

Modulation of the Hepatic Cytokine Response to Portal Endotoxaemia using the Probiotic *Lactobacillus Plantarum* 299v

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ABSTRACT

AIM: Therapies are required to reduce bacterial translocation in obstructive jaundice. This study aimed to determine the effect of *LP299v* in biliary obstruction.

METHODS: Rats were randomised into three test-feed groups- *LP299v*, placebo or water. Each received 2 mL daily test feed (day 1-7). All underwent laparotomy and bile duct ligation (day 8). Test feeding continued for another week (day 9-15), prior to isolated *in-situ* hepatic perfusion (day 16). Day 1 gavage with ¹⁴C PEG 4000 provided baseline intestinal permeability, with repetition on days 9 and 15. Isolated *in-situ* hepatic perfusion of Endotoxin (*E. coli*) was performed for 2 hours on day 16. Before and during perfusion samples of influent and effluent perfusate were taken for analysis. Finally, liver sections were processed for histological and TEM assessment.

RESULTS: There was no difference in weight loss, serum liver biochemistry, intestinal permeability, morphology, effluent perfusate TNF α and IL-6 between the three groups. AST and LDH were higher in the effluent perfusate of the placebo group at 80 minutes. The *LP299v* group demonstrated a lower IL-10 response to portal endotoxaemia compared to the other two groups.

CONCLUSION: *LP299v* slightly altered the inflammatory response to portal endotoxaemia. This mild inflammatory modulation may benefit patients undergoing interventional procedures.

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Key words: Cytokine response; Portal endotoxaemia; *Lactobacillus plantarum* 299v

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INTRODUCTION

The barrier function of the gastrointestinal (GI) tract is defective in obstructive jaundice resulting in an increase in bacterial translocation from the gut into the portal circulation^[1-4]. Reynolds *et al*^[5] concluded that biliary obstruction produces bacterial translocation which is greatly enhanced by surgical trauma and endotoxin. Clinical studies confirm dysfunction of the gut barrier in obstructive jaundice^[4-6]. The increased production of cytokines may tip the balance of homeostasis to either an enhanced pro or anti-inflammatory state leading to sepsis and ultimately multi-organ dysfunction syndrome (MODS). Therefore, therapies to enhance gut barrier function merit evaluation in the jaundiced patient^[7, 8].

Orally administered lactobacilli can colonise the GI tract, reducing the levels of pathogenic gram-negative bacteria and increasing the numbers of indigenous gram-positive bacteria^[9]. This is achieved by competition for nutrients and mucosal binding sites, immune stimulation and production of specific antibacterial substances^[10-13]. *LP299v* is a lactobacillus strain that has been well studied in multiple arenas such as intestinal permeability and inflammation. Its production of antimicrobial substances allow it to survive in the gastrointestinal tract. Treatment with *LP299v* is associated with reduced intestinal permeability^[4, 14, 15]. *Lactobacillus* in an isolated colonic loop model reduced intestinal permeability as measured by the lumen to blood clearance of dextran and mannitol^[16]. Similarly, pre-treatment with *LP299v* abolished the *E. coli*-induced increase in gut permeability, but did not change the intestinal passage of mannitol^[17].

The ability of *LP299v* to enhance gut barrier function may be particularly beneficial in the treatment of patients with obstructive jaundice about to undergo a therapeutic intervention. The 'two-hit' model of developing MODS proposes that an initial traumatic event primes the host, so that an innocuous second insult triggers an uncontrolled inflammatory response. This priming of inflammatory

cells may be by low doses of endotoxin, inflammatory mediators or cytokines. The development of obstructive jaundice, which leads to portal endotoxaemia as a result of increased intestinal permeability, is an initial priming event. A 'second hit' may be surgical intervention or in the experimental setting a further endotoxin challenge, leading to an uncontrolled inflammatory response. LP299v may be a useful treatment in preventing the priming of hepatic inflammatory cells by enhancing gut barrier function and thus reducing endotoxaemia.

The aims of this study were firstly to determine the effect of LP299v on intestinal permeability in experimental biliary obstruction and secondly to determine the effect of LP299v on hepatic pro and anti-inflammatory cytokine responses to 'a second hit' of portal endotoxaemia in experimental biliary obstruction.

MATERIALS AND METHODS

Male Wistar rats were randomised to one of three test-feed groups ($n=8$ in each group). The groups were: (1) LP299v in oatmeal; (2) placebo of oatmeal with heat-inactivated LP299v; and (3) water. Each group received the test feed (2 mls) by gastric gavage on a daily basis (day 1-7). The concentration of LP299v in the oatmeal was 2×10^9 cfu/mL. One week following commencement of test feed all rats underwent laparotomy and BDL (day 8). Test feeding was continued for a further 1 week (day 9-15) prior to isolated in-situ hepatic perfusion (day 15). On day 1 each rat was gavaged with ^{14}C PEG 4000 in order to measure baseline intestinal permeability. Measurement of intestinal permeability was repeated on day 9 and 15. Therefore, a measurement of intestinal permeability was performed at the start of the study, following BDL surgery and before isolated perfusion.

Isolated in-situ hepatic perfusion was performed for 2 hour on day 16. Endotoxin (*E. coli* serotype 0111:B4, Sigma Aldrich, UK) was perfused from time 10-20 minutes at a concentration of 1.6 $\mu\text{g}/\text{mL}$. A sample of blood was taken from the inferior vena cava prior to the commencement of perfusion. During the perfusion serial samples of influent and effluent perfusate were taken and placed in ice prior to being centrifuged, aliquoted and stored at -70°C for subsequent biochemical and cytokine analysis.

On completion of 2 hours of perfusion, liver sections were removed and placed in 10% buffered formalin and 2.5% glutaraldehyde. The samples were processed for histological and TEM assessment. Histological scoring was according to Mischinger's grading system for liver injury (Table 1). Ultrastructural assessment of the livers was carried out with the electron microscopist blinded to the particular treatment group. Throughout the study, the liver profile test of bilirubin, AST and LDH were performed in the sponsoring hospital biochemistry laboratory.

TNF α

Bioactive TNF α in plasma and perfusate was measured using an MTT tetrazolium cytotoxicity assay^[18] TNF α -sensitive WEHI-164 clone 13 cells which are murine Balb/c fibrosarcoma cells were used in this assay (European Collection of Animal Cell Cultures, Salisbury, Wiltshire, UK). RPMI-1640 was used as the tissue

culture medium, supplemented with 1 mM sodium pyruvate, 2 Mm L-glutamine (L-glu), 100 IU/mL penicillin/ 100 $\mu\text{g}/\text{mL}$ streptomycin (P/S) and 10% heat-inactivated foetal bovine serum (FBS) (Invitrogen, Paisley, UK). Trypsinization was used to detach the cells from the flask, which was confirmed by light microscopy. The TNF α -sensitive WEHI 164 cells were counted and the cell concentration adjusted to 2×10^5 cells/mL in WEHI culture medium.

Ten μL of cells and medium was taken from a T75 flask and mixed with 90 μL trypan blue dye (Sigma). This dye was used for cell counting as it determines the percentage of viable cells. The mix of cells and trypan was placed on a Mod-Fuchs haemocytometer (Merck Biochemicals, Dorset, England), which consisted of a grid divided into 16 small squares. The cell concentration per ml was then calculated.

One hundred μL of cells were seeded and allowed to adhere overnight in the CO₂ incubator. Perfusate and serum samples were diluted in Actinomycin D-supplemented (6 $\mu\text{g}/\text{mL}$) WEHI culture medium. Recombinant murine (rMu) (Genzyme Diagnostics, Cambridge, MA, USA) or recombinant human (rHu) (NIBSC, Potters Bar, UK) TNF α standard was serially diluted in the range 0-2000 pg/mL in Actinomycin D-supplemented WEHI medium and again 50 μL of each concentration were added to wells in duplicate.

The following day, 20 μL MTT (Sigma) was added to each well. Following incubation for 5 h, cell viability was determined by adding 50 μL 20% SDS in 10 mM HCL to each well. The next day the absorbance of each well was read on a Thermo-max microplate reader (Molecular Devices, CA, USA), at a wavelength of 570 nm.

IL-6

The IL-6 bioassay used murine B9 B-cell hybridoma cells, which are highly dependent on IL-6 for growth. The growth medium used for B9 cells was RPMI-1640 (Invitrogen) supplemented with 1 mM sodium pyruvate, 2 mM L-glu, P/S, 10% heat-inactivated FBS, 5 μM 2-mercapto ethanol (ME) and 5% Briclone (hybridoma cloning medium, IL-6 source, Archport Ltd., Dublin).

On the day of assay the B9 cells in tissue culture medium were placed in a 15 mL falcon tube and centrifuged at 900 rpm for 8 min at room temperature. Supernatant was discarded and the resultant cell pellet was resuspended in fresh RPMI-medium (without briclone) and the centrifugation step repeated. This procedure was repeated 3 times to ensure complete removal of IL-6. The cell pellet was resuspended in IL-6 free culture media and cells were counted as for the TNF α assay. The cell concentration was adjusted to a final concentration of 2.5×10^4 cells/mL.

One hundred μL of cells were seeded into each well of a 96-well microtitre plate. Serum and perfusate samples were diluted as appropriate using IL-6 - free diluent (RPMI and supplements minus Briclone) and 100 μL added to the wells in duplicate. The standard used was recombinant murine IL-6 standard which was serially diluted in the range 0-1000 pg/mL in IL-6-free culture medium with 100 μL of each concentration added to the wells in duplicate.

After 4 days incubation at 37°C , 20 μL MTT and, 5 hour later, 50 μL 20% SDS (in HCL), were added to each well. The plate was then incubated for a further 48 h at 37°C . Following this, the absorbance of each well was read at a wavelength of 570 nm.

IL-10

IL-10 was measured using a Quantikine M rat IL-10 Immunoassay kit (R&D Systems Europe Ltd, Oxon, UK). This assay employed the technique of quantitative sandwich enzyme immunoassay (ELISA). It contains *E. coli*-expressed recombinant rat IL-10 and antibodies

Table 1: Mischinger's histological grading system for liver injury.

Score	Degree of injury	Histological features
A	No injury	Sinusoidal lining cells slender and fusiform configuration, attached to the regularly arranged cords of hepatocytes
B	Slight injury	Sinusoidal cells round and denuded in configuration and detached from cords
C	Moderate injury	Sinusoidal lumen laced with sloughs of nuclear debris
D	Severe injury	Complete disruption of architecture

raised against the recombinant factor and can be used to measure rat IL-10 levels in cell culture supernatants, serum and plasma. The intensity of the colour measured, resulting from the enzyme reaction, is proportionate to the amount of IL-10. The sample values are read off the standard curve to give the amount of IL-10 in pg/mL. The minimal detectable dose of rat IL-10 is just below 10 pg/mL.

Statistical analysis

Pre-study sample size calculations indicated that a minimum of 8 animals per group would have 80% power to demonstrate statistical significance at 5%, based on previous experiments by Kennedy *et al*^[19] The data were recorded on SPSS (version 10.0, SPSS Inc., Chicago, USA) and then analysed. Non-parametric tests were used due to the small numbers. Related samples were analysed by Wilcoxon Signed-rank test, while Kruskal Wallis test or Friedman test as appropriate were used for unrelated samples. A *p* value of 0.05 was considered statistically significant.

RESULTS

Mortality

Of the initial 24 rats randomised to the three groups, there were 4 deaths and 4 failed perfusions. A further 12 rats were recruited and randomised. Therefore, in the final analysis involved 8 animals in each of the groups. Table 2 summarises the experimental outcome of all animals studied.

Table 2: Experimental outcomes in each group.

Group	Completed study	Died at or following		Not perfused
		first operation	perfusion	
LP299v	8	2	2	0
Placebo	8	0	1	3
Water	8	2	2	0
Total	24	4	5	3

Body weight

There was no difference in weights between the three groups at day 1, 8 and 16 (*p*=0.39, 0.88 and 0.53 respectively). However, there were significant weight changes within each group with time (LP299v: *p*=0.005, placebo: *p*=0.009 and water: *p*=0.005.). All three groups gained weight from the commencement of test feeding until the day of BDL surgery. This was followed by a fall in weight in all groups prior to perfusion (Figure 1).

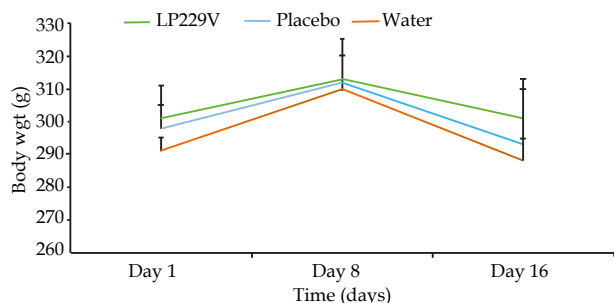


Figure 1 Changes in body weight in the three groups, median (IQR).

Serum liver biochemistry

There were no differences on day 16 in serum bilirubin, ALP and AST between the three groups (*p*=0.76, 0.98 and 0.39 respectively; figure 2).

Intestinal permeability

The baseline intestinal permeability measurement was approximately

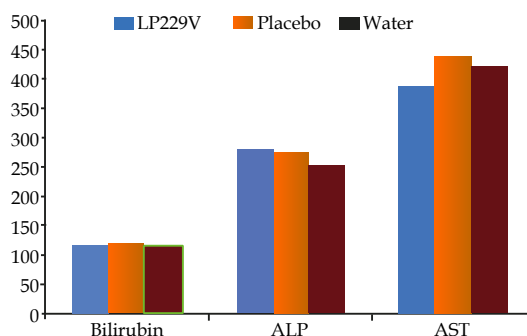


Figure 2 Serum liver biochemistry, showing median values for bilirubin, AST and LDH at day 16.

0.49% excretion. There was no difference in percentage excretion of ¹⁴C PEG between the three groups at day 1, 9 and 15 (*p*=0.28, 0.65 and 0.29 respectively). However, there was a significant difference in the percentage secretion of ¹⁴C PEG within each group over the three time points (LP299v: *p*=0.002, placebo: *p*=0.02, water: *p*=0.008, Friedman test). In both the placebo and water groups there was an increase in the percentage excretion of ¹⁴C PEG following BDL surgery (0.48±0.05 to 0.59±0.08 and 0.42±0.06 to 0.51±0.05 respectively; mean±SEM). In the LP299v group, there was a decrease in the percentage excretion of ¹⁴C PEG following BDL surgery compared to day 1 (0.54±0.05 to 0.49±0.03). These changes in intestinal permeability were not statistically significant. However, in all three groups percentage excretion of ¹⁴C PEG was significantly lower on day 15 compared to day 9 (LP299v: 0.25±0.05, *p*=0.012, placebo: 0.32±0.03, *p*=0.018, water: 0.12±0.05, *p*=0.012; figure 3a). The percentage change in ¹⁴C PEG excretion on day 9 and day 15 compared to day 1 is shown in figure 3b.

There was no difference in the volume of urine produced over each 24 hour time period between the three groups (day 1: *p*=0.59, day 9: *p*=0.573, day 15: *p*=0.37). However, there was a significant increase in the urinary volume within the LP299v and placebo groups with time (LP299v: *p*=0.007 and placebo: *p*=0.03).

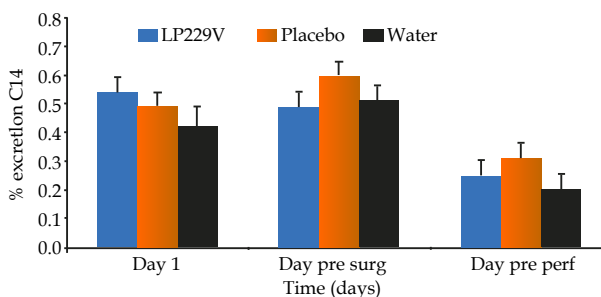


Figure 3a Percentage excretion of ¹⁴C PEG in each group. LP299v day 9 vs. day 15, *p*=0.012; placebo day 9 vs. day 15, *p*=0.018; water day 9 vs. day 15, *p*=0.012, Wilcoxon signed ranks test.

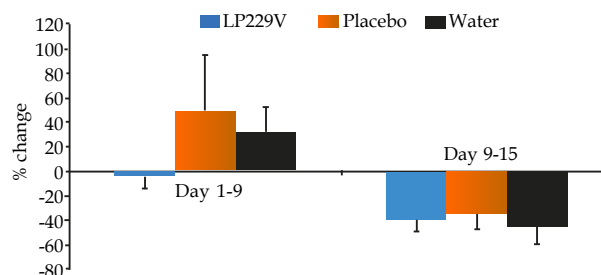


Figure 3b Percentage change in intestinal permeability in each group at Days 1-9 and Days 9-15.

Perfusate liver biochemistry

There was no difference in effluent perfusate bilirubin levels at 10, 40, 80 and 120 min. There was a significant difference in the effluent AST between the groups at 80 minutes ($p=0.01$). Post hoc analysis using the Mann Whitney U test confirmed that the placebo group had a significantly higher concentration of effluent AST and LDH compared to the other groups at 80 minutes (AST $p=0.01$; LDH $p=0.045$). Effluent perfusate pO_2 , pCO_2 and pH remained physiological throughout the perfusion with no significant difference between the groups.

Histology scoring and Transmission electron microscopy

Histological scoring showed that the majority of the livers had no evidence of moderate or severe injury (Figure 4). All the livers demonstrated features of increased ductal proliferation, sinusoidal dilatation and areas of intracytoplasmic vacuolation. There was evidence of Kupffer cell activation in all the sections examined but no clear difference in morphology between liver sections.

Effluent Perfusate $TNF\alpha$

Repeated measures analysis over time, between the three groups revealed no significant difference in the $TNF\alpha$ levels, although there appeared to be a reduction in concentration of effluent $TNF\alpha$ in response to portal endotoxaemia in the LP299v group compared to water and placebo ($p=0.5$; figure 5).

Effluent Perfusate IL-6 and IL-10

Repeated measures analysis over time between the three groups revealed no difference in the effluent perfusate IL-6 levels over time ($p=0.73$; figure 6). There was a significantly lower IL-10 concentration at time 100 minutes in the effluent perfusate of the LP299v group compared to the other two groups ($p=0.035$; figure 7)

DISCUSSION

Physiological Changes in each Test Feed Group

The loss of weight following surgery in all three groups before perfusion emphasises that jaundice is associated with a decline in general wellbeing. The administration of LP299v did not alter this catabolic response to surgery. White *et al*^[14] showed weight reduction in both LP299v and placebo fed animals but no inter-group difference. LP299v did not increase morbidity or mortality, nor did it offer any protection from the effects of weight loss and liver enzyme changes in the BDL group.

The Effects of LP299v on Intestinal Permeability

Intestinal permeability was measured by urinary excretion of ¹⁴C-PEG 4000. It is known that such repeated measurement does not result in urinary accumulation of ¹⁴C-PEG^[4,14]. No difference existed in the inter-group baseline intestinal permeability.

An increase in intestinal permeability was seen in the placebo and water groups compared to LP299v. Clinical studies have demonstrated that surgical insult may increase intestinal permeability^[20-23]. This may be attenuated by LP299v following surgical insult. The decreased intestinal permeability is difficult to explain as other studies have reported an increase in intestinal permeability following BDL^[4]. Changes in intestinal permeability may depend on other physiological circumstances. The volume of urine produced by the animals in each group was much lower at the time of the last intestinal permeability measurement. This may have been due to the effect of BDL on the animals decreasing their oral intake of fluid and causing dehydration. The renal function was not

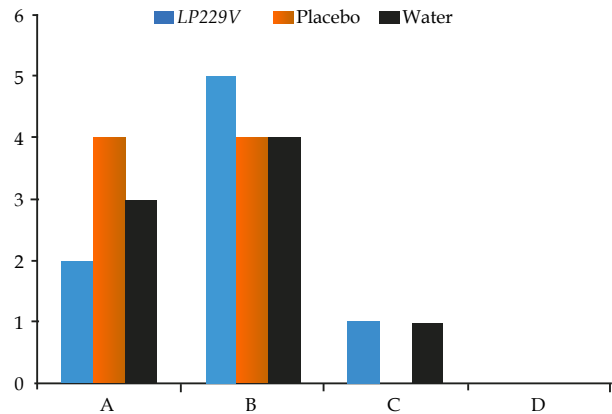


Figure 4 Histological scoring of perfused livers.

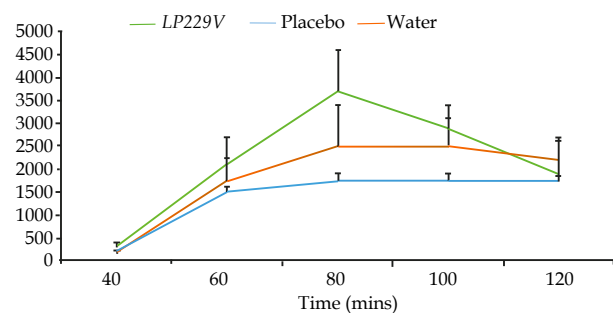


Figure 5 Effluent perfusate $TNF\alpha$ concentrations in each group, mean \pm SEM.

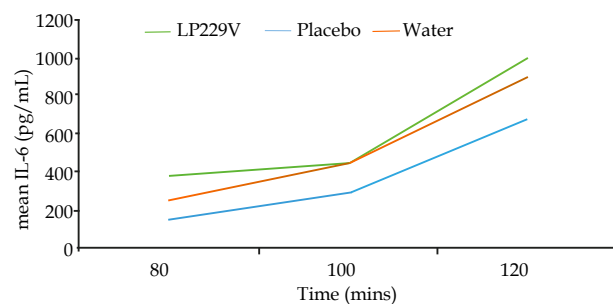


Figure 6 Effluent perfusate IL-6 concentrations in each group, mean \pm SEM.

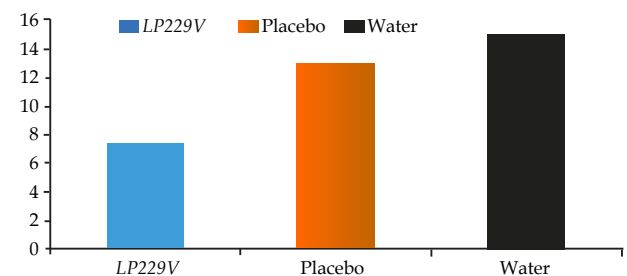


Figure 7 Effluent perfusate IL-10 concentrations in each group, mean \pm SEM.

tested in this study nor were the carcasses measured for radioactivity. All animals were allowed standard chow throughout and this may have influenced the intestinal permeability in the water group. One weakness in this study is that animals were not pair-fed. Conclusions are therefore difficult to make, as feeding with LP299v seemed to prevent an increase in intestinal permeability immediately following surgical intervention, but all groups of animals had a similar reduction in intestinal permeability following bile duct ligation.

White *et al.*^[14] showed a significant difference in intestinal permeability between LP299v and placebo-fed animals 1 week after BDL. LP299v protected against an *E. coli*-induced increase in intestinal permeability^[17]. A 53% increase in mannitol passage as a result of *E. coli* was abolished by pre-treatment with LP299v^[17].

Isolated Hepatic Perfusion

The pO₂ and pH in the effluent perfusate demonstrated that the portal pressure, flow rate and oxygenation of the Krebs-Henseleit buffer ensured a physiological environment. The effluent perfusate bilirubin levels remained at 2 µmol/L throughout the perfusion in all animals^[24]. The difference in effluent AST and LDH between the three groups at 80 minutes was lost at 120 minutes. However, the levels of effluent AST and LDH increased during the perfusion suggesting a degree of hepatic damage. On closer inspection of Alexander's data, perfusate AST and ALP levels were similar to this study during the first 2 hours of perfusion. However, Mischinger *et al.*^[25], looking at different perfusates in isolated perfusion, found lower levels of effluent LDH using Krebs Henseleit buffer

Centrilobular vacuolation and sinusoidal dilatation were present on histological examination, with slight injury. Ultrastructural assessment carried out on the liver samples from each group did not reveal specific features suggesting any benefit of LP299v. Although the electron microscopist was blinded to which livers being examined, there was no evidence of subtle changes in the Kupffer cell morphology between groups. This is not unusual as all livers came from jaundiced animals and therefore, showed evidence of marked Kupffer cell activity with increased number and size, as well as evidence of large secondary lysosomes. It was hoped that if LP299v did enhance gut barrier function and reduce endotoxaemia over the week prior to perfusion, there would be less Kupffer cell activity. However, in order to demonstrate this, a quantitative pathological assessment of the Kupffer cells would need to be carried out. The ingestion of LP299v should improve hepatic function, through the alteration in gut flora^[13,20,26] Zhang *et al.*^[11] concluded that LP ingestion assisted the return to active hepatic barrier function.

The Effects of LP299v on Hepatic Pro and Anti-inflammatory Cytokine Response to Portal Endotoxaemia

Probiotics enhance humoral immune responses and promote the intestine's immunological barrier^[27,28]. Several studies have demonstrated the activation of macrophages, increased phagocytosis and release of proinflammatory cytokines by orally administered species of *Lactobacillus*^[29-31]. Paradoxically, others suggest probiotics are capable of mediating suppression of lymphocyte proliferation and cytokine production by T-cells^[32,33].

The reduction in TNF α secretion with LP299v may attenuate an exaggerated inflammatory response to a second surge of endotoxaemia at the time of intervention in obstructive jaundice. There was no difference in the production of IL-6 in the three groups and a lower IL-10 production in the LP299v group at 100 minutes. This reduced IL-10 may be a direct consequence of reduced TNF α production, since IL-10 is known to reduce TNF α production^[34-36]. It may be that there is not the requirement for a significant IL-10 rise as a result of a diminished proinflammatory response. However, pre-feeding with LP299v influences the subsequent cytokine inflammatory balance to portal endotoxaemia. Gut barrier function is enhanced at the time of BDL surgery, which may partly explain the reduced inflammatory response. The change in the inflammatory balance as a result of feeding with LP299v may prevent a marked

anti-inflammatory response occurring.

LP299v may prevent the increase in intestinal permeability seen following surgical trauma and the development of obstructive jaundice. Animals fed LP299v before and after the development of jaundice had a slightly altered inflammatory response to portal endotoxaemia with a reduction in the proinflammatory cytokine TNF α and a reduction in the anti-inflammatory cytokine IL-10. The production of IL-6 is not influenced by feeding with LP299v. Since these results are not completely conclusive of effect, further studies are required to see if the administration of probiotics is beneficial pre and post intervention in patients with obstructive jaundice. Reduction in macrophage priming as a result of decreased portal endotoxaemia would potentially be of benefit at the time of a planned intervention in this group of patients.

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