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Interaction between the gut and its microbiota in inflammatory bowel disease

Sadaghian, Mehdi

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Document Version

Publisher's PDF, also known as Version of record

Publication date:

2015

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

Sadaghian, M. (2015). Interaction between the gut and its microbiota in inflammatory bowel disease [S.l.]: [S.n.]

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Riboflavin increases the abundance of *Faecalibacterium prausnitzii* and *Roseburia* in fecal samples of healthy volunteers and positively affects the overall microbial balance in the gut.

Mehdi Sadaghian Sadabad^{1,2}, Muhammad Tanweer Khan¹, Jan Maarten van Dijk¹, Hermie J. M. Harmsen¹

¹ Department of Medical Microbiology, University of Groningen, University Medical Center Groningen, Groningen, The Netherlands.

² Department of Gastroenterology and Hepatology, University of Groningen, University Medical Center Groningen, Groningen, The Netherlands.

Keywords: Riboflavin, intestinal microbiota, *F. prausnitzii*, microbial balance.

In modified form included in European patent application No. 12190948.5

Rijksuniversiteit Groningen, Stimulation of *Faecalibacterium prausnitzii*

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ABSTRACT

Objectives: The correlation between nutrition and intestinal microbiota and the role of intestinal microbiota in human health and disease is well established. *Faecalibacterium prausnitzii* is one of the most abundant bacteria in the human gut and decreased numbers are associated with Crohn's disease. *In vitro* studies revealed that *F. prausnitzii* uses riboflavin (vitamin B₂) for extracellular electron transfer to shuttle electrons to oxygen, unlike other butyrate producers, such as *Roseburia*. Here, we investigated the effect of riboflavin supplementation on the abundance of *F. prausnitzii* and on other members of microbiota in the feces of healthy volunteers.

Design: Healthy volunteers (n=11) were given a daily oral dose of 100 mg riboflavin for two weeks. Prior to the intervention, two fecal samples were collected with a one week interval. Two fecal samples were collected during the intervention and one sample 7 days after stopping the intervention. Fecal microbiota were evaluated using fluorescent *in situ* hybridization with group-specific probes.

Results: Numbers of *F. prausnitzii* increased in 8 volunteers upon riboflavin supplementation, however, this did not reach statistical significance ($p=0.131$) due to high inter-individual variability. The numbers of *Roseburia* were significantly increased in 10 volunteers ($p<0.05$). Fecal numbers of both *F. prausnitzii* and *Roseburia* dropped significantly after cessation of riboflavin supplementation ($p<0.05$). In contrast, riboflavin decreased the numbers of Enterobacteriaceae in 9 volunteers, which was observed in 6 volunteers with increased numbers of *F. prausnitzii*.

Conclusion: Riboflavin supplementation preferentially increases *F. prausnitzii* and *Roseburia* in feces of healthy volunteers. While previously established for *F. prausnitzii in vitro*, this was not detected for *Roseburia* before. Moreover, an inverse relationship was detected between numbers of *F. prausnitzii* and Enterobacteriaceae. Our results show the importance of riboflavin in the diet to support the beneficial bacteria such as *F. prausnitzii* and *Roseburia* and to possibly help to control pathosymbionts such as *E. coli* and restore symbiosis in patients with Crohn's disease.

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INTRODUCTION

The intestinal microbiota is under direct influence of several factors of which the diet is the most prominent one. The composition of the microbiota has a large impact on gut health, since it maintains intestinal homeostasis, provides nutrients for the host by metabolizing food ingredients and stimulates the maturation of the immune system^{1,2}. The complex interaction between microbiota and diet starts directly after birth, with the infant's first nutrition. It has been shown that breastfeeding results in a microbiota enriched with bifidobacteria and some lactic acid bacteria, while infants raised by bottle feeding will develop a more diverse microbiota with bifidobacteria but also *Bacteroides* spp., *Clostridium* spp. and facultative anaerobes, such as staphylococci, and *Enterobacteriaceae*³⁻⁵. The effect of diet on microbiota in adulthood is demonstrated by studies with prebiotics such as inulin that stimulates the growth and abundance of beneficial commensals, like *Bifidobacterium* and *Faecalibacterium*⁶ species that in turn will increase the production of short-chain fatty acids (SCFAs) within the intestinal tract⁷. The SCFA's, especially butyrate, are the preferred energy source for epithelial cells and enhance intestinal barrier function and directly affect mucosal immunity⁸.

The healthy gut harbors a vast amount of bacteria, which is comprised of a balance between beneficial bacteria and potential pathogens⁹. Loss of this balance may lead to the uncontrolled abundance of potential pathogens and so called dysbiosis that could lead to pathological conditions, like inflammatory bowel disease (IBD) and a variety of digestive tract diseases¹⁰.

Crohn's disease (CD), as a major form of IBD, is a Western lifestyle-related disease mainly determined by the interplay of microbiota, genetic susceptibility and environmental factors. CD patients with ileal disease show a dramatic decrease of the main butyrate-producing *Faecalibacterium prausnitzii* in the human gut, while there are increased numbers of pathogenic bacteria in these patients, like Adherent Invasive *E. coli* (AIEC)¹¹. Recently, we found that *F. prausnitzii* has the ability to use riboflavin (vitamin B₂) as a redox mediator for extracellular electron transfer, while other anaerobic bacteria like *Roseburia* do not show the same phenomenon *in vitro*. This will allow the growth of this strict anaerobic bacterium close to the mucus layer and the epithelial cells where oxygen penetrates from the blood circulation¹².

Considering the anti-inflammatory properties of *F. prausnitzii*¹³ and its role in producing SCFAs for epithelial cells⁸, increased abundance of this bacterium may be beneficial to maintain a healthy gut. Moreover, increased numbers of *F. prausnitzii* could change the dysbiosis in CD patients in a beneficial way. The ability of *F. prausnitzii* to deploy riboflavin to shuttle electrons and thereby stimulate its growth at the mucus layer, opens up possibilities for interventions to increase their numbers in the gut. Here, we investigated the effect of a daily oral riboflavin dose on the abundance of *F. prausnitzii*, as well as other

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major butyrate-producing bacteria, and the effects on the pathosymbiont *E. coli* and its relatives in fecal samples of healthy volunteers.

MATERIALS AND METHODS

Study cohort

Fresh fecal samples from 11 healthy volunteers (6 males and 5 females; mean [range] age, 32 [22 to 65] years) were obtained. The only exclusion criterion was the use of antibiotics 3 weeks prior to providing fecal samples. Volunteers were asked to take a daily oral dose of 100 mg riboflavin in a tablet form obtained from the local drug store (De Tuinen, The Netherlands). To record the natural variation in the fecal microbiota before the riboflavin intervention, volunteers were asked to deliver two fecal samples with one week interval before the start of the intervention (Samples 1 and 2; designated “before”). Volunteers delivered fecal samples 7 days after the intervention started and on day 14, the last day of intervention (Samples 3 and 4, designated “intervention”). One final sample was delivered 7 days after intervention was stopped (Sample 5, designated “after”). Without reporting any reason, 3 volunteers did not deliver after sample.

Fluorescent *in situ* hybridization (FISH)

Fresh fecal samples were diluted and fixed for FISH analysis as described¹⁴. Briefly, 2.5 g of feces was diluted 10-fold in filtered phosphate-buffered saline (PBS), homogenized and subsequently centrifuged at low speed (700×g) to remove debris. One milliliter of the supernatant was mixed with 3ml freshly prepared 4% paraformaldehyde in PBS and incubated overnight at 4°C to fix the bacteria. These 40-fold-diluted fecal samples were stored at -80°C until further analysis. FISH analyses were performed as described on different fecal dilutions in PBS ranging from 40- to 1,600-fold, depending on the relative amount of targeted bacteria. The analysis was performed with a set of probes for the several predominant groups of fecal bacteria. A list of probes used in the study and their target sequences is presented in Table 1. After visual counting using an epifluorescence microscope and extrapolation to the number of bacteria per gram feces, the median abundance and the average of samples 1 and 2 (before), samples 3 and 4 (intervention) were calculated. In addition, the median percentage of the targeted bacteria to the total bacteria as counted with the EUB probe and the average of the samples were calculated.

Statistics

Differences in the percentages of bacterial strains and their percentages related to the total bacterial counts were evaluated using the nonparametric Wilcoxon signed-rank test. Significance of differences was set to 0.05.

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Table 1: List of the probes used in the present study and their target sequence.

Target	Probe	Target Sequence 3'->5'
Universal probe	Eub338	TGAGGATGCCCTCCGTCG ¹⁵
<i>E. rectale</i> & <i>C. coccoides</i> group	Erec482	GCCATGTRACTGATTCTTCG ¹⁶
<i>F. prausnitzii</i> group	Fprau645	CAAAAAGAACTCATCAGTCTCC ¹⁷
<i>Roseburia</i> cluster	Rint623	TTCCAATGCAGTACCGGG ¹⁸
	Rint helper	GTTGAGCCCCGGGCTTT
Enterobacteriaceae	EC1531	CACCGTAGTGCTCGTCATC ¹⁹

RESULTS

Fecal samples of volunteers contain increased numbers of *F. prausnitzii*, *Roseburia* and the closely related *Eubacterium rectale* during daily supplementation with 100 mg of riboflavin. The number of *F. prausnitzii* prior to the riboflavin supplementation ("before" samples) were between 7.0×10^7 and 5.5×10^9 with an average of 1.3×10^9 per gram of feces. The average number of faecalibacteria (1.9×10^9 ; range 3.8×10^8 - 4.2×10^9) in fecal samples during supplementation were higher than those before and after supplementation in 8 out of 11 volunteers (Figure 1A). Taken as a group, the riboflavin-induced increase in *F. prausnitzii* numbers was just not significant (Wilcoxon signed-rank test $P=0.131$), but this is likely due to the limited numbers of volunteers and the large variation in faecalibacteria before the intervention. Moreover, *F. prausnitzii* numbers dropped significantly in all volunteers after cessation of riboflavin supplementation ($P < 0.05$). Similar to the absolute numbers of *F. prausnitzii* also the relative abundance of this bacterial species was enhanced during the riboflavin supplementation period (Figure 1 B).

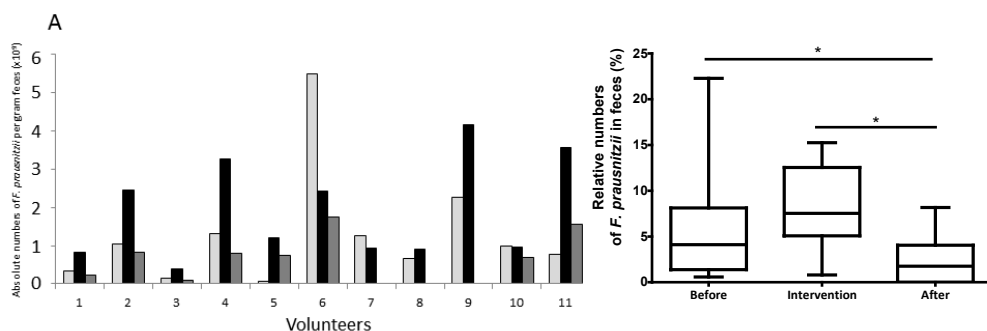


Figure 1. Increase in the abundance (A) and percentages (B) of *F. prausnitzii* in healthy volunteers upon riboflavin supplementation. Figure 1A shows the average of two before samples (light gray), average of two riboflavin intervention samples (black) and one after sample (dark gray) for each volunteer. Figure 1-B shows the percentages of *F. prausnitzii* to the total bacterial counts in before, intervention and after samples (*, $P < 0.05$).

Despite the large variation, there were no significant changes in the number of *Clostridium* cluster XIVa upon riboflavin supplementation. However, the butyrate-producing sub-

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cluster, the genus *Roseburia* and the closely related *Eubacterium rectale*, showed a significant increase during the intervention period in 10 out of 11 volunteers ($2.0 \times 10^8 - 2.7 \times 10^9$ with average of 1.3×10^9 for before samples and $5.6 \times 10^8 - 7.3 \times 10^9$ with average of 2.3×10^9) ($P < 0.05$). The abundance of *Roseburia* in volunteer 2 continued to increase after ceasing riboflavin supplementation (Figure 2A). Moreover, also the increases during intervention in percentages of the *Roseburia* were significant ($P < 0.05$) (Figure 2B). The abundance of *Enterobacteriaceae* (*E. coli* and relatives) was decreased during the riboflavin supplementation period in 9 of 11 volunteers ($2.4 \times 10^6 - 1.4 \times 10^8$ with average of 4.8×10^7 for before samples and $6.1 \times 10^5 - 8.8 \times 10^7$ with average of 3.4×10^7). Remarkably, this decrease coincided with an increase in abundance of *F. prausnitzii* in 6 cases (Figure 3). Both bacterial groups decreased in volunteer 6, while they both increased in volunteer 8 upon riboflavin supplementation.

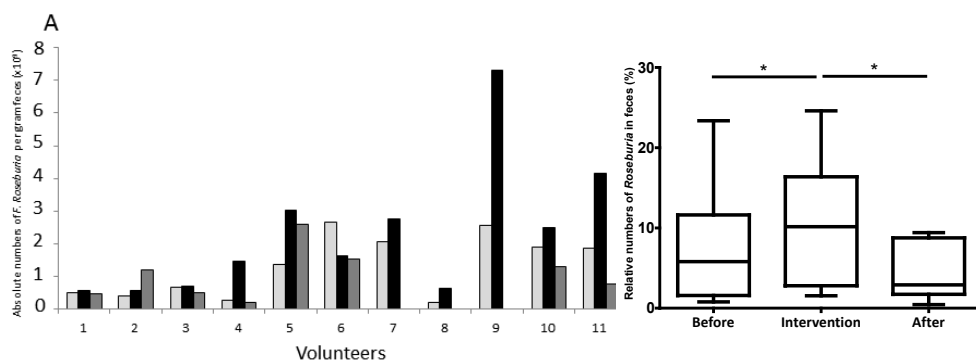


Figure 2. Increase in the abundance (A) and percentages (B) of *Roseburia* in healthy volunteers upon riboflavin supplementation. Figure 2-A shows the average of two before samples (light gray), average of two riboflavin intervention samples (black) and in one after sample (dark gray). Figure 2-B shows the percentages of *Roseburia* to the total bacterial counts in before, intervention and after samples (*, $P < 0.05$).

Volunteer 10 did not show a change in abundance of *F. prausnitzii* while the numbers of *Enterobacteriaceae* increased (Figure 3). Volunteer 7 showed a reverse counterbalance, an increase in *Enterobacteriaceae* and a decrease in faecalibacteria, possibly due to an unnoticed microbial infection.

DISCUSSION

The present study shows that daily oral intake of 100 mg riboflavin (vitamin B₂) rapidly increases the abundance of *F. prausnitzii* and *Roseburia* in feces of healthy volunteers. In most cases, the increase in number of faecalibacteria was associated with a decrease in numbers of *Enterobacteriaceae*.

The increase in abundance of *F. prausnitzii* is in line with the findings of Khan et al.^{20,21}, who found that flavins, such as riboflavin, act as redox mediators for faecalibacterial cells and

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stimulate their growth *in vitro* at oxic/anoxic interphases by extracellular electron transfer. However, despite the previous *in vitro* findings that indicated that this ability is specific for *F. prausnitzii* and not for other butyrate producers such as *Roseburia* sp. we demonstrate that *in vivo* also *Roseburia* benefits from the riboflavin supplementation.

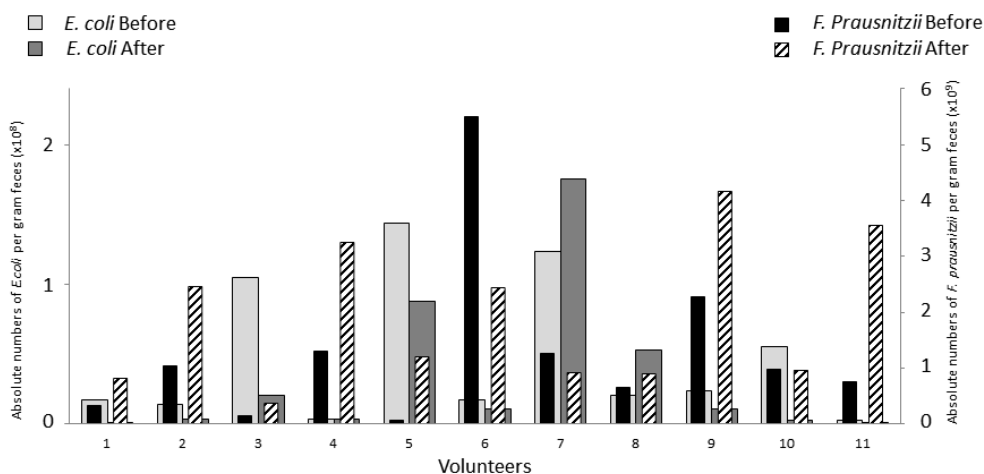


Figure 3. Counterbalance between the increased numbers of *F. prausnitzii* and decreased numbers of *E. coli* upon riboflavin intervention. This counterbalance is detected in volunteers 1, 2, 3, 5, 9 and 11. Numbers of *E. coli* are correlated with the left axis and *F. prausnitzii* are correlated with the right one.

Since it has not been shown that *R. intestinalis* can employ riboflavin as an electron transfer mediator, the mechanism(s) responsible for this increase and whether they directly benefit from riboflavin or not, remains speculative. One explanation could be that *Roseburia* benefits from an increased secretion of anti-oxidant thiols by epithelial cells as a consequence of a healthier intestinal epithelial layer due to the higher numbers of *F. prausnitzii*. Another possibility is that faecalibacteria lower the redox potential during the extracellular electron transfer or that they liberate or produce extra carbohydrates or amino acids that *Roseburia* feed on.

Importantly, the riboflavin-induced increase in abundance of butyrate-producing bacteria like *F. prausnitzii* along with the decrease in numbers of *Enterobacteriaceae* like *E. coli* may benefit Crohn's disease patients, since several studies have demonstrated that fecal *F. prausnitzii* levels are reduced in these patients, while concomitantly *E. coli* numbers are enhanced^{11,22}. It is important to elucidate how riboflavin supplementation decreases the abundance of *Enterobacteriaceae*, because this could be the key to unravel the origin of a microbial dysbiosis in the gut. One reason could be the reduction of redox potential and nutrients available for *Enterobacteriaceae* due to an increase of two major groups of

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butyrate-producing bacteria. Especially the role of *F. prausnitzii* that has the unique ability to colonize the fecal-mucosal interphase, the phase that *E. coli* cells are most likely to localize as well, is important. Alternatively, the niche for *E. coli* to grow specifically close to the mucus layer in the oxygenated zone could be diminished due to healthier epithelial cells that increase the secretion of thiols as a consequence of the increased butyrate and other anti-inflammatory substances produced by *Roseburia* and *F. prausnitzii*^{23,24}.

Our findings show that riboflavin supplementation can increase the abundance of butyrate-producing bacteria and decrease the numbers of *Enterobacteriaceae* and therefore support an anti-inflammatory balance in the intestinal tract. It could be beneficial for inflammatory bowel diseases, which are characterized by decreased numbers of *F. prausnitzii* and enhanced numbers of *E. coli* in the feces. Future studies should be directed towards maximizing the riboflavin effect. Now, most of the supplemented riboflavin is taken up in the ileum, designing a riboflavin delivery system to the colon would enable dose response studies.

In this study, a clear trend for growth stimulation of *F. prausnitzii* by riboflavin is detected. In addition, a strong positive effect is shown on the abundance of other butyrate-producing bacteria, i.e. *Roseburia*, and on the lower number of pathosymbionts like *E. coli* in the fecal samples of the healthy volunteers. This opens a new window for the use of vitamins as prebiotics and help to maintain a healthy microbial balance in general and improve the health of patients with a dysbiosis or inflammatory bowel disease.

ACKNOWLEDGEMENTS

We would like to thank Klaas Nico Faber and Gerard Dijkstra for their critical review of the manuscript.

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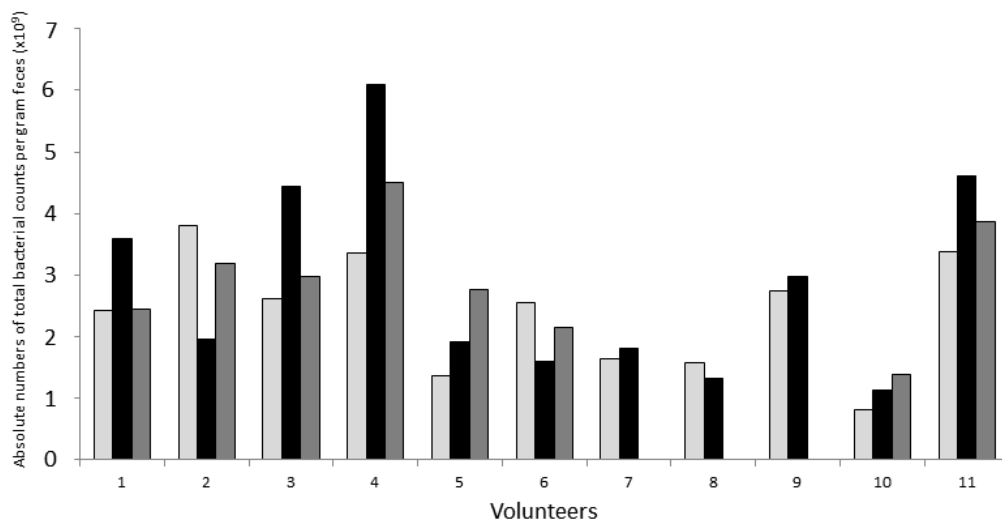
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Supplementary Data



Supplementary Figure 1. Total bacterial counts with EUB probe. Figure shows the average of two before samples (light gray), average of two intervention samples (black) and in one after sample (dark gray).