Small intestinal microbiota: the neglected stepchild needed for fat digestion and absorption

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ABSTRACT

Our recently published paper “Small Intestine Microbiota Regulate Digestive and Absorptive Adaptive Responses to Dietary Lipids” in Cell Host & Microbe explored the neglected small intestine microbiota and demonstrated its critical role as a regulator of fat digestion and absorption. This work generated the following important take home messages: 1) small intestinal microbes are particularly sensitive to high fat diets and turn on host processes regulating fat digestion and transport, 2) this action is very likely orchestrated by a consortium of microbes, each having different specific effects and targets, and 3) the actions of this consortium appear to be mediated by bacteria-derived small molecules or bioactive components. These findings are expected to provide insight into developing treatments for conditions of under- or over-nutrition. The goal of this addendum is to summarize our findings, address issues related to gut microbiota and gnotobiotic research specifically regarding technology and experimental design, discuss this work in the context of relevant literature, and lastly provide considerations for future research.

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Salient findings – section 1 (summary)

Small intestine microbiota – the neglected stepchild

Gut microbes have been previously implicated in regulating fat absorption. However, a comprehensive and mechanistic approach to understanding the complexities underlying the host-microbe interactions that regulate fat digestion and absorption pathways is still needed. Our recently published paper “Small Intestine Microbiota Regulate Digestive and Absorptive Adaptive Responses to Dietary Lipids” in Cell Host & Microbe identified mechanisms by which microbes regulate lipid digestion and absorption and also demonstrated that high fat diets affect this process, particularly through altering microbiota composition of the small intestine, that has been the neglected stepchild of gut microbiota research.

The small intestine is the major site of nutrient digestion and absorption and serves as a primary source for producing important enteroendocrine hormones that regulate the initial phases of nutrient processing and digestion. Despite this knowledge, little research has focused on host-microbe interactions occurring in this region of the gut. Therefore, the goal of our study was to 1) explore the basis for the resistance to diet-induced obesity and fat malabsorption in germ-free (GF) mice, 2) determine if and how high fat diets (HFD) affect small bowel microbiota membership and function, 3) determine if these changes increase the degree of fat digestion and absorption, and, if so, 4) identify the mechanisms by which microbes enhance these properties.

Our preliminary findings and work of others showed that GF mice are resistant to diet-induced obesity which may be related to a number of potential mechanisms. Our group, for instance, had reported that these mice exhibit severe disruption of both central and peripheral circadian networks, despite being exposed to the same light-dark conditions as their conventionally-raised counterparts (Leone, et al CHM 2015). This study further showed that Western diets promoted...
obesity by causing a different pattern of circadian disruption that led to changes in energy balance. However, in the current study, we also noted that GF mice have increased lipid in their feces compared to mice reared in normal conditions, suggesting that they malabsorb fat. To further examine this, we delivered radiolabeled triglyceride and cholesterol to SPF and GF mice and found that GF mice have severely impaired fat absorption over time compared to SPF mice. Next, we assayed the gut microbiota residing in the duodenum, jejunum, ileum and cecum under dietary pressure of a purified low fat (LFD) or (HFD) diet. Here it was found that HF diet had the greatest impact on gut microbiota structure in the jejunum and ileum. Next, we conducted transplant experiments using the jejunal microbiota to determine if this regional microbiota was capable of restoring fat absorption in germ free mice. Indeed, jejunal microbiota from HFD-fed mice increased lipid absorption in GF mice compared to that from LFD-fed mice.

One limitation of this experiment is that we did not directly compare jejunal microbiota to microbiota from more distal regions such as the cecum to directly compare the capacity of differing regional microbiota to increase lipid absorption. During the revision phase of the paper, we conducted a direct comparison of jejunal vs cecal microbiota. Due to time and resource limitations, we could only use chow-derived microbiota from specific pathogen free (SPF) mice, as we did not have material from HFD vs LFD-fed mice (usually collected from mice fed at least 4 weeks with these purified diets). In comparing jejunal vs cecal chow-derived microbiota there was no difference in lipid absorption. Therefore, future studies will be conducted to directly compare HFD-induced jejunal microbiota vs cecal or stool microbiota in their ability to increase lipid absorption and determine if either region elicits a greater level of absorption.

**Integrated role of microbes to impact lipid absorption**

Our study sheds light on the complexity of host-microbe interactions, as our study highlights the orchestrated actions of many different strains of microbes with unique functional roles to collectively impact various mechanisms involved in lipid absorption. For example, we found that the combination of two strains of bacteria increased Cckar expression in the pancreas after delivery to GF mice. We also found that either live *Clostridium bifermentans* or conditioned media from this bacterium induced Dgat2 expression in the duodenum and jejunum, while another bacterium also belonging to the Firmicutes phylum, *Lactobacillus rhamnosus* gg, induced Dgat1 expression in the jejunum. Neither strain had a significant impact on overall body weight and body fat compared to control mice. Therefore, it likely takes a consortium of microbial species to elicit overt changes in body weight and body fat by affecting different phases of host lipid digestive and absorptive processes. Notably, the strains selected in this study were reference strains purchased from ATCC to establish proof-of-concept that specific microbes regulate lipid absorption pathways. However, in future studies, we plan to cultivate specific indigenous human and/or murine small intestinal strains under HFD or LFD conditions and characterize them genomically and functionally.

Another consideration is that these supplementation experiments were conducted using antibiotic-treated SPF mice as opposed to GF mice. It is possible that monoassociations of GF mice have revealed significant changes compared to GF controls in other outcomes beyond Dgat2 and Dgat1 expression levels, as there were some trends identified in other outcomes measured but that did not reach significance. Notably, the mice were only supplemented once per week with these bacteria vs most probiotic studies where supplementation is performed daily. Therefore, more frequent dosing may have led to more dramatic outcomes as well. Even with our modest approaches, *C. bifermentans* significantly induced Dgat2 expression in the small intestine (either via weekly dose of CM or weekly dose of live bugs). Use of antibiotics is an important consideration for maintaining relevance to humans and avoiding developmental issues related to GF mice.

**Bioactivity of small molecules or bacterial components regulate fat absorption**

Because HFD increased the relative abundance of the family Clostridiaceae compared to LF diet in all regions, especially in the jejunum and ileum, we selected a representative reference strain from Clostridiaceae to determine if this strain or its
metabolic byproducts increased markers of lipid absorption in vitro or in vivo. Clostridium bifermens
tans was selected based on having high sequence similarity to OTUs found in the small intestine of mice after being fed the HFD. C. bifermens
tans increased the expression of Dgat2, an enzyme critical for fat transport, in vitro and in vivo. While it remains unclear if C. bifermens
directly increases adiposity under HFD conditions, our findings suggested the production of soluble bioactive components or molecules by this strain promotes oleic acid uptake and the expression of Dgat2 involved in TAG synthesis. The effect was specific and not seen in other representative intestinal strains. Future studies will focus on identifying indigenous microbial strains and how their bioactive molecules facilitate and orchestrate host processes that promote lipid digestion and absorption.

**Targeting small intestine microbiota to treat conditions of over- and undernutrition**

We believe our findings have broad and cross-cutting relevance to the extremes of human metabolic diseases, ranging from conditions of undernutrition to overnutrition. First, the small bowel microbiota are the first to encounter dietary components to which rapid adjustments must be made in membership and function. This process by our reckoning is not merely passive, but inherently important in promoting changes in host processes involved in lipid digestion and absorption. The collective impact of these changes stems from a consortium of small bowel microbes, each contributing in ways that help orchestrate multiple host processes to enhance dietary lipid processing. Under conditions of dietary restriction or starvation, we speculate that many of these key consortia that would otherwise be promoted by dietary fat become underrepresented, resulting in a physiological adaptation that downregulates host digestive and absorptive processes for dietary lipids. However, prolonged caloric restriction and malnutrition may result in irreparable changes and extinction of key lipid-sensing consortium effectors that compromise the ability of the host to adjust capacity necessary to adapt to dietary lipid refeeding. On the other hand, conditions of dietary excess and overnutrition may have the opposite effect where lipid-promoted small bowel consortia become overrepresented, thereby increasing capacity for processing and absorbing dietary lipids that only further upsets energy balance in ways to promote metabolic disease and obesity.

The finding that soluble, bioactive mediators may be mediating the effects of lipid-promoted small bowel microbial consortia that promote dietary lipid digestion and absorption also has important clinical and therapeutic implications. First, the identification of many of these molecules and their specific actions and host targets brings promise of developing novel and effective microbiome-based interventions to promote capacity for efficient dietary lipid processing in individuals in need of immediate nutritional restitution. These compounds could be specifically manufactured, formulated, delivered, and dosed to produce actions that would be microbe-independent and specifically tailored to individual needs. The alternative of trying to manipulate the small bowel microbiota would be more difficult and unpredictable. For overweight and obese individuals that continue to consume high fat, western diets, measures could be developed to either block the mediators or target specific members of microbial consortia that promote capacity for dietary fat digestion and absorption. Finally, the ability to monitor the levels of dietary lipid-promoted microbial effectors and their bioactive products becomes a means to titrate interventions to optimize interventions, whether they be therapeutic or preventative.

**New interpretation/speculation – section 2**

**Tools in microbiota research**

One thing we learned from this work is that new tools for assessing functional and individual microbe activity are needed. We are currently limited to large community profiles. One critique of our study was the lack of metagenomic or metatranscriptomic data from the small intestine. However, even large community profiles provide limited information regarding function. Metagenomic datasets only show the genetic capacity of microbial communities and predictions to the microbes to whom these belong. Additionally, functional tests are needed to interrogate the community or individual microbes within the community.
Because a focus of this research is to identify small molecules that mediate the actions of the small bowel microbiota, we aim to conduct unsupervised metabolomic screens to discover novel mediators. Once specific molecules are identified, high throughput assays to test many putative bioactive molecules on regulating lipid absorption will be needed.

Other approaches to identifying functional roles of bacteria on lipid absorption is by focusing on targeting the functional differences between gram positive vs gram negative or motile vs non-motile bacteria, as suggested recently by Lora Hooper’s group.\(^9\) Another approach in simplifying assessment of complex microbial communities is through using defined synthetic microbiota consortiums thereby simplifying the interpretation of their functional role. Along with this idea, is the continued use of gnotobiotic mice to examine how one microbe may change based on host diet in regards to their transcriptome and metabolome. However, the set-back in this scenario is that information is lost in how a single microbe communicates with the community to elicit host responses. Ultimately, it will take further creativity to find approaches that identify key microbial signals and their functional interaction with host cells and signaling pathways that regulate lipid digestion and absorption.

**Considerations for experimental design in microbiota research**

Our study raised important concerns regarding the control of experiments using conventionally-raised vs GF mice. For instance, a limitation of our study was that GF mice were housed in different conditions than SPF mice. For instance, GF mice were maintained in pine shavings while SPF mice were raised in corn cob bedding. This issue was raised by one of the reviewers and addressed in the latter revision phase of the paper. In order to address the housing caveat, the radiolabeled lipid absorption experiment comparing fat absorption in SPF vs GF mice was repeated and we found consistent results in that GF mice displayed impaired fat absorption. While the bedding conditions did not appear to be a confounding factor in this process, it remains unclear whether this factor was confounding for other physiological processes, and thus we encourage other researchers to incorporate a strict level of control in gnotobiotic experimental design.

A senior editor of Cell Host Microbe, Ella Hinson, (http://crosstalk.cell.com/blog/beyond-reviewer-3-a-peer-review-success-story?hs_preview=LIPjBTGO-5692804026) posted an highly informative blog detailing the events and the review process of our paper in specifically addressing housing issues in experiments comparing SPF and GF mice. Moving forward, particular attention to experimental conditions in SPF vs GF mice should be taken in gut microbiota studies. In addition to the need for standards in housing conditions (bedding, isolators, co- vs single-housing), it is also necessary to ensure control among animal diets and genetic background between SPF and GF mice. Because these mice are bred separately, studies should attempt to use ex-GF vs GF mice (in order to use litter-matched and genetically-similar mice) rather than SPF vs GF mice from different breeders. This issue was raised by one of our reviewers and while it was not feasible to repeat all SPF vs GF experiments using ex-GF mice, the conventionalization experiment was conducted using litter-match ex-GF mice and preliminary studies using ex-GF mice not reported in this study revealed significant changes in lipid absorption pathways (i.e., in an unreported experiment we found that conventionalization of GF mice restored Cck gene expression).

**Future directions/untapped mechanisms to be explored**

**Impact of fatty acid type on small intestine microbiota**

We initially began this study with the intention of investigating the influence of fatty acid composition of the diet on gut microbe interactions in host lipid absorption. However, this approach proved to be too complex for the initial stages of the work, particularly in determining mechanisms behind the microbial role in regulating digestive and absorptive pathways. Semova et al demonstrated that both short and long chain fatty acids are absorbed to a greater extent in Conv vs GF zebrafish.\(^2\) But it is still unclear how saturation status may affect this process such as using unsaturated vs saturated fatty acids or between mono-
and polyunsaturated fatty acids. Previous work from our group revealed that the fatty acid composition of the diet effects the distal gut microbiota\textsuperscript{4,5} but it is yet to be determined how diets varying in fatty acid composition impact the small intestine microbiota and how these changes might influence the degree of fat absorption. One potential connection in this process could be diet-mediated effects on bile acid profiles. For instance, Devkota et al. found that our obesogenic milkfat diet (used as the HFD for the study herein) increases the amount of taurine-conjugated bile acids that in turn facilitated an outgrowth of \textit{Bilophila wadsworthia}, resulting in increased incidence of colitis in IL10\/-\/- mice.\textsuperscript{4} This microbe was also identified to be elevated in abundance in humans consuming a diet rich in cheese and meat.\textsuperscript{6} In addition to dietary interactions, microbiota, or lack thereof, significantly impacts the bile acid profile throughout the body since they play a major role in bile acid de-conjugation (GF mice have increase conjugated bile acids compared to SPF mice).\textsuperscript{7,8} Thus, given that both the bile acid and microbiota composition are heavily influenced by diet, and that the presence of the microbiota also alters bile acid composition, this could have implications for the resultant host response.

**Conclusion**

This study demonstrates the essential role of gut microbes in regulating fat digestion and absorption. We found that HFD cause significant alterations in gut microbiota composition in the small intestine that increase fat absorption. Our findings have important implications for understanding how to target gut bacteria to increase fat absorption in conditions of undernutrition or decrease fat absorption in conditions such as obesity and diabetes.

This study underscores the importance of regionality, strain-level differences, function, and systems integration. This work opens the door for new research opportunities as many questions are left unanswered such as: What is the impact of fatty acid composition on the gut microbiota and level of fat absorption? What are the bioactive compounds produced by bacteria that interact with host cells to drive fat absorption? Which host receptors and pathways are receiving these cues? Which bacteria can be used to increase fat absorption or those that can be used to decrease fat absorption? Can we use pre- or pro-biotic therapies to manipulate this process in an effective yet safe manner? Taken together, this work represents an exciting area of research that is expected to lead to finding ways to treat or prevent under or over-nutrition.

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