

1 **Gut microbiota role in dietary protein metabolism and health-related outcomes:**  
2 **the two sides of the coin**

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26 **Abstract**

27 ***Background***

28 Human gut bacteria can synthesize proteinogenic amino acids and produce a range of  
29 metabolites via protein fermentation, some known to exert beneficial or harmful  
30 physiological effects on the host. However, the effects of the type and amount of dietary  
31 protein consumed on these metabolic processes, as well as the effects of the microbiota-  
32 derived amino acids and related metabolites on the host health are still predominantly  
33 unknown.

34 ***Scope and Approach***

35 This review provides an up-to-date description of the dominant pathways/genes  
36 involved in amino acid metabolism in gut bacteria, and provides an inventory of  
37 metabolic intermediates derived from bacterial protein fermentation that may affect  
38 human health. Advances in understanding bacterial protein fermentation pathways and  
39 metabolites generated at a global level via the implementation of ‘omics’ technologies  
40 are reviewed. Finally, the impact of dietary protein intake and high-protein diets on  
41 human health is discussed.

42 ***Key Findings and Conclusions***

43 The intestinal microbiota is able to synthesize amino acids, but the net result of  
44 amino acid production and utilization, according to dietary patterns still needs to be  
45 determined. The amount of ingested dietary protein appears to modify both the diversity  
46 and composition of the intestinal microbiota as well as the luminal environment of the  
47 intestinal epithelium and peripheral tissues. The understanding of the consequences of  
48 such changes on the host physiology and pathophysiology is still in an early stage but  
49 major progress is expected in the near future with the investigation of host-microbe  
50 omics profiles from well-controlled human intervention studies.

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54 **Key words:** Microbiota, microbiome, protein metabolism, high-protein diet

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## 76 **1. Introduction**

77 The dietary protein consumption level in humans is vastly different according to food  
78 availability and cultural dietary habits (Wu, et al., 2014). Although insufficient protein  
79 consumption remains a persistent problem in the developing world, the average daily  
80 protein intake in countries from Western Europe and the United States of America is  
81 generally higher than the recommended dietary intake of  $0.83 \text{ g protein} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$  for  
82 adults (EFSA Panel on Dietetic Products, 2012; Rand, Pellett, & Young, 2003). In  
83 individuals consuming a high-protein (HP) diet as a way to reduce their body weight,  
84 the protein consumption generally consists of approximately two to three times the  
85 recommended dietary intake; and can even represent five times this latter value (Pesta &  
86 Samuel, 2014). Such diets have been shown to increase satiety, modify lipid  
87 metabolism, and facilitate short- and medium-term weight reduction (Westerterp-  
88 Plantenga, Nieuwenhuizen, Tome, Soenen, & Westerterp, 2009). Although a reduction  
89 of body weight in overweight and obese individuals is obviously associated with  
90 favorable outcomes in terms of health (Papillard-Marechal, et al., 2012), such dietary  
91 modification are also associated with potentially deleterious effects in both healthy  
92 situations in the long-term and in some pathological situations, notably in kidney  
93 diseases (Juraschek, Appel, Anderson, & Miller, 2013) and in inflammatory bowel  
94 diseases (Jowett, et al., 2004).

95 Besides host physiological factors, recent evidence demonstrates that human gut  
96 microbiota in the small and large intestine also plays a role in host dietary protein  
97 metabolism. The interplay between host and gut microbial metabolism is complex, with  
98 microbes utilizing and even competing for dietary and endogenous proteins.  
99 Fermentation of amino acids by gut bacteria produces metabolites that can affect host  
100 protein/amino acid uptake (transport) and metabolism, as well as affect host cell

101 physiology (Davila, et al., 2013). Bacteria can also synthesize amino acids, which can  
102 be provided to the host (Metges, 2000). However, the net result of amino acid synthesis  
103 and degradation remains largely to be determined along with the role of the gut  
104 microbiota for the management of whole body nitrogen metabolism (Neis, Dejong, &  
105 Rensen, 2015). Such knowledge is important since it will yield information regarding  
106 the role of the microbiota in the utilization of amino acids from dietary origin in  
107 different physiological and pathological situations, as well as the role of the microbiota  
108 in the production of metabolites that could be available for the host and impact host  
109 metabolism and other physiological functions.

110       Despite the relatively rapid transit of the luminal content in the small intestine, part  
111 of the amino acid pool released from proteins through the action of pancreatic enzymes  
112 can be used by the host enterocytes (Davila, et al., 2013) as well as by the small  
113 intestinal microbiota (Dai, Zhang, Wu, & Zhu, 2010). Protein digestion in the  
114 mammalian digestive tract is a very efficient process, being generally equal to or even  
115 higher than 90% (Bos, et al., 2005). In the large intestine, where the microbiota  
116 concentration is much higher and the transit time is longer than in the small intestine,  
117 the remaining protein is broken down to peptides and amino acids via extracellular  
118 bacterial proteases and peptidases (Macfarlane, Cummings, & Allison, 1986). In  
119 contrast to the small intestine, however, the amino acids generated cannot be absorbed  
120 to any significant extent by the large intestine epithelium, except during the neonatal  
121 period in mammals (Darragh, Cranwell, & Moughan, 1994). Gut bacterial fermentation  
122 of amino acids thus results in an accumulation of various metabolic end-products in the  
123 luminal content, some of these metabolites being largely absorbed through the large  
124 intestinal epithelial cells, while others are released in feces in large amounts (Davila, et  
125 al., 2013). Several bacterial metabolites have also been shown to be active on colonic

126 epithelial cells, which, as detailed below, depending on their luminal concentrations,  
127 can exert beneficial or deleterious effects.

128 Bacterial metabolites which are not fully metabolized/detoxified by the colonic  
129 epithelial cells during their transcellular journey from the intestinal lumen to the  
130 bloodstream may reach the liver through the portal vein and then peripheral tissues  
131 where they can exert some biological effects, notably on kidney functions.

132 Investigations into the effects of microbially-derived metabolites on human health  
133 and the interaction of the microbiota with the human host have previously been limited  
134 due to the complexity of interactions between these two systems. The rapid advance of  
135 ‘omics’ technologies are beginning to expand our understanding of the relationships  
136 between the human host and gut microbiota by allowing a global analysis of the flow of  
137 host- and microbially-produced metabolites and genes involved in specific biochemical  
138 pathways (Qin, et al., 2010; Sridharan, et al., 2014). A thorough characterization and  
139 understanding of the bacterial pathways involved in amino acid metabolism and their  
140 derivatives is required for precise interpretation and prediction of dietary protein effects  
141 on the host health. Currently, a comprehensive review of those bacterial genes and  
142 metabolic routes is lacking.

143 This review merges up-to-date genomic information regarding amino acid-related  
144 metabolism in gut bacteria with their potential effects on human health. A description of  
145 the dominant pathways for bacterial amino acid biosynthesis as well as for amino acid  
146 degradation into metabolites that may play different roles in human health is provided.  
147 Moreover, information on the enzymes and homologous genes involved in these  
148 pathways as deduced from the KEGG database (Kanehisa, et al., 2014) is given. We  
149 then discuss the recent advances in understanding the effects of different dietary  
150 strategies (i.e. high-fat diet and HP diet) on the human gut microbiome and its role in

151 protein/amino acid metabolism based on metagenomic and metabolomic studies.  
152 Finally, we analyzed how this metabolic activity, notably in terms of bacterial  
153 metabolite production, may be responsible for the effects of dietary protein intake levels  
154 on health-related outcomes in both physiological and pathological situations as well as  
155 underline research areas that need new developments.

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## 158 **2. Bacterial synthesis of amino acids**

159 The effects of *de novo* production of amino acids by microbes on whole-body fluxes  
160 and human health are still not clearly understood. Bacterial production of amino acids  
161 that are accessible to the host may be useful to compensate indispensable amino acid  
162 deficiency in low quality protein diets. However, bacterially-produced amino acids  
163 could also have detrimental consequences in conditions such as insulin resistance in  
164 type 2 diabetes where systemic concentrations of amino acids such as aromatic and  
165 branched-chain amino acids are elevated (Neis, et al., 2015). A deeper understanding of  
166 the effects of microbially-produced amino acids on host health is warranted, as well as a  
167 revision of the biosynthetic pathways of amino acids in bacteria which is provided here.

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### 169 **2.1. Pathways/genes involved in *de novo* biosynthesis of amino acids**

170 Due to the high metabolic cost of synthesizing amino acids, the carbon backbone of  
171 all amino acids originates from common metabolic intermediates involved in processes  
172 such as the tricarboxylic acid cycle, the pentose phosphate pathway and glycolysis  
173 (Berg, Tymoczko, & Stryer, 2002). Among these intermediates,  $\alpha$ -ketoglutarate plays a  
174 central role in amino acid biosynthesis through its conversion to glutamate, as well as  
175 its participation in the biosynthetic pathways of other amino acids. Amino acids can be

176 grouped into families according to common starting products or use of common  
177 enzymes for synthesis. These consist of the following families: glutamate, serine,  
178 aspartate, pyruvate, and aromatic amino acid families, as well as several unique  
179 pathways for individual amino acids (Umberger, 1978). It is important to note that an  
180 overwhelming amount of the literature on bacterial metabolism has historically been  
181 focused on a few bacterial taxa, namely *Escherichia coli* and *Salmonella typhimurium*,  
182 and to a lesser extent *Bacillus subtilis* and recently *Corynebacterium glutamicum*, thus  
183 creating a potential bias towards mechanisms found in these organisms. Although many  
184 of these pathways are conserved across bacterial lineages including those inhabiting the  
185 intestine, diversity is found among different bacterial species at both the species and  
186 strain level. For example, whole genome analysis has revealed that the common gut  
187 bacterium *Clostridium perfringens* lacks numerous amino acid biosynthetic genes for  
188 glutamate, arginine, histidine, lysine, methionine, serine, threonine, aromatic and  
189 branched-chain amino acids (Shimizu, et al., 2002), while other *Clostridium* spp., such  
190 as *Clostridium acetobutylicum*, has a complete set of genes for amino acid biosynthesis  
191 (Nolling, et al., 2001). The gut bacterium *Lactobacillus johnsonii* also appears  
192 incapable of carrying out *de novo* biosynthesis of almost all amino acids due to a lack of  
193 complete biosynthetic pathways, and exhibits an apparent dependence on exogenous  
194 host amino acids/peptides for protein synthesis (Pridmore, et al., 2004). Other animal  
195 and human intestinal bacteria, including *Campylobacter jejuni*, *Helicobacter pylori*,  
196 *Enterococcus faecalis* and *Streptococcus agalactiae* have also lost certain amino acid  
197 biosynthetic pathways (Yu, Walker, Liu, & Zhang, 2009), suggesting a dependence on  
198 availability of exogenous peptides/amino acid sources. Furthermore, the sole presence  
199 of genes within a genome does not confirm their functionality. Genes for the  
200 biosynthesis of all 20 standard amino acids have been identified in the common gut



201 bacterium *Lactococcus lactis*, although supplemented sources of certain amino acids  
202 (isoleucine, valine, leucine, histidine, methionine, and glutamic acid) are also required  
203 for growth since genes in these biosynthetic pathways have been demonstrated to be  
204 non-functional due to point mutations (Bolotin, et al., 2001; Godon, et al., 1993). This  
205 may also be the case with another gut bacterium *Staphylococcus aureus*, as two distinct  
206 strains exhibited auxotrophy for different amino acids, despite the presence of complete  
207 sets of genes for biosynthetic pathways of these essential amino acids in both strains  
208 (Kuroda, et al., 2001). Given these different scenarios, it is likely that alternative  
209 intestinal bacterial co-metabolic pathways and regulation strategies remain to be  
210 discovered.

211

## 212 **Glutamate Family (glutamate, glutamine, proline, arginine)**

213 Glutamate and glutamine are both key nitrogen/amino group donors for amino acid  
214 synthesis and provide the major entry points of ammonia into bacterial metabolism (H.  
215 Shimizu & Hirasawa, 2007). These enzyme pathways are particularly important for  
216 some gut bacteria with a reliance on ammonia as a nitrogen source, such as many  
217 *Bacteroides* spp., which cannot replace ammonia with other nitrogen sources such as  
218 amino acids, peptides, urea or nitrate (reviewed in Fischbach & Sonnenburg, 2011).  
219 Glutamate is one of the most important central metabolites in all bacteria providing a  
220 link between carbon and nitrogen metabolism. Most enteric bacteria have two primary  
221 pathways for the synthesis of glutamate (Fig. 1a) involving either the enzyme glutamate  
222 dehydrogenase (GDH) or glutamine oxoglutarate aminotransferase (also called GOGAT  
223 or glutamate synthase) (Reitzer & Magasanik, 1987; H. Shimizu & Hirasawa, 2007).  
224 Preferential use of either pathway depends on both the energy state and intracellular  
225 ammonium concentrations in the cells. GDH is preferentially utilized for glutamate

226 synthesis when both energy and carbon sources are limited for the cell but ammonium  
227 and phosphate are present in excess, while the GOGAT pathway is preferred when the  
228 cell is not under energy limitation since this pathway requires the expenditure of ATP  
229 (Helling, 1994). However, exceptions to this strategy are seen in the common gut  
230 bacterium *Bacteroides fragilis* which contains 2 distinct glutamate dehydrogenase  
231 enzymes: a dual cofactor NAD(P)H-dependent (gene = *gdhB*) enzyme whose activity  
232 depends on high ammonia concentrations, and a NADH-specific enzyme whose activity  
233 depends on high peptide concentrations in which ammonia has no effect on activity  
234 (Abrahams & Abratt, 1998; Yamamoto, Saito, & Ishimoto, 1987). In the Gram-positive  
235 bacterium *Bacillus subtilis*, glutamate is exclusively synthesized by reductive amination  
236 of  $\alpha$ -ketoglutarate by the enzyme glutamate synthase encoded by the *gltAB* operon  
237 (Belitsky, 2002).

238 Glutamine is synthesized by the single reaction of glutamine synthetase (GS; EC  
239 6.3.1.2) in which ammonia is added to glutamate through ATP hydrolysis (Fig. 1a).  
240 Three distinct types of glutamine synthetases have been identified in bacteria: GSI is  
241 found in eubacteria and archaea including the ruminal *R. albus*, GSII is present only in  
242 eukaryotes and several soil bacteria: *Rhizobium*, *Frankia*, and *Streptomyces*, while  
243 GSIII has only been found in several unrelated bacteria *Bacteroides fragilis*, *Rhizobium*  
244 *leguminosarum*, and *Butyrivibrio fibrisolvens*, and *R. albus* (Brown, Masuchi, Robb, &  
245 Doolittle, 1994; Kim, Henriksen, Cann, & Mackie, 2014).

246 Several pathways exist for bacterial proline biosynthesis, however the most common  
247 pathway found in many groups involves a four-step process starting with the  
248 phosphorylation of glutamate (Fig. 1a) (reviewed in (Fichman, et al., 2014). Genomic  
249 analysis of proline biosynthesis has revealed that many eubacterial and archaeal species  
250 contain only one recognizable gene from this pathway (i.e.  $\Delta^1$ -pyrroline-5-carboxylate

251 reductase) while other species lack all genes from this pathway, suggesting that either  
252 these bacteria are dependent on exogenous proline sources for growth or that alternative  
253 pathways may exist that may not yet be elucidated (Fichman, et al., 2014).

254 Biosynthesis of arginine involves an eight-step process starting with the acetylation  
255 of the glutamate (Fig. 1a). This step occurs through the so-called “classical” pathway  
256 initially described for *E. coli* via the enzyme N-acetylglutamate synthase (NAGS) and  
257 encoded by the gene *argA* (reviewed in (Xu, Labedan, & Glansdorff, 2007). In many  
258 bacterial species, this step can also be achieved by an ornithine *N*-acetyltransferase  
259 (OAT) with dual functional activities (E.C. 2.3.1.35 / 2.3.1.1) that can also synthesize  
260 acetylglutamate *de novo* from acetyl-CoA and glutamate (Marc, et al., 2000; Xu, et al.,  
261 2007).

262

### 263 **Serine Family (serine, glycine, cysteine)**

264 Serine is an important metabolite and precursor to multiple amino acids in bacteria  
265 (glycine, cysteine, tryptophan). It is also a precursor for the metabolism of  
266 sphingolipids, folate, methane, sulfur, cyanoamino acid, and pyruvate, and also  
267 participates in the biosynthesis of purines and pyrimidines (Stolz, et al., 2007;  
268 Umbarger, 1978). Furthermore, L-serine plays a fundamental role in stabilizing blood  
269 sugar concentration in the liver (Remesy, Fafournoux, & Demigne, 1983). Synthesis of  
270 serine is a three-step process starting with the glycolytic intermediate 3-  
271 phosphoglycerate (Fig. 1b) (Peters-Wendisch, et al., 2005). Glycine is generated from  
272 serine by the single enzyme serine hydroxymethyltransferase (Fig. 1b) that catalyzes the  
273 reversible conversion of serine and tetrahydrofolate to glycine and 5,10-methylene  
274 tetrahydrofolate, which is a key intermediate in the biosynthesis of purine, thymidine,  
275 choline, glutathione and methionine (Trivedi, et al., 2002). In enterobacteria and

276 *Bacillus subtilis*, the synthesis of cysteine is a two-step reaction (Fig. 1b) that involves  
277 the substrates coenzyme A and hydrogen sulfide (H<sub>2</sub>S) (Kredich, 1996).  
278 *Bifidobacterium longum* lacks the genes for the final step with cysteine synthase and  
279 may use alternative pathways with several enzymes such as succinylhomoserine and the  
280 sulfur-containing compounds H<sub>2</sub>S or methanethiol produced by other colonic bacteria  
281 (Schell, et al., 2002). Several strains of *Bifidobacterium bifidum* have further displayed  
282 cysteine auxotrophy (Ferrario, et al., 2015), indicating a dependence of exogenous  
283 sources of this amino acid.

284

### 285 **Aspartate Family (aspartate, asparagine, lysine, threonine, methionine)**

286 Aspartate is an important precursor for the biosynthesis of numerous amino acids. It  
287 is synthesized from the transfer of an amino group from glutamate to oxaloacetate via  
288 the enzyme aspartate transaminase (Fig. 2). Asparagine is commonly made by one of  
289 two distinct asparagine synthetases in bacteria in which one enzyme utilizes ammonia  
290 while the other synthetase carries out a transamination reaction from glutamine to  
291 aspartate (Fig. 2) (Min, Pelaschier, Graham, Tumbula-Hansen, & Soll, 2002). Alternate  
292 pathways exist in some gut bacteria, such as *B. longum*, which lack both types of  
293 asparagine synthetases and likely use an asparaginyl-tRNA-dependent route (Schell, et  
294 al., 2002).

295 Two main pathways exist for lysine biosynthesis in bacteria, the diaminopimelic acid  
296 (DAP) and aminoadipic acid (AAA) pathways. The DAP pathway is used by most  
297 bacteria and some archaea and utilizes aspartate and pyruvate as starting material with  
298 meso-2,6-diaminopimelic acid as an intermediate (Fig. 2) (Patte, 1996). Four variations  
299 of this pathway have been identified in bacteria: the succinylase (most common) (Fig.  
300 2), acetylase, aminotransferase, and dehydrogenase pathways (Liu, White, & Whitman,

301 2010). No organism is known to possess both DAP and AAA pathways (Liu, et al.,  
302 2010). In *E. coli*, the first step in the DAP pathway employs three distinct aspartate  
303 kinase isozymes (ThrA, MetL, and LysC), each specific to one of three different  
304 biochemical pathways under regulation from lysine, methionine, and threonine  
305 (Vitreschak, Lyubetskaya, Shirshin, Gelfand, & Lyubetsky, 2004).

306 Synthesis of threonine shares the first two steps of the biosynthetic pathway of lysine  
307 (Fig. 2). In addition to the conversion of aspartate to 4-phospho-L-aspartate, ThrA and  
308 MetL isozymes also contain a homoserine dehydrogenase (Hom) domain that carries  
309 out the third step in the biosynthetic pathway in which aspartate 4-semialdehyde is  
310 converted to homoserine (Fig. 2) (Vitreschak, et al., 2004). Methionine shares the first  
311 three steps of the biosynthetic pathway of threonine (Fig. 2); after which several  
312 different pathways are utilized by different bacteria to attach a sulfur group to O-  
313 Succinyl-L-homoserine via either L-cysteine (Fig. 2) or incorporate inorganic sulfur  
314 with O-acetylhomoserine to form homocysteine (Rodionov, Vitreschak, Mironov, &  
315 Gelfand, 2004).

316

### 317 **Pyruvate Family (isoleucine, valine, leucine, alanine)**

318 The branched-chain amino acids (BCAA), valine, leucine and isoleucine share  
319 common biosynthetic pathways all stemming from intermediates from pyruvate  
320 metabolism. These pathways are so similar that many of the same enzymes are shared  
321 for biosynthesis of all these amino acids (Fig. 3). In *E. coli* and *S. enterica* serovar  
322 *Typhimurium*, three different isoenzymes of the enzyme acetolactate synthase (AHAS;  
323 EC 2.2.1.6;) are made up of a large and small subunit encoded by the respective genes  
324 *ilvIH*, *ilvBN*, and *ilvGM* (Umbarger, 1996). Isoleucine biosynthesis starts with the  
325 conversion of threonine to 2-oxobutanoate via the enzyme threonine deaminase and

326 then uses the same enzymes as those described for valine biosynthesis (Fig. 3). Leucine  
327 biosynthesis uses the last intermediate from the valine pathway, 2-oxoisovalerate, to  
328 carry out the initial reaction (Fig. 3) (Pátek, 2007). Leucine has also been demonstrated  
329 to be biosynthesized via the precursor SCFA isovalerate via carboxylation in the gut  
330 bacteria *Bacteroides fragilis* and *Prevotella ruminicola* (previously *Bacteroides*  
331 *ruminicola*) (Allison, Baetz, & Wiegel, 1984). Both of these bacteria also preferentially  
332 utilize 2-methylbutyrate as a precursor for isoleucine biosynthesis instead of carrying  
333 out *de novo* synthesis starting from glucose (Allison, et al., 1984). Several pathways  
334 exist for alanine biosynthesis, with starting precursors such as pyruvate and aspartate  
335 among the most common (Fig. 3). L-alanine is mainly synthesized from pyruvate and  
336 glutamate via an alanine transaminase (Oikawa, 2007).

337

### 338 **Aromatic Family (phenylalanine, tyrosine, tryptophan)**

339 Biosynthesis of aromatic amino acids typically follows the shikimate pathway and  
340 starts with the condensation of the glycolytic intermediate phosphoenolpyruvate and the  
341 pentose phosphate pathway intermediate erythrose 4-phosphate, via the enzyme 3-  
342 Deoxy-D-arabinoheptulosonate 7-phosphate (DAHP) synthase (Fig. 4) (Sprenger,  
343 2007). In *E. coli*, three distinct isoenzymes (called AroF, AroG and AroH) are involved  
344 in this step and each is specifically feed-back inhibited by the terminal end products  
345 tyrosine, phenylalanine and tryptophan, respectively (Sprenger, 2007). A single non-  
346 homologous DAHP synthase (AroA) is found in the Gram-positive *B. subtilis* (Panina,  
347 Vitreschak, Mironov, & Gelfand, 2003). The pathway proceeds for 6 steps to the  
348 common intermediate chorismate, which serves as a branchpoint to the individual  
349 pathways for phenylalanine, tryptophan and tyrosine (Fig. 4).

350 Alternative pathways exist for synthesis of each respective aromatic amino acid in  
351 different bacterial species, but commonalities within the pathways remain. The first  
352 reaction for both phenylalanine and tyrosine uses the enzyme chorismate mutase to  
353 convert chorismate to prephenate. In *E. coli*, chorismate mutases are bifunctional  
354 enzymes which can participate in the first two steps of both phenylalanine and tyrosine  
355 biosynthesis from chorismate (Fig. 4) (Sprenger, 2007). In *E. coli*, both pathways then  
356 use either the enzyme aromatic amino acid aminotransferase or aspartate  
357 aminotransferase (EC 2.6.1.1) to catalyze the transamination reaction into each  
358 respective  $\alpha$ -keto acid using glutamate as the amino group donor (Pittard, 1996) (Fig.  
359 4). Aromatic amino acid aminotransferase can also participate in an alternative pathway  
360 that catalyzes the conversion of prephenate to L-arogenate which can be further  
361 converted to either phenylalanine or tyrosine using prephenate dehydratase or  
362 cyclohexadieny/prephenate dehydrogenase, respectively. Instead of *de novo* synthesis of  
363 phenylalanine, several gut bacteria (*B. fragilis* and *P. ruminicola*) preferentially use  
364 phenylacetate as a precursor for this amino acid (Allison, et al., 1984).

365 Tryptophan biosynthesis is five-step pathway from chorismate (Fig. 4) in which  
366 glutamine or ammonia is used in the first step with anthranilate synthase (Nichols,  
367 1996; Pittard, 1996). In the final step, serine is used as the amino group donor for  
368 tryptophan synthase (Sprenger, 2007). The tryptophan biosynthesis pathway is quite  
369 conserved in different bacteria, although differences in gene order and the enzymatic  
370 reactions carried out by separate or fused enzyme units exist in different bacteria  
371 (reviewed in Xie, Keyhani, Bonner, & Jensen, 2003). For example, all of the genes in  
372 the biosynthetic pathway for tryptophan have been identified in the gut bacterium *B.*  
373 *longum* except for TrpF (phosphoribosylanthranilate isomerase), indicating a  
374 replacement of this gene with an unidentified homolog (Schell, et al., 2002).

375

## 376 **Histidine**

377 Histidine biosynthesis is a complex ten-step enzymatic pathway (Fig. 5) encoded by  
378 eight different genes, three of which (*hisD*, *hisB*, and *hisI*) encode bifunctional enzymes  
379 (reviewed in (Alifano, et al., 1996). Most of the work on the histidine biosynthetic  
380 pathway has mainly been studied in *E. coli* and *Salmonella typhimurium* and more  
381 recently in the industrially important *Corynebacterium glutamicum*, demonstrating large  
382 conservation along with some differences in the biochemical pathways between the  
383 different species (Kulis-Horn, Persicke, & Kalinowski, 2014). Whole genome studies in  
384 bacteria identified in the gut such as *L. lactis* confirm the presence of all the genes of  
385 this pathway (Bolotin, et al., 2001).

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## 388 **3. Overview of uptake systems and catabolism of proteins/amino acids in gut** 389 **bacteria**

390

### 391 **3.1. Protein hydrolysis and peptide/amino acid transporters**

392 Initial steps of bacterial protein catabolism include the extracellular hydrolysis of  
393 proteins via different bacterial proteases. According to the MEROPS peptidase database  
394 (Rawlings, Waller, Barrett, & Bateman, 2014), bacteria contain a highly diverse number  
395 of different proteases, present in many common gut microbiota species such as  
396 *Clostridium spp.*, *Bacteroides spp.*, *Lactobacillus spp.*, etc. containing up to hundreds of  
397 different identified proteases. Some bacteria, such as lactic acid bacteria, have  
398 developed sophisticated proteolytic systems to compensate their reduced capabilities for  
399 amino acid biosynthesis (Pessione, 2012). Proteolytic systems in lactic acid bacteria



400 consist of either extracellular or membrane-bound proteases (principally PrtP and CEP)  
401 that degrade proteins into oligopeptides, followed by their incorporation into the cell via  
402 peptide transporters (Opp, Dpp, Dtp, for oligopeptide, dipeptide, and di- and tripeptide,  
403 respectively) and finally numerous intracellular peptidases that degrade the peptides  
404 into short peptides and amino acids (reviewed in Liu, Bayjanov, Renckens, Nauta, &  
405 Siezen, 2010; Steiner, Naider, & Becker, 1995).

406 Amino acids and their derivatives are imported and exported from the bacterial cell  
407 via transmembrane proteins comprising ATP-dependent ABC transporters, several  
408 families of channel proteins, or secondary carriers relying on proton-motive force,  
409 sodium-ion motive force, solute-solute exchange, or uniport (Saier, 2000). Efflux  
410 systems for some amino acids such as lysine, arginine, threonine, cysteine, leucine,  
411 isoleucine, and valine are well-known in the bacteria *E. coli* and *Clostridium*  
412 *glutamicum* (Eggeling & Sahm, 2003), whereas to date no export systems for histidine  
413 have been described in any organism (Kulis-Horn, et al., 2014).

414

### 415 **3.2. Transamination, deamination and decarboxylation**

416 Bacteria may directly incorporate available amino acids as substrates for protein  
417 biosynthesis or may carry out catabolic reactions to use them as energy sources or to  
418 produce other metabolites. Under aerobic conditions, bacteria typically convert  
419 proteinogenic  $\alpha$ -amino acids to  $\alpha$ -oxo acids (aka.  $\alpha$ -ketoacids) or saturated fatty acids  
420 via transamination or deamination, which are further oxidized as energy sources in the  
421 tricarboxylic acid (TCA) cycle. However, in the absence of oxygen or other suitable  
422 electron acceptors, only strict or facultative anaerobic bacteria in the gut, such as  
423 Clostridia and Fusobacteria, are capable of utilizing amino acids as energy sources, thus  
424 fermenting amino acids to short-chain fatty acids (SCFAs), molecular hydrogen (H<sub>2</sub>),

425 carbon dioxide (CO<sub>2</sub>), and ammonia, with minor products of H<sub>2</sub>S, methylmercaptane,  
426 phenols, alcohols and organic acids (Davila, et al., 2013; J. Kim, Hetzel, Boiangiu, &  
427 Buckel, 2004). Several mechanisms for α-amino acid degradation exist for anaerobic  
428 bacteria, which includes the well-known Stickland reaction found in many proteolytic  
429 Clostridia which involves the coupled oxidation and reduction of two respective amino  
430 acids to organic acids. Other fermentation pathways found in various Clostridia as well  
431 as *Fusobacterium* spp. and *Acidaminococcus* spp. involve single amino acids that act as  
432 electron donors as well as acceptors (Fischbach & Sonnenburg, 2011; J. Kim, et al.,  
433 2004). The genus *Clostridium* contains unique amino acid degradation pathways, such  
434 as B12-dependent aminomutases, selenium containing oxidoreductases and oxygen-  
435 sensitive 2-hydroxyacyl-CoA dehydratases (Fonknechten, et al., 2010).

436 Amino acids can also be metabolized through decarboxylation reactions ultimately  
437 yielding amines and polyamines as products. Factors such as pH can influence the  
438 activity of deaminases and decarboxylases, ultimately affecting the accumulation of  
439 specific end products. Furthermore, many complex amino acids can undergo a series of  
440 metabolic reactions that produce a large variety of structurally-related metabolic end-  
441 products (Davila, et al., 2013).

442 Amino acid utilization may be achieved in a preferential manner, as Fonknechten et  
443 al. (2010) demonstrated that *Clostridium sticklandii* preferentially used threonine,  
444 arginine and serine for carbon and energy sources, but hardly utilized glutamate,  
445 aspartate and aromatic amino acids, even though these catabolic pathways are found in  
446 this organism. Furthermore, lysine degradation, which is a process that provides a major  
447 energy source, was only observed in stationary growth phases (Fonknechten, et al.,  
448 2010).

449

450 **3.3. Factors influencing protein fermentation:**

451 Many different factors may influence protein fermentation in the gut, such as  
452 substrate availability, transit time, pH and osmolarity. The ratio of available  
453 carbohydrates:protein determines substrate utilization by the gut microbiota (Smith &  
454 Macfarlane, 1996), and in humans, it has been shown that availability of complex  
455 carbohydrates lowers protein fermentation (Birkett, Muir, Phillips, Jones, & O'Dea,  
456 1996; De Preter, et al., 2008; Geboes, et al., 2006). When the main energy sources (i.e.  
457 fermentable carbohydrates) for microbiota are abundant, nitrogenous substrates can be  
458 used for biosynthetic (anabolic) process and bacterial growth. On the contrary, proteins  
459 are catabolized by bacteria when energy is scarce, leading to the production of amino  
460 acid-derived end products. Due to high carbohydrate fermentation in the proximal  
461 colon, there is a progressive decrease of carbohydrate availability in the distal colon,  
462 resulting in higher protein fermentation (Macfarlane, Gibson, & Cummings, 1992).  
463 Long transit time and elevated pH are also associated with high levels of protein  
464 fermentation (Cummings, Hill, Bone, Branch, & Jenkins, 1979; Macfarlane, Cummings,  
465 Macfarlane, & Gibson, 1989). Therefore, dietary carbohydrate and protein ratios may  
466 strongly influence the metabolic pathways activated in the large intestine and flow of  
467 metabolites generated.

468

469

470 **4. Key intermediate products from bacterial protein/amino acid catabolism and**  
471 **effects on host physiology/health**

472 Fermentation of amino acids derived from endogenous luminal and dietary protein  
473 by intestinal microbiota produces numerous metabolites with suspected or established

474 effects on host intestinal physiology, liver and peripheral tissues. Relevant examples of  
475 such metabolites are described below.

476

#### 477 **4.1. Microbially-produced compounds with neuroactive properties**

478 Recent evidence has shown that bacteria isolated from the mammalian gut have the  
479 capacity to synthesize neuroactive compounds including neurotransmitters, many of  
480 which result from the catabolism of amino acids. These compounds include GABA  
481 (produced by *Lactobacillus* spp., *Bifidobacterium* spp., and *Lactococcus lactis*);  
482 norepinephrine (produced by *Escherichia* spp. and *Bacillus* spp.); dopamine (produced  
483 by *Bacillus* spp.); histamine (produced by numerous bacterial genera); and serotonin  
484 (produced by *Streptococcus* spp., *Escherichia* spp. and *Enterococcus* spp.) (Sanders, et  
485 al., 1998; Wall, Ross, & Stanton, 2014). In addition, bacteria have been demonstrated to  
486 produce other neuroactive compounds from amino acid degradation such as nitric oxide  
487 and the biogenic amines tryptamine and phenethylamine. It is worth noting that for most  
488 of these bacterial metabolites, the understanding of their precise effects on the intestine  
489 and peripheral tissues remains in its infancy.

490  $\gamma$ -aminobutyrate (GABA), known as an inhibitory neurotransmitter, is microbially-  
491 produced by the decarboxylation of glutamate via the enzyme glutamate decarboxylase  
492 (Table 1). This enzyme makes up part of the glutamate decarboxylase (GAD) system  
493 found in several bacterial genera, which is implicated in acid tolerance by maintaining  
494 intracellular pH homeostasis through proton consumption (Feehily & Karatzas, 2013).  
495 Other factors in addition to acidic stress have been demonstrated to activate the GAD  
496 system, including sodium, polyamines and hypoxia (Feehily & Karatzas, 2013). GABA  
497 is subsequently exported from the cell via antiporters that import glutamate, or it  
498 remains in the cells and is metabolized to succinate via the GABA shunt pathway

499 (Feehily & Karatzas, 2013; Karatzas, Brennan, Heavin, Morrissey, & O'Byrne, 2010).  
500 Glutamate/GABA antiporters are found in numerous Gram-negative (*Escherichia*,  
501 *Shigella*, *Brucella*) and Gram-positive genera (*Listeria*, *Lactobacillus*, *Lactococcus*,  
502 *Clostridium*, *Bifidobacterium*) (Feehily & Karatzas, 2013). An *in vivo* study in mice  
503 also revealed that chronic administration of *Lactobacillus rhamnosus* (JB-1) induced  
504 changes in mRNA of GABA receptors B1b and A2, as well as reduced anxiety- and  
505 depression-related behavior (Bravo, et al., 2011), although the direct production of  
506 GABA by gut bacteria was not investigated. *In vivo* studies are required to evaluate  
507 whether gut microbiota derived GABA is active on the host.

508 Serotonin, a neurotransmitter involved in numerous processes including behavior,  
509 learning, appetite and glucose homeostasis, is produced in the human brain but also  
510 notably in intestinal enteroendocrine cells (El-Merahbi, Loffler, Mayer, & Sumara,  
511 2015). The key role of serotonin in the gut-brain axis has been extensively reviewed,  
512 indicating that peripherally it is involved in modulation of the gut immune system,  
513 gastrointestinal secretions, motility and visceral sensitivity and centrally in mood and  
514 cognition (O'Mahony, Clarke, Borre, Dinan, & Cryan, 2015). In humans, serotonin is  
515 produced from tryptophan degradation in a two-step process via the enzymes tryptophan  
516 5-monooxygenase (tryptophan hydroxylase 1) and aromatic-L-amino-acid decarboxylase  
517 (Table 1). It has been experimentally demonstrated *in vitro* that serotonin can also be  
518 produced by several bacterial genera isolated from the human gut, including  
519 *Streptococcus*, *Escherichia*, *Enterococcus*, *Lactococcus* and *Lactobacillus* (Wall, et al.,  
520 2014). However, the full mechanism of direct serotonin production by bacteria has not  
521 been clearly established. Analysis of the serotonin biosynthetic pathway in bacteria via  
522 the KEGG database reveals that several bacterial genera contain homologous genes for  
523 encoding aromatic-L-amino-acid decarboxylase used in the second step of this pathway

524 (Kanehisa, et al., 2014), but no homologous genes have presently been found for  
525 tryptophan 5-monooxygenase. Bacteria can also directly interact with the host to induce  
526 the production of serotonin by the host, with experimentally demonstrated effects on  
527 host physiology (Yano, et al., 2015). A comparison between germ-free mice and  
528 colonized mice (with either mouse or human gut microbiota) has shown that microbiota  
529 increased colonic expression of tryptophan hydroxylase 1, the rate limiting enzyme for  
530 mucosal serotonin synthesis, and thereby serotonin production, likely acting through  
531 SCFAs (Reigstad, et al., 2015).

532 Histamine and its receptors were first described as part of the immune and  
533 gastrointestinal systems, but their presence in the central nervous system and  
534 implication in behavior and energy homeostasis is gaining increasing attention  
535 (Baronio, et al., 2014). Production of histamine from the decarboxylation of the amino  
536 acid histidine has been well demonstrated in numerous Gram-positive and Gram-  
537 negative bacterial strains (Landete, De las Rivas, Marcobal, & Munoz, 2008). Gram-  
538 positive bacteria, such as lactic acid bacteria, use a pyruvoyl-dependent histidine  
539 decarboxylase (EC 4.1.1.22) (Table 1), whereas Gram-negative bacteria use a pyridoxal  
540 phosphate-dependent histidine decarboxylase (Landete, De las Rivas, et al., 2008). In  
541 *Lactobacillus* spp. histamine is readily exported from the cell using electrogenic  
542 histidine/histamine antiporters (Molenaar, Bosscher, ten Brink, Driessen, & Konings,  
543 1993). Bacterial histamine production can exert both positive and negative effects on  
544 human health. Recently, Thomas et al. (2012) demonstrated that histamine derived from  
545 a human gut isolate of the species *Lactobacillus reuteri* suppresses pro-inflammatory  
546 TNF- $\alpha$  production in isolated Toll-like receptor 2-activated human monocytoic cells,  
547 indicating a potential beneficial effect of microbially-derived compounds for  
548 inflammatory conditions. However, histamine production by bacteria has also been

549 implicated in food poisoning related to the ingestion of fish fermented by histamine-  
550 producing bacteria (scombrototoxin) (Bjornsdottir-Butler, Green, Bolton, & McClellan-  
551 Green, 2015).

552 Tyrosine is a precursor for several hormones including the thyroid hormones  
553 thyroxine (3,5,3',5'-tetraiodothyronine) and triiodothyronine, as well as a precursor to L-  
554 dihydroxyphenylalanine (L-DOPA) which is in itself a precursor to the catecholamine  
555 neurotransmitters dopamine, norepinephrine and epinephrine. Bacterial tyrosinases (EC  
556 1.14.18.1) (Table 1), which catalyze the conversion of tyrosine to L-DOPA, are widely  
557 found in many bacterial genera (Claus & Decker, 2006). Although *Escherichia* spp. and  
558 *Bacillus* spp. have been reported to produce norepinephrine (Wall, et al., 2014),  
559 enzymes necessary for production of the catecholamines norepinephrine and  
560 epinephrine have only been identified to date in animals, suggesting some mode of  
561 cooperation between host and gut microbes in the catecholamine biosynthetic process.  
562 However, catecholamine stress hormones have been demonstrated to promote both  
563 bacterial growth and virulence (Freestone, 2013). Some opportunistic pathogens  
564 inhabiting the intestinal tract (*Escherichia coli*, *Staphylococcus aureus*, etc.) are  
565 equipped with catechol siderophore uptake systems that facilitate the import Fe(III)-  
566 catecholamine complexes and, thereby, their growth under iron-restricted conditions  
567 (Beasley, Marolda, Cheung, Buac, & Heinrichs, 2011).

568 Tyramine is a biogenic amine capable of causing food poisoning if consumed at high  
569 levels and is involved in hypertension and migraine syndromes (Marcobal, De las  
570 Rivas, Landete, Tabera, & Munoz, 2012; Millichap & Yee, 2003). Tyramine is  
571 produced by the decarboxylation of tyrosine by the bacterial enzyme tyrosine  
572 decarboxylase (Table 1), which has been identified in Gram-positive bacteria such as  
573 lactic acid bacteria, but putative tyrosine decarboxylases in Gram-negative bacteria

574 remain to be characterized (Marcobal, et al., 2012). Tyramine is exported from bacterial  
575 cells via a tyrosine-tyramine antiporter (Linares, Fernandez, Martin, & Alvarez, 2009).  
576 In Gram-positive bacteria, high concentrations of tyrosine and acidic pH conditions  
577 have been demonstrated to increase the transcription of tyrosine decarboxylase and the  
578 tyrosine-tyramine antiporter (Linares, et al., 2009; Marcobal, et al., 2012). Gut  
579 microbiota derived tyramine has been shown to increase circulating serotonin levels by  
580 elevating its synthesis by enteroendocrine cells (Yano, et al., 2015) although this  
581 mechanism has not been defined.

582 Nitric oxide (NO) is produced from the amino acid arginine by many bacterial  
583 species via the enzyme nitric oxide synthase (NOS) (Table 1), although only several  
584 bacterial homologs of mammalian NOSs have been identified to date (Sudhamsu &  
585 Crane, 2009). Little is known about the extent that microbially-produced NO impacts  
586 the human host, notably at the intestinal mucosa level, although it is known that NO is  
587 involved in immune defense mechanisms and exerts cytotoxic effects at high  
588 concentrations.

589 Tryptamine is a  $\beta$ -arylamine neurotransmitter that has numerous biological roles  
590 including a ligand for the sigma-2 receptor, a trace amine-associated receptor that  
591 increases the inhibitory responses of cells to serotonin, and an inducer of serotonin  
592 release from enteroendocrine cells which can modulate gastrointestinal motility and  
593 may play a role in the pathology of inflammatory bowel diseases (Williams, et al., 2014  
594 and references therein). Recently it was demonstrated that tryptamine is produced by  
595 *Clostridium sporogenes* by the decarboxylation of tryptophan via a Trp decarboxylase  
596 (Table 1) (Williams, et al., 2014). Williams et al. (2014) also used a phylogenic-  
597 informed screening of other decarboxylases from the gut microbiota to show that a



598 second Trp decarboxylase appears in the Firmicute *Ruminococcus gnavus*, and similar  
599 enzymes were found in at least 10% of the human population.

600 Phenethylamine is a trace amine that acts as a neurotransmitter and, depending on the  
601 concentrations, is associated with specific psychological disorders (Irsfeld, Spadafore,  
602 & Pruss, 2013) and can affect satiety and mood (Pessione, 2012). Phenethylamine is  
603 produced in bacteria by the decarboxylation of phenylalanine (Diaz, Ferrandez, Prieto,  
604 & Garcia, 2001) via the enzyme aromatic-L-amino-acid decarboxylase (Table 1).

605 Potential physiological effects of microbially-derived phenethylamine on host health  
606 have yet to be elucidated.

607

#### 608 **4.2. Short- and branched-chain fatty acids**

609 The main short-chain fatty acids (SCFAs) produced in the large intestine by the  
610 mammalian gut microbiota are acetate, butyrate and propionate (Rechkemmer, Ronnau,  
611 & von Engelhardt, 1988). Although it is well known that dietary substrates for SCFA  
612 production are mainly fibers and resistant starches (Laparra & Sanz, 2010), isolated  
613 colonic bacteria growing *in vitro* on proteins as the only available carbon source have  
614 been demonstrated to produce SCFAs as well as branched-chain fatty acids (BCFAs)  
615 (Neis, et al., 2015). Bacterial fermentation of glycine, alanine, threonine, glutamate,  
616 lysine and aspartate can produce acetate; threonine, glutamate and lysine can produce  
617 butyrate, and alanine and threonine can produce propionate (Davila, et al., 2013).  
618 Nonetheless, the molecular pathways involved in production of these SCFAs are not  
619 well-defined.

620 Branched-chain fatty acids (BCFAs), namely isobutyrate, 2-methylbutyrate and  
621 isovalerate, are derived from branched-chain amino acids and are present at much lower  
622 concentrations in the large intestine luminal content (Liu, et al., 2014). Although a full

623 description of bacterial synthesis of BCFA from amino acids is beyond the scope of this  
624 article, many Gram-positive bacteria use the primers isovaleryl-CoA, isobutyryl-CoA,  
625 and 2-methylbutyryl-CoA derived from valine, isoleucine, and leucine to produce  
626 BCFA using a specialized branched-chain-keto acid dehydrogenase complex (Cronan &  
627 Thomas, 2009). The relative proportions of SCFAs and BCFAs depend on numerous  
628 factors including the type of protein degraded as well as the available concentrations of  
629 carbohydrates (Macfarlane & Macfarlane, 2012).

630 The SCFAs acetate, propionate and butyrate produced via bacterial fermentation of  
631 carbohydrates and/or amino acids have been proposed to exert various physiological  
632 effects, which have been recently reviewed (Hamer, et al., 2008). Briefly, for instance,  
633 butyrate provides energy to colonocytes and regulates cell proliferation and  
634 differentiation as well as the transcription of numerous genes involved in mucin  
635 production and hormone secretion (i.e. PYY, GLP-1, GLP-2) that influence gut  
636 integrity and transit, appetite and glucose metabolism (Daly, Cuff, Fung, & Shirazi-  
637 Beechey, 2005). Butyrate also plays an anti-inflammatory role via different  
638 mechanisms, including inhibition of LPS-mediated inflammatory cytokine secretion by  
639 intestinal epithelial cells and other immune cells and via induction of colonic regulatory  
640 T cells partly by an epigenetic modification of the forkhead box-P3 promoter  
641 (Furusawa, et al., 2013). Propionate and acetate undergo partial oxidation in  
642 colonocytes or can travel to the liver via the portal vein where they serve as substrates  
643 for gluconeogenesis and lipogenesis (Tremaroli & Backhed, 2012). Acetate is also a  
644 substrate for cholesterol synthesis, whereas propionate can decrease cholesterol  
645 synthesis (Demigne, et al., 1995). In addition, SCFAs are ligands for G protein-coupled  
646 receptors, namely Gpr41 and Gpr43, which are expressed in enteroendocrine L-cells of  
647 the distal small intestine and colon (Reigstad, et al., 2015). Gpr41 deficiency has been

648 shown to be associated with reduced expression of peptide YY (PYY), a gut hormone  
649 involved in satiety and gut motility (Samuel, et al., 2008). Direct administration of  
650 SCFAs in the colon increase PYY plasma level, and *in vitro* administration on primary  
651 colonic crypts also increase the release of PYY, suggesting a direct effect of SCFA on  
652 secretion (Chambers, et al., 2014; Cherbut, et al., 1998). Butyrate can also bind the  
653 GPR109a receptor expressed by intestinal macrophages and dendritic cells, thus  
654 activating production of the anti-inflammatory cytokine Il-10 (Pabst & Bernhardt,  
655 2010). BCFAs have also been shown to regulate electrolyte absorption and secretion  
656 (Musch, Bookstein, Xie, Sellin, & Chang, 2001). It has been proposed that SCFAs  
657 could exert effects beyond the gut via their interactions with Gpr expressed in different  
658 tissues and cellular types (e.g. adipocytes, pancreatic cells, neuronal cells) and thereby  
659 regulate metabolism by inducing energy expenditure and mitochondrial function (Gao,  
660 et al., 2009). Whether these effects could be mediated by the SCFAs generated by the  
661 gut microbiota requires further investigation.

662

### 663 **4.3. Sulfur-containing bacterial metabolites**

664 Fecal S-containing substances are a complex mixture of bacterial metabolites  
665 including methanethiol and the well-studied H<sub>2</sub>S, which is found at millimolar  
666 concentrations in the human colonic luminal contents and in feces (Macfarlane, et al.,  
667 1992). In addition to production of H<sub>2</sub>S via reduction of inorganic sulfate by intestinal  
668 sulfate reducing bacteria or microbial catabolism of intestinal sulfomucins, sulfide is  
669 produced by intestinal microbiota activity through fermentation of several S-containing  
670 amino acids of both dietary and endogenous origins (Blachier, et al., 2010). H<sub>2</sub>S is  
671 notably produced as a byproduct by the bacterial fermentation of cysteine via cysteine  
672 desulfhydrases, cystathione-B-lyase and tryptophanase (Table 1) (Awano, Wada, Mori,

673 Nakamori, & Takagi, 2005). Fermentation of methionine yields the sulfur-containing  
674 compound methanethiol (Davila, et al., 2013). Due to its lipophilic activity, H<sub>2</sub>S  
675 penetrates biological membranes (Reiffenstein, Hulbert, & Roth, 1992) and, when  
676 present in excess, inhibits mitochondrial cytochrome c oxidase activity reversibly with a  
677 binding constant similar to cyanide (Leschelle, et al., 2005). At lower concentrations,  
678 H<sub>2</sub>S is able to increase cellular respiration and ATP production by means of the  
679 mitochondrial sulfide-oxidizing unit (Bouillaud & Blachier, 2011). Although several  
680 lines of evidence suggest that excessive concentrations of sulfide in the intestinal  
681 luminal content may be implicated in the etiology of ulcerative colitis (Pitcher &  
682 Cummings, 1996) and in the risk of relapse after an inflammatory episode (Jowett, et  
683 al., 2004), there is also evidence that endogenously-formed low levels of H<sub>2</sub>S in  
684 intestinal mucosa may participate in the resolution of mucosal inflammation (Flannigan,  
685 et al., 2015). Experiments from colonocyte incubation and intra-colonic instillation  
686 with millimolar concentrations of NaHS, used as a H<sub>2</sub>S donor, indicate that hydrogen  
687 sulfide reversibly inhibits colonocyte oxygen consumption and increases the expression  
688 of hypoxia-inducible factor 1 alpha (HIF-1alpha) together with several inflammation-  
689 related genes, namely inducible nitric oxide synthase (iNOS) and interleukin 6 (IL-6)  
690 (Beaumont, et al., 2016). Importantly, it has been demonstrated that endogenously  
691 produced H<sub>2</sub>S maintains colon cancer cellular bioenergetics supporting colonic tumor  
692 growth (Szabo, et al., 2013).

693

#### 694 **4.4. Aromatic compounds**

695 In addition to neuroactive compounds, the catabolism of aromatic amino acids  
696 (phenylalanine, tyrosine and tryptophan) by the microbiota also produces phenolic and  
697 indolic compounds (Nyangale, Mottram, & Gibson, 2012). Recent support for a role of

698 gut microbiota in the production of various aromatic amino acid metabolites has  
699 recently been demonstrated by comparing metabolites between specific pathogen-free  
700 (SPF) mice and germ-free (GF) mice (Sridharan, et al., 2014). However, bacterial  
701 metabolism of aromatic compounds largely depends on the type of carbon availability,  
702 as fermentable carbohydrates largely inhibit aromatic amino acid fermentation (Smith &  
703 Macfarlane, 1996).

704 Phenol is produced from the conversion of tyrosine via the enzyme tyrosine phenol-  
705 lyase (Table 1). Optimal conditions for production of phenol by anaerobic bacteria in  
706 the human large intestine include a near-neutral pH of 6.8 and availability of free amino  
707 acids as opposed to peptides (Smith & Macfarlane, 1997). The types of intestinal  
708 bacteria may also play a significant role in production of phenol as aerobic bacteria  
709 tended to produce phenol from tyrosine degradation *in vitro* while anaerobic bacteria  
710 produced *p*-cresol (Bone, Tamm, & Hill, 1976). Phenol has been shown to decrease the  
711 integrity of the barrier function of colonocytes *in vitro* (Hughes, Kurth, McGilligan,  
712 McGlynn, & Rowland, 2008) and impaired the viability of human colonic epithelial  
713 cells at concentrations higher than 1.25 mM (Pedersen, Brynskov, & Saermark, 2002).

714 Formation of *p*-cresol (or 4 methylphenol) by microbes begins with the two-step  
715 conversion of tyrosine to 4-hydroxyphenylacetate (Meyer & Hostetter, 2012), although  
716 the genes involved in this conversion are unknown (Table 1) (Dawson, et al., 2011). In  
717 the final step, the bacterium *Clostridium difficile* uses the enzyme *p*-  
718 hydroxyphenylacetate decarboxylase (genes *hpdB*, *hpaC*, *hpdA*) to convert 4-  
719 hydroxyphenylacetate to *p*-cresol (Selmer & Andrei, 2001; Yu, Blaser, Andrei, Pierik,  
720 & Selmer, 2006). The *hpdBCA* operon is rarely found in gut microflora other than  
721 *Clostridium* spp. (Dawson, et al., 2011), and therefore it is unknown to what extent, if  
722 any, other gut genera play a role in *p*-cresol formation. Production of *p*-cresol is

723 stimulated by Fe(III) and competitive growth conditions in *Clostridium* spp. (Doerner,  
724 Mason, Kridelbaugh, & Loughrin, 2009; Selmer & Andrei, 2001). The *p*-cresol  
725 concentration in human feces averages approximately 0.4 mM (Gostner, et al., 2006;  
726 Lecerf, et al., 2012). These compounds are absorbed from the intestinal lumen to the  
727 portal bloodstream through colonocytes, metabolized in the liver and finally excreted by  
728 the kidneys with more than 90% of urinary phenolic compounds being recovered as *p*-  
729 cresol (Hughes, Magee, & Bingham, 2000). At millimolar concentrations, *p*-cresol  
730 inhibits human colonocyte proliferation and cell respiration and increases superoxide  
731 production (Andriamihaja, et al., 2015). Most importantly, in this latter study, *p*-cresol  
732 was found to be genotoxic towards human colonocytes. *p*-cresol can be conjugated in  
733 the colonic epithelium and in the liver, generating *p*-glucuronide and cresyl sulfate,  
734 (Evenepoel, Meijers, Bammens, & Verbeke, 2009). Phenolic compound sulphation has  
735 been shown to be impaired in the mucosa of ulcerative colitis patients (Ramakrishna,  
736 Roberts-Thomson, Pannall, & Roediger, 1991). Interestingly, *p*-cresyl sulfate promotes  
737 insulin resistance in chronic kidney disease (CKD) patients (Koppe, et al., 2013) and is  
738 found at elevated concentrations in the urine of autism patients (Heinken & Thiele,  
739 2015). It can also cause damage in renal tubular cells by induction of oxidative stress  
740 through activation of NADPH oxidase (Watanabe, et al., 2013).

741 Indole is produced from tryptophan via the enzyme tryptophanase (Table 1), which is  
742 found only in microbes (Meyer & Hostetter, 2012). After absorption through the  
743 intestinal epithelium, indole is transported to the liver where it undergoes hydroxylation  
744 by the host to 3-hydroxy-indole and finally sulfonated to indoxyl sulfate, a uremic  
745 toxin, via a sulfotransferase (Meijers & Evenepoel, 2011; Wikoff, et al., 2009). In  
746 contrast, indole also has a potential beneficial effect on host intestinal epithelial cells, as  
747 treatment of human enterocytes with indole was found to result in increased expression

748 of genes involved in the mucosal barrier functions and was associated with a reduction  
749 of inflammatory parameters (Bansal, Alaniz, Wood, & Jayaraman, 2010). Secretion of  
750 the incretin GLP-1 from enteroendocrine cells is increased during short exposures to  
751 indole via interference of voltage-gated K<sup>+</sup> channels, but reduced over long periods of  
752 exposure to indole via slowing ATP production by blocking NADH dehydrogenase  
753 (Chimerel, et al., 2014). Besides its role in glucose metabolism through increasing  
754 insulin secretion, GLP-1 is involved in inhibiting gastric secretion and motility; these  
755 phenomenons being associated with increased satiety (Steinert, Beglinger, & Langhans,  
756 2015). The indole derivative indole-3-aldehyde was also shown to regulate gut mucosal  
757 immune response through aryl hydrocarbon receptor as well as intestinal barrier  
758 function and inflammation through its sensing by pregnane X receptor (Venkatesh, et  
759 al., 2014; Zelante, et al., 2013).

760 Serum levels of the amino acid tryptophan itself also have a demonstrated role in  
761 host health, as decreases in this amino acid have been associated with increased immune  
762 activation, inflammatory diseases such as Crohn's disease, as well as cognitive deficit  
763 (Gupta, et al., 2012; Widner, et al., 1999). This may be particularly detrimental in  
764 elderly populations, as increased proportions of genes from gut microbiota involved in  
765 aromatic amino acid metabolism (including tryptophan), as well as decreased levels of  
766 tryptophan have been reported in elderly patients (Collino, et al., 2013; Rampelli, et al.,  
767 2013).

768

#### 769 **4.5. Polyamines**

770 Intestinal luminal polyamines (i.e. agmatine, putrescine, spermidine, cadaverine,  
771 etc.) can originate from dietary components, microbiota metabolism (Davila, et al.,  
772 2013), and endogenous origin, notably released from desquamated intestinal epithelial

773 cells. Gram-negative bacteria such as *E. coli* produce high concentrations of putrescine  
774 and spermidine in minimal media (Tabor & Tabor, 1985). Numerous amines (including  
775 polyamines) have been measured in the intestinal luminal contents at concentrations  
776 ranging from micro- to millimolar (Osborne & Seidel, 1990). Intestinal epithelial cells  
777 have the capacity to take up polyamines from the intestinal luminal contents (Blachier,  
778 et al., 1992).

779 Agmatine is formed by the decarboxylation of the amino acid arginine via the  
780 enzyme arginine decarboxylase (Table 1). Agmatine, which is produced and released  
781 by colonic bacteria as well as by desquamated intestinal epithelial cells and ingested in  
782 food, exerts inhibitory effects on colonocyte proliferation (Mayeur, et al., 2005).

783 Putrescine can be synthesized by bacteria from the amino acid arginine either through  
784 the intermediate ornithine or through agmatine (Nakada & Itoh, 2003). Agmatine is  
785 either directly converted to putrescine via the enzyme arginine decarboxylase, or goes  
786 through a two-step process which uses the enzymes agmatine deiminase and N-  
787 carbamoylputrescine amidase (Table 1). The agmatine deiminase pathway has been  
788 found in several bacterial genera such as *Pseudomonas*, *Enterococcus*, *Bacillus* and  
789 *Lactobacillus* (Landete, Arena, Pardo, Manca de Nadra, & Ferrer, 2008). Agmatine-  
790 putrescine antiporters have been identified in bacterial genera and function by importing  
791 agmatine and exporting putrescine (Polo, Gil-Ortiz, Cantin, & Rubio, 2012). Putrescine  
792 synthesis has been shown to be strictly necessary for colonic epithelial cell proliferation  
793 (Gamet, Cazenave, Trocheris, Denis-Pouxviel, & Murat, 1991; Mouille, Delpal,  
794 Mayeur, & Blachier, 2003).

795 In many bacteria including *E. coli*, spermidine is synthesized from putrescine and  
796 decarboxylated S-adenosylmethionine (SAM) via spermidine synthase (Tabor & Tabor,  
797 1985). Recently, bacteria lacking spermidine synthase orthologues were discovered to



798 have an alternate pathway in which spermidine is formed by the enzymes  
799 carboxynorspermidine dehydrogenase (aka carboxynorspermidine synthase) and  
800 carboxynorspermidine decarboxylase via the intermediate carboxyspermidine (Lee, et  
801 al., 2009). This alternative pathway of spermidine synthesis has been identified as the  
802 dominant pathway in the human gut microbiota as well as diverse human pathogens and  
803 is critical for growth in selected species (Hanfrey, et al., 2011). In host cells, SAM can  
804 react with spermidine via another enzyme, spermine synthase, to produce spermine.  
805 Prokaryotic cells do not appear to contain spermine synthase, but they can produce a  
806 different tetra-amine compound, thermospermine, that has been detected in lower  
807 eukaryotes and plants (Minguet, Vera-Sirera, Marina, Carbonell, & Blazquez, 2008).  
808 Spermidine and spermine are polycationic amines that are involved in numerous  
809 processes such as mitigating oxidative stress, and induction of autophagy to stimulate  
810 cellular longevity (Eisenberg, et al., 2009; Yamamoto, et al., 2012).

811 The polyamine cadaverine is synthesized from lysine in a one-step reaction with  
812 lysine decarboxylase (Table 1) and has been shown to provide an acid resistance  
813 mechanism in *E. coli* (Le Gall, et al., 2011). However, the effects of cadaverine on the  
814 colonic epithelial cells remain unknown.

815

#### 816 **4.6. Ammonia**

817 Ammonia provides the source of nitrogen for all amino acids and is primarily  
818 assimilated through either glutamate dehydrogenase or the glutamine  
819 synthetase/GOGAT cycle. It is also used directly in the biosynthesis of various amino  
820 acids including glutamate, glutamine, asparagine, valine, isoleucine, leucine,  
821 phenylalanine, tyrosine and tryptophan (Figs. 1-4). Ammonia can be produced by the  
822 microbial degradation of numerous amino acids to specific metabolites (i.e. arginine to

823 putrescine, tyrosine to phenol, tryptophan to indole, Table 1). Ammonia (taken as the  
824 sum of  $\text{NH}_3$  and  $\text{NH}_4^+$ ) is found at millimolar concentrations in the large intestine  
825 luminal content of mammals including humans (Mouille, Robert, & Blachier, 2004). In  
826 humans, the luminal ammonia concentration progressively increases from the ascending  
827 to the descending colon (Macfarlane, et al., 1992), in accordance with a higher rate of  
828 protein fermentation in the distal colon. The luminal ammonia concentration in the large  
829 intestine is primarily the net result of microbiota utilization and production through  
830 amino acid deamination, urea hydrolysis, and absorption from the luminal content to the  
831 portal blood, with the unabsorbed/unmetabolized ammonia being excreted in feces  
832 (Eklou-Lawson, et al., 2009). Although relatively large amounts of ammonia can be  
833 transferred from the intestinal lumen to the bloodstream, a part of this ammonia can be  
834 metabolized by colonocytes into citrulline and glutamine (Eklou-Lawson, et al., 2009;  
835 Mouille, et al., 2004), allowing control of the intracellular ammonia concentration in  
836 colonocytes during its transfer from the luminal content to the portal bloodstream.  
837 Ammonia inhibits mitochondrial oxygen consumption in a dose-dependent manner,  
838 leading to the concept that excessive luminal ammonia concentration behaves as a  
839 metabolic troublemaker towards colonocyte energy metabolism (Andriamihaja, et al.,  
840 2010). Accordingly, high millimolar concentrations of ammonia have been shown to  
841 markedly inhibit short-chain fatty acid oxidation in isolated colonocytes (Cremin, Fitch,  
842 & Fleming, 2003).

843

844

845 **5. Gut microbiome features related to amino acid metabolism as a function of the**  
846 **subject's metabolic phenotype and the diet**

847 Recent metagenomic studies have allowed a detailed examination of the metabolic  
848 capacity of the mammalian gut microbiome in metabolizing nitrogenous components,  
849 particularly amino acid-related compounds. An important insight from these studies  
850 revealed that the gut microbiome contains a large enrichment of genes involved in  
851 amino acid metabolism compared to the human genome (Gill, et al., 2006), thus  
852 expanding the human metabolic capacity to form a more diverse number of metabolites.  
853 In particular, the human microbiome had large enrichments of genes involved in the  
854 biosynthesis of lysine, phenylalanine, tyrosine, tryptophan, valine, leucine and  
855 isoleucine as well as enrichment in genes associated with the metabolism of alanine,  
856 aspartate, glutamate, histidine, methionine, glycine, serine, threonine and the urea cycle,  
857 with only slight to moderate enrichments of genes for the metabolism of other amino  
858 acids and non-protein amino acids (Gill, et al., 2006). Qin et al. (2010) also found an  
859 enrichment of genes involved in pathways such as the biosynthesis of lysine,  
860 phenylalanine, tyrosine, tryptophan, valine, leucine and isoleucine in the human gut  
861 microbiome compared to the host genome. Since biosynthetic pathways for essential  
862 amino acids do not exist in humans, it is not surprising that gut microbiota has  
863 developed a specialized set of genes and metabolic pathways for synthesizing these  
864 essential nutrients to ensure its survival.

865 Metagenomic sequencing analysis has shown that the metabolic phenotype (obese  
866 *versus* lean subjects) and the diet (e.g. high-fat diets, prebiotic intake, etc.) are  
867 associated with shifts in both specific microbiota taxonomic groups and functions of the  
868 mammalian gut microbiome, such as those involved in the metabolism of amino acids.  
869 Recent studies comparing the short-term effects of animal- and plant-based diets on the  
870 human gut microbiota and expression of metabolic-related genes using RNA-Seq  
871 revealed an increased expression of several catabolic amino acid genes for glutamine

872 and glutamate in animal-based diets, while increased expression of biosynthetic  
873 pathways for these amino acids was observed for plant diets (David, et al., 2014). David  
874 et al. (2014) also found that KEGG modules and pathways involved in methionine and  
875 leucine biosynthesis and cysteine metabolism were significantly associated with animal  
876 diets, while histidine biosynthesis and lysine and branched-chain amino acid  
877 degradation were significantly associated with plant diets.

878 In studies in rodents, Turnbaugh et al. (2006) found that KEGG pathways related to  
879 lysine biosynthesis and D-alanine metabolism were significantly enriched in the pooled  
880 cecal microbiome of *ob/ob* obese mice relative to the pooled lean cecal microbiome,  
881 while the KEGG pathways involved in the metabolism of glutamate, glycine, serine,  
882 threonine, cysteine, arginine and proline and the biosynthesis of phenylalanine, tyrosine  
883 and tryptophan were depleted in the *ob/ob* mouse microbiome relative to the lean one.  
884 Comparison of obese/lean sibling pairs of mice revealed that KEGG pathways involved  
885 in phenylalanine, tyrosine and tryptophan biosynthesis were also depleted in the cecal  
886 microbiomes of the obese mice (Turnbaugh, et al., 2006). Furthermore, using fecal  
887 samples from obese and lean human twin pairs, Turnbaugh et al. (2009) revealed a  
888 depletion of the KEGG pathway involved in tryptophan metabolism in obese twins  
889 compared to their lean counterparts. Recent studies by Everard et al. (2014) revealed  
890 that the clusters of orthologous group (COG) for amino acid transport and metabolism  
891 was enriched in both high-fat diet-fed (HFD) mice and high-fat diet-fed mice treated  
892 with the prebiotic oligofructose (HFD-Pre) compared to controls with and without  
893 prebiotic treatment, with the HFD-Pre mice yielding the highest enrichment of all  
894 groups. Nevertheless, the physiological meaning of those changes in gut microbiome  
895 amino acid metabolic pathways associated with genetically (*ob/ob*) or diet-induced  
896 (HFD) obesity remains unknown. Furthermore, to our knowledge there have not been

897 detailed metagenomic studies examining the effect of a high-protein (HP) diet in either  
898 murine models or humans.

899       Recent metabolomic studies have provided further evidence of the role of gut  
900 microbiota in amino acid metabolism. Zheng et al. (2011) compared metabolites  
901 produced by the host (rat) and microbiota during antibiotic administration and also after  
902 a 14-day recovery period and showed that the recovered mammalian gut microbiota  
903 alters the host's systemic metabolism in terms of production of short chain fatty acids,  
904 tryptophan and tyrosine and possibly indole-melatonin. Metabolomic analyses have  
905 also recently suggested that individual amino acids may play key roles in diet-related  
906 diseases. For example, high concentrations of branched-chain amino acids (BCAA) and  
907 their respective metabolites have been linked with obesity and type 2 diabetes, while  
908 aromatic amino acids and high ratios of glutamate/glutamine have also been associated  
909 with type 2 diabetes (reviewed in Heinken & Thiele, 2015). Although the association of  
910 BCAA and other amino acids with insulin resistance and type 2 diabetes has been well-  
911 known for decades, Newgard et al. (2009) recently performed metabolomic profiling of  
912 obese and lean humans to show that the addition of branched-chain amino acids (valine,  
913 leucine, isoleucine) and two aromatic acids (phenylalanine and tyrosine) to a high-fat  
914 diet contributes to the development of obesity-associated insulin resistance. Wang et al.  
915 (2011) also carried out metabolic profiling to investigate whether specific metabolites  
916 could predict diabetes development and discovered that the same five branched-chain  
917 and aromatic acids were strongly associated with the onset of diabetes, thus suggesting  
918 a potential role of amino acid metabolism in the pathogenesis of diet-related diabetes.  
919 Although the direct role of the gut microbiota in production of these amino acids has not  
920 yet been clearly defined *in vivo*, theoretically the gut bacteria have the capacity to

921 synthesize all of these essential amino acids and, thereby, play a role in those  
922 conditions. However, more work is needed to elucidate this connection.

923 In the case of increased dietary protein intake, the amount of undigested protein that  
924 is transferred to the large intestine markedly increases (Chacko & Cummings, 1988).  
925 Consequently, more substrate is available for bacterial amino acid catabolism. However,  
926 the consequences of this increased availability is not well described at a metabolic level  
927 as only a few studies have only partially examined microbial-derived metabolite profiles  
928 in HP diets by a non-targeted analytical approach in which all potential metabolites are  
929 analyzed. Russell et al. (2011) demonstrated that HP diets with low and moderate  
930 carbohydrate intake in humans displayed increased branched-chain fatty acids,  
931 phenylacetic acid and *N*-nitroso compounds compared to a weight-maintenance diet  
932 with moderate protein levels at the beginning of the intervention study. Reduced  
933 proportions of butyrate and antioxidant phenolic acids were also detected in the HP low-  
934 carbohydrate diets (Russell, et al., 2011) likely as a result of the low carbohydrate  
935 intake. Those changes in fecal metabolic profiles were associated with a decrease in  
936 proportions of the known butyrate-producing bacteria *Roseburia/Eubacterium rectale*  
937 (Russell, et al., 2011). Other studies in humans found that H<sub>2</sub>S fecal concentration was  
938 increased in humans fed a meat-rich diet (Magee, Richardson, Hughes, & Cummings,  
939 2000), and increased levels of phenol, *p*-cresol and phenylacetate have been detected in  
940 urine and feces of individuals receiving HP diets (Cummings, et al., 1979; Geypens, et  
941 al., 1997; Russell, et al., 2011). Increasing the amount of alimentary protein also results  
942 in an increase of the luminal and fecal ammonia concentration in humans (Geypens, et  
943 al., 1997). HP diets in humans also caused elevations in plasma levels and urinary  
944 excretion of indoxyl sulfate and urinary excretion of indoxyl glucuronide, kynurenic  
945 acid and quinolinic acid (Poesen, et al., 2015). Although several of these compounds are

946 strictly produced only by gut microbiota, no study has effectively examined the direct  
947 role of the gut microbiota in the production of these metabolites.

948 In murine models, HP diets have been shown to modify the composition and the  
949 diversity of the colonic microbiota in rats, with associated changes in the total amount  
950 of ammonia, SCFAs, H<sub>2</sub>S, branched-chain fatty acids, ethanol and several organic acids  
951 (L- and D-lactate, succinate) in the colonic luminal content (Liu, et al., 2014). Although  
952 this experiment did not determine the direct role of microbiota composition and  
953 increased substrate availability in the changes observed after HP diet ingestion, it  
954 appears that both parameters are likely to be involved in such changes. A metabolomic  
955 analysis comparing a HP, low-carbohydrate (HPLC) diet to a moderate  
956 protein/carbohydrate (MPMC) diet in a rat model revealed that the HPLC diet induced  
957 weight loss and reduced adipose weight, and the plasma metabolites glucose, insulin,  
958 triglyceride, linoleate, palmitate,  $\alpha$ -glycerophosphate and pyroglutamic acid and caused  
959 a significant increase in several plasma metabolites (i.e. urea, pyruvate,  $\alpha$ -tocopherol, 2-  
960 oxoisocaproate, and  $\beta$ -hydroxybutyrate) (Mu, Yang, Luo, & Zhu, 2015). In the plasma  
961 of mice, increases in tryptophan and other aromatic acid-derived metabolites such as  
962 indole-3-acetic acid, p-cresyl glucuronide, phenyl sulfate and phenylacetic acid were  
963 detected in HP diets (Poesen, et al., 2015). In the urinary metabolite profile, the HPLC  
964 urinary metabolite profile showed an increase in the fatty acids palmitate and stearate  
965 and a reduction of pantothenate and the TCA cycle intermediates citrate, 2-ketoglutarate  
966 and malate (Mu, et al., 2015). In addition to murine models, study designs in humans  
967 still need to be refined to integrate data from complementary functional omics-  
968 technologies to progress in the understanding of the flow of metabolites between the  
969 microbiota and the host derived from dietary protein and their physiological  
970 consequences on the metabolic phenotype.

971

## 972 **6. Impact of protein intake levels on health-related outcomes: possible**

### 973 **contribution of gut microbiota metabolic pathways**

974 Some of the metabolites produced by the microbiota from amino acids have been  
975 shown to be active on numerous host functions as illustrated above. These results  
976 suggest that some of the effects of HP diets on host metabolism and physiology may  
977 involve some of these metabolites and, therefore, microbiota-mediated metabolic  
978 pathways. In the following sections, the effects of high dietary protein intake observed  
979 in human and animal studies are summarized and suggest mechanisms that might  
980 implicate the gut bacteria-derived metabolites (Fig. 6).

981

### 982 **6.1. HP diet-induced satiety**

983 Two recent meta-analyses of clinical trials concluded that, when compared to  
984 normoproteic (NP) diet, a HP diet modestly reduces body weight, blood pressure, fat  
985 mass and triglyceride levels while sparing fat-free mass (Santesso, et al., 2012;  
986 Wycherley, Moran, Clifton, Noakes, & Brinkworth, 2012). Those effects are mainly  
987 attributed to dietary protein induced satiety (Westerterp-Plantenga, et al., 2009). In  
988 humans, HP diet-induced satiety is associated with an increase of PYY plasma levels  
989 (Batterham, et al., 2006). Implication of gut microbiota in those mechanisms, however,  
990 has not been investigated yet. Nevertheless, SCFAs, indole, tryptamine and tyramine are  
991 compounds produced by the gut microbiota from amino acid precursors and are  
992 candidates for mediating the effects of HP diet on enteroendocrine cell hormone  
993 secretion with an impact on satiety (Figure 6).

994

### 995 **6.2. HP diet effects on the intestinal mucosa**



996 In the rat model, HP diet consumption for 15 days resulted in a marked reduction in  
997 the height of the colonocyte brush-border when compared with control animals  
998 receiving a NP/hyperglucidic isocaloric diet (Andriamihaja, et al., 2010). This  
999 morphological change could be related to lower energy efficiency in HP rat colonocytes  
1000 due to proton leaks in the mitochondrial inner membrane (Andriamihaja, et al., 2010). A  
1001 reduction of the mucosal myeloperoxidase activity (representative of neutrophil  
1002 infiltration) together with a down-regulation of mucosal T<sub>helper</sub> cytokines was measured  
1003 in the ileum of rats receiving a HP diet (Lan, et al., 2015). This decreased inflammatory  
1004 status was associated with a hyperplasia of mucus-producing cells concomitant with an  
1005 increased expression of Muc2 at both the gene and protein levels (Lan, et al., 2015). A  
1006 HP diet also induced DNA damages in rat colonocytes, in association with an increase  
1007 in the bacterially-derived genotoxic metabolite *p*-cresol (Toden, Bird, Topping, &  
1008 Conlon, 2005).

1009 In an intervention study comparing volunteers receiving either HP diets or low  
1010 protein isocaloric diet for two weeks, no evidence was found for a role of protein  
1011 fermentation in gut toxicity in healthy human subjects despite identification of several  
1012 metabolites in fecal water with presumably cytotoxic and genotoxic effects towards  
1013 colonic epithelial cells (Windey, et al., 2012). SCFA, H<sub>2</sub>S, *p*-cresol and ammonia are  
1014 metabolites produced from amino acids that could interfere with colonocyte energy  
1015 metabolism and mucin secretion and may mediate the effects of HP diet on the  
1016 intestinal mucosa. These metabolites could partly result from the activity of gut  
1017 microbiota (Figure 6). However, the consequences of a HP intake on large intestine  
1018 mucosal health have never been directly investigated in humans.

1019

1020 **6.2.3. HP diet effects in inflammatory bowel diseases**

1021 SCFAs, H<sub>2</sub>S, indolic compounds, serotonin and histamine are all amino acid-derived  
1022 bacterial metabolites which are likely to interfere with the process of mucosal  
1023 inflammation due to their effects on the mucosal immune response and on the  
1024 maintaining of the epithelial barrier function (Figure 6). As reviewed by Halmos and  
1025 Gibson (2015), only a few studies have examined the impact of the level of protein  
1026 consumption on the risk of inflammatory bowel disease or risk of relapse in diagnosed  
1027 patients. Jowett et al. (2004) reported an increased risk of relapse over 1 year in  
1028 ulcerative colitis patients with the highest meat and protein consumption. A HP diet was  
1029 also associated with an increased risk of incident inflammatory bowel diseases  
1030 (Jantchou, Morois, Clavel-Chapelon, Boutron-Ruault, & Carbonnel, 2010), while Shoda  
1031 et al. (1996) demonstrated that increased consumption of animal protein was related to  
1032 increased incidence of Crohn's disease. However, another study did not find any  
1033 association between elevated protein intake and development of ulcerative colitis (Hart,  
1034 et al., 2008).

1035

#### 1036 **6.2.4. HP diet effects in colorectal cancer**

1037 Experimental studies with animal models of chemically-induced colonic preneoplastic  
1038 and neoplastic lesions have shown that dietary protein can influence the colonic  
1039 carcinogenesis process, depending on their quantity and quality (McIntosh & Le Leu,  
1040 2001). The production of amino-acid derived genotoxic metabolites produced by the  
1041 microbiota (e.g. *p*-cresol) could be implicated in this process. However, the complexity  
1042 of the Western diet makes the identification of alimentary compounds that impact the  
1043 risk of colorectal cancer a difficult task. In this context, it has been proposed that the  
1044 positive association between high consumption of red and processed meat and  
1045 colorectal cancer found in some epidemiological studies, may result from both the

1046 composition of meat (e.g. heme and protein) and from compounds generated by the  
1047 cooking process (Kim, Coelho, & Blachier, 2013). In support of this hypothesis, a  
1048 recent study has shown that hydrogen sulfide, which is produced by the gut microbiota  
1049 from L-cysteine released from dietary and endogenous protein, can drive mucin  
1050 denaturation and possibly increase the access of heme (a cytotoxic and genotoxic  
1051 compound) to colonic epithelial cells (Ijssennagger, et al., 2015).

1052

### 1053 **6.3. HP diet effects on kidney function**

1054 It was concluded from a systematic review that indoxyl sulfate and *p*-cresyl sulfate  
1055 are toxic for kidneys notably through reactive-oxygen species (ROS) generation  
1056 (Vanholder, Schepers, Pletinck, Nagler, & Glorieux, 2014). Moreover, it has been  
1057 proposed that *p*-cresol level in the blood is an indicator of CKD severity (Bammens,  
1058 Evenepoel, Keuleers, Verbeke, & Vanrenterghem, 2006). Recently, it has been shown  
1059 that CKD alters gut microbiota composition (Vaziri, Wong, et al., 2013) together with  
1060 an increased bacterial metabolic capacity for nitrogen utilization, as evidenced by the  
1061 increased abundance of urease, indole- and *p*-cresol-forming enzymes in CKD patients  
1062 (Wong, et al., 2014). Indeed, *p*-cresyl sulfate (or glucuronide) and indoxyl sulfate are  
1063 known conjugates of the gut microbiota-produced metabolites *p*-cresol and indole,  
1064 respectively, and are elevated in the serum of chronic kidney diseases (CKD) patients  
1065 (Vanholder & Glorieux, 2015). Furthermore, the elevated uremia in CKD induces an  
1066 important diffusion of urea into the intestinal lumen, and it has been shown that elevated  
1067 urea concentration impairs intestinal barrier function and induces inflammation in the  
1068 digestive tract, most likely through excessive ammonia production by bacterial ureases  
1069 (Vaziri, Yuan, & Norris, 2013), raising the view that CKD may impact gut health.  
1070 Those events may play a causative role in the establishment of CKD-associated

1071 dysbiosis. Moreover, CKD patients generally have a lower fiber intake, prolonged  
1072 transit time and protein malabsorption, all of which favor protein fermentation in the gut  
1073 (Evenepoel, et al., 2009).

1074 Moderate protein intake (0.6-0.8 g protein/kg/day) has been demonstrated to be  
1075 beneficial for CKD patients (Fouque, Pelletier, Mafra, & Chauveau, 2011). Reduced  
1076 renal exposure to gut microbiota amino acid-derived metabolites through control of  
1077 protein intake may contribute to better health outcomes. In healthy subjects, HP diets  
1078 increase glomerular filtration rate, but the long-term consequences for kidney health  
1079 remains unclear (Marckmann, Osther, Pedersen, & Jespersen, 2015). Thus, renal  
1080 consequences of increased colonic protein fermentation should be further studied in  
1081 healthy subjects.

1082

#### 1083 **6.4. Strategies to limit toxicity associated with the control of protein fermentation**

1084 Increased fermentable carbohydrate intake is one of the nutritional strategies that  
1085 may help to limit adverse effects of protein fermentation, as demonstrated in an  
1086 intervention study which has shown that dietary resistant starch lowers excretion of  
1087 ammonia and phenols (Birkett, et al., 1996). In healthy subjects, *p*-cresyl sulfate and  
1088 indoxyl sulfate levels are markedly lower in vegetarians than in omnivores, likely  
1089 because of a higher fiber intake and lower protein intake (Patel, Luo, Plummer,  
1090 Hostetter, & Meyer, 2012). In rats, supplementation with resistant starch protects  
1091 against HP diet-induced DNA damages in colonocytes in association with decreased  
1092 excretion of *p*-cresol (Toden, Belobrajdic, Bird, Topping, & Conlon, 2010). Resistant  
1093 carbohydrate supplementation also attenuates renal injury possibly through a decreased  
1094 production of amino acid-derived metabolites by the microbiota (Vaziri, et al., 2014). In  
1095 CKD patients, improved disease markers (including lower *p*-cresyl sulfate) were

1096 observed after resistant starch supplementation (Vanholder & Glorieux, 2015).  
1097 Unfortunately, CKD patients are advised to restrict their fruit and vegetable intake to  
1098 avoid potassium overload, thus excluding one of the main fiber sources. In that context,  
1099 the use of fibers may be an efficient tool to reduce protein fermentation (De Preter,  
1100 Hamer, Windey, & Verbeke, 2011). This has been demonstrated in human studies with  
1101 isotope-labeled biomarkers showing that fiber (lactulose) efficiently lowered ammonia  
1102 and *p*-cresol production by the gut microbiota (De Preter, et al., 2004).

1103 Consumption of specific probiotic bacteria belonging to the genera *Lactobacillus* and  
1104 *Bifidobacterium* has also been shown to decrease urinary *p*-cresol although to a lesser  
1105 extent than with prebiotics, and with no effect on ammonia (De Preter, et al., 2004;  
1106 Wutzke, Lotz, & Zipprich, 2010). In CKD patients, some probiotic bacteria used to  
1107 reduce the formation of gut-derived toxins have been investigated, but the level of  
1108 evidence remains low (Rossi, et al., 2014). Combining pre- and probiotics (known as  
1109 “synbiotics”) has also been considered an attractive approach, but requires further  
1110 clinical investigation (De Preter, et al., 2007).

1111 Other strategies such as elementary nutrition (free amino acid supplementation) or  
1112 the use of substrates that bind deleterious compounds derived from amino-acid  
1113 fermentation (e.g. zinc that binds H<sub>2</sub>S) have been proposed to minimize their effects in  
1114 medical conditions (e.g. CKD patients) (Mimoun, et al., 2012), but are out of the scope  
1115 of this review.

1116

1117

## 1118 **7. Conclusion and perspectives.**

1119 Dietary protein metabolism is the result of the interplay between host and gut  
1120 bacterial metabolic pathways but their respective roles and contributions to host

1121 physiology and metabolic health remain undefined. Several gut microbiota-derived  
1122 amino acid metabolites may theoretically have both beneficial and deleterious  
1123 physiological consequences on host cells and tissues, but so far their effects have only  
1124 been tested individually (i.e. in un-physiological conditions). The use of global-scale  
1125 omics technologies in tightly-controlled intervention studies will help to identify the  
1126 specific gut microbial metabolic pathways activated in response to dietary protein and  
1127 to disentangle the flow of metabolites between the host and the gut microbiota and their  
1128 contribution to host physiology. Moreover, since human diets consist of a wide range of  
1129 different protein sources (plant and animal) with different characteristics (digestibility,  
1130 amino acid composition); these differences also need to be taken into consideration for  
1131 interpretation of metadata obtained in the context of a varied diet. These future studies  
1132 will represent an important and necessary step for a better understanding of the complex  
1133 interplay between diet, microbiota and host metabolism and physiology and will  
1134 contribute to informing microbiome-based dietary recommendations.

1135

1136

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1140

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1897 **Figure Captions**

1898 **Figure 1: Prokaryotic biosynthetic enzymatic pathways of (A) glutamate and (B)**  
1899 **serine amino acid families.** Enzymes with associated gene names compiled from the  
1900 KEGG database are provided for each step in reaction pathways. Gene names include  
1901 orthologous and paralogous gene groups among different bacterial taxa. Intermediate  
1902 metabolites (yellow boxes) from the most common reaction pathways are provided for  
1903 each end product amino acid (blue boxes) from each enzymatic family.

1904

1905 **Figure 2: Prokaryotic biosynthetic enzymatic pathways of the aspartic acid amino**  
1906 **acid family.** Enzymes with associated gene names compiled from the KEGG database  
1907 are provided for each step in reaction pathways. Gene names include orthologous and  
1908 paralogous gene groups among different bacterial taxa. Intermediate metabolites  
1909 (yellow boxes) from the most common reaction pathways are provided for each end  
1910 product amino acid (blue boxes) from each enzymatic family.

1911

1912 **Figure 3: Prokaryotic biosynthetic enzymatic pathways of the pyruvate amino acid**  
1913 **family.** Enzymes with associated gene names compiled from the KEGG database are  
1914 provided for each step in reaction pathways. Gene names include orthologous and  
1915 paralogous gene groups among different bacterial taxa. Intermediate metabolites  
1916 (yellow boxes) from the most common reaction pathways are provided for each end  
1917 product amino acid (blue boxes) from each enzymatic family.

1918

1919 **Figure 4: Prokaryotic biosynthetic enzymatic pathways of the aromatic acid amino**  
1920 **acid family.** Enzymes with associated gene names compiled from the KEGG database  
1921 are provided for each step in reaction pathways. Gene names include orthologous and

1922 paralogous gene groups among different bacterial taxa. Intermediate metabolites  
1923 (yellow boxes) from the most common reaction pathways are provided for each end  
1924 product amino acid (blue boxes) from each enzymatic family.

1925

1926 **Figure 5: Prokaryotic biosynthetic enzymatic pathway of histidine.** Enzymes with  
1927 associated gene names compiled from the KEGG database are provided for each step in  
1928 reaction pathways. Gene names include orthologous and paralogous gene groups among  
1929 different bacterial taxa. Intermediate metabolites (yellow boxes) from the most common  
1930 reaction pathways are provided for each end product amino acid (blue boxes) from each  
1931 enzymatic family.

1932

1933 **Figure 6: High protein diet effects with potential involvement of gut microbiota**  
1934 **metabolism.** Left side: Peptides are degraded by the intestinal microbiota which release  
1935 amino acid derived metabolites in the lumen. Gut bacteria can also produce amino  
1936 acids. Right side: Host-functions affected by a high-protein diet demonstrated in human  
1937 and animal studies (capital letters and bold). High protein diets increase the amount of  
1938 protein available for fermentation by gut microbiota, which may mediate some of the  
1939 effects of high protein diets on host physiology. Candidate bacterial amino acid derived  
1940 metabolites for each effect of high protein diet are proposed. (*Illustrations are from*  
1941 *Servier Medical Art*)

1942

1943 **Table 1: Gut microbiota-produced metabolites from amino acid catabolism that**  
1944 **have potential effects on host physiology and health.** Genetic pathways for each  
1945 metabolite are described along with enzymes and respective genes for each step in  
1946 metabolic pathways. Gene names identified in the bacterium *E. coli* are highlighted in

1947 bold. Gene names include orthologous and paralogous gene groups among different  
1948 bacterial taxa. All gene names are compiled from KEGG database except where noted.  
1949 Amino acid abbreviations refer to glutamate (Glu); tryptophan (Tryp); histidine (His);  
1950 tyrosine (Tyr); arginine (Arg); phenylalanine (Phe); cysteine (Cys); methionine (Met);  
1951 lysine (Lys).



Metabolite	Amino Acid	Step	Substrate(s)	Product(s)	Enzyme (Enzyme Commission Number)	Gene(s)
<b><u>Neuroactive compounds</u></b>						
GABA	Glut	1	L-Glutamate	$\gamma$ -Aminobutanoate, CO <sub>2</sub>	Glutamate decarboxylase (4.1.1.15)	<i>gadA, gadB</i>
Serotonin (5-hydroxytryptamine)	Tryp	1	L-Tryptophan, Tetrahydrobiopterin, O <sub>2</sub>	5-Hydroxy-L-tryptophan, Dihydrobiopterin, H <sub>2</sub> O	Tryptophan 5-monooxygenase (1.14.16.4)	No genes found in prokaryotes
		2	5-Hydroxy-L-tryptophan	Serotonin, CO <sub>2</sub>	Aromatic-L-amino-acid decarboxylase (4.1.1.28)	<i>ddc, dcd, dcd1</i>
Histamine (pathway 1)	His	1	L-Histidine	Histamine, CO <sub>2</sub>	Histidine decarboxylase (4.1.1.22)	<i>hdc, hdcA, angH, pmsA, basG, dchS, vlmD</i>
Histamine (pathway 2)	His	1	L-Histidine	Histamine, CO <sub>2</sub>	Aromatic-L-amino-acid decarboxylase (4.1.1.28)	<i>ddc, dcd1</i>
L-DOPA	Tyr	1	L-Tyrosine, O <sub>2</sub>	3,4-Dihydroxy-L-phenylalanine, (DOPA), H <sub>2</sub> O	Tyrosinase (1.14.18.1)	<i>melO, melC1, melC2</i>
		2	2 N( $\omega$ )-Hydroxyarginine, NADH, H <sup>+</sup>	2 Nitric oxide, 2 L-Citrulline,	Nitric-oxide synthase	<i>nos, nosA, yflM, rplB,</i>

			O <sub>2</sub>	NAD <sup>+</sup> , 2 H <sub>2</sub> O	(1.14.13.165)	<i>sdaAB, txtD</i>
Tyramine (pathway 1)	Tyr	1	L-Tyrosine	Tyramine, CO <sub>2</sub>	Tyrosine decarboxylase (4.1.1.25)	<i>tyrDC, tdcA, mfnA, mfmA, gadD, gadB</i>
Tyramine (pathway 2)	Tyr	1	L-Tyrosine	Tyramine, CO <sub>2</sub>	Aromatic-L-amino-acid decarboxylase (4.1.1.28)	<i>ddc, dcd, dcd1</i>
Nitric Oxide	Arg	1	L-Arginine, NADH (or NADPH), H <sup>+</sup> , O <sub>2</sub>	N(ω)-Hydroxyarginine, NAD <sup>+</sup> (or NADP <sup>+</sup> ), H <sub>2</sub> O	Nitric-oxide synthase (1.14.13.165)	<i>nos, nosA, yflM, rplB, sdaAB, txtD</i>
Tryptamine	Tryp	1	L-Tryptophan	Tryptamine, CO <sub>2</sub>	Aromatic-L-amino-acid decarboxylase (4.1.1.28)	<i>dcd, dcd1, ddc</i>
Phenylethylamine	Phe	1	L-Phenylalanine	Phenethylamine, CO <sub>2</sub>	Aromatic-L-amino-acid decarboxylase (4.1.1.28)	<i>dcd, dcd1, ddc, tyrDC</i>
<b><u>Sulfide-containing metabolites</u></b>						
H <sub>2</sub> S (pathway 1)	Cys	1	L-Cysteine, H <sub>2</sub> O	H <sub>2</sub> S, Pyruvate, Ammonia	Cysteine desulfhydrase (4.4.1.1)	<i>cysA, metB, metC, yrhB, mceB,</i>
H <sub>2</sub> S (pathway 2)	Cys	1	L-Cysteine, H <sub>2</sub> O	H <sub>2</sub> S, Pyruvate, Ammonia	Cystathionine beta-lyase	<b><i>metC, metB, malY, patB</i></b>

Methanethiol	Met	1	L-Methionine, H <sub>2</sub> O	Methanethiol, Ammonia, 2-Oxobutanoate	(4.4.1.8) Methionine-gamma-lyase (4.4.1.11)	<i>mdeA, megL, metB, metZ</i>
<b><u>Aromatic compounds</u></b>						
Phenol	Tyr	1	L-Tyrosine, H <sub>2</sub> O	Phenol, Pyruvate, Ammonia	Tyrosine phenol-lyase (4.1.99.2)	<i>tpl</i>
<i>p</i> -cresol	Tyr	1	L-Tyrosine	unknown	unknown	Pathway unknown (Dawson et al., 2011)
		2	unknown	unknown	unknown	
		3	4-hydroxyphenylacetate, H <sup>+</sup>	4-methylphenol ( <i>p</i> -cresol), CO <sub>2</sub>	4-hydroxyphenylacetate decarboxylase (4.1.1.83)	<i>hpdB, hpdC, hpdA</i> (Yu et al., 2006)
Indole	Tryp	1	L-Tryptophan, H <sub>2</sub> O	Indole, Pyruvate, Ammonia	Tryptophanase (4.1.99.1)	<i>tnaA</i>
<b><u>Polyamines</u></b>						
Agmatine	Arg	1	L-Arginine	Agmatine, CO <sub>2</sub>	Arginine decarboxylase (4.1.1.19)	<i>speA, adiA, cad, pdaD, aaxB</i>
Putrescine (pathway 1)	Arg	1	L-Arginine	Agmatine, CO <sub>2</sub>	Arginine decarboxylase	<i>speA, adiA, cad, pdaD,</i>

					(4.1.1.19)	<i>aaxB</i>
		2	Agmatine, H <sub>2</sub> O	Putrescine, Urea	Agmatinase (3.5.3.11)	<i>speB</i> , <i>speB1</i> , <i>speB2</i> , <i>pah</i> , <i>gbh</i>
Putrescine (pathway 2)	Arg	1	L-Arginine	Agmatine, CO <sub>2</sub>	Arginine decarboxylase (4.1.1.19)	<i>speA</i> , <i>adiA</i> , <i>cad</i> , <i>pdaD</i> , <i>aaxB</i>
		2	Agmatine, H <sub>2</sub> O	N-Carbamoylputrescine, Ammonia	Agmatine deiminase (3.5.3.12)	<i>aguA</i> , <i>aguA1</i> , <i>aguA2</i>
		3	N-Carbamoylputrescine, H <sub>2</sub> O	Putrescine, CO <sub>2</sub> , Ammonia	N-carbamoylputrescine amidase (3.5.1.53)	<i>aguB</i>
Putrescine (pathway 3)	Arg	1	L-Arginine, H <sub>2</sub> O	L-Ornithine, Urea	Arginase (3.5.3.1)	<i>rocF</i>
		2	L-Ornithine	Putrescine, CO <sub>2</sub>	Ornithine decarboxylase (4.1.1.17)	<i>speC</i> , <i>speF</i>
Spermidine (pathway 1)	Arg	1	S-Adenosylmethioninamine, putrescine	5'-Methylthioadenosine, Spermidine	Spermidine synthase (2.5.1.16)	<i>speE</i>
Spermidine (pathway 2)	Arg	1	L-aspartate 4-semialdehyde, putrescine, NADPH, H <sup>+</sup>	carboxyspermidine, H <sub>2</sub> O, NADP <sup>+</sup>	Carboxynorspermidine synthase (1.5.1.43)	<i>cansdh</i>
		2	carboxyspermidine	spermidine, CO <sub>2</sub>	Carboxynorspermidine decarboxylase (4.1.1.96)	<i>nspC</i> , <i>cansdc</i>

Spermine	Arg	1	S-Adenosylmethioninamine, Spermidine	5'-Methylthioadenosine, Spermine	Spermine synthase (2.5.1.22)	SMS (not found in prokaryotes)
Cadaverine	Lys	1	L-Lysine	Cadaverine, CO <sub>2</sub>	Lysine decarboxylase (4.1.1.18)	<i>ldcC, cadA, speA, cad</i>

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