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Summary

Background and aim: This review examines to what extent high-protein diets (HPD), which may favor body weight loss and improve metabolic outcomes in overweight and obese individuals, may also impact the gut environment, shaping the microbiota and the host-microbe (co)metabolic pathways and products, possibly affecting large intestine mucosa homeostasis.

Methods: PubMed-referenced publications were analyzed with an emphasis on dietary intervention studies involving human volunteers in order to clarify the beneficial vs. deleterious effects of HPD in terms of both metabolic and gut-related health parameters; taking into account the interactions with the gut microbiota.

Results: HPD generally decrease body weight and improve blood metabolic parameters, but also modify the fecal and urinary contents in various bacterial metabolites and co-metabolites. The effects of HPD on the intestinal microbiota composition appear rather heterogeneous depending on the type of dietary intervention. Recently, HPD consumption was shown to modify the expression of genes playing key roles in homeostatic processes in the rectal mucosa, without evidence of intestinal inflammation. Importantly, the effects of HPD on the gut were dependent on the protein source (i.e. from plant or animal sources), a result which should be considered for further investigations.

Conclusion: Although HPD appear to be efficient for weight loss, the effects of HPD on microbiota-derived metabolites and gene expression in the gut raise new questions on the impact of HPD on the large intestine mucosa homeostasis leading the authors to recommend some caution regarding the utilization of HPD, notably in a recurrent and/or long-term ways.

Keywords: High-protein diet, microbiota, bacterial metabolites and co-metabolites, large intestine mucosa

1. Introduction

In a context of a high proportion of overweight and obese individuals, notably in populations from Europe and the USA (1), numerous different types of weight-loss diets are currently proposed and consumed (2). Among them, high-protein diets (HPD), which represent a heterogeneous group of diets with different composition (3), are all characterized by a higher proportion of protein (25 – 30 % of total energy intake) among the two other dietary macronutrients (i.e. carbohydrates and fat) when compared with the usual macronutrient proportion. These HPD are used by millions of individuals around the world for weight-loss (4). One of the main rationales for the consumption of HPD is that it is generally recognized that, on a basis of equal energy content, protein is more satiating than carbohydrates and fats (5). Considering that weight gain is primarily observed when energy recovered from food is superior to energy expenditure, notably in relationship with physical exercise (6), HPD, by reducing dietary energy intake, is likely to help, at least transiently, in the process of body weight reduction (7).

However, there is presently no definition of the maximal amount of dietary protein that can be consumed without short- and/or long-term metabolic and physiopathological side effects. Indeed, if the benefits of decreased body weight in overweight and obese individuals in terms of metabolic and general health outcomes appear obvious based on numerous studies (8), then the interest of HPD consumption for such outcomes must be confronted with possible undesirable effects upon different tissues and organs in a beneficial over deleterious ratio perspective. For instance, it is well known that HPD are contraindicated in individuals with chronic kidney diseases or at risk for such diseases, as HPD may accelerate kidney dysfunctions (9, 10). Regarding the impact of HPD on gut health, this remains an emerging but important topic.

The aim of the present review is to present the available evidence, including recent data obtained in the MyNewGut European research project, in order to balance the advantages of HPD for weight loss and metabolic health against the potential risks of such unbalanced diets focusing on the gut ecosystem homeostasis. As a matter of fact, there are indications from clinical and experimental studies that dietary changes may modify the large intestine luminal environment with a potential impact on the colonic mucosa (11).

PubMed-referenced publications were analyzed using the following terms in combination: [high-protein diet OR dietary protein OR protein source] / [intestinal microbiota OR bacterial metabolites OR co-metabolites] / [large intestine OR colon OR rectum] / [weight-loss OR overweight OR obesity]. Among the numerous papers found, priority was given for references related to dietary intervention studies with human volunteers, notably those reporting consequences in terms of intestinal physiology and physiopathology.

This review is part of a series of position paper of the MyNewGut project aiming at providing recommendations for dietary guidelines based on project results and the latest advantages in the field regarding insights gained in the role of the gut microbiome, as described in the introductory paper (12).

2. High-protein diet, weight loss, and metabolic effects

2.1 High-protein diet and weight loss

HPD can be defined in regards to the absolute amount of dietary protein (in grams) consumed per day, or to the proportion of dietary protein in the total energy intake; or to the amount of dietary protein per unit of body weight. A useful reference can be found on the recommended daily amount of dietary protein which has been determined to be equal to 0.83 g of protein per kg body weight per day ,(13) thus representing 58.1 g dietary protein per day for an individual weighting 70 kg. As a matter of fact, mean dietary protein consumption is largely above these recommended value for instance in France since it averages 87.3 g/day (average value for men and women) (14), and in the USA where it averages 82.8 g/day taking into account men and women dietary protein consumption (15), thus representing approximately 1.5 fold the recommended daily amount of protein. HPD can represent as much as 5 fold higher than the recommended daily amount (4), but it is generally considered that diets containing at least 25-30% of energy in the form of protein are HPD (16). As a matter of comparison, in France, 16.8% of the dietary energy comes from protein in typical diets (14). Incidentally, HPD are also largely consumed by athletes who wish to increase their muscle mass and performance, but this

aspect is out of the scope of the present review and will not be described here, although the readers are referred to excellent reviews on that topic (17, 18).

Two main types of controlled clinical intervention studies with HPD have been performed. The first one is the "*ad libitum*" studies in which volunteers consume the amount of HPD or control normoproteic diet (NPD) until they naturally stop their food consumption. In these studies, due to the satiating effects of HPD, volunteers on HPD generally eat less food than the control NPD subjects, and consequently significantly decrease their body weight compared to the body weight measured at the onset of the dietary intervention. In the study of Weigle et al. (19), HPD given *ad libitum* for 2 weeks resulted in a decrease of body weight. Johnstone et al. (20) also reported reduction of food intake and body weight following 4-week-consumption of HPD. A*d libitum* consumption of HPD for 6 months resulted in a marked decrease of body fat when compared with individuals receiving a NPD (21). In a study on weight loss maintenance after dietary energy restriction, it has been shown that HPD, when given for 12 weeks (22) or 12 months (23), is efficient for weight control. However, in the "real life" condition, a vast majority of individuals, after initial body weight reduction, recover their initial body weight in the long term (24), leading possibly to recurrent episodes of weight-loss HPD consumption. A study using *ad libitum* HPD has shown that meat-based HPD is not more efficient for body weight decrease than protein from plant origin (25).

The second type of HPD intervention studies consists of increasing the proportion of protein in the diet compared to the control, but in that case, the amount of energy consumed between groups is maintained constant. This is generally done by decreasing the relative proportion of another macronutrient in the diet, namely carbohydrates or fats. In that kind of isocaloric clinical protocol, the studies generally found no or little effect of such diets for body weight reduction (16, 26) corresponding to the view that the amount of dietary energy intake, at a constant level of physical exercise, is a major parameter for fixing the evolution of body weight for one given individual.

A third type of studies related to the use of HPD in obese patients are those related to the use of such diet for maintaining the lean mass in malnourished obese patients. Since we will not develop this aspect in our review, the readers are referred to a recent review paper on that topic (27).

137 *2.2 High-protein diet and metabolic parameters*

138 The interpretation of the effect of HPD on metabolic parameters can be somewhat complicated. For
139 instance, if a HPD is given to overweight individuals in an "*ad libitum*" protocol, it will be difficult to
140 determine what part the increased proportion of protein in the diet plays in the normalization of
141 metabolic parameters in comparison with the part played by the decrease of energy intake due to the
142 satiating effect of HPD and the resultant decrease of body weight. In overweight and obese
143 individuals, marked decrease of body weight, whatever the cause, allows the normalization of
144 metabolic parameters (8, 28).

145 In protocols in which the experimental diets are isocaloric, the HPD, as said above, are necessarily
146 decreased in another macronutrient, thus rendering it difficult to attribute the effects of HPD solely to
147 the increased content of protein and/or to the reduced amount of the other macronutrient. In a recent
148 randomized, parallel, double-blind controlled study in which the HPD (using milk casein or soy
149 protein as supplements) were given to volunteers for 3 weeks, no significant changes on any of the
150 biochemical and anthropometric parameters were measured in blood in fasting conditions when
151 compared with control subjects receiving a normoproteic isocaloric diet. A notable exception to this
152 lack of change in parameters was observed for systolic blood pressure, which was decreased in the
153 group of volunteers receiving the soy protein supplementation; an effect that was likely due to the
154 presence of protein-associated isoflavones in the protein extract (26). Thus, under condition of equal
155 energy consumption, HPD appear to exert no short-term sizeable effect on the metabolic and
156 anthropometric parameters.

157

158 3. **High-protein diet and changes in the gut ecosystem**

159 The process of protein digestion in the small intestine is a very efficient process with digestibility
160 usually ranging from 89 to 95%, depending on the nature of the protein (29, 30). Generally speaking,
161 proteins from animal sources are overall more digestible than proteins from plant sources (31). Some
162 sources of protein, for instance rapeseed protein, are known for their lower digestibility (32). In
163 addition, food cooking (33, 34) and food matrix structure (35) can impact protein digestibility.
164 Importantly, and as a result of incomplete digestion in the small intestine, a residual amount of

undigested protein and peptides, together with individual amino acids are transferred through the ileo-caecal junction in the large intestine (36). Based on a regular western diet, it has been determined that approximately 12 g of protein and peptide from both dietary and endogenous origin escape digestion in the small intestine, thus reaching the colonic lumen (37). This amount of nitrogenous material is increased nearly proportionally when the amount of dietary protein increases (29). From studies evaluating the proportion of dietary and endogenous protein which escape digestion and move from the ileum to the large intestine, it has been determined that the majority of the ileal nitrogen is originating from endogenous losses (1-2 g/day), while the nitrogen from dietary origin represents 0.7-1.2 g/day (36). The results obtained in animal models suggest that the part ascribed to endogenous protein is not vastly different according to the amount of protein consumed (38). Since the large intestine luminal content is characterized by a much more abundant microbiota than what is measured in the small intestine (39), and also by a much slower transit time (40), the proteins and peptides which enter the large intestinal luminal content undergo the catalytic action of bacterial proteases and peptidases which release sequentially shorter peptides and amino acids (41). The large intestinal epithelium, in contrast with the small intestinal epithelium which is very efficient for oligopeptide and amino acid absorption, is not believed to transfer any significant amount of amino acids from the lumen to the bloodstream, except in the neonatal period (42, 43). Therefore, protein and peptide-derived amino acids are metabolized by the large intestinal microbiota which use them for protein synthesis and catabolic pathways with the production of numerous intermediates and final metabolites (44); a net amount of these latter being able to accumulate within the luminal content (Figure 1). This process of protein degradation is more active in the distal part than in the proximal part of the large intestine (45). In the case of HPD consumption, the increased transfer of nitrogenous compounds in the large intestine is liable to modify the microbiota composition, and/or to change the microbiota diversity, and/or its metabolic activity, and finally to change the production of bacterial metabolites with possible consequences for the large intestinal mucosa metabolism, physiology and health (46-50) as described below.

3.1 High-protein diets and intestinal microbiota composition

193 Relatively few human intervention studies have examined the short-term (less than 4 weeks) effects of
194 HPD on the gut microbiota composition (Table 1). Two main factors preclude direct comparison
195 between the studies presented in Table 1: (i) differences in energy intake (e.g. calorie restriction) and
196 (ii) differences in fiber intake. These two parameters are known to have a profound influence on the
197 gut microbiota composition and should therefore be considered as important potential confounding
198 factors with the effects of dietary protein intake. Moreover, there are large variations between the
199 studies in terms of methods used to analyze the composition of the gut microbiota. With these
200 limitations in mind, it is still possible to propose some general conclusion regarding the effects of
201 dietary protein intake on the gut microbiota.
202 Two of the studies in Table 1 used HPD without modification of dietary fiber and energy intake. (26,
203 45). Using 16S rDNA sequencing for fecal or rectal biopsy samples, and denaturing gradient gel
204 electrophoresis (DGGE) for fecal samples, respectively, these two studies did not detect changes in the
205 gut microbiota composition after the HPD (Table 1). In a study by David et al. (51) a diet containing
206 dietary protein from animal origin containing almost no fibers was given *ad libitum* for 5 days. This
207 dietary intervention resulted in almost doubling the protein intake (i.e. 30.1% of energy intake) as
208 compared to the protein consumption at the onset of intervention, and was found to impact the
209 microbiota composition by increasing the abundance of bile-tolerant microorganisms (*Alistipes*,
210 *Bilophila*, and *Bacteroide*s), and by decreasing the levels of Firmicutes that metabolize plant
211 polysaccharides (*Roseburia*, *Eubacterium rectale*, and *Ruminococcus*). Such a HPD was found to
212 change the microbiota β-diversity within 2 days. However, this latter effect appeared to be transient, as
213 the β-diversity returned to the initial configuration within 2 days after the end of the intervention (51).
214 However, these changes could not be attributed solely to the level of protein intake since there was
215 considerable concomitant modification of fat intake (in addition to fiber intake) in this study.
216
217 The other studies presented in Table 1 used HPD with caloric restriction that resulted in weight-loss.
218 Two of them (different analysis of the same samples), showed that the HPD induced an alteration of
219 the gut microbiota composition with a decreased abundance of presumed beneficial bacteria such as
220 *Bifidobacterium* or *Rosburia/Eubacterium rectale (52, 53)* However, both resistant starch and total

carbohydrates were also lower in the high-protein/weight loss diet compared to the maintenance diet (52). This is an important point to consider as resistant starch has been positively associated with the abundance of *Bifidobacterium* and *Eubacterium spp (54, 55).*; and a reduction in carbohydrates led to decreases in both genera (56). In another study, a weight-loss HPD combined with an increase in fiber intake also induced a decrease in *Eubacterium rectale* but increased bacterial gene richness in individuals with low gene counts together with an increase abundance of bacteria considered protective such as *Faecalibacterium prausnitzii* and *Roseburi* (57). Lastly, two other studies using weight-loss HPD combined with a low fiber intake observed a decrease in the total bacterial biomass and in the abundance of *Bifidobacterium* and *Rosburia/Eubacterium rectale (56, 58).*

Overall, the studies presented in Table 1 show that HPD have a limited effect on the gut microbiota composition when they are not associated with calorie restriction or with a modification of fiber intake. This conclusion may also be connected to the observed relatively little changes in the microbiota composition according to the diet when compared with the inter-individual variations (< 10%) (53).

3.2 High-protein diets and impact on gut mucosa: potential role of bacterial metabolites

The mixture of bacterial metabolites in the intestinal content is complex (59) and far from being fully characterized. Among these compounds, numerous metabolites are produced by the intestinal microbiota from amino acid substrates (60). The concentrations of these metabolites are usually measured in the feces, which are related to the concentrations of the luminal content within the most distal part of the large intestine, namely in the rectum. These metabolite concentrations depend on the bacterial production from the available substrates, on the bacterial composition and overall metabolic activity, on the absorption through the large intestinal epithelium, and on the transit time (61). (Figure 1). Other parameters may influence the concentrations of the different forms of the bacterial metabolites within the large intestine content. For instance, the luminal pH, which will result from the overall acid/base balance in this compartment, will in turn determine the ratio of the different non-ionized and ionized forms of ionic bacterial metabolites (62), which will affect their uptake from the luminal content to the colonocyte intracellular content. In addition, the situation is complicated by the

fact that some bacterial metabolites (for instance hydrogen sulfide) can bind to fecal components, thus reducing the concentration of free (unbound) metabolites presumed to act on the epithelial cells (Figure 2) (63). We present below the effects of HPD on bacterial metabolites and their main effects observed in Humans and experimental animal models but the reader is referred to another recent review for more exhaustive description of the metabolites produced by the microbiota from amino acids (41).

3.2.1 Effects of high-protein diets on the fecal composition and effects of individual bacterial metabolites on colonic epithelial cells

Several intervention studies in humans have shown that HPD with different sources of dietary protein induce a shift from carbohydrate to protein degradation by the gut microbiota (26, 58, 59, 64), with an alteration of numerous bacterial metabolite concentrations in feces, thus indicating changes in the luminal environment of the colonic epithelial cells. In contrast with the high variability described above between human intervention studies regarding the effects of HPD on microbiota composition (Table 1), the effects of HPD on bacterial metabolites are more homogeneous despite differences in experimental design (Table 2). This observation emphasizes the importance of substrate availability, namely amino acids in our case, rather than taxonomic composition of the microbiota for determining the metabolic output in the large intestine. This could also be due to redundancy of functions and metabolic pathways in the microbiome, the collective genome of the microbiota (65).

Most of the studies in Table 2 reported that HPD consumption induced an increase in amino acid-derived short-chain fatty acids (SCFA) such as isobutyrate, isovalerate, and 2-methylbutyrate (26, 49, 58). In contrast, a decrease in the SCFA butyrate was consistently found after HPD consumption (26, 51, 56, 58) albeit several of these studies included decreases in fiber content among the HPD. However, in a recent study by Beaumont et al. (26) volunteers from the HPD and control groups consumed a similar amount of dietary fibers and energy than the NPD group thus suggesting that the reduction of fecal butyrate concentration in HPD can be attributed primarily to the amount of protein in the diet. As butyrate is well-known as a major oxidative substrate and a regulator of histone

276 acetylation, and thus of gene transcription in human colonocytes (66, 67), the measured decrease in its
277 fecal concentration after HPD is presumably detrimental for the rectal mucosa homeostasis.
278 Two studies in volunteers receiving a HPD found a marked increase in fecal ammonia concentrations
279 (59, 64), while two others did not (26, 58), likely due to the different experimental protocols. Also,
280 HPD were found to increase the concentrations of several S-containing metabolites (59, 68). For most
281 of these metabolites, there is surprisingly no indication on the impact of such changes on the
282 colonic/rectal epithelium renewal and functions. However, from *in vitro* studies with human or rodent
283 colonocytes, there are indications that several amino acid-derived bacterial metabolites including
284 hydrogen sulfide (H_2S), ammonia and *p*-cresol act as metabolic troublemakers towards colonocyte
285 mitochondrial energy metabolism within the range of concentrations that are measured in the colonic
286 content or in feces (69, 70).
287 In contrast, some bacterial metabolites derived from amino acids were found to exert beneficial effects
288 on the intestinal epithelial barrier (reviewed in 11). For instance, indole which is produced from L-
289 tryptophan has been shown to increase epithelial cell tight-junction resistance as will been detailed in
290 the part 3.3. Another bacterial metabolites derived from tryptophan, namely indole propionic acid, has
291 been shown recently to be efficient for decreasing the intestinal permeability in rodents (71). Thus, in
292 order to document the beneficial versus deleterious effects of the mixture of bacterial metabolites
293 contained within the intestinal content, it is clearly necessary to take into account the fact that these
294 contents contain compounds with both positive and negative effects on the intestinal mucosa.
295
296 3.2.2 Genotoxic and cytotoxic potential of fecal water recovered after high-protein diet consumption
297 In order to get information on the possible overall cytotoxic and genotoxic potential of fecal water-
298 soluble components after controlled dietary intervention, it is feasible to prepare the so-called “fecal
299 water” samples by diluting and homogenizing fecal samples in aqueous medium, and test the
300 supernatant on human colonocytes. Although fecal water samples do not contain all the luminal
301 compounds and dilute the bacterial metabolites, fecal water toxicity has been proposed to represent a
302 potential biomarker for intestinal disease risk (72). When an isocaloric HPD was given for 2 weeks to
303 healthy human subjects in a crossover design, the mixture of water-soluble components recovered

304 from the feces shown no increased genotoxicity or cytotoxicity potential towards human colonocytes
305 when compared to the NPD (45). Similarly, in a study by Benassi-Evans et al. (73), the authors
306 performed a nutritional intervention with HPD during 52 weeks using a parallel design with
307 overweight and obese volunteers. They found that the fecal water recovered from individuals
308 consuming HPD was not more genotoxic than ones recovered from control volunteers consuming
309 isocaloric NPD. In accordance with the results presented above, in a study by Beaumont et al. (26),
310 supplementation of the diet with either casein or soy protein for 3 weeks, did not result in higher
311 cytotoxic potential of the fecal water when compared with the results obtained from isocaloric NPD
312 volunteers. Thus, collectively, the available data indicate that the fecal water samples recovered from
313 volunteers consuming HPD in short- and medium terms show no increased genotoxic and cytotoxic
314 potential *in vitro* towards colonic epithelial cells than samples recovered from control NPD.
315
316 *3.3 High-protein diet and urinary metabolome*
317 Urinary metabolomic analysis is useful in order to identify the bacterial metabolites and cometabolites
318 (produced by the microbiota and metabolized by the host) which have been produced by the gut
319 microbiota, absorbed from the lumen to the bloodstream through the intestinal epithelium (with or
320 without metabolism in colonocytes), possibly further metabolized by the host in the liver or other
321 organs outside the splanchnic area, and finally excreted in the urine where they accumulate (Figure 3).
322 For instance, HPD ingestion results in the increased urinary excretion of the bacterial metabolite
323 phenol (64). This is of interest as phenol has been shown to act as a cytotoxic compound towards
324 colonocytes (74); and as impaired phenol detoxification has been associated with ulcerative colitis
325 (75).
326 In addition, the cometabolite *p*-cresyl sulfate is produced in the colon mucosa and the liver from the
327 bacterial metabolite *p*-cresol, which itself is produced by the microbiota from the amino acid L-
328 tyrosine (76). Urinary concentration of *p*-cresyl sulfate has been repetitively found to be increased
329 after HPD consumption (26, 59, 77) when compared with control NPD (Table 2). Since *p*-cresol has
330 been shown to inhibit colonocyte oxygen consumption, and to be genotoxic towards colonocytes (70),
331 *p*-cresyl sulfate synthesis has been hypothesized to correspond in colonic epithelial cells to a

detoxifying metabolic pathway for this bacterial metabolite. This possibility has been challenged by the fact that *p*-cresyl sulfate displayed pro-inflammatory and cytotoxic effects on renal tubular epithelial cells (78, 79), and that serum *p*-cresyl sulfate level may help in predicting progression of chronic kidney disease (80, 81).

In a study by Beaumont et al. (26), the relative concentration of another urinary cometabolite, namely indoxyl sulfate, increased after HPD (Table 2). Since indole, the precursor for the synthesis of indoxyl sulfate in the liver, has been shown to contribute to the maintenance of the colonic barrier function (82, 83) and to alleviate hepatic inflammation (84), this bacterial metabolite can be considered as beneficial for the host. However, in order to establish the beneficial vs. deleterious effects of indole on the colon epithelium, it is important to consider that this bacterial metabolite activates the aryl hydrocarbon receptor (AhR)-mediated transcription of Cyp 1a1 and Cyp 1b1 in human colonocytes (85, 86). These two enzymes belongs to the cytochrome P450 family which, apart from their role in the deactivation of deleterious compounds and xenobiotics, can catalyse the bioactivation of procarcinogen compounds into carcinogens (87-89). In addition, indoxyl sulfate is suspected to act as a uremic toxin contributing to renal disease progression (90-92).

Thus, the analysis of the urinary metabolome gives important information regarding the exposure of the intestinal mucosa to bacterial metabolites (Figure 3), even if the results obtained emphasizes the difficulty to predict how changes of a complex mixture of bacterial metabolites will impact the colonic/rectal mucosa according to the time of exposition and respective concentrations.

3.4 High-protein diets and gut mucosa inflammation

Although the results of epidemiological studies regarding the association between HPD consumption and risk of inflammatory bowel diseases (IBD) are heterogeneous (93), two studies have shown that a high amount of animal protein intake is associated with increased inflammatory bowel disease incidence and relapse (94, 95). However, short-term supplementation (3 weeks) with casein or soy protein, did not show any sign of rectal mucosal inflammation based on the measurement of pro-

inflammatory cytokines in rectal biopsies, and on the fecal concentrations of calprotectin and secreted IgA, when compared with an isocaloric NPD (26).

Participation of some bacterial metabolites on the process of mucosal inflammation in pre-disposed subjects may be related to a reduced capacity of the mucosa for deleterious metabolite detoxification. For instance, it has been reported that impaired H_2S detoxification in intestinal mucosa is associated with Crohn's disease (96) and ulcerative colitis (97). These results are important to be taken into account, knowing that increased protein consumption is correlated with increased H_2S fecal excretion in volunteers (68), and that excessive luminal H_2S decreases colonocyte respiration and increases the expression of several genes involved in IBD in a rodent model (98). It can therefore be predicted that there might be differences between individuals in terms of mucosal response to HPD according to individual detoxification capacities.

3.5 High-protein diets and gene expression in gut mucosa

The first experimental evidence using transcriptomic analysis which has shown that casein-containing HPD can modify gene expression in the colonic mucosa were obtained in the rat model by Mu et al. (99) using a 6-week- dietary intervention protocol with isocaloric experimental (HPD) and control (NPD) diets. Beaumont et al. (100) used a 2 week-intervention protocol with whole milk protein-containing HPD in the rat model to demonstrate that HPD down-regulates colonic epithelial cell gene expression notably in relationship with cell metabolism, NF-κB signaling, DNA repair, glutathione metabolism and cellular adhesion, when compared with gene expression in colonocytes recovered from isocaloric NPD. In this latter study, the HPD was found to up-regulate the expression of genes related to cell proliferation and chemical barrier function. These animal studies allow to establish the new proof of concept according to which increasing the amount of protein in the diet will result in a modification of gene expression in the colonic mucosa, and more specifically in the colonic epithelial cells. Further, a randomized controlled study with overweight volunteers reported that 3 week-dietary supplementation with either casein or soy protein resulted in small amplitude changes in the

385 expression of numerous genes in the rectal mucosa, notably for genes involved in homeostatic
386 processes such as cell cycle or cell death (26).
387
388 *3.6 The effects of high-protein diets on the fecal and urinary metabolome and on the large intestine*
389 *mucosa according to different protein sources*
390 It can be hypothesized that the source of protein used in the HPD studies may represent an important
391 parameter for modulating the colonic epithelium luminal environment and gene expression in the
392 rectal mucosa. First, as presented above, different dietary proteins displayed different digestibility
393 characteristics. Second, the differences in the amino acid composition between proteins provide the
394 intestinal microbiota with different amounts of individual amino acids as substrates for the microbiota
395 metabolic activity, thus potentially resulting in different fecal bacterial metabolite compositions and
396 urinary bacterial/host cometabolites in the urine. Up to now, this hypothesis has been little explored
397 but one recent study reported that when the habitual diet is supplemented with either milk casein or
398 soy protein, differences are observed in the fecal and urinary metabolome, with such differences
399 coinciding with changes in gene expression in the rectal mucosa (26). Indeed, in the case of
400 supplementation with casein, when compared with the isocaloric NPD group, the feces were
401 characterized by increased relative concentration of 2-methylbutyrate; while in the case of
402 supplementation with soy protein, an increase of this bacterial metabolite was also measured but
403 together with an increase of valerate, tyramine, and phenylacetate. Regarding the urinary metabolome,
404 casein supplementation resulted in increased urea, isobutyrate, 3-hydroxybutyrate, 3-
405 hydroxyisovalerate, *p*-cresyl sulfate, phenylacetylglutamine and indoxylsulfate relative concentration;
406 while supplementation with soy protein resulted in an increased of the same metabolites but not of the
407 uremic toxin *p*-cresyl sulfate, the co-metabolite produced from *p*-cresol (79).
408 More importantly, casein and soy protein HPD were found to differentially modify the expression of
409 genes playing key roles in the maintenance of the rectal mucosa homeostasis maintenance in general,
410 and in colonic health (gastrointestinal diseases and cancer) in particular. At the cellular level, the
411 casein diet was specifically associated with increased expression of genes related to extracellular
412 matrix, cell adhesion, and mucus production; while the soy protein diet was specifically associated

with modification of the expression of genes associated with oxidative stress and detoxification processes. Expression of other genes associated with cellular processes like apoptosis, cell cycle and proliferation, and cytoskeleton formation were modified by both casein and soy protein (26). To determine if such changes in gene expression impact the rectal epithelium renewal and functions, and/or if it corresponds to an adaptation towards a changing luminal environment, new experiments are required. Regarding this latter aspect, the fact that the expression of genes related to mucus production was solely increased in the rectal mucosa of volunteers after casein supplementation but not after soy protein supplementation, may indicate an adaptation of the rectal mucosa towards a more aggressive luminal environment following casein-based HPD consumption.

4. **Conclusion and perspectives**

Although it appears that HPD can help in diminishing the dietary intake, and thus favor weight loss, there are some results which raise new questions on the safety of their utilization. It must be recognized that, according to the available literature, there is no definitive evidence that such diets are deleterious for gut health in short- and medium- term intervention studies conducted so far.

Indeed, as presented above, short-term consumption of HPD by itself neither increases the inflammation of the large intestinal mucosa, nor increases the *in vitro* genotoxicity and cytotoxicity of the mixture of compounds contained in the fecal water extracts in healthy subjects. However, HPD have been shown in a repetitive manner to decrease fecal butyrate concentrations. Since butyrate is generally considered as a fuel substrate and a regulator of gene expression in the rapidly renewing colonic epithelial cells, this decrease must be seen as potentially deleterious for the colonic mucosa homeostasis. The same remark can be made regarding the finding that HPD consumption results in increased exposition of the intestinal mucosa to *p*-cresol, a bacterial metabolite with genotoxic and metabolic troublemaker characteristics towards colonocytes (70). In addition, *p*-cresol is the precursor of *p*-cresyl sulfate, a cometabolite with reported cytotoxic activity towards renal cells (78, 79) (Figure 3). Conversely, there is evidence that HPD increases the exposure of the large intestine mucosa to indole, a bacterial metabolite considered as an important player in the maintenance of the epithelial barrier function. However, this positive effect of indole on the intestinal epithelium must be

441 counterbalanced by the suspicion that indoxyl sulfate, a cometabolite of indole produced in the liver, is
442 also acting as a uremic toxin (90, 101) (Figure 3). Then, the different effects of bacterial metabolites
443 and cometabolites on different cell types, either within the intestinal mucosa as detailed in the present
444 paper, or at the periphery, makes it difficult to predict if one given compound in a mixture should be
445 considered as overall beneficial or deleterious. The finding that an increased consumption of dietary
446 protein modifies within 3 weeks the normal expression of genes known to be involved in processes
447 related to the maintenance of the rectal mucosal homeostasis (26), represents an important new finding
448 which should be taken into consideration before formulating any recommendation on HPD
449 consumption.
450 Regarding the effects of amino acid-derived bacterial metabolites on metabolic parameters, recent data
451 suggest that some of these metabolites might contribute to an improvement of some of these
452 parameters. For instance, indole has been shown *in vitro* to modulate the secretion of the incretin
453 glucagon-like peptide 1 (GLP-1) (102). Moreover, hydrogen sulfide produced by the gut microbiota
454 has been shown to lower blood pressure in rats (103), to improve glucose metabolism, and to increase
455 GLP-1 secretion in mice (104). Lastly, several neurotransmitters can be produced by the gut
456 microbiota from amino acids (41), and it can be speculated that this may contribute to the dietary
457 protein- induced satiety. Further studies, notably with larger groups of human volunteers, and of
458 longer duration are needed to determine whether the potential effects of amino acid-derived bacterial
459 metabolites, depending on the protein sources, could participate in the beneficial metabolic effects of
460 HPD associated with body weight reduction.
461
462 5. **Implications for dietary recommendation regarding high-protein diet consumption**
463 Although body weight reduction associated with *ad libitum* HPD consumption in overweight and
464 obese individuals is obviously associated with favorable outcomes, the data obtained principally from
465 clinical trials with human volunteers, dietary intervention in animal models, and *in vitro* experiments
466 with human colonic epithelial cells have shown that HPD modifies the luminal environment of the
467 rectal epithelium and impacts gene expression in the mucosa. We therefore recommend caution in the
468 utilization of HPD diets for body weight loss, taking into account the possible regain of body weight

after HPD consumption, which may lead to redundant and long-term utilization of HPD. Considering the most recent evidence showing that the effects of HPD on the gut depend on the protein source (i.e. from plant and animal sources), not only the quantity, but also the quality of dietary protein should be considered for further investigations and possibly for future dietary recommendations.

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Conflict of interest

FB, MB, KJP, NS, AL, MA, NK, MA, GA, RB, AMD, LA, SR, PB, DT, SPC, and YS declare no competing interest in relation to this paper.

Legends of figures:

Figure 1. Schematic view of the fate of undigested proteins in case of High-Protein Diet (HPD) consumption.

HPD diet consumption increases the transfer of dietary proteins from the ileum to the large intestine. The proteases and peptidases of the microbiota release amino acids which can be incorporated in the bacterial proteins or lead to a multitude of metabolic end products, notably in the distal parts of the large intestine. Some of these metabolites are known to be transferred by the colonic epithelial cells from the luminal content to the portal bloodstream with or without prior metabolism in the colonocytes. The concentrations of bacterial metabolites in the lumen are the net result of production/utilization by the microbiota, and absorption through the colonic epithelium. The metabolites measured in the feces is a reflection of the metabolites present in the rectum.

Figure 2. Schematic view of the entry and metabolism of bacterial metabolites in the colonic epithelial cells.

Several bacterial metabolites in the luminal content can enter colonocytes by processes of diffusion or transport. Although some of them can be released as such in the bloodstream, several bacterial metabolites are known to undergo intracellular metabolism leading to the production of co-metabolites. Bacterial metabolites and co-metabolites can be released in the portal bloodstream and reach the liver and peripheric organs outside the splanchnic area. Finally, these compounds can accumulate in urine after glomerular filtration and/or tubular secretion by kidneys.

Figure 3. Schematic view of the impact of high-protein diet (HPD) consumption on the bacterial metabolite and co-metabolite concentrations in feces and urine.

Undigested proteins and peptides enter the large intestine and are metabolized by the microbiota which produce various metabolites from amino acids. Some of these metabolites are partly absorbed through the large intestine epithelium, while the residual amount of metabolites are excreted in the feces. Absorbed metabolites reach the liver where some of them undergo further metabolism. Cometabolites and metabolites are finally excreted in the urine. HPD consumption results in measurable modifications of the concentration of bacterial metabolites in feces and urine. As indicated in the text, some compounds originating from the microbial metabolic activity (like butyrate and H_2S) are known to impact energy metabolism and gene expression in colonocytes, while some of them (like indole) are implicated in the maintenance of the epithelial barrier function. Some co-metabolites measured in urine (like indoxylsulfate and *p*-cresylsulfate) are suspected to act as uremic toxins.

Table 1: Effects of high-protein diet on intestinal microbiota composition. The main characteristics and findings from human intervention studies using high-protein diets are summarized. BMI: body mass index, DGGE: denaturing gradient gel electrophoresis, FISH: fluorescence in situ hybridization, % E: % of energy intake, g/d: grams/day. For carbohydrates and fat intake, the readers are referred to the original publications.

Study design	BMI	Duration	Protein intake	Protein source	Fiber intake	Calorie restricted	Method	Intestinal microbiota composition	Reference
n=12-13 Parallel	25 - 30	3 weeks	14 % E	Mixed	17.0 g/d	No	16S rDNA sequencing (feces and rectal biopsies)	Control diet	(26)
			34 % E	Mixed + casein	14.4 g/d	No		No detectable differences	
			31 % E	Mixed + soy protein	17.9 g/d	No		No detectable differences	
n=20 Cross-over	19 - 26	2 weeks	12 % E	Mixed	17.4 g/d	No	DGGE (feces)	No detectable differences	(45)
			15 % E	Mixed	16.3 g/d	No		Control diet	
			27 % E	Mixed	15.4 g/d	No		No detectable differences	
n = 10 Cross-over	19 - 32	5 days	10 % E	Plant protein	41.2 g/d	No	16S rDNA sequencing (feces)	↓ *Bilophila wadsworthia*	(51)
			16 % E	Mixed	21.1 g/d	No		Control diet	
			30 % E	Animal protein	0 g/d	No		↑ *Bilophila wadsworthia, Alistipes putredinis;* ↓ *Bifidobacterium adolescentis, Roseburia faecis, Ruminococcus bromii*	
n=14 Parallel	28 - 51	3 weeks	103.3 g/d	Mixed	27.7 g/d	No	Phylogenetic (HITchip) microarray (feces)	Control diet	(53)
			144.1 g/d	Mixed	25.1 g/d	Yes		↓ *Bifidobacterium, Aerococcus, Granulicatella, Dialister, Papillibacter cinnamivorans;* ↑ *Lactococcus, Bacteroides vulgatus, Anaerotruncus colihominis*	
n=14 Parallel	28 - 51	3 weeks	103.3 g/d	Mixed	27.7 g/d	No	16S rDNA sequencing, DGGE, qPCR (feces)	Control diet	(52)
			144.1 g/d	Mixed	25.1 g/d	Yes		↓ *Collinsella aerofaciens, Roseburia/Eubacterium rectale;* ↑ *Oscillibacter valericigenes*	
n=49 Non-randomized	33 (mean)	6 weeks	19 % E	Mixed	14.5 g/d	No	Metagenomic sequencing (feces)	Control diet	(57)
			37 % E	Mixed	19.0 g/d	Yes		↓ *Eubacterium rectale;* ↑ *Parabacteroides distasonis, Faecalibacterium prausnitzii, Bacteroides dorei, Parabacteroides merdae,Eubacterium eligens, Ruminococcus sp, Roseburia hominis, Odoribacter splanchnicus, Subdoligranulum sp*., Gene richness in low gene counts individuals	
n=17 Cross-over	30 - 49	28 days	13 % E	Mixed	21.9 g/d	No	FISH (feces)	Control diet	(58)
			28 % E	Mixed	12.8 g/d	Yes		↓ total bacteria	
			29 % E	Mixed	8.8 g/d	Yes		↓ total bacteria, *Bacteroides*, *Roseburia/Eubacterium rectale*	
n=20 Cross-over	30 – 42	28 days	94.4 g/d	Mixed	27.9 g/d	No	FISH (feces)	Control diet	(56)
			127.2 g/d	Mixed	11.7 g/d	Yes		↓ total bacteria, *Roseburia/Eubacterium rectale*, *Bifidobacterium*	
			119.5 g/d	Mixed	6.1 g/d	Yes		↓ total bacteria, *Roseburia/Eubacterium rectale, Bifidobacterium*	

Table 2: Effects of high-protein diet on the metabolic activity of the gut microbiota. The main characteristics and findings from human intervention studies using high-protein diets are summarized. BMI: body mass index, % E: % of energy intake, g/d: grams/day, NMR: nuclear magnetic resonance, GC: gas chromatography, MS: mass spectrometry, LC: liquid chromatography. For carbohydrates and fat intake, the readers are referred to the original publications.

Study design	BMI	Duration	Protein intake	Protein source	Fiber intake	Calorie restricted	Method	Intestinal microbiota metabolites	Reference
n=12-13 Parallel	25 - 30	3 weeks	14 % E	Mixed	17.0 g/d	No	NMR metabolomics, GC (feces)	Control diet	(26)
			34 % E	Mixed + casein	14.4 g/d	No		↓ butyrate; ↑ branched-chain amino acids, 2-methylbutyrate	
			31 % E	Mixed + soy protein	17.9 g/d	No		↓ butyrate; ↑2-methylbutyrate, isovalerate, valerate, phenylacetate, tyramine,	
n=12-13 Parallel	25 - 30	3 weeks	14 % E	Mixed	17.0 g/d	No	NMR metabolomics (urines)	Control diet	(26)
			34 % E	Mixed + casein	14.4 g/d	No		↑ isobutyrate, indoxylsulfate, phenylacetylglutamine, *p*-cresylsulfate	
			31 % E	Mixed + soy protein	17.9 g/d	No		↑ isobutyrate, indoxylsulfate, phenylacetylglutamine	
n = 10 Cross-over	19 - 32	5 days	10 % E	Plant protein	41.2 g/d	No	GC (feces)	No detectable differences	(51)
			16 % E	Mixed	21.1 g/d	No		Control diet	
			30 % E	Animal protein	0 g/d	No		↑ isobutyrate, isovalerate; ↓ acetate, butyrate	
n=14 Parallel	28 - 51	3 weeks	103.3 g/d	Mixed	27.7 g/d	No	GC (feces)	Control diet	(53)
			144.1 g/d	Mixed	25.1 g/d	Yes		↑ isobutyrate, isovalerate, lactate; ↓ Acetate, butyrate	
n=20 Cross-over	19 - 26	2 weeks	12 % E	Mixed	17.4 g/d	No	GC-MS metabolomics (feces)	No detectable differences	(45)
			15 % E	Mixed	16.3 g/d	No		Control diet	
			27 % E	Mixed	15.4 g/d	No		↑ isobutyrate	
n=20 Cross-over	19 - 26	2 weeks	12 % E	Mixed	17.4 g/d	No	GC-MS (urine)	No detectable differences	(45)
			15 % E	Mixed	16.3 g/d	No		Control diet	
			27 % E	Mixed	15.4 g/d	No		↑ *p*-cresol	
n=17 Cross-over	30 - 49	28 days	13 % E	Mixed	21.9 g/d	No	GC, LC-MS (Feces)	Control diet	(58)
			28 % E	Mixed	12.8 g/d	Yes		↑ isobutyrate, isovalerate, valerate, phenylacetate	
			29 % E	Mixed	8.8 g/d	Yes		↓ butyrate; ↑ isobutyrate, isovalerate, valerate, phenylacetate, phenylpropionate	
n=20 Cross-over	30 - 42	28 days	94.4 g/d	Mixed	27.9 g/d	No	GC (feces)	Control diet	(56)
			127.2 g/d	Mixed	11.7 g/d	Yes		↓ acetate, propionate, butyrate, valerate, lactate	
			119.5 g/d	Mixed	6.1 g/d	Yes		↓ acetate, propionate, butyrate, isovalerate, valerate, lactate; ↑ ammonia	

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Figure 1

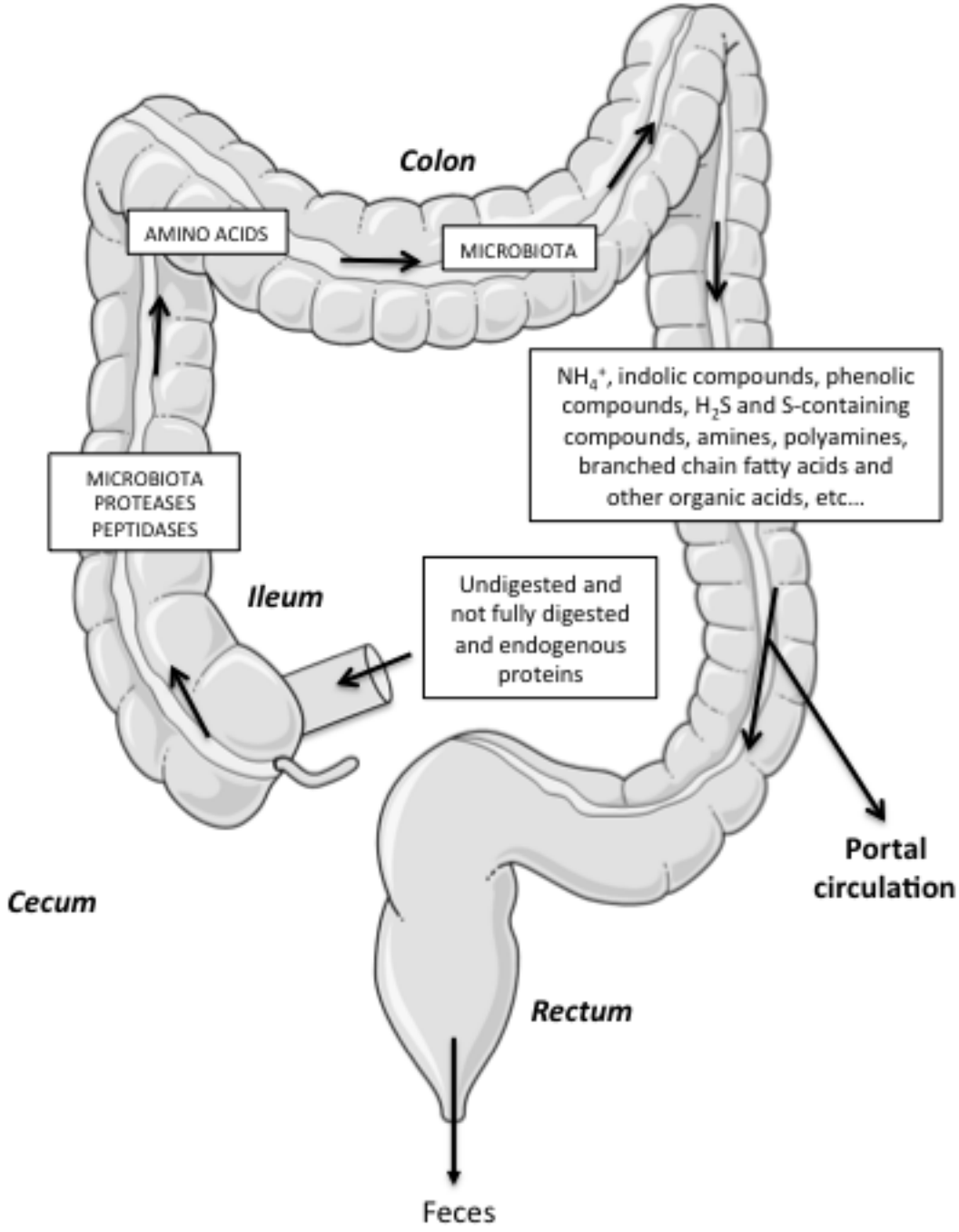

Colon
AMINO ACIDS
MICROBIOTA
NH_4^+, indolic compounds, phenolic compounds, H_2S and S-containing compounds, amines, polyamines, branched chain fatty acids and other organic acids, etc...
MICROBIOTA PROTEASES PEPTIDASES
Ileum
Undigested and not fully digested and endogenous proteins
Cecum
Portal circulation
Rectum
Feces

Figure 2

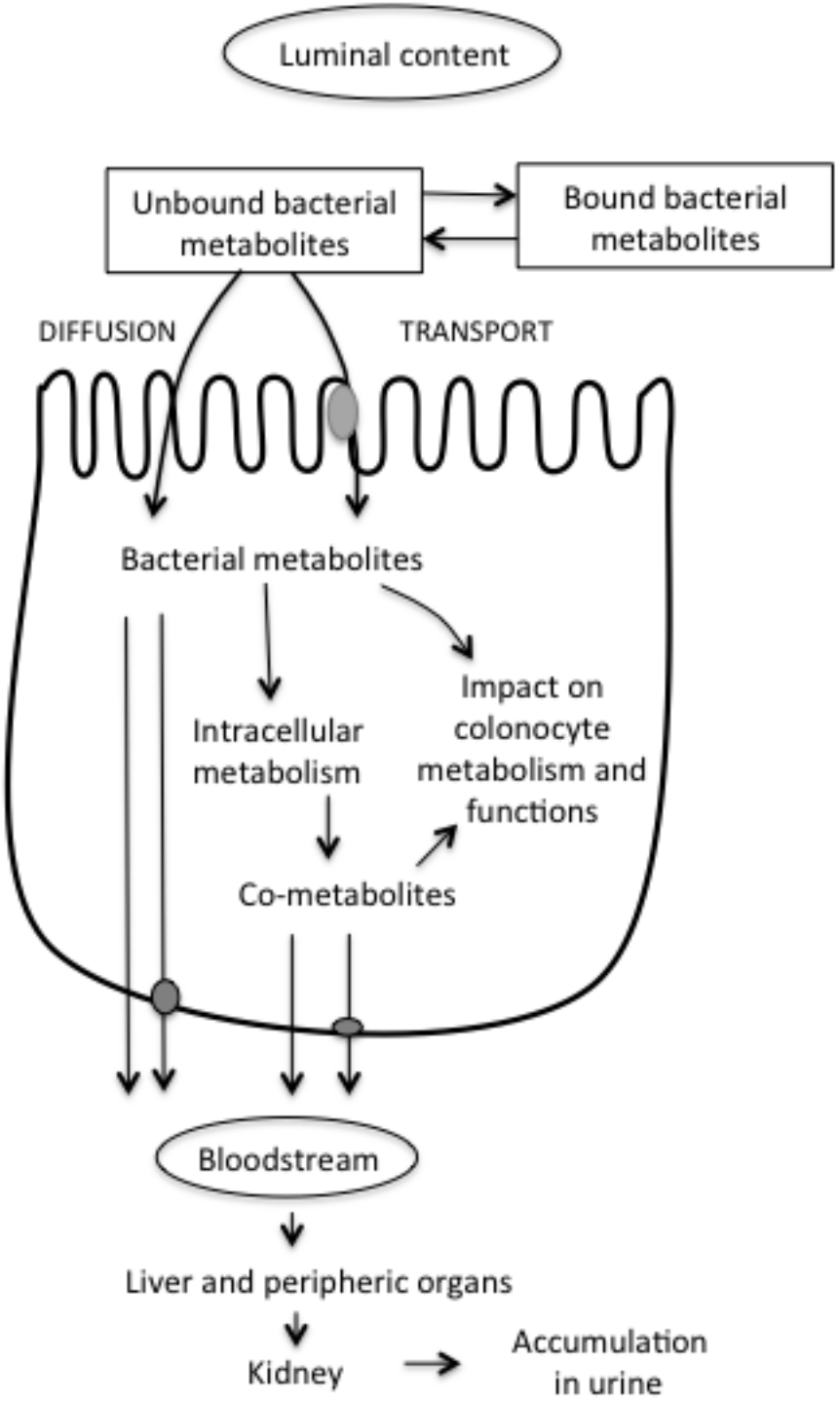
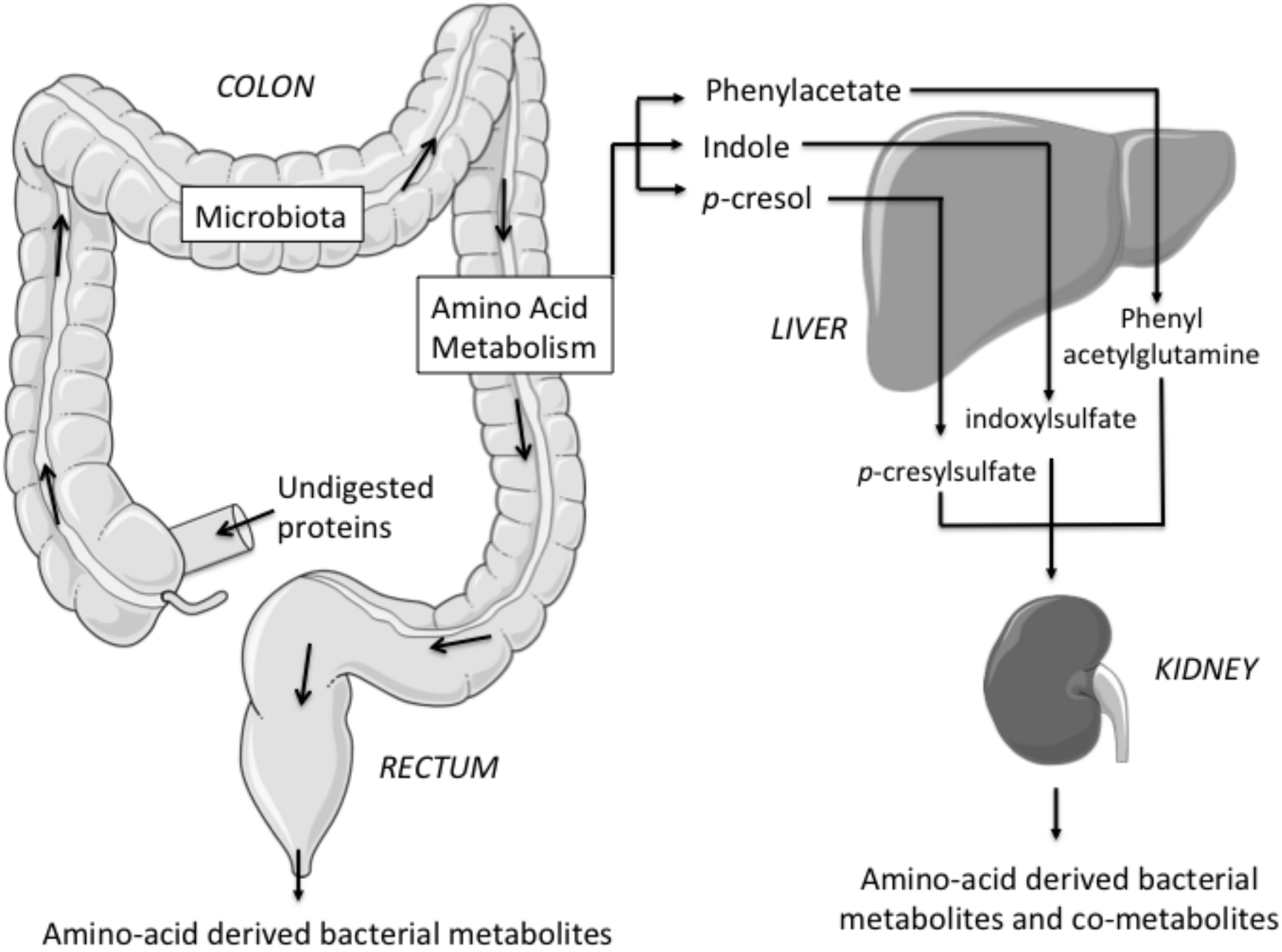

Luminal content
Unbound bacterial metabolites
Bound bacterial metabolites
DIFFUSION
TRANSPORT
Bacterial metabolites
Intracellular metabolism
Impact on colonocyte metabolism and functions
Co-metabolites
Bloodstream
Liver and peripheric organs
Kidney
Accumulation in urine

Figure 3


COLON
Microbiota
Amino Acid Metabolism
Undigested proteins
RECTUM
Amino-acid derived bacterial metabolites
Phenylacetate
Indole
p-cresol
LIVER
Phenyl acetylglutamine
indoxylsulfate
p-cresylsulfate
KIDNEY
Amino-acid derived bacterial metabolites and co-metabolites