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From correlation to causality: the case of *Subdoligranulum*

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ABSTRACT

Gut microbes are considered as major factors contributing to human health. Nowadays, the vast majority of the data available in the literature are mostly exhibiting negative or positive correlations between specific bacteria and metabolic parameters. From these observations, putative detrimental or beneficial effects are then inferred. *Akkermansia muciniphila* is one of the unique examples for which the correlations with health benefits have been causally validated *in vivo* in rodents and humans.

In this study, based on available metagenomic data in overweight/obese population and clinical variables that we obtained from two cohorts of individuals (n = 108) we identified several metagenomic species (MGS) strongly associated with *A. muciniphila* with one standing out: *Subdoligranulum*. By analyzing both qPCR and shotgun metagenomic data, we discovered that the abundance of *Subdoligranulum* was correlated positively with microbial richness and HDL-cholesterol levels and negatively correlated with fat mass, adipocyte diameter, insulin resistance, levels of leptin, insulin, CRP, and IL6 in humans.

Therefore, to further explore whether these strong correlations could be translated into causation, we investigated the effects of the unique cultivated strain of *Subdoligranulum* (*Subdoligranulum variabile* DSM 15176 ^{T}) in obese and diabetic mice as a proof-of-concept. Strikingly, there were no significant difference in any of the hallmarks of obesity and diabetes measured (e.g., body weight gain, fat mass gain, glucose tolerance, liver weight, plasma lipids) at the end of the 8 weeks of treatment. Therefore, the absence of effect following the supplementation with *S. variabile* indicates that increasing the intestinal abundance of this bacterium is not translated into beneficial effects in mice.

In conclusion, we demonstrated that despite the fact that numerous strong correlations exist between a given bacteria and health, proof-of-concept experiments are required to be further validated or not *in vivo*. Hence, an urgent need for causality studies is warranted to move from human observations to preclinical validations.

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Introduction

Human beings are superorganisms consisting of human cells coexisting with fungi, bacteria, and viruses, most of which reside in the gut. Recent human and animal studies have demonstrated that variations of the intestinal microbiota composition impact significantly on host physiology by influencing several processes including metabolism, immunity and inflammation, aging, and behavior.[1–4] Significant advances have been made in the study of gut bacteria-host interactions in the onset and progression of noninfectious diseases, particularly metabolic disorders such as obesity and insulin resistance, and diabetes. In this context, several bacterial taxa have been identified as being negatively (detrimentally) or positively (beneficially) correlated to metabolic health.[5] Unfortunately, most of the evidence so far is observational and associative in nature and the causal effect of specific species on the onset and development of obesity and associated metabolic disorders have been demonstrated only for a handful of species isolated from the human microbiota.[6–9]

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Therefore, given the potential role of specific bacteria in the physiologic regulation of host metabolic functions, identifying new bacterial taxa that mechanistically contributed to promote or maintain health can lead to the discovery of new targets for effective interventions in humans and bring new hope to slow down the metabolic disorders associated with obesity.

Among the novel probiotic candidates recently proposed, the most promising is *Akkermansia muciniphila*, for which the most advanced work has been done and both animal and human evidence have been published.[6,10–14] *A. muciniphila* is commonly found in human and mouse gut microbiome and we showed previously that its administration could counteract diet-induced obesity and related disorders, such as glucose intolerance, insulin resistance, hepatic steatosis, and gut permeability in rodents.[6,10,11] These data have been largely confirmed by others.[12] Recently, we demonstrated the proof-of-concept that *A. muciniphila* administration is not only safe but can also improve cardiometabolic risk factors when given as a probiotic to humans with overweight or obesity.[13]

In addition to showing that the administration of *A. muciniphila* can be causally linked with an improvement of several metabolic parameters, we noticed on several occasions that the higher abundance of *A. muciniphila* also co-occurs with the presence of another bacterium called *Subdoligranulum sp.* The genus *Subdoligranulum* belongs to the *Ruminococcaceae* family and is closely related to the *Faecalibacterium* genus. Indeed, we discovered that treatment of obese and diabetic mice with prebiotics (oligofructose) increased levels of *A. muciniphila* but that the levels of *Subdoligranulum* were also increased almost fourfold.[15] In obese humans, we noticed that a subgroup of the subjects with a mild improvement of their metabolic parameters during caloric restriction had significantly lower abundance of both *Akkermansia* and *Subdoligranulum* than the subgroup with a better metabolic response to the caloric restriction intervention.[16] Interestingly, the anti-diabetic drugs metformin and acarbose increase fecal *Subdoligranulum* relative abundance.[1718] Moreover, *Subdoligranulum* genus correlated negatively with glycated hemoglobin (HbA1c) and positively with HDL cholesterol.[18] Also, we found that alcoholic patients with poor gut integrity and high gut permeability had significantly reduced levels *Subdoligranulum*,[19] which confirmed previous observations who found less *Subdoligranulum* in cirrhotic compared to healthy individuals.[20,21] Later, it was described that *Subdoligranulum* was underrepresented in subjects with NAFLD and that *Subdoligranulum* was negatively correlated with different parameters related to metabolic risks such as C-reactive protein (CRP), Fatty Liver Index, and Homeostasis Model Assessment-Insulin Resistance (HOMA-IR).[5,22] Moreover, *Subdoligranulum variabile*, the only species of this genus isolated and described so far, was shown to produce butyrate,[23] a short-chain fatty acid, known for its health potential.[24] Several diseases, such as type-2 diabetes or inflammatory bowel diseases, were associated with lower abundance of butyrate producers like *Subdoligranulum*.[25,26]

Based on all these observations and associations found between *Subdoligranulum* and health, we postulated that *Subdoligranulum* could represent a promising probiotic candidate providing us with a handle to improve metabolic health. To make the leap from describing correlation to determining causation, we administered the only cultivated species of this genus to mice while inducing obesity by feeding them with a high-fat diet.

Results

Subdoligranulum is associated with Akkermansia muciniphila and correlates positively with a healthy metabolic status

Based on available metagenomics and qPCR data, and clinical variables from two previously published cohorts of individuals with overweight and obesity,[16,27,28] we built a co-occurrence network that represents taxa as interconnected nodes if a co-occurrence relationship exists between them (Figure 1a). This allowed us to identify several metagenomic species (MGS) that were associated with *A. muciniphila*, including *Subdoligranulum* (Figure 1a). Moreover, *Subdoligranulum* and *A. muciniphila* relative abundances correlated positively (R = 0,35, p = 0,0003, Spearman correlation). We explored the link between *Subdoligranulum* genus in fecal samples and found that its abundance profile was correlated positively with fecal microbiota richness, HDL-cholesterol levels and adiponectin levels (Figure 1b). Microbiota richness is considered a hallmark of gut health and stability and individuals with low HDL cholesterol have a greater risk of atherosclerosis and cardiovascular disease.[29] In

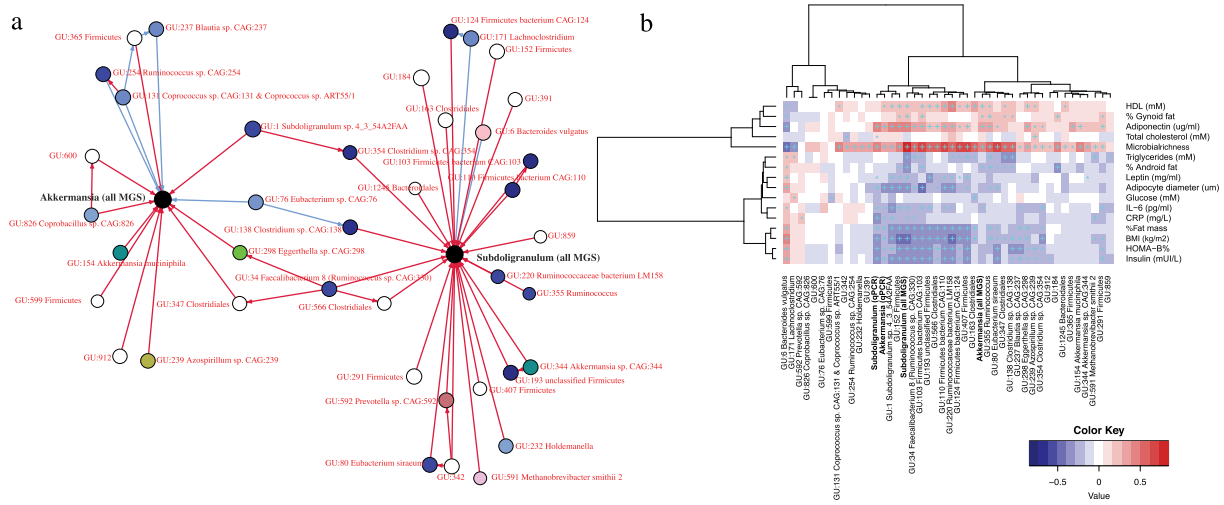


Figure 1. *Subdoligranulum* is associated with *Akkermansia muciniphila* and correlates positively with a healthy metabolic status. **(A)** Subset of the co-abundance network inferred using the SCS method related to the *Akkermansia* and *Suddoligranulum* genera. Nodes indicate metagenomic species and the two target genera, while edges indicate the inferred associations. Direction of the edges displays causal inference while the red and blue colors indicate respectively positive and negative associations. The colors of the vertices depict the different phylogenetic annotations at the family level – black being the two targets and white unknown phylogenetic annotations. **(B)** Heatmap of Spearman correlations between metagenomic species and clinical variables. '+' indicates significant associations resisting adjustment for multiple testing while '*,' associations which do not resist. N = 108.

addition, we observed negative correlations between *Subdoligranulum* and fat mass, adipocyte diameter, a series of surrogate of insulin resistance, and levels of leptin, insulin, CRP, and IL-6. Adiponectin is an adipose tissue-derived hormone that has been involved in protecting against insulin resistance/diabetes and atherosclerosis. Decreased adiponectin levels are thought to play a central role in the development of type-2 diabetes, obesity and cardiovascular disease in humans. In contrast, elevated levels of leptin, another adipokine produced by adipocytes, have been associated with various health conditions including hypertension, low-grade systemic inflammation, and metabolic dysfunction in obese humans. IL-6 and CRP are commonly used systemic inflammatory markers. Taken together the results of this association study indicate that *Subdoligranulum* genus is associated with a healthier metabolic status.

Impact of S. variabile supplementation does not impact on the prevention of diet-induced obesity in mice

Although the genus *Subdoligranulum* is apparently composed of several representative putative species, only one species has been isolated and cultivated so far. This is the strain *Subdoligranulum variabile* DSM 15176 ^{T}.

Therefore, to assess the *in vivo* effects of *Subdoligranulum variabile* we cultured this strain and administered 1.5×10^{9} cultivable cells (cc) of bacteria frozen in glycerol to adult mice fed a high-fat diet (HFD), 5 days a week for 8 weeks (HFD-Subdo group, n = 10). This group was compared to two other groups of mice that were forced-fed with glycerol 25% and were fed either a control diet (Control group, n = 11) or a high-fat diet (HFD group, n = 10) (Figure 2a).

The gavages and treatment were well tolerated, and mice did not display any abnormal signs of stress or discomfort.

After 8 weeks of follow-up, there was a significant difference in body weight gain between the control fed and the high-fat fed mice (Figure 2b). Analysis of the weekly NMR measurements indicated that this was primarily due to an increased fat mass gain (Figure 2c), rather than lean mass (Figure 2d), and that this was the consequence of an increased caloric intake (Figure 2e), confirming the effectiveness of the diet-induced obesity model. However, we found differences neither in body weight gain between the HFD and HFD-Subdo groups, nor in lean or fat mass accumulation.

These observations were corroborated by the data of the tissue dissection, which showed no differences in weight of isolated adipose tissues (inguinal, epididymal, visceral and brown) (figure

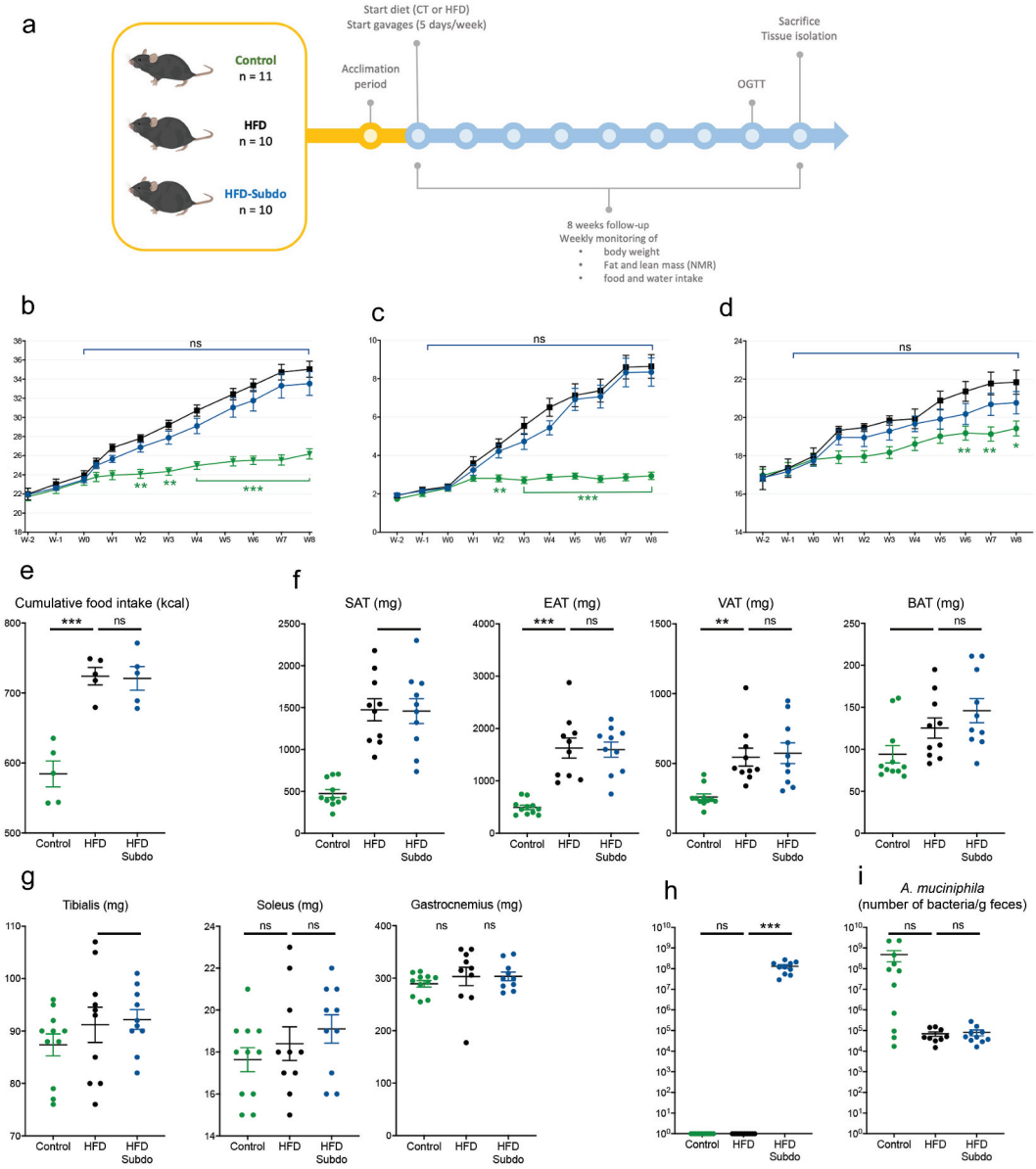


Figure 2. Impact of *Subdoligranulum variabile* supplementation on the prevention of diet-induced obesity in mice. **(A)** Study design: Mice were fed a high-fat diet (HFD) and gavaged with 1.5×10^9 cultivable cells (cc) of freshly prepared life bacteria, 5 days a week for 8 weeks (HFD-Subdo group, n = 10, blue). This group was compared to mice that were gavaged with vehicle and were fed either a control diet (Control group, n = 11, green) or a high-fat diet (HFD group, n = 10, black). **(B)** Body weight (g) **(C)** lean mass (g) **(D)** and fat mass (g) evolution during the 8 weeks follow-up. **(E)** Cumulative food intake in kcal during the 8 weeks of treatment. **(F)** Weight in mg of different fat depots at the end of the experiment. SAT: subcutaneous (inguinal), EAT: epididymal, VAT: visceral (mesenteric), BAT: brown adipose tissue. **(G)** Weight in mg of different types of muscles. **(H)** qPCR quantification of *Subdoligranulum* at the end of a 5-day period of gavage. Data were analyzed using one-way ANOVA followed by a Tukey post hoc test for E-H or using 2-way repeated measures ANOVA for B-C. '*' '**' and '***' indicate a significant difference versus HFD ($P < .05$, $P < .01$, $P < .001$, respectively).

2f) nor of selected muscles (*tibialis*, *soleus*, and *gastrocnemius*) (Figure 2g) at the end of the 8-weeks period.

We found that *Subdoligranulum* abundance was very low in mice. However, the mice treated with *S. variabile* exhibited a clear elevation of *Subdoligranulum* compared to the control group (Figure 2h). As expected, *A. muciniphila* was reduced by the HFD. Supplementation with *S. variabile* did not induce an increase of A. muciniphila in treated mice (Figure 2i).

S. variabile supplementation does not impact onlipid metabolism.

As we observed associations between *Subdoligranulum* and insulin levels as measures of insulin sensitivity in the overweight/obese group,

we subjected the mice to an oral glucose test (OGTT) in the last week of *S. variabile* administration (Figure 3a). Compared to the control group, mice fed the HFD had higher fasting glucose levels after fasting (time points −30 min and 0 min) and a higher blood glucose profile after being given a glucose load (time points 15 min to 90 min). However, we did not observe an improvement of glucose tolerance in mice supplemented with *S. variabile*, as evidenced by similar glucose profiles (Figure 3a) and corresponding areas under the curve (AUC, Figure 3c), suggesting that *S. variabile* does not impact on overall glucose metabolism in diet-induced obese mice.

HFD-fed mice were hyperinsulinemic in the fasted state, as they exhibited more than two-fold higher levels of plasma insulin as compared to control mice (Figure 3b). They also displayed higher insulin levels after glucose challenge. There was a small, but significant reduction of insulin levels 15 min after the glucose load in the *S. variabile* treated mice, resulting in a lower area under the curve (Figure 3d). However, this was not sufficient to alter the insulin resistance index in a significant manner (Figure 3e).

S. variabile supplementation does not impact on lipid metabolism

At the end of the 8 weeks of high-fat feeding and *S. variabile* administration, liver weight was comparable between all groups (Figure 4a). Total lipid contents of HFD-fed mice were somewhat increased, although this did not reach the significance threshold (p = 0,1) (Figure 4b). Hepatic triglyceride (p = 0,03), but not plasma triglycerides levels (p = 0,90) were increased by the higher dietary lipid supply in the HFD group (Figure 4c and d). Hepatic and plasma cholesterol levels were somewhat, but not significantly, elevated by HFD

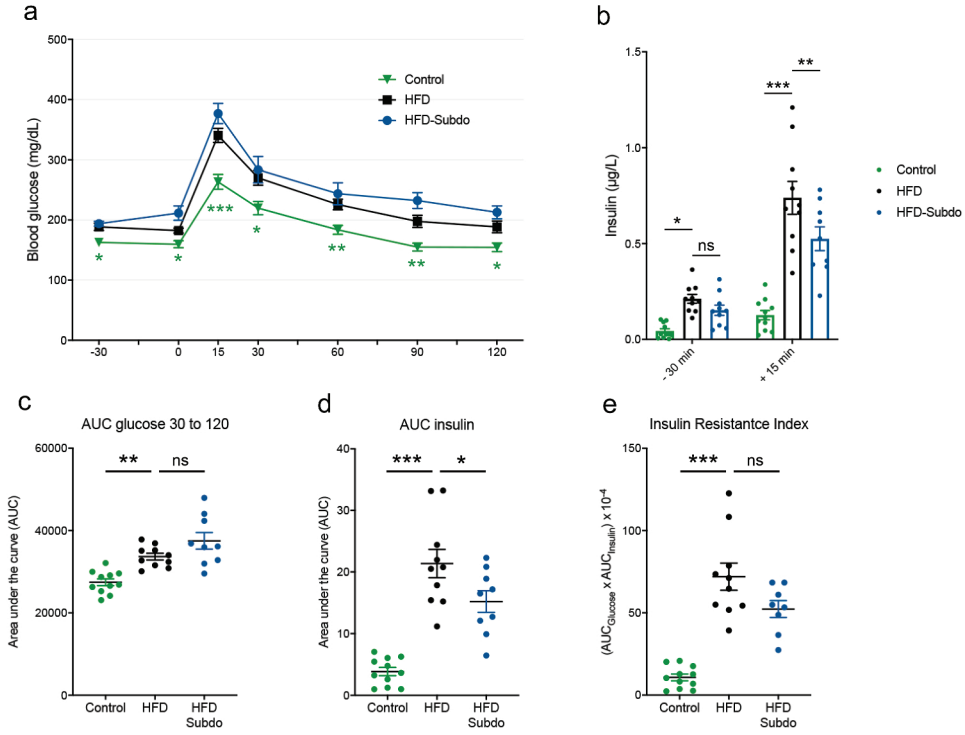


Figure 3. *Subdoligranulum variabile* supplementation does not impact on glucose metabolism. **(A)** Plasma glucose profile measured during an oral glucose tolerance test (OGTT). **(B)** Plasma insulin measured 30 min before and 15 min after glucose administration during the OGTT. **(C)** Mean area under the curve (AUC) of glucose measured during (OGTT). **(D)** Mean area under the curve of plasma insulin measured 30 min before and 15 min after glucose administration. (**E**) Insulin resistance index, calculated multiplying the area under the curve of both blood glucose (−30 to 120 min) and plasma insulin (−30 and 15 min) obtained following the oral glucose tolerance test. Data are represented as means ± SEM. Data were analyzed using 2-way repeated measures ANOVA for A or one-way ANOVA followed by a Tukey post hoc test for B-E.'*' '**' and '***' indicate a significant difference versus HFD ($P < .05$, $P < .01$, $P < .001$, respectively).

feeding (p = 0,08 and 0,07, respectively, Figure 4e and f). There were no overt improvements or worsening of these parameters by the supplementation with *S. variabile*, indicating that increasing the intestinal abundance of this bacterium is not involved in lipid metabolism in mice.

S. variabile supplementation does not impact on short-chain fatty acids or gut barrier

Since *Subdoligranulum* is a butyrate-producing organism and short-chain fatty acids (SCFA) have been linked to multiple beneficial health effects on host energy metabolism, we analyzed the SCFA concentrations in the cecal content of the mice. We found that the HFD slightly decreased the concentrations of the main SCFA: acetic acid, butyric acid, and propionic acid. Although most of the SCFA measured tended to be somewhat higher after *S. variabile* supplementation, none of the effects reached statistical significance (Figure 5a). Because butyrate is rapidly taken up by enterocytes where it serves as energy source[30] and where it enhances the intestinal barrier by regulating tight junction proteins,[31] we also measured some key markers of gut barrier integrity in the ileum. However, we found no increase in the mRNA expression of tight junction proteins (ZO-1, occludin and claudin 3), nor in the mRNA expression of intectin, a marker of intestinal epithelial cell renewal (Figure 5b).

Discussion

Different studies have started from observations and correlations between specific taxa and health, and then tentatively moved to causality by using different models. For example, mono-colonization of germ-free mice with specific bacterial strains, including *Enterobacter cloacae B9*,[32] *Clostridium ramosum*,[33] and *Lachnospiraceae* strain AJ110941[34] led to an increased propensity for obesity, identifying these strains as obesity-inducing pathogens. Another bacterium, *Bilophila wadsworthia* aggravated metabolic dysfunctions in conventional mice fed a high-fat diet.[8] In contrast, beneficial (probiotic) effects have been described for other bacterial strains, such as *Faecalibacterium prausnitzii*,[35] *Anaerobutyricum soehngenii* (formerly designated as *Eubacterium hallii*)[9] and *Christensenella minuta*.[7] Another key promising beneficial bacterium is *Akkermansia muciniphila* which has been extensively studied and finally confirmed in several proof-of-concept studies.[13]

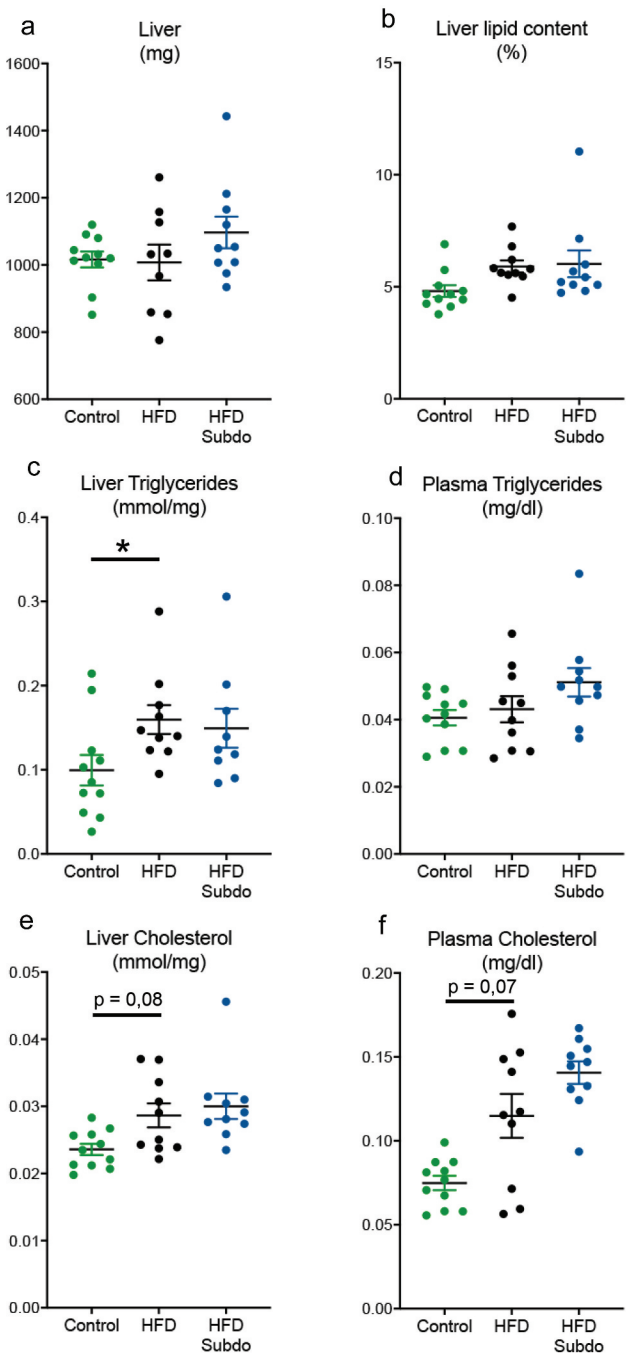


Figure 4. *Subdoligranulum variabile* supplementation does not impact on lipid metabolism. Effect of *Subdoligranulum variabile* administration on (**A**) liver weight (g), (**B**) liver lipid content (%), (**C**) liver triglycerides (mmol/mg), (**D**) liver cholesterol, (mmol/mg) (**E**) plasma triglycerides (mg/dl) and (**F**) plasma cholesterol (mg/dl). Data are represented as means ± SEM. Data were analyzed using one-way ANOVA followed by a Tukey post hoc test. '*' indicates a significant difference versus HFD ($P < .05$).

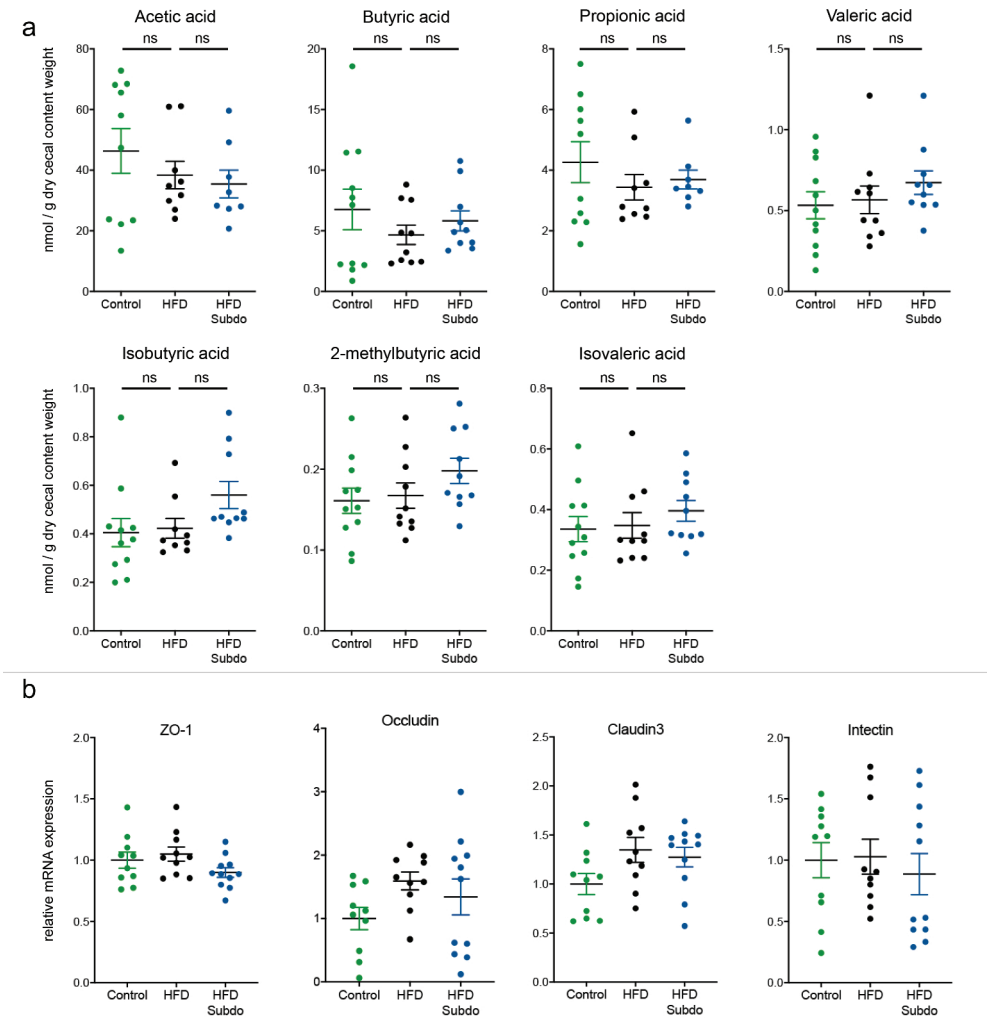


Figure 5. *Subdoligranulum variabile* supplementation does not impact on short-chain fatty acids or gut barrier. Effect of *Subdoligranulum variabile* administration on (**A**) short-chain fatty acids (nmol/g of dry cecal content) and (**B**) relative mRNA expression of key markers of gut barrier integrity. (ZO-1: Zonula occludens-1). Data are represented as means ± SEM. Data were analyzed using one-way ANOVA followed by a Tukey post hoc test.

In our search for novel beneficial bacteria candidates, we identified *Subdoligranulum*, a strictly anaerobic, non-spore-forming, butyrate-producing, Gram-negative staining organism. Only one species belonging to this genus was isolated from human feces in 2004 and has since then been found in the gut of several other vertebrates.[23] Although the precise physiological role of *Subdoligranulum* is unknown, several interesting findings suggested a promising probiotic effect on host metabolism. Indeed, a series of human studies revealed that the increase of *Subdoligranulum* was associated with improved metabolic health including in intervention with prebiotics.[16,19,20,22,36]

Thanks to a previous metagenomics approach in overweight/obese population, we discovered by using co-occurrence analysis that *Subdoligranulum MGS (metagenomics species)* was co-abundantly found with *A. muciniphila* and we confirmed the positive association between these two genera. Moreover, we found that *Subdoligranulum* was positively correlated with bacterial richness as well as with HDL cholesterol and surrogate measures of insulin sensitivity (HOMA). In addition, visceral fat mass was lower in individuals with high *Subdoligranulum* abundance. Taken together, our past and present data strongly supported the probiotic potential of *Subdoligranulum* and prompted us to investigate the role of this bacterium *in vivo*.

To firmly demonstrate causality, large, long-term clinical studies with well-defined criteria and protocols that yield robust outcomes are needed. However, initial exploratory research relies primarily on mechanistic studies performed in animals, in which confounding factors, such as age, diet, genetic background, environmental conditions, etc., can be omitted from the equation. Therefore, we treated mice with the only cultivated representative of this genus: *Subdoligranulum variabile* strain DSM 15176 ^{T} to determine whether it could counteract high-fat diet-induced obesity and associated metabolic disorders. After 8 weeks of *S. variabile* strain 15176^{T} administration to HFD-fed mice, levels of *Subdoligranulum* in the feces of the mice were significantly increased. However, using *Subdoligranulum variabile* no improvement was observed on any of the measured parameters (body weight, fat mass, glucose metabolism and, lipid metabolism). Although *S. variabile* produces butyrate, a short-chain fatty acid with health-promoting abilities, we found only a modest, non-significative increase of this metabolite. It is possible that the lack of effect is the result of the dose of bacterium administered, however, the doses we used and the abundance recovered in the feces are well within range of the doses deemed relevant in other studies.[6,37]

Therefore, the lack of effect of *S. variabile* DSM 15176 ^{T} could have other potential explanations. It is, for example, possible that putative effects of *Subdoligranulum* in humans did not translate directly to rodents or that *Subdoligranulum* only has beneficial effects when co-occurring with specific bacteria or within a certain microbial community. Indeed, our previous and present data show that *Subdoligranulum* correlated with *A. muciniphila*. Moreover, both *A. muciniphila* and *Subdoligranulum* are highly increased upon metformin treatment[17] or upon prebiotic treatment.[36] In this study, we found that administration of *S. variabile* did not result in a subsequent increase of *A. muciniphila* abundance. Another potential explanation would be that *Subdoligranulum variabile* is, in fact, not a determinant in obesity and that its correlation with improved metabolic parameters relies either on mere coincidence or on its inclination to thrive in a healthier microbial community. In other words, one may argue that *Subdoligranulum* is not a major bacterium acting on health but is rather an opportunistic bacterium growing either thanks to the presence of undigested compounds (e.g., fibers) or specific drugs (e.g., metformin, acarbose).

Importantly, it should be noted that in this study, we have tested only the impact of *Subdoligranulum variabile*, which is the only strain of this genus so far to be identified, isolated, and cultured. Meaning that several other, but unclassified, species within the *Subdoligranulum* genus may exist. Therefore, we may not rule out that the multiple correlations observed in humans are related to uncultured strains of *Subdoligranulum*. To support this hypothesis, we quantified the abundance of *Subdoligranulum variabile* versus *Subdoligranulum spp*. in human samples, and found that *Subdoligranulum variabile* only accounts for less than 1% of the total abundance of the *Subdoligranulum* genus in the human cohorts presented in this study (Supplementary Figure 1). Indeed, as depicted in Figure 1, several other species exist and may be responsible for the numerous and strong associations found between the *Subdoligranulum* genus and a healthier metabolic profile.

In conclusion, the present study strongly supports the need to continue to move from correlations to causation when investigating the gut microbiota. Our study illustrates that the species and the strain specificity may be an issue. This requires appropriate sequencing methods with species identification. Finally, our study underlines the necessity to assess the validity of an associative observation between certain microbial components and health or disease conditions and urges the scientific community to be careful when stating conclusions based solely on co-occurrence data and correlations.

Material and methods

Patients

Using qPCR and shotgun metagenomic sequencing,[38] we examined *Subdoligranulum* in two already published population with a broad range of BMI range and metabolic complication recruited at Research Center on Human Nutrition, Pitié-Salpêtrière Hospital in Paris, France. Details regarding these overweight, obese and severely obese populations have been described elsewhere.[16,27,29] Briefly

overweight and obese subjects were part of a dietary intervention (MICRO-Obes). Criteria to participate in the study included age between 25 and 65 years, body mass index (BMI) in the range of 27 to 38 kg/m^2, absence of chronic disease, stable weight for 3 months prior to recruitment, and no antibiotic intake for 2 months before dietary intervention and stool collection. And, severely obese subjects were part of a bariatric surgery program (MICROBARIA). Since recruited in the same center, clinical and biological measures were harmonized. Anthropometric measurements included BMI, waist and hip circumference, and waist-to-hip ratio (WHR). Body composition was determined using dual energy x-ray absorptiometry (DXA), for the estimation of total body fat, lean body mass, and gynoid and android fat proportions. Blood samples were collected after an overnight fast at baseline, enabling measurements included markers of glucose homeostasis, blood lipids, and systemic inflammation. Fasting glycemia, insulinemia, Non-esterified fatty acids (NEFA), TG, and cholesterol were used to calculate various indexes of insulin sensitivity: the Homeostasis Model Assessment insulin resistance index (HOMA-IR) was calculated using the HOMA2Calculator system developed by Levy et al., which uses mathematical modeling and a healthy population as reference to determine insulin sensitivity.

Fecal microbiota richness was calculated as the number of metagenomic species present in the sample as described in Le Chatelier et al.[29]

Culture of Subdoligranulum variabile

Subdoligranulum variabile DSM 15176 ^{T} was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ). The strain was routinely cultured in yeast extract-peptone medium supplemented with glucose, raffinose and anti-oxidants (detailed composition in supplementary Table 1). Attempts to enumerate *S. variabile* DSM 15176 ^{T} on solid medium failed despite separate autoclaving of phosphate and agar, as well as replacement of agar by gellan gum.[39,40] Thus, live and cultivable bacteria were enumerated using most probable number calculation associated to dilution to extinction method: bacterial culture was serially diluted to the appropriate concentrations according to optical density (OD) measurements at 680 nm, then 10 vials per dilution were inoculated with 1 ml of dilution. The number of vials with visible growing within the three following days allowed the calculation of the live bacteria concentration in the culture.[41] For the administration to the mice, bacterial culture was centrifuged at 4000 g during 20 min at 4°C. Then supernatant was removed, and the pellet was washed twice in anoxic PBS supplemented with antioxidants and finally resuspended in glycerol (25% vol/vol) and stored at −80°C.

Mice

Ten-week-old male C57BL/6 J mice were housed in pairs in specific pathogen-free conditions and in controlled environment (room temperature of 22 +/- 2°C, 12 h daylight cycle) with free access to food and water. After an acclimatization period of one week, mice were randomly assigned to one of the three conditions: Control diet (10 kcal% fat, D12450Ji, Research Diet, New Brunswick, NJ, USA) gavaged with vehicle (Control group, n = 11), high-fat diet (60 kcal% fat, D12492i Research Diet) gavaged with vehicle (HFD Group, n = 10), or high-fat diet gavaged with 1.5×10^9 live cells of *S. variabile* DSM 15176 ^{T} (HFD-Subdo group, n = 10). Gavages were performed 5 days a week (Monday to Friday) for 8 weeks.

Body weight, food, and water intake were recorded weekly. Body composition (lean and fat mass) was assessed by using 7.5 MHz time domain-nuclear magnetic resonance (TD-NMR) (LF50 Minispec, Bruker, Rheinstetten, Germany).

All mouse experiments were approved by and performed in accordance with the guidelines of the local ethics committee. Housing conditions were specified by the Belgian Law of May 29, 2013, regarding the protection of laboratory animals (agreement number LA1230314).

Oral glucose tolerance test (OGTT)

After 7 weeks of treatment, an oral glucose tolerance test (OGTT) was performed as previously described.[42] Briefly, 6 h-fasted mice were given an oral glucose load (2 g glucose per kg body weight) and blood glucose levels were measured at different time points: 30 min

before and 15, 30, 60, 90, and 120 min after oral glucose load. Blood glucose was measured with a standard glucose meter (Accu Check, Roche, Basel, Switzerland) on blood samples collected from the tip of the tail vein. Insulin resistance index was determined by multiplying the area under the curve of both blood glucose (−30 to 120 min) and plasma insulin (−30 and 15 min) obtained following the oral glucose tolerance test.

Insulin resistance index

Plasma insulin concentration was determined using an ELISA kit (Mercodia, Uppsala, Sweden) according to the manufacturer's instructions. Insulin resistance index was determined by multiplying the area under the curve of both blood glucose (−30 to 120 min) and plasma insulin (−30 and 15 min) obtained following the oral glucose tolerance test.[43] Glucose-induced insulin secretion was calculated as the difference between plasma insulin levels 30 min before and 15 min after oral glucose load.

Tissue sampling

At the end of the treatment period (week 10), all animals were anesthetized with isoflurane (Forene, Abbott, Queenborough, Kent, England). After exsanguination, mice were killed by cervical dislocation. Subcutaneous adipose tissue depots, intestines, muscles, cecum, and liver were precisely dissected, weighed, and immediately immersed in liquid nitrogen followed by storage at −80°C for further analysis.

Feces collection

Feces were collected early in the morning. Mice were briefly separated and transferred to empty cages without bedding. Droppings were immediately collected using sterilized forceps, put in a tube and frozen on dry ice. Samples were stored at −80°C until further analysis.

Subdoligranulum spp, Subdoligranulum variabile and Akkermansia muciniphila quantification by qPCR

Genomic DNA was extracted from mice feces using the QIAamp DNA Stool Mini Kit (Qiagen, Germany), including a bead-beating step. DNA concentration was determined and purity (A260/A280) was checked using a NanoDrop2000 (Thermo Fisher Scientific, USA). Samples were diluted to an end concentration of 10 and 0.1 ng/µl in TE buffer pH 8. Total bacteria qPCR were performed on the 0.1 ng/µl dilution and the *Subdoligranulum* spp and *Akkermansia muciniphila* qPCRs were performed on the 10 ng/µl dilution. A standard curve was included on each plate by diluting genomic DNA from pure culture. Primers sequences targeting 16S rRNA gene are presented in Table 1.

Measurement of short-chain fatty acids

For SCFA analysis, the cecal content (50–60 mg wet material) was homogenized in water followed by sonication in an ice water bath. Acetonitrile was used for protein precipitation (in the presence of valproic acid as internal standard). Following centrifugation, the supernatant was recovered and a derivatization step (using 3-nitrophenylhydrazine in the presence of EDC and pyridine) performed. Samples were purified using liquid-liquid extraction to remove the remaining reagents. After evaporation, the final residue was analyzed using an LTQ Orbitrap XL mass spectrometer coupled to an Accela HPLC system (ThermoFisher Scientific). A Hypersil GOLD PFP (100 x 2.1 mm; 1.9 µm) column using a gradient of water-acetonitrile-acetic acid and acetonitrile-acetic acid allowed separating the different isomers. For ionization, an APCI probe was used in positive mode. Calibration curves were prepared using the same conditions to determine sample content. Xcalibur® software was used for data analysis. For each cecal content, an aliquot was freeze-dried to determine a dry residue that was used for data normalization.

Table 1. Primers used for the qPCR.

Subdoligranulum spp	Forward	AGGCGGACCGGCAAGTTGG
	Reverse	CTACCTCTGCACTACTCAAGGCCAG
S. variabile	Forward	CAAGTCGAACGGAGTTATTTCGG
	Reverse	GTTCGCCACTAAGTTAAACCAG
Akkermansia muciniphila	Forward	CAGCACGTGAAGGTGGGGAC
	Reverse	CCTTGCGGTTGGCTTCAGAT
Total Bacteria	Forward	ACTCCTACGGGAGGCAGCAG
	Reverse	ATTACCGCGGCTGCTGG

Statistical analyses

Statistical analyses were performed using GraphPad Prism version 7.00 (GraphPad Software, San Diego, CA, USA) and R 3.5.1. Comparison between two groups was performed by Mann–Whitney–Wilcoxon test, comparison between three or more groups on one time-point was performed by one-way ANOVA followed by Tukey correction. Comparison between three or more groups at different time-points was performed by 2-way repeated measures ANOVA.

The co-abundance network was inferred using the Spectral Consensus Strategy (SCS) approach [44] with the *scalenet* R package applying default parameters, namely the "aracne" and "bayes_hc" inference modes. The dataset used to infer the network was composed of 776 variables (metagenomic species > 500 genes) plus two additional derived variables (i.e., targets) and 108 observations. Inferred edges were filtered by the estimated coefficient of mutual information and only those with $|MI| > 0.2$ were kept. From the global reconstructed, ecosystem only a subset was extracted with nodes that were directly linked to two targets. Both targets were computed as the sum of the relative abundance of all MGS annotated, respectively, as *Akkermansia* and *Subdoligranulum* at the genus level. The network was visualized with the *igraph* R package using Fruchterman-Reingold distribution of the vertices.

The heatmap was computed using Spearman correlations between metagenomic species and clinical information. The Manhattan distance along with the Ward algorithm were used for the hierarchical clustering of the variables.

Disclosure of potential conflicts of interest

P.D.C. is inventor of patent applications dealing with the use of *A. muciniphila* and its components in the context of obesity and related disorders. P.D.C. is co-founders of A-Mansia Biotech SA. K.C. is a consultant for Danone Research and LNC therapeutics for work unassociated with the present study.

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