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Assessment of Risk for Immune and Psychiatric Disorders Using qPCR-Based Monitoring of the *nan* Gene in Gut Microbiota: A Non-invasive Approach

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Authors' contributions

This work was carried out in collaboration among all authors. Author TF led the conception of this study, contributed significantly to the study design, data acquisition, and data interpretation, and played a major role in drafting and critically revising the manuscript. Authors MN, TK and SM were actively involved in sample collection, contributed to data interpretation, and provided crucial revisions to the manuscript to ensure accuracy and depth. Authors MN, TK, KF, EO, CM and TT further contributed to the overall conception and design of the study and provided thoughtful revisions to enhance the quality of the manuscript. Author YH played a central role in conceiving and designing the study, and critically appraising and refining the manuscript. All authors have reviewed and approved the final version of the manuscript and agree to be held accountable for all aspects of the study to ensure its integrity and accuracy. All authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

Aim: This study aimed to establish a non-invasive, rapid, and cost-effective method to assess the potential risk of immune and psychiatric disorders by quantifying *nan* gene levels in gut microbiota, specifically focusing on *Ruminococcus gnavus* and other *Lachnospiraceae* species associated with mucin degradation.
Methodology: We first designed a primer set targeting the consensus sequence of the *nanA* gene, which is highly conserved within *nan* gene clusters. To validate this primer set, we performed Next-Generation Sequencing (NGS) on the PCR-amplified fragments. To explore the association between *nan* levels and immune or psychiatric disorders, we conducted qPCR to quantify *nan* levels in the intestines, analyzing intestinal DNA from both allergy-induced mice with or without fructan treatment, and dogs with or without aggressive behavior. Additionally, to assess whether *nan* levels reflect the clinical status of immune disorders, fecal samples were collected from 45 patients with ulcerative colitis (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 members of the *Lachnospiraceae* family, including *Blautia* and *Dorea* species. The qPCR quantification of *nan* levels using this primer set revealed that allergy-induced mice treated with fructans, which are known to be associated with lower allergy scores compared to untreated mice, exhibited significantly reduced *nan* levels. Additionally, the *nan* levels of aggressive dogs were substantially higher than those of non-aggressive dogs. Notably, *nan* levels were also substantially elevated in patients with UC in comparison to the healthy control individuals.
Conclusion: qPCR-based measurement of *nan* levels in gut microbiota shows potential for selectively detecting pathogenic *nan*-harboring strains and may reflect the clinical status of immune and psychiatric disorders. This approach could provide a non-invasive, rapid, and cost-effective method for assessing the risk of these disorders.

Keywords: Aggression; allergy; nan gene; Ruminococcus gnavus; ulcerative colitis.

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*

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 (Barko et al. 2018). 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 (Ma et al. 2023). 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 (Martínez-Carrasco et al. 2021).

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 *Ruminococcus gnavus* (Crost et al. 2013, Crost et al. 2016, Bell et al. 2019, Kim 2023). 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 (Lawson 2015, Togo et al. 2018). 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.

A key gene in the *nan* gene 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) (Bell et al. 2019, Kim 2023). 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 (Hall et al. 2017). *R. gnavus* is also known for producing inflammatory polysaccharides such as glucorhamnan, which can activate dendritic cells through toll-like receptor 4 (TLR4) 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 (Henke et al. 2019). *R. gnavus* is also linked to other immune disorders. For example, allergy, which is an inappropriate immune response to harmless environmental substances (Galli et al. 2018), has been associated with *R. gnavus*. Studies have found that *R. gnavus* is more common in patients with allergies than in those without (Chua et al. 2018, Filippis et al. 2021).

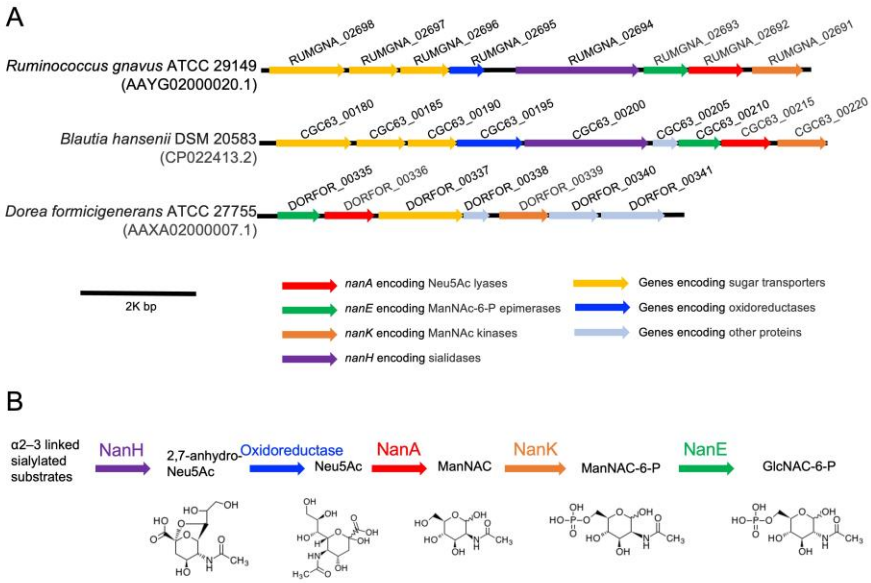

Fig. 1. (A) Depiction of the putative *nan* gene cluster in *Ruminococcus gnavus* ATCC 29149, *Blautia hansenii* DSM 20583, and *Dorea formicigenerans* ATCC 27755. Parentheses indicate GenBank accession numbers, arrows in the figure represent open reading frames, and the text above them indicates locus tags. (B) Proposed pathways for the catabolism of sialic acid in *Ruminococcus gnavus* ATCC 29149, as outlined in a previous study (Bell et al. 2019, Kim 2023). 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

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 (Vuong et al. 2017, Watanabe et al. 2021, Obrenovich 2018). 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 (Kirchoff et al. 2019).

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-effective approach to assessing the risk of these conditions. To this end, we have created a method 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 dogs 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 Research Subject

Fig. 2 presents a diagram of the microbiome analysis conducted in this study.

The analysis of *nan* levels in the ovalbumin-induced allergy mouse model involved four groups: five untreated allergy-induced mice, six mice treated with 1-kestose, six treated with inulin, and six treated with both 1-kestose and inulin, as described by Takahashi et al. 2023.

For the dog analysis, *nan* levels were quantified in toy poodles, excluding those with a history of gastrointestinal surgery, recent antibiotic use (within 2 months), or those who had been administered gastrointestinal, cranial nerve, or psychotropic drugs within 2 weeks prior to sample collection. The non-aggressive group comprised six healthy dogs (median age: 3.94 years; range: 3.03–3.94 years; 2 males, 4 females). The aggressive group included ten dogs, selected based on Wright and Nesselrote's criteria (Wright 1987), with a median age of 4.64 years (range: 2.79–6.31 years; 3 males, 7 females). Aggression was defined by behaviors such as lunging, snarling, growling, and biting directed at unfamiliar people or dogs, determined through a 5–10 min phone interview with clients, followed by a 1–3 h behavioral assessment session.

The microbiome analysis of fecal DNA samples from UC patients included qPCR to quantify *nan* levels and 16S rRNA gene sequencing. This analysis involved 45 participants with UC in remission for at least one year, including 25 males and 20 females, with a median age of 62 years (range: 39–88). Clinical Activity Index (CAI) scores were distributed as follows: 33 patients had a score of 1, ten had a score of 2, and two had a score of 3. Table 1 summarizes the characteristics of the 45 patients with UC.

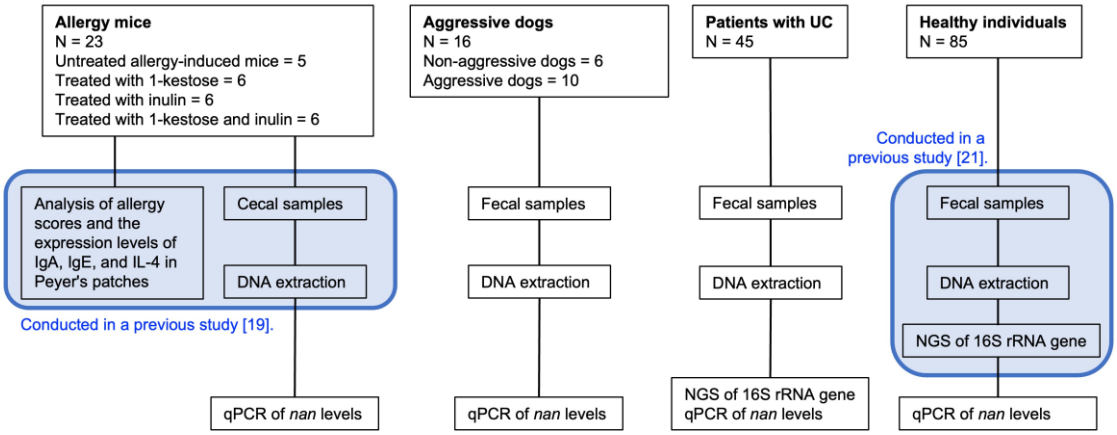

Fig. 2. A diagram of the microbiome analysis conducted in this study

Table 1. Characteristics of ulcerative colitis patients

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.

2.2 Intestinal DNA Samples

Cecal DNA samples from allergy-induced mice were obtained from a previous study by Takahashi et al. 2023.

For the dog study, fecal samples were collected using the Fecal Collection Kit FS-0017 (Techno Suruga Laboratory, Shizuoka, Japan) within 30 min of defecation. These samples were then stored at -20°C until DNA extraction, which was performed at the Techno Suruga Laboratory.

In the microbiome analysis of patients with UC, fecal DNA samples were collected using the Fecal Collection Kit FS-0017. DNA extraction was carried out using the QIAamp PowerFecal Pro DNA Kit (QIAGEN, Hilden, Germany), following the manufacturer's instructions. DNA samples from 85 healthy control individuals were obtained from a previous study by Fujii et al. 2024. For the analysis of amplified *nanA* sequences using next-generation sequencing (NGS), intestinal lavage fluids (ILFs) were collected during colonoscopies from four patients with UC. The group consisted of three men (aged 54, 55, and 65) and one woman (aged 20). DNA was then extracted from these ILF samples according to the previously described method (Fujii et al. 2024).

2.3 NGS Analysis

NGS analysis was performed on the amplified *nanA* gene fragments from a subset of 11 randomly selected intestinal DNA samples. These included three DNA samples from allergy-induced mice, three from aggressive dogs, and four from 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 (*Blautia wexlerae, Blautia luti,* and *Blautia coccoides*), 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. 3.

For the first PCR, a *nanA*-specific primer set (nanA_113F and nanA_113R, collectively referred to as the *nan* primer set) was designed to target conserved regions of the *nanA* gene, as detailed in Table 2. The forward primer, nanA_113F, binds at positions 61–77 bp, while the reverse primer, nanA_113R, binds at positions 152–173 bp of the *R. gnavus* ATCC 29149 *nanA* gene. For the second PCR, intended for NGS, a different primer set (nanA_for NGS_F and nanA_for NGS_R), also listed in Table 2, was utilized. Both PCRs adhered to previously established protocols targeting the 5α-reductase gene (Fujii et al. 2024). The PCR products were subsequently sequenced using NGS at Bioengineering Lab Co., Ltd., Kanagawa, Japan, as described in previous studies (Fujii et al. 2024). The resulting *nanA* sequences were analyzed for homology using BLASTn (version 2.15.0) on the NCBI website (https://www.ncbi.nlm.nih.gov/, accessed November 15, 2023). Phylogenetic analyses were conducted using Phylogeny.fr (http://www.phylogeny.fr/, accessed November 15, 2023).

Additionally, NGS analysis of the 16S rRNA gene and subsequent bioinformatics were performed on fecal DNA samples from 45 patients with UC, following previously described methods (Fujii et al. 2024). The sequencing data obtained from the MiSeq system was processed, analyzed statistically, and mapped using the EzBioCloud 16S database and microbiome pipeline provided by ChunLab Inc. (EzBioCloud 16S-based MTP app, available at https://www.EZbiocloud.net).

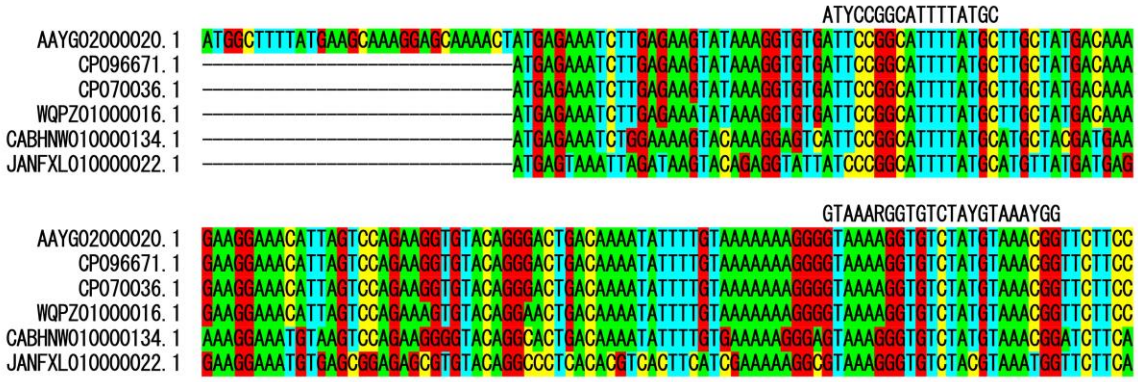

Fig. 3. N-terminal nucleotide sequence alignment of *nanA* gene homologs. The sequence shown above the alignment indicates the region selected for primer design. CP027002.1: *Ruminococcus gnavus* ATCC29149, CP096671.1: *Ruminococcus gnavus* strain CC55_001C, CP070036.1: *Ruminococcus gnavus* strain FDAARGOS_1342, WQPZ01000016.1: *Blautia wexlerae* strain MCC298, CABHNW010000134.1: *Blautia luti* isolate *Blautia_luti*_SSTS_Bg7063, JANFXL010000022.1: *Blautia coccoides* strain SL.3.07 GCCCFIEI_22

Table 2. PCR primers used in the present study

Primer name	Oligonucleotide sequence	T_m (°C)	Reference	Used for
nanA_113F	ATYCCGGCATTTTATGC	60.1	This study	amplification of *nanA*
nanA_113R	CCRTTTACRTAGACACCYTTTAC	59.6		
nanA_for NGS_F	ACACTCTTTCCCTACACGACGCTCTTCCGA TCT**ATYCCGGCATTTTATGC**	85.3	This study	NGS of *nanA*
nanA_for NGS_R	GTGACTGGAGTTCAGACGTGTGCTCTTCC GATCT**CCRTTTACRTAGACACCYTTTAC**	85.1		
F_Bact 1369	CGGTGAATACGTTCCCGG	66.6	(Fujii et al. 2024)	amplification of bacterial 16S rRNA gene
R_Prok1492	TACGGCTACCTTGTTACGACTT	61.9		

Bold sequences correspond to the sequences of nanA_113F or nanA_113R. The melting temperatures (T_m) were calculated using the Multiple Primer Analyzer (Thermo Fisher Scientific, Waltham, MA, USA)

2.4 Quantitative 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 20 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 (Fujii et al. 2024). 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 variability in 16S rRNA gene copies per bacterial genome, ranging from 1 to 15, with an average of about four copies (Lee et al. 2009).

2.5 Statistical Analysis

Statistical analysis of *nan* levels quantified by the qPCR and the relative abundance calculated by 16S rRNA gene NGS 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 *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. 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 exclusively homologous to *nanA* (see Fig. 4 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 *Blautia hansenii* 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 *Blautia hansenii* and 27% to *R. gnavus*.

3.2 qPCR Analysis of *nan* Levels on Intestinal DNA Samples from Allergy-induced Mice and Aggressive Dogs

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. 5A).

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. 5B).

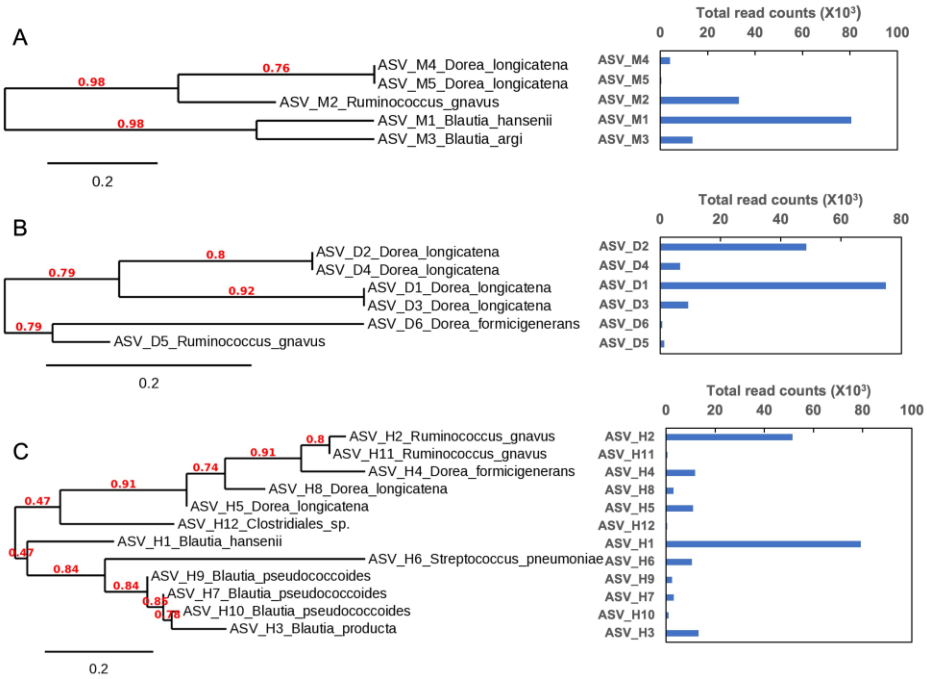

Fig. 4. Phylogenetic tree alongside read counts for amplicon sequence variants (ASVs) with more than 400 reads, as identified via next-generation sequencing analysis of *nanA* homologs in intestinal DNA samples from allergy-induced mice (A), aggressive dogs (B), and patients with ulcerative colitis (C). Bootstrap values in leaf annotations are indicated in red text

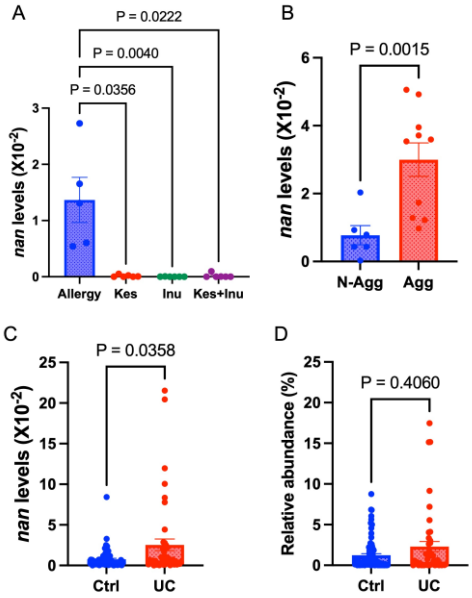

Fig. 5. The graphs display *nan* levels in intestinal DNA samples from allergy-induced mice (A), aggressive dogs (B), and ulcerative colitis (UC) patients (C), along with the relative abundance of *R. gnavus* in patients with UC (D). Each plot represents individuals, while bars indicate the mean ± SEM. 'Allergy' for the allergy-induced group. Kes, Inu, and Kes+Inu represent 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' is the aggressive dog group, 'Ctrl' stands for the healthy control group; and 'UC' is for the group of patients with UC

3.3 qPCR Analysis of *nan* Levels on Fecal DNA Samples from UC Patients

To evaluate whether *nan* levels correlate with the clinical status of immune disorders, we quantified *nan* levels by qPCR using the *nan* primer set on fecal DNA samples from patients with UC and healthy control individuals. The *nan* levels in the UC group were significantly higher than those in the control group (P = 0.0358), as shown in Fig. 5C.

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 healthy control group (Ctrl) have been previously reported (Fujii et al. 2024). 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. 6A), 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. 6B 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. 5D). 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, *Blautia luti*, *Blautia obeum*, and *Dorea longicatena*, were significantly less abundant in the UC group (Supplementary Data 4).

Fig. 6. 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 groups. 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, as well as psychiatric disorders like aggression, prompted the idea that measuring *nan* levels could help 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 *Blautia wexlerae* and *Blautia luti*, are frequently found in human fecal samples (Touyama et al. 2015), we designed primers to target the consensus sequence of the *nanA* gene across these *Blautia* species and *R. gnavus*.

While our primer set may not amplify all *nanA* genes, our primary goal was to ensure selective amplification of *nanA* genes across as broad a spectrum as possible of *Lachnospiraceae* species, including *R. gnavus*. 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 approximately 60°C, we intentionally reduced the annealing temperature to 50°C in our qPCR experiments to broaden the range of amplified *nanA* homologous genes. 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.

Additionally, we sought to confirm that qPCR-based measurement of *nan* levels in gut microbiota could serve as an indicator of the clinical status of immune and psychiatric disorders. To investigate the link between the *nan* levels 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 (Takahashi et al. 2023). Allergies are essentially abnormal immune responses to harmless environmental substances (Galli et al. 2008). 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 (Martínez-Carrasco et al. 2021). This involves the activation of TLRs on epithelial cells, which in turn release cytokines, chemokines, and antimicrobial peptides, activating the innate immune system (Lambrecht 2012). Mucin acts as a protective barrier, preventing allergens from interacting with TLRs, which is crucial for controlling allergic responses (Lambrecht 2012, Li et al. 2011). 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 the *nan* levels and psychiatric disorders, we conducted another qPCR analysis using intestinal DNA samples from aggressive and non-aggressive dogs. We found that *nan* levels were significantly higher in the aggressive dog group than the non-aggressive group. Recent studies highlight the role of gut microbiota in regulating stress responses through the microbiota-gut-brain axis in mice (Vuong et al. 2017). Additionally, another study suggests that the composition of the gut microbiota during early development can influence aggressive behavior in mice (Watanabe et al. 2021). 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 DNA samples from 45 patients with UC. The qPCR results showed that *nan* levels were significantly elevated in the UC group compared to the control group. These findings suggest that *nan* levels may reflect the clinical status of UC and potentially other immune disorders. However, unlike previous studies that have linked *R. gnavus* to IBD (Hall et al. 2017), our 16S rRNA gene 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 (Hall et al. 2017). Additionally, certain *R. gnavus* strains can degrade mucin glycans, while others cannot (Crost et al. 2013). Interestingly, our analysis also showed that *Lachnospiraceae* species, such as *Blautia luti*, *Blautia obeum*, 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

qPCR-based measurement of *nan* levels in gut microbiota shows potential for selectively detecting pathogenic *nan*-harboring strains and may reflect the clinical status of immune and psychiatric disorders. This approach could provide a non-invasive, rapid, and cost-effective method for assessing the risk of these disorders.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that generative AI technologies such as Large Language Models, etc. have been used during the writing or editing of manuscripts. This explanation will include the name, version, model, and source of the generative AI technology and as well as all input prompts provided to the generative AI technology Details of the AI usage are given below:

1. Name: ChatGPT
2. Version: GPT-4
3. Model: OpenAI
4. Purpose: Translation from Japanese to English for specific sections of the manuscript

CONSENT AND ETHICAL APPROVAL

The study involving patients with ulcerative colitis was approved by the Institutional Review Board of Fujita Health University (approval number: HM22-272), while the study involving healthy control individuals received approval under (approval number: 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 allergy-induced mice, all experimental animal procedures in this study were approved by the Laboratory Animal Care Committee of Nagoya University of Arts and Sciences (approval number: 128). All animal studies were conducted following the ARRIVE guidelines and the Animal Experimentation Guidelines of Nagoya University of Arts and Sciences.

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 (Li et al. 2016), and written informed consent was obtained from all participating dog owners (Japanese Ministry of Health, Labor and Welfare 1997).

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COMPETING INTERESTS

Authors have declared that they have no known competing financial interests or non-financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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