Ripening of gouda cheese using genetically modified *Lactococcus lactis* starters

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1. Introduction

Ripening of cheese is a complex process involving lactose/citrate metabolism, lipolysis, and proteolysis. The latter involves cleavage of caseins by proteolytic enzymes, first into peptides and finally into amino acids. The amino acids may then be further degraded to flavour compounds by other enzymes (1).

During recent years the proteolytic system of lactococci has been extensively characterized (2, 3, 4). From these investigations it is known, that the only extracellular caseinolytic enzyme of lactococci is the proteinase. It releases peptides from the α- and/or β-caseins. Peptidases are exclusively located in the cytoplasm and carry out the degradation of peptides released by the proteinase and transported into the cell by specific (oligo)peptide transport systems. During cheese ripening the peptidases must be released from the cells through cell lysis in order to contribute to proteolysis. However, the contribution to the level of free amino acids in the cheese matrix has yet to be established for the released peptidases.

Substrates for peptidases are peptides, which are cleaved with specificities and efficiencies different for each peptidase. While PepX preferentially liberates X-Proline dipeptides from longer peptides (5), PepT specifically cleaves tripeptides, as long as they do not possess proline in the middle position (6). PepN and PepC are general aminopeptidases (7, 8), cleaving N-terminal amino acids from a broad spectrum of peptides. PepO is an endopeptidase capable of cleaving e.g. casein fragments released from casein by the action of chymosin (9, 16).

In this communication the effects of deletions of single peptidase genes from the chromosome of starter strains are reported and the consequences of these alterations with respect to the amino acid levels and the flavour of cheeses produced with these starters are described.

2. Materials and Methods

2.1 Bacterial Strains

Lactococcus lactis subsp. *cremoris* MG1363(pLP712) (Prt^+ Lac^+) (10) was used as a single-strain starter for the production of control cheeses. Food-grade derivatives of this strain, each deleted for one the following peptidase genes, have been constructed previously: Δ*pepC*, Δ*pepN*, Δ*pepO* (11), Δ*pepT* (12), and Δ*pepX* (13).

2.2 Cheese Manufacture and Ripening

Gouda-like cheeses were produced from pasteurized milk in batches of 100 litres, each batch yielding four 2.5 kg cheeses for analysis of different stages of ripening (5 and 10 weeks). Always three batches were produced in parallel from the same cheese milk. For all cheese trials, two "GMO-cheeses" and one control ("wild-type") cheese were produced on a single day from one batch of milk for a direct comparison, thus avoiding any influence of milk quality on cheese ripening. The unmodified wild-type starter *L. lactis* subsp. *cremoris* MG1363 was used for the controls, peptidase-deficient derivatives of MG1363 were used for the "GMO-cheeses". Pelleted, deep-frozen preparations of the single strains were used as direct starters. Cheeses were ripened at 15°C and 90% relative humidity. For determination of cell counts, 10 g of cheese were homogenized in a Stomacher with 90 ml of quarter strength Ringer´s solution and plated on M17 medium for lactococci (starter counts) and MRS medium for lactobacilli (non-starter counts), each medium containing 0.5 % lactose.

Cheese manufacture was carried out in specifically constructed facilities to allow for production under contained conditions. Whey and all liquid waste was collected and thermally treated to reduce bacterial counts by at least 6 orders of magnitude.

2.3 Quantitative determination of free amino acids in cheese samples

45 ml of 0.07 M tri-sodiumcitrate (45°C) were added to 5 g of cheese. The sample was homogenised in a Stomacher for 5 min. To 3 ml of this suspension 3 ml of 0.6 M trichloroacetic acid (TCA) was added, shaken vigorously on a Vortex mixer, and centrifuged at 20000 rpm for 10 min at 4°C (rotor JA 20, Beckman Instruments). After a second extraction of the sediment with 3 ml of TCA and centrifugation, the supernatants were combined and filled up to a volume of 10 ml with distilled water. The samples were filtered through a 0.2 µm membrane filter and stored in aliquots at -20°C. HPLC of the samples was performed according to the method published by the manufacturer (Merck-Hitachi). 9-fluorenylmethoxycarbonyl- (FMOC-) and o-phtaldialdehyde- (OPA) derivatized amino acids were quantified by reversed phase-HPLC (14), using a Superspher 60 RP 8, 250 x 4 mm, column with fluorescence detection (Merck, Darmstadt, Germany).

2.4 Sensory analysis

Aroma and taste of the cheeses were evaluated in duplicate (winter and summer cheese batches) after 5 and 10 weeks of ripening by a taste panel of the Federal Research Centre for Nutrition and Food (Kiel, Germany) applying descriptive sensory techniques (DIN 10967 / ISO 11035). The cheese samples were coded for the analysis by someone not participating in the sensory analysis. Sensory data were not statistically treated. The scores for typical smell, bitter and flat taste ranged from 1-5 (very weak – very strong). The sensory values for winter and summer cheeses were averaged and the scores of the corresponding controls were subtracted, thus, some negative values are shown in Figure 3.

3. Results and Discussion

3.1 Construction of food-grade deletion mutants

L. lactis strains carrying deletions in peptidase genes were constructed by the gene replacement technique (11, 13, 15) as schematically outlined in Fig. 1. The peptidase genes were cloned in a vector plasmid carrying an erythromycin resistance gene (Em^R)

but lacking the *repA* gene encoding the RepA protein necessary for replication. Replication of this plasmid is enabled in specific bacterial host strains that provide the RepA protein in *trans* (13). The mutations were created within the peptidase genes by deleting internal restriction fragments, leaving sufficient cloned DNA on both sides of the deletions for subsequent recombination (Fig. 1a). Upon transformation of a deletion plasmid into a *L. lactis* strain carrying a wild-type copy of the respective peptidase gene (Fig. 1b), selection for erythromycin resistance yielded strains in which integration of the plasmid into the chromosome had taken place by homologous recombination between the chromosomal peptidase gene and one of the DNA regions flanking the deletion present on the plasmid (Fig. 1c). In the presence of antibiotic, only cells harbouring this co-integrate DNA structure will be able to grow. However, when the selective pressure is abolished by growing the cells in medium without antibiotic, cells will grow, in which the plasmid has been excised from the co-integrate by homologous recombination. This step is in principle a simple reversion of integration. However, if the homologous recombination events leading to integration and excision take place on different flanking regions of the deletions, as shown in Fig. 1d, the plasmid excised carries the wild-type copy of the *pep* gene, while the deletion derivative of this gene is left in the chromosome (Fig. 1e). The plasmid, which cannot replicate, will not be transmitted to daughter cells during subsequent growth, and single colonies lacking the plasmid are easily isolated. The cells of these colonies do no longer contain plasmid DNA. The proper excisants carry within a *pep* gene a mutation, which may as well have arisen by a natural mutation event.

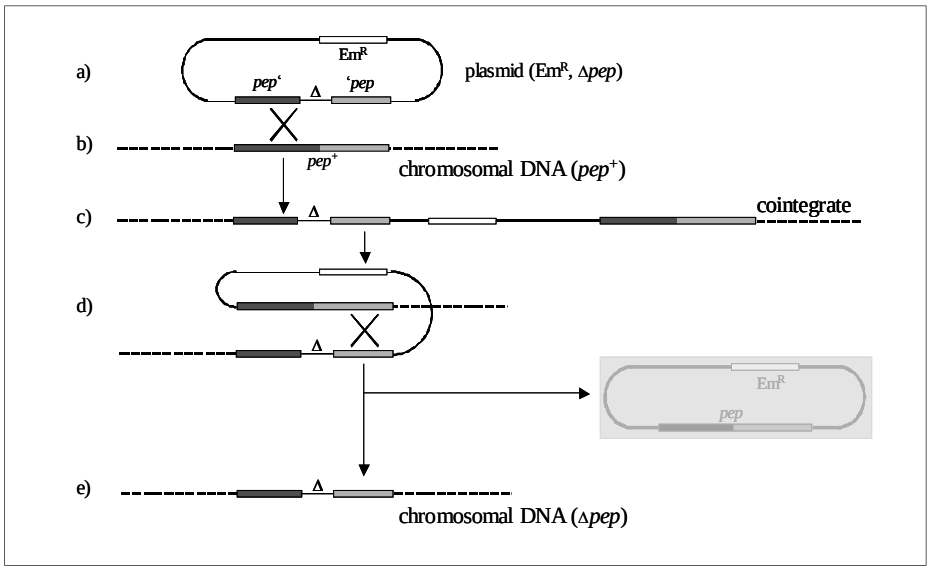

Fig. 1: Scheme showing the construction of chromosomal peptidase gene deletion mutants by gene replacement. (a) Deletions were constructed in peptidase genes (*pep*) cloned in a replication-deficient plasmid carrying an antibiotic resistance gene (Em^R) (11, 12, 13). (b) A peptidase deletion plasmid is used to transform the wild-type strain carrying the intact peptidase gene (pep$^+$). For convenience, left and right parts of the *pep* gene are shown in different shades of grey. By homologous recombination (×) the plasmid becomes integrated in the chromosomal DNA at the loaction of the wild-type pep gene. (c) The resultant lactococcal strain contains two copies of the *pep* gene, Δ*pep* and *pep*$^+$, separated by plasmid DNA carrying the Em^R gene. Only cells harbouring this cointegrate

can grow on the antibiotic erythromycin. (d) When the selective pressure is abolished by growing cells in the absence of the antibiotic, those cells will grow, that do no longer harbour the plasmid. The latter has been excised from the chromosome by homologous recombination, but has not been transmitted to the daughter cells due to replication deficiency. (e) Depending on which side of the deletion the homologous recombination event leading to either integration or excision has taken place, the Δ*pep* gene will be left in the chromosome while the *pep*$^+$ gene, located on the plasmid, is lost from the culture.

3.2 Cheese production and ripening

2 x 8 batches of experimental Gouda cheese were produced and ripened using peptidase deleted starter mutants (5 batches) and compared with the corresponding MG1363 (Prt$^+$ Lac$^+$) controls (3 batches). Acidification was similar for all strains including the control strain MG 1363 (Prt$^+$ Lac$^+$). The physical and chemical parameters of cheeses between batches showed little variation, which probably did not affect texture and aroma to a detectable extent (Table 1). The only cheese showing a higher water content and resulting lower values for dry matter, fat, and protein was produced with the *L. lactis* PepO$^-$ starter strain.

Tab. 1: Chemical and physical parameters of the experimental Gouda-type cheeses[a].

	wild-type	*pepX*$^-$	*pepN*$^-$	*pepC*$^-$	*pepT*$^-$	*pepO*$^-$
dry matter %	56.70	56.30	56.10	56.90	56.40	54.80
water %	43.30	43.70	43.90	43.10	43.60	45.20
fat %	26.10	26.00	26.00	26.40	26.20	24.90
protein %	23.90	23.60	23.50	23.90	23.80	23.10
salt %	1.60	1.60	1.60	1.50	1.50	1.60
total ash % (550°C)	4.10	4.20	4.20	4.00	4.20	4.00
calcium %	0.92	0.90	0.92	0.88	0.87	0.85
pH	5.28	5.25	5.27	5.25	5.18	5.22
fat in dry matter %	46.00	46.20	46.30	46.40	46.50	45.40
Wff %[b]	58.60	59.10	59.30	58.60	59.10	60.20

[a] Data on the other 4 wild-type cheeses (wt) and the duplicates of the mutant cheeses were comparable and are not shown
[b] Wff= water in fat free matter

Starter cell counts dropped from ca. 10^8 cfu × g^{-1} to >10^7 cfu × g^{-1} within 5 weeks of ripening. In some cheese batches produced in summer, starter counts increased, indicating that no or only little lysis had taken place (Table 2). Non-starter counts of the cheese batches produced in winter increased within 5 weeks from <10^4 cfu × g^{-1} to approximately 10^5 cfu × g^{-1} (Table 2). The non-starter counts of the summer cheese batches were higher after production and 5 weeks of ripening compared to the winter batches (ca. 10^6 cfu × g^{-1}).

After 10 weeks of ripening the starter counts dropped to approximately 10^6 cfu × g^{-1} (winter batches 1+2). The cell counts of the summer batches 3-5 at 10 weeks of ripening were different: cell counts of batch 3 (PepX$^-$, PepN$^-$) were >10^8 cfu × g^{-1}, batch 4 (PepC$^-$, PepT$^-$) >10^7 cfu × g^{-1}, and batch 5 ((PepC$^-$, PepO$^-$) >10^6 cfu × g^{-1}. The non-starter counts

of the winter batches were more or less unchanged (>10^5 cfu × g^{-1}), the summer batches showed increasing non-starter counts to >10^6 cfu × g^{-1}, except for PepC$^-$ and PepO$^-$ cheeses, where non-starter counts remained the same (>10^5 cfu × g^{-1}). Since the non-starter counts were higher after 10 weeks of ripening (ca. 10^7 cfu × g^{-1}; data not shown), the 5 week samples were chosen for comparison of "GMO" and "wild-type" cheeses in order to minimize the potential interference of results by growth and metabolism of non-starter (mostly *Lactobacillus* spec.).

Tab. 2: Production of 5 batches of experimental Gouda cheese[a)].

Type of starter	green cheese		5 weeks	
	starter	non-starter	starter	non-starter
		winter production		
control 1 (wt)	1.3 × 10^8	0.04 × 10^5	0.34 × 10^8	1.7 × 10^5
***pepX*$^-$**	1.0 × 10^8	0.02 × 10^5	0.20 × 10^8	0.4 × 10^5
***pepN*$^-$**	0.9 × 10^8	0.01 × 10^5	0.04 × 10^8	2.0 × 10^5
control 2 (wt)	0.9 × 10^8	0.01 × 10^5	0.2 × 10^8	0.9 × 10^5
***pepT*$^-$**	0.9 × 10^8	n.d.	0.3 × 10^8	0.3 × 10^5
***pepO*$^-$**	0.9 × 10^8	0.01 × 10^5	0.3 × 10^8	1.0 × 10^5
		summer production		
control 3 (wt)	1.6 × 10^8	0.04 × 10^5	4.6 × 10^8	11.4 × 10^5
***pepX*$^-$**	1.0 × 10^8	0.06 × 10^5	6.7 × 10^8	18.3 × 10^5
***pepN*$^-$**	1.2 × 10^8	0.07 × 10^5	11.0 × 10^8	8.7 × 10^5
control 4 (wt)	1.6 × 10^8	0.30 × 10^5	1.3 × 10^8	8.6 × 10^5
***pepT*$^-$**	1.7 × 10^8	0.60 × 10^5	0.9 × 10^8	44.1 × 10^5
***pepC*$^-$**	1.7 × 10^8	0.06 × 10^5	2.4 × 10^8	3.0 × 10^5
control 5 (wt)	1.6 × 10^8	0.20 × 10^5	0.2 × 10^8	2.9 × 10^5
***pepO*$^-$**	1.3 × 10^8	0.06 × 10^5	0.2 × 10^8	17.0 × 10^5
***pepC*$^-$**	0.9 × 10^8	0.04 × 10^5	0.6 × 10^8	3.0 × 10^5

[a)] Single strain starters were used for acidification. The wild-type L. lactis MG1363 (Pep$^+$ Lac$^+$) was used as control. Mutants are indicated by the deletion of the gene mentioned. One control (wild-type) and two experimental cheeses (pep) were each produced from the same batch of milk on a single day. The two winter and three summer batches were each produced within one week. For each starter, cheeses were produced at two different times of the year to distinguish seasonal and influences of milk quality from effects of the genetic modifications. Cell counts are expressed in colony forming units (cfu) per gram of cheese. Lactococci were plated on M17 medium, lactobacilli on MRS medium (wt = wild-type, n.d. = not determined; "green cheese= fresh cheese before salting).

Mierau et al. (11) have shown that deletion of *pep* genes from *L. lactis* MG1363 (Prt$^+$ Lac$^+$) impaired the ability of this starter culture to grow in milk. Deletion of single genes, however, resulted in slight reduction of the growth rates, only. Since we did not observe any systematic correlation of starter development with deletion of a *pep* gene, we conclude that the effects on growth as observed in milk are not relevant for starter development during ripening of cheese. In this respect, one has to bear in mind that (i) ripening times very much exceed the times of exponential growth in milk and (ii) the cheeses were only analysed after 5 weeks of ripening.

3.3 Levels of free amino acids in cheese as affected by genetic modification of starters

The concentrations of total free amino acids were 123 µM and 105 µM for the wild-type controls 1 and 2 (winter), respectively. Total free amino acids in cheese were much higher for all starters in the summer batches 3-5. Free amino acid concentrations were determined to be 198 µM, 207 µM and 275 µM for the controls 3-5 (summer). A reason for this is unclear. Lysis of starters was higher in winter batches, summer batches showed stronger growth of starters and non-starter after 5 weeks of ripening (Table 2).

As expected, deletion of single peptidase genes from the chromosome of *L. lactis* MG 1363 led to lower concentrations of amino acids in the experimental cheeses after 5 weeks of ripening (total amino acids compared to the controls: $PepX^-$ -19.5 %, $PepN^-$ -17.6 %, $PepT^-$ -23.4 %, $PepO^-$ -17.1 % and $PepC^-$ -31.6%). Due to, apparently, extended growth and metabolism of non-starter lactic acid bacteria, data were not as clear after 10 weeks of ripening (total amino acids compared to controls: $PepX^-$ -0.9 %, $PepN^-$ -5.7 %, $PepT^-$ -4.2 %, $PepO^-$ +0.4 % and $PepC^-$ -31.9%) and are not further discussed. It should be noted, though, that the $PepC^-$ cheese batches showed low concentrations of non-starter bacteria from the start of the experiment until 10 weeks of ripening: the difference in the amino acid levels in the experimental cheeses after 10 weeks compared to the controls were more or less the same as in the 5 weeks cheeses.

Deletion of PepC had the most pronounced effect on amino acid levels in cheese. Levels of all free amino acids in cheese were lower than in the control cheese after 5 weeks of ripening (Fig. 2 a+b). Amino acids of which the levels were most decreased were threonine, glycine, proline, asparagine, leucine and valine. A clear effect of the PepX deletion was the drastic reduction of free proline (Fig. 2a), while the concentrations of the other amino acids were more or less unchanged. In the latter case, reduction of proline can be attributed to the peptide cleavage specificity of PepX, as this peptidase preferentially cleaves X-Proline dipeptides (5). With regard to the other peptidases, such attributions cannot be made, since none shows such a preference for peptides containing specific amino acids as does PepX (6, 7, 8, 9).

3.4 Sensory analysis

Cheeses produced with $PepC^-$ starters showed the highest degree of bitter flavour compared to the control cheeses in all 4 sensory analyses (winter/summer, 5w/10w, Fig. 3). The most drastic influence on typical smell was observed for the $PepX^-$ and the $PepN^-$ cheeses. Even though the *pepN* deletion did not seem to have affected the amino acid levels much, the taste was described “flat” compared to controls in all 4 sensory analyes. A similar effect was observed for $PepT^-$ cheeses. Other effects were just found in single analyses and were therefore not considered to be clear differences to the controls.

The fact that starter strains deleted for a single peptidase did not improve the flavour is not surprising. However, our results show that in some cases the pattern of free amino acids within the cheese may be altered in specific ways, depending on the enzyme deleted (e.g. PepX).

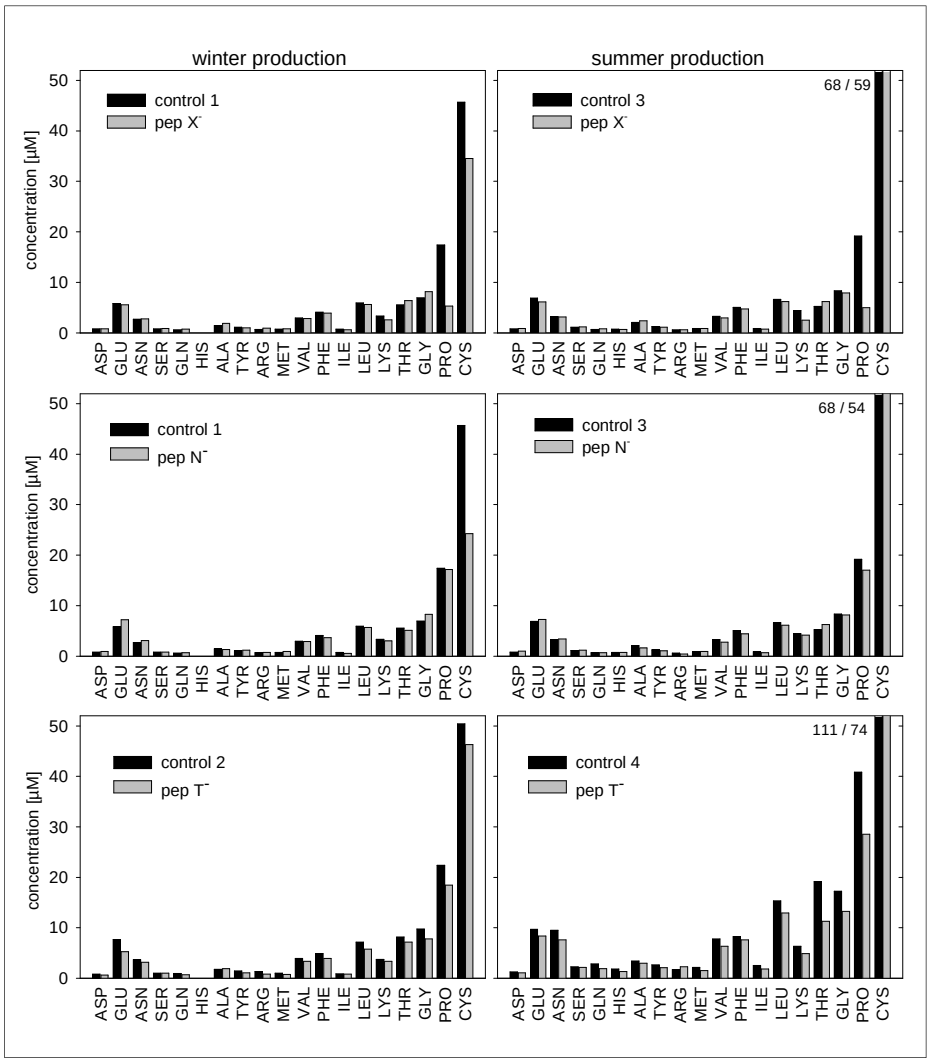

Fig. 2a+b: Liberation of amino acids in 5 sets of Gouda-like cheese batches after 5 weeks of ripening. The amino acids determined are indicated below the columns, using three-letter code. Concentrations (mM) are given for free amino acids in the cheese matrix. Tryptophane could be detected, too, however concentrations were near the detection limit and could not be quantified. Data represent the mean of two cheese extracts analyzed from each cheese batch by HPLC. The 5 different wild-type controls using wild type *L. lactis* MG1363 starters were produced from the same batch of cheese milk as the GMO cheeses. The values for the out of range bars of cysteine are printed next to the bars.

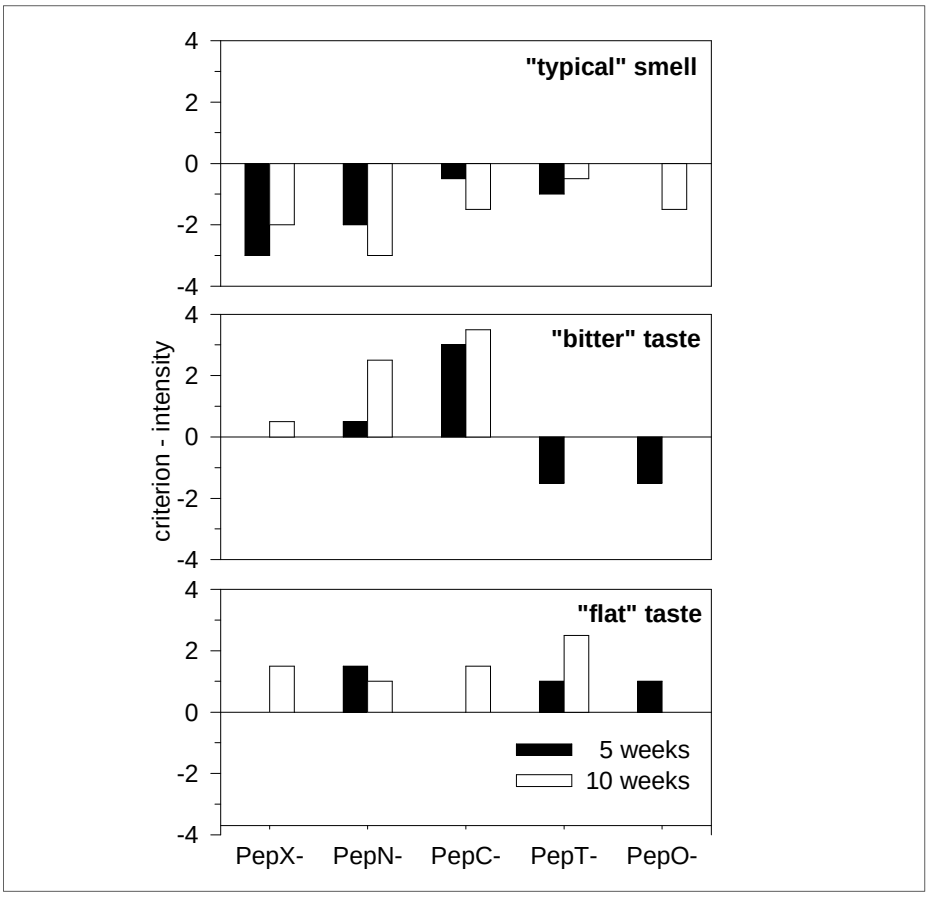

Fig. 3: Sensory analysis of experimental cheeses with the three descriptors “typical smell”, “bitter taste” and “flat taste”. The numerical score ranged from 0 to 5 (zero, very weak, weak, significant, strong, very strong). Cheeses were graded after 5 and 10 weeks with values of 2 productions (winter, summer) averaged. The values for the control cheeses were subtracted from the experimental cheeses produced with peptidase-deficient *Lactococcus* starters.

4. Conclusions

Amino acid concentrations and patterns were affected during cheese ripening by deletion of single peptidases. This influenced cheese flavour to some extent. An improvement of flavour was not observed. Since peptidases are intracellular enzymes, strains with good autolysing qualities should be used as hosts for genetic modifications affecting peptidase genes. Expression of additional peptidases or increased expression of existing ones in starter strains might improve flavour in a positive way by elevating free amino acid levels in cheese.

Acknowledgements

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6. Summary

Bockelmann, W., Golecki, S., Kok, J., Mierau, I., Heller. K.J.: **Ripening of gouda cheese using genetically modified *Lactococcus lactis* starters.** Kieler Milchwirtschaftliche Forschungsberichte **57** (4) 263-272 (2005)

26 Microbiology (cheese ripening; genetically modified starter; food-grade; peptidases; *L. lactis*)

Lactococcus lactis MG1363 (Lac^+, Prt^+) and isogenic derivatives, each deleted for one of peptidases PepC, PepN, PepO, PepT or PepX, were used as starters for cheese making. The genetic modifications introduced were food-grade to allow for organoleptic analysis of the experimental cheeses. No significant effects were observed for general chemical, physical and microbiological parameters. While the use of the PepX-deletion strain resulted in a specific reduction of the proline level, cheeses prepared with the other Pep-deletion starters showed reduced levels of all amino acids. This corresponded to high degrees of bitterness observed in sensory analysis. Compared to controls, all experimental cheeses exhibited a more flat odour and taste.

Zusammenfassung

Bockelmann, W., Golecki, S., Kok, J., Mierau, I., Heller, K.J.: **Reifung von Gouda Käse unter Einsatz von genetisch veränderten Starterkulturen *Lactococcus lactis*.** Kieler Milchwirtschaftliche Forschungsberichte **57** (4) 263-272 (2005)

26 Mikrobiologie (genetisch veränderte Starterkulturen, lebenmitteltaugliche Peptidasen, *L. lactis)*

Lactococcus lactis MG1363 (Lac^+, Prt^+) und isogene Derivate, in denen jeweils eines der Gene für die Peptidasen PepC, PepN, PepO, PepT oder PepX deletiert worden war, wurden als Starter zur Käseherstellung eingesetzt. Die genetischen Modifikationen wurden in lebensmitteltauglicher Weise vorgenommen, um die organoleptische Analyse der experimentellen Käse zu erlauben. Signifikante Effekte auf generelle chemische, physikalische und mikrobiologische Parameter wurden nicht beobachtet. Während der Einsatz des PepX-Deletionsstamms zu einer spezifischen Reduktion des Gehalts an Prolin führte, zeigte sich in den mit den anderen Pep-Deletionsstämmen hergestellten Käse eine generelle Reduktion aller Aminosäuren. Dieses korrelierte in der sensorischen Analyse mit hohen Werten für „Bitterkeit". Verglichen mit Kontrollkäsen zeigten alle experimentellen Käse eher flachen Geschmack und Geruch.

Résumé

Bockelmann, W., Golecki, S., Kok, J., Mierau, I., Heller, K.J.: **Maturation du fromage Gouda en utilisant des cultures de levain starter *Lactococcus lactis* génétiquement modifiées.** Kieler Milchwirtschaftliche Forschungsberichte **57** (4) 263-272 (2005)

26 Microbiologie (cultures de levain starter génétiquement modifiées, peptidases «food-grade» (homologuées pour le secteur alimentaire); peptidases; *L. lactis*)

Lactococcus lactis MG1363 (Lac^+, Prt^+) et des dérivatifs isogéniques, knock-out pour les peptidases PepC, PepN, PepO, PepT ou PepX, étaient utilisés comme levain starter dans la fabrication de fromage. Ces modifications génétiques, homologuées pour le secteur alimentaire, permettent des analyses organoleptiques des fromages expérimentaux. On n'a pas observé des effets signifiants pour les paramètres généraux chimiques, physiques et microbiologiques. Cependant l'utilisation de la souche PepX-knock-out menait à une réduction spécifique du niveau proline, tandis que pour des fromages préparés avec les autres starters Pep knock-out, un niveau réduit de tous les acides aminés était constaté. Ceci correspondait à un niveau élevé d'amertume relevé lors de l'analyse sensorielle. Comparé à des échantillons de contrôle , tous les fromages expérimentaux avaient une odeur et un goût moins prononcé.