Original Research Article

Assessment of risk for immune and psychiatric disorders using qPCR-based monitoring of the *nan* gene in gut microbiota: A non-invasive approach

REVIEW

ABSTRACT

Introduction. Gut health relies on the balance between mucin production in the intestines and its degradation by gut microbiota. Bacteria, such as *Ruminococcusgnavus*, possessing the *nan* gene (*nan*), degradate mucin for energy, potentially affecting immune and psychiatric disorders.

Hypothesis/Gap Statement. We hypothesize that bacteria, such as *R. gnavus*, along with other *Lachnospiraceae* species that possess *nan*, may undermine the integrity of the mucus barrier, potentially increasing the risk of immune and psychiatric disorders.

Aim. This study aimed to develop a non-invasive, rapid, and cost-effective approach for assessing the risk of immune and psychiatric disorders by measuringthe quantities of *nan* (*nan* levels)**.**

Methodology: We first designed a primer set (referred to as the *nan* primer set) targeting the consensus sequence of the *nanA* gene, which is highly conserved within *nan*. To validate this primer set, we performed Next-Generation Sequencing (NGS) on the PCR-amplified fragments. To investigate the link between our quantitative PCR (qPCR) method and immune and psychiatric disorders, we performedqPCR to measure *nan* levels in the intestine, analyzing intestinal DNA from animal studies on allergies and aggression. Additionally, to assess whether *nan* levels reflect the clinical status of immune disorders, fecal samples were collected from 45 patients with UC and analyzed for *nan* levels.

Results: NGS analysis of DNA fragments amplified from various intestinal samples using the *nan* primer set confirmed the presence of *nanA* sequences from *R. gnavus*and other *Lachnospiraceae* family members, including *Blautia* and *Dorea* species.Measuring *nan* levels using qPCR with this primer set showed that allergy-induced mice treated with fructans had significantly lower *nan* levels compared to untreated mice. Additionally, aggressive dogs were found to have significantly higher *nan* levels than non-aggressive dogs. In patients with UC, *nan* levels were also significantly higher than those in the control group, confirming that *nan* levels reflect the clinical status of immune disorders.

Conclusion: Measuring *nan* levels in gut microbiota using qPCR shows promise in selectively detecting potentially pathogenic *nan*-harboring strains.The findings suggest that monitoring *nan* levels in intestinal DNA using qPCR could serve as a non-invasive, rapid,

and cost-effective approach for assessing the increased risk of immune and psychiatric disorders.

Keywords:aggression; allergy; nan gene; Ruminococcusgnavus; ulcerative colitis

1. INTRODUCTION

The gut is a complex ecosystem containing trillions of microorganisms, collectively known as the gut microbiota. These microorganisms play a vital role in maintaining overall health by impacting various physiological processes [1]. Mucins are gel-like glycoproteins that form the mucus layer lining the gastrointestinal tract, serving as a protective barrier against pathogens and aiding in smooth digestion. Gut health depends on the integrity of this mucus layer, which is preserved by a delicate balance between mucin production in the intestines and its degradation by the gut microbiota [2]. When this balance is disrupted, it can lead to the breakdown of the mucus barrier, allowing bacterial antigens to penetrate and trigger inflammation in the intestinal mucosa—a hallmark of inflammatory diseases, such as inflammatory bowel disease (IBD).IBD, which includes Crohn's disease and ulcerative colitis (UC), is characterized by chronic inflammation of the gastrointestinal tract, leading to symptoms such as abdominal pain, diarrhea, and weight loss [3].
Certain gut bacteria have developed the ability to degrade mucin and use its carbohydrate components for energy. This process involves a set of enzymes encoded by the *nan* genes, which has been extensively studied in *Ruminococcusgnavus* [4, 5, 6]. Notably, *nan* genes are not unique to *R. gnavus*; they are also present in other members of the *Lachnospiraceae* family, such as *Blautia* and *Dorea* species (Fig. 1). *R. gnavus*has undergone several taxonomic changes over time. Although it was initially classified in the genus *Ruminococcus*, it has recently been reclassified under the genus *Mediterraneibacter* within the *Lachnospiraceae* family [7, 8]. However, as *R. gnavus* remains the most widely recognized name in the research community, we will use this name throughout the study to ensure consistency.

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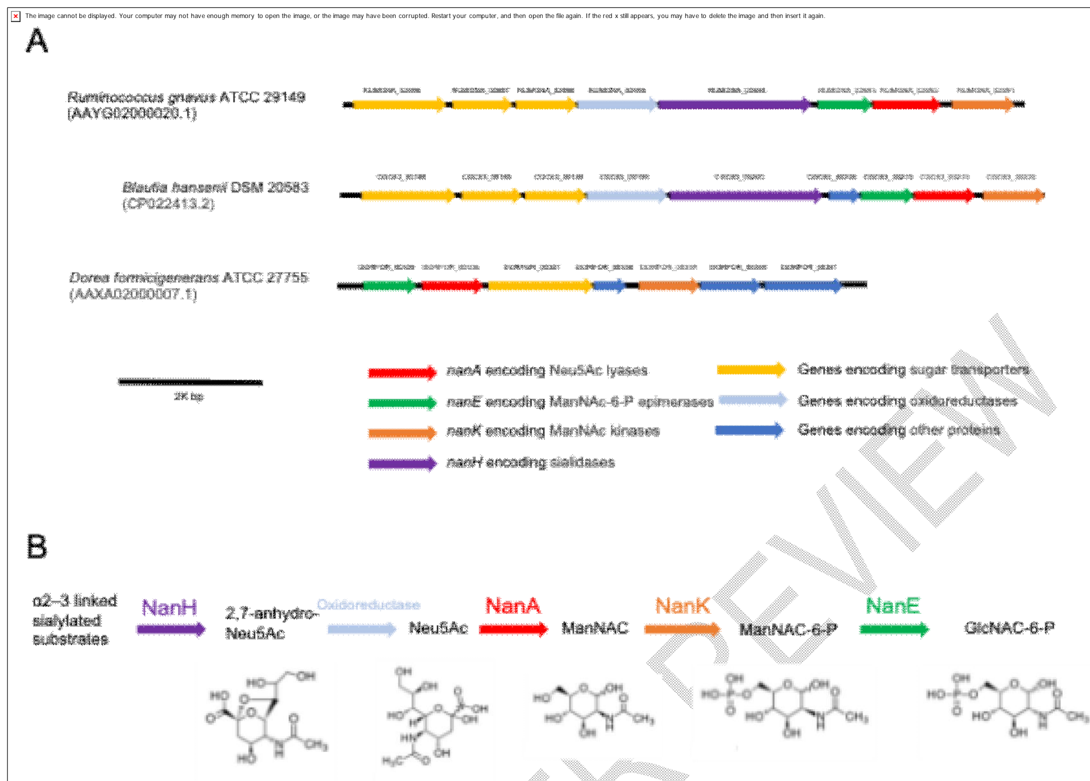

Fig. 1. (A) Depiction of the putative *nan* gene cluster in *Ruminococcusgnavus*ATCC 29149, *Blautiahansenii*DSM 20583, and *Dorea formicigenerans*ATCC 27755. Parentheses indicate GenBank accession numbers and text within the figure refers to locus tags. (B) Proposed pathways for the catabolism of sialic acid in *Ruminococcusgnavus*ATCC 29149, as outlined in a previous study (5, 6). Chemical structures are sourced from the KEGG COMPOUND Database (https://www.genome.jp/kegg/compound/). Abbreviations: ManNAc, N-Acetylmannosamine; GlcNAc, N-Acetylglucosamine; Neu5Ac, N-acetylneuraminic acid.

A key gene in this cluster, *nanA*, encodes the enzyme N-acetylneuraminate lyase, which breaks down sialic acid (a 9-carbon sugar) into pyruvate (a 3-carbon keto sugar) and N-acetylmannosamine (a 6-carbon amino sugar) [11]. The metabolic pathway, which relies on *nan* gene clusters, may play a role in disrupting the gut barrier, potentially leading to a "leaky gut" and related immune disorders.

In healthy individuals, *R. gnavus*generally makes up about 0.1% of the gut microbiota. However, its abundance can increase dramatically, reaching up to 69% in patients with IBD [9]. *R. gnavus* is also known for producing inflammatory polysaccharides such as glucorhamnan, which can activate dendritic cells through toll-like receptor 4 on the host. This activation triggers the release of inflammatory cytokines, such as tumor necrosis factor-alpha and interleukin-6, potentially contributing to the onset of Crohn's disease, a type of IBD [10]. *R. gnavus* is also linked to other immune responses, such as allergy, which is an inappropriate immune response to harmless environmental substances [11]. Studies have foundthat *R. gnavus*is more common in infants with than in those without allergies [12].

Beyond the role in immune responses, the gut microbiota and leaky gut also impact psychiatric conditions and behaviors, emphasizing a complex link between gut health and mental well-being [13, 14, 15]. Recent research in dogs has also found that specific changes in the gut microbiome are associated with aggression, a serious behavioral disorder in domestic dogs [16].

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We hypothesized that bacteria such as *R. gnavus*, along with other members of the *Lachnospiraceae* family carrying *nan*, may compromise the integrity of the mucus barrier, potentially increasing the risk of immune and psychiatric disorders. Our main objective is to develop a non-invasive, rapid, and cost-effectiveapproach for assessing the risk of these conditions. To this end, we have created a technique for measuring the quantities of *nan* genes (*nan* levels) in the intestine using quantitative PCR (qPCR). We validated this approach by analyzing intestinal DNA samples from animal studies on allergies in mice and aggression in dog and from patients diagnosed with UC—a form of IBD characterized by continuous inflammation of the inner lining of the large intestine.

2. METHODOLOGY

2.1 Ethics approval

The study involving DNA analysis from patients with UC was approved by the Institutional Review Board of Fujita Health University (HM22-272 and HM23-078). Informed consent was obtained from all participants, and the study was conducted in accordance with the principles of the Declaration of Helsinki and Good Clinical Practice guidelines.
For the study on aggressive dogs, ethical approval was granted by the Yamazaki University of Animal Health Technology as a clinical research project (20210427-001). This research followed the Japanese National Guidelines for the Humane Treatment of Animals [17], and written informed consent was obtained from all participating dog owners.

2.2DNA samples

In the study using the ovalbumin-induced allergy model in mice, cecal DNA samples were collected from groups comprising five allergy-induced mice, six allergy-induced mice administered 1-kestose, six allergy-induced mice administered inulin, and six allergy-induced mice given both 1-kestose and inulin, as described previously by Takahashi *et al.* [18].
For the study on dogs, fecal DNA samples were obtained from toy poodles aged 2–6 years. Fecal samples were collected using a Fecal Collection Kit FS-0017 (Techno Suruga Laboratory, Shizuoka, Japan) within 30 min of defecation, then stored at –20 °C until DNA extraction, which was performed at the Techno Suruga Laboratory. The study excluded dogs with a history of gastrointestinal surgery, recent antibiotic use (within 2 months of sample collection), or those administered with gastrointestinal, cranial nerve, or psychotropic drugs within 2 weeks before sample collection. The non-aggressive group consisted of 6 healthy dogs (median age: 3.94 years, range: 3.03–3.94 years; 2 males, 4 females). The aggressive group included 10 dogs selected based on criteria by Wright and Nesselrote [18], with a median age of 4.64 years (range: 2.79–6.31 years; 3 males, 7 females). The criteria involved a 5–10 min phone interview with clients, followed by a 1–3 h behavioral assessment session. Aggression was defined as agonistic behavior, including lunging, snarling, growling, and biting directed toward unfamiliar people or dogs.
For the UC patient study analyzing amplified *nanA* sequences using next-generation sequencing (NGS), intestinal lavage fluids (ILFs) were collected during colonoscopies from four patients with UC. The group included three men (aged 54, 55, and 65) and one woman (aged 20). DNA was then extracted from these samples, following the method previously described [19]. Additionally, fecal DNA samples for the clinical study were collected from 45 patients with UC, whose characteristics are detailed in section 3.3, at Fujita Health University using the Fecal Collection Kit FS-0017. The exclusion criteria were individuals who had used antibiotics, probiotics, or prebiotics within the preceding 7 days. DNA extraction was carried out using the QIAampPowerFecal Pro DNA Kit (QIAGEN, Hilden, Germany), according to the manufacturer's instructions. DNA samples from 85 healthy controls were sourced from a previous study by Fujii et al. [19].

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2.3NGS

NGS analysis was performed on the amplified *nanA*gene fragments from a subset of 11 randomly selected DNA samples. These included three samples from allergy-induced mice, three from aggressive dogs, and four from the ILFs of UC patients. To design the appropriate primer set, a Protein BLAST search for the NanA of *R. gnavus* ATCC 29149 was conducted on the NCBI website. This search identified four NanA variants from *R. gnavus* strains and three from *Blautia*strains (*Blautiawexlerae, Blautialuti,* and*Blautiacoccoides*), all showing high amino acid sequence identity to the NanA of *R. gnavus* ATCC 29149. The nucleotide sequences of these variants were then retrieved from GenBank and aligned using MAFFT on the GenomeNet website, as illustrated in Fig. 2.

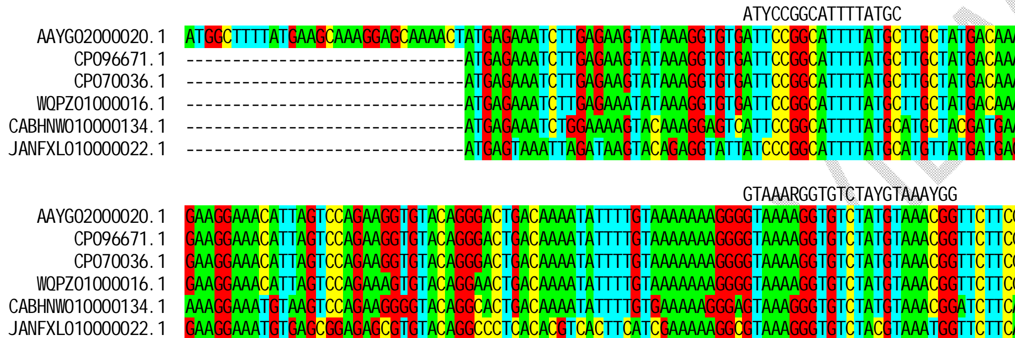

Fig. 2. N-terminal nucleotide sequence alignment of *nanA*gene homologs. The sequence shown above the alignment indicates the region selected for primer design. CP027002.1: *Ruminococcusgnavus*ATCC29149, CP096671.1: *Ruminococcusgnavus* strain CC55_001C, CP070036.1: *Ruminococcusgnavus* strain FDAARGOS_1342, WQPZ01000016.1: *Blautiawexlerae* strain MCC298, CABHNW010000134.1: *Blautialuti* isolate *Blautia_luti*_SSTS_Bg7063, JANFXL010000022.1: *Blautiacoccoides*strain SL.3.07 GCCCFIEI_22.

For the first PCR, a *nanA*-specific primer set (referred to as the *nan* primer set) was designed to target conserved regions of the gene. The forward primer, nanA_113F (5′-ATYCCGGCATTTTATGC-3′), binds to positions 61–77 bp, while the reverse primer, nanA_113R (5′-CCRTTTACRTAGACACCYTTTAC-3′), binds to positions 152–173 bp of the *R. gnavus* ATCC 29149 *nanA* gene. The melting temperatures (T_m) of these primers were calculated using the Multiple Primer Analyzer (Thermo Fisher Scientific, Waltham, MA, USA).

For the second PCR, which was used for NGS preparation, the primer sets nanA_forNGS_F (5′-ACACTCTTTCCCTACACGACGCTCTTCCGATCTATYCCGGCATTTTATGC-3′) and nanA_forNGS_R (5′-GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCCRTTTACRTAGACACCYTTTAC-3′) were employed. The underlined sequences in these primers correspond to the sequences of nanA_113F and nanA_113R. Both the first and second PCRs were conducted according to previously established protocols [20]. NGS for the PCR product was conducted at Bioengineering Lab Co., Ltd., Kanagawa, Japan, as shown previously [20]. Homology search was performed using BLASTn (version 2.15.0) on the NCBI website (https://www.ncbi.nlm.nih.gov/, accessed November 15, 2023), while phylogenetic analyses were conducted on Phylogeny.fr (http://www.phylogeny.fr/, accessed November 15, 2023).

In addition, NGS analysis of the 16S rRNA gene and subsequent bioinformatics analyses were performed on DNA samples from 45 patients with UC, using previously described methods [19]. The data output from the MiSeq sequencing system was processed, statistically analyzed, and mapped using the EzBioCloud 16S database and 16S microbiome

pipeline provided by ChunLab Inc. (EzBioCloud 16S-based MTP app, available at https://www.EZbiocloud.net).

2.4Quantitative PCR analysis

The quantities of *nanA* homologous genes and total bacterial 16S rRNA genes were analyzed using qPCR on the QuantStudio 3 system (Thermo Fisher Scientific). The reaction mixture for each DNA template was prepared using the PowerTrack SYBR Green Master Mix (Thermo Fisher Scientific), following the manufacturer's instructions. The *nan* primer set was used to amplify *nanA* homologous genes. The amplification protocol included an initial denaturation at 95°C for 2 min, followed by 40 cycles of denaturation at 95°C for 10 s, annealing at 50°C for 15 s, and extension at 72°C for 15 s, with a final extension at 72°C for 1 min. Absolute quantification was carried out using PCR fragments of the *nanA* gene from *R. gnavus* JCM 6515. The total bacterial 16S rRNA genes were quantified, as described previously [21]. To calculate the *nan* levels in the DNA samples, the number of *nanA* homologous genes was divided by the total number of 16S rRNA genes, then multiplied by four. This adjustment accounts for the variability in the number of 16S rRNA genes per bacterial genome, which ranges from 1 to 15 copies, with an average of approximately four copies per bacterium [22]. Statistical analysis of the qPCR results was performed using the Mann–Whitney test or the Kruskal–Wallis test for intragroup statistics followed by the post-hoc Dunn's multiple comparisons test in GraphPad Prism v.10.3.1, with statistical significance set at $P < 0.05$.

3. RESULTS

3.1 NGS of *nanA*homologs

To estimate the quantities of *nan* genes (*nan* levels) in *R. gnavus* and other members of the *Lachnospiraceae* family, we designed a *nan* primer set based on the consensus sequences of these *nanA*homolog genes. The forward and reverse primers had melting temperatures (T_m) of 60.1 and 59.6°C, respectively. To verify the specificity of this primer set for the *nanA* gene, we performed PCR amplification on the intestinal DNA samples from three allergy-induced mice, three aggressive dogs, and four UC patients, followed by NGS analysis. The total read counts for these DNA samples were 133,068 for allergy-induced mice, 141,337 for aggressive dogs, and 189,183 for patients with UC. NGS analysis showed that the amplified fragments contained sequences homologous to *nanA* (see Fig. 3 and Supplementary Data 1). Specifically, in the intestinal DNA of allergy-induced mice, amplicon sequence variants (ASVs) from *R. gnavus*, *Blautia*and *Dorea* species were identified, with 61% showing high similarity to *Blautiahansenii* and 25% to *R. gnavus*. In aggressive dogs, ASVs mainly originated from *Dorea*species and *R. gnavus*, with 53% closely related to *Dorea longicatena* and 34% to *R. gnavus*. For UC patients, the ASVs exhibited significant diversity, including sequences from *R. gnavus*, *Blautia*, *Dorea*, *Streptococcus*, and *Clostridiales* species, with 42% closely related to *Blautiahansenii* and 27% to *R. gnavus*.

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Fig. 3. Phylogenetic tree alongside read counts for amplicon sequence variants (ASVs) with more than 400 reads, as identified via next-generation sequencing analysis of *nanA* homologs in intestinal DNA samples from allergy-induced mice (A), aggressive dogs (B), and patients with ulcerative colitis (C). Bootstrap values in leaf annotations are indicated in red text.

3.2 qPCR analysis of *nan* levels on intestinal DNA from allergy-induced mice and aggressive dogs

Quantitative PCR (qPCR) using the *nan* primer set was performed to evaluate *nan* levels in the intestinal DNA of both allergy-induced mice and aggressive dogs. In allergy-induced mice treated with fructans (1-kestose, inulin, or their combination), a significant decrease in *nan* levels was observed when compared with that in the untreated allergy-induced group, with P-values of 0.0356, 0.0040, and 0.0222, respectively (Fig. 4A).
Similarly, qPCR analysis of fecal DNA from aggressive and non-aggressive dogs revealed that *nan* levels were significantly higher in the aggressive group, with a P-value of 0.0015 (Fig. 4B).

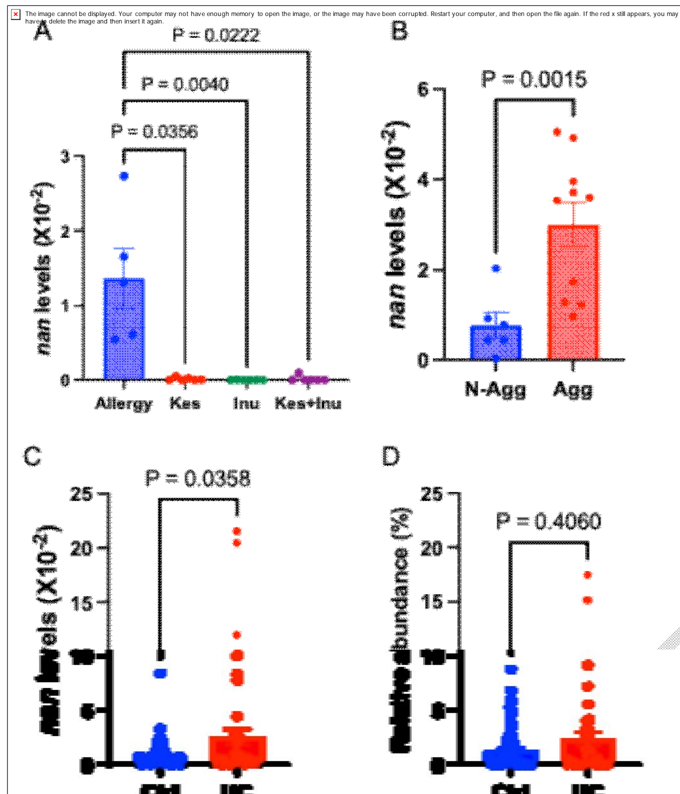

Fig. 4. The graphs display *nan* levels in intestinal DNA samples from allergy-induced mice (A), aggressive dogs (B), and ulcerative colitis (UC) patients (C), along with the relative abundance of *R. gnavus* in UC patients (D). Each plot represents individuals, while bars indicate the mean ± SEM. 'Allergy' for the allergy-induced group. Kes, Inu, and Kes+Inurepresent groups subjected to allergy induction with administration of each type of fructan: specifically 1-kestose, inulin, and a combination of both, respectively. 'N-Agg' denotes the non-aggressive dog group, 'Agg' the aggressive dog group, 'Ctrl' stands for the control group; and 'UC' for the group of UC patients.

3.3 qPCR analysis of *nan* levelson fecal DNA samples from patients with UC

To assess whether *nan* levels reflect the clinical status of immune disorders, fecal samples were collected from patients with UC and analyzed for *nan* levels. Table 1 summarizes the baseline characteristics of the 45 patients with UC in remission for at least one year who participated in the study. The group comprised 25 males and 20 females, with a median age of 62 years (ranging from 39 to 88). The clinical activity index (CAI) scores were as follows: one for 33 patients, two for 10 patients, and three for 2 patients. The control group (Ctrl) included 85 individuals, as reported in a previous study [19].

To estimate *nan* levels, qPCR using the *nan* primer set was performed on fecal DNA samples from patients with UC and healthy control subjects (Ctrl). The *nan* levels in the UC group were significantly higher than those in the Ctrl group (P = 0.0358), as shown in Fig. 4C, confirming that *nan* levels are reflective of the clinical status of immune disorders.

Table 1. Characteristics of ulcerative colitis patients.

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Characteristics	Ulcerative colitis (N = 45)
Median age, years (range)	52.4 (20–79)
Sex (male/female)	25/20
Median body weight, Kg (range)	62.0 (39–88)
CAI (1/2/3)	33/10/2
Types of ulcerative colitis (Pancolitis/Proctitis)	35/10

CAI, clinical activity index.

3.4 NGS of 16S rRNA genes on fecal DNA from UC patients

We conducted 16S rRNA gene NGS, a standard method for microbiome analysis, on DNA samples from UC patients to compare the findings with the results from qPCR analyses.This analysis produced an average of 22,403 ± 3,037 reads for the UC group (mean ± standard error of the mean). The 16S rRNA NGS results for the control group (Ctrl) have been previously reported [19]. Alpha diversity was significantly lower in the UC group than in the control group, as indicated by the Chao 1 index ($P = 0.011$) and the Shannon index ($P = 0.005$). The PCoA plot showed a clear separation between the UC and Ctrl groups (Fig. 5A), and PERMANOVA analysis confirmed a significant difference in beta diversity between their gut microbiomes ($P < 0.001$). The composition of the fecal microbiota at the family and species levels is shown in Fig. 5B and Supplementary Data 2, respectively. Further detailed species-level compositions are provided in Supplementary Data 3. Among the species from *R. gnavus* and those in the genera *Blautia* and *Dorea* with abundances greater than 0.5% in both groups, only *R. gnavus*showed an increase in the UC group (Supplementary Data 3). However, this increase was not statistically significant ($P = 0.4060$, Fig. 4B). Similarly, linear discriminant analysis effect size (LEfSe) at the species level revealed no significant difference in *R. gnavus* abundance between the UC and control groups (Supplementary Data 4). In contrast, LEfSe revealed that at the family level, *Lachnospiraceae*, and at the species level, *Blautialuti*, *Blautiaobeum*, and *Dorea longicatena*, were significantly less abundant in the UC group (Supplementary Data 4).

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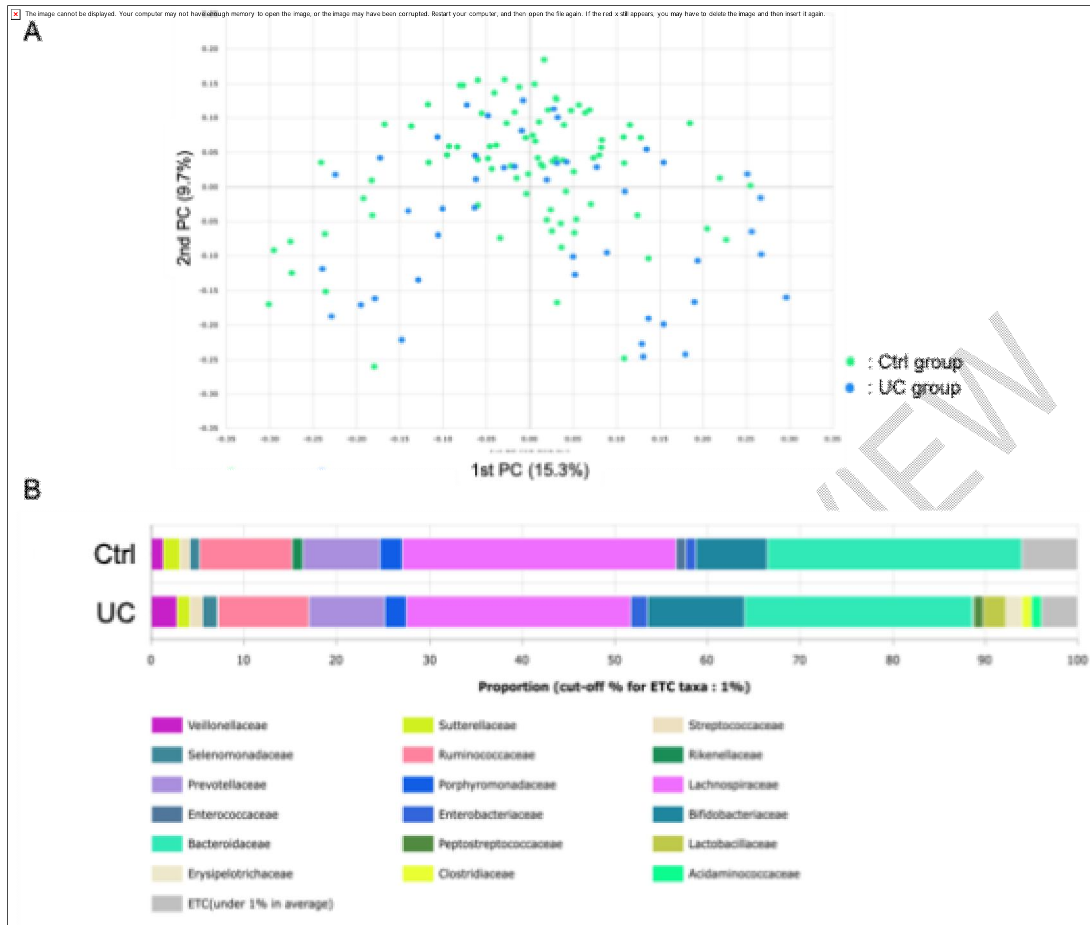

Fig. 5. Composition of the fecal microbiota in the control (Ctrl) and ulcerative colitis (UC) groups. (A) Principal coordinate analysis (PCoA) of beta diversity using Jensen–Shannon distances showing significant separation of microbial communities between the Ctrl and UC groups ($P < 0.001$). Each plot represents individuals. (B) Comparison of the average relative abundances at family level of intestinal bacteria between the Ctrl and UC group. A cut-off of 1% was applied for taxa categorized as 'etc.'

4. DISCUSSION

Studies linking mucin and *R. gnavus* to immune disorders such as IBD and allergies, and mental disorders such as aggression, suggest that measuring *nan* levels could help to assess the risk of these conditions. To explore this potential, we developed a method to estimate *nan* levels in *R. gnavus* and other members of the *Lachnospiraceae* family using qPCR targeting the *nanA* gene. We selected *nanA* as our target for PCR because it is highly conserved within the *nan* gene cluster of key *Lachnospiraceae* species closely related to *R. gnavus*. This conservation likely reflects its crucial role in mucin metabolism inside the bacterial cell, a process that does not depend on cross-feeding from other bacteria, unlike the secreted sialidase protein NanH. Since *Blautia* species, including *Blautiawexlerae* and *Blautialuti*, are frequently found in human fecal samples [23], we designed primers to target the consensus sequence of the *nanA* gene across these *Blautia* species and *R. gnavus*. Although our primer set may not amplify all *nanA* genes, our primary goal was to ensure that

the primer set selectively amplifies *nanA* genes across a wide range of *Lachnospiraceae* species, including *R. gnavus*.Additionally, we aimed to confirm that using qPCR to investigate its association with immune and psychiatric disorders potentially linked to mucin is a reasonable approach.

As expected, NGS analysis of DNA fragments amplified from various intestinal samples using the *nan* primer set revealed that, while the primers were designed to amplify *nanA* homologs in *R. gnavus* and *Blautia* species, we also detected many *nanA* homologs in *Dorea* species. Although the primer's T_m is around 60°C, we intentionally lowered the T_m to 50°C in our experiments to expand the range of *nanA* homologous genes amplified. This adjustment allowed for up to two-base mismatches between the primers and target sequences, likely increasing the diversity of homologous genes that could be detected. Ultimately, the NGS results of the actual PCR products confirmed both the specificity and wide-ranging applicability of our qPCR.

To investigate the link between our qPCR results and immune disorders related to mucin, we analyzed fecal DNA from animal studies. The qPCR analysis in mice revealed that nan levels were significantly higher in allergy-induced mice compared to those treated with both allergy induction and fructan.These changes in *nan* levels closely mirrored variations in allergy scores and the expression of IgA, IgE, and Interleukin-4, as reported previously [21]. Allergies are essentially abnormal immune responses to harmless environmental substances [11]. Recent research has revealed that the intestinal epithelium plays a critical role in initiating and regulating immune responses, rather than just serving as a simple barrier [3]. This involves the activation of toll-like receptors (TLRs) on epithelial cells, which in turn release cytokines, chemokines, and antimicrobial peptides, activating the innate immune system [24]. Mucin acts as a protective barrier, preventing allergens from interacting with TLRs, which is crucial for controlling allergic responses [25, 26].Our findings indicate that, in the fructan-treated group, reduced mucin degradation associated with lower *nan* levels may potentially decrease allergen binding to TLRs, which in turn may weaken the immune response.

To investigate the link between our qPCR results and psychiatric disorders,we conducted another qPCR analysis using intestinal DNA samples from aggressive and non-aggressive dogs in an animal study.We found that *nan* levels were significantly higher in the aggressive dog group when compared to the non-aggressive group. Recent studies highlight the role of gut microbiota in regulating stress responses through the microbiota-gut-brain axis in mice [13]. Additionally, another study suggests that the composition of the gut microbiota during early development can influence aggressive behavior in mice [14]. Along with these findings, our research suggests that *nan*-mediated mucin degradation may impact the gut-brain axis and could potentially contribute to aggressive behaviors.

To validate our qPCR method for measuring *nan* levels in a clinical setting, we analyzed fecal samples from 45 patients with UC. The qPCR results revealed that *nan* levels were significantly higher in the UC group when compared to the control group, confirming that *nan* levels reflect the clinical status of immune disorders. However, unlike previous studies that have linked *R. gnavus*to IBD [7], our NGS analysis did not find this association. This discrepancy may be due to our focus on UC patients in remission with relatively mild symptoms. Another key factor could be the diversity within *R. gnavus* itself. Research indicates that *R. gnavus* consists of various clades, some commonly found in IBD patients and others not, each performing different functions [9]. Additionally, certain *R. gnavus* strains can degrade mucin glycans, while others cannot [4]. Interestingly, our analysis also showed that *Lachnospiraceae* bacteria, such as *Blautialuti*, *Blautiaobeum*, and *Dorea longicatena*, which may harbor *nan* genes, were not increased in UC patients; in fact, they were significantly reduced. This unexpected finding highlights the limitations of conventional 16S rRNA gene analysis in accurately evaluating the intestinal microbiota and its potential pathogenicity. In contrast, our qPCR method for measuring *nan* levels offers a targeted

approach, selectively detecting potentially pathogenic strains that harbor the *nan* gene, thereby addressing the shortcomings of traditional 16S rRNA analysis.
This study offers valuable insights but also has several limitations. First, our primer set is specifically designed to detect certain *nan* genes, and it has not been validated for detecting *nan* genes beyond its target. While it effectively identifies nan from the main mucin degrader, *R. gnavus*, and related *Lachnospiraceae* species, other *nan* genes involved in mucin degradation may not be captured. Second, our study focuses on detecting the presence of the mucin-degrading *nan* gene rather than directly measuring mucin levels. To address this, we plan to conduct additional animal studies specifically designed to measure mucin levels. Lastly, while this study found significantly higher *nan* levels in groups with immune and psychiatric disorders compared to the control group, it remains unclear whether these levels are linked to disease severity. Future research will include patients with active ulcerative colitis to explore this further.

5. CONCLUSION

Measuring *nan* levels in gut microbiota using qPCR shows promise in selectively detecting potentially pathogenic *nan*-harboring strains. The findings suggest that monitoring *nan* levels in intestinal DNA using qPCR could serve as a non-invasive, rapid, and cost-effective approach for assessing the increased risk of immune and psychiatric disorders.

ETHICAL APPROVAL

This study was approved by the Institutional Review Board of Fujita Health University (HM22-272). Informed consent was obtained from all subjects involved in the study.

The study involving DNA analysis from patients with ulcerative colitis (UC) was approved by the Institutional Review Board of Fujita Health University (HM22-272 and HM23-078). Informed consent was obtained from all participants, and the study was conducted in accordance with the principles of the Declaration of Helsinki and Good Clinical Practice guidelines.

For the study on aggressive dogs, ethical approval was granted by the Yamazaki University of Animal Health Technology as a clinical research project (20210427-001). This research followed the Japanese National Guidelines for the Humane Treatment of Animals [13], and written informed consent was obtained from all participating dog owners.

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REFERENCES

1. Barko PC, McMichael MA, Swanson KS, Williams DA. The gastrointestinal microbiome: A review. J Vet Intern Med. 2018;32(1):9-25. https://doi.org/10.1111/jvim.14875
2. Ma T, Shen X, Shi X, Sakandar HA, Quan K *et al.*Targeting gut microbiota and metabolism as the major probiotic mechanism-An evidence-based review. Trends in Food Science & Technology.2023;138:178-98. https://doi.org/10.1016/j.tifs.2023.06.013
3. Martinez-Carrasco R, Argüeso P, Fini ME. Membrane-associated mucins of the human ocular surface in health and disease. Ocul Surf.2021;21:313-30. https://doi.org/10.1016%2Fj.jtos.2021.03.003

4. Crost EH, Tailford LE, Le Gall G, Fons M, Henrissat B *et al.*Utilisation of mucin glycans by the human gut symbiont *Ruminococcusgnavus* is strain-dependent. PLoS One. 2013;8(10):e76341. https://doi.org/10.1371/journal.pone.0076341
5. Crost EH, Tailford LE, Monestier M, Swarbreck D, Henrissat B *et al.* The mucin-degradation strategy of *Ruminococcusgnavus*: The importance of intramolecular trans-sialidases. Gut Microbes. 2016;7(4):302-12. https://doi.org/10.1080/19490976.2016.1186334
6. Bell A, Brunt J, Crost E, Vaux L, Nepravishta R *et al.* Elucidation of a sialic acid metabolism pathway in mucus-foraging Ruminococcusgnavus unravels mechanisms of bacterial adaptation to the gut. Nat Microbiol. 2019;4(12):2393-404. https://doi.org/10.1038/s41564-019-0590-7
7. Lawson PA, Finegold SM. Reclassification of*Ruminococcusobeum*as *Blautiaobeum* comb. nov. Int J Syst EvolMicrobiol. 2015;65(Pt 3):789-793. https://doi.org/10.1099/ijs.0.000015
8. Togo AH, Diop A, Bittar F, Maraninchi M, Valero R *et al.* Correction to: Description of *Mediterraneibactermassiliensis*, gen. nov., sp. nov., a new genus isolated from the gut microbiota of an obese patient and reclassification of *Ruminococcusfaecis*, *Ruminococcuslactaris*, *Ruminococcus torques*, *Ruminococcusgnavus* and *Clostridium glycyrrhizinilyticum*as *Mediterraneibacterfaecis*comb. nov., *Mediterraneibacterlactaris*comb. nov., *Mediterraneibacter torques* comb. nov.,*Mediterraneibactergnavus* comb. nov. and *Mediterraneibacterglycyrrhizinilyticus* comb. nov. Antonie Van Leeuwenhoek 2018;111(11):2129-130. https://doi.org/10.1007/s10482-018-1171-0
9. Hall AB, Yassour M, Sauk J, Garner A, Jiang X et al. A novel *R. gnavus*clade enriched in inflammatory bowel disease patients. Genome Med. 2017;9(1):103. https://doi.org/10.1186/s13073-017-0490-5
10. Henke MT, Kenny DJ, Cassilly CD, Vlamakis H, Xavier RJ et al. *Ruminococcusgnavus*, a member of the human gut microbiome associated with Crohn's disease, produces an inflammatory polysaccharide. Proc Natl Acad Sci. 2019;116(26):12672-7. doi.org/10.1073/pnas.1904099116
11. Galli SJ, Tsai M, Piliponsky AM. The development of allergic inflammation.Nature. 2008;454(7203):445-54. https://doi.org/10.1038/nature07204
12. Chua HH, Chou HC, Tung YL, Chiang BL, Liao CC *et al.* Intestinal dysbiosis featuring abundance of *Ruminococcusgnavus* associates with allergic diseases in infants. Gastroenterology. 2018;154(1):154-167. https://doi.org/10.1053/j.gastro.2017.09.006
13. Vuong HE, Yano JM, Fung TC, Hsiao EY. The microbiome and host behavior. Annu Rev Neurosci.2017;40:21-49. https://doi.org/10.1146/annurev-neuro-072116-031347
14. Watanabe N, Mikami K, Hata T, Kimoto K, Nishino R *et al.* Effect of gut microbiota early in life on aggressive behavior in mice. Neurosci Res. 2021;168:95-9. https://doi.org/10.1016/j.neures.2021.01.005
15. Obrenovich MEM. Leaky gut, leaky brain? Microorganisms. 2018;6(4). https://doi.org/10.3390/microorganisms6040107
16. Kirchoff NS, Udell MA, Sharpton TJ. The gut microbiome correlates with conspecific aggression in a small population of rescued dogs (Canis familiaris). PeerJ. 2019;7:e6103.
17. Japanese Ministry of Health, Labor and Welfare Good Clinical Practice. Ministry of Health, Labor and Welfare Ministerial Ordinance, No. 28 Dated March 27, 1997 and No. 24 Dated February 29, 2008. Tokyo, Japan.
18. Wright JC,Nesselrote MS. Classification of behavior problems in dogs: Distributions of age, breed, sex and reproductive status. Appl Anim Behav Sci. 1987;19:169-78. https://doi.org/10.1016/0168-1591(87)90213-9
19. Fujii T, Kuzuya T, Kondo N, Funasaka K, Ohno E et al. Altered intestinal *Streptococcus anginosus* and 5α-reductase gene levels in patients with hepatocellular carcinoma and elevated *Bacteroides stercoris* in atezolizumab/bevacizumab non-responders. J Med Microbiol. 2024;73(9). https://doi.org/10.1099/jmm.0.001878

20. Fujii T, Nakagawa Y, Funasaka K, Hirooka Y, Tochio T. Levels of 5α-reductase gene in intestinal lavage fluid decrease with progression of colorectal cancer. J Med Microbiol. 2024;73(6). https://doi.org/10.1099/jmm.0.001834
21. Takahashi H, Fujii T, Yamakawa S, Yamada C, Fujiki K *et al.* Combined oral intake of short and long fructans alters the gut microbiota in food allergy model mice and contributes to food allergy prevention. BMC Microbiol. 2023;23(1):266. https://doi.org/10.1186/s12866-023-03021-6
22. Lee ZM, Bussema C, Schmidt TM. rrnDB: documenting the number of rRNA and tRNA genes in bacteria and archaea. Nucleic Acids Res. 2009;37(Database issue):D489-93. https://doi.org/10.1093/nar/gkn689
23. Touyama M, Jin JS, Kibe R, Hayashi H, Benno Y. Quantification of *Blautia wexlerae* and *Blautia luti* in human faeces by real-time PCR using specific primers. Benef Microbes. 2015;6(4):583-90. https://doi.org/10.3920/bm2014.0133
24. Lambrecht BN, Hammad H. The airway epithelium in asthma. Nat Med. 2012;18(5):684-92. https://doi.org/10.1038/nm.2737
25. Li DQ, Zhang L, Pflugfelder SC, De Paiva CS, Zhang X *et al.*Short ragweed pollen triggers allergic inflammation through Toll-like receptor 4-dependent thymic stromal lymphopoietin/OX40 ligand/OX40 signaling pathways. J Allergy Clin Immunol. 2011;128(6):1318-1325.e1312. https://doi.org/10.1016/j.jaci.2011.06.041
26. Li J, Zhang L, Chen X, Chen D, Hua X *et al.*Pollen/TLR4 innate immunity signaling initiates IL-33/ST2/Th2 pathways in allergic inflammation. Sci Rep. 2016;6:36150. https://doi.org/10.1038/srep36150

ABBREVIATIONS

CAI, Clinical Activity Index; IBD,inflammatory bowel disease; *nan, nan* gene; *nan* levels, quantities of *nan* genes; LEfSe, linear discriminant analysis effect size; NGS, next-generation sequencing; PCoA, principal coordinate analysis; PERMANOVA, permutational multivariate analysis of variance; qPCR, quantitative PCR; T_m, melting temperatures; TLRs, toll-like receptors; UC, ulcerative colitis

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