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

Astrid Reichold, Sibylle A. Brenner, Astrid Spruss, Karin Förster-Fromme, Ina Bergheim, Stephan C. Bischoff

*Department of Nutritional Medicine, University of Hohenheim, Fruwirthstr. 12, 70599 Stuttgart, Germany*

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 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 (NF-κB) 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, NF-κB activation and finally inflammation in the liver.

© 2014 Elsevier Inc. All rights reserved.

Keywords: Obesity; Probiotics; Toll-like receptor 4; Nonalcoholic steatohepatitis

1. Introduction

Nonalcoholic fatty liver disease (NAFLD) is by now recognized as the 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-hydroxynonenal (4-HNE) protein adducts, inducible nitric oxygen synthase (iNOS) and heme oxygenase-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...
2.3. Liver histology, blood parameter and neutrophil staining was performed densitometrically in eight randomly selected fields, and representative were captured at a 200× magnification. Sections were scored using the microscope (Axio Vert 200M, Zeiss, Germany), representative photomicrographs stained with hematoxylin and eosin (H&E). Using a system incorporated in a hematoxylin (Sigma-Aldrich, USA) as described previously[28]. Analysis of staining

### Table 1

<table>
<thead>
<tr>
<th>Neutrophil counts in liver</th>
<th>C</th>
<th>WS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Data are shown as mean±S.E.M. (n=4–6; n=3–6 for ALT).</td>
<td></td>
<td></td>
</tr>
<tr>
<td>†</td>
<td>P=0.01 compared with C.</td>
<td></td>
</tr>
<tr>
<td>‡</td>
<td>P=0.01 compared with C+Rg.</td>
<td></td>
</tr>
<tr>
<td>‡</td>
<td>P=0.1 compared with C.</td>
<td></td>
</tr>
<tr>
<td>†</td>
<td>P=0.05 compared with C. *Determined from week 2 to 12; the first week was considered as adaption phase.</td>
<td></td>
</tr>
</tbody>
</table>
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+D). 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 such as elevated plasma transaminases, neutrophil count in the liver, activation of the TLR-4/MyD88 pathway and activation of NFκB. 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, NFκB) 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

---

**Figure 3.** Effect of *B.a.* supplementation on WS-induced hepatic lipid peroxidation and related parameters. (A) Densitometric analysis of 4-HNE and (B) iNOS staining. (C) Relative mRNA expression of HO-1 normalized to 18S mRNA expression. (D) Representative photomicrographs of expression of 4-HNE protein adducts (200×) and (E) iNOS protein expression (63× oil). Data are shown as mean±S.E.M.; **P<.01, ***P<.001.
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.

Acknowledgments

The authors thank Andreas Rings for his excellent technical assistance.

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× o]. Data are shown as mean±S.E.M.; *P<.05; **P<.01.


