PhD Thesis title: THE EFFECT OF EXOGENOUS AND ENDOGENOUS VITAMIN B9 AND B12 ON MICROBIAL GROWTH AND METABOLISM IN THE HUMAN GUT

summary:

B9 and B12 are essential micronutrients for all living cells, including gut bacteria. While *in silico* studies have identified the human gut microbiota as a potential source of B9 and B12 production, there is limited *in vitro* data on the actual production of B9 and B12 by gut microbial strains, especially on the different forms of B9 and B12 produced by them. Moreover, a considerable amount of undigested B9 and B12 from the diet reaches the colon, therefore, it is relevant to explore how the gut microbial community responds to this available supply. Specifically, it remains unclear whether the microbial communities depend on these exogenous B9 and B12 for their growth and metabolic needs or if they depend on endogenously synthesized B9 and B12, fostering potential cross-feeding interactions between prototrophic and auxotrophic species. To gain a comprehensive understanding of B9 and B12 production and requirements within the human gut microbiota as well as specific auxotrophic gut microbial species, further investigation was warranted to elucidate the specific B9 and B12 needs of the gut microbial community members.

In **chapter 2** of this thesis, the *in vitro* B12 production by human fecal microbiota and the effects of different levels of B12 (as cyanocobalamin) on composition and activity was investigated. The study utilized eight fecal microbial communities from healthy human adults, which were distributed over three enterotypes, dominated by *Firmicutes* (n = 5), *Bacteroides* (n = 1) or *Prevotella* (n = 2) taxa. Batch fermentations were conducted using Macfarlane medium with different B12 supplementation: low B12 medium (Control, 5 ng/ml), no B12 addition (NB12), and high B12 addition (ExtraB12, 2500 ng/ml). After 24 hours of incubation under strict anaerobic conditions, at 37° C, various measurements were taken to assess the microbiota community composition (qPCR, 16S rRNA metabarcoding), metabolic activity (HPLC-RI), and B12 levels (UHPLC-DAD). It was observed that all fecal microbial communities produced B12 in the NB12 condition after 24 h, with B12 concentrations ranging from 152 ± 4 to 564 ± 25 ng/ml. Interestingly, none of the B12 treatments had a significant impact on total bacterial growth, community richness, composition, and total metabolite production, compared to the low B12 control. However, a noteworthy finding was that the B12 supplementation (ExtraB12) showed a significant increase of propionate when compared to no supplementation treatment (NB12) in 2 out of 8 microbiotas. Furthermore, the specific taxonomic and metabolite changes observed in response to B12 treatments when compared to control incubations were donor-dependent, indicating that the effects of B12 supplementation were dependent on individual donors. Based on these *in vitro* results, it can be concluded that healthy human adult gut microbial communities have the capacity to produce B12 to meet their own requirements, regardless of the initial B12 content present in the donor's feces. Additionally, the supplementation of exogenous dietary B12 may have limited impact on the healthy human gut microbial community composition and function.

In **chapter 3**, a subsequent investigation was conducted to explore in greater depth whether B12 supplementation enhances propionate production by the commensal B12 auxotrophs *Akkermansia muciniphila* and *Bacteroides thetaiotaomicron*. The production of propionate by gut microbes, through carbohydrate metabolism, relies on the presence of B12 as a cofactor for the conversion of succinate to propionate. As mentioned before, B12 is expected in the human gut either from the unabsorbed dietary fraction or from *in situ* microbial production. However, there is limited experimental data for B12 production by single gut microbes, particularly, in

relation to the specific forms of B12 they produce. Furthermore, the promotion of propionate production by microbially produced and dietary B12 in the gut is not fully understood. These gaps are addressed in **chapter 3** of this thesis. At first, 12 gut microbial strains belonging to different genera and species were selected, including 8 predicted producers and 4 non producers, to evaluate their capacity to produce B12. Next, to gain a more comprehensive understanding of the effects of B12 supplementation on propionate production, single culture *in vitro* experiments using both, microbially produced B12 from inactivated bacterial (IB) preparations of *Blautia hydrogenotrophica, Marvinbryantia formatexigens,* and *Blautia producta*, and commercially available forms of B12 present in human diet (cyano-B12, adenosyl-B12, and hydroxyl-B12) in two doses (low, 1x (10 µg/l) and high, 20x (200 µg/l)) were conducted. By incorporating both sources, this thesis aimed to explore whether the origin of B12 (microbial or exogenous) influences propionate production, thus providing insights into the role of different B12 sources in modulating gut microbial propionate metabolism. B12 production was confirmed in 6 out of 8 predicted prototrophic strains. In addition, gut microbial produced B12 promoted the conversion of succinate to propionate by *A. muciniphila* and *B. thetaiotaomicron*, indicating that auxotrophic strains can use gut microbially produced B12. Notably, with 1x dose, only partial conversion of succinate to propionate were observed for *A. muciniphila* when grown with adenosyl-B12 (14.6 ± 2.4 mM and 18.7 ± 0.6 mM) and hydroxyl- B12 (13.0 ± 1.1 mM and 21.9 ± 1.2 mM) in comparison to cyano-B12 (0.7 ± 0.1 mM and 34.1 ± 0.1 mM). Higher dose (20x) of all the tested commercially available B12 forms resulted in significantly more succinate to propionate in both *A. muciniphila* and *B. thetaiotaomicron* compared to 1x dose. These findings are in line with the data obtained in **chapter 2** with B12 supplementation. To conclude, it was shown that different forms of B12 have different potential to impact the propionate metabolism of the commensal propionate producers in the gut.

Finally, in **chapter 4,** the *in vitro* B9 production in human gut bacterial strains as well as the impact of different forms of B9 was investigated on the growth of *Roseburia intestinalis* L1-82, a prevalent, butyrate producing B9 auxotrophic strain. At first, B9 production by six *in silico* predicted B9 prototrophic gut strains (*Marvinbryantia formatexigens* DSM 14469*, Blautia hydrogenotrophica* 10507T*, Blautia producta* DSM 14466, *Bacteroides caccae* DSM 19024, *Bacteroides ovatus* DSM 1896*,* and *Bacteroides thetaiotaomicron* DSM 2079T) was confirmed in their IB preparations using UHPLC-UV/FL. Further, the growth of B9 auxotroph *R. intestinalis* L1-82 was examined in the presence of different forms of B9 (tetrahydrofolate (THF), methyl-tetrahydrofolate (M-THF), and formyl-tetrahydrofolate (F-THF) and 10-formylfolic acid (FFA)), and folic acid. The results indicated that natural forms of B9 were consumed and metabolized more efficiently by *R. intestinalis* L1-82 compared to the synthetic form. To investigate the potential impact of B9 on human gut microbial communities, particularly the abundance of naturally occurring *R. intestinalis* spp., anaerobic batch fermentations were conducted on fecal microbiotas obtained from five healthy adults, supplemented with all B9 forms in low (50 µg/l) or high doses (200 µg/l), and no added B9 (NoB9). The qPCR and 16S rRNA metabarcoding analysis revealed that neither different forms of B9 nor their doses had a significant impact on the total bacteria, total *R. intestinalis* spp., or overall community composition after 48 hours of incubation. In summary, it was demonstrated that natural forms of B9 support the growth of butyrate producing *R. intestinalis* L1-82 in pure culture. However, B9 forms did not promote the growth of *Roseburia* spp., naturally present in fecal samples. Nonetheless, these findings indicate that the healthy adult human microbiota remains unaffected with different forms and doses of B9, and even in the absence of added B9.

To overall conclude, the studies conducted in **chapter 2** and **chapter 4** provided evidence that healthy human gut microbial communities do not depend on dietary B9 and B12 supply for their

B12- and B9-dependent metabolism and growth. This was mainly associated with the intrinsic capacity of gut microbes to produce these vitamins as shown for the gut microbial communities in **chapter 2** and for single gut microbial species in **chapter 3** and **chapter 4**. Additionally, the results indicate the existence of an intricate cross-feeding mechanism within the microbial community, allowing its members to sustain and maintain their own vitamin requirements. Overall, this thesis extended the knowledge about the effect of exogenous and endogenous vitamin B9 and B12 on the growth and metabolism of human gut microbiota.

Based on the findings obtained in this thesis, future research can focus on further validation of the observed treatments by including donors from different age groups or following specific diet, such as, vegan diet. Future studies should be extended to disease-associated dysbiotic human gut microbial communities to study the effect of supplementation. Moreover, mechanistic studies should be done to explore underlying mechanisms of vitamin cross feeding. Additionally, the findings on single auxotrophs (*R. intestinalis*, *A. muciniphila*, and *B. thetaiotaomicron*) can be applied for the industrial production of their live biotherapeutic products.

Publication 1

Title: Role of Dietary Micronutrients on Gut Microbial Dysbiosis and Modulation in Inflammatory Bowel Disease

Summary: In patients with inflammatory bowel diseases (IBD), dietary micronutrient intake is low and deficiencies are common. Besides the host, also the gut microbiota require micronutrients and low levels may disturb its functioning. Multi-omics studies indeed detected shifts in micronutrient-dependent microbial pathways in IBD. It is however not clear whether micronutrients may alleviate inflammation directly, by modulating the immune system, or also indirectly, by modulating the structure and function of the gut microbiota. The latter seems of particular interest, since the gut microbiota is one of the future therapeutic targets in IBD.

A review of the most recent available literature on relevant micronutrients in context of IBD and gut microbiota was conducted. An overview per relevant micronutrient on its role on gut bacterial growth, metabolism and host–microbe interactions during gut inflammation is provided.

Dietary micronutrients have potential to be part of future personalized microbiome-targeted therapies in IBD, considering both the micronutrient status of the host and the gut microbiota. However, cohort studies together with integrated multi-scale studies are needed to understand the mechanisms of micronutrient–microbiome–host interactions in IBD and to evaluate efficacy and safety of dietary micronutrient treatment strategies.

Publication 2:

Title: Healthy adult gut microbiota sustains its own vitamin B12 requirement in an in vitro batch fermentation model

summary: Vitamin B12 (cobalamin) is present in the human lower gastrointestinal tract either coming from the unabsorbed dietary fraction or from *in situ* production of the gut microbiota. However, it is unclear whether the gut microbial communities need exogenous B12 for growth

and metabolism, or whether B12 in low and high levels could affect gut community composition and metabolite production. Here, we investigated *in vitro* B12 production of human fecal microbiota and the effects of different levels of B12 (as cyanocobalamin) on composition and activity. Eight fecal communities from healthy human adults distributed over three enterotypes, dominated by *Firmicutes* (*n* = 5), *Bacteroides* (*n* = 1) or *Prevotella* (*n* = 2) were used to perform batch fermentations in Macfarlane medium supplemented with low B12 medium (Control, 5 ng/ml, within the tested fecal range), no B12 addition (NB12), and high B12 addition (ExtraB12, 2500 ng/ml). The microbiota community composition (qPCR, 16S rRNA metabarcoding), metabolic activity (HPLC-RI), and B12 levels (UHPLC-DAD) were measured after 24 h incubation at 37°C under strict anaerobic conditions. All fecal microbial communities produced B12 in the NB12 condition after 24 h, in the range from 152 ± 4 to 564 ± 25 ng/ml. None of the B12 treatments had an impact on total bacterial growth, community richness, diversity and total metabolite production, compared to the low B12 control. However, a significant increase of propionate was measured in ExtraB12 compared to NB12. Most taxonomic and metabolite changes compared to control incubations were donor-dependent, implying donor-microbiota-specific changes upon B12 treatments. Our *in vitro* data suggest that healthy human adult gut microbial communities have the capacity to produce B12 at levels fulfilling their own requirements, independently of the initial B12 content tested in the donor's feces. Further, supplementation of exogenous dietary B12 may have limited impact on the healthy human gut microbial community composition and function.

Publication 3

Title: Vitamin B12 analogues from gut microbes and diet differentially impact commensal propionate producers of the human gut

Summary:

To produce the health-associated metabolite propionate, gut microbes require vitamin B12 as a cofactor to convert succinate to propionate. B12 is sourced in the human gut from the unabsorbed dietary fraction and *in situ* microbial production. However, experimental data for B12 production by gut microbes is scarce, especially on their produced B12-analogues. Further, the promotion of propionate production by microbially-produced and dietary B12 is not yet fully understood. Here, we demonstrated B12 production in 6 out of 8 *in silico* predicted B12-producing bacteria from the human gut. Next, we showed *in vitro* that B12 produced by *Blautia hydrogenotrophica*, *Marvinbryantia formatexigens,* and *Blautia producta* promoted succinate to propionate conversion of two prevalent B12-auxotrophic gut bacteria, *Akkermansia muciniphila* and *Bacteroides thetaiotaomicron*. Finally, we examined the propiogenic effect of commercially available B12-analogues present in the human diet (cyano-B12, adenosyl-B12 and hydroxy-B12) at two doses. The low dose resulted in partial conversion of succinate to propionate for *A. muciniphila* when grown with adenosyl-B12 (14.6 ± 2.4 mM succinate and 18.7 ± 0.6 mM propionate) and hydroxy-B12 (13.0 ± 1.1 mM and 21.9 ± 1.2 mM), in comparison to cyano-B12 (0.7 ± 0.1 mM and 34.1 ± 0.1 mM). Higher doses of adenosyl-B12 and hydroxy-B12 resulted in significantly more conversion of succinate to propionate in both propionate-producing species, compared to the low dose. B12 analogues have different potential to impact the propionate metabolism of prevalent propionate producers in the gut. These results could contribute to strategies for managing gut disorders associated with decreased propionate production.

Publication 4:

Title: Microbially-produced folates support the growth of Roseburia intestinalis but not its

competitive fitness in fecal batch fermentations

Summary: Folate (vitamin B9) occurs naturally as tetrahydrofolate (THF), methyl-tetrahydrofolate (M-THF), and formyl-tetrahydrofolate (F-THF), and as dietary synthetic form (folic acid). While folate auxotrophy and prototrophy are known for several gut microbes, the specific forms produced by gut prototrophs and their impact on auxotrophs remains unexplored. Here, we quantified by UHPLC-FL/UV folate produced by six *in silico* predicted gut prototrophs (*Marvinbryantia formatexigens* DSM 14469*, Blautia hydrogenotrophica* 10507T*, Blautia producta* DSM 14466, *Bacteroides caccae* DSM 19024, *Bacteroides ovatus* DSM 1896*,* and *Bacteroides thetaiotaomicron* DSM 2079T) and investigate the impact of different folate forms and doses (50 and 200 µg/l) on the growth and metabolism of *R. intestinalis* in pure cultures and during fecal anaerobic batch fermentations (48 h, 37°C) of five healthy adults. Our results confirmed the production of different folates forms by all six gut strains (from 15.3 ng/ml to 205.4 ng/ml; THF (12.4 - 41.4 ng/ml) and 5-MTHF (0.2 - 113.3 ng/ml) being present in all samples). Natural folate forms but not folic acid promoted the growth kinetics and metabolism of the auxotroph *Roseburia intestinalis* L1-82, with dose-dependent effects. Folate forms and doses significantly impacted the concentration of total bacteria and *Roseburia* spp. in batch fermentations, compared to the control without folate addition, while community composition and metabolic activity was not affected. Our study demonstrates for the first time *in vitro* the production of different natural folate forms by predicted prototrophs and their stimulation on the growth of the folate auxotrophic butyrate-producing *R. intestinalis* L1-82. Folates did not impact fecal fermentations, suggesting that dietary folate may have limited effects on the human gut microbiota *in vivo*.

publication 5:
title: **Changes detected in the genome sequences of *Escherichia coli*, *Listeria monocytogenes*, *Vibrio parahaemolyticus*, and *Salmonella enterica* after serial subculturing**

Summary: Whole genome sequencing (WGS) is rapidly replacing other molecular techniques for identifying and subtyping bacterial isolates. The resolution or discrimination offered by WGS is significantly higher than that offered by other molecular techniques, and WGS readily allows infrequent differences that occur between 2 closely related strains to be found. In this investigation, WGS was used to identify the changes that occurred in the genomes of 13 strains of bacterial foodborne pathogens after 100 serial subcultures. Pure cultures of Shiga-toxin-producing *Escherichia coli*, *Salmonella enterica*, *Listeria monocytogenes*, and *Vibrio parahaemolyticus* were subcultured daily for 100 successive days. The 1st and 100th subcultures were whole-genome sequenced using short-read sequencing. Single nucleotide polymorphisms (SNPs) were identified between the 1st and final culture using 2 different approaches, and multilocus sequence typing of the whole genome was also performed to detect any changes at the allelic level. The number of observed genomic changes varied by strain, species, and the SNP caller used. This study provides insight into the genomic variation that can be detected using next-generation sequencing and analysis methods after repeated subculturing of 4 important bacterial pathogens.