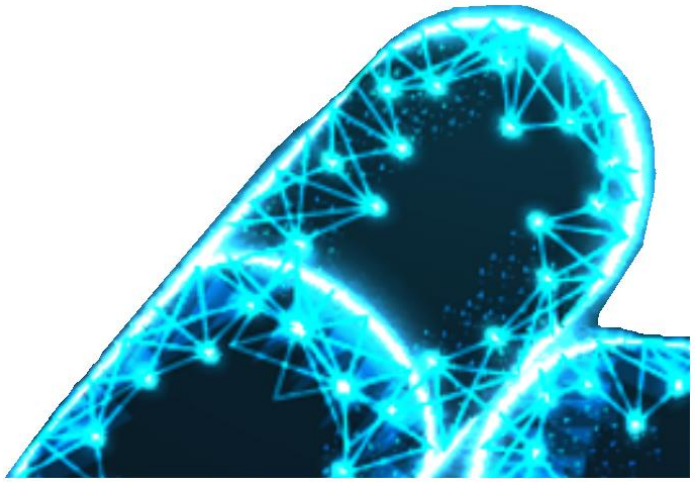
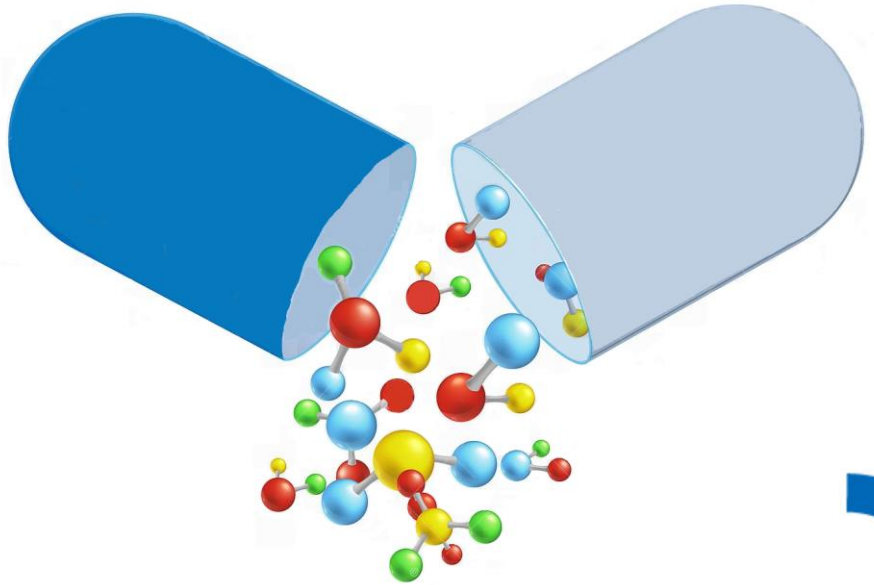
SIMBA Capsule

Small
Intestine
Micro**B**iome
Aspiration
Capsule





Metabolic Profiling of the Small Intestine

Metabolomic Profiling SIMBA capsules **NIMBLE Scientific** captures a complex metabolome measured by **HMT** that reveals significant interactions between diet, microbiota and the local microenvironment of the small intestine. Profiling and pathway analysis can allow physicians and researchers to better understand and treat intestinal diseases and dysbiosis.

Alexander Buko, PhD VP HMT-America

Introduction

- In a healthy colon, the microbiota produces vast amounts of **metabolites** that are essential to maintaining homeostasis in the colon microenvironment. In fact, these metabolites produced by the microbiota have been linked under certain conditions to diseases such as obesity, cardiovascular disease, and colorectal cancer.
- Microbiota and metabolites have location-specific signatures along the intestinal tract.
- The mucosal host-microbiota metabolic interactions along a healthy human intestinal tract are largely unknown. Although the microbiota and metabolome variations along the intestinal tract have been investigated in rodents and other animals, the dietary and anatomical differences between humans and these animals render these data less informative for humans.
- The **small intestine** is a highly specialized organ that is the major site for digestion and nutrient absorption as well as serving critical roles in oral drug uptake and metabolism, while also being a target of many pathogens and toxins. It has also been recognized that metabolic factors generated by the gut microbiome form gradients from the lumen to crypt that likely impact development, health and disease of the intestine as well as the organism as a whole. Despite a wealth of indirect evidence, the detailed role of various chemical factors and their concentrations within the crypt-villus microenvironment remains poorly understood **up to now**.
- **NIMBLEs' SIMBA** allows researchers to probe into microbiome metabolome within the small intestine.

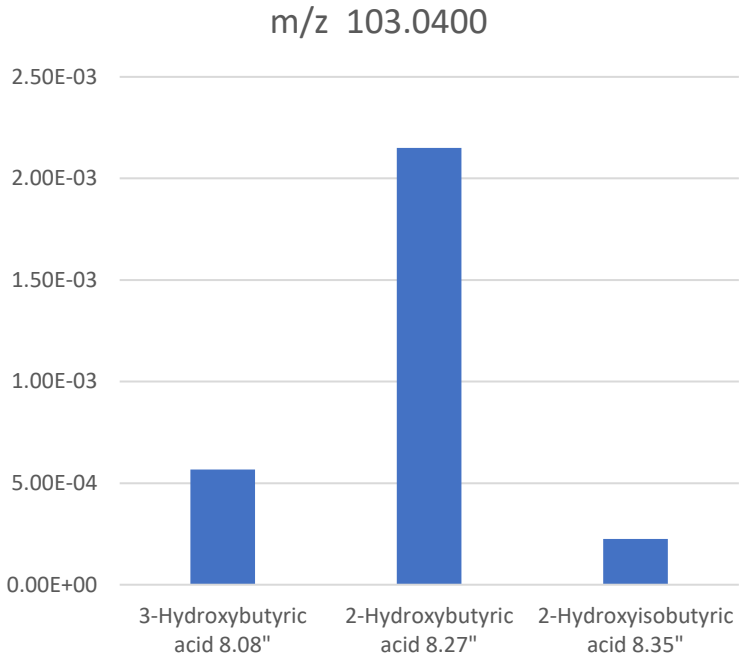
HMT OMEGA Scan advanced

- HMT is a metabolomics company specializing in small bioactive polar metabolomics.
- HMT has a polar metabolite library of almost 2,500 and lipid library of 1,500 in addition to the provision to produce raw data and metabolite ID to identify novel metabolites.
- HMT provides an extensive list of metabolites produced within the small intestine, utilizing 20 ul of capsule contents using our OMEGA Scan advanced method.
- OMEGA Scan Advdanced provides both quantitative and non quantitative measurements.
- OMEGA Scan uses proprietary capillary electrophoresis mass spectrometry (CE-MS) platform with high resolution and high sensitivity to detect over 950 metabolites in SIMBA capsule.
- CE-MS captures small polar metabolites that provide insights into food digestion, nutrient flux and bacterial metabolism.
- HMT provides dedicated experience scientists to provide support for statistical and biological/pathway interpretations for biomarker discovery.
- CE-MS has the highest separation / resolution of any chromatographic method allowing for highest specificity of identification of isobaric metabolites both structural and positional isomers, as well as, highest coverage of polar metabolites over HILIC and rpHPLC methods.

- Following examples from SIMBA data represent high resolution separation of structural and positional isomers using CEMS.

- With the possibility of 1000s of polar metabolites to be found in the small intestine, many will have the same mass and same elemental composition, but different structures, functions, sources, biology and health implications.

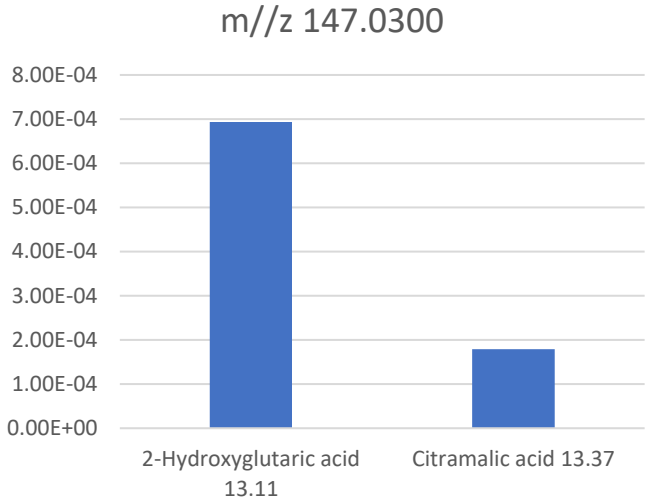
- Metabolite specificity is one key to understanding the wealth of information available in the SIMBA capsule.



3HBA – From BCAA catabolism – a ketone body high in diabetes.

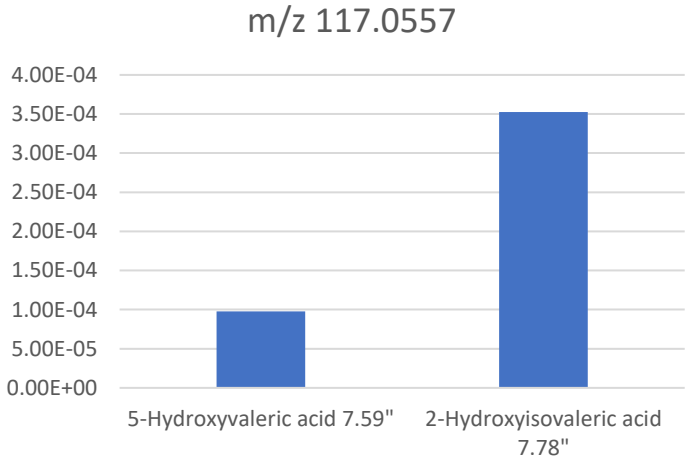
2HBA –from glutathione hepatic anabolism as oxidative marker

2HIBA -a natural product found in Salmonella enterica, Vitis vinifera, and other organisms



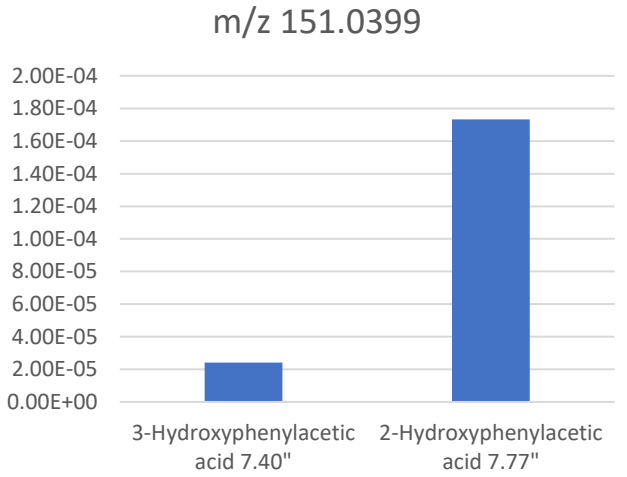
Citramalic acid can also be used as a urinary marker for gut dysbiosis

2-HG generated in high abundance when IDH is mutated in the TCA cycle.



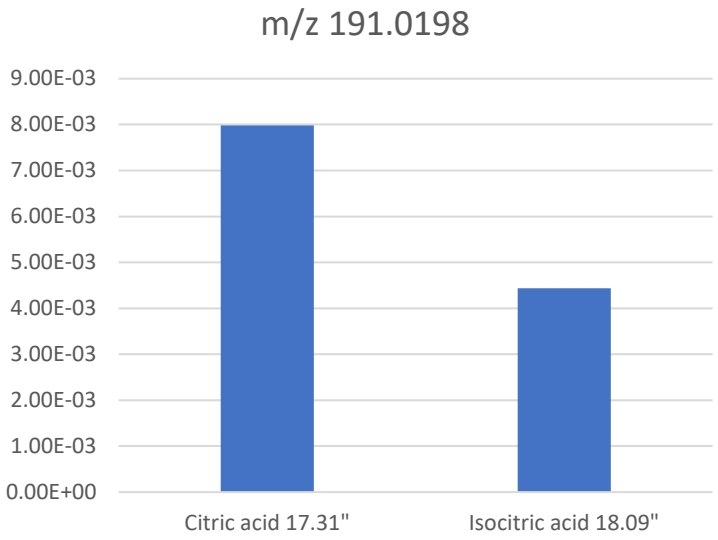
2HIVA patients with phenylketonuria, lactic acidosis and ketoacidosis from BCAA Metabolism.

5HVA is OMEGA fatty acid found in diet, oxidative stress.



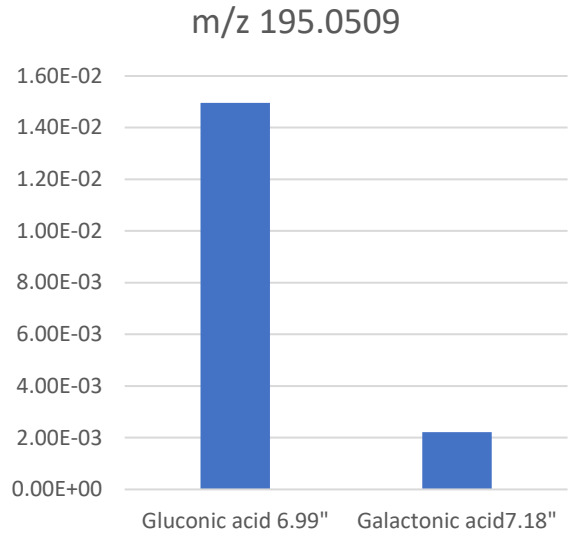
2-hydroxyphenylacetic acid is associated with phenylketonuria and also a microbial metabolite.

3-hydroxyphenylacetic acid is a marker of gut Clostridium species. Higher levels are associated with higher levels of Clostridia and a Tyrosine metabolite.



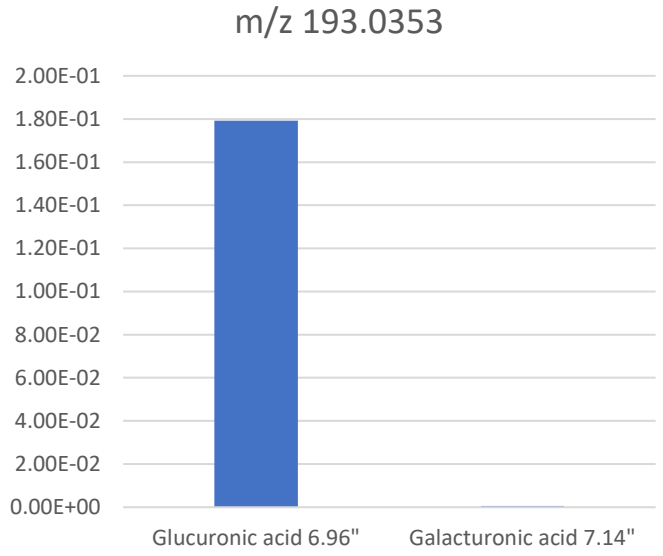
Citrate – intermediate in TCA cycle and exported from mitochondrial for Fatty acid synthesis, xenobiotic, chelator.

Isocitrate - acted upon by IDH to form 2-OG within mitochondrial matrix.



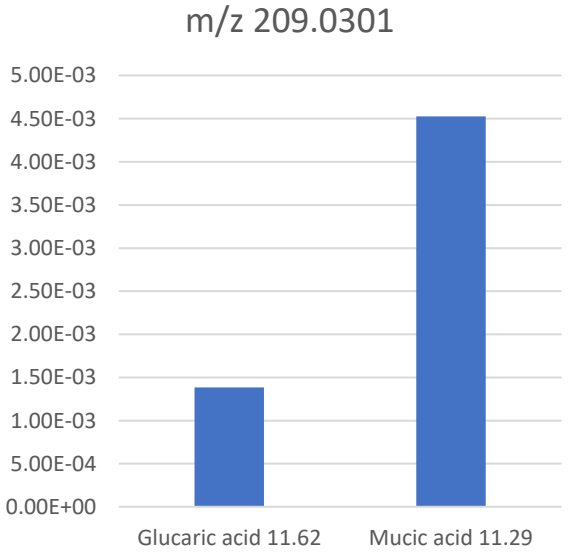
Gluconate – xenobiotic, cellular from pentose phosphate pathway

Galactonate - breakdown product of galactose, may be indicative of acidosis and liver dysfunction



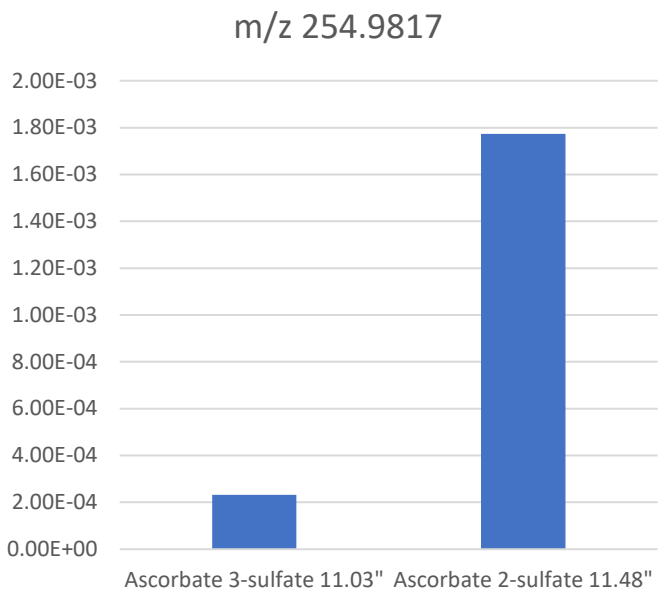
Glucuronate – source liver, detoxification of xenobiotics and waste products.

Galacturonate - oxidized form of d-galactose and xenobiotic.



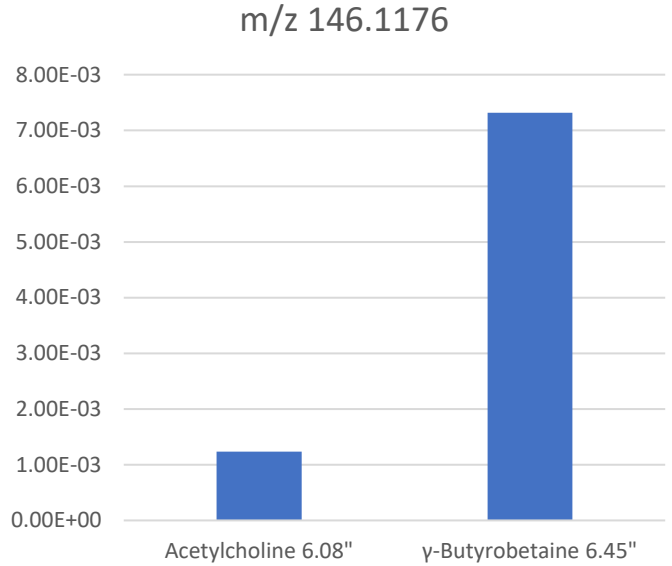
Glucarate - found in fruits, vegetables, and mammals from glucose oxidation

Mucic acid - oxidation of galactose, xenobiotic and osmoregulator



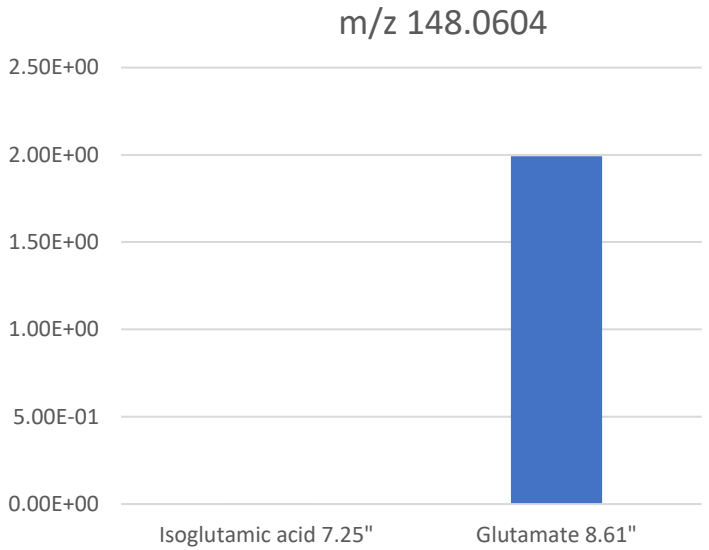
Ascorbate -2S : a Phase II metabolite of vitamin C arising from the action of a liver-derived sulfotransferase. Connected to collagen synthesis.

Ascorbate -3S : Whereas 2Sulfate is an endproduct of Ascorbate degradation, 3S may act as a sulfate reservoir for bacteria.



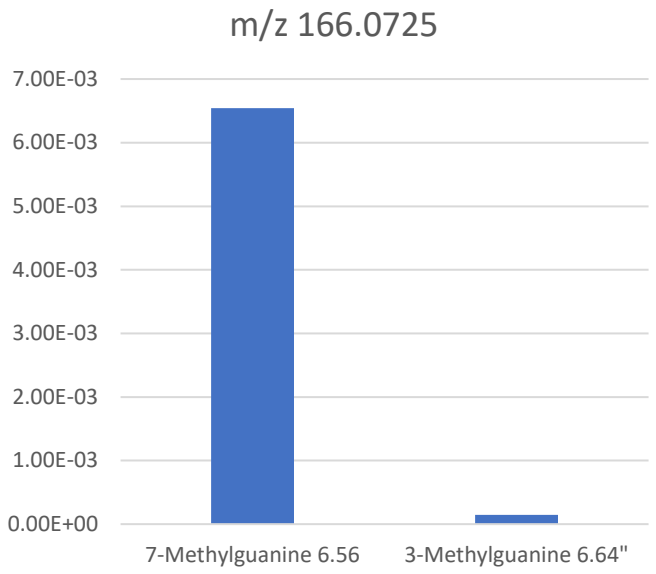
AC – while a neurotransmitter, also a microbial product produced by Lactobacillus

GBB – osmolyte and precursor to carnitine.



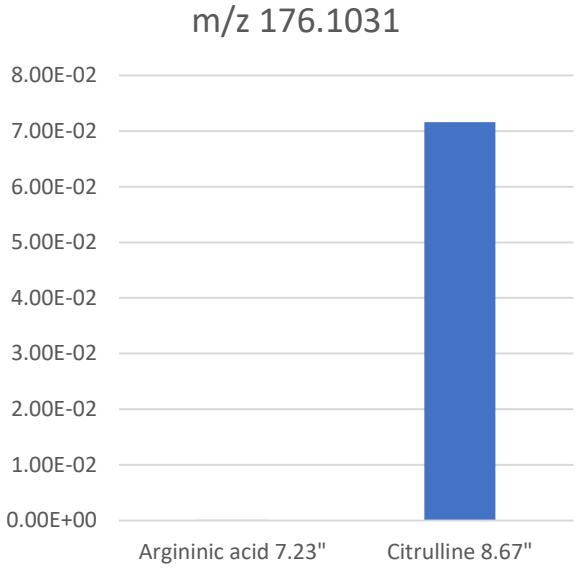
Glutamate is common amino acid found in diet and cells. Essential for many biological functions.

IsoGlutamate – histidine metabolite often found in bacterial cell walls, non standard amino acid.



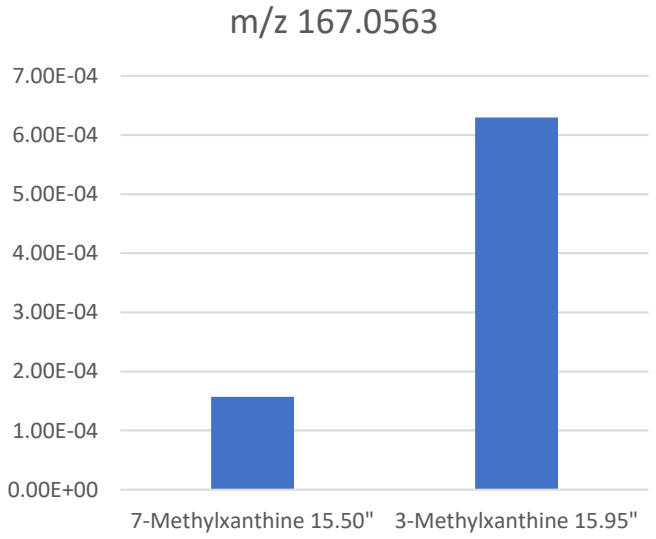
7MG - metabolite found in or produced by Saccharomyces cerevisiae , found higher in CRC.

3MG – minor purine observed in E. Coli.



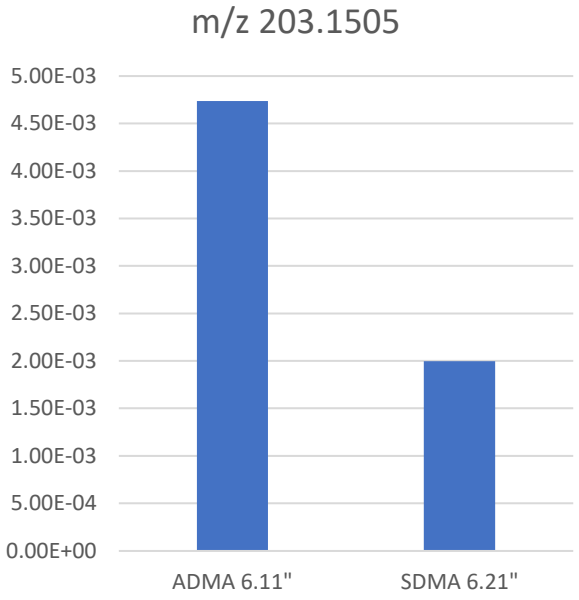
Argininic acid - deficiency of liver arginase, associated cirrhosis and uremia

Citrulline – bacterial source for synthesis of Arginine in liver



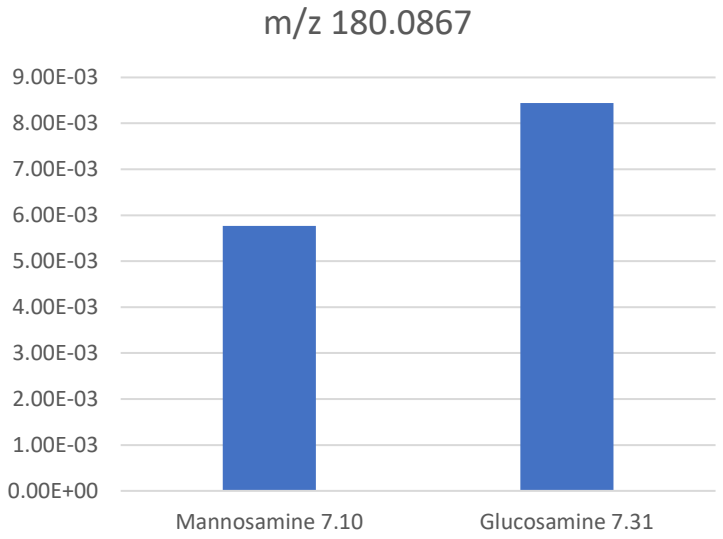
7MX – caffeine metabolite mainly catalyzed by CYP1A2 with activity to adenosine receptors. markers of abnormal purine metabolism.

3MX – Xenobiotic, phytochemical



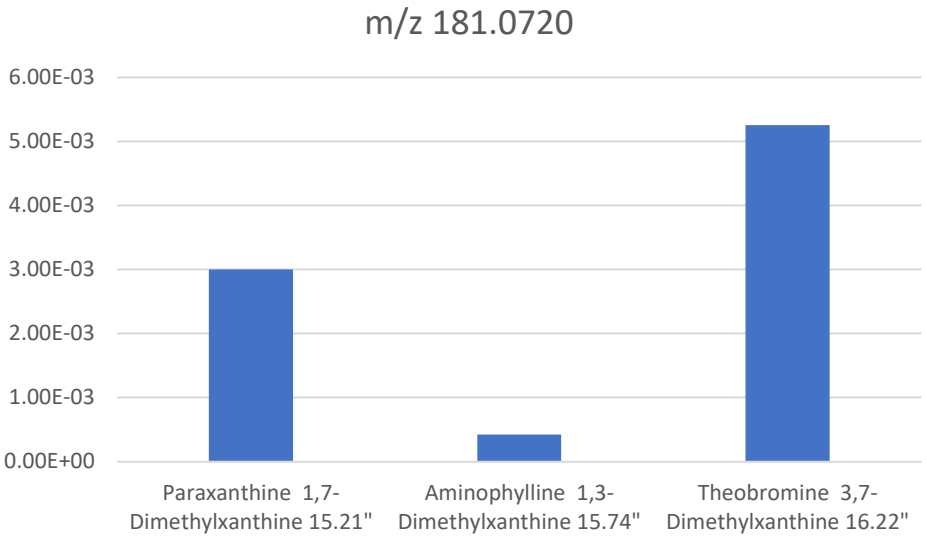
ADMA interferes with L-arginine in the production of nitric oxide, a key chemical to endothelial and hence cardiovascular health.

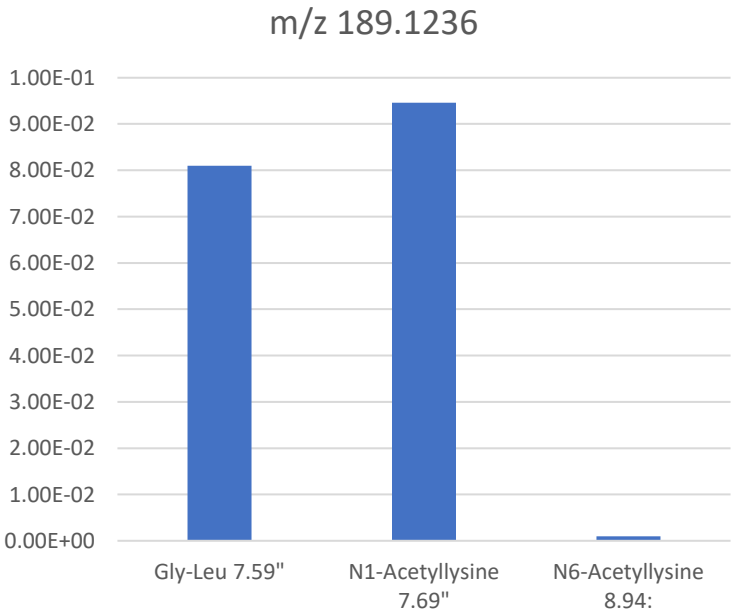
elevated levels of SDMA occur in patients with vascular disease, especially suffering end-stage renal disease.



D-Mannosamine is an amino monosaccharide.[1] It has been found as a component of LPS isolated from strains of *Salmonella* and *E. coli*.

D-Glucosamine is an amino monosaccharide and a precursor in the biosynthesis of UDP-N-acetyl-D-glucosamine. It inhibits bone erosion and loss of glycosaminoglycans and proteoglycans in joints.





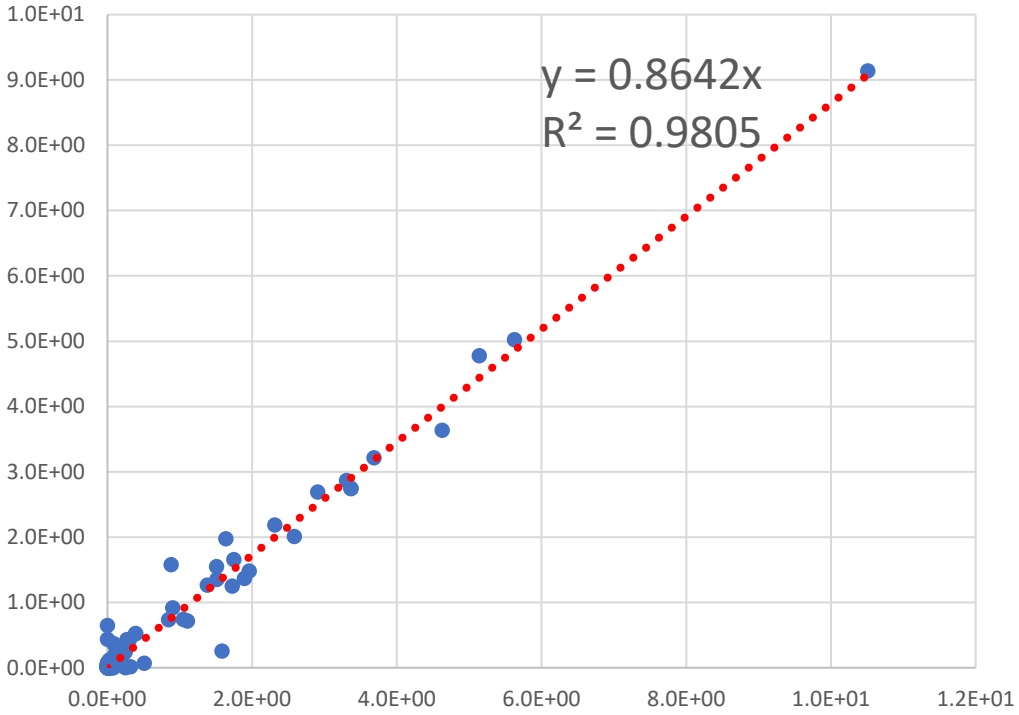
N-6-Acetyl-L-lysine can be released by an N-acylpeptide hydrolase from histones going through proteolytic degradation, implication in protein activation, gene activation.

N-1-Aceteyl –L-Lysine subject to both enzymatic and non-enzymatic acetylation and my be regulated by microbiota.

PX – caffeine metabolism catalyzed by CC P450 1A2

AP – Caffeine metabolites mediated by the CCP450 1A2, CC P450 3A4, CC P450 2C8, CC P450 2C9, and CC P450 2E1, acts as adenosine A2B receptor and blocks adenosine mediated bronchoconstriction. Xenobiotic.

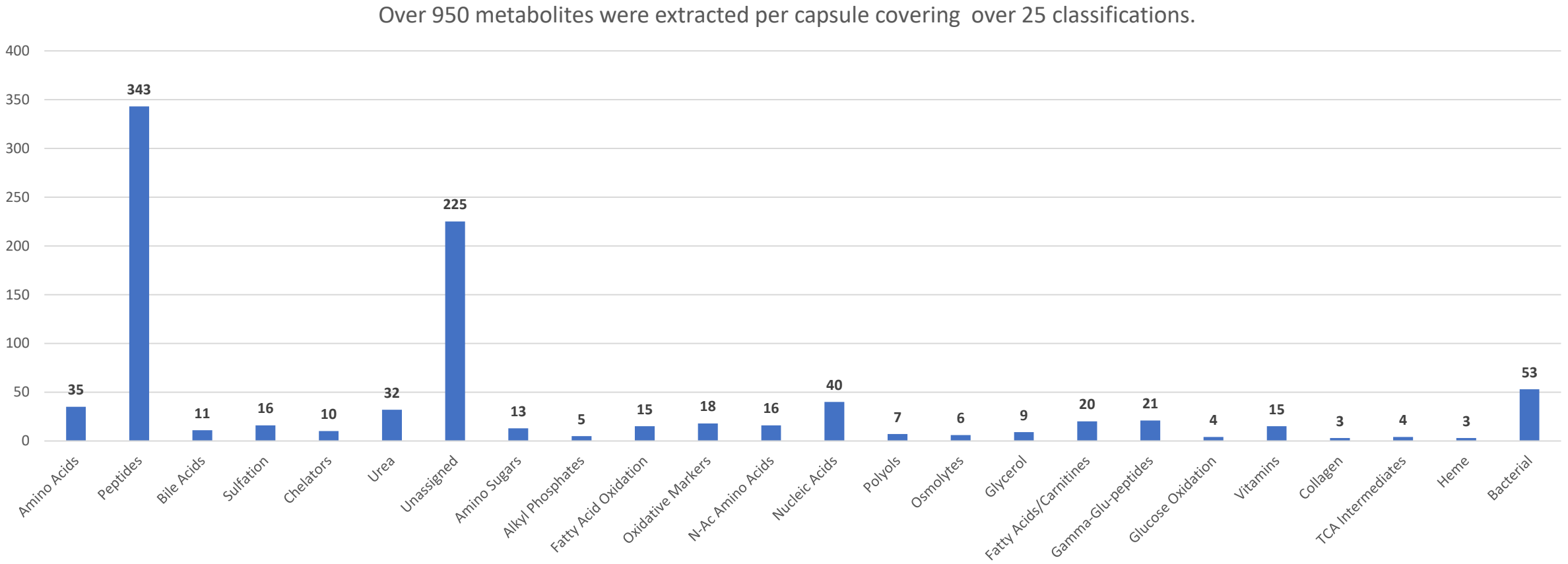
TB – phytochemical, principle alkaloid in Theobroma cacao. contributing factor in acid reflux



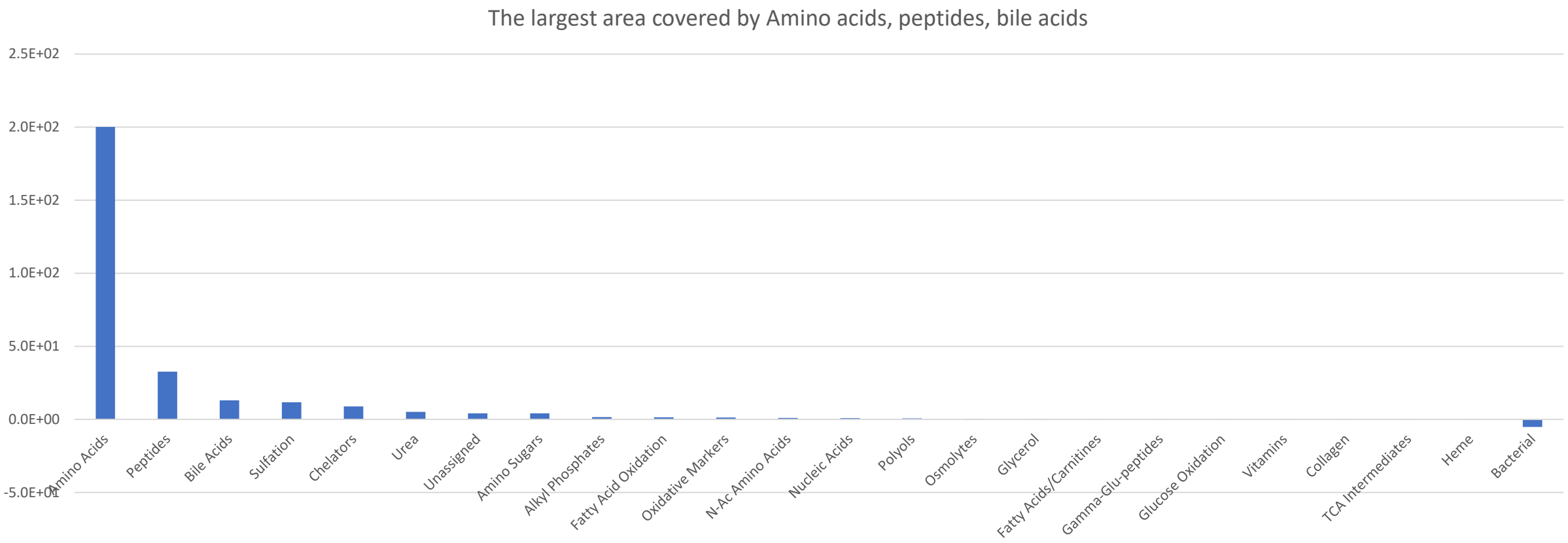
Reproducibility of both the SIMBA capture of small intestinal fluids and the HMT OMEGA scan is key to success.

Two capsules from same patient has **high correlation and reproducibility reflecting both on the SIMBA method and the metabolomics method capabilities.**

Each capsule contains 50 to 100 ul intestinal fluid, for which, 20 ul is required for metabolomic analysis, the remaining fluid can be used for orthogonal testing for protein, DNA and other macromolecules.



From 20 ul, over 950 validated metabolites are observed. These metabolites fall into one of over 20 classifications. Some are known from diet, others from host microenvironment, others specifically from bacterial metabolism while others are some combination. Many have specific metabolic pathways and biochemical implications that can be related to homeostasis, disease, aging or therapeutic effects of drugs or probiotics.



While the largest number of metabolites fall under peptides and class unassigned, the largest relative concentration of metabolites are amino acids and their degradation products, peptides (including anti inflammatory and anti oxidative peptides), then bile acids.

Functions of small intestine	Metabolite Classifications that support small intestine functions
Absorption Vitamins	Vitamins, Cholines
Absorption Minerals	Chelators
Lipid Sensing	Fatty acids, Glycerides Carnitines Amino Sugars, Bile Acids, Fatty Acid Oxidation products
Carbohydrate Sensing	Glucose oxidation, Polyols, Collagen
Protein Sensing	Amino Acids, Peptides Acetyl Amino Acids, Gamma Glu peptides, heme
DNA/RNA Sensing	Nucleic Acids
Ammonia Trafficing	Ureas
ROS	Oxidative markers
Osomtic Stress	Osmolytes
Microbiota	Bacterial

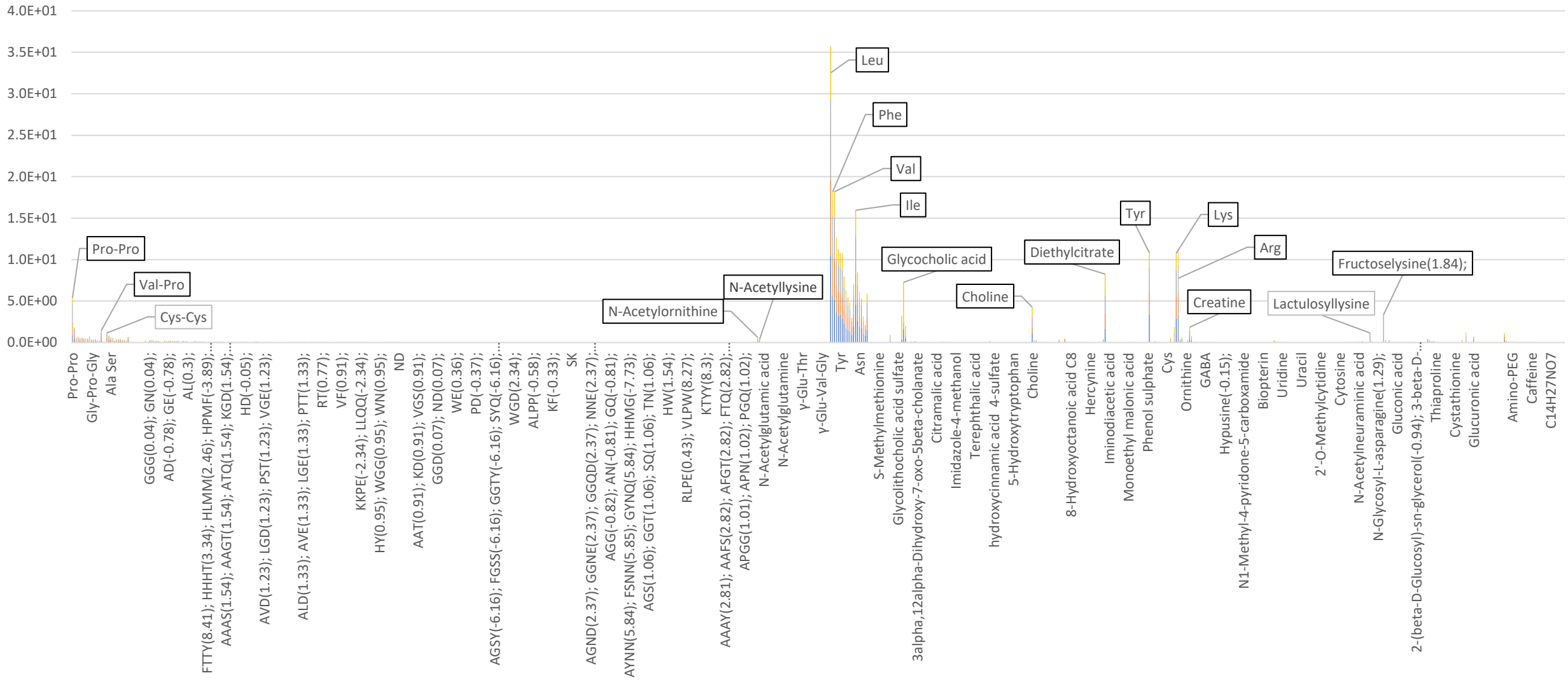
Functions of the small intestine include absorption of vitamins and minerals, lipid sensing, carbohydrate sensing, protein sensing. In addition, digestion of macromolecules such as proteins, carbohydrates and fats. Digestion of proteins create ammonia that may be disposed of in the colon. Such conditions within the small intestine would be expected to generate reactive oxygen species (ROS) and osmotic stress. Biodiversity within the small intestine helps the digestion process and maintaining healthy digestive system.

The metabolome observed in SIMBA capsule from healthy patients reflects these functions and how they work together.

Small intestine vs Colon

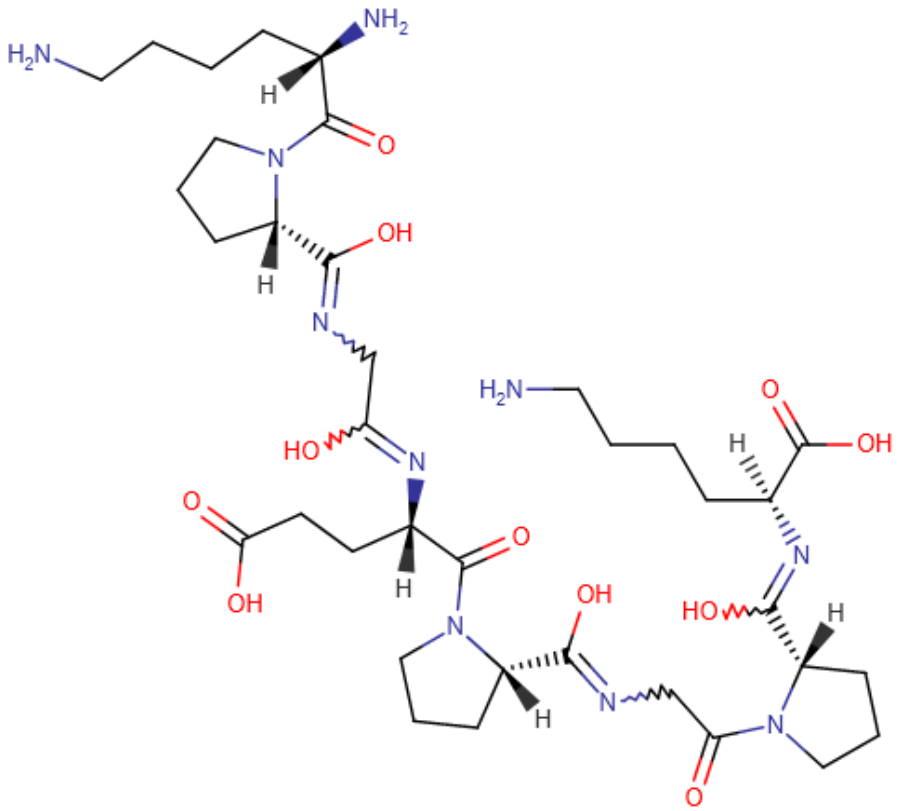
- Lower diversity and Lower pH in the small intestine
  - Duodenum 10^7 bacteria pH 5.6 - 8
  - Jejunum 10^7 bacteria pH 5.7 - 7.5
  - Ileum 10^11 bacteria pH 5.7 – 7.5
  - Vs
  - Colon 10^14 bacteria pH 6.7 – 8.5

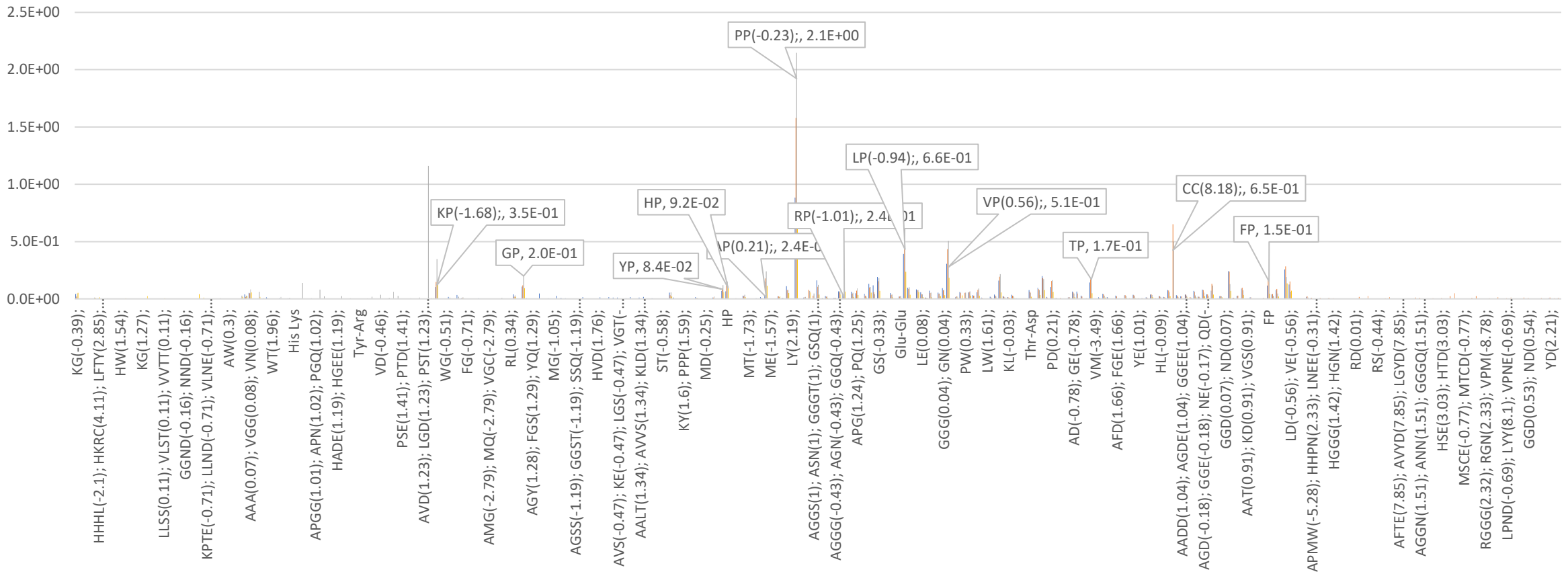
- In fact, too many bacteria in the small intestine can be problematic. SIBO – Small Bowel Bacterial Overgrowth is a disease of the short bowel.



967 Metabolites are observed from the capsule. Shown above are 745, those are abundant and consistent. Marked metabolites are the most abundant based on peak area. Most abundant metabolites include: Small peptides, BCAA/Aromatic/Basic amino acids, Acetylated Orn/Lys, Urea cycle intermediates, Choline pathway, Bile acids and Fructosyl/Lactosyl lysine.

Small Peptides



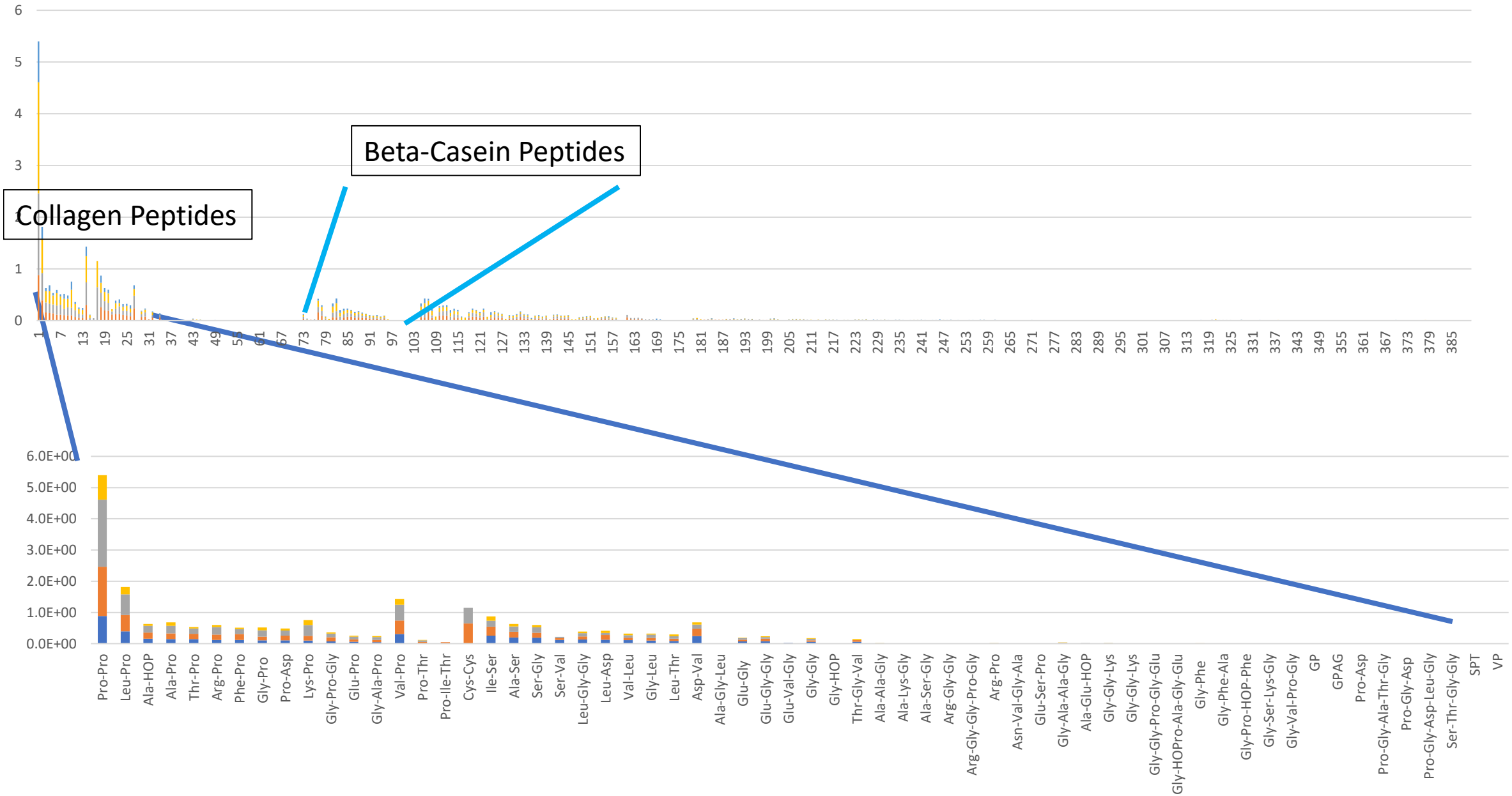


Over 350 small peptides are observed in small intestine. The most abundant are proline containing.

- Dietary proteins are, with very few exceptions, not absorbed. Rather, they must be digested into amino acids or small peptides. There are three sources that secrete proteolytic enzymes into the lumen of the digestive tract:
  - The stomach secretes pepsinogen, which is converted to the active protease pepsin by the action of low pH.
  - The pancreas secretes a group of potent proteases, chief among them trypsin, chymotrypsin and carboxypeptidases.
  - Microorganisms dispense various di and tripeptidases as well to digest endothelium tissues and proteins.
- Through the action of these proteases, dietary proteins are hydrolyzed within the lumen of the small intestine predominantly into small peptides and free amino acids.

- There is virtually no absorption of peptides longer than four amino acids into circulation. However, there is abundant absorption of di- and tripeptides from the small intestine. These small peptides are absorbed into the small intestinal epithelial cell by cotransport with H^+ ions via a transporter called PepT1.
- Once inside the enterocyte, the vast bulk of absorbed di- and tripeptides are digested into amino acids by cytoplasmic peptidases and exported from the cell into blood. A small number of these small peptides enter blood intact. Some of which have known biological activities such as antioxidants, neurostimulation or immunoregulation.

Out of over 350 peptides, the majority of the most abundant ones may come from Collagen and Casein.



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GPKGEPGAOGENGAPGQMGPRGLPGERGRPGAPGPAGARGNDGATGAAGPPGPTGPAGPP

GFPGAVGAKGEAGPQGPRGSEGPQGVRGEPGPPGPAGAAGPAGNPGADGQPGAKGANGAPG
IAGAPGFPGARGPSGPQGPGGPPGPKGNSGEPGAPGSKGDTGAKGEPGPVGVQGPPGPAGEE

GKRGARGEPGPTGLPGPPGERGGPGSRGFPGADGVAGPKGPAGERGAOGPAGPKGAOGE

AGRPGEAGLPGAKGLTGAOGAOGPDGKTGPPGPAGQDGRPGPPGPPGARGQAGVMGFPG

PKGAAGEPGKAGERGVPGPPGAVGPAGKDGEAGAQGPPGPAGPAGERGEQGPAGAOGFQG
LPGPAGPPGEAGKPGEQGVAPGPSGARGERGFPGERGVQGPPGPAGPRGANGAPGNDGAKG

DAGAPGAPGSQGAPGLQGMPGERGAAGLPGPKGDRGDAGPKGADGAOGKDGVRGLTGPI
GPPGPAGAPGDKGESGPSGPAGPTGARGAPGDRGEPGPPGPAGFAGPPGADGQPGAKGEPGD
AGAKGDAGPPGPAGPAGPPGPIGNVGAPGAKGARGSAGPPGATGFPGAAGRVGPPGPSGNAG

PPGPPGPAGKEGGKGPRGETGPAGRPGEVGPPGPPFKGAOGADGPAGAPGTPGPQGIAGQR
GVVGLPGQRGERGFPGLPGPSGEPGKQGPSGASGERGPPGPMGPPGLAGPPGESGREGAPGA

EGAOGRDGAOGAKGDRGETGPAGPPGAPGAPGARGGPGDS

Out of top 62 peptides most can be found in collagen include 10 hydroxyproline peptides.

These peptides cover around 64% of collagen Type 1 sequence.

Red amino acids are found in di, tri and tetrapeptides.

O denotes hydroxyproline

In normal tissue the submucosal layer consist almost entirely of collagen, the skeleton of the small intestine.

Aside from numerous proline contain dipeptides, these metabolites are also marker for collagen turnover and degradation.

Several G.I. diseases can change the collagen fiber structure. The collagen fiber content and its distribution outside the cell are closely related to some diseases. For example, systemic sclerosis (scleroderma) will replace smooth muscle and deposit large amounts of collagen instead. Gastrointestinal sclerdema is a common clinical disorder; however, due to its complicated etiology, most of them are not easily diagnosed and all the patients with such disease manifested distinct collagen fiber transformations. Obstructive diseases could also change the collagen structure and content.

The high percentage of proline containing dipeptides represents significant levels of collagen degradation. The major collagen type in healthy intestine was type I (68%), followed by types III (20%) and V (12%). **In normal tissue the submucosal layer consist almost entirely of collagen**, which is called the skeleton of the small intestine Intestinal strictures in Crohn's disease, for example, are therefore characterized by an accumulation of collagen, a proliferation of smooth muscle cells, and an increase in type V collagen, a collagen type produced in relatively large amounts by smooth muscle cells. The lining of the small intestinal mucosa is very highly specialized for **maximizing digestion and absorption of nutrients**.

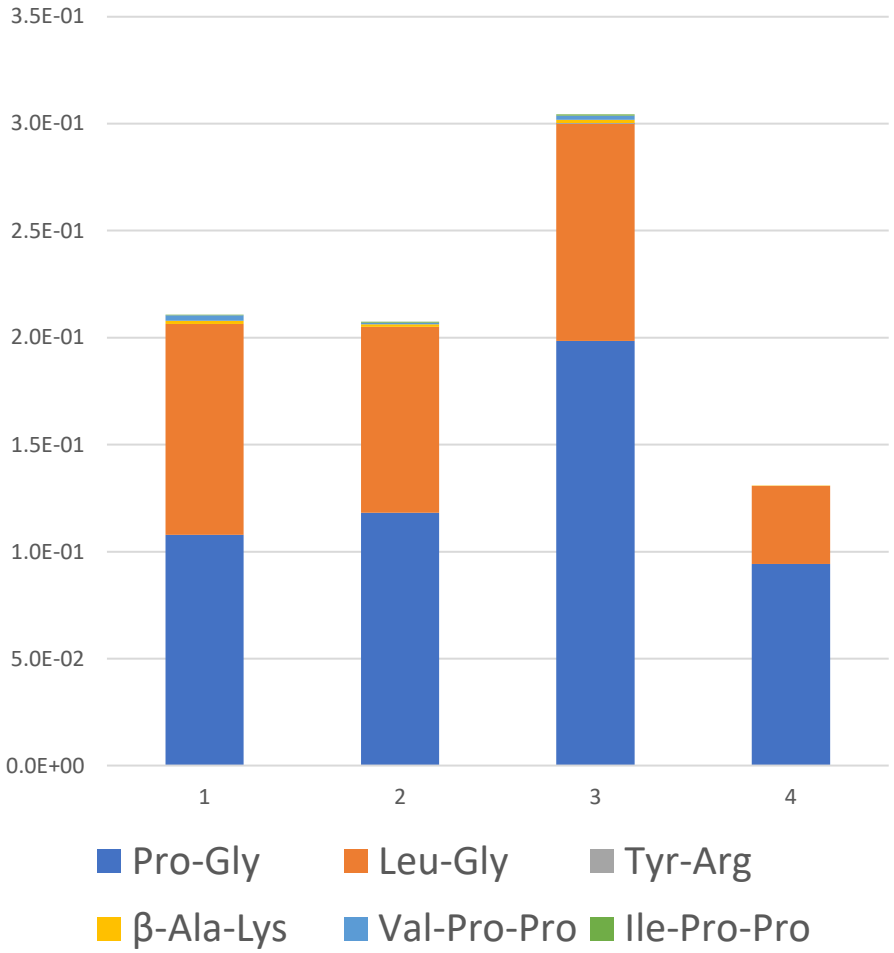
Monitoring small peptides provides insights in proper proteolytic digestion and collagen within the small intestine.

MKVLILACLVALALARELEELNVPGEIVESLSSSEESITRINKKIEKFQSEEQQQTEDELQDKIHPFAQTQ
SLVYPFPGPIPNSLPQNIPPLTQTPVVVPPFLQPEVMGVSKVKEAMAPKHKEMPFPKYPVEPFTESQSLT
LTDVENLHLPLPLLQSWMHQPHQPLPPTVMFPPQSVLSLSQSKVLPVPQKAVPYPQRDMPIQAFLLYQ
EPVLGPVRGPFPIIV

Digestion of proteins leads to the release of numerous peptides in the gastrointestinal tract, among them several bioactive peptides. Many come from abundant proteins such as β-casein, Collagen and Whey protein.

β-casein makes up ~30% of the total protein contained in bovine milk. Aside from Collagen peptides, the next most abundant small peptides may come from β-casein (above).

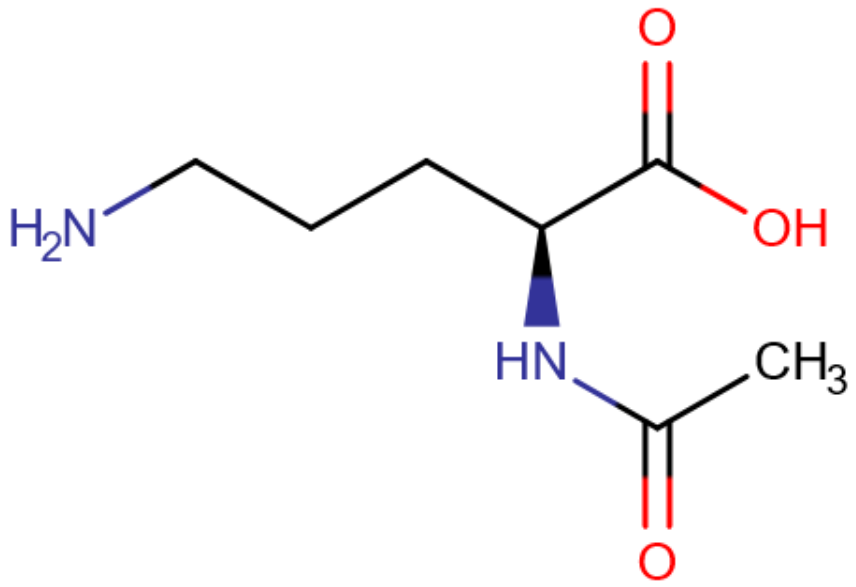
Bioactive peptides IPP and VPP from β-Casein, while VAP from α-Casein.

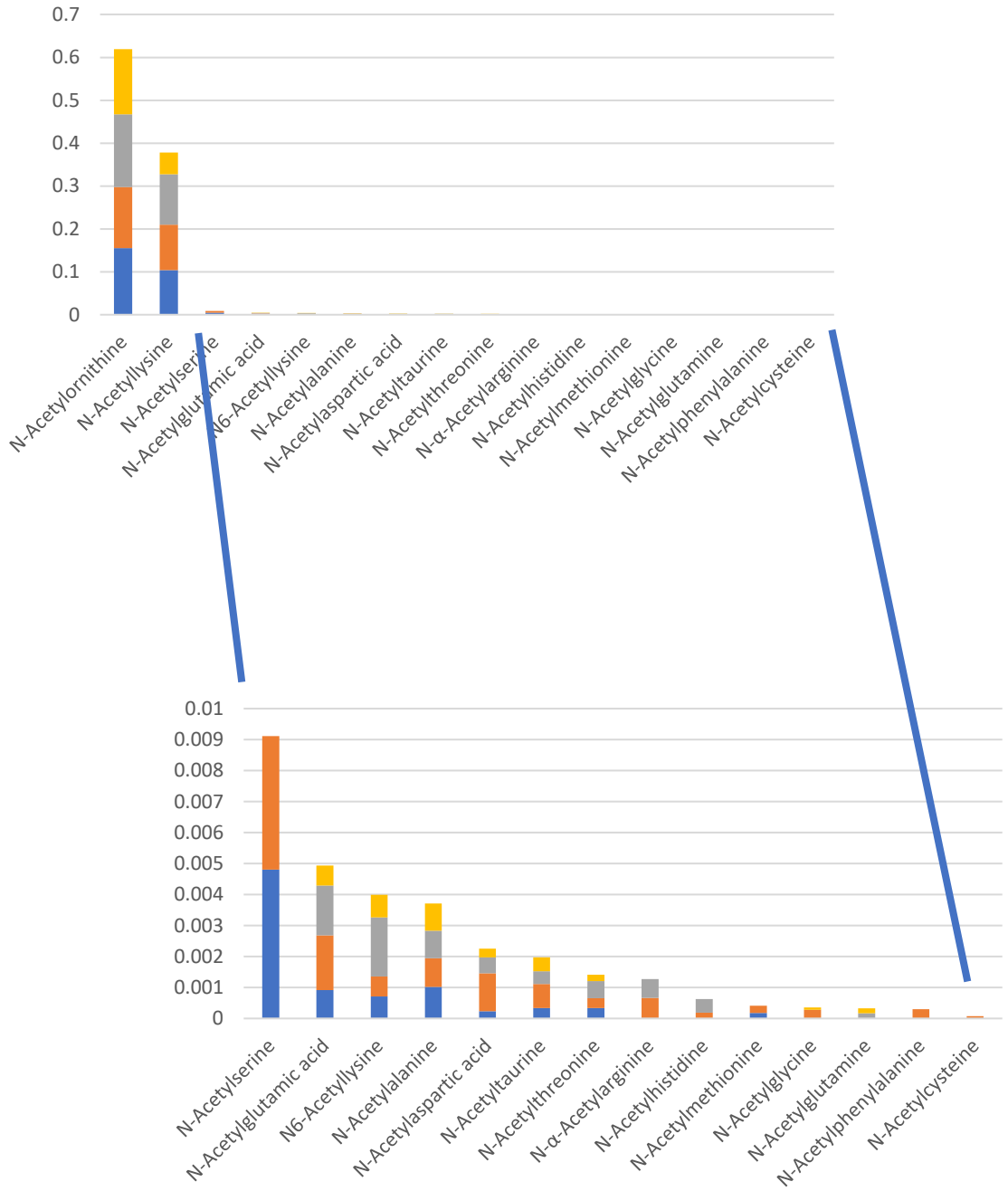


Small Peptides have been associated with specific activities.

- The dipeptide Pro-Gly promotes IGF-1 expression and secretion in HepG2 cells by activating JAK2/STAT5 signaling pathway through PepT1.
- The glycine-containing dipeptide leucine-glycine raises accumbal dopamine levels.
- Leucine and glycine dipeptides ameliorate physical fatigue through enhancing dopaminergic systems.
- Tripeptides Val-Pro-Pro and Ile-Pro-Pro Regulate the Proliferation and Migration of Vascular Smooth Muscle Cells.

Acetylated amino acids





14 Acetylated amino acids are observed in small intestine fluid.

The metabolic intermediates of acetate metabolism, such as high concentrations of acetyl-CoA, can provide acetyl for enzymatic (bacterial) amino acid acetylation.

Acylation may be a chemical event facilitated by pH and high concentrations of acetylphosphate as a non enzymatic pathway.

Most amino acids are acetylated by gut microbiota, including *N*-acetyl-alanine, *N*-acetyl-serine, *N*-acetyl-lysine, *N*-acetyl-cysteine, *N*-acetyl-leucine, *N*-acetyl-isoleucine, and *N*-acteyl-tyrosine. All these amino acids participate in TCA cycle and can be converted to pyruvate and/or acetyl-CoA by bacteria and host.

However, two acetyl amino acids (Ornithine and Lysine) stand out among all the rest.

***N*-acetylornithine** is an intermediary in the pathway for microbial **citrulline** and arginine synthesis from dietary glutamine and glutamate in the intestine. In the adult, endogenous arginine biosynthesis is an inter-organ: the net production of citrulline occurs almost **exclusively in the enterocytes of the small intestine,** but absorption of citrulline from the circulation and subsequent biosynthesis of arginine can take place in many tissues. Of these, the cortex of the kidney provides approximately 20% of whole-body requirements. AcetylOrnithine may be for efficient export of citrulline from gut to other organs and cells.

Glutamine and arginine are essential to gut mucosal integrity and restitution, hence gut bacterial synthesis of *N*-acetylornithine may have a major beneficial role in nutrition and may be related to a healthy gut barrier.

N-acetylornithine an Escherichia coli metabolite, a Saccharomyces cerevisiae metabolite. N-Acetylornithine has been observed lower in diabetes, but higher with aging. High Ac-Orn likely linked to Arg synthesis in small intestines.

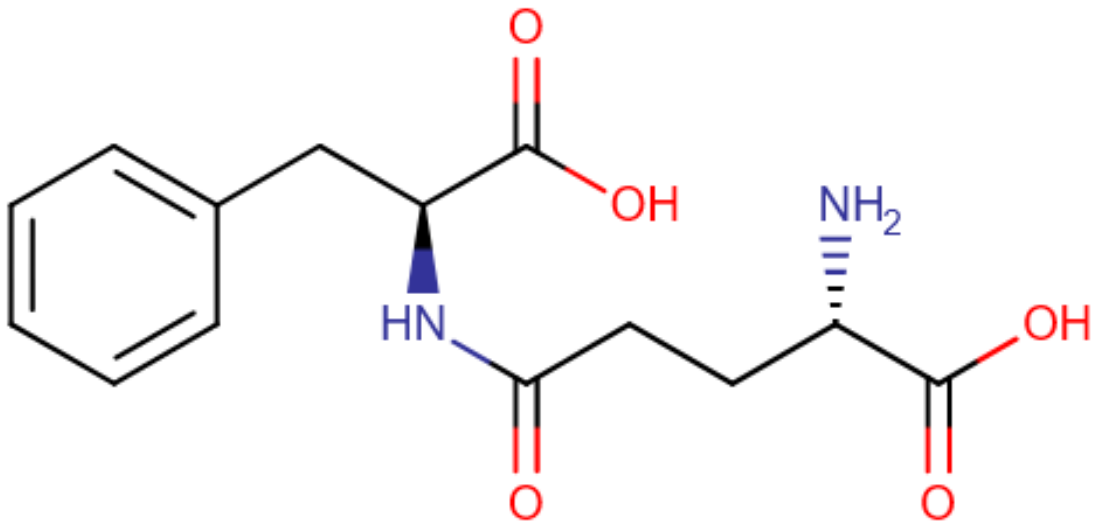
Lysine acetylation is an abundant post-translational modification in prokaryotes, regulating various microbial metabolic pathways.

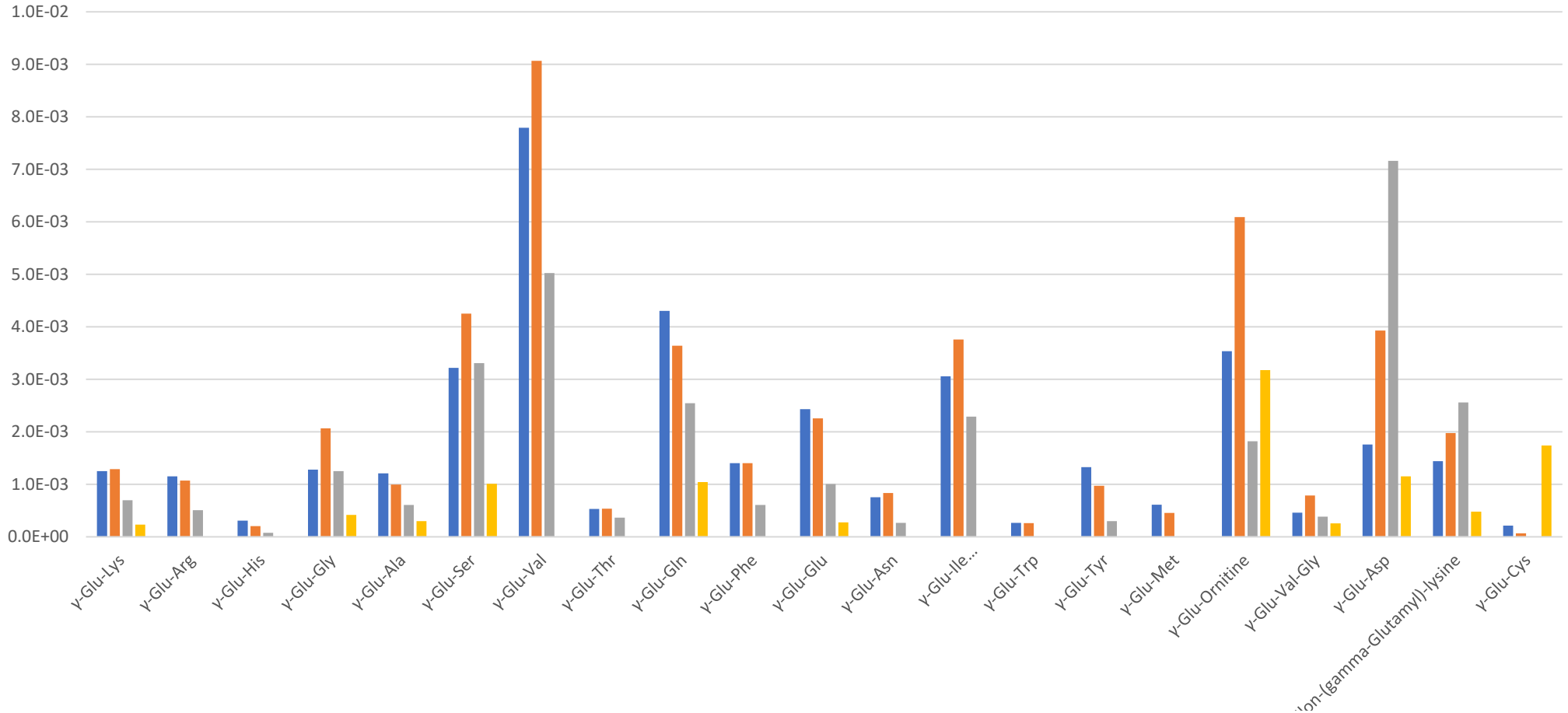
Lysine acetylation, an abundant post-translational modification (PTM) in prokaryotes, regulates various microbial metabolic pathways is widely distributed in gut microbial metabolic pathways, including anaerobic fermentation to generate **short-chain fatty acids SCFA-producing bacteria** e.g. Firmicutes. Widespread protein lysine acetylation observed to be lowered in patients with Crohn's disease.

Protein lysine acetylation has been characterized in several single bacterial species, including *Escherichia coli* , *Bacillus subtilis*, *Salmonella enterica*, and *Mycobacterium tuberculosis*, and widely implicated in various microbial processes, including chemotaxis, nutrient metabolism, stress response, and virulence.

Hence monitoring acetylated amino acids provides insights into ketogenic supply, SCFA producing bacteria, bacterial markers and arginine synthesis.

Gamma-Glu dipeptides/ Gamma glutamate transferases

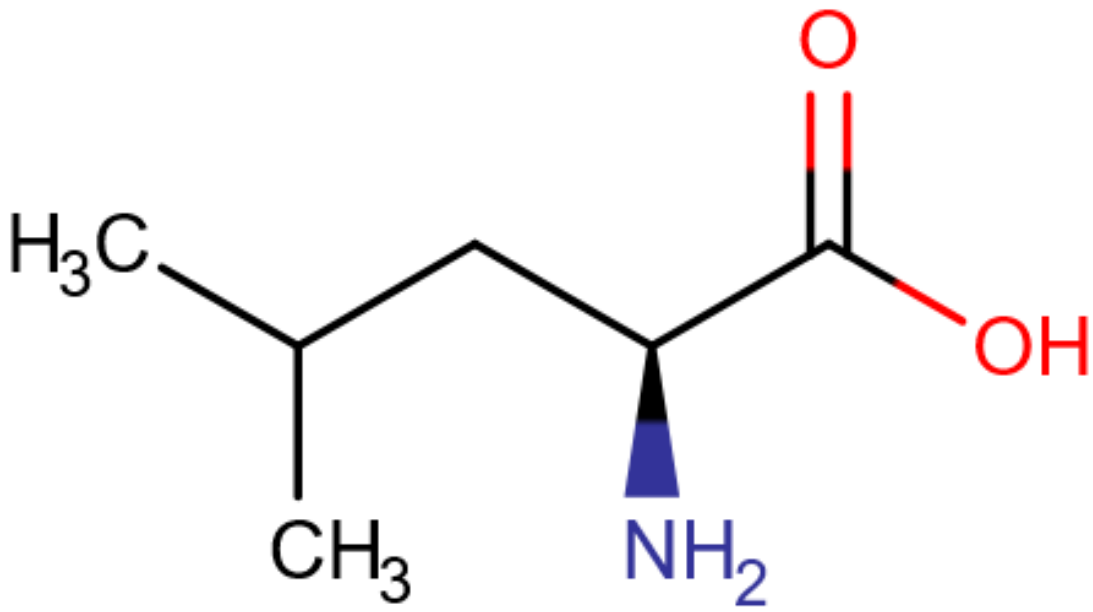


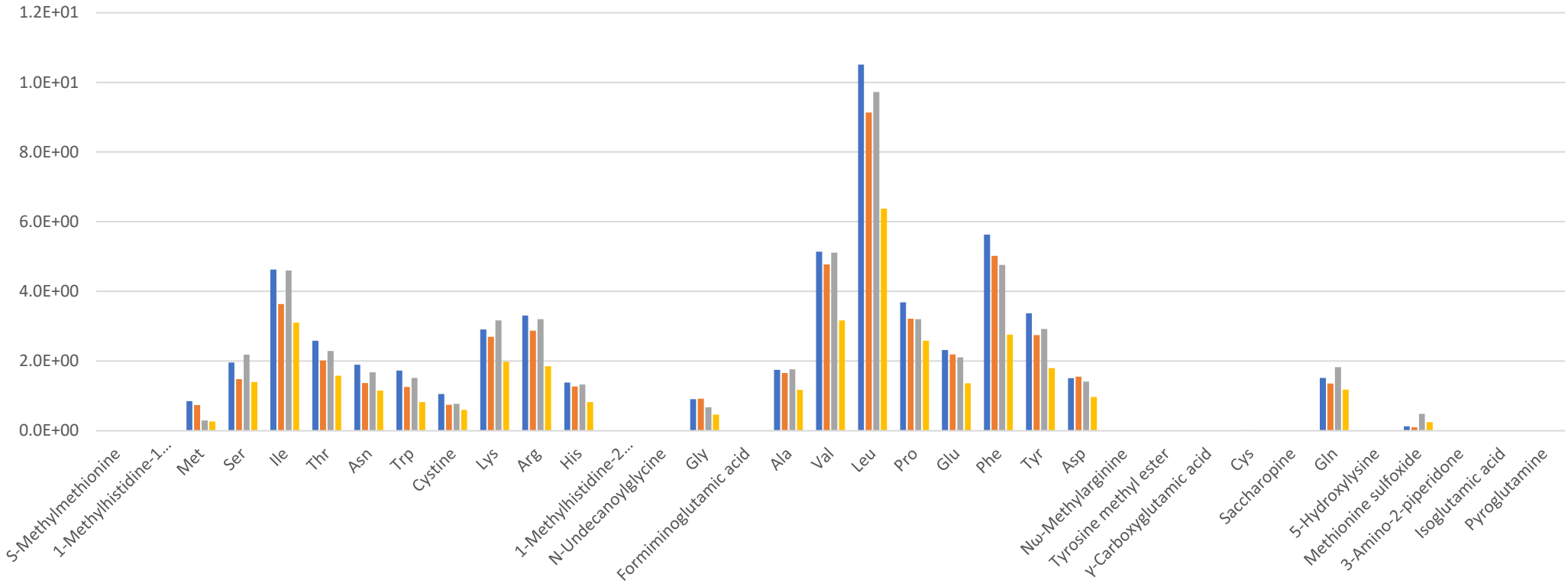


Gamma-Glu dipeptides are observed for all amino acids except Pro. While selected gamma-Glu peptides have been observed in tissues and biofluids under normal and disease conditions, it is unique to find all possible combinations within one sample type.

- **Gamma-glutamyl transpeptidase** (GGT) enzymes play a variety of roles. Higher eukaryotes mainly utilize GGT for glutathione degradation, and mammalian GGTs have implications in many physiological disorders.
- GGTs from unicellular prokaryotes serve different physiological functions in Gram-positive and Gram-negative bacteria.
- GGTs from Gram-negative bacteria like *Escherichia coli* use GGTs as glutathione degraders and from pathogenic species like *Helicobacter pylori* use GGTs as virulence factors.
- Bacterial GGTs are stable over a wide pH range (6.0–11.0) with maximum stability at alkaline pH range.
- Bacterial GGTs exhibit broad substrate specificity for amino acids and other amine-group containing compounds and can thus synthesize a variety of γ-glutamyl compounds.
- γ-Glutamyltranspeptidase (GGT) has been widely used as a marker enzyme of hepatic and biliary diseases. γ-glutamyl transferase (gGT) is a key bacterial virulence factor that is not only important for bacterial gastric colonization but also related to the development of gastric pathology.
- High levels of GGT may be a sign of liver disease or damage to the bile ducts.

Free Amino Acids





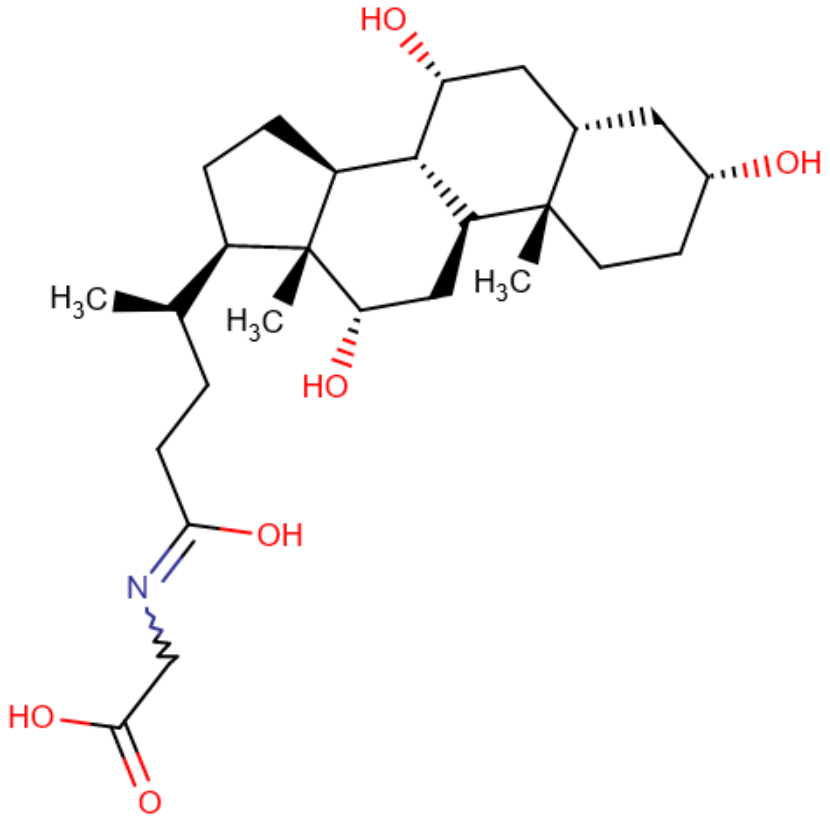
Large amount of **free amino acids** are found in capsule fluid, along with several degradation products. BCAA and aromatic amino acids are the most abundant. Compared to free amino acids in plasma, low abundant amino acids such as cysteine and aspartic acid are higher in this fluid.

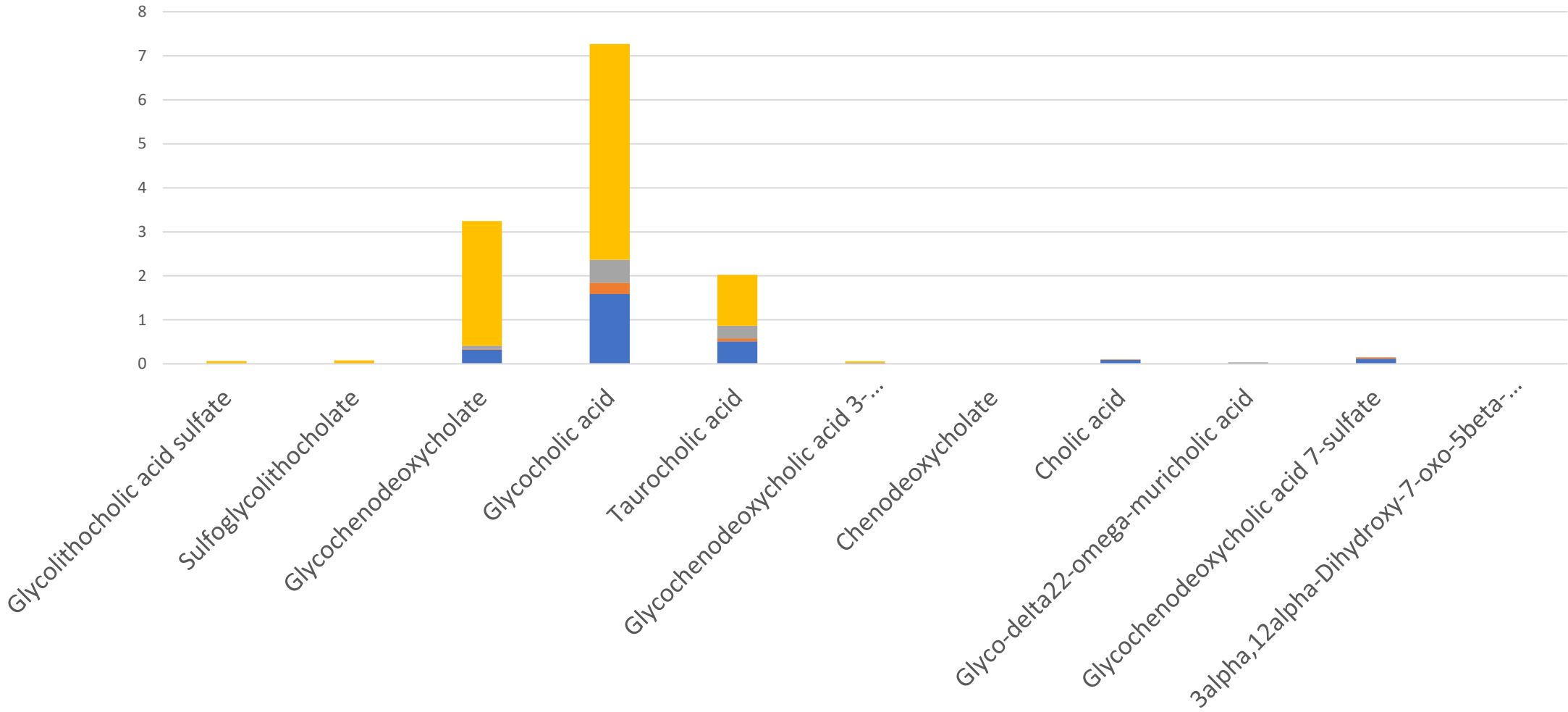
- The small intestine is not only responsible for terminal digestion and absorption of nutrients, but it also plays an important role in **amino acid catabolism** of arterial glutamine and dietary amino acids. Most of glutamine and almost all of glutamate and aspartate in the diet are catabolized by small intestinal mucosa, and CO2 accounts for 56-64% of their metabolized carbons.
- The small intestinal mucosa also plays an important role in degrading arginine, proline and branched-chain amino acids, and methionine, lysine, phenylalanine, threonine, glycine and serine in the diet, such that 30-50% of these dietary amino acids are not available to extraintestinal tissues.
- Dietary amino acids are major fuels for the small intestinal mucosa and are essential precursors for intestinal synthesis of glutathione, nitric oxide, polyamines, purine and pyrimidine nucleotides, and amino acids (alanine, citrulline and proline), and are obligatory for **maintaining intestinal mucosal mass and integrity**.
- Because intestinal amino acid catabolism plays an important role in modulating dietary amino acid availability to extraintestinal tissues, it has important implications for the utilization efficiency of dietary protein and amino acids in animals and humans.

- The mechanism by which **amino acids are absorbed into small intestine** is conceptually identical to that of monosaccharides.
- The lumenal plasma membrane of the absorptive cell bears at least four sodium-dependent amino acid transporters - one each for acidic, basic, neutral and amino acids. These transporters bind amino acids only after binding sodium. The fully loaded transporter then undergoes a conformational change that dumps sodium and the amino acid into the cytoplasm, followed by its reorientation back to the original form.
- Thus, absorption of amino acids is also absolutely dependent on the electrochemical gradient of sodium across the epithelium. Further, absorption of amino acids, like that of monosaccharides, contributes to generating the osmotic gradient that drives water absorption.
- The basolateral membrane of the enterocyte contains additional transporters which export amino acids from the cell into blood.

- Monitoring free amino acids provides insights into mucosal mass and integrity, amino acid catabolism and sodium transport within the small intestine.

Bile Acids

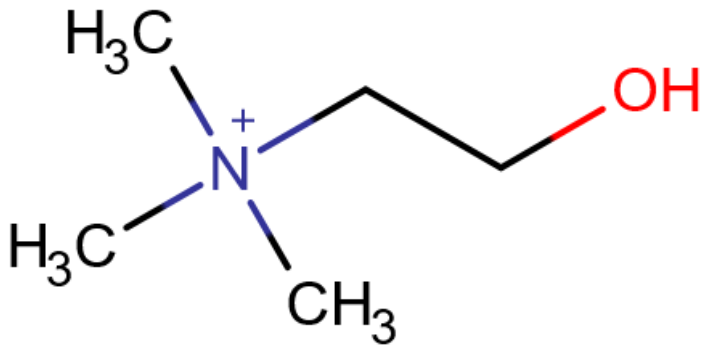


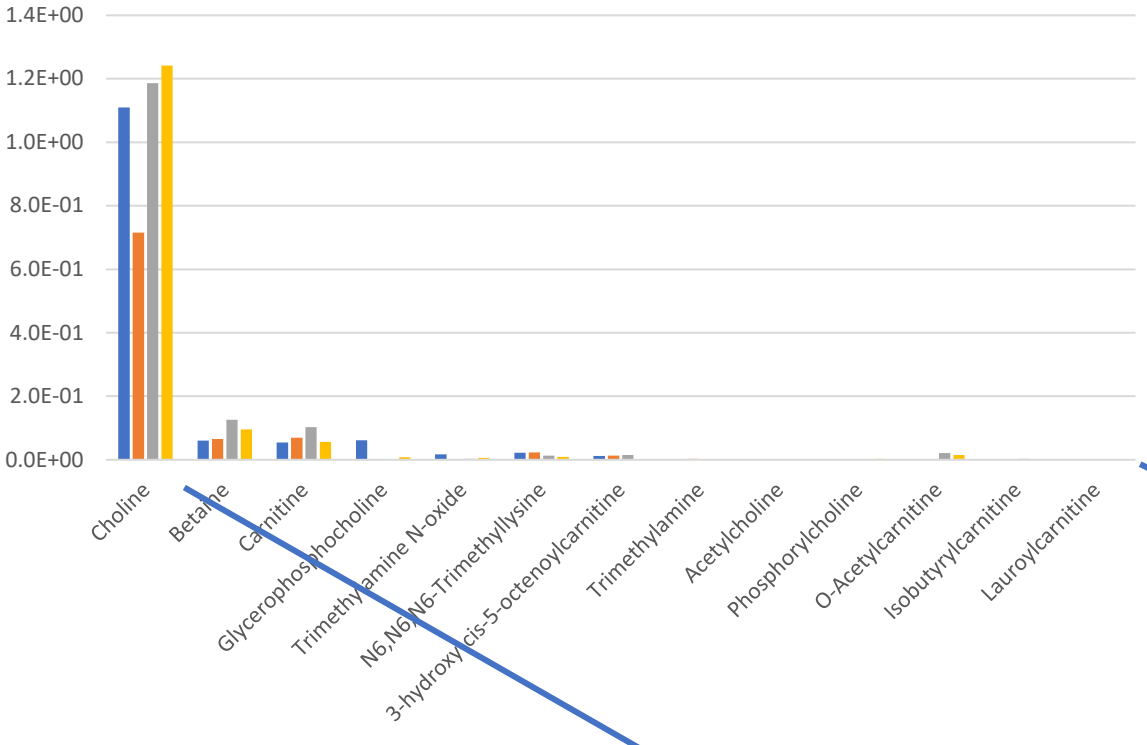


Several bile acids are observed in the capsulte, most are conjugated bile acids from the liver with Glycocholic, Glycochenodeoxycholic and Taurocholic being the most abundant.

- **Glycocholate, Glycochenodeoxycholate and Taurocholate** are the major bile acids among 11 observed.
- These are primary conjugated bile acids synthesized in liver that are secreted into the small intestine.
- Bile acids break down fats, absorb vitamins, and remove wastes. Glycocholate is a strong substrate for human hepatic bile salt uptake and efflux transporters. Glycocholate and Taurocholate use the same transporters and form similar binary micelles.
- Bile acids and bacteria have close relationships. The composition of the intestinal pool of bile acids is shaped by bacterial metabolism. In turn, bile acids play a role in intestinal homeostasis by controlling the size and the composition of the intestinal microbiota. As a consequence, alteration of the microbiome–bile acid homeostasis can play a role in hepatic and gastrointestinal pathological conditions.
- Because bile acids control the structure of the intestinal microbiome and the microbiome regulates the composition and size of the bile acid pool, alteration of the microbiome–bile acid homeostasis can have multiple pathological consequences. In cirrhotic patients a shrinking bile acid pool may alter the intestinal microbiome by increasing the size of bacterial populations that produce proinflammatory molecules, which trigger a feedback loop as inflammation downregulates bile acid synthesis in the liver.
- As cirrhosis progresses, decreased concentrations of bile acids in the small intestine permit bacterial overgrowth, which many contribute to cirrhosis complications like intestinal endotoxemia and hepatic encephalopathy. Microbiome-induced alteration of the bile acid pool may also play a role in NASH by impairing the activity of bile acid receptors and bile acid transporters.
- Bile acid metabolism and signaling is also impaired in cholestasis, which causes accumulation of bile acids in the liver with concomitant hepatocyte injury and inflammation. Patients with chronic cholestasis may be at higher risk of developing hepatocellular and bile duct cancer. This hypothesis is consistent with the observation that bile acids can promote cell proliferation by activating mitogenic pathways in the hepatobiliary tract.

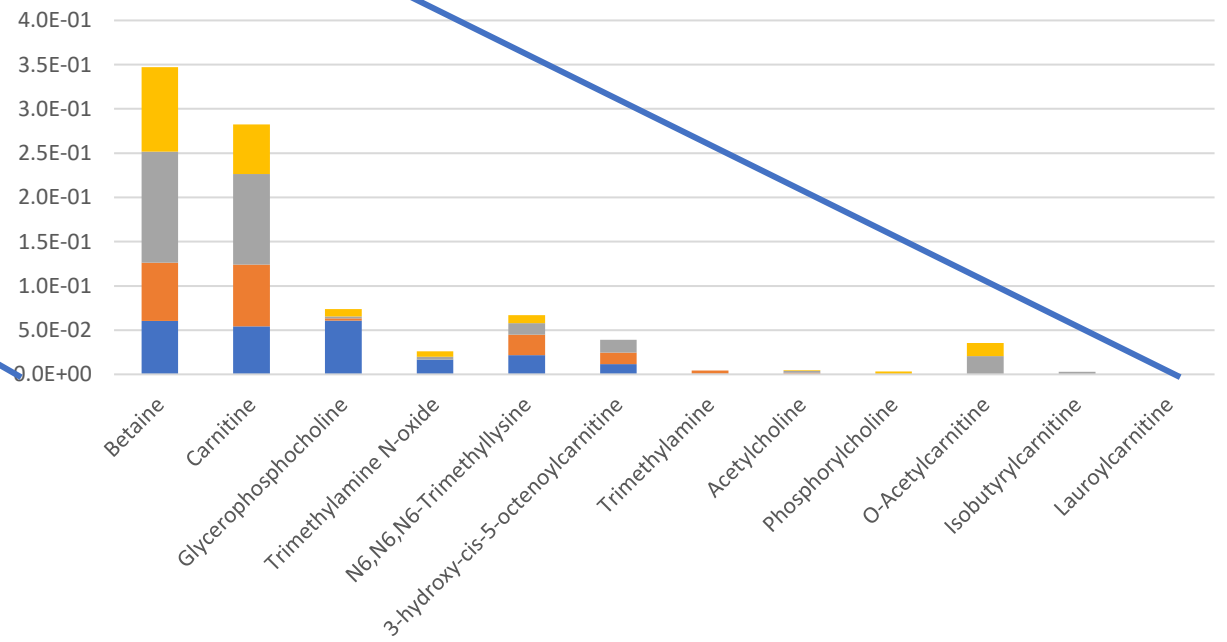
Choline Metabolism

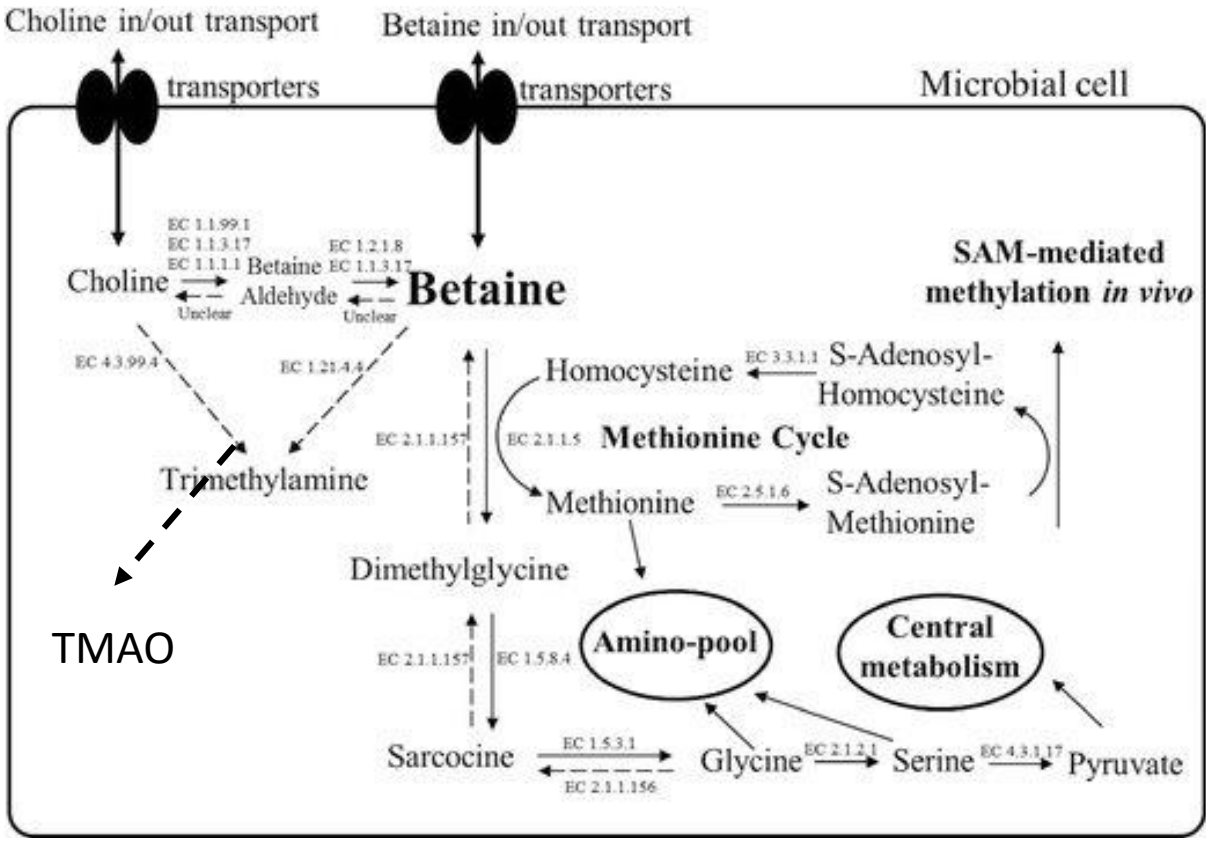




Choline is a dominate metabolite in the capsule fluid.

At lower levels, but still abundant, are choline degradation pathway metabolites such as Betaine, TMAO and Carnitine.





- **<u>Choline metabolism</u>** can be divided into four main pathways, which are involved in the synthesis of acetylcholine, betaine, phospholipids, and trimethylamine.

- Choline is catalyzed by choline acyltransferase into **acetylcholine**, which is key in cholinergic neurotransmission.

- Moreover, choline can be oxidized to obtain **betaine**, which is an important osmolyte, a methyl donor implicated in the epigenetic regulation of DNA, and a requirement in the synthesis of phosphatidylcholine, the most abundant phospholipid in the body, which is not only a major component of cellular membranes and needed for cell division and growth, but also plays a role in cell signaling as a donor to synthesize sphingomyelin from ceramide.

- **Choline** is an essential nutrient that must be obtained from the diet.

- Gut microbial metabolism of choline results in the production of trimethylamine (TMA), which, upon absorption by the host is converted into trimethylamine-N-oxide (**TMAO**) in the liver. The majority of TMAO is produced in the liver. Some bacteria from the phylum *Proteobacteria* are able to metabolize the TMAO into TMA via TMAO reductase using metabolic retroconversion. Other members of the order *Methanomassiliicoccales* are able to reduce TMAO to methane. Current area of research relies on the use of such microorganisms as potential probiotics, in order to reduce the circulating levels of TMAO.

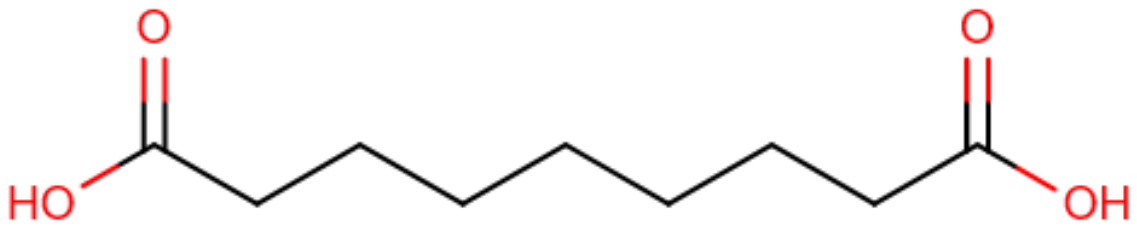
- A high accumulation of TMAO and TMA is related to cardiovascular disease, inflammatory bowel disease, non-alcoholic fatty liver disease, and chronic kidney disease. The presence of increased TMA and TMAO levels has been associated with higher activity of bacterial members of the phylum *Firmicutes* and *Proteobacteria*, which are known producers of this metabolite. TMA and TMAO levels have been linked to an elevated *Firmicutes*/*Bacteroidetes* ratio with higher levels of *Firmicutes* and lower levels of *Bacteroidetes* due to the inability of *Bacteroidetes* to produce TMA. The intestinal bacteria in charge of TMA production from choline include *Anaerococcus hydrogenalis*, *Clostridium asparagiformis*, *Clostridium hathewayi*, *Clostridium sporogenes*, *Desulfovibrio desulfuricans*, *Escherichia fergusoni*, *Ed. tarda*, *Klebsiella pneumoniae*, *Proteus penneri*, and *Providencia rettgeri.*

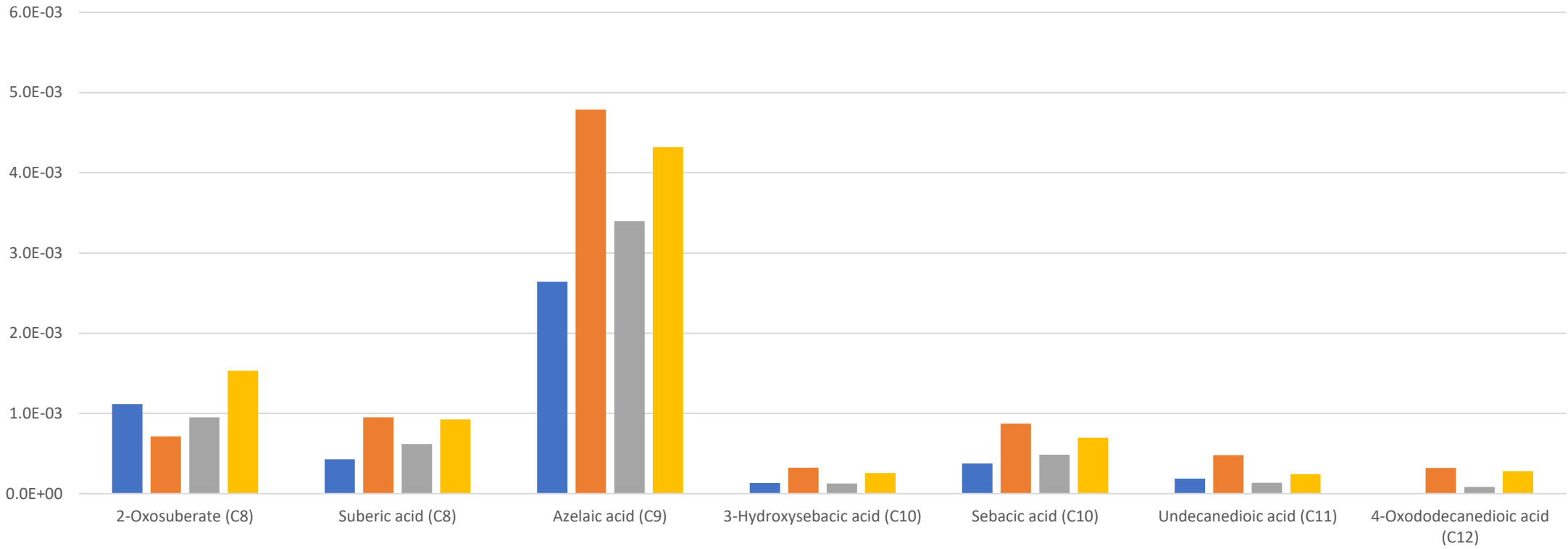
- **TMAO** is correlated with disease and all-cause mortality. Low serum TMAO induced by choline-deficits diets is associated with non-alcoholic steatohepatitis (NASH), while high concentrations of TMAO are associated with CVD and chronic kidney disease (CKD).

- The Choline metabolite**, Betaine,** improves intestinal function by enhancing the digestive enzymes, ameliorating intestinal morphology, and enriching intestinal microbiota. Betaine is also a cellular osmolyte.
- Betaine can be obtained by absorption through dietary intake as well.
- The primary role of betaine is to donate a methyl group in one-carbon metabolism in the liver.

- **Carnitine,** another Choline metabolite**,** has vital roles in the endogenous metabolism of short chain fatty acids. It can protect and support gut microbial species, and some dietary fibers can reduce the available iron involved in the bioactivity of carnitine. There is also an antagonistic relationship between high microbial populations and carnitine bioavailability.
- Carnitine can be synthesized in mammals, but not in bacteria, where carnitine or its immediate precursor (Trimethyllysine) are imported into the cells.

Omega Oxidized Middle Chain Fatty Acids





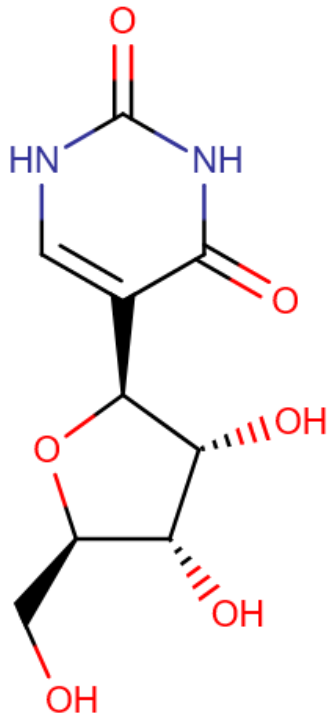
- Data support a role of the small intestine rather than the liver as a FAO sensor that can influence eating. Enterocytes in small intestine may sense dietary TAG-derived fatty acids via FAO and influence eating through changes in intestinal vagal afferent activities.

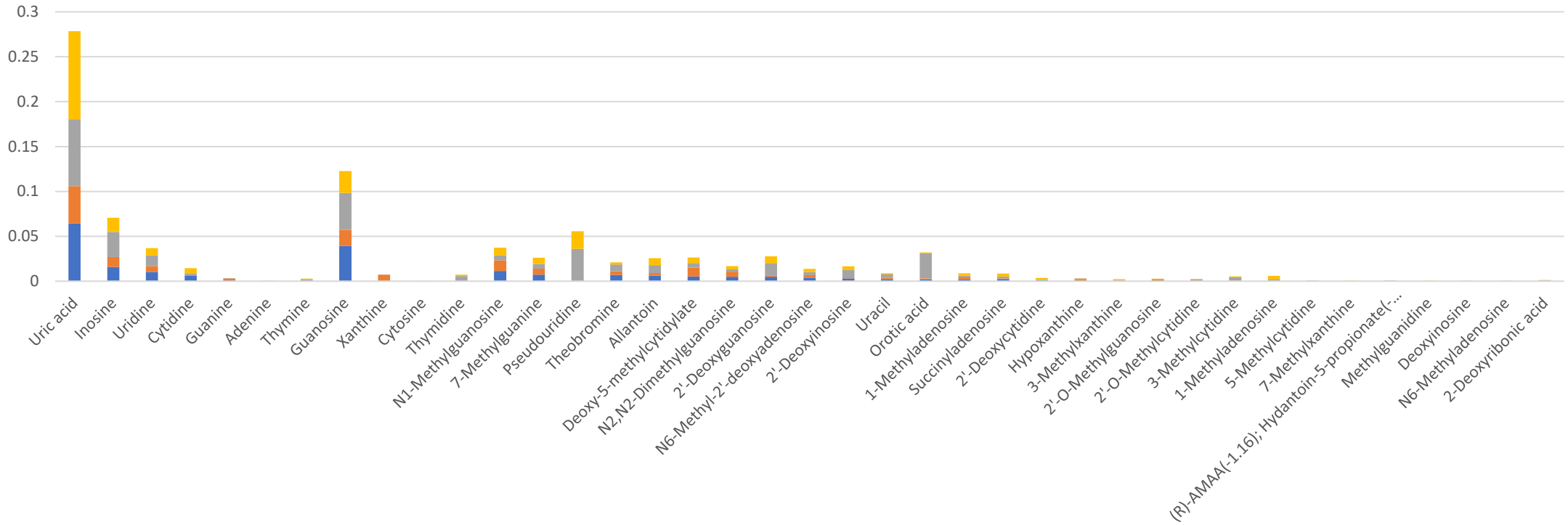
- FAO includes omega and beta fatty acid oxidation to Azelaic acid, Sebacic Acid, Suberic Acid from the decomposition of long chain fatty acid glycerides.

- In capsule fluid we observed several oxidized middle chain fatty acids C8 to C12.

- **MCFA**s are transported by blood from the alimentary tract to the liver where they are metabolized; therefore, they are not stored in the adipose tissue. MCFAs are able to permeate the blood-brain barrier.
- MCFAs derived from digestion of MCFA-containing triglycerides are predominantly degraded by hepatic mitochondrial β-oxidation.
- Depending upon the chain length, different MCFAs have been linked to different biology.
- In contrast to inhibiting glycolysis in hepatocytes, decanoate (C10), but not octanoate (C8), has been found to **stimulate glycolysis**, thus resulting in an enhanced release of lactate into the extracellular space.
- **Stimulation of acetyl-CoA carboxylase activity** depends on the chain length of the fatty acid . The stimulation magnitude increased from Octanoic (C8) to lauric (C12).
- Lipids also affect the gut microbiota both as substrates for bacterial metabolic processes, and by inhibiting bacterial growth by toxic influence.
- **Azelaic acid** has profound anti-inflammatory, antioxidative effects, and is bactericidal against a range of Gram-negative and Gram-positive microorganisms as well, including antibiotic-resistant bacterial strains. Several bacteria have been reported to be able to utilize azelaic acid as a unique source of carbon and energy,
- Medium-chain dicarboxylic acids (suberic and azelaic acid) and long-chain dicarboxylic acids (sebacic acids, dodecanedioic acid) are associated with microbiome health and enhanced fatty acid oxidation, perhaps to accelerate their movement into bacterial and host mitochondria and stimulate energy production.

Nucleic Acids

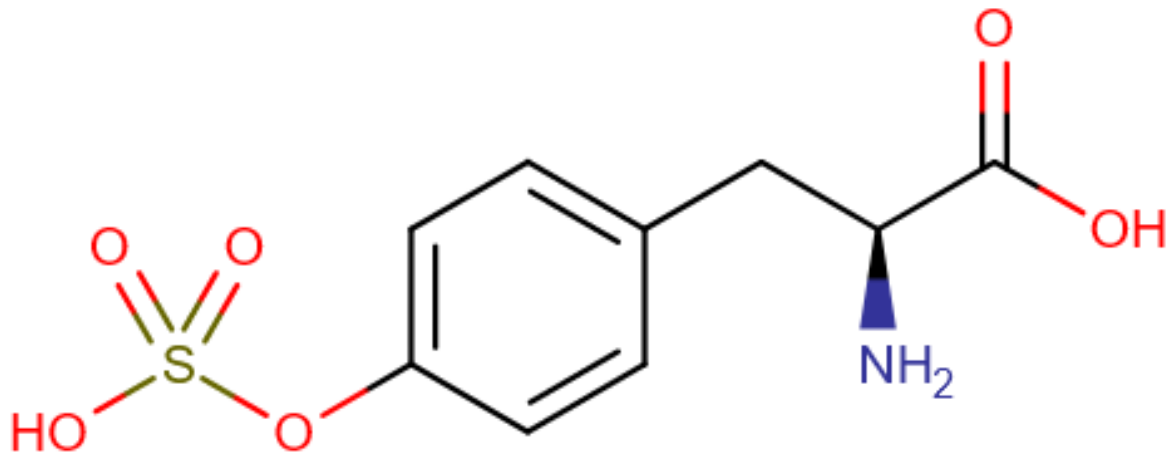


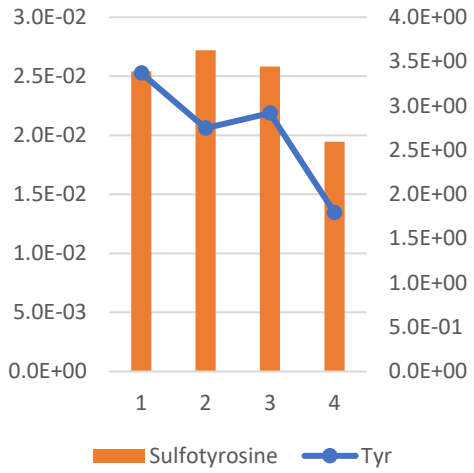


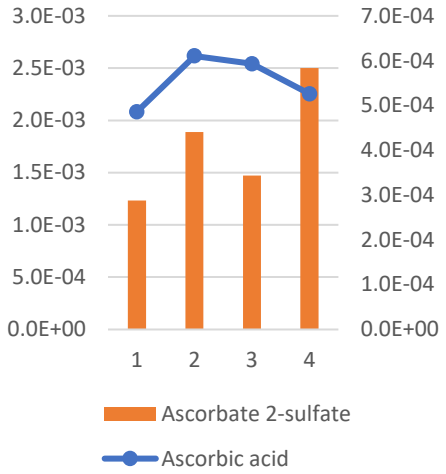
DNA and RNA are **digested in the small intestine with the help of both pancreatic enzymes and enzymes produced by the small intestine itself**. Ribonuclease and deoxyribonuclease break down RNA and DNA, respectively. 0ver 35 nucleic acids are observed in capsules. **Uric acid** is mainly synthesized in the liver, and **some is also produced in the small intestine**. It is the final product of human purine metabolism. Pseudouridine is the most abundant RNA modification in cellular RNA and one of the more abundant nucleic acids observed.

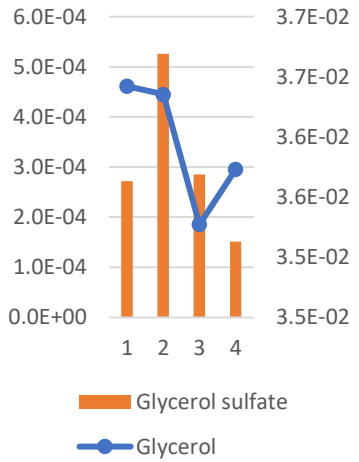
- An increase in **uric acid** level in blood, is presently the clinical diagnostic criteria for gout. Microbial genes for gout reveal a disorder in purine degradation and butyric acid biosynthesis in gout patients.
- In gout, *Bacteroides caccae* and *Bacteroides xylanisolvens* are enriched while *Faecalibacterium prausnitzii* and *Bifidobacterium pseudocatenulatum* are depleted which in turn changes the nucleic acid composition.
- Intestinal microbiota of gout are more similar to those of type-2 diabetes than to liver cirrhosis with observed changes in uric acid levels.

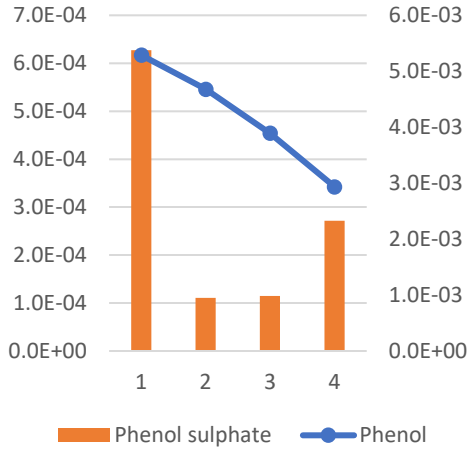
Sulfated Metabolites

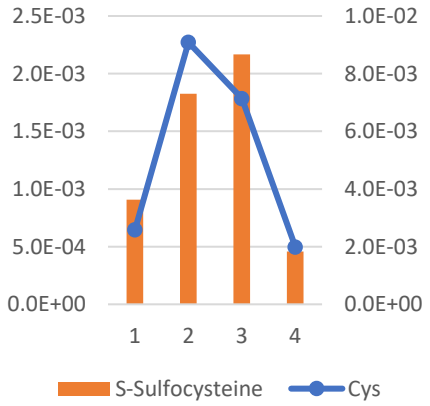










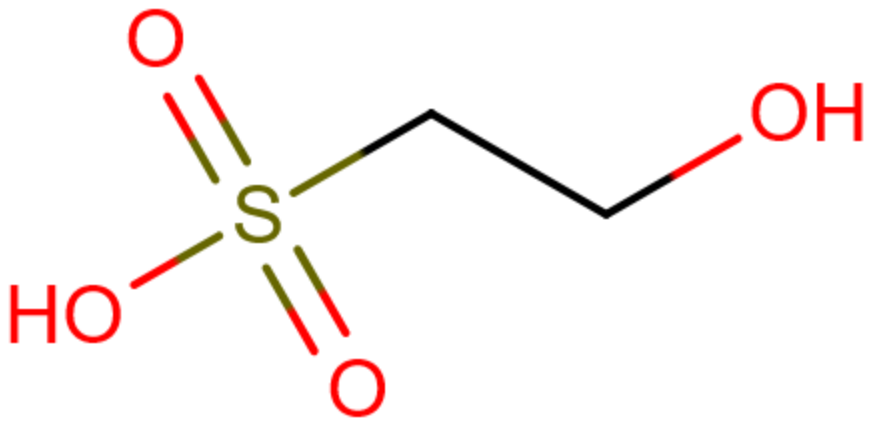


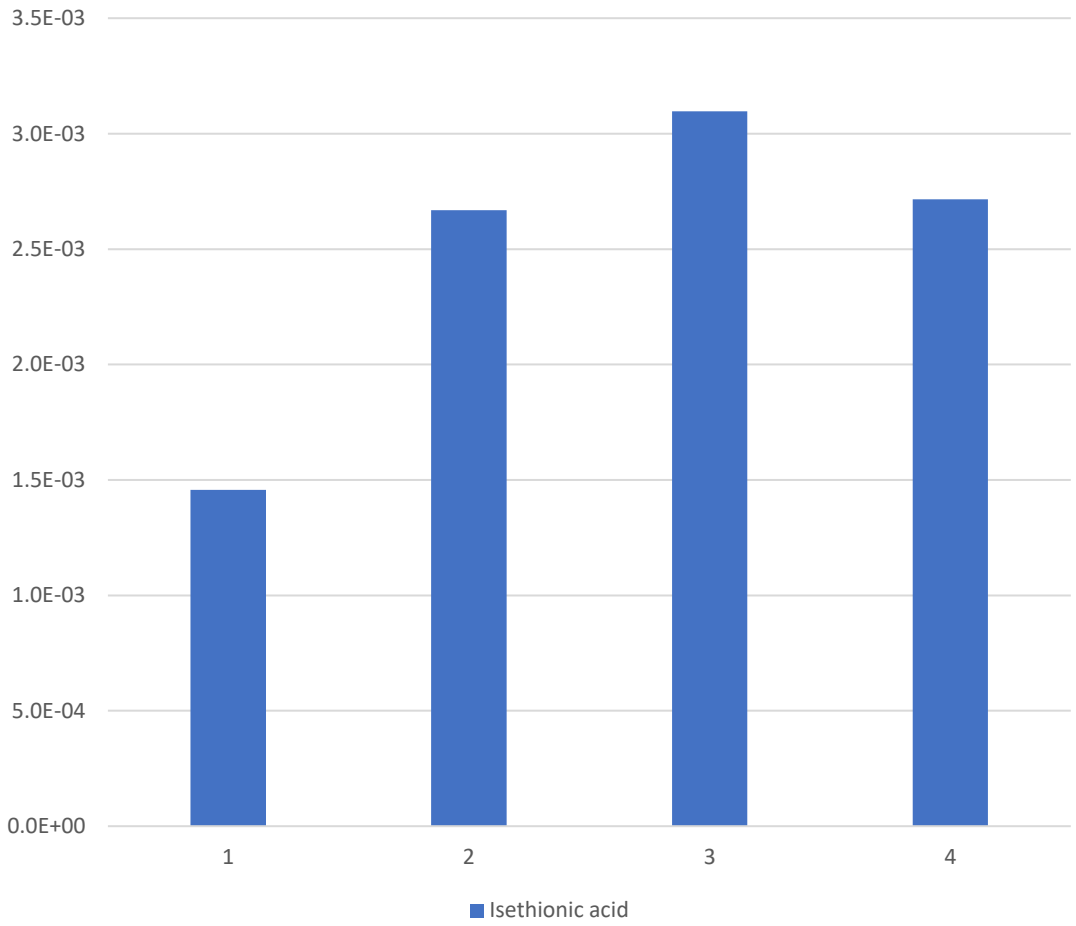
- **Sulfation** is a conjugation reaction essential for numerous biochemical and cellular functions in mammals. The 3'-phosphoadenosine 5'-phosphosulfate (PAPS) synthase 2 (PAPSS2) is the key enzyme to generate PAPS, which is the **universal sulfonate donor for all sulfation reactions**.

- There is an important role of PAPSS2-mediated sulfation in colitis and colonic carcinogenesis. Intestinal sulfation may represent a potential diagnostic marker and PAPSS2 may serve as a potential therapeutic target for inflammatory bowel disease and colon cancer.

- Observed in the small intestine is a strong correlation between sulfated and unsulfated species of amino acids, ascorbate, glycerol and phenol. The more the metabolite, the more the sulfated substrate, reflecting healthy distribution of PAPS.

Sulfonates





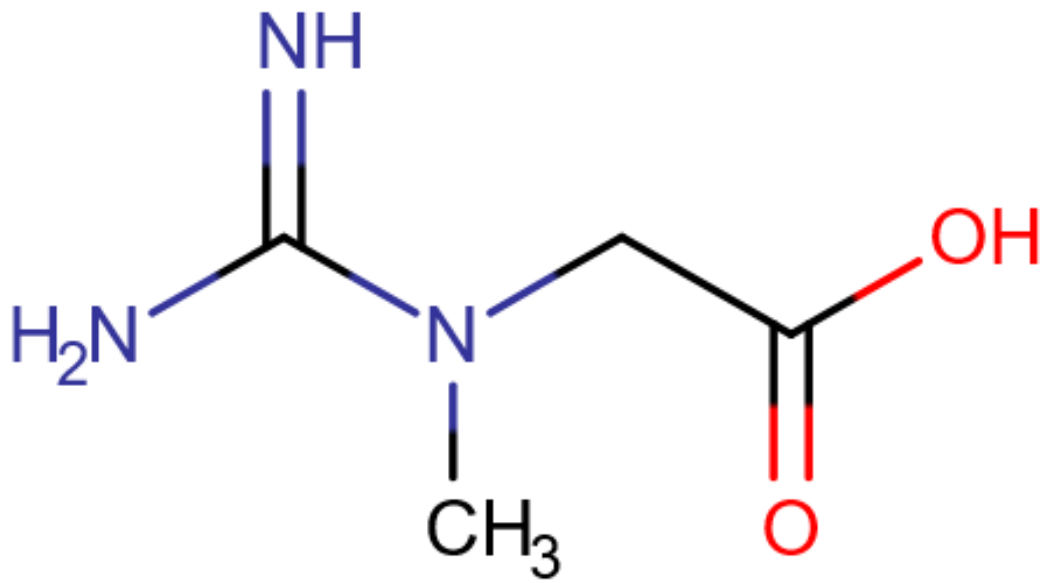
Enteric bacteria do not contain the putative cysteine importer. In oxic environments, molecular oxygen oxidizes environmental cysteine to cystine, which *E. coli* imports.

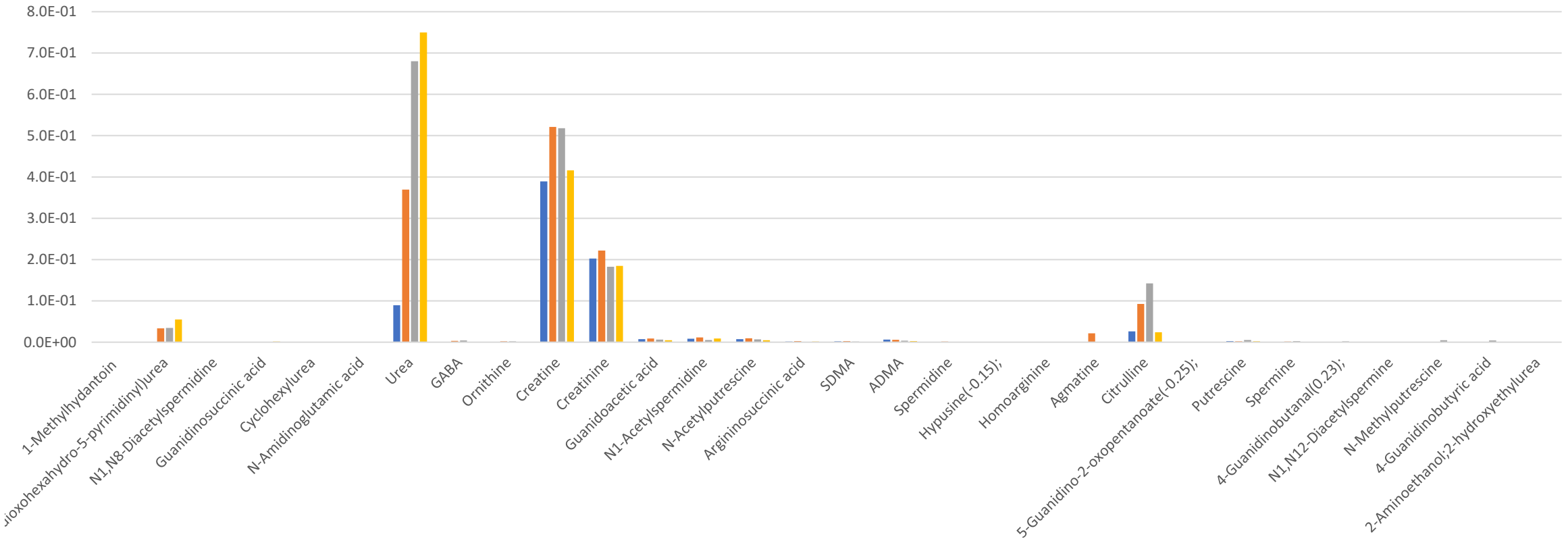
In anoxic environments where cysteine is stable, the cell chooses to **assimilate hydrogen sulfide** instead.

Isethionic acid ((2-hydroxyethanesulfonate) is a metabolite found produced by Escherichia coli that has incorporated hydrogen sulfide.

Isethionic acid is an osmoprotectant and biomarker for cellular distress.

Urea Cycle Metabolism



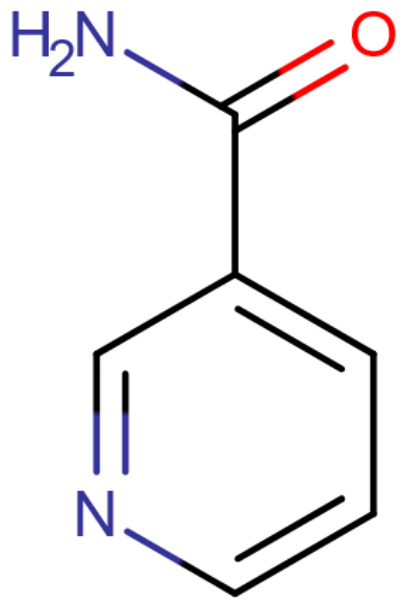


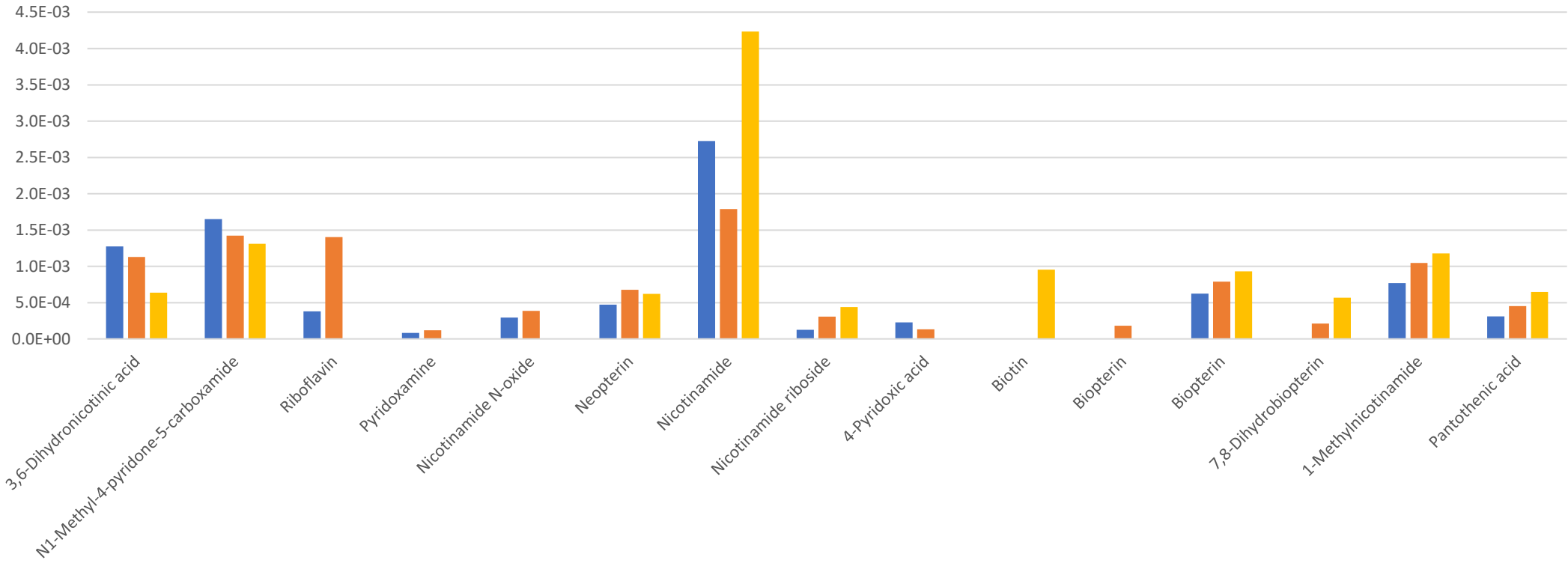
- **Ammonia** is being formed constantly from the deamination of amino acids derived from proteins, it is important that mechanisms exist to provide for the timely and efficient disposal. The liver is critical for ammonia catabolism because it is **the only tissue** in which all elements of the urea cycle are expressed, providing for the conversion of ammonia to urea.
- The **major contributor to plasma ammonia is the intestine, supplying about 50% of the plasma load.** Intestinal ammonia is derived via two major mechanisms. First, ammonia is liberated from urea in the intestinal lumen by ureases. **Ureases are not expressed by mammalian cells**, but are products of many bacteria, and convert urea to ammonia and carbon dioxide. Indeed, this provides the basis for a common diagnostic test, since *H. pylori*, which colonizes the gastric lumen and has been identified as a cause of peptic ulcer disease, has a potent urease.
- High amounts of creatine and creatine likely generated by the kidneys provide another route for excess ammonia.

- The chronic inflammatory disorder inflammatory bowel disease (IBD) can affect any part of the digestive system and is associated with a weakened intestinal barrier. Barrier integrity is mediated by tight junctions between intestinal epithelial cells. Maintaining tight junctions is an energy consumptive process. During times of high energetic demand, the **creatine-phosphocreatine cycle** can support ATP production. The cycle is dependent on intake of creatine by the creatine transporter. Previous research found creatine regulates energy distribution within cells and reduces the severity of colitis.
- **Creatine** maintains intestinal epithelial energy homeostasis and protects against colitis.

- **Citrulline** is a non-protein amino acid, and in humans its plasma content is derived largely from the amount produced in enterocytes of the **small bowel**.
- **Citrulline** has been described as a marker of intestinal function or absorption. Citrulline levels are correlated strongly with small bowel length in short bowel syndrome patients. Citrulline is strongly negatively correlated with intestinal disease severity with regards to enteropathies (coeliac disease, tropical enteropathy, Crohn’s disease, mucositis, acute rejection in intestinal transplantation.
- The epithelial cells of the small intestine produce citrulline, primarily from glutamine and glutamate, which is secreted into the bloodstream which carries it to the proximal tubule cells of the kidney, which extract the citrulline and convert it to arginine, which is returned to the blood. This means that impaired small bowel or renal function can reduce arginine synthesis and thus create a dietary requirement for arginine. For such a person, arginine would become "essential".

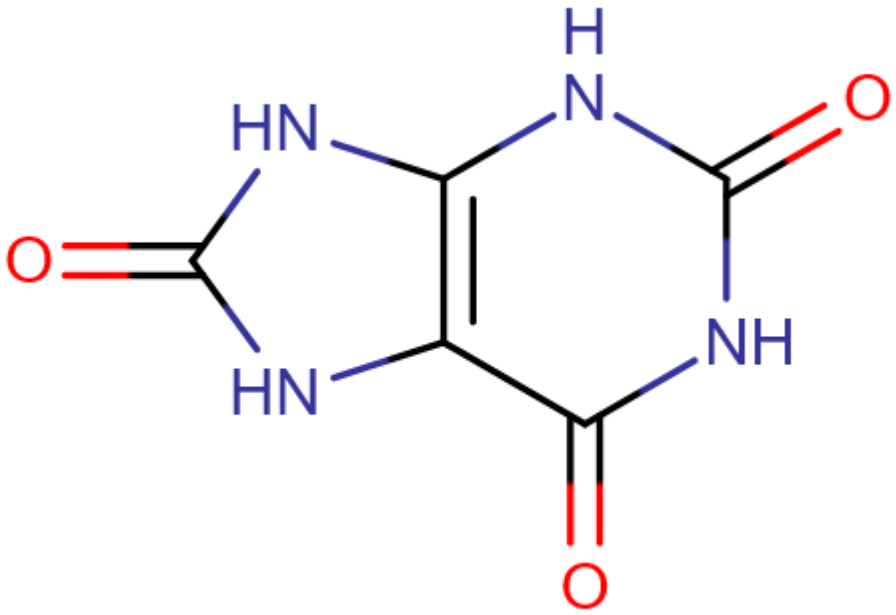
Vitamin B3 Metabolism

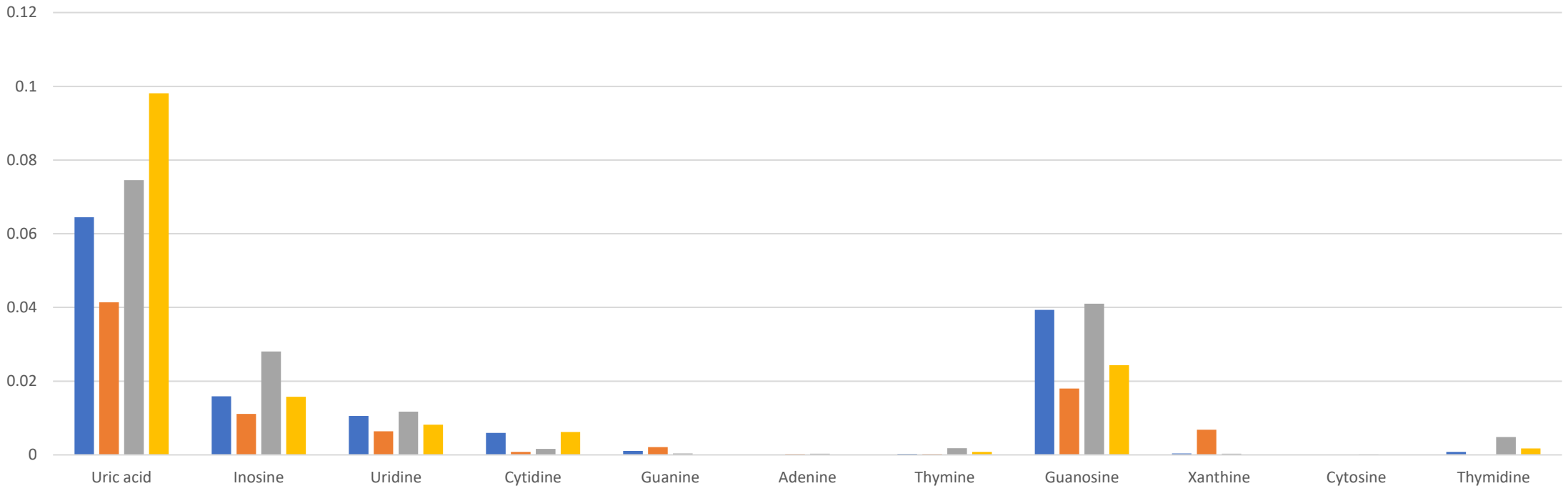




Nicotinamide, an amide form of vitamin B_3, is a key component of the metabolic pathway involved in the production of NAD+. One source of nicotinamide is the **diet.** A second source of nicotinamide is the metabolism of endogenous tryptophan in liver and kidneys. Nicotinamide can also be generated from niacin via the formation of NAD+. **Vitamin B3** is important for many digestive tract functions, including the breakdown of carbohydrates, fats, and alcohol.

Uric Acid

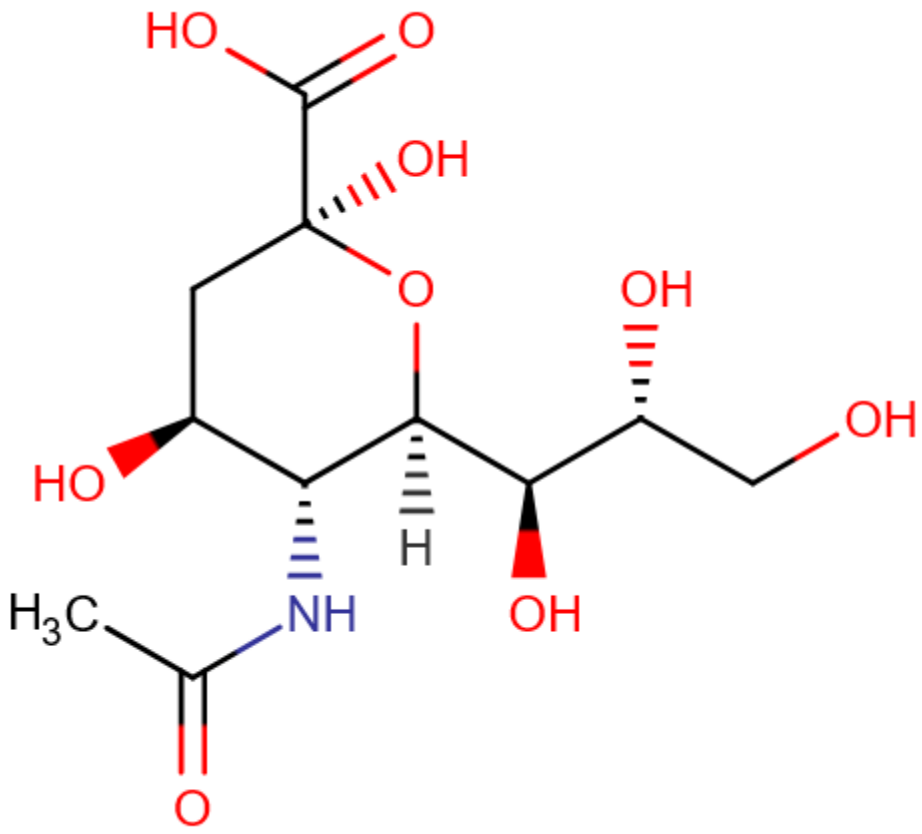


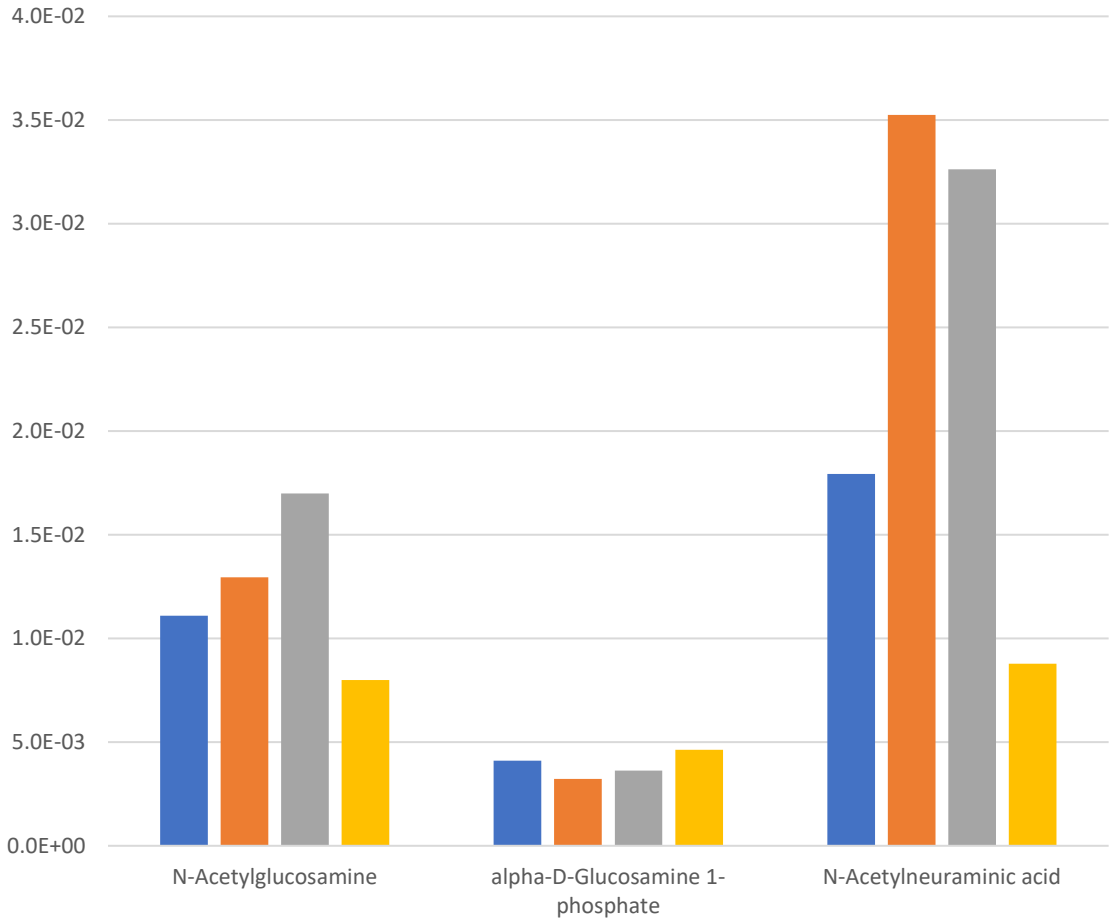


- The intestine is an important potential organ for the excretion of **uric acid** outside the kidneys. The excretion of uric acid of gut is mainly achieved through the action of uric acid transporters and the catabolism of intestinal flora, which plays an important role in the body's uric acid balance.
- Uric acid is the final product of human purine metabolism. Uric acid in the human body maintains a dynamic balance under the action of the liver, kidneys and intestines.

- Guanosine exerts beneficial effects in colitis, through modulation of colonic inflammation and downregulating of NFκB-mediated signaling.

Amino Sugar acids



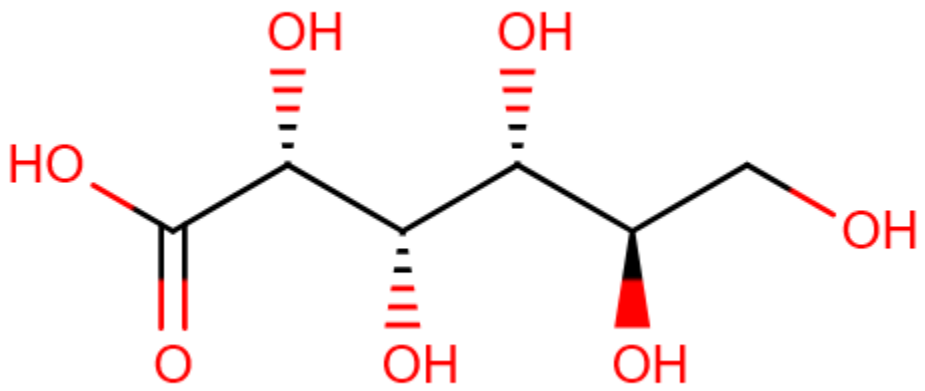


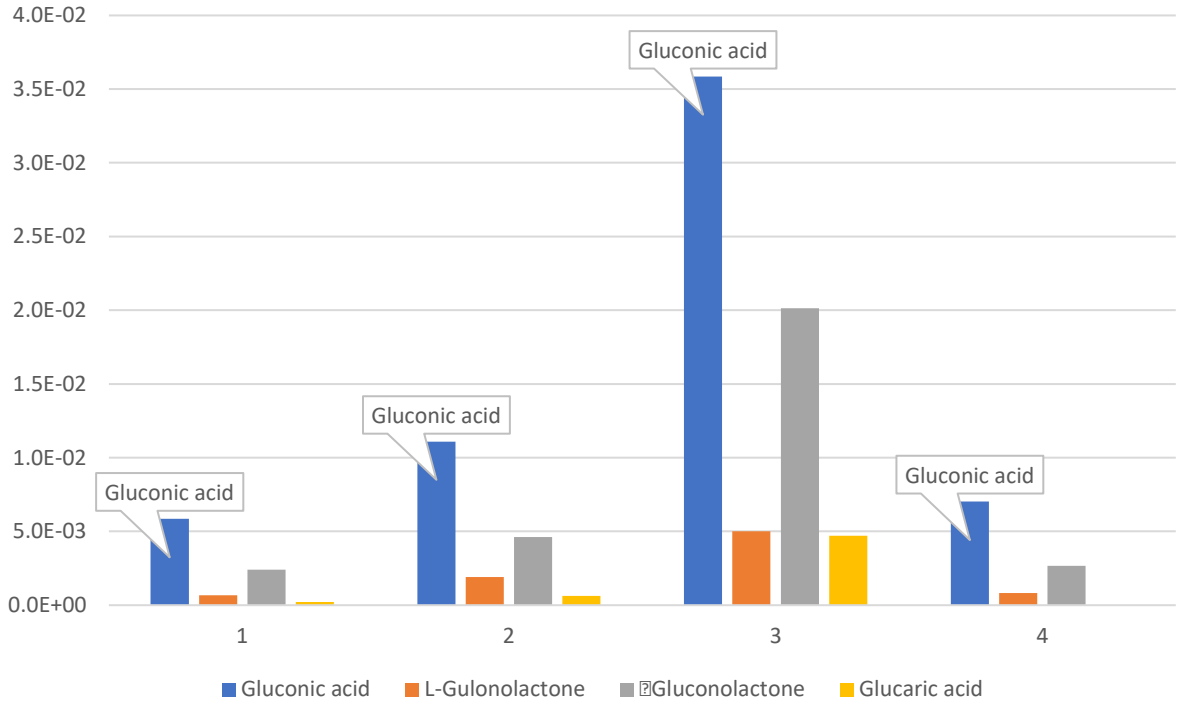
Oligosaccharide and lipoprotein digestion in the small intestine produces a host of small sugar like metabolites, as well as, amino acid modified metabolites.

NANA - *N*-Acetylneuraminic acid is the most abundant sialic acid (SA) in humans and is expressed as the terminal sugar on intestinal mucus glycans. Several pathogenic bacteria harvest and display host SA on their own surfaces to evade Siglec-mediated host immunity.

SA is recognized by immunoinhibitory sialic acid-binding immunoglobulin-like lectins (Siglecs) to prevent autoimmunity.

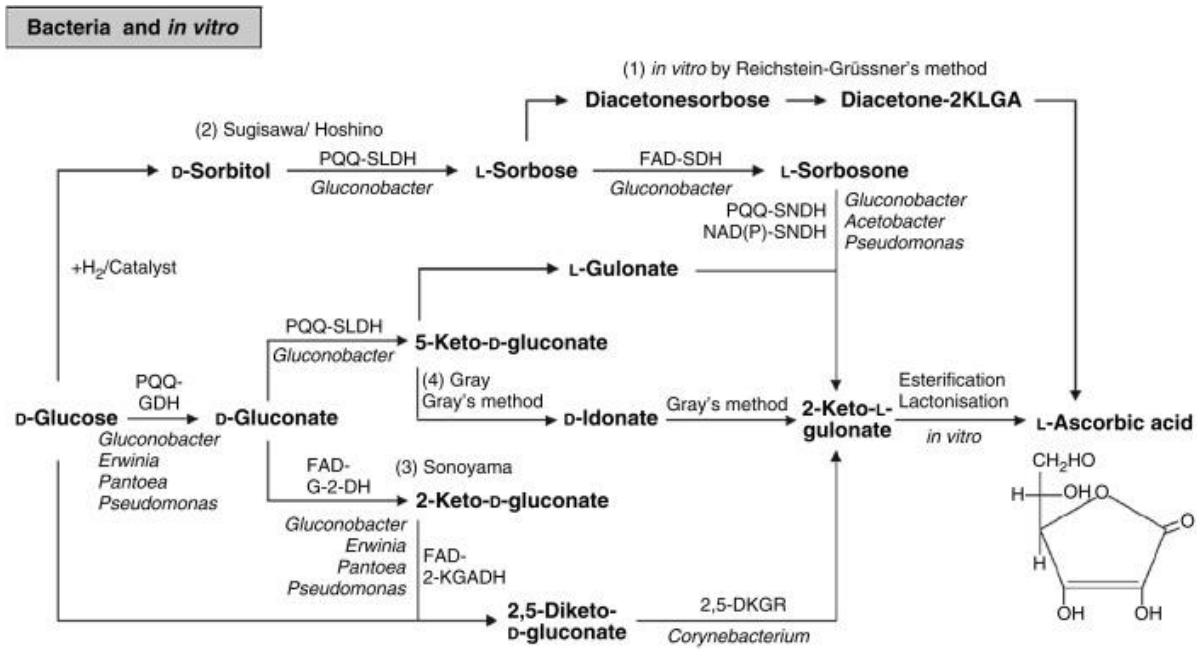
Gluconic Acid



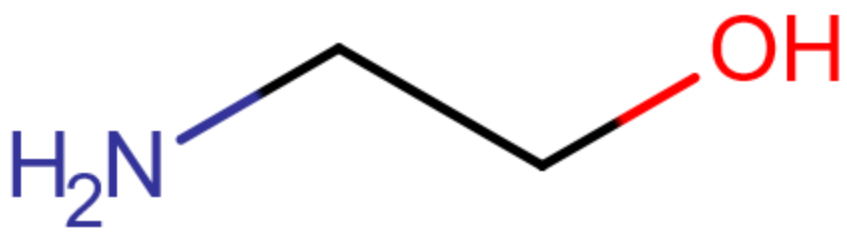


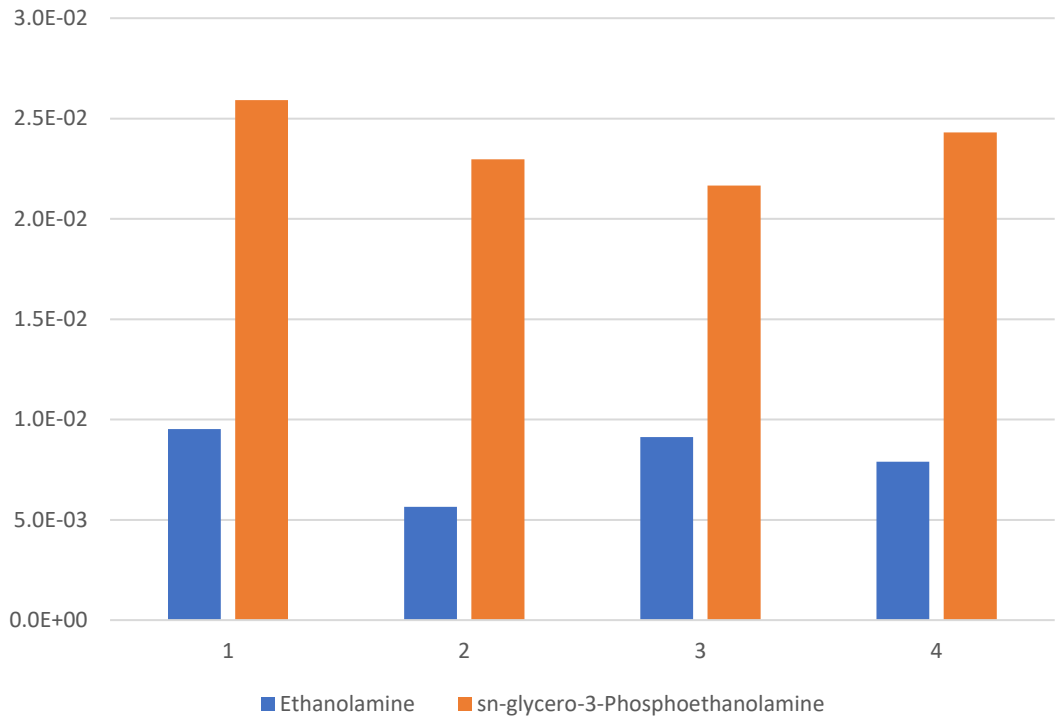
Gluconic Acid is only poorly absorbed in the small intestine and is primarly **fermented to butyric acid** in the lower gut.

Gluconic acid derives from the incomplete oxidation of glucose.



Ethanolamine





Glycero-**Phosphoethanolamine** is the second most abundant phospholipid in animal lipids, after **phosphatidylcholine,** and it is frequently the main lipid component of microbial membranes and a source for ethanolamine.

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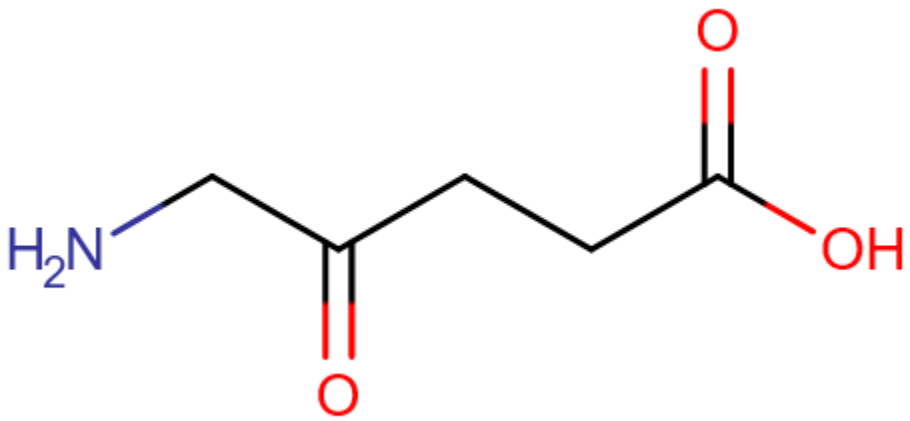
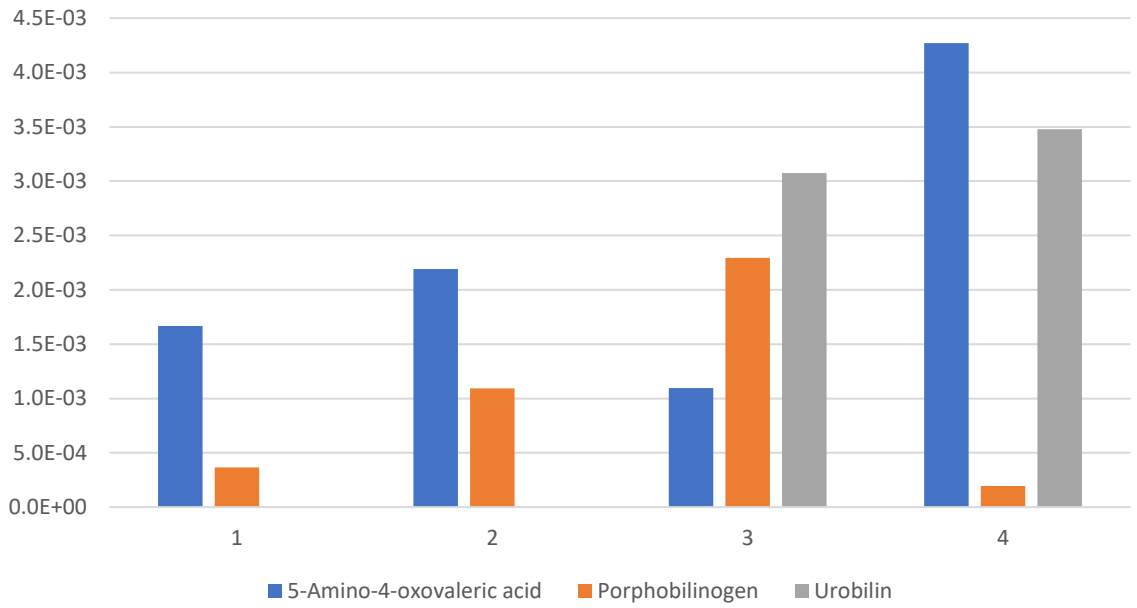
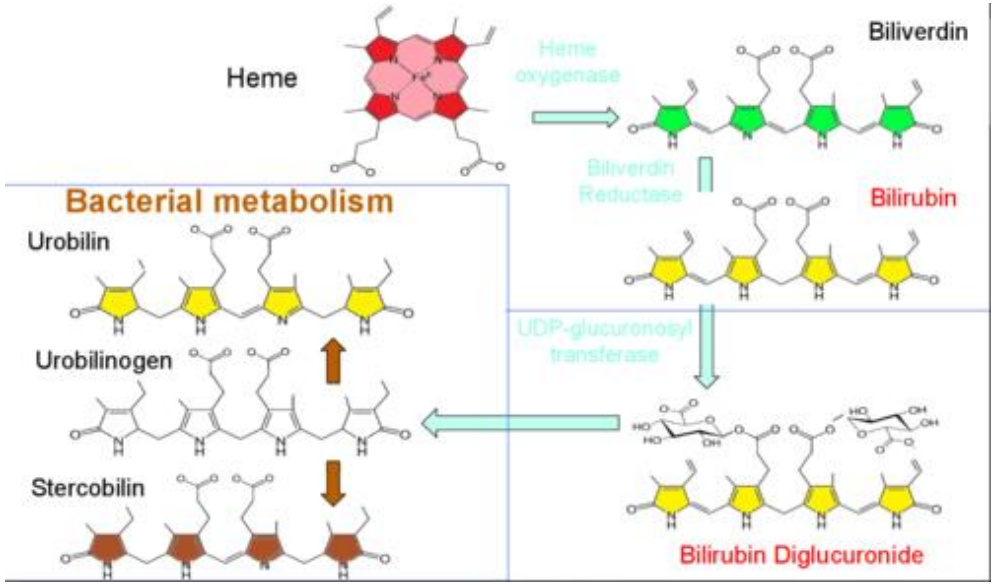
Ethanolamine is required for the proliferation of intestinal epithelial cells and bacteria, which is important for maintenance of the gut microbiome and intestinal development.

5-Aminolevulinic acid







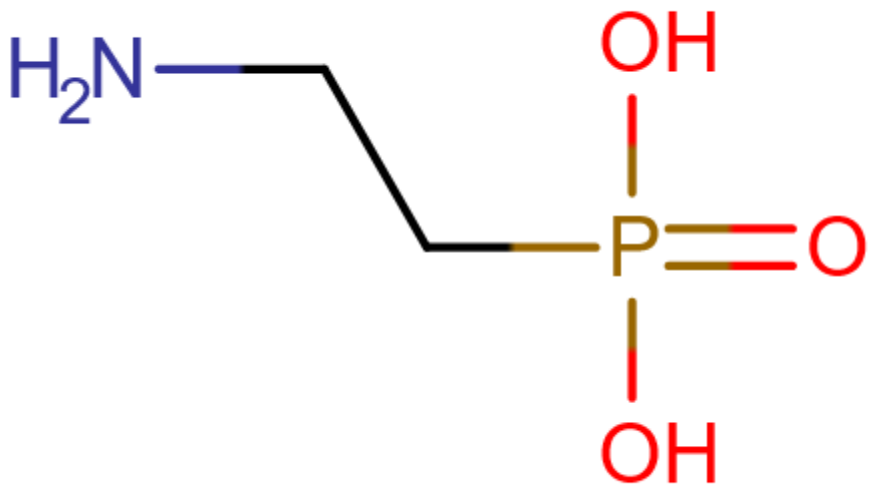
Porphobilinogen is a pyrrole derivative and an essential component of the heme synthesis pathway. It is formed in the cytoplasm from 5-Amino-4-oxovaleric acid (5ALA) or **5-Aminolevulinic acid**.

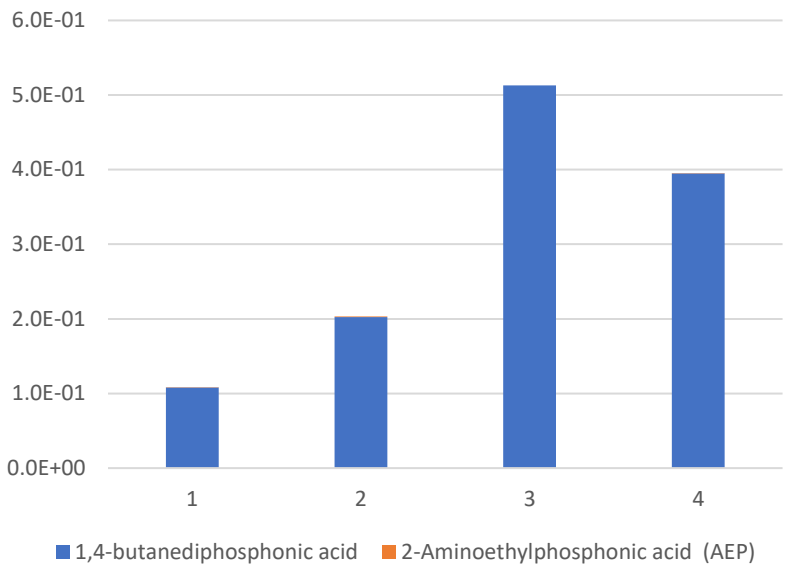
5-Aminolevulinic Acid is a Novel Therapeutic for Inflammatory Bowel Disease (IBD). 5-ALA has been shown to possess anti-inflammatory and immunoregulatory properties through upregulation of heme oxygenase-1 via enhancement of porphyrin, indicating 5-ALA is beneficial for the treatment of inflammatory conditions such as IBD , as well as, type 2 diabetes mellitus, endometriosis, and neurodegeneration.

5-ALA is largely gut-restricted, with low concentrations able to diffuse across the human colonic epithelium.

Bacteria, in turn break down heme into Urobilin.

2-Aminoethylphosphonate





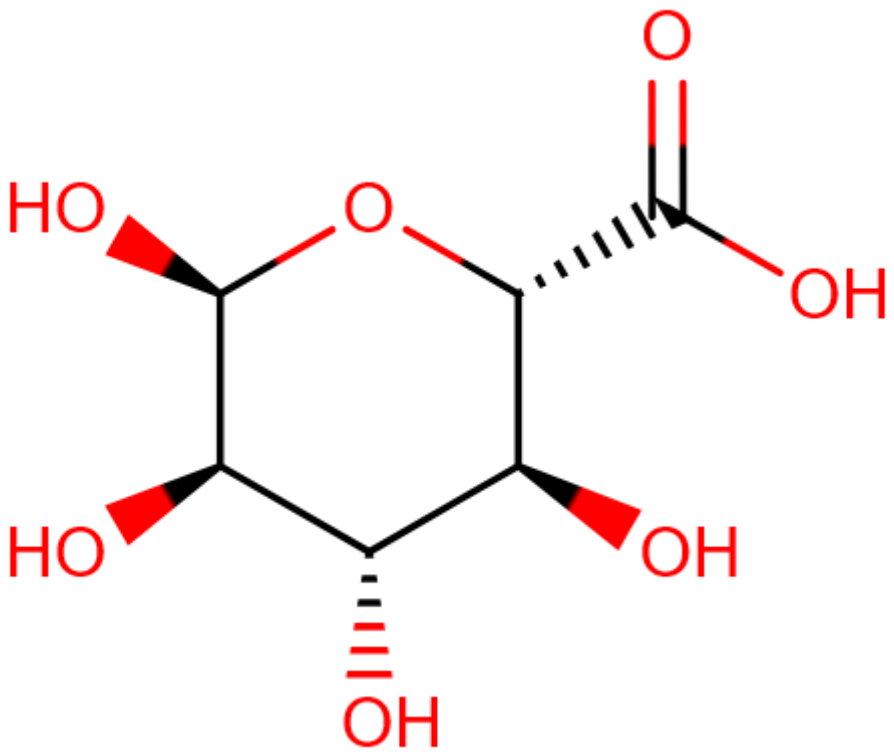
Phosphonates are bioactive natural products. Many bacterial phosphonate biosynthetic capacity is dedicated to tailoring cell surfaces with molecules like 2-aminoethylphosphonate (AEP). Phosphonates are compounds that contain the chemically stable carbon–phosphorus (C–P) bond. They are widely distributed amongst more primitive life forms and constitute a significant component of the organic phosphorus reservoir.

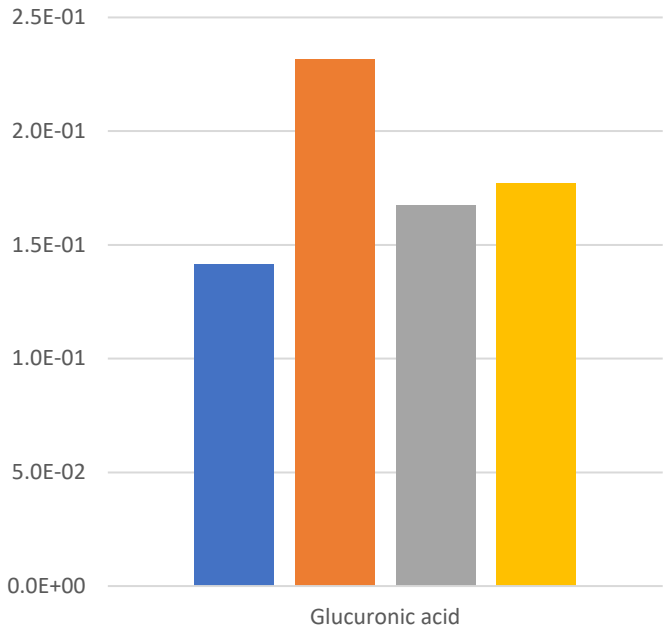
Phosphonyl tailoring cytidylyltransferases (PntCs) prefer AEP over phosphocholine (P-Cho) - a similar substrate used by the related enzyme LicC, which is a virulence factor in Streptococcus pneumoniae.

2-Aminoethylphosphonate (AEP) and its N-alkylated derivatives are the most abundant and ubiquitous of naturally occurring phosphonates. These are typically found as conjugates of glycans, lipids, and proteins, which in turn perform essential biochemical functions in **specialized lower organisms**. In pathogens, AEP conjugates are used for host infection and persistence. Thus, the enzymes responsible for AEP metabolism are prime targets for inhibitor development.

One Source of AEP is bacteria degradation of pyruvate.

Glucuronic Acid



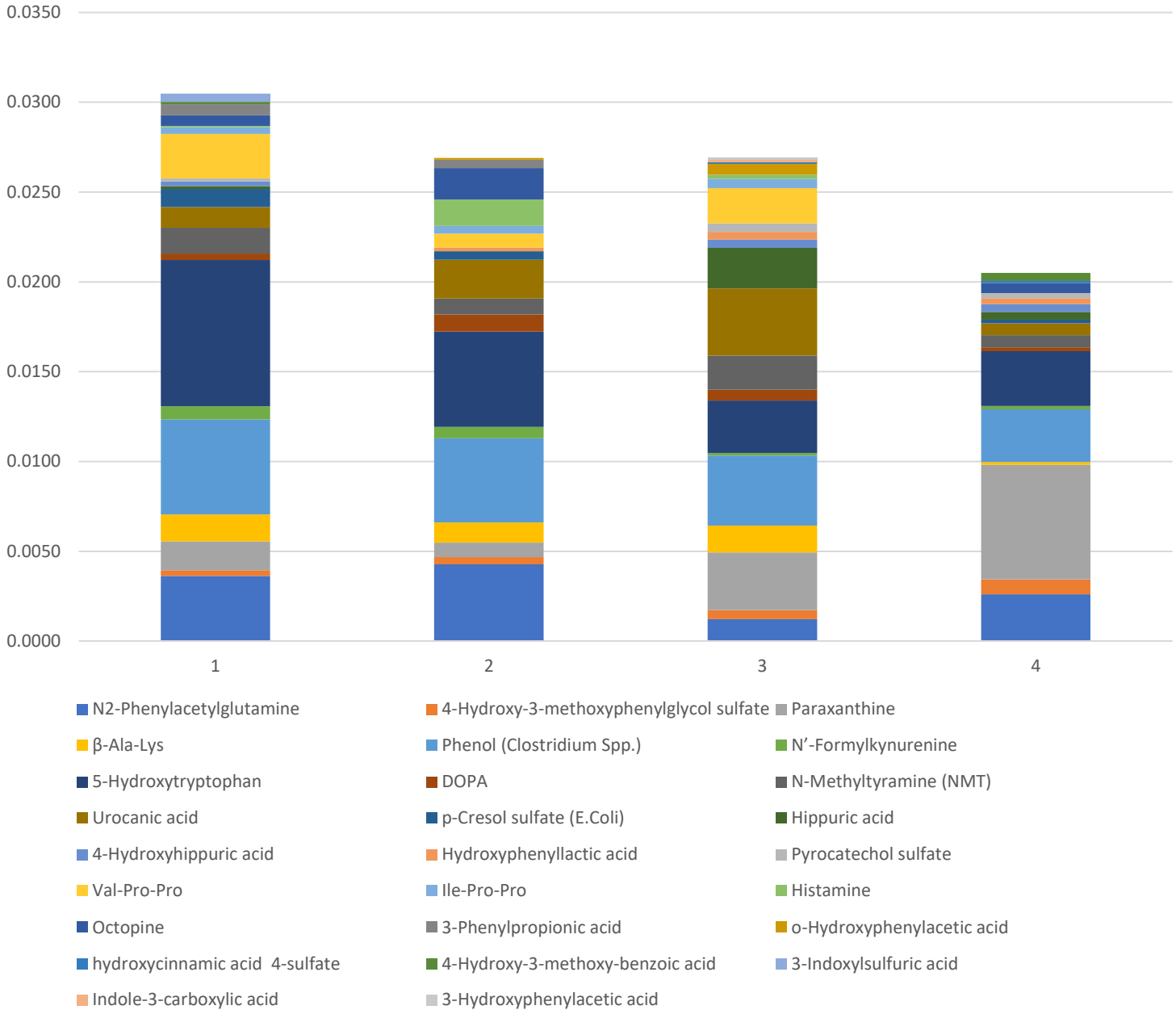


Mammalian systems inactivate endobiotic and xenobiotic compounds by linking them to a glucuronic acid sugar for GI excretion.

In the GI tract, the microbiota **express β-glucuronidase** enzymes that remove the glucuronic acid as a carbon source (or the terminal ends of polysaccharides) effectively reversing the actions of mammalian inactivation.

Probing the actions of microbial β-glucuronidases, and by understanding which substrate glucuronides they process, molecular insights into mammalian-microbial symbioses may be revealed amid the complexity of the intestinal tract.

Biodiversity / Healthy microbiome



Microbial panel to test Biodiversity and healthy biome.

Research suggests that having a wide array of microbes in our gut **makes our microbiome more capable and resilient**.

Many metabolites have been postulated as biomarkers for biome diversity. These biomarkers have been measured in fecal matter, urine and plasma. However, what would be those biomarkers in the small intestine?

Many biome related metabolites are observed in small intestine that match those often cited in healthy biome.

As such a biodiversity panel can be proposed to establish a range of accepted values.

HMT OMEGA Metabolomic Profiling

At HMT, our OMEGA Suite CEMS/LCMS profiles over 900 metabolites in human plasma. A subset of these metabolites appear to be influenced or generated by the gut microbiome reflecting biodiversity and the status of the biome-host relationship.

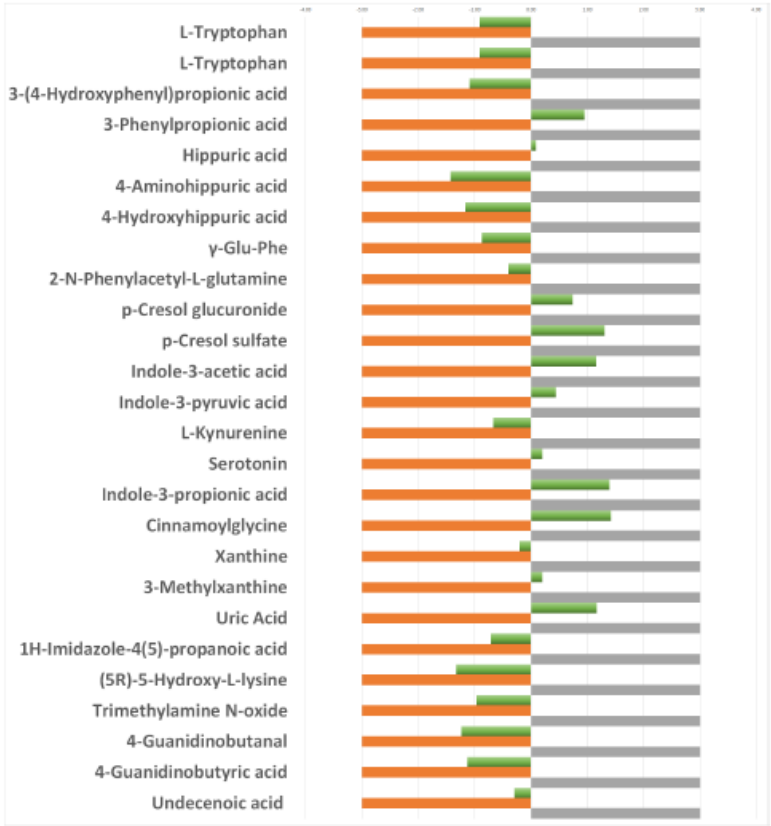
Despite the hundreds of associations between the gut microbiome and disease that have been identified over the past decade, we still do not yet understand what constitutes a 'healthy' microbiome–host relationship. It is likely that the connection between microbiome community structure and human health is highly contextual and complex, depending on diet, behavior, exposure to pathogens, history of antibiotic exposure, genetics and other factors. Understanding this interplay in a metabolomic context will enable future experimental work and the development of personalized multimodal interventions aimed at promoting wellness and treating disease (1).

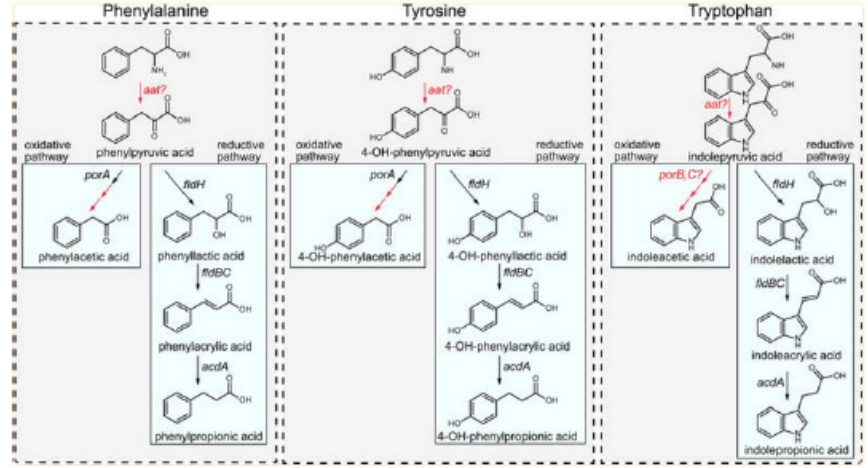
The human gut microbiota produces an unknown number of metabolites that accumulate in the bloodstream , where they can have systemic effects on the host. Among these metabolites are those from aromatic amino acid transformations. The gut symbiont *Clostridium sporogenes* generates aromatic amino acid metabolites. This pathway produces twelve compounds, nine of which are known to accumulate in host plasma. All three aromatic amino acids (tryptophan, phenylalanine and tyrosine) serve as substrates for this pathway, and it involves branching and alternative reductases for specific intermediates. Phenylalanine, tyrosine and tryptophan are all metabolized through the reductive pathway by the same enzymes.Gut bacteria-driven modulation of these plasma metabolites are thought to alter host immune activation and intestinal permeability(2).

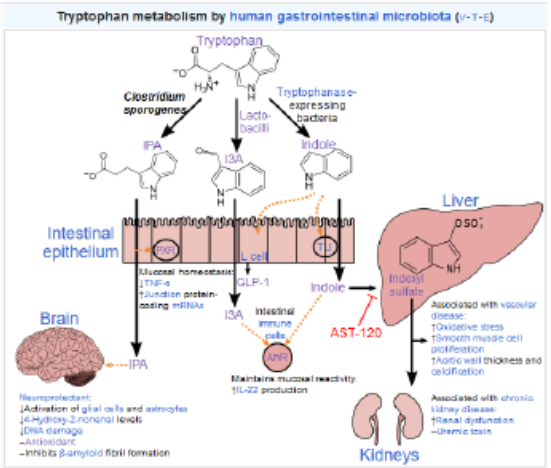
*Clostridium sporogenes*colonizes the human gastrointestinal tract, where it uses tryptophanto synthesize indoleand subsequently 3-indolepropionic acid(IPA)– a type of auxin (plant hormone)– which serves as a potent neuroprotective antioxidant within the human body and brain. IPA is an even more potent scavenger of hydroxyl radicals than melatonin. Similar to melatonin but unlike other antioxidants, it scavenges radicals without subsequently generating reactive and pro-oxidant intermediate compounds.*C. sporogenes* is the only species of bacteria known to synthesize 3-indolepropionic acid *in vivo* at levels which are subsequently detectable in the blood stream of the host (3).

(1)Dodd D, Spitzer MH, Van Treuren W, Merrill BD, Hryckowian AJ, Higginbottom SK, Le A, Cowan TM, Nolan GP, Fischbach MA, Sonnenburg JL. A gut bacterial pathway metabolizes aromatic amino acids into nine circulating metabolites. Nature. 2017 Nov 30;551(7682):648-652. doi: 10.1038/nature24661. Epub 2017 Nov 22. PMID: 29168502; PMCID: PMC5850949.
(2) Wilmanski, Tomasz & Rappaport, Noa & Earls, John & Magis, Andrew & Manor, Ohad & Lovejoy, Jennifer & Omenn, Gilbert & Hood, Leroy & Gibbons, Sean & Price, Nathan. (2019). Blood metabolome signature predicts gut microbiome α-diversity in health and disease. 10.1101/561209.
(3) From Wikipedia, the free encyclopedia

Biodiversity Metabolite Z- SCORE







HMT

Profiling Metabolic Biodiversity

Classifications	Biology Interest
Small Peptides	Proteolytic activity, Collagen turnover, Nutrient supply
Acetylated Amino Acids	Ketogenic potential, SCFA productivity, Bacterial Marker, Membrane absorbtion marker
Gamma-Glu Peptides	Bacterial virulence factors, gastric pathology
Free Amino Acids	amino acid catabolism, mucosla mass and intregrity, sodium balance
Bile Acids	Hepatic gastric condition, bacterial growth
Choline/Betaine/TMAO/Carnitine	Essential nutrients, TMAO toxicity, SCFA productivity
OMEGA Oxidized MCFAs	Fatty acid oxidation sensor, bacterial growth
Nucleic Acids	Purine metabolism, butyrate synthesis, bacterial growth
Sulfated Substrates	PAPS activity, sulfur metabolism
Isethionic Acid	Cellular stress, anoxia/oxia status
Urea/Creatine/Citrulline	Ammonia sensor, energy potential, membrane absorbtion
Vit B3	Essential nutrient, intestinal tract function
Uric Acid	Purine balance, nitrogen balance and export
Sialic Acid	Bacterial growh and diversity
Gluconic Acid	Butyric acid synthesis, oxidation potential
Ethanolamine	Microbial growth
5-AminoLevulinic Acid	Inflammatory marker
Phosphonates	Phosphate availability, bacterial growth
Glucuronic Acid	Host detoxification
BioDiversity Panel	Gut health, biodiversity

- In Summary, with 20 ul of the contents of one SIMBA capsule, over 950 metabolites can be observed.
- These metabolites can be classified into at least 20 categories.
- Each category has a potential to be informative of both host and microbiome health.