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Review Article

Low-Molecular Weight Bacterial Metabolites in Host-Microbial Interaction

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Abstract

The review gives an insight into inherent biological properties of bacterial metabolites - low-molecular weight Phenylcarboxylic Acids (PCAs), including Benzoic Acid (BA), *p*-Hydroxyphenyllactic Acid (HPLA), Phenyllactic Acid (PLA), *p*-Hydroxyphenylacetic Acid (HPAA), Phenylacetic Acid (PAA), and Phenylpropionic Acid (PPA). It has been demonstrated that bacteria from human microflora - predominantly anaerobes - can metabolize aromatic amino acids into PCAs, and PCAs are capable to suppress the growth and propagation of other bacteria, entering competitive interactions within microbial associations. The authors suggest that in the human colon, where concentrations of microbial metabolites reach biologically active level, PCAs may exert not only local, but also systemic effects, thus any deviation from existing composition of microbial associations may potentially result in the breakdown of habitual PCAs balance and emergence of PCAs with opposite biological properties. Available published data as well as findings from own research allowed us to substantiate a novel approach directed at the development of new therapeutic strategies based on regulation of local and systemic balance of microbial aromatic metabolites in the human body.

Keywords: Aromatic microbial metabolites; Benzoic acid; Clinical microecology; Host-microbe interaction; Low-molecular weight phenolic compounds; Metabolic profile; Microbiom; Microflora metabolomics; Monocarboxylate transporters; Phenylcarboxylic acids; Sepsis

Abbreviations

PCAs - Phenylcarboxylic Acids
BA - Benzoic Acid
HPLA - *p*-Hydroxyphenyllactic Acid
PLA - Phenyllactic Acid
HPAA - *p*-Hydroxyphenylacetic Acid
PAA - Phenylacetic Acid
PPA - Phenylpropionic Acid
BAA - Benzamino-Acetic Acid
AMM - Aromatic Microbial Metabolites
ATP - Adenosine Three-Phosphate
SB - Sodium Benzoate
ROS - Reactive Oxygen Species
LPS - Lipopolysaccharide
iNOS - Inducible NO-Synthase
MCTs - Monocarboxylate Transporters
MFS - Major Facilitator Super Family
HA - Hippuric Acid
NOAEL - No Observed Adverse Effect Level
MIC - Minimal Inhibitory Concentration
MBC - Minimal Bactericidal Concentration
MFC - Minimal Fungicidal Concentration

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Introduction

Well established and well balanced biological intercommunication between macro and microorganisms has been formed in the process of evolution. Meanwhile this phenomenon is hardly taken into account in the context of clinical research, as traditionally both the research and descriptions of biochemical and signaling processes are done separately for the macroorganism and inhabiting it microflora. It's due mostly to the inertness of medical science which keep replicating erroneous perception of diverged biochemical regulatory pathways of pro and eukaryotic organisms, neglecting their continuous coevolution.

We believe that further progress in clinical science is impossible without considering the role of human microflora vital activities, habitability and intercommunication with human metabolism, without discovering common signal pathways envisaging key roles of microbial metabolites in pathogenesis of both infectious and non-infectious (such as oncologic, endocrine, mental, etc.,) diseases. Clinical microecology, a novel domain in medical science, is the most appropriate term, encompassing all aspects listed above.

This deficit in knowledge of microecology is most painfully evident in anesthesiology and critical & emergency care medicine. Sepsis remains the leading direct cause of death in intensive care units, despite intensive use of multilevel and multi-component monitoring, most potent antimicrobials and hitech organ replacement technologies [1-5]. Active research of aromatic microbial metabolites and their potential role in tanatogenesis is being carried out in the Lab of human Metabolism in Critical States (MCS), Negovsky Scientific Research Institute of General Reanimatology [6].

It has been shown that simple chemical compounds act as signal molecules and bio-regulators in microbial community, representing the most archaic autoregulation and intercellular communication mechanism, so called quorum sensing [7]. In the process of evolution low molecular weight compounds have secured their principal role in the human metabolism, it's suffice to mention some hormones (such as endogenous catecholamines, thyroid hormones),

neurotransmitters (serotonin, γ-aminobutyric acid), tissue and mitochondrial metabolism autocrine regulators (NO), etc.

Simple chemical compounds may play important bridging role in the intercommunication between bacterial and human metabolism. For example, adrenaline and other catecholamines turned out to be involved into interbacterial communication, as well as into bacterial interaction with macro-organism [8,9]. Preliminary data on established profiles of live microorganisms exometabolites in human serum are already published [6,10,11]. Comprehensive studies of microbial metabolites in human biological liquids & tissues seem to be the most promising approach for future deeper insights into potential impact of micro-ecological derangements on human organism that instantly manifests via impaired balance of exometabolites.

Clinically Relevant Phenylcarboxylic Acids

In our previous publications we demonstrated the most sizable fluctuations in serum levels of the following hydroxylated and non-hydroxylated Phenylcarboxylic Acids (PCAs) (so called aromatic compounds) - *p*-Hydroxyphenyllactic Acid (HPLA), Phenyllactic Acid (PLA), *p*-Hydroxyphenylacetic Acid (HPAA), Phenylacetic Acid (PAA), Benzoic (BA) and *Phenylpropionic Acid* (PPA) - during sepsis (Figure 1) [12-14].

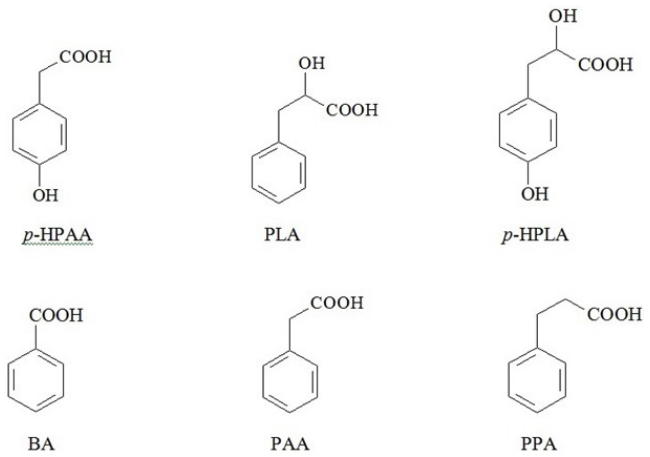

Figure 1: Sepsis Associated Phenylcarboxolic Acids [12,13].

Note: Low-molecular weight phenylcarboxylic acids detected in serum of critically ill patients. Most significant changes in serum PCAs profile were documented in patients with sepsis.

Moreover, we established direct correlation between cumulative serum content of PCAs and the severity of disease [10]. Quantification of some PCAs was successfully used in clinical practice for verification of sepsis (invention patent No 2423704 RU), although deeper insights and better understanding of specific roles and mechanisms of action of microbial exometabolites in human metabolism are still lying ahead [14-17].

Benzoic acid - represented to the fullest in available publications - has been chosen as a model for theoretic analysis of diverse biological PCAs properties.

Benzoic acid

Biologic properties: Pure benzoic acid (CAS No. 65-85-0; C_6H_5COOH, molecular weight 122.13) is a colorless and white crystalline substance with 122°C melting and 249°C boiling points, poorly soluble in water (2.9 g dissolves in 1 L of water at t°=20°C). Benzoic Acid (BA) and it's salts are commonly detected by spectrophotometry, gas and liquid chromatography methods [18].

BA is naturally synthesized by bacteria, plants and fungi. High BA concentrations are found in fermented dairy foods, considerable amounts of BA are produced by lactobacilli from hippuric acid and accumulated as the final product of phenylalanine biodegradation (Figure 2) [15,18,19].

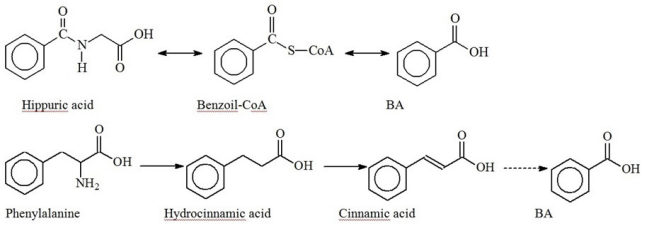

Figure 2: BA Sources in Fermented Diary Food [15,19].

Note: High BA concentrations are found in fermented dairy foods, due to production of BA by lactobacilli from hippuric acid and accumulation of BA as the final product of phenylalanine biodegradation.

BA concentration in yogurt varies from 9 to 56 mg/kg, in cheese it amounts up to 200 mg/kg and more [15]. BA is naturally found in tomatoes, beans, cereals, nuts, fruits, honey and mushrooms [20,21]. High BA concentrations up to 0.05% of total weight are found in different berries, in particular up to 4500 mg/kg in arctic cranberries and blueberries [22]. Instant increase of BA production in response to infection was established in plants [23]. Salicylic (ortho-hydroxybenzoic) acid is most common and best studied among herbal low-molecular weight PCAs. Ortho-hydroxybenzoic acid was shown to interfere with the expression of genes encoding mitochondrial proteins, and it's concentration was fluctuating proportionally to BA levels [24]. Fungi produce BA via biodegradation of phenylalanine [25]. In herbivorous BA was found in soft tissues and milk [19]. There's no published data or any reference in Human Metabolome Database (HMDB; www.hmdb.ca), indicating that BA is produced in human body, either as the final product of phenylalanine biodegradation or as a result of de novo synthesis from aliphatic compounds. Nevertheless, BA is also produced in the human body via benzaldehyde (present in food stuff) oxidation or benzyl alcohol (present in many medicinal drugs) oxidation [26,27], or polyphenolic products oxidation [28,29]. Knoop F was the first to demonstrate that BA is formed via β-oxidation of lateral PCAs chains with uneven number of carbon atoms (phenylpropionic and phenylvaleric acids), meanwhile β-oxidation of lateral PCAs chains with even number of carbon atoms (phenylbutyric, phenylcaproic acid) yields Phenylacetic Acid (PAA) [30]. Higher concentrations of BA, as compared to other PCAs, were found both in feces - 6.2 mg/L [31], and blood serum - 0.079 mg/L - of healthy volunteers [6,10]. BA found in human biological matrices & fluids originates predominantly from ingested food, as well as from the production by GIT microflora and from oxidation of polyphenoles [28,30]. Figure 3 shows production pathways of some PCAs in the human body from both, human and bacterial phenylalanine and tyrosine metabolism [14].

Although, bacteria utilize some PCAs, in particular Hydroxyphenylpropionic Acid (HPAA) and *p*-Hydroxyphenylpropionic Acid (*p*-HPPA) as precursors for phenylalanine, tyrosine and tryptophan synthesis [32].

Higher organisms were shown to lose their capacity to produce some metabolites in close co-habitation with microflora in the process of co-evolution. Figure 3 shows the example of anaerobic formation of

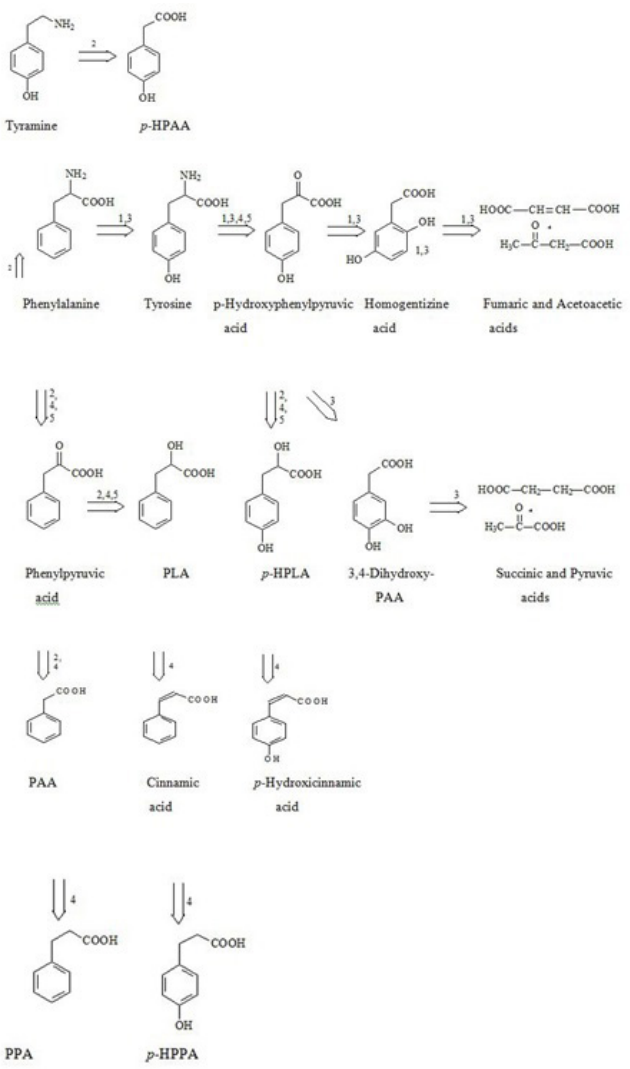

Figure 3: Interrelation between Endogenous and Microbial Catabolic Pathways of PCAS Synthesis from Phenylalanine and Tyrosyne in Humans [14].

cinnamic, hydroxicinnamic, phenylpropionic and hydroxyphenylpropionic acids exclusively by bacteria.

BA antimicrobial effects

Due to inherent antiseptic properties BA and it's salts are commonly used as preservatives (E210-E213) in food and cosmetic industry [15,18,23], BA salts such as sodium benzoate (CAS No. 532-32-1; C_6H_5COONa, molecular weight 144.1) are more popular for better water solubility (550 g/L at 20°C) [18]. Maximal permissible BA salts concentrations in different food products are varying from 150 to 2000 mg/kg (RF Sanitary Regulations and Norms N 2.3.2.1293-03) to provide adequate Minimal Inhibitory BA Concentrations (MICs) for the majority of listed in table 1 species of bacteria and fungi.

BA potential bacteriostatic and bactericidal effect on upper GIT tract microflora was demonstrated in experimental studies on piglets. Bacteriological studies of PCAs' potential to inhibit pure cultures of clinically significant strains showed that BA, as well as Phenylacetic Acid (PAA) and Phenylpropionic Acid (PPA), were inhibiting to greatest extent the growth of E. coli, with non-pathogenic ATCC 25992 E. coli strain being more resistant to BA/PCAs, than enteropathogenic

Microorganism	pH	MIC, mg/L
Esherichia coli [33]	6.0	100-200
Lactobacillus spp [15]	4.3-6.0	300-1800
Klebsiella pneumonia [33]	6.0	100-200
Pseudomonas aeruginosa [34]	5.0/7.0	250/1000
Pseudomonas aeruginosa [33]	6.0	200-500
Staphylococcus aureus [34]	5.0/7.0	500/1000
Staphylococcus aureus [33]	6.0	50-100
Streptococcus spp [15]	5.2-5.6	200- 400
Candida albicans [34]	5.0/7.0	130/>1000
Zygosaccharomyces bailii [33]	4.8	4500
Zygosaccharomyces bailii [33]	4.0	1200

Table 1: Minimal Inhibitory BA Concentrations for Some Bacterial and Fungal Species, mg/L.

Due to inherent antiseptic properties BA and its' salts are commonly used as preservatives (E210-E213) in food and cosmetic industry.

Note: 1 Principal endogenous pathway; 2-alternative endogenous pathway in patients with congenital abnormalities; 3-aerobic microbial pathway; 4- anaerobic microbial pathway; 5-catabolism by facultative anaerobes in aerobic conditions.

O157:H7 *E. coli.* A negligible effect of PCAs with one or two Hydroxyl Groups (3-hydroxy- 4-hydroxyl-3, 4-dihydroxy-substituted PCAs) in aromatic ring was documented with enteropathogenic strain, and no effect with non-pathogenic ATCC 25992 strain at ≤ 1000 mg/L concentrations. Hydroxybenzoic acids turned out to be more potent than BA in lactobacilli inhibition, while BA and derivatives were most effective inhibitors of pathogenic *Staphylococcus aureus* (strain EP167). PAA and PPA seem to be the supreme - as compared to their hydroxyl-derivates - inhibitors of *Lactobacilli* and *S. aureus* [35].

Pseudomonas aeruginosa PAO1 from gram -ve family showed resistance to BA and other PCAs at 1000 mg/L concentration. BA and PPA at 1000 mg/L only partially (by 16% and 29%, respectively) inhibited the *Candida albicans* MY1055. The authors suggested that different microorganism's sensitivity to PCAs depends predominantly on specific structure of cellular wall [36]. It's important to mention that all PCAs in this review inhibited propagation of microorganisms at concentration's values ranging within one order, which implies similar mechanisms of their action, according to the theory of weak organic acids (see below).

It has been suggested that BA belongs to so called allelochemicals, expressing allelopathy i.e., suppressing or inhibiting growth of other organisms in the environment [37]. This can be well applicable to other PCAs. We suggest that PCAs regulate to certain extent the diversity and propagation tempos of human microflora [36].

Jenner AM et al., identified significant amounts of different PCAs in human fecal waters with predominating PAA- 479 μM, PPA -166 μM, *p*- HPPA - 68 μM, 3,4-dihydroxy- cinnamic acid - 52 μM; BA - 51 μM, 3-hydrohy-phenylacetic acid- 46 μM; *p*-HPAA - 19 μM and 3,4- dihydroxy-PAA - 7 μM. Of importance, long-term monitoring of colonic PCAs' profile in participating volunteers showed consistent persistence of BA levels, varying within 23-25 μM in consecutive daily samples [31].

The results from own studies also confirm the potential of anaerobic bacteria derived from human microflora to produce PCAs [38]. Moreover, the identified PCAs profile was consistent with that of Jenner AM et al. (Table 2). Of importance, some PCAs in anaerobic

cultures were accumulated up to the levels, stated by other authors as concentrations inhibiting microbial propagation.

PCAs	*Lactobacillus fermentum*, 24 hour culture	*Bifidobacterium bifidum*, 24 hour culture	*Lactobacillus plantarum*, 72 hour culture	*Lactobacillus fermentum*, 72 hour culture
PPA	155.7 mg/L (1036.8 μM) Jenner AM [31]			
p-HPPA	123.2 mg/L (795.6 μM), Jenner AM [31]			
p-HPLA	13.7 mg/L (72.3 μM) Beloborodova N et al. [38]	(39.4 mg/L (216.28 μM) Beloborodova N et al. [36]	260 ± 13 μM, Francesca Valerio [40]	Undeterminable, Francesca Valerio [40]
PLA	35.4 mg/L (213 μM) Beloborodova NV et al. [38] 200-3500 μM Gerez CL [39]	64.9 mg/L (390 μM) Beloborodova NV et al. [38]	310 ± 19 μM, Francesca Valerio [40]	Insignificant amounts, Francesca Valerio [40]

Table 2: PCAs Production by Human Anaerobic Bacteria.

It has been confirmed, that Phenyllactic (PLA) and *p*-Hydroxyphenyllactic (*p*-HPLA), as well as other analyzed PCAs, were able to inhibit bacterial and fungal growth at 500 mg/L concentration, and that mold fungi and gram +ve bacteria were more sensitive to PLA and to *p*-HPLA [41-43].

Mechanisms of intracellular PCAs penetration

In earlier studies it has been shown that BA exhibits maximal antimicrobial potential in acidic environment, while in neutral environment it is significantly lower. [44,45]. Apart from BA, such acids as lactic (E270), acetic (E260), propionic (E280), and sorbic (E200) are commonly used as preserving agents. All these compounds are weak acids, in particular, pKa of BA is 4.21, i.e., at pH=7 the percentage of non-dissociated BA molecules is equal to 0.144%, while at pH=3 it rises up to 93.5% [15,44]. Table 3 shows MIC values for BA, lactic and acetic acids against some bacteria.

Microorganism	BA	Acetic acid	Lactic acid
B. cereus ATCC11778	296	2020	3480
B. subtilis ATCC6633	192	105	8320
E. coli ATCC25922	316	1550	3720
L. fermentum ATCC14931	2500	26300	25300
L. plantarum EH22G	2610	27500	30700

Table 3: MIC for Benzoic, Lactic and Acetic acids, mg/L [46].

Note: Comparison of antimicrobial effects of weak organic acids used as preservatives in food industry.

"Theory of weak organic acids" has been proposed to explain the antimicrobial potential of BA and other low-molecular weight organic acids [23]. This theory says that the proportion of non-dissociated BA molecules increases at low pH values of the solution/environment, making it possible for lipophylic BA molecules to penetrate through plasmatic cell membrane. Intracellular pH value is usually close to neutral, thus BA would dissociate in neutral environment with the release of H+ ion which would lead to intracellular acidification and inevitable impairment of cell functions. Similar penetration pattern is applicable to other weak organic acids [17,47]. Table 4 presents data on correlation between Minimal Inhibitory (MIC), Minimal Bactericidal (MBC) and Minimal Fungicidal (MFC) BA concentrations values and environmental pH values.

Effects on cell metabolism

Baker's yeast (*Saccharomyces cerevisiae*) is number one cell culture in experimental studies of BA effects on eukaryotic cell, as this species is known for it's inherent resistance to BA and is not able to utilize BA as the source of carbon [48]. *Saccharomyces cerevisiae* cell culture usually responds to BA addition by deceleration in cell mass growth with simultaneous up-regulation of glucose and oxygen consumption. Cytological studies showed linear correlation between rising consumption of oxygen and increasing mitochondrial volume. When BA concentration reaches its' threshold level, consumption of oxygen starts to decline with simultaneous up-regulation of enzymatic ethanol production [49]. Increased oxygen consumption and associated microbiostatic effect of BA is explained by increased ATP use for the removal of benzoates and protons from inside the cell to maintain physiological intracellular pH values [50,51]. BA is an osmotically active substance, thus intracellular BA accumulation is associated with acidification of intracellular medium and swelling of the cell. With rapid increase of BA concentration in culture media short-term peak of maximal oxygen consumption is usually followed by long-term depression in oxygen and glucose consumption due to inhibited Krebs' cycle enzymes (phosphofructokinaze, in particular) and, thus, inhibited glycolysis [15,49,52,53]. Moreover, BA causes depolarization of cell membrane and interferes with membrane transport [35].

In earlier study on murine mitochondrial culture BA at 0.1 μM concentration was shown to reduce membrane potential and calcium content to a significant extent, suppressing mitochondrial respiration (I complex of respiratory chain) and inhibiting oxidation of pyruvate presumably due to pyruvate dehydrogenase blockage. These effects of benzoate, viewed as toxic, were attenuated by menadione and dithiothreitole due to oxidation of thiol groups [38,54]. It was also found that BA and other PCAs inhibit production of Reactive Oxygen Species (ROS) in neutrophils, while ROS are known to impair phagocytic activity [38]. These results are consistent with other published data [55,56].

Sodium benzoate at 0.5-2 μM was reported to markedly suppress Lipopolysaccharide (LPS) - induced production of some cytokines (TNF-α, IL-1β), NF-κB and iNOS (inducible NO-Synthase) by microglia. Although exposure time to Sodium Benzoate (SB) (i.e., duration of microglia cells incubation with SB) before LPS addition to the culture medium was of critical importance for accomplishment of SB effects [57]. Spanish group also reported the inhibitory effect of other bacterial metabolites 3,4-dihydroxy-phenylpropinic and 3,4-dihydroxy-phenylacetic acids on production of pro-inflammatory cytokines (TNF-a, IL-1b and IL-6) in mononuclear cells [58].

Addition of SB to microglia cell culture was associated with down-regulated expression of superficial CD-markers and class II Major Histocompatibility Complex (MHC II). Similar phenomena were reported in experiments with human astrocytes [57].

Marked inhibition of fatty acids oxidation was induced in experimental setting by SB added to homogenized murine liver at 0.5-2 μM, meanwhile parenteral administration of 5-10 mmol/kg (1220-2440 mg/kg) SB to rats resulted in significant reduction of ATP, CoA and acetyl-CoA and increased levels of ammonium in liver tissue [59].

Microorganisms and pH values		*S. aureus* NCTC 4163		*P.aeruginosa* NCTC 6749		*B. subtilis* NCTC 10400		*C. albicans* ATCC 10231	
		pH=5	pH=7	pH=5	pH=7	pH=5	pH=7	pH=5	pH=7
BA	MIC	500	1000	250	1000	130	1000	130	>1000
	MBC/MFC	500	1000	250	>1000	130	1000	250	>1000
SB	MIC	390	6250	1560	25000	190	6250	12500	25000
	MBC/MFC	>50000	>50000	6250	25000	390	50000	25000	>50000

Table 4: Correlation between Media PH Levels and MIC, MBC and MFC Values for BA and Sodium Benzoate against Different Microorganisms, mg/L [34].

MIC - Minimal Inhibitory Concentration

MBC - Minimal Bactericidal Concentration

MFC - Minimal Fungicidal Concentration

Note: Effect of pH values on antimicrobial potential of BA and sodium benzoate. Lower BA and SB concentrations are required to suppress bacteria in acidic environment, which is consistent with weak organic acids theory [11].

Membrane transport of low-molecular weight metabolites

Maintenance of constant intracellular pH value in changing environment is one of principal cell functions to provide cell survival.

Lin J et al., reported glutamate-induced bacterial resistance to acidic environment as more effective mechanism than arginine-dependent resistance used by **E. coli** [60]. These mechanisms have not yet been clarified, but experiments with *E. coli* established enhanced expression of more than 30 proteins in response to BA challenge [61].

Membrane H+-ATPase is responsible for proton removal from intracellular space in *Saccharomyces cerevisiae*. This process is instantly intensified after BA addition to culture media [50]. *Saccharomyces cerevisiae*, in contrast to *Zygosaccharomyces bailii*, can't metabolize BA anions, thus they expel BA anions from the cell by transport carriers. Induction of Pdr12p- transporter synthesis is considered to be the principal mechanism of *Saccharomyces cerevisiae* adaptation to BA, providing benzoate removal by active transport mechanism [51].

Membrane carrier Pdr12p belongs to ABC-transporters super-family (ATP-binding cassette) and, apart from benzoate it also transports other anions of weak organic acids, including *p*-HPAA и PAA anions [17]. ABC-transporters were found in both prokaryotes and eukaryotes, including humans [62,63]. ABC-transporters play key role in bacterial resistance to antimicrobials in prokaryotic organisms, while in humans - in resistance to anticancer drugs [64].

BA anion transport in mammals and humans is executed by proton-dependent Monocarboxylate Transporters (MCTs), and Sodium-dependent Monocarboxylate Transporters (SMCTs) from MFS super family (Major Facilitator Super Family). MCTs family is represented by at least 14 membrane proteins, responsible for transportation of low-molecular weight monocarbonic acids, thyroid hormones, and such important for basal metabolism monocarboxylates as lactate, pyruvate and acetoacetate. SMCTs, harnessing sodium gradient, transport lactate, pyruvate and ketone bodies from extracellular environment in intestinal and renal epithelium and brain [65-67].

MCT1 is a universal transporter for the majority of tissues and organs, including BBB (blood-brain barrier), while some other MCTs are characterized by organ specificity. MCTs maintain intracellular pH by removal from cytosol of organic acids, produced via glycolysis and other metabolic processes. Muscular cells, erythrocytes and cancer cells are highly MCTs-dependent due to active glycolysis and intensive production of organic acids [68]. Liver and kidneys can utilize lactate for gluconeogenesis, while smooth cardiac and stripped skeleton muscles use lactate for "breathing" [69,70]. Depending on the tissue and it's functional activity MCTs either remove monocarboxylate organic acids from the cell, or transport them into the cell. In general, specific transport of mono- and C4-dicarboxylates plays key role in energy metabolism of eukaryotic cell, linking intracellular and systemic metabolic processes in the entire organism [71]. It has been demonstrated by now, that aromatic acids, such as BA and phenylpyruvic, can inhibit MCTs and interfere with cell capacity to maintain optimal intracellular pH value under elevated intracellular concentration of PCAs, thus changing enzymatic pathways inside the cell [68,72-76].

BA metabolism by microorganisms

Major metabolic pathways, participating enzymes, intermediary and final products of aerobic and anaerobic BA disintegration are shown in table 5 and figures 4 and 5 [77].

Pyrocatechin-as the key BA aerobic disintegration intermediate - is similar to endogenous catecholamines with inherent potential to raise blood pressure and dilate airways [83-86].

Fermenting bacteria do not get any energy benefit from aromatic ring breakdown. While nitrate-reducing bacteria and aerobes yet to greater extent utilize Acetyl-CoA in tri-carbonic acids cycle producing considerable amounts of ATP, thus over-compensating energy losses for BA metabolism. Formation of benzoyl-CoA also occurs in anaerobic degradation of PAA, phenol, *p*-cresol, aniline, BA precursors, and *p*-hydroxy BA [87-89].

In current study sepsis associated gram +ve and gram -ve bacteria produced a range of PCAs (predominantly PLA and *p*-HPLA, with the exception of *Pseudomonas aeruginosa* and *Acinetobacter baumanii* producing *p*-HPAA predominantly), although their potential to synthesize PCAs in pure culture is much lower than capacity of obligate anaerobes. As anaerobic bacteria do not get any energy benefits from aromatic ring breakdown, they had to develop some mechanisms of resistance to PCAs (biochemical or symbiotic) in order to tolerate accumulated amounts of PCAs in the environment.

PCAs excretion from human organism

p-hydroxyphenylpyruvic acid is the only PCA a human being can metabolize via homogentizine acid by aromatic ring breakdown. BA in humans and animals will be conjugated with glycine by glycine-transferase in kidney and liver mitochondria and excreted with urine as Hippuric Acid (HA) [90-92].

Pathway/Key Enzyme	Intermediary products	Final products
Aerobic		
β-keto-adipine pathway/dioxygenase intrinsic for both - bacteria and fungi. [6,78-80].	Catechol (pyrocatechine)→ ortho-disintegration Protocatechuic acid)→ ortho-disintegration into β-carboxy-cys-cys-muconic acid [77]. Catechol and protocatechuic acid→ meta-clevage	→ pyruvate and acetaldehyde [77].
Monooxygenase	4-hydroxybenzoic, 3,4- dihydroxybenzoic (protocatechuic), 2,5- dihydroxybenzoic (gentisine) acids [48,77,81]. Benzoyl-CoA→	Acetyl-CoA and succinyl-CoA [82].
Anaerobic		
	Benzoyl-CoA sequential reduction of double bounds in the ring and its' ultimate breakdown	Acetyl-CoA and carbon dioxide

Table 5: Major Metabolic Pathways of Aerobic and Anaerobic BA Disintegration.

Note: In anaerobic conditions the oxygen is not present to activate the aromatic ring. Anaerobic BA degradation starts form formation of Benzoyl-CoA, with further sequential reduction of double bonds in the ring and ring cleavage resulting in Acetyl-CoA and carbon dioxide as final products.

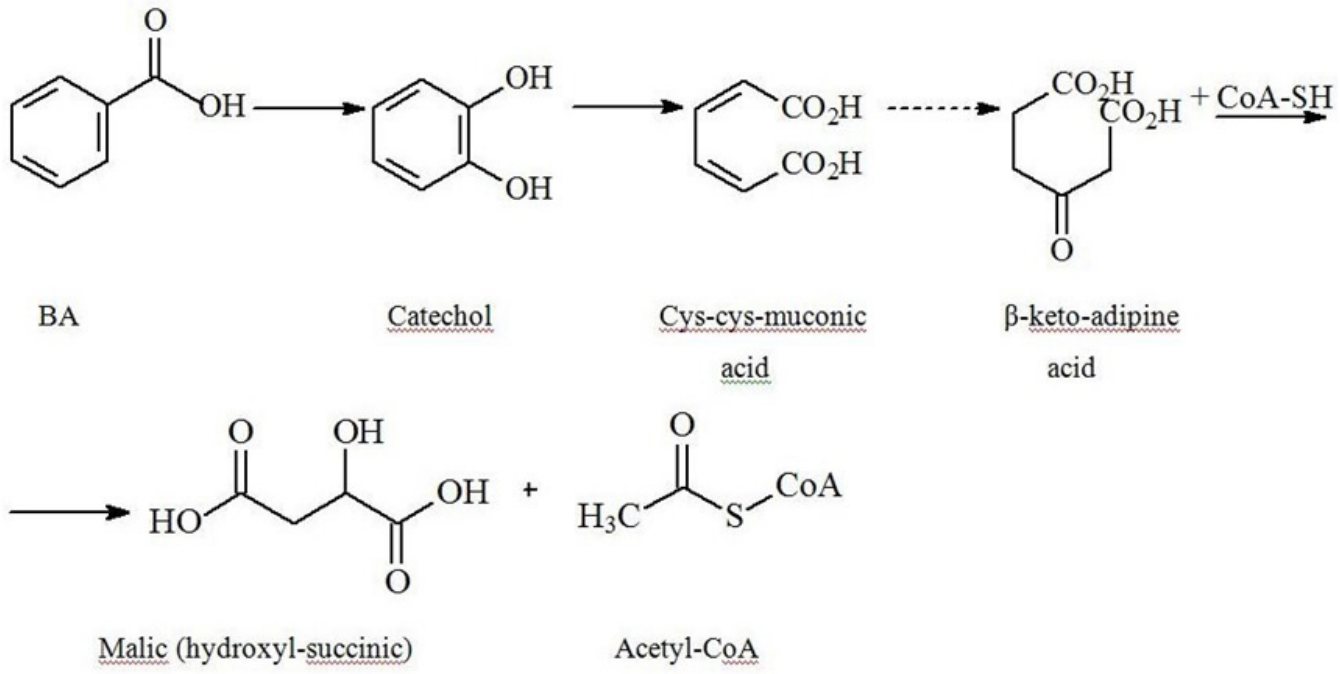

Figure 4: Aerobic and B-Keto-Adipine Pathways of Bacterial BA Degradation [77].

Note: Dioxygenase is required for aerobic - predominantly β-keto-adipine - pathway of BA degradation with catechol as intermediary product, which is further subjected to ortho-cleavage between two hydroxylated carbon atoms.

Besides, part of BA (20%) is excreted via conjugation with glucuronic acid [93]. Based on published data from human tissue cultures study average BA of glycine conjugation rate in human liver is equal to 254±90.5 nmol/min per 1 gram liver tissue (range 94.4 - 564), in renal cortex homogenates it is somewhat higher i.e., 321±99.3 nmol/min per 1 gram renal tissue (range 63.3 и 542) [94]. In humans PAA and *p*-HPAA are known to form conjugates with glycine and glutamine, PPA with glycine, although international metabolome databases give no references on *p*-HPAA and PAA.

In a healthy individual 70% of resulting Hippuric Acid (HA) after ingestion of a single BA test dose is excreted with urine within first 6 hours [84]. This is actually a description of the Quick's (Hippuric Acid) test used for liver failure assessment.

HA concentration will be considerably elevated in individuals with renal failure, moreover, HA is commonly recognized as a uremic toxin [95,96]. Of importance, C. *jejuni* from Campylobacter genus was recently identified as a pathogen causing gastroenteritis, and it differs from non-pathogenic *E. coli* by its' capacity to degrade HA to BA and glycine [97].

Results from our most recent experimental *in-vitro* study indicate that *Bacteroides spp*. are able to consume sepsis-associated *p*-HPLA and *p*-HPAA. This finding suggest that *Bacteroides spp*. the most predominant bacterial species in human gut microbiota may be involved in elimination of alternative tyrosine metabolic pathways products from the human body in parallel with endogenous mechanisms of detoxification [98]. This study was awarded the Sepsis Forum 2014 prize (Institute Pasteur, France).

PCAs dose-dependent effects in humans and animals

Peroral BA LD50 in rats 3040 mg/kg in mice is 1940-2263 mg/kg, while BA LD50 in cats is twice as low, because cats have lower glucuronidation capacity. Peroral SB LD50 in rats varies within 2100-4070 mg/kg according to data from different sources [18]. NOAEL (No Observed Adverse Effect Level) for long-term multiple

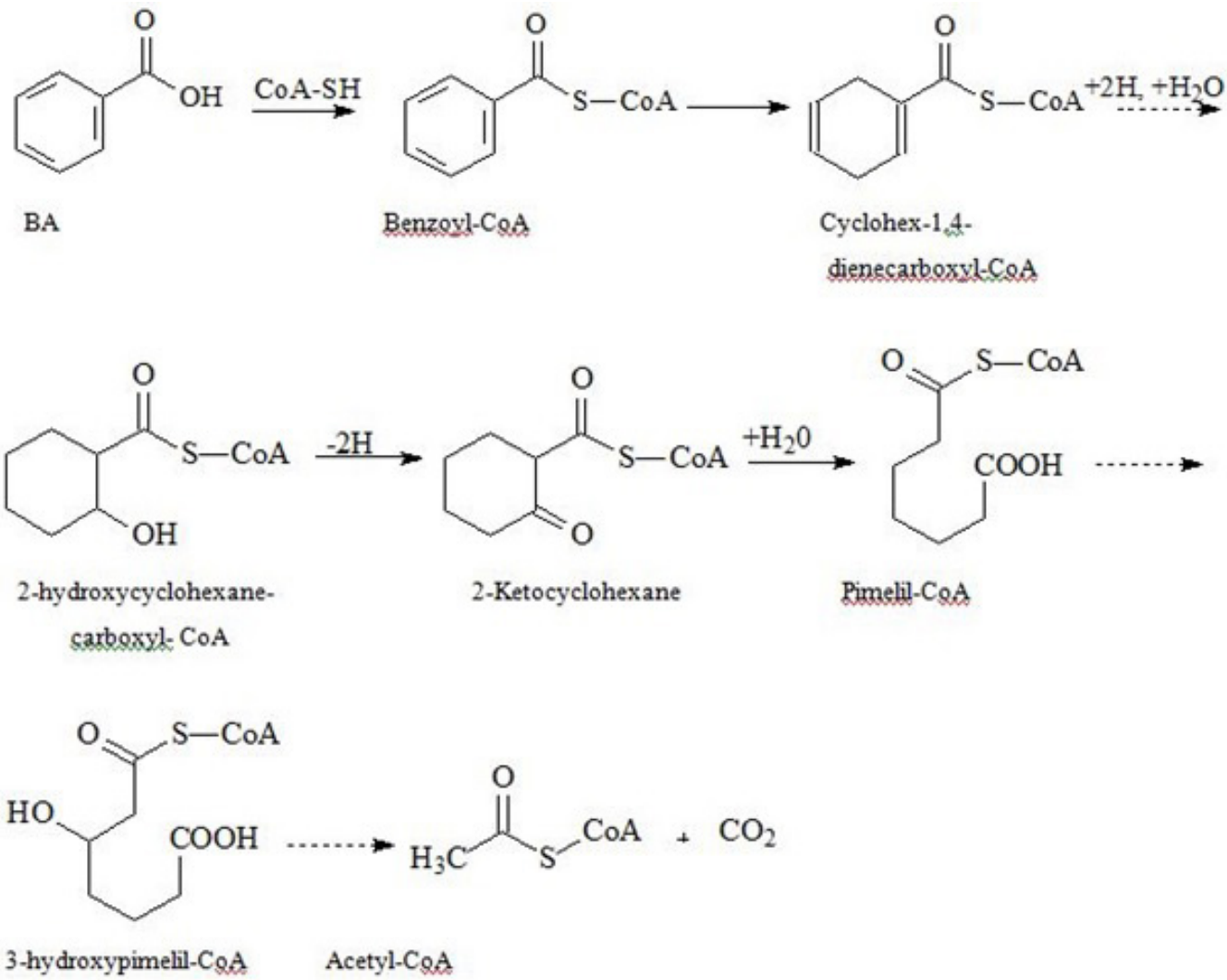

Figure 5: Aerobic Pathways of Bacterial BA Degradation [77].

peroral BA doses is 800 mg/kg/day. With higher doses liver and renal toxicity, weight loss and increasing morbidity were documented in experimental settings [93]. Meanwhile, supplementation of experimental diet with glycine is commonly known to enhance tolerability to benzoate toxicity [99].

Biotransformation rate of BA and of it's salts in humans varies within 17-29 mg/kg/h and does not seem to be dose-dependent [18]. BA peak plasma concentration is reached within 1-2 hours [18], and metabolic acidosis inevitably occurs after ingestion of BA 1000 mg/kg/day, usually associated with hypokalemia and hypocalcaemia. In a volunteer study incremental increase of BA doses up to 2500 mg/day was associated with nausea, headache, fatigue and heartburn. Manifestations of BA toxicities were documented when serum concentrations exceeded 800 mg/L (6.55 mM) [84].

In our cohort of patients with sepsis the documented average cumulative serum concentration of clinically significant PCAs was equal to 25.7 μM (with 25% and 75% inter-quartile range 13 μM and 59.2 μM, respectively) [10]. Published data indicate that in phenylketonuria cases cumulative levels of PLA can cross the threshold of 50 μM [100].

Sodium benzoate is administered as therapeutic agent perorally or as i/v infusions at 250-500 mg/kg/day to treat hyperammonemia in individuals with urea cycle disorders [101], and therapy is often associated with nausea [18]. SB brakes bilirubin-albumin bond, releasing bilirubin into circulation and increasing serum concentration of free bilirubin with inevitable manifestation of it's toxicity [102,103]. Therapy with SB is also associated with elevated levels of blood tryptophan and cerebral serotonin, behaving as hunger suppressants. In lab experiments trypsin benzoate induced modification of three-dimensional protein configuration [104]. Impaired HA synthesis in schizophrenia has been documented by some authors [105]. Impaired mental state and severe acidosis resulting in 2 deaths have been reported in 3 published cases of SB overdose [106].

FDA classifies BA and sodium benzoate as substances that are Generally Recognized as Safe (GRAS-listing). JECFA Committee (The Joint Fao/Who Committee on Food Additives) considers as Acceptable the Daily Intake (ADI) of BA and sodium benzoate equal to 0-5 mg/kg body weight [107].

Conclusion

Low-molecular weight aromatic acids such as benzoic and other phenylcarboxylic acids, known as intermediate and final products of bacterial metabolism, demonstrate bioregulatory activity directed

not only at micro but also at macro-organism (i.e., human body as a whole).

It has been stated that the severity of disease correlates with cumulative Aromatic Microbial Metabolites (AMM) load i.e., summative AMM concentrations in patient's blood serum [10,11]. Following the universal theory of weak organic acids, PCAs' mechanism of action is also universal and uniform: they cause acidification of intracellular medium, inhibit ATP synthesis and/or deplete intracellular ATP deposits, i.e., make certain input into development of cytopatic hypoxia in sepsis [108,109]. Microorganism's sensitivity to PCAs varies considerably, as it has been shown above, but there's a general trend to enhancing PCAs' toxicity with growing acidification of the environment (acidosis).

Summarizing published and own data, we outline the following statements and proposals for future research to better substantiate the hypothesis on two integrated (microbial and host's) metabolisms, and the role of microbial exometabolites in host's homeostasis [6]:

1. In periepithelial layers of human natural biocenoses, and at the borders of any infectious tissue lesion (in pericapillary and periendothelial space) PCAs levels reach the values, sufficient for local and/or systemic manifestation of their biological effects, leading to modified composition and biological activity of the microbiota, and modified reactivity of immune-competent cells and tissue-specific functions, etc.
2. In some clinical conditions (such as sepsis, shock, hypoxia, severe renal/hepatic failure, mitochondrial dysfunction and oths.) distribution patterns of PCAs' precursors, PCAs and their metabolites would considerably differ from the patterns found in a healthy individual. Any deviation from healthy pattern can results in modified biological activity of metabolites, also due to changes in their intracellular concentrations.
3. Accumulation of new data on PCAs would provide a background for future development of novel therapeutic strategies, such as: regulation of the composition and metabolic activity in natural microbiocenoses and pathologic biotopes (foci of infectious lesions) in the human body; stewardship of human metabolism; regulation of immune-cell reactivity via correction of metabolic profile, etc.

We need deeper insight into natural regulation of microbiocenosis, into signal pathways and mechanisms, which provide integration of two metabolisms, i.e., of the host and of it's microbiota. The main intention of this review is to draw attention of professionals to the development of new therapeutic approaches based on regulation of local and systemic balance of aromatic microbial metabolites in human organism, so that to improve clinical outcomes in most severe conditions, such as sepsis.

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