

**Dissertation**

**PROBIOTIC MODULATION OF GUT  
MICROFLORA IN CIRRHOSIS: INFLUENCE ON  
IMMUNE FUNCTION AND INFECTION**

submitted by

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## Declaration

I hereby declare that this thesis is my own original work and that I have fully acknowledged by name all of those individuals and organisations that have contributed to the research for this thesis. Due acknowledgement has been made in the text to all other material used. Throughout this thesis and in all related publications I followed the guidelines of “Good Scientific Practice”

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Ich erkläre ehrenwörtlich, dass ich die vorliegende Arbeit selbständig angefertigt und abgefasst, und jene Personen und Institutionen, die am Zustandekommen der Forschungsdaten beteiligt waren, namentlich genannt habe. Andere als die angegebenen Quellen habe ich nicht verwendet und die den benutzten Quellen wörtlich oder inhaltlich entnommenen Stellen habe ich als solche kenntlich gemacht. Die Arbeit an der Dissertation und daraus entstandener Publikationen wurde gemäß den Regeln der „Good Scientific Practice“ durchgeführt.

Graz, am 27.9.2016

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## Preface

Like a tropical rainforest the microflora of the intestine comprises thousands of known and unknown species that thrive on biodiversity and symbiosis. As self-regulative organs they both facilitate life within and beyond their borders. However, they are sensitive to external confounders, whether it is deforestation in the rainforest or poor lifestyle decisions (e.g.) for the microflora. Once biodiversity is destroyed, an irreplaceable treasure has been lost. In cirrhosis some of the consequences of an altered microflora can be subdued by antibiotics. However, the use of antibiotics has severe consequences itself; for example the rise of resistant pathogens. This study represents an approach to restore the microflora of cirrhotic patients by probiotic modulation.

For more convenient reading the thesis is split into three parts. The first part of the thesis describes the influence of probiotics on the microbiome, bacterial translocation and the innate immune system. The second part discusses interesting by-products of the study and preliminary data for future in-depth analysis. The third part deals with methodological problems and their solutions.



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## Abstract

Cirrhosis is associated with dysbiosis and bacterial translocation. Pro-inflammatory conditions and the overflow of endotoxin into the systemic circulation are major factors in the acquired immunodeficiency common in cirrhosis. Probiotics have beneficial effects on the gut barrier and inflammation. Therefore we hypothesized that the administration of a multispecies probiotic would restore the gut barrier of cirrhotic patients and thereby ameliorate bacterial translocation and innate immune dysfunction. To test this hypothesis a randomized, double-blind, placebo controlled study was conducted. Patients either received Ecologic® BARRIER (Winclove, Amsterdam, The Netherlands) for six months (n=44) or a placebo (n=36). After six months of intervention patients were observed for another six months. During the intervention the abundance of *Lactobacillus* was significantly increased and the invasion of *Veillonella* from the oral cavity into the intestine tended to decrease. We found that the administration of the probiotic increased neutrophil resting burst and serum neopterin levels in the probiotic group and boosted serum killing capacity of alcoholic cirrhotics. Furthermore, probiotics increased the conversion of phenylalanine to tyrosine which was reflected in an increase in quality of life and therefore in a better adherence to the study protocol in the probiotic group. Probiotics tended to improve liver function. Placebo did not show any effects. Probiotics did not influence gut permeability, endotoxemia, neutrophil phagocytosis, or cytokine levels.

In conclusion, probiotic administration was well tolerated and increased innate immune responses. It improved the general well-being of the patients and had a beneficial effect on liver function, although their influence on gut permeability, endotoxemia and cytokine expression was not detectable.

## Zusammenfassung

Zirrhose wird begleitet von typischen Änderungen im Mikrobiom. Eine schwache Darmbarriere und die damit einhergehende Inflammation und Endotoxinämie verursachen eine erworbene Immunschwäche, die häufig zu schweren Infektionen führt. Probiotika haben einen positiven Effekt auf die Darmbarriere und inflammatorische Prozesse. Deshalb wurde die Verabreichung eines Multispezies-Probiotikums auf ihre Wirkung auf die Darmbarriere, bakterielle Translokation und angeborene Immunreaktionen bei Zirrhose getestet. In einer randomisierten, doppelblinden, Placebo-kontrollierten Studie wurden Zirrhosepatienten entweder Ecologic® BARRIER (Winclove, Amsterdam, Niederlande) sechs Monate lang verabreicht (n=44) oder ein Placebo (n=36). Nach der Intervention wurden die Patienten noch weitere sechs Monate klinisch beobachtet. Die Intervention mit Probiotika erhöhte die Prävalenz von *Laktobazillen* und drängte eine vermutlich aus der

Mundhöhle einwandernde *Veillonella*-Spezies zurück. Probiotika verstärkten auch die Produktion von reaktiven Sauerstoffspezies in Neutrophilen und die Konzentration von Neopterin im Serum. Weiters verbesserte die Einnahme von Probiotika die Lebensqualität der Patienten und die Produktion von Tyrosin, was sich in wesentlich niedrigeren Dropout-Raten niederschlug. Probiotika zeigten auch einen positiven Effekt auf die Leberfunktion. Plazebos hatten keinen Einfluss auf diese Parameter. Darmpermeabilität, Endotoxinämie, Phagozytose von Neutrophilen und Zytokinkonzentrationen blieben von der Intervention weitgehend unberührt.

Zusammenfassend verstärkten die Probiotika die angeborenen Immunreaktionen, verbesserten die Lebensqualität und zeigten positive Effekte auf die Leberfunktion, obwohl sie keinen Einfluss auf die Darmbarriere, Endotoxinämie oder Zytokine zeigten.

## A. Probiotics in cirrhotics

### Introduction

#### Gut microbiome and liver disease

The symbiotic relationship between humans and microorganisms located on and in the human body exerts many beneficial functions for the host. This includes for example access and storage of nutrients extracted by microflora,(1) activation and education of the immune system,(2) or growth inhibition of pathogenic microorganisms.(3) Each person is colonized with an early microbiome at birth, which is developed and shaped throughout the person's life.(4) The beneficial relationship depends on a delicate balance of various species. Dysbiosis of the gut microbiome can cause diseases including various liver injuries - or vice versa.(5) Disease specific alterations of the intestinal microbiome can persist even when the primary cause (e.g. viral hepatitis infection) has been eradicated.(6)

Liver cirrhosis is the common end stage of different liver diseases. Culture free sequencing approaches to characterize the gut microbiome revealed systematic alterations caused by (or causing) cirrhosis. Reduced richness of species, paired with small intestinal bacterial overgrowth, increased abundance of Firmicutes, Proteobacteria and Fusobacteria but decreased abundance of Bacteroidetes with an invasion of the intestine by bacteria from the oral cavity (e.g. *Veillonella*, *Streptococcus*) have been described in cirrhotic patients.(7) The ratio between autochthonous and pathogenic taxa (Cirrhosis Dysbiosis Ratio) is associated with endotoxemia and infections.(8)

#### Gut permeability in liver cirrhosis

Between the gut microbiome and the host lies a multi-layer barrier that prohibits the translocation of bacteria into the circulation and therefore the certain death of the host.(9) The expression and regulation of tight junction proteins in the gut barrier protects the body against permeating pathogens by forming sealing cell-cell-connections. At the same time it allows the paracellular transport of small uncharged solutes and ions (pore pathway) as well as small amounts of larger molecules including lipopolysaccharides, also referred to as endotoxin (leak pathway).(10) Tight junction opening or sealing can be regulated by myosin light chain kinase (MLCK) and is associated with the increased permeation of the intestinal barrier.(11, 12) A low level of endotoxin leakage into the portal vein has been observed in healthy as well as diseased people and can be balanced by the phagocytic activity of the reticuloendothelial system (RES).(13) An excessive amount of these bacterial product translocating through a damaged intestinal barrier on the other hand can lead to inflammation.(14, 15) The disruption of tight junction function alone is not

enough to provoke disease; however, it can predispose the individual for the onset and progression of disease in the presence of another stimulus.(16, 17)

In the case of liver disease these stimuli can be manifold. Alcohol is one of the most discussed triggers of intestinal hyper-permeability and bacterial translocation.(18-21) Also, patients with chronic viral hepatitis B and C infections show increased levels of endotoxin, inflammation and enterocyte death.(22) Alterations in gut barrier function increase with the severity of liver disease.(22, 23) In fact, systemic endotoxemia is a well-recognized condition in cirrhosis,(13, 24) and increased intestinal permeability and bacterial translocation in cirrhosis have been verified in several studies.(23, 25-28)

### **The role of endotoxin in alcoholic liver cirrhosis**

Endotoxin has been identified as a major factor in the pathogenesis and progression of alcoholic liver disease.(21) Endotoxin is a pressure and heat-stable, pyrogenic component of the cell wall of Gram negative bacteria. It passes through the intestinal barrier and triggers a signalling cascade controlled by Toll like receptor (TLR) 4. TLR4 cannot directly bind endotoxin but it is crucial for the body's response.(29) Endotoxin is bound by lipopolysaccharide binding protein (LBP) and transferred to its principal cellular receptor, cluster of differentiation (CD) 14.(30, 31) CD14 then clusters with MD 2 and TLR4, which sets intracellular signalling in motion. Myeloid differentiation primary response gene (MyD) 88 dependent and MyD88 independent pathways have been described, evoking mostly nuclear factor (NF)- $\kappa$ B or interferon regulatory factor (IRF) 3 mediated transcription of pro-inflammatory cytokines, such as tumor necrosis factor (TNF)  $\alpha$ , interleukin (IL)-1, IL-6 and type 1 interferons (IFN  $\alpha/\beta$ ), as well as chemokines like IL-8 or monocyte chemotactic protein (MCP)-1.(32, 33)

The crucial role of endotoxin and TLR4 signalling in alcoholic liver disease has been demonstrated using TLR4 deficient and antibiotic treated animals: the use of antibiotics reduced blood endotoxin levels in rats and reduced liver pathology scores and AST after alcohol exposure; additionally, the knockout of TLR4 in mice ameliorated the effect of chronic alcohol exposure on liver pathology scores, ALT and TNF $\alpha$  despite elevated endotoxin levels.(34, 35) Gut-derived endotoxin reaches the liver via the portal vein and activates Kupffer cells.(36) In addition, the upregulation of cytochrome P4502E1, an enzyme involved in alcohol metabolism, can sensitize macrophages to endotoxin, enhance reactive oxygen species (ROS) production, and stabilize TNF $\alpha$  mRNA.(37) Treatment with antibiotics decreases portal endotoxin levels but has no effect on gut permeability.(38) This suggests that bacterial overgrowth with Gram negative organisms plays a key role in alcoholic liver injury. On the other hand, a complete sterilization of the intestine, as it is the case in germ-free mice, aggravates liver damage secondary to alcohol,(39) as well as in other types of experimental liver injury.(40) This supports the hypothesis that a eubiotic microflora

protects the host from liver toxicity and that the restoration of the microbiome might be a physiological therapeutic approach in liver cirrhosis.

Other TLRs are also involved in alcoholic liver disease. TLR2 and TLR9 have been shown to be pivotal for the induction of hepatic inflammation and neutrophil infiltration.(41) On the other hand, stimulation of TLR3 has been shown to be hepatoprotective in alcoholic liver injury.(42)

### **The role of endotoxin in non-alcoholic fatty liver disease (NAFLD)**

The upregulation of hepatic TLR4 in NAFLD has been demonstrated in genetic (43) and diet induced rodent models,(44-46) and confirmed in human liver biopsy studies.(47-50) The upregulation of the endotoxin binding machinery of the body (CD14, LBP, MD2),(49) increased gut permeability,(45, 51) and the rescue from NAFLD phenotype in TLR4 knock out animals(43) suggest an endotoxin mediated genesis of NAFLD. In addition, intestinal overgrowth with endotoxin producing bacteria such as *E.coli*,(49, 51) and amelioration of NAFLD after antibiotic treatment in rodents(45) also point towards the leaky gut hypothesis. However, it has been shown that the increased abundance of free fatty acids in the blood of NAFLD model organisms can also activate TLR4 signalling and lead to inflammation and lipid accumulation in the liver.(46, 52) High dietary cholesterol levels do not only result in TLR4 mediated inflammation but also in impulsivity and anxiety- and depression-like behaviour of mice. Substituting western style diet with regular chow showed significant improvement in TLR4 signalling in the liver and the central nervous system, as well as in behavioural parameters.(44)

### **Pro-inflammatory conditions and dysfunctional neutrophils in liver cirrhosis**

The activation of the innate immune system of the liver also has major implications for the systemic immune system. Cirrhotic patients often suffer from bacterial infections.(53, 54) A wide spectrum of dysregulated immune responses has been identified in these patients.(55, 56) Overexpression of cytokines (TNF $\alpha$ , IFN $\gamma$ , MCP1, IL-6, IL-8, IL-10, IL-12, G-CSF, MIP1 $\beta$ , among others) have been reported in liver cirrhotic patients.(57-59) A mismatch in cytokine expression is likely to disturb leucocyte behaviour.(60, 61) Cirrhotic patients exhibit impaired neutrophil function such as impaired migration,(62) inadequate activation,(62-64) and phagocytic dysfunction.(62, 64) Neutrophil dysfunction is linked to increased infection rates and poor outcome.(63, 65) Additionally, endotoxin can prompt cultured neutrophils to produce ROS and IL-8.(66) Furthermore, neutrophil dysfunction is reversible in vitro by removing endotoxin from patients' serum.(63) Endotoxemia has also been linked to reduced phagocytosis of the reticuloendothelial system.(67) However, not all studies agree with this association.(68) In fact, injections of endotoxin even enhance clearance of bacteraemia in vivo.(69) This suggests that endotoxin is not a sole factor in neutrophil dysfunction. Reduced liver function also results in insufficient expression of complement factors.(70) Oxidized albumin found in liver cirrhotic

patients does not bind endotoxin as sufficiently as non-oxidized albumin,(71) and lipoproteins are losing their anti-inflammatory effects on monocytes.(72) Constant production of ROS by innate immune cells contribute to low grade inflammation and oxidative stress.(73, 74)

### **Monoamine synthesis in low grade inflammation**

Low grade inflammation and oxidative stress have been implicated in the reduced production of monoamines, such as serotonin and dopamine. Low grade inflammatory conditions in aging, Alzheimer's disease, type 2 diabetes, pregnancy etc. are linked to decreased serotonin production and mood disorders.(75-80) Cirrhosis is a low-grade inflammatory disease and is associated with depression,(81) anxiety,(82) and sleep disorders.(83) Quality of life and mood disorders are negatively influenced by cirrhosis and pre-existing conditions such as viral hepatitis C infections, antiviral therapy and alcoholism.(84-86) Inflammatory conditions activate guanosine-triphosphate-cyclohydrolase (GTP-CH)-1 pathway that favours the production of neopterin and downregulates the production of tetrahydrobiopterin (BH4). Additionally, reactive oxygen species target BH4 which is pivotal as cofactor for phenylalanine-hydroxylase (PHA)(87) and tryptophan-hydroxylase (TPH).(88) Therefore the conversion of phenylalanine to tyrosine (and ultimately to dopamine), as well as the conversion of tryptophan to 5-hydroxytryptophan (and ultimately to serotonin) is inhibited in low-grade inflammation and oxidative stress conditions. In addition indolamine-2,3-dioxygenase (IDO) degrades tryptophan to kynurenine, diverting tryptophan from the serotonin pathway.(75) Gut microbiota have been implicated in the genesis of neuroinflammatory responses in cirrhosis and TLR4 mediated inflammation in the brain has been linked to sickness-behaviour and anxiety in NAFLD mice.(44, 89) However, in addition to oxidative stress and inflammation, low quality of life in cirrhosis can also be triggered by gastrointestinal pain,(82) low economic status(90, 91) and malnutrition.(92)

### **Probiotic supplementation in cirrhosis**

Probiotics are live microorganisms that exert health benefits for the host when applied in adequate amounts.(93) The use of probiotics is recommended in gastrointestinal diseases and for the prevention of allergies.(94-96) Studies have shown that the administration of probiotics could also be beneficial for liver cirrhotic patients because of their beneficial effect on liver function and hepatic encephalopathy.(97-99) In addition, probiotic intervention improved neutrophil function in alcoholic hepatitis patients.(100) The mechanisms by which probiotics improve the host's health are not fully understood yet. One possibility could be that the insertion of probiotic microorganism into the intestine restores the endogenous flora and therefore evokes its hepatoprotective characteristics.(40, 101) The main probiotic strains, Lactobacilli and Bifidobacteria, can proliferate in the intestinal mucosa and compete for niches of pathogenic species like *E.coli* or *Staphylococcus*.(97, 98, 101) Especially Lactobacilli can inhibit the growth of anaerobic species

and prevent bacterial overgrowth by reducing the pH in the intestinal lumen.(102) Another possibility of how probiotics aid the host's health would be the strengthening of the intestinal barrier. The beneficial effects of probiotics on the intestinal barrier have been demonstrated in vitro, in vivo, and in trained healthy men.(103-106) Probiotics can also modulate the intestinal immune system.(107) It is likely that more than one mechanism is employed in the beneficial effects of probiotics. Accordingly, multispecies probiotics are more effective than monostrain preparations in treating antibiotic-associated diarrhoea, and in promoting immune defences.(108) The probiotic used in the presented study is a multispecies preparation containing *Bifidobacterium bifidum*, *Bifidobacterium lactis*, *Lactobacillus acidophilus*, *Lactobacillus brevis*, *Lactobacillus casei*, *Lactobacillus salivarius*, *Lactococcus lactis* and *Lactococcus Lactis* in relatively high dosages. These species showed beneficial effects of varying degrees on strengthening epithelial monolayers after an infectious as well as an inflammatory stressor, inhibiting mast cell activation, stimulating IL10 expression and decreasing endotoxin load in vitro.(103)

## Hypothesis

We hypothesize that the administration of Ecologic BARRIER/OMNi-BiOTiC HETOX for six months will restore the intestinal microflora and gut permeability of cirrhotic patients and thereby reduce endotoxemia and proinflammatory conditions so that innate immune responses can be improved and infection rates reduced. A randomized, double-blind, placebo controlled study was conducted to test this hypothesis.

## Methods

Liver cirrhotic patients of any aetiology who visited the outpatient clinic at the University Hospital Graz (Department of Gastroenterology and Hepatology or the Department of Transplantation Surgery) between July 2012 and September 2013 were screened for eligibility. They were included in the study if all the inclusion criteria and none of the exclusion criteria were met (Table 1).

**Table 1: Inclusion and exclusion criteria of eligibility for the study**

Inclusion criteria	Exclusion criteria
<ul style="list-style-type: none"> <li>• Informed consent</li> <li>• Clinical and radiological evidence of cirrhosis, and/or biopsy proven cirrhosis of any aetiology</li> <li>• Between 18 and 80 years old</li> </ul>	<ul style="list-style-type: none"> <li>• Child-Pugh score over 11</li> <li>• Alcohol abstinence for more than two weeks at screening</li> <li>• Clinical evidence of active infection</li> <li>• Antibiotic treatment within seven days of enrolment (except for prophylactic treatment of spontaneous bacterial peritonitis)</li> </ul>

Inclusion criteria	Exclusion criteria
	<ul style="list-style-type: none"> <li>• Gastrointestinal bleeding within two weeks of enrolment</li> <li>• Use of immunomodulating agents, such as steroids, within one month of enrolment</li> <li>• Concomitant use of pre-, pro- or synbiotics</li> <li>• Renal failure with creatinine above 1.7mg/dl</li> <li>• Hepatic encephalopathy stage II or III</li> <li>• Pancreatitis</li> <li>• Other organ failure</li> <li>• Hepatic or extrahepatic malignancies</li> <li>• Pregnancy</li> <li>• Non-compliance to the study medication</li> </ul>

If eligible, patients were randomized in one of two parallel groups in a ratio of 1:1. The first group received a multispecies probiotic containing *Bifidobacterium bifidum* W23, *Bifidobacterium lactis* W52, *Lactobacillus acidophilus* W37, *Lactobacillus brevis* W63, *Lactobacillus casei* W56, *Lactobacillus salivarius* W24, *Lactococcus lactis* W19, and *Lactococcus lactis* W58 for six months. The daily dose was  $1.5 \times 10^{10}$  CFU in 6 grams of powder. The second group received a similar looking and tasting placebo. The medication was taken once daily by dissolving the powder in approximately 250ml of tap water or milk and drinking it after a ten-minute activation period. After six months of intervention patients were observed without intervention for additional six months. Patients were randomized in permuted blocks and stratified for aetiology and permanent antibiotic treatment using Randomizer® software (Institute of Medical Informatics, Medical University of Graz). Patients, care givers, investigators and outcome assessors were blinded to the allocation. Patients received boxes of consecutively numbered but otherwise blank sachets, one for every day. An external trial pharmacist kept an allocation list and disclosed it after the last patient had finished the study. Compliance to the study medication was documented by counting the returned sachets and regular phone calls were done by the study nurse to encourage the patients to keep on taking the study medication. Study visits were done at baseline, after three and six months of intervention and six months after the end of treatment (observation). Sample size calculation was based on the increase in phagocytic capacity of neutrophils (primary endpoint). Basis for the calculation was a pilot study in which probiotic administration restored neutrophil phagocytosis in alcoholic hepatitis patients.(100) A 25% increase was anticipated and with an alpha of 0.05 and a beta of 0.2 allowing a 20% dropout rate, 46 patients had to be included in each group (92 in total). Secondary and additional endpoints are listed in Table 2. Additional endpoints were added to the

protocol mostly to explain interesting or unclear results obtained from the pre-specified endpoints. Technical details including the SOP for sample preparation and storage are given in the Method Glossary.

**Table 2: Study endpoints**

Pre-specified and additional endpoints of the study with actually used mode of assessment in parentheses.

Primary endpoint	Secondary endpoints	Additional endpoints
<b>Phagocytic capacity (flow cytometry)</b>	Clinically significant infections (chart review and medical history) Endotoxin levels (cell based detection assay) Neutrophil oxidative burst (flow cytometry) Neutrophil toll like receptor expression (flow cytometry)* Albumin function (HPLC) <sup>o</sup> Inflammatory response (flow cytometry, routine biochemistry) Gut barrier function (NMR, ELISA) Bacterial flora (NG-sequencing) Quality of life (questionnaire)	Serum killing and growth retardation capacity (functional assay) Macrophage activation (ELISA) Tryptophan metabolism (HPLC) Serum neopterin (ELISA) Phenylalanine metabolism (HPLC) Oxidative stress (instead of albumin function) Ethylglucuronide (HPLC) Serum bile acid profile (HPLC)* Antimicrobial and acute phase proteins (ELISA, routine biochemistry)

\*not discussed in this thesis; <sup>o</sup>exchanged for different marker with similar statement

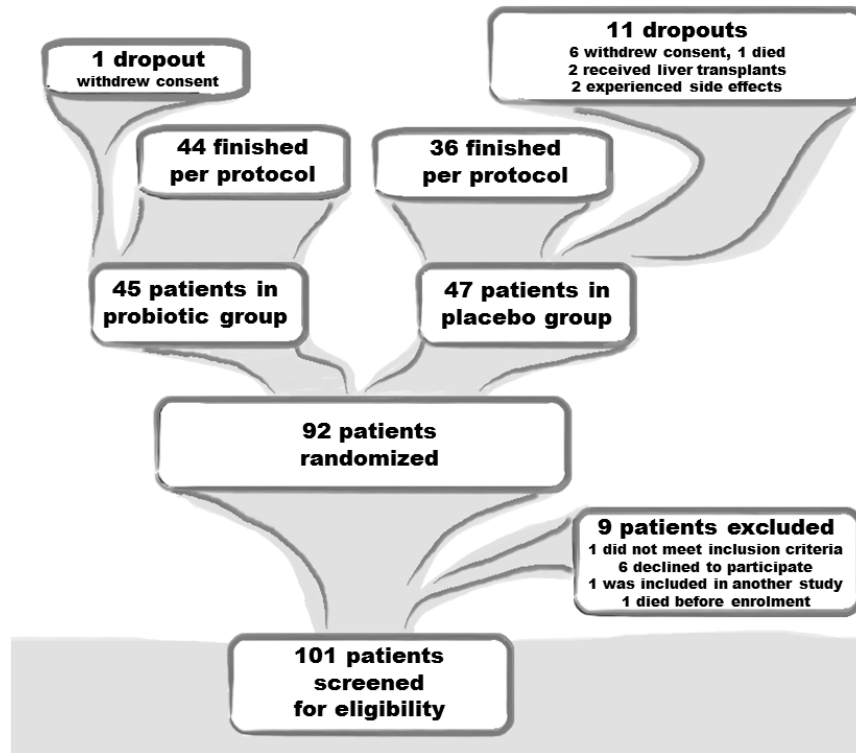
The study protocol was approved by the institutional ethics committee in Graz (23-096 ex 10/11) and performed according to the Declaration of Helsinki. The trial was registered at clinicaltrials.gov (NCT01607528).

Data was analysed and visualized using SPSS23 and GraphPadPrism6. Between-group differences of categorical variables were assessed by chi-square test/Fisher's exact test or McNemar test for unpaired and paired data respectively. Between-group differences of continuous variables were assessed by t-tests or ANOVA (both for unpaired and paired data) for comparisons of two or more groups respectively. For variables not matching the assumptions for those tests, non-parametric methods were used; Mann-Whitney/Wilcoxon signed rank tests and Kruskal-Wallis/Friedman tests for unpaired/paired data to compare two or more groups respectively. Post hoc test were done with multiple comparisons and Bonferroni correction. Microbiome abundance data were corrected using Benjamini and Hochberg procedure (False Discovery Rate) allowing for 5% false discoveries. All tests were performed on a 5% significance level.

## Results

### Recruitment and compliance

Starting in July 2012, 101 patients were screened for eligibility, 92 were randomized and 80 of them finished the study according to protocol. Details are given in Figure 1.



**Figure 1: Consort Flow Diagram**

Flow chart depicting enrolment and allocation of study patients freely based on CONSORT 2010 Flow Diagram(109)

Most of the dropout patients stopped the intervention between the baseline and the study visit after 3 months ( $n=5$ ) or between the visits after 3 and 6 months ( $n=4$ ). Dropout patients in this study refused to take the probiotic/placebo and withdrew their consent to any further examinations. Therefore, we do not have access to clinical data or samples after the patients dropped out of the study. Missing values were substituted with last observation carried forward procedure for the intention to treat analysis (ITT), a rather conservative method in a chronic progressive disease. Although the baseline values of liver function, albumin and neutrophil count would be better balanced with ITT, the imputation of values for the ITT would mask the natural progression of the disease since it is necessary almost exclusively in the placebo group (11 versus 1). Since ITT gave the same significant changes for the primary endpoint as well as the main findings (neopterin and neutrophil oxidative burst) within the groups as per protocol analysis (PPA), PPA with 44 analysed patients in the probiotic group and 36 in the placebo group is shown in the following.

Compliance to the study medication was excellent in both groups: Of 180 scheduled doses 176 were taken on average in the probiotic group and 179 in the placebo group. Possible adverse effects of the study medication were flatulence, gastric pain, diarrhoea and nausea. They were relatively mild and usually subsided after two weeks of intervention. The percentage of people affected by them was similar for both groups (41% and 33%, in probiotic and placebo group respectively,  $p=0.48$ ). Two patients dropped out of the study because of suspected adverse events (epistaxis with pre-existing arterial hypertension and nausea/flatulence). Both patients were allocated to the placebo group; therefore, an association with the study medication could be dismissed.

## Cohort characterization

### *General information*

Patients were diagnosed with cirrhosis on average seven years before they were enrolled in the study. Diagnosis was verified by liver biopsy (28 out of 80 patients). For the remaining patients, cirrhosis was verified by a combination of radiology, gastroscopy, and laboratory parameters. Cirrhosis was caused by alcohol in 44 patients (55%), by chronic hepatitis C virus infection in 13 patients (16.3%), and other causes in 23 patients (28.7%). Other causes were alpha 1-antitrypsin deficiency (n=1), primary biliary cholangitis (n=3), hemochromatosis (n=3), viral hepatitis B (n=4), Morbus Wilson (n=3), drugs (n=2), NASH (n=3), or unclear origins (n=4).

### *Liver function and related biochemical parameters*

Liver function related parameters stayed relatively stable throughout the study, except for albumin concentration. Albumin levels stayed stable during the intervention time but dropped significantly after six months of observation ( $p=0.007$ ) in the probiotic group; however, actual changes were small and clinical significance is doubtful. There was no change in the placebo group. Detailed characteristics are given in Table 3.

**Table 3: Description of study cohort and parameters of liver function**

Cohort characteristics and detailed liver function parameters before (baseline), during (3 months) and after (6 months) intervention and after 6 months of follow up (observation). (110)

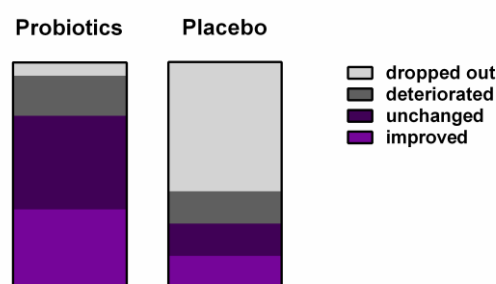
Parameter	Timepoint	Probiotics (n=44)	Placebo (n=36)	Controls (n=51)
Age (years)		60 (54; 64) <sup>#</sup>	56 (50; 63)	53 (44; 60)
Sex (m/f)		32/12 <sup>#</sup>	26/10 <sup>#</sup>	22/29
Aetiology (Alc/HCV/others)		24/8/12	20/5/11	
CPG (A/B+C)	baseline	28/16*	33/3§	
	3 months	31/13*§	33/3§	
	6 months	29/15*§	33/3§	

Parameter	Timepoint	Probiotics (n=44)	Placebo (n=36)	Controls (n=51)
	observation	24/20*	33/3§	
<b>CPS</b>	baseline	6 (5; 7)*	5 (5; 6)	
	3 months	5 (5; 7)*	5 (5; 6)	
	6 months	6 (5; 7)*	5 (5; 6)	
	observation	7 (5; 7)*	5 (5; 6)	
<b>MELD</b>	baseline	12 (9; 15)*	9 (8;13)	
	3 months	11 (9;14)*	9 (8; 11)	
	6 months	11 (9; 15)*	9 (8; 12)	
	observation	12 (9; 14)*	8 (7; 11)	
<b>HE (no/mild)</b>	baseline	39/5	35/1	
	3 months	40/4	34/2	
	6 months	38/6	34/2	
	observation	40/4	34/2	
<b>Ascites (no/mild)</b>	baseline	40/4	32/4	
	3 months	41/3	34/2	
	6 months	37/7	33/3	
	observation	38/6	34/2	
<b>ALT (U/l)</b>	baseline	36.5 (27.0; 1.25) <sup>#</sup>	32.5 (20.75; 46.25) <sup>#</sup>	21.0 (16.5; 27.5)
	3 months	34.5 (27.5; 48.5)	30.0 (22.0; 43.3)	
	6 months	38.5 (25.8; 52.3)	29.5 (22.0; 49.8)	
	observation	36.0 (26.0; 53.5)	30.0 (22.0; 42.5)	
<b>AST (U/l)</b>	baseline	49.0 (37.75; 69.5) <sup>#</sup>	42.5 (32.5; 56.5) <sup>#</sup>	22.0 (19.0; 27.0)
	3 months	44.5 (36.0; 59.0)	40.5 (31.5; 58.0)	
	6 months	53.5 (36.8; 70.0)*	37.5 (30.8; 59.0)	
	observation	49.5 (37.5; 68.3)	45.0 (29.8; 63.3)	
<b>GGT (U/l)</b>	baseline	125.0 (64.5; 234.8) <sup>#</sup>	107.5 (49.75; 175.3) <sup>#</sup>	20 (14.5; 31)
	3 months	107.0 (49.8; 246.0)	111.0 (45.5; 179.3)	
	6 months	114.5 (52.8; 205.8)	122.0 (47.3; 192.0)	
	observation	110.5 (58.0; 198.3)	109.5 (51.8; 244.0)	
<b>Crea (mg/dl)</b>	baseline	0.85 (0.73; 0.96)	0.81 (0.72; 0.94)	0.86 (0.77; 1.0)
	3 months	0.83 (0.73; 0.94)	0.78 (0.70; 0.98)	
	6 months	0.85 (0.74; 1.01)	0.80 (0.70; 0.88)	
	observation	0.85 (0.75; 0.98)	0.83 (0.71; 0.92)	
<b>Alb (g/dl)</b>	baseline	4.0 (3.3; 4.5) <sup>#§</sup>	4.3 (4.1; 4.7)	4.5 (4.4; 4.8)
	3 months	4.0 (3.4; 4.5)*	4.4 (4.0; 4.6)	

Parameter	Timepoint	Probiotics (n=44)	Placebo (n=36)	Controls (n=51)
Bili (mg/dl)	6 months	4.0 (3.4; 4.5)*	4.3 (4.0; 4.4)	
	observation	3.9 (3.3; 4.4)*	4.3 (3.9; 4.5)	
	baseline	1.38 (0.78; 2.41)#	1.11 (0.63; 1.42)#	
	3 months	1.29 (0.74; 2.25)	0.97 (0.74; 1.38)	0.50 (0.38; 0.61)
	6 months	1.32 (0.77; 2.69)	0.95 (0.68; 1.48)	
	observation	1.46 (0.88; 2.41)*	1.00 (0.64; 1.59)	
TG (mg/dl)	baseline	73.0 (55.0; 96.3)	91.0 (69.8; 112.0)	
	3 months	74.0 (62.8; 109.0)	80.0 (65.0; 113.3)	82.0
	6 months	74.5 (62.5; 104.5)	74.5 (63.0; 105.8)	(68.0; 112.5)
	observation	73.0 (57.0; 110.0)	95.0 (59.8; 118.0)	
INR	baseline	1.27 (1.14; 1.43)#	1.20 (1.12; 1.27)#	
	3 months	1.27 (1.18; 1.39)*	1.18 (1.09; 1.32)	1.01 (0.98; 1.05)
	6 months	1.28 (1.16; 1.48)*	1.18 (1.11; 1.25)	
	observation	1.30 (1.14; 1.45)*	1.14 (1.09; 1.25)	

Alc, alcoholic cirrhosis; HCV, viral hepatitis C induced cirrhosis; CPG, Child-Pugh grade; MELD, Model of End Stage Liver Disease; HE, hepatic encephalopathy; ALT, alanine aminotransferase; AST, aspartate transaminase; GGT, gamma glutamyl transferase; Crea, creatinine; Alb, albumin; Bili, total bilirubin; TG, triglycerides; INR, prothrombin time international normalized ratio; \* significant differences between test groups; # significant differences compared to healthy controls; \$ significant differences over time; § significant differences to expected distribution; significance level was 5%;

Note that the disproportionate dropout rate skewed the balance between groups regarding liver function. Baseline liver function was significantly worse in the probiotic group compared to the placebo group (Child-Pugh score  $p=0.02$  and MELD score  $p=0.05$ ). Patients that were included in the study with a Child-Pugh grade B or C tended to drop out of the study in the placebo group while comparable patients in the probiotic group remained in the study and were even more likely to improve their score than deteriorate after six months of intervention (Figure 2).



**Figure 2: Changes in Child-Pugh grade during intervention in patients with Child' B/C cirrhosis**  
 Patients with Child-Pugh grade B or C were more likely to improve than deteriorate in the probiotic group (n=16). Comparable patients in the placebo group tended to drop out of the study (n=7).

**Nutritional status**

Nutritional habits of the patients as assessed by a food frequency questionnaire (Clinical nutrition services, Medical University Graz) were not influenced by the intervention and stayed unchanged throughout the year. Height (174±9 cm), weight (82±16 kg) and body mass index (26.9±4.0 kg/cm<sup>2</sup>) were similar in both groups and did not change over time. Subjective global assessment(111) (SGA) attested a good nutritional status for 82% of the patients and marginal malnutrition for the remaining 18% at baseline. SGA status stayed constant over the study period; no differences between the groups could be observed. Total protein concentration was slightly lower in the probiotic group than in the placebo group (baseline: 7.49±0.62 vs. 7.82±0.65 mg/dl, respectively, p=0.024), but stayed constant within the group for the duration of the study.

**Blood count**

Leucocyte count, relative share of leucocyte subpopulations, erythrocyte count and thrombocyte count were similar in both groups and stayed constant over the course of the study. Also haemoglobin, mean cell haemoglobin concentration, haematocrit, mean cell volume and thrombocyte volume showed no differences between groups or over time. Due to cirrhosis thrombocyte counts were below normal. Details are given in Table 4.

**Table 4: Blood count parameters**

Blood count details for both test groups before (baseline), during (3 months) and after (6 months) intervention and after 6 months of follow up (observation). Norm values are given as reference.

<b>Blood count parameters (norm values)</b>	<b>Timepoint</b>	<b>Probiotics (n=44)</b>	<b>Placebo (n=36)</b>
<b>Leucocyte count (4.4-11.3G/l)</b>	baseline	4.3 (3.4; 5.5)	5.0 (3.9; 6.1)
	3 months	4.8 (3.7; 5.6)	5.1 (4.0; 6.5)
	6 months	4.5 (3.4; 5.7)	4.9 (3.8; 6.8)
	observation	4.7 (3.6; 5.6)	5.2 (3.7; 6.7)
→ % neutrophils (50-75%)	baseline	61 (53; 69)	60 (54; 65)
	3 months	61 (51; 67)	63 (52; 66)
	6 months	62 (53; 69)	59 (53; 67)
	observation	63 (54; 69)	60 (54; 64)
→ % monocytes (2-12%)	baseline	9 (7; 12)	9 (7; 11)
	3 months	10 (8; 12)	9 (7; 10)
	6 months	9 (7; 12)	9 (8; 11)

<b>Blood count parameters (norm values)</b>	<b>Timepoint</b>	<b>Probiotics (n=44)</b>	<b>Placebo (n=36)</b>
	observation	10 (7; 11)	9 (8; 11)
	baseline	24 (20; 32)	28 (23; 33)
→ % lymphocytes (20-40%)	3 months	28 (20; 35)	28 (21; 35)
	6 months	24 (19; 32)	27 (21; 33)
	observation	25 (21; 32)	27 (23; 32)
→ % eosinophils (-5%)	baseline	3 (2; 4)	3 (2; 4)
	3 months	3 (2; 3)	3 (2; 4)
	6 months	3 (2; 4)	3 (2; 4)
	observation	3 (2; 3)	3 (2; 4)
→ % basophils (-1%)	baseline	1 (0; 1)	1 (0; 1)
	3 months	1 (0; 1)	1 (0; 1)
	6 months	1 (0; 1)	1 (0; 1)
	observation	0 (0; 1)	1 (0; 1)
<b>Erythrocytes (4.10-5.10T/l)</b>	baseline	4.36 (3.90; 4.69)	4.57 (4.11; 4.91)
	3 months	4.40 (3.86; 4.74)	4.58 (4.14; 4.92)
	6 months	4.31 (3.94; 4.65)	4.58 (4.19; 4.86)
	observation	4.23 (3.88; 4.85)	4.49 (4.23; 4.85)
<b>Thrombocytes (140-440G/l)</b>	baseline	112 (76; 149)	116 (93; 167)
	3 months	113 (72; 147)	116 (90; 157)
	6 months	124 (64; 144)	120 (96; 155)
	observation	110 (62; 136)	115 (92; 168)
<b>Haemoglobin (12.0-15.3g/dl)</b>	baseline	13.5 (12.5; 14.7)	13.8 (12.8; 14.9)
	3 months	13.5 (12.5; 15.3)	14.2 (13.2; 14.8)
	6 months	13.8 (12.2; 14.6)	14.2 (13.2; 15.0)
	observation	13.6 (12.6; 14.7)	14.3 (13.1; 14.9)
<b>Mean cell haemoglobin concentration (33.0-36.0g/dl)</b>	baseline	35.6 (34.5; 36.2)	35.6 (34.5; 36.1)
	3 months	35.6 (34.2; 36.2)	35.5 (35.0; 36.2)
	6 months	35.8 (34.7; 36.5)	35.7 (35.0; 36.1)
	observation	35.5 (34.6; 36.0)	35.5 (34.6; 36.0)
<b>Haematocrit (35-45%)</b>	baseline	38 (36; 42)	39 (37; 43)
	3 months	39 (35; 42)	40 (37; 42)
	6 months	38 (35; 42)	39 (37; 42)
	observation	39 (36; 41)	40 (38; 42)
<b>Mean cell volume</b>	baseline	89 (84; 96)	87 (85; 92)

<b>Blood count parameters (norm values)</b>	<b>Timepoint</b>	<b>Probiotics (n=44)</b>	<b>Placebo (n=36)</b>
<b>(80-98fl)</b>	3 months	90 (85; 93)	88 (84; 92)
	6 months	89 (85; 94)	85 (83; 92)
	observation	89 (84; 93)	88 (84; 93)
<b>Thrombocyte volume (7-13fl)</b>	baseline	11 (10; 12)	11 (10; 12)
	3 months	11 (10; 12)	11 (10; 12)
	6 months	11 (10; 12)	11 (10; 12)
	observation	11 (10; 12)	11 (10; 12)

### *Electrolytes*

Calcium levels decreased significantly over the course of one year ( $p=0.001$ ) in the probiotic group. Similar changes occurred in the placebo group but did not reach statistical significance ( $p=0.180$ ). Chloride, phosphate, potassium and sodium concentrations did not show differences between groups or over time. Electrolyte concentrations are given in Table 5 for both test groups.

**Table 5: Serum electrolyte levels**

Serum electrolyte levels of both test groups before (baseline), during (3 months) and after (6 months) intervention and after 6 months of follow up (observation). Norm values are given as reference.

<b>Electrolytes (norm values)</b>	<b>Timepoint</b>	<b>Probiotics (n=44)</b>	<b>Placebo (n=36)</b>
<b>Calcium (2.20-2.65mmol/l)</b>	baseline	2.35 (2.27; 2.43) <sup>s</sup>	2.38 (2.32; 2.44)
	3 months	2.32 (2.24; 2.44)	2.36 (2.29; 2.44)
	6 months	2.30 (2.21; 2.35)*	2.36 (2.31; 2.41)
	observation	2.26 (2.19; 2.35)*	2.34 (2.29; 2.39)
<b>Chloride (95-110mmol/l)</b>	baseline	103 (101; 104)	102 (100; 104)
	3 months	103 (101; 105)	102 (100; 104)
	6 months	104 (101; 106)	104 (101; 104)
	observation	102 (100; 106)	103 (101; 104)
<b>Phosphate (0.84-1.45mmol/l)</b>	baseline	0.98 (0.88; 1.08)	0.98 (0.91; 1.10)
	3 months	1.01 (0.83; 1.10)	1.03 (0.89; 1.11)
	6 months	1.02 (0.85; 1.07)	0.98 (0.88; 1.09)
	observation	0.95 (0.87; 1.03)	0.95 (0.88; 1.07)
<b>Potassium (3.5-5.0mmol/l)</b>	baseline	4.0 (3.8; 4.2)	4.1 (3.8; 4.2)
	3 months	4.1 (3.8; 4.3)	4.1 (3.9; 4.3)
	6 months	4.0 (3.8; 4.2)	4.1 (3.9; 4.1)
	observation	4.0 (3.8; 4.1)	4.0 (3.8; 4.3)

<b>Electrolytes (norm values)</b>	<b>Timepoint</b>	<b>Probiotics (n=44)</b>	<b>Placebo (n=36)</b>
<b>Sodium (135-145mmol/l)</b>	baseline	140 (138; 141)	139 (137; 140)
	3 months	140 (137; 141)	140 (138; 141)
	6 months	139 (138; 141)	140 (138; 141)
	observation	138 (136; 141)	140 (137; 141)

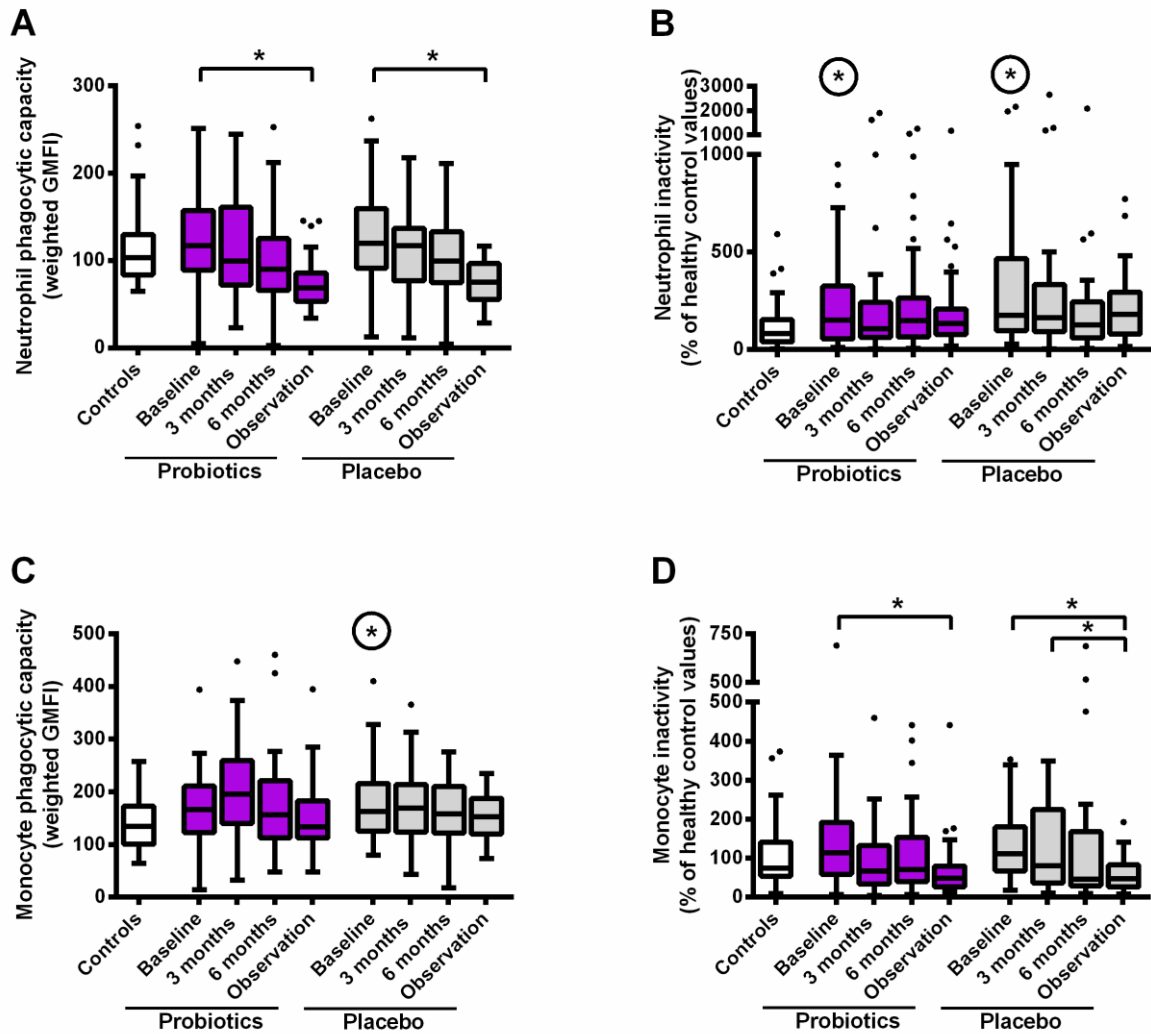
\* significant differences between test groups; § significant changes over time;

## **Effects of probiotics on immune function and infections**

### *Phagocytosis*

Neutrophil phagocytic capacity was comparable to controls at baseline but declined significantly in both groups during the study ( $p < 0.001$ ). Calcium is an important ion for phagocytosis and interestingly a contemporaneous decrease of calcium levels could be observed. However regression analysis revealed very little influence of the calcium drop on neutrophil phagocytosis (baseline:  $R^2 = 0.015$ ,  $p = 0.345$ ; 3 months:  $R^2 = 0.364$ ,  $p = 0.000$ ; 6 months:  $R^2 = 0.135$ ,  $p = 0.056$ ; observation:  $R^2 = 0.068$ ,  $p = 0.199$ ). Phagocytic inactivity of neutrophils stayed constant throughout the study.

Monocyte phagocytic capacity was slightly increased in the placebo group compared to healthy controls. In the probiotic group median capacity was also higher than in healthy controls, but the increase fell short of statistical significance. Probiotic intervention further increased phagocytic capacity of monocytes after 3 months (also not significant) and returned to baseline values afterwards. There was no change in the placebo group. Monocyte phagocytic inactivity decreased significantly over time in both groups. This could be a mechanism to balance the simultaneous loss of phagocytic capacity of neutrophils. For illustration see Figure 3. Technical details are given in the “Method glossary” under “Phagocytosis” and in “C. Methods in liver disease research” under “Phagoindex”.



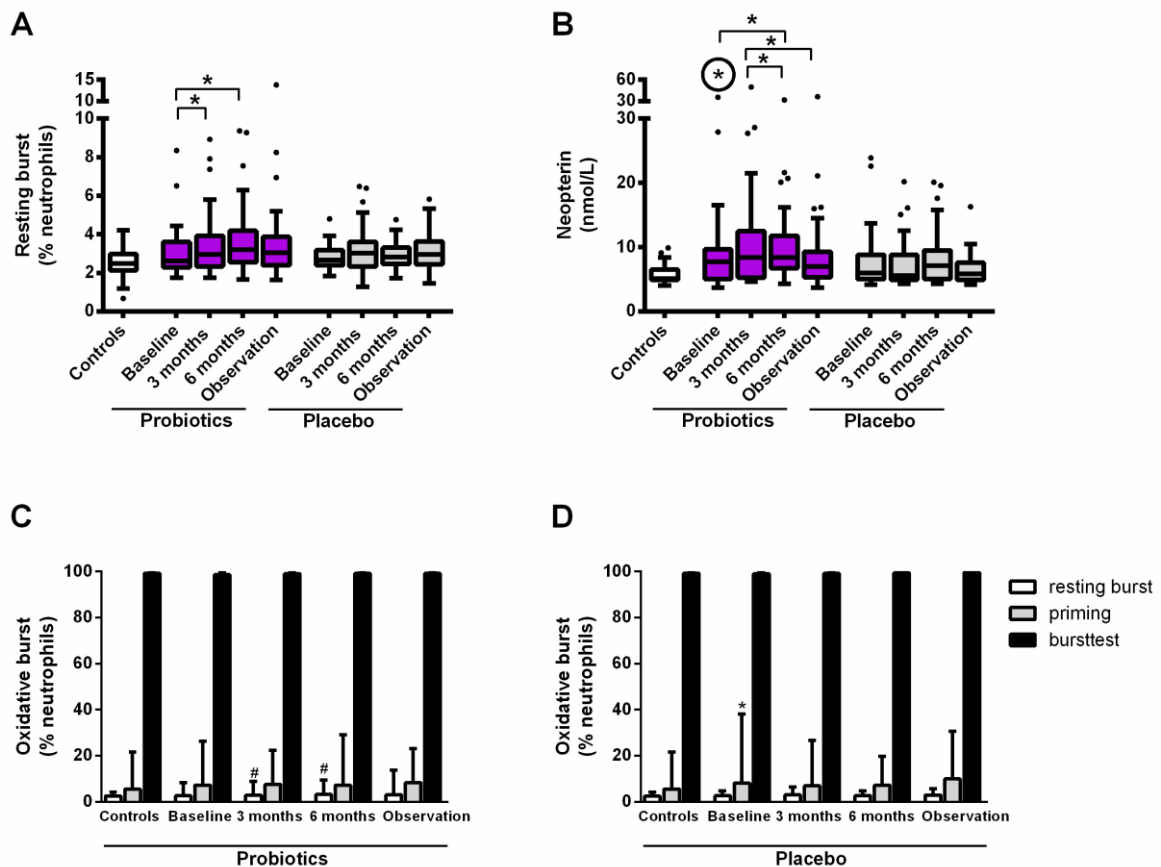
**Figure 3: Effects of probiotic intervention on phagocyte function.**

A Neutrophil phagocytic capacity calculated with Phagoindex; B Neutrophil phagocytic inactivity; C Monocyte phagocytic capacity calculated with Phagoindex; D Monocyte phagocytic inactivity,\* indicates significant differences between groups indicated by horizontal bars; \* in circle indicate a significant difference compared to healthy controls;(110)

### *Oxidative burst of neutrophils*

Oxidative burst function of neutrophils in reaction to *E. coli* in both groups was comparable to healthy controls and remained intact throughout the study. In the placebo group significantly more primed neutrophils were found compared to healthy controls ( $p=0.002$ ). Priming of both groups was unchanged over time. Resting burst did not show significant differences between groups at baseline. Probiotics increased neutrophil resting burst significantly after three and six months of intervention (2.6 to 3.0%,  $p=0.018$ ; and 2.6 to 3.2%,  $p=0.008$ , respectively). After the end of treatment resting burst decreased again. No significant changes in resting burst were found in the placebo group.

The antimicrobial molecule, neopterin, is a marker for macrophage activation and has been shown to induce ROS production in neutrophils.(112) Serum levels of neopterin were significantly increased at baseline in the probiotic group ( $p=0.001$ ). Probiotics further increased neopterin levels in serum after six months of intervention (7.8 to 8.4nmol/l,  $p=0.035$ ). This increase subsided after the end of treatment. Details are given in Figure 4. Technical details are given in “Method glossary” under “Oxidative burst” and “Parameters measured by collaborators”.



**Figure 4: Effects of probiotic intervention on oxidative burst function of neutrophils.**

A Neutrophil resting burst; B serum neopterin levels; C-D Oxidative burst profiles for probiotic (C) and placebo (D) group; \* significant differences between groups indicated by horizontal bars; \* in circle indicates a significant difference compared to healthy controls; in C/D: \* significant differences compared to controls; # significant differences to respective baseline values;(110)

Both resting burst and neopterin were increased during intervention and decreased after the end of treatment. The transient nature of the additional macrophage activation due to probiotics was confirmed by the stable expression of long term macrophage activation marker, sCD163. According to this marker macrophages are activated in patients compared to healthy controls, but there were no alterations between test groups or over time. The increase in resting burst did not impact on oxidative stress, assessed by advanced oxidation protein products (AOPP). AOPP levels

were significantly higher in the probiotic group compared to healthy controls at baseline (p=0.009) and decreased gradually but not significantly during the intervention. After the intervention had ended AOPP concentration returned to baseline values. In the placebo group AOPP concentration increased significantly in the last six months (p=0.044). The amount of ROS produced by activated neutrophils increased continuously in the probiotic as well as in the placebo group (p=0.014 and p<0.001 respectively). However, only the values in the probiotic group at the observation time point were significantly higher than in healthy controls. In primed neutrophils ROS production also increased significantly in the placebo group from baseline to observation (p=0.040). Increases in the probiotic group did not reach statistical significance; however, at the end of the observation period, both groups had significantly higher ROS production in primed neutrophils than healthy controls (p=0.018 and p=0.036 for probiotic and placebo group respectively). Also, in response to *E. coli* neutrophils of both test groups produced more ROS than healthy controls; most pronounced at the observation time point (p<0.001 and p=0.001 for probiotic and placebo group respectively). Details are given in Table 6. Technical details are given in the “Method glossary” under “Parameters measured by collaborators”.

**Table 6: Macrophage activation and ROS production during intervention**

Macrophage activation and ROS production for both test groups before (baseline), during (3 months) and after (6 months) intervention and after 6 months of follow up (observation) as well as healthy controls.

Parameters	Timepoint	Probiotics (n=44)	Placebo (n=36)	Controls (n=51)
<b>sCD163 (mg/l)</b>	baseline	5.53 (3.38; 8.80) <sup>#</sup>	4.23 (2.64; 5.89) <sup>#</sup>	
	3 months	4.96 (3.35; 8.80) <sup>#</sup>	4.32 (2.81; 5.80) <sup>#</sup>	1.35
	6 months	5.36 (3.59; 8.48) <sup>#</sup>	4.76 (2.70; 6.12) <sup>#</sup>	(1.12; 1.69)
	observation	4.98 (3.40; 7.18) <sup>#</sup>	4.29 (2.79; 5.45) <sup>#</sup>	
<b>Advanced oxidation protein products (µmol/l)</b>	baseline	42.6 (36.2; 46.7) <sup>#</sup>	36.9 (33.0; 46.2) <sup>§</sup>	
	3 months	40.8 (36.6; 45.9) <sup>#</sup>	39.5 (33.1; 45.3)	35.6
	6 months	39.2 (36.0; 47.4) <sup>#</sup>	36.6 (30.9; 44.6)	(30.2; 43.6)
	observation	41.9 (37.1; 48.1) <sup>#</sup>	40.6 (37.2; 48.4) <sup>#</sup>	
<b>ROS production through resting burst (GMFI)</b>	baseline	4.0 (3.3; 5.3) <sup>§</sup>	3.6 (3.3; 4.3) <sup>§</sup>	
	3 months	4.5 (3.6; 6.8)	4.4 (3.5; 5.2)	4.1
	6 months	4.6 (3.8; 6.7)	4.0 (3.4; 4.8)	(3.6; 4.8)
	observation	4.9 (4.3; 6.4) <sup>#</sup>	4.7 (4.1; 5.8)	
<b>ROS production by primed neutrophils (GMFI)</b>	baseline	4.8 (4.2; 6.1)	4.6 (3.9; 5.1) <sup>§</sup>	
	3 months	5.4 (4.2; 6.8)	5.2 (4.5; 6.0)	4.8
	6 months	4.9 (4.4; 5.8)	5.1 (4.1; 5.7)	(4.1; 5.4)
	observation	5.3 (4.7; 6.5) <sup>#</sup>	5.6 (4.7; 6.1) <sup>#</sup>	
<b>ROS production</b>	baseline	73.3 (45.9; 92.6)	58.9 (44.5; 80.8)	57.5

Parameters	Timepoint	Probiotics (n=44)	Placebo (n=36)	Controls (n=51)
<b>in response to</b>	3 months	84.5 (58.2; 117.5) <sup>#</sup>	72.8 (53.6; 90.6)	(41.7; 69.7)
<b><i>E. coli</i> (GMFI)</b>	6 months	81.1 (46.7; 101.0)	63.1 (47.0; 102.4)	
	observation	87.2 (63.5; 111.3) <sup>#</sup>	72.4 (59.9; 114.9) <sup>#</sup>	

GMFI stands for geometric mean of fluorescence intensity; # significant differences compared to controls; \$ significant change over time;

### *Antimicrobial molecules*

To rule out active infection as the reason for the increase in resting burst and neopterin levels, other antimicrobial molecules and acute phase proteins were examined. There were no changes in ferritin, fibrinogen, serum calprotectin, and serum zonulin. C-reactive protein did not show alterations over time in the probiotic group. In the placebo group an increase between the three months and the observation time point could be detected (p=0.028). With the average C-reactive protein concentration of 3.6mg/l, it was still well below the upper normal threshold of 5mg/l. Nitrite, a stable metabolite of nitric oxide, was significantly lower in both test groups compared to healthy controls. Nitrite levels stayed constant in the probiotic group and increased significantly from the six months to the observation time point in the placebo group (p=0.002). Details are given in Table 7. Technical details are given in the “Method glossary” under “Parameters measured by collaborators”, “Calprotectin” and “Zonulin”.

**Table 7: Antimicrobial molecules and acute phase proteins during intervention**

Antimicrobial molecules and acute phase proteins for both test groups before (baseline), during (3 months) and after (6 months) intervention and after 6 months of follow up (observation) as well as healthy controls;(110)

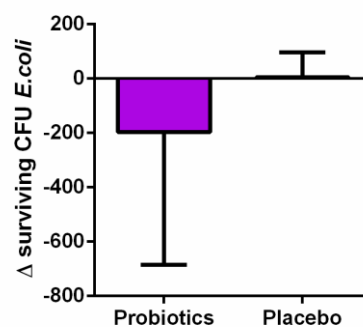
Parameters	Timepoint	Probiotics (n=44)	Placebo (n=36)	Controls (n=51)
<b>C-reactive protein (mg/l)</b>	baseline	2.0 (0.9; 4.7)	2.6 (1.2; 3.4) <sup>\$</sup>	
	3 months	1.9 (1.0; 5.2)	1.8 (0.7; 3.4)	1.4 (0.8; 2.0)
	6 months	2.5 (0.8; 5.2)	2.2 (0.9; 4.1)	
	observation	3.2 (0.9; 4.8)	2.6 (1.1; 4.0)	
<b>Ferritin (mg/dl)</b>	baseline	160.0 (59.3; 357.8)	97.5 (55.3; 221.3)	
	3 months	153.0 (50.3; 247.8)	92.0 (43.8; 166.0)	117.5
	6 months	152.5 (52.5; 104.5)	90.5 (47.3; 154.5)	(60.3; 218.5)
	observation	154.0(54.3; 246.0)	96.0 (40.5; 179.0)	
<b>Fibrinogen (mg/l)</b>	baseline	266.5 (216.3; 313.5) <sup>#</sup>	277.0 (241.3; 308.5)	302.0
	3 months	278.0 (212.5; 325.3)	283.5 (249.5; 325.0)	(254.0; 328.0)
	6 months	261.0 (214.3; 316.3)	292.0 (260.3; 318.5)	

Parameters	Timepoint	Probiotics (n=44)	Placebo (n=36)	Controls (n=51)
	observation	262.5 (203.5; 321.0)	283.0 (240.3; 329.3)	
	baseline	9.5 (5.2; 15.2) #	10.6 (6.0; 18.4) #§	
<b>Nitrite (<math>\mu\text{mol/l}</math>)</b>	3 months	8.0 (6.2; 13.6)#	9.1 (7.9; 14.3)#	19.4 (9.5; 32.4)
	6 months	9.1 (6.0; 14.7)#	7.9 (5.7; 13.7)#	
	observation	10.9 (6.7; 21.3)#	14.0 (9.3; 19.8)	
<b>Serum calprotectin (ng/ml)</b>	baseline	432.2 (326.6; 896.6)	627.9 (325.2; 798.5)	
	3 months	445.7 (240.1; 779.4)	456.2 (301.1; 847.5)	536.6 (334.0;
	6 months	460.9 (326.5; 824.7)	476.8 (297.3; 953.4)	779.0)
	observation	356.6 (214.0; 808.2)	482.6 (323.1; 626.2)	
<b>Serum zonulin (ng/ml)</b>	baseline	39.8 (23.1; 53.7)	46.2 (26.6; 63.5)	
	3 months	39.8 (23.3; 55.1)	44.8 (27.3; 63.0)	56.0 (48.0; 66.0)
	6 months	34.1 (22.0; 51.9)	44.7 (31.9; 61.0)	
	observation	37.8 (22.9; 58.2)	44.3 (28.5; 63.6)	

# significant differences compared to controls; § significant change over time;

### ***Killing capacity***

Increases in resting burst and neopterin did not have significant effects on functional tests regarding bacterial growth retardation or serum killing capacity. However, in the subgroup of alcoholic cirrhotics the impairment of serum killing capacity was markedly reduced after six months of probiotic intervention (less bacteria survived the serum challenge). There were no changes in the placebo group. Unfortunately, this observation did not reach statistical significance (Figure 5). For technical details see “Method glossary” under “Serum Killing capacity”.

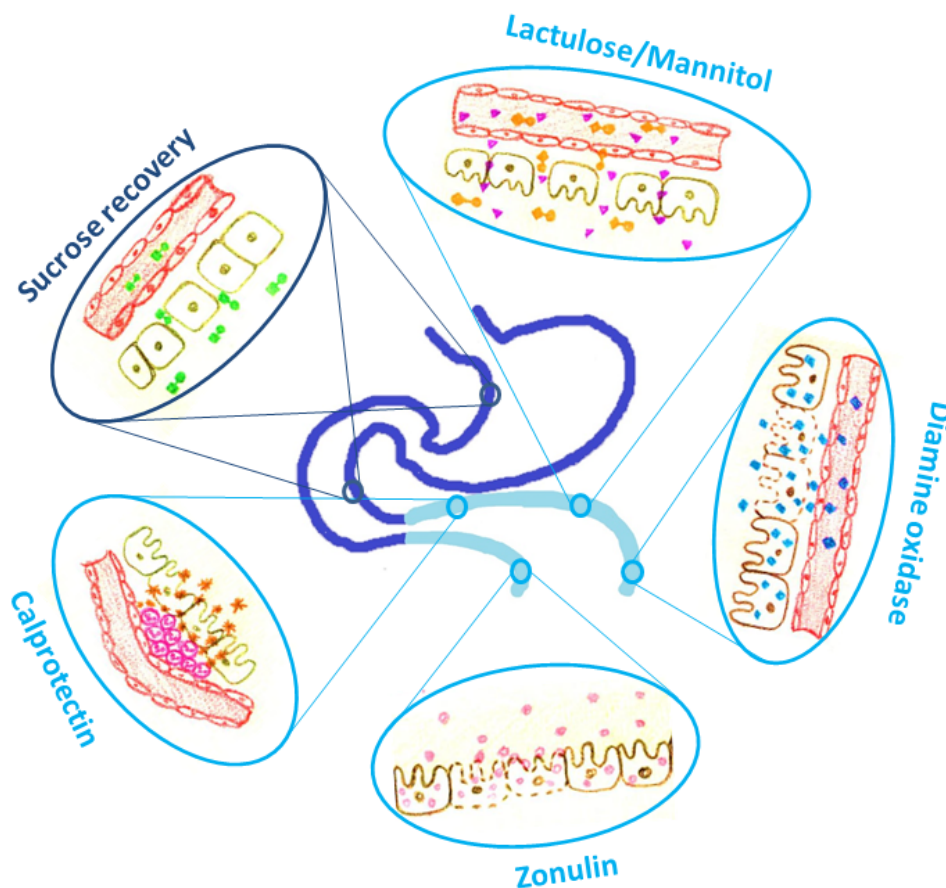


**Figure 5: Changes in serum killing capacity of alcoholic cirrhotics (n=44) after six months of intervention (110)**

A trend to a reduced number of mild infections in the probiotic group occurred in both phases of the study compared to the placebo group (15 vs 28 during intervention and 6 vs 11 during follow up, respectively). During the intervention with the probiotic no severe infection (hospitalization necessary) was documented while in the placebo group one patient suffered acute pancreatitis. In the follow up phase, five severe infections were observed in the probiotic group (pansinusitis, urinary tract infections (2), bronchitis, skin infection) and four infections in the placebo group (gastroenteritis, pneumonia, erysipelas, spontaneous bacterial peritonitis). The rise of severe infections occurred simultaneously with the decrease of phagocytic capacity of neutrophils.

### Effects of probiotics on gut permeability

In order to quantify gut barrier function, a panel of different markers reflecting gut permeability (lactulose-mannitol ratio, sucrose recovery), epithelial damage (diamine oxidase, stool zonulin), intestinal inflammation (stool calprotectin) and bacterial translocation (endotoxin, soluble CD14, lipopolysaccharide binding protein) was established. The panel is illustrated in Figure 6.



**Figure 6: Illustration of the gut permeability panel**

Gut permeability panel reflects gastroduodenal (dark blue) and small intestinal (light blue) barrier dysfunction. Sucrose in the urine results from translocation of intact molecules through the gastric or duodenal epithelium. Mannitol is consistently taken up in the small intestine and reflects the individual uptake rate of the patient. Lactulose can only pass a dysfunctional epithelium of the small intestine. Remaining

molecules are fermented in the colon. Excessive amounts of diamine oxidase originate from damaged cells in the small intestine (indicated with dashed lines) and are passing through the dysfunctional barrier. Zonulin in the stool either stems from dying cells (indicated with dashed lines) or by excessive secretion into the intestinal lumen in order to regulate tight junction functionality. Calprotectin in the stool originates from infiltrating neutrophil in case of an infection.

Differential sugar absorption was used to functionally assess permeability of the gastroduodenal (sucrose recovery) and small intestinal part (lactulose-mannitol ratio) of the gastrointestinal tract. Lactulose-mannitol ratio was significantly increased in patients with liver disease ( $p=0.017$ ) and was strongly dependent on liver function ( $p<0.001$ ). Child's A patients rarely show impairment in the small intestinal barrier, while Child's B/C patients show significant alterations in both mannitol absorption ( $p=0.015$ ) and lactulose recovery ( $p<0.001$ ). Sucrose recovery was unaltered in patient groups and healthy controls. Impairment in intestinal barrier function was mainly present in alcoholic cirrhosis. Mannitol absorption deteriorated significantly within one year in both groups ( $p=0.033$  and  $p=0.004$  for probiotic and placebo group respectively) but could be stabilized by probiotics for the duration of the intervention, while the placebo had no effect. Despite the stabilization of mannitol recovery, lactulose-mannitol ratio did not improve during intervention. For technical details see "Method glossary" under "Differential sugar absorption test" and "Parameters measured by collaborators".

Diamine oxidase (DAO) is a histamine cleaving enzyme with its main point of action in the small intestine. Gut epithelial cells contain a high amount of diamine oxidase to face food borne histamine. In the case of epithelial damage, the enzyme is released into the lumen and translocates through the permeable barrier into the blood stream. High serum levels of DAO therefore translate to epithelial damage and barrier breaches. Compared to healthy controls, cirrhotic patients had significantly increased DAO serum levels. A stepwise increase through Child-Pugh classes occurred ( $p<0.001$ ), while DAO concentrations were already significantly elevated in compensated cirrhosis ( $p=0.034$ ). In accordance with sugar absorption tests, alcoholic cirrhosis patients showed the steepest increase in DAO concentration. Probiotic intervention had no effect on serum DAO levels. Technical details are given in the "Method glossary" under "Diamine oxidase (DAO)".

Calprotectin is an antimicrobial protein predominantly found in neutrophil granulocytes. In the case of intestinal inflammation, neutrophils infiltrate the intestine and calprotectin can increasingly be found in the stool. Stool calprotectin levels in patients with liver cirrhosis were significantly higher compared to healthy controls. There was no statistically significant difference between Child-Pugh classes although calprotectin tended to be higher in patients with declining liver function. Alcoholic cirrhotics had the most pronounced phenotype of intestinal inflammation among the examined patient groups. Probiotic intervention had no effect on stool calprotectin levels. Technical details are given in the "Method glossary" under "Calprotectin".

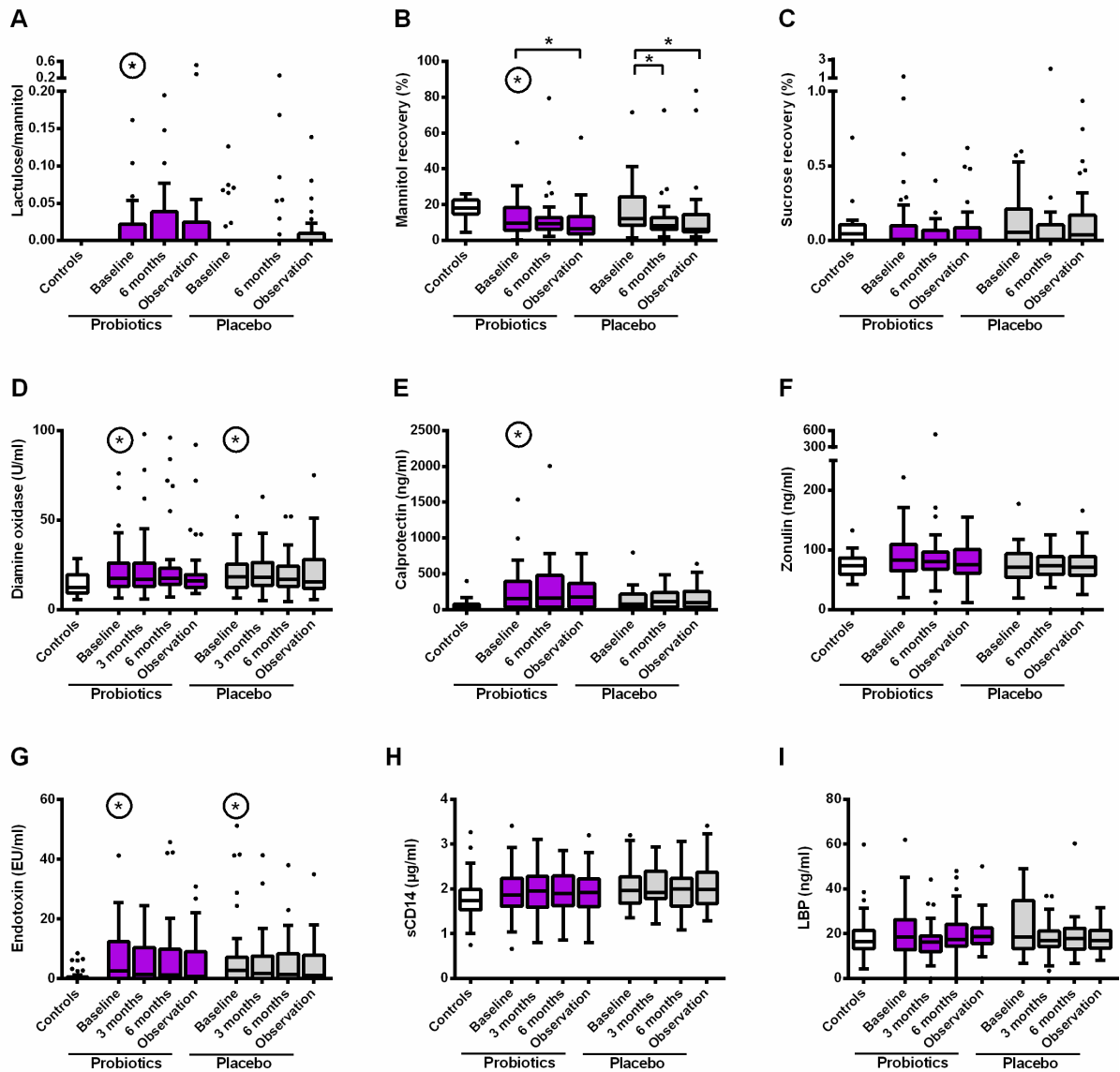
Zonulin (or Prehaptoglobin) has been found to regulate tight junction functionality in the intestinal epithelium. Therefore, epithelial cells contain a relatively high amount of this protein. The protein has to be secreted into the intestinal lumen to bind on apical receptors and affect the structural integrity of the tight junctions. In addition, when the gut barrier is compromised, epithelial cell mass is lost into the intestinal lumen and zonulin concentration in stool increases. No differences between healthy or cirrhotic patients, between compensated or decompensated patients or between aetiologies could be found. Probiotics did not influence zonulin concentration in stool. Technical details are given in the “Method glossary” under “Zonulin”.

Endotoxin can enter the portal vein from the intestinal lumen through a leaky gut barrier. Kupffer cells can filter endotoxins very efficiently in a healthy state. However, when liver tolerance is broken systemic overflow of endotoxin can be observed. Therefore, endotoxin levels in the systemic blood reflect a dual breach in functional clearance. Endotoxin levels were significantly elevated in both patient groups compared to healthy controls ( $p < 0.001$  for probiotic and placebo group). Alcoholic cirrhotics tended to have higher endotoxin levels. No differences between Child-Pugh grades could be observed. Probiotic intervention did not influence serum endotoxin levels. A correlation between serum endotoxin levels and phagocytic capacity of neutrophils could not be found ( $R^2 = 0.06$ ,  $p = 0.57$ ). Technical details are outlined in the “Method glossary” under “Endotoxin/Lipopolysaccharide” and in “C. Methods in liver disease research” under “Endotoxin”.

Baseline sCD14 levels were slightly but not significantly increased in both patient groups compared to healthy controls. No differences between Child-Pugh grades or aetiology could be observed. Probiotic intervention did not influence sCD14 concentrations. See also “Method glossary” under “Soluble cluster of differentiation (sCD) 14”.

Lipopolysaccharide binding protein concentrations were comparable to healthy controls in both test groups; they were not influenced by aetiology or liver function. No alterations during probiotic intervention could be found. See also “Method glossary” under “Lipopolysaccharide binding protein (LBP)”.

The entire gut permeability panel is illustrated in Figure 7.



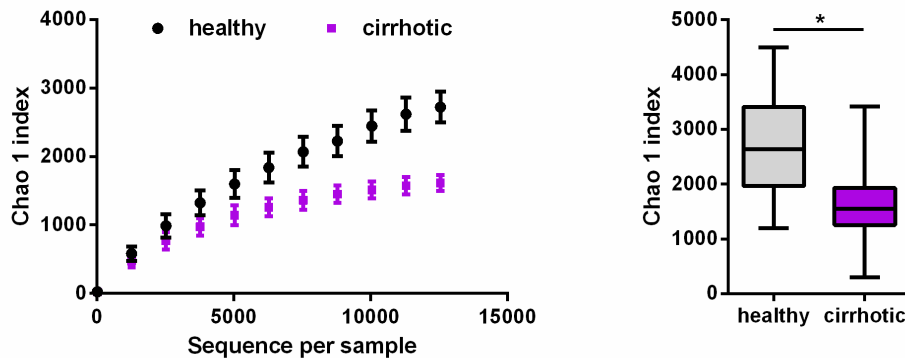
**Figure 7: The effect of probiotic intervention on gut permeability**

Gut permeability panel to assess the barrier damage in cirrhotic patients and the effects of the probiotic intervention on these markers. A Lactulose-mannitol ratio obtained from the differential sugar absorption test; B Mannitol recovery (differential sugar absorption test); C Sucrose recovery (differential sugar absorption test); D Diamine oxidase levels in serum; E Calprotectin levels in stool; F Zonulin levels in stool; G Endotoxin concentration in serum; H Plasma sCD14 concentrations; I Lipopolysaccharide binding protein levels in plasma; \* in circles indicate significant differences compared to healthy controls; \* significant differences between groups indicated by horizontal bars; (110)

## Effects of probiotic intervention on microbiome composition

### Baseline assessment

The microbiome of liver cirrhotic patients was substantially different from healthy controls. Alpha diversity was significantly reduced in patients compared to controls (chao1 index:  $1613 \pm 570$  and  $2723 \pm 890$ , respectively,  $p=0.001$ ). Details are depicted in Figure 8.



**Figure 8: Alpha rarefaction and diversity**

Alpha diversity of the gut microbiome of cirrhotic patients and healthy controls shown in a rarefaction plot (left) and as direct comparison drawn from 12552 reads per sample (right).

On the phylum level, no significant differences could be observed except for a higher abundance of unclassified bacteria in healthy controls ( $p=0.001$ ). Firmicutes to Bacteroidetes ratio was higher in cirrhotic patients compared to healthy controls; however, this observation did not reach statistical significance. Principal component analysis did not reveal specific clustering between groups, indicating a relatively similar microflora.

On the class level, cirrhotic patients had a significantly higher abundance of Bacilli ( $p=0.000$ ), and significantly lower abundance on another unclassified Firmicutes class ( $p=0.000$ ). The Verrucomicrobia class, Opitutae, showed significantly reduced copies in cirrhotic patients compared to healthy controls ( $p=0.001$ ); however, the abundance of this class was also minimal in healthy samples and clinical significance is questionable. A significantly higher abundance of a not further classified Proteobacteria ( $p=0.049$ ) and a Bacteroidetes ( $p=0.021$ ) as well as a decreased abundance of a not further identifiable Bacteria ( $p=0.006$ ) was evident in cirrhotic patients compared to controls.

On the order level, significantly higher abundance of Lactobacillales ( $p=0.000$ ), Actinomycetales ( $p=0.016$ ), and an unclassified Bacteroidetes ( $p=0.025$ ) were found in cirrhotic patients compared to controls. On the other hand, a significantly lower abundance of Puniceicoccales ( $p=0.005$ ), and

unclassified orders of Clostridia (p=0.000), Firmicutes (p=0.001), Actinobacteria (p=0.007) and Bacteria (p=0.007) were found in cirrhotic patients compared to controls.

On the family level, cirrhotic patients had significantly more Lactobacillaceae (p=0.001), Micrococcaceae (p=0.001), Streptococcaceae (p=0.001) and an unclassified family of Bacteroidetes (p=0.032) than healthy controls. Clostridiaceae 1 (p=0.000), Peptostreptococcaceae (p=0.001), Puniceicoccaceae (p=0.005), Clostridiales Incertae Sedis XIII (p=0.017), Oxalobacteraceae (p=0.026), and unclassified families of Clostridia (p=0.000), Clostridiales (p=0.003), Firmicutes (p=0.001), Actinobacteria (p=0.008) and Bacteria (p=0.009) were less abundant in patients compared to healthy controls.

On the genus level, cirrhotic patients significantly differed in 26 strains from healthy controls. More abundant in cirrhosis patients than in healthy controls were the genera *Veillonella*, *Blautia*, *Lactobacillus*, *Streptococcus*, *Rothia*, an unclassified genus of Peptostreptococcaceae and Bacteroidetes. Less abundant in patients were the genera *Clostridium sensu stricto*, *Clostridium XI*, *Turicibacter*, *Anaerofilum*, *Eubacterium*, *Mitsuokella*, *Coraliamargarita*, *Coprococcus*, *Finegoldia*, *Oxalobacter*, *Porphyromonas*, and unclassified genera of Clostridia, Clostridiaceae 1, Clostridiales, Clostridiales Incertae Sedis XIII, Firmicutes, Actinobacteria, Ruminococcaceae and Bacteria. For details see Table 8.

**Table 8: Changes on genus level of the microbiome due to cirrhosis**

Differentially abundant genera between cirrhotics and healthy controls; Abundance data (normalized to 12552 reads) are given as median (Q1; Q3).

Genus	Cirrhosis (n=87)	Controls (n=29)	p-value
<b>Veillonella</b>	9 (2; 85)	1 (0; 3)	0.001
<b>Blautia</b>	538 (260; 816)	224 (139; 357)	0.001
<b>Lactobacillus</b>	10 (2; 81.5)	1 (0; 7)	0.001
<b>Streptococcus</b>	82 (15; 333.5)	18 (5; 35)	0.002
<b>Rothia</b>	1 (0; 5.5)	0 (0; 1)	0.002
<b>Peptostreptococcaceae (unclassified)</b>	0 (0; 1)	1 (0; 4)	0.006
<b>Bacteroidetes (unclassified)</b>	0 (0; 2.5)	3 (0; 11)	0.047
<b>Clostridium sensu stricto</b>	4 (0; 27)	84 (15; 209)	0.001
<b>Clostridium XI</b>	20 (4; 80.5)	145 (40; 317)	0.001
<b>Turicibacter</b>	0 (0; 1)	6 (0; 26)	0.001
<b>Anaerofilum</b>	0 (0; 0)	0 (0; 0)	0.002
<b>Eubacterium</b>	0 (0; 0)	0 (0; 1)	0.002
<b>Mitsuokella</b>	0 (0; 0)	0 (0; 0)	0.003
<b>Coraliamargarita</b>	0 (0; 0)	0 (0; 0)	0.007

Genus	Cirrhosis (n=87)	Controls (n=29)	p-value
<b>Coprococcus</b>	10 (0; 42.5)	75 (17; 146)	0.008
<b>Finegoldia</b>	0 (0; 0)	0 (0; 0)	0.020
<b>Oxalobacter</b>	0 (0; 0)	0 (0; 8)	0.038
<b>Porphyromonas</b>	0 (0; 0)	0 (0; 0)	0.047
<b>Clostridia (unclassified)</b>	0 (0; 0.5)	2 (0; 6)	0.000
<b>Clostridiaceae 1 (unclassified)</b>	0 (0; 0)	1 (0; 4)	0.000
<b>Clostridiales (unclassified)</b>	95 (47; 249.5)	251 (170; 439)	0.004
<b>Clostridiales Incertae Sedis XIII (unclassified)</b>	0 (0; 2)	4 (1; 9)	0.000
<b>Firmicutes (unclassified)</b>	13 (5; 37.5)	81 (24; 174)	0.001
<b>Actinobacteria (unclassified)</b>	0 (0; 0)	0 (0; 0)	0.011
<b>Ruminococcaceae (unclassified)</b>	528 (257; 970.5)	962 (604; 1565)	0.027
<b>Bacteria (unclassified)</b>	112 (31; 257)	301 (119; 1225)	0.013

The 16S sequencing approach is not specific enough to classify bacteria according to their species. However, bacteria can be grouped within their own genus even though reliable identification is not possible. On this quasi-species level, cirrhotic patients showed a 7-fold higher abundance in a *Veillonella* strain (p=0.014) when compared to healthy controls (*Veillonella* are anaerobic, lactate fermenting, Gram negative opportunistic pathogens most abundant in the oral cavity.). A significantly lower abundance was found in the following: two strains of the genus *Clostridium XI*, one of *Clostridium sensu stricto*, one of *Coprococcus*, three different *Oscillibacter*, unclassified Lachnospiraceae, two unclassified Ruminococcaceae, unclassified Peptostreptococcaceae, three unclassified stains of Clostridiales, a no further classified Firmicutes and two bacteria that could only be identified to be a member of the kingdom bacteria. No specific strain of *Lactobacillus* or *Akkermansia* showed significant differences between the groups. For details see Table 9.

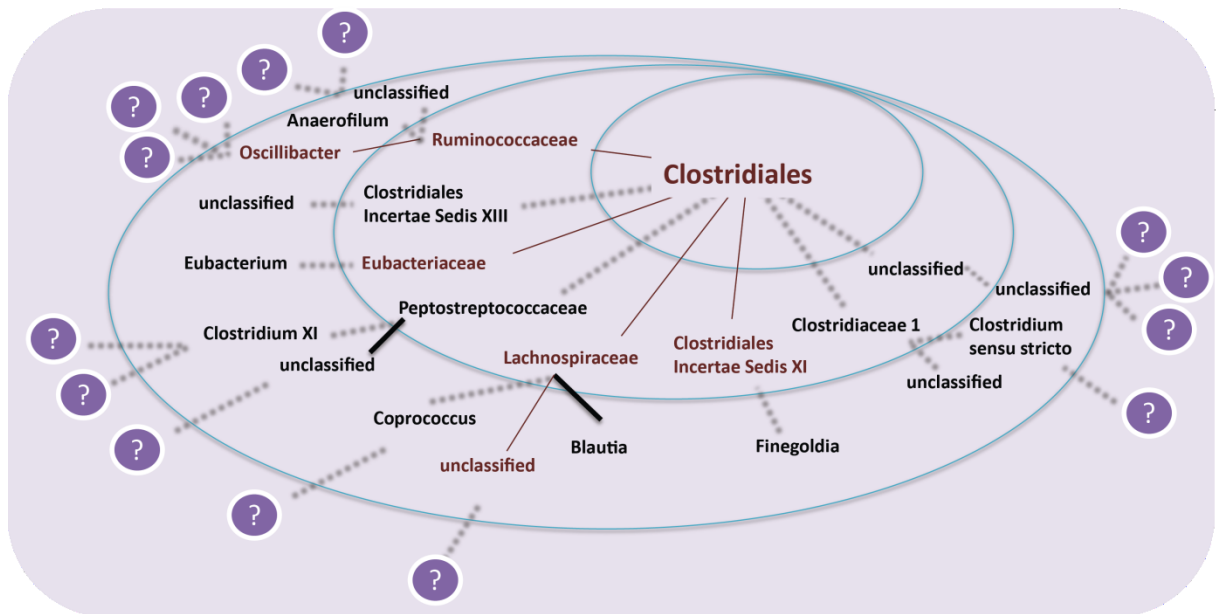
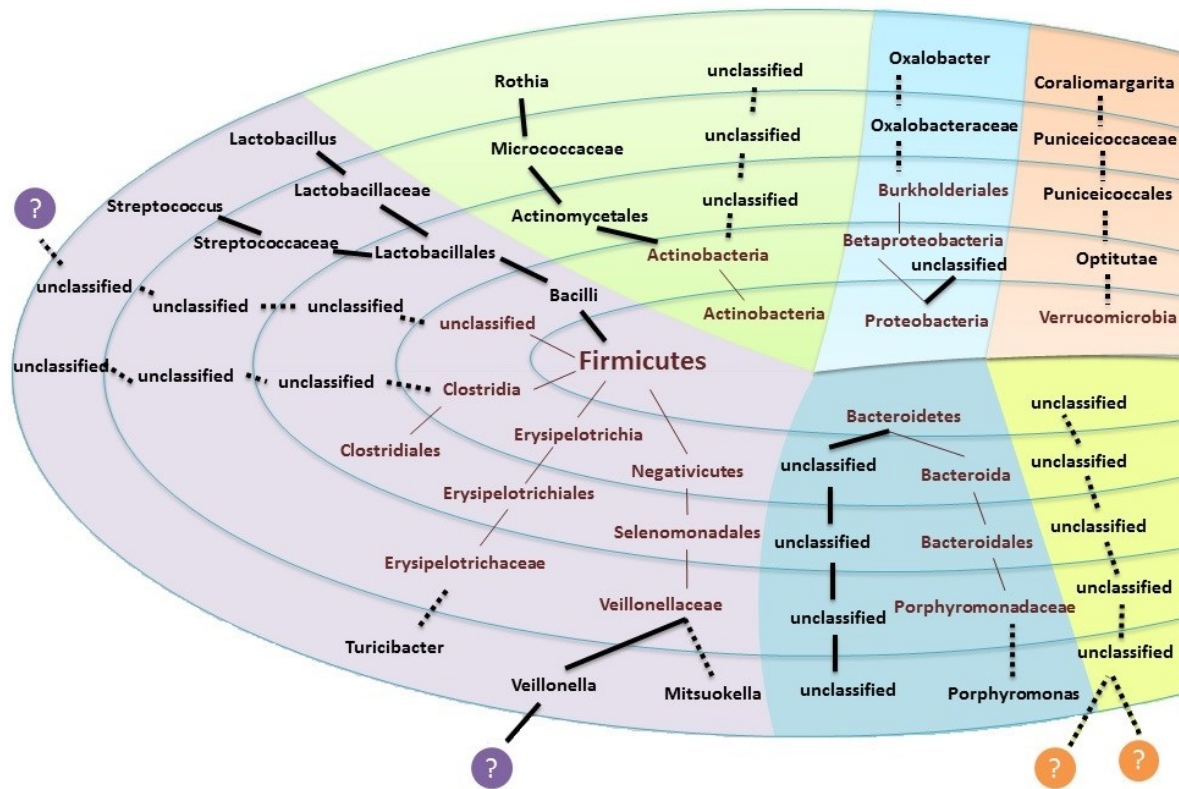
**Table 9: Changes on quasi-species level of the microbiome due to cirrhosis**

Differences in microbial bacteria between cirrhotic patients and healthy control on quasi-species level; Abundance data (normalized to 12552 reads) are given as median (Q1; Q3).

Bacteria	Cirrhosis (n=87)	Controls (n=29)	p-value
<b>Veillonella</b>	7 (2; 76)	1 (0; 2)	0.014
<b>Bacteria (I) (unclassified)</b>	0 (0; 0)	0 (0; 24.5)	0.020
<b>Bacteria (II) (unclassified)</b>	0 (0; 0)	0 (0; 9)	0.020

<b>Bacteria</b>	<b>Cirrhosis (n=87)</b>	<b>Controls (n=29)</b>	<b>p-value</b>
<b>Clostridiales (I) (unclassified)</b>	0 (0; 0)	0 (0; 22.5)	0.029
<b>Clostridiales (II) (unclassified)</b>	0 (0; 0)	1 (0; 6.5)	0.029
<b>Clostridiales (III) (unclassified)</b>	0 (0; 0)	0 (0; 2.5)	0.007
<b>Clostridium sensu stricto</b>	0 (0; 2)	14 (1; 105.5)	0.007
<b>Clostridium XI (I)</b>	1 (0; 17)	86 (16.5; 210)	0.003
<b>Clostridium XI (II)</b>	0 (0; 0)	2 (0; 4)	0.011
<b>Coprococcus</b>	0 (0; 0)	1 (0; 86.5)	0.007
<b>Firmicutes (unclassified)</b>	0 (0; 0)	0 (0; 6.5)	0.016
<b>Lachnospiraceae (unclassified)</b>	3 (0; 30)	40 (12; 178)	0.031
<b>Oscillibacter (I)</b>	0 (0; 1)	7 (0; 12)	0.030
<b>Oscillibacter (II)</b>	0 (0; 0)	0 (0; 3)	0.031
<b>Oscillibacter (III)</b>	0 (0; 0)	2 (0; 4)	0.003
<b>Peptostreptococcaceae (unclassified)</b>	0 (0; 0)	0 (0; 1)	0.047
<b>Ruminococcaceae (I) (unclassified)</b>	0 (0; 0)	0 (0; 8)	0.029
<b>Ruminococcaceae (II) (unclassified)</b>	0 (0; 0)	0 (0; 0.5)	0.046

Interestingly, nearly all differentially abundant species were Firmicutes (16/18) with most of the changes targeting the class of Clostridia. The remaining two species could not be identified beyond bacteria. These species are all reduced in cirrhotic patients with the notable exception of *Veillonella*. All differences are summarized in Figure 9.



**Figure 9: Summary of statistically significant changes in the microbiome of cirrhotic patients compared to healthy controls**

Only differentially abundant taxa are represented. Significant changes are indicated with bold black print, unchanged parent taxa are printed in dark red. An increase of a specific taxon is indicated by a continuous black line, a decrease with a dotted grey line. Elliptic intersections represent the taxonomic ranks starting with phylum in the upper graph and order in the lower graph. Differentially abundant unidentified species are illustrated by circled question marks coloured according to their phylogenetic affiliation.

Although Patients with Child's B/C cirrhosis showed less diversity within their microbiome, differences in Chao 1 index did not reach statistical significance. Beta diversity analysis on phylum level revealed that patients with Child's B/C cirrhosis showed a higher abundance of Fusobacteria compared to Child's A patients and healthy controls. On the other hand, the abundance of unclassified bacteria significantly decreased with the severity of the disease. Firmicutes to Bacteroidetes ratio increased markedly in Child's B/C patients, however not significantly. On the class level Deltaproteobacteria ( $p=0.048$ ) and an unclassified Bacteria class ( $p=0.012$ ) were significantly less abundant in Child's B/C patients than in Child's A. On the order level a no further identified Bacteria order showed significant decrease from Child's A to B/C ( $p=0.023$ ). There were no significant differences on the family level. On the genus level, compared to Child's A, Child's B/C showed significantly higher abundance in two Veillonellaceae genera, *Veillonella* ( $p=0.042$ ) and *Anaeroglobus* ( $p=0.042$ ); lower abundance was found of *Pseudoflavonifractor* ( $p=0.015$ ), *Parabacteroides* ( $p=0.042$ ) and an unclassified Bacteria genus ( $p=0.042$ ). The *Veillonella* strain identified on the quasi-species level that was significantly higher abundant in cirrhotics compared to healthy controls (see above) was more abundant in Child's B/C patients compared to Child's A patients (79 vs 5 respectively). The alteration did not reach statistical significance.

In regards to the aetiology of liver disease, no differences in alpha diversity could be detected. Fusobacteria were significantly more abundant in alcoholic cirrhosis patients than in "other" types. Hepatitis C infected patients had similar levels than alcoholic cirrhotics, but no significant differences could be detected compared to "others". Verrucomicrobia were least abundant in alcoholic patients and most abundant in "other" types of cirrhosis. Significant differences were found between these two groups. HCV patients did not show significant differences to either group. Firmicutes to Bacteroidetes ratio is highest in alcoholic patients, however this lacked statistical significance. There were no statistically significant differences on the class, order, family, or genus level. The *Veillonella* species of interest (see above) is most abundant in alcoholic patients, less abundant in HCV patients, and least abundant in patients with "other" types of cirrhosis. Differences were not statistically significant.

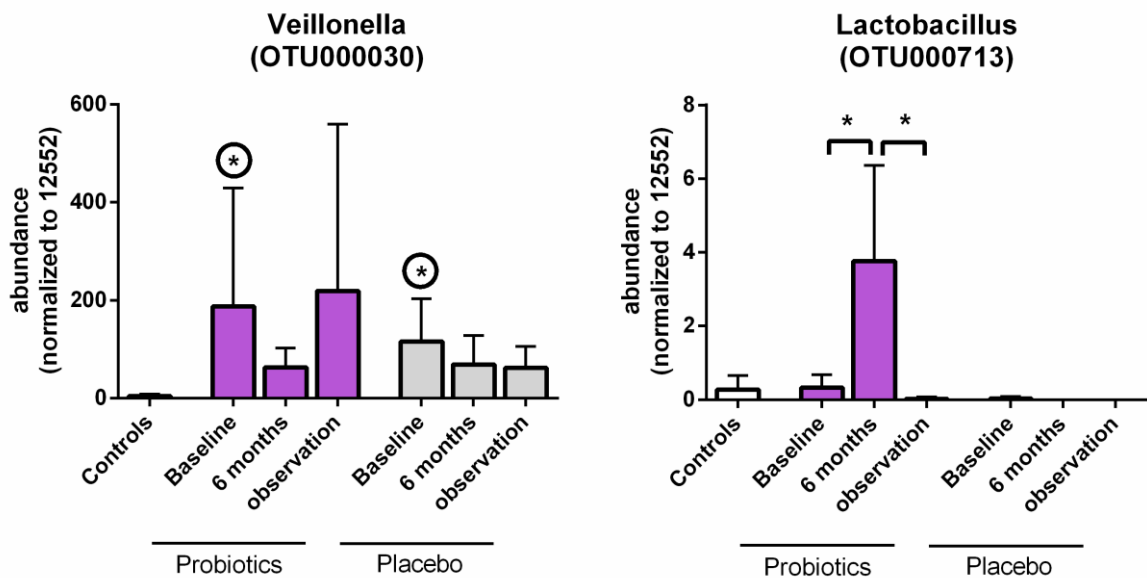
### ***Effects of probiotics on dysbiosis***

At baseline there was no difference between the probiotic and placebo groups in terms of alpha diversity ( $p=0.414$ ) or abundance of single operational taxonomic units (OTU). Probiotic intervention did not alter alpha diversity. Based on the main findings of the comparison between cirrhotic patients with healthy controls, we formed the a-priori hypothesis that the administration of the multispecies probiotic (described above) might abolish the invasion of the intestine by *Veillonella* (OTU000030). We found that after six months of probiotics patients showed a 43%

reduction of *Veillonella*, while the abundance stayed relatively stable in the placebo group. However, the reduction did not reach statistical significance and the abundance of this strain returned to baseline values six months after the intervention had stopped (Figure 10).

In a secondary unbiased approach to compare the microbiome between interventions, groups did not differ from each other before or after six months of probiotic intervention on phylum, class, order, family, or genus level. One OTU classified as *Lactobacillus* was significantly more abundant after treatment in the probiotic group than in the placebo group (3.76 vs 0, respectively,  $p=0.041$ ). The abundance ceased six months after the intervention had stopped (Figure 10). The sheer abundance of these two species did not correlate with each other ( $R^2=0.0$ ;  $p=0.988$ ).

Methodological details are given in the “Method glossary” under “Total DNA isolation, 16S library preparation and sequencing” and “Analysis of 16S sequencing data”.



**Figure 10: Abundance of *Veillonella* and *Lactobacillus* during probiotic intervention**

Abundance of *Veillonella* species (left) and *Lactobacillus* species (right) during the intervention with the probiotics versus placebo. \* significant difference compared to controls or as indicated

### Effects of probiotics on cytokine levels and production

Cytokines were measured in plasma (unstimulated) and after ex vivo stimulation of whole blood with 0.5µg endotoxin for four hours to investigate the inflammatory reaction of leucocytes. Measurements were done for every timepoint. Tumor necrosis factor (TNF)  $\alpha$  could not be detected in 84% of plasma samples in the probiotic group, 69% in the placebo group, and in 65% of healthy control samples. There were no significant differences between groups or over time (data not shown). When stimulated with endotoxin, both patient groups produced significantly less TNF $\alpha$

than healthy controls. In the probiotic group TNF $\alpha$  expression increased over time but fell short of significance ( $p=0.060$ ).

Interleukin (IL) 1 $\beta$  in plasma could not be detected in 59% of patients in the probiotic group, 53% of patients in the placebo group, and 76% of healthy controls. There were no significant differences between groups and over time. After stimulation IL1 $\beta$  was increasingly expressed in the probiotic group after six months of intervention compared to baseline and decreased after six months of observation, but this elevation also fell short of statistical significance ( $p=0.064$ ).

IL6 was not present in plasma of 75% of patients in the probiotic group, 69% of patients in the placebo group, and 80% of healthy controls. IL6 expression increased over time in both groups albeit non-significantly. At baseline, expression of both groups was comparable to healthy controls; however, after three months of intervention and six months of observation, the probiotic group showed significantly more IL6 than healthy controls ( $p=0.007$  and  $p=0.036$  respectively). After endotoxin stimulation IL6 production in the probiotic group increased significantly from baseline to the end of observation ( $p=0.022$ ). However, similar changes occurred in the placebo group and increased values were still comparable to healthy controls.

IL8 levels in the plasma were significantly higher in both groups compared to healthy controls ( $p<0.001$ ) throughout the study. There were no significant changes over time. When stimulated with endotoxin IL8 expression was comparable between all groups and over time.

Interferon (IFN)  $\alpha$  was not detectable in 50% of patients in the probiotic group, 47% of patients in the placebo group, and in 61% of healthy controls. Plasma levels were comparable between groups and over time. The same was true for IFN  $\beta$  with 32%, 31%, and 37% negative samples respectively. IFN  $\alpha$  and IFN  $\beta$  were not assessed after stimulation.

IFN  $\gamma$  was detectable in almost all samples. There were no differences in plasma levels between groups or over time. After six months of intervention with probiotics IFN  $\gamma$  expression doubled compared to baseline when stimulated with endotoxin and decreased at the end of observation ( $p=0.058$ ). Values were still comparable to healthy controls. Changes in the placebo group were much smaller.

IL10 levels were significantly higher in the plasma of patients in the probiotic group than in healthy controls ( $p=0.028$ ). Although there was no significant change detected in paired testing, significant differences between those two groups were lost after three months of probiotic intervention. The placebo group did not show alterations over time. After stimulation with endotoxin it became apparent that IL10 expression decreased gradually over the course of the study (significantly in the probiotic group ( $p=0.036$ ) and non-significantly in the placebo group). IL10 levels were

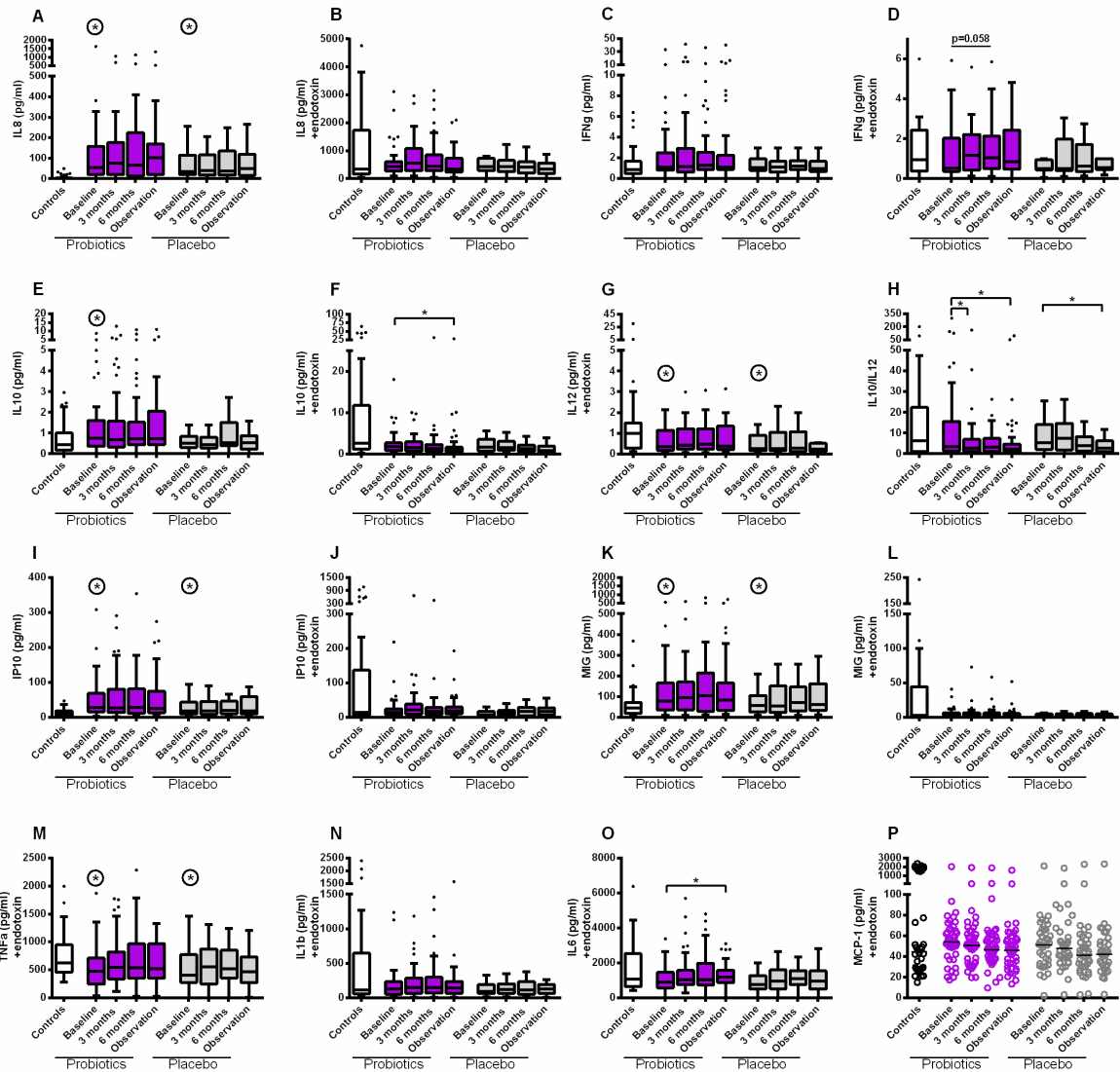
significantly lower than in healthy controls after three months until the end of the study in the probiotic group and after six months of intervention until the end of the study in the placebo group.

IL12 after stimulation with endotoxin was significantly less expressed in the placebo group compared to healthy controls ( $p=0.012$ ) throughout the study. There were no significant differences between test groups or over time. IL10-IL12 ratio after endotoxin stimulation decreased significantly in both groups over the course of the study ( $p=0.002$  and  $p=0.048$  for probiotic and placebo group respectively). In the probiotic group, there was an additional decrease after three months of intervention ( $p=0.040$ ).

Interferon gamma-induced protein (IP) 10 concentration was significantly higher in the plasma of both test groups compared to healthy controls ( $p<0.001$ ). Levels remained stable over time. After stimulation with endotoxin, patients and controls expressed comparable amounts of IP 10. There were no alterations over time.

Monokine induced by Gamma-Interferon (MIG) was significantly higher in the probiotic group than in healthy controls ( $p=0.017$ ). There were no significant differences over time. After endotoxin stimulation, differences were abolished and MIG expression stayed constant over time. No differences were detected in Monocyte Chemoattractant Protein (MCP)-1 after stimulation between groups or over time. Details are given in Figure 11 with the exception of  $TNF\alpha$ ,  $IL1\beta$ ,  $IL6$ ,  $IFN\alpha$  and  $IFN\beta$  plasma levels because of their high occurrence of negative samples.

Methods are described in the “Method glossary” under “Cytokines”.



**Figure 11: Cytokine and chemokine concentrations in plasma and after stimulation**

Cytokine and chemokine concentrations in plasma and after stimulation with endotoxin (+endotoxin) before (baseline), during (3 months) and after intervention (6 months) as well as after 6 months of follow up (observation). Healthy controls are given as reference. \* in circles indicate significant differences compared to healthy controls; \* significant differences between groups indicated by horizontal bars;

While cytokine concentrations in plasma were usually higher in cirrhotic patients compared to controls, a lower expression of cytokines by leucocytes after endotoxin stimulation were observable in patients compared to controls. This suggests that high concentrations of cytokines in cirrhosis originate from the liver rather than activated leucocytes.

### Effects of probiotics on quality of life

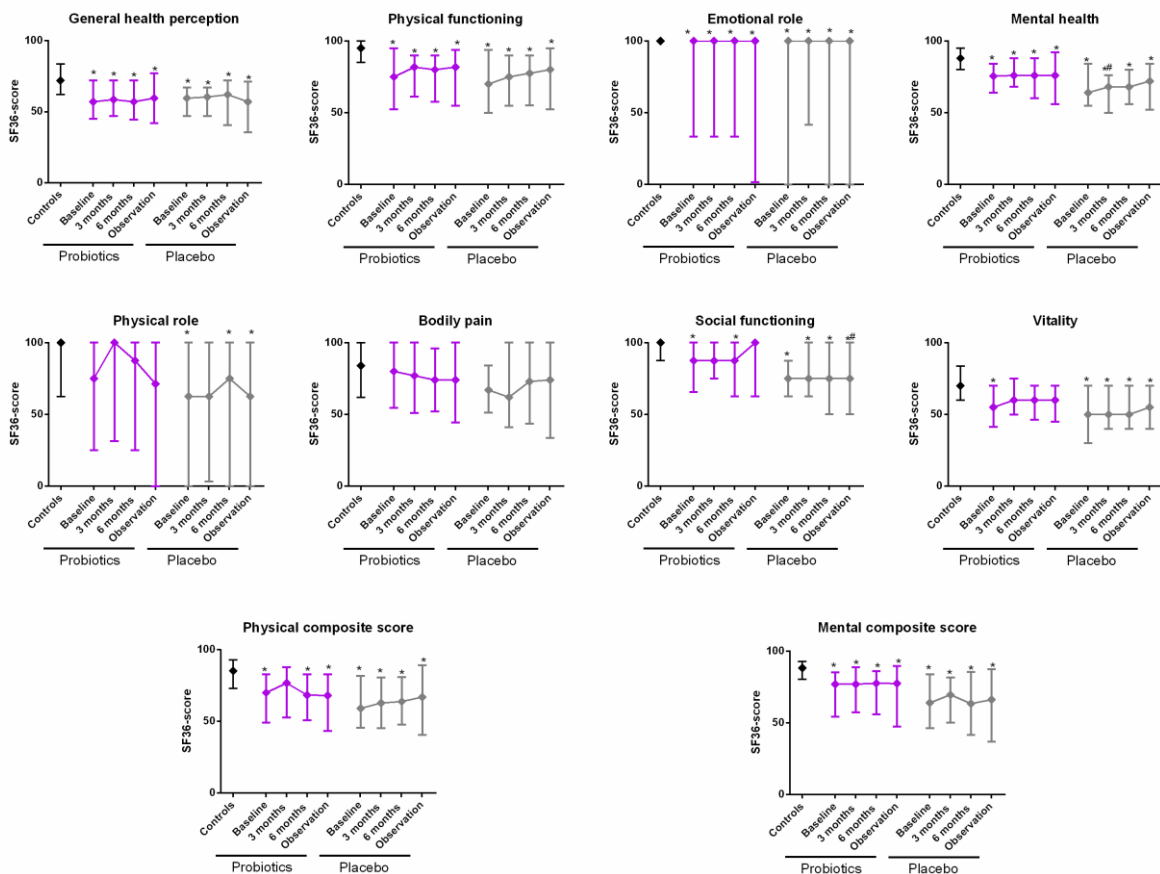
One of the most surprising findings was the exceptionally low dropout rate in the probiotic group. In order to clarify whether probiotics could have had an effect on the compliance of study patients,

quality of life and monoamine synthesis were examined. Quality of life as assessed by short form (SF) 36 questionnaire that consists of eight items and two composite scores. Physical and mental components are taken into consideration. SF36 items reflect the degree of impact that a certain health-related restriction has on quality of life. All items have possible values between 0 and 100 while higher values always reflect better quality of life. Items are explained in Table 10.

**Table 10: Description of SF36 items(113)**

<b>Item</b>	<b>Explanation</b>
<b>physical functioning</b>	describes to which extent health restrictions interfere with physical activities such as self-sufficiency, walking, taking stairs, bending down, picking something up, moderate or straining activities
<b>physical role</b>	describes to which extent physical health impairs work or other daily activities; for example achieve less than usual, restrictions in the type of activities possible or inability to perform certain activities
<b>bodily pain</b>	describes the extent of pain and the extent to which pain restricts normal work inside and outside of home
<b>general health perception</b>	describes the personal perception of health including present health status, future expectations of health and resistance against diseases
<b>vitality</b>	describes the ratio of feeling energetic and motivated to feeling tired and exhausted
<b>social functioning</b>	describes to which extent physical health and emotional problems restrict normal social interactions
<b>emotional role</b>	describes to which extent emotional problems restrict work or other daily activities including spending less time, achieving less than usual or being less careful than usual
<b>mental health</b>	describes the general mental health including depression, anxiety, emotional and behavioural control, general positive mind set

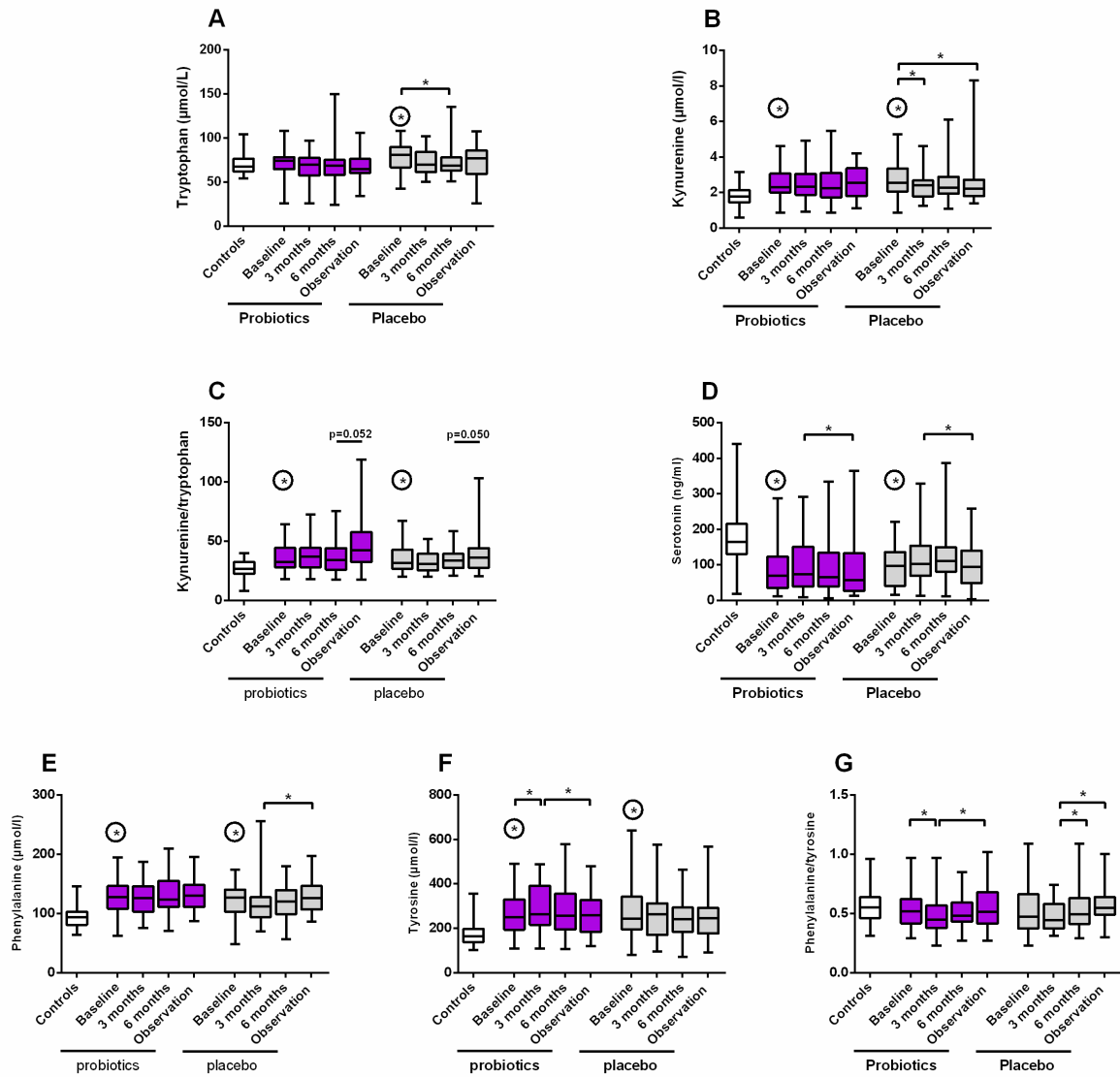
Physical role improved after three months of probiotic intervention to a degree that more than half of the patients achieved a perfect score. Because of the wide deviation statistical significance could not be reached. Similar changes were not observed in the placebo group. The physical composite score reflected these changes in a slight increase after three months of intervention with the probiotic but not with the placebo. Interestingly, social functioning increased after the intervention so that at the observation time point, more than half achieved perfect scores and the probiotic group had significantly higher scores than the placebo group. Vitality was significantly lower in patients than in healthy controls. In the probiotic group it increased slightly so that a significant difference between the patients and healthy controls was lost after three months of intervention and onwards. Physical functioning, general health perception, emotional role, mental health as well as mental composite score were significantly reduced in cirrhotic patients compared to controls and did not change during intervention in either group. Bodily pain had no significant influence on quality of life in cirrhotic patients. Details are illustrated in Figure 12.



**Figure 12: The effects of probiotic intervention on quality of life**

Quality of life scores before (baseline), during (3 months) and after intervention (6 months) as well as after 6 months of follow up (observation). Healthy controls are given as reference. \* indicate significant differences compared to healthy controls; # indicate significant differences between test groups; data are given as median and interquartile range;

Patients showed a shift in tryptophan and phenylalanine metabolism typical for low-grade inflammation. Serum levels of tryptophan were significantly higher in the placebo group compared to healthy controls at baseline ( $p=0.004$ ) and decreased significantly in the first six months to concentrations comparable to healthy values ( $p=0.005$ ). Kynurenine was significantly higher in both test groups compared to healthy controls ( $p<0.001$ ). In the placebo group, kynurenine decreased minimally but significantly after three months of intervention and six months of follow up ( $p=0.013$  and  $p=0.025$  respectively). However, actual changes were minimal so that biological significance is doubtful. Concentrations remained constant in the probiotic group. Kynurenine to tryptophan ratio was significantly increased in both test groups compared to healthy controls ( $p<0.001$ ). No significant changes over time could be found although there was a strong trend to a higher ratio at the observation time point compared to six months of intervention in the probiotic group ( $p=0.052$ ) and in the placebo group ( $p=0.050$ ). Accordingly, already low serotonin levels decreased significantly during the study period in both groups ( $p=0.014$  and  $p=0.018$  for probiotic and placebo group respectively). Phenylalanine levels were significantly higher in both patient groups compared to controls ( $p<0.001$ ) and further increased significantly in the placebo groups at the end of observation ( $p=0.010$ ). Tyrosine levels were significantly increased in patients compared to controls ( $p<0.001$ ). After three months of probiotic intervention, tyrosine levels increased significantly ( $p=0.016$ ) and then significantly decreased again until the end of observation ( $p=0.035$ ). Placebo did not show any effect. Phenylalanine to tyrosine ratio was significantly reduced by probiotics after three months of intervention ( $p=0.009$ ) and increased again at the end of observation ( $p=0.002$ ). In the placebo group, phenylalanine to tyrosine ratio significantly increased at the end of treatment ( $p=0.010$ ) and at the end of observation ( $p=0.003$ ) compared to three months of intervention. Details are given in Figure 13. There was no notable correlation between parameters of the tryptophan/phenylalanine metabolism and SF36 scores at baseline or after three months of intervention which is when most of the changes occurred. Technical details can be found in the “Method glossary” under “Parameters measured by collaborators”.



**Figure 13: The effect of probiotics on monoamine synthesis**

Parameters of monoamine synthesis before (baseline), during (3 months) and after intervention (6 months) as well as after 6 months of follow up (observation). Healthy controls are given as reference. \* in circles indicate significant differences compared to healthy controls; \* significant differences between groups indicated by horizontal bars;

## Discussion

Probiotic intervention increased neutrophil resting burst and serum neopterin levels, representing two antimicrobial strategies. It decreased the incidence of mild infections and improved serum killing capacity of alcoholic patients. Probiotics transiently suppressed the abundance of *Veillonella* in the intestine and increased the abundance of *Lactobacillus* at the same time. They improved quality of life and the phenylalanine to tyrosine conversion. During probiotic intervention patients were less likely to deteriorate in liver function and more likely to adhere to the study medication.

Although the primary endpoint (restoration of neutrophil phagocytosis) could not be reached, several other effects of probiotics could be detected. The increase in neutrophil resting burst and the upregulation of neopterin indicate an involvement of probiotics in the antimicrobial strategies of the innate immune system. The trend toward less infections and the improvement of serum killing capacity in alcoholic cirrhotics corroborate this hypothesis. Both resting burst and high neopterin levels have been linked to deranged immune responses and poor outcome; however, the increase found in the present study was well below the values reported in critically ill patients.(63, 114) It is not yet clear what implications low levels of resting burst and serum neopterin might have.

The deterioration of phagocytic capacity was independent of the intervention. To verify the loss of function, *in vitro* experiments were conducted where healthy neutrophils were conditioned with cirrhotic sera for three hours and phagocytic capacity assessed afterwards (data not shown). Dysfunction was partly transmissible onto healthy neutrophils, thus supporting the whole blood findings and the hypothesis that cirrhosis-associated neutrophil dysfunction is serum mediated.(63) Endotoxin has been suggested as a causative factor; however, this claim could not be verified in the present study. Therefore, the determining factor for neutrophil dysfunction is still unknown. With the deterioration of neutrophil phagocytosis more monocytes show phagocytic activity (a possible compensating mechanism). Phagocytic capacity of monocytes increased, although not significantly, after three months of probiotics. Since monocytes and macrophages are from the same myeloid lineage, the rise in neopterin should be examined as a possible activator in future studies.

The production of reactive oxygen species by neutrophils might also be triggered by neopterin(112), although no correlation could be found in the present study. Also, the mechanism by which neopterin is upregulated in this study cohort is unclear. It has been described that activated macrophages produce neopterin in response to IFN $\gamma$ .(115) This association could not be verified in the present study since neopterin and IFN $\gamma$  (in plasma or stimulated *ex vivo*) did not show any significant correlations. Also, the activation of macrophages could not be supported by the long-term activation marker, sCD163. A previous report has linked high neopterin levels with increased oxidative stress (increased levels of AOPPs).(116) Although we found elevated neopterin levels and increased ROS production, AOPP levels did not show significant elevation and even tended to decrease marginally during probiotic intervention. Active infections as a cause for the antimicrobial response have been excluded by examining acute phase proteins and other antimicrobial molecules. Furthermore, body temperature, respiratory rate and heart rate were within normal limits. Therefore, probiotics influenced innate immune responses without causing additional macrophage activation or oxidative stress.

As described before a possible target for probiotics is dysbiosis in the gut microbiome. Several significant changes in the microflora of cirrhotic patients compared to healthy controls have been found; most noteworthy being the high abundance of *Veillonella* in the stool of cirrhotic patients that could not be found in healthy controls. The invasion of the intestine by bacteria from the oral cavity is a known feature in cirrhosis.(7) Probiotic intervention tended to repress this *Veillonella* invasion during the time of intervention. The ability of probiotics to inhibit the growth of pathogens has been described before(102) and the significant proliferation of a *Lactobacillus* species in our patients supports this claim. All changes brought on by the probiotics were transient and vanished after the treatment had been discontinued. All taxa very robustly returned to baseline values. This observation and rather similar reports from different cohorts(7, 8) suggest that cirrhosis itself moulds the habitat for gut commensals in a very specific way. Also, since aetiology played only a minor role in the characterization of the microbiome, it seems to be cirrhosis itself rather than its underlying cause that shapes the microflora. Interestingly, this specific habitat does not seem to be particularly nurturing for Clostridiales. This is especially troubling because the reduction of intestinal Clostridiales taxa predisposes patients to nosocomial *Clostridium difficile* infections(117) and cirrhotic patients are poorly equipped to deal with *Clostridium* bacteremia.(118)

Certain (unknown) stimuli have been suggested as “tipping elements” that skew the abundance of a taxon in one way or another.(119) It would be interesting to know if the same tipping element that clears the intestine of cirrhotic patients from so many *Clostridiales* taxa also favours the proliferation of *Veillonella* or other xenobionts, or whether the invasion of xenobionts eradicates autochthonous taxa such as *Clostridiales*. Identifying this trigger could drive the development for therapeutic approaches.

Although significant changes in the microbiome were induced by the probiotics, no significant improvement in intestinal permeability could be observed. At baseline, cirrhotic patients showed a significant breach in intestinal barrier function localized in the small intestine (lactulose to mannitol ratio, DAO, Calprotectin). Gastroduodenal permeability was comparable to healthy controls. Probiotic intervention did not show any effects on gut permeability but during the intervention, a stabilization of mannitol recovery could be observed in the probiotic group. Even though this improvement was not reflected in the lactulose to mannitol ratio, it indicates a beneficial effect of the probiotics on the gut epithelium. Zonulin, a marker for epithelial damage, was not increased in the stool, although diamine oxidase (also a marker for enterocyte death) and several other markers for gut permeability were significantly elevated. The loss of epithelial cell mass is common in patients with cirrhosis and might account for the low zonulin concentrations found in the stool of cirrhotic patients. Even though zonulin was not increased, bacterial translocation was apparent in patients. Endotoxin was significantly higher in all patient groups compared to controls. The non-existent effect of the intervention on gut permeability was somehow

surprising given the aforementioned successful preclinical interventions. Probiotic intervention had no influence on endotoxemia in our study, contrary to previous reports.(120) Also, the pro-inflammatory cytokine profiles in plasma could not be ameliorated as suggested in other studies. This could be related to the unchanged conditions of the gut barrier. However, probiotics tended to improve the expression of cytokines IFN $\gamma$  and IL1 $\beta$  in response to endotoxin, which might be another sign of the activating effects of probiotics on leucocytes. Probiotic strains often have different effects, therefore it is possible that another product would show different results.

The study was not powered to detect improvements in liver function and therefore, these changes did not reach statistical significance. However, the reduction of MELD score of one point during the intervention concurs with a previous study on a larger cohort.(99) Hepatic encephalopathy was the main factor for the improvement in composite liver function scores in previous studies;(97, 121) in our study we could not identify a single factor for the improvement of liver function.

Anecdotal data suggest that patients drop out of the study when they felt overwhelmed with their health or personal life or were too weak to continue with the relatively extensive study protocol. Both reasons are more likely when liver function has deteriorated. Interestingly, even though the liver function in the probiotic group was worse than in the placebo group, patients found the motivation to adhere to the study. This phenomenon is reflected in an increase in quality of life and tyrosine, the first step in the production of dopamine, during the intervention. The same combination of probiotics as used in the present study but in a lower dose has shown beneficial effects on cognitive reactivity to sad mood before.(122) Note that probiotics have also been shown to relieve gastrointestinal pain(94) and migraine(123) which could be cofactors in the increase of overall well-being. At the moment, it is not possible to distinguish between the mechanisms by which probiotics increased quality of life but it will be a focus in future studies.

Changes in subjective parameters like quality of life might be subject to a placebo effect. Patients that complained about side effects or praised the product excessively during the intervention were all allocated to the placebo group. To test the robustness of the observed improvements, we asked the patients at the end of the study while they (and we) were still blinded to the allocation in which group they thought they had been. Interestingly, two thirds thought to be in the probiotic group independent of their true allocation. However, their guess regarding allocation did not influence quality of life, neither did their personal opinion on probiotic interventions and whether they should be recommended for cirrhotic patients (data not shown).

Many of the effects that probiotics had on the patients were subtle and transient. Our results suggest that a permanent effect of probiotics can only be achieved by continuous administration. Long-term administration of probiotics is still in need of clinical trials; however, the relatively long exposure in our study does not give reason for concerns. The product was very well tolerated and

side effects were mild and short term. Safety concerns raised before(124) were relativized in recent literature(125) and could also not be supported by the present study.

### **Limitations of the study**

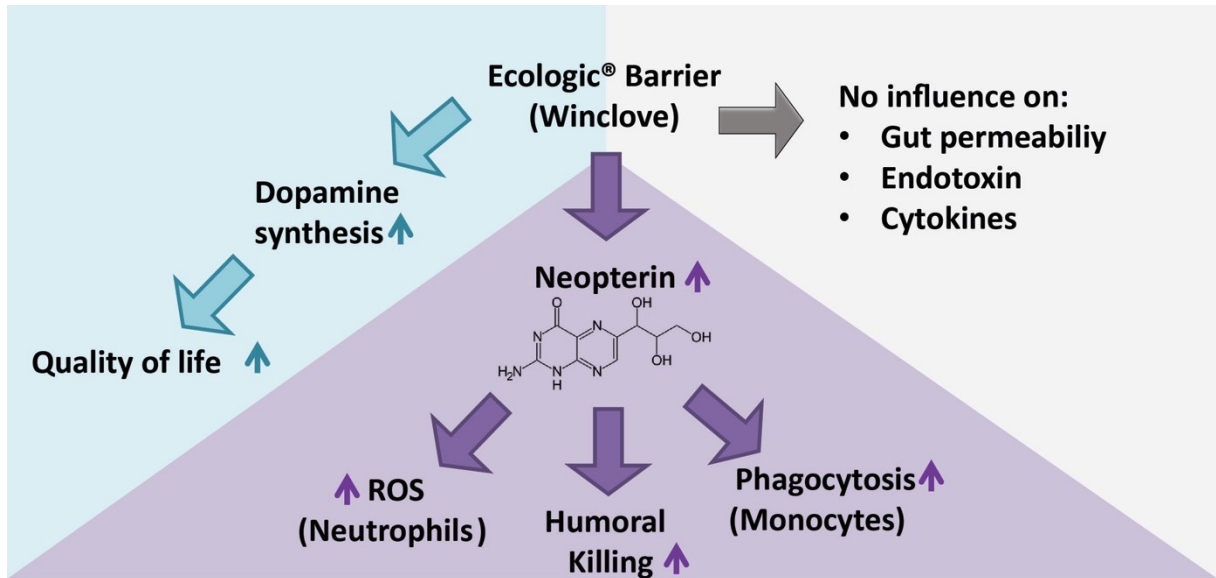
The study was conducted as single centre study; nevertheless, a relatively large number of patients could be recruited. To follow up on some results and to validate the efficacy of the probiotics, some adaptation in the protocol would be helpful. Firstly, stratification should include liver function so that a more representative control group could be established. Secondly, a broad spectrum of dysfunction only became apparent when liver function deteriorated. To better quantify improvements in neutrophil function or gut permeability, future studies should focus on patients with Child-Pugh scores above 6. Also including patients with more severe cirrhosis (Child-Pugh score above 11) could be helpful to verify the ability of cirrhotics to improve liver function and raise compliance. Granted, these patients have a higher mortality risk; however, an improvement in liver function might also increase their survival rate. Also the effect of probiotics on infection rates would best be examined in this patient cohort. Thirdly, differential sugar absorption test should be done in the hospital under strict supervision of a study nurse. Many patients did not have the mental capacity anymore to follow the descriptions at home. Errors made are discussed in “C. Methods in liver disease research” under “Differential sugar absorption test”. And lastly, more in-depth examination of quality of life should be employed including specific questionnaires for liver and gastrointestinal related conditions.

Microbiome abundance data was comprised from 16S DNA sequencing of spontaneous stool samples. Ethically, taking biopsies from cirrhotic patients cannot be endorsed because of the high risk of haemorrhagic complications. Therefore, stool samples represent the most accessible approximation. Although stool samples do not fully reflect the mucosa-associated microbiome, relative changes usually point in the same direction.(126) Another limitation of this method is the restricted resolution that neither allows identification of all observed taxonomic units nor the classification of taxa beyond the genus level. Nevertheless, this method gave us an overview of the changes in the microbiome of cirrhotic patients which can be followed up with more specific approaches.

The assessment of endotoxin in biological samples is always a matter of debate. We decided to use a cell based assay rather than the standard method (limulus amoebocyte lysate assay) because it reflects the level of bioactive endotoxin more accurately. The advantages and possible pitfalls are discussed in “C. Methods in liver disease research” under “Endotoxin”.

## Conclusion

In conclusion, probiotics are a safe and well-tolerated method to activate innate immune responses, partially restore dysbiosis and increase the general well-being of cirrhotic patients. Changes are independent of gut permeability, bacterial translocation or resting cytokine levels.



## **B. Liver cirrhosis ≠ liver cirrhosis**

This segment describes pilot experiments that will serve as preliminary data for follow up studies. During the extensive examination of innate immunity and gut permeability of cirrhotic patients in the study presented above it became apparent that most of the assessed parameters are influenced by liver function and/or aetiology. The three most striking findings were the overcompensating phagocytic capacity of neutrophils, high deviation of neutrophil function in alcoholic cirrhosis and the devastating effect of viral hepatitis C on the innate immune system.

### **Overcompensating for inactive neutrophils**

#### **Background**

During the analysis of the probiotic study gradual changes over time independent of the intervention could be observed. The most prominent deterioration was detectable in neutrophil phagocytic capacity, but also albumin levels, calcium concentration, mannitol recovery, serotonin production and the expression of IL10 deteriorated significantly over time. The expression of IL6, ROS and nitrite production, AOPPs, phenylalanine concentration and phenylalanine to tyrosine ratio gradually increased. Therefore, we tried to characterize those changes over time and determine the influence of liver function on them.

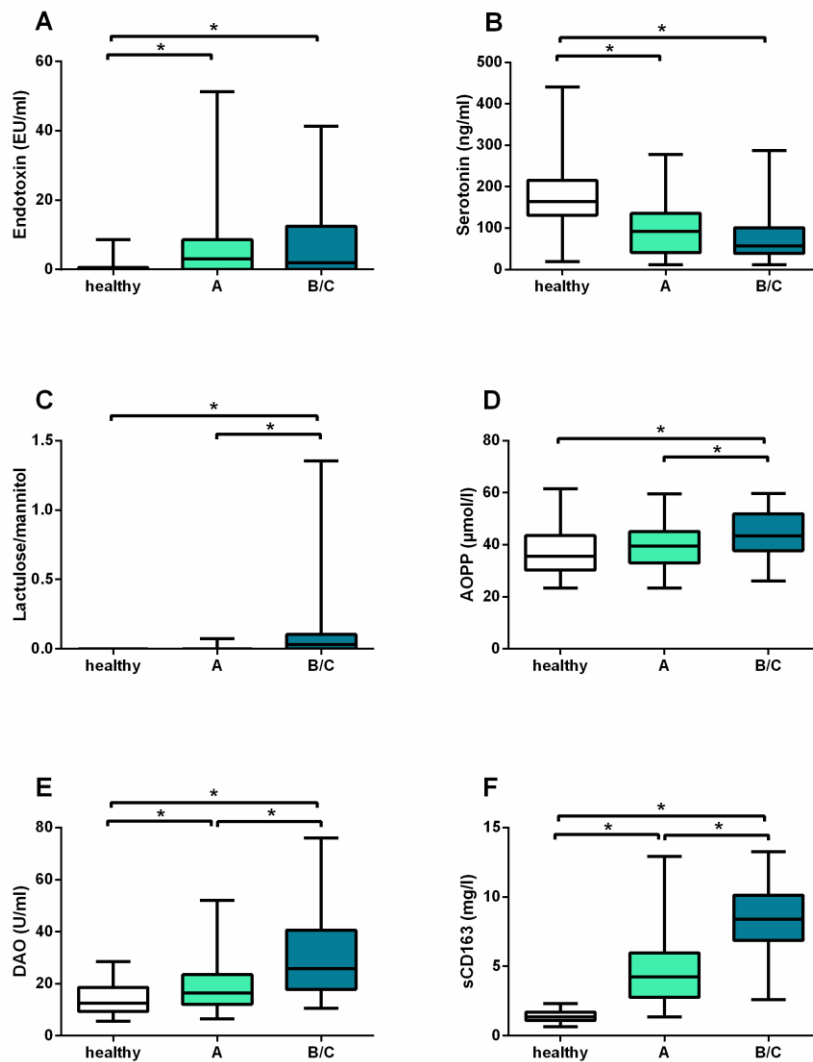
#### **Methods**

Liver cirrhosis is a progressive disease and severity of the disease can be reflected in many parameters. However, severity is usually quantified by Child-Pugh or MELD score. Both are composite scores that summarize key parameters in liver disease. For this assessment Child-Pugh score is used because of its universal usage in the clinics and practical grading system. All assessed parameters from the clinical study presented in “A. Probiotics in cirrhotics” were tested in patients with Child-Pugh score of five or six (n=72) compared to patients with a Child-Pugh score higher than six (n=29) and healthy controls (n=51).

#### **Results**

Most of the assessed parameters showed gradual one-directional alterations. Differences were found between healthy and cirrhotic patients (irrespective of severity) such as the percentage of primed neutrophils (p=0.006), endotoxin (p<0.001), plasma levels of IL8 (p<0.001) and IP10 (p<0.001), stool calprotectin (p=0.008), kynurenine (p<0.001), kynurenine to tryptophan ratio (p<0.001), neopterin (p<0.001), nitrite (p<0.001), phenylalanine (p<0.001), serotonin (p<0.001) or

growth retardation capacity ( $p < 0.001$ ); or the differences only become apparent when liver function has deteriorated such as AOPPs ( $p = 0.004$ ), phenylalanine to tyrosine ratio ( $p < 0.001$ ), mannitol recovery ( $p = 0.018$ ), lactulose recovery ( $p < 0.001$ ), lactulose to mannitol ratio ( $p < 0.001$ ), plasma levels of IL6 ( $p = 0.010$ ) or the overexpression of IL8 after endotoxin production ( $p = 0.003$ ). Also a stepwise increase like in DAO ( $p < 0.001$ ) or sCD163 ( $p < 0.001$ ) was observed. Examples are shown in Figure 14.

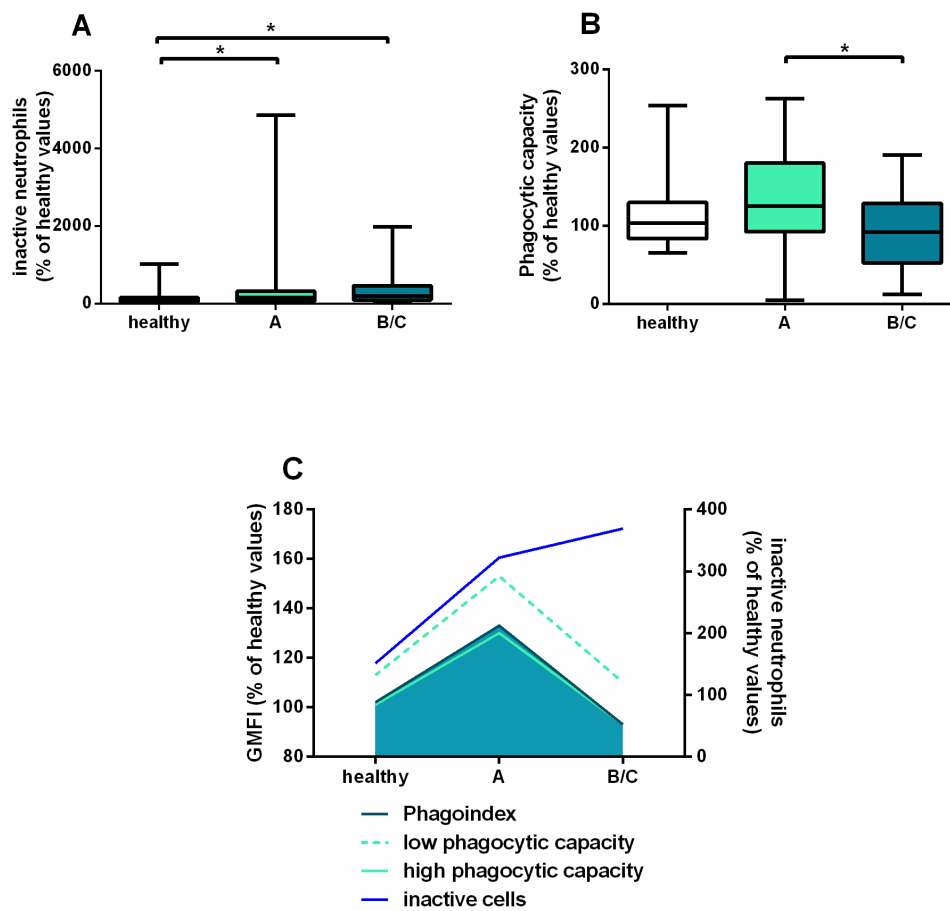


**Figure 14: Examples for one-directional changes in cirrhosis**

Examples for one-directional changes according to liver function. A+B Endotoxin and serotonin are altered in cirrhotic patients compared to controls. C+D Lactulose to mannitol ratio and advanced oxidation protein products are significantly deranged when cirrhosis is decompensated. E+F Diamine oxidase and sCD163 show stepwise increase with deteriorating liver function. \* significant changes as indicated by horizontal bars;

On the contrary to these gradual one-directional changes, compensating alterations can be observed in neutrophil phagocytic capacity. Patients of all Child-Pugh grades exhibit a significant increase in

inactive neutrophils. When cirrhosis is compensated, the decreased number of active cells can be compensated for by a significant increase of the phagocytic capacity of active cells. Low and high phagocytic cell populations alike increase the amount of bacteria they engulf in compensated cirrhosis and therefore boost the overall phagocytic capacity (Phagoindex). When the liver function deteriorates, this compensation is not possible anymore and phagocytic capacity decreases. Illustration is given in Figure 15.



**Figure 15: Compensatory increase in phagocytic capacity of neutrophils to balance the loss of active cells.**

A Inactive neutrophils increase with the onset of cirrhosis. B Phagocytic capacity can be overcompensated in compensated cirrhosis but decreases in more severe liver disease. C Schematic illustration of changes in neutrophils behaviour through different stages of liver cirrhosis: inactive neutrophils steadily rise with decreasing liver function (blue line). In compensated cirrhosis active neutrophils can match this increase with more capacity (turquoise lines). The possible amount of bacteria that can be engulfed (coloured area) is therefore increased in Child's A cirrhosis and drops in Child's B and C cirrhosis. A stands for Child-Pugh grade A; B/C stands for Child-Pugh grades B and C; \* significant changes as indicated by horizontal bars;

## Conclusions

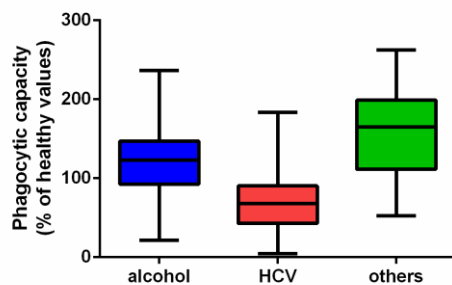
In early stages of liver disease the activation of neutrophils seems to have a beneficial effect on their phagocytic performance although early signs of neutrophil dysfunction – the rise of inactive cells – are already apparent. This overcompensation could account for the relatively low rate of severe infection in our study cohort compared to the literature.(53) It also explains the controversial reports on neutrophil function in liver cirrhosis that do not fully consent to decreased phagocytosis.

Overcompensation can in part be transferred onto healthy neutrophils by serum indicating that it is partly caused by serum components and partly by primed neutrophils. Primed neutrophils were found in our cohort of the clinical study (Figure 4).

## High variation of immune responses in alcoholic cirrhosis

### Background

Patients who suffer from cirrhosis secondary to alcohol show an especially high variation in neutrophil function that encompass all from very high to very low values (see Figure 16).



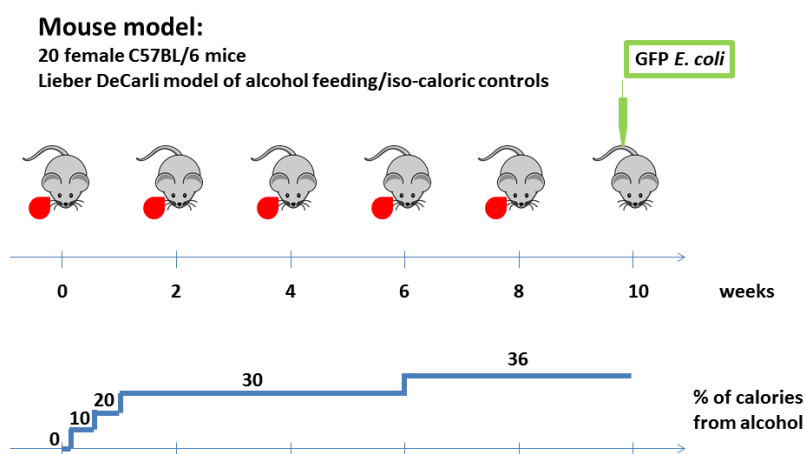
**Figure 16: Phagocytic capacity in different aetiologies of liver cirrhosis**

Based on our data of neutrophil phagocytosis being slightly higher than normal in compensated cirrhotics and deteriorating with liver function (see above) we hypothesise that the influence of alcohol on the innate immune system might be dependent on liver function. Thereby, alcohol should activate the immune system as long as the liver is fully functioning, but loses this property when liver injury becomes apparent. To test this hypothesis we utilized a Lieber-DeCarli mouse model of chronic ethanol feeding and subjected the animals to infectious stimuli, in vivo and ex vivo, to test their immune responses in case of bacterial infection.

## Methods and Materials

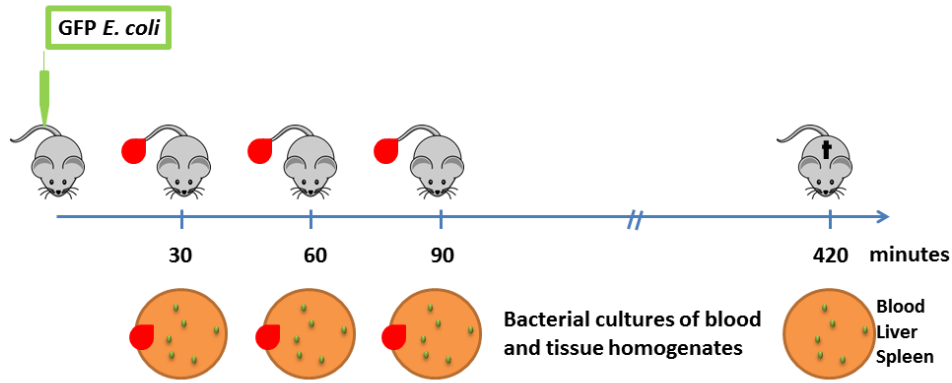
Lieber-DeCarli model of chronic ethanol feeding subjects test mice to a high fat liquid diet while the percentage of calories that come from alcohol increases gradually over ten weeks. The first day was alcohol free to accustom the mice to the liquid diet. The following three days, mice received 10% of their calories from alcohol (or maltodextrin for the control group). For the next three days, 20% of the calories came from alcohol/maltodextrin, and then 30% until the end of week six. For the remaining four weeks, 36% of calories were obtained from alcohol/maltodextrin. This method has been published before and reported to induce gut permeability, pro-inflammatory conditions but only mild alcoholic liver disease (ALD).(127) Alcohol and control diet as well as maltodextrin was purchased from TestDiet (St. Louis, USA); 200 Proof pure ethanol (Koptec, King of Prussia, USA) was used as alcohol source.

In three independent experiments, twenty female C57BL/6 wild type littermates in total with ten mice in each group were tested. Mice were weaned at three weeks of age and cohoused until the beginning of the feeding period. At the age of approximately nine weeks, mice were randomly allocated into two groups and put on a liquid diet with or without alcohol. Mice were caged in pairs and supplied with 20ml of diet per mouse and day. Diet was changed every three days and consummation was documented to ensure comparable calorie intake. During the feeding period, blood was drawn retro-orbitally at baseline and every second week using sterile heparinized glass pipettes (Fischerbrand Fisher Scientific, Pittsburgh, USA). Blood was tested for neutrophil and monocyte phagocytosis of live GFP-expressing bacteria as well as clearance capacity of whole blood. Bleeding became increasingly problematic due to the poor clotting during alcohol feeding. One mouse died from blood loss after six weeks of feeding. Feeding scheme and bleeding schedule are outlined in Figure 17.



**Figure 17: Feeding regime according to Lieber-DeCarli alcohol diet and bleeding time points**

At the end of the feeding period, mice were injected with  $5 \times 10^7$ - $2 \times 10^8$  GFP-expressing, chloramphenicol-resistant *E.coli* in the tail vein. Blood was taken after 30, 60 and 90 minutes from the tip of the tail. For this purpose, a 1mm long part of the tail was cut with sterilized scissors and a drop of blood was drained from the wound. Using a microtiter pipette, 2 $\mu$ l of blood were scooped from this drop and immersed in 200 $\mu$ l of sterile PBS (Fisher BioReagents Fisher Scientific, Pittsburgh, USA). After vigorous vortexing, the blood was plated in duplicates on chloramphenicol-positive Agar plates containing nutrient broth (Chloramphenicol was obtained from Sigma-Aldrich, St. Louis, USA; Difco Nutrient Broth from Difco Laboratories Inc, Livonia, USA; petri dishes from Falcon Corning, New York, USA). Mice were anesthetized for laparotomy with an intraperitoneal injection of a ketamine/xylazine cocktail seven hours after the infection (Ketaset/ketamine was obtained from Zoetis, Florham Park, USA; Anased/xylazine from Lloyd, Shenendoah, USA). Animals were sacrificed by draining blood from the inferior vena cava and removing liver and spleen from the abdomen. Blood was heparinized and 100 $\mu$ l and 200 $\mu$ l were plated undiluted on chloramphenicol-positive Agar plates containing nutrient broth. The remaining whole blood was centrifuged at 12000xg for 15 minutes. Plasma was shock-frozen in liquid nitrogen and stored at -80°C until further use. The right liver lobe was freed of its edges and the central part was cut into 8-10 pieces. One piece was weighed and homogenized in 200 $\mu$ l of sterile PBS (Fisher BioReagents Fisher Scientific, Pittsburgh, USA) using a bead beater and sterilized glass beads (Fisher Scientific, Pittsburgh, USA) of 1mm in diameter (600rpm, 2x1min). After homogenizing, tissue lysates were placed on ice immediately and total volume was filled up to 1ml. After vortexing, 100 $\mu$ l were plated in duplicates on chloramphenicol-positive Agar plates containing nutrient broth. The remaining pieces of the right lobe were shock-frozen in liquid nitrogen and stored at -80°C for RNA and protein extraction. The left and middle lobe of the liver were immersed in 10% formaldehyde (Sigma-Aldrich, St. Louis, USA) overnight, followed by 24 hours in 10% sterile sucrose solution (Sigma-Aldrich, St. Louis, USA) and 24 hours in 30% sterile sucrose solution to conserve the natural fluorescence of the injected bacteria. Afterwards, liver tissue was embedded in optimal cutting temperature (OCT) compound (Tissue-Tek Sakura, Alphen aan den Rijn, The Netherlands), frozen on dry ice, and stored at -80°C for immunofluorescent co-localization staining. Caudate lobe was frozen in OCT and stored at -80°C for oil red O staining. The spleen was cut in half, one part was weighed and homogenized/plated the same way as the liver (see above) and the other part was conserved for immunofluorescent staining (see above). Experimental setup is outlined in Figure 18.



**Figure 18: Experimental setup for induced bacteraemia at the end of 10 weeks of chronic alcohol feeding.**

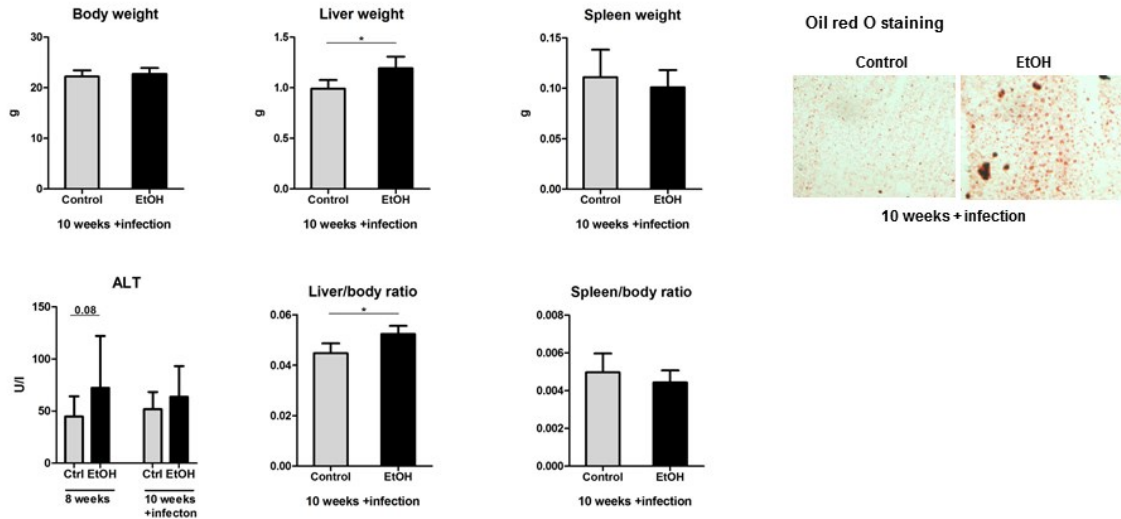
Bacterial culture conditions are described in the method glossary. Colonies growing on plates were selected by antibiotic resistance and GFP-expression was confirmed using fluorescence microscopy. Countable CFUs were used to monitor bacterial clearance from the blood stream and the redistribution of bacteria into the liver and the spleen.

Ex vivo experiments were also done with 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) treated mice on Lieber-DeCarli feeding for 6 weeks. This model induces massive liver injury even without alcohol. One mouse on iso-caloric control diet died from blood loss during the blood drawing.

Additional methods are given in the “Method glossary”.

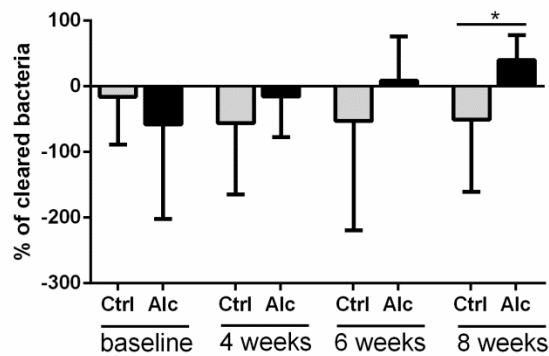
## Results

Lieber-DeCarli fed mice showed significantly increased liver weight, liver to body weight ratio, and lipid accumulation in the liver after ten weeks of alcohol diet and *E.coli* bacteraemia. Body weight and spleen weight did not differ between groups. ALT levels were relatively high in both groups and a strong trend was observed to higher ALT levels after eight weeks of alcohol compared to control mice. After ten weeks of feeding and bacteraemia, ALT levels were comparable in both groups. For details see Figure 19.



**Figure 19: Phenotype of alcoholic liver disease in alcohol (black bars) and iso-caloric control diet (grey bars) fed mice.**

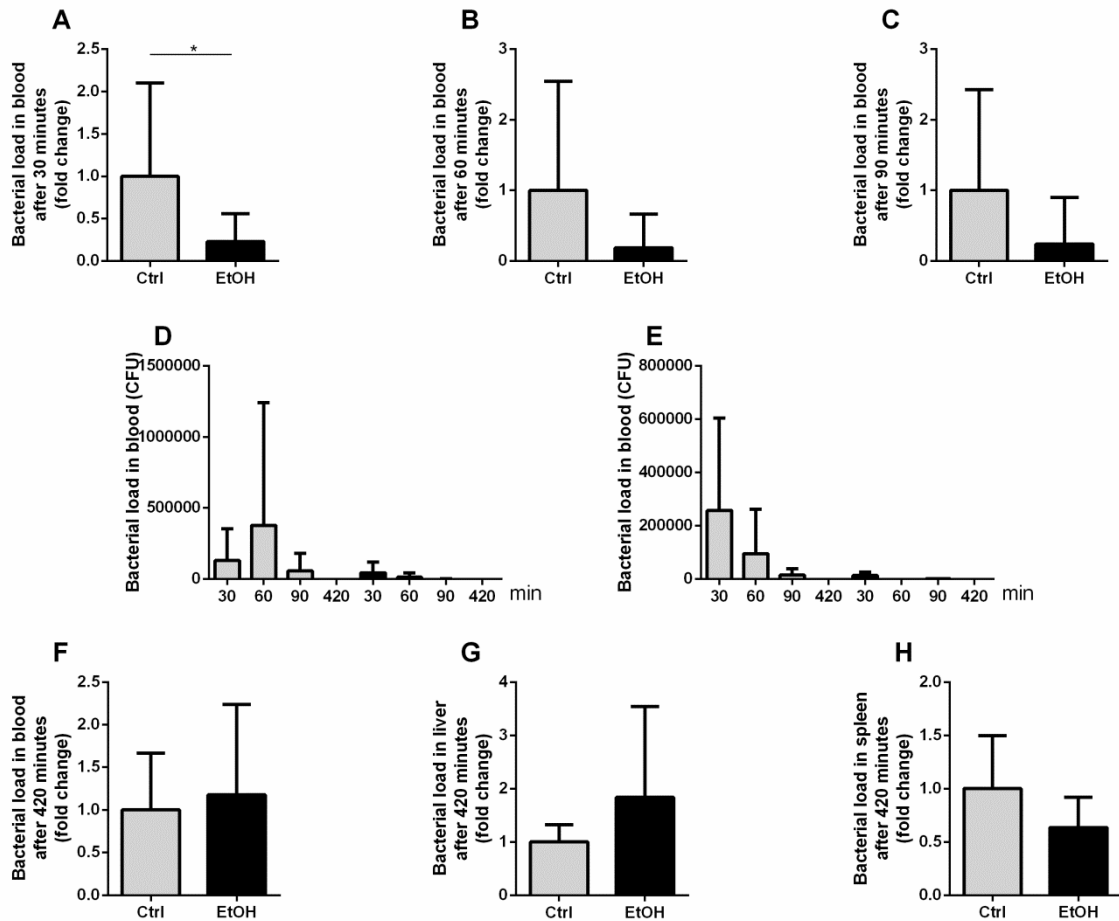
Ex vivo clearance capacity increased gradually during the feeding period in alcohol-fed mice, but not in control mice. After eight weeks, alcohol fed mice cleared *E.coli* significantly more efficiently than control mice (Figure 20). Neutrophil and monocyte activity did not change over time (data not shown).



**Figure 20: Ex vivo clearance capacity of whole blood in alcohol fed mice**

Ex vivo clearance capacity of whole blood is increased during alcohol diet (black bars) but not with control diet (grey bars).

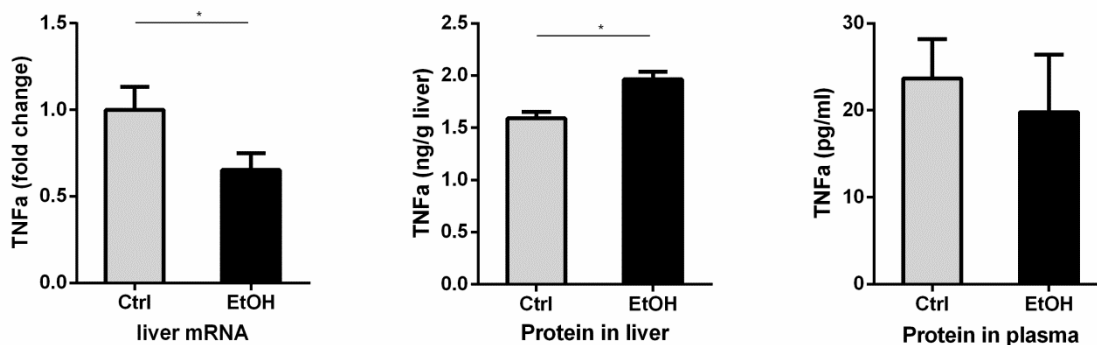
Alcohol-fed mice were also better equipped to clear experimental bacteraemia than control mice. Thirty minutes after injection, significantly fewer bacteria were found in the blood stream of alcohol fed mice compared to control mice. After sixty and ninety minutes, a similar trend was found (not significant). When the mice were sacrificed seven hours after injection, all advantages were evened out. No differences in bacterial load in blood, liver or spleen could be found. For details see Figure 21.



**Figure 21: Bacterial load after intravenous injection of *E. coli* at different time points**

A-C Bacterial load in the blood stream for alcohol fed mice (black bars) and control mice (grey bars) after thirty (A), sixty (B) and ninety (C) minutes given in fold change of the control mice of the respective experiment. D+E gives the same information in absolute terms per ml including the 7 hour time point grouped according to the injected bacterial load. Cohort 1 and 3 (D) received  $5 \times 10^7$  CFU and cohort 2 (E) was challenged with approximately  $2 \times 10^8$  CFU. F-H Bacterial load in 1ml of blood (F), whole liver (G) and whole spleen (H) seven hours after injection given as fold change of the control mice of the respective experiment.

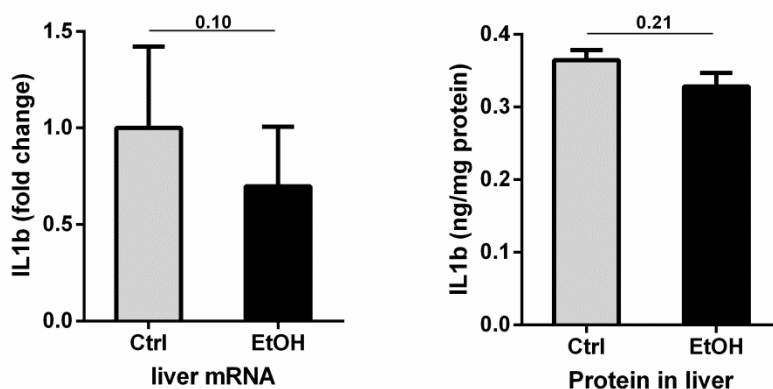
Activation of antimicrobial strategies in the liver did not show differences between groups seven hours after the infection; inducible nitric oxide synthetase (iNOS), regenerating islet-derived protein 3 gamma (Reg3g), Reg3b and F4/80 as marker for macrophage activation were equally expressed in both groups. TNF $\alpha$  was significantly less expressed on mRNA level but significantly higher on protein level in the livers of alcohol fed mice than of control mice. Plasma concentrations were similar in both groups. See also Figure 22.



**Figure 22: TNFα expression in the liver of alcohol-fed mice**

TNFα expression in the liver of alcohol-fed mice (black bars) and control mice (grey bars) seven hours after *E.coli* injection. \* significant differences

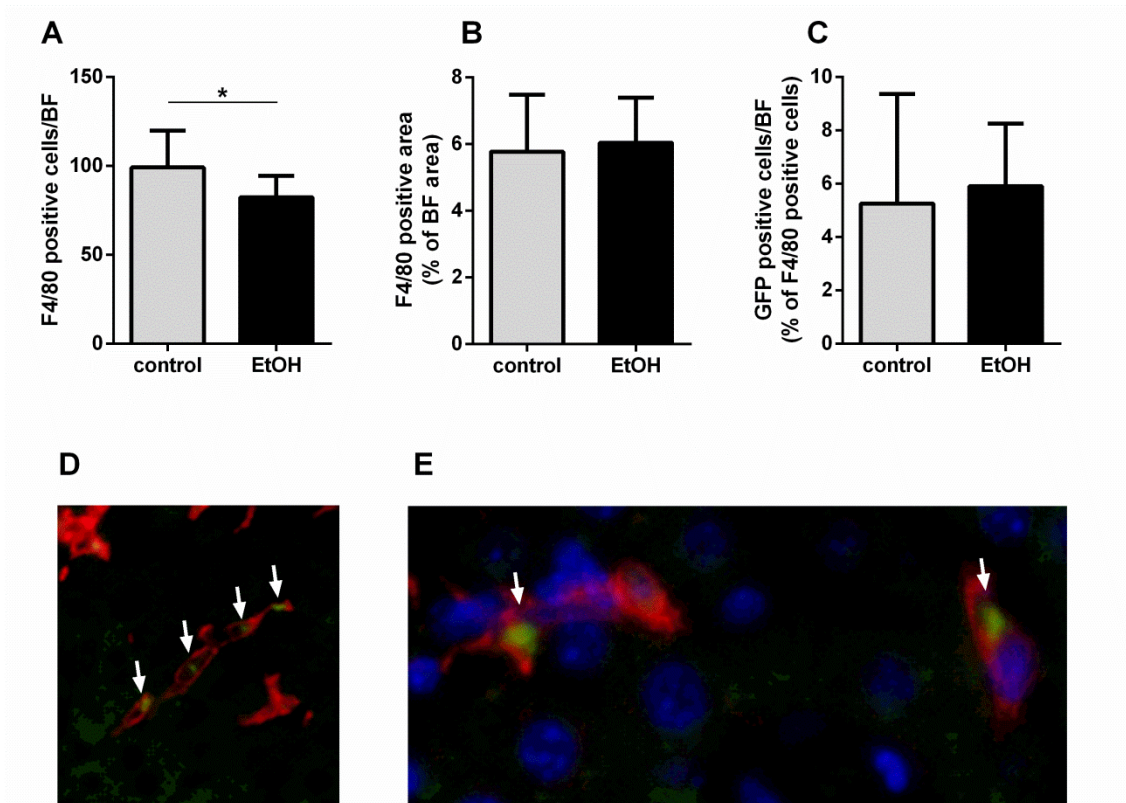
IL1b expression tended to be inhibited on mRNA and protein level which suggests an insufficient activation of the inflammasome in response to Gram negative bacteraemia. See also Figure 23.



**Figure 23: IL1b expression in the liver of alcohol-fed mice**

IL1b expression in the liver of alcohol-fed mice (black bars) and control mice (grey bars) seven hours after *E.coli* injection on mRNA (left) and protein level (right). Credit for tissue protein levels of IL1b in the liver goes to An-Ming Yang, University of California San Diego, La Jolla, USA

Kupffer cell expansion after the infection with *E.coli* is comparable in both groups. Kupffer cells however vary in size with bigger cells in the alcoholic group. Immunofluorescent staining of the liver for F4/80 (macrophage marker) revealed significantly less F4/80 positive cells in the alcohol group compared to the control group but no differences in F4/80 positive area. There were no differences between the F4/80 and GFP (bacteria) double positive cells. This suggests that phagocytosis of bacteria by macrophages in the liver is intact even after ten weeks of chronic alcohol feeding. Details are given in Figure 24.



**Figure 24: Kupffer cell expansion in the liver after infection with *E. coli***

A-C quantification of macrophages (A), of the area taken in by macrophages (B) and the percentage of macrophages with engulfed bacteria (C) for alcohol (black bars) and control fed (grey bars) mice. D+E Examples of macrophages (red) with engulfed bacteria (green) at 20x (D) and 40x (E) magnification. \* significant differences

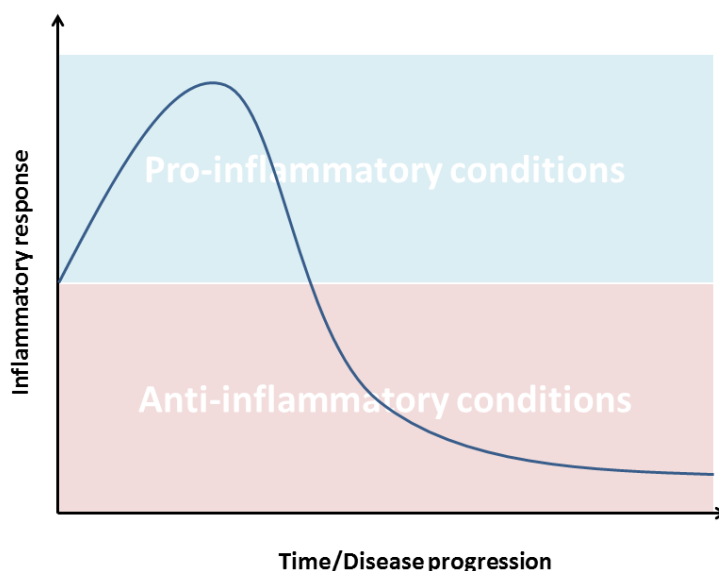
Ex vivo clearance capacity and phagocytosis capacity of neutrophils and monocytes assessed in mice treated with DDC before alcohol feeding were virtually non-existent in both alcohol- and control-fed groups (data not shown). Blood was provided by Cheng Peng, University of California San Diego, La Jolla, USA

## Discussion

Alcohol feeding over ten weeks in mice enhanced the immune response to *E.coli* bacteraemia and gave the mice an advantage in dealing with the pathogens compared to control-fed mice. This advantage was restricted to the early response phase and did not persist until the clearance of all bacteria.

A possible explanation would be that the administration of alcohol induced a sensitization of the innate immune system of the liver and therefore the alcohol-fed mice were somewhat prepared to deal with Gram negative bacteria. In many ways, alcoholic liver injury resembles the early response phase of bacterial infection, like the chemotactic infiltration and TLR4 mediated activation of leucocytes, especially Kupffer cells(128), the upregulation of pro-inflammatory

cytokines including TNF $\alpha$ (129) and the induction of NADPH oxidase.(37) Healthy mice could gain on this head start and dealt with the infection in a different dynamic. Mice with severe DDC liver injury were not able to cope with the bacteria ex vivo and showed no clearance or phagocytic activity. These results confer with weak in vivo clearance in toxic and cholestatic liver injury.(69) Further experiments were ethically not endorsable. However, the little information we received from the ex vivo experiments supports the hypothesis that the effect of alcohol on the innate immune system depends on the degree of liver injury. The pattern of initial immune activation and consequent immune paralysis is already known in sepsis. (130) Other than in sepsis, neutrophil dysfunction in cirrhosis is not directly mediated by cytokine imbalance, as our data suggest.(131) The liver is responsible for the expression of countless proteins. Once the liver loses the ability to produce them it is possible that the lack or relative overexpression of a protein or a set of proteins might dysregulate innate immunity. Nevertheless, the concept of SIRS/CARS as shown in Figure 25 can be helpful in explaining the reaction of the immune system to alcohol and liver disease.



**Figure 25: Simplistic scheme of SIRS/CARS in sepsis**

Initial strong pro-inflammatory conditions turn into an anti-inflammatory state of immune paralysis.

Blood-borne leucocytes were not affected by alcoholic liver disease in the mouse model although human neutrophils reacted rather strongly. Neutrophils are a major part of the innate immune system in man(132) while murine neutrophils and monocytes only make up a small portion of total white blood cells.(133)

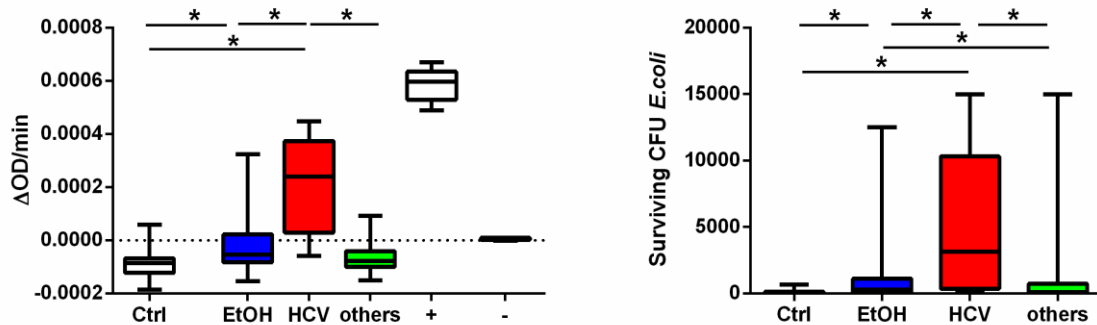
TNF $\alpha$  expression was lower in alcohol fed mice compared to control mice on mRNA level. This was also the case in alcohol-fed mice that did not undergo experimental bacteraemia. Despite the lacking mRNA TNF $\alpha$  protein levels were significantly higher in alcohol-fed mice compared to

controls. This result is consistent with the previously reported stabilization of TNF $\alpha$  mRNA by the alcohol-metabolizing enzyme Cytochrome4502E1.(37) IL1 $\beta$  expression in alcohol-fed mice did not match the expression in healthy mice after infection, although the administration of alcohol should have induced inflammasome activation and IL1b expression.(134)

## Chronic hepatitis C as an oppressor of neutrophils

### Background

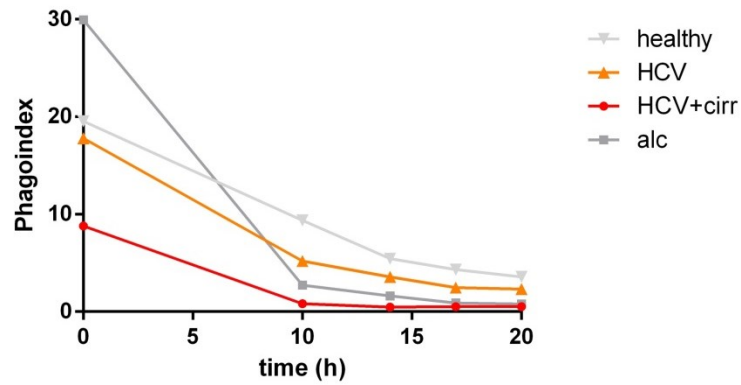
As seen in Figure 16, patients with chronic hepatitis C-induced cirrhosis have particularly low phagocytic capacity. This breach in innate immunity is also reflected in poor killing and growth retardation capacity of these patients (Figure 26).



**Figure 26: The impairment of growth retardation and serum killing capacity in cirrhosis according to aetiologies**

Hepatitis C-induced cirrhosis is accompanied by poor growth retardation (left) and serum killing capacity (right). \*significant differences between groups indicated by horizontal bars. + represents an uninhibited growth control; - is the negative control of broth without bacteria

Since neutrophil dysfunction was transmissible onto healthy neutrophils within minutes, serum mediated confounders seem likely to be the cause for the dysfunction. Gut permeability, serum endotoxin and liver function have been excluded as influential factor (data not shown). Hepatitis C cirrhotic sera have the strongest effect on the phagocytic capacity of neutrophils isolated from healthy donors. An immediate blockage of phagocytic activity is only transmitted by sera of patients with both cirrhosis and viral hepatitis C; either condition alone (hepatitis C without cirrhosis and alcoholic cirrhosis without hepatitis C) cannot induce neutrophil dysfunction. See also Figure 27.



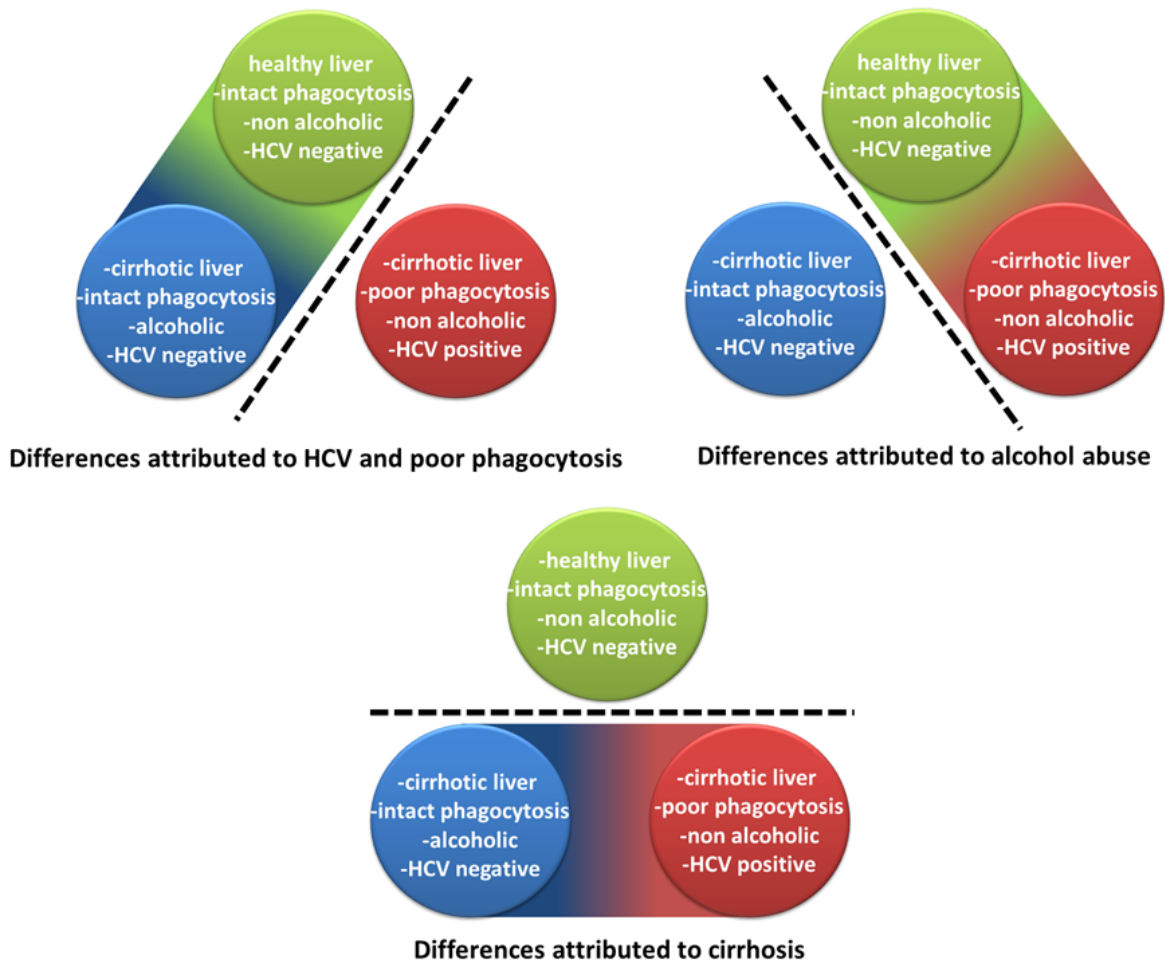
**Figure 27: Transmission of phagocytic dysfunction by serum onto healthy neutrophils**

Isolated healthy neutrophils acquire phagocytic dysfunction within minutes of incubation with diseased serum (representative graph). Sera from hepatitis C cirrhotic patients show particularly strong effects (red line).

To pinpoint possible serum components, a 2D gel electrophoresis/GC-MS approach was taken to compare sera from hepatitis C infected cirrhotic patients (HCV) to alcoholic cirrhotics and healthy controls.

### Methods

Three patients in each group were selected based on liver function (Child-Pugh score 8 or 9), gender (2 male, 1 female) and phagocytic capacity. Mean phagocytic capacity (in weighted GMFI) for HCV patients was 40, for alcoholic cirrhosis 131 and for healthy controls 110. The expected differences will be between healthy controls and cirrhotic patients, marking changes in serum proteome caused by cirrhosis. Differences between healthy controls/HCV patients and alcoholic patients will be attributed to alcoholism, and differences between healthy controls/alcoholic cirrhotics and HCV patients will in part be attributed to hepatitis C infection and in part to poor phagocytosis. The principle behind the experiment is outlined in Figure 28.



**Figure 28: Schematic explanation of expected outcome from the 2D gel electrophoresis**

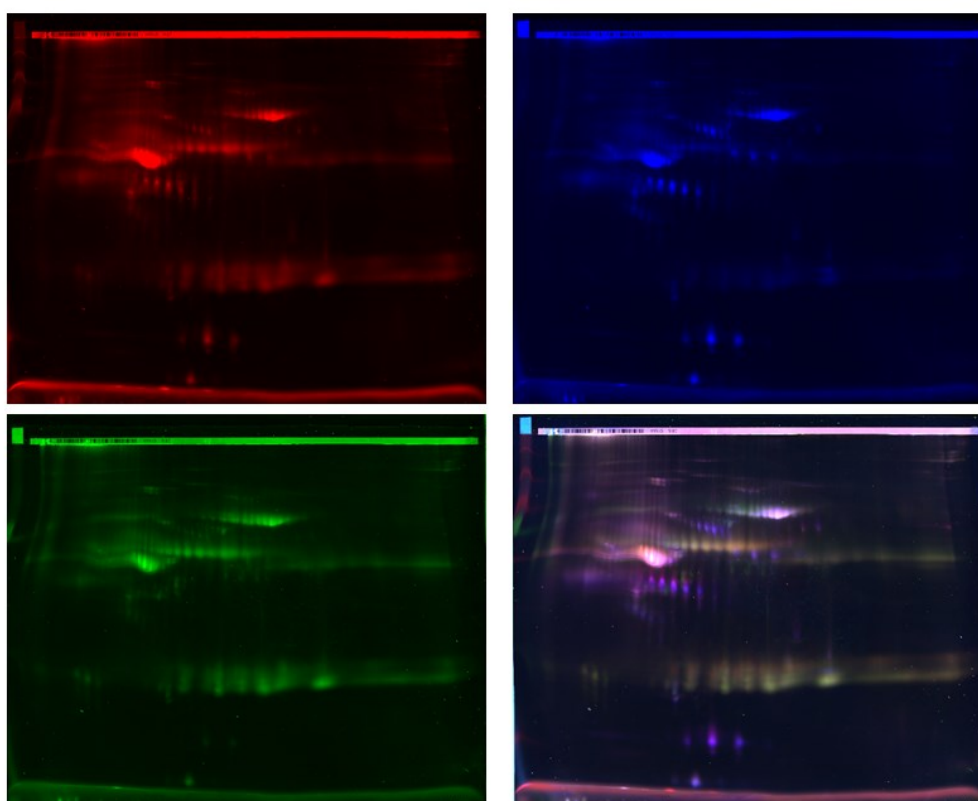
Schematic explanation of expected outcome from the 2D gel electrophoresis experiment comparing the serum proteome of cirrhotic patients and healthy controls; Proteins of interest will be common to healthy controls and alcoholic cirrhotics but differentially expressed in HCV cirrhosis. Green circles represent healthy controls, blue circles alcoholic cirrhosis and red circles HCV cirrhosis.

Patient serum was pooled within the groups and depleted from albumin and IgG using ProteoPrep® Immunoaffinity Albumin & IgG Depletion Kit (Sigma-Aldrich, Vienna, Austria). Proteins were precipitated with a 10% trichloroacetic acid (TCA)/aceton solution, washed with aceton and reconstituted in lysis buffer. 50µg of protein per group was fluorescently labelled with Amersham CyDye DIGE Fluors (GE Healthcare Life Science, Vienna, Austria) and mixed with 350µg of unlabelled protein. Healthy controls were labelled with Cy2 (yellow/green), HCV patients with Cy3 (red) and alcoholic cirrhotics with Cy5 (blue). All samples were pooled and brought on IPGstrips (GE Healthcare Life Science, Vienna, Austria) for isoelectric focusing (1<sup>st</sup> dimension). After 15 hours of run time, strips were taken from the IPGphor workstation (GE Healthcare Life Science, Vienna, Austria) and placed on a 12% SDS gel. A low molecular weight marker was placed on the left side of the strip and the strip was fixed with agarose. Electrophoresis ran at 600V, 15mA per gel and maximally 100 W as long as bromophenol blue front was still visible

(approximately 16 hours). Assessments were done in triplicates. Fluorescent scans of the gel were used to determine differentially expressed spots (at least 2-fold change). After scanning, gels were fixed with 30% ethanol and 10% acetic acid. Spots of interest were picked and proteins were tryptically digested. Peptides were identified by GC-MS which was done by Gerald Rechberger (Karl Franzens University of Graz, Austria).

## Results

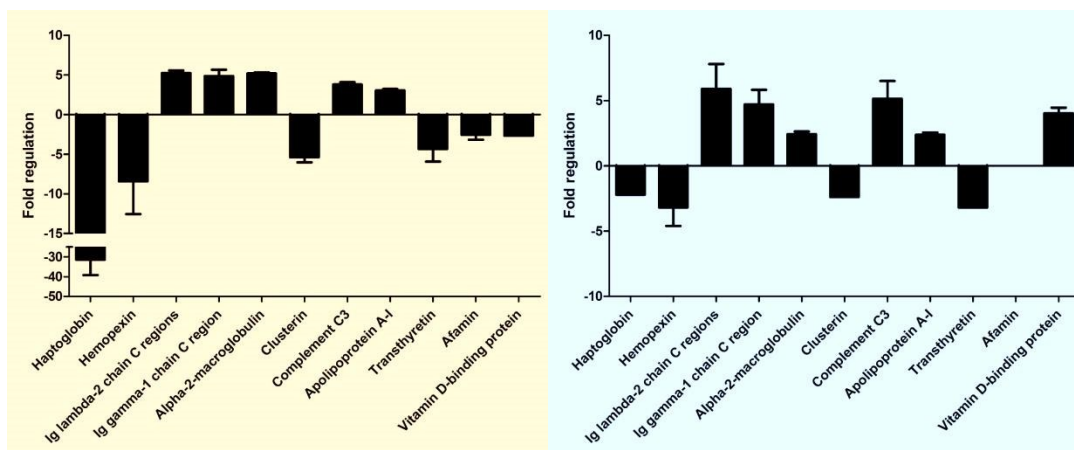
An average of 120 differentially expressed spots were found and analysed per gel. Representative scans are given in Figure 29. The colourful appearance of the merged picture already reflects the manifold alterations of the serum proteome between different diseases.



**Figure 29: Representative scans of a 2D gel of serum proteomics.**

The upper left scan depicts serum proteins of HCV patients (red), the upper right scan depicts alcoholic cirrhotics (blue), the lower left scan represents the healthy serum proteome and the lower right picture is a merge of all the channels. Credit goes to Christoph Nusshold (Medical University of Graz, Austria) who established the methods used in this section and helped to design the experiment.

The most prominent, differentially-expressed eleven proteins found mainly reflect changes attributable to cirrhosis. The two possible candidates that showed differential expression in HCV patients when compared to healthy controls and alcoholic cirrhotics were haptoglobin and afamin.



**Figure 30: Most prominent differences in serum proteoms of cirrhotics and healthy controls**  
Differentially expressed proteins in HCV cirrhosis compared to healthy controls (left) and alcoholic cirrhosis compared to healthy controls (right). Proteins of interest are haptoglobin (first protein from the left) and afamin (second protein from the right). Credit for this graph goes to Christoph Nussold (Medical University of Graz, Austria)

Haptoglobin scavenges haemoglobin in the blood and prevents its passage through the kidney. The reduced concentration of this protein has long been described in post-hepatitis patients.(135) However, a more recent study that compared hepatitis C-infected patients with different stages of fibrosis in a similar approach as ours found haptoglobin to be increased in advanced liver fibrosis compared to mild forms.(136) The expression of haptoglobin has not yet been verified in our cohort, however serum zonulin was assessed in the study cohort as an antimicrobial molecule and zonulin was previously identified as prehaptoglobin.(137) Therefore, serum zonulin was used as a proxy marker for haptoglobin in a preliminary analysis, knowing that values are not exactly transferable. An approximately 3-fold decrease of zonulin levels between HCV cirrhosis and healthy controls and a 2-fold decrease of zonulin between HCV cirrhotics and alcoholic cirrhotics could be found. Results are given in Table 11.

**Table 11: Zonulin concentration in serum of different types of cirrhosis and healthy controls**

Data are given in Median (Q1; Q3)

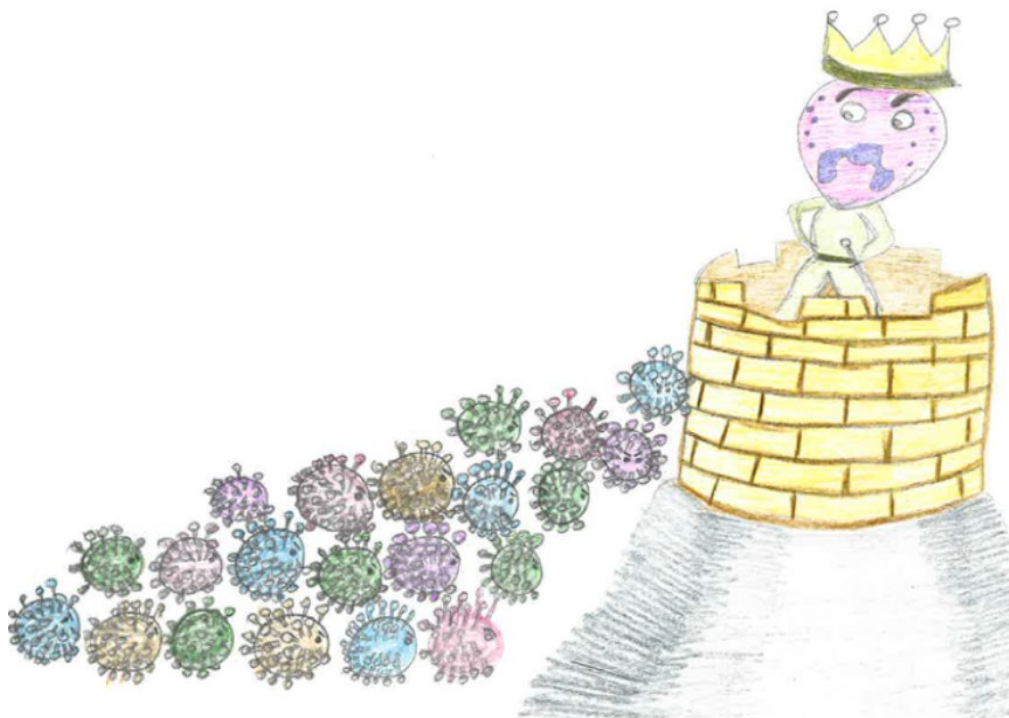
	Zonulin (ng/ml)
Healthy	56 (49; 66)
Hepatitis C cirrhosis	19 (10;22)
Alcoholic cirrhosis	37 (27; 54)
Other types of cirrhosis	65 (27; 78)

Changes in the phagocytic capacity reflect the regulation of serum zonulin. The variation of this protein only explains about 20% of the variation in phagocytosis (linear regression:  $R^2=0.22$ ,  $p<0.001$ ).

Afamin is a member of the albumin gene family and has been reported as a possible biomarker for ovarian cancer.(138) It is also implicated in other types of cancer and metabolic syndrome.(139) The physiological functions of afamin and its involvement in phagocytic dysfunction remain to be elucidated.

## **Conclusion**

Most changes in the serum proteome seem to be brought on by cirrhosis rather than a HCV-specific immunodeficiency. Both haptoglobin and afamin have been identified as a fibrosis marker in a proteomics study.(140) Whether these proteins also play a role in phagocytosis remains to be examined. An in-depth analysis would go beyond the scope of this thesis and will be part of a follow-up study. Dozens of proteins are still left to be investigated. The overwhelming volume of data comprised from this experiment lead to the conclusion that a multifactorial web of confounders influence the phagocytic capacity of neutrophils in HCV cirrhosis. Until this web is untangled, neutrophils will remain under the dominion of the hepatitis C virus.

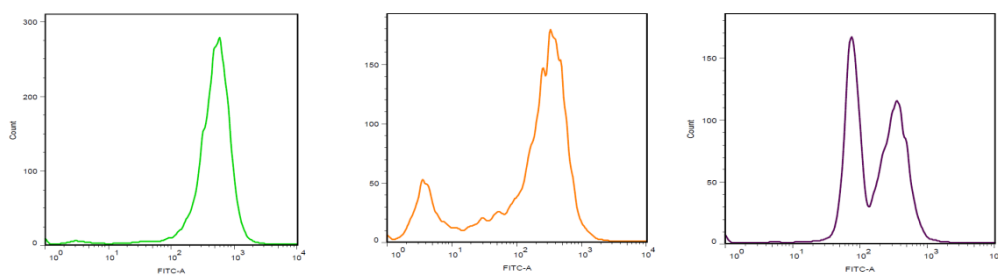


## C. Methods in liver disease research

Methodological approaches in liver cirrhosis include the characterisation of innate immune responses and the correct measurement of structurally diverse endotoxins. Limitations to standard methods and possible solutions are described here.

### Phagoindex

Phagotest kit by Glycotope or an adapted protocol with live GFP-expressing bacteria were used to assess the phagocytic dysfunction of neutrophils or monocytes. The technical details are described in M1. In short: A defined amount of blood or isolated cells are challenged in duplicates with bacteria labelled with a fluorochrome (FITC or GFP). One of the duplicates is placed on 37°C (test tube), the other one remains on ice (control vial). Analysis by flow cytometry gives fluorescence intensity values for each cell as a semi-quantitative measurement of how many bacteria the cell engulfed during the test. The readout of this test is usually assessed by phagocytic activity (percentage of FITC/GFP positive cells) or phagocytic capacity (fluorescence intensity of positive cells). This type of analysis is suitable when the dysfunction is relatively mild or it is either based on a reduction of capacity or activity but not both. Phagocyte dysfunction in liver disease is a combination of the accumulation of inactive cells and the reduction of capacity, resulting in diverse manifestations as seen in Figure 31. These manifestations strongly depend on liver function and aetiology. Especially the characteristic formation of a third, low phagocytic population makes phagocytic function hard to evaluate with a one-factorial approach.



**Figure 31: Histograms of different manifestations of phagocyte dysfunction in liver disease**

Intact function with mostly high phagocytic cells (green), accumulation of inactive cells (orange) and reduction of high phagocytic cells and overrepresentation of low phagocytic cells in the absence of inactive cells (violet).

### Multifactorial approach

Example 1: Given that patients with various types of phagocytic capacity shall be compared and that the most relevant readout is the amount of bacteria that a defined amount of cells can eliminate, a singular expression of either the percentage of FITC positive cells or the fluorescence intensity of positive cells present significant shortcomings. For a simple explanation, four fictional

cases are presented here with three phagocytes each. Case A shows full activity (100% positive cells) and high capacity (mean intensity of 10). Assuming the fluorescence intensity approximates the amount of bacteria engulfed, this would amount to 30 eliminated bacteria. Case B shows equal activity (100%) but reduced capacity (mean intensity of 4), which amount to 12 eliminated bacteria. Case C shows high capacity (mean intensity of 10) but reduced activity (33% positive cells). This would amount to 10 bacteria that were cleared from the environment. Case D presents low capacity (mean intensity of 4) as well as reduced activity (33% positive cells), adding up to 4 eliminated bacteria. This example already highlights that the four presented cases are not comparable with only one of the standard methods of analysis, either capacity or activity, and that the amount of bacteria engulfed would be the better readout.

The best approximation for the amount of engulfed bacteria would by definition be the sum of fluorescence intensity of every positive cell. This amount of data would not be feasible to handle, especially, when a simpler calculation technique could still estimate phagocyte function and be fast and easy to apply (even if not exactly accurate). Therefore, a very broad generalisation of this principle was trialled as a numerous approximation for phagocyte function. The “Phagoindex” was introduced as the mean phagocytic capacity weighted by the percentage of positive cells as outlined in Formula 1.

$$\text{Phagoindex} = p(\text{GMFI}) \times p(\text{rs})$$

**Formula 1: Calculation scheme for Phagoindex**

p stands for the FITC positive population, GMFI for geometric mean of fluorescence intensity, rs for relative share of the parent population expressed in percent;

Revisiting example 1, Phagoindex reflects the amount of bacteria engulfed more accurately than the percentage of FITC-positive cells or the fluorescence intensity of positive cells alone. Phagoindex is the only numeral approximation presented here that ranks the cases according to the theoretically engulfed bacteria (Table 12).

**Table 12: Comparison between standard methods to analyse phagotest readouts and the Phagoindex based on the fictional example 1 outlined above.**

	Eliminated bacteria (n)	Rank (n)	Capacity (c)	Rank (c)	Activity (a)	Rank (a)	Phagoindex (PI)	Rank (PI)
Case A	30	1	10	1	100%	1	1	1
Case B	12	2	4	3	100%	1	0.4	2
Case C	10	3	10	1	33%	3	0.33	3
Case D	4	4	4	3	33%	3	0.13	4

## Low phagocytic population

As mentioned above, patients with liver disease develop a population of low phagocytic cells in addition to high phagocytic cells. Example 2: The following example will demonstrate the limitations of the standard analysis when low phagocytic cells are observed. Three fictional cases are presented here with four phagocytes each. Case A has 100% activity with 50% of low phagocytic cells (mean intensity of 2) and 50% of high phagocytic cells (mean intensity of 4). The mean capacity is therefore 3 with 12 eliminated bacteria. Case B has 75% activity with 25% of low phagocytic cells (mean intensity of 2) and 50% of high phagocytic cells (mean intensity of 4), a mean capacity of 3.3 although the eliminated bacteria decreased to 10. Case C also shows a 75% activity with 50% of low phagocytic cells (mean intensity of 2) and 25% of high phagocytic cells (mean intensity of 4). The mean capacity is markedly reduced to 2.6 although the activity is the same as in case B, 75%. Eliminated bacteria amount to 8. The accumulation of low phagocytic cells is a feature of phagocyte dysfunction that should be reflected in the presented numeral approximation but should not extensively bias it. Therefore, both populations were analysed separately and the generalisation to estimate the sum of fluorescence intensity used above was extended to two populations. “Extended Phagoindex” was calculated according to Formula 2.

$$\text{Extended Phagoindex} = hp (GMFI) \times hp (rs) + lp (GMFI) \times lp (rs)$$

**Formula 2: Calculation scheme for Phagoindex with and without low phagocytic cells (reduced)**  
hp stands for high phagocytic cells, GMFI for geometric mean of fluorescence intensity, rs for relative share of the parent population expressed in percent, lp for low phagocytic; for convenience the Phagoindex is usually divided by 1000.

In the second example, Phagoindex is also superior to the standard methods; however the advantage to use the extended version is not immediately obvious. Both versions rank the cases correctly according to the theoretically engulfed bacteria (Table 13).

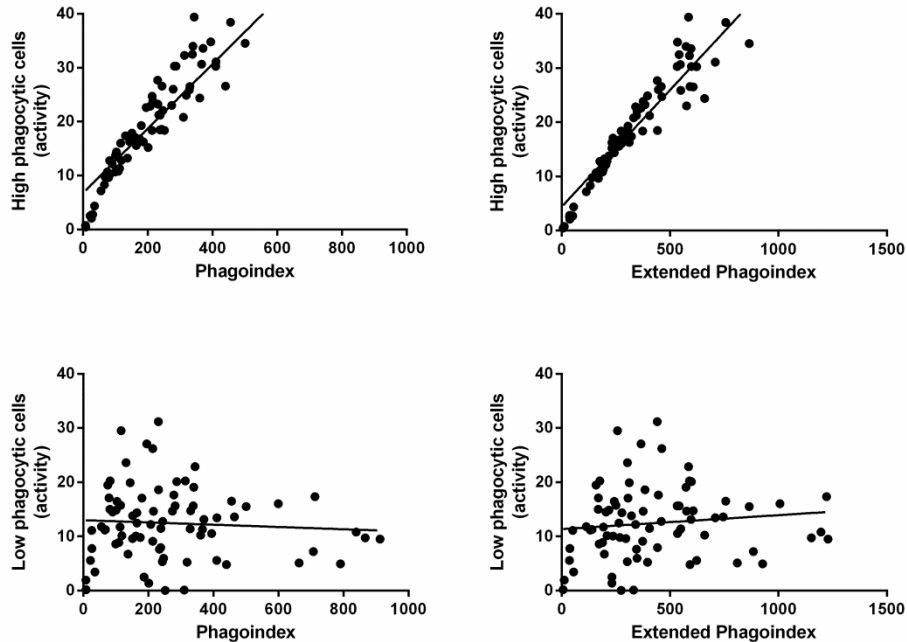
**Table 13: Comparison between standard methods to analyse phagotest readouts and two versions of Phagoindex based on the fictional example 2 outlined above.**

	Eliminated bacteria (n)	Rank (n)	Capacity (c)	Rank (c)	Activity (a)	Rank (a)	Phagoindex (PI)	Rank (PI)	Extended PI (PI)	Rank (PI)
Case a	12	1	3	2	100	1	0.300	1	0.300	1
Case b	10	2	3.3	1	75	2	0.247	2	0.250	2
Case c	8	3	2.6	3	75	2	0.195	3	0.200	3

## The extended version of the Phagoindex

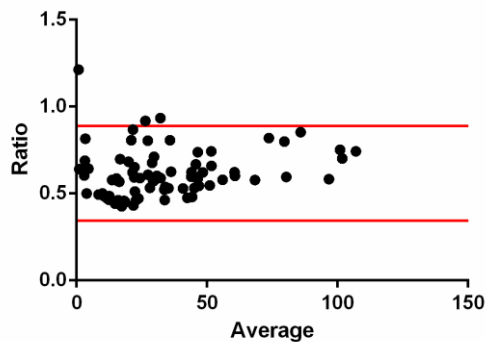
The most capable cells build the foundation of the phagocytic power; therefore, the Phagoindex in either version is strongly associated with the capacity and size of the high phagocytic population.

This is reflected in strong correlations between the Phagoindex/Extended Phagoindex and the size of the high phagocytic population ( $R^2=0.903$ ,  $p<0.0001$  and  $R^2=0.918$ ,  $p<0.0001$  respectively). On the other hand, Phagoindex/Extended Phagoindex is not dependent on low phagocytic cells ( $R^2=0.004$ ,  $p=0.566$  and  $R^2=0.013$ ,  $p=0.326$  respectively), shown in Figure 32.



**Figure 32: Correlations between phagocytic populations and the Phagoindex**  
 Strong association between the high phagocytic population but not the low phagocytic population and the Phagoindex as well as Extended Phagoindex; Data was collected from cultured neutrophils isolated from sodium citrate blood of six healthy donors cross incubated with 79 sera of cirrhotic patients.

As seen in the Bland-Altman plot in Figure 33, the two versions of Phagoindex are well comparable with some exceptions at the lower end. An explanation for this deviation might be that the use of geometric mean of the overall positive population favours lower values.



**Figure 33: Bland-Altman plot for Phagoindex and Extended Phagoindex**



### Figure 35: Simplistic example to explain the strengths of the Extended Phagoindex

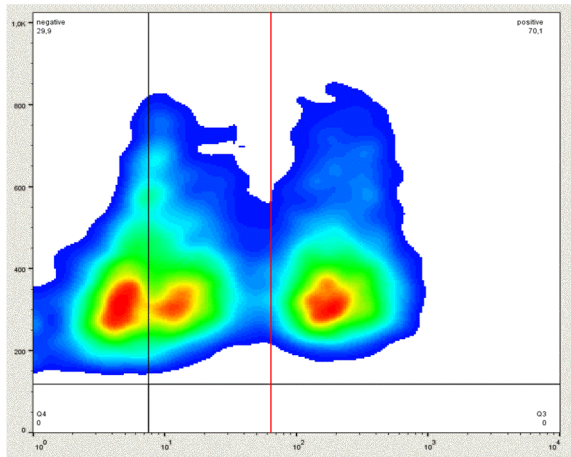
Simplistic example 3 to explain the strengths of the Extended Phagoindex compared to Phagoindex (PI) in case of pronounced low phagocytic population (A). The advantage is lost when the populations are more evenly distributed (B).

#### Gating strategy

A representative sample of neutrophils or monocytes (ideally  $n=10.000$ ) is gated according to forward-side-scatter characteristics. FITC intensity is then plotted on a logarithmic scale against the side scatter on linear scale in a smoothed pseudo-colour plot. Cells are divided into three populations: non-phagocytic, low-phagocytic and high-phagocytic. The non-phagocytic population is gated in the control vial with the gate line between the light and dark blue areas on the right side of the population. The high-phagocytic population is defined in the test vial as the farthest right population. It is gated at the left border between light and dark blue areas (see Figure 36). Low phagocytic cells are defined as all cells in between. One way to define them is to create an “OR”-gate that summarizes inactive and high phagocytic cells and then a “NOT”-gate to get all the cells that are excluded from the first gate. The relative share and geometric mean of fluorescence intensity is recorded for each population separately for the control and the test vial. Variations in auto-fluorescence are corrected by using the control vial as reference. The following values are calculated before inserted in the Extended Phagoindex according to Formula 2:

- The capacity of low-phagocytic cells,  $lp(\text{GMFI})$ , is calculated by subtracting the geometric mean fluorescence intensity (GMFI) of the inactive cells in the control tube from the GMFI of low phagocytic cells in the test tube.
- The activity of low-phagocytic cells,  $lp(\text{rs})$ , is calculated by subtracting the percentage of low phagocytic cells in the control tube from the percentage of low phagocytic cells in the test tube. Negative values are set to zero.
- The capacity of high-phagocytic cells,  $hp(\text{GMFI})$ , is calculated by subtracting the GMFI of the inactive cells in the control tube from the GMFI of high phagocytic cells in the test tube.
- The activity of high-phagocytic cells,  $hp(\text{rs})$ , is calculated by subtracting the percentage of high phagocytic cells in the control tube from the percentage of high phagocytic cells in the test tube.

These corrections also have to be done when the Phagoindex is calculated according to Formula 1. For  $p(\text{GMFI})$  subtract the GMFI of the inactive cells in the control tube from the GMFI of positive cells in the test tube. For  $p(\text{rs})$  subtract the percentage of positive cells in the control tube from the percentage of positive cells in the test tube. The percentage of inactive cells (non-phagocytic) is analysed separately as the uncorrected percentage of inactive cells in the test vial.



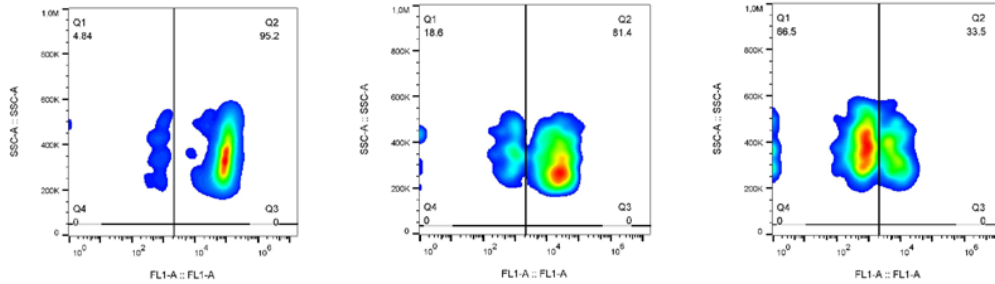
**Figure 36: Gating strategy for Extended Phagoindex**

FITC intensity is plotted on the abscissa and side scatter on the ordinate in a smoothed pseudo-colour plot. Non-phagocytic cells were defined in the control vial and the gate was assigned to the test vial (black line). A second gate was used to define high phagocytic cells in the test vial and was then assigned to the control vial (red line).

For analysis of a larger cohort that includes a change in bacteria batches, four to five batch controls have to be done for every lot of bacteria. Phagoindex and the percentage of inactive (non-phagocytic) cells are then related to the median of the appropriate batch controls. Batch controls consist of five healthy young people that are repeatedly tested with changing lots of bacteria. This is necessary because the fluorescence intensity and/or the amount of bacteria might vary between batches making it impossible to directly compare them to each other. Phagotest readouts are therefore presented as percentages of the median values derived from batch controls.

### **Phagoindex and Neutropenia**

Neutropenia is a common feature in liver cirrhosis. Patients presented in the clinical study in the first part of this thesis also show a significantly reduced neutrophil count compared to healthy controls. It is vital for the investigation of neutrophil function to examine the influence of neutrophil count on the responsiveness of phagocytes. Therefore neutrophils and monocytes were challenged with varying amounts of bacteria. These experiments were done in 25µl of heparinized murine whole blood with live GFP-expressing *E. coli* bacteria (10<sup>5</sup>, 10<sup>6</sup>, 10<sup>7</sup> CFU). Phagocytes reacted much stronger with phagocytosis when the amount of bacteria was higher than when the bacteria/phagocytes ratio was lower (Figure 37). In regard to neutropenia, these results would lead to the conclusion that patients with less neutrophils show better phagocytosis. This is not supported by the presented data. In conclusion, although the loss of neutrophils influences the phagotest readout, a correction for neutrophil count would only widen the gap between healthy and cirrhotic cohorts and add further inaccuracy to the measurements.



**Figure 37: Dependency of phagocytosis on the proportions of phagocytes and bacteria**

Murine neutrophils from 25µl heparinized whole blood in a FITC to SSC pseudo-colour plot after being challenged with 10µl PBS containing  $10^7$  (left),  $10^6$  (middle) or  $10^5$  (right) CFU of live GFP expressing E.coli for twenty minutes. It is clearly shown that the same amount of neutrophils react differently to varying amounts of bacteria.

## Endotoxin

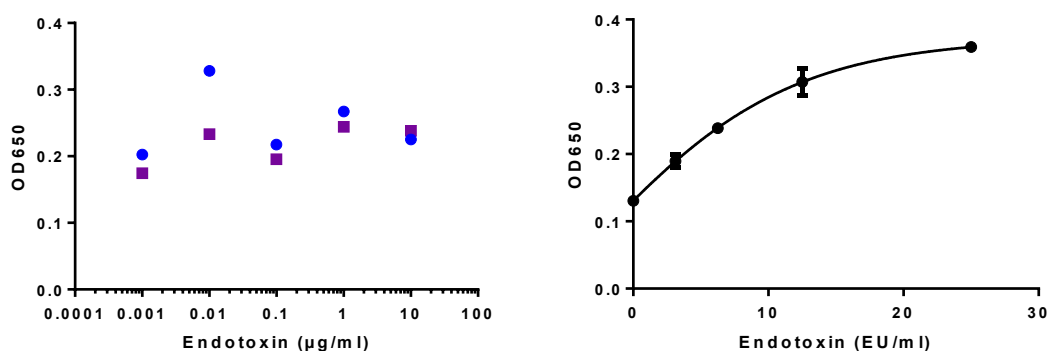
The standard method to measure endotoxin is the Limulus amoebocyte lysate (LAL) assay. However, none of the available kits are suitable for human samples. This paradox has been discussed before but LAL assay is still in use lacking valid alternatives.(24, 141)

Data discussed in the following comparison were obtained with Kinetic chromogenic LAL assay Endochrome-K™ (Charles River, Wilmington, USA). For technical details see “Method glossary” under “A. Limulus amoebocyte lysate (LAL) assay”. In addition, LAL Chromogenic Endpoint Assay (Hycult Biotech, Uden, The Netherlands) was tested with similar results (data not shown). Seven healthy control samples and endotoxin free water preparations with endotoxin spikes of 12.5 and 6.25 EU/ml were assessed with LAL assay. While the recovery of endotoxin was  $101 \pm 18.3\%$  in aqueous solutions, the recovery rate in serum samples was only  $0.16 \pm 0.09\%$ . Heat, dilution, or pre-treatment with Proteinase K were insufficient to improve recovery rates.

HEK-Blue™ LPS Detection Kit 2 (Invivogen, Toulouse, France) is a cell-based assay for qualitative and quantitative assessment of endotoxin in serum samples. It utilizes human embryonic kidney cells stably transfected with a TLR4 reporter cassette. TLR4 recognises structurally diverse endotoxins and activates a signal cascade that results in the expression of NFκB. In these cells, NFκB functions as a transcription factor for secreted embryonic alkaline phosphatase (SEAP). SEAP is secreted into the cell culture supernatant and facilitates a colour change of the detection medium that can then be assessed photometrically. However, the proposed protocol is unsuitable for reliable quantification. Furthermore, alkaline phosphatase disturbs the measurement which is problematic for the use in liver cirrhotic samples. The protocol was therefore adjusted to correct for these confounders.

## Reliability of standards and spikes

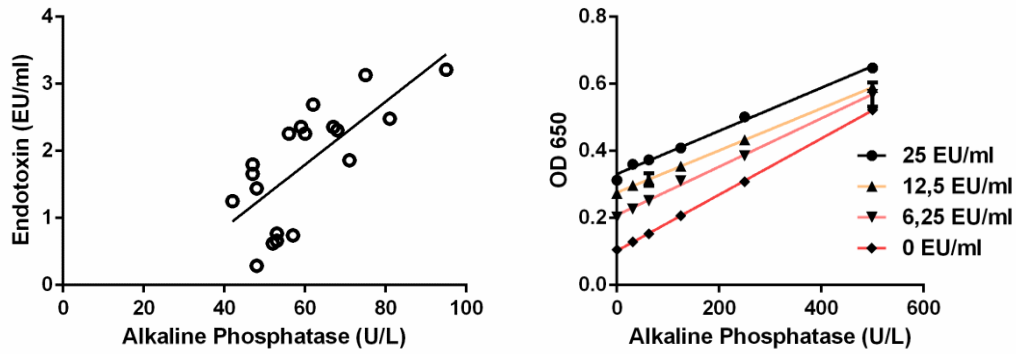
The first step in the adaptation was to achieve reliable quantification. A series of different culture conditions were tried and ultimately a density of  $5 \times 10^4$  cells per well in a 24 well plate with a 24 hour regeneration time after splitting produced the best fit in the standard curve. For technical detail see “Method glossary” under “B. HEK-cell based LPS detection”. Standard curve was prepared with Endosafe® *E.coli* Control Standard Endotoxin (Charles River, Wilmington, USA) ranging from 50 to 3.125 EU/ml in a 1:2 dilution series. The adapted protocol improved recovery rate compared to LAL assay. Seven healthy serum samples were spiked with 12.5 and 6.125 EU/ml and  $99.2 \pm 54.1\%$  were recovered. These rates can be improved by routinely handling the assay. For analysis, only batches with a recovery between 70 and 130% were used. This assay is not usable for aqueous solutions, no usable signal or no signal at all was recorded. Standard curves are shown in Figure 38.



**Figure 38: Exemplary standard curves assessed with adapted HEK-cell protocol** in water (left) or serum (right); curves in serum reach a coefficient of determination of 0.9924. Linear regression was done with the standard curves in water and reached coefficients of determination of 0.5155 (violet) or 0.0025 (blue).

## Dealing with alkaline phosphatase (AP)

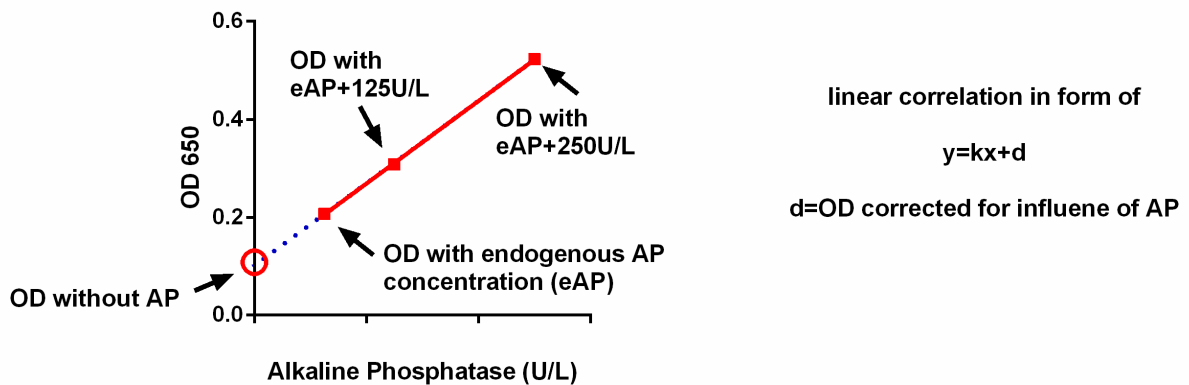
HEK-Blue™ LPS Detection Kit is sensible to AP. In assumingly endotoxin free, healthy controls endotoxin measurements show a strong correlation to AP levels ( $R^2=0.518$ ,  $p=0.0005$ ). Experiments with healthy serum plus endotoxin and AP spikes showed that with constant endotoxin levels, the influence of AP on the assay is linear. Correlations are shown in Figure 39. In addition, recombinant AP shows a linear association with photometric changes in cell free detection medium (data not shown).



**Figure 39: Correlation between assessed endotoxin levels and alkaline phosphatase concentration** in 21 assumingly endotoxin-free, healthy controls (left); Linear association between colour changes and alkaline phosphatase concentrations with constant endotoxin levels (right);

### Prospective and retrospective trouble shooting

The obvious influence of AP represents a fundamental weakness of this assay. Therefore two possible approaches were established to correct the readout according to AP levels. Since the correlation between AP and changes in OD650 is very stably linear as seen in Figure 39, spiking the samples with two different concentrations of recombinant AP allows calculating the unbiased optical density respective to 0U/L AP. The concept of this calculation is outlined in Figure 40.



**Figure 40: Calculation scheme to correct for the influence of alkaline phosphatase**

In short: serum is spiked with an equivalent of 125 and 250 U/L alkaline phosphatase and measured with the HEK-cell assay. Values for optical density at 650nm are plotted against the overall alkaline phosphatase concentrations. Linear regression is applied and the portion on the ordinate represents the corrected readout and can be used for endotoxin assessment.

This calculation technique works very well in patients with liver cirrhosis or healthy controls. However, in other liver diseases where AP concentration in the blood exceeds 500U/L, the signal from the AP is overpowering any signal coming from endotoxin. It is therefore not possible to use this technique in these patient cohorts. On the other hand, if using it on healthy controls with different AP levels, optical density can be matched almost perfectly, as seen in the following

example. Two healthy controls are given: one with an AP level of 152U/L and the other one with 140U/L. Both samples were spiked with endotoxin (25, 12.5 and 6.25 EU/ml) and measured with the cell-based assay using AP spikes. All data points were corrected according to the scheme in Figure 40. One control was used as a standard curve for the other one. Recovery rates were excellent ( $99.5\pm 5.1\%$ ). This method has also been successfully applied in healthy controls and PCOS patients (unpublished data).

The retrospective correction is applicable only when a small sample volume is available or the measurements were already performed without AP spikes. Here, the association between AP and Endotoxin assessments (more specifically  $y=0.047x+0.0204$  as shown in Figure 39) were used to correct for (known) AP levels. The principal is rather simple: If  $x$  in the equation stands for the AP concentration, the corresponding  $y$  will reflect the optical density that this sample should induce if it were endotoxin free. The difference between this value for optical density and the actually measured optical density (=residual) equals the change in optical density caused by endotoxin in the sample. Some of the samples can yield negative differences. The cause for this discrepancy is twofold. First, the correlation between AP and optical density in the endotoxin free, healthy controls is not perfect. It explains about half of the variation. While a big part of the unexplained variation should be caused by endotoxin levels, it is possible that there are other confounders that are not yet recognized (e.g. cytokine levels). And secondly, this assay is cell based which implies a certain biological variability. However, negative residuals are set to zero and only positive residuals are considered endotoxin positive. In the presented healthy controls the variability was so small that only traces of endotoxin could be found ( $<3.125\text{EU/ml}$ ). This method was also applied to the cirrhotic patient cohort of the clinical study presented in Chapter 1. While the absolute endotoxin levels were reduced, the correction did not change the relative results (probably also because AP levels did not exceed 500U/L and did not change over time).

### **Killing and Growth Retardation Assay**

The ability of the body to kill or inhibit the growth of bacteria in the blood or tissue has long been of interest in liver disease. Dysfunctional or downregulated complement factors have been described in alcoholic cirrhotics,(70) and the downregulation of antimicrobial peptides (AMP) in the intestine has been recently shown to play a pivotal role in the genesis of alcoholic liver disease.(142) Even though the issue has been recognized, the methodological advancements are limited. Only few protocols are published that allow the examination of serum killing capacity. They either involve the addition of complement factors,(143) are time- and work-intensive,(144) or are optimized for bacteria not able to grow in a liquid culture.(145) The problem in establishing an easy to handle protocol that reflects on the physiological properties of serum without additives might in fact lie in the choice of bacteria. Not all strains are vulnerable to AMPs. It has been

suggested that commensals in the gut harbor a resistance gene that allows them to survive and grow in the AMP rich environment of the intestine, for example.(146) The *E.coli* strain XL1blue is a serum sensitive strain and is therefore used in the Killing and Growth Retardation Assays described below.

### **Serum Killing Assay**

Serum Killing Assay is an adaptation of the killing assay proposed in the 1970s(147) that was established to test the intracellular killing of leucocytes and similar protocols have been published recently.(144, 145) The principle of the Serum Killing Assay is quite straight forward: Mix serum with a defined amount of bacteria, incubate at 37°C and plate the surviving bacteria for quantification. Technical details are given in “Serum Killing capacity” in the “Method glossary” of the method section.

The most important detail in this assay is the use of the correct amount of bacteria. Optical density at 600nm is used to estimate the biomass in the bacterial suspension. To ensure that most of the change in optical density comes from live bacteria, a 1:200 dilution of an overnight culture is grown to log phase for 2 hours before the bacteria are used. Results obtained from the Serum Killing Assay are well comparable to neutrophil phagocytosis, revealing a serious breach of the immune system in hepatitis C induced cirrhosis. This is outlined in “B. Liver cirrhosis  $\neq$  liver cirrhosis” under “Chronic hepatitis C as an oppressor of neutrophils”. However, it also highlights the limitations of the assay. While healthy serum can routinely clear the used amount of bacteria, plates of hepatitis C patients are generally overgrown. Reducing the amount of bacteria used in the test would result in the loss of accuracy of lower values and patients with better yet impaired killing capacity and healthy controls might not be distinguishable.

### **Growth Retardation Assay**

It was therefore necessary to improve the assay. In order to do that, optical density is utilized to monitor the growth of bacteria in the presence and absence of serum. Technical details are given in the “Method glossary” under “Growth retardation assay”. The principal of the assay is similar to the Serum Killing Assay but the readout is changed. In short: Bacteria are brought into log-phase by adding 100 $\mu$ l of the suspension into a sterile 96-well microtiter plate followed by 2 hours of incubation at 37°C. All the bacterial suspension needed for the test is prepared in one flask to avoid batch variation. Two wells are filled with fresh LB-broth to assure sterility of the growth medium. Serum is thawed at room temperature and diluted 1:10 in fresh LB-medium. After 2 hours of incubation 100 $\mu$ l of medium or serum dilution are added in duplicates to the plate so that the following information are obtainable:

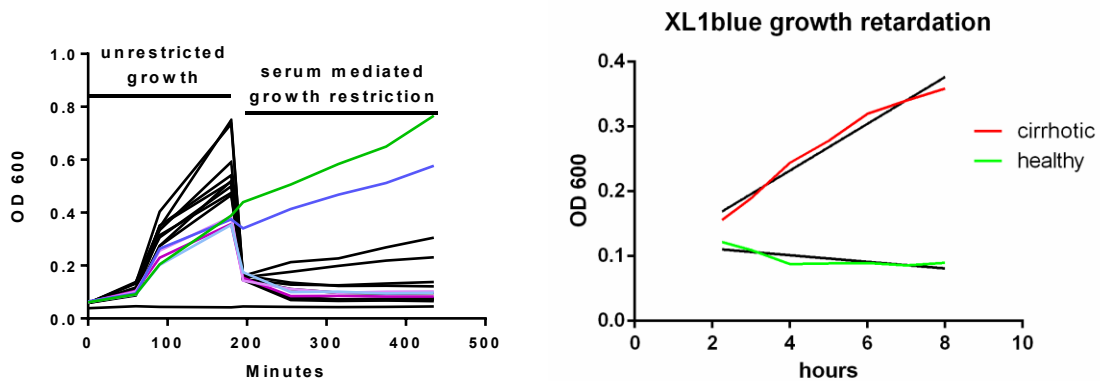
- negative control: add 100µl of fresh LB-broth to sterile wells containing only medium; this excludes possible changes of optical density due to contaminations;
- positive control: add 100µl of fresh LB-broth to bacteria in log phase; this provides undisturbed growth kinetics of the tested bacterial strain;
- test tubes: add 100µl of serum dilution to bacteria in log phase; changes in optical density provide information about the growth retardation facilitated by the serum;
- serum sterility: if there is enough serum sample another control can be established by adding serum dilutions to sterile LB-broth; this provides information about the sterility of the sample;

### **Measurements:**

Photometric measurements at 600nm are used to estimate the biomass in the solutions. Plates are read before and after the 2 hours log-phase incubation, immediately after adding the serum/controls, every one to two hours thereafter. In total, a test time of seven hours including log-phase incubation is sufficient for analysis. Plate is mixed in the photometer before assessment. Time since the first measurement is recorded at each read. Plate is incubated at 37°C during the measurements. Loose lids are used to avoid contamination, plates are not sealed (aerobic culture).

### **Analysis**

OD600 is plotted against the recorded time. Steep increase of OD should be observable in the first two hours followed by an incision after adding clear serum dilutions or medium. This second nadir represents the origin of a linear regression line fitted through the following data points. The slope of the individual regression line is used to compare growth retardation (for illustration see Figure 41). Small or negative values reflect intact killing capacity; higher values indicate impairment of the same. It is possible that the incision in optical density after adding the serum is not as pronounced when the sample is hemolytic, icteric or lipemic.

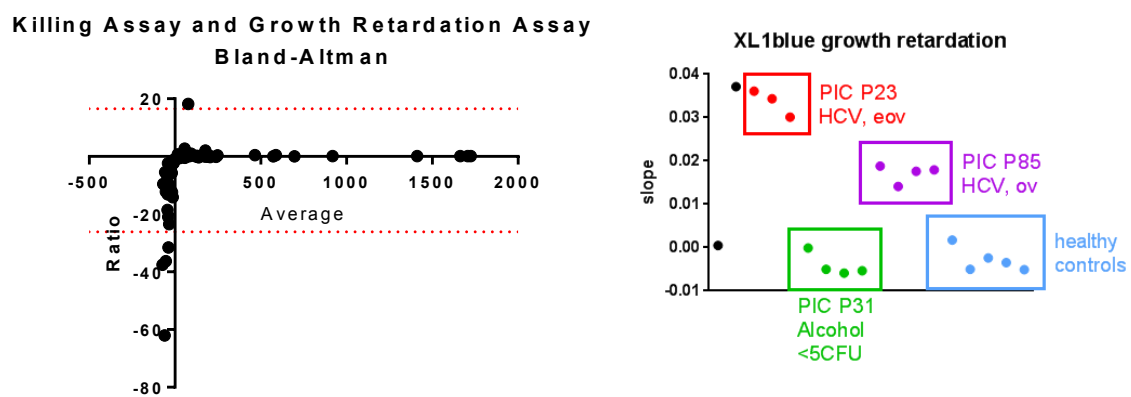


**Figure 41: Changes in optical density over time as measurement of bacterial growth in the presence and absence of serum**

Left figure shows the incision in optical density when serum is added after a period of unrestricted growth. Right figure illustrates a possible way to analyse the readout. Data points after the addition of serum are used to fit a linear regression curve. It is obvious that the optical density in case of the cirrhotic serum (lipemic) is not as reduced as by the healthy sample.

### Comparability to Serum Killing Assay

The results from the Growth Retardation Assay are well comparable to the Serum Killing Assay when the killing capacity is quite low (high values). When only a few CFU were found in the Serum Killing Assay and/or the readout of the Growth Retardation Assay was negative, relatively high deviations were found.



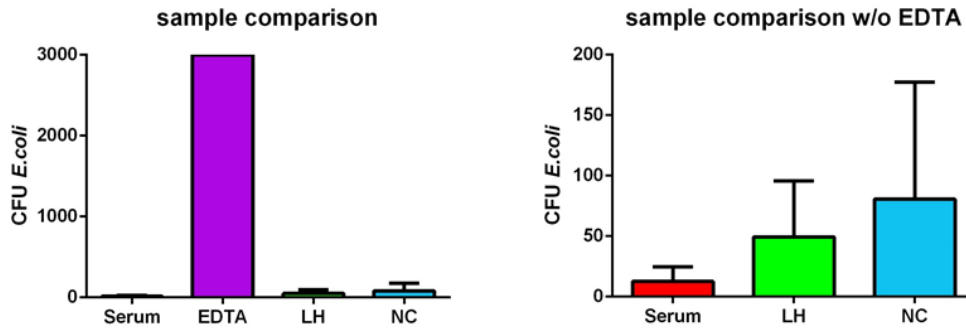
**Figure 42: Comparison between serum killing capacity and growth retardation assay**

Bland Altman plot for Serum Killing and Growth Retardation Assay (left) and general comparison of the two assay by representative examples; points represent the Growth Retardation readout and written in colour in the boxes are the results from the Serum Killing Assay. Eov stands for extremely overgrown dishes, ov for over grown dishes (right). Data was collected from the serum of 78 cirrhotic patients measured by both assays.

### Sample material

Serum, EDTA-, lithium heparin and sodium citrate plasma have been trialled for the assay. XL1blue is sensitive to all of the listed material except for EDTA-plasma in both assays. Results from Serum Killing Assay are given exemplary in Figure 43. It is clearly shown that EDTA-treated plasma is not able to kill the bacteria. Serum and the remaining plasma types are all capable but not comparable to each other. Therefore, the decision to use one of the tested materials has to be consistent.

Serum samples can undergo several freeze-thaw cycles and keep a stable killing capacity. Both assays were tested for their stability through repeated use of a sample. For the Growth Retardation Assay, samples thawed up to five times have been tested and results stayed consistent (data not shown).



**Figure 43: Comparison of Serum Killing capacity of serum and various types of plasma of four healthy controls. LH stands for lithium heparin, NC for sodium citrate.**

### Differential sugar absorption test

The test principle is based on ingestion and urinary recovery of sucrose, lactulose and mannitol. Sucrose passes the first part of the digestive system as a disaccharide and is subsequently cleaved into glucose and fructose in the jejunum. Therefore, a non-physiological translocation through a leaky gastric or duodenal epithelium can be assumed if intact sucrose molecules are detected in the urine. This way, sucrose recovery serves as a marker for gastroduodenal permeability.(28) Mannitol is a naturally occurring sugar in nutritious plants and animals but is also substituted in pharmaceutical products. Mannitol is either taken up paracellularly in the small intestine or metabolized by gut commensals. If it enters the body, mannitol is efficiently cleared from the blood stream and excreted through the urine in its original form. It has been suggested that mannitol uptake is dependent on the intestinal surface and morphological alterations in the intestine of cirrhosis patients might influence its recovery during the test. Lactulose is a non-absorbable, osmotically active disaccharide (fructose and galactose) and is frequently used as a laxative. If the gut barrier is weakened, lactulose can enter the blood stream through the small intestine similar to mannitol and since the human body cannot metabolize it, it is found in its original form in the urine.

This test has some drawbacks in liver disease. Mannitol and lactulose are steady components in the medication of our study cohort. The following pharmaceuticals containing mannitol were prescribed during the study and consequently also at the time of the differential sugar absorption tests: Acecamp (AstraZeneca, London, United Kingdom), Acemin (AstraZeneca, London, United Kingdom), Cal-D-Vita (Bayer, Leverkusen, Germany), Plavix (Sanofi Winthrop Industrie, Paris, France), Clonidin (Ratiopharm, Ulm, Germany), Lansoprazol (Laboratorios Liconsa, Madrid, Spain), Legalon (Madaus GmbH, Monza, Italy), Lisinopril (Salutas Pharma, Barleben, Germany), Pantoloc (Nycomed Oranienburg, Oranienburg, Germany), Pantoprazol (Hennig Arzneimittel GmbH&Co. KG, Floersheim/Main, Germany), Paspertin (Recipharm, Paretz, Sweden), Thyrex (Sandoz, Holzkirchen, Germany), Tratul (G.L.Pharma GmbH, Lannach, Austria), Dancor (Merck

KGaA, Darmstadt, Germany), Deflamat (Temmler, Marburg, Germany), Lamotrigin (G.L.Pharma GmbH, Lannach, Austria), Madopar (Roche Austria, Vienna, Austria), Paroxetin (Salutas Pharma, Barleben, Germany), Pramipexol (Salutas Pharma, Barleben, Germany), Pram (G.L.Pharma GmbH, Lannach, Austria), Supradyn plus (Delpharm Gaillard, Gaillard, France). Some medications also contained sucrose: Aldactone (Roche Austria, Vienna, Austria), Carvedilol (Pfizer Corporation Austria, Vienna, Austria and STADA Arzneimittel GmbH, Vienna, Austria), Cymbalta (Lilly, Indianapolis, USA), Noctor (Montavit, Absam, Austria), Enbrel (Wyeth Pharmaceuticals, Dallas, USA), Nervenruh forte (Roesch&Handel, St. Anton/Arlberg, Austria). Lactulose is present in Lasilacton (Famar L'Aigle, L'Aigle, France) and of course Laevolac (Fresenius Kabi, Graz, Austria). Although the patients were instructed not to take any medication the morning before the test, varying amounts of these sugars were found in the baseline urine collected prior to the sugar ingestion. There are several possible explanations for this disturbance. Firstly, there is the possibility that the patients ignored the instructions not to take their medication before the test. Secondly, it is possible that the reduced renal function in our patient cohort slowed down the excretion of the sugars that were ingested outside of the test. Thirdly, errors in the procedure are plausible since the patients had immense difficulty understanding how the test is performed. According to the nuclear magnetic resonance analysis, the following errors were made:

Patients did not collect baseline urine and filled both sample tubes with the urine collected after the sugar solution was ingested (2.9% of tests). In this case it was assumed that there was no sugar present and the sugar concentrations in the collective urine were used in uncorrected form.

Patients did not drink the sugar solution (2.9% of tests). Tests without the ingestion of the sugar solution were excluded from the analysis. The missing values were replaced with last-observation-carried-forward.

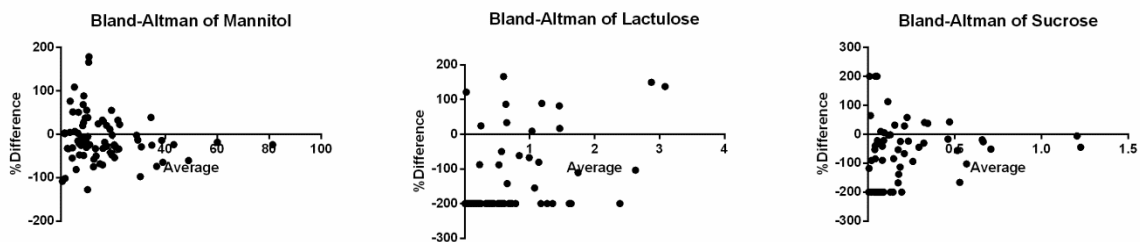
Patients confused the vials for baseline and collective urine (3.3% of tests). In this case, values were assumed to the other time point and analysed as planned.

Patients did not record the collected urine volume (22.8% of tests). When the urine volume was missing, it was substituted with the median volume of the respective time point (a learning process in the use of the test was assumed).

Patients did not fast or took their medication (0.8% of tests). Post-prandial patients were excluded and missing values were replaced using last-observation-carried-forward.

Initially the test was evaluated by high performance liquid chromatography (HPLC). See also "Parameters measured by collaborators" in the "Method glossary". However the measurements were not reliable, probably caused by a broken HPLC column. The instrument could not be repaired and therefore the evaluation was switched to nuclear magnetic resonance (NMR). See also

“Parameters measured by collaborators” in the “Method glossary”. Since the measurements with NMR and HPLC were not comparable as seen in Figure 44, all tests were evaluated a second time by NMR. All data presented in this thesis were measured by NMR.



**Figure 44: Comparison of mannitol, lactulose and sucrose recovery measured by HPLC and NMR**  
As seen in the plots, the differences can be up to 200%

## Method glossary

### Sample preparation

Blood samples were collected in pyrogen-free tubes (VACUETTE®, Greiner Bio-One, Kremsmuenster, Austria). Serum was kept on room temperature until clotting, plasma tubes were kept at 4°C until all tubes were centrifuged at 2000xg for ten minutes at 4°C. Plasma and serum was frozen at -80°C in 1ml aliquots in pyrogen-free cryotubes (Eppendorf, Hamburg, Germany). Blood sampling equipment and cryotubes were tested negative for endotoxin by LAL assay and HEK cell-based assay.

### Phagocytosis

Phagocytosis capacity and activity were quantified using Phagotest (Glycotope, Heidelberg, Germany). For human whole blood the test was used according to manufacturer's instructions. In short, 100µl of heparinized whole blood was challenged with 20µl of FITC-labelled *E.coli* (opsonized, unless stated otherwise) and incubated at 37°C for 10 minutes. Fluorescent signals from non-engulfed bacteria were then quenched with 100µl quenching solution. After washing cells twice erythrocytes were lysed for 20 minutes and washed twice. Before measurement, cells were re-suspended in 200µl DNA staining solution. Acquisition was done on LSRII (BD, New Jersey, USA).

For isolated neutrophils,  $5 \times 10^5$  to  $10^6$  cells were challenged with 10µl of bacteria, incubated for 10 minutes at 37°C, quenched, and washed twice before acquisition. No DNA staining was used.

For murine whole blood the test was adapted as follows: 25µl of heparinized whole blood were challenged with approximately  $10^6$  live *E.coli* expressing green fluorescent protein (GFP). For culture condition see "*E. coli* growth conditions". After incubating at 37°C for 20 minutes, extracellular GFP signal was quenched with 50µl trypan blue (Invitrogen, Carlsbad, USA) and cells were then washed twice with sterile PBS (Fisher Bioreagents Fishes Scientific, Pittsburgh, USA). Erythrocytes were lysed using red blood cell lysis buffer (Sigma-Aldrich, St. Louis, USA) for 20 to 30 minutes at room temperature. After washing with PBS, cells were re-suspended in 150µl PBS and acquired on an Accuri C6 (BD, New Jersey, USA).

Centrifugation steps were done at a speed of 350xg at 4°C. Incubations at 37°C were facilitated in a water bath. Serving as a negative control, a duplicate tube was used parallel to the test tube. It was treated exactly the same but left on ice during phagocytosis period. Analysis was done using FlowJo 7.6.5 (Tree Star Inc, Ashland, USA) and FCS 2.0 files. Cells were identified by forward-side scatter characteristics. Gating strategy is outlined in "C. Methods in liver disease research" under Phagoindex - Gating strategy

### **Oxidative burst**

Oxidative burst function was quantified using Phagoburst (Glycotope, Heidelberg, Germany). The test was used according to manufacturer's instructions. In short, 100µl of heparinized whole blood was challenged with 20µl of washing solution, N-Formylmethionine-leucyl-phenylalanine (fMLP), phorbol 12-myristate 13-acetate (PMA) or heat inactivated *E.coli* quantifying resting burst, priming state, high burst and oxidative burst, respectively. Cells were incubated at 37°C for 10 minutes. Dihydrorhodamine 123 acts as a substrate that is converted into a fluorescent molecule by reactive oxygen species. After lysing erythrocytes for 20 minutes at room temperature, cells were washed and re-suspended in 200µl of DNA staining. Acquisition was done on LSRII (BD, New Jersey, USA). Centrifugation steps were done at a speed of 350xg at 4°C. Incubations at 37°C were facilitated in a water bath. Cells were identified by forward-side scatter characteristics. Analysis was done using FlowJo 7.6.5 (Tree Star Inc, Ashland, USA) and FCS 2.0 files. Using the resting burst test tube, a gate distinguishing between FITC positive and negative cells was set. The gate was placed at the right border of the light blue density marker in a pseudo-colour plot with FITC on the abscissa and side scatter on the ordinate and transferred to all related tubes.

### **Calprotectin**

Calprotectin was measured in stool using the PhiCal Calprotectin ELISA Kit (Immundiagnostic AG, Bensheim, Germany) and in serum using IDK Calprotectin ELISA Kit (Immundiagnostic AG, Bensheim, Germany). Both sandwich ELISAs were done according to manufacturer's instructions. Calprotectin was detected by two selected monoclonal antibodies. Serum was diluted 1:100, it was not necessary to adjust this dilution. Stool samples were brought to room temperature and 15mg of feces were extracted with a stool sample tube (Immundiagnostic AG, Bensheim, Germany). Patients were randomized to a plate to avoid batch variations. All time points of one patient were measured on the same plate to keep them most comparable. For serum samples internal standards were run additionally for validation.

### **Zonulin**

Zonulin was measured in stool and serum using a competitive IDK Zonulin ELISA kits for stool and serum, respectively. These kits use a polyclonal antibody to detect zonulin. Serum samples were diluted 1:20, it was not necessary to adjust this dilution. Stool samples were brought to room temperature and 15mg of feces were extracted with a stool sample tube (Immundiagnostic AG, Bensheim, Germany). Patients were randomized to a plate to avoid batch variations. All time points of one patient were measured on the same plate to keep them most comparable. For serum samples internal standards were run additionally for validation.

### **Serum Killing capacity**

This assay was done with *E. coli* XL1blue (graciously provided by Dr. Martina Geier, Institute of Molecular Biotechnology, Graz University of Technology) Serum samples were thawed and vortexed. A 1:4 dilution with sterile PBS was prepared in autoclaved tubes (Eppendorf, Hamburg, Germany) and placed on ice. Ice cold bacterial suspension (preparation see above) was vortexed vigorously for at least 30 seconds and 100µl were added to the serum dilution. The mixture was kept on ice until all samples were prepared, then vortexed and placed in a 37°C water bath for 20 minutes. After incubation tubes were placed on ice immediately, and vortexed before 100µl were plated in duplicates on LB-Agar (Sigma-Aldrich, Vienna, Austria) prepared in 100x15mm Petri dishes (BD Biosciences, New Jersey, USA) containing 15ml of LB-Agar. For plating, 5-10 sterile glass beads with a diameter of 3mm (Medika, Graz, Austria) were placed on the cold agar and then the serum-bacteria mixture was added. By random shaking, the glass beads bounced off the walls of the dish and thereby distributing the liquid equally on the plate. Afterwards, the plates were turned with the agar side up and incubated overnight at 37°C. After incubation, plates were placed on room temperature and visible colonies were counted as assay outcome. The higher the amount of bacterial colonies on the plate, the weaker was the serum killing capacity.

### **Growth retardation assay**

This assay was done with *E. coli* XL1blue (graciously provided by Dr. Martina Geier, Institute of Molecular Biotechnology, Graz University of Technology) Serum samples were thawed and vortexed. A 1:10 dilution in sterile LB-broth (Sigma-Aldrich, Vienna, Austria) was prepared in autoclaved tubes (Eppendorf, Hamburg, Germany). Overnight culture of *E. coli* (growth conditions see below) was diluted 1:20 in fresh LB-broth. Then, 100µl of this solution were pipetted into the wells of a sterile 96-well plate (Thermo Scientific, Wilmington, USA) and incubated for 2 hours at 37°C with a loose lid to cover the plate. The plate was read before and after the incubation in a Spectrostar Omega (BMG Labtech, Ortenberg, Germany) at 600nm. Afterwards, 100µl of serum dilutions or LB-broth were added in duplicates to the bacteria, including positive and negative growth controls (for details see “C. Methods in liver disease research” under “Killing and Growth Retardation Assay”). The plate was read in the photometer again and incubated at 37°C for one hour. Measurements were repeated every hours until six timepoint after the addition of serum were acquired. In the meantime, the plate was always covered and incubated at 37°C. For details on how to analyse and interpret the data see “C. Methods in liver disease research” under “Killing and Growth Retardation Assay”.

### ***E. coli* growth conditions**

Bacteria were kept in Glycerol (Merck, Darmstadt, Germany) and LB-broth (Sigma-Aldrich, Vienna, Austria) at -80°C. For the overnight culture a sterile pipette tip was scratched over the

frozen surface of the glycerol stock of bacteria and dropped into 5ml of autoclaved LB-broth (20g/l) in a sterile 50ml Falcon tube (BD Biosciences Europe, Heidelberg, Germany). The tube was loosely closed with adhesive tape and incubated overnight at 37°C at 130-180rpm shaking. The next morning 500µl of the overnight culture was inserted in 200ml of fresh LB-broth in an autoclaved Erlenmeyer flask. The flask was loosely covered with sterilized tin foil and placed in a tightly fitting metal ring of the incubator (37°C, 130-180rpm). Bacteria were grown to log phase for 2 hours. After the incubation 50ml of the bacterial suspension were placed in a sterile 50ml Falcon tube and vortexed vigorously for at least 30 seconds. With a sterile pipette two times 200µl of the broth were transferred in a 96-well microtiter plate (Nalgene/Nunc, Rochester, USA) for photometric measurements (600nm). The remaining bacteria were spun down (4600xg, 10min, RT), supernatant was discarded and pelleted bacteria were re-suspended in sterile PBS in a density of 10<sup>8</sup> CFU/ml. Placed on ice immediately bacterial suspension keeps a stable CFU count for several hours.

### **Differential sugar absorption test**

Differential sugar absorption test is a widely used functional gut permeability test. For this study 20g sucrose (Sigma-Aldrich, Vienna, Austria), 10g lactulose (Laevolac, Fresenius Kabi, Graz, Austria) and 5g mannitol (Sigma-Aldrich, Vienna, Austria) were dissolved in 100ml of tap water. After overnight fasting, participants sampled the first urine of the day, drank the sugar solution and started to collect urine for the following 5 hours. Participants continued fasting until the test was over, only water and unsweetened tea was permitted. Samples of both urine fractions were frozen at -80°C with 1mg thimerosal (BioXtra, Sigma-Aldrich, Vienna, Austria) per ml urine.

Sugars were detected in the urine by high performance liquid chromatography (HPLC) and nuclear magnetic resonance (NMR). A comparison between the methods is given in “C. Methods in liver disease research” under “Differential sugar absorption test”. Assessment was outsourced, for details see “Parameters measured by collaborators”.

### **Diamine oxidase (DAO)**

DAO was measured by commercially available ready to use sandwich ELISA (Immundiagnostik AG, Bensheim, Germany) according to manufacturer’s instructions. DAO was detected by a polyclonal antibody. Serum samples in a 1:5 dilution were used. If values were out of range dilutions were adjusted stepwise up to 1:200. Patients were randomized to a plate to avoid batch variations. All time points of one patient were measured on the same plate to keep them most comparable.

## **Endotoxin/Lipopolysaccharide**

### ***A. Limulus amoebocyte lysate (LAL) assay***

Kinetic chromogenic LAL assay Endochrome-K™ (Charles River, Wilmington, USA) was used. Pyrogen free collected serum was diluted 1:10 in endotoxin free water in sterile glass tubes (Charles River, Wilmington, USA). Serum opsonisation of LPS was heat inactivated at 75°C for 30 minutes. A 1:10 serial dilution of Endosafe Endotoxin standards ranging from 50EU/ml to 0.005EU/ml were used for quantification. Standards, blanks and samples were pipetted in to a pyrogen free 96-well plate and spiked with 50µl of substrate solution. Data acquisition started immediately at 405nm every 10 minutes using a Spectrostar Omega (BMG Labtech, Ortenberg, Germany) at 37°C. Analysis was based on slope differences. Reagents, tubes and plates were all obtained f

### ***B. HEK-cell based LPS detection***

HEK-Blue™ LPS Detection Kit 2 (Invivogen, Toulouse, France) was adapted from a qualitative to a quantitative LPS detection tool. All reagents are provided in the Kit unless stated otherwise. HEK-Blue cells were cultured in DMEM containing 10% FCS, Normocin, and Selection-solution provided within the Kit. Cells were grown to a confluency of approximately 80% in a 75cm<sup>2</sup> culture flask (Corning, New York, USA) and detached using 1x 0.5% Trypsin-EDTA (Gibco Life Technologies, Carlsbad, USA). Cells were then seeded in 24-well plates (Nalgene/Nunc, Rochester, USA) at a density of 5x10<sup>4</sup> cells per well in 2ml selection medium and incubated for 24 hours for recovery. Medium was then removed and replaced by 950µl detection medium (provided within the Kit) and 50µl standards, blanks, spikes and samples. A 1:2 serial dilution of Endosafe Endotoxin standards (Charles River, Wilmington, USA) ranging from 50EU/ml to 3.13EU/ml were used for quantification. Each sample was measured in triplicates to correct for cell based biodiversity. Samples remained on the cells for 21 hours. In that time the expressed secreted embryonic alkaline phosphate (SEAP) induced a colorimetric change in the detection medium. Photometrical assessment of the supernatant in each well in technical duplicates at 650nm allows for quantitative LPS detection.

For more details and a systematic comparison between the two methods see “C. Methods in liver disease research” under “Endotoxin”. Endotoxin for the clinical study was measured using Hek-cell based LPS-detection.

### **Soluble cluster of differentiation (sCD) 14**

sCD14 was assessed by Human sCD14 Quantikine ELISA kit (R&D Systems, Minneapolis, USA), a sandwich ELISA using a monoclonal antibody against human CD14. Lithium heparin plasma was

diluted 1:200. Adjustment of the dilution was not necessary. Patients were randomized to a plate to avoid batch variations. All time points of one patient were measured on the same plate to keep them most comparable.

### **Lipopolysaccharide binding protein (LBP)**

LBP was measured by HK315 Human LBP ELSIA kit (Hycult biotech, Uden, The Netherlands) according to manufacturer's instructions. LBP levels were measured in EDTA plasma in a 1:1500 dilution.

### **Total DNA isolation, 16S library preparation and sequencing**

DNA extraction from stool and 16S sequencing was outsourced to the core facility for molecular biology at the centre for medical research in Graz. The following description was provided by Ingeborg Klymiuk:

“Total DNA from frozen stool samples was isolated with the MagnaPure LC DNA Isolation Kit III (Bacteria, Fungi) (Roche, Mannheim, Germany) according to manufacturer's instructions. Samples were thawed and homogenized in 500µl of bacterial lysis buffer (Roche, Mannheim, Germany). 250µl of homogenized stool sample was transferred to a Magna Lyser green bead tubes (Roche, Mannheim Germany) and bead beaten for mechanical lysis at 6500 rpm for 30 seconds two times in a MagNA Lyser Instrument (Roche, Mannheim Germany). After mechanic lysis enzymatic lysis with 20µl lysozyme at 37°C for 30 minutes and 30µl Proteinase K for 1.5 hours at 65°C was performed. Proteinase K was heat inactivated at 95°C for 10 minutes. The remaining steps were performed according to instructions from the Magna Pure DNA isolation kit and 250µl of the sample were used for DNA purification in a MagnaPure instrument. Total DNA was eluted in 100µl elution buffer and stored at -20°C until PCR amplification. For target specific PCR amplification of hypervariable regions the primers 27F and R357 were used and synthesized at Eurofins (MWG, Ebersberg, Germany) (27F-AGAGTTTGATCCTGGCTCAG; R357-CTGCTGCCTYCCGTA). 2µl of total DNA were used in a 25µ PCR reaction in triplicates with containing 1 x Fast Start High Fidelity Buffer (Roche, Mannheim, Germany), 1.25 U High Fidelity Enzyme (Roche, Mannheim, Germany), 200 µM dNTPs (Roche, Mannheim, Germany), 0.4 µM barcoded primers and PCR-grade water (Roche, Mannheim, Germany). Thermal Cycling 95°C for 3 minutes, 30 cycles: 95°C for 45 seconds, 55°C for 45 72°C for 1 minute, final extension at 72°C for 7 minutes. Triplicates were pooled, checked on a 1% agraose gel and 15µl of pooled PCR product were normalized according to manufacturer's instructions on a SequalPrep Normalization Plate (Life Technologies, Carlsbad, USA). 15µl of the normalized PCR product were used as template for indexing PCR in a 50µl single reaction (composition as described for the targeted PCR) to introduce barcode sequences to each sample (according to Kozich et al. 2013). Cycling conditions were the same as for the targeted PCR with only 8 cycles for amplification. After

indexing 5µl of each sample were pooled, 50µ of the unpurified library were loaded to a 1% agarose gel and purified from the gel with a Qiaquick Gel Extraction Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. The pool was quantified using the QuantiFluor ONE dsDNA Dye on the Promega Quantus instrument according to manufacturer's instructions and size of sequencing library was validation on an Agilent 2100 Bioanalyzer (Agilent) using a high sensitivity DNA assay according to manufacturer's instructions. The pool containing all samples was run at 6pM final concentration with version 3 600 cycles chemistry (Illumina, Eindhoven, Netherlands) according to manufacturer's and with 20% PhiX control DNA (Illumina, Eindhoven, Netherlands). FASTQ files were used for data analysis."

### **Analysis of 16S sequencing data**

Raw sequence data in fastq format were prepared for analysis using Mothur MiSeq SOP as described before.(148) In short: Sequence pairs (forward and reverse) were united and low quality reads (assembly errors, ambiguous bases) were removed. Identical sequences were merged and aligned using Silva-based bacterial references. Chimeric and non-bacterial sequences were excluded from analysis.

High quality pre-clustered reads were transferred to qiime. For statistical analysis "core diversity analysis" based on non-phylogenetic diversity was performed. Reads were normalized to 12552 randomly chosen data points. Chao 1 index was used to analyse alpha rarefaction and diversity. Beta diversity analysis between groups and over time was based on Bray Curtis index. Kruskal-Wallis tests were performed to compare groups. False discovery rate correction (FDR) was applied for multiple testing allowing for 5% of false discoveries. Significant differences were confirmed for paired data with Friedman tests using SPSS 23 (IBM Corporation, Armonk, USA).

### **Cytokines**

Cytokines in the clinical study were measured by ProcartaPlex® Multiplex Immunoassay (Affimetrix, Vienna, Austria) exactly according to manufacturer's instructions. A custom panel of 10 cytokines and chemokines was prepared by Affimetrix in a Mix&Match Assay.

### **Plasma**

Cytokines (TNF $\alpha$ , IL1 $\beta$ , IL6, IL8, IL10, IFN $\alpha$ , IFN $\beta$ , IFN $\gamma$ , IP10 and MIG) were assessed in undiluted sodium citrate plasma. Plasma was thawed on ice and used immediately after thawing. Beads were washed using a handheld magnetic separator block (Biorad, Hercule, USA). Standards and samples were mixed 1:1 with assay buffer before they were pipetted on the plate. Plates were assessed directly after the preparation on a Biorad Bioplex 200 (Biorad, Hercule, USA).

### ***Ex vivo cytokine stimulation***

100µl of heparinized whole blood were stimulated with 1ml of sterile PBS (Sigma-Aldrich, Vienna, Austria) containing 0.5µg of endotoxin (Sigma-Aldrich, Vienna, Austria). After careful vortexing, the blood was incubated in a water bath at 37°C for 4 hours in a 3ml FACS tube (Corning, New York, USA). After incubation, supernatant was carefully removed and transferred to a 1.5ml cryotube (Eppendorf, Hamburg, Germany). Supernatant was frozen immediately and stored at -80°C until further use. For cytokine assessment (TNF $\alpha$ , IL1 $\beta$ , IL6, IL8, IL10, IL12p70, IFN $\gamma$ , IP10, MCP-1 and MIG), supernatant was thawed on ice and used immediately after. Beads were washed using a handheld magnetic separator block (Biorad, Hercules, USA). Standards and samples were pipetted undiluted on the plate. After the assay, plates were stored at 4°C in the dark and were assessed on a Biorad Bioplex 200 (Biorad, Hercules, USA) the next morning after 30 minutes of shaking (500rpm) according to manufacturer's instructions.

### **Neutrophil isolation**

#### ***A. Isolation of neutrophils with Polymorphprep***

Heparinized blood drawn into a Vacuette container (Greiner Bio-One, Kremsmünster, Austria) was used for neutrophil isolation via polymorphprep gradient. Polymorphprep (Axis-Shield Diagnostics, Dundee, UK) was placed in a 15ml Falcon tube (Corning, New York, USA) and blood was carefully layered over it in a 1:1 ratio. Gradient was centrifuged for 35 minutes at 500xg at room temperature with the breaks turned off. PBMC layer was removed and discarded. PMN layer and the underlying polymorphprep phase were taken up and placed into a new tube. Cell suspension was diluted with HBSS (Gibco Life Technologies, Carlsbad, USA) to a total volume of 10ml. After gentle mixing, cells were pelleted (250xg, 10min, 4°C) and erythrocytes were lysed with red blood cell lysis buffer (Roche Diagnostics, Rotkreuz, Switzerland) for 10 minutes rolling. After pelleting and washing the cells, they were re-suspended in 3 ml and counted using a TC20™ Automated Cell Counter (BioRad, Hercules, USA).

#### ***B. Isolation of neutrophils with Percoll***

Forty ml of venous blood was drawn from a 21G needle "Ecoflo" (Dispomed Witt oHG, Gelnhausen, Germany) into a 20ml syringe very slowly. Blood was poured into a 50ml Flacon tube (Corning, New York, USA) containing warm 3.8% sodium citrate solution (Carl Roth, Karlsruhe, Germany). Platelet rich plasma was produced by centrifugation at 270xg, for 20 minutes at room temperature with the breaks turned off. Plasma was separated from the cell pellet and spun down at 1000xg for 20 minutes, producing platelet poor plasma (PPP). The cell pellet was treated with 6 ml of a 6% Dextran 500 solution in 0.9% NaCl (Serva AL Labor Technik, Heidelberg, Germany) and filled with 0.9% NaCl (obtained from the hospital's pharmacy) to a total volume of 50 ml.

Solutions were mixed by rolling and inverting the tube carefully. Bubbles and droplets in the lid and surface were removed and the tube was left standing upright for 30 minutes. Supernatant was placed in a fresh tube and red cell pellet was discarded. Cells were pelleted by centrifugation at 185xg for 6 minutes at room temperature. In the meantime, Percoll gradient (Sigma-Aldrich, Vienna, Austria) was prepared in PPP (51% and 42%) and over-layered in a 15ml Falcon tubes. After centrifugation, supernatant was discarded and cell pellet was re-suspended in approximately 2ml of PPP and placed on top of the Percoll gradient. Leucocytes were separated by centrifugation at 225xg for 11 minutes. PBMC layer was discarded and PMN layer was taken up and placed into a fresh 50 ml Falcon tube. Cells were re-suspended in 2ml PPP and diluted in HBSS without Calcium and Magnesium (Gibco Life Technologies, Carlsbad, USA) to a final volume of 40 ml and counted using a TC20™ Automated Cell Counter (BioRad, Hercules, USA). Neutrophils can be pelleted by centrifugation at 420xg for 5 minutes and cultured for approximately 24 hours in RPMI 1640 (Gibco Life Technologies, Carlsbad, USA) containing 10% FCS Clone (PAA Laboratories GmbH, Pasching, Austria) or human serum. Isolated neutrophils were cultured in 96-well clear vinyl microplates (Corning, New York, USA) at 37°C with 5% CO<sub>2</sub>.

Both methods are suitable to test neutrophil function, however they are not directly comparable. Three blood donors were tested for non-phagocytic cells and phagocytic capacity (given as Phagoindex) of isolated neutrophils obtained from Polymorphprep and dextran/percoll isolation protocols. Data are presented as average fold change to the respective parameters measured in whole blood of the same donors. See Table 14.

**Table 14: Comparison between neutrophil isolation techniques**

	<b>Polymorphprep</b>	<b>Dextran/Percoll</b>
<b>Anticoagulant</b>	Lithium heparin	Sodium citrate
<b>Amount of blood used (ml)</b>	7	40
<b>Time used for isolation (min)</b>	80	100
<b>Viability</b>	56%	48%
<b>Isolated neutrophils/ml blood</b>	3.26x10 <sup>6</sup>	3.05x10 <sup>6</sup>
<b>Phagocytic capacity (PI)</b>		
given in average fold change to	0.41 (0.12-0.84)	0.41 (0.17-0.56)
whole blood values with range		
<b>Non-phagocytic neutrophils</b>		
given in average fold change to	52 (10-111)	31 (5-66)
whole blood values with range		

### **Oil-red-O staining**

Frozen sections (5µm thick) of the caudate lobe of the murine livers in OCT were cut at -20°C and used for the assessment of lipid accumulations in the liver using Oil-red-O staining. Sections were placed on glass slips and thawed for one hour before use. Tissue was rinsed twice with PBS (Fisher BioReagents, Pittsburgh, USA) for 5 minutes and once with 60% Isopropanol (Fisher Chemicals, Pittsburgh, USA) for 1 minute. Slides were carefully dried and immersed in filtered Oil-red-O working solution (1.05g of Oil red, 300ml of Isopropanol and 100ml of distilled water). After 5 minutes in distilled water, slides were dried and mounted with glycerol. Oil-red-O powder was purchased from Sigma-Aldrich (St. Louis, USA) and glycerol from Fisher BioReagents (Pittsburgh, USA).

### **Ex vivo clearance capacity for mice**

Blood was drawn from the retro orbital venous plexus using autoclaved glass pipettes (Fisherbrand Fisher Scientific, Pittsburgh, USA) lined with heparin. GFP positive, chloramphenicol resistant *E. coli* bacteria(149) were used for this assay. They were grown as stated above with the exception that the pellet was resuspended to a density of  $10^7$  CFU/ml. For the assay, 25µl of heparinized whole blood was mixed with 10µl of bacterial suspension (containing  $10^5$  CFU in total). After vortexing, 10µl were taken out and diluted  $1:10^4$  and  $1:10^5$  in sterile PBS (Fisherbrand Fisher Scientific, Pittsburgh, USA) and plated on Chloramphenicol positive LB-Agar plates (see above). The remaining blood was incubated for 20 minutes in a water bath at 37°C. After incubation, tubes were placed immediately on ice. Dilution and plating of 10µl sample were repeated as described above. Dishes were incubated overnight at 37°C. The difference between the visible colonies before and after incubation were expressed as percentages of inserted bacteria and analysed as outcome of this assay. Therefore, positive values represent good clearance and negative values represent bacterial growth.

### **RNA extraction and qPCR**

RNA was extracted from murine liver tissue taken from the right lobe and shock frozen immediately after harvest in liquid nitrogen. Frozen tissue (approximately 30µg) was submerged in 1ml of TRIzol (Invitrogen, Carlsbad, USA) and placed on ice. An electrical tissue homogenizer was used to emulsify the tissue. The nozzle was rinsed with TRIzol and twice with DEPC water (Sigma-Aldrich, St. Louis, USA) between every sample. Homogenized samples were transferred to autoclaved tubes (Eppendorf, Hamburg, Germany) and put on room temperature for 5 minutes. Phase separation was done by the addition of 200µl of Chloroform (Fisher Scientific, Pittsburgh, USA) and 15 seconds of hand-held shaking. After 2-3 minutes at room temperature, tubes were centrifuged at 12000xg for 15 minutes at 4°C. Tubes were removed carefully and 400µl of the upper, aqueous phase was transferred to a new tube containing 500µl of Isopropanol (Fisher

Chemical, Pittsburgh, USA) for RNA precipitation. Tubes were inverted several times and left on room temperature for 10 minutes before centrifugation (12000xg, 10 minutes, 4°C). Supernatant was discarded as meticulously as possible and pellet was washed with 1ml of 75% ethanol (200 Proof pure ethanol, Koptec, King of Prussia, USA) in DEPC water (12000xg, 10 minutes, 4°C). Ethanol was removed from the tube and pellet was dried at room temperature to remove the last of the ethanol. Pellet was resuspended in 50µl of DEPC water and placed on ice. RNA concentration was determined using NanoDrop 2000 (Thermo Scientific, Wilmington, USA) and 10µg of RNA was diluted in DEPC water to a total volume of 44µl. DNase treatment (Ambion, Life Technologies, Carlsbad, USA) was done according to manufacturer's instructions. After the inactivator was added, tubes were vortexed and spun down at 3000rpm for 5 minutes at 4°C. The supernatant (40µ) was transferred to a fresh tube and RNA concentration was measured again. Reverse transcription was done using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, USA) according to manufacturer's instructions with 2µg of DNase treated RNA. Tubes with cDNA were spun down at 3000rpm for 5 minutes at 4°C. Samples were diluted 1:20 for qPCR assessments. A standard curve was prepared by pooling undiluted cDNA from every sample and diluting it 1:2.5 in DEPC water (Standard 1) for Standard 2-8 a 1:3 dilution series was applied. For an efficient distribution of sample volume between samples and standards the following formula was used:

$$20x = \frac{2}{3}(20 - x)2.5n$$

**Formula 3: Equal distribution of sample volume for the preparation of a standard curve for qPCR assessments**

x stands for the volume in µl that should be diluted 1:20 as sample of interest; 20-x stands for the volume that can be diverted for the standard pool and diluted 1:2.5; n stands for the number of samples;

iTaq Universal SYBR Green Supermix (BioRad, Hercules, USA) was used for the qPCR assessments which were done on a StepOne Plus Real Time PCR machine (Applied Biosystems, Foster City, USA). Primer sequences are given in Table 15.

**Table 15: Primer sequences for qPCR**

Gene	Primer sequences	
	5'-3'	3'-5'
<b>TNFα</b>	CCA CCA GGC TCT TCT GTC TAC	AGG GTC TGG GCC ATA GAA CT
<b>IL1β</b>	GGT CAA AGG TTT GGA AGC AG	TGT GAA ATG CCA CCT TTT GA
<b>iNOS</b>	TTC TGT GCT GCT CCA GTG AG	TGA AGA AAA CCC CTT GTG CT
<b>Reg3b</b>	GGC TTC ATT CTT GTC CTC CA	TCC ACC TCC ATT GGG TTC T
<b>Reg3g</b>	TCC ACC TCT GTT GGG TTC AT	AAG CTT CCT TCC TGT CCT CC
<b>18S</b>	AGT CCC TGC CCT TTG TAC ACA	CGA TCC GAG GGC CTC ACT A

### **TNF $\alpha$ and IL1 $\beta$ assessment from murine liver tissue and heparinized mouse plasma**

TNF $\alpha$  and IL1 $\beta$  proteins from murine tissue or plasma were assessed by ELISA. Reagents and antibodies were purchased from eBioscience (San Diego, USA) unless stated otherwise. Costar 3370 flat bottom assay plates (Corning, New York, USA) were coated with appropriate capturing antibodies. Therefore, capturing antibodies were diluted 1:250 in PBS and 100 $\mu$ l of this dilution was pipetted in each well. The plate was sealed tightly and incubated overnight at 4°C on a rocking plate. The next morning, the plate was washed three times with 200 $\mu$ l PBS-T (0.05% Tween 20 in PBS) and incubated with 200 $\mu$ l of assay diluent (1% bovine serum albumin in PBS, filtered) for 1 hour at RT. Tween 20 and PBS were purchased from Fisher Bioreagents (Pittsburgh, USA) and bovine serum albumin from Roche (Rotkreuz, Switzerland). Standards were prepared in assay diluent with STD1 containing 1000pg/ml reconstituted cytokines. A 1:2 dilution series was done for 7 standards in total. Assay diluent was used as blank. The plate was washed three times with 200 $\mu$ l PBS-T and standards (100 $\mu$ l), plasma (100 $\mu$ l) and liver homogenate (60 $\mu$ l) were placed on the plates in singlets. The plate was sealed and incubated for 2 hours at room temperature on a rocking board. Then the plate was washed 5 times with 200 $\mu$ l PBS-T and 100 $\mu$ l of Detection antibody was added. Incubation was 1 hour with the sealed plate at room temperature on a rocking board. Detection antibody was diluted 1:250 in assay diluent. The plate was washed 5 times with 200 $\mu$ l PBS-T and 100 $\mu$ l of Avidin-HRP was added for 30 minutes. Avidin-HRP was diluted 1:250 in assay diluent. The plate was washed 7 times in PBS-T and 100 $\mu$ l TMB substrate was added. The plate was incubated for 15 minutes before 50 $\mu$ l of 2N H<sub>2</sub>SO<sub>4</sub> (sulfuric acid 4N, Fisher Scientific, Pittsburgh, USA) was added and the reaction stopped. The plate was read on a SpectraMax Plus 384 Microplate Reader (Molecular Devices, Sunnyvale, USA) at 450nm.

The tissue used in this assay was homogenized as follows: Frozen liver pieces from the right lobe were weighted and placed in sterile screw cap tubes (Nalgene/Nunc, Rochester, USA) containing sterile glass beads with a diameter of 1mm. Extraction buffer (100mM Tris-HCl at pH 7.4, 150mM NaCl and protease inhibitor cocktail in ddH<sub>2</sub>O) was added (10 $\mu$ l/mg tissue) and tissue was homogenized in a Mini-Beadbeater-96 (BioSpec Products, Bartlesville, USA) on a frozen rack for 50 seconds. Tubes were placed on ice immediately and spun down at 18000xg for 10 minutes at 4°. Supernatant was used for the assessment. Tris was purchased from Fisher BioReagents (Pittsburgh, USA), HCl 6N from Ricca Chemical Company (Arlington, USA), NaCl from Sigma-Adrich, St Louis, USA), cOmplete Mini Protease Inhibitor Cocktail Tablets from Roche (Rotkreuz, Switzerland).

### **Immunofluorescent staining for F4/80**

This staining was done on the left and middle lobe of the murine livers that were conserved in formaldehyde (10%), sucrose (10%) and sucrose (30%) each for 24 hours and then frozen in OCT

compound (Tissue-Tek Sakura, Alphen aan den Rijn, The Netherlands). Formaldehyde and sucrose were purchased from Sigma-Aldrich (St. Louis, USA). Tissue was cut in 5µm thick sections at -20°C and thawed for approximately one hour. Slides were washed with distilled water twice for 2 minutes and carefully dried. Tissue sections were circled with a DAKO Cytomation pen (Agilent Technologies, Santa Clara, USA) and washed once with PBS (Fisher BioReagents, Pittsburgh, USA) for 2 minutes. Slides were tapped on paper and dried with tissue paper. DAKO protein block (Agilent Technologies, Santa Clara, USA) was added (2-3 drops) and incubated for 15 minutes in the dark at room temperature. Circles were tapped on paper and dried (not washed). A F4/80 antibody (rat/anti-mouse) purchased from eBioscience (San Diego, USA) was diluted 1:100 in DAKO Antibody diluent (Agilent Technologies, Santa Clara, USA). Approximately 25µl were added per circle and incubated for 2 hours at room temperature. Slides were washed twice with PBS-T (Tween 20, Fisher BioReagents, USA) for 5 minutes, tapped and dried and covered in secondary antibody. The secondary antibody (Invitrogen, Carlsbad, USA) was goat/anti-rat labelled with Cy3 (red) and diluted 1:200 in antibody diluent. After a 90 minute incubation at room temperature in the dark slides were washed twice with PBS-T for 5 minutes and mounted with Prolong® Gold antifade reagent with DAPI (Life Technologies, Carlsbad, USA). Slides were kept in the dark overnight and assessed the next day.

#### **Parameters measured by collaborators**

Neopterin was measured in serum by ELISA (BRAHMS Diagnostics, Hennigsdorf, Germany) according to manufacturer's instructions in serum in the lab of Dietmar Fuchs, Medical University of Innsbruck.

Mannitol, lactulose and sucrose were measured in urine after an oral sugar challenge by HPLC in the lab of Karl Öttl, Medical University of Graz,(150) and by NMR in the lab of Tobias Madl, Medical University of Graz.(110) Data presented in "Effects of probiotics on gut permeability" were acquired by NMR.

sCD163 was measured in EDTA plasma by ELISA in the lab of Henning Grønbaek, Denmark.(151)

AOPP were assessed by an absorbance assay(152) in apoB-depleted serum in the lab of Gunther Marsche, Medical University of Graz. This assay is disturbed by increasing amounts of Bilirubin, therefore linear regression was used to clean the AOPP measurements from the influence of bilirubin with Formula 4.

$$AOPPs = AOPPsignal - 0.40 \times BILI + 5,25$$

#### **Formula 4: Formula to clear AOPP signal from the influence of bilirubin**

AOPPs represent the corrected parameter, AOPPsignal represent the directly measured amount, BILI represents the bilirubin concentration in serum in µg/ml;

Nitrite, tryptophan, kynurenine, phenylalanine and tyrosine were measured in serum by HPLC in the lab of Dietmar Fuchs, Medical University of Innsbruck. Details are given elsewhere.(75)

Serotonin was measured in serum by ELISA in the routine laboratory of Barbara Obermayer-Pietsch, Medical University of Graz.

## Abbreviations

In alphabetical order:

Abbreviation	Description
ALD	alcoholic liver disease
ALT	alanine aminotransferase
AMP	antimicrobial peptides
ANOVA	analysis of variance
AOPP	advanced oxidation protein products
AP	alkaline phosphatase
AST	aspartate transaminase
BH4	tetrahydrobiopterin
CARS	compensatory anti-inflammatory response syndrome
CD	cluster of differentiation
CFU	colony forming unit
DAO	diamine oxidase
DDC	3,5-diethoxycarbonyl-1,4-dihydrocollidine
DEPC	diethyl dicarbonate
EDTA	ethylenediamine tritaacetic acid
ELISA	enzyme linked immunosorbent assay
EU	endotoxin unit
FACS	fluorescence associated cell sorting
FDR	false discovery rate
FITC	fluorescein isothiocyanat
fMLP	N-formylmetionyl-leucyl-phenylalanine
GC-MS	gas chromatography – mass spectrometry
G-CSF	granulocyte colony stimulating factor
GFP	green fluorescence protein
GMFI	geometric mean of fluorescence intensity
GTP-CH	guanosine-triphosphate-cyclohydrolase
HBSS	Hank's balanced salt solution
HCV	hepatitis C virus
HEK cells	human embryonic kidney cells
HPLC	high performance liquid chromatography
HRP	horseradish peroxidase
IDO	idolamine-2,3-dioxygenase
IFN	interferon
IgG	immunoglobulin G
IL	interleukin
iNOS	inducible nitric oxide synthetase
IP	interferon gamma-inducing protein
IPG	immobilized pH gradient
IRF	interferon regulatory factor
ITT	intention to treat

Abbreviation	Description
LAL	limulus amoebocyte lysate
LB	lysogeny broth
LBP	lipopolysaccharide binding protein
MCP	monocyte chemotactic protein
MELD	model of end stage liver disease
MIG	monokine induced by gamma-interferon
MIP	macrophage inflammatory protein
MLCK	myosin light chain kinase
MyD88	myeloid differentiation primary response gene 88
NADPH	nicotinamide adenine dinucleotide phosphate hydrogen
NAFLD	non-alcoholic fatty liver disease
NASH	non-alcoholic steatohepatitis
NFκB	nuclear factor-κB
NG-sequencing	next generation sequencing
NMR	nuclear magnetic resonance
OCT	optimal cutting temperature
OD	optical density
OTU	operational taxonomic unit
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCOS	polycystic ovary syndrome
PHA	phenylalanine-hydroxylase
PI	phagoindex
PMA	phorbol myristate acetate
PMN	polymorphonuclear cells
PPA	per protocol analysis
PPP	platelet poor plasma
Reg3	regenerating islet-derived protein 3
RES	reticuloendothelial system
ROS	reactive oxygen species
RT	room temperature
sCD	soluble cluster of differentiation
SDS	sodium dodecyl sulfate
SEAP	secreted embryonic alkaline phosphatase
SF36	short form 36
SGA	subjective global assessment
SIRS	systemic inflammatory response syndrome
SOP	standard operating procedure
STD	standard
TCA	trichloroacetic acid
TLR	Toll like receptor
TNF	tumor necrosis factor
TPH	tryptophan-hydroxylase

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## Appendix

The appendix contains the following documents:

- Study protocol
- Subjective global assessment form
- SF36 questionnaire
- Food frequency questionnaire

## **STUDY PROTOCOL**

VERSION 10

**2.10.2015**

# **Probiotic modulation of gut microflora in cirrhosis: Influence on immune function and infections**

**A randomized, double-blind, placebo controlled trial**

Study identification: PIC-2010

REC: 23-096 ex 10/11

Short title: Probiotics in cirrhosis

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## Background and state of the art

In the eight most economically leading countries in the world liver cirrhosis is the 10<sup>th</sup> most common cause of death (153, 154). Patients with end-stage liver disease are susceptible to a number of complications and have a markedly reduced life expectancy with one year mortality rates ranging between 10 and 82% depending on the stage of cirrhosis (155). The most serious complications include variceal haemorrhage, sepsis, hepatorenal syndrome and hepatic encephalopathy, often associated with a significant deterioration in liver function. Infection is the most common precipitant of this clinical decline termed acute on chronic liver failure. Long-term intestinal decontamination with norfloxacin reduces the incidence of infective episodes and complications of cirrhosis particularly hepatorenal failure (4).

The mechanisms of the increased susceptibility to infection are unclear but recent studies suggest that even in patients with compensated cirrhosis, innate immune response is defective (156, 157) We have recently shown that patients with cirrhosis have a defective neutrophil function, characterised by increased oxidative burst and decreased phagocytic capacity of the neutrophils as well as impaired albumin function, (158, 159) associated with increased risk of infection, progression to organ failure and mortality. (Figure 1) (63) Our studies have also shown that this functional defect is likely to be mediated by a humoral factor. In ex-vivo studies, we showed that treating patient's plasma with anti-CD14 antibodies, endotoxin removal columns or albumin but not with toll-like receptor antagonists prevented neutrophil phagocytic dysfunction, implicating endotoxaemia in pathogenesis. (Figure 2) (63)

Figure 1. A phagocytic capacity of less than 42%, which had the best sensitivity and specificity on AUROC analysis and was used to construct a Kaplan Meier survival curve; log-rank analysis shows a significantly higher mortality ( $p < 0.001$ ) in the group of patients with decreased phagocytic capacity (<42%).

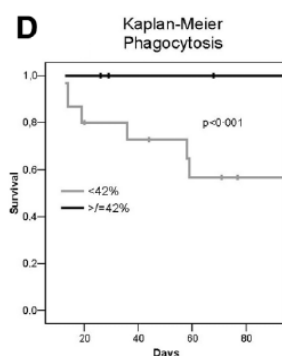
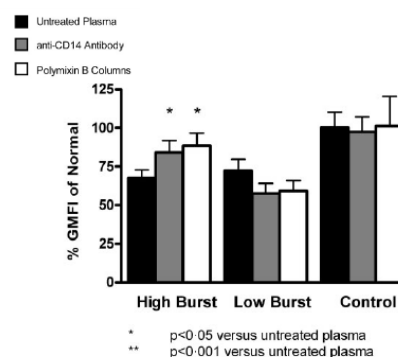


Figure 2. Impaired phagocytosis is reversible by passing high burst patient plasma over an endotoxin-removal column or incubation with anti-CD14 antibodies. Plasma from patients with low burst or from controls passed over the column or incubated with anti-CD14 antibodies does not influence phagocytosis.



Increased endotoxaemia in patients with cirrhosis is thought to be due to increased gut permeability leading to increased bacterial translocation and altered gut flora with a predominance of gram-negative organisms (160). It follows that alteration of the gut flora may favour decreased endotoxin levels, leading to a restoration of immune function in cirrhosis. Probiotics have been demonstrated to alter gut flora (160) and have shown positive effects on liver injury in experimental models of liver disease, dependent on the bacterial species used. (161) In patients with liver cirrhosis, probiotics have been shown to decrease hepatic encephalopathy, (162, 163) improve liver biochemistry (164) and decrease the rate of infection after liver transplantation. (165) Several meta-analyses have already supported the benefit of probiotics in preventing infections in the general hospital population, (166-168) however; the exact mechanism is still largely speculative. It has been suggested that a healthy flora promotes the integrity of the gut defence barrier by normalizing intestinal permeability and thereby controls intestinal inflammatory responses by modulating the release of cytokines. (169)

Results of a pilot controlled investigation leading up to this clinical study (100)

Twelve patients with alcoholic cirrhosis received food supplementation with *Lactobacillus casei* Shirota ( $6.5 \times 10^9$ ) 3 times daily for 4 weeks (100). Eight patients were included as disease controls. During treatment with *Lactobacillus casei* Shirota, no adverse events were noted and compliance to study medication was 86%. Baseline neutrophil function showed a significantly lower phagocytic capacity in patients compared with controls (73% versus 98%,  $p < 0.05$ ), which normalized at the end of the study. No improvement was seen in disease controls. TNF receptor (TNFR) 1 and 2 and interleukin (IL)-10 were significantly elevated in patient's plasma compared to healthy controls and did not change during the study. (Figure 4)  $\text{TNF}\alpha$ , IL-6 and IL-8 were either undetectable or at non-significant levels at baseline or following stimulation. Following ex-vivo stimulated neutrophil challenge experiments, levels of TNFR1, TNFR2 and IL-10 significantly decreased in supplemented patients ( $p < 0.05$ ). (Figure 5)

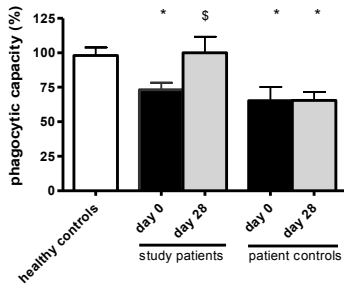


Figure 3: Phagocytic capacity of healthy controls, study patients and patient controls with alcoholic cirrhosis at baseline and at the end of the study. Phagocytic capacity is significantly reduced at baseline and increases significantly at the end of the study. \*p<0.05 versus healthy control, <sup>§</sup> p<0.05 versus baseline

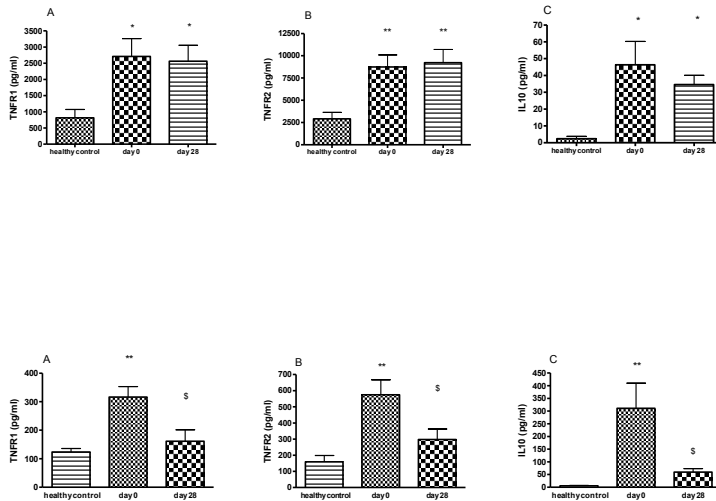
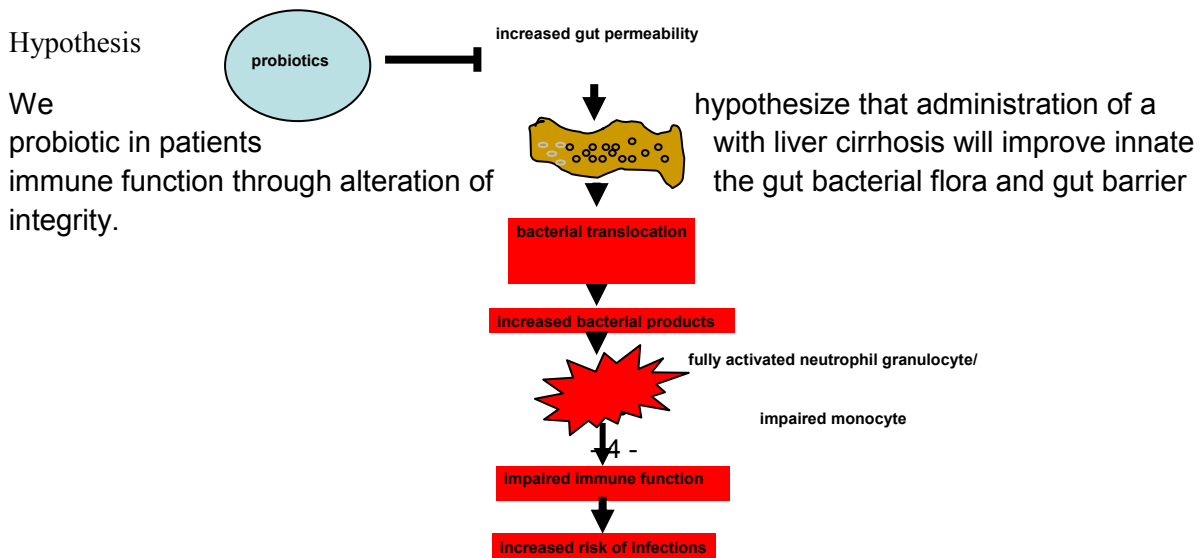


Figure 4: Plasma cytokines of healthy controls and patients with alcoholic cirrhosis at baseline and at the end of the study. TNFR1, TNFR2 and IL10 are significantly elevated in patients and do not change during treatment. \*p<0.05 versus healthy control, \*\*p<0.01 versus control

Figure 5: Ex vivo cytokine production in LPS-stimulated whole blood. TNFR1, TNFR2 and IL10 show a significant decrease at the end of the study as compared to baseline. \*\*p<0.01 versus healthy controls. <sup>§</sup> p<0.05 versus baseline

These data provide novel evidence that the functional phagocytic defect and the predominant anti-inflammatory stimulated cytokine production observed in cirrhosis can be restored with *Lactobacillus casei* Shirota.



### Specific aims

The primary aim of this randomised, controlled study is to assess whether food supplementation with a probiotic (Winclove-849) improves neutrophil phagocytic capacity in patients with cirrhosis. Secondary aims are to test whether food supplementation with a probiotic (Winclove-849) decreases the rate of clinically significant infections and improves neutrophil and albumin function, inflammatory response, gut barrier function or gut flora and quality of life.

### **End Points:**

#### Primary

Increase in neutrophil phagocytic capacity

#### Secondary

Clinically significant infections, endotoxin levels, neutrophil oxidative burst, neutrophil toll like receptor expression, albumin function, inflammatory response, gut barrier function, bacterial flora, quality of life

#### Innovative aspects and expected outcome

This study has the potential to enhance pathophysiological knowledge on impaired gut barrier function leading to an increased risk of infection and will be the proof of concept for a novel, low cost and low risk (except for specific patient groups as discussed below) therapeutic concept. Since infection in cirrhosis places a major burden not only on the individual patient but also on our health care system, this concept will satisfy an unmet clinical need. At the moment no other low risk prophylaxis to prevent infections in cirrhosis is established before patients develop their first complication. Thereafter antibiotic prophylaxis is well established (170) but it is our aim to start an intervention already one step ahead.

From previous studies we know that the use of a probiotic is well accepted by patients. If our hypothesis holds true, this will provide clinicians with an easily applicable prophylactic strategy for patients with cirrhosis.

#### Publication strategy

The study will be registered at [www.clinicaltrials.gov](http://www.clinicaltrials.gov) before recruitment of the first patient.

The results of this study will be disseminated in the scientific community and to the general public. It is our intent to publish the results derived from this project preferably in high-impact journals, abstracts and presentations at national and international meetings. All investigators will be authors of the manuscript and the manuscript will be approved by all authors.

Furthermore the knowledge derived from this project will be used to increase networking with national and international partners. Intellectual property submission will be done before publishing. The FWF Austrian Science Foundation will be acknowledged in these publications and presentations.

#### Potential additional aspects

##### *Implications for other branches of science*

The results of this study will be important for basic scientists (molecular biology, microbiology) working on gut barrier and gut flora and for food science since most probiotics are classified as food supplement. Furthermore these data will also be important for the understanding of sepsis, where a similar mechanism with increased gut permeability and endotoxemia, leading to immune paralysis, has been hypothesized. This study will provide the pathophysiological basis for the development of novel therapeutic strategies.

##### *Effects that will have implications beyond the field*

Since probiotics are generally considered to be a low risk therapeutic strategy (except for specific patient groups as discussed below) these results may have implications for people at risk to develop a disease (suboptimal health state) who are worried about their health state and consult a doctor for prophylactic advice. When our hypothesis holds true, it might be thinkable that in such cases, if gut permeability is high, probiotics can be recommended.

##### *Limitations*

It is important to note that the results of this study are only valid for the dose and probiotic strain used in our study and can not be generalized to other doses or strains. Also safety of this dose and probiotic strain has to be tested in other patient groups before being able to derive general conclusions.

#### Material and Methods

##### *Sample size calculation*

Phagocytic capacity in the patient group at baseline is  $73\pm 5\%$  and increases to  $100\pm 14\%$  at the end of the study. With an alpha of 0.05 and a beta 0.2 and assuming a 20% dropout rate, 92 patients are needed for the study (46 in each arm)

Additionally 20 healthy controls and 20 patients with alcohol dependence will be studied as controls

##### *Patient/ Control person recruitment*

Patients will be identified from the outpatient clinic at the Department of Gastroenterology and Hepatology or the Department of Transplantation Surgery, University Hospital Graz.

For this study healthy controls and patients with recent alcohol abuse will serve as controls for the baseline values of gut permeability. Healthy controls will be recruited from hospital staff by direct personal approach. Active alcoholics will be recruited from the emergency department of the Department of Internal Medicine or from the Department of Psychiatry.

#### *Randomisation*

After fulfilling inclusion criteria, 92 patients will be randomised into 2 groups:

**Group 1:** receiving a probiotic mixture (6 g of Winclove-849 containing Bifidobacterium bifidum W23, Bifidobacterium lactis W52, Lactobacillus acidophilus W37, Lactobacillus brevis W63, Lactobacillus casei W56, Lactobacillus salivarius W24, Lactococcus lactis W19, Lactococcus lactis W58 at a concentration of  $2.5 \times 10^9$  cfu/g)

**Group 2:** receiving a similar looking and tasting placebo without bacteria

Patient will be stratified according to their etiology of liver disease (alcoholic versus non-alcoholic) and if they take antibiotic prophylaxis for spontaneous bacterial peritonitis or not.

Each patient will be treated for 6 months.

Randomisation will be carried out using the “Randomizer” (Institute of Medical Informatics, Medical University of Graz) software after fulfilling the study criteria. The Randomizer provides a self-serve, easy to use, secure and 24 hour-a-day Randomization Service that runs exclusively on the Internet. Randomization will be performed by permuted blocks. All transactions are logged. The trial's audit trail and the list of randomizations can be downloaded and analyzed at any time by the trial monitor. At randomisation, patients will be stratified for aetiology into patients with alcoholic and non-alcoholic liver cirrhosis and for taking antibiotic prophylaxis or not.

#### *Study product*

The study product (Winclove-849) contains Bifidobacterium bifidum W23, Bifidobacterium lactis W52, Lactobacillus acidophilus W37, Lactobacillus brevis W63, Lactobacillus casei W56, Lactobacillus salivarius W24, Lactococcus lactis W19, Lactococcus lactis W58 at a concentration of  $2.5 \times 10^9$  cfu/g. The product has been developed to strengthen the intestinal barrier and to reduce translocation of harmful content from the lumen of the intestine into the body. The scientific background of the product is given in Appendix 1 and the product description in Appendix 2.

The product is licensed as a food supplement in the Netherlands as Ecologic® Barrier by Winclove (Amsterdam)

#### *Definitions*

**Significant Infection:** Infection will be defined as microbiologically proven for bacteria or fungi or suggested by radiological imaging or the diagnosis of spontaneous bacterial peritonitis. An infection will be considered ‘significant’ if this requires hospital admission.

Spontaneous bacterial peritonitis: Ascitic fluid infection without any evidence of an intra-abdominal surgically treatable source. The diagnosis is established by an elevated ascitic fluid absolute neutrophil count  $\geq 250$  cells/mm<sup>3</sup>.

*Inclusion/Exclusion criteria*

**Patients**

**Inclusion criteria**

Patients aged between 18-80 years

Clinical and radiological evidence of cirrhosis, and/or biopsy proven liver cirrhosis of any cause

Informed consent

**Exclusion Criteria:**

Child-Pugh score > 11

Abstinence from alcohol for < 2 weeks at the time of screening for inclusion

Clinical evidence of active infection

Antibiotic treatment within 7 days prior to enrolment (except for primary or secondary prophylaxis of spontaneous bacterial peritonitis)

Gastrointestinal haemorrhage within previous 2 weeks

Use of immunomodulating agents within previous month (steroids etc.)

Concomitant use of supplements (pre-, pro-, or synbiotics) likely to influence the study

Renal failure (such as hepatorenal syndrome), creatinine >1.7 mg/dL

Hepatic encephalopathy II to IV

Pancreatitis

Other organ failure

Hepatic or extra-hepatic malignancy

Pregnancy

Presumed non-compliance to the study medication

**Healthy Controls**

**Inclusion criteria**

Informed consent

**Exclusion criteria**

Malignancy, pregnancy, chronic inflammatory bowel disease, celiac disease, active alcohol abuse, any severe organ dysfunction

### **Alcoholic Controls**

#### **Inclusion criteria**

Informed consent

Active alcohol dependence (more than 15 units of alcohol (men) or 12 units of alcohol (women) within one week

Elevated AST and/or fatty liver on ultrasound

#### **Exclusion criteria**

Cognitive impairment due to alcohol abuse, malignancy, pregnancy, chronic inflammatory bowel disease, celiac disease, any severe organ dysfunction unrelated to renal dysfunction

#### *Study termination*

For individual patients, the study will be terminated after 6 months treatment and a follow-up period of further 6 months. The study can be terminated for individual patients due to a) a severe adverse event b) liver transplantation c) pregnancy d) complications of liver cirrhosis or diseases that do not allow the administration of the probiotic any more e) significant protocol violations f) withdrawal of consent d) lost of follow up e) any other situation that leads to the decision for the investigator to terminate the study. The whole trial can be stopped by the investigator if adverse events occur or other unforeseeable events might influence the safety or well-being of the study participants.

All patients will be recruited within 24 months, so the minimum follow-up period for the last patient entering the study is 12 months. After termination, all study patients will be followed up according to the follow-up policy of our institution for liver cirrhosis.

Table 1: Schedule of assessment

Day	-14 - 0	0	14					
Month				1	3	6	9	12
Visit no.	1	2	3	4	5	6	7	8
Inclusion / exclusion criteria	X							
Demographics / relevant medical history / current medical conditions	X	X	X	X	X	X	X	X
Prior concomitant meds/therapies	X	X	X	X	X	X	X	X
Physical examination	X				X	X		X
Body weight/ height/ waist-hip ratio/ midarm muscular circumference	X				X	X		X
Ultrasound abdomen and alpha-Fetoprotein	X							
Chest X-Ray	X							
Renal/Liver/Electrolytes/CRP/ Ferritin/ full blood count/coagulation/amylase/lipase	X				X	X		X
blood culture (aerobe and anaerobe, 30ml)	X				X	X		X
pregnancy test (in women of childbearing age)	X				X	X		X
12 ml lithium heparin blood	X				X	X		X
6 ml EDTA blood	X				X	X		X
8 ml Serum	X				X	X		X
4 ml sodium citrate plasma	X				X	X		X
Stool	X					X		X
Sucrose/Lactulose/Mannitol Test	X					X		X
Quality of life Questionnaire (SF36)	X				X	X		X
Food Frequency Questionnaire	X				X	X		X
Subjective Global Assessment (SGA) of nutritional status	X				X	X		X
Gastroscopy with gastric/duodenal biopsy (1 each) and gastric/duodenal aspirate (not mandatory)	X				X	X		X
Dosage administration record	Ongoing data capture (probiotic will be dispensed every 2 weeks)							
Adverse Events	Ongoing data capture							
Concomitant medications/therapies	Ongoing data capture							

Infection events	Ongoing data capture
Study Completion	Complete at any time if study drug is discontinued

### *Measurements and sampling*

All studies will be undertaken after obtaining informed consent. Study visits will be on an outpatient basis. Blood will be taken at the initial screening visit and after 3, 6 and 12 months. Routine biochemistry and haematology and blood cultures will be performed together with assays to neutrophil function (phagocytosis, oxidative burst, toll-like-receptor expression) endotoxin levels, bacterial DNA, plasma and ex vivo stimulated cytokine expression; macrophage activation will be estimated by sCD163 and neopterin. Additionally, tryptophan, kynurenine, tyrosine, phenylalanine, serotonin and nitrite were assessed as markers of monoamine synthesis associated with depression and sickness behaviour. In women of childbearing age a pregnancy test will be performed.

Gut permeability studies with sucrose/lactulose/mannitol testing will be performed at the beginning and after 6 and 12 months. Stool samples will be collected at the same timepoints for assessment of gut colonisation by the ingested probiotic species of bacteria and to determine the changes in gut microflora that results.

Assessment of nutritional status, food intake and quality of life will be performed at the initial screening visit and after 3, 6 and 12 months

In healthy controls and controls with alcohol dependence sampling and all tests are only performed once.

The probiotic or the placebo will be distributed to the patients by a study nurse at baseline and after 3 months. Patients will receive a 3-months supply in a box with one sachet containing 6 g of Winclove-849 or placebo for each day.

If a upper GI endoscopy is clinically indicated at any time point of the study, gastric and duodenal aspirate and one extra biopsy sample from duodenum and one from the stomach is collected for analysis of bacterial flora.

To ensure compliance and to confirm any infection events, the study nurse will call patients regularly to discuss any problems. The patients will receive a patient diary where they are asked to document any episode of illness during the study period.

All the assay investigators will be kept blinded to the randomisation of the sample subjects; a single operator (to limit methodological bias) will perform all non-routine measurements in batches. At the end of the study, the code will be broken and the results compared statistically.

### *Assessment of safety*

All adverse and serious adverse events will be documented and reported in accordance with standard guidelines. Serious complications requiring hospitalisation to be anticipated in end-stage liver disease include:

Hepatorenal syndrome

Gastrointestinal haemorrhage

Hepatic encephalopathy

Infection requiring hospitalisation

Ascites requiring paracentesis

Cardiovascular insufficiency requiring circulatory support

Expected adverse reactions of the treatment under investigation include:

- Flatulence
- Diarrhoea
- Abdominal discomfort
- Bloating

As a safety measurement blood will be collected into 3 pairs of aerobic/anaerobic blood culture bottles according to current guidelines. Blood will be sampled from a peripheral vein.

#### *Methodological Details about Measurements*

Neutrophil phagocytosis: The Phagotest<sup>®</sup> (Orpegen Pharma, Heidelberg, Germany) is used to measure phagocytosis by using FITC-labelled opsonized E. coli bacteria as described before. (63)

#### Neutrophil oxidative burst

The Phagoburst<sup>®</sup> kit (Orpegen Pharma, Heidelberg, Germany) is used to determine the percentage of neutrophils that produce reactive oxidants with or without stimulation according to the manufacturer's instructions as previously described using fluorescence activated cell sorting (FACS; Becton Dickinson FACScan, San Jose, USA; Cellquest<sup>™</sup> software) (63)

#### Toll-like receptors:

Surface expression of TLRs will be determined using the following fluorescein isothiocyanate (FITC)- and phycoerythrin (PE)-conjugated antibodies: TLR2-PE-Cy7, TLR4-APC, TLR9-PE, CD16-FITC (eBioscience, San Diego, CA). Cells will be incubated with the respective antibody pairs for 30 minutes, washed twice with PBS, lysed and fixed before FACS analysis. For TLR9 expression cells are permeabilized by incubating for 10 minutes with 500 µL of 1 x FACS Permeabilization Solution 2 (BD Biosciences, UK). Results will be confirmed by western blotting.

#### Gut permeability: Sucrose/lactulose/mannitol test

The patient drinks a solution of 200 ml water containing 20g sucrose, 10g lactulose and 5g mannitol. Urine is collected over 5 hours while fasting is continued for 3 hours after study

start. The urine volume collected at 5 hours is measured and 1 ml aliquots are frozen immediately at -80°C without preservative for subsequent analysis by NMR spectroscopy. The urinary recovery of the test sugars will be expressed as percentage of the dose ingested and the lactulose/mannitol ratio will be calculated.

#### Endotoxin levels: Limulus amoebocyte lysate assay

Heparinized whole blood is drawn with pyrogen-free needles into pyrogen-free tubes and the serum separated at 4°C and stored at -80°C in pyrogen-free polyethylene cryotubes (Nunc, Rochester, USA) on the day of collection. The chromogenic limulus amoebocyte lysate assay (Charles River Laboratories) is used for detection of endotoxin. (171) To measure endotoxin-binding capacity, plasma samples are spiked with 0.005, 0.05, 0.5, 5 and 50 EU of endotoxin and the percentage of recovery at the different concentrations is measured. Plasma from healthy control subjects is used as a reference.

#### HEK-Blue™ LPS Detection Kit

Blood is collected with pyrogen-free needles into pyrogen-free tubes and the serum separated at 4°C and stored at -80°C in pyrogen-free polyethylene cryotubes (Nunc, Rochester, USA) on the day of collection. HEK-Blue™ LPS Detection Kit (InvivoGen, Toulouse, France) with adapted protocol is used. In brief, cells are cultured in 24-well plates (5x10<sup>4</sup>/well). After 24 hours medium is discarded and replaced with samples/LPS standards and detection medium. Cells are incubated for 24 hours at 37°C and color intensity is measured at a wave length of 650nm.

Bacterial DNA: A PCR reaction for the universal amplification of a region of the 16S ribosomal RNA gene will be performed. Two microliters of template are added into a reaction mix containing 10 mmol/L Tris buffer (pH 8.3), 50 mmol/L KCl, 1.5 mmol/L MgCl<sub>2</sub>, 200 μmol/L of each deoxynucleoside triphosphate, 50 pmol of primers 5'-AGAGTTTGATCATGGCTCAG-3' and 5'-ACCGCGACTGCTGCTGGCAC-3' and 1.25 U BioTaq (Bioline, London, England) to complete a final volume of 50μL. The primers located at positions 7-27 and 531-514 (Escherichia coli numbering) are universal eubacterial primers that will amplify any known bacterial 16S ribosomal RNA gene. A 35-cycle PCR will be run in a GeneAmp 9700 (Applied Biosystems, Foster City, CA) using the following profile: 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 60 seconds. Total PCR reaction volume will be filtered with QIAquick Spin Columns (QIAquick PCR Purification Kit; QIAGEN) to remove rests of primers. Five microliters of purified products will be analyzed by 2% agarose gel electrophoresis and UV visualization.

#### Plasma cytokines and ex vivo cytokine production;

Soluble tumour necrosis factor receptor (sTNFR) 1 and 2 will be determined by ELISA from ethylene-diamine-tetraacetate anticoagulated plasma samples using commercially available antibody sets (R&D Systems, Abingdon, UK) in accordance with the manufacturer's instructions. Tumour necrosis factor alpha (TNFα), Interleukin (IL)6, IL-6R, IL8 and IL10 will be analysed by a cytometric bead array (BD Bioscience, Oxford UK) according to the manufacturers instructions. Samples will be analysed on a FACS Canto II (BD Bioscience, Oxford UK) with FACSDiva software for acquisition and FCAP array software for analysis. Pre-albumin will be measured using Cobas-MiraS.

Ex vivo stimulation of cytokines will be analysed in a whole blood assay after stimulation with endotoxin derived from E.coli (E.coli 0111:B4 Lot 085K4068, Sigma, Poole, UK) for 4 h at 37 °C.

#### Albumin oxidation in patients' plasma

Albumin fractions HMA, HNA-1 and HNA-2 are determined by means of high performance liquid chromatography (HPLC) (REF). Plasma samples are diluted 1:100 to a final volume of 1ml in sample-buffer (0.1M sodium phosphate, 0.3M sodium chloride, pH 6.87) and filtered through a Whatman 0.45µm nylon filter (Whatman International Ltd., Kent, UK). 20µl of the filtrate are then injected to the HPLC system. Separation process is performed by means of a Shodex Asahipak ES-502N 7C anion exchange column (Showa Denko Europe GmbH, Munich, Germany) using 50mM sodium acetate, 400mM sodium sulphate, pH 4.85 as the mobile phase. A gradient of 0 to 6% ethanol and a flow rate of 1ml/min is applied by a gradient pump (FLUX Rheos 4000; Spectronex GmbH, Vienna, Austria) and used for elution. The column is kept at a temperature of 35°C. Fractions are detected at fluorescence level at 280/340nm. As detector for fluorescence emission a Jasco 821FP detector (Spectronex GmbH, Vienna, Austria) and for UV a Waters 2487 UV/VIS detector (Waters GmbH, Eschborn, Germany) is used. Fraction quantification was done by comparing peak heights. Software used for this application is EZChrom Elite™ chromatography software (Scientific Software Inc., San Ramon, CA USA). Fractions are expressed in percent of total albumin.

#### Gut flora

Isolation of bacterial DNA is done from stool samples, gastric/duodenal aspirate or duodenal/stomach biopsies using QIAGEN stool DNA extraction kit according to the manufacturer's instruction. 16S rDNA variable region 4 is amplified from these DNA isolates by PCR using according primers. PCR is done in triplicates for each sample (á 20µl) according to the following protocol using Roche's FAST Start High Fidelity PCR system. PCR products are separated on a 1% 1xTAE agarose gel and specific bands (~300bp) are excised and gel-extracted using QIAGEN gel extraction kit (QIAGEN, Vienna). Purified PCR products are analysed on a BioAnalyzer 2100 DNA 1000 cassettes (Agilent Technologies) for integrity and DNA concentration is determined fluorometrically using Quantitect reagent (Invitrogen, Carlsbad, CA). An amplicon library is generated using aequimolar amounts of PCR products derived from the individual samples and bound to the sequencing beads at a one molecule per bead ratio. Long Read Sequencing using a 70x75 PicoTiter Plate (Roche Diagnostics) is done on a Genome Sequencer FLX system (Roche Diagnostics) according to the manufacturer's instruction. For analysis the freely available academic software packages UniFrac and DOTUR are used

#### Macrophage activation:

Neopterin will be measured by ELISA (BRAHMS Diagnostics, Berlin, Germany). sCD163 will be assessed by a self-engineered ELISA established in Henning Grønbaeks laboratory, Medicinsk Hepato-Gastroenterologisk, Aarhus Universitetshospital, Copenhagen, Denmark.

#### Monoamine synthesis:

Blood is collected with pyrogen-free needles into pyrogen-free tubes and the serum separated at 4°C and stored at -80°C in pyrogen-free polyethylene cryotubes (Nunc, Rochester, USA) on the day of collection. Free tryptophan, kynurenine, tyrosine and phenylalanine will be determined by high-performance liquid chromatography. Nitrite will be assessed in the cell-free, culture supernatant by the Griess reaction assay (Promega, Madison, Wisconsin). Serum levels of serotonin will be assessed by ELISA.

#### Quality of life:

SF-36 questionnaire. The SF36 (Short Form with 36 questions), a well-documented, self-administered QoL scoring system has been widely used and validated, also in liver disease. The questionnaire includes one multi-item scale that assesses eight health concepts: 1) limitations in physical activities because of health problems; 2) limitations in social activities because of physical or emotional problems; 3) limitations in usual role activities because of physical health problems; 4) bodily pain; 5) general mental health (psychological distress and well-being); 6) limitations in usual role activities because of emotional problems; 7) vitality (energy and fatigue); and 8) general health perceptions.

#### Food frequency questionnaire

A food frequency questionnaire developed by the dietology team of the Medical University of Graz will be used to assess changes in eating habits throughout the study. This questionnaire consists of 33 items concerning the intake of carbohydrates, milk products, meat, fish, fruit, vegetable, fat, drinks and snacks. Each item has 6 frequencies (never/rarely, 1-3 times per months, every week, 2-6 times per week, daily, more than once a day) and the patients are asked to tick the most appropriate box.

#### Subjective Global Assessment

Subjective global assessment is a simple and reliable assessment tool for the screening of malnutrition (111). The standard SGA comprises a nutritionist evaluation of height, weight (current, before illness, and weight range in the previous 6 months), nutritional history (appetite, intake, gastrointestinal symptoms), physical appearance (subjective assessment of fat loss, muscle wasting, edema and ascites) and existing conditions (encephalopathy, infections, renal insufficiency). Based on this evaluation, patients are classified into three groups: (i) well nourished, (ii) mild or moderately malnourished or (iii) severely malnourished. The SGA is available in German for the Austrian Society for Clinical Nutrition.

#### Data management, monitoring and archiving

For data management the ArchiMed software (Institute of Medical Informatics, Medical University of Graz) will be used. A data monitoring plan will be generated in cooperation with the study coordination center at the Medical University of Graz.

A Case Report Form (CRF) will be completed for each subject enrolled into the clinical study. Patient identifiers are kept confidential. Patient data are identified by a patient number and only the investigator will keep a list of patient identifiers and patient numbers. Patients will be informed about the collection of data and asked for their consent that their data might be

shown to representatives of the respective legal authorities or monitors who are also obliged to keep data confidential.

All original documents from this study will be archived for 15 years.

Statistical methodology and data analysis

All the data will be described as mean and standard errors. All clinical data will be analysed on an intention to treat basis but will also be described on 'as treated' basis. The primary analysis will be based on a chi-sq test or a Z-test for two proportions, as advised by the project statistician. Analysis of the secondary endpoints will be done as descriptive statistics, by t-test, Mann-Whitney test, Pearson and/or Spearman correlation as appropriate. Differences in the primary outcome measure will be described using a Kaplan-Meier analysis and using the Log rank test to determine the significance of difference between groups.

Possible pitfalls and strategies to overcome them

### Recruitment and patient adherence

One of the main problems of clinical studies is the difficulty to recruit enough patients. We have carefully assessed our capacities and found that we see 150 patients with cirrhosis potentially fulfilling the criteria for participating in this study. Therefore recruitment of 92 patients in 2 years seems to be feasible.

Once patients are recruited, the main problem is to ensure adherence to the study. The most important factors for this are that the patients are well informed, that the study is not too time consuming and that the patients feel well supported. In our study we will ensure that patients are well informed by providing written and personal information for the patients. The study has been designed with 4 visits to the outpatient clinic over a period of 12 months, which is approximately the number of visits the patients will have scheduled due to their disease. Therefore the study does not place any further burden on the patients. Between the visits patients will be supported by the study nurse, who will call patients regularly to ensure adherence to the study protocol.

### Is the effect due to the probiotic or due to improvement of nutrition?

Malnutrition is a common problem in liver cirrhosis (172). The therapeutic intervention with Winclove-849 adds only 20 kilocalories per day (341 kcal/100 gram) to the patients' diet, therefore it is unlikely to change the nutritional status of the patients significantly. However, we will perform a detailed analysis of nutrition using a food frequency questionnaire and we will also assess the nutritional status by the Subjective Global Assessment (111).

### Safety of probiotics in our patient cohort

Generally probiotics are considered as safe. Many probiotics are classified as food without any specific tests for safety. The product used in this study is commercially available since 1935 and has been used without any safety concerns. However, there have been concerns in treating patients with defective immunity with living bacteria (124, 173). Since patients with liver cirrhosis have a defective innate immunity (63), an increased awareness concerning safety of the treatment is necessary. We will perform a detailed assessment of adverse events at every study visit and the study nurse will call patients between the visits on a

regular basis to assess adverse events. The patients will also be informed about the importance to report adverse events to us. If necessary, the randomization code will be broken in order to know if the patient has received the active product or the placebo.

A previous study also suggests that the administration of *Lactobacillus casei* Shirota in patients with alcoholic cirrhosis is safe as shown by the lack of any adverse events, and the fact that markers of inflammation/infection such as white cell count and C-reactive protein remained unchanged over the study period. (100)

As a safety measurement blood cultures will be routinely taken every 3 months during the study period and at any time an infection is suspected a full workup according to the clinical presentation will be performed.

#### Work and Time Plan

Months \ Task	1-3	4-6	7-9	10-12	13-15	16-18	19-21	22-24	25-27	28-30	31-33	34-36
Study initiation	x											
Patient recruitment	x	x	x	x	x	x						
Patient follow up, assessment of quality of life, food frequency questionnaire, subjective global assessment, neutrophil function	x		x	x	x	x	x	x	x	x		
Endotoxin, bacterial DNA, cytokines, gut permeability, gut flora									x	x	x	
Evaluation of experiments; data preparation for publication												x
Data presentation on scientific meetings												x
Preparation of follow-up study												x

**Table 2:** Time schedule of proposed study

Ethical considerations

The use of a probiotic is expected to be safe for patients with liver cirrhosis. In the pilot study (19) no severe adverse events were noted, the only reported side effect was mild flatulence during the first days of therapy in a minority of the patients. The study itself only places a minimal burden on the patients. Usually patients with cirrhosis are seen in our liver outpatient clinic twice a year. When they are included in the study, two further visits within 12 months are planned. During the visits a blood sample is taken and the gut permeability test is performed. We believe that the potential benefit for the patients outweighs the minimal risk of having a blood sample taken and having to drink a sugar solution. The patients will benefit from a more detailed examination of immune function and if our hypothesis holds true, of the positive effect of the probiotic on immune function.

#### Quality control

The study will be performed in accordance to the ICH-GCP guidelines. The "Koordinierungszentrum für Klinische Studien der Medizinischen Universität Graz" has been consulted during the planning phase of the study and will be involved during the entire study period to assure compliance with the ICH-GCP guidelines. All documents will be checked and data monitoring will be performed.

#### Cooperation

aoUniv. Prof. Dr. R. Stauber (Department of Internal Medicine, Division of Gastroenterology and Hepatology, Medical University of Graz) and Dr. P. Stiegler (Department of Surgery, Division of Transplantation Surgery) will be involved in the recruitment of patients.

aoUniv. Prof. Dr. C. Högenauer (Department of Internal Medicine, Division of Gastroenterology and Hepatology, Medical University of Graz) will perform the gut flora analysis.

Assoz. Prof. Mag. Dr.rer.nat. Tobias Madl (Institute of Molecular Biology and Biochemistry, Medical University of Graz) will perform the gut permeability analysis.

Prof. H. Grønbæk (Medicinsk Hepato-Gastroenterologisk, Aarhus Universitetshospital, Denmark) will support the sCD163 measurements.

Univ-Prof., Dr. B. Obermayer-Pietsch (Division of Endocrinology and Metabolism, Medical University Graz) will perform serotonin assessments.

aoUniv. Prof. Dr. D. Fuchs (Division of Biological Chemistry, Biocentre, Innsbruck Medical University) will support the measurement of tryptophan, kynurenine, phenylalanine, tyrosine, neopterin and nitrite.

Institut Allergosan will provide the probiotic and the placebo at no cost for this study.

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#### Abbreviations

HMA human mercaptalbumin

HNA human non-mercaptalbumin

IL Interleukin

TNFalpha Tumor necrosis factor alpha

TNFR Tumor necrosis factor alpha receptor

TLR Toll like receptor

Signature page

Principal Investigator

Place, Date

Name

PD. Dr. V. Stadlbauer-Köllner

## Appendix 1

### Rationale for Winclove-849

#### Product proposal

Winclove Bio Industries has developed this product especially to strengthen the intestinal barrier and to reduce translocation of harmful content from the lumen of the intestine into the body.

Winclove-859 consists of the following 8 strains:

*Bifidobacterium bifidum* W23

*Bifidobacterium lactis* W52

*Lactobacillus acidophilus* W37

*Lactobacillus brevis* W63

*Lactobacillus casei* W56

*Lactobacillus salivarius* W24

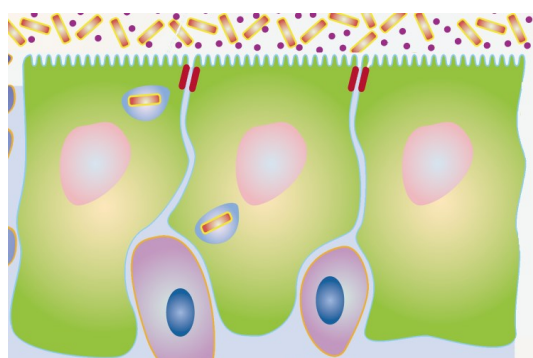
*Lactococcus lactis* W19

*Lactococcus lactis* W58

The product has a concentration of  $2,5 \cdot 10^9$  cfu/gram and the advised daily dosage is 2 grams.

#### Background

The intestinal mucosa is the largest interface between the outside world and the human internal milieu. Across a surface area that approximates the size of a soccer field, it is here where we prevent the highest concentration of bacteria from invading our internal environment while allowing nutrient and water absorption by a single cell layer of epithelium. The ability to control the invasion of harmful content from the lumen is called *intestinal*



*mucosal barrier function*. While the epithelial layer forms the most obvious physical boundary between inner and outer environment, the full complexity of factors that control intestinal barrier function reaches beyond the epithelium and is not fully understood. Throughout the intestine a single layer of epithelial cells covers the inner surface and is responsible for this barrier function.

Figure 1 : Intestinal epithelial cells play a crucial

role in barrier function

Tight junctions are protein structures that allow selective passage of ions and small molecules, but form, in healthy subjects, a tight barrier to protein sized molecules and bacteria. To make matters more complicated, the task of the epithelium is not only to keep bacteria and antigens out while absorbing nutrients, but also to allow contact between luminal contents and immune cells. This occurs through limited and highly controlled uptake of antigen and bacteria. This seemingly paradoxical task is, however, crucial in the induction of targeted and protective mucosal immune responses to pathogens as well as to the development of oral tolerance to commensals and food antigens

The barrier function of the intestine (before described as actions on level 2) can be influenced by different factors, like heredity, bacterial flora, diet, psychological stress, oxidative stress, exercise, and drugs (174). Increased permeability of the epithelial barrier has been associated with many gastrointestinal inflammatory disorders, like inflammatory bowel diseases (Crohn's disease, ulcerative colitis and pouchitis), celiac disease (175). An increased permeability can also lead to increased levels of endotoxins in the blood, which are linked to systemic inflammatory diseases, like metabolic syndrome, diabetes, atherosclerosis, chronic fatigue syndrome, autism, migraine and rheumatoid arthritis. Probiotics have proven capabilities to enhance the epithelial barrier, due to different working mechanisms (176).

#### Development Winclove-849

Winclove-849 is specially designed to improve the epithelial barrier and to increase the resistance to disturbances of the intestinal barrier. The probiotic strains were selected based on the following criteria:

*In vitro* strengthening of the epithelial barrier

Inhibition of mast-cell activation

Inhibition of pro-inflammatory cytokines

Decreasing lipopolysaccharide load

#### *In vitro* strengthening of the epithelial barrier

Trans epithelial electrical resistance (TEER) is an *in vitro* measurement of the movement of ions across the paracellular pathway. Maintenance of the intestinal integrity is critical for essential physiological processes. Therefore, a reduction in TEER may represent an early expression of cell damage and indicates that the barrier function of the intestine is decreased. Pathogenic bacteria, like *Salmonella enteritidis* 857 have shown to decrease the relative TEER in Caco-2 cells. In a small experiment with six bacterial strains, an epithelial cell-line (CaCo-2) was damaged by a pathogenic bacterium, *Salmonella enteritidis*. Three of the 6 strains were able to diminish the decrease in transepithelial resistance (and thus strengthen the barrier function) due to the *Salmonella* significantly, see figure 2. These three strains are present in Ecologic®Barrier.

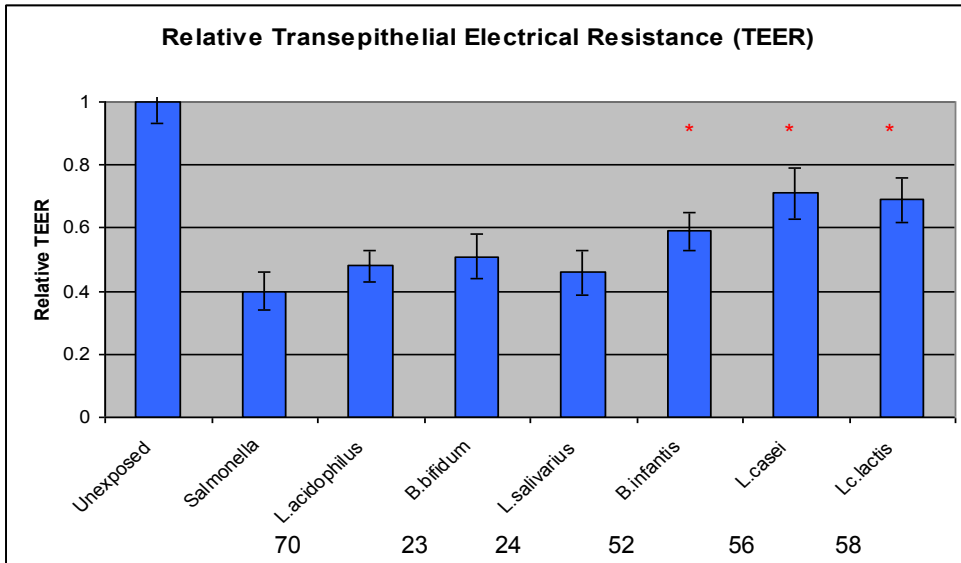
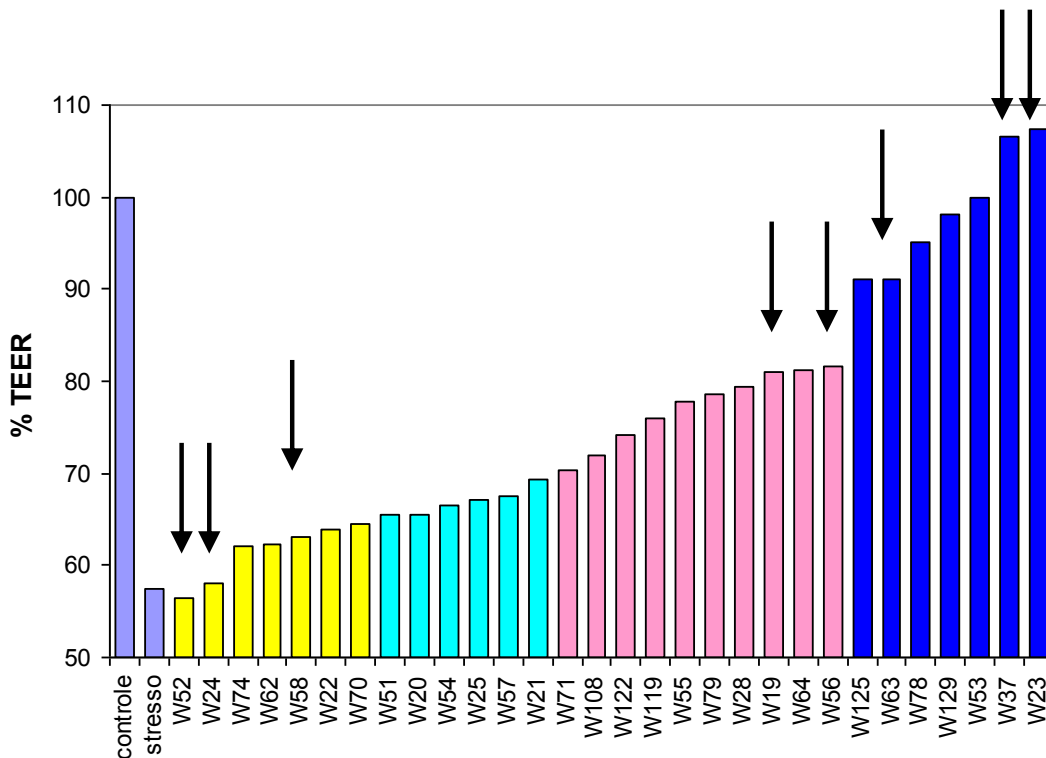


Figure 2: Probiotic effect of Salmonella-induced decrease in transepithelial electrical resistance..

A larger TEER screening of 31 strains with an inflammatory stressor (a combination of TNF- $\alpha$  and IL1- $\beta$ ) instead of *Salmonella enteritidis* has been performed. The cells were two hours pre-incubated with the probiotic strains, after which the inflammatory stressor was added. After 24 hours of incubation with the stressors, the TEER was measured (figure 3).

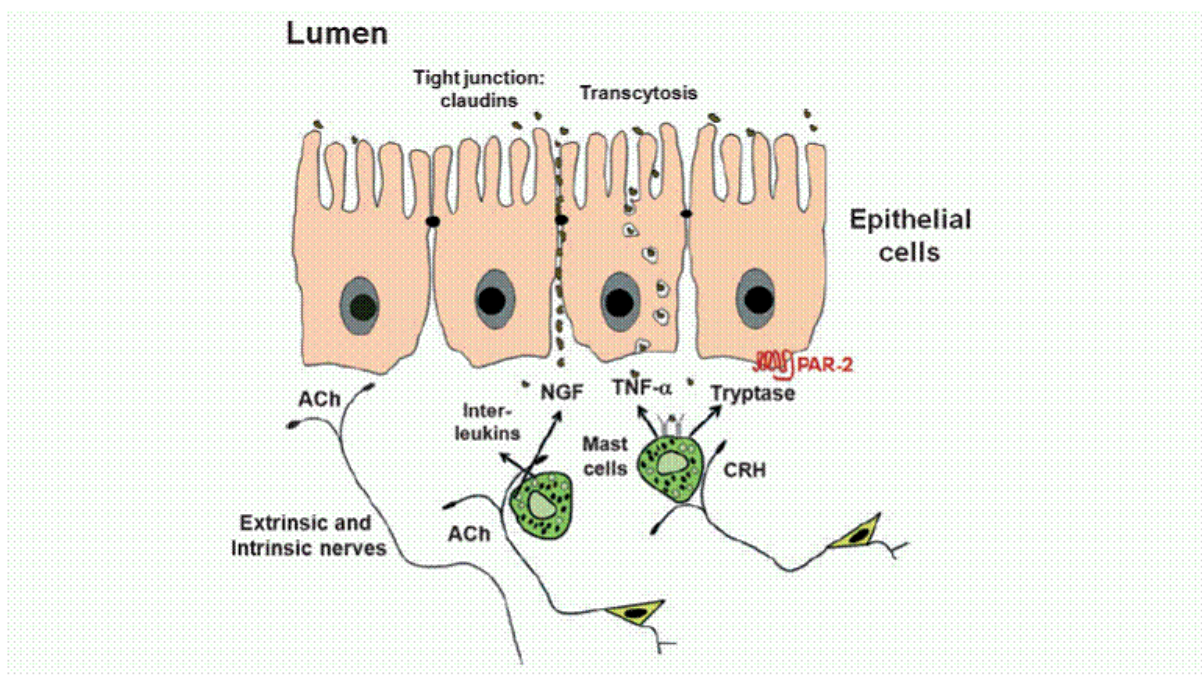


*Figure 3: : Effect of different probiotic strains on cytokine induced barrier dysfunction after 24 hours incubation with TNF- $\alpha$  and IL-1 $\beta$ . The value of the control, without the addition of any stressor, was set at 100%*

Three strains in Winclove-849 could for more than 90% protect the epithelial cell against the cytokine induced dysfunction of the barrier, whereas two others had a partial effect. 3 strains did not show an effect in this assay.

### Inhibition of mast-cell activation

Tight junctions are important proteins in the epithelial cells to maintain the barrier. They can open by the activation of mast cells. These mast cells can be triggered by the hormone Corticotropin-Releasing Hormone (CRH), which is released during stress. Triggering of the mast-cells by CRH results in granulation of mediators like nerve growth factor, different cytokines or  $\beta$ -hexosaminidase. These effects are presented in figure 5. Upon the release of these mediators, the tight junction will open resulting in increased permeability of the small intestine (177). Research has shown that certain probiotic strains can decrease the CRH-induced degranulation of mast-cells and thereby the opening of the epithelial barrier due to stress.



*Figure 5: A simplified scheme of potential mechanisms involved in neuro-immune modulation of the intestinal barrier function.*

In this experiment mast-cells were pre-treated with probiotic strains and subsequently stimulated with CRH. The amount of released  $\beta$ -hexosaminidase was measured to get insight on the protective effects of probiotics to inhibit the increase of the permeability. The results are shown in figure 6. The strains W23, W24, W52 and W56 (which are also selected to be present in Ecologic®Barrier) have a positive effect to prevent the release of  $\beta$ -

hexosaminidase upon CRH treatment. W19 had no preventive effect. The other strains of Winlove-849 were not tested in this assay.

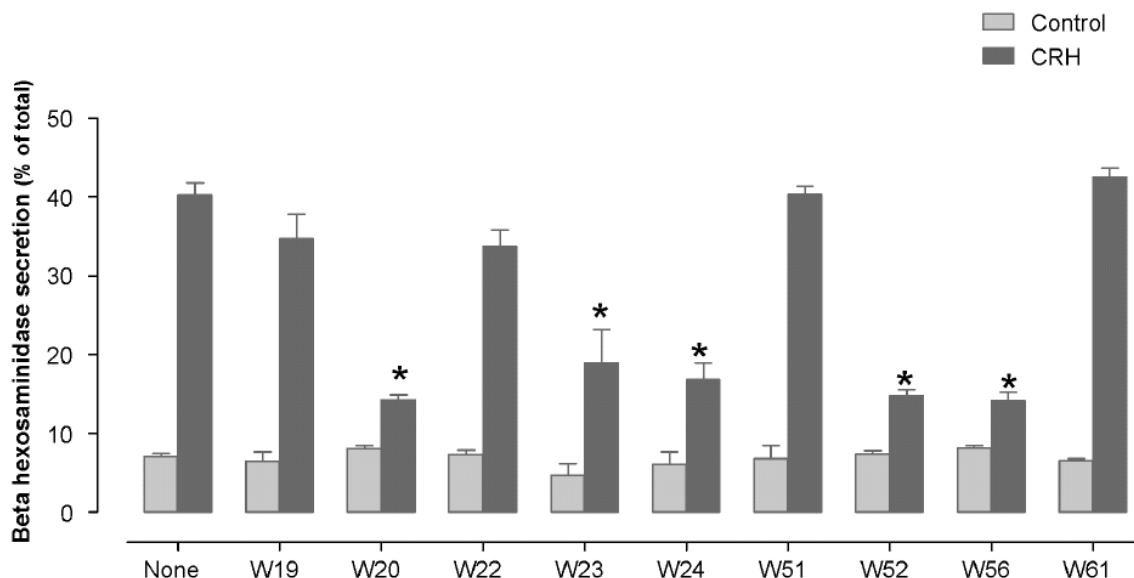


Figure 6: probiotic effect on mast-cells stimulated with CRH (from ref (178), chapter 10).

#### Inhibition of pro-inflammatory cytokines

A large part of the immune system (approximately 80%) is concentrated in and around the intestinal mucosa. The intestinal microbiota is involved in maturation of the immune system as demonstrated in studies in germ-free mice (179). In turn the microbiota in the intestine plays an important role in the regulation of functions in the immune system (180). The immune system can be modulated by probiotic bacteria and these effects are highly species- and strain-specific (180-182). Inflammatory signals cause disruption of the epithelial barrier. Interleukin-10 (IL-10) is an important cytokine to regulated immune responses and to prevent excessive pro-inflammatory responses. Induction of IL-10 by different probiotic strains was measured with an *in vitro* test with peripheral blood mononuclear cells (figure 7) .

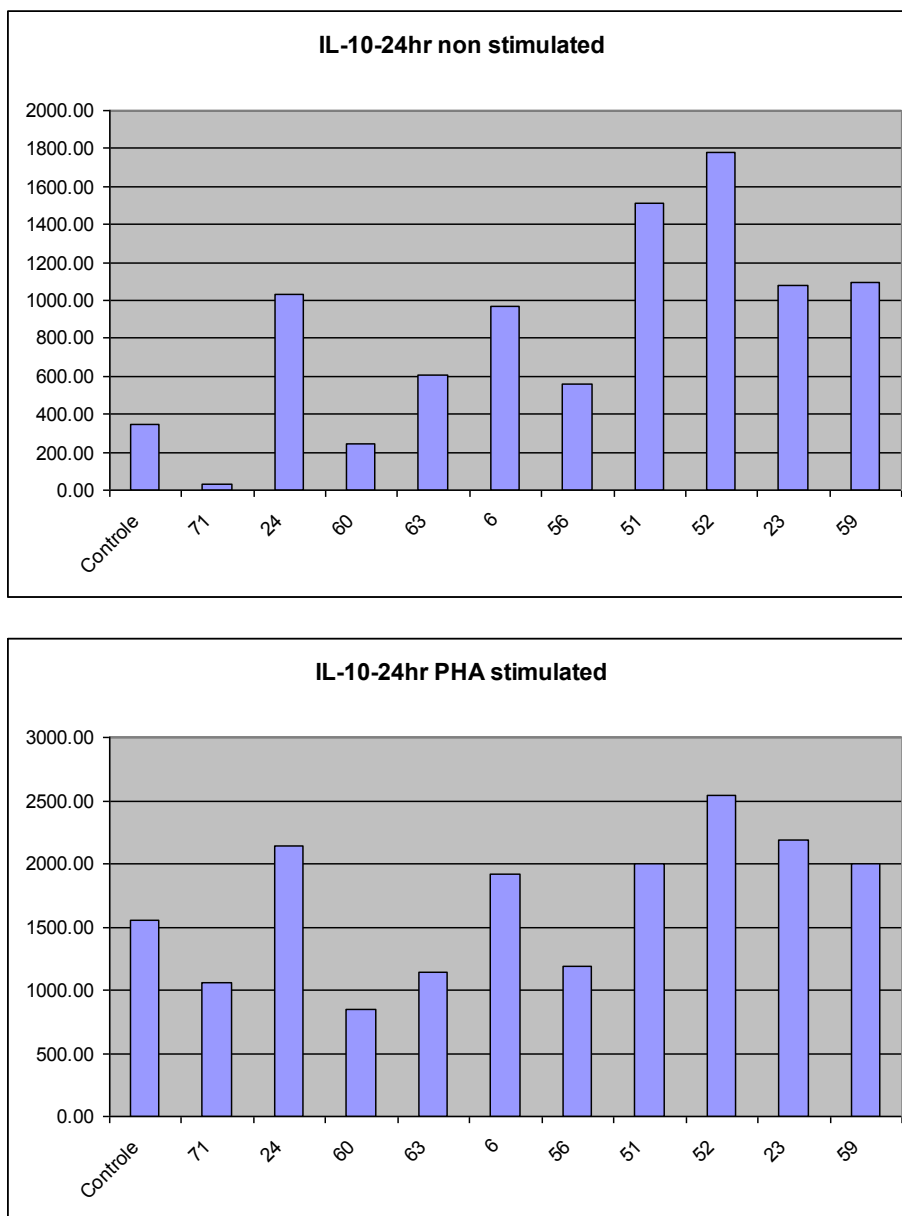


Figure 7: Stimulation of IL-10 production by bacterial strains.

Strains in Winclove-849 which stimulate IL-10 production are W23, W52 and W24.

#### Decreasing lipopolysaccharide (LPS) load

Lipopolysaccharide (LPS) is part of the cell wall of Gram-negative bacteria and is a very pro-inflammatory component. Leaky gut syndrome is associated with increased levels of LPS in blood plasma and lowering of the uptake of LPS is an important aspect of a good intestinal barrier function. Some probiotic bacteria have the capacity to break down LPS by the activity of the enzyme alkaline phosphatase. In an *in vitro* experiment the activity of this enzyme has been measured. In this assay the substrate p-nitrophenylphosphate is split into p-nitrophenol, which has a yellow colour. The colour intensity is measured with a spectrophotometer. The results are shown in figure 8. From the strains present in Winclove-849, W19, W23 and W37 are among the most active strains with regard to alkaline phosphatase activity.

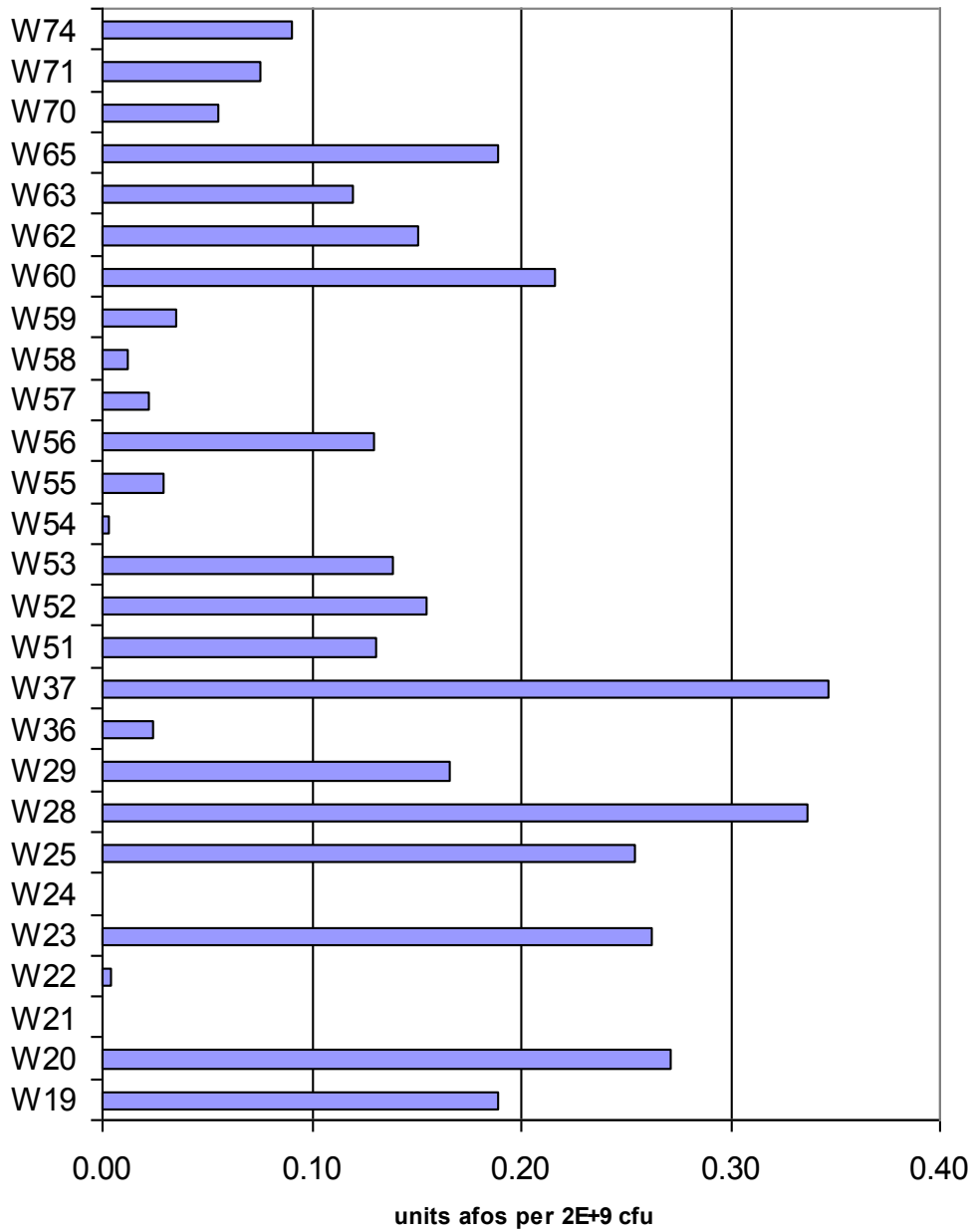


Figure 8: alkaline phosphatase activity of different strains.

## Conclusions

Probiotic bacteria can have an effect on the epithelial barrier via different molecular pathways and routes. Therefore a multispecies product is a very good choice to support these different routes. All the strains present in Winclove-849 were carefully selected based on their *in vitro* capacities to improve the epithelial barrier in at least two different assays. In the near future, the effect of combining this product with other active ingredients will be investigated.

A probiotic product which improve the epithelial barrier might have positive effects on systemic inflammatory diseases and diseases associated with increased pro-inflammatory cytokines, like migraine, autism, diabetes type II, arthritis, liver cirrhosis and inflammatory bowel diseases. There are plans to test the effect on epithelial barrier function *ex vivo*, or *in vivo*.

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## Appendix 2

### Product description



# Winclove 849

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**Product description:**

Light beige powder with good flow-properties. Reasonably dissolvable in water.

**Composition:**

Maize starch, Maltodextrins, Bacterial strains, Vegetable protein, Potassium chloride, Magnesium sulphate and Manganese sulphate.

**Bacterial strains:**

*Bifidobacterium bifidum* W23  
*Bifidobacterium lactis* W52  
*Lactobacillus acidophilus* W37  
*Lactobacillus brevis* W63  
*Lactobacillus casei* W56  
*Lactobacillus salivarius* W24  
*Lactococcus lactis* W19  
*Lactococcus lactis* W58

Total cell count lactic acid bacteria one year after production,  
in Winclove Bio Industries 25 kg drums stored below 25°C:  $\geq 2.5 \times 10^9$  cfu/g

**Physical analysis:**

Moisture  $< 5 \%$

**Microbiological analysis:**

Moulds & Yeasts  $< 500/g$   
*E. coli* absent in 0.1 g  
*Salmonella* absent in 25 g



## (1) SF36 - Fragebogen zum Gesundheitszustand

Name:		Unters.-Datum:	
Vorname:		ID-Nr.:	
Geb.-Datum:		Tel.-Nr.:	

*In diesem Fragebogen geht es um Ihre Beurteilung Ihres Gesundheitszustandes. Der Bogen ermöglicht es, im Zeitverlauf nachzuvollziehen, wie Sie sich fühlen und wie Sie im Alltag zurechtkommen.*

*Bitte beantworten Sie jede der folgenden Fragen, indem Sie bei den Antwortmöglichkeiten die Zahl ankreuzen, die am besten auf Sie zutrifft.*

**1. Wie würden Sie Ihren Gesundheitszustand im allgemeinen beschreiben? (Bitte kreuzen Sie nur eine Zahl an)**

- |               |   |
|---------------|---|
| Ausgezeichnet | 1 |
| Sehr gut      | 2 |
| Gut           | 3 |
| Weniger gut   | 4 |
| Schlecht      | 5 |

**2. Im Vergleich zum vergangenen Jahr, wie würden Sie Ihren derzeitigen Gesundheitszustand beschreiben? (Bitte kreuzen Sie nur eine Zahl an)**

- |   |   |
|---|---|
| Derzeit viel besser als vor einem Jahr      | 1 |
| Derzeit etwas besser als vor einem Jahr     | 2 |
| Etwa so wie vor einem Jahr                  | 3 |
| Derzeit etwas schlechter als vor einem Jahr | 4 |
| Derzeit viel schlechter als vor einem Jahr  | 5 |

**3. Im folgendem sind einige Tätigkeiten beschrieben, die Sie vielleicht an einem normalen Tag ausüben. Sind Sie durch Ihren derzeitigen Gesundheitszustand bei diesen Tätigkeiten eingeschränkt? Wenn ja, wie stark? (Bitte kreuzen Sie in jeder Zeile nur eine Zahl an)**

Tätigkeit	Ja, stark eingeschränkt	Ja, etwas eingeschränkt	Nein, überhaupt nicht eingeschränkt
a. anstrengende Tätigkeiten, z.B. schnell laufen, schwere Gegenstände heben, anstrengenden Sport treiben	1	2	3
b. mittelschwere Tätigkeiten, z.B. einen Tisch verschieben, staubsaugen, kegeln, Golf spielen	1	2	3
c. Einkaufstaschen heben oder tragen	1	2	3
d. mehrere Treppenabsätze steigen	1	2	3
e. einen Treppenabsatz steigen	1	2	3
f. sich beugen, knien, bücken	1	2	3
g. mehr als 1 Kilometer zu Fuß gehen	1	2	3
h. mehrere Straßenkreuzungen weit zu Fuß gehen	1	2	3
i. eine Straßenkreuzung weit zu Fuß gehen	1	2	3
j. sich baden oder anziehen	1	2	3

**4. Hatten Sie in den vergangenen 4 Wochen aufgrund Ihrer körperlichen Gesundheit irgendwelche Schwierigkeiten bei der Arbeit oder anderen alltäglichen Tätigkeiten im Beruf bzw. zu Hause? (Bitte kreuzen Sie in jeder Zeile nur eine Zahl an)**

Schwierigkeiten	Ja	Nein
a. Ich konnte nicht so lange wie üblich tätig sein	1	2
b. Ich habe weniger geschafft, als ich wollte	1	2
c. Ich konnte nur bestimmte Dinge tun	1	2
d. Ich hatte Schwierigkeiten bei der Ausführung (z.B. ich mußte mich besonders anstrengen)	1	2

**5. Hatten Sie in den vergangenen 4 Wochen aufgrund seelischer Probleme irgendwelche Schwierigkeiten bei der Arbeit oder anderen alltäglichen Tätigkeiten im Beruf bzw. zu Hause (z.B. weil Sie sich niedergeschlagen oder ängstlich fühlen)? (Bitte kreuzen Sie in jeder Zeile nur eine Zahl an)**

Schwierigkeiten	Ja	Nein
a. Ich konnte nicht so lange wie üblich tätig sein	1	2
b. Ich habe weniger geschafft, als ich wollte	1	2
c. Ich konnte nicht so sorgfältig wie üblich arbeiten	1	2

**6. Wie sehr haben Ihre körperliche Gesundheit oder seelischen Probleme in den vergangenen 4 Wochen Ihre normalen Kontakte zu Familienangehörigen, Freunden, Nachbarn oder zum Bekanntenkreis beeinträchtigt? (Bitte kreuzen Sie nur eine Zahl an)**

Überhaupt nicht	1
Etwas	2
Mäßig	3
Ziemlich	4
Sehr	5

**7. Wie stark waren Ihre Schmerzen in den vergangenen 4 Wochen? (Bitte kreuzen Sie nur eine Zahl an)**

Ich hatte keine Schmerzen	1
Sehr leicht	2
Leicht	3
Mäßig	4
Stark	5
Sehr stark	6

**8. Inwieweit haben die Schmerzen Sie in den vergangenen 4 Wochen bei der Ausübung Ihrer Alltagstätigkeit zu Hause und im Beruf behindert? (Bitte kreuzen Sie nur eine Zahl an)**

Überhaupt nicht	1
Etwas	2
Mäßig	3
Ziemlich	4
Sehr	5

9. In diesen Fragen geht es darum, wie Sie sich fühlen und wie es Ihnen in den vergangenen 4 Wochen gegangen ist. (Bitte kreuzen Sie in jeder Zeile die Zahl an, die Ihrem Befinden am ehesten entspricht).

Wie oft waren Sie in den vergangenen 4 Wochen ...

Befinden	Immer	Meistens	Ziemlich oft	Manchmal	Selten	Nie
a. ... voller Schwung?	1	2	3	4	5	6
b. ... sehr nervös?	1	2	3	4	5	6
c. ... so niedergeschlagen, daß Sie nichts aufheitem konnte?	1	2	3	4	5	6
d. ... ruhig und gelassen?	1	2	3	4	5	6
e. ... voller Energie?	1	2	3	4	5	6
f. ... entmutigt und traurig?	1	2	3	4	5	6
g. ... erschöpft?	1	2	3	4	5	6
h. ... glücklich?	1	2	3	4	5	6
i. ... müde?	1	2	3	4	5	6

10. Wie häufig haben Ihre körperliche Gesundheit oder seelischen Probleme in den vergangenen 4 Wochen Ihre Kontakte zu anderen Menschen (Besuche bei Freunden, Verwandte usw.) beeinträchtigt? (Bitte kreuzen Sie nur eine Zahl an)

Immer	1
Meistens	2
Manchmal	3
Selten	4
Nie	5

11. Inwieweit trifft jede der folgenden Aussagen auf Sie zu? (Bitte kreuzen Sie in jeder Zeile nur eine Zahl an)

Aussagen	Trifft ganz zu	Trifft weitgehend zu	Weiß nicht	Trifft weitgehend nicht zu	Trifft überhaupt nicht zu
a. Ich scheine etwas leichter als andere krank zu werden	1	2	3	4	5
b. Ich bin genauso gesund wie alle anderen, die ich kenne	1	2	3	4	5
c. Ich erwarte, daß meine Gesundheit nachläßt	1	2	3	4	5
d. Ich erfreue mich ausgezeichneter Gesundheit	1	2	3	4	5



# Ernährungsfragebogen

StudienID

Datum:

Wie häufig verzehren Sie folgende Lebensmittel und Getränke?						
Getreideprodukte	mehrmals täglich	täglich	mehrmals pro Woche	etwa 1 x pro Woche	mehrmals pro Monat	seltener/nie
Brot						
Müsli						
Vollkornprodukte						
Kartoffeln, Reis, Nudeln						
Milch und Milchprodukte	mehrmals täglich	täglich	mehrmals pro Woche	etwa 1 x pro Woche	mehrmals pro Monat	seltener/nie
Käsesorten mind. 45% Fett i. Tr.						
fettarme Käsesorten mit weniger als 45% Fett i. Tr.						
Milch, Topfen, Joghurt o.ä. mit 3,5% Fett						
Milch, Topfen, Joghurt o.ä. mit 1,5%Fett						
Sauerrahm, Obers, Creme fraîche						
Fleisch- und Fischprodukte	mehrmals täglich	täglich	mehrmals pro Woche	etwa 1 x pro Woche	mehrmals pro Monat	seltener/nie
Fleisch						
Fettreiche Wurstsorten (z.B. Leberstreichwurst, Salami, Bergsteiger, Mortadella, ...)						
Fettarme Wurstsorten (z.B. Putenwurst, Schinken, Krakauer,...)						
Fisch						
Fischkonserven						

<b>Obst und Gemüse</b>	mehrmals täglich	täglich	mehrmals pro Woche	etwa 1 x pro Woche	mehrmals pro Monat	seltener/nie
Gemüse und Salat						
Obst						
<b>Fette</b>	mehrmals täglich	täglich	mehrmals pro Woche	etwa 1 x pro Woche	mehrmals pro Monat	seltener/nie
Butter, Margarine						
Halbfettbutter oder -margarine						
Öle						
Schmalz						
<b>Extras</b>	mehrmals täglich	täglich	mehrmals pro Woche	etwa 1 x pro Woche	mehrmals pro Monat	seltener/nie
Süßigkeiten, Schokolade						
Kuchen, Kekse, Torten						
Eis, Pudding und süße Speisen						
In Fett gebackene Speisen (Wiener Schnitzel, Pommes frites,...)						
Chips, Erdnüsse, Knabbererien						
<b>Getränke</b>	mehrmals täglich	täglich	mehrmals pro Woche	etwa 1 x pro Woche	mehrmals pro Monat	seltener/nie
Mineralwasser, verdünnte Obstsaften, Wasser, Tee						
Limonaden, Cola						
Limonaden oder Cola mit Süßstoff						
Obst- und Gemüsesäfte unverdünnt						
Kakao, Milch, Milchshakes						
Kaffee						
Bier, Wein, sonstige alkoholische Getränke						
Energiedrinks						