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RESEARCH PAPER/REPORT

Gut microbiota profiles in critically ill patients, potential biomarkers and risk variables for sepsis

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ABSTRACT

Critically ill patients are physiologically unstable and recent studies indicate that the intestinal microbiota could be involved in the health decline of such patients during ICU stays. This study aims to assess the intestinal microbiota in critically ill patients with and without sepsis and to determine its impact on outcome variables, such as medical complications, ICU stay time, and mortality. A multi-center study was conducted with a total of 250 peri-rectal swabs obtained from 155 patients upon admission and during ICU stays. Intestinal microbiota was assessed by sequencing the V3-V4 hypervariable regions of the 16S rRNA gene. Linear mixed models were used to integrate microbiota data with more than 40 clinical and demographic variables to detect covariates and minimize the effect of confounding factors. We found that the microbiota of ICU patients with sepsis has an increased abundance of microbes tightly associated with inflammation, such as *Parabacteroides, Fusobacterium* and *Bilophila* species. Female sex and aging would represent an increased risk for sepsis possibly because of some of their microbiota features. We also evidenced a remarkable loss of microbial diversity, during the ICU stay. Concomitantly, we detected that the abundance of pathogenic species, such as *Enterococcus* spp., was differentially increased in sepsis patients who died, indicating these species as potential biomarkers for monitoring during ICU stay. We concluded that particular intestinal microbiota signatures could predict sepsis development in ICU patients. We propose potential biomarkers for evaluation in the clinical management of ICU patients.

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Introduction

The human gut hosts a massive and diverse number of microorganisms, known as the intestinal microbiota (IM), which coexists in a dynamic equilibrium with the host, relying on molecular cross-talk supported by the exchange of nutrients, metabolites, and protein–protein interactions.[1–3] The identification of such microbes and their abundance changes associated with diseases have been broadly described in the latest years. Population-based assessments have revealed a vast inter-individual variability in the human IM. Besides, the human IM is involved in the protection against pathogen arrival, regulation of the intestinal endocrine function, and acting as biofactories for the synthesis of vitamin and cofactors.[4,5]

Critically ill patients are physiologically unstable given their multiple organ dysfunctions, leading to a risk of death or severe systemic sequelae and thus requiring advanced and specialized life support with continuous monitoring. In critical illness, the gut is considered the driver of associated infectious complications because of the underlying deterioration of the intestinal epithelium, alteration of immune function, and infiltration of the endogenous IM.[6] Moreover, these patients exhibit a complex stress response

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mediated by neuronal, endocrine, and immune mechanisms that is fine balanced through cellular and physiological adaptations.[7] Notwithstanding, a dysregulated inflammatory response can produce organ damage and systemic infection, i.e., sepsis, hence increasing the risk of medical complications, longer hospital stays, mortality and additional cost for public health systems.[8,9]

When compared with healthy subjects, critically ill patients harbor pathogenic microorganisms that can out-compete indigenous species of the human IM, thus resulting in the loss of beneficial microbial species.[10] That unbalance between harmful and healthy microbes in the IM of critically ill patients is thought to be promoted by the host response to physiological stress and invasive and pharmacological medical care treatments prescribed (e.g. antibiotics, insertion of feeding tubes, surgery, etc.).[11,12] The changes in the IM diversity of critically ill patients after ICU admission seem to combine a reduction in strict anaerobes and increase in pathogenic species, thus associated with an increased mortality.[3,13] Furthermore, a previous report indicated that prolonged critical illness in ICU patients produces profound alterations in the structure of the IM leading to the microbial diversity vanishing to almost-null levels, with concomitant boosting of the virulence of those ultra-low diversity communities by opioid and antibiotic administration.[14]

The management of the IM on critically ill patients is a current trend of clinical research. All in all, it is considered as a pivotal factor that under control and surveillance could improve survival rates in ICU patients. Consequently, different IM-associated strategies to tackle adverse events in ICU patients have been proposed, mostly to prevent the growth of pathogens and improve patient prognosis;[15] e.g. the decontamination of the digestive tract (SDD), administration of probiotics and prebiotics alone or in combination (synbiotics), and fecal microbiota transplantation (FMT).[3,16,17]

The present study aims to describe the IM profiles in critically ill ICU patients, with and without sepsis, and try to shed light on the role of IM as contributing factor in the evolution of these patients and its influence on ICU stay time, development of different medical complications, and mortality. Additionally, we explored several variables recorded upon ICU admission and stay to determine IM covariates and potential confounding variables in this cohort. Longitudinal evaluation of patients with sepsis was aimed at determining predictive variables from different descriptors of the IM assessment.

Results

Table 1 summarizes the initial assessment of patients grouped according to sepsis status. Important differences were found in age and ICU stay time. As expected, differences in the CRP concentration and SOFA score were indicative of sepsis and supported the ICU patient grouping. Thirty-two out of the nonseptic patients (38.6%) developed a hospital-acquired infection with multiple origins. On the other hand, the pulmonary (56%) and abdominal (17%) infections were determined as the predominant origin of sepsis in such patients.

The IM of ICU patients upon admission

Initial assessment of the microbial diversity of M1 samples and the microbial community structure between groups was performed with alpha- and beta-diversity approaches, respectively, using the abundance and prevalence information of OTUs recovered. Taking into account all four alpha diversity parameters evaluated, we observed that sepsis samples had no meaningful differential distributions of diversity descriptors compared to their no-sepsis counterparts (Figure S1).

Beta-diversity assessment indicated that sepsis conditions could drive changes in the microbial community structure (PERMANOVA = 1.91, $p = .013$). However, we found that other variables could modulate the IM as well. As a result, sex and age seemed to influence the microbiota profiles to a larger extent than other parameters (PERMANOVA = 2.94, $p < .001$ and PERMANOVA = 1.97, $p = .003$ for sex and age, respectively) (Figure 1). Moreover, we found that variables such as housing (PERMANOVA = 1.29, $p = .083$) and ICU venue (PERMANOVA = 1.22, $p = .081$) could also shape the IM to a lesser extent. An extra matched case–control analysis was done to try replicating the above patterns

Table 1. Demographic, biochemical, and clinical evaluation of ICU patients.

Variable	Non-sepsis group N = 83[1]	Sepsis group N=72[1]	p-value[2]
Sex	Men = 51 (61.4%) Women = 32 (38.6%)	Men = 32 (44.4%) Women = 40 (55.6%)	0.051
Age	52.1 ± 18.6 Q1 = 39.5 - Q3 = 68.0	60.7 ± 18.8 Q1 = 50.7 - Q3 = 76.0	0.003
BMI	25.1 ± 3.9 Q1 = 22.1 - Q3 = 28.2	24.4 ± 4.0 Q1 = 22.4 - Q3 = 27.0	0.259
Housing	Rural = 20 (24.1%) Urban = 61 (73.5%) NA = 2 (2.4%)	Rural = 26 (36.1%) Urban = 46 (63.9%)	0.174
CRP[3]	11.5 ± 10.7 Q1 = 2.6 - Q3 = 17.9	19.2 ± 14.5 Q1 = 7.7 - Q3 = 26.4	< 0.001
Glucose[4]	9.33 ± 4.72 Q1 = 6.72 - Q3 = 10.18	10.11 ± 4.49 Q1 = 7.28 - Q3 = 11.72	0.081
SOFA	1.0 ± 0.0 Q1 = 1.0 - Q3 = 1.0	7.6 ± 3.9 Q1 = 5.0 - Q3 = 9.2	< 0.001
APACHE	16.0 ± 8.7 Q1 = 11.0 - Q3 = 19.8	20.5 ± 7.6 Q1 = 15.5 - Q3 = 25.0	< 0.001
ICU stay[5]	6.0 ± 8.7 Q1 = 4.0 - Q3 =12.0	10.0 ± 10.7 Q1 = 6.0 - Q3 = 18.0	0.004
Medical complications	Yes = 68 No = 15	Yes = 60 No = 12	1.000
ICU discharge	Alive = 62 (74.7%) Dead = 21 (25.3%)	Alive = 53 (73.6%) Dead = 19 (26.4%)	1.000

1 For continuous variables, the mean ± standard deviation (sd) is shown, as well as the interquartile distribution.
2 *p*-values reported from different statistical tests according to the nature of data (categorical or continuous variables normally or non-normally distributed – see methods).
3 Values reported as mg/L.
4 Values reported as mmol/L.
5 Values reported as days.
NA = not available.

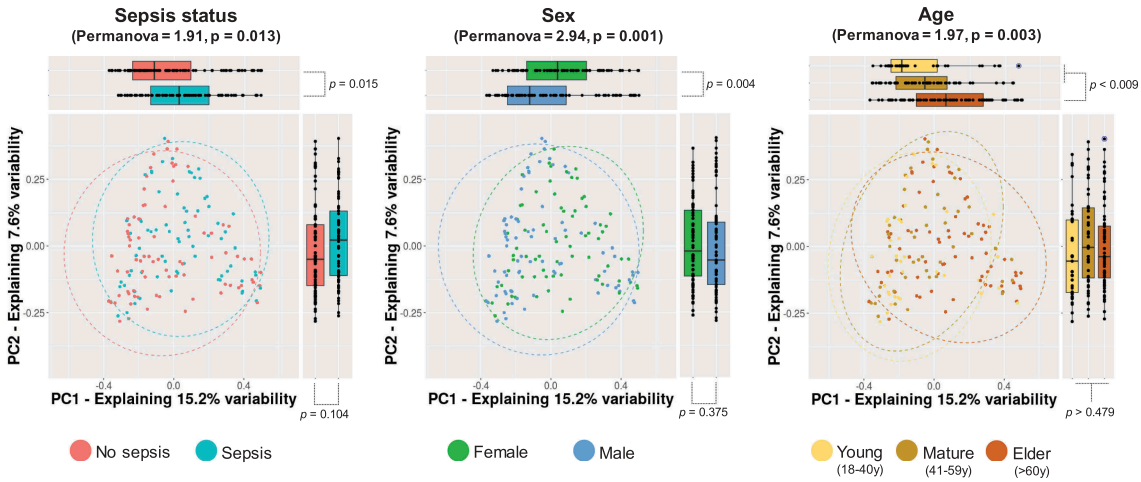

Figure 1. Microbial community structure of the study groups. Principal coordinate analysis (PCoA) of multidimensional data is drawn to display changes in microbial communities according to major variables retrieved to shape the IM, sepsis status, sex, and age. The x- and y-axes represent the two most informative principal coordinates (PCs) of the PCoA, and marginal boxplots describe the distribution of those values for the different groups. Color legends represent the respective variables under analysis. Blue-shaded points show outliers. A pairwise Wilcoxon rank-sum test was used to compare PC1 or PC2 values between groups, and *p*-values are shown beside marginal boxplots. The results of the permutation-based test (PERMANOVA) to compare dissimilarity indexes among samples are shown on top of plots accordingly.

when comparing homogenous groups (N = 140) in terms of age (p = .243) and sex distributions (p = .061). Consequently, we retained significant microbiota structures only when discriminated by sex (PERMANOVA = 2.18, p = .002) and age (PERMANOVA = 1.81, p = .006). Conversely, no

differential IM structure was distinguished in M1 samples by sepsis status (p = .098).

The application of an LMM for the integration of demographic, biochemical, and clinical records with microbiota data produced results similar to those from beta-diversity analysis, thus indicating that age and sex were essential covariates of the IM in the study subjects. Furthermore, we identified several OTUs tightly associated with other variables in the metadata (p < .01) (Table 2). For instance, housing, SOFA score, APACHE score, and ICU venue exhibited a strong association with a large number of the most abundant OTUs explored (Table 2). Minor associations with the IM were found for variables such as ICU stay, ICU discharge, BMI, CRP level, breastfeeding status during infancy, and pet ownership. No associations with medical complications or hospital-acquired infections were detected. A Venn analysis was used to discern overlapping OTUs influenced (according to the LMM results) by related clinical variables such as sepsis status, CRP level, SOFA score, and APACHE score. We identified a high specificity, indicating that OTUs are almost strictly

Table 2. Interaction among the most prevalent OTUs and recorded variables.

Variable	Groups[1]	Associated OTUs	Influence on associated OTUs[2]	Top OTUs associated[3]	p-value
Age	NA	45	4.41	OTU1: g_*Escherichia/Shigella* (1.28)	0.002
			(2.51/0.57)	OTU20: g_*Porphyromonas* (-1.76)	< 0.001
Sex	2	43	4.13	OTU63: g_*Sneathia* (0.48 for Women)	< 0.001
	(**Men**/Women)		(2.53/0.61)	OTU88: g_*Dialister* (-0.51 for Women)	< 0.001
Sepsis	2	27	4.43	OTU19: s_*Parabacteroides distasonis* (0.44 for Sepsis)	< 0.001
	(**No sepsis**/Sepsis)		(1.86/0.42)	OTU3: g_*Ezakiella* (-0.44 for Sepsis)	0.008
SOFA	NA	25	4.30	OTU19: s_*Parabacteroides distasonis* (0.67)	< 0.001
			(1.75/0.41)	OTU104: g_*Megasphaera* (-0.54)	0.002
APACHE	NA	21	6.61	OTU1: g_*Escherichia/Shigella* (0.87)	0.005
			(2.18/0.33)	OTU3: g_*Ezakiella* (-0.99)	0.007
Housing	2	19	6.25	OTU142: g_*Peptoniphilus* (-0.48 for Urban)	< 0.001
	(**Rural**/Urban)		(2.38/0.38)		
ICU stay	NA	8	5.27	OTU113: f_Christensenellaceae (0.36)	0.007
			(1.25/0.24)	OTU87: g_*Dialister* (-0.57)	< 0.001
CRP	NA	5	11.17	OTU313: g_*Cloacibacillus* (0.17)	0.007
			(2.35/0.21)	OTU121: f_Lachnospiraceae (-0.47)	< 0.001
ICU discharge	2	4	11.26	OTU108: g_*Parvimonas* (-0.35 for Dead)	0.007
	(Alive/Dead)		(2.24/0.20)		
BMI	NA	3	2.49	OTU261: g_*Bacteroides* (1.11)	0.009
			(0.44/0.18)	OTU471: s_*Bacteroides tethaiotaomicron* (-0.94)	0.009
Breastfeeding	2	3	5.23	OTU345: g_*Escherichia/Shigella* (-0.43 for "Yes")	0.008
	(**No**/Yes)		(1.06/0.20)		
Pets	2	1	6.66	OTU325: s_*Eubacterium coprostanoligenes* (0.20 for Yes)	0.009
	(**No**/Yes)		(1.03/0.15)		

1 When categorical variables were analyzed, the reference group for comparison is underlined. NA, not available.

2 Ratio obtained from the averaged explained variability of associated OTUs over the averaged explained variability of all OTUs analyzed. Percentages are shown between parentheses.

3 The taxonomy and variance (log-scale) associated with OTUs showing the extreme positive and negative associations with the variable are disclosed. The taxonomy assignment was assessed with SINA aligner and the SILVA database. Only reliable identifications based on the last-common-ancestor (lca) approach are presented disclosing family (f_), genus (g_), or species (s_) assignments.

associated with each variable except for sepsis status and SOFA comparison, for which 19 OTUs were found to be simultaneously influenced by both variables. Such a relationship was expected because of the dependence of sepsis diagnosis on SOFA scoring.

Finally, we attempted to discern a specific IM signature associated with sepsis status by removing the potential effect of all latent confounding variables (see methods). Consequently, after performing a new LMM analysis including significant covariates as random effects, we could retrieve a set of 24 OTUs specifically associated with the sepsis condition, and 7 out of them (OTU3 = *Ezakiella* spp., OTU104 = *Megasphaera* spp., OTU276 = *Allisonella* spp., OTU394 = unclassified, OTU453 = *Peptoniphilus* spp., OTU458 = *Prevotella* spp., OTU523 = *Prevotella copri*) were found to be in a lower proportion in samples from patients with sepsis than in those from patients without sepsis. Figure 2 shows the OTUs with extreme variance between groups (4 associated with sepsis and 2 with no sepsis). Moreover, we also identified that *Fusobacterium* species (OTU48, variance = 0.29, p = .003) were associated with ICU patients with sepsis, whereas *Prevotella* species (OTU458 and OTU523, variance = −0.27, $p < .001$) seemed to be more abundant in ICU patients without sepsis.

Short-term microbiota evolution in sepsis patients during ICU stays

A complementary LMM-based analysis was conducted to survey the evolution of the IM during an ICU stay. A total of 44 sepsis patients with 135 longitudinal samples (M1 = 44 samples and M2 = 91 samples) were evaluated to discern potential changes in the diversity of samples across the ICU stay and to detect covariates in the metadata recorded during the stay. The alpha diversity assessment strongly suggested that diversity profoundly declined in M2 samples from that observed in M1 specimens (Figure 3). A progressive pattern of microbial species loss was observed when observed OTUs and Chao's index were evaluated, reaching a decline of 850 phylotypes in M2.7 samples (Figure 3(a)). We calculated an average loss of 278 phylotypes from the 692 estimated average for M1 samples. A predictive death model based on logistic regression using the delta-observed richness (M2 minus M1 richness) indicated no exacerbated loss of microbial richness in patients that were dead upon ICU discharge.

In contrast, the main variables likely to shape the IM during follow-up were age, with 49 OTUs associated; the antibiotic treatment (grouped into cephalosporins, glycopeptides, macrolides, oxazolidinones, penicillins, and carbapenems), with 24 different OTUs associated; and the caloric intake reached by nutritional support, with 17 different OTUs associated (Table 3). The impact of sex seemed to influence the IM to a lesser extent in the longitudinal cohort.

We found only three OTUs associated with the ICU discharge variable ($p \leq 0.01$), whose association was retained after expanding the set of random variables in the LMM model to those that exhibited a large influence on microbiota data in either the initial assessment or the longitudinal assessment (see methods). Consequently, *Enterococcus* (OTU45, variance = 0.63, p = .006 and OTU46, variance = 059, p = .008) and *Salmonella* (OTU110, variance = 0.41, p = .010) species were strongly associated with death as result for ICU discharge. Figure 4(a) shows the abundance of OTU45 and OTU46 in samples across the ICU stay and shows a higher abundance and prevalence of such OTUs in samples of patients with sepsis who died than in samples of patients with sepsis who lived. Logistic regression was used to build a potential predictive model of death in the ICU based on changes associated with the abundance of *Enterococcus* species (OTU45 and OTU46). We observed that changes in the abundance of OTU46 better predicted the outcome than changes in OTU45 abundance, suggesting that an increase of one logarithmic unit in the abundance of this phylotype leads to an increase of 3.14-fold in the probability of death in the ICU by sepsis (p = .029) (Figure 4(b)).

Alternatively, we used the abundance changes of OTU110 as well as of OTU19 and OTU68, the last two exhibited extreme associations with sepsis status (Figure 2), to test alternative models, but their

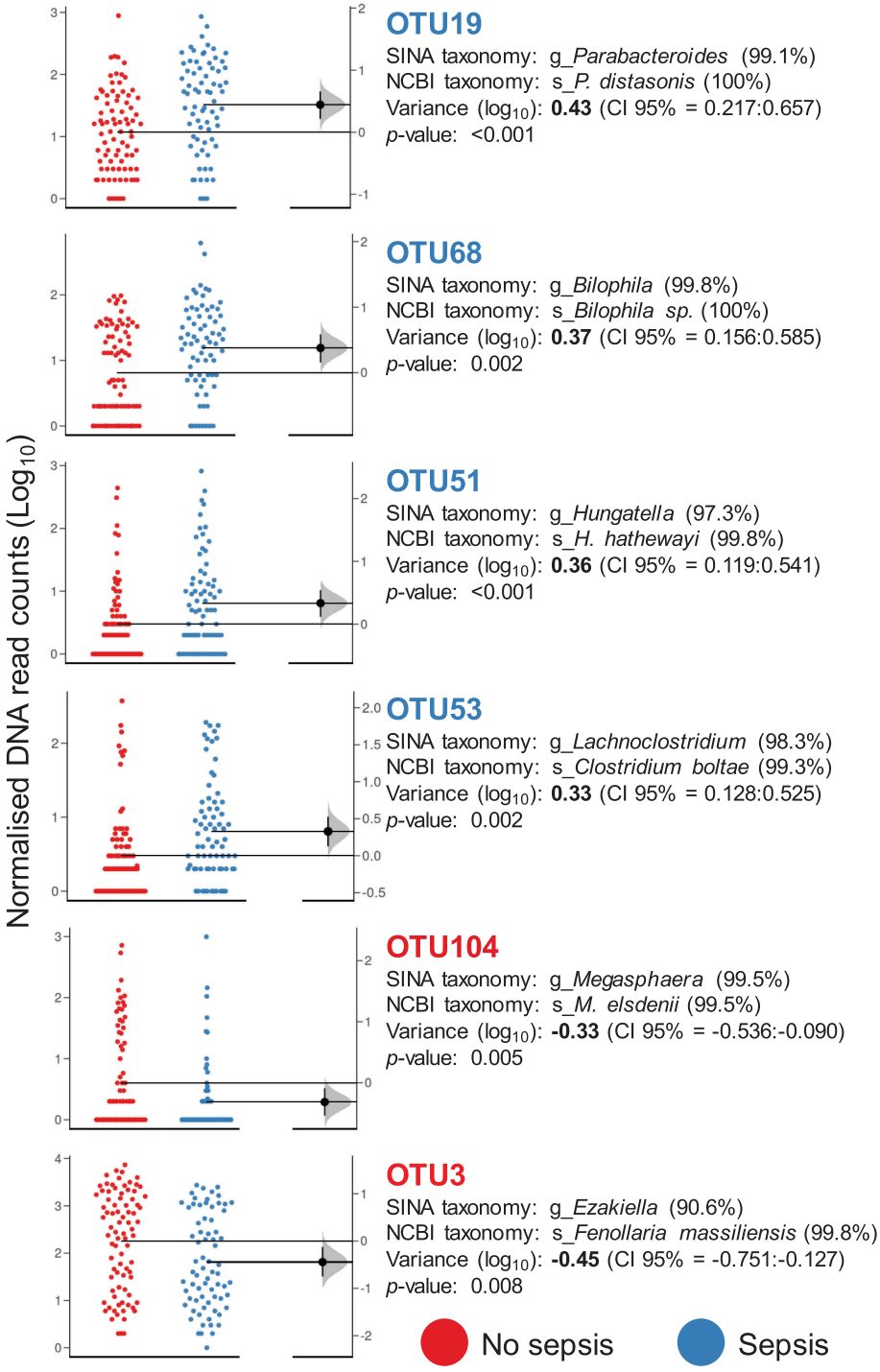

Figure 2. OTUs associated with the sepsis condition. Gardner–Altman estimation plots showing the distribution of the number of rarefied DNA reads obtained for OTUs with extreme variation between groups. In all cases, the variance is reported on a log scale and is referred to as observed in the "No sepsis" group ($p \leq 0.01$) and accompanied by confidence intervals (CI 95%). The color legend represents the primary variable of the study, sepsis status. SINA aligner (https://www.arb-silva.de/aligner/) with the SILVA database and a Blast-based search against the non-redundant NCBI 16S database (https://blast.ncbi.nlm.nih.gov/Blast.cgi?) were used as methods to disclose the taxonomy of selected OTUs. The sequence identity percentage is shown within parentheses. The distribution of unpaired mean differences between groups (based on 5000 replicates) is shown on the right of the respective Gardner–Altman plots.

longitudinal variation fitted poorly to a predictive model (OR = 1.12, 1.47 and 0.89; AIC = 40.4, 44.9 and 45.7; and p = .053, 0.362 and 0.835, respectively). We observed no differences between the ICU time, medical complications, and ICU discharge criteria in this longitudinal cohort.

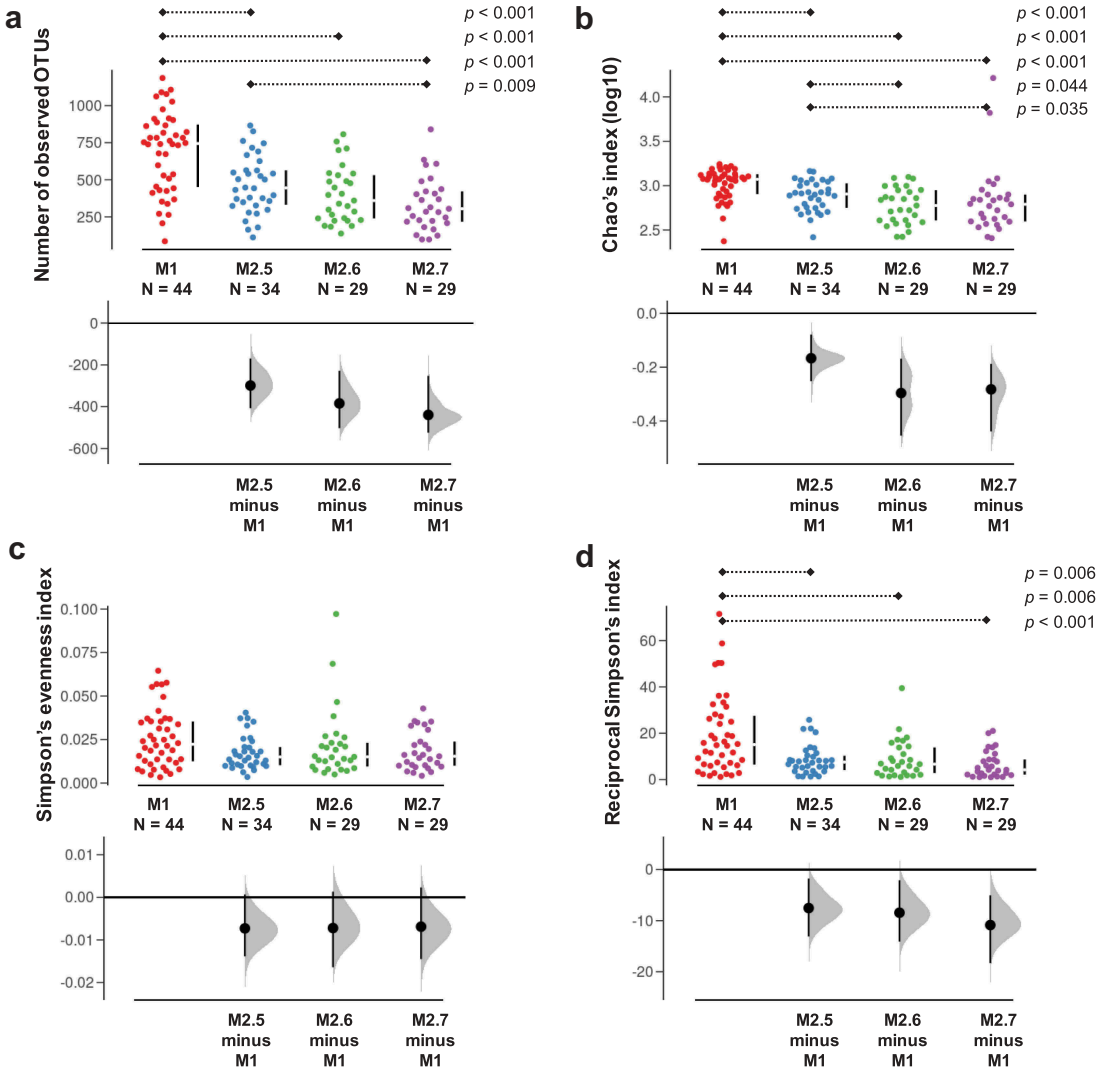

Figure 3. Alpha diversity analysis of longitudinal samples from the sepsis groups. The observed OTUs (a), Chao's index (b), Simpson's evenness (c), and Simpson's reciprocal index (d) were assessed across the M1, M2.5, M2.6, and M2.7 samples. Statistical assessment was carried out with the pairwise Wilcoxon rank-sum test for unpaired samples with the *post hoc* Benjamini–Hochberg method for multiple testing correction, and *p*-values derived from respective tests are depicted on top of Gardner–Altman estimation plots accordingly ($p \leq 0.05$). Distributions at the bottom of the plots show the unpaired median difference based on 5000 replicates.

Discussion

Critically illness promotes severe physiology alterations in patients, and it has been reported to drive drastic changes in the IM composition.[8] Currently, the IM is indicated as a key player in maintaining metabolic and immune homeostasis in humans, which indicates that any alteration during critical illness regarding the proper balance of species inhabiting the human gut could have devasting effects on ICU patient progression.[18] In the present study, we found no apparent differences in terms of alpha diversity descriptors of the IM between patients with and without sepsis upon ICU admission.

Unlike alpha diversity, beta diversity evaluation suggested that critically ill patients with sepsis have an intestinal microbial community structure that is distinct from that of those without sepsis ($p = .013$). Nevertheless, we found that variables such as age and sex definitively influence the IM to a larger extent than the sepsis condition itself (Figure 1 and Table 2), this last discarded to modulate, in a specific manner, the IM structure as a whole according to the matched control-case analysis.

Table 3. Covariates influencing the IM during follow-up of sepsis patients.

Variable	Groups[1]	Associated OTUs	Influence on associated OTUs[2]	Top OTUs associated[3]	p-value
Age	NA	49	4.54	OTU2: g_*Klebsiella* (2.08)	0.004
			(5.10/1.12)	OTU10: g_*Prevotella* (-2.97)	< 0.001
Antibiotic treatment	6 (**Carbapenems**, Cephalosporins, Glycopeptides, Macrolides, Oxazolidinones, Penicillins)	24	2.38	OTU26: g_*Pseudomonas* (2.24 for Oxazolidinones)	< 0.001
			(4.62[4]/1.94)	OTU122: g_*Sutterella* (-0.93 for Cephalosporins)	0.004
% caloric intake	NA	17	3.59 (1.46/0.41)	OTU150: g_*Megasphaera* (-0.50)	0.003
Nutritional support	2 (**Enteral**, Parenteral)	7	8.23 (3.99/0.49)	OTU8: s_*Bacteroides fragilis* (0.95 for Parenteral)	0.003
ICU time	NA	4	4.15	OTU92: f_Christensenellaceae (0.82)	0.006
			(1.57/0.38)	OTU406: g_*Sutterella* (-0.52)	0.008
ICU discharge	2 (**Alive**, Dead)	3	12.93 (4.91/0.38)	OTU45: g_*Enterococcus* (0.63 for Dead)	0.006
Sex	2 (**Men**/Women)	2	8.39	OTU1070: g_*Fusobacterium* (0.26 for Women)	0.008
			(4.02/0.48)	OTU28: g_*Finegoldia* (-0.48 for Women)	0.004

1 When categorical variables were analyzed, the reference group for comparison is underlined. NA, not available.
2 Ratio obtained from the averaged explained variability of associated OTUs over the averaged explained variability of all OTUs analyzed. Percentages are shown between parentheses.
3 The taxonomy and variance (log-scale) associated with OTUs showing the extreme positive and negative associations with the variable are disclosed when available. The taxonomy assignment was assessed with SINA aligner and the SILVA database. Only reliable identifications based on the last-common-ancestor (lca) approach are presented disclosing family (f_), genus (g_), or species (s_) assignments.
4 Average obtained from different groups when samples were categorized in more than 2.

The overlapping distribution in IM profiles among samples of sepsis patients, women, and elderly people in the PCoA (Figure 1) suggests that, for this Colombian population, the women older than 60 years condition would represent an elevated risk of developing sepsis after ICU admission.

Our results are consistent with previous findings of different IM surveys in elderly individuals that outlined a drastic change in their IM as a consequence of the intestinal physiology deterioration, poor diet, and permanent medication.[19,20] Therefore, a loss of microbial diversity and change in the microbiome functionality is frequently associated with age,[21,22] making this population more disease prone. However, a disparate IM between men and women has been previously reported, as well as its covariation with BMI.[23,24] Interestingly, this IM dissimilarity between men and women is proposed to underlie the gender-specific response to dietary interventions[25] and to potentially modulate disease onset.[26] Consequently, our data indicate that the IM of women could also be involved in sepsis predisposition in ICU patients. In any case, the co-variability of sex and age with the IM detected in our study reinforces the idea to take into account such information in the context of association-based research involving human microbiota data, with the aim to minimize the confounding factors generating spurious associations.[27]

After proper control of several covariates found to influence the IM of ICU patients, we were able to isolate a set of 24 OTUs with differential abundance between sepsis and non-sepsis subjects upon ICU admission. Nineteen of them were associated with sepsis samples, including OTU19 (*Parabacteroides distasonis*) and OTU68 (*Bilophila* spp.), which were the phylotypes exhibiting the most extreme association with sepsis samples (Figure 2). Whereas *P. distasonis* has been associated with endotoxin production, increased risk of mortality, antibiotic resistance,[28] and surface antigen-mediated attenuation of the immune response,[29] the genotoxic sulfide-producing *Bilophila* species are consistently associated

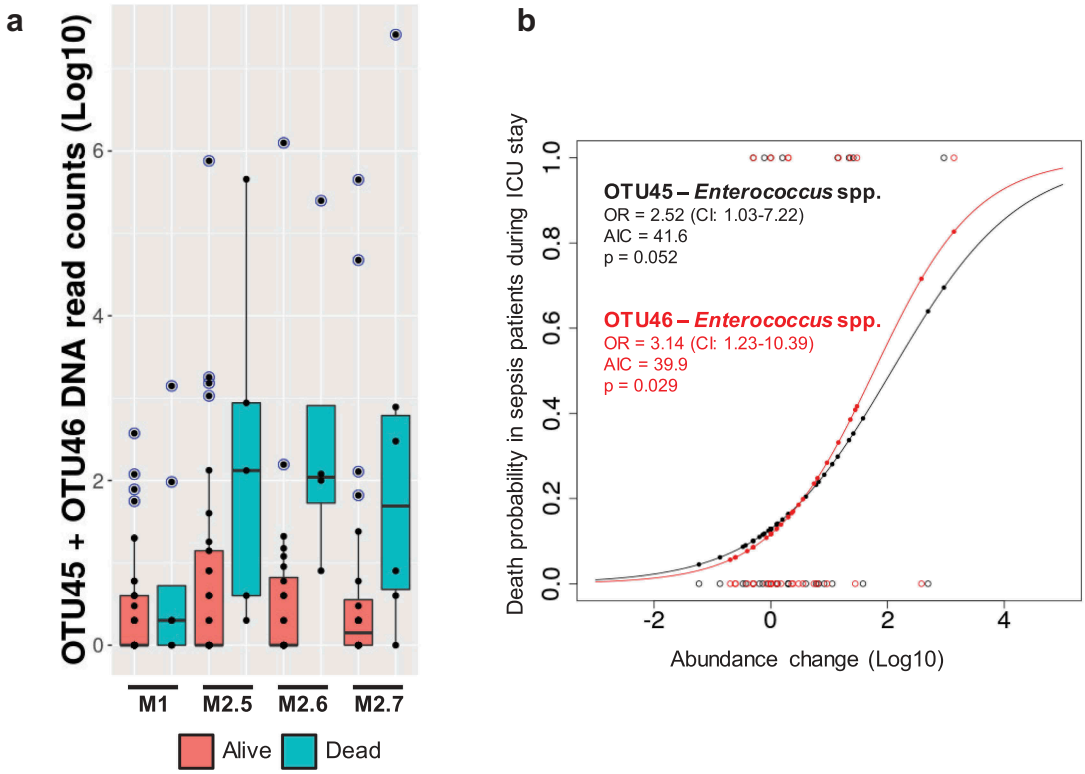

Figure 4. OTUs associated with death. A – The normalized DNA read counts (log10) for OTU45 and OTU46 are depicted in a boxplot manner for sepsis samples across the time (M1 to M2.7) of stay in the ICU. The color legend discriminates the samples from patients with ICU discharge as "alive" and "dead". Blue-shaded points indicate outliers. B – A logistic regression with data regarding OTUs potentially related to death of ICU patients with sepsis, based on their abundance changes across the ICU stay. Abundance changes were calculated as log[average(M2 samples)] – log[M1 samples]. The OTU information and the associated taxonomy as well as main parameters retrieved after logistic regression, such as the odds ratio (OR), Akaike information criterion (AIC), and *p*-values, are shown inside the plot.

with inflammation across studies.[30–32] Furthermore, the higher abundance of *Fusobacterium* species (OTU48) in sepsis samples was already reported for ICU patients with septic shock.[33] Accordingly, taking into account the association of *Bilophila* spp. and *Fusobacterium* spp. with the onset and progression of colorectal cancer,[34–36] we found that the IM of ICU patients with sepsis was enriched in harmful microbial species that would magnify the disruption of metabolic and immune homeostasis in these critically ill patients. Conversely, we found that 3 out of the 7 OTUs associated with non-sepsis samples were microbial species that are abundant in non-Westernized populations with traditional lifestyles. Therefore, the increased abundance of *Ezakiella*, the butyrate producer *Megasphaera*[37] (Figure 2) and *Prevotella* (and particularly *P. copri*) species could confer a protective role against sepsis. Consequently, strategies of plant-based enteral nutrition could raise the proportion of beneficial microbial species associated with complex carbohydrate metabolism in the IM of critically ill patients, thus ameliorating the intestinal barrier and function as a whole, reducing the risk of septic shock and improving prognosis during the ICU stay.[38]

We followed up a total of 44 out of the 72 sepsis patients (135 samples in sum) to determine the short-term evolution of their IM and its main shaping factors during the ICU stay. Strikingly, our follow-up survey in sepsis patients replicated previous results indicating a substantial loss of microbial diversity during ICU stay (Figure 3).[14] As a result, while being aware of the controversy on the definition,[39] we observed a clear dysbiosis understood as a drastic reduction in the proportion of microbial species along the time between samples from the same donor. Although we observed no acute diversity depletion as Zaborin and coworkers described, we did detect loss of up to 850 phylotypes (~71% of the initial observed

OTUs for a specific patient) after a maximum 7-day ICU stay and an average loss of 278 (~40%) phylotypes of the 692 observed for paired M1 samples on average. This loss of microbial diversity is expected given that the IM of sepsis patients is exposed to adverse effects, such as antibiotic administration, and drastic changes in dietary patterns during lengthened ICU stays. Globally, such a reduction in the effective number of microbial species in the ICU patient intestine is thought to break the equilibrium among species, reducing the number of beneficial commensals and promoting the arrival/proliferation of harmful and pathogenic bacteria.[40–42] In this regard, some reports have previously suggested the use of variations in the intestinal microbial diversity and its longitudinal variation as a biomarker for the prognosis of ICU patients.[43]

The data presented here strengthen the idea that a dramatic decrease in microbial diversity precedes the arrival of harmful bacteria in ICU patients. In our case, we detected the loss of microbial species, such as *Megasphaera* spp., *Prevotella* spp. and *Ezakiella* spp., in sepsis patients, gut microbes associated with a traditional diet, metabolism of complex carbohydrates, and being SCFAs producers. Notwithstanding, we disclosed unprecedently that such conditions would constitute a hallmark for the proliferation of pathogenic bacteria, such as *Parabacteroides distasonis, Bilophila* spp., *Fusobacterium* spp., and *Enterococcus* spp., and that the increased abundance over time would constitute a serious risk of mortality for ICU patients with sepsis (Figure 4).

Among the different variables explored to influence the IM of sepsis patients, we again found that age is the main variable to determine the evolution of the IM during an ICU stay (Table 3). The above finding was evidenced by retrieving a total of 49 different OTUs influenced by this variable ($p < .01$). We found a unique OTU associated positively with age, which indicates that *Klebsiella* (OTU2) species become more abundant as patients increase in age. In contrast, the abundance of species associated with complex carbohydrate metabolism and traditional diets, such as *Prevotella* spp.[44,45] (OTU10), were found to be less abundant in older patients than in younger patients. As expected, antibiotic treatment also seemed to exert an important influence in the IM of sepsis patients, and we found a total of 24 OTUs differentially associated with the seven classes of antibiotics recorded. Surprisingly, a higher abundance of *Pseudomonas* spp. (OTU26) and *Klebsiella* (OTU2) was correlated with the administration of oxazolidinones and glycopeptides, respectively. This result suggests the possibility of acquisition of respective resistant and pathogenic strains during treatment in the ICU. Contrary to that observed in the assessment of M1 samples, sex had a lesser extent impact on the IM in the follow-up data (Table 3). Nonetheless, *Fusobacterium* spp. (OTU1070) abundance was positively associated with women, thus emphasizing the risk of such condition to develop sepsis in the ICU in our study cohort.

Similarly, we could associate microbiota evolution with the main variables that were explored in the present study. Consequently, we retrieved particular associations between the microbiota and ICU stay time as well as between the microbiota and mortality, but no associations were observed for the microbiota with medical complications during an ICU stay. Remarkably, after controlling all possible covariates detected to influence the microbiota in the follow-up data, we retained the 3 OTUs associated with mortality, and two of them matched with *Enterococcus* species (OTU45 and OTU46) (Figure 4). An increased abundance of vancomycin-resistant *Enterococcus* (VRE) has been previously reported in critically ill patients when prior depletion of SCFA-producing bacteria occurs.[46] Indeed, the reduction in the concentration of butyrate, an effector molecule mediating anti-inflammatory signals and glucose and lipid metabolism, in the intestine has been specifically associated with the arrival of *Enterococcus* species.[10]

Enterococcus species have been reported to be recurrent members of the human IM, but they are also well-known nosocomial pathogens associated with urinary tract infections, bacteremia, and infectious endocarditis with elevated rates of mortality.[47] Although our study did not have the resolution to determine variability at the species or strain level, therefore failing to unveil if the enterococci boost was of endogenous or acquired origin, we did detect a progressive and significant increase in *Enterococcus* species abundance in the

intestine of ICU patients with sepsis who died, making it feasible to use it as a predictive biomarker, as previously stated.[42] Moreover, we could also infer some resistance patterns for such microbes because all deceased patients with sepsis (N = 8) were treated with beta-lactam antibiotics; 3 of them were additionally treated with glycopeptides (vancomycin), and 4 of them were additionally treated with macrolides or lincosamides. Accordingly, future studies should be conducted to profile the precise identity and resistance traits of the *Enterococcus* species exhibiting progressive growth in the intestine of ICU patients with sepsis.

Conclusions

Exhaustive metadata recording is essential to outline reliable associations and minimize the impact of confounding factors in human-associated microbiota assessments. Our multiple covariate-controlled multi-center study indicated that the IM in ICU patients could be informative to determine their progression during an ICU stay, as well as for calculating the risk for sepsis and mortality. We concluded that upon ICU admission, a microbiota evaluation could help to predict the risk of sepsis and mortality by measuring the abundance of certain harmful gut microbes. We observed that such a pathobiont-prone profile in sepsis ICU patients would worsen immune and metabolic homeostasis and exacerbate health deterioration. Overall, we concluded that declining IM diversity during an ICU stay deeply alters the delicate microbial equilibrium in critically ill patients and allows the emergence and proliferation of specific pathogens, aggravating their already weakened condition, even resulting in death. Finally, we encourage health-care institutions to adopt molecular protocols to follow up the evolution of the IM in critically patients during their ICU stay for monitoring the efficacy of treatments and making prognosis more reliable.

Methods

Study design and subjects

This is a descriptive observational, multi-center study carried out with patients from five hospitals located in Medellín and Rionegro (Colombia, South America). We used samples and data from a cohort of 155 adult patients admitted to the respective ICUs from fourth-level hospitals (the top-level at the Colombian health-care system) namely, *Hospital Pablo Tobón Uribe, Hospital San Vicente Fundación* venues in Medellín and Rionegro, *Hospital General de Medellín* and *Clinica Las Américas*. Patients or their relatives were personally informed of the study with the presence of the responsible clinician and signed informed consent forms. Eligible men and women were at least 18 years old and admitted to the ICU of their respective hospitals. ICU patients with terminal illnesses as well as those undergoing colostomy or ileostomy were excluded. Pregnant women or those with breastfed infants and homeless people were also excluded from this study. The sepsis criterion to discern the groups of study was adopted according to The Third International Consensus Definitions for Sepsis and Septic Shock.[48] During a 6-month window, a total of 83 ICU patients without sepsis and 72 diagnosed with sepsis were included in the study according to inclusion and exclusion criteria. Then, biological samples were obtained, and metadata were recorded to complete this assessment.

Sampling and data recording

Peri-rectal swabs were obtained at ICU admission upon inclusion criteria verification and before nutritional support, using brand-sealed sterile, polystyrene, and RNAse and DNAse free swabs (Deltalab, Barcelona, Spain. Cat# 300252). These rectal swabs were DNA-free certified (sterilized by ethylene oxide) and provided with a polystyrene-made protection tube containing no storage buffer that could alter the microbiota profiles. The peri-rectal swabs were immediately stored at −20°C in the respective hospitals and then transported in dry ice to a DNA extraction laboratory. Samples were stored at −80°C until DNA extraction. The initial sampling moment (M1) was set for all subjects included in the "no-sepsis" and "sepsis" groups. Follow-up sampling was performed for 44 out of the 72 sepsis patients who remained in the ICU until M2 sampling. The M2 samples consisted of peri-rectal swabs obtained on the fifth (M2.5), sixth (M2.6), and/or seventh (M2.7) day in the ICU, whereas the M3 samples consisted of

peri-rectal swabs collected at ICU discharge whenever possible (N = 3). Demographic, biochemical and clinical variables of interest were collected with respective M1 samples as patient-associated metadata, such as age, weight, height, BMI, sex, marital status, housing, pets, glucose, CRP level, sibling number, whether they were breastfed in infancy, ICU venue, sepsis status and the scores obtained from SOFA and APACHE-II assessments. Additional variables associated with the follow-up, such as the type of nutritional support, formula of nutritional support, antibiotic administration, and outcome variables (ICU stay [days], complications in the ICU, discharge condition), were also recorded. The staff from different ICUs collected the samples and information following consensus protocols agreed upon and approved by the clinicians and researchers involved in this study.

Fecal microbiota

The fecal DNA was obtained from peri-rectal swabs by using a QIAamp® DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Total DNA was quantified by UV absorbance methods (NanoDrop, Thermo Scientific, Wilmington, USA) and stored at −20°C until processing. The V3-V4 hypervariable regions of the 16S ribosomal ribonucleic acid (rRNA) gene were amplified using 1 µL of fecal DNA (25 ng on average) and 27 PCR cycles consisting of the following steps: 95°C for 20 sec., 55°C for 20 sec. and 72°C for 20 sec. Phusion High-Fidelity Taq Polymerase (Thermo Scientific, Wilmington, USA) and the barcoded primers S-D-Bact-0341-b-S-17 (TAGCCTACGGGNGGCWGCAG) and S-D-Bact-0785-a-A-21 (ACTGACTACHVGGGT ATCTAATCC) that target a wide variety of bacterial 16S rRNA genes[49] were used for PCR. Dual-barcoded PCR products were purified from triplicate reactions with an Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, UK) and quantified by fluorometric methods (Qubit 3.0 – Thermo Fisher Scientific, Waltham, MA, USA). A total of 250 samples were multiplexed in one sequencing run by combining equimolar quantities of amplicon DNA (~50 ng per sample) and sequenced in one lane of an Illumina MiSeq platform with a 2 × 300 PE configuration (Eurofins Genomics GmbH, Germany). Raw data were delivered in fastq files, and paired ends with quality filtering were assembled using *FLASH* software.[50] Sample de-multiplexing was carried out using sequence information from the respective DNA barcodes and *MOTHUR* v1.39.5 suite of analysis.[51] After assembly and barcode and primer removal, the sequences were processed for chimera detection and removal using the *UCHIME* algorithm[52] and the *SILVA* reference set of 16S sequences (release 128).[53] A rarefied subset of 12,000 sequences per sample was randomly selected after multiple shuffling (10,000X) from the original dataset for downstream analyses. Operational taxonomic unit (OTU) counts were retrieved by using this rarefied set of sequences and the *UCLUST* algorithm (clustered at 97% sequence identity) implemented in *USEARCH* v8.0.1623.[54] Common alpha diversity descriptors, including the observed OTUs, Chao's richness, Simpson's evenness, and Simpson's reciprocal index, were computed using *QIIME* v1.9.1[55] and the OTU abundance information. The beta diversity was also assessed with the respective algorithms implemented in *QIIME* v1.9.1, and evaluation of the community structure across the sample groups was assisted by principal coordinate analysis (PCoA) and Bray–Curtis dissimilarity indexes retrieved from sample pairwise comparisons.

Statistical analysis

Descriptive statistics were applied to assess compositional differences between groups regarding demographic, biochemical and clinical variables. The Shapiro–Wilk test was used to measure normality of the explored continuous variables before comparison using parametric (t-test) or non-parametric (Wilcoxon rank-sum test) methods. Similar approaches were adjusted for pairwise comparison of alpha diversity descriptors. Group differences in the distribution of categorical variables were analyzed using Pearson's chi-squared test. Permutation-based methods (PERMANOVA) were applied to measure changes in microbial communities associated with variables recorded as metadata.

A matched case–control approach was additionally executed to detect the impact of gut microbiota on sepsis condition by homogenizing sepsis and no-sepsis groups in terms of sex and age variables. Proper selection of controls (no sepsis) was carried out using the *matchControls* function of the e01071 *R* package. A total of 140 samples (70 sepsis and 70 no-sepsis samples) were included in this re-analysis. A linear mixed model (LMM – *nlme R* package) was used to measure the association of log-transformed OTU data, with individual variables recorded in the metadata. This analysis was performed on the most abundant OTUs (N = 377 with an average abundance >0.02%), accounting for 84% of the full diversity observed in the sample cohort. Idiosyncratic variation due to individual differences was set as a random effect for each variable analyzed (fixed effect). Covariates of the fecal microbiota were identified upon differential distribution of variables among groups supported by a p-value ≤0.01. OTUs specifically associated with the sepsis condition were retrieved by including age, sex, BMI, ICU venue (hospital center), APACHE, and housing as random effects in the LMM, in addition to individual variation. OTUs categorically associated with the ICU discharge condition were retrieved by including age, sex, BMI, caloric intake depending on nutritional support, ICU venue (hospital center), antibiotic treatment, and nutritional support as random effects in the LMM, in addition to individual variation. To establish possible relationships between changes in the abundance of OTUs and ICU discharge condition (alive or dead), a logistic regression model was applied, using the *glm* [family = "binomial"(link = "logit"))] function of R v3.5; ICU discharge condition was used as a binary outcome (1 = alive and 0 = dead) and changes in the relative abundance of different OTUs (abundance change = Log_{10} [average of normalized reads of M2 samples] – Log_{10} [normalized reads of M1 samples]) as an explanatory variable. Graphics (*ggplot2* and *dabestr* packages for boxplots and Garner–Altman estimation plots, respectively) and statistics were generated in *R* v3.5 (https://cran.r-project.org/).

List of abbreviations

AIC	Akaike information criterion
APACHE	acute physiologic assessment and chronic health evaluation
BMI	body mass index
CRP	c-reactive protein
DNA	deoxyribonucleic acid
FMT	faecal microbiota transplantation
ICU	intensive care unit
IM	intestinal microbiota
LMM	linear mixed model
OTU	operational taxonomic unit
OR	odds ratio
PC	principal coordinate
PCoA	principal coordinate analysis
PCR	polymerase chain reaction
RNA	ribonucleic acid
SDD	selective decontamination of the digestive tract
SOFA	sequential organ failure assessment.

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Authors' contributions

GMAO, BEVD, NAGG, and ABP designed the study, conducted the research, performed the experimental analyses, and managed and obtained the resources to carry out the assessment. ABP analyzed and interpreted the molecular data. AMJR, AGV, IAC, MAYM, and JBB selected patients and coordinated sampling, storage, and transport of the biological samples. ABP wrote the manuscript. ABP and GMAO have primary responsibility for the final content. All authors helped in manuscript preparation and read and discussed the results and conclusions included. All authors read and approved the final version of the manuscript.

Competing interests

The authors declare that they have no competing interests.

Data availability and material

The raw fastq sequences generated from the 16S amplicon sequencing of fecal DNA are publicly available at the

European Nucleotide Archive (ENA)[56] via the project number PRJEB33360.

Ethics approval and consent to participate

This study was conducted according to the guidelines laid down in the Declaration of Helsinki and was carried out in accordance with the principles of the Belmont Report and with the approval of the ethics committees from the University of Antioquia (Act 03 of 2015), Hospital Pablo Tobón Uribe, Hospital San Vicente Fundación, and Clínica Las Américas.

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