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Fergal P. Rattray:

PushLim – Flyt grænserne i osteproduktionen; brug af biobeskyttende kulturer til at reducere risikoen for ostefejl

PushLim – Pushing the limits in cheese manufacture and ripening; minimizing the risks through the use of bio-protective cultures

Mejeribrugets
ForskningsFond

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Final report

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1. Title of the project

Danish: Flyt grænserne i osteproduktionen; brug af biobeskyttende kulturer til at reducere risikoen for ostefejl.

English: Pushing the limits in cheese manufacture and ripening; minimizing the risks through the use of bio-protective cultures.

2. Project manager

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4. Sources of funding

The total budget of the project was 6.937.028 DKK, of which 2.774.811 DKK was from The Milk Levy Fund. The funding from The Milk Levy Fund (2.774.811 DKK) was used to primarily cover the costs of the Postdoc research scientist. Arla Foods provided “in kind” funding (736.488 DKK) to cover their own salary costs

5. Project period

Project period with DDRF funding: 02/2018 to 12/2021

6. Project summary

Danish:

Dette projekt tog fat på de udfordringer, som mejeriindustrien står over for, nemlig en stadigt stigende efterspørgsel efter produktionseffektivitet og sunde produkter samtidig med, at høj produktkvalitet og -sikkerhed opretholdes. Sådanne udfordringer omfatter (i) brugen af forhøjede modningstemperaturer for at fremskynde modningsprocessen (for at reducere omkostninger) og (ii) reduktion af saltindholdet (sundere produkter). Begge tiltag kan føre til en øget forekomst af ostedefekter såsom bismag, dannelse af spalter/revner, dannelse af biogene aminer og oppustning. Disse defekter skyldes en forstyrrelse af den normale ostemikroflora og udviklingen af en ugunstig, skadelig mikroflora under modningen. Derfor kan forhøjelse af modningstemperaturen og/eller reduktion af saltindholdet skubbe ostemodningen i en retning, hvor alvorlige og kostbare defekter let kan udvikle sig. Fokus for dette projekt var at kortlægge den skadelige mikroflora eksperimentelt ved hjælp af genotypiske (helgenomsekventering) og fænotypiske analysemetoder og at bruge denne kritiske information til at udvælge de mest egnede biobeskyttende kulturer for at flytte "ingen defekter"-grænsen ud over, hvad der normalt er muligt. Det er kun gennem anvendelsen af biobeskyttende kulturer, at modning ved forhøjede temperaturer og ost med reduceret saltindhold sikkert kan opnås. Pilotforsøg med ost (Cheddar) blev udført i pilotanlæg for at omsætte og anvende laboratorieforsøg i et "virkeligt" mejeriproduktionsmiljø.

English:

This project addressed the challenges facing the dairy industry in terms of an ever-increasing demand for production efficiencies and healthy products while at the same time maintaining high product quality and safety. Such challenges include, (i) the use of elevated temperature ripening to accelerate the ripening process (for cost reduction) and (ii) the reduction of salt levels (healthier products). Both measures may lead to an increased incidence of cheese defects such as off-flavour, slit/crack formation, biogenic amine formation and blowing. These defects are caused by a disturbance in the normal cheese microflora and through the evolution of an unfavourable detrimental microflora during ripening. Therefore, elevation of the ripening temperature and/or reducing the salt levels can push the cheese ripening in a direction in which serious and costly defects can readily develop. The focus of this project was to experimentally map the detrimental microflora using genotypic (Whole Genome Sequencing) and phenotypic analysis methods, and to use this critical information to select the most suitable bio-protective cultures for pushing the "no defect" limit beyond what is normally possible. Is it only through the application of bio-protective cultures that elevated temperature ripening and reduced salt cheese can be safely achieved. Pilot plant cheese trials (Cheddar) were performed to translate and apply the laboratory experiments into a "real-life" dairy manufacturing environment.

7. Project aim

Danish:

Formålene med projektet var som følger:

1. Bruge avancerede genotypiske og fænotypiske analysemetoder til at kortlægge mikrofloraen i en normal ost (kontrol) og i oste lavet med forhøjet modningstemperatur og lavt saltindhold.
2. Karakterisere og kvantificere kvaliteten af oste lavet med forhøjet modningstemperaturer og lavt salt indhold, herunder defekter som afsmag, teksturfejl (revner og flækker), produktion af biogene aminer og pustning af osten.
3. Afprøve grænserne for hvor langt vi kan gå med forhøjet modningstemperatur og lavt saltindhold før der ses en defekt.

4. teste og udvælge de bedst egnede biobeskyttende kulturer til at flytte grænsen før der ses en defekt.

English:

The objectives of the project were as follows:

1. To map using advanced genotypic and phenotypic analysis methods the microflora of normal cheese (control), elevated temperature ripened cheese and reduced salt cheese.
2. To characterize and quantify the quality and safety risks (i.e. defects such as off flavours, silt/crack formation, biogenic amines, blowing) associated with elevated temperature ripening and salt reduction in cheese.
3. To experimentally determine using cheese trials the “no defect” limit in cheese ripening.
4. To screen, select and apply bio-protective cultures to extend the “no defect” limit in cheese ripening.

8. Background for the project

In recent years the cheese industry is ever increasingly being challenged to move from the secure ground of historically “tried and tested” production methods into developing new innovative processes and recipes where the full effect of such changes on product quality and safety is not fully known or understood. This push comes from both internal and external sources, for example, the dairy companies ongoing challenges in reducing the ripening time of cheese to reduce costs, release tied (working) capital and be faster to market with new product development. Additionally, the dairy companies’ strategy to continually improve the nutritional value of their products for consumers through targeted reformulation which includes reduced salt levels. External pressure for reformulation may also come from customers and regulatory authorities in order to reduce salt content of the cheese and to reduce the negative impact on consumer health (Linares et al., 2011; Guinee and O'Kennedy, 2007). Elevating the ripening temperature and reducing salt levels has a dramatic effect on the way in which ripening and flavor develop in cheese. Changes to these key parameters constitute a quality and safety risk to the cheese producer and need to be fully investigated and understood before industrial implementation can be rolled out.

It is well established from the literature that elevation of ripening temperature is difficult to perform well, with frequent problems encountered such as off-flavours, bitterness and slit/crack formation. Similarly, reduction of salt content is challenging, in which bitterness, texture defect, silt/crack formation, production of biogenic amines (histamine and tyramine) and late blowing may be encountered (Møller et al., 2013). The common factor in all these problems is the changed microflora that develops during the ripening of cheese under conditions of elevated ripening temperature or reduced salt. These changes are illustrated in **Figure 1**, in which a Normal, Elevated Ripening Temperature, and Reduced Salt microflora are depicted.

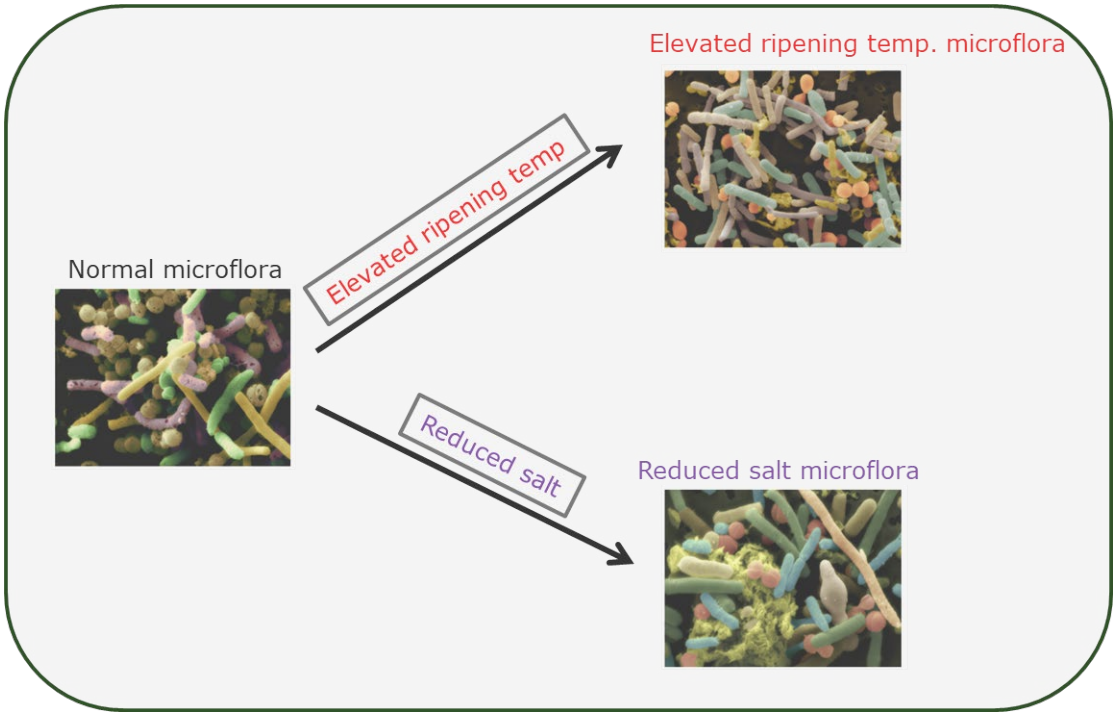

Figure 1. Microflora diversity in Normal, Elevated Ripening Temperature and Reduced Salt cheeses.

Microbial growth and populations are tightly controlled by ripening temperature and salt level (water activity) and many questions remain unanswered (Coolbear et al., 2008). What is the maximal level to which ripening temperature may be raised and salt can be reduced without causing quality defects such as slit/crack formation or off-flavour development and without increasing the risk of high biogenic amine (histamine and tyramine) levels? Can the dairy company aiming to innovate by pushing the normal limits in cheese manufacture and get the benefits of shorter ripening time (i.e. with elevated ripening temperature) and healthier cheese (i.e. reduced salt) while maintaining a high quality and safe product? Most importantly, what measures/practices are available to the dairy company to minimize these risks?

9. Sub-activities in the entire project period

The project consisted of five Work Packages (WPs) and was carried out over a period of 3 years. Overview of sub-activities for the project is presented in **Figure 2.**

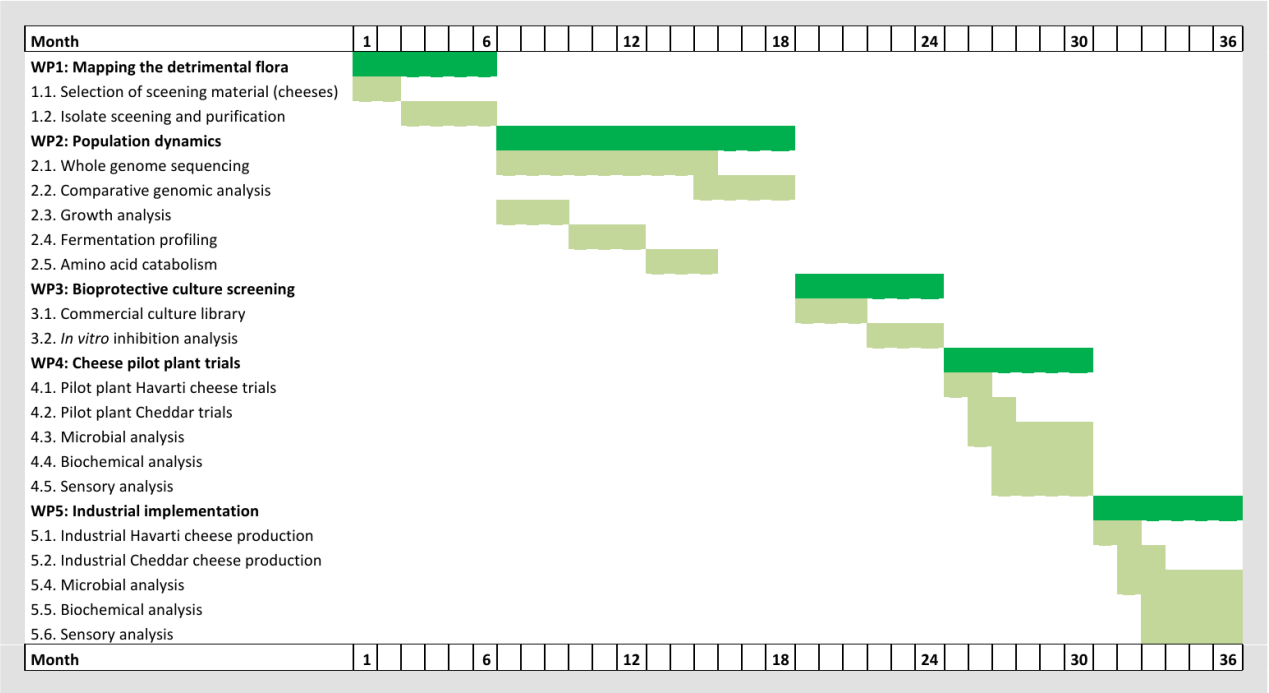

Figure 2. Overview of sub-activities for the project.

10. Deviations

No significant deviations were encountered with the execution of this project.

11. Project results

The project consisted of five Work Packages (WPs) which was carried out over a period of 36 months. Staffing for the project was a 1 Post-Doc researcher, 1 PhD student and 2 MSc students. Details of the various WPs are provided below together with detailed Gantt chart for the specific activities in Section 9.

WP1. Mapping the detrimental flora in cheese. Responsible; FOOD-KU and Arla Foods

In this work package cheeses displaying various defects, such as crack/slit formation, blowing or high levels of biogenic amines (histamine and tyramine) were collected from the Danish and wider European dairy industry and from commercial sources. The cheeses selected were likely to have been subjected to some form of elevated temperature ripening and/or salt reduction (see **Figure 3**).

These cheeses were used as the source material for purification of single isolates from the principal flora.

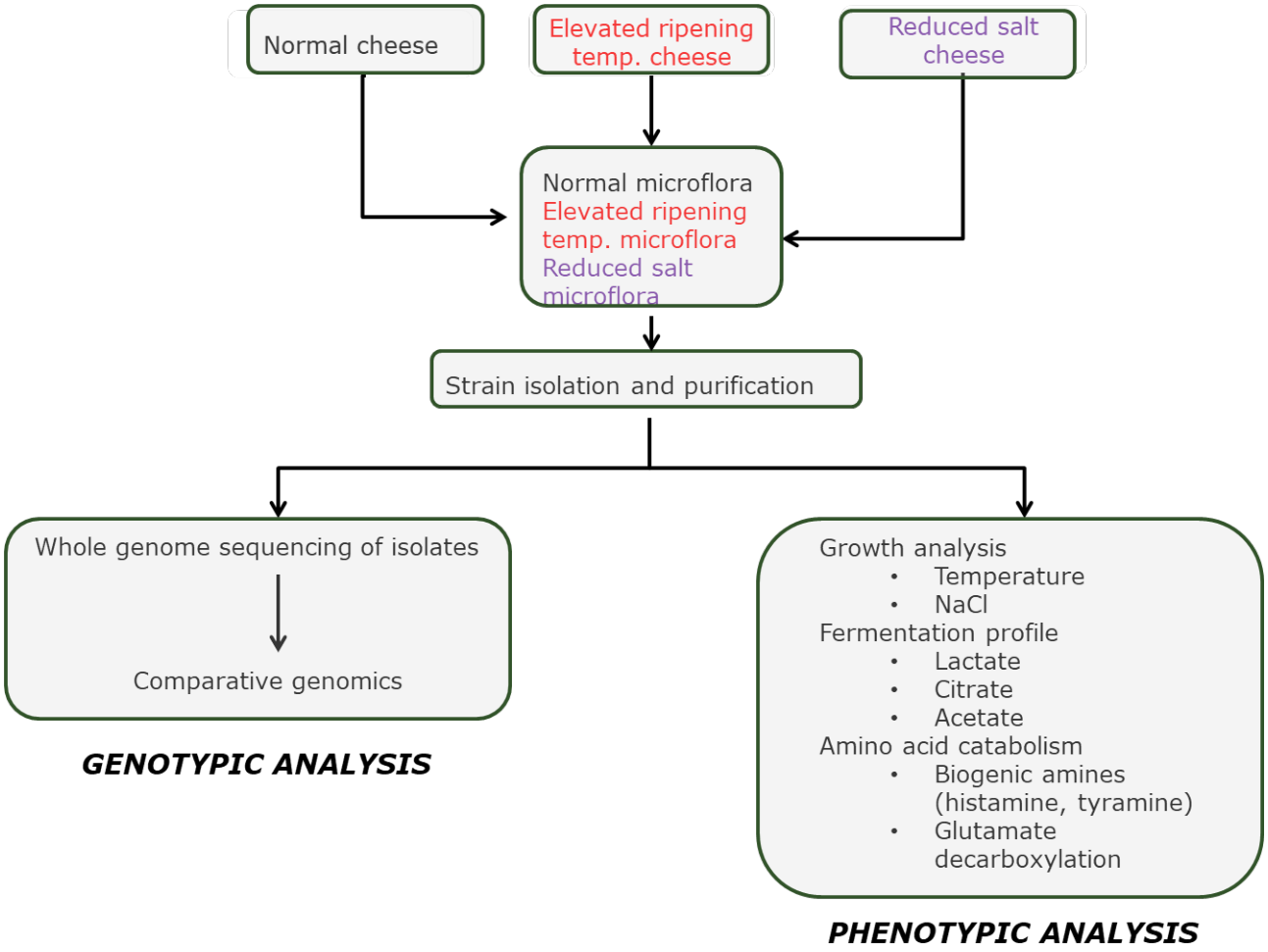

Figure 3. Advanced genotypic and phenotypic analysis of the microflora of normal cheese (control), elevated temperature ripened cheese and reduced salt cheese. **WP1 & WP2.**

The presence of histamine forming bacteria in commercially available cheeses as well as the histamine forming potential were evaluated using *in vitro* models. Five long–time-ripened cheeses made from different milk types were analysed for histamine producing bacterial isolates. The ability of the isolates to produce histamine was tested by incubation at 37 °C for five days in a restricted media with a pH indicator. Changes in the amino-compound profile were investigated using ultra performance liquid chromatography. Only eight of 106 isolates were able to produce compounds raising the pH and seven of those were confirmed to be histamine producers. Even though all isolates were obtained from the same vintage Danish Gouda cheese, made from raw cow milk, the amino-compound profile as well as the response to different environmental conditions diverged between the isolates. Rep-PCR and 16S rRNA gene sequencing was used to further characterize the isolates. The results indicated that evaluating the presence and concentration of histamine is not only a relevant parameter to evaluate quality and safety, but is also an important tool to classify histamine producers in cheese.

WP2. Population dynamics: effect of elevated ripening temperature and salt reduction on microbial populations (genotypic and phenotypic analysis methods) Responsible; FOOD-KU and Arla Foods.

Whole genome sequencing has evolved in the last 1-2 years to be a low-cost method in which as the name implies, involves the sequencing of the entire genome of the isolate. Whole genome sequencing of the isolates from WP1 was performed to establish the specific flora and the respective microbial pathways responsible for the defects in the cheese (see Figure 1). The method provided a detailed and complete fingerprint of the isolates and their metabolic potential,

and it was only by characterization of this that it became possible to identify remedy ensures to enable control of the detrimental flora. The DNA sequence information obtained in this WP was complimented with extensive phenotypic analysis such as growth analysis, fermentation utilization and amino acid catabolic profiling.

WP3. Culture screening for inhibiting/bio-protective cultures. Responsible; FOOD-KU

In this WP, the isolates obtained in WP1 and their whole genome sequence data from WP2, were used as a basis for selecting the most appropriate bio-protective cultures. Commercially available bio-protective cultures from the various culture suppliers (Chr. Hansen A/S, DuPont, Clerici-Sacco, CSK Food Enrichment or Bioprox) were used for in vitro screening to identify the most effective bio-protective culture(s) against the detrimental microflora. For example, bio-protective cultures against gas formers (responsible for crack/slit formation) were identified, as well as bio-protective cultures effective against biogenic amine producing cultures. Screening was performed in microtiter scale to screen large sample numbers and for various combinatorial studies.

Commercial bioprotective cultures have been used to screen for the ability to inhibit the growth of histamine producing isolates. The nisin-producing bioprotective (BS-10) culture inhibited the growth of histamine producing *Lentilactobacillus parabuchneri* KUH6. The nisin-producing bioprotective culture and histamine producing producing *Lentilactobacillus parabuchneri* KUH6 were tested for milk acidification activity to ensure that they would not affect normal cheese production. The promising bioprotective culture and histamine producer were identified as non-acidifiers and were selected for the cheese making trials.

WP4. Cheese pilot plant trials Responsible; FOOD-KU and Arla Foods.

Cheddar cheese was produced in pilot scale with the deliberate addition of various detrimental microflora. The effectiveness of the previously selected bio-protective cultures (from WP3) were added to the cheese milk; cheeses were produced with normal and reduced salt levels and ripened at normal and elevated (see Figure 3).

Thus, it was possible to study the bio-protective effects of the cultures at elevated and reduced salt conditions. Following manufacture of the cheeses, ripening was monitored by extensive microbial, biochemical and sensory analysis. It was keenly anticipated that at least some of the bio-protective cultures selected in the *in vitro* screening in WP3 would be effective in controlling the detrimental flora and reducing the incidence of defects.

January 2020 trials

The study was performed to investigate *Lentilactobacillus parabuchneri* KUH8, a histamine-producer (HP) in reduced-salt Cheddar cheese. Four cheeses were manufactured: 1) normal-salt (NS); 2) reduced-salt (RS); 3) normal-salt with HP (NS+HP); 4) reduced-salt with HP (RS+HP). Two replicates were produced with milk from the same batch, and the cheeses ripened at 10 and 15 °C. Cheeses were sampled immediately after manufacture and after 1, 3 and 6 months of ripening. Ultra-high-performance-liquid chromatography indicated that with the HP, histamine reached higher levels in reduced-salt cheeses (3.5–3.7 % S/M) at 15 °C (86, 1112, 2149 and 3149 mg kg−1), compared to normal-salt cheeses (5.4–6.3 % S/M) at 10 °C (78, 584, 593 and 1389 mg kg−1), at each respective cheese-sampling point. Higher salt-content reduced the growth rate of non-starter microbiota, but after six months the levels in all cheeses were similar, according to the ripening temperature: at 10 °C (8.05–8.30 $\log_{10}$ cfu g^{-1}), and at 15 °C (6.00–6.94 $\log_{10}$ cfu g^{-1}). A correlation between increased histamine levels, non-starter-cell development and pH was found. This study highlighted the importance of normal-salt content and low-ripening temperature as measures to control histamine-formation and to improve safety in cheese.

November 2020 trials

Cheddar cheeses were made for the second time with a different histamine producing isolate (*Lactobacillus parabuchneri pentosaceus* KUH6) in the dairy pilot plant at the University of Copenhagen. The cheese production experiments at a 4 x 100-liter scale were performed in duplicate. A total of 8 different vats of cheese were produced to investigate the effect of bioprotective culture (nisin producer), salt reduction, and ripening temperature on histamine production. The ripening of the cheese were followed over the next 6 months (see Figure 4).

The Cheddar cheese produced at a 4 x 100/liter scale with histamine producing *Lactobacillus parabuchneri* KUH6 was followed over 6 months of ripening. The four different treatments included in the low salt Cheddar cheese production: 1) Control low salt; 2) Nisin producer (*Lactococcus lactis* subps. *lactis,* BS-10)) in low salt; 3) Histamine producer (*Lactobacillus parabuchneri* KUH6) in low salt); and 4) Histamine producer (*Lactobacillus parabuchneri* KUH6) and nisin producer (*Lactococcus lactis* subps. *lactis*, BS-10) in low salt. Each block of Cheddar cheese was portioned to 12 smaller blocks and ripened in vacuum packaging at two different temperatures, 10°C and 15°C for six months. The cheese sampling was performed before ripening, and first, third, sixth months of ripening. The low salt Cheddar cheese samples subjected to chemical and microbiological analyses. The chemical analyses carried out before ripening period included; total solids, NaCl content, protein, and fat content. The pH was measured during the ripening period in each sampling time point. The microbiological analyses and amino compound profiling was performed before ripening, and first, third, sixth months of ripening.

The addition of the nisin producing culture (BS-10) seem to exert its activity against the adjunct culture (LH-32) rather than the histamine producer, *Lentilactobacillus parabucheri*. In these cheeses (with BS-10) the level of histidine was reduced, thus reducing the extent to which high histamine levels can be formed. These results indict that nisin producing culture present an interesting possibility for the reduction of biogenic amines in cheese.

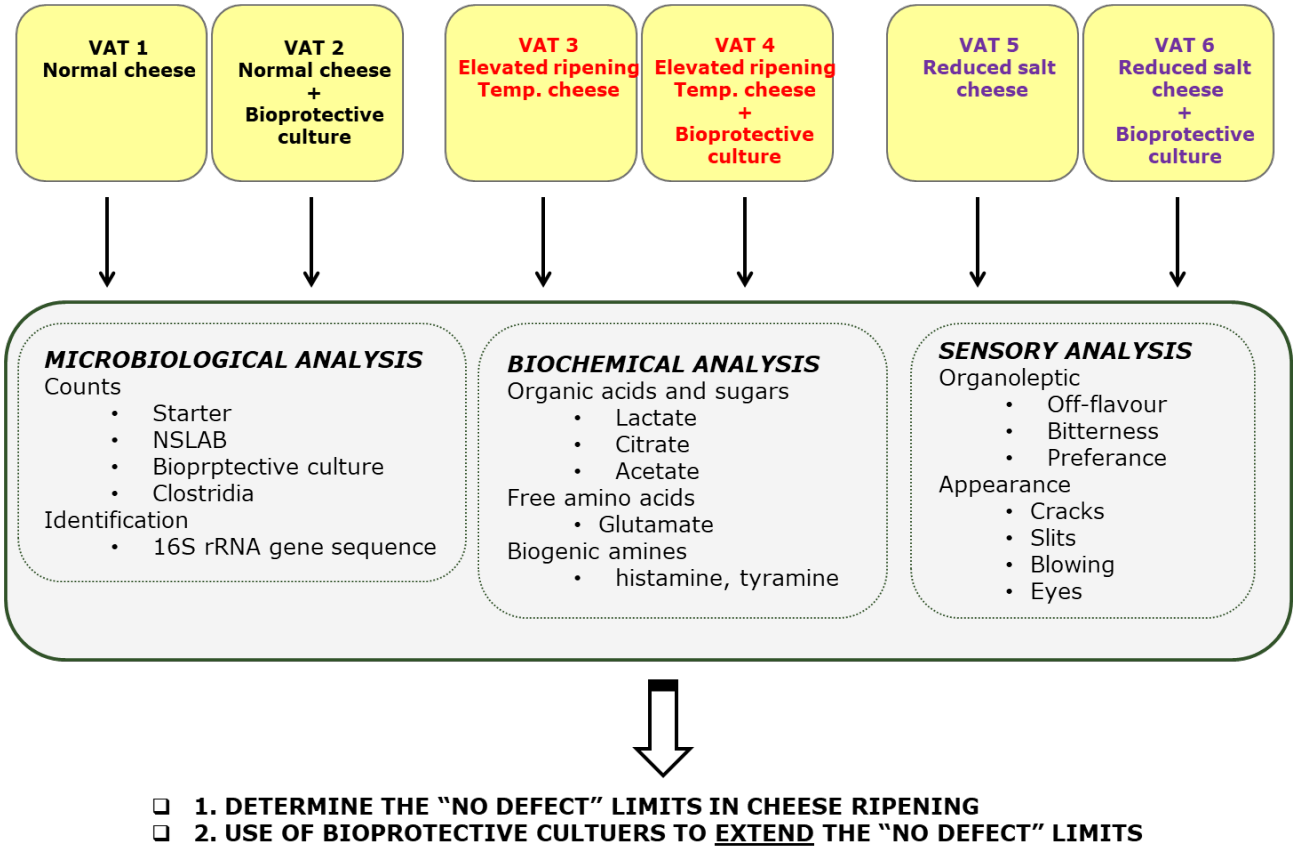

Figure 4. Pilot plant cheese trials; effect of bio-protective cultures on normal cheese (control), elevated temperature ripened cheese and reduced salt cheese challenged with detrimental microflora.

WP5. Industrial implementation Responsible; Arla Foods

No industrial cheese trials were conducted. However, the pilot cheese trials were all conducted using a commercially available bioprotective culture (BS-10) thus facilitating a rapid industrial implementation of this approach for controlling biogenic amine production in cheese.

12. The relevance of the results, including relevance for the dairy industry

These results will ensure that product quality of cheese in Denmark is maintained at the highest possible level. It has been established that reducing the salt content in cheese is not without its risks, and the knowledge and tools developed in the project enable a proper risk-benefit analysis. Specifically, regarding health-related issues (histamine production), it will be possible to add bioprotective cultures to safely produce cheeses with the lowest possible level of salt. Already, one such culture, BS-10 has been identified. The elevated temperature ripening is a potential way of shortening the ripening period. The accomplishment of defectless cheese production using elevated temperature ripening will induce a contribution to sustainability.

Overall, it has been a very satisfying project. Some challenges have been encountered along the way, but the project team has managed to successfully solve these issues. Support from the industry partner (Arla Foods) has been an integral part of the project. Furthermore, support from the starter culture suppliers (Chr. Hansen, Sacco, DSM, DuPont and CSK) will be crucial to the implementation of the results in the Danish cheese industry (450.000 tons of cheese in 2019).

This project's overall goal was to enable cheese to be produced with lower-than-normal levels of salt. Normally, this is not possible due to defects such as biogenic amines (histamine and tyraime) and slit/crack development. A natural solution through the use of bioprotective cultures now addressed these challenges. This approach is in full alignment of the expectations of modern consumers of "naturalness", while at the same time of keeping product quality high.

The outcome of this project will enable the dairy industry to create new products and processes for cheese which are healthier and more cost effective while maintaining control of quality and safety in cheese production. The abolition of the milk quota in 2015 has led to an increased milk production in Europe, and combined with this a global increase in the demand for high quality dairy products requires new and innovative ways of producing cheese. In 2014 the Danish cheese export had a value of 11.7 billion DKK making it the largest dairy product category exported out of Denmark. We believe that there is a potential for growth in this sector, and that the knowledge and its application will enable the dairy industry to facilitate that increase. These results will ensure that product quality of cheese in Denmark is maintained at the highest possible level.

13. Communication and knowledge sharing about the project

Papers in international journals:

Ücok E. F., Møller C.O. de A., Vogensen, F.K., Rattray, F.P. (2022) Reducing histamine production in reduced-salt Cheddar cheese using a nisin-producing *Lactococcus lactis*. SSRN. http://dx.doi.org/10.2139/ssrn.4041854

- The ability of *Lentilactobacillus parabuchneri* (a cheese isolate newly found to be histamine producer) was tested in low salt Cheddar cheese.
- A nisin producer was used as bioprotective culture to inhibit histamine formation.
- When only the histamine former was added, high levels of histamine were found through the six months of ripening.
- In presence of the nisin producer, the reduced levels of histamine were formed.
- The use of nisin producer in low salt cheese is suggested to avoid histamine formation.

Møller C.O. de A., Castro-Mejía, J. L., Krych, L., Rattray, F.P. (*2021*) Histamine forming ability of *Lentilactobacillus parabuchneri* in reduced salt Cheddar cheese. *Food Microbiology*.

- The histamine producer *L. parabuchneri* was tested in reduced-salt Cheddar cheese.
- The histamine formation was monitored during six months of ripening at 10 and 15 °C.
- Normal salt content limits the histamine formation at low temperature of ripening.
- Histamine was formed at high levels in reduced salt cheeses, and even higher at 15 °C.
- To improve safety in cheese, it was suggested: normal salt content and low ripening temperature.

Møller C.O. de A., Ücok E. F., Rattray, F. P. (2020) Histamine forming behaviour of bacterial isolates from aged cheese. *Food Research International* 128 (108719),1 – 12. https://doi.org/10.1016/j.foodres.2019.108719.

- Histamine producer isolates were obtained from cheese.
- The identity of the isolates was investigated with molecular biology techniques.
- The potential of histamine production was tested under different conditions.
- Classification proposed for histamine producers was based on amino-compound profile.

Oral presentations at scientific conferences, symposiums etc.:

Møller C.O. de A., Rattray F.P., Ücok E.F. (2021) Categorizing histamine producing bacteria from Gouda cheese according to amino compound profiles. MIFFI. https://miffi.org/wp-content/uploads/2024/10/CAP-Partner_MiFFI_programme2021_A4_finalWEB_17112021.pdf

Møller C.O. de A. (2020) The complexity of bacterial interaction in cheese ripening. Oral Presentation at Mejeriteknisk Selskab Seminar: "*Nyt om osteproduktion"*, 30th January, Billund, Denmark.

Møller C.O. de A., Ücok, E.F., Rattray F.P. (2018) Bioactive compounds as marker of quality and safety in fermented foods. Oral Presentation at the 1st ICBC International Congress on Bioactive Compounds, 23rd November 2018, Campinas, Brazil.

14. Contribution to master and PhD education

PhD project:

Elif F. Ücok (2018-2021) Investigation of microbial diversity of histamine producers in commercial aged cheeses and their antagonists bioprotective cultures, applied in cheese manufacture and evaluated during ripening time, through the project PushLim – Pushing the limits in cheese manufacture and ripening. Section of Microbiology and Fermentation, Department of Food Science, University of Copenhagen.

Master project:

- Geoffrey Johansen (2020-2021) Histamine formation potential in dairy isolated *Pediococcus pentosaceus*. Msc. in Microbiology and Fermentation, Department of Food Science, University of Copenhagen.
- Mathias Egholm Hørstedt Madsen (2020-2021) Investigating *Clostidium Sporogenes* ATTCC-15579 ability to produce tryptamine under conditions mimicking matured cheese. Msc. in Microbiology and Fermentation, Department of Food Science, University of Copenhagen.

15. New contacts/projects

This project showcased the new cheese making facilities (4 x 200 liter cheese vats, cheese press and ripening rooms) at the University of Copenhagen's pilot plant. Furthermore, a novel method for simultaneously measuring free amino acids and biogenic amines was developed. These facility and analysis improvements ensure that future collaborative projects with the Danish dairy industry are greatly enhanced.

16. References

Coolbear T, Crow V, Harnett J, Harvey S, Holland R, Martley F. (2008). Developments in cheese microbiology in New Zealand—Use of starter and non-starter lactic acid bacteria and their enzymes in determining flavour. International Dairy Journal, 18(7):705-713.

Guinee TP, O'Kennedy BT. (2007). Reducing salt in cheese and dairy spreads, Reducing salt in foods: Practical strategies. Kilcast D, Angus F, editors. Cambridge, UK: Woodhead; 2007. pp. 316–357.

Linares DM, Martín MC, Ladero V, Alvarez MA, Fernández M (2011). Biogenic amines in dairy products. Crit Rev Food Sci Nutr. 51(7):691-703. doi: 10.1080/10408398.2011.582813. PMID: 21793728.

Møller KK, Rattray FP, Bredie WLP, Høier E, Ardö Y (2013). Physicochemical and sensory characterization of Cheddar cheese with variable NaCl levels and equal moisture content. Journal of Dairy Science, 96 (4):1953-1971.