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Impact of digestive and oropharyngeal decontamination on the intestinal microbiota in ICU patients

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Abstract *Purpose:* Selective digestive microbial decontamination (SDD) is hypothesized to benefit patients in intensive care (ICU) by suppressing Gram-negative potential pathogens from the colon without affecting the anaerobic intestinal microbiota. The purpose of this study was to provide more insight to the effects of digestive tract and oropharyngeal decontamination on the intestinal microbiota by means of a prospective clinical trial in which faecal samples were collected from ICU patients for intestinal microbiota analysis. *Methods:* The faecal samples were collected from ICU patients enrolled in a multicentre trial to study the outcome of SDD and selective oral decontamination (SOD) in comparison with standard care (SC). Fluorescent in situ hybridization (FISH) was used to analyze the faecal microbiota. The numbers of bacteria from different bacterial groups were compared between the three regimens. *Results:* The total counts of bacteria per gram faeces did not differ between regimens. The *F. prausnitzii* group of bacteria, representing an important group among intestinal microbiota, was significantly reduced in the SDD regimen compared to the SC and SOD. The *Enterobacteriaceae* were significantly suppressed during SDD compared to both SOD and SC; enterococci increased in SDD compared to both other regimens. *Conclusions:* The composition of the intestinal microbiota is importantly affected by SDD. The *F. prausnitzii* group was significantly suppressed during SDD. This group of microbiota is a predominant producer of butyrate, the main energy source for colonocytes. Reduction of this microbiota is an important trade-off while reducing gram-negative bacteria by SDD.

Keywords Bacteria, anaerobic · Molecular diagnostic techniques · Prophylaxis, antibiotic · Critical care · Microbiota, faecal

Introduction

Selective (microbial) decontamination of the digestive tract (SDD), developed using immuno-compromised animal models [1, 2], was first clinically tested in severely immuno-compromised hemato-oncological patients and later applied to patients admitted to intensive care units (ICU) [3]. The concept of SDD is to selectively suppress potential pathogens, mostly Gram-negative bacteria, without disturbing the anaerobic intestinal microbiota.

Oral, non-absorbable antibiotics were combined with prophylactic systemic antimicrobial treatment (3rd generation cephalosporins) during the first four days to eradicate potential pathogens. In order to protect the anaerobic microbiota, the use of antibiotics with anti-anaerobic activity was discouraged.

Earlier studies were flawed by design [3] or lacked statistical power to detect a survival advantage. Meta-analyses showed a significant survival advantage [4, 5] one did not [6]. Also single centre randomized trials evaluating SDD showed reduction in mortality [7, 8].

A consistent finding across studies evaluating SDD in the ICU has been a reduction of the number of episodes of nosocomial infections—especially, of respiratory tract. As the prevention of ventilator-associated pneumonia (VAP) might play a dominant role in the mortality reduction of SDD, the oropharyngeal component of SDD, referred to as Selective Oropharyngeal Decontamination (SOD), has also been analysed in clinical trials [9]. Although a significant reduction in VAP was shown, historically no overall reduction in mortality by SOD [10] or non-selective oral decontamination [11] was observed.

A large multi-centre clinical trial of SOD and SDD was recently reported. In this trial both SDD and SOD showed a similar survival benefit compared to standard care (SC) [12]. As an adjunct to this study we investigated the impact of SOD and SDD regimes on the intestinal microbiota compared to SC.

The intestinal microbiota is a complex ecosystem which comprises more than 10^{11} bacteria per gram of faeces and more than 400 different species [13]. Some of the intestinal microbiota are beneficial and ways to promote their growth have been investigated [14]. With the use of SDD, the intestinal microbiota is believed to protect the human host by preventing increased colonisation with potential pathogens [15], mostly anaerobic bacteria that are difficult to isolate and identify by classical culture techniques. Quantification of the anaerobic microbiota based on culture methods is unreliable: selective media introduce bias; and many genera cannot be cultured in vitro. Molecular methods, such as fluorescent in situ hybridisation (FISH), yield absolute numbers of microorganisms [16, 17] instead of colony-forming units, which is the quantitative read-out of culture.

In the present study we evaluate, for the first time with molecular methods, the impact of SDD and SOD on the intestinal microbiota compared to SC in subjects admitted to ICU. We tested the hypothesis that SDD (or SOD) may execute its claimed beneficial effects by leaving the anaerobic intestinal microbiota unaffected.

Patients and methods

All patients consecutively admitted to the Medical ICU in our hospital, and that were evaluated within the framework of the Dutch multi-centre SDD-SOD study [12], were eligible. In this SDD-SOD study, participating centres followed three different regimens of treatment in their ICUs in a non-blinded random sequence:

1. SC: no prophylactic antimicrobials, no restrictions in the antibiotics used
2. SOD: prophylactic topical oropharyngeal antimicrobials, no restrictions in the antibiotics used.
3. SDD: prophylactic topical oropharyngeal and gastro-intestinal antimicrobials, with the addition of intravenous cefotaxime during the first four days. Selective use of antimicrobials was encouraged to avoid interference with the intestinal microbiota, in accordance with the concept of colonisation resistance.

The sequence of these courses allocated to our hospital was SC–SOD–SDD. For details of the trial and the antibiotic regimens used, we refer to the original article [12]. All included patients were scored for their severity of disease with the APACHE II classification system [18]. Other parameters that were recorded are demographics (age, gender etc.), concomitant diseases and the use of all antimicrobial products including the antimicrobials used for the prophylactic protocols (SOD and SDD).
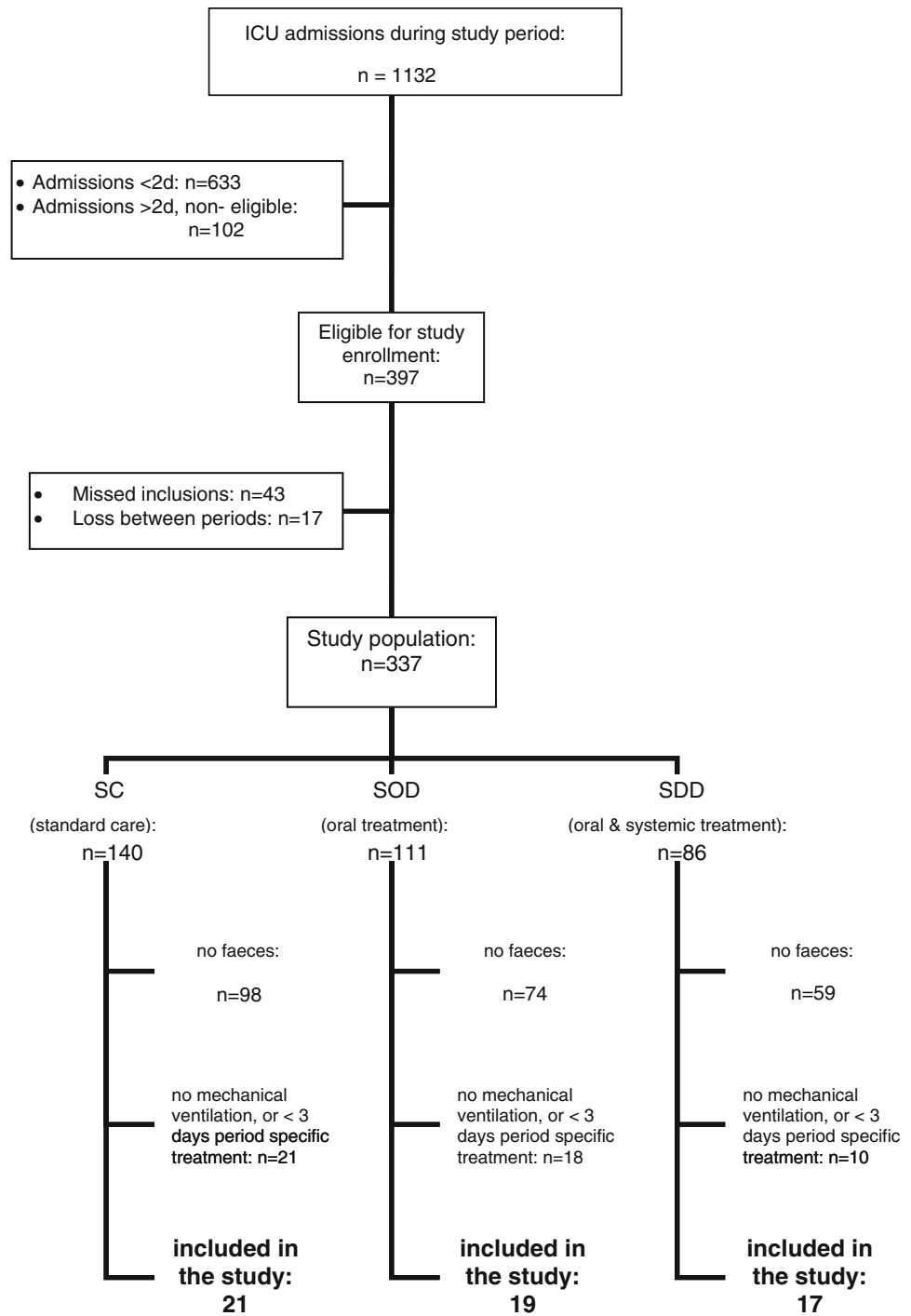
During all three consecutive trial periods, faecal samples were collected from the patients that produced faeces spontaneously or with the use of laxatives as on clinical indication alone. For every patient, only the first sample faecal that was passed after at least three days of period-specific treatment by patients on mechanical ventilation was used in order to avoid skewing of data (see Fig. 1).

For the multi-centre SOD-SDD study, a waiver from informed consent was provided by local and National Ethics Review Boards, as not patients but rather different standards of treatment protocols were compared in a randomized fashion. Samples and data were analyzed anonymously.

Materials and methods

All fresh faecal samples were stored in a refrigerator and processed for analysis within 24 h after collection. The processing procedure was as follows: after homogenization, from each faecal sample 1.0 g (wet weight) was taken and diluted in PBS (8 g/L NaCl, 0.2 g/L KCl, 1.44 g/L $Na_2HPO_4 \cdot 2H_2O$, 0.24 g/L KH_2PO_4, pH 7.4) 1:4 or 1:9 depending on faecal consistency. The faecal consistency was scaled on the Bristol Stool chart, where consistencies of stool type 1–6 were diluted 1:9, type 7 was diluted 1:4. These dilutions were processed for storage as described previously [19].

Fig. 1 Flow chart of patient selection

For quantification of the bacteria in faecal samples, multiple slides with 1 cm^2 wells were prepared for cell counting and hybridized as described previously [19]. Hybridization was performed using the 16S rRNA-targeted probes listed in table 1.

The probes were manufactured by Eurogentec (Seraing, Belgium). Together these probes detect approximately 90% of the expected hybridisable total amount of bacteria (Eub338) [17]. Additional to the probe set for detection of the normal intestinal microbiota, three probes were used to enumerate groups of potential pathogens. These are the EC1531 [20] probe for the *Enterobacteriaceae* and the Enfl84 and Enfm93 [21] to detect enterococci.

Table 1 Probes used for the detection of the intestinal microbiota

Target	Probe	Sequence
Total bacteria	EUB338	5′GCTGCCTCCCGTAGGAGT
Bacteroides/Prevotella	Bac303	5′CCAATGTGGGGGACCTT
E. rectale/Blautia coccoides	Erec482	5′GCTTCTTAGTCA(G/A)GTACCG
F. prausnitzii-group	Fprau645	5′CCTCTGCACTACTCAAGAAAAAC
Atopobium-group	Ato291	5′GGTCGGTCTCTCAACCC
Bifidobacteria	Bif164	5′CATCCGGCATTACCACCC
Ruminococci	Rbro730	5′TAAAGCCCAGYAGGCCGC
	Rfla729	5′AAAGCCCAGTAAGCCGCC
Roseburia cluster	Rint623	5′TTCCAATGCAGTACCGGG
	Rint helper	5′GTTGAGCCCCGGGCTTT
Enterobacteriaceae	EC1531	5′CACCGTAGTGCCTCGTCATCA
Enterococcus faecalis	Enfl84	5′CCTCTTTCCAATTGAGTGCA
Enterococcus faecium-group	Enfm93	5′GCCACTCCTCTTTTTCCGG

References to the probes can be found in the supplementary material

The fluorescent cells in the samples were counted using an automated microscope system [22]. The detection limit used with this method was 10^6 cells/g of faeces.

After analysis of the FISH results, minimal inhibitory concentrations (MIC) to the SDD/SOD antibiotics of a type strain of *F. prausnitzii* were performed. The A2-165 strain of *F. prausnitzii* was cultured in an anaerobic cabinet on YCFA agar-plates. YCFA medium consisted of (per 100 ml): 1 g casitone, 0.25 g yeast extract, 0.4 g NaHCO$_3$, 0.1 g cysteine, 0.045 g K$_2$HPO$_4$, 0.045 g KH$_2$PO$_4$, 0.09 g NaCl, 0.009 g MgSO$_4$.7H$_2$O, 0.009 g CaCl$_2$, 0.1 mg resazurin, 1 mg haemin, 1 μg biotin, 1 μg cobalamin, 3 μg *p*-aminobenzoic acid, 5 μg folic acid and 15 μg pyridoxamine. Final concentrations of short-chain fatty acids (SCFA) in the medium were 33 mM acetate, 9 mM propionate and 1 mM each of isobutyrate, isovalerate and valerate.

The MIC tests were performed using E-test® -strips containing tobramycin, colistin and cefotaxime, according to the instructions of the manufacturer (AB BIODISK, Solna, Sweden).

Statistics

The results were compared statistically between the three patient groups using SPSS® 16 statistical analysis software. For the continuous variables age and APACHE-scores, ANOVA analyses were performed, for nominal variables such as antibiotics use, a Chi-square cross tabulation with a Fisher's Exact test was used (see Table 2 for details).

The analysis of the numbers of bacteria was done with an ANOVA analysis after log-transformation to obtain a normal distribution. The normal distribution was checked with the Kolmogorov-Smirnoff test and by evaluation of P–P plots.

The numbers of enterococci however were still not normally distributed after log-transformation, therefore for these bacterial groups non-parametric tests (MWU) were performed.

Results

Patients: Figure 1 shows the flowchart of the patient sample selection. Faecal samples were collected for analysis from a total of 21 patients in the SC regimen, 19 patients in the SOD regimen and 17 patients in the SDD regimen.

The age distribution of patients did not differ significantly between the three study episodes; in the SC group, the mean age was 59.8, in the SOD group 63.7 and in the SDD group 56.7.

APACHE-II scores were also similar between groups: 14.3, 15.4 and 16.2 for the SC, SOD and SDD groups, respectively. The APACHE predicted (adjusted) death rate was higher in both the SOD and SDD regimen groups. These differences were not statistically significant. Furthermore enteral tube feeding and gastric retention did not differ statistically significantly between the three regimen groups, see table 2 for details.

Except for the use of cephalosporins (which are a part of the SDD regimen) the use of antibiotics did not differ significantly between the regimens. Because no statistical difference in the characteristics of the population was found, no further multivariate analysis was performed.

No significant impact of SDD was observed on the total number of bacteria of the colonic microbiota (Table 3). There is, however, a significant difference in the composition of the microbiota of the different regimens. Some differences are seen in the bacterial groups between the analysed regimens, but the numbers of the *F. prausnitzii* group in the SDD-regimen are significantly lower compared to both Standard Care and the SOD regimen. The *Eubacterium rectale* group shows lower numbers of bacteria in the SDD regimen compared to the SC regimen. A

Table 2 Characteristics of the patient population

Variable	SC	SOD	SDD
n	21	19	17
ANOVA			
Age (years)			
Mean	59.8	63.7	56.7
95% CI	53.3–66.2	55.2–72.3	50.0–63.5
APACHE			
Mean	14.3	15.4	16.2
95% CI	11.7–16.9	12.2–18.7	11.4–21.0
APACHE predicted death rate			
Mean	16.0	22.6	25.5
95% CI	10.8–21.3	14.1–31.0	11.7–39.1
Enteral feeding (ml/day)			
Mean	1807	1953	1912
95% CI	1701–1913	1779–2126	1802–2021
Gastric retention (ml/day)			
Mean	355	120	147
95%CI	120–590	16–224	35–259
Length of stay in the ICU before sampling (days)			
Mean	8.2	8.5	11.1
95% CI:	6.8–9.7	5.6–11.4	7.4–14.7
Median	8	5	5

	SC (%)	SOD (%)	SDD (%)	SCvsSOD (p)[a]	SCvsSDD (p)[a]	SODvsSDD (p)[a]
Fisher's exact-tests						
In-hospital mortality	38.1	26.3	47.1	0.51	0.74	0.30
Admission type						
Surgical	19.0	15.8	5.9	1.00	0.36	0.61
Medical	81.0	84.2	94.1			
Antibiotics before ICU admission	28.6	36.4	29.4	0.74	1.00	0.73
Systemic antibiotics in the ICU						
Beta-lactam	14.3	15.8	29.4	1.00	0.68	0.68
Beta-lactam + inhibitor	71.4	42.1	41.2	0.11	0.10	1.00
Cephalosporin	14.3	31.6	88.2	0.27	0.00	0.00
Quinolones	33.3	42.1	23.5	0.75	0.72	0.30
Aminoglycosides	0.0	5.3	0.0	0.48		1.00
Vancomycin	9.5	15.8	11.8	0.65	1.00	1.00
Clindamycin	4.8	0.0	0.0	1.00	1.00	
Metronidazole	0.0	5.3	5.9	0.48	0.45	1.00
Cotrimoxazole	9.5	5.3	0.0	1.00	0.49	1.00

[a] Fisher's exact-test

subgroup of *E. rectale*—the *Roseburia intestinalis* group—also shows lower numbers in the SDD regimen.

SDD had a significant impact on potential Gram-negative pathogen counts in the faecal microbiota to which it is targeted. *Enterobacteriaceae* in the SDD regimen were significantly reduced in numbers compared to both the SC-regimen and the SOD-regimen.

The gram-positive potential pathogens such as the enterococci increased significantly with the use of SDD, in comparison with both other regimens (Table 4). A significant rise in *Enterococcus faecalis* was also seen in the SOD regimen compared to the SC regimen. However, the numbers of *E. faecalis* in the SOD regimen were still significantly lower than when compared to the numbers of *E. faecalis* in the SDD regimen.

The MIC values of *F. prausnitzii* A2-165 for the SDD/SOD antibiotics were 4 µg/ml for tobramycin, >32 µg/ml for cefotaxime and >256 µg/ml for colistin.

Discussion

The major finding in this study was that the composition of the intestinal microbiota, as evidenced by stool analysis, was affected by the use of SDD: the *F. prausnitzii* group was significantly reduced in numbers in stools of subjects receiving SDD compared to subjects in both other regimens. This finding contrasts with the hypothesis of SDD that anaerobic microbiota would remain unaffected [15].

The group of bacteria detected with the Fprau-probe is one of the predominant bacterial groups in healthy volunteers, representing 10–15% of the intestinal microbiota on average [17, 23]. The *F. prausnitzii* group therefore plays an important role in maintaining the colonization resistance, normally protecting the human host from infections. *F. prausnitzii* has also been found to have anti-inflammatory effects which may play a

Table 3 Numbers and statistical analysis of the main intestinal microbiota groups

Variable	Regimen:					
	SC (21[a])		SOD (19[a])		SDD (17[a])	
	Mean	95% CI	Mean	95% CI	Mean	95% CI
Probe						
Total bacteria	3.7×10^9	2.2×10^9–6.2×10^9	1.6×10^9	7.8×10^8–3.4×10^9	1.9×10^9	8.7×10^8–4.3×10^9
Bacteroides	6.5×10^8	3.5×10^8–1.2×10^9	3.6×10^8	1.4×10^8–9.5×10^8	4.2×10^8	2.1×10^8–8.1×10^8
E. rectale[b]	5.1×10^8	3.0×10^8–8.5×10^8	1.4×10^8	5.4×10^7–3.4×10^8	6.2×10^7	2.6×10^7–1.4×10^8
R. intestinalis[b]	6.8×10^7	3.7×10^7–1.3×10^8	1.8×10^7	7.0×10^6–4.8×10^7	1.1×10^7	4.9×10^6–2.7×10^7
F. prausnitzii[c]	5.5×10^7	2.3×10^7–1.3×10^8	4.0×10^7	1.6×10^7–9.9×10^7	2.9×10^6	1.4×10^6–6.0×10^6
Atopobium	1.3×10^8	6.6×10^7–2.3×10^8	3.5×10^7	1.3×10^7–9.2×10^7	4.2×10^7	1.4×10^7–1.2×10^8
Bifidobacteria	4.4×10^7	1.6×10^7–1.2×10^8	1.6×10^7	5.4×10^6–4.6×10^7	5.8×10^7	1.8×10^7–1.8×10^8
Ruminococci	2.0×10^8	1.3×10^8–3.3×10^8	8.6×10^7	3.8×10^7–2.0×10^8	7.8×10^7	3.1×10^7–1.7×10^8
Enterobacteriaceae[c]	7.2×10^7	3.6×10^7–1.4×10^8	4.8×10^7	1.7×10^7–1.4×10^8	4.1×10^6	2.0×10^6–8.3×10^6

ANOVA test was used for statistical analysis

[a] Number of study subjects

[b] Indicates a significant difference between the SDD and SC regimens only

[c] Indicates a significant difference between SDD and both SC and SOD regimens

Table 4 Numbers and statistical analysis of enterococci per gram faeces

	SC ($n = 21$)	SOD ($n = 19$)	SDD ($n = 17$)	SC vs. SOD[a]	SC vs. SDD[a]	SOD vs. SDD[a]
E. faecalis	2.6×10^6	7.6×10^6	6.9×10^7	0.002	0.000	0.000
E. faecium	6.3×10^6	9.8×10^6	5.4×10^7	0.142	0.000	0.000

[a] Mann–Whitney U-tests, p values

dominant role in the development of Crohn's disease [24].

Furthermore, the *F. prausnitzii* group is considered to provide special health benefit for the human host because of its main fermentation product, butyrate [23, 25]. Butyrate is a short chain fatty acid that is the primary source of energy for the colonocytes [26]. In addition, butyrate promotes the growth of colonocytes, preventing mucosal atrophy. It also appears to lower the risk of malignant transformation of colonocytes in animal models [27]. The optimal concentration of butyrate is not known in vivo, but in vitro cell cultures show a growth arrest at concentrations below 10 mM [26]. In healthy volunteers, butyrate concentrations were also reduced during tube feeding [28], and in a blinded re-analysis of these faecal samples, the reduction of *F. prausnitzii* showed strong correlation with the reduction of butyrate measured in these faecal samples (manuscript submitted).

The *F. prausnitzii*-group of bacteria is difficult to culture in vitro and therefore could not be detected when SDD was first developed. Only culture-based methods were used to asses whether the intestinal microbiota remained intact in the early days of SDD. Therefore, this unintended effect could not have been foreseen when the regimen was first used. The MIC analysis of the SDD/ SOD antibiotics shows that *F. prausnitzii* is susceptible only to tobramycin at 4 μg/ml. This concentration of tobramycin is easily reached with the intestinal decontamination regimen of SDD. The reduction of *F. prausnitzii* is therefore most likely caused by the intestinal administration of tobramycin in the SDD-regimen group.

We show that the loss of *F. prausnitzii* is not compensated for by an increase of other important butyrate-producing bacteria. *Roseburia* spp., another important group of butyrate producing bacteria, are also present in significantly lower numbers during the SDD regimen compared to the SC regimen. Similar to *F. prausnitzii*, reductions in numbers of bacteria from the *Roseburia* spp. correlate to a reduction in the amount of butyrate which is produced [29]. Therefore, we postulate that butyrate production may be impaired in SDD patients due to the loss of these predominant butyrate-producing bacterial groups.

Clear evidence that the SDD regimen was given according to protocol is the fact that a significant reduction in the *Enterobacteriaceae* was found in the SDD regimen compared to both other regimens. The numbers of *Enterobacteriaceae* in the SC and SOD regimens were markedly higher than in healthy individuals [17].

Traditionally, cefotaxime been a component of the SDD regimen [8, 12, 30]. Although cefotaxime has been shown to have a moderate suppressive effect on Enterobacteriaceae [31, 32], we have reasons to believe that the effect of cefotaxime is negligible compared to the effect of the large amounts of non-absorbable antibiotics targeted specifically to *Enterobacteriaceae*.

Cefotaxime elimination is almost entirely by renal excretion; only 5% of elimination is by biliary excretion, and the amount of cefotaxime reaching the colon is exceedingly low compared to the amounts of non-resorbable tobramycin and colistin, both active against *Enterobacteriaceae*. Early studies haven shown that cefotaxime is almost entirely inactivated by faecal enzymes [33]. This is not the case with colistin and tobramycin, which makes it most likely that the effect seen is caused by these antibiotics instead. Furthermore the earlier studies of the effects of cefotaxime on the intestinal microbiota are based upon culturing methods with selective plates. Especially when antibiotics are used, these methods can lead to underestimation of the actual numbers of bacteria.

The second important finding is that SOD left the faecal microbiota relatively unaffected compared to stools from patients enrolled during the SC period of the study. As shown in the multi-center clinical trial [12], there is no significant difference in mortality between SDD and SOD, despite the fact that colonisation and infections with *Enterobacteriaceae* are significantly reduced in SDD compared to SOD. This reduction of *Enterobacteriaceae* is confirmed in our study. We speculate that the lack of further mortality reduction is partly explained by the negative effect of SDD on beneficial bacteria of the colonic microbiota. How the loss of these beneficial bacteria, which provide an important source of nutrition for colonocytes, translates into clinically significant effects in general, and in critically ill patients in particular, is presently unknown. The role of microbiota and the production and uptake of butyrate in particular to maintain colonic integrity in a range of conditions, including critical care settings with sepsis, should perhaps be studied in animal models.

The Bifidobacteria showed a mild increase in the SDD regimen that did not reach statistical significance. These Gram-positive bacteria are not susceptible to the antibiotics used in SDD.

A third consideration with the use of SDD is the increase of enterococci in the faeces [34]. Although the numbers of *E. faecalis* are also significantly higher in the SOD regimen compared to the SC regimen, the numbers of enterococci in the SDD regimen are an order of magnitude higher than in both other regimens. It is historically known that enterococci tend to increase in numbers under similar use of topical antibiotics [35], as these Gram-positive bacteria are naturally resistant to the SDD-antibiotics. Also, Gram-negative intestinal bacteria induce an antimicrobial peptide, Reg3g, at the luminal surface of intestinal cells by stimulating TLR4 [36], with growth limiting effects on enterococci. Elimination of Gram negative bacteria resulted in a decrease of Reg3g with subsequent increase and translocation of enterococci in a mouse model [36].

The enterococci were considered to be harmless when SDD was first introduced in the ICU and none of the included patients had bloodstream infections with enterococci. However, we are aware that enterococci cause serious nosocomial infections, spread easily, and acquire increased antibiotic resistance [37, 38]. Enterococci are the third leading cause of endocarditis, and nosocomial acquisition is associated with a poor prognosis [39, 40]. Furthermore, antibiotic resistance gene-transfer has been demonstrated in vivo between enterococci and other bacterial species [41].

Limitations to the study are the limited numbers of patients and samples. Also, by design of the study, no baseline samples could be obtained nor could the timing of the sampling be standardized, as all samples were produced spontaneously. Based upon the data provided in table 2 we believe this has not caused a major source of bias.

Conclusion

We show that the total numbers of bacteria of the faecal microbiota in patients in the ICU are not significantly influenced by SDD. *Enterobacteriaceae* dropped significantly in numbers in the SDD regimen compared to SC and SOD regimens, as expected.

SDD does have a significant impact on the composition of the anaerobic intestinal microbiota; the number of *F. prausnitzii*-group of bacteria is significantly reduced during the SDD regimen compared to both SC and SOD regimens. The bacteria from the *Enterococcus* groups are present in significantly higher numbers in the SDD compared to both SC and SOD regimens. The other groups of bacteria show some variations but these are not significant between both other regimens.

The hypothesis that SDD is unequivocally beneficial by only reducing *Enterobacteriaceae* while leaving the colonic microbiota unaffected has to be rejected.

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