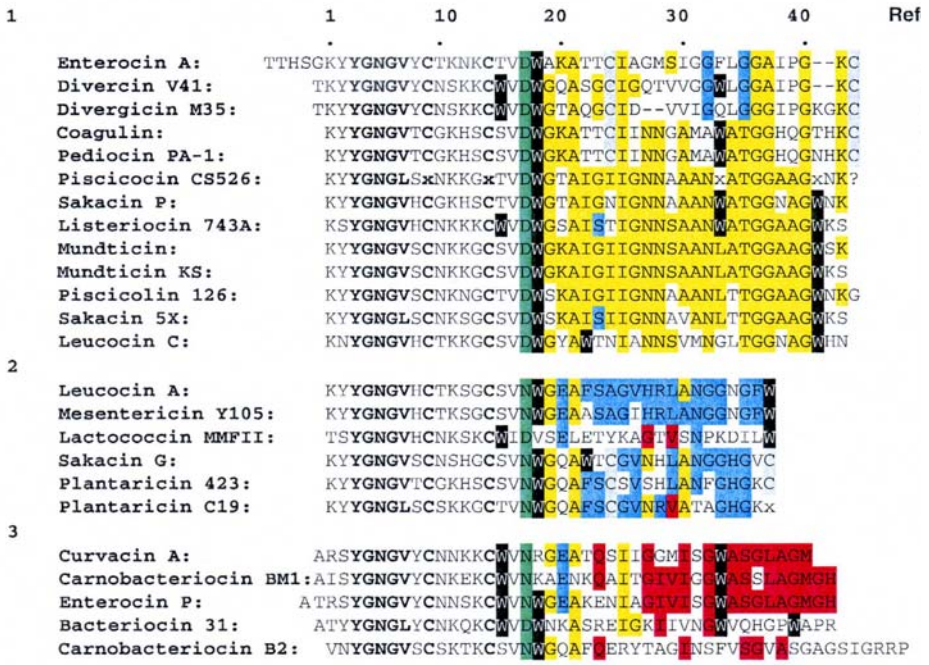

Figure 1.2 Multiple sequence alignment of pediocin-like (class IIa) bacteriocins. The classification of the peptide into subgroups 1 to 3 is based on sequence similarities and differences in the C-terminal half. Adapted from Nissen-Meyer et al. (2009).

Structural analyses by NMR spectroscopy have shown that the class II bacteriocins consist of an N-terminal β-sheet-like domain which is structurally stabilized by a conserved disulfide bridge formed between two cysteine residues, and a C-terminal domain consisting of one or two α-helices often ending with a structurally extended C-terminal tail (Fimland et al., 2005) (see Fig 1.3).

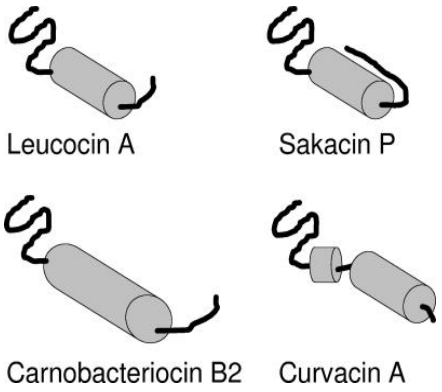

Figure 1.3 Schematic structure presentation of the class IIa bacteriocins for which 3D structures have been determined by NMR Adapted from Drider et al. (2006).

Class IIa bacteriocins have no structure in water but in a membrane mimicking environment the N-terminal part forms the three-stranded antiparallel β-sheet–like structure supported by the conserved disulfide bridge and the C-terminal part consisting of the one or two amphiphilic α-helices followed by the somewhat extended C-terminal tail of varying length. In

most bacteriocins, this C-terminal tail seems to fold back onto the α-helix region, forming a hairpin-like structure (see Fig. 1.3). The N-terminal β-sheet–like structure and the hairpin-like C-terminal parts are separated by a flexible hinge which allows the two parts to move relative to each other (Drider et al., 2006).

The conserved YGNGV sequence at the N-terminal part was proposed to be responsible for the antilisterial activity of class IIa bacteriocins, as all these bacteriocins share this or a variant of this sequence (see Fig. 1.2). Indeed, altering the residues in the YGNGV sequence led to reductions in potency (Quadri et al., 1997; Miller et al., 1998). The positively charged residues of class IIa bacteriocins are also located mostly in the N-terminal regions (Fig. 1.2) and a study on the affinity of pediocin PA-1 derived peptide fragments with target cells suggested that electrostatic interactions, and not the YGNGV region, mediated the initial binding of pediocin to target cells (Chen et al., 1997), and that Lys11 and His12 as part of the cationic patch in the N-terminal β-sheet are of special importance in these interactions (Chen et al., 1997; Kazazic et al., 2002; Vadyvaloo et al., 2004).

The hairpin-like C-terminal region of class IIa bacteriocins is diverse with respect to the number of residues and α-helices and the amino acid sequence and length of the C- terminal region following the helical segment(s). Studies have indicated that the C-terminal region penetrates the target bacterial cell membrane, thereby inducing leakage and causing cell death (Miller et al., 1998; Morisset et al., 2004; Fimland et al., 2005). Studies on the substitution of Trp residues in the bacteriocin sakacin P have shown that the Trp18 and Trp41 of sakacin P locate in the membrane-water interface of the target cell, whereas Trp33 locates in the hydrophobic part of the membrane, thereby creating the hairpin-like structure in the C-terminal part (Fig. 1.2) (Fimland et al., 2002). Thus, the hairpin-like C-terminal half of the molecule orients obliquely into the membrane, and the hydrophilic β-sheet–like N-terminal half is attached to the cell surface. The C-terminal region also seems to be important in determining the target cell specificity for class II bacteriocins. This was shown using hybrid bacteriocins constructed by combining N-terminal and C-terminal regions from different bacteriocins.

All class IIa bacteriocins whose modes of action have been studied permeabilise the cytoplasmic membrane. This is achieved by pore formation through insertion of the C-terminal region of the bacteriocins into the membrane. The membrane-located bacteriocin receptor was shown to be the enzyme IIC subunit of the mannose permease (EII_t^{Man}) (Ramnath et al., 2004), which belongs to the sugar phosphotransferase (PTS) system. The binding and insertion of the C-terminal halves of bacteriocin molecules leads to pore formation and induces leakage of K^+, inorganic phosphate, amino acids and other low molecular weight compounds from cells. It also dissipates the $\Delta\psi$ and the ΔpH and consequently leads to a breakdown of the proton motive force, which in turn induces a rapid depletion of intracellular ATP ultimately resulting in cell death (Chikindas et al., 1993; Drider et al., 2006). Figure 1.2 shows some of the well studied

class IIa bacteriocins including those that are produced by *L. plantarum* strains, i.e. plantaricin 423 and plantaricin C19.

1.2.3 Class IIb bacteriocins

The two-peptide class IIb bacteriocins consist of two different unmodified peptides, both of which must be present in about equal amounts in order for these bacteriocins to exert optimal antimicrobial activity (Oppegård et al., 2007). The sequences of two peptide bacteriocins are varied and homology is occasionally observed between different two-peptide systems (Garneau et al., 2002). The two peptide bacteriocins share many characteristics with one peptide bacteriocins, i.e., they are usually cationic and contain hydrophobic and/or amphiphilic regions. One or both peptides of some two-peptide bacteriocins (e.g., plantaricin E/F and J/K) may in fact individually display some, although low, antimicrobial activity, but high activity only occurs in combination with the complimentary peptide from the same two-peptide bacteriocin. In some cases, activity of one peptide can also be complimented with the second peptide from another bacteriocin system (Oppegård et al., 2007). For instance, the two complementary peptides that constitute lactococcin G are active at pico- to nanomolar concentrations when combined, but show no activity when tested individually at concentrations as high as 50 μM (Moll et al., 1996). The requirement of both complementary peptides for a potent antimicrobial effect clearly indicates that the two-peptide bacteriocins function together as one antimicrobial entity. Three of the two-peptide bacteriocins that have been identified and characterized, i.e. lactococcin G, lactococcin Q and enterocin 1071 show marked sequence similarity and are thus clearly evolutionary related.

Structure function studies have shown that the two peptides are unstructured in water and that there is no structural interaction between them in aqueous solution. However, they become structured upon exposure to membrane like entities and these induce the formation of an amphiphilic α-helix in the N-terminal and mid-region of both peptides. Furthermore, the peptides seem to interact upon contact with target membranes and this induces additional α-helical structures in each other (Hauge et al., 1998). The two complementary peptides thus appear to interact in a structure-inducing manner upon arrival at the target membrane resulting in the formation of an antimicrobial peptide complex with amphiphilical α-helical regions, and the synergistic antimicrobial effect is thus apparently due to the inter-peptide interactions, rather than the complementary peptides interacting separately at different sites on the target cell (Oppegård et al., 2007). The 3D structure of plantaricin E (33 residues) and plantaricin F (34 residues) peptides that constitute plantaricin EF have been analysed by NMR spectroscopy and peptide E forms two α-helix-like regions (residues 10 to 21 and 25 to 31) separated by a flexible GxxxG motif (residues 20 to 24), whereas peptide F forms one long helix from residue 7 to 32, with a kink and slightly more flexible region around Pro20. The E peptide has altogether two

putative helix-helix interaction GxxxG motifs, one at residue 5 to 9 and one at residue 20 to 24 (between the helices), while the F peptide has one such motif at residue 30 to 34. It has been suggested that the two peptides interact in parallel and staggered fashion relative to each other and form a helix-helix-structure involving the GxxxG motifs, similar to those of the lactococcin α- and β-peptides (Fimland et al., 2008; Oppegård et al., 2009).

The GxxxG motifs are common amongst nearly all class IIb bacteriocins, suggesting that membrane-penetrating helix-helix structures formed by two peptides might possibly be a common structure in most, if not all, two peptide bacteriocins. Lactococcin G perhaps is the most well characterized of the class IIb bacteriocins and a model for its structure function relationship was proposed (Rogne et al., 2008; Nissen-Meyer et al., 2009). As for plantaricin E/F NMR studies suggested α-helices in both the N-(residues 3-21) and C-(residues 24-34) terminal halves of the lactococcin G α-peptide in the presence of dodecylphosphocholine (DPC) micelles. Only one helix was found in the N-terminal half of the lactococcin β-peptide in the presence of DPC (Rogne et al., 2008). The α-peptide peptide has two GxxxG motifs (Gly7-Gly11 and Gly18 to Gly22) and the β-peptide has one (Gly18 to Gly22). The proposed structure thus again entails that the α- and β-peptides are orientated in the same direction and form a staggered helix-helix structure that spans the target cell membrane (Rogne et al., 2008). The helix-helix segment consists of the N-terminal half of the α-peptide (from about residue 3 to 22) and the C-terminal half of the β-peptide (from residue 13 to 32) (Fig. 1.4). The structural model also entails that the cationic C-terminal end (residues 35-39) (RKKKH) of the α-peptide is unstructured and forced through the target cell membrane by membrane potential (negative inside) thereby positioning the C-termini of the two peptides inside the target cell. The Trp-rich N-terminal end of the β-peptide is also proposed to be relatively unstructured and to position itself in the outer membrane interface thus forcing the N-termini of the two peptides to remain on the outer side of the target cell membrane and the helix-helix segment to transverse the membrane (Nissen-Meyer et al., 2009; Fig. 1.4).

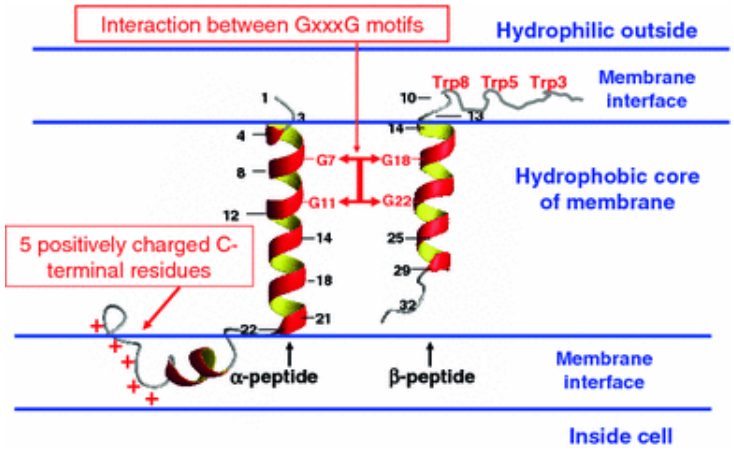

Figure 1.4 schematic representation of the structural model of lactococcin G and its orientation in target-cell membrane. The two peptides interact through the GxxxG motif. Adapted from Nissen-Meyer et al. (2009).

All two-peptide bacteriocins, similar to the one-peptide class IIa bacteriocins, render target cell membranes permeable to a variety of small molecules. These two peptide bacteriocins, however, appear to display some specificity with respect to which molecules they conduct across membranes. For example, lactococcin G permeabilizes target cell membranes for a variety of monovalent cations such as Na^+, K^+, Li^+, Cs^+, Rb^+ and choline, but not for divalent cations such as Mg^{2+} or anions. Plantaricin E/F and plantaricin J/K also permeabilize membranes for monovalent cations, including H^+, but not for divalent cations or anions. It appears, however, that plantaricin E/F conducts cations more efficiently than plantaricin J/K and vice versa for anions (Moll et al., 1999; Nissen-Meyer et al., 2009).

Well-studied two-peptide bacteriocins contain the *L. plantarum* bacteriocins plantaricin E/F, plantaricin J/K, plantaricin S (α, β peptides), plantaricin NC8 (α, β peptides) and plantaricin W (α, β peptides).

1.2.4 Bacteriocin production and transport

Bacteriocin genes may be either plasmid or chromosomally encoded. The gene encoding the bacteriocin peptide, i.e., the bacteriocin structural gene, generally encodes the bacteriocin as a prepeptide containing an N-terminal extension associated with transport. Both the class IIa and class IIb bacteriocins are encoded as prepeptides with an N-terminal extension which is removed by a site-specific proteolytic cleavage during export, and the mature bacteriocin or the bacteriocin peptides of class IIb bacteriocins are then secreted (Håvarstein et al., 1995, Nes et al., 1996). The presequence appears to play a dual role in that it serves to protect the cell from the cytosolic side from the antimicrobial effect of the bacteriocin by keeping the bacteriocin inactive, and it plays a role as a recognition signal during export and thus is important for trafficking of the prebacteriocins to the correct transporter.

Most class IIa and class IIb bacteriocins are secreted by a dedicated ATP binding cassette (ABC) transporter. Among the bacterial ABC transporters, the bacteriocin exporters make up a small subfamily, which are unique in that they have two protein domains (C-terminal and N-terminal domains) on the cytosolic side anchored to the membrane by an intervening transmembrane region (Håvarstein et al., 1995). The C-terminal cytosolic domain contains the ATP binding cassette, which upon ATP hydrolysis energizes the secretion of the peptide out of the cell, whereas the N-terminal cytosolic domain contains the proteolytic activity necessary for cleavage and maturation of the prebacteriocin (Havarstein et al., 1995; Aucher et al., 2005). The presequence is cleaved at the C-terminal side of a double Gly motif, thereby liberating the mature bacteriocin. The ABC transporter protein, as well as a further membrane located, so-called ‘accessory protein’, are required for export of the mature bacteriocin out of the cell (Franke et al., 1999; Ennahar et al., 2000). Because the cleavage signal appears to be the double Gly motif, the N-terminal extension involved in bacteriocin export is often termed the

'double-glycine-type leader peptides'. Some class IIa bacteriocins (e.g., enterocin P, bacteriocin 31, listeriocin 743A), instead have a *sec*-type leader sequence and these are translocated by the *sec*-dependent translocation system, while all class IIb bacteriocin peptides are encoded with a double-glycine-type leader sequence (Nissen-Meyer et al., 2009).

1.2.5 Bacteriocin immunity

For both the class IIa and class IIb bacteriocins, only one immunity peptide is required to protect the cell against the antimicrobial effect of the cognate bacteriocin. The immunity proteins are 85 to 118 amino acids in length, highly charged with most of them containing 25 to 35% charged residues. Bacteriocin immunity proteins differ substantially in sequence, nonetheless they have been classified into three groups (A, B and C). They are cytosolic proteins, with a minor fraction of the cellular pool perhaps loosely associated with the inside of the cell membrane (Quadri et al. 1995; Dayem et al., 1996). When expressed inside of sensitive cells, they strongly protect against the cognate bacteriocin, but when immunity peptide and bacteriocin are added externally, no protection is seen, indicating that the immunity peptides act inside the cell (Quadri et al., 1995; Sprules et al., 2004). The 3D-structures of carnobacteriocin B2, enterocin A and piscicolin 126 immunity proteins have been elucidated and they are globular proteins with a left-turning four-helix bundle protein motif. The four antiparallel α-helices are amphiphilic and connected through short loops (Fig. 1.5). They are orientated relative to each other so that their hydrophobic faces interact to form a core in the centre of the protein, whereas the hydrophilic and charged faces of the helices constitute the protein surface (Drider et al., 2006). This distribution of residues gives rise to a structurally stable and hydrophilic cytosolic protein. Homology modeling of further class IIa immunity peptides shows that the four-helix bundle is a conserved structural motif, that the length of the α-helices is relatively constant and that only minor differences exist in the loop regions (Johnsen et al., 2005a, b).

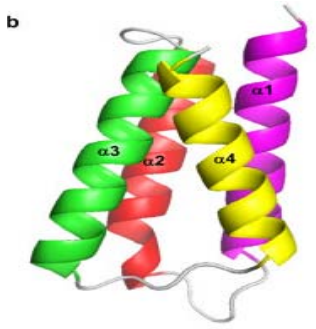

Figure 1.5 A ribbon diagram showing the overall structure of PedB, the pediocin immunity peptide. Adapted from Kim et al. (2006).

Despite the 3D structural similarities, the immunity proteins display strong specificity with respect to the bacteriocins they to which they confer resistance (Quadri et al., 1995; Fimland et al., 2002; Johnsen et al., 2004). The protection specificity was shown in studies with hybrid immunity peptides to be determined by the C-terminal region. Thus, it is hypothesized that the C-terminal, membrane interacting hairpin domain of class IIa bacteriocins is recognized by the C-terminal parts of the immunity proteins (Johnsen et al., 2005a,b). Accordingly, the bacteriocin and immunity proteins are probably located on opposite sides of the cell membrane, but there seems to be no direct contact between the two molecules (Fimland et al., 2005) and thus the membrane itself or a specific component in it plays a crucial role as a mediator in the recognition between the bacteriocin and the immunity protein (Drider et al., 2006). Recently, it was shown that the lactococcin G immunity protein recognizes specific regions in both peptides of the two-peptide (class IIb) bacteriocin lactococcin G, i.e., the N-terminal part of lactococcin α-peptide (residues 1-13), and the C-terminal part of the lactococcin β-peptide (residues 14-24) (Oppegård et al., 2009). The bacteriocin structural and immunity genes are generally located within the same operon and thus are expressed concomitantly.

1.2.6 Regulation of bacteriocin production

Production of some class IIa and class IIb bacteriocins produced by LAB is controlled by quorum sensing and thus a cell density-dependent regulation. Accordingly, a signal molecule (also termed induction peptide or pheromone) is secreted at a low but constant rate in most cells of the bacteriocin-producing population (Nes et al., 1996; Drider et al., 2006). The pheromone concentration thus reflects the cell density during growth and at a certain ‘threshold’ density. the pheromone-dependent regulatory system is activated The quorum sensing systems for bacteriocin production depend on three gene products and are accordingly termed ‘three component regulatory systems’ (Nes and Eijsinck, 1999). The three components include the (i) the inducer peptide (pheromone), (ii) the transmembrane histidine kinase (pheromone receptor)

and (iii) the cytosolic response regulator (Nes and Eijsinck, 1999; Drider et al., 2006). The inducer peptide is synthesized at a low level as a prepeptide, containing a double-glycine-type leader peptide which is cleaved and secreted through a dedicated bacteriocin ABC-transporter (Ennahar et al., 2000). At a certain concentration threshold of the externalized mature inducer peptide, the transmembrane histidine kinase is activated and this leads to the autophosphorylation of a conserved histidine residue at the cytosolic side of the transmembrane protein. Subsequently, the activated histidine kinase interacts with its cognate response regulator protein through transphosphorylation and the phosphate group of the histidine residue of the activated histidine kinase is transferred to a conserved Asp residue in the response regulator (Nes and Eijsinck, 1999; Ennahar et al., 2000). The phosphorylated and thus activated response regulator functions as a transcriptional activator and binds to bacteriocin gene-specific promoters and stimulates transcription.

The response activator also activates the genes encoding the three component system, and a positive feedback loop is thus initiated. At a certain time, all bacteriocin producers in the population may secrete bacteriocin and this sudden increase can have a great impact on the competing microbiota. The biosynthesis of several class II bacteriocins, in addition to cell density regulation, also were shown to vary with respect to growth temperature, ionic strength and pH (Cintas et al., 1997; Fimland et al., 2000; Drider et al., 2006).

1.2.7 *Lactobacillus plantarum* bacteriocin loci

In *L. plantarum* bacteriocin genes are often chromosomally encoded. Usually, more than one bacteriocin is encoded and genes required for biosynthesis, immunity, transport and regulation cluster together in a large bacteriocin locus of >10 kbp. The plantaricin bacteriocin locus in *L. plantarum* C11, isolated from a vegetable source, was first unraveled about 15 years ago, and since then different strains of *L. plantarum* (NC8, WCFS1, and J23) were found to harbor mosaic *pln* loci in their genomes (Fig. 1.6). Genes associated with bacteriocin production include the bacteriocin structural genes, immunity genes, and the genes encoding transporters, such as the ABC transporter and accessory protein genes. In the case of the *L. plantarum* strains C11 and WCFS1 bacteriocin gene loci, which have been intensively studied, inducible operons include those for *pln*EFI and *pln*JKL, which encode the two two-peptide bacteriocins plantaricin E/F and J/K and their cognate immunity proteins. The operon made up by the *pln*GH genes encodes an ABC-transporter (*pln*G) for transport of these bacteriocins as well as the accessory protein (*pln*H). Further downstream the operon, *pln*STUVW encodes proteins belonging to the type II CAAX amino protease family and their role in plantaricin biosynthesis are yet unknown (Diep et al., 2009). The *pln*ABCD operon is involved in regulation and encodes the three component regulatory system consisting of the inducer (*pln*A), the transmembrane histidine kinase (*pln*B) and the response regulator (*pln*CD). The *pln*MNOP codes for four

putative proteins of which PlnN seems to contain an N-terminal double-glycine leader consensus sequence. However, the synthetic peptide of the predicted mature part of PlnN did not show any bacteriocin activity (Anderssen et al., 1998). The *pln*OP encodes proteins with significant homology to family 2-glycosyl transferases and type II CAAX amino proteases, respectively (Diep et al., 2009).

The *pln* loci from NC8, J23 and J51 appear to be more divergent from the C11-*pln* locus, both in terms of gene content and organization. The J51 locus contains six operons, of which *pln*ABCD, *pln*EFI and *pln*GHSTUVW are found as in the C11-*pln* locus. The *pln*MNOP genes are not found in this locus and the *pln*JKLR appears to be truncated, as it lacks the bacteriocin genes *pln*JK and the *pln*R-like gene differs from its counterpart in C11-*pln*. Moreover this locus contains two other bacteriocin operons, i.e., *pl*NC8βαC and *orf*3-4-5. The regulatory operons in NC8-*pln* and J23-*pln* (Maldonado et al., 2003; Rojo-Bezares et al., 2007, 2008) are similar to each other, but different from those in C11-*pln*. Both loci contain a pheromone and a histidine protein kinase gene highly divergent from their C11 counterpart, and both possess only one response regulator gene *pln*D (see Fig. 1.6) (Diep et al., 2009). As in J51-*pln* the *pln*MNOP and *pln*JKLR operons are altered in either strain, the former being truncated in NC8, while the latter is missing the bacteriocin gene *pln*K in J23. The encoded J23-PlnJ is longer (73 amino acids) when compared to its counterpart (55 amino acids) in the other bacteriocin loci. The *pl*NC8βαC is also present in NC8-*pln* but not in J23. In some plantaricin loci, a gene *nap*A2 can be found (Fig. 1.6), which encodes a Na (+)/H (+) antiporter with unknown, if any, function in bacteriocin biosynthesis (Diep et al., 2009).

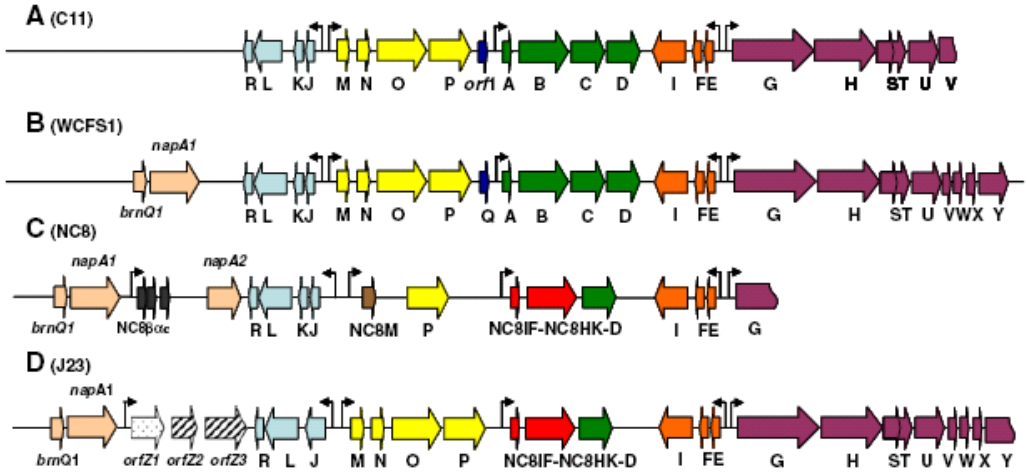

Figure 1.6 Genetic map of *pln* locus of different *L. plantarum* strains: A: *L. plantarum* C11; B: *L. plantarum* WCFS1; C: *L. plantarum* NC8; D: *L. plantarum* J23. The *pln* genes are represented by arrows with different colors corresponding to each operon. The promoter sequences are indicated by small black arrows and the putative orfZ123 promoter sequence is shown by a small black and discontinuous arrow. Adapted from Sáenz et al. (2009).

1.3 Application of protective cultures in meat biopreservation

In recent years, concerns about the safety and quality of foods have increased. The extent of microbiological problems in food safety was clearly reflected in the WHO food strategic planning meeting (WHO, 2002) as it was found that (i) the emergence of new pathogens and pathogens not previously associated with food consumption is a major concern and (ii) microorganisms have the ability to adapt and change, and changing modes of food production, preservation and packaging have therefore resulted in altered food safety hazards.

In industrialized countries, up to 10% of the population may suffer from foodborne diseases annually. To decrease the incidence of foodborne pathogens in foods, as well as high levels of chemical preservation of foods, the application of bacteriocin producing bacteria, especially LAB as starter or so-called 'protective' cultures, or their purified bacteriocins such as e.g., nisin, are recommend as alternative methods for 'biopreservation' of food. The bacteriocin or bacteriocin-producing LAB offer several desirable properties that make them suitable for food preservation: (i) are generally recognized as safe substances, (ii) are not active and non-toxic on eukaryotic cells, (iii) become inactivated by digestive proteases, having little influence on the gut microbiota, (iv) are usually pH and heat-tolerant, (v) they have a relatively broad antimicrobial spectrum, against many foodborne pathogenic and spoilage bacteria, (vi) they show a bactericidal mode of action, usually acting on the bacterial cytoplasmic membrane: no cross resistance with antibiotics (Gálvez et al., 2007). Especially, nisin is approved as a food additive not only in the Unite States, but also over 40 other countries for inhibiting the outgrowth of *Listeria* and *Clostridium* for over 50 years.

In studies of meat preservation, several bacteriocins or protective cultures have already been used to inhibit growth of *Listeria monocytogenes* or other pathogens. Raw meat, stored aerobically under chilled conditions, is usually spoiled by Gram-negative bacteria, predominantly pseudomonads, and LAB compete poorly under these conditions. Hence, very high inocula of LAB protective culture are generally required to observe an effect on the shelf life of aerobically stored raw meat. Pathogens of most importance in raw meat, e.g., *Salmonella*, *Campylobacter, E. coli* and *Yersinia enterocolitica* are Gram-negative and thus insensitive towards bacteriocins. Therefore the benefit of the protective culture on aerobically stored meat is mostly to control pathogens such as *L. monocytogenes* (Lücke, 2000). The micropopulations of anaerobically packaged, chilled raw meats, on the other hand, is dominated mainly by LAB and inoculation with selected psychrotrophic LAB can be used to extend the shelf life and to protect against *L. monocytogenes* (Lücke, 2000). An overview of studies on the effectiveness of bacteriocinogenic LAB in raw meats is given in Table 1.1.

Despite the successful results of some studies, the effectiveness of bacteriocinogenic cultures in food products and more specifically, in meat products can be limited by a range of factors. Firstly, some factors affect the efficacy of the bacteriocin itself, such as narrow activity

spectrum, poor solubility, limited diffusion and uneven distribution in the food matrix. Furthermore, inactivation through proteolytic enzymes can cause problems, as well as binding to food ingredients such as lipids or proteins. Problems of protective cultures may include poor adaptation to the substrate (pH, temperature and nutrients) and therefore poor growth and bacteriocin production (Buncic et al., 1997) as well as phage infections (Holzapfel et al., 1995).

Table 1.1 Examples of food application of bacteriocin producing protective cultures and their effect on the target pathogenic or spoilage bacterial strain.

Protective culture (Inoculation level, bacteriocin)	Product	Target	Effect (log_{10} CFU/g)	Reference
Pediococcus strains (10^8 CFU/g, pediocin-like bacteriocin)	Minced meat	*Y. enterocolitica* *L. monocytogenes*	7[a]	Skyttä et al. (1991)
Lactobacillus bavaricus MN (105 CFU/g, bavaricin MN)	Beef cubes	*L. monocytogenes*	+/- 5[a]	Winkowski et al. (1993)
Leuconostoc gelidum UAL187-22 (10^4/cm^2, leucocin A)	Beef	No inoculation of target cells	No negative effect on odour / appearance compared to control	Leisner et al., (1995)
Leuconostoc gelidum UAL187-22 (10^4/cm^2, leucocin A)	Beef	Sulfide-producing *L. sakei* strain (10^2/cm^2)	4[a] or 3.5[b]	Leisner et al., (1996)
L. sakei CTC 494 (10^6, sakacin K)	Chicken breast Minced raw meat	*L. innocua*	2.5-3[b]	Hugas et al. (1998)
L. sakei CTC494 & *E. faecium* CTC492 (10^5 CFU/g, sakacin and enterocin)	Model cooked pork	Slime producing *L. sakei* and *L. carnosum*	Partial prevention of ropiness	Aymerich et al. (2002)
Lactobacillus casei CRL 705 (10^6 CFU/ml spraying solution, 2 lactocins)	Beef	*L. monocytogenes* and *B. thermosphacta*	1.25[a] for *B. thermosphacta* and complete prevention of *L. monocytogenes* growth	Castellano and Vignolo (2004)
L. sakei CTC 4808 (10^7 CFU/g bacteriocin-like)	Sliced beef	*Enterobacteriaceae* *Pseudomonas* *B. thermosphacta*	1-2[a]	Katikou et al. (2005)
L. sakei CWBI-B1365 (sakacin G)	Raw beef	*Listeria monocytogenes*	2.5	Dortu et al. (2008)
E. faecium PCD71 (10^7 CFU/g, enterocin A,P, L50A/B)	Chicken meat	*Listeria monocytogenes*	0.7	Maragkoudakis et al. (2009)

[a] difference in cell count at the final storage day between the product containing the protective culture and non-inoculated control product.
[b] difference in cell count at the final storage day between the product containing the bacteriocinogenic LAB-strain and a control product containing non-bacteriocinogenic LAB strain.

1.4 Study Objective

This study was performed within the framework of a European Union (EU) funded study entitled '**Control and prevention of emerging and future pathogens at cellular and molecular level throughout the food chain**' or in short **'PathogenCombat'** (www.pathogencombat.com/) which belonged to the 6th framework program (priority 5: Food Quality and Food Safety). This PathogenCombat study was carried out by a consortium of 44 partners from research institutions (24) and the food industry (20) from 13 European countries. The overall objective of the PathogenCombat study was to provide new and essential information and methods to the food industry and public authorities on how to reduce the prevalence of new and re-emerging foodborne pathogens.

The overall objective of this study, which was part of the greater PathogenCombat study, was to evaluate the success of functional lactic acid bacteria starter cultures with bacteriocin activity in controlling foodborne pathogens in food. The bacteriocin producing strains chosen as protective cultures were initially screened using high throughput screening by Christian Hansen (Denmark) for antimicrobial activity. Both strains showed antimicrobial activity against *L. monocytogenes*, as well as other target indicator bacteria. The German industrial food sector partner in the project, who was linked to our institute, was '*Geflügelspezialitäten Ziegle*r' (Bammental, Baden Württemberg) and the project coordination required us to develop methods for this local food producer, i.e. to develop functional starter strains as protective cultures for the biopreservation of aerobically stored turkey meat. The target pathogens for this food commodity were *Listeria monocytogenes*, as well as *Salmonella enterica* subsp. *enterica* serovar Typhimurium (*S*. Typhimurium).

Specifically, this study aimed to:

- Characterize the bacteriocins produced by two bacteriocin-producing *Lactobacillus plantarum* strains BFE 5092 and PCS20, which were chosen by the consortium in an initial screening.
- To determine whether bacteriocin genes are located on the chromosome or whether they are plasmid located.
- To compare the bacteriocin gene loci between the two strains and to identify the bacteriocins associated with the bacteriocin activity.
- To assess the influence of low temperature on bacteriocin production in vitro and in vivo.
- To evaluate the influence of growth state (i.e. sessile versus planktonic) on bacteriocin production in vitro.

- To determine whether bacteriocin production is regulated at the transcriptional level.
- Assess the effectiveness of the protective *L. plantarum* BFE 5092 and PCS20 strains on inhibiting the foodborne pathogens *L. monocytogenes* or *S*. Enteritidis, or background spoilage microorganisms on fresh turkey meat.

CHAPTER 2.0
MATERIAL AND METHODS

The bacteria used in this study were cultured using the media and conditions described below. All media were from Merck (Darmstadt) and all chemicals from Roth (Karlsruhe) unless specified otherwise.

2.1 Culture media

2.1.1 Bacteriocin screening medium (BSM)

Composition (g/l): Meat extract 2.0; tryptone 10.0; yeast extract 4; Tween 80 1.0; citric acid di-ammonium salt 2.0; $MgSO_4.7H_2O$ 0.2; $MnSO_4.4H_2O$ 0.05; $K_2HPO_4.3H_2O$ 8.7; KH_2PO_4 8.0; glucose 2.0; agar 15.0. Medium for testing bacteriocin production among lactic acid bacteria.

2.1.2 Iso-Sensitest broth (Oxoid)

Composition (g/l): Hydrolysed casein 11.0; peptones 3.0; glucose 2.0; sodium chloride 3.0; soluble starch 1.0; di-sodium hydrogen phosphate 2.0; sodium acetate 1.0; magnesium glycerophophate 0.2; calcium gluconate 0.1. Medium for testing antibiotic sensitivity of bacteria, used together with 10% MRS broth (see below) to test antibiotic sensitivity of lactic acid bacteria.

2.1.3 LB broth (Luria-Bertani) broth

Composition (g/l): Tryptone 10.0; yeast extract 5.0; NaCl 10. Medium for growing *E. coli*.

2.1.4 LB Agar

LB broth containing 15 g/l agar.

2.1.5 M17 broth (Oxoid, Wesel)

Composition (g/l): Peptone from soy meal 5.0; peptone from meat 2.5; yeast extract 2.5; meat extract 5.0; ascorbic acid 0.5; sodium β-glycerophosphate 19.0; magnesium sulphate 0.25. Medium for cultivation of lactococci and streptococci.

2.1.6 M17 agar

M17 broth containing 15.0 g/l agar.

2.1.7 Malt Glucose agar

Composition (g/l): D(+)-glucose 4.0, Yeast extract 4.0; Malt extract 10.0; CaCo3 2.0, pH 7.0; Agar 15.0.

2.1.8 MRS (De Man, Rogosa and Sharpe) broth (Merck)

Composition (g/l): Peptone from casein 10.0; meat extract 8.0;yeast extract 4.0; D(+)-glucose 20.0; dipotassium hydrogen phosphate 2.0; Tween 80 1.0; diammonium hydrogen citrate 2.0; sodium acetate 5.0; magnesium sulphate 0.2; manganese sulphate 0.04. Medium for culturing lactic acid bacteria.

2.1.9 MRS agar

MRS broth containing 15.0g/l agar.

2.1.10 PALCAM agar supplemented with *Listeria* selective supplement (Oxoid). Composition (g/l): Peptone 23.0; starch 1.0; sodium chloride 5.0; agar 13.0; yeast extract 3.0; D(-)-mannitol 10.0; ammonium iron (III) citrate 0.5; esculin 0.8;glucose 0.5; lithium chloride 15.0; phenol red 0.08; pH, 7.0. The sterilised medium was cooled to 45-50°C and supplemented with 5.0 mg/l polymixin-B-sulphate; 10mg/l ceftacidim and 2.5mg/l acriflavine. Medium for culturing *Listeria* spp.

2.1.11 *Salmonella* / *Shigella* agar (Oxoid)

Composition (g/l): peptone 10.0; lactose 10.0; oxbile 8.5; sodium citrate 10.0; sodium thiosulphate 8.5; ammonium iron(III) citrate 1.0; brilliant green 0.0003; neutral red 0.025; agar 12.0. Dissolve by boiling. Medium for culturing *Salmonella* spp.

2.1.12 SOB broth medium

Composition (g/l): tryptone 20.0; yeast extract 5.0; NaCl 0.5. Dissolve in 950ml distilled water. Add 10ml of 250 mM KCl. adjust the volume of solution to 1 liter with distilled water and pH 8.0 with 5 N NaOH. Just before use, add 5ml of 2M $MgCl_2$. Medium used for *E. coli* in transformation experiments.

2.1.13 SOC medium

Filter sterilized 20 mM glucose (Merck) added to SOB medium after autoclaving. Medium used for *E. coli* in transformation experiments.

2.1.14 SOB agar

SOB broth medium containing 15g/l agar.

2.1.15 Standard I broth

Composition (g/l): peptone 15.0; yeast extract 3.0; sodium chloride 6.0; D(+)-glucose 1.0. General bacterial cultivation medium for aerobic, mesophilic bacteria.

2.1.16 Standard I agar

Standard I broth containing 15g/l agar

2.1.17 Tryptone Soya (TS) broth

Composition (g/l): Pancreatic digest of casein 17.0; enzymatic digest of soya bean 3.0; sodium chloride 5.0; di-potassium hydrogen phosphate 2.5. General bacterial cultivation medium for aerobic, mesophilic bacteria.

2.1.18 Violet red bile dextrose (VRBD) agar

Composition (g/l): peptone from gelatine 7.0; yeast extract 3.0; sodium chloride 5.0; D(+)-glucose 10.0; bile salt mixture 1.5; neutral red 0.03; crystal violet 0.002; agar-agar 13.0. Medium for cultivation of enterobacteria.

2.2 Buffers and solutions

2.2.1 DEPC-H_2O

DEPC (diethyl pyrocarbonate, Sigma) was added to bi-distilled water to a concentration of 0.1% to eliminate RNase. The solution was agitated overnight and then autoclaved to eliminate DEPC. DEPC-H_2O is RNase free.

2.2.2 EDTA (0.5 M)

181.6 g EDTA (ethylenediaminetetraacetic acid) in 800ml distilled water, adjusted to pH 8.0 with approx. 20 g NaOH to dissolve and adjusted to 1 liter.

2.2.3 GES solution

5 M guanidinium thiocyanate (Sigma); 100 mM EDTA; 0.5% Sarkosyl; pH 8.0.

2.2.4 Hybridisation buffers

2.2.4.1 *Alkaline Transfer Buffer*

0.4N NaOH;1M NaCl

2.2.4.2 *Denaturation solution*

1.5M NaCl; 0.5M NaOH

2.2.4.3 *Neutralising solution*

0.5M Tris.Cl (pH 7.2); 1M NaCl

2.2.5 Loading buffer

2.5mg/ml bromophenol blue dye, 50% (v/v) glycerol in 1X TE (pH 8.0)

2.2.6 Ringer solution (quarter-strength) (Merck)

For 1l quarter-strength Ringer's solution add two tablets to bi-distilled water, pH 7.0

2.2.7 TBE (10X)

Composition in (g/l): Tris.HCl (Roth) 121.1; boric acid 61.83; EDTA 0.76; final pH 8.0

2.2.8 TELS

25mM Tris, 10mM EDTA; 20% (w/v) sucrose; 20mg/ml lysosyme; 2U/ml mutanolysin (M9901 Sigma)

2.2.9 TER

20ug/ml Rnase (sigma) dissolved in 1 x TE (pH8.0) buffer

2.2.10 TERMLS

10mM EDTA, 0.2g/l D (+) glucose; 0.015g/ml lysozyme (L3790, Sigma); 100U/ml mutanolysin (M9901, Sigma); 25µg/ml RNase (R6513, Sigma)

2.2.11 Tris.HCl (1M)

121.1g Tris-base dissolved in 800ml distilled water, adjusted to pH 8.0 with approx. 42ml HCl (Merck), adjusted to 1L and autoclaved.

2.2.12 Tris EDTA (TE) Buffer pH 8.0 (10X)

100 mM Tris-Cl (pH8.0); 10mM EDTA (pH8.0)

2.2.13 20 X SSC

Composition (g/l): NaCl 175.3; sodium citrate 88.2; pH adjusted to 7.0 with 12 N HCl (Merck) and volume adjusted to 1 liter.

2.2.14 Solutions for small scale plasmid isolation from *E. coli* strains

2.2.14.1 *Alkaline lysis solution I*

50mM glucose; 25mM Tris-Cl (pH 8.0); 10mM EDTA (pH 8.0)

2.2.14.2 *Alkaline lysis solution II*

0.2N NaOH; 1% (w/v) SDS, prepared fresh and used at room temperature

2.2.14.3 *Alkaline lysis solution III*

5M potassium acetate 60ml; glacial acetic acid 11.5ml; H_2O 28.5ml. Stored at 4°C.

2.3 Plasmids used in the study, bacterial strains and growth conditions

The bacterial strains and plasmids used are shown in Table 2.1. *Lactobacillus plantarum* strains BFE 5092 (Mathara et al., 2004; Vizoso Pinto et al., 2006) and PCS20 used in this study were deposited in the 6th Framework EU-Project PathogenCombat culture collection and were originally isolated from a Kenyan traditional fermented milk product 'Kule naoto', and a Slovenian home made cheese product, respectively. These strains, and other LAB strains (Table 2.1), were routinely grown in MRS medium at 30°C for 18 h. As an exception, the *L. sakei* DSM20017^T strain used as an indicator in bacteriocin assays was grown in MRS broth at 25°C.

Table 2.1 Strains and plasmids used in this study.

Strains	Relevant characteristics	Source
L. plantarum BFE 5092	*pln*E$^+$, *pln*F$^+$, *pln*J$^+$, *pln*K$^+$ and *pln*N$^+$	Mathara et al. (2004), this study
L. plantarum PCS20	*pln*E$^+$, *pln*F$^+$	This study
L. johnsonii La1	Bacteriocin-negative control strain	Marteau et al. (1997)
L. plantarum 299V	Bacteriocin-positive control strain, *pln*E$^+$, *pln*F$^+$, *pln*J$^+$, *pln*K$^+$ and *pln*N$^+$	Adlerberth et al. (1996), this study.
L. sakei DSM 20017^T	Indicator strain in bacteriocin activity test	DSMZ[a]
L. pentosus DSM 20314	Indicator strain in bacteriocin activity test	DSMZ
E.faecalis FAIR-E24 (=BFE 900)	Bacteriocin-positive control stains, produces enterocins A and B	Franz et al. (1996)
L. monocytogenes EGDe serotype 1/2a	Indicator strain in bacteriocin activity test, pathogen used on turkey challenge test with protective cultures *L. plantarum* strains BFE 5092 and PCS20	Glaser et al. (2001)
S. enterica serovar Typhimurium S.TM SI 1344	pathogen used on turkey challenge test with protective cultures *L. plantarum* strains BFE 5092 and PCS20	*Salmonella* reference Laboratory, Robert Koch institute
Leuconostoc carnosum DMRICC 4010	Commercial protective culture used in biopreservation of meats, produces leucocin A and leucocin C like bacteriocins	Christian Hansen, Denmark
E. coli DH5α	Host strain for pUC19 Genotype: *fhu*A2 Δ(*arg*F-*lac*Z)U169 *pho*A *gln*V44 Φ80 Δ(*lac*Z)M15 *gyr*A96 *rec*A1 *rel*A1 *end*A1 *thi*-1 *hsd*R17	Life Technologies
E. coli top 10	Host strain for pUC19, pCHO1, and pCHO2 Genotype: F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lac*ZΔM15 Δ*lac*X74 *nup*G *rec*A1 *ara*D139 Δ(ara-leu)7697 *gal*E15 *gal*K16 *rps*L(Str^R) *end*A1 λ$^-$	New England Biolabs
Plasmid		
pUC19	LacZα, Amp^r, 2.7kb	New England Biolabs
pCHO1	pUC19 containing 5.5kb *Xba*I-*Eco*RI fragment from *L. plantarum* BFE 5092	This study
pCHO2	pUC19 containing 4 kb *Xba*I-*Eco*RI fragment from *L. plantarum* PCS20	This study

[a]DSMZ: Deutsche Sammlung von Mikroorganismen und Zellkulturen

Escherichia coli DH5α and *E. coli* top 10 were grown in Luria-Bertani broth (Sambrook et al., 1989) at 37°C with agitation at 200 r.p.m. *E. coli* top 10 cells were transformed by electroporation and transformants were selected on SOB agar plates supplemented with 150 µg/ml of ampicillin (Roth, Karlsruhe, Germany) using alpha complementation (blue white selection) as described by Sambrook et al. (1989). *Listeria monocytogenes* EGDe was grown in Standard I or Tryptone Soya broth at 37°C without agitation. *Salmonella* Typhimurium was grown in TSB (Roth) at 37°C. Stock cultures were kept at -80°C in the specific broth used for cultivation containing 20% (v/v) glycerol and were subcultured at least twice before use in experiments.

2.4 Bacteriocin activity tests

In order to determine presumptive bacteriocin activity, 5µl of the producer *L. plantarum* cultures were each spotted onto MRS agar plates and these were incubated at 30°C overnight. To minimize the acid effect on agar plates, the cultures were spotted onto BSM agar plates which were buffered with K_2HPO_4 and KH_2PO_4, contained less glucose than MRS medium (see 2.1.1 and 2.1.7), and these were incubated at 30°C overnight. To determine the effect of growth temperature on bacteriocin production, plates were also incubated at 30, 20, 15, 12, 10 and 8°C. To determine whether the inhibitory activity was proteinaceous in nature, 5 µl of a 20 mg/ml proteinase K solution was spotted next to the producer colony after growth and incubated a further 3 h at 37°C. After incubation, the plates were overlayered with soft (0.75%) MRS agar containing ca. 1 x 10^6 CFU/ml of the *L. sakei* DSM 20017^T for BSM plates and either *L. sakei* DSM 20017^T or *Listeria monocytogenes* EGDe indicator strain for MRS plates. The plates were incubated at 30°C overnight and then examined for zones of clearing around the producer colony.

2.5 Determination of antibiotic resistance profiles of *Lactobacillus* strains

The selected strains were investigated for their antibiotic resistance profile using the LAB susceptibility test medium (LSM) of Klare et al. (2005) which consists of Iso Sensitest broth containing 10% MRS broth. The two *L. plantarum* strains BFE 5092 and PCS20 were transferred in this medium at least two times. The strains were inoculated at a concentration of (1 X 10^8 CFU/ml) in LSM in 96 well microtiter plates, which contained a two-fold dilution series of each of the antibiotic being tested. The concentration range of antibiotic tested is shown in Table 2.2 for each antibiotic. Thus, seven different antibiotics were used to determine the resistance of these strains under aerobic condition at 30°C. The minimum inhibitory concentration (MIC) was determined as the concentration of antibiotic where no visible growth of the bacteria occurred.

Table 2.2 Antibiotics and their concentration range used for testing susceptibility of *L. plantarum* strains.

Antibiotic	Stock Solution	
	Concentration of stock solution and diluents used to prepare stock	Concentration range tested (µg/ml)
Erythromycin	50mg/ml in ethanol	0.064, 0.125, 0.25, 0.5, 1, 2, 4, 6, 8, 16, 32, 64
Gentamicin	50mg/ml in H_2O	0.25, 0.5, 1, 2, 4, 6, 8, 16, 32, 64, 128, 256, 512
Streptomycin	10mg/ml in H_2O	0.25,0.5,1,2,4,6,8,16,32,64,128,256,512
Ciprofloxacin	10mg/ml in H_2O	0.25,0.5,1,2,4,6,8,16,32,64,128,256,512
Vancomycin	50mg/ml in H_2O	0.25,0.5,1,2,4,6,8,16,32,64,128,256,512
Chloramphenicol	50mg/ml in ethanol	0.064,0.125,0.25,0.5,1,2,4,6,8,16,32,64
Tetracycline	10mg/ml in ethanol	0.25,0.5,1,2,4,6,8,16,32,64,128,256,512

2.6 Isolation of genomic DNA from *L. plantarum* strains and detection of known plantaricin genes by PCR amplification

The total genomic DNA of *Lactobacillus plantarum* strains BFE 5092 and PCS20 was isolated in small-scale preparations from 5 ml of overnight culture grown at 30°C in MRS broth according to the guanidinium thiocyanate method of Pitcher et al. (1989), as modified by Björkroth and Korkeala (1996) for Gram-positive bacteria. Briefly, overnight cultures were harvested by centrifugation and washed with TE buffer containing 0.5% NaCl. After centrifugation at 9,512x*g* for 10 min, the pellet was resuspended in 100 µl of TERMLS for digesting the Gram-positive cell wall. The cells were lysed using 500µl amount of the GES solution and the preparation was incubated on ice with 7.5M ammonium acetate. After centrifugation at 15 000x*g* for 10 min the upper fraction was collected and DNA was precipitated using ice cold 2-propanol. The DNA was washed using 70% ethanol, dried in a rotary evaporator and resuspended in 200µl of 10mM Tris-HCl (pH8). The concentration was measured spectrophotometrically at 260 nm as described in Sambrook et al. (1989).

For determining the presence of known plantaricin genes present in the genome of the *L. plantarum* strains, their structural genes were amplified were amplified in 50 µl volume PCR reactions each containing 100 ng template DNA, 10x *Taq* DNA polymerases buffer (GE Healthcare, Freiburg, Germany), 200 µM dNTP's (Peqlab, Erlangen, Germany), 25 pM of each plantaricin genes specific forward and reverse primers and 1.5 U *Taq* DNA polymerase (GE Healthcare). PCR amplification of the bacteriocin genes was carried out using the primers for all described plantaricin genes (PlnN, PlnEF, PlnJK, Pln423, PlnS, Pln1.25, and PlnNC8) and the amplification conditions shown in Table 2.3. The PCR reactions were performed with an initial denaturation step at 94°C for 3 min, followed by 32 cycles of denaturation at 94°C for 1 min,

annealing at the different primer annealing temperatures (see Table 2.3) for 1 min, and extension at 72°C for 30s, followed by a final extension step at 72°C for 6 min. PCR products were separated by electrophoresis using a 2% (w/v) agarose gel, which was stained with ethidium bromide. PCR products present in the gel were visualized using a Fluorchem Imager 5500 system (Alpha Innotech, USA) equipped with a UV light source. All PCR products were purified using PCR clean columns (Qiagen, Hilden, Germany) and subsequently commercially sequenced at GATC Biotech (Constance, Germany). The nucleotide sequences thus obtained were compared with sequences in the GenBank database using the BLAST algorithm. In addition, ClustalW nucleotide sequence alignments were done using the MegAlign module of the Lasergene software for DNA sequence analysis (version 7.0, DNASTAR).

Table 2.3 PCR primers used for amplification of plantaricin genes

Target	Positive control	Annealing temperature (°C)	Amplicon size (bp)	Primer sequence
*pln*JK	*L. plantarum* 299V	56	306	F[a]: ACG GGG TTG TTG GGG GAG GC R[b]: TTA TAA TCC CTT GAA CCA CC
*pln*EF	*L. plantarum* 299V	60	365	F: GGT GGT TTT AAT CGG GGC GG R: ACT TGA TGG CTT GAA CTA TCC
*pln*NC8	none	56	344	F: CAA ATT GAG GGC GGA TCA GTC R: TAA TCA CAC TGA ACA TCT CTA A
*pln*1.25	none	50	249	F: TTA GCA TTG ATT GAT GGA GGA R: GCA TCC TAT GTG AGG CTG CTG
*pln*S	none	54	460	F: ATG CTG TTA TCG GTG GGA A R: TCA TGC AAG GAG TGC CCA TGC
*pln*423	none	50	197	F: TAT GAT GAA AAA AAT TGA AAA AT R: CCA AAG ATA ATC CCC CCC CAT
*pln*N	*L. plantarum* 299V	50	160	F: GGG TTA GGT ATC GAA ATG G R: CTA ATA GCT GTT ATT TTT AAC C

[a]F: forward primer (5' to 3' direction), [b]R: reverse primer (3' to 5' direction)

For making DIG labeled probes, specific plantaricin genes (*pln*EF) were amplified by PCR using the DIG probe synthesis kit (Roche, Mannheim Germany). These plantaricin genes were amplified in 50 µl volumes each containing 50ng of template DNA, 1x PCR buffer, 200µM dA(CG)TP's, 130µM dTTP, 70µM DIG-dUTP, 25pM of each forward and reverse primer and 2.5U polymerase enzyme mix (Expand High Fidelity, Roche). The amplification condition for DIG PCR labeling and the primers used are show in Table 2.3. The PCR conditions were as described above for the respective genes.

2.7 Isolation of plasmid DNA from Gram-positive and Gram-negative bacteria

Plasmid DNA from *Lactobacillus* strains BFE 5092 and PCS20 was isolated to determine whether the bacteriocin loci were located on the chromosome, or whether they were

plasmid borne. Plasmids from Gram-negative bacteria were isolated to clone the bacteriocin loci from these *L. plantarum* strains for sequencing. Plasmid DNA from Gram-positive bacteria was extracted using the method of Birnboim and Doly (1979), as modified by van Belkum and Stiles (1995). Briefly, overnight cultures were harvested by centrifugation (9,512x*g*, 10 min) and washed with TE containing 0.5% NaCl as for the genomic DNA isolation method. After removing the supernatant, the pellets were resuspended in 100µl TELS solution. Two hundred µl of freshly prepared lysis solution (Sambrook et al., 1989) was added to disrupt the cells, after which 150µl of 3M potassium acetate (pH 5.0) was added and the sample was put on ice for 5 min. Next the protein was removed by phenol-chloroform and chloroform isoamyl extraction and precipitated with 2 volumes of absolute ethanol (Merck) and 1/9th volume of 3M sodium acetate (pH 5.2). The plasmid DNA was washed with 70% ethanol, dissolved in TER and incubated at 30°C for 30 min to remove the RNA. Plasmid DNA from Gram-negative bacteria such as *E. coli* was extracted using the alkaline lysis method exactly as described by Sambrook et al. (1989).

2.8 Cloning and characterisation of the plantaricin gene loci from *L. plantarum* strains BFE 5092 and PCS20

Large scale genomic DNA was isolated from 40 ml of an overnight culture in MRS broth grown at 30°C exactly as described by Quadri et al. (1994). Genomic DNA was digested in double restriction digests using *EcoR*I and *Xba*I restriction enzymes. The resulting fragments were separated on 1% agarose gel, stained with ethidium bromide in TBE buffer and photographed under UV light. The DNA in the agarose gel was subsequently transferred onto a nylon membrane (Hybond N+; Amersham Phamacia) using a vacuum blotter (Bio-Rad, Munich). The membrane was pre-hybridised for 6 hours at 68°C and then hybridized at 54°C with a *pln*EF-specific probe using the DIG- labeling and detection kit according to methods described in the manual (Roche, Mannheim). The *pln*EF probe was generated using the DIG PCR synthesis kit (Roche) and the primers and PCR conditions for amplification of the plantaricin EF genes as described in Table 2.3. The hybridisation with the probe was done to compare the location of the plantaricin genes on the chromosome and to locate the gene fragment bearing these genes for cloning experiments.

In addition, genomic DNA was also prepared and embedded into agarose plugs for PFGE analysis as described by Graves and Swaminathan (2001) to further compare the plantaricin loci in the different *L. plantarum* strains. The agarose plugs were washed, treated with proteinase K solution and again washed and digested with *Not*I (New England Biolabs, Frankfurt) as described by Huch et al. (2008). The agarose plugs were loaded onto a 1.2% Biozym Gold agarose (Biozym, Hessisch Oldendorf) gel which was subjected to electrophoresis in 0.5x TBE buffer at 14°C using a PFGE CHEF-DR III System (Bio-Rad). The switch times were 0.1-10s and the run time was 28 h. After electrophoresis, the gels were stained and visualised as described before (Kostinek et al., 2005) and subsequently blotted onto nylon N+

membrane using a vacuum blotter. The membrane was pre-hybridised and subsequently hybridized with the *pln*EF probe as described above, and the gene signal was detected using the DIG labeling and detection kit (Roche) also as described above.

The plantaricin gene loci of the *L. plantarum* BFE 5092 and PCS20 strains were cloned using the 'shotgun' cloning technique. Total genomic DNA of these strains was digested with *Xba*I and *EcoR*I restriction enzymes and separated on 1% agarose gels. For each strain, the DNA fragments ranging from 3 to 5-kbp were ligated into pUC19 (Table 2.1) using T4 DNA ligase (New England Biolabs) according to the manufacturer's instructions. The ligated mixture was used to transform *E. coli* top 10 electrocompetent cells (Invitrogen, Karlsruhe) by electroporation according to the methods of Sambrook et al. (1989). Colony blots were prepared using standard technique (Sambrook et al., 1989) and clones were checked whether they contained the plantaricin gene insert by hybridizing with the *pln*EF gene probe as described above. Positive colonies were selected, grown in Luria-Bertani supplemented with antibiotic, their plasmid DNA was extracted using the Qiagen (Hilden) midi kit and the insert was commercially sequenced at GATC Biotech using custom designed primers.

Sequences located further upstream or downstream of the cloned fragments were characterized by deriving primers from previously reported plantaricin operons and from PCR amplification of specific upstream and downstream regions from the respective strains genomic DNA. Furthermore, as the *L. plantarum* PCS20 region showed an atypical upstream region we also used the DNA walking *SpeedUp*™ kit of Seegene (Seegene, Biocat, Heidelberg) to amplify the unknown upstream region according to the kit manufacturer's instructions. All PCR generated sequences were sequenced bi-directionally. Regions exhibiting sequence differences to corresponding gene sequences in the databases were again amplified and sequenced to confirm these differences. Thus, well-characterised gene sequences of the plantaricin loci of approx. 16 kbp and 10 kbp were obtained from *L. plantarum* strains BFE 5092 and PCS20, respectively. The DNA sequences of these plantaricin loci from *L. plantarum* strains BFE 5092 and PCS20 were deposited in the GenBank database and received the accession numbers GU584090 and GU584091, respectively.

2.9 RNA isolation and *pln*EF gene expression studies using RT-PCR

To determine whether the plantaricin genes were actively transcribed in the *L. plantarum* strains, mRNA was reverse transcribed and the *pln*EF gene was amplified from the cDNA product. For this, total RNA was isolated from ca. 1 x 10^8 *L. plantarum* cells grown in MRS broth at 30°C to the end logarithmic growth phase (ca. 12 hours) using the GE RNA isolation kit (GE Healthcare Freiburg) and the Qiagen RNA protect solution according to the manufacture's instructions. The concentration of RNA was determined spectrophotometrically at 260nm in UV cuvettes (Sambrook et al., 1989). RNA was used as template for RT-PCR using the Qiagen OneStep RT-PCR kit (cat. no. 210212) and the *pln*EF genes were amplified using the primers in

Table 2.3. RNA digested with 10mg/ml RNAse (Sigma) at 37°C for 45 min served as a negative control in the RT-PCR experiment. The PCR program used for the one-step PCR is described in Table 2.4.

Table 2.4 PCR program for reverse transcription of the *pln*EF genes from *L. plantarum* strains BFE 5092 and PCS20

Step	Temperature	Time	Reaction
1	50°C	30min	reverse transcription
2	95°C	15min	activation of HotStar *Taq* DNA polymerase (Qiagen)
3 (32 cycles)	94°C	30sec	Denaturation
	52°C	30sec	Annealing
	72°C	1min	Extension
4	72°C	10min	final extension

2.10 Expression of 16S housekeeping, plantaricin and lactate dehydrogenase genes by *Lactobacillus plantarum* growing in liquid (planktonic growth) or on solid agar medium (sessile growth)

One of the aims of this study was to evaluate *L. plantarum* strains BFE 5092 and PCS20 for their suitability as protective cultures on turkey meat. As these bacteria would be growing on a meat surface, it was deemed important to know whether the plantaricin genes would be expressed also when the bacteria were sessile and, moreover, whether gene expression would be to a similar level when compared to growth in liquid culture. For this reason, the plantaricin gene expression was tested first in vitro in both liquid broth and on solid agar medium. The expression of the 16S rRNA, *pln*EF, *pln*G, *pln*JK, *pln*N and L-lactate dehydrogenase (*lldh*) genes by the *L. plantarum* strains PCS20 and BFE 5092 was determined when grown in TSB broth and when grown on TSB solidified with 2% agarose. For isolation of RNA from solid surfaces, the bacterial cells were spread-plated on large size (145 mm diameter) TSB agar plates (containing 2% agarose) and incubated for 48 hours at 30°C. The bacteria were harvested from the surface of the plates using 2 ml quarter-strength Ringers solution (Merck, Darmstadt, Germany) and a sterile glass spreader. For the liquid culture in TSB, the bacteria were inoculated (1% or ca. 1 x 10^6 CFU/ml) and then incubated overnight for 18 h. The initial cell concentration was adjusted to approximately 1 x 10^8 CFU for RNA isolation from cells isolated from both liquid and solid media, by measuring the optical density of the harvested bacteria at 580 nm and diluting the culture to an optical density value of 0.1 to 0.15 (of a 1:100 dilution in quarter-strength Ringer's solution), which corresponded to about 1 x 10^8 CFU/ml.

The Illustra RNAspin mini RNA isolation kit from GE Healthcare was used for RNA isolation. To stabilise RNA before extraction with the GE kit, the RNA Protect reagent (Qiagen) was added to the washed cell suspension in quarter-strength Ringers solution (2:1 v/v) and incubated at room temperature for 5 min. After this, cells were centrifuged at 9,500×g and the

cell wall was digested in 1x TE buffer containing 20mg/ml lysozyme. The pellet was resuspended and RNA was isolated according to the RNA kit manufacturer's instructions. After RNA isolation, total concentration of RNA was determined by spectrophotometry at 260nm in UV-cuvettes. Moreover, the ratio between the readings at 260nm/280nm (A_{260}/A_{280}) provided information on the purity of the nucleic acid, with pure preparations RNA ranged between 1.7 and 2.0.

The RNA was transcribed to cDNA using the iScript cDNA synthesis kit (Biorad). The RNA concentration used for cDNA synthesis was 100ng/µl and the reaction was done following the manufacture's instructions. The cDNA synthesis protocol is described in Table 2.5.

Table 2.5 PCR program used for cDNA synthesis using mRNA from *L. plantarum* strains BFE 5092 and PCS20 as template.

Step	Temperature	Time	Reaction
1	25°C	5min	Primer annealing
2	42°C	30min	cDNA synthesis
3	85°C	5min	Reverse transcriptase enzyme inactivation
4	4°C	Hold	Storage

Real time PCR is one of the most sensitive and reliable quantitative methods for gene expression analysis. Data derived from real-time PCR can be quantified absolutely and/or relatively. This method relies on the comparison between expression of a target gene in a control sample and the expression of the same target gene in reference sample (Yuan et al., 2006). To compensate for differences in expression as a result of differences in experimental setup such as e.g., efficiency of RNA extraction, the expression of the target gene is also compared relative to the expression of a housekeeping gene.

For real time PCR, the 16S rRNA gene was used as a housekeeping gene and a no template control (NTC) was used as negative control. Table 2.6 shows the primers used for amplification of the 16S, *pln*JK, *pln*N *pln*EF, *pln*GH, and *lldh* gene fragments by regular PCR, which were used as template for qRT-PCR to generate a standard curve. The table also shows the primers that amplify an internal fragment of these larger genes for quantitative real time PCR.

Table 2.6 Primers used to PCR amplify target genes as templates for generating a standard curve by conventional PCR and for quantitative PCR with primers designed to bind internal to the PCR product amplified by conventional PCR.

Gene Name	Primer name	Sequence	Size (base pair)
16S rRNA	16S fw, conventional PCR primer[a]	5' AG AGT TTG ATC MTG GCT CAG 3'	1539
	16S rev, conventional PCR primer	5' GG NTA CCT TGT TAC GAC TTC 3'	
	16S fw qRT primer[b]	5' TCA TGA TTT ACA TTT GAG TG 3'	121
	16S rev qRT primer	5' GAC CAT GCG GTC CAA GTT GTT 3'	
pln NJK	*pln*N fw conventional PCR primer[c]	5' CTA ATA GCT GTT ATT TTT AAC C 3'	1697
	pln JK rev conventional PCR primer	5' TTA TAA TCC CTT GAA CCA CC 3'	
	*pln*N fw qRT primer	5' CGT TGA AGG TGG AAA AAA CT 3'	94
	*pln*N rev qRT primer	5' CAT GCC ATG CAC TCG AAG TT 3'	
	*pln*JK fw qRT primer	5' TGA AGA ATT AAC TGC TGA CG 3'	84
	*pln*JK rev qRT primer	5' GAA CCA CCA AGC ACG GCC CG 3'	
Pln EF	*pln*EF fw conventional PCR primer	5' GGT GGT TTT AAT CGG GGC GG 3'	305
	*pln*EF rev conventional PCR primer	5' ACT TGA TGG CTT GAA CTA TCC 3'	
	*pln*EF fw qRT primer	5' CTA TTT CAG GTG GCG TTT TC 3'	93
	*pln*EF rev qRT primer	5' GTG GAT GAA TCC TCG GAC AG 3'	
pln G	*pln*GH fw conventional PCR primer	5' GAG ATG GAC TGT GGG GTC GC 3'	2954
	*pln*GH rev conventional PCR primer	5' GTC TCA ACA CTG TAC TTC GT 3'	
	*pln*G fw qRT primer	5'CCG GAG TTG CCC TTT TCT TT 3'	97
	*pln*G rev qRT primer	5'TGC TTT AAT AAG CTT GGA AT 3'	
lldh	*lldh* fw conventional PCR primer	5' GTT GTT ATT ACA GCC GGT GCG 3'	691
	lldh rev conventional PCR primer	5' TTT TTC AAA GTT GCG GCG A 3'	
	lldh fw qRT primer	5' TGT TGA TCC TCG TTT CGT TG 3'	137
	lldh rev qRT primer	5' AAA TCT TCG TCA GAA ACG CCT 3'	

[a] conventional PCR product is diluted in a decimal dilution series and used in qRT-PCR to obtain a standard curve

[b] qRT primer used in quantitative PCR reactions with conventional PCR product used in standard curve dilutions as template or with cDNA as template

[c] the conventional PCR product *pln*NJK contained the binding sites for qRT primers for both *pln*N and *pln*JK

The 16S is the 16S rRNA gene for *L. plantarum* species.

The *pln*EF, *pln*JK, and *pln*N are the plantaricin genes in *L. plantarum* C11 (Anderssen et al., 1998).

The *pln*G is the bacteriocin dedicated ABC transporter gene in *L. plantarum* C11 (Anderssen et al., 1998).

The *lldh* is L-lactate dehydrogenase gene in *L.plantarum* 423 (Ramiah et al., 2007).

The PCR protocol that was used for qRT-PCR is shown in Table 2.7. The qRT PCR was done in 96 well qRT PCR microtiter (Biorad) plates which were sealed with foil (Biorad). The PCR was done using a Biorad iQ5 qRT PCR cycler with SYBRGreen. In this method, the increase in PCR product is determined in real time by increase of the SYBR-Green fluorescens. SYBR-Green is excited by light with a wavelength of 494 nm and emits with a maximum of 521

nm. The intensity of the light emission is measured and the data are displayed by the Biorad iQ5 documentation software.

Table 2.7 Contents for the RT-PCR using cDNA as template

Reagents	Volume per reaction
IQ SYBR Green Supermix	12.5µl
Forward primer (50 pmol/ µl)	0.125 µl
Reverse primer (50 pmol/ µl)	0.125 µl
cDNA template (5ng/ µl)	3 µl
H_2O nuclease free water	9.25 µl
Total volume	25 µl

2.11 Quantitative Real Time PCR data analysis

2.11.1 Determining the gene copy number for quantification of absolute gene expression and establishing a standard expression curve

All PCR products were amplified in conventional PCR reactions from *L. plantarum* strains BFE 5092 or PCS20 genomic DNA to serve as templates for generating standard curves in qRT-PCR experiments. To show that the PCR reactions amplified the correct genes, the PCR products were subjected to gel electrophoresis on 1.5% agarose gels to confirm their expected sizes. In addition, all PCR products were partially or completely sequenced as to confirm their identity. To generate standard curves for expression studies of each of these specific genes, the conventional PCR products were measured at 260 nm using a by Nanodrop-Photometer (Peqlab) and the DNA concentration was determined from this optical density reading (Sambrook et al., 1989) in ng/µl. The molar mass of the PCR product was calculated on the basis of the number of nucleotides and their average molecular weight (600g/Mol/l) and the concentration of DNA determined. The molarity was established by dividing the determined concentration by the molar mass. As Avogadro's number states that one mole equates to 6.025 x 10^{23} molecules, the copy number of the PCR product could be determined by multiplying the determined molarity with Avogadro's number.

After every cycle of the qRT-PCR reaction, the iQ5 cycler used measures the fluorescence intensity of the SYBRGreen reporter dye. The detection software documents these fluorescence data in a coordinate system with the corresponding cell cycle in a semilogarithmic coordinate system. Thus a function is generated which shows the kinetics typical of the reaction. The amplification kinetics are only ideal in the exponential area, thus a quantification is only possible in this exponential range. The C_t value that is determined is a 'threshold value', i.e. the value where the signal clearly distinguishes itself from the background fluorescence and identifies the cycle at which this measurable fluorescence intensity is reached. Therefore, the higher a gene is expressed the higher its cDNA copy number and the earlier the C_t-value will be

reached. Thus, the lower the C_t-value, the higher the gene is being expressed.

For generating the standard curve, a suspension of PCR product of the target gene produced by conventional PCR, which contained about 10^7 copies/ul was prepared, and 10-fold serial dilutions were made down to 10^2 copies/ul. The diluted PCR product sample for making the standard curve was used in real-time PCR. Thus, a calibration curve was constructed by relating the C_t-value detected automatically by qRT cycler to the copy number and by linear regression of the standard curve data. In order to determine *absolute gene expression* levels, measured from the fluorescence obtained in the qRT-PCR reaction with cDNA. The C_t-values were used to calculate the copy number by entering the value into the linear regression equation and solving the equation to arrive at the for the copy number. An example of a linear regression of a standard curve is shown in Figure 2.1.

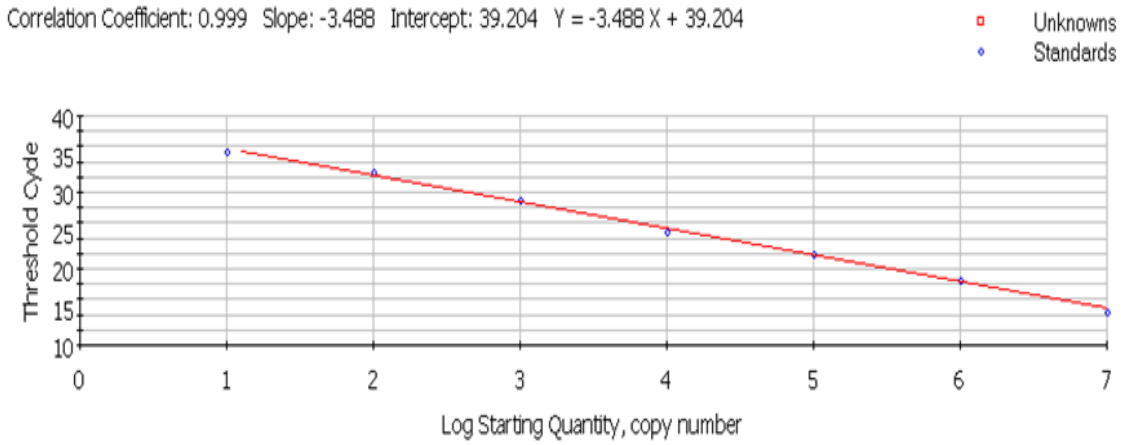

Figure 2.1 Example for Ct-values determined for generation of a standard curve using DNA from a decimally diluted PCR product sample with known copy numbers. The C_t value was plotted as a function of the logarithm of numbers of copies of template. The real expression copy number can be correlated from this curve after performing a linear regression.

2.11.2 Determining relative gene expression by quantitative PCR using the $\Delta\Delta C_t$ method

To determine the *relative gene expression,* the expression levels determined by qRT-PCR of a target gene were normalised to those of a non-regulated reference or housekeeping gene, in this study the 16S rRNA gene. Nomalisation against a housekeeping gene minimizes considerable deviations in transcription levels which result from experimental difficulties, i.e. for example in RNA isolation, and which should not be wrongly interpreted as real differences in transcription levels. The quantification of the relative expression in this work was by the $\Delta\Delta C_t$ method (Livak and Schmittgen, 2001). This method is based on the assumption of a 100% efficiency of the PCR reaction. In this method, the ΔC_t

value of the samples is first calculated from

$$\Delta C_t = C_{t\ \text{target gene}} - C_{t\ \text{reference gene}}$$

which relates the expression of the target gene relative to that of the reference gene. To relate the relative expression of the target gene under test condition to the reference gene under reference condition, the $\Delta\Delta C_t$-value is calculated as follows

$$\Delta\Delta C_t = \Delta Ct_{\text{sample)}} - \Delta Ct_{\text{control}}$$

The fold-change in expression (increase or decrease) for each gene from control to the sample can be calculated as follows

$$\text{relative expression} = 2^{-\Delta\Delta C_t}$$

In all qRT-PCR experiments all samples were pipeted three times and three measurements were obtained. The mean expression and the standard deviations were calculated.

2.12 Microbiological analysis of turkey meat and inoculation with protective culture and / or pathogenic bacteria

In order to test the *L. plantarum* strain BFE 5092 selected by the PathogenCombat coordinators as a protective culture, this strain was inoculated onto turkey breast meat in the presence or absence of pathogens. The turkey breast meat was obtained from a partner in the PathogenCombat Project (Ziegler, Bammental) who sent the meat on the same day the animal was slaughtered under cooled (on ice) conditions. The turkey meat (without skin, breast meat cut) was further cut into portions of ca 7 x 8 cm in a sterile laminar flow unit using a sterilised knife. Different samples were prepared, i.e., uninoculated control or sterilised turkey meat (see below) inoculated with only *L. plantarum* BFE 5092 as protective culture at approx 10^7 CFU/g, or sterilised turkey meat inoculated with *L. plantarum* BFE 5092 protective culture at 10^7 CFU/g and with the pathogen *L. monocytogenes* EGDe at a level of 10^5 CFU/g or with the pathogen *Salmonella* Typhimurium at a level of 10^5 CFU/g, or non-sterilised turkey meat with protective culture or pathogen, or sterilised turkey meat (see below) with only *L. monocytogenes* EGDe as pathogen at a level of 10^5 CFU/g or with only *Salmonella* Typhimurium at a level of 10^5 CFU/g. Before inoculation, the bacteria were grown in MRS broth (*L. plantarum*) or in Standard I broth (*L. monocytogenes*) at 30°C for 18 h. The optical density of the cultures was determined spectrophotometrically at 580 nm and the culture was washed with Ringers solution and diluted to obtain the required concentration. For some samples, turkey meat was sterilised using a linear accelerator at 10 kGy. The linear accelerator was a CIRCE III accelerator with 10MeV

energy and 15kW power (Getinge Linac Technologies, Orsay, France).

The turkey meat portions all were placed in Petri dishes and the bacteria were surface inoculated and spread on the surface using a sterile glass spreader. The turkey meat samples were stored at 8°C incubation temperature. This temperature was chosen as lower temperatures would not permit growth of the *L. plantarum* protective culture which was known to have a minimum growth temperature of 8°C. Microbiological sampling was done in duplicate at specific time intervals (every second or third day). For sampling, the turkey meat was transferred from the Petri dish into 50ml Ringer's solution and was pummeled with a stomacher for 1 min. After stomaching, 25ml of suspension was separately collected in falcon tubes for RNA isolation and then mixed with same volume of ice cold methanol (Roth) to prevent RNA degradation and to stop RNA expression. A further 1 ml sample of the suspension was removed to measure the pH. For enumeration of microorganisms, tenfold serial dilutions (10^{-1}–10^{-6}) were made for each sample and either 100µl or 10 µl of the appropriate dilutions was spread plated onto selective media for isolation of the different microbial groups. Thus Standard I agar was used for determining the total aerobic and mesophilic count at 30°C, MRS agar pH 6.4 was used for enumeration of total LAB at 30°C and Violet Red Bile Dextrose Agar (VRBD) (Merck) for enumeration of *Enterobacteriaceae* at 37°C under aerobic conditions. Potato Dextrose Agar (PDA) was used for the enumeration of yeasts at 25°C. An antibiotic cocktail consisting of penicillin G (100µg/ml), streptomycin (250µg/ml), vancomycin (25µg/ml), chloramphenicol (20µg/ml) and erythromycin (50µg/ml) was added to Potato Dextrose Agar to inhibit bacteria. Palcam agar supplemented with *Listeria* selective supplement (Oxoid) was used for enumeration of *L. monocytogenes* at 37°C, and *Salmonella / Shigella* agar for enumeration of *Salmonella* at 37°C.

2.13 Expression of antimicrobial genes and genes encoding surface proteins by *L. plantarum* strain BFE 5092 during growth on turkey meat using quantitative PCR

In order to determine the cause of any potential antimicrobial activity of the protective *L. plantarum* BFE 5092 culture in inhibiting pathogens in the challenge tests on turkey meat, the expression of genes associated with antimicrobial activity, i.e. L-lactate dehydrogenase genes and plantaricin EF, JK, N was determined by qRT-PCR as described above (see 2.12 and 2.13). Firstly, the study aimed to determine whether these genes were expressed at all on the turkey meat surface and secondly, as bacteriocin expression is a cell density regulated phenomenom, it was aimed to see whether RNA expression occurred at levels similar to those expression levels seen in vitro at similar cell numbers. Also, it was aimed to elucidate whether the presence of a Gram-positive pathogen (i.e. *Listeria monocytogenes*) would be able to enhance expression of the bacteriocin genes.

Before the expression of these genes could be measured using qRT-PCR, methods were needed to be established for isolating the bacteria from the food surface and isolating their

RNA. This was deemed difficult because of the presence of food components (especially protein) in the RNA isolation protocol and because of the presence of autochthonous bacteria whose RNA would be co-isolated. Total RNA was isolated by removing 25 ml of the food matrix suspension (see above) and by adding 25 ml of ice cold methanol to fix the cells and freeze RNA expression. The bacteria were harvested from the solution by low speed centrifugation at 200 x*g* for 5 min. This centrifugation left the bacteria in the supernatant while it served to pellet food components. To further decrease turkey meat components, the bacteria were centrifuged two more times at same low speed and the clear supernatant was transferred to a fresh Eppendorf tube. RNA was again stabilised by the addition of two volumes RNA Protect (Qiagen) After these steps, the RNA isolation proceeded with the General Electric Healthcare RNA isolation kit according to the manufacturers instructions and as mentioned above (see 2.9). In order to eliminate any remaining DNA contamination in the RNA sample, an additional DNase treatment step with DNAse from Ambion (Hilden) was performed. For this, 2U/µl was added to the sample and incubated at 37°C for 30 min. After incubation, the sample was mixed with DNase inactivation beads, and the remaining pure RNA was collected by centrifugation at 10,000 x*g* for 1.5min. The total RNA concentration was determined by measuring the absorbance at 260 nm using a NanoDrop spectrophotometer as previously described (Sambrook, 1989). To measure the expression of the target genes, the cDNA synthesis and qRT-PCR was performed as described above (see 2.11 and 2.12).

CHAPTER 3.0
RESULTS

3.1 Safety of protective cultures with respect to antibiotic resistance

According to EFSA (2005), bacteria used as starter cultures for food and feed should not contain transferable antibiotic resistances. For this reason, the *L. plantarum* BFE 5092 and PCS20 strains investigated in this study for development of potential protective cultures were investigated for their antibiotic resistance profile using the LAB susceptibility test medium (LSM) as suggested by Klare et al. (2005). These two *L. plantarum* strains did not show any conspicuous antibiotic resistances. According to the antibiotic resistant breakpoints from FEEDAP (2005) for gentamicin, streptomycin, tetracycline, chloramphenicol, vancomycin and and erythromycin for *L. plantarum* strains (see Table 3.1), these strains could be considered as susceptible to these antibiotics. Unfortunately FEEDAP does not list a breakpoint value for ciprofloxacin and the resistance towards this antibiotic was considerably high at 64 and 512 µg/ml for *L. plantarum* strains BFE 5092 and PCS20, respectively.

Table 3.1. Antibiotic resistance profile of protective *L. plantarum* strains BFE 5092 and PCS20.

Strain	Minimum inhibitory concentration (µg/ml)						
	ER	GM	SM	CI	VM	CL	TC
L. plantarum BFE 5092	0.032	0.064	16	64	256	1	8
L. plantarum PCS20	0.016	2	16	512	>512	2	8
Breakpoint value[a]	4	64	64	n.l.[b]	n.r.[c]	8	32

[a]:breakpoints according to FEEDAP (2005) ER: erythromycin, GM: gentamicin, SM: streptomycin. CI: ciprofloxacin, VM: vancomycin, CL: chloramphenicol, TC: tetracycline. The value '>512' means no growth inhibition occurred and this was the maximum concentration tested.
[b] n.l.: not listed by FEEDAP, [c] n.r.: not required by FEEDAP

3.2 Bacteriocin activity and PCR amplification of known bacteriocin genes

In order to detect whether the *L. plantarum* strains used in this study produced bacteriocins, the deferred inhibition assay with MRS agar was carried out, in which bacteriocin production by a producer colony on an agar plate is tested. The bacteriocin diffuses into the agar and is detected by the absence of growth of a lawn of indicator which is overlayered on top of the producer culture after it has grown. Furthermore, possible bacteriocin production was determined by PCR amplification of known bacteriocin genes using custom designed primers specific for these genes.

Using the deferred inhibition assay with *L. sakei* DSM 20017^T as a sensitive indicator, the zones of inhibition around the *L. plantarum* BFE 5092 and PCS20 producer strains were approx. 19 mm and 18 mm, respectively, measured from the edge of the colony (Fig. 3.1). By

spotting 3 µl of 10 µg/ml proteinase K solution next to the producing culture that was grown overnight, and then incubating a further 3 hours for proteinase K activity at 37°C, the inhibition zone was not visibly decreased in the vicinity of the proteinase K spot. This indicated that a bacteriocin activity based on a proteinaceous compound could not be detected.

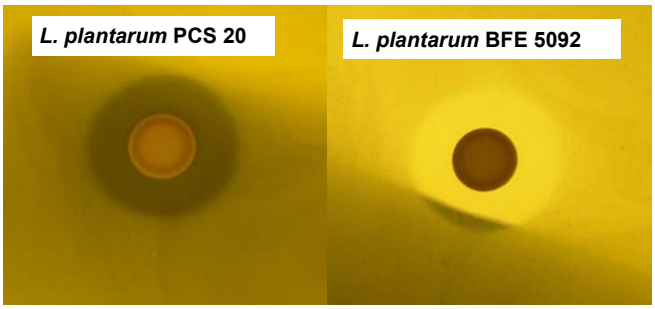

Figure 3.1 Deferred inhibition assay using *L. plantarum* strains BFE 5092 and PCS20 against *L. sakei* DSM20017^T. Overnight cultures were spotted onto MRS agar plates and overlayered with the *L. sakei* indicator strain in MRS soft agar. After re-incubation, positive strains showed a clear zone of inhibition surrounding the producer colony.

To diminish the effect of lactic acid produced by the different *L. plantarum* strains on the sensitive indicator strain *L. sakei* DSM 20017^T, buffered agar medium (BSM medium, see 2.1.1) containing 10x less glucose than in MRS agar, and more buffering substances such as $K_2HPO_4 \cdot 3H_2O$ and KH_2HPO_4, was used to test again for bacteriocin activity using the deferred inhibition assay. Using this assay, a zone of inhibition of approx. 5 mm measured from the edge of the colony was detected for the *L. plantarum* strains BFE 5092, while only a very slight zone of less than 1 mm could be detected for *L. plantarum* PCS20 (Fig. 3.2). In the case of *L. plantarum* BFE 5092, spotting 5 µl of a 10 mg/ml proteinase K solution next to the colony diminished the inhibition zone (Fig. 3.2) showing clearly that the activity was due to a proteinaceous compound such as a bacteriocin.

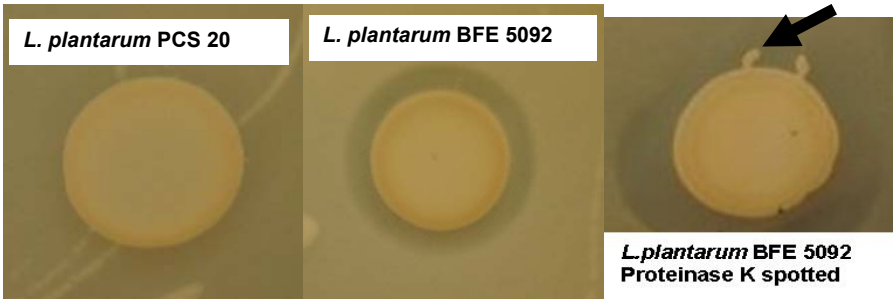

Figure 3.2 Deferred inhibition assay using *L. plantarum* strains BFE 5092 and PCS 20 against *L. sakei* DSM20017^T. Overnight cultures were spotted onto BSM agar plates and overlayered with the *L. sakei* indicator strain in BSM soft agar. After re-incubation at room temperature, positive strains showed a clear zone of inhibition surrounding the producer colony. The effect of proteinase K spotted next to producer strain resulted in a decrease in the size of the inhibition zone where the enzyme was spotted (see arrow).

Using custom-designed primers for known bacteriocin genes (see Table 2.3) and total genomic DNA from *L. plantarum* strain BFE 5092 and the control strain *L. plantarum* 299V, it could be shown that both these strains contained the structural genes for the bacteriocins PlnEF, PlnJK, and PlnN, which was confirmed by sequencing of the resulting PCR products (Fig. 3.3a). In contrast, the *L. plantarum* strain PCS20 contained only the structural genes for plantaricin EF (Fig. 3.3b).

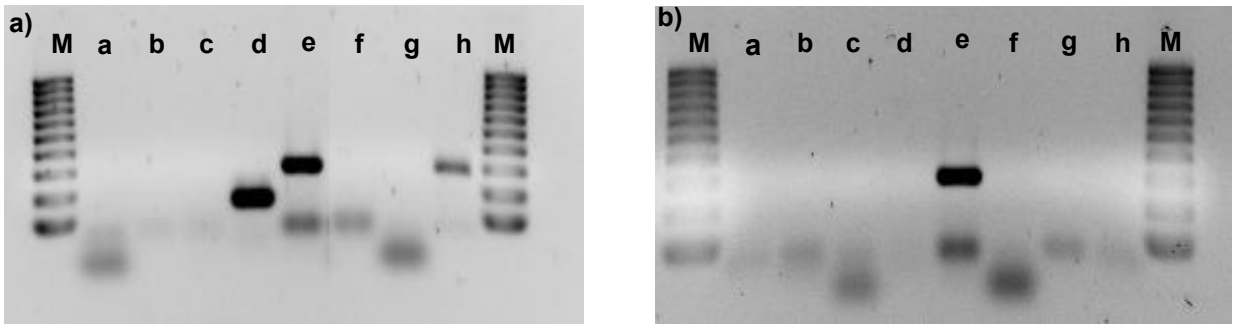

Figure 3.3 Agarose gel electrophoresis of PCR products obtained after PCR amplification of *L. plantarum* genomic DNA with custom designed primers for the known plantaricin genes a) DNA isolated from *L. plantarum* BFE 5092 M: standard 100 base pair marker a; plantaricin 1.25, b; plantaricin 4.23, c; pediocin AcH, d; plantaricin N, e; plantaricin EF, f; plantaricin NC8, g; plantaricin S, h; plantaricin JK b) DNA isolated form *L. plantarum* PCS20 A; plantaricin 423, b; plantaricin 1.25, c; plantaricin N, d; plantaricin NC8, e; plantaricin EF, f; plantaricin S, g; plantaricin JK, h; pediocin AcH. The lower (100 bp or smaller) bands on the gels correspond to primer or primer dimer bands.

The nucleotide sequences of the *pln*EF, *pln*JK and *pln*N PCR products, which were amplification products of the part of the gene encoding the mature bacteriocin peptides from *L. plantarum* strains BFE 5092, PCS20 (*pln*EF only) and 299V, showed identical sequences to those previously reported (Diep et al., 1996; Kleerebezem et al., 2003). Translation of nucleotide sequences of this partial sequence resulted in amino acid sequences constituting the mature parts of the PlnJK, PlnEF and PlnN peptides, which were identical to those reported previously for *L. plantarum* C11 (Diep et al., 1996) and *L. plantarum* WCFS1 (Kleerebezem et al., 2003) (results not shown).

3.3 Localisation and organisation of the plantaricin operons on the genomes of *L. plantarum* strains BFE 5092 and PCS20

Bacteriocin operons are known to be located either on the chromosome or on plasmid DNA. The localisation of the plantaricin genes produced by *L. plantarum* strains BFE 5092 and PCS20 was important firstly to compare the gene loci of the two strains and secondly to clone the gene loci to characterize them at a genetic level. In order to localize and preliminarily characterize the plantaricin gene loci in the different *L. plantarum* strains, plasmid DNA as well as the chromosomal DNA was isolated. The chromosomal DNA was cut with the restriction

enzymes *Xba*I and *Eco*RI and was run together with the isolated plasmid DNA fractions on an agarose gel. As shown in Fig. 3.4, *L. plantarum* BFE 5092 and *L. plantarum* PCS20 both contained plasmid DNA. Following electrophoresis, the gel was blotted and probed with a plantaricin EF probe. The hybridisation result showed that the plantaricin locus was located on the chromosome of these *L. plantarum* strains and not on the plasmid DNA. Hybridisation of the plantaricin EF probe with the *Xba*I and *Eco*RI digest of the chromosomal DNA furthermore showed that the loci appeared to be similarly localized on the chromosome, as a hybridisation signal occurred at the same position on the gel (Fig. 3.4). *L. plantarum* strain 299V, on the other hand, clearly showed a different localisation on the chromosome, as the hybridisation signal occurred at a different position. *L. johnsonii* La1 was used as negative control to detect any unspecific hybridisation signals, which did not occur (Fig. 3.4).

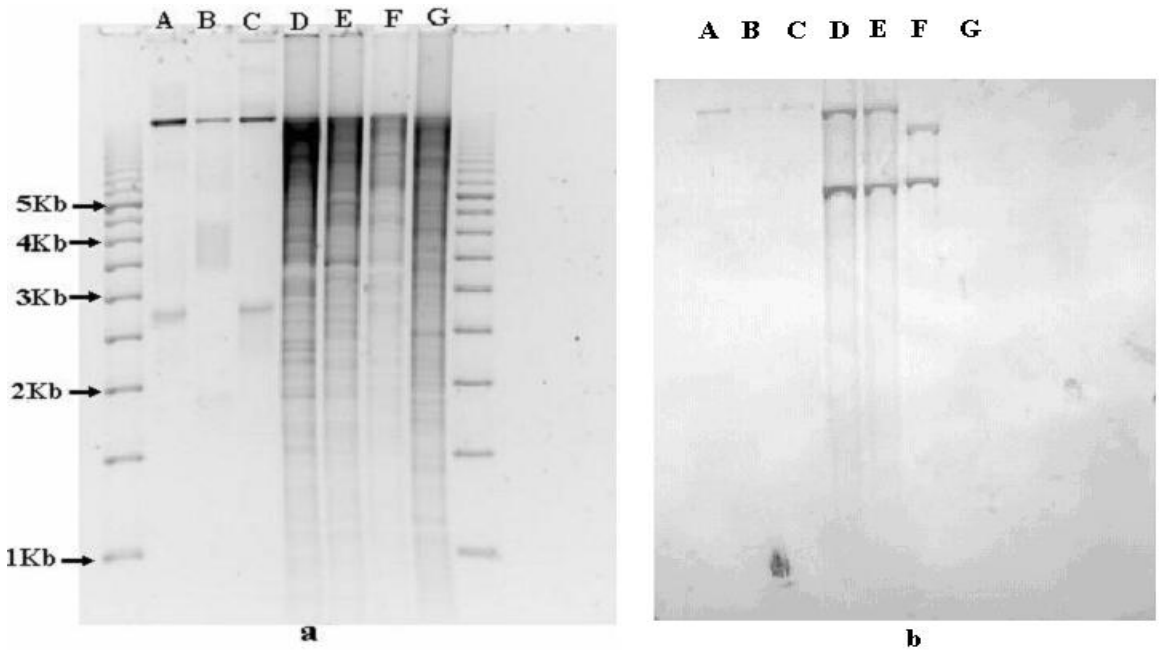

Figure 3.4 Photonegative of agarose gel with *Xba*I and *Eco*RI digested chromosomal and plasmid DNA (a) and corresponding membrane hybridised with *pln*EF probe (b). A: Plasmid from *L. plantarum* PCS20, B: Plasmid from *L. plantarum* BFE 5092, C: Plasmid from *L. plantarum* BFE 905 (positive plasmid control), D: *L. plantarum* BFE 5092 chromosomal DNA, E: *L. plantarum* PCS20 chromosomal DNA, F: *L. plantarum* 299V chromosomal DNA as positive control, G: *L. johnsonii* La1 chromosomal DNA as negative control.

PFGE with DNA restricted with *Not*I was also used to determine whether the plantaricin locus was located on different chromosomal locations in the two *L. plantarum* BFE 5092 and PCS20 strains. A completely different banding pattern of the PFGE fingerprint showed that these two *L. plantarum* strains were clearly not clonally related. In contrast to the hybridisation results obtained with the chromosomal DNA cut with *Xba*I and *Eco*RI restriction enzymes, the hybridisation of the PFGE gel showed that indeed the plantaricin loci in the two strains occurred at different sections of the chromosome, as the probe hybridized at two very different positions, i.e., with a band of about 97 kbp in *L. plantarum* BFE 5092, but with a smaller band in *L. plantarum* PCS20 (Fig. 3.5).

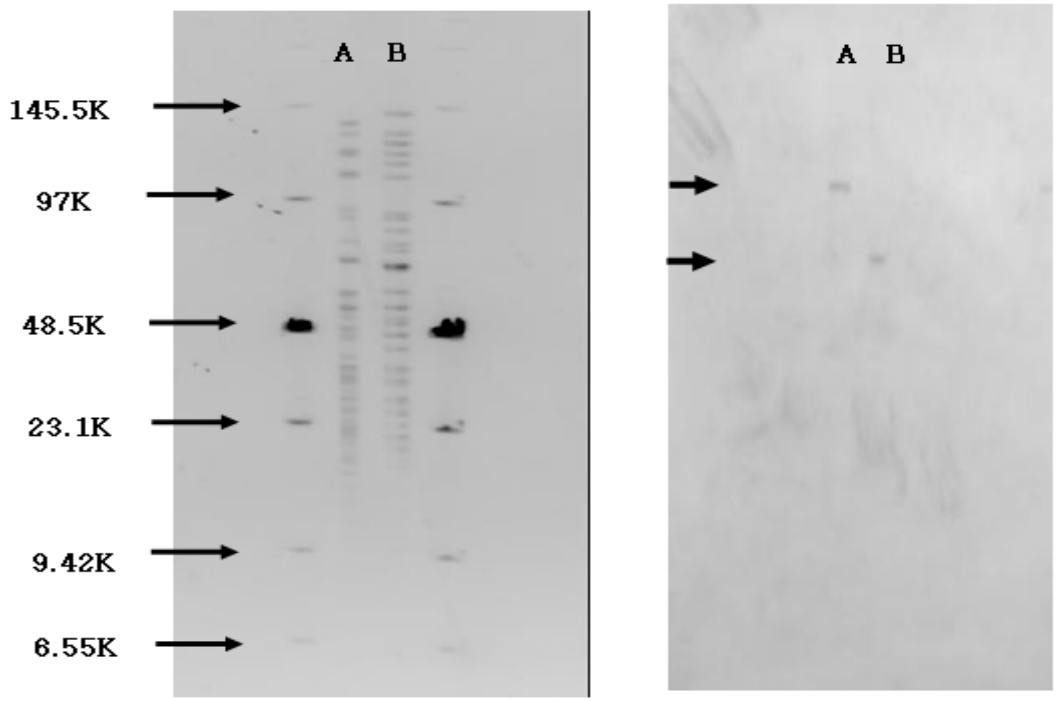

Figure 3.5 *Not*I digested total DNA fragments in pulsed field gel electrophoresis and corresponding hybridisation on nylon membrane. A*: L. plantarum* BFE 5092 B: *L. plantarum* PCS20. On the nylon membrane, *pln*EF DIG labeled probes were detected at different positions depending on the strains. Upper arrow (A) marker size neighboring 97 kb, lower arrow (B) between 97kb and 48.5kb.

3.4 Characterisation of the plantaricin loci of *L. plantarum* strains BFE 5092 and PCS20

The nucleotide sequence of the *pln*E and *pln*F genes of the *L. plantarum* strain BFE 5092 was 100% identical to that of the corresponding genes of *L. plantarum* strains C11, 299V or WCFS1 as deposited in the databank. Although the *pln*F gene produced by *L. plantarum* PCS20 was also identical to the corresponding gene in *L. plantarum* strains C11, 299V, WCFS1 and BFE 5092 (result not shown), the sequence of the *pln*E gene in strain PCS20 differed from the corresponding nucleotide sequences for this gene when compared to these other strains (Fig. 3.6). There was a deletion of one nucleotide 47 base pairs downstream of the *pln*E ATG start codon, which results in a frame shift of the open reading frame (ORF). Thus, the amino acids translated from the nucleotide sequence are initially identical for up to the first 15 amino acids, but differ by the next 24 amino acids. Two further deletions, one of two adjoining nucleotides and one of three adjoining nucleotides occurred downstream (Fig. 3.6). Thus, a total of 6 base pair deletions took place within the gene, which finally shifts the ORF back into the plantaricin-encoding frame, thus resulting in an identical 15 amino acid carboxyl end to plantaricin E as produced by *L. plantarum* strains C11 and WCFS1. Therefore, these deletions led to a hypothetically produced peptide which is 2 amino acids shorter than plantaricin E, and

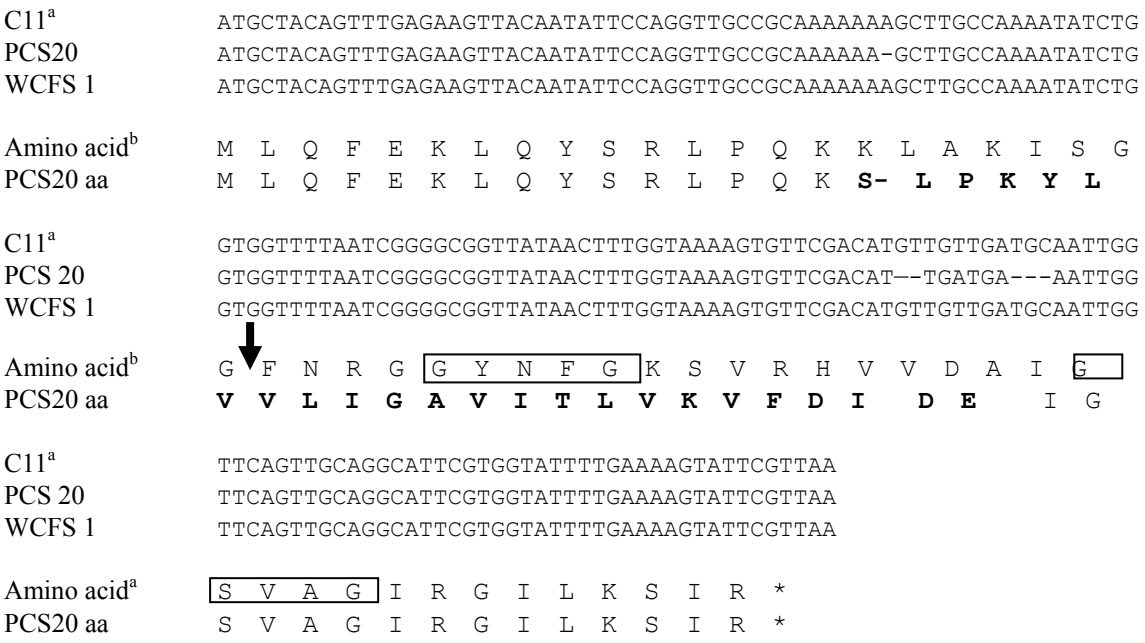

Figure 3.6 Nucleotide sequences and amino acid translations of plantaricin E gene in different *L. plantarum* strains selected in this work. Nucleotide deletions are shown, substitutions shown in bold print. Amino acid differences are also shown in bold print. Note the absence of a double-glycine leader sequence cleavage site (indicated by an arrow) for the *pln*E sequence of *L. plantarum* PCS20. Boxes show the conserved GxxxG sequences involved in peptide helix-helix interaction.

differs by 24 amino acids, while it shares 30 identical amino acids, i.e., 15 at the amino end and 15 at the carboxyl end of the hypothetical peptide (Fig. 3.6). As a consequence of the first deletion, the amino acid sequence is changed such that a double-glycine-type leader peptide is not encoded by the *L. plantarum* PCS20 *pln*E gene (Fig. 3.6).

The 16 kbp bacteriocin locus of the probiotic strain *L. plantarum* BFE 5092 was completely sequenced in this study in both directions and contained 20 genes (*pln*RLKJMNOPQABCDIFEGHTU) involved in biosynthesis, regulation, transport and immunity of 3 bacteriocin systems, i.e., the two-peptide bacteriocins plantaricin EF and plantaricin JK, as well as plantaricin N (Fig. 3.7). Of these, the translated products PlnR, PlnL, PlnK, PlnJ, PlnM, PlnO, PlnP, PlnQ, PlnA, PlnE, and PlnF were 100% identical to those reported for these gene products in *L. plantarum* strain WCFS1 (Kleerebezem et al., 2003). The other translation products associated with the genes from this bacteriocin locus also showed high similarities of 96.4, 98.0, 87.9, 98.4, 99.3 and 99.1 % identity for the PlnB, PlnC, PlnD, PlnI, PlnG and PlnH proteins, respectively. The *pln*T gene was unusual as it appeared to be a ‘fusion’ of the gene sequences of the *pln*S and *pln*T genes as found in *L. plantarum* strains WCFS1 and C11.

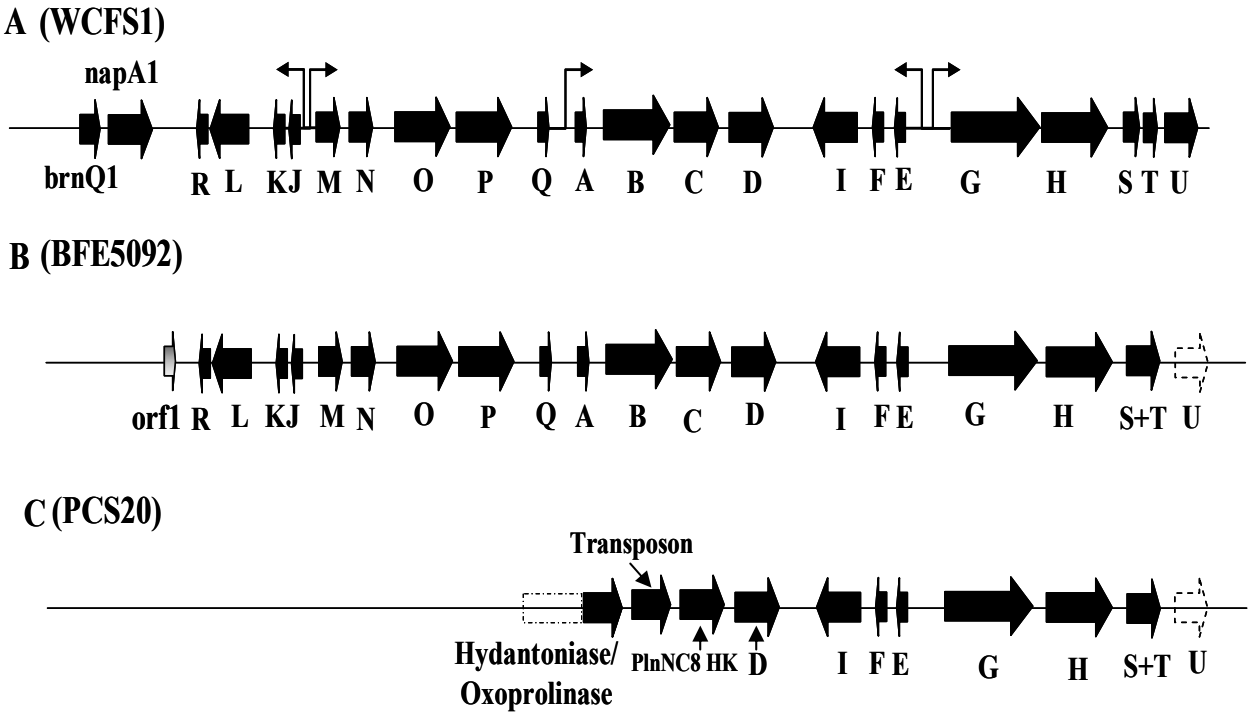

Figure 3.7 Genetic map of plantaricin locus of different *L. plantarum* strains (A) *L. plantarum* WCFS1 (GenBank accession number AL935253); (B) *L. plantarum* BFE 5092 (GenBank accession number GU584090, this study); (C) *L. plantarum* PCS20 (GenBank accession number GU584091, this study). The *pln* genes are shown by arrows corresponding to the approximate size of genes. In the case of *L. plantarum* PCS20, the hypothetical amino acid sequence in front of transposon gene is similar to a hydantoinase / oxoprolinase gene. The promoter sequences are indicated by small black arrows. Open reading frames shown by dotted line were only partially sequenced and the lines indicate the truncated part of the gene.

Nevertheless, a counterpart *pln*S/T gene with 96.9% sequence identity was found in *L. plantarum* strain V90 (Diep et al., 2009). In this study, only part of the *pln*U gene which flanks the *pln*T gen was PCR amplified. Sequencing did also not continue beyond the partial *pln*U sequence. It is known that the *pln*GHSTUVW genes are highly conserved in the *pln* gene locus (Diep et al., 2009). Moreover, the bacteriocin systems of *L. plantarum* strains WCFS1 and C11 are known to contain at least four more genes located downstream of *pln*STU, i.e., *pln*VWXY (Kleerebezem et al., 2003; Diep et al., 2009).

In contrast to the plantaricin locus of strain BFE 5092, the approx. 10.0 kbp locus of *L. plantarum* PCS20 sequenced in this study (Fig. 3.7) contained only the genes for the plantaricin EF peptides (mutated in the case of plantaricin E, see above), the gene encoding the plantaricin EF immunity peptide (*pln*I) and genes for bacteriocin transport (*pln*GHTU). Again, in this case only a partial sequence was obtained for *pln*U, because the gene was not completely PCR amplified and sequencing did not proceed beyond the *pln*U gene. The PlnI, PlnG and PlnH protein sequences from *L. plantarum* PCS20 were 100, 99.6, and 99.1% identical compared to the corresponding sequences of *L. plantarum* WCSF1. The *pln*T gene was similar (97.8% amino acid identity) to that of the BFE 5092 strain, and shared 95.6% sequence identity with the *pln*T

gene of *L. plantarum* strain V90 (Diep et al., 2009). Upstream of the *pln*EF genes there were genes involved in regulation of synthesis of the bacteriocin NC8, i.e., a histidine protein kinase gene, as well as a response regulator gene (Fig. 3.7), both of which have high homology to the response regulator genes *pl*NC8HK and *pln*D of *L. plantarum* NC8, which regulate production of the two-peptide bacteriocin plantaricin NC8 (Maldonado et al., 2004). However, the histidine kinase gene was not present as a complete gene but was disrupted by a transposon insertion from the 516th nucleotide of the corresponding *pl*NC8HK gene. Thus, the translated sequence when compared to that of the *pl*NC8HK sequence lacks the first 172 amino acids. The remainder of the translated amino acid sequences was 86.5% identical when comparing the translated gene product of the two strains. A direct repeat (AAATGAAA) was present at the end of the transposon, similar to that of the transposon inserted upstream of the *pln*Q gene in strain V90 (Diep et al., 2009). A further difference was that the NC8 induction factor gene (*pl*NC8IF), which occurs directly upstream of the NC8 histidine kinase gene in the genetic of *L. plantarum* NC8 (Maldonado et al., 2004), was absent (Fig. 3.7) and instead, the transposon gene interrupted the plantaricin NC8 histidine kinase (Fig. 3.7). The complete transposon gene was sequenced and this showed 99.2% similarity in amino acid sequence to the V90 transposon of the MULE superfamily as described to occur in the plantaricin V90 locus (Diep et al., 2009). Approx. 1 kbp was sequenced upstream of the transposon, but the remainder of the *pl*NC8HK gene, nor the *pl*NC8IF gene, could be detected. Instead, a gene encoding a hydantoinase/oxoprolinase gene was found upstream of the transposon (Fig. 3.7). Furthermore, the *pl*NC8IF gene could not be amplified with primers specific for this gene in this study (results not shown).

3.5 Expression of the *pln*EF genes in *L. plantarum* strains BFE 5092 and PCS20

Plantaricin expression studies were done to test whether plantaricin genes were being expressed by both strains. The observed deletions in the *pln*E gene of *L. plantarum* PCS20 were not thought to affect plantaricin gene expression, as these were inside the open reading frame and thus unlikely to affect possible regulatory sequences. Nevertheless, these experiments still could confirm that these mutations would not affect the *pln*EF expression. The expression of the *pln*EF genes was investigated by RT-PCR using the primers for amplification of the *pln*EF genes which amplify a PCR product of 306 bp (Table 2.3). The expression of the *pln*EF gene of *L. plantarum* strains BFE 5092 and PCS20 is shown in Figure 3.8. Both strains clearly expressed these genes, as the mRNA could successfully be transcribed to cDNA which served as template to amplify the 306 bp PCR product. As expected from the plantaricin loci sequence analyses of strains BFE 5092 and PCS20, the frameshift mutations observed in strain PCS20 did not affect gene expression and apparently a mutated *pln*E gene thus must have been expressed to a mutated PlnE peptide. It seems that the primers were able to bind in the beginning of open reading frame and the end of *pln*E region. Therefore, the mutation happened

within the reading frame and did not affect the transcription of the *pln*E locus.

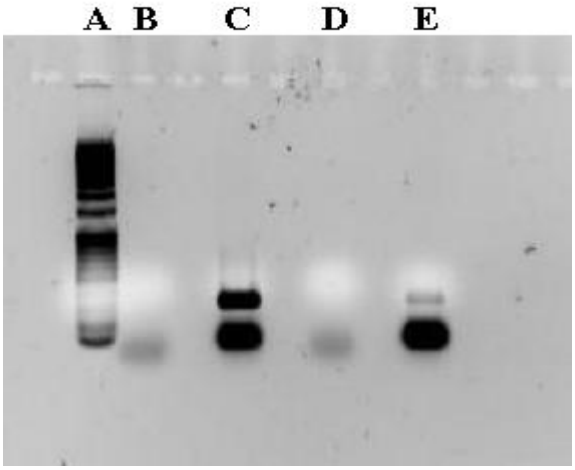

Figure 3.8 Amplification of plantaricin EF fragment using reverse transcriptase PCR A: Biorad 100 bp ladder B: RNase treated RNA from *L. plantarum* BFE 5092 used as negative control, C: RT-PCR product of the *pln*EF genes of *L. plantarum* BFE 5092 (upper band), D: RNase treated RNA from *L. plantarum* PCS20 used as negative control, E: RT-PCR product of the *pln*EF genes of *L. plantarum* PCS20 (upper band). The lower band at about 100 bp corresponds to primer.

3.6 Expression of plantaricin genes under different growth conditions

The *L. plantarum* strains BFE 5092 and PCS20 were aimed to be tested as protective cultures for preservation of turkey meat as requested by the PathogenCombat management, on grounds that the German national food sector partner (Geflügelspezialitäten Ziegeler) was a turkey producer. The *L. plantarum* strain PCS20 was included in this investigation despite the fact that the only plantaricin genes (*pln*EF) present in this strain were mutated and probably not functional, purely for comparison reasons, i.e., to compare the expression pattern of the two strains under the different test conditions. Before testing the strains for their biopreservative activity on turkey meat, their growth and gene expressions under growth conditions expected to occur in this food environment were tested in vitro. It was thus expected, that the bacteria would be growing at low temperature for storage of turkey meat, and that they would be sessile, i.e. growing on the meat surface as opposed to growth in liquid (planktonic). For this reason plantaricin and lactate dehydrogenase gene expressions were tested under such in vitro conditions.

3.6.1 Growth and inhibitory activity at different growth temperatures in vitro

Growth and inhibitory activity were tested for the protective *L. plantarum* strains PCS20 and 5092, as well as for the control strain 299V at different temperatures, in order to determine the lower growth limit and whether inhibition of indicator strains occurs at low temperature. For

testing the antimicrobial activity of the *L. plantarum* cells grown at different temperatures, the overnight producer *L. plantarum* cultures were grown at decreasing temperatures of 30, 20, 15, 12, 10 and 8°C. Eight degrees Celsius was determined to be the minimum growth temperature for *L. plantarum* strains PCS20 and BFE 5092 (Table 3.2), however growth as a colony on the plate was noticed to be slow and leading only to reduced colony growth. Antimicrobial activity of *L. plantarum* strains BFE 5092, PCS20 and 299v was determined using the deferred inhibition assay with the sensitive indicator strains *Lactobacillus pentosus* DSM 20314 or *L. sakei* DSM 20017^T and zones occurred when the producer cultures were grown at either 30, 20, 15, 12, 10 and 8°C. However, a progressive decrease in the zone of inhibition with decreasing temperature (Table 3.2) was noticed, and at 8°C only slight inhibition occurred.

Table 3.2 Growth and bacteriocin production of *L. plantarum* strains determined using the deferred inhibition assay with MRS agar at different temperatures. Inhibition zones were measured from the edge of the producer colony.

Strain	Zone of inhibition (mm) against *L. pentosus* DSM 20314 at different growth temperatures					
	30°C	**20°C**	**15°C**	**12°C**	**10°C**	**8°C**
299v	3	3	4	5	2	0
5092	3	3	3	4	2	0
PCS20	2	2	3	2	0	0
	Zone of inhibition (mm) against *L. sakei* DSM 20017^T at different growth temperatures					
299v	6	5	4	4	1	0-1
5092	5	6	4	4	1	0-1
PCS20	6	5	3	3	1	0-1

The expression of the lactate dehydrogenases (*lldh*) and plantaricin genes, i.e. the *pln*EF bacteriocin and *pln*G ABC transporter genes, were measured by qRT-PCR. In addition, the expression of the 16S housekeeping gene was also measured, as a reference gene for determining relative expression. Both strains expressed the *pln*EF, 16S, *lldh* and *pln*G genes at approximately similar copy numbers at both 8°C and 30°C when measured using absolute expression (Figs. 3.9, A & C). This correlated to the inhibitory activity observed on the agar plates, were zones of activity could be determined to as little as 8°C growth temperature (Table 3.2). Thus, by determining absolute gene expression, low temperature did not appear to affect gene expression levels when compared to optimum growth temperature of 30°C. Using the absolute gene expression data, the relative expression levels were calculated by comparing the expression levels of the genes to that of the 16S rRNA gene, and by comparing the expression at low temperature to that at optimal temperature of 30°C (normalised to 1 in this method, see Fig. 3.9) using the $\Delta\Delta C_t$ method described above. The relative expression data showed that the temperature seemed to be able to somewhat affect expression of the plantaricin genes in that expression at 8°C was approximately 0.75-fold less (Fig. 3.9B and D) to that at 30°C, against which was normalised. However, this is only a very small difference, when considering that a significant increase or decrease in expression should be at least two-fold change in expression.

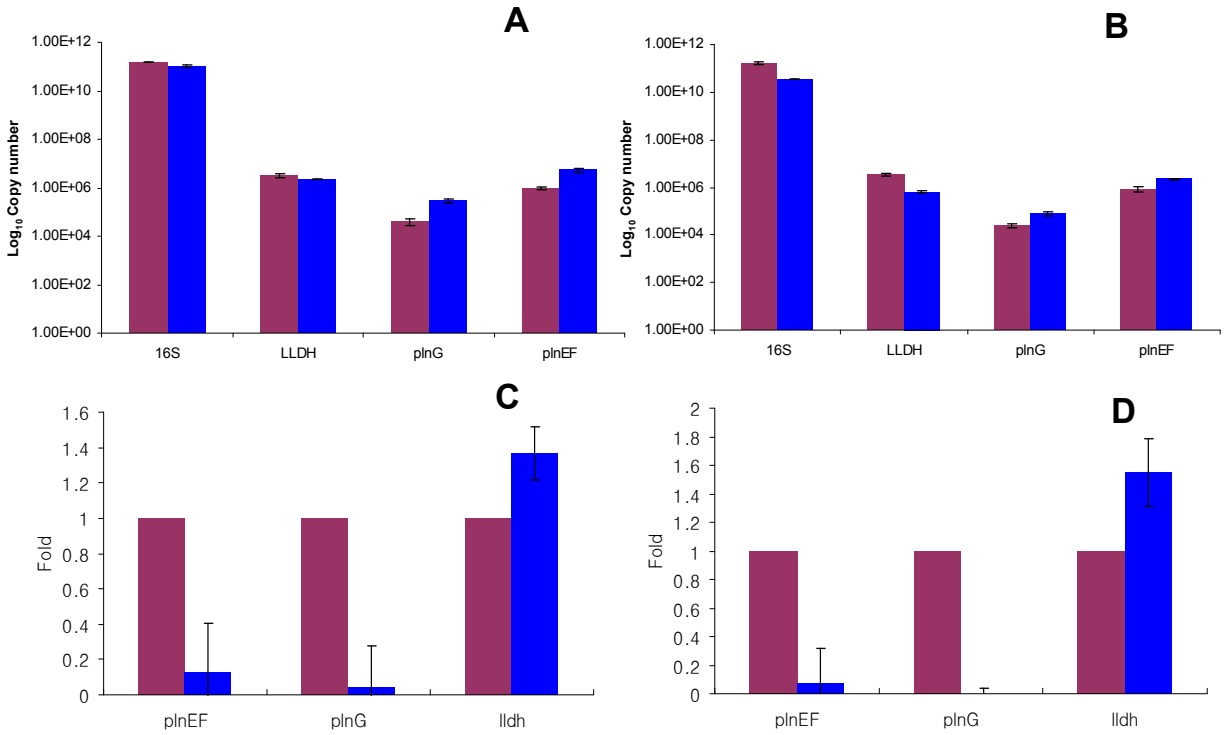

Figure 3.9 Absolute and relative expression of 16S, *lldh, plnEF* and *plnG* genes under different growth temperature conditions in *L. plantarum* strains PCS20 (A, C) and BFE 5092 (B, D) A, B: absolute quantification C, D: relative expression. 16S: 16S rDNA gene used as housekeeping gene for normalization (maroon bars) in relative expression determinations (blue bars). LLDH: L-lactate dehydroganase gene, *pln*EF: plantaricin EF gene, *pln*G: plantaricin ABC transporter gene. The reference gene expression for relative expression analysis was expression at 30°C (normalised to 1.0).

3.6.2 Expression of plantaricin-encoding genes by sessile and planktonic *Lactobacillus plantarum* strains

The expression of the lactate dehydrogenase (*lldh*), *pln*EF and *pln*G ABC transporter genes by sessile and planktonic strains BFE 5092 and PCS20 were also investigated in vitro using TSB broth (planktonic) and TSB agar containing 2% agarose (sessile). Cells were grown at 30°C, harvested at a cell density of 10^8 cells and their gene expression was quantified by qRT-PCR. Expression levels of *L. plantarum* PCS20 genes are shown in Fig. 3.10.

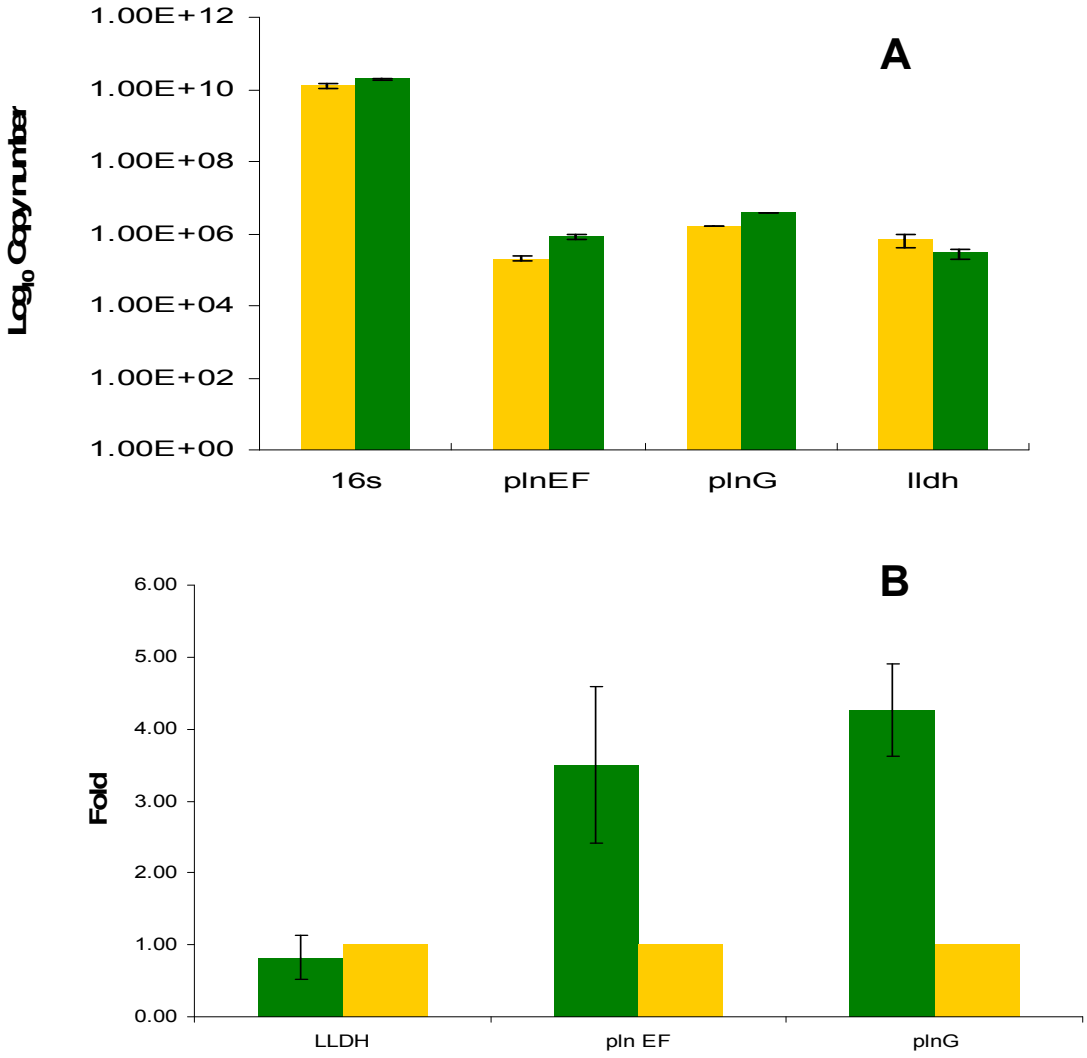

Figure 3.10 Absolute (A) and relative (B) expression of 16S, *lldh, plnEF* and *plnG* genes under different growth conditions such as planktonic (yellow bars) and sessile (green bars) in *L. plantarum* strain PCS20 16S: 16S rRNA gene used as housekeeping gene in relative expression determinations. LLDH: L-lactate dehydrogenase gene, *pln*EF: plantaricin EF gene, *pln*G: plantaricin ABC transporter gene. The reference gene expression for relative expression analysis was expression under planktonic growth (normalised to 1.0).

The expression levels of *lldh*, *pln*EF and *pln*G when quantified using absolute expression were overall quite similar during sessile and planktonic growth, but differed slightly for *pln*EF expression (approx. 0.5 log in copy number difference) (Fig. 3.10A). The relative expression levels of the *lldh*, *pln*EF and *pln*G genes are shown in Figure 3.10B. The *lldh* genes were clearly expressed to very similar levels. However, the *pln*EF and *pln*G genes appeared to be expressed at higher levels during sessile growth on solid medium, when compared to planktonic growth in liquid medium. The expression levels of the *pln*EF and *pln*G genes, however, were three-fold or four-fold higher during sessile growth on solid medium, when compared to that in broth, respectively. When taking the standard error into account, which was derived from 3 replicate experiments, it spanned in range about 1.5 gene expression folds.

Thus, it is doubtful whether the *pln*EF genes indeed were expressed higher during sessile growth on solid medium. Nevertheless, a clear elevation of gene expression was noted for the *pln*G gene expression, which is involved in bacteriocin transport (Diep et al., 1996) (Fig. 3.10B). In this case, the transporter gene was relatively three times more expressed when the strain was grown under sessile conditions.

Very similar results of absolute expression were obtained for *L. plantarum* BFE 5092 under both growth conditions (Fig. 3.11). In this experiment, the expression of all the plantaricin

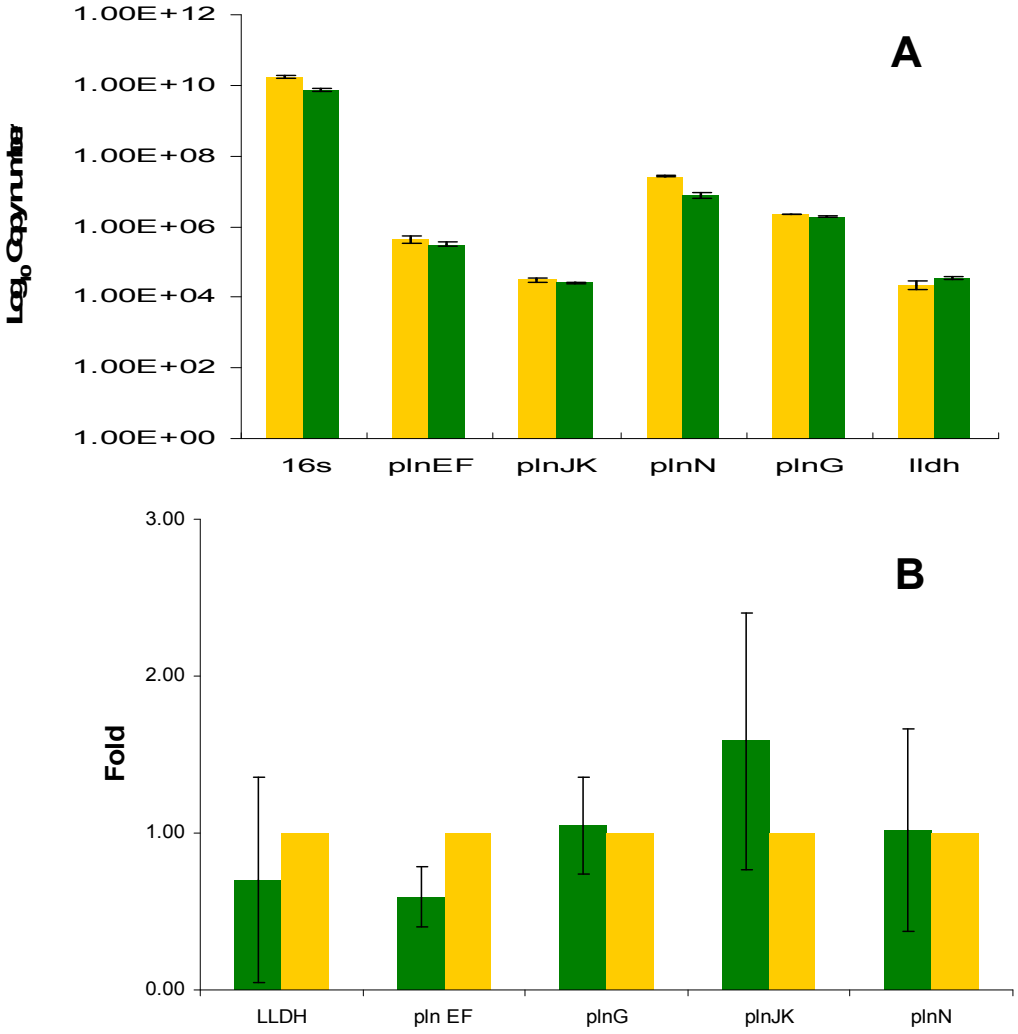

Figure 3.11 Absolute (A) and relative (B) expression of 16S, *lldh, plnEF*, *pln*JK, *pln*N and *plnG* genes under different growth conditions such as planktonic (yellow bars) and sessile (green bars) in *L. plantarum* strain BFE 5092 16S: 16S rRNA gene used as housekeeping gene in relative expression determinations. LLDH: L-lactate dehydroganase gene, *pln*EF: plantaricin EF gene, *pln*G: plantaricin ABC transporter gene, *pln*JK: plantaricin JK genes, *pln*N: plantaricin N gene. The reference gene expression for relative expression analysis was expression under planktonic growth (normalised to 1.0).

genes (i.e., *pln*EF, *pln*JK, *pln*N and *pln*G) present in this strain were evaluated, as well as the expression of the 16S rRNA and *lldh* genes. When the absolute expression levels were determined, again the expression of these genes generally appeared to be very similar under

the two growth conditions, with only slight differences in expression levels noticeable for example for the *pln*EF and *pln*N genes (Fig. 3.11A). When relative expression levels were determined and the standard error was taken into account, there was also no obvious difference in the expression levels of the plantaricin and *lldh* genes (Fig. 3.11B) under both growth conditions. Indeed the greatest difference noted in expression level was that of the *pln*JK gene, which was only 1.5-fold higher under sessile growth conditions and which is lower than the two-fold expression required signalling an obvious difference in gene expression.

3.7 Growth and gene expression of the *L. plantarum* BFE 5092 protective culture, and challenge tests with foodborne pathogens *L. monocytogenes* and *S. enterica* serovar Typhimurium on turkey meat at low temperature

The experiments with the protective culture for biopreservation of turkey meat were done using the strain *L. plantarum* BFE 5092 only, as it was shown that the *L. plantarum* PCS20 strain contained a mutated *pln*EF gene and thus was probably not capable of producing a functional two-peptide bacteriocin. In addition, this strain did not show the presence of other bacteriocin genes. The turkey meat for these experiments was provided by the designated national German partner in the PathogenCombat consortium with whom it was decided by the management that our institute should cooperate to test the protective cultures in their product. In these studies, the growth of *L. plantarum* BFE 5092 on turkey meat at low temperature was assessed and their expression of bacteriocin and adhesion genes was monitored. Also, the inhibitory effect of this protective culture against foodborne pathogens such as *L. monocytogenes* and *S*. Typhimurium was also assessed. Moreover, this inhibitory activity was compared to that of a commercial protective culture, i.e., *Leuconostoc carnosum* 4010 which is supplied commercially for bioprotection by Christian Hansen (Denmark).

3.7.1 Growth of *L. plantarum* BFE 5092 on turkey meat

First, we wanted to assess whether *L. plantarum* BFE 5092 was also capable of growing at low temperature on turkey meat. In the previous experiments it was shown that growth in MRS broth occurred down to 8°C but it needed to be confirmed that the bacteria would also survive and growth on turkey meat at this temperature. To prevent autochthonous LAB, which may be present naturally on turkey meat, to interfere with enumeration of the protective culture, the turkey meat was sterilized at 10kGy using a linear accelerator, which is present at our institute. It could be shown that this irradiation destroyed all background microbial populations (results not shown). The growth of *L. plantarum* BFE 5092 on turkey meat at 8°C is shown in Figure 3.12. The *L. plantarum* BFE 5092 culture was inoculated at approx. 10^7 CFU/g and did not grow well on the turkey meat at 8°C, as the count did eventually increase to only approx. 10^8 CFU/g after 9 days, this implied that the bacteria survived on the meat but were only

able to multiply slowly (Fig. 13.12). Interestingly, on non-sterilised meat there appeared to be background LAB populations, which increased in number from approx. 10^4 CFU/g to 10^9 CFU/g. This was a similar number as reached on turkey meat that was not irradiated but inoculated with *L. plantarum* BFE 5092 also at 10^7 CFU/g. Thus, it could be deduced that this increase in LAB numbers on inoculated but non-sterilised turkey meat was due also to autochthonous LAB, which clearly appear to be better adapted to this temperature and environment.

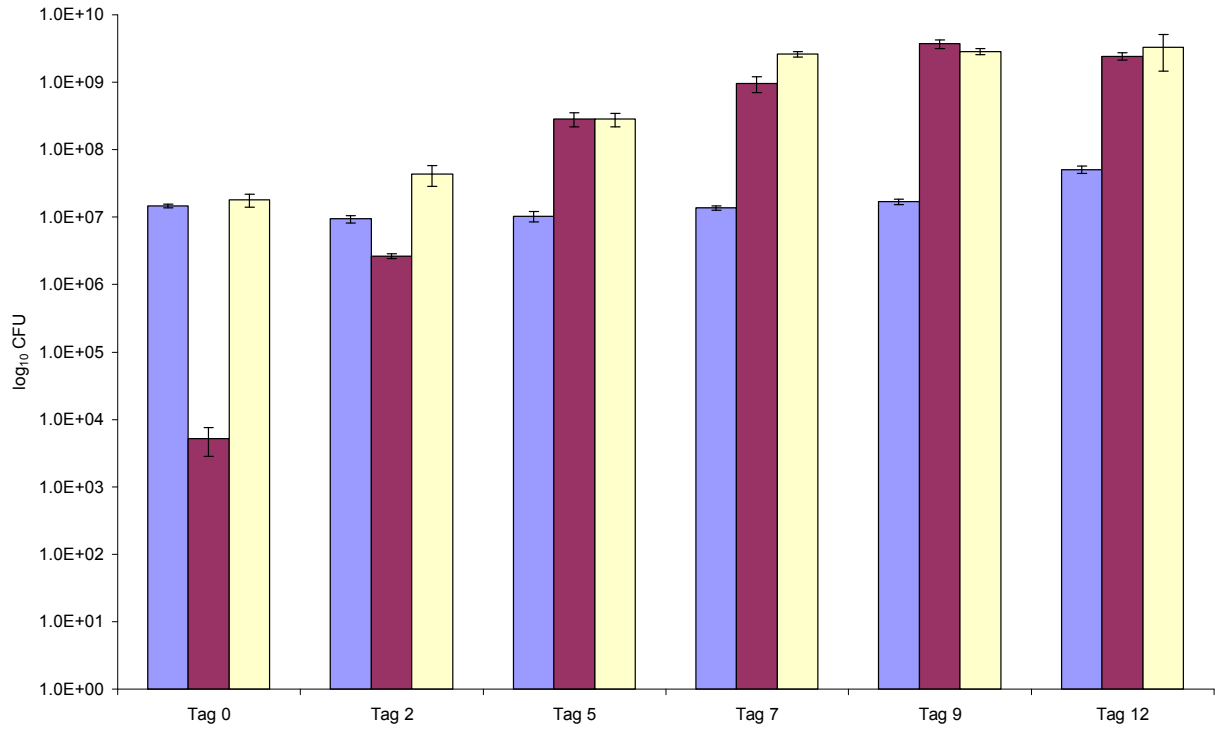

Figure 3.12 Total LAB $\log_{10}$ CFU/g number of *L. plantarum* BFE 5092 growing on irradiated (10kGy, blue bars) or non-irradiated turkey meat compared (yellow bars) compared to background lactic acid bacterial growth on turkey meat left uninoculated (maroon bars)

The development of the pH of the turkey meat inoculated with the protective culture, and that of the meat left uninoculated, is shown for the meat during storage at 8°C in Figure 3.13. The pH of the sterilised meat inoculated with *L. plantarum* BFE 5092 stayed constant at just below pH 6.0, indicating that the bacteria did not acidify the product, and thus also that the protective culture did not grow well. In contrast, the pH of the turkey meat stored at 8°C and left uninoculated increased to a level of ca. pH 8.0 after 5 days, indicating that bacteria were utilizing protein and metabolising this to compounds which increase the pH (e.g. ammonium).

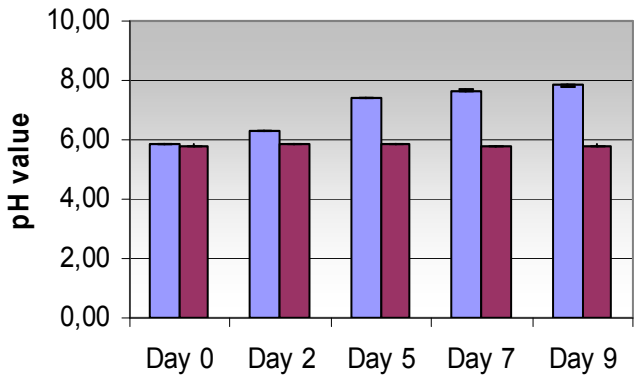

Figure 3.13 pH development of sterilized turkey meat inoculated with protective culture *L. plantarum* BFE 5092 (maroon bars) and of non-sterilised turkey meat left uninoculated (blue bars)

3.7.2 Growth of *L. plantarum* BFE 5092 and expression of bacteriocin genes when co-inoculated with *Salmonella* Typhimurium on turkey meat

The growth of the protective culture *L. plantarum* BFE 5092 on turkey meat and its potential inhibitory activity towards *Salmonella* Typhimurium was determined by spread plating and selective enumeration of LAB on MRS agar and *Salmonella* on *Salmonella/Shigella* agar. The turkey meat in this experiment was left un-inoculated and non-sterilised as a control, while sterilized turkey meat (linear accelerator at 10kGy) was inoculated with either *Salmonella* Typhimurium alone (10^5 CFU/g), *L. plantarum* BFE 5092 alone (10^7 CFU/g) and both *S.* Typhimurium and *L. plantarum* BFE 5092 at 10^5 and 10^7 CFU/g, respectively. Also, non-irradiated meat was inoculated with both the protective culture and *S.* Typhimurium at the same concentrations as above. The LAB counts determined on MRS agar are shown in Figure 3.14. *L. plantarum* BFE 5092 inoculated onto irradiated turkey meat stored at 8°C showed no growth and remained at a mean count of approx 10^7 CFU/g. In contrast, the LAB on non-sterilised meat grew to high numbers, from ca 10^6 CFU/g to 10^8 CFU/g. The non-sterilised turkey meat inoculated with the protective culture had a higher initial count of 10^7 CFU/g when compared to the non-inoculated, non-sterilised meat, and thus this was due to inoculation with this amount of bacteria. The increase in LAB count to 10^9 in this sample (non-sterilised, inoculated with protective culture), however, could not be described as a result of the growth of the protective culture, as clearly it did not grow on the sterilised meat, but rather was due to growth of the background LAB populations of the meat (Fig. 3.14).

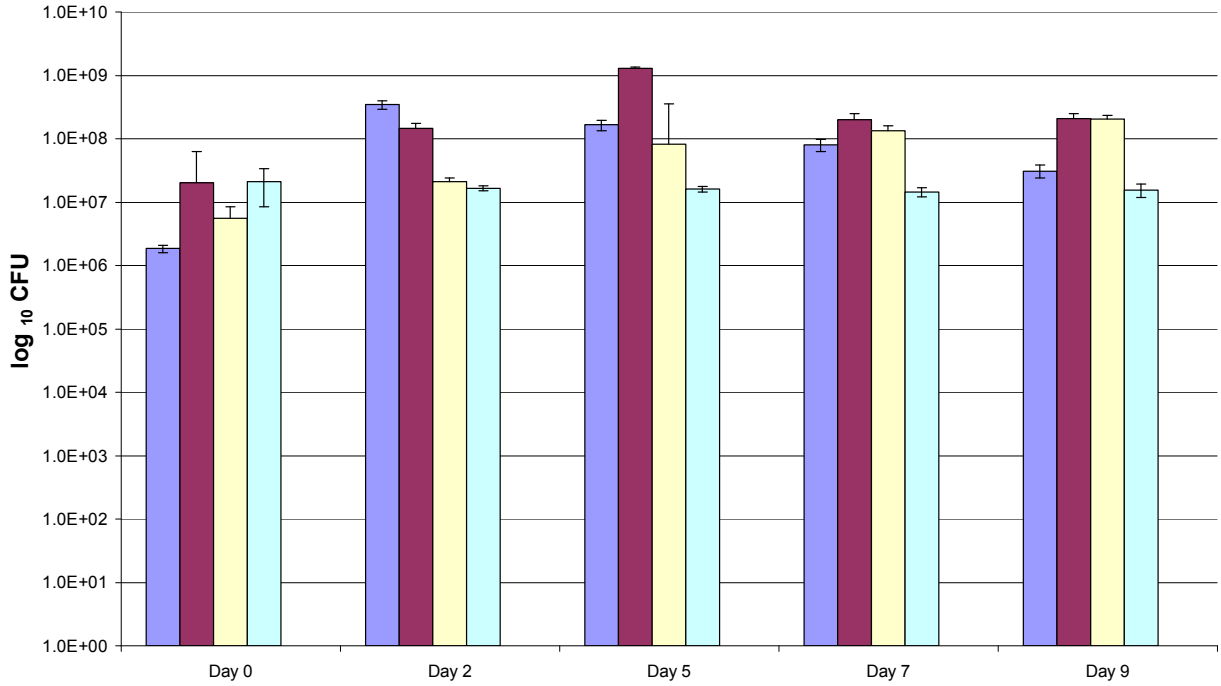

Figure 3.14 Numbers (log_{10} CFU/g) of LAB on turkey meat stored at 8°C determined on MRS agar. Turkey meat, non-sterilised with no cultures added, blue bars; non-sterilised meat with both protective culture and *Salmonella* added, maroon bars; sterlised turkey meat with both protective culture and *Salmonella* added, yellow bars; sterilised turkey meat with protective culture added, light green bars. Sterilised meat inoculated with *Salmonella* only did not show any growth as this pathogen does not grow on this medium. Counts shown are from triplicate determinations and the standard error is indicated.

Salmonella counts were assessed on *Salmonella* and *Shigella* agar, and showed that in the non-sterilised sample without added bacteria, *Salmonella* and possibly other bacteria able to grow on the *Salmonella* / *Shigella* agar were present at a level of 10^8 CFU/g already on day 0. This number increased further to 10^{10} CFU/g at day 5, after which it decreased to 10^9 CFU/g on day 7 and 9 (Fig. 3.15). It was not possible to distinguish *Salmonella* strains on the basis of colony characteristics on the *Salmonella* / *Shigella* medium alone, but it is very possible that other Gram-negative bacteria could also have grown. In both the samples sterilised and inoculated with only *Salmonella,* and sterilised and inoculated with both *Salmonella* and the protective culture, the *Salmonella* counts increased from an initial value of ca. 10^5 CFU/g to ca. 10^7 CFU/g (Fig. 3.15). The *L. plantarum* protective culture was previously tested for its ability to grow on *Salmonella / Shigella* agar and was incapable to do so (result not shown). Therefore, the count of the co-culture of protective culture and *Salmonella* was due to the presence of *Salmonella* only. The protective culture clearly had no inhibitory effect on the growth of *Salmonella* on the sterilised turkey meat when grown in co-culture, as the number of salmonellae was similar, if not somewhat higher on the sterilized turkey meat inoculated with both strains, when compared to sterilised turkey meat inoculated only with the *Salmonella* strain

(positive control) (Fig. 3.15).

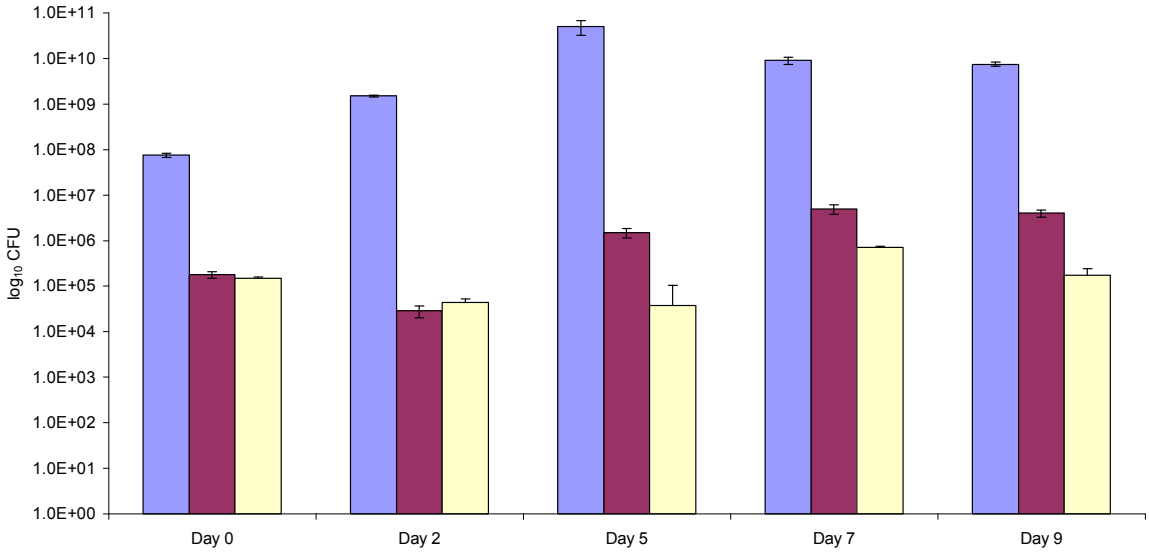

Figure 3.15 Numbers (log_{10} CFU/g) of *Salmonella* on turkey meat stored at 8°C determined on *Salmonella / Shigella* agar from turkey meat, non-sterilised with no cultures added, blue bars; sterilised meat with both protective culture *L. plantarum* BFE 5092 and *Salmonella* added, maroon bars; sterlised turkey meat inoculated with *Salmonella* only, yellow bar. Counts shown are from triplicate determinations and the standard error is indicated.

3.7.3 Growth of *L. plantarum* BFE 5092 and expression of bacteriocin genes when co-inoculated with *Listeria monocytogenes* EGDe on turkey meat

The growth of the protective culture *L. plantarum* BFE 5092 and its potential inhibitory activity against the foodborne pathogen *L. monocytogenes* EGDe on turkey meat was determined by spread plating and selective enumeration of LAB on MRS and *L. monocytogenes* on supplemented PALCAM agar. In the above experiment with *Salmonella*, a storage temperature of 8°C was used, and it was seen that the protective culture did not grow well. Therefore, in this experiment a slightly higher storage temperature of 10°C was used, which was hoped to allow better growth of the *L. plantarum* BFE 5092 strain. In addition, the expression of the plantaricin EF, JK and N genes was also determined from the protective culture cells growing on the turkey meat. Contrary to the above experiments with *Salmonella*, the turkey meat could not be sterilised in these experiments with *L. monocytogenes* as, unfortunately, the linear accelerator was damaged and became non-functional. This equipment remained non-functional and was subsequently decommissioned, making this convenient technique for sterilisation of turkey meat unavailable for further experimentation. The turkey meat in this experiment was thus left non-sterilised and un-inoculated as a control, while non-sterile turkey meat was inoculated with either *L. monocytogenes* EGDe alone (10^5 CFU/g), *L. plantarum* BFE 5092 alone (10^7 CFU/g) or with both *L. monocytogenes* EGDe and *L. plantarum* BFE 5092 (at 10^5 and 10^7 CFU/g, respectively). The LAB counts determined on MRS agar are

shown in Figure 3.16. The LAB count in un-inoculated, non-sterilised turkey increased from an initial of 10^6 CFU/g to 10^9 CFU/g within 8 days storage. Thus, the initial contamination with autochthonous LAB again was high and these bacteria were well adapted to grow at low temperatures in this environment. The LAB count of the non-sterilised turkey meat inoculated with *L. plantarum* protective culture was higher at 10^8 CFU/g at day 0, which was a result of the inoculation with the protective culture. These counts also increased to a similar level in a similar time frame when compared to the non-inoculated control, suggesting again that the growth of LAB observed was probably the result of the background, autochthonous populations. Unfortunately, since the linear accelerator was decommissioned, there was no possibility of monitoring the growth of only the protective culture at 10°C using sterilized turkey meat.

The un-inoculated turkey meat and the meat inoculated with only the protective culture, both showed that listeriae occurred naturally on the turkey meat and grew to levels of approx. 10^4 CFU/g after 8 days of storage at 10°C (Fig. 3.16). Clearly, the presence of the protective culture *L. plantarum* BFE 5092 did not have an effect on the growth of autochthonous listeriae. *L. monocytogenes* EGDe grew well from an initial level of ca. 10^5 CFU/g after inoculation to a high number of $>10^8$ CFU/g after 8 days, showing that the strain could grow well on this product and at the conditions of storage. This pathogen could also reach similar numbers ($>10^8$ CFU/g) in turkey meat when co-inoculated with the protective culture *L. plantarum* BFE 5092, indicating that this strain was not able to inhibit *L. monocytogenes* EGDe in this environment at 10°C (Fig. 3.16).

The Gram-negative bacteria also grew well from an initial count of 10^3 CFU/g at day 0 to 10^{11} CFU/g on day 8, on non-sterile turkey meat inoculated with both protective culture and *L. monocytogenes* EGDe, or with only *L. monocytogenes* EGDe. This indicated that, again, the protective culture also had no inhibitory effect against Gram-negative spoilage bacteria under the conditions of this study (Fig. 3.16).

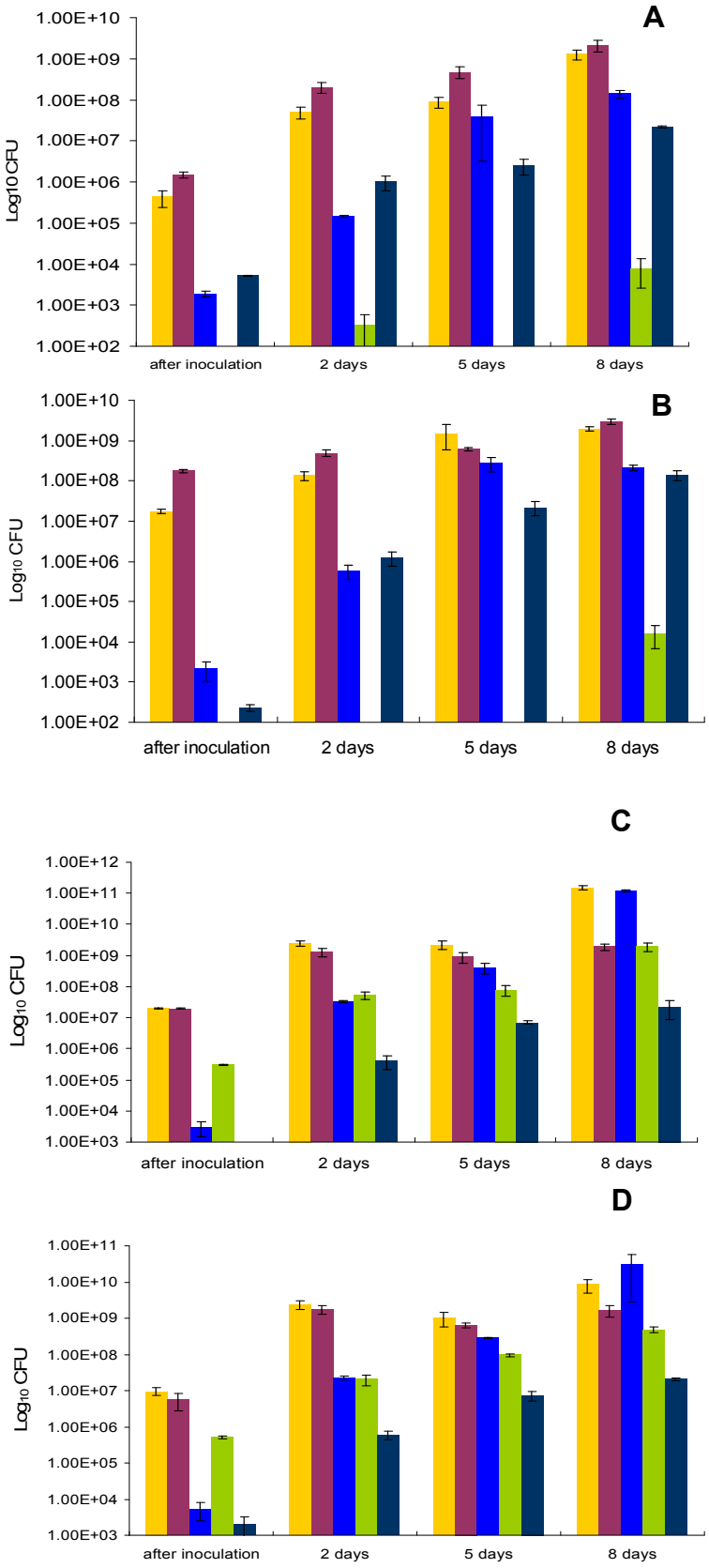

Figure 3.16 Numbers (log_{10}CFU/g) of naturally occurring bacteria on turkey meat (A), on turkey meat inoculated with protective culture BFE 5092 (B), on turkey meat inoculated with both *L. monocytogenes* EGDe and *L. plantarum* BFE 5092 (C) and on turkey meat inoculated only with *L. monocytogenes* EGDe (D). ST1 medium was used for enumeration of total of aerobic mesophilic bacteria (yellow bars), MRS for LAB (maroon bars), VRBD for enumeration of *Enterobacteriaceae* (blue bars), Palcam with *Listeria* selective supplement for listeriae (green bars) and malt glucose medium containing

antibiotic cocktail for yeasts (dark blue bars). Results are triplicate determinations and the standard error is shown.

Concurrent to determinations of cell counts, the expression of the *pln*EF, *pln*JK and *pln*N bacteriocin genes were determined during 'growth' of *L. plantarum* BFE 5092 on the turkey meat stored at 10°C in the experiment above. The *pln*EF genes expression in the co-culture of protective strain and pathogen was relatively low at days 0 and 5, and did not differ more than one-fold (Fig. 3.17). This relative expression was noticeably higher on day 2, at approximately five-fold higher expression of these genes by *L. plantarum* BFE 5092 inoculated together with the pathogen *L. monocytogenes* EGDe, than *L. plantarum* BFE 5092 being inoculated on turkey meat alone. However, the standard error was noticeably high, indicating quite some variation between the triplicate samples. The expression of the *pln*N gene appeared to be higher at days 0 and day 2 (2.96 and 1.85-fold expression) when the *L. plantarum* BFE 5092 strain was inoculated together with *L. monocytogenes* EGDe, as compared to *L. plantarum* BFE 5092 being inoculated on turkey meat alone. Again, the standard deviation was quite large and thus it could not be ruled out that the expression was quite high especially at

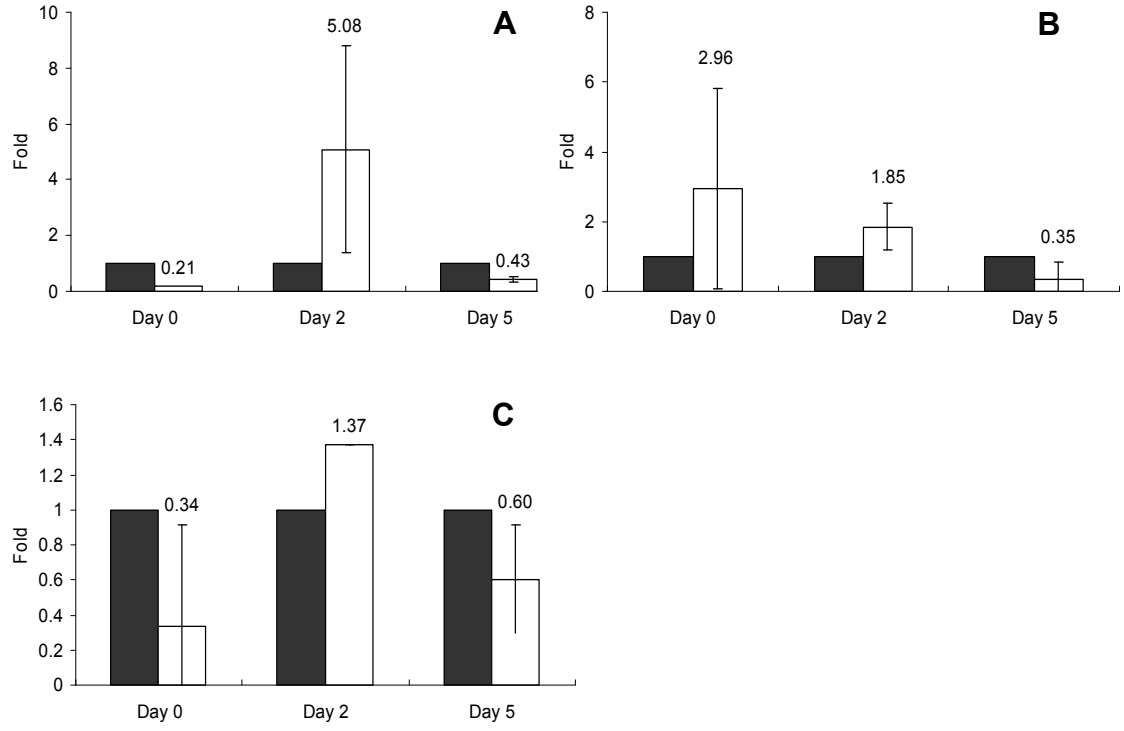

Figure 3.17 Relative expression of *plnEF* (A), *plnN* (B) and *pln*JK (C) genes during growth of *L. plantarum* BFE 5092 protective culture on non-sterilised turkey meat (black bars) and of the protective culture *L. plantarum* BFE 5092 growing together with *Listeria monocytogens* EGDe on non-sterilised turkey meat (white bars) The reference gene expression for relative expression analysis was expression of the genes on turkey meat only inoculated with *L. plantarum* BFE 5092 (normalised to 1.0).

day 0. At day 5 there was no noticeable difference in expression, as the difference in expression levels was less than one-fold. Expression of the *pln*JK genes appeared to be similar throughout the incubation period, as the difference in the expression levels of this gene between

the experiments with *L. plantarum* protective culture alone, or that of *L. plantarum* BFE 5092 together with *L. monocytogenes* EGDe on turkey meat, did not differ more than one-fold (Fig. 3.17).

3.8 Growth of the *Leuconostoc carnosum* 4010 protective culture and challenge tests with foodborne pathogen *S. enterica* serovar Typhimurium on turkey meat at low temperature

The *L. plantarum* strain BFE 5092 chosen by the consortium as protective culture for use in turkey meat biopreservation, as seen above, did not grow well at low temperatures. Also as seen above, the effect on the protective culture on inhibition of the foodborne pathogens *L. monocytogenes* EGDe and *S*. Typhimurium on turkey meat was negligible. For this reason, the commercial protective culture *Leuconostoc carnosum* 4010, marketed by Christian Hansen (Denmark), which is known to grow well at low temperature and produce two bacteriocins, leucocin A and leucocin C (Jacobsen et al., 2003), was used in biopreservation of turkey meat to determine whether the lack of success observed with the *L. plantarum* strain BFE 5092 was due to its failure to grow well at low temperature. These experiments were done when it was still possible to sterilize turkey meat with the linear accelerator, and the turkey meat was prepared as for the other experiments. The *L. carnosum* 4010 protective culture was inoculated at 10^7 CFU/g while the pathogen *S*. Typhimurium was inoculated at a level of 10^7 CFU/g. For this experiment, the gene expression of the leucocin genes was not determined, as these bacteriocins are similar to leucocin A and leucocin C but their genes have never been fully elucidated and no primers for amplifying their genes or for use in qRT-PCR are available.

Indeed, the *L. carnosum* strain 4010 showed good growth on sterilized turkey meat at 8°C growing from an initial of 10^7 CFU/g at day 0 to approx. 10^{10} CFU/g at days 5 and 8, as determined from the LAB count on MRS agar (Fig. 3.18). The LAB counts from sterilised and non-sterilised turkey meat were very similar, indicating that the majority of LAB growing on the non-sterilised turkey meats were the protective culture. The non-sterilised turkey meat left un-inoculated showed that the autochthonous LAB on the turkey meat occurred at a level of 10^4 CFU/g on day 0 and increased to only 10^8 CFU/g on day 8 (Fig. 3.18).

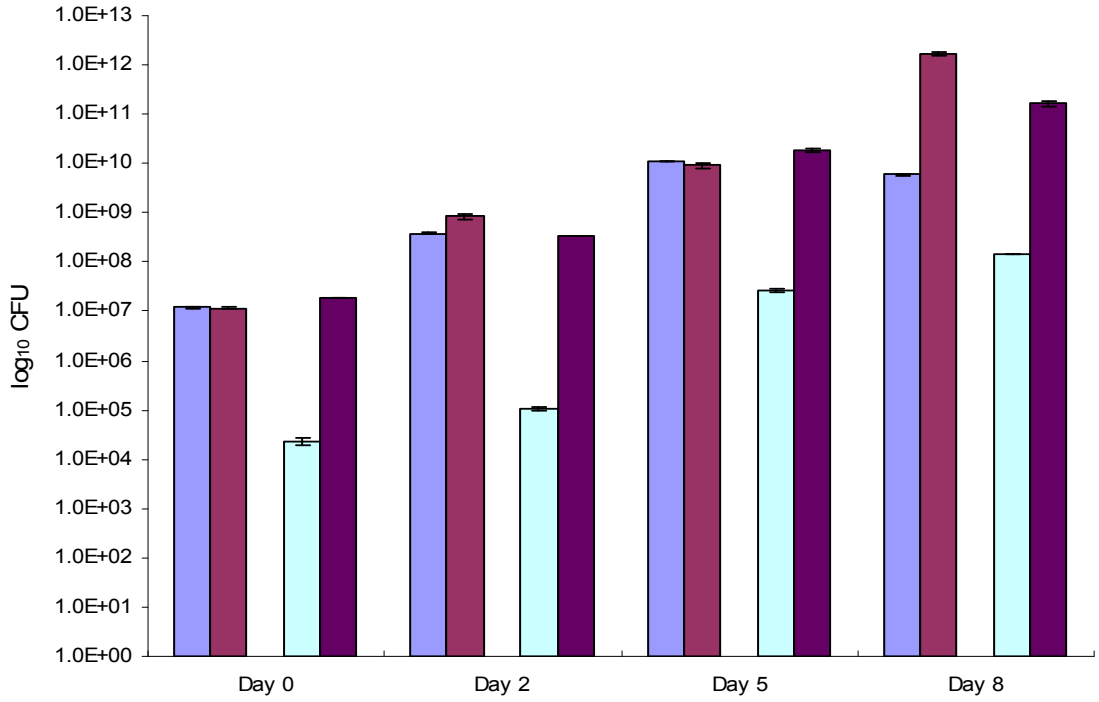

Figure 3.18 Numbers (log_{10} CFU/g) of LAB on turkey meat stored at 8°C determined on MRS agar. Sterilised turkey meat with *Leuconostoc carnosum* 4010, blue bars; sterilised meat with both protective culture and *Salmonella* added, maroon bars; non-sterlised turkey meat without inoculum, light green bars; non-sterilised turkey meat with both protective culture and *Salmonella,* violet bars. Sterilised meat inoculated with *Salmonella* only did not show any growth as this pathogen does not grow on this MRS medium. Counts shown are from triplicate determinations and the standard error is indicated.

S. Typhimurium again showed good growth on sterilized turkey meat when inoculated at a level of 10^7 CFU/g growing to approx. 10^9 CFU/g (Fig. 3.19). On both sterilized and non-sterilised turkey meat, *Salmonella* (inoculated together with *L. carnosum* 4010 on non-sterilised meat) grew to a level of 10^{10} CFU/g. This indicated that firstly, the turkey meat contained autochthonous Gram-negative bacteria which could grow on *Salmonella* / *Shigella* agar, and this resulted in a higher growth when compared to sterilised meat inoculated with only *S*. Typhimurium. Secondly, the protective culture *L. carnosum* 4010 obviously did not have a major effect on the growth of *Salmonella* or background Gram-negative bacteria, as the level of growth detected on this agar was the same as on agar inoculated with *S*. Typhimurium alone up to day 5, when the *Salmonella* count on *Salmonella* / *Shigella* agar reached approx. between 10^7 and 10^8 CFU/g (Fig. 3.19). After day 5, there was a ca 1 log_{10} CFU/g difference, indicating that the *Salmonella* count on the turkey meat inoculated with only *Salmonella* was ca. 1 log higher than the meat inoculated with both *Salmonella* and *L. carnosum* 4010 (Fig. 3.19).

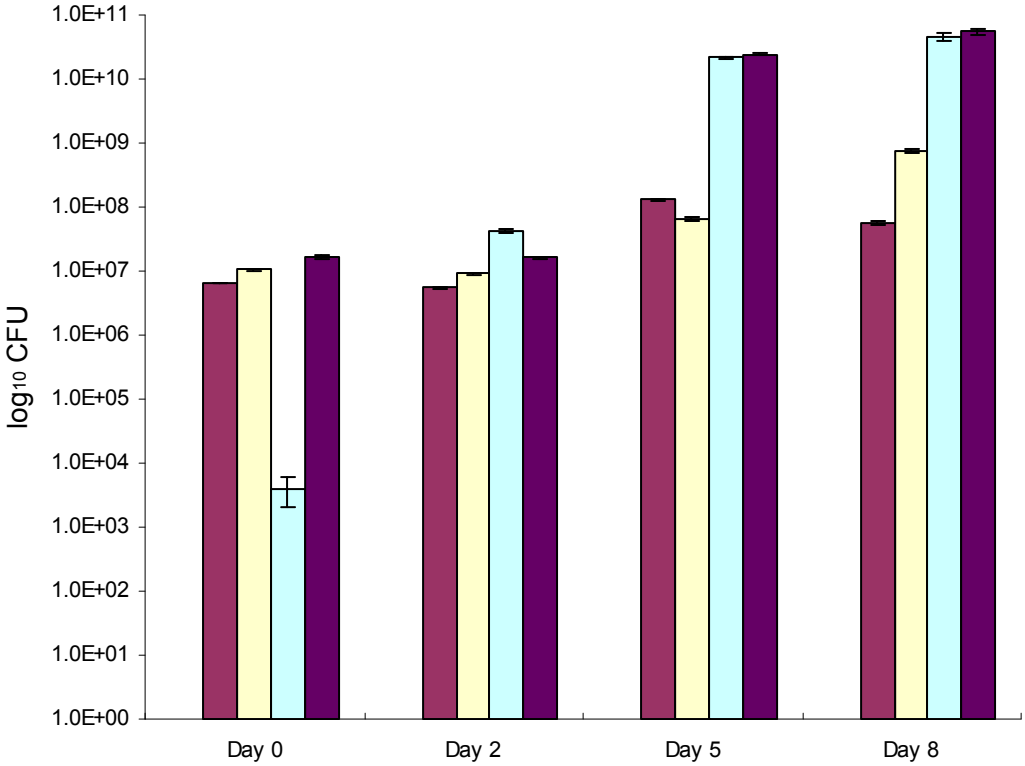

Figure 3.19: Numbers (log_{10} CFU/g) of *Salmonella* on turkey meat stored at 8°C determined on *Salmonella* / *Shigella* agar. Sterilised meat with both protective culture and *Salmonella* added, maroon bars; sterilised meat with *Salmonella* added, light yellow bars; non-sterilised turkey meat without inoculum, light green bars; non-sterilised turkey meat with both protective culture and *Salmonella,* violet bars. Counts shown are from triplicate determinations and the standard error is indicated.

The *Salmonella* count at day 8 was approx. 10^9 CFU/g. This difference in the *Salmonella* count occurring at day 8 was a clear difference, but occurred quite late in the storage period, i.e. at the last day at which the product was already terminally spoilt. Generally for all turkey experiments (at both 8 and 10°C) the turkey meat became noticeably spoiled (off odour and slimy appearance) on day 5 and later in the storage period. This noticeable spoilage was paralleled by a rise in pH (see Fig. 3.13) and an increase in microbiological counts, especially that of Gram-negative bacteria determined on either violet red bile dextrose (VRBD) or *Salmonella / Shigella* medium (Figs. 3.15, 3.16 and 3.19).

CHAPTER 4.0
DISCUSSION AND CONCLUSION

In a previous study, potentially probiotic LAB were isolated from '*kule naoto*' a Kenyan fermented milk product, and from homemade Slovenian cheese. The probiotic characteristics of *L. plantarum* strain BFE 5092 were investigated in depth (Mathara et al., 2004; Vizoso Pinto et al., 2006, 2007, 2009) *L. plantarum* is especially interesting in that it occurs in a wide variety of environmental niches, including plant-based, dairy and meat fermentations (Stiles and Holzapfel, 1997), African traditional fermented milks (Beukes et al., 2001; Mathara et al., 2004), and is a normal inhabitant of the human gastrointestinal tract (Tannock, 1999; Claesson et al., 2007). Its wide distribution and adaptability to different conditions prevailing in these environments is probably based on its large genome, which contains genes for utilization and transport (including 25 complete PEP-PTS sugar transport systems) of a wide variety of sugars and stress-related proteins, localised in a region close to the origin of replication, which has been termed a 'lifestyle island' and which probably arose from horizontal gene transfer events (Kleerebezem et al., 2003). Based on the versatility of these strains, their antimicrobial activity as determined in a high throughput screening assay by Danisco, and their known high capacity to colonise different environmental niches, the PathogenCombat consortium specifically selected two *L. plantarum* strains for development as possible functional strains in the PathogenCombat project. The fact that these strains showed antimicrobial activity was a decisive criterion for their use as protective cultures. Moreover, the well-described probiotic characteristics of *L. plantarum* BFE 5092 could make this strain interesting for use in foods which would not be heated, as the bacteria could play a dual role, i.e., as a protective culture in inhibiting food borne pathogenic bacteria in food and in delaying spoilage, and as probiotics after being ingested by the consumer. The PathogenCombat consortium was a very large consortium (more than 40 members) of many European nations, and the strains were targeted for development in different food products. This study was done together with a German national partner that was involved in production of raw turkey meat products. Thus, the strains in this study were investigated mainly for their role as a protective culture in the biopreservation of meat. As this raw meat would be heated before consumption, the probiotic aspect did not play a role in this study. However, it should be emphasized that it could play a role in biopreservation of other products such as cheese or fermented vegetables, and to investigate this was the objective of other study partners associated also with PathogenCombat.

4.1 Safety of protective *L. plantarum* BFE 5092 and PCS20 strains for biopreservation of turkey meat

Bacteria used as starter cultures for the production of foods, as protective cultures or as

probiotics could possibly contain antibiotic resistance genes (Mathur und Singh, 2005). In the past years, studies on the selection for and dissemination of antibiotic resistances have focused mainly on clinically relevant bacterial species. More recently, it was speculated that food bacteria may act as reservoirs of antibiotic resistance genes (Klein et al., 1998; Franz et al., 2005). Fermented foods, or other foods with intentionally added bacteria such as probiotics or starter cultures, therefore may be important vehicles for delivery of enormous amounts of living bacteria into the human body. These may carry transferable antibiotic resistances, which might be transferred to commensal or pathogenic bacteria. Recently, the European Food Safety Authority (EFSA) has taken responsibility to launch the European initiative towards a 'Qualified Presumption of Safety' (QPS) concept which, similar to the GRAS system in the USA, is aimed to allow strains with established history and safety status to enter the market without extensive testing requirements (EFSA, 2005). The presence of transmissible antibiotic resistance markers in the evaluation of strains thus is an important safety criterion and strains harbouring antibiotic resistances would not be regarded as safe. For this reason, the incidence of antibiotic resistances was determined for the *L. plantarum* strains BFE 5092 and PCS20 in this study, as these strains were selected by the PathogenCombat consortium for possible development as protective cultures for human food production.

Lactobacilli, leuconostocs and lactococci are generally susceptible to antibiotics which inhibit protein synthesis such as chloramphenicol, erythromycin and tetracycline, but more resistant towards the aminoglycosides such as neomycin, kanamycin, streptomycin and gentamicin (Danielsen and Wind, 2003; Gevers et al., 2003a, b; Katla et al., 2001, 2002; Temmerman et al., 2003; Delgado et al., 2005; Hummel et al., 2007; Ammor et al., 2007). The results of this study confirm this, as no resistances towards chloramphenicol, erythromycin and tetracycline could be determined, while the strains showed elevated resistance towards the aminoglycoside streptomycin. Specific strains of *Lactobacillus, Lactococcus lactis*, and *Pediococcus* have been shown to be highly resistant towards chloramphenicol, clindamycin, streptomycin, erythromycin and tetracycline (Temmerman et al., 2003; Ammor et al., 2007; Klare et al., 2007; Florez et al., 2008). This resistance has in many cases been attributed to the presence of resistance genes (Stroman et al., 2003; Florez et al., 2006; Ammor et al., 2007, 2008), but this was not the case in this study.

The two strains did, however, show a high vancomycin resistance. However, the leuconostocs, pediococci and several *Lactobacillus* spp., especially *L. rhamnosus, L. paracasei, L. plantarum* and *L. reuteri*, seem to be intrinsically resistant towards this antibiotic, whereas most *Lactococcus* and lactobacilli belonging to the *L. acidophilus* group appear to be sensitive (Danielson and Wind, 2003; Delgado et al., 2005; Ammor et al., 2007; Klare et al., 2007). The resistance of *Lactobacillus, Pediococcus* and *Leuconostoc* spp. to vancomycin was determined to be due to the presence of D-ala-D-lactate in their peptidoglycan rather than the D-ala-D-ala dipeptide (Klein et al., 2000). The observed high resistance to vancomycin for *L. plantarum*

strains BFE 5092 and PCS20 in this study thus appears to only reflect an intrinsic resistance and not a resistance based on a transferable trait. Lactobacilli are usually resistant to most nucleic acid synthesis inhibitors such as enoxacin, ciprofloxacin, perfloxacin, norfloxacin, nalidixic acid, sulphamethoxazole, trimethoprim and metrodinazole (Ammor et al., 2007) and such resistances appear to be intrinsic rather than acquired. Thus the high ciprofloxacin resistance was probably also an intrinsic resistance. Overall, therefore, the *L. plantarum* BFE 5092 and PCS20 strains in this study did not give cause to concern regarding antibiotic resistances which was considered as a prerequisite for obtaining QPS status and for further development as a protective culture.

4.2 Bacteriocin production by *L. plantarum* strains BFE 5092 and PCS20 and genetic characterisation of the bacteriocin loci

Many *L. plantarum* strains isolated from different environments are known to produce bacteriocin, often more than one (Ben Omar et al., 2008; Knoll et al., 2008; Rojo-Bezares et al., 2008; Settani et al., 2008; Diep et al., 2009; Sáenz et al., 2009). Bacteriocin production in this species may partly contribute to its success in colonizing a wide variety of niches such as fermenting wine and olives, fermented cheeses, vegetables and sausages, as well as the human saliva and gastrointestinal tract (Ehrmann, 2000; Holo et al., 2001; Maldonado et al., 2003; Ben Omar et al., 2008; Knoll et al., 2008; Rojo-Bezares et al., 2008; Trmcić et al., 2008; Müller et al., 2009; Diep et al., 2009; Sáenz et al., 2009). The genetic determinants for bacteriocins in most investigated *L. plantarum* strains, such as C11 (Diep et al., 1996), LMG 2379 (Holo et al., 2001), NC8 (Maldonado et al., 2003), J23 (Rojo-Bezares et al., 2008) and J51 (Navarro et al., 2008) are generally chromosomally encoded, and are organized in gene clusters. This was also true for the strains BFE 5092 and strain PCS20 in this study. The bacteriocin genes in both strains were chromosomally located, but PFGE analysis followed by hybridisation with a *pln*EF probe, showed that the genes occurred on different regions of the respective chromosomes. Indeed, this confirms results by Molenaar et al. (2005) who explored genome diversity of *L. plantarum* strains using microarrays and showed that regions encoding plantaricin biosynthesis varied between strains.

PCR screening for various known plantaricin genes showed that the *L. plantarum* strain BFE 5092 contained the genes for plantaricins EF, JK and N, while strain PCS20 was only positive for the *pln*EF genes. Both strains were negative for plantaricin genes such as plantaricin 423, 1.25β, NC8, W and S. The presence of genes encoding plantaricin S and plantaricin W are relatively rare among bacteriocinogenic *L. plantarum* strains, and these two bacteriocins were found in strains from olive and wine fermentation, respectively (Jimenez-Diaz et al., 1993; Holo et al., 2001).

The common feature of most plantaricin loci appears to be the presence of 1) a two-component regulatory system consisting of a membrane bound histidine protein kinase and a

cytoplasmic response regulator, 2) an inducing peptide, 3) a dedicated ABC transport system and 4) a number of adjacent bacteriocin-related peptides. The plantaricin loci of different bacteriocinogenic *L. plantarum* strains thus seem to be mosaic like structures with different modules and reorganizations presenting a high plasticity (Sáenz et al., 2009). In an analysis of the presence and arrangement of 27 plantaricin-related genes among 33 bacteriocinogenic *L. plantarum* strains from grape must, Sáenz et al. (2009) described seven genetic groups which they termed plantaritypes which together contained 18 subgroups. The most common group encountered, group 1, contained strains C11 and WCFS1, with strain C11 described as the 'type strain' of this group (Sáenz et al., 2009). This group contains genes of the *pln*ABCD regulatory system, as well as the *pln*MNO genes, but shows slight differences in the presence of some of the bacteriocin peptide genes, which allows strain classification within six subgroups. Both *L. plantarum* strains C11 and WCFS1 grouped into subgroup 1.1, as these have similar plantaricin gene combinations and a similar gene arrangement. The bacteriocin locus of *L. plantarum* strain BFE 5092 determined in this study could clearly be described as also belonging to plantaritype 1 (subgroup 1.1) as the gene combinations and arrangement were similar to those of strains C11 and WCFS1 (Figs. 1.6 & 3.6).

The plantaricin gene locus of *L. plantarum* strain PCS20, however, was unusual in that only the *pln*EF genes could be PCR amplified and that no PCR product was obtained for *pln*JK and *pln*N. Sequencing of the plantaricin locus of strain PCS20 showed that the *pln*EFI and *pln*GH genes were present. This arrangement, of absence of *pln*JK and *pl*nMNO genes, presence of *pln*EFI genes and combination with *pl*NC8HK and *pln*D genes is unusual and does not correspond to any of the plantaritypes as described by Sáenz et al. (2009). Furthermore, the transposon insertion into the *pl*NC8HK gene clearly disrupted the operon and the *pl*NC8IF gene could not be detected upstream of the transposon. Diep et al. (2009), when comparing the mosaic loci of plantaricin genes of different *L. plantarum* strains, concluded that the organization of the *pln* loci appeared to be bi-faceted, with one part (*pln*EFI and the transport operon *pln*GHSTUVW) being highly conserved, while the less conserved part includes the regulatory operon, and two or three other bacteriocin operons. Indeed, this 'minimalistic' plantaricin locus of strain PCS20 seems to confirm this, as the conserved genes *pln*EFI and *pln*GH are present, while the less conserved genes concerned with regulation appear to be disrupted and other bacteriocin operons are absent.

The nucleotide sequence analysis of the *pln*EFI operon revealed an interesting and unique gene sequence which translated resulted in a peptide which deviated significantly from the conserved PlnE amino acid sequence. Thus, a peptide results which theoretically is 2 amino acids shorter and differs by 24 amino acids while having 30 amino acids identical to that of PlnE described for strains WCFS1 and C11. This implies that there is a substantial difference (approx. 46%) to the regular PlnE peptide amino acid sequence. The RT-PCR results in this study clearly showed that the mutations which occur within the *pln*E gene sequence clearly do not affect

transcription of the *pln*EF genes. Nevertheless, it can be assumed that if the peptide is translated, a non-functional two-peptide bacteriocin would be produced. The reasons for this are two-fold. Firstly, the first deletion occurs in the double-glycine-type N-terminal extension sequence for the bacteriocin shifting the sequence out of frame. Thus, the double-glycines which serves as a recognition signal for the proteinase part of the dedicated ABC transporter (Håvarstein et al., 1995) and which are located at positions -2 and -1 relative to the cleavage site, are not encoded. In the PlnE peptides of *L. plantarum* strains C11 and WCFS1 these double glycines occur at positions 22 and 23 of the prepeptide (Fig. 3.6). Thus, it is doubtful whether the PCS20 mutated PlnE would be correctly processed and transported out of the cell by the ABC transporter. Secondly, recent three-dimensional structure studies on the PlnE two-peptide bacteriocin showed that PlnE has two GxxxG motifs, one at residues 5 to 9 and one at residues 30 to 40 of the mature peptide, which are thought to interact with the PlnF peptide through helix-helix interactions (Oppegård et al., 2007; Fimland et al., 2008). As a result of the first deletion in the *pln*E gene, the first of these two motifs is missing and the amino acid sequence in this region is significantly altered.

The insertion of the transposon into the histidine protein kinase gene at the site encoding amino acid 172 in the corresponding *pl*NC8HK gene resulted in a gene disruption, and therefore the production of a functional histidine protein kinase is also unlikely. Transposon insertion into a plantaricin locus was previously described for *L. plantarum* strain V90 (Diep et al., 2009), in which a transposon, which encodes a transposase of the so-called MULE superfamily, inserted just upstream of the *pln*Q gene (Diep et al., 2009) into a direct repeat target sequence. This target sequence was also found downstream of the transposon in the *pl*NC8HK gene in PCS20. Speculatively, the transposon insertion in the *pl*NC8HK-like gene in *L. plantarum* PCS20 possibly led to a disruption in bacteriocin regulation and activity, this selecting against the bacteriocin locus. The mutations in the *pln*E gene furthermore suggest that this bacteriocin locus in this strain is getting selected against and, therefore, appears to be becoming redundant.

When the strains were selected by the PathogenCombat consortium, nothing was known about which bacteriocins, if any, these strains produced. In this study, the detailed genetic analysis was successful in determining which plantaricin genes were present. Furthermore, the detailed analyses of the plantaricin gene locus of *L. plantarum* PCS20 also clearly showed mutations, which pointed towards a highly possible defect in bacteriocin production. For this reason, it was decided not to continue with the study of *L. plantarum* PCS 20 as a protective culture in the biopreservation of turkey meat, and *L. plantarum* BFE 5092 was the culture of choice for these studies, as this strain showed an intact bacteriocin locus. Moreover, it appeared to encode at least three different plantaricins, i.e., the two component plantaricins EF and JK, as well as the linear plantaricin peptide plantaricin N.

4.3 *L. plantarum* BFE 5092 as a protective culture in the biopreservation of raw turkey meat

Lactobacilli are extremely fastidious organisms, adapted to complex organic substrates such as carbohydrate for energy source, amino acid, and vitamins (Elli et al., 2000). The lactobacilli grow best in slightly acidic environments with an initial pH of 6.4-5.4 and the growth ceases when pH 3.6-4.0 is reached. Also, most lactobacilli grow best at mesophilic temperatures with an upper limit of around 40°C and some can grow below 15°C or even below 5°C. Obviously, the growth temperature has a great influence on the production kinetics of secondary metabolites. Maximum bacteriocin production is well known to occur at the optimum growth temperature of the bacterial strain (Leroy and De Vuyst, 1999). Clearly, different LAB grow in quite diverse environments and are adapted to very different environmental conditions such as temperature and pH. *Leuconostoc*, *Weissella* and *Carnobacterium* species, together with certain *Lactobacillus* spp. such as *L. sakei* and *L. curvatus*, for example, are adapted to grow in meats which are stored at low temperature and thus have a low growth optimum temperature between 20 and 30°C (Holzapfel et al., 2005; Hammes and Hertel, 2009). Therefore, bacteriocin production by these strains also usually occurs at a maximum at lower environmental temperatures. For example, bacteriocin production by *L. mesenteroides* E131, *L. mesenteroides* L124 and *L. curvatus* L442 was at an optimum at 25°C (Mataragas et al., 2004; Drosinos et al., 2005) *Lactobacillus plantarum*, on the other hand, grows at an optimum temperature of 30°C. Similarly, bacteriocin production by *Carnobacterium piscicolin* UAL26 isolated from meat was only produced at temperatures between 1 and 16°C, with a maximum at 10-16°C (Gursky et al., 2006). The protective culture *Leuconostoc carnosum* strain 4010 was shown to be able to produce bacteriocin even at the low temperature of 5°C (Budde et al., 2003).

Due to their higher growth optimum temperature, it was not clear at which lower limit bacteriocin production would still occur in the *L. plantarum* strains in this study. For this reason, bacteriocin production was studied at both the physiological as well as the genetic level at different low temperatures. It could thus be shown that the bacteriocin production, as determined by the deferred inhibition assay, occurred down to the lower growth limit of *L. plantarum*, i.e. 8°C. However, the inhibition zones detected were decreasing with lower temperature, indicating a decrease in antimicrobial activity. As *L. plantarum* also produces organic acids which can also play a role in the production of an inhibition zone, quantitative PCR was used to measure expression of the bacteriocin genes as a means to determine whether bacteriocin production would still occur at low temperatures. This was the case, as using qRT-PCR it could be established that the *pln*EF and the *pln*G genes were being expressed at a temperature as low as 8°C. Nevertheless, the growth of the microorganisms at the lower growth limit of 8°C was also noted to be diminished and the gene expression was slightly lower, although not more than one-fold lower (Fig. 3.9). When storing fresh meats, the temperature should be as low as possible. The results, however, suggested that below 8°C,

growth of *L. plantarum* BFE 5092 would probably not occur, and therefore there would also be no bacteriocin production below 8°C. Even at 8°C it was doubtful that bacteriocin would be produced in sufficient amounts or that the protective culture would establish itself as a predominant strain. Thus, we also used a slightly higher temperature of 10°C, which may occur in household refrigerators, although this would already indicate a temperature abuse. Taken together, these facts already indicated that the *L. plantarum* BFE 5092 strain would not be well suited as a protective culture for raw poultry meat. Nevertheless, the PathogenCombat management insisted on testing the use of this strain for biopreservation of turkey meat, as well as other food preservation purposes, despite misgivings to its effectiveness from this study.

A further complication may be that plantaricin production is a cell-density, regulated trait. The gene locus of *L. plantarum* BFE 5092 clearly showed all the genes associated with bacteriocin regulation (Fig. 3.7). As production of the bacteriocin thus is cell density dependant, these bacteria were inoculated in this study at high cell concentration (10^7 CFU/g) so that bacteriocin production would occur. Bacteriocin production may, however, also be dependant on whether bacteria are growing in liquid medium (planktonic growth) or on solid medium (sessile growth). Maldonado et al. (2003, 2004) showed that *L. plantarum* NC8 is unable to produce bacteriocin when inoculated as a pure culture in liquid medium regardless of the inoculum size and growth conditions. Thus, we tested in our study wherther bacteriocin production would occur under both planktonic and liquid growth conditions. This also would yield data on whether the strain would produce bacteriocin when growing on a turkey meat surface.

Clearly, our quantitative expression analysis results suggested that under both liquid and solid medium growth conditions, the *L. plantarum* bacteriocin genes were being expressed about equally well (Figs. 3.10 & 3.11). Interestingly, for *L. plantarum* strain PCS20 the *pln*G gene, which encodes the bacteriocin dedicated ABC transporter, appeared to be higher expressed on solid medium when compared to liquid medium. The *L. plantarum* PCS20 strain also contains bacteriocin regulatory genes (although incomplete) similar to the *L. plantarum* NC8 strain used in the studies of Maldonado et al. (2003, 2004). Speculatively, the inability of the *L. plantarum* strain NC8 to produce bacteriocin in liquid thus may be a result of the interaction of the response regulator with the promoter of the transport operon. This was never investigated by Maldonado et al. (2003, 2004) and would require further investigation. Unfortunately strain NC8 was not available in our study to investigate this further. The results of this study thus suggested that bacteriocin production by *L. plantarum* BFE 5092 at low temperatures of 8°C (or 10°C) would be possible, and that bacteriocin would be produced both under liquid as well as sessile growth conditions. Furthermore, as *L. plantarum* PCS20 contained a mutated plantaricin E gene and no further genes involved in bacteriocin production could be detected, it was decided to test the biopreservative activity of the *L. plantarum* strain on turkey meat only with *L. plantarum* BFE 5092.

The biopreservative potential of *L. plantarum* BFE 5092 was tested with turkey meat and using *Salmonella* Typhimurium or *L. monocytogenes* as pathogens which are typical for this type of product. The pathogens were inoculated at dosages which corresponded to their infective dose. The inoculation level chosen for the pathogens was considered higher than natural contamination, but this was done deliberately in order to be able to determine an inhibitory effect resulting from the use of the protective culture. Our results clearly showed that the 'protective' culture had no effect, i.e. it was unable to inhibit either *Salmonella* Typhimurium or *L. monocytogenes* on aerobical stored, raw turkey meat under the conditions of this study. This was expected for *Salmonella* as bacteriocins generally are not active towards Gram-negative bacteria. This is because Gram-negative bacteria have a second cell membrane which contains a lipopolysaccharide layer in the outer leaflet of the outer membrane, which bacteriocins cannot penetrate.

Bacteriocin producing LAB have been used with varying success in biopreservation of raw meat products. Skyttä et al (1991) noted a strong inhibitory effect of bacteriocin-producing *Pediococcus* strains against *Yersinia enterocolitica* and *L. monocytogenes* in minced beef. Winkowski et al. (1993) and Leisner et al. (1996) also could show good inhibition of *L. monocytogenes* or spoilage *L. sakei* strains in raw beef, respectively. When a bacteriocin-producing *L. sakei* strain was used as protective culture on chicken breast or mincer raw meat, Hugas et al. (1998) determined a 2.5 to 3 $\log_{10}$ reduction in numbers of co-inoculated *Listeria innocua*. On the other hand, Dortu et al. (2008) showed that bacteriocin-producing *L. sakei* and *Lactobacillus curvatus* strains could inhibit the growth of *L. monocytogenes* in raw beef, but not on raw chicken breast, even though the experiments were done using the same levels of inoculation of protective cultures and pathogen. These results were similar, therefore, to our study, even though the bacteriocin-producing *L. sakei* and *L. curvatus* used by Dortu et al. (2008) are probably better adapted to growth and bacteriocin production at low temperatures, as they are typically associated with meat products.

Dortu et al. (2008) also noted that under the conditions of their study, the bacteriocins produced did not seem to have noticeable activity, and they hypothesised that this could be due to either the absence of production of bacteriocin in the poultry meat, to its inactivation through binding to food ingredient, or possibly due to the activity of endogenous protease. Such explanations have been previously put forward for failure to see bacteriocin activity in food products (Aasen et al., 2003; Katla et al., 2002; Galvez et al., 2007; Katikou et al., 2005; Vermeiren et al. 2006a, b). Apart from the fact that the 'protective' *L. plantarum* BFE 5092 culture did not grow well at 8 or 10°C in our study, and hence probably was not a good competitor in this environment, the noted absence of any inhibitory activity could speculatively also have been due to two other factors. Firstly, the pH of the turkey breast meat was found to increase to levels above neutral after 2 days. This was probably a result of the fact that Gram-

negative bacteria were clearly highly competitive in this environment, grew to high levels and probably metabolised amino acids, liberating ammonium and thus increasing the pH. Psychrotrophic *Pseudomonas* spp. (especially the non-fluorescent group II *Pseudomonas* i.e., *P. fragi* and *P. lundensis*) are the dominant and metabolically most significant spoilage organisms of raw poultry meat (McMeekin, 1975; Balamatsia et al., 2006; Arnaut-Rollier, 1999; Charles et al., 2006). These dominant *Pseudomonas* spp. demonstrate a primary glycolytic phase of growth, where the metabolism of glucose occurs. This is followed by a secondary metabolic phase, where amino acids and lactate are degraded and ammonium is liberated, leading to a rise in pH. After these low molecular weight compounds become metabolised, these bacteria can synthesize and secrete extracellular proteinases, which degrade the muscle protein again to amino acids and ammonium (Greer, 1989).

Thus, even though bacteriocin may have been produced by the 'protective' culture *L. plantarum* BFE 5092 on turkey meat, it might not have resulted in antimicrobial activity as bacteriocins, are active in the acidic range at pH 5.0 or below, but not in the neutral or alkaline range (Messens and de Vuyst, 2002). Furthermore, the bacteriocins may have become degraded by extracellular proteinases of the naturally occurring, Gram-negative spoilage association. Lastly, the Gram-negative spoilage bacteria were also better adapted to the substrate, and showed a much quicker growth than the 'protective' culture, so that even a possible acid inhibition was not possible. Thus it could be concluded, that although the *L. plantarum* BFE 5092 strain possessed at least 3 bacteriocin systems, which were active and expressed at low temperature and during sessile growth on turkey meat, this strain was unsuitable for biopreservation of this meat product, most probably as a consequence of its low growth capability at low temperatures and its failure to compete in this environment. Possible inactivation of its bacteriocins by proteases from Gram-negative bacteria, or inactivity of bacteriocins at high pH could also have contributed to this failure to inhibit foodborne pathogens.

To determine whether the pH or possible inactivation by proteases could explain the inability of the protective *L. plantarum* BFE 5092 culture to inhibit pathogens, a protective control strain, which is commercially used for biopreservation of meats, was also used in our experiments. *Leuconostoc carnosum* strain 4010 (Christian Hansen) is known to grow well and to produce bacteriocins at low temperatures (Budde et al., 2003). Indeed, this strain also showed good growth at 8°C in our study, but was also not able to inhibit the growth of the background bacterial populations.

For future development, the *L. plantarum* BFE 5092 strain might still make an excellent multifunctional strain in the biopreservation of other food commodities such as fermented milk products (in the manufacture of yoghurt or cheese, for example). The strain could also possibly be considered as multifunctional, as it could serve not only as a protective culture in these food commodities, i.e., for the inhibition of foodborne pathogens such as *Listeria*, but when ingested it could also have a potential probiotic activity. Thus, although this strain was unsuitable for the

type of product used in this study, it may well be successful for biopreservation of other food commodities, which are produced and stored under conditions that may be better suited for growth. This possibility, as well as its previously determined probiotic potential, could not be further investigated in this study under the framework of the PathogenCombat project, but would make interesting future research.

5.0 References

Abee, T. 1995. Pore-forming bacteriocins of gram-positive bacteria and self-protection mechanisms of producer organisms. FEMS Micribiol. Lett. 1:1-10.

Adams, M.R. 1999. Safety of industrial lactic acid bacteria. J. Biotechnol. 68:171-178.

Adlerberth, I., Ahrne, S., Johansson, M.L., Molin, G., Hanson, L.A. and Wold, A.E. 1996. A mannose-specific adherence mechanism in *Lactobacillus plantarum* conferring binding to the human colonic cell line HT-29. Appl. Envrion. Microbiol. 62:2244-2251.

Aasen, I.M., Markussen, S., Møretrø, T., Katla, T., Axelsson, L. and Naterstad, K. 2003. Interactions of the bacteriocins sakacin P and nisin with food constituents. Int. J. Food Microbiol. 87:35-43.

Ammor, M.S., Florez, A.B. and Mayo, B. 2007. Antibiotic resistance in non-enterococcal lactic acid bacteria and bifidobacteria. Food Microbiol. 24:559-570.

Ammor, M.S., Guiemonde, M., Danielsen, M., Zygorec, M., van Hoek, A.H.A.M., de los Reyes-Gavilan, C.G., Mayo, B. and Margolles, A. 2008. Two different tetracycline resistance mechanisms, plasmid-carried *tet* (L) coexist in *Lactobacillus sakei* Rits. Appl. Environ. Microbiol. 74:1394-1401.

Anderssen, E.L., Diep, D.B., Nes, I.F., Eijsink, V.G. and Nissen-Meyer, J. 1998. Antagonistic activity of *Lactobacillus plantarum* C11: two new two-peptide bacteriocins, plantaricins EF and JK, and the induction factor plantaricin A. Appl. Environ. Microbiol. 64:2269-2272.

Arnaut-Rollier, I., De Zutter, L. and Van Hoof, J. 1999. Indentities of the *Pseudomonas* spp. In flora from chilled chicken. Int. J. Food Microbiol. 48 :87-96.

Aucher, W., Lacombe, C., Héquet, A., Frère, J. and Berjeaund, J.-M. 2005. Influence of amino acid substitutions in the leader peptide on maturation and secretion of mesentericin Y105 by *Leuconostoc mesenteroides*. J. Bacteriol. 187:2218-2223.

Axelsson, L. 2004. Lactic Acid Bacteria: Classification and Physiology. In: Lactic Acid Bacteria, Salminen, S., von Wright, A., Ouwehand, A. (eds.), Marcel Dekker inc. Pp1-66.

Aymerich, M.T., Garriga, M., Costa, S., Monfort, J.M. and Hugas, M. 2002. Prevention of ropiness in cooked pork by bacterocinogenic cultures. Int. Dairy J. 12:239-246.

Balamatsia, C.C., Paleologos, E.K., Kontominas, M.G. and Savviaidis, I.N. 2006. Correlation between microbial flora, sensory changes and biogenic amines formation in fresh chicken meat stored aerobically or under modified atmosphere packaging at 4°C: possible role of biogenic amines as spoilage indicators. Antonie van Leeuwenhoek. 89:9-17.

Ben Omar, N., Abriouel, H., Keleke, S., Sánchez Valenzuela, A., Martínez-Cañamero, M., Lucas López, R., Ortega, E. and Gálvez, A. 2008. Bacteriocin-producing *Lactobacillus* strains

isolated from poto poto, a Congolese fermented maize product, and genetic fingerprinting of their plantaricin operon. Int. Food Microbiol. 127:18-25.

Berger, B., Pridmore, R.D., Barretto, C., Delmas.Julien, F., Schreiber, K., Arigoni, F. and Brüssow, H. 2007. Similarity and differences in the *Lactobacillus acidophilus* group identified by polyphasic analysis and comparative genomics. J. Bacteriol. 189:1311-1321.

Beukes, E.M., Bester, B.H. and Mostert, J.F. 2001. The microbiology of South African traditional fermented milks. Int. J. Food Microbiol. 63:189-197.

Birnboim, H.C. and Doly, J. 1979. A rapid alkalin extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. 7:1513-1523.

Björkroth, J. and Korkeala, H. 1996. Evaluation of *Lactobacillus sakei* contamination in vacuum-packaged sliced cooked meat products by ribotyping. J. Food Prot. 59:398-401.

Buncic, S., Avery, S.M. and Moorhead, S.M. 1997. Insufficient antilisterial capacity of low inoculum *Lactobacillus* cultures on long-term stored meats at 4°C. Int. J. Food Microbiol. 34:157-170.

Budde, B.B., Hornbaek, T., Jacobsen, T., Barkholt, V. and Koch, A.G. 2003. *Leuconostoc carnosum* 4010 has the potential for use as a protective culture for vacuum-packed meats: culture isolation, bacteriocin identification and meat application experiments. Int. J. Food Microbiol. 83:171-184.

Cambell-Platt, G. 1987. Fermented foods of the world. Butterworths, London, UK.

Canchaya, C., Claesson, M.J., Fitzgerald, G.F., van Sinderen, D. and O'Toole, P.W. 2006. Diversity of the genus *Lactobacillus* revealed by comparative genomics of five species. Microbiology 152:3185-3196.

Castellano, P. and Vignolo, G. 2004. Protective effect of *Lactobacillus casei* CRL705 compared with the bacteriocins it produces during storage of refrigerated fresh meat under vacuum. Proceedings of the 19th International ICFMH symposium FOOD MICRO 2004, Portoroz (slovenia), 12-16 September, p.287.

Charles, N., Williams, S.K. and Rodrick, G.E. 2006. Effects of packaging systems on the natural microflora and acceptatility of chicken breast meat. Poult. Sci. 85:1798-1801.

Cintas, L.M., Casaus, P., Håvarstein, L.S., Hernάndez, P.E. and Nes, I.F. 1997. Biochemical and genetical characterization of enterocin P, a novel *sec*-dependent bacteriocin from *Enterococcus faecium* P13 with a broad antimicrobial spectrum. Appl. Environ. Microbiol. 63:4321-4330.

Claesson, M.J., van Sinderen, D. and O'Toole P.W. 2007, The genus *Lactobacillus* – a genomic basis for understanding its diversity. FEMS Microbiol. Lett. 269:22-28.

Claesson, M.J., van Sinderen, D. and O'Toole, P.W. 2008. *Lactobacillus* phylogenomics – towards a reclassification of the genus. Int. J. Syst. Evol. Microbiol. 58:1945-1954.

Cleveland, J., Montville, T.J., Nes, I.F. and Chikindas, M.K. 2001. Bacteriocins: Safe natural antimicribials for food preservation. Int. J. Food Microbiol. 71:1-20.

Cogan, T.M. 1995. The leuconostocs: milk products. In: Bacterial Starter Cultures for Food, Gilliland, S.E. (eds.), CRC Press: Boca Raton, pp. 25-40.

Danielsen, M. and Wind, A. 2003. Susceptibility of *Lactobacillus* spp. to antimicrobial agents. Int. J. Food Microbiol. 82:1-11.

Dayem, M.A., Fleury, Y., Devilliers, G., Chaboisseau, E., Girard, R., Nicolas, P. and Delfour, A. 1996. The putative immunity protein of the Gram-positive bacterium *Leuconostoc mesenteroides* is preferentially located in the cytoplasm compartment. FEMS Microbiol. Lett. 138:251-259.

Delgado, S., Florez, A.B. and Mayo, B. 2005. Antibiotic susceptibility of *Lactobacillus* and *Bifidobacterium* species from the human gastrointestinal tract. Curr. Microbiol. 50:202-207.

Dellaglio, F. and Felis, G.E. 2005. Taxonomy of lactobacilli and bifidobacteria. In: Probiotics and prebiotics: Scientific aspects (ed. G.W. Tannock).Caister Academic Press, U.K. pp.25-49.

Diep, D.B., Håvarstein, L.S. and Nes, I.F. 1996. Characterization of the locus responsible for the bacteriocin in *Lactobacillus plantarum* C11. J. Bacteriol. 178:4472-4483.

Diep, D.B., Straume, D., Kjos, M., Torres, C. and Nes, I.F., 2009. An overview of the mosaic bacteriocin *pln* loci from *Lactobacillus plantarum*. Peptides 30:1562-1574.

Dortu, C., Huch, M., Holzapfel, W.H., Franz, C.M. and Thonart, P. 2008. Anti-listerial activity of bacteriocin-producing *Lactobacillus curvatus* CWBI-B28 and *Lactobacillus sakei* CWBI-B1365 on raw beef and poultry meat. 2008. Lett. Appl. Microbiol. 47:581-586.

Drider, D., Fimland, G., Héchard, Y., McMullen, L. and Prévost, H. 2006. The continuing story of class IIa bacteriocins. Microb. Mol. Biol. Rev. 70:564-582.

Drosinos, E.H., Mataraga, M., Nasis, P., Galiotou, M. and Metaxopoulos, J. 2005. Growth and bacteriocin production kinetics of *Leuconostoc mesenteroides* E131. J. Appl. Microbiol. 99:1314-1323.

EFSA, 2005. Updating of the criteria used in the assessment of bacteria for resistance to antibiotics of human of veterinary importance, Question N° EFSA-Q-2004-079 adopted on 25 May 2005. The EFSA Journal 223:1-12.

Elli, M., Zink, R., Rytz, A., Reniero, R. and Morelli, L. 2000. Iron requirement of *Lactobacillus* ssp. in completely chemically defined growth media. J. Appl. Microbiol. 88:695-703.

Ennahar, S., Sashihara, T., Sonomoto, K. and Ishizaki, A. 2000. Class IIa bacteriocins: biosynthesis, structure and activity. FEMS Microbiol.Rev. 24:85-106.

FEEDAP. 2005. Opinion of the scientific panel on additives and products or substances used in animal feed on the updating of criteria used in the assessment of bacteria for resistance to antibiotics of human or veterinary importance. *EFSA J* 223, 1-12.

Felis, G.E. and Dellaglio, F. 2007. Taxonomy of *lactobacilli* and *bifidobacteria*. Curr. Iss. Intest. Microbiol. 8:44-61.

Fimland, G., Johnsen, L., Axelsson, L., Brurberg, M.B., Nes, I.F., Eijsink, V.G.H. and Nissen-Meyer, J. 2000. A C-terminal disulfide bridge in pediocin-like bacteriocins renders bacteriocin activity less temperature dependent and is a major determinant of the antimicrobial spectrum. J. Bacteriol. 182:2643-2648.

Fimland, G., Eijsink, V.G.H. and Nissen-Meyer, J. 2002. Mutational analysis of the role of tryptophan residues in an antimicrobial peptide. Biochemistry 41:9508-9515.

Fimland, G., Johnsen, L., Dalhus, B. and Nissen-Meyer, J. 2005. Pediocin-like antimicrobial peptides (class IIa bacteriocins) and their immunity proteins: biosynthesis, structure and mode of action. J. Pept. Sci. 11:688-696.

Fimland N., Rogne, P., Fimland, G., Nissen-Meyer, J. and Kristiansen, P.E. 2008. Three-dimensional structure of the two peptides that constitute the two-peptide bacteriocin plantaricin EF. Biochim. Biophys. Acta 1784:1711-1719.

Florez, A.B., Ammor, M.S., Delgado, S. and Mayo, B. 2006. Molecular analysis of a chromosome-carried *erm*(B) gene and its flanking insertion points in *Lactobacillus johnsonii* G41. Antimicrob. Agents Chemother. 50:4189-4190.

Florez, A.B., Ammor, M.S., Mayo, B., van Hoek, A.H.A.M., Aarts, H.J.M. and Huys, G. 2008. Antimicrobial susceptibility profiles of 32 type strains of *Lactobacillus*, *Bifidobacterium*, *Lactococcus* and *Streptococcus*. Int. J. Antimicrob. Agents 31:484-504.

Franke, C.M., Tiemersma, J., Venema, G. and Kok, J. 1999. Membrane topology of the lactococcal bacteriocins ATP cassette transporter protein LcnC. Involvement of LcnC in lactococcin A maturation. J. Biol. Chem 274:8484-8490.

Franz, C.M.A.P., Schillinger, U. and Holzapfel, W.H. 1996. Production and characterization of enterocin 900, a bacteriocin produced by *Enterococcus faecium* BFE 900 from black olives. Int. J. Food Microbiol. 29:255-70.

Gálvez, A., Abriouel, H., Lopez, R.L. and Ben Omar, N. 2007. Bacteriocin-based strategies for food biopreservation. Int. J. Food Microbiol. 30:51-70.

Garneau, S., Martin, N.I. and Vederas, J.C. 2002. Two-peptide bacteriocins produced by lactic acid bacteria. Biochimie. 84:577-592.

Garrity, G.M. and Holt, J.G., 2001. The Road Map to the Manual. In: Bergey`s Manual of Systematic Bacteriology, Boone, D.R., Castenholz, R.W. (eds.), New York, Springer Verlag, pp. 133-135.

Gevers, D., Danielsen, M., Huys, G. and Swings, J. 2003a. Molecular characteriyation of *tet*(M) genes in *Lactobacillus* isolated from different types of fermented dry sausage. Appl. Environ. Microbiol. 69:1270-1275.

Gevers, D., Masco, L., Baert, L., Huys, G., Debevere, J. and Swings, J. 2003b. Prevalence and diversity of tetracyyline resistant lactic acid bacteria and their *tet* gene s along the process

line of fermented dry sausages. Syst. Appl. Microbiol. 26:277-283.

Glaser, P., Frangeul, L., Buchrieser, C., Rusniok, C., Amend, A., Baquero, F., Berche, P., Bloecker, H., Brandt, P., Chakraborty, T., Charbit, A., Chetouani, F., Couvé, E., de Daruvar, A., Dehoux, P., Domann, E., Domínguez-Bernal, G., Duchaud, E., Durant, L., Dussurget, O., Entian, K.D., Fsihi, H., García-del Portillo, F., Garrido, P., Gautier, L., Goebel, W., Gómez-López, N., Hain, T., Hauf, J., Jackson, D., Jones, L.M., Kaerst, U., Kreft, J., Kuhn, M., Kunst, F., Kurapkat, G., Madueno, E., Maitournam, A., Vicente, J.M., Ng, E., Nedjari, H., Nordsiek, G., Novella, S., de Pablos, B., Pérez-Diaz, J.C., Purcell, R., Remmel, B., Rose, M., Schlueter, T., Simoes, N., Tierrez, A., Vázquez-Boland, J.A., Voss, H., Wehland, J. and Cossart, P. 2001. Comparative genomics of *Listeria* species. Science 294:849–852.

Gould, G.W. 1996. Methods for preservation and extension of shelf life. Int. J. Food Microbiol. 33: 51-64.

Graves, L.M. and Swaminathan, B. 2001. PulseNet standardized protocol for subtyping *Listeria monocytogenes* by macrorestriction and pulse-field gel electrophoresis. Int. J. Food Microbiol. 65: 55-62.

Greer, G.G. 1989. Red meats, poultry and fish. In: Enzymes of Psychrotrophs in Raw Food, (eds.), R.C. McKellar, CRC Press, Boca Raton, Fla. pp. 267

Gursky, L.J., Martin, N.I., Derksen, D.J., van Belkum, M.J., Kaur, K., Vederas, J.C., Stiles, M.E. and McMullen, L.M. 2006. Production of piscicolin 126 by *Carnobacterium maltaromaticum* UAL26 is controlled by temperature and induction peptide concentration. Arch. Microbiol. 186:317-325.

Hammes, W.P. and Hertel, C. 2009. Genus I. *Lactobacillus* Beijerinck 1901 212^{AL}. In: De Vos, P., Garrity, G.M., Jones, D., Krieg, N.R., Ludwig, W., Rainey, F.A., Schleifer, K.-H., and Whitmman, B (eds.). *Bergey's Manual of Systematic Bacteriology* (2nd ed.) Vol. III The *Firmicutes*. Springer, London, pp. 465-511.

Hauge, H.H., Nissen-Meyer, J., Nes, I.F. and Eijsink, V.G.H. 1998. Amphiphilic α-helices are important structural motifs in the α and β peptides that constitute the bacteriocin lactococcin G: enhancement of helix formation upon α-β interaction. Eur. J. Biochem 251:565-572.

Holo, H., Jeknic, Z., Daeschel, M., Stevanovic, S. and Nes, I.F. 2001. Plantaricin W from *Lactobacillus plantarum* belongs to a new family of two-peptide lantibiotics. Microbiology 147:643-651.

Holzapfel, W.H., Geisen, R. and Schillinger, U. 1995. Biological preservation of foods with reference to protective cultures, bacteriocins and food-grade enzymes. Int. J. Food Microbiol. 24:343-362.

Holzapfel, W.H. 1997. Use of starter cultures in fermentation on a household scale. Food Contr. 8:241-258.

Holzapfel, W.H. 2002. Appropriate starter culture technologies for small-scale fermentation in developing countries. Int. J. Food Microbiol. 75:197-212.

Holzapfel, W.H. 2005. Introduction to prebiotics and probiotics. In:Goktepe and V.K. Juneja (eds.), Probiotics in Food Safety and Human health, New York: Marcel Dekker, New York. pp. 1-31.

Huch, M., Hanak, A., Specht, I., Dortu, C.M., Thonart, P., Mbugua, S., Holzapfel, W.H., Hertel, C. and Franz, C.M.A.P. 2008. Use of *Lactobacillus* strains to start cassava fermentations for Gari production. Int. J. Food Microbiol. 128:258-267.

Hugas, M., Pagés, F., Garriga, M.and Monfort, J.M. 1998. Application of the bacteriocinogenic *Lactobacillus sakei* CTC494 to prevent growth of *Listeria* in fresh and cooked meat products packaged with different atmospheres. Food Microbiol. 15: 639-650.

Hummel, A.S., Hertel, C., Holzapfel, W.H. and Franz, C.M.A.P. 2007. Antibiotic resistances of starter and probiotic strains of lactic acid bacteria. Appl. Envrion. Microbiol. 73:730-739.

Jacobsen, T., Budde, B.B. and Koch, A.G. 2003. Application of *Leuconostoc carnosum* for biopreservation of cooked meat products. J. Appl. Microbiol. 95:242-249.

Jimenez-Diaz, R., Rios-Sanchez, R.M., Desmazeaud, M., Ruiz-Barba, J.L. and Piard, J.C. 1993. Plantaricins S and T, two new bacteriocins produced by *Lactobacillus plantarum* LPCO10 isolated from a green oiive fermentation. Appl. Envrion. Microbiol. 59:1416-1424.

Johnsen, L., Fimland, G., Mantzilas, D. and Nissen-Meyer, J. 2004. Structure-function analysis of immunity proteins of pediocin-like bacteriocins: the C-terminal parts of immunity proteins are involved in specific recognition of cognate bacteriocins. Appl. Environ. Microbiol. 70:2647-2652.

Johnsen, L., Dalhus, B., Leiros, I. and Nissen-Meyer, J. 2005a. 1.6-Å crystal structure of EntA-im: a bacterial immunity protein conferring immunity to the antimicrobial activity of the pediocin-like bacteriocin enterocin A. J.Biol.Chem. 280:19045-19050.

Johnsen, L., Fimland, G. and Nissen-Meyer, J. 2005b. The C-terminal domain of pediocin-like antimicrobial peptides (class Iia bacteriocins) is involved in specific recognition of the C-terminal part of cognate immunty protins and in determining the antimicrobial spectrum. J. Biol. Chem. 11:9243-9250.

Kandler, O. 1983. Carbohydrate metabolism in lactic acid bacteria. Antonie van Leeuwenhoek 49:209-224.

Katikou, P., Ambrosiadis, D., Georantelis, D., Koidis, P. and Georgakis, S.A. 2005. Effect of *Lactobacillus*-protective cultures with bacteriocin-like inhibitory substances producing ability on microbiological, chemical and sensory changes during storage of refrigerated vacuum-packaged sliced beef. J. Appl. Microbiol. 93:191-196.

Katla, A.-K., Kruse, H., Johnsen, G. and Herikstad, H. 2001. Antimicrobial susceptibility of starter culture bacteria used in Norwegian dairy products. Int. J. Food Microbiol. 67:147-152.

Katla, T., Moretro, T., Seveen, I., Aasen, I.M., Axelsson, L., Rorvik, L.M. and Naterstad, K. 2002. Inhibition of *Listeria monocytogenes* in chicken cold cuts by addition of sakacin P and sakacin P-producing *Lactobacillus sakei*. J. Appl. Microbiol. 93:191-196.

Kazazic, M., Nissen-Meyer, J. and Fimland, G. 2002. Mutational analysis of the role of charged residues in target-cell binding, potency and specificity of the pediocin-like bacteriocin sakacin P. Microbiology 148, 2019-2027.

Kim, I.-K., Kim, M.-K., Kim, J.-H., Yim, H,-S., Cha, S.-S. and Kang, S.-O. 2007. High resulution crystal structure of PedB: a structural basis for the classification of pediocin-like immunity proteins. BMC Struct. Biol. 2007 7:35.

Klare, I. Konstabel, C., Müller-Bertling, S., Reissbrodt, R., Huys, G., Vancanneyt, M., Swings, J., Goossens, H. and Witte, W. 2005. Evaluation of new broth media for microdilution antibiotic susceptibility testing of Lactobacilli, Pediococci, Lactococci and Bifidobacteria. Appl. Environ. Microbiol. 71:8982-8986.

Klare, I., Konstabel, C., Werner, G., Huys, G., Vankerckhoven, V., Kahlmeter, G., Hildebrandt, B., Müller-Bertling, S., Witte, W. and Goossens, H. 2007. Antimicrobial susceptibilities of *Lactobacillus*, *Pediococcus* and *Lactococcus* human isolates and cultures intended for probiotic or nutritional use. J. Antimicrob. Chemother. 59:900-912.

Kleerebezem, M., Boekhorst, J., van Kranenburg, R., Molenaar, D., Kuipers, O.P. and Leer, R. 2003. Complete genome sequence of *Lactobacillus plantarum* WCFS1. Proc. Natl. Acad. Sci. USA 100:1990-1995.

Klein, G., Pack, A., Bonaparte, C. and Reuter, G. 1998. Taxonomy and physiology of probiotic lactic acid bacteria. Int. J. Food Micribiol. 26:103-125.

Klein, G., Hallmann, C., Casas, I.A., Abad, J., Louwers, J. and Reuter, G. 2000. Exclusion of *van*A, *van*B and *van*C type glycopeptides resistance in strains of *Lactobacillus reuteri* and *Lactobacillus rhamnosus* used as probiotics by polymerase chain reaction and hybridization methods. J. Appl. Microbiol. 89:815-824.

Knoll, C., Divol, B. and du Toit, M. 2008. Genetic screening of lactic acid bacteria of oenological origin for bacteriocin-encoding genes. Food Microbiol. 25:983-991.

Konings, W.N. 2002. The cell membrane and the struggles for life of lactic acid bacteria. Antonie van Leeuwenhoek. 82:3-27.

Kostinek, M., Pukall, R., Rooney, A.P., Schillinger, U., Hertel, C., Holzapfel, W.H. and Franz, C.M.A.P. 2005. *Lactobacillus arizonensis* is a later heterotypic synonym of *Lactobacillus plantarum*. Int. J. Food Microbiol. 55:2485-2489.

Leisner, J.J., Greer, G.G., Dilts, B.D. and Stiles, M.E. 1995. Effect of growth of selected lactic acid bacteria on storage life of beef stored under vacuum and in air. Int. J. Food Microbiol.

62:1922-1927.

Leisner, J.J., Greer, G.G. and Stiles, M.E. 1996. Control of beef spoilage by a sulphide-producing *Lactobacillus sakei* strain with bacteriocinogenic *Leuconostoc gelidum* UAL187 during anaerobic storage. Appl. Environ. Microbiol. 62:2610-2614.,

Leroy, F. and De Vuyst, L. 1999. Temperature and pH conditions that prevail during fermentation of sausage are opitmal for production of the antilisterial bacteriocin sakacin K. Appl. Environ. Microbiol. 65:974-981.

Leroy, F. and De Vuyst, L. 2004. Lactic acid bacteria as functional starter cultures for the food fermentation industry. Trends Food Sci. Technol. 15:67-78

Lindgren, S.E. and Dobrogosz, W.Jren. 1990. Antagonistic activities of lactic acid bacteria in food and feed fermentations. FEMS Microbiol. Rev. 7:149-163.

Livak, K.J. and Schmittgen, T.D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-delta delta c(t)) method. Methods 25:402-408.

Lücke, F.K. 2000. Utilization of microbes to process and preserve meat. Meat Science 56:105-115.

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.-H., Diaz-Muniz, I., Dosti, B., Smeinaov, 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. and Mills, D. 2006. Comparative genomics of the lactic acid bacteria. Proc. Natl. Acad. Sci. USA 103:15611-15616.

Makarova, K.S. and Koonin, E.V. 2007. Evolutionary genomics of lactic acid bacteria. J. Bacteriol. 189: 1199-1208.

Maldonado, A., Ruiz-Barba, J.L. and Jiménez-Díaz, R. 2003. Purification and genetic characterization of plantaricin NC8, a novel coculture-inducible two peptide bacteriocin from *Lactobacillus plantarum* NC8. Appl. Environ. Microbiol. 69:383-389.

Maldonado, A., Jiménez-Díaz, R. and Ruiz-Barba, J.L. 2004. Induction of plantaricin production in *Lactobacillus plantarum* NC8 after coculture with specific gram-positive bacteria is mediated by an autoinduction mechanism. J. Bacteriol.186:556-1564.

Maragkoudakis, P.A., Mountyouris, K.C., Payrras, D., Cremonese, S., Fischer, J., Cantor, M.D. and Tsaklidou, E. 2009. Functional properties of novel protective lactic acid bacteria and application in raw chicken meat against *Listeria monocytogenes* and *Slmonella enteritidis*. Int. J. Food Microbiol. 15:219-226.

Marteau, P., Vaerman, J.P., Dehennin, J.P., Bord, S., Brassart, D., Pochart, P., Desjeux, J.F. and

Ranbaud, J.C. 1997. Effects of intrajejunal perfusion and chronic ingestion of *Lactobacillus johnsonii* strain La1 on serum concentrations and jejunal secretions of immunoglobulins and serum proteins in healthy human. Gastroenterol. Clin. Biol. 21:293-298.

Mataragas, M., Drosinos, E.H., Tsakalidou, E. and Metaxopoulos, J. 2004. Influence of nutrients on growth and bacteriocin production by *Leuconottoc mesenteroides* L124 and *Lactobacillus curvatus* L442. Antonie van Leeuwenhoek. 85:191-198.

Mathara, J.M., Schillinger, U., Kutima, P.M., Mbugua, S.K. and Holzapfel, W.H. 2004. Isolation, identification and characterisation of the dominant microorganisms of kule naoto: the Maasai tranditional fermented milk in Kenya. Int. J. Food Microbiol. 94:269-278.

Mathur, S. and Singh, R. 2005. Antibiotic resistance n food lactic acid bacteria – a review. Int. J. Food Microbiol. 105:281-295.

McMeekin, T.A. 1975. Spoilage association of chicken breast muscle. Appl. Environ. Microbiol. 29:44-47.

Messens, W. and de Vuyst, L. 2002. Inhibirory substances produced by *Lactobacilli* isolated from sourdoughs-a review. Int. J. Food Microbiol. 72:31-43.

Molenaar, D., Dringel, F., Schuren, F.H., de Vos, W,M., Siezen, R.J. and Kleerebezem, M. 2005. Exploring *Lactobacillus plantarum* genome diversity by using microarrays. J. Bacteriol. 197:6119-6127.

Moll, G., Ubbink-Kok, T., Hauge, H.H., Nissen-Meyer, J., Nes, I.F., Konings, W.N. and Driessen, A.J.M. 1996. Lactococcin G is a potassium ino-conducting, two component bacteriocin. J. Bacteriol. 178:600-605.

Moll, G., van der Akker, H.E., Hauge, H.H., Nissen-Meyer, J., Nes, I.F., Konings, W.N. and Driessen, A.J.M. 1999. Complementary and overlapping selectivity of the two-peptide bacteriocins EF and JK. J. Bacteriol. 181: 4848-4852.

Morisset, D., Bergeaud, J.M., Marion, D., Lacombe, C. and Frère, J. 2004. Mutational analysis of mesentericin Y105, an anti-Listeria bacteriocin, for determination of impact on bactericidal activity, in vitro secondary structure and membrane interaction. Appl. Environ. Microbiol. 70: 4672-4680.

Müller, D.M., Carrasco, M.S., Tonarelli, G.G. and Simonetta, A.C. 2009. Characterization and purification of a new bacteriocin with a broad inhibitory spectrum produced by *Lactobacillus plantarum* lp31 strain isolated from dry-fermented sauage. J. Appl. Microbiol. 106:2031-2040.

Navarro, L., Rojo-Beyares, B., Sáenz, Y., Díez, L., zarazaga, M., Ruiz-Larrea, F. and Torres, C. 2008. Comparative study of the *pln* locus of the quorum-sensing regulated bacteriocin-producing *L. plantarum* J51 strain. Int. J. Food Microbiol. 128:390-394.

Nes, I.F., Diep, L.S., Håvarstein, L.S., Brurberg, M.B., Eijsink, V. and Holo, H. 1996. Biosynthesis of bacteriocins in lactic acid bacteria. Antonie van Leeuwenhoek 70:113-128.

Nes, I.F. and Fijsink, V.G.H. 1999. Regulation of group II peptide bacteriocin synthesis by quorum-sensing mechanisms, In: Cell-cell signaling in bacteria. G.M. Dunny and S.C. Winans (eds.), American Society for Microbiology, Washington, D.C. pp.175-192.

Nissen-Meyer, J., Rogne, P., Oppegård, C., Haugen, H.S. and Kristiansen, P.E. 2009. Structure-function relationships of the non-lanthionine-containing peptide (class II) bacteriocins produced by gram-positive bacteria. Curr. Pharm. Biotechnol. 10:19-37.

Nout, M.J. and Motarjemi, Y. 1997. Assessment of fermentation as a household technology for improving food safety: a joint FAO/WHO workshop. Food Control 8:221-226.

Ohlsson, T. 1994. Minimal processing-preservation methods of the future: an overview. Trends Food Sci. Technol. 5:341-344.

Oppegård, C., Fimland, G., Thorbaek, L. and Nissen-Meyer, J. 2007. Analysis of the two-peptide bacteriocin lactococcin G and enterocin 1071 by site-directed mutagenesis. Appl. Environ. Microbiol. 73: 2931-2938.

Oppegård, C., Emanuelsen, L., Thorbek, L., Fimland, G. and Nissen-Meyer, J. 2009. The lactococcin G immunity protein recognizes specific regions in both peptides constituting the two-peptide bacteriocin lactococcin G. Appl. Environ. Microbiol. 76:1267-1273.

Pitcher, D.G., Saunders, N.A. and Owen, R.J. 1989. Rapid extraction of bacterial genomic DNA with guanidium thiocyanate. Lett. Appl. Microbiol. 8:151-156.

Poolman, B. 1993. Energy transduction in lactic acid bacteria. FEMS Micribiol. Rev. 12: 125-146.

Quadri, L.E.N., Sailer, M., Roy, K.L., Vederas, J.C. and Stiles, M.E. 1994. Chemical and genetic characterization of bacteriocins produced by *Carnobacterium piscicola* LV17B. J. Biol. Chem. 269:12204-12211.

Quadri, L.E.M., Sailer, M., Terebiznik, M.R., Roy, K.L., Vederas, J.C. and Stiles, M.E. 1995. Characterization of the protein conferring immunity to the antimicrobial peptide carnobacteriocin B2 and expression of carnobacteriocins B2 and BM1. J. Bacteriol. 177:1144-1151.

Quadri, L.E.N., Kleerebezem, M., Kuipers, O.P., de Vos, W.M., Roy, K.L., Vederas, J.C. and Stiles, M.E. 1997. Characterization of a locus from *Carnobacterium piscicola* LV17B involved in bacteriocin production and immunity: evidence for global inducer-mediated transcriptional regulation. J. Bacteriol. 179: 6163-6171.

Ramiah, K., van Reenen, C.A. and Dicks, L.M. 2007. Expression of the mucus adhesion genes Mub and MapA, adhesion-like factor EF-Tu and bacteriocin gene plaA of *Lactobacillus platnarum* 423, monitored with real-time PCR. Int. J. Food Microbiol. 116:405-409.

Ramnath, M., Arous, S., Gravesen, A., Hastings, J.W. and Héchard, Y. 2004. Expression of mptC of *Listeria monocytogenes* induces sensitivity to class IIa bacteriocins in *Lactococcus lactis.* Microbiology 150:1663-2668.

Ray, B. 2001. Fundamental Food Microbiology. CRC üress Bpca Raton, Florida, USA.

Rogne, P., Fimland, G., Nissen-Meyer, J. and Kristiansen, P.E. 2008. Three-dimensional structure of the two peptides that constitute the two peptide bacteriocin lactococcin G. Biochem. Biophys. Acta 1784:543-554.

Rojo-Bezares, B., Sáenz, Y., Navarro, L., Zarazaga, M., Ruiy-Larrea, F. and Torres, C. 2007. Coculture-inducible bacteriocin activity of *Lactobacillus plantarum* strain J23 isolated from grape must. Food Microbiol. 24:482-491.

Rojo-Bezares, B., Sáenz, Y., Navarro, L., Jiménez-Días, R., Zarazaga, M., Ruiz-Larrea, F. and Torres, C. 2008. Characterization of a new organization of the plantaricin locus in the inducible bacteriocin-producing *Lactobacillus plantarum* J23 of grape origin. Arch. Microbiol. 189:491-499.

Sáenz, Y., Rojo-Bezares, B., Navarro, L., Díez, L., Somalo, S., Zarazaga, M., Ruiz-Larrea, F. and Torres, C. 2009. Genetic diversity of the *pln* locus among oenological *Lactobacillus plantarum* strains. Int. J. Food Microbiol. 134:176-183.

Sambrook, J., Fritsch, E. F. and Maniatis, T. 1989. Molecular cloning: A laboratory manual, 2nd ed. Cold Spring Harbor, N.Y. Cold Spring Harbor Laboratory

Schleifer, K.H.and Ludwig, W. 1995. . Phylogenetic relationships of lactic acid bacteria. In: The Genera of Lactic Acid Bacteria (eds.), B.J.B. Wood and W.H. Holzapfel. Blackie Academic and Professional, Glasgow. pp 7-17.

Settanni, L., Valmorri, S., Suzzi, G. and Coresetti, A. 2008. Ther role of environmental factors and medium composition on bacteriocin-like inhibitory substance (BLIS) production by *Enterococcus mundtii* strains. Food Microbiol. 25:722-728.

Skyttä, E., Hereijgers, W. and Mattila-Sandholm, T. 1991. Broad spectrum antibacterial activity of *Pediococcus damnosus* and *Pediococcus pentosaceus* in minced meat. Food. Microbiol. 8:231-237.

Sprules, T., Kawulka, K.E. and Vederas, J.C. 2004. NMR solution structure of ImB2, a protein conferring immunity to antimicrobial activity of the type IIa bacteriocin, carnobacteriocin B2. Biochemistry 43:11740-11749.

Stiles, M.E. 1996. Biopreservation by lactic acid bacteria. Antonie van Leeuwenhoek 70:331-345.

Stiles, M.E. and Holzapfel, W.H. 1997. Lactic acid bacteria of foods and their current taxonomy. Int. J. Food Microbiol. 36:1-29.

Stroman, P., Müller, C.C. and Sorensen, K.I. 2003. Heat shock treatment increases the frequency of loss of an erythromycin resistance-encoding transposable element from the chromosome of *Lactobacillus crispatus* CHCC3692. Appl. Environ. Microbiol. 69:7173-7180.

Tannock, G.W. 1999. Analysis of the intestinal microflora: a renaissance. Antonie van Leeuwenhoek 76:265-278.

Temmerman, R., Pot, B., Huys, G. and swings, J. 2003. Identification and antibiotic susceptibility of bacterial isolates from probiotic products. Int. J. Food Microbiol. 81:1-10.

Vadyvaloo, V., Arous, S., Gravesen, A., Héchard, Y., Chauhan-Haubrock, R., Hastings, J.W. and Rautenbach, M. 2004. Cell-surface alterations in class IIa bactericin-resistant *Listeria monocytogenes* strains. Microbiology 150:3025-3033.

van Belkum, M.J. and Stiles, M.E. 1995. Molecular characterization of genes involved in the production of the bacteriocin leucocin a from *Leuconostoc gelidum*. Appl. Envirion. Microbiol. 61:3573-3579.

Vermeiren, L., Devlieghere, F. and Debevere, J. 2006a. Co-culture experiments demonstrate the usefulness of *Lactobacillus sakei* 10A to prolong the shelf-life of a model cooked ham. Int. J. Food Microbiol. 108:68-77

Vermeiren, L., Devlieghere, F., Vandekinderen, I. and Debevere, J. 2006b. The antagonistic interaction of the non-bacteriocinogenic *Lactobacillus sakei* 10A and lactocin S producing *Lactobacillus sakei* 148 towards *Listeria monocytogenes* on a model cooked ham. Food Microbiol. 23:511-518.

Vizoso Pinto, M.G., Franz, C.M.A.P., Schillinger, U. and Holzapfel. W.H. 2006. *Lactobacillus* ssp. with in vitro probiotic properties from human faeces and traditional fermented products. Int. J. Food Microbiol. 109:205-214.

Vizoso Pinto, M.G., Schuster, T., Briviba, K., Watzl, B., Holzapfel, W.H. and Franz, C.M.A.P. 2007. Adhesive and chemokine stimulatory properties of potentially probiotic *Lactobacillus* strains. J. Food Prot. 70:125-134.

Vizoso Pinto, M.G., Rodriguez Gómez, M., Seifert, S., Watzl, B., Holzapfel, W.H. and Franz, C.M.A.P 2009. *Lactobacilli* stimulate the innate immune response and modulated the TLR expression of HT29 intestinal epithelial cells in vitro. Int. J. Food Microbiol. 133:86-93.

WHO, 2002. Food safety strategic planning meeting: report of a WHO strategic planning meeting. WHO headquarters, Geneva, Switzerland, 20-22 February 2001 (http://whqlibdoc.who.int/hq/2001/WHO_SED_PHE_FOS_01.2.pdf). World Health Organization, Geneva.

Winkowski, K., Crandall, A. D. and Montville, T. J. 1993. Inhibition of *Listeria monocytogenes* by *Lactobacillus bavaricus* MN in beef systems at refrigeration temperatures. Appl. Envrion. Microbiol. 59:2552-2557.

Woese, C.R. 1987. Bacterial evolution. Microbiol. Rev. 51:221-71.

Yuan, J.S., Reed, A., Chen, F. and Stewart, C.N.Jr. 2006. Statistical analysis of real-time PCR data. BMC Bioinformatics 7:85.

Publication list

Book chapters

1. Franz, C.M.A.P., Gyu-Sung Cho and W.H. Holzapfel. 2010. *Probiotics: Taxonomy and Technological Feautures*. In: de Assis Fonseca Faria, J., Gomes da Cruz, A., and Shah, N. (eds.) Probiotic and Prebiotic Foods: Technology, Stability and Benefit to the Human Health. New Publisher, U.S.A. In press.
2. Franz, C.M.A.P., Gyu-Sung Cho, W.H. Holzapfel and A. Galvez. 2009. *Safety of Lactic Acid Bacteria*. In: Mozzi, F., Vignolo, G. and Raya, R. (eds.) Biotechnology of Lactic Acid Bacteria: Novel Applications. Research Signpost Editorial. In Press.

Publications in peer reviewed journals

1. Cho, G.-S., Hanak, A., Huch, M., Holzapfel, W.H. and Franz, C.M.A.P. 2009. Genetic analysis of the plantaricin EFI locus of *Lactobacillus plantarum* PCS20 reveals an unusual plantaricin E gene sequence as a result of mutation. *Int. J. Food Microbiol*. In press.
2. Nielsen, D.S., Cho, G.-C., Hanak, A., Huch, M., Franz, C.M.A.P. and Arneborg, N. 2010. The effect of bacteriocin-producing *Lactobacillus plantarum* strains on the intracellular pH of sessile and planktonic *Listeria monocytogenes* single cells. *Int. J. Food Microbiol*. In press.
3. Oguntoyinbo, F.A., Huch, M., Cho, G.-S., Holzapfel, W.H. and Franz, C.M.A.P. 2009. Diversity of *Bacillus* species isolated from *okpehe,* a traditional fermented soup condiment from Nigeria. *J. Food Prot.* In press.
4. Franz, C.M.A.P., Specht, I., Gyu-Sung, C., Graef, V. and Stahl, M.S. 2009. UV-C-inactivation of microorganisms in naturally cloudy apple juice using novel inactivation equipment based on Dean vortex technology. *Food Control*. 20:1103-1107.
5. Cho, G.-S., Do, H.K., Bae, C.Y., Cho, G.S., Whang, C.W. and Shin, H.K. 2006. Candidate probiotic bacteria isolated from Jeotgal: a traditional Korean fermented seafood. *J. Food Sci Nutr.* 11:140-145.
6. Cho, G.-S. and Do, H.K. 2006. Isolation and Identification of lactic acid bacteria isolated from a traditional Jeotgal product in Korea. *Ocean Science Journal.* 41:113-119.

Poster

1. Cho, G.-S., Huch, M., Hanak, A., Holzapfel, W.H., and Franz, C.M.A.P. 2009. Plantaricin locus of *Lactobacillus plantarum* strains PCS20 reveals an atypical plantaricin E gene as a result of mutation. 22-24 Juni 2009 Fachsymposium der Fachgruppe Lebensmittelmikrobiologie im Bildungzentrum Wildbad Kreuth.

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02.2004 – 02.2006	Master of Science (M. Sc.) at the Department of Life Science, Handong Global University, Pohang, South Korea
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03.1994 – 02.1997	Hanil high school graduation
03.1991 – 02.1994	Jinhae middle school graduation

Military service

1999 - 2001	Service in Donghae (Navy), Southkorea

Social service

08. 2003	Construction Volunteer service in Habitat for Humanity Korea (Kangwondo Samcheuck)
08. 2008	Construction Volunteer service in Habitat for Humanity Korea (Gyeongbook Chilgok)

AWARD

04. 2007 -05. 2010 DAAD Scholarship (Forschungsstipendien für Doktoranden)

Language

- Korean Native speaker
- English Good
- German Basic knowledge
  Deutsch als Fremdsprache B2-1 in Volkshochschule

PC-skills

- Microsoft Office: Word, Excel, Outlook, Powerpoint. Very good.
- DnaStar: Very good

Hobby

Playing soccer, cooking.

Karlsruhe, 10 March 2010 Gyu-Sung Cho