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Benedikte Grenov:

MicroGAM – Mælk og mikrobiota dysbiose i underernærede børn

MicroGAM – Milk and microbiota dysbiosis in malnourished children

Mejeribrugets
ForskningsFond

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Final report

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1. Title of the project

Danish: Mælk og mikrobiota dysbiose i underernærede børn

English: Milk and microbiota dysbiosis in malnourished children

2. Project manager

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4. Sources of funding

Danish Dairy Research Foundation

Co-financing of PhD student via China Scholarship Council (CSC)

5. Project period

Project period with DDRF funding: Jan 2019 – Dec 2021

Revised, if necessary: Jan 2019 – Dec 2022

6. Project summary

Danish:

Underernæring, der hæmmer højdevækst og mental udvikling hos børn, er stadig en stor udfordring i mange lavindkomstlande. En usund tarmmikrobiota kan være medvirkende årsag hertil. Vi undersøgte tarmmikrobiomet fra 1-5 årige ugandiske børn med væksthæmning før og efter de fik kosttilskud med og uden hhv. mælkeprotein og vallepermeat. Vi fandt at amning havde størst betydning for sammensætning af tarmmikrobiomet. Blandt ammede børn havde sojaprotein en effekt på tarmmikrobiomets sammensætning af bakterier, mens mælkeprotein havde en effekt blandt ikke-ammede børn. Vallepermeat havde kun begrænset effekt. I en delgruppe af børnene blev det undersøgt om de forskellige kosttilskud påvirkede tarmviromet (bakteriofager og andre vira), men det var ikke tilfældet. Projektets resultater kan fremover have betydning for udvikling af kosttilskud til underernærede børn.

English:

Malnutrition, which inhibits linear growth and cognitive development in children, is still a major challenge in many low-income countries. An unhealthy gut microbiota may be a contributing factor to this. We examined the gut microbiome from 1-5 year old Ugandan children with stunted growth before and after they received a nutritional supplement with and/or without milk protein and whey permeate. We found that breastfeeding had the greatest impact on the composition of the gut microbiome. Among breastfed children, soy protein had an effect on the gut microbiome's bacterial composition, while milk protein had an effect among non-breastfed children. Whey permeate had only limited effect. The gut virome was analysed in a subset of the children, but the nutritional supplements did not influence the gut virome differently. The project's results may in the future be important for the development of nutritional supplements for malnourished children.

7. Project aim

Danish:

Underernæring er fortsat en global udfordring. På trods af forbedringer i kvaliteten af ernæring er behandlingsresultaterne stadig lavere end forventet. Dårlig tarmfunktion og tarmmikrobiota dysbiose influerer formodentlig på absorption og udnyttelse af ernæringen. Projektets formål var at undersøge effekter af mælkekomponenter på reetablering af tarmmikrobiotaen i underernærede børn fra Uganda. Desuden blev det undersøgt, om reetablering af tarmmikrobiotaen var associeret med øget vækst.

English:

Malnutrition remains a global challenge. Despite improvements in the quality of nutritional treatment, the outcomes remain lower than expected. Poor gut function and gut microbiota (GM) dysbiosis may affect the absorption and utilization of food. The purpose of the project was to investigate the effect of milk components on recovery of GM dysbiosis in malnourished children from Uganda. In addition, we examined if recovery of the gut microbiota was associated with growth.

8. Background for the project

More than 40% of childhood deaths have been attributed to undernutrition (1). Despite development of therapeutic foods and guidelines designed for inpatient and community-based management of malnourished children, outcomes are still below expectations. Underlying causes of malnutrition seem not to be treated sufficiently with current protocols. Recent research has indicated that gut function and gut microbiota (GM) dysbiosis are involved in development of and/or maintaining acute malnutrition (2–4). A study followed a cohort of Malawian twins and evaluated the GM from twin pairs where one twin developed oedematous severe acute malnutrition (SAM;kwashiorkor) and the other did not (2). The GM from the twins that developed kwashiorkor had a lower diversity and when the GM from twin pairs was transferred to germ-free mice, the mice transferred fecal content (GM) from a child with kwashiorkor became malnourished when fed a maize diet similar to the diet of Malawian children. Another birth cohort was followed in Bangladesh and children who became malnourished had a delayed development of GM diversity compared to non-malnourished controls (3). Subsequent animal studies have further indicated a possible causal role of poor GM in development of malnutrition (5).

Diet can modulate the composition of the GM and especially during infancy and early childhood where development of a more stable and mature GM offers a window of opportunity for this (6). We have recently shown, that during treatment and subsequent recovery of a large cohort (n = 400) of children suffering from SAM, the GM undergoes profound and predictable changes as overall health improves (7). However, the effect of milk components on GM has to our knowledge not been investigated in malnourished children. However, animal studies have shown that casein and whey protein as well as the protein to carbohydrate ratio affected the GM in mice and whey protein isolate resulted in increased proportions of *Lactobacillacea* and decreased proportions of *Clostridiaceae* in the fecal GM (8). Studies in sows and piglets found a higher fecal content of lactobacilli and bifidobacteria and total bacteria after consumption of liquid whey in addition to standard feed compared to controls receiving standard feed and water (9) and another study in piglets found higher lactobacilli counts in caecum after adding lactose to the diet (10). A strong correlation was observed between GM and growth parameters in pre-pubertal rats. The rats were first fed ad libitum followed by 40% food restriction and then ad libitum feeding with either vegetable, casein or whey as the sole source of protein (11). Recently, we have completed the characterization of GM development during malnutrition of piglets (7 weeks with maize as only feed) followed by refeeding with corn soy blend fortified with phosphorus (CSB+), phosphorus and skim milk powder (CSB++), and phosphorus and whey permeate (CSB+P)). During malnourishment a very skewed GM with low diversity developed. Refeeding resulted in partly or near-complete restoration of GM diversity. GM recovery was superior in piglet receiving CSB with the skim milk or whey permeate (12).

In addition to high bacterial cell numbers and taxa diversity, the gut is home to massive numbers of bacteriophages. Their role is not fully understood, but they play important roles in shaping/maintaining the GM community also early in life (13). SAM has been shown to be associated with not only alterations/dysbiosis in the prokaryotic component of the gut, but also the gut virome is significantly altered and “immature for age” (i.e. being less developed, than one would expect, considering the chronological age of the child) (14). Reyes et al. (14) reported that in children suffering from SAM, refeeding with RUTF is not sufficient to fully restore GM biosis and push the gut virome back towards a “mature for age”-stage, but whether other types of refeeding such as supplementation with milk components will be able to do so remains unknown.

9. Sub-activities in the entire project period

The nutrition intervention trial was conducted between February and December 2020. Data was entered and cleaned between January and June 2021.

Faecal samples were sent from Uganda to Denmark in January 2021. During 2021, DNA from all faecal samples (close to 1500) was extracted, the quality was confirmed, libraries for 16S rRNA gene (V3 region) amplican sequencing was prepared and DNA sequencing was performed.

In 2022, gut microbiome profiles were associated with phenotypical and clinical data including intervention group, age, sex, height-for-age z-score, weight-for-age z-score, inflammation markers etc.

A manuscript has been prepared by the PhD student, and it is partly revised in collaboration with co-authors.

The gut virome from a subsample (n=230 faecal samples) was analysed by two master students under supervision of experienced lab technicians, Dennis Nielsen and Benedikte Grenov from December 2022 to June 2023. A virome manuscript is under preparation.

10. Deviations

Scientific deviations: In the application we wrote that faecal samples from 2 studies, MAGMAM and LARISAM would be analysed. However, unfortunatly, the LARISAM study was terminated before the samples were collected. Therefore, the number of samples from MAGMAM (later renamed MAGNUS) was increased and the full number of samples was unchanged.

Timeline deviations: The nutrition intervention study was delayed due to slow study approval process in Uganda. Faecal samples were collected and analysed more or less as planned. The PhD student has prepared a draft article based on 16S rRNA gene amplicon sequencing analyses. The finalizing and submission of the article has been delayed some, but it is expected that the manuscript will be ready for submission within 1-2 months.

Economical deviations: None

11. Project results

11.1 Study population

In total, 750 children from Eastern Uganda aged 1-5 years were recruited to 5 study groups - as shown in figure 1 below. The children were randomly assigned to one of 4 food supplements or no supplementation for a period of 12 weeks. One 100 g sachet of large-quantity lipid-based nutrient supplement was given per day. The composition of the supplements is shown in table 1 below.

	+ Milk protein isolate (MPI)	+ Soy protein Isolate (SPI)
Whey permeate (+ WP)	LNS-LQ + MPI + WP n=150	C. LNS-LQ + SPI + WP n=150
No whey permeate (-WP)	LNS-LQ + MPI – WP n=150	D. LNS-LQ + SPI - WP n=150

Family diet
E. Reference n=150

Figure 1: Four large-quantity lipid-based nutrient supplements (LNS-LQ) in a two-by-two factorial design. Reference group continues with family diet. n= number of children allocated. MPI: Milk protein isolate, WP: whey permeate, SPI: soy protein isolate

Table 1: Composition of large-quantity lipid-based nutrient supplements with milk or soy protein and whey permeate or maltodextrin

		MPI[1] + WP	MPI[1] - WP	SPI + WP	SPI - WP
Protein quality	DIAAS[2]				
6-35 months		0.93	0.93	0.78	0.78
36+ months		1.10	1.10	0.93	0.93
Nutrient	**Per 100 g**				
Macronutrients					
Energy	kcal	531	535	530	534
Carbohydrates	g	42	43	42	43
Lactose	g	15.7	0.4	15.3	0
Proteins	g	13.9	13.5	13.9	13.5
Milk	g	7.15	6.75	0.40	0
Vegetable	g	6.75	6.75	13.50	13.50
Lipids	g	33.7	33.7	33.7	33.7
Linoleic acid C18:2	g	3.0	3.0	3.0	3.0
Linoleic acid C18:3	g	0.5	0.5	0.5	0.5
Minerals					
Calcium	mg	691	594	691	594
Copper	mg	1.65	1.65	1.65	1.65
Iron	mg	12	12	12	12
Iodine	mg	127	113	127	113
Magnesium	mg	199.2	175.8	199.2	175.8
Manganese	mg	1.8	1.8	1.8	1.8
Phosphorous	mg	661	539	661	539

Potassium	mg	1315	985	1315	985
Sodium	mg	84	7	156	79
Selenium	µg	30	30	30	30
Zinc	mg	12.5	12.5	12.5	12.5
Vitamins[3]					
Vitamin A	µg	619	619	619	619
Vitamin B1	mg	1.2	1.1	1.2	1.1
Vitamin B12	µg	3.2	3.0	3.2	3.0
Vitamin B2	mg	3.1	2.8	2.7	2.4
Niacin	mg	14.9	14.6	14.9	14.6
Pantothenic acid	mg	5.7	4.5	5.7	4.5
Vitamin B6	mg	2.1	2.0	2.1	2.0
Biotin	µg	74.1	67.6	74.1	67.6
Folic acid	µg	223	223	223	223
Vitamin C	mg	67.9	67.6	67.9	67.6
Vitamin D	µg	16.9	16.9	16.9	16.9
Vitamin E	mg	18	18	18	18
Vitamin K	µg	30	30	30	30

[1] Milk protein isolate (casein and whey). [2] Digestible amino acid score. [3] Target values by the end of the products' shelf life.

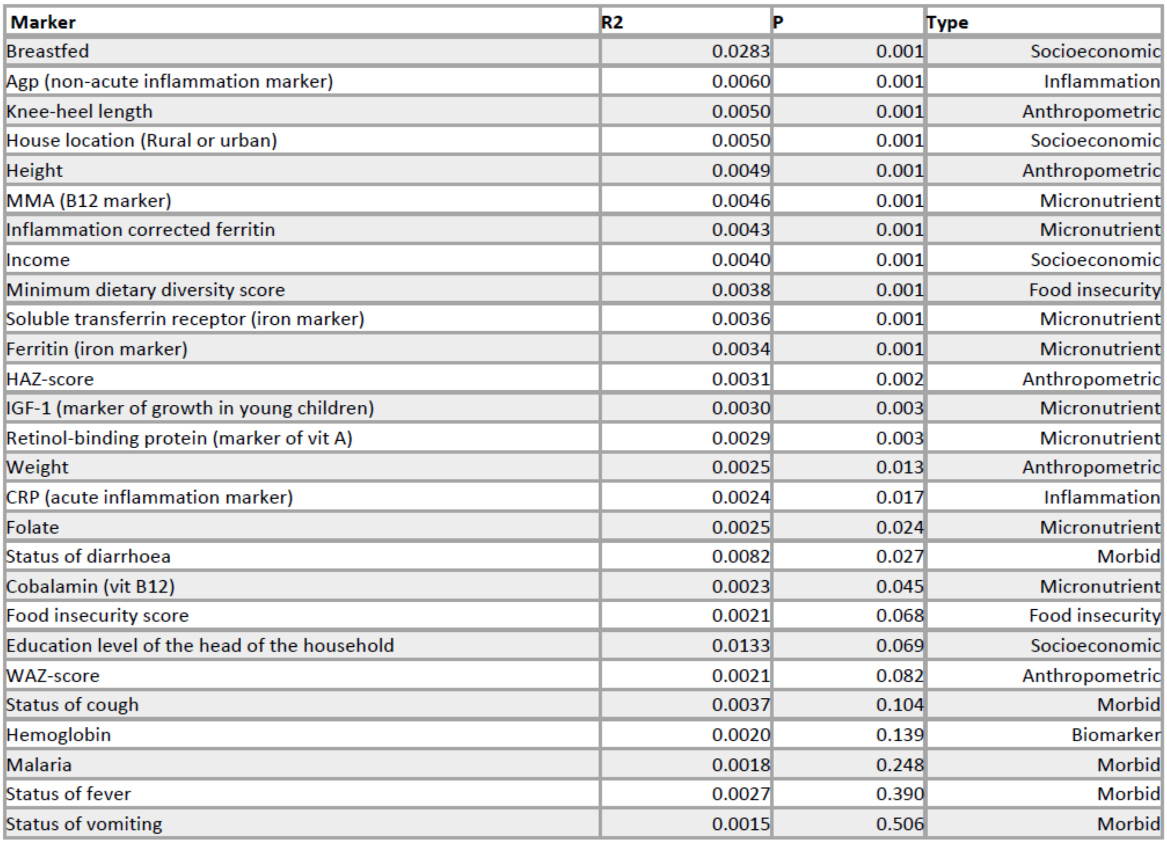
The median age (interquartile range) of the children was 30 (23, 41) months. Approximately half of the children were severely stunted (height-for-age < -3) and 13% were still breastfed (almost all below 2 years of age).

Stool samples were collected at baseline and after 12 weeks and the GM was analysed.

11.2 GM at baseline

Of all nutritional, clinical (including different blood markers) and demographic parameters analysed, breastfeeding was the most significant factor associated with GM bacterial composition at baseline (Table 2). Breastfed children had a lower bacterial diversity (Fig 2B, 2C), more bifidobacteria and lactobacilli and fewer *Prevotella* and *Faecalibacterium* than non-breastfed children (Fig 2A, 2E).

Table 2: Breast feeding status has strong impact on gut microbiome composition among Ugandian children up to the age of 5 years; db-RDA constrained-components based on Bray-Curtis dissimilarity metrics.



Marker	R2	P	Type
Breastfed	0.0283	0.001	Socioeconomic
Agp (non-acute inflammation marker)	0.0060	0.001	Inflammation
Knee-heel length	0.0050	0.001	Anthropometric
House location (Rural or urban)	0.0050	0.001	Socioeconomic
Height	0.0049	0.001	Anthropometric
MMA (B12 marker)	0.0046	0.001	Micronutrient
Inflammation corrected ferritin	0.0043	0.001	Micronutrient
Income	0.0040	0.001	Socioeconomic
Minimum dietary diversity score	0.0038	0.001	Food insecurity
Soluble transferrin receptor (iron marker)	0.0036	0.001	Micronutrient
Ferritin (iron marker)	0.0034	0.001	Micronutrient
HAZ-score	0.0031	0.002	Anthropometric
IGF-1 (marker of growth in young children)	0.0030	0.003	Micronutrient
Retinol-binding protein (marker of vit A)	0.0029	0.003	Micronutrient
Weight	0.0025	0.013	Anthropometric
CRP (acute inflammation marker)	0.0024	0.017	Inflammation
Folate	0.0025	0.024	Micronutrient
Status of diarrhoea	0.0082	0.027	Morbid
Cobalamin (vit B12)	0.0023	0.045	Micronutrient
Food insecurity score	0.0021	0.068	Food insecurity
Education level of the head of the household	0.0133	0.069	Socioeconomic
WAZ-score	0.0021	0.082	Anthropometric
Status of cough	0.0037	0.104	Morbid
Hemoglobin	0.0020	0.139	Biomarker
Malaria	0.0018	0.248	Morbid
Status of fever	0.0027	0.390	Morbid
Status of vomiting	0.0015	0.506	Morbid

Figure 2

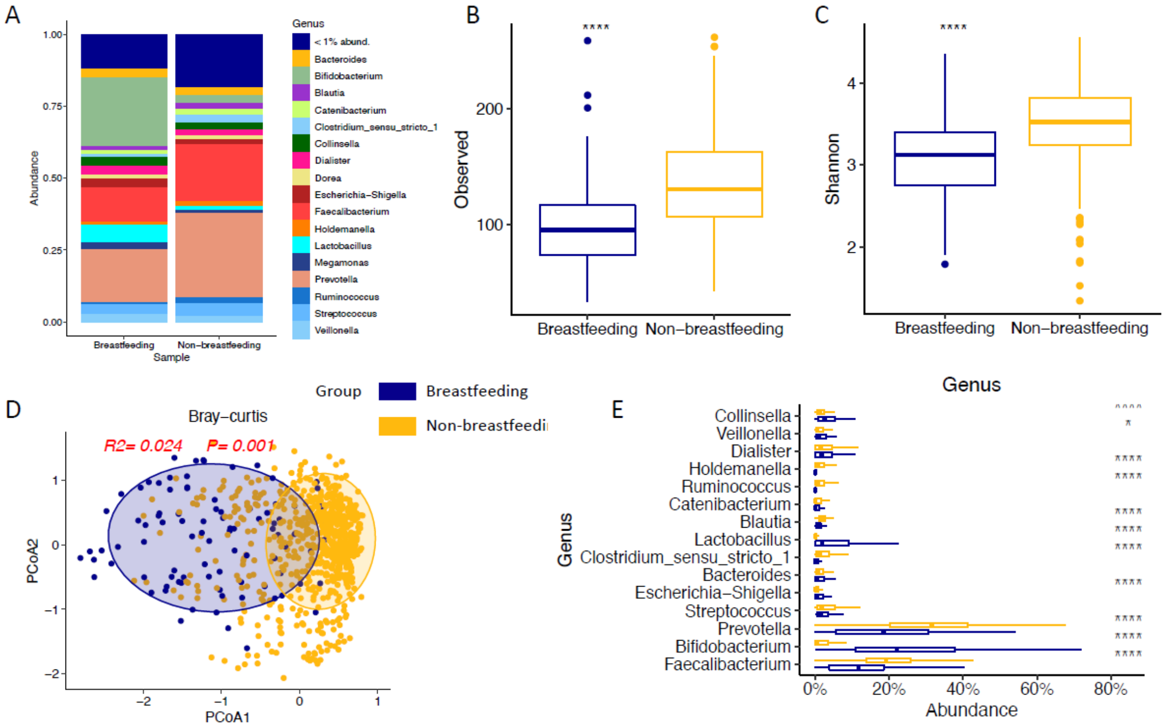


Figure 2: Breast feeding status has strong impact on gut microbiome composition among Ugandan children up to the age of 5 years**;** A) Distribution of taxa with relative abundance >1% in children either breast-fed or not; B, C) Number of observed species and Shannon diversity index for children either breast-fed or not; D) db-RDA constrained-components based on Bray-Curtis dissimilarity metrics for children either breast-fed or not; E) Taxa significantly different between children either breast-fed or not.

11.3 Effect of nutritional supplements on GM

The effect of the nutritional supplements on GM also depended on breastfeeding status (Fig 3). Among breastfed children, primarily soy protein had an effect on GM composition and function (Fig 4), while especially milk protein had effect in non-breastfed children (Fig 5). In non-breastfed children, the milk components of the intervention resulted in a GM composition associated with increased capacity to digest more diverse and complex food components. More specifically, the relative abundance of *Bifidobacterium* spp. decreased and the obligate anaerobic genus *Blautia*, which have been hypothezised to benefit host health by its ability to degrade complex carbohydrates increasing short chain fatty acid production increased in relative abundance (15). Of note, the predicted GM metabolic capacity only differed marginally between the different treatments (Fig. 6). Whey permeate had only limited effects on GM (Fig 4, 5).

11.4 Association between GM, age and growth

There was a clear positive association between child age and maturation of the GM, with the more mature GM e.g. being lower in bifidobacteria. However, the association with growth was less clear (data not shown)

Figure 3

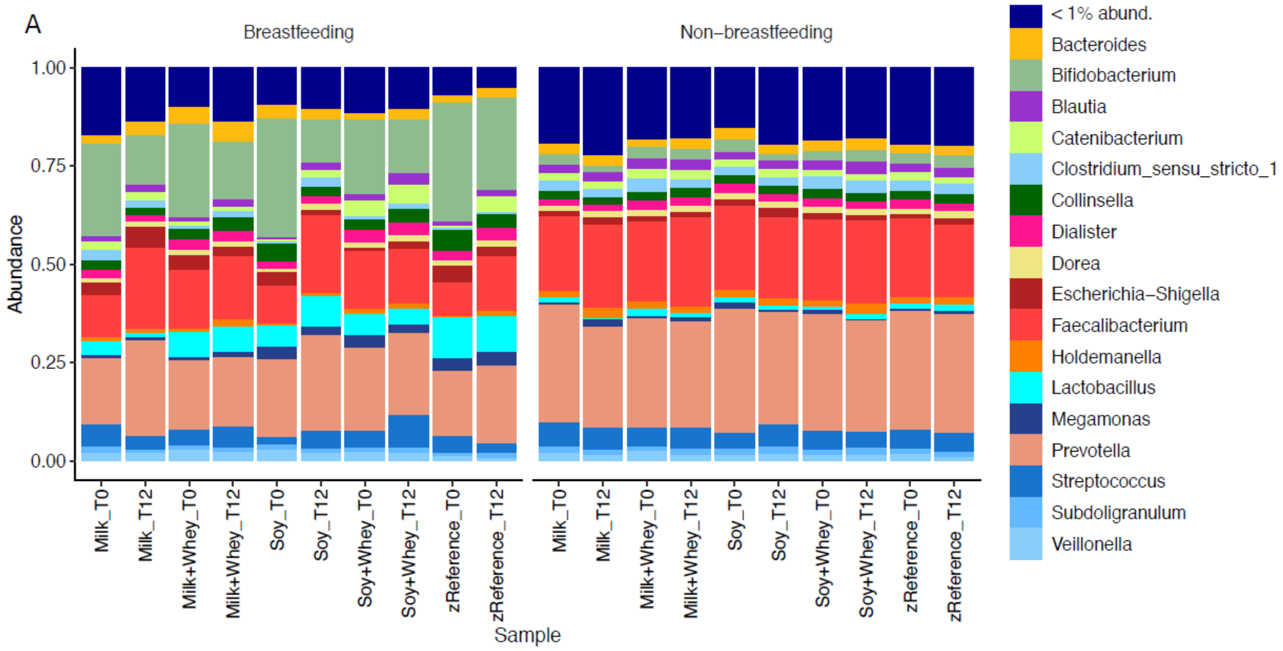


Figure 3: Distribution of taxa with relative abundance >1% before and after intervention in children either breast-feed or not.

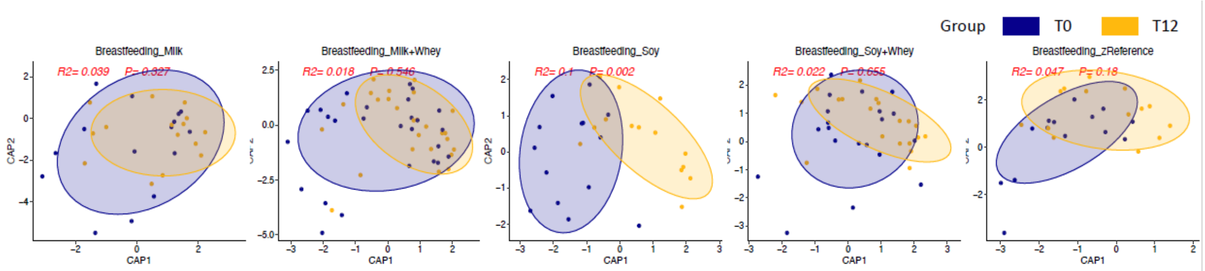


Figure 4: db-RDA constrained-components based on Bray-Curtis dissimilarity metrics for children still breastfed before and after 12 weeks of intervention. Inteventions from left to right: Milk protein; Milk protein and whey permeate; Soy protein; Soy protein and whey permeate; Reference (no intervention).

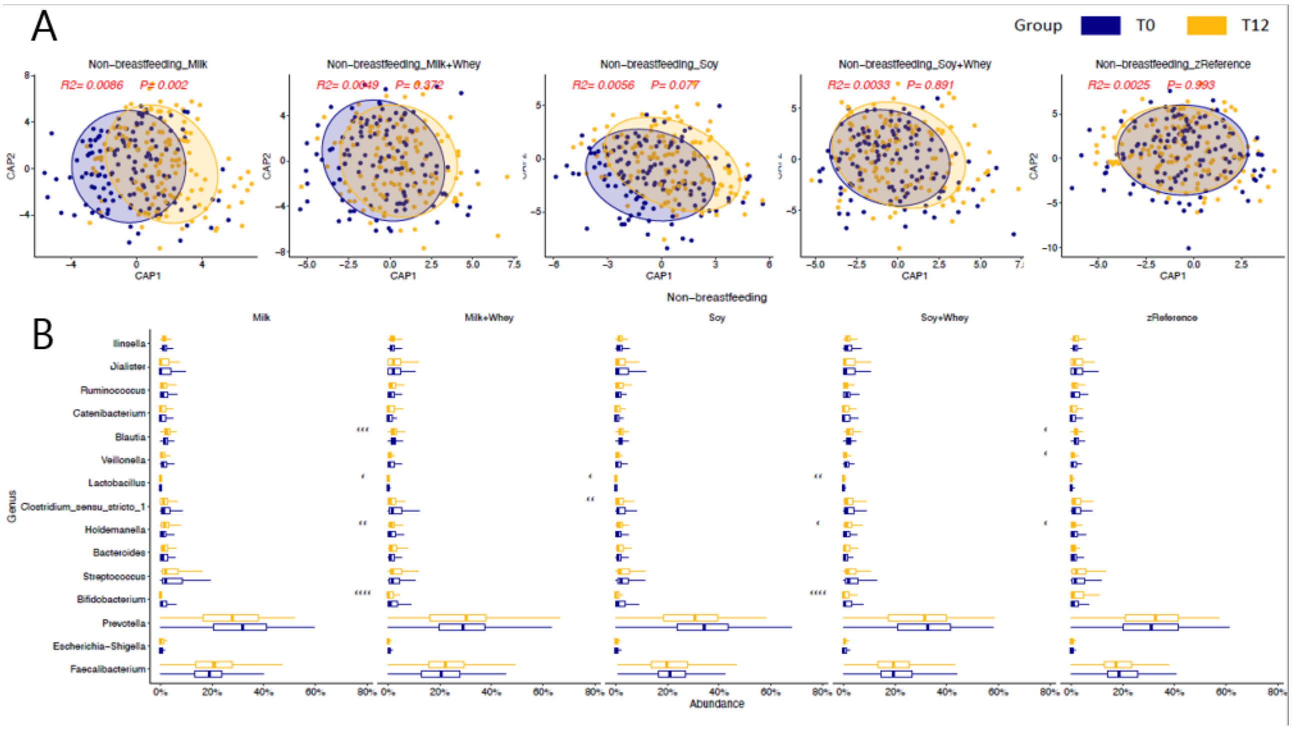


Figure 5: A) db-RDA constrained-components based on Bray-Curtis dissimilarity metrics for children not breastfed before and after 12 weeks of intervention. B) Taxa significantly different before and after intevention. Interventions from left to right: Milk protein; Milk protein and whey permeate; Soy protein; Soy protein and whey permeate; Reference (no intervention).

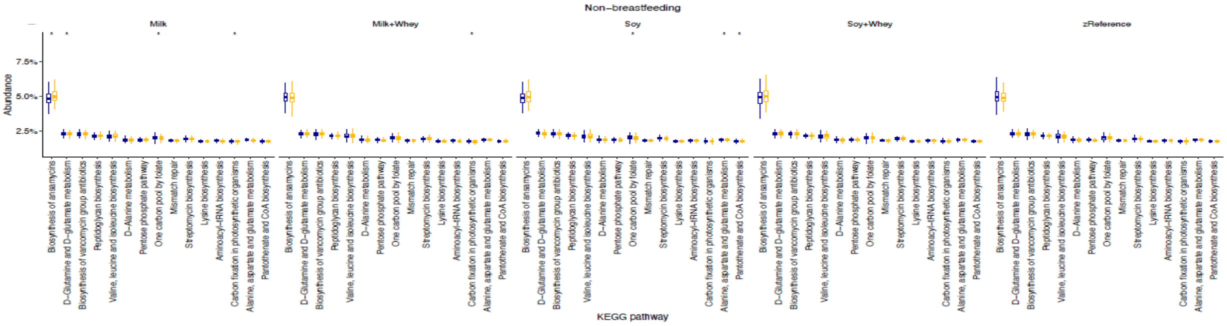


Figure 6: KEGG pathways significantly different before and after intervention. Interventions from left to right: Milk protein; Milk protein and whey permeate; Soy protein; Soy protein and whey permeate; Reference (no intervention).

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

The project contributes to the overall understanding of GM-diet (/dietary component) interactions in undernourished children. The results showed that breastfed and non-breastfed children respond differently to the nutritional supplement interventions. This may be important in relation to development of nutrition supplements in the future. Internationally, experts are aiming at developing nutrition supplements for undernourished children that specifically target improvement of their dysbiotic gut microbiota. The results of this project are important in this context.

Milk protein had a larger effect among non-breastfed children. Maybe because breastfed children already receive milk protein via breastmilk. The differential effect of milk components on GM in breastfed vs non-breastfed children may also be important for the dairy industry and development of new milk-based formulas or foods.

13. Communication and knowledge sharing about the project

Papers in international journals: Manuscript 1: Milk and microbiota dysbiosis in malnourished children, Yichang Zhang et al.; Manuscript 2: Interactions between dietary supplements and the gut virome on malnourished children, Yichang Zhang et al., in prep.

Easily read papers: Mælk, mikrobiota og underernæring - effekter af mælkeprotein og vallepermeat på etablering af en sund tarmmikrobiota hos ugandiske børn med hæmmet højdevækst, Mælkeritidende, Forskning 7/ 2021.

Mælk, mikrobiota og underernæring - effekter af mælkeprotein og vallepermeat på etablering af en sund tarmmikrobiota hos ugandiske børn med hæmmet højdevækst, Mælkeritidende, Forskning 6/ 2024.

Student theses: Master thesis: The Effects of Stunting and Lipid-Based Nutrient Supplementation on the Development of the Virome in Ugandan Children: A Sub-Analysis of the MAGNUS Trial.

Oral presentations at meetings: Meetings at the Danish Dairy Research Foundation, research meetings at University of Copenhagen.

14. Contribution to master and PhD education

Two master students in Human Nutrition, Marie-Claire Charlotte Nitschke and Grace Brigid Mongey, wrote their thesis based on the MicroGAM project: The Effects of Stunting and Lipid-Based Nutrient Supplementation on the Development of the Virome in Ugandan Children: A Sub-Analysis of the MAGNUS Trial.

One PhD student, Yichang Zhang, wrote his PhD thesis including data from the MicroGAM project. The title of his thesis was: Gut microbioata and child health.

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