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Microbial Origin of Phenylcarboxylic Acids in the Human Body

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Abstract—In previous studies we demonstrated increased amounts of phenylcarboxylic acids (PCA) in serum of patients with sepsis. This observation prompted the present study of the ability of the human microbiome bacteria to produce PCA *in vitro*. PCA were detected in culture media by gas chromatography–mass spectrometry. Increased amounts of phenyllactic and *p*-hydroxyphenyllactic acids were produced by *Klebsiella pneumonia*, *Escherichia coli*, and *Staphylococcus aureus*. Certain strict anaerobes (bifidobacteria, lactobacteria, eubacteria) have also been found to actively produce these PCA, but these bacteria are not etiologically linked to sepsis. Thus our results demonstrate the ability of sepsis-related bacteria to produce PCA and provide experimental support for the theory that the accumulation of PCA in the blood of patients with sepsis results from microbial degradation of phenylalanine and tyrosine.

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Key words: phenylcarboxylic acids, phenyllactic acid, *p*-hydroxyphenyllactic acid, endogenous microflora, facultative and strict anaerobes, sepsis, gas chromatography–mass spectrometry

Humans exist in a world of microbes; therefore, the study of mutual exchange of metabolic products on the boundary of two environments (internal sterile environment of a host organism and biocenoses intensively colonized by bacteria) is a subject of serious attention for the scientific community [1]. The basic biochemical processes in the human body have been studied rather thoroughly, thus making it possible to create a general database of human metabolites: Human Metabolome Database (HMDB; www.hmdb.ca). At the same time, the role of endogenous bacterial community, or "microbiome", in the maintenance of homeostasis of the internal human environment has been underestimated until recently [2]. Comparative analysis of metabolic profiles of plasma samples from germ-free (gnotobionts) and conventional animals has demonstrated a significant contribution of the microbiome to the composition of mammalian blood metabolites [3].

Previously we revealed that the content of some phenylcarboxylic acids (PCA) in serum of septic patients is 10-100 times higher than in healthy people [4, 5]. The findings have underlain the hypothesis that the revealed PCA are of microbial origin, whereas the HMDB refers them to the products of human metabolism. Various literature data about the toxic [6], antioxidant [6], carcinogenic [7], and anticarcinogenic [8] properties of some PCAs are the cause of increased interest in these substances.

The goal of this research was to find clinically significant microorganisms that can produce phenylcarboxylic acids. The study included the major representatives of human microflora: obligate anaerobic bacteria making the basis of endogenous microflora of a healthy people and forming colonization resistance (bifidobacteria, lactobacteria, eubacteria, and bacteroids) and aerobic and facultative anaerobic bacteria most often associated with the etiology of pyoinflammatory diseases and sepsis (*Staphylococcus aureus*, enterobacteria, and nonfermenting Gram-negative bacteria).

MATERIALS AND METHODS

Microbial cultures. Cultures of aerobic microorganisms and facultative anaerobes *Staphylococcus aureus*,

Abbreviations: GC-MS, gas chromatography-mass spectrometry; HMDB, Human Metabolome Database; HPAA, *p*-hydroxyphenylacetic acid; HPLA, *p*-hydroxyphenyllactic acid; HPPA, *p*-hydroxyphenylpropionic acid; PAA, phenylacetic acid; PCA, phenylcarboxylic acid; PLA, phenyllactic acid; PPA, phenylpropionic acid; TMS, trimethylsilyl.

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Staphylococcus epidermidis, *Staphylococcus hominis*, *Staphylococcus haemolyticus*, *Enterococcus faecalis*, *Enterococcus faecium*, *Klebsiella pneumonia*, *Seracia marcescens*, *Escherichia coli*, *Enterobacter cloacae*, *Acinetobacter baumanii*, and *Pseudomonas aeruginosa* were obtained prospectively from hemocultures of patients with bacteremia and sepsis. Three strains of each species were used in this work. Anaerobic cultures were taken from strain collection: *Eubacterium lentum* ATCC 43055, *Bacteroides fragilis* ATCC 25285, *Bacteroides thetaiotaomicron* ATCC 29741, *Clostridium perfringens* ATCC 13124, *Clostridium sporogenes* ATCC 19404; the strains of *Bifidobacterium bifidum* and *Lactobacillus fermentum* were isolated from Bifidumbacterin and Lactobacterin preparations produced by Microgen (Moscow, Russia). In total, 43 strains were investigated. All strains were identified and tested for purity using the BD BBL CRYSTAL system for microbial identification (Beckton Dickinson).

Growth conditions. *S. aureus*, *S. epidermidis*, *S. hominis*, *S. haemolyticus*, *E. faecalis*, *E. faecium*, *K. pneumonia*, *S. marcesceus*, *E. coli*, *E. cloacae*, *A. baumanii*, and *P. aeruginosa* were cultured under aerobic conditions; *E. lentum*, *B. fragilis*, *B. thetaiotaomicron*, *C. perfringens*, *C. sporogenes*, *B. bifidum*, and *L. fermentum* were cultured under anaerobic conditions. Anaerobic conditions were modeled using an anaerostat with the atmosphere of 80% N_2, 10% CO_2, and 10% H_2 placed into a thermostat at 37°C.

Bacterial strains were cultured on solid growth media for 24 h at 37°C. Aerobes were grown on meat–peptone agar, and anaerobes were grown on Schadler agar (Beckton Dickinson). Then, the suspension of each 24-h culture in the amount of $1.5 \cdot 10^8$ CFU (0.5 by MacFarland) was inoculated into test tubes with 8 ml of the liquid growth medium: GRM-broth for aerobes and Schadler broth for anaerobes and incubated for 24 h at 37°C. In 24-h cultures, the number of bacteria reached $(8\text{-}20) \cdot 10^8$ CFU/ml. For assessment of the background PCA content, control test tubes with sterile growth media were incubated simultaneously with the samples. The experiment with each culture was repeated three times to check reproducibility of the results.

Gas chromatography-mass spectrometry (GC-MS) analysis. The samples of 24-h bacterial cultures were centrifuged for 15 min at 800*g*; the internal standard for GC-MS analysis (10 μl of ethanol solution of 400 ng D_5-benzoic acid) was introduced into the collected supernatant (1 ml). Then, the sample was extracted with diethyl ether (2 × 1 ml) at pH 2; the ether extract was evaporated to dryness at 40°C. For obtaining trimethylsilyl (TMS) derivatives of PCA, the residue was treated with 20 μl of N,O-bis(trimethylsilyl)trifluoroacetamide (Fluka) at 80°C for 15 min. The resulting sample was dissolved in 80 μl of hexane and analyzed by the GC-MS method. Control samples were subjected to analogous treatment.

PCA in samples of 24-h microbial cultures was analyzed using an Agilent 6890/5973 gas chromato-mass spectrometer (Agilent Technologies, USA) in the full scan mode. The components were chromatographically separated in a HP5MS quartz capillary column, 0.2 mm in diameter, 25 m in length, with film thickness 0.33 μm. The carrier gas was helium; flow rate was 24 ml/min; flow rate through the column was 1.2 ml/min. The temperature conditions during analysis was as follows: evaporator temperature, 280°C; initial temperature of column thermostat, 80°C was held for 4 min; then, the temperature was increased to 240°C at 7°C/min and then to 320°C at 15°C/min, where it was held to the end of the analysis. Injected sample volume, 2 μl; total time of analysis, 35 min; detector operation delay time, 4 min. The list of detected compounds, retention times, and typical ions in the mass spectra are presented in Table 1.

The quantity of detected compound was estimated by comparing its peak area with the peak area for a known amount (400 ng) of standard compound, TMS derivative of D_5-benzoic acid (retention time 10.45 min), taking into account their molecular masses. The PCA content in the samples was assessed as the average value of three experiments.

RESULTS

Previously, in serum samples taken from a group of septic patients we found increased contents of phenyllactic (PLA), *p*-hydroxyphenyllactic (HPLA), and *p*-hydroxyphenylacetic (HPAA) acids out of the whole spectrum of detectable PCAs; this effect was the most pronounced in a group of patients with lethal outcome [5]. We suggested that these compounds are metabolites of endogenous bacteria, and the increase of their level in blood is associated with development of infectious complications of pneumonia and sepsis. In this work, we estimated by *in vitro* experiments the ability of endogenous bacteria to produce PCAs. Microorganisms were cultivated on enriched multicomponent media containing carbohydrates, proteins, amino acids, vitamins, etc. to create conditions simulating the internal environment of the human body.

Application of the GC-MS method for PCA analysis in a 24-h culture liquid revealed PLA and HPLA accumulation for most of the studied bacterial species (Table 2). On incubation under aerobic conditions, the greatest amount of these PCA was produced by Gram-negative enterobacteria: *Klebsiella* (30 μM PLA and 13 μM HPLA) and *E. coli* (10 μM PLA and 3.6 μM HPLA, respectively); besides, significant amounts of the acids were produced by *Staphylococcus aureus* (5.3 and 3.6 μM, respectively). Enterococci produced only PLA. Among the studied anaerobes, the most of PLA and HPLA was found in bifidobacteria (177 and 66 μM, respectively),

Table 1. Identified compounds and chromatographic and mass-spectral characteristics of their TMS derivatives

Compound	Retention time, min	Major ion, m/z	Additional ion, m/z
D_5-Benzoic acid	10.45	184	110
Benzoic acid	10.51	179	105
p-Hydroxybenzoic acid	18.27	267	223
2,4-Dihydroxybenzoic acid	20.75	355	281
3,4-Dihydroxybenzoic acid	20.84	193	370
Phenylacetic acid	11.71	164	91
p-Hydroxyphenylacetic acid	18.04	179	296
2-Hydroxyphenylacetic acid	15.22	179	147
Phenylpropionic acid	14.02	104	207
p-Hydroxyphenylpropionic acid	19.80	179	192
Cinnamic acid	16.29	205	131
p-Hydroxyphenylcinnamic acid	22.22	219	293
Phenyllactic acid	17.12	193	147
p-Hydroxyphenyllactic acid	22.02	179	147
Phenylpyruvic acid	16.85	147	293
p-Hydroxyphenylpyruvic acid	20.01	147	325
o-Hydroxyphenylacetic acid	16.75	147	253

lactobacteria (163 and 52 μM, respectively), clostridia *C. sporogenes* (62 and 22 μM, respectively), and eubacteria (18 and 2.1 μM, respectively). None of the studied microorganisms produced HPAA.

The studied strains of aerobic Gram-negative non-fermenting bacteria *A. baumanii* and *P. aeruginosa* and bacteroids showed no ability to produce PCA. It should be noted that on incubation of bacteroids for 48 h, we registered the formation of phenylacetic acid (PAA) in the culture medium (data not shown), which is in agreement with the data of other authors [9, 10]. The experiments with clostridia also confirmed the literature data [11], according to which the major products of phenylalanine and tyrosine metabolism under anaerobic conditions in *C. sporogenes* are phenylpropionic acid (PPA, 1.2 mM) and *p*-hydroxyphenylpropionic acid (HPPA, 0.8 mM), while *C. perfringens* does not produce these PCAs.

Thus, the experiments showed that some strains of clinically significant microorganisms actively produce PLA and HPLA. The highest production is observed in facultative anaerobes (*Klebsiella*, *E. coli*) and in some of the strict anaerobes (bifidobacteria, lactobacteria, eubacteria).

DISCUSSION

Normally, low molecular weight microbial metabolites continuously enter the blood flow in small amounts from the intestines, where they are formed as a result of food degradation by microflora; then they are detoxified in the liver and released with urine in native or modified form or as conjugates. The major PCA precursors in humans are aromatic amino acids: phenylalanine and tyrosine [12]. Previously, the following PCA have been identified in the serum of clinically healthy volunteers by the GC-MS method: benzoic, *p*-hydroxybenzoic, PAA, HPAA, PPA, HPPA, PLA, and HPLA [4]. According to the literature data of the 1980-90s, the study of tyrosine metabolism by the GC-MS method and isotopic analysis also showed the presence of HPLA and *p*-hydroxyphenylpyruvic acid in the blood plasma [13] and urine [14] of clinically healthy donors.

It was shown in the literature that there are two routes of tyrosine degradation by aerobic bacteria that are combined into pathway 3 in the figure. The first route found in Gram-negative microorganisms is similar to the normal endogenous tyrosine metabolism in human body (see figure, pathway 1), where the major intermediate is 2,5-dihydroxyphenylacetic acid (homogentisate) [15]. The major intermediate of the second route found in some Gram-positive microorganisms is 3,4-dihydroxyphenylacetic acid [16]. The mechanism of degradation of phenol-containing compounds under aerobic conditions involves the introduction of one or two hydroxyl groups into the phenol ring. Thus, the opening of the ring under the action of dioxygenases becomes easier and is followed by transformation to short-chain carbon compounds,

Table 2. Accumulation of phenylcarboxylic acids in 24-h culture medium of major clinically significant microorganisms

Bacterium	Phenylcarboxylic acid content, μM			
	PPA	HPPA	PLA	HPLA
Aerobes and facultative anaerobes				
Sterile medium (GRM-broth)	0.6	0.4	0.3	0.2
S. aureus	0.4	0.2	**5.3**	**3.6**
S. epidermidis	0.4	0.3	1.7	0.7
S. haemolyticus	0.5	0.4	3.1	1.6
S. hominis	0.5	0.3	3.9	1.7
S. marcescens	0.4	0.2	1.3	1.8
K. pneumoniae	0.4	0.2	**30**	**13**
A. baumanii	0.2	0.1	0.3	0.5
E. coli	0.4	0.2	**10**	**3.6**
E. faecalis	0.3	0.2	**1.8**	0.1
E. faecium	0.4	0.2	0.5	0.1
E. cloacae	0.5	0.3	1.1	0.6
P. aeuroginosa	0.5	0.3	0.3	0.2
Anaerobes				
Sterile medium (Schadler broth)	0.9	1.0	2.4	0.8
B. bifidum	0.5	0.3	**177**	**66**
L. fermentum	0.6	1.7	**163**	**52**
C. sporogenos	**1220**	**780**	**62**	**22**
C. perfringens	1.1	0.8	13	0.5
B. fragilis	0.1	0.1	0.5	0.1
B. thetaiotaomicron	0.1	0.1	0.4	0.1
E. lentum	0.1	0.1	**18**	**2.1**

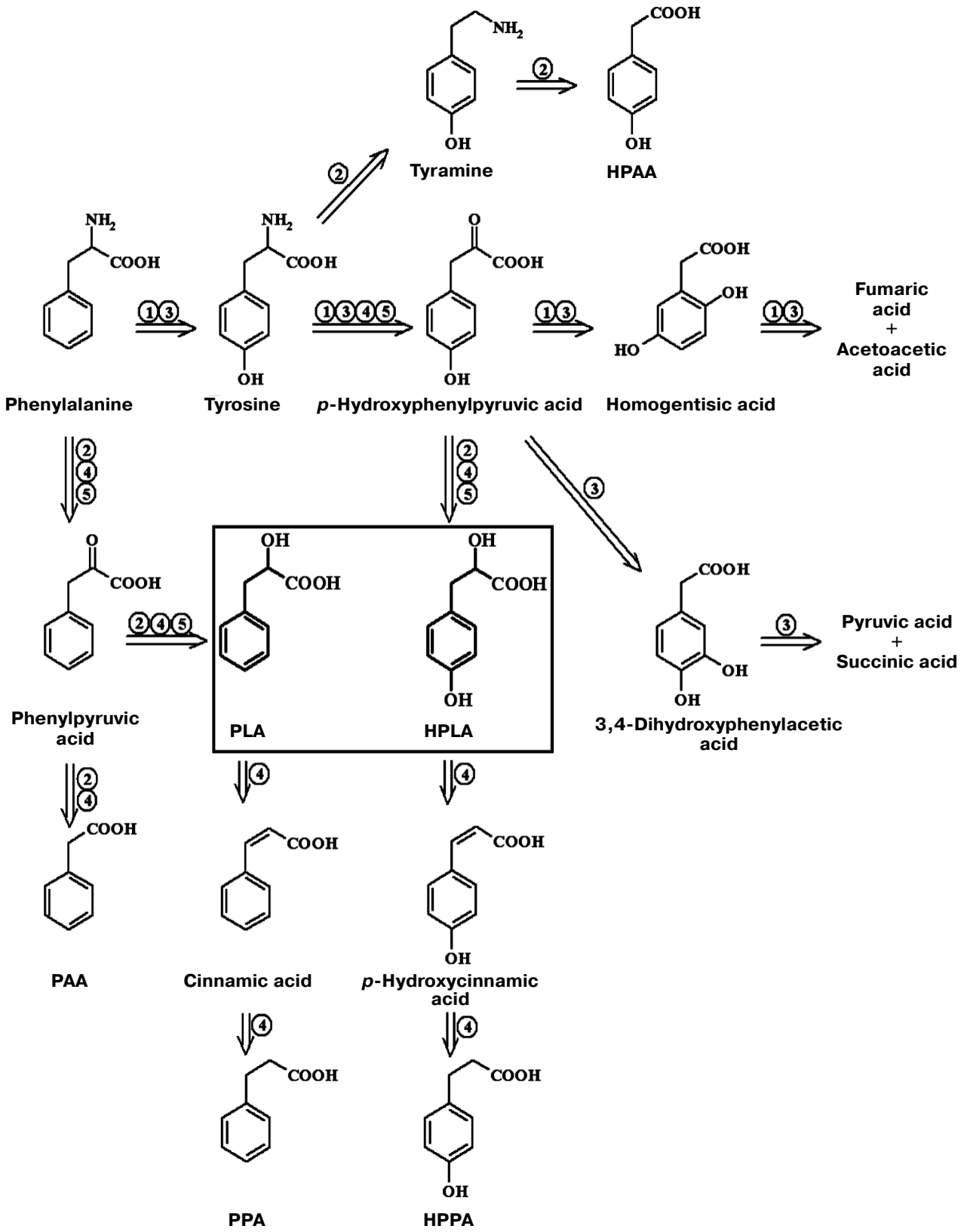
Note: PPA, phenylpropionic acid; HPPA, *p*-hydroxyphenylpropionic acid; PLA, phenyllactic acid; HPLA, *p*-hydroxyphenyllactic acid.

which can then enter the Krebs cycle or other metabolic pathways. The above-described mechanism explains the absence of PCA production in the aerobes studied in the present work: nonfermenting Gram-negative bacteria of the genera *Acinetobacter* and *Pseudomonas*.

On the other hand, during the metabolism of aromatic amino acids by anaerobic bacteria, the phenol ring usually remains intact and only the aliphatic side chain is transformed. For example, strict anaerobes *C. sporogenes* and *P. anaerobius* implement the following mechanism [11, 17]: first, deamination of tyrosine and phenylalanine by tyrosine- and phenylalanine-ammonium lyases with the formation of *p*-hydroxycinnamic and cinnamic acids, respectively. Then, these acids are reduced to *p*-hydroxyphenylpropionic (HPPA) and phenylpropionic (PPA) acids, respectively. When transported to the liver, HPPA and PPA undergo β-oxidation with the formation of benzoic acid, which is excreted with urine as hippuric acid [18].

Our data for *C. sporogenes* substantially supplement this picture. The accumulation of PLA and HPLA in 24-h culture medium revealed for this anaerobic bacterium implies that tyrosine and phenylalanine degradation can proceed in this medium through these intermediates as well (see figure, pathway 4). The accumulation of PLA and HPLA in the culture medium has been shown in this work for most of the facultative anaerobes, particularly Gram-negative enterobacteria and staphylococcus, cultivated under aerobic conditions (see figure, pathway 5).

Genetically determined disturbances of the normal endogenous metabolism of phenylalanine and tyrosine occur in the liver and kidneys, when the products of side degradation pathways are accumulated in blood (see figure, pathway 2). Normally, phenylalanine is converted



Interrelation of the endogenous and microbial catabolic pathways of phenylalanine and tyrosine: *1*) major endogenous pathway; *2*) alternative endogenous pathway; *3*) aerobic microbial pathway; *4*) anaerobic microbial pathway; *5*) catabolism by facultative anaerobes under aerobic conditions

into tyrosine; however, in phenylketonuria, due to the absence of phenylalanine hydroxylase, phenylalanine concentration in blood increases, resulting in the triggering of alternative catabolic pathways: phenylalanine aminotransferase is induced and phenylalanine is converted into phenylpyruvic acid, followed by reduction to phenyllactic acid and decarboxylation to phenylacetic acid [19]. The first stage of the normal endogenous pathway of tyrosine degradation is formation of *p*-hydroxyphenylpyruvic acid, which is hydroxylated with the formation of homogentisic acid by *p*-hydroxyphenylpyruvate-hydroxylase. The deficiency of this enzyme results in the development of tyrosinemia. Such patients demonstrate increased excretion of *p*-hydroxyphenylpyruvic acid, as well as HPLA and HPAA [20], which are formed as a result of triggering of alternative tyrosine degradation pathways. Increased PCA content can be observed also in patients with other pathological states. For example, PLA, HPAA, and *p*-hydroxybenzoic acid are accumulated in the blood plasma of patients with chronic renal failure [21]; enhanced excretion with urine of *p*-hydroxyphenylpyruvic acid, HPLA, HPAA, and *p*-hydroxybenzoic acid is observed in patients with liver cirrhosis [22]. Haan et al. [23] showed the microbial origin of PLA, HPAA, and HPLA excreted in the urine of patients with short gut syndrome.

When considering the causes of PCA accumulation in the blood of septic patients, the mechanisms associated with disturbance of activity of the enzymes of endogenous metabolism of phenylalanine and tyrosine in the liver and kidneys, by analogy with phenylketonuria and other genetically based diseases, can be excluded. As regards organ disturbances, it should be noted that sepsis in most patients is accompanied by multiple organ failure; however, the effect of impaired liver and kidney function is hardly decisive, because septic patients do not show accumulation in blood of other metabolites, especially *p*-hydroxyphenylpyruvic acid [4].

According to our data, PLA and HPLA found in septic patients are actively produced by Gram-negative enterobacteria and *Staphylococcus aureus*. Anaerobic bacteria (bifidobacteria, lactobacteria, and eubacteria), although capable of PLA and HPLA production, hardly contribute to the increase in PCA content in blood because they are known to be practically absent in the intestines of septic patients [24]. The latter is also shown by the absence in the blood of septic patients of PPA and HPPA, which are invariable products of anaerobic microbial metabolism [4].

Based on the above discussion, we consider that the increased PCA content in the blood of septic patients is associated with active metabolism of enterobacteria and *Staphylococcus aureus* etiologically linked with sepsis. The results of this research also demonstrate the possibility of using PLA and HPLA as markers for laboratory diagnostics of bacterial infections and sepsis. Another field of future research is establishment of the biological role of phenylcarboxylic acids under septic states.

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