Indian J Med Res 147, January 2018, pp 58-65
DOI: 10.4103/ijmr.IJMR_590_16

Quick Response Code:

Effects of synbiotics on ileal microbiota

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Received April 14, 2016

***Background & objectives*: Despite advancements in molecular-based methods, the composition of the human ileal microbiota and the effects of synbiotics/probiotics on its microbes remain poorly understood. The aim of this study was to determine the composition of the mucus microbiota in the human ileum and to assess the effects of oral administration of synbiotics on the microbiota.**

***Methods*: As part of a clinical trial for synbiotics treatment and surgical infection, ileal mucus was sampled when resection of the ileocecal portion was required. The microbiota composition was examined using 16S rRNA-targeted real-time-quantitative polymerase chain reaction.**

***Results*: A total of 33 samples from the synbiotics group and 39 from the control group were analyzed. Total numbers of bacteria in the ileum were $10^{8.5}$ cells/g in the synbiotics group and $10^{8.4}$ cells/g in the control group, in which obligate anaerobes were dominant over facultative anaerobes. The level of *Enterobacteriaceae* was significantly lower in the synbiotics group than in the control group. The administered probiotics species *Lactobacillus casei* strain Shirota and *Bifidobacterium breve* strain Yakult were detected in 42 and 76 per cent of the synbiotics group, respectively. No significant correlations were observed between tumour stage/size and the various microbes present, except for a negative correlation between tumour size and *Bifidobacterium*.**

***Interpretation & conclusions*: The present analysis of a substantial number of samples from surgically resected intestines showed an abundance of obligate anaerobes as a characteristic feature of the ileal mucus microbiota. Our results also indicated that the synbiotics intervention induced a prominent reduction in *Enterobacteriaceae* in the ileal microbiota.**

Key words Colonization resistance - dysbiosis - ileum - microbiota - probiotics - small intestine - synbiotics

Research on the gut microbiota in relation to human health and disease has been revolutionized by the use of molecular methods but is mainly based on the analysis of faecal samples that represent the luminal microbiota at the end of the large intestine. In contrast, limited information is available regarding the composition and population dynamics of the microbiota in the enclosed regions of the gastrointestinal (GI) tract, including the small intestine, mainly due to sampling difficulties caused by poor accessibility and rare opportunity.

Most of our knowledge about the small intestinal microbiota in humans has been derived from the

studies of ileal biopsies collected during colonoscopies or surgical intervention[1-10], or from samples obtained from sudden death victims at autopsy[11]. In addition, ileal effluent from ileostomy patients has been collected to study the diversity of the luminal microbiota of the ileum[12-17]. However, there are limitations associated with these studies, including small sample sizes and biases caused by the various disease states of the patients, most of whom had inflammatory bowel disease or a transplanted intestine. Only a few studies have described the composition of the small intestinal microbes under healthy or baseline conditions with relatively large sample sizes, by the use of molecular-based methods[6].

Probiotics are defined as live microorganisms that have positive effects on human health when ingested in sufficient amounts[18]. The effects of probiotic administration on the composition of the faecal microbiota have been extensively examined whereas other niches of the GI tract have been poorly studied[19,20]. The terminal ileum is specifically associated with the largest mass of lymphoid tissue in the human body: the gut-associated lymphoid tissue and Peyer's patches[21]. This region of the intestine and its indigenous microbiota may have a profound influence on host physiology and pathogenesis of immune-associated disorders. However, the linkage between probiotic treatment and the ileal microbiota is poorly understood.

This study was undertaken to analyze the microbial community within the human ileal mucus, using surgically resected specimens primarily from patients with colorectal cancers of relatively large size, and to assess the effects of oral administration of synbiotics, a combination of probiotics and prebiotics (dietary supplement of probiotics)[18], on the ileal microbiota.

Material & Methods

As part of our previous project for the randomized controlled trial in the department of Digestive Surgery of Nagoya Daini Red Cross Hospital (Nagoya city), Japan, ileal samples were obtained for examination, from June 2008 to September 2012, when resection of the ileocecal portion was required. The design and profile of the clinical trial, in which the primary outcome was the development of infectious complications after colorectal resection, were described elsewhere[22]. Briefly, patients scheduled for elective laparoscopic colorectal surgery were eligible to participate in this study and were enrolled between June 2008 and December 2013. Exclusion criteria were current routine use of probiotics or synbiotics, preferring or disliking taking probiotics or synbiotics and a schedule that did not allow for synbiotic intervention for more than seven days before surgery. Patients were randomly assigned in a 1:1 ratio to a synbiotics group or a control group. Randomization was performed using a computer-based randomization programme, without stratification or weighted minimization. Eligible patients were informed that 50 per cent of them would receive no treatment corresponding with synbiotic intervention (control group) and that assignment to the synbiotics or control groups would be based on a randomization programme, with written informed consent before participating in the trial. Every patient received standard pre- and post-operative care, including antibiotic treatment, regardless of which group the patient was assigned to. The study protocol was approved by the Institutional Review Board of the Nagoya Daini Red Cross Hospital. An English-language summary of the protocol was submitted (registration ID UMIN000003439) to the Clinical Trials Registry managed by the University Hospital Medical Information Networks in Japan (*http://www.umin.ac.jp/ctr/*). The sample collection of mucus of the surgically resected intestines and the analysis of mucosa-attached microbiota in this study were planned in the original protocol of the study, as a secondary outcome of the clinical trial.

Protocol for synbiotics intervention: Patients in the synbiotics group received a synbiotics formula that was previously found to be effective in preventing post-operative infectious complications after major hepatectomy for biliary cancer[18]. The reagents were as follows: one 80 ml bottle of Yakult Ace (Yakult Honsha Co., Ltd., Tokyo, Japan), which contained at least 3×10^{10} living *Lactobacillus casei* strain Shirota with 2.5 g galacto-oligosaccharides; one 100 ml bottle of MILMIL-S (Yakult Honsha), which contained at least 1×10^{10} living *Bifidobacterium breve* strain Yakult with 1.0 g galacto-oligosaccharides. The beverages were kept refrigerated during delivery and storage and were consumed within 14 days after production in the factory.

Patients in both groups were asked to avoid other probiotic products for the duration of the study. Patients in the synbiotics group were supplied with a diary sheet and asked to record their intake of the drink. All patients continued their normal diet. In the treatment group, the synbiotics were administered daily orally for 7-11 days before surgery (from the

time of study entry to the day before surgery). Patients were not blinded as to which group they were in, and no placebo product was used.

Surgery: Preoperative mechanical bowel preparation protocols were introduced in all patients using laxatives as follows: 34 g of magnesium citrate (MAGCOROL P, Osaka, Japan) dissolved in 180 ml water, one day before surgery, and 5 mg of sodium picosulphate hydrate (Laxoberon, Tokyo, Japan) two days before surgery. No oral non-absorbable antibiotic prophylaxis was administered. All patients received intravenous cephalosporin within one hour before the operation.

Usually, five trocars were introduced for ileocolic resection or right hemicolectomy. The colon and terminal ileum were mobilized laparoscopically in the medial to lateral fashion. Systematic lymph node clearance ranging from the trunk of the superior mesenteric artery to the pericolon was carried out under laparoscopic view, when curative resection for colorectal cancer was required. An additional incision was made to remove the specimen. Bowel reconstruction was performed by hand-sewn sutures or using an endoscopic linear and/or a circular stapler. An incision, required when the laparoscopic procedures for intestinal mobilization and/or lymph node clearance were considered not to be feasible, was defined as indicating conversion, regardless of its length.

Outcome measurement: Sample collection of ileal mucus: Microbiota was analyzed in the two study groups. Mucus on the terminal ileum was sampled immediately after the ileocolic region was resected. The mucus sample was placed directly into a tube (~1.0 g/tube) by the patients; it contained 2 ml of RNAlater (an RNA stabilization solution; Ambion, Austin, TX, USA). The samples were placed in a refrigerator at 4°C within 30 min of excretion. The samples were transported at −20°C to the Yakult Central Institute.

Outcome measurement: Bacteriologic tests: To quantify the bacteria present in the samples, total RNA fractions were extracted from mucus by a previously described method[23] and the gut microbiota composition was examined using the 16S or 23S rRNA-targeted real-time-quantitative polymerase chain reaction (RT-qPCR) using the Yakult Intestinal Flora-SCAN (YIF-SCAN®). Three serial dilutions of the extracted RNA sample were used for bacterial rRNA targeted RT-qPCR[23] and the threshold cycle values in the linear range of the assay were applied to the standard curve to obtain the corresponding bacterial cell counts for each nucleic acid sample. The data were used to determine the number of bacteria per sample. The specificity of the RT-qPCR assay using the group-, genus-, or species-specific primers was determined as described previously[23]. Quantitative analyses of *L. casei* strain Shirota[24] and *B. breve* strain Yakult[25] have been described previously.

Statistical analysis: Distinct changes in the amounts of ileal microbes were expected to be induced by the treatment, based on the findings of the previous study on faecal microbiota[26]. Given Cohen's *d* set at 0.8[27], an α value of 0.05 and a power of 0.8, the required sample size was estimated to be 27 in each arm. Consequently, 77 patients were enrolled in this study.

Statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS) version 22.0 (SPSS, Chicago, IL, USA) for Windows. The Chi-square test or the Fisher's exact test was used for the comparison of categorical variables. Continuous variables were compared using a *t* test when normally distributed or otherwise using the Mann–Whitney U-test. The Wilcoxon signed-rank test was used for non-parametric data when appropriate. Pearson's correlation coefficient was used to analyze the relationship between bacterial counts and faecal organic acid concentrations.

Results

From the beginning of the clinical trial to September 2012, 77 patients underwent ileocolic resection or right hemicolectomy and were randomized with 34 in the synbiotics group and 43 in the control group. Among these, 33 ileal samples from the synbiotics group and 39 from the control group were obtained and analyzed (Figure).

The patient and disease characteristics (age, sex, body mass index, the American Society of Anesthesiologists classification, smoking status, co-morbidities and prior abdominal surgery) and the operative findings (site, tumour stage, surgical procedure, operative time, blood loss, transfusion, wound status and conversion to laparotomy) were similarly distributed between the two groups (Table I).

Changes in ileal microbiota: The results of bacteriological tests of the ileal mucus are shown in Table II. Total numbers of bacteria in the ileal mucus were $10^{8.5}$ cells/g in the synbiotics group and $10^{8.4}$ cells/g in the control group. Numbers of obligate

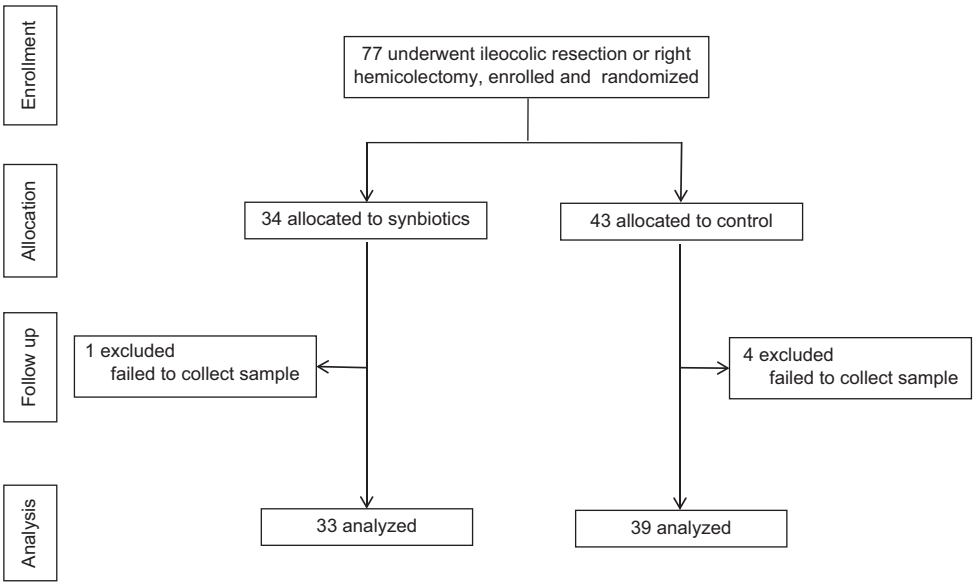

Figure. Flow chart showing study design.

anaerobes, such as *Clostridium* (*coccoides* group or *leptum* subgroup), *Bifidobacterium* or *Bacteroides fragilis* group, were dominant over facultative anaerobes, such as *Lactobacillus* species. The level of *Enterobacteriaceae* was significantly lower (reduced to 5% of the counterpart) in the synbiotics group than in the control group. The detection rate of the *L. casei* subgroup was significantly increased in the synbiotics group (100%), compared to the control group (5%). *L. casei* strain Shirota and *B. breve* strain Yakult, which were present in the synbiotics agent, were detected in 42 and 76 per cent, respectively, of the samples from the synbiotics group whereas these species were not detected in any samples from the control group (Table II).

Relationship between tumour status and ileal microbiota: No significant correlations were observed between tumour stage and the number of ileal microbes. No significant correlations were found between tumour size and the number of microbes, except for the negative correlation between tumour size and *Bifidobacterium* (Pearson correlation coefficient=−0.324, P=0.015).

Discussion

There has been a great deal of interest in the potential clinical and therapeutic implications of small intestinal dysbiosis in GI disorders, such as Crohn's disease[4,6,10], irritable bowel syndrome[29], pouchitis following ileal pouch-anal anasotomosis[30] or graft rejection after small bowel transplantation[14,15]. Heterogeneous and conflicting results concerning the composition of the human ileal microbiota have been reported previously, presumably due to the limited number of individuals, high inter-individual variations and differences in sampling methods or bias caused by the disease state. Furthermore, little information is available regarding the effects of synbiotics/probiotics on the ileal microbiota. This study was an attempt to examine the composition of the microbiota attached to or embedded in the mucus layer of the terminal ileum, using a relatively large number of samples and to assess the effects of oral administration of synbiotics on the ileal microbiota.

In this study, the total numbers of bacteria in the ileal mucus were $10^{8.5}$ cells/g for the synbiotics group and $10^{8.4}$ cells/g for the control group, which were consistent with the results of previous studies based on culture-dependent or independent methods[14]. There are a few published data concerning the survival or maintenance of probiotics strains in the small intestine[19]. *L. casei* strain Shirota and *B. breve* strain Yakult, administered as probiotics, were detected in 42 and 76 per cent, respectively, of the ileal samples from the synbiotics group, while these species were detected at about tenfold higher levels in 100 per cent of the faecal samples following synbiotics treatment[22]. Thus, it is likely that such probiotic species become highly abundant members of the population after passing through the terminal ileum into the colon.

Several previous studies showed that an increased abundance of *Enterobacteriaceae* or *Escherichia coli*

Table I. Clinicopathological characteristics and surgical factors of study patients

Characteristics	Synbiotics group (n=33)	Control group (n=39)
Sex (%)		
Male	14 (42.4)	23 (59.0)
Female	19 (57.6)	16 (41.0)
Age (yr)		
Mean (SD)	74.1 (10.9)	69.6 (11.0)
Median (range)	76 (41-92)	71 (30-85)
Body mass index (kg/m^2) [mean (SD)]	21.6 (2.5)	21.5 (2.5)
ASA classification (%)		
I (normal healthy patient)	13 (39.4)	17 (43.6)
II (mild systemic disease)	15 (45.5)	19 (48.7)
III (severe systemic disease)	5 (15.2)	3 (7.7)
Smoking status (%)		
Never	24 (72.7)	28 (71.8)
Quit	8 (24.2)	10 (25.6)
Current	1 (3.0)	1 (2.6)
Co-morbidities (%)		
Diabetes mellitus	3 (9.1)	3 (7.7)
Steroids	1 (3.0)	0
Previous abdominal surgery (%)	12 (36.4)	11 (28.2)
Disease (%)		
Cancer	33 (100)	38 (97.4)
Other	0	1 (2.6)
Target of operation (%)		
Cecum or ascending colon	29 (87.9)	36 (92.3)
Transverse colon	4 (12.1)	3 (7.7)
Stage of carcinoma (UICC 7th edition)[28] (%)		
0	1 (3.0)	5 (12.8)
I	13 (39.4)	11 (28.2)
II	13 (39.4)	12 (30.8)
III	5 (15.2)	11 (28.2)
IV	1 (3.0)	0
Tumour diameter (mm)		
Mean (SD)	36.3 (15.6)	34.4 (14.6)
Range	10-80	5-60
Operative time (min) [mean (SD)]	169.7 (39.9)	171.5 (56.2)
Blood loss (g) [mean (SD)]	38.1 (77.3)	33.2 (47.7)
Intraoperative transfusion (%)	0	0
Wound status (%)		
Clean-contaminated	33 (100)	39 (100)
Converted (%)	3 (9.1)	1 (2.6)

SD, standard deviation; ASA, American Society of Anesthesiologists; UICC, Union for International Cancer Control

correlated with Crohn's disease or acute graft rejection after transplantation, suggesting that the specific bacterial groups or species could contribute to the pathogenesis of the diseases[2,4,10,15]. A prominent reduction of *Enterobacteriaceae* was found in the synbiotics group, which indicated a therapeutic effect of probiotics in the small intestine.

The clinical trial including faecal sample analysis with the same series of patients demonstrated positive correlations among administered probiotics, the number of *Bifidobacterium* species and the concentrations of short-chain fatty acids, which were closely associated with decreases in potentially harmful species[22]. On the other hand, no significant differences in the numbers of *Bifidobacterium* species or other obligate (strict) anaerobes were found in the ileum of the groups studied. These observations suggest that some effects of synbiotics on gut microbiota emerge more prominently in the proximal colon downstream of the terminal ileum, where fermentation by enteric bacteria becomes accelerated in the slowed faecal flow.

Our results showed that the numbers of obligate anaerobes, such as the *Clostridium coccoides* group, *C. leptum* subgroup, *Bifidobacterium* or *Bacteroides fragilis* group, were dominant over facultative anaerobes. This composition is similar to those reported for the human ileum, mostly from biopsy samples, and likely represents the normal ileal microbial population[1,2,8,9,14]. It has been reported that

Table II. Effects of synbiotics on ileal mucosal microbiota

Microbes	Synbiotics group (n=33)		Control group (n=39)	
	Number of bacteria	Detection rate (%)	Number of bacteria	Detection rate (%)
Total microbes	8.5±1.3	100	8.4±1.4	100
Obligate anaerobes				
Clostridium coccoides group	8.0±1.3	88	7.7±1.2	87
C. leptum subgroup	7.9±1.3	85	7.3±1.1	90
Bacteriodes fragilis group	7.7±1.4	97	7.8±1.2	87
Bifidobacterium	7.1±1.4	91	6.7±0.8	67
Atopobium cluster	7.5±1.2	85	7.0±0.9	74
Prevotella	6.2±0.5	42	6.3±0.4	28
Clostridium difficile	5.1	3	5.1±1.0	8
C. perfringens	4.8±1.0	30	5.3±1.7	21
Facultative anaerobes				
Total *Lactobacillus*	5.9±0.9*	100	5.2±1.1	79
L. gasseri subgroup	4.6±1.0	61	4.8±1.1	67
L. brevis	<2.1	0	3.0	3
L. casei subgroup	5.5±0.9	100***	5.1	5
L. fermentum	5.5	6	5.2	5
L. fructivorans	<2.0	0	<2.0	0
L. plantarum subgroup	3.9±0.0	6	4.0±0.7	21
L. reuteri subgroup	4.6±0.8	67	4.4±1.1	49
L. ruminis subgroup	5.1±1.6	45	5.1±0.9	31
L. sakei subgroup	<3.0	0	4.0	3
Enterobacteriaceae	6.7±1.2***	85	8.0±1.2	85
Enterococcus	6.2±1.4	55	6.7±1.8	46
Staphylococcus	4.8±0.4	36	5.1±0.7	44
MRSA	<4.0	0	<4.0	0
MRCNS	<4.0	0	<4.0	0
MSSA	<4.0	0	<4.0	0
MSCNS	4.8±0.4	36	5.1±0.7	44
Aerobes				
Pseudomonas	4.9	3	5.0±0.7	15
Probiotics				
L. casei strain Shirota	6.4±0.6	42***	<5.6	0
B. breve strain Yakult	6.9±0.7	76***	<5.6	0

Values are mean±SD ($\log_{10}$ cells/g of faeces). P *<0.05, ***< 0.001 compared to control group. MRSA, methicillin-resistant *Staphylococcus aureus*; MRCNS, methicillin-resistant coagulase negative *Staphylococcus*; MSSA, methicillin-sensitive *Staphylococcus aureus*; MSCNS, methicillin-sensitive coagulase negative *Staphylococcus*; SD, standard deviation; *B. breve*, *Bifidobacterium breve*

facultative anaerobes constitute a substantial part of the ileal microbial community in studies examining ileal effluent from ileostomies[13,14]. The diffusion of oxygen inward from the ileostomy port may have influenced the growth of the obligate anaerobes, allowing facultative anaerobes to predominate[14]. Alternatively, retrograde flow of the contents of the large intestine into the small intestine may account for the higher levels of obligate anaerobes in the studies of patients without ileostomies[17]. In

addition, there is a possibility that the difference between surface-adherent and luminal microbial populations may have affected the results of the ileal microbiota[5].

In this study, we used surgically resected specimens mostly from patients with colorectal cancer. Thus, a limitation of the study was that the possibility could not be excluded that cancer itself or associated intestinal stasis might have influenced the composition of the ileal microbiota. However, such an influence appeared not to be prominent since no significant correlations were observed between tumour stage/size and the various microbes present, except for a negative correlation between tumour size and *Bifidobacterium*. Furthermore, the effects of synbiotics were estimated in comparison to randomized controls with similar backgrounds.

Another limitation of this study was that some microbial changes might have been induced by the laxative preparation or physical manipulation of the intestine, which are common problems with colonoscopy sampling and might have interfered with the accurate determination of the ileal microbiota in human individuals. In addition, the median age of the patients in each group was over 70 yr. Therefore, our microbiological data may represent the microbiome of elderly people because ageing profoundly affects the composition of intestinal microbiota[31]. Some other microbial species such as *Streptococcus* or *Veillonella* have also been reported to be characteristic in the ileal microbiota[13,16,17]. These species were not examined in our study.

In conclusion, a characteristic feature of the ileal mucus microbiota was disclosed, using a substantial number of samples from surgically resected intestines. These results also indicated that the synbiotic intervention elicited a prominent reduction in *Enterobacteriaceae* in the ileal microbiota. It is not easy to define a normal microbiota because of both the difficulty in collecting samples from healthy human ileum and substantial inter-individual variations. Further studies are needed to define the normal ileal microbiota and its compositional changes following a synbiotic/ probiotic intervention.

Acknowledgment

Authors acknowledge Norikatsu Yuki, Akira Takahashi and Yukiko Kado, of the Yakult Central Institute for their valuable assistance in performing the microbiota analyses and thank Kenta Murotani in the Division of Biostatistics, Clinical Research Center, Aichi Medical University for the valuable comments on the statistics.

***Financial support & sponsorship*:** Authors acknowledge the Yakult Central Institute, Japan, for financial support.

***Conflicts of Interest*:** S. Komatsu, E. Sakamoto and M. Nagino have no conflict of interest to declare. T. Asahara and K. Nomoto are researchers of Yakult Honsha Co., Ltd, Japan.

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