Invited review

Intestinal luminal nitrogen metabolism: Role of the gut microbiota and consequences for the host

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ABSTRACT

Alimentary and endogenous proteins are mixed in the small intestinal lumen with the microbiota. Although experimental evidences suggest that the intestinal microbiota is able to incorporate and degrade some of the available amino acids, it appears that the microbiota is also able to synthesize amino acids raising the view that amino acid exchange between the microbiota and host can proceed in both directions. Although the net result of such exchanges remains to be determined, it is likely that a significant part of the amino acids recovered from the alimentary proteins are used by the microbiota. In the large intestine, where the density of bacteria is much higher than in the small intestine and the transit time much longer, the residual undigested luminal proteins and peptides can be degraded in amino acids by the microbiota. These amino acids cannot be absorbed to a significant extent by the colonic epithelium, but are precursors for the synthesis of numerous metabolic end products in reactions made by the microbiota. Among these products, some like short-chain fatty acids and organic acids are energy substrates for the colonic mucosa and several peripheral tissues while others like sulfide and ammonia can affect the energy metabolism of colonic epithelial cells. More work is needed to clarify the overall effects of the intestinal microbiota on nitrogenous compound metabolism and consequences on gut and more generally host health.

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1. Introduction

The gut lumen contains a very complex mixture of compounds from alimentary and endogenous origins together with living microorganisms; collectively named the intestinal microbiota [1] Although the small intestine lumen contains a significant density of living bacteria (with density increasing from the proximal to the distal part of the small intestine), this density is much higher in the large intestine representing as much as 10^{13}–10^{14} cells g^{-1} of luminal content and belonging to thousands of bacterial taxa [2,3].

The intestinal microbiota is metabolically active and plays a significant role for the host physiology and metabolism. Just to give some examples, the intestinal microbiota is responsible for the luminal metabolism of undigested or not totally digested alimentary compounds, is involved in the synthesis of vitamins such as B and K as well as the metabolism of some micronutrients and some endogenous compounds such as bile acids, and in addition can provide resistance towards pathogens and modulate the mucosal immune system of the host [4–6].

In this review, we have focused on the role of microbiota in the small and large intestine for the metabolism of N-containing compounds, and tried to underline the possible consequences of this microbial biochemical activity for the host intestinal physiology and metabolism.

2. An overview of the bacterial utilization of proteins and related nitrogenous compounds

It remains difficult to get an extensive inventory of the pathways involved in the bacterial utilization of the nitrogenous compound since some reactions, especially in the first steps of catabolism, are highly specific according to the different species. Nevertheless, it is possible to give an overview of the general scheme of the bacterial metabolism of proteins and nitrogenous compounds in the gut (Fig. 1).

2.1. Protein hydrolysis

In the first steps of protein catabolism by the intestinal bacteria, these compounds are hydrolyzed by extracellular proteases and peptidases into amino acids and peptides. Provided that specific transporters are present, amino acids and peptides can be taken up into bacterial cells [7]. Then they undergo different fates which may be different according to physiological conditions.

2.2. Fate of amino acids

Amino acids can be directly incorporated into bacterial cells as building blocks of proteins.

They can also enter catabolic pathways. One of the major first steps is transamination or deamination that can be oxidative, reductive or coupled (*i.e.* Stickland reaction). The Stickland reaction is effective in most of the proteolytic clostridia in colon. This reaction involves a pair of amino acids, one of them being oxidized and decarboxylated (by oxidative deamination and decarboxylation) and the other one being reduced. The preferred H-donors are alanine, leucine, isoleucine, valine and histidine while the preferred H-acceptors are glycine, proline, ornithine, arginine and trytophane. In most cases, all these reactions yield the corresponding keto acids or saturated fatty acids that are related to central intermediates that can be easily degraded [7]. Many anaerobic bacteria metabolize them through the fermentation pathways where pyruvate is an important starting point followed by a series of reactions leading to the excretion of terminal H-acceptors such as short chain fatty acids (mainly acetate, propionate and butyrate), organic acids (mainly formate, lactate and succinate), ethanol and gazes (mainly H_2 and CO_2). Usually organic acids do not accumulate since they are rapidly further metabolized by other bacterial species to short chain fatty acids (SCFA). Primary amines can be deaminated by the same processes as amino acids. A special case is urea that is hydrolyzed in carbon dioxide and ammonia [8]. The ammonia generated by the deamination reactions can be utilized as a
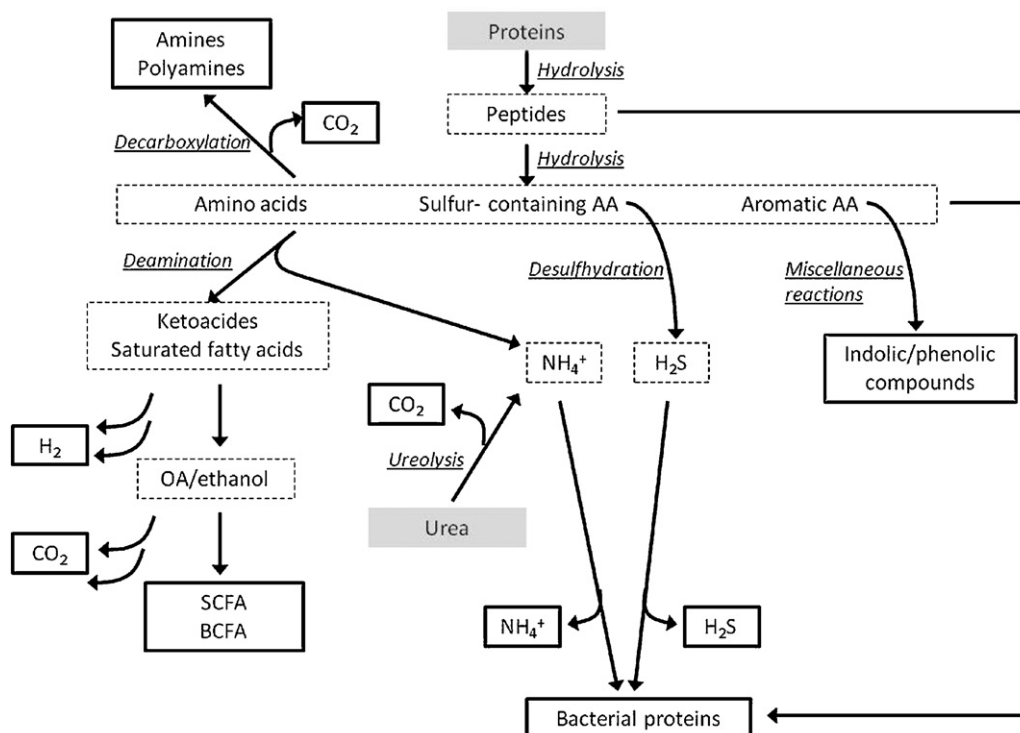

Fig. 1. Outline of pathways of protein metabolism by gut microbiota. ▨ : substrate; ⬚ : intermediary metabolite; ▢ : end product.

nitrogen source or excreted [7,9]. The gazes generated (H_2 and CO_2) can also be consumed by hydrogenotrophic microorganisms (mainly methanogenic archaea, acetogenic bacteria and sulfate reducing bacteria) to generate methane, acetate (by reductive acetogenesis) and hydrogen sulfide [10,11]. Acetate can be used as energy source by different types of epithelial cells and by other intestinal bacteria. Sulfides released by the microbiota can also be further metabolized by colonocytes [12] while methane is a stable end product that is not further metabolized in the gut [10].

Amino acids can also be metabolized *via* decarboxylation leading more or less directly to the production of amines and polyamines. Deaminases and decarboxylases are amino acid-specific enzymes. *In vitro*, the production of amino acids deaminases and decarboxylases are favoured respectively by alkaline and acid pH values. Many of the complex amino acids do not undergo solely these general reactions. Indeed, they are metabolized by a series of reactions including fission, deamination, decarboxylation oxidation and reduction, resulting in the production of a wide variety of metabolic end-products structurally related. Thus tyrosine gives rise to 4-ethylophenol, phenol and *p*-cresol whereas tryptophane results in the production of indole, skatole, kynunerine and onwards [8]. Sulfur-containing amino acids yield to the release of sulfide that can be excreted and utilized by colonocytes as described above or directly incorporated in *de novo*-synthesized amino acids.

2.3. De-novo biosynthesis of amino acids

Bacteria can synthesize *de novo* some if not all of the twenty amino acids required for protein biosynthesis. In fact, the human distal gut microbiome is enriched for a variety of clusters of orthologous groups of genes involved in essential amino acid biosynthesis [2]. The amino acids are formed from metabolic precursors derived from the central metabolism. Pyruvate, oxaloacetate and α-oxoglutarate are the starting point of 13 of the 20 amino acids. Among amino acids glutamate and glutamine occupy a prominent place as they are important intermediates in nitrogen metabolism [7].

2.4. Diversity and abundance of amino acid fermenting bacteria

The ability to metabolize peptides and amino acids is shared by a large number of bacteria ranging from the saccharolytic bacteria to obligate amino acid fermenters in gut microbiota. Culture based enumerations (MNP: most probable number) of five healthy volunteers show that amino acid fermenting bacteria account for $11.5 \log_{10} g^{-1}$ dry weight faeces [13]. Clostridia and peptostreptococci are the most frequent isolates in media containing single amino acids or Stickland pairs as energy and carbon sources. Nevertheless, a wide range of bacteria belonging to the genera *Fusobacterium*, *Bacteroides*, *Propionibacterium*, *Actinomyces*, and also including Gram-positive cocci (*e.g.* *Peptococcus*, *Streptococcus*, *Ruminococcus*, *Megasphaera*) are recovered. Enterobacteria are also cited in other studies as responsible for amino acid metabolism in the gut [14]. Peptides are the preferred substrates for many colonic bacteria probably due to kinetic advantages of peptide-uptake systems in comparison with those for free amino acids. The number of bacteria growing at the expense of alanine, aspartate and tryptophane do not exceed $10^6 g^{-1}$ dry weight faeces. In the last decades, the diversity of amino acid fermenting bacteria has also been evidenced all along the digestive tract in human and animals (ruminant and monogastric animals). Overall, bacteria associated to amino acid fermentation in the small intestine are very similar to those described in faeces [14].

3. Protein metabolism in the small intestinal lumen

Ingested dietary proteins and endogenous proteins are mixed in the small intestine lumen. The endogenous luminal proteins are originating from various sources including gastric and pancreatic secretory products, desquamated intestinal epithelial cells and mucous proteins [15,16]. These proteins are digested in the small intestinal lumen by proteases and peptidases originating from the exocrine pancreas. The resulting peptides can then undergo the process of final digestion through the catalytic activities of numerous peptidases present in enterocytes. Then, oligopeptides and amino acids are transported from the lumen to the portal bloodstream through a variety of transporters present in the brush border and baso-lateral membranes of the enterocytes [17,18]. A significant part of amino acids are metabolized during their transcellular journey through the absorptive intestinal cells. This metabolism corresponds to both local utilization of amino acids for protein synthesis and to the production of metabolites from several amino acids including for instance arginine [19], proline [20] and cysteine [21]. Some of these metabolites are used in the intestinal mucosa. It corresponds also to the production of metabolites that are used peripherally outside the intestinal mucosa. For instance ornithine and citrulline which are not present in proteins can be synthesized in enterocytes from several amino acids present in proteins and play roles in the interorgan metabolism [22]. In addition, the enterocytes use several amino acids (glutamine, glutamate and aspartate) [23] as fuels in the context of a high energy requirement for the cell renewal in the epithelial layer and for nutrient absorption [24] The first step of glutamine utilization by enterocytes is through the mitochondrial glutaminase which converts glutamine into ammonia and L-glutamate [25]. Some data suggest that the intestinal glutamine utilization is responsible for a significant part of the overall ammonia production in the body [26]. After absorption, the unmetabolized and *de novo* produced amino acids are recovered in the portal vein and captured by the liver where they are partly metabolized or released in the peripheral circulation.

Although it has not been directly measured *in vivo* yet, there are several reasons to consider that the small-intestinal microbiota should be taken into account when considering the metabolism of proteins in the small intestine (Fig. 2). Firstly, when comparing apparent amino acid absorption measured *in vivo* and metabolic capacities of isolated enterocytes towards amino acids measured *in vitro*, it appears that the microbiota is likely to participate in the significant catabolism of some indispensable amino acids in the small intestine lumen. For instance, lysine is very little, if not at all, oxidized in enterocytes isolated from piglets [27]. However, in milk-fed piglets, when expressed as a percentage of the enteral tracer input, there is a substantial first-pass intestinal metabolism of lysine (35%) [28]; of which only 18% being recovered in the intestinal mucosal proteins. As lysine catabolism in the intestinal mucosa is quantitatively greater than the amino acid incorporation into mucosal proteins, despite the low capacity of the enterocytes for lysine catabolism, it is tempting to propose that the intestinal microbiota [29] and/or other cell types than enterocytes in the intestinal mucosa are able to use lysine. Regarding this latter point, it is noteworthy that the intestinal mucosa contains a large number of immune cells in which amino acid catabolism appears to play a critical role in both innate and adaptive immunity in close relationship with the modulation of the gut barrier function [30]. The same reasoning can be made for other indispensable amino acids like methionine and phenylalanine which, although virtually not catabolized in piglet isolated enterocytes [27], appear to be utilized by the intestine and maybe partly by the microbiota. Indeed, in piglets, the net portal balance of methionine represents 48% of intake, suggesting that a part of the available methionine is consumed in the intestine [28]. Accordingly, the parenteral methionine requirement
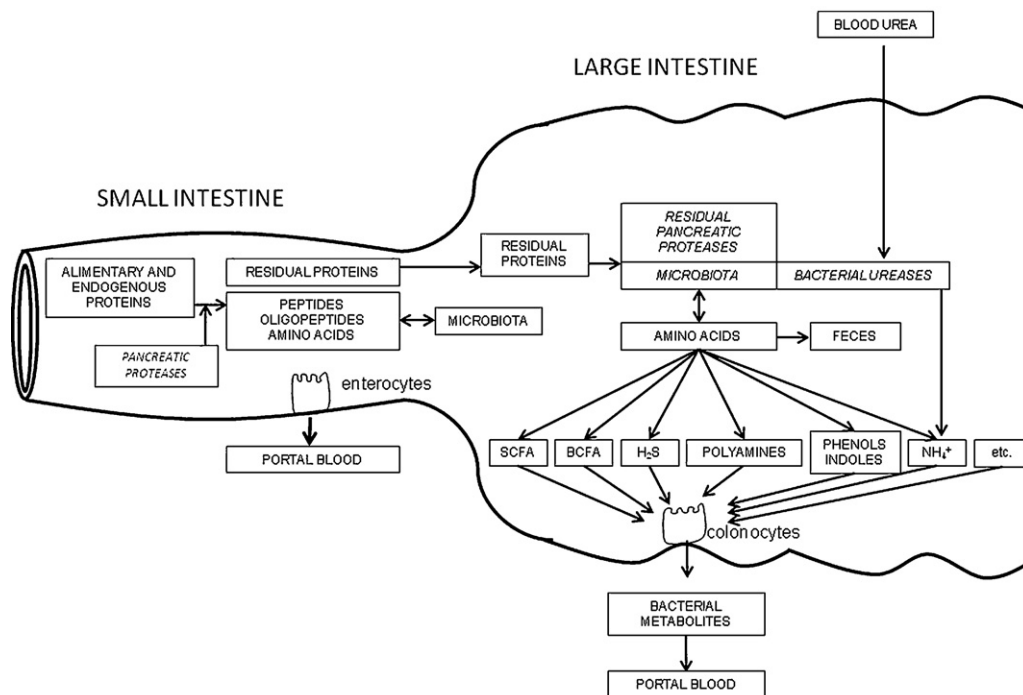
Fig. 2. Schematic view of the overall metabolism of luminal proteins in the small and large intestine. Alimentary and endogenous luminal proteins undergo the activities of exocrine pancreatic proteases which release peptides, oligopeptides and amino acids. The terminal digestion is occurring in enterocytes through the activities of peptidases. These small intestine epithelial cells are equipped to transport amino acids and oligopeptides and partially metabolize them during their transcellular journey from the lumen to the portal bloodstream. From the available data, it appears that the small intestine microbiota is likely to use amino acids but also to synthesize some of them. The net consequences of the microbial amino acid metabolism on the host and intestinal microbiota remain to be determined. Undigested residual luminal proteins can be transferred from the small to the large intestine and undergo the action of the microbiota and of the residual pancreatic proteases. The amino acids can then be metabolized by the microbiota producing a complex mixture of metabolic end products like short-chain fatty acids (SCFA), branched-chain fatty acids (BCFA), hydrogen sulfide (H_2S), polyamines, phenols, indoles, ammonium (NH_4^+), and many others metabolites which remain to be identified. Ammonium can also be produced from urea through the action of the bacterial ureases. Some of these bacterial metabolites can be transported inside colonocytes and exert both beneficial and deleterious effects on these epithelial cells depending on their luminal concentrations. Some of these latter are released in the portal blood and can exert various effects on the liver and in peripheral organs and tissues.

is approximately 70% of the enteral requirement in neonatal piglets [31]. The piglet gastrointestinal tract was reported to consume approximately 20% of the dietary methionine [32]. Regarding phenylalanine, it has been determined in milk-fed piglets that there is a marked first-pass metabolism of this amino acid (35% expressed as a percentage of the enteral tracer), of which only 18% is recovered in mucosal proteins [28] The situation of threonine is somewhat different. The metabolic capacity of pig isolated enterocytes for threonine catabolism is virtually absent [27]. The mucins from pig intestine are glycoproteins very rich in threonine [33]; a fact which likely partly explains that in the piglet model, the threonine requirement during total parenteral nutrition is approximately 45% of the mean enteral requirement [34]. In growing pigs, the catabolism through the threonine dehydrogenase pathway does not account for the relatively high first pass extraction rate of dietary threonine by the portal-drained viscera (which includes stomach, small and large intestines, pancreas and spleen) [35]. In piglets, although the absolute amounts of systemic and dietary threonine utilized by the portal-drained viscera was reduced in protein-restricted diet, the percentage of dietary threonine intake used by the portal-drained viscera did not differ much between groups (normal-protein diet *vs* low-protein diet), with a measured value being above 85% [36]. As expected, luminal, rather than systemic threonine, is preferentially utilized for protein synthesis in the piglet mucosa. Thus, the portal-drained viscera requirement for threonine is high and the high rate of utilization by the piglet mucosa is largely due to the incorporation of this amino acid into the proteins of the mucosa [36] although the utilization of this amino acid by the intestinal microbiota *in vivo* cannot be excluded and remains likely. The metabolic fate of branched-chain amino

acids (BCAA) (*i.e.* leucine, isoleucine and valine) in the intestine is also of interest. In milk-fed piglets, 32% of leucine in the diet is extracted by the portal-drained viscera in the first pass; with 21% of the extracted leucine being utilized for protein synthesis in the intestinal mucosal [28]. Overall, it has been estimated that 44% of total BCAAs are extracted by first-pass splanchnic metabolism in neonatal piglets [37]. The catabolism of BCAAs in enterocytes isolated from developing piglets has been studied. In these cells, BCAAs are extensively transaminated and between 15 and 50% of the decarboxylated branched-chain alpha-ketoacids are oxidized depending on the age of piglets[27]. Enterocytes isolated from post-weaning pigs also actively degrade BCAAs [38] raising the view that a part of the intestinal utilization of BCAAs originates from their catabolism in enterocytes. From these overall and indirect arguments, it can be proposed that indispensable and dispensable luminal amino acids are utilized by the intestinal microbiota. However, the relative participation of the enterocytes (and/or other cells present in the lamina propria) and the intestinal microbiota in this process remains to be determined.

The dietary and endogenous proteins are likely to provide amino acids for microbiota than can be used for protein synthesis, generation of metabolic energy and recycling of reduced co-factors. It also appears that the microbiota is able to provide amino acids to the host for protein synthesis, raising the view that the amino acid exchange between the microbiota and the host can proceed in both directions. From previous studies measuring the contribution of microbial amino acids to amino acid homeostasis in the host, it appears that the microbiota is indeed able to provide amino acids to the host [39] For instance, authors [40] have examined the ability of germ-free and conventional rats to incorporate ^{15}N from

[15]NH$_4$Cl into body lysine (an amino acid which is not transaminated in mammalian tissues) to establish whether or not the ^{15}N enrichment found in the lysine was due to absorption of lysine synthesized by the intestinal microbiota. The authors concluded that all the ^{15}N-lysine measured in the host was from microbiota origin. The site of lysine absorption was studied by returning the ^{15}N-labeled digesta into the ileum of unlabeled pigs [41], allowing to determine that 75% or more of the total lysine produced by the microbiota was absorbed in the small intestine. On the other hand, when [15]NH$_4$Cl is given *per os*, ^{15}N-labeled ammonia is likely to be rapidly incorporated into microbial amino acids [42]. In the pig model, it appears that lysine produced by the microbiota is mainly used for protein synthesis in the splanchnic area *i.e.* intestine and liver [43]. In human infants, it has been determined that amino acids in plasma can derive from urea after hydrolysis and utilization of nitrogen by the intestinal microbiota [44] although the mechanisms by which amino acids synthetized by the microbiota enter the systemic amino acid pool remained to be determined. The metabolic fate of ^{15}N when given as urea or ammonia to human volunteers was also investigated showing that threonine from intestinal microbiota origin appears in the blood plasma [42] even if this contribution to the whole body threonine metabolism could not be estimated in this latter study. In fact, threonine biosynthetic genes have been identified in the core of functional genes present in the human gut microbiome recently [45].

Another important parameter which needs to be taken into account is the ileal losses of nitrogen and amino acids since this parameter is necessary for determining the amino acid requirements. Endogenous losses of amino acids in humans have been characterized in ileostomized volunteers receiving a protein-free diet [46]. In this study, the authors found that between 14 and 61% of the current indispensable amino acid requirement were lost in the ileostomy fluid. Authors [47] have determined in humans that after the ingestion of a protein-containing meal, significant amounts of both indispensable and dispensable amino acids are lost in the ileal effluents even if it remains unclear if these amino acids are partly used and/or absorbed by the large intestine mucosa and/or used by the microbiota. As pointed out in 2008 [48], a high ileal digestibility of proteins is relevant for reducing the amount of endogenous and alimentary proteins and peptides entering the large intestine.

In pigs fed with milk-protein, 30–60% of essential amino acids are detected in portal blood depending on the amino acid that is considered with only 10–20% being recovered in mucosal proteins [28]. Importantly, it is known that bacteria substantially assimilate or catabolize proteins and amino acids [49–51] in the small intestine. A recent study by [49], using isotope dilution technique after continuous infusion, reported on the contribution of urea, endogenous protein and dietary protein for the utilization of valine for the intestinal microbiota protein synthesis in growing pigs. They show that in normally nourished pigs, more than 90% of the microbial valine originates from preformed amino acids in dietary and endogenous proteins. Dietary proteins account for approximately 70% and 20% of microbial valine in ileal digesta and ileal mucosa respectively. The authors also determined that in the same conditions, about 70% of the ammonia in the ileal digesta was generated by microbial fermentation of proteins; while about 30% came from urea hydrolysis. Interestingly, the ability of small-intestinal bacteria to metabolize free amino acids was also evidenced *in vitro* [50] in physiological conditions [51]. In this latter work, single free amino acids in millimolar concentrations were metabolized within 3 h by monocultures of small-intestinal bacteria (*Escherichia coli*, *Klebsiella* sp., *Streptococcus* sp.) or mixed bacterial cultures derived from jejunal and ileal samples of growing pigs. Threonine, glutamine, arginine, lysine, and leucine were rapidly and extensively metabolized. Oxidation to CO$_2$ accounts for less than 10% of the amino

acid utilization. Protein synthesis and other metabolic routes (*e.g.* SCFA and polyamines production) are the main metabolic fates for amino acids. Nevertheless the rates and metabolic fate of amino acids were dependent on the specific bacterial species and their compartment in the gut. With the same *in vitro* model, the authors described the potential regulatory role of glutamine and arginine for amino acids utilization by the small-intestinal bacteria [52,53]. In agreement with these results, the analysis of the genome of *Lactobacillus johnsonii*, a common inhabitant of the small intestine, shows that this microorganism completely lacks the genes encoding for the biosynthetic pathways involved in amino acid production [54]. In addition, this bacteria does not appear to assimilate ammonium, nor to possess the metabolic pathways involved in sulfur assimilation. In contrast, *L. johnsonii* displays an extracellular protease, three oligopeptide transporters, more than 25 cytoplasmic peptidases and about 20 amino acid-permease type transporters. These characteristics strongly suggest that *L. johnsonii* depends on exogenous amino acids and/or peptide supply for protein synthesis. However, it is obviously not possible to generalize these observations to all other bacterial populations present in the small intestine.

Taken together these results suggest that in normally nourished host, *de novo* synthesis of amino acids from microorganisms scarcely contributes to subsequent host amino acid supply. Indeed, small-intestinal microbiota might play an important role in first pass metabolism of amino acids and nitrogen recycling in the small intestine. From the limited information available regarding amino acid metabolism by the microbiota, it is only fair to say that the net result of amino acid utilization and production by the intestinal microbiota remains to be deciphered (Fig. 2). Lastly, in future works, it may be useful to take into account major differences between the proximal and distal parts of the small intestine. Indeed, if in the duodenum/jejunum, the luminal concentrations of proteins, peptides and amino acids are relatively high [55] and bacterial concentrations are relatively low, it is exactly the opposite in the ileum.

4. Protein metabolism in the large intestinal lumen. Production of bacterial metabolites and consequences for the host

Alimentary protein digestion followed by amino acid and peptide absorption through the small intestinal epithelium can be considered as an efficient process [56–60]. Nevertheless, substantial amounts of nitrogenous compounds from both alimentary and endogenous origins can enter the large intestine through the ileocaecal junction [61]. Indeed, even highly digestible proteins may partly escape digestion in the small intestinal lumen [62] and substantial quantities of nitrogenous material are transferred from small intestine to large intestine lumen [63–65]. This nitrogenous material, which consists mainly in proteins and peptides [66], undergoes proteolysis by the large intestine microbiota and residual pancreatic proteases resulting in peptide and amino acid release followed by the production of a multitude of bacterial metabolites, some of them representing intermediary metabolites and others representing end-products [14] (Fig. 2). Although some amino acids are detected at millimolar concentration in the human colonic content, most of them are present at concentration below 0.01 mM [13]. The undigested and partially digested proteins from both alimentary and endogenous sources (*e.g.* pancreatic secretory products, desquamated enterocytes, mucous proteins [15]) enter the caecum and then are transferred to the ascending, to the transverse and descending colon before reaching the sigmoid colon and rectum [67]. In contrast to its rapid passage in the small intestine, the transit of the luminal material in the large intestine is considerably slower coinciding with the co-existence of a large

population of bacteria [68]. A longer transit time in the large intestine has been associated with a more extensive proteinaceous substrate breakdown [69].

Surprisingly proteins and peptides breakdown by colonic microbiota has been scarcely studied. Yet bacteria commonly utilize proteins as nitrogen, carbon and energy sources. Little is known about the eco-physiological significance of these phenomena in gut even though they are favoured in colonic conditions by the rarefaction of readily fermentable substrates (carbohydrates) from the proximal colon [70]. The amino acid-derived bacterial metabolites have been only partially characterized, and most of them have not been tested for their effects upon colonic epithelial cells.

4.1. Hydrolysis of proteins and peptides in the large intestine. The fate of amino acids

Bowel is a site of heavy proteolytic activity, largely mediated by the microbiota. Around 12–18 g proteins enter the large bowel every day, which corresponds to 2–3 g nitrogen per day, with 10–15% of urea, ammonia, nitrate and amino acids, 48–51% proteins and 34–42% peptides [66]. Ammonia concentration has been continuously found in millimolar concentration in a relatively large range (from 3 mM to 44 mM) [71]. By means of ^{15}N perfusion experiments, Wrong et al. [72] showed that the ammonium does not originate from the host urease activity but only from the proteolytic and microbial urease activities. The fact that proteases are more active at neutral or slightly alkaline pH than more acidic pH [73], together with the fact that the luminal pH is more acid in the ascending than in the descending colon, may partly explain the higher protein degradation in the distal than in the proximal part of the large intestine [74].

The proteolytic activity in large intestine has been mainly attributed to the genera *Bacteroides*, *Clostridium*, *Propionibacterium*, *Fusobacterium*, *Streptococcus*, and *Lactobacillus* [74]. *Bacteroides* spp. are known to secrete proteases in the intestinal with presumed activity near the brush border of absorptive cells. These proteases do act like elastase and when they are abundantly secreted (for instance in the case of *Bacteroides* species overgrowth), may degrade maltase and sucrase enzymes in the enterocyte brush borders. Surprisingly, these proteases do not affect the alkaline phosphatase activity. Thus they have specific proteolytic activities [75]. More recent studies describe the participation of microbial proteases in inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS). In such pathological conditions, potentially pathogenic bacteria such as *Staphylococcus aureus*, *Clostridium difficile*, *Clostridium sordelii*, *Clostridium perfringens* enterohaemorrhagic *E. coli*, enterotoxigenic *E. coli*, *Helicobacter pylori* and *Bacteroides fragilis* display proteases as virulence factors targeting the host's epithelium [76,77]. Proteases act in two ways: (i) controlling the quality of unfolded proteins generated in adverse host environment and (ii) controlling the proteolysis of regulatory proteins in response to the alteration of environmental factors. The case of the Lon proteases of members of the Firmicutes phylum (*S. aureus*, *Listeria monocytogenes*) represents a good example of such actions of bacterial proteases [78].

It is generally considered that colonic luminal amino acids are not significantly absorbed by the colonic epithelium except in a relatively short period of time after birth [79]. Then, this would mean that amino acids generated from non digested alimentary and endogenous protein (and not metabolized by the microbiota) would be lost for protein synthesis in the body. The same reasoning can be made regarding the net production of amino acids by the colonic microbiota that would also be lost for the host if not transported inside colonocytes. In such a case, the amino acids necessary for colonocyte metabolism would then be available exclusively from the arterial blood. Colonic epithelial cells

possess the capacity for degrading several amino acids including arginine [80] which is catabolized into ornithine and nitric oxide; and glutamine [81] which is used as a fuel substrate. Interestingly, increasing the level of dietary protein intake in rats is associated with an increased capacity of colonic epithelial cell to convert arginine into ornithine and urea [82] a phenomenon that may be related to an elevated requirement of ornithine in the liver urea cycle needed for the elimination of increased ammonia concentration in the portal blood. Some evidences suggest however that it is not possible to totally exclude amino acid absorption in the large intestine [6,39]. Indeed, experiments performed on the pig model indicate that after endoluminal injection of proteins or amino acids into the large intestine lumen, an improvement of the whole body N balance is measured which may be tentatively interpreted as an increased amino acid absorption at this site [83]. In addition, some absorption of microbial amino acids from the pig colon was also suggested based on the appearance of ^{15}N-labeled amino acids in the portal venous blood after endoluminal injection of ^{15}N-labeled bacteria into the caecum [84]. As discussed above, recent works have documented that amino acids synthesized by the intestinal microbiota may be absorbed [40,42,44] in the small intestine and/or maybe to a small extent through the large intestine epithelium in humans. The putative absorption of amino acids in the large intestine is supported by the detection of the ATBo+ neutral and cationic amino acid transporter in the luminal membranes of colonocytes [85]. Another neutral and cationic amino acid transporter B+ system is expressed on the apical surface of colonic absorptive cells [86]. However these studies do not prove the possible role of the transporters described in amino acid absorption by colonocytes. The implication of a peptide transporter for amino acid absorption by the colonic epithelium has been proposed [87]. However, in humans, data indicate that the di/tripeptide transporter hPepT1 is not expressed in normal colon but is expressed only in situation of chronic inflammation [88]. It is also possible but not proven, that luminal amino acids would enter colonocytes through the apical transporters, be used in these cells and would thus not appear in the portal blood. In other words, in such situation, luminal amino acids would not be available for the rest of the body but only for protein synthesis and other pathways of amino acid metabolism inside colonocytes.

In contrast, there is a large body of literature indicating that amino acids are intensely metabolized by luminal bacteria, and this represents likely the major fate of amino acids in the large intestine. Genomic and physiologic studies have shown that intestinal microbiota possesses specialized enzymes for the utilization of amino acids [6]. According to the anatomy and physiology of the colon, putrefactive processes become quantitatively more important in the distal bowel [89]. Limited information is however available on the effects of increased ingestion of alimentary proteins on the colonic luminal composition; and on the effects of amino acid-derived metabolites on the colonic epithelial cells and mucosal physiology/physiopathology. However, there is no doubt that, as presented below, several bacterial metabolites derived from amino acids are able, at physiological concentrations, to exert marked effects on colonic epithelial cell metabolism and physiology.

4.2. Ammonia

Ammonia is found at millimolar concentrations in the large intestine lumen [82,90,91], and increasing the amounts of alimentary proteins results in a spectacular increase of the luminal and faecal ammonia [92]. In humans, the ammonia luminal concentration progressively increases from the ascending to the descending colon [93] in accordance with a higher rate of protein fermentation in the distal than in the proximal colon. The two environmental characterics of the proximal colon (low pH and high carbohydrate

availability) explain the reduced net production of ammonia [13]. The luminal ammonia concentration in the large intestine lumen is primarily the net result of the microbiota production through amino acid deamination and urea hydrolysis [94], microbiota utilization of ammonia, and ammonia absorption through the epithelial cells [95–101]. Urea hydrolysis in the intestinal lumen is performed through the microbiota urease activities. Although urease activity in *H. pylori* has been intensely studied, there is little available data regarding urease activities in the large intestine microbiota. Urea transporters are expressed in the colonic mucosa [102] where they are likely to participate in the transfer of urea from the circulation to the intestinal lumen. A recent work suggests that part of ammonia is condensed with L-glutamate through the activity of glutamine synthetase (which is relatively high in colonocytes compared to enterocytes) to allow glutamine synthesis [95]. This likely allows control of ammonia intracellular concentration in colonocytes. Another metabolic pathway allowing ammonia detoxification in these cells is related to the presence of arginase, carbamoylphosphate synthetase I (CPS I) and ornithine carbamoyl transferase (OCT) activities in these cells [82,103]. Ammonia is firstly condensed with bicarbonate allowing carbamoylphosphate synthesis; then carbamoyphosphate is condensed with ornithine to allow the synthesis of the metabolic end product citrulline.

Large amounts of ammonia can be absorbed through the large intestine mucosa [96]. Ammonia has been considered as a metabolic troublemaker since this compound is able to inhibit in a dose-dependent manner the mitochondrial oxygen consumption [104]. In addition, high millimolar concentrations of ammonia inhibit short-chain fatty acid oxidation [105,106] in colonic epithelial cells. In human colonic epithelial cells of adenocarcinoma origin, ammonia at millimolar concentrations increases the volume of vacuolar lysosomes and represses markedly cell proliferation without affecting cell viability [107]. Using the model of colonic adenocarcinoma cells Caco-2, Hughes et al. [108] have shown that ammonia increases paracellular permeability. Endoluminal injection of 75 mM NH_4Cl in the isolated colon for 7 days resulted in a greater number of mitoses per crypt, a result which was interpreted as a compensatory phenomenon for the deleterious effect of ammonia towards colonocytes [109].

4.3. Hydrogen sulfide

Hydrogen sulfide (H_2S) is found at millimolar concentrations in the human colonic luminal content as well as in faeces [93,110,111]. However, since a large part of sulfide is presumed to be bound to luminal compounds, the unbound sulfide in the colonic content is likely to be in the micromolar range [112,113]. H_2S is a bacterial metabolite produced through fermentation of sulfur-containing amino acids (methionine and cysteine), through the reduction of inorganic sulfate and sulfited additives and through the catabolism of intestinal sulfomucins [111,114,115]. Bacteria that are able to derive energy from the carbon chains of cysteine possess specific desulfhydrases (*i.e.* cysteine desulfhydrase) that lead to the production of sulfide [116,117]. In bacteria, methionine can be converted to α-ketobutyrate, ammonia and methanethiol [117,118]. These metabolic capabilities are not attributed to a specific taxonomic group of bacteria and they have been poorly studied specifically for components of the gut microbiota. However, such metabolic capabilities have been evidenced in bacteria like *E. coli*, *Salmonella enterica*, *Clostridium* spp. and *Enterobacter aerogenes* [116,119] which are commonly found in the large intestine. Bacterial groups such as enterococci, enterobacteria, peptostreptococci, fusobacteria and eubacteria are able to ferment sulfur-containing amino acids [117].

Interestingly, there is a correlation between the level of meat intake and the level of faecal excretion of sulfide [110].

Furthermore, an increase in dietary protein leads to an increase in faecal volatile S-containing substances [92]. H_2S at excessive concentrations inhibits colonic epithelial cell respiration [120] and provokes genomic DNA damage [121], [122]. Because of its lipophilic property, H_2S penetrates biological membranes [123] and inhibits the cytochrome c oxidase catalytic activity (the terminal oxidase activity of the mitochondrial electron transport chain), with a binding constant similar to that measured with cyanide [124]; raising the view that hydrogen sulfide is, like ammonia, acting as a metabolic troublemaker towards colonocyte fuel utilization [120]. In contrast, when low micromolar concentrations of sulfide are infused to permeabilized colonocytes, a mitochondrial sulfide oxidation is observed which is maintained as long as the sulfide flux does not exceed the cellular oxidation capacity. This latter result suggests that hydrogen sulfide represents the first mineral fuel for human colonocytes [125]. In colonocytes, the mitochondrial Sulfide Oxidizing Unit (SOU), which is responsible for sulfide detoxification, appears to involve the sulfide quinone reductase (SQR), which oxidizes sulfide and donates electrons to the coenzyme Q in the respiratory chain; and two other enzymes the dioxygenase ETHE1 and the thiosulfate sulfur transferase (TST, also known as one isoenzyme of the rhodanese) which allow the production of thiosulfate [12]. Accordingly, it has been shown that the transfection of CHO cells with a mammalian expression vector containing the cDNA for the human SQRDL gene homologous to the bacterial enzyme, led to an increased capacity for sulfide oxidation [126]. In a recent work, it has been shown that the sulfide oxidative capacity is higher in differentiated than in more proliferative colonic epithelial cells [12]. In these cells, the respiratory capacity and SOU activity appear to represent major determinants allowing sulfide detoxification in colonic epithelial cells. Authors [127] showed that cysteine addition to a simulator of the human intestinal microbial ecosystem (SHIME) inoculated with a representative cultures of human intestinal microbiota increased sulfide concentration and favored the chemical conversion of nitrite to NO. They concluded that dietary reduced sulfur compounds such as sulfur-containing amino acids may contribute to colonocyte damages through excessive NO formation.

4.4. Polyamines

Polyamines (putrescine, spermidine, spermine and their acetylated derivatives) are polycationic molecules which are produced in low amounts by normal colonocytes from the different amino acid precursors (arginine, ornithine and methionine) [103,128]. In these cells, the intracellular content is thus likely mainly derived from the luminal content [129]. In contrast, colonic epithelial cells originating from colonic cancer are characterized by a very high capacity for polyamine synthesis, a situation which is related to the high polyamine requirement of neoplastic cells for continuous mitosis [130].

Colonic bacteria represent a source of polyamines and of other amines since the microbiota is able to produce various compounds including putrescine, agmatine, cadaverine, tyramine and histamine from their respective amino acid precursors that are ornithine/arginine, arginine, lysine, tyrosine and histidine [74,131]. In bacterial cells, polyamines can interact with many cellular polymers or structures. Thus they are involved in a wide variety of cellular reactions that are essential for growth, multiplication and survival. In the past ten years, their role in a series of physiological processes such as the response to physiological stress, the biofilm formation, bacteriocin production and the microbial pathogenesis was also established [132–134]. Microbial polyamine requirements can be satisfied through uptake and *de-novo* synthesis. Intracellular concentrations are widely variable from one micro-organism to another. They are also affected by a series of environmental

factors (*e.g.* pH, oxygen concentration, presence and concentration of the precursor amino acids) as well as by the physiological state of cells (*e.g.* growth phase, cellular stress). In fact, the intracellular concentrations seem to be finely balanced by biosynthesis, degradation, uptake and excretion even if the regulatory mechanisms are not clearly known [133]. The biosynthetic pathways are well established for putrescine, agmatine, cadaverine and spermidine. Spermine *de novo* synthesis is not definitively established in bacteria [132]. In the last decades, polyamine metabolism was extensively studied in *E. coli*, and, more recently it has been deciphered in human pathogens. With regard to colonic ecosystem, several studies demonstrated the *in vitro* ability of representative colonic bacteria to produce polyamines under colonic-type environmental conditions[135]. This is the case for some species of the genera *Bacteroides*, *Lactobacillus*, *Veillonella*, *Bifidobacterium* and *Clostridium*. *In vivo*, urinary excretion of cadaverine (exclusively from bacterial origin) is decreased throughout an antibiotic treatment that eliminates a significant part of the intestinal microbiota which evidences its role in cadaverine production [136]. The concentration of spermidine is increased in the luminal contents of conventional rats when compared to germ-free rats fed the same diet [137]. However there is still scarce information concerning the factors that affect polyamine concentrations and partitioning throughout colon. Obviously one of these factors is microbiota composition as revealed in a study of Noack et al. [138] where the luminal contents of gnotobiotic rats colonized with different associations of bacteria exhibited different polyamine profiles. Nutritional factors also play a role and for example an increase of dietary amino acids at the expense of proteins leads to putrescine and cadaverine decrease respectively in the caecum and colon of early-weaned pigs [139].

Apart from putrescine and agmatine, little is known about the effects of other biogenic amines on the colonic mucosa. Putrescine is strictly necessary for cancerous colonic epithelial cell mitosis [140–142] and agmatine is able to slow down cancerous colonocyte mitosis in *in vitro* experiments [143].

4.5. Indolic and phenolic compounds

The degradation of aromatic amino acids (phenylalanine, tyrosine, tryptophane) by the microbiota produces phenolic and indolic compounds [144–150]. Nevertheless, the metabolism of aromatic amino acids was poorly investigated with regards to role of specific components of the intestinal microbiota. Yet some of the metabolites generated as phenol and indole are suspected to act respectively as co-carcinogen and colon cancer promoter [151]. The main studies were carried by Smith and Macfarlane at the end of the 90s. Anaerobes that are known to ferment aromatic amino acids are among *Bacteroides*, *Lactobacillus*, *Bifidobacterium*, *Clostridium* and *Peptostreptococcus*. MNP (Most Probable Number) counts revealed that 10% of total anaerobes are able to produce indolic and phenolic compounds [148]. Aromatic amino acids are metabolized slowly by bacteria when compared with other amino acids. They yield a series of phenolic and indolic compounds as end-products including *p*-cresol, indole, phenol, skatole (Fig. 3). During human slurries fermentation, tyrosine yields mainly phenol and *p*-cresol; while phenylalanine yields phenylacetate and tryptophane yields indoleacetate and indole [152]. The analysis of the colonic contents of four sudden death victims revealed that indolic compounds could not be detected in such conditions. Phenylacetate and hydroxyphenylpropionate dominate in the proximal colon while phenol is present in trace amounts. In distal colon, these latter metabolites increase more than four fold. If phenol dominates (50% of total metabolites), *p*-cresol and hydroxyphenylpropionate are also present at high levels. Concentrations of phenolic compounds increase markedly in the distal colon, as does the relative proportions of p-cresol and phenol; providing further evidence of the higher amino acid fermentation in the distal region of the large intestine [89]. Concentrations of indolic and phenolic metabolites depend on the balance between the rates of microbial production and colonic absorption. During *in vitro* incubations of proximal bowel contents, the endogenous substrate fermentation resulted in higher rates of production for phenol ($1 \, \mu mol \, h^{-1} \, (g \, gut \, content)^{-1}$). Phenylacetate, phenylpyruvate, phenylpropionate, hydroxyphenylpropionate, *p*-cresol and hydroxyphenylacetate production rates are in the $0.2–0.5 \, \mu mol \, h^{-1} \, (g \, gut \, content)^{-1}$ range. The production rates of phenyllactate, indole and indoleacetate do not exceed $0.1 \, \mu mol \, h^{-1} \, (g \, gut \, content)^{-1}$ [148]. Phenolic compounds appear to be largely absorbed from the colon luminal content. They are partly metabolized during their transfer from lumen to blood and in the liver, before being finally excreted in urine [153]. More than
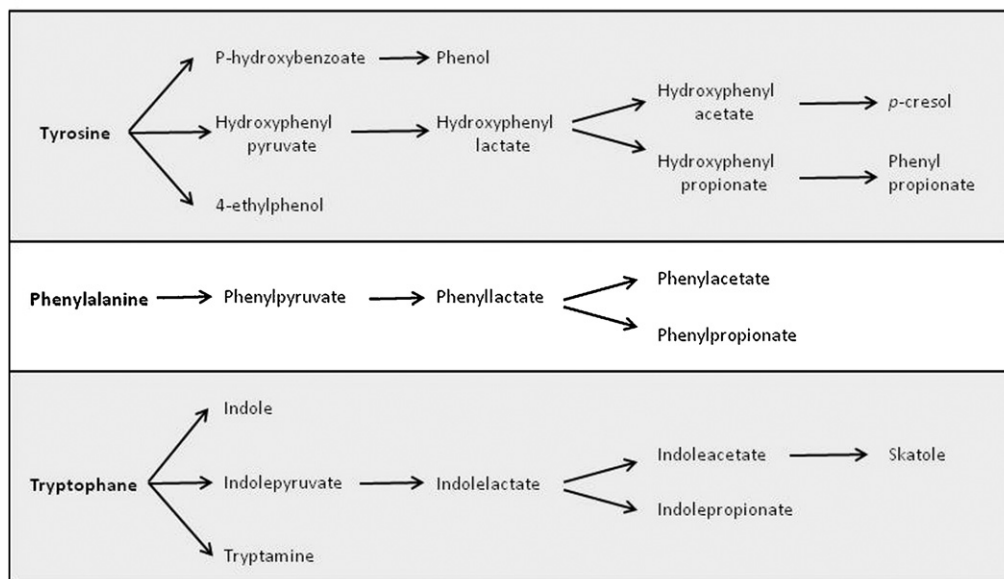

Fig. 3. Schematic diagram of the pathways of aromatic amino acids metabolism by gut microbiota.
Adapted from Smith and Macfarlane, 1996.

90% of urinary phenolic compounds are present as *p*-cresol [145]. A recent comparative analysis of blood plasma metabolites of germ free mice and conventional mice revealed profound significant differences between these two groups of animals [154]. Tryptophan and tyrosine levels are increased by at least 1.5-fold in germ-free mice. The products of aromatic amino acid bacterial metabolism (indoxyl-sulfate, phenyl sulfate, *p*-cresol sulfate, phenylpropionyl-glycine) are exclusively found in conventional mice. Hippuric acid and phenylacetylglycine, that can be processed either by cooperation between the host and gut microbiota, or by the host alone; were increased in conventional mice. According to the available literature, the main environmental parameters affecting aromatic amino acid fermentation are pH, carbohydrates availability as well as the type or availability of the nitrogen sources. Aromatic amino acid fermentation is favored at neutral pH *vs* acidic pH. The concentrations of microbial metabolites and the relative rates of production are both modified when the amount of carbohydrates are changed. Free aromatic amino acids, peptides and proteins can be fermented by the microbiota. However, the amounts and profiles of metabolites depend largely on the nature of the available nitrogenous compounds [117,155]. Interestingly, an increase of the protein intake raises the faecal and urinary concentration of *p*-cresol [92,156]. Recently, authors [157] showed that high protein diet increased phenylacetate concentration. Undoubtedly the composition of microbiota is another prominent parameter affecting the fate of aromatic amino acids in the gut lumen that could account for the great inter-individual variability as highlighted by Smith and Macfarlane [148].

Very little is known regarding the effects of the phenolic and indolic compounds on the colonic epithelial cells. Phenol has been shown to decrease *in vitro* the integrity of the barrier function [108]. Phenol, at concentrations higher than 1.25 mM, impaired the viability of human colonic epithelial cells [158].

4.6. Short chain fatty acids

Short-chain fatty acids (SCFAs), mainly acetate, butyrate and propionate are end products of bacterial fermentation in the large intestine in man and other mammals [159,160]. It is well known that substrates for short-chain fatty acid production are mainly fibers and resistant starch [6]. However, it is largely omitted that undigested proteins represent another important substrate for SCFA production [161].

Indeed, several amino acids released from proteins in the large intestine are precursors for SCFA synthesis. Acetate can be produced by the microbiota from glycine, alanine, threonine, glutamate, lysine and aspartate [162,163]. Butyrate can be produced from glutamate and lysine [74] and propionate can be synthetized from alanine and threonine [164]. The branched-chain fatty acids (BCFA) (isobutyrate, 2-methylbutyrate and isovalerate) are derived from valine, isoleucine and leucine respectively [74]. They are present in lower quantities than SCFA in the large intestine content [165]. They originate exclusively from the breakdown of proteins and are not produced from carbohydrates [166,167] representing therefore good markers of protein breakdown in the intestinal lumen. Thus, five amino acids among the nine indispensable amino acids are used for short-chain and branched-chain fatty acid production by the microbiota.

Culture based enumerations (MNP) performed with faecal sample of five healthy volunteers showed that acetate, propionate and butyrate-producing bacteria (from amino acid fermentation) equated with total amino acid fermenting population that is $11.5 \log_{10} g^{-1}$ dry weight faeces. Forty percent of the total amino acid fermenting population is isobutyrate forming. The valerate, isovalerate and isocaproate forming bacteria are much less represented (respectively $8.5 \log_{10}$; $9.3 \log_{10}$ and $4.8 \log_{10} g^{-1}$ dry weight

faeces) and exhibit a great inter-individual variability [13]. The amount and pattern of SCFAs produced depend largely on substrate availability (which is related to nutritional conditions), on bacterial composition of the microbiota and on the intestinal transit time [168]. In *in vitro* experiments, dissimilatory metabolism of amino acids and peptides was reduced when fermentable carbohydrates were available and when pH was low (5.5 *vs* 6.8). In addition the profile of branched chain fatty acids was qualitatively changed in presence of carbohydrates. These observations explain in large part that dissimilatory metabolism of amino acids is mainly associated with the distal colon [13]. In rats, some of the SCFAs and BCFAs were found to be increased following hyperproteic diet ingestion in rats [104]. However, high protein diet, associated with low carbohydrate content utilized for weight loss purpose in obese men, induced a significant decrease in total short chain fatty acid level in faeces when compared to a maintenance diet. This decrease concerned mainly butyrate and also acetate (respectively 50% and 36% decrease) and to a lesser extent propionate. Simultaneously isovalerate and isobutyrate levels were increased. The consumption of the high protein diet was associated with a sharp decline of the butyrate-producing *Roseburia*/*E. rectale* group [157]. The interpretation of the results on human dietary intervention should take into account that the low carbohydrate content was balanced by both a higher protein and fat content.

The three SCFAs acetate, propionate and butyrate are well known to be oxidized and used as fuels by the colonic epithelial cells. Among these latter, butyrate has been highly studied since, in addition to represent a fuel for colonocytes (which substitutes for L-glutamine and D-glucose oxidation [169]); this SCFA is known to be transported inside colonic epithelial cells and to act on intracellular targets in colonocytes. The part of butyrate which is unmetabolized is recovered in the portal blood [170,171]. Acetate, propionate and butyrate are mainly utilized by muscles, liver and colonic mucosa respectively [172]. Much less is known regarding metabolism and effects of branched-chain fatty acid metabolism on colonocytes except some regulatory effects of these compounds on electrolyte absorption and secretion [173–177].

5. Energy expenditure and gain

Data regarding the microbiota contribution to the host energy expenditure or gain are scarce [178]. The fact that antibiotics are widely used to promote growth and feed use efficiency in farm animals like pigs and chickens [179,180] suggests an energetic cost for the host to maintain microbiota. Although the mechanisms involved in such an effect remain unclear, it has been proposed that the competition between bacteria and host would partly explain the results observed. The part played by amino acids among other compounds in such a competition remains to be determined.

6. Conclusion

Although limited, the available experimental evidences strongly suggest that the intestinal microbiota is involved in the utilization and catabolism of some indispensable and dispensable amino acids originating from the alimentary and endogenous proteins in the intestinal lumen. Other experimental evidences suggest that the intestinal microbiota is also able to provide amino acids to the host raising the view that the amino acid exchange between the microbiota and host can proceed in both directions. However, the net result of such exchanges remain to be determined *in vivo* from both a quantitative and qualitative points of view according to various parameters like the nutritional status, the parts of the intestine involved, the availability of the nitrogen sources and of other nutrient sources, the composition and concentration of the microbiota,

the overall metabolic capacity of the microbiota, the transit time, the luminal pH *etc.*

From recent publications, it appears that schematically, the main anatomical distinction regarding the intestine should be done between the small and large intestine where the situation is much contrasted. Regarding the large intestine, it appears that amino acids are usually not absorbed by the colonic mucosa, but rather are used by the microbiota which produces numerous metabolic end-products which have been not exhaustively characterized.

Finally, new experimental works is required in order to progress in the knowledge of the nutritional and physiological consequences of the intestinal microbial metabolism of nitrogenous compounds on the host. The effects of the metabolites produced by the microbiota from different amino acids on the colonic and rectal epithelial cells are better characterized including both beneficial and deleterious consequences. The effects of intestinal bacterial metabolites at peripheral level (liver and other organs) are more largely unknown and require further investigations.

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