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ORIGINAL SCIENTIFIC REPORT

A Four-Probiotics Regimen Reduces Postoperative Complications After Colorectal Surgery: A Randomized, Double-Blind, Placebo-Controlled Study

Katerina Kotzampassi[1] · George Stavrou[1] · Georgia Damoraki[2] · Marianna Georgitsi[2] · George Basdanis[1] · Georgia Tsaousi[1] · Evangelos J. Giamarellos-Bourboulis[2]

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Abstract

Background Heterogeneous results of published studies led to conduct a randomized clinical trial to assess the efficacy of a new formulation of four probiotics as prophylaxis for complications after colorectal surgery.

Methods A double-blind, placebo-controlled randomized study was conducted enrolling patients undergoing colorectal surgery for cancer. Capsules of placebo or of a formulation containing *Lactobacillus acidophilus*, *L. plantarum*, *Bifidobacterium lactis* and *Saccharomyces boulardii* were administered starting one day before operation and continuing for another 15 days postoperatively. Patients were followed up for 30 days with the development of postoperative complications as the primary outcome. Gene expression and serum levels of cytokines were measured on postoperative day 4 (www.clinicaltrials.gov NCT02313519).

Results The study was prematurely stopped after enrolment due to efficacy in the primary outcome. Administration of probiotics significantly decreased the rate of all postoperative major complication (28.6 vs. 48.8 % of the placebo arm, p 0.010, odds ratio 0.42). Major benefit was found in the reduction of the rate of postoperative pneumonia (2.4 vs. 11.3 %, p 0.029), of surgical site infections (7.1 vs. 20.0 %, p 0.020) and of anastomotic leakage (1.2 vs. 8.8 %, p 0.031). The time until hospital discharge was shortened as well. Gene expression of *SOCS3* was positively related with gene expression of *TNF* and of circulating IL-6 in the probiotic group but not in the placebo group.

Conclusions The studied probiotic formulation significantly decreased the risk of postoperative complications, namely mechanical ventilation, infections and anastomotic leakage. Modulation of the gene expression of *SOCS3* is one suggested mechanism (www.clinicaltrials.gov NCT02313519).

Introduction

Major colorectal surgery is accompanied by an unacceptably high morbidity of 15–23.2 % [1–3] leading to an increase in the number of ventilation support days, prolongation of total hospital stay, mortality and great medical costs [4,5]. Most of postoperative infectious complications are linked with gut microbiota; it is proposed that modulation of the gut microbiota with probiotics may prevent postoperative infections [6,7]. Several recent randomized controlled studies in limited number of patients undergoing elective abdominal surgery have demonstrated that the perioperative use of probiotics is safe and reduces both the incidence of postoperative infections as well as the duration of hospital stay and the length of antibiotic therapy [8–12].

One limitation of all published studies is the lack of homogeneity. This refers to the type of used probiotics and the administered regimen. Some trials have tested the efficacy of single probiotic preparations, whereas others have

✉ Katerina Kotzampassi
kakothe@yahoo.com

[1] Department of Surgery, Aristotle University of Thessaloniki, Medical School, 54 635 Thessaloniki, Greece

[2] 4th Department of Internal Medicine, University of Athens, Medical School, 124 62 Athens, Greece

tested the efficacy of mixtures of different probiotics. The type of administered probiotics seems to be of salient importance since according to their diversity they interact with the immune system of the host [13].

The lack of large-scale randomized clinical studies to assess the clinical efficacy of mixed preparations in colonic surgery led to the conduct of the present randomized controlled trial to investigate the impact of a preparation of four probiotics on postoperative morbidity after open elective colonic surgery. Since the development of postoperative infections is partly linked with the activation of the innate immune responses that can be modulated by probiotics, the current study assessed the effect of feeding probiotics on the expression of genes regulating cytokine production as well.

Patients and methods

Patients

The present randomized, double-blind, placebo-controlled trial was conducted in the Department of Surgery of the AHEPA University Hospital of Thessaloniki after protocol approval by the Institutional Ethics Committee. Written informed consent was provided from all patients scheduled to be included, independent of final inclusion. All adult patients consecutively admitted to our Department from April 2013 until July 2014 and scheduled to undergo elective, open, colonic resection with primary anastomosis were initially considered eligible for participation in the study. The final study inclusion was based on the operation completeness, i.e. radical tumour resection and anastomosis construction. The trial is registered (www.clinicaltrials.gov NCT02313519).

The day prior to surgery, patients were randomized by the sealed envelope method to either placebo or probiotic perioperative treatment. The allocated sequence was prepared at a 1:1 ratio by a biostatistician. Both surgeons and physicians in charge, participants and study investigators were blinded to this randomization.

Inclusion criteria were (a) both genders; (b) age $\geq$ 18 years old; (c) acceptable nutritional status (i.e. serum albumin $>$ 3.5gr/dL, NRS 2002 score $\leq$ 3) and (d) programmed for open surgery for colorectal cancer. All enrolled patients were operated on by the same consultant either as surgeon or as the primary assistant.

Exclusion criteria were (a) age $<$ 18 years; (b) denial or inability to consent; (c) need for emergency or palliative surgery; (d) American Society of Anaesthesiologists (ASA) class IV or V; (e) pregnancy or lactation; (f) inflammatory bowel disease; (g) use of antibiotics the last 10 days before surgery; (h) recent steroid therapy or preoperative neoadjuvant chemotherapy or radiotherapy; (i) signs of bacterial infection (defined by white cell count and body temperature) and (k) infection by hepatitis B or C virus by human immunodeficiency virus and by cytomegalovirus.

Study design

The afternoon prior to surgery and following bowel cleansing, individuals were randomized to receive an initial loading dose of four capsules of placebo or probiotics. On the day of operation and for the next 14 consecutive days, all patients continued receiving placebo or probiotics in a dose of one capsule twice a day with 100 ml of drinking water given by the nursing staff. If the patient remained intubated postoperatively, blind treatment was given through the nasogastric tube.

The probiotic preparation consisted of a combination of four probiotics: *Lactobacillus acidophilus* LA-5 1.75×10^9 cfu, *Lactobacillus plantarum* 0.5×10^9 cfu, *Bifidobacterium lactis* BB-12 1.75×10^9 cfu and *Saccharomyces boulardii* 1.5×10^9 cfu per capsule (LactoLevure®, UniPharma, Athens, Greece). Placebo consisted of identical capsules of powdered glucose polymer, and they were constructed by the same industry that manufactures the probiotics capsules.

In all patients, following general anaesthesia, bowel anastomosis was performed by the use of a circular stapler, straight, or articulated depending on the level of anastomosis. The peritoneum and aponeurosis were closed with a continuous suture technique.

After surgery and recovery from anaesthesia, all subjects were transferred to the surgical ward. When a patient was considered unstable, he was transferred to the Intensive Care Unit where mechanical ventilation was continued until the patient was ready for tracheal extubation according to the judgement of the attending anesthesiologists. Fluid therapy was similar to all patients, while no artificial nutrition was given in all uncomplicated patients. However, in the case of an anastomotic leakage or in any complication leading to reintubation and mechanical ventilation (i.e. after the sixth postoperative day), patients were switched to total parenteral nutrition (Smof-Kabiven, Fresenius Kabi, Oberursel, Germany) without discontinuation of probiotics.

Patients' follow-up

An attending physician, belonging to the investigators group and totally independent of the surgeons involved in the operation, was responsible for daily data collection for 30 continuous postoperative days; he was strictly forbidden to be involved in patient manipulation and treatment. Data were recorded daily in an electronic case report form (eCRF); eCRFs were monitored by a monitor blind to the allocated treatment. Recorded information was demographics and comorbidities, medical history, type of surgical procedures, supplementary medical treatment, haematology and

biochemistry, baseline POSSUM (Physiological and Operative Severity Score for the Enumeration of Mortality and Morbidity) scores, need for re-intubation and ventilation support, advent of postoperative infections, any anastomotic leakage, discharge from drainage tubes, bowel movement, defecation and the total number of in-hospital days as well as days in ICU. In case of discharge before day 30, follow up on day 30 was done by telephone calls. Definition of postoperative infections and severe sepsis was done with internationally accepted criteria [14,15].

For the first 30 enrolled patients, 6 ml of whole blood was collected after venipuncture of one peripheral vein under aseptic conditions on the third postoperative day. A volume of 2.5 ml was dispensed into PAXgene collection tubes containing stabilizing agent (PAXgene® Blood RNA Tubes; PreAnalytiX GmbH) followed by gentle inversion for 8–10 times. Tubes were kept for two hours at room temperature. They were then transferred overnight to −20 °C followed by −80 °C storage until RNA isolation procedures were performed. The remaining was collected into one sterile tube without anticoagulant and centrifuged in room temperature. Serum was kept at −80 °C until assayed.

Laboratory procedures

Total RNA was extracted from the PaxGene RNA collection tubes using nucleic acid purification kit (PaxGene® Blood RNA kit; PreAnalytiX GmbH). Complementary DNA (cDNA) was synthesized from 1 μg total RNA using the iScript cDNA Synthesis kit (BioRad, Hercules, CA, USA). Gene expression was assessed by the iQ™5 cycler system (BioRad,Hercules, CA, USA) using 2 μl cDNA, 10 μl iTaq™Universal SYBR® Green Supermix, (BioRad,Hercules, CA, USA), 6 μl nuclease-free water and 0.1 mg/ml sense and antisense primers to a final volume of 20 μl. Primer sequences were for *β*$_2$*-microglobulin*: sense 5′-ATG AGT ATG CCT GCC GTG TG-3′ and antisense 5′-CCA AAT GCG GCA TCT TCA AAC-3′; for *TNF* 5′-TGG CCC AGG CAG TCA GA-3′ and antisense 5′-GGT TTG CTA CAA CAT GGG CTA CA-3′ and for *SOCS3* sense 5′-TGC GCC TCA AGA CCT TCA G-3′ and antisense 5′-GAG CTG TCG CGG ATC AGA AA-3′. *β*$_2$*-microglobulin* was selected as the housekeeping gene.

Concentrations of tumour necrosis factor-alpha (TNFα), interleukin (IL)-6 and IL-10 in serum were measured in duplicate by an enzyme immunoassay (R&D Minneapolis, USA). The lower detection limits were 20 pg/ml for TNFα, 5 pg/ml for IL-6 and 10 pg/ml for IL-10.

Outcome measures

The primary study endpoint was the occurrence of major postoperative complications within 30 days. These complications comprised any anastomotic leakage, abdominal wound infection and dehiscence, and any infection accompanied or not by severe sepsis.

The secondary study endpoints were (a) the occurrence of minor postoperative complications within 30 days. These complications comprised: peripheral vein thrombosis, pulmonary embolism, acute heart failure and acute renal failure; (b) the time until development of complications within 30 days; (c) days on mechanical ventilation, duration of postoperative ileus and total hospital days and (c) gene expression and serum cytokines.

Power of the study

The study was powered for the primary endpoint. Taking into consideration that the total rate of major complications in the study site is 48 % and making the hypothesis that this would be decreased by 30 % in the probiotic group, it was anticipated that 208 patients per group were required in order to achieve an 80 % power with a 2-sided p value of less than 0.05. An interim analysis was planned after inclusion of 40 % of the calculated sample size, with a premature end to the study, if any difference in the primary end point was shown, according to the O'Brien–Fleming approach [16].

Statistical analysis

Baseline demographics between the two groups were compared by the Chi square test for qualitative variables and by the Student's "t test" for quantitative variables. Primary outcomes were compared between the two groups by the Chi square test; odds ratio (OR) and 95 % confidence intervals (CI) were calculated by Mantel and Haenzel's statistics. The time until development of an event was compared between groups by the log-rank test. Correlations between the relative copies of genes and serum cytokines were done separately for each group by the Spearman's rank of order. Any value of p below 0.05 after adjustment for multiple comparisons was considered significant.

Results

Primary outcome

The study reached successfully the primary endpoint (Table 1). The overall complication rate was 48.8 % in the placebo group and 28.6 % in the probiotics group (p 0.010). Reduction of complications in the probiotics group involved significant reduction of the incidence of (a) postoperative infections mainly of lower lung infections

and of surgical site infections, (b) of anastomotic leakage and (c) the need for postoperative intubation and mechanical ventilation.

However, it should be underscored that the successful achievement of the study primary endpoint was shown at the premature stop of the study. More precisely, at the planned interim analysis after inclusion of 40 % of the calculated sample size, this significant difference for the primary outcome was found and the study was stopped prematurely. At this stage, 164 patients who fulfilled inclusion criteria were randomly assigned to the two groups: 80 in the placebo group and 84 in the probiotics group (Fig. 1). The relevant patient characteristics and surgical procedure details are summarized in Table 2, showing a well-balanced distribution of patients between groups.

Regarding the development of infectious complications, the most frequently isolated organisms were (a) *Acinetobacter baumannii* [8 (10.0 %) in the placebo group; 3 (3.7 %) in the probiotics group, p 0.099)]; (b) *Pseudomonas aeruginosa* [4 (5.0 %) in the placebo group; 2 (2.3 %) in the probiotics group, p 0.135)] and methicillin-resistant *Staphylococcus aureus* (MRSA) [3 (3.8 %) in the placebo group; 1 (1.2 %) in the probiotics group, p 0.335)].

Secondary outcomes

The time until development of first major complication was shorter in the placebo arm than in the probiotics arm. The time until first bowel movement and until first defecation was shorter for the probiotics group (Fig. 2).

Regarding the development of minor complications, one and nil patients from the placebo arm and the probiotic arm, respectively, developed massive pulmonary embolism. The length of hospital stay was shorter in the probiotics group; the time until alive discharge was shorter compared to the placebo arm (Fig. 2). Median time until hospital discharge was 10 days in the placebo group and 8 days in the probiotics group.

Circulating cytokines and expressions of assessed genes were correlated separately in the placebo group and in the probiotics group on day 4. Positive correlations were found between the gene expression of *SOCS3* and the gene expression of *TNF* as well as between the gene expression of *SOCS3* and circulating IL-6 in the probiotics groups. This finding indicates that both gene expression of *TNF* and circulating concentrations of IL-6 were under the control of *SOCS3* in the probiotics group (Fig. 3). A similar correlation was absent in the placebo group showing that the gene expression of *TNF* and the circulating concentrations of IL-6 were not below any regulation by *SOCS3*.

Discussion

The present randomized controlled study evidenced a considerable reduction of overall major complications and of infectious complications within the first 30 days after colorectal surgery with a combined probiotic formula of four probiotics (*L. acidophilus* LA-5, *L. plantarum*, *B. lactis* BB-12 and *S. boulardii*) over placebo. The benefit did not involve only the reduction of the absolute complication rate but also shortening of the time until hospital discharge. Although, the four strains used in our study are widely used in nutritional and clinical practice, it is the first time they are used in combination in a randomized study of colon surgery.

Modern surgical fast tract protocol fully supports the avoidance of mechanical bowel cleansing and advice for laparoscopic intervention [17,18]; our department still insists on traditional surgical techniques; this is why the study design involved start of probiotics the day before surgery, immediately after termination of bowel cleansing. Despite these old-fashioned attitudes, the benefit described in this study resembles the findings reported by Zhang et al. [19]. They reported a reduction of infectious complications from 33.3 % in 30 patients to 10 % in another 30 patients

Table 1 Primary study outcomes

	Controls (n = 80)	Probiotics (n = 84)	OR (95 % CIs)	p
Any major complication (n, %)	39 (48.8)	24 (28.6)	0.42 (0.22–0.80)	0.010
Any infectious complication (n, %)	23 (28.7)	10 (11.9)	0.33 (0.15–0.76)	0.009
Pneumonia	9 (11.3)	2 (2.4)	0.19 (0.04–0.92)	0.029
Surgical site infections	16 (20.0)	6 (7.1)	0.31 (0.11–0.83)	0.020
Urinary tract infection	6 (7.5)	4 (4.8)	0.62 (0.17–2.27)	0.528
Bacteremia	8 (10.0)	6 (7.1)	0.69 (0.22–2.09)	0.583
Severe sepsis	4 (5.0)	1 (1.2)	0.23 (0.02–2.09)	0.192
Anastomosis leakage (n, %)	7 (8.8)	1 (1.2)	0.13 (0.01–0.99)	0.031
Need for mechanical ventilation (n, %)	28 (35.0)	17 (20.2)	0.47 (0.23–0.96)	0.037

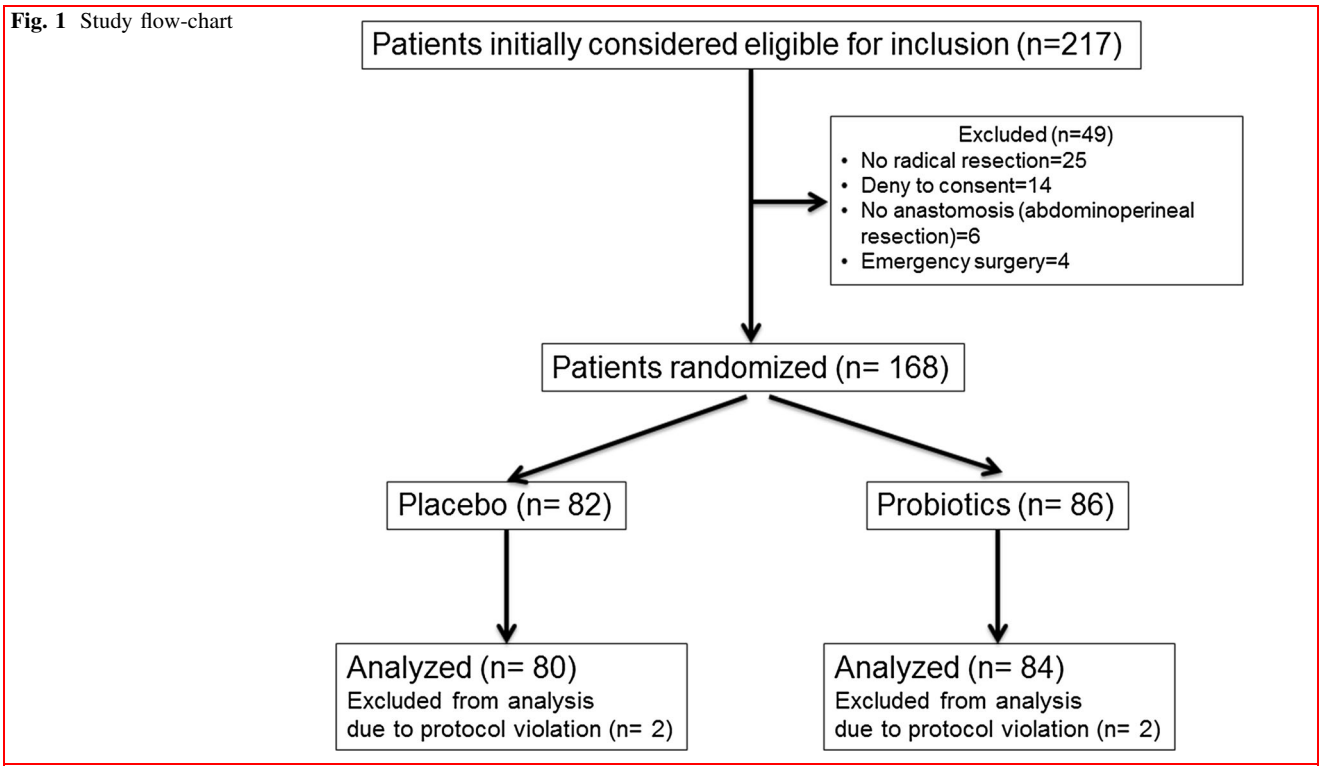

Fig. 1 Study flow-chart

Table 2 Baseline characteristics of the study population

	Controls ($n = 80$)	Probiotics ($n = 84$)	p
Age (years, mean ± SD)	66.4 ± 11.9	65.9 ± 11.5	0.812
Male/female (n, %)	58 (72.5)/22 (27.5)	57 (67.5)/27 (32.1)	0.609
POSSUM score (mean ± SD)			
Physiological	20.4 ± 6.9	19.5 ± 5.0	0.382
Operational	13.7 ± 3.0	13.6 ± 2.2	0.756
Comorbidities (n, %)			
Type 2 diabetes mellitus	19 (23.8)	22 (26.2)	
Heart failure	21 (26.2)	24	0.955
Chronic renal disease	11 (13.8)	12	
Type of surgery (n, %)			
Low anterior resection	36 (45.0)	41 (48.8)	
Recto-sigmoidectomy	20 (25.0)	22 (26.2)	0.895
Right hemicolectomy	15 (18.8)	14 (16.7)	
Total colectomy	9 (11.2)	7 (8.3)	

who received a formulation by three species of *Bifidobacteria*. On the opposite, in a study enrolling a total of 75 patients randomized to the oral preoperative and postoperative intake of either placebo or of *L. plantarum* 299 V, the rate of postoperative complications was not significantly altered [20], probably implying the need to use mixture of probiotics. The described benefit is in line with the results of the meta-analysis by He et al.; according to them preoperative intake of probiotics can help in the prevention of postoperative pneumonia [21].

The mechanism of action of probiotics may be related either with the earlier bowel movement preventing bacterial translocation from the gut or with modulation of the innate immune responses. Studying a subgroup of patients on postoperative day 4, a full derangement of the regulation of the expression of pro-inflammatory responses by *SOCS3* was

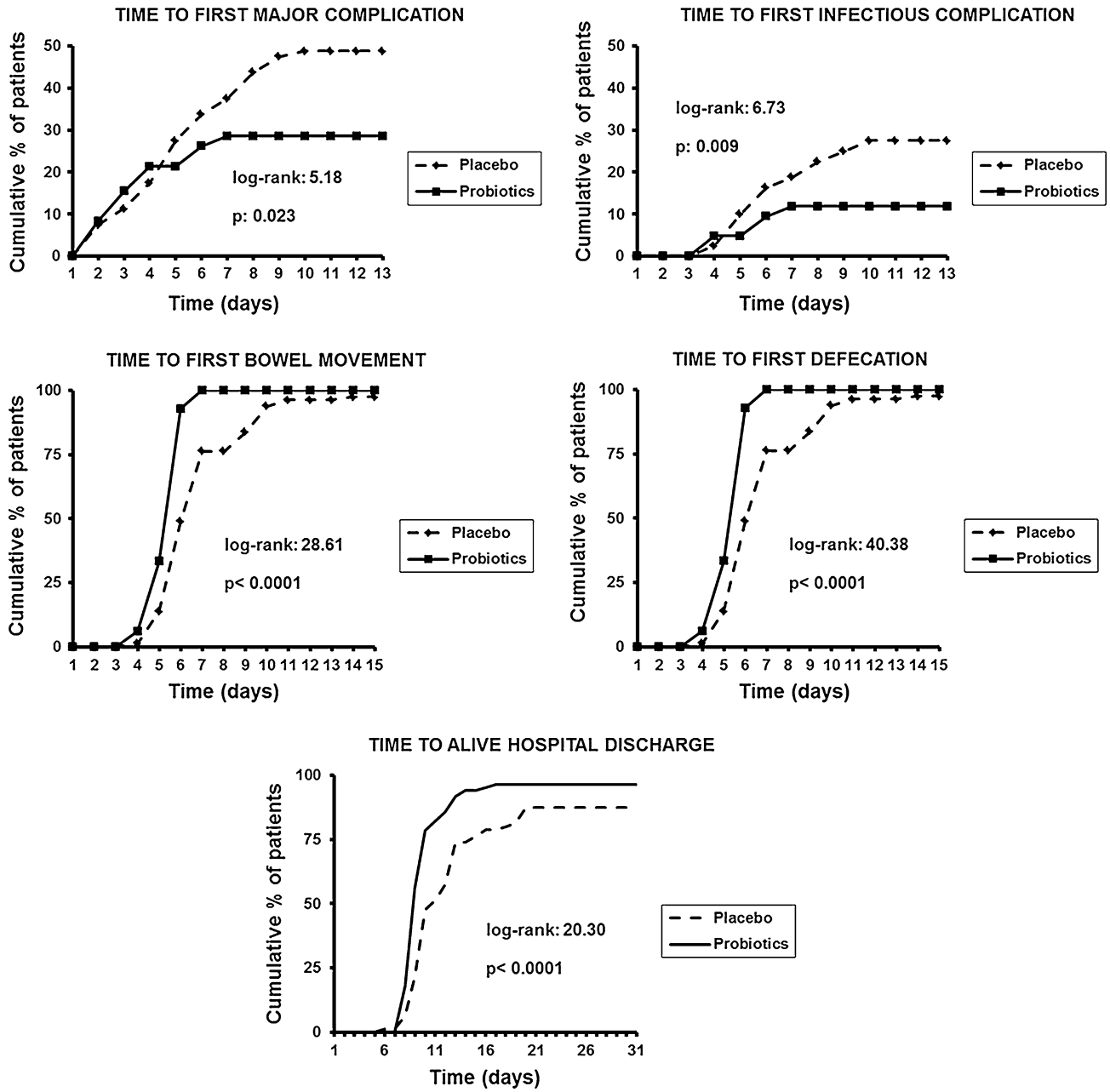

Fig. 2 Secondary study outcomes *p* values refer to statistical comparisons between placebo-treated patients and probiotics-treated patients

found. *SOCS3* encodes for the protein SOCS3 (suppressor of cytokine stimulation-3) that suppresses over-whelming cytokine responses [22]. Among patients assigned to the probiotics group, cytokine production was under the control of *SOCS3*. Modulation of *SOCS3* expression by the same mixture of probiotics has also been described by our group in a murine infection model [23].

Results of the current randomized, double-blind, placebo-controlled trial suggest that intake of a formulation of *L. acidophilus* LA-5, *L. plantarum*, *B. lactis* BB-12 and *S. boulardii* starting one day before major colorectal surgery and continuing for 15 days postoperatively significantly decreased the risk of postoperative complications, namely mechanical ventilation, infections and anastomotic

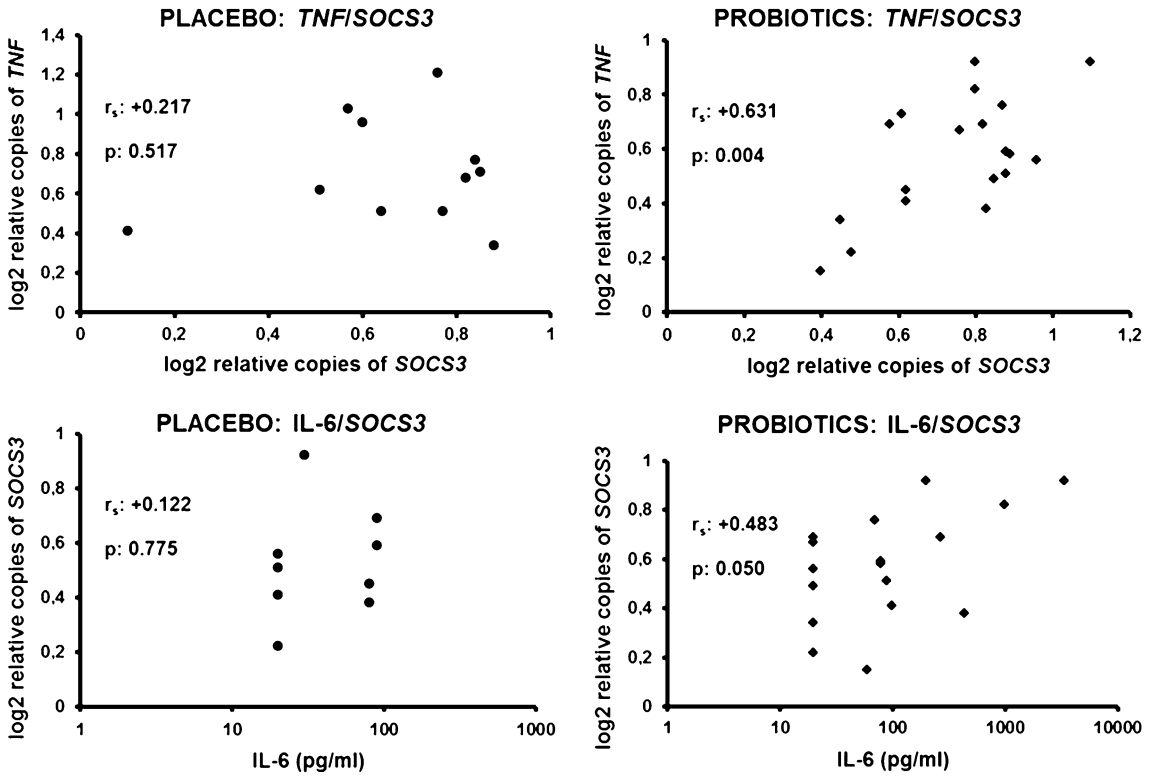

Fig. 3 Modulation of *SOCS3* gene expression in each group of treatment The correlations between gene expression of *TNF* and of *SOCS3* as well as between serum IL-6 and gene expression of *SOCS3* on day 4 are designed separately for the placebo group and for the probiotics groups. *p* values of correlations are provided

leakage. Modulation of the gene expression of *SOCS3* is involved as a mechanism underling clinical benefit.

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Conflict of interest None of the authors has any conflict of interest related to this study.

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