Abstract book

Potential of *Lactobacillus* in Northern European cheeses

Symposium in Tallinn, Estonia

23-25 April 2007

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Monday 23.04

12.00 – 14.00 Registration
13.00 – 14.00 Lunch

14.00 – 14.30 Symposium opening
Tiiu Maie Laht, Head of arrangement committee
Siv Skeie, Coordinator of the NordOst network
Some official person

14.30 – 18.00 Session A: *Lactobacillus* in Nordic cheese
Chairman: Tiiu Maie Laht

Key note speaker:

14.30 – 15.15	*Lactobacillus* in Nordic cheese Ylva Ardö, University of Copenhagen, Denmark.
15.15 – 15.25	Discussion

Oral presentations:

15. 25 – 15.55	Interaction between starter bacteria and adjunct Lactobacillus plantarum INF15D on the degradation of citrate, asparagine (Asn) and aspartate (Asp) in a washed curd cheese S. Skeie, A. Kieronczyk, S. Eidet, M. Mjånes, K. Olsen, H. Østlie
15.55 – 16.15	Coffee Break
16.15 – 16.45	Key Aroma Compounds in Cheeses Produced with *Lactobacillus helveticus* C. Varming, M. A. Petersen, Y. Ardö
16.45 – 17.15	Properties of NSLAB in Estonian cheeses K. Adamberg, S. Adamberg, T.M. Laht

17.15 – 17.30 Poster presentations:

Identification of NSLAB from Finnish Emmental Cheese by PCR
M. Immonen, J. Tanskanen
Diversity of *Lactobacillus* in Swedish hard cheese
U. Rehn, F.K. Vogensen, Y. Ardö
Identification of aminopeptidases in *Lactobacillus paracasei*
M. S. Nielsen[1*], Y. Ardö[1]

18.00 – 20.00 Spa evening
20.00 – 21.30 Dinner Buffet

Tuesday 24.04

9.00 – 12.45 Session B: *Lactobacillus* as cheese ripening cultures

Chairman: Inga Ciprovica

Key note speaker:

09.05 – 09.50 *Lactobacillus* as cheese ripening cultures,
Tom Beresford, Teagasc, Ireland.

09.50 – 10.00 Discussion

Oral presentations:

10.00 – 10.30 Functional properties of non-starter lactic acid bacteria related to cheese
Hilde Østlie, Norwegian University….

10.30 – 11.00 Influence of Lactobacillus helveticus strains on amino acid composition in a cheddar cheese model system
M.P. Jensen, F.K.Vogensen, Y.Ardö, J. Steele

11.00 – 11.20 Coffe

11.20 – 11.50 Milk thermal treatment: influence on the growth of lactic starters
I. Stulova, A. Kazarjan, T. Kriščiunaite, T.-M. Laht, R. Vilu

11.50 – 12. 45 Starter culture companies: Presentation of their contribution to the range of *Lactobacillus* cultures for use in Northern European cheeses.

- CSK by Hans Brandsma
- Chr. Hansen by Ilka Eppert, Mikkel Laust Broe og Niels Kristian Sørensen
-

12.45 – 13.45 Lunch

13.45 – 17.30 Session C: Improving bioactivity of cheese,

Chairman: Daiva Leskauskaite

Key note speaker:

13.45 – 14.30 Improving bioactivity of cheese,
Rosina Lopez, Instituto de Fermentaciones Industriales, Spain

14.30 – 14.40 Discussion

Oral presentations:

14.40 – 15.10 The influence of spices on the microbial flora of cheese during ripening.
M. Skemaite, R. Venskutonis, T. Molland, S. Skeie

15.10 – 15.40 The effect of hydrogen peroxide on the growth characteristics of thermophilic lactic starters and rheological properties of acid milk gels.
T. Kriščiunaite, I. Stulova, T.-M. Laht

15.40 – 16.00 Coffe Break

16.00 – 16.20 Poster presentations:

Phosphopeptides in low-fat, semi-hard cheese with *Lactobacillus helveticus*
Y. Ardö, M. Zakora

Antimicrobial effects of plant extracts on undesirable bacteria, yeast and lactic acid bacteria in milk.
A. Sipailiene, T. Faye, P. R. Venskutonis, S. Skeie

Savoury flavour in Cheddar cheese: Impact of Lactobacillus as adjunct culture on Taste and Peptide Profile.
L.T. Andersen, W.L.P. Bredie, L. Stahnke, N.K. Sørensen, Y. Ardö

The dynamics of nitrates, nitrites and important groups of microorganisms in Edam-type cheeses.
A. Elias, P. Elias

16.20 – 17.00 Poster session and discussion

17.30 – 23.00 **Estonian spring experience**

18.00: Guided tour in old Tallinn

Dinner at **Eesti Maja** a traditional Estonian Restaurant

Wedensday 25.04
09.00 – 12.20
Session D: Application of genomics data for *Lactobacillus* in the production of cheeses

Chairman: Ylva Ardö

Key note speaker:

09.05 – 09.50	Applications of genomics data for *Lactobacillus* in the production of cheeses, Jim Steele, University of Wisconsin, US.
09.50 – 10.00	Discussion

Oral presentations:

10.00 – 10.30	The nomenclature and the identification of the species in the *Lactobacillus casei* complex F. K. Vogensen
10.30 – 10.50	Coffee
10.50 – 11.20	*Lactobacillus* lipoteichoic acids: Structures and bioactivities. T. Alatossava
11.20 – 11.50	Protective cultures for semi-hard cheese production Š. Tůma, Y. Ardö, F.K. Vogensen, M. Plocková
11.50 – 12.20	Summing up and closing of the symposium S. Skeie

12.30 – 13.30 Lunch

14.00 -	For those who are interested: Visit at the new research facilities at Tallinn Technical University.

Session A – Key note speaker:

Lactobacillus in Northern European cheeses

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Introduction

Mesophilic *Lactobacillus* were early isolated from Northern European cheese varieties by Orla-Jensen (8, 9) and Troili-Petersson (11), and they were considered to be important for cheese ripening because they were found in high numbers. Orla-Jensen suggested a taxonomy for *Lactobacillus* with three groups similar to the taxonomy still used (Table 1). *Lactobacillus* strains have also been isolated from mesophilic dairy starters (3, 6). However, for a long time the culture producers have mainly considered *Lactobacillus* as contaminants in mesophilic cultures of *Lactococcus* and some times also *Leuconostoc*. These starter cultures are commonly declared as free of *Lactobacillus*. Fortunately, the beneficial *Lactobacillus* species belong commonly to the microflora of the milk and dairy environments in Northern Europe, and they enter the cheese vat spontaneously. *Lactobacillus* of the *paracasei/casei* complex that are the most commonly found species do not grow very well in milk, but they are excellent survivors, and the cheese environment are selective for them in competition to other bacteria, and they dominate the microflora of most cheese varieties during ripening.

Table 1. Nomenclature of dairy *Lactobacillus* (9, 10)

Homofermentative	Facultative heterofermentative	Obligate heterofermentative
Lb. acidophilus *Lb. delbrueckii* subsp. *bulgaricus* *Lb. delbrueckii* subsp. *lactis* *Lb. helveticus*	*Lb. casei* *Lb. curvatus* *Lb. paracasei* subsp. *paracasei* *Lb. paracasei* subsp. *tolerans* *Lb. plantarum* *Lb. rhamnosus*	*Lb. brevis* *Lb. buchneri* *Lb. fermentum* *Lb. kefir* *Lb. reuteri*

Homofermentative *Lactobacillus*

Thermophilic *Lactobacillus* of group 1 are traditionally used as starter bacteria in Southern and Mid-Europe as for instance in Emmental and Parmesan production. Traditionally, these bacteria were not commonly present in the milk and dairy environment in the Northern Europe, and they were first introduced recently as cultures for high temperature cooked cheeses, as Finish Emmental.

Newly developed low fat, semi-hard cheese varieties with 10 - 30 % fat in dry matter are produced with adjuncts of homofermentative *Lactobacillus*. Amino acid content is increased and flavour is improved. A spray-dried powder of heat-treated *Lb. helveticus* is used in *e.g.* Kadett (20 % fat in dry matter) to considerable accelerate the peptide breakdown. The amount of amino acids produced during ripening is the double or even higher as compared to similar normal-fat cheese. This intense breakdown of small peptides influences the consistency to become shorter and more similar to more ripened cheese. The flavour is mild, clean, nutty and aromatic with a sweet note.

Facultative homofermentative *Lactobacillus*

Ripened cheese varieties of Northern Europe are traditionally made with a mesophilic starter, and non-starter facultative heterofermentative *Lactobacillus* (FHL) contribute to development of quality and specificity during ripening. The non-starter microflora has been studied during the last decade in the Norwegian cheeses Jarlsberg (7) and Norveiga (7) and the Swedish Herrgård (1, 7), Grevé (7) and Präst (12), the Danish Danbo cheese (2, 3) and the Estonian Eesti Kuldne, Eesti, Saare and Arensburg cheese (5). *Lb. paracasei/casei* are not only the main group of NSLAB found in the Northern European cheeses, but also in several other cheeses in the world. They may have different roles in cheese ripening including contributing to flavour formation and controlling activities of detrimental bacteria. Some of them also have a potential as probiotics.

Obligate heterofermentative *Lactobacillus*

Lactobacillus species of group 3 are mainly known as detrimental contaminants in Northern European cheeses. They produce gas from lactose as well as from amino acids and may thereby have detrimental influence on texture and potential to blow the cheeses. *Lb. brevis* and *Lb. buchnerii* are able to decarboxylate amino acids and produce gas and amines, of which some are biogenic, e.g. histamine and tyramine (4). Amines contribute to a biting sharp flavour sensation. Obligate heterofermentative *Lactobacillus* may also contribute to specific flavour notes in some cheeses e.g. Prästost. *Lb. danicus* that are found in mesophilic starter cultures (3) have the potential to dominate in some cheeses with mild and clean flavours and thereby inhibit other non-starter bacteria to grow without interfering with the flavour.

References

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Keywords: *Lactobacillus*, cheese microflora, cheese ripening, Northern European cheese varieties

Interaction between starter bacteria and adjunct Lactobacillus plantarum INF15D on the degradation of citrate, asparagine (Asn) and aspartate (Asp) in a washed curd cheese

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Background

Aromatic mesophilic DL starters which contain citrate degrading bacteria are generally used in Dutch type cheeses and in Scandinavian cheese varieties. In Cheddar type cheeses, the degradation of citrate is not considered important for flavour formation and eyes should be absent, and homofermentative O-cultures are used. However, the concentration of citrate normally decreases during Cheddar cheese ripening, and this decrease is attributed to the presence of non starter lactic acid bacteria (NSLAB). In Swiss type cheeses, the propionic acid bacteria produce gas for the eye formation and thermophilic starter cultures are used due to high cooking temperatures of the cheese curd. The degradation of citrate in Swiss type cheeses has been related to the presence facultative heterofermentative NSLAB. In dairy products, diacetyl and acetoin may be produced from citrate metabolism performed by Cit^+ lactic acid bacteria or by aspartate (Asp) metabolism by mesophilic *Lactobacillus* sp. Many lactic acid bacteria (LAB) have GDH activity, which make them capable to deaminate glutamate (Glu) to α-KG which is used for amino acid transamination. Thage, Broe, Petersen, Petersen, Bennedsen, & Ardo (2005) found GDH activity in a commercial DL starter, CHN-11 from Chr. Hansen. Helinck, Le Bars, Moreau & Yvon (2004) showed that GDH activity was common in *Sc. thermophilus.*

Lb plantarum INF15D has previously shown some interesting properties as an adjunct to cheese (Skeie *et al.*, *submitted;* Skeie *et al.*, 2001; Kieronczyk *et al.*, 2004; Kieronczyk *et al.*, 2001). It is GDH positive, degrade Asp to acetoin and diacetyl, degrades Ser and citrate and in cheese, the strain degraded Ser to acetate and citrate to aspartate, which was degraded further to acetoin during early ripening. The objective of this work was to examine the influence of *Lb. plantarum* INF15D in combination with different commercial starters on the degradation of citrate and Asp in a washed curd cheese.

Materials and Methods

Washed-curd brine salted cheeses were made in 4 replicate blocks with 4 different starters; citrate degrading (Cit^+) heterofermentative mesophilic DL-starters (CHN-11 and CHN-19), citrate negative (Cit^-) mesophilic homofermentative O-starter (R-704) and a thermophilic starter TCC-20, all with the addition of an adjunct *Lactobacillus plantarum* INF15D. The cheese was sampled and analysed 2 days after cheesemaking and then after 1, 4, 8 and 12 weeks of ripening. Microbial counts, dry matter, pH, organic acids and volatile compounds were measured at all sampling points, fat at 4 weeks, salt at 8

weeks and amino acids from 4 weeks on. Significant differences ($P<0.05$) between replicate block, treatment factor (starter) and age were found by using the Proc Mixed procedure with repeated measurements using SAS/Stat 8.2 package. A toeplitz covariance structure was used when analysing the data.

Results

Citrate degradation by *Lb. plantarum* INF15D in cheese varied according to the type of starter used. In cheese made with Cit^+ DL-starters, the degradation of citrate and Asp by the adjunct *Lb. plantarum* INF15D was less expressed as diacetyl and acetoin were produced from citrate via α-acetolactate by Cit^+ *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis* and/or *Leuconostoc. mesenteroides* subsp. *cremoris*. In the presence of a Cit^- O-starter, *Lb. plantarum* INF15D degraded citrate mainly to Asp which was further converted to acetoin and diacetyl. However, in the presence of a thermophilic starter, *Lb. plantarum* INF15D degraded citrate mainly to succinic acid. Both *Lb. plantarum* INF15D and the starter cultures were capable to deaminate Asn to Asp, with *Lb. plantarum* INF15D being the most effective. The highest degradation of Asn was found in cheese made with the Cit^- O-starter (R-704) and adjunct *Lb. plantarum* INF15D. Both *Lb. plantarum* INF15D and the starter cultures were capable to deaminate Asn to Asp.

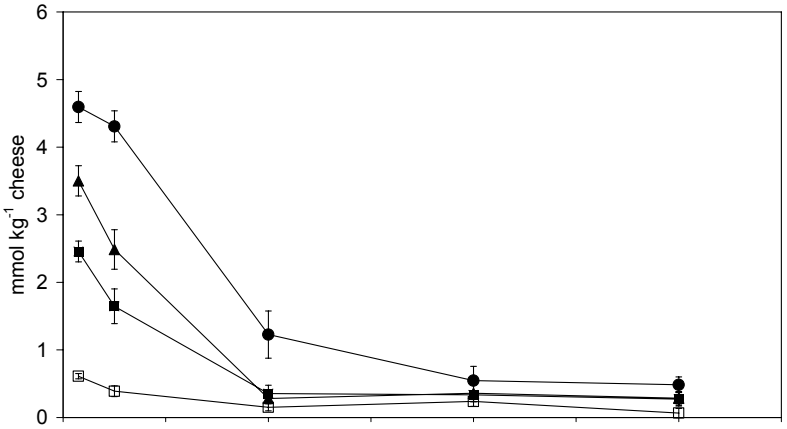

Figure 1. Development of citrate (mmole kg^{-1}) during ripening of cheese made with the starters CH N-11 (■), CH N-19 (□), R-704 (●) and TCC-20 (▲). (Each data point represents mean ± standard deviation of 4 cheeses).

Conclusion

The degradation of citrate, Asp and Asn by the adjunct *Lb. plantarum* INF15D in cheese varied according to the type of starter used.

Keywords: Cheese, starter, *Lactobacillus plantarum*, citrate, aspartate, asparaginase, asparagine.

References

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Session A - oral presentations

Key Aroma Compounds in Cheeses Produced with *Lactobacillus helveticus*

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Background

In maturation of semi-hard cheese, the addition of *Lactobacillus helveticus* as an adjunct has been shown to enhance the cheese flavour characteristics [1]. The breakdown of peptides by *Lb. helveticus* remove bitterness in cheese, and increase the amount of amino acids that in terms can be converted to aroma compounds [2, 3].

Aroma compounds are responsible for the odour of cheese, however, of the vast amount of aroma compounds present only a fraction is contributing to its odour. GC-Olfactometry (GC-O) was used to identify the key aroma compounds that are most important for the odour of three commercially available cheeses produced with *Lb. helveticus* as adjunct culture.

Materials & methods

Kadett (semi-hard) with 10% fat was obtained from Arla Foods (Sweden), and Dubliner (Cheddar type) with 32% fat, Kerrygold (Ireland) and Prima Donna (Gouda type) with 29% fat, (The Netherlands) were obtained from local retailers.

Volatile compounds were isolated from the cheese matrix by dynamic headspace collection on Tenax TA traps. Thirty grams of cheese, 65 ml of water and 1.00 ml of internal standard (4-methyl-1-pentanol) was homogenised. The sample homogenate was equilibrated to 40°C in a water-bath, and purged with N_2 (200 ml/min) under magnetic stirring for 60 min. The collected volatiles were thermally desorbed using an Automated Thermal Desorber and analysis was carried out by a gas chromatograph-mass spectrometer (GC-MS) equipped with a DB-Wax column. Identification of compounds was carried out by probability-based matching with library mass spectra and authentic reference standards. In GC-Olfactometry the effluent of the GC-column was split between the sniffing port and a Flame Ionization Detector (FID). Eight judges sniffed each of the three cheese isolates once during a session of 41 minutes. The judges noted starting and ending time of the odors and gave free choice descriptions of the odor qualities. The sum of the number of judges observing an odour is termed Nasal Impact Frequency (NIF).

Results

A total of 16 odours were observed by GC-O in all of the three cheeses, and of these 13 could be identified (Table 1). Twenty additional odours were observed in one or two of the cheeses only (not shown). The majority of the key compounds identified have previously been reported to be important odorants in cheese [4]. However, by GC-MS a total of more than 80 aroma compounds were identified in the cheeses (not shown); hence the majority of these have no impact on the cheese odour.

The key aroma compounds of the three cheeses represent several chemical classes with a broad range of odour characteristics (Table 1). Seven of the identified compounds could be derived from amino acids: 2-methylbutanal, 3-methylbutanal, diacetyl, 2-acetyl-1-pyrroline, dimethyl trisulfide, methional, and phenyl acetaldehyde [5]. These compounds, however, may also originate from the metabolism of other bacteria than *Lb. helveticus*. Also other compounds, like esters, lactones, and unsaturated

aldehydes contribute to the odour of the examined cheeses. According to their NIF-values, the key aroma compounds contribute to varying degree to the aroma of the cheeses.

Table 1. Key odorants in cheeses produced with *Lactobacillus helveticus*

NIF-value[a]			Odour description[b]	Compound
Prima Donna	Dub-liner	Kadett		
6	6	4	solvent, permanent marker, butyric acid	2-methylbutanal[c] and 3-methylbutanal[c]
7	6	6	caramel, diacetyl, butter, cheese	2,3-butandione (diacetyl) [c]
6	2	6	fruit, pineapple, ethyl butanoate	ethyl butanoate[c]
4	4	4	sweetish, fruity, nasty, fermented	ethyl hexanoate[c] and (*Z*)-4-heptenal[d]
4	5	6	dirty socks, popcorn, sweetish, baked	2-acetyl-1-pyrroline[e]
6	7	7	fermented, onion, cheese-like	dimethyl trisulfide[c]
4	3	3	weak, hamburger, fermented	unknown[f]
4	2	4	flower, sweetish, fermented	unknown[f]
5	6	4	butter, dairy, earth, cucumber	(*E*)-2-octenal[c]
8	7	7	potato, cabbage**,** fermented	methional[c]
5	7	7	flower, hyacinth, cheese	phenyl acetaldehyde[c]
5	6	7	vitamin tablet, soup, cheese	2-methyl-3-(methyl dithio)furan[d]
4	6	2	fruity, gas, sourish	(*E,E*)-2,4-nonadienal[d]
5	6	4	coconut, dill, aromatic plant	δ-decalactone[c]
5	3	3	fruit, sweetish, coriander	δ-dodecalactone[c]
2	4	3	bad breath, cigarette smoke	unknown[f]

[a] Number of judges observing the odour. [b] Only odours observed in all of the three cheeses are included in the table, and only odours observed in one cheese by at least three panellists are reported. [c] Mass spectra, odour and Retention Index (RI) agreed with authentic standards. [d] No interpretable MS-signal; RI and odour properties agreed with authentic standards. [e] No interpretable MS-signal; RI and odour properties agreed with literature. [f] No interpretable MS-signal and no RI and odour properties agreed with literature.

Conclusion

In the three cheeses produced with *Lb. helveticus* as an adjunct, 16 key odours were observed by GC-O. These represented a broad range of different classes of compounds and half of these identified aroma compounds could be derived from amino acid catabolism.

Acknowledgment: We thank the Innovation Law, Danish Dairy Research Foundation, Arla Foods and Thise Dairy.

References

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Keywords: key aroma compounds, GC-Olfactometry, *Lactobacillus helveticus*

Properties of NSLAB from Estonian cheeses

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Background: Lactic acid bacteria are common habitats in dairy products. Dairy ecosystems have been developing in thousands of years giving us wonderful collection of different type of cheeses. On the other side modern technology provoke the loss of variability. The only way to keep biological processes under control is to study physiology of microorganisms and relationships between of microorganisms systematically. This work focused on the physiological properties of non-starter lactic acid bacteria (NSLAB) isolated from Estonian cheeses.

Materials & methods: The species of lactobacilli were identified using TaqI restriction analysis of amplified 16S-23S spacer region compared with the reference strains (Jacobsen et al., 1999). Strains of *Lactobacillus* were distinguished by PFGE as described by Jacobsen et al. (1999). Carbohydrate fermentation patterns were determined by using API 50 CHL system, in accordance with manufacturer's instructions (BioMérieux). The effect of water activity was studied by growing the strains (inoculum 2%) in MRS broth containing different concentrations of NaCl (0, 2, 4, and 6.5%) and glycerol (0, 10, 20, and 30%). Survival of the strains during heating (30 min. at 60ºC), acid (pH 2-3) and bile stress (bile salt concentrations up to 0.4%) were also studied. Two novel cultivation methods were used: D-stat (Kasemets et al., 2003) to study the effect of galactose limitation and pH-auxoaccelerostat (Drews et al., 1998) to study the effect of environmental conditions (temperature, pH and water activity (a_w)) on the growth of non-starter lactobacilli.

Results: Most of the isolates from the Estonian cheeses originated from 2 dairies belonged to species *L. paracasei*, while only few isolates of *L. curvatus* and *L. danicus* were found. However, the latter species was found to dominate in several Estonian cheeses. According to API 50 CHL strains of *Lb. paracasei* had the widest sugar fermentation abilities and highest growth rates (up to 0.57 h^{-1}) in MRS medium. On the contrary, *Lb. danicus* fermented only seven sugars and had low maximum growth rates (0.26-0.38 h^{-1}) and lower temperature optimum (< 30 ºC).

Results showed that NSLAB were well adapted to environmental conditions of ripening cheese. Biomass yield (Y_{ATP}) of *Lb. paracasei* E1H3 was not affected by smooth change of pH from 5.5 to 4.4 and temperature from 30ºC to 6ºC. Y_{ATP} decreased twice by smooth change of a_w from 0.99 to 0.95 and NaCl concentration from 0 to 6 g L^{-1}. *Lb. paracasei* was the most tolerant species to low water activities (growth down to a_w 0.89) and heating at 60ºC within 30 min. *Lb. curvatus* strains showed the highest growth rates under conditions characteristic to ripening cheese (6.5 % NaCl, a_w 0.95). *Lb. danicus* strains were acid and heat sensitive and did not grow at salt concentrations above 4% and water activities below 0.93.

The carbohydrate limitation similar to cheese environment was simulated in the D-stat experiments. Experiments with gradual decrease of galactose concentration in the high tryptone feeding medium

containing arginine showed that the biomass yield based on carbohydrate consumption ($Y_{X/HEX}$) increased with the decrease of galactose feeding. At the same time the gradual increase of acetate/lactate ratio was observed. We suppose that directing the pyruvate flow from lactate to acetate will improve the ATP and growth yield based on carbohydrate consumption. Some strains produced actively ornithine from arginine showing active arginine-deiminase (ADI) pathway.
Simple test to screen out probiotic potential of non-starter lactobacilli showed 46 to 77% survival of *L. paracasei* strains within 3 hours at pH 3. However, all isolates of *L. danicus* lost viability already during one hour incubation at pH 3. The numbers of *L. curvatus* SSR4 and SSR6 decreased from 10^9 to 10^5 - 10^6cfu ml^{-1} during the first hour and were very low (10 and 20 cfu ml^{-1}, respectively) after 3 hours incubation. Some *L. paracasei* strains (SSR5, EER1, E1R6, E1H3) were relatively resistant to bile salts, while others were quite sensitive. Sequential pH and bile stress (30 min at pH 3 followed by 0.4 % bile salt addition for 30 min) decreased viability of *Lb. paracasei* SSR5 by 1.5 log to 6 log (30 min at pH 3 followed by 0.2 % bile salts) that is comparable to similar experiments with known probiotic strain *Lb. acidophilus* La5.

Conclusions: The isolates from the Estonian cheeses studied belong to species *L. paracasei* (majority of isolates), *L. danicus* and *L. curvatus*. Their proportions and numbers are dynamically changing during ripening. Although the different species have different growth characteristics (specific growth rate, acidification ability, final cell number) they are well adapted to changing environmental parameters of ripening cheese (carbohydrate limitation, low temperatures and water activities). Some strains showed also good resistance to acid and bile indicating their possible survival in upper intestinal tract while ingested. The behaviour of selected strains of NSLAB and cheese populations will be studied in gastrointestinal tract model.

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Keywords: NSLAB, identification, physiology, cheese

Session A – poster

Identification of NSLAB from Finnish Emmental Cheese by PCR

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The number of non-starter lactic acid bacteria (NSLAB) increases during cheese ripening and these bacteria may constitute a dominant population in the mature cheese. The NSLAB diversity, their metabolism and interactions with starter bacteria have an effect on the ripening process of the cheese.

The diversity of NSLAB in a Finnish Emmental cheese was studied using species-specific PCR. Emmental cheeses were produced in Valio Ltd cheese factory from March to May. Seven cheeses were ripened for 6 months and the samples were aseptically taken from the centre of the cheese block. Both chemical and microbiological analyses were performed. Cheese homogenates were serially diluted and plated to MRS agar. Five bacterial isolates of each morphological group were chosen for further analysis, the total number of bacterial isolates was 189. Crude DNA extracts were used for species-specific PCR and PCR products were analyzed using agarose gel electrophoresis. Genomic DNA was isolated from cheese and qPCR-analyses were performed using species-specific primers. Growth of lactobacilli in one cheese was followed during the whole ripening period of 12 months.

Three *Lactobacillus* species were found in the Emmental cheeses studied. The NSLAB species were identified as *Lactobacillus rhamnosus*, *Lactobacillus casei* and *Lactobacillus fermentum*. The total number of lactobacilli in the cheeses ranged from 10^6 to 10^9 cfu g^{-1}. *Lactobacillus rhamnosus*, which included the adjunct starter, dominated in the cheeses. *Lactobacillus fermentum* represented the minority of NSLAB in all cheeses. The number of lactobacilli increased during the first 90 days of ripening. *Lactobacillus fermentum* was detected only after 24 days of ripening. All of the species studied were found in the mature cheese.

Although the Emmental cheeses were produced in the same production plant and within two months time the NSLAB varied in diversity, number and dominance.

Keywords: non-starter lactic acid bacteria (NSLAB), cheese ripening, PCR, qPCR

Session A – poster

Diversity of *Lactobacillus* in Swedish hard cheese

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Background

The microbiological composition of cheese changes during ripening. As the starter culture undergoes autolysis, a considerable amount of intracellular enzymes is released together with compounds that functions as nutrients for the NSLAB (Crow et al. 1995). This enhances the growth and activity of NSLAB, which is often found in high numbers in long ripened cheeses. Analysis of the microflora of ripened cheese has shown that it is most often dominated by *Lactobacillus* strains. The different strains can vary in their metabolism, which may be reflected in the flavour development of the cheese (Thage 2003; 2004a; 2004b; 2005). Diversity of the NSLAB strains in cheese may therefore be of importance for a satisfactory aroma development during ripening (Steele et al. 2006).

The aim of this work was to investigate the numbers and diversity of the NSLAB flora in a Swedish hard cheese during ripening and relate it to the overall sensory properties.

Materials & methods

Swedish hard cheese was obtained from the producer after 3, 12, 26, 48 and 56 weeks of ripening. Cheese samples were spread on De Man, Rogosa and Sharpe (MRS) agar, in which pH was adjusted to 6.5 and 5.4. MRS pH 5.4 plates were incubated at 37°C and separated *Lactobacillus* sp. from the total number of lactic acid bacteria, which grow on MRS pH 6.5 incubated at 30°C.

A number of 20 colonies were randomly selected from MRS pH 5.4/37°C plates from 26 and 56 weeks old cheeses. Repetitive sequence based PCR (rep-PCR) analysis was used on the isolates in order to investigate the number of different DNA profiles among the lactic acid bacteria in the cheeses (Versalovic et al. 1994). Enumeration and rep-PCR results were evaluated together with the results from the sensory analysis performed by the producer.

Results

All cheeses were dominated by starter bacteria until three weeks of age. After three weeks, the cell counts on MRS pH 6.5/30°C decreased towards the highest bacteria number on MRS pH 5.4/37°C, which indicated autolysis of the starter bacteria. The total amount of lactic acid bacteria reached maximum numbers of 10^7-10^8 CFU/g cheese after three weeks. The counts on MRS pH 5.4/37°C reached a maximum of approximately 10^6 CFU/ g cheese after 26 weeks.

The results of rep-PCR on the isolates from MRS pH 5.4/37°C demonstrated that all cheeses were dominated by *Lactobacillus* species after 26 weeks of ripening. A variation in rep-PCR profiles and number of profiles varied among the samples. None of the same profiles were found in more than one cheese. However, in each of the cheeses, the microflora had a tendency to be dominated by one *Lactobacillus* profile.

For some of the cheeses, the dominating profile occurred both after 26 and 56 weeks of ripening, but the diversity of profiles increased with ripening time. For one of the cheeses, the bacteria number was relatively low all through the ripening period. This cheese also had a weak aroma development, which is in accordance with earlier studies of numbers of NSLAB versus aroma development during ripening (Antonsson et al. 2001). This cheese also had a lower number of different profiles, which further suggest a relationship between low strain diversity and aroma development during ripening.

Conclusions

The microflora of this Swedish hard cheese was dominated by NSLAB after three weeks of ripening. The rep-PCR on cheese isolates demonstrated that the NSLAB flora was dominated by *Lactobacillus*. The diversity of different profiles varied among the cheeses and the results indicated a correlation between high diversity and strong aroma development. Furthermore, the diversity of different rep-PCR profiles increased between 26 and 56 weeks of ripening.

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Keywords: NSLAB, rep-PCR, *Lactobacillus*, cheese ripening

Session A – poster

Identification of aminopeptidases in *Lactobacillus paracasei*

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Background

Lactobacillus paracasei strains isolated from cheese belong to the group of non starter lactic acid bacteria (NSLAB), and may contribute to cheese ripening and flavour formation through amino acid release and amino acid conversion (Ardö, 2006, Thage *et al.*, 2005). The objective of this study was to investigate the complexity and specificity of the aminopeptidases in a *Lb. paracasei* strain using specific artificial substrates and kinetic studies of enzymes in crude cell-free extract (CFE) and anion exchange chromatography fractions.

Materials and methods

A crude CFE of a *Lb. paracasei* strain isolated from cheese was analysed for specific aminopeptidase activity using eight chromophore substrates of amino acid para-nitroanilides (*p*NA). The selected substrates included Leu-*p*NA, Val-*p*NA, Lys-*p*NA, Glu-*p*NA, Phe-*p*NA), Pro-*p*NA, Gly-*p*NA, and Gly-Pro-*p*NA. Determination of specificity was made from results of the activity measured after 1 and after 24 h using five repetitions.

Enzymes of the crude CFE were separated by fast anion exchange chromatography (AIE-FPLC). The fractions were collected and screened for aminopeptidase activity using the substrates, for which the crude CFE showed specific activity. A few interesting fractions holding specific activity were selected to be investigated further by kinetic measurements.

Results

The crude CFE got specific aminopeptidase activity for substrates with branched-chain, basic and prolyl amino acids as well as the X-prolyl dipeptide (Table 1). No specific activity was obtained on Gly-*p*NA or Glu-*p*NA substrates and only very low on Phe-*p*NA, which were only analysed in the crude extract.

Table 1. Specific activity and kinetic properties of identified aminopeptidase enzymes in a *Lb. paracasei* strain. Label A-F is identified in Fig. 1.

Enzyme preparation; Lable	Specificity from highest to lowest activity	K_M-value, mM Leu-AP	K_M-value, mM X-propyl-AP
Crude extract	Leu, Lys, Gly-Pro, Val, Pro, Phe	0.30	0.29
F 3; Void	Leu/Lys, Val/Gly-Pro, Pro	NI	NI
F 8; A	Gly-Pro, Leu, Val, Lys, Pro	0.15	NI
F 22/23; B	Gly-Pro	NI	0.24
F 24; C	Lys, Leu	0.24	NI
F 25; C+D	Lys/Leu	0.11	NI
F 28; D	Leu, Val, Pro (Lys, no peak)	0.04	NI
F 29; E	Lys, Leu (shoulder)	0.20	NI
F 33; F	Lys	NI	NI

F, FPLC fraction; NI, not investigated

FPLC peak fractions with maximum peptiolytic activities for the selected substrates were labelled as enzymes A, B, C, D, E and F (Fig. 1). Their specificity for different substrates differed (Table 1). However, at least four of them as well as the void fraction got activity on Leu-*p*NA, and those fractions as well as the crude extract were selected for kinetic examinations. Lineweaver-Burk estimation of K_M-values confirmed the presence of four different peptidase enzymes in F8, F25, F28 and F29, respectively, with ability to degrade Leu-*p*NA. The most active aminopeptidase that degraded branched-chain amino acids with the lowest K_M-value for leucine degradation was found in fraction 28 (Enzyme D). Interestingly, the K_M-value of the crude extract was higher than of all fractions degrading Leu-*p*NA, which indicate the presence of inhibitory substances in the crude extract (Table 1). The second most actively degraded substrate was Gly-Pro-*p*NA, which got the same K_M in fraction 22/23 (Enzyme B) as in the crude CFE. Enzymes C, E and F got their highest activity against the basic substrate (Lys-*p*NA). In totally 10 strains of *Lb. paracasei* Enzyme C and D were found in all, B and E in 6, and five other enzymes were only found in one of the strains, respectively (data not shown).

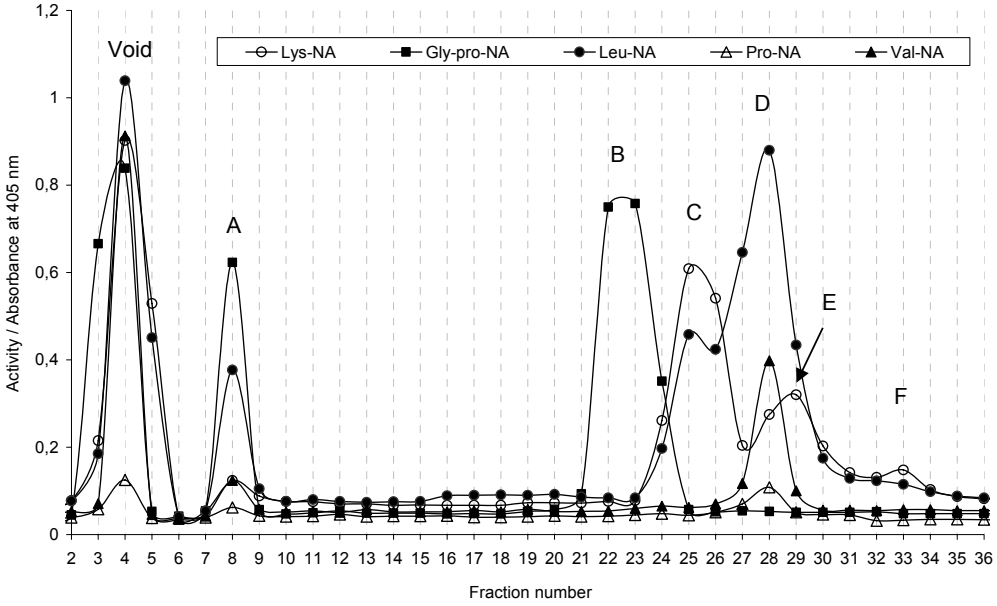

Figure 1. Specific aminopeptidase activities measured after 24 hours incubation in the fractions of anion exchange chromatography (AIE-FPLC) fractions of CFE of *Lb. paracasei*. Labels A-F represents activity peaks.

Conclusion

The results indicate the presence of at least six different aminopeptidases in the *Lb. paracasei* strain. The specificity for Leu-*p*NA was found in four different enzymes of which one enzyme got its main activity against it. Of the other enzymes was one highly specific for Gly-Pro-pNA, and for three of them, Lys-*p*NA was the preferred substrate. By using these three kinds of aminopeptidases, the *Lb. paracasei* strain is highly useful to degrade casein peptides during cheese ripening.

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Keywords: *Lb. paracasei* subsp. *paracasei*, aminopeptidase activity, enzyme kinetics

Session B – Key note speaker

Lactobacillus as Cheese Ripening Cultures

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Cheese ripening consists of a complex series of chemical and biochemical reactions that contribute to texture and flavour development. Many of these reactions are the result of microbial activity within the ripening cheese. The microbial flora of most cheese varieties is complex and consists of starter and secondary flora. Lactobacilli are key members of the microbial flora of all ripened cheeses and can be present either as part of the starter or secondary flora. Lactobacilli form a genetically diverse group of organisms and can be isolated from a wide range of environments. They are traditionally divided into 3 groups on the basis of being either (I) obligatory homofermentative, (II) facultatively heterofermentative, or (III) obligatory heterofermentative. The lactobacilli used as cheese starter cultures are generally members of Group I while the secondary flora of cheese contains primarily Group II lactobacilli. Both starter and non-starter lactobacilli are associated with cheese manufactured in the Nordic region.

Acid production during cheese manufacture is the primary function of starter cultures; however, as they form the primary biomass, in particular in young cheese, they contribute significantly to the ripening process. *Lactobacillus delbrueckii* subsp. *lactis, Lb. delbrueckii* subsp. *bulgaricus* and *Lb. helveticus* are all used as starter cultures and are particularly associated with Swiss- and Italian- type cheeses that are heated to high temperatures during the manufacturing process. Selection of starter cultures for flavour improvement is an important target in cheese research. Thus, the capacity of strains to impact on proteolysis, amino acid catabolism and lipolysis are key criteria for consideration in strain selection. However, wide differences in the expression of proteinases, peptidases, amino acid converting enzymes, lipases and esterases exist between starter strains, as well as their autolytic abilities. The autolytic behaviour of *Lb. helveticus* strains in Cheddar cheese was recently studied and considerable strain to strain variation was observed. While there appears to be some correlation between levels of autolysis and cheese flavour, it was noted that cheese manufactured with the most autolytic strain did not receive the highest flavour scores, suggesting that other factors such as enzyme activity also contribute to the ripening process. Genomic approaches to study such activity will greatly accelerate progress in this area. A number of genome sequencing projects on starter lactobacilli are recently completed or ongoing. The sequence of a highly autolytic cheese strain, *Lb. helveticus* DPC4571, was recently completed by our group. Analysis of the 2.08 Mb genome has indicated a close genetic relationship between this dairy culture and members of the acidophilus complex that are natural inhabitants of the gastrointestinal tract. However, it appears that DPC4571 has acquired genes involved in fatty acid and amino acid metabolism that may represent adaptation to the dairy niche while loosing a number of genes reported to be important for survival in the gastrointestinal tract.

Non-starter lactobacilli are not required to contribute to acid production during manufacture but grow in the cheese during ripening. Many species of non-starter lactobacilli have been isolated from cheese, but those most frequently encountered are *Lb. casei*/*Lb. paracasei*, *Lb. plantarum*, *Lb. rhamnosus*, and *Lb. curvatus*. The non-starter lactobacilli are adventitious micro-organism gaining entry to the cheese either from the milk or the processing plant during manufacture. They grow from low numbers in young cheese to represent a major portion of the biomass of many mature cheese varieties and thus are

likely to contribute to the ripening process. Studies on a range of cheeses, including Cheddar and Herrgård have demonstrated that the composition of the non-starter lactobacillus population changes during ripening and no strong evidence of a factory specific flora has emerged. Such variation in the non-starter lactobacillus population may contribute to batch to batch variations often noted in cheese flavour. Many studies have reported attempts to elucidate the role of non-starter lactobacilli on cheese flavour development either by attempting to control the growth of adventitious non-starter lactobacilli or through the addition of strains as starter adjunct. Techniques to control the growth of non-starter lactobacilli have included the manufacture of cheese under controlled microbiological conditions, addition of antibiotics or bacteriocins, or controlling the ripening temperature. None of these have been fully successful and this has made interpretation of the resulting data difficult. The effect of adding adjunct cultures of non-starter lactobacilli are equivocal with some studies showing positive effects while others report negative effects on flavour formation. The reason for the equivocal nature of the finding probably results from the flavouring potential of the isolates selected combined with growth of adventitious strains during ripening. However, most of the more recent studies on this topic have indicated a positive effect of adjunct strains on flavour. Complete genome sequences are now available for a number of species of non-starter lactobacilli. Application of this information will enable a more structured approach to strain selection and will likely lead to a clearer understanding of the role of these organisms in cheese ripening and the selection of strains capable of positively impacting on cheese quality.

Session B - oral

Functional properties of non-starter lactic acid bacteria related to cheese

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Background

The direct addition of selected starters to raw materials has been a breakthrough in the processing of fermented foods, resulting in a high degree of control over the fermentation process and standardization of the end product. However, the selection of commercial starters is based on rapid acidification and phage resistance and is not very flexible with regard to the desired properties and functionality of the end product. The biodiversity of commercial starters has therefore become limited. Moreover, many products obtain their typical flavour intensity from the NSLAB flora developing in the product through maturation. A collection of NSLAB has recently been isolated from commercial Norvegia cheeses (Østlie et al., 2004). All these strains are characterized by phenotypic and molecular based identification methods, however, further characterization of functional properties related to cheese is needed. This presentation will focus on properties of selected strains from the isolated NSLAB cheese flora to apply them as functional adjunct cultures in cheese production and development. The present work aimed to test properties such as autolytic properties, antimicrobial activity, probiotic properties and growth and metabolism in milk.

Materials & methods

The 16 different selected strains used were isolated from Norvegia cheese after 90, 180 and 270 days of ripening Growth and autolysis were followed during a 10 week period. Bacterial growth and autolysis were determined in MRS by absorbance measurements at 600 nm (Abs_{600}). Growth and metabolism were done in reconstituted skim milk (10 %) inoculated with 1% (v/v) of a washed stationary phase culture. The inoculated milk was incubated at 30°C for 0 to 7 days. The viable microbial counts were determined in the incubated milk after 0, 24, 48, and 96 h and pH, volatile compounds, organic acids and bacteriocin activity after 0, 24, 48, 96 and 168 h incubation. Volatile compounds were analysed by headspace gas chromatography and organic acids by high pressure liquid chromatography (HPLC). Screening for antimicrobial activity against different lactic acid bacteria, *Clostridium* sp. and *Listeria* sp. was done according to Faye et al. (2000). The acid resistance was examined in MRS broth adjusted with hydrochloric acid (HCl) to obtain a final pH of 2.0 and 3.0 and the bile tolerance in MRS broth containing 0.3 % (w/v) bile salts (Sigma), both experiments using overnight culture (1%) as inoculum. Resistance of bile salts was assessed in terms of viable colony counts, enumerated after incubation for 0, 3 and 24 h and resistance of acid was assessed after incubation for 0 and 3 h

Results

Growth and autolysis of 16 strains of NSLAB were measured in MRS broth. Variations in growth occurred among the strains, however, maximal autolysis was low and little variations were observed among strains. Maximal absorbance in MRS broth of the strains varied from 8.3 to 12.4 and was reached after 1 to 6 days. Maximal autolysis of the strains varied from 0 to 16%.

Preliminary results from the testing of probiotic properties showed strain variations. All the strains survived incubation at pH 3 for 3 h, showing about equal cell numbers during the incubation period

However, after incubation at pH 2.0 for 3 h a marked reduction in viability of the cells was seen. Regarding bile salts, most of the strains were resistant to 0.3 % bile after both 3 and 24 h incubation. The growth and metabolism experiment performed in milk showed also strain variations. The strains reduced pH from 6.6 to between 4.35 and 6.15 after 24 h incubation. Most of the strains attained maximum viable cell numbers after 48 h incubation ranging between 7.9-9.14 log cfu/ml. All the strains showed the same level of viable cells during the 96 h incubation period. The amount of lactate produced varied from 2357 to 14919 mg/kg, and *Lactobacillus plantarum* INF-15 D produced the lowest amount. Fourteen of the strains produced lactate in the range 10000-15000 mg/kg. All the strains continued lactate production during the fermentation period, except one. The amount of acetate produced varied between 146-705 mg/kg. *Lactobacillus plantarum* 15 D started to produce acetate after 96 h incubation. The initial level of citric acid in the milk was around 2000 mg/kg. Most of the strains metabolized small amounts of citrate (2-15%), however, variations were observed among the strains. After 168 h incubation, the *Lb. paracasei* INF-450 strain metabolized 18% citrate, the *Lb. paracasei* INF-1055 strain metabolized 39.8% and the *Lb. paracasei* INF-460 strain metabolized 64%.

Strains of the NSLAB flora unique at the different cheese plants may offer an important base for cheese production and innovation.

Keywords: Adjuncts, non-starter lactic acid bacteria, cheese, autolysis, probiotic properties, antimicrobial activity

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Session B - oral

Milk thermal treatment: influence on the growth of lactic starters

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Introduction

Microorganisms are essential for manufacture and ripening of cheeses. Today commercially produced starter cultures are usually added to the vat milk and their initial numbers are easy to control.
However, the growth in milk and survival of starter bacteria in cheese is also affected by milk pretreatment, which influences the association of proteins and activity of milk enzymes, especially plasmin. It is important that the fermentation in the vat commences rapidly and produces sufficient amount of bacterial cells before gel formation, as the growth in cheese is depending on the starter counts at the beginning of gel formation.

Calorimetric measurements allow to draw up energy balances for the running chemical reactions during the growth process. The slopes of the power-time and heat-time curves permit conclusions about the growth rate, the occurrence of limiting conditions and they give hints about the formation of metabolites *(Winkelmann et al., 2003)*. Thus the calorimetric measurement is clearly a key factor in understanding thermodynamics of life processes. The on-line measured heat signals could also be used together with other on-line data for bioprocess monitoring, optimization and control *(Liu et al., 1999)*.

Calorimetry has the advantages of being unspecific, non-invasive and insensitive to the electrochemical and optical properties of the investigated system. The activity of the living cells within a microbial culture is characterized by the produced thermal power and therefore measurable with different calorimetric methods *(Winkelmann et al., 2003)*.

Batch microcalorimetry was chosen to monitor the influence of environmental composition on cell growth. Thermogenic curves obtained from microcalorimeter contained a lot of information shown and analyzed in current investigation.

Materials and Methods

Inoculum preparation

A frozen *Streptococcus thermophilus* culture was thawed and pre-cultured successively on Petri dishes with M-17 Agar (LAB M) medium for 24 h at 40^{o}C. One colony from the pre-cultured Petri dish was taken and placed into 10 mL sterilized reconstituted skim milk (Kalev Paide Tootmine AS, Paide, Estonia) at 40°C and left till coagulation. Skim milk powder was reconstituted with thorough mixing for 1 h in distilled water to yield a final concentration of 10% (w/v) milk solids.

A frozen *Lactobacillus paracasei* S1R1 culture isolated from Estonian cheese (Kask et al., 2003) was thawed and pre-cultured successively on Petri dishes with MRS Agar (LAB M) medium for 24 h at 35^{o}C. One colony from the pre-cultured Petri dish was taken and placed into 10 mL sterilized MRS Broth (LAB M) medium at 35°C and pre-grown for 14-16 h to reach mid exponential phase. For both strains the inoculum was prepared by dilution of the pre-grown culture in peptone water (NaCl, 8.5 g L^{-1}; Bacteriological peptone (LAB M), 1 g L^{-1}).

Sample preparation and growth condition
Milk samples were obtained from commercial dairies:
(a) sterilized reconstituted skim milk; (b) pasteurized at 78^{o}C for 15 s commercial milk (Tere) with 2.5% fat content (Tere AS, Tallinn, Estonia); (c) commercial UHT treated milk (Milla) with 3.5% fat content (Kalev Paide Tootmine AS, Paide, Estonia); (d) commercial UHT treated milk (Milla) with 0.05% fat content commercial milk (Kalev Paide Tootmine AS, Paide, Estonia). 1% (v/v) of starter culture was added into each sample. Three ampoules were filled per sample.

Experimental measurements
Batch experiments were run at 35°C in 3 mL ampoules. Samples were placed into the TAM III Thermal Activity Monitor (Thermometric, Järfälla, Sweden), which was electrically calibrated before the experiment initiation and data were baseline corrected.

Results
Heat production and microbial development was followed in isothermal batch microcalorimeter. Power-time curves of *Streptococcus thermophilus* and *Lactobacillus paracasei* S1R1 strains in various milk samples were registered. The power-time curves of 3 replicates fit well. Total amount of heat evolved during the bacterial growth, lag-phase duration and maximal growth rate of used strains in various milk samples were measured by integration of this power-time curve.

Our experiments showed that the fastest bacterial growth was observed in the commercial milk (Tere) with 2.5% fat content. The shortest lag-phase duration, highest maximal growth rate and total amount of heat evolved during bacterial growth was observed in this sample. The longest lag-phase duration was in commercial UHT milk (Milla) with 3.5% fat content. Maximal growth rate and total amount of heat evolved during bacterial growth in other milk samples was almost equal. Lag-phase duration depends on the used milk sample.

Conclusions
Some relationship between experimentally measured parameters and various milk samples was found. Data demonstrated that isothermal microcalorimetry was a useful technique to monitor and analyze microbial growth in milk.

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Keywords: Milk; Microbial growth; Isothermal microcalorimetry

Session C – Key note Speaker

Improving Bioactivity of Cheese

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During recent years, consumers have become increasingly concerned not only about safety, quality and nutritional value of foods, but also about the relationship between food and health. The recognition that certain food products provide health benefits beyond inherent basic nutrition, based upon emerging clinical evidences, has boosted the consumption of the so-called functional foods. The functional food market is large and in a phase of rapid growth, especially in USA, Europe and Asia.

Milk is a major provider of important nutrients, such as calcium, protein and riboflavin, but it has often been postulated that the consumption of dairy products, including cheese, is associated with a high risk of coronary heart disease, mainly because of their content in saturated fats. Therefore, while some existing and traditional dairy products, such as milk and yogurt are considered functional foods, in that they impart health benefits, cheese is not. However, study results have been conflicting, with a somewhat surprising beneficial association between the intake of dairy products and the metabolic syndrome. Unlike milk and butter, cheese does not seem to increase plasma cholesterol, moreover, the use of specific bacterial strains for fermentation has been shown to offer rather moderate cholesterol-reducing properties. There is also good evidence that certain microorganisms, particularly *Lactobacillus helveticus*, provide a mildly decreasing effect on blood pressure, probably through the production of bioactive peptides. Cheese is, therefore, a good candidate for new processing practices to improve its positive impact on heath. Taken as a whole, cheese has a positive nutritional image and, in many parts of the world, cheeses are frequently consumed at least once a day, making cheese an excellent carrier for bioactive compounds because of the high daily consumption.

Thus, cheese is a good matrix for the consumption of probiotics, defined as “living micro-organisms, which upon ingestion in certain numbers, exert health benefits beyond inherent basic nutrition”. Most commonly they have been lactobacilli, such as *Lactobacillus acidophilus* and bifidobacteria. These bacteria beneficially affect human health by improving the balance of intestinal microflora and improving mucosal defences against pathogens. Fermented milk drinks and yogurts are the most popular and optimised food carriers for probiotic bacteria. However, there are numerous challenges related to the instability of some intestinal strains of probiotic bacteria in fermented milk products and cheese has been suggested as an alternate vehicle to deliver probiotic bacteria in sufficient numbers as compared with fermented milk. The oxygen level, resulting in an almost anaerobic environment, high pH and buffering capacity, the high fat content and the solid matrix of cheese may protect bacteria more efficiently than a fluid environment, not only during the storage of the food, and but also during its passage through the gastrointestinal tract. However, in contrast to the short shelf life of probiotic fermented milks and yogurts, hard cheeses have long ripening periods that compromise the viability of probiotic strains until consumption.
The successful incorporation of probiotic bacteria into cheeses requires that the bacteria remain viable throughout the shelf-life of the product and do not adversely affect the composition, flavour, texture, and other sensory characteristics of the traditional cheese. This requires some technological changes

both in the selection of the appropriate species and strains and in the modification of the cheese processing to promote a suitable environment. If the probiotic cheese can be manufactured with little or no alteration of the traditional cheese making technology, this would make the development of probiotic cheeses attractive for commercial production.

The term conjugated linoleic acid (CLA) refers to a collection of 28 positional and geometrical isomers of octadecadienoic acid. CLA is found in the milk fat and so it is present naturally in dairy products, such as cheese. The different CLA isomers exhibit different biological effects, such as reduction of body weight and fat mass, decreased glucose levels, antiatherogenic effects, inhibition of carcinogenesis and even modification of the immune system and bone metabolism. However, to sustain many of these properties only animal studies and often contradictory results are available, and it remains to be established whether CLA may exhibit beneficial effects in humans, as well as the related mechanisms. Studies have demonstrated that increasing the CLA content in the human diet increases its content in human milk, plasma and cellular lipids. In turn, the CLA content of the milk can be increased up to eight fold simply by dietary manipulation of the lactating ruminant. High values often occur with the feeding of fresh pasture. Similarly, different dietary oil treatments, mainly using plant or fish oils, have been shown to exert different degrees of enrichment of milk fat with CLA. Possibilities to increase the CLA content of dairy products with microbial cultures which are able to convert linoleic acid to CLA have been identified. Such strains have the potential to enhance CLA levels in dairy products during fermentation or contribute to CLA production *in situ* in the intestine after ingestion.

A modified fatty acid profile can influence several of the physical and chemical properties of dairy products, as it is well known that a high content of unsaturated fatty acids increases the risk of oxidation and off-flavours. However, most studies on modified milk do not indicate any adverse result on the sensory properties. Processing of milk to cheese appears to have no effect on the final content of CLA in cheeses and no changes have been found in the total content or isomer profile of CLA during ripening. On the other hand, certain properties of cheeses produced from CLA-enriched milk have been shown to differ from control cheeses. CLA-enriched milk has a reduced fat globule and casein micelle size, as well as an altered protein distribution of the casein micelles, which may affect its cheese making properties leading, for instance, to cheeses softer than normal. However, there are several studies that report no changes in the organoleptic characteristics of cheeses made from CLA-enriched milk.

In recent years it has been recognized that dietary proteins provide a rich source of biologically active peptides. Such peptides are inactive within the sequence of the parent protein and can be released by hydrolysis. Upon oral administration, bioactive peptides may affect the major body systems, namely, the cardiovascular, digestive, immune and nervous systems. Today, milk proteins are considered the most important source of bioactive peptides and an increasing number of sequences (with various bioactivities *in vitro*, e.g. mineral-carrying, antimicrobial, antihypertensive and immunostimulatory) are being identified in cheese, as arising from the concerted proteolytic action of the starter and non-starter bacteria and the other enzymes present. Thus, the occurrence of bioactive peptides in cheese appears to be dependent on the ripening stage and the equilibrium between their formation and the degradation by the proteolytic systems involved in the maturation process. On the other hand, *in vitro* experiments have demonstrated that, apart from the generation during ripening, more bioactive peptides are likely to be formed in the gastrointestinal tract upon ingestion of a piece of cheese.

Keywords: cheese, bioactivity, probiotics, CLA, bioactive peptides

Session C: Oral presentation

The influence of spice ethanol extracts on the lactic acid microbial flora of cheese during ripening

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Background and objective

Traditionally spices have been used to add flavour to fresh and ripened cheeses, and the addition of new spice varieties to cheese attracts increased interest among cheese producers and consumers. Many spices are known to have antimicrobial effects and some studies have been reported on antimicrobial properties of different species and their extracts including black pepper, clove, coriander, caraway and cumin (Nasar-Abbas & Halkman, 2004). Possible effects of the spices added to the cheese curd on cheese ripening may be associated with the presence of microorganisms in the spices and their potential antimicrobial effect on the flora of starter lactic acid bacteria and non starter lactic acid bacteria (NSLAB) in cheese. However, the knowledge on how spices used in cheese, influence the microflora of lactic acid bacteria in is limited.

The objective of this study was to examine the influence of some spices used in cheese on the growth and survival of lactic acid bacteria. The microbial population of a variety of cheeses from Lithuania with caraway as well as the Norwegian cheese Nøkkelost produced with clove and cumin were characterised. The isolates of lactic acid bacteria from these cheeses were isolated and the strain identified by API identification.

Materials and methods

The populations of lactic acid bacteria (LAB) in five different Lithuanian cheeses and Nøkkelost cheese from Norway were estimated by traditional agar plating techniques, and initial identification of LAB isolates was performed by API 50CH tests.

The antibacterial activities of clove (*Eugenia caryophyllata*), caraway (*Carum carvi*), coriander (*Coriandrum sativum*), cumin (*Cuminum cyminum*) and black pepper (*Piper niger*) ethanol extracts were determined by a micro dilution broth method using microtiter plates. The minimal inhibitory concentration (MIC) was calculated by determining the concentration of extracts that suppressed bacteria growth as measured by optical density of the microtiter plate wells (Holo, Nilssen, & Nes, 1991). A microtiter plate assay was also made with some selected strains from the strain collection at the Norwegian University of Life Sciences; *Lb. parcasei* INF 448*, Lb. plantarum* INF15D, *Lb. casei* ATCC 393, *Lc. cremoris* P2*, Lc. lactis* L2.

Results

The minimum inhibitory concentration of ethanol extracts ranged between 5.83 and 85.71 mg ml^{-1}. Among the selected strains from the University collection, the *Lactococcus* starter strains were more inhibited by the ethanol extracts than the *Lactobacillus* strains, with clove being the most inhibitive extract.

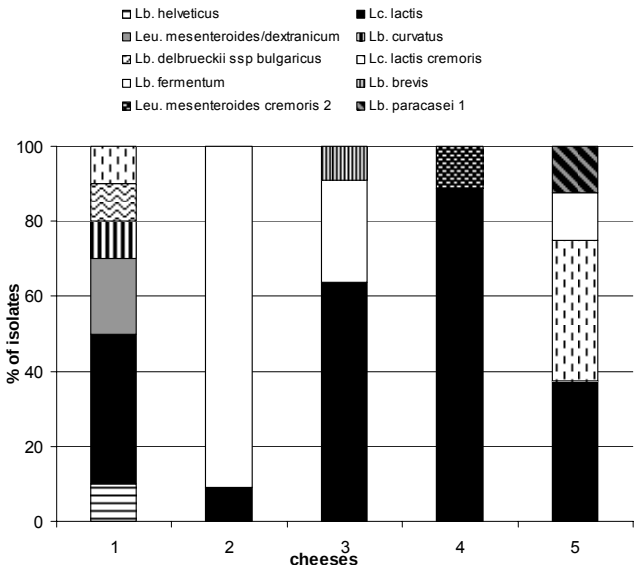

Figure 1. Strains of lactic acid bacteria isolated from different types of Lithuanian cheeses: 1. Tilziukas with caraway; 2. Massdam; 3. Semi – hard Dvaro; 4. Kelmes varskes (fresh curd); 5. Dziovintas Dvaro (dried curd).

The strains isolated from the Lithuanian and Norwegian cheeses were tested by the same microtiterplate assay. Both mesophilic and thermophilic species were found in all Lithuanian cheeses. Most of the strains remained from the starter, but also the NSLAB's *Lb. fermentum* 1, *Lb. brevis* 3 and *Lb. curvatus* were found in all tested cheeses (Fig. 1).

The MIC of the strains showed a variable degree of antimicrobial activity and no clear pattern could be found between the species and strains isolated from Lithuanian cheeses in terms of their inhibition of the extracts however the clove and cumin extracts were more inhibitive than the caraway, black pepper and coriander extracts.

Different strains isolated from Nøkkelost cheese with clove and cumin was analyzed after 1 and 2 months of ripening. In 1 month old Nøkkelost mainly starter strains were found, however *Lb. plantarum* which is rarely found in cheese, was found in all the investigated cheeses. Obviously *Lb. plantarum* is a "house" strain at this dairy or it may originate from the spices. *Lb*. *paracaseii* ssp. *paracaseii* was only found in one cheese after 1 month of ripening.

After 2 months of ripening, the flora was still mostly dominated by the starter strains *Lc. lactis* ssp. *lactis 2* and *Leuc. mesenteroides* ssp. *messenteroides/dextranicum 2*, while *Lb. plantarum* was not found in the cheese after 2 months of ripening. *Lb. paracasei* ssp. *paracasei 3* and *Lb. curvatus* was found in one cheese each. The MIC of the spice ethanol extracts that suppressed the growth of the isolated lactic acid bacteria ranged between 11.58 and 44.78mg ml^{-1}

Conclusion

The microbial population of the Lithuanian cheeses varied with various species originating both from the starter and from NSLAB. No clear pattern could be found due to the addition of caraway to those cheeses, however in the ripened cheeses with caraway a broader variety of species was found, most likely due to the starter composition. During ripening of Nøkkelost the composition of the microbial flora altered from mainly starter strains at 1 month to more NSLAB species after 2 and 3 months of ripening.

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Keywords: lactic acid bacteria, cheese, spice extracts, antimicrobial effect

Session C – Oral presentation

The effect of hydrogen peroxide on the growth characteristics of thermophilic lactic starters and rheological properties of acid milk gels

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Physical properties of milk protein gels are important quality attributes of fermented milk products, including cheese. The starter growth is one important parameter to control the texture formation. Thermophilic starters are known to be very sensitive to inhibitory substances. In the present work the effect of strong oxidant, hydrogen peroxide, which is routinely used as sanitizer in dairy industry, on the growth characteristics of thermophilic lactic acid bacteria was studied. The dynamics of growth and biochemical activity of starters will reflect an array of stress reactions in response to the changing environmental conditions due to oxidizing effect of hydrogen peroxide.
Dynamic rheology tests are useful to study structure development of acid milk gels. The onset of gel formation could relate to lag phase of starter growth.

Materials and Methods

Milk and starters
Commercial UHT milk (fat content 0.05% or 3.5%) trade name Milla, produced in Kalev Paide Tootmine AS, Paide, Estonia was used in all experiments. Liquid bulk starters (Chr. Hansen XY-11) were purchased from small dairy Nõmmiku Talu, Estonia.

Hydrogen peroxide addition, sample pretreatment
H_2O_2 solutions were prepared from stock 30% solution (Riedel-de Haën, Seelze, Germany), and 75 µl of appropriate dilutions were added to 50 ml of UHT milk to yield final concentrations of 0, 10, 25, 50, 75, 100, 150, 200 and 250 mg L^{-1}. Then, milk samples were left at 40°C for at least 1 h before starter addition (1%).

Dynamic rheological measurements
The rheological measurements were performed using a Physica MCR 301 (Anton Paar GmbH, Germany) with the direct strain oscillation (DSO) option, the Peltier temperature control unit C-PTD200 and coaxial cylinder measuring system CC27 (outer and inner ∅ 28.92 and 26.66 mm). After the addition of starter (1%) at 40°C, sample was stirred and then an appropriate volume was transferred to the measuring system. Gelation assays were performed at 40°C and lasted for 18 or 24 h for samples with H_2O_2 concentrations of 0, 10, 25, 50 and 75, 100, 200, 250 mg L^{-1} respectively. Samples were oscillated at a frequency of 0.1 Hz and the strain applied was 0.01, which was within the linear viscoelastic region. Samples were tested every 10 minutes after starter inoculation during the complete experimental run. To prevent evaporation, vegetable oil was added to the surface of milk sample. Measurements were replicated on three individual milk samples. The storage modulus (G'), loss modulus (G'') and loss tangent (tan δ) were determined. Gelation time (t_{gel}) was arbitrary defined as the

time necessary for the rheometer response (torque) to reach the value of 0.01 μNm, considered to be the first reliable increase in viscosity that exceeded the signal noise obtained when the milk started to gel. G' curves were smoothed with 1% relative smoothing range and differentiation against time (dG'/dt) was performed with RHEOPLUS/32 V2.66 software (Anton Paar GmbH, Germany). A gelation rate parameter was obtained from the maximum rate of increase in G' over time, $(dG'/dt)_{max}$.

Statistic analysis

All experimental results were submitted to single-factor analysis of variance (ANOVA), and the differences among means were determined by Fisher's least significant difference test. Paired Student's *t* tests were run to compare the rheological properties of different fat content milks. Differences were considered significant when *p* values were less than 0.05.

Results

Dynamic low amplitude oscillatory measurements showed that there was a significant prolongation in gel formation, t_{gel}, with increasing H_2O_2 concentration for both UHT milks. Samples with higher fat level seemed to have somewhat longer gelation times. The lowest observed H_2O_2 concentration (10 mg L^{-1}) already prolonged the lag phase of gel formation at least 1 h; at 100 mg L^{-1} H_2O_2 concentration gelation time reached roughly 17 h, therefore extending the onset of gel formation fourfold compared to control samples ($t_{gel} \approx$ 4 h). With ≥150 mg L^{-1} H_2O_2 the onset of gelation was not observed during 24 hours.

For Milla 3.5% G' increased faster during the initial stage of gelation in control samples and with stable decreasing rate for H_2O_2 concentrations of 10-75 mg L^{-1}, being slowest with 75 mg L^{-1} H_2O_2; then, the gelation rate increased again with 100 mg L^{-1} H_2O_2. The effect of hydrogen peroxide on gelation rate of Milla 0.05% samples was not statistically significant.

Dependence of loss tangent (tan δ) values 6 h after onset of gelation of Milla 3.5% and Milla 0.05% gels on H_2O_2 concentration showed clear differences between high fat and low fat milk samples. However, both milks (skimmed and 3.5% UHT) exhibited an increase in tan δ up to 75 mg L^{-1} H_2O_2 content, and then at concentration of 100 mg L^{-1} diminished up to the loss tangent values in control gels.

Conclusions

Hydrogen peroxide addition to milk one hour before starter inoculation showed the significant influence on the onset of milk gelation and affected rheological characteristics of resulting gels. Assuming that the majority of hydrogen peroxide should have been reacted with milk biomolecules by that time, it could be concluded that starter growth would be affected by the oxidative changes in substrates or growth factors.

Keywords: Hydrogen peroxide; starter growth; viscoelasticity, milk gels

Session C - poster

Phosphopeptides in low-fat, semi-hard cheese with *Lactobacillus helveticus*

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Background

A high content of organic bound phosphate associated with calcium in cheese has a beneficial impact on human health. The phosphopeptides are released during cheese ripening from casein that contains several sequences with repeated residues of serine phosphate. The content of phosphopeptides in cheese depends on the variety; *e.g.* 65 phosphopeptides with the serine-P groups of casein intact have been identified in ten months old semi-hard Herrgård cheese made with mesophilic starter, while only few phosphopeptides have been found in two years old cheese made with thermophilic starter (Ferranti et al., 1997; Lund & Ardö, 2004). The organic bound P was 84 % in the ripened Herrgård cheese and only 20 % in the high cooked cheese, which suggests that organic bound phosphate groups were released mainly in the latter. Cultures of the thermophilic *Lactobacillus helveticus* are used to improve flavour and texture of semi-hard low-fat cheese, and the objective of this study was to investigate influence on the composition of phosphopeptides.

Materials and methods

Semi-hard (61 % moisture), low-fat (15 % fat), round-eyed cheese (8 Kg low, square formed, 1.7 % NaCl) were made with addition of heat-treated *Lb. helveticus* (Enzobact, Medipharm, Kågeröd, Sweden) and without as control. Pasteurised milk, calf rennet and mesophilic undefined DL- starter (Flora Danica, Chr. Hansen, Denmark) were used. The cheeses were ripened for two months.

Water soluble phosphopeptides from cheese were isolated with the affinity IMAC-Fe(III) chromatography using a pre-purification step of cation exchange chromatography to remove basic peptides, which may interfere. The elute was collected in three pools, which content of peptides was analysed using reversed phase LC-ESI mass spectrometry. (Lund & Ardö, 2004)

Results

The three pools that were collected from the IMAC elute corresponded to three main peaks of the chromatogram. LC-MS analysis of those three pools showed that Pool 1 contained peptides with one phosphate group, Pool 2 mainly with two and Pool 3 with four. The same complex composition of phosphopeptides as in ten months old semi-hard Herrgård cheese was found both in cheeses without and with heat-treated *Lb. helveticus*. The control contained larger phosphopeptides that were hydrolysed to smaller peptides by the *Lb. helveticus* enzymes (Fig. 1). The large α_{s1}-casein peptides with 2P, (Peak 39 - 41, Fig 1) were efficiently broken down to several smaller peptides (Peak 26, 29, 30 & 33) by the heat-treated *Lb. helveticus*. On the other hand, the groups of serine-P were intact, and there was no change in content of inorganic P during two months of ripening. It was concluded that heat-treated *Lb. helveticus* could be used to accelerate release of peptides from casein in low-fat cheese without loosing organic bound P due to phosphatase activity.

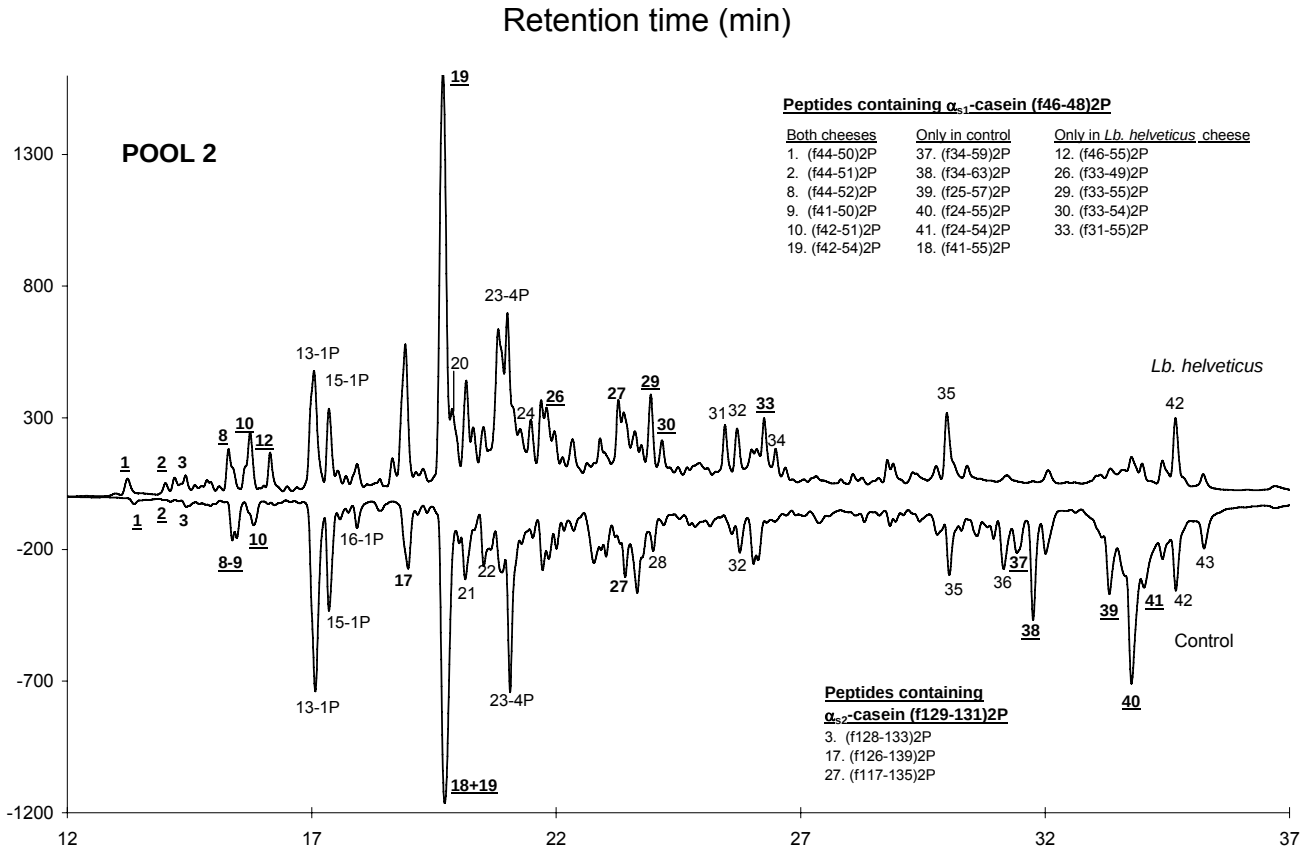

Figure 1. HPLC-MS of Pool 2 of phosphopeptides from cheese with heat treated *Lb. helveticus* and from Control cheese without. Phosphopeptides in the numbered peaks have been tentatively identified using MS. Underlined numbers refer to peaks containing α_{s1}-casein (f46-48).

Conclusion

Phosphopeptides identified from semi-hard, low-fat cheese covered all the phosphorylation sites of β-, α_{s1}- and α_{s2}-casein, and phosphate groups were still bound to all the originally phosphorylated serine residues of the peptides. Addition of heat-treated *Lactobacillus helveticus* accelerated breakdown of the phosphopeptides, but not any removal of phosphate groups.

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Key words: phosphopeptides, heat treated *Lactobacillus helveticus*, cheese

Session C- Poster

Antimicrobial effect of plant extracts on undesirable bacteria and yeast in milk

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Introduction

Microbial spoilage of milk is mainly associated with the growth of detrimental bacteria (Cousin, 1982; Bishop and White, 1966). Inhibition of pathogenic and food spoilage microorganisms by essential oils has been the subject of a great number of studies, ranging from specified studies testing the activity of single compounds against a few microorganisms to large scope studies, testing a wide range of essential oils and/or extracts on a broad spectra of microorganisms. Regardless abundant data (Holley & Patel, 2005; Smith-Palmer et al., 2001) of the antimicrobial effect of natural compounds on food pathogens and spoilage microorganisms, their commercial use is still very limited. Foods are usually more complex matrices than the testing media; therefore higher doses of essential oils are often required for foods to achieve any relevant effect. In case of cheese, Smith-Palmer et al. (2001) reported that the antimicrobial effect was stronger when the content of fat was reduced; however this effect was less significant in all types of cheese compared to the effects in a standard media. All oils applied in low fat cheese exhibited antimicrobial effect at 1 % doses, with clove oil being the most effective; while in the *in vitro* studies less than 0.1 % doses of the same oils were efficient against bacteria. Herbs and spices are added to food products to impart spicy flavour to the product; however an additional effect on food microorganisms should also be expected. A great number of milk products are fermented by lactic acid bacteria; consequently the ideal herbs in this respect would be those which contribute to a pleasant flavour, inhibit food spoilage bacteria and do have as little as possible effect on the growth of lactic acid bacteria. The objective of this study was to assess the potential use of acetone and ethanol plant extracts in combination with lactic acid bacteria as natural preservatives in milk against *Staphylococcus (Staph.) aureus*, *Enterococcus (E.) faecalis*, *Debaryomyces (D.) hansenii*, *Saccharomyces (S.) cerevisiae*. The minimum inhibitory concentration (MIC) of the extracts was tested on the lactic acid bacteria, *Lactobacillus* and the pathogenic bacteria in addition to growth assessments in milk.

Materials and methods

Food spoilage bacteria (*E. faecalis* ATCC 29212*, Staph. aureus* ATCC 25923, *E. coli* ATCC 25922) and yeasts (*D. hansenii*, *S. cerevisiae*, *Candida (C.) parapsilosis*), non-starter lactic acid bacteria (NSLAB) isolated from cheese and identified as *Lactobacillus* strains, as well as dairy starter bacteria (*Lactococcus (Lc.) lactis* subs. *lactis* biovar. *diacetylactis* NCDO 176*, Lc. lactis* subs. *lactis* INF L2*, Lc. lactis* subs. *cremoris* INF 393) and a commercial dairy starter R-704 (consist of *Lc. lactis* subs. *lactis, Lc. lactis* subs. *cremoris*) (Chr. Hansen, Hørsholm, Denmark)) were used in this study. The minimum inhibitory concentration (MIC) was determined by a microtiter plate assay according to Holo et al. (1991). For growth assessment, 1 % of overnight culture suspensions of the food spoilage bacteria or yeasts and a starter mix (CH-R704) were inoculated in 10 ml of sterile milk (skimmed milk powder, TINE BA, Norway). Acetone plant extracts of savory, rosemary and curcuma and pure ethanol were added at a level of 0.94 µl/ml, according to the MIC results obtained by the microtiterplate assay. Inoculated suspensions without extracts served as control. Ethanol was added to one suspension instead of extract. Serial dilutions were plated on M17 agar and selective media (VRB Agar, Bile Esculin Azide Agar, Baird-Parker Agar Base with Egg Yolk-Tellurite Emulsion, Rose-Bengal

Chloramphenicol Agar Base CM549 with Chloramphenicol Selective Supplement SR78) after inoculation (0h) and after 8 and 24 h incubation at 30°C and 37°C. The plates with yeasts and starter cultures were incubated at 30 °C for 72 h and 48 h, respectively, while pathogenic bacteria were incubated at 30 and 37 °C for 24 h.

Results

The obtained results showed that the sensitivity of various spoilage bacteria to the added extracts differed, causing a wide range of MIC. In general, ethanol extracts exhibited the lowest inhibitory effect while acetone extracts were more effective. Savory extracted by acetone (SA) had the lowest MIC value on the spoilage bacteria, while curcuma extracted with acetone (CA) had the lowest MIC against the spoilage yeasts. A problem with the curcuma extract was that the wells with spoilage bacteria became turbid and it was difficult to measure any effect. A very high concentration of rosemary acetone extract (RA) was needed to inhibit *E. coli* and *Staph. aureus*, while RA was very effective against *E. faecalis*. In general, the extracts had lower MIC on lactic acid bacteria than on the spoilage bacteria, meaning that the extracts were more repressive against the lactic acid bacteria tested than the spoilage bacteria tested. The growth of *Lc. lactis* ssp. *cremoris* was retarded by the extracts while *Lc. lactis* ssp. *lactis* biovar *diacetylactis* was more resistant. The effect of the extracts on the starter mix and *Lc. lactis* ssp. *lactis* was quite similar. The results showed that the NSLABs were sensitive to SA, RA and acetone extract of marjoram (MA). A high concentration of ethanol extracts was needed to inhibit the NSLAB and ranged from 11.25 to 1.40 μg/ml. It is known, that the presence of fat, carbohydrate, protein, salt and pH value influence the effect of plant extracts in food (Holley, 2005). In milk, all extracts reduced the growth of *E. faecalis*, and RA at 37°C had bactericidal effect. During the first 8h of growth, the starter mix R704 did not have an inhibitory effect on *E. faecalis*, however during further incubation at 37°C the growth of E. faecalis was suppressed, and after 24 h the same cell count was obtained as with SA and CA extracts. The extracts, ethanol and the starter mix had increased bactericidal effect on *Staph. aureus* when incubated at 37°C compared to incubation at 30°C. Bactericidal effect at 30°C was achieved by only by RA, while the other treatments resulted only in growth inhibition. As measured by the microtiter plate assay, the MIC of SA was lower than 0.94 μl/ml, while that of RA was remarkably higher than 0.94 μl/ml; however during incubation in milk, 0.94 μl/ml of RA was considerably more effective than 0.94 μl/ml of SA in suppressing and reducing *Staph. aureus*. Inoculation of *D. hansenii* with starter mix R704 resulted in a better growth of this yeast as compared with the control culture. CA exhibited bactericidal effect, while SA and RA suppressed the growth of *D. hansenii*. Acidic conditions are not favourable for *S. cerevisiae*; in this case the starter mix R704 suppressed the growth of this yeast in a similar way as SA and CA, while RA did not show any effect on the growth of *S. cerevisiae*.

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Keywords: plant extract, antimicrobial activity, lactic acid bacteria, food spoilage bacteria, yeasts

Session C – poster

Savoury flavour in Cheddar cheese: Impact of Lactobacillus as adjunct culture on Taste and Peptide Profile

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Background

The flavour of Cheddar cheese develops during processing and most predominately in the subsequent ripening period. During ripening, proteolysis of caseins forms the basis for generating a wide range of peptides and amino acids, which are known to influence cheese flavour. These changes results from enzymatic activities of residual coagulant, indigenous milk proteinases (e.g. plasmin), and microbial proteinases and peptidases from both starter and non-starter lactic acid bacteria.

A good balance between proteolysis and peptidolysis are known to prevent development of bitterness and insure formation of the general background flavour of Cheddar, towards a brothy and savoury flavour (McSweeney, 1997). However, the components causing savoury flavour are not completely known, and there are contradictory results on the contribution of small peptides and amino acids (van den Oord *et al.,* 1997).

The objective of this investigation was to characterize the effect of thermophilic *Lactobacillus* used as adjunct culture on the level of savoury flavour evaluated by sensory evaluation and analysing the composition of the water soluble non-volatile fraction of two Cheddar cheeses.

Materials & methods

Two commercially available aged Cheddar cheeses were selected for this study; cheese A age 12 months and cheese B age 10 months. The latter, contained a thermophilic *Lactobacillus* as adjunct culture. Standard analytical methods for cheese were used to measure pH, dry matter, fat and salt content. The amino acid composition were analysed by RP-HPLC.

The water soluble components of the Cheddar cheeses were extracted, freeze-dried and dissolved before fractionation by gel permeation on a Superdex peptide column using water as eluent. Three identical runs for each cheese were obtained. The fractions were pooled and freeze-dried before dissolved in 0.3 ml of water. Peptide profiling was then preformed both on the water soluble extract and the fractions from the gel permeation using a LC-MS ion trap system equipped with a C-18 column.

The sensory properties of the cheeses were characterized by descriptive sensory analysis using both a trained sensory panel and a group of cheese experts. The sensory evaluations focused on descriptors related to savoury flavour and an evaluation of the overall level of savouriness. Moreover, the fractions obtained by gel permeation were screened for taste properties.

Results

Cheese B added a thermophilic *Lactobacillus* as adjunct culture, had more intense savoury flavour characterised as 'umami' and 'bouillon-like' than cheese A. The two cheeses also varied significantly in other sensory attributes, like 'nutty' and 'fruity'.

Total content of amino acids was considerable higher in cheese B added a thermophilic *Lactobacillus* as compared to cheese A. Leucine, phenylalamine, arginine, aspargine, and glutamine decreased relatively to the total amount of amino acids expressed as molar%, while proline, lysine, and glutamine increased. These differences in the amino acid profile indicate the presence of thermophilic *Lactobacillus* in cheese B and are in accordance with results obtained by Ardö *et al*., 2002. In relation to the taste of umami the total amount of glutamic acid expressed as mmol/kg cheese were higher in cheese B as compared to cheese A.

The peptide composition of the water soluble extract of cheese A revealed accumulation of larger peptides from β-casein and peptides originating from secondary proteolytic activity on the peptide α_{s1}-casein *f*(1-23). This accumulation of peptides was not observed in the water soluble extract of cheese B which was dominated by primarily amino acids and small hydrophilic peptides. This altered peptide profile is properly related to the activity of thermophilic *Lactobacillus*.

The water soluble extracts were further resolved into six fractions for cheese B and eight fractions for cheese A by gel permeation. By screening for taste properties, savoury flavour was identified by terms of 'umami' and 'cheese-like' in two fractions from each cheese. Peptide profiling showed that the fractions with identified savoury flavour from cheese B were dominated by the presence of primarily amino acids and small hydrophilic peptides, while the fractions of cheese A had a more complex peptide profile.

In future characterization, key components of hydrophilic nature causing savoury flavour will be identified by LC-MS and further related to the activity of thermophilic *Lactobacillus*

Conclusion

The results of the present study indicated differences in the intensity of savoury flavour, amino acid composition and peptide profile which could be related to the presence of a thermophilic *Lactobacillus.*

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Keywords: Thermophilic *Lactobacillus*, Savoury flavour, Peptide profile, Cheddar

Session C – poster

The dynamics of nitrates, nitrites and important groups of microorganisms in Edam-type cheeses

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Introduction

Nitrates are supplemented to bulk milk in cheesemaking to prevent late blowing. Due to this, nitrate ions 0.2 to 104 mg/kg can be found in cheese. According to the data obtained by Heechen *et al.* (1991), supplementation of 15 g nitrate to 100 kg bulk milk increased the nitrate content in cheese up to 56 mg/kg. During ripening it decreased to 30 mg/kg, which is below the permissible standard of 50 mg/kg. Microorganisms play a significant role in natural oxidation and reduction processes of nitrates and nitrites. However, there is only fragmentary information available regarding the effect of cheese microbial populations, comprising technology- and contamination-induced bacteria, on the nitrate and nitrite content transformation dynamics. The objective of the present study was to determine the dynamics of nitrates, nitrites and main groups of microorganisms in Edam-type cheeses made in Estonia, and to find out their potential interactions.

Material and Methods

Cheese samples (0.7-1.0 kg) were taken after pressing and after ripening from the production batches of cheese industry. The samples were wrapped in plastic film, chilled to the temperature 0 to 4°C, and isothermally transported to the laboratory.

The samples were homogenized, nitrates and nitrites were extracted with acetonitrile-containing hot water. The samples were filtered and the filtrate was transferred into the column Alltech $C_{18.}$

The nitrates and nitrites were determined by using liquid chromatograph Shimadzu LC 10 AS and UV detector Shimadzu SPD-10A operated at 215 nm and analytical column Allsep Anion Column.

The smallest amount of nitrates determined was 10 mg NO_3^-/kg and that of nitrites 5mg NO_2^-/kg.

This method has been validated by the Swedish National Food Administration and acknowledged as Nordic Standard NMKL No. 165, 2000 (Pentchuk *et al*., 1986).

The samples for microbiological analyses were prepared according to standards EVS 633:1994 and EVS 639:1994. The total bacterial count was determined according to the IDF Standard 100A: 1987, using LAB 115 culture medium. Mesophilic lactococci were identified at 25°C in culture medium M17 (LAB 92) (Collins *et al*., 1995), mesophilic lactobacilli were determined at 30°C in culture medium LAB 93 (culture medium MRS, IDF Standard 117A:1988).

To detect spores of anaerobic bacteria, the respective one-tenth dilutions of cheeses were heated in water bath for 10 min at 80°C, then rapidly cooled down to 30 °C and inoculated into Petri dishes a´ 1 cm^3.

The inoculations, made in culture medium (*Reinforced clostridial agar*), were cultivated at 30°C in plastic sacks in household gas atmosphere, i.e. in anaerobic conditions. All formed colonies were counted, whereas it was calculated that there was one viable spore per each colony (LAB M, 1995).

The dilution liquid, used in microbiological analyses, was prepared according to the IDF 100A:1987.

Results and Discussion

After pressing, the fresh Eesti cheese contained 55 mg $NaNO_3$/kg on the average. During ripening for 3-4 weeks, the content of nitrates decreased by 10 mg/kg, maintaining the average level of 45 mg $NaNO_3$/kg in fully ripened cheeses. After pressing, the average nitrate content of fresh Pühajärve cheeses was 69.8 mg $NaNO_3$/kg. During the ripening period of 14 days, similarly to that of Eesti cheese the content of nitrates decreased by 10 mg/kg. Thus, the average level of nitrates 59.8 mg $NaNO_3$/ kg (range 37-88 mg/kg) in ripe Pühajärve cheeses exceeded the EU limits (50mg/kg) by 9.8 mg/kg. The content of nitrites in all the studied cheese batches of both Eesti and Pühajärve cheeses was <5 mg $NaNO_2$/kg, i.e. in compliance with the norms set by the EU.

In Eesti cheese after pressing the average total bacterial count was 480 million cfu/g, of which lactococci represented 360 million cfu/g, for lactobacilli the count was 3800 cfu/g and the counts of anaerobic spores 31.4 cfu/g respectively. During ripening, both the total bacterial count and content of lactococci dropped, retaining the level of 7.8 million cfu/g and 3.1 million cfu/g, respectively. The numbers of lactobacilli and spores of anaerobic bacteria increased, reaching in ripened cheeses the average of 4.7 million cfu/g and 229 cfu/g , respectively.

The average total bacterial count of Pühajärve cheese after pressing was 98 million cfu/g, the count of lactococci, lactobacilli, and spores of anaerobic bacteria was 75 million cfu/, 14 thousand cfu/g and 170 cfu/g, respectively. During ripening the total bacterial count and count of lactococci decreased, ranging between 9.9 million cfu and 8.2 million cfu per gram, on the average, whereas the count of lactobacilli and spores of anaerobic bacteria increased up to 12 million and 201 cfu per gram, respectively.

No significant differences were observed between Eesti and Pühajärve cheese, regarding the development of microorganisms in general. It appeared, however, that in Eesti cheese the higher development activity of spores of anaerobic bacteria was probably due to the lower nitrate content of cheese.

Statistical analysis of the obtained data (*Spearman correlation*) revealed that higher content of starter lactococci inhibited the development of anaerobic bacteria at the ripening stage ($P<0.01$). The nitrate content of cheeses also suppressed the development of anaerobic bacteria and lactobacilli, whereas it did not affect the growth of lactococci of starter cultures ($P<0.05$).

In cheesemaking, active starter cultures should be used to elevate the levels of lactococci in cheese. At the same time the milk with the lowest count of spores of anaerobic bacteria should be chosen to reduce supplementation of nitrates and keep the content of nitrates as low as possible in ripened cheese.

Keywords: nitrates, nitrites, cheese

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Session D – Key note speaker

Application of Genomics Data for *Lactobacillus* in the Production of Cheeses

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Research has shown that the lactic acid bacteria (LAB) present in the cheese matrix have a central role in flavor development. Therefore, it is likely that effective strategies to accelerate or intensify cheese flavor can be derived from a more fundamental understanding of LAB physiology in milk and cheese environments. Although many details must still be elucidated, current knowledge indicates LAB influence cheese flavor development via several key mechanisms including proteolysis, amino acid metabolism, lipase/esterase activity and citrate utilization. This presentation will focus on how genomic sequences of *Lactobacillus helveticus* CNRZ32 and *Lactobacillus casei* ATCC334 are being utilized to enhance our understanding of how LAB influence cheese flavor development.

L. helveticus CNRZ32 has been used as a culture adjunct to reduce bitterness, as well as, enhance and accelerate cheese flavor development since the 1980's. It is believed that the proteolytic enzyme system of CNRZ32 plays an essential role in this strain's demonstrated ability to influence cheese flavor development. Therefore, the Steele research group has focused on this enzyme system for the past fifteen years. Prior to genome sequencing, the nucleotide sequences of 12 CNRZ32 genes encoding proteolytic enzymes were known. Analysis of the genome sequence of CNRZ32 resulted in the discovery of more than 20 new genes encoding for proteolytic enzymes. This illustrates the capacity of genomics to dramatically accelerate cheese flavor research. Significantly, functional analysis of three newly discovered endopeptidases, which were of particular interest because endopeptidases have a central role in hydrolysis of bitter peptides, indicates that one of these enzymes is likely a critical component of the debittering activity found in this strain. Recently, differences in global gene expression of CNRZ32, when in grown in milk versus a rich laboratory medium, were examined. Growth of CNRZ32 in milk induced genes encoding cell-envelope proteinases, oligopeptide transporters, endopeptidases, as well as enzymes for serine-phosphate catabolism, lactose utilization and cysteine biosynthesis pathways, *de novo* synthesis and/or salvage pathways for purines and pyrimidines, and other functions. These results led to experiments that suggest that cheese-derived phosphoserine-containing peptides may serve as an energy source for *L. helveticus* CNRZ32 in milk. This illustrates the capacity of genomics to advance our fundamental understanding of LAB physiology in milk.

The non-starter lactic acid bacteria biota of cheese has an important role in the development of cheese flavor. Typically, *L. casei* is a dominant component of this microbiota. The genome sequence of *L. casei* ATCC 334, the proposed neotype strain for this species, which was isolated from cheese, is currently publicly available. To probe the genetic diversity, evolution and phylogeny of *L. casei* we have utilized this genome sequence with genomotyping and multilocus sequence typing approaches to examine a collection of forty *L. casei* strains isolated from different environments. The genomotyping analysis demonstrated that this strain collection contains a high level of genetic diversity with approximately 6.65% of the genes encoding metabolic enzymes present in ATCC334, being absent in a strain isolated from silage. The multilocus sequence typing approach yielded an evolutionary tree that suggests that the major cluster of cheese isolates is quite distinct from strains isolated from other environments. This suggests that significant biodiversity is available within *L. casei* to select strains

with distinct influences upon cheese flavor development. We have also utilized the ATCC334 genome sequence and a model cheese ripening system to identify putative metabolic pathways important for these organisms' ability to grow in ripening cheese. The results from these analyses indicate that citrate, residual carbohydrates, serine-phosphate containing peptides, and perhaps DNA serve as energy sources for the growth of these organisms in cheese. The results from these analyses will hopefully greatly enhance our understanding of how these organisms grow in ripening cheese. It is anticipated that this information will result in the development of strategies to control wild-strains of non-starter lactic acid bacteria in cheese and hence control cheese quality.

Keywords: *Lactobacillus*, genomics, cheese, flavor

Session D – Oral presentation

The nomenclature and the identification of the species in the *Lactobacillus casei* complex.

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The nomenclature of the species in the so-called *Lactobacillus casei* complex has not been clearly solved, and this has let to confusion of the identification belonging to the complex. The species names at present used within this complex are *L. casei, L. zeae, L. paracasei,* and *L. rhamnosus*. A literature review shows that only 3 species, not 4, should be recognized in the complex of which *L. rhamnosus* is one of them. It is also clear that the present type strains for *L. casei* (ATCC 393^T) and *L. zeae* (ATCC 15820^T) belong to the same species, while the proposed neotype strain for *L. casei* for (ATCC 334^T) and the type strain for *L. paracasei* (NCIMB 700151^T (formerly NCDO 151^T)) belong to a second species. However the discussion about which name is the correct is still running. I will here present evidence for that the correct names in the complex should be *L. casei, L. paracasei*, and *L. rhamnosus*, while *L. zeae* should be transferred to *L. casei*.

Background references to the discussion on the nomenclature can be found on the following homepage: http://www.bacterio.cict.fr/index.html (List of Prokaryotic names with Standing in Nomenclature).

The discussion is related to which species is described first, *L. casei* or *L. zeae*. As the *L. casei* type strain ATCC 393^T (according to the ATCC homepage) is the "*Streptobacterium casei* strain 7" originally described by S. Orla-Jensen in 1919 in his monograph "The Lactic Acid Bacteria" (later renamed to *Lactobacillus casei*), while the name *L. zeae* was published by Kuznetsov in 1959. This has been overlooked by Dellaglio and co-workers (1,2) when they suggested that *L. casei* should have ATCC 334 as neotype strain, and that ATCC 393 should be transferred to *L. zeae*, while the *L. paracasei* name should be rejected. I will present data that allow for a clear identification to species level for the three species.

Conclusion:
Since the species *Lactobacillus casei* with ATCC 393 as type strain was published before *L. zeae*, *L. zeae* should be transferred to *L. casei*, and the name *L.* zeae rejected. *L. paracasei* should be kept as a separate species with NCIMB 700151 as type strain and ATCC 334, should be renamed *L. paracasei* ATCC 334.

Keywords: *Lactobacillus casei, Lactobacillus paracasei, Lactobacillus zeae*, nomenclature, identification

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Session D - oral presentation

Lactobacillus lipoteichoic acids: Structures and bioactivities.

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Lipoteichoic acids (LTAs) of Gram-positive eubacteria have been suggested to take part in number of biological activities, such as cell-cell adhesions, various immunomodulatory activities, regulation of cell wall autolysins and as phage receptor components. Most of the LTA studies have been done using *Bacillus subtilis* and *Staphylococcus aureus* as model bacteria. However those bioactivities listed above have been associated with *Lactobacillus* LTAs, too, although so far quite limited studies with some controversial data are available. One reason for the discrepancy of published data on LTA promoted bioactivities could be the LTA isolation and purification methods employed in various studies.

In this presentation the most recent published and unpublished data on structures and bioactivities of *Lactobacillus* LTAs will be discussed. This presentation deals with *(i)* the isolation methods for LTAs, *(ii)* unpublished data on *Lactobacillus delbrueckii* LTA structures, *(iii) L. delbrueckii* LTAs as phage receptor components, *(iv)* published and unpublished data on *Lactobacillus* LTAs as immonostimulatory agents, and finally *(v)* effects of D-Alanine and glycosyl substitutions of LTA backbones on *Lactobacillus* LTA-promoted bioactivities.

Keywords: *Lactobacillus*, lipoteichoic acids, structure, bioactivity

Session D - oral presentation

Protective cultures for semi-hard cheese production

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In order to suppress the undesirable microorganisms in cheese, different protective techniques can be used. The promising possibility would be the use of lactic acid bacteria, especially lactobacilli as protective cultures.

Non-starter lactobacilli constitute the majority of the NSLAB population in semi-hard cheese varieties during ripening and many strains are able to inhibit food spoiling bacteria, moulds, pathogens, due to the production of antimicrobial compounds (lactic acid, carbon dioxide, bacteriocin, diacetyl, hydrogen peroxide, ethanol, organic acid, etc.).

Some of the lactobacilli isolated from Czech and Danish semi-hard cheeses exhibited inhibition against *Fusarium proliferatum* M 5689, followed by *Penicillium* sp. DMF 0006 and *Aspergillus niger* DMF 0801. They showed also anticlostridial activity against *Cl. tyrobutyricum*, *Cl. butyricum* and *Cl. sporogenes*. All lactobacilli with protective potential were examinated for the growth-rate, proteolytic, enzymatic and acidification activities in milk. The strains were tested for the production of biogenic amines and allergenicity. These properties are necessary for successful implementation of protective lactobacilli in semi-hard cheese production. The application of protective lactobacilli as adjunct cultures in semi-hard cheese production is however often limited due to its narrow activity spectrum and its inactivation due to the interaction with cheese ingredients.

The new isolates were identified by Rep-PCR assay as *Lactobacillus paracasei*.

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