

RESEARCH PAPER

 OPEN ACCESS 

Impact of intensive lifestyle intervention on gut microbiota composition in type 2 diabetes: a *post-hoc* analysis of a randomized clinical trial

Shaodong Wei^{a,b}, Asker Daniel Breyndrod^{a,c}, Urvish Trivedi ^a, Martin Steen Mortensen^a, Mette Yun Johansen^d, Kristian Karstoft ^{d,e}, Allan Arthur Vaag^{d,f}, Mathias Ried-Larsen ^d, and Søren Johannes Sørensen ^a

^aSection of Microbiology, Department of Biology, University of Copenhagen, Copenhagen, Denmark; ^bNational Food Institute, Technical University of Denmark, Kgs Lyngby, Denmark; ^cSkaggs School of Pharmacy and Pharmaceutical Sciences, La Jolla, CA, USA; ^dCenter of Inflammation and Metabolism and Center for Physical Activity Research, Copenhagen University Hospital - Rigshospitalet, Copenhagen, Denmark; ^eDepartment of Clinical Pharmacology, Bispebjerg Hospital, University of Copenhagen, Copenhagen, Denmark; ^fSteno Diabetes Center Copenhagen, Gentofte, Denmark

ABSTRACT

Type 2 diabetes (T2D) management is based on combined pharmacological and lifestyle intervention approaches. While their clinical benefits are well studied, less is known about their effects on the gut microbiota. We aimed to investigate if an intensive lifestyle intervention combined with conventional standard care leads to a different gut microbiota composition compared to standard care alone treatment in individuals with T2D, and if gut microbiota is associated with the clinical benefits of the treatments. Ninety-eight individuals with T2D were randomized to either an intensive lifestyle intervention combined with standard care group (N = 64), or standard care alone group (N = 34) for 12 months. All individuals received standardized, blinded, target-driven medical therapy, and individual counseling. The lifestyle intervention group moreover received intensified physical training and dietary plans. Clinical characteristics and fecal samples were collected at baseline, 3-, 6-, 9-, and 12-month follow-up. The gut microbiota was profiled with 16S rRNA gene amplicon sequencing. There were no statistical differences in the change of gut microbiota composition between treatments after 12 months, except minor and transient differences at month 3. The shift in gut microbiota alpha diversity at all time windows did not correlate with the change in clinical characteristics, and the gut microbiota did not mediate the treatment effect on clinical characteristics. The clinical benefits of intensive lifestyle and/or pharmacological interventions in T2D are unlikely to be explained by, or causally related to, changes in the gut microbiota composition.

ARTICLE HISTORY

Received 8 June 2021
Revised 19 October 2021
Accepted 8 November 2021

KEYWORDS



exercise; gut microbiota; lifestyle intervention; metformin; physical activity; standard care; type 2 diabetes


Introduction

The prevalence of type 2 diabetes (T2D) has increased rapidly over the past decades.¹ Successful management of T2D improves individuals' life expectancy and quality.² The basic principles of T2D management are based upon a combination of pharmacological treatments and lifestyle interventions.³ The medical approach includes the preferred first-line medication metformin and potentially second-line medications, *e.g.* glucagon-like peptide-1 (GLP-1) analogue, to manage blood glucose levels. Healthy lifestyle involves increased physical activity levels and dietary changes aiming at a substantial weight loss. Many studies have reported that intensive lifestyle interventions can reduce the incidence of T2D,^{4–6} prevent further

deterioration of impaired glucose tolerance,^{5,7} decrease the hemoglobin A1c (HbA1c) level,⁸ and reduce the medication use in overt T2D.⁹

Accumulating evidence suggests that the gut microbiota have specific signatures for individuals with T2D,¹⁰ hence it raises the question if these two treatments (pharmacological treatments or intensive lifestyle interventions) have different impacts on the gut microbiota. For example, the first-line glucose-lowering medication (GLMED) metformin was shown to significantly affect gut microbiota, *e.g.* increase of *Escherichia* spp., *Akkermansia muciniphila*, and decrease of *Intestinibacter* spp.,^{10–12} and it has been suggested that the therapeutic benefits might be partly mediated *via* the gut

CONTACT Søren Johannes Sørensen  sjs@bio.ku.dk  Section of Microbiology, Department of Biology, University of Copenhagen, Universitetsparken 15, Bldg. 1, Copenhagen DK-2100, Denmark

 Supplemental data for this article can be accessed [here](#).

© 2021 The Author(s). Published with license by Taylor & Francis Group, LLC.

This is an Open Access article distributed under the terms of the Creative Commons Attribution License (<http://creativecommons.org/licenses/by/4.0/>), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

microbiota.¹³ Similarly, lifestyle interventions with a healthy diet and/or physical exercise training have also been shown to impact the gut microbiota composition. Diet, in particular, is a key determinant of the gut microbiota composition as dietary patterns (western or vegetarian diet), specific foods (fruits, grain, or vegetables), and food constituents (fiber, protein, or fat) can all lead to changes in the gut microbiota.¹⁴ Increased exercise levels are associated with enhanced fuel mobilization, muscle glucose uptake, and fat oxidation,¹⁵ and affect gastric emptying through interleukin-6 (IL-6), leading to reduced postprandial glycemia.¹⁶ However, it is still unclear how exercise influences gut microbiota.¹⁷ Professional athletes with extreme exercise (e.g. rugby training athletes) and associated diets have higher gut microbial diversity, with enhanced production of amino acids and short-chain fatty acids (SCFAs) (likely contributed by genus *Roseburia* and family XIII Incertae Sedis), compared to more sedentary individuals.^{18,19} Recently, a study has linked extreme exercise (marathon running) with increased gut microbe *Veillonella atypica*,²⁰ although further validation is needed.²¹ However, only few randomized clinical trials have evaluated the effects of regular exercise on the microbiome in human populations, especially in individuals with T2D, and in these studies, the effects of exercise have been limited.^{22–24}

We previously showed that an intensive lifestyle intervention in individuals with T2D maintained glycemic control while reducing the need for GLMED, and improved clinical characteristics compared to the standard care.⁹ In this *post hoc* analysis, we aimed to investigate how an intensive lifestyle intervention with a subsequent reduction in GLMED impacts the gut microbiota composition compared to standard care in individuals with T2D, and whether gut microbiota composition changes may contribute to the observed clinical treatment benefits.

Results

Taxonomic composition differences between treatments

At baseline 0 month (M0), the gut microbiota compositions of individuals in the two treatment groups were not significantly different and no taxa differed in

their relative abundances between groups at any taxonomic levels (from phylum to amplicon sequence variant [ASV], adjusted P (P_{adj}) > .05). Specifically, at the phylum level, Firmicutes was the most abundant phylum (71.2% [lifestyle, median] vs. 74.3% [standard care, median], $P_{adj} = .921$), followed by Actinobacteria (5.6% vs. 5.5%, $P_{adj} = .909$), Proteobacteria (2.6% vs. 2.6%, $P_{adj} = .842$), Bacteroidetes (2.6% vs. 0.7%, $P_{adj} = .755$), and Verrucomicrobia (1.0% vs. 0.6%, $P_{adj} = .839$). These five phyla accounted for the majority of the gut microbiota abundances (98.9% [lifestyle] and 99.2% [standard care]).

To investigate if the two treatments shifted gut microbiota similarly, we compared the changes in observed richness and Shannon diversity between groups at all time windows (Figure 1a, from baseline to 3 months [M0-M3], to 6 months [M0-M6], to 9 months [M0-M9], and to 12 months [M0-M12]). However, none of these changes were significantly different between groups ($P > .05$, shown with “ns”). Next, we assessed the changes in alpha diversity for each treatment. From M0 to M12, richness increased 17.1% in the lifestyle group (105.0 ± 30.3 [M0] vs. 123.0 ± 38.7 [M12], mean \pm SD; $17.1\% = [(123.0 - 105.0)/105.0] \times 100$) and 42.9% in standard care (91.8 ± 33.3 [M0] vs. 131.2 ± 30.3 [M12]; $42.9\% = [(131.2 - 91.8)/91.8] \times 100$). Specifically, both treatments had increased richness at M3 compared to M0 (lifestyle, 105.0 ± 30.3 [M0] vs. 122.1 ± 30.4 [M3], mean \pm SD, $P < .001$; standard care, 91.8 ± 33.3 [M0] vs. 110.8 ± 31.2 [M3], $P = .014$), then slightly reduced until M9 (119.7 ± 34.9 [lifestyle], $P = .007$; 107.3 ± 34.5 [standard care], $P = .022$), thereafter increased quickly from M9 to M12. We also investigated ASVs (shown at the genus level) that were responsible for the increased richness (Figure S1) and found that they were mainly from the genera *Bacteroides*, *Ruminococcaceae* UCG 014, *Alistipes*, and *Roseburia*. From M0 to M12, Shannon diversity increased 11.3% in the lifestyle intervention (3.28 ± 0.51 [M0] vs. 3.65 ± 0.61 [M12], mean \pm SD; $11.3\% = [(3.65 - 3.28)/3.28] \times 100$) and 23.5% in standard care (3.06 ± 0.53 [M0] vs. 3.78 ± 0.50 [M12]; $23.5\% = [(3.78 - 3.06)/3.06] \times 100$). Only individuals in the lifestyle group showed increased Shannon diversity at M3 compared to M0 (3.28 ± 0.51 [M0] vs. 3.55 ± 0.47 [M3], mean \pm SD, $P <$

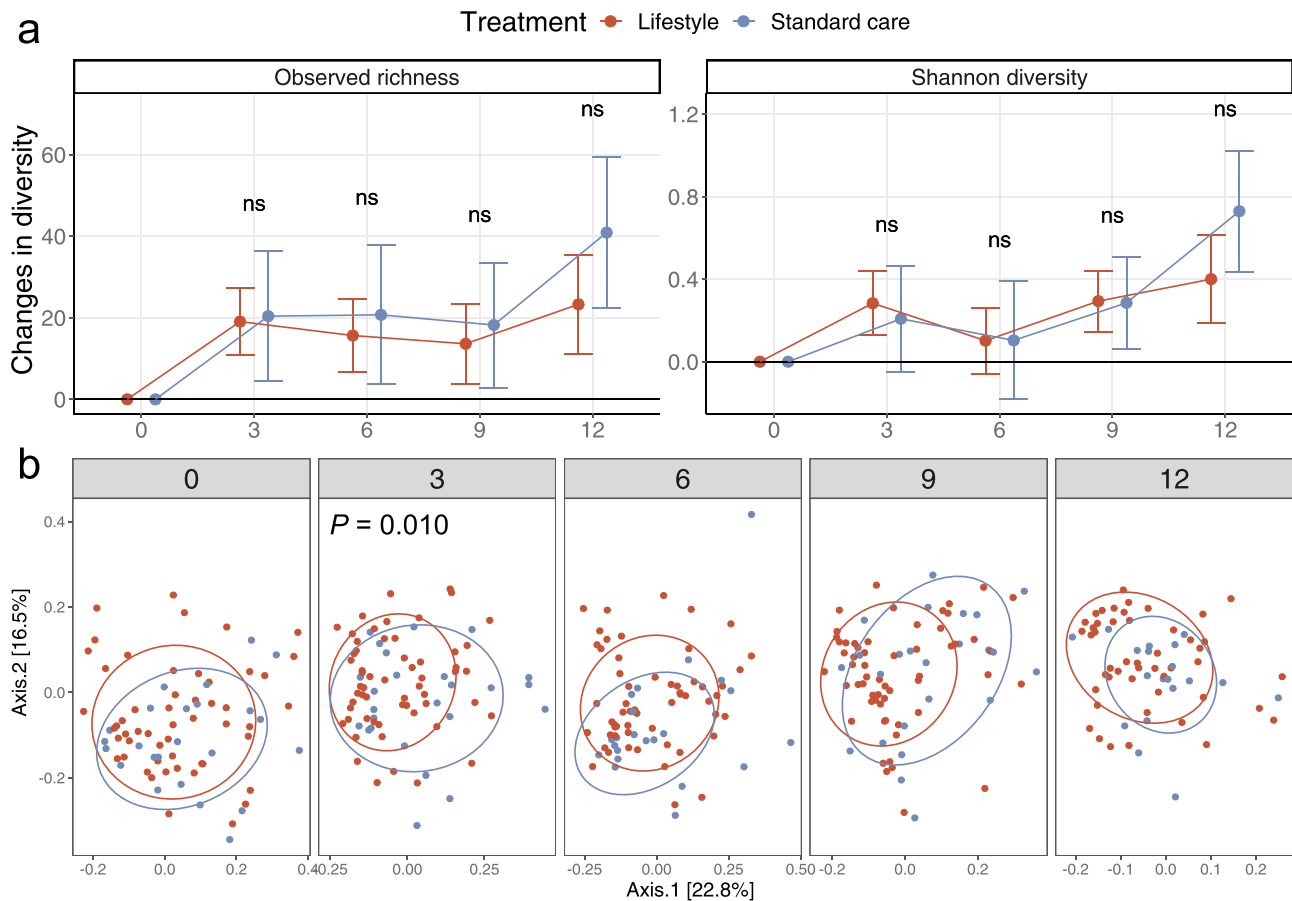


Figure 1. Taxonomic composition differences between treatments over time (0, 3, 6, 9, 12 months). (a) Change of alpha diversity for each individual averaged within groups compared to baseline (M0). The dots refer to the mean and error bars refer to the 95% confidence interval. For the sake of interpretation, if the error bar overlaps the horizontal line (value of zero), it indicates that the change of alpha diversity is not significantly different from zero (no change). The comparison alpha diversity between a time point and baseline was performed with paired two-sided t-test. The comparison of changes in alpha diversity between treatments was performed with two-sided t-test and the significance was shown as “ns” ($P > .05$). (b) Distribution of samples based on weighted UniFrac distance visualized with principal coordinates analysis (PCoA) with ellipses indicating 75% confidence regions for clusters. The P value is from the multivariate permutational analysis of variance (PERMANOVA).

.001) and standard care did not induce significant difference during the first three months (3.06 ± 0.53 [M0] vs. 3.28 ± 0.70 [M3], $P = .106$). Both treatments increased Shannon diversity significantly at M9 (119.7 ± 34.9 [lifestyle], $P < .001$; 3.33 ± 0.63 [standard care], $P = .014$) and M12.

Additionally, we assessed the gut microbiota composition based on the weighted UniFrac distance, visualized by principal coordinates analysis (PCoA), stratified by time point (Figure 1b). Two treatments did not lead to different gut microbiota compositions at M12 ($P = .127$) and other time points ($P = .138$ [M6], $P = .063$ [M9]), except for M3 ($P = .010$). We also compared the distance for each patient at different time windows as a way to assess the changed beta diversity over time (Figure

S2). However, none of the changed beta diversities were significantly different between groups at all time windows ($P > .05$).

We then compared the group variance based on weighted UniFrac distance, by assessing the change of group dispersion (mean UniFrac distance to group centroid in multivariate space) over time (Figure S3). When comparing the differences between treatments in the change of sample dispersion, no significant difference was observed at M12 or any other time points ($P > .05$, shown with “ns”, in all conditions). From M0 to M12, both treatments led to reduced group dispersion (0.276 ± 0.103 [M12] vs. 0.316 ± 0.085 [M0], $P = .022$, lifestyle, [mean \pm SD]; 0.232 ± 0.058 [M12] vs. 0.316 ± 0.085 [M0], $P = .001$, standard care). Specifically, for the

lifestyle intervention, the group dispersion was significantly reduced at M3 (0.271 ± 0.068 , $P = .003$), M9 (0.270 ± 0.091 , $P = .006$), and M12 (0.276 ± 0.103 , $P = .022$), compared to M0 (0.316 ± 0.085). For standard care, no significant change was observed at M3 (0.300 ± 0.072 , $P = .532$), M6 (0.308 ± 0.109 , $P = .743$), or M9 (0.311 ± 0.084 , $P = .743$), compared to M0 (0.316 ± 0.085).

Next, we found that due to the large group dispersion, the inter-individual variation at M0 was higher than the intra-individual development between M0 and M12 (Figure S4, 0.442 ± 0.159 [M0] vs. 0.404 ± 0.206 [M0-M12], $P = .019$, lifestyle, median \pm IQR [interquartile range]; 0.460 ± 0.185 [M0] vs. 0.320 ± 0.201 [M0-M12], $P = .010$, standard care, median \pm IQR), or M0 and M3 (0.442 ± 0.159 [M0] vs. 0.302 ± 0.189 [M0-M3], $P < .001$, lifestyle; 0.460 ± 0.185 [M0] vs. 0.352 ± 0.205 [M0-M3], $P = .001$, standard care), based on the weighted UniFrac distance.

Finally, we compared the change of taxa abundances between treatments at different taxonomic levels (from phylum to ASV) and different time windows (M0-M3, M0-M6, M0-M9, and M0-12). However, no taxa differed in the changes of their relative abundances at any time windows ($P_{adj} > .05$, in all conditions).

Functional composition differences between treatments

Based on the phylogenetic investigation of communities by reconstructing unobserved states (PICRUSt, version 2) program,²⁵ we assessed the imputed functional composition of microbial communities. A total of 428 enzyme-catalyzed reactions (ECs) and 147 KEGG orthologs (KOs) were selected and their changes of relative abundances in time windows between treatments were compared, with the same method as the taxa abundances comparison. It showed that no ECs and KOs had significantly different changes in their relative abundances at any time windows ($P_{adj} > .05$, in all conditions). In addition, we also grouped enzyme-catalyzed reactions into pathways and the changes of relative abundances for 193 pathways were compared. Again, no pathways had significantly different changes in their abundances at any time windows ($P_{adj} > .05$, in all conditions).

Clinical characteristics and differences between treatments

In contrast to the similar changes in gut microbiota taxonomic and functional compositions between treatments, clinical characteristics, as reported earlier, changed differently during the 12-month intervention (M0 vs. M12).^{9,26–28} Specifically, individuals randomized to lifestyle intervention decreased their HbA1c levels by 3.4 ± 7.5 mmol/mol (mean \pm SD) and reduced their need for and use of pharmacological treatments (GLMED score decreased by 1.6 ± 1.6). Intensive lifestyle interventions were in addition associated with increased physical fitness by 6.5 ± 5.7 ml/kg/min, and improved body composition by increasing lean mass (0.7 ± 2.4 kg) and decreasing fat mass (6.3 ± 6.5 kg) (Table S1). In contrast, individuals randomized to standard care either did not progress toward improved health status (e.g. GLMED score increased 0.5 ± 1.9), or to a less extent (e.g. physical fitness increased 0.3 ± 4.9 ml/kg/min, fat mass decreased 1.5 ± 4.7 kg).

To further describe the changing trend of clinical characteristics, we assessed the change of characteristics over time compared to M0 within each individual and grouped by treatments (Figure S5). We found that the change in clinical characteristics largely occurred during the first three months for individuals in the lifestyle intervention group. In contrast, the characteristics of individuals allocated to the standard care were quite stable during the whole intervention period. Additionally, we also compared the difference in the change of clinical characteristics between treatments, and most of them started being different between treatments from M3 and remained until M12 (Figure S5).

Gut microbiota composition was shifted in both treatments

Next, we performed a distance-based redundancy analysis (dbRDA) to assess the change of gut microbiota composition from M0 to M3 or M12, and interrogated if such changes could be correlated with clinical characteristics and/or treatments (Figure 2). Of all assessed characteristics (Figure S5, Table S2), only GLMED score significantly explained a proportion of variance at M3. Besides,

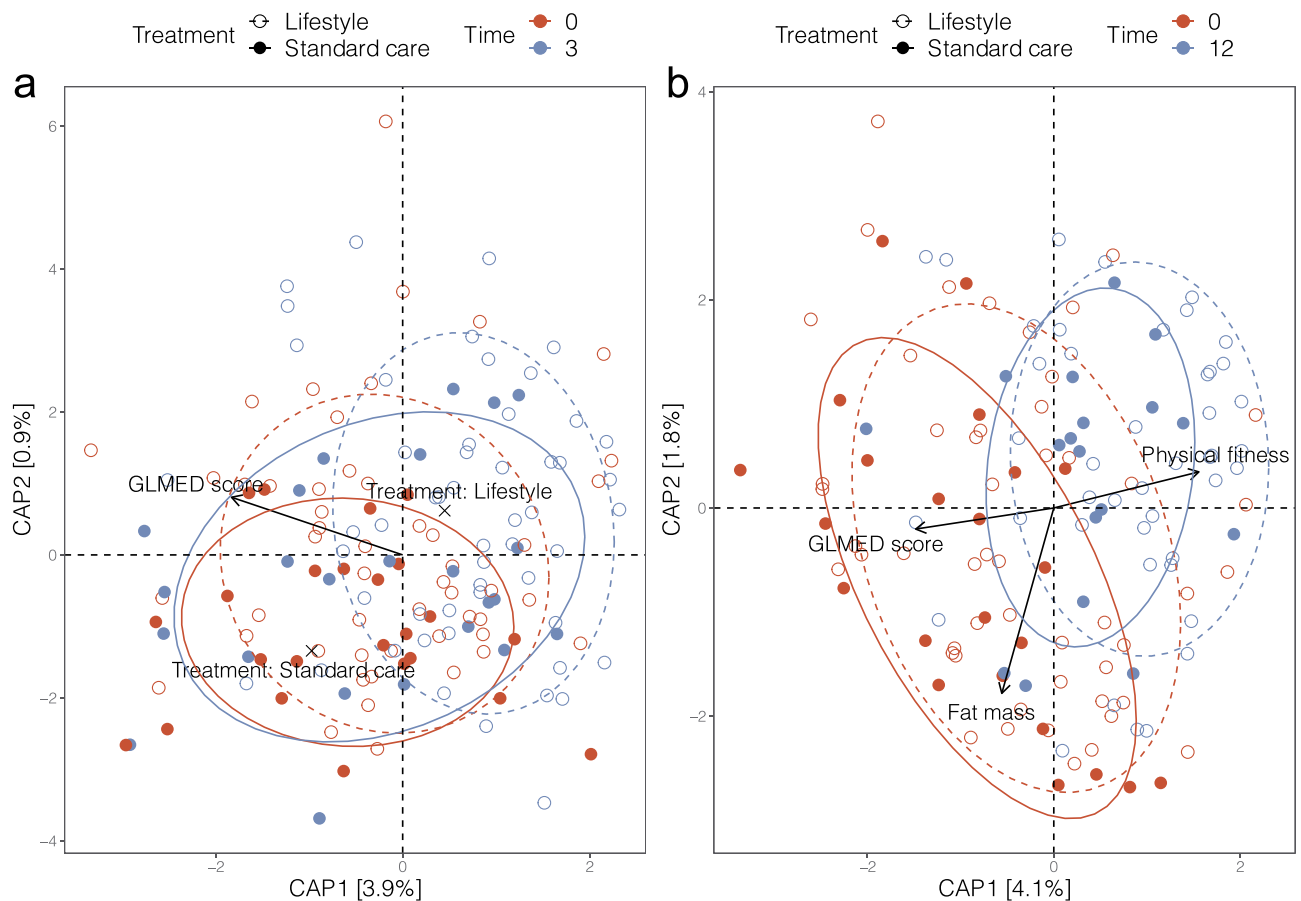


Figure 2. The weighted UniFrac distance-based redundancy analysis (dbRDA) showing the distribution of samples at M0 and M3 (a) or M0 and M12 (b), and the associations with clinical characteristics and/or treatments. Each dot is a sample. Colors refer to different time points. Solid dots and solid lines refer to lifestyle intervention; hollow dots and dashed lines refer to standard care. Ellipses indicate 75% confidence regions for clusters. The direction and magnitude of arrows indicate which clinical characteristics that taxa abundance responds to the most strongly. The black crosses in (a) indicate the direction and magnitude of treatments.

the diabetes treatments (lifestyle intervention and standard care) were also significantly correlated (Figure 2a). While, at M12, significantly correlated factors were GLMED score, physical fitness, and fat mass (Figure 2b). In agreement with the arrows' direction (characteristics increasing gradient), individuals from the lifestyle intervention significantly reduced the GLMED score, fat mass, and significantly increased physical fitness over time (Figure S5). Individuals from the standard care also tended to change in the beneficial direction (e.g. increased physical fitness, decreased fat mass), though none of these characteristics changed significantly over time.

Samples from both treatments largely overlapped with each other both at M0 ($R^2 = 0.010$, $P = .613$) and M12 ($R^2 = 0.022$, $P = .130$), except

M3 ($R^2 = 0.032$, $P = .010$, also shown in Figure 1b). However, they all differed significantly from their baseline at both M3 ($R^2 = 0.040$, $P < .001$, M0 vs. M3, lifestyle; $R^2 = 0.047$, $P = .026$, M0 vs. M3, standard care) and M12 ($R^2 = 0.074$, $P < .001$, M0 vs. M12, lifestyle; $R^2 = 0.105$, $P < .001$, M0 vs. M12, standard care). To investigate which taxa were responsible for the changed beta diversity at M3 and M12 in comparison with M0, we compared the relative abundances between time points for each treatment (Table S3). We observed that many genera changed their abundances significantly at M3 or M12, such as *Bacteroides*, *Blautia*, *Roseburia*, *Escherichia*, and *Faecalibacterium*. Besides, we also observed a sharp reduction of Firmicutes to Bacteroidetes ratio for both treatments at M3 and M12 (26.9 ± 62.2 [M0, median \pm IQR], 8.0 ± 10.4

[M3], 4.7 ± 6.1 [M12], $P < .001$ for both M3 and M12 against M0, lifestyle; 63.9 ± 92.1 [M0], 8.1 ± 15.2 [M3, $P = .003$], 7.8 ± 5.5 [M12, $P < .001$], standard care).

Gut microbiota alpha diversity does not correlate with clinical characteristics

The gut microbiota alpha diversity has been suggested as an indicator of health status.^{29,30} To validate if this notion is reflected in our study, we assessed the correlations between the shift in gut microbiota alpha diversity and the change in clinical characteristics for each treatment (pooling all time windows together), which is shown in Figure 3. Overall, almost none of these correlations was significant ($P > .05$, Table S4). In detail, body fat-related characteristics, such as android fat mass,

gynoid fat mass, fat percentage, and fat mass tended to be inversely correlated with gut microbiota alpha diversity in the lifestyle group; The lean mass, fat-free mass, and physical fitness positively correlated with alpha diversity in the lifestyle group. However, these correlations were almost inverted in the standard care group. HbA1c showed quite small correlation coefficients in both groups and were far from being significant (Table S4). GLMED score showed negative correlations with observed richness and positive correlations with Shannon diversity in both groups, but none of them were significant.

Mediation analysis of gut microbiota

Our study showed clear relevant changes in a number of clinical characteristics (Figure S5). However, it remains unknown the extent to which

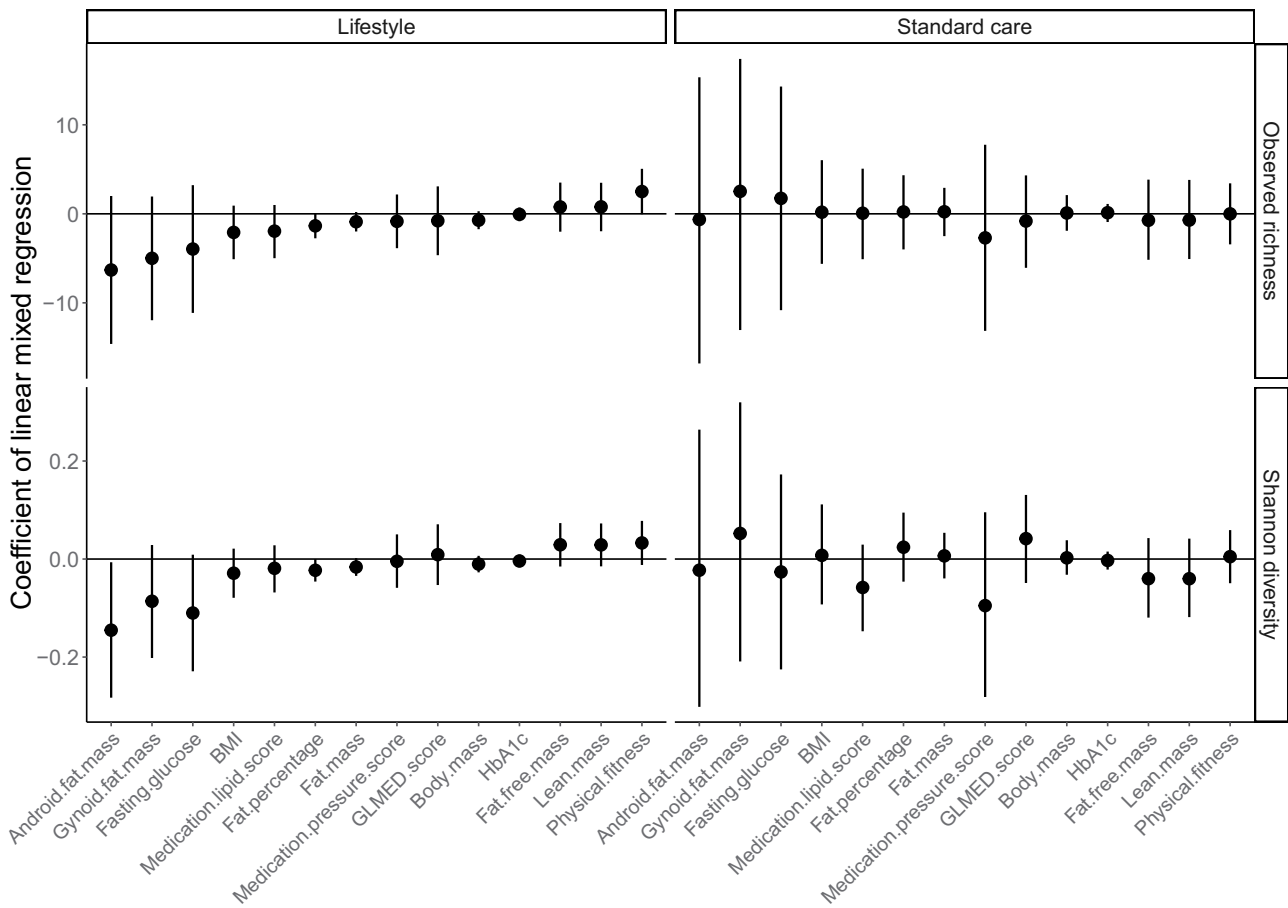


Figure 3. The correlations between the change of clinical characteristics and the change of alpha diversity. The Y axis is the correlation coefficient obtained from the linear mixed models, the effects of age, gender, and time windows were adjusted, and patient was used as the random effect. X axis is the clinical characteristic. Vertical lines are the 95% confidence interval of the correlation coefficient mean. A correlation is significant if its confidence interval does not overlap the black horizontal line (value of zero referring to no correlation).

gut microbiota may mediate any of these clinical benefits and/or differences under the treatment effects. We, therefore, applied a robust distance-based mediation analysis to evaluate the overall mediation effect of gut microbiota. Among all mediation conditions (see Materials and methods), only one mediation came out as statistically significant, namely the treatments' effect on GLMED score at M3, which appeared to be significantly mediated by the gut microbiota at this specific time point M3 ($P = .030$). However, this specific effect was not further validated by another mediation method "SparseMCM" ($P = .505$, overall mediation effect).

Gut microbiota differences stratified by GLMED score change

Based on our dbrDA (Figure 2) and mediation analysis, the clinical characteristic GLMED score possibly best reflected gut microbiota change related to treatments. When considering a reduction of GLMED score at M12 as an indicator of a successful lifestyle change, 17.6% of individuals treated with standard care (three out of 17 persons reduced GLMED score) changed their lifestyle without supervision. Similarly, 23.3% of individuals in the lifestyle intervention (10 out of 43 persons did not reduce GLMED score) failed to change lifestyles. Therefore, to assess the actual impact of lifestyle interventions on gut microbiota, we regrouped individuals into GLMED score groups: Same ($N = 8$), Increase ($N = 16$), and Decrease ($N = 36$) based on the change of GLMED score (individuals not having samples at both M0 and M12 were removed) (Figure S6). To assess if the direct effect of GLMEDs confounded our GLMED score grouping, we assessed the influence of different intensities of medication treatment on the gut microbiota by comparing the alpha diversity between different medication intensities (GLMED score 1 to 6) and medication discontinuation (GLMED score 0) (Figure S7, Table S2). In most of the conditions, GLMED did not lead to different gut microbiota alpha diversity compared to GLMED free individuals ($P > .05$).

By comparing the GLMED groups (decrease *vs.* increase), we observed that the changing trend of gut microbiota Shannon diversity only differed at

M3 (Figure S8a), the change of group dispersion differed at M3 and M9 (Figure S8b), gut microbiota beta diversity differed at M3 ($P = .002$, Figure S8c), the change of relative abundance of KOs, ECs, and metabolic pathways did not differ at any time windows. Among all assessed taxonomic levels, only very few taxa changed their relative abundances differently between groups (Table S5).

Discussion

After the 12-month trial, we did not observe statistical differences in the change of gut microbiota composition between the intensive lifestyle intervention and standard care. The gut microbiota alpha diversity did not correlate with the clinical characteristics, and the gut microbiota did not mediate the treatment effects on the clinical characteristics.

Two treatments led to similar gut microbiota changes

After 12 months of intervention, the two groups did not differ in their gut microbiota in any relevant aspects. (i) The change of alpha diversity was not different between groups at M12 (Figure 1a). (ii) The beta diversity was not different between groups at M12 (Figure 1b). (iii) The change of group dispersion was not different between groups at M12 (Figure S3). (iv) The change of taxa abundance at all assessed taxonomic levels was not different between groups at M12. (v) The change of functional abundance was not different between groups at M12. (vi) Based on our GLMED score grouping, potentially a way to better reflect actual lifestyles, almost none of the above-mentioned outcome measurements differed between GLMED score groups (increase *vs.* decrease) at M12 (Figure S8).

However, we did observe some weak differences in the gut microbiota at M3 between treatments, such as the changing trend of Shannon diversity compared to baseline (Figure 1a), beta diversity between groups (Figure 1b), change of group dispersion compared to baseline (Figure S3), and the dbrDA analysis (Figure 2a). Other than gut microbial changes during the first three months, the shift of clinical characteristics also largely occurred during the first three months (Figure S5). Individuals

allocated to lifestyle interventions almost improved all these clinical characteristics during this time window compared to standard care. Thereafter, the improvement in clinical characteristics remained stable and barely further improved.

Importantly these time-wise co-occurred changes of gut microbiota and clinical characteristics at M3 do not necessarily imply that gut microbiota and clinical characteristics were associated, since we almost did not observe any significant associations between the change of the gut microbiota alpha diversity and the change of clinical characteristics, and many of these associations were not consistent between treatments (positive or negative) (Figure 3). Indeed, the lack of differences in gut microbiota composition between treatments after 12 months makes any causal effects of gut microbiota on clinical outcomes highly unlikely.

Do the two treatments beneficially alter gut microbiota?

After a 12-month intervention, both treatments were associated with extensive and potentially beneficial gut microbiota changes compared to the baseline pre-treatment gut microbiota profiles. The changes included increased richness (17.1% [lifestyle] and 42.9% [standard care]) and Shannon diversity (11.3% [lifestyle] and 23.5% [standard care]). Generally, increased gut microbial diversity and richness have been considered metabolically beneficial,^{29,30} which in turn could have contributed to the beneficial clinical outcomes for both treatments after 12 months. In support of this, the dBRDA analysis revealed that along with the change of gut microbiota composition over time, the physical fitness was improved and the fat mass and GLMED score were reduced (Figure 2b).

A number of taxa were significantly shifted over time (Table S3), which likely reflected the clinical benefits of treatments. For example, *Bacteroides* has been suggested to be beneficial for glucose metabolism in mice,^{31,32} and negatively associated with T2D in humans.^{33–35} In our study, both lifestyle intervention and standard care led to increased occurrence and abundance of *Bacteroides* (Figure S1, Table S3). *Roseburia* was reported to be higher in abundances in healthy control than diabetic individuals.^{10,36} In the present study, *Roseburia* was the top genus contributing to the increased richness and was more abundant at M3 and M12 than baseline (Figure S1, Table S3).

Other genera were possibly also involved in the improvement of clinical outcomes, such as *Blautia*, *Escherichia*, and *Faecalibacterium*.^{37,38} To be noted, though two diabetes treatments (lifestyle intervention and standard care) indeed changed the gut microbiota composition along with potential beneficial effects on clinical outcomes, there are few data, if any, to suggest that the clinical benefits are caused directly by the changes in the gut microbiota composition.³⁹

Besides, although controversial,^{37,40} the ratio between Firmicutes and Bacteroidetes has been positively associated with obesity and T2D in previous studies.^{41–45} In this study, the Firmicutes to Bacteroidetes ratio in both treatments decreased dramatically in the supposed beneficial direction.

Treatments likely do not impact clinical characteristics through gut microbiota

Although the GLMED score was observed to be significantly mediated by the gut microbiota at M3 based on the distance-based mediation analysis, such mediation effect was not confirmed using an alternative method SparseMCM (P = .505). Therefore, we do not have sufficient statistical evidence to prove a mediation effect. Even if we consider GLMED score as being mediated by the gut microbiota, the mediation effect of gut microbiota does not seem to be strong, considering that 14 clinical characteristics (shown in Figure S5) were assessed, but only GLMED score was significant. A better application of mediation analysis would be to assess the mediation effect between the change of gut microbiota and the change of clinical characteristics; however, to our knowledge, such a longitudinal mediation method is not yet available.⁴⁶

Difficulties in inferring general principles

In our study, it seems that general and strong associations were not present between gut microbiota and clinical characteristics or treatments. A few reasons may have contributed: (i) We observed that the differences between individuals at baseline M0 were larger than the differences within individuals between M0 and M3, or between M0 and M12, based on weighted UniFrac distance (Figure S4). It is consistent with the study by Taniguchi

et al.²⁴ that the effect of exercise was not greater than the individual variation. Thus, high inter-individual variation may lead to reduced detection power. But our paired t-test, paired Wilcoxon rank sum test, and linear mixed model have avoided or taken into account such inter-individual variation. A good control of influencing factors (*e.g.* medications, diet) for gut microbiome would help decrease the large individual variation. (ii) Small effect size of physical exercise on the gut microbiota makes it difficult to detect differences between treatments. Prior studies only reported “subtle”²³ and “modest”²² effect of exercise on the gut microbiota. Increasing the sample population and the intensity of lifestyle interventions would facilitate the detection of microbial differences. (iii) Lack of adherence to treatments, derived from that *e.g.* not all patients in lifestyle intervention group changed their lifestyles and not all patients in standard care group continued their lifestyles, decreased the ability to differentiate changed lifestyles from constant lifestyles. However, this is alleviated by regrouping individuals based on the GLMED score change and consistent results were observed as compared to the intention-to-treat therapy-based grouping (Figure S8). A direct measurement of lifestyles for both treatments, such as daily calories intake, foot-steps, and physical activity intensities, would help regroup and compare patients based on the actual lifestyles. (iv) The use of the 16S rRNA gene sequencing technique has limited us to estimate the microbial functions (though can be predicted with PICRUSt2, but still with limitations, <https://github.com/picrust/picrust2/wiki/Key-Limitations>) and does not provide enough resolution (*e.g.* strain level) to detect differences. Including other “omics” techniques would certainly increase the assessment dimensions.

Conclusions

In this *post-hoc* analysis, we did not observe significant differences in the change of gut microbiota composition between intensive lifestyle and standard care interventions after 12 months, except for a weak difference observed at the three-month follow-up. Gut microbiota did not correlate with assessed clinical characteristics and did not appear to mediate the treatment benefits on clinical

characteristics. Overall, our results imply that clinical benefits of intensive lifestyle interventions and/or conventional standard care with multifactorial and pharmacological treatments, were unlikely to be attributable to the observed changes in the gut microbiota.

Materials and methods

Study design, participants, and randomization

This is a *post-hoc* analysis of a randomized clinical trial^{9,26–28} where the primary aim was to investigate the effect of an intensive lifestyle intervention on glycemic control. A total of 878 individuals were screened for inclusion (Figure S9). Based on the inclusion criteria (*e.g.* above 18 years old, T2D diagnosis for less than 10 years) and exclusion criteria (*e.g.* HbA1c level greater than 9%, insulin-dependence), 98 participants were included in the study. Participants were randomized to either standard care alone or intensive lifestyle and standard care in a ratio of 1:2, respectively (stratified by sex). To reduce the confounding influence of dysregulated baseline HbA1c levels, GLMED was standardized to obtain glycemic control at least six weeks prior to baseline assessment. A full protocol paper has been published.⁴⁷ This study was performed in Region Zealand and the Capital Region of Denmark from April 2015 to August 2016. This study was approved by the Scientific Ethical Committee at the Capital Region of Denmark (clinicaltrials.gov registration: NCT02417012). All participants provided oral and written informed consent.

Outcomes for the post-hoc microbiome analysis

The outcomes were: (i) Differences in gut microbiota taxonomic and functional compositions (or changes in compositions) between treatments at each time point (or time window). (ii) Gut microbiota changes from baseline to 12 months for each lifestyle and standard care treatment. (iii) Correlation and mediation analysis of gut microbiota with the treatments and/or the clinical characteristics.

Interventions

The intensive lifestyle intervention has been previously described in detail elsewhere.⁴⁷ Briefly, all participants received standard care, such as education in T2D and blinded treat-to-target medical regulation based on a pre-specified algorithm. Participants from both groups were encouraged to be physically active in their leisure time ($\geq 10,000$ steps per day).

In addition, the intensive lifestyle group participants additionally completed five to six weekly aerobic sessions whereof two to three sessions were combined with resistance training. All exercise sessions were supervised during the first four months, and supervision was gradually reduced throughout the intervention period. Also, participants in the lifestyle intervention group were given a hypocaloric diet with a macronutrient distribution of 45% to 60% carbohydrate, 15% to 20% protein, and 20% to 35% fat ($< 7\%$ saturated fat). During the first 4 months the total energy intake was restricted (-500 kcal/day relative to individually calculated energy need) and thereafter an isocaloric diet was given, aiming for a body mass index of 25 kg/m^2 . Participants were also asked to register their diet and weigh their food. Individual and group-based dietary counseling were offered by clinical dietitians and progressively reduced over time. Food frequency questionnaire was used for both groups at baseline and 12 months. Participants in the standard care group were not monitored for their daily diet or physical activity.

Assessment of outcomes and fecal samples

The gut microbiota was assessed by 16S rRNA gene sequencing of the fecal samples collected at baseline (M0), 3- (M3), 6- (M6), 9- (M9), and 12-month (M12) follow-up (Figure S9). Basic techniques and principles used to study the microbiota in a clinical context were previously reviewed.⁴⁸ The clinical characteristics were collected at the same time point as the fecal samples and the baseline clinical characteristics are shown in Table S6.

The fecal samples were taken by the participants themselves at home with OMNIgene-GUT|OM-200 kit and immediately stored in the freezer at -20°C . The samples were transported (30–45 min) in

insulated envelopes alongside a freezer pack and brought to the lab by participants along with the visit. The feces collection date was as close to participants' scheduled visits as possible and not earlier than 48 hours. The samples were immediately stored at -80°C upon arrival in the lab.

Sample population

In total, 413 fecal samples were collected from 98 individuals, of which 389 were successfully sequenced ($19,884 \pm 9,180$ sequences [mean \pm SD], samples lower than 2,000 sequences were removed, Figure S10),⁴⁹ from 86 individuals (age [54.3 ± 8.9 , years], mean \pm SD; sex male 52.7%; $N = 60$ [lifestyle] vs. $N = 26$ [standard care]), having at least three samples among the five time points (M0, M3, M6, M9, and M12) (Figure S9). Baseline characteristics of the included individuals by groups are shown in Table S6.

DNA extraction, sequencing, and bioinformatics

The total microbial DNA was extracted using the PowerMag® Soil DNA Isolation Kit on the EpMotion® 5075vt automated pipetting system (Eppendorf). The V3-V4 region of 16S rRNA gene was amplified by a two-step PCR procedure (30 + 15 cycles) using the modified broad range primers Uni341F (5'-CCTAYGGGRBGCASCAG-3') and Uni806R (5'-GGACTACHVGGGTWTCTAAT-3').^{50,51} The PCR BIO HiFi polymerase was used for PCR with the recommended reaction setup and cycling conditions. The amplified products were purified with Agencourt AMPure XP Beads (Beckman Coulter Genomics, MA, USA), normalized with SequalPrep™ Normalization Plate (96) Kit (Invitrogen), and pooled in sequencing libraries with up to 192 samples, including positive (mock communities, Figure S11a) and negative (blank DNA extraction and blank PCR, Figure S11b) controls. The concentration of the pooled libraries was then determined using the Quant-iT™ High-Sensitivity DNA Assay Kit (Life Technologies). Paired-end sequencing was performed on the Illumina MiSeq System (Illumina Inc., Ca, USA) with 5.0% PhiX. All reagents used were from the MiSeq Reagent Kits v2 (Illumina Inc., CA, USA). Adaptors and sequencing primers of raw FASTQ

files were removed using “cutadapt” (version 1.15).⁵² Trimmed reads were denoised and assembled into amplicon sequence variants (ASVs) using a modified QIIME2 (version 2018.2) implementation of the DADA2 pipeline, where 8 nucleotides were removed at the 5′ end of both forward and reverse, without 3′ truncation, the default overlap length of forward and reverse reads was decreased to six nucleotides, and all other parameters were set as default.^{53,54} Taxonomy was assigned against the Silva database (SSU Ref NR 99, release 132).⁵⁵

Statistical analysis

Alpha diversity (observed richness and Shannon diversity, at the ASV level) was assessed with R-package “phyloseq” after sample size rarefaction (function “rarefy_even_depth”),⁵⁶ the change of alpha diversity compared to baseline within each treatment was tested with paired t-test, and the comparison of changes between treatments was performed with t-test. Their associations with clinical characteristics were performed with a linear mixed effect model with R-package “lmerTest” (age, gender, and time point were included as covariates; patient was included as the random effect).⁵⁷ For beta diversity (at the ASV level), weighted UniFrac distance was calculated with function “diversity beta-phylogenetic” in QIIME2,⁵³ tested with permutational multivariate analysis of variance (PERMANOVA) with “adonis” (R-package “vegan”).^{58–60} The dispersion of samples was assessed with the function “betadisper” (R-package “vegan”). The change of dispersion within treatments and comparison of changes between treatments was tested with the same method as for alpha diversity. The comparison of individual variation and individual development based on UniFrac distance was performed with Wilcoxon rank sum test (R-package “stats”). The functional potential of the gut microbiota was predicted with phylogenetic investigation of communities by reconstruction of unobserved states (PICRUSt2) and identified as enzyme-catalyzed reaction (EC), KEGG ortholog (KO), and metabolic pathways (MetaCyc).^{25,61–63} ECs, KOs, and metabolic pathways were removed before further analysis when their relative abundances were

lower than 0.1%. The comparison change in functional composition and metabolic pathways between groups was performed with Wilcoxon rank sum test. The weighted UniFrac distance-based redundancy analysis (dbRDA) was performed with the function “ordinate” in R-package “phyloseq” to correlate gut microbiota with clinical characteristics. The clinical characteristics that significantly explain proportions of variance in the constrained ordination were selected with the function “ordistep” in R-package “vegan”. The change of clinical characteristics within treatments and the comparison of changes between treatments was tested in the same way as for alpha diversity. The mediation analysis was performed with R-package “MedTest”,⁶⁴ to assess if treatment effect on the outcome is mediated by the mediator. Specifically, the mediation analysis was performed at each time point with standard care and lifestyle intervention as the treatments, the value of each clinical characteristic as the outcome (assessed characteristics are shown in Figure S5), and the weighted UniFrac distance as the mediator gut microbiota. Besides, we also permuted the mediator and outcome at different time points, which means that the mediator gut microbiota at a time point was tested against clinical characteristics at all time points (e.g. gut microbiota at M3 can be tested against GLMED score at M12) to account for possible delayed mediation effect. The significant results derived from the MedTest were validated with another mediation method “SparseMCM” (R-package SparseMCM),⁶⁵ done at the genus level and only for genera having higher than 0.1% of relative abundances, the *P* value was obtained with 100 times permutations. The comparison of taxa abundances or Firmicutes to Bacteroidetes ratio between time points within treatments was performed with paired Wilcoxon rank sum test. The comparison of alpha diversity across the glucose-lowering mediation (GLMED) score was tested with paired Wilcoxon rank sum test. The Benjamini-Hochberg correction to control false discovery rate for multiple testing⁶⁶ was only applied for taxonomic, functional potential and metabolic pathway analysis (shown as P_{adj}). The significance level was set at 0.05 (two-sided statistical tests).

The ASVs whose prevalence lower than 1% (present in less than 4 samples) were not included in all the analyses. Most plots were generated with R-package “ggplot2”.⁶⁷

Acknowledgments

The authors thank all participants and their families. The authors thank the Danish Diabetes Association and the staff at the Center for Physical Activity Research for their assistance. The authors thank the intervention assistants, physical trainers, and clinical dietitians for their contribution to this study. The authors thank China Scholarship Council for funding SW.

Data availability statement

Sequencing data are available from Sequence Read Archive under bioproject ID PRJNA751485 (<https://www.ncbi.nlm.nih.gov/sra/PRJNA751485>). All individual-level data from cohort participants are protected under Danish and European law and cannot be made openly available. However, relevant data can be made available to other researchers under a data transfer agreement as a collaboration effort.

Disclosure statement

The authors declare that they have no conflict of interest.

Funding

The Center for Physical Activity Research (CFAS) is supported by grants from TrygFonden (grants ID 101390 and ID 20045). During the study period, the Center of Inflammation and Metabolism (CIM) was supported by a grant from the Danish National Research Foundation (DNRF55). The project is also supported by funding from Augustinus Foundation and Rigshospitalet (MYJ).

ORCID

Urvish Trivedi  <http://orcid.org/0000-0003-1541-6212>
 Kristian Karstoft  <http://orcid.org/0000-0002-6596-4199>
 Mathias Ried-Larsen  <http://orcid.org/0000-0002-8388-5291>
 Søren Johannes Sørensen  <http://orcid.org/0000-0001-6227-9906>

Contributors

MRL, MYJ, KK, and AAV performed the trial and collected clinical characteristics and fecal samples from participants. SW and MSM carried out the 16S rRNA gene

sequencing of fecal samples. SW, ADB, and MSM contributed to the bioinformatics and statistical analysis. SJS, SW, ADB, UT, MSM, MYJ, and MRL contributed to the concept and interpretation of the data. SW wrote the manuscript. All authors made a substantial contribution to the revision of the manuscript.

References

1. World Health Organization (WHO). Global report on diabetes [Internet]. 2016. <https://www.who.int/publications/i/item/9789241565257>
2. Franco OH, Steyerberg EW, Hu FB, Mackenbach J, Nusselder W. Associations of diabetes mellitus with total life expectancy and life expectancy with and without cardiovascular disease. *Arch Intern Med*. 2007;167(11):1145–1151. doi:10.1001/archinte.167.11.1145.
3. American Diabetes Association. 9. Pharmacologic approaches to glycemic treatment: standards of medical care in diabetes—2020. *Diabetes Care*. 2020;43(Supplement 1):S98–110. 10.2337/dc20-S009.
4. Knowler WC, Barrett-Connor E, Fowler SE, Hamman RF, Lachin JM, Walker EA, and Nathan DM. Diabetes prevention program research group. Reduction in the incidence of type 2 diabetes with lifestyle intervention or metformin. *N Engl J Med*. 2002;346:393–403. doi:10.1056/NEJMoa012512.
5. Ramachandran A, Snehalatha C, Mary S, Mukesh B, Bhaskar AD, Vijay V, Indian Diabetes Prevention Programme (IDPP). The Indian diabetes prevention programme shows that lifestyle modification and metformin prevent type 2 diabetes in Asian Indian subjects with impaired glucose tolerance (IDPP-1). *Diabetologia*. 2006;49(2):289–297. 10.1007/s00125-005-0097-z
6. Gong Q, Gregg EW, Wang J, An Y, Zhang P, Yang W, Li H, Li H, Jiang Y, Shuai Y, et al. Long-term effects of a randomised trial of a 6-year lifestyle intervention in impaired glucose tolerance on diabetes-related microvascular complications: the China Da Qing diabetes prevention outcome study. *Diabetologia*. 2011;54(2):300–307. 10.1007/s00125-010-1948-9.
7. Tuomilehto J, Lindström J, Eriksson JG, Valle TT, Hämäläinen H, Ilanne-Parikka P, Keinänen-Kiukaanniemi S, Laakso M, Louheranta A, Rastas M, et al. Prevention of type 2 diabetes mellitus by changes in lifestyle among subjects with impaired glucose tolerance. *N Engl J Med*. 2001;344(18):1343–1350. 10.1056/NEJM200105033441801.
8. Umpierre D, Ribeiro PAB, Kramer CK, Leitão CB, Zucatti ATN, Azevedo MJ, Gross JL, Ribeiro JP, Schaan BD. Physical activity advice only or structured exercise training and association with HbA1c levels in Type 2 diabetes. *JAMA*. 2011;305(17):1790–1799. doi:10.1001/jama.2011.576.

9. Johansen MY, MacDonald CS, Hansen KB, Karstoft K, Christensen R, Pedersen M, Hansen LS, Zacho M, Wedell-Neergaard A-S, Nielsen ST, et al. Effect of an intensive lifestyle intervention on glycemic control in patients with Type 2 diabetes: a randomized clinical trial. *JAMA*. 2017;318(7):637–646. doi:10.1001/jama.2017.10169.
10. Forslund K, Hildebrand F, Nielsen T, Falony G, Le Chatelier E, Sunagawa S, Prifti E, Vieira-Silva S, Gudmundsdottir V, Krogh Pedersen H, et al. Disentangling type 2 diabetes and metformin treatment signatures in the human gut microbiota. *Nature*. 2015;528(7581):262–266. doi:10.1038/nature15766.
11. de La Cuesta-zuluaga J, Mueller NT, Corrales-Agudelo V, Velásquez-Mejía EP, Carmona JA, Abad JM, Escobar JS. Metformin is associated with higher relative abundance of mucin-degrading *Akkermansia muciniphila* and several short-chain fatty acid-producing microbiota in the gut. *Diabetes Care*. 2017;40(1):54–62. doi:10.2337/dc16-1324.
12. Wu H, Esteve E, Tremaroli V, Khan MT, Caesar R, Mannerås-Holm L, Ståhlman M, Olsson LM, Serino M, Planas-Fèlix M, et al. Metformin alters the gut microbiome of individuals with treatment-naive type 2 diabetes, contributing to the therapeutic effects of the drug. *Nat Med*. 2017;23(7):850–858. doi:10.1038/nm.4345.
13. Sun L, Xie C, Wang G, Wu Y, Wu Q, Wang X, Liu J, Deng Y, Xia J, Chen B, et al. Gut microbiota and intestinal FXR mediate the clinical benefits of metformin. *Nat Med*. 2018;24(12):1919–1929. doi:10.1038/s41591-018-0222-4.
14. Graf D, Di Cagno R, Fåk F, Flint HJ, Nyman M, Saarela M, Watzl B. Contribution of diet to the composition of the human gut microbiota. *Microb Ecol Health Dis*. 2015;26:26164. doi:10.3402/mehd.v26.26164.
15. Sigal RJ, Kenny GP, Wasserman DH, Castaneda-Sceppa C. Physical activity/exercise and type 2 diabetes. *Diabetes Care*. 2004;27(10):2518–2539. doi:10.2337/diacare.27.10.2518.
16. Lang Lehrs kov L, Lyngbaek MP, Soederlund L, Legaard GE, Ehses JA, Heywood SE, Wewer Albrechtsen NJ, Holst JJ, Karstoft K, Pedersen BK, et al. Interleukin-6 delays gastric emptying in humans with direct effects on glycemic control. *Cell Metab*. 2018;27(6):1201–1211.e3. doi:10.1016/j.cmet.2018.04.008.
17. Cerdá B, Pérez M, Pérez-Santiago JD, Tornero-Aguilera JF, González-Soltero R, Larrosa M. Gut microbiota modification: another piece in the puzzle of the benefits of physical exercise in health? *Front Physiol*. 2016;7:51. doi:10.3389/fphys.2016.00051.
18. Barton W, Penney NC, Cronin O, Garcia-Perez I, Molloy MG, Holmes E, Shanahan F, Cotter PD, O'Sullivan O. The microbiome of professional athletes differs from that of more sedentary subjects in composition and particularly at the functional metabolic level. *Gut*. 2018;67(4):625–633. doi:10.1136/gutjnl-2016-313627.
19. Clarke SF, Murphy EF, O'Sullivan O, Lucey AJ, Humphreys M, Hogan A, Hayes P, O'Reilly M, Jeffery IB, Wood-Martin R, et al. Exercise and associated dietary extremes impact on gut microbial diversity. *Gut*. 2014;63(12):1913–1920. doi:10.1136/gutjnl-2013-306541.
20. Scheiman J, Lubert JM, Chavkin TA, MacDonald T, Tung A, Pham L-D, Wibowo MC, Wurth RC, Punthambaker S, Tierney BT, et al. Meta-omics analysis of elite athletes identifies a performance-enhancing microbe that functions via lactate metabolism. *Nat Med*. 2019;25(7):1104–1109. doi:10.1038/s41591-019-0485-4.
21. Turpin-Nolan SM, Joyner MJ, Febbraio MA. Can microbes increase exercise performance in athletes? *Nat Rev Endocrinol*. 2019;15(11):629–630. doi:10.1038/s41574-019-0250-2.
22. Cronin O, Barton W, Skuse P, Penney NC, Garcia-Perez I, Murphy EF, Woods T, Nugent H, Fanning A, Melgar S, et al. A prospective metagenomic and metabolomic analysis of the impact of exercise and/or whey protein supplementation on the gut microbiome of sedentary adults. *mSystems*. 2018;3(3):1–17. doi:10.1128/mSystems.00044-18.
23. Kern T, Blond MB, Hansen TH, Rosenkilde M, Quist JS, Gram AS, Ekstrøm CT, Hansen T, Stallknecht B. Structured exercise alters the gut microbiota in humans with overweight and obesity-A randomized controlled trial. *Int J Obes (Lond)*. 2020;44(1):125–135. doi:10.1038/s41366-019-0440-y.
24. Taniguchi H, Tanisawa K, Sun X, Kubo T, Hoshino Y, Hosokawa M, Takeyama H, Higuchi M. Effects of short-term endurance exercise on gut microbiota in elderly men. *Physiol Rep*. 2018;6(23):e13935. doi:10.14814/phy2.13935.
25. Douglas GM, Maffei VJ, Zaneveld JR, Yurgel SN, Brown JR, Taylor CM, Huttenhower C, Langille MGI. PICRUSt2 for prediction of metagenome functions. *Nat Biotechnol*. 2020;38(6):685–688. doi:10.1038/s41587-020-0548-6.
26. MacDonald CS, Nielsen SM, Bjørner J, Johansen MY, Christensen R, Vaag A, Lieberman DE, Pedersen BK, Langberg H, Ried-Larsen M, et al. One-year intensive lifestyle intervention and improvements in health-related quality of life and mental health in persons with type 2 diabetes: a secondary analysis of the U-TURN randomized controlled trial. *BMJ Open Diabetes Res Care*. 2021;9(1):e001840. doi:10.1136/bmjdr-2020-001840.
27. MacDonald CS, Johansen MY, Nielsen SM, Christensen R, Hansen KB, Langberg H, Vaag AA, Karstoft K, Lieberman DE, Pedersen BK, et al. Dose-response effects of exercise on glucose-lowering

- medications for Type 2 diabetes: a secondary analysis of a randomized clinical trial. *Mayo Clin Proc.* 2020;95(3):488–503. doi:10.1016/j.mayocp.2019.09.005.
28. Ried-Larsen M, Johansen MY, MacDonald CS, Hansen KB, Christensen R, Wedell-Neergaard A-S, Pilmark NS, Langberg H, Vaag AA, Pedersen BK, et al. Type 2 diabetes remission 1 year after an intensive lifestyle intervention: a secondary analysis of a randomized clinical trial. *Diabetes Obes Metab.* 2019;21(10):2257–2266. doi:10.1111/dom.13802.
 29. Le Chatelier E, Nielsen T, Qin J, Prifti E, Hildebrand F, Falony G, Almeida M, Arumugam M, Batto J-M, Kennedy S, et al. Richness of human gut microbiome correlates with metabolic markers. *Nature.* 2013;500(7464):541–546. doi:10.1038/nature12506.
 30. Brahe LK, Le Chatelier E, Prifti E, Pons N, Kennedy S, Blædel T, Håkansson J, Dalsgaard TK, Hansen T, Pedersen O, et al. Dietary modulation of the gut microbiota—a randomised controlled trial in obese postmenopausal women. *Br J Nutr.* 2015;114(3):406–417. doi:10.1017/S0007114515001786.
 31. Yang J-Y, Lee Y-S, Kim Y, Lee S-H, Ryu S, Fukuda S, Hase K, Yang C-S, Lim HS, Kim M-S, et al. Gut commensal *Bacteroides acidifaciens* prevents obesity and improves insulin sensitivity in mice. *Mucosal Immunol.* 2017;10(1):104–116. doi:10.1038/mi.2016.42.
 32. Gauffin Cano P, Santacruz A, Á M, Sanz Y. *Bacteroides uniformis* CECT 7771 ameliorates metabolic and immunological dysfunction in mice with high-fat-diet induced obesity. *PLoS One.* 2012;7(7):e41079. doi:10.1371/journal.pone.0041079.
 33. Zhang X, Shen D, Fang Z, Jie Z, Qiu X, Zhang C, Chen Y, Ji L. Human gut microbiota changes reveal the progression of glucose intolerance. *PLoS One.* 2013;8(8):e71108. doi:10.1371/journal.pone.0071108.
 34. Lippert K, Kedenko L, Antonielli L, Kedenko I, Gemeier C, Leitner M, Kautzky-Willer A, Paulweber B, Hackl E. Gut microbiota dysbiosis associated with glucose metabolism disorders and the metabolic syndrome in older adults. *Benef Microbes.* 2017;8(4):545–556. doi:10.3920/BM2016.0184.
 35. Yamaguchi Y, Adachi K, Sugiyama T, Shimozato A, Ebi M, Ogasawara N, Funaki Y, Goto C, Sasaki M, Kasugai K. Association of intestinal microbiota with metabolic markers and dietary habits in patients with Type 2 diabetes. *Digestion.* 2016;94(2):66–72. doi:10.1159/000447690.
 36. Salamon D, Sroka-Oleksiak A, Kapusta P, Szopa M, Mrozińska S, Ludwig-Słomczyńska AH, Wołkow PP, Bulanda M, Klupa T, and Małecki MT, et al. Characteristics of gut microbiota in adult patients with type 1 and type 2 diabetes based on next-generation sequencing of the 16S rRNA gene fragment. *Polish Arch Intern Med.* 2018;128:336–343. doi:10.20452/pamw.4246.
 37. Gurung M, Li Z, You H, Rodrigues R, Jump DB, Morgun A, Shulzhenko N. Role of gut microbiota in type 2 diabetes pathophysiology. *EBioMedicine.* 2020;51:102590. doi:10.1016/j.ebiom.2019.11.051.
 38. Zhang Q, Hu N. Effects of metformin on the gut microbiota in obesity and Type 2 diabetes mellitus. *Diabetes Metab Syndr Obes.* 2020;13:5003–5014. doi:10.2147/DMSO.S286430.
 39. Meijnikman AS, Gerdes VE, Nieuwdorp M, Herrema H. Evaluating causality of gut microbiota in obesity and diabetes in humans. *Endocr Rev.* 2018;39(2):133–153. doi:10.1210/er.2017-00192.
 40. Magne F, Gotteland M, Gauthier L, Zazueta A, Pesoa S, Navarrete P, Balamurugan R. The firmicutes/bacteroidetes ratio: a relevant marker of gut dysbiosis in obese patients? *Nutrients.* 2020;12(5):1474. doi:10.3390/nu12051474.
 41. Turnbaugh PJ, Ley RE, Mahowald MA, Magrini V, Mardis ER, Gordon JL. An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature.* 2006;444(7122):1027–1031. doi:10.1038/nature05414.
 42. Ley RE, Bäckhed F, Turnbaugh P, Lozupone CA, Knight RD, Gordon JL. Obesity alters gut microbial ecology. *Proc Natl Acad Sci U S A.* 2005;102(31):11070–11075. doi:10.1073/pnas.0504978102.
 43. Ley RE, Turnbaugh PJ, Klein S, Gordon JL. Microbial ecology: human gut microbes associated with obesity. *Nature.* 2006;444(7122):1022–1023. doi:10.1038/4441022a.
 44. Sasaki M, Ogasawara N, Funaki Y, Mizuno M, Iida A, Goto C, Koikeda S, Kasugai K, Joh T. Transglucosidase improves the gut microbiota profile of type 2 diabetes mellitus patients: a randomized double-blind, placebo-controlled study. *BMC Gastroenterol.* 2013;13(1):81. doi:10.1186/1471-230X-13-81.
 45. Louis S, Tappu R-M, Damms-Machado A, Huson DH, Bischoff SC. Characterization of the gut microbial community of obese patients following a weight-loss intervention using whole metagenome shotgun sequencing. *PLoS One.* 2016;11(2):e0149564. doi:10.1371/journal.pone.0149564.
 46. Xia Y. Mediation analysis of microbiome data and detection of causality in microbiome studies [Internet]. 2021. 457–509. doi:10.1007/978-3-030-67951-4_16
 47. Ried-Larsen M, Christensen R, Hansen KB, Johansen MY, Pedersen M, Zacho M, Hansen LS, Kofoed K, Thomsen K, and Jensen MS, et al. Head-to-head comparison of intensive lifestyle intervention (U-TURN) versus conventional multifactorial care in patients with type 2 diabetes: protocol and rationale for an assessor-blinded, parallel group and randomised trial. *BMJ Open.* 2015;5(12):e009764 doi:10.1136/bmjopen-2015-009764.

48. Sarangi AN, Goel A, Aggarwal R. Methods for studying gut microbiota: a primer for physicians. *J Clin Exp Hepatol.* 2019;9(1):62–73. doi:10.1016/j.jceh.2018.04.016.
49. Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Lozupone CA, Turnbaugh PJ, Fierer N, Knight R. Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *Proc Natl Acad Sci U S A.* 2011;108(Supplement_1):4516–4522. doi:10.1073/pnas.1000080107.
50. Takai K, Horikoshi K. Rapid detection and quantification of members of the archaeal community by quantitative PCR using fluorogenic probes. *Appl Environ Microbiol.* 2000;66(11):5066–5072. doi:10.1128/AEM.66.11.5066-5072.2000.
51. Klindworth A, Pruesse E, Schweer T, Peplies J, Quast C, Horn M, Glöckner FO. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids Res.* 2013;41(1):e1. doi:10.1093/nar/gks808.
52. Martin M. Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet.journal.* 2011;17(1):10. doi:10.14806/ej.17.1.200.
53. Bolyen E, Rideout JR, Dillon MR, Bokulich NA, Abnet CC, Al-Ghalith GA, Alexander H, Alm EJ, Arumugam M, Asnicar F, et al. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nat Biotechnol.* 2019;37(8):852–857. doi:10.1038/s41587-019-0209-9.
54. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP. DADA2: high-resolution sample inference from Illumina amplicon data. *Nat Methods.* 2016;13(7):581–583. doi:10.1038/nmeth.3869.
55. Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, Peplies J, Glöckner FO. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res.* 2013;41(D1):D590–6. doi:10.1093/nar/gks1219.
56. McMurdie PJ, Holmes S. phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS One.* 2013;8(4):e61217. doi:10.1371/journal.pone.0061217.
57. Kuznetsova A, Brockhoff PB, and Christensen RHB. lmerTest package: tests in linear mixed effects models. *J Stat Softw.* 2017;82(13). doi:10.18637/jss.v082.i13.
58. Anderson MJ. A new method for non-parametric multivariate analysis of variance. *Austral Ecol.* 2001;26:32–46. doi:10.1111/j.1442-9993.2001.01070.pp.x.
59. Lozupone C, Knight R. UniFrac: a new phylogenetic method for comparing microbial communities. *Appl Environ Microbiol.* 2005;71(12):8228–8235. doi:10.1128/AEM.71.12.8228-8235.2005.
60. Oksanen J, Kindt R, Legendre P, O’Hara B, Stevens MHH, Oksanen MJ, Suggests M. The vegan package. *Community Ecol Packag.* 2007;10:719.
61. Bairoch A. The ENZYME database in 2000. *Nucleic Acids Res.* 2000;28(1):304–305. doi:10.1093/nar/28.1.304.
62. Kanehisa M, Sato Y, Kawashima M, Furumichi M, Tanabe M. KEGG as a reference resource for gene and protein annotation. *Nucleic Acids Res.* 2016;44(D1):D457–62. doi:10.1093/nar/gkv1070.
63. Caspi R, Billington R, Keseler IM, Kothari A, Krummenacker M, Midford PE, Ong WK, Paley S, Subhraveti P, Karp PD. The MetaCyc database of metabolic pathways and enzymes - a 2019 update. *Nucleic Acids Res.* 2020;48(D1):D445–53. doi:10.1093/nar/gkz862.
64. Zhang J, Wei Z, Chen J. A distance-based approach for testing the mediation effect of the human microbiome. *Bioinformatics.* 2018;34(11):1875–1883. doi:10.1093/bioinformatics/bty014.
65. Wang C, Hu J, Blaser MJ, Li H. Estimating and testing the microbial causal mediation effect with high-dimensional and compositional microbiome data. *Bioinformatics.* 2020;36(2):347–355. doi:10.1093/bioinformatics/btz565.
66. Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J R Stat Soc Ser B.* 1995;57:289–300. doi:10.1111/j.2517-6161.1995.tb02031.x.
67. Villanueva RAM, Chen ZJ. ggplot2: elegant graphics for data analysis (2nd ed.). *Meas Interdiscip Res Perspect.* 2019;17(3):160–167. doi:10.1080/15366367.2019.1565254.