Università degli Studi di Sassari

Scuola di Dottorato di Ricerca

Scienze dei Sistemi Agrari e Forestali e delle Produzioni Alimentari

Direttore: Prof. Giuseppe Pulina

Indirizzo

Biotecnologie microbiche e agroalimentari

Coordinatore: Marilena Budroni

XXII Ciclo – Triennio Accademico 2007-2009.

STUDIO DI PEPTIDASI COINVOLTE NELL'ATTIVITÀ PROTEOLITICA DI *LACTOBACILLUS PLANTARUM*

STUDY OF PEPTIDASES INVOLVED ON PROTEOLYTIC ACTIVITY OF *LACTOBACILLUS PLANTARUM*

Tesi di dottorato: Dott. Mauro Forteschi

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Introduction

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Lactic acid bacteria

Lactobacillus is the largest genus among the genera that comprise the lactic acid bacteria (LAB) (1), with over 100 species described to date (October 2006; http://www.ncbi. nlm.nih.gov/Taxonomy). The *Lactobacillus plantarum* species is a common constituent of the microflora of many fermented foods, such as dairy products, meat, vegetables and plants, and it has also been isolated from a wide variety of nutrient-rich habitats and environmental niches (2) (3) (4) (5). Metabolically, it is a facultative heterofermentative bacterium that has an elaborate proteolytic system for peptide degradation, while it can also synthesise most amino acids *de novo* (6). Moreover, *L. plantarum* has often been isolated from the human gastrointestinal tract, and the strain *L. plantarum* 299v is considered as a probiotic that can provide beneficial effects for the health of the consumer. Several studies have focused on the use of *L. plantarum* as a vehicle for the delivery of therapeutic compounds due to its ability to survive in the human gastrointestinal tract (7).

The complexity of the LAB proteolytic system has been highlighted by detailed studies over the last 20 years. The number of different proteinases and peptidases with different and wide specificities and cellular distributions are the main aspect of this complexity. The proteolytic system of LAB is the main tool used by this bacteria to establish a selective advantage for growth in complex environments.

The *L. plantarum* WCFS1 genome was the first *Lactobacillus* to be sequenced (6). No genes coding for peptidases, the enzymes responsible for the first steps in protein and large peptide degradation, have been found in the *L. plantarum* genome. However, *L. plantarum* has peptide-uptake systems (Opp and Dtp), and once internalised, the peptides are subjected to the actions of a number of peptidases.

Many peptidases have been extensively studied in lactococci and lactobacilli over the last 10 years (28, 29). In the *L. plantarum* genome, 19 genes code for peptidases (Table 1). With the exception of the pathways leading to the branched-chain amino acids, valine, leucine and isoleucine, the complete pathways for the biosynthesis of all other amino acids is present in the *L. plantarum* genome. These genes are organised into large clusters, or operons (8).

The *L. plantarum* metabolic pathways have been reconstructed (9) (8) in the LACPLANTCYC (www.lacplantcyc.nl) database, and 129 pathways are available as reference systems for pathway predictions for other LAB. Other genome-based studies of *L. plantarum* include the characterisation of four prophage elements (10), and the prediction of co-regulated transcriptional units (11).

Proteolytic system

Proteinases

The first step in protein assimilation by LAB is protein and large peptide degradation; this step is carried out by the cell-envelope proteinase (CEP). Several enzymes have already been cloned and characterised from LAB (e.g. PrtP from *L.*

lactis) (12-17). In lactococci, the prtP enzyme can be encoded by a plasmid or by the chromosome genes ,whereas lactobacilli CEPs are only genome encoded. LAB usually have just one CEP. The CEPs of LAB are synthesised as pre-proproteins of *ca.* 2,000 amino acids, which are organised into distinct functional domains (16) (Fig. 1): the pre-prodomain (PP) of CEPs is a signal sequence (~40 residues) that is required for secretion; the prosequence (~150 residues), which is removed by autocatalytic processing; the catalytic domain (serine protease) (PR) (~500 residues); the insert domain (I) (~150 residues), which is responsible for CEP substrate specificity; the A domain (~400 residues), with still unclear functions; the B domain (~500 residues), which might be involved in processes of stabilisation of the protein and its activity/ specificity; the helix domain (H) (~200 residues), which is responsible for spatial positioning of the A and B domains to the external side of the cell envelope; and a W domain (~100 residues), which functions as a cell-wall spacer. Some differences between the domain organisation have been descibed: for example, the B domain that is found in most of the CEPs is not present in PrtS of *S. thermophilus*, and the H domain is present only in PrtP (210 aa), PrtS (367 aa), and PrtH (72 aa).

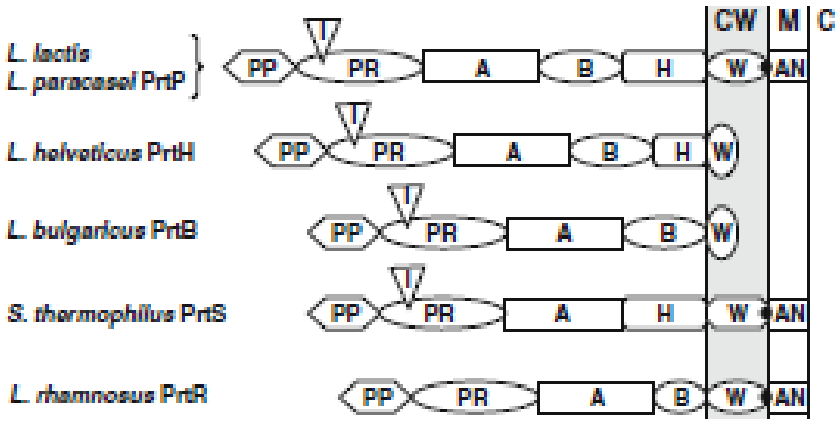

Figure 1. Schematic representation of the CEPs of different LAB strains, modelled according to Siezen (1999) (16). CW, Cell wall; M, membrane; C, cytoplasm; PP, pre-prodomain; PR, catalytic domain; I, insert domain; A, A domain; B, B domain; H, helix domain; W, cell-wall spacer domain; black dot, sorting signal; and AN, anchor domain (20).

In *L. lactis*, the PrtP gene is preceded by a gene coding for a membrane-bound lipoprotein (PrtM) that has been shown to be essential for autocatalytic maturation of PrtP (2324). Similar genetic organisation has been demonstrated for two genes in *L. paracasei* (13). Recently, investigations into the PrtP-like proteinase have shown the presence of its gene in the *L. plantarum* BGSJ3-18 natural strain (25). This gene showed 95% identity with the *L. paracasei, L. casei* and *L lactis* PrtP gene. The *L. plantarum* PrtP-like gene is located on a plasmid and this could be due to horizontal gene transfer between the species. This transfer might be the result of the contemporary presence of different LAB species in the same environmental niche, like cheese or sourdough (25). To date, no data are available concerning the structure and specificity of this PrtP-like proteinase.

After protein degradation, peptide uptake is the second step of nitrogen assimilation. This step consist of the transport of peptides in to the cells, where they are further hydrolysed by endocellular peptidases. Peptide uptake is controlled by the oligopeptide permease proteins (Opp), which are members of the

ATP-binding cassette (ABC) transporter superfamily. The Opp transporter is composed of five proteins: one oligopeptide-binding protein (OppA), two integral membrane proteins (OppB and OppC), and two nucleotide-binding proteins (OppD and OppF). The DtpT transporter, which is a secondary transporter, belongs to the PTR family of peptide transporters. The Dpp system (previously designated as DtpP) is an ABC transporter that depends on ATP or a related energy-rich phosphorylated intermediate. The transcriptional repressor CodY senses the internal pool of branched-chain amino acids (isoleucine, leucine and valine) that inhibit the transcription of the Opp genes, among others. In *L. lactis* MG1363, the gene that codes for the second peptide-binding protein of the Dpp system, DppP, contains nonsense and frameshift mutations, which are indicated by asterisks in Figure 2.

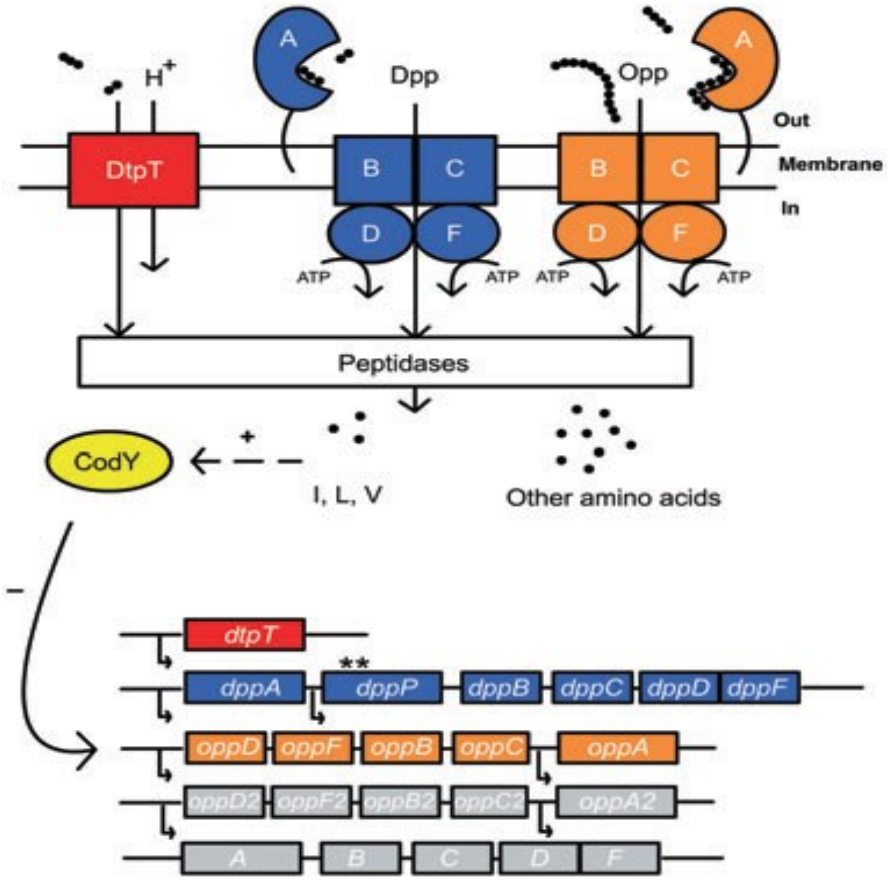

Figure 2. Schematic overview of function, regulation and genetic organisation of the peptide transporters in *L. lactis* MG1363 (26).

Presumably originating from a duplication of the Opp operon, a second set of oligopeptide transporter genes has been described for *L. lactis* MG1363, and the genes are designated as oppD2F2B2C2A2 (28) (Fig. 2). An OppA deletion mutant impaired the ability of the mutant strain to use oligopeptides, which indicates that in *L. lactis* MG1363, this second Opp system is either not expressed or non-functional. The oppA knock-out complementation with the peptide-binding protein OppA2 restored the ability of the strain to use oligopeptides, providing strong evidence of the functional interaction between the OppA2 protein and the OppBCDF translocator.

Analysis of the *L. lactis* MG1363 genome has revealed that next to opp and opp2, other dipeptides/ oligopeptides/ nickel transporter homologues are present (Fig. 2). If this system is expressed and whether or not it is functional as a peptide transporter is not yet clear.

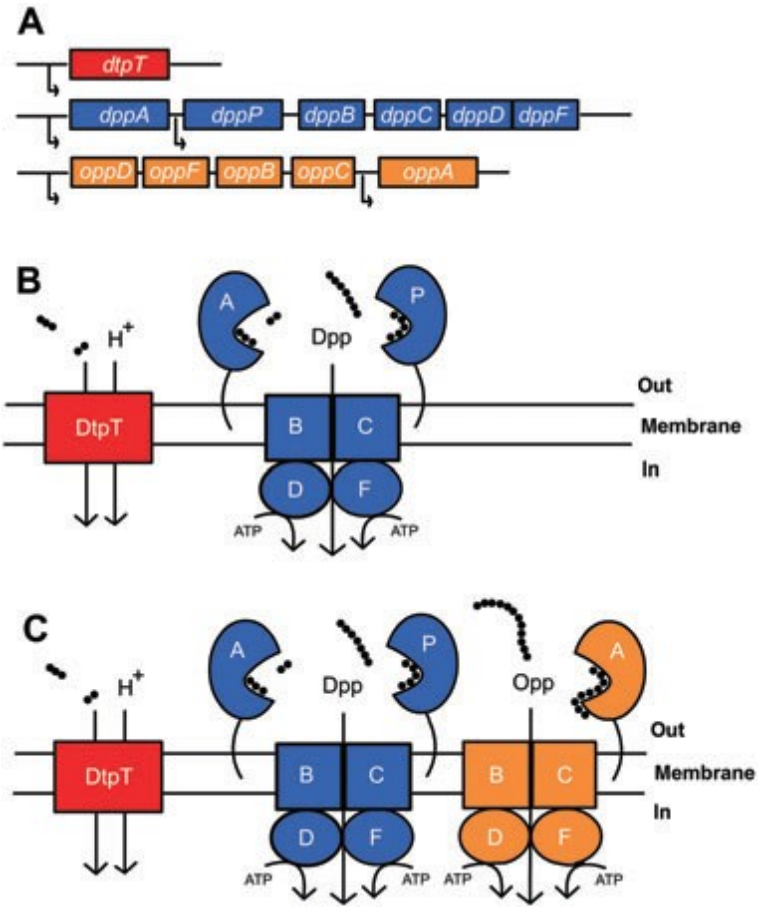

Figure 3. Schematic overview of the genetic organisation of peptide transporter genes and function in *L. lactis* IL1403 and other lactococcal strains. DtpT is presumed to be functional in all lactococcal strains. A. Genetic organisation of peptide transporter genes in IL1403. The dppP gene does not contain the nonsense and frameshift mutations observed in MG1363. B. In IL403 and SKM6, the Dpp system is used for the uptake of di/tripeptides (DppA) and oligopeptides (using DppP). The Opp system is not used due to a lack of OppA production. C. Strains SK11 and Wg2 are equipped with at least two functional oligopeptide transporters, Opp and Dpp, and the latter uses DppP as an oligopeptide-binding protein (25)

Peptidase

After the peptides are taken up by the LAB, they are degraded by the concerted actions of peptidases that have differing and partially overlapping specificities

(32) (Fig. 4). Peptidases from *Lactococcus* and *Lactobacillus* strains have been purified and biochemically characterised, and in most cases, the corresponding genes have been cloned and sequenced (Table 1). No enzyme with carboxypeptidase activity has been reported to date for any LAB. General aminopeptidases (PepN and PepC), and the X-prolyl dipeptidyl aminopeptidase (PepX) are the first enzymes to act on peptides. Collectively, these enzymes can remove the N-terminal amino acids from a peptide, with their specificity depending on the peptide length and the nature of the N-terminal amino-acid residue (37) (38). The pathways for use of peptides follow a series of intracellular events that are carried out by peptidases in the cytoplasm (Fig. 4). Di/tripeptides generated by endopeptidases, general aminopeptidases, and PepX are then subjected to additional cleavage by the tripeptidase, PepT, and dipeptidases, PepV and PepD (Table 1). These enzymes show greater activities towards peptides containing hydrophobic amino acids, including leucine, methionine, phenylalanine and glycine. A common feature of endopeptidases is their inability to hydrolyse intact casein, while they can hydrolyse internal peptide bonds of casein-derived peptides.

L. lactis PepF can cleave oligopeptides that are from 7 to 17 residues long; moreover, this enzyme is also important for protein turnover under conditions of nitrogen starvation in *L. lactis* (35) (36). An enzyme with specificity towards di/tripeptides with N-terminal leucine residues and dipeptides containing proline was characterised biochemically from *L. bulgaricus* (41). Other peptidases with more specific substrate specificities include: PepA, which liberates N-terminal acidic residues from peptides that are three to nine residues long; PepP,

which is selectively specific for tripeptides with proline in the middle position; PepR and PepI, which act on dipeptides containing proline in the penultimate position; PepQ, which cleaves dipeptides with proline in the second position; and PepS, which shows specificity for peptides containing two to five residues, with Arg or aromatic amino-acid residues in the N-terminal position (37) (38) (42) (Table 1).

Table 1. Catalytic classes of peptidases, according to sequence analysis and biochemical characterisation. M, metallopeptidase; C, cysteine-peptidase; S, serine peptidase. ↓ indicates the cleavage site (20).

Peptidase	LAB strain	Type[a]	Reference
	Endopeptidases NH2–Xn↓Xn–COOH		
PepO	*L. lactis P8-2-47*	M	Mierau et al. 1993
	L. lactis SSL135	M	Tynkkynen et al. 1993
	L. helveticus CNRZ32	M	Chen and Steele 1998
	L. rhamnosus HN001	M	Christensson et al. 2002
PepO2	*L. lactis IL1403/NCDO763*	M	Nardi et al. 1997
PepF1	*L. lactis NCDO763*	M	Monnet et al. 1994
PepF2	*L. helveticus CNRZ32*	M	Nardi et al. 1997
PepO2	*L.lactis IL1403/NCDO763*	M	Chen et al. 2003
PepO3	*L. helveticus CNRZ32*	M	Sridhar et al. 2005
PepE	*L. helveticus CNRZ32*	C	Fenster et al. 1997
PepE2	*L. helvetiucs CNRZ32*	C	Sridhar et al. 2005
PepF	*L. helvetiucs CNRZ32*	M	Sridhar et al. 2005
PepG	*L. delbrueckii subsp. lactis DSM7290*	C	Klein et al. 1997
PepO	*S. thermophilus A*	M	Chavagnat et al. 2000
	Aminopeptidases NH2–X↓Xn–COOH		
PepN	*L. lactis Wg2*	M	Strøman 1992
	L. lactis MG1363	M	Tan et al. 1992
	L. delbrueckii subsp. lactis DSM7290	M	Klein et al. 1993
	L. helveticus CNRZ32	M	Christensen et al. 1995
	L. helveticus 53/7	M	Varmanen et al. 1994
	S. thermophilus A	M	Chavagnat et al. 1999
PepC	*L. lactis AM2*	C	Chapotier et al. 1993
	L. delbrueckii subsp. lactis DSM7290	C	Klein et al. 1994a
	L. helveticus CNRZ32	C	Fernández et al. 1994
	L. helveticus 53/7	C	Vesanto et al. 1994
	S. thermophilus A	C	Chapot-Chartier et al. 1994
	Aminopeptidases NH2–Glu/Asp↓Xn– COOH		
PepS	*S. thermophilus A*	M	Fernandez-Espla 1999
PepA	*L. lactis FI1876*	M	I'Anson et al. 1995
PepL	*L. delbrueckii subsp. lactis DSM7290*		S Klein et al. 1995
	Tripeptidases NH2–X↓X–X–COOH		
PepT	*L. lactis MG1363*	M	Mierau et al. 1994
	L. helveticus 53/7	M	Savijoki and Palva 2000
	Dipeptidases NH2–X↓X–COOH		
PepD	*L. helveticus 53/7*	C	Vesanto et al. 1996
	L. helveticus CNRZ32		C Dudley et al. 1996
PepV	*L. lactis MG1363*	M	Hellendoorn et al. 1997
	L. delbrueckii subsp. lactis DSM 7290	M	Vongerichten et al. 1994
	Proline-specific NH2–X↓Pro–COOH		
PepQ	*L. delbruecki subsp. lactis DSM7290*	M	Stucky et al. 1995
	L. bulgaricus B14	M	Rantanen and Palva 1997
	L. bulgaricus CNRZ 397	M	Morel et al. 1999
	NH2–Pro↓Xn–COOH		
PepI	*L. bulgaricus CNRZ397*	S	Gilbert et al. 1994
	L. delbrueckii subsp. lactis DSM7290	S	Klein et al. 1994b
	L. helveticus 53/7	S	Varmanen et al. 1996a
	NH2–Pro↓X–COOH		
PepR	*L. helveticus CNRZ32*	S	Dudley and Steele1994
	L. helveticus 53/7	S	Varmanen et al. 1996b
	L. rhamnosus 1/6	S	Varmanen et al. 1998
	NH2–X–Pro↓Xn–COOH		
PepX	*L. lactis NCDO763*	S	Nardi et al. 1991
	L. delbrueckii DSM7290	S	Meyer-Barton et al. 1993
	L. helveticus CNRZ32	S	Yüksel and Steele 1996
	L. helveticus 53/7	S	Vesanto et al. 1995
	L. rhamnosus 1/6	S	Varmanen et al. 2000a

	S. thermophilus ACA-DC4	S	Anastasiou et al. 2002
	NH2–X↓Pro–Xn–COOH		
PepP	*L. lactis*	M	Matos et al. 1998

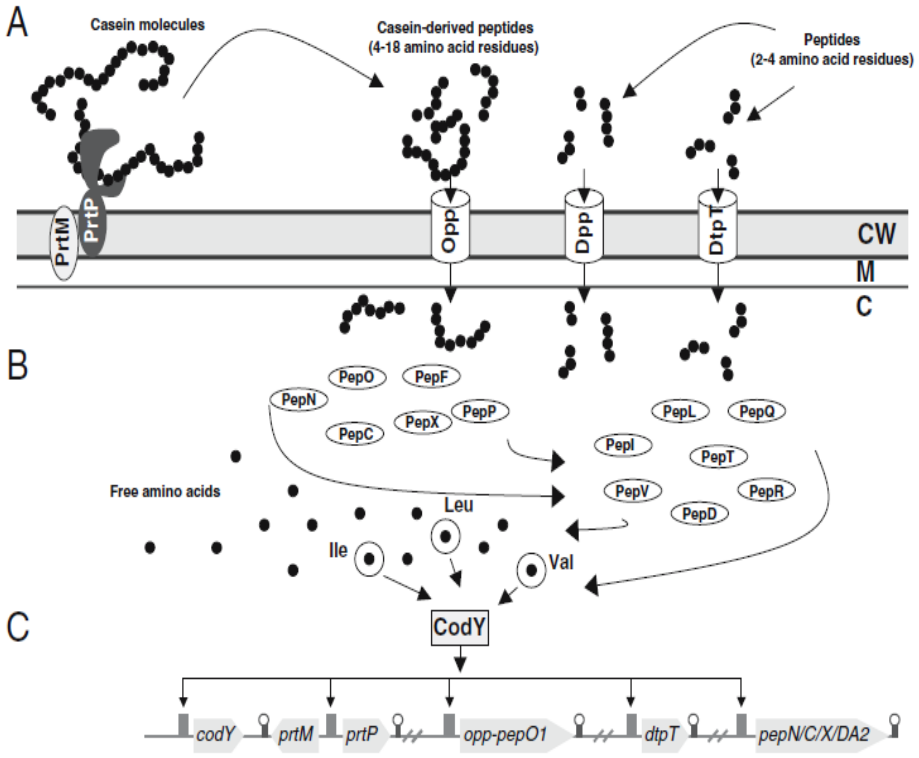

Figure 4. Simplified representation of function and regulation of the proteolytic system of lactococci for casein breakdown (37) (38) (26). A. PrtP, cell-envelope proteinase; Opp, oligopeptide permease; DtpT, the ion-linked transporter for dipeptides and tripeptides; and Dpp, the ABC transporter for peptides containing two to nine amino-acid residues. B. Intracellular peptidases. PepO and PepF, endopeptidases; PepN/PepC/PepP, general aminopeptidases; PepX, X-prolyl dipeptidyl aminopeptidase; PepT, tripeptidase; PepQ, prolidase; PepR, prolinase; PepI, proline iminopeptidase; and PepD and PepV, dipeptidases D and V. C. The transcriptional repressor CodY senses the internal pool of branched-chain amino acids (isoleucine, leucine and valine); using these residues as cofactors, CodY represses the expression of the genes that comprise the proteolytic system in *L. lactis*. (20)

Distribution of the proteolytic system components in sequenced LAB genomes

An overview of the distribution of the components of the proteolytic system identified in 22 completely sequenced LAB was given by Siezen in 2010 (43). Some of the enzymes, such as the cell-wall bound proteinase (PrtP), are only

found in a few LAB strains. Indeed, PrtP was only found in the chromosomes of *L. acidophilus*, *L. johnsonii*, *L. bulgaricus*, *L. casei*, *L. rhamnosus* and the *S. thermophilus* LMD9 strain, as well as on the plasmid of *L. lactis* subsp. *cremoris* SK11 (44), while the endopeptidase PepE/PepG and the proline endopeptidase PepI/PepR/PepL superfamilies are absent from both lactococci and streptococci. On the other hand, most of the peptidases appear to be indispensable for bacterial growth and survival, as they are coded for in all of the LAB genomes. Moreover, some LAB genomes have two homologue peptidases, that possibly have the same function, e.g. two PepC homologues (GI codes: 42518641 and 42518638) in *L. johnsonii*. Essential peptidases that are found in all of the LAB genomes, such as endopeptidase PepO and dipeptidase PepV, are coded for by multiple paralogous genes. *L. acidophilus*, *L. brevis*, *L. casei*, *L. rhamnosus* and *L. lactis* strains have all three of the known LAB peptide transport systems, i.e. the di/tripeptide Dpp and DtpT systems and the oligopeptide Opp system (37). Several peptide transporters or peptidases fall into larger protein superfamilies. Examples include: (i) the oligopeptide-binding protein OppA and the di/tripeptide-binding proteins DppA/DppP, in the same peptide-binding protein family; (ii) the aminopeptidase PepC, together with endopeptidases PepE and PepG, belong to MEROPS peptidase family C1-B; (iii) the proline peptidases PepI, PepR and PepL, belong to the MEROPS family S33; and (iv) the aminopeptidase PepM, together with proline peptidases PepP and PepQ, belong to the MEROPS family M24. These large protein families can be divided into subfamilies with putatively different substrate specificities using a comparative genomics approach. Two large peptidase families (PepP/PepQ/PepM, and PepI/PepR/PepL) will be discussed in detail in the following sections.

	peptidase	Family	Substrate/ Annotation	LAC [d]	LJO	LGA	LDB	LBU	LHE	LPL	LBE	LRF	LSK	LSL	LCA	LRH	PPE	OOE	LME	LLX	LLA	LLM	STH	STU	STM
Proteinase																									
Cell-wall bound proteinase	PrtP	S8-A	maturation protein for PrtP (adjacent PrtP)	1	1	0	1	1	0	0	0	0	0	0	2 (1p)	2	0	0	0	0	1 [b]	0	0	0	1
	PrtM			0	1	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	1 [b]	0	0	0	0
Peptides transporters																									
Oligopeptides ABC transport system	OppA		Oligopeptide-binding protein	3	4	4	2	2	1	0	1	0	0	0	1	1	0	1	0	1	2 [b]	2	4 (2p)	3 (1p)	2
	OppB		permease protein	1	1	1	1	1	1	0	1	0	0	0	1	1	0	1	0	1	2 [b]	2	1	1	1
	OppC		permease protein	1	1	1	1	1(p)	1	0	1	0	0	0	1	1	0	1	0	1	2 [b]	2	1	1	1
	OppD		ATP-binding protein	1	1	1	1	1	1	0	1	0	0	0	1	1	0	1	0	1	2 [b]	1	1	1	1
	OppF		ATP-binding protein	1	1	1	1	1	1	0	1	0	0	0	1	1	0	1	0	1	2 [b]	1	1	1	1
Di/tripeptides ABC transport system	DppA/P		di/tripeptide-oligopeptide-binding protein	3	0	0	6	5	1	4	3	0	1	1	3	2	2	3	2	3	3	2	1(p)	1(p)	1(p)
	DppB		permease protein	1	0	0	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1(p)	1(p)	1(p)
	DppC		permease protein	1	0	0	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	2(p)	2(p)	1(p)
	DppD		ATP-binding protein	1	0	0	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1(p)	1(p)	1(p)
	DppF		ATP-binding protein	1	0	0	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1
di/tripeptides ion-linked transporter	DtpT		PTR family	1	1	1	0	0	0	1	1	1	1	1	1	1	1	1	0	0	1	1	1	1	1
Peptidases																									
Aminopeptidase	PepC	C1-B	X|(X)n	1	1(2) [a]	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	PepN	M1	X|(X)n	1(1) [a]	1	1	1	1	1(1) [a]	1	1	1	1	1 [b]	1	1	1	1	1	1	1	1	1	1	1
(unique aminopeptidases)	PepM	M24-A	Met|(X)n	1	1	1	1	1	1	1	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	PepA	M1	Glu/Asp|(X)n	1	1	1	1	1	1	0	0	0	0	0	0	1	0	0	1	1	1	1	1	1	1
	Pcp	C15	pyroGlu|(X)n	1	1	1	1	1	1	0	1	0	0	0	1	1	0	0	0	0	1 [b]	1	0	0	0
endopeptidase	PepE/PepG	C1-B	(X)m|(X)n	3	3	3	2	2	3	1	1	1	1(p)	0	1	1	0	1	0	0	0	0	0	0	0
	PepO	M13	(X)m|(X)n	2	3	3	1	1	3	1	1	1	1	1	2	2	1	1	1	1	2 [b]	2	1	1	1
	PepF	M3-B	(X)m|(X)n	1	1	1	1	1	1	2	2	0	2	2	2	3	1	3	2	1	2 [b]	1	1	1	1
dipeptidase	PepD	C69	X|X	5 (1p)	6	4	3 (1p)	3 (1p)	5 (1p)	4	5	5	5	2	4	3	4	1	0	2	2 (1p)	2	1(p)	1(p)	1 (p)
	PepV	M20-A	X|X	1	1	2	1	1	1	1	2	2	2	1	2	2	1	2	2	1	1	1	1	1	1
tripeptidase	PepT	M20-B	X|X-X	2	2	2	2	2	2	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1
proline peptidase	PepX	S15	X-Pro|(X)n	1	1	1	1	1	1	1	1	1	1	1	1	1	1(1) [a]	1	1	1(1) [a]	1	1	1	1	1
	PepI	S33	Pro|X-(X)n	1	0	0	1	1	1	1	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0
	PepR	S33	Pro|X	1	1	1	1	1	1	1	1	1	1	0	1	1	1	0	0	0	0	0	0	0	0
	PepL [c]	S33	Leu|(X)n	1	0	1	0	0	0	1	0	0	0	0	1	1	0	3	0	0	0	0	0	0	0
	PepP	M24-B	X|Pro-(X)n	1	1	1	1	1	1	1	1	1	0	1	1	1(1) [a]	0	1	1(1) [a]	1	1	1	1	1	1
	PepQ	M24-B	X|Pro	1	1	1	2	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

A: paralogs: The number in brackets indicates the number of extra paralogs present, which do not belong to the same ortholog group as all other protein family members.
B: plasmid-encoded proteins: PrtP, PrtM, Pcp and one of the PepF, PepO, Opp transport system encoding genes of LLA, as well as PepN from LSL
C Including The *L.plantarum* and *L. casei* proteins in the intermediate group in the PepL/PepR family which are not orthologs of the PepR family
D: strains name are : LAC *L. acidophilus* NCMF; LJO, *L. johnsonii* NCC533; LGA *L. gasseri* ATCC 33323; LDB *L. bulgaricus* ATCC 11842; LBU, *L. bulgaricus* ATCC BAA365; LHE, *L. helveticus* DPC 4571; LPL, *L. plantarum* WCSF1; *L. brevis* ATCC 367;LRF, *L. reuteri* F275; LSK *L.sakei* 23K; LSL *L.salivarius* UCC118; LCA, *L. casei* ATCC 334; LRH, *L. rhamnosus* GG; PPE, *P. pentosaceus* ATCC 25745; OOE *O oeni* PSU1; LME, *L. mesenteroides* ATCC 8293; LLX *L. lactis* sub *lactis* IL 1403; LLA *L. lactis* subsp *cremoris* SK11; LLM *L.lactis* subsp *cremoris* MG1362; STH, *S. thermophilus* LMG 18311; STM, *S. thermophilus* LMD9 p.pseudogenes (e.g. with truncations or frame shift

Figure 5. Distribution of proteinases, peptide transporters and peptidases of the proteolytic system in LAB. The number of genes identified is indicated. The MEROPS families are indicated for proteinases and peptidases. Colour shading shows the absence of a gene (white), a single gene (yellow) or multiple genes (green). (43)

Subfamilies of the PepP/PepQ/PepM peptidase family

PepP, PepQ and PepM belong to the M24 MEROPS peptidase family, a family of enzymes that requires metal ions for their catalytic activity. PepM is a methionyl aminopeptidase that can cleave N-terminal methionine from proteins. PepP is a proline peptidase, which cleaves off an oligopeptide from the N-terminal side of the proline reside. PepQ is also a proline peptidase, which has specificity for dipeptides (its cleavage site is Xaa-Pro, where Xaa represents any amino acid). Phylogenetic analysis has demonstrated that PepP, PepQ and PepM are members of three distinct subgroups. In agreement with the differences in their catalytic activities and the different substrate specificities of each of these peptidases, PepP and PepQ appear to be more similar than either with PepM on the basis of the family tree. PepM belonging to the M24A subfamily, and is an aminopeptidase that typically requires cobalt ions for catalysis, while PepP and PepQ belong to the M24B subfamily, which requires manganese (38). In each LAB genome, except in *L. sakei* and *Pediococcus pentosaceus*, one gene that codes for the PepP subgroup has been found. The absence of the pepP genes in both the *L. sakei* and *P. pentosaceus* genomes is very likely due to a gene-loss event. An experimentally verified pepP gene from *L. lactis*, and the encoded protein have been purified and characterised (45). Moreover, LAB-derived pepP genes are always flanked on the chromosome by an elongation factor for a protein translation gene. As suggested by Matos et al. (45), the conserved gene context of pepP is consistent with the physiological role of PepP in protein maturation. In the PepM subgroup (aminopeptidase), *L. brevis* has an extra paralogous gene, which is similar to the *L. plantarum* pepM gene. Except the pepM gene from *L. plantarum* and both the paralogues from *L. brevis*, analysis of the flanking region

of pepM suggests that these genes share the same neighbour genes in all *Lactobacillus* strains. The *L. plantarum* pepM gene (LPL_28377183) is flanked by a methionine-metabolism-related operon (cysK_cblB/cglB_cysE). Therefore, the pepM gene in *L. plantarum* may have a broader function, probably using proteins and peptides as the methionine pool, in addition to the classic PepM function for N-terminal maturation of proteins. Based on the protein family tree, it is hypothesised that an extra pepM gene was acquired first in the ancestor of *L. brevis* and *L. plantarum*, after which one gene was lost from *L. plantarum*. One of the *L. brevis* pepM genes (LBE_116334483) is located in the same operon as a transposase

Subfamilies of peptidase family PepI/PepL/PepR

The subfamilies of the praline-specific enzymes PepI/PepL and PepR can cleave di-tri-peptides towards the N-terminal side of proline. The proline iminopeptidase PepI has aminopeptidase activity toward N-terminal proline peptides, preferably tri-peptides. Prolinase PepR has a broad specificity for dipeptides, including Pro-Xaa dipeptides (38), while the only characterised PepL from *L. delbrueckii* subsp. *lactis* DSM7290 has a high specificity for di-/tri-peptides with N-terminal leucine residues (33). Interestingly, the same α/β hydrolase superfamily includes the PepI/PepR/PepL family and the esterase EstA family. In fact, BLASTP analysis of PepI/PepR/ PepL members against the non-redundant protein database show homologues from the EstA family. The presence of large insertions and deletions in the homologues sequences of the EstA and PepI/PepLand PepR subfamilies, plus the presence of regions of the proteins that share very low sequence

similarities, mean that multiple sequence alignments are not reliable. Therefore, analysis by superposition of the 3D structures of the representative proteins have included proline iminopeptidases from *Thermoplasma acidophilum* (PDB ID: 1MTZ) (46), *Xanthomonas campestris* pv. *citri* (PDB ID: 1AZW) (47) and *Serratia marcescens* (PDB ID: 1WM1) (48) as members of the PepI/R/L family, and an esterase A (PDB ID: 2UZ0) from *Streptococcus pneumonia* (49) as a member of the EstA subfamily (Fig. 6). These studies have highlighted a highly similar catalytic domain, which shows a typical canonical α/β hydrolase topology that consists of an eight-stranded β-sheet, and hasa non-conserved cap domain.

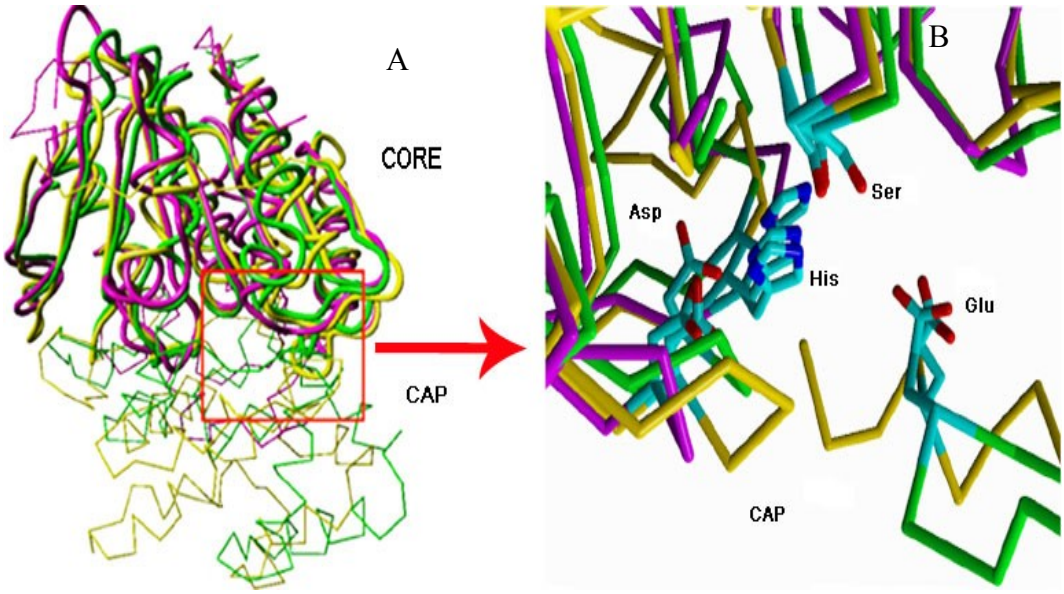

Figure 6. Superposition of the 3D structures of the proline iminopeptidases 1WM1 (yellow) and 1MTZ (green), and esterase 2UZ0 (purple). A. The four conserved structural core segments are shown as thick tubes, and the variable segments as thin sticks connecting C-alpha atoms. The variable large cap regions of the peptidases, which do not superpose, are in the bottom half of the imagine. Note that the esterase has a much shorter connecting segment in this cap region. The red frame indicates the position of the active site, which is shown as the zoomed-in view in (B). B. The catalytic site is shown with catalytic residues Ser, His and Asp. The active site is enlarged and rotated by about 180° relative to (A). A short stretch of the cap region in both peptidases is shown, bearing the Glu residues that interact with the positive charge of the peptide substrate N-terminus. Note that the side chains of the two Glu residues superimpose very well, despite coming from different (non-superimposable) parts of the cap region (43).

Four conserved structural regions in the catalytic domain that are separated by variable loops were identified based on the structure alignment. The cap domain shows instead large structural variation, and the esterase EstA has a much smaller cap domain than the peptidases (Fig. 6). The cap regions of the peptidases cover and close the substrate-binding region, allowing only the N-terminal proline of a peptide to fit into the substrate-binding pocket.

Lactobacillus plantarum WCSF1 genome

The ecological flexibility of *L. plantarum* arises as this species has one of the largest genomes known among LAB (6). The complete genome sequencing was performed in 2003, and analysis of the putative biological functions assigned to 2,120 (70%) of the 3,052 predicted protein-encoding genes have been carried out. Genome annotation, however, is only the first step to understand the function of genes. To understand their biological functions, individual genes need to be studied in a biological context, including metabolic pathways. Databases and tools that aid in metabolic reconstruction can be found on the Internet. These resources differ considerably, and unfortunately, most of them are either not organism specific or have been generated automatically and hence contain many errors. There are, however, well-curated databases, of which EcoCyc is a prime example (50). The *L. plantarum* WCFS1 genome contains a single, circular chromosome of 3,308,274 bp, two small, cryptic plasmids (2,365 and 1,917 bp) and a larger plasmid (36,069 bp) that code for genes involved in conjugal plasmid transfer and several other functions. The chromosome G-C content is 44.5%, whereas a slightly lower G-C content was found in the plasmid (6).

Proteolytic system of *L. plantarum*

Taxonomically, *L. plantarum* has been grouped as a facultative heterofermentative lactobacillus, which indicates that sugars can be fermented via the Embden–Meyerhoff–Parnas (EMP) pathway or the phosphoketolase pathway, leading to homolactic and heterolactic fermentation profiles, respectively (52).

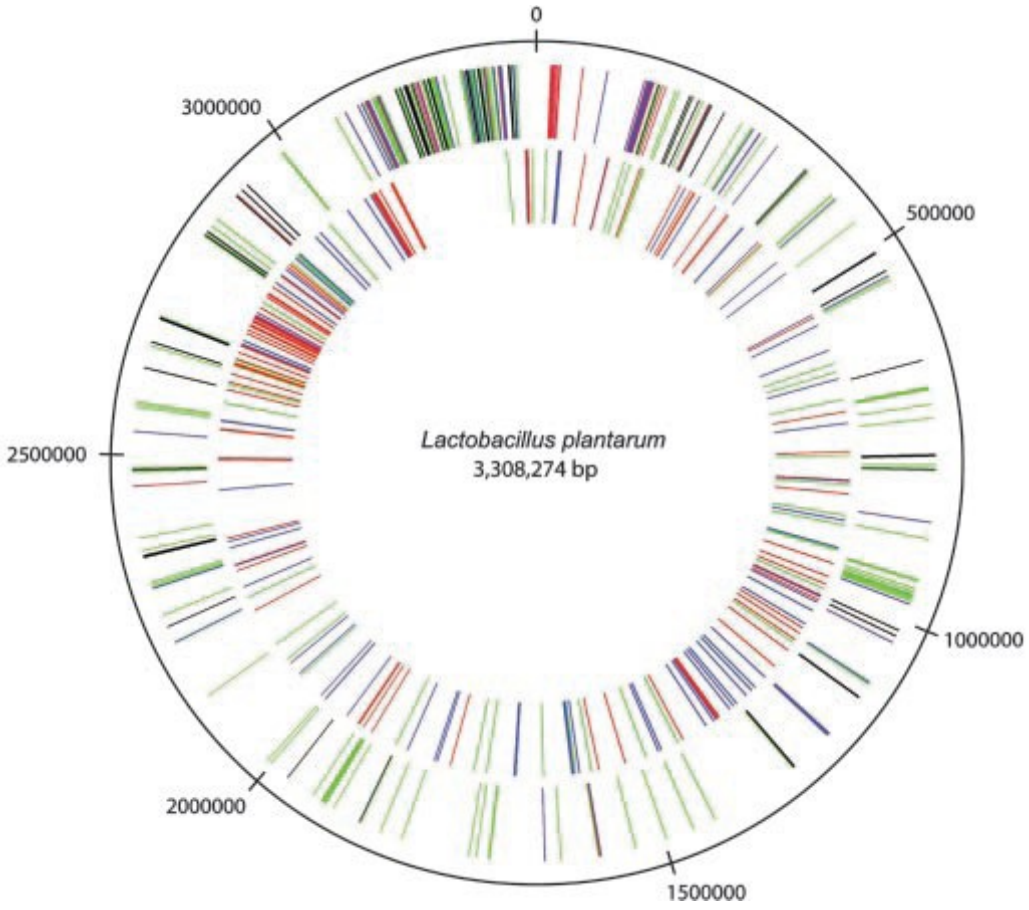

Figure 7. Non-random distribution of genes belonging to specific functional categories in the *L. plantarum* chromosome. The outer circle contains all of the genes that code for proteins involved in sugar transport (PTS, black; other transporters, blue), sugar metabolism (green), and biosynthesis and/or degradation of polysaccharides (red). The inner circle contains all of the genes predicted to code for secreted proteins; see also Table 1. Red, signal peptides; green, N-terminal lipoprotein anchor; blue, N-terminal signal anchor sequence. In particular, the 213-kb region from 3,072,500 to 3,285,500 codes almost exclusively for proteins for sugar transport, metabolism, and regulation. Moreover, this entire region has a lower GC content (41.5%) than the rest of the genome (Fig. 8), suggesting that many genes may have been acquired by horizontal gene transfer. This would be in agreement with the hypothesis that this part of the *L. plantarum* chromosome represents a lifestyle-adaptation region that is used to effectively adapt to the changes in conditions encountered in the numerous environmental niches in which this microbe is found. The *L. plantarum* genome codes for 30 transporter systems that have been predicted to be involved in the transport of carbon sources. Once internalised, sugars are used as a carbon source for growth and for generation of energy through fermentation. (6)

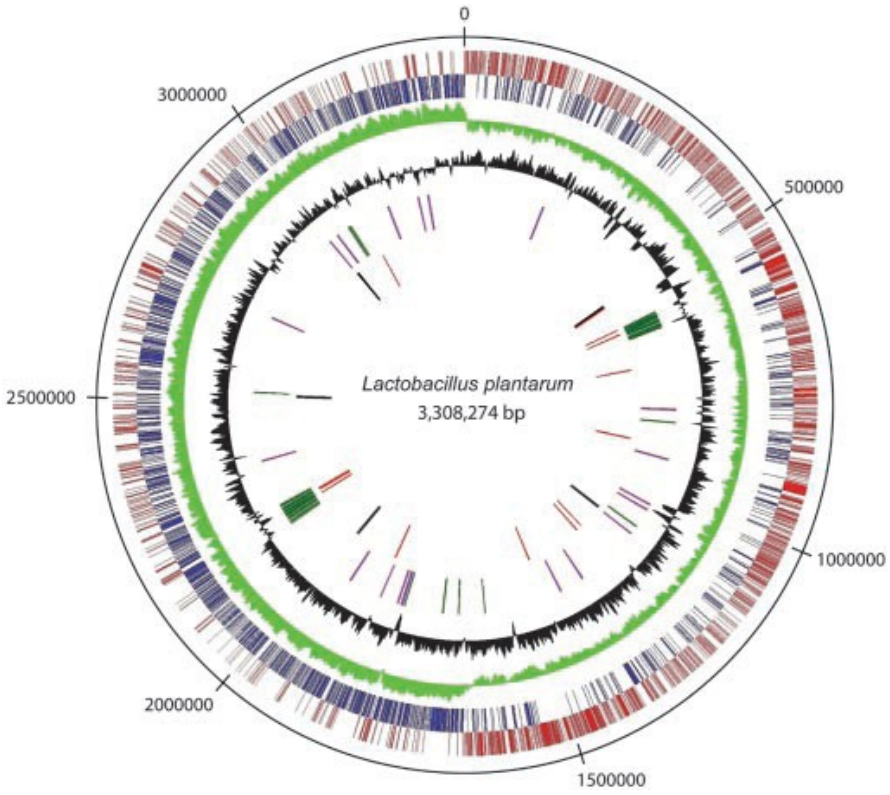

Figure 8. Genome-atlas view of the *L. plantarum* WCFS1 chromosome, with the predicted origin of replication at the top. The outer to inner circles show (i) positive strand ORFs (red); (ii) negative strand ORFs (blue); (iii) GC-skew (green); (iv) G/C content (black); (v) prophage-related functions (green) and IS-like elements (purple); and (vi) rDNA operons (black) and tRNA coding genes (red). The G/C% and GC skew (C-G)/(C/G) were calculated in a window of 4,000 nt, in steps of 75 nt. The G/C percentage is plotted as the number of G/C nucleotides in the plus strand divided by the window size, i.e., (G/C)/4,000; the lowest and highest values are 30.8% and 51.8%. The upper and lower values of the GC skew were 0.22 and -0.27. (6)

A wide range of proteolytic enzymes has been found in the *L. plantarum* genome, and their specificities cover a wide range of very different substrates that can be used as nitrogen sources. Despite this, the genome does not contain any gene encoding proteinases or cell-envelope proteinases (CEPs), which are the primary enzymes required for protein and large polypeptide degradation (6). In *L. plantarum*, the Opp and Dtp peptide uptake systems have been found. Once internalised, the peptides that are transported inside the cell are degraded by

peptidases. Most of these have already been studied in lactococci and lactobacilli (37), although none have yet been described for *L. plantarum*. In the *L. plantarum* genome, 19 genes code for intracellular peptidases (Table S06, www.cmbi.kun.nl_lactobacillus) and their specificities cover a wide diversity of substrates. Between *L. plantarum* WCSF1 and *Lactococcus lactis* IL1403, the biggest difference is seen as the number of peptidases that can cleave N-terminal proline residues, wile *L. plantarum* has three genes (*pep*I, *pep*R *pep*R2), in *L. lactis* none of these enzymes are founded. Complete pathways for biosynthesis of most amino acids, except valine, leucine and isoleucine, have been found in the *L. plantarum* genome, where these genes are generally organised in large clusters, or operons.

Peptidase	specificity	Gene	genes in *Lactococcus lactis* IL1403	genes in *Lb. plantarum* WCFS1
Cell-envelope proteinase	(X)n | (X)n	prtP	0	0
Endopeptidases				
Oligoendopeptidase F	(X)n | (X)n	Pepf	1	2
Endopeptidase	(X)n | (X)n	pepO	2	1
Aminopeptidases				
aminopeptidase M (Met)	Met | (X)n	pepM	1	1
aminopeptidase A (Glu/Asp)	Glu/Asp | (X)n	pepA	1	0
aminopeptidase C	X | (X)n	pepC	1	2
aminopeptidase N	X | (X)n	pepN	1	1
Tri/di-peptidases				
Tripeptidase	X | X-X	pepT	1	1
Dipeptidase	X | X	pepD	2	4
Dipeptidase	X | X	pepV	1	1
Proline-specific peptidases				
aminopeptidase P	X | Pro-(X)n	pepP	1	1
Xaa-Pro dipeptidyl aminopeptidase	X-Pro | (X)n	pepX	1	1
proline iminopeptidase	Pro | X-(X)n	pepI	0	1
Pro-Xaa dipeptidase (prolinase)	Pro | X	pepR	0	2
Xaa-Pro dipeptidase (prolidase)	X | Pro	pepQ	1	1
Total			14	19

Table 2. The ' | ' indicates the cleavage site (6).

LAB proteolysis during sourdough fermentation can help celiac patients

Sourdough fermentation is a traditional process for improving bread quality and producing different wheat and rye breads (53) (54). The advantages of sourdough baked food technology are the decrease in the pH during fermentation, the enhanced gas retention, and the strengthening of the gluten network. All these features, and often the inhibition of amylases, lead to a better taste and flavour (55), and a longer shelf life of the sourdough-derived products (56) (54). A variety of genera and species of LAB have been found and identified in sourdoughs.

These are mainly constitutes by members of the genus *Lactobacillus* (57), the sourdough microflora that is represented by heterofermentative bacteria (58), including the species *L. pontis* (59) (60), *L. sanfranciscensis* (61) (62), *L. fermentum*, *L. reuteri* (63) and *L. panis* (64). The effective maltose metabolism, the ability to use fructose as an electron acceptor, and the glucose accumulation all go towards explaining the dominance of the heterofermentative LAB in sourdough (58).

During sourdough fermentation, proteolytic activity of LAB leads to the specific sensory properties of the products (65).The proteolytic activity was first attributed to flour enzymes, such as aminopeptidase (AP), carboxypeptidase and endopeptidase (EP) (66). Later, proteolysis by sourdoughs was found to be higher than in yeasted and unstarted doughs (67). This enhanced proteolysis during sourdough fermentation can be attributed to the proteolytic activity of sourdough LAB. The protein constituent in wheat sourdough can be divided into four major classes: albumins, globulins, gliadins and glutenins (68). Gluten proteins (gliadins and glutenins) are responsible for the rheological properties of doughs. Indeed, they can contribute to gas retention during fermentation, which is reflected in the textural properties of the bread (69). LAB proteolysis during fermentation releases small peptides and free amino acids into the environment, providing the substrates for microbial growth, which are important for acidification rate and as precursors for flavour development in the final products (70).

Furthermore, this proteolytic activity has been investigate as a tool to reduce allergen compounds present in foods derived from gluten, which are recognised as the first step in the pathogenesis of celiac disease (71). Gliadins are

the principal toxic components of wheat gluten, and they are members of the prolamins family, which are rich in proline and glutamine (72). During endoluminal digestion, the prolamins of wheat (σ, β, γ and ω-gliadin subgroups), rye (e.g. secalin) and barley (e.g. hordein) release this family of proline-rich and glutamine-rich polypeptides, which trigger an inappropriate T-cell-mediated response (73)

A number of small peptides are derived from the digestion of α-gliadine, among which there is a relatively large fragment that is the most noted, and it is known as the 33-mer peptide, LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF (residues 57 to 89). Two reasons make this peptide particularly interesting: (i) this peptide remains intact regardless of prolonged exposure to proteases, while most of the other fragments are cleaved to smaller fragments when reaction times are extended. (ii) It contains three distinct T-cell epitopes; namely PFPQPQLPY, PQPQLPYPQ (three copies) and PYPQPQLPY (two copies) (72), which were identified in T-cell proliferation assays. There are also a few praline-rich and glutamine-rich short sequences (e.g. P-S-Q-Q and Q-Q-Q-P sequences). Studies with these fragments of α-gliadin have clearly indicated that they are toxic to celiac patients, and they can cause inflammatory responses in the small intestinal mucosa.

The resistance of the 33-mer gliadin peptide and the other smaller peptides to gastrointestinal breakdown is due to the location and abundance of the proline residues. Kosla (72) hypothesised that a prolyl endopeptidase enzyme would catalyse the breakdown of this peptide, thus diminishing its toxic effects. Prolyl endopeptidase (PEP) from *Flavobacterium meningosepticum* was then used

in preliminary *in-vitro* studies with short gliadin peptides, and the results supported this hypothesis (74). *In-vitro* and *in-vivo* studies regarding the ability of this PEP to cleave the 33-mer gliadin peptide were performed (Fig. 9A, B). Evidence of synergistic effects of PEP and brush-border membrane peptidases was provided by the authors, and a rapid decrease in the T-cell stimulatory potential of the PEP-treated peptide was shown (Fig. 9C).

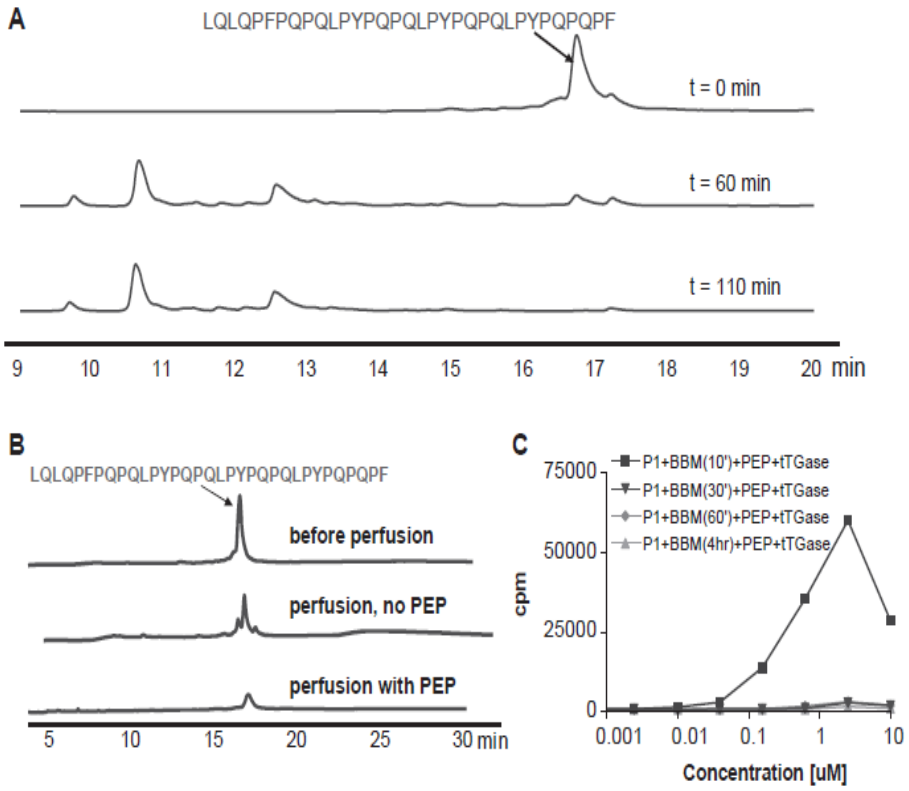

Figure 9. Breakdown and detoxification of the 33-mer gliadin peptide with PEP. A. RPLC-UV215 traces of the 33-mer gliadin peptide incubated *in vitro* with PEP. B. RPLC-UV215 traces of the *in-vivo* digested 33-mer gliadin peptide with and without PEP in the rat small intestine. C. Stimulation of the T-cell clone TCC 380.E2 (specific for QPFPQPELPY) by the 33-mer gliadin peptide after PEP and brush-border-membrane enzyme treatment for different durations, followed by tTGase treatment (72).

The great quantity of the Pro-Xaa-Pro motif in the 33-mer peptide (75) and the preference of PEP for this substrate (76) support the hypothesis that peptidase treatment can be used for gluten detoxification in Celiac Sprue patients.

Several studies have investigated the proteolytic sourdough microflora with the aim of reducing the impact of the toxic gluten-derived peptides. The proteolytic activity of sourdough LAB was described by Di Cagno and coworkers (77). LAB strains were shown to hydrolyse albumins, globulins and gliadins during sourdough fermentation. Higher levels of proteolysis in the fermented sourdoughs compared to the chemically acidified sourdoughs was reported in this study, providing strong evidence that the sourdough microflora is mainly responsible for the proteolysis. The large liberation of free amino acids during the fermentation further supported these findings.

These findings have encouraged the use of LAB for hydrolysis of these gliadin-derived peptides that are involved in celiac disease. Enzyme preparations of lactobacilli can hydrolyse the 31-43 fragment of α-gliadin (sequence L-G-Q-Q-Q-P-F-P-P-Q-Q-P-Y) after 4 h of treatment. Proteinase and peptidase activities were tested separately, to demonstrate the hydrolytic activities of the cell-wall-associated proteinases, which might have easier access to the peptide substrates during sourdough fermentation. The cytoplasmic preparation containing peptidase showed the highest activity and several differences in hydrolysis patterns were found among the strains, .

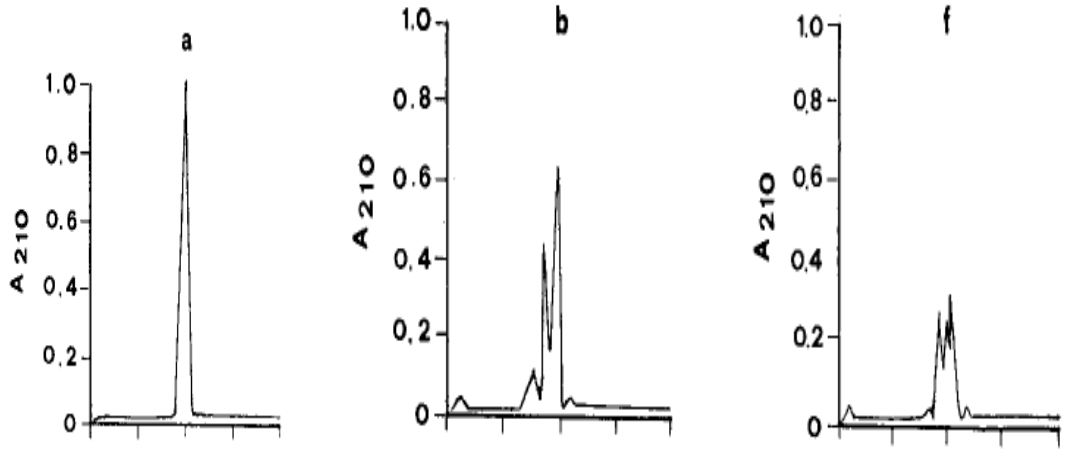

Figure 10. RP-FPLC chromatograms of the α-gliadin 31-43 fragment (a), from cell wall and cytoplasmic enzyme preparations of *L. alimentarius* 15M (b, f) (77).

In the agglutination test performed by Di Cagno and coworkers (77), they found that compared to the whole 31-43 fragment of α-gliadin, the peptide treated with the enzyme preparations of selected lactobacilli showed considerably lower agglutination activity, thus indicating a suitable enzyme substrate specificity and excluding the generation of more toxic peptides.

In 2004, it was then demonstrated by Di Cagno and coworkers (78) that selected lactobacilli can hydrolyse the 33-mer peptide, and starting from a mixture of normal and non-toxic flour, it is possible to produce non-toxic bread (78). Specialised peptidases from a pool of lactobacilli composed of *L. alimentarius* 15M, *L. brevis* 14G, *L. sanfranciscensis* 7A, and *L. hilgardii* 51B can hydrolyse all of the different peptide bonds that potentially include the amino-acid proline. The hydrolysis operated by the four lactobacilli used in the study involved oligopeptides, like fragment 62-75 of α-gliadin and the 33-mer peptide.

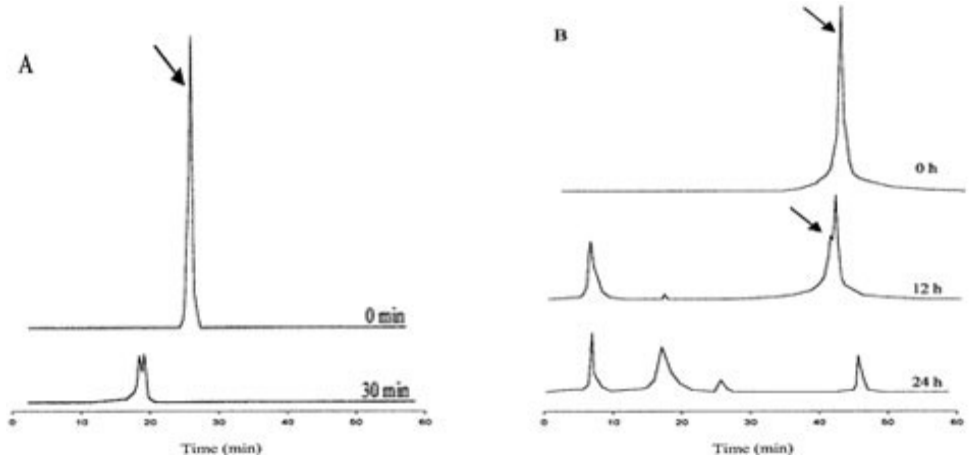

Figure 11. Hydrolysis of peptides by pooled cells of the four selected lactobacilli. A. Fragment 62-75 of α-gliadin before and after 30 min of hydrolysis, as indicated. B. The 33-mer peptide before and after 12 and 24 h of hydrolysis. Arrows indicate the substrate (78).

The proteolytic system of sourdough-isolated *L. plantarum* strains was investigated with respect to their hydrolysis of the toxic 31–43 gliadin fragment in 2005 (81). In this study, chromogenic substrates were used to check the enzyme activity regarding this specificity: amino peptidase, proline iminopeptidase X-prolyl-dipeptidyl aminopeptidase endopeptidase, dipeptidase and tripeptidase activities. This demonstrated that *L. plantarum* strains can hydrolyse the chromogenic substrates, and moreover, it showed that they can hydrolyse the 31-43 toxic fragment of α-gliadine (73% hydrolysis in 4 hours).

In 2005, an interesting study was published (82) that was aimed at an evaluation of the growth and metabolic activities of sourdough pediococci and heterofermentative lactobacilli strains in a gluten-based medium. Medium was prepared with 9% wheat flour gluten, 2% glucose, 1% KH_2PO_4, 1% K_2HPO_4, and 1% Tween-80. One strain of *L. brevis,* one of *Lactobacillus curvatus,* one of *Lactobacillus fermentum,* nine of *L. plantarum,* five of *L. reuteri*, two of

Pediococcus acidilacticci and 18 of *P. pentosaceus* were tested in the study. The result was that 13 LAB strains (nine lactobacilli and four pediococci) of 42 screened were found to grow in gluten media broth using only gluten as their nitrogen source. Based on the observed peptide profile changes *P. pentosaceus* was the most proteolytic strain among these pediococci, and a similar activity on gluten was reported for *L. plantarum* too. The results of this study provided the evidence that sourdough-isolated pediococci strains are proteolytically active on gluten. Furthermore, the ability of *L. plantarum* to hydrolyse a synthetic peptide homologous to the α-gliadin 31–43 amino-acid sequence was demonstrated, and this provided evidence that selected sourdough LAB strains can reduce gluten-allergen compounds during sourdough fermentation.

Recently, a complementary approach of using a glutamine-specific endoprotease (EP-B2, a cysteine endoprotease from germinating barley seeds) in conjunction with the prolyl endopeptidase enzyme that had demonstrated rapid detoxification of gluten under simulated gastrointestinal conditions (83) (84).

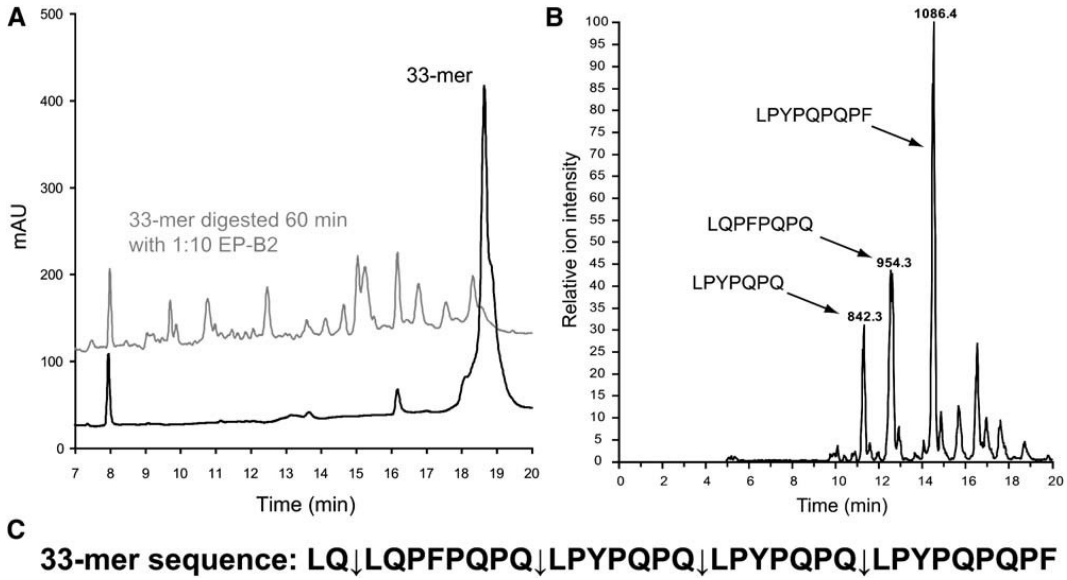

Figure 12. Activity of EP-B2 against the proteolytically resistant 33-mer peptide from α-gliadin. A. Reverse phase HPLC UV215 traces of the intact synthetic 33-mer and of the 33-mer following 60 min of digestion by EP-B2 at a molar ratio of 1:10 proenzyme: substrate (pH 3.0). B. Liquid chromatography mass spectroscopy (LC-MS) mass chromatogram of the digested sample from (A). Peaks labelled as digestion fragments were identified by their MS2 spectra. C. Sequence of the 33-mer peptide showing the sites of EP-B2 catalysed cleavage (↓), as determined by LC-MS/MS (83).

Because its pharmacological relevance and repetitive sequence, the 33-mer peptide, LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF, was tested in efficacy studies. Incubation of the 33-mer peptide with catalytic amounts of proEP-B2 (1:10 molar ratio, enz:sub) at pH 3.0 for 60 min resulted in almost complete breakdown of the peptide. At a gluten/ EP-B2/ PEP weight ratio of 75:3:1, commercial gluten (obtained from a grocery store) was fully detoxified within 10 min of simulated duodenal conditions, as highlighted by chromatographic analysis, T-cell proliferation assays, and a commercial anti-gluten antibody test (84). The extent of gluten proteolysis was determined in the stomach and small intestine for a variety of gluten loads (1-2 g) and digestion times (90-210 min) (85) (Fig.13).

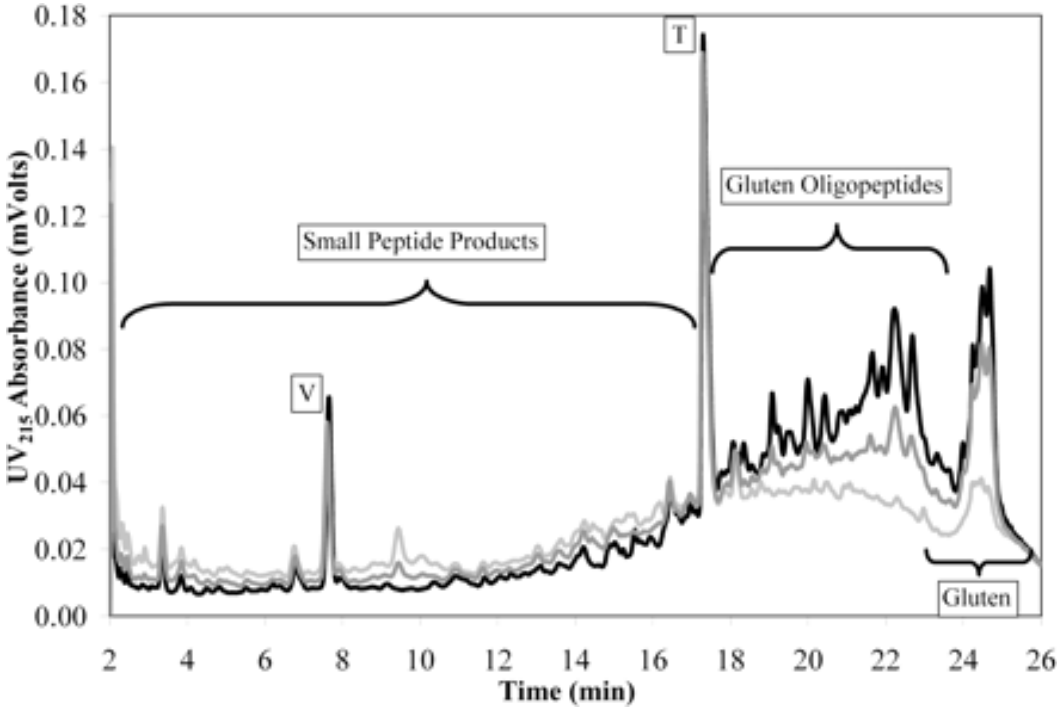

Figure 13. Gastric contents of rats fed with a gluten (1 g) test meal with varying levels of EP-B2 enzyme, followed by 90-min digestion time. Dark grey line, control (no EP-B2); medium grey line, low-dose EP-B2 (10 mg); and light grey line, high-dose EP-B2 (40 mg). The HPLC traces are divided into the early peaks (representing smaller peptide products), middle peaks, and late peaks (representing larger undigested gluten-derived peptides and soluble gluten protein). The approximate elution time of the 33-mer peptide was 25 min (83)

In a 2008 study (86), the peptidase activities of sourdough-isolated lactobacilli and pediococci strains were evaluated for their ability to hydrolyse the chemically synthesised α-gliadin fragments: 31-43, 62-75 and 57-89, which are considered to be allergenic for celiac patients, and to determine the relationships between the enzymatic activities and the reduction of these allergens in the α-gliadin fragments. *L. plantarum* hydrolysed more than 85% of the α-gliadin fragments 31-43 and 62-75 after 2 h, while *L. reuteri* did not hydrolysed these fragments. Ninety percent hydrolysis was observed for the 62-75 α-gliadin fragment with *P. pentosaceus* after 6 h, but this strain did not hydrolyse the 31-43 fragment at all. After 4 h, a reduction of 50% in the levels of the 31-43 α-gliadin

fragments was observed, whereas the 62-75 fragment was totally degraded in 30 min by this pool of LAB (77). Good efficiency for the hydrolysis of the 31-43 α-gliadin fragment was shown by he *L. plantarum* strains (>85% in 2 h), while lower efficiency was shown by all of the strains for the hydrolysis of the 62-75 fragment (<80% in 2 h), with respect to that reported by Di Cagno et al. (2002) (77). No hydrolysis of the 57-89 α-gliadin fragment was observed by any of the strains, even after 24 h. Seven different mixtures of LAB strains were evaluated for their degradation of this fragment. Varying degrees of hydrolysis of the 57-89 fragment were observed using these combinations. The mixture of *P. pentosaceus* and *L. plantarum* showed the greatest degradation (56%-60% after 8 h). An assay using a chromogenic substrate showed high aminopeptidase activities on Q-pNA, endopeptidase activities on QF, and dipeptidase activities on L-L, The smallest level of degradation was obtained using a mixture of two strains of *L. reuteri*, as only 21%-30% of hydrolysis was observed for the 57-89 fragment, which showed low tripeptidase activity and an absence of endopeptidase and dipeptidase activities, as confirmed by chromogenic substrates.

At the end of 2009 (87), the cytoplasmic extracts of the 10 sourdough LAB were pooled, (*Lactobacillus sanfranciscensis*, 7 strains, *Lactobacillus alimentarius*, 1 strain, *Lactobacillus brevis*, 1 strain, and *Lactobacillus hilgardii*, 1 strain) and several peptidases were partially purified and used alone or in combination to hydrolyse the 33-mer epitope .

As shown in Table 3, no single peptidase from the tested LAB pool hydrolysed the 33-mer peptide without generating toxic epitopes, with at least three peptidases (PepN, PepX and PepO) were needed eliminate the 33-mer

allergenicity. The first step in the 33-mer hydrolysis was the action of four peptidases (PepN, PepO, PEP, and PepX) that hydrolysed the 33-mer to dipeptides and tripeptides. Then, further hydrolysis that result in liberation of free amino acids was carried out by PepT, PepV, PepQ and PepR.(4, 33). The activity of only one (e.g., PepO) or a combination of two (e.g., PepN and PepO) peptidases caused maintained/ increased toxicity of the native 33-mer due to the liberation of more immunogenic epitopes. Complete hydrolysis was achieved only within 12-24 h and only when the complementary specificity of most of the peptidases of the LAB pool were used. No differences were seen when a synthetic peptide was used in the experiment. In conclusion, the authors said that there are no single strains or peptidases that can completely hydrolyse the 33-mer peptide and reduce its toxicity, although efficient hydrolysis and detoxification can indeed be achieved by a variety of strains carrying different peptidases with different specificities.

Table 3. *In-vitro* hydrolysis of the 33-mer epitope by partially purified peptidases from the pooled cytoplasmic extract of 10 sourdough lactobacilli (87). *a* PepN, general aminopeptidase type N; PepI, proline iminopeptidase; PepX, X-prolyl dipeptidyl aminopeptidase; PepO, endopeptidase; PEP, prolyl endopeptidyl peptidase; PepT, tripeptidase; PepV, dipeptidase; PepQ, prolidase; PepR, prolinase. *b* Peptides derived from the 33-mer epitope. For instance, PepO cleaved the internal peptide bond Q2L of the 33-mer epitope and liberated four different peptides, and three of these peptides were immunogenic epitopes. The combination of PepN and PepI or of PepN and PepX liberated only leucine (L) from the NH_2 terminus of the 33-mer epitope. *c* The presumptive immunogenic epitope row shows the number position of the presumptive immunogenic epitopes that are contained in the native sequence of the 33-mer epitope (37). The fragments are indicated by their number positions (e.g., 2-33 is the fragment containing positions 2 to 33 of the native sequence of the 33-mer epitope). Presumptive immunogenic epitopes can have the lowest size of 7-9 amino-acid residues. When the size is 7 amino-acid residues, epitopes should be contained in a larger fragment to be immunogenic (88).

Epitope or peptidase *a*	Sequence(s) of epitope or peptide fragment(s)*b*	No. of epitopes	Free a a
Epitopes			
33-mer epitope	L_1QLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF$_{33}$		
Presumptive immunogenic epitopes*c*	[2-33], [3-10], [4-10], [6-33], [25-33], [11-17], [18-24], [6-12], [10-33]		
Peptidases			
PepN	QLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF	1	L
PepI	LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF	1	
PepX	LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF	1	
PepO	LQ; LQPFPQPQ; 2 LPYPQPQ; LPYPQPQPF	3	
PEP	LQLQP; FPQPQLPYPQPQLPYPQPQLPYPQPQPF	1	
PepT	LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF	1	
PepV	LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF	1	
PepQ	LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF	1	
PepR	LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF	1	
PepN/PepI	QLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF 1		L
PepN/PepX	QLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF 1		L
PepN/PepO	LQ; QPFPQPQ; LPYPQPQ; LPYPQPQ; LPYPQPQPF	3	L
PepN/PEP	QLQP; FPQPQLPYPQPQLPYPQPQLPYPQPQPF	1	L
PepN/PepT/PepV/PepQ/PepR	QLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF	1	L
PepI/PepX	LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF	1	
PepI/PepO	LQ; LQPFPQPQ; LPYPQPQ; LPYPQPQ; LPYPQPQPF	3	
PepI/PEP	LQLQP; FPQPQLPYPQPQLPYPQPQLPYPQPQPF	1	
PepI/PepT/PepV/PepQ/PepR	LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF	1	
PepX/PepO	LQ; LQPFPQPQ; LP; YP; QPQ; LP; YP; QPQ; LP; YP; QP; QPF	1	
PepX/PEP	LQLQP; FP; QP; QLPYPQPQLPYPQPQLPYPQPQPF	1	L
PepX/PepT/PepV/PepQ/PepR	LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF	1	
PepO/PEP	LQ; LQLQP; FPQPQ; LPYPQPQ; LPYPQPQ; LPYPQPQPF	3	
PepO/PepV	LQPFPQPQ; LPYPQPQ; LPYPQPQ; LPYPQPQPF	3	L; Q
PepO/PepT/PepQ/PepR L	Q; LQPFPQPQ; 2 LPYPQPQ; LPYPQPQPF	3	
PEP/PepT/PepV/PepQ/PepR	LQLQP; LQLQ; FPQPQLPYPQPQLPYPQPQLPYPQPQPF	1	
PepN/PepI/PepX	QLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF	1	L
PepN/PepI/PepO	LQ; QPFPQPQ; 2 LPYPQPQ; LPYPQPQPF	3	L
PepN/PepI/PEP	QLQP; FPQPQLPYPQPQLPYPQPQLPYPQPQPF	1	L
PepN/PepI/PepT/PepV/PepQ/PepR	QLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF	1	L
PepN/PepX/PepO	LQ; QP; FP; QPQ; LP; YP; QPQ; LP; YP; QPQ; LP; YP; QP;QPF		L
PepN/PepX/PEP	QLQP; FP; QP; QLPYPQPQLPYPQPQLPYPQPQPF	1	L
PepN/PepX/PepT/PepV/PepQ/PepR	QLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF	1	L
PepI/PepX/PepO	LQ; LQPFPQPQ; LP; YP; QPQ; LP; YP; QPQ; LP; YP; QP; QPF	1	
PepI/PepX/PEP	LQLQP; FP; QP; QLPYPQPQLPYPQPQLPYPQPQPF	1	
PepI/PepX/PepT/PepV/PepQ/PepR	QLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF	1	
PepX/PepO/PEP	LQPFPQPQ; LP; YP; QPQ; LP; YP; QPQ; LP; YP; QP; QPF	1	
PepX/PepO/PepT/PepV/PepQ/PepR	LQ; LQPFPQPQ; LP; YP; QPQ; LP; YP; QPQ; LP; YP; QP; QPF	1	
PepN/PepI/PepX/Pep	O LQ; QP; FP; QPQ; LP; YP; QPQ; LP; YP; QPQ; LP; YP; QP;QPF		L
PepN/PepI/PepX/PEP	QLQP; FP; QP; QLPYPQPQLPYPQPQLPYPQPQPF	1	L
PepN/PepI/PepX/PepT/PepV/PepQ/PepR	QLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF	1	
PepN/PepI/PepX/PepO/PEP	LQ; QP; FP; QPQ; LP; YP; QPQ; LP; YP; QPQ; LP; YP; QP;QPFL		
PepN/PepI/PepX/PepO/PEP/PepT/PepV/PepQ/PepR		13P; 10Q; 5L; 3Y; 2F	
PepN/PepX/PepO/PEP/PepV/PepQ		13P; 10Q; 5L; 3Y; 2F	

Wild strains of LAB

Different types of Sardinian traditional bread are produced using sourdoughs (in Sardinia, they are called ‘‘framentarzu’’). The sourdoughs used to produce four different typical breads from specific area of Sardinia (the thin crisp Carasau and Zichi breads, the thin soft Spianata, and a crumb bread called Moddizzosu) were studied using molecular techniques and statistical methods, for the characterisation and identification of the isolates and to investigate the diversity, structure and composition of the LAB communities in traditional Sardinian sourdough (89). Within and among the bread types, great differences in species compositions where found. With the exception of sourdoughs used to produce Moddizzosu, which were dominated by *L. pentosus*, most samples were dominated by facultative heterofermentative lactobacilli (*L. plantarum, L. pentosus*), in contrast to what has been shown for other sourdoughs (90) (see Table 4).

Table 4. Number of isolates for each bread, and number of sourdough samples containing a given species (89).

Bacterial species	Carasau	Moddizzosu	Spianata	Zichi	Sourdough samples containing a given species
Lactobacillus casei	3				1
Lactobacillus zeae	1				1
Pediococcus pentosaceus				8	1
Lactobacillus sakei	2	5			5
Lactobacillus pentosus	17	50	4	9	19
Lactobacillus plantarum	5		16		5
Lactobacillus alimentarius	13	1	3	4	6
Lactobacillus farciminis			19	6	1
Lactobacillus brevis	2			21	9
Lactobacillus sanfranciscensis	4		1	1	3
Weissella confusa				6	5
Leuconostoc citreum				8	1
Isolates	47	56	43	63	

.

The metabolic effects of sourdough bread in subjects with impaired glucose tolerance (pre-diabetes) (91) was investigate using *L. plantarum* isolates from this study. The overall diet pattern (rather than single nutrients) was suggested as mixed meals comprehensive of sourdough bread, and these were evaluated to determine synergistic or antagonistic effects on the health of pre-diabetes and healthy subject. The glycaemic index was reduced in patients who were feed with sourdough bread, compared to bakers' yeast bread. A reduced insulin requirement was the consequence of this finding. This is of great importance, as it has been reported that even a short peak of hyperglycaemia can be very dangerous to diabetic patients. Delays in the evolution of impaired glucose tolerance to diabetes can be obtained using a diet programme that includes sourdough bread. Moreover, this type of bread provided a lower postprandial hyperglycaemia, which would result in fewer macrovascular complications in patients with diabetes.

Aim

The proteolytic activities of LAB have been widely investigate in many fields, with their ability to hydrolyse the toxic gluten-derived peptides and thus to provide beneficial contributes for celiac patient being the main field of study here over the last decade. The aim of this thesis was to investigate the ability of *L. plantarum* to hydrolyse gluten or gliadine, and to determine the enzymes that are responsible for this activity.

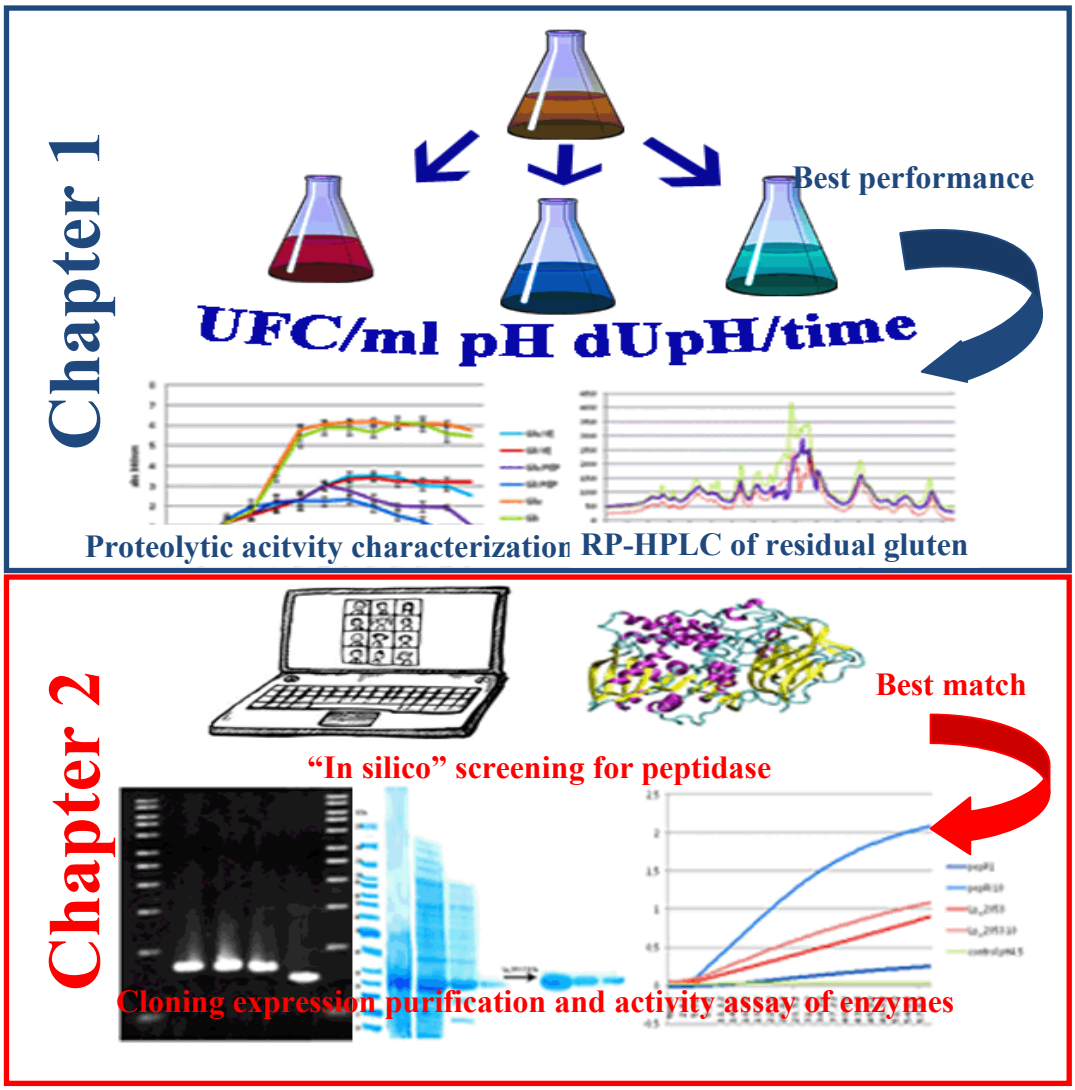

Figure 14 Sperimental design. *L. plantarum* strains were tested for their ability to use gluten/gliadine as nitrogen source, proteolytic activity was measure and characterized in the best condition of growth then gluten hydrolysis was assayed by RP-HPLC. In the second chapter "in

silico" screening for proline peptidase was performed, enzymes was cloned expressed and purified in order to check their activity and specificity

Chapter 1

Proteolytic activity of *L. plantarum* reference strain WCSF1 and the wild-sourdough-isolated strains Sb5c and Sb7b

Materials and methods

Strains

The strains used in this study were the *L. plantarum* WSCF1 reference strain, and the *L. plantarum* Sb5c and Sb7b wild strains (89) (91) (92).

Media

All of the strains were pre-cultured overnight at 30 °C on deMan, Rogosa, Sharpe (MRS) medium (Oxoid, Basingstoke, UK). Recombinant *E. coli* strains were selected on Luria–Bertani (LB) medium (Oxoid) with added 50 µg/mL kanamycin or ampicillin..

Table 5. Media compositions.

Media name	Glucose	Gluten	Gliadine	Yeast Extract	Peptone	Tween 80	note
MRS	Oxoid UK						
Glu MRS	MRS composition with gluten (28g/L) NO yeast extract peptone and tryptone						
Gli MRS	MRS composition with gliadine (28g/l) NO yeast extract peptone and tryptone						
Gluten (Glu)	20 g/l	10g/l					
Gliadine (Gli)	20 g/l		10g/l				
Glu YE	20 g/l	10g/l		0,5g/l			
Gli YE	20 g/l		10g/l	0,5g/l			
Glu PEP	20 g/l	10g/l			0,5g/l		
Gli PEP	20 g/l		10g/l		0,5g/l		
Hy glu	20 g/l	10g/l			Pepsin 10.000 Units agitation 37°C overnight		
Hy gli	20 g/l		10g/l		Pepsin 10.000 Units agitation 37°C overnight		
Glu TW80	20 g/l	10g/l				1%	
Gli TW80	20 g/l		10g/l			1%	

Several media were used to evaluate the ability of the strains to grow using gluten or gliadine as their nitrogen source. These media compositions are listed in Table 5. Glucose, gluten, gliadine and pepsin were from Sigma (St Louis, USA), yeast

extract and peptone was from Biolife (Milano, Italy), Tween 80 was from Acros Organics (Geel, Belgium)

The media were standardised at pH 7 with NaOH prior to sterilisation, to minimize the differences in the starting condition during the acidification rate measurements. Furthermore, to ensure the same amount of protein in the media, gluten and gliadine were added separately to the liquid phase prior to sterilisation. All of the strains were precultured on MRS overnight at 37 °C, then washed four times in 0.8% NaCl (in water). The number of cells was then standardised by measuring the absorbance at 600 nm, and the cell suspensions were used as inoculi for assays for grow ratios, pH measurements and proteolytic activities.

Growth curves

The strains were grown on the media listed on Table 1 at 37 °C under static conditions, with vital counts performed every 3 h by plating serial dilutions on MRS agar. The plates where incubated for 48 h at 37 °C under anaerobic conditions. The colony-forming unit (CFU) calculations were carried out using the ISO method N° 4833:1991, using the following formula:

$$N = \Sigma c/(n_1 + 0.1n_2)\, d$$

where **N** is the CFU, **Σc** is the summation of the colonies counted on the plates with more than 15 and less than 300 colonies, $\mathbf{n_1}$ is the number of counted plates in the lowest dilution, $\mathbf{n_2}$ is the number of counted plates in the higher dilution, **d** corresponds to the lower dilution.

Acidification rates

The apparatus for the automatic pH measurements and the automatic data acquisition was described by Spinnler and Corrieu (93). We preferred to filter the signals before acquisition, as follows: one measurement was made every 1 s, and after every 90 s the mean of the 90 measurements was calculated and stored. From these data we calculated the acidification rates (dpH/dt) expressed as pH milliunit/s. At the end of the incubation period the following three parameters were calculated: maximum acidification rate (V_m) in pH milliunits/min (pH mU/min); time at which V_m was observed (T_m); pH at which V_m was observed (pH_m).

Proteolytic activity measurements

Gluten/ gliadine proteolysis was evaluated using the o-phthaldialdehyde (OPA) method described by Churck et al. (1983), with slight modifications. The OPA reagent was prepared as follows: 25 mL 100 mM sodium tetraborate; 2.5 mL 20% (w/v) SDS; 40 mg OPA (dissolved in 1 mL methanol); and 100 μL β-mercaptoethanol, diluted to a 50-mL final volume with water. This OPA reagent was prepared fresh daily. Aliquots of 200 μL of samples where taken every 3 h, briefly centrifuged (12,000 rpm, 2 min) to eliminate gluten/ gliadin debri, and then the supernatant was filtered through a 0.22-μm siringe-driven filter (Millipore, Billerica, MA, USA). To assay for proteolysis, a small aliquot of filtrate sample (usually 50 μL containing roughly 100 μg protein) was added directly to 1.0 mL OPA reagent in a 1.5 mL cuvette; the solution was mixed briefly by inversion and incubated for 2 min at room temperature, and the

absorbance at 340 nm was then measured in a spectrophotometer (Agilent Palo Alto, CA, USA).

Proteolytic activity characterisation

To characterise the proteolytic activity of the strains, a chromogenic assay was performed with the substrates listed in Table 2. The strains where grown on gluten and gliadine alone and on gluten gliadine with yeast extract and peptone (see Table 1) at different times (0, 4 h, 8 h, 12 h and 24 h), and then the cells were centrifuged and the pellet was washed twice in milli-Q sterile water. The washed cells where resuspended in acetate buffer pH 6, 500 mM NaCl, 10 mg/mL lysozyme (Sigma, St Louis, USA) and incubated for 1 h at 37 °C with strong agitation prior to disrupting the cells. Cell disruption was carried out using a Fast Prep-24 cell disruptor (MP Biomedicals, Solon, OH, USA) with 0.5-mm glass beads (Biospec, Bartlesville, OK, USA). Cell lysates were produced using five cycle of disruption (30 s each), with 5 min chilling on ice between the cycles. The cell lysates were then clarified by centrifugation at 17,770 rpm for 40 min. The supernatants were collected and assayed for total protein concentrations using the Bradford assay with bovine serum albumin as the standard. Protein concentrations were then standardised to 1.2 mg/mL. Chromogenic assays were performed using 0.2 mM substrate with 0.5 mg total protein from the clarified lysate, in acetate buffer pH 4.5, in a final volume of 0.5 mL, with incubation at 37 °C. The activity was monitored using a spectrophotometer (Agilent, Palo Alto, CA, USA) to monitor the kinetics as increasing absorbance at 410 nm, over 120 min. The activity was also monitored at 3 h, and at 15 h (overnight).

Table 6. Substrates used in this study and the activities that were detected.

Substrate	*Activity detected*	*Supplier*
leu-pNa	Aminopeptidase	Sigma (St Louis MO USA)
Pro-pNa	Pro-iminopeptidase	Sigma (St Louis MO USA)
gly –pro-pNa	X-Prolyl-dipeptidyl-aminopeptidase	Sigma (St Louis MO USA)
gly-pro-ala-pNA	Endopeptidase	Sigma (St Louis MO USA)
Z-gly-gli-leu-pNa	Prolyl-endopeptidyl-peptidase	Sigma (St Louis MO USA)
Suc-ala-pro-pNa	Prolyl-endo-peptidase	Bachem (Torrance CA USA)

To analyse the ability of the peptidases to hydrolyse the 33-mer peptide (LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF), this peptide was synthesised using boc/HBTU chemistry on a solid-phase, then purified by RP-HPLC, and stored at -20 °C following lyophilisation. For the proteolytic assays, the peptide was resuspended in H_2O prior to use.

Evaluation of gluten/gliadine hydrolysis

Gluten and gliadine media where assayed by RP-HPLC to evaluate the protein degradation following the bacteria growth. An aliquot of each medium was taken from the culture batches at different times (time zero, 1 h, 2 h, 4 h) and stored at -80 °C until analysis. Uninoculated aliquots of each media were stored and used as the controls. All of the aliquots were then centrifuged at 13,000 rpm for 10 min and the supernatant was filtered (0.22-µm pore size). The protein concentrations of the samples where determined by Bradford assays and standardised to 7 µg/µL, to obtain comparable chromatograms. Fifty µL of each sample (350 µg total protein) where applied to an Aglient 1200 series HPLC sysem equipped with a C4 4.6/25 5µ RP column, using HPLC-grade water with 0.1% trifluoroaceic acid (TFA) as solvent A, and acetonitrile with 0.1% TFA as solvent B. A linear

gradient from 24% to 50% of solvent B was run in 60 min at a 1 mL/min flow rate. Absorbance at 210 nm and 220 nm was detected using an Agilent 1200 series diode array detector.

Data processing and statistical analysis

All measurements were performed as three independent assays, and the mean values with standard deviations (SD) are presented. The data were compared by one-way analysis of variance (ANOVA) and using Dunnett's t-test. Statistical significance ($P < 0.05$) was determined with the Minitab-12 software (State College, PA, USA).

Results

Grow rate on gluten media

The bacterial growth rates were examined as previously described (Materials and methods), with all of the strains showing vitality when precultured on MRS medium at 37 °C overnight (data not shown).

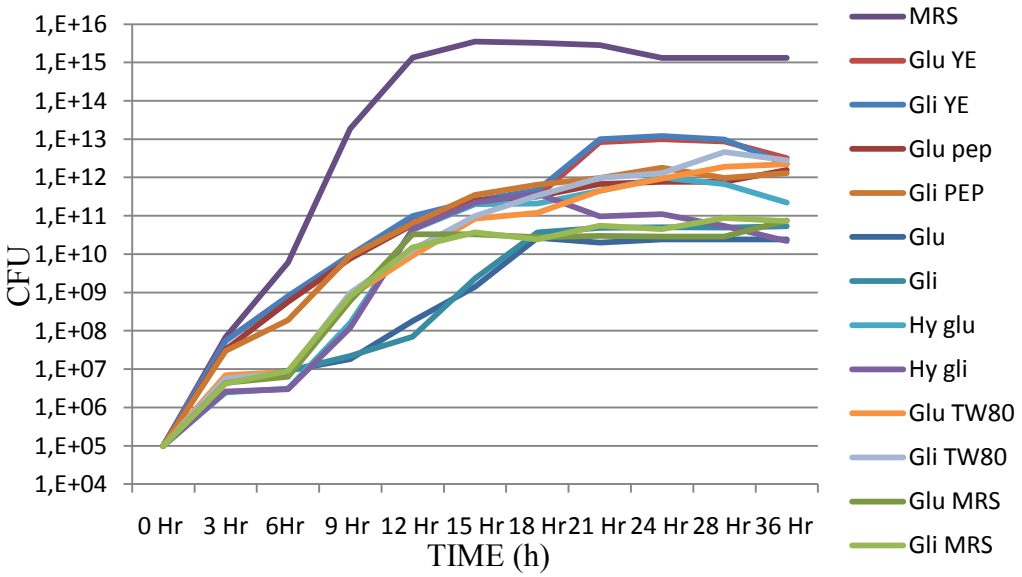

Figure 14. Reference strain WCSF1growth rates for the media indicated (listed in Materials and methods)..

Growth in the MRS medium was monitored as positive control reference, with a maximum density of 3.5 $\times 10^{15}$ CFU/mL was observed after 15 h of incubation. A longer lag phase was observed in the gli media, with the starting of the logarithmic phase only after the 12 h of incubation. The logarithmic phase ended at 18 h, when the strain reached 3.7 $\times 10^{10}$ CFU/mL and remained constant until the end of the experiment (maximum CFU/mL, 5.0 $\times 10^{10}$ at 24 h). The WCSF1 strain also showed the ability to grow on the glu media; under these conditions, the strain remained at 10^{8} CFU/mL (2 logs more) to 12 h, and then grew relatively slowly for 12 h (maximum, 2.4 $\times 10^{10}$CFU/mL at 24 h), with no

decrease observed until the end of the experiment. On Hy gli and Hy glu media, a lag phase was seen over the first 6 h, showing 3.0 and 3.2 $\times 10^6$ CFU/mL for Hy gli and Hy glu, respectively. The logarithmic phase started at 9 h (at 1.3 $\times 10^8$ CFU/mL for Hy gli, and 1.7 $\times 10^8$ CFU/mL for Hy glu) and stopped at 18 h, reaching 3.8 $\times 10^{11}$ for Hy Gg. With the Hy glu medium, there was a longer log phase that ended at 24 h, reaching 9.9 $\times 10^{11}$ CFU/mL. On the Glu TW80, the lag phase finished at 6 h (8.5 $\times 10$ CFU/mL), with the logarithmic phase from 9 h to 28 h, when the maximum density was reached (1.9 $\times 10^{12}$ CFU/mL). On Gli TW80 medium, the strain behaved as in the Glu TW80 medium: the logarithmic phase also started at 9 h of incubation, and ended at 24 h, when they reached 1.3 $\times 10^{12}$ CFU/mL (maximum density, 4.5 $\times 10^{12}$ CFU/mL at 28 h). In the Glu PEP medium, from 3.2 $\times 10^7$ CFU/mL at 3 h of incubation, the logarithmic growth was observed for 12 h, with a final density of 1.6 $\times 10^{12}$ CFU/mL at 36 h. Similar growth was seen on the Gli PEP medium, where the WCSF1 strain finished the lag phase in 3 h and started to grow in a logarithmic way to reach a maximum density at 24 h of 1.8 $\times 10^{12}$ CFU/mL. In Glu YE medium, the strain grew in a logarithmic way from 3 h to 21 h, when it reached 8.3 $\times 10^{12}$ CFU/mL. After this time, a stationary phase was observed and the density stayed roughly constant. Similar growth was observed on the Gli YE medium, where a longer logarithmic phase was observed that started at 3 h and ended at 24 h, when 1.2 $\times 10^{13}$ CFU/mL was counted. Glu MRS and Gli MRS did not show any interesting differences from the gluten or gliadine minimal media.

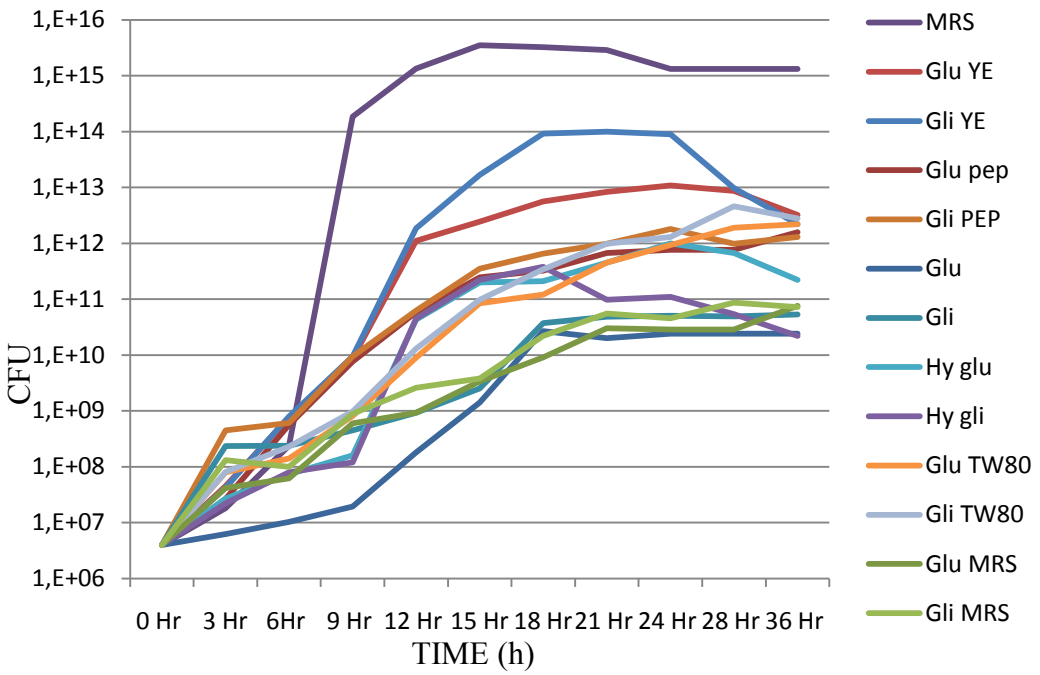

Figure 15. Natural strain Sb5c growth rates for the media indicated (listed in Table 1 in the Materials and methods).

Figures 15 and 16 show the Sb5c and Sb7b strain grow rates (CFU/mL) on the media listed in the Materials and methods. These were in good agreement with the experiments performed previously, with both strains showing the ability to grow on all of the media. The general concepts described for the reference strain WCSF1 are still valid for these strains, although between these strains themselves, we can see some slight differences in the grow rates. A longer lag phase was observed for both of these strains in the Glu medium, where we can see the starting of the logarithmic phase only after 9 h of incubation. The logarithmic phase ended at 24 h, when the strains reached 10^{10} CFU/mL. Both of these strains also showed the ability to grow on Gli medium, and under this condition both of these strains started quickly, reaching 10^{8} CFU/mL (2 logs more) in 3 h; they then

grew relatively slowly for 12 h (to 10^9 CFU/mL), and then they increased again to 10^{10} CFU/mL at 18 h of incubation and reached the stationary phase.

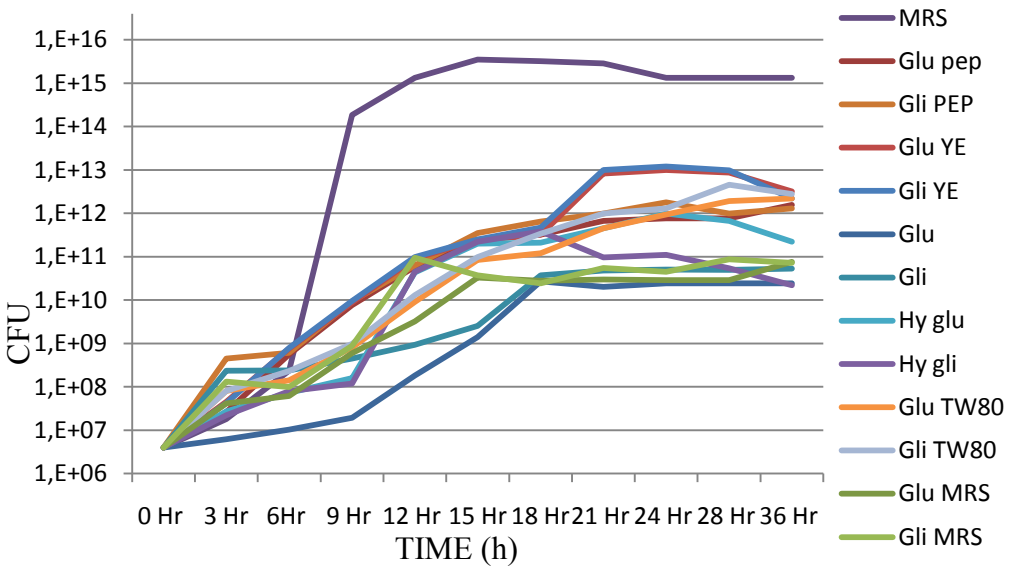

Figure 16. Natural strain Sb7b growth rates for the media indicated (listed in Maerials and methods).

When the nitrogen source in the media was subjected to hydrolysis (Hy gli and Hy glu media), lag phases were shown by both strains in the first 3 h of the experiment (2.5 log of increment); this increment was followed by the logarithmic phase that ended at 18 h for Hy gli, reaching 5.8 $\times 10^{11}$ CFU/mL and 4.5 $\times 10^{11}$ CFU/mL for the strains Sb5c and Sb7b, respectively. After this peak, the number of viable cells immediately started to decrease. The Hy glu medium allowed the strains to grow in logarithmic phase until 24 h and o reach 10^{12} CFU/ml. After the end of the logarithmic phase, a short stationary phase was observed and the number of viable cells started to decrease after 28 h from the beginning of the fermentation. For the Glu TW80 and Gli TW80 media, there was a very short lag phase and both strains were able to reach 10^{11} CFU/mL in 15 h of growth. After this time point, we observe differences between the Glu TW80 and Gli TW80

media. For the former, the logarithmic phase continued until 28 h and the strains reach 4.5 $\times 10^{12}$ and 2.6 $\times 10^{12}$ CFU/mL for the strains Sb5c and Sb7b, respectively. For the latter (Gli TW80 medium), the logarithmic phase ended at 15 h, to reach the stationary phase where the strains counted as 3.8 $\times 10^{10}$ and 9.8 $\times 10^{9}$ for the Sb5c and Sb7b strains, respectively. On the media with peptone, almost no lag phase was observed. For the Glu PEP medium, both strains reached 10^{9} CFU/mL in 2 h of incubation (3 logs). The strains then grew in a logarithmic way to 28 h, and reached final densities of 9.5 $\times 10^{12}$ CFU/mL for strain Sb5c, and 7.8 $\times 10^{12}$ CFU/mL for strain Sb7b. The stationary phase in this medium was observed until the end of the experiment, and no significant decreases in the numbers of viable cells were seen. In the same way, on Gli PEP medium, the strains grew to reach 10^{9} CFU/mL in 2 h, and the logarithmic phase of growth finished as in the Glu PEP medium (28 h), although at the end of this the strains counted as 1.2 $\times 10^{11}$ CFU/mL and 9.8 $\times 10^{10}$ CFU/mL for the Sb5c and the Sb7b strains, respectively. On the Glu YE and Gli YE media, the strains showed the best growth performances. In these media, there was almost no lag phase detected, and the strains grew in a logarithmic way for 12 h, showing no differences between the Glu YE and the Gli YE media and between the strains (10^{10} CFU/mL). On the Gli YE media, the strain Sb5c continued to grow in a logarithmic way until 18 h, to reach 9.4 $\times 10^{13}$ CFU/mL. On the Glu YE medium, the Sb5c strain reached 5.6 $\times 10^{12}$ at 18 h of incubation, and 1.1 $\times 10^{13}$ at 24 h. The strain Sb7b showed a different behaviour: after 12 h, there was no increase in the density, and this reached 2.3 $\times 10^{11}$ CFU/mL at 18 h. After this time, growth is observed, and this strain reached 1.2 $\times 10^{13}$ CFU/mL and 9.9 $\times 10^{12}$ CFU/mL at 24

h. The media made using the MRS base (Glu MRS and Gli MRS) did not show any interesting differences from the gluten or gliadine minimal media.

Acidification rate

Reference strain WCSF1

Acidification rates were measured as described in the Materials and methods. As shown in Figure 17, we see that the reference strain WCSF1 reduced the pH for all of the media used in this study. From the data in Figure 17, we calculated the acidification rates expressed in mU pH/min (Fig. 18). Both Figures show a wide range of acidification rates .With the aim to provide a reference to compare the data obtained, we calculated the acidification parameters on MRS media: the lowest pH on this was 4, and the V_m value of -10.0 mU pH/min was reached after 8 h and 6 min from the starting of the culture. Between the gluten/ gliadine-based media, the best performance was observed in the gliadine yeast-extract medium, where there was a lower pH value (3.16), the highest (in absolute value) acidification rate of -12.359 mU pH/min, and the short T_m value of just 3 h and 12 min.

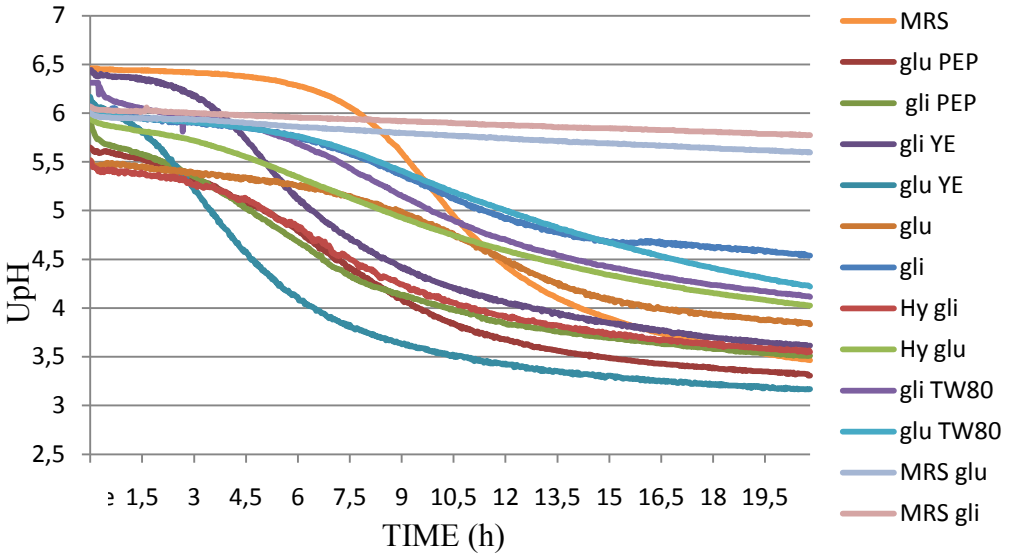

Figure 17. pH variations during the WCSF1 strain fermentation on the different media.

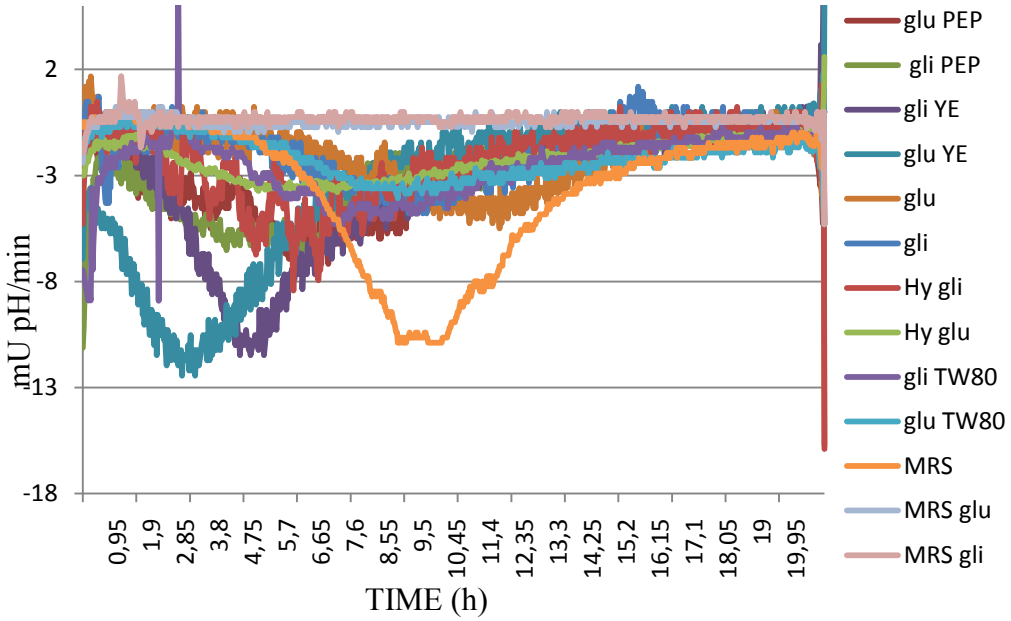

Figure 18. Acidification rates for the WCSF1 strain on the different media, calculated as the first derivates of mU pH/min.

On the media with peptone and gluten or gliadine, the pH reached values of 3.30 and 3.50, high V_m values were observed (-7.766 and -7.340 mU pH/min) and a rapid T_m; indeed, the V_m value was reached 6 h and 7 min and in 6 h and 13 min in the media with gluten and gliadine, respectively. An intermediate behaviour was shown when the strain WCSF1 was inoculated on gluten yeast-extract media. On this media we observed a very good V_m value (-11.522 mU pH/min) and also a good T_m value (4 h 45 min), but the minimal pH was 3.59. The most poor media with only gluten or gliadine as the nitrogen source showed minimal pH values of 3.30 and 4.52, respectively, V_m values of -5.76 mU pH/min and 5.04 mU pH/min, and T_m values of 10 h and 39 min and of 9 h and 4 min when on gluten and gliadine, respectively. The other media showed a intermediate behaviours, and the summarised data from those runs are listed on Table 8.

Table 7. Summarised review of the minimun pH, ΔpH, V_m and T_m calculated from the automatic pH measurements for the WCSF1 strain. The data are means ±standard deviations from three independent experiments

Medium	**Minimum pH**	**ΔpH**	**Maximun acidification rate, Vm** (mU pH/min)	**Time when Vm was observed, T_m** (hh:mm:ss)
Glu PEP	3.30 ±0.15	2.34 ±0.14	-7.766 ±1.2	06:07:30
Gli PEP	3.50 ±0.16	2.42 ±0.13	-7.347 ±1.4	06:13:30
Glu YE	3.59 ±0.17	2.99 ±0.10	-11.522 ±2.1	04:45:00
Gli YE	3.16 ±0.11	2.84 0±0.10	-12.359 ±1.8	03:12:00
Glu	3.83 ±0.15	1.67 ±0.17	-5.766 ±2.5	10:39:00
Gli	4.52 ±0.19	1.54 ±0.14	-5.014 ±2.1	09:04:00
Hy glu	3.84 ±0.12	1.96 ±0.13	-8.066 ±1.5	05:57:00
Hy gli	3.54 ±0.18	1.96 ±0.14	-5.790 ±1.3	05:55:00
Gl TW80	3.87 ±0.14	2.19 ±0.20	-9.577 ±1.9	04:46:30
Gli TW80	3.67 ±0.15	1.78 ±0.14	-5.571 ±2.0	06:33:00
MRS	4.00 ±0.11	2.99 ±0.13	-10.903 ±1.4	08:06:00
MRS glu	5.59 ±0.25	0.41 ±0.20	-0.5047 ±1.3	07:24:00
MRS Gli	5.77 ±0.25	0.29 ±0.19	-0.7571 ±1.2	08:24:00

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Strain Sb5c

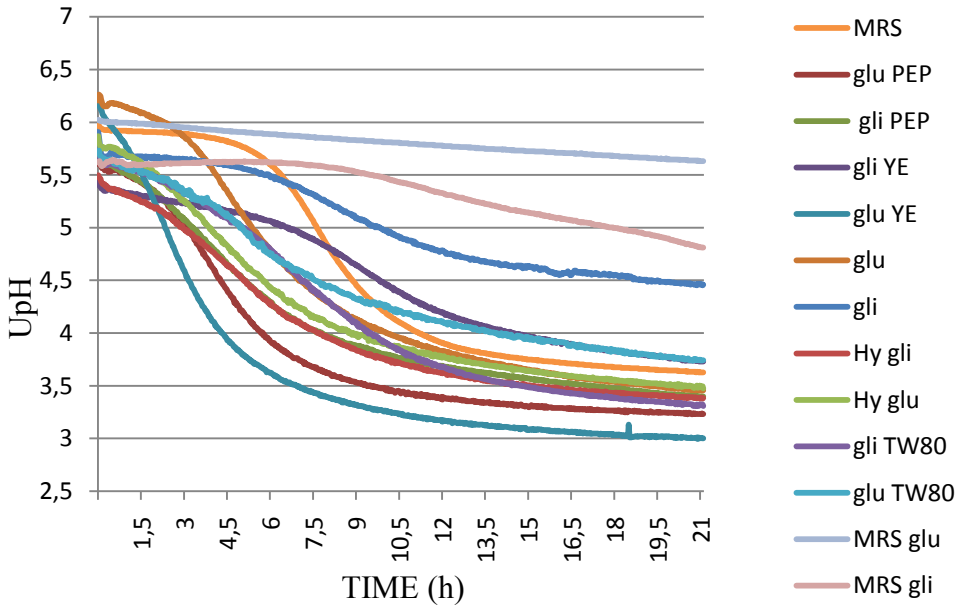

Figure 19. pH variations during the Sb5c strain fermentation on the different media.

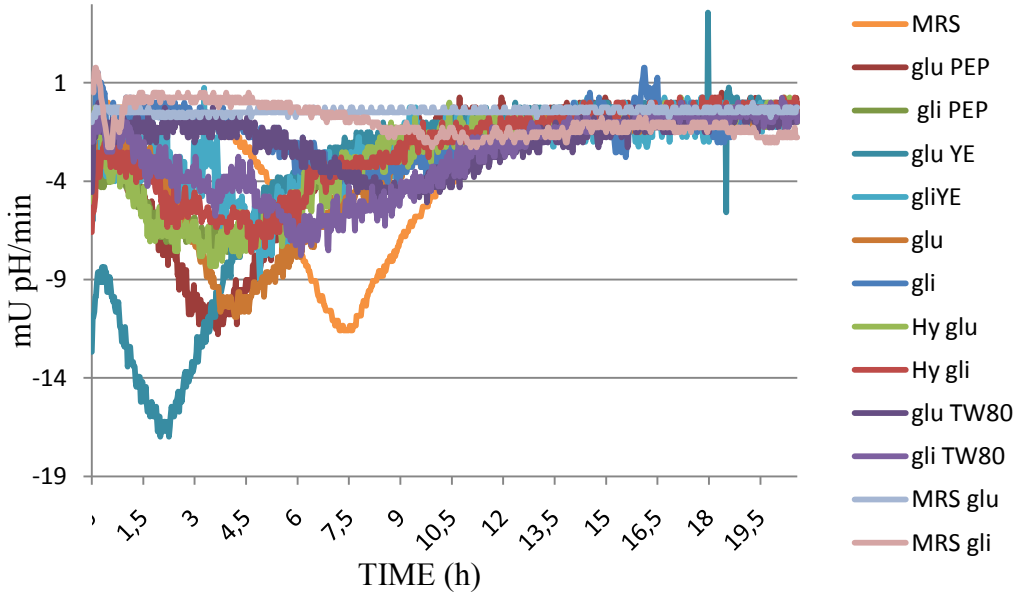

Figure 20. Acidification rates for the Sb5c strain on the different media, calculated as the first derivates of mU pH/min.

From the Figures 19 and 20, we can see that strain Sb5c shows the best performances for acidification and metabolic activity in the medium containing yeast extract and peptone. The MRS medium was used as the reference and strain Sb5c showed a V_m of -11.59 mU pH/ min, with the T_m as 7 h and 15 min, and the pH decrease (ΔpH) of 2.354 U pH. In the medium with gluten and yeast extract we observed the highest acidification rate (-16.981 mU pH/min) and the shortest T_m (only 2 h and 1.5 min). Also, the ΔpH was the highest in the range of these media used (3.199 U pH). Good performance was also seen in the medium with gluten and peptone, where this strain reached a V_m of -11.776 mU pH/min, also wih a good T_m of 3 h and 42 min, and a ΔpH of 2.393 U pH. On the gliadine yeast extract medium, this Sb5c strain also showed a remarkably high V_m (-9.019 mU pH/min) which was reached in 4 h and 55 min (ΔpH, 1.978 U pH). The medium with gliadine and peptone showed a good V_m (-7.857 mU pH/min) and T_m (3 h and 45 min), with a ΔpH of 2.728 U pH.

Table 8. Summarised review of the minimun pH, ΔpH, V_m, and T_m calculated from the automatic pH measurement for the Sb5c strain. The data are means ±standard deviations from three independent experiments.

Medium name	**Minimum pH**	**ΔpH**	**Maximun acidification rate Vm** (mUpH/min)	**T_m Time when Vm was observed** (hh:mm:ss)
Glu PEP	3,23	2,393	-11,776	3:42:00
Gli PEP	3,39	2,728	-7,8571	3:45:00
Glu YE	3,00	3,199	-16,981	2:01:30
Gli YE	3,74	1,978	-9,019	4:55:30
Glu	3,45	2,746	-10,9	4:12:00
Gli	4,45	1,447	-4,057	7:31:30
Hy glu	3,38	2,392	-8,31905	3:28:30
Hy gli	3,47	2,113	-7,6	4:40:30
Gl TW80	3,72	1,724	-5,070	8:19:30
Gli TW80	3,30	2,341	-7,766	6:07:30
MRS	3,62	2,354	-11,59	7:15:00
MRS glu	5,63	0,391	-0,75	1:51:00
MRS Gli	4,80	0,809	-2,2809	9:57:00

The medium with gliadine and peptone showed a good Vm (-7.857 mU pH/min) and Tm (3 h and 45 min) (□pH 2.728). On the minimal media with only gluten and gliadine, strain Sb5c showed the lower Vm values of -5.070 mU pH/min on gluten media and -4.057 mU pH/min on gliadine media, with the time when the Vm is reached (Tm) reflecting this behaviour: on the gluten medium the Tm was 8 h and 19 min, and on the gliadine medium it was 7 h and 31 min, with □pH values of 1.724 and 1.447, respectively. Intermediate behaveiour was shown for the gluten and gliadine hydrolysed media. In these media, the Vm values were -8.319 mU pH/min (hydrolysed gluten) and -7.600 mU pH/min (hydrolysed gliadine). Also, more interestingly, the Tm values were 3 h and 28 min (hydrolysed gluten) and 4 h and 40 min (hydrolysed gliadine), and the □pH values were 2.392 U pH and 2.113 U pH, respectivelly. Good performance was also shown when Tween 80 was added to the minimal media. In the case of the gluten plus Tween 80 medium, a very good Vm was observed (-10.900 mU pH/min), the Tm was 4 h and 12 min, and the □pH was 1.724 U pH. On the gliadine plus Tween 80 medium, the Vm was -7.766 mU pH/min and the Tm was 6 h and 7 min (□pH, 2.341). The MRS-based media showed very low pH changes (see Table 9).

Strain Sb7b

The natural strain Sb7b also had the ability to lower the pH of the media where it grows. Figures 21 and 22 shown the pH variations during these fermentations on the medium described in the Materials and methods (Fig. 21) and the metabolic activities derived from the pH measurements (Fig. 22)

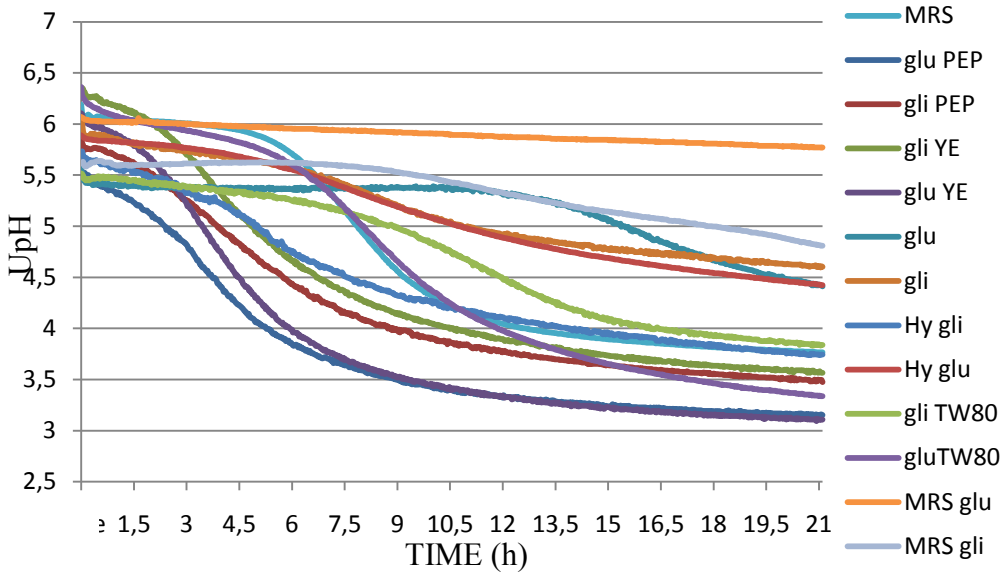

Figure 21. pH variations during the Sb7b strain fermentation on the different media.

For the strain Sb7b on the MRS medium, we found a V_m of -7.771 mU pH/min and a T_m of 4 h and 54 min, with a ΔpH of 2.423. The best performance of this strain was observed on gluten yeast-extract medium, where we measured the highest V_m (-13.861 mU pH/min) and the T_m of 3 h and 12 min; the ΔpH was 2.99 U pH.

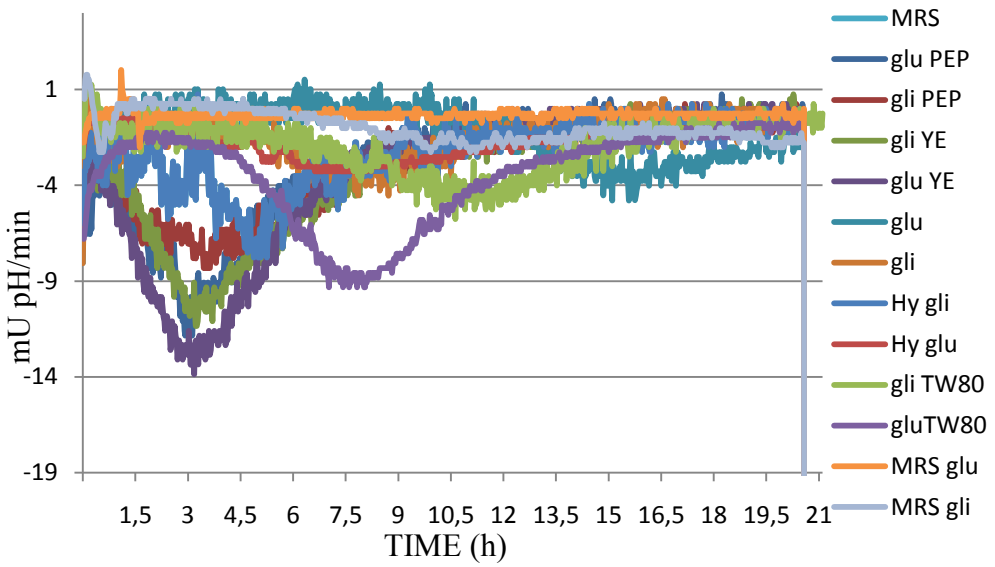

Figure 22. Acidification rate for the Sb7b strain on the different media, calculated as the first derivates of mU pH/min.

Also, a good performance was found on the gluten peptone medium, which showed a V_m of -11.842 mU pH/min and the shortest T_m (2 h and 57 min), and a ΔpH of 2.34 U pH. A good behaviour was also observed on gliadine media with yeast extract and with peptone; in these cases we observed V_m values of -11.338 mU pH/min and -7,809 mUpH/min, respectively. The T_m values were 3 h and 16 min for the gliadine yeast-extract medium, and 3 h and 10 min for the medium with peptone (ΔpH values, 2,.68 and 2.39 U pH, respectively). On the gluten medium, we observed a V_m of -4.79 mU pH/min at 15 h and 7 min (T_m), and a ΔpH of 1.164 U pH. When the strain Sb7b was grown on gliadine minimal medium, the V_m was -4.538 mU pH/min, which was observed after 8 h and 45 min (ΔpH, 1.465 U pH). In general, the hydrolysed substrates revealed better performances than the non-hydrolysed ones: we found V_m values of -5.771 mU pH/min and -5.2761 mU pH/min on the gliadine and gluten hydrolysed media,

respectively. The T_m values were 4 h and 54 min, and 6 h and 39 min for the gliadin and gluten media, respectively (ΔpH, 1.97 U pH, and 1.45 U pH, respectively). The Tween 80 media also showed better performances than the minimal gliadine and gluten media; indeed, we observed V_m values of -9.323 mU pH/min and -8.766 mU pH/min for these gluten and gliadine media, respectively. The T_m values were also improved (5 h and 30 min for gluten plus Tween 80, and 4 h and 15 min for gliadine plus Tween 80), with ΔpH of 3.03 U pH for the gluten plus Tween 80 medium, and 1.67 U pH for the gliadine plus Tween 80 medium. The MRS gluten and gliadine media showed very low pH decreases (ΔpH, 0.296 U pH and 0.809 U pH, respectively)(see Table 10).

Table 9. Summarised review of the minimun pH, ΔpH, V_m and T_m calculated from the automatic pH measurement for the strain Sb7b. The data are means ±standard deviations from three independent experiments.

Medium name	**Minimum pH**	**ΔpH**	**Maximun acidification rate Vm** (mUpH/min)	**T_m Time when Vm was observed** (hh:mm:ss)
Glu PEP	3,15	2,349	-11,8429	2:57:00
Gli PEP	3,47	2,392	-7,8095	3:10:30
Glu YE	3,11	2,995	-13,8619	3:12:00
Gli YE	3,56	2,688	-11,3381	3:16:30
Glu	4,41	1,164	-4,7904	15:07:30
Gli	4,60	1,465	-4,5381	8:45:00
Hy glu	4,42	1,465	-3,27619	6:39:00
Hy gli	3,74	1,978	-7,77143	4:54:00
Gl TW80	3,37	3,027	-9,32381	7:30:00
Gli TW80	3,39	1,678	-5,76667	10:15:30
MRS	3,76	2,423	-7,77143	4:54:00
MRS glu	5,77	0,296	-0,75714	2:42:00
MRS Gli	4,80	0,809	-0,50476	7:01:30

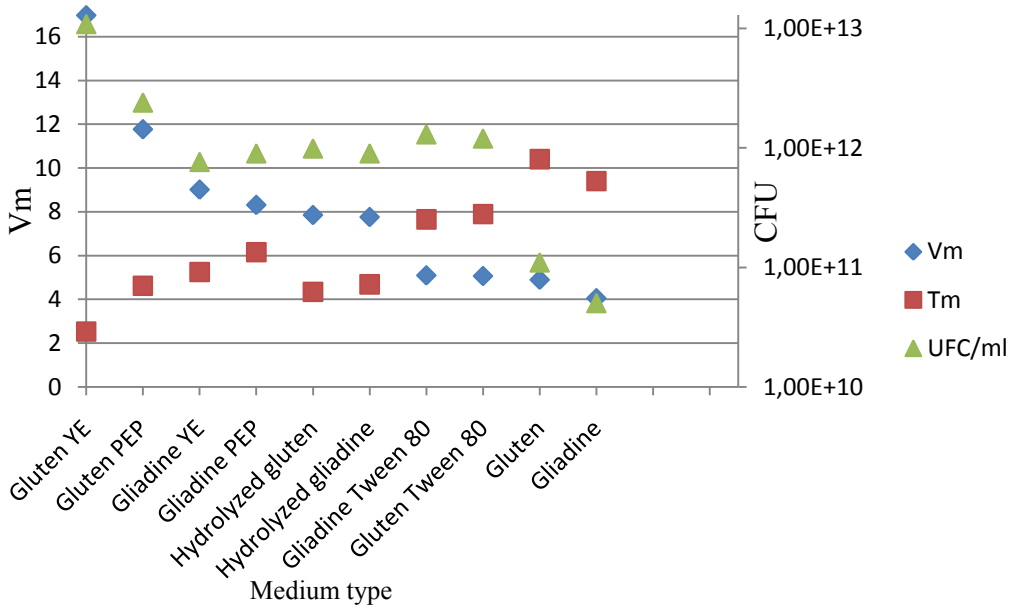

Figure 23. Representation of the Sb5c strain acidification parameters and CFU/mL, medium (abscissa) are listed from the highest to the lower Vm value best performance is shown by glu YE media and increment of CFU is showed when tween 80 is added to the media, decreasing in Tm value is visible to the Hy media. Same behavior was shown to the reference strain WCSF1 and the wild strain SB7B (data not shown)..

Proteolytic activity

Proteolytic activity measurements were performed to evaluate the levels of proteolytic activity at different times and under different conditions of growth. Figure 24 shows the proteolysis levels (absorbance at 340 nm, as arbitrary units [AU]) for the strain WCSF1 measured during the bacterial growth. Based on these data, we can observe that on the gli and glu media, the proteolysis reaches the highest levels (7.0 and 7.2 AU for glu and gli, respectively).

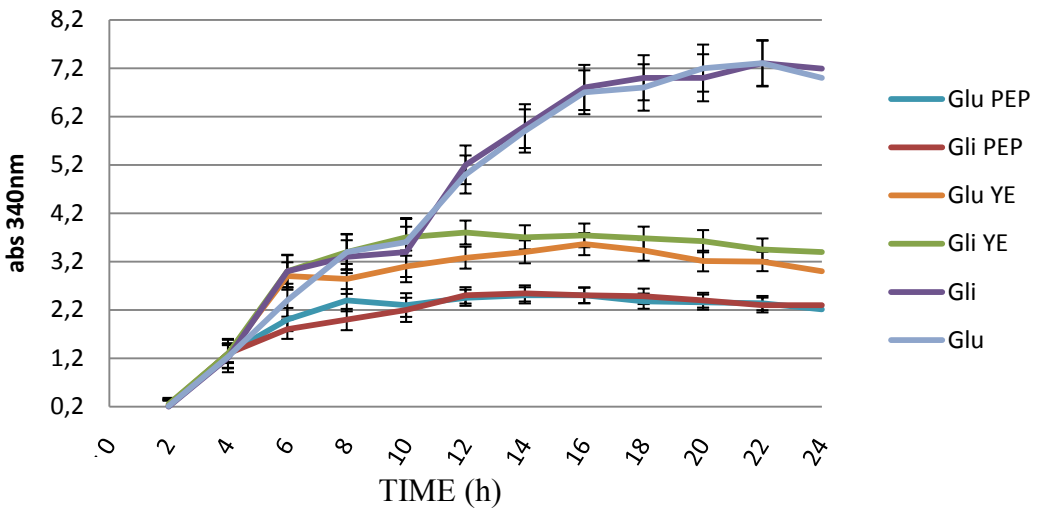

Figure 24. Proteolytic activity of the strain WCSF1 measured as absorbance at 340 nm (AU) assayed every 2 h. The data are means ±standard deviations from three independent experiments..

Indeed, in these media, the proteolytic activities of the strain WCSF1 appear to start later on compared to the Gli YE and Glu YE media, where the highest levels (around 0.55 AU for both medium) are reached in 12 h, which then remain roughly constant until the end of the experiment. On the contrary, after 10 h of growth in the glu and gli media, we see improvements in the proteolysis that remain constant to 24 h. In the Glu PEP and Gli PEP media, the proteolysis levels did not reach particularly higher levels.

Figures 25 and 26 show these proteolysis levels as reached by the wild strains Sb5c and Sb7b. As for the reference strain WCSF1, we see the highest proteolysis levels on the minimal media gli and glu; indeed, in both of these media, both wild strains reached proteolysis levels of around 6 AU after 10 h of fermentation, with only slight differences between these media.

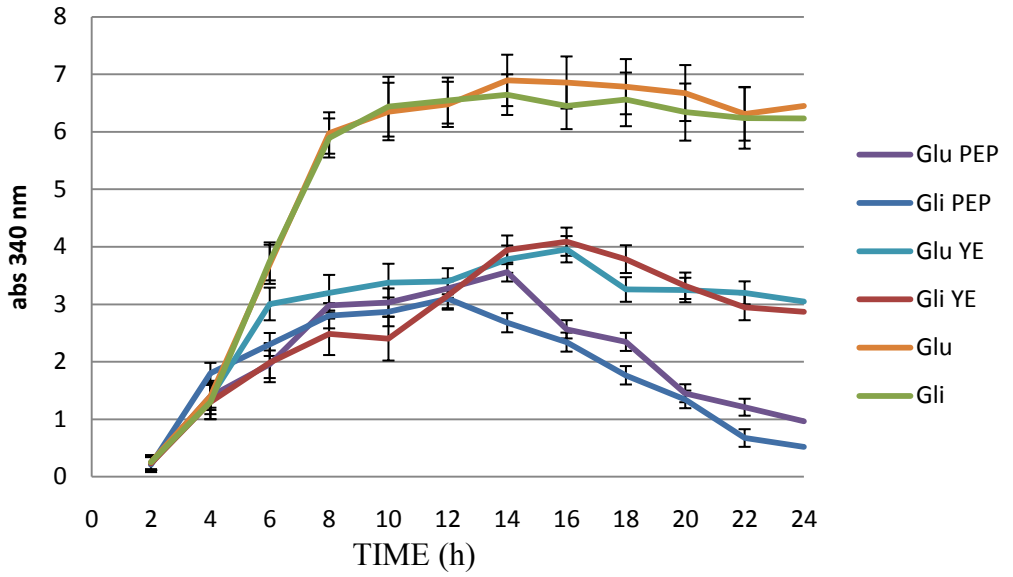

Figure 25. Proteolytic activity of the strain Sb5c measured as absorbance at 340 nm (AU) and assayed every 2 h. The data are means ±standard deviations from three independent experiments.

In the Glu PEP and Gli PEP media, in contrast, we found lower levels of proteolysis, which reached maximum levels at 12 h (3 AU); then after this time point, differences can be see between the Gli PEP and Glu PEP media.

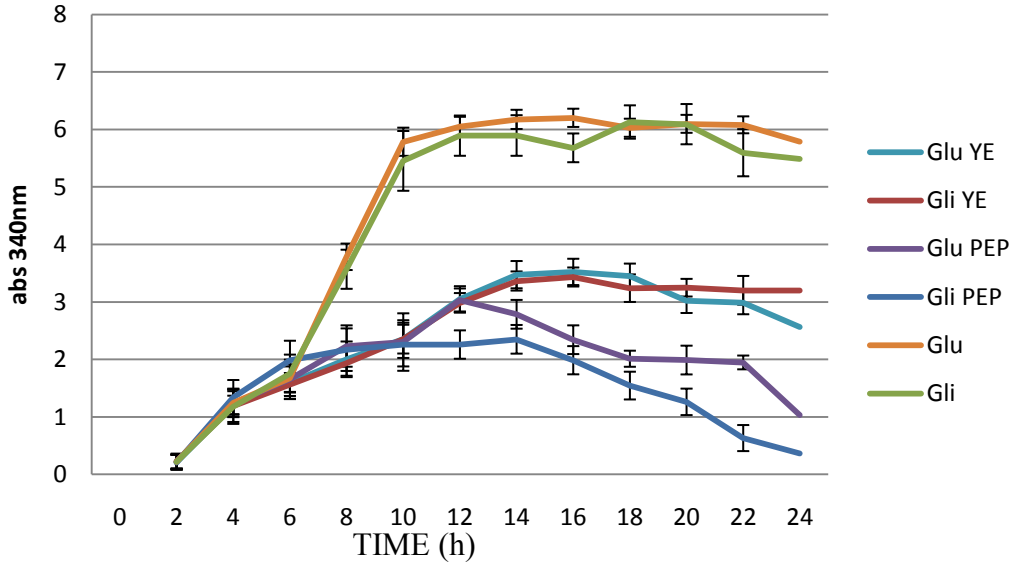

Figure 26. Proteolytic activity of the strain Sb7b measured as absorbance at 340 nm (AU) and assayed every 2 h. The data are means ±standard deviations from three independent experiments.

In Glu PEP medium, the proteolysis was roughly 1 AU higher than for the Gli PEP medium. In both of these media, the proteolysis levels later decreased, until the end of the experiment, and reached final levels of 0.36 AU for Gli PE and 1.04 AU for Glu PEP. Different behaviours were observed in the media with yeast extract, where the proteolysis levels were intermediate between the two previously described media. The highest proteolysis levels in these media were reached in 16 h, with the SB5c strain reaching 3.63 AU and 3.79 AU, while the Sb7b strain reached 3.43 AU and 3.52 AU in the Gli YE and Glu YE media, respectively. After this time point, the proteolysis levels remained roughly constant until the end of the experiment.

Proteolytic activity characterisation

During cell growth, a number of proteolytic enzymes have to be expressed at different times, to ensure a nitrogen source for cell metabolism. Proteolytic activity characterisation was perfomed to investigate the enzymatic activities in relation to the media where the cells were growing. As described in the Materials and methods, we performed 2-h kinetic activity assays on the cell lysates, although no detectable activities were observed using this method (data not show). A 3-h incubation time was also considered not to be enough time to complete the colour development in the cuvette, so in this section the data are only reported for a 15-h incubation.

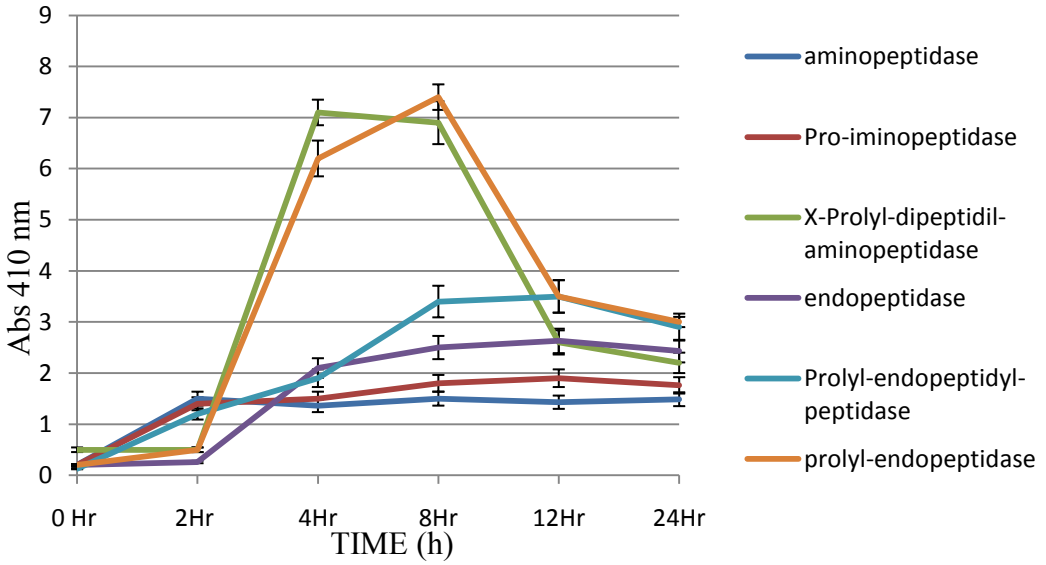

Figure 27. Proteolytic activity characterisation of the WCSF1 strain on gluten media. The data are means ±standard deviations from three independent experiments. (see Materials and methods).

Based on the results showed on Figure 27 we can see that aminopeptidase and pro-iminopeptidase enzymes revealed with the leu-pNa and pro-pNa substrates (with absorbance measured at 410 nm, as AU), respectively, were

present in the early phase of grow (2 h), and then remained roughly constant until the end of the experiment. The prolyl-endopeptidyl-peptidase enzymes revealed with the substrate Z-gly-gly-leu-pNa (with absorbance measured at 410 nm, as AU) are detectable at good levels at the 2-h time point (1.2 AU); these increased to reach a peak at 12 h (3.5 AU) and then decreased (2.9 AU). The endopeptidase enzyme family revealed with the substrate gly-pro-ala-pNa (with absorbance measured at 410 nm, as AU) showed an increment from the 2-h time point and 4 hours timepoint (2.1 AU at 4 h, and 2.6 AU at 12 h), later than the other activities described above, and then remained roughly constant until the last time point (2.4 AU at 24 h). The most interesting aspects were the enzyme family of prolyl endopeptidase, and X-prolyl-dipeptidyl-aminopeptidase, which were revealed by the gly-pro-pNa and suc-ala-pro-pNa substrates, respectively (with absorbance measured at 410 nm, as AU). For these two categories of enzymes, a high peak of activity was detected at 4 h (6.2 AU for gly-pro-pNa) and 8 h (6.9 AU for suc-ala-pro-pNa) of growth. The activities of these prolyl endopeptidase family enzymes remained roughly constant between 4h and 8 h of growth (7.4 AU), and after this time drammatic decreases in the activities was showed for both of these enzyme family. Figure 28 shows what happened when the same strain was inoculated on the media with yeast extract.

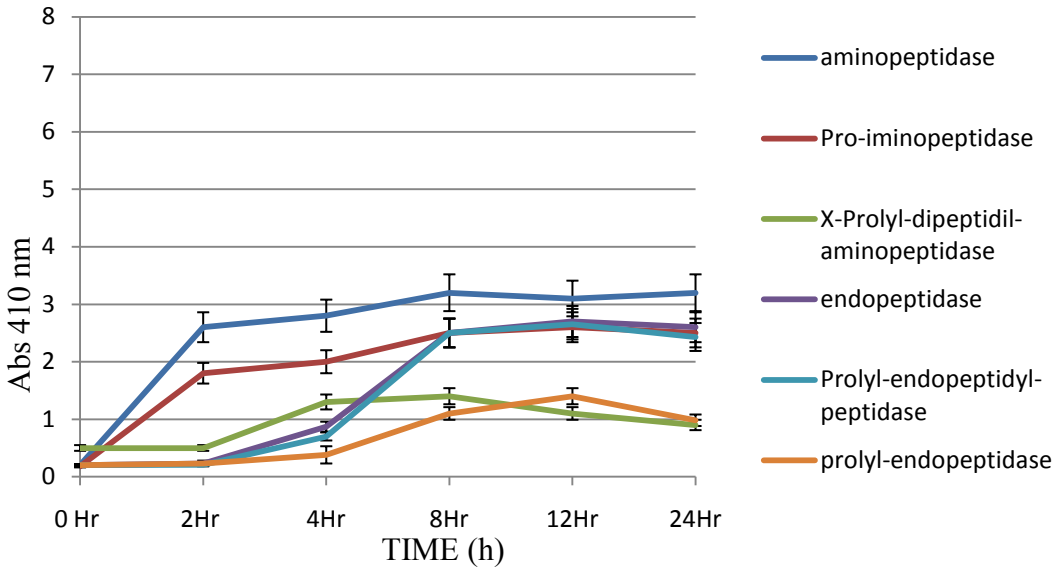

Figure 28. Proteolytic activity characterisation of the WCSF1 strain on media with 0.05% yeast extract. The data are means ±standard deviations from three independent experiments. (see Materials and methods).

A general depression of all of these activities is seen here for the prevalence of the aminopeptidase and pro-iminopeptidase enzymes measured. The most relevant difference is for the behaviour of the prolyl endopeptidase and X-prolyl-dipeptidyl-aminopeptidase, which did not showed any significant large in creases in activity, but just a small increase in the late phase of growth.

The wild strains Sb5c and Sb7b showed similar behaviours, although with generally lower levels of the activities detected. In terms of the activities detected on the glu media, the results shown on Figures 29 and 30 allow us to see that aminopeptidase and pro-iminopeptidase activities are revealed at the beginning of the growth, as happened for the reference strain WCSF1. Indeed, at this time, the relevant substrates for this enzymes showed the highest absorbances (Sb5c, 1.3 AU and 1.5 AU; Sb7b, 1.3 AU and 0.9 UA, for the aminopeptidases and pro-iminopeptidases, respectively). These activities increased to a maxima at 8 h

(Sb5c, 2.4 AU and 2.8 AU; Sb7b, 2.1 AU and 2.5 AU, for leu-pNA and pro-pNA, respectively), and slowly decreased until the end of the experiment (Sb5c, 2.1 AU and 2.3 AU; Sb7b, 1.8 AU and 2.2 AU, for aminopeptidases and pro-iminopeptidases, respectively). The prolyl-endopeptidyl-peptidase enzymes are hardly detectable in the early phase of grow (Sb5c, 0.3 AU; Sb7b, 0.4 AU). The enzymes family of endopeptidase was poorly detectable at 2 h, and they showed their highest activities from 4 h (Sb5c, 2.8 AU; Sb7b, 2.3 AU) to 8 h (Sb5c, 2.5 AU; Sb7b, 2.8 AU). These activities were also well detectable at 12 h (Sb5c, 2.3 AU; Sb7b, 2.1 AU), while at 24 hours they showed lower activities (Sb5c, 1.8 AU; Sb7b, 1.5 AU).

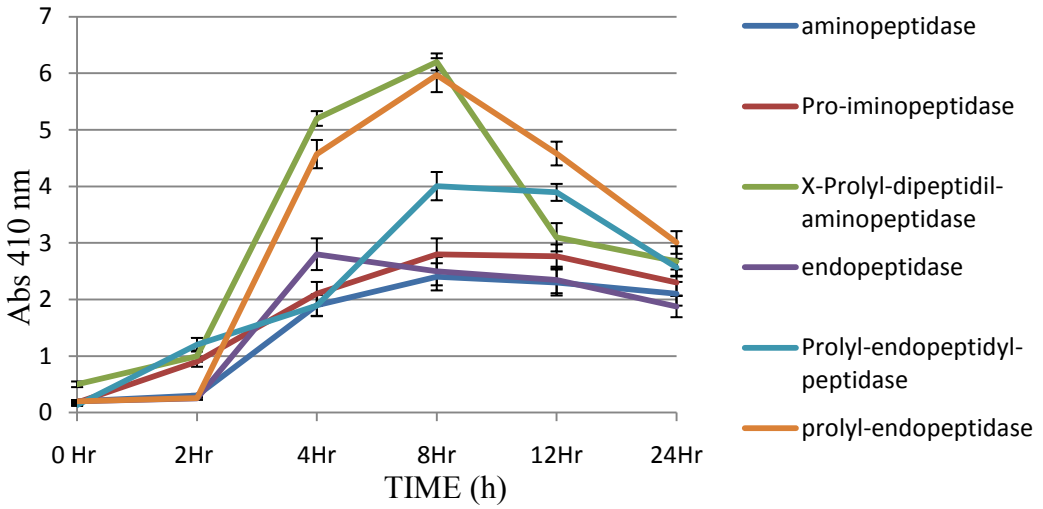

Figure 29. Proteolytic activity characterisation for the Sb5c strain growing on gluten media. The data are means ±standard deviations from three independent experiments. (see Materials and methods).

After 8 hours, the activity start to increase, to reach the highest levels at 8 h (Sb5c, 4.00 AU; Sb7b, 3.00 AU), and this level of activity remained constant to the next time point (Sb5c, 3.8 AU; Sb7b, 2.9 AU), and then started to decrease, to reach 2.5 AU for Sb5c and 2.21 AU for Sb7b at 24 h.

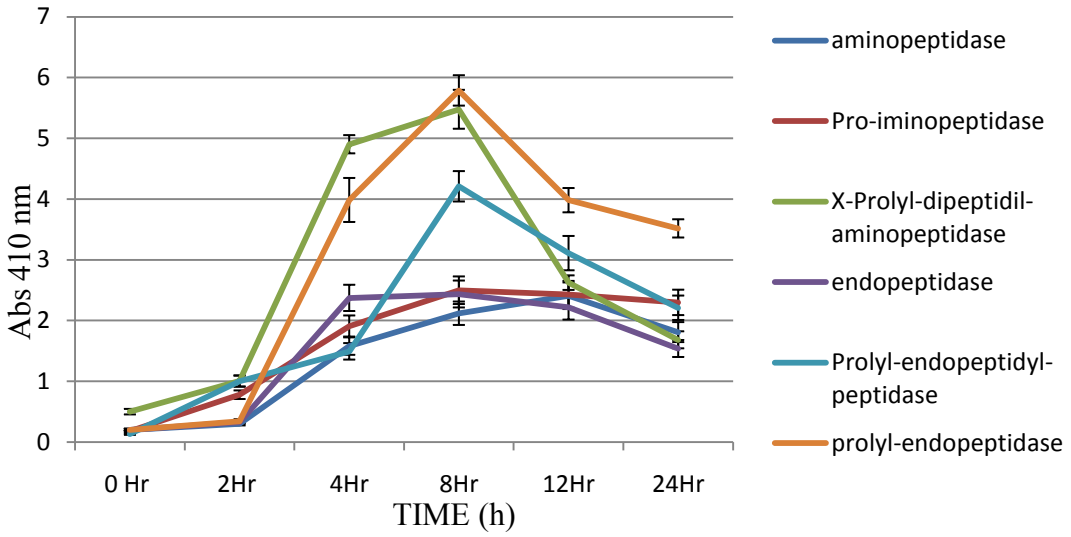

Figure 30. Proteolytic activity characterisation of the Sb7b strain grown on gluten media. The data are means ±standard deviations from three independent experiments (see Materials and methods).

The enzyme families of the prolyl endopeptidases and X-Prolyl-dipeptidil-aminopeptidases were again the most active in this experiment. These two families of enzymes showed high peaks of activity detectable at 4 h. At this time, the prolyl endopeptidase enzymes reached 4.0 AU for the strain Sb5c, and 3.8 AU for the strain Sb7b; these high activities remained to the next time point with little difference (Sb5c, 4.1 AU; Sb7b, 4.02 AU). High activities for this enzyme were found until the end of the experiment, when the absorbance of 3.0 AU and 2.6 AU were measured for the Sb5c and Sb7b strains, respectively. The X-prolyl-dipeptidyl-aminopeptidase family enzymes also showed large activities between 4 h and 8 h, with the maximun activity detected at 4 h, rather that the 8 h seen for prolyl endopeptidase. At 4 h, we detected 3.2 AU and 3.6 AU for the Sb5c and Sb7b strains, respectively; this activity was increased further for the Sb5c strain (4.02 AU), while there was a small decreasing for the Sb7b strain (3.2 AU). In a similar way to the prolyl endopeptidase, X-prolyl-dipeptidyl-

aminopeptidases then decreased their detectable activities, to reach 2.6 AU for the Sb5c strain, and 1.7 AU for the Sb7b strain.

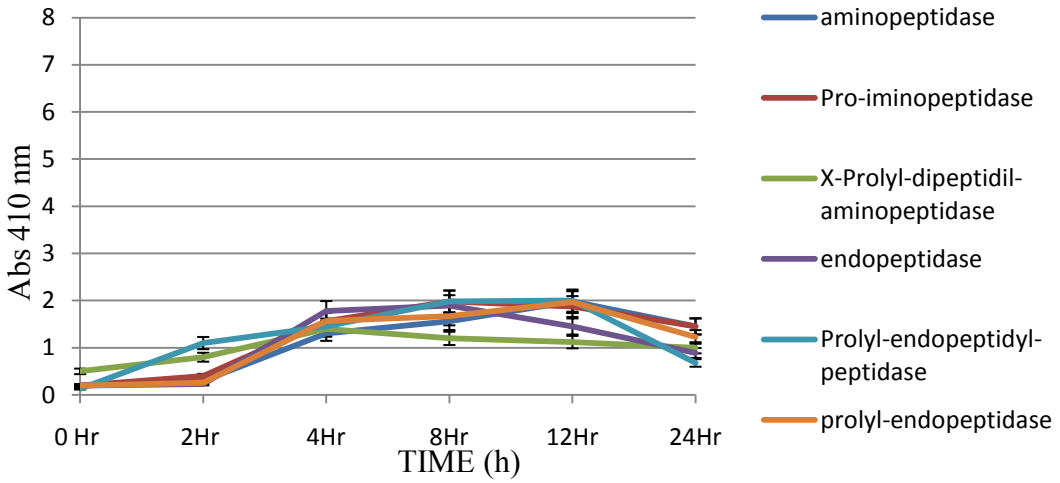

Figure 31. Proteolytic activity characterisation for the Sb5c strain on the media with 0.05% yeast extract. The data are emans ±standard deviations from three independent experiments. (see Materials and methods).

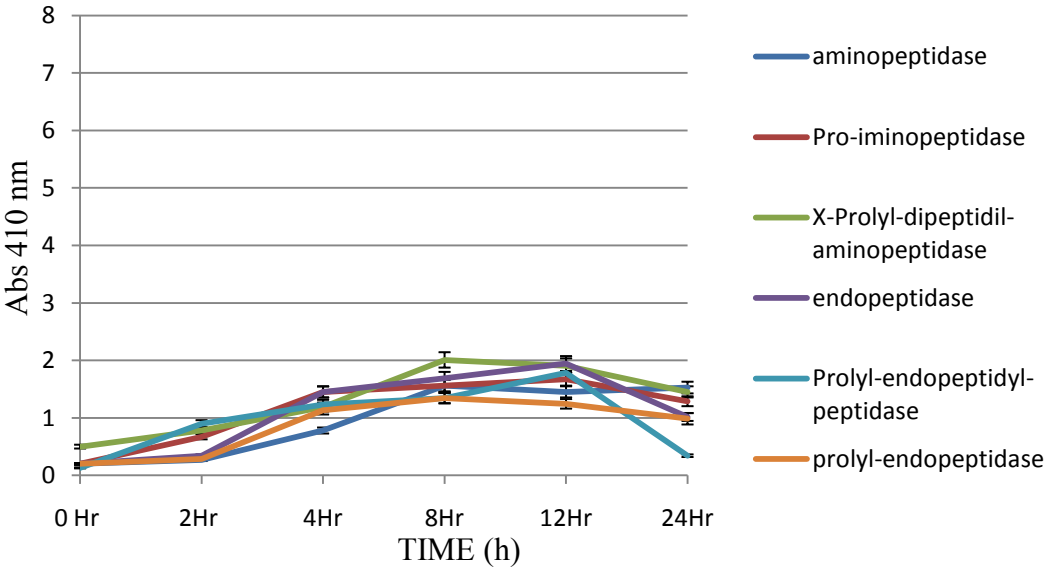

Figure 32. Proteolytic activity characterisationfor the Sb7b strain on the media with 0.05% yeast extract. The data are means ±standard deviations from three independent experiments. (see Materials and methods).

With the same aim, we also performed these experiments with gluten yeast-extract media (Figs. 31 and 32). Under these growth conditions we had

already found lower levels of general proteolysis for both of these strains. As can easily be seen in Figures 31 and 32, the level of specific proteolysis here lower than in the gluten media.

Gluten and gliadine hydrolysis

To evaluate the actual consumption of gluten by the strains, and what part of he gluten protein complex this bacteria can hydrolyse, the gluten was extracted from the gluten media at the zero time point and at the end of these incubations (24 h). These were then assayed by RP-HPLC, and the resulting chromatograms for absorbance at 210/220 nm are showed in the following Figure (Fig. 33).

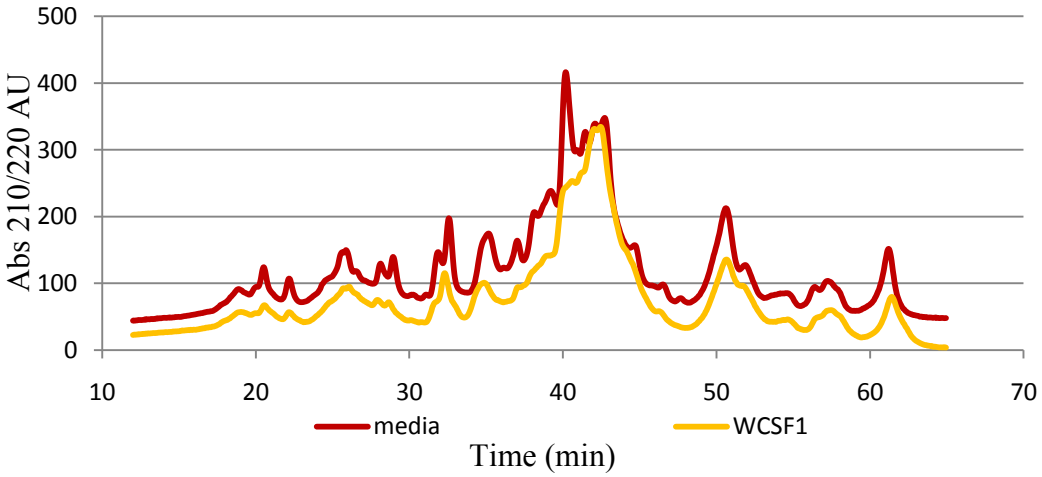

Figure 33. Chromatogram comparisons for the gluten extracted from the media (GYE, 0.05% yeast extract) (brown line) and from the same media after strain WSCF1 had grown on it (yellow line).

RP-HPLC analysis of the gluten extract from the media after the bacterial growth showed several significant differences between the reference media and the media on which the strains had been grown. In the chromatogram (Fig. 33) we can see decreases in the fractions corresponding to the ω gliadine (retention time, 20 min), high-molecular-weight glutenin (retention times, 25 min and 28 min), α

gliadine (retention time, 42 min) and γ gliadin (retention time, 62 min). When the strain WCSF1 grew on minimal media containing only gluten as the nitrogen source (Fig. 34), there was a dramatic decrease in the fraction corresponding to α gliadin (retention time, 42 min), which was greater than that observed when the media with yeast extract was used. There was also a strong reduction in the peak at 45 min retention time, corresponding to β gliadine, which was not seen previously.

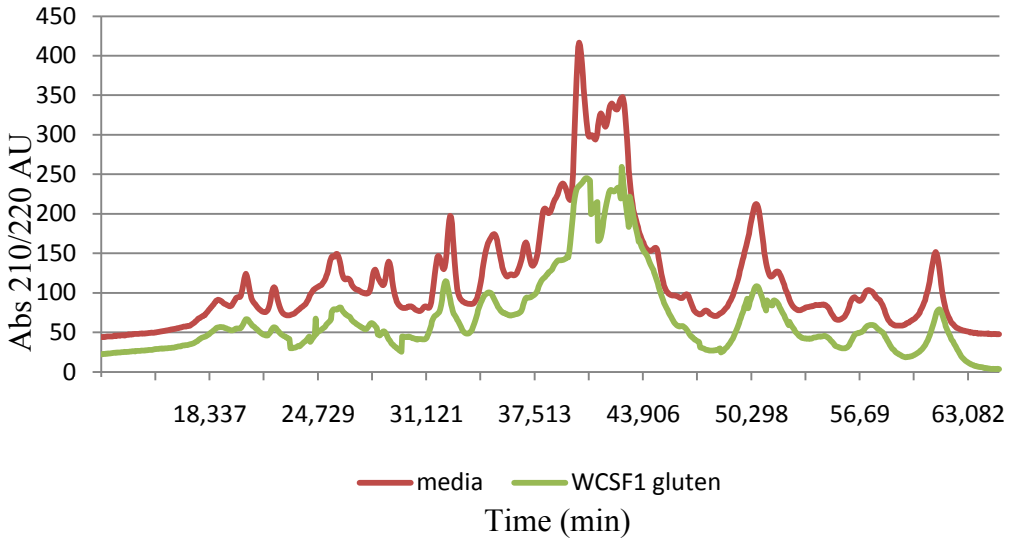

Figure 34. Chromatograms of the gluten extract from the minimal medium (1% gluten, 2% glucose) and of the same medium after the strain WCSF1 had grown on it.

The wild strains Sb5c and Sb7b were also evaluated regarding their ability to hydrolyze gluten, and to assess which part of the gluten protein complex these strains were able to hydrolyse.

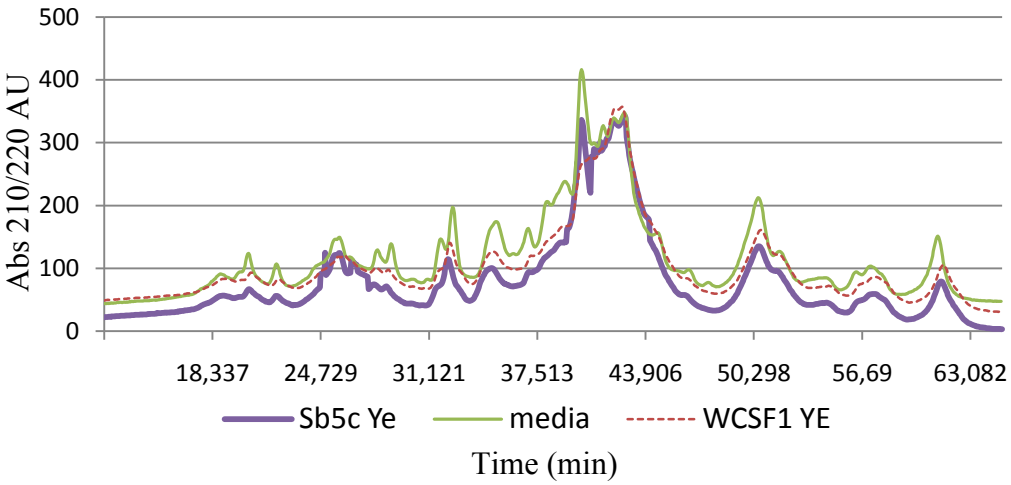

Figure 35. Chromatograms of the gluten extracts. Green line, from the gluten and 0.05% yeast extract medium; purple line, the same media after the strain Sb5c had grown on it; red dotted line, as reference, from the same medium after the strain WCSF1 had grown on it.

RP-HPLC analysis of the residual gluten showed differences between the amounts of hydrolysed substrates across the strains. Residual gluten from the Glu YE media showed low hydrolysis levels, mostly related to the α-gliadine fraction (retention time, 42 min) and the high molecular weight glutenin (retention times, 25 and 38 min). Although both strains acted similarly, some differences were still detectable (Figs. 35, 36).

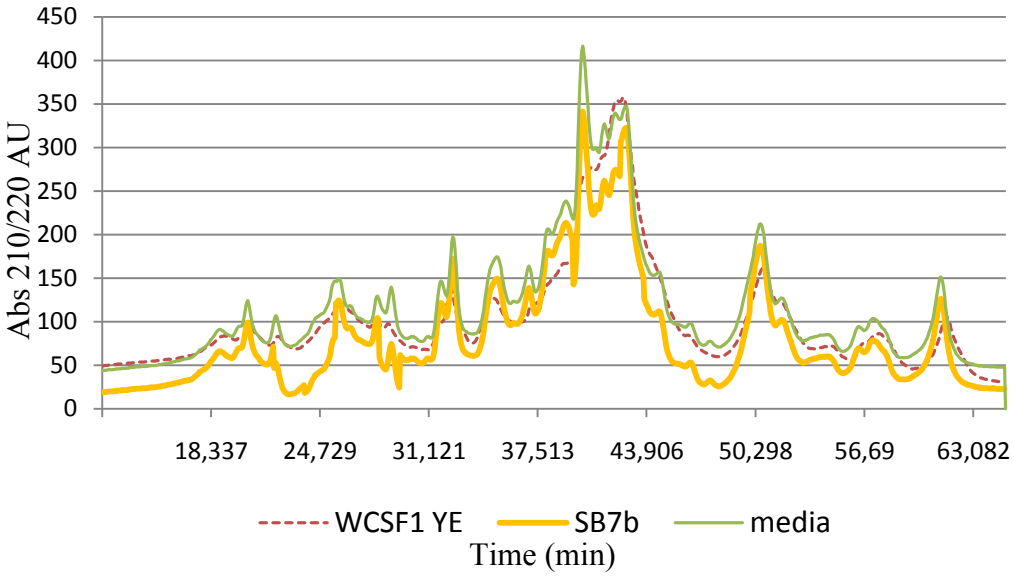

Figure 36. Chromatograms of the gluten extracts. Green line, from the gluten and 0.05% yeast extract medium; orange line, same media after the strain Sb7b had grown on it; red dotted line, as reference, from the same media after the strain WCSF1 had grown on it (see Chapter 1, Fig. 1).

Proteolysis from strain Sb5c applied mainly to the α-gliadine fraction and the high-molecular-weight glutenin (retention times, 42 and 38 min, respectively), while only poor activity was detected against the fraction at 25 min retention time (high-molecular-weight glutenin). Strain Sb7c instead showed high levels of proteolysis of the high molecular weight glutenin at 25 min retention time, while the α-gliadine fraction and the high-molecular-weight glutenin at 38 minutes retention time showed lower hydrolysis levels compared to the strain Sb5c.

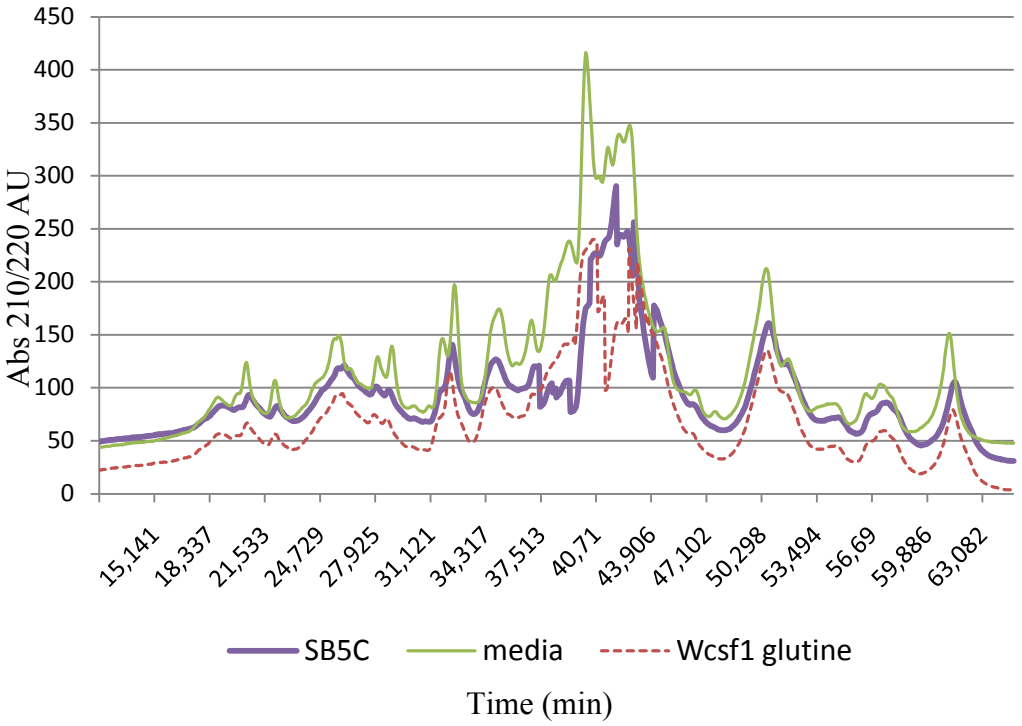

Figure 37. Chromatograms of the gluten extracts. Green line, from the gluten minimal medium; purple line, same media after the strain Sb5c had grown on it; red dotted line, as reference, from the same medium after the strain WCSF1 had grown on it..

When the same procedure was applied to the minimal gluten medium, we observed dramatic changes in the chromatograms (Figs. 37, 38). In both cases, the highest levels of hydrolysis were easy seen, with the fractions corresponding to α-gliadine and β-gliadine (retention time, from 40 to 45 min) were strongly reduced by both strains. The remarkable difference between the strains is the level of α-gliadine hydrolysis, which was higher with the strain Sb5c. High proteolysis was also seen for the high-molecular-weight glutenin, and only for the strain Sb7b, the γ-gliadine peak at 62 min retention time was divided into two peaks.

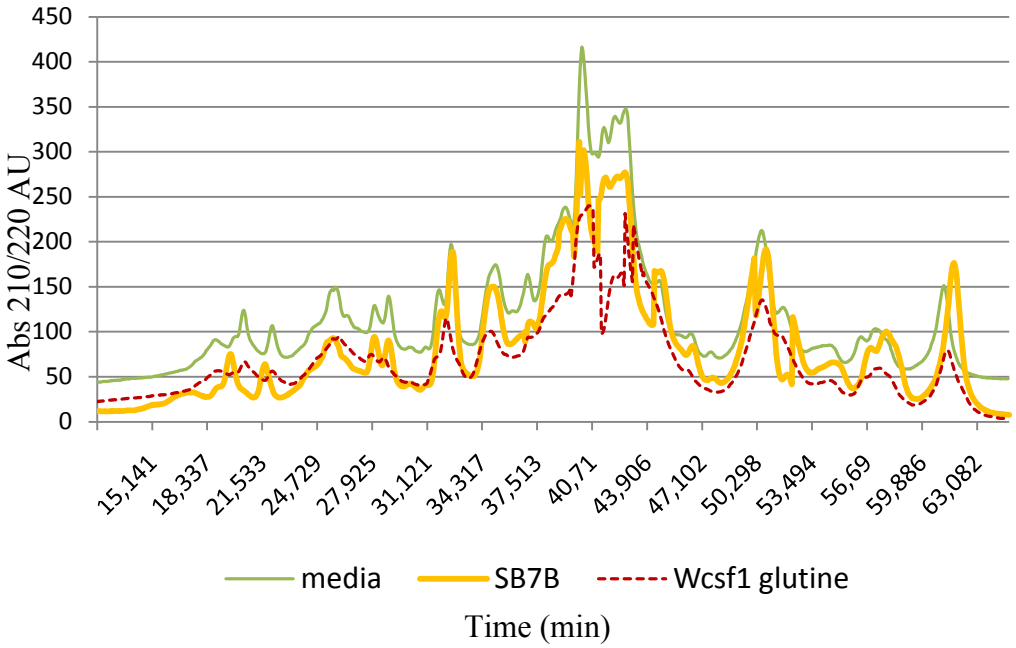

Figure 38. Chromatograms of the gluten extracts. Green line, from the gluten minimal medium; orange line, same medium after the strain Sb7b had grown on it; red dotted line, as reference, from the same medium after the strain WCSF1 had grown on it

Table 11 shows the levels of gluten proteolysis (as percentages of the uninoculated media) promoted by the strains. The proteolysis was measured on the total media and on α–β-γ gliadine and low-molecular-weight (LMW) glutenin and high-molecular-weight (HMW) glutenin.

Table 10. Hydrolysis levels of gluten and the gluten fractions on YE Glu and Glu media. The data are expressed as the means ±standard deviations of three independent experiments.

	TOT Hy	α gliadine	βgliadine	γ gliadine	LMW glutenin	HMW glutenin
WCSF1 GLUYE	26% ± 1,5	3,64% ±0,4	19,08% ±1,3	33,16% ±2,6	34,99% ±2,4	13,55% ±1,6
WCSF1 GLU	39,35% ±2,1	42,03% ±2,6	42,11% ±2,1	34,99% ±2,4	59,66% ±2,8	29,01% ±1,45
Sb5c GLUYE	25,7% ±1,8	5,86% ±1,1	21,59% ±1,2	29,77% ±1,6	31,27% ±1,4	29,6% ±2,4
Sb5c GLU	35,6% ±2,6	25,24% ±2,1	33,21% ±2,4	46,25% ±2,4	32,23% ±1,5	52,62% ±2,6
Sb7b GLU YE	4,57% ±0,48	3,54% ±0,8	10,32% ±0,4	9,32% ±0,8	12,07% ±0,15	10,51% ±1,3
Sb7b GLU	11,27% ±1,12	13,16% ±0,7	17,66% ±1,3	15,08% ±1,2	25,71% ±1,8	22,22% ±1,8

The reference strain WCSF1 was the most proteolytic (26% and 39.4% total hydrolysis in both media), with its proteolytic activity mainly directed against the LMW glutenin that are hydrolysed by 35.0% and 59.7% in the Glu YE and Glu media, respectively. α/β-Gliadine was poorly hydrolysed on Glu YE media (3.6% and 19.1% for α-gliadine and β-gliadine, respectively), and was highly hydrolysed on Glu media (42.0% and 42.1% for α-gliadine and β-gliadine, respectively), with γ-gliadine hydrolysed with slightly difference of intensities in both media (33.2% and 35.00% on Glu YE and Glu media, respectively).

Similarly, but less intense, behaviour was detected for the natural strain Sb5c. This strain showed high hydrolysis levels (25.7% and 35.6% for Glu YE and Glu, respectively), with LMW glutenin the preferred substrate on Glu YE medium (31.3% hydrolysis) and α/β-gliadine showing the highest levels of hydrolysis in this medium (5.9% and 21.6% for α- and β-gliadine, respectively). On The Glu media, HMW glutenin was the preferred substrate (52.6% hydrolysis) and α/β gliadine showed 25.2% and 33.2% hydrolysis. Remarkably, on this media, the levels of hydrolysis of γ-gliadine reached 46.3%.

The strain Sb7b showed the lowest proteolysis levels, with only 4.6% and 11.3% total hydrolysis in the Glu YE and Glu media, respectively. LMW glutenin is the preferred substrate for both media (12.1% and 25.7% for Glu YE and Glu media, respectively), and in this medium α/β-gliadine showed the lowest hydrolysis levels in the experiment (3.5% and 10.3% for α- and β-gliadine, respectively). Due to the generally higher proteolysis, on Glu medium, α/β-gliadine was hydrolysed at 13.2% and 17.7%, while γ-gliadine showed 15% hydrolysis. The general consideration indicates that glutenin is the preferred

substrate for this strains, the protein that was indeed the most hydrolysed under all of the conditions of growth. When the media contained yeast extract, the gluten proteolysis was directed principally against glutenin, with only small effects on gliadine. Among the gliadines, γ-gliadine was preferred on Glu YE media, showing levels of hydrolysis higher than α/β-gliadine for all of the strains. When only gluten was present in the medium, proteolysis was much higher. General considerations are still valid (LMW glutenin was always preferred), but in this case, hydrolysis of gliadine increased (up to 13-times higher in the case of α-gliadine hydrolysed by the strain WCSF1), and reach values in the order of magnitude of 40% for the most proteolytic strain WCSF1.

Discussion

Growth rates and proteolytic activities of *L. plantarum* were assayed in this first chapter. The growth rates of the three strains were evaluated on different media, using MRS as he reference rich substrate. Even if all of the strains showed the ability to grow on all of the media (the lowest CFU/mL was 2.2×10^{10} on Hy Gli medium), some differences between the growth rates in the different media were found. The biggest significant differences ($p < 0.05$) were shown between the media that contained only gluten or gliadine as the nitrogen source (Glu; Gli; Glu TW80; Gli TW80, Hy Glu and Hy Gli) and the media with yeast extract or peptone (Glu PEP; Gli PEP; Glu YE, Gli YE). When the nitrogen source is only gluten or gliadine, we observed differences in the early phase of growth. As already shown by Rollan et al. (94), the ability of *L. plantarum* to grow on gluten-based media is a strain-related property, and wide differences can be found between the strains. Confronting the data here with those showed by this Rollan et al. (94) study, we can say that all of the strains used in the present study reached higher densities at 12 h and 24 h of growth. Based on the observed acidification performances, we can say that the Glu YE medium is the medium where the strains gave the best performance. The highest numbers of colonies (CFU/mL) and the shortest T_m was in fact observed in this media for all of the strains. We can observe that the Hy glu and Hy gli media do not improve the V_m and the growth, although in this media the T_m decreased as the hydrolysis provided a number of small peptides that can be used by strains in their early phase of growth. Adding Tween 80 to the media on the other hand, showed effects on the grow of the bacteria but not on the acidification rate, V_m, which was indeed low and comparable to the glu and gli media. In the glu and gli media, the strains showed lower concentrations (CFU/mL) and lower V_m values.

The highest level of proteolysis were detected on the glu medium, even if based on the acidification parameters and growth-rate measurements this appears not to be the favorite medium of the strains. Surprising, the most proteolytically active strain was the reference strain WCSF1. Low proteolytic activity in media containing yeast extract has already been seen by Thiele in 2002 (53), and can be explained as a kind of inhibition of the proteolytic activity that is due to the peptides and amino acids contained in the yeast extract. Moreover, we found significantly lower activities in chromogenic assays. Here, we found that the most expressed family of enzymes were X-prolyl-dipeptidyl aminopeptidase and prolyl endopeptidase, which reached maximum activity after 4-8 h of incubation. This result is in agreement with a number of other studies that have described the consumption of the small peptides available on the media at the beginning of the fermentation (iminopeptidases and pro-iminopeptidases are active from the first 2 hours, and stay constant to 24 h) and the synthesis of enzymes that can hydrolyse the larger peptides remaining in the media (82, 20, 66, 81, 37, 94). HPLC analysis of the residual gluten gave us an answer about the fraction of gluten that is actually hydrolysed by these microorganisms. This is the first example of this kind of assay in experiments of gluten hydrolysis by such microorganisms, and it gave us a quantitative and qualitative profile of gluten consumption by the strains (Table 11). We found that when gluten is the only nitrogen source, all of the strains showed their highest level of proteolysis.

According to Zotta et al (94), LMW glutenin appears to be the favorite proteolytic substrate in both media (94), but when gluten is the only nitrogen source, proteolysis became widely distributed across all of the protein complex. In

this case, α/β-gliadine were subjected to dramatic significant reductions ($p < 0.05$), while LMW glutenin did not showed significant differences between the media ($p > 0.05$). These data are in agreement with some other studies that noted proteinases with high activities against gluten toxic fragments (mainly contained in the α/β-gliadine fraction) that lost their activities when other substrates were present in the environment (95) (97) (98). According to De Angelis et al. (87) and Yoshimoto et al. (96), and based on the data produced in the present study, the higher levels of α/β-gliadine proteolysis can be attribute to the X-prolyl-dipeptidyl-aminopeptidase and prolyl endopeptidase family of enzymes that act in a synergistic way to hydrolyse the α/β-gliadine complex. These two families of enzymes act as esopeptidases and endopeptidases, respectively (96), and their synergic activity was noted especially for the hydrolysis of proteic complexes such as casein (94). Their catalytic activity is not related to the chymotrypsin or subtilisin family of enzymes, but instead represents a new family of serine peptidases that are involved in the maturation and degradation of neuropeptides and hormones involved in several diseases, such amnesia and Alzheimer's disease (97)

Chapter 2

‘In silico’ screening, cloning, expression, purification, and activity assays of proline-specific peptidase.

Materials and methods

'*In-silico*' analysis

'*In-silico*' analyses where performed to obtain suitable indications about the putative praline-specific genes available in the proteome. The prolyl-endopeptidase pattern:

D-x(3)-A-x(3)-[LIVMFYW]-x(14)-G-x-S-x-G-G-[LIVMFYW] (2)

was obtained through the Swiss-Prot database. The Pattinprot software was used to match the prolyl-endopeptidase pattern with the entire proteome of WSCF1 that was available on the database, using 80% sensitivity.

Cloning of putative esterase-protease-encoding genes

Genomic DNA for the strains was isolated from overnight cultures (30 °C, no agitation, 10 mL volume) on MRS medium, using DNeasy Blood and Tissue kits (QIAGEN). The cell pellets were shaken on 20 mg/mL lysozyme overnight at 4 °C, and then the manufacturer instructions where followed. Primer-directed mutagenesis was used to introduce NdeI and SpeI restriction sites on the flanking region of the genes. The primers used are listed on Table 12.

Table 11 Primer sequence for amplification of the SpeI and NdeI genes, with restriction sites colored red.

Gene	primer
pepI;	5'- GGGCATATG/GACGTGAAGAAGGATACAEGCCATTT-3 5'- GGGACTAGT/CTAATCATGGGCTGCTAGCCAGTGA -3'
pepRII	5'- GGGCATATG/AAAACGTGACGAATTTTAACGCTT-3' 5'- GGGACTAGT/TTAGCGGCCCAATTGATCAGAAAAATA3'
pepRI	5'- GGGCATATG/AAACAAGGAACGACAATCATCACGCTG-3' 5'- GGGACTAGT/TTATTTTTGATTAAAGCTGCCATCTCTTCCA-3'
Lp_2953	5'- GGGCATATG/ACATCGATGGAATTTAAGATTAAACGG-3' 5'- GGGACTAGT/TCATTTAAACGCGGCCAGTGCTAATTGGAT-3'

Fifty µl PCR reaction mixtures were prepared as follows: 30 µM of each primer; 5 µL 10× Pfu buffer (Stratagene); 200 µM dNTPs (Invitrogen); 3 ng template; and 0.8 µM Taq polymerase Hotstart Turbo (Stratagene). The PCR reactions were carried out under the following conditions: 10 min at 99 °C for Taq activation, followed by 35 cycles (45 s at 98 °C; 45 s at 55 °C; 90 s at 72 °C) and a final elongation step at 72 °C for 10 min. The PCR products were cloned into the TOPO plasmid (Invitrogen) and then introduced via heat-shock transformation of XL1 blue chemically competent cells (Invitrogen). The transformant *E. coli* XL1 blue chemically competent cells where then plated on LB medium with 50 µg/mL ampicillin and left to grow overnight at 37 °C. To purify a reasonable amount of plasmid carryng the right sequence of the gene from every transformant *E. coli* strain, seven colonies where taken from the plates an allowed to grow in 5 ml of LB medium with 50 µg/ml of ampicillin at 37 °C overnight. The cultures where centrifuged birefly and the plasmids were then purified using the PureYield plasmid miniprep system kit (Promega), following the manufacturer instructions. The purified plasmids where then sequenced by BMR genomics service, using an M17 (registred) primer set. The insert with the desired sequence (verified by the BLAST software) where then excised by the recombinant TOPO plasmids, using

the *Spe*I and *Nde*I endonucleases. The 6-His-tagged expression vector pXL49 was chosen to clone, express and purify with nickel-nitriloacetic acid (Ni-NTA) resin. Fifty µl of restriction reaction mixture were prepared as follows: 3 µL NdeI, 3 µL SpeI, 4 µL buffer 2, 0.5 µL bovine serum albumin 100×, and 8 µL and 10 µL for insert and vector, respectively; the reactions where performed at 37 °C for 4 h. After the restriction reaction, all of the reaction products where loaded onto 1% agarose gels stained with ethidium bromide, and run at 45 V for 60 min. After the run, the bands corresponding to the linearized vectors and the inserts where excised from the gels and purified using the Zymoclean kit (Zyomoresearch), with the manufacturer instructions followed. The ligation reactions between the insert and the vector were performed using 6 µL purified insert, 6 µL purified vector, 3 µL 5× buffer, and 1 µl T4 DNA ligase high concentration (5 U/µl invitrogen). The reactions where performed at room temperature for 1 . The recombinant pXL49 plasmids were transformed in BL21 for the expression of the proteins of interest.

Expression and purification of recombinant proline-specific proteases from the *L. plantarum* WSCF1 strain

The amplified inserts were digested with *Nde*I and *Spe*I and cloned into the expression vector, pET28b (Novagen), to yield a construct encoding the desired proteases with C-terminal His_6 tags. The resulting plasmids, pPEPI, pPEPRII, pPEPRI and pLp2953 were verified by DNA sequencing and introduced into the *E. coli* BL21 cells (Novagen) via transformation. A 5 mL inoculum was grown for 12-16 h with shaking at 37 °C. One liter of LB medium containing 50 µg/mL

kanamycin was inoculated with the 5 mL inoculum, and was grown at 37 °C. At an optical density of 600 nm (OD_{600}) of 0.6, the expression of the recombinant proteases was induced by addition of 0.5 mM isopropyl β-D-thiogalactoside (IPTG) (Sigma). The cultures were incubated at 18 °C to 22 °C for an additional 6-12 h. The induced cells were harvested by centrifugation at 5,000× *g* for 20 min, in a J2-21M Beckman centrifuge. The cell pellets were resuspended in 40 mL disruption buffer (200 mM sodium phosphate, pH 7.0; 200 mM NaCl; 2.5 mM DTT; 1.5 mM benzamidine; 2.5 mM EDTA; 2 mg/L pepstatin; 2 mg/L leupeptin; 30% [v/v] glycerol), and lysed by sonication with a Branson Sonifier 450. The lysed cells were further clarified by centrifugation at 45,000× *g* for 60 min, and the supernatants bound to 3 mL 1:1 Ni-NTA resin:ethanol slurry (Qiagen) for 1 h at 4 °C. The resin was packed into a column, and washed with 20-times the column volume with 50 mM phosphate buffer, 5 mM imidazole (pH 6.0 for pepR1; pH 7.0 for pepR2; and pH 7.5 for Lp_2953 and pepI), until no protein was detected in the wash by Bradford assay. The bound protein was then eluted with 3-times the column volume of 50 mM phosphate buffer, 200 mM imidazole, (pH 6.0 for pepR1; pH 7.0 for pepR2; pH 7.5 for Lp_2953 and pepI). The eluted fractions containing the desired protein, as assessed by SDS-PAGE, and buffer exchanged with 50 mM phosphate buffer pH 6 using an Amicon Ultra concentrator (Millipore). The final protein concentration was determined by the Bradford assay. The enzyme preparations were standardised at 7 μg/μl final concentration, and then stored at 0 °C on ice, or at -20 °C in a freezer.

Lp2593 purification of from inclusion bodies

Induced cells were harvested by centrifugation at 5,000× *g* for 20 min in a J2-21M Beckman centrifuge. The cell pellet was resuspended in 40 mL disruption buffer (200 mM sodium phosphate, pH 7.0; 200 mM NaCl; 2.5 mM DTT; 1.5 mM benzamidine; 2.5 mM EDTA; 2 mg/L pepstatin; 2 mg/L leupeptin;and 30% [v/v] glycerol) and lysed by sonication with a Branson Sonifier 450. The lysed cells were centrifuged at 45,000× *g* for 60 min. The Lp_2953 inclusion bodies contained in the pellet were rinsed twice with water, and solubilised by addition of 8 M urea, 50 mM Tris-Cl, 2 mM β-mercaptoethanol, pH 8.0. The protein solution was further clarified by centrifugation at 45,000× *g* for 60 min, and the supernatant was bound to 10 mL 1:1 Ni-NTA resin:ethanol slurry (Qiagen) for 2 h at room temperature. Five ml of resin was packed into a column, and washed with 7 M Urea, 50 mM Tris-Cl, 2 mM β-mercaptoethanol, pH 8.0, until no protein was detected in the wash by the Bradford assay. The bound protein was then eluted with 7 M Urea, 50 mM Tris-Cl, 2 mM β-mercaptoethanol, 200 mM imidazole, pH 8.0. The eluted fractions containing the desired protein, as assessed by SDS-PAGE, were dialyzed against 100 sample volumes of refolding/ storage buffer (100 mM Tris-Cl, pH 8.0, 5 mM EDTA, 15% [v/v] glycerol, 2 mM β-mercaptoethanol) at 4 °C for 3 h, followed by a second dialysis against a fresh portion of refolding buffer overnight. Alternatively, the elution fractions containing the desired protein were diluted into refolding buffer to a final concentration of 30 µg/mL, gently agitated overnight at 4 °C, and concentrated again. The final protein concentration was determined by the Bradford assay. The enzyme preparations were stored at 0 °C on ice, or at -20 °C in a freezer.

Activity measurement

Activity assay was performed against the chromogenic substrates gly-pro-pNa and suc-ala-pro-pNa, supplied from Bachem. All of the purified proteins were standardised to the same concentration (7 µg/µL). The assays were performed at pH 6.0 and pH 4.5 (both in acetate buffer) using 1 mg and 10 mg of enzyme (final concentrations). The final concentration of the substrate was 3 µM (2.6 AU maximum absorbance expected). The 33-mer rich peptide hydrolysis was carried out at pH 6 and pH 4.5 using 200 µM peptide and 20 µM of each enzyme. The reactions were carried out at 37 °C for 60 min in 100 µL total volume. The digestion was analyzed by HPLC (solvent A, water and 0.1% TFA; solvent B, acetonitrile+0.1% TFA) using a 5µ C18 column.

Lp_2953 characterization

Esterase activity was determined by monitoring the release of p-nitrophenol from p-nitrophenyl butyrate (pNPC4) at 348 nm in a spectrophotometer (Agilent, Palo Alto, CA, USA) for 5 min at room temperature. To carry out the reaction, a stock solution of 100 mM pNPC4 was prepared in isopropanol. Substrate specificity was assessed using the following p-nitrophenyl esters: acetate (pNPC2), butyrate (pNPC4), caprylate (pNPC8), caprato (pNPC10), laurate (pNPC12), myristate (pNPC14), and palmitate (pNPC16), as substrates. A stock solution of each p-nitrophenyl ester was prepared in isopropanol. The substrates were emulsified to a final concentration of 1 mM in 50 mM phosphate buffer, pH 6.0, containing 4.4 mg/mL Triton X-100. The reaction mix consisted of 998 µL emulsified substrate and 2 µL enzyme solution (1 µg total protein). The reactions were carried out at

room temperature, as described above. Esterase activities were measured as functions of the different concentrations of pNPC4 and pNPC2 (0.1, 0.2, 0.3, 0.4, 0.5, 1.0, and 3.0 mM). One unit of esterase activity was defined as the amount of enzyme that released 1 μmol p-nitrophenol per minute. The Michaelis–Menten substrate affinity constant (K_m), maximum velocity (V_{max}), and the turnover number (k_{cat}) were calculated using Graph Pad Prism 5.01 software. The catalytic efficiencies (k_{cat}/K_m) were also determined.

Results

'In -silico' screening for proline specific proteases

In-silico analyses where performed to investigate the proline-specific proteases in the proteome of the WSCF1 strain. Using the SWISSPROT database, the pattern was as follows:

D-x(3)-A-x(3)-[LIVMFYW]-x(14)-G-x-S-x-G-G-[LIVMFYW] (2)

Were serine is the catalytic residue and position 24 to 29 is the entire active site. The results are showed in Table 13.

Table 12. Prolyl-endopeptidase pattern matched with the *L. plantarum* WCSF1 proteome: the four best matches are showed.

Protein/ gene name	S.L.	Sequence	Bad position (mismatch)
pep R1/LP_0853	60 %	**yDYFlDEVeEVRQKLGLDNFYLIGQSWGGaL**	1 'D', 3 'A', 5 '[LIVMFYW]', 13 '[LIVMFYW](2)'
pepI/LP_0088	66%	**aAMWvAELrALRTYLDLPEIHLLGQSWGGML**	1 'D', 3 'A', 5 '[LIVMFYW]'
pepR2/LP_2919	66%	**eDYYiSEVdEVRQQLGYKHCYLAGHSWGGML**	1 'D', 3 'A', 5 '[LIVMFYW]
Lp_2953	84%	**eIADAKAVLDEALTLHYDHIVLAGHSQGGVV**	1 'D'

Among the proteases listed in Table 13, three of them (pepR1; pepI; pepR2) were already listed as putative praline-specific enzymes on the WSCF1 proteome database. On the other hand, even if Lp_2953 shows the higher levels of similarity (84% similarity level), it is listed in the WSCF1 proteome database as a putative esterase. Moreover, the only bad position showed in the pattern is

aspartic acid instead glutamic acid. This residue substitution allows us to consider actual differences between the Lp_2953 enzyme and the pattern, which will be lower than the calculated levels.

Gene cloning and sequence analysis

Specific primers where designed for the respective genes, including for the NdeI and SpeI endonuclease restriction sites. PCR reaction where performed to amplify the genes on all three strains used in this study.

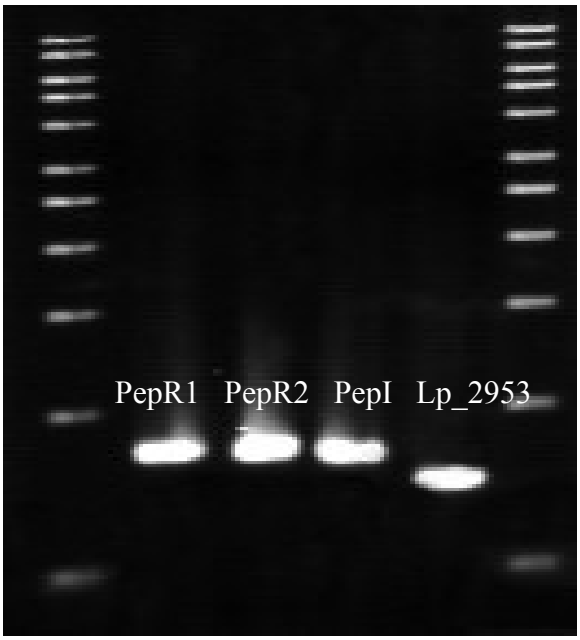

Figure 39. PCR products of WCSF1 reference strain in 1% agarose gel lanes: 1/6 marker 1 Kb Biorad; 2. pepR1. 3. pepR2. 4. pepI. 5. lp_2953. The same results were obtained for the wild strains, Sb5c and Sb7b .

The size of all the amplicons corresponded to the data available from the database. All of the amplicons were cloned on the TOPO cloning plasmid (see Materials and methods) and then sequenced. The sequences of all of the genes

were translated into the protein sequences, which were compared to the database available, with sequence alignment using the Clustal W2 software (100)

```
LP_2953-DATABASE
MTSMEFKIKRDGLALQARLETPAAPSSTLVILMHGFTADMGYDTTQFVPQLAQALVAHGL 60
LP_2953-WSCF1
MTSMEFKIKRDGLALQARLETPAAPSSTLVILMHGFTADMGYDTTQFVPQLAQALVAHGL 60
LP_2953-SB5C
MTSMEFKIKRDGLALQARLETPAAPSSTLVILMHGFTADMGYDTTQFVPQLAQALVAHGL 60
LP_2953-SB7B
MTSMEFKIKKDGLALQARLETPAAPSSTLVILMHGFTADMGYDTTQFVPQLAQALVAHGL 60

*********:**************************************************
LP_2953-DATABASE
AVLRFDFNGHGCSEGRFQDMTVINEIADAKAVLDEALTLHYDHIVLAGHSQGGVVASMLA 120
LP_2953-WSCF1
AVLRFDFNGHGCSEGRFQDMTVINEIADAKAVLDEALTLHYDHIVLAGHSQGGVVASMLA 120
LP_2953-SB5C
AVLRFDFNGHGCSEGRFQDMTVINEIADAKAVLDEALTLHYDHIVLAGHSQGGVVASMLA 120
LP_2953-SB7B
AVLRFDFNGHGCSEGRFQDMTVINEIADAKAVLDEALTLHYDHIVLAGHSQGGVVASMLA 120

************************************************************
LP_2953-DATABASE
GYYPDVVDKLILMAPAATLKSDAQQGVLQGATYDPQHIPAYLNIRDGLKVGGFYLRTAQQ 180
LP_2953-WSCF1
GYYPDVVDKLILMAPAATLKSDAQQGVLQGATYDPQHIPAYLNIRDGLKVGGFYLRTAQQ 180
LP_2953-SB5C
GYYPDVVDKLILMAPAATLKSDAQQGVLQGATYDPQHIPAYLNIRDGLKVGGFYLRTAQQ 180
LP_2953-SB7B
GYYPDVVDKLILMAPAATLKSDAQQGVLQGATYDPQHIPAYLNIRDGLKVGGFYLRTAQQ 180

************************************************************
LP_2953-DATABASE
LPIYEVAQQYAGSVTLIHVTADTVVSPQASEKYHEVYQHSQLHWVQDGGHRFSGDARATA 240
LP_2953-WSCF1
LPIYEVAQQYAGSVTLIHVTADTVVSPQASEKYHEVYQHSQLHWVQDGGHRFSGDARATA 240
LP_2953-SB5C
LPIYEVAQQYAGSVTLIHVTADTVVSPQASEKYHEVYQHSQLHWVQDGGHRFSGDARATA 240
LP_2953-SB7B
LPIYEVAQQYAGSVTLIHVTADTVVSPQASEKYHEVYQHSQLHWVQDGGHRFSGEARATA 240

************************************************:*****
LP_2953-DATABASE
IQLALAAFK 249
LP_2953-WSCF1
IQLALAAFK 249
LP_2953-SB5C
IQLALAAFK 249
LP_2953-SB7B
IQLALCAFK 249
*****.***
```

SeqA	Name	SeqB	Name	Score
1	LP_2953-DATABASE	2	LP_2953-WSCF1	100
1	LP_2953-DATABASE	3	LP_2953-SB5C	99
1	LP_2953-DATABASE	4	LP_2953-SB7B	98
2	LP_2953-WSCF1	3	LP_2953-SB5C	99
2	LP_2953-WSCF1	4	LP_2953-SB7B	98
3	LP_2953-SB5C	4	LP_2953-SB7B	99

Figure 40. Alignment of the deduced amino-acid sequence of the Lp_2953-coded protein with the registered bio cyc database, and the translated amino-acid sequences obtained from gene sequencing of Lp_2953 amplicons from the WCSF1 strain and the wild strains, Sb5c and Sb7b.

```
PEPr1-database
MKQGTTIITLDNGYHLWTNTQGKGDIQLLCLHGGPGGNHEYWENFGEELADLGVQVSMYD 60
pepR1-WCSF1
MKQGTTIITLDNGYHLWTNTQGKGDIQLLCLHGGPGGNHEYWENFGEELADLGVQVSMYD 60
pepR1-SB5C
MKQGTTIITLGNGYHLWTNTQGKGDIQLLCLHGGPGGNHEYWENFGEELADLGVQVSMYD 60
pepR1-SB7B
MKQGTTIITLGNGYHLWTNTQGRGDIQLLCLHGGPGGNHEYWENFGEELADLGVQVSMYD 60

**********.**********:**********************************
PEPr1-database
QLGSWYSDQPDYSDPEIAKKYLTYDYFLDEVEEVRQKLGLDNFYLIGQSWGGALTMMYAL 120
pepR1-WCSF1
QLGSWYSDQPDYSDPEIAKKYLTYDYFLDEVEEVRQKLGLDNFYLIGQSWGGALTMMYAL 120
pepR1-SB5C
QLGSWYSDQPDYSDPEIAKKYLTYDYFLDEVEEVRQKLGLDNFYLIGQSWGGALTMMYAL 120
pepR1-SB7B
QLGSWYSDQPDYSDPEIAKKYLTYDYFLDEVEEVRQKLGLDNFYLIGQSWGGALTMMYAL 120

************************************************************
PEPr1-database
KYGQHLKGAIISSMVDNIEEYVVNVNKCREEALPADAVAYMKQKEAEGNWNDPQYQKYVD 180
pepR1-WCSF1
KYGQHLKGAIISSMVDNIEEYVVNVNKCREEALPADAVAYMKQKEAEGNWNDPQYQKYVD 180
pepR1-SB5C
KYGQHLKGAIISSMVDNIEEYVVNVNKCREEALPADAVAYMKQKEAEGNWNDPQYQKYVD 180
pepR1-SB7B
KYGQHLKGAIISSMVDNIEEYVVNVNKCREEALPADAVAYMKQKEAEGNWNDPQYQKYVD 180

************************************************************
PEPr1-database
VLNAGYVDRKQPTSIRHLINTTATPVYNAFQGDNEFVITGKLKEWDIRDQIHNIKVPTLL 240
pepR1-WCSF1
VLNAGYVDRKQPTSIRHLINTTATPVYNAFQGDNEFVITGKLKEWDIRDQIHNIKVPTLL 240
pepR1-SB5C
VLNAGYVDRKQPTSIRHLINTTATPVYNAFQGDNEFVITGKLKEWDIRDQIHNIKVPTLL 240
pepR1-SB7B
VLNAGYVDRKQPTSIRHLINTTATPVYNAFQGDNEFVITGKLKEWDIRDQIHNIKVPTLL 240

************************************************************
PEPr1-database
TFGEHETMPLASARRMARDIPNSRLVTTPNGGHHHMIDNAPVYFDHLKQFIRDVEDGSFN 300
pepR1-WCSF1
TFGEHETMPLASARRMARDIPNSRLVTTPNGGHHHMIDNAPVYFDHLKQFIRDVEDGSFN 300
pepR1-SB5C
TFGEHETMPLASARRMARDIPNSRLVTTPNGGHHHMIDNAPVYFDHLKQFIRDVEDGSFN 300
pepR1-SB7B
TFGEHETMPLASARRMARDIPNSRLVTTPNGGHHHMIDNAPVYFDHLKQFIRDVEEGSFN 300

************************************************************:****
```

```
PEPr1-database      QK 302
pepR1-WCSF1         QK 302
pepR1-SB5C          QK 302
pepR1-SB7B          QK 302
                    **
```

SeqA	Name	SeqB	Name	Score
1	PEPr1-database	2	pepR1-WCSF1	100
1	PEPr1-database	3	pepR1-SB5C	99
1	PEPr1-database	4	pepR1-SB7B	99
2	pepR1-WCSF1	3	pepR1-SB5C	99
2	pepR1-WCSF1	4	pepR1-SB7B	99
3	pepR1-SB5C	4	pepR1-SB7B	99

Figure 41. Alignment of the deduced amino-acid sequences of pepR1-coded protein with the registered bio cyc database, and the translated amino-acid sequences obtained from gene sequencing of pepR1 amplicons from the WCSF1 strain and the wild strains Sb5c and Sb7b.

```
pepR2-DATABASE
MKNVTRILTLSNGYHLWSHTSNLGGRTKLLCLHGGPGDTHEVFERFGPELADLDIEVTMY 60
pepR2-WCSF1
MKNVTRILTLSNGYHLWSHTSNLGGRTKLLCLHGGPGDTHEVFERFGPELADLDIEVTMY 60
pepR2-SB5C
MKNVTRILTISNGYHLWSHTSNLGGRTKLLCLHGGPGDTHEVFERFGPELADLDIEVTMY 60
pepR2-SB7B
MKNVTRLLTLSNGYHLWSHTSNLGGRTKLLCLHGGPGDTHEVFERFGPELADLEIEVTMY 60

******:**:****************************************:******
pepR2-DATABASE
DQLGSWYSDTPNWDDDAIRQQYLTEDYYLSEVDEVRQQLGYKHCYLAGHSWGGMLAMTYA 120
pepR2-WCSF1
DQLGSWYSDTPNWDDDAIRQQYLTEDYYLSEVDEVRQQLGYKHCYLAGHSWGGMLAMTYA 120
pepR2-SB5C
DQLGSWYSDTPNWDDDAIRQQYLTEDYYLSEVDEVRQQLGYKHCYLAGHSWGGMLAMTYA 120
pepR2-SB7B
DQLGSWYSDTPNWDDDAIRQQYLTEDYYLSEVDEVRQQLGYKHCYLAGHSWGGMLAMTYA 120

************************************************************
pepR2-DATABASE
ADHQDQLDGLIIISMIDNIADYLKRMHAIRTAEFSPAENAFMLAIEKRQQWNNPHYRQLI 180
pepR2-WCSF1
ADHQDQLDGLIIISMIDNIADYLKRMHAIRTAEFSPAENAFMLAIEKRQQWNNPHYRQLI 180
pepR2-SB5C
ADHQDQLDGLIIISMIDNIADYLKRMHAIRTAEFSPAENAFMLAIEKRQQWNNPHYRQLI 180
pepR2-SB7B
ADHQDQLDGLIIISMIDNIADYLKRMHAIRTAEFSPAENAFMLAIEKRQQWNNPHYRQLI 180

************************************************************
pepR2-DATABASE
THLYHQYINRCHPSMMQHQLDIQAKPVYNHFQGDNEFVVYGVLDDWDFSDTLATIQVPTL 240
pepR2-WCSF1
THLYHQYINRCHPSMMQHQLDIQAKPVYNHFQGDNEFVVYGVLDDWDFSDTLATIQVPTL 240
pepR2-SB5C
THLYHQYINRCHPSMMQHQLDIQAKPVYNHFQGDNEFVVYGVLDDWDFSDTLATIQVPTL 240
pepR2-SB7B
THLYHQYINRCHPSMMQHQLDIQAKPVYNHFQGDNEFVVYGVLDDWDFSDTIATIQVPTL 240

**************************************************:*********
pepR2-DATABASE
LMFADHETMPLATAERMQQRMPNAKLVVTPDSGHNHMVDNPAVFFTYLRNYFSDQLGR 298
pepR2-WCSF1
LMFADHETMPLATAERMQQRMPNAKLVVTPDSGHNHMVDNPAVFFTYLRNYFSDQLGR 298
pepR2-SB5C
LMFADHETMPLATAERMQQRMPNAKLVVTPDSGHNHMVDNPAVFFTYLRNYFSDQLGR 298
pepR2-SB7B
LMFADHDTMPLATAERMQQRMPNAKLVVTPDSGHNHMVDNPAVFFTYLRNYFSDQLGR 298
```

******:**

SeqA	Name	Len(aa)	SeqB	Name	Len(aa)	Score
1	pepR2-DATABASE	298	2	pepR2-WCSF1	298	100
1	pepR2-DATABASE	298	3	pepR2-SB5C	298	99
1	pepR2-DATABASE	298	4	pepR2-SB7B	298	98
2	pepR2-WCSF1	298	3	pepR2-SB5C	298	99
2	pepR2-WCSF1	298	4	pepR2-SB7B	298	98
3	pepR2-SB5C	298	4	pepR2-SB7B	298	98

Figure 42. Alignment of the deduced amino-acid sequences of the pepR2-coded protein with the registered bio cyc database, and the translated amino-acid sequences obtained from gene sequencing of the pepR2 amplicons from the WCSF1 strain and the wild strains, Sb5c and Sb7b

Based on the above results, no significant differences were found between the enzyme sequences, and so we decide to carry out the expression and purification of the enzymes derived from the strain WCSF1.

Enzyme purification

Following denaturing Ni-NTA affinity chromatography, Lp_2953 was eluted as a single band from the column at ~27 kDa, with a purity of >90%, as assessed by SDS-PAGE (Fig. 43). This is in good agreement with the predicted molecular weight (27.07 kDa) of the recombinant Lp_2953 protein based on its sequence.

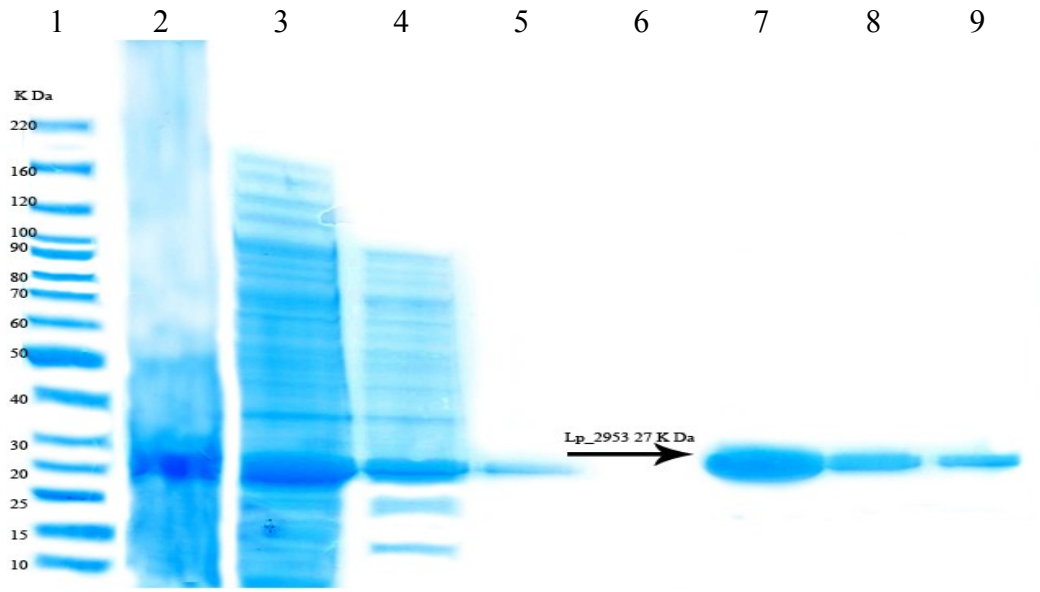

Figure 43. Lp_2953 denaturation-purification. Lane 1, benchmark molecular weight ladder; lane 2, denaturated pellet; lane 3, flow through; lane 4, first wash; lane 5, second wash; lane 6, third wash; lane 7, first elution; lane 8, second elution; lane 9, third elution.

Pep R2, Pep R1 and Pep I also showed their expected sizes as a single band on the SDS-PAGE (34.5 kDa, 34.4 kDa, and 33.3 kDa, respectively). The purifications were performed as described in the Materials and methods, with the purification process shown through the respective SDS_PAGE stages in Figures 44 and 45.

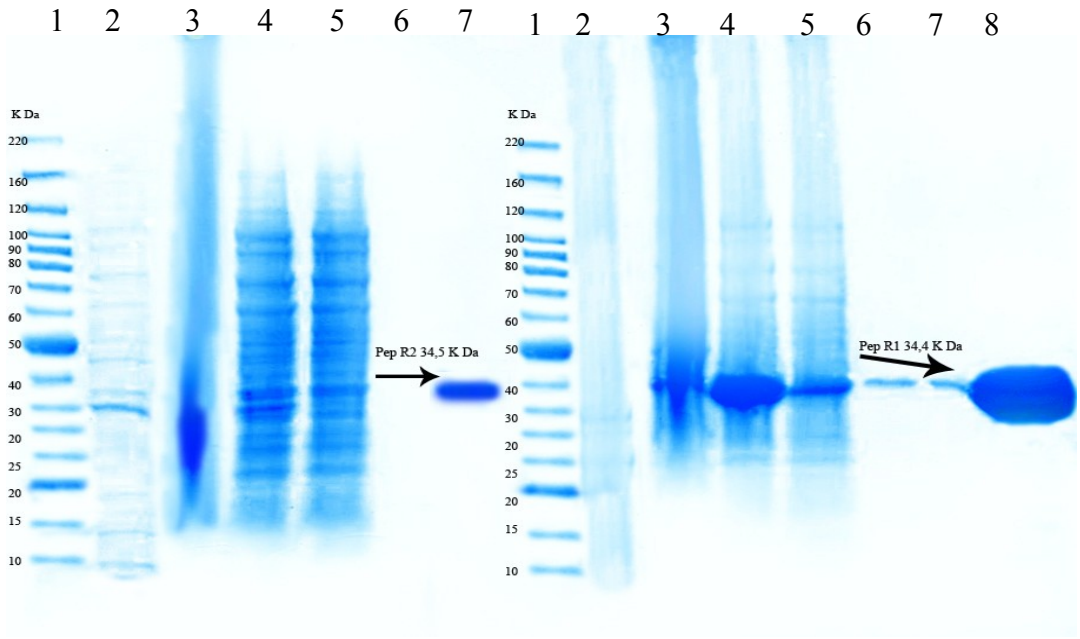

Figure 44. Pep R2, Pep R1 denaturation-purification. Lanes as given in the legend to Figure 43. From the gels, it can be seen that both enzymes are pure and of the expected size (Pep R2 34.5 kDa, and Pep R1 34.4 kDa).

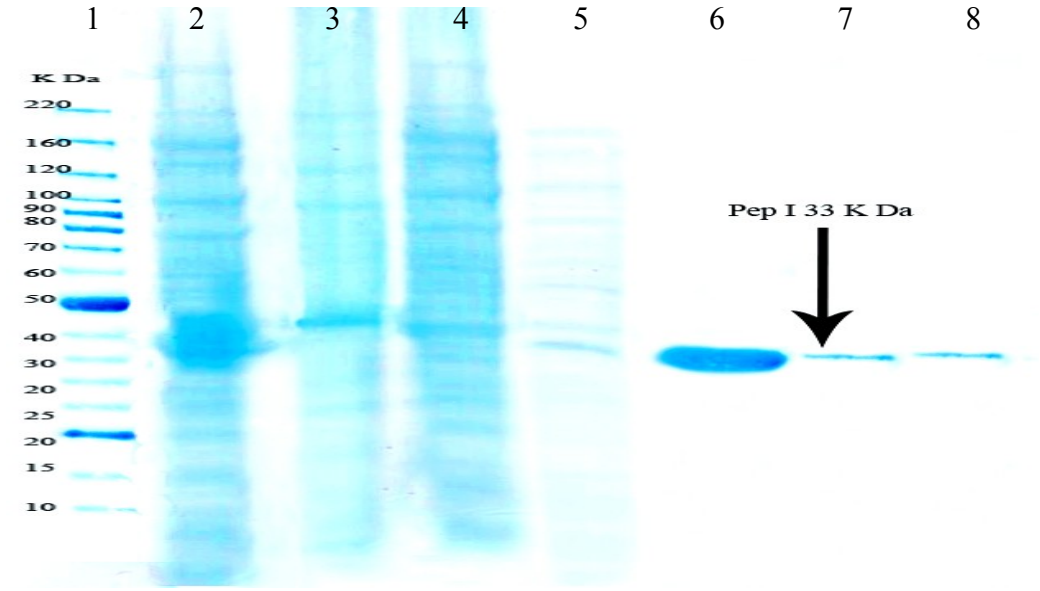

Figure 45. Pep 1 denaturation-purification. Lanes as given in the legend to Figure 43. From the gel, it can be seen that the enzyme is pure and of the expected size (33.3 kDa).

Activity on chromogenic substrates and proline-rich peptide:

The activity of the expressed proteins where measured first against the chromogenic substrate gly-pro-pNa at pH 4.5 and pH 6. Figure 46 shows the activities at pH 4.5 using 1 mg/mL and 10 mg/mL enzyme concentrations.

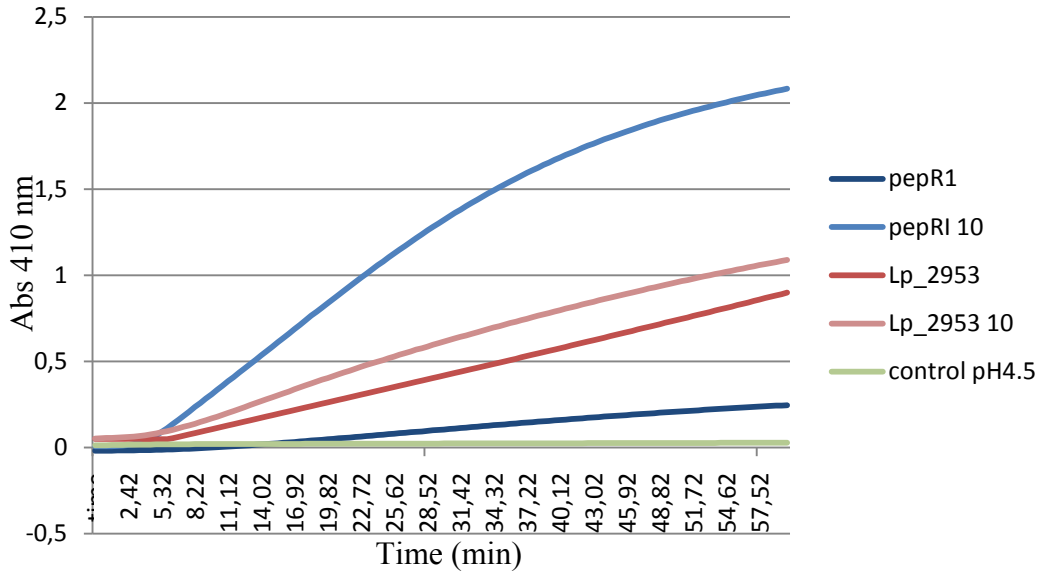

Figure 46. Activities of the different proteases at pH 4.5, as indicated. The absorbance at 410 nm was measured for the kinetics over 60 min..

As shown in Figure 46, the behaviour of these enzymes was widely different, PepI and PepR2 precipitated out of solution at pH 4.5, with the consequent loss of any activity (Table 14).

Table 13. Relative activities of the purified enzymes on the gly-pro-pNa substrate. The data are means ±standard deviations of three independent experiment, with the relative activities calculated as residual activities at pH 4.5 compared to pH 6.

	Activity pH 6	S D	Activity pH 4,5	S D	residual activity
pepRI	100%	±8	100%	±10	78%
pepRII	20%	±12	0%	NA	0
pepI	15	±14	0%	NA	0
Lp_2953	80	±16	75%	±13	43%

The activity of the best enzymes, Pep R1 and Lp_2953, where checked also on the proline-rich long 33-mer peptide. The hydrolysis was evaluated by RP-HPLC.

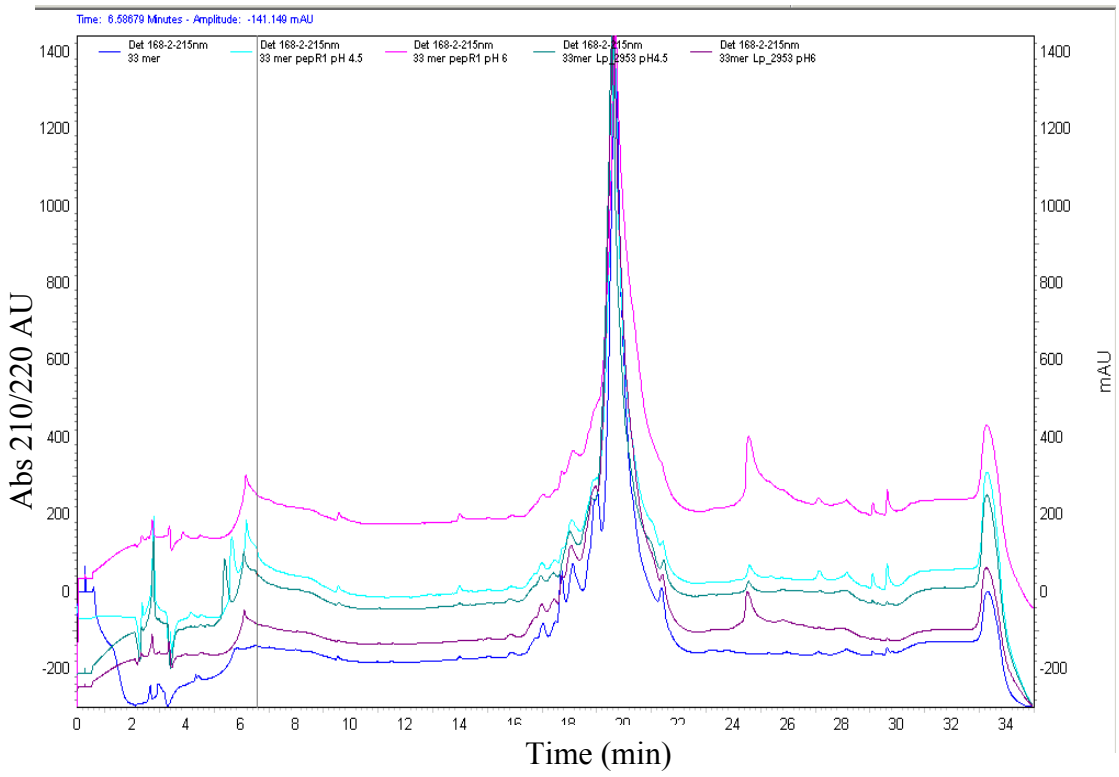

Figure 47. HPLC analysis of the long-chain praline-rich peptide after hydrolysis by the PepR1 and Lp_2953 enzymes. Blue line, native 33-mer peptide; pink line, digested by PepR1 at pH 6; light blue line, digested by PepR1 at pH 4.5; purple line, digested by Lp_2953 at pH 6; green line, digested by Lp_2953 at pH 4.5.

In the chromatogram shown in Figure 47, it can be seen that there was no hydrolysis of the 33-mer substrate by the PepR1 and Lp_2953 enzymes, which indicated that these enzymes cannot cleave this long proline-rich peptide.

Lp_2953 partial characterisation

The Lp_2953 substrate specificity was investigated by testing is enzymatic activities against p-nitrophenyl esters of different chain lengths, at pH 6.0 and room temperature.

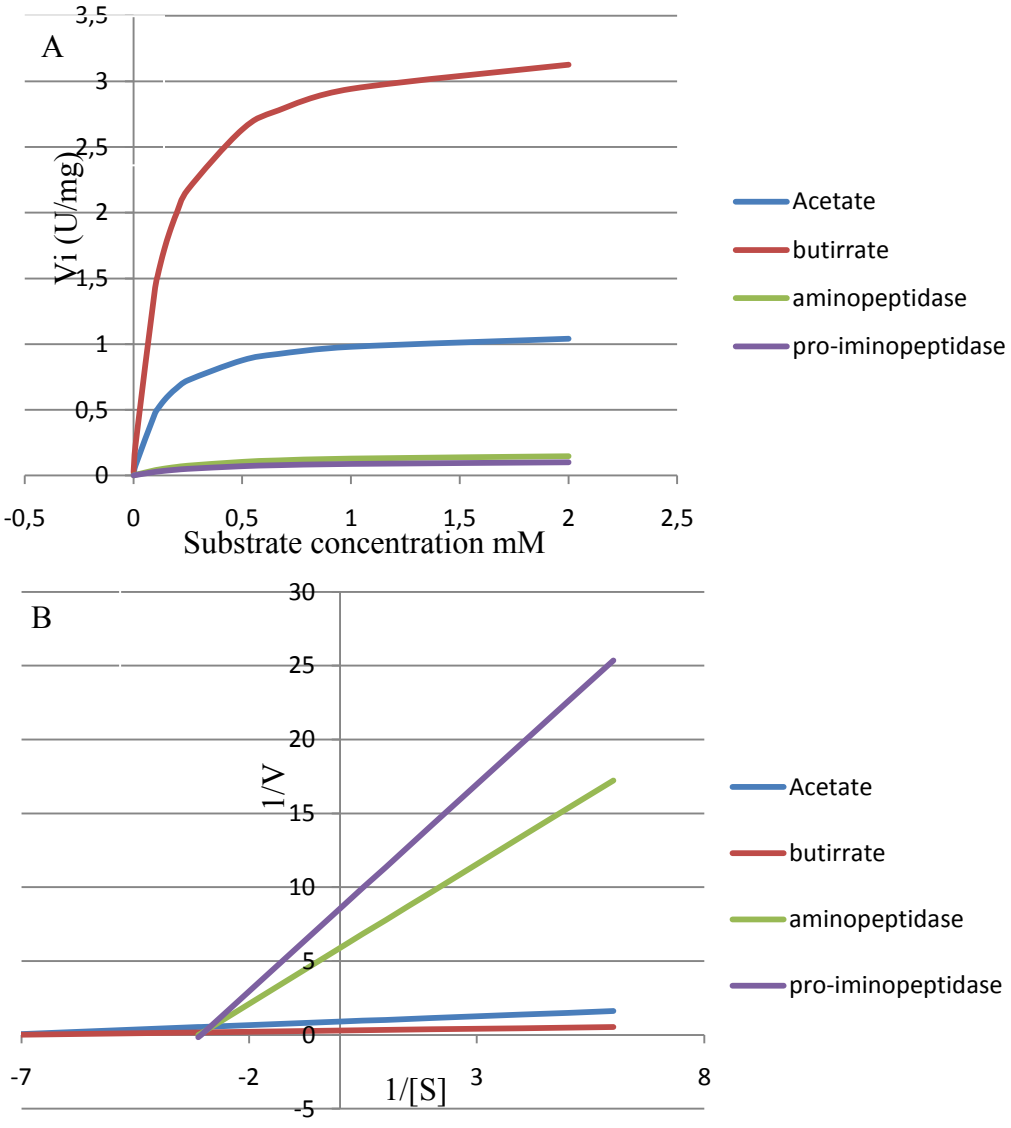

Figure 48. A. Michaelis–Menten plots for the recombinant Lp_2953 esterase from *L. plantarum*. These kinetic data were measured spectrophotometrically using pNPC2 pNPC4 leu-pNa and pro-pNa as substrates. B. Double-reciprocal Lineweaver–Burk plot of the transformed data

The recombinant esterase showed the highest activity for p-nitrophenyl-butyrate (pNPC4) (294.1 AU/mg proein), followed by p-nitrophenyl-acetate

(pNPC2) (98.0 AU/mg protein). No activity was detected for the other p-nitrophenol esters under these conditions tested. Lower activities were detected when aminopeptidase (leu-pNa) and pro-iminopeptidase (pro-pNa) were used as substrates (12.8 AU/mg protein and 8.8 AU/mg protein, respectively). Simple hyperbolic Michaelis–Menten kinetics were seen for these four substrates. V_{max}, K_m, k_{cat}, and k_{cat}/K_m were all calculated and are presented in Table 15. It can be seen that the recombinant esterase has the same selectivity for the acetate and butyrate substrates, with a K_m of 0.13 for both of these substrates. Despite this, it showed a higher V_{max} for the butyrate substrate than for the acetate. However, the k_{cat} and k_{cat}/K_m values showed that the recombinant esterase is more efficient in hydrolysing butyrate than acetate. Regarding the proteolytic activity of this enzyme, we can detect aminopeptidase and pro-iminopeptidase activities, but the calculated kinetic parameters showed lower efficiencies for these kinds of catalysis.

Table 14. Kinetic parameters of recombinant Lp_2953 esterase from *L. plantarum*. Enzyme activities determined at room temperature in 50 mM phosphate buffer (pH 6.0). Results are mean ± standard deviation from three independent experiments.

Substrate	**V_{max}** (μM/min/mg)	**K_m** (μM)	**K_{cat}** (S^{-1})	**K_{cat}/K_m** (S^{-1} μM^{-1})
Acetate	1111.1 ±2.2	0.1333 ±0.004	18.51 ±5.5	138.88 ±10.5
Butyrate	3333.3 ±2.5	0.1333 ±0.008	55.55 ±1.6	416.66 ±12.4
Leu-pNa	169.7 ±1.6	0.3208 ±0.015	2.82 ±0.4	8.8183 ±1.6
Pro-pNA	116.8 ±1.3	0.3271 ±0.02	1.94 ±0.01	5.9523 ±1.2

Discussion

To investigate the enzymes involved in gluten proteolysis, we performed an '*in-silico*' analysis of the genes coding for the proline-specific protease in the *L. plantarum* genome. The analysis was performed using the *L. plantarum* WCSF1 proteome as the template, and the prolyl-endopeptidase as the pattern. Of the four putative proline-specific proteases, three of them (pepRI; pepRII and pepI) are listed as praline-specific peptidase on the *L. plantarum* database, while Lp_2953 is listed on the same database as a putative esterase. This is apparently in contrast with our finding of high similarity in the catalytic domain that was seen on three-dimensional overpositioning of the proline iminopeptidase family proteins and the Est 1 esterase family (43). All of these four genes were cloned and sequenced, and their deduced protein sequences were aligned and compared using the Clustal W2 software (100). The gene cloned from the WCSF1 strain showed 100% identity between the sequence annotated on the database and the cloned enzyme, while high similarity (>98%) was found for the natural strains Sb5c and Sb7b. These genes were then cloned on expression plasmids and expressed in *E. coli* BL21 cells. All of the recombinant proteins were successfully purified by Ni-NTA affinity chromatography under native and denaturing conditions (Lp_2953). To date, no peptidases have yet been cloned and purified from these *L. plantarum* strains, and these are the first data available in the literature (20) (43) (30) (94). These enzymes was then assayed for their activities using gly-pro-pNa as substrate at pH 6 and pH 4.5. In this case, two different performances were observed: PepI and PepR2 showed low activities at pH 6 and no activities a pH 4.5, while the PepRI and Lp_2953 enzymes showed activities at pH 6 that remained at pH 4.5 (see Table 14). Compared to the data available for similar peptidases, we noted a high stability at low pH values; to date, no peptidase from

LAB has shown activity at pHs below 5.5 (36) (34) (33) (42) (12) (97). At these pHs, PepR1 showed concentration-dependent activity that indicated good specificity, although with a low turnover number for the enzyme; Lp_2953 showed not concentration-dependent activity. Although these two enzymes showed the ability to cleave the pNa derivate, they did not hydrolyse the long-chain immunogenic 33-mer peptide (see Figure 47). This findings is in good agreement with the literature, where a number of studies have reported the specificities of proline-specific peptidases related to the length of the substrate (85) (87) (74). In a recent study, De Angelis et al. (87) obtained partial 33-mer hydrolysis by synergic actions of at least three enzymes from a pool of ten lactobacilli (pepX pepN and pepO). Further hydrolysis resulted in only free amino acids with the addition of the pepR and pepI enzymes, suggesting that the enzymes found in our study might have roles in a second phase of 33-mer hydrolysis.

The Lp_2953 enzyme was found to be similar to the proline-specific enzymes, although it is listed as an esterase in the database. This finding is only relatively unexpected, as Siezen et al. (43) noted high similarities in the three-dimensional structure of the active site of the proline-iminopeptidase superfamily and the Est 1 family of esterases. In the *in-silico* analysis, we found that the only difference between the prolyl edopeptidase pattern and the matched Lp-2953-coded protein is in the substitution of aspartic acid for glutammic acid in the first position of the pattern. This substitution of two similar amino acids in this position of the pattern does not influence the protein structure, and therefore their functions and activities (96).

Based on the results obtained for the chromogenic proline-specific substrates, where we detected activities on the praline-specific peptidase substrates, partial characterisation of Lp_2953 was carried out. Several p-nitrophenyl esters were used as substrates and the enzyme showed the highest activity for the butyrate (pNPC4) (294.1 AU/mg protein), followed by p-nitrophenyl-acetate (pNPC2) (98.03 AU/mg protein). No activity was detected for the other p-nitrophenol esters under the conditions tested. Comparisons using several proline specific substrates were carried out, and the results show that the activity as a proteolytic enzyme is detectable, but not comparable with the esterase detected activity (see Table 14). The esterase nature of this enzyme is clearly shown by the kinetic parameters, and the proteolytic activity can be considered as an 'accessory effect' for this enzyme, which has shown bifunctional activities (101); nevertheless, it clearly showed an esterase nature. Characterisation of an esterase from *L. plantarum* ATCC 8014 was published by Brod et al. in 2010 (102). In this study, the lp_0973 gene was cloned and the encoded esterase was purified and characterised. The authors found high specificity for acetate, followed by butyrate substrates. Our findings for the *L. plantarum* esterase enzyme with specificity for butyrate and with kinetic parameters similar to those that were seen by Brod et al. (2000) is evidence of the flexibility of this microorganism. As Lp_2953 esterase showed catalytic activity as a peptidase, even if this was low if compared to its esterase activity, this makes us believe that we are considering a bifunctional enzyme. Bifunctional enzymes are enzymes that have two distinct catalytic activities within the same polypeptide chain. Bifunctional enzymes have been found in a variety of environments. Chorismate mutase/ prephenate dehydrogenase from *Haemophilus influenza* Rd KW20 is an

example of a bifunctional enzyme, and it catalyses the rearrangement of chorismate to prephenate, plus the NAD(P)+-dependent oxidative decarboxylation of prephenate to 4-hydroxyphenylpyruvate in tyrosine biosynthesis (103). In rat liver, UDP-GlcNAc 2-epimerase/ ManNAc kinase catalyses the biosynthesis of N-acetylneuraminic acid. This enzyme is present as polypeptide that self-associates as a dimer and as a hexamer, with apparent molecular masses of 150 kDa and 450 kDa, respectively (104). Peroxisomal 2-enoyl-CoA hydratase/ 3-hydroxyacyl-CoA dehydrogenase, formiminotransferase/ cyclo-deaminase and ATP sulfurylase/ adenosine 5-phosphosulfate kinase are three other known bifunctional enzymes that catalyse two subsequent steps of metabolic pathways (105) (106).

Conclusions

In this thesis, we have evaluated the ability of *L. plantarum* to grow on gluten-based media, and how gluten is hydrolysed by this microorganism. To do this, several media were examined, and we show that this bacterial grow is best supported by gluten medium with yeast extract. Proteolysis levels were then assayed in this medium and in gluten medium, and we show that the yeast extract acts as a proteolysis inhibitor for this bacteria, while in the medium with only gluten, proteolysis reached the highest levels. Using a RP-HPLC assay for the residual gluten, we noted that LMW glutenin is the preferred substrate for this bacteria, while α/β-gliadine were hydrolysed to signifincant levels only in gluten medium. The proteolytic activity characterisation assay allow us to believe that the X-prolyl-dipeptidyl-amonipeptidase and prolyl endopeptidase enzymes might be the responsible of the α/β-gliadine hydrolysis.

In a second step, we aimed at an investigation of the enzymes involved, to clone, express and purify the enzymes that showed the highest similarities in the '*in-silico*' screening. Four enzymes were cloned, and two of these showed interesting results, with their activities at low pH never having been reported for similar enzymes before. Unfortunately, none of these enzymes hydrolysed the toxic 33-mer peptide alone, and to date no LAB enzymes have been shown to do this alone.

Interestingly, one of the cloned enzyme was listed in the database as a 'putative esterase' and this led us to perform a partial characterisation of this enzyme. The results of this was that Lp_2953 shows two distinct catalytic activities: an esterase activity and a peptidase activity. The former (the most

expressed) was directed against the butyrate derivate, the latter was an iminopeptidase activity with specificity for the C-bond of the leu-pNa substrates.

In this thesis, we have opened several topics of research on the *L. plantarum* proteome. The first is that to date no peptidase from *L. plantarum* has been cloned and expressed; our characterization of such an expressed peptidase sheds some light on its proteolytic system in general, and indicates the features of this enzyme, especially regarding its ability to work at low pH. Secondly, assays of this peptidase and some of the other 19 listed peptidases in *L. plantarum*, when used either alone or in combination, indicate that they may indeed provide a catalytic cocktail that can hydrolyse the toxic gluten peptides. This kind of assay can be performed using reference strains and wild strains, to determine whether horizontal gene transfer or environment-linked mutations might have introduced difference between these. Thirdly, a further investigation of the Lp_2953-coded esterase can be done, as a structural analysis relating to its bifunctional activity and structure, which could easily be done in the near future.

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