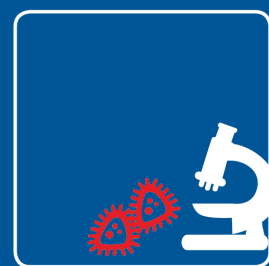


Clarissa Schwab:

BioDairy – Sundheds-biomarkører for mælkeindtag

BioDairy – Health biomarkers of dairy intake



Final report

for collaborative projects funded via the Danish Dairy Research Foundation (DDRF)

1. Title of the project

Danish: Sundheds-biomarkører for mælkeindtag (BioDairy)

English: Health biomarkers of dairy intake (BioDairy)

2. Project manager

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3. Other project staff

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4. Sources of funding

Mælkeafgiftsfonden, co-financing Brødrene Hartmann Fonden, Novo Nordisk Foundation

5. Project period

Project period with DDRF funding: 1.1.2023-30.6.2024

Revised, if necessary: 1.1.2023-31.12.2024 (cost neutral extension)

6. Project summary

Danish:

Baggrund: Laktat er en nøgleforbindelse i fermenterede mejeriprodukter; med en standard portionsstørrelse kunne der indtages mere end 1 g laktat pr. måltid. Efter indtagelse kan yderligere laktat fremstilles ud fra laktose. På trods af det regelmæssige indtag af fermenterede mejeriprodukter er der overraskende lille mekanistisk forståelse af den immunologiske og mikrobielle tarmrespons på kostens laktose og/eller laktat, der potentielt kan have indflydelse på tarmens sundhed.

Formål: BioDairy havde til formål at skabe en platform af værktøjer til at identificere mikrobielle og immunologiske biomarkører for effekten af indtag af fermenterede mejeriprodukter indeholdende laktose og laktat.

Resultater: Ved at kombinere *in vitro* batchfermenteringer af fækal mikrobiota, enkeltkulturstudier med knock-out-mutanter og *in vivo* kohortedata, undersøgte vi tarmmikrobiotaens evne til at fermentere laktose til laktat og videre, vha cross-feeding, til andre metabolitter. Vi identificerede bakterieslægterne *Anaerobutyricum* og *Faecalibacterium* som laktat-fermenterende og butyrat-producerende. Butyrat er erkendt at have sundhedsmæssige effekter. *In vitro*-batchfermenteringer viste at laktat, via cross-feeding førte til dannelse af buyrat eller valerat, hvis repræsentanter af slægten *Megasphaera* var til stede. *In vitro*-data viste endvidere, at tilstedeværelsen af starterkulturen *Streptococcus thermophilus* havde ringe indflydelse på laktoseudnyttelsen, muligvis på grund af manglende evne til at konkurrere med den fækale mikrobiota. I en human kohorte-interventionsundersøgelse førte indtagelse af drænet yoghurt med et højt indehold af laktose og laktat til højere laktat- og valeratniveauer,

Parallelt hermed undersøgte vi den immunmodulerende virkning af laktat og sammenlignede virkningen af laktat med virkningen af butyrat og andre kortkædede fedtsyrer (SCFA) på dendritiske cellers antigenpræsenterende kapacitet. Vi viste, at meget højere koncentration af laktat er påkrævet for at påvirke cellernes kapacitet til at reagere på forskellige mikrobielle stimuli end de koncentrationer der kræves for SCFA, hvilket er i overensstemmelse med receptorernes respektive bindingsaffinitet. Dette indikerer, at i al fald når det gælder påvirkning af immuncellerne, er fermentering af laktat til butyrat eller valerat vigtig for en effektiv modulering af immunsystemet. Vi har endvidere etableret teknikker til at vurdere virkningen af laktat og laktose på epitelbarrieren og på immunsystemet og indsamlet tarmprøver, der nu analyseres for barrierens genekspression, immunpolarisering, SCFA-produktion og mikrobiotasammensætning.

Konklusion: Alt i alt identificerede BioDairy vigtige immunologiske og mikrobielle biomarkører, der relaterer sig til forbruget af fermenteret mejeri.

English:

Background: Lactate is a key compound of fermented dairy; with a standard serving size more than 1 g lactate could be ingested per meal. Following ingestion, additional lactate can be produced from lactose. Despite its regular consumption, there is surprisingly little mechanistic understanding of the immunological and microbial intestinal response to dietary lactose and/or lactate with potential impact on gut intestinal health.

Aim: BioDairy aimed at generating a platform of tools to identify microbial and immunological biomarkers in response to fermented dairy consumption containing lactose and lactate.

Results: Combining *in vitro* fecal microbiota batch fermentations, single culture studies including knock-out mutants and *in vivo* cohort data, we investigated the ability of intestinal microbiota to digest lactose and to cross-feed on the important fermentation intermediate lactate. We identified the bacterial genera *Anaerobutyricum* and *Faecalibacterium* as utilizers of lactose that produce the metabolite butyrate, which has been associated with health benefits. Based on *in vitro* data, lactate cross-feeding led to the formation of buyrate, or to valerate if representatives of the genus *Megasphaera* were present. Also based on *in vitro* data, the presence of the starter culture *Streptococcus thermophilus* had little impact on lactose utilization possibly due to low competitiveness against the fecal microbiota. In a human cohort intervention study, consumption of drained yogurt containing lactose and lactate led to higher lactate and valerate levels transferring *in vitro* observations to *in vivo*.

In parallel, we investigated the immunomodulatory effect of lactate and compared the effect of lactate with the effect of butyrate and other short chain fatty acids (SCFA) on the antigen presenting capacity of dendritic cells (DC). We demonstrated that much higher levels of lactate were required to affect the DCs capacity to

respond to various microbial stimuli compared to the SCFA which is in accordance with the receptors' respective binding affinity. This indicates that fermentation of lactate to butyrate or valerate is important for an efficient modulation of the immune system, at least in respect to the immune cells. We have established techniques to assess the effect of lactate and lactose on the epithelial barrier and on the immune system and collected intestinal samples to be assed for SCFA production and microbiota composition.

Conclusion: Together BioDairy developed analytical tools and identified important immunological and microbial biomarkers that relate to the consumption of fermented dairy.

7. Project aim

Danish: Det overordnede mål med BioDairy var at bestemme virkningen af laktose og laktat leveret gennem fermenteret mejeri/eller som et fødevarerilsætningsstof/eller som en del af et synbiotikum på mikrobielle interaktioner og værtsimmunrespons.

English: It was the overall objective of BioDairy to determine impact of lactose and lactate delivered through fermented dairy/or as a food additive/or as part of a synbiotic on microbial interactions and host immune response.

8. Background for the project

Fermented dairy products can deliver lactate as well as lactose, which in turn can be fermented to lactate by viable lactate forming starter cultures, probiotics, or the gut microbiota. Lactate may act as a regulating molecule on host cells inducing regulatory cells and acting anti-inflammatory (iScience 2021;24:103067; Front Microbiol 2019;10:2839). Lactate acts primarily through binding to the receptor GPR81, which belongs to the same family as the short chain fatty acids (SCFA) receptors GRPR41 and GPR43, exerting the same type of effects. The specific contribution of lactate and SCFA, respectively, on immune regulation and gut health has not been completely resolved, and whether SCFA and lactate interact and contribute equally is not known. As both lactose and lactate may be converted into the SCFA propionate and butyrate through microbial metabolite cross-feeding, it is difficult to deduce whether the lactose and lactate from the gut acts directly on host cells or indirectly upon conversion to propionate and butyrate.

The concentration in gut tissue is unknown and surprisingly little information on lactate absorption processes exists. In sheep, absorption of lactate depended on concentration and pH (J Exp Zool A Comp Exp Biol 2003;295:29); in pigs, absorption behavior was more similar to glucose than to short chain fatty acids (SCFA, Livestock Sci 207;109:118). Concurrently, lactate is produced endogenously in host cells and is secreted from actively metabolizing tissue (such as active muscles). Therefore, it may be difficult to estimate the exogenous lactate present in circulation and hence estimate the amount of absorbed lactate. The normal plasma lactate concentration is 0.3–1.3 mmol/L and may depend on specific conditions (Cont Edu Anaest Crit Care Pain 2006;6:128).

In order to assess lactose, lactate and foods containing these ingredients for their direct or indirect effects on immune-regulation and gut barrier integrity, we want to establish a research platform that can characterize the effects, e.g. assessing whether the effects are specific to the lactate or primarily caused by the microbial conversion of lactate to SCFA. We will investigate the role of dietary lactose on starter culture driven lactate production using a novel approach combining B-GAL and LDH inactivation mutants and concurrent lactose feeding in wild-type and GPR81 mouse models.

If lactate proves to play a key role in immune regulation, lactate and lactose and foods containing these molecules may represent important alternatives to consumers that of various reasons have difficulties eating a high fiber diet (e.g. IBS patients, elderly with low appetite). Moreover, in contrast to most fibres, lactose is soluble and can be hydrolyzed by the activity of a single enzyme (B-GAL). Lactose may therefore be converted into lactate in the upper part of the gastrointestinal tract affecting a larger proportion of tissue.

9. Sub-activities in the entire project period

Task 1. Develop and characterize a mouse model not responding to lactate.

Deliverable: We identify microbial and immunological biomarkers that differ between wild-type and lactate receptor deficient mice.

Task 2. Generation of starter culture LDH deletion mutants through microbial engineering.

Deliverable: We will provide B-GAL and LDH deletion mutants that are characterized in detail.

Task 3. Determine the influence of dietary lactate on immune regulation.

Deliverable: We will reveal to which extent dietary lactate exerts a direct effect on the gut mucosa and/or the mucosal immune system.

Task 4. Impact of microbial and dietary manipulation on intestinal microbiota and major fermentation metabolites in WT and GPF81 mice.

Deliverable: We will identify microbial and host biomarkers of lactose/lactate intake.

10. Deviations

Task 1. We realized that by including a GPR43KO and/or a GPR109a KO mouse we will be able to assess whether the mouse reacts to lactate or whether it primarily reacts to the butyrate that can be formed from lactate. We have applied for and received permission from the Danish Animal Testing Authority to work with the KO mice, and are now conducting a pilot study with wild type mice, where we test the effect of lactose in order to test all analyses of a feeding trial. The work with KO mice will be carried out in 2025 when the mice are ready. (Scientific and time-table related deviation)

Task 2. To delete the *ldh* gene, we generated plasmids for homologous recombination of *ldh*. Since transformation using the method described by Blomqvist et al (Lett Appl Microbiol 2010;50:314), which exploits natural competence of *S. thermophilus* for transformation, was not successful, we established a method to produce electrocompetent cells that successfully transformed the strains. We had problems with the selection marker (kanamycin) as *S. thermophilus* adapted to this antibiotic and stopped the experiment.

11. Project results

Task 3. Determine the influence of dietary lactate on immune regulation.

Background and Aim: To study and compare direct and indirect effects we established an antigen presenting cell model where we can measure the effect of lactate, butyrate and other SCFA on the inflammatory response *in vitro*, which includes effects on the inflammatory potential as well as their differentiation state. Animal feeding experiments have been performed to compare effects *in vitro* with effects observed *in vivo*.

Results: The results suggest that the major effect of lactate may be through the degradation products butyrate and valerate as the effect of these compounds is more than 100-fold stronger than the effect of lactate. Moreover, we found that the cells in the presence of butyrate but not of lactate attain a highly tolerogenic phenotype still able to present antigens. Various protocols and analytical methods have been established to assess the effects of lactose/lactate on the gut barrier, the underlying gut-associated lymph nodes and the systemic immune response, and samples from the *in vivo* effect of lactose feeding are currently being analyzed. Two manuscripts are in preparation.

Task 4: The effect of microbial and dietary manipulation on the gut microbiota and key fermentation metabolites

Substudy 1:

Background and Aim: The consumption of fermented food has been linked to positive health outcomes possibly due to interactions of food components with the intestinal microbiota. To investigate whether dairy-related nutritional and microbial modulation impacted intestinal microbiota composition and activity, we employed static fecal microbiota batch fermentations and a dairy model system consisting of lactose and *Streptococcus thermophilus* wildtype and β -galactosidase deletion mutant in combination with single culture studies to investigate of selected strains to use lactose and express the enzyme β -galactosidase.

Results: 16S rRNA gene-based microbial community analysis showed that supplementation of lactose increased the abundance of the families *Bifidobacteriaceae*, and of the genera *Anaerobutyricum* and *Faecalibacterium* spp. The supplied lactose was hydrolysed within 24 h of fermentation and led to higher expression of β -galactosidase enzymes of the fecal microbial community. Targeted protein analysis using LC-MS confirmed that bifidobacteria contributed most β -galactosidases together with other taxa including *Escherichia coli* and *Anaerobutyricum hallii*. Lactose addition led to up to 1.8-fold higher levels of butyrate compared to controls likely due to lactate-based crossfeeding and direct lactose metabolism by butyrate producing *Anaerobutyricum* and *Faecalibacterium* spp. Representative strains of both genera used lactose to produce butyrate when cultivated in single cultures. When supplemented at around $5.5 \log \text{ cells mL}^{-1}$, *S. thermophilus* or its beta-galactosidase negative mutant outnumbered the indigenous *Streptococcaceae* population at the beginning of fermentation but had no impact on lactose utilisation and final SCFA profiles.

Conclusion: This study brings forward new insight on how major constituents of fermented dairy affect intestinal microbial ecology and activity when supplied together or alone. We provide evidence that lactose availability increased the production of butyrate by fecal microbiota through cross-feeding and did not observe contribution of starter cultures to lactose metabolism possibly due to a lack of competitiveness. The methodological set-up that was used in this study can be implemented in future studies to investigate the impact of other fermented food and their major components on intestinal microbiota composition and activity.

Substudy 2:

Background and Aim. The human gut hosts an ecosystem of microbes contributing to the production of SCFA through fermentation and chain elongation. The SCCA with five carbons in the backbone, valerate, is frequently detected in human fecal samples, but little is known about the microbial activities that drive intestinal valerate formation. In this study, we combined co-culture studies and *in vitro* fecal microbiota batch fermentations ($n=20$, human donors) with the analysis of a female cohort ($n=49$) that monitored and modified dairy consumption, to study the role of lactose and lactate in the microbial formation of valerate.

Results. In co-culture with the lactose-utilizing and lactate-producing *Streptococcus thermophilus* LMG 18311 valerate was formed by *Megasphaera elsdenii* confirming cross-feeding activities with a food culture. *In vitro*, valerate was produced by the majority of fecal microbiota in the control medium. Lactose addition increased valerate formation of fecal microbiota that harboured *Megasphaera*, while valerate formation was decreased when *Megasphaera* was low or not detectable. The addition of *M. elsdenii* to fecal fermentations increased valerate production in 90% of donors suggesting the possibility of microbiota modulation. *In vivo*, the occurrence of *Megasphaera* in the cohort participants was higher in women that consumed fermented dairy compared to non-consumers. Consumption of fermented dairy (intervention and regular consumers) during the three-months study period increased the number of *Megasphaera* positive carriers. Intervention with drained yogurt (skyr) led to significantly higher fecal microbial diversity and relative abundance of *Streptococcaceae* likely due its presence in drained yogurt. Compared to the control group; fecal levels of lactate and valerate were significantly higher in the intervention group. Both *in vitro* and *in vivo*, there was co-occurrence of the lactate-producer *Lactobacillus* and *Megasphaera*.

Conclusion. This study brings forward new mechanistic understanding of the intestinal microbial formation of the SCCA valerate during fermented dairy consumption. Our findings identified *Megasphaera* as a key microbe in lactate-driven valerate production.

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

Fermented dairy is a regular component of the (Danish) meal plan (Nutrients 2018;10:1085). BioDairy provides and tested an experimental platform that contributed in the deciphering of the individual role of compounds of fermented dairy (e.g. lactose, lactate and starter cultures) on the complex interplay of host and intestinal microbiota. The mechanistic understanding gained by BioDairy will allow to sustain the market position of dairy producers. BioDairy is especially linked to the DDRF Strategy Focus Point 'Foods for a healthier life' providing the scientific background on how the major components of fermented dairy, lactose and lactate contribute to health through microbial and host interactions in the gastrointestinal system.

13. Communication and knowledge sharing about the project

Papers in international journals: 1 accepted, 1 under review, 2 in preparation:

Qing Li, Angeliki Marietou, Freja Foget Andersen, Jiri Hosek, Carsten Scavenius, Jianbo Zhang, Clarissa Schwab. '*In vitro* investigations on the impact of fermented dairy constituents on fecal microbiota composition and fermentation activity'. Accepted for publication in Microbiology Spectrum, January 2025

Lucía Huertas-Díaz, Mense Elvan, Angeliki Marietou, Jiri Hosek, Line Thams, Line Barner Dalgaard, Mette Hansen, Clarissa Schwab. 'Megasphaera contributes to lactate-driven valerate production in the human gut'. In preparation

Zhuqing Xu, Danny Blichfeldt Eriksen, Peter Riber Johnsen, Dennis Sandris Nielsen, Hanne Frøkiær. 'The effect of microbial metabolites from colonic protein fermentation on bacteria-induced cytokine production in dendritic cells' has been successfully submitted online and is presently being given full consideration for publication in 'Molecular Nutrition and Food Research' Submitted

Ida B Christensen, Mads T Bruun, Conan C Cassidy, Hanne Frøkiær 'Immune modulating effect of Lactobacillus spp.-derived metabolites and short chain fatty acids on dendritic cells stimulated with *Staphylococcus aureus* Clonal Complex-type 1 associated with atopic dermatitis'. In preparation

Easily read papers: 1 contribution to Mælkeritidende:

Schwab Clarissa, Frøkiær Hanne. 'Gut Microbes love lactose'. 2024

Student theses: 2 bachelor theses, 2 Master thesis

Freja Foget Andersen. 'The role of lactase active starter cultures on intestinal lactose utilization'. AU Bachelor Thesis in Biotechnology 2023

Kathrine Bendixen. 'Generation of *Streptococcus thermophilus* *ldh* deletion mutant'. AU Bachelor Thesis in Biotechnology 2023

Conan C. Cassidy and Mads Tranholm Bruun. 'Lactate - An immunomodulating endogenous and exogenous component' KU master thesis in Biotechnology 2023

Evelyn Pil: 'Immunomodulatory effects of the gut metabolites lactate and butyrate' KU master thesis in Immunology and Inflammation, 2024

Oral presentations at scientific conferences, symposiums etc.:

Angeliki Marietou, AU-BCE, Poster presentation at Danish Microbiology Conference 2023

Qing Li, AU-BCE, Poster and Pitch Presentation at Arla Food for Health 2023

Lucía Huertas-Díaz, AU-BCE, Poster and Pitch Presentation at Arla Food for Health 2024

Lucía Huertas-Díaz, AU-BCE, Poster and Pitch Presentation at Seeon Conference, Germany, 2024

Clarissa Schwab, AU-BCE, Invited Presentation at Arla Amba Minisymposium, 2023

14. Contribution to master and PhD education

KU, 3 Master theses, 2 Master theses that started August 2024. 1 PhD student since 2024

AU, 2 Bachelor theses, 1 PhD student contributing experiments and student supervision in 2023 and 2024

15. New contacts/projects

We have received funding for follow-up from Arla amba and the Leo Foundation. We have established collaboration with the STENO Center Aarhus and the Inselspital Bern in Switzerland.