Functional consequences of microbial shifts in the human gastrointestinal tract linked to antibiotic treatment and obesity

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The microbiomes in the gastrointestinal tract (GIT) of individuals receiving antibiotics and those in obese subjects undergo compositional shifts, the metabolic effects and linkages of which are not clearly understood. Herein, we set to gain insight into these effects, particularly with regard to carbohydrate metabolism, and to contribute to unravel the underlying mechanisms and consequences for health conditions. We measured the activity level of GIT carbohydrate-active enzymes toward 23 distinct sugars in adults patients (n = 2) receiving 14-d β-lactam therapy and in obese (n = 7) and lean (n = 5) adolescents. We observed that both 14 d antibiotic-treated and obese subjects showed higher and less balanced sugar anabolic capacities, with 40% carbohydrates being preferentially processed as compared with non-treated and lean patients. Metaproteome-wide metabolic reconstructions confirmed that the impaired utilization of sugars propagated throughout the pentose phosphate metabolism, which had adverse consequences for the metabolic status of the GIT microbiota. The results point to an age-independent positive association between GIT glycosidase activity and the body mass index, fasting blood glucose and insulin resistance ($r^2 \geq 0.95$). Moreover, antibiotics altered the active fraction of enzymes controlling the thickness, composition and consistency of the mucin glycans. Our data and analyses provide biochemical insights into the effects of antibiotic usage on the dynamics of the GIT microbiota and pin-point presumptive links to obesity. The knowledge and the hypotheses generated herein lay a foundation for subsequent, systematic research that will be paramount for the design of "smart" dietary and therapeutic interventions to modulate host-microbe metabolic co-regulation in intestinal homeostasis.

Introduction

The human gastrointestinal tract (GIT) is home to trillions of finely tuned, interacting microbes, which are defined as the gut microbiota.[1] Their combined genomes provide us additional functions and molecules.[2-4] The phylogeny, age and geographical origin of a host[5] and other factors, such as obesity,[6] diet,[7] pregnancy,[8] and antibiotic usage,[9,10] strongly shape gut microbial diversity. The insights gained from a series of studies emphasizing the contribution of colonic bacteria in modulating the host health status (e.g., by participating in liver development and in the metabolism of bile acids and hormones) underscore the importance of the GIT microbiome for gut homeostasis.[10-15]

The richness, diversity and evenness of bacterial taxa and gene content of the GIT microbiota have been shown to be altered in obese and antibiotic-treated subjects.[6,10,16] In humans, long-term exposure to antibiotics has been associated with weight gain and acquired obesity,[17,18] and a link between antibiotic use and obesity was proposed.[19] Indeed, antibiotic treatments have long been used to promote weight gain and increase body mass index (BMI) in animals.[20] While research efforts to elucidate the impact of antibiotic treatment and obesity on GIT microbiota have mainly focused on community shifts and metagenomic data,[21,22] little is known about the functional consequences of these shifts on the cross-talk between gut microbial metabolism and host responses.

Our recent study in fecal samples from a patient subjected to a β-lactam intravenous therapy suggested that antibiotics may ultimately alter the energy balance in the GIT.[10] A number of reports suggest that the relative contribution glycoside-hydrolases (GH) or glycosidases is indicative of the capacity for sugar metabolism and energy production and conversion in the GIT microbiome,[23,24] and that its alteration may stimulate weight gain.[25] Therefore, in the study herein presented, we set to assess the functional differences and consequences of microbial shifts in the human gastrointestinal tract in relation to antibiotic treatment and obesity. To this end, we systematically collected glycosidase activity data in fecal bacterial proteins from unrelated adult patients (n = 2) upon 14 d antibiotic therapy, plus obese (n = 7) and lean (n = 5) adolescents who did not present any intestinal disorders and had not taken antibiotics (for details see Table S1). The variations in fecal bacterial glycosidase activities were complemented with: (1) a metaproteome-wide integrated metabolic analysis to quantify the impact of the activity shifts on the GIT metabolism itself; and (2) a comparative analysis between activity levels and anthropometric and biochemical parameters to find presumptive correlation variables. With this joint functional approach we laid a foundation to study unto what degree antibiotic use and obesity correlate with the acquisition and stability of functional characteristics of the GIT microbiota. We believe that future attempts to manipulate gut microbiome will capitalize, among other, on the knowledge of GH as potential targets to benefit human health through therapeutic and dietetic interventions.

Results

Shifts in carbohydrate turnover metabolism in antibiotic-treated patients. **Figure 1** displays the specific activities (units/g total protein), tested against 23 sugars, of the intra-cellular bacterial proteins from fecal samples (FS) from patients P1 (68-y-old male) and P2 (73-y-old female), prior (day 0; FS-0) and on days 3 (FS-3), 6 (FS-6), 11 (FS-11) and 14 (FS-14) of β-lactam intravenous therapy. As regards to the activity values per each of the sugars, P1 and P2 could not be distinguished from each other (interpersonal variations ranging from 1.69×10^{-5} to 1.0%, depending on the sugar); accordingly, results are presented as mean values ± standard errors (**Fig. 1**). There were several notable findings.

First, a 2-fold increase in total glycosidase activity occurred on day 3 that recovered on days 6 and 11 (**Fig. 1A**, inset), time points characterized by a decrease in total bacterial taxa and richness.[10] Interestingly, on day 14, when bacterial community were significantly less active at the level of RNA,[10] a 3-fold activity increase was observed compared with FS-0 (**Fig. 1A**, inset).

Second, the specific activity ratios for the most to least efficiently hydrolyzed carbohydrates were 385 (for FS-6), 410 (for FS-11), 496 (for FS-0), 683 (for FS-14) and 3,081 (for FS-3), with β- and α-galactose being the most efficiently hydrolyzed carbohydrates and β-glucuronide, β-acetylglucuronide and β-cellobiose being hydrolyzed at the lowest levels (**Fig. 1A**). Accordingly, β-galactosidases (ranging from 47% [for FS-11]

to 81% [for FS-3]) of the total activity) and glucuronidases and cellulases (accounting for < 1% of total activity) were the most and least active enzymes (**Table S2**), respectively, independently of the duration of the treatment. These data agree with the generally low fermentability of insoluble fibers such as celluloses and hemicelluloses.[26] Note that β-fucose, α-mannose and α-arabinopyranose were preferentially hydrolyzed over α-fucose, β-mannose and β-arabinopyranose, with altered relative activity levels between isomers depending on the time point (**Fig. 1A**; **Table S2**). This result contrasted with the overall similar hydrolytic capacities for α- and β-xylose, which were not significantly affected by the antibiotic therapy.

Third, different trends toward alterations of the metabolism of specific carbohydrates, was observed throughout the therapy. According to VEGAN package,[27] five sugar clusters were evident (**Fig. 2**). The hydrolytic capacity for the sugars included in cluster one decreased during therapy, reaching a minimum on day 11; these sugars included α-glucose, α-maltopentose, α-xylose and β-arabinopyranose. For the cluster two, the activity level was maximal on days 3 and 14 and minimal on day 11, including α- and β-galactose, β-cellobiose and α-arabinofuranose. The activities for sugars in cluster three decreased on day 3 but rapidly increased to peak on day 14; these activities involved β-glucose, β-mannose, β-lactose, β-fucose and α-maltose. In cluster four, the activities oscillated, reaching maximum on day 6, including β-xylose, β-glucuronide, β-acetylglucuronide, α-rhamnose and α-fucose. Finally, α-maltohexose, α-acetylneuraminic acid, N-acetyl-β-D-glucosaminide and α-mannose, were the only sugars hydrolyzed at the highest level on day 11; these four carbohydrates formed cluster five.

Shifts in carbohydrate turnover metabolism between lean and obese. We further evaluated the glycosidase activity profiles in the fecal microbiota of lean (n = 5) and obese (n = 7) adolescents aged 13–16 y. As regards to the activity values per each of the sugars, no statistically significant differences (p < 0.05; Student's t-test) were found between the subsets of each of the two groups; same as for P1 and P2, results in **Figure 1** are presented as mean values ± standard errors. From the data shown in **Figure 1B**, obese subjects were characterized by a higher total sugar metabolism capacity, with a net global increase for the 23 carbohydrate tested from 38.0 ± 0.6 to 426.5 ± 3.2 units/g total protein. In addition, in comparison with lean subjects, obese had a less balanced GIT biochemical environment with declining relative activity levels toward α-acetylneuraminic acid (Obese/Lean: 0.01%/0.26%), N-acetyl-β-D-glucosaminide (0.02%/0.31%), β-fucose (1.1%/5.3%), α-arabinopyranose (1.3%/4.0%) and α-rhamnose (1.6%/4.2%), while having drastic improvements for α-arabinofuranose (11.0%/1.1%) and α-glucose (7.8%/1.4%) activities (**Table S2**).

Shared and distinct functional changes: antibiotic-treated adults vs. obese and lean adolescents. Comparison of the glycosidase activities in obese and antibiotic-treated patients yielded two common characteristics. First, a 14 d antibiotic treatment promoted the total GIT glycosidase activity (approximately 3-fold) as compared with the admission day, a trait that was also observed albeit at higher level (11-fold) in obese as compared with
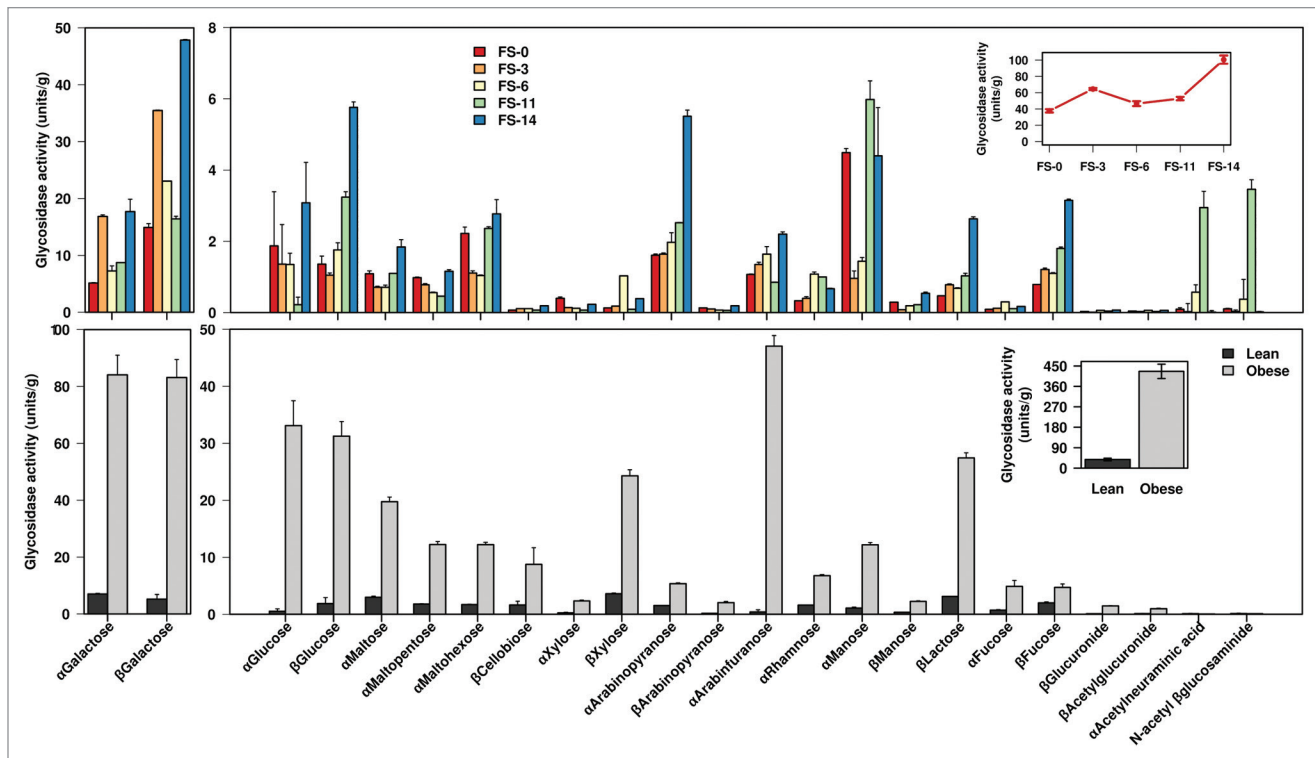
Figure 1. Shifts in carbohydrate turnover profile. Enzyme activities (units per gram total protein) from the total faecal microbiota against 23 different sugar substrates were quantified by measuring the release of *p*NP in triplicates, as described in Materials and Methods section. (**A**) Glycosidase profile for both β-lactam-treated adult patients P1 and P2 in the time span investigated. (**B**) Glycosidase profile for lean (n = 5) and obese (n = 7) adolescents. A single plot (with mean values ± SD, estimated for each group of samples using three independent measurements each) is shown for P1/P2 (panel **A**) and lean and obese subjects (panel **B**), as no statistically significant differences in activity values were discernible within each set of samples. The insets in panels (**A**) and (**B**) represent the cumulative activity (total units per gram ± SD) for all sugars being hydrolyzed for each of the samples. Note: α- and β-galactosidase activities were separated, as the activity levels were significantly higher as compared with the other activities and are shown in the left panels.

lean (**Fig. 1**). Second, nine out of 23 substrates were also preferentially processed in both obese and 14 d antibiotic-treated subjects, including α-glucose, β-glucose, α-maltohexose, α-galactose, β-galactose, α-arabinopyranose, α-arabinofuranose, α-manose and β-fucose (**Fig. 1** and **Table S2**).

Similarly to antibiotic-treated adult patients, high levels of activity toward α- and β-galactose characterized both lean- and obese-adolescents and accounted for 32% and 39% of the total normalized activity, respectively (**Fig. 1**; **Table S2**). This suggests that the Human GIT microbiome is particularly enriched in both activities, independently of the subject and the age. By contrast, activity toward α-acetylneuraminic acid and N-acetyl-β-D-glucosaminide accounted for < 0.6% (or < 0.22 ± 0.13 units/g) of the total normalized activity in obese and lean subjects as well as in patients before the antibiotic treatment (**Fig. 1**; and **Table S2**), while their contribution at day 11 of the antibiotic intervention accounted for ~12% (or 6.4 ± 0.73 units/g). This indicates that both activities play secondary roles in the GIT microbiota in the absence of antibiotics, while they become particularly enriched as a consequence of the antibiotic treatment.

GIT glycosidase activity associated with BMI and glucose level. We further evaluated whether subject characteristics (**Table S1**) such as BMI may be strong predictor of the capacity of

the GIT microbiota to metabolize carbohydrates, that is, whether a relation between the GIT glycosidase activity (**Fig. 1**) and BMI could be established. Results shown in **Figure 3A** provide evidences of the goodness of a sigmoid ($r^2 = 0.98$; BMI_{50} value of 24) model to adequately describe this dependence for all subjects investigated but one (P1). Noteworthy, comparison of the glycosidase activity between adult P2 (weight [Kg], 54.0; BMI [Kg/m²], 33.5) the admission day prior to the antibiotic treatment and the adolescents with similar BMI (weights [Kg], 40.9–65.9; BMI [Kg/m²], 17.94–23.57) yielded essentially the same results (**Fig. 3A**). As the host fasting glucose and homeostasis model assessment-estimated insulin resistance (HOMA-IR) indexes[28] were available for the obese subjects, a similar analysis was performed; we found a linear relation ($r^2 \geq 0.94$) between both data types and GIT glycosidase activity (**Fig. 3B**); note that this correlation was only observed for patients with ≥ 83 mg/dL fasting blood glucose. Other biochemical data (**Table S1**) related to host lipid and nitrogen metabolisms did not correlate with glycosidase activity.

Antibiotics promote host mucus hydrolyzing activities when richness and biodiversity decrease. The census of GH-like enzymes (183 in total from 37 families) in P1 fecal bacterial protein extracts, determined through quantitative shotgun
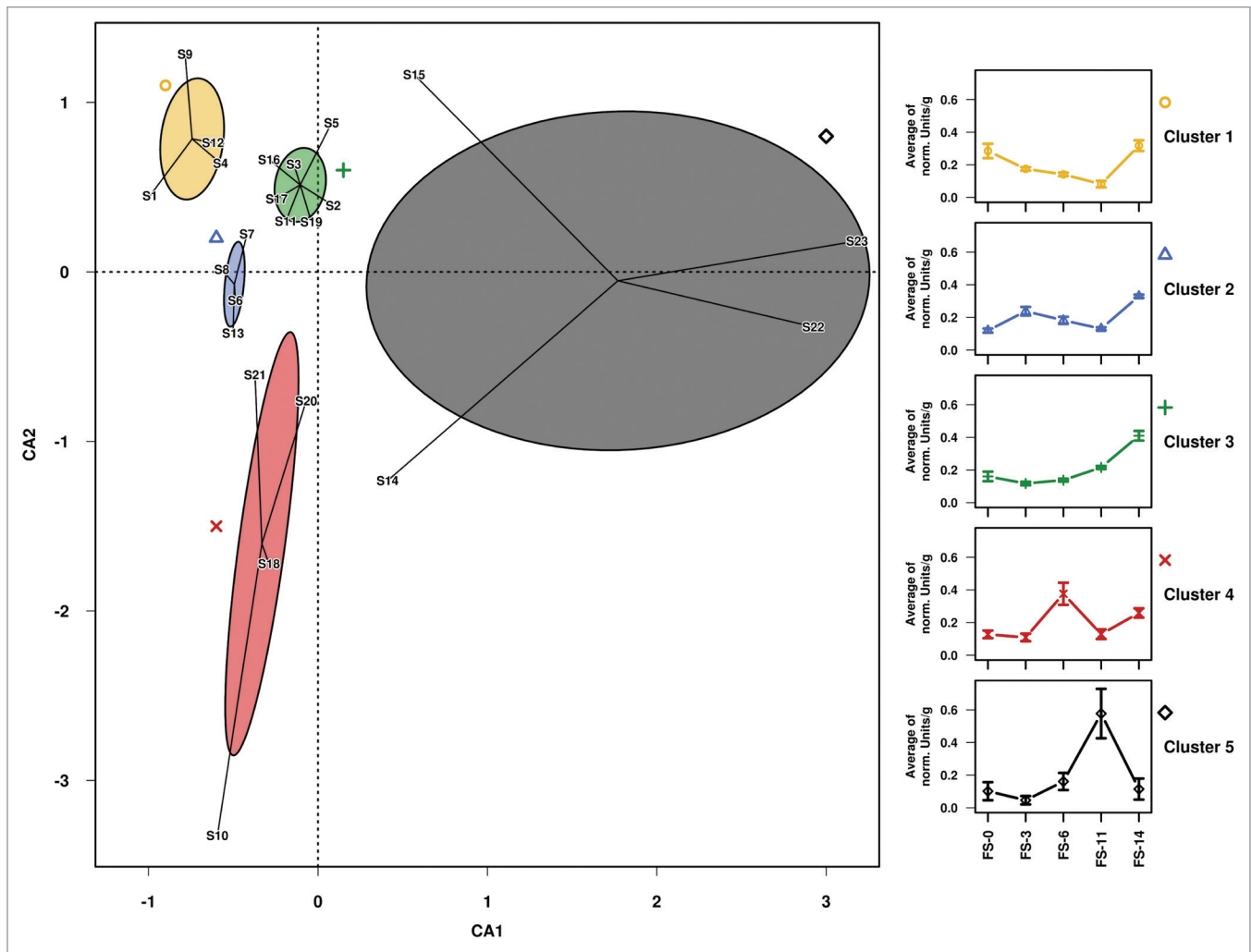
Figure 2. Correspondence analysis of the carbohydrate turnover profile. The relative proportion of glycosidase activity (units/g total protein) for each of 23 different sugars in the different samples was considered for the analysis. Five distinct clusters (color-coded in the figure), which are based on shifts in the turnover profiles toward the different sugars, are evident. Cluster one included α-glucose (S1), α-ᴅ-maltopentose (S4), α-xylose (S9) and β-arabinopyranose (S12). Cluster two included β-ᴅ-cellobiose (S6), α-ʟ-galactose (S7), β-ᴅ-galactose (S8) and α-arabinofuranose (S13). Cluster three included β-ᴅ-glucose (S2), α-maltose (S3), β-ᴅ-mannose (S16), β-lactose (S17) and β-fucose (S19). Cluster four included β-xylose (S10), α-ʟ-rhamnose (S14), α-fucose (S18), β-glucuronide (S20) and β-acetylglucuronide (S21). Cluster five included α-ᴅ-maltohexose (S5), α-mannose (S15), α-acetylneuraminic acid (S22) and N-acetyl-β-ᴅ-glucosaminide (S23). The mean values for each group of samples were calculated accordingly to data provided in **Figure 1**.

metaproteome measurements,[10] showed no significant differences in GH-like enzymes counts (**Fig. S1**). However, their expression was, by contrast, greatest on day 11 when the expression of other enzymes (193 classes according to their EC numbers) was minimal (**Fig. 4A**). This is a result of the high expression levels of mucin glycan degrading families 2 and 20 β-N-acetylhexosaminidases (EC 3.2.1.52)[29] in FS-11 with respect to the level of total GH-like enzymes (**Fig. 4B**) and total enzyme content (**Fig. 4C**). This agrees with the fact that FS at day 11 showed a marked increase of activity against α-acetylneuraminic acid and N-acetyl-β-ᴅ-glucosaminide (**Fig. 1**). Note that FS-14 was characterized by the lowest expression of GH-like enzymes (**Fig. 4A**), with a major fraction (~16% of the total) expressed at levels < 0.2% of the relative concentration (**Fig. 4B**).

Metabolic shifts. Using the method described in Materials and Methods, we further integrated protein expression data

available for patient P1,[10] into a network model that collected the metabolic routes that might be present in the microbiome. These network models showing changes in the metabolic reactions are shown in **Figure S2**. We observed that compared with FS-0, the significant reduction of enzyme expression observed at day 11 of the antibiotic therapy (**Fig. 4A**) had a direct metabolic consequence: 121 reactions were partially (from 1.5- to 20-fold) and 47 totally deactivated (**Table S3**), as compared with the beginning of the therapy. A comparison of FS-11 network topologies, with reduced sugar hydrolysis capacity (**Fig. 1A**) correlated with the strong downregulation of reactions linked to glycolysis (32-fold) and pentose phosphate (18-fold) pathways compared with FS-0 (**Tables S4 and S5**). In addition, the global attenuation of carbohydrate metabolism was followed to a lesser extent by changes in amino acid metabolism (**Tables S4 and S5**). Thus, in sample FS-11, 7 out of 11 pathways of amino acid metabolism

were highly deactivated by meaning of the lower expression level (2.6- to 117-fold) of proteins assigned to those pathways, compared with FS-0. Other striking differences observed on day 11 included nucleotide metabolism (average 53-fold lower protein expression), oxidative phosphorylation (3.3-fold lower protein expression) and the tricarboxylic acid cycle (4.5-fold lower protein expression) (**Tables S4 and S5**).

Using a similar approach, network models (**Fig. S3**) were created for two of the adolescents herein investigated (Obese111 and LeanCE01) according to their available metaproteome measurements.[6] Forty and 38 reactions were found to be potentially up and downregulated in obese as compared with lean subjects, respectively; additionally, 14 reactions were only found in obese and 8 in lean. The results confirmed that bacterial protein extracts from the obese had a significant improved contribution of proteins linked to the pentose phosphate metabolism (25.3-fold) and the tricarboxylic acid cycle (8.9-fold). To note is that both pathways were also significantly upregulated (7.8- and 52.3-fold, in the same order) in the subject P1 at the end of the antibiotic therapy (day 14) as compared with the admission day prior to the antibiotic treatment (**Table S5**). Additionally, the fructose and mannose (14.4-fold), galactose (21.2-fold), starch and sucrose (31.9-fold) and amino sugar and nucleotide sugar (14.0-fold) metabolisms were also upregulated in the obese, in agreement with the higher activity of obese GIT for these types of sugars (**Fig. 1B**).

Discussion

The results presented here enabled us to gain valuable insights into the biochemical and metabolic-wide effects of antibiotic usage and obesity in the gut microbiota. The study also allowed us to propose presumptive links between these variables. First, there were significantly increased rates of sugar metabolism in both 14 d β-lactam treated patients and obese, albeit at different levels, which correlated with the strong activation of network reactions linked to glycolysis and pentose phosphate pathways. Second, as compared with non-treated patients and leans, both antibiotic-treated and obese subjects had a less balanced GIT functional environment with significant alteration in the hydrolytic capacity of ~40% of all sugars tested. These factors all together promote the digestion and energy extraction from dietary polysaccharides and the fermentation of their final products. As shown earlier,[30] these are major factors contributing to the diversity of human GIT microbiota and its impact on host metabolism, metabolic syndrome and obesity. In addition, the findings presented here point to an age-independent correlation between GIT glycosidase activity and (1) BMI for ten out of 11 subjects investigated and (2) blood glucose levels and insulin resistance in obese patients. Finally, our data support the hypothesis that antibiotic therapy additionally altered the active fraction of enzymes controlling the thickness, composition and consistency of the mucin glycans.

The duration of the antibiotic treatment was found to be an important factor affecting the activity and stability of the GIT carbohydrate metabolism. Although our native microbiota is considerable resilient due to redundancy among its constituent taxa, antibiotic perturbation disrupts our microbial ecosystem and imprints a long-term selection of new community members that possibly deploy or activate species-encoded functional processes. This hypothesis is underscored by the fact that β-lactam treatment was shown to have marked effects on the capacity to anabolize at least 40% of all sugars investigated. Another observation of the altered functional community was an improvement on the mucin "scavenging" capacity when the total biodiversity reached a minimum as a consequence of the antibiotic therapy. It is plausible that some gut microbial species rely on host glycans in the absence of energetic input because of the impaired utilization of dietary carbohydrates and depression of carbohydrate metabolism in the GIT at intermediate stages of the antibiotic therapy, as has been demonstrated in absence of dietary inputs.[31] Together with previous observations, our findings support the hypothesis that antibiotic effects should be significant factors in the symptoms related to carbohydrate maldigestion,[25,32] and the progression of infections and diseases. The fact that the altered composition of mucus is likely a key factor in determining which microorganisms physically associate with this layer, copes with the last two hypotheses.[31,33] Further studies are required to ascertain whether the antibiotic-induced changes at the level of microbial composition and function have immediate or long-lasting collateral effects and whether they are preventable; this should be of practical importance since this phenomenon linked to antibiotic therapy may be one of the mechanistic factors involved in infancy to adulthood obesity. Indeed, early-life treatment with antibiotics altered murine metabolic homeostasis and increased adiposity in mice.[34]

Another interesting result of this study was the high anabolic capacity toward dietary sugars in obese patients. In addition, compared with lean, obese microbiota have a preferential capacity to anabolize polysaccharides containing α-glucose and α-arabinofuranose while having a relative lower capacity to metabolize α-rhamnose, β-fucose, α-arabinopyranose and β-glucose (an essential plant component). Accordingly, it is plausible, that a mechanism that promotes weight gain and insulin resistance or type 2 diabetes in obese,[35,36] may be the increased level of enzymes aimed at the digestion of highly refined carbohydrates, particularly α-polyglucoses, the "western diet" hypothesis.[19] Recently, the role of gut microbiota in the pathophysiology of insulin resistance and obesity was demonstrated because the transfer of lean microbiota increased insulin sensitivity in male recipients with metabolic syndrome.[35] Despite this bulk of data, there is no known functional mechanism to explain this link. Our data provide for the first time a metabolic connection between gut microbiota and host glucose metabolism as we observed a positive correlation between gut glycosidase activities and biochemical parameters such as BMI for all subjects but P1 (see below) and fasting blood glucose level and HOMA-IR index in obese subjects. The sigmoid model proposed for BMI and GH activity pose an intriguing question on the causes that trigger gut GH anabolic capacity at BMI higher than 24, an event that significantly enhance energy extraction from dietary α-polyglucoses. Food choices, social components, physical performance and
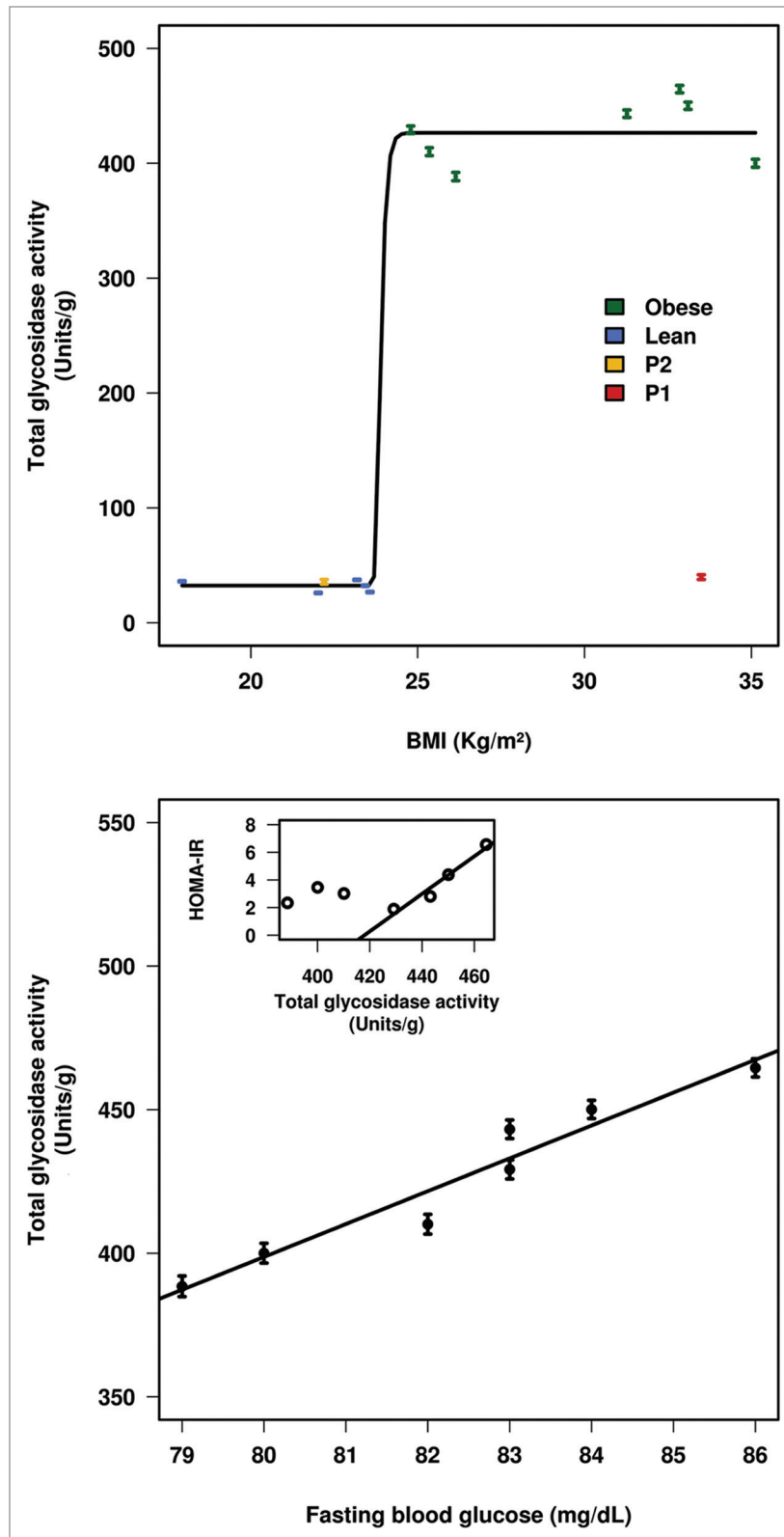
Figure 3. Correlation between metabolic GIT activity and anthropometric and biochemical parameters. (**A**) Association between the BMI and total glycosidase activity. Note: no correlation was found for P1 (for additional comments see Discussion), and its data are not considered for the regression. (**B**) Association between the fasting blood glucose level and total glycosidase activity in obese subjects; inset in (**B**) represents the association between HOMA-IR index[28] (calculated by an automatic web calculator; http://www.hcvsociety.org/files/HOMACalc.htm) and total glycosidase activity. The mean values for each group of samples were calculated using three independent measurements and according to the method and data described in **Figure 1**.

many other possible reasons may underlie this effect. Obviously, we are aware that these correlations were determined for a very small number of subjects and that thus require further confirmation with a higher set of individuals and conditions. However, we believe that the correlations observed support our conclusions reasonably well and that it is plausible that the changes in the GIT microbiota capacity to metabolize carbohydrates may be an origin factor to develop obesity, which in turn may be preventable at a higher level than previously thought.

Altogether, the knowledge and the hypotheses generated herein lay a foundation for subsequent, systematic research that will ultimately enable the design of: (1) personalized diets based on actual potential digestibility of dietary polysaccharides to regulate weight gain and; (2) intervention strategies at defined stages of antibiotic therapies to counterbalance their negative collateral effects toward intestinal mucus barrier. As for (1), it is plausible, for instance, that subsequent studies will allow defining GIT "enzymotypes." Accordingly, this can help identifying sets of missing enzymes and sugar-degrading capacities that are needed to stabilize the GIT microbiota and which, for example, can be added as prebiotics particularly enriched in specific, missing enzyme components. This is of particular importance as carbohydrates are converted by GIT bacterial communities to a variety of organic acids and alcohols.[37] As for (2), mucosal protection therapy could be added as a part of normal therapeutic guidelines at define stages.

By resorting on an integrated, multidisciplinary metabolic analysis, we mapped and assessed the impact of the activity shifts on the GIT metabolism itself. We have contributed to establish a biochemical baseline of the metabolic functioning of the gut microbiota, through which we have suggested potential biochemical links between antibiotic use and obesity, between anthropometric and biochemical parameters and GIT carbohydrate metabolism. However, these results should be seen under the light of an explorative study, as there are several limitations that prevent us to take definitive conclusions, in particular with regard to the links between metabolism dynamics of antibiotic perturbation and obesity. First, we did not examine a separate cohort of subjects that would serve as controls to evaluate the effect of aging on the GIT biochemical environment structure. Second, we only measured the effect of antibiotics without considering the diet of each of the subjects investigated. It is unknown to what degree aging and alterations of diet components correlate with the biological functions herein examined. However, we believe that the impact of aging and diet on our results would be comparatively minor because we did not find significant interpersonal variations in hydrolytic rates within each of the three different groups of unrelated individuals investigated; also, the fact that all adolescents and one adult (P2) with similar BMI do have similar total glycosidase activity (independently of the age and diet) agree with this hypothesis. Third, we should stress the attention to the fact that no correlation between GIT glycosidase activity and BMI was found for P1; to note is that subject P1 is a non-insulin-dependent diabetes mellitus patient daily treated with glimepiride (together with amlodipine, ramipril, hydrochlorothiazide and Marcumar).[10] This suggests additional side effects

of the antidiabetic drug, the in deep consequences of which may be further evaluated.

We advocate that biochemically-driven, systems-biology approaches[15] investigating different antibiotics and subjects with different characteristics (defined, for example, by age and clinical and biochemical parameters) and different dietary variations and diseases will be required to properly establish the linkage between GIT biochemical shifts and human health status according to different environmental factors. The fact that the present study indicates that biodiversity evenness and richness and protein abundance and expression may not be taken as indicators of metabolic activities, supports the importance of such functional analysis. Finally, the results suggest that biochemical data could be used as a potential marker for comparative purposes in future studies on the Human Microbiome Project.

Materials and Methods

Subjects and sampling. Written, informed consent was obtained from all of the subjects. After Institutional Review Board (IRB) review, this study was exempted from IRB approval. The study related to P1 and P2 was approved by the Ethical Board of the Medical Faculty of the Christian-Albrecht-University, Kiel, Germany. Both patients were admitted to the Department of Internal Medicine at the University Hospital Kiel (Germany) because of an infected cardiac pacemaker. For P1, antibiotic therapy was initiated upon admission with a combined intravenous therapy of ampicillin/sulbactam (2 × 750 mg) and cefazolin (3 × 2 g/day) in a single dose and continued with cefazolin alone intravenously for the next 14 d.[10] For P2, antibiotic therapy was initiated upon admission with an intravenous therapy of cefazolin (3 × 2 g/day) for seven days and continued with ampicillin/sulbactam (2 × 750 mg) for next seven days. FS from both patients, who had not taken antibiotics within the previous three months and did not present any intestinal disorders, were collected on 14 d time span.

Clinical examination data of P1 has been described elsewhere.[10] A clinical examination of P2 identified inflamed skin and subcutaneous tissue around the pacemaker, and laboratory findings revealed an elevated C-reactive protein level (CRP 18.1 mg/l; normal value < 8 mg/l) and a full blood count within normal ranges. The pacemaker had been placed to treat the patient's atrio-ventricular block III, first diagnosed in 1994. She had no further cardiovascular risk factors and did not take any regular medication. The patient's CRP level returned to normal within eight days after the beginning of AB therapy.

Fresh FS collected from seven obese (four males and three females) and five lean adolescents (two males and three females), with ages ranging from 13 to 16 y-old, who did not present any intestinal disorders and had not taken antibiotics within the previous 12 mo, were examined. Subjects were selected according to their BMI index [weight (kg)/height (m²)] from the panel of adolescents collected during the course of the EVASYON study.[38] The EVASYON study was designed to develop a multidisciplinary obesity treatment program that was assessed and controlled by pediatric services in Spain. All participants in
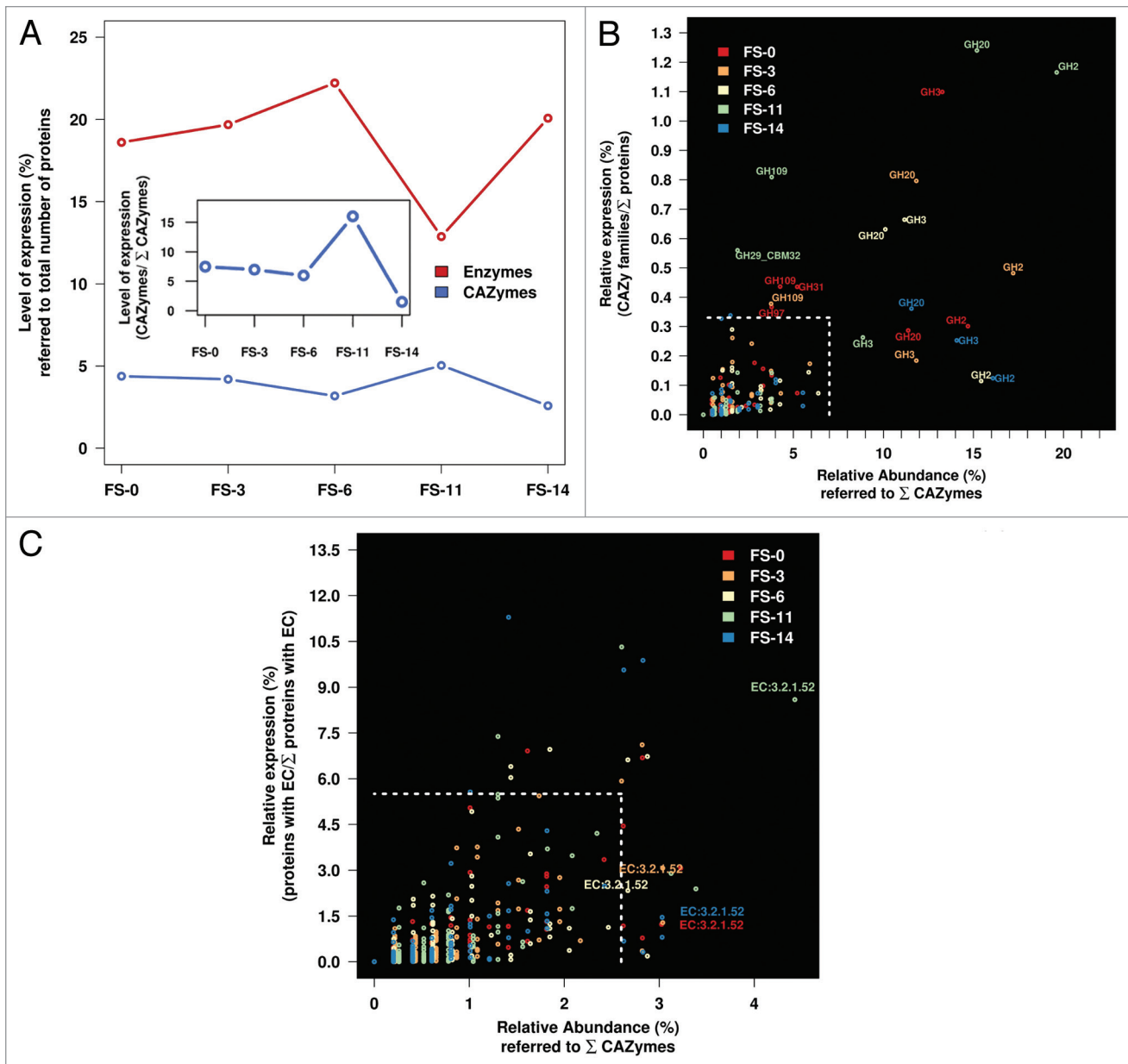
Figure 4. Relative abundance and expression levels of GH-like enzymes (referred as CAZymes, carbohydrate active enzymes) and other enzymes (proteins with EC numbers) compared with the total amount of protein recovered from each of the shotgun metaproteomes of the GIT microbiota of patient P1. The results are the average of the shotgun metaproteome measurements from two independent technical replicates per sample.[10]

EVASYON respect the following international conventions and declarations.[38]

The anthropometric, clinical and biochemical data are shown in **Table S1**.

Biochemical experimental setting. Fresh stool samples were collected from each subject, frozen immediately and stored at −80°C until further processing. Protein extracts were isolated, as described previously.[6,10] Briefly, FS were resuspended in phosphate-buffered saline solution (PBS). The samples were then centrifuged at 2,000 g at 4°C for 2 min to remove fecal debris. The supernatant was centrifuged at 13,000 g for 5 min to pellet the bacterial cells, which were lysed in BugBuster®

Protein Extraction Reagent (Novagen, Darmstadt, Germany) for 30 min at 4°C, with further disruption by sonication (using a pin Sonicator® 3000; Misonix) for 2.5 min (10 W) on ice (5 cycles × 0.5 min). The extracts were then centrifuged for 10 min at 12,000 g to separate cell debris and intact cells and the supernatants carefully aspirated (to avoid disturbing the pellet) and transferred to a new tube. Proteins were stored at −80°C at a ~0.22 mg/ml concentration until use.

Glycosidase activity was quantified in triplicates in 96-well plates using a BioTek Synergy HT spectrophotometer in a colorimetric assay with a set of 23 structurally diverse sugars (as p-nitrophenol [pNP] derivatives, all from Sigma Chemical Co.)

that represented the core structure of common glycans and under previously described conditions,[39] with minor modifications. Briefly, reactions contained 4.0 μg total protein and 1 mg/ml sugar substrate (from a 10-mg/ml stock solution) in a 20-mM glycine buffer (pH 9.0, T = 30°C in a final volume of 150 μl). Reactions were followed every 10 min by measuring the release of pNP at 410 nm for 130 min. In all cases, one unit (U) of enzyme activity was defined as the amount of protein producing 1 μmol of reducing sugars for 1 min under the assay conditions.

Proteome-scale metabolic reconstruction. Available network reconstructions in the literature are generally organism-specific. Genome-scale metabolic networks provide a more accurate description of the metabolic processes of specific organisms, typically including manually curated physiological information as well as in silico algorithmic refinements. Apart from the reactions and their associated information (reversibility, compartment, EC number and genes), additional experimental information may be involved. Accordingly, metabolic reconstruction typically starts from an annotated genome, which is then integrated with reaction databases and reported physiological evidence, as well as gene transcript levels or protein expression values, to produce a functional computational model. In our case, the reconstruction was slightly different because we aimed to reconstruct the entire gut metabolic network, where multiple proteins from multiple organisms are co-expressed. Herein we used the expression values of the set of proteins that were unambiguously quantified in the shotgun metaproteomes in the same set of samples used for activity tests (P1, Obese111 and LeanCE01),[6,10] to produce metabolic reconstructions of the entire gut metabolic network, where multiple proteins from multiple organisms are co-expressed. The enzyme commission number (EC number) was used as a numerical classification scheme for enzymes based on the metabolic reactions they catalyze, with each EC class exclusively defining the function performed by the enzyme.[40] EC numbers in this analysis are exclusively used to refer to specific enzymes. Using appropriate bioinformatics tools, the Kyoto Encyclopedia of Genes and Genomes (KEGG) was used to obtain the names and EC codes of the enzymes corresponding to the metabolic reactions represented in the entire metabolic network.[41,42] Customized computer programs written in R[27] and Perl (http://www.perl.org/) were used to obtain enzyme information from local copies of KEGG. To explore the variation in the full metabolic network that represented the metabolic activities of the microbiome, we pooled them to ensure that the same EC code originating from different organisms in each of the considered samples was considered linked to the same metabolic function. This information was further integrated with a generic metabolic map, which was constructed using information from KEGG,[41,42] which provided an overview of the complete metabolism of the biological system.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Supplemental Materials

Supplemental materials may be found here:
www.landesbioscience.com/journals/gutmicrobes/article/25321

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