**Bifidobacterium adolescentis** protects from the development of nonalcoholic steatohepatitis in a mouse model☆☆☆

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Abstract

To investigate the hypothesis that an oral supplementation of *Bifidobacterium adolescentis* protects against a diet-induced nonalcoholic steatohepatitis in a mouse model, C57BL/6 mice were fed either a Western-style or a control diet fortified with *B. adolescentis* (5×10⁸ cfu/ml) ad libitum for 12 weeks. Mice fed a Western-style diet gained significantly more weight than mice fed a control diet and developed a mild steatohepatitis. Western-style diet-fed groups concomitantly treated with *B. adolescentis* had significantly decreased liver damage, whereas portal endotoxin levels and toll-like receptor-4 protein levels as well as myeloid differentiation factor 88 mRNA were increased in livers of both Western-style diet-fed groups. The protective effects of the *B. adolescentis* were associated with a significant attenuation of the formation of reactive oxygen species, activation of nuclear factor κB (NFκB) and induction of markers of inflammation in the liver. Taken together, our data suggest that an oral supplementation of the *B. adolescentis* attenuates diet-induced steatohepatitis, and this effect is associated with prevention from lipid peroxidation, NFκB activation and finally inflammation in the liver.

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1. Introduction

Nonalcoholic fatty liver disease (NAFLD) is by now recognized as one of the most common liver diseases in Western countries [1,2]. The spectrum of the disease ranges from simple steatosis to nonalcoholic steatohepatitis (NASH), fibrosis and cirrhosis [3]. Lately, it has become more obvious that steatosis, long thought to be a relatively benign state of injury, is a state of liver disease in which the liver is more vulnerable to injury from various causes [4]. It is known by a number of studies that oxidative stress and lipid peroxidation play a crucial role in the pathogenesis of NAFLD/NASH (reviewed in [5]). In this context, markers like 4-hydroxyxynenal (4-HNE) protein adducts, inducible nitric oxygen synthase (iNOS) and heme oxygense-1 (HO-1) act as a marker of lipid peroxidation [6–10]. Moreover, development of NAFLD/NASH is associated with the development of insulin resistance and cardiovascular disease, further emphasizing the relevance of these diseases [11,12]. Since mechanisms underlying NAFLD are still poorly understood, therapeutic and preventive options are basically limited to weight control. There is cumulative evidence that, in the early phases of NAFLD, bacterial overgrowth in the intestine, impaired intestinal barrier function and an increased translocation of bacterial endotoxin may be involved in the development of the liver damage (reviewed in [13]). Human studies confirmed that patients with different stages of NAFLD suffer from endotoxemia and have higher prevalence of bacterial overgrowth in the small intestine. Such pathologies are associated with an increased expression of the endotoxin receptor toll-like receptor (TLR)-4 in the liver [14–16]. Interestingly, studies in rodent models of NAFLD suggested that the development of NAFLD is markedly reduced by treatment with prebiotics [17], antibiotics [18] and possibly selected probiotics [19–21]. Bifidobacteria are part of the ‘normal’ human intestinal microbiota. Their presence is influenced by factors such as diet, and age; their functions comprise protection against endotoxin translocation and support of mucosal barrier functions [22,23]. The use of *Bifidobacterium* spp. as oral supplementations in human studies has been encouraging in terms of potential benefit to the host, both through improving the microbiota pattern and through enhancing the immune response to bacterial and virus challenge [24–26]. The purpose of the present study was to test the hypothesis that
**Bifidobacterium adolescentis** protects against the development of Western-style diet-induced NASH in a mouse model.

### 2. Materials and methods

#### 2.1. Animals and treatments

Eight-week-old C57BL/6j mice (n=4–6 per group; Janvier S.A.S., Le-Genes-St-Isle, France) were housed in a specific pathogen-free barrier facility in individually ventilated cages. Animals were either fed a control diet (C; control diet to TDB8137, ssnff Spezialitäten GmbH, Soest, Germany) or Western-style diet (WS; TDB8137 modified-Western type diet and CDB8137 modified, ssnff Spezialitäten GmbH, Soest, Germany); tap water fortified with *B. adolescentis* ATCC 15705 (B.a.) at 5×10⁷ cfu/ml ad libitum for 12 weeks. The *Bifidobacterium* was cultured in brain heart broth (Merck, Germany) under anaerobe conditions. For the treatment, B.a. was washed and diluted with phosphate-buffered saline to adjust the appropriate concentration. All procedures were approved by the local Institutional Animal Care and Use Committee. Body weight and consumption of chow were assessed once a week. As B.a. survives for at least 2 days in drinking water (own unpublished data), drinking solution with B.a. supplementation was changed and assessed every second day to ensure that the animals had the same B.a. intake. The concentration of the bacteria was used previously [27]. After 10 weeks, mice were fasted for 6 h, and blood was taken from the tail vein to determine the fasting glucose. At sacrifice, animals were anesthetized with 80 mg ketamine and 6 mg xylazine/kg body weight by intraperitoneal injection, and blood was collected from the portal vein. Portions of liver tissue were frozen immediately in liquid nitrogen and stored at −80 °C, while others were fixed in neutral-buffered formalin or frozen-fixed in Tissue Tek O.C.T. compound (Sakura Finetek Europe, Netherlands) for sectioning and mounting on microscope slides.

#### 2.2. Oil Red O staining

To determine hepatic lipid accumulation, frozen sections of liver (10 μm) were stained with Oil Red O (Sigma-Aldrich, USA), washed and counterstained with hematoxylin (Sigma-Aldrich, USA) as described previously [28]. Analysis of staining was performed densitometrically in eight randomly selected fields, and representative pictures of the Oil Red O staining at 400× magnification were taken using a microscope (Axiovert 200M, Zeiss, Germany).

#### 2.3. Liver histology, blood parameter and neutrophil staining

For assessment of liver histology, paraffin-embedded sections of liver (5 μm) were stained with hematoxylin and eosin (H&E). Using a system incorporated in a microscope (Axio Vert 200M, Zeiss, Germany), representative photomicrographs were captured at a 200× magnification. Sections were scored using the *NAFLD Activity Score* (NAS) as described by Kleiner et al. [29]. Fasting glucose was determined using a glucometer (Bayer Vital, Germany). Plasma alanine aminotransferase (ALT) activity was measured using a commercially available kit (Beckman Coulter, Germany).

**Neutrophils** were stained using a commercially available Naphthol AS-D Chloroacetate-Esterase-Kit (Sigma-Aldrich, USA). To determine the neutrophil counts, staining was assessed in eight randomly selected fields using the microscope (63×0.75) (Leica, Germany).

#### 2.4. RNA isolation and real-time polymerase chain reaction (RT-PCR)

Total RNA was extracted from liver samples using pegGOLD TriFast (PEQLAB, Germany). RNA concentrations were determined spectrophotometrically, and 1 μg total RNA was reverse transcribed according to the manufacturer's instructions of the reverse transcription system (Promega, Germany). PCR primers for myeloid differentiation factor 88 (MyD88), plasminogen activator inhibitor-1 (PAI-1), HO-1, chemokine (C-C motif) ligand 2 and 19 (CCL2 and CCL19) and 18S (Table 1) were designed using Primer3 software (Whitehead Institute for Biomedical Research, USA). SYBR Green Universal PCR Master Mix (Applied Biosystems, Germany) was used to prepare the PCR mix. The amplification reactions were carried out in an iCycler (BioRad Laboratories, Germany) with an initial hold step (95 °C for 3 min) and 50 cycles of a three-step PCR (95 °C for 15 s, 60 °C for 15 s, 72 °C for 30 s). The fluorescence intensity of each sample was measured at each temperature change to monitor amplification of the target gene. The comparative Cₚ method was used to determine the amount of target, normalized to an endogenous reference (18S) and relative to a calibrator (2⁻ΔΔCt). The purity of PCR products was verified by melting curves and gel electrophoresis.

#### 2.5. Endotaxin assay and nuclear factor κB (NF-κB) activity

Using a commercially available liquid amebocyte lysate assay (Charles River, France) with a concentration range of 0.015–1.2 EU/ml, the concentration of endotaxin in portal plasma of mice was determined as previously described [30]. Using a Trans-AM enzyme-linked immunosorbent assay (ELISA)-based kit (Active Motif, UK), activity of NF-κB was measured in nuclear extract isolated from liver samples. For the isolation of the nuclear fraction, lysis buffer (1 M HEPES, 50% glycerol, 5 mM NaCl, 0.5 M EDTA and 1 mM DTT) containing a protease and phosphatase inhibitors mix (Sigma-Aldrich, USA) was used following the instructions of the manufacturer.

#### 2.6. Immunostaining for 4-hydroxybenzoic acid (4-HNE) protein adducts, inducible nitric oxide synthase, toll-like receptor 4 protein in liver and occludin and zona occludens 1 protein in duodenum

Paraffin-embedded liver sections (5 μm) were used for the detection of 4-HNE protein adducts (1:1000, 30 min, AG Scientific, USA), TLR-4 (1:1100, overnight, humid 4 °C, Abcam plc, UK) and iNOS (1:2250, overnight, humid 4 °C, Invitrogen; USA). Tissue sections were incubated with a peroxidase-linked secondary antibody and diaminobenzidine (DAKO, Germany). To determine the concentration of 4-HNE protein adducts (200×), TLR-4 and iNOS (both 63×) in oil in liver and occludin as well as ZO-1 (both 63×) in duodenum sections, staining was assessed densitometrically in eight randomly selected fields using a microscope (Axio Vert 200M, Zeiss, Germany).

#### 2.7. Statistical analyses

All results are presented as mean±S.E.M. (standard error of the mean). One-way analysis of variance with the consequent Tukey test was applied for the determination of significance levels (GraphPad Software, CA, USA). Differences were considered as significant if the P≤0.05.
3. Results

3.1. Effect of B.a. on diet-induced liver damage

The intake of B.a. per day was quite stable in both B.a. groups (C + B.a., WS + B.a.), while B.a. was delivered via drinking water ad libitum and ranged between 1 and 2×10^9 cfu per week (Table 2). Despite not being protected against the increased fasting glucose levels and the increased liver weight as well as liver to body weight ratio, massive accumulation of fat and mild inflammation found in mice only fed a WS were markedly attenuated in WS-fed mice concomitantly treated with B.a. (Fig. 1A–D). In line with these findings, levels of transaminases were also markedly higher in mice only exposed to WS diet, an effect of the WS diet that was markedly attenuated in mice concomitantly treated with B.a. (Table 2). However, as values varied considerably within groups, differences did not reach the level of significance.

3.2. Effect of B.a. on portal endotoxin levels and the TLR-4 cascade in the liver

Portal endotoxin levels were twofold higher in both groups exposed to WS diet compared to both groups receiving control diet, but not changed by B.a. supplementation (Fig. 2A). WS diet also caused an enhanced concentration of TLR-4 protein and of the mRNA encoding for the TLR adaptor protein MyD88. Although portal endotoxin levels were not affected by B.a., the probiotic treatment attenuated TLR-4 expression and markedly reduced MyD88 mRNA expression in mice receiving WS (Fig. 2B–D).

3.3. Effect of B.a. treatment on markers of hepatic lipid peroxidation

The chronic intake of a WS diet was associated with a sixfold increase of 4-HNE protein adducts in the liver compared to the respective controls. However, hepatic 4-HNE protein expression was almost at the level of the respective control in WS diet-fed mice concomitantly treated with B.a. (Fig. 3A+D). Similar results were obtained for iNOS protein expression and basically normalized following concomitant treatment with B.a. (Fig. 3B+E). Apart from iNOS, we measured mRNA expression of HO-1, another hepatic marker of inflammation considered as a kind of adaptive response against oxidative damage, which might be critical in the progression of the disease. HO-1 mRNA expression was about threefold increased in mice receiving WS diet compared to control diet. This increase was virtually absent in mice receiving WS diet together with B.a. (Fig. 3C).

3.4. Effect of B.a. supplementation on markers of inflammation: PAI-1, CCL2, CCL19 and NFκB activity

Apart from iNOS, a marker of lipid peroxidation induced by proinflammatory markers that is induced by lipid peroxidation, we measured other hepatic markers of inflammation such as NFκB activity and mRNA expression of PAI-1 as well as the chemokines CCL2 and CCL19. All four markers of inflammation were clearly increased in mice fed WS diet compared to control diet (NFκB activity ~3.0-fold, PAI-1 ~6.3-fold, CCL2 ~3.5-fold, CCL19 ~2.9-fold). Treatment with B.a. almost fully protected against the diet-induced increase in NFκB activity and CCL19 mRNA expression and clearly attenuated the...
increase in PAI-1 and CCL2 mRNA expression (Fig. 4A–D). The effects of B.a. on these markers of inflammation were not statistically significant because of variations of the results obtained from rather small animal groups, but the tendency was consistent.

3.5. Effect of B.a. supplementation on intestinal barrier function

Although there were no differences regarding the endotoxin levels in the portal plasma of WS diet-fed mice with or without B.a. supplementation; the concomitant treatment with B.a. resulted in higher occludin (~2.5-fold, \( P < 0.05 \)) and ZO-1 (~1.9-fold, \( P < 0.01 \)) protein concentrations in the duodenum of WS diet-fed mice concomitantly treated with B.a. (Fig. 5). The other parts of the small intestine did not show differences between WS diet-fed groups (data not shown).

4. Discussion

Our present study shows for the first time that the probiotic B.a. attenuates liver damage induced by a WS diet. We provide data showing that diet-induced fat accumulation in the liver of mice is lower when treated concomitantly with B.a. In parallel, transaminases were lower in WS diet-fed mice treated with B.a. compared to mice without probiotic treatment. Which mechanisms could underlie such an effect of a probiotic bacterium? The present study and studies from other groups [31,32] provide evidence for the hypothesis that diet-induced NAFLD could be a result of inflammatory responses triggered by bacterial endotoxin derived from the intestine. Possibly, the WS diet causes by yet unclear mechanisms an impairment of the intestinal barrier, allowing enhanced influx of endotoxin into the liver, which results in liver inflammation and fat accumulation. Indeed, our data confirm that fat accumulation in the liver is accompanied by signs of liver inflammation, neutrophil count in the liver, activation of the TLR-4/MyD88 pathway and activation of NF\( \kappa \)B. Most interestingly, some of these inflammatory parameters could be reduced at least by following oral treatment of mice with B.a. Although our data did not reach the level of significance, we showed that there is an obvious potential of B.a. to reduce inflammation relating to a WS diet-induced NASH. But there are further studies needed concerning duration of treatment with B.a. and/or using different concentrations or administration methods, like gavage.

Our study confirms and extends previous reports suggesting a beneficial effect of particular probiotics in animal models of alcoholic
and other forms of chronic liver disease [19–21,33] and in analogous liver diseases in humans [6,34]. As humans, mice respond to overfeeding with fatty liver disease as well as liver inflammation documented for example by the increase of transaminases and the formation of reactive oxygen species (ROS) [35,36]. We show that treatment of mice on a WS diet with B.a. improved not only clinical and chemical parameters of NAFLD (e.g., transaminases and liver pathology) but also hepatic markers of inflammation (e.g., PAI-1, CCL2, CCL19, NF\(\kappa\)B) and thus NASH.

In previous studies, we [37] and others (reviewed in [38]) could show that mouse feeding models reflect to a large extent human NAFLD and related diseases, and therefore, our experimental data might have implications also for the human situation, although they cannot be easily extrapolated to humans. It has been discussed that some beneficial effects of probiotics like Bifidobacterium ssp. result from the stabilization of the intestinal barrier and from changes in the composition of the intestinal microbiota. For example, results obtained in a mouse model showed that Bifidobacterium ssp. lowered gut endotoxin concentration and enhanced mucosal immunity [22,39,40]. Furthermore, results of Chen et al. [41] suggested that modifying gut microbiota in favor of Bifidobacterium ssp. could be useful in reducing the adverse effects of high-fat diet on markers of the metabolic syndrome like insulin resistance. Although we could not show a reduction of endotoxin levels in WS diet groups with B.a. supplementation, the intestinal barrier in the duodenum of mice fed a Western-style diet with B.a. treatment could be improved.

Results of several studies have shown that oxidative stress and lipid peroxidation play important roles in the pathogenesis of NASH (for overview, see [5]). In agreement with these studies, we show here that a WS diet compared to a control diet causes enhanced...
hepatic lipid peroxidation, iNOS protein concentration and HO-1 mRNA expression. Most interestingly, we could show that B.a. supplementation not only reduces liver damage but also attenuates other parameters associated with oxidative stress. This suggests that one mode of action of B.a. could be prevention from oxidative stress and subsequent lipid peroxidation in the liver. Whether this effect is a result of reduced liver challenge with proinflammatory triggers such as endotoxin from the intestine capable of inducing iNOS [30] or rather because of an enhanced clearance of ROS (e.g., through antioxidative defence [42–44] cannot be answered by the present data.

In summary, our data indicate that an oral B.a. supplementation attenuates NASH in a mouse model of diet-induced liver disease. The protective effect likely results from prevention of lipid peroxidation in the liver by yet unclear mechanisms. In particular, the identification of the molecular structures within the probiotic responsible for the protective effects is required. Concerning the apparent safety of this probiotic already used successfully in a human study [45] and naturally present in the normal healthy intestine [46], future human trials could be considered testing this probiotic strain for its possible preventive effect against NAFLD in humans.

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References

Fig. 5. Effect of B.a. supplementation on intestinal barrier function. (A) Densitometric analysis of occludin and (B) ZO-1 staining. (C) Representative photomicrographs of expression of occludin protein and (D) ZO-1 protein expression (both 63× oil). Data are shown as mean±S.E.M.; *P < 0.05; **P < 0.01.


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