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Christian Solem:

Optimering af smagsdannelse i hårde oste

Optimization of flavor formation in hard cheeses

Mejeribrugets
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Final report

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1. Title of the project

In Danish: Optimering af smagsdannelse i hårde oste
In English: Optimization of flavor formation in hard cheeses

2. Project manager

Christian Solem, DTU Food, Building 201, Kemitorvet, 2800 Lyngby, 30585533, chso@food.dtu.dk

3. Other project staff

Peter Ruhdal Jensen, DTU Food, perj@food.dtu.dk
Jun Chen, DTU Food (no longer employed)
Robin Dorau, DTU Food (no longer employed)
Lin Chen, DTU Food (no longer employed
Jianming Liu, DTU Food (no longer employed)

4. Sources of funding

Danish Dairy Research Foundation
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5. Project period

Project period with DDRF funding: 01-01-2017 until 31-5-2020
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6. Project summary

In Danish:

I forbindelse med ostefremstillingen varmes ostemassen for at udskille valle, og det er gennem denne såkaldte eftervarmning at ostens fasthed bestemmes. En højere eftervarmning er således en mulighed for at opnå en fastere ost, men desværre kan de mælkesyrebakterier, der benyttes i forbindelse med ostefremstillingen, ikke altid tåle denne temperaturpåvirkning. Til fremstilling af danske ostevarianter såsom Danbo og Havarti benyttes såkaldte mesofile kulturer, som ikke kan tåle meget mere end 37-39°C's varmepåvirkning, alt afhængig af kulturens sammensætning. I nærværende projekt har vi undersøgt, om mælkesyrebakterierne kan tilpasses højere temperaturer ved gradvist, over en længere periode, at øge den temperatur,

som de vokser ved. Håbet var, at denne tilpasning ville kunne muliggøre anvendelse af kraftigere eftervarmning. Som udgangspunkt benyttede vi mejeristammen SD96 (*Lactococcus lactis*), som kan vokse i mælk ved 39°C. Denne stamme blev dyrket i steril mælk, ved gradvist højere temperaturer i op til halvandet år, hvilket resulterede i en række stammer, som kunne vokse bedre ved høje temperaturer. Således kunne stammen RD07, der blev isoleret efter halvandet år, vokse fint ved 41°C. Flere af stammerne er blevet undersøgt i detaljer, bl.a. er deres arvemateriale blevet bestemt. En af stammerne, RD01, er blevet testet af Arla Foods, som benyttede stammen til at fremstille Havartiost med. Konklusionen var, at stammen var bedre egnet til ostefremstilling ved høj eftervarmning end den kommercielle ostekultur, Flora Danica.

Bakterievirus (fager) er normalt en udfordring for mejerier, da de kan inaktivere mælkesyrebakterierne. Normalt håndteres fagudfordringen ved at benytte kulturer med mange forskellige mælkesyrebakteriestammer samt ved at lave kulturrotation. Fager er selektive, og hvis der er mange forskellige mælkesyrebakterier til stede, så har de vanskeligt ved at inficere dem alle. Kulturrotation er en metode til at forhindre fager i at tilpasse sig de forskellige mælkesyrebakterier; kulturen som anvendes udskiftes med jævne mellemrum, hvilket forhindrer fagerne i at tilpasse sig en given kultur. Udfordringen med kulturrotation er en vis produktvariation, da anvendelse af forskellige kulturer resulterer i oste, der smager forskelligt. Vi er fremkommet med en løsning, hvor mælkesyrebakterierne opdyrkes i steril mælk tilsat osteløbe, hvilket indkapsler dem i ostemasse, som giver 100% resistens over for fager.

Ud over temperaturtolerance, så har vi arbejdet på at optimere mælkesyrebakteriernes smøraromaproduktion, da smøraroma er en af de aromaer, der påvirkes negativt ved høj eftervarmning. Vi isolerede naturlige varianter af stammen RD01, som var ude af stand til at lave mælkesyre. En af disse varianter, RD1M5, var særdeles effektiv til at producere smøraroma ud fra mælkesukker. Vi optimerede på produktionsbetingelserne, fx beluftning og pH og kunne effektivt producere smøraroma fra lavværdi-mejerisidestrømme. Arla Foods Ingredients leverede diverse sidestrømme, som vi testede.

Vores konklusion er, at det er fuldt ud muligt at benyttes mesofile kulturer til at fremstille hårdere ostevarianter med god smag. Det er muligt, at eftervarmningstemperaturer så høje som 45-50°C kan benyttes – dette skal dog eftervises i større skala. Der åbner sig dog muligheder for at helt nye ostevarianter kan blive fremstillet. Mht. smøraroma, så har vi vist, at denne kan fremstilles mere bæredygtigt ud fra lavværdi-sidestrømme.

In English:
During the cheese manufacturing process, the cheese curd is heated to expel whey, the so-called cooking step, and the temperature used dictates the firmness of the final cheese. Thus, in principle, a firmer cheese can be obtained merely by using a higher cooking temperature, however, there is a limitation to this, i.e., the heat tolerance of the lactic acid bacteria (LAB) used to make the cheese. For making the Danish cheese varieties, Danbo and Havarti, mesophilic cultures are used, which are composed of LAB that prefer moderate temperatures (around 30°C), and cooking typically is done at 37-39°C, depending on the microbial composition of the starter culture. In the present project, we have explored whether the LAB can be adapted into tolerating higher temperatures, by, gradually and over time, increasing the temperature at which they grow. Our hope was that the adapted strains could be used to prepare cheeses cooked at higher temperatures. As a starting point, the dairy strain SD96 (*Lactococcus lactis*), which is able to grow slowly at 39°C, was used. SD96 was grown in sterile milk for one and a half year, at gradually increasing temperatures. One of the strains obtained in this adaptive laboratory evolution was RD07, which is able to grow well at 41°C.

Several of the heat tolerant strains were characterized, for instance by scrutinizing their genomes. One isolate, RD01, was tested in cheese trials at Arla Foods' R&D facility, where Havarti type cheeses were prepared using RD01 and the commercially available Flora Danica culture. Based on this it was concluded that RD01 was superior in terms of producing cheeses with good flavor.

Bacterial viruses (phages) are normally a challenge for dairies since they can infect and kill the LAB and thus ruin the cheese fermentation. The phage issue can be managed by using starters composed of many strains and by frequently changing the culture used (culture rotation). Phages are selective, and by using a blend of different strains, some will remain uninfected and carry out the task at hand. By using culture rotation, the phages are unable to adapt to a particular culture, which they eventually do if the same culture is used continuously. The challenge with both approaches is that the microbial composition of the starter changes over time, and this results in product inconsistency. To address this challenge, we developed a method that in principle allows the use of single strain starters. By growing the starter in sterile milk (no phages) to which is added rennet, it is possible to encapsulate the LAB in cheese curd. We have demonstrated that the LAB encapsulated in cheese curd are 100% resistant to phages, and the method could lead to more sustainable cheese production by eliminating failed cheese fermentations.

Besides temperature tolerance, we have also focused on butter aroma production since this is affected negatively by high cooking temperatures. We have isolated natural variants of RD01, which is unable to produce lactic acid. One of these, RD1M5, was efficient at producing butter aroma from the sugar in milk (lactose). We optimized the conditions for producing butter aroma, for instance aeration and pH, and managed to demonstrate butter aroma production using low-value dairy side streams provided by Arla Foods Ingredients.

To conclude, mesophilic cultures can be adapted into performing well at higher temperatures. One of the adapted strains was tested by Arla Foods, and it was indeed possible to prepare good cheese using this strain. It is plausible that cooking temperatures as high as 45-50°C can be applied, however, this remains to be demonstrated in larger-scale trials. We expect that novel cheese variants can be manufactured using the thermally robust strains. Regarding butter aroma, we have demonstrated that it can be produced in a more sustainable manner from low-value dairy side streams.

7. Project aim

In Danish:
Vi ønskede at finde løsninger, der tillod, at mesofile starterkulturer kan benyttes ved højere eftervarmningstemperaturer. Vi planlagde at benytte flere forskellige fremgangsmåder, hvor en gik ud på at ændre ostefermenteringsprocessen ved at fjerne ilt. Vi vidste fra egen tidligere forskning, at tilstedeværelse af ilt ved højere temperaturer har en skadelig effekt på *Lactococcus lactis* (*L. lactis*), som er den organisme, der dominerer den mesofile starterkultur. Vi planlagde at optimere den mesofile starterkultur på forskellige måder, så den bedre er tilpasset de højere temperaturer, ved fx at tilsætte forskellige additiver eller ved at ændre på vækstbetingelser forud for podning af mælken. Endeligt ønskede vi at skabe en mere temperaturrobust version af den mesofile starterkultur gennem adaptiv evolution/mutagenese. I samarbejde med Arla Foods var det ønsket at teste de nye ostefremstillingsprocesser/starterkulturer i forskellige ostefremstillingsforsøg.

In English:
We wanted to find solutions so that the mesophilic starter can operate successfully at elevated cooking temperatures. We planned to use several approaches, where one involved changing the cheese fermentation process by removing oxygen. We knew from our previous research that the presence of oxygen is detrimental for *L. lactis*, the dominant organism in mesophilic starters. We also wished to optimize the mesophilic starter in different ways to accommodate its use at elevated temperatures. For instance, the effect of different additives or changed growth conditions prior to inoculation in milk was planned. Finally, we wanted to create more temperature-robust versions of the mesophilic starter through adaptive evolution/random mutagenesis. Testing of the novel processes/starter cultures in cheese production was planned in collaboration with Arla Foods.

8. Background for the project

The aim is to solve a real-life challenge faced by the Danish dairy industry, namely a desire to produce harder cheese variants using mesophilic starters, as the thermophilic starters normally used fail to produce the desired flavor compounds, such as lactic acid and butter aroma (personal communication Søren Lillevang, Arla Foods). In this project, we focus on the cheese process used to make typical Danish semi-hard cheeses like Danbo or Havarti, and which can be shortly summarized as follows: 1) addition of rennet/starter culture to the pre-heated pasteurized milk, 2) milk gel formation due to the rennet, 3) cutting gel/stirring/cooking, 4) pressing of the cheese curds (where main acidification occurs). In addition to these steps, there is a salting and a maturation step. An overview is provided in Fig. 1 below.

In cheese production, the optimization of cheese moisture, yield, and quality is very important, and these parameters are strongly influenced by the condition under which syneresis takes place. Syneresis is the process where the whey is expelled from the curd matrix due to its contraction caused by rearrangements in the paracasein network. Syneresis is normally induced by an increase in the temperature (cooking) at a specific time-point after formation of the milk gel, where it has obtained a certain firmness, that ensures a sufficient retainment of fat but also allows for the desired amount of whey to be expelled.

The starter culture used for making the cheese is affected by the curd cooking. Mesophilic starters are mainly comprised of different *Lactococcus lactis* strains, which grow optimally at around 30°C. When the temperature is elevated during cooking, these lactic acid bacteria are dramatically affected; some strains completely stop growing, while others are hampered significantly, and this happens during most cheese making. After cooking, when the temperature has been lowered, it will take considerable time for the LAB to recover and resume fast growth and acidification. The higher the cooking temperature, the longer this delay will be, and in most cases mesophilic cultures are not compatible with cooking above 39°C. We have previously demonstrated that it is possible to adapt *L. lactis* into growing at higher temperatures, by using an approach termed adaptive laboratory evolution. In the current project, the same approach was applied to a dairy isolate of *L. lactis*, which over the course of time would be adapted to grow well in milk at temperatures exceeding 40°C. Furthermore, we planned to carry out experiments to enhance the butter flavor forming ability of *L. lactis* by natural means, i.e., without using genetic engineering approaches.

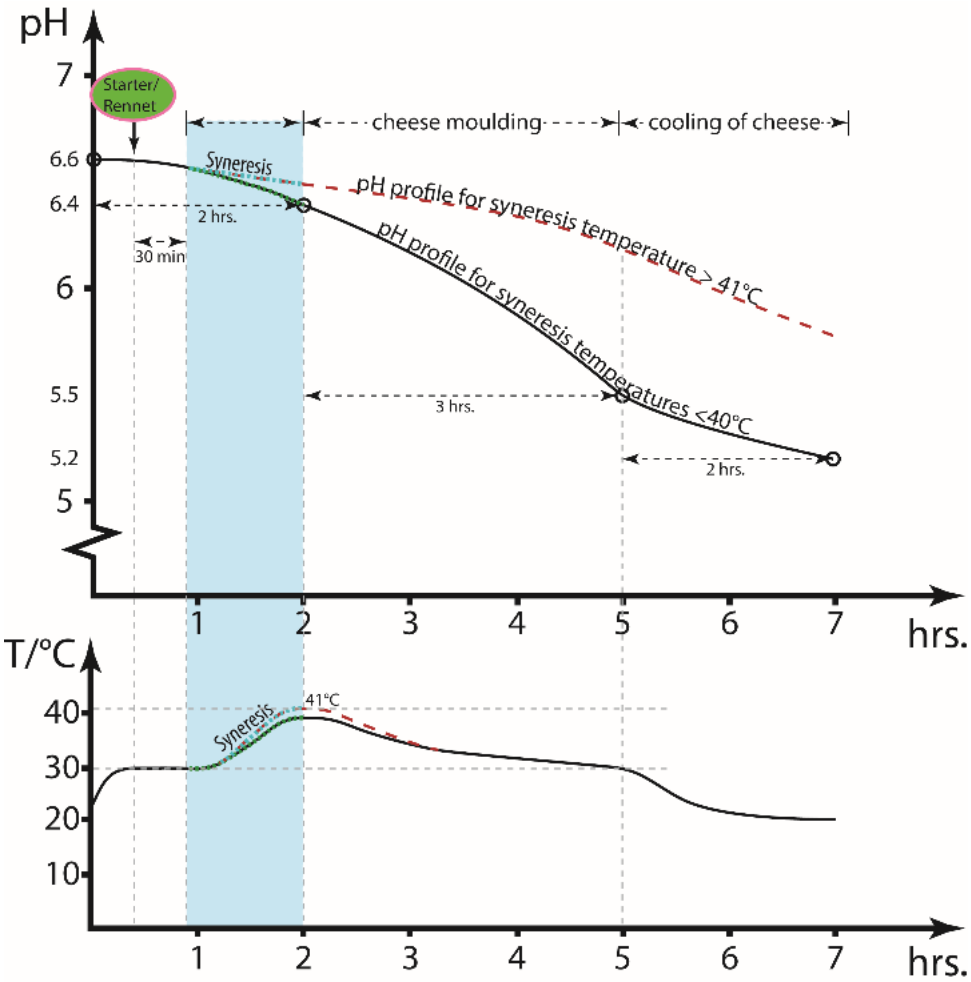


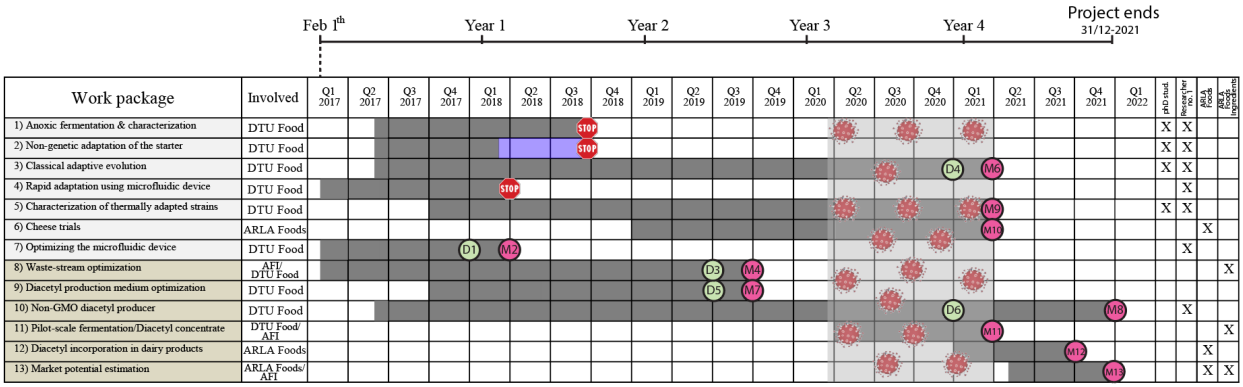
Figure 1. The typical pH and temperature profiles normally observed for cheese production using mesophilic starters at Arla Foods (personal communication Søren Lillevang, Arla Foods). The milk in the cheese vat is heated to 30°C and the starter culture is added at the same time as the rennet. After approximately 30 minutes the coagulation is complete, and the cheese gel is cut, and the cheese curds are stirred slowly. To facilitate expulsion of whey, the temperature is increased to approximately 36-39°C (syneresis). After syneresis the cheese grains are transferred to cheese molds and pressed into large cheeses. At this point, the temperature drops, and acidification rate increases rapidly. If the syneresis temperature exceeds 39°C the starter is affected negatively, resulting in a lag in growth/acidification and flavor formation (red dashed line), interfering with cheese production and quality.

9. Sub-activities in the entire project period

Work Packages (WP) are briefly described below:
WP 1: Here it was investigated whether complete absence of oxygen could make the cheese starter more robust towards high temperatures.
WP 2: We investigated whether pre-adaptation of the starter to elevated temperatures could increase the robustness towards heat.
WP 3: Here long-term adaptive evolution to higher growth temperatures was carried out.
WP 4: Similar to WP 3, just using a microfluidic device, which in principle could speed up the adaptation.
WP 5: Characterization of adapted strains physiologically and by using genomics and transcriptomics.
WP 6: Cheese trials at Arla Foods, testing the thermotolerant strains.

WP 7: Optimizing a microfluidic device for adaptation experiments.
WP 8: Different side streams from Arla Foods Ingredients (AFI) were explored as fermentation substrate for LAB.
WP 9: Media comprised of side streams from AFI were optimized for diacetyl production.
WP 10: A natural butter aroma producing LAB was generated by classical mutagenesis and screening.
WP 11: Pilot-scale fermentations were carried out at AFI.
WP 12: Diacetyl concentrates was incorporated into different dairy products.
WP 13: The market for at butter aroma concentrate was assessed by AFI.

Time schedule:



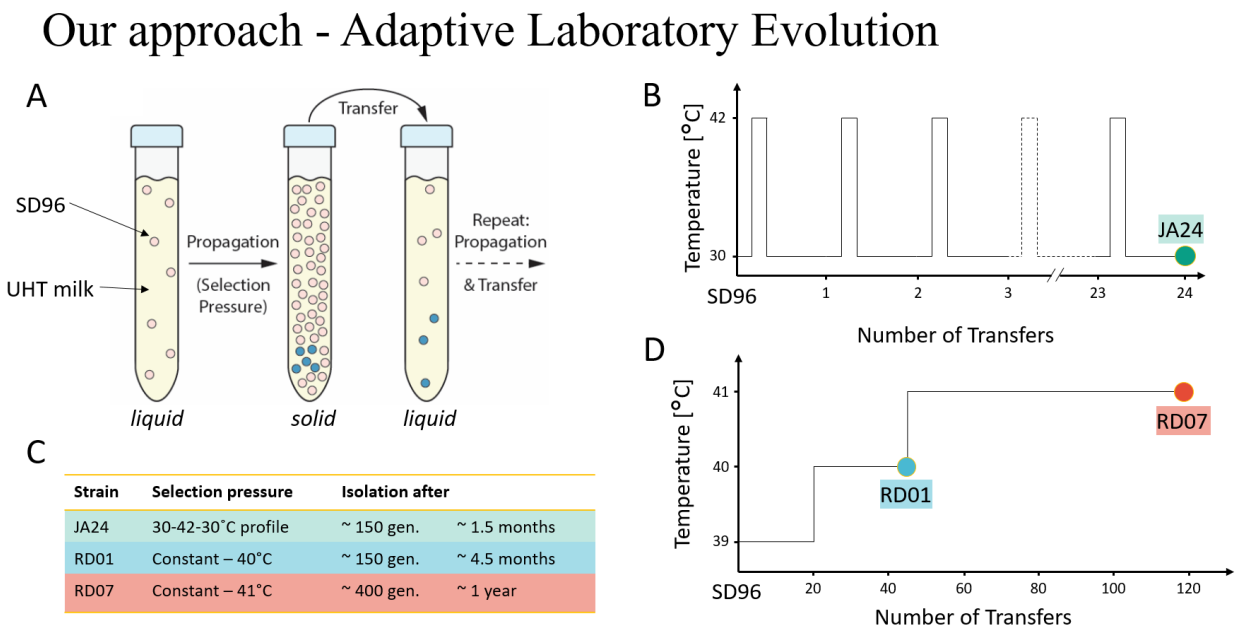
10. Deviations

No larger scientific deviations. Delays in hiring the PhD student extended the project by six months and co-funding from Innovation Fund Denmark by an additional 6 months. The work packages were adjusted accordingly. Changes in personnel, from 2 to 1 researcher, enabled us to extend the project until the end of 2021.

11. Project results

Adaptation of Lactococcus lactis to growth at elevated temperatures

Cooking has a negative effect on the cheese starter culture, and at temperatures above 39°C, a lag of several hours is observed in terms of acidification. We attempted different approaches for reducing the lag phase, e.g., pre-adaptation to high temperatures, however, this had little effect. We have previously seen that heat tolerance can be enhanced by adapting bacteria to elevated growth temperatures, which is why we carried out a classical adaptive laboratory evolution experiment: the dairy strain *Lactococcus lactis* SD96, kindly provided by Sacco Srl., was grown in sterile milk at gradually increasing temperatures for an extended time period. The adaptive laboratory evolution approach is described in Figure 2.



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Strain	Selection pressure	Isolation after	
JA24	30-42-30˚C profile	~ 150 gen.	~ 1.5 months
RD01	Constant – 40°C	~ 150 gen.	~ 4.5 months
RD07	Constant – 41°C	~ 400 gen.	~ 1 year

Figure 2. Adaptive Laboratory Evolution (ALE) of *L. lactis* SD96. A) *L. lactis* SD96 was grown continuously in sterile milk (UHT milk), at gradually increasing temperatures, for up to 1.5 years using a serial dilution approach, which resulted in the more heat tolerant strains RD01 and RD07. B) Alternatively, SD96 was exposed to short periods of high temperature (42°C), effectively emulating a cheese fermentation with an unusually high cooking temperature, which resulted in JA24. C) Table providing an overview of the three strains JA24, RD01 and RD07 isolated in the two ALE's. D shows the temperature profile over time (number of transfers).

We focused on two strains with enhanced tolerance to elevated growth temperature: RD01, which was obtained after 4.5 months of adaptation, grew well at 40°C and RD07, isolated after 1.5 years of adaptation, could grow at 41°C. The strains RD01 & RD07 were characterized in detail, and a surprising bonus of the adaptation was discovered: the adapted strains autolyzed more rapidly after depleting the fermentable sugars. Autolysis normally takes place in the cheese during the ripening and is accelerated by the salt introduced during brining. Figure 3 shows autolysis of the different strains over time.

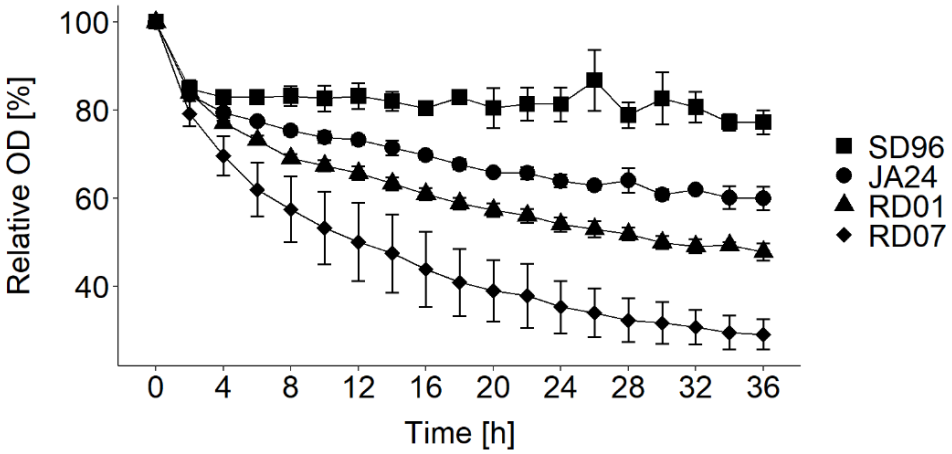

Figure 3. Autolysis of the wild-type *L. lactis* strain SD96 and three derivatives obtained in ALE. Cells were re-suspended in a buffer and cell density (OD or optical density at 600 nm) was measured over time. It can be seen that all adapted cells autolyze more rapidly as compared to the wild-type strain, and in particular RD07 is almost fully autolyzed in 36 hours.

Cheese trials carried out at Arla Foods R&D facility demonstrated that RD01 was superior, in terms of acidification and flavor formation, when a high cooking temperature of 39.5°C was used to prepare a Havarti type cheese (Flora Danica used as control). Genome sequencing revealed that both strains had accumulated various mutations, e.g. both RD01 & RD07 had a mutation is a gene encoding an enzyme involved in cell wall biosynthesis. RD01 had a mutation upstream *codY*, encoding a regulator of branched chain amino acid metabolism, whereas RD07 had a mutation in *purR*, a gene encoding a regulator of purine metabolism. A transcriptomic analysis revealed that both RD01 & RD07 had a greatly upregulated proteolytic system, as the cell envelope bound protease PrtP was upregulated 3 & 4 times, respectively.

In parallel with adaptation to heat tolerance, we investigated the possibility of enhancing butter aroma production potential of *Lactococcus lactis*. As a starting point we used RD1, which grew well in milk and media composed of by-products from Arla Foods Ingredients' whey processing facility. First, we attempted to eliminate the lactic acid forming capacity of RD1 by chemical mutagenesis. Using agar plates containing triphenyl tetrazolium chloride (TTC), we managed to isolate natural varieties unable to generate lactic acid, which appeared as red colonies on TTC agar plates. Characterization revealed that one particular isolate, RD1M5, was particularly well-suited, almost producing no lactic acid. When grown with aeration (in shake flasks), the main fermentation product was acetoin, a compound with a pleasant buttery aroma. The acetoin product yield was > 90%, which was surprising as production of acetic acid, to some extent, was expected.

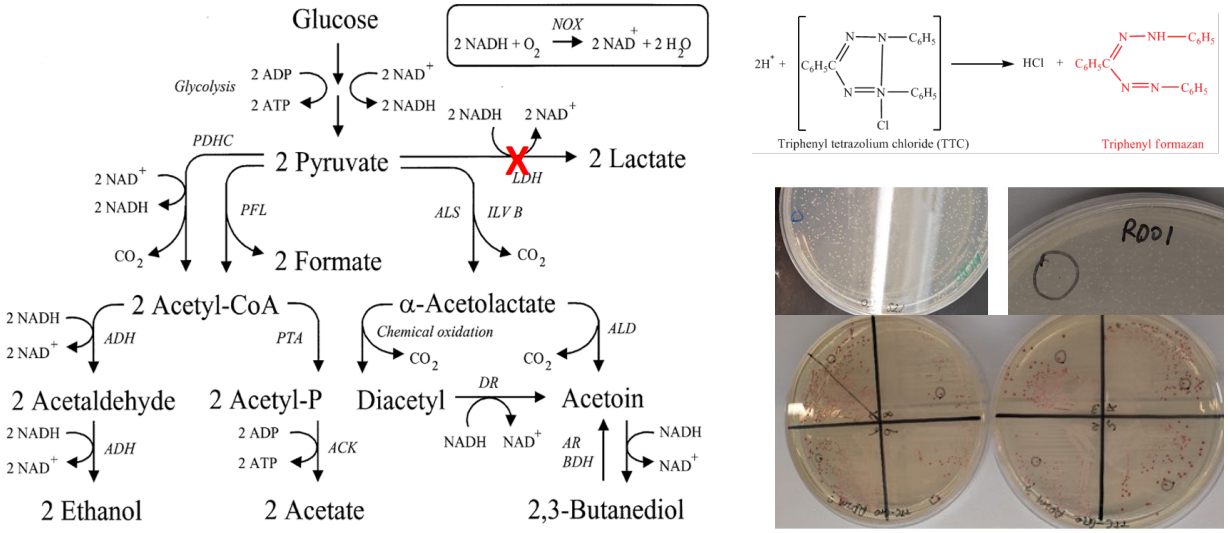

Figure 4. To the left, the central metabolism of *L. lactis* is shown. *L. lactis* generally metabolizes fermentable carbohydrates into lactic acid, and typically > 90% of the sugar metabolized ends up as lactic acid. Minor amounts of formic acid, acetic acid and ethanol are also formed. When *L. lactis* cultures are aerated, other products can be formed, for instance acetoin and diacetyl. The red X indicates that the gene encoding lactate dehydrogenase has been inactivated, thereby eliminating formation of lactic acid. To the top right is shown the chemical structure of TTC (triphenyl tetrazolium chloride), a compound that can be used to screen for strains unable to form lactic acid. To the bottom left is shown red colonies, which are derivatives of *L. lactis* RD01 unable to produce lactic acid.

Acetoin production from whey processing by-products (whey mother liquor, a by-product from lactose production) was explored with a successful outcome. Furthermore, we explored production of diacetyl, a more

potent butter aroma compound, using RD1M5. We knew, from previous work, that that the enzyme acetolactate decarboxylase (ALD), plays a critical role in diacetyl production. When the ALD activity is high, little diacetyl and mostly acetoin is generated, and reducing the activity of this enzyme was therefore deemed crucial. Knocking out the gene encoding ALD turned out to be difficult, which is why we changed strategy into whole-cell catalysis. We discovered that the ALD activity could be reduced merely by reducing the pH of substrate into which the cells were suspended. By growing the cells to high density, and subsequently resuspending them in a substrate with low pH, more than 1 g/L of diacetyl could be generated. Since the diacetyl content of butter and cheese is in the 1-4 mg/kg range, 1liter fermented dairy "waste" provides enough butter aroma for 250-1000 kg of butter or cheese, and optimization is surely possible. Figure 4 provides an overview of the chemistry of the butter flavor production.

During the project, at steering group meetings, we were routinely confronted with the concern that cultures composed of single strains might not be compatible with dairy operations due to the omnipresence of bacteriophages. To address this concern, we elaborated a novel strategy for handling the phage issue. UHT milk is sterile and does not contain any phages. By adding the single-strain starter and rennet to the UHT milk, we managed to encapsulate the bacteria in "cheese curd". This is similar to what is done during the cheese fermentation, where starter and rennet is added almost simultaneously, and the milk gel is formed within approximately 30 minutes. Thus, in cheese fermentations, most of the bacterial growth takes place in the curd. In our case the curd used to encapsulate the starter is free from phages, in contrast to the mildly pasteurized cheese milk and the often phage infected cheese vat. This approach was validated in the laboratory.

In Figure 5 below, curd encapsulated starter was added to milk supplemented with a phage. It is seen that for the non-encapsulated starter the pH drop is small, whereas the protected, curd-encapsulated starter results in a rapid acidification.

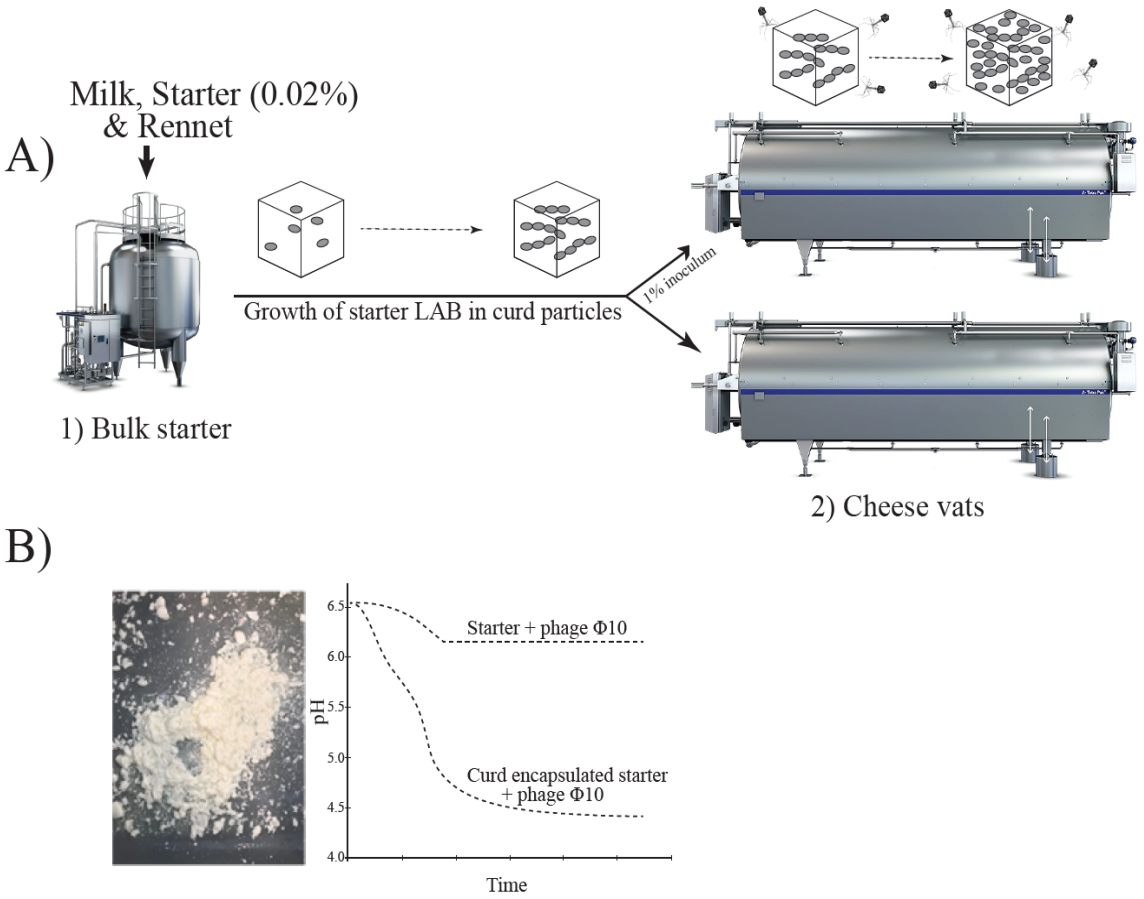

Figure 5. Protecting the cheese fermentation from failure due to phage infection. Cheese cultures are frequently pre-grown in a tank (the bulk starter) to reduce costs. Typically, the main cheese fermentation is inoculated with 1% of the bulk starter culture. By using sterile/high temperature pasteurized milk in the bulk starter vat, it is possible to eliminate or reduce the number of phages sufficiently to avoid significant phage infection. A) By coagulating the milk in the bulk starter tank, the lactic acid bacteria are protected from phages when transferred to the cheese vat. B) Demonstration in the lab where milk containing a lytic phage is inoculated with either unprotected starter or starter encapsulated in cheese curd. As clearly seen, the encapsulated starter rapidly acidifies the milk, whereas the non-protected starter is rapidly killed.

Overall, the project had a successful outcome. We managed to demonstrate that mesophilic starters indeed have potential to be used at elevated temperatures. Adaptive Laboratory Evolution is an excellent approach for increasing the thermal robustness of these starters. We demonstrated a novel application for the by-products of cheese whey processing, namely butter aroma production, and strain RD1M5 turned out to be an excellent production platform for both acetoin and diacetyl.

Finally, we provided a solution for a challenge that since long has plagued the dairies: phages! A minor adjustment of the cheese manufacturing process can completely eliminate any phage issues, which currently is quite costly to handle (excessive cleaning routines etc.).

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

The results obtained should be highly relevant for the dairy industry. In general, curd cooking hampers growth and performance of lactic acid bacteria during most cheese making, and thus, starters with elevated

heat tolerance should be of general interest, and not just for making “Hushållsost”. For an example, in Cheddar cheese fermentations, cooking at 39°C is common. Thus, using thermo-tolerant starters should make cheese production more efficient. Mesophilic starters able to tolerate cooking at temperatures above 45°C could be used for producing novel types of harder cheese variants, thereby expanding the market for cheese.

Through real cheese trials we demonstrated that our single strain was able to produce cheeses superior in flavor and taste to cheeses made with the commercial Flora Danica culture, which demonstrates that there is no need for complex undefined cultures; a single strain can provide all the cheese flavor needed.

Converting low-value side streams into butter aroma is also of relevance. Butter aroma is needed in a wide range of foods, not only dairy foods. For butter, two approaches are currently used to introduce butter aroma; classical butter fermentation, where the sweet butter is fermented using special cultures, and the NIZO method where a concentrated butter aroma concentrate is prepared using special media supplemented with citric acid. For both methods, butter aroma is generated when citric acid is present. In our case, butter aroma is generated from lactose, which allows us to achieve far higher concentrations of diacetyl and acetoin than possible from citric acid.

The final challenge we addressed related to phages. We demonstrated that phages can be pacified, merely by encapsulating the lactic acid bacteria in sterile cheese curd. Failed fermentations due to phages still happen, despite using culture rotation schemes. One disadvantage when using culture rotation is that the cheeses usually end up having different properties, e.g., taste and texture, and this can be avoided when using curd encapsulated cultures.

From the scientific perspective, we have learned a lot about what limits growth of the cheese starter in milk, how the LAB can adapt to higher growth temperatures and that curd encapsulation can slow down diffusion of phages sufficiently to eliminate failed fermentations/dead vats.

We found that adaptation to high growth temperatures resulted in strains that autolyzed more rapidly, a property that could have a great impact on cheese ripening. Further studies on the effect that these strains have on cheese ripening are merited, as cheese ripening is the costliest part of cheese production. Also, it would be interesting to test the curd encapsulation technology in large scale at a dairy. Regarding butter aroma producing strains, we have only scratched the surface. It should be possible to achieve far greater titers, e.g., by strain and fermentation optimization.

13. Communication and knowledge sharing about the project

Papers in international journals:

1. Liu, J., Chan, S. H. J., Chen, J., Solem, C., Jensen, P. R. 2019. Systems Biology - A Guide for Understanding and Developing Improved Strains of Lactic Acid Bacteria. Front. Microbiol. 10:876
2. Dorau, R., Chen, J., Jensen, P. R., Solem, C. 2020. Complete Genome Sequence of *Lactococcus lactis* subsp. *lactis* bv. diacetylactis SD96. Microbiol. Resour. Announc. 9(3)
3. Dorau, R., Chen, L., Liu, J., Jensen, P. R., Solem, C. 2019. Efficient production of α-acetolactate by whole cell catalytic transformation of fermentation-derived pyruvate. Microb. Cell Fact. 18(1):217

4. Liu, J. M., Chen, L., Dorau, R., Lillevang, S. K., Jensen, P. R., & Solem, C. (2020). From Waste to Taste-Efficient Production of the Butter Aroma Compound Acetoin from Low-Value Dairy Side Streams Using a Natural (Nonengineered) *Lactococcus lactis* Dairy Isolate. *Journal of agricultural and food chemistry*, *68*(21), 5891–5899
5. Liu, J.-M., Solem, C., Jensen, P. R. 2020. Harnessing biocompatible chemistry for developing improved and novel microbial cell factories. Microb. Biotechnol. 13(1):54-66
6. Liu, J. M., Chen, L., Jensen, P. R., & Solem, C. (2021). Food grade microbial synthesis of the butter aroma compound butanedione using engineered and non-engineered Lactococcus lactis. *Metabolic engineering*, *67*, 443–452
7. Dorau, R., Chen, J., Liu, J., Ruhdal Jensen, P., & Solem, C. (2021). Adaptive Laboratory Evolution as a Means to Generate Lactococcus lactis Strains with Improved Thermotolerance and Ability To Autolyze. *Applied and environmental microbiology*, *87*(21), e0103521

Easily read papers:
Hårde oste med bedre smag/Mælkeritidende, indsendt 29-09-2017, publiceret 23. marts 2018.

Student theses:
1. Xiaohang Sun (2021-01-25 til 2021-06-25), kandidatspeciale, Udvikling af *Lactococcus lactis* til produktion af aromastoffer.
2. Mengying Xie (2020-03-20 til 2020-08-20), kandidatspeciale, Genetisk karakterisering af termotolerante *Lactococcus lactis* stammer.
3. Monika Karolina Golabek (2017-02-20 til 2017-05-26), specialkursus, Udvikling af aromaproduktion i *Lactococus lactis.*

Oral presentations at scientific conferences, symposiums etc.:
Conference 1. 2017, 12th International Symposium on Lactic acid bacteria, the Netherlands:
Posters:
1. Liu JM, Solem C, Jensen PR. Combining metabolic engineering and biocompatible chemistry for high-yield production of food ingredient and fine chemicals.
2. Liu JM, Kandasamy V, Solem C, Jensen PR. Engineering Lactococcus lactis for growth-coupled production of diacetyl, acetoin isomers and 2,3-butanediol isomers with high titer and high yield.

Conference 2. 2017, 7th International Conference on Biomolecular Engineering, San Diego, USA:
Oral presentation: Liu JM, Solem C, Jensen PR. Combining metabolic engineering and biocompatible chemistry for high-yield production of food ingredient and fine chemicals.

Other:
A book chapter in Metabolic Engineering – Concepts and Applications: Metabolic Engineering of Lactic Acid Bacteria.

14. Contribution to master and PhD education

PhD students: Robin Dorau
Master students: Xiaohang Sun and Mengying Xie.

15. New contacts/projects

New collaborations with Thise and Mammen dairies focusing on cheese ripening.