

## High-protein diets for weight management: Interactions with the intestinal microbiota and consequences for gut health. A position paper by the my new gut study group

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23	Summary									
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*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.

29 Methods: PubMed-referenced publications were analyzed with an emphasis on dietary intervention 30 studies involving human volunteers in order to clarify the beneficial vs. deleterious effects of HPD in 31 terms of both metabolic and gut-related health parameters; taking into account the interactions with the 32 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.

40 *Conclusion:* Although HPD appear to be efficient for weight loss, the effects of HPD on microbiota-41 derived metabolites and gene expression in the gut raise new questions on the impact of HPD on the 42 large intestine mucosa homeostasis leading the authors to recommend some caution regarding the 43 utilization of HPD, notably in a recurrent and/or long-term ways.

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

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#### 1. Introduction

In a context of a high proportion of overweight and obese individuals, notably in populations from 54 55 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 56 with different composition (3), are all characterized by a higher proportion of protein (25 - 30 % of57 total energy intake) among the two other dietary macronutrients (i.e. carbohydrates and fat) when 58 59 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 60 is generally recognized that, on a basis of equal energy content, protein is more satiating than 61 carbohydrates and fats (5). Considering that weight gain is primarily observed when energy recovered 62 from food is superior to energy expenditure, notably in relationship with physical exercise (6), HPD, 63 by reducing dietary energy intake, is likely to help, at least transiently, in the process of body weight 64 65 reduction (7).

However, there is presently no definition of the maximal amount of dietary protein that can be 66 67 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 68 69 general health outcomes appear obvious based on numerous studies (8), then the interest of HPD 70 consumption for such outcomes must be confronted with possible undesirable effects upon different 71 tissues and organs in a beneficial over deleterious ratio perspective. For instance, it is well known that 72 HPD are contraindicated in individuals with chronic kidney diseases or at risk for such diseases, as 73 HPD may accelerate kidney dysfunctions (9, 10). Regarding the impact of HPD on gut health, this 74 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: [highprotein 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.

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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).

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

95 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, 96 97 or to the proportion of dietary protein in the total energy intake; or to the amount of dietary protein per 98 unit of body weight. A useful reference can be found on the recommended daily amount of dietary 99 protein which has been determined to be equal to 0.83 g of protein per kg body weight per day (13) 100 thus representing 58.1 g dietary protein per day for an individual weighting 70 kg. As a matter of fact, 101 mean dietary protein consumption is largely above these recommended value for instance in France 102 since it averages 87.3 g/day (average value for men and women) (14), and in the USA where it 103 averages 82.8 g/day taking into account men and women dietary protein consumption (15), thus 104 representing approximately 1.5 fold the recommended daily amount of protein. HPD can represent as 105 much as 5 fold higher than the recommended daily amount (4), but it is generally considered that diets 106 containing at least 25-30% of energy in the form of protein are HPD (16). As a matter of comparison, 107 in France, 16.8% of the dietary energy comes from protein in typical diets (14). Incidentally, HPD are 108 also largely consumed by athletes who wish to increase their muscle mass and performance, but this

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

111 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 112 normoproteic diet (NPD) until they naturally stop their food consumption. In these studies, due to the 113 satiating effects of HPD, volunteers on HPD generally eat less food than the control NPD subjects, and 114 115 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 116 117 resulted in a decrease of body weight. Johnstone et al. (20) also reported reduction of food intake and 118 body weight following 4-week-consumption of HPD. Ad 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 119 120 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" 121 condition, a vast majority of individuals, after initial body weight reduction, recover their initial body 122 123 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 124 125 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).

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#### 137 2.2 High-protein diet and metabolic parameters

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

In protocols in which the experimental diets are isocaloric, the HPD, as said above, are necessarily 145 146 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 lack of change in parameters was observed for systolic blood pressure, which was decreased in the 152 group of volunteers receiving the soy protein supplementation; an effect that was likely due to the 153 154 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.

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

The process of protein digestion in the small intestine is a very efficient process with digestibility usually ranging from 89 to 95%, depending on the nature of the protein (29, 30). Generally speaking, proteins from animal sources are overall more digestible than proteins from plant sources (31). Some sources of protein, for instance rapeseed protein, are known for their lower digestibility (32). In addition, food cooking (33, 34) and food matrix structure (35) can impact protein digestibility. Importantly, and as a result of incomplete digestion in the small intestine, a residual amount of 165 undigested protein and peptides, together with individual amino acids are transferred through the ileo-166 caecal junction in the large intestine (36). Based on a regular western diet, it has been determined that 167 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 168 increased nearly proportionally when the amount of dietary protein increases (29). From studies 169 170 evaluating the proportion of dietary and endogenous protein which escape digestion and move from 171 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 0.7-172 1.2 g/day (36). The results obtained in animal models suggest that the part ascribed to endogenous 173 174 protein is not vastly different according to the amount of protein consumed (38). Since the large 175 intestine luminal content is characterized by a much more abundant microbiota than what is measured 176 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 177 178 peptidases which release sequentially shorter peptides and amino acids (41). The large intestinal 179 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 180 lumen to the bloodstream, except in the neonatal period (42, 43). Therefore, protein and peptide-181 182 derived amino acids are metabolized by the large intestinal microbiota which use them for protein 183 synthesis and catabolic pathways with the production of numerous intermediates and final metabolites 184 (44); a net amount of these latter being able to accumulate within the luminal content (Figure 1). This 185 process of protein degradation is more active in the distal part than in the proximal part of the large 186 intestine (45). In the case of HPD consumption, the increased transfer of nitrogenous compounds in 187 the large intestine is liable to modify the microbiota composition, and/or to change the microbiota 188 diversity, and/or its metabolic activity, and finally to change the production of bacterial metabolites 189 with possible consequences for the large intestinal mucosa metabolism, physiology and health (46-50) 190 as described below.

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#### 192 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 HPD on the gut microbiota composition (Table 1). Two main factors preclude direct comparison 194 195 between the studies presented in Table 1: (i) differences in energy intake (e.g. calorie restriction) and (ii) differences in fiber intake. These two parameters are known to have a profound influence on the 196 gut microbiota composition and should therefore be considered as important potential confounding 197 factors with the effects of dietary protein intake. Moreover, there are large variations between the 198 199 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 dietary intervention resulted in almost doubling the protein intake (i.e. 30.1% of energy intake) as 207 208 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, Bilophila, and Bacteroides), and by decreasing the levels of Firmicutes that metabolize plant 210 polysaccharides (Roseburia, Eubacterium rectale, and Ruminococcus). Such a HPD was found to 211 change the microbiota  $\beta$ -diversity within 2 days. However, this latter effect appeared to be transient, as 212 the  $\beta$ -diversity returned to the initial configuration within 2 days after the end of the intervention (51). 213 However, these changes could not be attributed solely to the level of protein intake since there was 214 considerable concomitant modification of fat intake (in addition to fiber intake) in this study. 215

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The other studies presented in Table 1 used HPD with caloric restriction that resulted in weight-loss. Two of them (different analysis of the same samples), showed that the HPD induced an alteration of the gut microbiota composition with a decreased abundance of presumed beneficial bacteria such as *Bifidobacterium* or *Rosburia/Eubacterium rectale (52, 53)* However, both resistant starch and total 221 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 222 223 abundance of Bifidobacterium and Eubacterium spp (54, 55).; and a reduction in carbohydrates led to 224 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 225 individuals with low gene counts together with an increase abundance of bacteria considered 226 227 protective such as Faecalibacterium prausnitzii and Roseburi (57). Lastly, two other studies using 228 weight-loss HPD combined with a low fiber intake observed a decrease in the total bacterial biomass 229 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).

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#### 236 3.2 High-protein diets and impact on gut mucosa: potential role of bacterial metabolites

237 The mixture of bacterial metabolites in the intestinal content is complex (59) and far from being fully 238 characterized. Among these compounds, numerous metabolites are produced by the intestinal 239 microbiota from amino acid substrates (60). The concentrations of these metabolites are usually 240 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 241 242 bacterial production from the available substrates, on the bacterial composition and overall metabolic 243 activity, on the absorption through the large intestinal epithelium, and on the transit time (61). (Figure 244 1). Other parameters may influence the concentrations of the different forms of the bacterial 245 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-246 247 ionized and ionized forms of ionic bacterial metabolites (62), which will affect their uptake from the 248 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).

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

258 Several intervention studies in humans have shown that HPD with different sources of dietary protein 259 induce a shift from carbohydrate to protein degradation by the gut microbiota (26, 58, 59, 64), with an 260 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 261 above between human intervention studies regarding the effects of HPD on microbiota composition 262 263 (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, 264 namely amino acids in our case, rather than taxonomic composition of the microbiota for determining 265 266 the metabolic output in the large intestine. This could also be due to redundancy of functions and 267 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-268 derived short-chain fatty acids (SCFA) such as isobutyrate, isovalerate, and 2-methylbutyrate (26, 49, 269 58). In contrast, a decrease in the SCFA butyrate was consistently found after HPD consumption (26, 270 271 51, 56, 58) albeit several of these studies included decreases in fiber content among the HPD. 272 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 273 274 reduction of fecal butyrate concentration in HPD can be attributed primarily to the amount of protein 275 in the diet. As butyrate is well-known as a major oxidative substrate and a regulator of histone acetylation, and thus of gene transcription in human colonocytes (66, 67), the measured decrease in itsfecal 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, HPD were found to increase the concentrations of several S-containing metabolites (59, 68). For most 280 of these metabolites, there is surprisingly no indication on the impact of such changes on the 281 282 colonic/rectal epithelium renewal and functions. However, from in vitro studies with human or rodent colonocytes, there are indications that several amino acid-derived bacterial metabolites including 283 hydrogen sulfide (H<sub>2</sub>S), ammonia and *p*-cresol act as metabolic troublemakers towards colonocyte 284 285 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.

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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 supernatant on human colonocytes. Although fecal water samples do not contain all the luminal 300 301 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 consuming HPD was not more genotoxic than ones recovered from control volunteers consuming 308 309 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 volunteers consuming HPD in short- and medium terms show no increased genotoxic and cytotoxic 313 potential in vitro towards colonic epithelial cells than samples recovered from control NPD. 314

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#### 316 *3.3 High-protein diet and urinary metabolome*

Urinary metabolomic analysis is useful in order to identify the bacterial metabolites and cometabolites 317 318 (produced by the microbiota and metabolized by the host) which have been produced by the gut microbiota, absorbed from the lumen to the bloodstream through the intestinal epithelium (with or 319 320 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 colonocytes (74); and as impaired phenol detoxification has been associated with ulcerative colitis 324 325 (75).

In addition, the cometabolite *p*-cresyl sulfate is produced in the colon mucosa and the liver from the bacterial metabolite *p*-cresol, which itself is produced by the microbiota from the amino acid Ltyrosine (76). Urinary concentration of *p*-cresyl sulfate has been repetitively found to be increased after HPD consumption (26, 59, 77) when compared with control NPD (Table 2). Since *p*-cresol has been shown to inhibit colonocyte oxygen consumption, and to be genotoxic towards colonocytes (70), *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 336 indoxyl sulfate, increased after HPD (Table 2). Since indole, the precursor for the synthesis of indoxyl 337 338 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 339 beneficial for the host. However, in order to establish the beneficial vs. deleterious effects of indole on 340 the colon epithelium, it is important to consider that this bacterial metabolite activates the aryl 341 342 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 343 the deactivation of deleterious compounds and xenobiotics, can catalyse the bioactivation of 344 procarcinogen compounds into carcinogens (87-89). In addition, indoxyl sulfate is suspected to act as 345 346 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.

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#### 352 *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 proinflammatory cytokines in rectal biopsies, and on the fecal concentrations of calprotectin and secretedIgA, when compared with an isocaloric NPD (26).

360 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. 361 For instance, it has been reported that impaired H<sub>2</sub>S detoxification in intestinal mucosa is associated 362 with Crohn's disease (96) and ulcerative colitis (97). These results are important to be taken into 363 364 account, knowing that increased protein consumption is correlated with increased H<sub>2</sub>S fecal excretion in volunteers (68), and that excessive luminal H<sub>2</sub>S decreases colonocyte respiration and increases the 365 expression of several genes involved in IBD in a rodent model (98). It can therefore be predicted that 366 367 there might be differences between individuals in terms of mucosal response to HPD according to 368 individual detoxification capacities.

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#### 371 *3.5 High-protein diets and gene expression in gut mucosa*

372 The first experimental evidence using transcriptomic analysis which has shown that casein-containing 373 HPD can modify gene expression in the colonic mucosa were obtained in the rat model by Mu et al. 374 (99) using a 6-week- dietary intervention protocol with isocaloric experimental (HPD) and control 375 (NPD) diets. Beaumont et al. (100) used a 2 week-intervention protocol with whole milk protein-376 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 377 metabolism and cellular adhesion, when compared with gene expression in colonocytes recovered 378 from isocaloric NPD. In this latter study, the HPD was found to up-regulate the expression of genes 379 380 related to cell proliferation and chemical barrier function. These animal studies allow to establish the 381 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 382 cells. Further, a randomized controlled study with overweight volunteers reported that 3 week-dietary 383 384 supplementation with either casein or soy protein resulted in small amplitude changes in the expression of numerous genes in the rectal mucosa, notably for genes involved in homeostaticprocesses such as cell cycle or cell death (26).

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# 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 rectal mucosa. First, as presented above, different dietary proteins displayed different digestibility 392 393 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, 3hydroxyisovalerate, *p*-cresyl sulfate, phenylacetylglutamine and indoxylsulfate relative concentration; 405 406 while supplementation with sov 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).

More importantly, casein and soy protein HPD were found to differentially modify the expression of genes playing key roles in the maintenance of the rectal mucosa homeostasis maintenance in general, and in colonic health (gastrointestinal diseases and cancer) in particular. At the cellular level, the casein diet was specifically associated with increased expression of genes related to extracellular matrix, cell adhesion, and mucus production; while the soy protein diet was specifically associated 413 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 414 415 proliferation, and cytoskeleton formation were modified by both casein and soy protein (26). To 416 determine if such changes in gene expression impact the rectal epithelium renewal and functions, 417 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 418 419 production was solely increased in the rectal mucosa of volunteers after casein supplementation but 420 not after soy protein supplementation, may indicate an adaptation of the rectal mucosa towards a more 421 aggressive luminal environment following casein-based HPD consumption.

422

#### 423 4. Conclusion and perspectives

424 Although it appears that HPD can help in diminishing the dietary intake, and thus favor weight loss, 425 there are some results which raise new questions on the safety of their utilization. It must be 426 recognized that, according to the available literature, there is no definitive evidence that such diets are 427 deleterious for gut health in short- and medium- term intervention studies conducted so far.

428 Indeed, as presented above, short-term consumption of HPD by itself neither increases the 429 inflammation of the large intestinal mucosa, nor increases the *in vitro* genotoxicity and cytotoxicity of 430 the mixture of compounds contained in the fecal water extracts in healthy subjects. However, HPD 431 have been shown in a repetitive manner to decrease fecal butyrate concentrations. Since butyrate is 432 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 433 434 homeostasis. The same remark can be made regarding the finding that HPD consumption results in 435 increased exposition of the intestinal mucosa to p-cresol, a bacterial metabolite with genotoxic and 436 metabolic troublemaker characteristics towards colonocytes (70). In addition, p-cresol is the precursor 437 of *p*-cresyl sulfate, a cometabolite with reported cytotoxic activity towards renal cells (78, 79) (Figure 438 3). Conversely, there is evidence that HPD increases the exposure of the large intestine mucosa to 439 indole, a bacterial metabolite considered as an important player in the maintenance of the epithelial 440 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 also acting as a uremic toxin (90, 101) (Figure 3). Then, the different effects of bacterial metabolites 442 443 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 considered as overall beneficial or deleterious. The finding that an increased consumption of dietary 445 protein modifies within 3 weeks the normal expression of genes known to be involved in processes 446 447 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 suggest that some of these metabolites might contribute to an improvement of some of these 451 452 parameters. For instance, indole has been shown in vitro to modulate the secretion of the incretin glucagon-like peptide 1 (GLP-1) (102). Moreover, hydrogen sulfide produced by the gut microbiota 453 has been shown to lower blood pressure in rats (103), to improve glucose metabolism, and to increase 454 455 GLP-1 secretion in mice (104). Lastly, several neurotransmitters can be produced by the gut microbiota from amino acids (41), and it can be speculated that this may contribute to the dietary 456 457 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

Although body weight reduction associated with *ad libitum* HPD consumption in overweight and obese individuals is obviously associated with favorable outcomes, the data obtained principally from clinical trials with human volunteers, dietary intervention in animal models, and *in vitro* experiments with human colonic epithelial cells have shown that HPD modifies the luminal environment of the rectal epithelium and impacts gene expression in the mucosa. We therefore recommend caution in the utilization of HPD diets for body weight loss, taking into account the possible regain of body weight

469	after HPD consumption, which may lead to redundant and long-term utilization of HPD. Considering
470	the most recent evidence showing that the effects of HPD on the gut depend on the protein source (i.e.
471	from plant and animal sources), not only the quantity, but also the quality of dietary protein should be
472	considered for further investigations and possibly for future dietary recommendations.
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478	
479	Conflict of interest
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481	competing interest in relation to this paper.
482	
483	Legends of figures:
484	Figure 1. Schematic view of the fate of undigested proteins in case of High-Protein Diet (HPD)
485	consumption.
486	HPD diet consumption increases the transfer of dietary proteins from the ileum to the large intestine.
487	The proteases and peptidases of the microbiota release amino acids which can be incorporated in the
488	bacterial proteins or lead to a multitude of metabolic end products, notably in the distal parts of the
489	large intestine. Some of these metabolites are known to be transferred by the colonic epithelial cells
490	from the luminal content to the portal bloodstream with or without prior metabolism in the
491	colonocytes. The concentrations of bacterial metabolites in the lumen are the net result of
492	production/utilization by the microbiota, and absorption through the colonic epithelium. The
493	metabolites measured in the feces is a reflection of the metabolites present in the rectum.
494	Figure 2. Schematic view of the entry and metabolism of bacterial metabolites in the colonic epithelial

495 cells.

496 Several bacterial metabolites in the luminal content can enter colonocytes by processes of diffusion or 497 transport. Although some of them can be released as such in the bloodstream, several bacterial 498 metabolites are known to undergo intracellular metabolism leading to the production of co-499 metabolites. Bacterial metabolites and co-metabolites can be released in the portal bloodstream and 500 reach the liver and peripheric organs outside the splanchnic area. Finally, these compounds can 501 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.

504 Undigested proteins and peptides enter the large intestine and are metabolized by the microbiota which 505 produce various metabolites from amino acids. Some of these metabolites are partly absorbed through 506 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 507 and metabolites are finally excreted in the urine. HPD consumption results in measurable 508 509 modifications of the concentration of bacterial metabolites in feces and urine. As indicated in the text, 510 some compounds originating from the microbial metabolic activity (like butyrate and H<sub>2</sub>S) are known to impact energy metabolism and gene expression in colonocytes, while some of them (like indole) are 511 implicated in the maintenance of the epithelial barrier function. Some co-metabolites measured in 512 513 urine (like indoxylsulfate and *p*-cresylsulfate) are suspected to act as uremic toxins.

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**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	BMI	Duration	Protein	Protein	Fiber	Calorie	Method	Intestinal microbiota composition	Reference
design				source		restricted	1(0 D))		(26)
n=12-13			14 % E	Mixed	17.0 g/d	No	16S rDNA	Control diet	(26)
Parallel	25 - 30	3 weeks	34 % E	Mixed + casein	14.4 g/d	No	sequencing (feces	No detectable differences	
			31 % E	Mixed + soy protein	17.9 g/d	No	and rectal biopsies)	No detectable differences	
n - 20	19 - 26 er	2 weeks	12 % E	Mixed	17.4 g/d	No	DGGE (feces)	No detectable differences	(45)
Cross over			15 % E	Mixed	16.3 g/d	No		Control diet	
Cross-over			27 % E	Mixed	15.4 g/d	No		No detectable differences	
	19 - 32	5 days	10 % E	Plant protein	41.2 g/d	No	16S rDNA sequencing (feces)	↓ Bilophila wadsworthia	(51)
n = 10			16 % E	Mixed	21.1 g/d	No		Control diet	
Cross-over			30 % E	Animal protein	0 g/d	No		$\uparrow$ Bilophila wadsworthia, Alistipes putredinis; $\downarrow$ Bifidobacterium adolescentis,	
				-	-			Roseburia faecis, Ruminococcus bromii	
n-14	28 - 51	3 weeks	103.3 g/d	Mixed	27.7 g/d	No	Phylogenetic	Control diet	(53)
n=14 Dorollol			144.1 g/d	Mixed	25.1 g/d	Yes	(HITchip)	$\downarrow$ Bifidobacterium, Aerococcus, Granulicatella, Dialister, Papillibacter	
Falallel							microarray (feces)	cinnamivorans; † Lactococcus, Bacteroides vulgatus, Anaerotruncus colihominis	
		3 weeks	103.3 g/d	Mixed	27.7 g/d	No	16S rDNA	Control diet	(52)
II=14 Dam=11-1	28 - 51		144.1 g/d	Mixed	25.1 g/d	Yes	sequencing, DGGE,	$\downarrow$ Collinsella aerofaciens, Roseburia/Eubacterium rectale; $\uparrow$ Oscillibacter	
Parallel							qPCR (feces)	valericigenes	
			19 % E	Mixed	14.5 g/d	No		Control diet	(57)
n=49	33 (mean)	6 weeks	37 % E	Mixed	19.0 g/d	Yes	Metagenomic sequencing (feces)	↓ Eubacterium rectale; ↑ Parabacteroides distasonis, Faecalibacterium prausnitzii,	
Non-								Bacteroides dorei, Parabacteroides merdae, Eubacterium eligens, Ruminococcus sp,	
randomized								Roseburia hominis, Odoribacter splanchnicus, Subdoligranulum sp., Gene richness	
								in low gene counts individuals	
	30 - 49	28 days	13 % E	Mixed	21.9 g/d	No		Control diet	(58)
n=17			28 % E	Mixed	12.8 g/d	Yes	FISH (feces)	↓ total bacteria	
Cross-over		2	29 % E	Mixed	8.8 g/d	Yes		↓ total bacteria, Bacteroides, Roseburia/Eubacterium rectale	
20	30-42	28 days	94.4 g/d	Mixed	27.9 g/d	No		Control diet	(56)
n=20			127.2 g/d	Mixed	11.7 g/d	Yes	FISH (feces)	↓ total bacteria, Roseburia/Eubacterium rectale, Bifidobacterium	
Cross-over		-	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	BMI	Duration	Protein	Protein	Fiber	Calorie	Method	Intestinal microbiota metabolites	Reference
design			intake	source	intake	restricted			
m-12 12	25 - 30	3 weeks	14 % E	Mixed	17.0 g/d	No	NMR metabolomics, GC (feces)	Control diet	(26)
II=12-15 Derellal			34 % E	Mixed + casein	14.4 g/d	No		↓ butyrate; ↑ branched-chain amino acids, 2-methylbutyrate	
Falallel			31 % E	Mixed + soy protein	17.9 g/d	No		$\downarrow$ butyrate; $\uparrow$ 2-methylbutyrate, isovalerate, valerate, phenylacetate, tyramine,	
n=12.13	25 - 30	3 weeks	14 % E	Mixed	17.0 g/d	No	NMR metabolomics (urines)	Control diet	(26)
Dorollal			34 % E	Mixed + casein	14.4 g/d	No		$\uparrow$ isobutyrate, indoxylsulfate, phenylacetylglutamine, <i>p</i> -cresylsulfate	
1 araller			31 % E	Mixed + soy protein	17.9 g/d	No		↑ isobutyrate, indoxylsulfate, phenylacetylglutamine	
n = 10			10 % E	Plant protein	41.2 g/d	No	GC	No detectable differences	(51)
II = 10	19 - 32	5 days	16 % E	Mixed	21.1 g/d	No	(feces)	Control diet	
Closs-over			30 % E	Animal protein	0 g/d	No		↑ isobutyrate, isovalerate; ↓ acetate, butyrate	
n=14	28 - 51	3 weeks	103.3 g/d	Mixed	27.7 g/d	No	GC	Control diet	(53)
Parallel			144.1 g/d	Mixed	25.1 g/d	Yes	(feces)	↑ isobutyrate, isovalerate, lactate; ↓ Acetate, butyrate	
n-20			12 % E	Mixed	17.4 g/d	No	GC-MS	No detectable differences	(45)
Cross-over	19 - 26	2 weeks	15 % E	Mixed	16.3 g/d	No	metabolomics	Control diet	
C1033 0VC			27 % E	Mixed	15.4 g/d	No	(feces)	↑ isobutyrate	
n-20	19 - 26	2 weeks	12 % E	Mixed	17.4 g/d	No	GC-MS (urine)	No detectable differences	(45)
Cross-over			15 % E	Mixed	16.3 g/d	No		Control diet	
C1033 0VC1			27 % E	Mixed	15.4 g/d	No		$\uparrow p$ -cresol	
n-17		28 days	13 % E	Mixed	21.9 g/d	No	GC, LC-MS	Control diet	(58)
Cross-over	30 - 49		28 % E	Mixed	12.8 g/d	Yes		↑ isobutyrate, isovalerate, valerate, phenylacetate	
C1035-0701			29 % E	Mixed	8.8 g/d	Yes	(rees)	$\downarrow$ butyrate; $\uparrow$ isobutyrate, isovalerate, valerate, phenylacetate, phenylpropionate	
n-20	30 - 42	42 28 days	94.4 g/d	Mixed	27.9 g/d	No	GC (feces)	Control diet	(56)
Cross-over			127.2 g/d	Mixed	11.7 g/d	Yes		↓ acetate, propionate, butyrate, valerate, lactate	
C1035-0701			119.5 g/d	Mixed	6.1 g/d	Yes		$\downarrow$ acetate, propionate, butyrate, isovalerate, valerate, lactate; $\uparrow$ ammonia	

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Figure 1



Figure 2

Luminal content





Amino-acid derived bacterial metabolites

Figure 3