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# High-protein diet modifies colonic microbiota and luminal environment but not colonocyte metabolism in the rat model: the increased luminal bulk connection

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**Liu X, Blouin JM, Santacruz A, Lan A, Andriamihaja M, Wilkanowicz S, Benetti PH, Tomé D, Sanz Y, Blachier F, Davila AM.** High-protein diet modifies colonic microbiota and luminal environment but not colonocyte metabolism in the rat model: the increased luminal bulk connection. *Am J Physiol Gastrointest Liver Physiol* 307: G459–G470, 2014. First published June 26, 2014; doi:10.1152/ajpgi.00400.2013.—High-protein diets are used for body weight reduction, but consequences on the large intestine ecosystem are poorly known. Here, rats were fed for 15 days with either a normoproteic diet (NP, 14% protein) or a hyperproteic-hypoglucidic isocaloric diet (HP, 53% protein). Cecum and colon were recovered for analysis. Short- and branched-chain fatty acids, as well as lactate, succinate, formate, and ethanol contents, were markedly increased in the colonic luminal contents of HP rats ($P < 0.05$ or less) but to a lower extent in the cecal luminal content. This was associated with reduced concentrations of the *Clostridium coccoides* and *C. leptum* groups and *Faecalibacterium prausnitzii* in both the cecum and colon ($P < 0.05$ or less). In addition, the microbiota diversity was found to be higher in the cecum of HP rats but was lower in the colon compared with NP rats. In HP rats, the colonic and cecal luminal content weights were markedly higher than in NP rats ($P < 0.001$), resulting in similar butyrate, acetate, and propionate concentrations. Accordingly, the expression of monocarboxylate transporter 1 and sodium monocarboxylate transporter 1 (which is increased by higher butyrate concentration) as well as the colonocyte capacity for butyrate oxidation were not modified by the HP diet, whereas the amount of butyrate in feces was increased ($P < 0.01$). It is concluded that an increased bulk in the large intestine content following HP diet consumption allows maintenance in the luminal butyrate concentration and thus its metabolism in colonocytes despite modified microbiota composition and increased substrate availability.

protein; microbiota; SCFA; transport; metabolism

HIGH-PROTEIN (HP) diets are commonly used for body weight loss (36, 38). In fact, such diets have been shown to increase satiety, to modify lipid metabolism, and to facilitate short- and middle-term weight reduction (24, 61, 72). However, the consequences on gut microbiota composition, on microbial-derived metabolites in the large intestine luminal content, and on colonocyte epithelial cell metabolic capacities have been little studied.

Increasing protein intake induces an increase in the proportion of nitrogenous compounds, mainly protein, peptides, and to a lesser extent amino acids, entering the large intestine (13, 23, 25, 51, 64). They are subjected to luminal proteolysis and subsequent metabolism by the large number of microorganisms of the large intestine microbiota (about $10^{11}$ to $10^{12}$ viable bacteria/g content) (29) leading to the production of numerous amino acid-derived metabolites (53), including phenols, indoles, amines, sulfide, ammonia, and monocarboxylic acids. Monocarboxylic acids include short-chain fatty acids (SCFA), branched-chain fatty acids (BCFA) and several other organic acids. In contrast to SCFA, which are produced from indigestible carbohydrates, other metabolites such as BCFA are produced by the microbiota exclusively from amino acids (55). Changes in diet composition may affect the composition and metabolic activities of the microbiota that adapts to the new colonic luminal environment and substrate availability. Phyla composing the microbiota are known to be quite stable over their lifetime, but the dietary composition influences the repartition of species and functional groups (69). This has been particularly documented for carbohydrates, fibers, and more recently for fat-rich diets (35, 48). In contrast, few studies have addressed the influence of protein-rich diet on the composition and activity of the gut microbiota (8).

The colonic luminal environment may influence colonic epithelial cell metabolic capacities. SCFA and other monocarboxylic acids like lactate are absorbed from the lumen to the colonocyte cytosol by transport processes involving the monocarboxylate transporter 1 (MCT1), the protein CD147 (that participates to the apical expression of MCT1), and the sodium monocarboxylate transporter 1 (SMCT1) (11, 57). The absorbed components are partly metabolized mainly through oxidation pathways and used as energy substrates by colonic epithelial cells, with the unmetabolized fractions being recovered in the portal blood plasma and used by other tissues (3, 45). These bacterial metabolites, and particularly butyrate, are involved in numerous processes regarding colonocytes metabolism and physiology, including expression of MCT1 (10, 16), sodium-dependent fluid absorption (6), cell proliferation (20), histone acetylation, and gene expression (1, 20). Indeed, butyrate is considered as the preferential oxidative substrate for colonocytes (19, 58). In contrast to butyrate and propionate, which are mainly metabolized by the intestinal mucosa and liver, acetate is recovered in significant amount in the systemic blood (45).

The present study aims to evaluate the influence of HP diet on the composition of the intestinal microbiota, on the com-

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Table 1. *Composition of the experimental diets*

| | NP | HP |
|---|---|---|
| Ingredients, g/kg | | |
| Whole milk proteins | 140 | 530 |
| Sucrose | 100 | 45.7 |
| Corn starch | 622.4 | 287 |
| Mineral mixture AIN 93-M[#] | 35 | 35 |
| Vitamin mixture AIN 93-V[#] | 10 | 10 |
| Soybean oil | 40 | 40 |
| Cellulose | 50 | 50 |
| Choline | 2.3 | 2.3 |
| Energy, kJ/g | 14.6 | 14.6 |

NP, normoproteic diet; HP, high-protein diet.[#]Data from Ref. 56.

position of SCFA, BCFA, and other organic acids in cecal and colonic contents, and on putative modifications of energy metabolism in colonic epithelial cells. The central role of butyrate on colonocyte physiology suggests the necessity of an exquisite regulation of butyrate availability for both energy production and gene expression in these cells (1, 2, 34, 62, 65). Thus, we evaluated if the modifications of the gut ecosystem following HP diet ingestion are associated with metabolic changes toward butyrate (and other energy substrates) in isolated colonic epithelial cells or if, conversely, metabolic homeostasis is maintained in these cells after such a dietary modification.

## MATERIALS AND METHODS

*Animals, diets, and colonocyte isolation.* Wistar male rats (150 g) were acclimatized for 6 days to receive a standard rodent diet containing 16% (wt/wt) protein. Then they were randomly allocated to either normoproteic diet (NP) diet containing 14% (wt/wt) protein or HP isocaloric diet (by decreasing digestible carbohydrates) containing 53% (wt/wt) protein (Table 1). After 15 days of experimental diet, animals were anesthetized with pentobarbital sodium (40 mg/kg body wt), and total cecal and colonic luminal contents were collected by expulsion. Luminal contents (0.2 g) were taken after homogenization for bacterial DNA extraction, and the remaining was frozen in liquid nitrogen and kept at −80°C for subsequent analysis. The whole colon was used for colonocyte isolation using the perfusion method with EDTA as described (52). The present protocol received written agreement from the local animal ethical committee (COMETHEA at Jouy-en-Josas, France, Nos. 11/042 and 12/090).

*Bacterial DNA extraction.* Bacterial DNA extraction of fresh cecal and colonic luminal contents was performed with a QIAamp DNA Stool Mini kit (Qiagen). The DNA concentration and purity were measured with Nanodrop. The DNA integrity was confirmed by electrophoresis on agarose gel with Gelred staining. Extracted bacterial DNA was normalized to 30 ng/μl for subsequent real-time qPCR assay.

*Analysis of microbiota composition by real-time qPCR.* The main bacterial groups of the intestinal microbiota were quantified by real-time qPCR using specific primers (Table 2) and SYBR Green I Master mix (Roche Diagnostics) in a LightCycler 480 instrument (Roche Diagnostics) as previously described (59). The bacterial groups included total bacteria, *Bifidobacterium*, *Lactobacillus* group, *Bacteroides fragilis* group, *Clostridium coccoides* group, *C. leptum* group, *Enterobacteriaceae*, *Desulfovibrio* spp., and *Faecalibacterium prausnitzii*. Standard curves of each bacterial group were generated from serial dilutions of a known copy number of the target gene cloned into a plasmid vector. For each reference strain, the 16S rRNA gene was cloned into a pGEM-T Easy Vector System (Promega). An *Escherichia coli* strain was transformed with the recombinant plasmid, and plasmid DNA was extracted from *E. coli* by the miniprep method.

*Denaturing gradient gel electrophoresis.* PCR amplifications with universal primers and denaturing gradient gel electrophoresis (DGGE) analysis were performed as described (60). Renyi's entropy (diversity profile) was used to explore differences in bacterial species detected by DGGE and DNA sequencing between the different diets. The formula of Renyi's entropy is:

$$H\alpha = \frac{\ln\left(\sum_{i=1}^{S} P_i^{\alpha}\right)}{1-\alpha}$$

where $S$ is the number of species in the sample and $P_i$ is the proportion of the species $i$ in the sample. Diversity profiles are calculated by changing the value of α from zero to infinity. Their graphical display provides diversity ordering of samples. A sample profile always above the profile of another sample indicates a higher diversity. The shape of the profile is an indication of evenness. The flatter the curve is, the higher the evenness is. Renyi's entropy also provides information on

Table 2. *Primers used for bacterial DNA amplification for microbiota composition analysis*

| Target Bacterial Group/Species | Designation | Primer (5′-3′) Squence | Annealing Temperature, °C |
|---|---|---|---|
| Total bacteria | HAD F | TGGCTCAGGACGAACGCTGGCGGC | 59[a] |
| | HAD R | CCTACTGCTGCCTCCCGTAGGAGT | |
| *Bifidobacterium* | g-Bifid F | CTCCTGGAAACGGGTGG | 55[b] |
| | g-Bifid R | GGTGTTCTTCCCGATATCTACA | |
| *Lactobacillus* group | Lac F | AGCAGTAGGGAATCTTCCA | 61[c] |
| | Lac R | ATTYCACCGCTACACATG | |
| *Bacteroides fragilis* group | Bfra 531 F | ATAGCCTTTCGAAAGRAAGAT | 50[b] |
| | Bfra 766 R | CCAGTATCAACTGCAATTTTA | |
| *Clostridium coccoides* group | Ccoc F | AAATGACGGTACCTGACTAA | 50[b] |
| | Ccoc R | CTTTGAGTTTCATTCTTGCGAA | |
| *C. leptum* group | Clep F | GCACAAGCAGTGGAGT | 50[d] |
| | Clep R | CTTCCTCCGTTTTGTCAA | |
| *Enterobacteriaceae* | Entero F | CATTGACGTTACCCGCAGAAGAAGC | 63[c] |
| | Entero R | CTCTACGAGACTCAAGCTTGC | |
| *Defulfovibrio* spp. | Desulf.spp F | GGTACCTTCAAAGGAAGCAC | 58[e] |
| | Desulf.spp R | GGGATTTCACCCCTGACTTA | |
| *Faecalibacterium prausnitzii* | F.praus F | CCCTTCAGTGCCGCAGT | 61[e] |
| | F.praus R | GTCGCAGGATGTCAAGAC | |

F, forward primer; R, reverse primer. The program of PCR was as follows: initial denaturation at 95°C for 5 min; 40 cycles of denaturation at 95°C for 10 s, annealing at primer-specific temperature for 10 s and extension at 72°C for 30 s; and melting curve at 95°C for 5 s, 65°C for 1 min, and 98°C continuous followed by cooling to 40°C. Data from Refs. 70 (a), 49 (b), 12 (c), 50 (d), and 47 (e).



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three further diversity index values: species richness for $\alpha = 0$, Shannon index for $\alpha = 1$, and Simpson index for $\alpha = 2$. (68).

*Analysis of SCFA, BCFA, and other organic acids in luminal contents and in feces.* Cecal and colonic contents recovered at death were immediately weighted. The feces were recovered during the 3 days before death. All of the organic acids and ethanol were extracted from contents and feces by vigorous homogenization with diluted ultrapure water followed by centrifugation (14,000 *g*, 15 min at 4°C). The SCFA and BCFA in the supernatants were then derivatized by esterification and analyzed with a gas chromatograph equipped with a capillary column (30 m, 0.32 mm ID; RestekRtx 502.2) and fitted with a flame ionization detector using a modification of the method of Kristensen et al. (39). The amounts of SCFA and BCFA were determined by external standards with reference to internal standards. Formate, L- and D-lactate, succinate, and ethanol were measured in cecal and colonic samples using the Megazyme biochemical assay kits (Megazyme) according to the manufacturer's instructions. To calculate the percentages of ingested energy lost in feces as SCFA, we used the corresponding values as defined by Livesey and Elia (42) with acetate representing 3.48 kcal/g, propionate representing 4.93 kcal/g, and butyrate representing 5.92 kcal/g.

*Metabolic assays in isolated colonocytes.* Isolated colonocytes were resuspended in Krebs-Henseleit bicarbonate-buffered medium (pH 7.4), enriched with 10 mg/ml BSA. The incubation medium was equilibrated against a mixture of $O_2$-$CO_2$ (19:1, vol/vol). D-Glucose, L-glutamine, n-butyrate, and L-lactate oxidation by isolated colonocytes was carried out at 37°C in the incubation medium containing 5 mM D-glucose containing tracer amounts of D-[1-$^{14}$C]glucose, 1 mM L-glutamine containing tracer amount of L-[U-$^{14}$C]glutamine, 2 mM sodium butyrate containing tracer amount of n-[1-$^{14}$C]butyrate (sodium salt), or 2 mM L-lactate containing tracer amount of L-[U-$^{14}$C]lactate. The incubation was stopped with perchloric acid (3% final concentration), and radioactive $CO_2$ was measured by liquid scintillation after trapping in methylbenzethonium hydroxide. Lactate production by colonocytes from 5 mM D-glucose was determined in the incubation medium using a colorimetric system as described by the manufacturer's instructions (Biovision/Clinisciences).

*Gene expression analysis in colonocytes.* Total RNA was extracted from isolated colonocytes using Trizol extraction derived from the method of Chomczynski and Sacchi (14). A subsequent step of DNase and purification was done with the RNeasy Mini Kit and DNase I (Qiagen). Next, sample quality was controlled by the 260-to-280 nm absorption ratio measured with Nanodrop (between 1.8 and 2.0) and checked on 1% agarose gel. For real-time RT-PCR analyses, 1 μg total RNA was first reverse transcribed using the High Capacity cDNA Archive kit (Applied Biosystems). Reverse-transcribed RNA (5 ng of cDNA) were amplified on a thermal cycler (ABI prism 7900 HT; Applied Biosystems) using the SYBR green fluorescence method and specific primers (Table 3). Results were analyzed with the SDS 2.1 real-time detection system software. Quantification of RNA was carried out by comparison of the number of cycles required to reach reference and target threshold values ($\delta$-$\delta C_t$ method) and was normalized using Ribosomal Protein L13a (*Rpl13a*) mRNA.

*Western blot analysis of MCT1 and SMCT1 in colonocytes.* The cell surface expression of MCT1 and SMCT1 was achieved using the cell surface protein isolation kit (Pierce Biotechnology). Briefly, surface proteins of isolated colonocytes were biotinylated with the membrane-impermeant reagent Sulfo-NHS-LC-Biotin during 30 min at 4°C. The reaction was quenched, and, after a washing step, membrane proteins were solubilized by incubation in Triton X-100-containing buffer. Biotinylated proteins were bound to ImmunoPure immobilized streptavidin (Pierce), washed three times, and then dissolved in 62.5 mM Tris·HCl, pH 6.8, with 50 mM DTT.

Equal amounts of protein were loaded on 4–12% NuPAGENovexBis-Tris gel (Invitrogen) and run using 1× NuPage MES buffer. The XCellSureLock Mini Cell (Invitrogen) was used for electrophoresis. Protein was then transferred to Hybond ECL membranes that were blocked for 2 h at room temperature using 1× Tris-buffered saline (TBS, pH 7.5), 0.05% Tween 20, and 5% (wt/vol) nonfat dry milk and incubated overnight (4°C) with polyclonal antisera raised against rat MCT1 (1:5,000; Millipore) or rat SMCT1 (1:500; Santa Cruz Biotechnologies) diluted in 1× TBS (pH 7.5), 0.05% Tween 20, and 5% (wt/vol) BSA. Nitrocellulose membranes were then washed with 1× TBS and 0.05% Tween 20 and incubated for 1 h at room temperature with horseradish peroxidase-linked secondary antibody anti-chicken IgG to reveal MCT1 or anti-goat to reveal SMCT1. Bound antibody was visualized using the ECL (Enhanced ChemiLuminescence) system according to the manufacturer's instructions (Pierce Biotechnology).

*Immunofluorescence analysis of MCT1 and SMCT1 in colon.* For immunohistochemical studies, segments of colon were immediately fixed in 4% paraformaldehyde and embedded in paraffin. Four-micrometer tissue sections were subjected to deparaffinization and washed with TBS plus 0.025% Triton X-100. Nonspecific binding sites were then blocked for 2 h at room temperature with TBS plus 1% BSA and 10% normal serum. Next, the sections were incubated with MCT1 antibody (1:500) and SMCT1 antibody (1:100) diluted in TBS plus 1% BSA in a humidified chamber at 4°C overnight. Subsequently, the sections were washed two times in TBS plus 0.025% Triton X-100 and exposed for 1 h to AlexaFluor 488-conjugated secondary antibodies diluted in TBS plus 1% BSA. After being washed in TBS, sections were mounted using Vectashield mounting medium (Vector Laboratories).

*Statistical analysis.* The results are expressed as mean values ± SE. Statistical analyses were performed by using SAS 9.1 (SAS Institute, Cary, NC). Rats' body weight and food intake analysis were performed using the mixed model for repeated-measure analysis, and the differences between NP and HP groups at each time point are revealed by Tukey's post hoc test. The Welch's *t*-test was used to compare two groups. The difference in microbiota composition was analyzed by Mann-Whitney-Wilcoxon test. The prevalence of bacterial species identified by PCR-DGGE was analyzed by Fisher's test. Differences with $P$ values $<0.05$ were considered as statistically significant.

## RESULTS

*Body weight gain and weight of the cecal and colonic contents.* Differences in rat weight gain of the HP group compared with the NP group were significantly more marked in the second half of diet intervention (Fig. 1*A*), whereas the differences in their daily food intake were more marked in the

Table 3. *Primers used for gene expression analysis in isolated rat colonocytes*

| RNA | Accession No. | Forward Primer (5′-3′) Sequence | Reverse Primer (5′-3′) Sequence |
|---|---|---|---|
| *Mct1* | NM 012716.2 | GCAACGACCAGTGAAGTGT | AAGCCCAAGACCTCCAAT |
| *Smct1* | NM 001191987.1 | CTGGGCTTGTTTTCTTTGG | CGTTGTGCGTGCTGTTAC |
| *Cd147* | NM 001109882.1 | AGTGAGGGAGAGTTTGTGAAG | TCAGTGCCGTTGGAGATAG |
| *Rpl13a* | NM 173340.2 | GGATCCCTCCACCCTATGACA | CTGGTACTTCCACCCGACCTC |

*Mct1*, monocarboxylate transporter 1; *Smct1*, sodium monocarboxylate transporter 1. The program of PCR was as follows: 95°C for 10 min, followed by 40 cycles of 15 s at 95°C, 1 min at 60°C.



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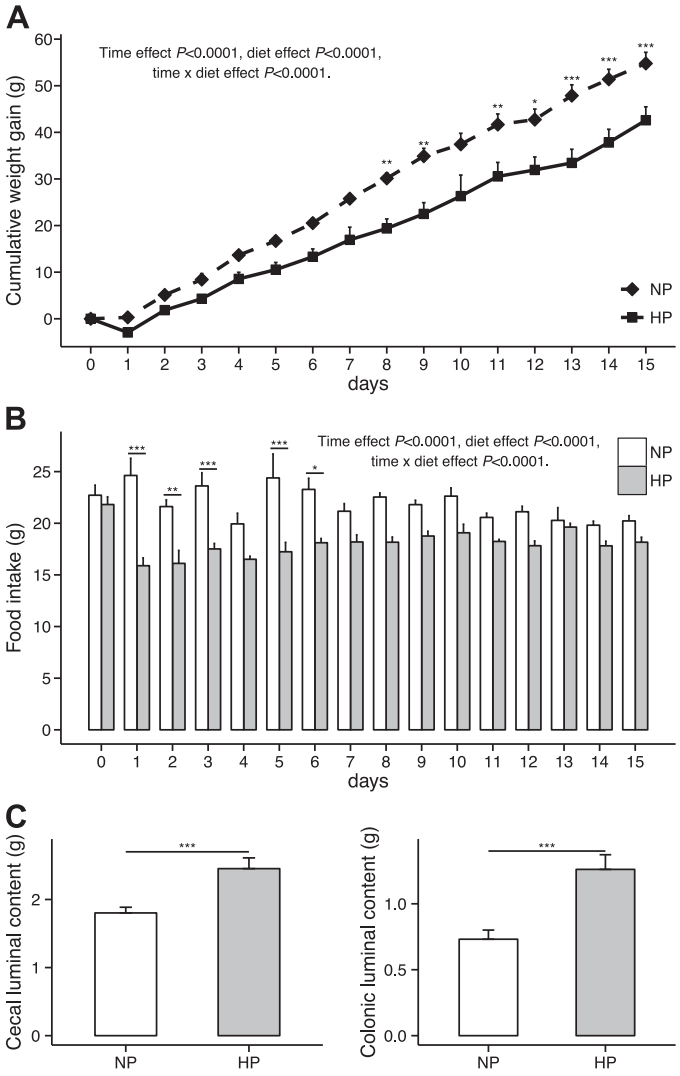



Fig. 1. Characteristics of animals after normoproteic diet (NP)/hyperproteic-hypoglucidic isocaloric diet (HP) feeding for 15 days. Cumulative body weight gain (*A*), food intake (*B*), and weight of the cecal and colonic luminal contents (*C*) recovered from rats. The results were obtained from 8 animals in each group and are expressed as means ± SE. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

first half (Fig. 1*B*). The weight of the cecal and colonic luminal contents was increased by 136 and 172%, respectively, in HP animals compared with NP animals (Fig. 1, *C* and *D*).

*Microbiota composition, biodiversity, and species prevalence.* The results of the effects of the HP diet on gut microbiota composition determined by real-time PCR are shown in Table 4. The total bacterial concentration was slightly but significantly decreased (1.3-fold) in the colon of HP animals compared with NP animals. However, total bacterial concentrations detected in the cecal luminal contents were similar between NP and HP animals. *C. coccoides* group gene copy numbers had a 4.3- and 1.6-fold decline in cecum and colon of HP animals, respectively, compared with NP diet. A marked decrease of *C. leptum* group gene copy numbers was also detected in cecum and colon of the HP group with, respectively, 2.2- and 3.4-fold change compared with NP animals. Finally *F. prausnitzii* gene copy numbers were dramatically decreased in both cecum (2.3-fold) and colon (4.6-fold) after HP diet. The concentrations of *Bifidobacterium*, *Lactobacillus* group, *B. frafilis* group, *Enterobacteriaceae*, and *Desulfovibrio* spp. did not differ significantly between the HP and NP groups.

Bacterial community fingerprint in the cecal and colonic luminal contents was analyzed by PCR-DGGE. The DNA banding pattern obtained was used to determine the species diversity with the Renyi's entropy (Fig. 2). HP diet consumption compared with NP diet increased diversity, richness, and evenness in cecum. By contrast, HP diet consumption decreased diversity and richness in colon but had no effect on evenness. Whatever the diet, gut microbiota displayed greater diversity and richness in cecal contents than in colonic contents. With NP diet, evenness was lower in cecum than in colon. With HP diet, evenness became similar in cecum and colon.

The assignment of PCR-DGGE bands to bacterial species (Table 5) shows an increased number of different bacterial species identified by PCR-DGGE (>90% identity in the GenBank using the BLAST algorithm) in cecal content than in colonic content. Several of these bacterial species had significantly higher or lower prevalence with the HP diet depending on the bacterial species. *Clostridium* sp. had significantly lower prevalence (0%) in both colonic and cecal contents with the HP diet than with the NP diet. These results are consistent with that obtained by the absolute quantification by real-time PCR, where two important groups of *Clostridium* were decreased. *B. vulgatus* was also identified in both cecal and colonic contents, with an increased prevalence after the HP diet only in cecal content. The prevalences of *B. dorei* and *Fusobacterium* sp. were different according to the diet and opposite depending on the large intestine segment considered. Indeed, their prevalence increased significantly in cecal content, whereas in colonic content their prevalence decreased. *E. coli* was identified only in cecal content, where its prevalence was significantly lower in HP- than in NP-fed animals.

*Organic acids in cecal and colonic luminal contents.* The total quantity of organic acids in the luminal contents mainly follows the mass of the luminal contents. When calculating their quantity in the whole cecal contents, the total amount of acetate, isobutyrate, isovalerate, formate, and succinate was significantly higher, whereas the total amount of L-lactate, D-lactate, and ethanol was not significantly different in the HP group compared with the NP group (Fig. 3, *A* and *B*, and Table 6). For the quantity in the whole colonic content, the total amounts of acetate, propionate, butyrate, valerate, isobutyrate, isovalerate, L-lactate, D-lactate, succinate, and ethanol were significantly higher except formate in the HP group compared with the NP group (Fig. 3, *C* and *D*, and Table 6). However, when these results were expressed as concentrations in cecal and colonic luminal contents, the concentrations of SCFA were not significantly different after HP diet compared with NP diet (Table 7).

In the feces, the total quantity in acetate, propionate, and butyrate recovered for the three last days was 1.9-, 2.6-, and 2.1-fold higher for HP animals compared with NP animals (Fig. 3*E*). Figure 3*F* shows the relative amounts of energy lost as SCFA in feces expressed as percentages of energy intake.

*Energy substrate metabolism and expression of monocarboxylic and sodium transporters in isolated colonocytes.* As indicated in Fig. 4*A*, the oxidation of butyrate was not signif-



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Table 4. *Microbiota composition in the cecal and colonic luminal contents recovered from rats after NP/HP feeding for 15 days determined by real-time qPCR*

| Bacterial Group | NP | | HP | | *P* Value |
|---|---|---|---|---|---|
| | Median | IQR | Median | IQR | |
| Cecal luminal contents | | | | | |
| Total bacteria | 13.3 | 13.1–13.5 | 13.3 | 13.2–13.4 | 0.910 |
| *Bifidobacterium* | 6.6 | 5.6–7.9 | 7.0 | 6.6–7.5 | 0.307 |
| *Lactobacillus* group | 8.5 | 8.0–8.9 | 8.3 | 7.9–8.8 | 0.495 |
| *Bacteroides fragilis* group | 11.1 | 10.9–11.2 | 11.2 | 11.2–11.3 | 0.124 |
| *C. coccoides* group | 10.7 | 10.6–10.8 | 9.2 | 7.7–10.2 | 0.028* |
| *C. leptum* group | 10.9 | 10.7–11.0 | 10.1 | 9.8–10.3 | 0.006* |
| *Desulfovibrio* spp. | 10.2 | 10.0–10.3 | 10.1 | 9.9–10.1 | 0.248 |
| *F. prausnitzii* | 8.5 | 8.3–8.9 | 7.7 | 7.4–8.0 | <0.001* |
| *Enterobacteriaceae* | 10.6 | 10.4–10.7 | 10.6 | 10.2–10.9 | 0.909 |
| Colonic luminal contents | | | | | |
| Total bacteria | 12.4 | 12.4–12.5 | 12.2 | 12.1–12.3 | 0.002* |
| *Bifidobacterium* | 7.4 | 7.1–7.6 | 7.0 | 6.9–7.2 | 0.052 |
| *Lactobacillus* group | 9.2 | 8.7–9.5 | 8.6 | 8.1–9.2 | 0.218 |
| *B. fragilis* group | 11.6 | 11.3–11.8 | 11.4 | 11.1–11.6 | 0.248 |
| *C. coccoides* group | 11.0 | 11.0–11.1 | 10.5 | 10.4–10.7 | <0.001* |
| *C. leptum* group | 11.8 | 11.7–11.8 | 10.6 | 10.5–11.1 | <0.001* |
| *Desulfovibrio* spp. | 9.1 | 8.9–9.3 | 9.1 | 9.0–9.3 | 0.739 |
| *F. prausnitzii* | 6.7 | 6.6–6.9 | 5.2 | 5.1–5.5 | 0.001* |
| *Enterobacteriaceae* | 10.7 | 10.5–10.9 | 10.6 | 10.2–10.8 | 0.436 |

IQR, interquartile range. Results are expressed as log of gene copy numbers of the different bacterial groups/species per gram of wet luminal content and were obtained from 8 animals in each group. *Statistically significant difference.

icantly different in colonocytes isolated from NP and HP animals and neither was the oxidation of the substrates originating from blood, i.e., L-glutamine and D-glucose. Furthermore, the production of L-lactate from D-glucose in isolated colonocytes was similar in NP and HP animals (241 ± 27 and 232 ± 42 nmol·mg protein$^{-1}$·30 min$^{-1}$, respectively, $n = 9$ in each experimental group). Last, the oxidation of L-lactate was also found to be similar in colonocytes from NP and HP animals, averaging, respectively, 3.41 ± 0.87 and 4.44 ± 1.87 nmol·mg protein$^{-1}$·30 min$^{-1}$ ($n = 3$ in each experimental group).

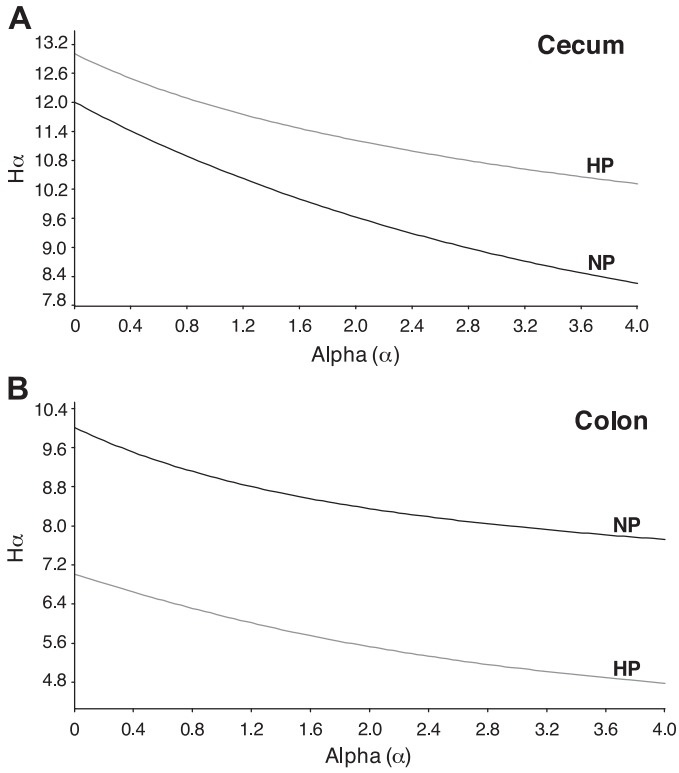



Fig. 2. Renyi's diversity profiles of microbiota in luminal content of rats after NP/HP feeding for 15 days. *A*: diversity profiles in the cecal luminal contents; *B*: diversity profiles in the colonic luminal contents. Results were obtained from 8 animals in each group.

As indicated in Fig. 4*B*, the mRNA levels of the two monocarboxylic acid transporters *Mct1* and *Smct1*, as well as that of *Cd147* (CD147 involved in the apical expression of MCT1 participating in the transport of monocarboxylic acids in colonic epithelial cells), were not significantly different between NP and HP groups. As shown in Fig. 4*C*, with the use of Western blot analysis of cell membrane proteins recovered from colonocytes isolated after NP and HP diet consumption, the amounts of MCT1 and SMCT1 were similar in both groups of animals. Last, by visualizing MCT1 and SMCT1 using immunofluorescence technique, our results indicate similar immunolocalization of both monocarboxylic acid transporters in NP and HP colonocytes (Fig. 4*D*).

## DISCUSSION

The results obtained in the present study clearly indicate that rats, after the consumption of the HP diet compared with the NP diet, were characterized in the large intestine by an increase in the mass of its luminal content, a shift in microbiota composition, and marked changes in the amount of organic acids, particularly in the colon.

Analysis of bacterial biodiversity with PCR-DGGE strongly suggests that the gut microbiota is deeply reshaped following HP diet ingestion. The fluctuations of biodiversity markers (diversity and richness) suggest changes at the species level resulting from adaptation of the microbiota to the new intestinal environment created by the HP diet. The large intestine microbiota of rats submitted to HP diet had to cope with the



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Table 5. *Prevalence of bacterial species identified in the cecal and colonic luminal contents by PCR-DGGE analysis and subsequent DNA sequencing*

| Bacterial Species | Accession No. | Identity, % | No. of Positive Cases/Total Cases (%) | | *P* Value |
|---|---|---|---|---|---|
| | | | NP | HP | |
| Cecal luminal contents | | | | | |
| *Actinobacteium* sp. | HQ675413 | 98 | 4/8 (50) | 7/8 (87) | 0.282 |
| *Alistipes massiliensis* | JF824799 | 95 | 3/8 (37) | 0/8 (0) | 0.100 |
| *Bacteroides bacterium* | GU957728 | 97 | 0/8 (0) | 4/8 (50) | 0.076 |
| *B. chinchilla* | AB547637 | 94 | 4/8 (50) | 8/8 (100) | 0.076 |
| *B. dorei* | JF 298878 | 98 | 3/8 (37) | 8/8 (100) | 0.025* |
| *B. sartorii* | AB572597 | 95 | 4/8 (50) | 1/8 (12) | 0.282 |
| *B. uniformis* | JF298891 | 97 | 2/8 (25) | 4/8 (50) | 0.363 |
| *B. vulgatus* | EFG22256 | 99 | 0/8 (0) | 7/8 (87) | 0.001* |
| *Blautia luti* | AJ133124 | 99 | 4/8 (50) | 4/8 (50) | 1.000 |
| *Clostridium* sp. | AB610549 | 96 | 4/8 (50) | 0/8 (0) | 0.025* |
| *Escherichia coli* | JQ219679 | 100 | 8/8 (100) | 3/8 (37) | 0.025* |
| *Fusobacterium* sp. | AF342858 | 99 | 0/8 (0) | 6/8 (75) | 0.006* |
| *Prevotella* sp. | DQ273884 | 98 | 7/8 (87) | 5/8 (62) | 0.569 |
| *Rhodospirillum* sp. | HM998908 | 91 | 0/8 (0) | 4/8 (50) | 0.076 |
| *Ruminococcus* sp. | DQ882650 | 98 | 2/8 (25) | 0/8 (0) | 0.466 |
| *Verrumicrobia bacterium* | JN65682 | 93 | 1/8 (12) | 4/8 (50) | 0.282 |
| Colonic luminal contents | | | | | |
| *Actinobacterium* sp. | HQ675413 | 98 | 7/8 (87) | 8/8 (100) | 0.335 |
| *Bacteroides bacterium* | GU957728 | 96 | 0/8 (0) | 4/8 (50) | 0.076 |
| *B. dorei* | EU722737 | 92 | 5/8 (62) | 0/8 (0) | 0.025* |
| *B. fibrisolvas* | AJ222769 | 97 | 3/8 (37) | 0/8 (0) | 0.100 |
| *B. sartorii* | AB572597 | 95 | 6/8 (75) | 5/8 (62) | 0.641 |
| *B. vulgatus* | DQ100446 | 96 | 3/8 (37) | 2/8 (25) | 0.641 |
| *Clostridium* sp. | FJ625862.1 | 93 | 5/8 (62) | 0/8 (0) | 0.025* |
| *Enterococcus* sp. | AY683831 | 95 | 1/8 (12) | 5/8 (62) | 0.118 |
| *Fusobacterium* sp. | AF342858 | 98 | 7/8 (87) | 2/8 (25) | 0.040* |
| *Lachnospiraceae* | AB186803 | 91 | 2/8 (25) | 0/8 (0) | 0.466 |
| *Ruminococcus bromii* | DQ882649 | 94 | 5/8 (62) | 2/8 (25) | 0.314 |

PCR-denaturing gradient gel electrophoresis (DGGE) of DNA from the luminal contents recovered from rats after NP/HP feeding for 15 days, using the universal primers HAD-F and HAD-R. *Statistically significant difference.

increased proportion of protein while ensuring the energetic stability of the ecosystem. For instance, HP diet did not influence *B. fragilis* group gene copy number; nevertheless, it increased the prevalence of *B. vulgatus* and *B. dorei* in cecum. *B. vulgatus* is one of the most proteolytic species among the *B. fragilis* group, and it is also known that *Bacteroides* are one of the gut colonizers that obtains the energy from amino acid fermentation contributing to SCFA generation (30). Recently the *Bacteroides* enterotype has also been associated with increased intake of dietary protein in humans (75). In both segments of the large intestine, gene copy numbers of *C. coccoides* group, *C. leptum* group, and *F. prausnitzii* (a major member of the *C. leptum* group) were significantly decreased. The HP diet did not change the other bacterial groups analyzed, but, in colon, the gene copy numbers of total bacteria were slightly but significantly decreased under the HP diet even if the difference measured is close to the variation window of real-time PCR. The changes in clostridia are important since these clusters account for >90% of *Firmicutes*, which is one of the two main phyla colonizing the human gut (21). Some butyrate-producing bacteria are known to belong to the clostridial cluster XIVa, *C. coccoides* group (4), and *F. prausnitzii* is also considered as a butyrate-producing bacteria (43). The reduction of these bacterial group numbers in response to low-carbohydrate diets has also been related to reduction in SCFA generation in humans. More recent studies of the fecal microbiota of human vegetarian and omnivores have also reported that the butyryl-CoA-transferase gene, which is associated with microbial butyrate production, highly correlated with *Clostridium* cluster XIVa and *Roseburia-E. rectale* abundance and to a lesser extent with *C. leptum* and *F. prausnitzii* abundance and with crude fiber intake (37). The fact that, in the present study, these bacteria were decreased after HP diet consumption at the expense of carbohydrates is coherent with previous studies. However, the understanding of the specific microbial groups involved in protein metabolism and their relation to SCFA generation in the large intestine is rather limited, and other taxa and genes may be switched on to take over the role that seems to be played by members of clostridial cluster XIVa under a high-carbohydrate diet in the context of a HP diet. Thus, from our results, it appears difficult to assign to what extent the modified luminal content in bacterial metabolites is due to changes in substrate availability compared with changes in microbiota composition.

The enhancement of diversity, richness, and evenness (which was found in cecum) is perceived as beneficial for microbial ecosystems while their reduction (which was found in colon) is considered as unfavorable. In cecum, protein fermentation is believed to be lower than in the colon and particularly compared with the distal colon. However, the fact that we measured a significant amount of BCFA (which derive exclusively from amino acid fermentation) in the cecum indicates that protein fermentation already occurs there.

It is worth noting that the decrease of *C. leptum* and *C. coccoides* groups and *F. prausnitzii* in gut microbiota was previously associated with the active phase of inflammatory



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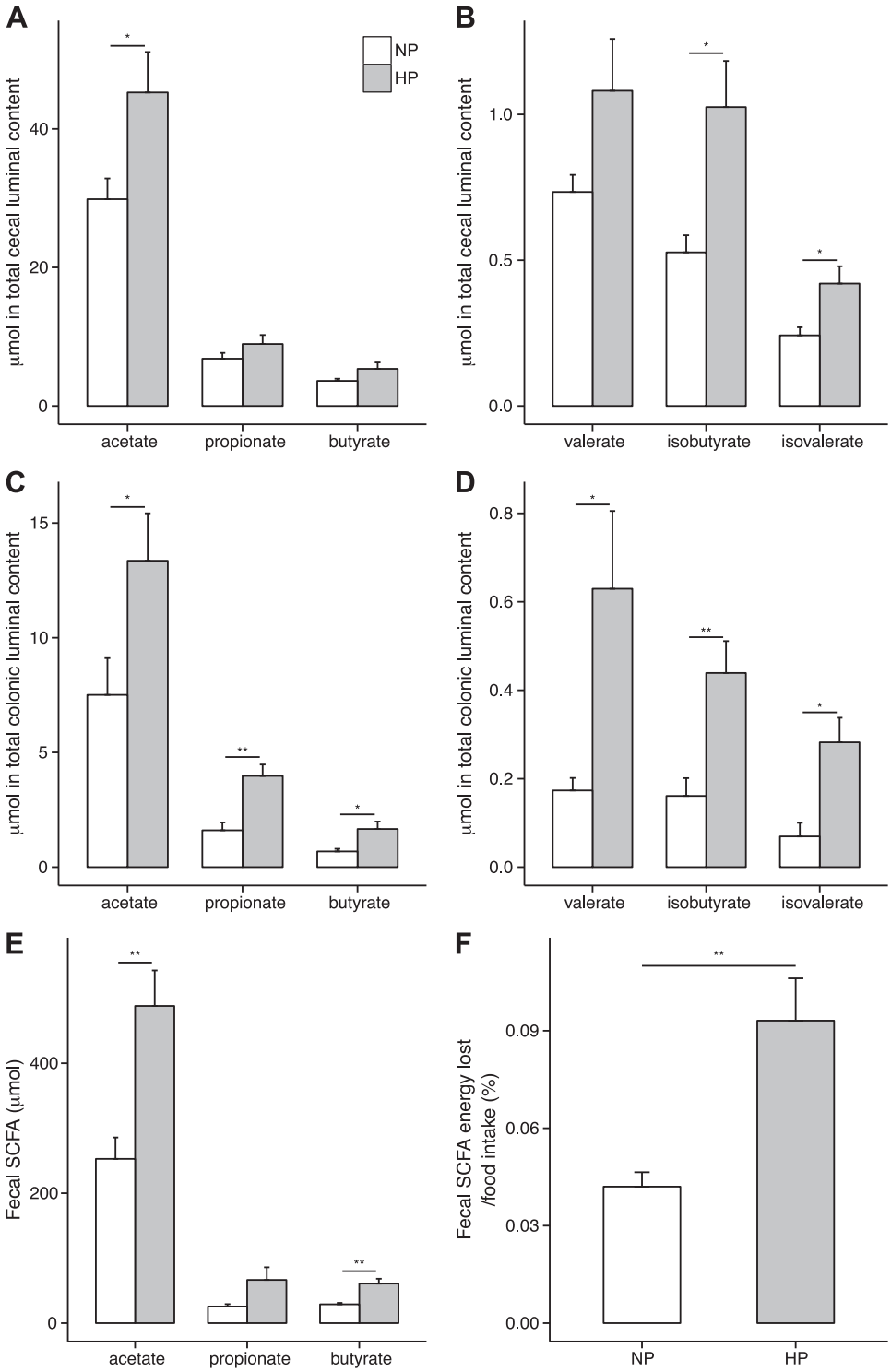



Fig. 3. Total amounts of short-chain fatty acids (SCFA) in the large intestine's luminal contents and in feces recovered from rats after NP/HP feeding for 15 days. *A* and *B*: SCFA and branched-chain fatty acids (BCFA) in the cecal luminal contents; *C* and *D*: SCFA and BCFA in the colonic luminal contents; *E*: total fecal SCFA; and *F*: fecal SCFA expressed as percentages of energy loss. The feces were recovered for the last 3 days before death. To calculate the energy loss as SCFA, total energy in the food ingested is taken as 100%. Results are presented as means ± SE and were obtained from 8 animals in each group, $*P < 0.05$ and $**P < 0.01$.

bowel diseases (67). In addition, anti-inflammatory effects of *F. prausnitzii* in the trinitrobenzene sulfonate-induced colitis model were also reported (66). Next, it can be hypothesized that HP diet ingestion may create an unfavorable luminal condition in terms of microbiota composition in the large intestine in the case of subsequent inflammatory episodes. Further work, out of the scope of the present study, is necessary to document this new working hypothesis.

HP rats showed a marked increase of the total content of almost all the measured monocarboxylic acids in the colon luminal contents. In cecal content, the situation was not vastly different but less marked.

These results are compatible with the view that, despite lower food intake in the HP group, more proteins and then amino acid substrates were available to the microbiota in the large intestine for the synthesis of SCFA (from alanine, aspar-



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Table 6. *Total amounts of organic acids and ethanol in the total cecal and colonic luminal contents recovered from rats after NP/HP feeding for 15 days*

| | NP | | HP | | |
|---|---|---|---|---|---|
| μmol in Total Luminal Contents | Mean | SE | Mean | SE | *P* Value |
| Cecal luminal contents | | | | | |
| L-Lactate | 0.214 | 0.036 | 0.390 | 0.088 | 0.095 |
| D-Lactate | 0.098 | 0.014 | 0.145 | 0.043 | 0.325 |
| Succinate | 3.452 | 0.585 | 7.505 | 0.845 | 0.002* |
| Formate | 0.565 | 0.027 | 0.947 | 0.114 | 0.015* |
| Ethanol | 27.590 | 8.388 | 23.352 | 11.821 | 0.775 |
| Colonic luminal contents | | | | | |
| L-Lactate | 0.053 | 0.006 | 0.210 | 0.029 | <0.001* |
| D-Lactate | 0.004 | 0.002 | 0.008 | 0.002 | 0.040* |
| Succinate | 0.905 | 0.625 | 7.064 | 1.066 | <0.001* |
| Formate | 0.064 | 0.013 | 0.177 | 0.044 | 0.052 |
| Ethanol | 0.796 | 0.142 | 1.756 | 0.340 | 0.031* |

Results were obtained from 6–8 animals in each group. *Statistically significant difference.

tate, glutamate, glycine, lysine, threonine), BCFA (from isoleucine, leucine, valine), and other organic acids (from deamination and fermentation of amino acids) (5, 22, 44, 45). This will correspond to a loss of amino acids for the host since the colonic epithelium (except in the neonatal period) cannot absorb a significant amount of amino acids. Among the 10 monocarboxylic acids that were markedly increased in colonic content, some of them (butyrate, acetate, propionate, and L-lactate) have been shown to represent important luminal oxidative substrates providing energy in the form of ATP for the colonic epithelial cells. In the case of succinate, this metabolite has been shown to be transported within the colonic mucosa (74) and to increase oxygen consumption in colonocytes (31). Because the large intestine epithelial layer is renewed within a few days (with important corresponding anabolic ATP-consuming pathways) (54), together with consumption of ATP by colonocytes through the membrane-bound ATPase involved in colonic electrolyte transport (7), ATP has to be synthesized at a rate matching its cellular utilization to maintain a constant ATP cell content, a necessary condition to maintain the viability of epithelial cells (41). The other monocarboxylic acids (valerate, isobutyrate, isovalerate, formate, and D-lactate), which have not yet been tested for their capacity to be oxidized in colonocyte, are present in much lower amounts than SCFA and succinate. Interestingly, the amount of ethanol in colonic content recovered from HP animals was markedly increased compared with NP animals. It remains, however, to be determined if ethanol is absorbed through the colonic epithelium with or without metabolism during its transfer from the lumen to the bloodstream. Ethanol, succinate, formate, and lactate, as metabolic intermediates in microbiota metabolism, usually do not accumulate to any substantial extent in the large intestine content. However, the production of these latter compounds can be increased under specific circumstances (33, 45).

Microbiota-derived energy substrates, which are absorbed in the process of transfer from the intestinal lumen to the portal vein blood, are available first for the liver and then, for some of them, for other peripheral tissues. In other words, transport from the lumen inside the colonocytes and further metabolism in these cells will primarily determine the amount of energy substrates available for liver and peripheral tissues in host. From this point of view, our results showing that the expression of the monocarboxylic acid transporters MCT1 and SMCT1 is not significantly modified after HP diet consumption represent strong evidence indicating similar capacities for luminal transport of energy substrates originating from the microbiota by the colonic epithelium. More evidence in favor of similar transport of monocarboxylic acids in colonocytes from HP and NP animals originates from the calculation of SCFA concentrations in the colonic lumen. Indeed, when we calculate acetate, propionate, and butyrate concentrations, they are not

Table 7. *SCFA concentrations in the cecal and colonic luminal contents recovered from rats after NP/HP feeding for 15 days*

| | NP | | HP | | |
|---|---|---|---|---|---|
| μmol/g of Wet Luminal Contents | Mean | SE | Mean | SE | *P* Value |
| Cecal luminal contents | | | | | |
| Acetate | 15.186 | 1.046 | 16.848 | 1.636 | 0.409 |
| Propionate | 3.446 | 0.292 | 3.282 | 0.331 | 0.716 |
| Butyrate | 1.886 | 0.161 | 1.986 | 0.288 | 0.768 |
| Valerate | 0.378 | 0.022 | 0.399 | 0.058 | 0.733 |
| Colonic luminal contents | | | | | |
| Acetate | 9.714 | 2.610 | 10.413 | 1.430 | 0.819 |
| Propionate | 2.065 | 0.554 | 3.291 | 0.573 | 0.147 |
| Butyrate | 0.905 | 0.205 | 1.358 | 0.274 | 0.208 |
| Valerate | 0.168 | 0.055 | 0.436 | 0.130 | 0.090 |

Results were obtained from 8 animals in each group.



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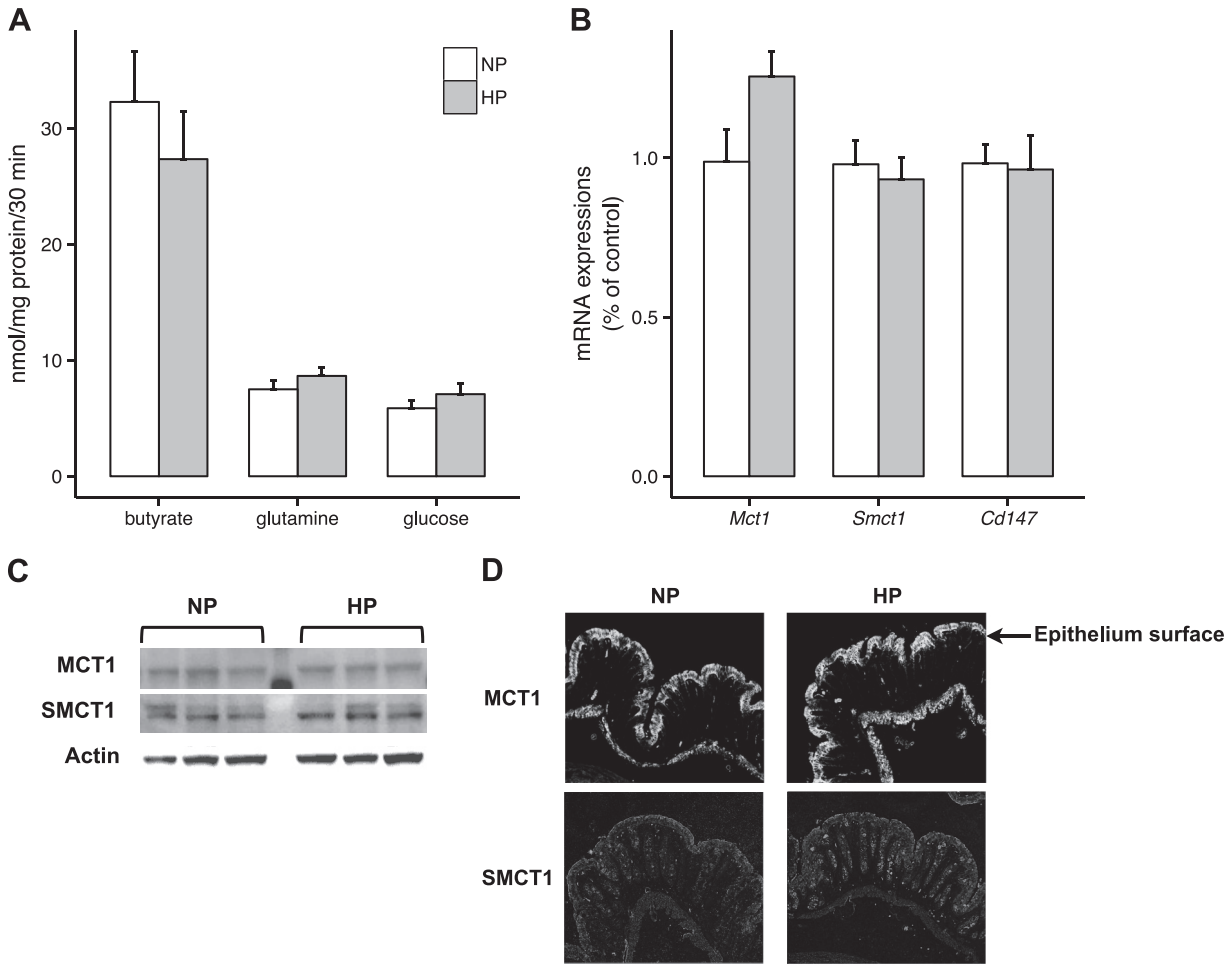



Fig. 4. Oxidation of energy substrates and expression of monocarboxylate transporters in isolated colonocytes and colonic sections from rats after NP/HP feeding for 15 days. *A*: oxidation of butyrate (2 mM), L-glutamine (1 mM), and D-glucose (5 mM) by the isolated colonocytes recovered from rats after NP/HP feeding for 15 days. Results are expressed as means ± SE and were obtained from 8–9 animals in each group. *B*: gene expression of monocarboxylate transporter 1 (*Mct1*), sodium monocarboxylate transporter 1 (*Smct1*), and *Cd147* in isolated colonocytes. The mRNA expression was determined by real-time PCR, and the results were normalized with *Rpl13a* mRNA and are obtained from 5–6 animals in each group; *C*: Western blot analysis of membrane MCT1 and SMCT1 in isolated colonocytes. Images are representative immunoblots of MCT1, SMCT1, and β-actin from 5 animals in each group; *D*: Immunofluorescence staining of MCT1 and SMCT1 in colonic sections. The immunoblots are representative of results obtained from 5 animals in each group.

significantly different in the colonic lumen of NP and HP animals.

Incidentally, the increased weight of the colonic luminal contents can be largely explained by increased water retention after HP diet consumption (42). From these results, and the low-affinity $K_m$ value of butyrate transport in colonic epithelial cells (40), we can predict that transport of SCFA from the lumen to the colonocyte will be similar between HP and NP animals. In further support of this proposition, we found that colonocytes from HP and NP animals oxidized butyrate and lactate to the same extent. Because we found that, in colonocytes isolated from HP and NP animals, the oxidation of the substrates L-glutamine and D-glucose (originating from the arterial blood) are similar (as well as the conversion of D-glucose to lactate), our results indicate similar energy metabolism capacity of the colonocytes whatever the diet consumed.

The present results strongly suggest that the increase of the bulk of the colonic content induced by the protein-rich diet allows the concentrations of luminal energy substrates to remain constant. Such maintenance, in the condition of modified luminal environment, is presumably important taking into account that, as suggested by previous studies (9, 63), the profile of luminal energy substrates available to colonic epithelial cells and metabolic characteristics of these cells play a major role in the control of processes such as cell proliferation/differentiation, which are central for the epithelium renewal and its homeostasis. Because the capacity for SCFA transport and metabolism by the colonic epithelium after HP diet consumption remained unchanged, we predicted that a part of these luminal energy substrates will be lost by the host (46). This was exactly what we observed with a more than twofold increase of the amount recovered in the feces of HP animals compared with NP animals. However, in HP animals, the part of energy lost in feces as SCFA remains quite modest, averaging ~0.1% of the energy ingested. A beneficial counterpart to amino acid and energy wasting is likely related to the physiology of the colonic epithelium. Indeed, among SCFA, butyrate plays a unique role by serving both as an energy substrate in colonocytes and as a regulator for intracellular



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gene targets, including those involved in colonic epithelial cell proliferation and migration (1, 73).

Thus, the same butyrate transport and oxidation in mitochondria of colonocytes recovered from HP and NP animals likely represents a way to maintain butyrate intracellular concentration within a narrow range compatible with its action upon its targets, including nuclear ones related to the epigenetic regulation of gene expression (15, 71). Because the expression of the MCT1 transporter in the colonocyte brush-border membrane is sensitive to the butyrate concentration (10, 16), the maintenance of the luminal concentration of butyrate despite increased protein consumption and butyrate production by the microbiota would thus allow maintaining metabolic homeostasis in the colonic epithelium. In conclusion, the data presented in this study are compatible with the view that an increased bulk in the large intestine contents following HP diet ingestion allows some kind of homeostasis regarding bacterial metabolites produced by the microbiota in a context of modified composition and increased substrate availability. Particularly, the present study indicates that, despite a marked increase of the amount of butyrate in the colonic luminal contents, a rather constant concentration is associated with unchanged capacity of colonic epithelial cells for its transport and metabolism. Because of the complexity of metabolic reactions and interconversions involving microbiota and its environment, further studies particularly involving high-throughput analysis are necessary to better explain the taxonomic and functional differences resulting from the HP diet and their long-term consequences on host-microbiota interactions.

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## DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

## AUTHOR CONTRIBUTIONS

Author contributions: X.L., J.-M.B., A.S., A.L., M.A., S.W., and P.-H.B. performed experiments; X.L. and J.-M.B. analyzed data; X.L., J.-M.B., and A.S. prepared figures; X.L., F.B., and A.-M.D. drafted manuscript; X.L., J.-M.B., A.S., A.L., M.A., S.W., P.-H.B., D.T., Y.S., F.B., and A.-M.D. edited and revised manuscript; X.L., J.-M.B., A.S., A.L., M.A., S.W., P.-H.B., D.T., Y.S., F.B., and A.-M.D. approved final version of manuscript; Y.S., F.B., and A.-M.D. conception and design of research; F.B. and A.-M.D. interpreted results of experiments.

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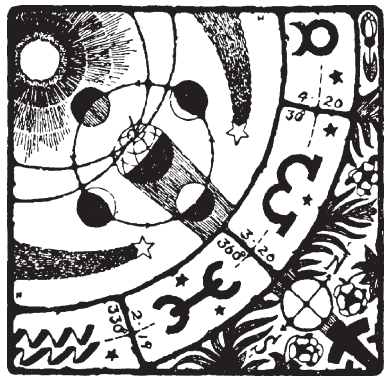
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