High-fiber and high-protein diets shape different gut microbial communities, which ecologically behave similarly under stress conditions, as shown in a gastrointestinal simulator

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Abstract

The aim of this work was to investigate the relationship between the structure of gut microbial communities fed with different diets (i.e. high-protein - HP - vs. high-fiber - HF - diet) and their functional stability when challenged with mild and acute doses of a mix of amoxicillin, ciprofloxacin and tetracycline. We made use of the Simulator of the Human Intestinal Microbial Ecosystem (SHIME®) - a continuous model of the gastrointestinal tract - coupled with 16S-targeted Illumina and metabolomics (i.e. UHPLC-HRMS) analyses. Independently of the diet, the sudden exposure to an acute stress led to a modification of the microbial community structure, selecting for species belonging to *Bacillus* spp.; *Clostridium* cluster XIVa; Enterococci; Bacteroides; and Enterobacteriaceae. The antibiotic treatment led to a decrease in the number of OTUs (at least -10%). Cluster analysis of untargeted metabolic data showed that the antibiotic treatment affected the microbial activity. The impact on metabolites production was lower when the community was pre-exposed to mild doses of the antibiotic mix. This effect was stronger in the proximal colon for the HF diet and in the distal colon for the HP diet. Different diets shaped different gut microbial communities, which ecologically behaved similarly under stress conditions.

Introduction

The human body and especially the gastrointestinal tract (GIT) host a highly complex microbial community, which is deeply involved in influencing several health-associated parameters [1]. As for any other environment, several abiotic and biotic factors normally concur in shaping the structure and composition of these communities, which, on their side, can reach high levels of organization by means of specific interactions, cross-signalling, cross-feeding, and aggregation [2, 3]. For the GIT, a key factor is the diet, which is known to have an effect on the composition of the gut microbial community and on its functional stability (i.e. saccharolytic vs. proteolytic fermentation) [4]; [5]. More specifically, the stability concept refers to the functional properties of the community rather than to its composition, which is normally dynamic [6]. The functional stability of the gut microbiota is of great importance for the host's health. In fact, gut microbiota malfunctioning (i.e. dysbiosis) has been already associated with several detrimental conditions, e.g. chronic inflammation, obesity, allergy, etc. [7] [8-10]. In a simplified study, in which all the species of the community were involved in the same functionality, it has been shown that community structure and functional stability are deeply interconnected under changing environmental conditions [11]. However, the evaluation of the effect of environmental perturbations on the composition and activity of a microbial community may be highly challenging when working in more complex environments (i.e. GIT) and fundamental research is much needed.

The aim of this work was to investigate the relationship existing between the structure of gut microbial communities fed with different diets (i.e. high-protein - HP - vs. high-fiber - HF - diet) and their functional stability. In order to challenge the functional stability of the different communities, we applied an antibiotic treatment to investigate the capacity of the gut microbiota in counteracting an acute stress (e.g. high dose of antibiotics). In other

words, if different diets are able to shape different microbial communities (both at composition and functional level), does this affect the stability of the ecosystem in presence of a sudden stress? As a secondary endpoint, we evaluated whether or not a microbial community that has been pre-exposed to a similar mild stress (e.g. low dose of antibiotics) can better cope with the acute stress (i.e. priming effect, as defined by [12]).

In order to reach these goals, we made use of the Simulator of the Human Intestinal Microbial Ecosystem (SHIME®) - a continuous model of the gastrointestinal tract that allows the simulation of the main physiological and microbiological processes occurring in the GIT under representative environmental conditions [13]; [14]; [15]; [16] [17] - coupled with 16S-targeted Illumina and metabolomics (i.e. UHPLC-HRMS based) analyses.

Materials and Methods

Simulator of the human intestinal microbial ecosystem (SHIME®)

The reactor setup was adapted from the SHIME® (ProDigest and Ghent University, Belgium), representing the GIT of the adult human, as described by Molly *et al.* [18] and Worametrachanon *et al.* [19]. Four modified SHIME systems were run in parallel, each consisting of a succession of 1 reactor per diet simulating stomach and small intestine (temporal succession), and 2 proximal (PC) and distal (DC) colon compartments (Figure 1). The PC was characterized by a pH of 5.6-6.0, a volume of 500 mL and a retention time of 20h; the DC had a pH of 6.5-6.9, a volume of 800 mL and a retention time of 32h. The colonic compartments were inoculated with the fecal microbiota of a healthy 35-year old volunteer who had no history of antibiotic treatment in the 6 months prior to the faecal sample collection. From the beginning of the experiment (start-up period of 2 weeks) each arm of the SHIME (i.e. HF or HP, Fig. 1) was fed with a medium either representative of a high-fiber or a high protein diet (Table 1). Both diets provided a total amount of 18.7 g/L

of ingredients. Following the start-up period each arm of the SHIME was split in 2 sub-arms for 2 weeks of either control (NS = non stress) or mild antibiotic treatment (MS = mild stress) and then 1 week of acute stress (NSS and MSS = acute stress) (Figure 2). The antibiotics used to induce either a mild or an acute stress were amoxicillin, ciprofloxacin and tetracycline, respectively at 10, 10, 2.5 mg/L final colonic concentration (i.e. mild stress) and 40, 40, 10 mg/L final colonic concentration (i.e. acute stress) and were dosed in each proximal colon compartment, as indicated in Fig. 2. The detailed schedule and sampling scheme is shown in Fig. 2. Samples have been named as follows: either P (= protein) or F (= fiber) for the type of diet; P (= proximal) or D (= distal) for the colonic location; NS 1 to 5 for the samples collected in the 'control' arm of the SHIME; MS 1 to 5 for the samples collected in the 'mild stress' arm of the SHIME; NSS 6 to 9 for the samples collected during the acute stress phase, in the control arm of the SHIME; finally, MSS 6 to 9 for samples collected during the acute stress phase, in the arm of the SHIME that previously underwent the 'mild stress' conditions. All samples (5 mL each) for metabolites analyses were immediately stored at -80°C till further analysis. Samples for DNA extraction (2 aliquots of 1 mL) were centrifuged and the pellet stored at -80°C.

Illumina metagenomic sequencing

Metagenomic DNA was extracted and purified using the FastPrep®-24 Instrument (MP Biomedicals; Illkirch, France) as previously described by Vilchez-Vargas *et al.* [20]. The V1-2 region of the 16S rRNA gene was amplified as previously described [21]. However, in a first 20 cycles PCR reaction the 16S rDNA target was enriched using the well-documented 27F and 338R primers [22, 23] as previously specified [24]. Libraries were sequenced in a MiSeq platform (Illumina). Reads were clustered allowing for two mismatches, as previously described [26]. The data-set was then filtered to consider only

those phylotypes that were present in at least one sample at a relative abundance >0.1% or were present in all samples at a relative abundance >0.001%, as previously described [21]. Samples were re-sampled to the minimum sequencing depth (19448 reads) using the package phyloseq from the R program. A total of 1,400,256 reads were obtained, and grouped into 745 phylotypes. Rarefaction curves and statistics were generated using the package vegan from the R program and network analysis was performed with cytoscape considering the pearson correlation range of -0.8<rho<0.8. All phylotypes were assigned a taxonomic affiliation based on the naïve Bayesian classification (RDP classifier) with a 80% of threshold [27].

Metabolites analyses

Short-chain fatty acids (SCFAs) and ammonium were determined as described by Van de Wiele *et al.* [13]. The validated metabolomics analysis was performed on a UHPLC (ultra-high performance liquid chromatography) Accela system hyphenated to an Orbitrap-HRMS (high resolution mass spectrometer) (Thermo Fisher Scientific, San José, CA, USA) [28]. Chromatographic separation of the SHIME metabolites was achieved on an Acquity HSS T3 C18 column (1.8 µm, 150 mm x 2.1 mm, Waters) kept at 45°C. Additionally, a vanguard pre-column (1.8 µm, 5 mm x 2.1 mm, Waters) with identical stationary phase was used to guarantee a longer column lifetime. Elution was carried out using a binary solvent system consisting of ultrapure water (A) and acetonitrile (B) both acidified with 0.1% formic acid at a constant flow rate of 0.4 mL/min. A gradient profile with the following proportions (v/v) of solvent A was applied: 0-1.5 min at 98%, 1.5-7.0 min from 98% to 75%, 7.0-8.0 min from 75% to 40%, 8.0-12.0 min from 40% to 5%, 12.0-14.0 min at 5%, 14.0-14.1 min from 5 to 98%, followed by 4.0 min of re-equilibration. A 10 µl aliquot of each sample was injected for analysis. HRMS analysis was performed on

an Exactive™ stand-alone benchtop mass spectrometer, equipped with a heated electrospray ionization source (HESI-II), operating in polarity switching mode. Ionization source working parameters were optimized and were set to a sheath, auxiliary and sweep gas of 50, 25 and 5 arbitrary units (au), respectively, heater and capillary temperature of 350 °C and 250 °C and tube lens, skimmer, capillary and spray voltage of 60 V, 20 V, 90 V and 5 kV (+/-), respectively. A scan range of *m/z* 50-800 was chosen and the resolution was set at 100,000 FWHM at 1 Hz (1 scan per second). The automatic gain control (AGC) target was set at balanced ($1 \times E^6$ ions) and the maximum injection time was 50 ms.

The SHIME samples were extracted as follows: 1.5 mL of suspension was centrifuged (5 min at 13,300 rpm) and the supernatant filtrated over a PVDF filter (pore size of 0.22 μm). Finally, the extract was diluted (1:5) with ultrapure water and transferred to a glass HPLC-vial [28].

The first step for chemometric data analysis was conducting extensive data pre-processing of the obtained full scans HRMS data files with Sieve™ 2.1 software (Thermo Fischer Scientific). This included automated peak extraction, peak alignment, deconvolution and noise removal. To increase the diversity of the detected metabolites, the negative and positive ionization mode were considered separately [29]. Next, a logarithmic transformation and Pareto scaling ($1/\sqrt{SD}$, where SD is the standard deviation) were performed for inducing normality and standardizing the range of independent X-variables, respectively [30]. Finally, multivariate regression techniques (Simca 13.5.0, Umetrics, Sweden) were used to display the differentiation between the metabolomes derived from the SHIME samples. This study relied on principal component analysis (PCA) to reveal outliers, groups and trends, whereas (orthogonal) partial least square analysis ((O)PLS) was used for constructing a prediction model that could explain and predict the Y-variable (antibiotic treatment)) from the X-matrix (SHIME metabolome with metabolite

abundances). Model diagnostics in terms of fit (R^2X, R^2Y) and predictive ability (Q^2) were calculated to see how good the model fits the data and plots were validated by ANOVA of the cross-validated residuals (CV-ANOVA) [31]. Finally, in order to discover the number of significantly altered metabolites we relied on permutation tests with Benjamini Hochberg's False Discovery Rate (FDR) to correct for multiple comparisons. An adjusted $P<0.05$ was considered to be statistically significant.

Results

Analysis of the microbial community composition

The prolonged exposure of the original fecal inoculum to the 2 diets and to the different physiological conditions of proximal and distal colon led to the selection of microbial communities characterized by a different composition. As shown in Figure 3, in presence of a HF diet, the communities of both colon compartments were dominated by bacteria belonging to the Bacteroidetes phylum (>50%), followed by Firmicutes (approx. 30%) and Proteobacteria. The HP diet led to a higher percentage of Proteobacteria (44% in the PC and 35% in the DC) at the expense of both Firmicutes and Bacteroidetes. The fecal material of the donor itself was already characterized by a high percentage of Proteobacteria (data not shown). Independently of the composition and the diet, the analysis of the rarefaction curves (Fig. S1) showed that the presence of a mild or acute stress had a negative impact on the richness (i.e. number of OTUs) of the community. In fact, with the exception of the PC samples in the HP SHIME pre-treated with a mild stress, the administration of antibiotics resulted in at least 10% decrease in the number of OTUs.

To further analyze the impact of diet and stress on the different communities and to maximize the differences observed among the samples, the Illumina data were processed by means of a PCA focused on those OTUs that changed in abundance during the

experiment. As shown in Figure 4, samples from all colon compartments clustered according to the level of stress to which they were exposed. Moreover, samples previously exposed to mild stress (i.e. MS) and subsequently exposed to acute stress (i.e. MSS) clustered together (black dashed clusters, Fig. 4). The main changes at the level of community composition are shown in Fig. S2 (proximal colon) and S3 (distal colon). Overall, independently of the diet, samples pre-exposed to mild stress (MS) clustered with those exposed to acute stress (MSS). On the contrary, NS and NSS samples ended up in separate clusters. Exposure to the antibiotic stress selected for similar changes in common microbial groups, independently of the used diet. Among them, both in the PC and DC, it was observed an increase in the concentration of *Bacillus* spp.; *Burkholderia* spp.; *Clostridium* cluster XIVa; Enterococci; Bacteroides; and Enterobacteriaceae.

Finally, during the experiment, we analyzed the structure of the various communities by investigating the network of positive interactions in the whole database, based on a Pearson correlation matrix (Fig. S4 and S5). Keystone OTUs - selected as the top 3 with the highest values of betweenness centrality (i.e. indicator of a node's centrality in a network) - differed among the samples and were dominated by bacteria belonging to the phyla of Firmicutes and Bacteroidetes (Table 2). At phylogenetic level, it was possible to observe that a HF diet coinciding with a mild or acute stress had the tendency to promote the role of Firmicutes (i.e. *Clostridium* XIVa and *Phascolarctobacterium* spp.) in the proximal colon and the role of some *Bacteroides* spp. in the distal colon. In contrast, the presence of a HP diet and external stress favored the role of *Bacteroides* spp. both in the proximal and the distal colon. Finally, the total number of positive interactions (i.e. suggested level of mutualistic interactions) for each network is summarized in Table 3. In all cases, with the exception of the distal colon in presence of a HP diet, the addition of mild stress led to a decrease of the number of positive interactions (NS vs. MS in Table 2). In presence of the

HF diet, the total number of positive interactions in the proximal colon - when exposed to acute stress - increased in absence (NSS) or presence (MSS) of the exposure to the mild stress of 63% and 250%, respectively. In contrast, in the distal colon, the exposure to acute stress led to a decrease in positive interactions of 89% (NSS) and 65% (MSS). In presence of the HP diet, this trend was different, as compared to the HF diet. Positive interactions increased with 20% in the MSS samples in the proximal colon and with 192% in the NSS samples in the distal colon. Finally, samples from the PC under NSS conditions showed a 65% decrease and those from the distal colon under MSS conditions had a 13% decrease in positive interactions.

Analysis of the metabolites production

SCFA and ammonium were measured as markers for saccharolytic and proteolytic activity. SCFA profiles consisted mainly of acetate, propionate and butyrate with small amounts of other acids such as isobutyric, valeric, isovaleric and caproic acid (data not shown). The evolution of the total amount of SCFAs in the different colon compartments of the SHIME reactor is shown in Figure 5a. With both diets, it was possible to observe a common trend: during the first 2 experimental weeks the concentration of SCFA remained stable in both the proximal and distal colon compartments of the SHIME arms that acted as control. On the contrary, the presence of a mild stress led to an initial decrease in the concentration of total SCFAs and a stabilization of the concentration at lower levels (i.e. grey and dotted lines in Fig. 5a) as compared to the respective control arm (i.e. black and dashed lines in Fig. 5a). In presence of the acute stress (black arrow, Fig. 5a), the concentration of total SCFA in the original control arm dropped (i.e. black and dashed lines in Fig. 5a), while the arms that previously underwent the mild stress conditions remained unaffected (i.e. grey and dotted lines in Fig. 5a). The two diets led to a different recovery following acute stress.

In presence of the HF diet the concentration of SCFA returned within 2 days to levels similar to those present prior to the acute stress. In contrast, for the HP diet, the lost capability in saccharolytic fermentation was not recovered up to 5 days following stress. The concentration of ammonium during the first 2 experimental week (i.e. control arm vs. mild stress arm) was not affected (Fig. 5b). The application of the acute stress led to a build-up in the concentration of ammonium in all arms of the SHIME, independently of a pre-exposure to the stress. This transient peak in ammonium occurred 1 day after the stress in presence of HP diet (up to 500 mg/L in the DC) and 2 days after the stress in presence of the HF diet (up to 400 mg/L in the DC).

Figure 6 shows the OPLS score plot output of the total dataset of the untargeted metabolomic fingerprints of the proximal (Fig. 6a) and distal (Fig. 6b) colon. In both cases, it is possible to observe that the samples primarily clustered according to the diet along the x-axes. Sub-clusters were then formed in both compartments between NS and MS samples. Finally, exposure to an acute dose of antibiotics led to the formation of a separate joint cluster for both the NSS and MSS samples (along the y-axes). In the proximal colon supplemented with the HF diet, exposure to mild stress led to a temporal adaptation of metabolites, as indicated by the red arrow in Fig. 6a. In response to this adaptation, the samples exposed to the acute stress formed two separate sub-clusters(dotted circles). During the experiment - according to a 2-sided permutation test with multiple comparisons - 28% and 26% of the metabolites significantly changed in the proximal colon with a HF and a HP diet, respectively. In the distal colon, 34% and 42% of the metabolites significantly changed in presence a HF and a HP diet, respectively.

Data from Fig. 6 have been further analyzed in order to consider only the main metabolites responsible for the differential clustering (multivariate regression). Seventy-two untargeted metabolites for the PC and 66 for the DC - selected from the S-plot - have been used to

create the dendograms shown in Fig. S6. Both in the PC (Fig. S6a) and in the DC (Fig. S6b), samples formed separate clusters based on the diet. Within each of the clusters, the MS and NS 2 to 5 samples (cfr. Fig. 2) were more similar among each other according to the sampling point (i.e. MS 2 and NS 2 clustered together, MS 3 and NS 3 clustered together, etc…). Following the acute stress, samples in the PC tended to mix 'temporal sampling' point and 'preliminary exposure (MSS) or not (NSS) to the stress'. On the contrary, in the distal colon MSS and NSS samples formed different clusters (the effect was more pronounced in presence of the HP diet). Finally, a targeted approach was applied to a selected number of samples to highlight the effect of the experimental conditions on 66 selected metabolites. Data are shown as clustering of heat maps for the HF (Fig. 7a) and the HP (Fig. 7b) diets, with a similarity cut-off of 85%. Both with the HF and the HP diet, samples from the proximal and the distal colon formed separate clusters. Within the HF cluster of the proximal colon, it was possible to observe a small sub-cluster containing samples MS5, MSS7 and NSS7. On the contrary, in the distal colon, samples clustered according to the sampling time. In presence of the HP diet, all samples from the proximal colon clustered together, while in the distal colon it was possible to observe a small sub-cluster containing samples MS5, MSS7 and NSS7. In terms of specific metabolites, main differences could be observed between proximal and distal colon, independently of the diet: D-fructose, maltose, galactose, mannose and malic acid were more abundant in the PC, while hydroxybenzoic acid, chenodeoxycholic acid and ursodeoxycholic acid were more abundant in the DC. Finally, metabolites with a possible negative impact on human health, such as indole and cadaverine, were preferentially produced in presence of the HP diet while higher levels of glucose were correlated with a HF diet. Among the 66 selected metabolites, no significant differences could be specifically attributed to the presence of a mild/acute antibiotic stress.

Discussion

The objective of this study was to evaluate how a high-protein and high-fiber diet may impact the composition of the gut microbiota and affect its functional stability (i.e. capacity of a microbial community in counteracting external stress - mild vs. acute dose of antibiotics) from an ecological point of view. We already showed in the past that the SHIME, in line with other chemostat models inoculated with a fecal microbial community, allows the creation of highly reproducible conditions when similar environmental factors are applied [32, 33]. In this way, by inoculating the system with the fecal material of a single donor, we could increase the number of variables in the system (i.e. HP and HF diets; mild and acute stress; different colon region-specific conditions). For the purpose of this work, the design has to be considered as working with 1 individual either pre-exposed or not to mild stress. In the current study, HF and HP diets led to the development of microbial communities characterized by a different composition (Fig. 3) and metabolic activity (Fig. 5, 6 and 7). HF and HP diets originally shaped - starting from the same fecal sample - different microbial communities in the simulated proximal and distal colon compartments at the end of the stabilization period (Fig. 3). This is in line with what reported by Turnbaugh and colleagues [34], who showed that the community composition can be altered over short time intervals when humanized mice were switched to different diets. HP diet was associated with an increased relative proportion of bacteria belonging to the phylum of Proteobacteria. Proteobacteria are a major phylum of gram-negative bacteria, which include a wide variety of pathogens and have been considered as a signature of dysbiosis in gut microbiota [35].

We then applied a stress to evaluate whether or not a different diet had an impact on the developed communities. The choice of stress (i.e. antibiotics) was based on its

relevance for the gut microbiota and was designed to induce an environmental fluctuation to challenge the functional stability of the different communities. We opted for a combination of antibiotics with a broad antimicrobial-spectrum in order to avoid an outcome of the study merely linked to the inhibition/modification of a limited number of microbial groups. However, the possible drawback of this approach is that the differences observed between the two diets might have been underestimated. Moreover, we decided to prime the microbial communities with a similar stress as it has already been shown that a primary stress can improve the capacity of a microbial community to cope with a subsequent severe stress only if the applied stresses are similar [36, 37].

Despite the already mentioned different composition of the gut microbiota induced by the diets in the proximal and distal colon, the 16S targeted Illumina analysis showed a similar pattern of changes in response to the mild and acute stresses, independently of the administered diet (Fig. 4, S2 and S3). In fact, samples collected from the SHIME arm that was pre-exposed to the mild stress (MS) underwent a lower number of changes – as compared to those from the control arm (NS -> NSS) – when exposed to the acute stress (MSS clustered with MS). The exposure to the antibiotic stress selected for similar changes in common microbial groups and led to a general decrease of richness (Fig S1), independently of the used diet. In this respect, we suggest that - despite the fact that the priming ability was present in the gut microbiota - this occurred with a fitness cost leading to a selection and survival of the most effective species, as postulated by Rilling and colleagues [12]. In the simulated colon compartments, different diets selected for different numbers of positive interactions and taxa (Table 2 and 3). Scientific literature has already shown that long-term diets rich in protein and animal fat are associated - in controlled-feeding study - with enterotypes rich in levels of Bacteroides [4] while a diet high in fiber content has been correlated with a decrease in the levels of Firmicutes and an enrichment

in Prevotella [43]. In our study, the high-fiber diet led to a higher number of interactions in the proximal colon (main area of fiber fermentation) and the pre-exposure to a mild stress further boosted the number of positive interactions under acute stress. Keystone genera in these networks were bacteria belonging to *Clostridia* cluster XIVa, which includes a high number of butyrate producers, and *Phascolarctobacterium* spp., subdominant members of the gut microbiota specialized in using succinate generated by other bacterial species [44]. The HP diet led to a different development of the microbial communities by primarily selecting for keystone species belonging to the genus Bacteroides. Moreover, with a HP diet, pre-exposure to a mild stress was less important in preserving positive interactions among bacteria.

We then investigated whether or not the priming effect observed at the level of the microbiota could also be observed at metabolic level. It is known from literature that a typical western diet provides the colonic microbiota with approx. 50 g/day of fermentable substrate (i.e. mainly dietary fiber and 20% of protein) [38]. Carbohydrates are the principal carbon and energy source for colonic microbes and are mainly fermented in the proximal colon. Indeed, many of the health benefits potentially associated with fibers are related to their fermentation by gut microbiota leading to the production of SCFA [39]. In contrast, dietary proteins, which are fermented more distally in the colon, are correlated with putrefactive fermentation products and possible development of bowel diseases, despite the fact that they are an important source of essential amino acids for the body and nitrogen for bacteria [40]. Data on SCFA, ammonium (Fig. 5) and metabolome (Fig. 6 and 7) showed a clear impact of both the mild and acute stress on the metabolism of the microbial community. It has been reported in literature that patients who underwent an antibiotic treatment showed significant disturbances in fecal SCFA pattern [42]. For the acute stress, the impact was different depending on whether or not the microbial

community had been previously exposed to a mild dose of the same stress. In fact, pre-exposure to stress increased the resistance of the community to the acute stress with respect to this specific metabolic activity. Finally, in presence of a HF diet and when focusing on SCFA, the gut microbiota showed a higher resilience (as compared to the HP diet) both in the proximal and distal colon (Fig. 5a). Ammonium has been measured as a marker for proteolytic activity. For this specific metabolite we did not observe any adaptation following the mild stress. On the contrary, the acute stress led to ammonium accumulation and the two diets led to a different pattern. On one side, ammonium is one of the endpoints of the microbial proteolytic activity. On the other side, ammonium can be assimilated by bacteria as nitrogen source and incorporated into amino acids and nucleic acids [45]. In this respect, the accumulation of ammonium is the result of a lower activity of assimilation by bacteria due to the stress induced by the antibiotic. The effect was stronger and faster with the HP diet as the ammonium production in presence of protein is higher as compared to a diet which favours a saccharolytic fermentation (i.e. HF).

Finally and as expected, the analysis of the metabolomic data showed a strong effect of the type of diet and colonic sector (i.e. PC or DC) on clustering. Sugars were increased in the PC as result of the saccharolytic activity while compounds originated from proteolytic activity (i.e. ammonium, indole, cadaverine) were preferentially produced in presence of the HP diet. Despite the fact that the untargeted analysis showed that the application of the antibiotic stress had an effect on metabolites production, the targeted analysis did not allow the identification of any specific metabolite that was consistently affected by the stress. Interestingly, the effect of the stress mainly occurred in the main areas of fermentation of the diets: proximal colon for fibers, distal for proteins.

In conclusion, different dietary habits are known to have an impact on the host wellbeing by influencing, among others, glycaemic response [46], modulation of

atherogenic pathways [47], risk for metabolic syndrome [48] and renal disease progression [49]. Moreover, fiber consumption is apparently inversely associated with cardiovascular disease and cancer mortality [50]. It is also known that different diets can modify the gut microbiota composition and activity [34]. In this work, we have shown that different microbial communities fed with a HF and a HP diet, ecologically behaved similarly under stress conditions. Independently of the diet, the sudden exposure to an acute stress led to a modification of the microbial community structure, a decrease in richness and a strong modification of the profile of the metabolites that are produced. Some distinctive characteristics were also associated to the diets. In fact, following stress, different diets led to the development of microbial networks dominated by different OTUs/phyla that were better fit for the specific stress. Moreover, the HF diet was characterized by a higher production and stability of SCFA and a quicker recovery after the acute stress, as compared to the HP diet. The pre-exposure to a similar mild stress led to an adaptation of the composition of the microbiota and a subsequent smaller impact of the acute stress on its composition. In terms of functionality, a mild stress showed a minor impact on the profile of the produced metabolites. This led to a higher functional stability following an acute stress in the main area of fermentation of the diet (i.e. PC for HF diet and DC for the HP diet). The long-term implications for such changes are not yet well understood and further *in vivo* work is needed to determine the impact of these findings on health parameters.

Author contributions

MM and TVdW designed the study and wrote the paper; MM performed the SHIME experiments with the help of PT and RA; RJ and DHP performed the Illumina sequencing; RVV analyzed the Illumina data; JVB and LV produced and analyzed the metabolomics

data. All authors read and approved the paper. None of the authors declares any conflict of interest.

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Figure legends

Figure 1 – Design of the SHIME® setup. A TWINSHIME setup has been modified to create 2 arms (each fed with either a high fiber - HF - or high protein - HP- diets). Each arm, characterized by a single stomach/small intestine compartment (S1 or S2), was subdivided in order to simulate two parallel proximal (PC) and distal (DC) colon compartments. The lower arms acted as a control for 2 weeks (CTRL 2wks - NS) while the upper arms were challenged with a mild dose of antibiotics and thus stress (MILD 2wks - MS). During the third experimental week all arms were challenged with a single acute dose of the antibiotic mix.

Figure 2 – Experimental design and samplings. Each of the arms described in Fig. 1 was subdivided in 2 parallel colon compartments which acted as a control for 2 weeks in absence of stress (NS) or which underwent mild stress (MS). In both cases, during the third experimental week an acute stress was dosed to the system (NSS and MSS). Black dotted circles indicate the days on which the SHIME was challenged with mild stress. Full black circles indicate the challenge with the acute stress. The black arrows with the related numbers indicate when samples were collected. On a day highlighted by a circle, samples were always collected before the stress challenge. Capital letters indicate the days of the week from Monday to Sunday.

Figure 3 – Relative abundance (%) of the main bacterial phyla in samples collected from the proximal and distal colon compartments of the 2 SHIME arms (HF = high-fiber diet; HP = high-protein diet) after the start-up period and prior to the subdivision of each arm in NS and MS reactors. Concentrations of Actinobacteria were very low, ranging between 0.005% and 0.001%.

Figure 4 – Principal component analysis of the full Illumina dataset. Data are presented for the proximal and the distal colon of the SHIME arms treated with the high-fiber (HF) or high-protein (HP) diets. Samples have been named according to the scheme presented in Fig. 2. Samples clustered according to the dose of stress: 0 = no stress/control; low = mild stress; high = acute stress.

Figure 5 – Total SCFA and ammonium profiles. Concentration of (a) total SCFA (mmol/L) and (b) ammonium (mg/L) in the proximal (PC) and distal (DC) colon of the SHIME treated with high-fiber (HF) and high-protein (HP) which either acted as a control (NS) before acute challenge (NSS) or as mild stress primed system (MS) before acute challenge (MSS). Full black circles indicate the day of challenge with a mild stress only for the MS samples. The black arrows indicate the challenge with the acute stress both for the MSS and the NSS reactors. Numbers on the x-axes indicate the experimental day following the 2 weeks of start-up period.

Figure 6 – OPLS analysis of the full metabolomic dataset for proximal (A) and distal (B) colon of the SHIME arms treated with the high-fiber (HF) or high-protein (HP) diets. Samples have been named according to the scheme presented in Fig. 2. In the proximal colon supplemented with the HF diet, exposure to the mild stress led to a temporal adaptation of metabolites, as indicated by the red arrow. In response to this adaptation, the samples exposed to acute stress formed two separate sub-clusters (dotted circles).

Figure 7 - Heat map (GENE-E software, http://www.broadinstitute.org/cancer/software/GENE-E/index.html) visualizing a selected

number of 66 known metabolites detected in colonic SHIME suspension fluids - treated with the HF (a) and the HP (b) diet - with hierarchical clustering of the different samples. Samples have been named according to Fig. 2. In the name of the samples the first F or P indicates either HF or HP diet; subsequently, P or D indicate either proximal or distal colon.

Table 1: Composition of the high fiber (HF) and high protein (HP) diets. All ingredients were purchased at Sigma-Aldrich (Schnelldorf, Germany). The HF and HP diets have been adapted starting from the standard SHIME diet as reported in Van de Wiele et al. [13]. We used the same ingredients of the standard SHIME diet but their relative proportion was modified in the direction of either a HF or HP diet.

	HF (g/L)	HP (g/L)
Starch	1.0	1.0
Arabinogalactan	1.0	0.5
Pectin	2.0	0.5
Xylan	1.0	0.5
Glucose	0.4	0.2
Arabinoxylan	4.0	1.0
Yeast extract	3.0	3.0
Peptone	0.6	2.5
Bacto tryptone	0.6	2.5
Casein	0.6	2.5
Mucin	4.0	4.0
Cysteine	0.5	0.5

Table 2: Betweenness centrality (BC) values of the top 3 OTUs in each of the positive network interactions (see Fig. S4 and S5). Samples have been named as follows: either P (= protein) or F (= fiber) for the type of diet; P (= proximal) or D (= distal) for the colonic location; NS = samples collected in the 'control' arm of the SHIME; MS samples collected in the 'mild stress' arm of the SHIME; NSS samples collected during the acute stress phase, in the control arm of the SHIME; MSS samples collected during the acute stress phase, in the arm of the SHIME that previously underwent the 'mild stress' conditions.

	BC	OTU	Hit Phylum	Hit Genus		BC	OTU	Hit Phylum	Hit Genus
FPNS	0.51	5	Firmicutes	Phascolarctobacterium[100%]	**FPMS**	0.37	10	Firmicutes	Clostridium XlVa[92%]
	0.49	119	Bacteroidetes	Bacteroides[100%]		0.35	17	Proteobacteria	Enterobacteriaceae[100%]
	0.44	25	Proteobacteria	Achromobacter[100%]		0.20	3	Firmicutes	Clostridium XlVa[99%]
FPNSS	0.51	35	Bacteroidetes	Bacteroides[100%]	**FPMSS**	0.68	191	Firmicutes	Clostridium XlVa[98%]
	0.51	69	Firmicutes	Clostridium XlVa[94%]		0.51	5	Firmicutes	Phascolarctobacterium[100%]
	0.43	38	Proteobacteria	Escherichia/Shigella[88%]		0.42	10	Firmicutes	Clostridium XlVa[92%]
FDNS	0.29	5	Firmicutes	Phascolarctobacterium[100%]	**FDMS**	0.67	34	Bacteroidetes	Bacteroides[100%]
	0.27	44	Bacteroidetes	Bacteroides[100%]		0.50	3	Firmicutes	Clostridium XlVa[99%]
	0.25	92	Firmicutes	Phascolarctobacterium[100%]		0.30	101	Bacteroidetes	Bacteroides[100%]
FDNSS	1.00	162	Bacteroidetes	Bacteroides[100%]	**FDMSS**	0.53	80	Bacteroidetes	Bacteroides[100%]

	0.47	50	Firmicutes	Bacillus[92%]		0.52	171	Bacteroidetes	Bacteroides[100%]
	0.32	44	Bacteroidetes	Bacteroides[100%]		0.40	24	Firmicutes	Clostridium XlVa[93%]
PPNS	0,53	101	Bacteroidetes	Bacteroides[100%]	**PPMS**	0,52	5	Firmicutes	Phascolarctobacterium[100%]
	0,52	57	Bacteroidetes	Bacteroides[100%]		0,50	187	Bacteroidetes	Bacteroides[100%]
	0,51	52	Bacteroidetes	Bacteroides[100%]		0,39	1	Bacteroidetes	Bacteroides[100%]
PPNSS	0,29	11	Firmicutes	Enterococcus[100%]	**PPMSS**	0,60	50	Firmicutes	Bacillus[92%]
	0,25	46	Bacteroidetes	Bacteroides[100%]		0,36	13	Proteobacteria	Enterobacteriaceae[100%]
	0,19	34	Bacteroidetes	Bacteroides[100%]		0,20	1	Bacteroidetes	Bacteroides[100%]
PDNS	1.00	92	Firmicutes	Phascolarctobacterium[100%]	**PDMS**	0.36	3	Firmicutes	Clostridium XlVa[99%]
	0.24	32	Proteobacteria	Pseudomonas[100%]		0.22	156	Bacteroidetes	Bacteroides[100%]
	0.21	73	Firmicutes	Erysipelotrichaceae [96%]		0.22	2	Bacteroidetes	Bacteroides[100%]
PDNSS	0.29	69	Firmicutes	Clostridium XlVa[94%]	**PDMSS**	0.55	52	Bacteroidetes	Bacteroides[100%]
	0.26	43	Firmicutes	Clostridium XlVa[89%]		0.53	49	Proteobacteria	Escherichia/Shigella[99%]
	0.22	105	Bacteroidetes	Bacteroides[100%]		0.33	4	Bacteroidetes	Bacteroides[100%]

Table 3: Total number of positive interactions in the microbial networks (see Fig. S4 and S5). HF = high-fiber diet; HP = high-protein diet; PC and DC = proximal and distal colon compartments. NS = samples collected in the 'control' arm of the SHIME; MS samples collected in the 'mild stress' arm of the SHIME; NSS samples collected during the acute stress phase, in the control arm of the SHIME; MSS samples collected during the acute stress phase, in the arm of the SHIME that previously underwent the 'mild stress' conditions.

			HF	
	NS	**NSS**	**MS**	**MSS**
PC	664	1080	522	1829
DC	4404	470	3838	1355
			HP	
	NS	**NSS**	**MS**	**MSS**
PC	1297	459	1091	1312
DC	2076	6064	2117	1837

Supporting information legends

Supporting Information Available, including data on rarefaction curves (Figure S1) the heat map showing the enrichment of the genera most affected during the experiment (Figures S2 and S3), the networks of positive interactions of the whole Illumina dataset (Figures S4 and S5) and the differential clustering (multivariate regression) of untargeted metabolites (Figure S6).

Figure S1: Rarefaction curves (a) and number of OTUs (b) in the samples.

Figure S2: Heat map of the main changes at the genus level in community composition in the proximal colon. Samples have been named according to Fig. 2

Figure S3: Heat map of the main changes at the genus level in community composition in the distal colon. Samples have been named according to Fig. 2.

Figure S4: Network of positive interactions of the whole Illumina dataset based on Pearson correlation matrix with HF diet. Node size = betweenness centrality; Green to red = low to high interactions. Networks have been generated with CytoScape 3.1. Samples have been named according to Fig. 2

Figure S5: Network of positive interactions of the whole Illumina dataset based on Pearson correlation matrix with HP diet. Node size = betweenness centrality; Green to red = low to high interactions. Networks have been generated with CytoScape 3.1. Samples have been named according to Fig. 2

Figure S6: Differential clustering (multivariate regression) of untargeted metabolites for the proximal (A) and the distal (B) colon compartments. Samples have been named according to Fig. 2. Color code: red/orange = HP diet; Blue/light blue = HF diet.