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Inhibition of miR122a by *Lactobacillus rhamnosus* GG culture supernatant increases intestinal occludin expression and protects mice from alcoholic liver disease

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HIGHLIGHTS

- Ethanol exposure increases miR122a in the intestine of mice.
- Intestinal tight junction occludin is decreased by ethanol exposure in mice.
- Increase miR122a down-regulates epithelial occludin expression.
- LGGs decreases miR122a and increases occludin in the intestine of mice with ALD.

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ABSTRACT

Alcoholic liver disease (ALD) has a high morbidity and mortality. Chronic alcohol consumption causes disruption of intestinal microflora homeostasis, intestinal tight junction barrier dysfunction, increased endotoxemia, and eventually liver steatosis/steatohepatitis. Probiotic *Lactobacillus rhamnosus* GG (LGG) and the bacteria-free LGG culture supernatant (LGGs) have been shown to promote intestinal epithelial integrity and protect intestinal barrier function in ALD. However, little is known about how LGGs mechanistically works to increase intestinal tight junction proteins. Here we show that chronic ethanol exposure increased intestinal miR122a expression, which decreased occludin expression leading to increased intestinal permeability. Moreover, LGGs supplementation decreased ethanol-elevated miR122a level and attenuated ethanol-induced liver injury in mice. Similar to the effect of ethanol exposure, overexpression of miR122a in Caco-2 monolayers markedly decreased occludin protein levels. In contrast, inhibition of miR122a increased occludin expression. We conclude that LGGs supplementation functions in intestinal integrity by inhibition of miR122a, leading to occludin restoration in mice exposed to chronic ethanol.

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1. Introduction

Alcoholic liver disease (ALD) is one of the major chronic liver diseases (Adachi and Brenner, 2005). ALD ranges from alcoholic fatty liver (steatosis) to alcoholic steatohepatitis to fibrosis and cirrhosis, and is often co-morbid with other diseases including HIV, hepatitis B (HBV) and hepatitis C (HCV). There are limited therapeutic approaches, and alcohol abstinence is the most

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effective treatment for ALD. However, abstinence may not totally reverse all the deleterious effects of excess alcohol exposure. Thus, developing targeted and effective treatments for liver and systemic injury is urgent.

Probiotics are defined as live microorganisms, which, when administered in adequate amounts, confer health benefits to the host (Reid, 2005). Probiotics have clinical utility in many of gastrointestinal diseases. *L. rhamnosus* Gorbach–Goldin (LGG) is a Gram-positive bacteria which is well-recognized for its advantageous effects including immunoregulation, lipid modulation, and gene expression in diseases such as ALD, non-alcoholic-liver disease, inflammatory bowel disease and neuronal disorders. Previous studies have demonstrated that LGG supplementation can provide an effective treatment for or prevention of ALD in patients and experimental animal models (Forsyth et al., 2009; Kirpich and McClain, 2012; Wang et al., 2011, 2012). However, adverse events noted with probiotic use include bacteremia (Land, 2005), fungemia (Riquelme et al., 2003), and worsened outcomes in severe pancreatitis, with an increased incidence of bowel ischemia and mortality (Besselink et al., 2008). In addition, probiotics may not be effective in intestinal disorders with altered epithelium, as the bacteria must colonize in the intestine to be effective.

As an alternative, heat-killed bacteria and probiotic-produced nonviable soluble proteins have been demonstrated to be effective in recent studies (Ueno et al., 2011; Yan et al., 2007). Bacteria-free LGG culture supernatant (LGGs) was shown to protect intestinal epithelial cells from apoptosis and promote proliferation (Yan et al., 2007), while our group recently showed that LGGs pretreatment prevented acute alcohol-induced hepatic fat accumulation (Wang et al., 2012). The beneficial effect of LGGs is associated with improved intestinal barrier function and reduced endotoxemia (Wang et al., 2012). However, the regulatory mechanism underlying the protective effect of LGGs on intestinal tight junction proteins in response to alcohol exposure is still not clear. Previous studies indicated that the intestinal tight junction protein, occludin, may be regulated by microRNA122a (miR122a) at the level of transcription (Ye et al., 2011) in response to TNF-α challenge, which is increased in the intestine of mouse exposed to alcohol. This led us hypothesized that LGGs could be effective in chronic alcoholic liver disease by positive modulation of intestinal tight junction via miR122a regulation. To test our hypothesis, LGGs and alcohol were co-administered to mice for 4 weeks. Hepatic steatosis and inflammation and intestinal miR122a expression and occludin regulation were examined. The effect of miR122a manipulation on occuldin expression in Caco-2 cells was also studied.

2. Material and methods

2.1. Lactobacillus rhamnosus *GG culture*

LGG was purchased from American Type Culture Collection (ATCC 53103; Rockville, MD) and cultured in *Lactobacillus* MRS broth (Difco; BD, Sparks, MD) at 37 °C in accordance with ATCC guidelines. LGG was cultured to reach the bacterial density of 10^9 colony-forming units/mL. The culture suspension was then centrifuged at 1000 g for 10 min. The LGG culture supernatant (LGGs) was removed and filtered through 0.22-μm filters. This procedure yielded the LGGs from the culture at a concentration of 10^9 colony-forming units/mL bacterial cells. LGGs was stored at −80 °C for later use (Wang et al., 2012).

2.2. Alcohol feeding

8–10 week-old male C57BL/6N mice were purchased from Harlan Laboratories (Indianapolis, IN). The mice were pair-fed liquid diet (Lieber DeCarli) for 4 weeks. In brief, animals received the diet containing 17% of energy as protein, 40% as corn oil, 7.5% as carbohydrate, and 35.5% as either alcohol (5% v/w, alcohol-fed, AF) or isocaloric maltose dextrin (pair-fed, PF). The alcohol concentration in the diet was kept at 1.6% (v/w) for three days and 3.3% (v/w) for another three days before 5% alcohol feeding for 4 weeks. LGGs at a dose equivalent to 10^9 CFU/day/mouse was given to the mice in the liquid diet. Each group had 4–10 mice. At the end of the experiments, plasma and tissue samples were collected for assays. All mice were treated according to protocols reviewed and approved by the Institutional Animal Care and Use Committee of the University of Louisville.

2.3. Tissue staining

Formalin-fixed hepatic samples were embedded in paraffin and stained with hematoxylin-eosin using standard protocols (Wang et al., 2012).

2.4. Caco-2 cell culture

Caco-2 cells are colon carcinoma cells, which undergo a process of spontaneous differentiation in culture and fully differentiate into mature enterocytes in 21 days (Sambuy et al., 2005). Caco-2 cells obtained from the ATCC were cultured in Eagle's minimal essential medium supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, and 10% fetal bovine serum at 37 °C in a 5% CO_2 environment. Cells were cultured in Transwell inserts (Costar, Corning) for 21 days to polarize and differentiate into epithelial cell-like monolayer. EtOH was added at the concentrations indicated and incubated for 24 h. LGGs, prepared from 10^9 CFU/mL culture, was added to the cells at a concentration of 1% along with 5% EtOH. Fluorescein isothiocyanate–dextran-4 (FD-4) was added to the apical top of the culture and incubated for 30 min. The basal medium was collected for Caco-2 cell permeability measurement using a microplate fluorescence reader with an excitation wavelength of 485 nm and an emission wavelength of 530 nm.

2.5. Transfection of Caco-2 cells with pre-miR122a and hsa-miR122

To overexpress or to inhibit the miR122a, polarized and differentiated Caco-2 cells were cultured with serum-free medium, and transfected with pre-miR122a precursor molecules or hsa-miR122 (Life Technologies, Carlsbad, CA) or the negative controls (pre-miR-control or Anti-miR negative control) using the Lipofectamine 2000 (Invitrogen, Carlsbad, CA) following the manufacturer's instructions.

The effective concentrations of pre-miR-122a and pre-miR-control were determined from the concentration range of 10–50 nM for 24 h. hsa-miR122 and the negative control were used at a concentration of 100 nM. The levels of miR-122a were then measured by qRT-PCR.

2.6. Ex vivo *intestinal permeability assay*

Ileum from mice treated with and without alcohol was freshly isolated, and the permeability to FD-4 was measured as described in our previous study (Kirpich et al., 2012; Wang et al., 2012).

2.7. Liver TNF-α *analysis*

Liver tissue was homogenized (50 mg/mL) in RIPA buffer. TNF-α was measured using the Infinity Assay Kit (BD) according to the manufacturer's instructions.

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