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Title:

Synbiotic therapy reduces the pathological gram-negative rods caused by an increased acetic acid concentration in the gut

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Key words:

Pseudomonas, Probiotics, Enteral nutrition

Ventilator-associated pneumonia, Critical illness

Abstract

Background The mechanisms for the improvement of the gut flora and the intestinal environment by synbiotic therapy is unclear.

Aims This study evaluated the changes in the gut flora and the intestinal environment after synbiotic therapy, and tried to clarify the mechanisms by which synbiotic therapy reduces pathological bacteria in the gut.

Methods A total of 47 enteral feeding patients with long term mechanical ventilation support were enrolled in the study. Patients were randomly assigned to synbiotic and control groups, at a two to one ratio. Patients in the synbiotic group were administrated *Lactobacillus*, *Bifidobacterium*, and galactooligosaccharides as synbiotics for 8 weeks.

Results The characteristics of the patients were not significantly different between the control (n=16) and synbiotic (n=31) groups. In the synbiotic group, the counts of *Bifidobacterium* and *Lactobacillus* in the gut increased significantly to 100 times the initial level following synbiotic treatment. The acetic acid concentration increased (71.1 ± 15.9 vs. $46.8 \pm 24.1 \mu \text{ mol/g}$) and pH decreased in the gut in comparison with the control group. The concentration of acetic acid in the gut was increased in proportion to the *Bifidobacterium* counts. The counts

of pathological gram-negative rod decreased significantly to one-tenth of the initial level in inverse proportion to the *Bifidobacterium* counts. Furthermore, the amount of *Pseudomonas aeruginosa* in the lower respiratory tract decreased significantly after synbiotic therapy compared to the controls.

Conclusion Synbiotic therapy reduces the pathological gram-negative rods by increasing the acetic acid concentration in association with an increased counts of *Bifidobacterium*.

Introduction

Previous reports have described the effects of probiotics/synbiotics for general infectious complications in various patients, such as liver transplantation [1,2], major abdominal surgery [3-5], severe acute pancreatitis [6-8], trauma [9,10], and critically ill patients [11-13]. However, these reports generally did not examine changes in the gut flora following probiotic/synbiotic therapy [1-3,6-12]. Klarin et al [14] pointed out that there are often insufficient effects of probiotics/synbiotics in these studies that reported negative results. We believe that the changes in gut flora need to be confirmed in probiotics/synbiotics studies in order to demonstrate that a sufficient number of bacteria have been ingested and were viable.

Recently, several reports have described the effects of probiotic/synbiotic therapy for Ventilator-associated pneumonia (VAP) [15,16]. VAP is an important complication in patients on mechanical ventilation and is caused by various pathological bacteria; especially virulent gram-negative aerobes such as *Pseudomonas aeruginosa* and *Enterobacteriaceae* [17,18]. Forestier et al. [15] indicated that probiotic therapy delayed respiratory tract colonization by *P. aeruginosa*. Knight et al [16] demonstrated that synbiotic

therapy did not affect the oropharyngeal microbial flora and did not reduce the incidence of VAP. However, changes in gut flora were not examined in these studies [15,16]. It is unknown whether probiotics/synbiotics actually affected the gut flora of the patients in these studies [15,16].

The mechanisms by which synbiotic therapy improves the gut flora and the intestinal environment, and reduces pathological bacteria are unclear. The present study investigated the changes in the gut flora and the intestinal environment after synbiotic therapy in patients on long term mechanical ventilation support, and tried to clarify the mechanisms by which synbiotic therapy reduces pathological bacteria in the gut.

Materials and methods

The present study was approved by the Institutional Review Board of Hokkaido University Hospital and Inoue Hospital. The clinical trial registry number is UMIN: 000004085 (<http://www.umin.ac.jp/ctr/index.htm>). Informed consent was obtained from each of the patients or the patient's family. The present study

included patients who received mechanical ventilation support and administration of enteral tube feeding for more than one month at Inoue Hospital in December of 2008. Patients were excluded if they were under 18 years of age or had a terminal illness. Patients who had received selective digestive decontamination, or prebiotic and probiotic therapies were also excluded. After inclusion in the present study, the patients were randomly assigned to the synbiotic or control groups (at a 2:1 treatment-to-control allocation). Yakult BL Seichōyaku (Yakult Honsha, Tokyo, Japan) 1g and Oligomate S-HP (galactooligosaccharides, Yakult Honsha, Tokyo, Japan) 5g were administered three times a day as the synbiotic therapy. One gram of Yakult BL Seichōyaku contains 1×10^8 living *Bifidobacterium breve* strain Yakult and 1×10^8 living *Lactobacillus casei* strain Shirota. The synbiotic therapy was continued during the 8 week study period. Enteral feeding was completely established before the assignation of each patient. In both groups, Medief (100kcal, protein 4.5g, fat 2.8g, carbohydrate 14.2g, dietary fiber 1.2g in 100ml) (Ajinomoto, Tokyo, Japan) was used as the enteral feed solution for the patient's requirements during the study period. General laboratory examinations were performed at the assignation (week 0) and every week during the study period. Culture

surveillances of pharynx swabs and those of the sputum aspirated in the lower respiratory tract were performed on weeks 0, 4, and 8. The amount of bacteria was also assessed by a semi-quantitative method on agar plates (0, no bacteria on the agar plate; +1, less than one-third of the agar plate covered; +2, two-thirds of the agar plate covered; +3, the agar plate was completely covered with the bacteria).

Fecal samples for bacteriological and organic acid analyses were acquired from the patients on weeks 0, 4, and 8. The fecal samples were put into test tubes containing 1ml RNAlater (Ambion, Inc., Austin, TX, USA), a ribonucleic acid (RNA) stabilization solution, prior to bacteriological analysis. Samples for fecal organic acid analysis were put into test tubes containing 1ml of 1% perchloric acid. The samples were stored at -20 °C until analysis.

The fecal samples for bacteriological analysis were incubated for 5 minutes at room temperature. RNA was isolated using the method described elsewhere [19,20]. Finally, the nucleic acid fraction was suspended in 1 mL nuclease-free water. A standard curve was generated with reverse transcription-quantitative polymerase chain reaction (RT-qPCR) (using the threshold cycle [C_T] value, the cycle number when the threshold fluorescence

was reached) and the corresponding cell count, which was determined microscopically with 4,6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA) staining for dilution series of the standard strains as described elsewhere [19,20]. In order to determine the type of bacteria present in the samples, three serial dilutions of an extracted RNA sample were used for RT-qPCR, and the C_T values in the linear range of the assay were applied to the standard curve to obtain the corresponding bacterial cell count in each nucleic acid sample. These data were then 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 [19-21]. Quantitative analysis of *L. casei* strain Shirota has also been described previously [21].

The samples for the fecal organic acid analysis were homogenized and centrifuged at 12,000 rpm at 4°C for 10 minutes. The supernatant was put in a glass tube, and allowed to stand at 4°C for 12 hours. The suspension was then passed through a filter with a pore size of 0.45 μm (Millipore Japan, Tokyo). The sample was analyzed for organic acids by high-performance liquid chromatography, and the concentrations of organic acids were calculated with

the use of external standards. The reproducibility and stability of these measurements have been described previously [22].

Unless otherwise indicated, all measurements are expressed as the mean \pm standard deviation. The SPSS 15.0J statistical software package (SPSS Inc., Chicago, Illinois) was used for all statistical analyses. Comparisons between the two groups were made using the Mann-Whitney U test, the chi square test or the two-way repeated measure ANOVA. The relationship between two variables was investigated by a simple regression analysis. A value of $P < 0.05$ was considered to be statistically significant.

Results

Forty-seven patients who needed long term mechanical ventilation support were included in this study. Sixteen patients were assigned to the control group and 31 patients were assigned to the synbiotic group. All patients were able to complete the study. The characteristics of the patients were not statistically different between the control and synbiotic groups (Table 1). All patients had undergone tracheotomy, were hemodynamically stable, and had no infection. No

patient had any severe renal dysfunction which would thereby require the same renal replacement therapies.

Table 2 presents the changes of the gut flora in the patients during the 8 week study period. At the initiation of the study (week 0), the gut flora of the patients in both groups had been disturbed. The level of beneficial bacteria (*Bifidobacterium* and *Lactobacillus*) significantly decreased, while the number of pathological bacteria (*Enterobacteriaceae*, *Enterococcus*, and *Pseudomonas*) significantly increased in comparison to both groups with healthy subjects as described in a previous study [20]. However, the gut flora did not differ between each group at the initiation of the study. After 4 weeks of treatment in the synbiotic group, *Bifidobacterium* and total *Lactobacillus* were observed to have increased significantly to 100 times the initial level compared to the controls ($P < 0.001$ by the two-way repeated measure ANOVA). Although *L. casei* strain Shirota was not detected at all at week 0, it was detected after 4 weeks of treatment in the patients in the synbiotic group ($P < 0.001$ by the two-way repeated measure ANOVA). Of interest, *Enterobacteriaceae* and *Pseudomonas* decreased significantly to one-tenth of the initial levels after 4 weeks of symbiotic treatment compared to the controls ($P < 0.001$ and 0.002 by the two-way

repeated measure ANOVA, respectively).

Table 3 shows the changes of the fecal organic acids in the patients during the 8 week study period. At week 0, although the feces were alkalized in both groups, the concentrations of fecal organic acids were not different between the two groups. After 4 weeks of treatment in the synbiotic group, the concentrations of acetic acid in the gut had increased significantly ($P = 0.004$ by the two-way repeated measure ANOVA). Furthermore, the pH in the gut decreased significantly after 4 weeks in the patients in the synbiotic group ($P = 0.007$ by the two-way repeated measure ANOVA).

The relationship between the *Bifidobacterium* counts and other variables are shown in Fig. 1. The acetic acid concentration increased in proportion to the *Bifidobacterium* counts in the gut, therefore the pH in the gut decreased. The counts of *Enterobacteriaceae* and *Pseudomonas* also decreased in inverse proportion to the *Bifidobacterium* counts.

Figure 2 shows the amount of *P. aeruginosa* in the sputum aspirated in the lower respiratory tract in the two groups. Although the amount of *P. aeruginosa* in the lower respiratory tract increased gradually in the control group, the amount in the synbiotic group gradually decreased ($P = 0.017$ by the two-way

repeated measure ANOVA). The amounts of *P. aeruginosa* in the pharynx were not significantly different between the two groups.

Pneumonia was observed in 5 patients from the synbiotic group and in 3 patients from the control group during the 8-week study period. Pneumonia related to *P. aeruginosa* was observed in 3 patients and 2 patients from the synbiotic and control groups, respectively. The frequency of fever, diarrhea, and other infectious complications were not different between the two groups. Frequency of antibiotic treatments was not different between the two groups. The results of general laboratory examinations were also not different between the two groups during the 8 weeks. No adverse effects of synbiotic therapy were observed during the study period.

Discussion

We demonstrated the following points: 1) The synbiotic therapy increased the *Bifidobacterium* and total *Lactobacillus* counts in the gut flora. 2) The increase in the acetic acid concentration and decrease of the pH in the gut were observed as a consequence of the increase in *Bifidobacterium* counts. 3) The counts of

pathological gram-negative rod in the gut decreased in parallel with the increase in the acetic acid concentration. 4) The synbiotic therapy decreased the colonization of *P. aeruginosa* in the lower respiratory tract of the patients compared to the controls.

Many previous investigations about probiotic/synbiotic therapy have been reported [1-13]. However, most of these previous reports did not examine the changes in the gut flora that resulted from the synbiotic therapy [1-3,6-12]. We have herein clarified the effects of synbiotic therapy on the gut flora. At the beginning of the study, the level of beneficial bacteria (*Bifidobacterium* and *Lactobacillus*) was significantly decreased, while the number of pathological bacteria (*Enterobacteriaceae*, *Enterococcus*, and *Pseudomonas*) was significantly increased in comparison of the both groups with healthy subjects in the previous study [20]. In the synbiotic group, *Bifidobacterium* and total *Lactobacillus* increased to 100 times the counts before the synbiotic therapy. Furthermore, the numbers of *Bifidobacterium* and total *Lactobacillus* at week 8 in the synbiotic group were greater than those in the healthy subjects [20].

The *B. breve* strain Yakult and *L. casei* strain Shirota were administrated as probiotics in this study. We confirmed that the majority of the *Lactobacillus* in

the gut was *L. casei* strain Shirota after the symbiotic therapy. Although we did not have a method to confirm the strain of *Bifidobacterium*, a previous study, which used the same synbiotics as our study, demonstrated that *B. breve* strain Yakult reached the same level as *L. casei* strain Shirota after symbiotic therapy [4,5]. Another previous study, which used *L. casei* strain Shirota and galactooligosaccharides as synbiotics, showed that there was an increase of endogenous *Bifidobacterium* in the gut without an administration of exogenous *Bifidobacterium* [23,24]. Based on these results, we propose that the endogenous *Bifidobacterium* was mainly increased in the present study.

In the present study, the acetic acid concentration in the gut significantly increased with synbiotic therapy. The increase in acetic acid resulted in an increase in the total organic acids and a decrease of the pH in the gut. Acetic acid is the main fermentation product of *Bifidobacterium* [25,26] and accounts for 60% of total organic acids in the gut. In the present study, the increase of *Bifidobacterium* by synbiotic therapy likely resulted in the increase in the acetic acid concentration in the gut.

At the beginning of the present study, pathological bacteria (*Enterobacteriaceae*, *Enterococcus*, and *Pseudomonas*) in the patients of the

both groups were already increased in comparison to healthy subjects in the previous study [20]. Of these pathological bacteria, only the gram-negative rod, such as *Pseudomonas* and *Enterobacteriaceae*, were decreased to one-tenth of the initial level by the synbiotic therapy. In the present study, the acetic acid concentration in the gut increased with synbiotic therapy. Acetic acid has an antimicrobial effect on *Pseudomonas* and *Enterobacteriaceae* [27,28]. Furthermore, because the pH in the gut is significantly decreased, the undissociated acetic acid level is significantly increased. The antimicrobial effect of undissociated acetic acid is 10-600 times greater than that of dissociated acetic acid [27]. Therefore, the marked increase in undissociated acetic acid might have resulted in the reduction of *Pseudomonas* and *Enterobacteriaceae* after the synbiotic therapy. However, because gram-positive cocci (*Staphylococcus* and *Enterococcus*) are tolerant to acetic acid [29], this may explain why the levels of these bacteria did not decrease after the synbiotic therapy.

Patients on long term mechanical ventilation support have a high risk of colonization of pathological bacteria in the lower respiratory tract and subsequent VAP [18]. The major pathological bacteria are virulent

gram-negative aerobes such as *P. aeruginosa* and *Enterobacteriaceae* [17,18].

In the present study, *P. aeruginosa* were observed in the lower respiratory tract in many patients at the beginning of the study. In the synbiotic group, the amount of *P. aeruginosa* in the lower respiratory tract gradually decreased. However, there was no difference in the frequency of infectious complications in both groups, because pneumonia rarely appeared either group.

Several review articles concerning the effects of probiotics/synbiotics on nosocomial pneumonia and VAP [30-32] have been published. For example, McNabb et al. [31] described that probiotic products reduced the pathologic colonization of the host, although there is currently insufficient theoretical evidence about how they reduce the pathologic colonization. The present study may clarify one of the mechanisms underlying the reduction in the colonization of *P. aeruginosa* wherein the increase in beneficial bacteria leads to changes in the organic acid concentration and microbicidal activity against *P. aeruginosa*. Siempos et al. [32] also reported that probiotic therapy decreased the incidence of VAP and colonization of the respiratory tract with *P. aeruginosa*. This review by Siempos et al. [32] supported the results obtained from our present study.

Recently, the safety of probiotics/synbiotics has been under debate [33].

Besselink et al. [8] indicated that probiotic prophylaxis increased the frequency of bowel ischemia and mortality in patients with severe acute pancreatitis. Probiotic therapy also apparently accelerates bowel movements [23]. However, in the study by Besselink et al., the synbiotics and enteral nutrition were forcibly administered via a nasojejunal tube, and the patients with severe acute pancreatitis already had a high risk of bowel ischemia [8]. Besselink et al. [8] speculated that the local oxygen demand was increased by probiotic bacteria. In the present study, no patients with high risk of bowel ischemia were included, and no adverse events related the synbiotic therapy observed.

There are several limitations associated with our study. The present study was not blinded. Because the incidence of pneumonia was very low in the present study, this study did not have sufficient power to measure any differences in the incidence of pneumonia for each group. Furthermore, we used a semi-quantitative method to assess the amount of *P. aeruginosa* in the lower respiratory tract. If a quantitative method had been used to assess the pathologic colonization, then the difference between each group may have been more obvious.

In conclusion, synbiotic therapy increased the counts of *Bifidobacterium*

in the gut flora. The *Bifidobacterium* increased the acetic acid concentration in the gut. The counts of pathological gram-negative rods decreased in inverse proportion to the acetic acid concentration, because of the antimicrobial effect of acetic acid.

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Figure legend

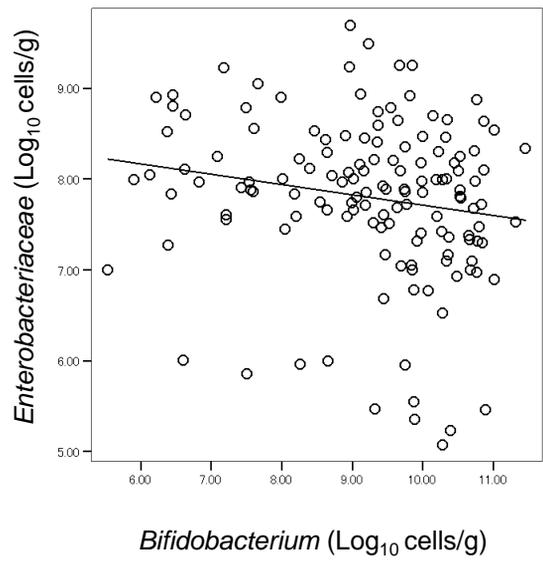
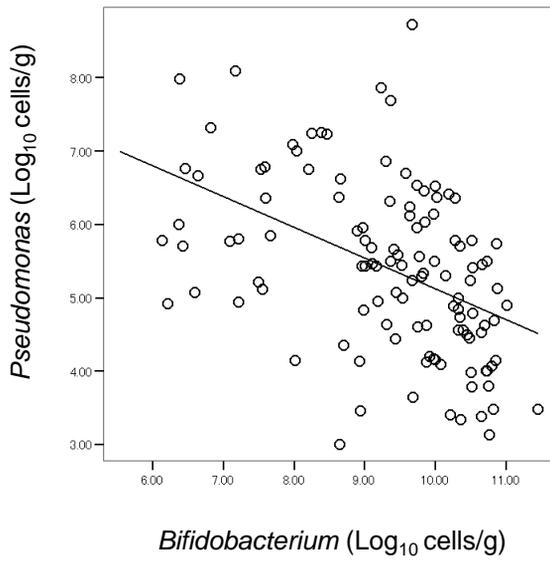
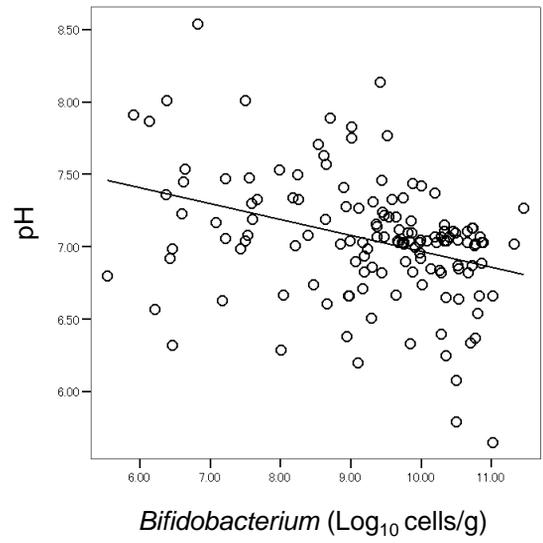
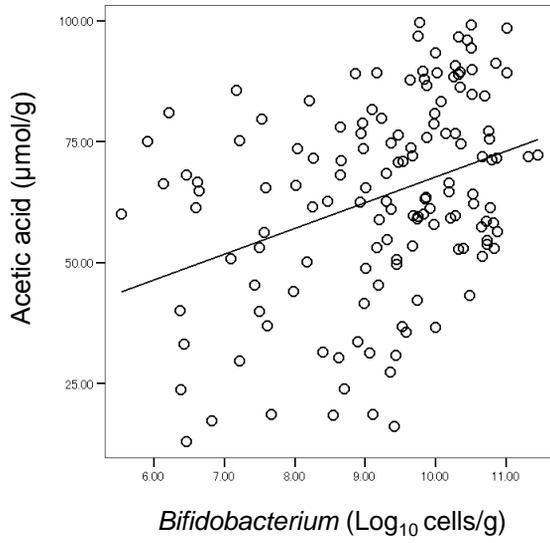
Fig. 1 The relationship between the *Bifidobacterium* counts and the acetic acid concentration, pH, *Pseudomonas* counts, *Enterobacteriaceae* counts in the gut.

A simple regression analysis revealed the correlation between the *Bifidobacterium* counts and other variables. Upper left: the correlation between the *Bifidobacterium* counts and the acetic acid concentration in the gut ($r^2 = 0.12$, $P < 0.001$). Upper right: the correlation between the *Bifidobacterium* counts and the pH in the gut ($r^2 = 0.12$, $P < 0.001$). Lower left: the correlation between the *Bifidobacterium* counts and the *Pseudomonas* counts in the gut ($r^2 = 0.21$, $P < 0.001$). Lower right: the correlation between the *Bifidobacterium* counts and the *Enterobacteriaceae* counts in the gut ($r^2 = 0.03$, $P = 0.041$).

Fig. 2 The amount of *Pseudomonas aeruginosa* in the sputum aspirated from the lower respiratory tract.

Although amount of *P. aeruginosa* in the lower respiratory tract increased gradually in the control group, that in the synbiotic group gradually decreased (P

= 0.017 by the two-way repeated measure ANOVA). The error bar represents the standard deviation. +1 less than one-third of the agar plate covered by bacteria, +2 two-thirds of the agar plate covered by bacteria, +3 the agar plate was completely covered with bacteria.



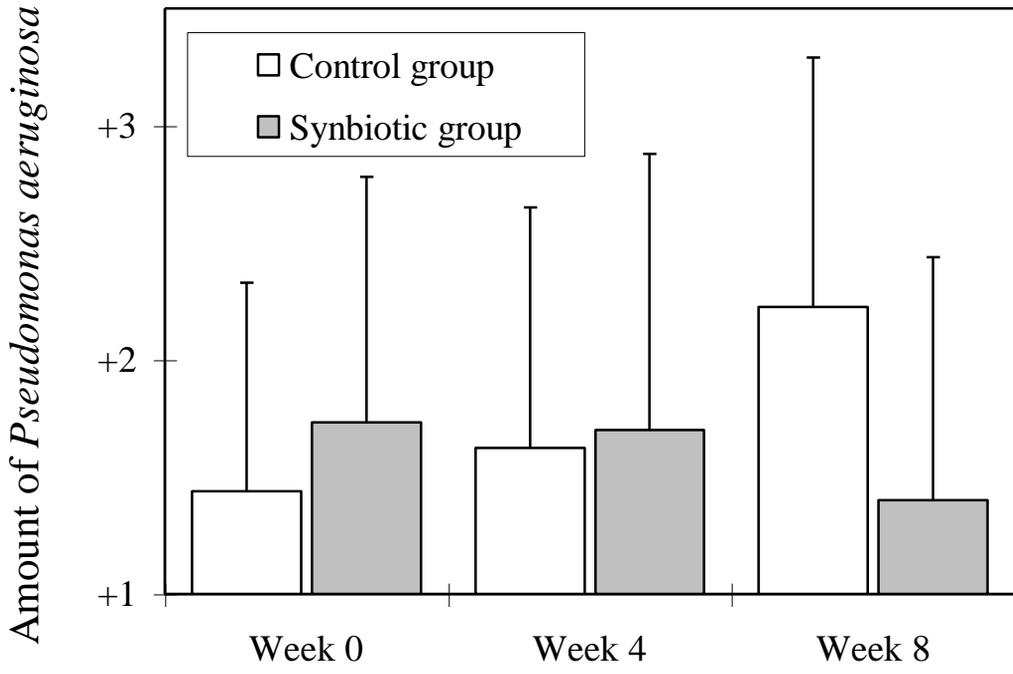


Table 1 Characteristics of the patients at the beginning of the study

	Control group (N=16)	Synbiotic group (N=31)	P value
Sex (male / female)	12 / 4	14 / 17	NS
Age (years)	75 ± 7	74 ± 14	NS
Duration of mechanical ventilation (months)	23 ± 16	13 ± 20	NS
Cause of mechanical ventilation			
Neurological disorder	12	14	
Respiratory failure	2	13	NS
Neuromuscular disease	2	4	
Feeding route			
Nasogastric tube	0	2	
Gastrostomy	15	27	NS
Jejunostomy	1	2	
Amount of <i>Pseudomonas aeruginosa</i> on week 0			
The pharynx	0.8 ± 0.8	1.1 ± 1.0	NS
The lower respiratory tract	1.4 ± 0.9	1.7 ± 1.0	NS

All measurements were presented number or the mean ± SD. NS, not significant.