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Sepsis-Associated Metabolites and Their Biotransformation by Intestinal Microbiota

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Summary

High serum levels of microbial metabolites of aromatic amino acids (AMM) stands as a prognostically unfavorable factor, indicating the progression of multiple organ dysfunction and an increased risk of death in patients with sepsis and septic shock. This study is based on a hypothesis that excess of sepsis-associated AMM in patients with sepsis is caused by metabolic alterations (dysfunction) in the intestinal microbiota.

The aim of this study was to compare the potential of normobiota and pathobiota to bio-transform sepsis-associated metabolites of aromatic amino acids tyrosine and phenylalanine, such as phenyllactic acid (PLA) and 4-hydroxyphenyllactic acid (4-HPLA).

Materials and methods. Samples of intestinal contents of patients with septic shock (N=10, pathobiota) and healthy volunteers (N=9, normobiota) were placed in test tubes with the omnipurpose thioglycol medium. The clinical model of excessive inflow of sepsis-associated AMM into the intestine (for example, from blood or sites of inflammation) was reproduced in the *in vitro* experiment by adding PLA or 4-HPLA in clinically significant concentrations (25 μM) into each test tube with pathobiota and normobiota. After incubation in a thermostat (37°, 24 hours), AMM concentrations were measured in the samples with pathobiota and normobiota using GC-MS analysis.

Results. Concentration of AMM decreased within 24 hours in the tubes with normobiota after PLA or 4-HPLA were added. In the tubes with pathobiota, no decrease in AMM concentrations was documented after loading with PLA or 4-HPLA. Concentrations of PLA (P=0.002) and 4-HPLA (P<0.001) were statistically significantly higher in pathobiota samples compared to normobiota.

Conclusion. The *in vitro* experiment demonstrates that after excessive load with sepsis-associated metabolites (PLA, 4-HPLA), the microbiota of healthy people is capable to bio-transform such metabolites to the end products of microbial metabolism, while pathobiota of septic patients exhibits altered biotransformational potential. This data demonstrate that microbiota dysfunction may contribute to the pathogenesis of sepsis.

Keywords: sepsis; pathobiota; aromatic microbial metabolites; phenyllactic acid; 4-hydroxyphenyllactic acid; phenylpropionic acid; microbiota; biomarkers; aromatic amino acid metabolites

Conflict of interest. The authors declare no conflict of interest.

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Introduction

Sepsis remains a continuing challenge of the 21st century, and intensive research into new pathogenetic therapies to reduce its incidence and mortality is ongoing [1–4]. Contemporary studies revealing the impact of the gut microbiota to critical illness and sepsis development attract special attention [5–7]. In patients with sepsis, severe disturbances in the taxonomic composition of the intestinal microbiota, increased permeability of the intestinal wall, changes in immunoreactivity, and the development of antibiotic resistance in microorganisms are observed, all of which lead to metabolomic perturbations [8, 9]. For example, significant accumulation of metabolites of aromatic amino acids phenylalanine and tyrosine in blood serum has been found in patients with sepsis of various etiologies, such as severe pneumonia, abdominal diseases, postoperative complications of cardiac surgery, etc. [6, 10]. High serum levels of microbial metabolites of aromatic amino acids (Aromatic Microbial Metabolites, AMM [6, 10, 11]) such as phenyllactic acid (PLA), 4-hydroxyphenyllactic acid (4-HPLA), and 4-hydroxyphenylacetic acid (4-HPAA) have been shown to most frequently correlate with disease severity and mortality. This has led to the introduction of the term «sepsis-associated metabolites» for these metabolites [11]. Previous studies have shown that several aromatic metabolites are predominantly of microbial origin [12–14]. Biotransformation of aro-

matic amino acids takes place in the gut, normally hundreds of bacterial species of healthy microbiota feed on metabolic intermediates, and as a result, in a healthy organism, small amounts of the end products of microbial metabolism, mainly phenylpropionic acid (PPA) and phenylacetic acid (PAA), enter the systemic circulation [15]. In sepsis, microbial metabolism of aromatic amino acids occurs both in the gastrointestinal tract and at sites of inflammation. Therefore, the intermediates of metabolism enter the bloodstream in excess, leading to an increase in the levels of circulating sepsis-associated metabolites, PLA, 4-HPLA, and 4-HPAA.

Metabolomic monitoring of patients with sepsis has shown that even after surgical treatment of purulent foci, the high levels of PLA and 4-HPLA often do not decrease, but even increase, which is associated with progression to multiorgan failure and an unfavorable prognosis. We have proposed that this may be due to metabolic dysfunction of the microbiota. When the pool of normal anaerobic bacteria is depleted in a septic patient, aromatic amino acids and their intermediates are not fully biotransformed into end products, contributing to the accumulation of sepsis-associated microbial metabolites in the body.

Our study was based on the idea that an important function of a healthy microbiota consists in utilizing potentially toxic products by biodegrading them to metabolic end products under the conditions of a normal microbial community functioning in the gut of a healthy individual («normobiota»). The working hypothesis of the study was that the microbiota of a septic patient is unable to eliminate the excess of sepsis-associated metabolites that originate from the blood and/or are produced in the intestinal lumen and do not undergo biotransformation. This hypothesis was tested in an in vitro model experiment by culturing the intestinal microbiota with the addition of sepsis-associated metabolites to simulate their excessive entry into the intestine from the blood or from inflammatory foci, with subsequent measurement of metabolites.

Thus, the aim of this study was to compare the ability of normobiota and pathobiotato biotransform sepsis-associated metabolites of the aromatic amino acids tyrosine and phenylalanine using PLA and 4-HPLA as examples.

Materials and Methods

The biomaterial for the model experiment included

- Blood serum from healthy donors (*N*=48) and patients with sepsis (*N*=10);
- Intestinal contents from healthy volunteers (*N*=9) and patients with septic shock (*N*=10).

Sepsis patients. The study included 10 patients (9 men and 1 woman) admitted to the Sklifosovsky Research Institute for Emergency Medicine in December 2022.

Inclusion criteria were:

- age older than 18 and younger than 80 years;
- sepsis;
- septic shock.

Exclusion criterion was terminal condition with life expectancy less than 24 hours.

Patients were admitted with the diagnosis of combined trauma (*N*=6), closed traumatic brain injury (*N*=1), non-traumatic subarachnoid and parenchymal intraventricular hemorrhage (*N*=1), and acute abdominal disease (*N*=2). The mean age of the patients was 43 (34–60) years. Pulmonary sepsis predominated in 60% of the patients included in the study, with abdominal and mixed (cerebral/pulmonary) sepsis accounting for 20% each. All patients received mechanical ventilation, intensive therapy including combinations of antibiotics, inotropic support and others. Serum samples were collected from all patients to determine biomarkers of sepsis. For the in vitro experiment, samples of intestinal contents were collected once from all patients.

Healthy donors. Blood serum samples from healthy donors (*N*=48) were obtained from the Federal State Budgetary Institution N.N. Burdenko Main Military Clinical Hospital (Moscow, Russia). The age of the donors was 39 (33–45) years, 35 were men and 13 were women. Healthy donors had no general clinical signs of acute inflammation and no chronic liver or kidney disease.

Determination of serum biomarkers of inflammation and sepsis. Serum biomarkers of inflammation and sepsis, including protein S100, IL-6, NT-proBNP, and PCT, were measured using the Cobas e411 electrochemiluminescence analyzer (Roche, Basel, Switzerland). Elecsys S100/NT-proBNP/IL-6/PCT (Roche Diagnostics) reagent kits were used for the measurement.

Evaluation of microbiota composition in samples using the Colonoflor-16 (Biocenosis) test system. Qualitative and quantitative composition of obligate and opportunistic microorganisms of the microbiota was evaluated by real-time PCR with fluorescence detection using the Colonoflor-16 (Biocenosis) test system (AlfaLab, Russia). The system allowed to detect 23 parameters, including 21 groups/species of microorganisms and total bacterial count.

Description of the *in vitro* experimental model. Samples of healthy gut microbiota or microbiota from sepsis patients were placed in nutrient medium with the addition of one of the sepsis-associated PLA or 4-HPLA metabolites. After 24 hours of incubation in a thermostat, the qualitative and quantitative composition of AMM was measured and evaluated.

Culturing of intestinal samples in thioglycol medium with PLA or 4-HPLA addition. Universal

thioglycol medium (TGM) was used to create *in vitro* growth conditions for facultative anaerobic and anaerobic bacteria. The experimental scheme for each intestinal content sample is shown in Fig. 1. AMM composition was investigated in healthy volunteers (*N*=9) and sepsis patients (*N*=10) before and after incubation of intestinal contents in TGM supplemented with sepsis-associated microbial metabolites at clinically relevant concentrations (25 µM PLA or 4-HPLA) for 24 hours at 37°C. After incubation, the tubes were vortexed, centrifuged at 1000 rpm for 10 minutes, and the supernatant was frozen at –20°C. The concentration of metabolites in the intestinal contents of healthy volunteers and sepsis patients before and after incubation in TGM was measured by GC-MS.

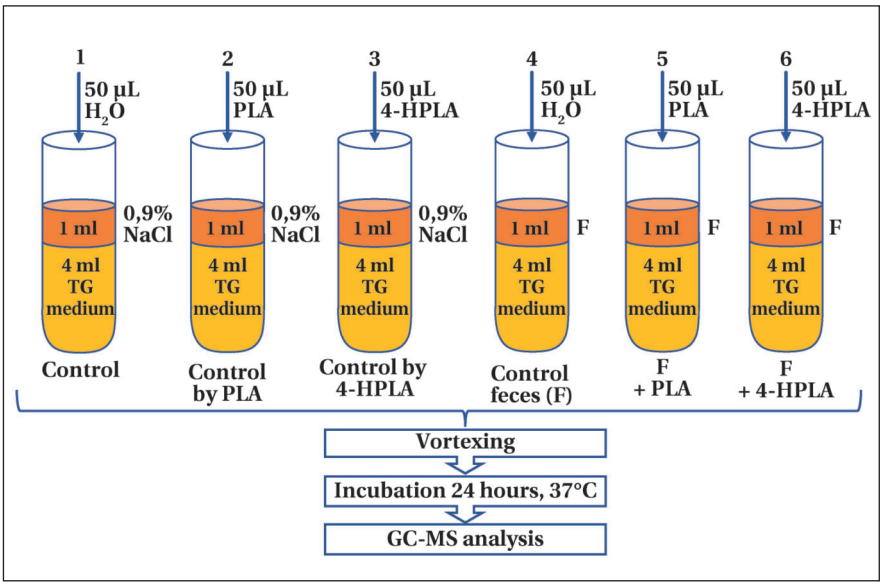

Fig. 1. Scheme of the experiment.
Note. A series of 6 tubes was formed for each sample of intestinal contents (IC), for a total of 19 rows. In each row, four control tubes were assigned: 1) to record the baseline TGM without added reagents, 2) and 3) to record the addition of sepsis-associated metabolites of PLA or 4-HPLA after incubation without IC, 4) to record the baseline values of metabolites in IC without added reagents. Two additional tubes (5 and 6) contained IC and added corresponding sepsis-associated metabolites of PLA or 4-HPLA. After incubation, microbiota metabolites in the TGM were measured by GC-MS analysis.

Analysis of metabolites in intestinal contents and serum using GC-MS. A GC-2010 Plus gas chromatograph and a GCMS-QP2020 mass spectrometer (both from Shimadzu, Japan) were used for metabolite analysis. The test sample was extracted twice with diethyl ether, then evaporated to dryness and derivatized with N,O-bis(trimethylsilyl)trifluoroacetamide. The resulting solution was diluted with n-hexane, and 2 µL of the final solution was injected. For quantitative data analysis, relative signals were calculated as the ratio of the TMS peak area of the target compound derivatives to the peak area of the surrogate internal standard. The concentrations calculated from the calibration graphs were used to present the results. To calculate the relative acid content in percent, the following formula was used:

$$\text{Relative content,\%} = 100\times\left(\frac{\text{relative signal of an acid}}{\Sigma\,\text{relative signals of all measured acids}}\right)$$

Statistical analysis. Statistical analysis of the data was performed using Microsoft Excel 2010 and IBM SPSS Statistic 27. Descriptive statistics were presented as median *(Me)* and interquartile range (IR, 25–75%). The *T*-Wilcoxon test was used for comparison between groups of paired samples. The Mann-Whitney test was used for between-group comparisons of independent samples. The differences between groups were considered significant at two-sided $P<0.05$, where p is the probability of a first-order error when testing the null hypothesis.

Results

Biomarkers and metabolites. We assessed the severity of the disease in the patients at the time of enrollment using several scales. The values were 30 (23–38) points for the APACHE II and 11 (9–15) points for the SOFA. Markers of inflammation and bacterial activity, such as IL-6 and procalcitonin, the heart failure marker NT-proBNP, the brain damage marker S100 (Table 1), and AMM (Table 2), increased multifold.

The sum of concentrations of three metabolites associated with sepsis, namely PLA, 4-HPLA, and 4-HPAA, was found to be more than six times higher in sepsis patients compared to healthy individuals. Such microbial metabolites as phenylpropionic acid, 4-hydroxyphenylpropionic acid, and hydroxybenzoic acid were not detected above the lower limit of quantification, except in one case where the serum concentration of 4-hydroxyphenylpropionic acid was 15.8 µM.

Regarding the microbial metabolites of aromatic amino acids found in the intestinal contents, there was a notable difference between healthy volunteers (*N*=9) and sepsis patients (*N*=10), as shown in Fig. 2. The main metabolites observed in healthy volunteers were PLA and PAA, accounting for 16–86% of the total determined metabolites. In contrast, the proportion of sepsis-associated aromatic metabolites in healthy controls did not exceed 5%. However, in patients with sepsis, the proportion of sepsis-associated phenolic metabo-

Table 1. Biomarkers in patients with sepsis (*N*=10).

Biomarker	Normal value	Result
IL-6, pg/L	<7	235 (126–3320)
Procalcitonin, ng/mL	<0.25	24 (10–49)
NT-proBNP, pg/mL	<125	1404 (728–31368)
S100, pg/mL	<0.1	0.47 (0.26–1.42)
Neutrophil to lymphocyte ratio (NLR)	<4	16 (7–47)

Table 2. Microbial metabolites of aromatic amino acids in serum of healthy donors and sepsis patients, μM.

Metabolites	Concentration, μM		*P*-value
	Donors, *N*=48	Patient with sepsis, *N*=10	
PPA	<0.5 (<0.5–0.5)	<0.5	—
PLA	<0.5	2.0 (1.0–2.3)	—
4-HPPA	<0.5	<0.5	—
4-HPAA	<0.5	2,1 (1.7–7.0)	—
4-HPLA	1.3 (1.0–1.6)	4,6 (2.5–12.3)	<0.001
*∑ (PLA, 4-HPAA, 4-HPLA)	1.9 (1.4–2.2)	12.9 (5.2–27.8)	<0.001

Note. *∑ (PLA, 4-HPAA, 4-HPLA), sum of three levels of clinically significant sepsis-associated acids, μM.

lites was significantly higher, amounting to 40% of the total metabolites.

The taxonomic composition of the intestinal contents was quantified using real-time PCR. In patients with sepsis, a quantitative increase of so-called «proinflammatory» microorganisms such as *Proteus vulgaris/mirabilis*, *Staphylococcus aureus*, *Fusobacterium nucleatum*, *Enterobacter* spp., *Klebsiella pneumoniae* was detected. The anaerobic imbalance coefficient (*Bacteroides fragilis group/Faecalibacterium prausnitzii* ratio) was found to be several times higher, amounting to 3750 (400 — 13750) with reference values lower than 100. The levels of lactobacilli and bifidobacteria were also reduced compared to the reference values in more than 60% of patients.

Changes in the concentrations of AMM in the intestinal contents of healthy volunteers and sepsis patients after incubation

The mean concentrations of AMM after incubation are shown in Fig. 3. Control measurements of PLA and 4-HPLA levels after incubation in TM were performed without the addition of intestinal contents. When 25 μM 4-HPLA (Fig. 3, *b*) or PLA (Fig. 3, *b*) was added to normobiotic medium, a decrease in these metabolites and an increase in PPA (P=0.028, the T-Wilcoxon criterion) were observed after 24 hours compared with the control (assays 3 and 2, respectively). Addition of PLA and 4-HPLA to the pathobiota(Fig. 3, *d*, *e*) did not reduce the levels of these acids after 24 hours, PPA was not found at levels above the lower limit of quantification, except in two cases where PPA concentrations were 8 and 15 μM. PLA (P=0.002) and 4-HPLA (P<0.001) levels were significantly higher and PPA (P=0.003) levels were significantly lower in pathobiota samples compared to normobiotic samples.

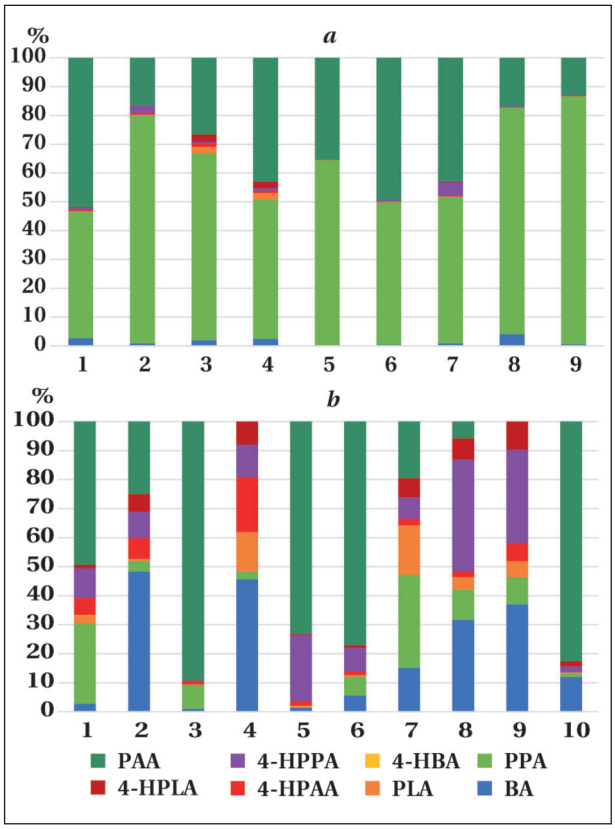

Fig. 2. Composition of microbial metabolites of aromatic amino acids in intestinal contents based on relative acid content.
Note. *a* — in healthy volunteers (*N*=9); *b* — in patients with sepsis (*N*=10).

Discussion

In an *in vitro* experiment, we observed a decrease in the levels of sepsis-associated aromatic microbial metabolites when «healthy» gut microbiota was present. Conversely, we observed an increase in these metabolites when the medium contained pathobiota from sepsis patients. These findings support the hypothesis that the gut microbiota plays a crucial role in the biotransformation of microbial metabolites and the maintenance of homeostasis under normal conditions. Furthermore, our results suggest that this function is impaired in sepsis.

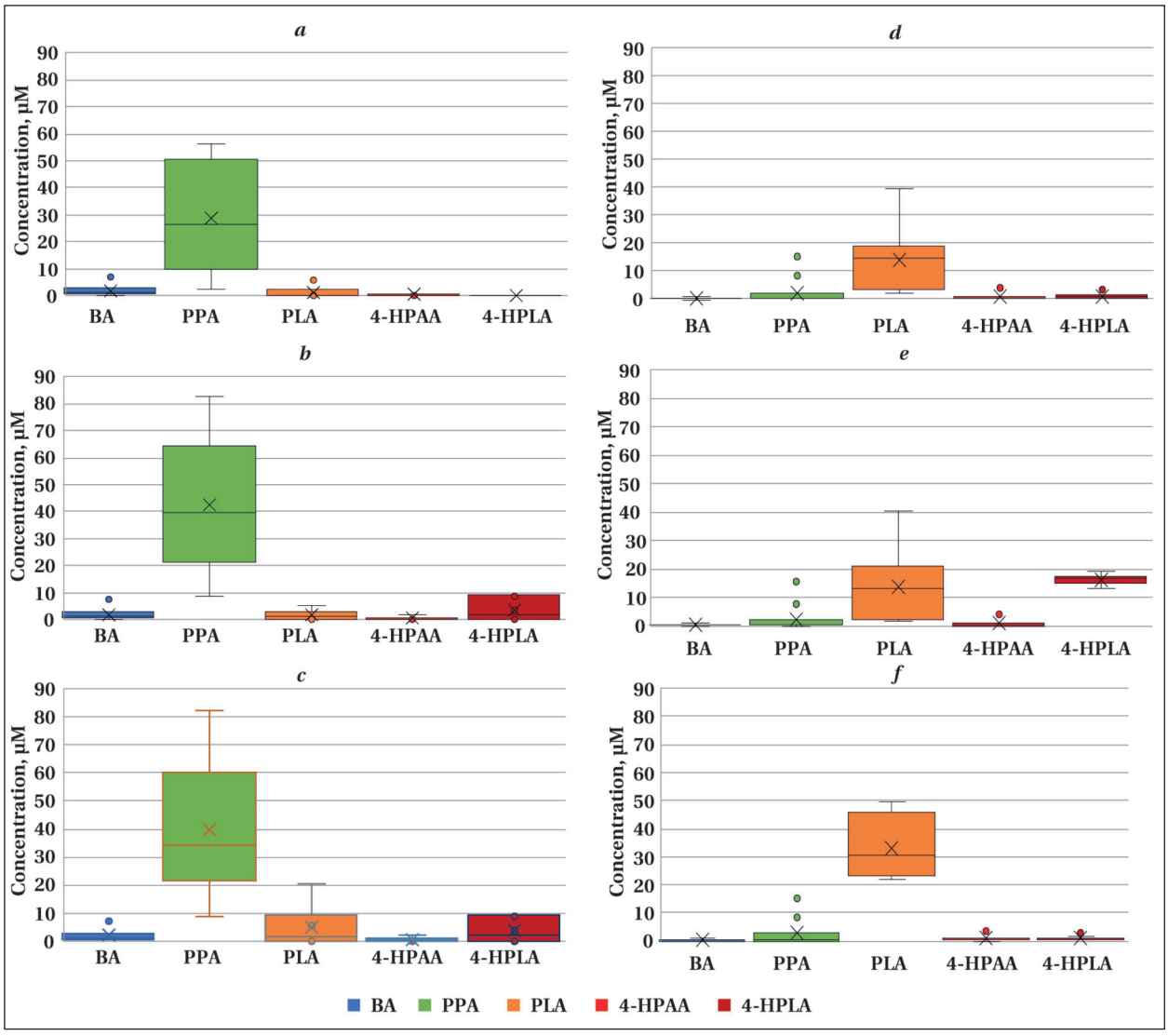

Fig. 3. The levels of microbial metabolites of aromatic amino acids.
Note. Median (IR, 25–75%) in «TGM + normobiota» and «TGM + pathobiota» after 24 h incubation; *a* and *d*: normobiota and pathobiota after incubation without addition of sepsis-associated acids; *b* and *f*: normobiota and pathobiota with added 25 μM 4-HPLA; *c* and *f*: normobiota and pathobiota with added 25 μM PLA.

In addition to the manifold increase in reference values of markers of inflammation and bacterial infection (IL-6, procalcitonin), heart failure (NT-proBNP) and nerve damage (protein S100), reflecting the severity of the patients' condition and the risk of complications [16, 17], we found an almost sevenfold increase in the sum of concentrations of three sepsis-associated serum metabolites, namely PLA, 4-HPAA and 4-HPLA. PLA and 4-HPLA have previously been shown to be metabolites of a number of aerobic and anaerobic bacteria, including the most common pathogens such as Gram-negative bacteria of the Enterobacteriaceae family (e.g., *Escherichia coli, Klebsiella pneumoniae*, etc.) and Gram-positive bacteria (e.g., *Staphylococcus aureus*) [13]. Experimental and clinical studies confirm the biological activity of these metabolites, in particular the inhibition of mitochondrial, neutrophil and platelet function, inhibitory effect on Na^+/K^+ATPase activity [11, 18, 19]. PLA suppressed cell proliferation in rat pancreas, liver and kidney tissue culture and bacterial culture proliferation [20]. Elevated levels of 4-HPAA have been associated with altered bacterial metabolism [21, 22], disruption of the catecholamine synthesis pathway, and development of hemodynamic disturbances in sepsis [10]. The concentration of another metabolite, phenylpropionic acid, one of the end products of microbial metabolism, was significantly reduced or undetectable in the blood of patients with sepsis compared to healthy subjects. This observation is consistent with the fact that only Clostridium sporogenes bacteria were able to produce PPA in the experiment [11, 23]. A positive correlation of one of the phenylpropionic acid precursors, 4-HPPA, with Gram-positive bacteria (including the families *Christensenellaceae*,

Oscillospiraceae, and the genus *Ruminococcus*) representing a healthy microbiome has also been described [6]. The profile of microbial aromatic metabolites in the gut was not identical to that in the serum, but the following trend persisted: sepsis-associated metabolites in healthy individuals did not exceed 5%, whereas their proportion increased manifold and reached 40% in patients with septic shock.

This study was based on the assumption that impaired microbiota composition in sepsis leads to microbiota dysfunction, namely the loss of the ability to utilize excess aromatic metabolites. Indeed, culturing clinically relevant concentrations of 4-HPLA or PLA with normal microbiota was associated with a decrease in the levels of these metabolites and an increase in PLA levels compared to the baseline control, while the pathobiotalacked the ability to biotransform sepsis-associated metabolites. The biochemistry of aromatic acid catabolism by Escherichia coli has been previously studied in vitro in sufficient detail [24, 25]. *Escherichia coli* maintains its ability to grow under aerobic conditions by enzymatic cleavage of phenolic acids to simple compounds in media where aromatic acids are the sole carbon source. The maximum *in vitro* production of PLA and 4-HPLA was observed for *Enterobacteriaceae* and *Staphylococcus aureus*. For example, a 60-fold accumulation of 4-HPLA and 100-fold accumulation of PLA occurred in 24-hour nutrient medium during *Klebsiella* culture compared with the control [12]. Apparently, the biotransformation of metabolites such as PLA and 4-HPLA is performed by healthy human microbiota under the conditions of existing microbial biodiversity, when the excess of metabolites produced as a result of the activity of some bacterial species can be metabolized by other bacterial species according to the «pipeline» principle, resulting in the generation of end products of microbial metabolism, such as PLA [11, 26].

The inability to further metabolize occurs due to changes in the composition and forms of microorganisms under unfavorable conditions and extensive antibiotic therapy. PCR analysis of the taxonomic composition of patients' intestinal contents revealed an anaerobic imbalance, an increase in «proinflammatory», and a decrease in «anti-inflammatory» microorganisms. This finding is consistent with previous studies characterizing the intestinal microbiota of sepsis patients [6, 27, 28]. The microbiota composition changes rapidly within hours of the onset of critical illness [29, 30]. The normal microbiome transforms into an abnormal one dominated by monotonous communities of multidrug-resistant microorganisms [31–34]. The presence of bacterial forms called persisters, which temporarily lose metabolic activity, may contribute to metabolic dysfunction and are challenging to detect using traditional microbiological methods [35, 36]. While a complete transition of the entire population to a persistent state is possible under nutrient starvation [37, 38], we did not specifically investigate this phenomenon.

The experiment showed that redundant sepsis-associated acids in the intestinal contents of healthy humans are partially eliminated by the normobiota, leading to a decrease in the levels (biotransformation) of these acids. The results are consistent with the concept of metabolic interaction potential, based on genomic metabolic reconstructions, which shows that microbial communities contain metabolically interdependent groups. Cooperating groups can efficiently utilize limited resources through metabolite exchange, providing a survival advantage and allowing coexistence in different niches compared to smaller microbial communities [39]. Widespread use of antibiotics disrupts existing metabolic connections and leads to dysfunction of the microbial community as a single organism. Maintaining the biodiversity of the microbiota is an important task because this diversity of species provides continuous biotransformation to the final microbial metabolites that are «useful and safe» for the host.

Conclusion

The accumulation of sepsis-associated metabolites in the blood is not only due to their excessive entry from bacterial growth sites (purulent and inflammatory infection sites), but also largely due to the inability of the gut microbiota to metabolically biotransform these compounds. Comparison of the metabolomic profiles of normobiota and pathobiotain an in vitro experiment showed that when loaded with sepsis-associated metabolites of PLA and 4-HPLA, the microbiota of a healthy individual biotransforms them into the end products of microbial metabolism, whereas the pathobiotaof a septic patient is unable to perform this function. Thus, in sepsis, along with other signs of decompensation of vital functions such as respiration, circulation, brain function, etc., there is a disturbance in microbiota metabolism that contributes to the progression of sepsis and increases the risk of a fatal outcome. Targeting the gut microbiota to eliminate metabolic dysfunction may be a promising strategy for the prevention and treatment of sepsis.

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