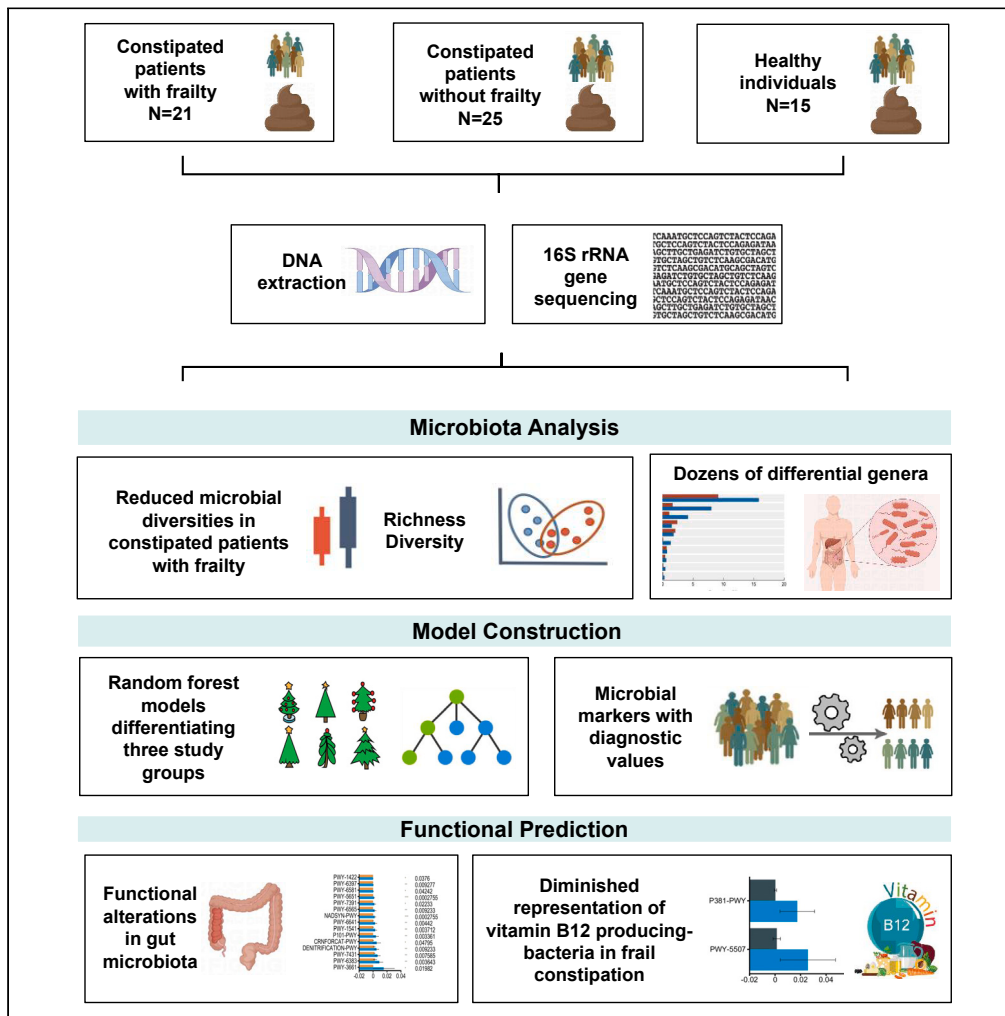# iScience

Article

Diminished representation of vitamin-B12-producing bacteria in constipated elders with frailty

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Highlights

Constipated frail patients show reduced gut microbiota diversity

Microbial markers can differentiate between frail and non-frail constipation

Frail constipation microbiota has diminished vitamin B12 synthesis

Article

Diminished representation of vitamin-B12-producing bacteria in constipated elders with frailty

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SUMMARY

Constipation and frailty are associated with intestinal dysbiosis. This study aims to identify intestinal microbial signatures that can differentiate between constipated elders accompanied by frailty and those without frailty. We collected stool samples from 61 participants and conducted 16S rRNA gene sequencing. Constipated patients with frailty (Constipation_F) exhibited reduced gut microbial diversities compared to constipated patients without frailty (Constipation_NF) and healthy individuals (C). From differential genera, random forest models identified 14, 8, and 5 biomarkers for distinguishing Constipation_F from Constipation_NF, Constipation_F from C, and Constipation_NF from C, respectively. Functional analysis revealed that pathways (P381-PWY and PWY-5507) related to vitamin B12 synthesis were reduced in Constipation_F, which aligns with the decreased abundances of vitamin-B12-producing *Actinomyces* and *Akkermansia* in this group. Our study unveils substantial differences in gut microbiota between constipated elders with frailty and those without, underscoring the diagnostic and therapeutic potential of genera involved in vitamin B12 synthesis.

INTRODUCTION

Constipation, a common gastrointestinal condition among older individuals, poses various challenges such as discomfort, reduced mobility, and potential complications.[1–3] A multicenter study reported that the prevalence of constipation among individuals aged 65 years and older is 17.60% in China,[4] highlighting the substantial burden this condition imposes on the elderly population. Given the worldwide aging demographic trends, addressing the prevalence and healthcare burden of constipation in the elderly is of paramount importance for public health planning and resource allocation.[3,5] Further research and targeted interventions are essential to mitigate the impact of constipation on the well-being of the elderly population.

Frailty, characterized by diminished physiological reserves and increased vulnerability to stressors,[6–8] has been proved to be associated with a higher prevalence of constipation among older individuals.[9,10] The constipation experienced by frail elderly individuals tend to be severe and persistent, significantly impacting their quality of life.[9] Constipation in this population is characterized by distinct features that reflect the complex interactions of physiological and lifestyle factors inherent in aging and frailty.[11,12] Several factors contribute to these interactions. Frailty often involves alterations in muscle tone, reduced physical activity, and changes in dietary habits, all of which can impact the normal functioning of the gastrointestinal tract.[13] On the other hand, frailty has been associated with disturbances in the gut microbiota.[14–16] Frailty is frequently linked to systemic inflammation and immune dysregulation,[17,18] both affecting the composition and diversity of the gut microbiota.[19] Thus, gut dysbiosis may participate in the pathomechanism of constipation with frailty.

In fact, dysbiosis has been implicated in constipation in general.[20,21] Changes in the abundance of specific bacterial species and overall microbial diversity can impact nutrient absorption efficiency, modulate gut motility, and influence the production of short-chain fatty acids (SCFAs), all of which play a role in bowel motility and transit time.[13,22,23] Although the associations of gut microbiota both with constipation and with frailty have been extensively investigated, a significant gap persists in our understanding of the microbial features in constipated patients with frailty.

Therefore, the current study aims to examine the microbial structures and functions associated with constipation with or without frailty. We identified microbial features that can precisely and efficiently differentiate between constipation with and without frailty. Further, functional analysis revealed that the microbial features in constipation with frailty are characterized by reduced capacity in the production of vitamin B12. Our findings could potentially lead to more effective interventions and therapies tailored to address constipation in frail individuals.

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Table 1. Characteristics of patients with constipation and healthy controls

Characteristic	Constipation_F (n = 21)	Constipation_NF (n = 25)	C (n = 15)	p value[a]
Age, mean (SD), year	66.67 (5.55)	67.16 (5.25)	66.93 (4.36)	0.95
Sex				
Female	16 (76.2)	18 (72.0)	10 (66.7)	0.82
Male	5 (23.8)	7 (28.0)	5 (33.3)	
BMI, mean(SD)	24.41 (1.85)	23.58(1.85)	23.92 (1.49)	0.66

[a]$p < 0.05$ was considered statistically significant.

RESULTS

Characteristics of patients with constipation and healthy controls

Detailed characteristics for the three study groups Constipation_F, Constipation_NF, and C are summarized in Table 1. No difference was observed regarding age, BMI, and gender ratio among the study groups.

The microbial ecology of constipation with and without frailty: An overview

All samples (n = 61) were adequately sequenced, with at least 34,518 sequencing reads per sample. From the raw sequencing reads, 1,382 ASVs in Constipation_F; 2,501 ASVs in Constipation_NF; and 1,771 ASVs in C were identified (Figure 1A). ASV abundance was significantly lower in Constipation_F compared to Constipation_NF or C. The four dominant phyla—Firmicutes, Bacteroidota, Proteobacteria, and Actino-bacteriota—accounted for 99.67%, 98.42%, and 96.72% of the microbiomes in Constipation_F, Constipation_NF, and C, respectively (Figures 1B and S1).

Compared to Constipation_NF or C, Constipation_F exhibited lower levels of Firmicutes and Actinobacteriota. The abundance of Firmicutes and Actinobacteriota progressively decreased with the occurrence of constipation and frailty. A small amount of Verrucomicrobiota (≤ 2.5%) was also detected in Constipation_NF and C. It is worth noting that Constipation_NF had a relatively high amount of Proteobacteria, while Constipation_F showed a higher abundance of Bacteroidota. These findings highlight significant structural differences in the microbiome between constipated patients with and without frailty (Figure 1B).

Microbial diversities of the study groups were evaluated at the ASV level (Figures 1C and S2; Table S1). With the Pd metric of α-diversity based on phylogenetic distances, significant reduction was observed in Constipation_F compared to Constipation_NF or C (Figure 1C). However, no significant difference was observed between Constipation_NF and C (Figure 1C). For diversities of the gut microbiota at the community level (β-diversity), principal coordinate analysis (PCoA) utilizing unweighted UniFrac distance revealed that β-diversities were different among the study groups and that the majority of the samples clustered by health status (Figure 1D; $R^2 = 0.1952$, $p = 0.001$; Table S2), indicating that health status was a major effect factor for the phylogenetic composition of these samples. From the clustering of the samples, it is apparent that the β-diversity of the Constipation_F group was distanced from those of the other groups, which is consistent with the changes in α-diversities. These findings demonstrate the presence of dysbiosis in Constipation_F.

Microbial features in Constipation_F and Constipation_NF

To identify microbial features associated with the disease status, the relative abundances of each genera were compared among the study groups. Figure 1E shows the top 10 differentially abundant bacterial genera ($p < 0.05$) in the comparisons between Constipation_F and Constipation_NF, between Constipation_F and C, and between Constipation_NF and C, respectively.

Between Constipation_F and Constipation_NF, most of the differentially abundant genera, such as *Akkermansia* and *Faececalibacterium*, were significantly lower ($p < 0.05$) in Constipation_F, whereas *Megamonas*, *Streptococcus*, and *norank_f_norank_o_Clostridia_UCG-014* were more abundant in Constipation_F ($p < 0.05$) (Figure 1E; Table S3).

Similarly, between Constipation_F and C, eight out of ten differential genera were less represented in Constipation_F, including *Acidaminococcus* and *Akkermansia*. In contrast, the abundance of *Bacteroides* and *Phascolarctobacterium* showed an increase in Constipation_F (Figure 1E; Table S4).

Notably, many differential genera were observed between Constipation_NF and C (Figure 1E; Table S5), despite similar α-diversities and β-diversities between these two groups.

Akkermansia and *Streptococcus* were significantly different in both Constipation_F versus C and Constipation_F versus Constipation_NF, suggesting that they are characteristic genera for Constipation_F.

Microbial markers in the gut of constipated patients with or without frailty

We used random forest models to identify microbial markers that can distinguish between every two study groups. To this end, the entire samples (n = 61) were partitioned into training cohorts (n = 44, consisting of 15 constipation-frail patients, 18 constipation-non-frail patients, and 11 healthy individuals) and test cohorts (n = 17, including 6 constipation-frail patients, 7 constipation-non-frail patients, and 4 healthy

A The amount of differential ASVs

B Relative proportions of microbiota at Phylum level

C α-diversity of the study groups

D β-diversity of the study groups

E Relative Abundances of the differential bacterial genera
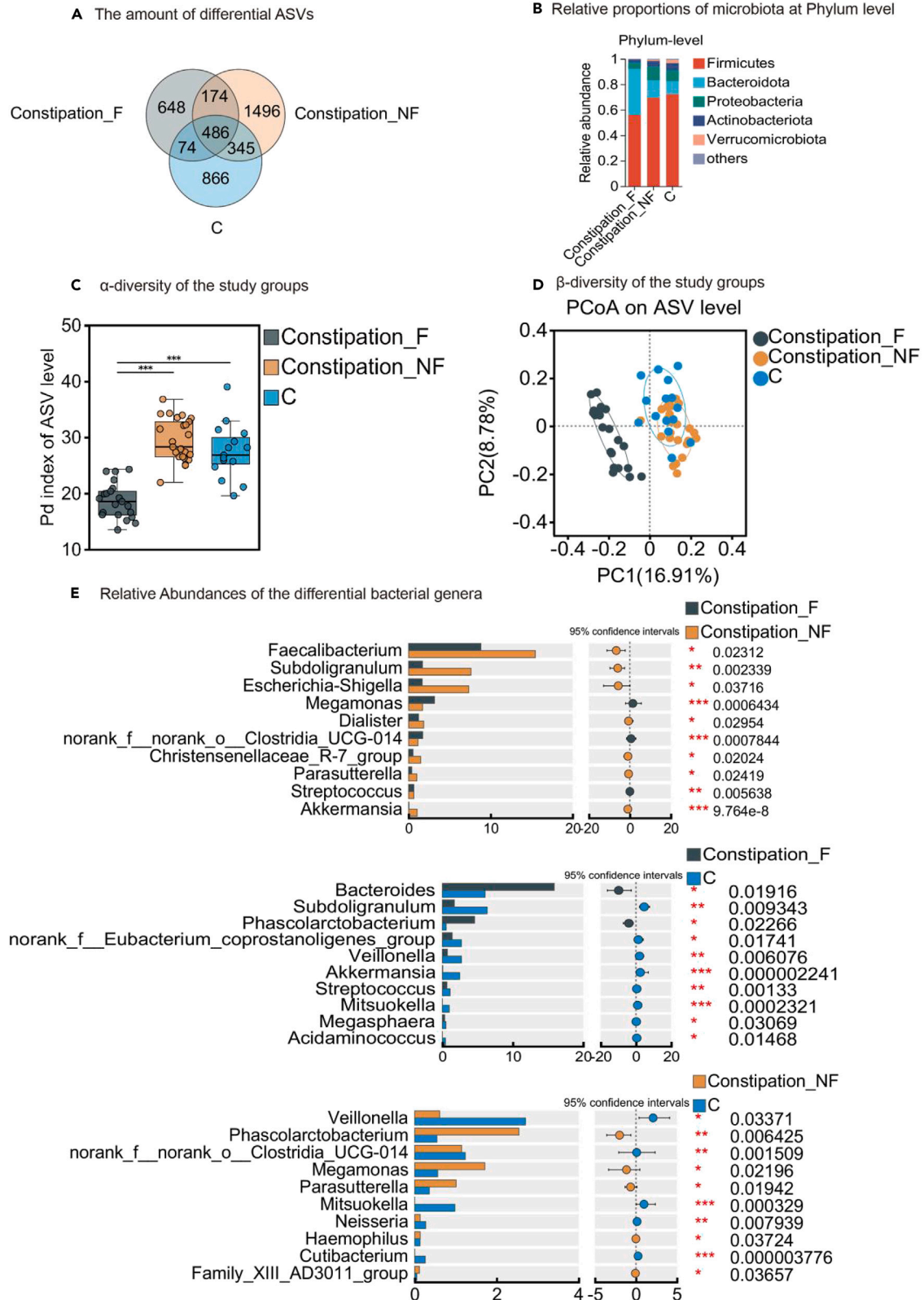
Figure 1. Compositional differences in the gut microbiota among patients with constipation with frailty, constipation without frailty, and healthy control

(A) The Venn diagram shows the total number of differential ASVs in the three study groups (Constipation_F, Constipation_NF, and C) and the amount of overlap between every two groups.

(B) Relative proportions of microbiota of Constipation_F, Constipation_NF, and C at phylum level.

(C) α-diversities of the study groups. Plotted are phylogenetic distance (Pd)-based measurement of α-diversities. Boxplots represent the 25th–75th percentile of the distribution; the median is shown as a thick line in the box; the error bars represent 1.5 times of IQR. Results for other α-diversity metrics are provided in a supplementary file.

(D) UniFrac-based principle coordinates analysis: gut microbiomes clustered by health status. The β-diversity of the study cohort was evaluated by UniFrac-based analysis.

(E) Relative abundances of the differential bacterial genera between every two study groups. The relative abundances of the genera were compared between Constipation_F and Constipation_NF, between Constipation_F and C, and between Constipation_NF and C. Plotted are top 10 abundant genera among the differential genera. The bar graphs on the left side indicate the relative proportions of the differential bacteria in the study groups, with 95% confidence intervals and p values utilizing Wilcoxon rank-sum test shown on the right side. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

individuals). With the training cohorts, 57, 79, and 62 differentially abundant genera ($p < 0.05$) were identified between Constipation_F and Constipation_NF, between Constipation_F and C, and between Constipation_NF and C (Figure S3; Tables S6, S7, and S8). To identify microbial markers distinguishing Constipation_F from Constipation_NF, random forest model was constructed with the differential genera between these two groups, as well as patient metadata features including age, sex, and BMI. Through stratified 10-fold cross-validation, 14 biomarkers were identified for distinguishing Constipation_F from Constipation_NF (Figure 2A). Similarly, eight and five biomarkers were identified for distinguishing between Constipation_F and C and between Constipation_NF and C, respectively (Figures 2B and 2C).

Diagnostic values of the microbial markers

Next, we evaluated the diagnostic values of the identified microbial genera markers. Here, the diagnostic capability of the 14 genera markers described previously (Figure 2A) was evaluated for differentiating between Constipation_F and Constipation_NF, in an RF model constructed with the 14 biomarkers (Figure 2D). With 10-fold cross-validation using the same training and test cohorts described previously, the AUCs for all training cohorts were 0.98 and above (Figure S4), indicating substantial responsiveness of the models to the biomarkers. Concurrently, the AUCs in the test cohort was 0.98, demonstrating excellent generalizability and reliability of the RF model (Figure 2D). Similarly, the diagnostic capabilities of the eight genera markers for Constipation_F versus C and five genera markers for Constipation_NF versus C were evaluated, achieving AUCs of 0.75 and 0.71, respectively (Figures 2E and 2F).

Functional alterations in the gut of constipation with or without frailty

The gut microbiota plays a crucial role in regulating and sustaining host physiological functions.[24] Given the compositional alterations in the gut microbiome of patients with Constipation_F and Constipation_NF, we postulated that microbial functions in these patients were altered accordingly. Therefore, the microbial functions in the study groups were assessed with PICRUSt2 and compared between the study groups.

Dozens of differential pathways were identified through comparison between Constipation_F and Constipation_NF, between Constipation_F and C, and between Constipation_NF and C (Figures 3A–3C; Tables S9, S10, and S11). These differential pathways were predominantly associated with metabolic functions including the synthesis and metabolism of sugars, fatty acids, vitamins, amino acids, and nucleotides. Particularly noteworthy were the common differential pathways, P381-PWY and PWY-5507, in Constipation_F versus Constipation_NF and Constipation_F versus C, both linked to vitamin B12 synthesis (Figures 3A and 3B). Vitamin B12 is a vital water-soluble vitamin known to ameliorate megaloblastic anemia, alleviate neurological lesions, and participate in fatty acid metabolic processes.[25]

Previous studies revealed that *Actinomyces* and *Akkermansia* have the capability to produce vitamin B12.[26,27] In our study, *Actinomyces* and *Akkermansia* were identified as biomarkers in Constipation_F versus Constipation_NF, with lower abundances in Constipation_F for both genera. To validate the reliability of the model, we performed ROC analysis for each of the biomarkers and found AUCs of 0.86 and 0.56 for *Akkermansia* and *Actinomyces* in the test cohort, respectively (Figure S5; Table S12). Additionally, it is noteworthy that the abundance of *Akkermansia* and *Actinomyces* exhibited a gradual decrease from C, to Constipation_NF, and then to Constipation_F (Figure 3D).
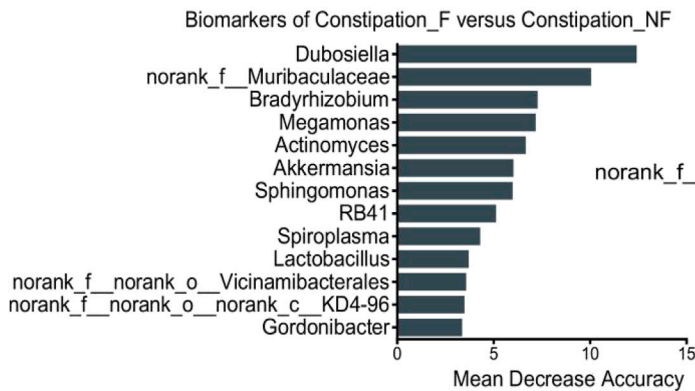
DISCUSSION

In this study, we observed that the structures and functions of the gut microbiota were significantly different between constipated elderly patients with frailty and those without. We then identified disease-status-specific microbial markers that displayed potential diagnostic values for distinguishing among Constipation_F, Constipation_NF, and healthy individuals with RF models. Importantly, the compositional and microbial function analysis consistently suggested reduced capacity in vitamin B12 production in the gut microbiota of frail constipation. Our findings support a potential mechanism for reduced vitamin B12 mediating a causal role of dysbiosis in the pathogenesis of constipation. Further investigation in this direction may lead to vitamin-B12-related, diagnostic markers and therapeutic targets for frail constipation.
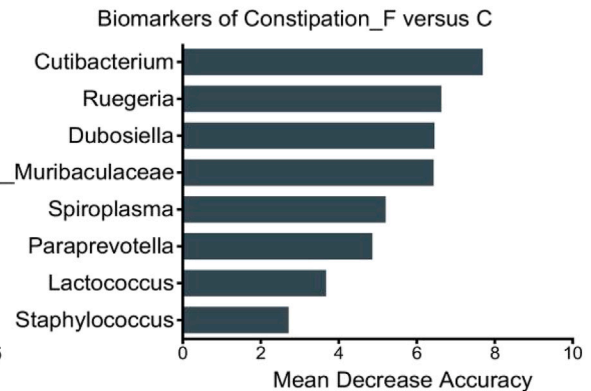
Taxonomic analyses identified multiple microbial markers specific for frail constipation. Among these, two microbial genera, *Akkermansia* and *Actinomyces*, have the capacity to distinguish Constipation_F from Constipation_NF, demonstrating potential value as microbial indicators of frail constipation. Another outstanding observation was significant differences in the abundances of multiple genera between Constipation_F and Constipation_NF, emphasizing distinct gut microbial profiles associated with frail and non-frail constipation. Additionally,
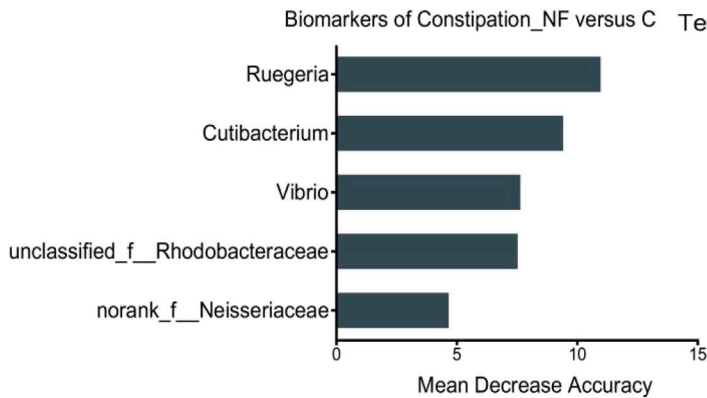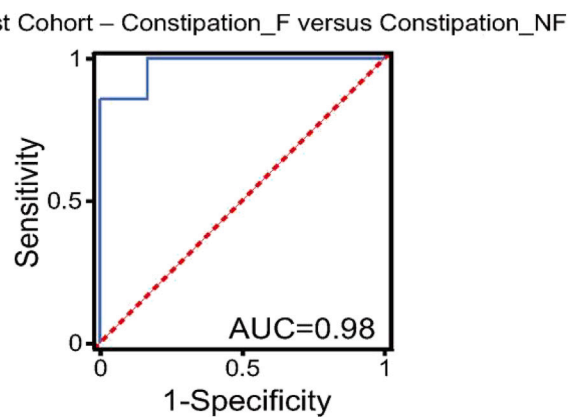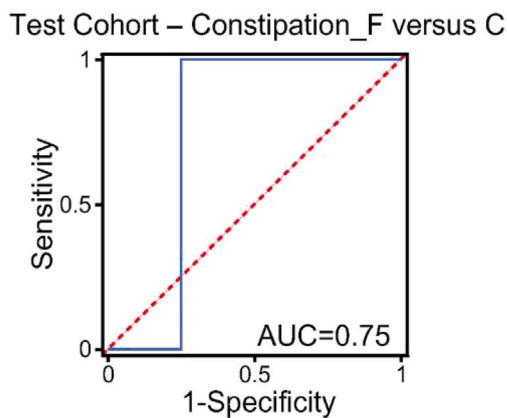
Figure 2. Identification and evaluation of the microbial markers for Constipation_F and Constipation_NF

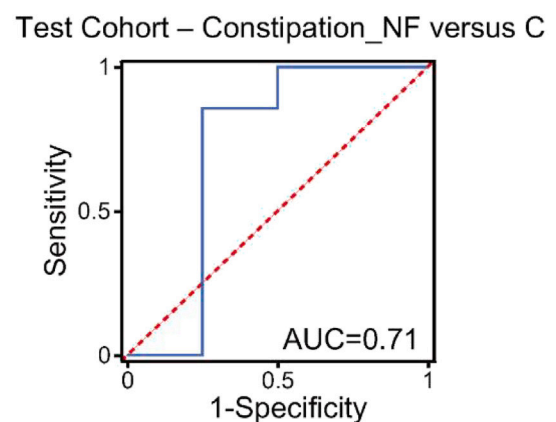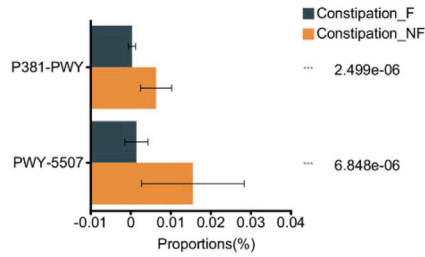(A–C) Microbial markers of the study groups selected using random forest models. The three bar charts show the performances of the biomarkers in random forest models for Constipation_F versus Constipation_NF, Constipation_F versus C, and Constipation_NF versus C.

(D–F) Receiver operating characteristic curves of test cohorts for distinguishing between Constipation_F and Constipation_NF, between Constipation_F and C, and between Constipation_NF and C.

A Two key microbial pathways betwwen Constipation_F and Constipation_NF

B Two key microbial pathways betwwen Constipation_F and C

C Differential microbial pathways betwwen Constipation_NF and C

D The abundances of Akkermansia and Actinomyces in the study groups

Figure 3. Functional alterations in the gut microbiota of Constipation_F and Constipation_NF
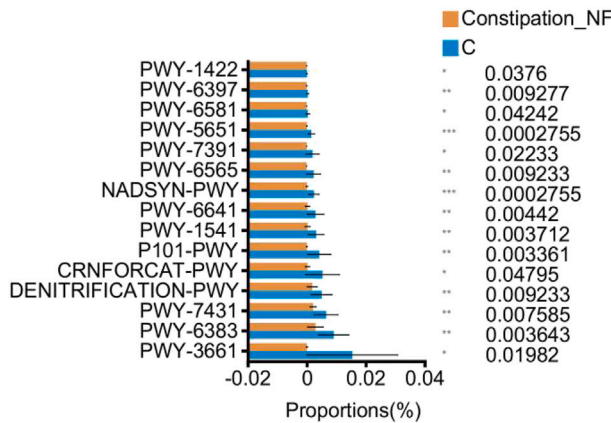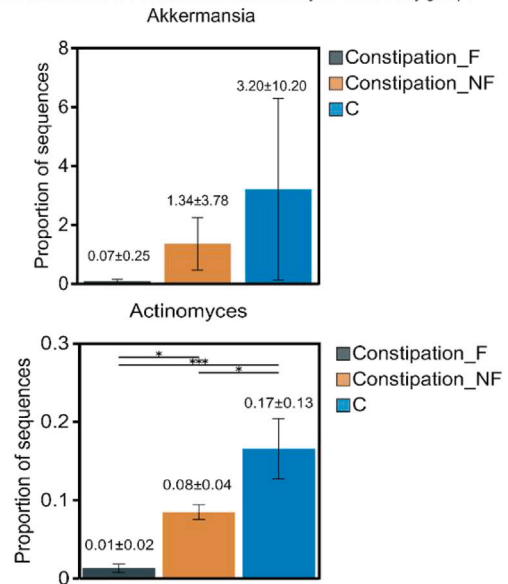
(A) Differential pathways for Constipation_F versus Constipation_NF with vitamin B12 relevant pathways highlighted.

(B) Differential pathways for Constipation_F versus C with vitamin B12 relevant pathways highlighted.

(C) Differential microbial pathways between Constipation_NF versus C.

(D) The abundances of *Akkermansia* and *Actinomyces* in the study groups.*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; Wilcoxon rank-sum test.

it is interesting to note that the microbiota in non-frail constipation represents an intermediate state between those with frail constipation and those participants who are healthy. These observations underscore the complexity of gut microbiota dynamics in constipated patients with or without frailty.

With RF models, we identified a series of biomarkers associated with Constipation_F, Constipation_NF, and C. These markers could be utilized for predicting and screening constipated patients with or without frailty. Notably, certain gut microbes, such as *Cutibacterium* (which distinguishes C from the two constipation groups) and *Dubosiella* (which distinguishes Constipation_F from the other two groups), demonstrate potential for broader applications (Figures 2A–2C and S3).

Two biomarkers, *Cutibacterium* and *Ruegeria*, were common for distinguishing Constipation_F versus C and Constipation_NF versus C and thus may serve as general markers for constipation (Figures 2B and 2C). *Cutibacterium* was significantly less represented in patients with constipation (Figure S3), and it is closely associated with skin diseases such as acne.[28] Vanesa et al. propose that it has a significant correlation with lactate producers and protein degraders.[29] *Ruegeria* was considered as a gut pathogen, but its association with constipation remains to be investigated.[30]

Three common biomarkers were observed in Constipation_F versus Constipation_NF and Constipation_F versus C, namely *Dubosiella*, *norank_f_Muribaculaceae*, and *Spiroplasma* (Figures 2A, 2B, and S3). Capable of distinguishing Constipation_F from the other two groups, these three biomarkers were identified as Constipation_F-specific markers. Studies have shown that *Dubosiella* has anti-aging functions, such as reducing oxidative stress, improving vascular endothelial function, and reshaping gut microbiota.[31] These functions precisely explain the susceptibility of the elderly to constipation with frailty. *Muribaculaceae*, a member of Bacteroidota, specializes in the fermentation of complex polysaccharides and propionate production. Byron et al. discovered that the propionate produced by *Muribaculaceae* is associated with better gut health and increased longevity in mice.[32] *Spiroplasma*, a member of the Tenericutes, is believed by some researchers to be a pathogen for transmissible spongiform encephalopathies, but its role in gut microbiota remains to be investigated.[33]

There was no common microbial marker for distinguishing Constipation_F versus Constipation_NF and Constipation_NF versus C (Figures 2A and 2C).

Functional analysis provides explanations for the differential microbial structures associated with distinct disease status. Of particular importance, pathways P381-PWY and PWY-5507, both of which displayed reduced abundances in Constipation_F when compared to C as well as Constipation_NF, are closely linked to vitamin B12 synthesis. This result brought our attention to the reduced abundances of *Actinomyces* and *Akkermansia* in Constipation_F because these genera have the capability to synthesize vitamin B12.[26,27] These results consistently demonstrated reduced capability in vitamin B12 production in the microbiota of constipated elder patients with frailty.

Vitamin B12 plays a crucial role in various physiological processes, including its potential impact on gastrointestinal function, closely intertwined with constipation. The mechanistic relationship between vitamin B12 and constipation involves several pathways. (1) Neural regulation: vitamin B12 is indispensable for the proper functioning of the nervous system, including the enteric nervous system that regulates bowel movements.[34] Deficiency in vitamin B12 may lead to neurological impairments, potentially affecting the motor function of the gastrointestinal tract.[35] (2) Fatty acid metabolism: vitamin B12 is intricately involved in fatty acid metabolism, a process known to play a crucial role in intestinal motility.[36,37] The deficiency of vitamin B12 may impact fatty acid metabolic processes, potentially leading to alterations in the efficiency of myofiber metabolism and muscle mass.[38,39] (3) SCFAs and gut microbiota: vitamin B12 deficiency may influence the production of SCFAs.[40,41] SCFAs, such as propionate, are crucial for maintaining gut health and regulating motility.[42] Vitamin B12 is also closely associated with frailty.[43,44] In individuals with insufficient levels of vitamin B12, symptoms like weakness, fatigue, and lethargy may arise due to the compromised ability of the body to produce a sufficient number of healthy red blood cells.[45] Anemia resulting from vitamin B12 deficiency can lead to reduced oxygen transport to tissues, exacerbating feelings of weakness and fatigue.[46–49] Additionally, neurological consequences of vitamin B12 deficiency may manifest as numbness, tingling, and muscle weakness.[35,50] Adding to these findings, our research reveals an altered microbiome structure, with reduced capability in vitamin B12 production in the gut of frail constipated patients, supporting the notion that diminished abundance in microbes producing vitamin B12 is a significant factor contributing to frail constipation in elderly patients. Therefore, supplementing with vitamin B12 or specific bacterial strains known for synthesis of vitamin B12 may be a potential approach in treating patients with frail constipation.

Frail constipation, especially in elderly patients, can have a significant impact on their quality of life. Based on this study, we propose several potential avenues for future research. First, multi-center cohort studies exploring the correlation between frailty, aging, and constipation should be conducted, in order to investigate the relationship between aging and the prevalence of frail constipation and the effect of frail constipation on the elderly. Second, the functions and mechanisms of specific gut microorganisms, such as how vitamin-B12-synthesizing bacteria affect frail constipation, can be explored through intervention studies. In addition, the development of innovative microbe-mediated therapeutic modalities is a very promising research direction. Gut microbiota with vitamin-B12-synthesizing function can be used as a potential direction for the development of therapeutic drugs such as prebiotic drugs and microbiota transplantation.

In conclusion, our study introduces a novel perspective by focusing on the interplay between constipation, frailty, and the gut microbiota, particularly in highlighting the diminished representation of vitamin-B12-producing bacteria in the gut of constipated elderly individuals with frailty. While the relationship between gut microbiota and constipation has been explored, our research uniquely identifies specific microbial

signatures that differentiate between constipated elderly with and without frailty. This targeted approach provides a more nuanced understanding of intestinal dysbiosis in the context of frailty and may lead to tailored interventions for this vulnerable population. The identification of biomarkers through random forest models and the functional analysis of microbial pathways related to vitamin B12 synthesis represent original contributions to the field.

Limitations of the study

Several limitations in the current study warrant consideration. Firstly, the cross-sectional nature of our study design hinders our ability to establish causation or infer temporal relationships. Secondly, with 61 participants in total, there is a potential for overfitting with the area under the curve (AUC) analysis. The high AUC values observed in our test data, while promising, may not be indicative of the model's performance in a broader population. To mitigate this risk, we have implemented stratified 10-fold cross-validation, which has shown robustness in our training and test datasets. However, we recommend that future studies with larger sample sizes be conducted to confirm the generalizability of our identified microbial markers and to ensure that the models developed are not overfitted to our specific cohort. Thirdly, while our functional analysis hints at potential pathways associated with vitamin B12 synthesis, additional experimental validation is necessary to confirm these predictions.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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  - 16s rRNA gene sequencing
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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2024.110403.

AUTHOR CONTRIBUTIONS

L.Z. performed the experiments and wrote the manuscript; N.D. and J.L. collected samples; X.D., Y.L., and Y.M. contributed to data collection; L.Z., J.W., D.W., and Y.L. analyzed and interpreted data; S.W., X.J., D.D., and X.Z. performed result visualization.; L.Z. and S.Z. conceived and designed the experiments and reviewed and edited the manuscript. All authors approved the final version of the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

1. Camilleri, M., Ford, A.C., Mawe, G.M., Dinning, P.G., Rao, S.S., Chey, W.D., Simren, M., Lembo, A., Young-Fadok, T.M., and Chang, L. (2017). Chronic constipation. Nat. Rev. Dis. Primers 3, 17095. https://doi.org/10.1038/nrdp.2017.95.

2. Barberio, B., Judge, C., Savarino, E.V., and Ford, A.C. (2021). Global prevalence of functional constipation according to the rome criteria: a systematic review and meta-analysis. Lancet. Gastroenterol. Hepatol. 6, 638–648. https://doi.org/10.1016/S2468-1253(21)00111-4.

3. Chang, L., Chey, W.D., Imdad, A., Almario, C.V., Bharucha, A.E., Diem, S., Greer, K.B., Hanson, B., Harris, L.A., Ko, C., et al. (2023). American gastroenterological association-american college of gastroenterology clinical practice guideline: pharmacological management of chronic idiopathic constipation. Gastroenterology 164, 1086–1106. https://doi.org/10.1053/j.gastro.2023.03.214.

4. Du, X., Liu, S., Jia, P., Wang, X., Gan, J., Hu, W., Zhu, H., Song, Y., Niu, J., and Ji, Y. (2022). Epidemiology of constipation in elderly people in parts of china: a multicenter study. Front. Public Health 10, 823987. https://doi.org/10.3389/fpubh.2022.823987.

5. Chang, A.Y., Skirbekk, V.F., Tyrovolas, S., Kassebaum, N.J., and Dieleman, J.L. (2019). Measuring population ageing: an analysis of the global burden of disease study 2017. Lancet Public Health 4, e159–e167. https://doi.org/10.1016/S2468-2667(19)30019-2.

6. Dent, E., Martin, F.C., Bergman, H., Woo, J., Romero-Ortuno, R., and Walston, J.D. (2019). Management of frailty: opportunities, challenges, and future directions. Lancet 394, 1376–1386. https://doi.org/10.1016/S0140-6736(19)31785-4.

7. Dent, E., Morley, J.E., Cruz-Jentoft, A.J., Woodhouse, L., Rodriguez-Manas, L., Fried, L.P., Woo, J., Aprahamian, I., Sanford, A., Lundy, J., et al. (2019). Physical frailty: icfsr international clinical practice guidelines for identification and management. J. Nutr. Health Aging 23, 771–787. https://doi.org/10.1007/s12603-019-1273-z.

8. Morley, J.E., Vellas, B., van Kan, G.A., Anker, S.D., Bauer, J.M., Bernabei, R., Cesari, M., Chumlea, W.C., Doehner, W., Evans, J., et al. (2013). Frailty consensus: a call to action. J. Am. Med. Dir. Assoc. 14, 392–397. https://doi.org/10.1016/j.jamda.2013.03.022.

9. Arco, S., Saldana, E., Serra-Prat, M., Palomera, E., Ribas, Y., Font, S., Clave, P., and Mundet, L. (2022). Functional constipation in older adults: prevalence, clinical symptoms and subtypes, association with frailty, and impact on quality of life. Gerontology 68, 397–406. https://doi.org/10.1159/000517212.

10. Asaoka, D., Takeda, T., Inami, Y., Abe, D., Shimada, Y., Matsumoto, K., Ueyama, H., Matsumoto, K., Komori, H., Akazawa, Y., et al. (2020). The association between frailty and abdominal symptoms: a hospital-based cross-sectional study. Intern. Med. 59, 1677–1685. https://doi.org/10.2169/internalmedicine.4399-19.

11. Al Nou'mani, N.J., Al Alawi, A.A., Al-Maqbali, J.S., Al Abri, A.N., and Al Sabbri, S.M. (2023). Prevalence, recognition, and risk factors of constipation among medically hospitalized patients: a cohort prospective study. Medicina 59, 1347. https://doi.org/10.3390/medicina59071347.

12. Liu, X., Wang, Y., Shen, L., Sun, Y., Zeng, B., Zhu, B., and Dai, F. (2023). Association between frailty and chronic constipation and chronic diarrhea among american older adults: national health and nutrition examination survey. BMC Geriatr. 23, 745. https://doi.org/10.1186/s12877-023-04438-4.

13. Ticinesi, A., Nouvenne, A., Cerundolo, N., Catania, P., Prati, B., Tana, C., and Meschi, T. (2019). Gut microbiota, muscle mass and function in aging: a focus on physical frailty and sarcopenia. Nutrients 11, 1633. https://doi.org/10.3390/nu11071633.

14. Cui, G., Li, S., Ye, H., Yang, Y., Jia, X., Lin, M., Chu, Y., Feng, Y., Wang, Z., Shi, Z., and Zhang, X. (2023). Gut microbiome and frailty: insight from genetic correlation and mendelian randomization. Gut Microb. 15, 2282795. https://doi.org/10.1080/19490976.2023.2282795.

15. Lim, M.Y., and Nam, Y.D. (2023). Gut microbiome in healthy aging versus those associated with frailty. Gut Microb. 15, 2278225. https://doi.org/10.1080/19490976.2023.2278225.

16. Wen, C.J., Koh, Y.C., Tung, Y.C., Ho, P.Y., Hsieh, S.C., Lo, Y.C., Tsai, J.S., and Pan, M.H. (2023). Cross-sectional study: new approach for diagnostic identification of non-robust older adult. Mol. Nutr. Food Res. 67, e2300056. https://doi.org/10.1002/mnfr.202300056.

17. Ferrucci, L., and Fabbri, E. (2018). Inflammageing: chronic inflammation in ageing, cardiovascular disease, and frailty. Nat. Rev. Cardiol. 15, 505–522. https://doi.org/10.1038/s41569-018-0064-2.

18. Tran, V.H.E., De Glas, N.A., Portielje, J., Van Heemst, D., Van Den Bos, F., Jochems, S.P., and Mooijaart, S.P. (2023). Biomarkers of the ageing immune system and their association with frailty - a systematic review. Exp. Gerontol. 176, 112163. https://doi.org/10.1016/j.exger.2023.112163.

19. Piggott, D.A., and Tuddenham, S. (2020). The gut microbiome and frailty. Transl. Res. 221, 23–43. https://doi.org/10.1016/j.trsl.2020.03.012.

20. Zhu, L., Liu, W., Alkhouri, R., Baker, R.D., Bard, J.E., Quigley, E.M., and Baker, S.S. (2014). Structural changes in the gut microbiome of constipated patients. Physiol. Genom. 46, 679–686. https://doi.org/10.1152/physiolgenomics.00082.2014.

21. Zhang, S., Wang, R., Li, D., Zhao, L., and Zhu, L. (2021). Role of gut microbiota in functional constipation. Gastroenterol. Rep. 9, 392–401. https://doi.org/10.1093/gastro/goab035.

22. Adak, A., and Khan, M.R. (2019). An insight into gut microbiota and its functionalities. Cell. Mol. Life Sci. 76, 473–493. https://doi.org/10.1007/s00018-018-2943-4.

23. Dimidi, E., Christodoulides, S., Scott, S.M., and Whelan, K. (2017). Mechanisms of action of probiotics and the gastrointestinal microbiota on gut motility and constipation. Adv. Nutr. 8, 484–494. https://doi.org/10.3945/an.116.014407.

24. Dominguez-Bello, M.G., Godoy-Vitorino, F., Knight, R., and Blaser, M.J. (2019). Role of the microbiome in human development. Gut 68, 1108–1114. https://doi.org/10.1136/gutjnl-2018-317503.

25. Wolffenbuttel, B.H., Owen, P.J., Ward, M., and Green, R. (2023). Vitamin b$_{12}$. BMJ Br. Med. J. (Clin. Res. Ed.) 383, e071725. https://doi.org/10.1136/bmj-2022-071725.

26. Saunders, A.P., Otto, R.H., and Sylvester, J.C. (1952). The production of vitamin b12 by various strains of actinomycetes. J. Bacteriol. 64, 725–728. https://doi.org/10.1128/jb.64.5.725-728.1952.

27. Kirmiz, N., Galindo, K., Cross, K.L., Luna, E., Rhoades, N., Podar, M., and Flores, G.E. (2020). Comparative genomics guides elucidation of vitamin b$_{12}$ biosynthesis in novel human-associated akkermansia strains. Appl. Environ. Microbiol. 86, e02117-19. https://doi.org/10.1128/AEM.02117-19.

28. Dréno, B., Pecastaings, S., Corvec, S., Veraldi, S., Khammari, A., and Roques, C. (2018). Cutibacterium acnes (propionibacterium acnes) and acne vulgaris: a brief look at the latest updates. J. Eur. Acad. Dermatol. Venereol. 32, 5–14. https://doi.org/10.1111/jdv.15043.

29. Rocha Martin, V.N., Schwab, C., Krych, L., Voney, E., Geirnaert, A., Braegger, C., and Lacroix, C. (2019). Colonization of cutibacterium avidum during infant gut microbiota establishment. FEMS Microbiol. Ecol. 95, fiy215. https://doi.org/10.1093/femsec/fiy215.

30. Zheng, Z., Xiao, Y., Ma, L., Lyu, W., Peng, H., Wang, X., Ren, Y., and Li, J. (2022). Low dose of sucralose alter gut microbiome in mice. Front. Nutr. 9, 848392. https://doi.org/10.3389/fnut.2022.848392.

31. Liu, T.H., Wang, J., Zhang, C.Y., Zhao, L., Sheng, Y.Y., Tao, G.S., and Xue, Y.Z. (2023). Gut microbial characteristical comparison reveals potential anti-aging function of dubosiella newyorkensis in mice. Front. Endocrinol. 14, 1133167. https://doi.org/10.3389/fendo.2023.1133167.

32. Smith, B.J., Miller, R.A., and Schmidt, T.M. (2021). Muribaculaceae genomes assembled from metagenomes suggest genetic drivers of differential response to acarbose treatment in mice. mSphere 6, e0085121. https://doi.org/10.1128/msphere.00851-21.

33. Bastian, F.O. (2014). The case for involvement of spiroplasma in the pathogenesis of transmissible spongiform encephalopathies. J. Neuropathol. Exp. Neurol. 73, 104–114. https://doi.org/10.1097/NEN.0000000000000033.

34. Calderón-Ospina, C.A., and Nava-Mesa, M.O. (2020). B vitamins in the nervous system: current knowledge of the biochemical modes of action and synergies of thiamine, pyridoxine, and cobalamin. CNS Neurosci. Ther. 26, 5–13. https://doi.org/10.1111/cns.13207.

35. Green, R., Allen, L.H., Bjørke-Monsen, A., Brito, A., Guéant, J., Miller, J.W., Molloy, A.M., Nexo, E., Stabler, S., Toh, B., et al. (2017). Vitamin b$_{12}$ deficiency. Nat. Rev. Dis. Primers 3, 17040. https://doi.org/10.1038/nrdp.2017.40.

36. Xiao, Y., and Kashyap, P.C. (2022). Microbially derived polyunsaturated fatty acid as a modulator of gastrointestinal motility. J. Clin. Invest. 132, e161572. https://doi.org/10.1172/JCI161572.

37. Boachie, J., Adaikalakoteswari, A., Gazquez, A., Zammit, V., Larque, E., and Saravanan, P. (2021). Vitamin b12 induces hepatic fatty infiltration through altered fatty acid metabolism. Cell. Physiol. Biochem. 55, 241–255. https://doi.org/10.33594/000000368.

38. Huang, Y.H., Chiu, W.C., Hsu, Y.P., Lo, Y.L., and Wang, Y.H. (2020). Effects of omega-3

fatty acids on muscle mass, muscle strength and muscle performance among the elderly: a meta-analysis. Nutrients *12*, 3739. https://doi.org/10.3390/nu12123739.

39. Choi, S., Chon, J., Lee, S.A., Yoo, M.C., Chung, S.J., Shim, G.Y., Soh, Y., and Won, C.W. (2023). Impact of vitamin b12 insufficiency on the incidence of sarcopenia in korean community-dwelling older adults: a two-year longitudinal study. Nutrients *15*, 936. https://doi.org/10.3390/nu15040936.

40. Guetterman, H.M., Huey, S.L., Knight, R., Fox, A.M., Mehta, S., and Finkelstein, J.L. (2022). Vitamin b-12 and the gastrointestinal microbiome: a systematic review. Adv. Nutr. *13*, 530–558. https://doi.org/10.1093/advances/nmab123.

41. Zheng, Y., Xiang, S., Zhang, H., Ye, K., Zhang, Y., Ge, Y., Feng, X., Bao, X., Chen, J., and Zhu, X. (2021). Vitamin b12 enriched in spinach and its effects on gut microbiota. J. Agric. Food Chem. *69*, 2204–2212. https://doi.org/10.1021/acs.jafc.0c07597.

42. Blaak, E.E., Canfora, E.E., Theis, S., Frost, G., Groen, A.K., Mithieux, G., Nauta, A., Scott, K., Stahl, B., van Harsselaar, J., et al. (2020). Short chain fatty acids in human gut and metabolic health. Benef. Microbes *11*, 411–455. https://doi.org/10.3920/BM2020.0057.

43. Soh, Y., and Won, C.W. (2020). Association between frailty and vitamin b12 in the older korean population. Medicine (Baltim.) *99*, e22327. https://doi.org/10.1097/MD.0000000000022327.

44. Ates, B.E., Soysal, P., Aydin, A.E., Dokuzlar, O., Kocyigit, S.E., and Isik, A.T. (2017). Vitamin b12 deficiency might be related to sarcopenia in older adults. Exp. Gerontol. *95*, 136–140. https://doi.org/10.1016/j.exger.2017.05.017.

45. Mohamed, M., Thio, J., Thomas, R.S., and Phillips, J. (2020). Pernicious anaemia. BMJ Br. Med. J. (Clin. Res. Ed.) *369*, m1319. https://doi.org/10.1136/bmj.m1319.

46. Socha, D.S., Desouza, S.I., Flagg, A., Sekeres, M., and Rogers, H.J. (2020). Severe megaloblastic anemia: vitamin deficiency and other causes. Cleve. Clin. J. Med. *87*, 153–164. https://doi.org/10.3949/ccjm.87a.19072.

47. Green, R., and Datta, M.A. (2017). Megaloblastic anemias: nutritional and other causes. Med. Clin. North Am. *101*, 297–317. https://doi.org/10.1016/j.mcna.2016.09.013.

48. Azzini, E., Raguzzini, A., and Polito, A. (2021). A brief review on vitamin b_{12} deficiency looking at some case study reports in adults. Int. J. Mol. Sci. *22*, 9694. https://doi.org/10.3390/ijms22189694.

49. Esposito, G., Dottori, L., Pivetta, G., Ligato, I., Dilaghi, E., and Lahner, E. (2022). Pernicious anemia: the hematological presentation of a multifaceted disorder caused by cobalamin deficiency. Nutrients *14*, 1672. https://doi.org/10.3390/nu14081672.

50. Stabler, S.P. (2013). Clinical practice. Vitamin b12 deficiency. N. Engl. J. Med. *368*, 149–160. https://doi.org/10.1056/NEJMcp1113996.

51. Mearin, F., Lacy, B.E., Chang, L., Chey, W.D., Lembo, A.J., Simren, M., and Spiller, R. (2016). Bowel disorders. Gastroenterology *150*, 1393–1407. https://doi.org/10.1053/j.gastro.2016.02.031.

52. Morley, J.E., Malmstrom, T.K., and Miller, D.K. (2012). A simple frailty questionnaire (frail) predicts outcomes in middle aged african americans. J. Nutr. Health Aging *16*, 601–608. https://doi.org/10.1007/s12603-012-0084-2.

53. Vandeputte, D., Falony, G., Vieira-Silva, S., Tito, R.Y., Joossens, M., and Raes, J. (2016). Stool consistency is strongly associated with gut microbiota richness and composition, enterotypes and bacterial growth rates. Gut *65*, 57–62. https://doi.org/10.1136/gutjnl-2015-309618.

54. Magoč, T., and Salzberg, S.L. (2011). Flash: fast length adjustment of short reads to improve genome assemblies. Bioinformatics *27*, 2957–2963. https://doi.org/10.1093/bioinformatics/btr507.

55. Callahan, B.J., Mcmurdie, P.J., Rosen, M.J., Han, A.W., Johnson, A.J., and Holmes, S.P. (2016). Dada2: high-resolution sample inference from illumina amplicon data. Nat. Methods *13*, 581–583. https://doi.org/10.1038/nmeth.3869.

56. Bolyen, E., Rideout, J.R., Dillon, M.R., Bokulich, N.A., Abnet, C.C., Al-Ghalith, G.A., Alexander, H., Alm, E.J., Arumugam, M., Asnicar, F., et al. (2019). Reproducible, interactive, scalable and extensible microbiome data science using qiime 2. Nat. Biotechnol. *37*, 852–857. https://doi.org/10.1038/s41587-019-0209-9.

57. Douglas, G.M., Maffei, V.J., Zaneveld, J.R., Yurgel, S.N., Brown, J.R., Taylor, C.M., Huttenhower, C., and Langille, M. (2020). Picrust2 for prediction of metagenome functions. Nat. Biotechnol. *38*, 685–688. https://doi.org/10.1038/s41587-020-0548-6.

STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Stool samples	Collected from participants	N/A
Critical commercial assays		
QIAamp DNA Microbiome Kit	Qiagen (Hilden, Germany)	Cat#51704
Agilent 2100 Bioanalyzer	Agilent (Germany)	RRID: SCR_018043
2× Phanta Max Master Mix	Vazyme (Nanjing, China)	Cat#P515-01
GeneAmp 9700 polymerase chain reaction system	ABI (USA)	RRID: SCR_018436
Deposited data		
Microbiome 16S rRNA sequence data	European Nucleotide Archive	PRJEB71623
Oligonucleotides		
Barcode-indexed primers 341- F: 5''-ACTCCTACGGGAGGCAGCAG-3'; 806-R: 5'-GGACTACHVGGGTWTCTAAT-3'	Sangon Biotech	Cat#F22FTSNCKF1957_BACtclvM
Software and algorithms		
Qiime2 (version 2022.2) pipeline	Bolyen, E., 2019	https://qiime2.org
mothur-1.30	Majorbio (Shanghai, China)	https://mothur.org/wiki/calculators/
R-3.3.1 (Vegan) package	Majorbio (Shanghai, China)	https://cran.r-project.org/
Python 2.7 package	Majorbio (Shanghai, China)	https://www.python.org/downloads/
R-3.3.1 (stat) package	Majorbio (Shanghai, China)	https://cran.r-project.org/
R-3.3.1 (random Forest) package	Majorbio (Shanghai, China)	https://cran.r-project.org/
Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt2, v2.2.0-b).	Douglas, G.M., 2020	http://huttenhower.sph.harvard.edu/galaxy
Other		
Illumina MiSeq platform	Illumina (San Diego, USA)	https://cgi.uconn.edu/illumina-miseq/
Majorbio Cloud platform	Majorbio (Shanghai, China)	https://cloud.majorbio.com

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources should be directed to and will be fulfilled by the lead contact, Dr.Lixin Zhu (zhulx6@mail.sysu.edu.cn).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- Data are available from the lead contact and corresponding authors. 16S rRNA gene reads are publicly available from the European Nucleotide Archive under study project accession number PRJEB71623 (European Nucleotide Archive: PRJEB71623).
- No original code was reported in this article.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Human subject

Patients with constipation and healthy people as control were enrolled in this study at Beijing Hospital of Traditional Chinese Medicine, Capital Medical University from December 2021 to February 2023.

Selection criteria for our constipated patients included the following: age between 60 and 80 years, and diagnosis with functional constipation based on Rome IV criteria (Mearin et al., 2016[51]).The healthy controls were age, gender, and body mass index (BMI) matched with the constipated patients.

All of the following were excluded: (1) stomach, small intestine, colon or rectum resection, colon or rectum cancer, inflammatory bowel disease, insulin-dependent diabetes, and other diseases or conditions that strongly affect colonic transit; (2) used drugs that cause constipation; (3) underwent abdominal surgery within 3 months or plan to undergo abdominal surgery during the trial period; (4) had a history of mental illness; (5) alcohol or drug abuse; (6) psychological, mental, or cognitive abilities, language expression abilities, and other conditions that have been assessed by researchers as unable to cooperate in completing the task.

The diagnosis of frailty was based on an established frail scale (Morley et al., 2012[52]), which consisted five items. Patients were classified as frail if they exhibited three or more positive indicators on the frail scale. Thus, constipated patients were divided into two study groups: constipated patients with frailty (Constipation_F) and constipated patients without frailty (Constipation_NF).

Ethics statement

The protocol involving participants in this study was approved by the Ethics Committee of Beijing Hospital of Traditional Chinese Medicine, Capital Medical University (2022BL02-003-01). All subjects were voluntarily recruited and informed of the nature of the study before sample collection with written informed consent obtained.

METHOD DETAILS

Clinical sample collection

The stool samples of constipated patients and healthy individuals were collected. Demographic information including age, gender, and BMI was obtained during subject recruitment. We estimated the sample size based on previous research data. In total, 46 constipated patients (21 Constipation_F and 25 Constipation_NF) and 15 healthy controls (C) were included in this study.

16s rRNA gene sequencing

Stool samples were collected and stored in a sterile container at -80°C before microbiome analysis (Vandeputte et al., 2016[53]). DNA extraction was performed utilizing the QIAamp DNA Microbiome Kit from Qiagen (Hilden, Germany). The concentration of bacterial DNA was determined using an Agilent 2100 Bioanalyzer (Agilent, Germany). The DNA samples were stored at -80°C until all samples were prepared for sequencing. The V3-V4 region of the bacteria's 16S rRNA was amplified through polymerase chain reaction with barcode-indexed primers (341-F: 5′-ACTCCTACGGGAGGCAGCAG-3′ and 806-R: 5′-GGACTACHVGGGTWTCTAAT-3′) using 2× Phanta Max Master Mix (Vazyme, P515-01, Nanjing, China) on an ABI GeneAmp 9700 polymerase chain reaction system (USA). Agencourt AMPure XP beads were employed for the purification of DNAs, which were subsequently dissolved in an Elution Buffer. Subsequently, the purified amplification products were combined, and the paired ends were sequenced on an Illumina MiSeq platform (Illumina, San Diego, USA). Raw data underwent demultiplexing, quality filtering, and splicing to yield clean data.

QUANTIFICATION AND STATISTICAL ANALYSIS

Microbiome analysis was conducted using the Majorbio Cloud platform (https://cloud.majorbio.com).

Analysis of 16S rRNA gene sequencing data

Following demultiplexing, the resultant sequences underwent quality filtering using fastp (v0.19.6) and were then merged with FLASH v1.2.7 (Magoc et al., 2011[54]). Subsequently, the high-quality sequences underwent de-noising utilizing the DADA2 (Callahan et al., 2016[55]) plugin within the Qiime2 (version 2022.2) pipeline with recommended parameters (Bolyen et al., 2019[56]). This process achieves single-nucleotide resolution based on error profiles within the samples. The sequences de-noised by DADA2 are commonly referred to as amplicon sequence variants (ASVs). To mitigate the impact of sequencing depth on alpha and beta diversity measurements, the number of sequences from each sample was rarefied to 34518, maintaining an average Good's coverage of 99.99%. Taxonomic assignment of ASVs was conducted using the Naive Bayes consensus taxonomy classifier implemented in Qiime2 and silva138/16s_bacteria.

Statistical analysis

Employing mothur-1.30 and based on ASV information, we computed alpha diversity, encompassing the Pd metric (based on phylogenetic distances), Sobs richness, ACE richness, Chao richness, Shannon index, Simpson index, and Good's coverage. Upon implementing unweighted-unifrac and adonis with 999 permutations, Principal Coordinates Analysis (PCoA) was utilized to evaluate the microbial community similarity across diverse samples using the R-3.3.1 (Vegan) package. We analyzed the composition of community structures at the phylum level across different groups and visually depicted the microbial abundance of each group using a community bar diagram. This visualization was crafted using the Python 2.7 package. By conducting the Wilcoxon rank-sum test on the Majorbio Cloud Platform, differential genera between two groups were determined, utilizing the R-3.3.1 (stat) package.

A Random Forest (RF) model analysis was conducted on the Majorbio Cloud Platform. After generating an ASVs table for all participants, a training cohort comprising 15 Constipation_F, 18 Constipation_NF, and 11 C was used for model construction. A test cohort, including 6 Constipation_F, 7 Constipation_NF, and 4 C, was employed to validate the initial findings. Following the analysis of differentially abundant ASVs at the genus level, we constructed the RF model using the R-3.3.1 (random Forest) package. The model utilized stratified 10-fold cross-validation to distinguish between Constipation_F, Constipation_NF, or C. The features employed for model development including patient metadata and differential ASVs at the genus level. Patient metadata features encompassed age, sex, and BMI. Stratified 10-fold cross-validation was implemented to configure training and testing datasets. The "important features" were identified from the top-performing model, consisting of both patient metadata and microbial features, while the top microbial features were designated as "biomarkers". Ultimately, the model's performance was evaluated using the Area Under the Curve (AUC).

The Function Prediction was carried out on the Majorbio Cloud Platform with ASV representative sequences as the basis, utilizing the Kyoto Encyclopedia of Genes and Genomes (KEGG) database through PICRUSt2 (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States, v2.2.0-b) (Douglas et al., 2020[57]).