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The formation and antimicrobial activity of nisin and plant derived bioactive components in lactic acid bacteria fermentations

Doctoral Dissertation

Marja Tolonen

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The formation and antimicrobial activity of nisin and plant derived bioactive components in lactic acid bacteria fermentations

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Abstract

This study focused on the formation of nisin, a 34-residue antibacterial polypeptide, and plant-derived biomolecules and their antimicrobial properties in lactic acid bacteria (LAB) fermentations. The role of the genes *nisB* and *nisC* in the nisin biosynthetic pathway (*nisA/ZBTCIPRKFEG*) was investigated. Nisin was produced in M17G medium and in cabbage juice by fermenting with a wild-type *Lactococcus lactis* N8 and the mutant strains LAC48 and LAC67. The fermentation types were: 1) pH not controlled, 2) adjusted at pH5.5, 3) recycling of *L. lactis* cells (pH5.5) (circulated batch), and 4) cabbage (sauerkraut) fermentation without pH control. Besides nisin, the focus was on the formation of plant-derived molecules, especially glucosinolate breakdown products in cabbage fermentation. The cabbage fermentations were inoculated with the following starter cultures: *Lactobacillus plantarum*, *L. sakei*, *Leuconostoc mesenteroides*, *Pediococcus pentosaceus, P. dextrinicus, Lactococcus lactis* N8, and *L. lactis* LAC67.

Nisin was produced in different types of fermentations. The nisin titer achieved in batch fermentations in M17G medium was 2200 - 2500 IU ml^{-1}. In the case of LAC48 the concentration remained high also after the logarithmic growth phase, whereas nisin production by the strain N8 was more typical: the nisin titer decreased after the maximum growth. The result showed that nisin production in the case of LAC48 was not directly related to biomass formation and was not associated with growth, which is not typical of nisin production. In order to study end-product inhibition in nisin production, a system (circulated batch) was built for nisin adsorption during fermentation. The adsorbent Amberlite XAD-4 was found to bind nisin effectively. Cells of the strains LAC48 and N8 were able to compensate the removed nisin throughout fermentation, indicating that nisin production also occurs in the stationary phase. A high concentration of nisin (8000 IU ml^{-1}) was achieved by eluting nisin from the XAD-4 resin. Nisin was produced in sauerkraut fermentation up to 1400 IU ml^{-1} and was detectable (250 IU ml^{-1}) in sauerkraut even after 13 days' fermentation.

The role of the proteins NisB and NisC in nisin maturation has been unclear to the present day. This study showed that NisB is required for dehydration and NisC for lanthionine formation in post-translational modification of

nisin. Nisin precursors from the strain lacking NisB activity were totally unmodified, whereas from the strain lacking NisC activity, but having NisB activity, were dehydrated but lacked normal lanthionine formation. This is the first reported experimental result indicating the role of NisB and NisC in correct modification of nisin.

In addition to nisin, plant-derived biomolecules have been shown to possess antimicrobial properties and also to exhibit potential health effects. However, formation and/or degradation of these biomolecules in fermentation process has not been well known. In the present study, plant-derived biomolecules, glucosinolates, were decomposed during cabbage fermentation, forming beneficial breakdown products such as isothiocyanates (ITC), indole-3-carbinol (I3C) and sulforaphane. The research showed that lactic acid bacteria may affect the degradation of glucosinolates and the formation of breakdown products in cabbage fermentation. When *L. sakei* was used as the starter culture, the concentrations of 3-methylsulphinylpropyl-ITC, allyl-ITC and sulforaphane were significantly higher compared with the end-products using other (single strain) starter cultures. Goitrin was found in substantially high concentrations in all end-products of starter-culture fermentation. Sulforaphane nitrile, allyl-nitrile, and goitrin were found in small quantities in the end-products of mixed-culture (*L. mesenteroides* + *P. dextrinicus*) and spontaneous fermentation.

Inhibitory effects of fermented cabbage juices were observed using the indicator strains *Escherichia coli*, *Listeria monocytogenes*, and *Candida lambica*. The strongest inhibitory effects of cabbage juice were achieved by juice fermented with the strains *Lactobacillus sakei* and *Leuconostoc mesenteroides*, which also had the highest AITC concentration. Further, the juice fermented with *L. sakei* was capable of effectively inhibiting the growth of *C. lambica* and *E. coli*, but not of *L. monocytogenes*.

The results indicate that concentrated nisin (8000 IU ml^{-1}) can be produced by using Amberlite XAD-4 adsorbent and that bioactive glucosinolate breakdown products are formed in cabbage fermentation. A combination of these compounds may be used to extend food shelf-life, offering potential for the biopreservation of foods. In order to maximize the production of glucosinolate breakdown products potentially beneficial to health, the use of cabbage cultivars containing a high concentration of glucosinolates sinigrin, glucobrassicin, and indolyl glucosinolates is suggested. Further study is needed to optimize the cabbage fermentation process (starter culture, pH, temperature, etc.). The inhibitory effects of the combinations of nisin and glucosinolate breakdown products on food spoilage microbes should be further characterized in future research by using a wide variety of food pathogens and spoilage microbes.

Key words: cabbage, sauerkraut, glucosinolate, glucosinolate breakdown product, flavonoids, nisin, lantibiotic, fermentation, isothiocyanate

Foreword

This work was carried out at the Department of Applied Chemistry and Microbiology, University of Helsinki; VTT Biotechnology; and MTT Agrifood Research Finland, during 1998-2001. I wish to thank the heads of the above institutes for providing excellent facilities for my research.

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This doctoral thesis was written while I was working at Rovaniemi Polytechnic. I wish to express my sincere thanks to Rovaniemi Polytechnic and especially to Director Matti Lempiäinen for giving me the opportunity and financial assistance to complete the thesis. My colleagues at Rovaniemi

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Helsinki, February 2004

Marja Tolonen

List of original articles

The thesis is a summary and discussion of the following articles, which are referred to by their Roman numerals:

I Koponen O., Tolonen M., Qiao M., Wahlström G. and Saris P. E. J. **2002**. NisB is required for the dehydration and NisC for the lanthionine formation in the post-translational modification of nisin. Microbiol. 148:3561-3568.

II Tolonen M., Siika-aho M. and Saris P.E.J. **2004**. Production of nisin with continuous adsorption to Amberlite XAD-4 resin using *Lactococcus lactis* N8 and *L. lactis* LAC48. *Appl.* Microbiol. Biotechnol. doi: 10.1007/s00253-003-1413-5 (in press).

III Tolonen M., Taipale B., Viander B., Pihlava J-M., Korhonen H. and Ryhänen E-L. **2002**. Plant derived biomolecules in fermented cabbage. J. Agric. Food Chem. 50(23):6798-6803.

IV Tolonen M., Rajaniemi S., Pihlava J-M., Johansson T., Saris P.E.J. and Ryhänen E-L. **2004**. Formation of nisin, plant derived biomolecules and antimicrobial activity in starter culture fermentations of sauerkraut. Food Microbiol. 21(2):167-179.

The author's contributions in articles:

I: Experimental work: repeating all essential experiments including nisin bioassay, Western analysis, and purification of the His-tagged nisin. Writing the paper and interpretation of the results.

II: Main responsibility for planning and experimental work, data handling, writing the paper, and interpretation of the results.

III: Processing of data, writing the paper and interpretation of the results.

IV: Main responsibility for planning and experimental work, data handling, writing the paper, and interpretation of the results.

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Symbols and abbreviations

ABC	ATP-binding cassette
ADI	acceptable daily intake
AhR	aryl hydrocarbon receptor
ATCC	American Type Culture Collection
ATP	adenosine triphosphate
BDP	breakdown product
bp	base pair
Dha	dehydroalanine
Dhb	dehydrobutyrine
DNA	deoxyribonucleinic acid
DW	dry weight
e.g.	exempli gratia, for example
ery	erythromycin
FW	fresh weight
GC	gas chromatography
GC-MS	gas chromatography mass spectrometry
GS	glucosinolate
HPLC	high performance liquid chromatography
i. e.	id est, that is to say
in situ	in its original place
in vitro	taking place e. g. in a test tube or in culture dish
in vivo	taking place in a living organism
ITC	isothiocyanate
I3C	indole carbinol
kb	kilo base
kDa	kilo Dalton
LAB	lactic acid bacteria
Lan	lanthionine

MeLan	methyllanthionine
Met	methionine
MIC	minimum inhibitory concentration
min	minute
M17G	modified (rich in nutrients) growth medium for *L. lactis* cells, G; glucose
N-terminal	aminoterminal
PMF	proton motive force
ppm	parts per million, e.g. mg kg^{-1}
TSA	tryptic soy agar
WHO	World Health Organization

Contents

1 Introduction

Lactic acid bacteria (LAB) produce a variety of low molecular mass compounds including acids, alcohols, carbon dioxide, diacetyl, hydrogen peroxide, and small antimicrobial proteins called bacteriocins (reviewed by McAuliffe et al. 2001). Nisin, a post-translationally modified bacteriocin produced by certain strains of *Lactococcus lactis*, belongs to a group of lantibiotics containing unusual amino acids as well as lanthionine rings. Nisin is widely used as a food preservative in many countries (Turtell and Delves-Broughton 1998). LAB are utilized in the industrial production of lactic acid and nisin, and also in the processing of fermented foods, such as various milk products, dough, beverages, sausages, and fermented vegetables. The compounds produced by LAB affect the flavor, structure, and shelf-life of these food products (reviewed by Caplice and Fitzgerald 1999 and Ross et al. 2002).

Cruciferous plants, e.g. *Brassica* vegetables, contain bioactive molecules such as glucosinolates (reviewed by Rosa et al. 1997) and polyphenolic compounds such as flavonoids (reviewed by Ross and Kasum 2002). Glucosinolates are of interest because their enzymatic degradation releases physiologically active compounds which contribute to the bioactivity of cruciferous plants and to the characteristic flavor and taste in vegetable food products. The biosynthesis of these compounds is modified due to the environmental growth conditions. Moreover, glucosinolates are reported to play a role in defending the plant against general herbivore attack. Glucosinolates are modified by autolysis and food processing methods such as cooking, and fermenting, and by cutting or chewing; the components are degraded and/or decomposed and a variety of breakdown products (BDP) are formed. The hydrolysis products of glucosinolates include isothiocyanates (ITC), indole-3-carbinol (I3C), sulforaphane, goitrin, and nitriles, which have been shown to have pronounced biological effects in animal models, in humans and *in vitro* studies (Fahey et al. 2002; Brandi et al. 2003; Nachshon-Kedmi et al. 2003). The compounds may induce detoxification enzymes in mammalian cells, thereby acting as a cancer-preventing agent. On the other hand, in high concentrations, some of them may cause goitrogenic or other toxic effects in animals and humans. In addition to the reported health effects, many of these small compounds possess antimicrobial activities against certain harmful microbes, e.g. food spoilage bacteria (Shofran et al. 1998; Lin et al. 2000).

Several recent reports have described the identification, properties, activity, and degradation of bioactive compounds such as nisin and plant-derived biomolecules. However, the effect of processing on the decomposition of these molecules and the stability of the formed compounds is not well known. On the other hand, the nisin gene cluster (*nisA/ZBTCIPRKFEG*) has

been cloned and sequenced, and the roles of the encoded proteins in nisin biosynthesis have been partially characterized. The proteins NisB and NisC are associated with the formation of lanthionines in nisin biosynthesis, but the functions of the genes encoding these proteins have not been clearly shown. The research described in this thesis was conducted to investigate the role of the genes *nisB* and *nisC* in the biosynthesis of nisin (in maturation), the production of nisin, and the formation of plant-derived bioactive molecules in selected lactic acid fermentations, and to evaluate the potential antimicrobial properties of cabbage juice fermented with specific lactic acid bacteria.

2 Literature review

2.1 Nisin

Several lactic acid bacteria (LAB) produce small proteins or peptides called bacteriocins, which either disable or kill closely related bacteria. Bacteriocins are divided into subclasses I, II and III (Table 1) (Klaenhammer 1993). Class Ia bacteriocins are called lantibiotics (Nes et al. 1996). They are elongated, cationic peptides up to 34 residues in length that show similarities in the arrangement of their lanthionine (Lan) bridges. These cationic and hydrophobic peptides form pores in the membranes of the target cells and have a more flexible structure compared with other bacteriocins. The most prominent member of class Ia is nisin (nisin A, nisin Z and nisin Q), which is produced by certain strains of *Lactococcus lactis* (Hurst 1981; Zendo et al. 2003). Nisin-producing *L. lactis* strains have been found in various sources of food (Table 2). Nisin is, in fact, widely used in food applications in several countries (Turtell and Delves-Broughton 1998).

2.1.1 The structure of the nisin molecule and the gene cluster

Nisin is a ribosomally synthesized 34-residue polypeptide (3.35 kDa) produced by some *Lactococcus lactis* strains (Table 2). Three naturally occurring variants, nisin A (Dodd et al. 1990; Steen et al. 1991; Kuipers et al. 1993), nisin Z (Graeffe et al. 1991; Mulders et al. 1991; Immonen et al. 1995) and nisin Q (Zendo et al. 2003) have been characterized. Variants A and Z differ in a single amino acid residue at position 27: asparagine in nisin Z is replaced by histidine in nisin A (Graeffe et al. 1991). The effect of this single base-pair substitution on the properties of the peptide has been reported to be minimal (de Vos et al. 1992). The variant nisin Q differs in four amino acids with nisin A and in three with nisin Z; further studies are needed to investigate the effects of these substitutions (Zendo et al. 2003).

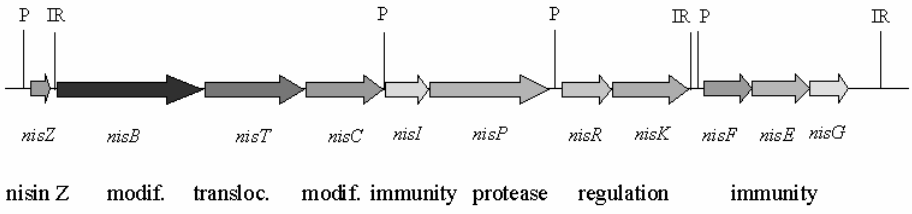

Figure 1. Organization of nisin biosynthetic, regulatory and immunity genes. P denotes a mapped promoter. An inverted repeat is indicated by IR.

Table 1. Classification of bacteriocins. (Modified from Klaenhammer 1993).

Group	Features	Representing bacteriocins	Reference
Class I Lantibiotics			
I a	Lantibiotics, small (< 5 kDa) peptides containing lanthionine (Lan) and β-methyl lanthionine (MeLan), flexible molecules compared to I b group	nisin, lacticin 481	Kaletta and Entian 1989; Dodd et al. 1990; Steen et al. 1991; Engelke et al. 1992; Kuipers et al. 1993; Graeffe et al. 1991; Mulders et al. 1991; Piard et al. 1992; Zendo et al. 2003
I b	Globular peptides with no net charge or net negative charge	mersacidin	
Class II Non-lantibiotics, small heat stable peptides			
II a	Small (< 5 kDa) heat-stable peptides, non-Lan peptides, synthesized in a form of precurcor which is processed after two glycine residues, active against *Listeria*, have a concensus sequence of YGNGV-C in the N-terminal	pediocin PA-1, sakacins A and P, leucocin A, carnobacteriocins	Gonzalez and Kunka 1987; Henderson et al. 1992; Marugg et al. 1992; Schillinger and Lucke 1989; Holck et al. 1992; Tichaczek et al. 1992; Stoffels et al. 1992
II b	Two component system: two different peptides required to form an active poration complex	lactococcins G and F, lactacin F, plantaricin EF and JK	Nissen-Meyer et al. 1992; Klaenhammer et al. 1992
II c	*Sec*-dependent secreted bacteriocin	acidocin B	
Class III Large heat-labile proteins			
III	Large (> 30 kDa) molecules sensitive to heat	helveticins J and V-1829, acidophilucin A, lactacins A and B	Joerger and Klaenhammer 1990; Klaenhammer et al. 1992

Table 2. Examples of bacteriocin nisin isolated from some foods.

Source	Strain	Active against	Reference
Radish	*L. lactis* sp. *cremoris* R	*Clostridium, Staphylococcus, Listeria and Leuconostoc* spp.	Yildirim and Johnson 1998
Bean-sprouts	*L. lactis* sp. *lactis* (NisZ)	*L. monocytogenes* Scott A	Cai et al. 1997
Irish kefir grain	*L. lactis* DPC3147	*Clostridium, Enterococcus, Listeria, Leuconostoc* spp.	Ryan et al. 1996
Dry fermented sausage	*L. lactis* (NisA)	*L. monocytogenes*	Rodriguez et al. 1995
Sauerkraut	*L. lactis* subsp. *lactis* (Nis)	*L. monocytogenes*	Hechard et al. 1992

The nisin gene cluster (size about 15 kb) is located in a gene cluster on the nisin-sucrose transposon and comprises eleven genes *nisABTCIPRKFEG* (Figure 1). The gene cluster has been cloned and sequenced (Kaletta and Entian 1989; Steen et al. 1991; Kuipers et al. 1993; van der Meer et al. 1993; Engelke et al. 1994; Immonen et al. 1995; Siegers and Entian 1995; Immonen and Saris 1998). The structural gene *nisA/Z* encodes the 57-amino acid pre-peptide, which is biologically inactive and carries an N-terminal leader peptide (23 amino acids) (Figure 2). The gene cluster also includes the genes responsible for intracellular post-translational modification reactions (*nisBC*) in which amino acids are dehydrated and lanthionines are formed (Figure 2, Figure 3). The peptide is secreted by an export protein encoded by the gene *nisT* (Engelke et al. 1992; Kuipers et al. 1993; Qiao and Saris 1996), *nisP* encodes an extracellular subtilisin-like serine-protease involved in precursor cleavage (van der Meer et al. 1993; Qiao et al. 1996), *nisI* encodes a lipoprotein for self-immunity (Kuipers et al. 1993; Qiao et al. 1995), and *nisFEG* encodes a putative ATP-binding cassette exporter (ABC-transporter), which also plays a role in self-immunity (Kuipers et al. 1993; Engelke et al. 1994; Siegers and Entian 1995; Immonen and Saris 1998; Ra et al. 1999). Moreover, *nisR* and *nisK* have been shown to be involved in a two-component regulation system in nisin biosynthesis: *nisR* encodes a response regulator NisR and *nisK* a histidine kinase NisK (van der Meer et al. 1993; Engelke et al. 1994; Immonen et al. 1995; Siegers and Entian 1995; Qiao et al. 1996).

2.1.2 Dehydration and formation of lanthionine rings

Nisin, like other lantibiotics, is produced on ribosome as a prepeptide which undergoes extensive post-translational modification to form a mature, extracellular, biologically active peptide. The term “lantibiotics” is due to the presence of unusual amino acids (dehydroalanine, Dha; dehydrobutyrine, Dhb; lanthionine, Lan; methyllanthionine, MeLan) not normally found in nature (Sahl et al. 1995). The chemical reactions required for the synthesis of these amino acids were first postulated by Ingram (1970). The process involves dehydration of serine (to dehydroalanine, Dha) and threonines (to dehydrobutyrin, Dhb) and the nucleophilic attack of the electrophilic centers with neighboring nucleophilic groups (Figure 3) forming five intramolecular thioether rings. The thioether Lan is formed when the double bond in Dha is attacked by the thiol (-SH) group of a neighboring cysteine residue (Ingram 1970; Matteuzzi et al. 2004). MeLan is formed due to a similar reaction with Dhb, respectively. The intramolecular bridges formed in this reaction are called Lan rings (rings A-E in Figure 2). In addition, a number of other unusual amino acid -derived residues have been identified in other lantibiotics, e.g. in biosynthesis of epidermin and lactocin S (reviewed by McAuliffe et al. 2001).

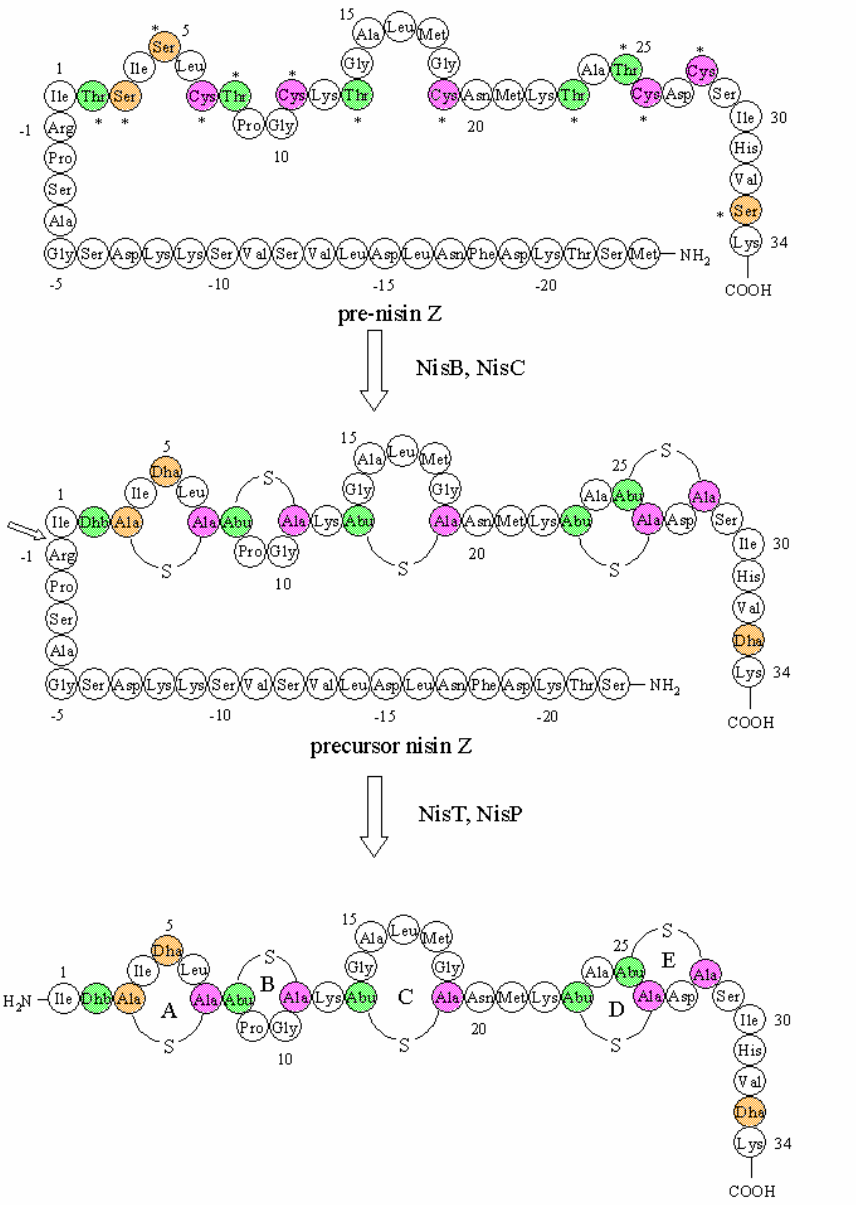

Figure 2. Schematic outline of the biosynthesis of nisin Z. Lanthionine rings are labeled A-E. Asterisks indicate residues that will be modified in post-translational reactions. The small white arrow indicates processing of the leader peptide by the action of NisP. Dha = dehydroalanine; Dhb = dehydrobutyrine; Ala-S-Ala = lanthionine, Abu-S-Ala = β-methyllanthionine. (Adapted from Kuipers et al. 1995).

The role of NisB and NisC in the biosynthesis of nisin and comparable proteins of other linear lantibiotics, such as subtilin, epidermin, gallidermin

and Pep5, has been reviewed by McAuliffe et al. (2001). Subtilin, epidermin and gallidermin share structural similarities with nisin. In addition to a functional analysis, a comparison of these genes with each other and with genes of a known function identifies two genes, *lanB* and *lanC*, found only in the gene clusters needed for the biosynthesis of lantibiotics. These genes potentially encode the membrane-associated enzymes involved in the unique reactions of lantibiotic biosynthesis, i.e. the dehydration of serines and threonines of the precursor molecule, leading to the formation of Dha and Dhb (Engelke et al. 1992; Kuipers et al. 1993; Siegers et al. 1996). Dehydrated amino acid residues and lanthionines or methyllanthionine rings in nisin are reported to be important for the activity (Kuipers et al. 1992; van Kraaij 2000) and essential for the inhibition of spore outgrowth in the case of nisin and subtilin (Liu and Hansen 1993; Chan et al. 1996).

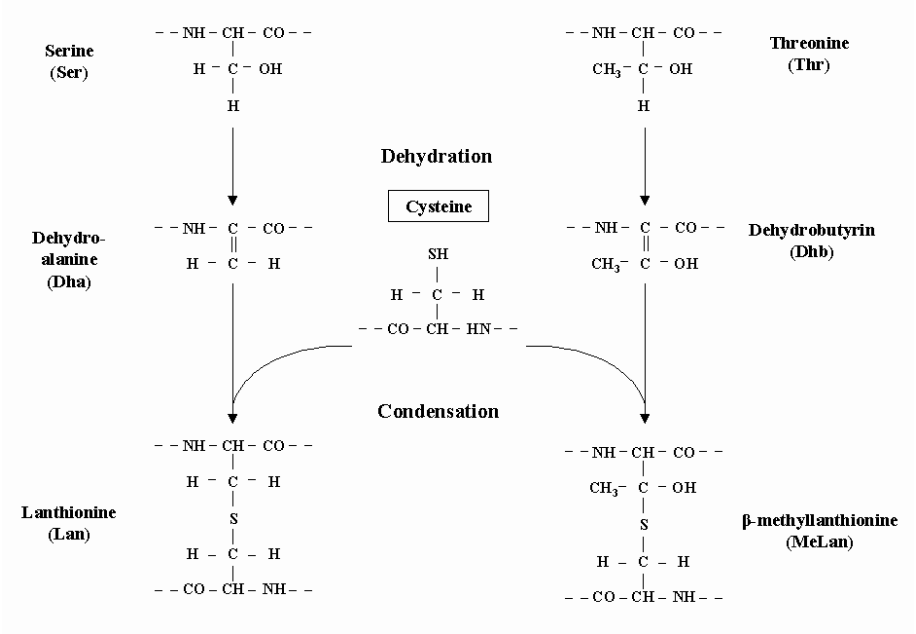

Figure 3. Mechanism for the synthesis of the unusual amino acids dehydroalanine, dehydrobutyrine, lanthionine and β-methyllanthionine. (Based on Ingram 1970).

Experimental evidence for the importance of *lanB* and *lanC* genes in the maturation process has been reported by Meyer et al. (1995). Pep5 precursors from PepB and PepC mutant strains were purified, and the analysis of these precursors showed that a lack of PepB activity resulted in a lack of dehydration, whereas a lack of PepC activity yielded secreted precursors that had been correctly dehydrated but contained only one lanthionine out of three. These results showed that PepC is not required for the dehydration reaction but seems to be involved in correct lanthionine formation. In the case of nisin, Sen et al. (1999) suggested that NisB affects the dehydration

reaction of the nisin precursor; over-expression of the *nisB* gene increased the efficiency of the dehydration reaction.

2.1.3 Biosynthesis – modification, regulation and immunity

The modification (dehydration and formation of the lantionines) is followed by translocation of pre-nisin and cleavage of the leader part. The fully modified nisin precursor is translocated across the membrane by the ABC transporter NisT (Qiao and Saris 1996). Siegers et al. (1996) have shown that NisB, NisC, NisT and pre-nisin form a protein complex which is bound to the cell membrane (Figure 4). The N-terminal leader sequence is cleaved from the 57-residue pre-nisin by an extracellular subtilisin-like serine protease (NisP) (van der Meer et al. 1993; Qiao et al. 1996), and the mature 34-residue nisin is released into the growth medium. The protein complex NisBCT together with the protease NisP is responsible for the modification, transport and processing of the pre-peptide in nisin biosynthesis.

The proteins NisR and NisK are reported to be involved in the regulation of nisin biosynthesis (van der Meer et al. 1993; Kuipers et al. 1993; Engelke et al. 1994; Immonen et al. 1995; Siegers and Entian 1995; Qiao et al. 1996) (Figure 4). Kuipers et al. (1993) have shown that the *nisA* transcription product is abolished when a 4-base-pair deletion is introduced into the structural *nisA* gene. However, the transcription is restored by adding a sub-inhibitory amount of nisin to the growth medium. The results indicated that fully modified nisin can induce the transcription of its own structural gene as well as of the downstream genes by limited read-through, via a two-component NisR/NisK system (Kuipers et al. 1993). The protein NisK, a histidine kinase, senses the presence of nisin in the growth medium and causes autophosphorylation (Engelke et al. 1994). The phosphate group is transferred to a response regulator NisR, which acts as a transcriptional activator, followed by mRNA synthesis and ribosomal synthesis of unmodified nisin and other biosynthetic proteins (van der Meer et al. 1993; Qiao et al. 1996).

Interestingly, Chandrapati and O'Sullivan (2002) showed that the induction of the *nisA* promoter can also occur independently of the NisRK system. They characterized the galactose-mediated induction by determining the *nisA* start site during growth in galactose, which was observed to be identical to the *nisA* start site upon nisin induction. Based on the results they also speculated that the Leloir pathway or some component of it plays a role in the induction of the *nisA* promoter (Chandrapati and O'Sullivan 2002).

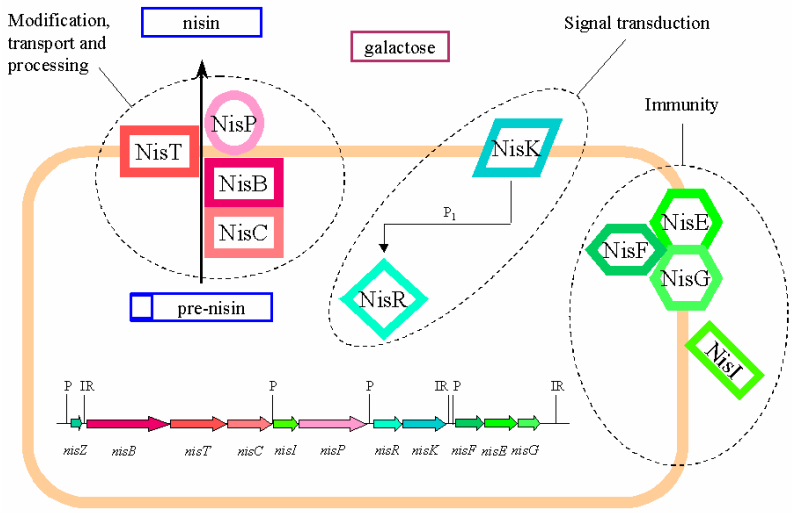

Figure 4. Proposed model for regulation of nisin production in *Lactococcus lactis*. (Modified from Quadri 2002; Immonen and Saris 1998).

The nisin-producing cells must necessarily be immune to the antimicrobial peptide they produce. To date, the mechanism by which these proteins confer immunity remains relatively unknown. However, two distinct systems of self-immunity have been identified: the membrane-associated lipoprotein NisI (Kuipers et al. 1993; Qiao et al. 1995) and the ABC-transport proteins NisFEG protect the cell from the bacteriocidal action of nisin (Siegers and Entian 1995; Dodd et al. 1996; Ra et al. 1999). NisI may have an important function for nisin immunity and evidently cooperates with *nisFEG* -encoded proteins to provide a high level of immunity (Ra et al. 1999). In addition to lipoprotein-form, a part of the produced NisI by *L. lactis* N8 cells is released as a lipid-free form (LF-NisI), which may be bound to nisin followed by diffusion into the medium (Koponen et al. 2004). LF-NisI enhances the activity of nisin against several indicator strains and may also have a role in protection against nisin (nisin immunity) (Koponen et al. 2004).

2.1.4 Antimicrobial mechanism

In general, the action of the bacteriocins produced by Gram-positive bacteria is directed primarily against other Gram-positive species. The lantibiotic nisin is bactericidal against a wide range of Gram-positive bacteria, including *Bacillus* (Liu and Hansen 1993), *Mycobacterium* (Montville et al. 1999), *Clostridium*, *Lactococcus*, *Listeria*, *Staphylococcus* and *Streptococcus* strains (reviewed by Cleveland et al. 2001). Further, nisin has been shown to prevent the outgrowth of spores of *Bacillus* (Liu and Hansen 1993) and *Clostridium* strains (Wessels et al. 1998). Under normal circumstances, bacteriocins produced by Gram-positive bacteria do not have a bactericidal effect on Gram-negative species. However, in some cases, activity against Gram-

negative cells, e.g. *Salmonella*, can be observed when the outer cell membrane is weakened or disrupted (Stevens et al. 1991).

The primary target for many of these small cationic peptides (type-A lantibiotics), e.g. nisin, is the cytoplasmic membrane of sensitive cells (Kordel and Sahl 1986). Nisin is suggested to form pores in the cytoplasmic membrane and, accordingly, to destroy the proton motive force (PMF) of the target cell (Bruno and Montville 1993) (Figure 5). The PMF, which is composed of a chemical component (pH gradient ΔpH), and an electrical component (membrane potential ΔΨ), drives ATP synthesis and the accumulation of ions and other metabolites through a PMF-driven transport system in the membrane. A collapse of the PMF, induced by bacteriocin action, causes membrane permeability, allows the leaking of small compounds (e.g. ATP, amino acids, ions) through the membrane, and leads to cell death (Bruno and Montville 1993; reviewed by McAuliffe et al. 2001). Molecules larger in size than 500 Da have not been observed to diffuse the membrane due to nisin treatment (Sahl et al. 1987; Garcera et al. 1993).

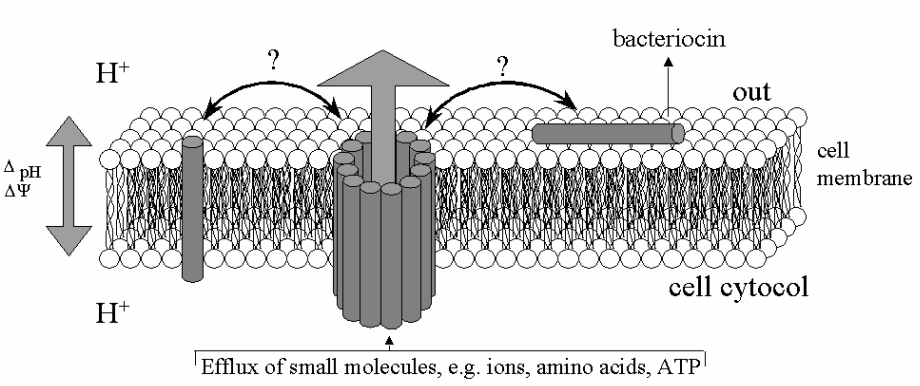

Figure 5. General model of the mechanism of pore formation by peptides (e.g. bacteriocin nisin). (Modified from Jack et al. 1997).

It has long been known that nisin inhibits peptidoglycan biosynthesis and that it interacts with cell wall precursors, either lipid I or lipid II (Reisinger et al. 1980; Jack et al. 1997). The degree of association is mainly dependent on the type of lipids present, especially the charge carried by those lipids. Later studies have indicated that nisin is capable of pore formation; nisin uses lipid II as an integral part of the pore formation process (Brötz et al. 1998). The activity of nisin is dependent on the concentration of lipid II (undecaprenyl-pyrophosphoryl-MurNAc-(pentapeptide)-GlcNAc) in the membrane of sensitive cells (Breukink et al. 1999; Breukink and de Kruijff 1999). Lipid II appears to be the sole docking molecule for the high-affinity binding of nisin (Breukink et al. 1999). In the absence of lipid II, significantly higher concentrations of nisin are required to form target-independent pores (Wiedemann et al. 2001). Due to the cationic properties of nisin, it is most

effective in the presence of a high percentage of anionic membrane lipids (Martin et al. 1996; Breukink et al. 1997). Further, while the binding of nisin to lipid II promotes pore formation, it can also independently block cell-wall biosynthesis (Chan et al. 1996; Breukink et al. 1999; Wiedemann et al. 2001).

2.2 Bioactive components in *Brassica* vegetables

2.2.1 Glucosinolates and their breakdown products

Brassica vegetables are widely used around the world as raw material for fermented vegetables (e.g. sauerkraut and kimchi). *Brassica* is a genus of a larger group of cruciferous plants (*Brassicaceae*, *Capparaceae*, *Resedaceae*) and comprises many commonly consumed species, e.g. white cabbage, Brussels sprouts, broccoli, cauliflower and kohlrabi. All cruciferous plants contain secondary metabolites called glucosinolates; over 100 different glucosinolate compounds have been identified (reviewed by Rosa et al. 1997). There are commonly 10 – 20 glucosinolates in *Brassica* vegetables, of which only a few have been investigated thoroughly. Glucosinolates are negatively charged sulfur-containing compounds derived from amino acids and usually occur in plants as potassium salts. All glucosinolates possess a common basic structure containing a β-D-thioglucose group, a sulfonated oxime moiety (C=N-OSO_3-) and a variable side chain (R) (Figure 6). The side chains are divided into three different classes (Table 3): aliphatic glucosinolates (sinigrin, glucoiberin, glucoraphanin) derived from methionine, indole glucosinolates (glucobrassicin, 4-OH-glucobrassicin, 4-MeO-glucobrassicin) derived from tryptophan, and aromatic glucosinolates (glucotropaeolin, gluconasturtin, sinalbin) derived from phenylalanine and tyrosine (reviewed by Rosa et al. 1997).

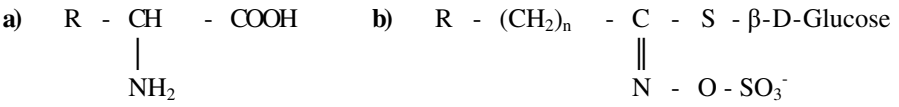

Figure 6. General structure of amino acids (a) and glucosinolates (b) (R = side chain).

Glucosinolate biosynthesis in plants has three important stages, namely carbon side-chain (R) elongation, formation of N-hydroxy amino acid, and chain modifications (reviewed by Halkier and Du 1997). The first step in the biosynthesis of glucosinolates from amino acids includes the elongation of the carbon side-chain, which is followed by the formation of hydroxy-amino acids from methionine, tryptophan, phenylalanine and tyrosine (or chain-elongated homologues thereof, e.g. homophenylalanine). Next, the hydroxy-amino acids are modified into aldoxime intermediates by the following enzymes: oxygen-demanding mono-oxygenases type cytochrome P450 and

FMO (mono-oxygenase bound to flavine) and peroxidase. Formation of the aldoxime is followed by conjugation reactions which introduce sulfur to form thiohydroximic acid (glucosylation; S-glucosyl transferase and sulfation; 3'-phosphoadenosine-5'-phosphosulfate). The last step involves the modification of side chains (R). Glucosinolate biosynthesis is controlled mainly at the beginning of the process by activating, substrate-specific enzymes and, thus, the quality of the forming glucosinolate is strongly influenced by that initiation stage (reviewed by Halkier and Du 1997). Besides substrate components, the formation of glucosinolates in the plant is dependent on the pH, the presence of ferro-ions and epithio-specific proteins, and other unknown factors (reviewed by Tookey et al. 1980).

Table 3. Glucosinolates commonly founds in cruciferous vegetables. (Fenwick et al. 1983a; Rosa et al. 1997)

Trivial name	Chemical name (R-side chain)	Major source [2]
Aliphatic glucosinolates		
Sinigrin	2-propenyl	a, c, d, e, f
Gluconapin	3-butenyl	a, b, c, d, e, f
Glucobrassicanapin	4-pentenyl	b, c, d
Glucoiberverin	3-methylthiopropyl	a, c, d, e, f
Glucoerucin	4-methylthiobutyl	a, c, d, e, f
Glucoiberin	3-methylsulfinylpropyl	a, c, d, e, f
Glucoraphanin	4-methylsulfinylbutyl	a, b, c, d, e, f
Glucoalyssin	5-methylsulfinylpentyl	b, e, f
Glucoerysolin	4-methylsulfonylbutyl	a, f
Gluconapoleiferin [1]	2-hydroxy-4-pentenyl	c, d
Progoitrin/epiprogoitrin [1]	2-hydroxy-3-butenyl	a, b, c, d, e, f
Aromatic glucosinolates		
Glucotropaeolin	Benzyl	a
Gluconasturtin	2-phenylethyl	b, c, d, e, f
(Gluco)sinalbin	p-hydroxybenzyl	g
Indolyl glucosinolates		
Glucobrassicin	3-indolylmethyl	b, c, d, e, f
Neo-glucobrassicin	1-methoxy-3-indolylmethyl	a, f
4-Methoxyglucobrassicin	4-methoxy-3-indolylmethyl	a, b, c, d, e, f
4-Hydroxyglucobrassicin	4-hydroxy-3-indolylmethyl	a, b, c, d, e, f

(1) Glucosinolates yielding goitrogenic oxazolidine-2-thiones on myrosinase treatment
(2) a = cabbage, b = Chinese cabbage, c = cauliflower, d = broccoli, e = Brussel sprouts, f = white cabbage, g = white mustard

The content of glucosinolates in plants varies between cultivars, plant individuals and part of the plants, due to factors such as genetics, environment and plant nutrients. The content of glucosinolates in *Brassica* vegetables has been reviewed extensively by Rosa et al. (1997). Glucosinolates can be found in the roots, seeds, leaf and stem of the plant, while the youngest tissues contain the highest amount. Glucosinolates have several functions in the plant: plant defense against fungal diseases and pest infestation, sulfur and nitrogen metabolism, and growth regulation (reviewed by Bones 1990; Koritsas et al. 1991; Birch et al. 1992). They may also play a role in plant defense against the effects of stress from extreme temperatures in cold or warm climates (Ludvick-Müller et al. 2000), and may enhance plant seedling (Kelly et al. 1998). The flavor and odor typical of *Brassica* vegetables is mainly due to the glucosinolates and their BDPs, such as volatile isothiocyanates, thiocyanates and nitriles (Heaney and Fenwick 1995; Kassahun et al. 1996; van Doorn et al. 1998; Kim et al. 2002). Isothiocyanates are known to be pungent and goitrin contributes to bitterness (Fenwick et al. 1983b; van Doorn et al. 1998). According to van Doorn et al. (1998), consumers preferred Brussels sprouts with a low sinigrin and progoitrin content. The presence of a high content of sinigrin in white cabbage caused a pungent and bitter flavor which was stronger near the heart of the cabbage than in the outer leaves (van Doorn et al. 1998). The flavor and odor can be influenced by processing; most nitriles (e.g. allyl-, 3-butenyl- and 3-methylthiopropyl cyanides), for example, are volatile and can be minimized or completely lost by cooking (Kassahun et al. 1996).

The nature and level of glucosinolates vary in different plant species, with seed containing up to 5% and leaf tissue as little as 0.1% of fresh weight (FW). The molarity of glucosinolates in plants is relatively small (1 - 250 μmol g^{-1}), depending on the compound and the species. However, the concentration can be even 10% of plant dry weight (DW) (Rosa et al. 1997). The total glucosinolate content in fresh white cabbage is reported to vary between 10.9 and 21.4 μmol g^{-1} DW (Table 4) (Tiedink et al. 1988; Kushad et al. 1999; Verkerk 2002). Reported levels of glucosinolate content in fresh vegetables vary considerably in different studies and even in the same study among parallel samples or cabbage heads. The differences may be partly due to different analysis methods and environmental and agronomic factors such as soil type, plant spacing, light, temperature, and application of sulfate and nitrogenous fertilizer (Fenwick et al. 1983a; Rosa 1997; Kushad et al. 1999). Identical cultivars may differ in their total glucosinolate content up to 200% due to these growth condition effects (Kushad et al. 1999). Table 4 presents the glucosinolates typically found in white cabbage.

Table 4. Glucosinolate content in fresh white cabbage (concentrations in μmol g^{-1} DW).

Glucosinolate	Tiedink et al. 1988 Mean (Range)	Kushad et al. 1999 Mean (Range)	Verkerk 2002 Mean (Range)
Sinigrin	4.2 (nd)	7.8 (7.6-7.9)	9.6 ± 2.6
Glucoiberin	6.8 (nd)	0.0 (0.0)	5.4 ± 0.1
Glucobrassicin	3.4 (nd)	0.9 (0.8-1.0)	2.9 ± 0.0
Progoitrin	0.2 (nd)	0.2 (0-0.4)	1.1 ± 0.1
Glucoraphanin	0.2 (nd)	0.1 (0-0.7)	0.04 ± 0.0
Gluconapin	-	0.7 (0.3-1.1)	1.3 ± 0.1
4-OH-Glucobrassicin	-	0.3 (0.0)	0.5 ± 0.1
4-MeO-Glucobrassicin	0.4 (nd)	0.3 (0.2-0.4)	0.5 ± 0.1
Total	15.2	10.9	21.4

nd; not determined

Glucosinolates are degraded and decomposed by the enzyme myrosinase (a thioglucosidase; thioglucoside glucohydrolase, EC 3.2.3.1), which is stored with glucosinolates either separately in plant tissues (Kelly et al. 1998) or in the same cell in myrosin grains (Bones and Rossiter 1996). When the plant cells are damaged or wounded (e.g. by cutting, chewing or cooking) myrosinase is released and activated, causing enzymatic hydrolysis of glucosinolates and formation of BDPs (Figure 7; Table 5). The degradation of glucosinolates and the rate of formation of breakdown products (BDPs) are affected by the pH and depend on the amount of glucosinolate substrate, temperature and pressure, and presence of enzyme-inhibitors and activators, such as ascorbic acid and metal ions (Wilkinson et al. 1984; Ludikhuyze et al. 2000).

Glucosinolate degradation may also occur as a result of long storage, e.g. freezing and refrigerating (reviewed by Verkerk 2002), fermentation (Daxenbichler et al. 1980), cooking (Rosa and Heaney 1993), and pulping (de Vos and Blijleven 1988; Verkerk et al. 2001). On the other hand, processing and storage may enhance the production of some glucosinolates (Rodrigues and Rosa 1999; Verkerk et al. 2001). Verkerk et al. (2001) found that chopping and storage of *Brassica* vegetables in air raised the amount of indolyl glucosinolates. For example, 4-methoxyglucobrassicin increased 15-fold in white cabbage during storage (48 h). Similarly, the amount of 4-hydroxyglucobrassicin grew unexpectedly when broccoli was storaged at room temperature (Rodrigues and Rosa 1999). A possible explanation for these observations may be that chopping the cabbage initiates a defense mechanism – i.e. the formation of glucosinolates – which also occurs due to wounding by insects or slugs (Koritsas et al. 1991; Birch et al. 1992).

Table 5. Hydrolytic products of different glucosinolates present in *Brassica* vegetables [Collected from Bradfield and Bjeldanes 1987; Bones and Rossiter 1996; Kassahun et al. 1996; Kushad et al. 1999) (N = nitrile; ITC = isothiocyanate)].

Glucosinolate	Breakdown products
Aliphatic glucosinolates	
Sinigrin	allyl-ITC
	allyl-cyanide, N
	1-cyano-2,3-epithiopropane, N
	2-propenyl-ITC
	thiocyanates
Gluconapin	3-butenyl-ITC
	1-cyano-3,4-thiobutane
Glucoerucin	4-methylthiobutyl-ITC
	4-methylthiobutanenitrile, N
	thiocyanates
Glucoiberin	3-methylsulphinylpropyl-ITC (iberin)
	1-cyano-3-methylsulphinylpropane, N (iberin nitrile)
	3-methylsulfinylpropyl cyanide
Glucoraphanin	4-methylsulfinylbutyl-ITC (sulforaphane)
	5-(methylsulfinyl)-pentane nitrile, N (sulphoraphane nitrile)
Progoitrin	1-cyano-2-hydroxy-3-butene, N
	1-cyano-3,4-epi-thiobutane, N
	1-cyano-2-(S)-hydroxy-3(R)(S)-epi-thiobutane, N
	5-vinyloxazolidine-2-thione (goitrin)
Indolyl glucosinolates	
Glucobrassicin	Indole-3-carbinol
	ascorbigen
	3-indoleacetonitrile, N
	thiocyanates
Neo-glucobrassicin	indoles and thiocyanates (*)
4-OH-Glucobrassicin	indoles and thiocyanates (*)
4-MeO-Glucobrassicin	indoles and thiocyanates (*)

*) as in the case of glucobrassicin, via intermediates

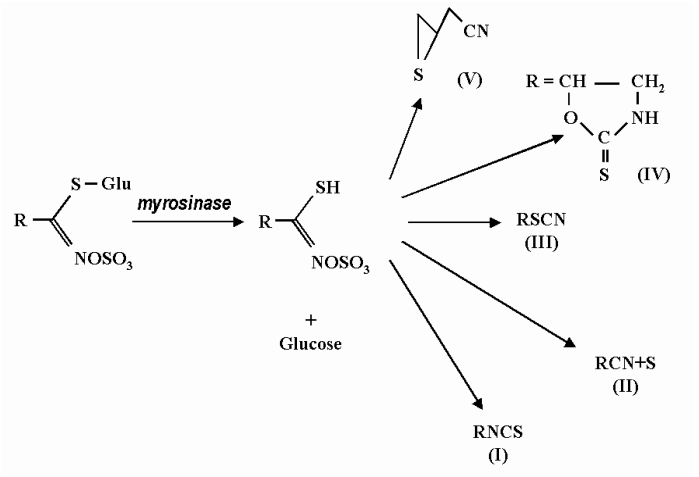

Figure 7. Degradation of glucosinolates. After hydrolysis catalysed by myrosinase, the subsequent rearrangement of the unstable intermediate in dependent on side chains and hydrolysis conditions. The major degradation products are isothiocyanates (I), which are formed at pH>7, and nitriles (II), which are formed at pH<4. 2-propenyl-, benzyl- and 4-(methylthio)-butylglucosinolates form thiocyanates (III). The presence of beta-hydroxylated side chains results in spontaneous cyclization to produce oxazolidine-2-thiones (IV). A terminal double bond in the side chain results in the formation of an epithionitrile (V), if an epithio-specified protein and Fe2+ ions are present. Abbreviations: Glu = glucose, R = variable side chain (Halkier and Du 1997).

Biochemically, the enzyme-catalyzed hydrolysis of glucosinolates initially involves cleavage of the thioglucoside linkage, yielding D-glucose and an unstable thio-hydroximate-O-sulfonate which spontaneously rearranges (reviewed Halkier and Du 1997) (Figure 7). Aliphatic glucosinolates are degraded to isothiocyanates (ITC) at close-to-neutral pH (pH5 – pH7). Indolyl glucosinolates firstly yield an unstable indolyl-ITC, and further degrade with a loss of sulfur to form indole-3-carbinol (I3C) and related compounds; I3C may form adducts with ascorbic acid to generate ascorbigen, or it can undergo self-condensation reactions resulting in the production of di-indolylmethane and indolocarbazole. A low pH enhances the forming of nitriles rather than isothiocyanates; progoitrin is hydrolyzed to nitrile 1-cyano-2-hydroxybut-3-ene (Tookey et al. 1980), glucobrassicin to 3-indoleacetonitrile (Searle et al. 1982), glucoraphanin to sulforaphane nitrile (Kushad et al. 1999); and sinigrin to allyl-cyanide (Kassahun et al. 1996). Kassahun et al. (1996) found a high concentration (209 – 1551 mg^{-1} kg DW) of allyl-nitrile in commercial sauerkraut after treatment (cooking) of the samples in acetic acid or in water. Further, ferrous ions depress isothiocyanate formation under acidic conditions, but not at neutral pH. Thiocyanate ions can be formed during hydrolysis of allyl-, benzyl- and 4-methylthiobutyl glucosinolates (reviewed by Halkier and Du 1997). ITCs

may further react with amines, aminoacids, alcohols and thiols to form their corresponding thioureas, thio hydantoins, thiocarbamates and dithiocarbamates (Ashworth 1975). Amines and amides, in turn, can further react with nitrite, forming carcinogenic N-nitroso compounds in acidic conditions (Tiedink et al. 1988; Walters 1992).

2.2.1.1 Effect of processing and storage on glucosinolates

As mentioned above, any process that disrupts the cellular structure (e.g. cutting, chewing, cooking, fermenting or freezing) of *Brassica* vegetables results in glucosinolate hydrolysis or thermal degradation and formation of several BDPs (Rosa and Heaney 1993; Yen and Wei 1993; Verkerk et al. 1997; Ciska and Kozlowska 2001). Although *Brassica* vegetables are usually eaten fresh, most are processed in some way, e.g. cut, blanched or cooked, before being consumed.

Cooking influences the glucosinolates and their hydrolysis products; water-soluble compounds leach into the cooking water (Rosa and Heaney 1993; Kassahun et al. 1996; Ciska and Kozlowska 2001) and myrosinase activity decreases due to thermal inactivation (Ludikhuyze et al. 1999; Verkerk 2002). Reported optimal temperatures for myrosinase activity vary from 40ºC (Verkerk 2002) to 60 ºC (Yen and Wei 1993). Cooking of cabbage leaves has been found to reduce the content of glucosinolates by more than 50% (Rosa and Heaney 1993). For example, Howard et al. (1997) detected no glucosinolates or their BDPs in steam-blanched broccoli. Verkerk (2002), however, found that microwave treatment of vegetables led to an exceptionally high total concentration of glucosinolates. One reason for this may have been the absence of leaching of glucosinolates into the cooking water, which occurs in conventional cooking of vegetables (Verkerk 2002).

Both the glucosinolates and their hydrolysis products in fresh broccoli or cabbage are degraded or decomposed during storage due to spontaneous autolysis (Howard et al. 1997; Rangkadilok et al. 2002; Verkerk 2002). Storing at 20 ºC resulted in a 55% loss of glucoraphanin concentration during the first 3 days in broccoli stored in open boxes and a 56% loss by day 7 in broccoli stored in plastic bags (Rangkadilok et al. 2002). The concentration decreased less in a modified atmosphere (Rangkadilok et al. 2002). Frozen storage has been shown to significantly reduce the level of alkyl ITC, but increase the levels of alkyl cyanide (Valette et al. 2003).

Glucosinolates are also affected by the cabbage fermentation process (sauerkraut production); they are totally degraded and several BDPs are formed (Daxenbichler et al. 1980; Tolonen et al. 2000). Gail-Eller and Gierscher (1984) demonstrated that the fermentation temperature also affects the degradation of glucosinolates: at 19 ºC the glucosinolates were degraded totally in three days, whereas at 5 ºC in eight days. Tolonen et al. (2000) used

a temperature of 20 ºC in cabbage fermentation, whereas Daxenbichler et al. (1980) did not mention the temperature or their other process parameters. Further, ionic radiation before the fermentation of white cabbage has been speculated to enhance glucosinolate degradation (Gail-Eller and Gierscher 1984).

Overall, food processing is likely to affect the glucosinolates for the following reasons: partial or complete inactivation of myrosinase; heat degradation of glucosinolates and BDPs; enzymatic breakdown of glucosinolates; loss of enzymatic co-factors (ascorbic acid, iron); and leaching of glucosinolates, BDPs and myrosinase into the cooking water (reviewed by Verkerk 2002). It is evident that glucosinolates are also decomposed in industrial vegetable processing, which involves more or less extensive handlings, like washing, cutting, blanching, addition of chemicals, drying, fermenting, freezing, canning, and sterilizing (reviewed by de Vos and Blijleven 1988; Verkerk 2002).

2.2.1.2 Role of microbiota

Many studies indicate that specific microbiota, including LAB, possess myrosinase-like activity (Tani et al. 1974; Nugon-Baudon et al. 1990; Smits et al. 1993; Palop et al. 1995; Elfoul et al. 2001; Krul et al. 2002; Cheng et al. 2004). On the other hand, Gail-Eller and Gierscher (1984) reported that the addition of a LAB starter culture in sauerkraut processing had no effect on the degradation of glucosinolates. Table 6 summarizes the results of glucosinolate degradation by microbiota.

The enzyme myrosinase has been identified and cloned from plant sources (Xue et al. 1992) and, more recently, from the microbes *Caulobacter crescentus* (NC 002696) and *Escherichia coli* O157:H7 EDL933 (NC 002655) (Perna et al. 2001). The strains *Lactobacillus acidophilus* (Nugon-Baudon et al. 1990), *Lb. agilis* (Palop et al. 1995), *Bacteroides thetaiotaomicron* (Elfoul et al. 2001) and *Bifidobacterium* sp. (Cheng et al. 2004) are able to degrade glucosinolates and to form BDPs such as isothiocyanates (ITC) *in vivo* or *in vitro* (Table 6). The microbes *Aspergillus niger, A. clavatus* (Smits et al. 1993), *Fusarium oxysporium* and *Enterobacter cloacae* (Tani et al. 1974) have also been reported to possess myrosinase-like activity *in vitro*.

Table 6. Degradation of glucosinolates by members of the microbiota. AITC; allyl-ITC, BzITC; benzyl-ITC, BCN; 3-butene-nitrile, PhACN; phenyl-acetonitrile.

Strain	Glucosino-late substrate	Detected breakdown products	Degra-dation % (A)	Incubation time (days); *in vivo*/ *in vitro*	Source
Lb. acidophilus	sinigrin	AITC	13-28	5 d, *in vitro*	B
Lb. agilis	sinigrin	AITC	100	1 d, *in vitro*	C
Bacteroides thetaiotaomicron	sinigrin	AITC	13	*in vivo*	D
Bifidobacterium pseudocatenulatum	Sinigrin, glucotro-paeolin	AITC		1-2 d, *in vitro*	E
B. adolescentis	Sinigrin, glucotro-paeolin	BCN, PhACN AITC, BzITC		1-2 d, *in vitro*	E
B. longum	Sinigrin, glucotro-paeolin	BCN, PhACN AITC, BzITC		1-2 d, *in vitro*	E

A) of initial glucosinolate
B) Nugon-Baudon et al. 1990
C) Palop et al. 1995
D) Elfoul et al. 2001
E) Cheng et al. 2004

The degradation potential of strains of Bifidobacterium sp. have been tested *in vitro* in a large-intestinal model by Cheng et al. (2004) (Table 6). They tested the degradation of glucosinolates using the *Bifidobacterium* growth medium and a cell-free cytocol extract as a source of myrosinase-like activity. AITCs were the main degradation products at close-to-neutral pH, whereas acidic conditions enhanced the production of nitriles. Cheng et al. (2004) concluded that *Bifidobacterium* sp. may digest glucosinolates to form organic nitriles in the human digestive tract due to a low pH in the intestine.

2.2.1.3 Potential health effects of glucosinolates and their breakdown products

Epidemiological investigations since the 1960s have indicated that some cancers, particularly cancers of the colon, rectum and thyroid, occur less frequently in individuals who regularly consume *Brassica* vegetables, like Brussels sprouts, cabbage, cauliflower, and broccoli (Manousos et al. 1983; Voorrips et al. 2000; Seow et al. 2002; Fowke et al. 2003). Wattenberg's (1978) study led to the identification of sulfur-containing compounds (glucosinolates, isothiocyanates, dithiolthiones, indoles, sulfonates, and vitamins) in cole crops that provide protection against cancer. Evidence for the anticarcinogenic effects of *Brassica* vegetables in humans is strongly supported by evidence obtained with experimental animal models (Grubbs et al. 1995; Oganesian et al. 1999; Hecht 1999, 2000; Fahey et al. 2002;

Munday and Munday 2002) or experiments with cell lines *in vitro* (Leoni et al. 1997; Bonnesen et al. 2001; Brandi et al. 2003; Nachshon-Kedmi et al. 2003). ITCs have been found to reduce breast cancer among Chinese women (Fowke et al. 2003), risk of lung cancer among cigarette-smoking men (London et al. 2000), and incidence of bladder cancer (Munday and Munday 2002). Sulforaphane may prevent stomach tumors and inhibit *Helicobacter pylori* bacteria (Fahey et al. 2002). Dietary I3C has been documented to inhibit tumorigenesis in various target organs – including mammary tissue (Grubbs et al. 1995), liver (Oganesian et al. 1999), prostate (Nachshon-Kedmi et al. 2003), and breast (Ju et al. 2000; Brandi et al. 2003).

Table 7. Glucosinolates and breakdown products suggested possess potential anti-carcinogenic properties. (Source: Talalay and Zhang 1996). (-NCS- basic structure of glucosinolate).

Protective isothiocyanates
α-naphthyl-NCS, β-naphthyl-NCS
phenyl-$[CH_2]_n$-NCS, where n = 0, 1, 2, 3, 4, 5, 6, 8, 10 (=2-phenylethyl, from gluconasturtin)
$PhCH(Ph)CH_2$-NCS, $PhCH_2CH(Ph)$-NCS (=2-phenylethyl, from gluconasturtin)
$CH_3[CH]_n$-NCS, where n = 5, 11
$CH_3[CH_2]_3CH(CH_3)$-NCS
Sulphoraphane from glucoraphanin, $CH_3S(O)[CH_2]$4-NCS2-Acetylnorbornyl-NCS (3 isomers)
Protective glucosinolates
Indolylmethyl glucosinolate (glucobrassicin)
Benzyl glucosinolate (glucotropaeolin)
4-hydroxybenzyl glucosinolate (glucosinalbin)
Carcinogens *(substance causing tumour in target organ)*
3'-Methyl-4-dimethylaminoazobenzene
4-Dimethylaminoazobenzene
N-2-Fluorenylacetamide, acetylaminofluorence
7,12-Dimethylbenz[a]antracene (DMBA)
Benzo[a]pyrene
Methylazoxymethanol acetate
N-nitrosodietylamine
4-(Methylnitroamino)-1-(3-pyridyl)-1-butanone (NNK)
N-nitrosobenzylmethylamine (NBMA)
N-butyl-N-(4-hydroxybutyl)nitrosamine
Tumour target organs
Rat: liver, lung, mammary gland, bladder, small intestine/colon, oesophagus
Mouse: lung, forestomach

Biochemically, glucosinolate BDPs, indoles and isothiocyanates have been reported to prevent cancers and to modulate biotransformation enzyme activity by several mechanisms (reviewed by Lampe and Peterson 2002). Isothiocyanates (ITC) and indole-3-carbinol (I3C) are considered to modulate phase-I and phase-II biotransformation enzyme activity, and thus possibly to influence the processes related to chemical carcinogenesis, such as the metabolism, DNA-binding and mutagenic activity of promutagens. Phase-I enzymes (e.g. cytochrome P450 enzymes and flavin-dependent mono-oxygenases) involve oxidation, reduction and hydrolysis reactions, thereby making xenobiotics more hydrophilic as well as susceptible to detoxification. Phase-I enzymes may also enhance the bioactivation of a number of carcinogens, like benzo[a]pyrene and aflatoxin B1. Phase-II enzymes (e.g. quinone-reductases, glutathione-S-transferases and sulfotransferases) primarily catalyze conjugation reactions which make phase-I metabolites more polar and readily excretable. These enzymes can, at least partly, alter the toxicity, mutagenicity and tumorigenicity of specific chemicals (Table 7).

Some ITCs induce phase-I enzymes, others only phase-II enzymes, and some induce both. Sulforaphane, the ITC of glucoraphanin, is the most potent naturally occurring inducer of phase-II enzymes (Fahey and Talalay 1999; Pereira et al. 2002). The induction of phase-II enzymes has been shown to protect cells against toxic electrophiles (Zhang and Talalay 1994; Hecht 1999). Phenethyl-ITC (from gluconasturtin) prevents malignancies and kills cancer cells by modifying the cellular metabolism so as to inhibit phase-I enzymes and promote phase-II enzymes (Hecht 1999). Furthermore, I3C and ITC are reported to cause additive induction of detoxifying enzymes when used in combination (Staack et al. 1998; Bonnesen et al. 2001).

Besides their beneficial effects, *Brassica*s, glucosinolates and their derivatives have also shown adverse effects including mutagenicity, carcinogenicity, toxicity, and goitrogenicity (Talalay and Zhang 1996; reviewed by Verhoeven et al. 1997). Many feeds containing a high concentration of rape or crambe seeds (high in glucosinolate concentrations) have been shown to decrease feed intake and growth rate, and to cause goitrogenity, enlarged livers, kidneys, and thyroid and adrenal glands among different animal species (rat, mouse, trout) (Table 8). This may be due to a too high concentration of glucosinolates and their derivatives in the diet. Goitrin and AITC may cause enlargement of the thyroid gland (rewieved by Verhoeven et al. 1997), AITC and indole-3-carbinol mutagenicity and carcinogenicity in animal models and, in the case of goitrin, also among humans. Furthermore, indoles, which are the BDPs of indolyl glucosinolates, can be easily nitrosated by nitrite, forming carcinogenic N-nitroso compounds (Yang et al. 1984; Walters 1992; reviewed by Jongen 1996). However, the nitrosated products are stable only in the presence of large amounts of free nitrite (Jongen 1996). Some genotoxic effects were also observed in bacterial gene mutation assays when cells were treated with

phenethyl-ITC and especially with allyl-ITC (Kassie and Knasmuller 2000). However, no kinds of toxic effects of ITCs have been observed in humans. It is difficult to extrapolate the results of animal studies to humans, since the dose of both the anticarcinogenic and the carcinogenic compounds used in animal studies often far exceed the estimated level of the compound in a normal human diet. Furthermore, the release and adsorption of these compounds in the intestinal tract may be complex and remains unclear.

Table 8. Toxity of hydrolytic products of glucosinolates present in *Brassica* vegetables. (Reviewed by Verhoeven et al. 1997).

Glucosinol.	Product	Animal	Dose	Effect (*
Sinigrin	Allyl-ITC	Rat	2-4 mg (oral, 60 days)	1, 2, 3, 9
Gluconapin	1-cyano-3,4,-thiobutane	Rat	LD_{50} (sc) = 109 mg kg^{-1} (b.w.)	2, 3
Glucoiberin	3-methylsulphinyl-ITC	Rat	LD_{50} (sc) = 90 mg kg^{-1} b.w.	2, 3
Progoitrin	a) 1-cyano-2-(S)-hydroxy-3-butene (N)	Rat	LD_{50} (sc) = 109 mg kg^{-1} b.w.	4, 5
		Rat	200 mg (oral, 4 days)	6
		Mouse	LD_{50} (oral) = 170 mg kg^{-1} b.w.	
	b) 1-cyano-2-(S)-hydroxy-3(R)(S)-epi-thiobutanes (N)	Rat	75-300 ppm in diet (90 days)	5, 7
		Mouse	LD_{50} (oral) = 178-240 mg kg^{-1} b.w.	
	c)1-cyano-3,4-epi-thiobutane (N)	Rat	125 mg day^{-1} (oral, 3 days)	7, 8
	d) goitrin (= 5-vinyloxyazolidinethione)	Human	50-200 mg (oral)	9
		Rat	0.23% in diet (90 days)	1
		Mouse	50 µg (oral)	1
		Mouse	LD_{50} (oral) = 1260-1415 mg kg^{-1} b.w.	
Gluco-brassicin	Indole-3-carbinol	Trout	0.1% in diet	10
		Rat	0.1% in diet	11

b.w. = body weight; N = nitrile; LD50 = 50% lethality
*) Toxicological effects: 1 = thyroid enlargement, goitre; 2 = embryonal deaths; 3 = decreased foetal weights; 4 = adrenal enlargement; 5 = hepatic lesions/necrosis; 6 = pancreatic lesions; 7 = renal lesions; 8 = lesions of the fore stomach; 9 = inhibition of iodine uptake of thyroid; 10 = cancer promoter after aflatoxin B1 initiation; 11 = cancer promoter after dimethylhydrazine initiation

2.2.2 Flavonoids

Flavonoids (e.g. quercetin, kaempferol, catechins, antocyanins) are the most common group of plant polyphenols and provide much of the color and flavor to fruits and vegetables (reviewed by Ross and Kasum 2002). More than 4000 flavonoids are known and they have been divided into at least 12 subclasses according to their molecular structure. Polyphenols, found in the woody and external parts of plants, are involved in plant growth and reproduction, provide resistance to pathogens and predators, and protect crops from disease and preharvest seed germination (Bravo 1998). Some flavonoids (catechins, quercetin, hesperetin) are known to possess antifungal (Goetz et al. 1999), antiviral (Malhotra et al. 1996), and antimicrobial activity (reviewed by Murphy Covan 1999). Recently, interest in the possible health benefits of polyphenols, particularly flavonoids, has increased owing to their antioxidant and bioactivity properties (Madhavi et al. 1998; reviewed by Ross and Kasum 2002).

*Brassica*ceae plants are reported to be a weak source of flavonols and flavonoids, which are commonly found in concentrations below 10 mg $100g^{-1}$ (Herrmann 1988). However, quercetin and kaempferol have occurred in concentrations of 110 mg kg^{-1} and 200 mg kg^{-1} FW in white cabbage, and kaempferol in a concentration of 60 mg kg^{-1} FW in broccoli (Chu et al. 2000). In studies by Hertog et al. (1992a), fermented cabbage contained below 1 mg kg^{-1} FW of quercetin and below 2 mg kg^{-1} FW kaempferol. The concentration of flavonoids is known to be generally much higher in the leaves than in other tissues of the same plant; the flavonol concentration decreases significantly from the outer to the inner leaves in cabbage (Herrmann 1988). Flavonoid content in plants is affected by environmental factors (e.g. light, temperature) (Hietaniemi et al. (1999), agronomic factor (e.g. fertilizers, soil humidity) and genetic factors, (e.g. specie, subspecie, part of plant) (reviewed by Keskitalo 2001).

Processing and storage have a reported influence on flavonoids; flavonoids in berries were found susceptible to losses during storage of nine months (Häkkinen et al. 2000). Moreover, when the berries were crushed and storaged overnight at +4 ˚C - +6 ˚C, the concentration of quercetin decreased, argued as being due to the chemical and enzymatic reactions that occur when the cell membrane of berries is destroyed (Häkkinen et al. 2000). It has also been shown that flavonoids mainly remain in the plant fiber matrix and are not transferred into berry or fruit juice (van der Sluis et al. 1999; Häkkinen et al. 2000). Flavonoids may also be influenced by gastrointestinal microbiota (Aura et al. 2002). The decomposition of flavonoid quercetin was tested *in vitro* in an anaerobic fecal fermentation model; biologically active phytoestrogens – precursors of hormone-like compounds in the mammalian system – could rapidly deconjugate quercetin derivatives to hydroxyphenylacetic acid by microbiota. It was concluded that colonic

microbiota have potential to transform flavonoids into lower-molecular-weight phenolics, which have been reported to have beneficial health effects, e.g. by reducing the occurrence of cardiovascular disease and cancer (reviewed by Ross and Kasum 2002).

2.3 Lactic acid bacteria (LAB) fermentations

2.3.1 General

Fermentation has been used as a method for the preservation of food and beverages for thousands of years (reviewed by Ross et al. 2002). The development of large-scale fermentation processes has led to commercial production of fermented foods and alcoholic beverages, with the most widely used microorganisms, such as yeast for the production of beer, wine and spirits, and lactic acid bacteria (LAB) for a variety of dairy, vegetable, and meat fermentations (Table 9). The raw material for fermentation provides the substrates for the metabolites necessary for the extension of food shelf-life and for high quality of the end-products.

The fermentation process involves the oxidation of carbohydrates to generate organic acids, alcohol and carbon dioxide, as well as the flavor compounds diacetyl and acetaldehyde (reviewed by Caplice and Fitzgerald 1999). Compounds that may have positive health implications – including vitamins, antioxidants, and bioactive peptides – are also formed in fermentation.

LAB can be subdivided into two distinct groups based on their carbohydrate metabolism (reviewed by Caplice and Fitzgerald 1999). The homo-fermentative group, comprising *Lactococcus*, *Pediococcus*, *Enterococcus*, *Streptococcus* and some lactobacilli, use the Embden-Mayerhof-Parnas pathway to generate 2 mol of lactate per mole of glucose and derive approximately double the amount of energy per mole of glucose compared to heterofermenters. Heterofermentative bacteria produce equal amounts of lactate, acetate, carbon dioxide, and ethanol from glucose via the hexose monophosphate or pentose pathway. Members of this group include *Leuconostoc*, *Weissella* and some lactobacilli.

Table 9. Examples of fermented food products and starter organisms used in food fermentations. (Collected from Hammes 1991; Caplice and Fitzgerald 1999; Ross et al. 2002; Barrangou et al. 2002; Choi et al. 2003)

Fermented product	Starter strain
Dairy products	
Yogurt	*Lactobacillus delbruckii* sp. *bulgaricus, Streptococcus thermophilus*
Cheese	*Lactococcus lactis* sp. *lactis, L. lactis* sp. *cremoris, Propionibacterium* sp., *Lb. delbruckii* sp. *bulgaricus, Carnobacterium piscicola, Brevibacterium linens, S. thermophilus, Lb. delbruckii, Lb. helveticus*
Sour milk	*Lb. acidophilus, Lb. kefir, Lb. delbruckii* sp. *bulgaricus*, *Lactococcus* sp., probiotic bacteria
Vegetables	
Cabbage (sauerkraut)	*Leuconostoc mesenteroides, L. fallax, Pediococcus pentosaceus, P. acidilactici, Lb. plantarum, Lb. sake, Lb. cellobiosus, Lb. brevis, Lb. curvatus*
Kimchi	*Lactobacillus* sp.*, Leuconostoc citreum*
Olives	*L. mesenteroides, Lb. plantarum*
Pickles	*Lb. plantarum, P. cerevisiae* (*P. pentosaceus*)
Meat products	
Sausage	*Lb. curvatus, Lb. sake*
Beverages	
Wine	*Leuconostoc oenos*
Beer	*Saccaromyces cerevisiae*, LAB
Cereals	
Sour dough	*Lb. sanfrancisco, Lb. brevis, Lb. fructivorans, Lb. fermentum*
White bread	*Saccaromyces cerevisiae*, LAB

2.3.2 Cabbage fermentation - sauerkraut processing and microbiology

Sauerkraut, the most common fermented *Brassica* product, can be manufactured from a variety of cabbage (*Brassica oleracea*) cultivars, e.g. Lennox. Harris (1998) has summarized the manufacturing process as follows: before processing, the cabbage heads are trimmed to remove outer green leaves and the core, and sliced into fine shreds. The raw material is mixed, weighed, dry-salted, either inoculated (starter) or not inoculated (spontaneous), and packed into tanks for unaerobic fermentation. Once the tank has been filled to the proper level its top is usually covered with a sheet of plastic. Brine begins to form as soon as the cabbage is salted, as a result of

the osmotic extraction of water from the cut cabbage tissue. In a relatively short time, conditions within the tank become anaerobic due to the depletion of oxygen and production of carbon dioxide by the growing lactic acid bacteria. Fermentation is usually allowed to proceed for as little as a few weeks to as long as a year before packaging (reviewed by Harris 1998).

The ideal temperature and salt (NaCl) concentration for sauerkraut fermentation have been reported to be approximately 18 ºC and 1.8 - 2.25% NaCl, respectively (Pederson and Albury 1969). The desirable sequence of lactic acid bacteria is initiated at or near this temperature and salt concentration. The typical concentration of fermentable sugars in the cabbage raw material is about 5%, with approximately equal concentrations of fructose and glucose (2.5%) and a smaller amount of sucrose (Haila et al. 1992; Rosa et al. 2001).

At the beginning of fermentation, once brine has covered the salted cabbage, spontaneous fermentation begins; the first stage is called the gaseous phase and is initiated typically by heterofermentative *Leuconostoc mesenteroides* (Fleming et al. 1988; Font de Valdez et al. 1990). Heterofermentative lactic acid bacteria metabolize the cabbage sugars, principally glucose and fructose, to a mixture of lactic acid, acetic acid, ethanol, mannitol and carbon dioxide, accompanied by a rapid decrease of pH. The forming of carbon dioxide replaces air and creates an anaerobic atmosphere in the tank. Carbon dioxide stimulates the growth of many other lactic acid bacteria. *L. mesenteroides* rapidly die off as the fermentation proceeds, due to their relative sensitivity to acidic conditions (McDonald et al. 1990). In addition to *L. mesenteroides* a variety of *Leuconostoc fallax* strains has been recently found in industrial sauerkraut fermentation (Barrangou et al. 2002), which may indicate that the heterofermentative stage of sauerkraut is more complicated than has been previously reported. The gaseous stage is followed by a non-gaseous phase – the final stage of fermentation – in which the homofermentative bacteria *Lactobacillus* sp. and *Pediococcus* sp. (Fleming et al. 1988; Font de Valdez et al. 1990) form large quantities of lactic acid from the remaining carbohydrates, further lowering the pH to a final level of pH3.5 – 3.8 (titratable acidity 2.0 – 2.5%). The strains *Lactobacillus sake*, *Streptococcus faecalis* and *Lactococcus lactis* have also been isolated from spontaneously fermented sauerkraut, but the role of these bacteria in the fermentation process is not fully clear (Harris et al. 1992a; reviewed by Bűckenhuskes 1997).

Natural (spontaneous) vegetable fermentations rely on the microbial populations present in the raw materials, based on the microbiota natural to the vegetable cultivation area (reviewed by Bűckenhuskes 1997). Vegetables are typically dominated by Gram-negative aerobic bacteria and yeasts, while lactic acid bacteria make up a minor portion of the initial population (0.15 – 1.5% of the total population). Growth of other bacteria species – such as

clostridia, enterobacteria or other spoilage bacteria – in the cabbage raw material during the early stages of vegetable fermentation before growth and acid production by LAB, may cause spoilage problems. However, due to the production of acids, the pH drops rapidly and, in combination with added NaCl, usually effectively inhibits undesirable Gram-negative organisms (Aukrust et al. 1994). When fermentation is completed, oxidative yeasts and moulds may grow on the surface of the sauerkraut if suitable sugars are available during storage (reviewed by Bűckenhuskes 1997; Gardner et al. 2001).

In controlled (starter culture) fermentation, cabbage is cultivated with a LAB starter culture, thus decreasing the risk of early-stage spoilage (Fleming et al. 1995; Gardner et al. 2001). The starter cultures used in cabbage fermentations are selected according to criteria which include homo- or heterofermentation and carbon dioxide production, rate of production of organic acids and their configurations, temperature range, flavor production, acid and salt tolerance, cell sedimentation (Nout and Rombouts 1992), and competitiveness between the starter culture and the natural microbiota (Gardner et al. 2001). Several LAB strains, such as *Leuconostoc mesenteroides*, *Pediococcus pentosaceus*, *P. acidilactici*, *Lactobacillus plantarum*, *Lb. sake*, *Lb. brevis*, *Lb. cellobiosus* and *Lactococcus lactis*, have been used as starters in cabbage fermentations (Keipper et al. 1932; Breidt et al. 1995; Gardner et al. 2001; reviewed by Oliver et al. 1999). Pure or mixed starter cultures of *L. mesenteroides*, *Lb. brevis* and *Lb. plantarum* have been reported to improve the organoleptic and structural properties of sauerkraut compared to a spontaneously fermented end-product (Gardner et al. 2001).

2.3.3 Nisin production in *Lactococcus lactis* fermentation

The production of nisin in *L. lactis* fermentations has been widely studied (reviewed by de Vuyst and Vandamme 1994; Taniguchi et al. 1994; Yang and Ray 1994; Kim et al. 1997; Guerra and Pastrana 2002). For example, studies have focused on the effects of culture conditions – such as pH, temperature, aeration, carbon, nitrogen and phosphate source and calcium ion concentration – on nisin production. The development of effective nisin production systems using LAB has been another field of interest. The most challenging problem in nisin production is its proteolytic degradation (Parente and Hill 1992; Guerra and Pastrana 2002). Most bacteriocins are produced during active growth, and there is a more or less sharp decrease at the end of the growth stage (de Vuyst and Vandamme 1992, 1993; Daba et al. 1993) or the production of the bacteriocin (nisin) stops completely when cells enter the stationary phase (de Vuyst and Vandamme 1992). Maximum nisin production is directly related to biomass formation (primary metabolite) and production is closely associated with growth (end-product inhibition) (Kim et al. 1997; Amiali et al. 1998; Guerra and Pastrana 2002)

Several researchers have attempted to optimize the production of nisin by using different kinds of fermentors and developing optimal processing conditions (de Vuyst and Vandamme 1992, 1993; Yang and Ray 1994; Desjardin et al. 2001; Guerra and Pastrana 2002). Research has focused on the influence of the cultivation medium, source of carbon, nitrogen and phosphorus, pH, temperature, aeration, and type of fermentor. The use of pH-controlled batch cultures in rich media, for example, resulted in nisin titer in the range from 2500 to 4000 IU ml^{-1} (de Vuyst and Vandamme 1992; Chinachoti et al. 1997). Immobilization technology was used to increase cell density in the fermentor, and to carry out cultivation in stable continuous cultures with high volumetric nisin productivity (Zezza et al. 1993; Chinachoti et al. 1995; Wan et al. 1995; Cho et al. 1996; Chinachoti et al. 1997; Sonomoto et al. 2000; Desjardins et al. 2001). Fermentation by immobilized LAB cells enables the continuous production of nisin, but maximum nisin production in the broth does not increase as compared with free-cell continuous cultures; nisin concentration is 5- to 10-fold lower than that measured for a free-cell pH-controlled batch culture for the same strain (Taniguchi et al. 1994; Amiali et al. 1998; Desjardins et al. 2001). The highest nisin concentration (8200 IU ml^{-1}) has been achieved by a pH-controlled repeated-cycle batch culture immobilized in κ-carrageenan/locust bean gum gel beads (Bertrand et al. 2001).

It has further been reported that a high lactate concentration and low pH limit the growth of nisin-producing cells, thereby decreasing nisin productivity (Taniguchi et al. 1994; Shimizu et al. 1999; Yu et al. 2002). It has been speculated that there is a direct link between lactate removal and improved nisin production (Taniguchi et al. 1994; Yu et al. 2002). Removal of lactate in continuous production of nisin in a reactor system with a microfiltration module (anionic-exchange resin) increased nisin productivity more than fourfold compared with that obtained in a batch culture (Yu et al. 2002). Further, lactate removal from the cultivation medium was shown to preclude the inhibition of cell growth, thus extending the fermentation period (Taniguchi et al. 1994; Chinachoti et al. 1995).

Nisin can also be produced in mixed culture systems. Shimizu et al. (1999) produced nisin in a fermentor in a mixed culture consisting of *L. lactis* and *Kluyveromyces marxianus* to control the pH and the amount of lactate. Lactate was produced by *L. lactis* and assimilated by *K. marxianus*; as a result, a high level (98 μg ml^{-1}; 3920 IU ml^{-1}) of nisin accumulated in the medium as compared to a system using other pH-control strategies, e.g. alkali (58 μg ml^{-1}; 2320 IU ml^{-1}), or a process without pH-control (Shimizu et al. 1999). Nisin has also been produced in a paired culture system in cabbage (sauerkraut) fermentation to affect the different growth phases (Harris et al. 1992b; Breidt et al. 1995). In mixed culture fermentations performed with *L. lactis* sp. lactis and the nisin-resistant strains *Leuconostoc mesenteroides* and *Lb. plantarum*, nisin delayed the growth of the homolactic strains (*Lb.*

plantarum) and extended the heterolactic phase (*L. mesenteroides*), thus improving the formation of the organoleptic properties of sauerkraut. Furthermore, up to 700 IU ml^{-1} of nisin was produced in the cabbage brine but its biological activity was rapidly lost; after 72 hours, no nisin activity was detectable any longer.

2.4 Applications of antimicrobial compounds

2.4.1 Nisin

Many LAB – including *Lactobacillus*, *Lactococcus*, *Pediococcus*, *Streptococcus*, and *Leuconostoc* spp. – excrete antimicrobial proteins and peptides, called bacteriocins (Table 1). Most of them have been extensively examined for applications in microbial food safety (reviewed by Caplice and Fizgerald 1999 and Ross et al. 2002) and for the treatment of human and animal infections (reviewed by Twomey et al. 2002).

Table 10. Examples of effective use of nisin in food production.

Food product	Target organism	Effective nisin concentration (IU ml^{-1})	Reference
Cottage cheese	*Listeria monocytogenes*	2000	Ferreira and Lund 1996
Ricotta cheese	*L. monocytogenes*	100	Davies et al. 1997
Skim milk	*Bacillus cereus* spores	4000	Wandling et al. 1999
Bologna-type sausage	*Lactobacillus sake* *Lb. curvatus*	1000	Davies et al. 1999
Lean beef	*Brochothrix thermosphacta*	400	Cutter and Siragusa 1998
Kimchi	*Lactobacillus* sp.	100	Choi and Park 2000
Rainbow trout	*L. monocytogenes*	4000 - 6000	Nykänen 2001

Nisin (E234) is the only bacteriocin allowed as a food additive and is currently used in the food industry in over 50 countries (Turtell and Delves-Broughton 1998). Bacteriocins from food-grade lactic acid bacteria are antimicrobial mainly against closely related bacteria, but some of them may protect against a broader variety of microbes (reviewed by Cleveland et al. 2001). Nisin has a wide spectrum of bactericidal activity against bacteria including the strains *Lactococcus*, Streptococcus, *Staphylococcus*, *Listeria*, and *Mycobacterium*, and is successfully used against food pathogens such as *Listeria monocytogenes*, *Clostridium botulinum*, *Staphylococcus aureus*, *Streptococcus* sp., *Bacillus cereus*, and other spore-forming bacteria (Klaenhammer 1988; Liu and Hansen 1993; reviewed by Cleveland et al.

2001). To date, nisin has been tested as a food preservative in processed cheese (Ferreira and Lund 1996), skim milk (Wandling et al. 1999), sausage processing (Davies et al. 1999), lean beef (Cutter and Siragusa 1998), kimchi (Choi and Park 2000), and fish (Nykänen 2001) (Table 10). The concentrations of nisin in the various studies differ considerably (100 - 4000 IU ml^{-1}) and depend on the application and the target organism (Table 10). The maximum allowed concentration of nisin in food applications varies depending on the product (100 IU ml^{-1} – No limit) and the country (reviewed by Cleveland et al. 2001).

2.4.2 Glucosinolates and their breakdown products

The inhibitory effects of glucosinolates and their BDPs have been studied by using pure commercial compounds or by degrading glucosinolates *in vitro* (Kyung and Fleming 1994b, 1997; Brabban and Edwards 1995; Shofran et al. 1998; Lin et al. 1999; Lin et al. 2000). Testing of different sulfur compounds derived from raw cabbage and cabbage fermentation products showed that pure sinigrin was relatively innocuous to bacteria and yeasts up to 1000 ppm (Brabban and Edwards 1995; Kyung and Fleming 1997). AITC, the BDP of sinigrin, was inhibitory to the growth of both oxidative and fermentative yeasts (*Saccaromyces cerevisiae* Y6, *Torulopsis etchellsii* Y24, *Hansenula mrakii* Y27, *Pichia membranefaciens* Y20); the minimum inhibitory concentration (MIC) was between 1 and 4 ppm. AITC was found to inhibit the bacteria *Salmonella typhimurium* (100 ppm), *L. monocytogenes* (200 ppm) (Kyung and Fleming 1997), and *E. coli* B34 (50 - 200 ppm) (Kyung and Fleming 1997; Shofran et al. 1998). ITCs, thiocyanates and nitriles caused inhibition of the growth of *Bacillus subtilis* (80 ppm), and the yeast *Saccaromyces cerevisiae* proved the most sensitive (49 ppm) in the study by Brabban and Edwards (1995). Furthermore, the MICs of AITC vapor against *E. coli*, *Salmonella typhimurium*, and *Salmonella enderitidis* were determined to be as low as 34, 54, and 110 ng ml^{-1}, respectively, and 54 ng ml^{-1} against both *Vibrio papahaemolyticus* and *Pseudomonas aeruginosa* (Shofran et al. 1998).

Gram-positive *Listeria monocytogenes* was more resistant to the hydrolysis product of ITC than Gram-negative *E. coli* O157:H7 and *Salmonella* Montevideo (Lin et al. 2000). AITC was added into the bacterial medium at different growth stages in concentrations of 500, 1000 and 2500 μg ml^{-1} (ppm), and its effectiveness was compared to the antibiotics streptomycin sulfate, polymyxin B, and penicillin G. AITC showed effective antimicrobial activity against the test bacteria at all growth stages. Based on the results, Lin et al. (2000) concluded that the bactericidal mechanism of AITC was not limited to its effect on the biosynthesis of macromolecules, which occurs actively during the exponential growth phase. Moreover, it has been speculated that ITCs inhibit the growth of Gram-negative organisms by

binding ITCs to sulfydryl (-SH) groups of enzymes responsible for cell-wall synthesis (in biosynthesis of the cell) (Fenwick et al. 1983a).

In their study on the antibacterial properties of fresh and heated cabbage juice (unfermented), Kyung and Fleming (1994a) found that fresh cabbage juice exhibited antibacterial activity against the LAB strains *Lactobacillus plantarum* ATCC14917, *Leuconostoc mesenteroides* C33, *L. mesenteroides* ATCC2893, *L. mesenteroides* NCK293, and *Lactococcus lactis* NCK400. However, in the case of *Pediococcus pentosaceus* ATCC33316 and *Lb. plantarum* WSO, no inhibition occurred. After the juice was heat-treated, all bacteria were able to grow and no inhibition was observed. Antibacterial activity, therefore, appeared to be heat-labile, but the possible effective compounds were not identified in the study (Kyung and Fleming 1994a).

2.4.3 Organic acids and other components

Besides the antimicrobial peptides, bacteriocins, a wide variety of small inhibitory components are either present or are formed in LAB fermentations: bacterial metabolites such as organic acids (e.g. acetic and lactic acid), hydrogen peroxide, diacetyl, ethanol, acetaldehyde, organosulfur phytochemicals, and plant polyphenols, such as flavonoids or their conjugates, in addition to glucosinolates and their BDPs (Kyung and Fleming 1997; Ryu et al. 1999; reviewed by Caplice and Fizgerald 1999). The organic acids can inhibit a broad range of microorganisms (Nout and Rombouts 1992), especially when undissociated. Their inhibitory properties are believed to result from the action of the acids on the bacterial cytoplasmic membrane, which interferes with the maintenance of membrane potential and inhibits active transport. Acetic acid is more effectively bactericidic than other food-grade organic acids, like lactic, malic, and citric acid, and can inhibit the growth of yeasts, moulds, and bacteria (Blom and Mortvedt 1991; Ryu et al. 1999). Diacetyl, acetaldehyde, and ethanol are produced in food systems at concentrations so low that the contribution to antimicrobial activity is minimal (reviewed by Caplice and Fitzgerald 1999).

The organosulfur phytochemicals found in cabbage: methyl methanethiosulfinate (MMTSO), s-methyl-L-cysteine sulfoxide (SMCSO), dimethyl sulfide (DMS), dimethyl disulfide (DMDS), and dimethyl trisulfide (DMTS), have been reported to possess antimicrobial properties (Kyung and Fleming 1994b; Kyung and Fleming 1997). The tested strains were *Pediococcus pentosaceus*, *Leuconostoc mesenteroides*, *Lactobacillus* sp., *Listeria monocytogenes*, *Staphylococcus aureus*, *Escherichia coli*, *Enterobacter aerogenes*, *Bacillus subtilis*, *Salmonella typhimurium*, *Saccaromyces cerevisiae* Y6, *Torulopsis etchellsii* Y24, *Hansenula mrakii* Y27, and *Pichia membranefaciens* Y20. SMCSO and MMTSO were not inhibitory at 500 ppm to any of the tested bacteria and yeasts. DMDS at 500 ppm retarded some strains, but did not prevent the growth of any of the test

microorganisms. The most efficient compound was MMTSO, whose MIC was between 50 and 200 ppm for all bacteria, and between 6 and 10 ppm for all yeasts tested (Kyung and Fleming 1997). Nitrates and nitrites may also reduce the growth of microorganisms; Hyytiä et al. (1997) found that nitrate (686 mg kg^{-1}) and nitrite (166 mg kg^{-1}) effectively reduced the growth of *Clostridium botulinum* but did not inhibit it. The effects of nitrate and nitrite was strongly dependent on the concentration used (Hyytiä et al. 1997).

2.4.4 Antimicrobial effects of different compounds and treatments on food applications

Traditional bacteriocins have some limitations that reduce their effectiveness in food applications: they are usually ineffective against spoilage and pathogenic Gram-negative bacteria, yeasts, and fungi. They kill a narrow range of host microbes and bacteriocin-resistant strains in the food matrix and can occur even in the presence of bacteriocins (reviewed by Helander et al. 1997). EDTA has been reported to influence the permeability of the cell membrane of Gram-negative cells, thereby broadening the antibacterial spectrum of bacteriocins (Stevens et al. 1991). Because EDTA is not suitable for food applications, some studies have investigated the synergistic effects of bacteriocins and the use of other bioactive molecules as well as various treatments (hurdle technology; e.g. in packaging and processing) in food applications (Table 11). Studies have also focused on the effects of nisin combined with other bacteriocins, such as pediocin (Hanlin et al. 1993); modified atmosphere (Fang and Lin 1994; Szabo and Cahill 1998; Nilsson et al. 2000; Cabo et al. 2001); different temperatures (Rodriguez et al. 1997; Szabo and Cahill 1998); lactic acid and lactate (Ariyapitipun et al. 1999, 2000; Nykänen 2001); and garlic extract (Singh et al. 2001). Ariyapitipun et al. (1999; 2000) and Nykänen et al. (2000) used nisin in combination with lactic acid to demonstrate an increased effect when the preservatives were used together to inhibit Gram-negative organisms. It has been speculated that the effectivity is due to the chelating ability of lactate, which results in an increased permeability of the cell membrane to nisin (Cutter and Siragusa 1995). According to these reports, several synergistic antimicrobial properties can be achieved by using these combinations, and food shelf-life can be elongated.

Moreover, the glucosinolate BDP, allyl-ITC, has been reported to extend the shelf-life of fresh beef, sliced raw tuna, and cheese (Isshiki et al. 1992; Muthukumarasamy et al. 2003). Kim et al. (2002) tested the shelf-life of cooked rice by treating it with allyl-ITC in combination with acetic acid. They observed an excellent antimicrobial effect, which suggests that acetic acid in combination with AITC has potential as an agent to extend the shelf-life of cooked rice. AITC also inhibited the most important spoilage fungi (*Penicillium commune*, *P. roqueforti*, *Aspergillus flavus*, and *Endomyces fibuliger*) in a modified atmosphere packaging of bread for more than two

weeks when AITC was applied at 1 µl into the 250 ml microbial culture flask (Nielsen and Rios 2000).

Table 11. Increased activity of nisin when used in combination with other biomolecules or special treatment. (Modified from Cleveland et al. 2001).

Bacteriocin	Other factors	Effect	References
Nisin A	N_2; CO_2; low temperature	Effect on *L. monocytogenes*: increase in the lag phase (400 IU ml^{-1}); inhibition of growth (1250 IU ml^{-1})	Szabo and Cahill 1998
Nisin A	milk lactoperoxidase (LP) and low temp.	Nisin-producing *L. lactis* acts synergistically with LP in reduction of *L. monocytogenes*	Rodriguez et al. 1997
Nisin A	calcium alginate gel	Gel-immobilized nisin is delivered more effectively than pure nisin and suppresses growth of *Bro. thermosphacta* on beef carcasses	Cutter and Siragusa 1998
Nisin	sucrose fatty acid esters	Synergy against *L. monocytogenes, B. cereus*, *Lb. plantarum* and *S. aureus*	Thomas et al. 1998
Nisin	CO_2	Synergistic when used against wild-type and nisin-resistant *L. monocytogenes*	Nilsson et al. 2000
Nisin	pulsed electric field	Synergistic activity against *B. cereus*	Pol et al. 2000
Nisin	modified atmosphere packaging	Combination was more effective than either treatment alone at preventing growth of *L. monocytogenes*	Fang and Lin 1994
Nisin	garlic extract	Combination showed synergistic effect against five *L. monocytogenes* strains	Singh et al. 2001
Nisin	lactic acid/ lactate	Combination effectively inhibited *L. monocytogenes* and improved sensory quality of cold-smoked rainbow trout	Nykänen 2001
Nisin	CO_2	Increased significantly shelf-life of pizza compared to commercially stored samples; complementary effects of nisin and CO_2 against LAB and yeast	Cabo et al. 2001

3 Aims of the study

The main goal of this study was to investigate the formation of active nisin, glucosinolates and their breakdown products, and flavonoids in specific lactic acid bacteria (LAB) fermentations. Futhermore, the aim was to evaluate nisin activity in cabbage fermentations and the potential antimicrobial properties of fermented cabbage juice. The detailed objectives of the research were to:

1. Produce nisin by *Lactococcus lactis* N8 and *L. lactis* LAC48 in M17G medium by using pH-controlled, non-pH-controlled batch fermentation and pH-controlled fermentation with continuous removal of nisin from the fermentor into the binding resin (circulated batch) (II).

2. Analyze the role of the cytosolic proteins NisB and NisC of *L. lactis* N8 in the post-translational modification of nisin by purification and analysis of the modification intermediates from strains lacking NisB or NisC activity (I).

3. Evaluate the production of nisin during *L. lactis* N8 cabbage fermentation (IV).

4. Characterize the content of plant-derived bioactive components (flavonoids and glucosinolates) and glucosinolate breakdown products in the cabbage raw material, in spontaneously fermented cabbage, and in cabbage fermented with specific LAB starter cultures (III, IV).

5. Evaluate the potential antimicrobial activity of cabbage juice containing plant-derived biomolecules and/or nisin, both prior to fermentation and after fermentation (IV).

4 Materials and methods

The the plasmids used in present study are described in Table 12, and growth media and bacterial strains in Table 13. The analysis methods used in this study are presented in Table 14 and the fermentations in Table 15. More itemized descriptions are presented in Papers I-IV.

Table 12. Plasmids constructed and used in this study.

Plasmid	Relevant properties	Description	Reference, manufacturer
pLEB22	*erm*	Paper I	Axelsson et al. 1988
pLEB36	'*nisTCIP*'	Paper I	Immonen et al. 1995
pKTH1984	*nisZ*	Paper I	Graeffe et al. 1991
pCRII	T/A cloning vector	Paper I	Invitrogen
pLEB124	*L. lactis* expression vector with P_{45} promoter	Paper I	Qiao et al. 1995
pLEB384	pLEB124 + *cat*	Paper I	Qiao et al. 1996
pTC*lux*Hb	Vector used for the *nisC* complementation plasmid	Paper I	Jacobs et al. 1995
pLEB406	*erm*, '*nisC*', integration plasmid	Paper I	This study
pLEB507	P_{45} + *nisC*, the *nisC* complementation plasmid	Paper I	This study
pLEB544	His-tagged *nisZ*	Paper I	This study
pLEB561	pLEB124 + His-tagged *nisZ*	Paper I	This study
pLEB563	pLEB384 + His-tagged *nisZ*	Paper I	This study
pLEB599	P_{45} + *nisR nisK* + *gfp* gene under control of P_{nisF} promoter	Paper IV	Reunanen and Saris 2003

Table 13. The bacterial strains, cultivation media, and temperatures used in this study.

Microbial species	Relevant properties/Source of LAB (S)	Cultivation medium (*	Reference, source
Escherichia coli			
DH5α	host strain for plasmid construction/ for antimicrobial test	LB	Hanahan 1983
Lactococcus lactis			
N8	nisin Z producer	M17G	Graeffe et al. 1991
LAC48	nisin Z producer, spontaneous mutant	M17G	Qiao et al. 1997
LAC67	*nisZ* mutant	M17G (ery5)	Qiao et al. 1997
LAC53	*nisB* mutant	M17GS	Qiao et al. 1996
MG1614	no plasmids, no nisin genes	M17G	Gasson 1983
NZ9800	*nisA* mutant	M17GS	Kuipers et al. 1993
LAC104	*nisC* mutant	M17GS	This study
LAC208	NZ9800 + pLEB561	M17GS	This study
LAC212	LAC104 + pLEB563	M17GS (ery5)	This study
LAC214	LAC53 + pLEB563	M17GS (ery5)	This study
LAC240	pLEB599 with *gfp* gene	M17G (ery5)	Reunanen and Saris 2003
Lb. plantarum DSM14485	S: fermented cucumber	MRS	MTT (A
Lb. sakei DSM15298	S: fermented cabbage	MRS	MTT (A
L. mesenteroides DSM14486	S: fermented cabbage	MRS	MTT (A
L. monocytogenes EELAL215	for antimicrobial test	TSA	EELA (B
M. luteus A1 NCIMB86166	nisin-sensitive strain	LB	NCIMB (C
P. pentosaceus DSM14488	S: fermented grass	MRS	MTT (A
P. dextrinicus sp. 9/PX3	S: fermented cabbage	MRS	MTT (A
Candida lambica VTTC-00360	for antimicrobial test	YG, YGC	VTT (D

*) LB: Luria Bertani, MRS deMan-Rogosa-Sharpe, TSA: Tryptic Soya Agar, YG: Yeast Glucose,
A) MTT: Agrifood Research Finland
B) EELA: National Veterinary and Food Research Institute, Finland
C) NCIMB: National Collection of Industrial and Marine bacteria
D) VTT: Technical Research Centre of Finland
YGC: YG + chloramphenicol, M17GS: M17 + glucose + sucrose
DSM: Deutsche Sammlung von Microorganismen und Zellkulturen GmbH

Table 14. The methods used in this study are described as follows.

Analysis	Description	Reference
Genetic analyses		
DNA manipulations	Paper I	See: Table 12 and Table 13
Mass spectrometry	Paper I	Saarinen et al. 1999
N-terminal amino acid sequencing	Paper I	Kalkkinen and Tilgman 1988; Saarinen et al. 1999
Purification of His-tagged prenisin	Paper I	Pharmacia Biotech (1
Western analysis	Paper I	Stratagene, Giagen (1
Fermentation parameters		
Biomass and dry weight	Papers II, III, IV	This study
Glucose in cabbage brine	Paper IV	Boehringer – Mannheim
Lactic and acetic acids	Papers II, III, IV	Boehringer - Mannheim
Microbial counts	Papers II, III, IV	This study
Microbial identification (APICHL)	Paper III, IV	BioMerieux sa
pH	Papers II, III, IV	This study
Titratable acids	Papers III, IV	Hart and Fisher 1971
Total glucose	Papers III, IV	Li and Schumann 1980; Haila et al. 1992 (1
Dietary and bioactive compounds		
Ascorbic acid (Vitamin C) (2	Paper IV	Speek et al. 1984
Flavonoids (3	Paper III	Hertog et al. 1992b
Glucosinolate breakdown products (BDP) (4	Papers III, IV	Daxenbichler and van Etten 1977 (1 Spencer and Daxenbichler 1980 (1
Glucosinolates (GS) (5	Paper III	British standard 1996
(Iso)thiocyanate-anion (6	Papers III, IV	Lyons et al. 1991
Nitrate (6	Papers III, IV	Lyons et al. 1991
Nitrite (6	Papers III, IV	Lyons et al. 1991
Antimicrobial assays		
Agar diffusion assay; nisin bioassay	Papers II, IV	Qiao et al. 1996 (1
Antimicrobial activity in cabbage juice	Paper IV	This study
Quantification of nisin by green fluorescent protein (GFP) method	Paper IV	Wahlström and Saris 1999 (1 Reunanen and Saris 2003 (1

1) used with slight modifications.
2, 3) HPLC
4) volatile breakdown products, GC-MS; Quantified against commercial standard or in the case of lacking standard the ion ratios were measured
5) glucosinolates as a desulpho-forms, HPLC
6) HPLC with reverse UV detection

Table 15. The starter strain, type of fermentor, temperature, and culture medium used in the present study.

Ferm. No.	Starter strain	Type of fermentor	T (˚C)	Medium[D]	Description
Nisin production [A]					
F1	*L. lactis* N8	batch, pH not controlled	30	M17G	Paper II
F2	*L. lactis* N8	batch, pH 5.5	30	M17G	Paper II
F3	*L. lactis* N8	circulated batch, pH 5.5	30	M17G	Paper II
F4	*L. lactis* LAC48	batch, pH not controlled	30	M17G	Paper II
F5	*L. lactis* LAC48	batch, pH 5.5	30	M17G	Paper II
F6	*L. lactis* LAC48	circulated batch, pH 5.5	30	M17G	Paper II
Sauerkraut production					
F7	Spontaneous [B]	batch, pH not controlled	20	cabbage	Paper III
F8	*L. mesenteroides* + *P. dextrinicus* [B]	batch, pH not controlled	20	cabbage	Paper III
F9	*Lb. plantarum* [C(*]	batch, pH not controlled	20	cabbage	Paper IV
F10	*L. mesenteroides* [C(*]	batch, pH not controlled	20	cabbage	Paper IV
F11	*P. pentosaceus* [C(*]	batch, pH not controlled	20	cabbage	Paper IV
F12	*Lb. sakei* [C(*]	batch, pH not controlled	20	cabbage	Paper IV
Nisin and sauerkraut production					
F13	*L. lactis* N8 [C]	batch, pH not controlled	20	cabbage	Paper IV
F14	*L. lactis* LAC67 [C]	batch, pH not controlled	20	cabbage	Paper IV

A) fermented in programmable 1.5-L fermentor, liquid volume 1 L; two parallel fermentations
B) Series A; fermented in 50-L plastic vats, weight of raw cabbage 20 kg, four parallels
C) Series B; fermented in 0.5-L glass vats, weight of raw cabbage 500 g, three parallels
D) M17G: added glucose 2% (w/V), cabbage: no added glucose; total glucose in raw cabbage 2.6% (w/w)
*) performed twice (in consecutive years)

4.1 Statistical analysis

Statistical analysis (Papers III and IV) was conducted using SAS Software Release 8.2 (TS1MO). Colony-forming units (cfu) were converted to logarithms, and an analysis of variance was performed with SAS statistical software. The means were compared by a t-test (glucosinolate BDPs, cfu).

5 Results and discussion

5.1 Role of NisB and NisC in the biosynthesis of nisin (I)

5.1.1 Construction of *nisB* and *nisC* mutants and His-tagged nisin

To study the role of the NisB and NisC proteins in nisin biosynthesis, in post-translational modification, *nisB* and *nisC* mutant strains were needed. The *nisB* mutant strain LAC53 has been constructed in a previous study (Qiao et al. 1996). In the constructed *nisB* mutant strain LAC53, the downstream genes (*nisTCIPRK*) are transcribed from the promoter of the *erm* gene located downstream of the *nisB* mutation (Qiao et al. 1996), which makes it usable for an analysis of the NisB function. The *nisC* mutant strain was constructed as described in Paper I.

For an analysis of the nisin precursors expressed in the *nisB* and *nisC* mutant strains (Table 12, Table 13), the precursors needed to be purified. To aid the purification steps, a His-tag (six histidins) coding sequence was added to the C-terminus of the *nisZ* gene in the *L. lactis* expression vector with the P45 promoter (pLEB124) (Paper I). This constructed plasmid pLEB561 was transformed into the *nisA* mutant strain, and named LAC208. Without nisin induction, the cells of LAC208 did not produce nisin, but induction with 25 ng ml^{-1} nisin resulted in the secretion of active nisin (Figure 3, Paper I). This result indicates that the His-tagged nisin precursor is a functional substrate for the nisin modification enzymes and transport protein, but not a functional inducer of the positively autoregulated nisin operons (Kuipers et al. 1995; Qiao et al. 1996; Ra et al. 1996). Therefore, the plasmid pLEB563, identical to pLEB561 except for the chloramphenicol resistance marker, was transformed into the *nisB* and *nisC* mutant strains LAC53 and LAC104, resulting in LAC214 and LAC212, in order to produce partially modified nisin (with the His-tag) for the characterization of the function of the NisB and NisC enzymes.

5.1.2 Role of NisB and NisC in dehydration and lanthionine formation

The constructed strains LAC214 (for NisB) and LAC212 (for NisC) were grown in M17GS supplemented with 25 ng ml^{-1} nisin in order to induce the expression of a His-tagged nisin precursor. No nisin activity was observed either in the growth media or in the cell cytosol of the LAC214 and LAC212 strains. However, a His-tag-nisin polypeptide was detected from the cell cytosol by using the Western analysis (Figure 4, Paper I). The result of the SDS-PAGE (Figure 5, Paper I) showed that the purified putative His-tagged nisin precursor migrated as a polypeptide of approximately 6.9 kDa,

indicating that the nisin leader remained uncleaved. Accordingly, the polypeptides were further purified, analyzed by N-terminal amino acid sequencing, SDS-PAGE and mass analysis (MALDI) (Figures 5 and 6, Paper I). Sequencing of the N-terminal of the His-tagged nisin precursor verified that the purified polypeptide was the His-tagged nisin precursor with the N-terminal leader and that no modified amino acid residues were present in the leader. The C-terminus was likely to be intact as the polypeptide could be purified using the HisTrap column and detected using the Western analysis with a His-tag-specific antiserum.

The result of the mass analysis showed (Figure 6, Paper I) that the purified nisin precursor isolated from LAC212 (*nisC* mutant) could potentially contain all lanthionines typical of active nisin. However, the leader peptide was needed to be digested to confirm the functionality of peptide (van der Meer et al. 1993). Therefore, the isolated nisin precursors (1 mg) and a control (growth supernatant of LAC71; *nisP* mutant, Qiao et al. 1996) were treated with trypsin; nisin activity was observable only in the control sample. This result indicated that the N-terminal leader was not cleaved or if cleaved, peptide was not fully modified. It has been demonstrated earlier that all lanthionins are necessary for nisin activity, and that if the N-terminal leader is not cleaved, the nisin will be inactive (van der Meer et al. 1993; Qiao et al. 1995). In this study, as expected, the nisin-induced expression of the His-tagged nisin precursor in the LAC214 and LAC212 strains did not yield any active nisin either inside or outside of the cells, due to lack of complete modification of nisin. Further, the expression of the His-tagged nisin was initiated only if the cells were induced with intact nisin. His-tagged nisin seemed to damage the inductive capacity of nisin. The results showed that if NisB and NisC did not modify the nisin precursor, the precursor remained in the cell cytosol and was not transported out of the cell.

Mass analysis, SDS-PAGE, and N-terminal amino acid analysis of the His-tagged nisin precursors purified from the LAC214 and LAC212 strains all agreed that the N-terminal leader was not digested from the nisin precursor inside the cell. This indicates that the nisin precursor is protected from intracellular proteases as long as it is not completely modified, because in a *nisT* mutant strain in which the transport of the modified nisin was blocked the leader was digested and active nisin could be isolated from inside of the cells (Qiao and Saris 1996).

The mass analysis of the His-tagged prenisin was performed to determine the degree of dehydration of serines and threonines (Figure 6, Paper I). The mass of the nisin precursor decreases by 18 Da as a result of each dehydration step (a water molecule cleaved) and can be analyzed by mass spectrometry. The mass analysis of the His-tagged nisin precursor from the LAC214 strain (NisC functional but no NisB activity due to the mutation) showed that the mass corresponded to a His-tagged nisin precursor with none of the serine

and threonine residues dehydrated (Figure 6b, Paper I). This showed that NisB is needed for the dehydration reaction to occur. The same analysis using the nisin precursor purified from the LAC212 strain (NisB functional but no NisC activity due to the mutation) showed that the His-tagged nisin precursor was not as heavy as when isolated from the LAC214 strain (Figure 6a, Paper I). The results of the mass analyses clearly indicated that NisB was responsible for the dehydration reaction and that NisB did not need NisC for the dehydration reaction. However, the results showed that NisB was not effective for dehydrating all the serine and threonine residues as the majority of the nisin precursors were only partly dehydrated. The structural gene of the His-tagged nisin was located on a multicopy plasmid in the LAC214 strain, resulting in potentially too high levels of the nisin precursor for the NisB enzyme to dehydrate all the potential sites. Another explanation for the partial dehydration is that lack of NisC has an effect on the activity of the NisB enzyme, which is known to form a complex with NisC and NisT (Siegers et al. 1996). Clearly, the observed inefficient dehydration by the NisB enzyme did not hinder the function of the nisin biosynthetic machinery, as active His-tagged nisin could be secreted by the *nisA* mutant strain containing the plasmid pLEB561 (LAC208).

The relative intensities in the mass analysis of the pre-nisins showed some inaccuracy (Figure 6, Paper I). The width of the mass peak (approx. 7000 – 7120 Da) of the prenisin of LAC214 (NisC functional, NisB mutated) could be explained by a mixture of nisin precursors which are potentially incorporated with salt (potassium or sodium) in MALDI analysis. The same analysis using the nisin precursor purified from LAC212 (NisB functional, NisC mutated) indicated that the dehydration of prenisin could occur by degrees; the majority of the peptides being partially dehydrated and the minority possessing fully dehydrated residues (dehydroalanine Dha, dehydrobutyrine Dhb). It is not possible to judge by mass analysis whether any of the potentially dehydrated residues had reacted with cysteine to form lanthionine (Figure 6, Paper I). However, if all lanthionines (Lan, MeLan) had been formed, then cleavage of the leader should have yielded active nisin. As mentioned above, no nisin activity was observed after trypsin treatment of the nisin precursor isolated from the LAC212 strain, indicating that NisC is needed for correct lanthionine formation.

5.2 *L. lactis* N8, *L. lactis* LAC48 and *L. lactis* LAC67 fermentations (II, IV)

5.2.1 Nisin production

5.2.1.1 Batch fermentation

In our earlier studies we found a spontaneous mutant *Lactococcus lactis* LAC48 possessing an increased nisin-production capacity (on a laboratory scale) and an exceptionally high level of nisin resistance, up to 5000 IU ml^{-1} (Qiao et al. 1997). The strain might be a potential candidate for use in industrial fermentation, and so we tested the production of nisin in a larger-scale fermentation (volume 1 litre) using the LAC48 strain (Table 16; F4, F5), and compared it to nisin productivity of the strain *L. lactis* N8 (F1, F2). The results showed that LAC48 grew slower than N8 but produced more nisin per cell (Fig 2a and 2b, Paper II). Additionally, LAC48 cells showed higher maximum nisin yield (2.5×10^6 IU g^{-1} dcw, after 8 hours) than N8 cells (1.5×10^6 IU g^{-1} dcw, after 4 hours). When nisin was produced by the strain N8, the nisin titer gradually decreased to zero level after reaching the maximum peak value, as reported also in earlier studies (de Vuyst and Vandamme 1992; 1993). In the current study, nisin production per cell (LAC48) remained above 2×10^6 IU g^{-1} dcw through 22 hours, after which it gradually decreased to a level of 0.5×10^6 IU g^{-1} dcw. There was no sharp reduction in nisin activity in the LAC48 cultivations, which would imply that maximum nisin production by LAC48 is neither directly related to biomass formation nor associated with growth – contrary to what has been speculated in earlier studies (Kim et al. 1997; Amiali et al. 1998; Desjardins et al. 2001). This discrepancy may be partly explained by the exceptionally high resistance of LAC48 to nisin, which enhances the long-lasting production of this peptide (Qiao et al. 1997). It is also possible that proteases were either completely absent or present only at a low level in the fermentation of LAC48. The results of this study showed that the LAC48 cells were not inhibited by the concentration of nisin they produced, potentially reducing cell death and lysis, which would release intracellular proteases. The observed decrease in nisin titer during prolonged fermentation, as in the case of N8, is possibly caused by intracellular proteases.

5.2.1.2 Nisin adsorption and elution

To avoid the potential inhibition of cells by too high concentrations of nisin (feedback inhibition), nisin was removed from the fermentor by binding it to an adsorbing resin. Different materials potentially capable of binding nisin were tested in a small scale (test tube, 2 ml). Based on preliminary experiments and literature, sodium acetate buffer (pH 5.5, 15 mS cm^{-1}) was selected for optimal binding solutions (data not shown). Of the tested adsorbing materials (Amberlite XAD-1180, Amberlite XAD-4, Streamline

SP, Streamline DEAE, Streamline Phenyl, Bentonite, and kieselguhr), the hydrophobic Amberlite XAD-4 adsorbed nisin most efficiently (Table 1, Paper II). The results indicated that over 90% of nisin was bound into the XAD-4 resin and, therefore, it was selected for the elution tests. Based on the small-scale binding test, 1 M NaCl in 20% ethanol (Table 2, Paper II) was chosen for the elution of nisin from the Amberlite XAD-4 particles in a column test.

The adsorption and elution of nisin (1 litre volume) in the Amberlite XAD-4 column showed that the major part of nisin was adsorbed onto the column at the beginning of binding, after which adsorption gradually weakened. This may indicate that the capacity of the XAD-4 column was limited; approximately 20% (300,000 IU) of initial nisin (total 1.500,000 IU) titer did not bind to the column. However, high activity of nisin (850,000 IU) could be eluted (Fr.1: 5000 IU ml^{-1}; Fr. 2: 8000 IU ml^{-1}; Fr. 3: 4000 IU ml^{-1}) from the column. The bound portion of nisin which was unelutable in the used elution conditions was calculated to be approximately 350,000 IU (remaining in the column). This nisin portion was potentially either degraded or bound irreversibly into the column. On the basis of this result, approximately 80% of nisin could be removed and 57% harvested from 1 litre of cultured broth using the Amberlite XAD-4 column.

Elution of nisin from the Amberlite XAD-4 column in circulated batch fermentation (F3 and F6) resulted in fractions with relatively low nisin activity (200 – 2000 IU ml^{-1}). This finding was in disagreement with the result of the test mentioned above, in which high activity of nisin (8000 IU ml^{-1}) was eluted. To study if the circulation of spent medium had an effect on nisin activity – which might explain the observed discrepancy – we tested the adsorption and elution of nisin in Amberlite XAD-4 columns in three similar columns; the medium containing the initial nisin concentration 1200 IU ml^{-1} was conducted through the columns followed by 1) immediate elution, 2) elution after two hours' circulation, and 3) elution after five hours' circulation. The spent medium (without nisin) was used as a reference. The concentrations of nisin in the elutions were as follows: 1) 5000 – 8000 IU ml^{-1}, 2) 100 IU ml^{-1}; and 3) no detectable nisin in the eluted fraction. Increased circulation obviously resulted in a higher loss of nisin activity, which explains why only low nisin activity could be eluted from the columns onto which nisin had been adsorbed during fermentation. The loss of nisin activity could result from the fact that, when bound to XAD-4, nisin may be an easier target for the proteases present in the fermentation broth than plain nisin in solution. To solve the problem of potential degradation of nisin, different protease inhibitors could be used in the medium or the medium could be heated to inactivate the proteases, as was done in the column test in our study.

Table 16. Type of fermentation, strains, temperature, pH, cfu, glucose, lactic acid, and nisin activity in nisin and cabbage (sauerkraut) fermentation.

Ferm No.	Type of fermentation	t (d)	pH		LAB (cfu ml^{-1})		Glucose (g l^{-1})		Lactic acid (g l^{-1})		Nisin (IU ml^{-1})
			t = 0 h [1]	final	t = 0 h [1]	final	t = 0 h [1]	final	t = 0 h [1]	final	
***Lactococcus lactis* N8** [A]											
F1	Batch	2	6.6 ± 0.0	4.6 ± 0.2	2.2 x 10^8	n.d.	19.9 ± 0.3	9.8 ± 0.2	~ 0	7.6 ± 0.1	2200 ± 100
F2	Batch, pH 5.5	2	6.6 ± 0.0	5.5 ± 0.1	1.4 x 10^8	n.d.	20.2 ± 0.4	8.0 ± 0.2	~ 0	7.7 ± 0.1	2500 ± 200
F3	Cycle batch (Amberlite XAD-4)	3	6.6 ± 0.0	5.5 ± 0.1	2.8 x 10^8	n.d.	20.3 ± 0.3	10.1 ± 0.4	~ 0	12.1 ± 0.3	2500 ± 200 (8000) [4]
***L. lactis* LAC48** [A]											
F4	Batch	2	6.6 ± 0.0	4.6 ± 0.1	2.1 x 10^8	n.d.	20.0 ± 0.2	10.0 ± 0.2	~ 0	4.3 ± 0.1	2500 ± 200
F5	Batch, pH 5.5	2	6.6 ± 0.0	5.5 ± 0.1	1.2 x 10^8	n.d.	20.1 ± 0.3	9.0 ± 0.2	~ 0	4.2 ± 0.1	2500 ± 200
F6	Cycle batch (Amberlite XAD-4)	3	6.6 ± 0.0	5.5 ± 0.1	1.9 x 10^8	n.d.	19.9 ± 0.2	9.5 ± 0.2	~ 0	6.2 ± 0.1	2500 ± 200
***L. lactis* N8** [B]											
F13	Cabbage, batch	14	6.0 ± 0.2	4.2 ± 0.1	1.3 x 10^7	8.2 x 10^8	26.1 ± 0.3 [2]	23.3 ± 0.2 [3]	n.d.	4.4 ± 0.1	1400 ± 200
***L. lactis* LAC67** [B]											
F14	Cabbage, batch	14	5.9 ± 0.2	4.3 ± 0.1	8.2 x 10^6	8.4 x 10^8	26.3 ± 0.2 [2]	23.4 ± 0.3 [3]	n.d.	5.3 ± 0.2	0

1) Sample taken immediately after inoculation
2) Total glucose in raw material
3) Free glucose (in cabbage brine)
4) Eluted from the Amberlite XAD-4 column by NaCl-ethanol (in the column test)
A) Fermentations performed in duplicate
B) Fermentations were performed in triplicate
n.d. Not determined

5.2.1.3 Nisin production in cabbage fermentation

To investigate the production of nisin and other potentially bioactive molecules formed in sauerkraut manufacturing, we inoculated the cabbage with the known nisin-producer *L. lactis* N8. Nisin is not classified as "natural" preservative when it is applied in a concentration that exceeds the amount found in food that has been naturally fermented with a nisin-producing culture (Cleveland et al. 2001). Thus, one interesting factor is the ability of lactic acid bacteria to produce the desired compounds in situ (in this case to inhibit spoilage microbes in sauerkraut). Cabbage fermented with the spontaneous mutant *L. lactis* LAC67, which is incapable of producing nisin, was used as a reference fermentation.

The growing of *L. lactis* N8 cells in cabbage fermentation showed a labile growth curve and unstable nisin production (Figure 1, Paper IV). The highest cell count achieved within one day (107 cfu ml^{-1}) decreased in the period between days 1 and 4, but, interestingly, rose again after day 4, and gradually declined to a level of 10^2 cfu ml^{-1}. At the same time, the concentration of nisin reached a peak value of 1400 IU ml^{-1} in the brine after one day's fermentation, dropped to a level of 1000 IU ml^{-1}, then increased again, and finally, after 13 days, decreased to a level of 250 IU ml 1^{-1}. The concentration was substantially high if compared with previous research: Breidt et al. (1995) reported a nisin concentration of approximately 700 IU ml^{-1} after one-day cabbage fermentation (total time two weeks), whereas no nisin was detectable in the brine on day 5 although the *L. lactis* cell count was still 10^5 cfu ml^{-1}. Nisin activity between days 9 and 25 remained unknown because nisin was not analyzed between these fermentation days. Moreover, the results of nisin concentrations in the previous studies are not fully comparable due to different analysis methods.

5.2.1.4 Comparison of nisin production in different fermentations

The comparison of the nisin productivities in M17G medium, using strains *L. lactis* N8 (F1 – F3) and *L. lactis* LAC48 (F4 – F6) is presented in Paper II, and in cabbage fermentations with *L. lactis* N8 (F13) and *L. lactis* LAC67 (F14) in Paper IV. The maximum volumetric activity in M17G medium varied between 2200 and 2500 IU ml^{-1}; in cabbage fermentation (F13) the maximum concentration was 1400 IU ml^{-1}. The strain *L. lactis* LAC67 was unable to produce nisin, as could be expected due to the mutated *nisZ* structural gene. There was a sharp decline in nisin activity after maximum production with N8 in all of the tested fermentation types, with nisin activity gradually achieving the level of zero, except in the case of cabbage fermentation (F13). Nisin was produced through cabbage fermentation with N8, as described above (Chapter 5.2.1.3), but at a lower level than in M17G medium. In fermentation with LAC48, nisin productivity continued at an

approximately constant level (2000 – 2500 IU ml^{-1}) through 48 hours in the pH-controlled circulated batch and no decrease occurred. As speculated earlier, LAC48 showed long-lasting nisin productivity, possibly due to its exceptionally high resistance to nisin (Qiao et al. 1997). Further, the circulation of the fermentation broth through the XAD-4 column did not lower the concentration of nisin titer in the broth in the fermentations with N8 and LAC48. With N8 the nisin titer remained at the same level as without circulation and binding, whereas in the LAC48 fermentation the titer stayed high throughout the 48-hour fermentation (no decrease occurred), as mentioned above. This indicated that when nisin was removed during fermentation by adsorption to the column, the cells of LAC48 were capable of substituting the lost quantity to the fermentation broth, thereby reaching the nisin concentration achievable under the used conditions.

The maximum production of nisin by *L. lactis* N8 was delayed in cabbage fermentation (F13), resulting in a lower level of nisin (1400 IU ml^{-1}, 24 h) than in M17G meduim (2500 IU ml^{-1}, 10 – 12 h). It was to be expected as M17G represents a modified medium, rich in nutrients and optimal for growth of *L. lactis* and for nisin production, whereas cabbage is an example of a "natural" growth medium for nisin production in situ. However, nisin was produced (1300 – 1400 IU ml^{-1}) for a relatively long time (7 days) in cabbage fermentation, although at an unstable rate, as shown in Figure 1 (Paper IV). Nisin activity decreased after maximum production similarly as in nisin production in M17G medium, but increased again prior to dropping close to zero. As speculated in the case of nisin production in the circulation batch (Amberlite XAD-4 column), the peptide may be exposed to proteolytic degradation due to long fermentation. Nisin production can also be restricted by end-product inhibition (Kim et al. 1997; Amiali et al. 1998; Guerra and Pastrana 2002). Therefore, it was possibly due to nisin degradation in cabbage fermentation that *L. lactis* N8 was able to grow again and produce active nisin in desired conditions (decreased end-product inhibition).

The results of the present study confirm that the *L. lactis* LAC48 strain may be a potential candidate for use in industrial fermentation to achieve a high nisin titer, as suggested in a previous study (Qiao et al. 1997). An advantage of LAC48 is that the decrease from the maximum nisin titer is not sharp, giving more flexibility as to when nisin should optimally be harvested. Other known nisin producers, including N8, show a sharp drop in the nisin titer after the maximum value (Parente and Hill 1992; de Vuyst and Vandamme 1992; Kim et al. 1997).

Development of new applications for nisin in the food industry is limited by the low yield and low concentrations achieved during fermentation. Consequently, the processes result in relatively a high cost of purified nisin. It is, therefore, important to develop new processes, e.g. by producing concentrated nisin titers or combining nisin with other bioactive

(antimicrobial) substances usable in food applications. A high-concentration solution of nisin (8000 U ml^{-1}) in NaCl-ethanol, as obtained in this study, could potentially be used directly in food applications, as was also speculated by Brewer et al. (2002). Further, the present study showed that nisin could be produced in situ, e.g. in sauerkraut production. Accordingly, the combined effects of nisin and other antimicrobial substances in sauerkraut may be synergistic, extending the shelf-life of food. The use of bacteriocin-producing cultures in food might be advantageous compared with purified bacteriocin preparations, which could be considered food additives. In addition, the use of the fermentation broth as an ingredient or a processing aid for food applications as such or in a freeze-dried form will be difficult if the bacteriocin concentration in the broth is low. The present study also emphasized the importance of controlling the effect of protease activities in fermentation when high nisin titers are to be produced. The adsorption of nisin onto Amberlite XAD-4 resin and its elution into the fermentation broth could be a good way of increasing the nisin titer in food applications. For this to be successful, the broth entering the column should be heated in order to inactivate the proteases, and the flow rate and column capacity should be higher than those used in this study.

5.2.2 Formation of biomass, glucose consumption and lactic acid production

L. lactis N8 and *L. lactis* LAC48 produced equal amounts of biomass in M17G medium, achieving the maximum (1.5 g g^{-1} dcw) after 10 hours of fermentation without showing any significant decrease towards the end of fermentation. This was also observed when the adsorption column was connected to the cultivation, indicating that the Amberlite XAD-4 did not remove any nutrients required for cell growth from the medium. Therefore, the difference in nisin yields between the N8 and LAC48 strains cannot be explained by a difference in biomass. The mutation on *L. lactis* LAC48, which has an effect on growth rate, did not influence the final biomass.

Glucose concentration at the end of the N8 (F1 – F3) and LAC48 (F4 – F6) fermentations varied from 8.0 to 10.1 g l^{-1} (0.8 – 1.0%), indicating that a lack of glucose did not limit nisin production or biomass formation. The nisin-producer strains of *L. lactis* are typically weak producers of lactic acid (de Vuyst and Vandamme 1994; Harris 1998) and, therefore, not used for lactic acid production. Accordingly, nisin producers are not preferred in food fermentation where the fast production of lactic acid is desirable. In this study, lactic acid was produced as follows: 7.6 – 12.1 g l^{-1} (N8 in M17G), 4.2 – 6.2 g l^{-1} (LAC48 in M17G), 4.4 g l^{-1} (N8 in cabbage) and 5.0 g l^{-1} (LAC67 in cabbage). Production was accelerated when the Amberlite XAD-4 column was connected to the fermentations (Figure 4, Paper II). The concentration of lactic acid was approximately 50% higher at the end of these fermentations compared to fermentations without the column. The reason for this is not

known, but it is possible that substances inhibitory to lactic acid production were adsorbed on the XAD-4 column. *L. lactis* LAC48 yielded about half the amount of lactic acid produced by using *L. lactis* N8, showing that the mutation of LAC48 has a pleiotropic effect. It seemed to increase nisin production and nisin and erythromycin resistance, but decreased the growth rate and the production of lactic acid.

Table 17. Dry weight, time of fermentation, glucose and acid concentrations, titratable acidity, and number of lactic acid bacteria (LAB) in sliced raw cabbage and in fermentation end products (in brine). Series A (Paper III), Series B (Paper IV).

Ferm. No.	Culture used in cabbage fermentation	t (d)	Free glucose (g l^{-1}) (1)	Titratable acidity (%)	Lactic acid (D+L) (g l^{-1})	Acetic acid (g l^{-1})	Total LAB (cfu ml^{-1})
	Sliced raw cabbage (n = 6) (2)		11 ± 1.0 (total: 26) (3)	0.1	n.d.	n.d.	$10^2 – 10^4$
Series A							
F7	Spontaneous (n = 4)	5	n.d.	n.d.	n.d.	n.d.	9.2×10^9
F8	*L. mesenteroides* + *P. dextrinicus* (n = 4)	3	n.d.	n.d.	n.d.	n.d.	9.1×10^9
Series B							
F9	*Lb. plantarum* (n = 3)	6	12.5 ± 0.2	1.0 ± < 0.1	11.5 ± 0.3	0.5 ± < 0.1	2.5×10^9
F10	*L. mesenteroides* (n = 3)	3	12.3 ± 0.5	1.1 ± < 0.1	4.8 ± 0.2	2.7 ± < 0.1	3.8×10^9
F11	*P. pentosaceus* (n = 3)	9	17.8 ± 0.1	0.7 ± < 0.1	5.2 ± 0.1	0.1 ± < 0.1	2.5×10^7
F12	*Lb. sakei* (n = 3)	3	9.2 ± 0.3	0.5 ± < 0.1	6.5 ± 0.2	0.3 ± < 0.1	9.2×10^9
F13	*L. lactis* N8 (n = 3)	14	23.3 ± 0.4	0.4 ± < 0.1	4.4 ± 0.1	0.1 ± < 0.1	8.1×10^8
F14	*L. lactis* LAC67 (n = 3)	14	23.4 ± 0.5	0.8 ± < 0.1	5.3 ± 0.2	0.8 ± < 0.1	8.2×10^8

(1) Concentration of free glucose (in brine)
(2) Average number of samples collected immediately after inoculation of fermentations F9 – F14
(3) Total glucose in untreated cabbage
n.d. Not determined

5.3 Sauerkraut fermentations (III, IV, and unpublished results)

5.3.1 Fermentation process and microbiology

The starter fermentations in this study were initiated and controlled by different starter cultures of LAB (F7 – F14; inoculation 10^6 –10^7 cfu ml^{-1}) (Table 17). The starter cultures (Series A; Paper III, Series B; Paper IV) were selected based on our preliminary studies, in which certain LAB were shown to produce organoleptically and microbiologically good-quality sauerkraut (data not shown). The LAB count in the fermented end-product (sauerkraut brine) varied from 10^7 to 10^9 cfu ml^{-1}. The brine pH decreased rapidly, gradually reaching the appropriate level (below pH 4; varied for 3 – 14 days) recommended for typical cabbage fermentation (Pederson and Albury 1969; Fleming et al 1988; Harris 1998). In the *L. lactis* fermentations (F13, F14) the total LAB counts never exceeded 10^8 cfu ml^{-1} and the brine pH remained above 4.2, as expected; as already noted, *L. lactis* nisin-producers are known to be inefficient producers of lactic acid (reviewed by de Vuyst and Vandamme 1994). On the other hand, nisin was produced at a level which potentially reduced the growth of natural microbiota (LAB) that cause acidifying and lowering of the pH in natural (spontaneous) fermentation.

The growth of *L. lactis* N8 cells and other bacteria strains in F13 was predominant by turns (Figure 1, Paper IV). At the beginning, *L. lactis* N8 dominated the fermentation process and, further on, fermentation was undertaken by presumably nisin-resistant strains, as these were tolerant to a nisin concentration as high as 1400 IU ml^{-1}, and alternately by *L. lactis* N8. Preliminary studies showed that the LAB strains used in the present study – *Lb. plantarum*, *L. mesenteroides*, and *Lb. sakei* – were able to grow in the presence of pure (commercial) nisin at concentrations of 200 – 2000 IU ml^{-1} (data not shown). However, only total LAB were plated through the *L. lactis* cabbage fermentation and the *L. lactis* N8 count was determined, whereas the other strains were not identified. Potentially nisin-resistant LAB strains (or strains capable of surviving in the presence of nisin) were predominant when the count of the nisin producer was low (day 4 and day 13). Nisin produced in situ or added to brined cabbage has been shown to affect the bacteria population in fermentations by delaying (e.g. 300 IU ml^{-1}) or preventing (e.g. 10,000 IU ml^{-1}) the growth of naturally present LAB (Breidt et al. 1995). Moreover, Harris et al. (1992b) found that *Lactobacillus plantarum* was slightly suppressed by a nisin level of 100 AU ml^{-1} and completely inhibited by a level of 600 AU ml^{-1} in a mixed-culture fermentation.

Vegetables, the raw material, are typically dominated by Gram-negative bacteria and yeasts (reviewed by Bűskenhuskes 1997), which may spoil sauerkraut. The yeasts, moulds, and coliformic bacteria were plated in order to define the hygienic quality of the sauerkraut in the present study. Yeasts

and moulds were found at low levels (10^1 -10^3 cfu ml^{-1}) in raw cabbage and in the end-product of the mixed-culture fermentation (F8; *P. dextrinicus* + *L. mesenteroides*), but not in the spontaneous fermentation (F7) or in any of the end-products of the starter (pure) culture fermentations (F9 – F14). Coliformic bacteria were not found in any of the end-products. The results indicated that the inoculation of the cabbage fermentations was sufficient to control the process and prevent spoiling of sauerkraut, and that the hygienic quality of the fermented products was good.

Acids and sugar concentrations in the cabbage fermentations are presented in Table 17 and dry weights in Table 3 (Paper IV). The total sugar concentrations (free + bound in the cabbage matrix) in raw cabbage corresponded to those typical of cabbage cultivated in Finland (glucose 2.6%, fructose 2.1%, and sucrose 0.7% of FW) (Haila et al. 1992). The free glucose increased slightly towards the end of the fermentations, except in the case of the *Lb. sakei* fermentation (data not shown). It is probable that glucose was released from the fiber matrix into the brine during fermentations because of the softening of the cabbage texture and osmotic extraction of water from the cut cabbage tissue (Hammes 1991; Harris 1998). Glucose was also formed due to degradation of glucosinolates; when the thioglucoside linkage is cleaved, glucose and thio-hydroximate-O-sulphonate are released. A part of the released glucose is converted to organic acids (6.1 -12.0 g l^{-1}) by bacteria strains during the fermentation process. It must be noted, however, that only free glucose (glucose in brine) was analyzed during fermentation in the present study and that some glucose may have remained in the cabbage fiber matrix.

The titratable acidity of the sauerkraut (series B) remained at a lower level (0.4 – 1.1%) in this study than typically reported (2.0 – 2.5%) for cabbage fermentations (reviewed by Oliver et al. 1999). *L. mesenteroides* exhibited the strongest acidifying potential in this study, as the titratable acidity rose up to 1.1% (Table 17). The acidity data obtained in the fermentations is in agreement with the literature, where *L. mesenteroides* has been reported to acidify starter culture (single strain) fermentations most effectively, whereas other strains are weaker acid producers (reviewed by Oliver et al. 1999). Mild acidity and low concentration of salt have been reported to enhance the quality of sauerkraut (Fleming et al. 1995). The ascorbic acid found in the fermented cabbage (45 ± 3 mg $100g^{-1}$ FW) in the present study resembled the typical concentration in white cabbage (Fleming et al. 1988; Hägg et al. 1994; Rosa et al. 2001).

5.3.2 Content of glucosinolates

The predominant glucosinolates (GSs) in white cabbage (cv. Lennox) in the present study were sinigrin (35 – 48% of total) and glucoiberin (29 – 30%). Smaller amounts of glucobrassicin (3 – 11%), progoitrin (5 – 9%), glucoraphanin (6 – 8%) and gluconapin (4 – 5%) were found. Glucoraphanin was found in the cabbage at a higher concentration (1.28 μmol g^{-1} DW) than has been reported in the literature (below 0.2 μmol g^{-1} DW) (Tiedink et al. 1988, Kushad et al. 1999; Verkerk 2002). However, there was high variation in the GS concentrations in cabbage during the years 1998 – 2000 (Table 4, Paper IV), possibly due to factors like cultivation soil type, plant spacing, light, temperature, and application of fertilizers, as reported in the literature (Fenwick et al. 1983a; Rosa 1997; Kushad et al. 1999; Verkerk 2002). Kushad et al. (1999) observed that significant differences occurred between white cabbage cultivars in the contents of sinigrin, gluconapin, and progoitrin, whereas the concentrations of indole glucosinolates were relatively similar among the tested cabbage cultivars. Overall, the glucosinolate content may vary depending on the type of treatment, genetic and environmental factors, extent and nature of processing, as well as packaging and storage conditions (Verkerk 2002).

In the present study, the glucosinolate (GS) content was determined in spontaneous (F7) and mixed-culture fermentation (F8) (series A). GS content in fermentations F9 – F14 (series B) was not characterized. As shown in Figure 8, the content of aliphatic GSs sinigrin and glucoiberin increased after spontaneous fermentation was initiated (t = 0 h), achieved the maximum content at 8 hours and then gradually decreased to zero. One sample was taken from the fermentation chambers at day 14 (sauerkraut refrigerated after fermentation completed) to check whether any intact GSs were left in the sauerkraut; 4-MeOH-glucobrassicin was found in small quantities in both fermentations (0.1 μmol g^{-1} DW). The finding that the amount of aliphatic glucosinolates increased in this study differs from data reported by Verkerk et al. (2001). They found that cutting of several *Brassica* vegetables and storage in air resulted in a notable increase especially in indolyl glucosinolates. Because of the different processing conditions – fermentation vs. chopping and air storage – the findings are not comparable. The higher content of aliphatic glucosinolates could be due to the influence of the microbiota, if bacteria affected or interfered with GS degradation and decomposition during the fermentation process.

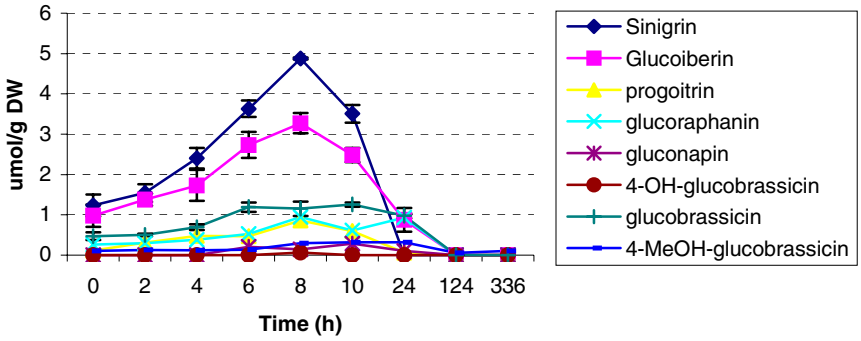

Figure 8. Content of glucosinolates in spontaneous fermentation F7 (n = 3) of white cabbage (cv. Lennox). Sauerkraut was refrigerated when fermentation was complete (pH3.9, 124 h). Time t = 0 refers to GS of untreated cabbage heads.

5.3.3 Formation of glucosinolate breakdown products

To determine the content of glucosinolate breakdown products (BDPs) in fermented cabbage (sauerkraut), we characterized the BDPs in sauerkraut produced by spontaneous fermentation (F7) and fermented with a mixed culture (*P. dextrinicus* + *L. mesenteroides*; F8) (series A; white cabbage cv. Lennox, cultivation year 1998, batch 20 kg, four parallels). As mentioned earlier, the strains used in fermentation trials were selected based on preliminary studies in which the cultures were shown to produce good-quality sauerkraut (unpublished results). No significant differences were found in the content of individual BDPs between the mixed-culture and spontaneous fermentation end-products, which was not surprising as the mixed-culture strains had been previously isolated from spontaneous cabbage fermentation. Thus, the strains *L. mesenteroides* and *P. dextrinicus* might have existed also in the microbiota of spontaneous fermentation (F7). The large size of the fermentation batches (20 kg cabbage each) may have caused the substantially high standard deviation (SD) found in the content of BDPs in sauerkraut in fermentations F7 and F8; raw cabbage was unlikely to be homogeneous in all four batches (parallels) and local differences in microbiota were likely to occur over the fermentation processes.

However, interesting BDPs – AITC, sulphoraphane, and iberin – were found in series A fermentations (F7 and F8). Accordingly, we further studied the decomposing of glucosinolates in the presence of a starter culture and the potential role of bacteria in degradation by using single-culture fermentations (series B; white cabbage cv. Lennox, cultivation years 1999 and 2000, batch 0.5 kg, three parallels): *Lactobacillus plantarum*, *Lb. sakei*, *Leuconostoc mesenteroides*, *Pediococcus pentosaceus*, *Lactococcus lactis* N8, and *L. lactis* LAC67 (F9 – F14) (Table 15). The fermentations (*Lb. sakei*, *Lb.*

plantarum, *L. mesenteroides*, *P. pentosaceus*/*P. dextrinicus*) and the analyses of the BDPs were performed in consecutive years (1999, 2000) and *L. lactis* N8 and LAC67 in year 2000.

The glucosinolate BDPs detected in this study were formed from sinigrin (AITC, allyl cyanide), glucoiberin (iberin), glucoraphanin (sulforaphane), glucobrassicin (I3C), and progoitrin (goitrin). Significant differences ($P < 0.05$) were observed in the content of BDPs between different starter-culture fermentations (F9 – F14, Figure 9), indicating that the degradation of GSs may be affected by the starter bacteria (myrosinase-like activity in bacteria) in addition to the myrosinase endogenous to the plant. The results of the BDP contents in consecutive years showed similarities, which supports the assumption of the role of bacteria. However, plant myrosinase has also been reported to highly active, and so it is not possible to conclude what the role of these two (microbial and endogenous) was based on the data available in the present study.

When *Lb. sakei* was used as the starter culture (F12), the total BDP concentration in the end-products was two- or threefold that of the other strains in series B (Figures 2 and 3, Paper IV). In particular, the concentrations of 3-methylsulphinylpropyl-ITC, allyl-ITC, and sulforaphane were significantly higher ($P < 0.05$) in the fermented end-product of *Lb. sakei* fermentation (F12) than in the other end-products of fermentation series B. Based on the results shown in this study it is presumable that *Lb. sakei* possesses higher myrosinase-like activity in the given conditions than the other strains in this study. The lactic acid bacteria *Lactobacillus acidophilus* (Nugon-Baudon et al. 1990), *Lb. agilis* (Palop et al. 1995), and *Bifidobacterium* sp. (Cheng et al. 2004) have also been found to exhibit myrosinase-type activity (literature review, Table 6) *in vitro* or in the intestinal tract. It is worth noting that the substrate used here differs from conditions reported in the literature, where endogenous myrosinase has not been present.

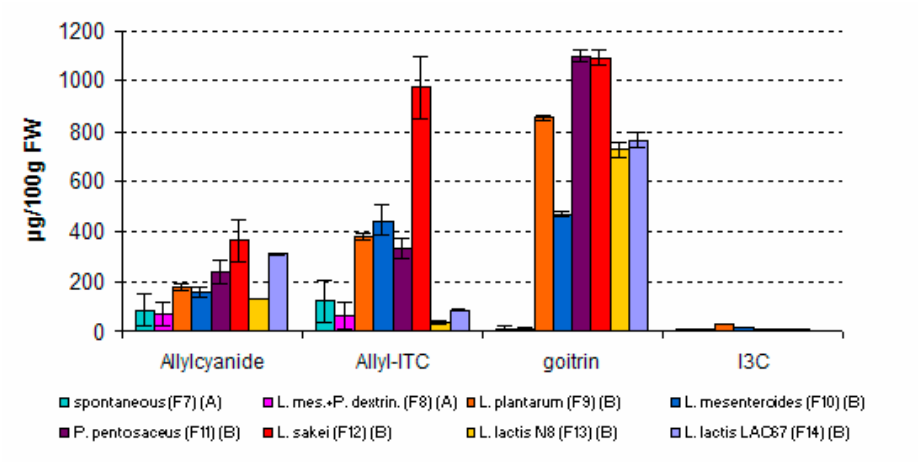

Figure 9 a. Quantified BDPs of glucosinolates (µg 100 g^{-1} FW) in fermentation end products in series A (F7, F8) and series B (F9-F14). Mean value and standard deviation, n=4 (F7, F8); n=3 (F9–F14). ITC=isothiocyanate, I3C=indole-3-carbinol.

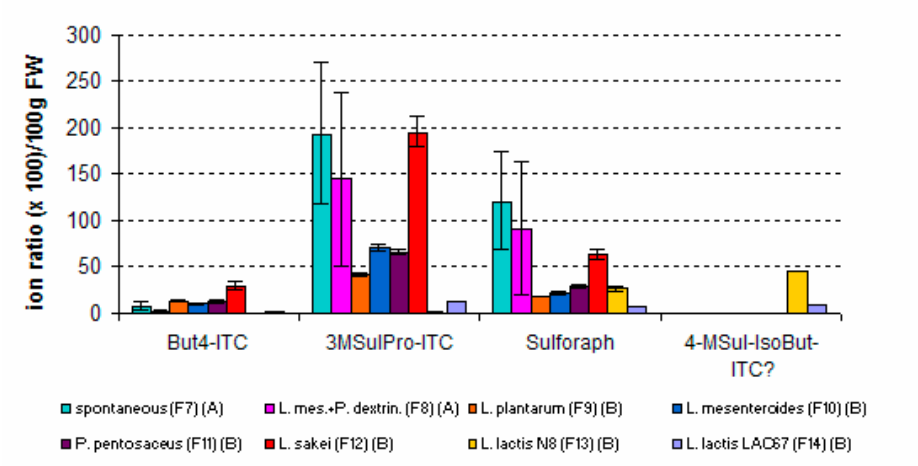

Figure 9 b. Relative ion ratios (x 100) 100 g^{-1} FW of glucosinolate BDPs in fermentation end products in series A (F7, F8) and series B (F9-F14). Mean value and standard deviation, n=4 (F7, F8); n=3 (F9–F14). But=butyl, ITC=isothiocyanate, MsulfPro=methylsulfinylpropyl, sulforaph=sulforaphane.

The total content of BDPs in the end-products of *L. lactis* cabbage fermentation (F13, F14) was low, which may indicate that the glucosinolates had been volatilized or degraded further, forming other BDPs (not analyzed in this study), as suggested by Ashworth (1975) and Heaney and Fenwick (1987). Because of the long-lasting (14 d) *L. lactis* fermentations, a variety of LAB species may have occurred in the course of the fermentation process, but their role in the formation of glucosinolate BDPs remains unclear. The relatively low concentrations of the quantified BDPs in the end-products of the mixed-culture (F8) and spontaneous (F7) fermentations may be due to the low initial concentration of glucosinolates in the raw material, only about 20 – 30% of the level in the raw material of the starter-culture fermentations (F9 – F14). Furthermore, the possible volatilization of some BDPs during freeze storage and the long delay prior to analysis (in the spontaneous and mixed-culture fermentations) can also lower the concentration of glucosinolates (Rangkadilok et al. 2002). The quantities of iberin and sulforaphanin in the fermentations are not known due to lack of commercial standards (Figure 9b).

Indole glucosinolate I3C (from glucobrassicin) was found only in low quantities (below 40 µg in 100 g FW) in all the sauerkraut fermentations (Figure 9a). Glucobrassicin was present at a concentration of 1.7 µmol g^{-1} DW in the cabbage raw material in the year 2000; theoretically, 2500 µg of I3C could have been formed in 100 g of fresh cabbage. Indole glucosinolates may occur partly as ascorbigens and especially as di-indoles (McDanell 1987; Agerbrick 1998), but these were not analyzed in this study. The absence or low concentration of indole glucosinolates may also be due to poor stability of I3C in an acidic solution (Bradfield and Bjeldanes 1987). Nitriles were formed only from sinigrin (allyl-cyanide) in this study. One explanation for the low nitrile amount could be the considerably high pH at the beginning of fermentation, and there was no GS left for degradation when the pH reached the level below pH4, which could enhance nitrile formation. However, Kassahun et al. (1996) have reported 10 times higher nitrile concentrations in fermented cabbage. The thiocyanate ion concentration was below the detection limit in all the fermentations in the current study.

The concentration of AITC was 1000 µg 100 g^{-1} FW (10 ppm) in cabbage fermented with *Lb. sakei*, and 350 – 450 µg 100 g^{-1} FW (3.5 – 4.5 ppm) in cabbage fermented with *Lb. plantarum*, *L. mesenteroides* and *P. pentosaceus*. This indicates that *Lb. sakei* may possess more GS degradation activity than the other strains used in this study and may be a potential strain for ITC production in food applications (Mäki et al. 2003). Further studies are needed to investigate the GS degradation ability (myrosinase-like activity) of these strains by using pure glucosinolates as substrates and, moreover, to compare the activity to the plant endogenous myrosinase.

One point of interest in the present study was to characterize BDPs in a natural substrate, sauerkraut, which is widely used and referred to as a health food. It has been reported that ITCs and I3C (*Brassica* vegetables) in the diet enhance the synthesis of detoxification enzymes and that the induction of detoxification enzymes is dose-dependent (Staack et al. 1998; Bonnesen et al. 2001). Dietary I3C has been shown to cause a bifunctional induction of detoxifying enzymes, acting as an anticarcinogen in concentrations of 50 – 1000 ppm (5 - 100 mg 100 g^{-1} FW) in the diet (Guo et al. 1992; Staack et al. 1998). Accordingly, the low level of I3C (10 - 40 μg 100 g^{-1} FW = 0.1 – 0.4 ppm) found in this study is obviously not alone sufficient to enhance the synthesis of detoxifying enzymes. However, the mixture of glucosinolate BDPs (ITC, I3C and sulforaphane) in the present study may have health benefits; combinations of indoles and isothiocyanates have been suggested to give greater health benefit than the use of a monofunctional inducing agent on its own (Staack et al. 1998; Bonnesen et al. 2001). Staack et al. (1998) found that 3-methyl-sulphinylpropyl-ITC (38 ppm) and phenylethyl-ITC (0.1 ppm) showed no detoxification (induction) effects when alone, whereas they caused additive induction effects when used in combination with I3C (56 ppm) (rats, dosing 7 days).

The genotoxic effects of glucosinolate BDPs in the present study are not worth consideration, as these occur in humans only at very high doses, more than 100 times the exposure level found in an average diet (Kassie and Knasmuller 2000). AITC has been reported to be toxic to rats at a concentration of 2 – 4 mg (oral, 60 days) (reviewed by Stoewsand 1995). Further, goitrin may cause toxic effects (inhibits iodine uptake of the thyroid gland in humans) in concentrations of 50 – 200 mg (oral) (Stoewsand 1995). Goitrin was found at a concentration of 1 mg 100g^{-1} FW in the present study, far from a toxic concentration in humans.

It is possible that fermentation enhances the bioavailability of these compounds (glucosinolate breakdown product, BDPs) in humans. However, the intake of bioactive compounds varies considerably depending on the diet, and the release and adsorbtion of BDPs in the intestinal tract is not known and could be complicated. Thus, the influence of fermentation on these compounds in the human body need to be studied.

5.3.4 Content of other molecules

5.3.4.1 Nitrate and nitrite

The nitrate content in the raw material of the starter-culture fermentations (F9 – F12) was 130 ± 20 mg kg^{-1} FW throughout the fermentations. The concentration dropped below the detection limit (10 mg kg^{-1} FW) at the end of the fermentation process, except in the case of *Lb. sakei* (F12) in which roughly 50% of the initial nitrate concentration was degraded (60 mg kg^{-1} FW in the end-product). In the spontaneous (F7) and mixed-culture (F8) fermentations, the nitrate concentration remained at the same level throughout the fermentations (100 – 200 mg kg^{-1} FW). Furthermore, the concentration of nitrite was below the detection limit (10 mg kg^{-1} FW) in all fermentations. Nabrzyski et al. (1989) have reported that the concentration of nitrate dropped to 42% of the initial level after 7 days' fermentation, but no significant changes were observed in the content of nitrites in the course of the fermentation process.

It is worth noting that indole compounds and nitrite were found at very low levels in the present study, which may indicate the formation of N-nitroso compounds, but these were not analyzed in this study. Nitrate may convert to nitrite and further act as a precursor of nitroso compounds, which are reported to have carcinogenic properties (Table 7) (Yang et al. 1984; Walters 1992; reviewed by Jongen 1996). However, it is reported in the literature that nitrosated products are stable only in the presence of large amounts of free nitrite (Jongen 1996). In any case, the concentrations of nitrate and nitrite in the present study were below ADI (acceptance daily intake; 3.65 mg nitrate and 0.2 mg nitrite per kg body weight), and, therefore, do not represent any risk to human health (WHO 1974; 1980).

5.3.4.2 Flavonoids

The content of flavonoids was measured both from the raw material and the end-product of the spontaneous (F7) and mixed-culture fermentations (F8). *Brassica*ceae plants are reported to be a weak source of flavonols and flavonoids (Herrmann 1988). In the present study, kaempferol was detected at a concentration of 0.9 mg kg^{-1} FW, but no other flavonoids or flavonols were found. Hertog et al. (1992a) have reported an amount of kaempferol approximately equal to the present study, whereas Chu et al. (2000) found flavonoid concentrations as high as 100 – 200 mg kg^{-1} FW (quercetin and kaempferol) in cabbage. The kaempferol concentration in the present study remained at a constant level in the cabbage (in solid portion) throughout fermentation and no flavonoids were detected in juice, indicating that the flavonoids stayed in the cabbage fiber matrix over the fermentation process and were not leaked into the brine. This finding is in agreement with earlier studies according to which flavonoids mainly remain in the plant fiber matrix and are not transferred into (berry or fruit) juice (van der Sluis et al. 1999;

Häkkinen et al. 2000). Flavonoids in vegetables and berries have been studied to some extent, but there are no reports on the effect of fermentation on degradation of flavonoids in cabbage.

5.3.5 Antimicrobial activity of cabbage juice

The antimicrobial effects of cabbage juice (fermented with *Lb. plantarum*, *Lb. sakei*, *L. mesenteroides*, *L. lactis*, *P. pentosaceus*) on the indicator microbes in this study are shown in Figure 4 a-f (Paper IV). *Candida lambica*, *E. coli*, and *L. monocytogenes* were used as the indicator strains to illustrate the antimicrobial effects of cabbage juice containing nisin and/or glucosinolate BDPs. *C. lambica* is a typical spoiling yeast in fermented cabbage, *E. coli* represents Gram-negative bacteria, and the food pathogen *L. monocytogenes* sp. is known to be sensitive to nisin and is widely used in studies of the antimicrobial properties of nisin (Table 10). Furthermore, *L. monocytogenes* is reported to grow in cabbage and in cabbage juice (Beuchat et al. 1986; Conner et al. 1986), and may cause food poisoning, e.g. via cole slaw (Conner et al. 1986).

Figure 4 (Paper IV) shows the different types of inhibition caused by the fermented end-products on the indicator strains. No antibacterial effects were observed in any juice pressed at the beginning of fermentation (t = 0 h) or in fermented cabbage juices in dilutions of 1:10. Fermented cabbage juice (in dilutions of 1:1 and 1:5) showed dose-related inhibitory effects. *C. lambica* was most effectively inhibited by the juice from the *Lb. sakei* and *L. mesenteroides* fermentations, which might be explained by the high concentration of ITCs (*Lb. sakei*: AITC 10 ppm and *L. mesenteroides* AITC: 4.5 ppm in sauerkraut) (Figure 9a and b). However, it is worth noting that glucosinolate BDPs were analyzed from sauerkraut (cabbage + brine) whereas the antimicrobial tests performed in juice. Thus, the content of BDPs in juice is not accurately known. AITC has been reported to strongly inhibit the growth of both oxidative and fermentative yeasts at a concentration (MIC) as low as 1 - 4 ppm (Kyung and Fleming 1997). AITC also inhibited the bacteria *Salmonella* typhimurium (100 ppm), *L. monocytogenes* (200 ppm) (Kyung and Fleming 1997), and *E. coli* B34 (50 - 200 ppm) (Kyung and Fleming 1997; Shofran et al. 1998). In the present study, *E. coli* was strongly inhibited in all juices and *L. monocytogenes* in all juices except that fermented with *L. sakei*. This indicates that *L. monocytogenes* was not sensitive to ITC in the conditions used in the present study.

The antimicrobial properties of commercial nisin and nisin produced by *L. lactis* N8 and *L. lactis* LAC67 were evaluated in Paper IV (Figure 5). *E. coli* and *C. lambica* did not show sensitivity to commercial nisin (unpublished results) in cabbage juice, whereas *L. monocytogenes* was sensitive at concentrations of 10 - 100 IU ml^{-1}. It was not possible to draw conclusions about the synergistic antimicrobial properties of nisin and glucosinolate

BDPs on the strain *L. monocytogenes* EELA L215. However, it must be kept in mind that only one strain of each indicator microbe was used. Different *L. monocytogenes* strains have different sensitivity to nisin; e.g. a lower fatty acid ratio of C15:C17 in the *L. monocytogenes* membrane structure results in increased tolerance to nisin (reviewed by Cleveland et al. 2001). Further, the effectivity of nisin is dose-dependent; the concentrations of nisin used in this study (0.1, 1, 5, 10, 100 IU ml^{-1}) were lower than those commonly used in food application tests (Table 10). In a real food system, there are many factors such as emulsifier, phospholipids, and fatty acids, that appear to interfere with nisin (Mazzotta and Montville 1997), and higher concentrations are needed to effectively inhibit food-borne pathogens in the food matrix. Nykänen (2001) reported, e.g., that injecting of nisin into cold smoked salmon should be optimized so that nisin and other ingredients are sufficiently scattered in the products and to ensure that their concentrations are appropriate. However, a concentration as low as 100 IU ml^{-1} has been shown to act effectively against some strains of *L. monocytogenes* in ricotta cheese (Davies et al. 1997) and in kimchi (Choi and Park 2000).

The results of this study indicate that the fermented cabbage juice possessed inhibitory effects. Whether the effective compounds were glucosinolate BDPs was not shown, but neither can they be excluded. The role of some compounds that have been reported to cause antimicrobial effects – e.g. diacetyl, hydrogen peroxide, organosulfur phytochemicals, and bioactive peptides – were not determined in the present study. Diacetyl has, however, been found in fermented foods at concentrations of 0.2 - 1.5 ppm (Helander et al 1997), which are too low to effectively inhibit microorganisms. (reviewed by Caplice and Fitzgerald 1999). Further, hydrogen peroxide (H_2O_2) was not likely to occur in the anaerobic cabbage fermentations, as H_2O_2 is formed in presence of oxygen in aerobic conditions. The organosulfur components methyl methanethiosulfinate, s-methyl-L-cysteine sulfoxide, and sulfides are found in unfermented cabbage and have been reported to possess antimicrobial properties on some strains (Kyung and Fleming 1994b; Kyung and Fleming 1997). The effect of nitrate and nitrite on the inhibition of *E. coli* and the yeast *C. lambica* (in juice fermented with *L. sakei*) was not worth consideration due to the low concentration (nitrate 60 ppm; nitrite below detection limit); nitrate is reported to reduce the growth of *Clostridium botulinum* at a concentration of 680 ppm (Hyytiä et al. 1997). Further, it has been shown that organic acids (acetic and lactic acid) are compounds which can inhibit a broad range of microorganisms (Nout and Rombouts 1992), especially when undissociated. In the present study, the effect of the pH on inhibitory activity was eliminated by buffering the cabbage brine samples from pH~4 to pH 6.5 before antimicrobial analysis.

The LAB used in the fermentation trials were tested for their ability to produce bacteriocins; only *L. lactis* N8 was capable of producing bacteriocin (nisin) under the conditions used in this study. However, the presence of

other bacteriocins in *L. lactis* fermentations cannot be precluded; a variety of cell morphology was observed during the *L. lactis* fermentations (data not shown), and bacteriocin-producing strains may have occurred. The strains were not identified or classified in the *L. lactis* fermentations. For example, the strain *Lactobacillus sakei* has been reported to produce bacteriocins such as sakacin P (Katla et al. 2002), sakasin A (Schillinger and Lucke 1989), and lactocin S (Mortverdt et al. 1991) to inhibit, e.g., other lactobacilli and *L. monocytogenes*.

6 Summary and conclusions

1. In this study, nisin was produced in M17G medium at concentrations of 2200 - 2500 IU ml^{-1} by *L. lactis* N8 and *L. lactis* LAC48 in the batch fermentors and in a circulated batch. A high concentration of nisin (8000 IU ml^{-1}) was achieved by binding and eluting nisin from Amberlite XAD-4 resin. *L. lactis* N8 showed a sharp decline in the production of nisin in M17G medium, as is typical of nisin production. In the case of LAC48, however, the decrease from the maximum nisin titer was not sharp, giving more flexibility as to when nisin should optimally be harvested. Maximum nisin production by LAC48 was not directly related to biomass formation, nor was it associated with growth. N8 and LAC48 were capable of substituting the lost quantity of nisin (harvested by Amberlite XAD-4 binding resin) to the fermentation broth, thereby reaching the highest achievable nisin concentration under the used conditions.

2. The proteins NisB and NisC were needed for the modification and secretion of fully active nisin. NisB was required for dehydration and NisC for correct lanthionine formation in the post-translational modification of nisin. NisB seemed to be responsible for the dehydration reaction, not requiring NisC for the reaction.

3. Nisin was produced during the *L. lactis* N8 cabbage fermentation (14 days) at concentrations of 250 – 1400 IU ml^{-1}. The growth of the food spoilage bacteria *Listeria monocytogenes* EELA L215 was reduced in cabbage juice fermented with *L. lactis* N8.

4. Sinigrin, glucoiberin, and glucobrassicin were the main glucosinolates found in the white cabbage, cv. Lennox, used in this study, and kaempferol was the sole flavonoid. Glucosinolates were decomposed during the cabbage fermentation process to form several potentially beneficial BDPs such as isothiocyanates (ITCs) and sulforaphane. Besides endogenous myrosinase, lactic acid bacteria may also affect the degradation of glucosinolates and the formation of BDPs in cabbage

fermentation. Cabbage juice fermented with *Lb. sakei* showed the highest amount of isothiocyanates.

5. The cabbage juices exhibited inhibitory effects on the indicator strains *E. coli*, *L. monocytogenes*, and *C. Lambica*. The strongest inhibitory effects of cabbage juice were achieved by juice fermented with the strains *Lb. sakei* and *L. mesenteroides*, which also possessed the highest concentration of ITCs. Further, the juice fermented with *Lb. sakei* was capable of effectively inhibiting the growth of *C. lambica* and *E. coli*, but not of *L. monocytogenes*.

Based on the results of this research it is suggested that fermented cabbage could be studied further as a potential functional food containing health-promoting agents. Beside functionality, the combined effects of glucosinolate BDPs and nisin (8000 IU ml^{-1} in NaCl-ethanol obtained in this study) could be utilized for other food applications – e.g., to inhibit food spoilage microbes, thus extending the shelf-life of food and enhancing food safety. However, more research is needed to optimize the production of these compounds, investigate the amount and stability of glucosinolate BDPs, as well as to determine the effects of these compounds on food product safety.

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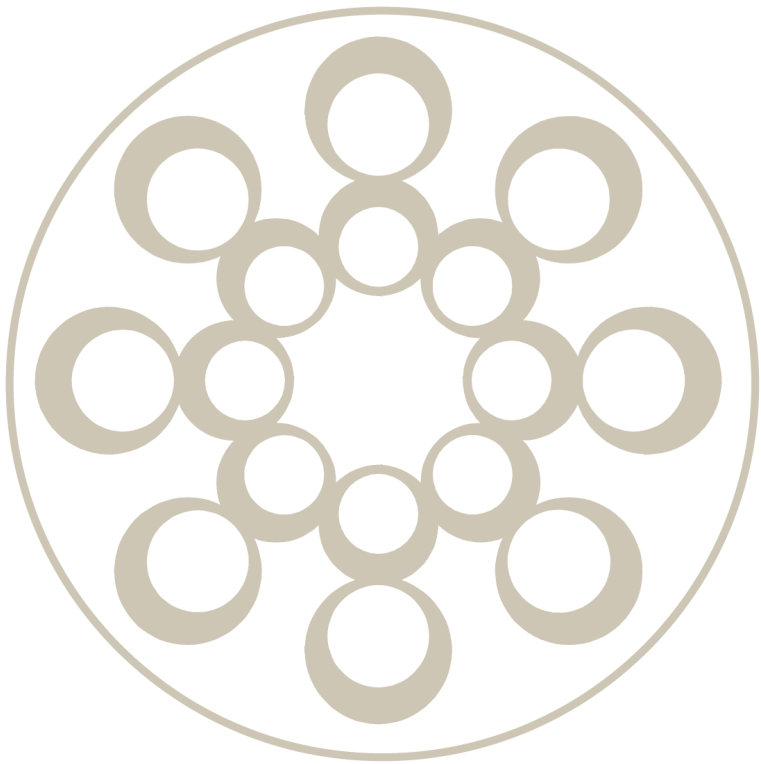

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