nature portfolio

Peer Review File

The gut methanotroph Methylocystis intestini modulates intestinal peristalsis and fat metabolism via reducing methane levels

Corresponding Author: Professor Zheng Yu

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Thank you for the oppertunity to review this paper. I congratulate the authors for such a novel study.
The isolation and characterization of Candidatus Methylocystis intestini, a novel human-associated methanotroph, is both novel and biologically significant.It fills a major knowledge gap: while methanogens have been widely studied, methanotrophs in the gut have not. The authors convincingly show that methane impairs intestinal motility and fat metabolism in mice and that this is reversed by methanotroph administration. Multi-cohort validation showing reduced methanotroph abundance in constipated and obese individuals strengthens the clinical relevance.

I have a few comments:
A key missing element: actual methane concentrations in feces, breath, or intestinal lumen are not measured. This is essential to prove that methanotrophs are effectively lowering methane levels in vivo.

Association between methanotroph gene abundance and clinical phenotype (constipation, obesity) is correlative. This should be clearly stated to avoid overinterpretation. Reality is that correlation of methanogenesis with bowel habit changes is well established but the association with obesity has not been strong.

While gavage experiments suggest colonization, no quantitative data (e.g. CFU/g feces or FISH/ISH quantification post-gavage) are shown to demonstrate sustained presence over time

Reviewer #2

(Remarks to the Author)
As a microbial physiologist, I read the study by Zhao et al with excitement that methanotrophic bacteria are found in human fecal metagenomic data and can be isolated from fecal material, and that the addition of methanotrophs can decrease CH4-associated phenotypes (constipation) in murine models. Methanotrophs can be found wherever their specialist carbon source is available, so it is not surprising that they are found in the mammalian gut where methanogenesis is known to occur. The authors isolate what appears to be a novel Methylocystis species, an alphaproteobacterial methanotroph genus commonly found in soil and readily isolated following well-established methanotroph isolation protocols. This is an impactful contribution should methanotrophs be involved in human health and disease. I have a few comments about the isolation and isolate.
• The authors seemed to have followed the standard protocol of methanotroph isolation at 30C, which I found curious given that gut methanotrophs would be expected to be at 37C consistent with the host.
• Several details related to the isolation are lacking. Were different colony morphologies observed? How were colonies chosen for passage? How many passages were required to achieve an axenic culture? These are relevant given that several different types of methanotrophs were enriched from the fecal material (Fig. 1B).

The Methylocystis isolate genome suggested it may have metabolic capabilities not typical of other methanotrophs (e.g. sulfiredoxin and phosphoadenosine phosphosulphate reductase), which the authors suggest may be linked to adaptation to

the gut. Does the strain express these enzymes and use these substrates?
• The unique genomic potential of this isolate should be validated via growth experiments or biochemically using model methanotroph controls.

Previous studies have correlated elevated gut CH4 to slow intestinal peristalsis, but the underlying mechanisms are not understood. CH4 is an inert gas, so one would hypothesize that its physiological effects are physical (pressure) or indirect via its metabolism. Following this logic, one could further hypothesize that increased gut CH4 may increase gut methanotrophs, which would be a positive correlation between methanotrophs and constipation. The authors conduct a series of murine experiments wherein they introduced CH4 with or without the Methylocystis isolate to mice via gavage. They present compelling data that the addition of CH4 increases constipation and that including the Methylocystis isolate relieves the phenotypes. However, several questions remain, and, if addressed, would make the correlative phenotypes more compelling.
• How much does the CH4 concentration increase in the experimental system?
• Does the Methylocystis introduced use the CH4? The authors conclude their “findings suggest that gut methanotrophs regulate fat metabolism by reducing methane levels” (line 156-157), but there is no data to support the bacteria are assimilating CH4/this conclusion.
• What is the effect of increased CH4 on the gut microbiome or intestinal epithelia? Are methanotrophs enriched?
• What is the effect of introduction of the Methylocystis isolate on the gut microbiome or intestinal epithelia? Could the Methylocystis isolate be competing with a CH4-enriched microbe or dysbiosis that disrupts normal gut peristalsis to improve constipation outcomes?
• Prior studies (Jensen et al., Nat. Comm, 2021) have shown that methanotroph, Methylococcus capsulatus, cell lysates have anti-inflammatory activity. Does the Methylocystis inoculated here need to be viable to observe the phenotypes? Perhaps the authors should heat-kill or lyse cells to test. Again, the methanotroph-associated phenotypes presented here may not be related to CH4 consumption.

The study analyzed a series of prior metagenomic sequencing data and shows that methanotrophs are commonly found in the gut, but at very low abundance. Thus, high sequence depth is required, and the limited depth of many studies could explain why they have not been previously identified. In 550 samples, ~60% had at least ten reads and ~27% > 50 reads correlated to the particulate methane monooxygenase genes unique to methanotrophs. Based off data presented in Fig. 4E, the methanotroph gut microbiome relative abundance is 10-5 (although I calculated a maximum of 10-7 using data in Fig. 4C), with minimal (although statistically significant) variations between healthy and obese or constipated individuals. At these low abundances, it is hard to envision that minor variances in methanotroph population could be responsible for the observed disease phenotypes. The rate of methane consumption by this amount of methanotrophs would be quite low. The authors should address and discuss these limitations. Nonetheless, there are questions that remain that are likely embedded in the data.
• What is the phylogenetic distribution of the identified metagenomic MMO sequences? Are similar genera identified in the murine model isolation found in humans? Is Methylocystis commonly found, and is there any additional correlation to specific genera and disease (although I am not sure confident correlations can be made with such low sequence abundance)?

This study contains meritorious evidence to support that the addition of the Methylocystis can lessen constipation phenotypes associated with increased CH4 in a murine model. The data also supports that methanotrophs are in the human gut, albeit at very low abundance. The study and significance of the findings could be improved if alternative hypothesis were investigated. In particular, the effects of CH4 and/or the addition of Methylocystis (or other gut methanotrophs) on the gut microbiome and/or epithelium to provide mechanistic insight into the presented correlations.

Reviewer #3

(Remarks to the Author)
Key results
Zhao et al. newly isolated methane-oxidizing bacteria (MOB) from human fecal samples and investigated their impact on host physiology. Whole-genome sequencing of the isolated strain revealed a series of genes predicted to encode methane-metabolizing enzymes. The authors also performed oral administration of the isolated MOB to mice and evaluated its effects in the context of methane-induced impairment of intestinal peristalsis and increased fat deposition. Their results demonstrated that MOB administration improved intestinal motility and reduced fat accumulation.

Validity
It is commendable that the authors successfully isolated a novel strain of potential methane-oxidizing bacteria and performed gene prediction related to methane metabolism. However, although the authors conclude that the strain possesses methane-oxidizing capability, this claim is not fully substantiated, as methane-derived metabolites such as CH_3OH were not directly measured in this study. Therefore, some statements regarding the metabolic activity appear to be overstated.
Furthermore, in the animal experiments, the current data demonstrate correlations but do not provide direct evidence of causality between methane oxidation by MOB and the observed effects on intestinal motility or fat deposition. While the authors designed their study based on the premise that methane treatment increases lipid accumulation in adipose tissue, there are no or few previous reports supporting this phenomenon could be identified. Therefore, it is important for the authors to provide a more thorough explanation and justification for this observation.

Significance

The authors newly isolated a bacterial strain harboring genes predicted to encode methane-oxidizing enzymes. Methane is typically produced by certain archaea species and is generally considered to negatively impact host physiology. The authors propose a novel hypothesis that gut-associated methane-oxidizing bacteria may mitigate these effects by reducing methane levels through oxidation. While this concept is novel, the current version of the manuscript does not provide direct evidence of methane oxidation or its causal impact on host physiological functions.

Data and methodology and Clarity and context
Certain aspects of the experimental procedures and data presentation lack clarity and transparency. These areas should be addressed and improved to enhance the overall rigor and reproducibility of the study. These points were also described in the following Suggested improvement.

Suggested improvements
In line 88, the authors state that they used NMS1 medium to culture methane-oxidizing bacteria. However, the abbreviation "NMS1" is undefined. For clarity and reproducibility, the full name and composition of the NMS1 medium should be provided. Also, it could be helpful to reader to show the actual name and the composition.
In Figures S4, 1, 2, and 3, the authors claim that Methylocystis intestini exhibits methane-metabolizing capability. However, this claim appears to be based solely on gene annotation. To substantiate this claim, direct detection of methane-derived metabolites such as methanol (CH_3OH) or formaldehyde (CH_2O) in vitro culture supernatants or in intestinal/fecal samples would be necessary. Without such metabolic validation, the assertion regarding methane oxidation remains speculative and should be stated more cautiously.
In line 104 and subsequent sections, the authors refer to the newly isolated strain as "Candidatus Methylocystis intestini". However, the designation "Candidatus" is typically reserved for uncultured or not-yet-cultivable organisms (e.g., Candidatus Arthromitus sp. SFB-mouse). Given that the strain appears to be cultured in vitro (authors should clarify this as well), it is recommended to omit the "Candidatus" designation and clearly describe the culturing conditions.
In Figure 1F, although the authors mentioned a type II methanotroph-specific intracytoplasmic membrane (ICM) structure, the presented image lacks sufficient resolution to clearly visualize these structures. It is advisable to replace the current image with a higher-resolution version and to include a comparative image of a type I methanotroph (which typically exhibits stacked disc-like ICMs) to aid the reader's understanding.
Regarding Figures 2 and 3, it remains unclear whether the observed host phenotypes are truly driven by methane oxidation mediated by M. intestini. To address this, generating a ΔpmoCAB mutant strain (if feasible) would provide direct evidence. If mutant generation is not possible, a comparison with a closely related non-methanotrophic strain would help to the authors' claim.
In Figures 2A and 3A, the authors should clearly explain the experimental procedures used to administer methane and bacteria to the mice—whether by oral gavage, inhalation, or another route—in the main text.
In Figure S7, the body weights of mice (~40 g) appear to be higher than typical values for standard inbred strains like C57BL/6. This may be due to the use of an outbred or larger strain, which should be specified. Additionally, the authors should investigate whether M. intestini can similarly influence methane-induced adiposity and gene expression in a widely used inbred strain such as C57BL/6. The age of the mice at the start of the experiment should also be stated clearly.
In line 146, the authors report that M. intestini reduced methane-induced blood LDL levels; however, Figure 3C does not show statistical significance.
In Figure 3D, it is unclear which specific adipose tissue was analyzed and at what time point the gene expression analysis was performed. These details should be clearly stated. If the gene expression was assessed at the end of the experiment, the observed transcriptional profiles likely reflect the outcome of the physiological changes, rather than the mechanism underlying them. To better understand the mechanism of action, it would be informative to examine gene expression at earlier time points, such as week 1.
In Figure 3E, the authors report that methane treatment upregulated the expression of Fabp1, Cd31 and Apoe4. It is unclear whether these findings have been previously reported or are novel to this study. The authors should clarify this point and discuss the potential mechanisms by which methane may induce the expression of these genes.
Regarding Figure 3E, the authors should discuss a mechanism by which methanotrophs suppress methane-induced transcriptional changes. It may be on reducing methane but at least, it would be mentioned on what is the upregulated genes (colored in red in the lower pannel) in CH4+MOB vs CH4. It might be also useful to explain the venn's diagram and/or see the PCA-plot including all groups (control, CH4 and CH4+MOB) together. Particularly, in Figure3F, the meaning of the numbers "1", "1", and "3" in the Venn diagram should be explained in the main text and figure legend.
In line 156, the statement "These findings suggest that gut methanotrophs regulate fat metabolism by reducing methane levels" may be an overstatement, especially given the absence of direct methane measurements and the lack of experiments using a ΔpmoCAB strain. The conclusion should be more cautiously worded.
In line 163, the authors mention the gene cluster mmoXYZBCD, but do not describe its function. A brief explanation of its role in methane oxidation and its specific step in the pathway should be added, ideally alongside Figure S4.
Another point, the methane-oxidizing reaction requires oxygen; therefore, the source of oxygen should be discussed. According to Figure 2B, M. intestini primarily colonizes the lumen of the small intestine. One possibility is that M. intestini utilizes trace amounts of residual oxygen that are present in the small intestinal environment. Another possibility is that the bacteria obtain oxygen from host tissues, such as from epithelial cells.

As for Method section,
Line 374: "0.2 ml of methane-containing bubble water and 0.2 ml of methane gas were injected into animals."
→ The method of methane administration is unclear. Did the authors apply these two forms simultaneously? Clarification of the route (e.g., oral, rectal) and timing is necessary.
Line 375: "For gut methanotroph intervention, gavage with 0.5 ml Candidatus M. intestini (OD600 > 1, washed with PBS) was applied per mouse."

→ Please specify the frequency and duration of the bacterial administration (e.g., daily for how many days?).
Line 377: “For diet-intervention, the high-level fat was provided with 0.4 ml lard by gavage per mouse.”
→ This implies that the high-fat intervention was performed via lard gavage rather than a standardized high-fat diet. This point should be explicitly clarified in both the methods and the figure legends to ensure reproducibility and transparency.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Thank you for addressing my comments.

Reviewer #2

(Remarks to the Author)
The authors have included several additional data that significantly improve the manuscript by filling knowledge gaps identified in the prior version. The R1 manuscript now successfully links M. intestini active CH4 consumption to associated phenotypes. I have one major comment and a few minor points for the authors’ consideration:
Major comment:
The CH4-induced microbiome dysbiosis is striking. The data is presented as alpha- and beta-diversity to highlight the phenotype. However, perhaps additional analysis that show the altered bacterial phyla/other taxonomic groups could be included in the supplement to provide insight into the CH4-induced effects. What is the authors’ hypothesis for CH4-induced dysbiosis? This is an important finding of the study and, thus, the discussion related thereto should be expanded.
Minor comments:
• Highlighted text should be grammatically edited throughout.
• line 88. The isolation results should be placed in context. I recommend starting the paragraph with “To isolate methanotrophs from the human intestine, we inoculated NMS1 medium with human fecal material” or something similar to contextualize followed by the “Growth of bacteria…” sentence.
• line 112 “with methane consumption capability” is unclear and should be rephrased or removed.
• line 119 the timepoint should be added to “the intestine within X after gavage”.
• line 124-125, ensure the figure designations correspond correctly and that all figure panels are referenced in the text throughout (Fig. 2F, 2G, and 2I are missing in the text).
• lines 145-147 should be moved after the sentence starting with “These effects were reversed by…”

Reviewer #3

(Remarks to the Author)
The authors have provided a thorough revision that addresses the large majority of my previous concerns. In cases where they were unable to provide compelling new mechanistic insights, they have appropriately tempered their conclusions. The manuscript has improved considerably, and I have no further comments.

Response to reviews

Reviewer 1#

Comment:

Thank you for the opportunity to review this paper. I congratulate the authors for such a novel study. The isolation and characterization of Candidatus *Methylocystis intestini*, a novel human-associated methanotroph, is both novel and biologically significant. It fills a major knowledge gap: while methanogens have been widely studied, methanotrophs in the gut have not. The authors convincingly show that methane impairs intestinal motility and fat metabolism in mice and that this is reversed by methanotroph administration. Multi-cohort validation showing reduced methanotroph abundance in constipated and obese individuals strengthens the clinical relevance.

Response:

Thank you very much for your hard work and valuable comments on our work. We appreciate the reviewer's comprehensive evaluation and the opportunity to revise our study. We will reply to your questions point by point in the following answers and modify the manuscript.

Comment:

A key missing element: actual methane concentrations in feces, breath, or intestinal lumen are not measured. This is essential to prove that methanotrophs are effectively lowering methane levels in vivo.

Response:

We appreciate the reviewer's insightful comment. We fully agree that direct quantification of methane would provide the most definitive evidence that methanotrophs are actively lowering methane levels in vivo.

In response, we have now included additional data on methane concentrations. We measured methane concentrations in exhaled breath of mice. Our results demonstrate that animals colonized with methanotrophic bacteria exhibited significantly lower methane levels compared with CH4 group (Figure S10, copy below):

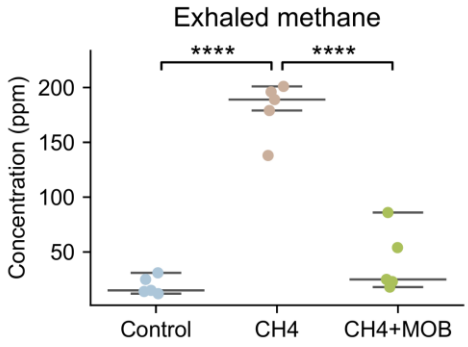


Figure S10. Exhaled methane concentrations of mice in different groups. ****: P-value < 0.0001; Mann-Whitney-Wilcoxon test, two-tailed. Benjamini/Hochberg correction.

This provides direct in vivo evidence that methanotrophs actively reduce methane availability within the host. To further support this observation, we conducted headspace gas analysis in bacterial cultures. Methanotrophic isolates showed a marked reduction in methane concentration (Figure S8, copy below), confirming their methane-oxidizing capacity:

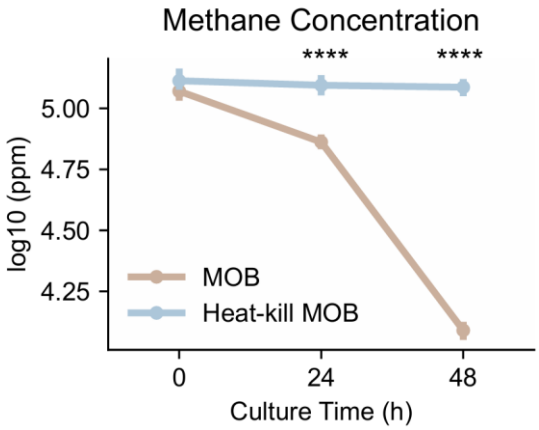


Figure S8. Methane concentration in headspace gas of the cultured bottle with NMS1 medium. MOB: *M. intestini*. ****: P-value < 0.0001; Mann-Whitney-Wilcoxon test, two-tailed.

Together, these results provide both in vivo and in vitro evidence that methanotrophs effectively lower methane levels. We have added these data and their interpretation to the revised Results sections:

Line 111, "In addition, we found *M. intestini* grew better at 37°C than at 30°C (Figure S7) and with methane consumption capability (Figure S8)." has been added.

Line 120, “Exhaled gas analysis after one week of intervention demonstrated that oral methane administration significantly increased the methane concentration in mice, whereas colonization with *M. intestini* markedly reduced the elevated methane levels (Figure S10).” has been added.

Comment:

Association between methanotroph gene abundance and clinical phenotype (constipation, obesity) is correlative. This should be clearly stated to avoid overinterpretation. Reality is that correlation of methanogenesis with bowel habit changes is well established but the association with obesity has not been strong.

Response:

We appreciate the reviewer’s valuable comment and fully agree that the associations reported in our study are correlative. To avoid any overinterpretation, we have revised the text throughout the Results to explicitly state that methanotroph abundance correlates with clinical phenotypes rather than implying causation:

Line 116, “Methane has been reported to slow intestinal peristalsis” has been changed to “Methane has been reported to be related to slow intestinal peristalsis”

Line 138, “These findings suggest that gut methanotrophs can counteract the inhibitory effects of methane on intestinal motility. Methane is known to disrupt smooth muscle function and interfere with normal peristaltic activity, and our results provide direct evidence that reducing methane concentrations through methanotroph intervention can restore gastrointestinal function. This offers a potential therapeutic strategy for methane-associated constipation, distinct from existing treatments.” has been removed.

Line 139, “Regulation of Fat Metabolism by Gut Methanotrophs” has been changed to “Association of Gut Methanotrophs with Fat Metabolism”.

Line 167, “These findings suggest that gut methanotrophs regulate fat metabolism by reducing methane levels. The relationship between the gut microbiota and metabolic diseases, particularly obesity, has been well established, and our results indicate that methanotrophs may play a key role in restoring metabolic balance by mitigating the effects of excessive methane.” has been removed.

In addition, we have clarified in the revised Discussion that the evidence regarding obesity is more limited. We have now added references to highlight this distinction and to ensure a balanced interpretation:

Line 200, “This study provides exploratory evidence that active gut methanotrophs can modulate CH_4-associated physiological readouts.” has been added.

Line 217, “Some studies have linked slower intestinal motility to obesity[15,16]. Excessive methane production in the gut has also been associated with metabolic disturbances and overweight [8,9]. At present, it remains unclear whether the methane-induced intestinal motility links to the metabolic disturbances, or whether methane exerts a direct effect. Our data show that methane exposure is associated with alterations in fat metabolism, which was similar with previous study[17]. These changes were reversed upon the intervention of *M. intestini.* Although there are no reports on the direct mechanism of methane regulation of fat metabolism, our study found that after methane excess, genes related to the fat digestion and absorption pathway in the intestinal tissue were upregulated, including *Fabp1*[11], *Cd36*[18], *Apoa4*[19] and *Clps*[20]. These genes have established roles in enterocyte lipid uptake, intracellular trafficking and lipoprotein assembly, and are regulated in part by PPAR-family nuclear receptors. Moreover, after intestinal colonization with *M. intestini*, methane levels in the body decreased and were accompanied by the downregulation of these genes. This reversal effect, to a certain extent, further emphasizes the potential function of methane. Here, *M. intestini* colonization also increased expression of *Pla2g3*, an enzyme capable of generating lysophospholipid signaling molecules that may influence intracellular trafficking responses[21,22]. We speculate that when fat absorption related pathways are perturbed, the intestinal epithelium upregulates *Pla2g3* to hydrolyze membrane phospholipids, thereby releasing fatty acids or lysophospholipids as a means to maintain intracellular lipid supply or remodel membrane components in the short term.

In addition, similar reversal effect after *M. intestini* intervention was also reflected in the gut microbial community structure in the lower part of the intestine. Gut microbiota dysbiosis has been linked to metabolic disorders[23], however, it is

unclear whether this methane-induced dysbiosis is an indirect or direct result of methane-induced constipation. ” has been added.

Comment:

While gavage experiments suggest colonization, no quantitative data (e.g. CFU/g feces or FISH/ISH quantification post-gavage) are shown to demonstrate sustained presence over time

Response: We thank the reviewer for this constructive suggestion. In the revised manuscript, we have now included quantitative data to demonstrate the persistence of methanotrophic bacteria following gavage. Specifically, we performed qPCR targeting the methanotroph-specific 16S rRNA gene sequence in fecal samples from the CH_4+MOB group and control group. Absolute abundances were quantified at day 1, day 3, and day 5 post-gavage.

Our results show that methanotroph abundance was significantly higher in the CH_4+MOB group compared to controls at all three time points, indicating successful colonization and sustained presence of the administered strains in the gut (Figure S9, copy below). And we added this result into the Result section:

Line 118,” Using metagenomic sequencing, we found the *Candidatus M. intestini* established a gradient distribution in the intestine after gavage (Figure 2B)” has been changed to “Using metagenomic sequencing and RT-qPCR, we found the *M. intestini* can successfully colonize in the intestine after gavage (Figure 2B and Figure S9).”

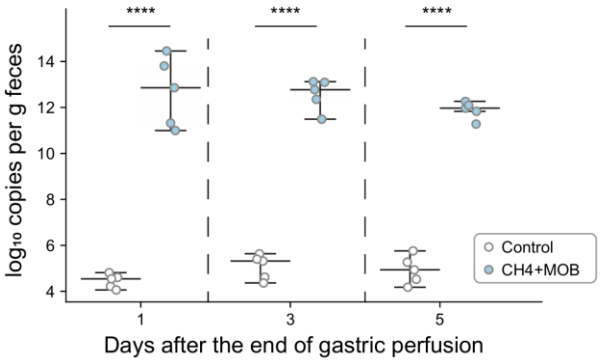


Figure S9. RT-PCR of the abundance of *M. intestini* in feces of mice from different groups. ****: P-value < 0.0001; Mann-Whitney-Wilcoxon test, two-tailed. Benjamini/Hochberg correction.

Reviewer 2#

Comment:

As a microbial physiologist, I read the study by Zhao et al with excitement that methanotrophic bacteria are found in human fecal metagenomic data and can be isolated from fecal material, and that the addition of methanotrophs can decrease CH4-associated phenotypes (constipation) in murine models. Methanotrophs can be found wherever their specialist carbon source is available, so it is not surprising that they are found in the mammalian gut where methanogenesis is known to occur. The authors isolate what appears to be a novel Methylocystis species, an alphaproteobacterial methanotroph genus commonly found in soil and readily isolated following well-established methanotroph isolation protocols. This is an impactful contribution should methanotrophs be involved in human health and disease. I have a few comments about the isolation and isolate.

Response:

We sincerely thank the reviewer for the thoughtful, encouraging, and constructive assessment of our work. We greatly appreciate the recognition of the potential impact of studying methanotrophs in the context of human health and disease, as well as the time and effort the reviewer has devoted to carefully evaluating our study. In the following responses, we will answer the reviewer's specific comments.

Comment:

The authors seemed to have followed the standard protocol of methanotroph isolation at 30C, which I found curious given that gut methanotrophs would be expected to be at 37C consistent with the host.

Response: We thank the reviewer for this insightful comment. Indeed, our initial isolation was performed at 30 °C following the standard protocol widely used for methanotrophs, as most previously described methanotroph species exhibit optimal growth around this temperature.

In response to the reviewer's suggestion, we conducted additional growth experiments comparing the performance of our intestinal methanotroph strain (*M. intestini*) at 30 °C and 37 °C. The results revealed that the strain exhibited faster entry

into the logarithmic phase and overall better growth at 37 °C compared with 30 °C (Figure S7):

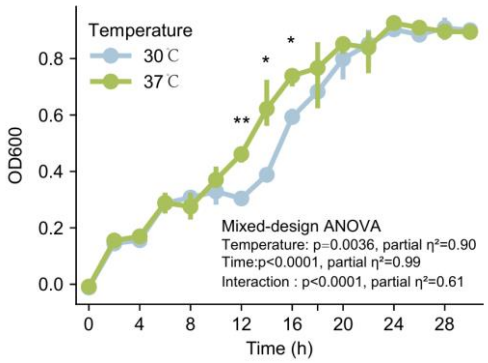


Figure S7. The growth curve of *M. intestini* at 30 and 37 degrees Celsius. The mixed-design ANOVA test is used.

This finding not only supports the reviewer's expectation but also provides indirect evidence that the intestinal methanotroph is well adapted to the host gut environment.

We have included these new data in the Result to highlight the relevance of temperature adaptation to gut colonization:

Line 111, "In addition, we found *M. intestini* grew better at 37°C than at 30°C (Figure S7) and with methane consumption capability (Figure S8)." has been added.

Comment:

Several details related to the isolation are lacking. Were different colony morphologies observed? How were colonies chosen for passage? How many passages were required to achieve an axenic culture? These are relevant given that several different types of methanotrophs were enriched from the fecal material (Fig. 1B).

Response:

We appreciate the reviewer's insightful comment and provide more detailed information regarding the isolation process. Because the abundance of methanotrophs in the gut is relatively low, we first performed liquid enrichment for approximately one month in NMS1 medium with methane as the sole carbon source. During this period, the culture was transferred about five times to gradually eliminate non-methanotrophic microorganisms and enrich for intestinal methanotrophs. The

enriched culture was then streaked onto NMS1 agar plates.

At this stage, we observed colonies with different morphologies, including methanotrophs and other methylotrophs such as *Methylorubrum rhodesianum* (pink colonies that can grow on methanol but not on methane). These organisms were initially difficult to separate. To ensure successful isolation, nearly all colonies were tested by re-inoculation into NMS1 liquid medium under methane. Only true methanotrophs could be further enriched under these conditions. After approximately 15–20 rounds of streaking and enrichment, we obtained a stable axenic culture. Importantly, all methanotroph colonies shared a uniform morphology, appearing as small white pinpoint colonies on NMS1 agar plates.

While Fig. 1B shows the genus-level community composition of the enrichment culture, further analysis (Fig. S1) revealed that only a single methanotroph strain containing the complete *pmo* gene cluster was present, which corresponds to the strain we isolated (Figure S2, ANI>99%, copy below) and designated as *Methylocystis intestini*.

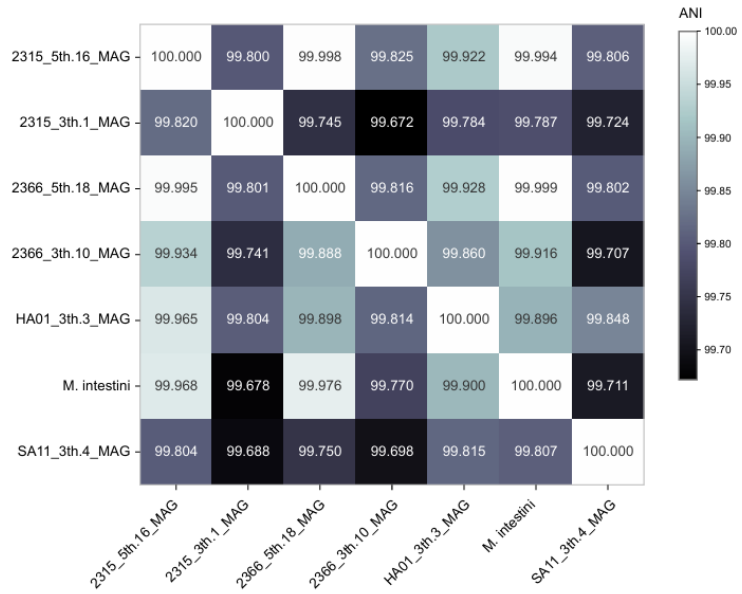


Figure S2. The average nucleotide identity (ANI) between the *M. intesini* and MAGs with methane oxidizing genes.

We have additional included more methodological details in the revised Methods section:

Line 302, “After five subsequent serial dilutions, bacteria were plated onto

solid NMS1 medium containing 1% Agarose (Sangon Biotech Co., Ltd.). The plates were incubated at 30 °C in an atmosphere containing 25% methane in gas-tight cylinders. Single colonies were restreaked repeatedly. First, strain purity was confirmed using phase-contrast microscopic techniques and plating onto complex LB medium (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl) that served as a negative control [17]. Second, the purity was confirmed by sequencing the 16S rRNA gene fragment of the isolate amplified by using 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') bacterial universal primers and the Sanger technology. Only one sequence was recovered." has been changed to "During this period, the culture was transferred about five times (approximately every 5-7 days by inoculating fresh NMS1 medium) to gradually eliminate non-methanotrophic microbes and enrich for intestinal methanotrophs.

After five subsequent serial dilutions in liquid medium, the enriched culture was then streaked onto NMS1 plates with 1% Agar (Sangon Biotech Co., Ltd.) which incubated at 30 °C in an atmosphere containing 25% methane in gas-tight cylinders for isolation of individual colonies. At this stage, we observed colonies with different morphologies, including potential methanotrophs and other methylotrophs such as *Methylorubrum rhodesianum* (*M. rhodesianum* which formed pink colonies capable of growing on methanol but not on methane as the sole carbon source). These organisms were initially difficult to separate purely based on colony morphology. To ensure successful isolation of true methanotrophs, a rigorous purification protocol was followed. Specifically, nearly all distinct colony types were picked and tested by re-inoculation into fresh NMS1 liquid medium under a methane atmosphere. Growth was monitored over 1-2 weeks**.** Only true methanotrophs could be further enriched under these strict conditions. After confirming growth in liquid medium, cultures derived from methanotroph colonies were subsequently re-streaked onto fresh NMS1 agar plates to obtain single colonies. This cycle of liquid enrichment followed by solid-phase isolation was repeated. After approximately 20 rounds of streaking and enrichment, we obtained a stable axenic culture. Purity of the isolate was confirmed by phase-contrast microscopy, by the inability to grow on complex LB medium (10

g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl)[18] ,and by sequencing of the 16S rRNA gene using 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') bacterial universal primers and the Sanger technology, which yielded a single sequence. The final isolation shared a uniform morphology, appearing as small, white, pinpoint colonies on NMS1 agar plates after extended incubation."

Comment:

The Methylocystis isolate genome suggested it may have metabolic capabilities not typical of other methanotrophs (e.g. sulfiredoxin and phosphoadenosine phosphosulphate reductase), which the authors suggest may be linked to adaptation to the gut. Does the strain express these enzymes and use these substrates?

Response:

We sincerely thank the reviewer for this insightful comment. As the reviewer correctly points out, our statement regarding sulfiredoxin and phosphoadenosine phosphosulphate reductase was based solely on genomic prediction without direct functional validation. To avoid overinterpretation, we have removed these speculative statements from the revised manuscript. We believe this change improves the clarity and rigor of our work:

"And among them, 20 genes were located on the genomic island, indicating potential horizontal transfer. One of these genes encodes a sulfiredoxin protein, involved in oxidative stress response, which may be involved in adaptation to low oxygen environment. Other genes encode proteins related to phosphoadenosine phosphosulphate reductase involved in metabolism of 3'-phosphoadenosine-5'-phosphosulphate (PAPS). PAPS is a relatively abundant chemical in human intestine participating in sulphation of intestinal mucin" has been removed.

Line 109, "Compared with other *Methylocystis spp.*, more than 15% genes (573/3,776) in *Candidatus M. intestini* were unique." has been revised to "More than 15% genes (573/3,776) in *M. intestini* were less than 70% amino acid sequence identity compared with other *Methylocystis* spp. (Figure S5)."

Comment:

Previous studies have correlated elevated gut CH4 to slow intestinal peristalsis, but the underlying mechanisms are not understood. CH4 is an inert gas, so one would hypothesize that its physiological effects are physical (pressure) or indirect via its metabolism. Following this logic, one could further hypothesize that increased gut CH4 may increase gut methanotrophs, which would be a positive correlation between methanotrophs and constipation. The authors conduct a series of murine experiments wherein they introduced CH4 with or without the Methylocystis isolate to mice via gavage. They present compelling data that the addition of CH4 increases constipation and that including the Methylocystis isolate relieves the phenotypes. However, several questions remain, and, if addressed, would make the correlative phenotypes more compelling.

Response:

We sincerely thank the reviewer for this thoughtful and constructive summary of our study. We also fully acknowledge the reviewer's point that, despite these observations, additional evidence is needed. In the revised manuscript, we have carefully addressed the reviewer's concerns point by point.

Comment:

How much does the CH4 concentration increase in the experimental system?

Response:

We thank the reviewer for this valuable comment. To assess the increase of CH_4 in our experimental system, we measured CH_4 levels in the exhaled breath of mice following gavage. Compared with baseline (air control), CH_4 administration led to a significant elevation in exhaled CH_4 concentration, with an average increase of approximately 7 folds (Figure S10, copy below).

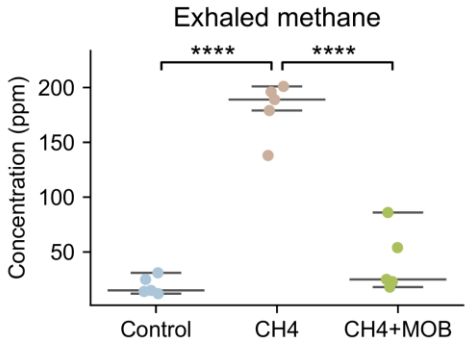


Figure S10. Exhaled methane concentrations of mice in different groups. ****: P-value < 0.0001; Mann-Whitney-Wilcoxon test, two-tailed. Benjamini/Hochberg correction.

We chose exhaled CH_4 as a readout because it is a widely accepted and noninvasive surrogate marker of intestinal CH_4 production and accumulation, as demonstrated in human breath tests. The increase observed in breath CH_4 therefore indicates that our intervention effectively elevated gut CH_4 levels in vivo. These data support the validity of our experimental model and have been included in the revised manuscript:

Line 120, "Exhaled gas analysis after one week of intervention demonstrated that oral methane administration significantly increased the methane concentration in mice, whereas colonization with *M. intestini* markedly reduced the elevated methane levels (Figure S10)." has been added.

Comment:

Does the Methylocystis introduced use the CH4? The authors conclude their "findings suggest that gut methanotrophs regulate fat metabolism by reducing methane levels" (line 156-157), but there is no data to support the bacteria are assimilating CH4/this conclusion.

Response:

We thank the reviewer for this important comment. To address this point, we have now included measurements of exhaled CH_4 concentration in mice after colonization with *Methylocystis intestini*. Compared with the CH_4 group, mice receiving both CH_4 and *M. intestini* exhibited significantly reduced breath CH_4 levels (Figure S10, copy

below), indicating that the introduced strain lowered CH_4 levels in vivo.

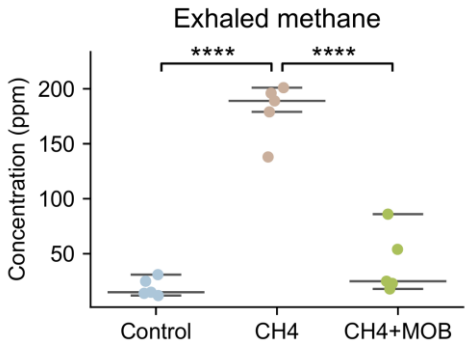


Figure S10. Exhaled methane concentrations of mice in different groups. ****: P-value < 0.0001; Mann-Whitney-Wilcoxon test, two-tailed. Benjamini/Hochberg correction.

Comment:

What is the effect of increased CH4 on the gut microbiome or intestinal epithelia? Are methanotrophs enriched? What is the effect of introduction of the Methylocystis isolate on the gut microbiome or intestinal epithelia? Could the Methylocystis isolate be competing with a CH4-enriched microbe or dysbiosis that disrupts normal gut peristalsis to improve constipation outcomes?

Response:

We sincerely thank the reviewer for these thoughtful and multifaceted questions. We added additional Histological evaluation (H&E staining) of intestinal tissues revealed no overt differences in epithelial morphology or inflammation between CH_4-treated and control mice, suggesting that the observed constipation phenotype was not associated with structural epithelial damage (Figure S12, copy below):

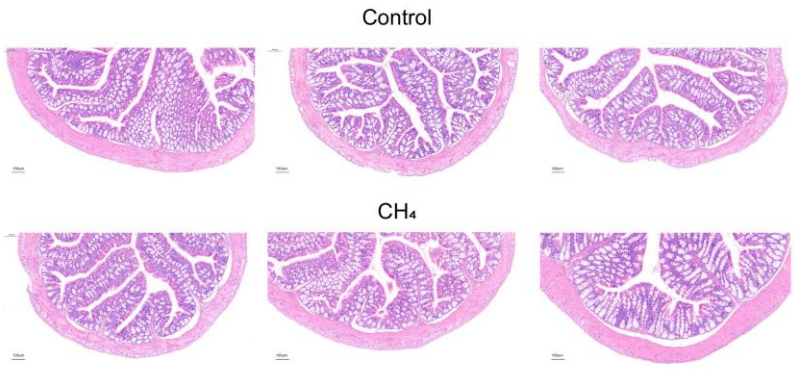


Figure S12. The Hematoxylin-Eosin (H&E) staining of mice colon in Control and

CH_4 groups.

And shotgun metagenomic sequencing of intestinal contents from the ileum, cecum, and colon demonstrated that CH_4 administration significantly reduced α-diversity (Shannon index) in the colon. Supplementation with *Methylocystis intestini* restored α-diversity to control levels (Figure S13A, copy below). β-diversity analysis (NMDS with Bray–Curtis distance) showed that in the upper intestinal tract (ileum and cecum), CH_4 exposure did not significantly alter microbial composition beyond inter-individual variation, whereas the introduction of *M. intestini* led to distinct community shifts. This may reflect preferential colonization of *M. intestini* in the proximal gut. In contrast, in the colon, CH_4 exposure significantly altered microbial composition, and *M. intestini* supplementation partially reversed these CH_4-induced changes (Figure S13B, copy below). Regarding the methane-related microbe enrichment, we did not detect an increased abundance of endogenous methanotrophs in the gut following CH_4 administration. Likewise, gavage with *M. intestini* did not result in significant suppression of resident methanogens, suggesting that its beneficial effects are not due to direct competition with methanogenic archaea (Figure S13C, copy below):

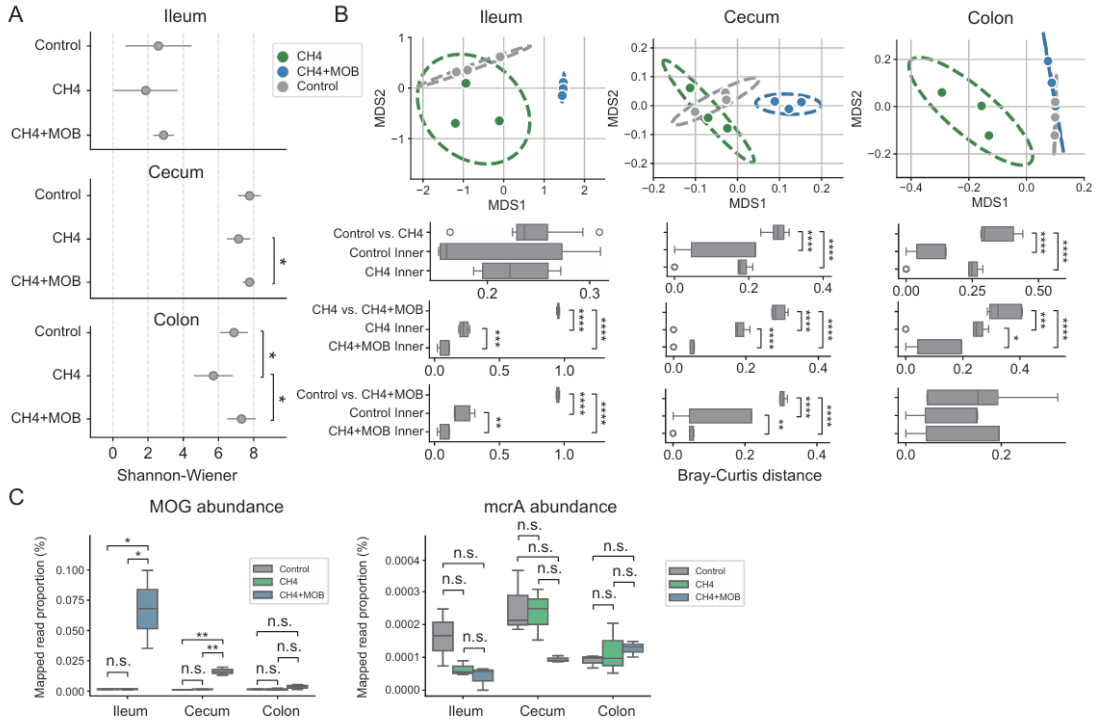


Figure S13. The gut microbiome in different groups. **(A)** The alpha-diversity, 95%

Confidence interval are shown. **(B)** The non-metric multidimensional scaling analysis. The box plot below shows the Bray-Curtis distance between different groups and within groups. **(C)** The proportion of the number of read compared between methane oxidation gene (MOG) and *mcrA* gene in different groups. *: P-value < 0.05; **: P-value < 0.01; ***: P-value < 0.001; ****: P-value < 0.0001, T test, two-tailed, Benjamini/Hochberg correction.

Together, these findings indicate that elevated CH_4 disrupts gut microbial diversity and community structure, particularly in the colon, while *M. intestini* supplementation mitigates these effects and restores microbial balance. The data suggest a modulatory rather than competitive role for *M. intestini* in improving CH_4-associated constipation phenotypes. We have incorporated these results and interpretations into the revised manuscript:

Line 125, "And these effects were not alleviated by the intervention of heat-kill methane-oxidizing bacteria or other bacteria (Figure S11). Additionally, histological examination revealed no obvious differences in epithelial morphology between the control and CH_4 groups (Figure S12). Shotgun metagenomic sequencing showed that CH_4 significantly reduced the α-diversity and disrupted the microbial community structure in the colon, whereas supplementation with *M. intestini* restored diversity to control levels (Figure S13A, Figure S13B). And we did not observe enrichment of methanotrophs after CH_4 administration, nor suppression of methanogens following *M. intestini* gavage (Figure S13C)." has been added.

Comment:

Prior studies (Jensen et al., Nat. Comm, 2021) have shown that methanotroph, Methylococcus capsulatus, cell lysates have anti-inflammatory activity. Does the Methylocystis inoculated here need to be viable to observe the phenotypes? Perhaps the authors should heat-kill or lyse cells to test. Again, the methanotroph-associated phenotypes presented here may not be related to CH4 consumption.

Response:

We greatly appreciate the reviewer's insightful comment. To directly address this question, we performed two additional control interventions: (i) gavage with

Methylorubrum rhodesianum, a methanol-oxidizing bacterium isolated alongside *Methylocystis intestini* but lacking methane-oxidizing capacity, and (ii) gavage with heat-killed *M. intestini*. In both cases, mice did not exhibit the alleviation of CH_4-associated constipation or fat accumulation phenotypes that were observed with viable *M. intestini* administration (Figure S11 and Figure S16):

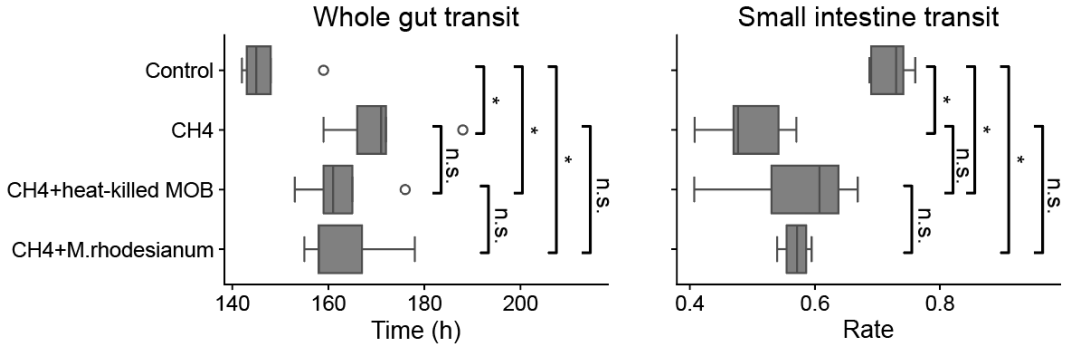


Figure S11. Left: The whole gut transit time. Right: Small intestine transit rate. MOB: *M. intestini*. M. rhodesianum: *Methylorubrum rhodesianum*. n.s.: non-significance; *: P-value < 0.05; T test, two-tailed, Benjamini/Hochberg correction.

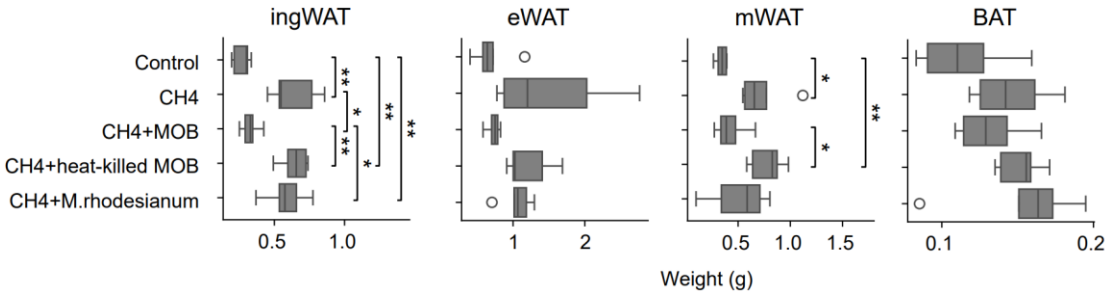


Figure S16. Comparisons among different groups in adipose tissue weight from different depots in KM mice. (WAT: white adipose tissue; BAT, brown adipose tissue; eWAT: epididymal WAT; ingWAT: inguinal WAT; mWAT: mesenteric WAT.) *: P-value < 0.05; **: P-value < 0.01, T test, two-tailed, Benjamini/Hochberg correction.

These findings indicate that the observed phenotypic effects require viable *M. intestini* and are not reproduced by a non-methane-oxidizing methylotroph or by inactivated cells. This suggests that the beneficial effects are linked to the metabolic activity of live methanotrophs, consistent with their ability to modulate CH_4 levels in

vivo. We have incorporated these new data and discussion into the revised manuscript.

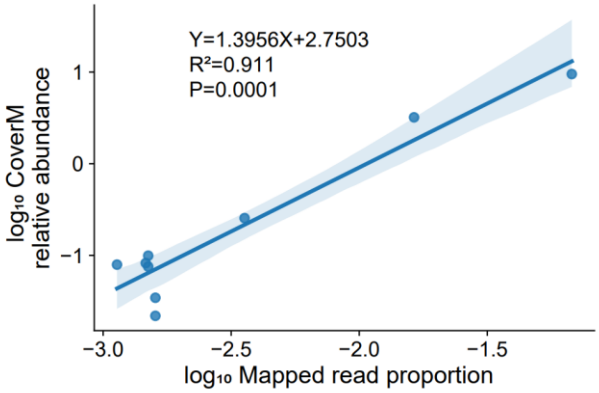
Comment:

The study analyzed a series of prior metagenomic sequencing data and shows that methanotrophs are commonly found in the gut, but at very low abundance. Thus, high sequence depth is required, and the limited depth of many studies could explain why they have not been previously identified. In 550 samples, ~60% had at least ten reads and ~27% > 50 reads correlated to the particulate methane monooxygenase genes unique to methanotrophs. Based off data presented in Fig. 4E, the methanotroph gut microbiome relative abundance is 10^{-5} (although I calculated a maximum of 10^{-7} using data in Fig. 4C), with minimal (although statistically significant) variations between healthy and obese or constipated individuals. At these low abundances, it is hard to envision that minor variances in methanotroph population could be responsible for the observed disease phenotypes. The rate of methane consumption by this amount of methanotrophs would be quite low. The authors should address and discuss these limitations. Nonetheless, there are questions that remain that are likely embedded in the data.

Response:

We thank the reviewer for raising this important point. We agree that methanotrophs are detected in the gut at low relative abundance, however, we think the functions of these low-abundance microbes have been still importance to balance methane levels in the gut.

In addition, we would like to clarify that our abundance estimation approach may underestimate the true levels of methanotrophs. In the current study, we primarily relied on the presence of methane monooxygenase (MMO) genes to estimate relative abundance. This gene-centric method is highly specific but may yield lower relative abundance values compared to genome-based approaches. Indeed, in our murine dataset, we compared abundance estimates obtained using CoverM (which maps reads to full reference genomes) with our MMO-based pipeline, and found that MMO-based estimates were consistently lower, though well correlated across samples:



Correlation of the abundance of methanotrophs determined using different methods. The ordinate represents the relative abundance of methanotrophs obtained using CoverM, and the abscissa represents the abundance of methanotrophs obtained using the same method as Figure 4.

This suggests that while both methods are valid for comparative analysis across groups, gene-centric quantification may underestimate absolute abundances in individual samples. To avoid confusion, we have revised Fig. 4 and its legend to explicitly present values as “Mapped read proportion” rather than “Relative abundance”.

Finally, we acknowledge that the abundance of methane oxidated bacteria is currently low. We have revised the Discussion to highlight this as a limitation:

Line 249, “Methanotrophs were found to thrive in widely ecological niche where methane serves as a dedicated carbon source[24], therefore, their presence in the human gut, which was colonized methanogenic microbes[25], is not unexpected. Our data indicate that metagenomic samples in which methane oxidation genes were undetectable often had lower sequencing depth, and that the relative abundance of gut methanotrophs is likely low, which helps explain why these organisms have long been overlooked within the complex gut microbial community. A major contributing factor is the insufficient sequencing depth to capture rare microbial populations[26,27]. Moreover, the genome of the human-derived methanotroph in our study showed only about 85% similarity with currently characterized members of the genus, suggesting a relatively unique genomic background that has not yet been represented in existing

microbial genome databases. This genomic distinctiveness may further account for the historical underestimation of methanotrophs in the human gut." has been added.
Line 275, "In addition, the abundance of methanotrophs in the human gut appears to be relatively low, which may be as a limitation factor of their functional impact within the complex microbial community. Thus, further studies are warranted to clarify the extent to which gut methanotrophs contribute to host physiology and to determine the conditions under which their effects become biologically significant." has been added.

Comment:

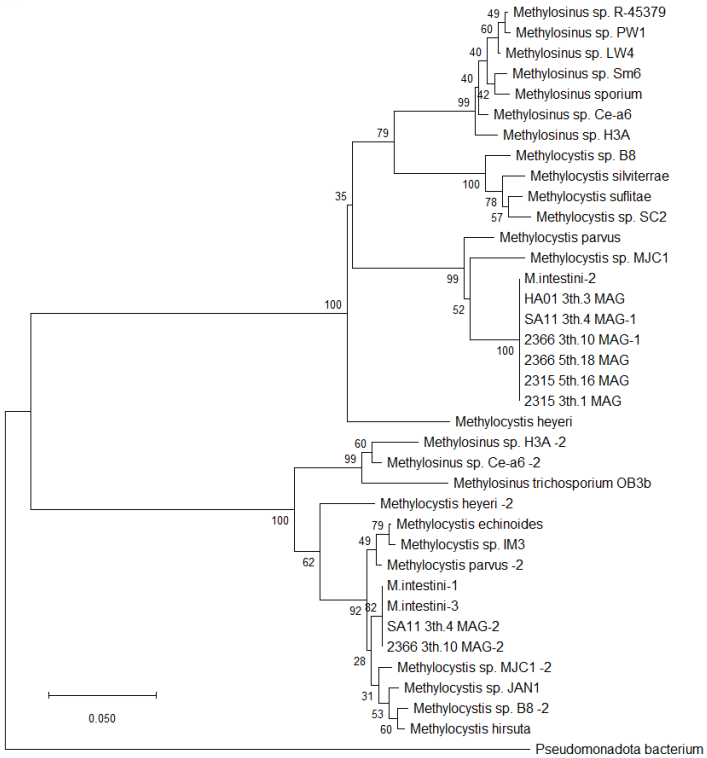
What is the phylogenetic distribution of the identified metagenomic MMO sequences? Are similar genera identified in the murine model isolation found in humans? Is Methylocystis commonly found, and is there any additional correlation to specific genera and disease (although I am not sure confident correlations can be made with such low sequence abundance)?

Response:

We thank the reviewer for these valuable comments. To address these points, we performed additional phylogenetic and database analyses:

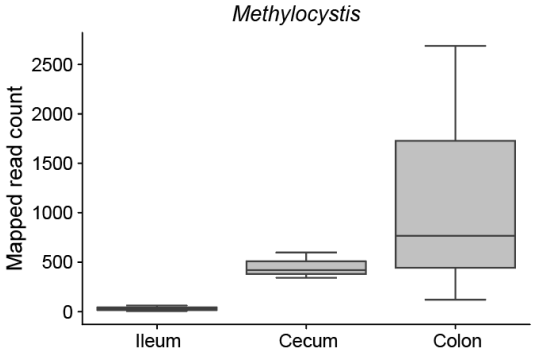
1. Phylogenetic distribution of MMO sequences

   We reconstructed a phylogenetic tree using the eight *pmoA* genes obtained from six MAGs, three pmoA copies extracted from the genome of our isolate *M. intestini*, and several pmoA sequences from other reference strains. The resulting tree (copy below) shows that both pmoA copies from *M. intestini* cluster within the same branch as our MAGs, and appear closely related to an unclassified *Methylocystis sp. MJC1*:



2. Presence in the murine model

   Even in control mice (without gavage of *M. intestini*), we detected methanotroph-related sequences belonging to the *Methylocystis* genus, suggesting that phylogenetically related taxa are present in the murine gut:



3. Global distribution of *Methylocystis*

   To evaluate whether *Methylocystis* is commonly found, we queried MicrobeAtlas (doi:10.1101/2025.07.18.665519). The data show that *Methylocystis* has been isolated worldwide across diverse habitats, supporting its wide ecological distribution and potential to colonize different hosts:

Copy from MicrobeAtlas

4. Associations with other microbes or disease

   We further examined potential associations of *Methylocystis* with other microorganisms or diseases using GloBI (doi:10.1016/j.ecoinf.2014.08.005) and GMrepo (doi:10.1093/nar/gkab1019). These searches did not reveal additional documented associations between *Methylocystis* and specific microbial interactions or disease states.

Taken together, these results show that the pmoA sequences identified in metagenomes are phylogenetically consistent with our murine isolate *M. intestini* and also belong to *Methylocystis* genus, that *Methylocystis* is globally distributed. While widely present, it currently has no documented additional associations with specific diseases beyond those explored in this study.

Comment:

This study contains meritorious evidence to support that the addition of the Methylocystis can lessen constipation phenotypes associated with increased CH4 in a murine model. The data also supports that methanotrophs are in the human gut, albeit at very low abundance. The study and significance of the findings could be improved if alternative hypothesis were investigated. In particular, the effects of CH4 and/or the addition of Methylocystis (or other gut methanotrophs) on the gut microbiome and/or epithelium to provide mechanistic insight into the presented correlations.

Response:

We sincerely thank the reviewer for highlighting the importance of exploring mechanistic insight. We agree that this is a crucial direction to strengthen the study.

To partially address this point, we have now included additional data on the effects of CH_4 and *M. intestini* inoculation on the gut microbiome and intestinal

epithelium. Specifically, hematoxylin and eosin (H&E) staining of intestinal tissues showed no overt histological differences between CH_4-treated and control groups. However, metagenomic sequencing of ileum, cecum, and colon samples revealed that CH_4 exposure significantly decreased α-diversity (Shannon index) in the colon, while *M. intestini* gavage restored this diversity toward control levels. Furthermore, NMDS analysis based on Bray-Curtis distances showed that CH_4 altered microbial community composition most markedly in the colon, whereas *M. intestini* partially reversed this shift. Interestingly, while *M. intestini* did not significantly suppress endogenous methanogens, its presence appeared to regulate the methane content to modulate overall community stability.

We have revised the Results and Discussion sections to incorporate these findings, which suggest that the beneficial effects of *M. intestini* may be mediated, at least in part, through microbiome modulation rather than direct effects on the intestinal epithelium. We also acknowledge the limitation that further mechanistic studies, and we have added this as an important future direction in the discussion.

Reviewer 3#

Comment:

Zhao et al. newly isolated methane-oxidizing bacteria (MOB) from human fecal samples and investigated their impact on host physiology. Whole-genome sequencing of the isolated strain revealed a series of genes predicted to encode methane-metabolizing enzymes. The authors also performed oral administration of the isolated MOB to mice and evaluated its effects in the context of methane-induced impairment of intestinal peristalsis and increased fat deposition. Their results demonstrated that MOB administration improved intestinal motility and reduced fat accumulation.

Response:

We thank the reviewer' hard work and for the clear and accurate summary of our study and for highlighting its significance. We have further reinforced these conclusions with additional data in the revised manuscript.

Comment:

It is commendable that the authors successfully isolated a novel strain of potential methane-oxidizing bacteria and performed gene prediction related to methane metabolism. However, although the authors conclude that the strain possesses methane-oxidizing capability, this claim is not fully substantiated, as methane-derived metabolites such as CH_3OH were not directly measured in this study. Therefore, some statements regarding the metabolic activity appear to be overstated.

Furthermore, in the animal experiments, the current data demonstrate correlations but do not provide direct evidence of causality between methane oxidation by MOB and the observed effects on intestinal motility or fat deposition. While the authors designed their study based on the premise that methane treatment increases lipid accumulation in adipose tissue, there are no or few previous reports supporting this phenomenon could be identified. Therefore, it is important for the authors to provide a more thorough explanation and justification for this observation.

Response:

We sincerely thank the reviewer for this constructive comment. We agree that our original manuscript may have overstated the evidence regarding methane oxidation

activity of the isolated strain.

In this study, we did not directly measure intermediates such as methanol or formaldehyde, as these compounds are typically transient metabolites in methanotrophs and their concentrations are extremely low and difficult to capture. While growth in NMS1 medium (which contains no other carbon source) logically suggests that survival requires methane utilization, we recognize that this alone cannot be taken as definitive proof of methane consumption. To strengthen our evidence, we therefore measured methane concentrations directly in the headspace of cultures and observed a clear decrease (Figure S8, copy below):

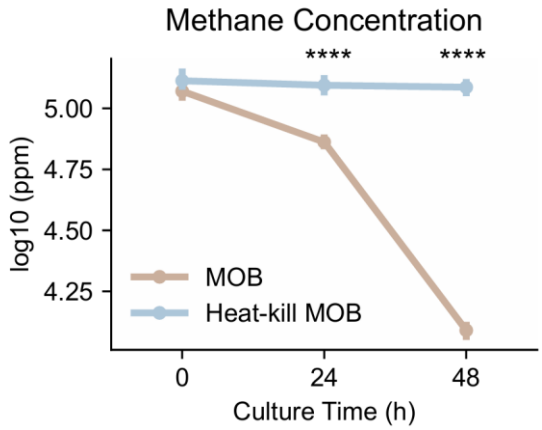
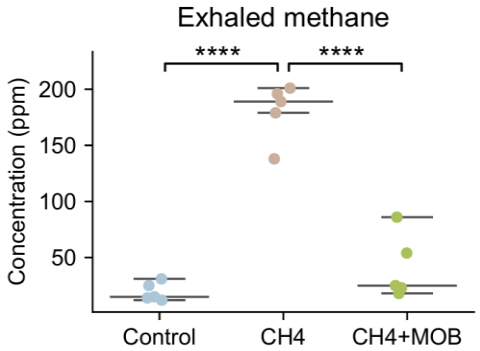


Figure S8. Methane concentration in headspace gas of the cultured bottle with NMS1 medium. MOB: *M. intestini*. ****: P-value < 0.0001; Mann-Whitney-Wilcoxon test, two-tailed.

Moreover, in vivo colonization experiments demonstrated that mice gavaged with *M. intestini* exhibited significantly reduced exhaled CH_4 concentrations. Together, these results provide functional support for methane oxidation capability of the isolate (Figure S10, copy below).



Regarding the relationship between methane exposure and fat deposition, we agree that previous reports are limited. Our study provides experimental evidence showing that continuous methane gavage increases lipid accumulation in murine adipose tissue, and that this phenotype can be alleviated by administration of *M. intestini*. Although the underlying mechanism remains unclear, we have revised the Discussion to emphasize that these findings should be interpreted as correlative and exploratory:

Line 203, “Methane has been implicated in slowing gastrointestinal motility, with prior studies linking its accumulation in the gut to decreased intestinal peristalsis and the onset of constipation[10]. Methane has been shown to disrupt smooth muscle function and interfere with normal peristaltic activity in vitro and in vivo [12,13]. Our results support these findings by showing that the introduction of methane-oxidizing bacteria into mice led to a significant reduction in methane levels in the gut, as well as an improvement in gastrointestinal motility. Although inactivated *Methylococcus capsulatus*, a type of methylotroph, has been reported to possess anti-inflammatory properties that may combat intestinal inflammation [14], our supplemental experiments showed that inactivated methanotrophs cannot relieve intestinal motility abnormalities caused by methane. And no significant gut tissue damage after methane introduction promoted us to hypothesize that *M. intestini* restored normal gastrointestinal function by counteracting methane's inhibitory effects through the reduction of its concentration in the gut.

Some studies have linked slower intestinal motility to obesity[15,16]. Excessive methane production in the gut has also been associated with metabolic disturbances and overweight [8,9]. At present, it remains unclear whether the methane-induced intestinal motility links to the metabolic disturbances, or whether methane exerts a direct effect. Our data show that methane exposure is associated with alterations in fat metabolism, which was similar with previous study[17]. These changes were reversed upon the intervention of *M. intestini.* Although there are no reports on the direct mechanism of methane regulation of fat metabolism, our study found that after methane excess, genes related to the fat digestion and absorption pathway in the

intestinal tissue were upregulated, including *Fabp1*[11], *Cd36*[18], *Apoa4*[19] and *Clps*[20]. These genes have established roles in enterocyte lipid uptake, intracellular trafficking and lipoprotein assembly, and are regulated in part by PPAR-family nuclear receptors. Moreover, after intestinal colonization with *M. intestini*, methane levels in the body decreased and were accompanied by the downregulation of these genes. This reversal effect, to a certain extent, further emphasizes the potential function of methane. Here, *M. intestini* colonization also increased expression of *Pla2g3*, an enzyme capable of generating lysophospholipid signaling molecules that may influence intracellular trafficking responses[21,22]. We speculate that when fat absorption related pathways are perturbed, the intestinal epithelium upregulates *Pla2g3* to hydrolyze membrane phospholipids, thereby releasing fatty acids or lysophospholipids as a means to maintain intracellular lipid supply or remodel membrane components in the short term.

In addition, similar reversal effect after *M. intestini* intervention was also reflected in the gut microbial community structure in the lower part of the intestine. Gut microbiota dysbiosis has been linked to metabolic disorders[23], however, it is unclear whether this methane-induced dysbiosis is an indirect or direct result of methane-induced constipation. Whatever, these results provide a potential explanation for methane-related obesity: *M. intestini* may counteract the interference of methane on the microbial community structure by regulating the methane concentration in the intestine, restoring the microbial diversity in the lower intestine. At the same time, it weakens the overactivation of host fat absorption-related PPAR signaling pathways, and ultimately leading to the downregulation of fat metabolism genes, thereby achieving the regulation of host fat metabolism." has been added.

We hope that these revisions appropriately address the reviewer's concerns.

Comment:

The authors newly isolated a bacterial strain harboring genes predicted to encode methane-oxidizing enzymes. Methane is typically produced by certain archaea species and is generally considered to negatively impact host physiology. The authors propose a novel hypothesis that gut-associated methane-oxidizing bacteria may mitigate these

effects by reducing methane levels through oxidation. While this concept is novel, the current version of the manuscript does not provide direct evidence of methane oxidation or its causal impact on host physiological functions.

Response:

We thank the reviewer for highlighting this important issue. We agree that the current study does not provide direct chemical evidence of methane oxidation intermediates (e.g., methanol, formaldehyde), and thus the claim of methane oxidation must be interpreted with caution. To address this limitation, we performed additional experiments in which we measured methane concentrations in both the culture headspace and the exhaled breath of colonized mice. In both cases, *Methylocystis intestini* significantly reduced methane levels, providing functional support for its methane-oxidizing capability. Nevertheless, we acknowledge that these data remain indirect, as methane-derived metabolites were not quantified, and we have revised the Discussion accordingly to clearly state this limitation.

In addition, our murine experiments confirmed that continuous methane exposure increased constipation-associated phenotypes and lipid accumulation, while administration of *M. intestini* alleviated these effects. According your useful comments, we also introduced two additional control groups: one receiving heat-killed *M. intestini* and the other receiving a methanol-oxidizing bacterium (*Methylorubrum rhodesianum*, lacking methane-oxidizing capacity). Neither of these groups showed the beneficial effects observed with live *M. intestini*, but instead exhibited phenotypes similar to the methane-exposed group. These findings strengthen the interpretation that the observed effects are specifically related to the methane-oxidizing activity of *M. intestini*, while reducing the likelihood of confounding factors such as general microbial colonization or non-specific bacterial components. That said, we fully agree with the reviewer that the current data are not sufficient to demonstrate a direct causal relationship between methane oxidation and the observed host phenotypes. In the revised manuscript, we have carefully modified our wording to present the findings as correlative and exploratory, rather than causal, and we have emphasized the need for further mechanistic studies.

Comment:

Certain aspects of the experimental procedures and data presentation lack clarity and transparency. These areas should be addressed and improved to enhance the overall rigor and reproducibility of the study. These points were also described in the following Suggested improvement.

Response:

We thank the reviewer for this constructive suggestion. We fully agree that clarity and transparency in both experimental procedures and data presentation are essential for rigor and reproducibility. In the revised manuscript, we have carefully revised the Methods section to provide more detailed descriptions of the isolation process, culture conditions, gavage experiments, and quantification methods. In addition, we have improved data presentation by expanding figure legends, clarifying statistical analyses, and reorganizing supplemental materials to ensure that all datasets are clearly described and easily traceable. We believe that these changes enhance the transparency and reproducibility of our study, and we appreciate the reviewer's guidance in helping us strengthen the manuscript.

Comment:

In line 88, the authors state that they used NMS1 medium to culture methane-oxidizing bacteria. However, the abbreviation "NMS1" is undefined. For clarity and reproducibility, the full name and composition of the NMS1 medium should be provided. Also, it could be helpful to reader to show the actual name and the composition.

Response:

We thank the reviewer for pointing out the lack of clarity regarding the NMS1 medium. In the revised manuscript, we have now defined the abbreviation "NMS1" at first mention and provided the full name and detailed composition of the medium in the Table S1 Methods section:

Line 281, "To isolate the gut microbes that can metabolize methane, the NMS1 medium was utilized and methane was used as the only carbon source" has been

revised to “To isolate the gut microbes that can metabolize methane, the Nitrate Mineral Salts Medium (NMS1) medium (Table S1) was utilized and methane was used as the only carbon source”

Table S1. Composition of NMS1 medium

	Weight
$MgSO_4 \cdot 7H_2O$	0.2g
$CaCl_2 \cdot 2H_2O$	0.14g
KNO_3	1g
KH_2PO_4	0.26g
Na_2HPO_4	0.51g

Dissolve the above substances in 1L of water

Comment:

In Figures S4, 1, 2, and 3, the authors claim that *Methylocystis intestini* exhibits methane-metabolizing capability. However, this claim appears to be based solely on gene annotation. To substantiate this claim, direct detection of methane-derived metabolites such as methanol (CH_3OH) or formaldehyde (CH_2O) in vitro culture supernatants or in intestinal/fecal samples would be necessary. Without such metabolic validation, the assertion regarding methane oxidation remains speculative and should be stated more cautiously.

Response:

We thank the reviewer for this insightful comment. We agree that our original claim of methane oxidation was primarily based on genome annotation and therefore required more caution. As correctly noted, direct detection of methane-derived intermediates such as methanol or formaldehyde would provide the definitive validation; however, these metabolites are short-lived as the intermediates of methane oxidation and typically present at very low concentrations, making them technically challenging to quantify in our culture conditions.

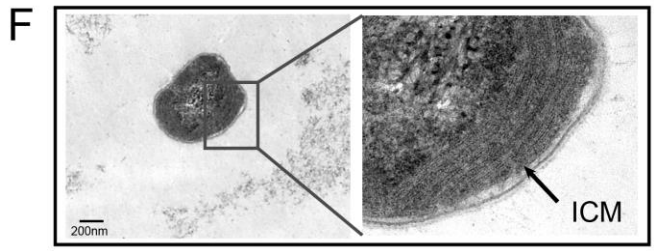
To strengthen our evidence, we performed additional experiments measuring methane concentrations directly in both the culture headspace and in exhaled breath of colonized mice. In both cases, *Methylocystis intestini* significantly reduced methane levels compared to controls, providing functional support for its methane-metabolizing capacity.

Comment:

In Figure 1F, although the authors mentioned a type II methanotroph-specific intracytoplasmic membrane (ICM) structure, the presented image lacks sufficient resolution to clearly visualize these structures. It is advisable to replace the current image with a higher-resolution version and to include a comparative image of a type I methanotroph (which typically exhibits stacked disc-like ICMs) to aid the reader's understanding.

Response:

We thank the reviewer for this valuable suggestion. We agree that the resolution of the current image in Figure 1F is not sufficient to clearly visualize the type II methanotroph-specific intracytoplasmic membrane (ICM) structures. In the revised manuscript, we have replaced the panel with a clearer (TEM) image, in which the characteristic peripheral ICM organization can be more clearly observed (copy below, Figure 1F):



In addition, to aid comparison, we have included a representative image of a type I methanotroph (with stacked disc-like ICMs) from published references as figure S4, so that the distinction between type I and type II morphologies is apparent to readers. We believe these modifications improve the clarity of Figure 1:

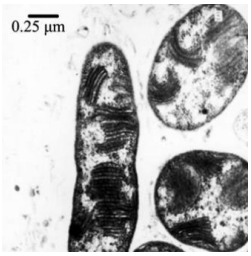


Figure S4. Electron micrograph of a cross-section of a type I methanotroph copy

from Murrell [1] with a flat type of inner cellular membranes (ICM).

[1] Murrell, J. C. "The aerobic methane oxidizing bacteria (methanotrophs)." *Handbook of hydrocarbon and lipid microbiology*. Springer, Berlin, Heidelberg, 2010. 1953-1966.

Comment:

Regarding Figures 2 and 3, it remains unclear whether the observed host phenotypes are truly driven by methane oxidation mediated by *M. intestini*. To address this, generating a ΔpmoCAB mutant strain (if feasible) would provide direct evidence. If mutant generation is not possible, a comparison with a closely related non-methanotrophic strain would help to the authors' claim.

Response:

We thank the reviewer for this insightful suggestion. We agree that generating a ΔpmoCAB mutant strain would provide the most direct genetic evidence to establish whether the observed host phenotypes are indeed mediated by methane oxidation. However, *M. intestini* is a newly isolated strain, and at present, genetic manipulation systems for methanotrophs remain limited and technically challenging. To address this limitation, we performed additional control experiments. Specifically, we introduced two groups of mice: one receiving heat-killed *M. intestini* and another receiving a methanol-oxidizing bacterium (*Methylorubrum rhodesianum*) that lacks methane-oxidizing capability. Neither of these groups alleviated methane-induced phenotypes, in contrast to the live *M. intestini* group, suggesting that the beneficial effects are indeed dependent on methane oxidation activity rather than general microbial colonization or the presence of microbial biomass (Figure S10 and Figure S15):

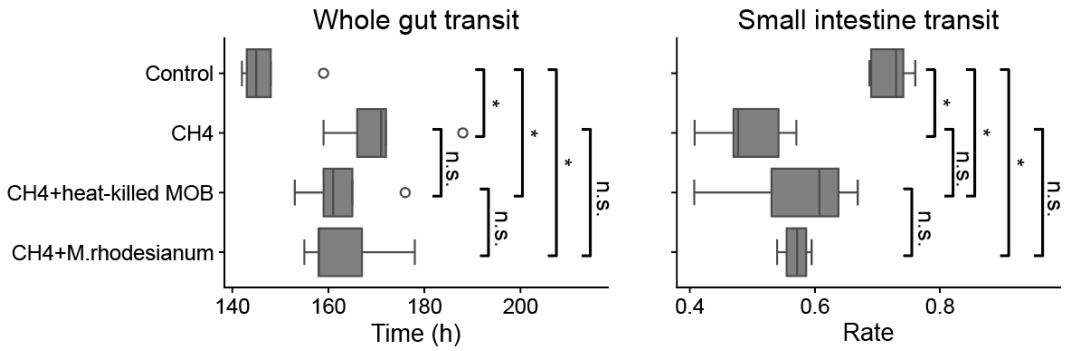


Figure S10. Left: The whole gut transit time. Right: Small intestine transit rate. MOB: *M. intestini*. M. rhodesianum: *Methylorubrum rhodesianum*. n.s.: non-significance; *: P-value < 0.05; T test, two-tailed, Benjamini/Hochberg correction.

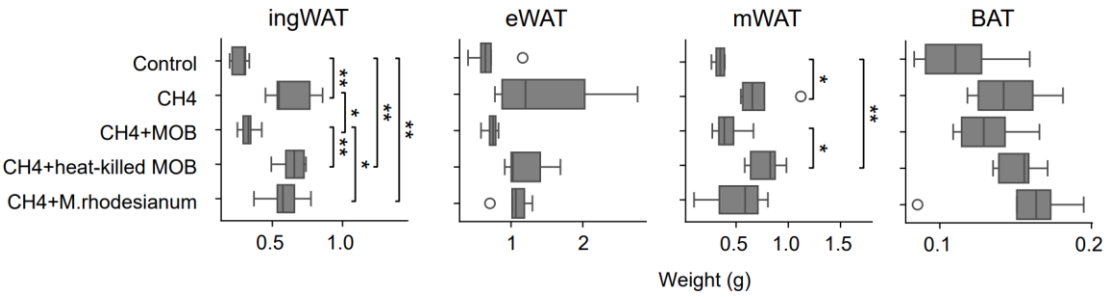


Figure S15. Comparisons among different groups in adipose tissue weight from different depots in KM mice. (WAT: white adipose tissue; BAT, brown adipose tissue; eWAT: epididymal WAT; ingWAT: inguinal WAT; mWAT: mesenteric WAT.) *: P-value < 0.05; **: P-value < 0.01, T test, two-tailed, Benjamini/Hochberg correction.

However, that these results do not substitute for direct genetic evidence. Accordingly, we have revised the manuscript to present our conclusions more cautiously, framing them as correlative and exploratory.

Comment:

In Figures 2A and 3A, the authors should clearly explain the experimental procedures used to administer methane and bacteria to the mice—whether by oral gavage, inhalation, or another route—in the main text.

Response:

We thank the reviewer for pointing out this lack of clarity. In the revised manuscript, we have clarified in both the Methods section that methane and bacterial suspensions were administered to mice via oral gavage:

Line 466, “0.2ml of methane-containing bubble water and 0.2ml of methane gas were injected into animals. Large-caliber syringes were used to prevent gas from escaping. For gut methanotroph intervention, gavage with 0.5 ml *Candidatus M. intestini* (OD600>1, washed with PBS) was applied per mouse.” has been changed to “For CH4 group, MRS (0.2 ml) and methane gas (0.2 ml) were both administered to

animals by oral gavage using large-caliber syringes to prevent gas from escaping. For CH4+MOB and CH4+ M. rhodesianum group, 0.5 ml of *M. intestini* or *M. rhodesianum* suspension (OD600 > 1, washed with PBS) was additionally administered by oral gavage 4-5 hours after methane intervention. For CH4+ heat-kill MOB group, the live 0.5ml *M. intestini* was replaced by the same bacteria after PBS wash but sterilized by autoclaving at 121 °C for 20 min. For Control group, we also administered 0.2ml PBS and 0.2ml air by oral gavage. These interventions were administered twice daily until the end of the experiment. After one week of adaptive feeding (at 6-week-old), the KM and C57BL/6 mice began to start the experiment intervention."

We hope this additional detail will help to ensure that the experimental procedures are fully transparent and reproducible.

Comment:

In Figure S7, the body weights of mice (~40 g) appear to be higher than typical values for standard inbred strains like C57BL/6. This may be due to the use of an outbred or larger strain, which should be specified. Additionally, the authors should investigate whether *M. intestini* can similarly influence methane-induced adiposity and gene expression in a widely used inbred strain such as C57BL/6. The age of the mice at the start of the experiment should also be stated clearly.

Response:

We thank the reviewer for this helpful comment. The relatively higher body weights (~40 g) shown were due to the use of the Kunming (KM) mouse strain, which is indeed larger than the used C57BL/6 strain. Following the reviewer's suggestion, we additionally performed parallel experiments using C57BL/6 mice. The results were consistent with the phenotypes observed in the KM strain. These new data have been incorporated into the revised manuscript (Figure S17, copy below). We believe these results further strengthen the generalizability of our findings.

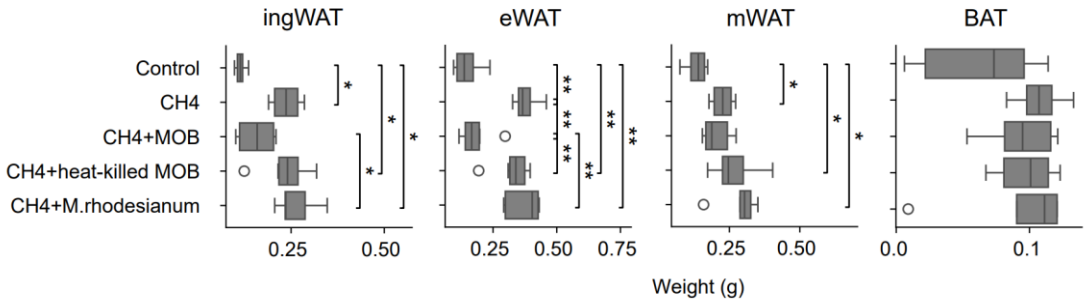


Figure S17. Comparisons among different groups in adipose tissue weight from different depots in C57BL/6 mice. (WAT: white adipose tissue; BAT, brown adipose tissue; eWAT: epididymal WAT; ingWAT: inguinal WAT; mWAT: mesenteric WAT.) *: P-value < 0.05; **: P-value < 0.01, T test, two-tailed, Benjamini/Hochberg correction.

And we revised the method to make the age of the mice at the start of the experiment clearer:

Line 475, "After one week of adaptive feeding (at 6-week-old), the KM and C57BL/6 mice began to start the experiment intervention." has been added.

Comment:

In line 146, the authors report that *M. intestini* reduced methane-induced blood LDL levels; however, Figure 3C does not show statistical significance.

Response:

We thank the reviewer for pointing this out. We carefully re-examined the data presented in Figure 3C and confirmed that the reduction in blood LDL levels upon *M. intestini* supplementation did not reach statistical significance. We agree that our previous wording may have overstated this result. Accordingly, we have revised the text to clarify that a decreasing trend, but not a statistically significant reduction, was observed:

Line 154, "methane exposure significantly increased fat deposition in inguinal white adipose tissue (ingWAT) and elevated blood cholesterol and low-density lipoprotein (LDL) levels (Figure 3B, 3C)." has been changed to "In addition, we found the blood cholesterol levels decreasing after methane exposure. Although we noted a decreasing trend in blood LDL levels in the group treated with *M. intestini*,

this change did not reach statistical significance (Figure 3C)”

Comment:

In Figure 3D, it is unclear which specific adipose tissue was analyzed and at what time point the gene expression analysis was performed. These details should be clearly stated. If the gene expression was assessed at the end of the experiment, the observed transcriptional profiles likely reflect the outcome of the physiological changes, rather than the mechanism underlying them. To better understand the mechanism of action, it would be informative to examine gene expression at earlier time points, such as week 1.

Response:

We thank the reviewer for pointing out this issue. We apologize for the confusion caused by our original description. To clarify, the gene expression analysis shown in Figure 3D was performed on ileum tissue, not adipose tissue. The samples were exactly collected at week 1 (This is the tissue we obtained by killing mice at week 1 after detecting the transit rate of the small intestine) of intervention. We deliberately chose this early time point to capture potential transcriptional changes in the intestinal epithelium that may underlie the phenotype, rather than measuring only late-stage consequences after prolonged methane exposure.

We have revised the main text, figure legend, and Methods section to explicitly state that the transcriptomic data in Figure 3D are derived from the ileum, and that sampling was performed after one week of treatment. We agree with the reviewer that this clarification is essential for proper interpretation of the results and figure legends:

Line 158, “Next, we performed RNA sequencing of the ileum collected after one week of intervention. The result showed that CH4-treated samples separated from the control group, while CH4+MOB samples shifted toward the control group (Figure S18).” has been add.

Line 160, “Gene set enrichment analysis of the ‘Fat digestion and absorption' pathway in different groups.” has been changed to “Gene set enrichment analysis of the ‘Fat digestion and absorption’ pathway based on ileum transcriptomes collected

after 1 week in different groups."

Comment:

In Figure 3E, the authors report that methane treatment upregulated the expression of Fabp1, Cd31 and Apoe4. It is unclear whether these findings have been previously reported or are novel to this study. The authors should clarify this point and discuss the potential mechanisms by which methane may induce the expression of these genes.

Response:

We thank the reviewer for this valuable comment. We have carefully reviewed the existing literature and to our knowledge, there are no previous reports directly linking methane exposure to the upregulation of Fabp1, Cd36, or Apoa4 expression in intestinal tissues. Previous studies have mainly described associations between intestinal methane and altered gastrointestinal motility or obesity-related phenotypes (DOI:10.1007/s10620-009-1012-0; DOI: 10.1177/205064061774445).

Mechanistically, these genes are well known to play central roles in lipid metabolism. Fabp1 and Cd36 are involved in fatty acid uptake and transport in enterocytes, and both are regulated by nuclear receptors such as PPAR (DOI: 10.1126/scisignal.272re3). Apoa4 is a key apolipoprotein involved in lipoprotein metabolism and chylomicron synthesis (DOI: 10.1073/pnas.83.22.8457). One possible explanation for our findings is that methane, by altering intestinal motility or microbial signaling, may secondarily activate PPAR pathways, leading to increased Fabp1, Cd36, or Apoa4 expression. Alternatively, methane-associated microbial or host signals could directly modulate epithelial transcriptional regulators, we added these in the discussion of the manuscript:

Line 217, "Some studies have linked slower intestinal motility to obesity[15,16]. Excessive methane production in the gut has also been associated with metabolic disturbances and overweight [8,9]. At present, it remains unclear whether the methane-induced intestinal motility links to the metabolic disturbances, or whether methane exerts a direct effect. Our data show that methane exposure is associated with alterations in fat metabolism, which was similar with previous study[17]. These changes

were reversed upon the intervention of *M. intestini*. Although there are no reports on the direct mechanism of methane regulation of fat metabolism, our study found that after methane excess, genes related to the fat digestion and absorption pathway in the intestinal tissue were upregulated, including *Fabp1*[11], *Cd36*[18], *Apoa4*[19] and *Clps*[20]. These genes have established roles in enterocyte lipid uptake, intracellular trafficking and lipoprotein assembly, and are regulated in part by PPAR-family nuclear receptors. Moreover, after intestinal colonization with *M. intestini*, methane levels in the body decreased and were accompanied by the downregulation of these genes. This reversal effect, to a certain extent, further emphasizes the potential function of methane. Here, *M. intestini* colonization also increased expression of *Pla2g3*, an enzyme capable of generating lysophospholipid signaling molecules that may influence intracellular trafficking responses[21,22]. We speculate that when fat absorption related pathways are perturbed, the intestinal epithelium upregulates *Pla2g3* to hydrolyze membrane phospholipids, thereby releasing fatty acids or lysophospholipids as a means to maintain intracellular lipid supply or remodel membrane components in the short term.

In addition, similar reversal effect after *M. intestini* intervention was also reflected in the gut microbial community structure in the lower part of the intestine. Gut microbiota dysbiosis has been linked to metabolic disorders[23], however, it is unclear whether this methane-induced dysbiosis is an indirect or direct result of methane-induced constipation. Whatever, these results provide a potential explanation for methane-related obesity: *M. intestini* may counteract the interference of methane on the microbial community structure by regulating the methane concentration in the intestine, restoring the microbial diversity in the lower intestine. At the same time, it weakens the overactivation of host fat absorption-related PPAR signaling pathways, and ultimately leading to the downregulation of fat metabolism genes, thereby achieving the regulation of host fat metabolism.

In addition, we agree with the reviewer that these interpretations remain speculative, and our current study does not establish a causal link between methane

and the transcriptional regulation of these genes. To address this, we have revised the manuscript to clarify that our findings are correlative and exploratory.

Comment:

Regarding Figure 3E, the authors should discuss a mechanism by which methanotrophs suppress methane-induced transcriptional changes. It may be on reducing methane but at least, it would be mentioned on what is the upregulated genes (colored in red in the lower pannel) in CH4+MOB vs CH4. It might be also useful to explain the venn's diagram and/or see the PCA-plot including all groups (control, CH4 and CH4+MOB) together. Particularly, in Figure3F, the meaning of the numbers "1", "1", and "3" in the Venn diagram should be explained in the main text and figure legend.

Response:

We thank the reviewer for this helpful comment. We apologize for the unclear explanation in our original figure legend. In fact, the volcano plot in Figure 3E was specifically designed to highlight genes within the "Fat digestion and absorption" pathway, and therefore not all differentially expressed genes were annotated. We have clarified this point in both the legend and main text. The red dot in the lower panel represents *Pla2g3*, which was significantly upregulated in the CH4+MOB group compared with CH4 alone. We have now annotated this gene in Figure 3E. The reason it was not previously highlighted is that *Pla2g3* was not significantly altered in CH4 vs. Control; our original annotation strategy emphasized showing how CH4-induced genes shifted upon methanotroph intervention (whether upregulated genes decreased or downregulated genes increased). In addition, as suggested, we have included a PCA plot (Figure S17, copy below) in the supplementary materials, which shows that CH4-treated samples separated from the control group, while CH4+MOB samples shifted toward the control group, supporting a partial reversal effect.

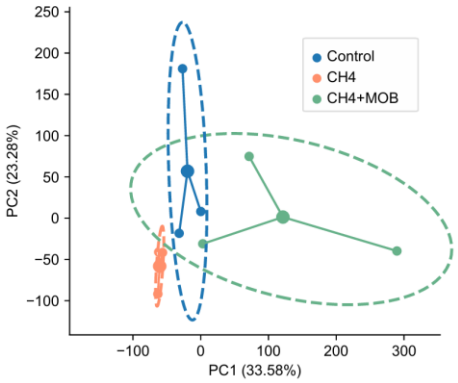


Figure S17. Principal Component Analysis (PCA) of three groups of RNA sequencing data of the ileum collected after one week of intervention.

We have also revised the Venn diagram and its legend to clarify the meaning of the numbers and prevent misunderstanding:

Line 758, "Volcano plot highlighting different genes belonging to the 'Fat digestion and absorption' pathway" has been added.

Line 760, "Venn diagram showing the number of unique and overlapping genes between the different gene sets belonging to the 'Fat digestion and absorption' pathway CH4 vs. Control and CH4+MOB vs. CH4. The left circle represents CH4 vs. Control. The right circle represents CH4+MOB vs. CH4." has been added.

Line 152, "Next, we performed RNA sequencing of the ileum collected after one week of intervention. The result showed that CH4-treated samples separated from the control group, while CH4+MOB samples shifted toward the control group (Figure S17)." has been added.

Line 157, "Pla2g3 was the unique gene with upward expression in CH4+MOB group compared CH4 group. The four genes (Fabp1, Apoa4, Clps, and Cd36) that were significantly upregulated after methane intervention all showed a downregulation trend after additional MOB intervention, among which the Fabp1 gene showed a significant downward trend (Figure 3E and Figure 3F)." has been added.

Comment:

In line 156, the statement "These findings suggest that gut methanotrophs regulate fat

metabolism by reducing methane levels" may be an overstatement, especially given the absence of direct methane measurements and the lack of experiments using a Δ pmoCAB strain. The conclusion should be more cautiously worded.

Response:

We thank the reviewer for this valuable suggestion. We agree that some of our original statement was overstated. We have carefully revised the manuscript text to emphasize that our observations should be interpreted as exploratory and correlative, rather than establishing direct causality.

"These findings suggest that gut methanotrophs regulate fat metabolism by reducing methane levels" has been removed.

Comment:

In line 163, the authors mention the gene cluster mmoXYZBCD, but do not describe its function. A brief explanation of its role in methane oxidation and its specific step in the pathway should be added, ideally alongside Figure S4.

Response:

We thank the reviewer for this valuable suggestion. To avoid confusion, we have removed the mention of the *mmoXYZBCD* gene cluster from the Results section. As the reviewer noted, this operon encodes the soluble methane monooxygenase (sMMO); however, it was not identified in the genome of our isolated strain *M. intestini*. Since our current study cannot determine whether other gut-associated methanotrophs beyond *M. intestini* are present, we initially included *mmoXYZBCD* in the reference database when constructing the methane oxidation gene set. We appreciate the suggestion of adding *mmo* genes into Figure S4, but given that they were not found in *M. intestini*, we believe this could mislead readers. Instead, we have clarified this point and provided a more detailed explanation in the Methods section to improve transparency and readability:

Line 549, "The sequences included the pmo genes (pmoA, pmoB, pmoC) and mmo genes (mmoX, mmoY, mmoZ, mmoB, mmoC, mmoD). Both particulate methane monooxygenase (pMMO, encoded by pmoABC) and soluble methane

monooxygenase (sMMO, encoded by mmoXYZBCD) catalyze the initial and rate-limiting step of methane oxidation, namely the conversion of methane (CH_4) to methanol (CH_3OH). The pmo complex is membrane-bound and more commonly found in type II methanotrophs, whereas mmo is cytoplasmic and expressed in some methanotrophs under copper-limited conditions." has been added.

Comment:

Another point, the methane-oxidizing reaction requires oxygen; therefore, the source of oxygen should be discussed. According to Figure 2B, *M. intestini* primarily colonizes the lumen of the small intestine. One possibility is that *M. intestini* utilizes trace amounts of residual oxygen that are present in the small intestinal environment. Another possibility is that the bacteria obtain oxygen from host tissues, such as from epithelial cells.

Response:

We thank the reviewer for this insightful comment. Indeed, methane oxidation requires molecular oxygen as the initial electron acceptor. Although the intestinal lumen is generally considered a low-oxygen environment, several studies have demonstrated the presence of trace amounts of residual oxygen, particularly in the proximal small intestine, due to diffusion from epithelial cells and luminal mixing. Consistent with this, Figure 2B shows that *M. intestini* primarily colonizes the small intestinal lumen, where residual oxygen levels are relatively higher compared with the colon. Therefore, one possibility is that *M. intestini* utilizes these low but detectable oxygen concentrations to perform methane oxidation. Another possibility, as the reviewer suggested, is that oxygen may be supplied indirectly from the host epithelium or through localized microenvironments where oxygen tension fluctuates (e.g., at the mucosal interface). We have added this discussion in the revised manuscript:

Line 262, "In addition, methane oxidation by methanotrophs requires the presence of oxygen. Although the intestinal lumen is predominantly anaerobic, previous studies have demonstrated the existence of oxygen gradients along the mucosal surface and the intestinal axis, giving rise to localized microaerophilic

niches, particularly in the proximal small intestine[28]. Our observation that methanotroph abundance was highest in the small intestine following gavage supports this possibility. An alternative explanation is that methanotrophs may acquire oxygen directly from host epithelial cells. Nevertheless, the precise source of oxygen that supports methane oxidation in the gut remains to be clarified." has been added.

Comment:

Line 374: "0.2 ml of methane-containing bubble water and 0.2 ml of methane gas were injected into animals."

→ The method of methane administration is unclear. Did the authors apply these two forms simultaneously? Clarification of the route (e.g., oral, rectal) and timing is necessary.

Response:

We thank the reviewer for pointing out this lack of clarity. We apologize for the confusing wording in our original description. To clarify, methane was administered by oral gavage. Specifically, we prepared methane-rich saline by dissolving methane gas in sterile PBS (pH 7.4) under a pressure of 0.4–0.6 MPa for 8 hours. Each mouse received 0.2 ml of methane-rich saline and 0.2 ml of methane gas simultaneously by gavage once per day. The use of large-caliber syringes was necessary to prevent gas escape during gavage. We have revised the Methods section to explicitly describe the route and timing of methane administration:

Line 480, "For CH4 group, MRS (0.2 ml) and methane gas (0.2 ml) were both administered to animals by oral gavage using large-caliber syringes to prevent gas from escaping. For CH4+MOB group, 0.5 ml of *M. intestini* suspension (OD600 > 1, washed with PBS) was additionally administered by oral gavage 4-5 hours after methane intervention. For Control group, we also administered 0.2ml PBS and 0.2ml air by oral gavage."

Comment:

Line 375: "For gut methanotroph intervention, gavage with 0.5 ml Candidatus *M. intestini* (OD600 > 1, washed with PBS) was applied per mouse."

→ Please specify the frequency and duration of the bacterial administration (e.g., daily for how many days?).

Response:

We thank the reviewer for this suggestion. We apologize for not providing enough details in the original description. We have now revised the Methods section to clearly state both the frequency and duration of bacterial administration:

Line 480, "For CH4 group, MRS (0.2 ml) and methane gas (0.2 ml) were both administered to animals by oral gavage using large-caliber syringes to prevent gas from escaping. For CH4+MOB group, 0.5 ml of *M. intestini* suspension (OD600 > 1, washed with PBS) was additionally administered by oral gavage 4-5 hours after methane intervention. For Control group, we also administered 0.2ml PBS and 0.2ml air by oral gavage. These interventions were administered twice daily until the end of the experiment."

Comment:

Line 377: "For diet-intervention, the high-level fat was provided with 0.4 ml lard by gavage per mouse."

→ This implies that the high-fat intervention was performed via lard gavage rather than a standardized high-fat diet. This point should be explicitly clarified in both the methods and the figure legends to ensure reproducibility and transparency.

Response:

We thank the reviewer for pointing this out. Indeed, in this study the high-fat intervention was performed by oral gavage of 0.4 ml lard per mouse once daily and the high-level sugar was provided with 60% (w/v) sucrose by ad libitum drinking, rather than by providing a standardized high-fat diet. We have revised the corresponding figure legends and methods to explicitly clarify this, in order to improve transparency and reproducibility:

Line 764, "The experimental design for two weeks intervention of additional diet." has been changed to "Experimental design for the two-week diet intervention. Mice

were subjected to a high-fat and high-sugar treatment by oral gavage of 0.4 ml lard per mouse per day and ad libitum access to high-concentration sucrose water for two weeks."

Response to reviews

Reviewer #2:

Comment:

The authors have included several additional data that significantly improve the manuscript by filling knowledge gaps identified in the prior version. The R1 manuscript now successfully links M. intestini active CH4 consumption to associated phenotypes. I have one major comment and a few minor points for the authors' consideration:

Response:

Thank you very much for your hard work and valuable comments on our work. We have revised the manuscript according to your comments.

Comment:

The CH4-induced microbiome dysbiosis is striking. The data is presented as alpha- and beta-diversity to highlight the phenotype. However, perhaps additional analysis that show the altered bacterial phyla/other taxonomic groups could be included in the supplement to provide insight into the CH4-induced effects.

Response:

We thank the reviewer for this constructive suggestion. In the revised manuscript, we have now included phyla taxonomic data as Figure S13C (Copy below), and revised the according expression.

##Line 128, "Shotgun metagenomic sequencing showed that CH_4 significantly reduced the α-diversity and disrupted the microbial community structure in the colon, whereas supplementation with *M. intestini* restored diversity to control levels (Figure S13A, Figure S13B). And we did not observe enrichment of methanotrophs after CH_4 administration, nor suppression of methanogens following *M. intestini* gavage (Figure S13C)." has been changed to "Shotgun metagenomic sequencing showed that CH_4 significantly reduced the α-diversity and disrupted the microbial community structure in the colon, whereas supplementation with *M. intestini* restored diversity to control levels (Figure S13A, Figure S13B, Figure S13C). And we did not observe enrichment of methanotrophs after CH_4 administration, nor suppression of methanogens following *M. intestini* gavage (Figure S12C)."

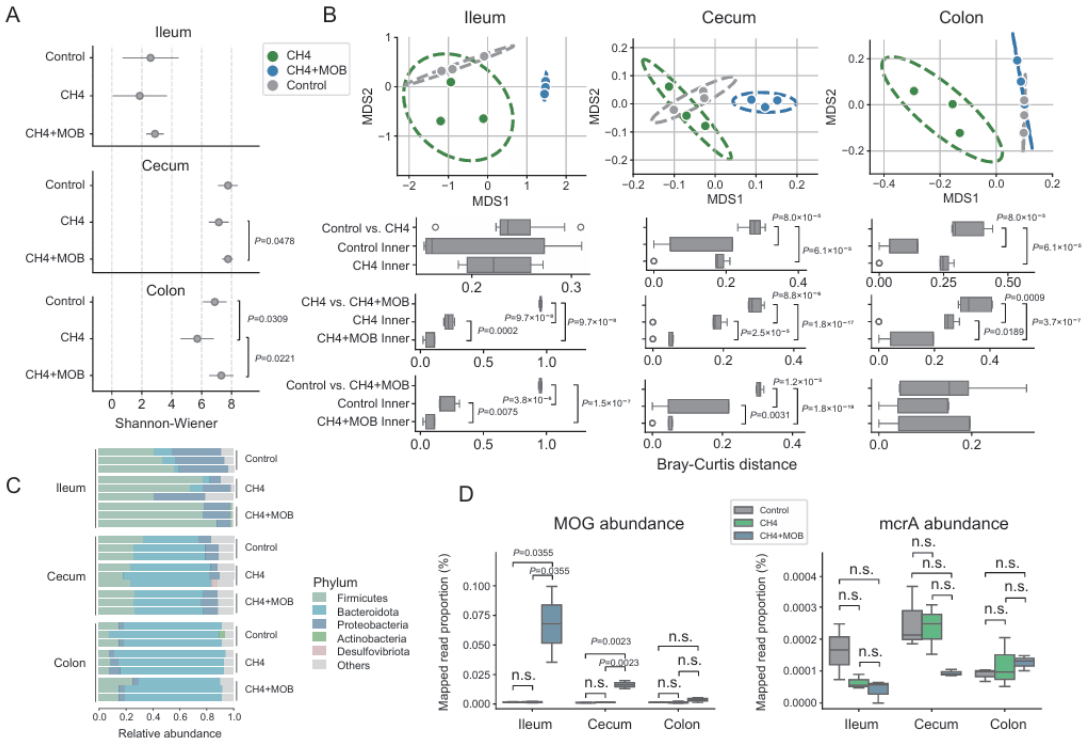


Figure S12. The gut microbiome in different groups. **(A)** The alpha-diversity, 95% Confidence interval are shown. **(B)** The non-metric multidimensional scaling analysis. The box plot below shows the Bray-Curtis distance between different groups and within groups. **(C)** The proportion of gut microbial composition at phylum level. Only the top three phyla are displayed in each sample, and the rest are classed as 'Others'. **(D)** The proportion of the number of read compared between methane oxidation gene (MOG) and *mcrA* gene in different groups.

Comment:

What is the authors' hypothesis for CH4-induced dysbiosis? This is an important finding of the study and, thus, the discussion related thereto should be expanded.

Response:

We sincerely thank the reviewer for this comment. We hypothesize that methane-

induced dysbiosis may primarily arise from methane-mediated alterations in intestinal motility. Specifically, excessive methane slows down gut peristalsis, thereby prolonging the retention and fermentation time of luminal contents. This delayed transit favors the rapid depletion of simple carbohydrates and provides a competitive advantage to *Bacteroides*, which are efficient carbohydrate degraders. As a result, the microbial community shifts toward a *Bacteroides*-dominated and less diverse composition, a dysbiotic pattern resembling that observed in patients with constipation. We have expanded the Discussion section accordingly to elaborate on this hypothesis and its potential implications for methane-associated gut microbial imbalance:

##Line 242, “A possible explanation for this might be that prolonged retention likely alters substrate availability within the gut, leading to the depletion of simple carbohydrates and favoring the expansion of Bacteroidota, a phylum that has a very broad metabolic potential. This shift toward a Bacteroidota-enriched and altered diverse microbial community mirrors the compositional changes observed in individuals with chronic constipation. Therefore, methane-induced slowing of intestinal transit may indirectly shape the microbial ecosystem, establishing a feedback loop between impaired motility and microbial imbalance.” has been added.

Comment:

Highlighted text should be grammatically edited throughout.

Response:

We appreciate the reviewer’s comment. The highlighted text has been comprehensively revised to correct grammatical issues and improve overall fluency.

Line 94, “Analysis of draft genomes revealed the presence of complete methane oxidation gene cluster, pmoCAB in only several Methylocystis draft genomes (Figure S1).” has been changed to “Analysis of draft genomes identified a complete *pmoCAB* methane oxidation gene cluster in only a subset of *Methylocystis* genomes (Figure S1).”

##Line 99, “Based on the average nucleotide identity (ANI), we confirmed that the isolated single colony and the enriched bacteria containing methane oxidation genes were the same strain (ANI<99%, Figure S2). However, genome comparisons with the known Methylocystis revealed an ANI of less than 85.6%, suggesting that the new strain represented a potential novel species (Figure S3). We tentatively named it Methylocystis intestini. Transmission electron microscopy revealed a unique intracytoplasmic membrane (ICM) system characteristic of the type II but not type I methanotroph (Figure 1F and Figure S4).” has been changed to “Based on average nucleotide identity (ANI) analysis, we confirmed that the isolated single colony and the enriched culture containing methane oxidation genes represented the same strain (ANI > 99%; Figure S2). However, genome comparison with known *Methylocystis* species revealed an ANI of less than 85.6%, suggesting that this strain represents a potential novel species (Figure S3). We tentatively designated it *Methylocystis intestini*. Transmission electron microscopy further revealed a distinct intracytoplasmic membrane (ICM) system characteristic of type II, but not type I, methanotrophs (Figure 1F and Figure S4).”

Line 109, “More than 15% genes (573/3,776) in M. intestini were less than 70% amino acid sequence identity compared with other Methylocystis spp. (Figure S6). In addition, we found M. intestini grew better at 37°C than at 30°C (Figure S7) and with methane consumption capability (Figure S8).” has been changed to “More than 15% of the genes (573 out of 3,776) in *M. intestini* shared less than 70% amino acid sequence identity with those of other *Methylocystis* species (Figure S6). In addition, *M. intestini* exhibited optimal growth at 37 °C compared with 30 °C (Figure S7) and demonstrated clear methane consumption capability (Figure S8).”

##Line 145, “And this result didn’t change by the intervention of heat-kill methane-oxidizing bacteria or other bacteria (Figure S16). In addition, we observed similar result in another mouse strain (Figure S17).” has been changed to “This effect was not altered by the intervention with heat-killed methane-oxidizing bacteria or by other bacterial treatments (Figure S16). Moreover, a similar pattern was observed in an

additional mouse strain (Figure S17)."

Line 148, "In addition, we found the blood cholesterol levels decreasing after methane exposure. Although we noted a decreasing trend in blood LDL levels in the group treated with M. intestini, this change did not reach statistical significance (Figure 3C)." has been changed to "In addition, we observed a reduction in blood cholesterol levels following methane exposure. Although the group treated with *M. intestini* exhibited a decreasing trend in circulating LDL levels, the change did not reach statistical significance (Figure 3C)."

Line 152, "Next, we performed RNA sequencing of the ileum collected after one week of intervention. The result showed that CH4-treated samples separated from the control group, while CH4+MOB samples shifted toward the control group (Figure S18)." has been changed to "To further investigate the molecular responses to methane and *M. intestini* intervention, we performed RNA sequencing of ileal tissue collected after one week of treatment. Principal component analysis revealed that CH_4-treated samples were clearly separated from the control group, whereas the CH_4 + MOB group shifted closer to the control group (Figure S18)."

Line 158, "Pla2g3 was the unique gene with upward expression in CH4+MOB group compared CH4 group. The four genes (Fabp1, Apoa4, Clps, and Cd36) that were significantly upregulated after methane intervention all showed a downregulation trend after additional MOB intervention, among which the Fabp1 gene showed a significant downward trend (Figure 3E and Figure 3F)." has been changed to "*Pla2g3* was the only gene that exhibited increased expression in the CH_4 + MOB group compared with the CH_4 group. In contrast, four genes that were significantly upregulated following methane exposure (*Fabp1*, *Apoa4*, *Clps*, and *Cd36*) showed a consistent trend of downregulation after additional MOB intervention, among which *Fabp1* displayed a statistically significant decrease (Figure 3E and Figure 3F)."

Line 198, "In this study we isolated a human-derived methanotroph, Methylocystis

intestini. Our data showed that M. intestini carried methane-oxidizing genes with ability to consume methane both in vitro and in vivo. And we found CH_4 administration in mice was associated with delayed intestinal transit and increased adiposity, whereas colonization with viable M. intestini partially reversed these phenotypes. This study provides exploratory evidence that active gut methanotrophs can modulate CH_4-associated physiological readouts." has been changed to "In this study, we isolated a human-derived methanotroph, *Methylocystis intestini*. Our results demonstrate that *M. intestini* harbors a complete set of methane-oxidizing genes and is capable of consuming methane both in vitro and in vivo. Administration of CH_4 in mice was associated with delayed intestinal transit and increased adiposity, whereas colonization with viable *M. intestini* partially reversed these phenotypes."

##Line 205, "Methane has been shown to disrupt smooth muscle function and interfere with normal peristaltic activity in vitro and in vivo 12,13. Our results support these findings by showing that the introduction of methane-oxidizing bacteria into mice led to a significant reduction in methane levels in the gut, as well as an improvement in gastrointestinal motility. Although inactivated Methylococcus capsulatus, a type of methylotroph, has been reported to possess anti-inflammatory properties that may combat intestinal inflammation 14, our supplemental experiments showed that inactivated methanotrophs cannot relieve intestinal motility abnormalities caused by methane. And no significant gut tissue damage after methane introduction promoted us to hypothesize that M. intestini restored normal gastrointestinal function by counteracting methane's inhibitory effects through the reduction of its concentration in the gut." has been changed to "Experimental evidence further indicates that methane can disrupt smooth muscle contractility and impair peristaltic activity both *in vitro* and *in vivo* [12,13]. Consistent with these findings, our results showed that introducing methane-oxidizing bacteria into mice significantly reduced intestinal methane levels and improved gut motility. Although an inactivated methylotroph (*Methylococcus capsulatus*) has been reported to exert anti-inflammatory effects that may alleviate intestinal inflammation [14] , our supplemental

experiments demonstrated that heat-killed methanotrophs failed to reverse methane-induced motility abnormalities. Moreover, no substantial intestinal tissue damage was observed following methane exposure, leading us to hypothesize that *M. intestini* primarily restores normal gastrointestinal function by counteracting the inhibitory effects of methane through its metabolic consumption."

Line 248, "Whatever, these results provide a potential explanation for methane-related obesity: M. intestini may counteract the interference of methane on the microbial community structure by regulating the methane concentration in the intestine, restoring the microbial diversity in the lower intestine." has been changed to "Collectively, these findings provide a potential mechanistic explanation for methane-associated metabolic alterations. *M. intestini* may mitigate methane-induced dysbiosis by regulating intestinal methane concentrations, thereby restoring microbial diversity in the distal gut"

Comment:

line 88. The isolation results should be placed in context. I recommend starting the paragraph with "To isolate methanotrophs from the human intestine, we inoculated NMS1 medium with human fecal material" or something similar to contextualize followed by the "Growth of bacteria…" sentence.

Response:

We thank the reviewer for this helpful suggestion. We have revised the paragraph to include a contextual introductory sentence to better describe the isolation process. The revised text now reads as follows:

Line 88, "To isolate methanotrophs from the human intestine, we inoculated NMS1 medium with human fecal material" has been added.

Comment:

line 112 "with methane consumption capability" is unclear and should be rephrased

or removed.

Response:

We appreciate the reviewer's insightful comment. We agree that the phrase "with methane consumption capability" was unclear. We have revised the sentence to more precisely describe our observation and measurement of methane oxidation activity. The revised text now reads as follows:

Line 122, "with methane consumption capability" has been changed to "And we found that the methane concentration in the headspace gas of the culture bottles inoculated with *M. intestini* decreased significantly, indicating active methane oxidation"

Comment:

line 119 the timepoint should be added to "the intestine within X after gavage".

Response:

We thank the reviewer for pointing out this omission. We have now added the specific time point for intestinal sampling after gavage.

##Line 119, "Using metagenomic sequencing and RT-qPCR, we found the *M. intestini* can successfully colonize in the intestine after gavage" has been changed to "Using metagenomic sequencing and RT-qPCR, we found the *M. intestini* can successfully colonize in the intestine within five days after gavage"

Comment:

line 124-125, ensure the figure designations correspond correctly and that all figure panels are referenced in the text throughout (Fig. 2F, 2G, and 2I are missing in the text).

Response:

We appreciate the reviewer's careful observation. We have checked all figure

references throughout the text and ensured that each figure panel (including Fig. 2F, 2G, and 2I) is properly cited and described in the revised manuscript.

Comment:

lines 145-147 should be moved after the sentence starting with "These effects were reversed by…"

Response:

We appreciate the reviewer's helpful suggestion. We have revised the text accordingly, moving lines 145–147 to follow the sentence "These effects were reversed by methane-oxidizing bacteria intervention", to improve logical flow and readability.