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Effects of probiotics, lactitol and rifaximin on intestinal flora and fecal excretion of organic acids in cirrhotic patients

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AIM: The aim of the present study was to assess fecal organic acid excretion and gut flora changes in a group of patients with compensated liver cirrhosis without hepatic encephalopathy by comparing probiotic therapy with more common therapeutic approaches.

METHODS: Thirty patients with compensated Child B liver cirrhosis were allocated into one of three matched groups, which were randomly given one of three 3-week oral treatments: (i) lactitol 20 g t.i.d.; (ii) 400 mg rifaximin b.i.d.; or (iii) the synbiotic SCM-III (Microflorana-F, Named, Lesmo, Italy) 10 mL t.i.d. Stool samples were collected at both the time of entry into the study and at the end of the trial period for the assessment of intestinal bacterial flora and for the determination of fecal pH and of organic acid concentration.

RESULTS: All three tested compounds significantly increased the total anaerobic bacterial count to the same extent. The change was mainly due to a reduction in the *Bacteriodes* population and an expansion of the bifidobacteria population. However, only SCM-III significantly decreased the total count of *Bacteroides* and *Clostridium*. Lactitol and SCM-III decreased (to a similar extent) the fecal pH compared with healthy controls and with pretreatment values ($P < 0.05$). Both lactitol and SCM-III produced a significant increase in the fecal concentration of acetic acid and lactic acid. However, only SCM-III decreased the fecal concentration of toxic short-chain fatty acids.

CONCLUSIONS: In the present clinical study, we confirmed the findings from an *in vitro* study of enhanced-non-toxic organic acid recovery from stools during treatment with nonabsorbable disaccharides. In the present study, we found that lactitol did not produce any significant effect on *Bacteroides* and *Clostridium*, whereas the specific bacterial counts of such species significantly decreased only in the group treated with the synbiotic. These data suggest a potential role of synbiotics in the long term treatment of chronic liver disease.

KEY WORDS: cirrhosis, intestinal flora, lactitol, probiotics, rifaximin, SCM-III.

INTRODUCTION

Lactulose and lactitol are the most frequently used agents in the treatment of hepatic encephalopathy because of their efficacy and the small number of reported mild side-effects.[1–3] *In vitro* fecal incubation studies have shown that the production of C4-6 fatty acids, which primarily arise from the breakdown of amino acids, are inhibited by either acidification or the presence of lactulose in the buffered medium.[4] Rifaximin, a virtually nonabsorbable erythromycin derivative, has a broad antibacterial activity against Gram-positive bacteria, and has been shown to improve hepatic encephalopathy to the same extent as other poorly absorbed antibiotics.[5,6] Although gut

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flora and fecal pH manipulations with beneficial effects are among the targets of the above therapeutic measures, only a few studies have looked at the use of probiotics in a clinical environment before overt encephalopathy occurs.[7,8] Recently, we demonstrated that a novel symbiotic (SCM-III) can significantly reduce endotoxinemia with a parallel improvement in liver damage,[9] whereas ongoing clinical studies show similar promising results (F. Marotta, unpubl. data). The aim of the present study was to gain further insights into fecal organic acid excretion and gut flora changes in a group of patients with compensated liver cirrhosis without hepatic encephalopathy by comparing probiotics and more common therapeutic approaches.

MATERIALS AND METHODS

Patients

Our study population consisted of 30 patients with compensated Child B liver cirrhosis (i.e. serum bilirubin level >2 mg/dL, serum albumin <3.5 g/dL, prolonged prothrombin time, transient encephalopathy, ascites responsive to treatment). Patients were aged 58–74 years, with a male to female ratio of 19/11. Patients had no history of prior encephalopathy episodes or gastrointestinal bleeding. There was no history of ongoing alcoholism nor of administration of intravenous albumin, antibiotic, nonabsorbable disaccharides, probiotics or amino acid supplements in the previous 3 months. A dietary history, with emphasis on recording fiber and protein intake, was taken. Afterwards, patients were allocated to one of three groups that had been previously matched for clinical, medication and dietary details. Groups were then randomly given one of the three following 3-week oral treatments: (i) lactitol 20 g t.i.d.; (ii) 400 mg rifaximin b.i.d.; or (iii) SCM-III (*Lactobacillus acidophilus*, *Bifidobacterium*, *Lactobacillus bulgaricus* in an ion/vitamin/phytochemical extracts-enriched medium [Microflorana-F, Named, Lesmo, Italy]) 10 mL t.i.d. Stool samples were collected both at entry into the study and at the end of the trial period. Stool specimens were also collected from 10 healthy subjects of comparable age.

Assessment of intestinal bacterial flora

Determination of bacterial flora was carried out as reported by Mitsuoka *et al.*[10] Briefly, 9 mL of a diluent was added to 1 g of the fecal sample, the mixture was vigorously shaken and tenfold serial dilutions of the suspension were prepared. Each dilution was set in aliquots of 0.05 mL onto agar plates of media that was appropriate for the target organisms as shown in Table 1. The organisms grown were identified and counted after incubation for 48 h at 35°C for aerobes and for 72 h at 35°C for anaerobes in an anaerobic tube. Bacterial identification was based on the morphology of the colonies, microscopic examination of Gram-stained slides, tests for growth under aerobic conditions and appropriate biochemical tests. A peptone yeast extract solution was used to examine the *bifidus* factors derived from non-carbon sources. The bacterial cells grown were sedimented at 7000 *g* for 10 min, washed three times with 5 mL each of sterile physiological saline (0.85% NaCl, 0.1% L-cysteine-HCl and 0.1% sodium thioglycolate) and finally suspended in 5 mL of reduced physiological saline. The number of organisms per gram of feces was calculated, the lower limit of detection being 2×10^2 colony forming units per g for each isolate.

Table 1. Percentage distribution of fecal bacterial flora: effect of lactitol, rifaximin and SCM-III pre- and post-treatment

	Lactitol	Rifaximin	SCM-III
Aerobes			
Pre-treatment	9	5	6
Post-treatment	3	2	2
Bacteroides			
Pre-treatment	43	42	40
Post-treatment	28*	29*	25*
Bifidobacterium			
Pre-treatment	21	22	23
Post-treatment	39*	36*	42*
Eubacterium			
Pre-treatment	8	14	11
Post-treatment	16*	20*	19*
Others			
Pre-treatment	19	17	20
Post-treatment	14	13	12

*$P < 0.05$ *vs* pretreatment values.

Determination of fecal pH and organic acid concentration

Fecal pH was measured by a pH-meter electrode (F-22, Horiba, Kyoto, Japan), which was inserted deep into the feces immediately after collection. The remaining fecal samples were stored in sealed sterilized tubes at −80°C to determine the organic acids concentration. Later, after thawing, the frozen fecal samples were assayed for organic acid concentration by using a specific high-performance liquid chromatography analytical system (Shimazu Seisakusho, Tokyo, Japan) as described by Hara *et al.* with minor modifications.[11]

Table 2. Fecal flora assessment (log no. species per g wet feces): effects of lactitol, rifaximin and SCM-III pre- and post-treatment (showing only relevant data)

Bacterial species	Lactitol	Rifaximin	SCM-III
Total aerobes			
Pre-treatment	8.14 ± 0.22	8.26 ± 0.20	8.23 ± 0.12
Post-treatment	8.08 ± 0.18	8.19 ± 0.11	8.15 ± 0.16
Total anaerobes			
Pre-treatment	9.77 ± 0.17	9.23 ± 0.31	9.51 ± 0.26
Post-treatment	11.02 ± 021*	10.28 ± 017*	11.13 ± 011*
Lactobacillus			
Pre-treatment	8.15 ± 0.11	8.01 ± 0.07	7.89 ± 0.22
Post-treatment	8. 63 ± 0.23*	8. 33 ± 0.17*	8. 41 ± 0.09*
Eubacterium			
Pre-treatment	8.66 ± 0.25	8.71 ± 0.31	8.59 ± 0.25
Post-treatment	9.44 ± 0.15*	9.33 ± 0.11*	9.51 ± 0.16*
Bifidobacterium			
Pre-treatment	9.34 ± 0.12	9.12 ± 0.20	9.43 ± 0.31
Post-treatment	10.10 ± 0.11*	10.08 ± 0.10*	11.66 ± 0.24*
Bacteroides			
Pre-treatment	9.50 ± 0.27	9.75 ± 0.24	9.69 ± 0.17
Post-treatment	9.39 ± 0.31	9.52 ± 0.34	9.11 ± 0.20*
Clostridium			
Pre-treatment	9.50 ± 0.29	9.18 ± 0.21	8.51 ± 0.09
Post-treatment	8.93 ± 0.32	9.10 ± 0.13	7.26 ± 0.22*

*$P < 0.05$ *vs* pretreatment values.

One gram of fecal sample was homogenized in a solution containing 2 mL of 10% sodium tungstenate, 2 mL of 1 N sulfuric acid and 1 mL of 15 mM 2-ethylbutyric acid as an internal standard and then centrifuged for 10 min at 7000 *g*. The volatile fatty acids and lactic acids in the supernatant were converted to their respective 2-nitrohydrazides using 1-ethyl-3-(3-dimethyl amino propyl) carbodimide hydrochloride for high-performance liquid chromatography analysis.

Statistical analysis

Significance was established by analysis of variance and the level of significance was determined by using Duncan's multiple-range test. Data are expressed as means (SD) and a probability value of <0.05 indicates significance.

RESULTS

Assessment of intestinal bacterial flora

Compared with pretreatment values, all three tested compounds significantly increased total anaerobic bacterial count to the same extent. The change was mainly due to a decrease in the *Bacteriodes* population and an increase in bifidobacteria (Table 1). However, only SCM-III significantly decreased the total count of *Bacteroides* and *Clostridium* when compared with pretreatment values and the results of other groups ($P < 0.05$ *vs* pretreatment and *vs* lactitol and rifaximin; Table 2).

Table 3. Fecal pH: effect of lactitol, rifaximin and SCM-III administration

Group	Pre-treatment	Post-treatment
Lactitol	7.0688 ± 0.097	6.5053 ± 0.133*
Rifaximin	7.1989 ± 0.122	7.0689 ± 0.112
SCM-III	7.2803 ± 0.108	6.1894 ± 0.105†
Healthy controls	7.1473 ± 0.113	

*$P < 0.05$ *vs* healthy controls and *vs* pretreatment value. †$P < 0.01$ *vs* healthy controls and *vs* pretreatment value.

Determination of fecal pH and organic acid concentration

Both lactitol and SCM-III decreased fecal pH to an equal extent as compared with both healthy controls and with pretreatment values ($P < 0.05$), whereas rifaximin didn't produce any appreciable effect. Both lactitol and SCM-III brought about a significant increase in the fecal concentration of acetic acid and lactic acid ($P < 0.05$ *vs* pretreatment values; Table 3).

Table 4. Fecal concentration of organic acids (mg/g; only those that changed) pre- and post-treatment

Organic acid	Lactitol	Rifaximin	SCM-III
Acetic acid			
Pre-treatment	5.12 ± 0.22	5.62 ± 0.31	5.41 ± 0.18
Post-treatment	5.92 ± 0.24*	5.33 ± 0.34	5.76 ± 0.14*
Lactic acid			
Pre-treatment	2.26 ± 0.14	2.54 ± 0.22	1.99 ± 0.19
Post-treatment	2.62 ± 0.17*	2.32 ± 0.23	2.51 ± 0.12*
Butyric acid			
Pre-treatment	1.74 ± 0.14	1.75 ± 0.23	1.74 ± 0.19
Post-treatment	1.68 ± 0.18	1.70 ± 0.21	1.56 ± 0.13*
Iso-butyric acid			
Pre-treatment	0.26 ± 0.05	0.27 ± 0.07	0.28 ± 0.03
Post-treatment	0.25 ± 0.09	0.24 ± 0.05	0.22 ± 0.06*
Propionic acid			
Pre-treatment	1.63 ± 0.04	1.61 ± 0.08	1.62 ± 0.06
Post-treatment	1.60 ± 0.05	1.57 ± 0.07	1.51 ± 0.03*
Valeric acid			
Pre-treatment	0.28 ± 0.03	0.29 ± 0.02	0.32 ± 0.03
Post-treatment	0.25 ± 0.09	0.25 ± 0.07	0.19 ± 0.05*

*$P < 0.05$ *vs* pretreatment values.

However, only SCM-III produced a decreased fecal concentration of some toxic short-chain fatty acids (isobutyrate, butyrate, propionate and valerate; $P < 0.05$ *vs* pretreatment values; Table 4). Rifaximin treatment did not significantly affect the fecal output of organic acids.

DISCUSSION

Hepatic encephalopathy is one of the major complications of advanced liver cirrhosis. Short-chain (C2–C6) fatty acids represent the major anions of luminal colonic content, accounting for 60–70% of the whole. However, whereas acetate is non-toxic, C(3)4–C5 fatty acids (proprionate, butyrate, isobutyrate, valerate and isovalerate) have been implicated as potential toxic products involved in the pathogenesis of hepatic encephalopathy in humans. As compared with the recent study of Murawaki *et al.*,[12] which was carried out in a limited number of patients, the present research confirms the findings from an *in vitro* study of enhanced-non-toxic organic acid recovery from stools during treatment with nonabsorbable disaccharides.[13,14] Further, unlike the findings from *in vitro* studies,[14,15] oral administration of lactitol didn't prove to decrease toxic organic acid production. These data are in agreement with the results achieved by administering lactulose in the study of Murawaki *et al.*[12] Lactitol did not produce any significant effect on *Bacteroides* and *Clostridium*, which are NH3-producing bacteria, which is contrary to what has been found in healthy volunteers.[16] This issue is a matter of some controversy because whereas lactulose has been shown to quantitatively decrease the growth of *Bacteroides* and *Clostridium* in cirrhotic patients,[15] lactitol, another chemically similar nonabsorbable disaccharide, has not been found to exert any appreciable effect in the same clinical set up.[17] Indeed, Ballongue *et al.*[16] have shown that lactulose and lactitol have different effects on colonic microflora and metabolism. Although the effects of lactulose and lactitol were not significantly different in the treatment groups, the pH-lowering capacity exerted by lactitol in the present study was not as effective as the one exerted by the probiotics; similar results have been found in other studies when lactitol is compared with lactulose.[16,18]

Bifidobacterium and *Lactobacillus* have been long known to produce bacteriocines and unfavorable adhesion conditions for Gram-positive species.[19,20] In the present study, all three tested compounds produced a reduction in the Gram-positive population in favor of an expansion of *Bifidobacterium* and *Lactobacillus*. However, the specific bacterial count of *Bacteroides* and *Clostridium* significantly decreased only in the group treated with the synbiotic. Both pH-lowering and a reduction in short-chain fatty acid (SCFA) production exert an additive inhibitory effect on the growth of *Bacterioides and* other Gram-negative and coliform bacteria.[21] This might explain the failure of rifaximin to substantially change the concentration

of *Bacterioides and* other Gram-negative and coliform bacteria. These findings are of particular interest in light of a study conducted by Liu, who showed that that higher concentrations of *Enterococcus* and *Clostridium* in fecal samples is associated with impending hepatic coma or recent recovery from it.[22] A study by Floch *et al.* from 1970 indicates that the qualitative and quantitative composition of gut flora in chronic liver disease patients is comparable to that of healthy subjects;[23] however, more recent observations seem to contradict this.[23,24] Although the role of disaccharides and antibiotics in the acute phases of liver disease is well established, the most appropriate option for a constant gut ecosystem manipulation in otherwise clinically stable chronic liver disease awaits further clarification, especially when considering that the protracted use of rifaximin results in antibiotic resistance, as has been shown both *in vitro* and *in vivo*.[25] Further, it is of interest that antibiotics such as neomycin and polimixin B have been found to worsen liver fibrosis in an experimental alcohol/carbon tetrachloride rat model,[26] whereas in an alcohol-rat model we obtained a significant histological improvement by the use of the synbiotic SCM-III.[9] In only a few studies has the issue of the potential beneficial effect of gut flora manipulation in cirrhotic patients by means of probiotics been addressed, and some studies have lacked an objective gut ecosystem analysis.[7,8,27,28] Our preliminary studies have shown that this synbiotic preparation remains viable through the gut, by using pulsed-field gel electrophoresis (F. Marotta, unpubl. data). Although no definitive clinical implications can be inferred from the present study and a number of limitations have to be taken into account (such as the need to tailor the dose of disaccharide in order to have a comparable laxative effect, the optimal dosage of probiotic, and the inner variability of the gut microecology), the present data suggest a potential role for synbiotics in the long-term treatment of chronic liver disease.

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