Exploring Microbial Metabolism

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Why are there anti cancer drugs in plants?

Vinblastine from Periwinkle plant

Paclitaxel from Pacific Yew

Camptothecin from China’s “Happy Tree”

What are there anti bacterial drugs in soil?

Penicillins

Macrolides such as Erythromycin

Amino glycosides such as Streptomycin

What is the composition of the metabolome found in blood?

From where do these metabolites arise from?   And where are they going?

What are those metabolites in blood that come from our microbiome?

	% Cardiac Output	ml/min
Brain	14.00%	700
Heart	4.00%	200
Lung	2.00%	100
Kidneys	22.00%	1100
Liver	27.00%	1350
Muscle	15.00%	750
Bone	5.00%	250
Skin	6.00%	300
Thyroid gland	1.00%	50
Adrenal gland	0.50%	25
Other	3.50%	175

The liver, kidneys, muscle and brain have the highest blood flows of 1500, 1100, 750 and 700 ml/min.

Are there unique metabolites associated with these specific organs?
What contribution do these organs make to circulating blood metabolites?

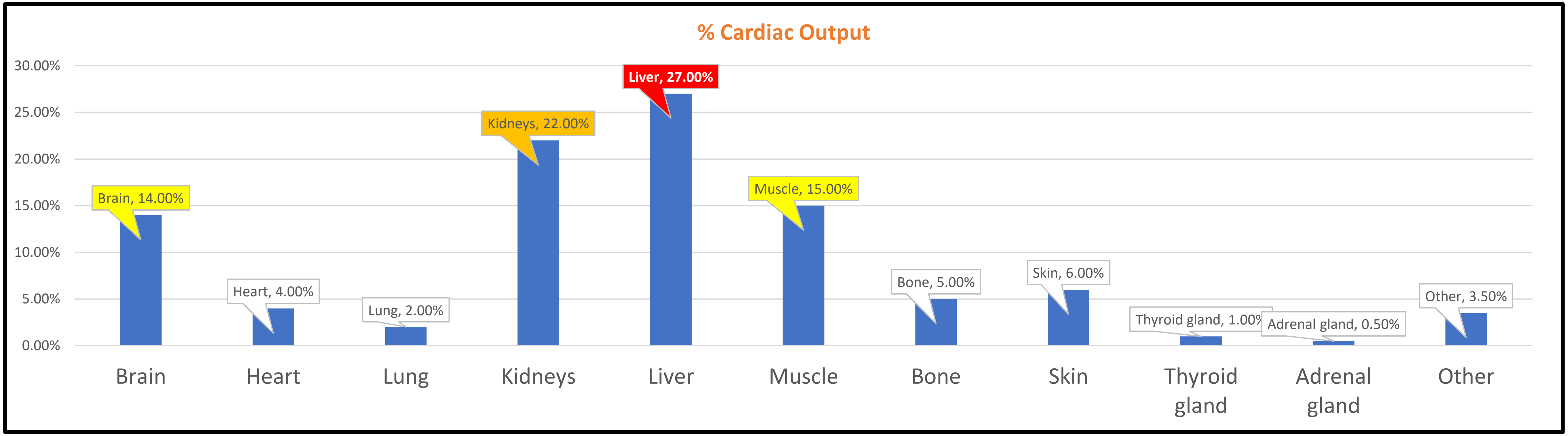
~70% of liver blood supply from portal vein hence continuous interaction with gut bacteria and metabolites.

How does this blood flow impact signature of liver diseases such NASH? NAFLD?

The liver and kidneys combined represent 50% of the blood flow, will then their contribution to the blood metabolome be significantly greater than lung and heart?

Would we expect metabolite contributions from brain and muscle be greater than lung and heart?

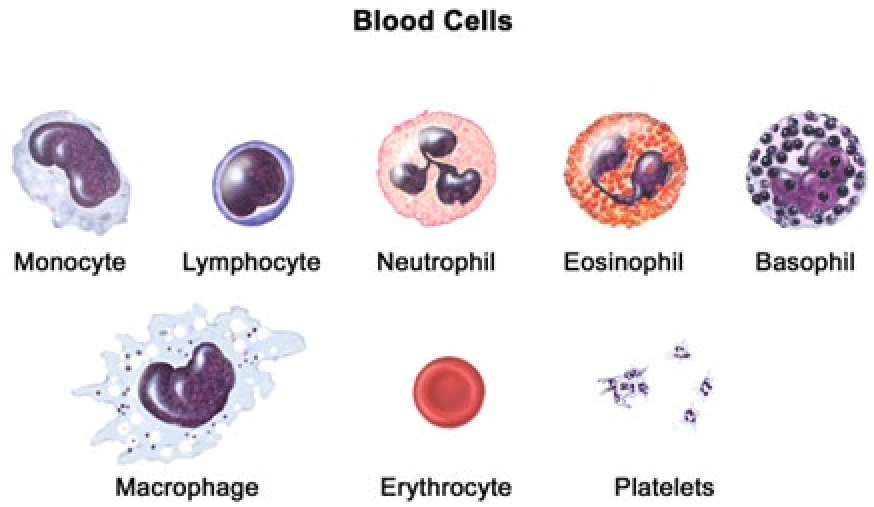
What other factors may influence metabolite exchange between blood and organs? Total surface area? Membrane transporters? Disease and Aging?



Blood contains floating cells of many types

How do these cells contribute to plasma metabolome?

Are there unique metabolites associated with specific blood cell types?



Blood Cells	cells/ul	uM size
RBC	5,000,000	7.5
Neutrophil	12,000	11
Eosinophil	300	23.5
Basophil	30	12
Lympohcyte	2,000	10
Monocyte	500	18
Platlets	300,000	3

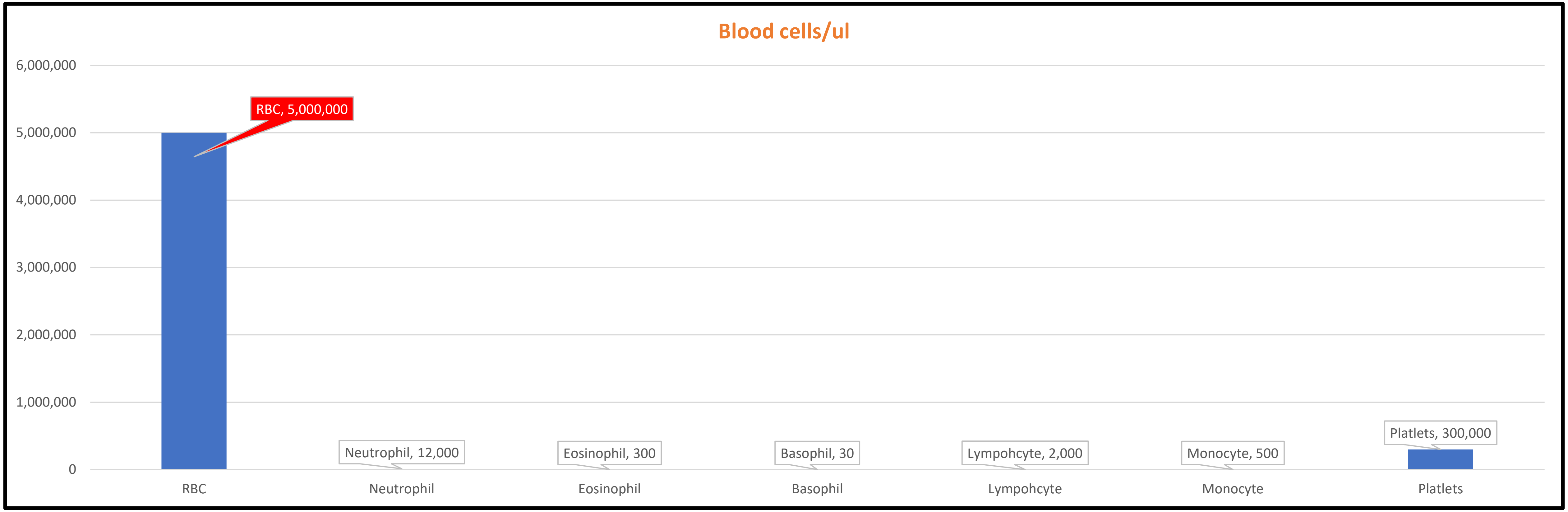
Blood contains 25 Trillion RBCs, the largest contribution of blood cells.

How do red blood cells contribute to plasma metabolome?

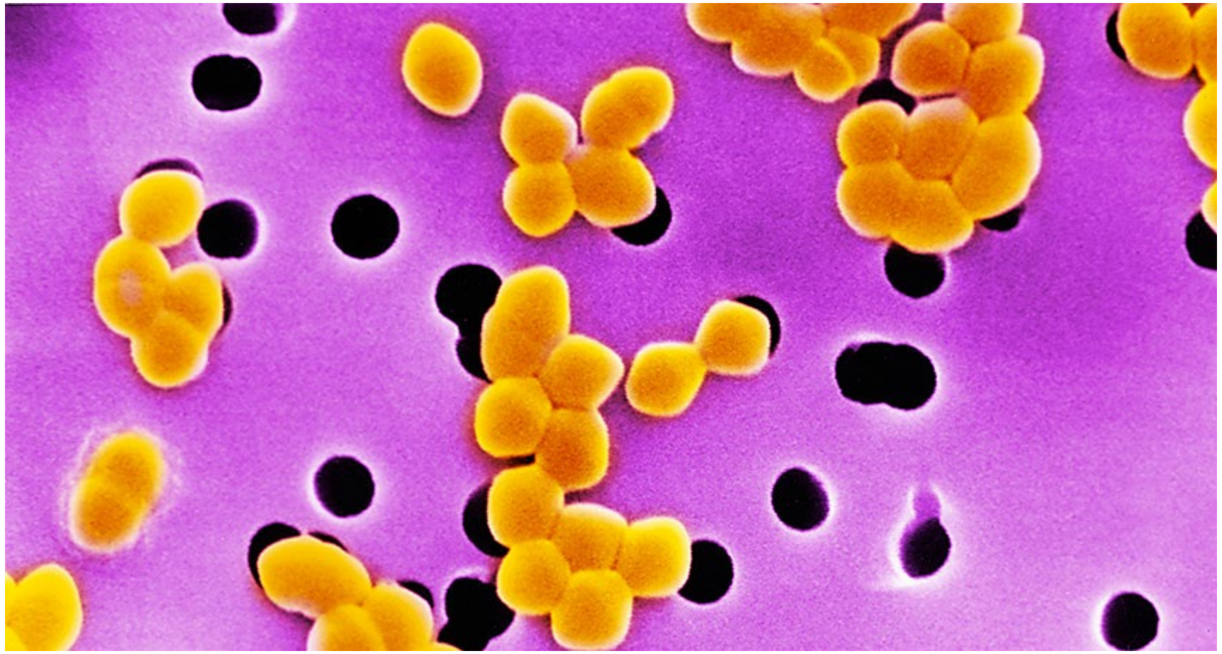
Are there unique metabolites associated with RBCs?

What is the contribution of other cell types?

With 5 Million RBC cells per ul blood, what metabolites in blood would be affected by RBC turnover or cell dysfunction?



Aside from Organs, tissues, bone, blood the human body contains non-human cells. What contribution do these make to blood?



		No bacterial cells/g	time hr	Size
Stomach		10,000	2	
Jejunum		100,000	4	10 feet
Ileum		10,000,000	4	12 feet
Colon		1,000,000,000,000	60	5 feet
Skin		100,000,000,000		22 sq. feet

There are 40 trillion non-human cells in the human body; most are in the intestinal track

This is compared to around 30 trillion human cells.

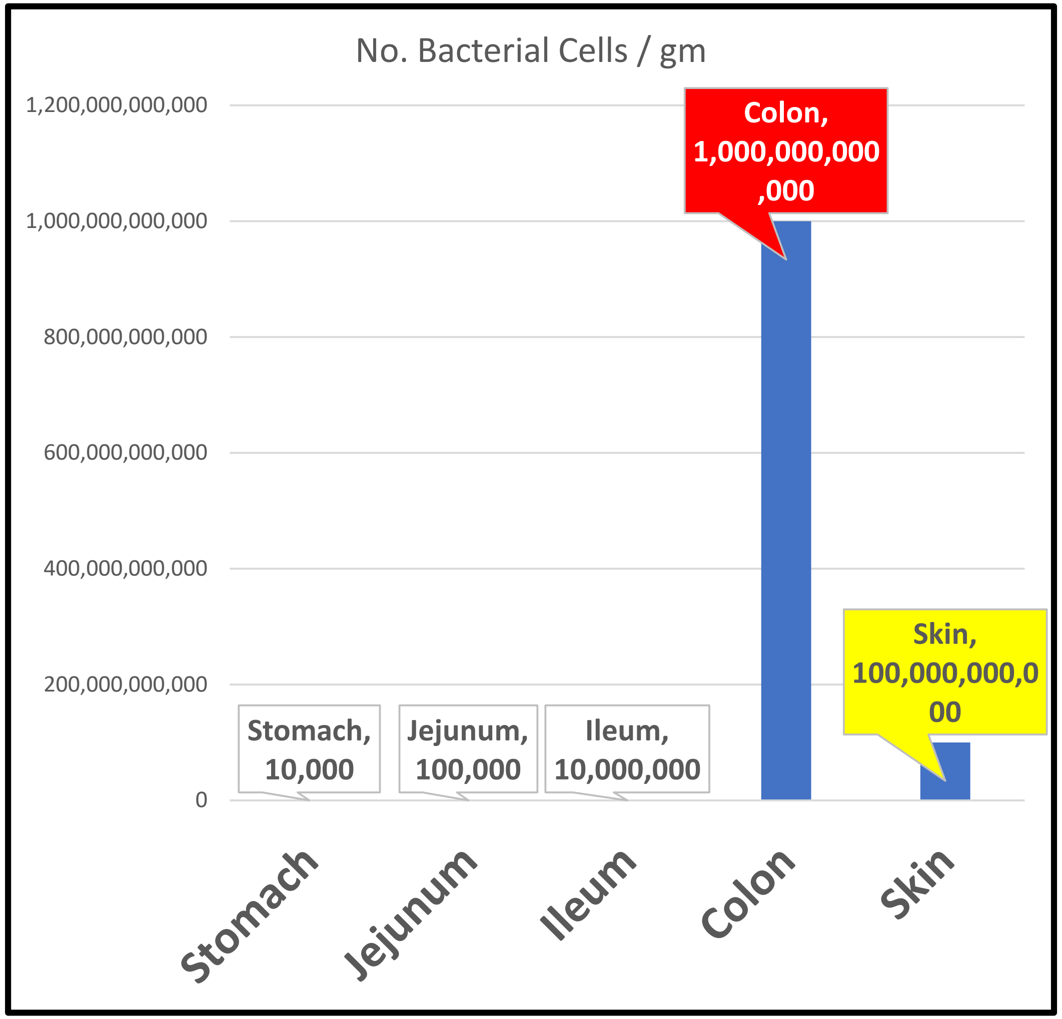
A 220 lb human would have between 2 to 6 lb of microbial cells.

Second most microbial populated area is skin.

Skin provides the first line of external defense, while the gut provides internal defense.

How does this microbiome defend or protect our health?

Is there a dysfunctional microbiome that is harmful?



The largest concentration of microbiota is in the colon, skin is second.

For 220 lb human there would be about 33 lb of skin and 12 lb of intestines representing almost 20% of body mass and majority of the microbiota.

Does this balance change with diseases such as Obesity, Cancer, diet, and exposure?

How do these skin and gut non-human cells contribute to blood?

How to they communicate with each other and the human host?

HMT 2nd to blood, urine and feces contain the most aging related metabolites, as well as, exposed to the highest concentration of non-human cells from the intestinal track.

Such exposure has been linked to developing a healthy immune system.

Researchers calculate that more than 10,000 microbial species occupy the human ecosystem.

The author in 1958 Building his microbiome

Bacteria Travels Outside Intestinal track

- Aside from intestinal track and skin, microbiota are found in eyes– ears – breasts – bladder – nose – belly button, virtually everywhere. The lowest concentrations may be in lungs, brain and circulating blood.

- Bacteria can also harbor and thrive within tumors and within inflamed tissue. Are these pathogens adding to disease severity or acting as anti-inflammatory, immune stimulants, protective and defensive?

Humans have 26K protein coding genes

Where the human genome carries some 26,000 protein-coding genes, researchers estimate that the human microbiome contributes some 8 million unique protein-coding genes or 360 times more bacterial genes than human genes There may be 46 million microbial genes in just the mouth and gut.

~50% of these genes are shared genes that cover many basic functions– carbon metabolism, proteolysis, energy metabolism needed for survival to derive nutrients and energy.

~50% are Unique genes that cover antibody resistance, building microbial cell walls, adaptations to specific pH or temperature.

Humans don't have all the enzymes needed to digest our own diet. Microbes in the gut help break down many of the proteins, lipids and carbohydrates in our diet into nutrients that we can then absorb. Moreover, the microbes produce beneficial compounds, like vitamins and anti-inflammatories that our own genome cannot produce.

Summary

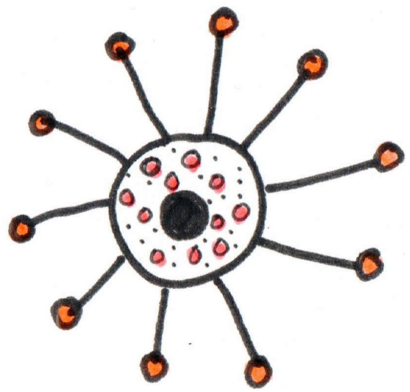
- Blood circulates around major organs like liver, kidney, muscle, as well as, other tissues and organs carrying human and non human metabolites to and from.
- Blood itself contains trillions of RBCs and other cells that contribute to the blood metabolome.
- Cells from microbiota out number human cells and are present in almost all areas of human body, with vast numbers in the intestinal track and skin providing nutrients, vitamins and other needed metabolites for health and well being.
- Microbiota contain vast numbers of metabolic enzymes outnumbering human genome many with unique functions.
- It stands to reason that the influence of the microbiome is vital and significant and a large of the human metabolome is influenced by the microbiota.
- Lastly, how the influence of the microbiome changes under stress, aging, disease and other clinical dysfunctions is yet to be fully measured and understood.

Ancient Biology – how did this start?

- Among all cells on Earth, bacteria are the most abundant and they comprise the most diverse domain in terms of physiology and metabolism and are generally regarded as ancient organisms.

- How much do we know about their biology and communication within us?

- What is the origins of this symbiotic relationship and how does this help us understand their biology within us?

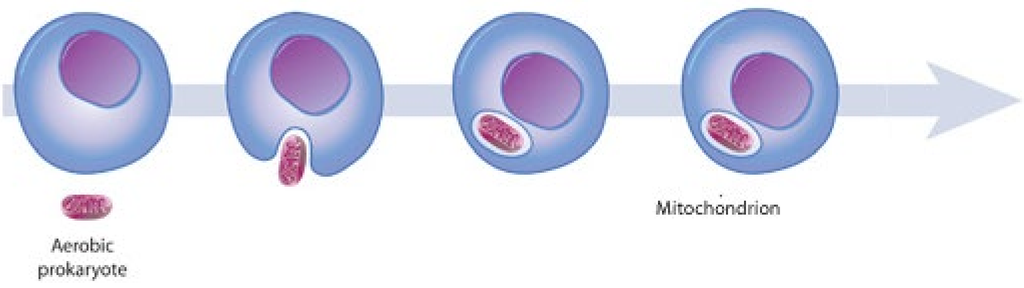


Cellular parasites

- Aerobic bacteria had been domesticated by living cells into a symbiotic relationship within the cell in a unique codependent biology starting early in evolutionary history.

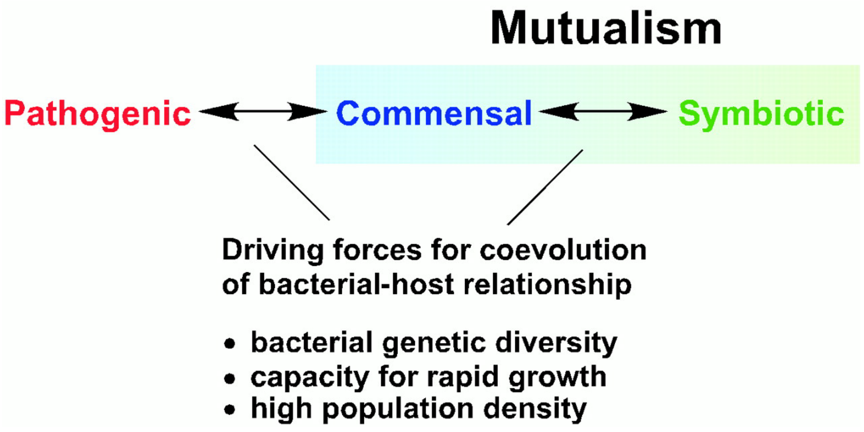
- Our health depends upon a healthy gut and skin microbiome and the microbiome relies upon our providing a nutritional and healthy microenvironment in which they can grow and survive.

- The gut & skin microbiome interact with human cells and organs via signaling and functional metabolites that influence REDOX balance, immune response, inflammation and cell signaling.



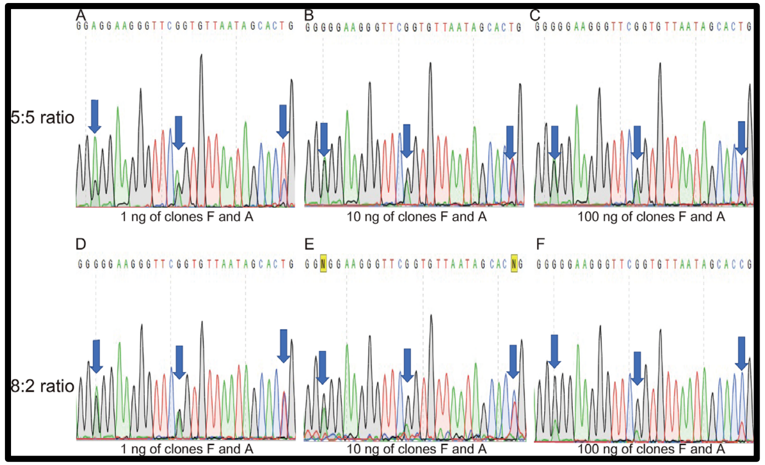
Plentiful and Beneficial

- Next to plants, bacteria account for most of the biomass on Earth.
- Microorganisms biosynthesize metabolites required for the growth and maintenance of cellular functions.
- Metabolites are required for survival and offer a selective competitive advantage to the community.
- The interactions of these secondary metabolites with their biological hosts have been optimized by evolution.



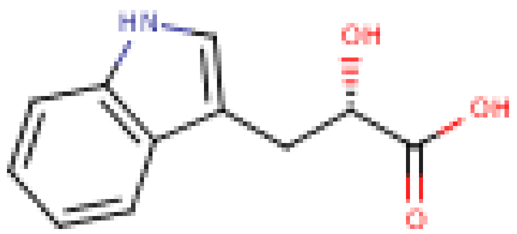
Technology Advancements – DNA/RNA

- Genomic sequencing, 16S and whole genome sequencing, have transformed the ability to identify the diversity within the microbiome and created large databases of DNA and RNA sequences for identification and functional analysis.
- . Due to the functional constancy and highly conserved nature, the 16S rRNA gene is the most commonly used phylogenetic marker for genus and species identification and taxonomic significance in bacteria and archaea. Three targets which encompasses the 18S rRNA gene and two internal transcribed spacers (ITS) have been widely used for a successful identification in the broadest range of fungi.
- Bacterial whole genome sequencing can be divided into bacterial genome *de novo* sequencing and bacterial genome resequencing, which is a method to obtain the complete genome sequence of bacteria.
- Bioinformatics analysis services explore the genetic structure and functions, evolutionary relationships of microbial populations, and the unique biological characteristics of a strain.



Technology Advancements - Metabolomics

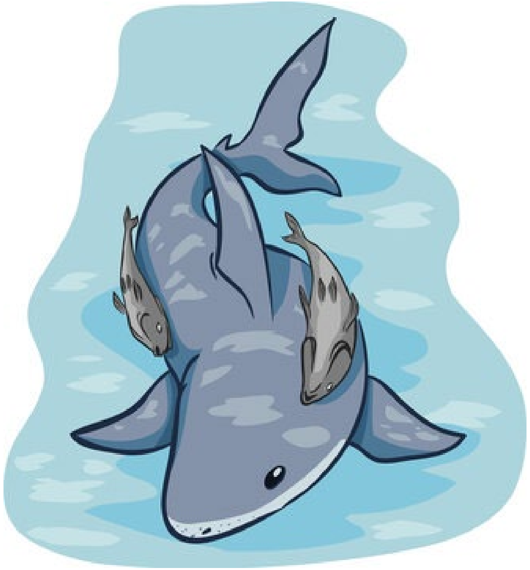
- Metabolomic databases and technologies have also grown during past 20 years, building large libraries of metabolites that have been identified and quantitated in multiple biological sources, including many specific to originate within the microbiome.



- Bioinformatics has allowed the identification and construction of metabolic pathways and functions.

Natural defensives

- Bacterial metabolites providing the host with chemical defenses and antioxidants.
- Many have suspected or proven roles in immune and oxidative defense and have attracted much attention as sources for new therapeutics.
- Many of these know microbial metabolites can be potential drugs. Their low natural abundance, however, represents a major obstacle to drug development.
- Many bioactive microbial metabolites have yet to be discovered or have their bioactivities understood.



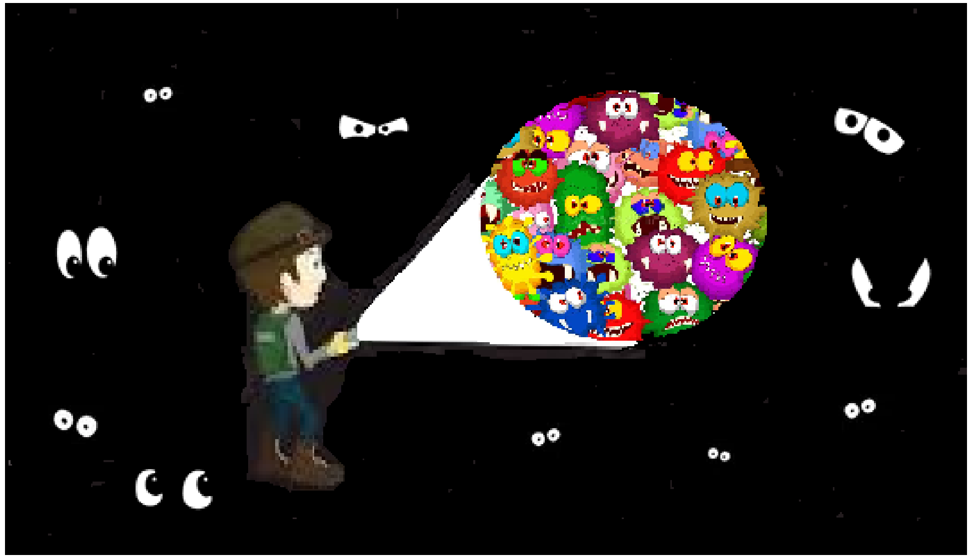
What do we know?

- We are only beginning to understand the microbiota-host relationship. Expressed genes and observed metabolites are mostly not well understood.

- How good are we at measuring such a diversity of metabolites that come from different types of samples and with a large variation in size and shape?

- Is there one solution to identify microbial metabolites or do we need several technologies to cover this landscape?

- Every metabolomic method has its own bias as to what is can measure and measure well.



Biological Diversity and Function

- The biological roles of this vast chemical microbiome created diversity that we observe in fluids and tissues is <u>largely a mystery</u>. There is much to learn about how bacteria adapt to their microenvironments and how they interact with their host.

For example, we just recently understood how bacteria actually move.

“University of Virginia: Ending a 50-Year Mystery, UVA Reveals How Bacteria Can Move” Sept 2022.

Chemical Diversity overwhelms Biodiversity

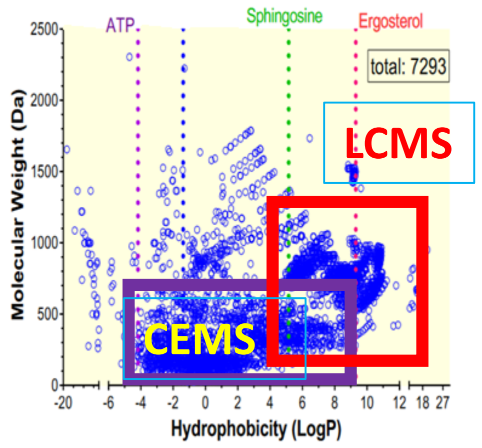
- The metabolome consists of thousands of small molecules (under 1500 mass). Over half in which are under 500 in mass and vary in greatly in their physical properties->
- The most often used metabolomic approaches using LCMS methods covers a portion of chemical space dominated by lipid like or non-polar metabolites ->
- These non-polar metabolites cover a large range of mass from under 100 to over 1000.
- While polar metabolites are expected to dominate the landscape and are mostly under mass 500.
- Capillary Electrophoresis Mass Spectrometry (CEMS) covers a majority of this small polar metabolites chemical space and complements that LCMS methods that cover largely the non-polar space.

The metabolome is chemically diverse which makes it hard to study (Unlike other macromolecules like nucleic acids and proteins)

Hydrophobic
e.g. fatty acids, lipids, cholesterol

Hydrophilic Positive
e.g. amino acids, amines

Hydrophilic negative
e.g. sugars, organic acids



Polar

Lipid

New Methods for Discovery

- Human Metabolome Technologies (HMT) has unique one-of-a-kind capillary electrophoresis-mass spectrometry (CE-MS) services recently upgraded to even higher performance.

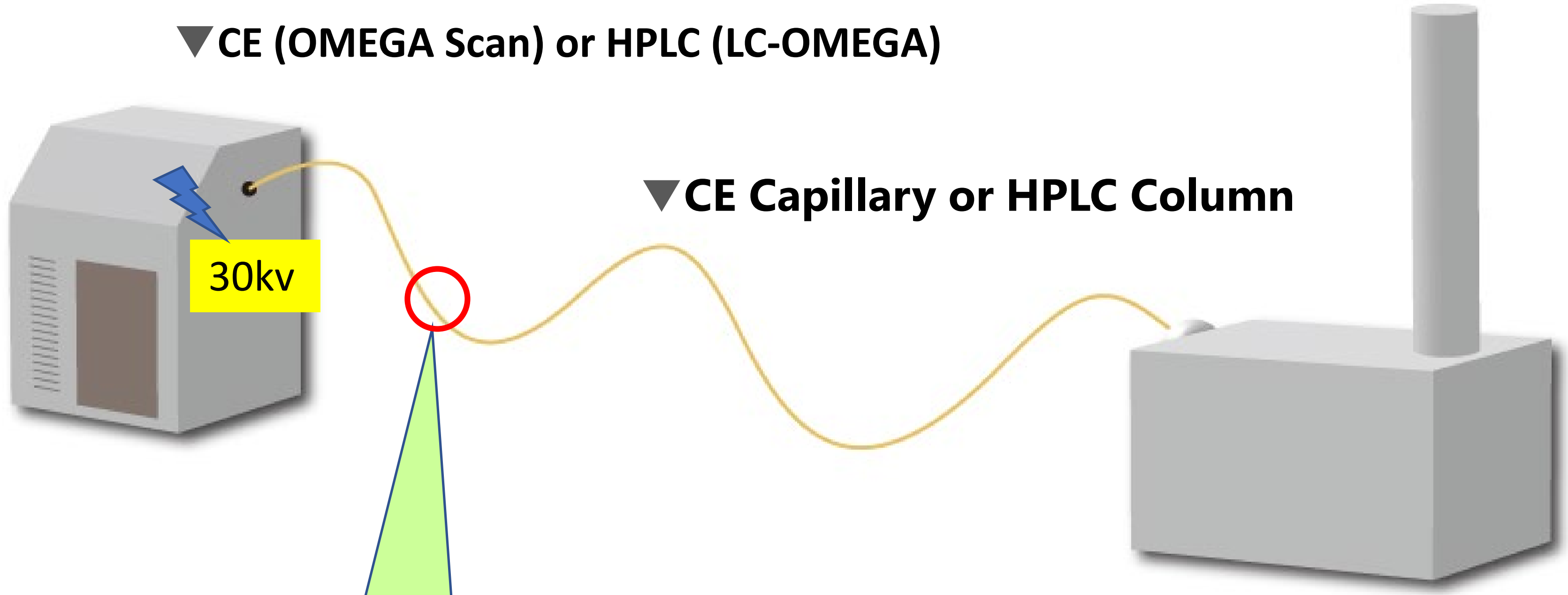
- No other CRO uses CE-MS untargeted profiling to cover the chemical diversity from the microbiome, as well as, endogenous biochemical pathways.

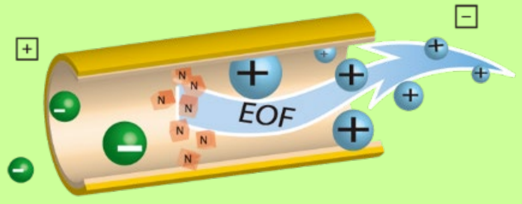
- This methodology offers a new look into the chemical world of the microbiome. What are we observing?

OMEGA 2 adv Unique CE-MS Technology

▼CE (OMEGA Scan) or HPLC (LC-OMEGA)

▼CE Capillary or HPLC Column





In CE, compounds are separated based on their charge and ionic radius (size) using an electric gradient. Touch free.

In LC, compounds are separated based on their hydrophobicity using a stationary phase. On / Off mechanism.

CEMS consists of a high resolving capillary electrophoresis unit attached to a Q-Exactive Plus high performance mass spectrometer.

Separation of metabolites is accomplished using an open fused silica capillary tube with a large electrical field applied across the tube to allow metabolites to separate according to charge, mass and physical properties.

HMT has unique patents on the construction of their CEMS platforms.

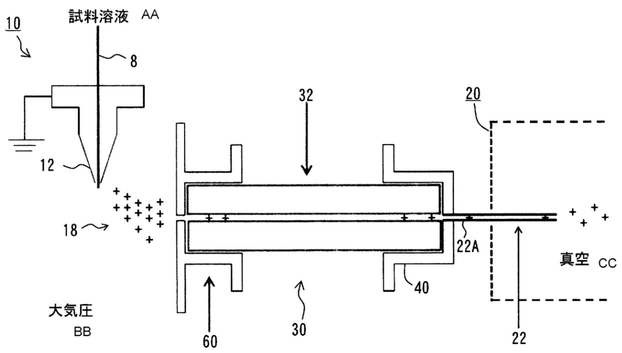
OMEGA-Scan – Highest Sensitivity and Resolution

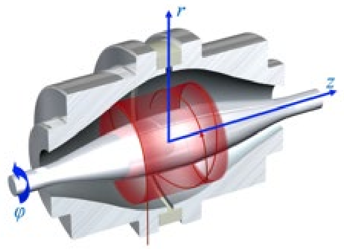
- This Unique platform combining CE's high separation resolution for ionic metabolites and Q-Exactive Plus high sensitivity & mass resolution are unique and covered by patent protections.

- HMT is the only CRO to offer this technology for untargeted metabolomic profiling and as such makes unique and novel discoveries.

HMT Patent in Japan (6106864)
HMT international Patent (PCT/JP2017/012095) as "Ion source adaptor"

Metabolomics Platform with Capillary Electrophoresis Coupled with High-Resolution Mass Spectrometry for Plasma Analysis, Analytical Chemistry, December 2018



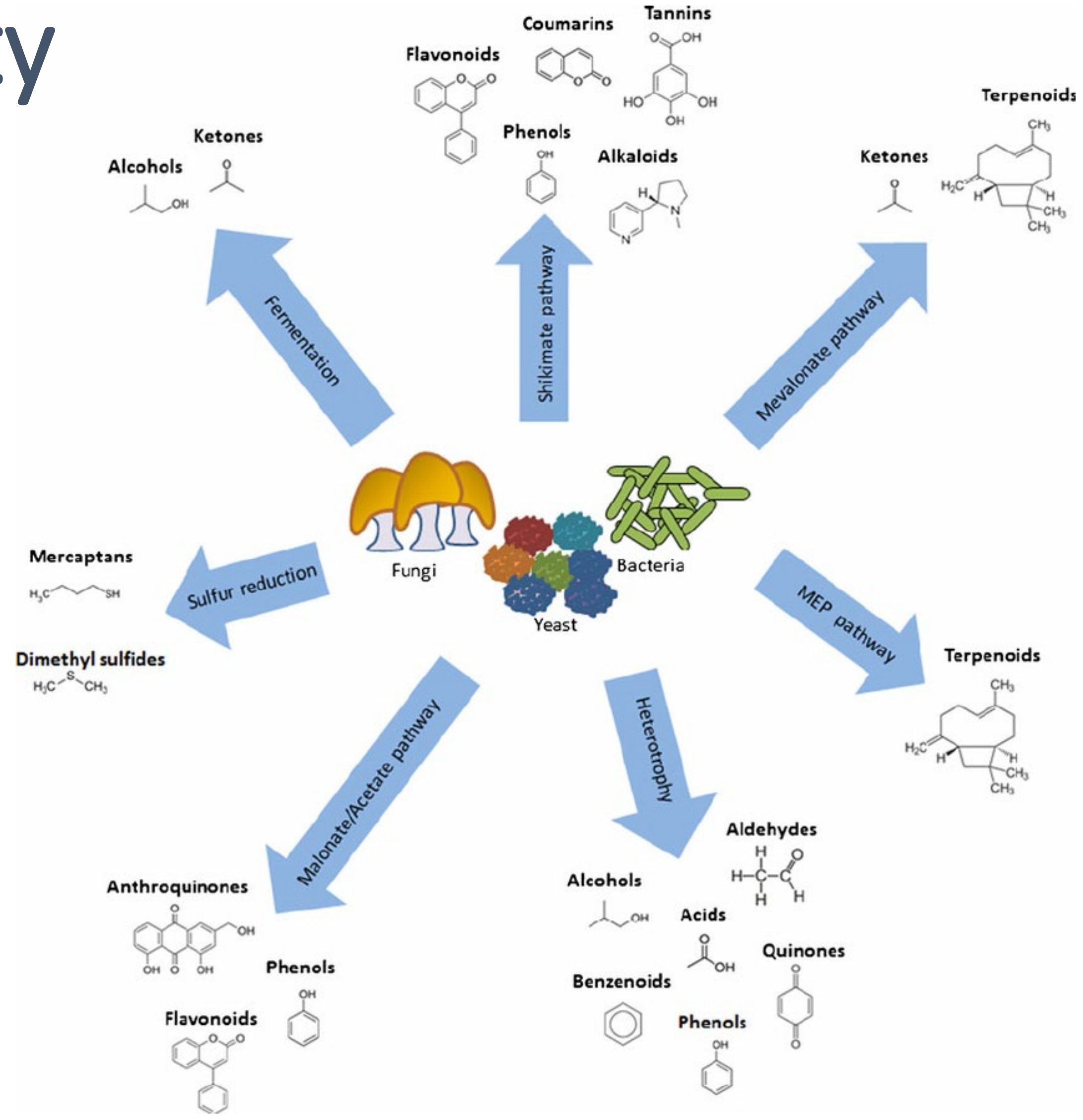


Metabolite Class Diversity

- Bacteria transform many common biochemical compounds and macromolecules into new metabolites for fuel, biomass production, survival & protection.

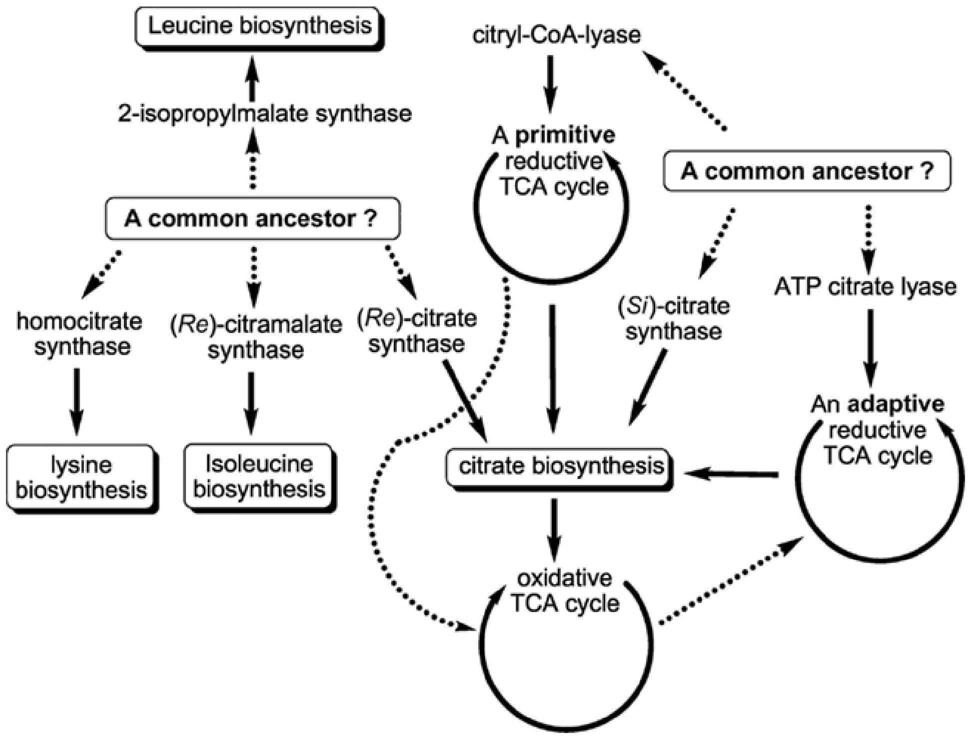
- While some of these transformations and pathways are common to endogenous biology, others are very unique to individual microbial species and families, signatures of specific bacteria and fungi.

- These metabolites cover a wide range of polarity, size and physical properties.



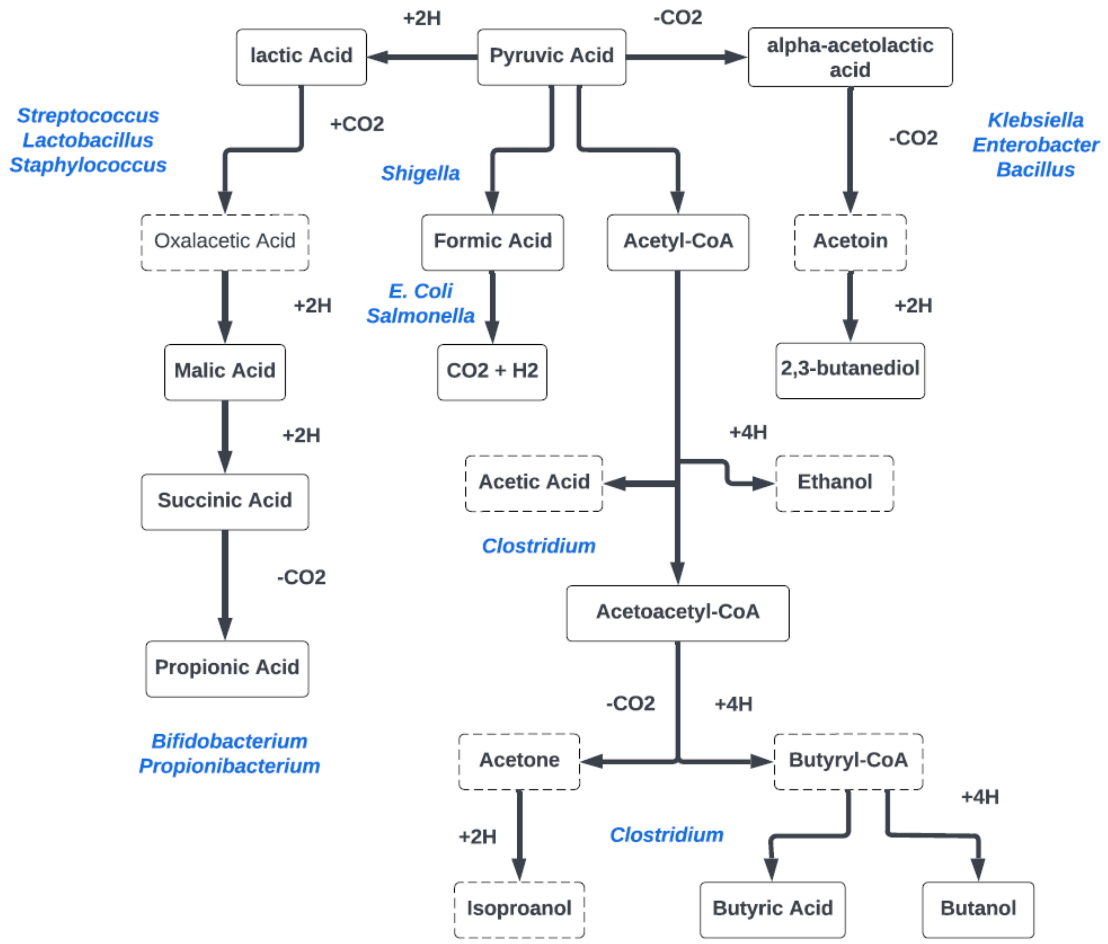
Defining Metabolite Pathways

- Human and microbiota share many common biochemical transformations include:
  - Central carbon (glucose)metabolism
  - ATP generation
  - Antioxidant response
  - Biomass synthesis
- However, there aresignificant differences in specific pathways generating novel metabolites between human and non-human.
- For example, while BCAA amino acids and Lysine are essential in human diet, they can be synthesized by bacteria.



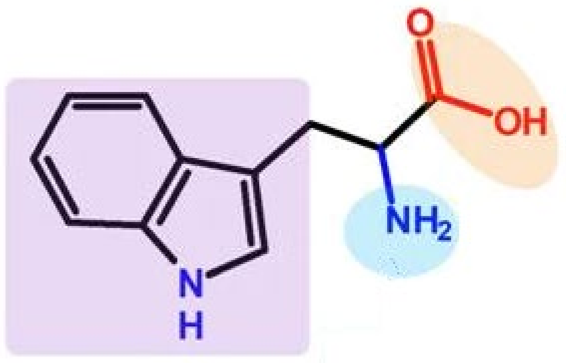
Pyruvate & SCFA Synthesis

- A common class of metabolites linked to bacteria are the small chain fatty acids (SCFAs), that are linked to health and well being.
- These bioactive metabolites can be produced from multiple different pathways from pyruvate with relative amounts depending upon the bacteria diversity.
- Measuring the relative amounts of SCFA may suggest a certain bacterial profile.
- There are major differences in other pathways as well including amino acid synthesis and degradation, vitamin biosynthesis and many others.



Tryptophan Metabolism as Example

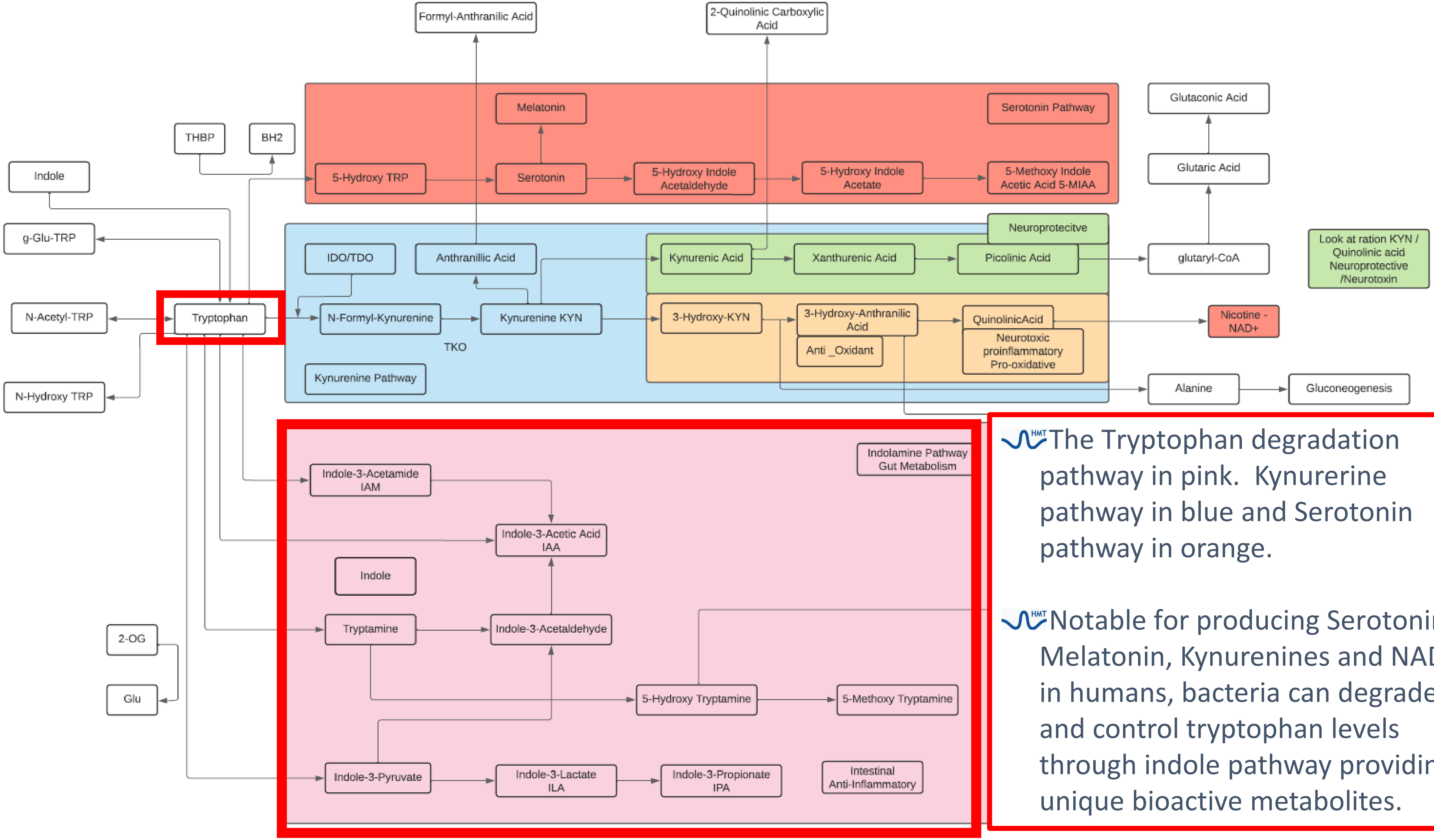
- While Tryptophan is the least abundant amino acid in proteins, tryptophan metabolites have many diverse functions. Many of these tryptophan microbial metabolites are bioactive, highly regulated and are linked to human health.



- Five genera, *Clostridium*, *Burkholderia*, *Streptomyces*, *Pseudomonas*, and *Bacillus* are enriched in the tryptophan metabolism pathways, suggesting a higher potential of these bacterial groups to metabolize tryptophan in gut and influence our neurochemistry, immunological response and health.

- Tryptophan metabolites are AhR ligands – receptors that are linked to immune function and stem cell maintenance at barrier tissues like the gut, skin and lung.

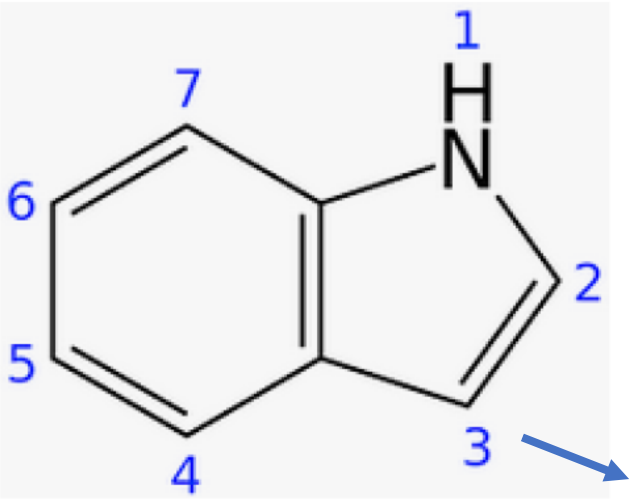
- The number of these metabolites from Tryptophan continues to grow, most with unknown biology.



- The Tryptophan degradation pathway in pink. Kynurerine pathway in blue and Serotonin pathway in orange.

- Notable for producing Serotonin, Melatonin, Kynurenines and NAD+ in humans, bacteria can degrade and control tryptophan levels through indole pathway providing unique bioactive metabolites.

5,6-Dihydroxy
5-Hydroxy
5-Acetyoxy
5-Methoxy



4-amino
4-Phosphate

Protonation not on N, but on C-3
Most electrophilic aromatic substitution C-3

2-Carboxy
2,3-dione
2,3-dihydroxy
2-Acetic

3-ethane amino
3-ethane dimethylamino
3-methyl
3-cyano
3-alanyl
3-acrylate
3-sulfate
3-carboxylate
3-methylacetate
3-acetonitrile
3-acetoxy
3-acetaldehyde
3-acetamide
3-carboxyl
3-lactoyl
3-pyuvoyl
3-propionyl
3-butyryl

Pos or Neg health benefits

Neuroprotective
Antioxidative
Oxidative
Inhibit fibrl formation
Reduce DNA damage
Increase IL-22

Liver
Brain
Kidneys
Intestinal Epithelium

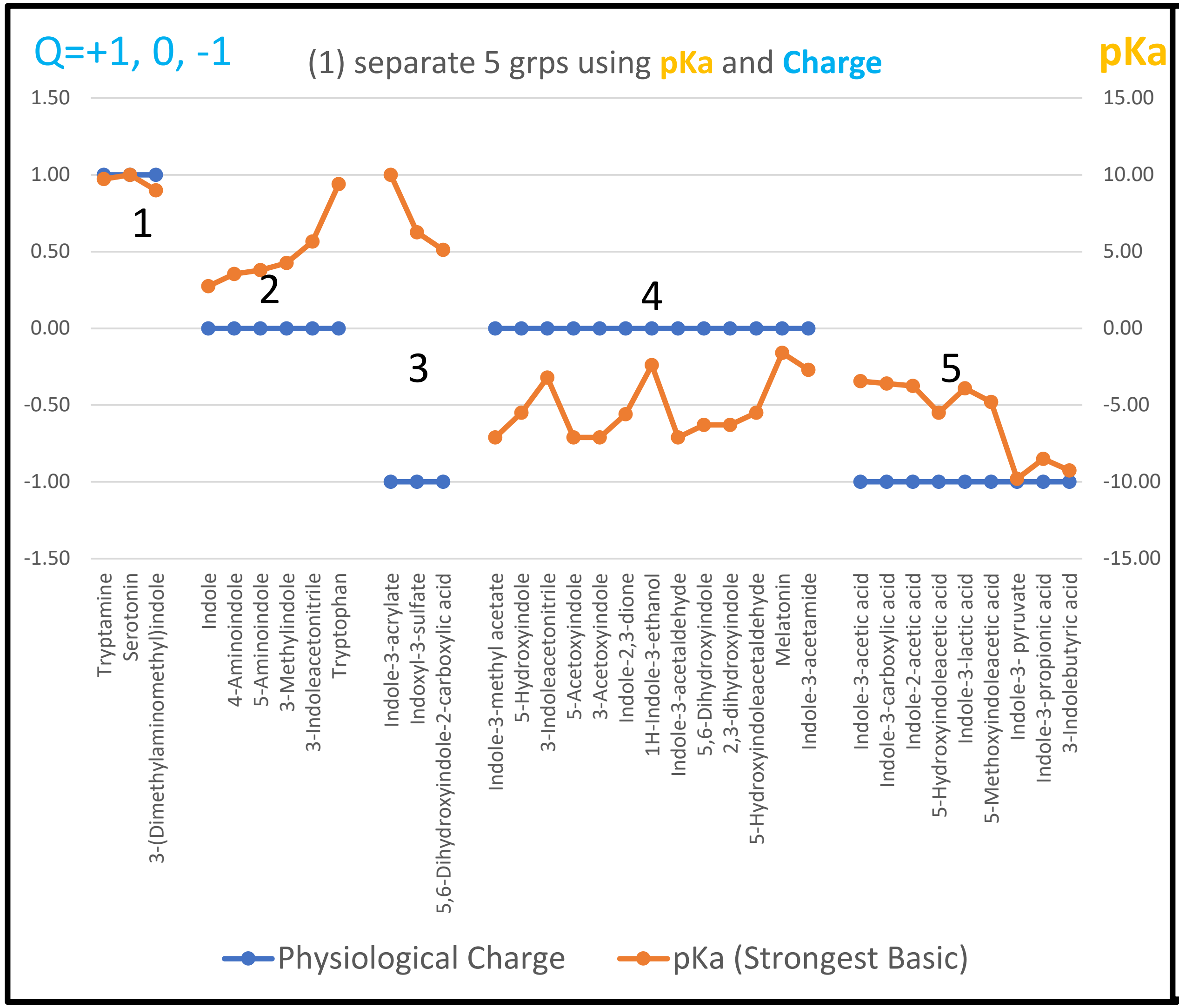
- The Indole pathway generates many different metabolite species.
- Some of these metabolites have had research on their bioactivities.
- Some are neuroprotective, some are toxic, but the activities of most are unknown.
- Some are thought to be essential to human health.
- CEMS has identified over 50 tryptophan derivatives, including over 40 indoles.
- Most of the chemistry is at position 3.

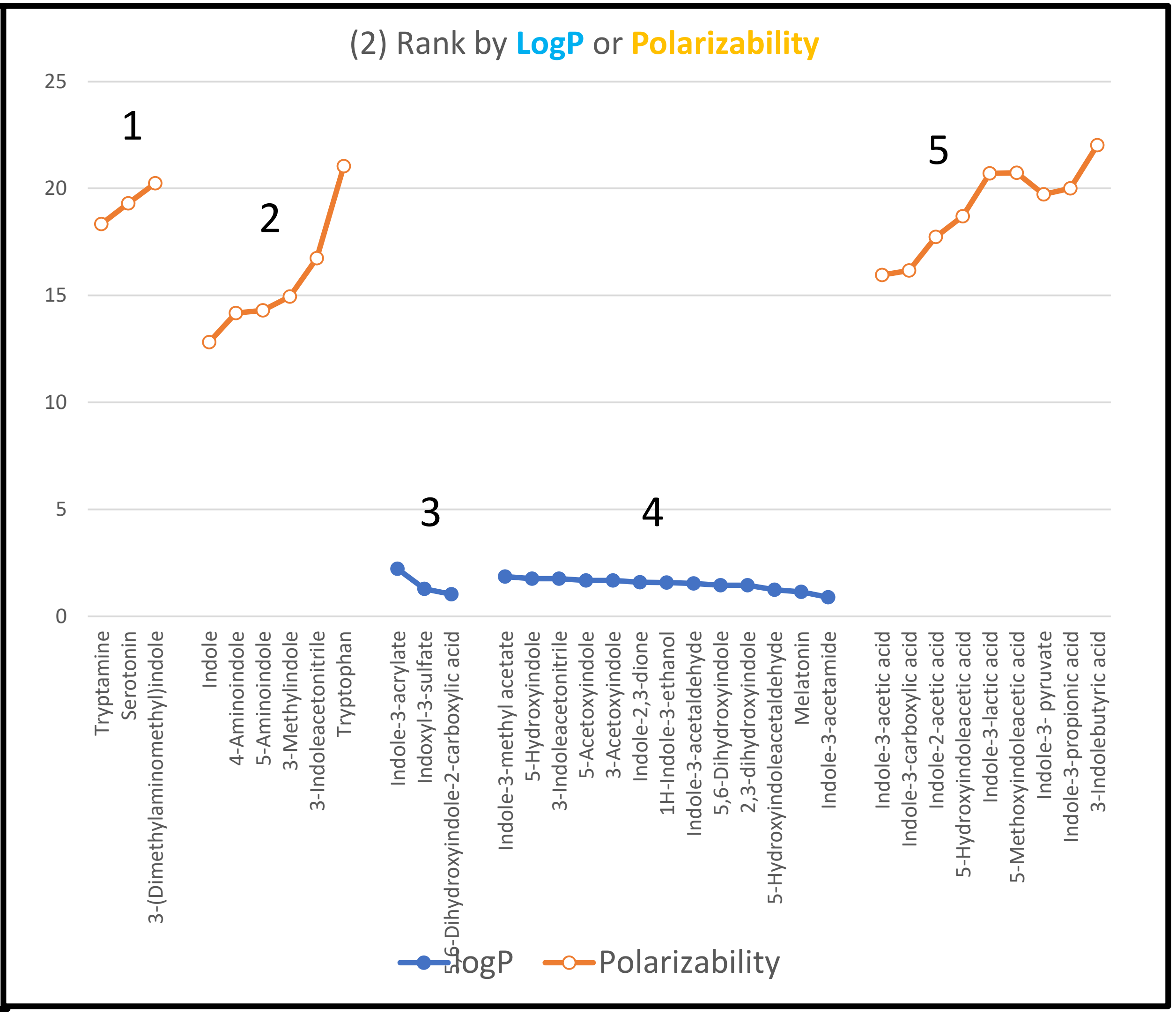
How does HMT identify new indoles (or any new polar metabolite)?

- Step **One:** Polar Extraction and separation of metabolites using capillary electrophoresis (CE)
- Step **Two**: Measure an exact Mass (< 3 ppm) with high performance MS (Q-Exactive Plus).
- Step **Three**: Determine possible empirical formulae and potential structures from the calculated exact mass.
- Step **Four**: Match potential structures against migration times for <u>other similar cmpds</u> to limit possibilities and resolve isomers (A process we call **OMEGA search**). Similar structures should migrate in similar mode and time as other related structures (Fatty acids with fatty acids, nucleic acids with nucleic acids). A confirmed and published observation unique to capillary electrophoresis! This migration time prediction is based on the physical chemical properties of each structure (polar surface area, pKa, charge etc), which can be calculated for each structure.
- Step **Five**: Confirm with certified reagents and fragmentation patterns.

OMEGA search depends upon the predictability of migration time elution **based on metabolite physical properties like pkA, LogP, Polarizability**.

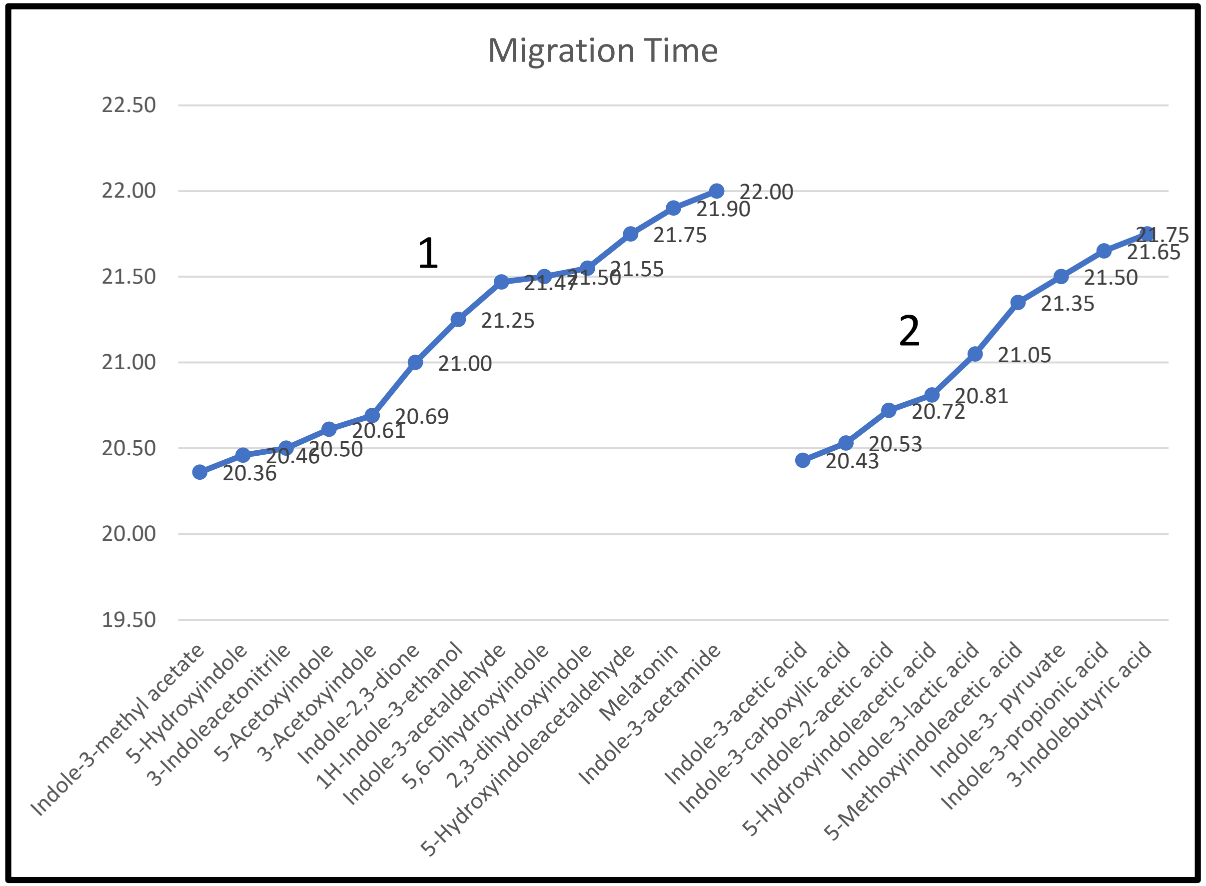
38 indoles can be observed to elute with two simple rules.
(1) First create 5 groups based on pKa and Charge (Q) (left)
(2) Then sort members in each group by LogP or Polarizability (right)

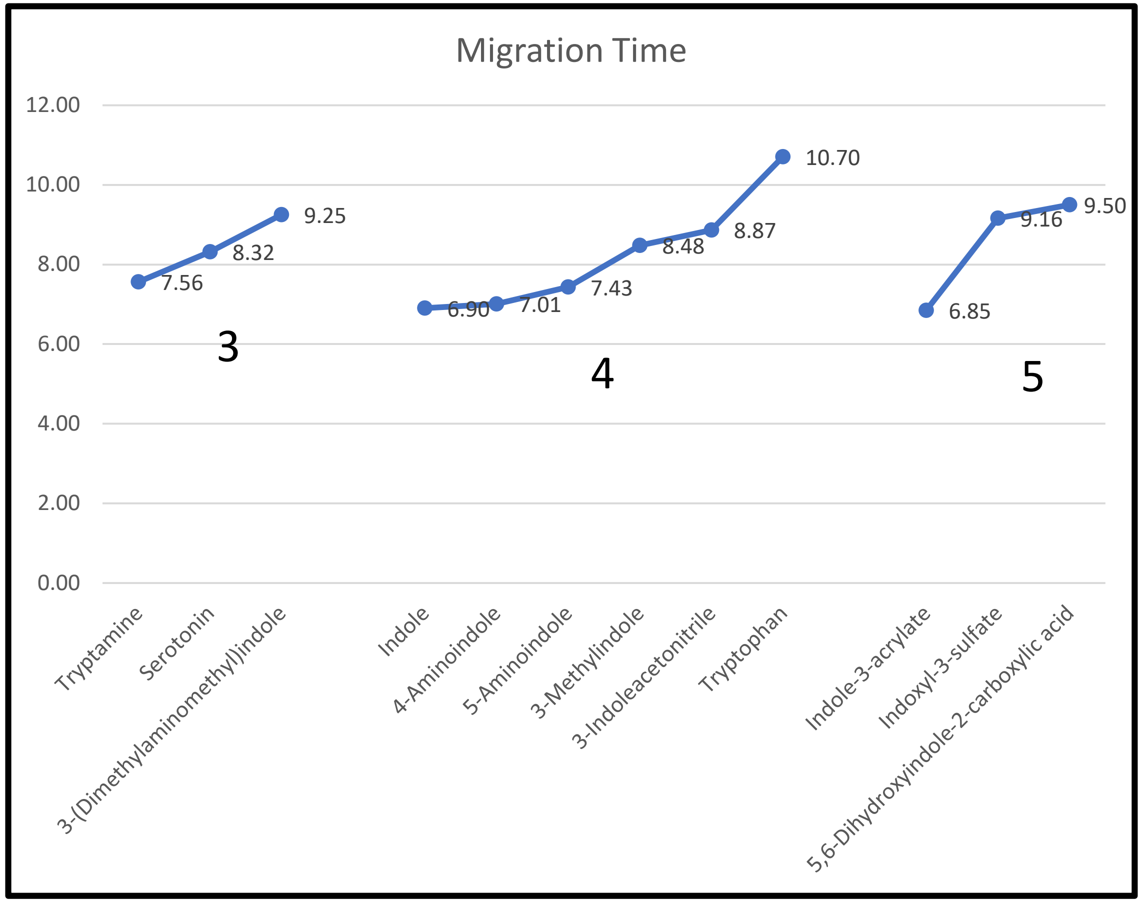




The result is that these metabolite **elution times now line up within each group**.

Any new metabolite that matches these same physical properties, if correct, will have a migration time within that group.





Expanding OMEGA search to other classes

- This same process works for all metabolite classes, including nucleic acids, fatty acids, amino acids, small peptides, organic acids.

- This is a unique and critical feature of CEMS.

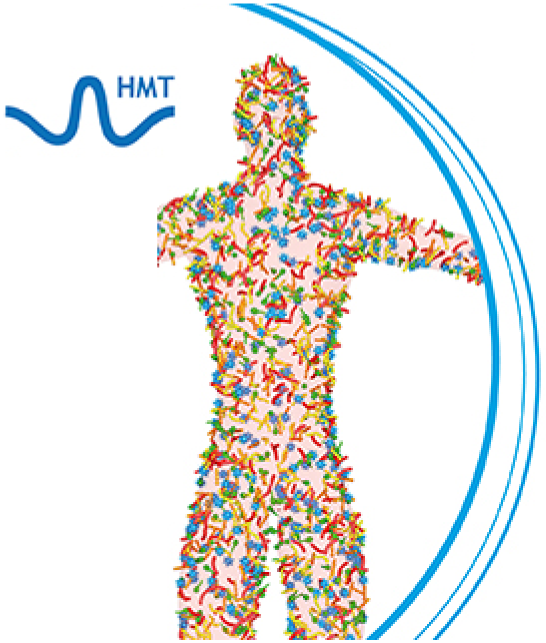
- Over 114 modified SCFAs and over 75 middle chain fatty acids have been observed using CEMS, many with known biological activities such as:
  - Induction and maintenance of intestinal regulatory T cells
  - Epithelial energy source
  - Inhibition of autophagy
  - Maturation of microglia
  - Inhibition of pro-inflammatory gene expression in macrophages
  - Signatures of fatty acid oxidation

CE also provides higher resolution

High resolution CE provides **Specificity** in addition to the discovery of novel compounds.

- As with several indole examples, many metabolites exist as isomers – same chemical composition, but different structure.

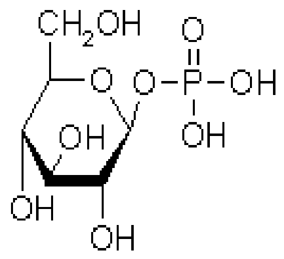
- The high resolving power of CE allows HMT to identify many isomers that LC methods are unable to identify in the profiling or untargeted mode.



Isomer Resolution Examples

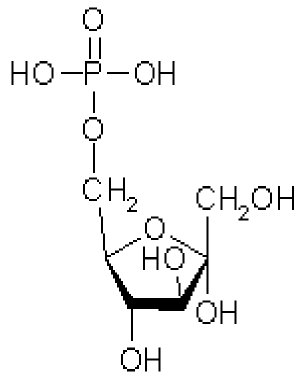
- Using the high resolution and selectivity of HMT CE-MS service, it is possible to measure all glycolytic sugar phosphate intermediates and resolve their isomeric equivalents for quantitative measurements.
- CE differentiates ribose-5P from ribulose-5P, 3-Phosphoglycerate from 2-Phosphoglycerate. 2-Hydroxybutyrate from 3-Hydroxybutyrate to name a few.
- Such metabolites are critical to both human and microbiota central carbon metabolism and glucose metabolism studies.

Glucose 1-phosphate 14.22”



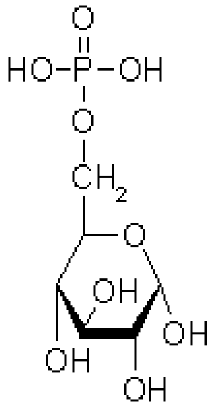
Positional isomers

Fructose 6-phosphate 14.45”



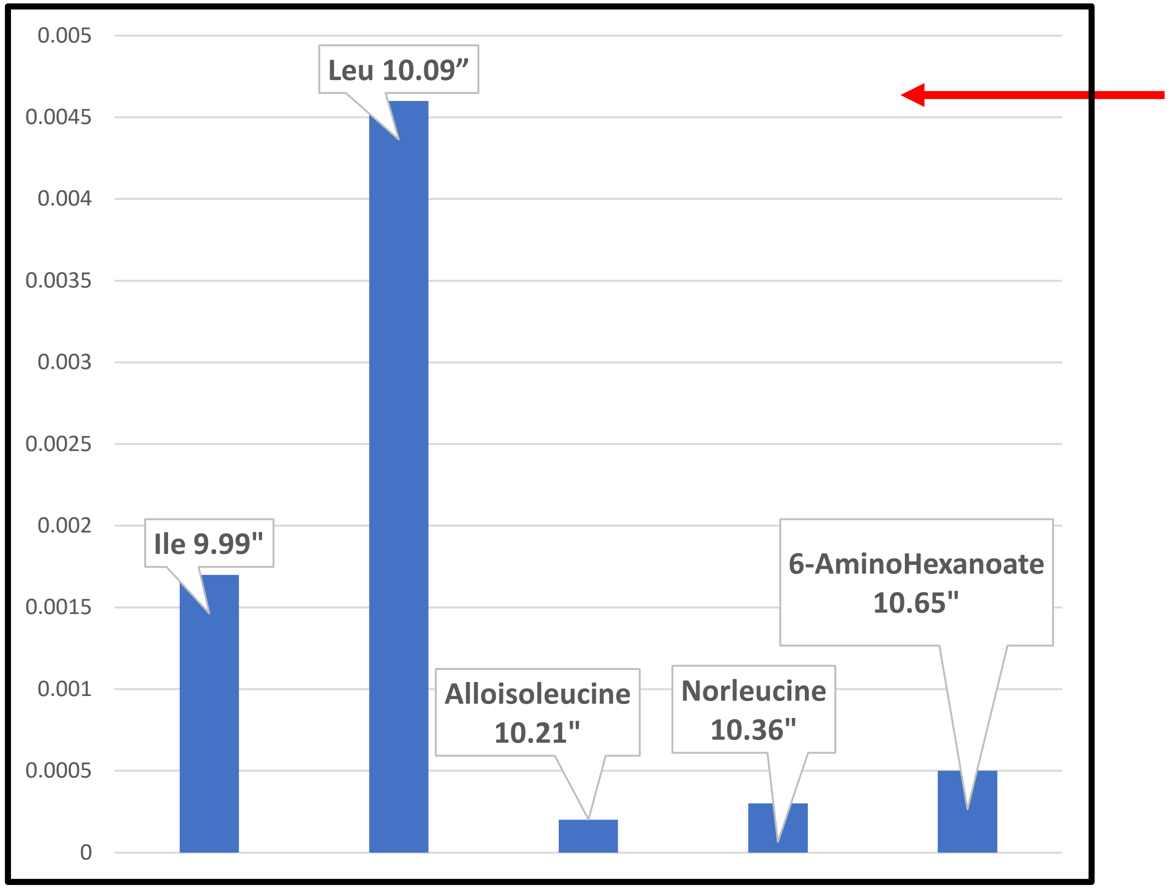
Structural isomers

Glucose 6-phosphate 14.55”



Glycolytic intermediates and their migration times.

Amino Acid Isomers are essential to identify



- **Branched and unbranched metabolites** isomers are separated by Capillary electrophoresis. Leucine has 4 other isomers for example each separated by CEMS.

- While D and L amino acids require a different platform, such differentiation is critical to fully understanding the microbiome including D- and L- BCAA amino acids.

- **D-leucine, D-*allo*-isoleucine, and D-valine** has been observed in the growth medium of a lactic acid bacterium, *Lactobacillus otakiensis.*

- D-Leu, D-Ile, and D-Val are reportedly involved in regulation of cell wall remodeling in *Vibrio cholerae*, while D-Leu is involved in controlling **biofilm** dispersal in *Bacillus subtilis* .

Biofilm development

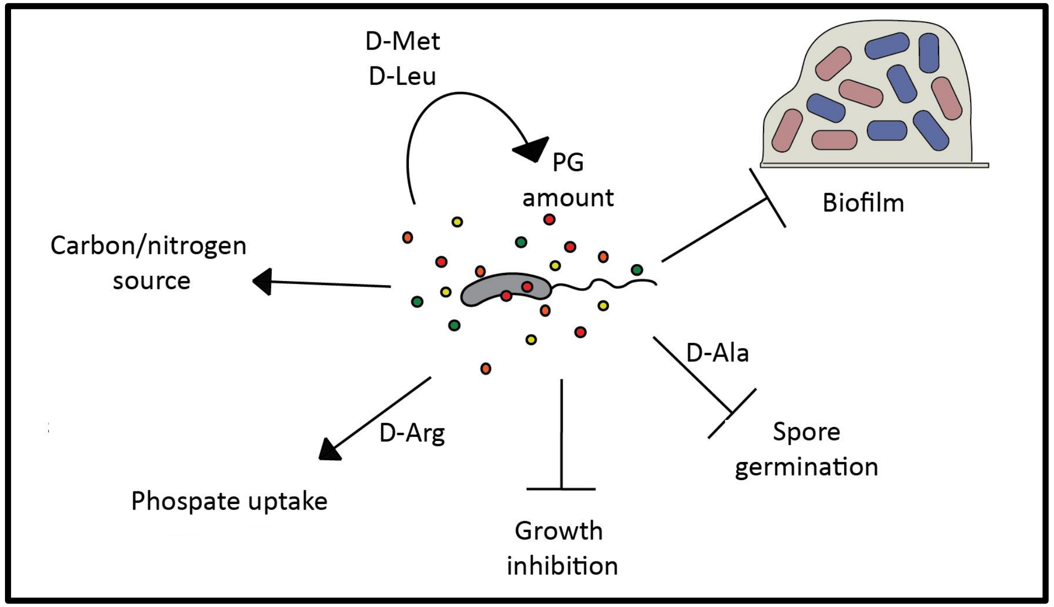
- Bacteria have a large capacity to utilize and catabolize all amino acids and to create racemic mixtures of D and L.

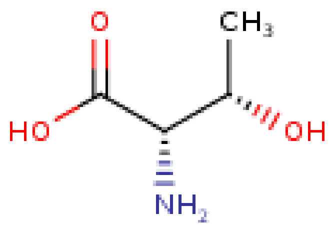
- Bacteria have been known to synthesize more than 10 kinds of D-amino acids.

- D-amino acids have been associated with biofilm development, cell wall remodeling, spore germination and signaling.

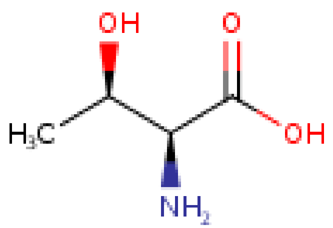
- How or do these D-amino acids interact with human biology?

- HMT has a unique platform to measure D and L amino acids.





L-allothreonine

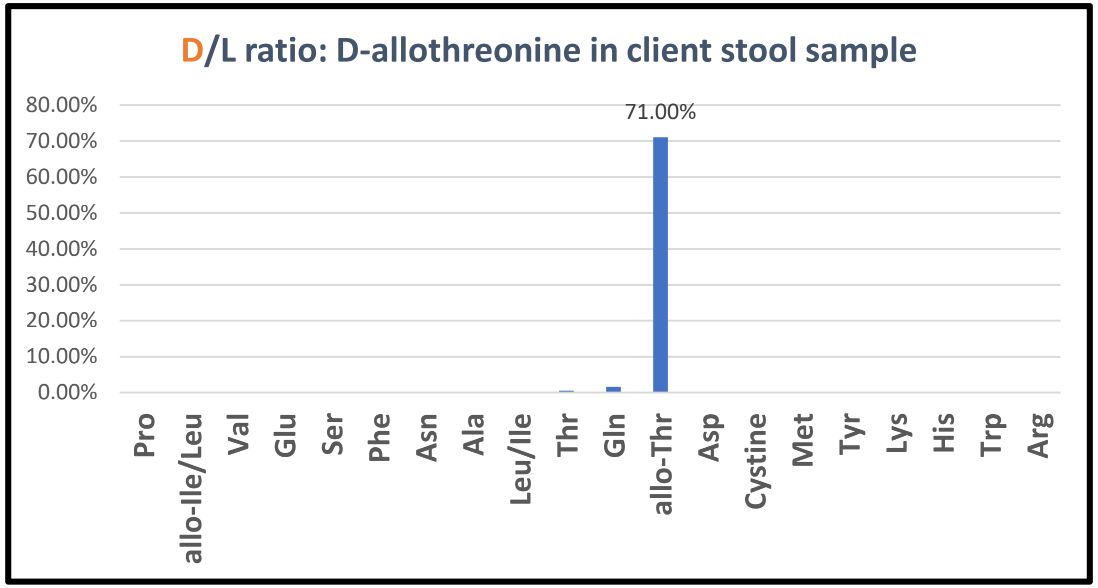


L-Threonine

- **L-allothreonine** is the L-enantiomer of allothreonine. It has a role as an *Escherichia coli* metabolite and a *Saccharomyces cerevisiae* metabolite occurring as a component of peptido-lipids in certain bacteria.

- The **D isomer** also exists in bacteria and found in human feces.

- Can D and L amino acids differentiate bacterial species and how do they interact with **human host**?



Lysine Pathway

- Lysine is an essential amino acid in humans but produced in bacteria from aspartate through the diaminopimelate (DAP) pathway.

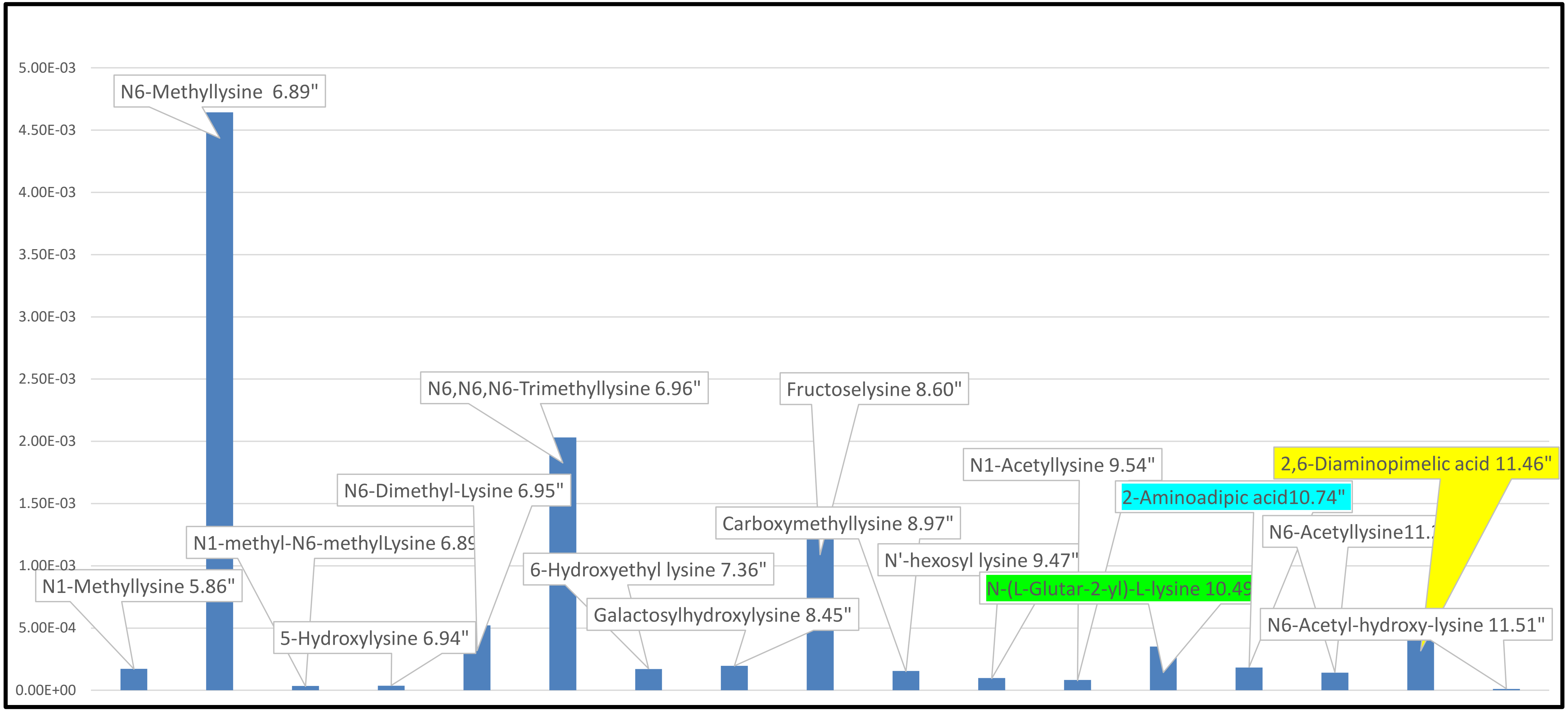
- DAP is not only a direct precursor of lysine, but it is also an important constituent of the cell wall peptidoglycan.

- The first step in the lysine catabolic pathway is the formation of saccharopine and then 2-aminoadipic acid, processes that are mitochondrial and potential biomarkers for liver and kidney disease.

- Carboxymethylysine (CML) is a potential biomarker of UV damage in skin.

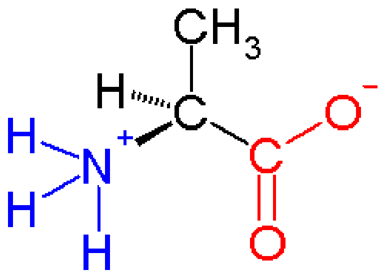
Lysine Pathway

Over 17 Lysine derivatives can be observed by CEMS in stool and tissues endogenous and bacterial.

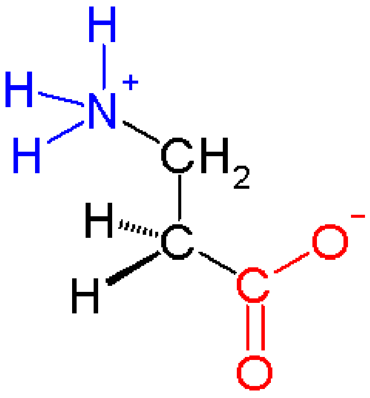


Beta Amino Acids are identified by CEMS

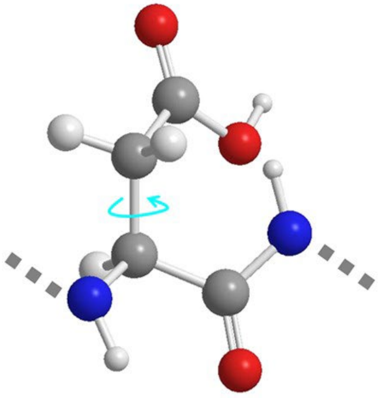
- Beta-Ala and Beta-Asp peptides are two prominent alternative amino acids observed in human samples
- Bacterial enzymes cleave beta-amino acids and releases beta-amino acids and beta-dipeptides
- Bacterial enzymes can also synthesize beta amino acids from alpha amino acids
- Alzheimer's disease (AD) has also been associated with the formation of the β-Asp metabolites



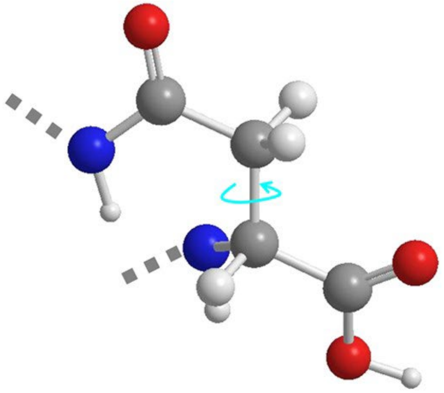
L-α-alanine



β-alanine



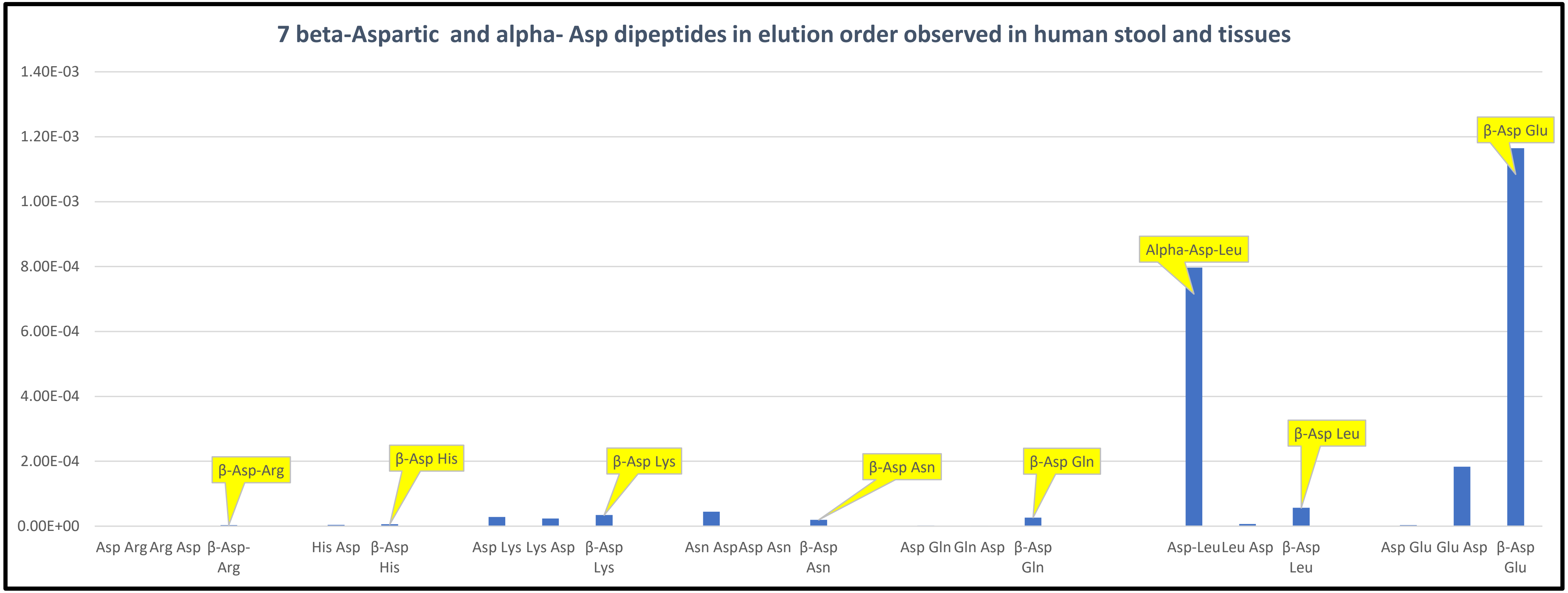
L-α-Asp



D-β-Asp

Beta Amino Acids are identified by CEMS

- Beta-Asp dipeptides are potential biomarkers in skin for UV damage and aging.
- CEMS separates beta from alpha dipeptides.



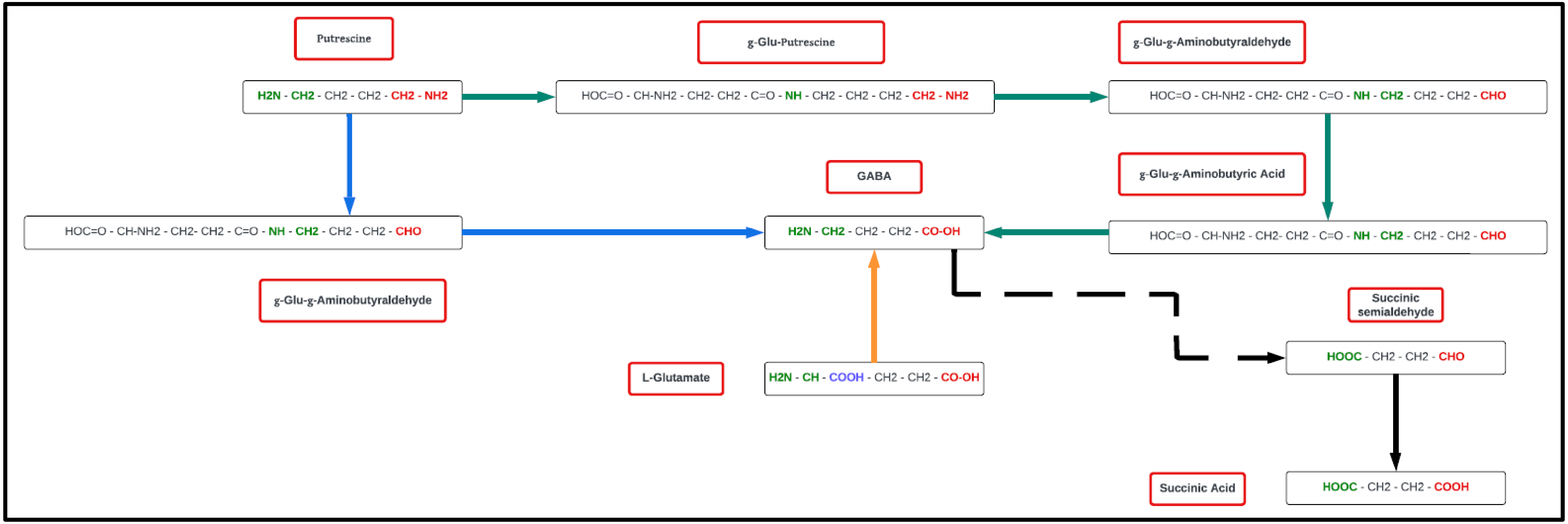
GABA synthesis an important bacterial pathway

- γ**-aminobutyric acid (GABA)** – is not just a CNS neurotransmitter synthesized in neurons, but also a bacterial metabolite created in the gut.

- Notable among the **Probiotics** metabolome is the observation of γ-aminobutyric acid (GABA), which plays an essential role in the prevention of neural diseases, type 1 diabetes, cancer, immunological disorders and asthma.

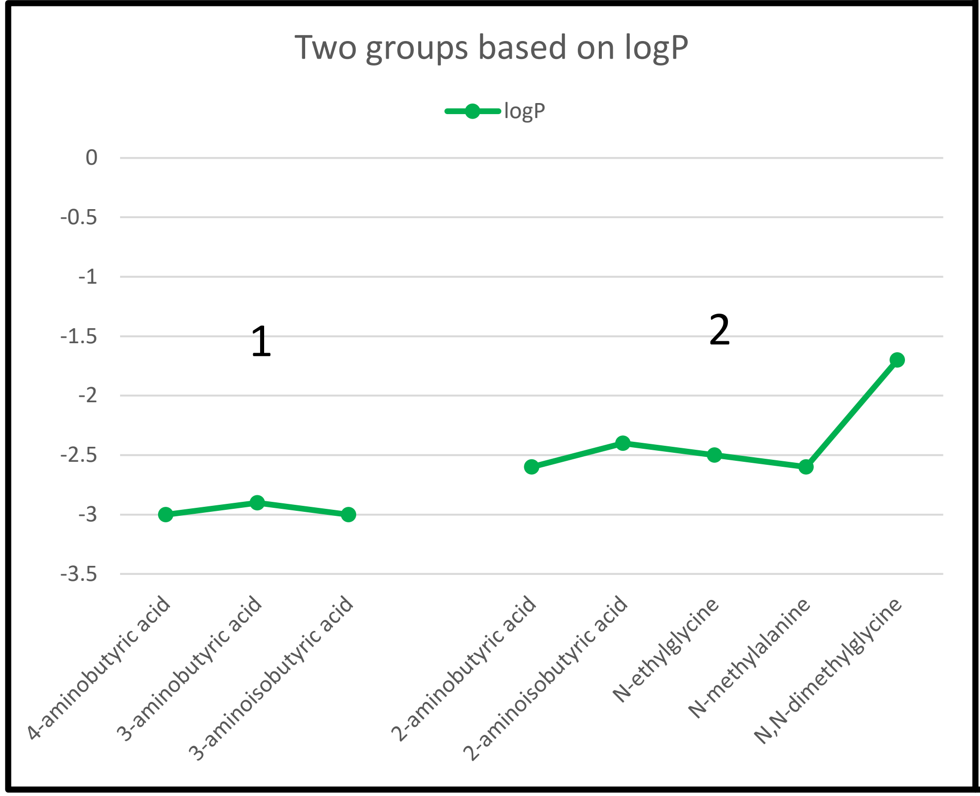
GABA synthesis an important bacterial pathway

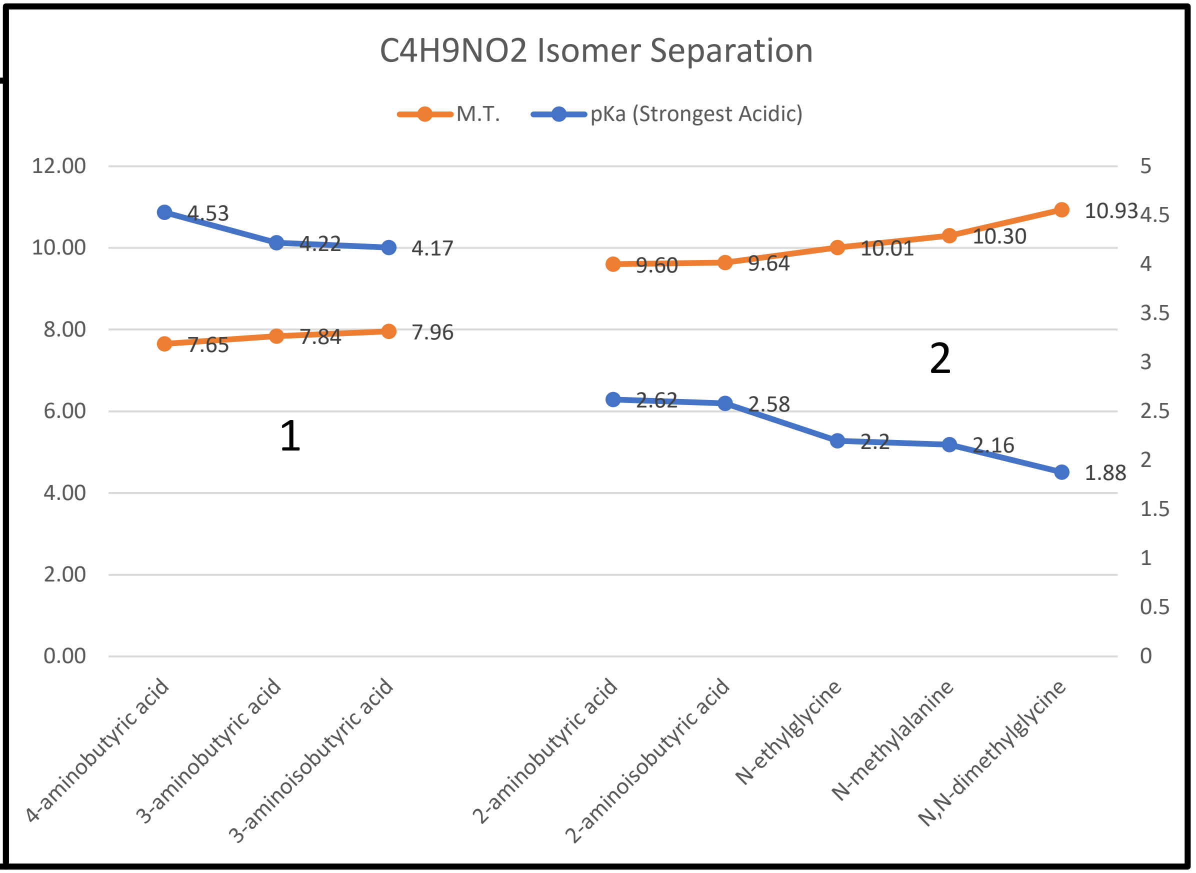
- Generally, γ–aminobutyric acid is produced by **lactic acid bacteria**, using γ-glutamyl-transferases through two pathways.



There are 8 isomers of GABA

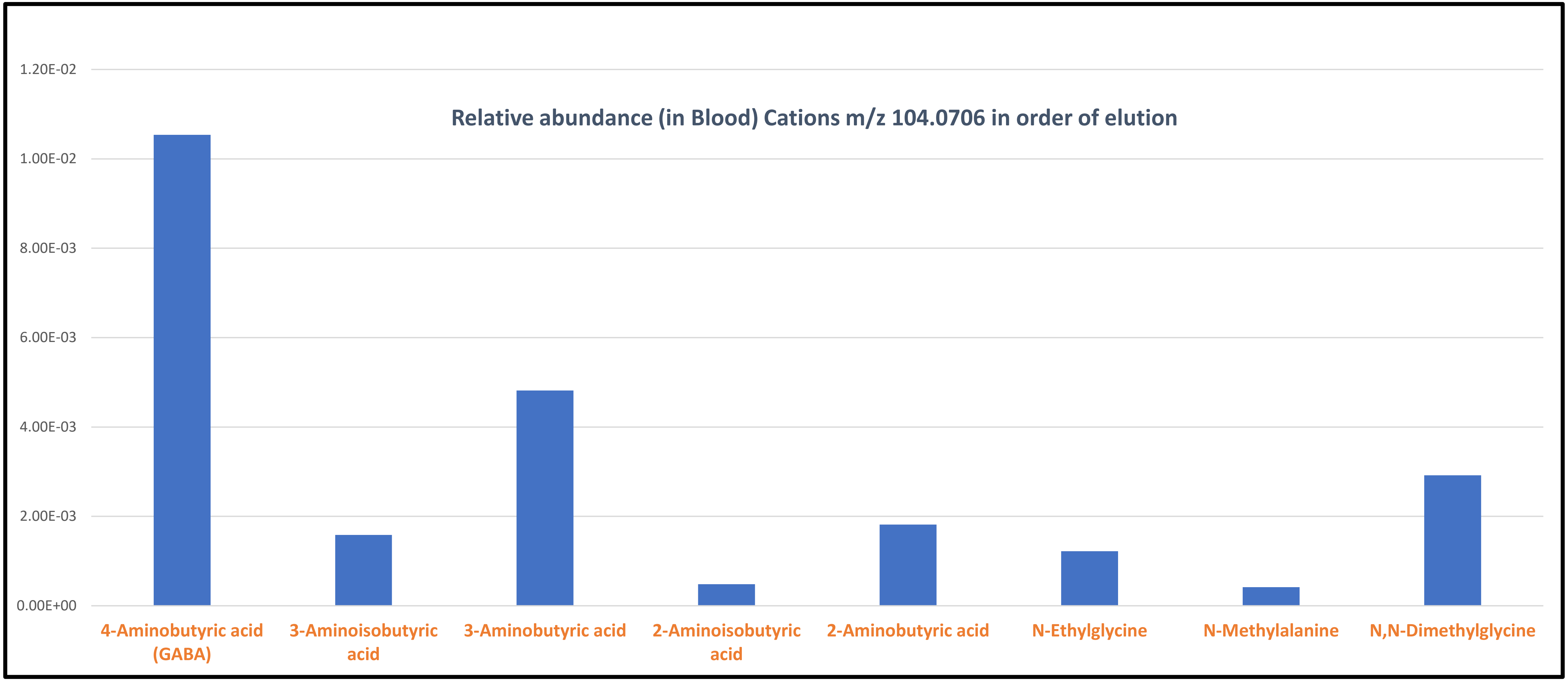
- **CE-MS** is able to resolve all 8 GABA isomers providing specificity and quantitation in any sample type.
- Separation of these isomers is linked to differences in their physical properties:pKa and LogP, dividing them into 2 groups.





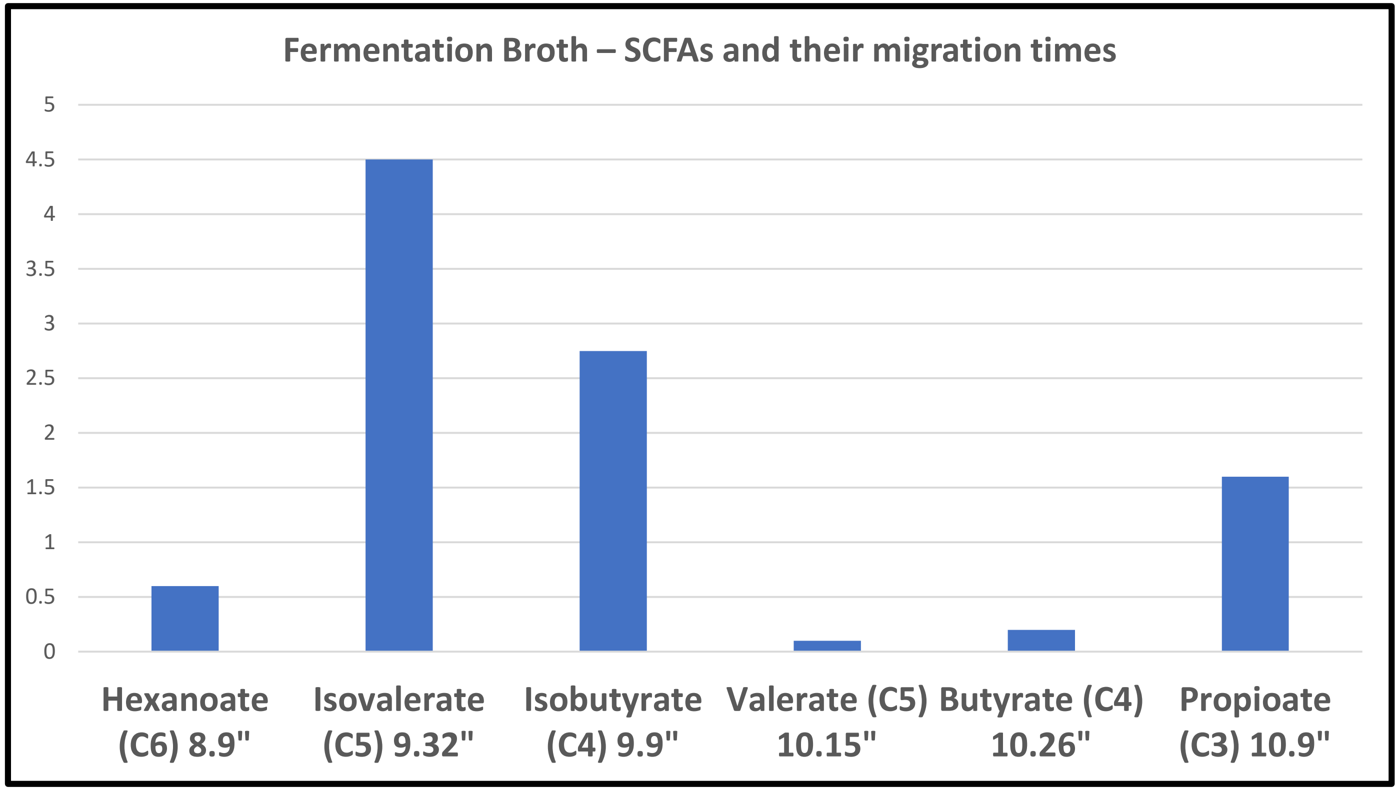
Isomer Specificity achieved using CEMS

- Each isomer can be resolved and quantitated in any sample with GABA eluting first and N,N-dimethylglycine last.
- Positional (4 vs 3 vs 2) and structural (linear vs branched (Iso) are based on different physical properties.

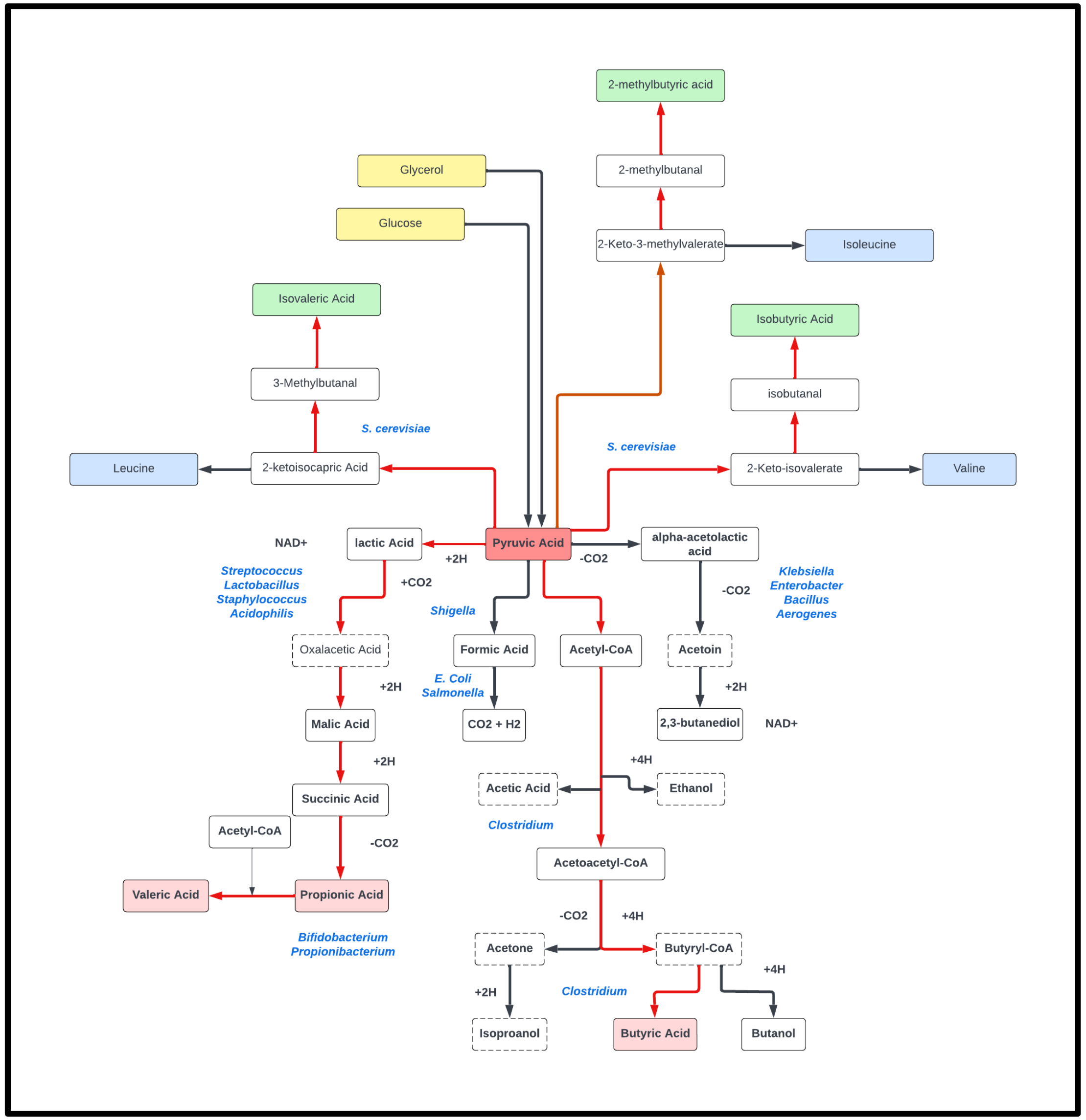


SCFA Resolution and Specificity

- In a similar way, CE-MS is can resolve SCFAs and SCBFA (short chain branched fatty acids).
- Such variations are seen in adipose tissue from patients with high BMI, fermentations broth and cell media.

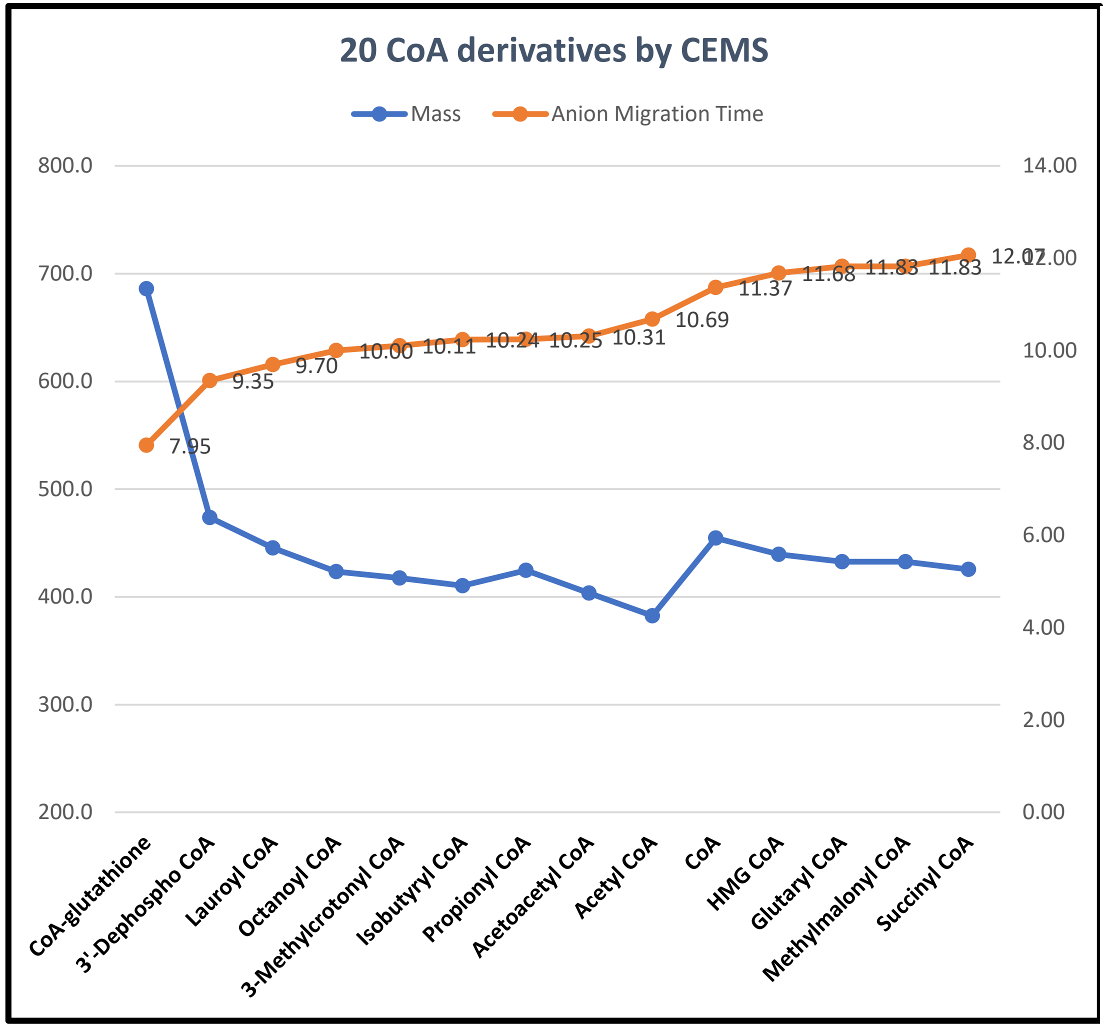


SCFA Resolution and Specificity



- Understanding metabolomic data includes creating pathway maps that represent observations and knowledge as well as to show differences between human and microbiota processes.
- HMT Bacterial pathway maps present how bioprocessing by different bacteria leads to different outcomes.

Many other examples



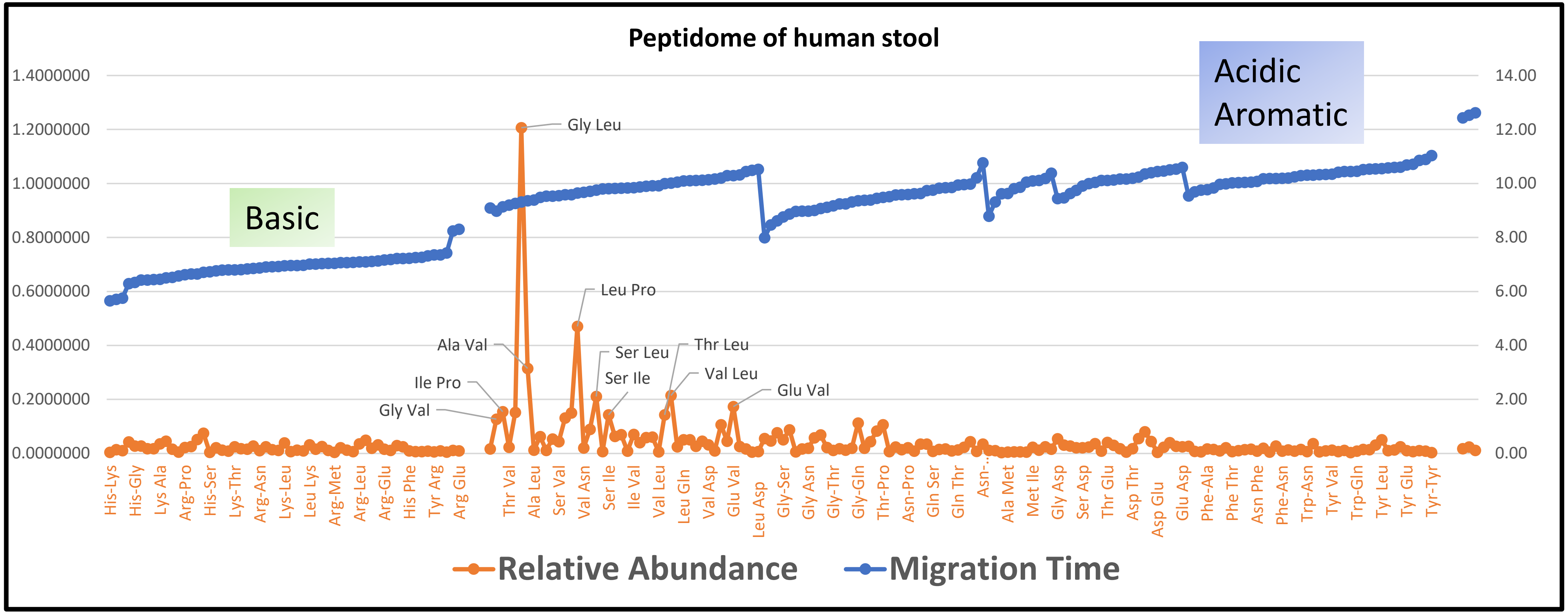
Resolution, uniqueness of discovery & specificity from CEMS analysis has lead to HMT identifying and contributing to a growing list of potential microbiota related metabolites and adducts:

- Fructosyl-X,
- Alkenyl-glycosides
- X-lactosyl
- X-ethanolamine
- CDP-X, CTP-X,
- Succinyl-X,
- Many X-Cysteine (8 related to aging)
- X-Glutathione
- > over 20 X-CoA
- Hundreds Di/Tri peptides

The unexplored peptidome

- The large distribution of small peptides is observed in stool, blood, tumors, tissues that apparently varies with disease, age and drug treatment. Only a small percentage of these have been show to have biological activity. In K-12 E.Coli for example, **Gly-Leu acts as growth inhibitor. Majority of these are not observed using traditional LCMS analysis.**

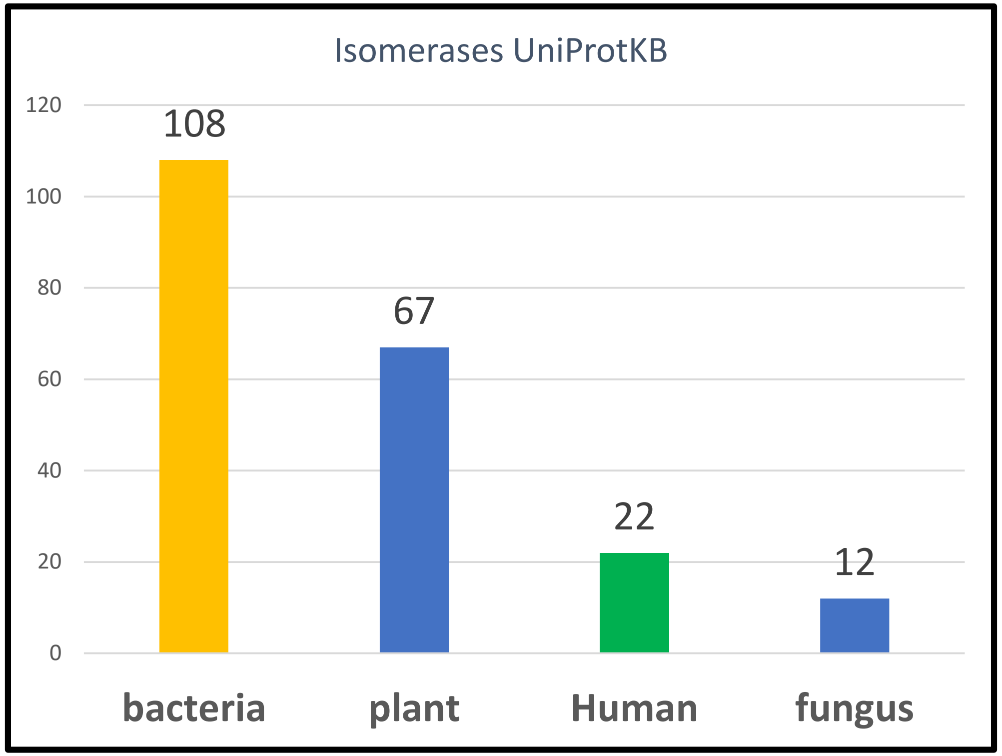
- Bacteria contain a large variety of dipeptidases, tripeptidases and aminopeptidases for cell growth and development.

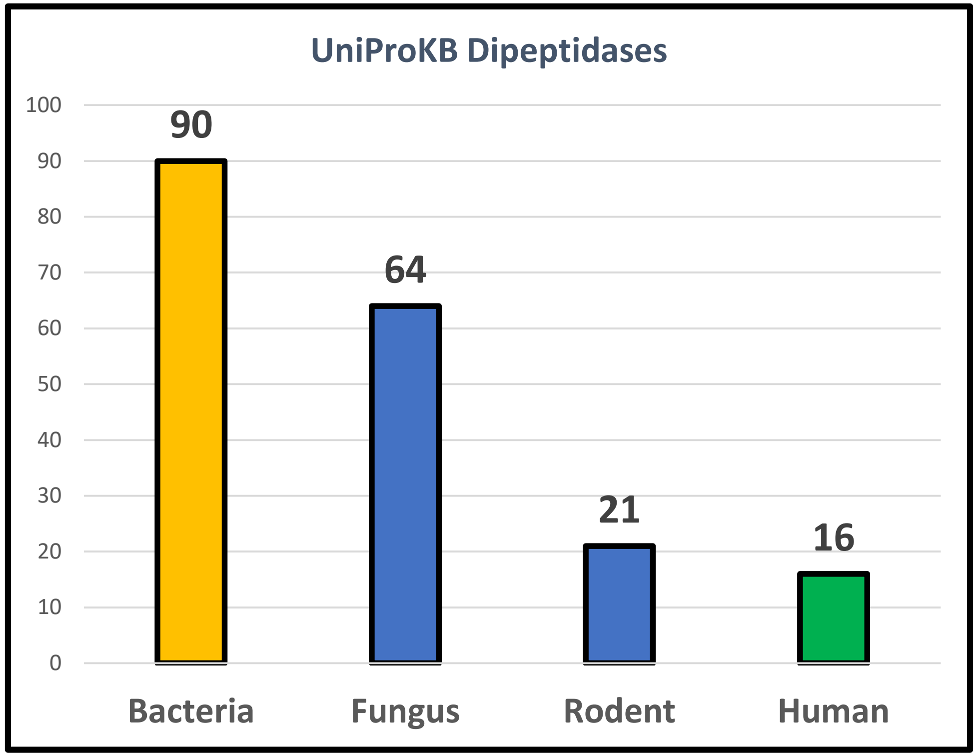


Migration Time Differentiates acidic from basic and from neutral peptides.

Isomerase and Dipeptidases are extensive in Bacteria

- Isomerases are plentiful in bacteria and plant Genomes vs Human Genome.
- Dipeptidases in bacteria and fungus also out number human.
- What is the effect of these transformational enzymes on human health?
- Are certain patterns of the products of these enzymes biomarkers for disease or specific biodiversity?



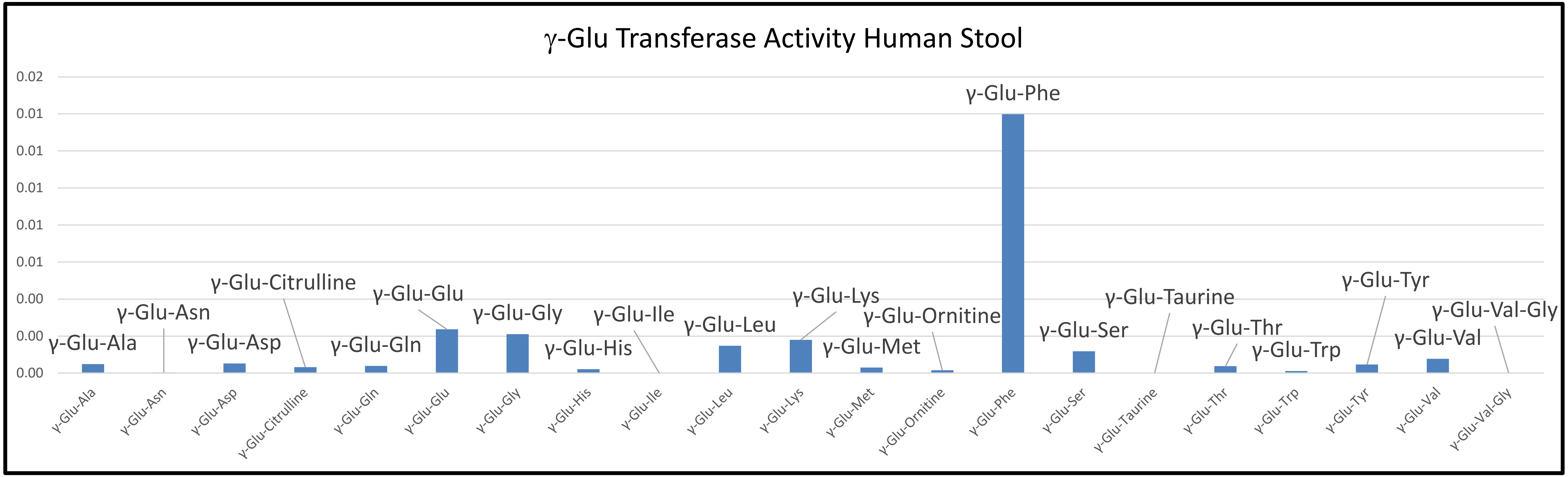


The Gamma-Glutamylome

- **Gamma-glutamyl transpeptidases (GGTs)** are ubiquitously present in all life forms and plays a variety of roles in diverse organisms.

- Higher eukaryotes mainly utilize GGTs for glutathione degradation.

- GGTs from unicellular prokaryotes, however, can serve different physiological functions in Grampositive and Gram-negative bacteria as glutathione degraders and as **virulence factors**.



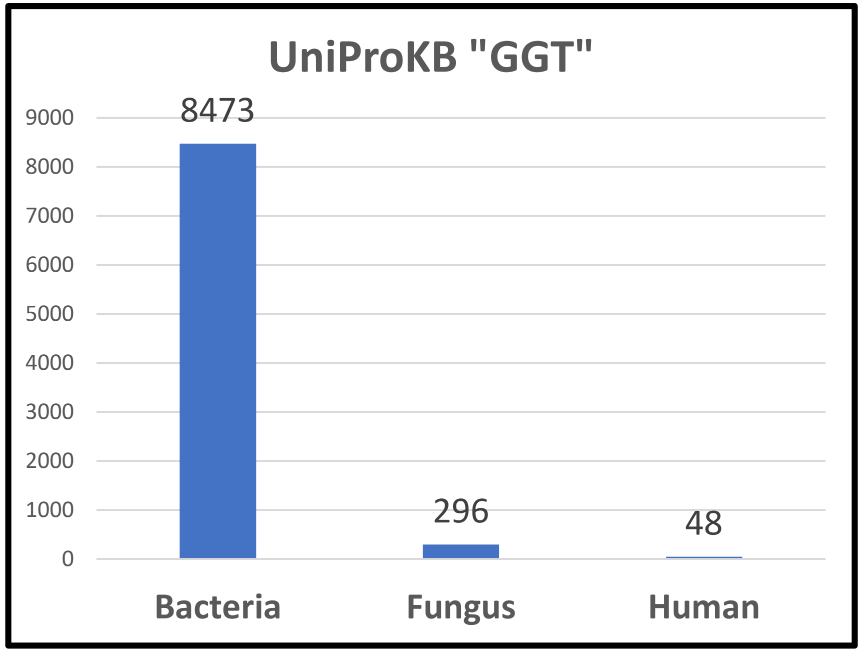
GGTs – functions and diversity

- Despite the observations of many of these gamma glu dipeptides, little is known about their specific activities. Many have been observed to be dysregulated in liver diseases and under oxidative stress conditions. Are they storing or transposing amino acids or have unique activity of their own?

- γ-**Glu-Trp** is an **immunomodulatory dipeptide** with broad spectrum stimulatory activities that boost immune response.

- γ-**Glu-Taurine** has many physiological effects on the CNS.

- This diversity is represented in the bacteria **genome by the large number of related genes compared to human**.



GGTs – functions and diversity

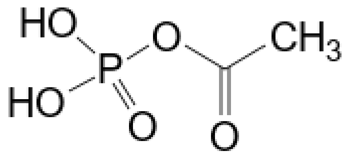
- γ–glu di and tri peptides are observed in most any biological sample, with large numbers in plasma, stool, tumors.
- What contribution may come from human cells versus bacterial cells and what are their roles?

GGT group and associated organisms	Expression and localization	Putative Physiological role
1. Glutathione degrading GGT – mainly present in glutathione producing Gram-negative bacteria		
E. coli	Periplasmic expression	Glutathione utilization as a source of amino acid and nitrogen under nutrient limiting conditions
Proteus mirabilis	Localized on the outer cytoplasmic membrane facing the periplasm	
2. GGT as virulence factor – mainly present in pathogenic Gram-negative bacteria		
Helicobacter pylori	Periplasmic expression	Utilization of host's glutathione and glutamine as a source of glutamate; Inhibition of T-cell proliferation in host
Campylobacter jejuni	ND#	
Fransicella tularensis	ND	Utilization of cytosolic glutathione and γ-glutamyl cysteine peptides present in host cytosol for cysteine acquisition
Neisseria meningitidis	Expressed on the inner cytoplasmic membrane facing cytoplasm	Utilization of cytosolic γ-glutamyl cysteine peptides for cysteine acquisition
3. PGA hydrolyzing GGT – mainly present in PGA producing Gram-positive bacteria		
Bacillus subtilis (natto)	Extracellular secretion	Degradation of PGA into glutamate as a source of nitrogen under nutrient-starved conditions
Bacillus licheniformis	Extracellular secretion	
*Bacillus anthracis**	Membrane-bound expression	Covalent anchorage of PDGA to peptidoglycan layer to maintain capsule integrity; Depolymerization of PDGA inside the cytoplasm

Not determined.

**Bacillus anthracis is also a pathogenic strain and GGT is involved in its virulence; however, it has been placed in the third group as it is a Gram-positive Bacillus with PDGA as a virulence factor.*

The Acetylome



AcP

- ***N*-acetyltransferases** (GNATs) are a predominant transformational family present in bacteria and present across bacterial phylogeny and modify a wide range of macromolecule substrates, including many metabolites. *Listeria monocytogenes* contains ~14 GNATs, whereas, *Streptomyces lividans* containing ~72 GNATs.

- **Non-enzymatic acetylation** is also possible with the **bacterial metabolite acetyl phosphate (AcP) which** can non-enzymatically acetylate large and small substrates. AcP is the **predominant acetyl donor in E. Coli.**

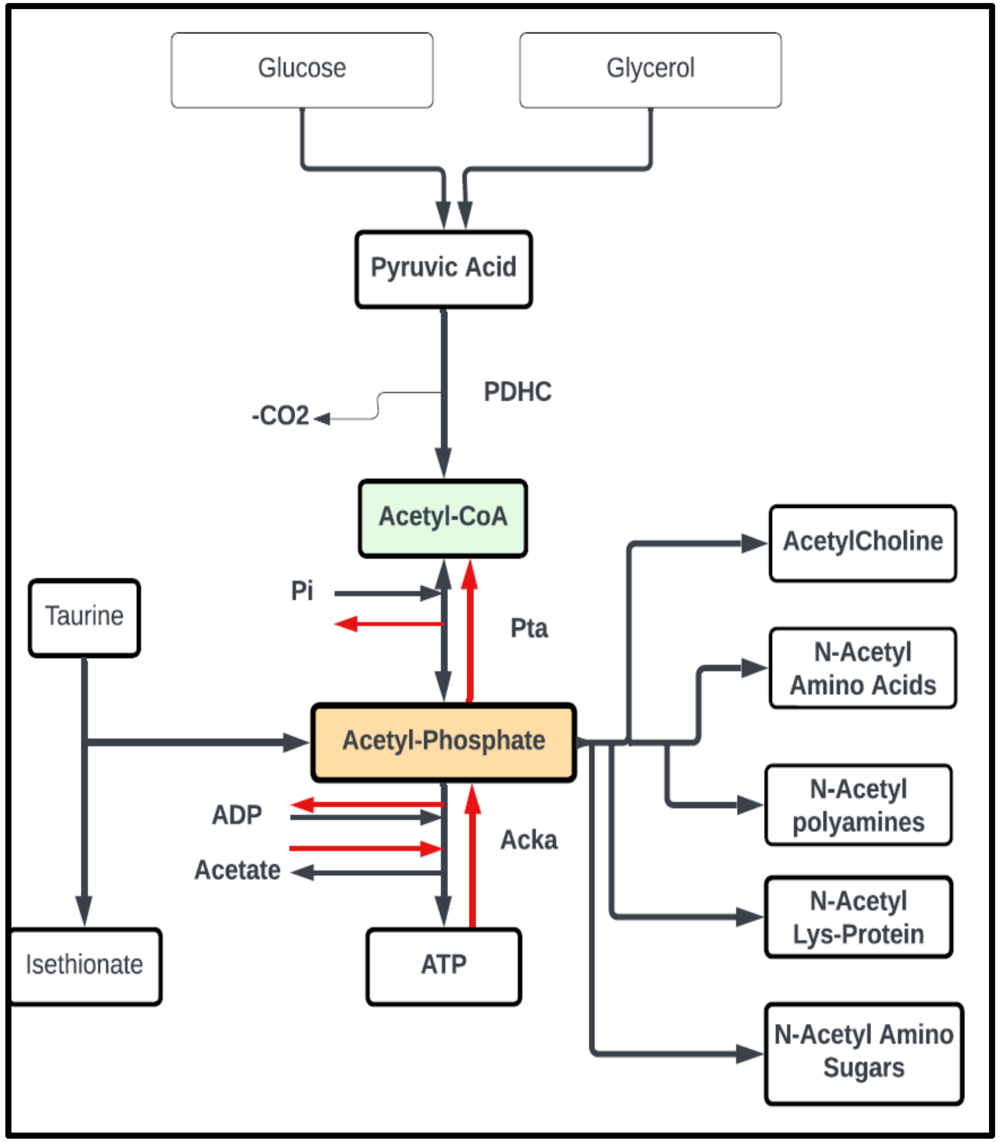
- Basic **pH** promotes N-acetylation, but also increases the rate at which AcP and AcCoA dissociate. Thus bacterial regulation of N-acetylation may depend on the spacial context of the local microenvironment. AcP is thought to be a **global regulator** in some bacteria.

- What does N-acetylation tell us about the microenvironment and its role in local biology?

- N-Acetylation may serve as a **feedback mechanism to slow an excessive carbon flux by storing two carbon units.** While there are designated carbon storage polymers, such as starch in plants or glycogen in animals, for bacteria, perhaps N-acetylation is a **primordial form of carbon storage**.

Acetyl-P Pathway

- Acetyl-P pathway converts ADP to ATP.
- The reversibility of this pathway permits both acetyl-CoA synthesis (acetate activation) and acetate evolution (acetogenesis).
- Ac-P is also linked to cholinesterase activity and taurine metabolism.
- Role of non-enzymatic acetylation and the possibility of unknown acetyltransferases are yet to be explored and fully understood.



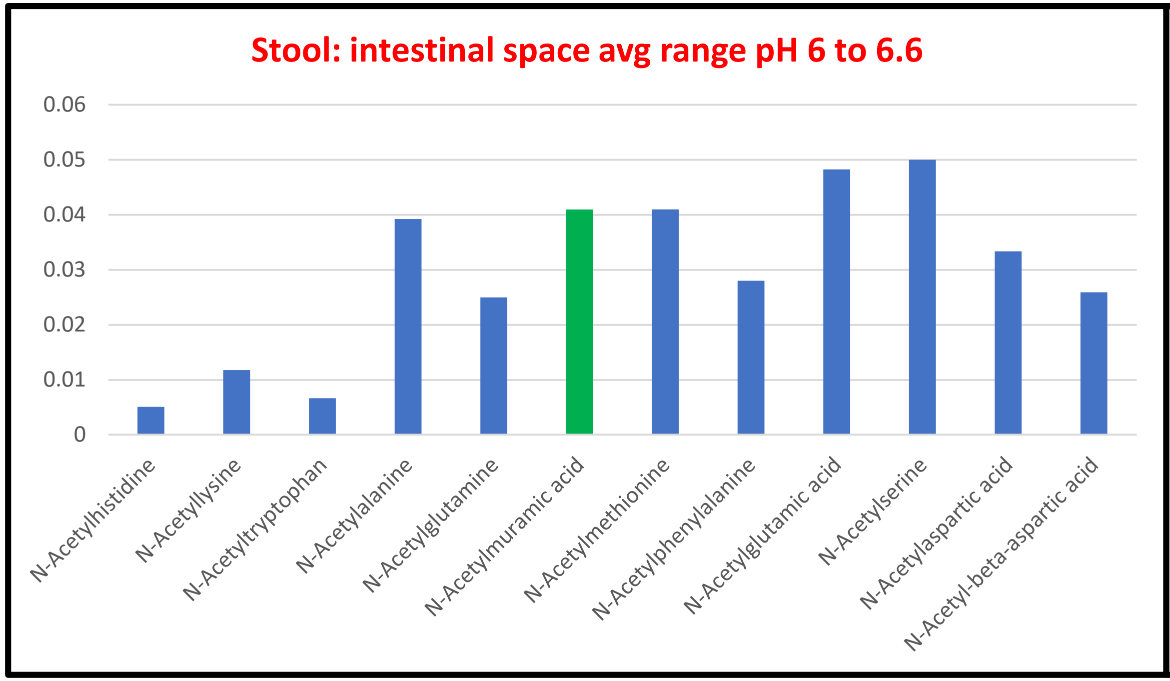
Amino Acid Acetylome

- What does monitoring N-Acetylation of amino acids tells us about carbon flux or pH in local environment? The N-acetylated pattern in the more acidic stool is unlike that in the more neutral or basic plasma.

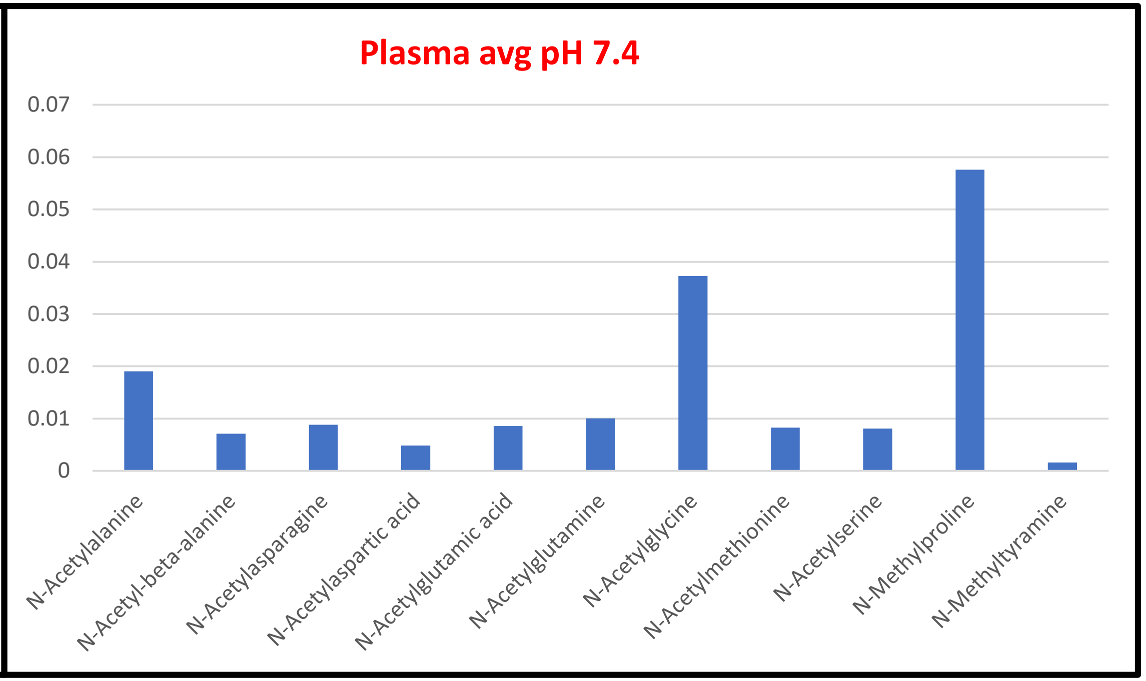
- Other amines (amino sugars, polyamines) are also acetylated. Is there a pattern of behavior linked to health?

- There are 22 Acetylated amines in MetaboAging database.

Basic, acid amino acids amino sugars



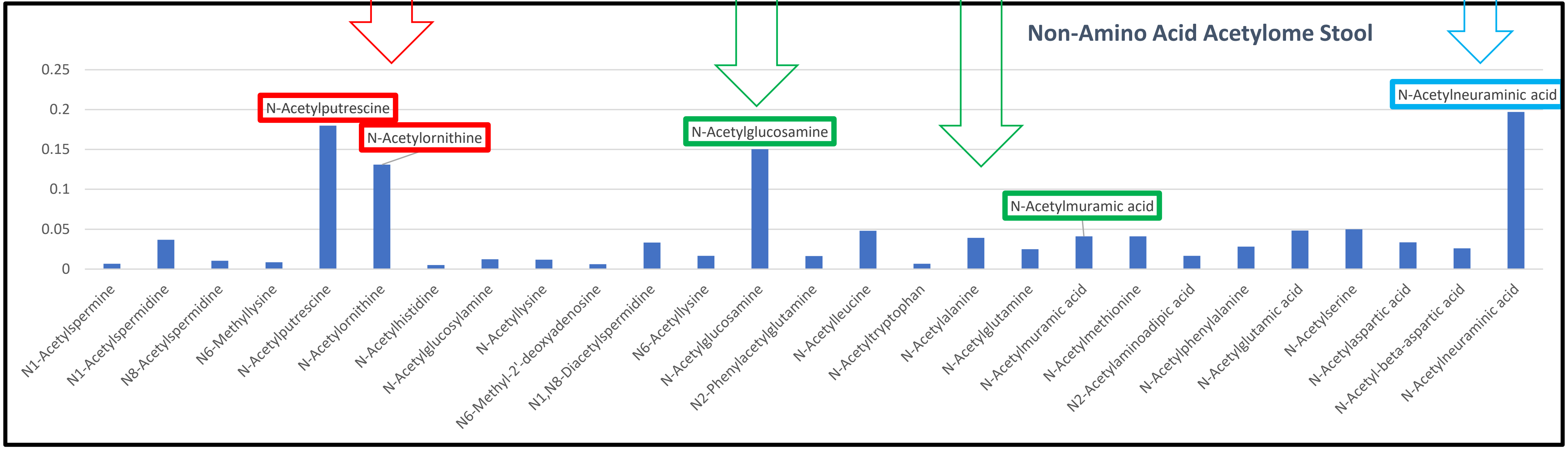
Neutral amino acids



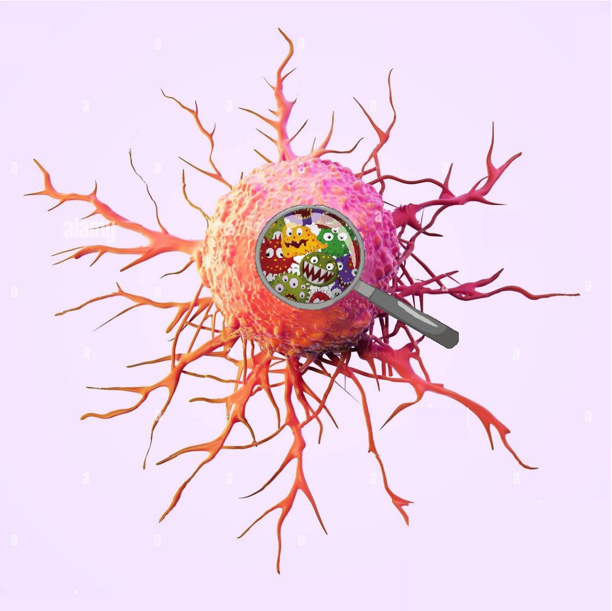
- Putrescine is required for bacterial cell motility and biofilm formation in human bacterial pathogens *P. mirabilis and Y. pesti*.
- **Polyamines** are hydrophilic molecules that cannot passively diffuse across that bacterial plasma membrane.
- Putrescine act as a signal for bacterial cell-cell communication and for pathogen-host interactions.

- The **peptidoglycan in the bacterial cell wall** is built from alternating units of N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc).

- N-acetylneuraminic acid is the most abundant sialic acid in humans and is generally expressed as the terminal sugar on intestinal mucus glycans.
- Several pathogenic bacterial species harvest sialic acid from the mucus and display this sugar on their own surface to evade Siglec-mediated host immune surveillance.



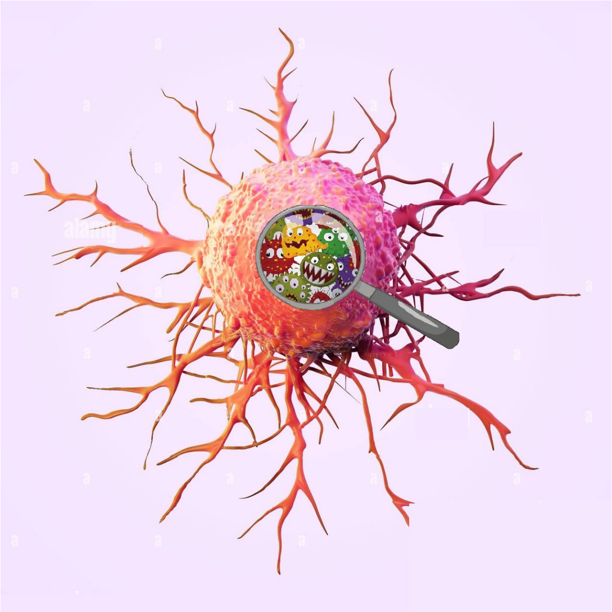
Bacteria and Tumor Tissue



- What role does bacteria play in tumors?
- How does microbiota move into tumor tissue?
- Does microbiota contribute immune response or antioxidant response?
- Do bacteria feed cancer cells with essential amino acids and vitamins?
- The tumor microbiome has been identified in a variety of cancers such as pancreatic, lung, and breast cancers with different compositions in different tumors & with different effects on tumors. Breast cancer has the highest degree of biodiversity.
- The microbiome can play an important role in the formation of the tumor microenvironment, regulation of local immunity, and modification of tumor cell biology, and directly affect the efficacy of drug treatment for tumors.

Bacteria and Tumor Outcome

- The abundant blood supply carries both metabolites and bacterial cells into the tumor hypoxic environment bringing abundant nutrients. Reduced immune cells may help the bacteria to colonize within the tumor tissue.
- Bacteria can also enter through ducts, for example, from the duodenum directly into the pancreas.
- The presence of bacteria can affect tumor characteristics by increasing gene mutations, regulating the function of immune cells, modulating signaling pathways, influencing drug resistance, affect collagen degradation or anaerobic respiration.
- The tumor bacteria are found in the cytoplasm and nucleus and are present in both cancer and immune cells (e.g. macrophages and leukocytes).



What specific technologies does HMT have to address comprehensive metabolite discovery, identification and pathway analysis?

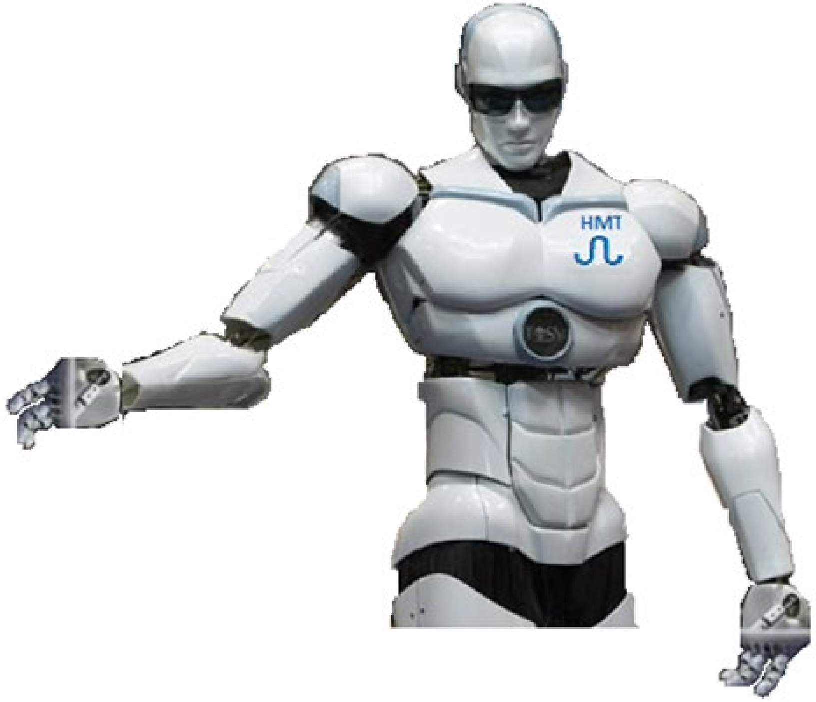
OMEGA SEARCH – pathway to identify novel metabolites

- **Prediction of metabolite identity from accurate mass, migration time prediction and isotopic pattern information in CE-TOFMS data.**

- *Electrophoresis. 2010 Jul;31(14):2311-8. doi: 10.1002/elps.200900584.*
- *Sugimoto M, Hirayama A, Robert M, Abe S, **Soga T**, Tomita M.*

- **Metabolomics-based approach for ranking the candidate structures of unidentified peaks in capillary electrophoresis time-of-flight mass spectrometry.**

- Electrophoresis. 2017 Apr;38(7):1053-1059. doi: 10.1002/elps.201600328. Epub 2017 Jan 17.
- Yamamoto H, Sasaki K.



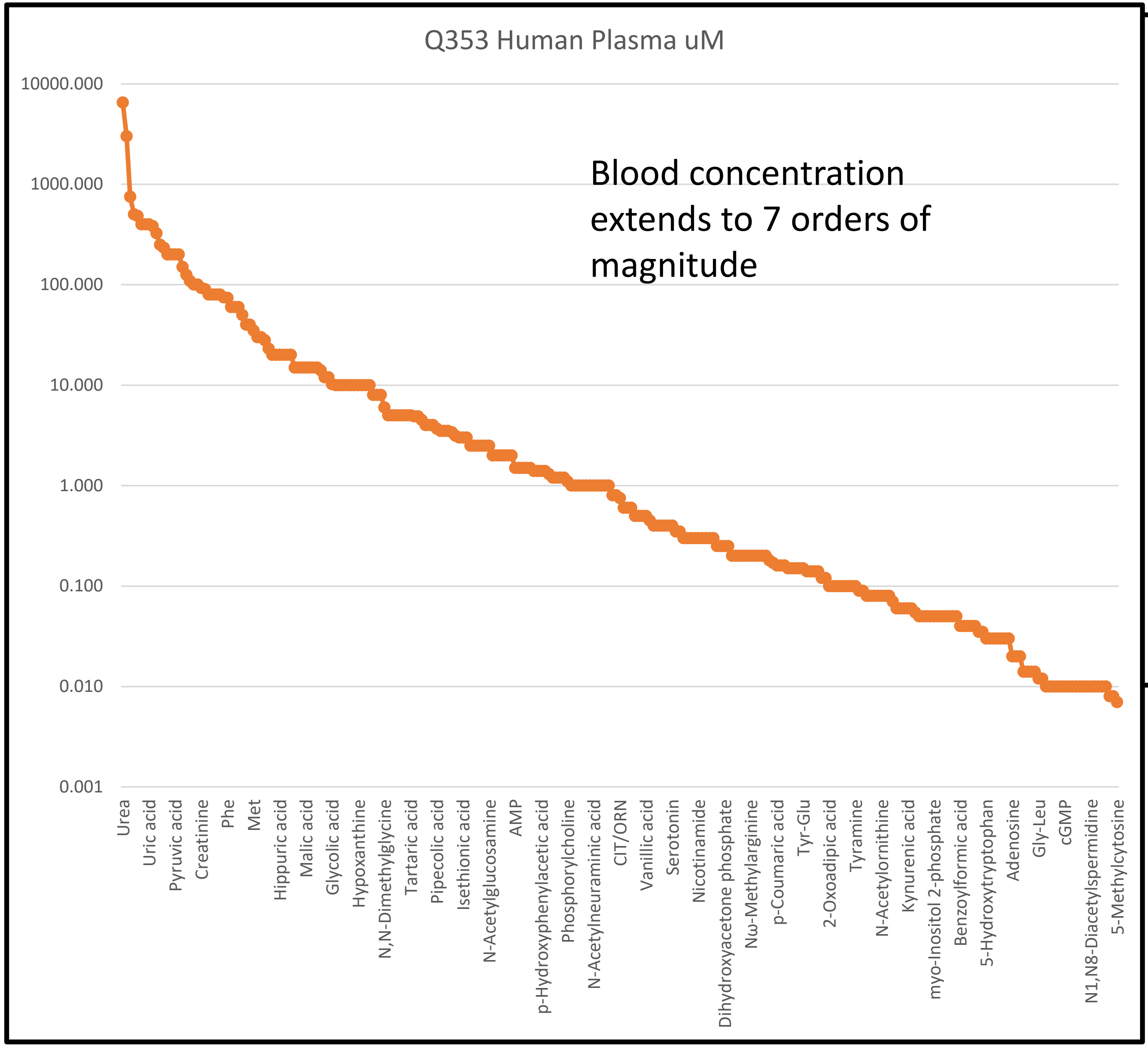
Q-353 quantitative panel

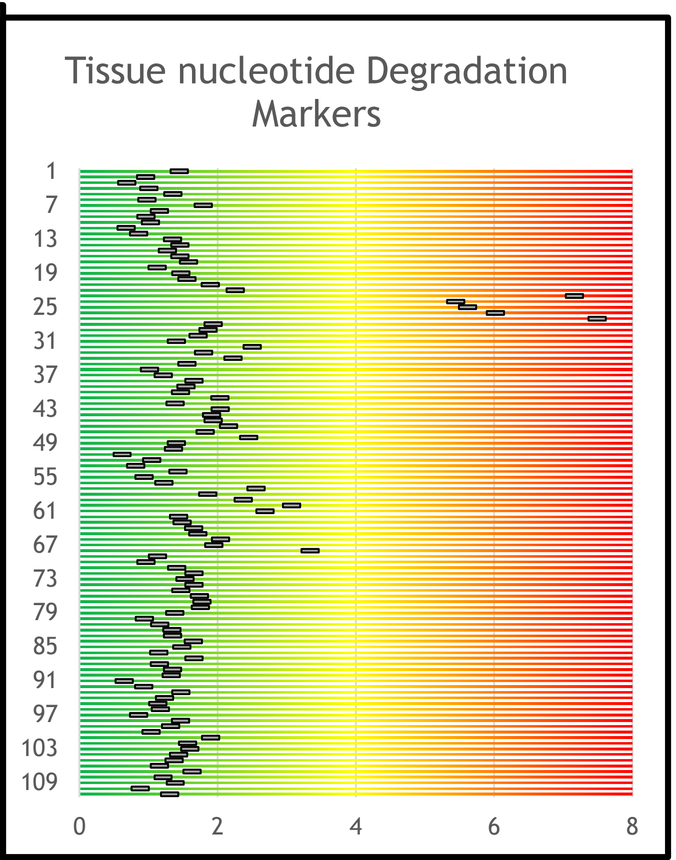
Quantitation can be a key to measurement accuracy, batch to batch normalization and quality control.

- HMT offers a **Q353** package that supplements untargeted metabolomic profiling. The Q353 provides **quantitation** of 353 metabolites that includes amino acids, nucleic acids, ATP, ADP and representative bacterial metabolites like Serotonin.

- The Q353 platform comes with representative ranges (upper and lower) for human serum, plasma and other sample types.

- Adding a standard quantitation panel to untargeted profiling is unique to HMT.





Unique to HMT – QC protocol to check on individual and group sample quality in tissues and in blood products.

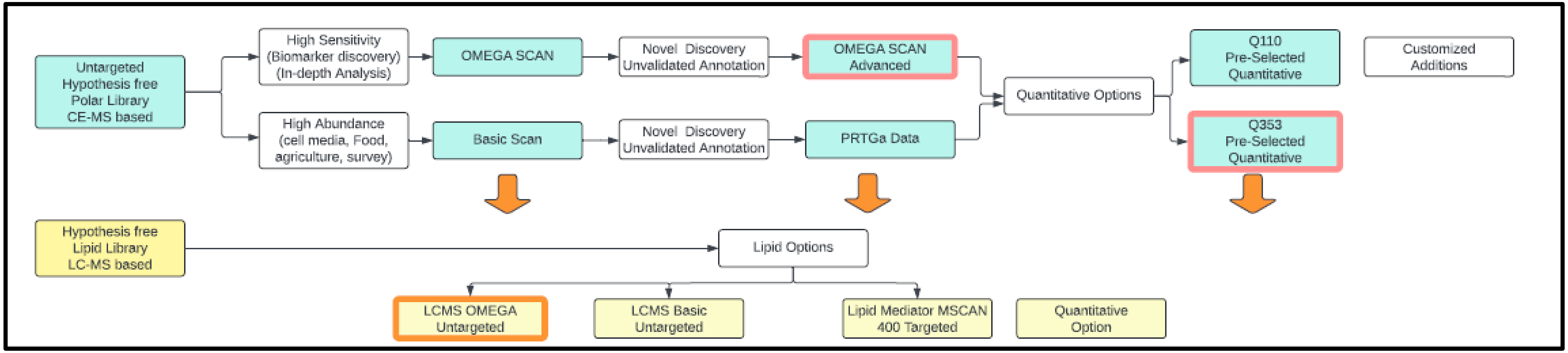
OMEGA scan – 500 or more annotated polar metabolites amino acids, SCFA, MCFA, Nucleic acids, Vitamins
Advanced report – 500 or more unvalidated, proposed metabolites generally under 600 mass.

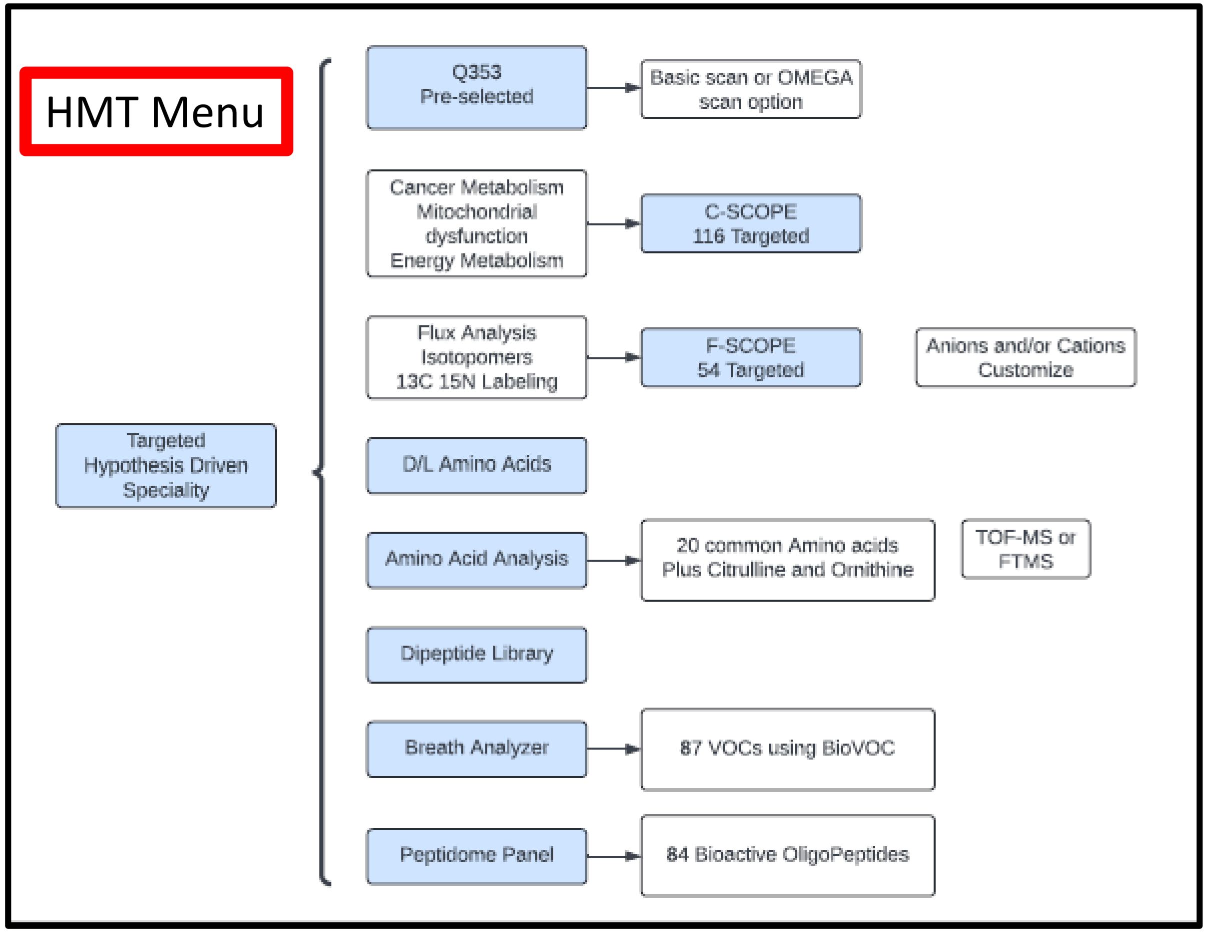
LC-OMEGA – 400 or more lipids, long chain fatty acids, bile acids, steroids, Vitamins, Phytochemicals under 1000 mass.

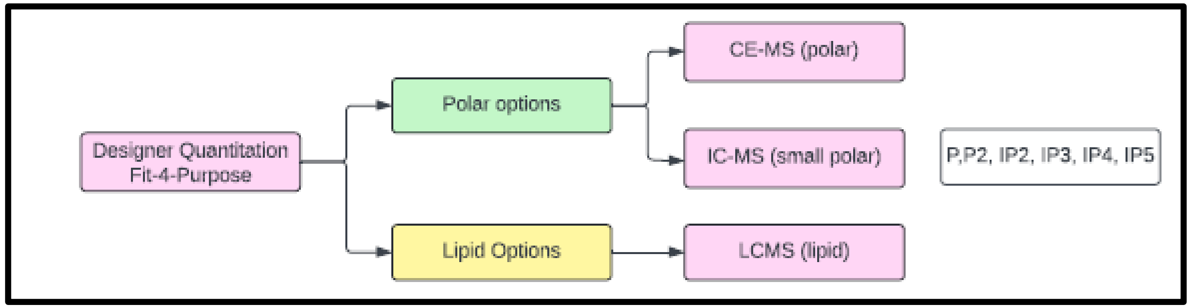
Lipidome – Extensive profiling complex lipids and diacylglycerides, many over 1000 mass

Q353 – 353 pre-selected quantitative measurements included with untargeted report

HMT Menu allows to choose different combinations of platforms







Other options include:

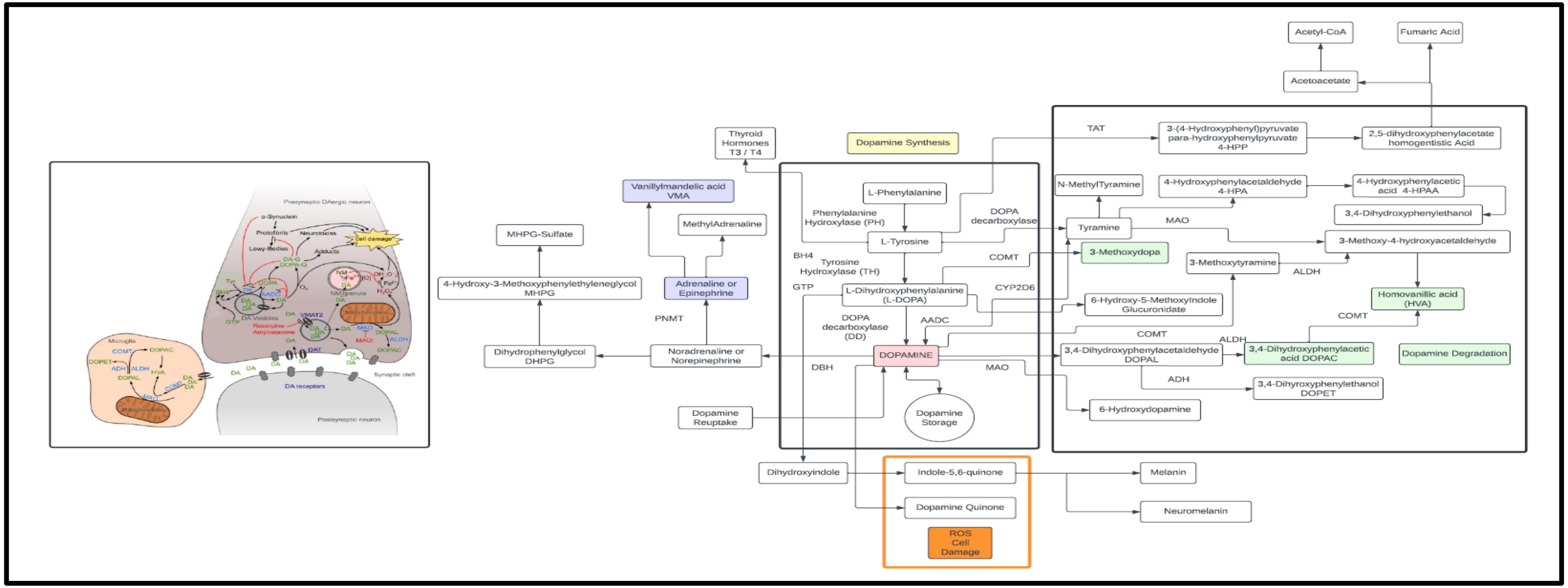
- Glycolytic, PPP, TCA cycle, Urea cycle quantitative panel
- 13-C, 15-N isotopomer analysis
- D- L- Amino acid analysis
- Dipeptide library
- Bioactive Oligopeptide scan
- Fit -4 purpose quantitative panels
- Pi, PPi quantitative analysis
- Ipx quantitative analysis
- S1P quantitative analysis

Exploring new territories with OMEGA$^{2\ adv}$

- Beta-amino acids & isomerases
- Acetylome & *N*-acetyltransferases
- γ-glutamyl-transferases
- Peptidome – Dipeptidases and Tripeptidases
- Novel indole metabolism, lysine metabolism, GABA metabolism, SCFA metabolism
- Novel metabolites from host and bacterial sources
- Novel biochemical pathways and interactions

What is next?

- What are the relevant pathways? Communications?
- How does metabolomic change correlate with health, outcomes, disease?
- We have over 50 original pathway maps individualized for client research.



Summary

- We have highways of chemistry going through us largely influenced by the microbiome.
- We inherited this microbiome from plants and soil and evolution.
- We know microbes play a very important role in our health and disease and how we age.
- How much do we know of this “communication”?
- How do we best measure and discover bioactive metabolites from the microbiome?

Summary

- Lesson 1 - Microbes produce all sorts of cool stuff, and much of it isn't annotated or understood.

- Lesson 2 - Single analytical methods don't detect all of this – being that's the case, we need new methods to visualize chemical diversity.

- Lesson 3 – We need a multi omics approach combing high resolution high sensitivity CEMS with lipid profiling using LCMS including raw data (Advanced options) with genome sequencing to assemble the parts to this puzzle.