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Akkermansia muciniphila and improved metabolic health during a dietary intervention in obesity: relationship with gut microbiome richness and ecology.

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Key words: *Akkermansia muciniphila*, obesity, glucose metabolism, gut ecosystem.
Abbreviations: T2D, type 2 diabetes; CR, calorie restriction; WS, weight stabilization; WHR, waist-to-hip ratio; DXA, dual energy x-ray absorptiometry; NEFA, non-esterified fatty acids; TG, triglycerides; hs CRP, high sensitivity C-reactive protein; IL-6, interleukin-6; LPS, lipopolysaccharide, AST, aspartate transaminase; ALT, alanine transaminase; GGT, gamma-glutamyl transpeptidase; HOMA-IR, Homeostasis Model Assessment of Insulin Resistance Index; OGTT, oral glucose tolerance test; scWAT, subcutaneous white adipose tissue; SRV, statistical recoupling variables; QM, quantitative metagenomics; MGS, metagenomic species; LGC, low gene count; HGC, high gene count; MAR, mean adequacy ratio; NAR, nutrient adequacy ratio; BIC, Bayesian information criterion; FODMAP, fermentable oligosaccharides, disaccharides, monosaccharides and polyols.
Word Count: 3993

ABSTRACT

Objective: Individuals with obesity and type 2 diabetes differ from lean and healthy individuals in their abundance of certain gut microbial species and microbial gene richness. Abundance of *Akkermansia muciniphila*, a mucin-degrading bacterium, has been inversely associated with body fat mass and glucose intolerance in mice, but more evidence is needed in humans. The impact of diet and weight loss on this bacterial species is unknown. Our objective was to evaluate the association between fecal *A. muciniphila* abundance, fecal microbiome gene richness, diet, host characteristics, and their changes after calorie restriction (CR).

Design: The intervention consisted of a 6-week CR period followed by a 6-week weight stabilization (WS) diet in overweight and obese adults (N=49, including 41 women). Fecal *A. muciniphila* abundance, fecal microbial gene richness, diet and bioclinical parameters were measured at baseline and after CR and WS.

Results: At baseline *A. muciniphila* was inversely related to fasting glucose, waist-to-hip ratio, and subcutaneous adipocyte diameter. Subjects with higher gene richness and *A. muciniphila* abundance exhibited the healthiest metabolic status, particularly in fasting plasma glucose, plasma triglycerides and body fat distribution. Individuals with higher baseline *A. muciniphila* displayed greater improvement in insulin sensitivity markers and other clinical parameters after CR. These participants also experienced a reduction in *A. muciniphila* abundance, but it remained significantly higher than in individuals with lower baseline abundance. *A. muciniphila* was associated with microbial species known to be related to health.

Conclusion: *A. muciniphila* is associated with a healthier metabolic status and better clinical outcomes after CR in overweight/obese adults. The interaction between gut microbiota ecology and *A. muciniphila* warrants further investigation.

SUMMARY BOX:

What is already known about this subject?

- Evidence suggests that gut microbiota diversity and metabolic function plays an important role in the development of obesity and related metabolic disorders.
- Dietary changes including calorie restriction can profoundly impact the gut microbiota.
- *Akkermansia muciniphila* is associated with healthier glucose metabolism and leanness in mice but this is less conclusive in humans.

What are the new findings?

- Higher *A. muciniphila* abundance is associated with a healthier metabolic status in overweight/obese humans.
- There is an interaction between gut microbiome richness, certain metagenomic species and *A. muciniphila*, whereby higher abundance of this species together with greater microbial gene richness are associated with a healthier metabolic status.
- Higher abundance of *A. muciniphila* at baseline is associated with greater improvement in glucose homeostasis, blood lipids and body composition after calorie restriction.

How might it impact on clinical practice in the foreseeable future?

- Our findings demonstrate the need for further investigation to ascertain the therapeutic applicability of *A. muciniphila* in the treatment of insulin resistance.
- *A. muciniphila* may be identified as a diagnostic or prognostic tool to predict the potential success of dietary interventions.

INTRODUCTION

Altered gut microbiota composition and function contribute to the development of obesity in mice and its associated comorbidities in both mice and humans.[1–5] There is increasing evidence showing interactions between environmental factors, gut microbiota, metabolic diseases and cardiovascular risks.[5–7] Specific bacterial groups have been implicated in obesity and related metabolic diseases, and may therefore be considered as therapeutic targets. As such, *Akkermansia muciniphila*, a mucin-degrading bacterium, was proposed to be a contributor to the maintenance of gut health[8–10] and glucose homeostasis.[11] We, and others, have shown in mouse studies a causative role for this species in lowering body fat mass, improving glucose homeostasis, decreasing adipose tissue inflammation, and increasing gut integrity.[12–14] The latter was demonstrated following oral administration of *A. muciniphila* that led to increased mucin layer thickness, decreased metabolic endotoxemia,[12] and increased number of goblet cells.[13]

In humans, the role of *A. muciniphila* remains ambiguous. One study reported that *A. muciniphila* was more abundant in subjects with normal glucose tolerance compared to a pre-diabetic group.[15] The opposite relationship was seen by others, where *A. muciniphila* was enriched in patients with type 2 diabetes (T2D) compared to non-diabetic controls.[16] These two studies were conducted in lean/overweight Chinese adult populations with a wide age range. A third study in 70-year old normal weight European women showed that *A. muciniphila* was not among the species applicable to classify women as having T2D.[17] This discrepancy may be due to differences in study design, methodology, and population characteristics such as ethnicity, age and diet.[18]

Studying changes in *A. muciniphila* after an intervention known for improving metabolic health offers stronger evidence of its role than measuring cross-sectional relationships. Weight

loss through calorie restriction (CR) or bariatric surgery has a profound effect on gut microbiota.[19,20] Characteristics of the gut ecosystem, such as high microbial gene richness, have been associated with better cardiometabolic health and improvements in clinical characteristics after a diet-induced weight loss intervention.[21,22] Limited available evidence suggests that *A. muciniphila* increases with bariatric surgery in both humans and mice,[23–26] but there is no evidence on the effects of CR.

We have previously published results from this dietary intervention,[21,27] where overweight and obese individuals underwent weight loss through CR followed by weight stabilization (WS). In the same cohort, we herein aim to evaluate the potential associations between *A. muciniphila* with microbial gene richness, diet, host anthropometric and metabolic parameters, and further address their changes after the intervention.

MATERIALS AND METHODS

Study population

This dietary intervention was conducted at the Institute of Cardiometabolism and Nutrition (ICAN), Pitié-Salpêtrière Hospital in Paris, France. The 49 participants were overweight (N=11) or obese (N=38) (male:female = 8:41), and have been previously described in detail.[21,27] A smaller sample size has been specified when there is missing data. Briefly, subjects had no diabetes, chronic or inflammatory diseases. No antibiotics were taken for 2 months before stool collection. Details of the dietary intervention, which consisted of a 6-week CR diet enriched with fibers and protein followed by a 6-week WS period have been previously described.[27] The study was reviewed and authorized by the Ethical Committee (CPP N°1 Hôtel Dieu Hospital) and all participants signed an informed consent. The study has been registered on clinicaltrials.gov: NCT01314690.

Body composition and biochemical parameters

Anthropometric measurements included BMI, waist and hip circumference and their ratio (WHR). Total body fat, fat free mass, gynoid and android fat proportions were determined using dual energy x-ray absorptiometry (DXA), as previously described.[28]

Blood samples were collected after a 12-hour fast at baseline, week 6 and 12. Measurements included blood lipids, namely non-esterified fatty acids (NEFA), triglycerides (TG), total, LDL and HDL-cholesterol. Inflammatory and endotoxemia markers included high sensitivity C-reactive protein (hs CRP), interleukin-6 (IL-6)[29] and lipopolysaccharide (LPS),[30] as described previously.[27] Aspartate transaminase (AST), alanine transaminase (ALT) and gamma-glutamyl transpeptidase (GGT) were measured as part of a clinical blood panel (laboratory-established normal ranges: 20-32 IU/L, 20-35 IU/L, and 8-36 IU/L, respectively).

The Homeostasis Model Assessment of Insulin Resistance Index (HOMA-IR) was calculated using the HOMA2Calculator developed by Levy et al, which uses mathematical modeling and a healthy reference population to determine insulin sensitivity.[31] Glucose and insulin AUC from the oral glucose tolerance test (OGTT) were calculated, and the Disse index[32] was derived using the formula: $Disse = 12\,x\,\left[\,2.5\,x\,\left(\frac{HDL}{Total\ Chol}\right) - NEFA\right] - insulin$

Adipocyte morphology and adipose tissue macrophages

Subcutaneous white adipose tissue (scWAT) samples were obtained at baseline, week 6 and 12 by needle biopsy from the periumbilical region under local anesthesia.[33] Adipocyte diameter was quantified as previously described.[34] Adipocyte morphology in relation to fat mass was measured using the curve fitting model developed by Spalding et al to describe

associations between adipocyte volume, number and body fat.[35,36] The formula with re-estimated parameters is:

$$Theoretical\ Adipocyte\ volume\ (pl) = \frac{(40.7 * \text{Kg Fat Mass})}{(1 + (0.025 * \text{Kg Fat Mass})}$$

Observed adipocyte volume[37] was calculated with the formula:

$$Observed\ Adipocyte\ volume\ (pl) = [\left(\frac{\pi}{6x10^3}\right) x\ (Adipocyte\ Diameter, \mu m)^3]$$

HAM56 was measured as a marker of scWAT macrophages with monoclonal antibody (DakoCytomation). HAM56 positive cells were quantified as a percentage of total adipocyte number.[38]

Metabolic phenotyping of serum by ^{1}H NMR spectroscopy.

Serum samples were prepared and analysed on a NMR spectrometer (Bruker) operating at 600.22 MHz ^{1}H frequency as previously described,[39] using 350 µL of sample mixed with 350 µL of buffer before centrifugation at 12000g at 4°C for 5 min. The ^{1}H NMR spectra were pre-processed and metabolic signals were recovered using statistical recoupling of variables (SRV).[40]

Fecal microbiota

A quantitative metagenomics (QM) approach was used to characterize the fecal microbiota with high resolution. Briefly, high-throughput SOLiD sequencing was performed on total fecal DNA as described in Cotillard et al.[21] Reads were mapped and counted onto the 3.9 million gene catalog,[41] after cleaning for quality, human, plant and cow origin using the Meteor Studio platform. The metagenomic species (MGS) catalog published by Nielsen et al was used to cluster gene profiles in the current study. We used the Le Chatelier et al[22] methodology implemented in the MetaOMineR pipeline to compute MGS tracer profiles, where we calculated the mean of the 50 most correlated bacterial genes after filtering at 20% presence

and used only large MGS with more than 500 genes to focus on potential bacterial species. The taxonomic annotation is an updated version of the published dataset. The methodology for stratification as a function of gene richness (low gene count, LGC and high gene count = HGC) was as formerly described and is based on the first metagenomics catalog.[21,22]

A. muciniphila quantification

A. muciniphila was quantified with qPCR as described in Everard et al.[12] Briefly, DNA was extracted from fecal samples,[27] and qPCR (Applied Biosystems) was done using the 16S rRNA primers for *A. muciniphila* detection and amplification: forward CAGCACGTGAAGGTGGGGAC, and reverse CCTTGCGGTTGGCTTCAGAT. Total 16S rRNA was also quantified and used to normalize *A. muciniphila* using bacterial universal primers: forward ACTCCTACGGGAGGCAGCAG, and reverse ATTACCGCGGCTGCTGG. Each assay was performed in duplicate. The cycle threshold of each sample was then compared with a standard curve (performed in triplicate) made by diluting genomic DNA (fivefold serial dilution) (DSMZ).

A. muciniphila was also quantified using QM (GU:154), as some of the analysis included direct comparisons between qPCR and QM data, and good agreement was found between the two methods (**Supplementary Figure 1 and 2**).

Diet Mean Adequacy Ratio (MAR)

Diet was assessed with 7-day unweighted food records completed just before baseline, week 6 and 12, as previously described.[34] We used the Mean Adequacy Ratio (MAR) as an indicator of global nutrient adequacy of the diet.[42,43] The MAR is the mean nutrient adequacy ratio (NAR) for 16 nutrients (proteins, fiber, retinol equivalents, thiamine, riboflavin, niacin, vitamin B6, folates, vitamin B12, ascorbic acid, vitamin D, vitamin E, calcium, potassium, iron and magnesium). Each NAR was calculated as the mean intake of a nutrient divided by the

French Recommended Dietary Allowance[44] and multiplied by 100. To avoid compensation of high intake of one nutrient for low intake of another, each NAR was truncated at 100. The MAR ranges from 0 to 100; the higher the score, the better global nutrient adequacy of the diet.

Bayesian network

A Bayesian network was constructed in order to simultaneously study associations between relevant variables and *A. muciniphila* qPCR abundance. Bayesian networks are probabilistic graphical models used to represent complex associations. The variables are the vertices in the graph, and the edges are the direct dependencies between them. We applied the Hill Climbing algorithm, which belongs to a family of local search techniques that performs a heuristic search based on scoring metrics. The Bayesian Information Criterion (BIC) was used as a scoring function. These procedures were conducted using the bnlearn R package, version 3.6.[45]

Statistical analysis

Normally distributed data were analyzed using parametric tests (paired *t test* and ANCOVA with age and sex as covariates). For variables with a skewed distribution or when conducting analysis of groups with small sample size (i.e. Akk LO/HI vs. LGC/HGC) non-parametric tests were conducted (Wilcoxon rank sum test, or Kruskal-Wallis followed by multiple signed rank sum tests for individual comparisons with Bonferroni correction). Spearman analysis was used to determine correlation between variables. Values in tables are reported as mean (SE), or adjusted mean (SE) in the case of ANCOVA. In figures data are reported as box plots or as means or adjusted means ± SE. Statistical significance was set as alpha=0.05, except in post hoc analysis with Bonferroni correction. OGTT curve analysis was done using repeated measures ANOVA. Microbiome analyses were performed using the

MetaOMineR package (Prifti and Le Chatelier, in preparation). SAS 9.3 for Windows (SAS Institute, Cary, NC) and R was used for all statistical analyses.

RESULTS

Baseline comparison between Akk LO and Akk HI groups

A. muciniphila is associated with a healthier metabolic status

The $\log_{10}$ transformed *A. muciniphila* was normalized to $\log_{10}$ total bacterial content and we refer to this measurement as *A. muciniphila*. There was no difference in fecal *A. muciniphila* abundance between overweight and obese subjects (-2.57 ± 2.18 and -2.38 ± 1.72, p=0.97, respectively). *A. muciniphila* abundance had a bimodal distribution, consistent with that seen in QM (**Supplementary Figure 1**). Therefore, baseline *A. muciniphila* abundance was categorized around the baseline median and groups were defined as having lower (Akk LO, abundance < median, N=24) or higher abundance (Akk HI, abundance ≥ median, N=25). Sex and average age did not differ between Akk LO and Akk HI groups (**Table 1**). However, there was a higher number of younger subjects (age ≤ median, AgeLO, N=17) in the Akk HI group than older subjects (age > median, Age HI, N=8). Further analyses were subsequently adjusted by age and sex.

Subjects in the Akk HI group had a healthier metabolic status, as shown by a lower WHR, leptin and surrogates of insulin sensitivity (**Table 1**). The Akk HI group had lower fasting blood glucose and insulin. Fasting blood glucose was inversely associated with *A. muciniphila* (**Supplementary Figure 3**). Both HOMA-IR and Disse index suggested higher insulin sensitivity in Akk HI compared to Akk LO (**Table 1 and Figure 1A**). Furthermore, there was an inverse association between glucose AUC during OGTT and *A. muciniphila* abundance (**Figure 1C**). Glycaemia at T15 and T60 were significantly higher in Akk LO. Both AST and GGT were

lower in the Akk HI group and average values were in the normal range while they were elevated in Akk LO patients (**Table 1**).

A. muciniphila is inversely associated with adipocyte size

ScWAT Adipocyte diameter, but not total fat mass, was inversely associated with *A. muciniphila* abundance (**Figure 2A and B**), and Akk HI had lower mean adipocyte size (**Table 1**). When fitting the formula developed by Spalding et al to describe the association between adipocyte volume and fat mass[35] the Akk HI group tended to fall below the theoretical curve (**Figure 2C**) as quantified in a residual plot (**Figure 2D**), suggesting increased adipocyte hyperplasia in Akk HI subjects.

Signature associated with *A. muciniphila* abundance

To study associations between relevant variables simultaneously at baseline, and examine the strongest associations with *A. muciniphila* abundance, a Bayesian network was built (**Figure 1B**). Corroborating the observations from the univariate analysis, the clinical factors most dependent (d) with baseline *A. muciniphila* abundance are fasting glucose (d=0.86), HOMA-IR (d=0.66) and mean adipocyte diameter (d=0.84).

Changes with calorie restriction intervention

Akk HI group had greatest benefits from the dietary intervention

There was no difference in weight loss between the Akk HI and LO groups (data not shown). While there was a decrease in *A. muciniphila* abundance in the Akk HI group after CR and the total intervention period, it remained consistently and significantly higher than the Akk LO group (more than 100 times difference, **Figure 3A** and **Supplementary Figure 3**), although the range of abundance became more spread out after CR and WS in both groups (**Supplementary Figure 4A**). The change in *A. muciniphila* abundance was different between the two groups after CR and the 12-week period (**Figure 3B**). The Akk HI group remained

metabolically healthier throughout the dietary intervention, with a tendency for a higher Disse index after CR and WS (**Figure 4A and E**), a greater improvement of total and LDL cholesterol after CR and total intervention period (**Figure 4C, D and G, H**), and a continued decrease in WC during the WS period (**Figure 4B and F**).

Serum acetate correlates with *A. muciniphila* at baseline

A. muciniphila is a producer of SCFA, primarily acetate and propionate.[46,47] The latter is not usually detectable in serum by ^{1}H NMR spectroscopy, but serum acetate was positively correlated with *A. muciniphila* abundance (**Figure 5A**). There was a reduction in serum acetate throughout the dietary intervention in the total population as well as the Akk LO and HI groups. Although it remained higher in Akk HI group, the difference in variation in serum acetate concentrations did not reach significance when compared between groups (**Figure 5B and C**).

***A. muciniphila* and the microbial ecosystem**

It is likely that the association between fecal microbiota and health indicators is not attributable to a single microbe, but rather to an ecosystem that influences the complicated interaction between host biology and environment. As such, we studied *A. muciniphila* abundance in relation to the microbiome-wide MGS abundance and microbial gene richness.

A. muciniphila and MGS abundance

There were 27 large MGS (> 500 genes) associated with *A. muciniphila* abundance throughout the intervention ($p<0.01$, including the *A. muciniphila* MGS, 13 Firmicutes, 5 Bacteroidetes, 1 Actinobacteria and 1 Euryarchaeota) (**Figure 6A**). Nineteen of these MGS (70%) were more abundant in the Akk HI group. Some of the 26 MGS remained associated with *A. muciniphila* abundance throughout the intervention, while for others this association was lost at week 6, or lost and then regained at week 12. These 26 MGS represented less than 20% of the microbiome at all times when considering the large MGS as a reference (**Figure 6B**).

Individuals with higher *A. muciniphila* and gene richness have healthiest metabolic profile

We previously reported that high fecal gene richness was associated not only with healthier baseline metabolic status but also with better outcomes from the dietary intervention.[21] We therefore studied the relationship between *A. muciniphila* abundance and bioclinical parameters in the context of gene richness, leading to the definition of four groups: Akk LO, LGC; Akk HI, LGC; Akk LO, HGC; and Akk HI, HGC. The Akk HI, HGC group had the best metabolic status with the lowest median % android fat, fasting glucose and triglycerides, and the highest median % gynoid fat (**Figure 7A-D**). Most importantly, after the CR and WS phases, this group remained metabolically healthier (**Supplementary Figure 5**). Linear regression analysis showed that the interaction term had the largest effect size for body fat distribution and triglycerides, while Akk LO/HI had the biggest effect size for glucose (**Supplementary Table 1**).

***A. muciniphila* and dietary intake**

At baseline, dietary intake did not greatly differ between the Akk LO and HI groups. However, age was identified as a confounder for diet, with older subjects having a healthier diet than younger subjects, i.e. higher consumption of dairy products, fruits and vegetables and fish, and lower consumption of sugary drinks.[48] There were no significant differences in the 16 NARs and the MAR between the Akk LO and HI groups (**Figure 8A and Supplementary Table 2**), but older subjects tended to have higher NARs of several nutrients (data not shown) and had a significantly higher MAR than younger subjects (**Figure 8B**). During the WS period, older subjects experienced a greater increase in MAR (**Figure 8D**).

When studying the change in MAR, there was no difference in diet quality between Akk LO and Akk HI at any time point (**Figure 8A and Supplementary Table 2**). These results did not change after adjustment for total energy intake. As expected, in either categorization (age or

A. muciniphila abundance) MAR significantly decreased during the CR period and increased after the WS period.

DISCUSSION

We herein show in overweight and obese individuals that higher *A. muciniphila* abundance is associated with a healthier metabolic status, particularly with higher insulin sensitivity at baseline and improvement after CR and WS, thus confirming in humans what had been observed in murine models.[12–14] Subjects with higher *A. muciniphila* and gene richness are metabolically healthier before and after the dietary intervention, thus demonstrating an interaction between gut bacterial richness and *A. muciniphila* abundance.

Murine studies showed not only a positive correlation between *A. muciniphila* and health, but established causality, where induced *A. muciniphila* expansion led to improved metabolism[12–14]. Our results show an association between *A. muciniphila* and a healthier insulin sensitivity profile, and indicate that higher *A. muciniphila* abundance is linked to better outcomes after weight loss through CR. Importantly, *A. muciniphila* abundance in the Akk HI group remained approximately 100 times higher than in the Akk LO group throughout the intervention even if there was an intriguing reduction in the Akk HI group (**Figure 3**). We suggest that there may be a range of *A. muciniphila* abundance associated with a healthier metabolic status and better outcomes after CR.

Adipocyte hypertrophy is associated with chronic pro-inflammatory cytokine secretion[49] and greater risk for insulin resistance.[36] Adipocyte diameter, glucose and surrogates of insulin sensitivity appear tightly linked with *A. muciniphila* in the Bayesian network (**Figure 1B**). Primary defects in glucose homeostasis were observed at fasting and during early OGTT time points, which reflect more hepatic insulin sensitivity, rather than peripheral glucose

disposal.[50,51] Therefore, our results suggest that the glucose homeostatic defect in Akk LO individuals is primarily hepatic. In line with this, hepatic biology was solely impaired in Akk LO patients (**Table 1**). Clamp studies are needed to validate this hypothesis more precisely.

A. muciniphila produces a variety of fermentation products, including SCFA, through mucin degradation. These substrates may serve as energy sources both for other bacteria and the host.[46] It is possible that through this cross-feeding[18] *A. muciniphila* may contribute to the expansion of other beneficial species, while it may itself have a direct effect on host metabolism, consistent with rodent studies.[12] Serum SCFA analysis showed an association between *A. muciniphila* abundance and acetate at baseline. Acetate plays a role in prevention of weight gain through an anorectic effect, inflammation, metabolic dysregulation, and it is the most predominant gut-produced SCFA in peripheral blood.[52,53] However, it is unclear to what extent *A. muciniphila* contributes to circulating acetate. Indeed, while there is a strong correlation between *A. muciniphila* abundance and serum acetate concentration at baseline, this was not maintained throughout the dietary intervention.

Our results shed new light on the relationship between *A. muciniphila*, the gut ecosystem, and host health. The healthiest metabolic status was seen in subjects with higher *A. muciniphila* abundance in the context of greater bacterial gene richness in this French population. *A. muciniphila* was also found more abundant in HGC individuals in a Danish population.[22] Furthermore, we show that *A. muciniphila* was associated with 26 MGS, which represent up to 20% of the microbiome. Of interest, one of these MGS is *Methanobrevibacter smithii*, believed to be a producer of mucin-like glycans, as proposed by [54], while an association with mucin-degrader Ruminococcaceae was also observed. The latter was increased in abundance when NOD mice, which spontaneously develop type-1 diabetes, were fed a diabetes-protective diet.[55]

In a study where germ free mice with or without *A. muciniphila* gavage were infected with *Salmonella typhimurium*, the presence of *A. muciniphila* exacerbated the infection,[56] which suggested that the effect of an unregulated growth of *A. muciniphila* without competition from other species led to a deleterious modification of the gut environment and thinning of the mucosal layer, enabling the infection. Conversely, a recent study shows *in vitro* that *A. muciniphila* may adhere to the intestinal epithelial cells, thereby contributing to strengthen the monolayer integrity.[57]

Dietary patterns influence gut microbiota diversity, although little is known about the effect of diet on *A. muciniphila*.[18,58] Consumption of various types of dietary fiber has yielded different results: an increase of *A. muciniphila* with oligofructose [12,59] and fermentable oligosaccharides, disaccharides, monosaccharides and polyols (FODMAP) diet,[60] but a decrease with pectin or guar gum when compared to mice fed a fiber-free diet.[61]. We did not observe significant differences in baseline nutrient intake between Akk groups. Even though subjects increased consumption of fiber (particularly inulin-type fructans) during CR, this study design prevents us from reaching conclusions regarding *A. muciniphila* and diet. We can conclude, however, that the Akk HI group experienced greater metabolic improvement than Akk LO, while there was no difference between groups in weight loss, or MAR score. However, since MAR does not include saturated fats, sodium, or simple sugars intakes it is not a complete diet quality indicator. Studies specifically designed to assess the effect of diet, particularly fiber intake, on *A. muciniphila* abundance in a population homogenous in age and health status are warranted.

The relatively narrow range of glucose intolerance phenotype in this population constitutes a limitation of this study. Further investigation should focus on more diverse populations ranging from lean healthy to glucose intolerance or insulin resistance to overt T2D.

Even though we have shown that higher baseline *A. muciniphila* abundance is associated with better clinical outcomes after CR, and literature suggests an increased abundance of *A. muciniphila* after gastric bypass,[23–26] a direct comparison between the effect of energy restriction versus bariatric surgery should also be implemented to establish a link between energy restriction, nutrient malabsorption, *A. muciniphila* modifications, and improved glucose metabolism.

From the present study we cannot conclude whether fecal bacterial abundance is directly proportional to abundance in the gut. Microbiota in the mucus layer differs from that of the intestinal lumen,[62] and *A. muciniphila* is closely associated to the gut mucosal layer. The observed differences in abundance of *A. muciniphila* into feces may be due to actual changes in bacterial numbers, or alterations of the mucosal layer and gut architecture. Host genetics, may also play a role in how dietary interventions influence gut microbiota and metabolic health, as previously shown in mice, where different strains had notably different gut microbial composition and intestinal environment that correlated with a variety of cardiometabolic profiles.[63] The host's innate and adaptive immune system may also influence the composition of gut microbiota.[64] A recent study showed greater prevalence of *A. muciniphila* in the absence of pressure from the adaptive immune system in Rag1(-/-) immunodeficient mice.[65] Furthermore, while dietary interventions have been proven to greatly impact gut microbiota characteristics,[19,20] the stability of gut microbiota modifications after a dietary intervention needs to be assessed to verify whether gut microbiota changes are related to the maintenance of metabolic benefits over time. In conclusion, we demonstrated a significant association between *A. muciniphila* abundance and metabolic health and we provide a first view of *A. muciniphila* association with the gut ecosystem. Collectively, these observations demonstrate the importance of studying *A. muciniphila* in the context of the gut environment, as it may drive a favorable or

deleterious contribution of *A. muciniphila* to health. The underlying mechanisms explaining these associations should be investigated in future studies.

TABLES

Table 1. Comparison between clinical variables categorized into *A. muciniphila* abundance groups.

			Akk LO (N=24)	Akk HI (N=25)	p-value
	Sex, N(%)	F	19 (79.2)	22 (88.0)	0.4
		M	5 (20.8)	3 (12.0)	
	Age (y)		45 (12)	39 (12)	0.18
	Age categorization around the median, N(%)	Age LO (≤ 49 y)	8 (32.0)	17 (68.0)	**0.02**
		Age HI (> 49 y)	16 (66.7)	8 (33.3)	
Body composition	BMI (kg/m^2)		33.0 (0.9)	32.5 (1.0)	0.63
	Waist circumference (cm)		108.8 (2.2)	105.7 (2.3)	0.27
	Hip circumference (cm)		113.4 (2.0)	115.0 (2.1)	0.51
	WHR		0.96 (0.01)	0.92 (0.02)	**0.04**
	Fat mass (%)		35.6 (1.0)	34.2 (1.1)	0.30
	Lean mass (%)		61.5 (1.0)	62.7 (1.1)	0.33
	% of android fat (DXA)		61.1 (1.3)	59.5 (1.4)	0.33
	% of gynoid fat (DXA)		36.3 (1.3)	37.6 (1.4)	0.42
	Adipocyte Diameter (µm)		111.5 (1.6)	104.8 (1.8)	**0.002**
Glucose homeostasis	Glucose (mmol/L)		5.4 (0.1)	5.2 (0.1)	**0.02**
	Insulin (µIU/ml)		11.3 (0.9)	8.9 (0.9)	**0.03**
	HOMA-IR		1.5 (0.1)	1.2 (0.1)	**0.03**
	Disse index		-9.2 (1.0)	-6.0 (1.1)	**0.02**
Liver enzymes	Alanine transaminase (ALT) (IU/L)		38.2 (3.3)	31.5 (3.5)	0.11
	Aspartate transaminase (AST) (IU/L)		39.5 (3.7)	29.0 (3.9)	**0.03**
	Gamma-glutamyl transpeptidase (GGT) (IU/L)		57.0 (5.6)	35.3 (6.0)	**0.004**
Blood lipids	LDL-c (mmol/L)		3.4 (0.2)	3.3 (0.2)	0.66
	Triglycerides (mmol/L)		1.2 (0.9 - 1.7)	1.0 (0.8 - 1.2)	0.08
	Non-esterified fatty acids (NEFA) (mmol/L)		0.42 (0.04)	0.41 (0.04)	0.76
Systemic inflammation	hs CRP (mg/L)		4.6 (1.7 - 7.2)	2.4 (0.9 - 6.9)	0.11
	IL-6 (pg/ml)		1.3 (0.7 - 2.9)	1.6 (1.1 - 2.3)	0.93

	LPS (pg/ml)	1.7 (1.2 - 2.7)	2.1 (1.2 - 2.9)	0.80
scWAT macrophage markers	HAM56 (%)	13.6 (8.2 - 22.9)	10.0 (6.5 - 17.5)	0.18
	%HAM56 / Adipocyte Diameter	0.13 (0.02)	0.10 (0.02)	0.23
Adipokines	Leptin (ng/ml)	44.1 (3.6)	30.9 (3.9)	**0.005**
	Adiponectin (µg/ml)	15.1 (5.9 - 20.0)	14.7 (11.5 - 17.4)	0.77

For variables with a skewed distribution (triglycerides, CRP, IL-6, LPS, %HAM56 and adiponectin): Wilcoxon rank sum test, median (Q1-Q3) shown. For other variables: ANCOVA adjusting for age and sex, adjusted mean (SE) shown. Akk LO = *A. muciniphila* below the median; Akk HI = *A. muciniphila* at or above the median.

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AUTHOR CONTRIBUTIONS

KC and SR designed the overall clinical research study and managed it; PDC and AE generated the *A. muciniphila* qPCR results; JD and FL generated the quantitative metagenomics results and EP analysed association between *A. muciniphila* and MGS; EOV was involved in analysis and interpretation of dietary data; BK and JAW were involved in analysis and interpretation of

clinical results; MCD managed this project and implemented data integration and statistical analysis; NS created the Bayesian Network and contributed to statistical analysis; MED, JC and LH generated NMR acetate results; MCD, JAW, EP, EOV, BK and KC wrote the manuscript. All authors provided input on the analysis and interpretation of the results, and preparation of the manuscript.

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FIGURE LEGENDS

Figure 1. Association between *A. muciniphila* abundance and markers of insulin sensitivity.

A: Comparison of fasting glucose, insulin, HOMA-IR and Disse index between Akk LO and Akk HI groups. **B:** Bayesian network showing the dependencies between variables selected based on their association with *A. muciniphila*. The thickness of the edges connecting the vertices (variables) represents the weight of dependencies between variables. Akk = *A. muciniphila*, WHR = waist-to-hip ratio, Adip_Diam = adipocyte diameter, TG = triglycerides, Chol = total cholesterol, HOMA-IR = Homeostasis Model Assessment of Insulin Resistance Index, Disse = Disse index, AST = aspartate transaminase, ALT = alanine transaminase, GGT = gamma-glutamyl transpeptidase. **C** and **D:** OGTT glucose and insulin curves, respectively (included times: 0, 15, 30, 60, 90, and 120 minutes), with comparison in glucose AUC between Akk LO (N=18) and Akk HI (N=22) by ANCOVA adjusting for age and sex. Spearman correlation between glucose or insulin AUC and *A. muciniphila* abundance is shown. Akk LO = *A. muciniphila* below the median; Akk HI = *A. muciniphila* at or above the median.

Figure 2. Relationship between adipocyte volume and body fat mass according to *A. muciniphila* groups.

A: Spearman correlation between *A. muciniphila* and kg fat mass. **B:** Spearman correlation between *A. muciniphila* and adipocyte diameter. **C:** Association between adipocyte volume and body fat mass in relation to fitted curve, with black circles representing the Akk HI group and white circles the Akk LO group. **D:** Residuals of data points in part C. Akk LO = *A. muciniphila* below the median; Akk HI = *A. muciniphila* at or above the median.

Figure 3. Changes in *A. muciniphila* abundance with dietary intervention.

A: Paired *t test* was used to measure the within-group change in *A. muciniphila* abundance, mean (SE) is shown; *p<0.05 with paired *t test*; #p<0.01, ##p<0.001 and ###p≤0.0001 with *t test* between Akk LO and HI at each time point. **B:** ANCOVA adjusting for age and sex was used to compare the change between Akk LO and Akk HI groups, adjusted mean change (SE) is shown; p<0.05. CR=calorie restriction; WS=weight stabilization; Akk LO = *A. muciniphila* below the median; Akk HI = *A. muciniphila* at or above the median.

Figure 4. Comparing the effect of dietary intervention on bioclinical parameters between *A. muciniphila* groups.

A-D: Paired *t test* was used to measure the within-group change in Disse index (A), waist circumference (B), and total and LDL cholesterol (C, D); mean (SE) is shown. **E-H:** ANCOVA adjusting for sex, age and baseline value was used to compare the change between Akk LO and Akk HI groups in Disse index (E), waist circumference (F), and total and LDL cholesterol (G-H); adjusted mean change (SE) is shown. *p≤0.05; **p ≤ 0.01; ***p ≤ 0.001; ****p ≤ 0.0001; CR=calorie restriction; WS=weight stabilization; Total = T0 to W12. Akk LO = *A. muciniphila* below the median (gray bars and lines); Akk HI = *A. muciniphila* at or above the median (black bars and lines).

Figure 5. Serum acetate and *A. muciniphila*.

A: Spearman correlation between serum acetate and *A. muciniphila* abundance. **B:** Within-group change in serum acetate assessed by paired *t test*, mean (SE) shown, * p≤0.05. **C:** Comparison of change in serum acetate between Akk groups, mean (SE) shown; *t test*. Akk LO = *A. muciniphila* below the median; Akk HI = *A. muciniphila* at or above the median.

Figure 6. Association between *A. muciniphila* and metagenomic species

A: Barcodes indicating the presence and abundance of the MGS that are significantly abundant between Akk LO and Akk HI (Wilcoxon $p<0.01$) in a given time point. White is absent and abundance increases from light blue to dark red. Samples are sorted by *A. muciniphila* baseline abundance. Green text indicates MGS that are more abundant in the Akk HI group at baseline and in brown in the Akk LO group. P-values in red indicate MGS that are correlated with gene richness; # significant q-value; ‘ $p<0.05$; * $p<0.01$. **B:** Cumulative abundance load of the *A. muciniphila* MGS (red) and the 26 associated MGS (yellow) compared to the rest of the MGS (with more than 500 genes) in gray.

Figure 7. Clinical parameters that differ across *A. muciniphila* and gene richness groups.

A. muciniphila x gene count groups were compared: **A:** % android fat; **B:** % gynoid fat; **C:** fasting plasma glucose; and **D:** fasting plasma triglycerides. Akk LO = *A. muciniphila* below the median; Akk HI = *A. muciniphila* at or above the median; HGC = high gene count; LGC = low gene count. Kruskal-Wallis followed by Wilcoxon Rank Sum test for individual comparisons with Bonferroni adjustment. Sample sizes are Akk LO, LGC N=9; Akk HI, LGC N=9; Akk LO, HGC N=11; Akk HI, HGC N=16 ($p=0.56$, Fisher’s Exact test).

Figure 8. Change in MAR diet quality score by *A. muciniphila* abundance and age over the different stages of the dietary intervention.

A-B: Paired *t test* was used to measure the within-group change in MAR. **C:** ANCOVA adjusting for age, sex and baseline MAR value was used to compare the change between Akk categories. **D:** ANCOVA adjusting for sex and baseline MAR value was used to compare the change between age categories. In A-B mean (SE), and in C-D adjusted mean change (SE) is

shown. *p≤0.05; **p ≤ 0.01; ***p ≤ 0.001; ****p ≤ 0.0001. CR=calorie restriction; WS=weight stabilization. Akk LO = *A. muciniphila* below the median, N=15; Akk HI = *A. muciniphila* at or above the median, N=21. Age LO = Age below population median, N=18; Age HI = Age at or above the population median, N=18.

Supplementary Figure 1. *A. muciniphila* abundance distribution comparison between qPCR and QM.

Supplementary Figure 2. Comparison of changes in *A. muciniphila* abundance throughout dietary intervention between qPCR and QM.

Supplementary Figure 3. Correlation matrix depicting the baseline association between relevant variables.

Supplementary Figure 4. Individual variations in *A. muciniphila* abundance and fasting glucose throughout the dietary intervention.

A: *A. muciniphila* individual kinetics. **B:** Fasting glucose individual kinetics.

Supplementary Figure 5. Clinical parameters that differed across *A. muciniphila* and gene count groups at different time points.

Kruskal-Wallis for trend followed by Wilcoxon Rank Sum test for individual comparisons with Bonferroni adjustment. Akk LO = *A. muciniphila* below the median; Akk HI = *A. muciniphila* at or above the median; HGC = high gene count; LGC = low gene count. Sample sizes are Akk LO, LGC N=9; Akk HI, LGC N=9; Akk LO, HGC N=11; Akk HI, HGC N=16.