JOURNAL OF FUNCTIONAL FOODS 10 (2014) 117–127

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Evaluation of therapeutic properties of fermented vegetables extract (OM-X®) in the model of colitis induced by *Citrobacter rodentium* in mice

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ARTICLE INFO

Article history:
Received 15 March 2014
Received in revised form 28 May 2014
Accepted 3 June 2014
Available online 20 June 2014

Keywords:
Citrobacter rodentium
Inflammation
Microbiota
Epithelium
Tight junction

ABSTRACT

Infection of mice with *Citrobacter rodentium* serves as a model to study human intestinal infections. *C. rodentium* infection leads to increased production of inflammatory cytokines, immune cell infiltration and damage to the gut barrier. We used this model of colitis to evaluate the therapeutic properties of OM-X®, an extract prepared by fermentation of vegetables, seaweeds, fruits and mushrooms. Administration of OM-X® to *C. rodentium*-infected mice reduced damage to the intestinal epithelium, lowered inflammation scores, increased IL-10 expression and maintained FoxP3 gene expression. OM-X® also partially prevented bacterial translocation, increased expression of tight junction genes and increased proliferation of epithelial cells. PCR analysis of stool samples showed that OM-X® significantly reduced the populations of bacteria harboring *buk* gene (mostly *Clostridium* species). It is suggested that alterations of microbiota composition, following OM-X® consumption, contribute to protection against infection and epithelial damage, and lead to an increased expression of anti-inflammatory cytokines.

1. Introduction

The mouse pathogen *Citrobacter rodentium* belongs to the family of attaching and effacing (A/E) bacterial pathogens (Schauer & Falkow, 1993), which also includes the important human pathogens enterohemorrhagic *Escherichia coli* (EHEC) and enteropathogenic *E. coli* (EPEC) (Mundy, MacDonald, Dougan, Frankel, & Wiles, 2005). EPEC causes watery diarrhea, fever and nausea and is an important cause of infant diarrheal disease

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http://dx.doi.org/10.1016/j.jff.2014.06.003

in developing countries (Kapar, Nataro, & Mobley, 2004). In contrast, EHEC infection is associated with sporadic outbreaks across industrialized countries, due to the consumption of contaminated food or water supplies (Welinder-Olsson & Kaifser, 2005).

C. rodentium infection in mice can be used as a model to determine the role of early host factors, such as innate immune functions and effector cells, in intestinal inflammation. Oral *C. rodentium* infection of normal adult mice causes transient colonization of the caecum and colon, which peaks after 1 week, and resolves after 3–4 weeks (Maaser et al., 2004; Simmons et al., 2003; Wiles et al., 2004). In pathologic terms, mild colitis with epithelial hyperplasia, tight junction disruption, loss of goblet cells and mucosal infiltration of neutrophils and T cells were reported (Guttman, Samji, Li, Vogi, & Finlya, 2006; Rodrigues, Sousa, Johnson-Henry, Sherman, & Gareau, 2012; Wiles et al., 2004).

The possible curative effect of probiotics on mice infected by *C. rodentium* has been investigated (Chen, Louie, Shi, & Walker, 2005; Rodrigues et al., 2012). Probiotics are live microorganisms that are ingested to promote beneficial effects on health by altering indigenous microbiota and modulating host immune response (Ashraf, Vasiljevic, Day, Smith, & Donkor, 2014; Sanders et al., 2013). Preadministration and coadministration of probiotic mixture containing *Lactobacillus helveticus* and *Lactobacillus rhamnosus* improved *C. rodentium*-induced barrier dysfunction, epithelial hyperplasia, and binding of the pathogen to host colonocytes (Chen et al., 2005). Furthermore, some effects of prebiotics were reported. Prebiotics are defined as "non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, thus improving host health". Some prebiotic substances reduce intestinal inflammation and contribute to the recovery of damaged colonic mucosa (Al-Sheraji et al., 2013; Lara-Villoslada et al., 2006; Peshev & Van den Ende, 2014; Yousef, Pichyangkura, Soodvilai, Chatsudthipong, & Muanprasat, 2012).

This study was aimed to evaluate the therapeutic properties of oral administration of vegetable fermented extract (OM-X®) in a model of colitis induced by *C. rodentium* in mice. OM-X® is a mixture of vegetables, fruits, seaweeds and mushrooms naturally fermented using 12 strains of lactic acid bacteria and bifidobacteria. Final fermentation product is a paste rich in live lactic acid bacteria, metabolites and nutrients. OM-X® has potential probiotic and prebiotic activities; its safety for human consumption has been demonstrated (Spierings, Walshe, & Pescatore, 2013), and its beneficial effects on bone health (Kawakami et al., 2003), oral ulcerations (Hashim, Rahman, & Philip, 1999), and acute non-bloody diarrhea (local study) have been shown in human clinical studies. We hypothesized that pre-treatment with OM-X® before *C. rodentium* inoculation would exert a protective effect against infection and improve mucosal immune response, contributing to enhanced protection and attenuation of *Citrobacter*-mediated intestinal injury. Furthermore, in order to evaluate the prebiotic effect of OM-X®, we looked at changes of gut flora composition in animals treated with OM-X®, *vs.* untreated animals. We used an approach based on the PCR quantification of functional genes, *but* gene coding for butyryl-CoA:acetate CoA-transferase which is involved in the synthesis of butyrate and mostly found in species like *Faecalibacterium*, *Roseburia*, *Eubacterium*, and *buk* gene coding for butyrate kinase, mostly present in *Clostridium* species (Vital et al., 2013).

2. Materials and methods

2.1. Test product

OM-X® sample was provided by BIOBANK Co., Ltd. (Okayama, Japan). OM-X® is a naturally fermented mixture of vegetables, fruits, seaweeds and mushrooms using 12 strains of lactic acid bacteria and bifidobacteria. After 5 years of fermentation, the fermented mixture contains probiotics, prebiotics such as oligosaccharides and dietary fiber, trace amounts of vitamins, minerals, short-chain fatty acids, and amino acids (see Supplementary Table S1 in the online version at doi:10.1016/j.jff.2014.06.003).

2.2. Mice, treatment protocols

Female Balb/c mice (Charles River) aged 9 weeks were maintained in accredited animal facility at the Institut Pasteur of Lille according to governmental guidelines. Mice were infected by oral gavage with 0.1 ml of an overnight culture in Luria broth containing approximately 5×10^8 CFU of wild-type *C. rodentium* (formerly *Citrobacter freundii* biotype 4280 strain DBS100). OM-X® was resuspended in 0.5% carboxymethylcellulose (CMC) just before oral gavage and administered to mice at 500 mg/kg/day. Mice were divided into four groups: 1) Control mice treated with vehicle, 2) Control mice treated with OM-X®, 3) *C. rodentium*-infected mice treated with vehicle, 4) *C. rodentium*-infected mice treated with OM-X®. OM-X® or vehicle were administered for 3 weeks before infection of mice by *C. rodentium*. Treatment was continued for 10 days, then animals were sacrificed by cervical dislocation.

2.3. Mortality rate, body weight evolution

Mortality rate and body weight evolution were evaluated for all four groups, twice a week before infection, daily after infection.

2.4. Bacterial counting (detection of C. rodentium in stool samples)

Stools were collected from controls and infected mice at day 2 post-infection, placed in 1.5 ml of PBS, and homogenized. Homogenates were serially diluted in PBS, plated onto Luria broth agar plates, incubated overnight at 37 °C, and bacterial colonies were counted on the following day.

2.5. Intestinal permeability

Mice received by gavage 20 ml/kg body weight of phosphate-buffered saline (pH 7.4) containing 22 mg/ml fluorescein isothiocyanate-conjugated dextran (FITC-dextran, molecular mass 4.4 kDa; Sigma, St. Louis, MO, USA). A blood sample (~150 μl) was obtained in a capillary tube 2 h after

administration of the markers by retro-orbital puncture. Blood was centrifuged (1200 g at 4 °C) for 20 min. The concentration of fluorescein was determined by spectrophotofluorometry (CytoFluo 2300, Millipore, Bedford, MA, USA) with an excitation wavelength of 485 nm and an emission wavelength of 530 nm using serially diluted samples of the marker as standard.

2.6. Sacrifice, collection of samples

Uninfected mice or mice at day 10 post-infection were anesthetized with Halothane and killed by cervical dislocation, and colons were resected for further analysis; the colons were divided in half to separate the proximal and distal portions. Tissues were immediately placed in 4% paraformaldehyde for histological analysis, or stored at –86 °C for subsequent RNA extraction. Serum from each animal was collected at the time of sacrifice. Blood samples were collected by cardiac puncture, placed in a 1.1 ml Z-Gel micro tube (Sarstedt, Nümbrecht, Germany). Tubes were inverted five times and samples were then allowed to clot for 1 hour at room temperature. Tubes were centrifuged at 2300 g over 10 min at 20 °C. Serum (supernatant) was collected into a fresh pre-chilled tube and stored at –80 °C. Stool samples from each animal were collected on day –21 (before first gavage) and on day 10, before sacrifice.

2.7. Histological assessment of inflammation

Paraffin-embedded colonic tissue sections (5 mm) stained with hematoxylin and eosin were examined by two blinded observers. The severity of inflammatory lesions was quantified according to Ameho's criteria (Ameho et al., 1997): tissue sections were assessed for submucosal edema; epithelial hyperplasia (score based on percentage above the height of the control); epithelial integrity (score based on number of epithelial cells shedding per lesion, epithelial ulceration, epithelial ulceration with severe crypt destruction); neutrophil and mononuclear cell infiltration. Results were expressed as a mean ± SEM score.

2.8. Bacterial translocation

Upon dissection mesenteric lymph nodes (MLN) and spleen were collected and conserved for up to 2 hours in Ringer/Tween 80 medium under anaerobic conditions. After homogenization, samples were incubated in Brain Heart Broth. Ten and hundred time dilutions were performed in Ringer cystein medium. These dilutions were spread out on CS ANA plates (Columbia agar enriched with 5% defibrinated horse blood) and Mc Conkey plates, and incubated at 37 °C under aerobe or anaerobe atmosphere. Colonies were counted and identified 2 and 7 days after spreading.

2.9. MPO quantification

Myeloperoxydase (MPO) is an enzyme contained in polymorphonuclear neutrophil primary granules and is used as a marker of neutrophil infiltration. Proteins were extracted from the whole colon using an extraction buffer (200 mM NaCl, 5 mM ethylenediaminetetraacetic acid (EDTA), 10 mM Tris, 10% glycerin, 1 mM PMSF, 1 μg/ml leupeptin and 28 μg/ml aprotinin, pH 7.4). Proteins were quantified, MPO level was measured by ELISA (Clinisciences, Nanterre, France) according to manufacturer's instructions.

2.10. Evaluation of gene expression in the colonic wall by real-time qPCR

Total RNA was isolated from colonic tissue using RNeasy kit (Macherey Nagel, Hoerdt, France) according to manufacturer's instructions. RNA concentration was determined by spectrophotometry. After treatment at 37 °C for 30 min with 20–50 units of RNase-free DNase I (Roche Diagnostics Corporation, Indianapolis, IN, USA), oligo-dT primers (Roche Diagnostics Corporation) and "High capacity cDNA reverse transcription kit" (Applied Biosystems, Gent, Belgium) were used to synthesize single-stranded cDNA. PCR was performed using SYBR green Master Mix (Applera, Courtaboeuf, France) with murine specific oligonucleotides (see Supplementary Table S2 in the online version at doi:10.1016/j.jff.2014.06.003) in a GeneAmp Abiprism 7000 (Applera). In each assay, calibrated and no-template controls were included. Each sample was run in triplicate. SYBR green dye intensity was analyzed using the Abiprism 7000 SDS software (Applera). All results were normalized to the housekeeping gene GAPDH.

2.11. Proliferation and apoptosis of colonic epithelial cells

Colonic tissue was immediately placed in 4% paraformaldehyde solution for histological studies. Evaluation of epithelial cell proliferation was performed by immunohistochemistry using a rabbit polyclonal anti-PCNA antibody (Santa Cruz Corporation, Santa Cruz, CA, USA). Tissues were incubated with the primary anti-PCNA antibody at a concentration of 1/100e diluted in PBS + 0.5% Tween 20 and 3% goat serum overnight at 4 °C. A secondary antibody (anti-goat rabbit Alexa-fluor 488, 1/100e, 1 h) was used. Hoechst staining was used to stain the cell's nucleus. Quantification (green staining-PCNA normalized to blue staining-Hoechst) was performed using ImageJ software (Abramoff, Magalhaes, & Ram, 2004).

2.12. Quantification of buk and but gene in the stool samples

Dried stool samples were weighted and total DNA extracted using QIAamp DNA stool kit (Qiagen). DNA was eluted in 200 μl elution buffer. Purity was checked by measuring 260/280 and 260/230 OD ratios. Typically 5–15 μg total DNA were obtained from each sample; extracted genomic DNA was stored at –20 °C. Primers sequences were as described in Supplementary Table S3 in the online version at doi:10.1016/j.jff.2014.06.003. *but*-expressing bacteria include several species. Because of significant sequence variations in *but* gene itself it was not possible to design a single set of primers able to cover all these species. Two different primer pairs were used, one corresponding to *but* sequences found in bacteria related to *Faecalibacterium*, and one corresponding to the sequences of *Roseburia*-related species. PCR mix was ABsolute Blue QPCR SYBR Green (ABgene, Pittsburgh, PA, USA). Optimal hybridization temperature and primer concentrations were determined for this reagent and are re-

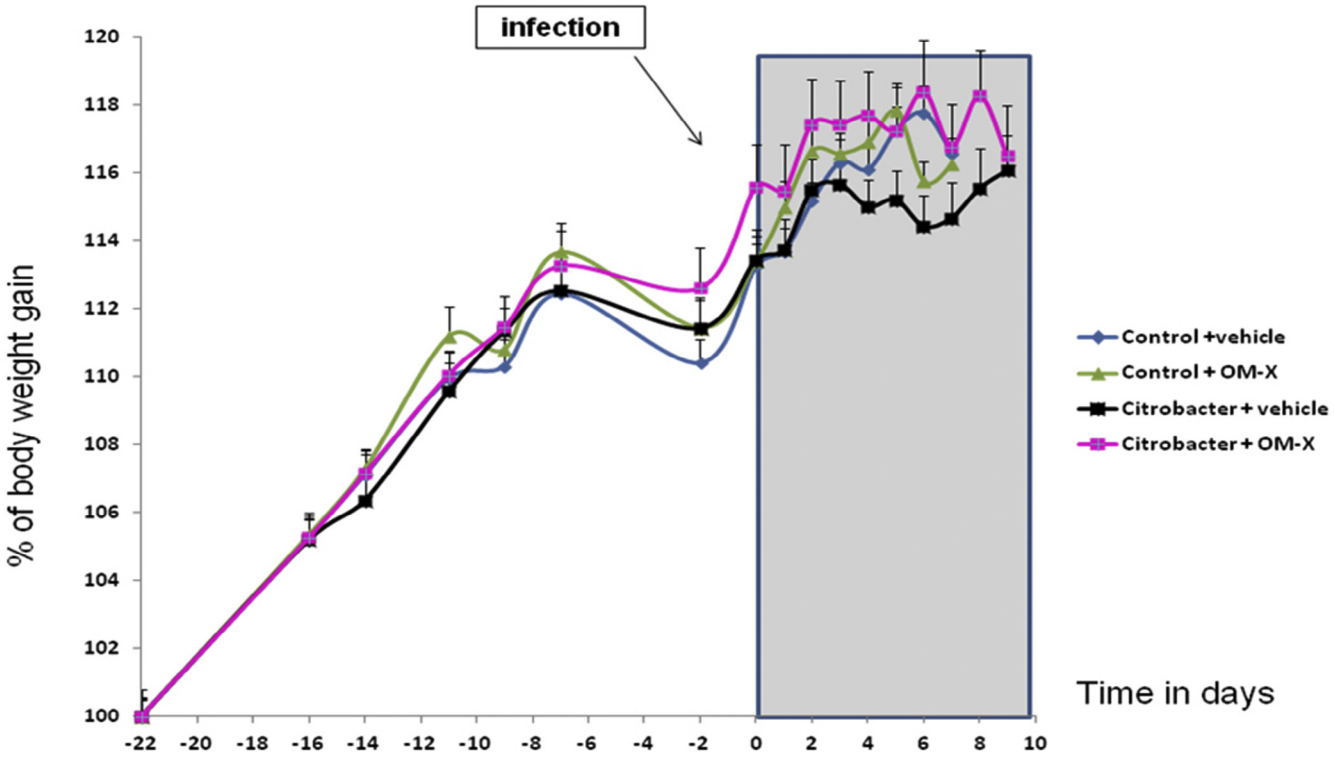

Fig. 1 – Effect of treatment on body weight evolution. Number of animals per group: control + vehicle *n* = 10; control + OM-X® *n* = 10; Citrobacter + vehicle *n* = 15; Citrobacter + OM-X® *n* = 15.

ported in the Supplementary Table S3 in the online version at doi:10.1016/j.jff.2014.06.003.

Quantative PCR was performed with a Mastercycler Realplex thermocycler (Eppendorf, Hamburg, Germany). Samples (100 ng DNA) were amplified in a total reaction volume of 25 µl containing 12.5 µl ABsolute Blue qPCR SYBR green (2×) and 2.5 µl of each primer. Thermocycling was done as follows: 15 min at 95 °C then 40 cycles 20 s at 95 °C, 20 s at individual annealing temperature and 30 s at 72 °C. Bacterial DNA was extracted from reference strains using Nucleospin DNA extraction kit, Macherey Nagel; serial 10-fold dilutions were used to build a standard curve. The following bacterial strains were used as references; *buk*: *Clostridium perfringens* LAB (from University of Lille), *but*: *Faecalibacterium prausnitzii* (DSMZ 17677) and *Roseburia intestinalis* (DSMZ 14610).

2.13. *Statistical analysis*

All comparisons were performed using the permutation test for two independent samples. Statistics have been calculated using the StatXact software (Cytel Inc, Cambridge, MA, USA). Differences were considered statistically significant when *p* value was <0.05.

3. Results

3.1. *OM-X® is safe and well tolerated*

The weight of the animals was monitored twice a week before *Citrobacter* infection, and daily after infection. Presence of *C. rodentium* in the stool of infected animals was confirmed by culturing stool samples collected on day 2 post-infection; no *C. rodentium* was detected in non-infected controls. Administration of OM-X® did not affect the growth of the animals (Fig. 1). Histological analysis did not reveal any inflammation or intestinal lesions in mice which had received OM-X®. As expected, oral infection by *C. rodentium* induced a significant colitis at the histological level with a mean score of 2.5 ± 0.21. Inflammatory lesions tended to be less severe in mice treated with OM-X®; however, this protective effect was not significant ($p = 0.07$) (Fig. 2).

MPO activity in the intestine can be used to quantitate inflammation. In this study, mice infected with *C. rodentium* had

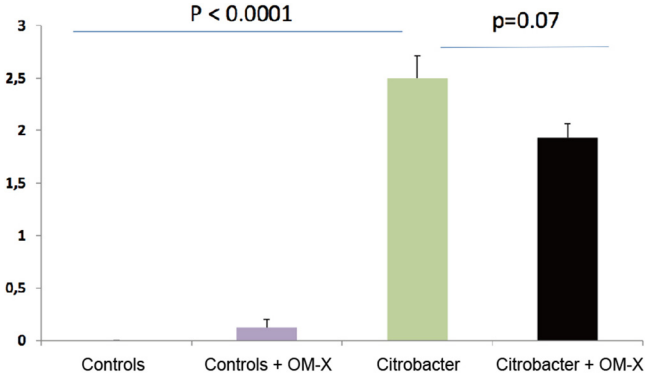

Fig. 2 – Effect of treatment on histological inflammatory scores. Unit is Ameho's score, as defined in Materials and methods section.

significantly higher MPO levels compared with healthy control mice, but this increase was not prevented by OM-X® administration (data not shown).

3.2. OM-X® inhibits bacterial translocation

Colonic inflammation leads to a modification of intestinal barrier integrity. Increase of intestinal permeability allows bacteria to cross the intestinal barrier from the lumen to mesenteric lymph nodes (MLN) and to the spleen through systemic blood circulation. Evaluation of bacterial translocation was performed in MLN and spleen of each infected mice after homogenization of the tissues and culture on selective media: CS ANA, a non-selective medium, and Mc Conkey which is more specific for enterobacteria like *C. rodentium*. Translocation levels in control mice were below the limit of detection, therefore only results for *C. rodentium*-infected mice are provided. A high level of translocation was observed in the spleen of infected mice receiving the vehicle, with 64% of the animals showing evidence of translocation. In mice treated with OM-X®, the translocation of enterobacteria following *Citrobacter* infection was reduced (28.5% *vs.* 64.3%). In MLN, bacterial translocation was observed in 42% of the infected mice receiving only the vehicle, compared with only 21% of the mice treated with OM-X®. Taken together these results indicate that OM-X® exerts a protective effect against bacterial translocation from the intestinal lumen to the MLN and the systemic blood circulation (Table 1).

3.3. OM-X® administration alters cytokine responses in the intestinal mucosa

To investigate the influence of OM-X® treatment on pathogen-induced cytokine response in the intestinal mucosa, we examined the expression of cytokine genes in colonic tissue (Fig. 3). In control mice without colitis, there was no induction of pro-inflammatory cytokines following OM-X® treatment; however, a significant induction of IFNγ was observed. *C. rodentium* caused a significant induction of TNF-α, which was not prevented by OM-X®. A significant effect of OM-X® was seen in infected mice, with a substantial induction of anti-inflammatory IL-10. Consistent with this induction of IL-10, OM-X® also had a significant effect on FoxP3 expression, which was maintained at control levels in OM-X®-treated animals, whereas animals infected with *Citrobacter*, but not treated by OM-X®, showed a significant drop of FoxP3 expression. FoxP3 is specifically expressed by regulatory T cells (Treg cells), which are anti-inflammatory regulatory cells present in the mucosa, and strong producers of IL-10. Our results suggest a role for OM-X® in immune regulation during enteric bacterial infection.

3.4. OM-X® administration alters expression of tight junction related genes

Tight junction proteins such as claudins and zonula occludens bind epithelial cells together and are crucial to maintain the integrity of the membrane. Dysregulation of their expression is an indication of membrane damage and increased permeability. Inflammation has been shown to decrease the expression of tight junction proteins. In control mice without colitis, OM-X® administration significantly induced the expression of genes coding for tight junction proteins ZO-1, ZO-2, and claudin-2 (Fig. 4). Induction was also perceptible in infected animals but less significant. For claudin-4 the effect was not significant in control animals, but OM-X® could significant suppress the decrease of clandin-4 in infected mice ($p = 0.04$). Our results suggest that OM-X® leads to the reinforcement of the intestinal barrier by increasing the expression of tight junction proteins.

3.5. Effect of OM-X® on intestinal epithelial cell proliferation

Cellular proliferation can be evaluated by PCNA (proliferation cell nuclear antigen) staining. Quantification using ImageJ software showed higher staining in OM-X®-treated mice than in controls. In *C. rodentium* infected mice, there was virtually no PCNA staining; however, proliferation again increased following treatment with OM-X® (Fig. 5).

3.6. Investigation of butyrate-producing bacterial communities

We investigated the effect of OM-X® on gut microbiota composition (Fig. 6). It has indeed been shown that colonic microbiota can influence the susceptibility to infectious colitis (Ghosh et al., 2011). We made the hypothesis that OM-X® could favor the growth of specific commensal species like *Faecalibacterium*, *Roseburia*, *Eubacterium*, *Acidaminococcus*, which are heavy producers of butyrate. Butyric acid is a short-chain fatty acid (SCFA) acting as an essential source of energy for colonocytes, stimulating their proliferation, and exerting anti-inflammatory effects (Pryde, Duncan, Hold, Stewart, & Flint, 2002).

Instead of focusing on specific bacterial species, we used an alternative approach based on the quantification of functional genes as described previously (Vital et al., 2013). The

Table 1 – Bacterial translocation in control, infected mice *vs.* OM-X®-treated, infected mice.

CS ANA (ns)	MLN	Infected mice	Infected mice + OM-X
	+	6	3
	–	8	11
	% positive mice	42.8%	21%
Mc Conkey (eb)	MLN	Infected mice	Infected mice + OM-X
	+	3	2
	–	11	12
	% positive mice	21%	14%

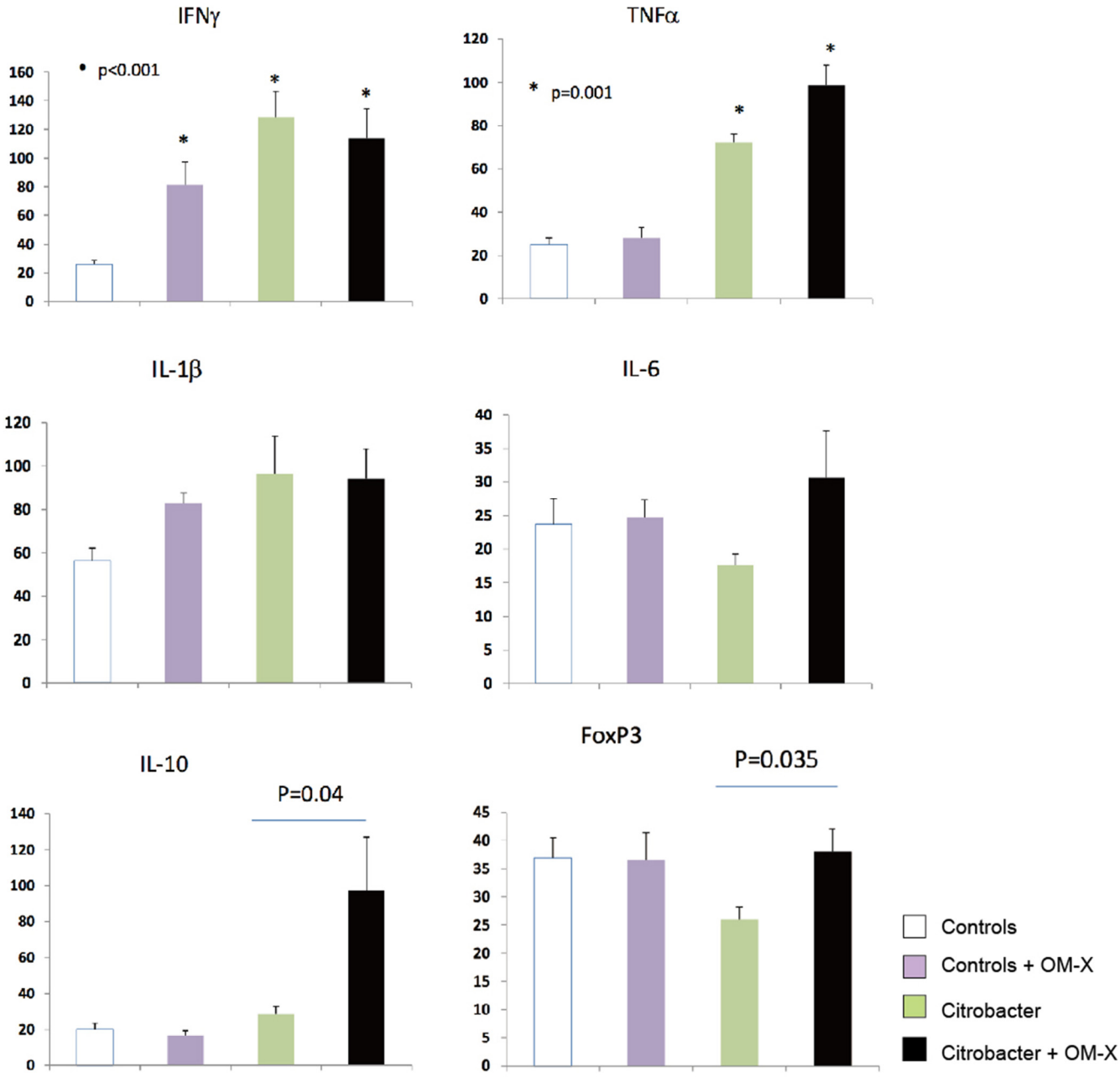

Fig. 3 – Expression of cytokine genes in colon tissue (real-time qPCR). Relative units: ratio target gene expression over GAPDH expression.

principle of this approach is to quantify key genes involved in butyrate synthesis pathways. The enzyme butyryl-CoA:acetate CoA-transferase (*but*) is involved in the synthesis of butyrate from butyryl-CoA; it is found mostly in species like *Faecalibacterium*, *Roseburia*, *Eubacterium* (Duncan, Barcenilla, Stewart, Pryde, & Flint, 2002). Another possible pathway of butyrate synthesis involves butyrate kinase (*buk*), whose gene is mostly present in *Clostridium* species (*Clostridium acetobutyricum*, *C. perfringens*) (Hartmanis, 1987; Louis et al., 2004). The *but* pathway appears to be the predominant pathway of butyrate synthesis in healthy humans. It yields more butyrate than the *buk* pathway; it requires acetate as a substrate and therefore may prevent the accumulation of acetate and lactate in the gut (Louis & Flint, 2009).

The average count of *buk* bacteria in stool samples from control mice was 450,000 cfu/g stool. However, in controls treated with OM-X®, *buk* count values significantly dropped to an average of 220,000 cfu/g stool ($p = 0.044$). *C. rodentium*-infected mice showed a high variability in their *buk* bacteria counts. OM-X® was able to decrease the average *buk* bacteria count from 346,000 cfu/g (infected mice, OM-X®-treated) to 250,000 cfu/g (infected mice, not treated); however, because of the high variability between animals this decrease did not reach statistical significance.

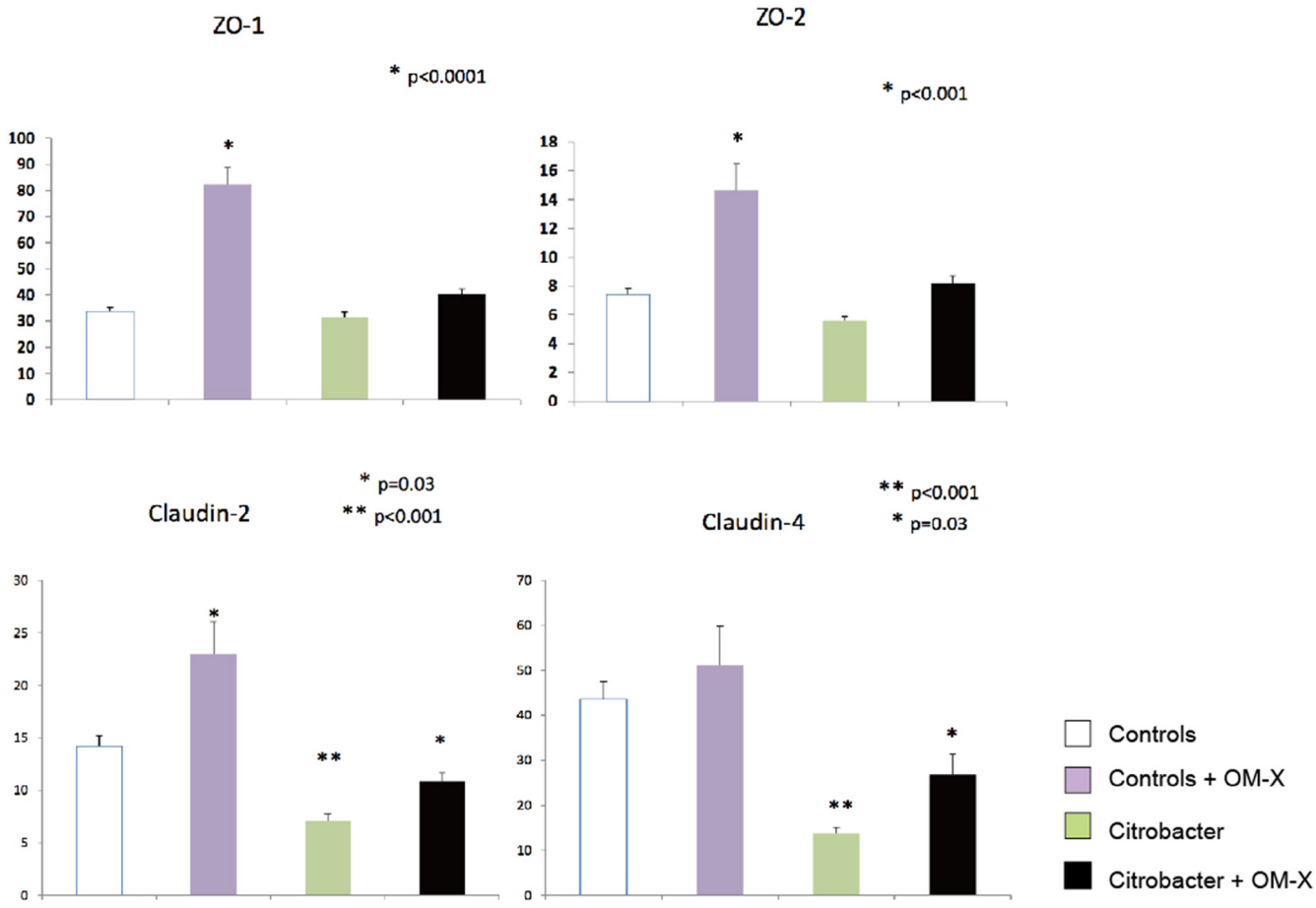

Fig. 4 – Expression of tight-junction genes in colon tissue (real-time qPCR). Relative units: ratio target gene expression over GAPDH expression.

but bacteria were clearly dominant compared with *buk* bacteria, with typical count numbers between 5 and 15 billion cfu/g stool for the *Faecalibacterium*-related species, and 2–4 billion for *Roseburia*-related species. There was a moderate increase of *but* bacteria related to *Faecalibacterium* in control mice treated with OM-X®, compared with controls that received only vehicle. The counts of *Roseburia*-related species were similar in both groups. Using both primer sets, a highly significant drop of *but*

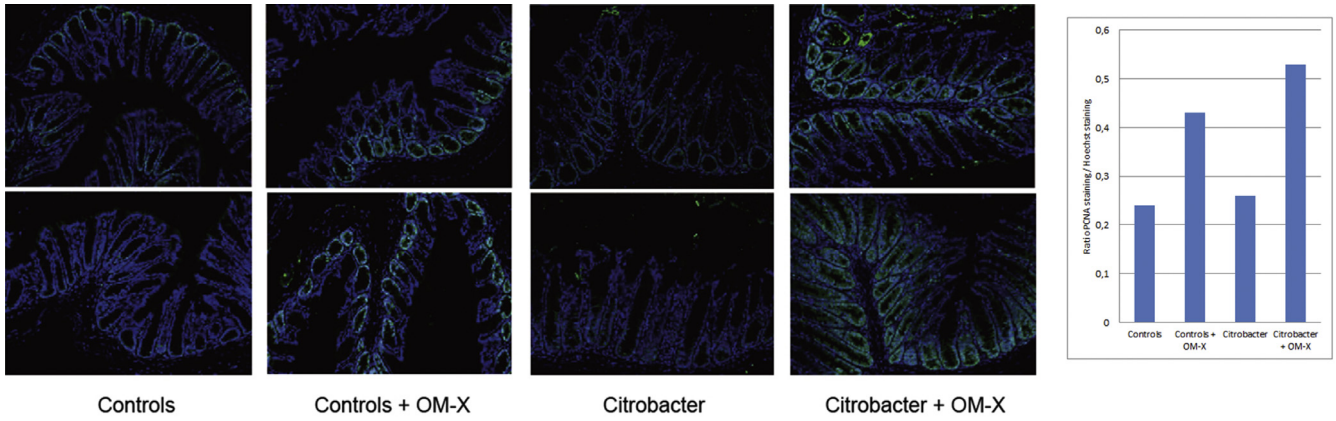

Fig. 5 – Evaluation of cellular proliferation by PCNA staining. Blue: Hoechst staining; green: PCNA staining, indicating proliferation. Quantification is provided as ratio of green staining over blue staining, using ImageJ software (Abramoff et al., 2004).

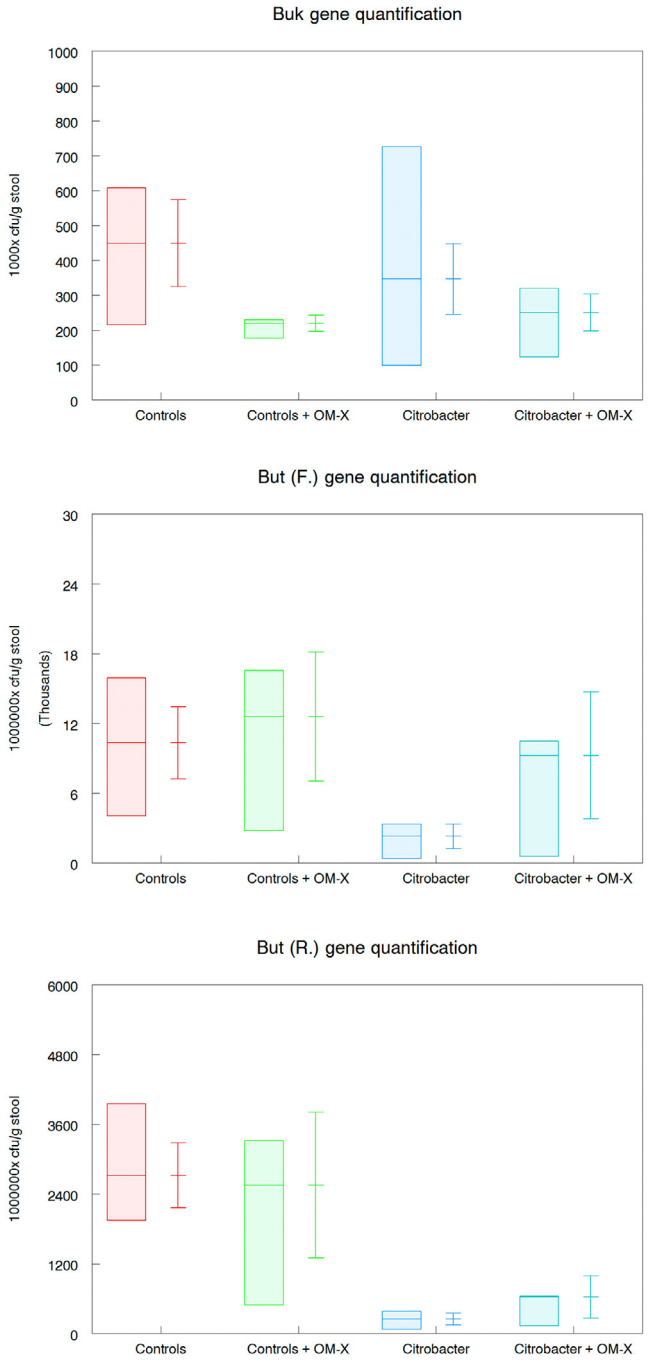

Fig. 6 – *buk* and *but* bacterial gene quantification in mouse stool samples. Expressed in cfu/g stool.

bacteria counts was observed following *C. rodentium* infection (from 10.3×10^9 to 2.28×10^9 cfu/g stool, $p = 0.028$ for *Faecalibacterium*-related species; from 2.7×10^9 to 0.25×10^9 cfu/g stool, $p = 0.0007$ for *Roseburia*-related species). Treatment of infected animals with OM-X® tended to prevent this drop. The mean count was back to 9.25×10^9 cfu/g stool for *Faecalibacterium*-related species, and to 0.63×10^9 cfu/g stool for *Roseburia*-related species. However because of the variability between animals these trends did not reach significance.

4. Discussion

Although histological analysis of the intestinal wall did not reveal any significant improvement of *C. rodentium*-induced colitis in mice treated with OM-X® (a trend could be observed, but not statistically significant, $p = 0.07$), a significant effect was observed at the molecular level since infected mice treated with OM-X® showed an increased expression of the anti-inflammatory cytokine IL-10. Major producers of IL-10 are Treg cells, which are crucial effectors of tolerogenic and anti-inflammatory pathways in the gut membrane (Geiger & Tauro, 2012; Murai et al., 2009). *Citrobacter* infection reduced the colonic expression of FoxP3, a specific marker of Treg cells. The levels of FoxP3 expression were however restored in infected mice which had received OM-X®. Increased expression of FoxP3 indicates full maturation of Treg cells and activation of their suppressive capacity, and/or could also result from an increased number of Treg cells in the mucosa (recruitment).

In *Citrobacter* infection, like in any other type of infection, the immune system must keep a balance between activation that clears up the pathogen, and excessive inflammation that would lead to tissue damage. Induction of Treg had already been observed in the context of *Citrobacter* infection (Symonds et al., 2009) and was interpreted as a mechanism to prevent excessive colitis and epithelium destruction. Our results suggest that OM-X® treatment will reinforce the suppressive arm of the immune response, leading to a reduction of inflammation and better protection of membrane integrity. Interestingly induction of IFNγ, which was observed in mice treated with OM-X®, could have the same biological relevance. Although IFNγ is considered as pro-inflammatory, it has indeed been shown to exert a crucial homeostatic function that limits inflammation-related tissue damage, through multiple mechanisms including inhibition of matrix metalloproteinases and complement components (Hu & Ivashkiv, 2009). This protective action of IFNγ has been described in particular in the context of mouse colitis (Thelemann et al., 2014).

Maintenance of intestinal barrier function is crucial to prevent microorganisms and luminal antigens from entering the body. Proper barrier function depends on epithelial cells renewal, as well as on the capacity of cells to tightly adhere to each other in order to form a continuous lining. The secretion of mucus by specialized cells in the mucosa also contributes to membrane impermeability. We found that OM-X® administration resulted in a significant decrease of bacterial translocation to spleen and MLN, which is an indication of improved intestinal barrier function. Furthermore, OM-X® was able to increase significantly the expression of tight junction proteins (ZO-1, ZO-2, claudin-2, and claudin-4), which are playing an important role in the reinforcement of the gut membrane (Fanning & Anderson, 2009; Liu et al., 2013; Tsukita et al., 2009).

An intriguing result of our study was the increased proliferation of colonic epithelial cells observed in infected animals treated with OM-X®. Although hyperplasia is a hallmark of *Citrobacter* infections, in our experiment very little proliferation was observed in infected animals. Most probably the Balb/c mice used in our study, which are known to display little

sensitivity to *Citrobacter* infection (Mundy et al., 2005), had already cleared most of the pathogens; the increased proliferation associated with OM-X®, which was associated with decreased translocation and improvement of barrier function markers, should therefore be interpreted as part of a gut mucosa repair process, contributing to wound healing and maintenance of barrier function. Failure to properly repair lesions in the gut mucosa indeed leads to disruption of the barrier and is a key feature of colitis (Iizuka & Konno, 2011).

Bacterial translocation is a natural process but excessive translocation can lead to infections and various inflammation-related disorders (Balzan, de Almeida-Quadros, de Cleva, Zilberstein, & Cecconello, 2007; Ilan, 2012; Klatt, Funderburg, & Brenchley, 2013). The ability of OM-X® to restore membrane integrity and reduce bacterial translocation is therefore highly beneficial, and opens possibilities of applications for the prevention of several health disorders. It is known that butyrate produced by commensal bacteria belonging to Clostridium IV and XIVa clusters can exert beneficial effects including increased IL-10 expression, recruitment of Treg cells in the gut membrane and proliferation of epithelial cells (Arpaia et al., 2013; Atarashi et al., 2011, 2013; Furusawa et al., 2013). The abundance of each of these groups is estimated by fluorescent *in situ* hybridization at around 2–15% of the total microbiota (Louis & Flint, 2009). However, both clostridial clusters comprise several bacterial species, some butyrate producers and some non butyrate producers. In order to quantify the populations of butyrate-producing bacteria we used an approach based on analysis of functional genes related to butyrate synthesis pathways, instead of focusing on bacterial 16S rRNA sequences (Vital et al., 2013). Real-time PCR analysis was performed to quantify the amount of *but* sequences (present in *Faecalibacterium*, *Roseburia*, *Eubacterium*) and *buk* sequences (present in *Clostridium* s.s. species). OM-X® was able to reduce *buk* populations in control mice, and this effect was significant ($p = 0.044$). A similar trend (however not significant, because of sample variability) was observed in *C. rodentium*-infected mice.

Bacteria containing *but* sequences were detected at much higher levels than bacteria containing *buk* sequences, and therefore represent the predominant butyrate-synthesizing organisms in healthy mice. *C. rodentium* infection induced a dramatic drop of butyrate-producing organisms, possibly contributing to the inflammation and increased permeability seen in infected mice. In both controls and *Citrobacter*-infected mice, administration of OM-X® tended to increase the counts of *but* bacteria; however, this effect was not significant.

Taken together these results are encouraging, demonstrating the capacity of OM-X® to improve the composition of the gut microbial composition in mice, by significantly decreasing the amount of *buk* bacteria and slightly favoring the growth of *but* populations. These results therefore suggest a prebiotic effect of OM-X®. During manufacturing of OM-X®, which is a non heated process, the Maillard reaction results in the accumulation of melanoidin (data not shown). Melanoidin is an end-product typically found in fermented food, and its capacity to alter gut microbiota composition has already been reported (Fogliano & Morales, 2011). Modification of the *buk*/*but* bacterial balance in animals consuming OM-X® may therefore be the result of melanoidin ingestion.

A particularly interesting finding is the capacity of OM-X® to reduce the counts of bacteria carrying the *buk* gene. *Clostridium difficile* and *C. perfringens* are two carriers of *buk* gene (Aboulnaga et al., 2013), known to produce toxins which can alter intestinal permeability, predominantly by opening the mucosa tight junction (Goldstein et al., 2009; Hansen et al., 2013). These results confirm previous (Y. Miyake, 2006) data showing the *in vitro* anti-microbial effect of OM-X® against *C. difficile*. The capacity to inhibit these pathogenic organisms is an interesting property, which could find applications such as the prevention or treatment of *C. difficile* infections following antibiotic treatment.

Acknowledgment

We would like to thank Dr Iichiro Ohhira for fruitful discussions.

Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.jff.2014.06.003.

REFERENCES

Aboulnaga, H., Pinkenburg, O., Schiffels, J., El-Refai, A., Buckel, W., & Selmer, T. (2013). Effect of an oxygen-tolerant butyril-coenzyme A dehydrogenase/ electron-transferring flavoprotein complex from *Clostridium difficile* on butyrate production in *Escherichia coli*. *Journal of Bacteriology*, *195*, 3704–3713.

Abramoff, M. D., Magalhaes, P. J., & Ram, S. J. (2004). Image processing with ImageJ. *Biophotonics International*, *11*(7), 36–42.

Al-Sheraji, S. H., Ismail, A., Manap, M. Y., Mustafa, S., Yusof, R. M., & Hassan, F. A. (2013). Prebiotics as functional foods: A review. *Journal of Functional Foods*, 5, 1542–1553.

Ameho, C. K., Adjei, A. A., Harrison, E. K., Takeshita, K., Morioka, T., Arakaki, Y., Ito, E., Suzuki, I., Kulkarni, A., Kawajiri, A., & Yamamoto, S. (1997). Prophylactic effect of dietary glutamine supplementation on interleukin 8 and tumour necrosis factor alpha production in trinitrobenzene sulphonic acid induced colitis. *Gut*, *41*, 487–493.

Arpaia, N., Campbell, C., Fan, X., Dikiy, S., van der Veeken, J., de Roos, P., Liu, H., Cross, J. R., Pfeffer, K., Coffer, P. J., & Rudensky, A. Y. (2013). Metabolites produced by commensal bacteria promote peripheral regulatory T-cell generation. *Nature*, 504, 451–455.

Ashraf, R., Vasiljevic, T., Day, S. L., Smith, S. C., & Donkor, O. N. (2014). Lactic acid bacteria and probiotic organisms induce different cytokine profile and regulatory T cells mechanisms. *Journal of Functional Foods*, *6*, 395–409.

Atarashi, K., Tanoue, T., Oshima, K., Suda, W., Nagano, Y., Nishikawa, H., Fukuda, S., Saito, T., Narushima, S., Hase, K., Kim, S., Fritz, J. V., Wilmes, P., Ueha, S., Matsushima, K., Ohno, H., Olle, B., Sakaguchi, S., Taniguchi, T., Morita, H., Hattori, M., & Honda, K. (2013). Treg induction by a rationally selected mixture of *Clostridium* strains from human microbiota. *Nature*, 500, 232–236.

Atarashi, K., Tanoue, T., Shima, T., Imaoka, A., Kuwahara, T., Momose, Y., Cheng, G., Yamasaki, S., Saito, T., Ohba, Y., Taniguchi, T., Takeda, K., Hori, S., Ivanov, I. I., Umesaki, Y.,

Itoh, K., & Honda, K. (2011). Induction of colonic regulatory T cells by indigenous *Clostridium* species. *Science*, *331*, 337–341.
Balzan, S., de Almeida-Quadros, C., de Cleva, R., Zilberstein, B., & Cecconello, I. (2007). Bacterial translocation: Overview of mechanisms and clinical impact. *Journal of Gastroenterology and Hepatology*, *22*, 464–471.
Chen, C. C., Louie, S., Shi, H. N., & Walker, W. A. (2005). Preinoculation with the probiotic *Lactobacillus acidophilus* early in life effectively inhibits murine *Citrobacter rodentium* colitis. *Pediatric Research*, *58*, 1185–1191.
Duncan, S. H., Barcenilla, A., Stewart, C. S., Pryde, S. E., & Flint, H. J. (2002). Acetate utilization and butyryl coenzyme A (CoA):acetate-CoA transferase in butyrate-producing bacteria from human large intestine. *Applied and Environmental Microbiology*, *68*, 5186–5190.
Fanning, A. S., & Anderson, J. M. (2009). Zonula occludins-1 and -2 are cytosolic scaffolds that regulate the assembly of cellular junctions. *Annals of the New York Academy of Sciences*, *1165*, 113–120.
Fogliano, V., & Morales, F. J. (2011). Estimation of dietary intake of melanoidins from coffee and bread. *Food & Function*, *2*, 117–123.
Furusawa, Y., Obata, Y., Fukuda, S., Nakato, G., Takahashi, D., Nakanishi, Y., Uetake, C., Kato, K., Kato, T., Takahashi, M., Fukuda, N. N., Murakami, S., Miyauchi, E., Hino, S., Onawa, S., Fujimura, Y., Lockett, T., Clarke, J. M., Topping, D. L., Tomita, M., Hori, S., Ohara, O., Morita, T., Koseki, H., Kikuchi, J., Honda, K., Hase, K., & Ohno, H. (2013). Commensal microbe-derived butyrate induces the differentiation of colonic regulatory T cells. *Nature*, *504*, 446–450.
Geiger, T. L., & Tauro, S. (2012). Nature and nurture in Foxp3(+) regulatory T cell development, stability, and function. *Human Immunology*, *73*, 232–239.
Ghosh, S., Dai, C., Brown, K., Rajendiran, E., Makarenko, S., Baker, J., Ma, C., Halder, S., Montero, M., Ionescu, V. A., Klegeris, A., Vallance, B. A., & Gibson, D. L. (2011). Colonic microbiota alters host susceptibility to infectious colitis by modulating inflammation, redox status, and ion transporter gene expression. *American Journal of Physiology. Gastrointestinal and Liver Physiology*, *301*, G39–G49.
Goldstein, J., Morris, W. E., Loidl, C. F., Tironi-Farinati, C., McClane, B. A., Uzal, F. A., & Miyakawa, M. E. (2009). *Clostridium perfringens* epsilon toxin increases the small intestinal permeability in mice and rats. *PLoS ONE*, e7065.
Guttman, J. A., Samji, F. N., Li, Y., Vogi, A. W., & Finlya, B. B. (2006). Evidence that tight junctions are disrupted due to intimate bacterial contact and not inflammation during attaching and effacing pathogen infection *in vivo*. *Infection and Immunity*, *74*, 6075–6084.
Hansen, A., Alston, L., Tulk, S. E., Schenck, L. P., Grassie, M. E., Alhassan, B. F., Veermalla, A. T., Al-Bashir, S., Gendron, F. P., Altier, C., MacDonald, J. A., Beck, P. L., & Hirota, S. A. (2013). The P2Y6 receptor mediates *Clostridium difficile* toxin-induced CXCL8/IL-8 production and intestinal epithelial barrier dysfunction. *PLoS ONE*, *8*, e81491.
Hartmanis, M. G. (1987). Butyrate kinase from *Clostridium acetobutylicum*. *The Journal of Biological Chemistry*, *262*, 617–621.
Hashim, B. Y., Rahman, R. A., & Philip, K. (1999). Lactic acid bacteria is beneficial for oral aphthous ulcerations. *Annals of Dentistry*, *1*, 44–47.
Hu, X., & Ivashkiv, L. B. (2009). Cross-regulation of signaling and immune responses by IFN-gamma and STAT1. *Immunity*, *31*(4), 539–550.
Iizuka, M., & Konno, S. (2011). Wound healing of intestinal epithelial cells. *World Journal of Gastroenterology*, *17*, 2161–2171.
Ilan, Y. (2012). Leaky gut and the liver: A role for bacterial translocation in nonalcoholic steatohepatitis. *World Journal of Gastroenterology*, *18*, 2609–2618.
Kapar, J. B., Nataro, J. P., & Mobley, H. L. (2004). Pathogenic *Escherichia coli*. *Nature Reviews. Microbiology*, *2*, 123–140.
Kawakami, M., Ohhira, I., Araki, N., Inokihara, K., Iwasaki, H., & Matsubara, T. (2003). The influence of lactic acid bacteria (OM-X) on bone structure. *The Journal of Applied Nutrition*, *53*, 2–8.
Klatt, N. R., Funderburg, N. T., & Brenchley, J. M. (2013). Microbial translocation, immune activation, and HIV disease. *Trends in Microbiology*, *21*, 6–13.
Lara-Villoslada, F., Debras, E., Nieto, A., Concha, A., Gálvez, J., López-Huertas, E., Boza, J., Obled, C., & Xaus, J. (2006). Oligosaccharides isolated from goat milk reduce intestinal inflammation in a rat model of dextran sodium sulfate-induced colitis. *Clinical Nutrition*, *25*, 477–488.
Liu, X., Yang, G., Geng, X. R., Cao, Y., Li, N., Ma, L., Chen, S., Yang, P. C., & Liu, A. (2013). Microbial products induce claudin-2 to compromise gut epithelial barrier function. *PLoS ONE*, *8*, e68547.
Louis, P., Duncan, S. H., McCrae, S. I., Millar, J., Jackson, M. S., & Flint, H. J. (2004). Restricted distribution of the butyrate kinase pathway among butyrate-producing bacteria from the human colon. *Journal of Bacteriology*, *186*, 2099–2106.
Louis, P., & Flint, H. J. (2009). Diversity, metabolism and microbial ecology of butyrate-producing bacteria from the human large intestine. *FEMS Microbiology Letters*, *294*, 1–8.
Maaser, C., Housley, M. P., Iimura, M., Smith, J. R., Vallance, B. A., Finlay, B. B., Schreiber, J. R., Varki, N. M., Kagnoff, M. F., & Eckmann, L. (2004). Clearance of *Citrobacter rodentium* requires B cells but not secretory immunoglobulin A (IgA) or IgM antibodies. *Infection and Immunity*, *72*, 3315–3324.
Mundy, R., MacDonald, T. T., Dougan, G., Frankel, G., & Wiles, S. (2005). *Citrobacter rodentium* of mice and man. *Cell*, *7*, 1697–1706.
Murai, M., Turovskaya, O., Kim, G., Madan, R., Karp, C. L., Cheroutre, H., & Kronenberg, M. (2009). Interleukin 10 acts on regulatory T cells to maintain expression of the transcription factor Foxp3 and suppressive function in mice with colitis. *Nature Immunology*, *10*, 1178–1184.
Peshev, D., & Van den Ende, W. (2014). Fructans: Prebiotics and immunomodulators. *Journal of Functional Foods*, *8*, 348–357.
Pryde, S. E., Duncan, S. H., Hold, G. L., Stewart, C. S., & Flint, H. J. (2002). The microbiology of butyrate formation in the human colon. *FEMS Microbiology Letters*, *217*, 133–139.
Rodrigues, D. M., Sousa, A. J., Johnson-Henry, K. C., Sherman, P. M., & Gareau, M. G. (2012). Probiotics are effective for the prevention and treatment of *Citrobacter rodentium*-induced colitis in mice. *The Journal of Infectious Diseases*, *206*, 99–109.
Sanders, M. E., Guarner, F., Guerrant, R., Holt, P. R., Quigley, E. M., Sartor, R. B., Sherman, P. M., & Mayer, E. A. (2013). An update on the use and investigation of probiotics in health and disease. *Gut*, *62*, 787–796.
Schauer, D. B., & Falkow, S. (1993). Attaching and effacing locus of a *Citrobacter freundii* biotype that causes transmissible murine colonic hyperplasia. *Infection and Immunity*, *61*, 2486–2492.
Simmons, C. P., Clare, S., Ghaem-Maghami, M., Uren, T. K., Rankin, J., Huett, A., Goldin, R., Lewis, D. J., MacDonald, T. T., Strugnell, R. A., Frankel, G., & Dougan, G. (2003). Central role for B lymphocytes and CD4+ T cells in immunity to infection by the attaching and effacing pathogen *Citrobacter rodentium*. *Infection and Immunity*, *71*, 5077–5086.
Spierings, E. L. H., Walshe, T., & Pescatore, F. (2013). Safety and tolerability of Dr Ohhira's OM-X capsules in healthy volunteers. *Integrative Medicine*, *12*, 38–42.
Symonds, E. L., Riedel, C. U., O'Mahony, D., Lapthorne, S., O'Mahony, L., & Shanahan, F. (2009). Involvement of T helper type 17 and regulatory T cell activity in *Citrobacter rodentium*

invasion and inflammatory damage. *Clinical and Experimental Immunology*, *157*, 148–154.

Thelemann, C., Eren, R. O., Coutaz, M., Brasseit, J., Bouzourene, H., Rosa, M., Duval, A., Lavanchy, C., Mack, V., Mueller, C., Reith, W., & Acha-Orbea, H. (2014). Interferon-gamma induces expression of MHC class II on intestinal epithelial cells and protects mice from colitis. *PLoS ONE*, *9*(1), e86844.

Tsukita, S., Katsuno, T., Yamazaki, Y., Umeda, K., Tamura, A., & Tsukita, S. (2009). Roles of ZO-1 and ZO-2 in establishment of the belt-like adherens and tight junctions with paracellular permselective barrier function. *Annals of the New York Academy of Sciences*, *1165*, 44–52.

Vital, M., Penton, R. C., Wang, Q., Young, B. V., Antonopoulos, A. D., Sogin, L. M., Morrison, G. H., Raffals, L., Chang, B. E., Huffnagle, B. G., Schmidt, M. T., Cole, R. J., & Tiedje, M. J. (2013). A gene-targeted approach to investigate the intestinal butyrate-producing bacterial community. *Microbiome*, *1*, 8.

Welinder-Olsson, C., & Kaifser, B. (2005). Enterohemorrhagic *Escherichia coli* (EHEC). *Scandinavian Journal of Infectious Diseases*, *37*, 405–416.

Wiles, S., Clare, S., Harker, J., Huett, A., Young, D., Dougan, G., & Frankel, G. (2004). Organ specificity, colonization and clearance dynamics *in vivo* following oral challenges with the murine pathogen *Citrobacter rodentium*. *Cellular Microbiology*, *6*, 963–972.

Yousef, M., Pichyangkura, R., Soodvilai, S., Chatsudthipong, V., & Muanprasat, C. (2012). Chitosan oligosaccharide as potential therapy of inflammatory bowel disease: Therapeutic efficacy and possible mechanisms of action. *Pharmacological Research*, *66*, 66–79.